

Effects of *BRAF* Mutations and *BRAF* Inhibition on Immune Responses to Melanoma

Kristina M. Ilieva¹, Isabel Correa¹, Debra H. Josephs^{1,2}, Panagiotis Karagiannis¹, Isioma U. Egbuniwe¹, Michiala J. Cafferkey¹, James F. Spicer², Mark Harries³, Frank O. Nestle¹, Katie E. Lacy¹, and Sophia N. Karagiannis¹

Abstract

Malignant melanoma is associated with poor clinical prognosis; however, novel molecular and immune therapies are now improving patient outcomes. Almost 50% of melanomas harbor targetable activating mutations of *BRAF* that promote RAS–RAF–MEK–ERK pathway activation and melanoma proliferation. Recent evidence also indicates that melanomas bearing mutant *BRAF* may also have altered immune responses, suggesting additional avenues for treatment of this patient group. The small molecule inhibitors selective for mutant *BRAF* induce significant but short-lived clinical responses in a proportion of patients, but also lead to immune stimulatory bystander events, which then subside with the emergence of resistance to inhibition. Simultaneous *BRAF* and MEK inhibition, and especially combination of *BRAF* inhibitors with new immunotherapies such as checkpoint blockade antibodies, may further enhance immune activation, or counteract immunosuppressive signals. Preclinical evaluation and ongoing clinical trials should provide novel insights into the role of immunity in the therapy of *BRAF*-mutant melanoma. *Mol Cancer Ther*; 13(12); 2769–83. ©2014 AACR.

Therapeutic Approaches for Melanoma

Cutaneous melanoma is an aggressive and potentially lethal form of skin cancer originating from the malignant transformation of melanocytes in the basal layer of the epidermis of the skin. The incidence of malignant melanoma has trebled worldwide since the 1970s (1). In 20% of cases, patients develop locoregional or distant metastases and historic median survival for those diagnosed with distant metastases is only 6 to 9 months (2). Treatment options for malignant melanoma have been limited until recently. For metastatic disease, chemotherapeutic agents such as dacarbazine (DTIC) were the standard of care for over 30 years, but do not significantly improve median overall survival (OS). Immunotherapy with high-dose interferon (IFN α 2b) is approved in the adjuvant setting and for resected advanced disease, but suffers from sig-

nificant associated toxicities and only a small portion of patients derive clinical benefits. Because of associated severe toxicities, high-dose IL2 is only administered for stage IV disease in limited numbers of specialized centers worldwide, with around 5% of the patients achieving long-term clinical responses (3).

The clinical landscape has been transformed with the approval of new therapies in the last 2 years, which include pathway inhibitor drugs and an mAb. Vemurafenib, approved by the FDA in 2011, is a kinase inhibitor selective for the commonest mutant form of the *BRAF* kinase (*BRAF*^{V600E}) designed to target transformation and proliferation of melanoma cells. Two new pathway inhibitor drugs dabrafenib (also an inhibitor of mutant *BRAF*) and trametinib (an MEK inhibitor) were approved in 2013. Vemurafenib, dabrafenib, and trametinib are all associated with potent, but often short-term responses (4–6). Ipilimumab, an anti–CTLA-4 (CTL antigen; ref. 4) mAb blocking a T-cell regulatory pathway designed to promote activated immunity, was approved in 2011 (7) as a second-line treatment for advanced melanoma. More recently, it has been approved for first-line therapy. Ipilimumab treatment is characterized by durable responses but only in a minority of patients.

The success of ipilimumab has enhanced appreciation of the potential of immune responses to influence patient outcomes. Importantly, emerging studies suggest that *BRAF*-mutant melanomas and *BRAF* inhibition can also alter immune inflammatory mechanisms associated with tumors. Here, we review evidence of associations between *BRAF*-mutant melanoma and *BRAF*

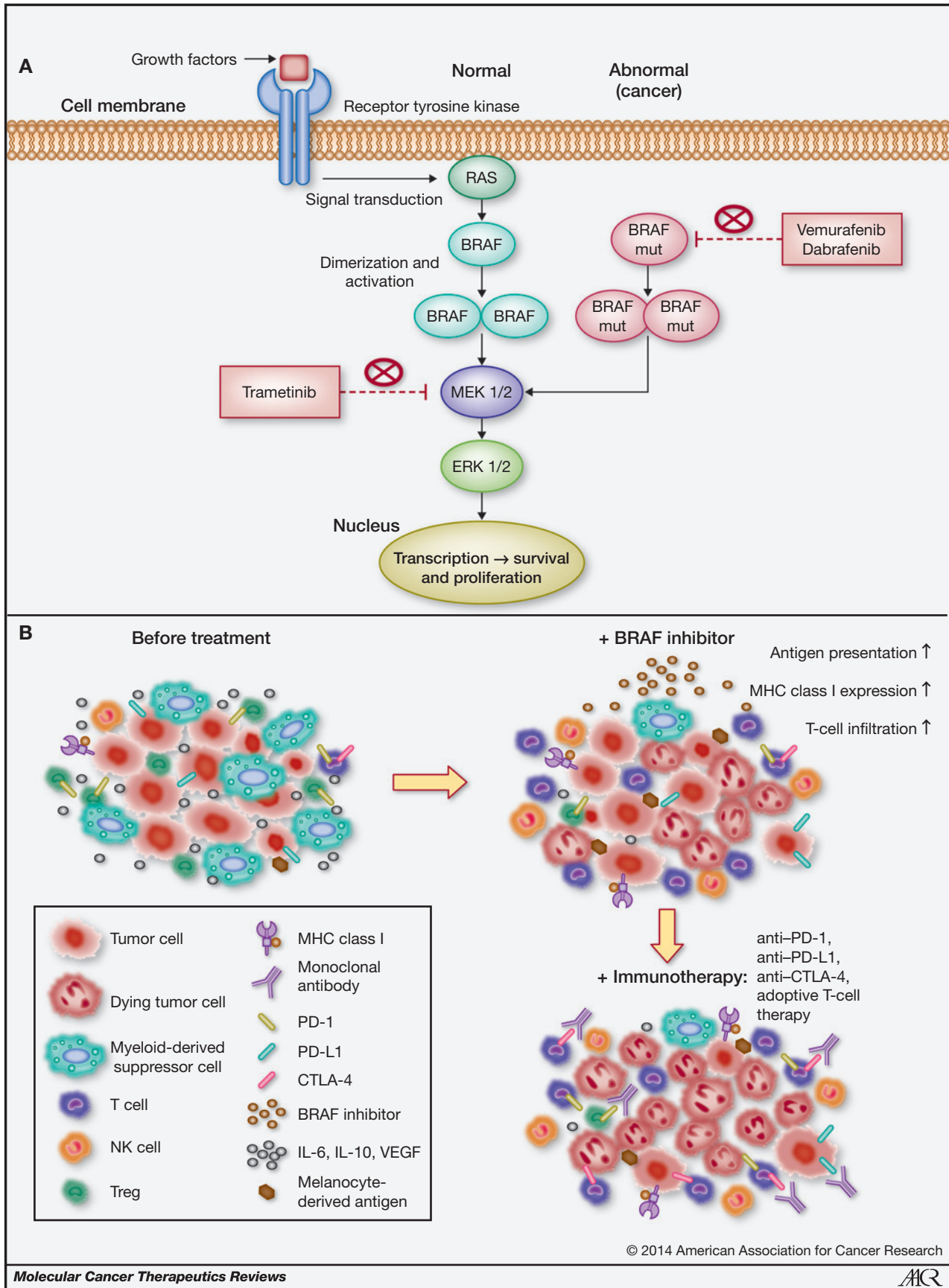
¹St. John's Institute of Dermatology, Division of Genetics and Molecular Medicine and NIHR Biomedical Research Centre at Guy's and St. Thomas's Hospitals and King's College London, London, United Kingdom.

²Department of Research Oncology, Division of Cancer Studies, King's College London, Guy's Hospital, London, United Kingdom. ³Clinical Oncology, Guy's and St. Thomas's NHS Foundation Trust, Guy's Hospital, London, United Kingdom.

Corresponding Author: Sophia N. Karagiannis, St. John's Institute of Dermatology, Division of Genetics and Molecular Medicine, King's College London and NIHR Biomedical Research Centre at Guy's and St. Thomas's Hospitals and King's College London, Guy's Hospital, Tower Wing, 9th Floor, London, SE1 9RT, UK. Phone: 44-0-20-7188-6355; Fax: 44-0-20-7188-8050; E-mail: sophia.karagiannis@kcl.ac.uk

doi: 10.1158/1535-7163.MCT-14-0290

©2014 American Association for Cancer Research.



pathway inhibition with immunity and discuss their potential translational implications, including exploring the merits of combination strategies to strengthen immune responses or to counteract tumor-associated immune escape mechanisms.

Activating Immune Responses

Melanoma elicits immune responses, a notion supported by clinical and experimental evidence such as partial regressions in some melanoma lesions, T-cell infiltration in tumors correlating with better clinical outcomes, higher incidence of melanoma in immunosuppressed individuals, and the discovery of melanoma-specific antigens and spontaneous T-cell and antibody responses against melanoma-associated antigens in patients (8). However, immune activation is counteracted by immune evasion mechanisms orchestrated by tumors on multiple levels. These may include recruitment of regulatory T cells (Treg), secretion of immunosuppressive mediators such as IL10, VEGF, and TGF β and redirecting T- and B-cell responses in lesions and the circulation (9–13). Through reeducating their environment, tumors may recruit immunosuppressive cells such as Tregs, alternatively activated (M2d) macrophages and myeloid-derived suppressor cells (MDSC) but also promote exhaustion, reduce antitumoral functions, and suppress maturation of important immune sentinels such as dendritic cells (DC), CTLs, and macrophages (14–16).

Various therapeutic strategies have been based on the premise that immune responses could be directed against melanoma to restrict tumor growth, if immune escape mechanisms can be counteracted or neutralized. Immunotherapy has made considerable advances in the past years with a diverse range of "immune potentiators" developed for therapy. The CTLA-4 and the programmed cell death 1 (PD-1) are transmembrane proteins on T cells that transduce inhibitory signals and reduce antigen-specific T-cell responses. The mAbs ipilimumab and nivolumab bind to CTLA-4 and PD-1, respectively, designed to reverse these checkpoint mechanisms in T cells (17). In a phase III trial, ipilimumab treatment at 3 mg/kg doses resulted in a median OS of 10 months, and of 10.1 months when given in combination with a gp100 peptide, whereas

the median OS for patients given gp100 treatment alone was 6.4 months (18). In a subsequent phase III trial, OS with high-dose ipilimumab (10 mg/kg) plus dacarbazine (11.2 months) was higher than dacarbazine treatment alone (9.1 months). High-dose (up to 10 mg/kg) treatments are reported to result in 4-year survival rates of 19.7% to 28.4% in previously treated patients, and 37.7% to 49.5% in treatment-naïve patients (19). Ipilimumab treatment 4us characterized by slow onset but durable response rates in a proportion of patients. Treatment is also associated with immune-related toxic side-effects arising from the universal activation of CTLA-expressing T cells irrespective of antigen specificity. These toxicities are observed in approximately 50% to 60% of patients and include mainly inflammatory skin and gastrointestinal colitis symptoms, which can be managed with corticosteroid treatment. Despite associated toxicities and long-term survival benefits in only subsets of patients, antibodies blocking negative immune signals via CTLA-4, PD-1, and other molecules (e.g., CD40 and CD137) have demonstrated that it is possible that clinical benefits could be harnessed with activation of immunity in the context of cancer. The emergence of such antibodies has reinvigorated interest in the translation of cancer immunotherapies to the clinic.

Constitutively Activated BRAF

Melanoma is one of the richest cancers with respect to mutations per mb of DNA and exhibits a versatile genetic profile across patients and anatomic locations of tumors (20). The B-raf gene is mutated in up to 66% of human malignant melanomas. Its protein product, the BRAF kinase, is a key player in the RAS–RAF–MEK–ERK proliferative pathway (Fig. 1A), which is widely dysregulated in various cancers, including melanoma (21, 22). B-raf-activating mutations are located in the kinase domain; this is also the case for the common amino acid substitution at position V600E, a valine (V) to a glutamic acid (E), the mutant form targeted by vemurafenib therapy (23, 24). B-raf-activating mutations may lead to a disrupted conformation of the kinase domain, which dramatically enhances BRAF activity and leads to constitutive ERK activation (25). This mechanism was proposed on the basis of X-ray crystal structure data of the wild-type and mutant (BRAF^{V600E}) forms of BRAF in their inactive

Figure 1. A, the RAS–RAF–MEK–ERK cellular signaling cascade couples extracellular signals to transcription factors, regulating gene expression. Extracellular signal molecules (i.e., growth factors) bind to their respective RTKs, which in turn recruit and activate the GTPase RAS. RAS phosphorylates and promotes the dimerization and activation of the RAF family (ARAF, BRAF, and CRAF) of protein kinases. Activated RAF is responsible for the subsequent signal transmission through MEK1/2 and ERK1/2 and the transcription of genes involved in cell-cycle regulation. Mutations in the B-raf gene cause disruptions in the kinase domain and constitutive activation of the BRAF kinase, promoting cell-cycle dysregulation, cell survival, and proliferation. Kinase inhibitors (vemurafenib and dabrafenib) specific for mutant BRAF result in high response rates but short overall median survival of patients with melanoma due to the emergence of resistance. MEK inhibitors (e.g., trametinib) constitute a strategy in battling BRAF inhibitor resistance. B, BRAF inhibitor treatment may condition tumor microenvironments in favor of immune activation. Top left, melanoma tumors may promote conditions contributing to ineffective antitumoral immunity, for example, infiltration of immunosuppressive cells (e.g., Treg and MDSC); immunosuppressive cytokines (IL6, IL10, and VEGF); reduced MHC class I and tumor-specific antigen expression; effector cell exhaustion (e.g., detected by expression of PD-1, CTLA-4 on immune cells and PD-L1 on tumor cells). Top right, BRAF inhibitors cause tumor cell death, reducing the associated immunosuppressive signals: favoring infiltration of T and NK cells, decreasing immunosuppressive cells and restoring tumor antigen expression and presentation via MHC class I. Bottom right, BRAF inhibitor treatment may condition tumor microenvironments in support of immunotherapy: maintain enhanced immune cell activation in adoptive T-cell therapy; counteract T-cell exhaustion signals with anti-PD-1, anti-PD-L1, anti-CTLA-4 blockers; maintain loss of immunosuppressive elements with anti-CTLA-4 blockade of Treg infiltrates.

conformations as part of the complex with a nonspecific *BRAF* inhibitor (sorafenib). A later study revealed the crystal structure of *BRAF*^{V600E} in a complex with a selective inhibitor in active conformation and suggested another model for constitutively activated *BRAF*^{V600E}. This model was based on a negatively charged glutamate at position 600, mimicking the conformation of the phosphorylated wild-type protein, which is necessary for kinase activation (26). This was thought to result in constitutively activated *BRAF* kinase, which is likely to promote RAS–RAF–MEK–ERK network-supported proliferation and tumor growth. This led to the concept that oncogenic mutations in the RAS–RAF–MEK–ERK pathway may provide therapeutic opportunities to target the mutant forms of molecules like *BRAF*^{V600E} in melanoma (22).

Treatments with Small Molecule Inhibitors to *BRAF*-Mutant Forms

One of the first *BRAF* inhibitors tested in clinical trials was sorafenib, a multikinase inhibitor, which does not distinguish between mutant and wild-type *BRAF*. Although combined sorafenib and DTIC treatment resulted in improved response rates and progression-free survival (PFS) in early trials, it failed to meet expectations in a phase III clinical trial when compared with standard chemotherapy (27, 28). After sorafenib, a new generation of *BRAF* inhibitors selective for mutant *BRAF* was designed. Vemurafenib, a V600E/K mutation-selective *BRAF* inhibitor (Fig. 1A), was approved by the FDA in 2011 and by the European Medicines Agency in 2012. In the pivotal phase III trial of vemurafenib compared with DTIC, vemurafenib increased the median OS from 9.6 months for DTIC-treated patients to 13.2 months, and was associated with a response rate of 48%, compared with 5% with standard chemotherapy (DTIC). However, PFS was only 5.3 months due to the appearance of drug resistance (29, 30). In an extended follow-up study, the agent was shown to improve survival for patients with the most common V600E as well as those with the less common V600K *BRAF*-mutant forms (31).

Resistance is thought to be attributed to a number of factors, including induction of alternative splice variants of *BRAF* or *de novo* mutations in *NRAS* or *MEK*. Upregulation of signaling through receptor tyrosine kinases (RTK) in alternative proliferative pathways such as the PI3K/AKT pathway is also thought to be associated with both innate as well as acquired resistance (32). In addition, even following therapy, constitutively active *BRAF* has been reported to still activate the MAPK pathway through dimerizing with *CRAF* (33–36). *BRAF* inhibition is also associated with dermatologic side effects such as skin photosensitivity, rashes, squamoproliferative lesions, including keratoacanthomas and squamous cell carcinomas (SCC) or, more rarely, *de novo* primary melanomas and secondary melanomas. Cases of *NRAS* leukemia and *KRAS*-mutant colorectal cancer have also been reported (35). These paradox-

ical oncogenic effects of *BRAF* inhibitor treatment, often manageable by careful monitoring, are thought to arise from inhibitor recognition of wild-type *BRAF*. This may lead to *BRAF*–*CRAF* dimerization along with enhanced RAS, resulting in MAPK pathway activation. In cells with either mutant RAS acquired from external stimuli such as UV exposure or when wild-type RAS is activated through external growth factor signals, *BRAF* inhibition may also support proliferation and migratory properties through focal adhesion kinase (FAK/ERK) signaling (37).

Dabrafenib, a small molecule *BRAF*^{V600E} kinase inhibitor approved by the FDA in 2013, also acts similarly to vemurafenib (4), but has a different side-effect profile, in particular reduced phototoxicity (38). As is the case with vemurafenib, however, most patients go on to develop resistance to dabrafenib (median PFS—5.1 months). Trametinib is an MEK1/2 kinase inhibitor that functions downstream of *BRAF* in the same pathway, triggering G₁ cell-cycle arrest, apoptosis, and reduced cell proliferation (39). In a phase III clinical trial, the agent demonstrated favorable PFS (4.8 months) compared with dacarbazine (1.5 months) and OS rates of 81% compared with 67% with dacarbazine (40).

BRAF inhibitors, thus, seem to induce significant but short-term clinical responses. Clinical trials testing alternative *BRAF* inhibitors are also underway (Table 1), and further in-depth analyses of resistance mechanism pathways and strategies to counteract these are needed.

Mutant *BRAF* and Immune Responses

Evidence for immunogenicity of the mutant *BRAF* protein in melanoma

A number of studies suggest that mutant forms of *BRAF* may be recognized by host immunity and could be involved in antitumor immune responses. *Ex vivo* stimulation of lymphocytes derived from patients with melanoma with a synthetic *BRAF* peptide carrying the V600E mutation led to the generation of MHC class II–restricted CD4⁺ T cells specific for this peptide; these cells recognized tumor cells expressing mutant *BRAF* (41). Andersen and colleagues (42) also reported the presence of HLA-B*2705–restricted CTL responses in the blood of patients with melanoma against a synthetic mutant but not against the wild-type *BRAF*. Another study reported that stimulation with *BRAF* peptides carrying the V600E mutation *in vitro* induced HLA-A*0201–restricted proliferation of T cells derived from patients with *BRAF*^{V600E}-positive melanomas, these sensitized T cells were cytotoxic against *BRAF*^{V600E}/HLA-A*0201–positive melanoma cells. Furthermore, HLA-A*0201–restricted *BRAF*^{V600E} peptides stimulated proliferation of T cells from HLA-A2–positive patients with *BRAF*^{V600E}-positive melanoma and cytotoxicity against *BRAF*^{V600E}-positive melanoma cells. T cells from healthy controls and patients with *BRAF*^{V600E}-negative lesions did not respond to mutated epitope challenge (43). As the HLA-A*0201 haplotype is present in 50% of patients with melanoma, vaccination studies aimed at activating immunity against the mutant

Table 1. Registered clinical trials in the United States and Europe for *BRAF* inhibitors alone or in combination with alternative kinase inhibitors (selected from the following sources: <http://www.clinicaltrials.gov>; <http://public.ukcrn.org.uk>; <https://www.clinicaltrialsregister.eu>)

Category	Drug/intervention	Drug type	Sequence of drug administration	Stage/cancer type	Identifier	Phase	
<i>BRAF</i> inhibitor only	RO5212054 (PLX3603)	<i>BRAF</i> V600 kinase inhibitor	N/A ^a	Advanced solid tumors	NCT01143753 (US)	I	
	LGX818	<i>BRAF</i> V600 kinase inhibitor	N/A ^a	Locally advanced or metastatic melanoma	NCT01436656 (US)	I	
	CEP-32496	<i>BRAF</i> V600E kinase inhibitor	N/A ^a	Advanced solid tumors (phase I) advanced melanoma and metastatic colorectal cancer (phase II)	NCT01877811 (US)	I/II	
	RAF265 (CHIR-265)	<i>BRAF</i> and VEGFR-2 inhibitor	N/A ^a	Locally advanced or metastatic melanoma	NCT00304525 (US)	I/II	
	LGX818	<i>BRAF</i> V600 kinase inhibitor	N/A ^a	Stage IV or unresectable stage III melanoma	NCT01894672 (US)	II	
	GSK2118436 (dabrafenib)	<i>BRAF</i> V600E/K kinase inhibitor	N/A ^a	Metastatic melanoma to the brain	NCT01266967 (US)	II	
	Vemurafenib	<i>BRAF</i> V600 kinase inhibitor	N/A ^a	Metastatic melanoma to the brain	NCT01378975 (US)	II	
	Vemurafenib	<i>BRAF</i> V600 kinase inhibitor	N/A ^a	Surgically incurable and unresectable stage IIIC or stage IV <i>BRAF</i> V600 mutation-positive melanoma	NCRN324 BRIM-P (UK) (Pediatric patients) (US)	I	
	GSK2118436 (dabrafenib)	<i>BRAF</i> V600E/K kinase inhibitor	N/A ^a	Previously treated metastatic (Stage IV) <i>BRAF</i> V600E/K mutation-positive cutaneous melanoma	2009-015297-36 (EU)	II	
	Vemurafenib	<i>BRAF</i> V600 kinase inhibitor	N/A ^a	High-risk <i>BRAF</i> V600 mutation-positive cutaneous melanoma (Stage IIC or III) after surgical resection	NCRN442 BRIM 8 (UK)	III	
	Vemurafenib	<i>BRAF</i> V600 kinase inhibitor	N/A ^a	<i>BRAF</i> V600 mutation-positive unresectable or metastatic melanoma	NCRN530 ZeSS (UK)	IV	
	<i>BRAF</i> inhibitor + alternative kinase inhibitor	XL281 (1) ± famotidine (2)	Multiple <i>RAF</i> kinase inhibitor (1) H2 receptor antagonist (2)	XL281 administered once daily, famotidine administered concomitantly in a single dose during weeks 2, 3, and 4 of first cycle of trial	Non-small cell lung cancer, colorectal cancer, papillary thyroid cancer, and melanoma	NCT00451880 (US)	I
		Vemurafenib (1) + GDC-0973 (cobimetinib) (2)	<i>BRAF</i> V600 kinase inhibitor (1) MEK1 inhibitor (2)	Vemurafenib—oral repeated dose; GDC-0973—oral repeated dose	Locally advanced/unresectable or metastatic melanoma	NCT01271803 (US)	I
PLX3397 (1) + vemurafenib (2)		RTK inhibitor of KIT, CSF1R and FLT3 (1) <i>BRAF</i> V600 inhibitor (2)	PLX3397 administered once daily; vemurafenib administered twice daily	Unresectable or metastatic melanoma	NCT01826448 (US)	I	
PX-866 (1) + vemurafenib (2)		PI-3K inhibitor (1) <i>BRAF</i> V600 kinase inhibitor (2)	PX-866 and vemurafenib coadministered daily in 28-day cycles	<i>BRAF</i> -mutant cancers, including advanced melanoma	NCT01616199 (US)	I/II	
Vemurafenib (1) + P1446A-05 (2)		<i>BRAF</i> V600 kinase inhibitor (1) cyclin-dependent kinase 4 (CDK4) inhibitor (2)	Vemurafenib twice daily; coadministered with P1446A-05 once daily	Advanced or inoperable malignant melanoma	NCT01841463 (US)	I/II	

(Continued on the following page)

Table 1. Registered clinical trials in the United States and Europe for *BRAF* inhibitors alone or in combination with alternative kinase inhibitors (selected from the following sources: <http://www.clinicaltrials.gov>; <http://public.ukcrn.org.uk>; <https://www.clinicaltrialsregister.eu>) (Cont'd)

Category	Drug/intervention	Drug type	Sequence of drug administration	Stage/cancer type	Identifier	Phase
	GSK2141795 (1) + dabrafenib (2)	Akt inhibitor (1) <i>BRAF</i> V600E/K kinase inhibitor (2)	GSK2141795 once daily coadministered with dabrafenib twice daily on days 1–28	<i>BRAF</i> -mutant cancer, including recurrent, stage IIIc and stage IV melanoma	NCT01902173 (US)	I/II
	LEE011 (1) + LGX818 (2)	CDK 4/6 inhibitor (1) <i>BRAF</i> V600 kinase inhibitor (2)	LEE011 administered once daily for 21 consecutive days followed by a 7-day break (28-day cycle); LGX818 administered once daily on a continuous dosing schedule (28-day cycle)	Locally advanced or metastatic melanoma	NCT01777776 (US)	I/II
	Dabrafenib (1) + trametinib (2)	<i>BRAF</i> V600E/K kinase inhibitor (1) MEK I/II inhibitor (2)	Dabrafenib administered twice daily on days 1–28; Trametinib added on days 15–28, followed by surgery on days 28–30	Presurgical model of advanced, operable melanoma	NCT01701037 (US)	II
	Dabrafenib (1) + trametinib (2)	<i>BRAF</i> V600E/K kinase inhibitor (1) MEK I/II inhibitor (2)	Dabrafenib administered twice daily; trametinib administered once daily	Metastatic melanoma that is refractory or resistant to <i>BRAF</i> inhibitor	NCT01619774 (US)	II
	Dabrafenib (1) + trametinib (2)	<i>BRAF</i> V600E/K kinase inhibitor (1) MEK I/II inhibitor (2)	Dabrafenib administered twice daily; trametinib administered once daily	Metastatic melanoma that is refractory or resistant to <i>BRAF</i> inhibitor	NCT01619774	II
	Dabrafenib (1) + trametinib (2)	<i>BRAF</i> V600E/K kinase inhibitor (1) MEK I/II inhibitor (2)	Dabrafenib administered twice daily; trametinib administered once daily; repeated in 3-week cycles	Unresectable stage III and stage IV melanoma	NCT01726738 (US)	II
	Dabrafenib (1) + trametinib (2)	<i>BRAF</i> V600E/K kinase inhibitor (1) MEK I/II inhibitor (2)	Single dose of dabrafenib alone on day 1; Continuous repeat doses of trametinib on days 2–15; second single dose of dabrafenib administered concomitantly with trametinib on day 15; no medication administered on days 16–28 (washout period)	Metastatic melanoma	NCT01072175 (US)	II
	LGX818 (1) + MEK162 (2) vs. LGX818 (1) + LEE011 (3) vs. LGX818 (1) + BGJ398 (4) vs. LGX818 (1) + BKM120 (5) vs. LGX818 (1) + INC280 (6)	<i>BRAF</i> V600 kinase inhibitor (1) MEK1/2 inhibitor (2) CDK 4/6 inhibitor (3) FGFR inhibitor (4) PI3K inhibitor (5) c-Met inhibitor (6)	Single agent treatment with LGX818 followed by "rational combination" with other agents following disease progression on LGX818 alone	Locally advanced or metastatic melanoma	NCT01820364 (US)	II
	Dabrafenib (1) + trametinib (2)	<i>BRAF</i> V600E/K kinase inhibitor (1) MEK I/II inhibitor (2)	Dabrafenib administered twice daily; trametinib administered once daily for 12 months	High-risk melanoma after surgical resection	NCT01682083 (US)	III
	Dabrafenib (1) + trametinib (2) vs. dabrafenib (3) monotherapy	<i>BRAF</i> V600E/K kinase inhibitor (1) MEK I/II inhibitor (2) <i>BRAF</i> V600E/K kinase inhibitor (3)	Dabrafenib administered twice daily; trametinib administered once daily	Unresectable (stage IIIc) or metastatic (stage IV) melanoma	NCT01584648 (US)	III

(Continued on the following page)

Table 1. Registered clinical trials in the United States and Europe for *BRAF* inhibitors alone or in combination with alternative kinase inhibitors (selected from the following sources: <http://www.clinicaltrials.gov>; <http://public.ukcrn.org.uk>; <https://www.clinicaltrialsregister.eu>) (Cont'd)

Category	Drug/intervention	Drug type	Sequence of drug administration	Stage/cancer type	Identifier	Phase
	LGX818 (1) + MEK162 (2) vs. LGX818 monotherapy vs. vemurafenib (3) monotherapy	<i>BRAF</i> V600 kinase inhibitor (1) MEK1/2 inhibitor <i>BRAF</i> V600 kinase inhibitor (3)	LGX818 administered once daily; MEK162 administered twice daily	Unresectable or metastatic melanoma	NCT01909453 (US)	III
	Dabrafenib (1) + trametinib (2) vs. vemurafenib (3) monotherapy	<i>BRAF</i> V600E/K kinase inhibitor (1) MEK I/II inhibitor (2) <i>BRAF</i> V600 kinase inhibitor (3)	Dabrafenib administered twice daily; Trametinib administered once daily	Unresectable (stage IIIC) or metastatic (Stage IV) melanoma	NCT01597908 (US)	III
	Vemurafenib (1) + GDC-0973 (cobimetinib) (2) vs. vemurafenib monotherapy	<i>BRAF</i> V600 kinase inhibitor (1) MEK1 inhibitor (2)	Vemurafenib administered twice daily on days 1–28 of each 28-day cycle; GDC-0973 administered once daily on days 1–21 of each 28-day cycle	Unresectable locally advanced or metastatic melanoma	NCT01689519 (US)	III
	GSK2118436 (dabrafenib) (1) + GSK1120212 (trametinib) (2) vs. GSK2118436 (dabrafenib) + placebo	<i>BRAF</i> V600E/K kinase inhibitor (1) MEK I/II inhibitor (2)	N/S ^b	Previously treated advanced, unresectable (stage IIIC) or metastatic (stage IV) BRAF V600E/K mutation-positive cutaneous melanoma	2012-005569-10 (EU)	II
	LGX818 (1) + MEK162 (2) vs. LGX818 (1) + LEE011 (3) vs. LGX818 (1) + BGJ398 (4) vs. LGX818 (1) + BKM120 (5) vs. LGX818 (1) + INC280 (6)	<i>BRAF</i> V600 kinase inhibitor (1) MEK1/2 inhibitor (2) CDK 4/6 inhibitor (3) FGFR inhibitor (4) PI-3K inhibitor (5) c-Met inhibitor (6)	N/S ^b	Locally advanced or metastatic melanoma	2012-004798-17 (EU); NCT01820364 (USA)	II
	Dabrafenib (1) + trametinib (2) vs. vemurafenib (3) monotherapy	<i>BRAF</i> V600E/K kinase inhibitor (1) MEK I/II inhibitor (2) <i>BRAF</i> V600 kinase inhibitor (3)	N/S ^b	Unresectable (stage IIIC) or metastatic (stage IV) BRAF V600E/K mutation-positive cutaneous melanoma	NCRN423 COMBI-V (UK - Closed – in follow-up); 2011-006088-23 (EU - Ongoing)	III
	Dabrafenib (1) + trametinib (2) vs. placebo	<i>BRAF</i> V600E/K kinase inhibitor (1) MEK I/II inhibitor (2)	N/S ^b	High-risk BRAF V600E/K mutation-positive cutaneous melanoma after surgical resection	NCRN427 COMBI-AD (UK - Ongoing); 2012-001266-15 (EU - Ongoing)	III
	Vemurafenib (1) + GDC-0973 (cobimetinib) (2) vs. vemurafenib monotherapy	<i>BRAF</i> V600 kinase inhibitor (1) MEK1 inhibitor (2)	N/S ^b	Previously untreated BRAFV600 mutation-positive, unresectable locally advanced (stage IIIC) or metastatic (stage IV) melanoma	NCRN510 CO-BRIM (UK)	III
	GSK2118436 (dabrafenib) (1) + GSK1120212 (trametinib) (2) vs. GSK2118436 (dabrafenib) monotherapy	<i>BRAF</i> V600E/K kinase inhibitor (1) MEK I/II inhibitor (2)	N/S ^b	Unresectable (stage IIIC) or metastatic (stage IV) BRAF V600E/K mutation-positive cutaneous melanoma	NCRN286 COMBI-d (UK-Closed – in follow-up); 2011-006087-49 (EU -Ongoing)	III
	LGX818 (1) + MEK162 (2) vs. LGX818 monotherapy vs. vemurafenib (3) monotherapy	<i>BRAF</i> V600 kinase inhibitor (1) MEK1/2 inhibitor <i>BRAF</i> V600 kinase inhibitor (3)	N/S ^b	Locally advanced, unresectable or metastatic BRAF V600-mutant cutaneous melanoma	2013-001176-38 (EU)	III

NOTE: Trials are ongoing unless otherwise stated.
^aN/A, not applicable.
^bN/S, not stated.

$BRAF^{V600E}$ form in this patient group were suggested. In concordance with these findings, blocking of the $BRAF$ -MAPK pathway in $BRAF$ signaling-addicted melanoma cells *in vitro* triggered enhanced recognition of tumor cell antigens by tumor-infiltrating T lymphocytes and the authors suggested that $BRAF$ blockade and adoptive T-cell therapies may confer synergistic effects (44).

Taken together, these studies suggest that immune responses against the mutant form of $BRAF$ may be present or harnessed in melanoma and that the selective immunogenicity of the mutant forms may provide the basis for the development of new strategies to overcome immunologic tolerance.

$BRAF$ -mutant forms and immune escape mechanisms

In addition to immunogenic responses against mutated $BRAF$, a number of studies suggest that the mutant $BRAF$ protein kinase may conversely be associated with tumor-induced immune escape mechanisms. Increased expression of immunosuppressive mediators (IL6, IL10, and VEGF) by $BRAF^{V600E}$ melanoma cells has been reported. These cytokines can promote recruitment of inflammatory cell subsets such as MDSCs and Tregs in tumor micro-environments. These effects were reduced through targeted mutant $BRAF$ protein inhibition or through RNAi strategies, implying that eliminating mutant $BRAF$ -expressing tumor cells could result in some control of tumor-associated immunosuppressive signals (45). In addition, it was demonstrated that under these conditions, $BRAF^{V600E}$ melanoma cells could impair maturation of DC, and suppress their capacity to produce IL12 and TNF α (45). Another study reported increased transcription of IL1 (IL1 α and IL1 β) by melanoma cell lines transduced to express $BRAF^{V600E}$. IL1 α/β -stimulated tumor-associated fibroblasts could suppress melanoma-specific CTL functions by upregulation of the checkpoint ligand molecules COX-2, PD-L1, and PD-L2. IL1 protein overexpression was reversed by targeted $BRAF^{V600E}$ inhibition both in cell lines and in tumors from patients treated with the $BRAF^{V600E}$ inhibitor vemurafenib (46). Although IL1 cytokines play immunostimulatory as well as immunosuppressive functions in different contexts, these findings suggest that $BRAF$ inhibition could influence these immune signals in tumors. Another immunosuppressive effect of mutant $BRAF$ may relate to the downregulation of MHC class I molecules by melanoma cells. MHC class I expression is reduced in A375 melanoma cells overexpressing mutant $BRAF^{V600E}$, and MHC class I and II expression triggered by IFN γ and IFN α 2b can be enhanced after $BRAF$ inhibition with vemurafenib only in homozygous, but not in heterozygous, $BRAF^{V600E}$ -mutant cells (47).

Despite evidence to support that immune responses against mutant $BRAF$ could be triggered under certain contexts, associations of $BRAF^{V600E}$ -expressing melanoma cells with immunosuppressive signals indicate that melanomas may use adaptive mechanisms to avoid immune clearance (Fig. 1B).

Could $BRAF$ inhibitors be toxic to host immunity?

An important question relates to the potential of $BRAF$ inhibitors to exert toxic effects on immune cells. Several studies have indicated that $BRAF$ inhibitors do not appear to have direct adverse effects on lymphocytes. Comin-Anduix and colleagues (48) reported that clinical concentrations of the $BRAF^{V600E}$ inhibitor vemurafenib, which are cytotoxic to melanoma cells, do not affect the viability or function of lymphocytes from healthy donors or from patients with melanoma. Importantly, there was a significant gap between the therapeutic concentrations of the inhibitor and the concentrations needed to observe any toxic effects of the agent on lymphocytes. In addition, treatment of different melanoma cells lines and primary melanoma tumor digests with a selective $BRAF^{V600E}$ inhibitor resulted in enhanced expression of melanocyte differentiation antigens, important for immune recognition (e.g., gp100 and MART-1), while not adversely affecting melanoma patient T-cell function (48–50). Another study confirmed that a $BRAF$ inhibitor selective for $BRAF^{V600E}$, $BRAF^{V600K}$, and $BRAF^{V600G}$ did not affect cancer patients' immunity in relation to a number of clinical parameters, such as serum cytokine levels, peripheral blood mononuclear cell counts and frequencies of different leukocyte subsets (B cells, T cells, NK cells, monocytes, DCs, and Tregs; refs. 51). A subsequent study reported no adverse effects of vemurafenib on cytokine production by CD4⁺ and CD8⁺ T cells in a $BRAF$ wild-type ($BRAF^{WT}$) mouse model, and no deleterious effects on T-cell-mediated antitumoral functions.

However, despite significant evidence to suggest that $BRAF$ inhibitors do not have a deleterious effect on host immune cells, Hooijkaas and colleagues (52) reported some adverse effects of vemurafenib on the immune response in $BRAF^{V600E}$ -positive melanoma mouse models. Treatment was associated with significant decreases in tumor-infiltrating T cells, natural killer (NK) cells, monocytes, and MDSCs, and infiltration of these cells could not be rescued by addition of an anti-CTLA-4 antibody. The same study reported that combination treatments with anti-CTLA-4 antibodies did not confer additional tumor restriction above those observed with vemurafenib alone. The findings may be interpreted as a negative impact of vemurafenib on immune responses in the local tumor microenvironment. An alternative explanation may be that vemurafenib-induced tumor cell death leads to reduced immunosuppressive cytokine production and reduced numbers of immunoregulatory cell infiltrates such as Treg or MDSCs. The latter may explain the absence of additive tumor-restrictive effects with anti-CTLA-4 antibody treatment, which is thought to also function by targeting immune effector cell responses against CTLA-4-expressing tumor-associated Treg (53, 54).

$BRAF$ inhibitor influence on host immune responses

Although $BRAF$ inhibitor drugs are not designed to directly activate antitumor immune responses, there is increasing evidence to indicate enhanced antitumor

immunity with use of these agents and correlation with clinical responses. Wilmott and colleagues (55) demonstrate increased CD4⁺ and CD8⁺ T-cell infiltration in melanoma patient biopsies from patients in the early stages of treatment with vemurafenib and dabrafenib. Immune cell infiltrate rates in biopsies from patients who relapsed following treatment resembled those observed in pretreatment samples, and correlated with the appearance of resistance against *BRAF* inhibitor treatment. These observations imply that *BRAF* inhibitors appear to reverse some tumor-associated immunosuppressive signals and that the immunostimulatory effects observed with response to treatments subside with disease progression. Another study also revealed increases in the frequency of tumor antigen-specific CD8⁺ T cells and modest increases in circulating levels of TNF α associated with *BRAF*^{V600} inhibitors (51). Furthermore, *BRAF*^{V600E} inhibition has been reported to restore maturation of DC and production of TNF α and IL12 without any adverse effects on DC viability or capacity to prime T cells (45, 56). *BRAF* inhibitor treatment of patients with metastatic melanoma resulted in reduced generation and differentiation of MDSC, known to be promoted by tumor-induced expression of immunosuppressive cytokines such as IL6 (57).

In a *BRAF*^{V600E}/Pten-inducible mouse model of melanoma, the immunosuppressive effects of *BRAF*^{V600E} melanoma tumors were manifested through accumulation of immunoregulatory cell subsets such as FoxP3⁺ Tregs and CD11b⁺/Gr-1⁺ MDSCs, reduced frequencies of CD4 T cells producing IFN γ , TNF α , and IL2 and lower expression of the costimulatory ligand CD40L (58). Compromised CD40L-CD40 signaling was linked to loss of maturation signals necessary for antigen presentation, resulting in concentration of immature DCs, and of macrophages featuring alternatively activated M2 phenotypes in tumors. *BRAF*^{V600E} inhibitor treatment reduced tumor growth and increased CD8 and CD4 T-cell infiltration. Importantly, enhanced expression of CD40L and production of IFN γ by CD4 T cells along with reduced frequencies of FoxP3⁺ Tregs were observed and specific blocking of IFN γ and CD40L signaling were individually shown to impede *BRAF*^{V600E} inhibitor effects. These findings support key contributions of host immunity to *BRAF* inhibitor functions. In a mouse model of *BRAF*^{V600E} melanoma, Vemurafenib treatment decreased expression of the regulatory chemokine (C-C motif) ligand 2 (CCL2) by melanoma cells and this was associated with tumor growth reduction. Reduced tumor growth was associated with higher NK cell infiltrates and increases in the CD8⁺ T cell to Treg ratios in tumors (59). In the same study, it was shown that combining *BRAF*^{V600E}-targeted therapy with antibodies to immune modulatory molecules such as CD137 and CCL2 with could confer additional benefits in restricting tumor growth and in suppressing *de novo* tumorigenesis. These findings further support the notion that activating immunity alongside pathway inhibition could be beneficial (Fig. 1B).

In summary, some studies suggest that *BRAF*-MAPK pathway inhibition may not have a negative impact on the immune system of patients with cancer, but may influence host immune responses systemically and in tumor microenvironments in multiple ways, counteracting immunosuppressive pathways and often favoring immune activation (Fig. 1B). These effects may be directly attributed to reduced tumor cell viability and consequently to reduced tumor-induced immunomodulation. The subsequent appearance of resistance to *BRAF* inhibitors and restoration of tumor growth and the tumor-induced immunosuppressive equilibrium at the time of disease progression support a link between mutant *BRAF* dysregulation and alterations in immune signals in cancer (46, 47).

Combination Treatments: Possible Synergistic Benefits with Enhancing Immunity?

Pathway inhibitor combinatory approaches

It has been suggested that combination therapies with *BRAF* and alternative inhibitors of the MAPK pathway might be a strategy to overcome resistance and to prolong patient PFS, with the aim of improving the short-lived clinical benefits of *BRAF*-targeted monotherapies (35). *BRAF* inhibitor-resistant melanomas have been reported to feature elevated expression of the checkpoint molecule ligand PD-L1, which could be reduced with subsequent treatment with MEK and PI3K inhibitors (60). These findings may indicate additive effects of combined pathway inhibitors on known checkpoint immune modulatory mechanisms. Results of an open-label combination phase I/II clinical trial between the newly FDA-approved *BRAF*^{V600E/K} inhibitor dabrafenib and the MEK1/2 inhibitor trametinib provided some optimism with a nonsignificantly reduced frequency of cutaneous SCCs detected, possibly due to reduction of MAPK signaling, which may counteract the effects of *BRAF* inhibitors on wild-type cells. The study showed significantly increased median PFS and response rates compared with those with single agents (61); however, clinical efficacy for combination treatments remains to be determined. A potential future direction may entail the use of inhibitors to RAF and ERK alongside *BRAF* blockade, which may also prevent the paradoxical activation of the MAPK pathway in wild-type cells. Immune response activation may also be enhanced in these combinatory strategies, as hallmarks of immunosuppression associated with resistance to *BRAF*-targeted therapy characterized by lower CD8⁺ T cell infiltration and reduced tumor antigen expression at the time of progression were reversed with *BRAF* and MEK combination therapies (62). Tumor biopsies from patients with metastatic melanoma treated with either a *BRAF* inhibitor (vemurafenib) alone or *BRAF* plus MEK inhibitor combinations (dabrafenib + trametinib) were taken before and during therapy and at the point of relapse. *BRAF* alone and *BRAF* plus MEK combination treatments were associated with increased tumor antigen expression, CD8⁺

T-cell infiltrates, and reduced inflammatory cytokine levels (IL6 and IL8). In biopsies from patients treated with *BRAF* inhibitor treatment alone, upregulation of the exhaustion markers PD-1 and TIM-3 in T cells and of PD-L1 (PD-1 ligand) on melanoma cells (62) might help explain subsequent loss of immune activatory signals and reversal toward immune suppression in *BRAF* inhibitor-resistant disease. Clinical trials of combination therapies with a *BRAF* inhibitors and different MEK, Cdk, PI3K, and Akt inhibitors may provide novel clinical and mechanistic insights in future (Table 1).

Pathway inhibitors and immunotherapy: aiming to overcome immune suppression

BRAF inhibitors may initially condition the tumor microenvironment to favor immune activation, perhaps rendering these agents synergistic partners to strategies specifically targeting the immune response. The proposition that the clinical efficacy observed with small molecule inhibitors might be enhanced if combined with immunotherapies is currently under investigation in a number of preclinical and clinical settings (Fig. 1B; Table 2).

Preclinical studies showed that *BRAF* inhibitors may enhance the potency of adoptive immune cells by promoting tumor antigen expression, antigen recognition, and T-cell infiltration in tumors (44). In a mouse model of melanoma, vemurafenib in combination with adoptive lymphocyte transfer therapy resulted in enhanced tumor cytotoxicity and cytokine secretion by tumor-infiltrating adoptively transferred T cells (63). In another *in vivo* model, vemurafenib treatment resulted in increased T-cell infiltration into tumors and was attributed to loss of VEGF expression by destruction of melanoma cells. In this study, the findings were consistent with reduced VEGF expression in biopsy samples of patients treated with *BRAF*^{V600E} inhibitor therapy (64). These findings provide rationale for synergistic effects when combining *BRAF* inhibition and T-cell immune activatory therapy strategies and inspired early clinical trials for this concept.

Another approach would be to combine the increased intratumoral immune activity following *BRAF* inhibitor administration with the functions of checkpoint blockade inhibitor antibodies to overcome T-cell exhaustion (65). However, a phase I clinical trial featuring concurrent treatment with ipilimumab (anti-CTLA-4 antibody) and vemurafenib (*BRAF*^{V600E/K} inhibitor) reported asymptomatic hepatotoxicity effects reversible upon discontinuation of the drug combinations or with glucocorticoid treatment, resulting in termination of the study (66). Phase I clinical trials with the *BRAF*^{V600E/K} inhibitor dabrafenib with or without trametinib (MEK1/2 inhibitor) in combination with ipilimumab are on-going (Table 2). Other suggested treatment partners of *BRAF* inhibitors include IFN α 2b, IL2, antibodies to PD-L1, CD137, and IL1 blockers, which may act as adjuvants (47, 59). Clinical trials to test different combinations of vemurafenib with IL2 or IFN α 2b are on-going and are expected to shed light on the merits of future combination therapies (Table 2). Howev-

er, because IFN α 2b or IL2 monotherapies are associated with high reported toxicities, a cautious approach to such combinatory strategies would be paramount.

Translational Considerations and the Future of Combination Treatments

A number of factors, relating to efficacy and safety, may be taken into account when considering implementation of combination strategies; these may include dose, timing, and sequence of administration. For these there is currently little precedence, but perhaps understanding and taking into account host immune responses may be important in this context.

It has been proposed that patients with mutant *BRAF*-expressing tumors with highly symptomatic disease, in particular those with acutely life-threatening metastases such as brain metastases, should be prescribed, a *BRAF* inhibitor treatment based on capacity to trigger quick clinical responses. Considering the quick impact on tumor growth restriction and the positive overall effect of *BRAF* inhibitors on immunity, treatment with a *BRAF* inhibitor might perhaps be followed by immunotherapy such as a checkpoint blocker to neutralize T-cell inhibitory signals (67). On the other hand, a case for immunotherapy preceding *BRAF* inhibitor therapy may be supported by early reports that significant proportions (around half) of patients who do not respond to *BRAF* inhibitor treatment generally have a worse clinical outlook compared with untreated patients. It is, therefore, worth exploring whether identifying and treating these patients with immunotherapy such as with ipilimumab as early as possible may be beneficial (68, 69). It may also be reasonable for asymptomatic patients to be treated with immunotherapy, because of the potential to confer long-term, durable responses. The same patients may then receive *BRAF* inhibitor treatment as a salvage therapy on disease progression (70). This, however, may not be widely practical, because in some countries, including the United Kingdom, ipilimumab is not yet routinely funded for first-line therapy for metastatic melanoma; therefore, patients with early disease may not be able to access treatment.

In future, the clinical landscape may feature pathway inhibitor simultaneous combinations with immunotherapies, including checkpoint blockade agents. Treatment combinations may be informed by elucidating tumor escape mechanisms associated with pathway inhibitors and by designing complementary immune intervention strategies to overcome these. With increasing numbers of patients now treated with pathway inhibitor drugs, the critical mechanisms of immune response cross-talk with *BRAF*-mutant melanomas and *BRAF* inhibitors and how these are linked to the almost inevitable rise of resistant disease require further in-depth investigation. Under the selective pressure of human immune responses and pathway blockade interventions, tumors may evolve to activate alternative downstream interacting signaling pathways and can escape destruction through clonal selection.

Table 2. Registered clinical trials in the United States and Europe for *BRAF* inhibitors in combination with immunotherapies with or without different kinase inhibitors and/or chemotherapeutic agents (selected from the following sources: <http://www.clinicaltrials.gov>; <http://public.ukcrn.org.uk>; www.clinicaltrialsregister.eu)

Drug/intervention	Drug type	Sequence of drug administration	Stage/cancer type	Identifier	Phase
Ipilimumab (1) ± dabrafenib (2) ± trametinib (3)	Anti-CTLA-4 mAb (1) <i>BRAF</i> V600E/K kinase inhibitor (2) MEK I/II kinase inhibitor (3)	Oral dabrafenib twice daily for 25 days; ± oral trametinib once daily for 25 days; ± i.v. ipilimumab repeated every 3 weeks for four courses	Unresectable or metastatic melanoma	NCT01940809 (US)	I
Vemurafenib (1) + young TILs ^a (2) + cyclophosphamide (3) + fludarabine (4) + aldesleukin (5)	<i>BRAF</i> V600 kinase inhibitor (1) TILs (2) chemotherapeutic agent (3) chemotherapeutic agent (4) IL2 (5)	Once cryopreserved, autologous TIL ^a available, patients commence oral vemurafenib administered twice daily; i.v. cyclophosphamide on days 7 and 6; i.v. fludarabine on days 5 through 1; infusion of 1×10^9 - 2×10^{11} young TIL ^a on day 0; followed by i.v. infusion of high-dose aldesleukin	Metastatic melanoma	NCT01585415 (US)	I
Dabrafenib (1) ± trametinib (2) + ipilimumab (3)	<i>BRAF</i> V600E/K kinase inhibitor (1) MEK I/II kinase inhibitor (2) anti-CTLA-4 mAb (3)	Oral dabrafenib administered twice daily; ± oral trametinib administered once daily; i.v. ipilimumab repeated every 3 weeks for four courses	Unresectable or metastatic melanoma	NCT01767454 (US)	I
Ipilimumab (1) + dabrafenib (2) + trametinib (3) vs. Ipilimumab (1) + trametinib (3) vs. Ipilimumab (1) + dabrafenib (2) vs. Ipilimumab (1) monotherapy	Anti-CTLA-4 mAb (1) <i>BRAF</i> V600E/K kinase inhibitor (2) MEK I/II inhibitor (3)	Oral dabrafenib twice daily for 25 days; oral trametinib once daily for 25 days; followed by i.v. ipilimumab repeated every 3 weeks for four courses	Unresectable or metastatic melanoma	NCT01938703 (US)	I
Vemurafenib (1) + IL2 + INFα2b	<i>BRAF</i> V600 kinase inhibitor (1)	Oral vemurafenib administered twice daily for a 21-day cycle; i.v. IL2 administered on days 2-5 of a 21-day cycle; subcutaneous INFα2b administered on days 1-5 of a 21-day cycle	Metastatic melanoma	NCT01603212 (US)	I/II
Ipilimumab (1) + vemurafenib (2)	Anti-CTLA-4 mAb (1) <i>BRAF</i> V600 kinase inhibitor (2)	Oral vemurafenib administered twice daily; i.v. ipilimumab repeated every 3 weeks	Metastatic melanoma	NCT01400451 (US)	I/II

(Continued on the following page)

Table 2. Registered clinical trials in the United States and Europe for *BRAF* inhibitors in combination with immunotherapies with or without different kinase inhibitors and/or chemotherapeutic agents (selected from the following sources: <http://www.clinicaltrials.gov>; <http://public.ukcrn.org.uk>; www.clinicaltrialsregister.eu) (Cont'd)

Drug/intervention	Drug type	Sequence of drug administration	Stage/cancer type	Identifier	Phase
Lymphodepletion using fludarabine (1) and cyclophosphamide (2) ACT ^b with TIL ^a Infusion + Vemurafenib (3) HD ^c IL2 (aldesleukin)	Chemotherapeutic agent (1) chemotherapeutic agent (2) <i>BRAF</i> V600 kinase inhibitor (3)	Combination of vemurafenib followed by lymphodepletion with fludarabine and cyclophosphamide; ACT ^b with TIL ^a infusion followed by high-dose IL2	Metastatic melanoma	NCT01659151 (US)	II
Vemurafenib (1) + aldesleukin (2)	<i>BRAF</i> V600 kinase inhibitor (1) IL2 (2)	Oral vemurafenib administered twice daily; i.v. infusion of aldesleukin administered as per the study protocol	Metastatic or unresectable melanoma	NCT01754376 (US)	II
HD ^c IL2 + vemurafenib (1)	<i>BRAF</i> V600 kinase inhibitor (1)	Initial course of vemurafenib followed by high-dose IL2 (patients discontinue vemurafenib before treatment with IL2 and resume dosing afterward)	Metastatic melanoma	NCT01683188 (US)	IV
Vemurafenib (1) + pegylated INF α 2b + IL2	<i>BRAF</i> V600 kinase inhibitor (1)	N/S ^e	Unresectable stage III or stage IV BRAF mutation-positive cutaneous melanoma	2013-000773-71 (EU)	II
Chemotherapy ^d + INF α 2b + vemurafenib (1) vs. chemotherapy + INF α 2b	<i>BRAF</i> V600 kinase inhibitor (1)	N/S ^e	Unresectable (stage III) or metastatic (stage IV) BRAF mutation-positive melanoma vs. unresectable (stage III) or metastatic (stage IV) BRAF mutation-negative melanoma	2013-000280-84 (EU)	II

NOTE: Trials are ongoing unless where otherwise stated.
^aTIL, Tumor-infiltrating lymphocytes.
^bACT, adoptive cell therapy.
^cHD, high dose.
^dUnspecified chemotherapy.
^eN/S, not stated.

This may give rise to *BRAF*-resistant melanomas with enhanced capacity to manipulate immunity.

Patients with mutant *BRAF* or *NRAS* melanomas at later disease stages (III and IV)—the cohort most likely to be offered pathway inhibitor drugs—have a worse prognosis when compared with other patient groups

(71, 72). *BRAF*-resistant melanomas are able to maintain or reactivate important signaling pathways MAPK and PI3K. Indeed, genomic analyses identified mutant *BRAF* amplification, alternative splice variants and *de novo* RAS gene alterations as well as mutations in the PI3K/Pten/Akt pathway associated with *BRAF*-

resistant tumors (73, 74). Under the selective pressure of pathway inhibitor drugs, MAPK pathway–promoted immunosuppressive mediators VEGF, IL6, and IL10 could constitute particularly important tools for emerging resistant tumors to maintain or reestablish command of their microenvironments and to reeducate host immunity (45). Enhanced expression of PD-L1 by the reactivated MAPK pathway in *BRAF* inhibitor–resistant melanomas would also support capability to reclaim suppression of host immune sentinels like T cells (60). It is, therefore, possible that *BRAF*-mutant melanomas constitute more aggressive tumors better able to establish effective suppression of host immunity along with *BRAF* inhibitor resistance.

Exploring resistance as a function of pathway network dysregulation rather than in relation to particular mutations on individual molecules, together with monitoring immunosuppressive or activatory signals, may help elucidate specific signatures associated with disease progression and lead to the identification of targets for immunotherapy. For instance, if tumor escape is associated with enhanced VEGF production and PD-L1 expression by T cells, possible combinations of VEGF and/or PD-L1 blockade strategies with pathway inhibitors may prevent or restrict melanoma progression. Furthermore, therapeutics that could support or complement pathway inhibitor functions by "waking up" dormant immune-activating signals such as enhancing CD40–CD40L interactions to promote antigen-presenting cell activation may help target "Achilles heel" elements of melanomas and augment pathway inhibitor drug effects. Toxicities observed in the ipilimumab plus vemurafenib combinatory trial, perhaps due to paradoxical MAPK pathway activation of wild-type cells alongside autoimmune effects of ipilimumab in the presence of enhanced *BRAF* inhibitor–induced antigen presentation, mandate careful design of therapeutic strategies less likely to attack healthy tissues. Combinations with tumor antigen–specific antibodies or T cells that selectively target tumor cells, or attacking modulatory elements in tumor microenvironments associated with tumor resistance to *BRAF* inhibitors such as the PD-1/PD-L1 axis, may merit consideration. The complexities of the interactions between resistance to pathway inhibition and immunomodulation may be addressed with emerging and novel bioinformatics tools. Activatory immunologic signatures associated with specific melanoma subtypes have already been shown to predict more favorable prognosis. Furthermore, immunosuppressive molecular signatures predict worse clinical outcomes (71, 75). These indicate double-edged sword roles for immunity in melanoma disease progression, but equally support the rationale for monitoring immunity alongside clinical course and clinical responses to treatments.

Concluding Thoughts

Novel insights from the laboratory and the clinic support links between pathway dysregulation with different

components of immune responses. *BRAF* inhibitors exhibit immune-activating functions, which alone, may not be sufficient to counteract tumor-associated escape mechanisms. Thus, combination treatments with different inhibitors of the RAS–RAF–MEK–ERK proliferative pathway, and also with immunotherapies may help enhance these circumscribed immunologic and clinical responses. Future translational directions may take advantage of dysregulated pathway molecules such as *BRAF* being recognized by host immunity and of molecular pathway cross-talk with specific molecules associated with immune suppression to develop rational targeted immunotherapies such as vaccines and therapeutic antibodies. Bioinformatics tools such as gene clustering and pathway analyses in large patient datasets are revealing differential classification of melanomas, including specific immunologic signatures associated with good or bad prognoses. As both checkpoint blockade antibodies and small molecule inhibitors are in clinical use, it is important now to elucidate whether we can link clinical responses with immune activation or with counteracting immunosuppressive signals to improve treatment. New approaches may consider molecular heterogeneity and pathway dysregulation together with monitoring immunologic parameters such as antigen presentation, effector cell activation, or immunosuppressive elements in tumors before and during therapy and while in remission. These could in future provide additional criteria with which to predict clinical benefits, facilitate stratification, and guide optimal monotherapy or combinatory approaches for different subsets of patients.

Disclosure of Potential Conflicts of Interest

M. Harries is an advisory board member for Roche. No potential conflicts of interest were disclosed by the other authors.

Disclaimer

The views expressed are those of the author(s) and not necessarily those of the NHS, the NIHR, or the Department of Health. The authors are solely responsible for study design, data collection, analysis, decision to publish, and preparation of the article.

Grant Support

The authors acknowledge support from Cancer Research UK (C30122/A11527; C30122/A15774; to S.N. Karagiannis, D.H. Josephs, and J.F. Spicer); KCL Experimental Cancer Medicine Centre, jointly funded by Cancer Research UK, the National Institute for Health Research, Welsh Assembly Government, HSC R&D Office for Northern Ireland and Chief Scientist Office, Scotland (to J.F. Spicer, S.N. Karagiannis, and F.O. Nestle); CR UK/EPSRC/MRC/NIHR KCL/UCL Comprehensive Cancer Imaging Centre (C1519/A10331; to J.F. Spicer, D.H. Josephs, and S.N. Karagiannis); the Medical Research Council (MR/L023091/1; to S.N. Karagiannis and F.O. Nestle); and the Dermatrust (to K.E. Lacy, I.U. Egbuniwe, and F.O. Nestle). The research was supported by the National Institute for Health Research (NIHR) Biomedical Research Centre based at Guy's and St Thomas' NHS Foundation Trust and King's College London (to S.N. Karagiannis, P. Karagiannis, F.O. Nestle, K.E. Lacy, K.M. Ilieva, and I. Correa). The authors acknowledge support from KAPA-Privatstiftung Wien (to K.M. Ilieva and P. Karagiannis).

Received April 10, 2014; revised July 24, 2014; accepted August 21, 2014; published OnlineFirst November 10, 2014.

References

- Garbe C, Leiter U. Melanoma epidemiology and trends. *Clin Dermatol* 2009;27:3–9.
- Balch CM, Gershenwald JE, Soong SJ, Thompson JF, Atkins MB, Byrd DR, et al. Final version of 2009 AJCC melanoma staging and classification. *J Clin Oncol* 2009;27:6199–206.
- Hauschild A. Adjuvant interferon alfa for melanoma: new evidence-based treatment recommendations? *Curr Oncol* 2009;16:3–6.
- Ballantyne AD, Garnock-Jones KP. Dabrafenib: first global approval. *Drugs* 2013;73:1367–76.
- Bollag G, Tsai J, Zhang J, Zhang C, Ibrahim P, Nolop K, et al. Vemurafenib: the first drug approved for BRAF-mutant cancer. *Nat Rev Drug Discov* 2012;11:873–86.
- Wright CJ, McCormack PL. Trametinib: first global approval. *Drugs* 2013;73:1245–54.
- Sznol M. Advances in the treatment of metastatic melanoma: new immunomodulatory agents. *Semin Oncol* 2012;39:192–203.
- Shimanovsky A, Jethava A, Dasanu CA. Immune alterations in malignant melanoma and current immunotherapy concepts. *Expert Opin Biol Ther* 2013;13:1413–27.
- Fujimura T, Ring S, Umansky V, Mahnke K, Enk AH. Regulatory T cells stimulate B7-H1 expression in myeloid-derived suppressor cells in metastatic melanomas. *J Invest Dermatol* 2012;132:1239–46.
- Song S, Wang Y, Wang J, Lian W, Liu S, Zhang Z, et al. Tumour-derived IL-10 within tumour microenvironment represses the antitumour immunity of Socs1-silenced and sustained antigen expressing DCs. *Eur J Cancer* 2012;48:2252–9.
- Gilbert AE, Karagiannis P, Dodev T, Koers A, Lacy K, Josephs DH, et al. Monitoring the systemic human memory B-cell compartment of melanoma patients for anti-tumor IgG antibodies. *PLoS ONE* 2011;6:e19330.
- Karagiannis P, Gilbert AE, Josephs DH, Ali N, Dodev T, Saul L, et al. IgG4 subclass antibodies impair antitumor immunity in melanoma. *J Clin Invest* 2013;123:1457–74.
- Jayaraman P, Parikh F, Lopez-Rivera E, Hailemichael Y, Clark A, Ma G, et al. Tumor-expressed inducible nitric oxide synthase controls induction of functional myeloid-derived suppressor cells through modulation of vascular endothelial growth factor release. *J Immunol* 2012;188:5365–76.
- Wang T, Ge Y, Xiao M, Lopez-Coral A, Azuma R, Somasundaram R, et al. Melanoma-derived conditioned media efficiently induce the differentiation of monocytes to macrophages that display a highly invasive gene signature. *Pigment Cell Melanoma Res* 2012;25:493–505.
- Jacobs JF, Nierkens S, Figdor CG, de Vries IJ, Adema GJ. Regulatory T cells in melanoma: the final hurdle towards effective immunotherapy? *Lancet Oncol* 2012;13:e32–42.
- Schlecker E, Stojanovic A, Eisen C, Quack C, Falk CS, Umansky V, et al. Tumor-infiltrating monocytic myeloid-derived suppressor cells mediate CCR5-dependent recruitment of regulatory T cells favoring tumor growth. *J Immunol* 2012;189:5602–11.
- Simeone E, Ascierto PA. Immunomodulating antibodies in the treatment of metastatic melanoma: the experience with anti-CTLA-4, anti-CD137, and anti-PD1. *J Immunotoxol* 2012;9:241–7.
- Hodi FS, O'Day SJ, McDermott DF, Weber RW, Sosman JA, Haanen JB, et al. Improved survival with ipilimumab in patients with metastatic melanoma. *N Engl J Med* 2010;363:711–23.
- Wolchok JD, Weber JS, Maio M, Neyns B, Harmankaya K, Chin K, et al. Four-year survival rates for patients with metastatic melanoma who received ipilimumab in phase II clinical trials. *Ann Oncol* 2013;24:2174–80.
- Curtin JA, Fridlyand J, Kageshita T, Patel HN, Busam KJ, Kutzner H, et al. Distinct sets of genetic alterations in melanoma. *N Engl J Med* 2005;353:2135–47.
- Keshet Y, Seger R. The MAP kinase signaling cascades: a system of hundreds of components regulates a diverse array of physiological functions. *Methods Mol Biol* 2010;661:3–38.
- Roberts PJ, Der CJ. Targeting the Raf-MEK-ERK mitogen-activated protein kinase cascade for the treatment of cancer. *Oncogene* 2007;26:3291–310.
- Greenman C, Stephens P, Smith R, Dalgleish GL, Hunter C, Bignell G, et al. Patterns of somatic mutation in human cancer genomes. *Nature* 2007;446:153–8.
- Garnett MJ, Marais R. Guilty as charged: B-RAF is a human oncogene. *Cancer Cell* 2004;6:313–9.
- Wan PT, Garnett MJ, Roe SM, Lee S, Niculescu-Duvaz D, Good VM, et al. Mechanism of activation of the RAF-ERK signaling pathway by oncogenic mutations of B-RAF. *Cell* 2004;116:855–67.
- Xie P, Streu C, Qin J, Bregman H, Pagano N, Meggers E, et al. The crystal structure of BRAF in complex with an organoruthenium inhibitor reveals a mechanism for inhibition of an active form of BRAF kinase. *Biochemistry* 2009;48:5187–98.
- Eisen T, Marais R, Affolter A, Lorigan P, Robert C, Corrie P, et al. Sorafenib and dacarbazine as first-line therapy for advanced melanoma: phase I and open-label phase II studies. *Br J Cancer* 2011;105:353–9.
- Hauschild A, Agarwala SS, Trefzer U, Hogg D, Robert C, Hersey P, et al. Results of a phase III, randomized, placebo-controlled study of sorafenib in combination with carboplatin and paclitaxel as second-line treatment in patients with unresectable stage III or stage IV melanoma. *J Clin Oncol* 2009;27:2823–30.
- Chapman PB, Hauschild A, Robert C, Larkin JMG, Haanen JBAG, Ribas A, et al. Updated overall survival (OS) results for BRIM-3, a phase III randomized, open-label, multicenter trial comparing BRAF inhibitor vemurafenib (vem) with dacarbazine (DTIC) in previously untreated patients with BRAFV600E-mutated melanoma. *J Clin Oncol* 2012;30(suppl; abstr 8502).
- da Rocha Dias S, Salmonson T, van Zwielen-Boot B, Jonsson B, Marchetti S, Schellens JH, et al. The European Medicines Agency review of vemurafenib (Zelboraf®) for the treatment of adult patients with BRAF V600 mutation-positive unresectable or metastatic melanoma: summary of the scientific assessment of the Committee for Medicinal Products for Human Use. *Eur J Cancer* 2013;49:1654–61.
- McArthur GA, Chapman PB, Robert C, Larkin J, Haanen JB, Dummer R, et al. Safety and efficacy of vemurafenib in BRAF(V600E) and BRAF(V600K) mutation-positive melanoma (BRIM-3): extended follow-up of a phase 3, randomised, open-label study. *Lancet Oncol* 2014;15:323–32.
- Mao M, Tian F, Mariadason JM, Tsao CC, Lemos RJr, Dayyani F, et al. Resistance to BRAF inhibition in BRAF-mutant colon cancer can be overcome with PI3K inhibition or demethylating agents. *Clin Cancer Res* 2013;19:657–67.
- Rushworth LK, Hindley AD, O'Neill E, Kolch W. Regulation and role of Raf-1/B-Raf heterodimerization. *Mol Cell Biol* 2006;26:2262–72.
- Hatzivassiliou G, Song K, Yen I, Brandhuber BJ, Anderson DJ, Alvarado R, et al. RAF inhibitors prime wild-type RAF to activate the MAPK pathway and enhance growth. *Nature* 2010;464:431–5.
- Gibney GT, Messina JL, Fedorenko IV, Sondak VK, Smalley KS. Paradoxical oncogenesis—the long-term effects of BRAF inhibition in melanoma. *Nat Rev Clin Oncol* 2013;10:390–9.
- Flaherty KT. Dividing and conquering: controlling advanced melanoma by targeting oncogene-defined subsets. *Clin Exp Metastasis* 2012;29:841–6.
- Halaban R, Zhang W, Bacchicocchi A, Cheng E, Parisi F, Ariyan S, et al. PLX4032, a selective BRAF(V600E) kinase inhibitor, activates the ERK pathway and enhances cell migration and proliferation of BRAF melanoma cells. *Pigment Cell Melanoma Res* 2010;23:190–200.
- Hauschild A, Grob JJ, Demidov LV, Jouary T, Gutzmer R, Millward M, et al. Dabrafenib in BRAF-mutated metastatic melanoma: a multicentre, open-label, phase 3 randomised controlled trial. *Lancet* 2012;380:358–65.
- Gilmartin AG, Bleam MR, Groy A, Moss KG, Minthorn EA, Kulkarni SG, et al. GSK1120212 (JTP-74057) is an inhibitor of MEK activity and activation with favorable pharmacokinetic properties for sustained in vivo pathway inhibition. *Clin Cancer Res* 2011;17:989–1000.
- Flaherty KT, Robert C, Hersey P, Nathan P, Garbe C, Milhem M, et al. Improved survival with MEK inhibition in BRAF-mutated melanoma. *N Engl J Med* 2012;367:107–14.

41. Sharkey MS, Lizee G, Gonzales MI, Patel S, Topalian SL. CD4(+)T-cell recognition of mutated B-RAF in melanoma patients harboring the V599E mutation. *Cancer Res* 2004;64:1595–9.
42. Andersen MH, Fensterle J, Ugurel S, Reker S, Houben R, Guldberg P, et al. Immunogenicity of constitutively active V599EBraf. *Cancer Res* 2004;64:5456–60.
43. Somasundaram R, Swoboda R, Caputo L, Otvos L, Weber B, Volpe P, et al. Human leukocyte antigen-A2-restricted CTL responses to mutated BRAF peptides in melanoma patients. *Cancer Res* 2006;66:3287–93.
44. Donia M, Fagone P, Nicoletti F, Andersen RS, Hogdall E, Straten PT, et al. BRAF inhibition improves tumor recognition by the immune system: potential implications for combinatorial therapies against melanoma involving adoptive T-cell transfer. *Oncoimmunology* 2012;1:1476–83.
45. Sumimoto H, Imabayashi F, Iwata T, Kawakami Y. The BRAF-MAPK signaling pathway is essential for cancer-immune evasion in human melanoma cells. *J Exp Med* 2006;203:1651–6.
46. Khalili JS, Liu S, Rodriguez-Cruz TG, Whittington M, Wardell S, Liu C, et al. Oncogenic BRAF(V600E) promotes stromal cell-mediated immunosuppression via induction of interleukin-1 in melanoma. *Clin Cancer Res* 2012;18:5329–40.
47. Sapkota B, Hill CE, Pollack BP. Vemurafenib enhances MHC induction in BRAF homozygous melanoma cells. *Oncoimmunology* 2013;2:e22890.
48. Comin-Anduix B, Chodon T, Sazegar H, Matsunaga D, Mock S, Jalil J, et al. The oncogenic BRAF kinase inhibitor PLX4032/RG7204 does not affect the viability or function of human lymphocytes across a wide range of concentrations. *Clin Cancer Res* 2010;16:6040–8.
49. Vosganian GS, Bos R, Sherman LA. Immunologic effects of an orally available BRAFV600E inhibitor in BRAF wild-type murine models. *J Immunother* 2012;35:473–7.
50. Boni A, Cogdill AP, Dang P, Udayakumar D, Njauw CN, Sloss CM, et al. Selective BRAFV600E inhibition enhances T-cell recognition of melanoma without affecting lymphocyte function. *Cancer Res* 2010;70:5213–9.
51. Hong DS, Vence L, Falchook G, Radvanyi LG, Liu C, Goodman V, et al. BRAF(V600) inhibitor GSK2118436 targeted inhibition of mutant BRAF in cancer patients does not impair overall immune competency. *Clin Cancer Res* 2012;18:2326–35.
52. Hooijkaas A, Gadiot J, Morrow M, Stewart R, Schumacher T, Blank CU. Selective BRAF inhibition decreases tumor-resident lymphocyte frequencies in a mouse model of human melanoma. *Oncoimmunology* 2012;1:609–17.
53. Simpson TR, Li F, Montalvo-Ortiz W, Sepulveda MA, Bergerhoff K, Arce F, et al. Fc-dependent depletion of tumor-infiltrating regulatory T cells co-defines the efficacy of anti-CTLA-4 therapy against melanoma. *J Exp Med* 2013;210:1695–710.
54. Peggs KS, Quezada SA, Chambers CA, Korman AJ, Allison JP. Blockade of CTLA-4 on both effector and regulatory T cell compartments contributes to the antitumor activity of anti-CTLA-4 antibodies. *J Exp Med* 2009;206:1717–25.
55. Wilmott JS, Long GV, Howle JR, Haydu LE, Sharma RN, Thompson JF, et al. Selective BRAF inhibitors induce marked T-cell infiltration into human metastatic melanoma. *Clin Cancer Res* 2012;18:1386–94.
56. Ott PA, Henry T, Baranda SJ, Fleta D, Manches O, Bogunovic D, et al. Inhibition of both BRAF and MEK in BRAF(V600E) mutant melanoma restores compromised dendritic cell (DC) function while having differential direct effects on DC properties. *Cancer Immunol Immunother* 2013;62:811–22.
57. Schilling B, Sucker A, Griewank K, Zhao F, Weide B, Gorgens A, et al. Vemurafenib reverses immunosuppression by myeloid derived suppressor cells. *Int J Cancer* 2013;133:1653–63.
58. Ho PC, Meeth KM, Tsui YC, Srivastava B, Bosenberg MW, Kaech SM. Immune-based antitumor effects of BRAF inhibitors rely on signaling by CD40L and IFN γ . *Cancer Res* 2014;74:3205–17.
59. Knight DA, Ngiow SF, Li M, Parmenter T, Mok S, Cass A, et al. Host immunity contributes to the anti-melanoma activity of BRAF inhibitors. *J Clin Invest* 2013;123:1371–81.
60. Jiang X, Zhou J, Giobbie-Hurder A, Wargo J, Hodi FS. The activation of MAPK in melanoma cells resistant to BRAF inhibition promotes PD-L1 expression that is reversible by MEK and PI3K inhibition. *Clin Cancer Res* 2013;19:598–609.
61. Flaherty KT, Infante JR, Daud A, Gonzalez R, Kefford RF, Sosman J, et al. Combined BRAF and MEK inhibition in melanoma with BRAF V600 mutations. *N Engl J Med* 2012;367:1694–703.
62. Frederick DT, Piris A, Cogdill AP, Cooper ZA, Lezcano C, Ferrone CR, et al. BRAF inhibition is associated with enhanced melanoma antigen expression and a more favorable tumor microenvironment in patients with metastatic melanoma. *Clin Cancer Res* 2013;19:1225–31.
63. Koya RC, Mok S, Otte N, Blacketer KJ, Comin-Anduix B, Tumei PC, et al. BRAF inhibitor vemurafenib improves the antitumor activity of adoptive cell immunotherapy. *Cancer Res* 2012;72:3928–37.
64. Liu C, Peng W, Xu C, Lou Y, Zhang M, Wargo JA, et al. BRAF inhibition increases tumor infiltration by T cells and enhances the antitumor activity of adoptive immunotherapy in mice. *Clin Cancer Res* 2013;19:393–403.
65. Lemech C, Infante J, Arkenau HT. Combination molecularly targeted drug therapy in metastatic melanoma: progress to date. *Drugs* 2013;73:767–77.
66. Ribas A, Hodi FS, Callahan M, Konto C, Wolchok J. Hepatotoxicity with combination of vemurafenib and ipilimumab. *N Engl J Med* 2013;368:1365–6.
67. Cooper ZA, Frederick DT, Ahmed Z, Wargo JA. Combining checkpoint inhibitors and BRAF-targeted agents against metastatic melanoma. *Oncoimmunology* 2013;2:e24320.
68. Altomonte M, Di Giacomo A, Queirolo P, Ascierto P, Spagnolo F, Bajetta E, et al. Clinical experience with ipilimumab 10 mg/kg in patients with melanoma treated at Italian centres as part of a European expanded access programme. *J Exp Clin Cancer Res* 2013;32:82.
69. Ackerman A, Klein O, McDermott DF, Wang W, Ibrahim N, Lawrence DP, et al. Outcomes of patients with metastatic melanoma treated with immunotherapy prior to or after BRAF inhibitors. *Cancer* 2014;120:1695–701.
70. Jang S, Atkins MB. Which drug, and when, for patients with BRAF-mutant melanoma? *Lancet Oncol* 2013;14:e60–9.
71. Mann GJ, Pupo GM, Campain AE, Carter CD, Schramm SJ, Pianova S, et al. BRAF mutation, NRAS mutation, and the absence of an immune-related expressed gene profile predict poor outcome in patients with stage III melanoma. *J Invest Dermatol* 2013;133:509–17.
72. Long GV, Menzies AM, Nagrial AM, Haydu LE, Hamilton AL, Mann GJ, et al. Prognostic and clinicopathologic associations of oncogenic BRAF in metastatic melanoma. *J Clin Oncol* 2011;29:1239–46.
73. Shi H, Hugo W, Kong X, Hong A, Koya RC, Moriceau G, et al. Acquired resistance and clonal evolution in melanoma during BRAF inhibitor therapy. *Cancer Discov* 2014;4:80–93.
74. Shi H, Hong A, Kong X, Koya RC, Song C, Moriceau G, et al. A novel AKT1 mutant amplifies an adaptive melanoma response to BRAF inhibition. *Cancer Discov* 2014;4:69–79.
75. Jonsson G, Busch C, Knappskog S, Geisler J, Miletic H, Ringner M, et al. Gene expression profiling-based identification of molecular subtypes in stage IV melanomas with different clinical outcome. *Clin Cancer Res* 2010;16:3356–67.