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*J Immunol* 2014; 192:2505-2513; Prepublished online 31 January 2014; doi: 10.4049/jimmunol.1302616
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Dynamics of Chemokine, Cytokine, and Growth Factor Serum Levels in BRAF-Mutant Melanoma Patients during BRAF Inhibitor Treatment

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The purpose of this study is to profile the changes in the serum levels of a range of chemokines, cytokines, and growth and angiogenic factors in MAPK inhibitor–treated metastatic melanoma patients and to correlate these changes with clinical outcome and changes in melanoma tissue biopsies taken from the same patients. Forty-two chemokine, cytokine, angiogenic, and growth factors were measured in the sera of 20 BRAF inhibitor–treated and four combination BRAF and MEK inhibitor–treated metastatic melanoma patients using a multiplex chemokine assay. The changes were correlated with Ki-67 and CD8+ tumor-infiltrating lymphocytes in the tumor biopsies taken at the same time points, as well as clinical outcome, including response rate, progression-free survival, and overall survival. Serum levels of IFN-γ, CCL4, and TNF-α were significantly increased, whereas CXCL8 significantly decreased from pretreatment (PRE) to early during treatment (EDT) serum samples. The decrease in serum CXCL8 levels from PRE to EDT significantly correlated with decreases in markers of melanoma proliferation (Ki-67) and increases in cytotoxic tumor-infiltrating T cells in corresponding tumor biopsies. In addition, a greater fold reduction in CXCL8 serum levels from PRE to EDT serum samples was associated with decreased overall survival. These results suggest that BRAF inhibition causes decreased CXCL8 secretion from melanoma cells and induce an immune response against the tumor associated with increased IFN-γ, CCL4, and TNF-α. Further studies are needed to determine if CXCL8 is predictive of response and to confirm the functions of these chemokine and cytokine in BRAF-mutant melanoma under BRAF inhibition. The Journal of Immunology, 2014, 192: 2505–2513.

Mutations of the serine/threonine-protein kinase B-raf (BRAF) gene occur in ~40–50% of all patients with metastatic melanoma (1, 2). These BRAF mutations alter the structure and function of the kinase domain, causing its constitutive activation, which results in the continuous stimulation of the MAPK pathway (1). Melanomas rely on MAPK overstimulation to promote proliferation, reduce apoptosis, and sustain an immunosuppressive microenvironment (3–6). As a consequence, BRAF-mutant melanomas are highly sensitive to treatment with targeted BRAF inhibitors (BRAFi), with patient biopsies taken early during BRAFi therapy showing reduced tumor cell proliferation and an increase in the density of intratumor immune cell infiltration (7–11).

The BRAFi dabrafenib and vemurafenib have proven highly active (12–14) and improve progression-free survival (PFS) and overall survival (OS) in patients with BRAF-mutant metastatic melanoma (12, 15). Similarly, the combination of a downstream MEK inhibitor (MEKi) with a BRAFi can improve response rates and extend PFS (16). However, only a minority of patients achieve a complete response, with the majority of patients experiencing dramatic initial results followed by subsequent drug resistance. Multiple mechanisms of BRAFi resistance have been described that act predominantly via MAPK reactivation and PI3K pathway upregulation (17–22). Recently, attention has turned to the potential of the tumor microenvironment to augment patient responses to BRAFi treatment in light of studies by Straussman et al. (23) and Khalili et al. (11), which demonstrated the importance of the tumor microenvironment in contributing to BRAFi resistance and sustaining immunosuppression.

Melanoma cells and their associated stroma alter the tumor microenvironment by secreting chemokines and cytokines that promote an oncogenic and immunosuppressive microenvironment (24). Chemokines are a large family of small signaling proteins that bind to G-protein–coupled receptors on the surface of cells,
thereby forming intercellular communication networks that can regulate the microenvironment (24). In melanoma, the overstimulation of the MAPK pathway through BRAF mutations causes the overproduction of many chemokines, cytokines, and growth factors such as IL-6, CXCL5/IL-8, IL-10, and vascular endothelial growth factor (VEGF), which can promote proliferation, migration, invasion, angiogenesis, and immune evasion and act as autocrine growth factors (10, 25–27). However, little is known about the dynamics of systemic chemokine and cytokine serum levels in association with tumor response and patient outcome to BRAFi treatment.

Therefore, we sought to profile changes in the levels of a range of chemokines, cytokines growth factors, and angiogenic factors in the serum of BRAF-mutant, BRAFi-treated melanoma patients for associations with changes in tumor proliferation and immune cell infiltration in tumor biopsies. Additionally, we sought to evaluate sera levels of chemokine and cytokine as a predictor of patient response and outcome to BRAFi therapy.

Materials and Methods

Patients, tumor biopsies, and serum samples

This study comprised a cohort of metastatic melanoma patients who received BRAFi inhibitors as part of clinical trials at Melanoma Institute Australia or Westmead Hospital between 2009 and 2012 (12, 15, 16, 28–31). Physicians treated all patients with American Joint Committee on Cancer stage IV BRAF-mutant melanoma and were treated with either single-agent BRAFi (dabrafenib or vemurafenib) or combination BRAFi (dabrafenib and MEKi, trametinib). Clinical and follow-up details were collected and analyzed on all patients as approved by the Westmead and Royal Prince Alfred Hospital Human Research Ethics Committees (protocol numbers X10-0305 and HREC/10/RPAH/339). Tumor biopsies and serum samples were collected before the commencement of the BRAFi (pretreatment [PRE]), after 3–15 d on treatment (early during treatment [EDT]), and at clinical disease progression (PROG). Serum samples were collected in plain tubes, allowed to clot, centrifuged for 10 min at 3000 × g in a refrigerated centrifuge at 4˚C, and then the serum was aliquoted, snap frozen in liquid nitrogen, and stored in a –80˚C freezer. The time the sample was in –80˚C storage was recorded for each sample.

Assessment of response and outcomes

Objective response to MAPK inhibitor (MAPKi) treatment was assessed by computed tomography scans performed at 6–9-wk intervals using Response Evaluation Criteria in Solid Tumors (RECIST) criteria (32, 33). Best RECIST response was analyzed categorically (complete response [CR], partial response, stable disease, and progressive disease) as well as continuously (the percent change in target lesions compared with baseline). Clinical outcome was assessed using RECIST-defined PFS and OS from commencement of BRAFi.

Multiplex cytokine/chemokine array

For 15 patients, sera was analyzed using a Milliplex MAP Human Cytokine/Chemokine Panel (MPXHCYT060; Millipore) 42plex cytokine/chemokine panel for the following chemokines, cytokine, growth, and angiogenic factors (lower limits of detections): epidermal growth factor (5.3 pg/ml), etoxacin (2.1 pg/ml), fibroblast growth factor-2 (6 pg/ml), Fli3 ligand (6.1 pg/ml), fractalkine (7.6 pg/ml), G-CSF (3.9 pg/ml), GM-CSF (2.3 pg/ml), GROα (11.4 pg/ml), IFN-α2 (7.2 pg/ml), IFN-γ (0.2 pg/ml), IL-1α (1.5 pg/ml), IL-1β (0.7 pg/ml), IL-1β antagonist (2.3 pg/ml), IL-2 (0.4 pg/ml), IL-3 (9.8 pg/ml), IL-4 (0.6 pg/ml), IL-5 (0.1 pg/ml), IL-6 (0.4 pg/ml), IL-7 (1 pg/ml), CXCL8 (0.3 pg/ml), IL-9 (1.1 pg/ml), IL-10 (0.3 pg/ml), IL-12 (p40) (12.3 pg/ml), IL-12(p70) (0.9 pg/ml), IL-13 (0.3 pg/ml), IL-15 (0.6 pg/ml), IL-17A (0.4 pg/ml), CXCL10 (1.3 pg/ml), CCL2 (1.2 pg/ml), MCP-3 (5.2 pg/ml), CCL22 (2.4 pg/ml), CCL3 (6.6 pg/ml), CCL4 (3.2 pg/ml), CCL5 (2.2 pg/ml), CCL7 (7.5 pg/ml), TGF-α (1.4 pg/ml), TNF-α (0.2 pg/ml), TNF-β (4.1 pg/ml), VEGF (10.1 pg/ml), platelet-derived growth factor (PDGF)-AA (0.3 pg/ml), PDGF-AB/BB (12.2 pg/ml), and CCL5 (1.6 pg/ml). An additional nine patients were assessed on the narrower cytokine/chemokine panel 10plex (MPXHCYT060; Millipore), which included; IFN-α2, IFN-γ, IL-10, IL-6, CXCL8, CXCL10, CCL4, TNF-α, VEGF, and CCL5. Both panels were performed in duplicate according to the manufacturer’s instructions using a common Millipore-supplied standard. Millipore quality controls were supplied and used as per the manufacturer’s instructions. All serum samples were tested undiluted, except when analyzed for CCL5, PDGF-AA, and PDGF-AB/BB, when they were diluted 1:200. The cytokine and chemokines were analyzed on a Luminex 200 (Luminex). The data obtained were autoanalyzed with Milliplex analyst and 3.1 xPonent software (Millipore), which uses five-parameter logistic regression.

Tumor biopsy morphological assessment

Tumor biopsies were assessed for the percentage of Ki-67–immunoreactive tumor cells (proliferative marker) and density of CD8+ T cell tumor infiltration, as described in previous studies (7, 9). The biopsies were fixed in 10% buffered formaldehyde. After overnight fixation, they were embedded in paraform wax, and 4-μm-thick sections were cut. All immunohistochemical staining was performed on a Leica Bond-Max autostainer (Leica Microsystems, Bannockburn, IL) according to the manufacturer’s protocol. Ag retrieval was performed at pH 9 using Epitope Retrieval 2 solution (Leica Microsystems) for 20 (CD8) and 30 min (Ki-67) at 100˚C. Slides were then incubated for 15 min at room temperature with the respective primary Abs at the following dilutions: CD8 (B1-44B), 1:100 (M7103; DakoCyto- tomation, Glostrup, Denmark); and Ki-67 (MM1), 1:100 (PA0118; Leica Microsystems). Ab detection was performed using the Bond Polymer Refine Red Detection system as per the manufacturer’s instructions (DS9390; Leica Microsystems). Slides were then counterstained with hematoxylin.

The slides were examined independently by two investigators (J.S.W. and R.A.S.) blinded to patient outcome. The percentage of intratumoral immunoreactive CD8+ T cells and Ki-67–positive tumor cells was semi-quantitatively assessed as per previous studies (7, 9).

Statistical analysis

Statistical analysis was performed using IBM SPSS statistics v21.0 (SPSS Inc). Raw changes from PRE to EDT were calculated as: (PRE value − EDT value). Fold-change of immunohistochemical (IHC) measurements from PRE to EDT were calculated as: (EDT value/PRE value). Wilcoxon signed-rank test was used to test for significant changes in serum levels of chemokine and cytokine between treatment stages. Correlation analysis was performed using a Spearman rho test, and the Mann–Whitney U test was used to test for associations of scalar and categorical variables. Multivariate time to event analysis was conducted for continuous absolute PRE, EDT, raw change PRE–EDT change, and PRE–EDT fold change in serum levels of all chemokine and cytokine covariates while adjusting for serum –80˚C storage time using the Cox proportional hazards method. The following outcomes were investigated: PFS (defined as the interval between commencement of the MAPKi and disease progression) and OS (defined as the interval between commencement of the MAPKi and death). All patient deaths in this cohort were caused by melanoma. The storage duration for the serum was recorded and entered into any survival analyses to adjust for degradation of analytes over time (34).

Results

Patient details

Sera and tumor samples were collected from 24 melanoma patients whose tumor had a BRAFV600E mutation (21 BRAFV600E and 3 BRAFV600K). Twenty patients were treated with a single-agent BRAFi (18 dabrafenib and 2 vemurafenib), and 4 patients received combination BRAFi and MEKi therapy consisting of dabrafenib and trametinib (Supplemental Table I, Table I). The dosage and schedule for 24 patients in this study, 2 patients achieved a complete response, 16 had partial responses, 5 had stable disease, and 1 patient had progressive disease as best RECIST response.

Significant changes in the levels of serum chemokine and cytokine in melanoma patients during BRAFi treatment

Eleven patients had PRE, EDT, and PROG triplicate serum samples, and 13 patients had PRE and EDT paired samples only. Significant increases were observed in the level of serum IFN-γ, CCL4 (p < 0.05), and, to a lesser extent, TNF-α (p = 0.083) from PRE to EDT samples (median 3.9 versus 6.5, 33.7 versus 40.1, and 9.1 versus 7.9 pg/ml, respectively; Fig. 1A–C). However, the median TNF-α serum level was lower at EDT compared with the PRE levels as a result of the elevated pretreatment level of patient 10, the cause of which is unknown, and this contrasts with the increases experienced by the remaining patients. Additionally, there

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was a significant positive correlation between increased serum levels of IFN-γ, CCL4, and TNF-α (all \( p < 0.01 \)). Conversely, CXCL8 significantly decreased in serum samples from PRE to EDT (median 43.5 versus 26.7 pg/ml, respectively; \( p = 0.001 \); Fig. 1D).

**Associations among changes in the levels of serum chemokine, cytokine, tumor biopsy proliferative rate, and immune cell infiltrate from PRE to EDT samples**

Changes in the serum levels of chemokine and cytokine from PRE to EDT were analyzed for associations with changes in CD8+ T cell tumor infiltration and percentage of Ki-67–immunoreactive tumor cells from PRE to EDT. A greater fold reduction in the serum concentrations of CXCL8 from PRE to EDT significantly correlated with reduced percentage of Ki-67–immunoreactive tumor cells from PRE to EDT biopsies (\( r = 0.592; p = 0.043 \) (Fig. 2A). Likewise, a greater fold reduction in CXCL8 was significantly correlated with an increase in the density of intratumoral CD8+ T cells (tumor-infiltrating lymphocytes) in corresponding tumor biopsies (\( r = -0.545; p = 0.029 \) (Fig. 2B).

**Associations among serum levels of chemokine, cytokine, and RECIST response to BRAFi inhibition**

Pretreatment serum levels of chemokine and changes from PRE to EDT were analyzed for associations with best RECIST response and best percentage change of RECIST target lesions. Higher pretreatment serum levels of CCL2 correlated with a lower degree of RECIST response (\( r = 0.618; p = 0.043 \) (Fig. 2C). The majority of patients who achieved complete or partial response had increased serum levels of CCL2 from PRE to EDT (median change 31 pg/ml) compared with those with stable or progressive disease (median change −62 pg/ml), all of whom also had decreased serum levels of CCL2 (\( p = 0.008 \); Fig. 2D). Pretreatment serum levels of IL-10 were significantly lower in patients who achieved complete or partial responses compared with those with stable or progressive disease (\( U = 24; p = 0.033 \).

**Correlation of serum levels of chemokine and cytokine and survival in patients treated with BRAFi**

However, a greater fold reduction in the serum levels of CXCL8 from PRE to EDT in serum samples was associated with a shorter OS (fold change CXCL8 PRE-EDT, hazard ratio [HR] 0.078, 95% confidence interval [CI] 0.007–0.829; \( p = 0.034 \); Table II). Additionally, a higher percentage of Ki-67–positive immunoreactive tumor cells in pretreatment tumor biopsies was associated with decreased OS (HR 1.074, 95% CI 1.020–1.131; \( p = 0.007 \), yet a greater reduction the percentage of Ki-67–immunoreactive tumor cells from PRE to EDT tumor tissue biopsies correlated with decreased OS (HR 0.94, 95% CI 0.891–0.983; \( p = 0.008 \).
Correlation of serum levels of CXCL8 and tumor burden

In view of our finding that serum levels of CXCL8 were associated with OS, we sought to test for correlations between CXCL8 and markers of tumor burden. Patients who had elevated serum levels of lactate dehydrogenase (LDH) at pretreatment also had significantly higher pretreatment serum levels of CXCL8 compared with those with a normal LDH (median 55.2 versus 26 pg/ml, respectively; $U = 19; p = 0.001$; Fig. 3A). Similarly, higher pretreatment serum...
levels of CXCL8 significantly correlated with higher pretreatment sum of the diameters of RECIST target lesions ($r = -0.623$; $p = 0.001$, Fig. 3B). Likewise, a greater decrease in both raw change and fold change in serum levels of CXCL8 from PRE to EDT serum samples correlated with an increased sum of diameters of all RECIST target lesions at baseline ($r = 0.761$ and $r = 0.656$, respectively; both $p < 0.001$; Fig. 3C). A greater raw change and fold change in CXCL8 from PRE to EDT also correlated with higher pretreatment baseline serum levels of CXCL8 ($r = -0.803$ and $r = -0.517$, respectively; both $p < 0.01$; Fig. 3D).

**Discussion**

In this study, we assessed changes in serum levels of chemokine and cytokine in $BRAF$-mutant melanoma patients in response to BRAFi treatment and correlated serum levels with tumor response and patient outcome data. The serum levels of the majority of chemokines and cytokines were unaffected by BRAFi treatment. This likely reflects the fact that many chemokine and cytokine are acting locally within the tumor microenvironment and are not associated with changes in systemic serum levels. Nevertheless, in analyzing the systemic serum, we attained an overview of the dynamics of chemokine and cytokine levels as a result of the entire patient’s tumor burden. Furthermore, analysis of patient serum provides a minimally invasive method for exploring potential novel biomarkers of response and outcome for BRAFi-treated patients.

In the current study, high pretreatment serum levels of CCL2 (MCP1) were found to correlate with a reduced RECIST response, suggesting CCL2 may play a protumorigenic role in these patients. High serum levels of CCL2 are known to correlate with poorer prognosis in breast and pancreatic cancer patients (35, 36). Recently Knight et al. (37) showed that $BRAF$-mutant melanoma cell lines have reduced CCL2 mRNA and protein production following treatment with a BRAFi. They also showed that $BRAF^{Y600E}$-driven mouse models treated with a combination of a BRAFi and anti-CCL2-neutralizing Abs had a greater suppression of tumor growth compared with single-agent BRAFi (37). These protumorigenic functions of CCL2 relate to its ability to recruit tumor-associated macrophages, which release factors that promote tumor angiogenesis.
migration, and invasion (35, 36, 38–41). Conversely, the current study also found an increase in CCL2 from PRE to EDT correlated with better RECIST response. The increase in CCL2 from PRE-EDT in the good RECIST responders most likely represents a BRAFi-induced immune response, which we have previously shown correlates with a reduction in tumor size (9, 42). CCL2 is known to be released during immune responses causing stimulation of macrophages and T cells (43, 44). Recent studies by Li et al. (45) found CCL2 may have a functional role in inducing a host immune response in early low-volume disease while promoting growth and survival in established high-volume disease. Therefore, CCL2 may have both a protumorigenic or proimmunosurveillance role in BRAFi-treated patients, which is expression level, microenvironment, and tumor burden specific.

We found a significant increase in the serum levels of IFN-γ, CCL4, and TNF-α and a significant decrease in CXCL8 from PRE to EDT serum samples. The results are concordant with prior studies showing an increase in TNF-α in serum (46) and a decrease in CXCL8 in tissue biopsies (10) taken from patients before and during treatment with BRAFi. The increased serum levels of chemokine and cytokine in the current study may be indicative of an induced immune response, as CCL4 and IFN-γ are secreted by activated NK cells in the early phase of immune responses and by CD4/8 T cells in later phases, leading to the secretion of TNF-α by APCs and an antitumor immune response (47). These changes serum levels of chemokine and cytokine may be more pronounced in the local tumor environment as opposed to the systemic sera, as they are generally locally acting inflammatory mediators (48). BRAFi have been shown to increase antigenicity of melanoma cells, thereby inducing immune cell infiltration, which has been proposed to contribute to the efficacy of BRAFi (9–11, 49).

Conversely, recent studies show that TNF-α and IFN-γ may also contribute to immune evasion of melanoma. TNF-α secretion by macrophages can result in the downregulation of melanocytic Ag expression by melanocytes, whereas the secretion of IFN-γ causes the upregulation of the potent T cell inhibitor programmed cell death ligand I on melanoma (10, 11, 50). Additionally, TNF-α is a potent stimulator of CXCL8 secretion by melanoma cells. The latter is known to promote the intratumoral accumulation of myeloid-derived suppressor cells, which are a major component of the immunosuppressive tumor microenvironment and act via the induction of regulatory T cells and inhibition of effector T cell function (51–53). CXCL8 signaling can also promote autocrine growth in melanomas by binding to its G-protein–coupled receptors CXCR1 and CXCR2 on the melanoma cell surface. Such binding can stimulate the PI3K and MAPK signaling pathways (25), which are known to be the major pathways activated following the acquisition of tumor resistance to BRAFi therapy. Thus, the dynamics and role of these chemokines and cytokines in melanomas under BRAFi treatment warrants further investigation, as they may contribute to immune and tumor response.

This is the first study, to our knowledge, to show that changes in sera correlated with changes in melanoma tissue, a reduction in the sera levels of CXCL8 from PRE to EDT significantly correlated with a reduction in tumor proliferation (Ki-67), and an increased immune response (CD8+) in corresponding BRAFi-treated patient tumor biopsies. Additionally, we found that a greater reduction in serum levels of CXCL8 from PRE to EDT serum samples was associated with poorer OS. The latter finding may be a reflection of an association between tumor burden and prognosis, as the patients who had the greatest reduction in CXCL8 from PRE to EDT had high pretreatment levels of CXCL8 and a higher sum of RECIST lesion diameters at baseline. This greater pretreatment tumor burden probably led to their reduced OS. Indeed, patients with a lower pretreatment tumor burden have a significantly longer OS on vemurafenib (54, 55). Additionally, when subgroup analysis was performed, the association between the fold change in CXCL8 from pretreatment to early during treatment and OS was found to depend heavily upon two patients who had low volume of disease at baseline.

A greater reduction in the proliferative marker Ki-67 from PRE–EDT tumor biopsies was also found to associate with a shorter OS. Initially, this association seems counterintuitive, as it might be predicted that a greater reduction in proliferation may correlate with increased survival. However, as we have previously shown, BRAFi are universally antiproliferative, but their effect diminishes over time, and the proliferative rate is regained at disease progression (7). Therefore, the patients who have higher PRE Ki-67 scores may show a large decrease in Ki-67 in the short term; however, upon disease progression, their tumors regain their initial highly proliferative phenotype, which ultimately leads to the reduced OS, regardless of the initial antiproliferative tumor response to MAPKi.

In conclusion, the results of this study demonstrate that the serum levels of chemokines and cytokines that are indicative of an immune response are significantly upregulated in the serum of
BRAF-mutant melanoma patients under BRAF inhibition. These chemokines and cytokines are capable of triggering either a potent immunostimulatory or an inhibitory function in melanoma. Therefore, their precise role in augmenting the immune response following BRAFi treatment may provide biological insights into the effectiveness of the combination of targeted BRAF and immune checkpoint inhibitors that are currently being investigated in the treatment of metastatic melanoma patients. Serum levels of CXCL8 correlated with immune response within BRAF-treated melanoma metastases, reflective of tumor burden, to BRAF inhibition was prognostic. The findings point to the need for additional studies to determine the functions of these chemokines and cytokines in BRAF-mutant melanoma under BRAF inhibition.

Acknowledgments
We thank Robyn Saw, Kerwin Shannon, Andrew Spillane, Jonathan Stretch, Michael Quinn, Katherine Carson, Maria Synnott, Tracy Liaw, Valerie Jakrot, and the clinical trials and biospecimen banking teams at Westmead and Melanoma Institute Australia. We also thank Dr. Donna Lai at the Bosch Institute Core facility of the University of Sydney for training and advice for the execution of the chemokine multiplex arrays.

Disclosures
A.M.M. received honoraria and travel support from Roche and received travel support from GlaxoSmithKline. J.F.T. is a consultant advisor and received travel support from GlaxoSmithKline and Proventus. R.F.K. is a consultant advisor and received honoraria from Novarits and GlaxoSmithKline and is a consultant advisor and received honoraria and travel support from Roche. R.A.S. is a consultant advisor for GlaxoSmithKline, Roche, and Novarits. G.V.L. is a consultant advisor for GlaxoSmithKline, Bristol-Myers Squibb, and Novarits and is a consultant advisor and received honoraria and travel support from Roche. The other authors have no financial conflicts of interest.

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