The Paradoxical Activation of MAPK in Melanoma Cells Resistant to BRAF Inhibition Promotes PD-L1 Expression that is Reversible by MEK and PI3K inhibition

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Statement of Translational Relevance

The availability of the selective BRAF inhibitor vemurafenib and immune checkpoint blockade with ipilimumab have dramatically altered treatment for patients with metastatic melanoma in offering survival advantages. Overcoming resistance to BRAF inhibition in melanoma is currently a critical area of investigation. Furthermore, the integration of targeted therapies with immune therapies presents novel opportunities for patients. There remain many questions in how best to combine such therapies to improve patient outcomes. In the current study, we identify a molecular mechanism that provides the basis for BRAF inhibitor resistant melanoma cells to evade the immune system via upregulation of the inducible ligand PD-L1. Subsequently, we identify potential molecular means to reverse this through MEK inhibition. These findings have particular implications for therapeutic strategies in the development of targeted combinations as well as combinations of targeted small molecules and immune checkpoint blockade.
ABSTRACT:

Purpose: Selective BRAF inhibition (BRAFi) provides a paradigm shift for melanoma treatment. The duration of benefit is typically limited before resistance develops. Interest remains in combining targeted and immune therapies to overcome resistance and improve durability of clinical benefit. One mechanism of evading immune destruction is PD-L1 expression by tumors that results in potent anti-tumor immune suppression.

Experimental Design: BRAFi resistant melanoma cells were examined for changes in PD-L1 expression by immunoblot and flow cytometry. Signaling pathways involved in altering PD-L1 expression were examined. Strategies to maximize the effect of the BRAFi therapy were studied including MEKi, MEKi combinations, and additional pathways including PI3K.

Results: Melanoma cells resistant to BRAFi exhibit increased MAPK signaling and promotion of PD-L1 expression. PD-L1 expression is transcriptionally modulated by c-Jun and augmented by STAT3. MEK inhibition (MEKi) regains down regulation of MAPK signaling and suppresses the production of PD-L1. MEKi in melanoma cells demonstrates duel therapeutic effects with simultaneous suppression of PD-L1 expression and induction of apoptosis. By combining MEKi with BRAFi, an additive effect on the inhibition of PD-L1 expression results.

Conclusions: We report a novel mechanism that suppresses pre-existing immune responses in melanoma patients receiving BRAFi therapy. BRAFi resistance leads to increased expression of PD-L1 in melanoma cells, mediated by c-Jun and STAT3. MEKi may be feasible to counteract BRAFi resistance of MAPK reactivation and also for the...
additive effect of PD-L1 suppression. Potential therapeutic benefits of combining targeted inhibitors and immune modulation to improve patient outcomes should be investigated.
INTRODUCTION

Use of the selective BRAF inhibitor (BRAFi) vemurafenib (PLX4032/RG7204) has altered the paradigm for treating metastatic melanoma tumors with V600E mutations, demonstrating high response rates and survival advantage over chemotherapy(1-3). However, the median progression-free survival to treatment with vemurafenib is approximately 7 months. As a result, an active area of investigation remains identification of means to improve the durability of selective BRAFi treatment. The combination of BRAF plus MEK inhibition is currently being investigated as a means to overcome BRAFi resistance. In addition, the success of CTLA-4 blockade with ipilimumab at improving survival with a subset of patients experiencing durable benefit(4, 5), suggests that combinations with immune therapy may provide significant improvement to BRAFi alone. As a result, improved understanding of the molecular interactions with immune regulation provides the basis for the development of rational combinations for patients.

Several mechanisms of resistance to vemurafenib have been identified as leading to downstream activation of MAPK. These include: 1. Deregulated receptor tyrosine kinase (RTK) activity, e.g., platelet-derived growth factor receptor β (PDGFRβ) or insulin-like growth factor 1 receptor (IGF1R)(6, 7); 2. Mutational activation of \( \text{NRAS} \) and MEK1(8); 3. Switching among RAF isoforms(7); 4. Dimerization of aberrantly spliced BRAF (V600E)(9). Taken together, these studies indicate the reactivation of the downstream kinases in the MAPK pathway as the mechanism of resistance development. Oncogenic signaling may alter the ability of the host to mount an effective immune response against certain cancers. As a result, therapies targeting oncogenic mutations may subsequently influence both the immunogenicity of the tumor as well as the ability of the host to mount an effective immune response.
response. *In vitro* data suggests that BRAFi does not significantly affect viability or function of lymphocytes\(^{(10)}\) and that vemurafenib induces a lymphocytic infiltrate in melanoma tumor deposits in treated patients\(^{(11, 12)}\).

Programmed death-1-ligand 1 (PD-L1), also denoted as B7-H1, is a cell surface protein of the B7 family\(^{(13-15)}\). PD-L1 is one of the two ligands for program death receptor 1 (PD-1), a co-stimulatory molecule that negatively regulates T cell immune responses. PD-L1 is an inducible ligand expressed on a variety of tissues including solid tumors, by which cancer cells acquire resistance to T cell killing. Specifically, ligation of PD-L1 expressed on cancer cells to PD-1 expressed on T cells suppresses T cell activation, proliferation, and induces T cell apoptosis. The critical role of PDL1-PD1 interaction has been depicted in both preclinical models and clinical outcomes\(^{(16)}\). PD-L1 blockade using antibodies has anti-tumor activity in numerous preclinical models. Furthermore, PD-L1 expression is correlated with poor clinical prognosis for a number of cancers including renal, breast cancers and esophageal. As a result, increased PD-L1 expression by cancer cells remains a fundamental escape mechanism from host immunity. Understanding the molecular mechanisms that modulate PD-L1 expression would likely lead to improved treatments for patients.

The regulation of PD-L1 expression is complex and regulated by multiple signaling pathways, some that have been investigated including MAPK\(^{(17)}\), phosphoinositide 3-kinase/protein kinase B (PI3K/PKB), and the Janus kinase/signal transducers and activators of transcription (JAK/STAT)\(^{(18)}\). Factors that influence PD-L1 expression may also be dependent on the cell type. In the current study, established BRAFi-resistant melanoma cell lines were examined for relevant changes in PD-L1 expression.
Further determination of molecular targets that influence PD-L1 expression in melanoma cells and potential rational combinations for improved efficacy were explored.

MATERIALS AND METHODS

Cell Culture and Mutational Characterization. Melanoma cell lines including K028, M34, and K029 were previously established and expanded from fresh tissues. Tumor samples were obtained from patients on Dana-Farber Cancer Institute/Harvard Cancer Center institutional review board-approved protocols. Melanoma cell lines SK-Mel-5 and A375 were purchased from ATCC. Cell cultures were maintained in DMEM containing 10% (v/v) fetal bovine serum. Mutational analyses of NRAS, BRAF and MEK were done by direct PCR product sequencing(19). The selection of these cell lines in the study are based on detection of mutation at BRAFV600E before and after establishment of BRAFi resistance, whereas no mutations of NRAS and MEK1 have been identified in all of BRAFi resistant cell lines using direct PCR product sequencing on the mutated hotspots of these genes.

Small Interfering RNA Transfections. Cells were transfected with siRNAs (20nM) using Lipofectamine TM RNAiMAX reagent (Invitrogen) as per the manufacturer's instructions. siRNA constructs were purchased from Dharmacon including RPS6; STAT3; c-Fos; human c-Jun; STAT5A; and STAT5B. siRNA constructs of ERK1/2, JNK and p38 were purchased from Cell Signaling Technology, Inc. siRNA constructs were diluted in OPTI-MEM I reduced serum medium (GIBCO) to a final concentration of 20 nM and mixed with LipofectamineTM RNAiMAX reagent. Non-target siRNA (QIAGEN) was used as a control.
Drug Treatment. The following compounds and reagents were used: PLX4032 was provided by the laboratory of Dr. Nathanael Gray at Dana-Farber Cancer Institute (Shanghai Haoyuan Chemexpress Co., Ltd.). STAT3 inhibitor Nifuroxazide was purchased from Santa Cruz Biotech. LY294002 and U0126 were purchased from Cell Signaling Technology. RAD001 was purchased from Selleck Chem. A stock solution was prepared in DMSO or water at a concentration of 10 mmol/L or 10mg/ml and maintained at –20°C. Drug was diluted in DMSO or water for working solutions and used at concentrations ranging from 0.1 nmol/L to 25 μmol/L for the treatment of cell lines.

Quantitative Real-time PCR for Relative RNA Levels of PD-L1. Total RNA was extracted using the Rneasy Mini Kit (Qiagen), and reverse transcription reactions were performed using the SuperScript First-Strand Synthesis System (Invitrogen). Real-time PCR analyses were performed using the ABI 7300 real-time PCR machine (Applied Biosystems). The primers used for qPCR is AGTGGTAAGACCACCACCACCAAT (Forward) and TCATTGAGGATGTCCAGAGGT (Reverse). To discriminate specific from nonspecific cDNA products, a melting curve was obtained at the end of each run. Data were normalized to β-actin levels in the samples in triplicates. Relative expression is calculated using the delta-Ct method using the following equations: ΔCt (Sample) = Ct (Target) – Ct (Reference); relative quantity = 2−ΔCt.

Human Phospho-MAPK Array. The BRAFi-resistant melanoma lines M34 and K028 were plated in DMEM with 10% fetal bovine serum and allowed to adhere overnight. Media was then exchanged containing PLX4032 at 2 μM and incubated at 37°C, 5% CO2. The parental melanoma lines M34 and K028 treated with DMSO served as controls. Cell
lysates (300 μg total proteins per array) were applied following the manufacturer's instructions (R & D Systems).

**Flow Cytometry Analysis.** Cells were harvested and stained with APC-conjugated anti-human CD274 (Clone 29E.2A3, Biolegend), Annexin V-FITC (BD Pharmingen) or isotype-matched control antibody (BD Pharmingen). Samples were analyzed on a FACS Caliber Flow Cytometer using CellQuest (BD) utilizing FlowJo (TreeStar) software. Standardized fluorescence intensities were calculated by dividing the median fluorescence intensities of specific antibodies by the median fluorescence intensities of isotype controls.

**Immunoblot Analyses.** Cells were seeded in six-well plates in DMEM containing 10% fetal bovine serum. At 60% to 70% confluence, cells were then treated with the relative agent for 30 minutes to 72 hours at various concentrations. Cells were lysed in Cell Lysis Buffer (Cell Signaling Technology) containing protease inhibitor cocktail (Roche Diagnostics). Proteins were separated on 7.5–12.5% SDS-polyacrylamide gels. immunoblot analysis was performed as described previously(19). The following antibodies were purchased from Cell Signaling Technology, Inc: phospho-p44/42 mitogen-activated protein kinase (Thr202/Tyr204); phospho-AKT/protein kinase B (Ser473); phospho-S6 ribosomal protein (Ser240/Ser244); phospho-STAT3 (Tyr705); phospho-Stat3 (Ser727); phospho-STAT5 (Tyr 694); phospho-c-Jun, total AKT/protein kinase B; total STAT5; total JNK, total STAT3, phospho-STAT5; total c-Jun. Human B7H1/PDL1 polyclonal was purchased from R&D System; Total p44/42 mitogen-activated protein kinase and total S6 ribosomal protein (H-4) were from Santa Cruz Biotechnology, Inc; and actin was from Sigma.
**AP1 ELISA Assay** The BRAFi-resistant melanoma lines A375, K028, M34, SK-Mel-5 and K029 were plated in DMEM with 10% fetal bovine serum and allowed to settle down overnight. The cells were then changed to fresh DMEM with 10% fetal bovine serum containing PLX4032 at 2 μM and incubated at 37°C, 5% CO2 for 45 minutes. The parental melanoma lines without treatment of PLX4032 as controls. The nuclear portions of protein were prepared using the nuclear extraction kit (Panomics). The AP1 activation was quantified using the transcription factor ELISA kit following the manufacture’s in instructions (Panomics).

**Statistical Analysis.** Experiments were repeated in triplicate, with the exception of the comparisons of PD-L1 expression before and after knockdown of c-Jun, which had two replicates. Data are expressed as mean ± SD. Comparisons of group means were assessed using general linear models. For multiple pair wise comparisons against a control within a cell line, Dunnett’s correction was used to preserve the overall type-1 error. Statistical significance was defined as $P < 0.05$.

**RESULTS**

**PD-L1 expression is increased in melanoma cell lines resistant to PLX4032.** A panel of melanoma cell lines harboring BRAF V600E mutations were analyzed by immunoblot for PD-L1 expression before and after the establishment of BRAFi resistance (Figure 1A) and confirmed for cell surface expression by flow cytometry (Figure 1B, C and D). Although all of the parental melanoma lines showed detectable levels of mRNA for PD-L1, only one line (M34) expressed significant level of PD-L1 pre-treatment (94.67±4.99%). Following the establishment of resistance to PLX 4032, all of melanoma lines expressed
significantly higher levels of PD-L1 than their parental line counterparts, 6.2-fold to 10.9-fold increase by the frequency of PD-L1 positive cells and 7.8-fold increase in the median florescence intensity for M34 cells (Figure 1C and D). PD-L1 expression remained increased in the resistant cell lines in the absence of PLX4032 for up to three months following drug withdrawal.

**Acquired BRAFi-resistant melanoma cell lines exhibit increased signaling of multiple pathways.** We assessed status of signaling pathways in melanoma cell lines before and after establishment of BRAFi resistance. A combined analyses of the Phospho-MAPK Array and immunoblot revealed that in the presence of 10%FCS containing medium level concentration of PLX4032 (2uM), the BRAFi-resistant cell lines developed dramatic increased activation in MAPK signaling pathways at multiple levels including extracellular signal-regulated kinase (ERK1/2), c-Jun N-terminal kinase (JNK1, 2 and 3) and p38 (α-, β-, δ and γ- isoforms) (Supplemental Figure 1A and B). In addition, further characterization of these BRAFi-resistant lines revealed c-Jun phosphorylation and phosphorylation of STAT3 and 5 enhanced compared to parental lines (Supplemental Figure 1C and D).

**PI3K-mTOR-S6 signaling pathway influences PD-L1 expression in melanoma cells.** To assess the molecular pathways involved in PD-L1 expression in melanoma cells following BRAFi, we began to investigate the effects on PI3K signaling due to its potential influence on inducible cell surface receptors. Most of the BRAFi resistant cell lines exhibited an increase of phosphorylation of S6 related to PI3K signaling (Supplemental Figure 2A). We next sought a pharmacologic effect of PI3K inhibition on PD-L1 expression. Treatment of BRAFi-resistant melanoma lines with PI3K inhibitor LY294002 decreased pAKT in all lines as expected. Chemical PI3K inhibition also significantly decreased PD-L1
transcript abundance and protein expression in BRAFi-resistant cell lines (Supplemental Figure 2B and C). These results have further confirmed by immunoblot staining (Data not shown).

We then tested the effects of ribosome protein (S6) inhibition on PD-L1 expression. Treatment of the BRAFi-resistant melanoma cells with the mTOR inhibitor RAD001 did lead to substantial decrease of S6 phosphorylation in the three BRAFi-resistant lines K028PR, M34PR and K029PR, however, such an inhibition failed to elicit any inhibitory effect on the expression of PD-L1 (Supplemental Figure 2D). Furthermore, a completed depletion of S6 using siRNA did not inhibit PD-L1 expression (Supplemental Figure 2E).

Taken together, while pharmacologic inhibition of PI3K partially reduces PD-L1 expression, inhibition downstream of PI3K by either pharmacologic inhibition of mTor or siRNA knockdown of S6 fails to significantly reduce PD-L1 expression. This suggests that the PI3K-mTOR-S6 pathway may not have significant impact in the modulation of PD-L1 expression in BRAFi-resistant melanoma cells.

**Increased activation of MAPK promotes PD-L1 expression in the BRAFi-resistant melanoma cells.** Having examined the PI3K-mTOR-S6 pathway involvement in PD-L1 expression, we next examined the possible contribution of the MAPK pathway. Treatment with the MEK inhibitor U0126 led to a prolonged decrease mRNA level of PD-L1 lasting for 24 hours (Figure 2A). Correspondingly, treatment of the BRAFi-resistant cells with U0126 led to a inhibition on phosphorylation of ERK1/2 (Supplemental Figure 3), which resulted in significant decrease of PD-L1 expression (Figure 2B).

We further confirmed the role of ERK-MAPK pathway in modulation of PD-L1 expression using siRNA technology. With substantial depletion of ERK1/2, knockdown of
ERK1/2 led to nearly 3.6-fold decrease of PD-L1 expression in K028 resistant line, 4.3-fold decrease in M34 parental line, and 1.7-fold decrease in M34 resistant lines, respectively (Figure 2C and D). In the resistant cells, PD-L1 expression decreases by flow cytometry, specifically in the cells that are not undergoing apoptosis. These results, in line with inhibition of PD-L1 expression induced by treatment of melanoma cells with MEKi U0126, supports the notion that ERK-MAPK signaling plays a critical role in modulation of PD-L1 expression in melanoma cells.

**c-Jun promotes PD-L1 expression in melanoma cells which can be enhanced via cooperation of STAT3.** Having defined the significant role of MAPK signaling in modulation of PD-L1 expression in melanoma cells, factors involved in modulation of PD-L1 expression at transcriptional level was investigated next. The BRAFi-resistant melanoma cells had an increased activity of c-Jun (Figure 3A), a transcriptional factor primarily driven by MAPK signaling. This suggested a role of c-Jun in modulation of PD-L1 expression. We further tested the effects of MEKi(U0126) on the activity of c-Jun. Treatment of the BRAFi-resistant cells with U0126 led to a significant inhibition of both the levels of phosphorylation and protein (Figure 3B). Together, this evidence supported further investigation of c-Jun activity in the transcriptional regulation of PD-L1.

To test the role of c-Jun as the transcriptional factor of PD-L1 in the BRAFi-resistant melanoma cells, we knocked down c-Jun using siRNA (Figure 3C) and determined the effects on PD-L1 expression (Figure 3D and E). Knockdown of c-Jun led to significant decrease of PD-L1 in K028 resistant and parental M34 line, as well as near complete inhibition of PD-L1 expression in M34 resistant line (Figure 3D and E).
Previous studies have shown that c-Jun acts as enhancer to modulate the expression of their targets forming a complex with its co-factor STAT3(20). Along with the inhibition of c-Jun activity by MEK inhibition, treatment of the BRAFi resistant melanoma cell lines by U0126 also led to dephosphorylation of STAT3 at sites of both of Tyr705 and Ser 727 (Figure 4A). We then examined the role of STAT3 in modulation of PD-L1 expression in the BRAFi-resistant melanoma cells. Knockdown of STAT3 alone induced significant decreases of PD-L1 expression in both of the parental M34 cells (3.3-fold decreased) and the BRAFi-resistant M34 cells (6.6-fold decreased) (Figure 4B). Moreover, combined with knockdown of c-Jun, knockdown of STAT3 led to a synergistic inhibition of PD-L1 expression in the parental M34 lines and the BRAFi-resistant M34 line. Treatment of the BRAFi-resistant cells with the STAT3 inhibitor Nifuroxazide also led to significant decrease of PD-L1 expression (Figure 4C). These data indicated that STAT3 may be involved in the modulation of PD-L1 with co-operation of c-Jun. Both knockdown of STAT5 or pharmacological inhibition of STAT5 failed to decrease the expression of PD-L1 in both parental and resistant lines (Figure 4D).

**Duel effects of MEKi or combination limits the induction of PD-L1 expression while inducing melanoma cell apoptosis.** MEKi is an important therapeutic strategy for BRAF mutated melanoma cells either as a single agent or in combination with BRAFi. The MAPK pathway may have dual effects on tumor growth/survival and host immunity. We first tested the effect of inhibition of MAPK signaling via BRAFi, MEKi, or the combination on ERK1/2 and c-Jun signaling (Figure 5A). MEKi, BRAFi, or the combination markedly decreased the expression of PD-L1 in parental melanoma cells by immunoblot (Figure 5B).
These effects on PD-L1 were confirmed as well by flow cytometry as well as the significant induction of apoptosis by either agent (Figure 5C).

To investigate whether MEK inhibition has similar effects in BRAFi-resistant cells as compared to parental melanoma cells, immunoblot of resistant cell lines treated with U0126, PLX4032, or the combination demonstrated reduction in both ERK1/2 and c-Jun signaling (Figure 5D). Treatment of BRAFi-resistant melanoma cell lines demonstrated reduction in PD-L1 expression with exposure to U0126 (Figure 6E). The combination of BRAFi and MEKi further decreased PD-L1 expression in the K028 and K029 resistant cell lines (Figure 5E). Combined MEKi with BRAFi in the BRAFi-resistant melanoma cells significantly decreased PD-L1 expression by flow cytometry and decreased apoptosis in M34R cells (Figure 5F).

**Proposed signaling mechanism by which c-Jun and STAT3 influence PD-L1 expression in melanoma cells.** In BRAFi resistant V600E melanoma cells, activation of MAPK and PI3K pathways lead to c-Jun and STAT3 dependent induction of PD-L1 (Figure 6). The AP-1 dependent enhancers and JAK3/STAT3 dependent PD-L1 promoter subsequently results in increased PD-L1 expression. MEKi and STAT3 inhibition can counteract this resulting in decreased PD-L1 expression in melanoma cells.

**DISCUSSION**

Accumulating evidence suggests that re-activation of signaling downstream to RAF in the MAPK pathway is a major mechanism of resistance to BRAF inhibition in melanoma therapeutics(21, 22). In the present study, we found that the re-activation of MAPK leads to increased expression of the inducible ligand PD-L1. This upregulation
involves c-Jun and STAT3 signaling as a consequence of BRAFi resistance. This is the first evidence that demonstrates that re-activation of MAPK signaling is not only essential to BRAFi resistance, but also provides an additional mechanism of therapeutic resistance via modulation of host immune responses. Since fully human monoclonal antibodies to PD-1 and PD-L1 have demonstrated significant clinical efficacy that appears associated with tumor expression of PD-L1 as a biomarker(23-25), interventions that alter PD-L1 expression may have additional implications for immune therapy.

In addition to enhanced activation of ERK-MAPK that occurs in BRAFi-resistant melanoma cells(6, 9, 26-29), JNK-MAPK and P38-MAPK signaling were also enhanced. The role of these two sub-MAPK pathways in BRAFi resistance remains poorly defined. While ERK-MAPK is generally involved in the control of cell proliferation, JNK signaling usually mediates responses to various forms of cellular stresses, such as damage repair mechanism, cell growth arrest and cell death(30). Indeed, upon substantial depletion of ERK1/2 or JNK, a single knockdown is sufficient to elicit significant apoptosis, especially in the melanoma cells with BRAFi resistance. Furthermore, synergistic induction of apoptosis is able to be reached upon combined knockdown of ERK1/2 with JNK, suggesting an important role for these sub-pathways in melanoma cell survival. Interestingly, both ERK1/2 and JNK are direct kinases that modulate the activity of c-Jun, an inducible transcription factor that directs changes of gene expression such as PD-L1(31), suggesting these two kinases may be involved in the modulation of PD-L1 in response to multiple extracellular stimuli. Either the inhibition of activity of its upstream kinase MEK with MEKi(U0126) or the physical knockdown of ERK1/2 or JNK are able to reduce PD-L1 expression in melanoma cells(32-35). Taken together, these data suggest both ERK1/2 and JNK may modulate PD-L1
expression, especially the de novo up-regulation of PD-L1 induced by BRAFi via the paradoxical activation of MAPK. In agreement with this observation, the present study has shown that depletion of ERK1/2 and JNK synergistically suppresses PD-L1 expression, but fails to induce similar effect on the induction of apoptosis.

The increased activity of c-Jun in melanoma cells with BRAFi resistance is closely related to levels of PD-L1. Moreover, knockdown of c-Jun is necessary and sufficient to suppress expression of PD-L1 on melanoma cells either sensitive or resistant to BRAF inhibition. We further observed that a combined depletion of c-Jun and STAT3 resulted in a synergistic inhibition of PD-L1 while a single knockdown of either c-Jun or STAT3 only led to partial inhibition, indicating a co-operation of these two factors in modulation of PD-L1 expression in the melanoma cells without established BRAFi resistance. A similar transcriptional synergy of c-Jun and STAT3 was also observed in the BRAFi-resistant cells; however, in this case, a single knockdown of c-Jun is sufficient to inhibit PD-L1 expression, suggesting that the BRAFi-resistant melanoma cells are more dependent on c-Jun for PD-L1 expression.

In the present study, we have identified that enhanced phosphorylation of STAT3 at Tyr 705 as well as Ser 727 are induced in the BRAFi-resistant melanoma lines. How the activity of STAT3 is elevated in BRAFi resistant melanoma cells requires further investigation. Interestingly, the knockdown of c-Jun alone or STAT3 alone is sufficient to reduce PD-L1 expression in both parental and BRAFi-resistant melanoma cells. However, this failed to induce significant apoptosis.

The role of the PI3K-AKT/PKB-mTOR pathway in BRAFi resistance has been previously studied. Jiang et al.(36) reported that activation of the PI3K/AKT pathway
accounts for, at least in part, the MEK-independent survival of the BRAFi-resistant melanoma cells. The increased PI3K/AKT activation is the result of extra-cellular stimuli in some cases. Previous reports in glioma, breast, and prostate carcinoma(37, 38) suggest that the expression of PD-L1 is dependent on the PI3K-AKT-mTOR-S6K1 pathway. In the present study, we found that blockade of this pathway either by PI3Ki(LY940002), mTORi(Rad001), or by depletion using siRNA has moderate effects on PD-L1 expression both in melanoma cells sensitive or resistant to BRAFi.

An important model of tumor homeostasis incorporates host immune surveillance to prevent tumor outgrowth during the earliest stages of tumor growth(39, 40). Prolonged immunosuppression is associated with high occurrence of secondary malignancies (e.g. immunosuppressive treatment not only fosters growth of pre-existing tumors but also promotes incidences of secondary cancers in kidney transplant recipients)(39). One of the adverse effects of selective BRAF inhibition treatment in melanoma is the development of secondary squamous cell carcinomas (keratoacanthoma-type) of the skin. These lesions harbor preexisting H-RAS mutations that upon driving of the paradoxical activation of MAPK lead to accelerated growth of the lesions and squamous-cell carcinomas(41). It would also be of interest to investigate a potential role of PD-L1 expression via the above mechanism in the formation of secondary neoplasms. In agreement with this possibility, Cao et al. found that over-expression of PD-L1 promotes carcinogenesis in a methylcholantrene (MCA)-induced model of squamous cell carcinoma (SCC), and that blockade of PD-1/PD-L1 signaling reverses the incidence of the secondary squamous cell carcinoma(42, 43).

The expression of PD-L1 has been associated with MAPK signaling in other tumor types. PD-L1 expression was found to be regulated by MEK/ERK signaling in anaplastic
large cell lymphoma and Hodgkin lymphoma(35). In acute myeloid leukemia, protection of blasts from cytotoxic T cells can be reversed by MEK inhibition(44). The importance of molecularly driven expression of PD-L1 is further exemplified through 9p24.1 amplification in nodular sclerosing Hodgkin lymphoma and primary mediastinal large B-cell lymphoma that result in increased expression of PD-L1 and induction by JAK2(45). This strongly suggests that the oncogenic drivers for malignant transformation also result in expression of the inducible ligand PD-L1 as a generalizable principle to the development of the malignant phenotype.

Much of the characterization of the mechanisms of the BRAFi resistance in melanoma suggested that targeting downstream effects of MAPK may prove effective, such as the targeting of MEK. This has led to a number of clinical investigations administering a MEK inhibitor following a BRAF inhibitor, or giving the combination of a MEK inhibitor and BRAF inhibitor to improve clinical outcomes over BRAF inhibition alone. Interestingly, the present study reveals that inhibition of ERK-MAPK by MEKi not only suppresses PD-L1 expression but also induces apoptosis on the melanoma cells. This suggests that MEK inhibition may not only have an effect on tumor cell death, but also strongly influences PD-L1 inducible ligand that would make it more susceptible to immune destruction. This not only supports the further study of BRAF inhibition plus MEK inhibition in BRAF mutated tumors, but suggests a role of MEKi in immune modulation that deserves further exploration. There remains concern of the effects of these small molecule inhibitors on immune cell function. Investigators have suggested a dual role to these inhibitors. Specifically, vemurafenib may improve T cell function through the paradoxical activation of MAPK. It has been demonstrated that vemurafenib at least preserves the viability and function of
lymphocytes(10). There is greater concern that MEKi may be more detrimental to T cell specific immune function. The dual roles of these inhibitors in modulating tumor cell expression of PDL1 and T cell function require further clinical investigation. Further clinical exploration of combinatorial approaches of BRAFi and/or MEKi with blockade of the PD-1/PD-L1 pathway is also warranted.

The influences of c-Jun and STAT3 on PD-L1 expression reveal the importance of signaling pathways in determining the immune evasion potential of a cancer cell. As a mechanism, these findings further support the observations of AP-1 dependent enhancer for PD-L1 expression and the JAK3/STAT3 dependent PD-L1 promoter. AP-1 dependent PD-L1 expression in Hodgkin Lymphoma was recently described whereby constitutive AP-1 activity in the presence of EBV infection induces PD-L1 expression(31). In addition, amplification of 9p24.1 in subtypes of non-Hodgkin lymphoma results in increased JAK2 expression and associated PD-L1 promoter activity(45). The enhancer/promoter elements of inducible immune regulatory ligands may play a critical role in cancer immune evasion. Furthermore, MEK/ERK signaling has been previously described to regulate PD-L1 expression in anaplastic large cell and Hodgkin lymphoma(35). MEK inhibition has also been shown to reverse PD-L1 mediated inhibition of cytotoxic T cell function against acute myeloid leukemia cells(44). These observations along with the current report confirm the generalized importance of MAPK signaling in influencing PD-L1 expression. As a result, molecular mechanisms for immune evasion by tumor cells and subsequent contribution to the immunosuppressive tumor microenvironment can be established.

In melanoma, the significance of therapeutically targeting the oncogenic driver mutation BRAF has dramatically altered treatment for patients(1, 3). Selective BRAF
inhibition has been shown to enhance T-cell recognition of melanoma(11) as well as induce a brisk infiltration of T-lymphocytes into metastatic deposits(12). In the current study, the development of BRAFi resistance results in the melanoma cell avoiding destruction through immune evasion by increasing PD-L1 expression. It will be important to characterize this phenomenon further to examine the relationship of PD-L1 and PD-1 in those infiltrates. It has also been reported that BRAF (V600E) expression in melanoma further induces immune suppression by the induction of IL-1 in stromal fibroblasts(46). The current study adds to the fundamental understanding of the molecular mechanisms involved in tumor immune evasion within the tumor microenvironment. Importantly, as significant numbers of pharmacologic tools now exist to alter these signaling pathways, such influence must be appreciated in order to engineer better combinatorial therapeutic approaches.

In summary, we have identified a novel mechanism that contributes to clinical resistance of BRAFi in patients with metastatic melanoma. Molecular resistance to BRAFi as exemplified by increased MAPK signaling prompts PD-L1 expression via enhancing the activity of c-Jun and its co-factor, STAT3. Based on these findings, molecular induction of PD-L1 in tumors may have dramatic effects on clinical efficacy. Appreciating the dual roles of small molecule inhibition on tumor cell survival as well as immune recognition will be an important basis for future clinical investigation strategies for combination of small molecules and combinations of small molecules with immune checkpoint blockade in order to improve patient outcomes.
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Figure 1. Increased PD-L1 expression in BRAFi-resistant melanoma cells. (A) Immunoblot analysis of melanoma parental (P) and PLX4032 resistant (R) melanoma lines demonstrate increased PD-L1 expression in resistant lines. The established BRAFi resistant cell lines A375, K028, M34, SK-Mel-5, and K029 were challenged with 10%FBS DMEM containing PLX4032 (2 μM) for 72 hours. The corresponding parental melanoma lines grew without treatment of PLX4032 as controls. (B) Flow cytometry of parental and PLX4032 resistant cell lines confirm changes in PD-L1 expression on the cell surface. Cells were gated for PD-L1 positivity (y-axis) and scc staining (x-axis). The flow profile is representative of three independent experiments. The red population represents PD-L1 positive cells. (C) Flow cytometry comparisons for PD-L1 expression before (P) and following (R) establishment of BRAFi resistance. The data are the average of three independent experiments. (D) Due to the high percentage staining in the M34 parental line, changes in the median fluorescence intensity (MFI) as a function of developing BRAFi resistance were examined. Represented is the fold change in parental and PLX4032 resistant M34 cells expression of PD-L1. The data are the average of three independent experiments.

Figure 2. The MAPK pathway influences changes in PD-L1 expression in PLX4032 resistant melanoma cells. (A) The MEK inhibitor U0126 decreases PD-L1 expression at the mRNA level in melanoma cells. Both the high PDL1 expressing M34 parental line and the BRAFi-resistant lines K028, K029, and M34 were treated with U0126(U) (20μM) for 4 hours. Cells were treated with DMSO(D) for controls. PD-L1 mRNA levels were analyzed by qRT-PCR. The data are the average of three independent experiments. (B) MEKi with
U0126 inhibits PD-L1 expression at the protein level. PD-L1 frequencies were analyzed by flow cytometry at 72 hours as described in A. The data are the average of three independent experiments. (C) Effects on PD-L1 expression by knockdown of ERK1/2 and JNK using siRNA. The parental M34 line and the PLX4032-resistant lines K028 and M34 were treated with siRNAs (10nM) targeting ERK1/2 alone, JNK alone, or the combination for 48 hours and analyzed by immunoblotting. JNK was not targeted for K028R since it was not seen to be upregulated in the kinase array. Cells were treated with non-target siRNA (NT) as controls. The blot is representative of three independent experiments. Example of flow cytometric analyses of PD-L1 expression and apoptosis on melanoma cells following knockdown of ERK1/2 alone, JNK alone, or the combination (right). (D) Flow cytometry profiling of PD-L1 expression before and after knockdown of ERK1/2 alone, JNK alone, or the combination in parental M34 and PLX4032 resistant K028 and M34 cell lines. The data are the average of three independent experiments. NT = nontarget siRNA. Represented are percent cells staining positive for PD-L1 (left) and the percent cells positive for Annexin V evaluation for apoptosis (right). P-values are relative to NT for each cell line. (* represents p < 0.001)

Figure 3. c-Jun promotes PD-L1 expression relevant to BRAFi resistance. (A) AP-1 activity assay in parental (P) and PLX4032 resistant (R) cell lines. The data are the average of three independent experiments with duplicated wells of each sample. (B) Immunoblot analyses of ERK1/2 and c-Jun in the PLX4032-resistant melanoma cells lines without treatment (DMSO, D) or in the presence of the MEK inhibitor U0126(U) (20 μM) for 30 minutes. (C) Effects on PD-L1 expression by knockdown of c-Jun using siRNA. The parental
line M34 and PLX4032-resistant lines K028 and M34 were treated with siRNAs (10nM) targeting c-Jun for 48 hours and subjected to immunoblotting. Cells were treated with non-target siRNA(NT) as controls. The blot is representative of three independent experiments. (D) Examples of flow cytometric analysis of PD-L1 expression as well as apoptosis by Annexin V staining following 96 hours of treatment. The flow cytometry profiles are representative of three independent experiments. (E) Comparisons of PD-L1 expression before and after knockdown of c-Jun in the parental M34 line, the PLX4032 resistant M34 and K028 cell lines. The data are the average of two independent experiments. NT = nontarget siRNA. Represented are the percentage of cells that stain positive for PD-L1 and the percentage of cells positive for Annexin V evaluation for apoptosis.

Figure 4. STAT3 influences PD-L1 expression in conjunction with c-Jun. (A) Immunoblot analyses of STAT3 activity in BRAFi-resistant melanoma cell lines without treatment (DMSO, D) or in the presence of U0126(U) (20 μM) for 30 minutes. (B) Effects on PD-L1 expression by knockdown of c-Jun, STAT3, or the combination using siRNA. Both parental and BRAFi-resistant lines (M34) were treated with siRNA (10 μM) targeting c-Jun alone, STAT3 alone, or the combination for 48 hours. Cells were treated with non-target siRNA (NT) as controls. Immunoblot analyses of c-Jun and STAT3 (top left). The blot is representative of three independent experiments. Examples of flow cytometric analysis of PD-L1 expression as well as apoptosis (right). The flow cytometry profiles are representative of three independent experiments. Comparison of PD-L1 expression before and after knockdown of c-Jun alone, STAT3 alone, or the combination in parental and PLX4032 resistant M34 cell lines (bottom left). The data are the average of three
independent experiments. NT = nontarget siRNA. Represented are percent cells that stain positive for PD-L1 and percent cells positive for Annexin V evaluation of apoptosis. (C) Pharmacological suppression of STAT3 activity decreases PD-L1 expression in melanoma cells. Both parental and BRAFi-resistant cell lines (M34) were treated with Nifuroxazide (5μM) for 30 minutes (immunoblot) or 72 hours (flow cytometry). Cells were treated with DMSO as a control. Immunoblot analysis of STAT3 activity (top). The blot is representative of three independent experiments. Flow cytometric analyses of PD-L1 expression as a function of drug exposure (bottom). The flow profile is representative of three independent experiments. (D) The effect on PD-L1 expression by knockdown of STAT5 in melanoma cells. The same cell lines as described in (A) were treated with siRNAs (10nM) targeting STAT5. Cells treated with non-target siRNA(NT) were used as controls. Immunoblot analysis of STAT5 (top). The blot is representative of three independent experiments. Flow cytometric analysis of PD-L1 expression and apoptosis (bottom left). The flow cytometry profile is representative of three independent experiments. Statistical analysis of PD-L1 expression before and after knockdown of STAT5 in parental and PLX4032 resistant M34 cell lines (bottom right). The data are the average of three independent experiments. NT = nontarget siRNA. Represented are percent cells that stain positive for PD-L1 and percent cells positive for Annexin V evaluation for apoptosis. (* represents p < 0.001)

**Figure 5. MEKi decreases PD-L1 expression in BRAFi resistant cells.** (A) Immunoblot analyses of the activities of ERK1/2 and c-Jun in the K028, M34, and K029 parental lines treated with U0126(U) (20 μM), PLX4032(PLX) (10 μM), or the combination of U0126 (20 μM) and PLX4032 (10 μM) for 30 minutes. Cell were treated with DMSO(D) as controls.
(B) Immunoblot example demonstrating decrease in PD-L1 expression in parental M34 cells treated with U0126, PLX4032, or the combination. (C) Flow cytometry profiling for PD-L1 expression and apoptosis. The data are average of three independent experiments. (D) The same characterization as in (A) in the BRAFi-resistant lines K028, M34, and K029. (E) Immunoblot demonstrating the change in PD-L1 expression in BRAFi-resistant cell lines to U0126, PLX4032, or the combination. (F) Flow cytometry analyses of PD-L1 expression and apoptosis induced on the samples described in (D). The data are average of at least three independent experiments. P-values are relevant to DMSO(D) controls.

**Figure 6.** Proposed signaling mechanism for c-Jun and STAT3 influence on PD-L1 expression in BRAFi resistant V600E melanoma cells. Pathways that converge with c-Jun include MAPK and JNK. Reactivation of MAPK and PI3K signaling leads to c-Jun and STAT3 dependent enhancement/promotion of PD-L1 expression. This likely involves AP-1 dependent enhancer and JAK3/STAT3 dependent PD-L1 promoter. MEKi and STAT3 inhibition decreases PD-L1 expression.
Figure 2

A) Fold change of PD-L1 mRNA

B) %PD-L1+

C) Western blot analysis of ERK1/2 and JNK phosphorylation

D) %Annexin V+
Figure 4

A

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%PD-L1+

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Annexin V

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* P>0.05
Figure 5
Figure 6

RTK

PLX

BRAF
(V600E)

NRAS

RAF1

SRC

PI3K

MEK1/2

ERK1/2

c-Jun

STAT3

AP-1

c-Jun

STAT3

PD-L1
The Paradoxical Activation of MAPK in Melanoma Cells Resistant to BRAF Inhibition Promotes PD-L1 Expression that is Reversible by MEK and PI3K inhibition


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