The BRAF and MEK Inhibitors Dabrafenib and Trametinib: Effects on Immune Function and in Combination with Immunomodulatory Antibodies

Targeting PD-1, PD-L1, and CTLA-4

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Abstract

Purpose: To assess the immunologic effects of dabrafenib and trametinib in vitro and to test whether trametinib potentiates or antagonizes the activity of immunomodulatory antibodies in vitro.

Experimental Design: Immune effects of dabrafenib and trametinib were evaluated in human CD4⁺ and CD8⁺ T cells from healthy volunteers, a panel of human tumor cell lines, and in vivo using a CT26 mouse model.

Results: Dabrafenib enhanced pERK expression levels and did not suppress human CD4⁺ or CD8⁺ T-cell function. Trametinib reduced pERK levels, and resulted in partial/transient inhibition of T-cell proliferation/expression of a cytokine and immunomodulatory gene subset, which is context dependent. Trametinib effects were partially offset by adding dabrafenib. Dabrafenib and trametinib in BRAF V600E/K, and trametinib in BRAF wild-type tumor cells induced apoptosis markers, upregulated HLA molecule expression, and downregulated certain immunosuppressive factors such as PD-L1, IL1, IL8, NT5E, and VEGFA. PD-L1 expression in tumor cells was upregulated after acquiring resistance to BRAF inhibition in vitro. Combinations of trametinib with immunomodulators targeting PD-1, PD-L1, or CTLA-4 in a CT26 model were more efficacious than any single agent. The combination of trametinib with anti–PD-1 increased tumor-infiltrating CD8⁺ T cells in CT26 tumors. Concurrent or phased sequential treatment, defined as trametinib lead-in followed by trametinib plus anti–PD-1 antibody, demonstrated superior efficacy compared with anti–PD-1 antibody followed by anti–PD-1 plus trametinib.

Conclusion: These findings support the potential for synergy between targeted therapies dabrafenib and trametinib and immunomodulatory antibodies. Clinical exploration of such combination regimens is under way.

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Introduction

Immunotherapies and targeted therapies have distinctly different mechanisms of action and have both been shown to be efficacious in patients with advanced cancers (1, 2). It is expected that combinations of both modalities may create synergies with increased benefit for patients with cancer. To enable such combinations, it is critical to determine how targeted therapies affect immune function in the tumor microenvironment and peripheral systems. Immunogenic cell death, characterized by secretion of cell damage–associated hallmark molecules consisting of calreticulin (CRT), HSP70 and 90 proteins, HMGB1, and ATP, increased expression of tumor antigens and HLA molecules, and decreased expression of immunosuppression factors are desirable features for potential immune sensitization (3, 4). These effects may allow targeted agents to not only directly inhibit tumor growth, but also further enhance immune response by immunotherapy, through either tumor cell intrinsic or extrinsic immunomodulatory mechanisms, thus making the cancer therapy more effective and durable.

Inhibition of oncogenic MAPK signaling by dabrafenib, trametinib, or the combination of dabrafenib and trametinib has been an effective strategy and approved for the treatment of metastatic melanoma tumors bearing BRAF V600E and V600K mutations (5). The first generation of checkpoint immunomodulatory antibodies targeting either CTLA-4 or PD-L1/PD-1 has demonstrated impressive clinical activity resulting in durable responses in subsets of patients with various cancer types (1, 6, 7). BRAF inhibitors, such as vemurafenib, have been shown to increase immune response and efficacy in combination with immunomodulators in preclinical models (8, 9). However, MEK inhibitors, including trametinib, have been reported to be immunosuppressive in vitro, which has limited the in vitro assessment of MEK inhibitor combinations with immunotherapies (10). In this study, we assessed the immunologic effects of dabrafenib and trametinib at clinically relevant exposures on both immune and
tumor cells in vitro. The effect on immune cells by dabrafenib was consistent with the literature reports for BRAF inhibitors (10, 11). However, we found that the effect of trametinib on immune cells was both complex and context dependent. The antiproliferative effect of trametinib on T cells was partial and transient in vitro. Furthermore, we tested trametinib alone and in combination with several immunomodulatory antibodies in an immunocompetent syngeneic mouse model. Our in vivo data demonstrate the superior efficacy by the combination of trametinib with anti–PD-1 antibody concurrently or sequentially phased, when first treated with trametinib followed by trametinib plus anti–PD-1 antibody. These findings support clinical exploration of both trametinib and dabrafenib in combination with specific immunomodulatory antibodies.

Materials and Methods

Cell lines and reagents

Human melanoma cell line, A375PF11, was derived from a clonal isolate of the A375 cell line obtained from the American Type Culture Collection (ATCC). Human melanoma line YUSIT1 was obtained from Yale Dermatology Cell Culture Facility (12). Human melanoma lines: SK-MEL-24, CHL-1, HMVII, and SK-MEL-2; human non–small cell lung cancer (NSCLC) lines: Calu6, A549, and H358; and mouse colon carcinoma line, CT26, were obtained from the ATCC and cultured in RPMI with 10% fetal bovine serum (FBS) media. All cell lines were characterized by genotypic and RNA expression analyses using the Affymetrix 500K SNP chip and HG-U133Plus2 chip, respectively (Affymetrix, Inc.) for human lines, and using Exome Seq and RNA-Seq (Illumina) for CT26, and kept in culture for <3 months. 12R5-1, 12R5-3, 16R6-3, 16R5-5, and 16R6-4 were dabrafenib-resistant clones derived from A375PF11 (referred to henceforth as A375; ref. 13).

Human CD4+ and CD8+ T cells were isolated from whole blood using STEMCELL Technologies RosetteSep Human T Cell Enrichment Cocktails (STEMCELL Technologies) and Lympo-Prep density gradient medium according to the manufacturer's protocol. Human whole blood was obtained from GlaxoSmithKline’s blood donation unit (Upper Providence site, PA) under the Institutional Review Board (IRB) approval.

In vitro T-cell assays

Human CD4+ and CD8+ cells were activated with anti–CD3/anti–CD28 antibodies either in bead- or plate-bound forms. Trametinib and dabrafenib were added at same time or sequentially with activation. T-cell proliferation, cytokine secretion, and apoptosis induction, cell signaling, surface markers, and gene expression levels were measured. Protocols are described in the Supplementary Methods.

Human and mouse tumor cell assays

The expression levels of immunomodulators, HLA molecules, and tumor-associated antigens from tumor cells were determined by NanoString, RT-PCR, flow cytometry, and/or Western blot analyses. Procedures, antibodies, and reagents are described in the Supplementary Methods.

In vivo evaluation in CT26 murine carcinoma syngeneic mouse model

Female BALB/C mice (Charles River) received food and water ad libitum and were housed in GlaxoSmithKline in compliance with the recommendations of the Guide for Care and Use of Laboratory Animals. Tumors were established by subcutaneously implanting 5 × 10^4 CT26 cells in suspension into the right flank of mice. Tumor weights were calculated using the equation (l × w^2)/2, where l and w refer to the larger and smaller dimensions collected at each measurement. Treatments began at day 11 or 12 with tumor size 40 to 100 mm^3. Tumors were monitored and mice were euthanized when an endpoint was reached, defined as tumor volume greater than 2,000 mm^3, tumor ulceration, or study end (21 or 68 days after initial dosing), whichever came first. Tumor regressions, median tumor volume, and treatment tolerability were also considered.

Percentage tumor growth inhibition (% TGI) was defined as the difference between the mean tumor volume (MTV) of the designated control group and the MTV of the drug-treated group, expressed as a percentage of the MTV of the designated control group: % TGI = [1 – (MTV_{drug-treated}/MTV_{control})] × 100. The Kaplan–Meier method was carried out to estimate the survival probability of different treatment groups at a given time. The median time to endpoint (TTE) and its corresponding 95% confidence interval (CI) were calculated.

For pharmacodynamic analysis, fresh tumors, lymph nodes, spleen, serum, and whole blood were collected 4 and 24 hours after last dose on days 3 or 7. Flow cytometric analysis of lymphocytes from mouse blood, tumor tissues, lymph nodes, and spleens, cytokine analysis from serum, and gene expression and immunohistochemistry (IHC) analyses from tumor tissues are described in the Supplementary Methods.

Statistical analysis of the results was performed by contrast analysis following one-way ANOVA, and described in the Supplementary Methods.

Results

Trametinib, but not dabrafenib, partially and transiently inhibits T-cell proliferation and cytokine production in vitro

We used CD4+ and CD8+ cells isolated from healthy volunteers to assess whether dabrafenib and trametinib could affect
T-cell proliferation and function in vitro. T cells were activated with anti-CD3/anti-CD28 antibodies coated either on beads or plates. Trametinib and dabrafenib were added either simultaneously or sequentially with activation. At clinically relevant exposures, both dabrafenib and trametinib had little to no effect on naïve T cells (data not shown). At clinically relevant concentrations, dabrafenib alone did not significantly inhibit proliferation, apoptosis, or cytokine production of activated CD4⁺ and CD8⁺ T cells (Fig. 1). Unlike dabrafenib, trametinib alone resulted in partial inhibition of CD4⁺ T-cell proliferation after 3 days, but not after 7 days of treatment if CD4⁺ T cells were dosed with compound 24 hours before activation (Fig. 1A, Drug>Act., top). At 7 days, partial growth inhibition was apparent only at trametinib concentrations greater than 100 pg/mL.

Figure 1.
Trametinib transiently inhibits T-cell proliferation and reduces activation-induced apoptosis. A, the proliferation of CD4⁺ T cells as measured by CFSE following treatment with increasing concentrations of dabrafenib and trametinib at the indicated time points following addition of compounds. B, caspase-3/7 activity in CD4⁺ T cells following treatment with dabrafenib (370 nmol/L), trametinib (37 nmol/L), or the combination of dabrafenib and trametinib (dabrafenib + trametinib, 370 nmol/L/37 nmol/L) for 24 hours. C, levels of soluble cytokines in the media of CD4⁺ T cells after treatment with dabrafenib (370 nmol/L), trametinib (37 nmol/L), or the combination of dabrafenib and trametinib (370 nmol/L/37 nmol/L) for 72 hours. Drug>Act. signifies the addition of drug 24 hours before addition of CD3/CD28 activation beads. Act.>Drug signifies the addition of drug 24 hours after activation with CD3/CD28 activation beads. Average of six individual donors are shown.
nmol/L, which is above clinical exposure levels. The partial inhibitory effect was not observed if CD4⁺ T cells were activated before adding trametinib (Fig. 1A, Act.→Drug, bottom). In addition, treatment with trametinib alone (Drug→Act. and Act.→Drug) and in combination with dabrafenib (Act.→Drug) for 24 hours resulted in decreased activation-induced apoptosis (AID) measured by caspase-3/7 activity following T-cell activation (Fig. 1B). This was despite similar levels of proliferation in T- and dabrafenib/trametinib–treated cells at the time and concentration tested. The effects of trametinib on cytokine production were variable depending on the cytokine analyzed, result which was consistent with previously reported paradoxical effects of BRAF inhibitors in BRAF wild-type (WT) cells (14); with no observed changes in pAKT and pS6 protein levels (Fig. 2A and data not shown). In contrast, trametinib alone reduced pERK levels, but not pAKT and pS6 expression levels, as compared with controls (Fig. 2A and data not shown).

Furthermore, immune gene expression profiling using the NanoString nCounter gene expression system demonstrated unique gene signatures associated with both CD4⁺ and CD8⁺ T-cell activation by anti-CD3/CD28 antibodies (Fig. 2B). We identified genes with expression levels changed equal to or greater than 3-fold upon activation. Of the 525 genes in the panel, in CD4⁺ T cells, 88 genes (17%) were upregulated and 55 genes (10%) were downregulated; however, in CD8⁺ T cells, 39 genes (7%) were upregulated and 88 genes (17%) were downregulated. Interestingly, more genes were downregulated in CD8⁺ cells upon activation, whereas more genes were upregulated in CD4⁺ cells. Ontology enrichment indicated activation modulated sets of genes with specific functions related to the activity of the T-cell receptors, accompanied by genes encoding cytokines, chemokines, as well as genes involved in cell proliferation, transcription, and growth (Supplementary Tables S2A, S2B, and S2C). When trametinib was added simultaneously with T-cell activators, it partially offset the upregulation of 10 genes, CCL3, CCL4, GZMB, IL2, IL3, IL9, IL10, IL17A, IL17F, and IL23R, mostly cytokines and chemokines. It also enhanced the expression of three upregulated cytokines, IL4, IL5, and IL13, while it attenuated the downregulation of FCER1A, MX1, and RARRES3 genes in CD4⁺ cells. Similarly, trametinib showed an offset of 11 upregulated genes, CCL3, CCL4, GZMB, IFNG, IL2, IL3, IL10, CD82, IL1R1, TNF, and XCL1, and three downregulated genes CD244, CXC4R, and SIGIRR in CD8⁺ cells in response to T-cell activation. Dabrafenib alone had little to no effect on activated CD4⁺ and CD8⁺ T cells. No apoptosis genes, including CASP1, CASP8, BCL2, and TNFSF10 (TRAIL), were modulated by trametinib and dabrafenib alone and in combination with trar immunomodulatory genes associated with activated T cells [FOXP3, CD274 (PD-1), TNFRSF4 (OX40), ICOS, CTLA4, TNFRSF9 (4-1BB), CD25, and IFNG] were not affected substantially by dabrafenib and trametinib (<2-fold) in CD4⁺ cells. However, OX40, ICOS, CTLA-4, 4-1BB, and IFNγ were partially reduced by trametinib alone and in combination with dabrafenib (≈2-fold) in CD8⁺ cells. Both dabrafenib and trametinib had little-to-no effect on naïve CD4⁺ and CD8⁺ T cells (data not shown).

Multicolor flow cytometry confirmed cell-surface expression changes of CD69, CD25, PD-1, OX40, and CTLA-4 in CD4⁺ and CD8⁺ cells (Fig. 2C and data not shown). Dabrafenib effects were similar to vehicle control–treated samples. Treatment with trametinib decreased the expression of CD25, CD69, OX40, and PD-1 in CD4⁺ and CD8⁺ T cells, and CTLA-4 in CD4⁺ T cells only. However, the expression levels of CD69 and OX40 in trametinib–treated cells were still well above nonactivated T cells. Combining dabrafenib with trametinib partially offset the inhibitory effects seen with trametinib alone (Fig. 2C and Supplementary Tables S3A and S3B).

Dabrafenib and trametinib alone and in combination reduce the expression of tumor suppression factors and increase the expression of HLA-class I molecules and tumor antigens in BRAF V600E–mutant melanoma cell lines

To determine how MAPK pathway inhibition affects the expression levels of immunoregulatory genes/proteins in BRAF V600E or V600K–mutant melanoma cells, we first treated A375 melanoma cells with dabrafenib and trametinib either alone or in combination with the absence and presence of IFNγ. As shown in Fig. 3A, IFNγ was able to induce both PD-L1 and HLA-A expression in A375 cells. Dabrafenib and trametinib (either alone or in combination) decreased PD-L1 and increased HLA-A regardless of IFNγ exposure. Interestingly, the inhibition of PD-L1 by dabrafenib, trametinib, and dabrafenib + trametinib was transient in vitro, and did not track with the activation status of the pathway over time. In A375 cells treated with dabrafenib, trametinib, or dabrafenib + trametinib over a 30-day time course, PD-L1 mRNA levels increased steadily up to day 30 after an initial reduction through day 8 (Supplementary Fig. S3A). In contrast, DUSP6 mRNA levels, a reliable surrogate of MAPK activation, remained low throughout the 30-day time course (Supplementary Fig. S3B), indicating an uncoupling of PD-L1 expression and MAPK activation status in cancer cells chronically exposed to dabrafenib, trametinib, or dabrafenib + trametinib. In addition, we observed that a number of BRAF inhibitor resistant clones (12RS-1, 12RS-3, 16R6-3, 16R5-5, and 16R6-4; ref. 13), which developed from the parental line A375, expressed high levels of PD-L1 as determined by Western blot analysis, flow cytometry, and RT-PCR (Fig. 3A–B and C and data not shown). In A375 dabrafenib-resistant clones, increased PD-L1 protein expression tended to correlate with increased pSTAT levels (Fig. 3C), a result which was consistent with a previous report (15). IFNγ expression was below the level of detection in all cell samples tested and adding IFNγ did not further increase PD-L1 expression in 12RS-1–resistant line (data not shown). In the 12RS-1 cell line, PD-L1 expression was still partially responsive to MAPK pathway inhibition as PD-L1 mRNA levels were reduced...
by 39%, 34%, and 77%, respectively, in response to trametinib, dabrafenib, and dabrafenib + trametinib treatment. We also used a NanoString custom-built codeset (302 genes) to measure the expression of genes including tumor antigens, HLA molecules as well as markers associated with immunomodulation, apoptosis, and MAPK signaling in both A375 and
SK-MEL-24 BRAF V600E–mutant cells. As shown in Fig. 3C and Supplementary Table S4A for A375 cells, MAPK inhibition by dabrafenib and trametinib upregulated tumor antigens NY-ESO-1, BAGE, TRP1, gp100, HLA-class I and class II molecules, immunomodulation factors such as CD40, ICOSLG, IL15, IRF1, OX40L, SPP1, STAT1/3, TOX, B7-H3, PDCD2, and pro-apoptosis/tumor suppression genes including BCL2L11 (BIM1), DPP4, PIK3IP1, RARRES3, and TP53IP, and downregulated a subset of immunosuppressive factors such as PD-L1, VEGFA, IL1A, and NT5E (CD73). Similar results were seen in SK-MEL-24 cells.

**Figure 3.**
Immunomodulation by dabrafenib and trametinib in A375 BRAF-mutant melanoma cells. A, RT-PCR quantification of PD-L1 and HLA-A in A375 cells treated with trametinib/dabrafenib with and without IFNγ for 48 hours. B, PD-L1 protein expression from flow cytometry and (C) Western blot analyses in A375 parental and dabrafenib acquired resistant cell lines. D, differential gene expression from A375 cells treated with trametinib (10 nmol/L), dabrafenib (300 nmol/L), and the combination of trametinib and dabrafenib (10 nmol/L/300 nmol/L) for 48 hours (P < 0.05; ≥ 2-fold change).
Dabrafenib and Trametinib Effect on Immune Function

Table 1. In vitro effects on human tumor cell lines

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<th>Cell lines</th>
<th>PD-L1 baseline (RT-PCR)</th>
<th>T (IC50, nmol/L) CTG</th>
<th>Gene expression changes by T at 10 nmol/L, 48 h of treatment*</th>
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<td>BRAF-mutant melanoma</td>
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<tr>
<td>A549</td>
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<td>Low</td>
<td>26</td>
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NOTE: Gene expression levels were evaluated by NanoString and/or RT-PCR.
# Cells were treated with trametinib (10 nmol/L) alone in all lines or in combination with dabrafenib (300 nmol/L) in 12R5-1 line for 48 hours.
*Expression increase by trametinib treatment was only detected by RT-PCR, but not NanoString due to below detection range.
| , designated decrease with <0.5-fold of control.
| #, designated increase with >2-fold of control.
| —, designated no change, with less than 2-fold of changes vs. control or below the detection range.

(Table 1 and Supplementary Table S4B). In addition, down-regulation of PD-L1 and upregulation of HLA-I and II molecules were confirmed in A375 and SK-MEL-24 and observed in 12R5-1, but not in YUSIT BRAF V600K-mutant cells, by RT-PCR and/or flow cytometry analyses (Table 1 and data not shown). Furthermore, out of these four tested lines, tumor antigen NY-ESO-1 mRNA was increased ≈2-fold in A375 cells measured by NanoString, but not by Taqman, and at the protein level by Western blot analysis; whereas MART1 upregulation by MAPK inhibition was observed at mRNA level (≈6-fold) in YUSIT and both mRNA (37–93-fold) and protein levels in SK-MEL-24 cells.

Trametinib increases apoptosis markers and the expression of HLA-molecules in non-BRAF-mutant tumor cells
To evaluate the immunomodulatory effects of trametinib on non-BRAF-mutant tumor cells, we monitored the same key markers with the NanoString custom-built codest in three BRAF WT melanoma lines, HMVII and SK-MEL-2 (both have a NRAS mutation and are sensitive to trametinib), CHL1 (resistant to trametinib), and three KRAS-mutant NSCLC lines, Calu6, H358, and A549 (sensitive to trametinib). The representative genes, PD-L1, DUSP4, DUSP6, HLA-I (A, B, C), HLA-II (DMA, DPA, DRA), IL6, and IL8 were confirmed with RT-PCR. In addition, surface protein expression levels of PD-L1 and HLA-I molecules were evaluated by flow cytometry analysis. The data are shown in Supplementary Table S4B (NanoString) and summarized in Table 1. Baseline level of PD-L1 is low in most of the lines, except H358 with moderate expression. Trametinib displayed mixed effects on PD-L1 expression with upregulation in Calu-6 and A549 (only detected by RT-PCR), downregulation in H358 NSCLC line, and no change in three out of three melanoma lines tested. IFNγ increased the expression levels of PD-L1 and HLA-I in all tested cell lines (data not shown). DUSP4 and DUSP6 expression level was monitored as a measure of MAPK pathway inhibition by compound treatment. Trametinib induced an upregulation of apoptosis markers, PIK3IP1, TP53INP1, BCL2L11 (BIM1), and HLA-I and/or II expression in five out of six cell lines tested except in the CHL-1. Whereas it decreased IL8 levels in two out of three melanoma lines (HMVII and SK-MEL-2) and reduced the expression of NTE5 (CD73) and/or VEGFA in all three lung lines. It also reduced TGFA, EREG, and AREG genes in two out of three lung lines (H358 and Calu6). Baseline level of IL6 was extremely low in all lines except in CHL1. RT-PCR, but not NanoString, detected some level of IL6 induction by trametinib in two out of the six lines (A549 and Calu6). All cell lines except CHL1 showed a reduction in DUSP4/6 levels by compound treatment, indicating that upregulation of markers involved in apoptosis, HLA-I/II, and downregulation a subset of immunosuppression IL8, NTE5, VEGFA may be associated with MAPK pathway inhibition by trametinib. In addition, trametinib dose dependently increased MART1, GPI100, TRP-1, and TYR (3–10-fold at 3–10 nmol/L) tumor antigen gene expression in HMVII BRAF WT melanoma line, but not in the rest of the lines tested.

Combinations of trametinib with immunomodulatory antibodies targeting PD-1, PD-L1, or CTLA-4 in CT26 murine syngeneic tumor models are superior to single agents
The in vivo antitumor effect of trametinib was evaluated in the murine immunocompetent BALB/C syngeneic CT26 tumor model. CT26 mouse colorectal tumor cells harbor the homozygous KRAS G12D mutation and MAPK1 and MET amplifications (16). In vivo, trametinib caused dose-dependent inhibition of cell proliferation with a mean IC50 value of 20 nmol/L and blocked MAPK signaling measured by pERK (Fig. 4A). In vivo, as shown in Fig. 4B, 18 days of daily trametinib monotherapy at 1 mg/kg resulted in moderate antitumor activity with 61% TGI.
Anti-mouse PD-1, PD-L1, and CTLA-4 antibodies dosed alone showed minimal to low efficacy with 2%, 18%, and 32% TGI, respectively. However, concurrent combinations beginning with the first dose of trametinib with anti-mouse PD-1, PD-L1, or CTLA4 antibodies demonstrated much more profound activity with 80%, 81%, and 84% TGI, respectively. No overt toxicity, as defined by weight loss, unkempt appearance, mortality and behavior, was observed in any of the groups during the course of treatment.
Figure 5.
Trametinib alone and/or in combination with anti-PD-1 increased intratumoral CD4+ and CD8+ T cells in CT26 murine model. A, representative figures of flow cytometry gating and quantification for CD4+ and CD8+ (top), and Treg (CD25+/FoxP3+) (bottom) in tumors from each treatment group. B, flow cytometry quantification of tumor-infiltrating immune cells (mean ± SEM; n = 3; *, P < 0.05 vs. untreated and IgG2a+ vehicle controls). C, representative pERK and total ERK IHC staining tumor sections from each treatment group. D, heatmap generated by clustering of 77 genes with ≥1.5-fold of tumor gene expression changes by any treatment group. Un; untreated; IgG2a, nonspecific isotype control for anti-PD-1; α-PD-1, anti-PD-1 antibody treatment; MEK, trametinib treatment.
of treatment. The above data indicate that concurrent combinations of trametinib with immunomodulatory antibodies targeting PD-1, PD-L1, and CTLA-4 potentiate antitumor activity as compared with the single agents at their tolerated doses.

Next, we explored whether the combination of trametinib with the anti–PD-1 antibody in different lead-in sequences would affect in vivo efficacy. As shown in Fig. 4C and D and Supplementary Fig. S1 (tumor growth curves from individual mice), three combination regimens were evaluated: trametinib and anti–PD-1 antibody given concurrently starting in the first week (MEK-1st+PD-1-1st); trametinib given in the first week as single agent followed by adding anti–PD-1 antibody in the second week with continued trametinib dosing (MEK-1st+PD-1-2nd); and anti–PD-1 antibody given in the first week as single agent followed by adding trametinib in the second week with continued anti–PD-1 dosing (PD-1-1st+MEK-2nd). All three combination regimens showed inhibition of tumor growth more effectively than their single-agent controls during the initial 2 to 3 weeks of treatment (Fig. 4C). However, only two of three combination treatment groups, concurrent MEK-1st+PD-1-1st and trametinib lead-in followed by trametinib + PD-1 (MEK-1st+PD-1-2nd) produced profound delay in median tumor growth with TIE of 39 and 49 days, two out of 10 and four out of 10 68-day survivors, respectively, at the end of the study, and differed significantly from untreated and vehicle/isotype controls by log-rank survival analysis (P < 0.05; Fig. 4D). Conversely, all PD-1-1st+MEK-2nd showed minimal benefit in long-term survival. Finally, it is worth noting that both PD-1-1st monotherapy and PD-1-1st+MEK-2nd treated groups had one out of 10 68-day survivors although majority of the groups showed no response. All treatments were well tolerated.

Trametinib alone or in combination with anti–PD-1 antibody in vivo reduced immunosuppression factors, increased HLA-class II genes and lead to increased intratumoral CD4+ and CD8+ T cells

Given that trametinib can potentiﬁce the efﬁcacy of immunomodulators in vivo, we investigated potential immunomodulatory mechanisms of trametinib in combination with an anti–PD-1 blocking antibody in lymphocyte tissues and CT26 tumors in vivo. We collected whole blood, tumors, spleens, and lymph nodes after 7 days of treatment with trametinib and anti–PD-1 antibody alone, or in concurrent combination. In tumors, trametinib alone and in combination with anti–PD-1 antibody alone, but not anti–PD-1 antibody alone, signiﬁcantly increased CD4+ T cells by 4.3- and 3.4-fold, respectively, compared with untreated and non-speciﬁc IgG2a controls (P < 0.05) measured by ﬂow cytometry (Fig. 5A and B and Supplementary Table S3). Only the combination of trametinib and anti–PD-1 antibody signiﬁcantly increased CD8+ T cells by 4.7-fold vs. untreated (P < 0.05) and 3.6-fold vs. non-speciﬁc IgG2a control (P = 0.05; Fig. 5B). CD69+ cells from CD4+ population were reduced by 53% from trametinib treatment (P < 0.05 vs. untreated). CD69+ cells from CD8+ population were reduced by 57% from the combination of trametinib with anti–PD-1 antibody (P < 0.05 vs. untreated). Other immune cell subtypes, such as Treg, measured by Foxp3+/CD25+ from CD4+ population, PD-1+, PD-L1+, Ox40+, and ICOS+ from both CD4+ and CD8+ cells were changed less than 2-fold or not statistically signiﬁcant (P > 0.05) by any treatment (Supplementary Table S5). There were no signiﬁcant alterations in the numbers and expression levels of CD39+, CD4+, CD25+, CD69+, and PD-1+ cells from spleens and lymph nodes (data not shown). Of note, these immune surface markers and circulating cytokines from peripheral blood samples were variable from mouse to mouse, and marginally (< 2-fold) or not signiﬁcantly affected by any treatment in the study (data not shown). IHC analysis revealed that treatment with trametinib alone and in combination with anti–PD-1 antibody led to 70% to 75% inhibition of pERK in the tumor (Fig. 5C and Supplementary Tables SSA and SSB), demonstrating effective MAPK signaling inhibition by trametinib in the CT26 tumor model in immunocompetent mice.

In addition, tumor gene expression was proﬁled using a NanoString-based immunology panel and follow-up with quantitative RT-PCR (qRT-PCR). Among the 561 mouse genes proﬁled, 77 showed a ≥1.5-fold change by trametinib, anti–PD-1, or the combination of the two in comparison with untreated and non-speciﬁc IgG2a controls (Supplementary Table S6). A heatmap was generated by gene clustering of these 77 genes (Fig. 5D). The group I genes are those upregulated by trametinib alone and/or trametinib in combination with anti–PD-1 but not by anti–PD-1 alone. Interestingly, three MHC class II genes, H2-Ea-ps, CD74, and H2-Eb1, along with CD4 and IL12b were upregulated most signiﬁcantly by the combination treatment. Group II includes 12 genes mainly associated with T-cell IFNγ inducible cytotoxic factor (GZMB), immunoregulators (CD274-PD-L1 and IL2RA-CD25), chemokines (CXCL9, CXCL11, CXCL10, CXCL12, CCL5-RENTES, and CCL8-MCP-1), and other immune factors (CFF, CD36, and C3). Although not statistically signiﬁcant, tumor gene expression from 2 out of 3 mice treated with anti–PD-1 antibody alone showed unique and noticeable upregulation of all of these genes, implying a gene signature of anti–PD-1 effect on tumors. Group III has 42 genes that were downregulated by trametinib alone and in combination with anti–PD-1. These genes include inﬂammation factors (e.g., SELE-E-selectin, PTGS2-COX-2, MIF, TNF, and PLAIR), chemokines (CCL2, CCL3, CCL4, CCL6, CCL7, CCL9, CCL11, CCL12, CXCL1, CXCL3, and CXCR2), pro-inﬂammatory IL1 family cytokines mostly associated with immunosuppression (IL33, IL1RII, IL1RN, IL1B, IL2, and IL18RAP), tumor metastasis factors (S100A8 and S100A9, PLA1, and ITGA6), markers associated with immunosuppression (TGFBl), and markers for monocytes (CD163 and CD14), macrophage and dendritic cells (MSR1 and CD209G) and myeloid cells (TREM1). A few selected genes were further evaluated using qRT-PCR that has more sensitive detection and showed consistent results with NanoString. As illustrated in Supplementary Fig. S2, anti–PD-1 antibody increased IFNg and GZMB by itself, and PD-1, ICOS, and CTLA-4 with and without trametinib. Only the combination of trametinib and anti–PD-1 increased CD4, CX40, and PD-1. trametinib alone and in combination with anti–PD-1 showed reduction of CCL2 (consistent with NanoString data), DUSP6 (correlated with pERK IHC) and IL6 (only detected by RT-PCR, Supplementary Fig. S2). All data generated demonstrated that trametinib in concurrent combination with anti–PD-1 downregulated immunosuppression factors, upregulated HLA molecules, and increased immune response in tumors with little or no immune phenotypical/functional changes from circulating cell populations and immune organs in vivo. All of these effects contribute to and support the rationale that trametinib in combination with immunomodulatory antibodies targeting PD-1, PD-L1, and CTLA-4 is a more effective antitumor therapeutic approach.
Discussion

Although the mechanisms of action for BRAF and MEK inhibitors, such as dabrafenib and trametinib, regarding tumor growth inhibition are well studied, their impact on immune cells and the tumor microenvironment is less understood. Preclinical and limited clinical findings have suggested that immunomodulatory effects in the tumor microenvironment and on circulating immune cells by a BRAF inhibitor alone and the combination of a BRAF inhibitor with a MEK inhibitor is context-dependent (8, 17–20). On the other hand, concerns have been raised regarding the potential immunosuppressive activity of MEK inhibitors due to their immunosuppressive activity in vitro (10). However, the studies have been limited in scope and lack in vivo validation in mouse models. Here, we present data showing that the immunomodulatory effects of trametinib on activated T cells were multifaceted and context dependent. Changes that could be considered immunosuppressive were only observed in vitro, were transient, and did not translate in vivo using an immunocompetent mouse tumor model. The differences between data from this study and those of prior reports may be explained by a number of factors, including (i) the in vitro experiments done by others had a short period of time exposure to MEK inhibitors without monitoring longer-term effects, therefore only the transient suppressive activity by MAPK inhibition was captured; (ii) MEK inhibitors transiently block the MAPK signaling during the initial T-cell activation, thus delay the kinetics of T-cell activation and cytokine secretion in vitro; (iii) the circulating T cells collected in vivo are mostly in a naïve state, reflected in this study by the low number of activated T cells from blood and immune organs with and without anti–PD-1 antibody treatment in mice and much less active than T cells activated by anti-CD3/CD28 in vitro. Our preclinical data are complementary to the recent study showing that both dabrafenib and trametinib alone or the combination increased tumor infiltration of lymphocytes, and enhanced the antitumor effect with adoptive T-cell immunotherapy in the syngeneic murine model of BRAF V600E–mutant melanoma (21). Our studies indicate that trametinib alone increases tumor infiltrating CD4+ lymphocytes (TIL), does not negatively affect the prevalence of CD8+ TILs while significantly increasing CD8+ TILs when combined with anti–PD-1 antibody. Most importantly, combining trametinib with anti–PD-1 either concurrently or in phased sequence, with trametinib administered first followed by trametinib plus anti–PD-1, resulted in much more effective and durable antitumor activity than both single agents in the KRAS-mutant CT26 colorectal tumor syngeneic mouse model. Interestingly, phased sequence of anti–PD-1 antibody dosed first, followed by anti–PD-1 antibody plus trametinib showed minimal benefit in long-term survival compared with single-agent treatment. The observation suggests that blockage of oncogenic MAPK signaling by trametinib is critical and effective to prime and synergize tumors in response to immunotherapy through the induction of apoptosis markers, upregulation of HLA molecules, and reduction of immunosuppression factors from tumors. Our data support the rationale to evaluate the sequencing of these agents, including giving the targeted therapy as a lead-in to an immunomodulatory antibody or concurrently in clinical trial design to maximize antitumor efficacy and with the hope of minimizing side effects.

Our data show that PD-L1 was not only induced by IFN-γ in all cell lines tested, but was also upregulated in cell lines with acquired resistance to dabrafenib. Conversely, dabrafenib, trametinib, and the combination partially downregulated PD-L1 expression in a subset, but not all cell lines tested, an observation similar to data from recent publications (15, 22, 23). Interestingly, we also observed that trametinib could induce PD-L1 expression in two of the NSCLC lines with low baseline level of PD-L1 expression. Our data in A375 cells chronically exposed to dabrafenib, trametinib, or dabrafenib + trametinib, suggests that PD-L1 expression may be regulated via a compensatory pathway in response to sustained inhibition of MAPK signaling. Although the mechanisms of PD-L1 regulation appear to be multifactorial, MAPK signaling and feedback regulation by MAPK inhibition likely contribute, in part, to the expression levels of PD-L1 in tumors. However, PD-L1 expression level could be affected by multiple pathways including the MAPK pathway, and the change of PD-L1 expression is accompanied by multiple other genetic and morphologic changes that collectively contribute to tumor growth and metastasis (24). As a result, PD-L1 may serve as a marker, but may not be the driver determining patient response to BRAF inhibitor treatment. Future studies should further investigate the compensatory pathways responsible for PD-L1 regulation in the context of MAPK inhibition. As reported, during the initial treatment with either BRAF inhibitor alone or BRAF+MEK inhibitor in patients BRAF-mutant melanoma, both PD-L1 and CD8+ T-cell infiltrates were increased in treated tumors (18). However, CD8+ T-cell infiltrates decreased when these patients progressed from the treatment. It was also noted that PD-L1 expression increased when patients became refractory to BRAF or BRAF + MEK inhibitor treatment. The evidence of PD-L1 modulation data from both preclinical and clinical studies indicates that downregulation of PD-L1 may be a marker of acquired resistance to BRAF, MEK, or BRAF + MEK inhibition, and may provide rationale for PD-L1 inhibitor use in BRAF-resistant patients.

In this study, we also demonstrated that inhibition of MAPK by trametinib, dabrafenib, or the combination of trametinib and dabrafenib in BRAF-mutant melanoma and trametinib in BRAF WT melanoma and NSCLC lines increased the expression of apoptosis markers and HLA-class I and/or II molecules and decreased a subset of immunosuppression factors in vitro. The increase of apoptosis markers PIK3P1, TP53INP1, and BCL2L11 (BIM1) may sensitize tumor cell killing by cytotoxic T cells. Tumor cells secrete IL1, IL8, and VEGFA soluble factors that promote immunosuppression (25, 26). NTF5 (CD73) gene is highly expressed in many human solid tumors. It encodes an ectoenzyme generating extracellular adenosine that induces potent immunosuppressive effects and impaired anti-tumor T-cell responses (27, 28). EGFR ligands, such as TGFα, EREG, and AREG, can activate EGRF, promote tumor metastasis, and enhance regulatory T cell-suppressive function via the EGFR (29). HLA-class I molecules are often downregulated or lost in tumor cells and play a key role in immune escape (30–32). A study by Carreto and colleagues (33) showed that higher HLA class I gene expression was observed in regressing but not in progressing metastases from microdissected tumor regions, supporting the idea that the nature of HLA class I alterations in tumor cells may contribute to antitumor effects of therapeutic interventions. Our observation of MAPK inhibition induced apoptosis, increase of MHC class I and/or II along with the decrease of immunosuppression factors in most of the human tumor cell lines, and in CT26 tumors when combined with anti–PD-1 antibody in vivo is intriguing. These effects may potentially increase immune response when MAPK pathway...
inhibitors combine with immunotherapy and may lead to therapeutic synergy.

These data taken together further expand the understanding of BRAF and MEK inhibitor effects on the immune system and provide scientific evidence to support the investigation of the combination of trametinib, with or without dabrafenib, with immunomodulators in the clinic. Clinical studies are under way to test the combination of these important agents in patients with metastatic melanoma.

Disclosure of Potential Conflicts of Interest

A. Hoos and J. Jing have ownership interests (including patents) in GlaxoSmithKline. No potential conflicts of interest were disclosed by the other authors.

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