

## Research Article

**MEK Inhibition, Alone or in Combination with BRAF Inhibition, Affects Multiple Functions of Isolated Normal Human Lymphocytes and Dendritic Cells**Laura J. Vella<sup>1</sup>, Anupama Pasam<sup>1</sup>, Nektaria Dimopoulos<sup>1</sup>, Miles Andrews<sup>1</sup>, Ashley Knights<sup>1</sup>, Anne-Laure Puaux<sup>3</sup>, Jamila Louahed<sup>3</sup>, Weisan Chen<sup>1,2</sup>, Katherine Woods<sup>1</sup>, and Jonathan S. Cebon<sup>1</sup>**Abstract**

Combination therapy with BRAF and MEK inhibition is currently in clinical development for the treatment of BRAF-mutated malignant melanoma. BRAF inhibitors are associated with enhanced antigen-specific T-lymphocyte recognition *in vivo*. Consequently, BRAF inhibition has been proposed as proimmunogenic and there has been considerable enthusiasm for combining BRAF inhibition with immunotherapy. MEK inhibitors inhibit ERK phosphorylation regardless of BRAF mutational status and have been reported to impair T-lymphocyte and modulate dendritic cell function. In this study, we investigate the effects on isolated T lymphocytes and monocyte-derived dendritic cells (moDC) of a MEK (trametinib) and BRAF (dabrafenib) inhibitor combination currently being evaluated in a randomized controlled clinical trial. The effects of dabrafenib and trametinib, alone and in combination, were studied on isolated normal T lymphocytes and moDCs. Lymphocyte viability, together with functional assays including proliferation, cytokine production, and antigen-specific expansion, were assessed. MoDC phenotype in response to lipopolysaccharide stimulation was evaluated by flow cytometry, as were effects on antigen cross-presentation. Dabrafenib did not have an impact on T lymphocytes or moDCs, whereas trametinib alone or in combination with dabrafenib suppressed T-lymphocyte proliferation, cytokine production, and antigen-specific expansion. However, no significant decrease in CD4<sup>+</sup> or CD8<sup>+</sup> T-lymphocyte viability was observed following kinase inhibition. MoDC cross-presentation was suppressed in association with enhanced maturation following combined inhibition of MEK and BRAF. The results of this study demonstrate that MEK inhibition, alone or in combination with BRAF inhibition, can modulate immune cell function, and further studies *in vivo* will be required to evaluate the potential clinical impact of these findings. *Cancer Immunol Res*; 2(4); 351–60. ©2014 AACR.

**Introduction**

As a result of the identification of mutations in the BRAF kinase as an oncogenic driver in cutaneous melanomas (1, 2), BRAF kinase inhibitors (BRAFi) have now become the standard of care for treatment of patients with metastatic melanoma bearing V600 mutations (3). Unfortunately, clinical benefit is generally short-lived and BRAFi-resistant disease develops with a median of 6 to 7 months (4, 5). Multiple mechanisms have been described, but, importantly, each of the resistance mechanisms has the common theme of reactivating mitogen-

activated protein kinase (MAPK) pathway signaling or activating parallel signaling pathways that drive cell proliferation and survival (6–14).

Trametinib (GSK1120212; GlaxoSmithKline Pharmaceuticals) is an orally available, small-molecule, selective inhibitor of MEK1 and MEK2 (15). Monotherapy with trametinib improved progression-free and overall survival when compared with chemotherapy administered to patients who had BRAF-mutant melanoma, validating trametinib as a therapeutic approach (16). In an attempt to extend the duration of clinical benefit, MEK inhibitors (MEKi) have been combined with BRAFi therapy (17), with the combination of trametinib with dabrafenib (GSK2118436; GlaxoSmithKline Pharmaceuticals) that resulted in a 3.5-month prolongation of progression-free survival when compared with dabrafenib monotherapy. The addition of trametinib with dabrafenib, therefore, represents an effective strategy for delaying the emergence of BRAFi resistance (17).

Immunotherapies have also shown considerable promise in the treatment of metastatic malignancy, and inhibitory antibodies that target the molecular immune checkpoints CTLA-4 and PD-1 have been reported to induce durable clinical remissions as well as prolong survival in responding patients (18–20). The anticancer effects of these agents are mediated by cellular effectors of the immune system. This has provided

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a rationale for combining MAPK pathway inhibitors with immunotherapy, as BRAFi can sensitize BRAF-mutant melanoma cells to immune recognition and relieve immune suppression (21–26). Amid the speculation as to the role for combined MAPK pathway inhibition and immunotherapy, the degree to which kinase inhibition, specifically dabrafenib and/or trametinib, may directly affect immune function remains poorly defined.

To optimally combine kinase inhibitors with immunotherapy, it is critical to understand how the combination of dabrafenib and trametinib influences immune cell subpopulations. We assessed the effects of dabrafenib and trametinib, alone and in combination, on healthy donor T lymphocytes and monocyte-derived dendritic cells (moDC).

## Materials and Methods

### Reagents

BRAF inhibitor (GSK2118436, dabrafenib) and MEK1/2 inhibitor (GSK1120212, trametinib) were provided by GlaxoSmithKline (GSK) and solubilized in 100% dimethyl sulfoxide (DMSO) at 10 and 2.5 mmol/L, respectively. Stock solutions were stored at  $-20^{\circ}\text{C}$  and physiologically relevant concentrations tested (27, 28). All phenotyping antibodies were from BD Biosciences, unless indicated otherwise. NY-ESO-1-specific CD8<sup>+</sup> T-lymphocyte clones were generated as described (29, 30). Complete media contained RPMI-1640 with 20 mmol/L HEPES, 60 mg/L penicillin, 12.5 mg/L streptomycin, 2 mmol/L L-glutamine, 1% nonessential amino acids, and 10% heat-inactivated fetal calf serum (Invitrogen). All flow cytometry was performed on a BD FACSCanto II, and data analyses were performed using the FlowJo software (TreeStar Inc).

### Isolation of CD4/CD8 T lymphocytes

Peripheral blood mononuclear cells (PBMC) from buffy coat preparations from healthy donors (Red Cross Blood Bank) were prepared by Ficoll-Paque (GE Healthcare) density gradient centrifugation. T cells were isolated by positive selection using magnetic anti-CD4 or -CD8 microbeads (Miltenyi Biotech). Cells were counted and selection confirmed by flow cytometry (purity confirmed to be  $\geq 90\%$ ).

### Proliferation assay

Purified CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes were labeled with 10  $\mu\text{mol/L}$  carboxyfluorescein succinimidyl ester (CFSE) dye (Invitrogen) at  $37^{\circ}\text{C}$ , washed, and plated at  $1 \times 10^5$  cells per well in a 96-well U-bottom plate (BD Falcon). Cells were stimulated with beads coated with anti-CD3/CD28 antibodies (Invitrogen) at a 2:1 ratio for 5 days, with or without the indicated concentration of MAPK inhibitors. Media and inhibitors were replenished on day 3. After staining with a violet live/dead fluorescent viability dye and anti-CD3, anti-CD4, and anti-CD8 fluorescent antibodies, cells were analyzed on the BD FACSCanto II, gating for lymphocytes with FSC/SSC parameters. Data analysis was performed using the FlowJo proliferation tool (TreeStar). The "proliferation index" was used to define a proliferation value. This value, which represents the average number of cell divisions undergone by the responding cells, more faithfully reflects the biology of the system by considering

only the fraction of proliferating cells (<http://www.flowjo.com/v7/html/proliferation.-html>).

### Cytokine production assay

PBMCs were plated at  $1 \times 10^5$  cells per well in a 96-well U-bottom plate. Cells were cultured in the presence and absence of the mitogens phorbol myristate acetate (PMA, Sigma Aldrich; 5 ng/mL) and ionomycin (1  $\mu\text{mol/L}$ ; Sigma-Aldrich) for 24 hours, with or without the indicated concentrations of the BRAF and/or MEK inhibitors. Golgi plug (CD8<sup>+</sup> culture) and Golgi STOP (CD4<sup>+</sup> culture) was added for the final 12 hours to halt cytokine secretion. Cells were then stained with anti-CD3, -CD4, and -CD8 antibodies, permeabilized and stained for IFN- $\gamma$ , TNF- $\alpha$ , and interleukin (IL)-17, and analyzed using a BD-FACSCanto II. Data analyses were performed using FlowJo, gating on viable CD3<sup>+</sup>CD4<sup>+</sup> and CD3<sup>+</sup>CD8<sup>+</sup> cytokine-producing T cells.

### Dendritic cell culture

Immature moDCs were generated by culturing CD14<sup>+</sup> monocytes with granulocyte macrophage colony-stimulating factor (20 ng/mL) and IL-4 (10 ng/mL) for 4 days. On day 5, moDCs were plated at  $1 \times 10^5$  cells per well in a 96-well U-bottom plate with or without BRAF and/or MEK inhibitors in the presence of 100 ng/mL lipopolysaccharide (LPS; Sigma Aldrich) for 24 hours. Phenotypic analysis was performed using the following antibodies: anti-HLA-DR, anti-CD86, and anti-CD83 in combination with a violet live/dead fluorescent dye (Invitrogen). Cells were analyzed using a BD FACSCanto II and data analyses were performed using FlowJo, gating on viable moDCs (live/dead dye exclusion).

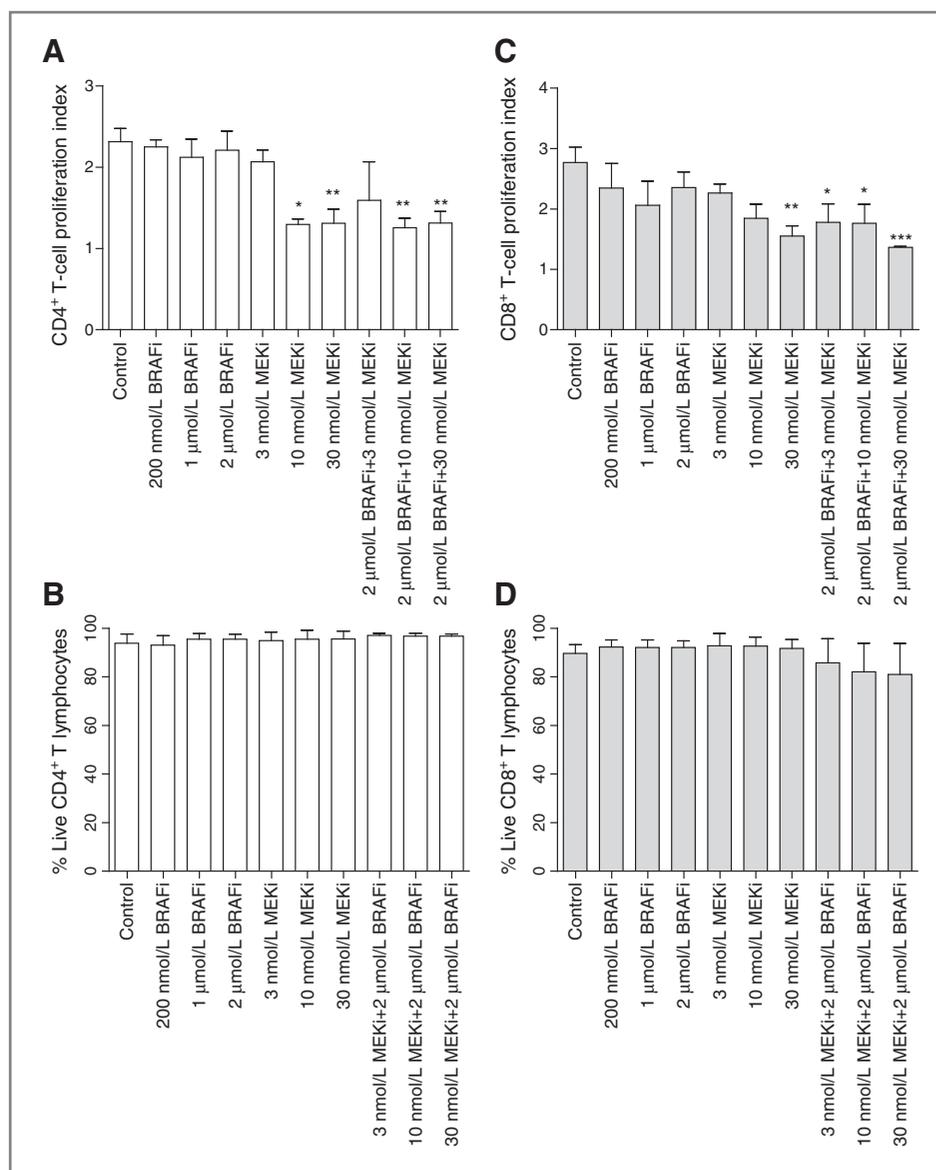
### Tumor antigen cross-presentation assay

*In vitro* antigen presentation experiments were performed with moDCs pulsed for 16 hours with (i) immune complexes formed with purified recombinant NY-ESO-1 protein produced in *Escherichia coli* (GSK Vaccines; refs. 30, 31) and anti-His<sub>6</sub> Ab (mouse monoclonal MAB050; R&D Systems); (ii) control recombinant NY-ESO-1 protein and anti-IgG1 isotype control (mouse monoclonal MAB002; R&D Systems); and (iii) the cognate peptide recognized by the respective T-cell clone in the absence or presence of different concentrations of BRAF and MEK inhibitors. After 16 hours, dendritic cells (DC) were washed and used as antigen-presenting cells (APC) for NY-ESO-1-specific CD8<sup>+</sup> T-cell clones (HLA-A2/NY-ESO-1<sub>157-165</sub>) at a 1:10 T cell-to-APC ratio with GolgiPlug for 4 hours. T-cell activation was measured by intracellular cytokine staining (ICS) for IFN- $\gamma$ . Where indicated, before the ICS assessment, DCs were pulsed for 1 hour with 1  $\mu\text{g/mL}$  of the NY-ESO-1 peptide followed by washing.

### Viability

Cells were treated with media containing the indicated kinase inhibitors or equal amount of DMSO for the indicated time. Viability was assessed using violet live/dead fluorescent dye (Invitrogen) and analyzed using a BD FACSCanto II and data analysis performed using the FlowJo software (TreeStar Inc.).

**Figure 1.** Trametinib and combination decrease T-lymphocyte proliferation. The proliferation index (A and C) and viability (B and D) of CD3/CD28-activated CFSE-labeled CD4<sup>+</sup> (A and C) and CD8<sup>+</sup> (B and D) T cells treated for 5 days with dabrafenib or trametinib, alone or in combination. Each point represents the mean value from the results obtained with 4 healthy donors; error bars show the SEM. One-way ANOVA with a Dunnett post test against the untreated control was performed. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ . The proliferation index was determined using the proliferation tool in the FlowJo software package. This value represents the average number of cell divisions that the cells underwent by considering only the fraction of proliferated cells. Dabrafenib, BRAFi; trametinib, MEKi. Representative flow cytometry data are presented in Supplementary Fig. S1.



### ***In vitro* T-lymphocyte expansion using specific peptide**

HLA-A2<sup>+</sup> healthy donor PBMCs were plated at  $1 \times 10^6$  cells per well in a 48-well plate in culture media to which 1 μg/mL (final concentration) of EBV BMLF1<sub>280-288</sub> (GLCTLVAML) peptide were added with 25 IU recombinant hIL-2, in the presence or absence of the BRAF and MEK inhibitors. This treatment was repeated every 3 days until day 10. On day 10, the inhibitors were removed, and the cells were washed thoroughly and then restimulated with or without 1 μg/mL BMLF1 peptide for 4 hours in the presence of Golgi-stop. T-cell activation was measured by ICS for IFN-γ. Flow cytometry was performed using the BD FACSCanto II and data were analyzed using FlowJo, gating on viable CD3<sup>+</sup>CD8<sup>+</sup> cytokine-producing T cells.

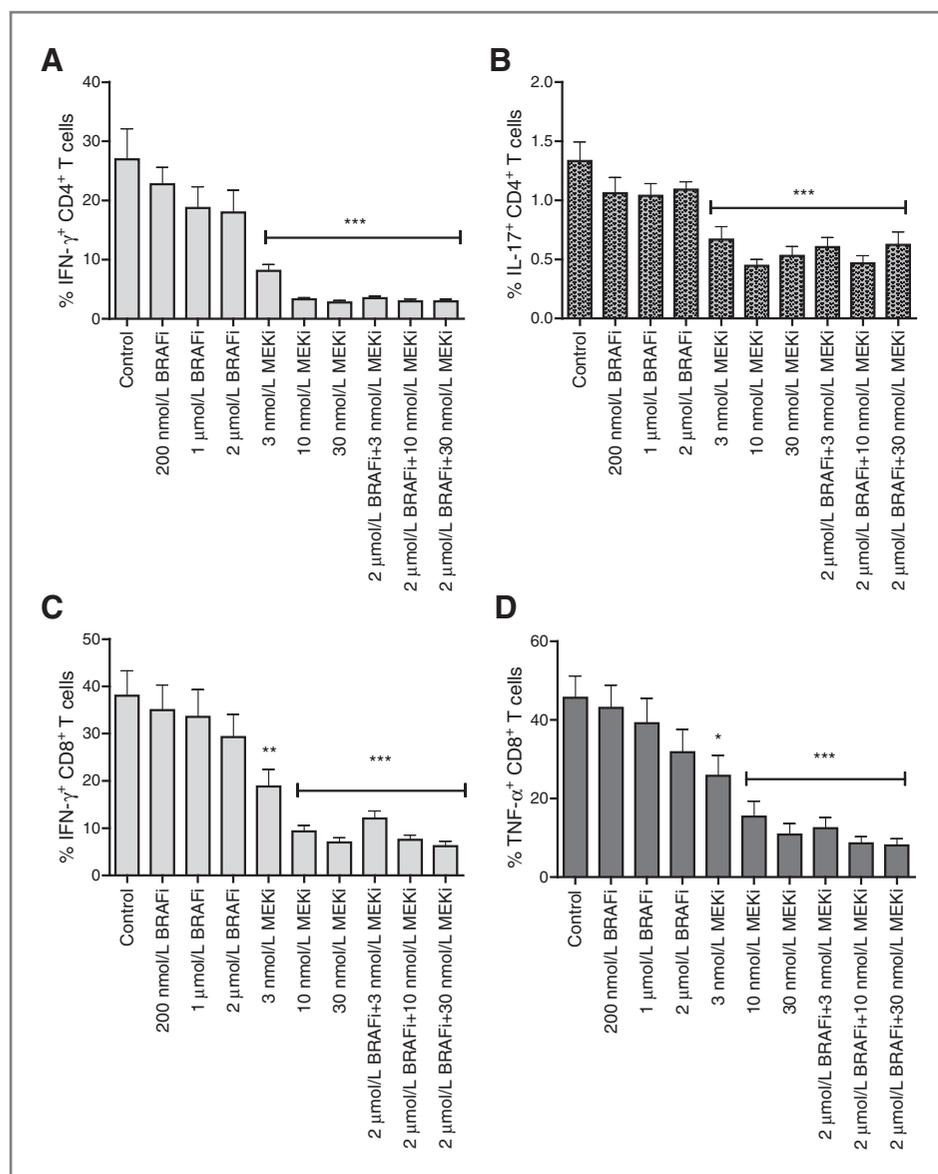
### **Results**

The BRAFi vemurafenib and its derivative PLX4720 do not have a negative effect on healthy donor T lymphocytes (21, 32),

whereas MEK inhibitors, U0126, PD0325901 (21), and PD98059 (33, 34), have been shown to have negative effects on T-lymphocyte proliferation and cytokine secretion. The effect of dabrafenib and trametinib, alone and in combination, on healthy human T lymphocytes has not yet been reported. Therefore, we examined the effects of trametinib and dabrafenib, alone and in combination, on stimulated CD8<sup>+</sup> and CD4<sup>+</sup> T lymphocytes by assessing cellular proliferation, viability, and cytokine secretion.

### **Trametinib alone and in combination with dabrafenib decreases T-lymphocyte proliferation and the percentage of cytokine-producing cells**

To investigate the effects of kinase inhibition on lymphocyte proliferation and viability, purified CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes were isolated from normal donor blood, labeled with the membrane-incorporating dye CFSE, and activated with



**Figure 2.** Trametinib and combination decrease T-lymphocyte cytokine production. The percentage of IFN- $\gamma$  (A) and IL-17 (B) positive CD4<sup>+</sup> T cells, and IFN- $\gamma$  (C) and TNF- $\alpha$  (D) positive CD8<sup>+</sup> T cells following treatment with dabrafenib or trametinib, alone or in combination was assessed by ICS flow cytometry 24 hours after activation by PMA-ionomycin. The percentage of cytokine-producing cells is shown as a fraction of total CD3<sup>+</sup> CD4<sup>+</sup> (A and B) or CD3<sup>+</sup> CD8<sup>+</sup> (C and D) T cells. Mean values with SEM from 8 healthy donors are shown. One-way ANOVA with a Dunnett post test against the untreated control was performed. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ . Dabrafenib, BRAFi; trametinib, MEKi. Representative flow cytometry data are presented in Supplementary Fig. S2.

anti-CD3/CD28 beads in the presence of dabrafenib or trametinib alone or in combination. After 5 days, the proliferation index and viability for each treatment condition and population was determined by Flow cytometry. Proliferation plots for activated CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes are shown in Fig. 1A and B. A statistically significant decrease in proliferation of CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes was observed following trametinib or concurrent treatment, whereas following dabrafenib alone proliferation was comparable with that of the control. No effect on CD4<sup>+</sup> or CD8<sup>+</sup> T-lymphocyte viability was observed (Fig. 1C and D).

Cytokine-producing CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes were enumerated by ICS 24 hours following nonspecific activation with PMA-ionomycin in the presence of kinase inhibitors (Fig. 2). Significant differences were seen for IFN- $\gamma$ - and IL-17-producing CD4<sup>+</sup> T lymphocytes (Fig. 2A and B), and

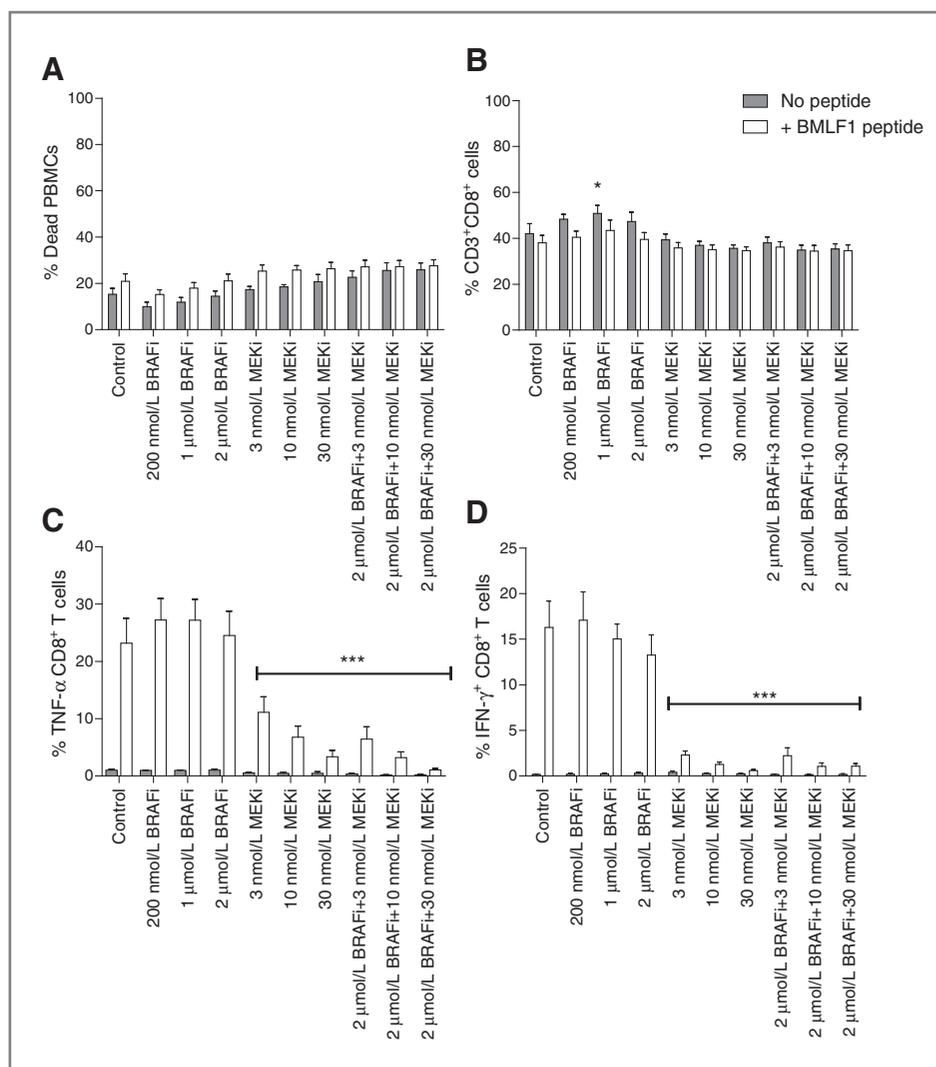
IFN- $\gamma$ - and TNF- $\alpha$ -producing CD8<sup>+</sup> T lymphocytes (Fig. 2C and D). Trametinib alone or in combination reduced the frequency of cytokine-producing cells even at the lowest concentration (3 nmol/L; Fig. 2).

Taken together, these data demonstrate that treatment of human T lymphocytes with trametinib alone or in combination with dabrafenib both constrains proliferation and inhibits cytokine production by both CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes.

#### **Trametinib alone and in combination with dabrafenib impairs expansion of antigen-specific T lymphocytes *in vitro***

To investigate the effect of dabrafenib, trametinib, and their combinations on lymphocyte activation and proliferation, *in vitro* peptide antigens were used to stimulate

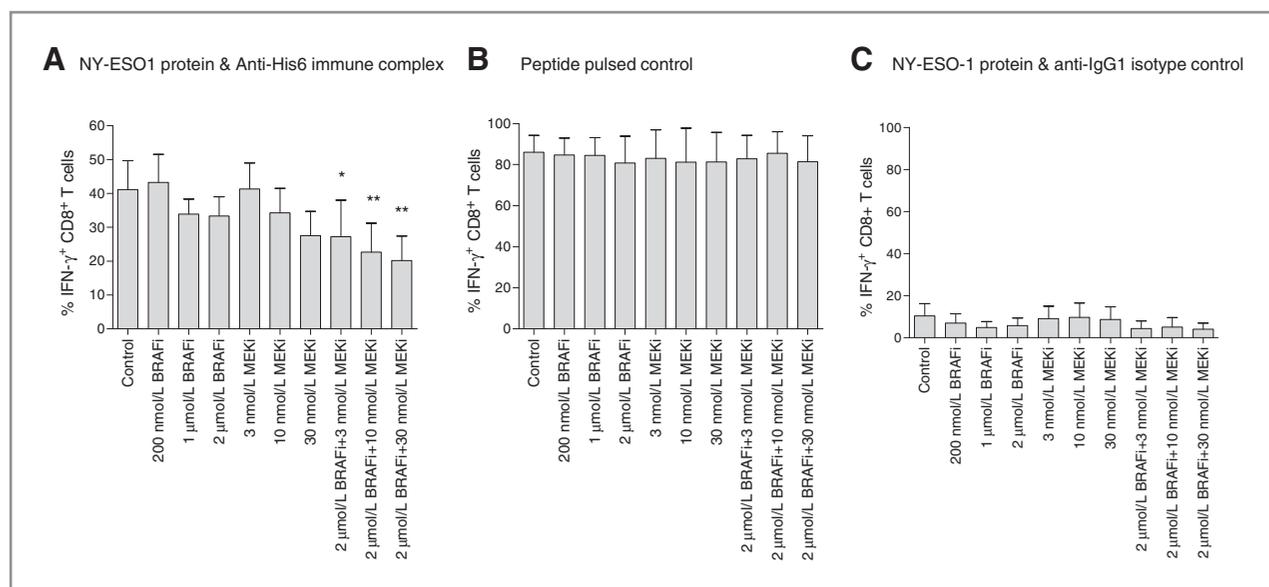
**Figure 3.** Trametinib and combination affect the proliferation of antigen-specific T lymphocytes. The effect of kinase inhibition on *in vitro* stimulation of peptide-specific CD8<sup>+</sup> T cells was assessed 10 days after stimulation of PBMCs with an HLA-A2–restricted synthetic peptide for EBV BMLF1 (280–288). The percentage of dead PBMCs (A) and percentage of CD3<sup>+</sup>CD8<sup>+</sup> T cells in the viable PBMC population (B) following 10 days of kinase inhibition are shown. Specific T cells were detected by ICS using fluorescently tagged antibodies to TNF- $\alpha$  (C) and IFN- $\gamma$  (D). The percentage of TNF- $\alpha$  or IFN- $\gamma$  producing CD8<sup>+</sup> T cells was determined by fluorescence-activated cell sorting analysis. Each point represents a mean value from multiple stimulations performed with samples from 6 healthy donors and the mean with SEM is indicated. Two-way ANOVA with a Bonferroni post test against the DMSO control was performed. \*,  $P < 0.05$ ; \*\*\*,  $P < 0.001$ . Dabrafenib, BRAFi; trametinib, MEKi. Representative flow cytometry data are presented in Supplementary Fig. S3.



antigen-specific CD8<sup>+</sup> T lymphocytes in whole PBMC cultures. An immunodominant epitope from the Epstein-Barr virus (EBV) BMLF1 protein, (peptide position 280–288) was selected to stimulate memory responses, as the majority of healthy HLA-A2<sup>+</sup> donors generally have a preexisting immune responses to this epitope (35). PBMCs were stimulated *in vitro* with BMLF1 in the presence of titrating concentrations of the inhibitors. After 10 days in culture, cells were restimulated with the BMLF1 peptide and PBMCs (Fig. 3A), CD4<sup>+</sup> and CD8<sup>+</sup> (Fig. 3B) viability was determined, and reacting CD8<sup>+</sup> T lymphocytes were enumerated by ICS for TNF- $\alpha$  (Fig. 3C) or IFN- $\gamma$  (Fig. 3D). Figure 3C and D show a concentration-dependent decrease in the generation of antigen-specific T lymphocytes with trametinib alone and in combination (6 donors). No statistically significant change with any tested dose of dabrafenib was observed when compared with the DMSO control (Fig. 3C and D). This result suggests that the MEK inhibitor trametinib specifically inhibits the activation of antigen-specific T lymphocytes.

#### Trametinib alone and in combination with dabrafenib reduces cross-presentation of a tumor antigen

If dabrafenib and trametinib are to be employed in combination with immunotherapeutics, it is critical to understand their potential effect not only on T-cell activation and function but also on the capacity of the DCs to process and present antigen. Therefore, we studied the effects of trametinib and dabrafenib alone and in combination on the ability of human moDCs to process and present the cancer–testis antigen NY-ESO-1 (30, 31). MoDCs were isolated from HLA-A\*0201<sup>+</sup> donors and incubated with NY-ESO-1 as an immune complex (20  $\mu$ g/mL anti-His<sub>6</sub> antibody), in the presence or absence of inhibitors (Fig. 4A). NY-ESO-1<sub>157–165</sub> peptide-pulsed DCs (Fig. 4B) and NY-ESO-1 protein with anti-IgG1 isotype control (Fig. 4C) was used as a control. The HLA-A\*0201/NY-ESO-1<sub>157–165</sub> epitope was processed and presented efficiently as assessed by the response by an epitope-specific CD8<sup>+</sup> T-cell clone in a 6-hour ICS assay for IFN- $\gamma$  (Fig. 4A). There was significantly reduced, dose-dependent presentation of this epitope in the presence of trametinib when combined with dabrafenib ( $P < 0.05$ ).



**Figure 4.** Dabrafenib and trametinib in combination inhibit cross-presentation *in vitro*. Immature HLA-specific moDCs were treated for 18 hours with dabrafenib or trametinib, alone or in combination in the presence or absence of NY-ESO-1 peptide (positive control) and NY-ESO-1 protein and His-tag antibody. After 24 hours, an NY-ESO-1-specific, HLA-restricted CD8<sup>+</sup> T-cell clone was added to the treated and peptide-pulsed moDC to assess cross-presentation of NY-ESO-1 epitope in a standard recognition assay (no drug was present during the 4-hour APC-T-cell coculture) measuring specific cytokine secretion by the T-cell clone upon activation. Non-antigen-pulsed moDCs were used as the negative control (not shown). A, the capacity of HLA-A2<sup>+</sup> moDCs to cross-present NY-ESO-1-derived epitope (157–165) to the NY-ESO-1-specific CD8<sup>+</sup> T-cell clone (157-165/HLA-A2) *in vitro*. B, positive control; following drug treatment, moDCs were pulsed for 1 hour with the synthetic peptide for which the T-cell clone was specific. C, NY-ESO-1 protein with anti-IgG1 isotype control. Mean values with SEM from 4 healthy donors are shown. One-way ANOVA with a Dunnett post test against the control was performed; \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ . Dabrafenib, BRAFI; trametinib, MEKI.

To confirm these findings using a second epitope, the ability of HLA-Cw3 moDCs to cross-present NY-ESO-1 epitope (92–100) was examined (31, 36). Similarly, but to a lesser extent, the presentation of this epitope by moDC was reduced by 30 nmol/L trametinib and the combinations containing 10 and 30 nmol/L trametinib (data not shown).

#### Trametinib alone and in combination with dabrafenib promotes LPS-induced moDC maturation

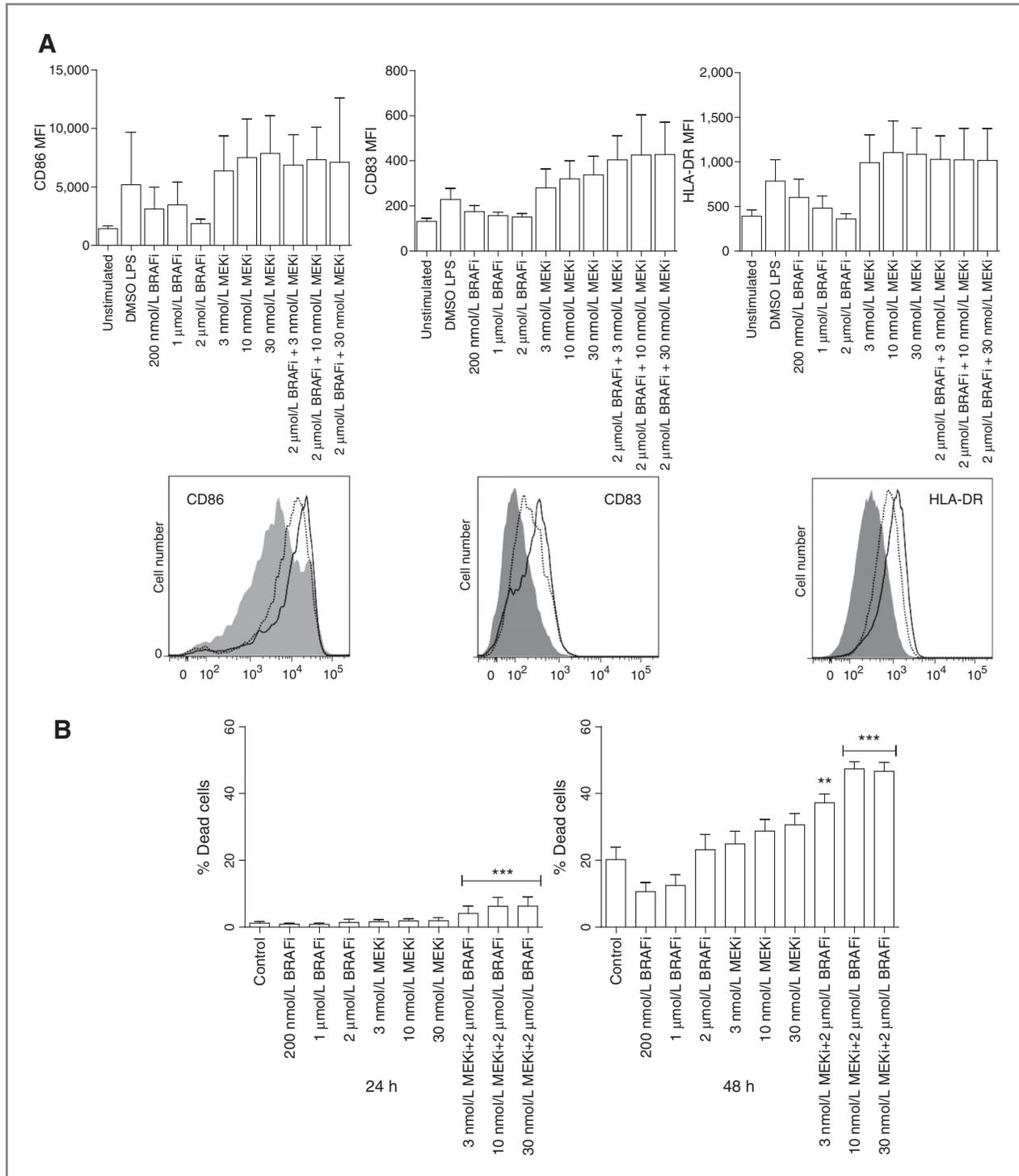
It has been shown previously that inhibition of ERK signaling potentiates DC maturation (37–40) and mature DCs are less effective at taking up antigen for processing than immature DCs. We therefore sought to investigate this further as a potential explanation for the negative impact of kinase inhibition on antigen cross-presentation. MoDCs were incubated with LPS in the presence of kinase inhibitors for 24 hours. LPS alone upregulated the expression of CD86, CD83, and HLA-DR that was further augmented by trametinib alone or in combination with dabrafenib (Fig. 5A). The effect of trametinib was reflected in both the percentage of positive cells and the median fluorescent intensity (MFI) of the whole population of cells, demonstrating that inhibition of the ERK signal transduction pathway enhances phenotypic maturation as has been shown previously by others (refs. 39, 41; Fig. 5A). No effect on the normal maturation pathway was observed when the MEKi was used alone or in combination (in the absence of LPS; data not shown). The toxicity exerted by the individual and combined

inhibitors was assessed using a fluorescent live/dead dye. There was a significant increase in the number of dead moDCs following 24- and 48-hour combination treatment and a dose-dependent increase (trend, not significant) in cell death when incubated with trametinib for 48 hours (Fig. 5B).

#### Discussion

Targeting oncogenic BRAF with kinase inhibitors has been one of the great success stories in the treatment of melanoma (3, 5, 42), and combination with MEK inhibition has further prolonged the survival of patients with metastatic disease (16, 17). Unfortunately, relapse is the rule rather than the exception, and the addition of effective agents will be required to build on these early successes (43). Among the most promising other therapies are the immunotherapeutics, and antibodies that target immune checkpoints such as CTLA-4 and PD-1/PD-L1/PD-L2 have attracted considerable attention (18–20). Clinical approaches combining kinase inhibitors with immunotherapeutics are a logical and evolving next step. To inform these approaches, it is helpful to better understand the effects of kinase inhibition on immune cell function.

There are limited data showing the effect of dabrafenib, trametinib alone, or their combination on immune cell function. Because the efficacy of immunotherapy is dependent on immune cell function, it is useful to ascertain the effects of these kinase inhibitors alone and in combination on these cell subsets before attempting any combination strategies. The



**Figure 5.** Trametinib and combination promote moDC maturation and decrease moDC viability. **A**, moDCs were incubated with the indicated concentrations of individual inhibitors or combinations, for 24 hours, in the presence of LPS. Cells were stained with fluorescently labeled live/dead stain, CD86, CD83, or anti-HLA-DR antibodies. MFI of bound antibody was determined by flow cytometry. Representative single-parameter histograms show the expression of CD80, CD83, and CD86 by MoDCs (gray) after the addition of LPS (dotted black line) or 30 nmol/L trametinib plus LPS (solid black line). **B**, moDCs were incubated with the indicated concentrations of individual inhibitors or combinations, for 24 or 48 hours. The toxicity exerted by the individual and combined inhibitors was assessed using a fluorescent live/dead dye. Mean values with SEM from 4 healthy donors are shown. One-way ANOVA with a Dunnett post test against the control was performed. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ . Dabrafenib, BRAFi; trametinib, MEKi.

current study sought to assess the effects of these drugs alone and in combination on isolated immune cells obtained from healthy donors. We show that while dabrafenib had no demonstrable inhibitory effects on human CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes or moDCs, trametinib inhibited or modulated a variety of functions.

The idea of combining BRAFi with immunotherapy arose following the discovery that expression of melanoma differentiation antigens (MDA; refs. 21, 32) and other tumor antigens, including cancer–testis antigens (22), on melanoma cells increased in response to vemurafenib (or its derivative, PLX4720). The increase in MDA and tumor antigen expression was associated with enhanced antigen-specific T-lymphocyte recognition *in vitro* (21, 22) and in clinical trial patients (24), providing the first evidence for combining vemurafenib treatment with T-lymphocyte-directed immunotherapy. BRAFi also enhances the levels of MHC molecules on melanoma cells, reverses immune suppression of T lymphocytes by inhibiting IL-1 $\alpha$  and IL-1 $\beta$  (25), and inhibits VEGF secretion from tumor cells (26), providing an additional explanation for why T-cell recognition is enhanced.

Regarding the direct effect of kinase inhibition on healthy T lymphocytes, previous *in vitro* studies have shown that vemurafenib and its derivative (PLX4720) do not compromise T-lymphocyte function (21, 32), whereas MEKi U0126 and PD0325901 are detrimental. We asked whether the function of normal healthy donor T lymphocytes was affected by direct dabrafenib or trametinib or combined treatment. Trametinib at a monotherapy dose of 10 nmol/L or in combination (2  $\mu$ mol/L dabrafenib combined with 3 nmol/L trametinib) significantly decreased T-lymphocyte proliferation and resulted in the dose-dependent suppression of cytokine-producing CD4<sup>+</sup> T lymphocytes and CD8<sup>+</sup> T lymphocytes. In contrast, dabrafenib had no effect on T-lymphocyte proliferation or the percentage of cytokine-positive cells. This result parallels the *in vitro* findings with the other BRAFi (vemurafenib) and its derivative (PLX4720), which have no effect on PBMC viability or proliferation (32), T-lymphocyte proliferation (21), or cytokine secretion (21, 23).

MEK inhibitors are not selective and inhibit ERK phosphorylation regardless of genotype or cell type. The MEK inhibitors U0126 and PD0325901 have been shown to have deleterious effects on T-cell viability, proliferation, and IFN- $\gamma$  production (21), whereas trametinib suppressed TNF- $\alpha$  and IL-6 production from PBMCs and reactivation of antigen-specific memory T lymphocytes (44). We demonstrate that trametinib has deleterious effects on T-lymphocyte proliferation and activation of antigen-specific T lymphocytes.

In response to maturation stimuli, inhibition of ERK activation can result in enhanced DC maturation with higher expression of MHC class II, costimulatory, and adhesion molecules (38, 39), indicating that ERK signaling helps in the maintenance of the immature state of DCs (37). The MEK inhibitors U0126 and PD98059 have been shown to promote the maturation of moDCs in the presence of inducing agents LPS or TNF- $\alpha$  (37, 39). Similarly, we found that in the presence of LPS, trametinib and the combined inhibitors enhanced phenotypic maturation. In a recent article, Ott and colleagues

report on their finding that maturation is inhibited by U0126; however, they suggest that the differences in the maturation stimuli are one important factor that might account for some of the inconsistencies (45).

The fact that trametinib and combined inhibitors enhanced phenotypic maturation provides a possible explanation why the NY-ESO-1 cross-presentation was impaired when moDCs were pretreated with trametinib and the combined inhibitors. Previous studies with PD98059 illustrated that blockade of the MAPK–ERK pathway not only potentiates phenotypic maturation but also enhances the loss of endocytic activity. This suggests that moDCs matured in the presence of trametinib and combined inhibitors lose their ability to capture and/or internalize NY-ESO-1–antibody immune complexes resulting in reduced cross-presentation of both NY-ESO-1<sub>92–100</sub> and NY-ESO-1<sub>157–165</sub> epitopes.

*In vivo*, BRAF inhibition has no detectable negative impact on existing systemic immunity or the *de novo* generation of tumor-specific T lymphocytes in patients with BRAF-mutant melanoma (46). *In vivo* BRAF inhibitors increase both the number of tumor-infiltrating lymphocytes (24, 47) and the intratumoral CD8<sup>+</sup> T:FoxP3<sup>+</sup>CD4<sup>+</sup> T-cell ratio, which has been linked to the downregulation of tumor CCL2 gene expression and production (48), and they inhibit the generation of myeloid-derived suppressor cells. Together, these studies demonstrated that BRAF inhibition engages a host immune response and that future trials combining BRAF inhibitors with immunotherapy would not be expected to impair but rather prolong clinical response. Immunotherapeutics are inducing durable remissions in a significant proportion of patients (18–20), and clinical trials that combine kinase inhibition with immunotherapeutics are a potentially promising strategy; however, these approaches should incorporate a careful evaluation of any effects on host immunity, as interference with immune function has the potential to compromise long-term outcomes.

#### Disclosure of Potential Conflicts of Interest

J.S. Cebon has received honoraria from the speakers' bureau and serves as a consultant/advisory board member for GlaxoSmithKline. No potential conflicts of interest were disclosed by the other authors.

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## MEK Inhibition, Alone or in Combination with BRAF Inhibition, Affects Multiple Functions of Isolated Normal Human Lymphocytes and Dendritic Cells

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