Combined BRAF, MEK, and CDK4/6 Inhibition Depletes Intratumoral Immune-Potentiating Myeloid Populations in Melanoma

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Abstract

Combined inhibition of BRAF, MEK, and CDK4/6 is currently under evaluation in clinical trials for patients with melanoma harboring a BRAFV600E mutation. While this triple therapy has potent tumor-intrinsic effects, the impact of this combination on antitumor immunity remains unexplored. Here, using a syngeneic BrafV600E/Cdkn2a−/−Pten−/− melanoma model, we demonstrated that triple therapy promoted durable tumor control through tumor-intrinsic mechanisms and promoted immunogenic cell death and T-cell infiltration. Despite this, tumors treated with triple therapy were unresponsive to immune checkpoint blockade (ICB). Flow cytometric and single-cell RNA sequencing analyses of tumor-infiltrating immune populations revealed that triple therapy markedly depleted proinflammatory macrophages and cross-priming CD103+ dendritic cells, the absence of which correlated with poor overall survival and clinical responses to ICB in patients with melanoma. Indeed, immune populations isolated from tumors of mice treated with triple therapy failed to stimulate T-cell responses ex vivo. While combined BRAF, MEK, and CDK4/6 inhibition demonstrates favorable tumor-intrinsic activity, these data suggest that collateral effects on tumor-infiltrating myeloid populations may impact antitumor immunity. These findings have important implications for the design of combination strategies and clinical trials that incorporate BRAF, MEK, and CDK4/6 inhibition with immunotherapy for the treatment of patients with melanoma.

Introduction

Inhibition of the MAPK/ERK pathway using small-molecule inhibitors of mutant BRAFV600E and MEK is a standard-of-care therapy for treating BRAF-mutant melanoma. Such therapy is typified by exceptional short-term clinical responses followed by the eventual emergence of resistance in most patients (1). Immune checkpoint blockade (ICB) of the inhibitory receptors PD1 and CTLA4 on T cells, is an additional treatment option for patients with melanoma (2); however, the immunomodulatory effects of MAPK/ERK-targeted therapy can impact the efficacy of this therapy. For example, short-term BRAF inhibition can enhance tumor susceptibility to ICB by increasing the immunogenicity of tumors (3, 4), while resistance to long-term BRAF inhibition correlates with a loss of tumor-infiltrating lymphocytes essential for ICB efficacy (4–6).

Cyclin-dependent kinases 4 and 6 (CDK4/6), are aberrantly activated in approximately 90% of melanomas (7), and inhibiting these kinases improves the efficacy of dual BRAF and MEK inhibition (8, 9). As a result, triple inhibition of BRAF, MEK, and CDK4/6 is now being evaluated in clinical trials for the treatment of melanoma (NCT01820364, NCT02065063). Similar to BRAF- and MEK-targeted therapies, CDK4/6 inhibitors also have immunomodulatory effects (10, 11). However, the impact of the triple therapy on antitumor immunity remains unexplored.

Here, we used our recently developed syngeneic mouse model of BrafV600E/Cdkn2a−/−Pten−/− melanoma (12), to examine the immunomodulatory effects of BRAF, MEK, and CDK4/6 inhibition. While superior to dual BRAF and MEK inhibition in controlling tumor growth, we found that continuous administration of triple therapy depleted the tumor microenvironment of proinflammatory macrophages and cross-priming CD103+ dendritic cells (DC) that may be required for optimal antitumor T-cell immunity. These data have important clinical implications for therapy scheduling and the stratification of patients with melanoma between trials of this triple therapy and available immunotherapy options.

Materials and Methods

Mice and in vivo growth and therapy studies

Animal work was performed in agreement with the National Health and Medical Research Council (NHMRC) Australian code for care and use of animals for scientific purposes 8th edition (2013) with approval from the Peter MacCallum Animal Experimentation Ethics Committee. A total of 2 × 106 YOVAL1.1 cells in PBS were injected subcutaneously on the right flank of C57BL/6 (purchased from Walter and Eliza Hall Institute of Medical Research, Parkville, Australia) or...
YOVAL1.1 cells were cultured in RPMI1640 (Thermo Fisher Scientific, catalog no. 11875093) supplemented with 10% FBS (Thermo Fisher Scientific, catalog no. 10100147), 20 mmol/L HEPES, boiled for 5 minutes, and quantified using DC protein assay (Bio-Rad, catalog no. 5000112) as per the manufacturer’s protocol. Equal amounts of protein in 5× SDS sample buffer [0.05% (w/v) 313 mmol/L Tris HCl pH 6.8, 10% (w/v) SDS, 50% [volume for loading buffer] glycerol, 10% (w/v) β-mercaptoethanol, bromophenol blue] were boiled for 5 minutes and resolved via SDS-PAGE using precoated gels (Bio-Rad) with running buffer [25 mmol/L Tris (VWR), 190 mmol/L glycine (Astral Scientific), 0.1% (w/v) SDS]. Precision-plus protein dual color standard (Bio-Rad, catalog no. 1610374) was used as a molecular weight marker. Proteins were transferred onto methanol-activated polyvinylidene difluoride membranes (Millipore) using the Trans-Blot Turbo semidry transfer system (Bio-Rad). Antibodies used were: anti-phospho-44/42 MAPK (pErk1/2; 845-15C12, Cell Signaling Technology, catalog no. 4370S), anti-p44/42 MAPK (ERK1/2; EPR3924, Cell Signaling Technology, catalog no. 9102S), anti-Phospho-RB (Ser807/811; Cell Signaling Technology, catalog no. 9308S), anti-RB1 (G3-245; BD Pharmingen, catalog no. 354136), anti-α-Tubulin (DM1A; Merck Millipore, catalog no. 05-829), anti-Mouse IgG (H + L)-HRP Conjugate (Bio-Rad, catalog no. 1706516), anti-Rabbit IgG (H + L)-HRP Conjugate (Bio-Rad, catalog no. 1706515).

In vivo drug assays
YOVAL1.1 cells were seeded at a density of 500 cells per well in 96-well plates and allowed to adhere for 24 hours. After 24 hours, confluency was measured using Incucyte Zoom (Essen Biosciences). Targeted therapies were then added to each well using five technical replicates per treatment group. Assay plates were then incubated under standard cell culture conditions. Live cell confluency was measured every 12 to 24 hours for 2 weeks using Incucyte Zoom. Media was removed and replaced with fresh media plus drugs after 1 week.

Immunoblot
Cells were lysed in 2% SDS buffer containing 0.5 mmol/L EDTA and 20 mmol/L HEPES, boiled for 5 minutes, and quantified using DC protein assay (Bio-Rad, catalog no. 5000112) as per the manufacturer's protocol. Equal amounts of protein in 5× SDS sample buffer [0.05% (w/v) 313 mmol/L Tris HCl pH 6.8, 10% (w/v) SDS, 50% (volume for loading buffer) glycerol, 10% (w/v) β-mercaptoethanol, bromophenol blue] were boiled for 5 minutes and resolved via SDS-PAGE using precoated gels (Bio-Rad) with running buffer [25 mmol/L Tris (VWR), 190 mmol/L glycine (Astral Scientific), 0.1% (w/v) SDS]. Precision-plus protein dual color standard (Bio-Rad, catalog no. 1610374) was used as a molecular weight marker. Proteins were transferred onto methanol-activated polyvinylidene difluoride membranes (Millipore) using the Trans-Blot Turbo semidry transfer system (Bio-Rad) with tris-glycine transfer buffer [50 mmol/L Tris, 0.375% (w/v) SDS, 40 mmol/L glycine, 20% (v/v) methanol]. Membranes were blocked in 5% skim milk (Diploma Instant) in TBS-T [TBS-T+0.1% Tween-20 (Sigma-Aldrich)] for 1 hour prior to probing with primary antibody overnight at 4°C, and horseradish peroxidase (HRP)–conjugated secondary antibody for 1 hour at room temperature. Immunoblots were washed for 10 minutes in TBS-T three times after each antibody incubation. Proteins were detected using ECL Western blotting substrate (GE Healthcare) and imaged using the ChemiDoc Imaging System (Bio-Rad). Antibodies used were: anti-phospho-44/42 MAPK (Erk1/2; Thr202/Tyr204; D13.14.4E) XP (Cell Signaling Technology, catalog no. 4370S), anti-p44/42 MAPK (ERK1/2; Cell Signaling Technology, catalog no. 9102S), anti-Phospho-RB (Ser807/811; Cell Signaling Technology, catalog no. 9308S), anti-RB1 (G3-245; BD Pharmingen, catalog no. 354136), anti-α-Tubulin (DM1A; Merck Millipore, catalog no. 05-829), anti-Mouse IgG (H + L)-HRP Conjugate (Bio-Rad, catalog no. 1706516), anti-Rabbit IgG (H + L)-HRP Conjugate (Bio-Rad, catalog no. 1706515).

ICH
Tumors were fixed in 10% neutral buffered formalin (NBF) overnight and paraffin embedded, followed by 4-μm-thick sectioning onto glass slides. Slides were dewaxed in xylene, followed by antigen retrieval in 10 mmol/L citrate buffer (Sigma; pH 6) at 125°C for 3 minutes. PerkinElmer OPAL reagents were used for staining as per...
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Software suite tidyverse from R was used to perform all data manipulation and visualization. All sequencing data have been deposited into the Gene Expression Omnibus (GEO) under the accession number GSE162467.

For analyses of Sade-Feldman and colleagues’ dataset (18), transcripts per million (TPM) values were downloaded from the GEO (accession ID GSE120575 and processed using the R statistical software language (version 3.6.1) with the Seurat package (version 3.1.2; ref. 13). The top 2,000 variable genes were selected using the FindVariableFeatures function with the variance stabilizing transformation method. The selected genes were then scaled using the ScaleData function, using the fraction of total counts belonging to mitochondrial genes and the log total unique molecular identifier (UMI) counts for each cell as variables to regress out. PCA of the scaled genes was then calculated using the RunPCA function. The top 20 principal components were used to calculate Uniform Manifold Approximation and Projection (UMAP; ref. 19) values using the uwot R package (version 0.1.5). Cells were clustered using the Louvain algorithm (with resolution parameter 0.8) using the FindClusters function applied to the SNN network calculated using the FindNeighbors function. Cosine distance metric and 30 nearest neighbors were used for both UMAP and SNN algorithms. Signature AUC scores were calculated using the AUC R package (version 1.8.0). Clusters were labelled with putative cell types informed by the scMatch algorithm (20). Patients where total myeloid cells were less than 1% were excluded from analysis. Cells in the monocytic cluster with CD103+ DC AUC score greater than 0.2 were classified as CD103+ DCs.

**The Cancer Genome Atlas analysis**

RNA-Seq by Expectation Maximization (21) scaled expression values for The Cancer Genome Atlas (TCGA) were downloaded from the GDAC Firehose website (22). Counts were normalized to TPM values with a pseudocount of 2. ENTZ gene IDs were mapped to HGNCS gene symbols using the biomaRt R package (version 2.4.20; ref. 23) and collapsed to unique values per gene symbol by selecting the most variable entrez ID among all samples for each gene symbol. Primary and, where a primary sample was unavailable, metastatic tumor samples from the skin cutaneous melanoma (SKCM) dataset (n = 469; Supplementary Table S1) were selected using TCGAbiolinks R package (version 2.14.0; ref. 24) and were matched with overall survival (OS) endpoints from TCGA Pan-Cancer Clinical Data Resource (25). WDFY4 and XCR1 expression was separated in “low” (bottom quartile) and “int-high” (top 3 quartiles) categories, then used to fit Kaplan–Meier and Cox regression models using the survival R package (version 3.1-8; ref. 26).

**Statistical analysis**

One-way ANOVA with Tukey multiple comparisons tests, log-rank (Mantel–Cox) test, and unaired t tests were performed using GraphPad Prism. All experiments other than single-cell RNA sequencing (scRNA-seq) were performed in at least three biological replicates and error bars show ± SEM. Significance was determined as *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001.

**Results and Discussion**

Combined BRAF, MEK, and CDK4/6 inhibition has antitumor and immunomodulatory activity

To determine whether the CDK4/6 inhibitor, palbociclib, enhances the efficacy of dual BRAF and MEK inhibition in a synergetic setting, we first tested the in vitro activity of this triple therapy on our immunogenic BrafV600ECdkn2a−/−/Pten−/− mouse melanoma cell line,
YOVAL1.1 (12). Consistent with human melanoma cell lines (8, 9), triple therapy potently suppressed both phosphorylation of retinoblastoma (Supplementary Fig. S1A) and proliferation of YOVAL1.1 cells compared with dual BRAF and MEK inhibition (BRAFi+MEKi) or CDK4/6 inhibition (CDK4/6i) alone (Fig. 1A). Triple therapy also led to significant upregulation of the HMC Class I molecule H-2Kb (Fig. 1B; Supplementary Fig. S1B), which presents the YOVAL1.1 immunogenic peptide derived from ovalbumin (12), and induced surface exposure of CRT, an indicator of immunogenic cell death (Fig. 1C; Supplementary Fig. S1B; ref. 27). These effects were predominately mediated through BRAFi+MEKi, consistent with previous reports (28). In vivo, triple therapy resulted in immediate tumor regression and significantly improved survival compared with BRAFi+MEKi or CDK4/6i alone (Fig. 1D; Supplementary Fig. S1C). Notably, treatment of YOVAL1.1 tumors with triple therapy recapitulated the potent and prolonged tumor control observed with dual BRAFi and CDK4/6i in xenograft models of human BrafV600E/Cdkn2a−/− melanoma (8, 9).

To examine the immunomodulatory effects of triple therapy, mice bearing YOVAL1.1 tumors were treated with vehicle, BRAFi+MEKi, CDK4/6i, or triple therapy for 7 days, after which the tumors were harvested and the immune compartment analyzed by flow cytometry. No significant difference in the total number of tumor-associated CD45.2+ immune cells was observed across groups (Supplementary Fig. S1D and S1E), however, there was a notable shift in the distribution of the lymphoid and myeloid compartments in response to the triple therapy (Fig. 1E–J; Supplementary Fig. S1D–S1I). Specifically, these tumors contained a significantly higher frequency of both CD4+ and CD8+ T cells compared with all other treatment groups (Fig. 1F–G; Supplementary Fig. S1F), while the frequency of NK and B cells remained relatively unchanged (Supplementary Fig. S1G and S1H). Interestingly, despite a significant increase in CD4+ T cells following triple therapy, the frequency of CD4+ regulatory T cells (Treg) was not increased (Fig. 1F), likely due to the reported anti-proliferative effects of CDK4/6 inhibitors on this T-cell subset (10). The increase in lymphocyte frequency following triple therapy was coupled with a concurrent decrease in the frequency of myeloid cells. We observed a significant loss of tumor-associated CD11b+ cells, which was exclusive to tumors treated with triple therapy (Fig. 1H–J). This encompassed a reduction in all myeloid subsets analyzed, including monocytes (CD11b+Ly6C+Ly6G−), mononuclear myeloid-derived suppressor cells (MDSC; CD11b+Ly6C+Ly6G−/CD11c−), and tumor-associated macrophages (Ly6C+Ly6G−/CD11c+CD14+) (Fig. 1J; Supplementary Fig. S1D). No changes were seen in polymorphonuclear MDSCs (CD11b+Ly6C+Ly6G+), although the frequency of these cells across all groups was negligible (Supplementary Fig. S1I). Collectively, these data highlight that, in addition to potent tumor-intrinsic activity, triple therapy leads to profound remodeling of the tumor immune microenvironment.

Tumors treated with BRAFi, MEK, and CDK4/6 inhibition are unresponsive to ICB

To investigate the contribution of host immunity to the antitumor activity of triple therapy, we treated tumors grown in fully immunodeficient NSG mice, Rag2−/−, B2m−/−, and Cd8−/− mice, which lack functional T, B, and NK cells, and wild-type mice depleted of CD8+ T or NK cells. We observed no difference in the efficacy of triple therapy in these mice compared with immunocompetent mice, suggesting that the in vivo antitumor activity of this combination was predominately mediated by tumor-intrinsic mechanisms (Supplementary Fig. S2A and S2B), likely due to selective and potent inhibition of the clinically relevant oncogenic drivers in these tumor cells (12). While triple therapy was superior at controlling YOVAL1.1 tumor growth and delayed the emergence of drug resistance compared with dual BRAFi+MEKi, tumors did eventually escape approximately 80 days into treatment in a third of the mice (Fig. 1D; Supplementary Fig. S2C). Another third demonstrated complete tumor clearance for several weeks, but tumors rapidly reestablished upon therapy withdrawal (Supplementary Fig. S2C). Therapy resistance and the need for continued treatment are two common clinical challenges of targeted therapies. Consequently, several clinical trials are currently examining the benefits of combining MAPK/ERK- or CDK4/6-targeted therapies with ICB as a strategy to overcome such challenges (NCT02130466, NCT04075604). ICB is reportedly more efficacious in settings where tumor burden is low (29), tumor-lymphocyte infiltration is high (30) and immunosuppressive populations, such as Tregs and MDSCs, are low (Fig. 2A; refs. 31, 32). Notably, all these factors shifted favorably following triple therapy (Fig. 2B and C). We have previously shown the YOVAL1.1 model is amenable to anti-PD1+anti-CTLA4 (12), and as such we sought to determine whether ICB could be used to promote immune-mediated clearance or control of tumors following withdrawal from triple therapy. To investigate this, mice with established YOVAL1.1 tumors were treated short term (1–2 weeks) with triple therapy ± ICB (anti-CTLA4+anti-PD1). Multiple scheduling combinations were tested (Fig. 2D–G) to examine both the capacity of ICB to enhance triple therapy–mediated tumor regression up front, or to promote clearance of residual tumors following triple therapy. However, in all instances, tumors failed to respond to ICB, with no significant difference in tumor growth or survival (Fig. 2D–G).

To determine whether triple therapy had induced tumor-intrinsic resistance to ICB, residual tumors were harvested from mice treated with triple therapy and reimplanted into treatment-naïve recipients (Fig. 2H). Once the tumors reached an equivalent size at which they were resistant to ICB following triple therapy, mice were treated with either ICB or an isotype control (Fig. 2H). In this setting, ICB led to complete tumor clearance in 8/9 mice (Fig. 2H). Taken together, these findings suggest that the immunomodulatory activity of triple therapy promotes a host tumor microenvironment that is unresponsive to ICB.

Triple therapy depletes tumor-associated proinflammatory macrophages and CD103+ DCs

To investigate changes in the tumor immune microenvironment that might account for the unresponsiveness of triple therapy–treated YOVAL1.1 tumors to ICB, we further analyzed the tumor-associated immune compartment using scRNA-seq and paired TCR sequencing. YOVAL1.1 tumors were harvested from mice after 7 days of treatment with either vehicle or triple therapy, followed by isolation and droplet encapsulation of the CD45.2+ cells for sequencing (Fig. 3A). Dimensionality reduction tSNE analysis delineated 10 broad immune subsets including B cells, DCs, innate lymphoid cells, macrophages, monocytes, neutrophils, NK cells, CD4+ and CD8+ T cells, and Tregs (Fig. 3B and C). In accordance with our earlier analyses (Fig. 1), we detected an almost complete loss of myeloid populations following triple therapy, coupled with a corresponding increase in the lymphoid compartment (Fig. 3C and D). Both CD8+ and CD4+ T cells were transcriptionally and clonally similar across untreated and treated samples, with comparable expression of genes associated with effector function (e.g., Ifng, Prf1; Fig. 3E–G). However, T cells from tumors

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Figure 1.
Triple therapy has antitumor and immunomodulatory activity. A, YOVAL1.1 cell confluence measured over time in vitro in the presence of vehicle or the indicated drug(s); this experiment is representative of n = 3. B and C, YOVAL1.1 cells analyzed by flow cytometry after 72 hours of treatment; one-way ANOVA Tukey multiple comparisons test, n = 3–5. D, YOVAL1.1 tumor growth and survival in C57BL/6 mice; n = 7–9 per group, log-rank (Mantel–Cox) test. Analysis of YOVAL1.1 tumors by flow cytometry (E and F) and IHC (G) pretreatment or after 7 days of treatment. E, H, and I, tSNE clustering based on equal numbers of CD45.2+ cells pooled from all treatment groups; plots are representative of n = 4 concatenated samples per group. M-MDSC, mononuclear MDSC; PMN-MDSC, polymorphonuclear MDSC. F and J, Indicated population frequency of CD45.2+; one-way ANOVA Tukey multiple comparisons test, n = 4–9. G, Representative of n = 3; quantification in Supplementary Fig. S1F. All gating strategies are shown in Supplementary Fig. S1B and S1D. Error bars show ± SEM. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001. BRAFi, dabrafenib; CDK4/6i, palbociclib; MEKi, trametinib; triple, BRAFi+MEKi+CDK4/6i.
Figure 2.
Tumors treated with triple therapy are unresponsive to ICB. A, Schematic showing tumor features associated with response/resistance to ICB (29–32). B, Tumor weights after 7 days of treatment; unpaired t-test, n = 6–9. C, Indicated population frequency of CD45.2+ cells compared with pretreatment samples from Fig. 1E–J; columns show individual mice. D–G, YOVAL1.1 tumor growth and survival in C57BL/6 mice; n = 3–6 per group. H, Residual YOVAL1.1 tumors were isolated following 1 week of triple therapy, transferred into naive C57BL/6 recipients, and treated with ICB when tumors reached an equivalent size at which they were harvested; graphs show tumor growth and survival from time of tumor inoculation, with ICB administered where indicated; n = 9–10 per group, log-rank (Mantel–Cox) test. Error bars show ± SEM. ***, P < 0.001. BRAFi, dabrafenib; CDK4/6i, palbociclib; ICB, anti-CTLA4+anti-PD1; MEKi, trametinib; triple, BRAFi+MEKi+CDK4/6i.
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Figure 3.
Triple therapy depletes proinflammatory macrophages and CD103⁺ DCs. ScRNA-seq on CD45.2⁺ cells isolated from YOVAL1.1 tumors following 7 days of treatment with vehicle or triple; n = 10 mice per group pooled. A, Schematic of experimental setup; tumors from 10 mice per treatment group were pooled, and CD45.2⁺ immune cells were isolated by FACS for scRNA-seq. B and C, tSNE clustering and annotation based on gene expression. Endoth/Fibro, endothelial cells/fibroblasts; ILC, innate lymphoid cell. D, Cluster frequencies. E, Gene expression of CD8⁺ and CD4⁺ T cells. F, Number of unique TCR rearrangements. G, Frequency of top 100 abundant clones plus remaining clones (other). H, Gene expression of macrophages. Frequency (I) and gene expression (J) of DC clusters 1–4 (DC_c1–4) delineated from analysis in C. K, Expression of CD103⁺ DC gene signature (40) in DC clusters. L, Frequency of CD103⁺ DCs (DC_c2) as a percentage of all myeloid cells identified by scRNA-seq. Triple, BRAFi (dabrafenib) + MEKi (trametinib) + CDK4/6i (palbociclib).
Tumor-associated myeloid cells are prognostic for clinical responses to ICB and required for inducing T-cell immunity

To investigate the potential clinical impact of depleting WDFy4 and Xcr1+ DCs we analyzed TCGA datasets, and found that low expression of WDFy4 and Xcr1 significantly correlated with poor survival in patients with melanoma (Fig. 4A). To investigate whether CD103+ DCs were impacted by triple therapy, we analyzed a previously published scRNA-seq dataset of tumor-infiltrating immune populations from patients with melanoma taken prior to or during ICB (18). Using the CD103+ DC signature that was applied to our scRNA-seq analyses (Fig. 3K; ref. 40), we identified a rare population within the myeloid compartment of patient samples that was enriched for this gene set (Fig. 4B; Supplementary Fig. S4B–S4D). In accordance with our scRNA-seq dataset, this population was also enriched for WDFy4 and Xcr1 expression, with XCR1 expression exclusive to this immune subset (Fig. 4B; Supplementary Fig. S4D). Importantly, a significantly higher proportion of this CD103+ DC subset was present within the myeloid population in patients that responded to ICB compared with nonresponders, as defined by REGIST (Fig. 4C; ref. 42). Likewise, patients with low levels of intratumoral CD103+ DCs (less than 5% of the myeloid population) had much poorer response rates than patients with higher CD103+ DC frequencies (20% vs. 50% response rate, respectively; Fig. 4D). This correlation appeared stronger for patients treated with anti-CTLA4 or anti-CTLA4+anti-PD1 than anti-PD1 alone (Fig. 4C). In contrast to anti-PD1 therapy, which blocks inhibitory interactions between T cells and tumor cells, anti-CTLA4 functions primarily through derepressing cross-priming interactions between T cells and DCs (43). CD103+ DCs might therefore be expected to correlate more strongly with ICB responses in the context of anti-CTLA4 therapy, consistent with our findings. Furthermore, we have previously shown that treatment-naive YOVAL1.1 tumors are responsive to anti-CTLA4 and anti-CTLA4+anti-PD1, but not anti-PD1 alone (12). This suggests that poor responsiveness to ICB in this model is mediated through mechanisms that infer resistance to anti-CTLA4, such as a loss of CD103+ DCs following triple therapy.

To further validate whether CD103+ DCs were among the dominant myeloid subsets depleted following triple therapy, YOVAL1.1 tumors were harvested from mice after 7 days of triple therapy and analyzed by flow cytometry using surface markers specific for this population (Supplementary Fig. S4E; ref. 40). Unsupervised tSNE clustering delineated a distinct cluster expressing MHC II, CIITA, CD4, and CD11c, which we identified as being CD103+ DCs using published gating strategies (Fig. 4E and F; Supplementary Fig. S4E; ref. 40). In support of our scRNA-seq analyses, both CD103+ DCs and macrophages were markedly depleted in tumors treated with the triple therapy compared with vehicle controls (Fig. 4F and G), with a significant reduction in the frequency and total number of these myeloid populations (Fig. 4G; Supplementary Fig. S4F). Importantly, the frequency of both populations was significantly higher in treatment-naïve tumors of an equivalent size (Fig. 4G), which were previously confirmed to be responsive to ICB (Fig. 2H; ref. 12). This further indicated that depletion of these myeloid subsets by triple therapy may be contributing to the poor responsiveness of these tumors to ICB. Interestingly, depletion of these subsets was not seen after only 2 days of triple therapy, while MDSCs were still depleted efficiently in this time frame (Supplementary Fig. S5A and S5B). Furthermore, following 7 days of triple therapy, depleted MDSCs rapidly repopulated tumors when therapy was withdrawn, while antigen-presenting subsets failed to recover in the 5 days following therapy withdrawal (Supplementary Fig. S5C and S5D). This suggests that short intermittent dosing of triple therapy may be needed to preserve immune-potentiating myeloid populations within tumors.

As tumor-associated CD103+ DCs and proinflammatory macrophages were depleted following triple therapy, and are critical components of antitumor T-cell immunity (35, 40), we hypothesized that the CD45.2+ immune population from these tumors would fail to stimulate optimal T-cell responses. To test this,
Figure 4.
Intratumoral myeloid populations depleted by triple therapy are required for optimal T-cell immunity. 

A, Kaplan-Meier OS of 469 patients with melanoma from TCGA stratified by top 75% (int-high) versus bottom 25% (low) expression of indicated gene; log-rank P values are indicated. 

B–D, Analysis of scRNA-seq on tumor-infiltrating immune cells from patients with melanoma treated with ICB (18). B, UMAP clustering based on gene expression, showing myeloid populations only (full plots in Supplementary Fig. S4B–S4D). CD103+ DCs identified using signature from Fig. 3K (40). C, CD103+ DC frequency of all myeloid cells in responders versus nonresponders; unpaired t test. Tx, treatment. D, Proportion of responders among patients with low (<5% of myeloid population) or high (>5% of myeloid population) CD103+ DCs. 

E–G, Analysis of YOVAL1 tumors by flow cytometry after 7 days of treatment; gating strategy shown in Supplementary Fig. S4E. E and F, ISNE clustering based on equal numbers of CD45.2+ cells pooled from both treatment groups; plots are representative of n = 8 concatenated samples per group. G, Frequency of indicated population; one-way ANOVA Tukey multiple comparisons test, n = 8–10. H, Schematic of experimental setup; CD45.2+ cells were isolated from tumors treated with vehicle or triple for 7 days, pulsed with OVA222–239, and cocultured with CTV-labeled CD4+ T cells from transgenic OT-II mice. I, Representative plot of CTV measured after 90 hours of coculture (left) and percentage proliferating based on CTV dilution (right); unpaired t test, n = 3. J, Representative plot and flow cytometric analysis of activation markers; one-way ANOVA Tukey multiple comparisons test, n = 3. K and J, n = 3 independent cocultures with CD45.2+ cells from 3 mice for each coculture. K, Schematic showing tumor features associated with response/resistance to ICB and the microenvironment of tumors following treatment with triple. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. Triple, BRAFi (dabrafenib) + MEKi (trametinib) + CDK4/6i (palbociclib).
CD45.2<sup>+</sup> immune cells isolated from tumors treated with vehicle or triple therapy were pulsed with the cognate antigen, bivalent antibody (OVA<sub>252-264</sub>) and cocultured with CT26-labeled naive CD45<sup>+</sup> OT-II T cells (Fig. 4H). Strikingly, OT-II<sup>+</sup> T cells cocultured with CD45.2<sup>+</sup> cells from tumors treated with the triple therapy failed to proliferate or upregulate activation markers (CD44, CD69, and CD25; Fig. 4I and J). Taken together, these data suggested that tumor-associated myeloid populations depleted by triple therapy are important for the induction of optimal T-cell responses.

In conclusion, we demonstrate that combined inhibition of BRAF, MEK, and CDK4/6 leads to profound remodeling of the tumor immune microenvironment. The changes observed are reminiscent of those reported in tumors that respond to ICB (such as low tumor burden, increased lymphocyte frequency, and a reduction in immunosuppressive cells; Fig. 4K). However, our data demonstrate that this triple combination also depletes immune-potentiating myeloid populations, including proinflammatory macrophages and cross-priming CD103<sup>+</sup> DCs, and in doing so may reduce these tumors unresponsive to ICB (Fig. 4K). As a result, this promising therapy, while effective due to potent tumor-intrinsic activity, may impede the ability of the immune system to contribute to the overall antitumor response.

Authors’ Disclosures

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