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 × 10^5 YOVA1.1 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

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C57BL/6-Rag2<sup>−/−</sup> (bred in house) male mice (6–10 weeks old) and tumors were measured twice per week. Tumor volumes were calculated as $0.5 \times \text{tumor length} \times \text{tumor width}^2$. NOD scid gamma (NSG) mice were bred in house and C57BL/6 OT-II were obtained from the Peter Doherty Institute (Melbourne, Australia). For survival, end-point tumor volume was $>1,200 \text{mm}^3$. Unless otherwise indicated, mice were randomized for treatment when tumors reached approximately 100 mm$^3$. For targeted therapies, mice were dosed with dabrafenib/trametinib (Ark Pharm, catalog no. AK174048 and Focus Bioscience, catalog no. HY-10999, respectively; 300/0.3 mg/kg in 0.5% hydroxypropylmethyl cellulose, 0.2% Tween 80 in H$_2$O), palbociclib (Pfizer; 80 mg/kg in 50 mmol/L sodium lactate), or the combination.

The targeted therapies were administered daily by oral gavage 6–7 days per week. Anti-PD1 (Bio X Cell, clone RMP1-14, catalog no. BE0146), anti-CTLA4 (Bio X Cell, clone 2A3, catalog no. BE0089, or polyclonal Syrian hamster, catalog no. BE0087, respectively) were corresponding isotype controls (Bio X Cell, catalog no. BE0131). For survival, or polyclonal Syrian hamster, catalog no. BE0087, respectively) were corresponding isotype controls (Bio X Cell, clone 2A3, catalog no. BE0089, or polyclonal Syrian hamster, catalog no. BE0087, respectively) were given by intraperitoneal injection 4 days apart (first dose 200/150 μg, subsequent doses 150/100 μg). CD8 and natural killer (NK)–depleting antibodies, anti-CD8 (Bio X Cell, clone YTS169.4, catalog no. BE0117) and anti-asialo GM1 (Novachem, catalog no. 986-10001), respectively, were administered at 250 μg/mouse on day 1 and 0 and 150 μg/mouse on day 4, 8, and weekly ongoing, with day 0 being the day of tumor inoculation.

**Tumor preparation, flow cytometry, and sorting**

Tumors were harvested, weighed, and digested with a scalpel followed by incubation with collagenase IV (1.6 mg/mL; Worthington Biochemical Corporation, catalog no. LS004188) + DNase (2 U/mL; Merck, catalog no. 11284932001) in DMEM, high glucose, pyruvate (Thermo Fisher Scientific, catalog no. 11965092) for 45 minutes at 37°C with agitation. Digests were then filtered through a 70 μm filter and washed before staining with relevant antibodies for flow cytometry analysis or FACS. Immune cells were isolated from tumors by FACS on live CD45.2<sup>+</sup> cells using BD FACSaria Fusion. Antibodies were sourced from Thermo Fisher eBioscience—H–2Kb (AF6-88.5.5.3), CD3 (17A2), CD4 (GK1.5), FOXP3 (FJK-16A), Ly6C (HK1.4), Ly6G (1A8), and CD103 (2E7)—BioLegend—CD8 (53–67.6), CD19 (6D5), NKI.1 (PK136), CD11b (M1/70), MHC-II (1-A/1-E; M5/114.15.2), F4/80 (BM8), CD24 (M1/69), CD44 (IM7), CD69 (H.2F3), and CD25 (PC61)—Tombo Biosciences CD45.2 (104)—and Abcam—calreticulin (CRT; EPR39294), Fixable yellow (Invitrogen, L34959) was used to stain live/dead cells. Fluorescence was measured on a BD FACSFlow cytometer or a BD LSR Fortessa X-20 cytometer (BD Biosciences) and data analyzed using FlowJo, LLC software. For t-distributed stochastic neighbor embedding (tSNE) analysis, subpopulations of equal numbers of CD45.2<sup>+</sup> cells were generated from representative samples using the DownSample V3 plugin. Subpopulations from all groups were then concatenated into one file for dimensionality reduction using the tSNE function, with all antibody markers plus FSC and SSC as parameters. Different groups within the concatenated file were then redelineated on the basis of preassigned sample IDs.

**Cell lines and in vitro drug assays**

YOVAL1.1 cells were developed in-house (described in ref. 12; 2019) and used for in vivo work within 8 days (two passages) of thawing, or for in vitro work within 14 days (four passages) of thawing. 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 (Merck, catalog no. H0887), 1% GlutaMAX (Thermo Fisher Scientific, catalog no. 35050061), 1 mmol/L Minimal Essential Medium Non-Essential Amino Acids (Thermo Fisher Scientific, catalog no. 11140050), 0.1% 2-mercaptoethanol (Merck, catalog no. M6250) and 1 mmol/L Sodium Pyruvate (Thermo Fisher Scientific, catalog no. 11360070), at 37°C in 5% CO$_2$. Cells were confirmed negative for *Mycoplasma* by PCR and used within 1 week of testing. For proliferation assays, cells were cultured in various combinations of 2 mmol/L dabrafenib, 0.2 mmol/L trametinib, or 2 μmol/L palbociclib. For flow cytometry and Western blot cells were cultured in various combinations of 20 mmol/L dabrafenib, 2 mmol/L trametinib, or 1 μmol/L palbociclib for 72 hours. DMSO was used as a vehicle for all *in vitro* drug assays.

**Proliferation 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 volume (v/v)] glycerol, 10% (v/v) β-mercaptoethanol, bromophenol blue] were boiled for 5 minutes and resolved via SDS-PAGE using precast 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 + 0.1% Tween-20 (Sigma-Aldrich)] 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; DB Pharrmingen, catalog no. 554136), 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).

**IHC**

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...
the manufacturer’s protocol (Opal 7-Color Manual IHC Kit, catalog no. NE811001KT). Anti-CD3 (clone SP7, Abcam) with Opal 690 Fluorophore and Dapi (Invitrogen, catalog no. D1306) were used for staining. PerkinElmer Vectra 3 microscope was used to obtain images. Analysis was performed with InForm v2.4.0 (PerkinElmer) and HALO v2.3 (Indica).

**Primary cell isolation, CTV labeling, and cocultures**

Spleens from C57BL/6 OT-II transgenic mice were filtered through a 70 μm filter and red blood cells were lysed with red cell lysis buffer (150 mMol/L NH₄Cl, 10 mMol/L KHCO₃, 0.1 mMol/L Na₂EDTA). Naive CD4⁺ T cells were isolated using a mouse naive CD4⁺ T-cell Isolation Kit (EasySep, catalog no. 19765) and incubated with CellTrace Violet (CTV; Thermo Fisher Scientific) 1:1,000 in PBS for 20 minutes. CTV-labeled cells were washed with PBS and cultured 10:1 with CD45.2⁺ cells isolated from tumors. Cocultures were performed in 96-well plates (1 well per biological replicate) at 37°C in 5% CO₂ RPMI1640 supplemented with 10% FBS, 20 mMol/L HEPEs, 1% GlutaMAX, 1 mMol/L Minimal Essential Medium Non-Essential Amino Acids, 0.1% 2-mercaptoethanol, and 1 mMol/L Sodium Pyruvate.

**Single-cell RNA sequencing and T-cell receptor sequencing**

Sorted cells were washed with PBS, counted, and diluted to approximately 1,000 cells/μL in PBS supplemented with 1% molecular grade BSA (Thermo Fisher Scientific, catalog no. B14) and 200 U/mL RNase inhibitor (Merck, catalog no. 3335399001). Cells were then loaded onto the 10x Chromium instrument (10x Genomics) to generate single-cell Gel Beads-in-Eulsion (GEM) and capture/barcode cells. Samples were processed and libraries prepared using 10x Genomics Single Cell V(D)J kit as per the manufacturer’s instructions (10x Genomics, catalog no. PN-1000014). T-cell receptor (TCR) libraries were prepared using the 10x Chromium Single Cell V(D)J Enrichment Kit (10x Genomics catalog no. PN-1000071).

**Single-cell RNA sequencing and TCR sequencing analysis**

For RNA analysis, sequencing reads were harmonized, mapped to the mm10 mouse genome and gene transcription abundance was quantified using the CellRanger suite 3.0 (10x Genomics) with default parameters. The software suite Seurat (13) was used for the analysis and manipulation of gene transcript abundance data. Droplets were excluded from further analyses if including less than 200 detected transcripts and if including a fraction of mitochondrial-derived reads higher than 0.1. Cell-cycle state was inferred for each cell using the CellCycleScoring function with default parameters. Principal component analysis (PCA) was used to reduce the dimensionality of the data. PCA revealed the presence of unwanted variation generated from the cell-cycle phase. Transcript abundance was normalized and the unwanted variation was removed using regularized negative binomial regression (SCT algorithm; ref. 13). Cells were assigned to clusters using the shared-nearest neighbor (SNN) method using default resolution. The algorithm SingleR (14) with Blueprint reference was used to label each cell cluster with a cell-type identity. All differential transcript abundance analyses were conducted using FindMarkers function with the parameters min.pct = 0.1 and only.pos = TRUE. Heatmaps were drawn using tidyHeatmapwrapper for the ComplexHeatmap algorithm (15). For TCR analyses, sequencing reads were trimmed for adapters using trimgalore, and aligned to the mm10 mouse genome using STAR (16) with default parameters. Mixrr (17) was used to quantify TCR proportions for each sample pool. The 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 monocyte 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 GDC Firehose website (22). Counts were normalized to TPM values with a pseudocount of 2. Entrez gene IDs were mapped to HGNC gene symbols using the biomart R package (version 2.4.0; 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 unpaired 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 syngeneic setting, we first tested the in vitro activity of this triple therapy on our immunogenic Braf⁰⁰⁰⁰⁰⁰₁₆ Dclk2 Δ/− Xcr1−/− mouse melanoma cell line,
YOVAL1.1 (12). Consistent with human melanoma cell lines (8, 9), triple therapy potently suppressed both phosphorylation of retino-blasta (Supplementary Fig. S1A) and proliferation of YOVAL1.1 cells compared with dual BRAFi and MEKi inhibition (BRAFi+MEKi) or CDK4/6 inhibition (CDK4/6i) alone (Fig. 1A). Triple therapy also led to significant upregulation of the MHC 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 (ref. 27; Fig. 1C; Supplementary Fig. S1B). 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 BRAfioxCdkn2a-/- 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 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 (Supplementary Fig. S1D), likely due to the reported anti-inflammatory effects of CDK4/6 inhibitors on this T-cell subset (10). Indeed, tumors treated with CDK4/6i alone had significantly fewer Tregs than all other treatment groups (Fig. 1F).

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–I). This encompassed a reduction in all myeloid subsets analyzed, including monocytes (CD11b$$^{high}$$Ly6c$$^{high}$$) and mononuclear myeloid-derived suppressor cells (MDSC; CD11b$$^{high}$$Ly6c$$^{high}$$Ly6G$$^{low}$$), DCs (Ly6c$$^{low}$$CD11c$$^{+}$$MHC-II$$^{+}$$CD80/CD86$$^{+}$$), and tumor-associated macrophages (Ly6c$$^{low}$$CD11c$$^{+}$$MHC-II$$^{+}$$CD80$$^{+}$$; Fig. 1J; Supplementary Fig. S1D). No changes were seen in polymorphonuclear MDSCs (CD11b$$^{high}$$Ly6c$$^{high}$$Ly6G$$^{high}$$), although the frequency of these cells across all groups was negligible (Supplementary Fig. S1J). 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 BRAF, 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 immuno-deficient NSG mice, Rag2$$^{-/-}$$ 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 (refs. 31, 32; Fig. 2A). Notably, all these factors shifted favorably following triple therapy (Fig. 2B and C). We have previously shown that 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, 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
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 cells analyzed by flow cytometry after 72 hours of treatment; one-way ANOVA Tukey multiple comparisons test, \( n = 3–5 \). **D**, YOVAL1 tumor growth and survival in C57BL/6 mice; \( n = 7–9 \) per group, log-rank (Mantel–Cox) test. Analysis of YOVAL1 tumors by flow cytometry (**E** and **F**–**J**) 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. **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 \( \pm \) 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.

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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.
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).
treated with triple therapy expressed slightly lower levels of exhaustion-associated genes (e.g., Lag3, Pdcd1, Tigit, Toxic) and higher levels of memory/stem-like-associated genes (e.g., Tgfβ, Il7ra; Fig. 3E), and exhibited greater numbers of unique TCR clones and overall clonal diversity (Fig. 3F and G; Supplementary Fig. S3A); all features favorable for ICB efficacy (33). By flow cytometry, there was no change in the overall frequency of PD1+ CD8+ T cells following triple therapy, suggesting that the unresponsiveness of these tumors to anti-PD1 is not due to a lack of PD1 expression on tumor-infiltrating T cells (Supplementary Fig. S3B). Taken together, these data indicate that the functional capacity of T cells to respond to ICB is not impaired, but potentially enhanced, following triple therapy.

Transcriptional profiling of the intratumoral myeloid compartment revealed that both immunosuppressive [defined by expression of Mrc1 (34)] and proinflammatory [defined by expression of Cxcl10 and Cxcl9 (35)] macrophage subsets were depleted following triple therapy (Fig. 3H). Proinflammatory macrophages underpin the success of T-cell–directed therapies, including ICB, as these cells cooperate with T cells to promote tumor clearance through phagocytosis and the production of inflammatory cytokines, such as TNFα (36). In addition, macrophage-derived Cxcl10 and Cxcl9 are essential for the recruitment of T cells to the tumor site following ICB (35), and while tumors treated with triple therapy were host to an abundance of lymphocytes, clonal replacement of intratumoral T cells is suggested to be critical for the success of ICB (37). Indeed, proinflammatory Cxcl9+ and Cxcl10+ macrophages are associated with improved survival and clinical responses to ICB in patients with melanoma (35). Hence, the absence of this cellular subset following triple therapy may impact the efficacy of ICB through reduced capacity to stimulate both existing and new antitumor T-cell responses.

DCs are another critical component of antitumor immunity, including that mediated by dual BRAFi+MEKi (28). Our tSNE analysis delineated four distinct DC clusters, of which cluster 2 was most markedly depleted by triple therapy (Fig. 3I). This DC subset expressed high levels of genes associated with antigen cross-presentation (e.g., Wdfy4, Xcr1; refs. 38, 39; Fig. 3J), suggesting that triple therapy may strip the tumor immune compartment of the ability to cross-present antigens and stimulate T-cell activity. Indeed, a specific subset of antigen-presenting CD103+ DCs is critical for promoting intratumoral T-cell immunity (40), and for enhancing the therapeutic efficacy of combined BRAFi and anti-PDL1 therapy (41). To investigate whether CD103+ DCs were impacted by triple therapy, we applied a previously published gene signature for this specific subset (40) to our scRNA-seq analyses and found enrichment of this signature specifically in the Wdfy4+Xcr1+ DC cluster (Fig. 3K), identifying this cluster as CD103+ DCs. The intratumoral ratio of CD103+ DCs to other myeloid populations is strongly prognostic for survival across a range of cancers (40), and importantly our scRNA-seq analyses showed this ratio was diminished in tumors treated with triple therapy (Fig. 3L). This suggested that triple therapy may impair antitumor immunity and responses to ICB via potently depletion of CD103+ DCs.

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+Xcr1+ CD103+ 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; Supplementary Fig. S4A). To examine this more specifically in the context of ICB, we analyzed a 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 RECIST (ref. 42; Fig. 4C). 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-naïve 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, CD11c, CD24, and CD103, which we identified to be 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,
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% (int-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, tSNE 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 OVA323-339, 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. I and J, n = 3 independent cocultures with CD45.2+ cells pooled 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 < 0.001; ****P < 0.0001. Triple, BRAFi (dabrafenib) + MEKi (trametinib) + CDK4/6 (palbociclib).
CD45.2+ immune cells isolated from tumors treated with vehicle or triple therapy were pulsed with the cognate antigen, OVA232-339, and cocultured with CTV-labeled naïve CD4+ OT-2 T cells (Fig. 4H). Strikingly, OT-2 T cells cocultured with CD45.2+ 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+ DCs, and in doing so may render these tumors unresponsive to ICB (Fig. 4K). As a result, this promising therapy, while effective due to potent tumor-intrinsnic activity, may impede the ability of the immune system to contribute to the overall antitumor response.

Authors’ Disclosures

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