Mutant BRAF and MEK Inhibitors Regulate the Tumor Immune Microenvironment via Pyroptosis

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ABSTRACT

Combinations of BRAF inhibitors and MEK inhibitors (BRAFi + MEKi) are FDA-approved to treat \textit{BRAF\textsuperscript{V600E/K}}-mutant melanoma. Efficacy of BRAFi + MEKi associates with cancer cell death and alterations in the tumor immune microenvironment; however, the links are poorly understood. We show that BRAFi + MEKi caused durable melanoma regression in an immune-mediated manner. BRAFi + MEKi treatment promoted cleavage of gasedermin E (GSDME) and release of HMGB1, markers of pyroptotic cell death. GSDME-deficient melanoma showed defective HMGB1 release, reduced tumor-associated T cell and activated dendritic cell infiltrates in response to BRAFi + MEKi, and more frequent tumor regrowth after drug removal. Importantly, BRAFi + MEKi-resistant disease lacked pyroptosis markers and showed decreased intratumoral T-cell infiltration but was sensitive to pyroptosis-inducing chemotherapy. These data implicate BRAFi + MEKi-induced pyroptosis in antitumor immune responses and highlight new therapeutic strategies for resistant melanoma.

SIGNIFICANCE: Targeted inhibitors and immune checkpoint agents have advanced the care of patients with melanoma; however, detailed knowledge of the intersection between these two research areas is lacking. We describe a molecular mechanism of targeted inhibitor regulation of an immune-stimulatory form of cell death and provide a proof-of-principle salvage therapy concept for inhibitor-resistant melanoma.

See related commentary by Smalley, p. 176.

INTRODUCTION

Melanoma represents a small fraction of cutaneous malignancies yet accounts for the majority of skin cancer-related mortalities (1). Agents targeting the MEK–ERK1/2 pathway or immune checkpoints have emerged as effective treatment modalities that significantly improve progression-free survival and overall survival for patients with stage III and stage IV melanoma (2–4). Targeted therapy elicits high response rates with the majority of patients with \textit{BRAF\textsuperscript{V600E/K}}-mutant melanoma exhibiting tumor shrinkage in response to the combination of BRAF and MEK inhibitors (BRAFi + MEKi). A limitation of targeted therapies is that tumors frequently recur within 13 months (5). Acquired resistance is often due to reactivation of the MEK–ERK1/2 pathway caused by mechanisms including NRAS mutation, increased BRAF copy number, and aberrant mutations of cell cycle regulators (6). Immune checkpoint inhibitors have come to the forefront of melanoma treatment, as they reverse dysfunctional antitumor T-cell states and induce durable antitumor responses in approximately 50% of patients (8).

Given the clinical momentum in combining these two classes of therapies, it is important to understand the actions of targeted therapies on the tumor immune environment. BRAFi and/or MEKi are known to induce antitumor immune responses. BRAFi increase MHC expression and induce CD4\textsuperscript{+} and CD8\textsuperscript{+} T cell–dependent antitumor immunity (9–19). Furthermore, MEKi improve antitumor T-cell responses by impairing T-cell receptor (TCR)–mediated apoptosis of tumor antigen–specific T cells (19–23). Generally, BRAFi and/or MEKi efficacy correlates with T-cell infiltration of tumors, whereas the loss of intratumoral CD8\textsuperscript{+} T cells and influx of tumor-associated macrophages are associated with acquired resistance in metastatic melanoma (10, 17, 19, 24).

Despite this knowledge, the mechanisms by which targeted inhibitors affect the phenotype and function of tumor-associated T cells are incompletely understood. Furthermore, the functional relationship between BRAFi + MEKi–mediated tumor cell death and alterations in the tumor immune environment remains to be elucidated.

It is well established that BRAFi and/or MEKi cause programmed cell death of \textit{BRAF\textsuperscript{V600E/K}}-mutant melanoma cells. Mechanistically, inhibition of MEK–ERK1/2 signaling induces BIM-EL and BIM-mediated mitochondrial depolarization, leading to cytochrome C release and activation of caspase-3 (16, 25–27). It has recently been shown that the intrinsic apoptotic pathway intersects with a distinct form of cell death termed pyroptosis that is gasedermin-mediated and involves pore-based release of immune-stimulatory factors (28–31). We and others have demonstrated that caspase-3 cleavage leads to pyroptosis by inducing gasedermin E (GSDME, or DFNAS5) cleavage and subsequent pore formation within...
the plasma membrane (31–34). This pore formation causes the release of immune stimulants, including HMGB1, which are able to induce dendritic cell (DC) activation and, in turn, propagate antitumor T-cell activity (32, 33, 35). Cleaved GSDME also permeates the mitochondria to positively feedback to the intrinsic apoptotic pathway (32, 34). Recent evidence shows MEKi-induced GSDME cleavage in lung cancer cell lines (36); however, how these effects contributed to antitumor immune responses remained unclear. We hypothesized that targeted inhibitor-mediated pyroptosis leads to activation of antitumor immune responses in BRAF-mutant melanoma.

In this study, we used human and syngeneic mouse melanoma models to analyze GSDME-associated pyroptosis as it relates to efficacy of BRAFi + MEKi treatment and modulation of the tumor immune microenvironment. We demonstrated that therapeutic efficacy of BRAFi + MEKi is modulated by a functional immune system, specifically CD4+ and CD8+ T cells. Treatment-induced HMGB1 release, tumor-associated T-cell alterations, and tumor eradication were dependent on GSDME. Conversely, BRAFi + MEKi–resistant tumors did not undergo pyroptosis and lacked robust T-cell responses. Finally, restoring GSDME cleavage and HMGB1 release delayed the growth of BRAFi + MEKi–resistant tumors. These data define a novel mechanism connecting BRAFi + MEKi–induced pyroptosis to immune responses and present new salvage options for targeted therapy–resistant melanoma.

RESULTS
Therapeutic Efficacy of BRAFi + MEKi Combination Treatment In Vivo Depends on an Intact Immune System

Acquired resistance to BRAFi + MEKi treatment is accompanied by reduced intratumoral infiltration of T cells (17). To ascertain the functional contribution of the immune system in BRAFi + MEKi therapeutic efficacy, we compared tumor responses in syngeneic BrafV600E mouse melanoma allografts of D4M3.A and YUMM1.7 cells (37, 38). Intratumoral dermal cells were established in either immunocompetent (C57BL/6 mice) or immunodeficient [NOD/SCID gamma (NSG)] mice and mice treated with/without BRAFi + MEKi. D4M3.A tumors in either immunocompetent C57BL/6 mice or immunodeficient NSG mice showed robust tumor regression following BRAFi + MEKi treatment (Fig. 1A). However, BRAFi + MEKi induced prolonged tumor regressions in C57BL/6 mice, with tumors taking an average of 138 days to regrow to 200 mm3 compared with short-term regressions averaging 57.4 days in NSG mice (Fig. 1A). In a second model, YUMM1.7 tumors took an average of 104.2 days to regrow to 200 mm3 compared with initial regressions in C57BL/6 mice compared with 16.8 days in NSG mice (Fig. 1A and B). Immunocompetent mice lacking palpable lesions after ≥90 days of treatment regrew tumors when taken off BRAFi + MEKi, indicating the presence of residual disease (Fig. 1A and B, dark blue dots; Supplementary Fig. S1A). These tumors regressed upon readministration of BRAFi + MEKi (cyan dots). The differences in tumor growth kinetics in different mouse strains were not attributed to altered baseline tumor growth rates (Supplementary Fig. S1B). Importantly, we observed extended overall survival of BRAFi + MEKi–treated C57BL/6 mice compared with NSG mice when utilizing both the D4M3.A and YUMM1.7 models (Fig. 1C). Together, these data suggest that an intact immune system significantly contributes to the therapeutic efficacy of BRAFi + MEKi.

T Cells Are Required for Sustained Tumor Growth Inhibition by BRAFi + MEKi

Next, we determined how ERK1/2 pathway inhibition affected the immune cell composition in patient tumors. RNA-sequencing (RNA-seq) analysis of BRAFi-treated patient tumors (European Genome-phenome Archive, EGAS00001000992; ref. 39) highlighted gene signatures consistent with increased expression of T-cell and DC infiltration in on-treatment samples (Fig. 2A). These findings were contrasted by lower intratumoral immune transcript abundance upon tumor relapse. Expression levels of genes associated with T cells and plasmacytoid DCs positively correlated with percent tumor response in this patient population (Fig. 2B; Supplementary Fig. S1C). These data suggest that tumor-associated T cells and DCs associate with the efficacy of ERK1/2 pathway inhibition in patients with BRAF-mutant melanoma.

To further characterize alterations, we analyzed the immune cell infiltrates of syngeneic tumors harvested either pretreatment or after four days of BRAFi + MEKi treatment. Compared with untreated controls, the proportion of CD8+ and CD4+ cells among CD3+ T cells was increased in YUMM1.7 and D4M3.A tumors treated with BRAFi + MEKi (Fig. 2C and D). The overall amount of CD3+ cells slightly decreased within the tumor and the percentages of CD8+ and CD4+ T cells of total cells were not affected (Supplementary Figs. S2 and S3A). Furthermore, we observed higher levels of activated (CD44+) and proliferating (Ki-67+) T cells in tumors of BRAFi + MEKi–treated mice compared with tumors from untreated mice (Fig. 2E; Supplementary Fig. S2). Treatment-associated changes in T-cell abundance were selective to the tumor in that they were not observed in the spleen (Supplementary Fig. S3B and S3C). Markers of T-cell function, IFNγ and IL2, were unchanged following BRAFi + MEKi treatment and, in contrast, the production of TNFα from intratumoral and splenic CD4+ and CD8+ T cells was decreased (Supplementary Fig. S3D and S3E).

To assess the functional contribution of T cells to tumor regression and acquired resistance following BRAFi + MEKi, we depleted CD4+ and CD8+ T cells in YUMM1.7 tumor–bearing mice (Supplementary Fig. S3F and S3G). Concurrent depletion of CD4+ and CD8+ T cells significantly shortened the time to tumor growth (Fig. 2F) and reduced survival of mice following BRAFi + MEKi (Fig. 2G). These data suggest that T cells contribute to maintaining tumor regressions caused by BRAFi + MEKi therapy.

To characterize other effects of BRAFi + MEKi on the tumor immune microenvironment, we analyzed myeloid-derived cells and a panel of markers for immune activity (MHC-I, MHC-II, PD-L1, IDO-1, FasL, GalS9, and OX40L). Both tumor-associated macrophages (TAM) and myeloid-derived suppressor cells (MDSC) were decreased intratumorally during BRAFi + MEKi treatment (Supplementary Fig. S3H and S3I). We also observed a consistent decrease in the expression of IDO-1, FasL, GalS9, and OX40L on CD45.2-negative and CD45.2-positive cells within tumors (Supplementary
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Figure 1. Time to acquired resistance of BRAFi + MEKi is immune-mediated. A, Male C57BL/6 or NSG mice were intradermally implanted with D4M3.A (3 × 10⁵) mouse BrafV600E melanoma cells. Tumors were grown to approximately 50–250 mm³ after which animals were given either control (AIN-76A) or PLX4720 and PD0325901 (200 ppm PLX4720 and 7 ppm PD0325901 in AIN-76A) laced chow (BRAFi + MEKi). Tumor growth, represented as the change in volume (mm³) over time, is shown from the start of treatment. Dark blue dots indicate removal of combination chow due to lack of visible tumors, and cyan dots indicate when combination chow was restarted due to recurrent, visible tumors. B, Same as A, except that YUMM1.7 (2.5 × 10⁵) cells were injected. C, Survival curves of D4M3.A or YUMM1.7 tumor-bearing, C57BL/6 or NSG mice treated with BRAFi + MEKi. Significance was determined by a log-rank test. ***, P < 0.001. Xs indicate if mice died for a non–experiment-related reason.

Fig. S3J–S3M). Together, these data suggest that BRAFi + MEKi treatment reduces the immune-suppressive cells in the microenvironment of BrafV600E melanomas.

BRAFi + MEKi Induces Markers of Immune-Stimulatory Cell Death in BrafV600E Melanomas

The basis of BRAFi + MEKi-mediated T-cell activation is unclear. We detected increased expression of the activation marker MHC-II on tumor-infiltrating DCs following BRAFi + MEKi treatment from both YUMM1.7 and D4M3.A tumors (Fig. 3A; Supplementary Fig. S4A). These data are consistent with the possibility that activated DCs contribute to the T-cell expansion observed during BRAFi + MEKi treatment. Next, we analyzed the release and cell-surface expression of immune stimulators from dying cells that could potentiate antitumor immune responses in part via effects on DCs, specifically HMGB1 and calreticulin (24, 25). As expected, treatment of YUMM1.7 and D4M3.A monocultures with BRAFi + MEKi in vitro increased cell death as determined by annexin V staining and propidium iodide (PI) uptake compared with DMSO-treated cells (Fig. 3B). BRAFi + MEKi-mediated tumor cell death correlated with release of HMGB1 from cells (Fig. 3C; Supplementary Fig. S4B). The release of additional inflammatory factors has been associated with pyroptotic cell death (40, 41), and we did detect BRAFi + MEKi-dependent release of another inflammatory mediator, IL1α, from melanoma cells (Fig. 3C). In addition, BRAFi + MEKi treatment increased cell-surface expression of calreticulin (Fig. 3D). Similar results were obtained in human BrafV600E melanoma cells including A375, a frequently used research.

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Figure 2. BRAFi + MEKi is T-cell mediated. A, Heat map of gene set variation analysis (GSVA) scores for immune cell gene sets from patient tumors before and during BRAFi, and after the onset of resistance to BRAFi. Patient indicator numbers are included below. B, Scatter plots of GSVA scores and percent tumor regression data for on-treatment samples. Pearson correlation coefficient ($r$) and $P$ values are displayed. C, YUMM1.7 (Y1.7, black) or D4M3.A (D4M, blue) tumor-bearing mice were treated PLX4720 (1 μmol/L) and PD0325901 (35 nmol/L). Cohorts of mice were sacrificed pretreatment or after four days of BRAFi + MEKi treatment, and intratumoral T cells were assessed by FACS. Representative FACS plots of tumor-associated CD8$^+$ and CD4$^+$ T cells (of CD3$^+$ cells). D, Quantification of FACS plots of YUMM1.7 (Y1.7, black) or D4M3.A (D4M, blue) tumors. E, Phenotype of tumor-associated T cells; CD44$^+$, activated; Ki-67$^+$, proliferating. F, Tumor growth during BRAFi + MEKi of CD4- and CD8-depleted mice compared with their appropriate isotype controls. G, Kaplan-Meier survival plot of mice from F. Significance was determined by a log-rank test. *, $P < 0.05$. 

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Figure 3. BRAFi + MEKi induces immune-stimulatory cell death. **A**, Activation status (ratio of MHC-II MFI in tumors compared with spleens) of F4/80-negative, CD11C-positive, CD11B-negative DCs in tumors during BRAFi + MEKi treatment. **B**, YUMM1.7 or D4M3.A cells were treated with PLX4720 (1 μmol/L) and PD0325901 (35 nmol/L) in vitro \((n = 3)\) for 72 hours \((B)\) or 24 hours \((C\) and \(D)\). Cell death as indicated by % Annexin V+ and PI+ cells after 72 hours of treatment (representative gating on left; quantitation on right). **C**, Levels of HMGB1 and IL1α in supernatant from YUMM1.7 or D4M3.A cells treated with BRAFi + MEKi for 48 hours \((n = 3)\). Coomassie stained gel as loading control. **D**, Calreticulin surface expression of YUMM1.7 and D4M3.A mouse melanoma cell lines \(\text{left shows representative plots; right shows percentages}\). **E**, Same as **C** for A375 cells or short-term patient tumor cells \(\text{TJUMEL57}\) treated with BRAFi + MEKi for 48 hours \((n = 3)\). **F**, Same as **D** for human cells treated with BRAFi + MEKi for 48 hours. **G**, Pmel-1 T cells were expanded ex vivo for 5 days in the presence of supernatant from YUMM1.7 or D4M3.A cells treated with DMSO or PLX4720 (1 μmol/L) and PD0325901 (35 nmol/L) for 48 hours. Shown is the cell number represented as a ratio of BRAFi + MEKi-treated:control supernatants \((n = 3)\). Statistical analysis was completed by Student t test. *, \(P < 0.05\); **, \(P < 0.01\).
cell line, and TJUMEL-57, a short-term ex vivo culture of a patient melanoma tumor (Fig. 3E and F; Supplementary Fig. S4C). These data suggest that combination BRAFi + MEKi treatment induces immune-stimulatory forms of cell death.

Next, we determined whether tumor cell death was immune-stimulatory in terms of inducing T-cell expansion. To this end, we cultured splenocytes from Pmel-1 mice (a transgenic mouse model with T cells specific for the melanoma antigen gp100) with conditioned medium from BRAFi + MEKi–treated tumor cells (42). This system incubates antigen-presenting cells, like DCs, with the immune stimulants present in the supernatant of BRAFi + MEKi–treated melanoma cells, allowing us to test for promotion of T-cell expansion. The addition of conditioned medium from BRAFi + MEKi–treated YUMM1.7 or D4M3.A cells increased T-cell proliferation compared with medium from DMSO-treated cells (Fig. 3G). These data suggest that during BRAFi + MEKi treatment, dying melanoma cells release factors that promote T-cell expansion.

**BRAFi + MEKi Mediates GSDME-Dependent Pyroptosis**

Given our data indicating that BRAFi + MEKi–induced release of factors promotes T-cell expansion, we hypothesized that BRAFi + MEKi causes pyroptotic cell death. From RNA-seq datasets from BRAFi + MEKi–treated melanoma patient samples (EGAS0000001000992; ref. 39), we determined that expression of a pyroptosis gene set positively correlated with percent tumor response to targeted therapy (Fig. 4A), further suggesting that BRAFi + MEKi induces pyroptotic cell death.

Expression of pyroptosis genes was increased on-treatment in partial and complete responders (Supplementary Fig. S4D). We examined a second dataset (GSE999898; ref. 43), containing patient-matched pretreatment, on-treatment, and progression samples but lacking initial response data. This analysis also showed upregulation of the pyroptosis signature on-treatment that was decreased following onset of resistance (Supplementary Fig. S4E). We have recently shown that pyroptosis can be initiated by caspase-3–mediated GSDME cleavage in response to various apoptotic stimuli (32, 34). Since BRAFi + MEKi treatment induces caspase-3 activation (44), we tested for GSDME processing in melanoma cell lines. In addition to decreased phospho-ERK1/2 and increased cleavage of caspase-3, BRAFi + MEKi treatment caused the production of the 35 kDa GSDME cleavage fragment in mouse and human melanoma cells (Fig. 4B; Supplementary Fig. S5A). Cleavage of caspase-3 and GSDME by the combination was dose-dependent (Supplementary Fig. S5B), and BRAFi and MEKi were individually able to induce GSDME cleavage but to a lesser extent than the combination (Supplementary Fig. S5C).

To test for requirement, we reduced GSDME expression by knockdown (siRNA) and GSDME knockout (KO) using two different GSDME CRISPR/Cas9 guide sequences. GSDME knockdown/KO decreased the release of HMGB1 from melanoma cells into the supernatant, indicating inhibition of pyroptosis (Fig. 4C and D; Supplementary Fig. S5D–S5G). GSDME KO did not alter the level of cellular HMGB1 expression (Fig. 4C). To further test whether GSDME was required for pyroptosis, we measured PI uptake in BRAFi + MEKi–treated GSDME-KO D4M3.A and YUMM1.7 cell lines (32, 34). GSDME-KO cells internalized less PI than control cells, suggesting a reduction of pyroptosis levels (Fig. 4E). Together, these data imply that BRAFi + MEKi induced GSDME-mediated pyroptotic cell death.

To determine whether the antitumor immune responses observed during BRAFi + MEKi were dependent on mediators of pyroptosis, we compared tumor-infiltrating lymphocyte (TIL) populations from control (CTL) or GSDME-KO tumors treated for four days with BRAFi + MEKi. Higher levels of T cells and activated DCs (MHC-IIhi, CD11Bhi) were detected in CTL tumors in comparison with the GSDME-KO counterparts (Fig. 4F; Supplementary Fig. S5H). Furthermore, dialyzed medium from BRAFi + MEKi–treated GSDME-KO cells was ineffective at promoting T-cell expansion (Fig. 4G). These data provide evidence that BRAFi + MEKi–induced antitumor immune responses are dependent on pyroptotic cell death.

In support of this notion, RNA-seq analysis of cutaneous melanoma samples from The Cancer Genome Atlas (TCGA) displayed a positive correlation between T-cell genes and pyroptosis genes (Fig. 4H). To confirm whether GSDME affects tumor growth during treatment, we measured the size of BRAFi + MEKi–treated CTL and GSDME-KO D4M3.A tumors (Supplementary Fig. S5I). There was no noticeable difference in the initial regression of CTL or GSDME-KO tumors treated with BRAFi + MEKi (Fig. 4I). However, when testing for the regrowth of residual disease by removing drug from mice lacking palpable lesions, only 1 of 6 (16.7%) of CTL tumors regrew, whereas 5 of 6 (83.3%) of GSDME-KO tumors regrew (Fig. 4I). These data suggest that GSDME-dependent pyroptosis is associated with lower levels of residual disease after BRAFi + MEKi treatment.

**Figure 4.** BRAFi + MEKi induces GSDME-dependent pyroptosis. A, Pyroptosis genes (left) and scatter plot of pyroptosis genes GSEA scores and percent tumor regression data for BRAFi and BRAFi + MEKi on-treatment samples from patient tumors. Pearson correlation coefficient (r) and P values are displayed (right). **B**, Levels of pERK1/2, cleaved caspase-3, and GSDME in the cell lysates from mouse YUMM1.7 and D4M3.A treated for 24 hours and human A375 cells treated for 48 hours with PLX4720 (1 µmol/L) and PD0325901 (35 nmol/L). HMGB1 and GAPDH were used as loading controls. Full-length GSDME runs at 55 kDa and cleaved GSDME runs at 35 kDa. **C**, Full-length GSDME and cellular HMGB1 levels in empty-vector controls (CTL) and GSDME CRISPR knockout (KO1 and KO2) YUMM1.7 or D4M3 A cells analyzed by Western blot. GAPDH was used as loading control; n = 3–4. **D**, Level of secreted HMGB1 in supernatants from CTL, KO1, or KO2 YUMM1.7 or D4M3.A cells treated with PLX4720 (1 µmol/L) or PD0325901 (35 nmol/L) for 16 hours. Comassie-stained gel shows protein loading. n = 3. **E**, PI incorporation over time, normalized to 0 hours for CTL, KO1, or KO2 cell lines treated with either DMSO or BRAFi + MEKi (n = 3; data are representative of two independent experiments). Significance determined by area under the curve. ***P < 0.001. **F**, T cells (CD3+) and activated CD11B+ DCs (F4/80+, CD11B+, CD11C+, MHC-IIhi) in CTL or KO1 YUMM1.7 tumors four days after beginning BRAFi + MEKi treatment. Statistical analysis was completed by Student t test. *P < 0.05. **G**, T-cell expansion stimulated by dialyzed supernatants from control and GSDME-KO–treated cells. Fold change of Pmel-1 T-cell numbers after 5 days is shown (n = 3). Statistical analysis by Student t test. *P < 0.05. **H**, Scatter plot comparing GSA signature scores using RNA-seq data from TCGA cutaneous melanoma patient samples. Pearson correlation coefficient (r) and P values are displayed. **I**, Tumor growth of CTL or KO1 D4M3.A treated with BRAFi + MEKi in C57BL/6 mice. Mice were removed from BRAFi + MEKi at day 91 (CTL) and day 92 (sg1GSDME) when tumors were undetectable.
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**A** Pyroptosis gene set

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**B**

- BRAFi + MEKi
- D4M
- A375

- pERK1/2
- Cleaved casp-3
- GSDME
- GAPDH

**C**

- YUMM1.7
- D4M3.A

- BRAFi + MEKi
- CTL KO1
- KO2

- GSDME
- HMGB1
- GAPDH

**D**

- YUMM1.7
- D4M3.A

- BRAFi + MEKi
- CTL KO1
- KO2

- HMGB1

**E**

- Fold change
- BRAFi + MEKi (hrs)
- YUMM1.7
- D4M3.A

**F**

- BRAFi + MEKi–treated GSDME-KO YUMM1.7 tumors
- T cells
- Activated CD11B− dendritic cells

**G**

- T-cell expansion
- Cell number (fold change)
- DMSO
- BRAFi + MEKi

**H**

- TCGA SKCM
- T-cell genes

- r = 0.74
- P = 1.58E-82

**I**

- CTL D4M3.4 tumors, BRAFi + MEKi
- GSDME-KO1 D4M3.A tumors, BRAFi + MEKi

- 1/6 Tumors regrow
- 5/6 Tumors regrow

- Cessation of BRAFi + MEKi
BRAFi + MEKi Combination–Resistant Cells Do Not Undergo Pyroptosis

Acquired resistance to combination BRAFi + MEKi in patient samples is associated with T-cell exclusion (5, 17), findings we corroborated using available datasets (Fig. 2A and B). To assess pyroptosis in the setting of BRAFi + MEKi resistance, we treated established YUMM1.7 and D4M3.A tumors in C57BL/6 mice with BRAFi + MEKi until they became resistant. Cell lines generated from combination-resistant tumors (CRT) did not take up PI or generate cleaved caspase-3 when treated with BRAFi + MEKi (Fig. 5A–C). These effects were associated with weak induction of the proapoptotic BH3 proteins BIM-EL and BMF in CRTs compared with parental cells (Supplementary Fig. S6A). BRAFi + MEKi also failed to induce GSDME cleavage or HMGB1 release, or increase calreticulin surface expression in CRT cells (Fig. 5B–E; Supplementary Fig. S6B). Similar findings were obtained in human melanoma A375-derived CRT cells upon treatment with the combination therapy (Fig. 5F and G). Importantly, expression of the GSDME N-terminal sequence (amino acids 1–270), and to a lesser extent full-length GSDME, in BRAFi + MEKi-resistant CRT34 cells was sufficient to induce both the release of HMGB1 and IL1α as well as PI uptake (Fig. 5H and I). These data show that expression of pore-forming GSDME in BRAFi + MEKi-resistant cells is sufficient to cause release of proinflammatory mediators and melanoma cell death.

As GSDME cleavage was associated with intratumoral immune responses (Fig. 4G), we compared TILs from YUMM1.7 CRTs and D4M3.A CRTs to TILs from treatment-naïve tumors taken at similar sizes. Whereas responsive tumors had increased DC activation and T-cell counts during BRAFi + MEKi (Figs. 2 and 3), there was no difference in the anti-PD-1/PD-L1 response (Fig. 4G). BRAFi + MEKi also induced CD45.2+ naïve tumors taken at similar sizes. Whereas responsive tumors had increased DC activation and T-cell counts during BRAFi + MEKi (Figs. 2 and 3), there was no difference in the anti-PD-1/PD-L1 response (Fig. 4G). BRAFi + MEKi also induced CD45.2+ naïve tumors taken at similar sizes. Whereas responsive tumors had increased DC activation and T-cell counts during BRAFi + MEKi (Figs. 2 and 3), there was no difference in the anti-PD-1/PD-L1 response (Fig. 4G). BRAFi + MEKi also induced CD45.2+ naïve tumors taken at similar sizes. Whereas responsive tumors had increased DC activation and T-cell counts during BRAFi + MEKi (Figs. 2 and 3), there was no difference in the anti-PD-1/PD-L1 response (Fig. 4G). BRAFi + MEKi also induced CD45.2+ naïve tumors taken at similar sizes. Whereas responsive tumors had increased DC activation and T-cell counts during BRAFi + MEKi (Figs. 2 and 3), there was no difference in the anti-PD-1/PD-L1 response (Fig. 4G). BRAFi + MEKi also induced CD45.2+ naïve tumors taken at similar sizes. Whereas responsive tumors had increased DC activation and T-cell counts during BRAFi + MEKi (Figs. 2 and 3), there was no difference in the anti-PD-1/PD-L1 response (Fig. 4G). BRAFi + MEKi also induced CD45.2+ naïve tumors taken at similar sizes. Whereas responsive tumors had increased DC activation and T-cell counts during BRAFi + MEKi (Figs. 2 and 3), there was no difference in the anti-PD-1/PD-L1 response (Fig. 4G). BRAFi + MEKi also induced CD45.2+ naïve tumors taken at similar sizes. Whereas responsive tumors had increased DC activation and T-cell counts during BRAFi + MEKi (Figs. 2 and 3), there was no difference in the anti-PD-1/PD-L1 response (Fig. 4G). BRAFi + MEKi also induced CD45.2+ naïve tumors taken at similar sizes. Whereas responsive tumors had increased DC activation and T-cell counts during BRAFi + MEKi (Figs. 2 and 3), there was no difference in the anti-PD-1/PD-L1 response (Fig. 4G). BRAFi + MEKi also induced CD45.2+ naïve tumors taken at similar sizes. Whereas responsive tumors had increased DC activation and T-cell counts during BRAFi + MEKi (Figs. 2 and 3), there was no difference in the anti-PD-1/PD-L1 response (Fig. 4G). BRAFi + MEKi also induced CD45.2+ naïve tumors taken at similar sizes. Whereas responsive tumors had increased DC activation and T-cell counts during BRAFi + MEKi (Figs. 2 and 3), there was no difference in the anti-PD-1/PD-L1 response (Fig. 4G). BRAFi + MEKi also induced CD45.2+ naïve tumors taken at similar sizes. Whereas responsive tumors had increased DC activation and T-cell counts during BRAFi + MEKi (Figs. 2 and 3), there was no difference in the anti-PD-1/PD-L1 response (Fig. 4G). BRAFi + MEKi also induced CD45.2+ naïve tumors taken at similar sizes. Whereas responsive tumors had increased DC activation and T-cell counts during BRAFi + MEKi (Figs. 2 and 3), there was no difference in the anti-PD-1/PD-L1 response (Fig. 4G). BRAFi + MEKi also induced CD45.2+ naïve tumors taken at similar sizes. Whereas responsive tumors had increased DC activation and T-cell counts during BRAFi + MEKi (Figs. 2 and 3), there was no difference in the anti-PD-1/PD-L1 response (Fig. 4G). BRAFi + MEKi also induced CD45.2+ naïve tumors taken at similar sizes. Whereas responsive tumors had increased DC activation and T-cell counts during BRAFi + MEKi (Figs. 2 and 3), there was no difference in the anti-PD-1/PD-L1 response (Fig. 4G).

DISCUSSION

The salient findings of this study are that (i) an intact immune system is required for in vivo BRAFi + MEKi efficacy in BRAF-mutant melanoma; (ii) T lymphocytes are required for the sustained therapeutic effects of BRAFi + MEKi; (iii) T-cell activation and tumor regression are contingent on GSDME-dependent pyroptosis of tumor cells; and (iv) reinduction of pyroptosis may offer an effective salvage therapy for BRAFi + MEKi-resistant tumors. These findings provide a novel mechanistic link between BRAFi + MEKi-induced pyroptosis, regulation of the tumor immune microenvironment, and antitumor immunity (Fig. 7). In addition, we provide proof-of-principle evidence for a salvage therapy for BRAFi + MEKi-resistant BRAF-mutant melanoma.

Despite their clinical efficacy and evidence of inhibitor-induced T-cell infiltration, it remains unclear how BRAFi + MEKi induce antitumor T-cell responses. Furthermore, links between pyroptosis and tumor-associated immune infiltrates remain unclear. It is known that caspase-3 activation cleaves GSDME to induce pore formation and pyroptosis...
**Figure 5.** BRAFi + MEKi–resistant cell lines (CRT cells) do not undergo pyroptosis. YUMM1.7 CRT cells (CRT47L, 47R, 49N, and 54LR), D4M3.A CRT cells (CRT53L), or A375 CRT cells (CRT34) were treated with PLX4720 (1 μmol/L) and PD0325901 (35 nmol/L) for 72 (A) or 24 (B–G) hours. A, Cell death as indicated by PI uptake of cells after BRAFi + MEKi treatment. B, Levels of cleaved caspase-3 and GSDME in YUMM1.7 CRT cells after treatment. GAPDH as loading control. Full-length GSDME runs at 55 kDa and cleaved GSDME runs at 35 kDa. C, Same as B for parental D4M3.A cells and D4M3.A CRT cells. D, Levels of HMGB1 in supernatants from parental YUMM1.7 and CRT cells. Coomassie-stained gel showing protein loading. E, Same as D for D4M3.A CRT cells. F, Same as B for parental A375 and derived CRT cell lysates. G, Same as D for human cell lines. H, A375 CRT34 cells were transfected with pLenti3-hygro vector (Vec), full-length human GSDME (FL), or amino acids 1-270 of GSDME (N) for 24 hours. Cell lysates (left) and supernatant (right) were analyzed by Western blotting with indicated antibodies. GAPDH or Coomassie-stained gel served as loading control. I, Cell death, as measured by PI uptake (n = 3). Statistical analysis for this figure was completed by Student t test. *, P < 0.05. J, Activation status of dendritic cells in control treated (C) and CRT tumors. Ratio of MHC-II MFI in tumors compared with spleens. K, Tumor-asssociated CD8+ and CD4+ T cells as percent of CD3+ cells.
**Figure 6.** BRAFi + MEKi–resistant cell lines are susceptible to pyroptosis. YUMM1.7 CRT cells (CRT47R, 49N, and 54LR) or A375 CRT cells (CRT34) were treated with 37.5 μM etoposide or 1 μM doxorubicin for 24 hours. A, Levels of HMGB1 in supernatant from mouse cell lines. Coomassie-stained gel was used as loading control. Full-length GSDME runs at 55 kDa and cleaved GSDME runs at 35 kDa. B, Cleavage of caspase-3 and GSDME in mouse cells after treatment. GAPDH was used as loading control. C, PI uptake over time during etoposide treatment; n = 3. **, P < 0.01. D, Same as A and B for human cells. E, YUMM1.7 CRT cells (CRT47R) were transiently transfected with either control siRNA for 24 hours and then treated with etoposide (37.5 μM/L) for a further 24 hours. Cell lysates and supernatant were analyzed by Western blotting with indicated antibodies. GAPDH or Coomassie-stained gel serve as loading controls. F, As above, except that cell death is indicated by PI uptake of cells after etoposide treatment; n = 3. *, P < 0.05. G, As above, T-cell expansion stimulated by diafiltered supernatants was analyzed. Fold change of Pmel-1 T-cell numbers after 5 days is shown (n = 3). Statistical analysis by Student t test, *, P < 0.05; **, P < 0.01. H, Mice were started on BRAFi + MEKi treatment one day before tumor cell implantation. YUMM1.7 CRT47R cells (2.5 × 10⁶) were intradermally implanted on day 0. On day 5, mice were removed from BRAFi + MEKi chow and etoposide treatment was administered as described in Supplementary Fig. S7E and S7F. Shown are tumor growth (left) and percent survival (right) of treated mice.
Targeted Inhibitors Induce Pyroptosis in Melanoma

**Figure 7.** Proposed model of BRAFi + MEKi-induced pyroptosis. 

A. BRAFi + MEKi treatment blocks ERK1/2 signaling, inhibiting growth and survival of 

B. ERK1/2 pathway blockade results in activation of caspase-3, leading to cleavage of GSDME. The N-terminal cleaved region translocates to the plasma membrane leading to pore formation. 

D. After BRAFi + MEKi-induced GSDME pore formation, HMGB1 and other DAMPs are released from the cell. 

E–G. Extracellular DAMPs lead to the activation of dendritic cells, which induce T-cell proliferation and contribute to antitumor effects following BRAFi + MEKi treatment.

(32, 33). Here, we show that BRAFi + MEKi induced caspase-3 activation and GSDME cleavage, and that GSDME is required for the release of the damage-associated molecular pattern (DAMP) molecule HMGB1 from melanoma cells. Released HMGB1 is known to promote inflammation through its binding to toll-like receptor 4 on dendritic cells (45, 46). Although previous work demonstrated that the combination of BRAFi plus HDAC inhibitors induced release of HMGB1 from melanoma (47), our data underscore the mechanistic role of GSDME in HMGB1 release and effects on antimelanoma immunity. Our findings are supported by a recent study that found that MEKi promote GSDME cleavage in lung cancer (36), although again antitumor immune responses were not assessed in that report. We demonstrate that GSDME cleavage is required not only for pyroptosis but also for antitumor T-cell responses observed following BRAFi + MEKi administration. Thus, our data suggest that GSDME-dependent pyroptosis may be an indispensable mediator of immune-driven therapeutic response in BRAF-mutant melanoma. Consistent with this notion, lack of GSDME and pyroptosis led to enhanced regrowth of residual disease after removal of BRAFi + MEKi. MEKi efficacy...
is known to be T cell–dependent via impaired TCR-driven apoptosis in CD8+ T cells (19–23); thus, GSDME-mediated pyroptosis during BRAFi + MEKi may be working in tandem with impaired T-cell apoptosis to induce robust immune responses. Taken together, our data define a new functional intersection between BRAFi + MEKi–induced pyroptosis and T-cell responses to melanoma.

The development of resistance to BRAFi + MEKi in metastatic melanoma remains a significant challenge in the clinic. Although several melanoma cell-autonomous mechanisms of resistance to BRAFi and/or MEKi have been established (5, 6, 48), it has remained unclear how antimelanoma immune responses in the tumor microenvironment can be leveraged to overcome treatment resistance. Resistance to BRAFi + MEKi is linked to loss of intratumoral T-cell responses (17), data corroborated here using an independent human dataset and mouse models. These findings are increasingly important as BRAFi + MEKi therapy in combination with immune checkpoint inhibition is being tested in patients with advanced melanoma with promising efficacy albeit toxicity challenges (49–51). In addition, in preclinical models, CSF1R inhibitors targeting macrophage accumulation improved efficacy of BRAFi (18), and the efficacy of MEKi can be improved by targeting the PD-1/PD-L1/L2 axis (19, 22). The prevalence of resistance to targeted therapies and the relative lack of insight regarding the immune system’s role in this process underscores the importance of our findings. We demonstrated that combination inhibitor–resistant tumor cells do not undergo pyroptosis with BRAFi + MEKi, resulting in a loss of antitumor immune responses. These data may help to explain the loss of intratumoral CD8+ T cells in patients who are no longer responsive to BRAFi + MEKi (17). Furthermore, reinduction of pyroptosis with etoposide in BRAFi + MEKi–resistant melanomas provides proof-of-concept that targeting this programed cell death pathway represents a potential strategy for salvage therapy for patients with melanoma who are resistant to BRAFi + MEKi.

In summary, this study establishes the requirement for T cells on the immune-mediated mechanisms of resistance to BRAFi + MEKi. Furthermore, we link ERK1/2 pathway inhibition to the induction of pyroptosis through cleavage of GSDME to produce a more productive antitumor immune response. Expanding on this knowledge may lead to new salvage therapies for patients with BRAFi + MEKi–resistant metastatic melanoma.

**METHODS**

**Cell Culture**

D4M3.A cells (derived from Tyr::CreER, Braf<sup>F508<sup>+</sup></sup>;Pten<sup>−/−</sup> mice; cells donated by Dr. Constance E. Brinckerhoff, Dartmouth University, Hanover, NH; 2016) were cultured in DMEM/F12 with 5% FBS, 1% penicillin/streptomycin, and 1% l-glutamine. YUMM1.7 cells (Braf<sup>F508<sup>+</sup></sup>;Pten<sup>−/−</sup>; Cldn2<sup>−/−</sup> ; donated by Dr. Marcus Rosenberg, Yale University, New Haven, CT; 2014) were cultured in DMEM/F12 50/50 with 10% FBS, 1% penicillin/streptomycin, and 1% nonessential amino acids. A375 cells (purchased from ATCC in 2005) were cultured in DMEM with 10% FBS. Cell lines were short tandem repeat analyzed, confirmed for BRAFi/Braf<sup>F508<sup>+</sup></sup> mutation, and IMPACT III PCR pathogen tested (IDEXX) to authenticate them and determine that they were pathogen-free. CRT cells were isolated from tumors, cultured in the same medium as parental cells with the addition of PLX4720 (1 μmol/L) and PD0325901 (35 nmol/L), and utilized within 5 passages for experiments. Drug concentrations utilized are close to published GI50 values for PLX4720 and PD0325901 (52, 53). Inhibitor levels maintain the same BRAFi to MEKi ratio as used for in vivo experiments. A375-derived CRT cells were published previously (54).

**In Vivo Tumor Growth Studies**

Animal experiments were approved by the Institutional Animal Care and Use Committee and performed at Thomas Jefferson University (Philadelphia, PA) in a facility accredited by the Association for Assessment & Accreditation of Lab Animal Care International. Male C57BL/6 mice (Jackson Laboratory; 6–12 weeks) were used unless denoted. Tumors were implanted intradermally in 100 μL HBSS. Six-week-old male or female NSG mice were provided by Dr. Timothy Mанс (Thomas Jefferson University). Tumor volume was tracked with a caliper: volume = (length × width)<sup>2</sup> × 0.52. When the volume reached approximately 50 to 250 mm<sup>3</sup> animals were fed with either vehicle control chow or combination BRAFi + MEKi chow (200 ppm PLX4720 plus 7 ppm PD0325901). For etoposide experiments, mice were treated with intraperitoneal injections of 17 μmol/L/animal of etoposide following the schedule outlined in Supplementary Fig. S7E and S7F (55). PLX4720 and PD0325901 were generously provided by Plexxikon Inc., and chow was purchased from Research Diets Inc.

**Western Blot Analysis and Cell Supernatant Collection**

Protein lysates were prepared in Laemmli sample buffer, separated by SDS-PAGE, and proteins were transfected to polyvinylidene difluoride membranes. Immunoreactivity was detected using HRP-conjugated secondary antibodies (CalBioTech) and chemiluminescence substrate (Thermo Fisher Scientific) on a Versadoc Imaging System (Bio-Rad). Primary antibodies, all from Cell Signaling Technology unless otherwise stated, were as follows: anti-phospho-ERK1/2 (T202/Y204), anti-ERK2 (Santa Cruz Biotechnology), anti-GSDME (Abcam), anti-cleaved caspase-3, anti-BIM/BOD (Enzo Life Sciences), anti-IL1α (Santa Cruz Biotechnology), anti-HMGB1, and anti-GAPDH. Cell supernatants were harvested in the absence of FBS in culture medium to avoid distortion of SDS-PAGE. After centrifugation to remove cell debris, cell supernatants were concentrated 10× using Amicon Ultra 10K (Sigma-Aldrich). Concentrates were mixed with Laemmli sample buffer (Bio-Rad) and analyzed via Western blotting. For T-cell culture, concentrates were further washed twice with PBS and once with RPMI-1640 medium by spin, and sterilized by filtration through a 0.2-μm filter. Protein gel staining was performed using Coomassie Brilliant Blue R-250.

**Calreticulin Surface Expression**

Cells were treated with BRAFi + MEKi for the indicated times. Adherent cells were washed and stained with live/dead stain (Zombie UV, BioLegend) per company instructions. Cells were then primary stained with live/dead stain (Zombie Green, BioLegend) per company instructions. Cells were then secondary stained with the appropriate anti-rabbit AF488 antibodies (BioLegend) per company instructions. Cells were then primary stained with live/dead stain (Zombie Green, BioLegend) per company instructions. Cells were then secondary stained with the appropriate anti-rabbit AF488 antibodies (BioLegend) per company instructions. Cells were then primary stained with live/dead stain (Zombie Green, BioLegend) per company instructions. Cells were then secondary stained with the appropriate anti-rabbit AF488 antibodies (BioLegend) per company instructions.

**Annexin V/PI Analysis**

Cells were treated with BRAFi + MEKi for 72 hours. Adherent cells were washed and incubated with 5 μL Annexin V-APC (BD Biosciences) in 100 μL of binding buffer and then incubated with 0.02 mg/mL PI for 15 minutes at room temperature. Cells were analyzed on the FACSCalibur or BD LSR II flow cytometers. Experiments were performed in triplicate, and statistical analysis was completed using a two-tailed t test assuming equal variance with error bars representing SEM.
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IncuCyte (Essen Bioscience) imaging of PI uptake was used to measure cell death. Cells were treated with the drugs indicated and PI (10 μg/mL). Red fluorescence was imaged and quantified with the IncuCyte. Experiments were performed in triplicates, and statistical analysis performed by calculating the areas under the curve and a two-tailed t-test assuming equal variance with error bars representing SEM.

**siRNA Transfections**

Cells were transfected for 24 hours with siRNAs at a final concentration of 25 nmol/L using LipoFectamine RNAiMAX (Invitrogen). Nontargeting control (5′-UGGGUUACAUUGGCACUAU-3′) and GSDME-targeting (D-041196-01-0005, Dharmacon) were used. After transfection, cells were treated with the indicated drugs for another 24 hours.

**Flow Cytometry of Tumor, Spleen, and Blood Samples**

Spleens were processed mechanically using a 70-μm nylon filter and a syringe plunger. For immunogenicity and TIL studies, tumors were removed and dissociated into single-cell suspensions by placing tumors in digestion medium: HBSS (CellGro), 10% FBS, 0.3–0.5 mg/mL collagenase 1A (Sigma), and 60 U/mL DNase I (Sigma), and mincing using the gentleMACS Octo Dissociator using C Tubes (Miltenyi Biotec). Minced tumors were incubated at 37°C for 30 minutes while shaking, minced again, washed with T-cell medium (RPMI-1640, with 1-glutamine + 10% FBS + 1% penicillin/streptomycin and 5 × 10^-5 M 2-CDA), and filtered through a 70-μm nylon filter. Blood was collected in heparin-coated tubes, and stained for flow cytometry.

**RNA-seq Data Analysis**

RNA-seq data were collected from European Genome–phenome Archive, EGAS0000100000992 (39) and from the GSE98988 dataset (43). TCGA cutaneous melanoma RNA-seq V2 normalized gene expression and mutation call data were retrieved from the latest Broad GDAC Firehose data run (stddata__2016_01_28). The expression and mutation call data were retrieved from the latest Broad GDAC Firehose data run (stddata__2016_01_28). The immune cell gene set analysis was performed as published previously (56). Gene set scores were calculated using the GSVA package (version 1.28.0) in R (version 3.5.1; ref. 57).

**Pyropoptosis Gene Set**

The pyropoptosis gene set was determined using http://amigo.geneontology.org/amigo/term/GO:0070269 and the recent Molecular mechanisms of cell death: recommendations of the Nomenclature Committee on Cell Death 2018 (58). Genes used were GSDMA/B/C/D/E, CASP1/2/3/4/5/8, NLRC4, IL1B, and IL18.

**Radiotherapy**

Ionizing radiation was administered at doses ranging from 5 to 20 Gy using a 250-kVp X-ray machine (PanTak) with 50 cm source-to-skin distance and a 2 mm aluminum filter. The dose rate was approximately 3.6 Gy/minute.

**In Vitro Drug Treatments**

Cells were treated with BETi (2 μmol/L PLX51107 provided by Plexikon Inc.), CDK4/6i (1 μmol/L palbociclib), etoposide...
(37.5 μmol/L, Sigma), dacarbazine (20 μmol/L, Sigma), tamoxifen (1 μmol/L, Sigma), ERK1/2i (1 μmol/L SCH772984, SelleckChem), or paradox-breaking BRAFi (500 nM PLX8394 provided by Plexxikon Inc.) for 24 hours. Etoposide (37.5 μmol/L) and doxorubicin (1 mmol/L, Fisher Scientific) concentrations were based upon previous publications (59–61).

Disclosure of Potential Conflicts of Interest

D.A. Erkes is an assistant editor of Cancer Immunology Research at the American Association for Cancer Research, is an independent consultant for SRIN Therapeutics, and has ownership interest in an intraskeletal CMV-based cancer vaccines patent, 6107-1088 (378375). U. Rodeck reports receiving commercial research grants from Advaxis Immunotherapies and Akrevia Therapeutics, has ownership interest (including patents) in Akrevia Therapeutics, and is an unpaid consultant/advisory board member for Akrevia Therapeutics. A.E. Aplin is a consultant at SpringWorks Therapeutics and Fortress Biotech, reports receiving a commercial research grant from Pfizer Inc., and has ownership interest in patent number 9880150. No potential conflicts of interest were disclosed by the other authors.

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References

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