Received: 18 January 2023 | Revised: 17 October 2023 | Accepted: 3 November 2023

DOI: 10.1002/ijc.34807

IJC INTERNATIONAL JOURNAL of CANCER

RESEARCH ARTICLE

Cancer Therapy and Prevention

BRAF and MEK inhibitor combinations induce potent molecular and immunological effects in NRAS-mutant melanoma cells: Insights into mode of action and resistance mechanisms

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Abstract

About 25% of melanoma harbor activating NRAS mutations, which are associated with aggressive disease therefore requiring a rapid antitumor intervention. However, no efficient targeted therapy options are currently available for patients with NRAS-mutant melanoma. MEK inhibitors (MEKi) appear to display a moderate antitumor activity and also immunological effects in NRAS-mutant melanoma, providing an ideal backbone for combination treatments. In our study, the MEKi binimetinib, cobimetinib and trametinib combined with the BRAF inhibitors (BRAFi) encorafenib, vemurafenib and dabrafenib were investigated for their ability to inhibit proliferation, induce apoptosis and alter the expression of immune modulatory molecules in sensitive NRAS-mutant melanoma cells using two- and three-dimensional cell culture models as well as RNA sequencing analyses. Furthermore, NRAS-mutant melanoma cells resistant to the three BRAFi/MEKi combinations were established to characterize the mechanisms contributing to their resistance. All BRAFi induced a stress response in the sensitive NRAS-mutant melanoma cells thereby significantly enhancing the antiproliferative and proapoptotic activity of the MEKi analyzed. Furthermore, BRAFi/MEKi combinations upregulated immune relevant molecules, such as ICOS-L, components of antigen-presenting machinery and the “don't eat me signal” molecule CD47 in the melanoma cells. The BRAFi/MEKi-resistant, NRAS-mutant melanoma cells counteracted the molecular and immunological effects of BRAFi/MEKi by upregulating downstream mitogen-activated protein kinase pathway molecules, inhibiting

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Funding information
Bundesministerium für Bildung und Forschung, Grant/Award Numbers: 01ZX1913A, 01ZX1913C; Deutsche Forschungsgemeinschaft, Grant/Award Numbers: SE 581/33-1, SE 581/34-1; Deutsche Krebshilfe, Grant/Award Number: 70113450; Else Kröner-Fresenius-Stiftung; NCT Scholarship for Medical Doctoral Thesis

[Correction added on 08 March 2024, after first online publication: The copyright line was updated.]

apoptosis and promoting immune escape mechanisms. Together, our study reveals potent molecular and immunological effects of BRAFi/MEKi in sensitive NRAS-mutant melanoma cells that may be exploited in new combinational treatment strategies for patients with NRAS-mutant melanoma.

KEYWORDS
BRAF, MEK, melanoma, NRAS mutation, resistance

What's new?

Patients with NRAS-mutant melanoma often have aggressive disease requiring rapid intervention. Here, the authors investigated the activity of BRAF/MEK inhibitor combinations in sensitive and resistant NRAS-mutant melanoma cells. They found that BRAF/MEK inhibitors act in a synergistic manner to inhibit proliferation and activate the mitochondrial pathway of apoptosis in sensitive NRAS-mutant melanoma cells while also upregulating immune modulatory molecules. Meanwhile, the resistant NRAS-mutant melanoma cells appear to acquire their resistance by counteracting both the molecular and immunological effects of the inhibitors. The findings may pave the way for the development of new combinational treatment strategies in patients with NRAS-mutant melanoma.

1 | INTRODUCTION

NRAS mutations occur in about 25% of melanoma lesions and are often associated with a rapid tumor progression. Treatment options include immune checkpoint inhibitor therapy, for example, monoclonal antibodies against programmed cell death protein 1 (*PD-1*) *or combined antibodies against PD-1 and* cytotoxic T lymphocyte-associated protein 4 (*CTLA-4*), which achieve long-term survival in 40%–50% of all melanoma patients. However, 50% of patients irrespective of their tumor mutational status do not benefit from this treatment strategy due to intrinsic resistance.[1] Since currently direct inhibitors against NRAS are not available, targeted therapy options involve the inhibition of the mitogen-activated protein kinase (MAPK) pathway further downstream of NRAS. This includes the MEK inhibitor (MEKi) binimetinib, which displayed a moderate antitumor activity in patients with NRAS-mutant melanoma, however, the duration of response (DOR) was limited due to the occurrence of MEKi resistance.[2] Interestingly, efficacy of MEKi was higher in patients with immunotherapy pretreatment. A retrospective study evaluated MEKi as second-line therapy after failure of immunotherapy and observed a response rate of 18.2%, but a median progression-free survival (PFS) of only 2.8 months.[3] Therefore, there remains an urgent need for efficient second-line therapy options after failure of anti-PD1/anti-CTLA-4 antibodies for patients with NRAS-mutant melanoma.

Various preclinical and translational studies investigated combinational treatment strategies with MEKi such as inhibitors against bromodomain and extra-terminal domain, phosphatidylinositol 3-kinase/protein kinase B, extracellular signal-regulated kinase (ERK) or cyclin-dependent kinase 4/6.[4–8] Although some treatment strategies looked promising, no dramatic improvement of the antitumor activity was achieved and combinational treatment options often resulted in increased toxicity.

In a previous study from our lab we characterized NRAS-mutant melanoma cells treated with combinations of MEKi and BRAF inhibitors (BRAFi), which is a clinically approved and well-tolerated treatment strategy for patients with BRAF-mutant melanoma.[9] We showed that the BRAFi encorafenib induced MAPK pathway-independent endoplasmic reticulum (ER) stress thereby

significantly potentiating the antiproliferative and proapoptotic effects of the MEKi binimetinib in NRAS-mutant melanoma cells, organotypic skin cultures and patient-derived tumor slice cultures. Furthermore, the combination of BRAFi and MEKi did not trigger phosphorylation of ERK and thus paradoxical activation of the MAPK pathway.[9] The latter is a mechanism that occurs in BRAF-wildtype (wt)/NRAS-mutant melanoma treated with BRAFi alone and has been associated with the development of squamous and basal cell carcinoma.[10–12]

In addition to the molecular effects, MEKi also induce immunological effects in BRAF-wt/NRAS-mutant tumors, which promote an immune stimulatory microenvironment, foster recognition of tumor cells by T cells, increase T cell infiltration into the tumor and boost T cell activity.[13–17]

Together, our own and published data indicate various molecular and immunological effects of BRAFi and MEKi that may support the usage of BRAFi/MEKi possibly in combination with immunotherapy as a treatment option for NRAS-mutant melanoma. However, no systematic approach of testing the three possible BRAFi/MEKi combinations has yet been undertaken. The aim of our study was to investigate the impact of the three BRAFi/MEKi combinations encorafenib/binimetinib, vemurafenib/cobimetinib and dabrafenib/trametinib on growth, apoptosis, signal transduction and immunogenicity of sensitive and BRAFi/MEKi-resistant NRAS-mutant melanoma cells.

2 | MATERIALS AND METHODS

2.1 | Cell culture

The NRAS-mutant WM1366 (purchased from Rockland, Pottstown, USA via BIOMOL GmbH, Hamburg, Germany, RRID: CVCL_6789) and SK-MEL-147 (a kind gift from Keiran Smalley, Moffitt Cancer Center, RRID: CVCL_3876) melanoma cell lines were cultured in RPMI medium 1640 supplemented with 10% fetal calf serum (FCS). The cell lines have been authenticated using STR profiling within the last 3 years. Cells were subjected to regular mycoplasma testing and all experiments were performed with mycoplasma-free cells.

2.2 | Establishment of resistant cell lines

Three BRAFi/MEKi-resistant NRAS-mutant melanoma cell lines were generated from the parental/sensitive WM1366 cell line using an intermittent treatment regimen with a 48 h treatment period followed by culturing cells without inhibitors until recovery. This procedure was repeated until the cells were able to proliferate in the presence of the inhibitor combinations. Single clone cells were isolated from the resistant bulk cells by serial dilution. Single clones and bulk cells were compared for their morphology, ability to proliferate (MUH assay) and to resist induction of apoptosis (apoptosis assay) after BRAFi/MEKi treatment (Figure S1). Images comparing the morphology of single clones and bulk cells were taken at ×100 magnification. The images were then cut in size, reduced to gray scale and adjusted in brightness using Photoshop v20.0.9 (Adobe, San Jose, CA). As the bulk cells and single clones had a very similar behavior, one single clone per cell line (G7 for EB-resistant, H1 for VC-resistant, E5 for DT-resistant) was chosen for further analysis. The three WM1366 resistant cell lines have been authenticated using STR profiling within the last 3 years and match the parental cell line.

2.3 | Treatment of cells

Cells were treated for 48 h with 200 U/mL recombinant human IFN-γ from PAN-biotech (No. P2060100, Aidenbach, Germany). The inhibitors encorafenib, binimetinib, vemurafenib, cobimetinib, dabrafenib and trametinib were purchased from Selleck Chemicals (Houston, TX). The reagents were initially dissolved in DMSO and then diluted in cell culture media to obtain the final concentrations. Media containing the inhibitors was added to 40% confluent cells, following an incubation at 37°C for the indicated times. Controls were incubated with DMSO alone. For culture of the resistant cell lines, inhibitor containing media was continuously used. The concentration of inhibitors used for the flow cytometry, Western blot and quantitative PCR (qPCR) experiments as well as to create the resistant cell lines was chosen according to the following criteria: (a) the concentration causes >50% inhibition of proliferation in the sensitive NRAS-mutant melanoma cells (our study) and (b) the concentration is similar or lower than the maximal plasma concentration observed in treated BRAF-mutant patients.[18] Therefore, 2.5 μM encorafenib, 0.25 μM binimetinib, 10 μM vemurafenib, 0.5 μM cobimetinib, 0.625 μM dabrafenib and 0.0625 μM trametinib were used. As such, encorafenib/binimetinib and dabrafenib/trametinib combination treatments were administered in a 10:1 ratio and vemurafenib/cobimetinib combination treatments in a 20:1 ratio, correlating with BRAFi/MEKi ratios used in other in vitro studies.[19,20]

2.4 | Organotypic skin cell culture model

For dermal reconstruction, a 24-well standing insert was placed in a 24-well plate (pores 0.4 μm, No. PIHP01250, Millipore) and coated with 250 μL of a cell-free collagen solution, containing collagen (No. 354236, Corning, Corning, NY) at a final concentration of 3.2 mg/mL in gel neutralization solution (66 mM HEPES, 18.75 ng/μL chondroitin-4-sulfate, 18.75 ng/μL chondroitin-6-sulfate, 2% dialyzed FCS in ×2 DMEM). After incubation for 30 min at 37°C, 500 μL of a collagen solution (as above) containing 1×10^5 normal human dermal fibroblasts (No. 2509, Lonza, Walkersville, MD) were added to the insert. After 1 h incubation at 37°C to allow setting of the gel, the fibroblast/collagen gel was overlaid with CnT-Prime Fibroblast Proliferation Medium (No. CNT-PR-F, CELLnTEC, Bern, Switzerland). After 4 days incubation at 37°C with a medium change after 2 days, the collagen gel had contracted to generate a crater at the top for seeding the epidermal cells. For epidermal reconstruction, 1×10^5 neonatal human epidermal keratinocytes (No. FC-0007, Cell Systems, Troisdorf, Germany) and 5.7×10^4 sensitive or resistant WM1366 melanoma cells (ratio of 1:1.75) were resuspended in 50 μL DermaLife K media (No. LM0007, Cell Systems) containing 2%

dialyzed FCS and added onto the dermis. After 1 h incubation at 37°C, the dermal/epidermal reconstruct was submerged in DermaLife K media containing 2% dialyzed FCS and was incubated for 5 days at 37°C with a medium change after 2 days. Afterwards, the DermaLife K media was removed from the reconstruct, the 24-well insert placed in a 6-well plate and 1 mL of CnT-PR-FTAL5 medium (No. CnT-PR-FTAL5, CELLnTEC) containing 2% dialyzed FCS was added to the bottom of the well. Following a 12 day incubation period at 37°C with a medium change every 2 days, the medium was changed to CnT-PR-FTAL5 containing 2% dialyzed FCS including BRAFi/MEKi (concentrations see above). After incubation for 6 days at 37°C with changing the medium on day 4, the skin model was fixed in 4% formalin and embedded paraffin. Hematoxylin & eosin (H&E) or immunostaining with antibodies against cytokeratin (No. M3515, Agilent, Santa Clara, CA), Ki-67 (No. M7240, Agilent) or cleaved PARP (No. 5625, Cell Signaling Technology, Danvers, MA) were performed on 1 µm sections as previously described.[21] Images were taken with the KEYENCE BZ-X800E microscope (KEYENCE, Osaka, Japan) at ×100 and ×400 magnification, which were adjusted for white balance and cropped using Photoshop v20.0.9 (Adobe). Stained images were evaluated by a certified dermato-pathologist (Mi.Se.).

2.5 | Proliferation assay

Cell proliferation was determined with a 4-methylumbelliferyl heptanoate (MUH) assay as described previously.[18]

2.6 | Apoptosis assay (subG1 analysis of cell-cycle)

Apoptosis was determined by analyzing the subG1 population of the cell cycle as described previously.[18]

2.7 | Western blot analysis

Western blot analysis was performed as described previously.[21] A detailed description is given in the Data S1.

2.8 | Flow cytometry for the detection of immunological molecules

For detection of ICOS-L, OX40-L, CD137-L, CD47 and B7-H3, 1×10^6 cells per 10 cm dish were seeded and treated for 48 h at 37°C with the inhibitors or DMSO. After detaching the adherent cells with 10 mM EDTA for 30 min at 37°C followed by washing with PBS, the cells were stained for 15 min at 4°C with the following antibodies: PE-anti-human ICOS-L (No. 552502), BV421-anti-human OX40-L (No. 563766) from BD Biosciences or APC-anti-human CD137-L (No. 311506) AF700-anti-human CD47 (No. 323126), PE/Cy7-anti-human CD276 (B7-H3, No. 351008) from Biolegend (San Diego, CA). The viable cell fraction was determined by staining with 7-amino-actinomycin D (No. 559925, BD Biosciences, Franklin Lakes, NJ). Stained cells were measured with the FACSCanto II (BD Biosciences) and analyzed using the Flowlogic (Inivai Technologies, Mentone, Victoria, Australia) software to determine the absolute mean fluorescence intensity (MFI) (CD47, B7-H3) or percentage of positive cells (ICOS-L, OX40-L and CD137-L).

For detection of HLA class I, HLA class II and PD-L1 surface expression, cells were seeded and treated with the inhibitors or DMSO for 48 h at 37°C. Harvested cells were then stained with the following antibodies: APC-anti-human HLA-ABC (No. 17-9983-42, eBioscience, San Diego, CA), APC-anti-human PD-L1 (No. 17-5983-42, eBioscience), APC-anti-human HLA-DR,DQ,DP (No. 130-123-843, Miltenyi Biotec, Bergisch Gladbach, Germany) and APC-msIgG2a isotype control (No. 17-4727-81, eBioscience). Stained cells were measured using the Navios (Beckman Coulter, Brea, CA) and analyzed with Kaluza. For HLA class I and PD-L1, the relative MFI was calculated from the MFI of the total cell population divided by the MFI of the isotype control. For HLA class II, the percentage of positive cells in the isotype control was subtracted from the percentage of positive HLA class II cells.

2.9 | RNA isolation for cDNA synthesis and quantitative PCR

A detailed description is given in the Data S1 and Table S1.

2.10 | RNA isolation for RNA sequencing and bioinformatics analyses

A detailed description can be found in the Data S1.

2.11 | Statistical analyses

Results from the proliferation assay were either displayed as a 3D bar graph of the mean using Excel2016 (Microsoft, Redmond, WA) or plotted as mean ± SEM using Prism v9 (GraphPad, San Diego, CA). Results from the flow cytometry, Western blot, RNA sequencing and qPCR analyses were displayed as mean ± SEM using Prism v9 (GraphPad). Volcano plots and gene set enrichment analysis (GSEA) signature graphs were created using Prism v9 (GraphPad). Significance was defined as $P < .05$ based on a two-tailed non-paired Student's *t*-test using Excel2016 (Microsoft).

3 | RESULTS

3.1 | BRAFi enhance the antitumor cell activity of MEKi in sensitive NRAS-mutant melanoma cells

To investigate the antiproliferative effects of BRAFi and MEKi in NRAS-mutant melanoma cells, sensitive WM1366 cells treated with varying concentrations of BRAFi and MEKi alone and in combination

(encorafenib/binimetinib, vemurafenib/cobimetinib, dabrafenib/trametinib) were analyzed with a 4-methylumbelliferyl-heptanoate (MUH) proliferation assay. All three BRAFi alone did not inhibit proliferation at any concentration in this BRAF-wt/NRAS-mutant cell line (Figure 1A), which is to be expected as these inhibitors are specifically designed to target the mutated, but not wild-type BRAF protein. The three MEKi alone caused efficient antiproliferative effects, with cobimetinib and trametinib inhibiting the growth up to 60%. Combined BRAFi/MEKi treatment further potentiated this antiproliferative effect in a synergistic manner, leading to growth inhibition rates of up to 86% in the encorafenib/binimetinib-, 78% in the dabrafenib/trametinib- and 68% in the vemurafenib/cobimetinib-treated cells, respectively. Comparable antiproliferative effects of BRAFi/MEKi combinations were observed in the sensitive SK-MEL-147 NRAS-mutant melanoma cell line (Figure S2A).

To determine the effects of BRAFi and MEKi on the MAPK proliferation pathway, the effector ERK as well as its phosphorylated form, pERK, were investigated by Western blot. BRAFi treatment alone led to an increase in pERK (Figure 1B upper panel, Figure S3A) indicative of paradoxical activation of the MAPK pathway. The addition of MEKi to BRAFi overcame the paradoxical activation resulting in hardly any remaining pERK expression in the sensitive NRAS-mutant melanoma cells.

Using flow cytometry, the apoptotic cell fraction of BRAFi- and MEKi-treated, sensitive WM1366 and SK-MEL-147 NRAS-mutant melanoma cells was analyzed, demonstrating comparable effects to the proliferation assay: the three BRAFi alone did not trigger apoptosis and MEKi alone caused moderate apoptotic rates of 25%–55%, the combined BRAFi/MEKi treatment resulted in apoptosis rates ranging between 50% and 80% (Figures 1C and S2B). Furthermore, the proapoptotic Bcl-2 proteins BimEL, L and S were upregulated after MEKi treatment, which was even more pronounced upon combination of BRAFi/MEKi (Figure 1B middle panel, Figures S3B and S4). The cleavage product of the initiator and executioner caspase-3 and -9 as well as the caspase product PARP were induced after MEKi treatment, which was significantly increased after combined BRAFi/MEKi treatment.

Since the three BRAFi enhance the antiproliferative and proapoptotic activities of the MEKi, but single treatment regimens did not trigger these effects, the pathways contributing to the BRAFi-induced antitumor cell activity in the sensitive NRAS-mutant cells were further investigated. BRAFi has been proposed to induce ER stress in BRAF- and NRAS-mutant melanoma cells.[9,22] Accordingly, all three BRAFi alone and in combination with MEKi induced the expression of the activating transcription factor 4 (ATF4) and the CCAAT-enhancer-binding protein homologous protein (CHOP) (Figure 1B lower panel, Figure S3C). In contrast, ER stress sensors such as the PKR-like ER kinase (PERK) and the activating transcription factor 6 (ATF6) were not altered upon BRAFi and/or MEKi treatment, while the inositol-requiring enzyme 1 alpha (IRE1α) was downregulated in the MEKi alone and combination treatments. Furthermore, we also assessed the integrated stress response marker α-subunit of the eukaryotic initiation factor 2 (eiF2α), which, in addition to ER stress, also detects amino acid and heme deprivation as well as viral infection. Phosphorylated eiF2α (peIF2α) was induced in the BRAFi alone and BRAFi/MEKi combination treatments. Of note, the induction of ATF4, CHOP and peiF2α was slightly lower after BRAFi/MEKi compared to BRAFi alone treatment, indicating a possible rescue effect of MEKi.

Taken together, the stress-inducing effects of BRAFi act synergistically with the antiproliferative and proapoptotic effects of MEKi to induce a potent antitumor cell activity in NRAS-mutant melanoma cell lines.

3.2 | BRAFi/MEKi combinations trigger antitumor cell activities in sensitive, but not in resistant NRAS-mutant melanoma cells

To characterize the molecular mechanisms induced by BRAFi and MEKi in NRAS-mutant melanoma cells, WM1366 cells resistant to each of the three BRAFi/MEKi combinations were established. The encorafenib/binimetinib-resistant, vemurafenib/cobimetinib-resistant and dabrafenib/trametinib-resistant WM1366 cells were termed EB-resistant, VC-resistant and DT-resistant, respectively.

In the first step, the proliferative capacity of sensitive and resistant WM1366 cells after treatment with BRAFi/MEKi was compared. As shown above, proliferation of sensitive cells was successfully inhibited with increasing concentrations of BRAFi/MEKi combinations (Figure 2A). In contrast, the resistant cells continued proliferating in presence of BRAFi/MEKi, even at higher concentrations than those used for generation of the resistant phenotypes.

Furthermore, sensitive WM1366 cells displayed a significant reduction in pERK levels following treatment with vemurafenib/cobimetinib and dabrafenib/trametinib (Figure 2B upper panel, Figure S5A), confirming the above results. In contrast, pERK levels were not completely inhibited in the VC-resistant and DT-resistant cells treated with vemurafenib/cobimetinib and dabrafenib/trametinib. In addition, the basal pERK levels were significantly higher in the DMSO-treated VC-resistant and DT-resistant cells compared to the sensitive counterparts ($P = .014$ and $P = .001$, respectively), indicating a resistance mechanism to BRAFi/MEKi treatment. Interestingly, in sensitive and EB-resistant cell lines pERK levels were increased after treatment with encorafenib/binimetinib, suggesting that binimetinib may not be strong enough to overcome the encorafenib-induced paradoxical activation of the MAPK pathway.[18]

The apoptotic cell cycle fraction and the activation of apoptotic proteins were analyzed upon treatment with BRAFi/MEKi of the sensitive and resistant WM1366 cell lines. As shown above, all three combination treatments increased the apoptotic fraction, induced the expression of the proapoptotic Bcl-2 family members BimEL, L, S and the cleavage of caspase-3 and -9 as well as the caspase product PARP in the sensitive WM1366 cells (Figure 2B middle panel and Figures 2C and S5B). In contrast, the three resistant cell lines did not display significant apoptotic rates and showed a considerably reduced expression of the apoptotic proteins in comparison to the sensitive cells.

Interestingly, in contrast to apoptotic proteins, there was no downregulation, but even a slight upregulation of IRE1α, peIF2α, ATF4 and CHOP in the resistant compared to the sensitive melanoma

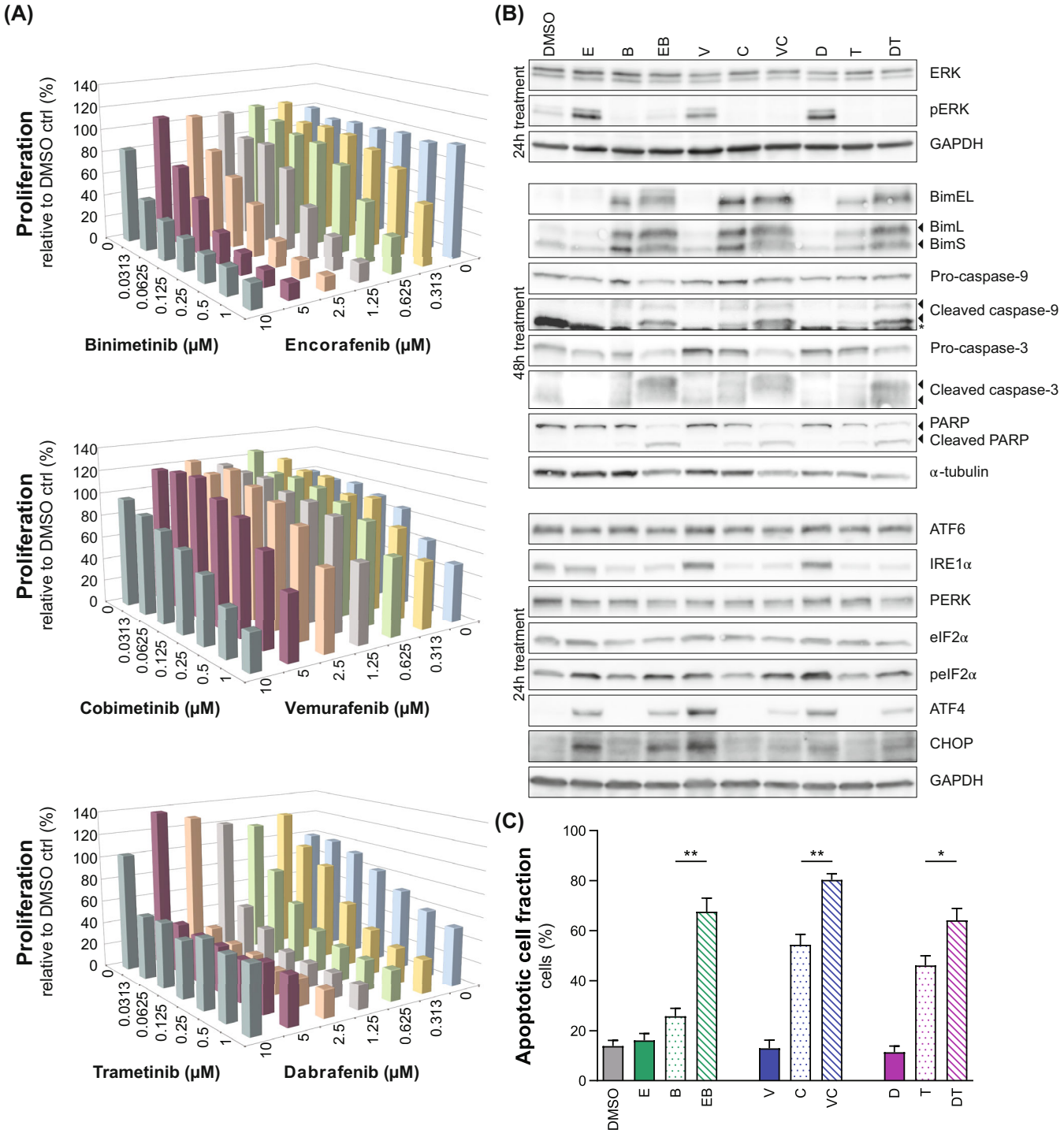

FIGURE 1 BRAF inhibitors enhance the antitumor activity of MEK inhibitors in sensitive NRAS-mutant melanoma cells. (A) Sensitive WM1366 cells were treated with the indicated concentrations of BRAF inhibitors (BRAFi) and MEK inhibitors (MEKi) for 72 h and analyzed in a 4-methylumbelliferyl-heptanoate (MUH) proliferation assay as described in Materials and Methods. Values represent the mean of three independent experiments and are displayed in a 3D column chart as percentage of proliferation (vertical axis) relative to the DMSO control (ctrl). The increasing MEKi (binimetinib, cobimetinib, trametinib) concentrations (0–1 μM) are depicted on the horizontal axis. Increasing BRAFi (encorafenib, dabrafenib, vemurafenib) concentrations (0–10 μM) are displayed on the depth axis using the following coloring: 0 μM blue, 0.313 μM yellow, 0.625 μM green, 1.25 μM light gray, 2.5 μM orange, 5 μM red, 10 μM dark gray. SEM and significance were omitted for simplicity. (B) Western blot analyses of signal transduction (upper panel), apoptotic (middle panel) and stress sensor (lower panel) proteins in sensitive WM1366 cells after 24 or 48 h treatment with the BRAFi/MEKi; *, non-specific band; one representative image of three independent experiments, quantification is shown in Figure S3. (C) Sensitive WM1366 cells were treated with BRAFi/MEKi for 72 h and analyzed by flow cytometry for their apoptotic cell fraction as described in Materials and Methods. Values represent the mean ± SEM of three independent experiments. A two-sided unpaired Student's *t*-test was carried out to determine the significance between samples treated with MEKi alone versus BRAFi/MEKi: $^{*}P < .05$, $^{**}P < .01$; E, encorafenib (2.5 μM); B, binimetinib (0.25 μM); V, vemurafenib (10 μM); C, cobimetinib (0.5 μM); D, dabrafenib (0.625 μM); T, trametinib (0.0625 μM).

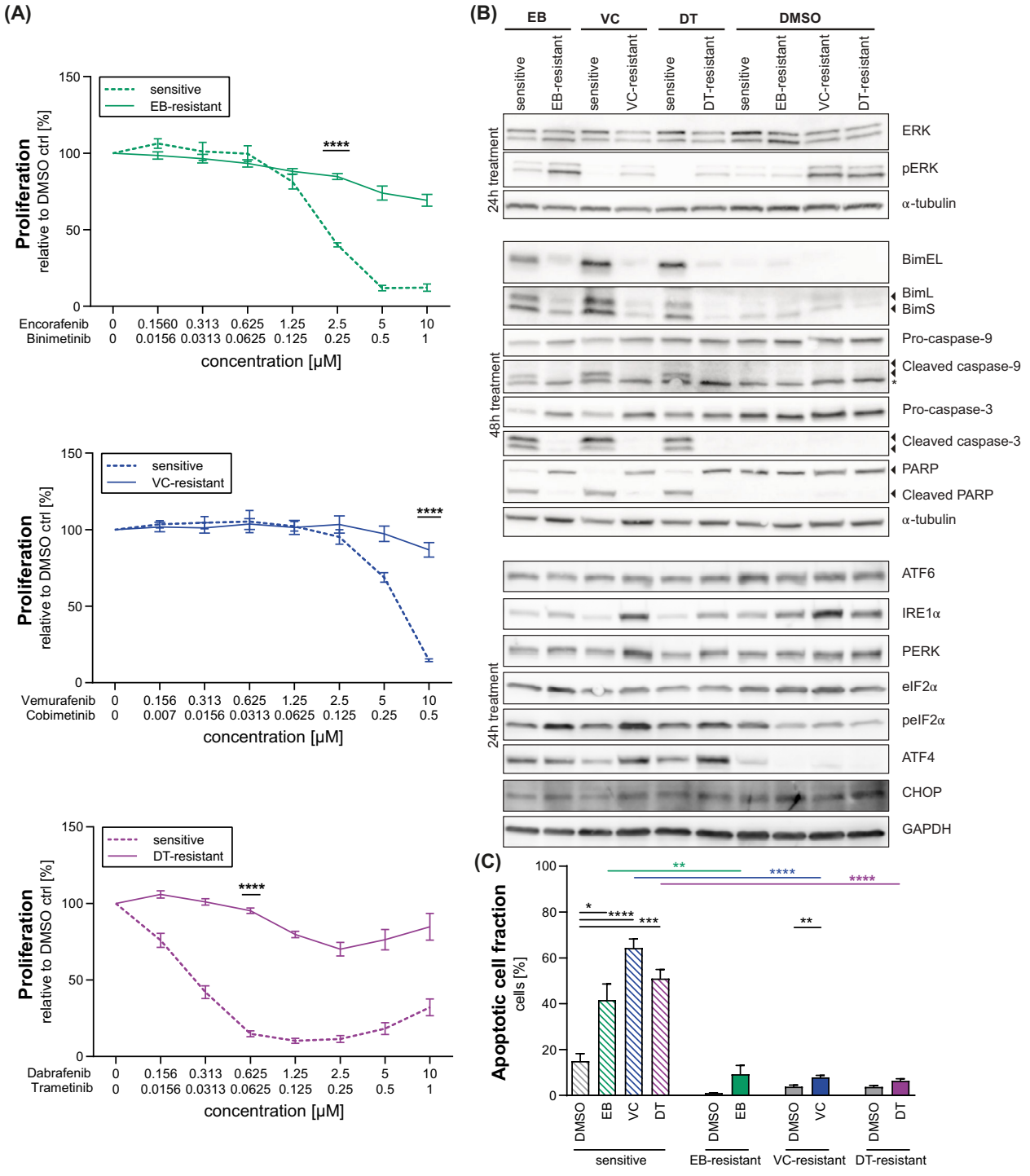

FIGURE 2 Resistant NRAS-mutant melanoma cells act against the antiproliferative and apoptosis-inducing, but not stress-inducting activity of BRAF and MEK inhibitors. (A) Sensitive and resistant WM1366 cells were treated with the indicated concentrations of BRAF inhibitors (BRAFi) and MEK inhibitors (MEKi) for 72 h and analyzed in a 4-methylumbelliferyl-heptanoate (MUH) proliferation assay as described in Materials and Methods. Values represent the mean ± SEM of four independent experiments relative to the DMSO control (ctrl). A two-sided unpaired Student's *t*-test was carried out to determine the significance between sensitive and resistant cells at the BRAFi/MEKi concentration used to establish the resistant cell lines: ****$P < .0001$. (B) Western blot analyses of signal transduction (upper panel), apoptotic (middle panel) and stress sensor (lower panel) proteins in sensitive and resistant WM1366 cells after 24 or 48 h treatment with the BRAFi/MEKi; *, non-specific band; one representative image of three or four (ERK, pERK) independent experiments, quantification is shown in Figure S5. (C) Sensitive and resistant WM1366 cells were treated with BRAFi/MEKi for 72 h and analyzed by flow cytometry for their apoptotic cell fraction as described in Materials and Methods. Values represent the mean ± SEM of four independent experiments. A two-sided unpaired Student's *t*-test was carried out to determine the significance between the following samples: DMSO-treated and BRAFi/MEKi-treated cells (black stars) as well as BRAFi/MEKi-treated sensitive and resistant cells (green stars for EB, blue stars for VC, magenta stars for DT): *$P < .05$, ***$P < .001$. E, encorafenib (2.5 μM); B, binimetinib (0.25 μM); V, vemurafenib (10 μM); C, cobimetinib (0.5 μM); D, dabrafenib (0.625 μM); T, trametinib (0.0625 μM).

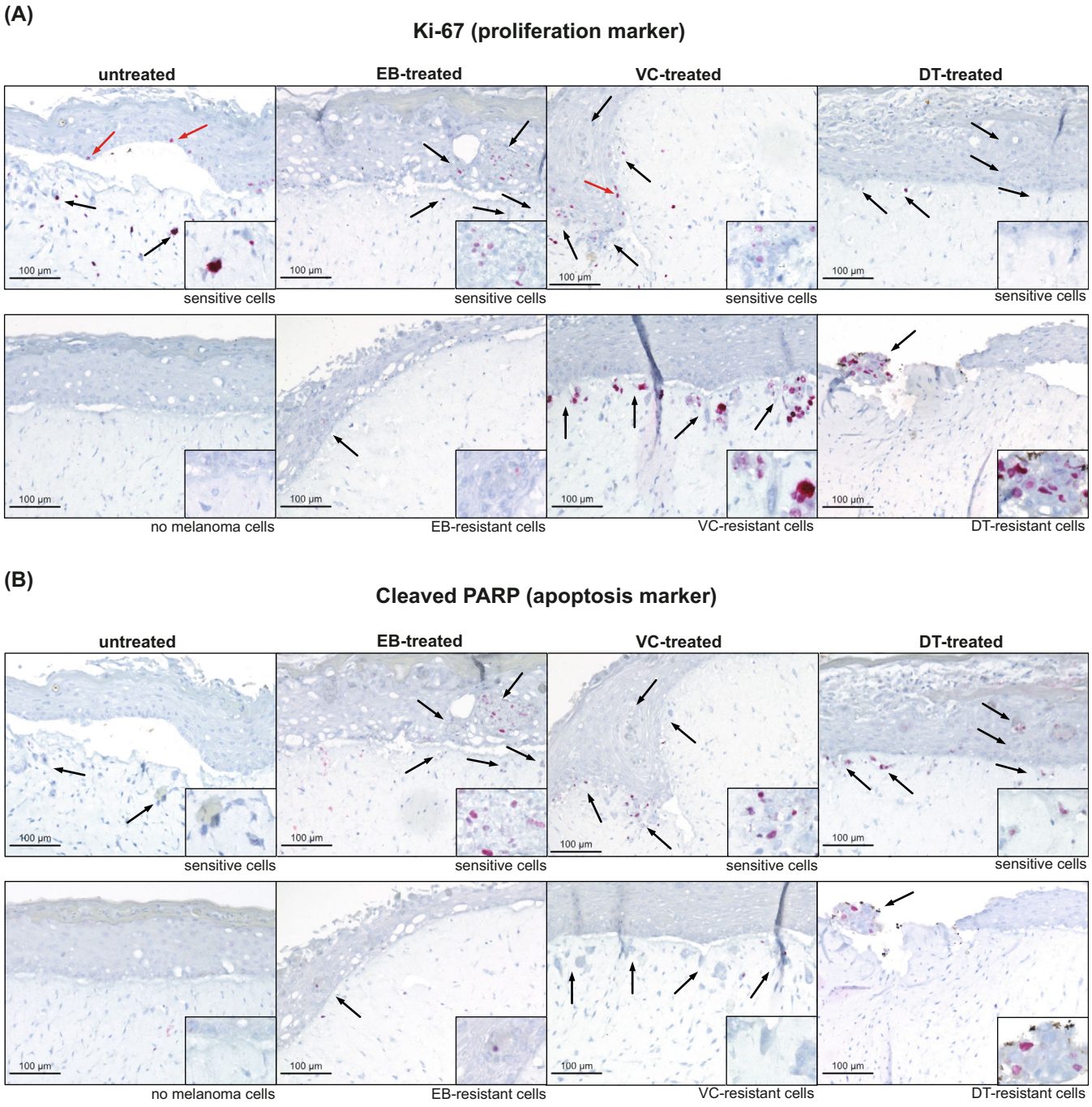

FIGURE 3 Sensitive but not resistant NRAS-mutant melanoma cells seeded into organotypic skin culture models display decreased proliferation and increased apoptosis after BRAF and MEK inhibitor treatment. Sensitive and resistant WM1366 cells were grown in organotypic skin culture models and treated with BRAF inhibitors (BRAFi) and MEK inhibitors (MEKi). Models were fixed with 4% formalin, embedded in paraffin and stained with antibodies against Ki-67 (proliferation marker) (A) or cleaved PARP (apoptosis marker) (B). Representative images of three independent experiments. Images were taken at ×100 and ×400 magnification (inlay). Scale bar indicates 100 µm. E, encorafenib (2.5 µM); B, binimetinib (0.25 µM); V, vemurafenib (10 µM); C, cobimetinib (0.5 µM); D, dabrafenib (0.625 µM); T, trametinib (0.0625 µM). Melanoma cells grew either as nests in the epidermis or infiltrated the dermis as single cells (black arrows). Ki-67 stained images also detected proliferating keratinocytes in the basal layer (red arrows). See also Figure S6 for Hematoxylin & Eosin and cytokeratin stained images.

cells after combined BRAFi/MEKi treatment (Figure 2B lower panel, Figure S5C), indicating that resistance may not be directed against BRAFi-induced activation of the stress pathways.

To confirm the two-dimensional (2D) cell line data in a more physiological context, the growth of the sensitive and resistant cells after BRARi/MEKi treatment was assessed in an organotypic skin model (Figure 3). Melanoma cells grew as cell nests in the epidermis or as single cells infiltrating the dermis (Figures 3 and S6, indicated with black or white arrows). Untreated, sensitive melanoma cells were positive for the proliferation marker Ki-67 and negative for the

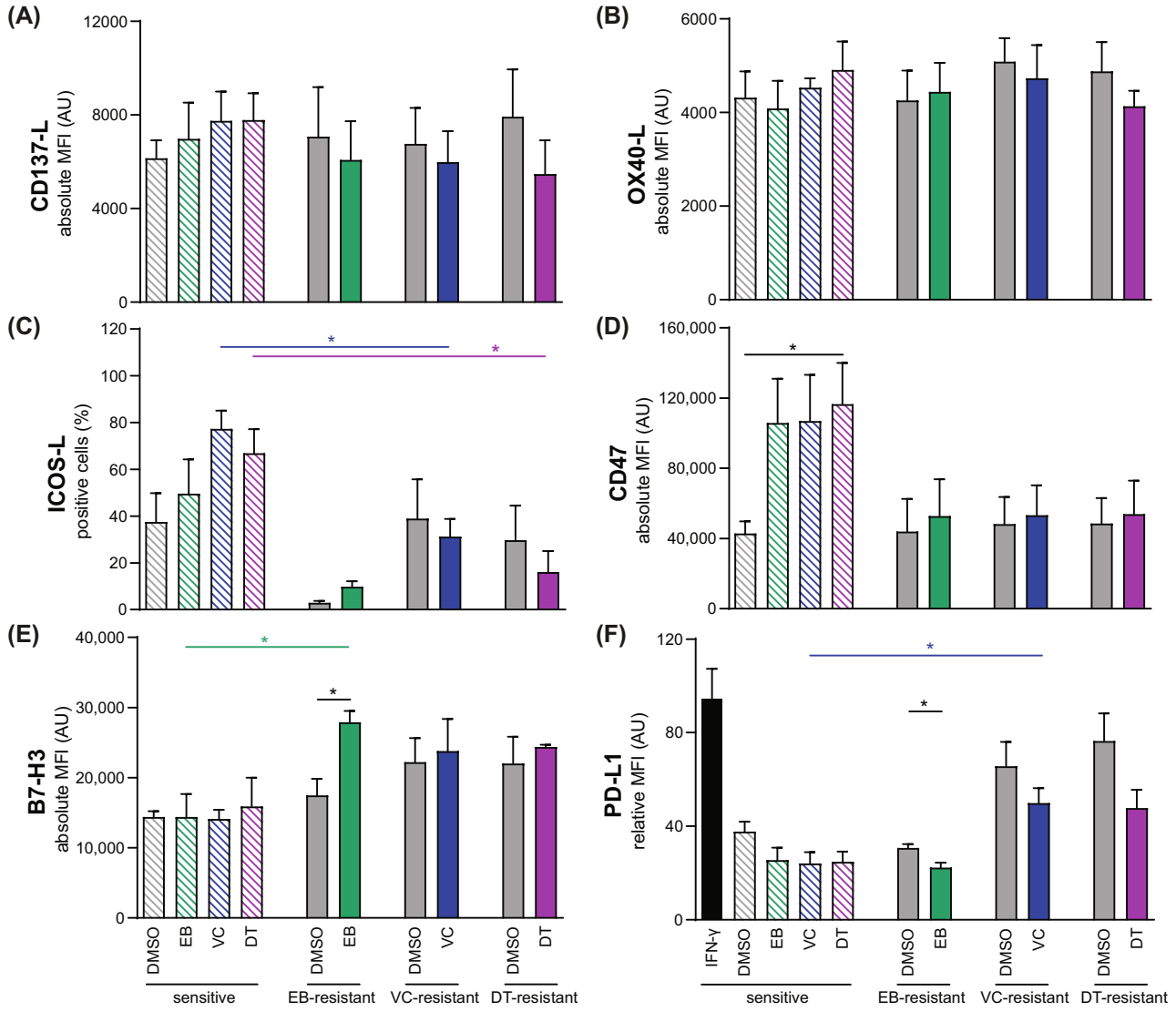

FIGURE 4 BRAF inhibitor and MEK inhibitor combinations induce immunological effects in sensitive, but not in resistant NRAS-mutant melanoma cells. (A-F) Sensitive and resistant WM1366 cells were treated with combinations of BRAF inhibitors (BRAFi) and MEK inhibitors (MEKi) for 48 h and analyzed by flow cytometry for the surface expression of immunological molecules as described in Section 2. Values represent the mean ± SEM of three independent experiments of the absolute mean fluorescence intensity (MFI) (A,B, D,E), the MFI relative to the isotype control (F) or % positive cells (C). A two-sided unpaired Student's *t*-test was carried out to determine the significance between the following samples: DMSO-treated and BRAFi/MEKi treated cells (black stars) as well as BRAFi/MEKi-treated sensitive and resistant cells (green stars for EB, blue stars for VC, magenta stars for DT): $^{*}P < .05$, $^{**}P < .01$; E, encorafenib (2.5 μM); B, binimetinib (0.25 μM); V, vemurafenib (10 μM); C, cobimetinib (0.5 μM); D, dabrafenib (0.625 μM); T, trametinib (0.0625 μM); AU, arbitrary units; IFN-γ, interferon γ.

apoptosis marker cleaved PARP (Figure 3). In comparison to the untreated cells, the BRAFi/MEKi-treated sensitive melanoma cells displayed only minimal proliferation, but clear signs of apoptosis (Figure 3, Ki-67 and cleaved PARP staining, respectively). In addition, the treated cells, especially in the cell nests, showed an early necrotic phenotype characterized by loss of cellular detail and nuclear dissolution (Figure S6, H&E staining). Intriguingly, the BRAFi/MEKi-resistant cells displayed pronounced proliferation (except EB-treated cells), but only minimal expression of apoptotic markers, indicating resistance to treatment.

In summary, BRAFi/MEKi combinations inhibit proliferation and induce apoptosis in sensitive, but not in BRAFi/MEKi-resistant cells in 2D and 3D cell culture models. This effect may be partially caused by an upregulation of pERK and downregulation of mitochondrial apoptotic proteins in the resistant cells.

3.3 | BRAFi/MEKi combinations induce an immune stimulatory phenotype of sensitive, but not of resistant NRAS-mutant melanoma cells

In addition to the effects on MAPK and apoptosis signaling pathways, BRAFi and MEKi can also induce immunological effects in melanoma.[13]

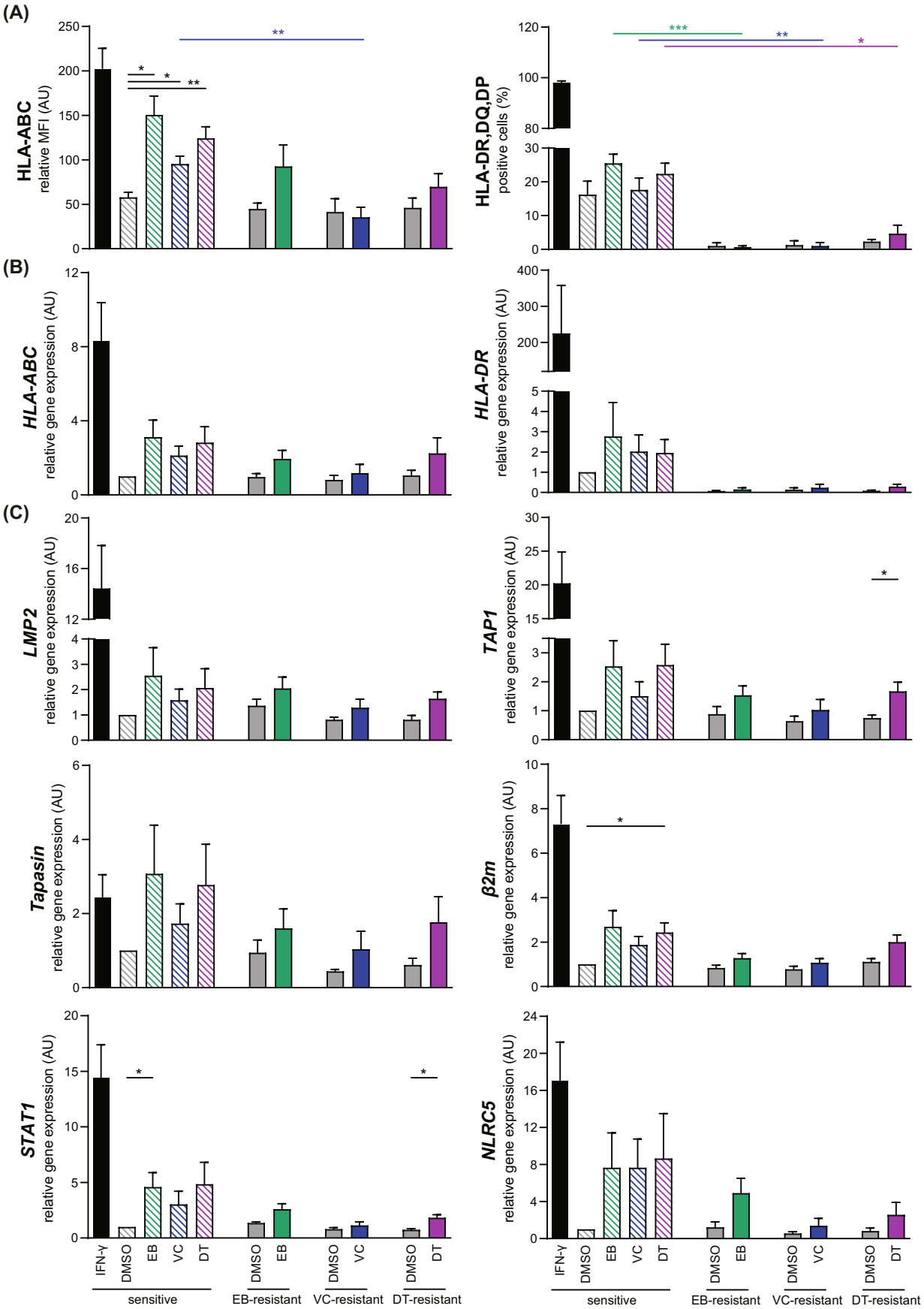

FIGURE 5 Legend on next page.

An altered expression of immune stimulatory and/or inhibitory molecules as well as members of the human leukocyte antigen (HLA) class I antigen-presenting machinery (APM) is often detected in tumor cells compared to non-neoplastic cells, and may thus influence the activity of infiltrating immune cells. Using flow cytometry, transcriptome analyses and quantitative PCR, we investigated whether these molecules are indeed expressed and are altered during BRAFi/MEKi treatment thereby influencing the immunogenicity of the sensitive and resistant NRAS-mutant cell lines.

Monitoring of OX40-L, CD137-L and ICOS-L surface expression on NRAS-mutant cells, known to interact with their corresponding receptors on T cells to co-stimulate immune responses[23] demonstrated no changes in the expression of OX40-L and CD137-L in the sensitive and resistant NRAS-mutant cells in response to BRAFi/MEKi treatment (Figure 4A,B). In contrast, the frequency of ICOS-L positive cells was increased in the sensitive cells treated with BRAFi/MEKi, but was significantly reduced in the resistant cells (Figure 4C). Interestingly, the expression of CD47, which is considered as a "don't eat me signal" for macrophages,[24] was also induced by BRAFi/MEKi treatment in sensitive, but not in resistant cells (Figure 4D).

Regarding co-inhibitory molecules, the expression of programmed death ligand 1 (PD-L1) (B7-H1) and B7-H3, known to suppress T cell responses,[25] was slightly reduced in the sensitive cells after BRAFi/MEKi treatment (Figure 4E,F), while resistant cells displayed a trend for increased expression levels of both molecules (eg, PD-L1 surface expression of DMSO-treated DT-resistant versus sensitive cells: $P = .038$).

In addition, the surface (Figure 5A) and gene expression (Figure 5B) levels of HLA class I (HLA-ABC) and HLA class II (HLA-DR,DQ,DP) antigens and components of the HLA class I APM were investigated (Figure 5C). These include LMP2, a subunit of the immunoproteasome; TAP1, which transports peptides from the ER into the cytosol; tapasin that facilitates optimal peptide loading onto HLA class I molecules; β_2m, a non-covalently associated component of the HLA class I complex as well as STAT1 and NLRC5 as transcriptional regulators of HLA genes and APM components.[26] The expression of all these molecules was induced in the sensitive cells by BRAFi/MEKi treatment (Figure 5). In contrast, in resistant cells, the increase of their expression after inhibitor treatment was much lower. Particularly, the number of resistant cells presenting HLA-DR,DQ,DP on their surface and the *HLA-DR* gene expression levels were significantly decreased in the resistant compared to the sensitive cells.

To determine, which of the two inhibitors confers the immune-related phenotype, we tested the sensitive WM1366 cells for alterations in the gene and protein expression of immune modulating molecules after single treatment with either dabrafenib or trametinib, respectively. As shown in Figure S7A–C, the increased surface expression of ICOS-L, CD47 and HLA-ABC after BRAFi/MEKi treatment was found upon MEKi, but not upon BRAFi treatment. The same trend was detected by analysis of the gene expression levels of APM components (Figure S7D–I).

RNA sequencing of all WM1366 cell lines was performed to compare differentially expressed genes between the sensitive and each individual resistant line (Figure 6A–C, Tables S2 and S3). To focus especially on genes related to immune activation and immune inhibition, "gene set enrichment analysis" (GSEA) was employed demonstrating decreased antigen presentation via HLA class II signature expression in both VC-resistant as well as EB-resistant cells compared to the sensitive cell line (gene ontology term signatures: antigen processing and presentation of exogenous peptide antigen via MHC class II; MHC class II protein complex binding) (Figure 6D,E). These results mainly correspond to the inactivation of immune stimulatory HLA genes (*HLA-DP* and *HL-DR*), and the activation of immune inhibitory genes PD-L1 (*B7-H1*), *B7-H3* and *B7-H6* in the resistant cell lines (Figure 6F,G). Interestingly, although there was a similar decrease in gene expression of *HLA-DP* and *HLA-DR* genes in all three resistant cells, the EB-resistant melanoma line exhibited no increased gene expression of *PD-L1* or *B7-H6*.

Altogether, BRAFi/MEKi induce immune stimulatory molecules and APM components in sensitive NRAS-mutant melanoma cells, while the expression of these molecules is reversed in the resistant NRAS-mutant melanoma cells.

4 | DISCUSSION

4.1 | Molecular effects of BRAFi/MEKi treatment in sensitive NRAS-mutant melanoma cells

In our study, the effects of BRAFi/MEKi combinations on sensitive NRAS-mutant melanoma cells were investigated, demonstrating that treatment of these cells with all three MEKi alone moderately inhibited the MAPK proliferation pathway and induced the mitochondrial

FIGURE 5 BRAF inhibitor and MEK inhibitor combinations induce the HLA antigen-presenting machinery in sensitive, but not in resistant NRAS-mutant melanoma cells. (A) Sensitive and resistant WM1366 cells were treated with different concentrations BRAF inhibitors (BRAFi) and MEK inhibitors (MEKi) for 48 h and analyzed by flow cytometry for the surface expression of HLA class I (HLA-ABC) and HLA class II (HLA-DR,DQ,DP) molecules as described in Materials and Methods. Values represent the mean ± SEM of three independent experiments of the mean fluorescence intensity (MFI) or % positive cells relative to the isotype control. (B,C) Quantitative PCR analyses of the HLA related genes in sensitive and resistant WM1366 cells after 48 h treatment with the BRAFi/MEKi as described in Materials and Methods. Values represent the mean ± SEM of three independent experiments relative to the housekeeping gene and displayed as x-fold gene expression over the DMSO control. A two-sided unpaired Student's *t*-test was carried out to determine the significance between the following samples: DMSO-treated and BRAFi/MEKi-treated cells (black stars) as well as BRAFi/MEKi-treated sensitive and resistant cells (green stars for EB, blue stars for VC, magenta stars for DT): $^*P < .05$, $^{**}P < .01$, $^{***}P < .001$; E, encorafenib (2.5 μM); B, binimetinib (0.25 μM); V, vemurafenib (10 μM); C, cobimetinib (0.5 μM); D, dabrafenib (0.625 μM); T, trametinib (0.0625 μM); AU, arbitrary units; IFN-γ, interferon γ.

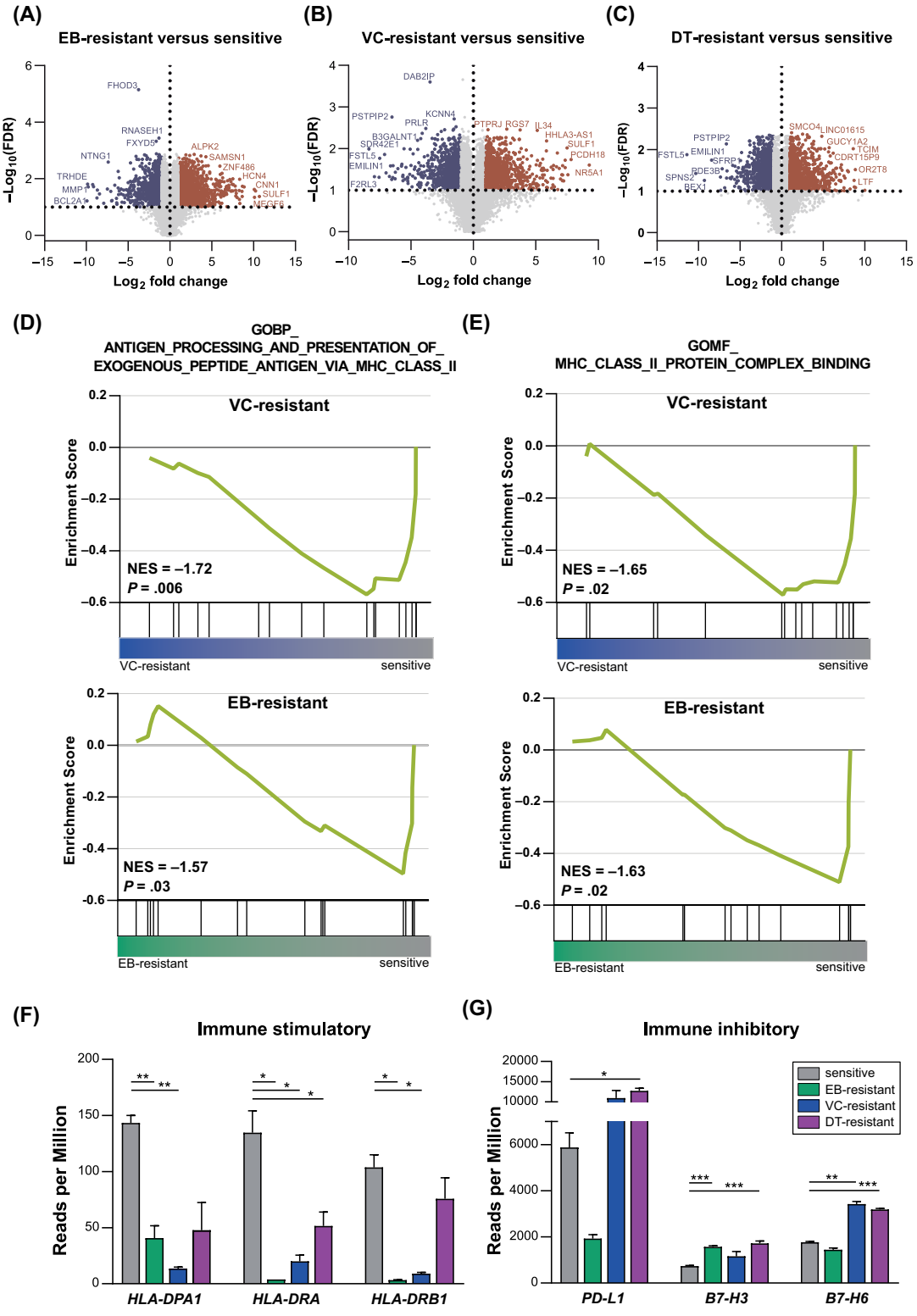

FIGURE 6 BRAFi/MEKi-resistant melanoma cells exhibit decreased expression of immune stimulatory genes and increased expression of immune inhibitory genes. (A–C) Volcano plots indicating the differentially expressed genes (DEG) between EB-resistant (A), VC-resistant (B) and DT-resistant (C) compared to sensitive melanoma cells as determined by RNA sequencing. Highlighted are genes which were significantly decreased in the resistant cells in blue (FDR < 0.1, $\log_2$ fold change <−1) and increased in resistant cells in red (FDR < 0.1, $\log_2$ fold change >1). (D,E) GSEA showing decreased antigen presentation via MHC class II signature expression in VC-resistant and EB-resistant compared to sensitive cells. (F,G) Summary of mRNA expression of indicated immune stimulatory (F) and immune inhibitory (G) genes from RNA sequencing. Values represent the mean ± SEM of three independent experiments. A two-sided unpaired Student's *t*-test was carried out to determine the significance between the sensitive and resistant cells: **P* < .05, ***P* < .01, ****P* < .001.

pathway of apoptosis. The data obtained correlated well with published reports from our and other laboratories, showing a suppression of proliferation via ERK inhibition and an induction of apoptosis through activation of Bim and PARP in NRAS-mutant melanoma cells after treatment with MEKi alone.[9,27,28]

When combining MEKi with BRAFi, the antiproliferative and proapoptotic effects were significantly increased, suggesting a synergistic activity of these inhibitors in sensitive NRAS-mutant cells, a phenomenon, which was not observed in BRAF-mutant cells after combined treatment.[18] Previous data from our own laboratory showed that the BRAFi encorafenib potentiated the antitumor activity of the MEKi binimetinib by inducing intracellular stress through ATF4 upregulation. ATF4 thereby connected to the mitochondrial pathway of apoptosis via the proapoptotic Bcl-2 protein Puma.[9] Data from our study revealed that not only encorafenib, but also vemurafenib and dabrafenib led to an upregulation of ATF4, CHOP and peiF2α, suggesting a similar mechanism for all three BRAFi in sensitive NRAS-mutant melanoma cells.

Overall, our data demonstrated a potent antitumor cell activity of the BRAFi/MEKi combinations in NRAS-mutant melanoma cells, organotypic skin cultures and patient-derived tumor slice cultures (our study and[9]). Furthermore, ERK phosphorylation, which was induced by BRAFi alone, was reduced with vemurafenib/cobimetinib and dabrafenib/trametinib, indicating a sufficient control of the BRAFi-inducing paradoxical activation of MAPK pathway. Accordingly, skin toxicities such as cutaneous squamous cell carcinoma and skin papilloma occurred less frequently in patients treated with the BRAFi/MEKi combination compared to BRAFi alone.[29] It is noteworthy that due to the weak antitumor cell activity in NRAS-mutant melanoma cells,[18] binimetinib may not be an ideal partner in the BRAFi/MEKi combinations. Altogether, these data provide a good rationale for implementing BRAFi/MEKi combinations as a treatment strategy for patients with NRAS-mutant melanoma, but it needs to be ensured that the BRAF-inducing paradoxical activation is completely eliminated. Accordingly, a preclinical study indicated a benefit of panRAF inhibitors (panRAFi), which do not lead to paradoxical activation of the MAPK pathway, in combination with MEKi in NRAS-mutant melanoma cell lines.[30] Clinical trials are currently investigating this combination strategy (eg, NCT04417621).

4.2 | Immunological effects of BRAFi/MEKi treatment in sensitive NRAS-mutant melanoma cells

Next to the effects on growth properties, signal transduction pathways and apoptosis, BRAFi/MEKi combinations also influenced the expression of immune modulatory molecules in NRAS-mutant melanoma cells. These included the upregulation of the immune stimulatory molecule ICOS-L and components of the HLA class I and II APM on the surface of melanoma cells. On the other hand, a trend in downregulation of immune inhibitory molecules, such as PD-L1, was observed. BRAFi/MEKi treatment also caused an increase of the "don't eat me signal" CD47 on the melanoma cell surface. Interestingly, the immune stimulatory effects were promoted by MEKi alone in NRAS-mutant melanoma cells, indicating a connection between the immunological effects and the effects on the MAPK and apoptotic pathways.[31]

These results confirm published data showing that MEKi enhanced the immunogenicity of BRAF-wt tumors.[13–17] Comparable results were also obtained in BRAF-mutant melanoma treated with BRAFi and/or MEKi, suggesting that the underlying mechanisms might be similar.[13] Whether the observed alterations in NRAS-mutant melanoma cells can ultimately cause an activation of T cells and trigger T cell killing remains to be elucidated. However, it was shown that MEKi treatment upregulated HLA molecules on cancer cells including BRAF-mutant melanoma cells, which was accompanied by an increased T cell killing.[31]

These data suggest that the immunological effects of MEKi in NRAS-mutant melanoma may lead to an activation of the immune system, proposing MEKi as a backbone for combined treatment with immunotherapies. The data are in line with two in vivo KRAS tumor models demonstrating an increased T cell infiltration and durable tumor regression upon combined treatment of MEKi and anti-PD-1/-L1.[15,16] Despite these encouraging experimental in vivo data, a clinical trial investigating the MEKi cobimetinib in combination with the anti-PD-L1 antibody atezolizumab in BRAF-wt melanoma did not improve PFS over monotherapy with the anti-PD-1 antibody pembrolizumab (5.5 months for cobimetinib/atezolizumab vs 5.7 months for pembrolizumab).[32] This indicates that perhaps another therapeutic drug in addition to MEKi and immunotherapy is needed.

In addition to the immunological effects of MEKi, the synergistic antiproliferative and proapoptotic activity of combined BRAFi and MEKi provide a strong rationale of combining BRAFi/MEKi with immunotherapy. For instance, the upregulation of CD47 upon BRAFi/MEKi treatment, which presents a "don't eat me signal" to macrophages (our study and[33]), may be exploited therapeutically by additionally blocking CD47 to increase phagocytosis of melanoma cells and thus improve T cell responses.[34] As such, monoclonal antibodies against CD47 showed promising results in clinical trials in solid tumors and hematological malignancies.[24] Ongoing clinical studies on BRAF-mutant metastatic melanoma provided the first indication of prolonged DOR and PFS in patients treated with BRAFi/MEKi combined with PD-1 compared to the double combination, with further improvement after a longer follow-up.[35,36] However, triple combinations also showed a significant increase in grade 3/4 adverse events, so that questions regarding the reduction of toxicities and the best timing of the drugs remain. Our in vitro data suggest a short run-in phase with BRAFi/MEKi double combinations followed by a switch to immunotherapy to avoid BRAFi/MEKi-resistance mechanisms and decrease toxicity. A clinical trial using the panRAFi belvarazumab in combination with the MEKi cobimetinib and the PD-L1 antibody atezolizumab for NRAS-mutant melanoma is currently recruiting and results are eagerly awaited (NCT04835805).

4.3 | Decreased molecular and immunological effects in resistant NRAS-mutant melanoma cells after BRAFi/MEKi treatment

Data from our study showed that the three BRAFi/MEKi-resistant NRAS-mutant melanoma cell lines acquire resistance by counteracting

the MEKi-induced molecular as well as immunological effects. This occurred by upregulation of the MAPK pathway molecule ERK and preventing activation of the mitochondrial apoptosis proteins Bim, caspase-3/9 and PARP. In addition, immune escape mechanisms were promoted by inhibiting the expression of immune stimulatory molecules, such as ICOS-L and components of the HLA APM. A trend to increased expression of immune inhibitory molecules, such as B7-H3 and PD-L1, in the resistant cells was also observed.

In accordance with our findings, similar MEKi resistance mechanisms in NRAS-mutant melanoma cells were shown in other studies, including an increase in pERK5 and an upregulation of prosurvival Bcl-2 family members.[7,37] In addition, an increased expression of the immune inhibitory molecule B7-H3 was associated with a reduced sensitivity to BRAFi and MEKi therapies in BRAF-mutant melanoma cells.[38] Overall, BRAF-mutant and NRAS-mutant melanoma may thus share common resistance mechanisms under treatment with BRAFi and/or MEKi.[39–42]

To overcome BRAFi/MEKi-resistance, NRAS-mutant melanoma cells could potentially be treated with ERK inhibitors. In line with this, co-treatment of MEK and ERK5 inhibitors prevented growth of NRAS-mutant melanoma cells and NRAS-mutant tumors in mice.[7] This was also observed in other cancer cell lines, in which combined MEK and ERK inhibition prevented the generation of resistances as well as overcame resistance to MEKi.[43] Furthermore, if resistance mechanisms to BRAFi/MEKi treatment include an increased expression of immune inhibitory molecules, anti-B7-H3 or anti-PD-L1/PD-L2 antibodies may be used to re-sensitize BRAFi/MEKi-resistant cells and improve T cell responses.[38,44]

5 | CONCLUSION

Our study pursued a systematic approach of treating sensitive and resistant NRAS-mutant melanoma cells with all six available BRAFi and MEKi alone as well as in combination (encorafenib/binimetinib, vemurafenib/cobimetinib and dabrafenib/trametinib) and comparing their effects on growth signaling and apoptotic pathways as well as antigen-presentation and expression of immune modulating molecules. The analyses revealed that all three BRAFi induce stress regulators such as ATF4 and enhance the antitumor cell activity of the three MEKi. The BRAFi/MEKi thus act in a synergistic manner to inhibit proliferation and activate the mitochondrial pathway of apoptosis in sensitive NRAS-mutant melanoma cells. The inhibitors also upregulate immune modulatory molecules, such as ICOS-L, CD47 and HLA class I APM components in the sensitive NRAS-mutant cells. Intriguingly, the BRAFi/MEKi-resistant NRAS-mutant melanoma cells appear to acquire their resistance by counteracting both, the molecular and immunological effects of the inhibitors, for example, by upregulating downstream MAPK pathway molecules, inhibiting mitochondrial apoptosis proteins and downregulating immune stimulatory molecules. Based on these data, we suggest new therapeutic approaches for NRAS-mutant melanoma, including treatment with BRAFi/MEKi in combination with antibodies against CD47. Overall, these data encourage further studies that promote the design of efficient treatment strategies for patients with NRAS-mutant melanoma.

AUTHOR CONTRIBUTIONS

The work reported in the article has been performed by the authors, unless clearly specified in the text. **Lisa Dinter:** Methodology, formal analysis, data curation, writing – original draft. **Paula C. Karitzky:** Methodology, formal analysis, data curation, writing – original draft. **Alexander Schulz:** Formal analysis, data curation, writing – review & editing. **Alexander A. Wurm:** Methodology, formal analysis, writing – review & editing. **Marie-Christin Mehnert:** Methodology, data curation. **Mildred Sergon:** Methodology, formal analysis. **Antje Tunger:** Methodology, formal analysis, data curation. **Mathias Lesche:** Methodology, formal analysis, writing – review & editing. **Rebekka Wehner:** Methodology, writing – review & editing. **Anja Müller:** Methodology, formal analysis, data curation. **Theresa Käubler:** Methodology, data curation. **Heike Niessner:** Methodology, writing – review & editing. **Andreas Dahl:** Resources. **Stefan Beissert:** Resources, writing – review & editing. **Marc Schmitz:** Formal analysis, resources, writing – original draft. **Friedegund Meier:** Conceptualization, formal analysis, writing – original draft, supervision. **Barbara Seliger:** Methodology, formal analysis, resources, writing – original draft. **Dana Westphal:** Conceptualization, methodology, formal analysis, data curation, writing – original draft, supervision.

ACKNOWLEDGMENTS

We would like to thank Maria Heise for excellent secretarial help. We are grateful to Daniela Aust, Gustavo Baretton, Birgit Liebscher, Sylvia Strohbach, Diana Ludwig and colleagues from the Department of Pathology in Dresden for providing excellent infrastructure and IHC staining. The authors of this publication were supported by a Federal Ministry of Education and Research BMBF e:Med Junior Research Alliance Grant (D.W., grant number: 01ZX1913A, R.W., grant number: 01ZX1913C), a NCT scholarship for medical doctoral thesis (L.D.), the Else Kröner Promotionskolleg (P.C.K.), the DFG (grant numbers: SE 581/34-1, SE 581/33-1) (B.S.) and the Deutsche Krebshilfe (grant number: 70113450) (B.S.). Open Access funding enabled and organized by Projekt DEAL.

CONFLICT OF INTEREST STATEMENT

The authors declare not conflict of interest.

DATA AVAILABILITY STATEMENT

The RNA sequencing data are available at Gene Expression Omnibus (GEO) under accession number GSE245262. Further information is available from the corresponding author upon request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Dinter L, Karitzky PC, Schulz A, et al. BRAF and MEK inhibitor combinations induce potent molecular and immunological effects in NRAS-mutant melanoma cells: Insights into mode of action and resistance mechanisms. *Int J Cancer*. 2024;154(6):1057-1072. doi:10.1002/ijc.34807