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Original Article

Aberrant apoptotic machinery confers melanoma dual resistance to BRAF^{V600E} inhibitor and immune effector cells: immunosensitization by a histone deacetylase inhibitor

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Abstract: BRAF^{V600E}-inhibitors (BRAFi; e.g., vemurafenib) and modern immune-based therapies such as PD-1/PD-L1 and CTLA-4 checkpoints blockade and adoptive cell transfer (ACT) have significantly improved the care of melanoma patients. Having these two effective (BRAFi and immunotherapy) therapies raises the question whether there is a rational biological basis for using them in combination. We developed an *in vitro* model to determine whether tumor resistance mechanisms to a small molecule inhibitor of a driver oncogene, and to cytotoxic T lymphocyte (CTL)- and natural killer (NK) cell-delivered apoptotic death signals were exclusive or intersecting. We generated melanoma sublines resistant to BRAFi vemurafenib and to CTL recognizing the MART-1 melanoma antigen. Vemurafenib-resistant (VemR) sublines were cross-resistant to MART CTL and NK cells indicating that a common apoptotic pathway governing tumor response to both modalities was disrupted. Pretreatment of VemR melanomas with a histone deacetylase inhibitor (HDACi) restored sensitivity to MART CTL and NK apoptosis by skewing the apoptotic gene programs towards a proapoptotic phenotype. Our *in vitro* findings suggest that during the course of acquisition of BRAFi resistance, melanomas develop cross-resistance to CTL- and NK-killing. Further, aberrant apoptotic pathways, amenable by an FDA-approved chromatin remodeling drug, regulate tumor resistance mechanisms to immune effector cells. These results may provide rational molecular basis for further investigations to combine these therapies clinically.

Keywords: Vemurafenib, MAPK, BRAF^{V600E} kinase inhibitor, apoptosis, immunotherapy, signal transduction, adoptive cell transfer, HDACi, SAHA, gene expression, sensitization, melanoma, NK cells, TCR transgenic CTL

Introduction

Activating somatic BRAF^{V600E} mutation is found in nearly half of human melanomas and results in sequential phosphorylation and activation of MEK1/2 and ERK1/2 leading to apoptosis resistance, increased survival, and proliferation of melanomas [1, 2]. The BRAF^{V600E}-inhibitor (BRAFi), vemurafenib, selectively binds to BRAF^{V600E} in its active conformation, effectively inhibiting its kinase activity [3, 4], blocking constitutively active ERK1/2 pathway, and inducing apoptosis in melanomas. It induces cell cycle arrest and apoptosis of BRAF^{V600E} harboring cells, inhibits tumor growth and increases survival of experimental animals in melanoma xenograft models [5]. In clinical trials, treatment with optimal concentration of vemurafenib results in complete or partial tumor regression in >80% of BRAF^{V600E} harboring melanoma patients [6]. However, progression free survival is limited due to the development of resistance to vemurafenib over a 6-8 months period [7].

The principal BRAFi escape mechanisms include recovery of ERK1/2 phosphorylation through activating NRAS mutations, paradoxical MAPK activation, COT kinase reactivation, PTEN loss, AKT amplification/mutation, CRAF dimerization, BRAF genomic amplification, BIM

suppression, cyclin D1 induction, and overexpression of various receptor tyrosine kinases (RTK) [8-18]. These bypass mechanisms provide melanomas with the advantage to resist apoptosis and proliferate [19]. Thus, combining vemurafenib with strategies that impede these compensatory/resistance mechanisms is an area of active basic and clinical research [20, 21].

Remarkable clinical responses have also been seen in patients with metastatic melanoma with modern immune-based approaches CTLA-4 and PD-1/PD-L-1 checkpoints blockade, adoptive cell transfer (ACT), as well as earlier experiences with high-dose IL-2 [21]. The proximal mediators of these therapies are tumor-reactive cytotoxic T lymphocytes and natural killer (NK) cells. Various resistance mechanisms to immune-mediated apoptotic death signals have been described including phenotypic changes and effector cell exhaustion [22], functional tolerance, deficiencies in antigen processing and presentation, and mutation or down-regulation of antigenic epitopes. Considering that the immune system eradicates tumors via apoptosis, a more fundamental property of tumors that may limit the efficacy of immunotherapy-resistance to apoptosis- may also be a determining factor [23, 24].

Vorinostat (suberoylanilide hydroxamic acid, SAHA) is the first Food and Drug Administration (FDA) approved histone deacetylase inhibitor (HDACi) with well-established clinical efficacy in cutaneous T-cell lymphoma (CTCL) patients [25], and other cancer cell types. As single agent or combined with other agents, HDACi has some anti-melanoma activity *in vitro* and *in vivo* [26]. Given the aberrant expression profile of apoptosis-associated genes in drug- and immune-resistant tumors, and the ability of SAHA to negatively regulate these resistance mechanisms [26], the efficacy of SAHA in conjunction with vemurafenib and/or immunotherapy in melanoma treatment warrants further investigation.

The availability of these two clinically effective melanoma therapies, which deliver death signals through different mechanisms, presents the obvious opportunity for their use in combination. We developed an *in vitro* model to understand the mechanisms of tumor-acquired resistance to BRAFi and to immune effector cells and whether these resistant pathways intersect. Our *in vitro* model consists of a melanoma cell line M249 which harbors $BRAF^{V600E}$ and expresses the melanoma antigenic epitope $MART\text{-}1_{27\text{-}35}$ in the context of HLA A*0201; this cell line is sensitive to both vemurafenib and to MART-specific CTL (F5 CTL). A second melanoma cell line M238, lacking $MART\text{-}1_{27\text{-}35}$ and HLA A*0201 yet sensitive to both vemurafenib and NK cells, was also used. Serial exposure of M249 and M238 cells to vemurafenib yielded vemurafenib-resistant [M249(VemR), M238 (VemR)] sublines, which were completely resistant to MART CTL despite having unaltered MART-1/A*0201 expression, and NK cell killing, respectively. This cross-resistance incriminates disruption of common intracellular apoptotic machinery by both modalities. Pretreatment of M249(VemR) and M238(VemR) sublines with SAHA restored sensitivity to MART CTL and NK killing, respectively. These results imply that a common apoptosis machinery regulates resistance, which can be restored by an epigenetic modifier, thus, sensitizing melanomas to immune effector cells.

Materials and methods

Cell lines and clones

Human melanoma lines were established from surgical specimens as described [23, 24]. For the generation of CTLR sublines, parental cells were grown in the presence of step-wise increasing numbers of F5 CTLs (E:T 20:1, 40:1, 60:1) for a total of 8 weeks (2-3 weeks for each E:T). Thirty percent to 50% of melanoma cells survived the first cycle of selection (20:1, 2 weeks), percentage of which drastically reduced during subsequent selection cycles until no further killing was observed. Remaining viable melanoma cells were then subjected to two consecutive rounds of limiting dilution analysis. Single cells were propagated and maintained in RPMI-1640 supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS). After immunoselection, cells were maintained in medium containing excess (10:1) F5 CTLs, but were grown in F5 CTL-free medium at least 1 week prior to analysis. Vemurafenib-resistant sublines were established by the growth of vemurafenib-sensitive parental lines in the presence of step-wise increasing concentrations of vemurafenib (0.1-10 μM for 3 month). Cells were grown in Vemurafenib-free medium

at least one week prior to analysis. Cultures were incubated in controlled atmosphere incubator at 37°C with saturated humidity at 0.3-0.5 × 10^6 cells/mL and were used at 50% to 70% growth confluency for each experiment. Cultures were routinely (once/month) checked for mycoplasma contamination (Lonza).

Reagents

Vemurafenib (PLX4032) was purchased from Selleck (Houston, TX). Stock vemurafenib was stored at –80°C at 10 mM in DMSO prior to being used in experiments. MART-1, actin and tubulin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), Millipore (Temecula, CA) and Sigma. Antibodies to various receptor tyrosine kinases (RTK) were purchased from Cell Signaling Technology (Beverly, MA).

ELISA

The amount of cytokine release was measured using ELISA assay kits (eBiosciences) according to manufacturer's instructions.

Transduction of CD8 CTLs with F5 MART-1 TCRα/β retrovirus

Nonadherent population of healthy donor human peripheral blood mononuclear cells (PBMC) was isolated after obtaining informed consent form and Institutional Review Board (IRB) approval and cultured in AIM-V media supplemented with 5% human AB serum, OKT3 (50 ng/mL), and IL-2 (300 IU/mL) for 48 hours. $CD16^+CD56^+$ NK cells and $CD3^+CD8^+$ CTLs were both isolated by EasyStep Negative Selection enrichment kits (Stem Cell Technologies) according to manufacturer's instructions. CTLs were transduced with MSCV MART-1 TCR as described [35, 36]. $CD8^+$ CTLs with more than 95% MART-1 TCRα/β expression and purified NK cells were used in all experiments.

Drug sensitivity and proliferation assay (XTT)

Inhibition of proliferation was assessed using the standard XTT assay kit (Roche, Indianapolis, IN) that measures the metabolic activity of viable cells. The percentage of proliferation (viability) was calculated using the background-corrected reading as follows: % of Control = [(OD of sample wells/OD of untreated cells)] × 100 [37].

Expression analysis of apoptotic genes by quantitative real-time PCR (qPCR)

Samples were analyzed with iQ SYBR Green Supermix using iCycler Sequence Detection System (BioRad). Gene expression analysis was performed using RT^2 profiler apoptosis PCR arrays. Total RNA was extracted from 10^7 cells for each condition with RNeasy mini kit (Qiagen) and quantified by 3.1.2 NanoDrop ND-1000 spectrophotometer. Three micrograms of total RNA was reverse transcribed to first-strand cDNA for 1 hour at 42°C with 200 units SuperScript II RT and 20 μM random hexamer primers. Amplification of 2.5 μL of cDNAs was performed using gene-specific primers.

Cell-mediated cytotoxicity assay

Melanoma cultures were trypsinized for 5 minutes, washed once in cold PBS and labeled with 100 μCi of $Na_2{}^{51}CrO_4$ for 1 hour (37°C/5% CO_2). After 3X washes, 10^4 cells were added to V-bottom 96-well plates and used immediately as described [23, 24]. Percentage of specific ^{51}Cr-release was measured as: % cytotoxicity = (experimental release - spontaneous release)/(total release - spontaneous release) × 100.

Flow cytometric analysis for evaluation of active caspase-3 levels (apoptosis)

Levels of active caspase-3 were evaluated for the measurement of apoptosis [37]. Melanoma cells were treated under the conditions routinely used for PI staining and cell cycle analysis. At the end of the incubation period, the cells were washed once with ice-cold 1 × PBS/0.1% BSA and were resuspended in 100 μl ice-cold 1 × PBS/0.1% BSA. Fifty microliters of cell suspension (containing 2 × 10^6 cells) were aliquoted to each sample and fixed with the perm/fix solution (PharMingen) for 20 min. Thereafter, the cells were washed twice with 1 × perm/wash (PharMingen) solution and stained with the FITC-labeled anti-active caspase-3 antibody for 30 min (light protected). Thereafter, the samples were washed once with 1 × perm/wash solution followed by flow cytometric analysis (Coulter Electronics, Miami, FL). As negative control, the cells were stained with isotype control (pure IgG1) under the same conditions described above.

Immunoblot analysis

A total of 10^7 cells were grown in complete medium (± inhibitors), lysed at 4°C in RIPA buffer [50 mmol/L Tris-HCl (pH 7.4), 1% NP-40, 0.25% sodium deoxycholate, 150 μmol/L NaCl] supplemented with protease inhibitor cocktail (Complete Mini; Roche) and subjected to immunoblot analysis as described [23, 24].

BRAF and NRAS mutational analysis

Genomic DNA extracted from melanoma cell lines was subjected to polymerase chain reaction (PCR) using primer sets that were designed to amplify specific regions of exon 15 (activation domain) of BRAF, and exon 1 and 2 of NRAS. PCR products were first analyzed on agarose gels to determine single band amplicons, then purified and submitted for genotyping analysis (UCLA, Sequencing Core Facility) of both strands by Sanger sequencing. Final sequences were analyzed for novel and known mutations within genomic hot-spots.

Statistical analysis

Assays were set up in duplicates or triplicates and results were expressed as mean ± standard error of the mean (SEM). Statistical analysis and *P* values were calculated by two-tailed paired t test with a confidence interval (CI) of 95% for determination of significance of differences between treatment groups ($P < 0.05$: significant). ANOVA was used to test significance among the groups using InStat 2.01 software.

Results

Cytotoxic effects of BRAFi vemurafenib (PLX4032) and MART TCR-transduced CTL on human melanoma cell lines

The M249 melanoma cell line harbors the $BRAF^{V600E}$ oncogenic mutation, expresses the MART-1 melanoma antigen, is stably transfected with HLA A*0201 allowing M249 melanomas to present the MART-1 epitope in the context of this class-I restricting element and be recognized and killed by $MART\text{-}1_{27\text{-}35}$ TCR-engineered human T cells (F5 CTL). The $BRAF^{V600E}$/MART-1 negative/A*0201 negative melanoma line M238 cannot be recognized and killed by F5 CTL (**Figure 1A**). M249 and M238 melanomas were exposed to various concentrations of the BRAFi vemurafenib (0.0001-10 μmol/L, 72 hr) and an XTT proliferation assay was used to determine inhibition of proliferation and survival rate; both cell lines showed dramatic sensitivity to the cytostatic effect of vemurafenib with an IC_{50} value between 0.1 and 1.0 μmol/L at 72 hr post treatment (**Figure 1B**). Vemurafenib treatment (0.01-2.5 μmol/L, 8-72 hr) induced apoptosis in both cell lines in a dose- and time-dependent manner as measured by active caspase-3 levels, albeit with different kinetics (**Figure 1C**).

Generation of vemurafenib-resistant (VemR) melanomas

Serial exposure of M249 and M238 cells to increasing concentrations of vemurafenib (0.1-10 μmol/L) over a three month period yielded multiple tumor sublines resistant to the cytostatic (**Figure 2A**) and apoptotic (**Figure 2B**, **2C**) effects of vemurafenib, sublines which we term VemR. Sequence analysis showed that M249 (VemR) and M238(VemR) sublines retained $BRAF^{V600E}$ mutation and acquired no secondary NRAS mutations (**Figure 2D**). Protein levels of EGFR, c-KIT, Met, and PDGFRβ were upregulated, and IGFRβ was downregulated in M249 (VemR) sublines. However, in M238(VemR) sublines c-Kit levels were undetectable, EGFR levels were significantly upregulated whereas PDGFRβ levels were reduced. Protein levels of Met and IGFRβ remained unchanged (**Figure 2E**).

Recognition and killing of VemR sublines by immune effector cells

The M249(VemR) sublines could be recognized (as measured by IFN-γ and IL-2 release) (**Figure 3A**) but not killed (using ^{51}Cr-release assay) by F5 CTL (**Figure 3B**), whereas, M238(VemR) sublines were neither recognized nor killed by F5 CTLs. While the M238 cells were sensitive to NK cell-mediated killing, the VemR sublines were resistant (**Figure 3C**). Slight induction in the expression of MART-1 protein levels in the M249(VemR), but not M238(VemR), sublines were observed (**Figure 3D**). These results indicate that the recognition machinery (peptide/MHC complex) of M249(VemR) cells was intact, but these cells have developed cross-resistance to apoptotic death signals delivered by F5 CTL. Similarly, while the MART-1 negative,

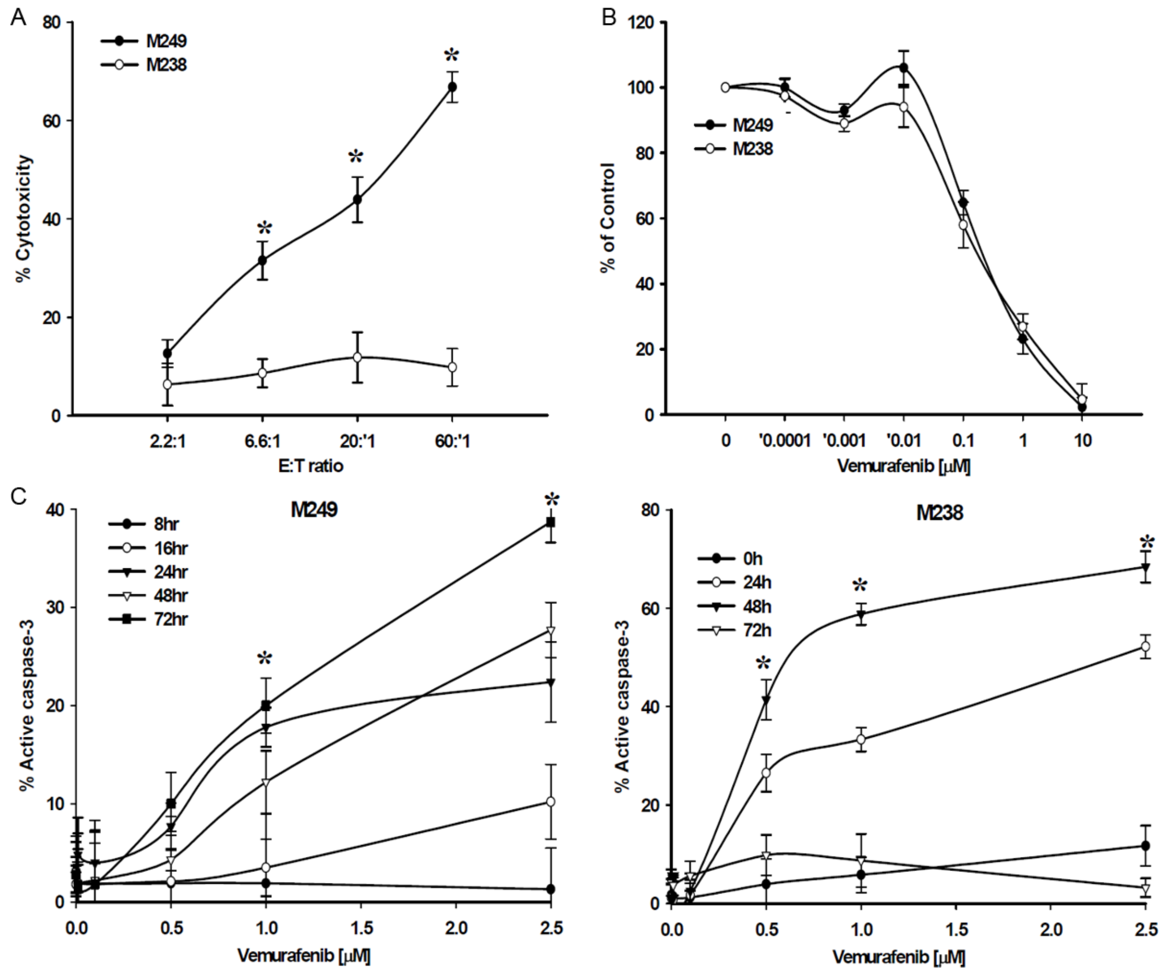

Figure 1. Cytotoxic and cytostatic effects of Vemurafenib and F5 CTL on M249. (A) Cytotoxic activity of F5 CTLs against M249 and M238. ^{51}Cr-labeld tumor cells were co-incubated with F5 CTLs at various E:T ratios in a 6 hr standard ^{51}Cr-release assay. Cytostatic (B) and Apoptotic (C) effects of vemurafenib on M249 and M238. Cells were treated with (B) Vemurafenib (0.0001-10 µmol/L) for 72 hours, or (C) vemurafenib (0.1-2.5 µmol/L) for 8-72 hours and percent proliferation (% of control) and apoptosis were assessed by XTT proliferation assay (IC_{50} 0.1-1.0 µmol/L) and active caspase-3 levels by FACS analysis, respectively. Samples were set up in triplicates and the results are represented as mean ± SEM of three independent experiments. **P* values < 0.05.

A*0201 negative M238 cells were sensitive to NK cells, its VemR derivatives were NK-resistant.

F5 CTL-resistant M249 cells are not sensitive to vemurafenib

We have recently described the generation of several F5 CTL-resistant melanoma sublines and have characterized their mechanism of resistance, which includes constitutive activation of survival pathways and over expression of anti-apoptotic factors [23, 24]. Two sublines of M249 cells completely resistant to F5 CTL [M249(CTLR1, R2)] were generated by serial exposure to these cytotoxic TCR-transgenic $CD8^+$ T cells. F5 CTL resistant cells [M249 (CTLR1, R2)] were exposed to vemurafenib over a range of concentrations (0.1-2.5 µmol/L) for various time points (24-72 hr). These CTL-resistant tumor cells were resistant to BRAFi as measured by active caspase-3 levels (**Figure 4A**, **4B**). Reinforcing this observation of cross-resistance to apoptotic death signals, CTL-resistant melanoma targets were resistant to F5 CTL and vemurafenib in combination (**Figure 4C**).

The HDACi SAHA reverses resistance to immune effector cells

HDACi regulate the expression pattern of apoptotic genes rendering tumors more susceptible

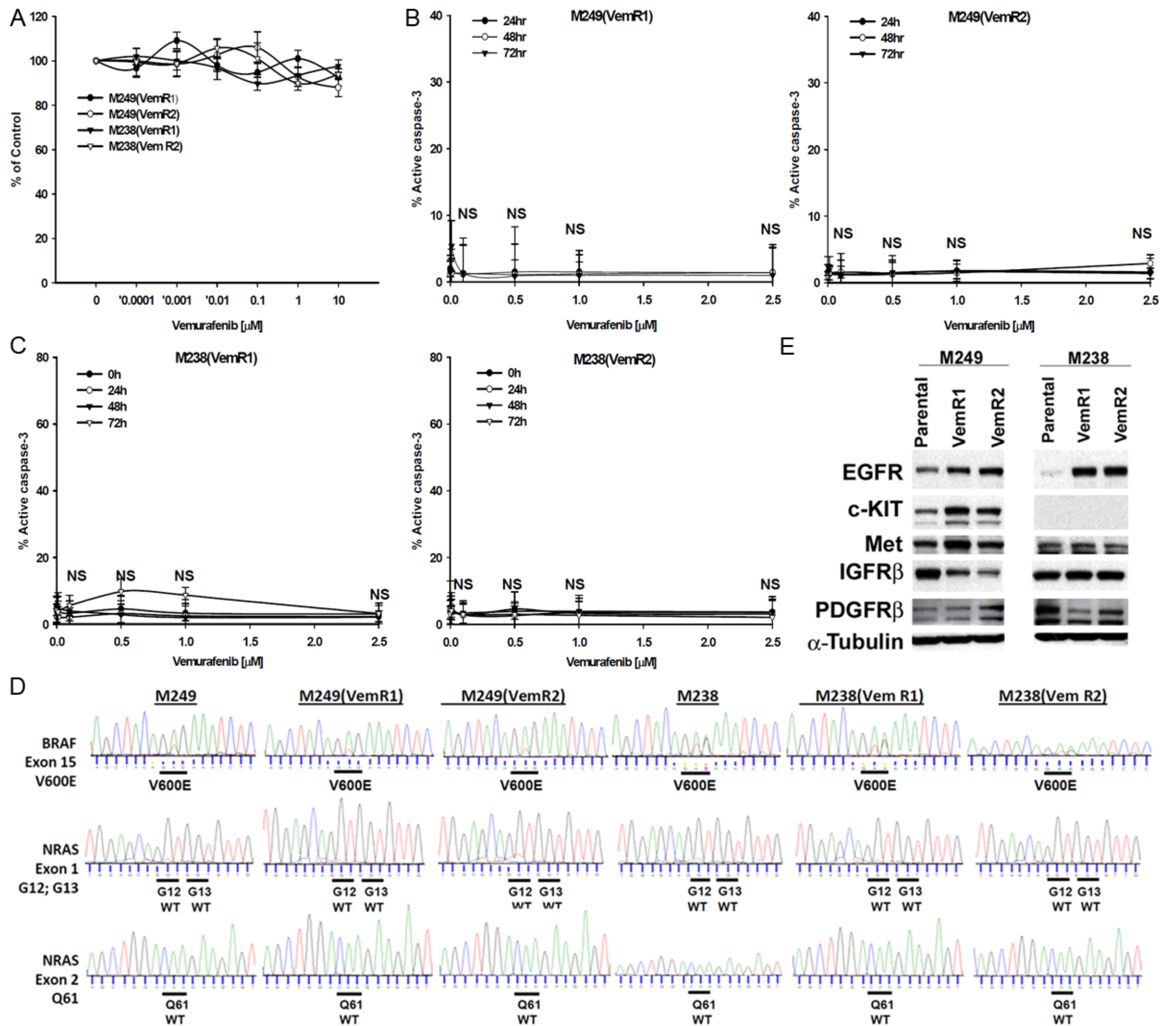

Figure 2. Generation of VemR sublines. M249 and M238 cells were grown in the presence of increasing concentrations of Vemurafenib (0.1-10 μmol/L) for 3 months and subjected to LDA analysis. (A) Cytostatic effects of Vemurafenib on M249(VemR) and M238(VemR) sublines. Cells were grown in the presence of vemurafenib (0.0001-10 μmol/L, 72 hr) and percentage of proliferation was assessed by XTT assay. Samples were set up in triplicates and results are presented as mean ± SEM of three independent experiments. Sensitivity of (B) M249(VemR) and (C) M238(VemR) sublines to vemurafenib-mediated apoptosis. M249(VemR1, VemR2) and M238(VemR1, VemR2) sublines were grown in the presence of Vemurafenib (0.1-2.5 μmol/L) for various time points (24-72 hr) and percent apoptosis was assessed by measuring the levels of active caspase-3. Experiment was repeated three times with similar results. Higher vemurafenib concentrations (up to 10 μmol/L) had no apoptotic effect (not shown). NS: not significant. (D) Mutational Analysis of Vemurafenib-resistant sublines. Genomic amplification and sequence analysis of VemR sublines compared to their parental counterparts. VemR sublines harbor $BRAF^{V600E}$ mutation in exon 15, do not harbor NRAS mutations (wild type NRAS) and no secondary NRAS genomic hot-spot mutations at exon 1 [G12, G13] and exon 2 [Q61] were identified. (E) Expression of various receptor tyrosine kinases (RTK) in VemR sublines by western blot analysis. Results are representative of two independent experiments.

to apoptotic stimuli [26] and overcome BRAFi-resistance [27]. Incubation of M249(VemR) and M249(CTLR) sublines with 1 μmol/L SAHA for 48 hr largely reversed their resistance to F5 CTL killing (**Figure 5A**). We confirmed these findings with two patient-derived MART-specific CTLs (**Figure 5B**). Similarly, pretreatment of M238(VemR) sublines with SAHA reversed their resistance to NK killing (**Figure 5C**). Pretreatment of either of these categories of resistant sublines (VemR and CTLR) with SAHA, however, did not restore their sensitivity to vemurafenib (**Figure 5D**).

Expression of apoptotic genes in M249 sensitive and resistant sublines

Focused apoptosis array qPCR analysis showed that the expression of several groups of apop-

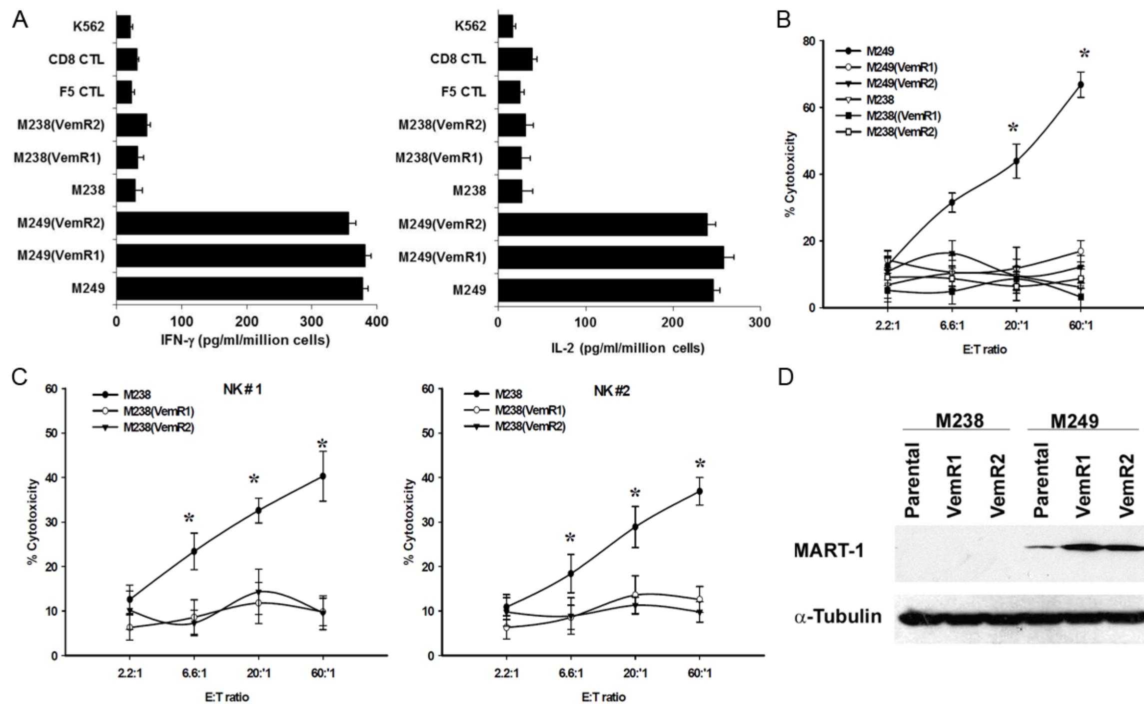

Figure 3. Recognition and killing of M249(VemR) and M238(VemR) sublines by F5 CTLs and NK cells. (A) M249 parental and M249(VemR) sublines express comparable levels of surface MART-1/ HLA A*0201 complex. 10^6 tumors were co-incubated overnight with F5 CTLs at 1:1 E:T ratio. IL-2 and IFN-γ released was measured using ELISA. Samples were set up in quadruplicate; results are presented as mean ± SEM of two independent experiments. F5 CTL, CD8 CTL and K562 cells were used as control. (B) M249(VemR) and (C) M238(VemR) cells exhibit resistance to F5 CTL- and NK-mediated killing. Cells were used in a 6 hr ^{51}Cr-release assay. Samples were set up in duplicate, results presented as mean ± SEM of two independent experiments. **P* values < 0.05. (D) MART-1 protein expression. Whole cell extract (40 μg) were subjected to immunoblotting. Levels of α-tubulin were used for equal loading (n = 3).

tosis-associated genes was altered in M249(VemR) and M249(CTLR) sublines compared to their parental cells. The expression levels of positive regulators of apoptosis (e.g., Apaf-1, BAD, CIDE-A, -B), various caspases (e.g., caspases-2, -3, -5, -6, -8), TNF/TNFR superfamily and death domain proteins (e.g., TNFSF10, TNFRSF10B, 11B), DNA damage (GADD45) were reduced, while the expression levels of several negative regulators of apoptosis (e.g., Bcl-2 members) were increased, consistent with a resistant phenotype (**Table 1A**). Treatment of these resistant sublines (VemR and CTLR) with SAHA increased the expression levels of positive regulators of apoptosis, caspases, TNF/TNFR family and death domain proteins and decreased the expression levels of several negative regulators of apoptosis (**Table 1B**), consistent with a proapoptotic gene program. In comparison, a larger number of apoptotic genes were modified in M238(VemR) (± SAHA) sublines, however, a trend very similar to M249(VemR) was observed (Supplemental Table 1).

Discussion

Remarkable clinical responses in patients with metastatic melanoma can be achieved with BRAFi vemurafenib as well as immune-based approaches such as CTLA-4 and PD-1/PD-L1 checkpoints blockade, and adoptive cell transfer (ACT) [21, 28, 29]. Having two effective therapeutic modalities - small molecule BRAFi and immunotherapy - raises the question whether there is a biological rationale to use them in combination.

Here we report that in the course of acquisition of resistance to BRAFi, VemR sublines were recognized, but not killed, by highly avid and specific melanoma-reactive CTLs. Similarly, CTL-resistant sublines developed cross-resistance to vemurafenib indicating the inversion of a common apoptotic machinery. Exposure to

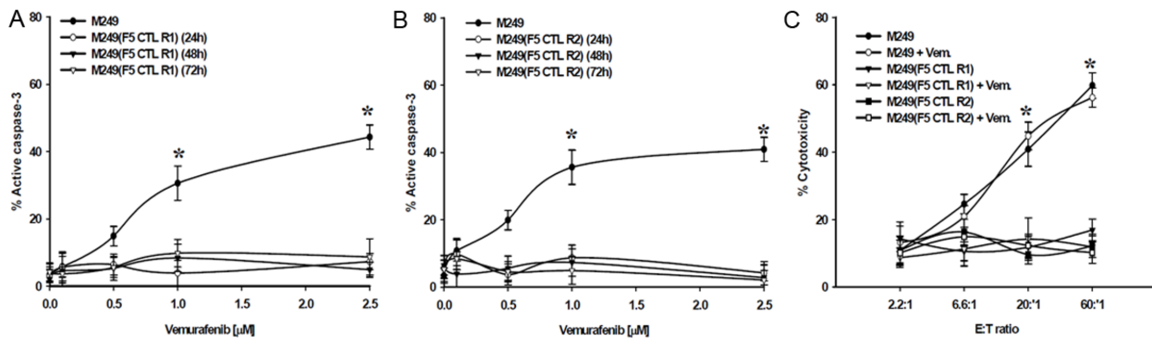

Figure 4. Effects of Vemurafenib on (A) M249(CTLR1) and (B) M249(CTLR2) sublines. Cells were grown in the presence of vemurafenib (0.1-2.5 μmol/L) for various time points (24-72 hr). Percentage of apoptosis was assessed by measuring the levels of active caspase-3. (C) Inability of vemurafenib to immunosensitize. M249(F5 CTLR) sublines were treated with 1 μmol/L vemurafenib (24, 48 hr), washed twice, labeled, and co-incubated with F5 CTLs at various E:T ratios in a 6 hr standard ^{51}Cr-release assay. Only results of 24 hr Vemurafenib pretreatment are shown. Similar results were obtained at 48 hr vemurafenib pretreatment. Samples were set up in duplicates; results are presented as mean ± SEM of two independent experiments. Parental M249 cells (± vemurafenib) were used as control. *P values < 0.05.

SAHA largely restored sensitivity of both M249(VemR) and M249(CTLR) sublines to TCR engineered and to naturally occurring patient-derived MART-1 CTLs by increasing the expression profile of proapoptotic genes. The observation of cross-resistance was further reinforced by using a second melanoma line, M238, and its vemurafenib-resistant derivatives. The MART-1_{27-35}/A*0201 negative M238(VemR) sublines had altered expression profile of apoptotic genes consistent with a resistant phenotype, and developed cross-resistance to NK killing; a phenomenon that was largely reversed by SAHA. These *in vitro* data support the requirement of intact apoptotic machinery in tumors for the full execution of apoptotic death signals delivered by otherwise robust immune effector cells. However, other factors such as tumor heterogeneity and the effects of tumor microenvironment and immune suppressive cytokines might be important contributing factors influencing the outcome of immunotherapy *in vivo*.

The effectiveness of both BRAFi and immune therapies raises the obvious question whether these can be combined. The ability of BRAFi to upregulate MART-1 and gp-100, resulting in increased *in vitro* recognition by TCR transgenic antigen-specific human CTL without negatively affecting the viability and function of T cells is shown [30-32]. However, similar to our results, these reports found vemurafenib unable of sensitizing BRAF^{V600E} melanomas to CTL killing. In contrast, improved efficacy of ACT by vemurafenib in animal models of melanoma, however, has been recently reported [33]. The discrepancy might be explained by use of different cell lines and experimental settings (*in vitro* versus *in vivo*), and contributions of the tumor microenvironment.

Upregulation of the immune checkpoint molecule CTLA-4 on activated T cells and its interaction with CD80/86 blocks T cell activation. The fully humanized mAb ipilimumab blocks this interaction resulting in sustained T cell stimulation [21]. Likewise, the programmed death receptor 1 (PD-1) is another member of the B7:CD28 family of costimulatory molecules that regulate T cell activation, whose ligand (PD-L1) is expressed on melanomas. The human mAb, MDX-1106 (BMS-936558), directed against PD-1 plays a role in breaking tolerance [34]. Similarly, ACT uses antigen-specific autologous T cells in eradicating melanomas [35, 36]. These modern immune therapies have shown remarkable improvement in the treatment of metastatic melanoma [21, 28]. The efficacy of these immune therapies is dependent on the cytotoxic potential of CTL and NK cells [21], and would be predicted to have limited efficacy in patients whose melanomas developed resistance to vemurafenib. In fact, combination of vemurafenib and CTL was ineffective to overcome CTL-resistance [30-32].

Upregulation of various RTKs has been implicated in vemurafenib-resistance [16-20]. Protein expression analysis of various RTKs

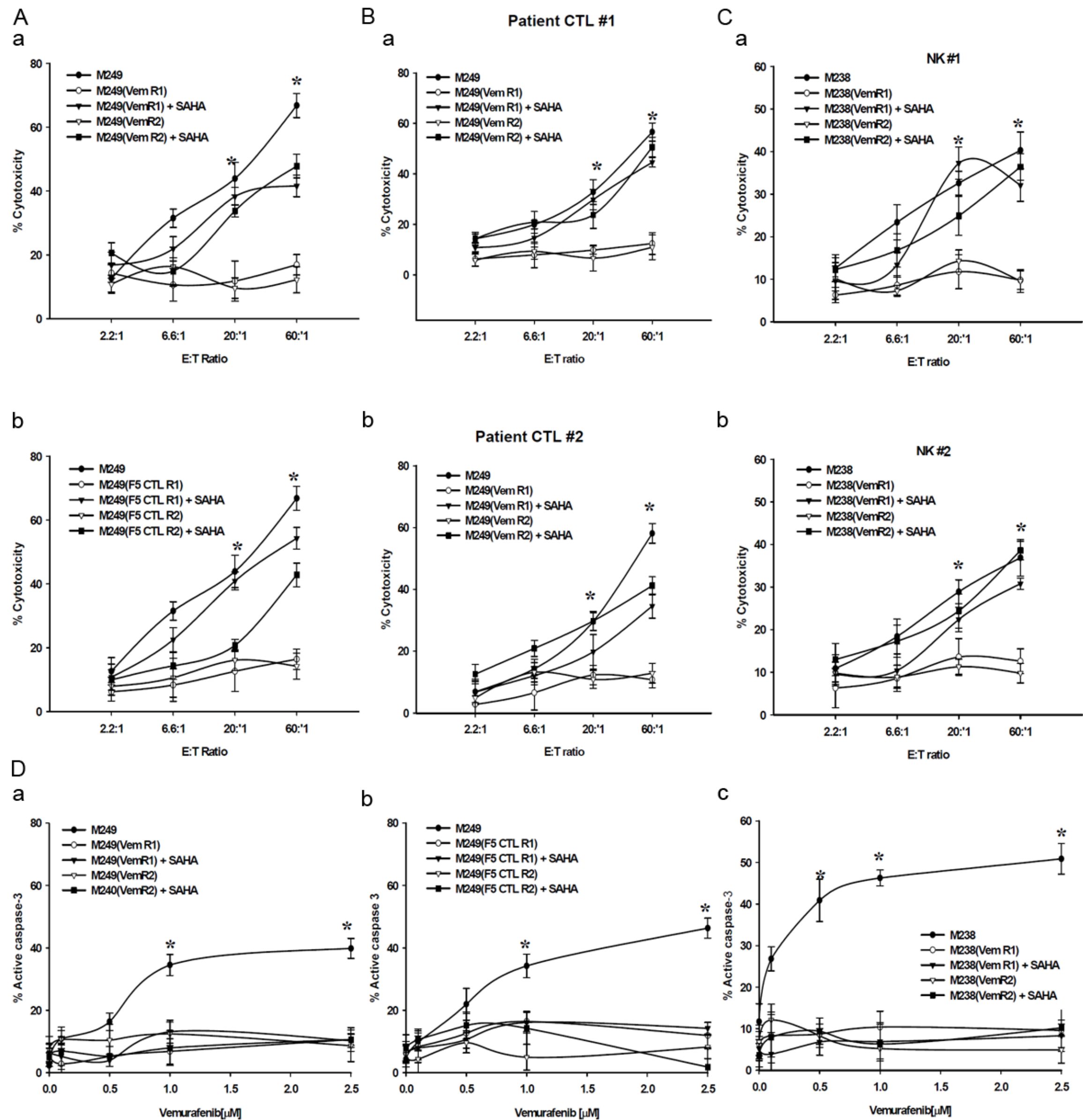

Figure 5. Immunosensitization of M249 VemR, F5 CTLR and M238(VemR) sublines by SAHA. Cells (10^6) were left either untreated or pretreated with SAHA (1 μmol/L-48 hr) and used in ^{51}Cr-release assay using various effectors: (A) MART TCR transgenic CTL, (B) two patient derived MART-1 specific CTLs, (C) two freshly-isolated NK cells. (D) Inability of SAHA to sensitize: (a) M249(VemR), (b) M249(F5 CTLR), (c) M238(VemR) sublines to vemurafenib. SAHA pretreated cells (1 μmol/L-48 hr) were washed, grown in the presence of vemurafenib (0.1-2.5 μmol/L), and apoptosis was assessed by measuring levels of active caspase-3. Untreated M238 and M249 cells were used as control. Samples were set up in duplicates and results are presented as mean ± SEM (n = 4). **P* values < 0.05.

revealed upregulation of EGFR, c-KIT, Met, and PDGFRβ, and downregulation of IGFRβ in M249(VemR) sublines. In contrast, in M238 (VemR) sublines c-Kit levels were undetectable, levels of EGFR were significantly upregulated and PDGFRβ were reduced, while IGFRβ and Met remained unchanged. These data suggest that the two melanoma lines used in our studies utilize different means to develop resistance to vemurafenib. The activation status and contribution of these changes in vemurafenib-resistance, however, requires further scrutiny. Nonetheless, irrespective of the aberrant upstream signaling operative in VemR sublines, activation of any of the above pathways eventually leads to “resistance to apoptosis”. In

Table 1. Differential expression of apoptotic genes in (A) M249(VemR) and (B) M249(CTLR) and their regulation by SAHA as measured by real time qPCR analysis using apoptosis arrays (representative of two independent experiments, genes with > 2.5 fold mRNA changes were considered significant)

A. M249(VemR)

		M249(VemR) vs M249	M249(VemR) + SAHA vs M249(VemR)
	Gene name	Fold mRNA change	Fold mRNA change
Positive apoptosis regulators	TBP53BP2	-4.3	2.5
	APAF-1	-2.6	3.9
	BAD	-3.2	4
	CIDEA	-7.2	9.8
	CIDEB	-3.5	2.4
	FADD	-2.7	3.5
	AKT1	2.6	-1.9
Negative apoptosis regulators	BAG1	8.9	-6
	BCLAF1	3.8	-2.8
	BCL2L1	3.6	-4.2
	BCL2	2.8	-3
	NAIP(BIRC1)	-2.8	2.1
Caspases and caspase activators/regulator	CASP2	3.7	-1.3
	CASP3	-4.1	2.5
	CASP5	5.5	-4.82
	CASP6	-3.2	3.14
	CASP8	-4.8	2.8
	TP73	-3.7	4.6
	CARD6	-6.5	9.8
	CARD8	-10.3	3.8
Death domain and TNF/TNFR family	MGC:45012/TRAP/TRAF2	-3.3	2.1
	CART-1/MLN62/TRAF4	-2.8	3.4
	TNFRSF5/CD40	-1.3	2.6
	TNFSF10/TRAIL	-3.4	7.3
	TNFRSF10B/CD262	-7.2	11.3
	TNFRSF11B/OPG	-5	2.3

B. M249(CTLR)

		M249(CTLR) vs M249	M249(CTLR) + SAHA vs M249(CTLR)
	Gene name	Fold mRNA change	Fold mRNA change
Positive apoptosis regulators	AKT1/PKB	-2	5.3
	BBC2/BCL2L8	-5.1	8.2
	BAK/BAK Like	-3	5.3
	BCL2L4/BAX	-5.3	5
	BCLXs	-3.3	5
	BCL-B/BOO	-2.7	6.6
	CIDE-A	-1.5	2.5
	CIDE-B	-1.6	6.3
	TRP53/LFS-1	-6.4	8.7
	APO-1/ALPS1A	-4.4	3.5
Death domain proteins	FADD/GIG3/MORT-1	-2.8	6.9
	TNFRSF10a/CD261	-2.15	10
	TNFRSF10B/CD262	-4.5	7.6
	TNFRSF11B/OPG	-52.5	57.3
	TNFRSF21/DR6	-1.7	4.5
TNF/TNFR superfamily	APO2L/TNFSF10	-23.5	7.3
	TNFRSF1A/Hs.89862	-2.15	3.5
	MGC:45012/TRAP/TRAF2	-3.1	4
	CAP-1/CD40BP/TRAF3	-1.5	2.5
	CART-1/MLN62/TRAF4	-3	4.5

Caspases and activators	CASP1/ICE/IL1BC	-4.6	13.1
	CASP2/ICH-1L	-2	7.3
	CASP4/ICE(rel)III	-3	2.5
	CASP7(CMH-1/LAP3)	-1.5	2.8
	APAF1	-2.1	4
	FADD/GIG3/MORT-1	-2.8	6.9
Negative apoptosis regulators	DAPK/DKFZP78	3.6	-4.5
	DFF45/DFF1	2.5	-4.4
	DP5/HARAKIRI	6.5	-6.7
	BNIP-2/NIP2	2.8	-2.8
	P73	-3	3.1
	ACC-1/ACC-2 A1	-4.4	3.1
	BCL2L2/BCL-W	4.1	-6.22
	BCL2L3/EAT/MCL1	1.7	-2.5
	LT/TNFB	6.1	-10.1
DNA damage	GADD45/DDIT1	-2.2	3.3

fact, the expression of several gene categories including positive regulators of apoptosis, inducers of apoptosis, death domain and TNF/TNFR family, caspases and regulators, were decreased while anti-apoptosis genes were upregulated in M249(VemR) and M249(CTLR) sublines, which might account for their resistance phenotype. Although a larger number of apoptotic genes were modified in M238(VemR) sublines, however, a trend similar to M249 (VemR) was observed.

Pretreatment of both CTL- and vemurafenib-resistant sublines with clinically achievable low micromolar concentration of SAHA largely restored their CTL-sensitivity. TCR transgenic MART CTL was used as a reliable and reproducible source of specific CTL [36, 37]. Cross-resistance of VemR and CTLR sublines and immune sensitization by SAHA were also confirmed using naturally occurring MART CTL derived from metastatic melanoma patients. The observed cross-resistance was not limited to M249(VemR) sublines. In fact, VemR derivatives of the MART-$1_{27\text{-}35}$/A*0201 negative M238 line, which cannot be targeted by F5 CTLs, developed cross-resistant to NK cell killing. SAHA regulated the expression pattern of several groups of apoptotic genes simultaneously: there was a general trend that anti-apoptotic genes were reduced while positive apoptosis regulators were upregulated indicating that SAHA sensitizes through cooperative collaboration among multiple groups of apoptotic genes. Although, the precise nature of the resistant factor(s) remains to be identified, by lowering the threshold of apoptosis and generating a pro-apoptotic intracellular setting, SAHA dooms the resistant cells to undergo apoptosis upon immune effector (CTL and NK) encounter.

Upon adoption of alternative resistant mechanisms, VemR sublines lose their survival/growth dependency on $BRAF^{V600E}$, which may explain the inability of SAHA to restore vemurafenib sensitivity. CTL and NK killing is independent of $BRAF^{V600E}$, therefore, SAHA-mediated modulation of apoptotic machinery sensitizes both VemR and CTLR sublines to CTL-mediated, but not vemurafenib, killing.

These *in vitro* results suggest that modulation of aberrant apoptotic machinery via inclusion of SAHA to $BRAF^{V600E}$ targeted therapy as adjuvant will overcome the acquired dual resistance to vemurafenib and CTL and will immunosensitize $BRAF^{V600E}$-harboring melanomas to CTL and NK killing. These results may provide a rationale molecular basis for future investigations to combine these therapies.

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Disclosure of conflict of interest

The authors claim no conflicts of interest.

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Supplemental Table 1. Differential expression of apoptotic genes in (A) M238(VemR1) and M238(VemR2) and (B) and their regulation by SAHA as measured by real time qPCR analysis using apoptosis gene arrays (representative of two independent experiments, genes with > 2.5 fold mRNA changes are considered significant)

A. M238(VemR1) and M238(VemR2) compared to M238

		M238(VemR1) vs M238	M238(VemR2) vs M238
	Gene name	Fold mRNA change	
DNA damage	CIDEA (CIDE-A)	12.85	17.4
	CIDEB	-4.17	-4.68
Other	GADD45A (RP5-975D15.1, DDIT1, GADD45)	-7.67	-8.41
Antiapoptosis	AKT1 (AKT, PKB, RAC)	-9.1	-11.78
	BAG1 (RP11-326F20.2, BAG-1, HAP)	-3.79	-6.06
	BAG3 (BAG-3, BIS, CAIR-1)	-5.51	-6.21
	BAG4 (BAG-4, SODD)	-5.99	-6.67
	BCL2L1 (RP5-857M17.3, BCL-XL/S, BCL2L)	2.79	3.14
	BCL2L2 (BCL-W, BCL2-L-2, BCLW)	-6.04	-6.95
	BIRC3 (AIP1, API2, CIAP2)	3.93	3.58
	BIRC8 (ILP-2, ILP2, hILP2)	6.7	7.35
	BNIP1 (TRG8, NIP1, SEC20)	-6.25	-5.1
	BNIP2 (BNIP-2, NIP2)	-6.72	-8.49
	BNIP3 (NIP3)	-2.76	-2.93
	DAPK1 (DAPK)	3.36	3.21
	FAS (RP11-399O19.7, ALPS1A, APO-1)	-3.41	-4.46
	MCL1 (BCL2L3, EAT, MCL1-ES)	-4.15	-4.73
	NAIP (BIRC1, NLRB1, PSINAIP)	-3.41	-5.15
	RIPK2 (WUGSC:H_RG437L15.1, CARD3, CARDIAK)	-3.57	-3.99
	XIAP (RP1-315G1.5, API3, BIRC4)	-4.71	-5.36
Negative regulation	BCL10 (RP11-234D19.2, CARMEN, CIPER)	-3.02	-3.66
	DFFA (H13, DFF-45, DFF1)	-3.95	-4.89
	TP53 (BCC7, LFS1, P53)	-4.07	-4.4
	TP73 (P73)	3.35	2.46
Positive regulation	ABL1 (RP11-83J21.1, ABL, JTK7, BCR/ABL)	-2.62	-3.06
	BAD (BBC2, BCL2L8)	-6.39	-10.39
	BAK1 (BAK, BAK-LIKE, BCL2L7)	-5.05	-6.67
	BAX (BCL2L4)	-6.15	-8.16
	BCLAF1 (BTF, BK211L9.1)	-4.68	-7.67
	BID (FP497)	-2.47	-3.22
	CD70 (CD27L, CD27LG, TNFSF7)	-6.46	-5.9
	FADD (GIG3, MORT1)	-12.13	-14.8
	HRK (DP5, HARAKIRI)	4.34	3.04
	NOD1 (CARD4, CLR7.1, NLRC1)	-2.68	-2.89
	PYCARD (ASC, CARD5, TMS)	10.06	9.33
	TP53BP2 (53BP2, ASPP2, BBP)	-3.13	-3.96
	TRAF2 (MGC:45012, TRAP, TRAP3)	-8.5	-10.23
	TRAF3 (CAP-1, CAP1, CD40BP)	-4.2	-4.99
	TRAF4 (ICE, IL1BC, P45)	-4.13	-4.04
TNF/TNFR Domain Proteins	CD40 (BP50, CDW40, TNFRSF5)	5.4	5.91
	TNFSF10 (APO2L, APO-2L, CD253, TRAIL)	16.95	3.98
	TNFSF8 (CD153, CD30L, CD30LG)	2.55	2.89
	TNFRSF9 (4-1BB, CD137, CDW137)	6.15	7.22
BIR Domain Proteins	BIRC2 (API1, HIAP2, HIAP-2)	-3.93	-4.98
Death domain Proteins	TNFRSF10B (DR5, CD262, KILLER)	-7.41	-7.34
	TNFRSF11B (OCIF, OPG, TR1)	-1.87	-3.71
	TNFRSF1A (CD120A, FPF, MS5)	-6.16	-8.53
	TNFRSF21 (UNQ437/PRO868, BM-018, CD358)	-8.54	-7.86
	TNFRSF25 (RP4-650H14.2, APO-3, DDR3)	2.55	1.59
	TRADD (HS.89862)	-3.49	-4.73

Caspases	CASP1 (ICE, IL1BC, P45)	6.67	5.03
	CASP10 (ALPS2, FLICE2, MCH4)	4.94	4.62
	CASP2 (CASP-2, ICH1, NEDD-2)	-15.96	-24.81
	CASP3 (CPP32, CPP32B, SCA-1)	-6.64	-9.21
	CASP6 (MCH2)	-7.54	-12.73
	CASP7 (RP11-211N11.6, CASP-7, CMH-1)	-4.53	-3.7
	CASP9 (RP11-265F14.3, APAF-3, APAF3)	-2.98	-3.89
Caspase activators	APAF1 (APAF-1, CED4)	-5.58	-5.95
	CARD8 (CARDINAL, DACAR, DAKAR)	-3.6	-4.91

B. M238(VemR1) + SAHA and M238(VemR2) + SAHA compared to M238(VemR1) and M238(VemR2)

		M238 (VemR1) + SAHA vs M238 (VemR1)	M238 (VemR2) + SAHA vs M238 (VemR2)
	Gene name	Fold mRNA change	
DNA damage	CIDEA (CIDE-A)	-4.42	1.44
	CIDEB	3.18	3.21
Other	GADD45A (RP5-975D15.1, DDIT1, GADD45)	3.24	3.65
Antiapoptosis	AKT1 (AKT, PKB, RAC)	10.33	13.51
	BAG3 (BAG-3, BIS, CAIR-1)	4.6	5.03
	BAG4 (BAG-4, SODD)	3	2.51
	BCL2A1 (ACC-1, ACC-2, BCL2L5)	2.96	2.02
	BCL2L2 (BCL-W, BCL2-L-2, BCLW)	3.56	3.77
	BIRC3 (AIP1, API2, CIAP2)	-8.76	-17.8
	BNIP1 (TRG8, NIP1, SEC20)	2.72	2.17
	BNIP2 (BNIP-2, NIP2)	3.27	4.32
	BNIP3L (BNIP3A, NIX)	1.61	2.61
Negative regulation	TP53 (BCC7, LFS1, P53)	1.92	2.98
Positive regulation	ABL1 (RP11-83J21.1, ABL, JTK7, BCR/ABL,)	2.08	2.53
	BAD (BBC2, BCL2L8)	5.84	8.23
	BAK1 (BAK, BAK-LIKE, BCL2L7)	3.93	4.55
	BAX (BCL2L4)	5	5.9
	CD70 (CD27L, CD27LG, TNFSF7)	4.33	4.8
	FADD (GIG3, MORT1)	9.53	11.23
	HRK (DP5, HARAKIRI)	-2.05	3.15
	TRAF2 (MGC,45012, TRAP, TRAP3)	3.27	3.61
	TRAF4 (ICE, IL1BC, P45)	4.44	5.07
Death domain proteins	TNFRSF10B (DR5, CD262, KILLER)	4	3.68
	TNFRSF11B (OCIF, OPG, TR1)	-2.39	-2.63
	TNFRSF21 (UNQ437/PRO868, BM-018, CD358)	4.98	5.79
Caspases	CASP2 (CASP-2, ICH1, NEDD-2)	6.4	9.26
	CASP3 (CPP32, CPP32B, SCA-1)	2.85	3.63
	CASP6 (MCH2)	3.72	6.91
	CASP7 (RP11-211N11.6, CASP-7, CMH-1)	2.69	1.84
	CASP9 (RP11-265F14.3, APAF-3, APAF3)	2.38	3.34
	CFLAR (CASH, CASP8AP1, CLARP)	-5.03	-4.31
Caspase activators	APAF1 (APAF-1, CED4)	6	5.64