

# Molecular Mechanism of MART-1<sup>+</sup>/A\*0201<sup>+</sup> Human Melanoma Resistance to Specific CTL-Killing Despite Functional Tumor-CTL Interaction

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## Abstract

Durable responses in metastatic melanoma patients remain generally difficult to achieve. Adoptive cell therapy (ACT) with *ex vivo* engineered lymphocytes expressing high affinity T-cell receptors (TCR $\alpha/\beta$ ) for the melanoma antigen MART-1<sub>27-35</sub>/HLA-A\*0201 [recognized by F5 cytotoxic T lymphocytes (F5 CTL)] has been found to benefit certain patients. However, many other patients are inherently unresponsive and/or relapse for unknown reasons. To analyze the basis for the acquired resistance and strategies to reverse it, we established F5 CTL-resistant (R) human melanoma clones from relatively sensitive parental lines under selective F5 CTL pressure. Surface MART-1<sub>27-35</sub>/HLA-A\*0201 in these clones was unaltered and F5 CTLs recognized and interacted with them similar to the parental lines. Nevertheless, the R clones were resistant to F5 CTL killing, exhibited hyperactivation of the NF- $\kappa$ B survival pathway, and overexpression of the antiapoptotic genes B cell lymphoma protein 2 (Bcl-2), Bcl-2 related gene (long alternatively spliced variant of Bcl-x gene; Bcl-x<sub>L</sub>), and myeloid cell differentiation 1 (Mcl-1). Sensitivity to F5 CTL-killing could be increased by pharmacological inhibition of the NF- $\kappa$ B pathway, Bcl-2 family members, or the proteasome, the latter of which reduced NF- $\kappa$ B activity and diminished antiapoptotic gene expression. Specific gene-silencing (by siRNA) confirmed the protective role of antiapoptotic factors by reversing R clone resistance. Together, our findings suggest that long-term immunotherapy may impose a selection for the development of resistant cells that are unresponsive to highly avid and specific melanoma-reactive CTLs, despite maintaining expression of functional peptide:MHC complexes, due to activation of antiapoptotic signaling pathways. Though unresponsive to CTL, our results argue that resistant cells can be resensitized to immunotherapy with coadministration of targeted inhibitors to antiapoptotic survival pathways. *Cancer Res*; 71(4); 1406–17. ©2010 AACR.

## Introduction

Early stage melanoma is curable, but advanced malignant melanoma is generally fatal and is rising in incidence. Treatment with single agents, combination chemotherapy, and combination of chemotherapy and immunomodulatory agents all remain unsatisfactory (1), highlighting the urgent need for alternative modalities. The utilization of cancer

vaccines and immunotherapy such as dendritic cells (DC), interleukin 2 (IL-2), and adoptive cell therapy (ACT) using autologous lymphocytes have emerged as the most effective treatments for patients with metastatic melanoma with objective tumor regression in about 50% of patients. Yet, these approaches are limited due to toxic side effects, relative unresponsiveness, and lack of specificity (2).

The transfer of T cell receptor (TCR) genes is required and sufficient to endow recipient T cells with donor cell specificity (3). T cells with genetically engineered TCR recognize target antigen leading to effective immune responses to viral and tumor challenges in *in vivo* and *in vitro* models (4,5). *In vivo*, T cells redirected by TCR gene transfer are fully functional after transfer into mice and expand dramatically upon encountering their cognate antigen (Ag; ref. 6), conferring new Ag specificity, and functional activity to CD8<sup>+</sup> cytotoxic T lymphocytes (CTL; ref. 7). The clinical utilization of TCR engineering is based on the premise that an effective and specific cytotoxic immune response mounted against a defined tumor associated antigen (TAA), presented in the context of appropriate major histocompatibility (MHC) complex, will eradicate resistant tumor cells (2).

The HLA-A\*0201-restricted epitope Melan-A/MART-1<sub>27-35</sub> (AAGIGILTV), is a melanoma-melanocyte differentiation Ag

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The gene expression data have been deposited with the GEO repository with the accession number GSE25436 (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE25436>).

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found in >90% of melanomas, recognized by the TCR complex and serves as an ideal target for CTL-based immunotherapy. Rosenberg and colleagues have designed a retroviral vector encoding high affinity TCR $\alpha/\beta$  chains termed F5 MART-1 TCR (8). Adoptive transfer of genetically engineered T cells into lymphodepleted patients has achieved objective clinical responses in a subset of patients (13%; ref. 9) but the overall result of such treatment has been modest, underlying the need for strategies to optimize immune-based approaches in the clinical treatment of melanoma.

Selective outgrowth of immune-resistant variants (also resistant to other modalities) is a common phenomenon following initial immunotherapy (10); such variants remain a major hurdle in successful cancer therapy. Postulated underlying contributing mechanisms of resistance include: development of functional tolerance (11), antigenic ignorance (low levels of Ag expression; ref. 12), downregulation of Ag presentation associated molecules (13), tolerance induction to target Ag (14), and/or effector cell exhaustion (15). Tumors can also qualitatively or quantitatively alter their Ag expression by mutation/downregulation of Ag epitopes leading to regulation of peptide:MHC interaction and TCR binding (16) or via complete Ag-loss (17).

Compared to other immune-based approaches, administration of F5 CTL to metastatic melanoma patients shows superior efficacy (9). Despite its modest clinical efficacy, a subpopulation of patients, via an elusive mechanism, does not respond to F5 CTL therapy and/or acquires resistance upon long-term therapy. An alternative explanation for the failure of immunotherapy may be independent of the common Ag-loss variants and may be due to inherent/acquired properties of tumors. We hypothesized that development of F5 CTL-resistance is due to tumor's failure to respond to F5 CTL signaling. Further, unresponsiveness of the cells to immunotherapy may be due to hyperactivation of survival pathways and up-regulation of resistant-factors.

Soon after its discovery (18) NF- $\kappa$ B recognition sequences ( $\kappa$ B site) were discovered in promoters of many genes regulating cell differentiation, proliferation, survival, and apoptosis (19). NF- $\kappa$ B activation by various stimuli is partly responsible for transcriptional activation and expression of antiapoptotic B cell lymphoma protein 2 (Bcl-2) and inhibitors of apoptosis (IAP) family members, which rescue tumor cells from apoptotic stimuli delivered by cytotoxic agents as well as by immune effector cells (20–23). NF- $\kappa$ B activation has been observed in melanomas (22–27) as a major mechanism protecting tumor cells from death receptor-induced apoptosis (20–22). Hence, pharmacological inhibition of this pathway is an attractive approach to circumvent resistance in various models (19–23) which has proven successful in enhancing the apoptotic effects of TNF- $\alpha$  and CPT-11 resulting in tumor regression *in vivo* (28).

To recapitulate various aspects of acquired resistance, F5 CTL-resistant (R) clones were generated (29). Others have also investigated possible mechanisms of CTL-resistance *in vitro* (30, 31). Using a battery of functional and biochemical assays, clones were compared to parental (P) cells to examine alterations in F5 CTL effects. To test the above hypotheses, we

investigated: (1) phenotypic and functional properties of R clones (e.g., differences regarding HLA-A\*0201 surface and MART-1 expression, proliferation, ability of F5 CTL to recognize/interact with tumors), (2) immunosensitivity and reversal of immune resistance of the clones (immunosenitization) using specific pharmacological inhibitors, (3) activation status of NF- $\kappa$ B pathway, and (4) expression/functional significance of Bcl-2 members. The results are concordant with our hypotheses and reveal that R clones display different biochemical and functional properties compared to P cells.

## Materials and Methods

### Cell lines and clones

Human melanoma lines were established from surgical specimens as described in ref. 32. For the generation of R clones, P 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, clones 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. Cultures were incubated in controlled atmosphere incubator at 37°C with saturated humidity at  $0.5 \times 10^6$  cells/mL and were used at 50% to 70% confluency for each experiment. Cultures were routinely (once/month) checked for mycoplasma contamination (Lonza).

### Reagents

Mouse anti-Bcl- $\kappa$ L, anti-Mcl-1, and anti-Bcl-2 mAbs and rabbit anti-p65 and anti-p-p65 polyclonal Abs were purchased from Santa Cruz Biotechnology and DAKO, respectively. Mouse anti-p-I $\kappa$ B- $\alpha$  and antiactin mAbs were obtained from Imgenex and Chemicon, respectively. [E-3-(4-butylphenyl sulfonyl) 2-propenitril] (Bay11–7085) was purchased from Calbiochem. Rabbit anti-p-IKK $\alpha/\beta$  [Ser<sup>180/181</sup>] Ab and 2-methoxyantimycin-A3 (2MAM-A3) were obtained from Cell Signaling and Biomol, respectively. Bortezomib, procured commercially, was diluted in dimethyl sulfoxide (DMSO). DMSO concentration did not exceed 0.1% in any experiment.

### Transduction of CD8<sup>+</sup> CTLs with F5 MART-1 TCR $\alpha/\beta$ retroviral construct

Nonadherent population of healthy donor human peripheral blood mononuclear cells (PBMC) was cultured in AIM-V media supplemented with 5% human AB serum in the presence of anti-CD3 Ab (OKT3, 50 ng/mL) and IL-2 (300 IU/mL) for 48 hours. CD8<sup>+</sup> CTLs were isolated by Easy Step Negative selection human CD8<sup>+</sup> T cell enrichment kit according to manufacturer's instructions (Stem Cell Technologies). Using retronectin (Takara Bio) coated 6-well plates, CD8<sup>+</sup> CTLs were

transduced twice with 4 mL of retroviral vector MSCV-MART-1 F5 TCR supernatants by centrifugation at  $1,000 \times g$ ,  $32^\circ\text{C}$  for 10 minutes, cells were incubated for 16 hours at  $37^\circ\text{C}$  incubator with 5%  $\text{CO}_2$ . Next day, procedure was repeated and cells were maintained in AIM-V medium supplemented with 30 IU/mL IL-2 (7–9). Transduction efficiency was evaluated 48 hours post-transduction by MART-1 tetramer staining of  $\text{CD3}^+\text{CD8}^+$  population using antihuman CD3, CD8 Abs (BD Biosciences), and MART-1 tetramer (ELAGIGILTV; Beckman-Coulter) by fluorescence activated cell sorter (FACS) analysis. Minimally activated (30 IU/mL IL-2)  $\text{CD8}^+$  (nontransduced CTLs expressing endogenous TCR) cells were used as control (7–9).

### Immunoblot analysis

Cells ( $10^7$ ) were either grown in complete medium or medium supplemented with various inhibitors and were lysed at  $4^\circ\text{C}$  in radioimmuno-precipitation assay (RIPA) buffer [50 mM Tris-HCl (pH 7.4), 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl] supplemented with one tablet of protease inhibitor cocktail (Complete Mini; Roche). A detergent-compatible (DC) protein assay kit (Bio-Rad) was used to determine protein concentration. An aliquot of total protein was diluted in equal volume of  $2\times$  SDS sample buffer, boiled for 10 minutes, lysates were electrophoresed on 12% SDS-PAGE gels. Immunoblot was carried out as described (33). The relative intensity of bands, hence, relative alterations in protein expression was assessed by densitometric analysis of digitized images obtained from multiple independent experiments.

### Cell-mediated cytotoxicity

Overnight cultures of melanoma cells were trypsinized for 5 minutes, collected, washed once in fresh PBS and labeled with  $100\ \mu\text{Ci}$  of  $\text{Na}_2^{51}\text{CrO}_4$  for 1 hour at  $37^\circ\text{C}$  and 5%  $\text{CO}_2$ . Cells were then washed  $3\times$  in medium,  $10^4$  cells were added to V-bottom 96-well culture plates (Costar) and used immediately in cytotoxicity assay. Effector cells ( $100\ \mu\text{L}$ ) were then added at indicated E:T ratio. Plates were incubated for 5–7 hours at  $37^\circ\text{C}$  and 5%  $\text{CO}_2$ . Following incubation,  $150\ \mu\text{L}$  of supernatant was harvested from each well and counted in a Beckman  $\gamma$ -4000 gamma counter (Beckman). Total  $^{51}\text{Cr}$ -release was determined by lysing target cells with  $50\ \mu\text{L}$  of 10% SDS (Sigma) and collecting  $150\ \mu\text{L}$  for count. Spontaneous release was determined by collecting  $100\ \mu\text{L}$  of supernatant from targets from each treatment in the absence of effectors. Percentage of cell-specific  $^{51}\text{Cr}$ -release was determined as follows: % cytotoxicity = (experimental release – spontaneous release)/(total release – spontaneous release)  $\times 100$ . Data are presented as effector cell-mediated killing at each E:T ratio.

### Immune-complex kinase assay

Alteration in kinase activity of inhibitor of kappa B (I $\kappa$ B) kinase complex (IKK) in R1 cells was assessed by its ability to phosphorylate I $\kappa$ B- $\alpha$  (Ser<sup>32/36</sup>) as described in ref. 34.

### RelA/p65 transcription activity and cytokine release

Transcriptional activity of nuclear p65 and cytokine release were measured by TransAM p65 (Active Motif)

and ELISA assay kits (eBiosciences) according to manufacturer's instructions.

### Application of small interfering RNA (siRNA)

R1 clones were seeded in the wells of 6-well plates in 2 mL antibiotic-free growth medium and incubated until cell confluence reached 50%. A total of  $8\ \mu\text{L}$  (40 nM) of Bcl-x<sub>L</sub>, Mcl-1, or Bcl-2 siRNA or a relevant amount of a control siRNA solution was mixed with  $4\ \mu\text{L}$  of Lipofectamin 2000 (Invitrogen) in OptiMeM solution (Invitrogen). After 6 hours supernatant was aspirated, 2 mL of fresh medium was added and transfection was performed for 80 hours according to the manufacturer's instructions (Santa Cruz Biotechnology; ref. 35). Harvested cells were further subjected to immunoblot for confirmation of specific gene-knockdown and cytotoxicity assay.

### Quantitative real-time PCR (qPCR)

Samples were analyzed in triplicate with iQ SYBR Green Supermix using iCycler Sequence Detection System (BioRad). 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-stranded cDNA for 1 hour at  $42^\circ\text{C}$  with 200 units SuperScript II RT and  $20\ \mu\text{M}$  random hexamer primers. Amplification of  $2.5\ \mu\text{L}$  of cDNAs was performed using gene-specific primers. Internal control for equal cDNA loading was assessed using G3PDH primers. Percentages of expression of each molecule were calculated with the assumption that control samples were considered as 100%.

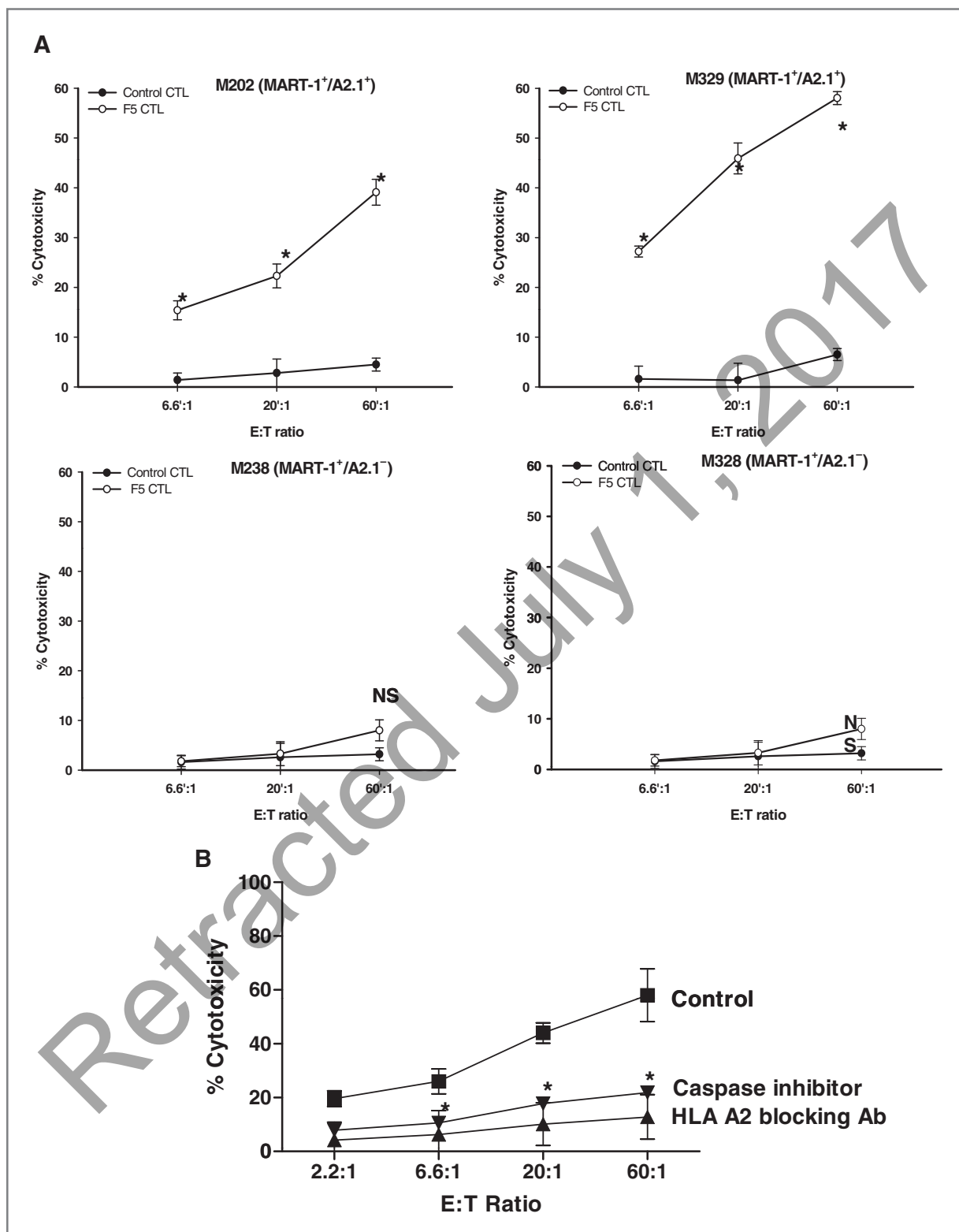
### Statistical analysis

Assays were set up in duplicates or triplicates and results were expressed as mean  $\pm$  standard error of the mean (SEM). Statistical analysis and *P* values were calculated by two-tailed paired *t* test with 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

### F5 MART-1 CTLs kill MART-1<sup>+</sup>/HLA-A\*0201<sup>+</sup> human melanomas in an MHC-restricted manner

$\text{CD8}^+$  CTLs were isolated from PBLs and subjected to two consecutive rounds of transduction with F5 MART TCR  $\alpha/\beta$  retrovirus.  $\text{CD8}^+$  CTLs with >95% MART-1 TCR $\alpha/\beta$  expression (F5 CTLs; Supplementary Fig. S1A) were used in subsequent experiments. F5 CTLs specifically release large quantities of interferon gamma (IFN- $\gamma$ ) upon recognition of surface MART-1/HLA-A\*0201 complex (Supplementary Fig. S1B). We then assessed the efficiency and specificity of F5 CTLs in killing melanomas compared to nontransduced CTLs expressing endogenous TCR (control CTLs). F5 CTLs efficiently kill melanoma targets only when both MART-1 and HLA-A\*0201 are coexpressed on cell surface (M202, M329) but do not kill tumor cells lacking MART-1 and/or HLA-A\*0201 (M238, M328; Fig. 1A). Pretreatment of tumors with either



**Figure 1.** A, specificity and efficacy of F5 CTLs in killing MART-1<sup>+</sup>/HLA-A\*0201<sup>+</sup> melanomas. <sup>51</sup>Cr-labeled tumors were incubated with F5 CTLs at various E:T ratios in 6 hours <sup>51</sup>Cr-release assay. Minimally activated nontransduced CD8<sup>+</sup> were used as control. B, F5 CTL-mediated killing of melanomas is MHC-restricted and mainly via apoptosis. Tumors were left either untreated or pretreated with MHC-I blocking mAb (20 μg/mL for 20 minutes) or pan caspase inhibitor zVAD-fmk (1 μM for 18 hours) and used in <sup>51</sup>Cr-release assay. Samples were set up in duplicates, results are represented as mean ± SEM of two independent experiments. \*, *P* values < 0.05, significant compared with control. NS: not significant.

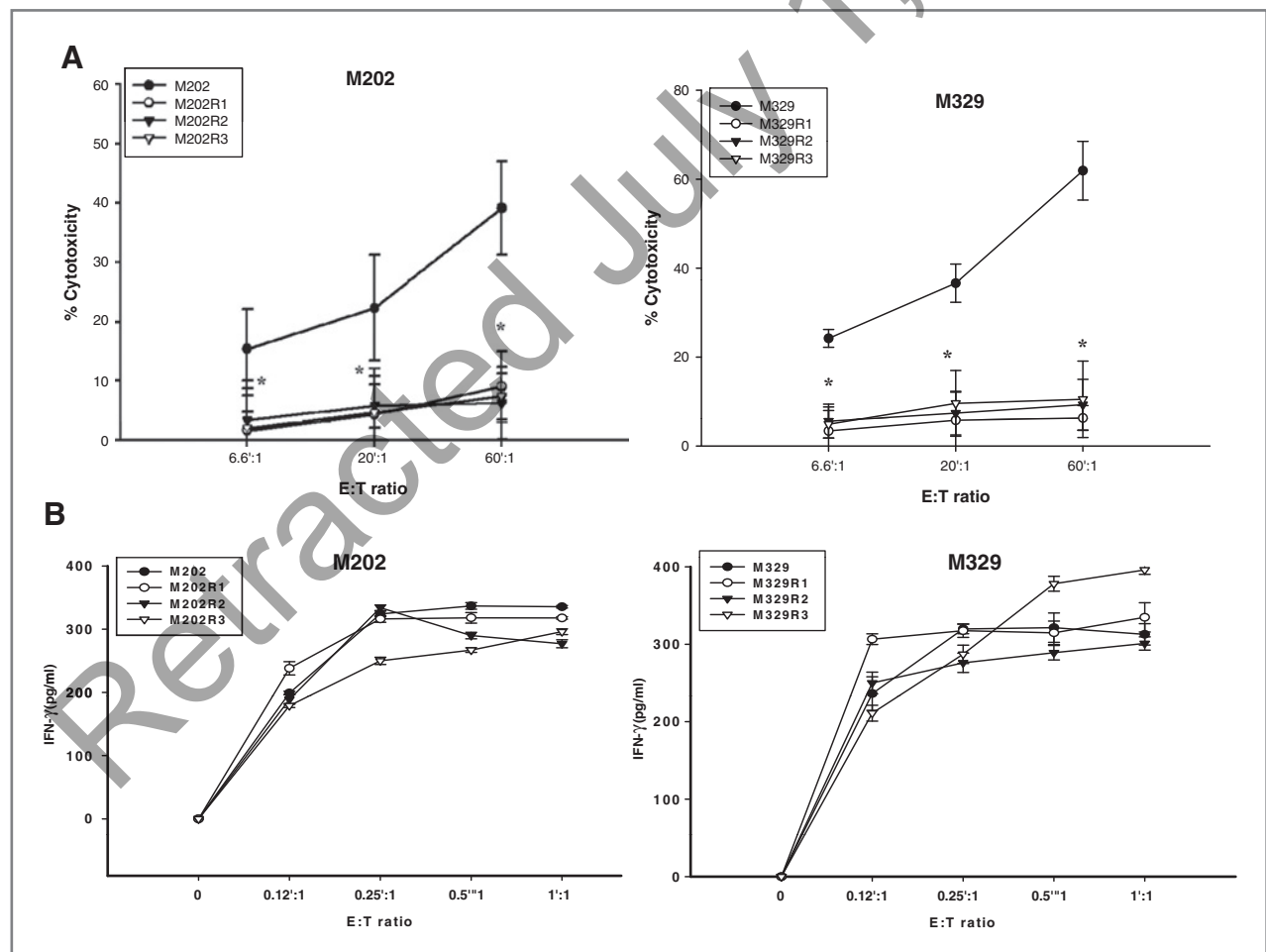


MHC-I blocking mAb or general caspase inhibitor significantly reduced the level of killing (Fig. 1B). To further confirm that F5 CTLs kill targets through apoptosis, levels of active caspase-3 in melanomas were measured. Significant levels of active caspase-3 were accumulated in melanomas after coincubation (6 hours) with F5 CTLs (Supplementary Fig. S1C). These results show that F5 CTLs, in an MHC-restricted fashion, use a caspase-dependent apoptotic pathway in killing MART-1<sup>+</sup>/HLA-A\*0201<sup>+</sup> human melanomas.

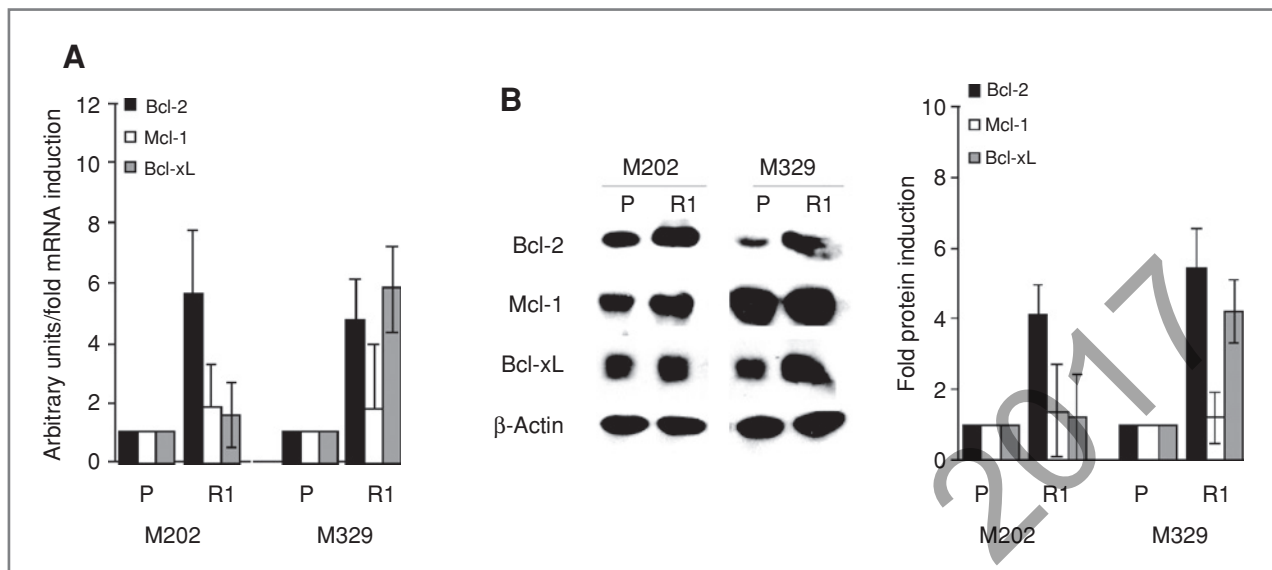
#### Generation of F5 CTL-resistant melanoma cell lines

We established an *in vitro* model of F5 CTL-resistant melanomas using two MART-1<sup>+</sup>/A\*0201<sup>+</sup> melanoma lines (M202, M329), by continuous growth of relatively sensitive parental (P) cells in the presence of varying numbers F5 CTLs for several weeks. Bulk cells exhibited resistance to F5 CTL-mediated killing as early as 4 weeks post selection and by 8 weeks cells were highly resistant (Supplementary Fig. S2A). Subsequently, bulk cultures were subjected to two consecutive

rounds of limiting dilution analysis (LDA) to acquire a homogeneous population. Three R clones were generated from each line, all of which had higher growth rates (114.7% to 148.6%) compared to P lines (Supplementary Fig. S2B), retain the resistant phenotype for about 3–4 weeks in the absence of selective pressure and exhibit significantly higher resistance to F5 CTL-killing (Fig. 2A, Supplementary Fig. S2B). These R clones neither lose surface HLA-A2 nor MART-1 expression (Supplementary Fig. S3A–C). As there are currently no reagents available to quantitate surface MART-1/HLA-A\*0201 complexes, we performed a recognition assay to measure specific cytokine secretion upon F5 CTL recognition of melanomas. The findings show that P and R clones are both capable of triggering F5 CTLs to secrete IFN- $\gamma$  and IL-2, suggesting that they present comparable levels of MART-1/HLA-A\*0201 to F5 CTLs (Fig. 2B, Supplementary Fig. S3D). These results also suggest that the development of R clones is not due to the development of antigen (Ag) or MHC-loss variants.



**Figure 2.** A, R clones exhibit resistance to F5 CTL-mediated killing. Cells were used in a standard 6-h  $^{51}\text{Cr}$ -release assay. Samples were set up in duplicate, results presented as mean  $\pm$  SEM of two independent experiments. B, P and R clones express comparable levels of surface MART-1/HLA-A\*0201 complex.  $10^6$  tumors were coincubated overnight with various E:T of F5 CTLs. IFN- $\gamma$  released was measured using ELISA. Samples were set up in quadruplicate, results are represented as mean  $\pm$  SEM.



**Figure 3.** Overexpression of antiapoptotic Bcl-2 members in R clones. A, 2.5  $\mu$ g cDNA was used in qPCR analysis. Levels of G3PDH were confirmed for equal loading. Results are represented as mean  $\pm$  SD of triplicate samples. B, WCEs (40  $\mu$ g) were subjected to immunoblotting. Levels of  $\beta$ -actin were used for equal loading ( $n = 2$ ).

#### Overexpression of Bcl-2, Bcl-xL, and Mcl-1 in R clones

To gain insight into the mechanism of F5 CTL-resistance observed in R1 clones we performed preliminary microarray analysis which revealed that expression of regulators of apoptosis (among other genes) was higher in M202R1 clones compared to parental M202 (data not shown). Previous findings also established Bcl-xL as an important CTL-resistant factor (23). Thus, we decided to evaluate expression levels of antiapoptotic Bcl-2 members in R clones by quantitative real-time PCR analysis. R1 clones exhibited increased mRNA expression of Bcl-2 (4.8- to 5.6-fold), Bcl-xL (1.6- to 5.8-fold), and Mcl-1 (1.8- to 1.9-fold) (Fig. 3A). Immunoblot analysis confirmed that R clones express higher Bcl-2, Bcl-xL, and Mcl-1 protein levels (~1.2- to 5.4-fold) compared to P cells (Fig. 3B). Interestingly, levels of other pro- and antiapoptotic factors (Bcl-1, Bad, Bid, c-IAP-1, -2, survivin, XIAP, NOXA, Puma) were unaltered (Supplementary Fig. S4A). These results show that R clones express higher levels of antiapoptotic factors, which may explain their unresponsiveness to F5 CTL-mediated apoptosis.

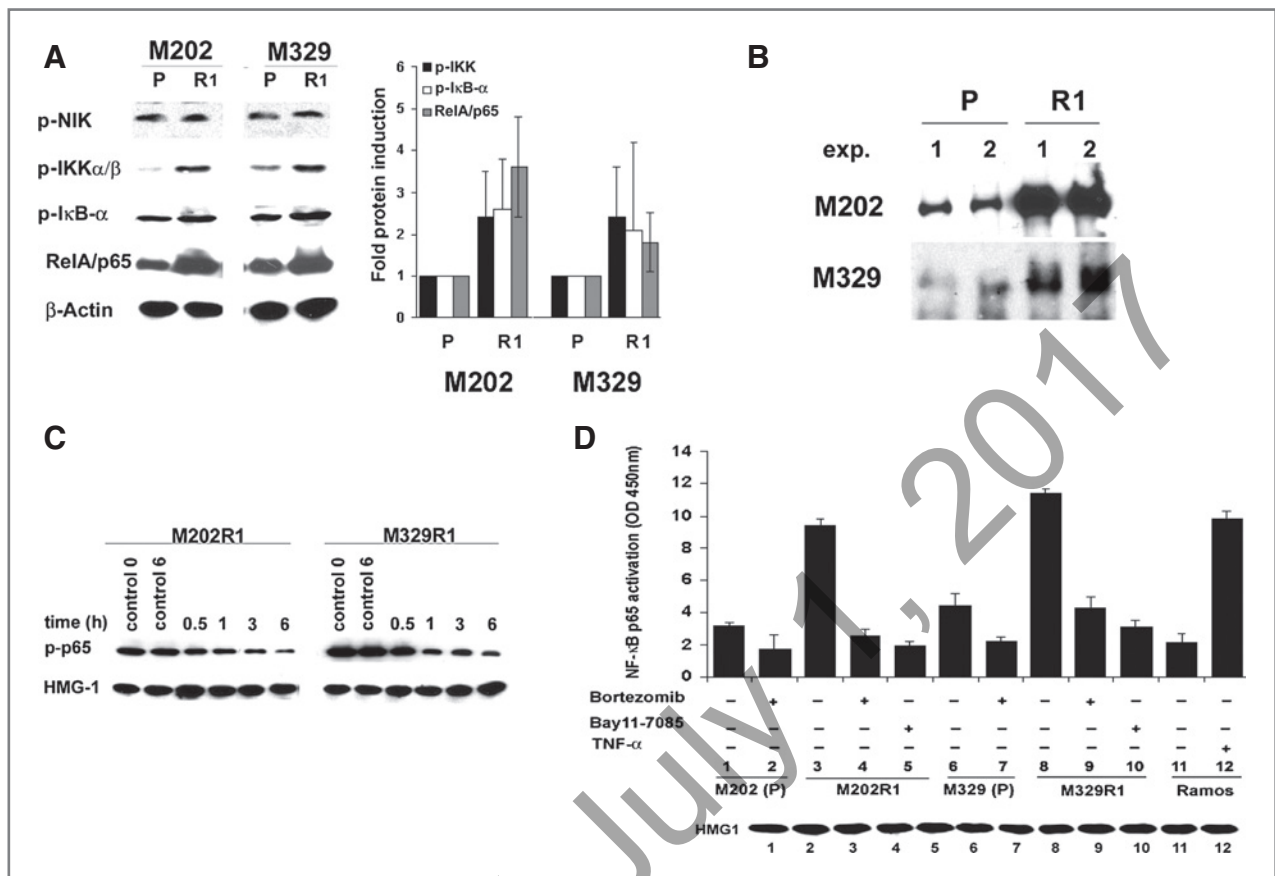
#### Hyperactivation of the NF- $\kappa$ B pathway in R clones

The above findings suggest that dynamics of signaling pathways, which regulate the expression of these resistant-factors, are altered in the clones. The expression of these resistant-factors is regulated mainly through NF- $\kappa$ B pathway (19). We hypothesized that NF- $\kappa$ B is hyperactivated in R clones. Whole cell extracts of P cells and R clones were subjected to immunoblot analysis for components of the NF- $\kappa$ B pathway. The phosphorylation-dependent state of IKK- $\alpha/\beta$ , I $\kappa$ B- $\alpha$  (~2.1- to 3.6-fold) as well as p65 NF- $\kappa$ B subunit was significantly higher in R1 clones than P cells, while there was no difference in p-NIK levels (Fig. 4A). Basal (total) levels of these molecules remained unaffected (data not shown).

To ascertain the observed hyperphosphorylation results in increased activity of the NF- $\kappa$ B pathway, we performed immune-complex kinase assays. R clones showed significantly increased IKK kinase activity as assessed by increased ability of lysates to phosphorylate its specific substrate (I $\kappa$ B- $\alpha$ , 1-50 S<sup>32/36</sup>) (Fig. 4B). This phenomenon was not observed by I $\kappa$ B- $\alpha$ <sup>S32/36A</sup> (data not shown). Thus, increased phosphorylation of signaling molecules culminates in higher kinase activity of NF- $\kappa$ B in R clones. Similarly, nuclear levels of p-p65 were much higher in R1 clones (Fig. 4C). EMSA showed that compared to parental cells NF- $\kappa$ B DNA-binding activity (DBA) is increased in all clones derived from these lines. Specificity of EMSA was confirmed using appropriate controls as bortezomib (36) and another NF- $\kappa$ B specific inhibitor Bay11-7085 (37, 38), which preferentially reduced NF- $\kappa$ B DBA while [2-(2'-amino-3'-methoxyphenyl)-oxanaphthalen-4-one] (PD098059) [activator protein-1 (AP-1) inhibitor] had no effect (data not shown). Also, analysis of p65 transcriptional activity revealed higher activity in the R clones compared to the P cells. Both bortezomib and Bay11-7085 significantly reduced p65 transcriptional activity (Fig. 4D). Collectively, results show that NF- $\kappa$ B pathway is constitutively hyperactivated in R clones and denote the inability of cells to negatively regulate the activity of this pathway in the clones unlike P cells. NF- $\kappa$ B hyperactivation leads to enhanced transcription of its respective antiapoptotic target genes leading to higher immune-resistance of the clones.

#### Direct involvement of NF- $\kappa$ B in resistance

The NF- $\kappa$ B pathway is hyperactivated in R clones leading to overexpression of Bcl-2, Bcl-xL, and Mcl-1 levels, prompting us to investigate whether inhibition of NF- $\kappa$ B pathway or Bcl-2 members can reverse F5 CTL-resistance. Since NF- $\kappa$ B has higher



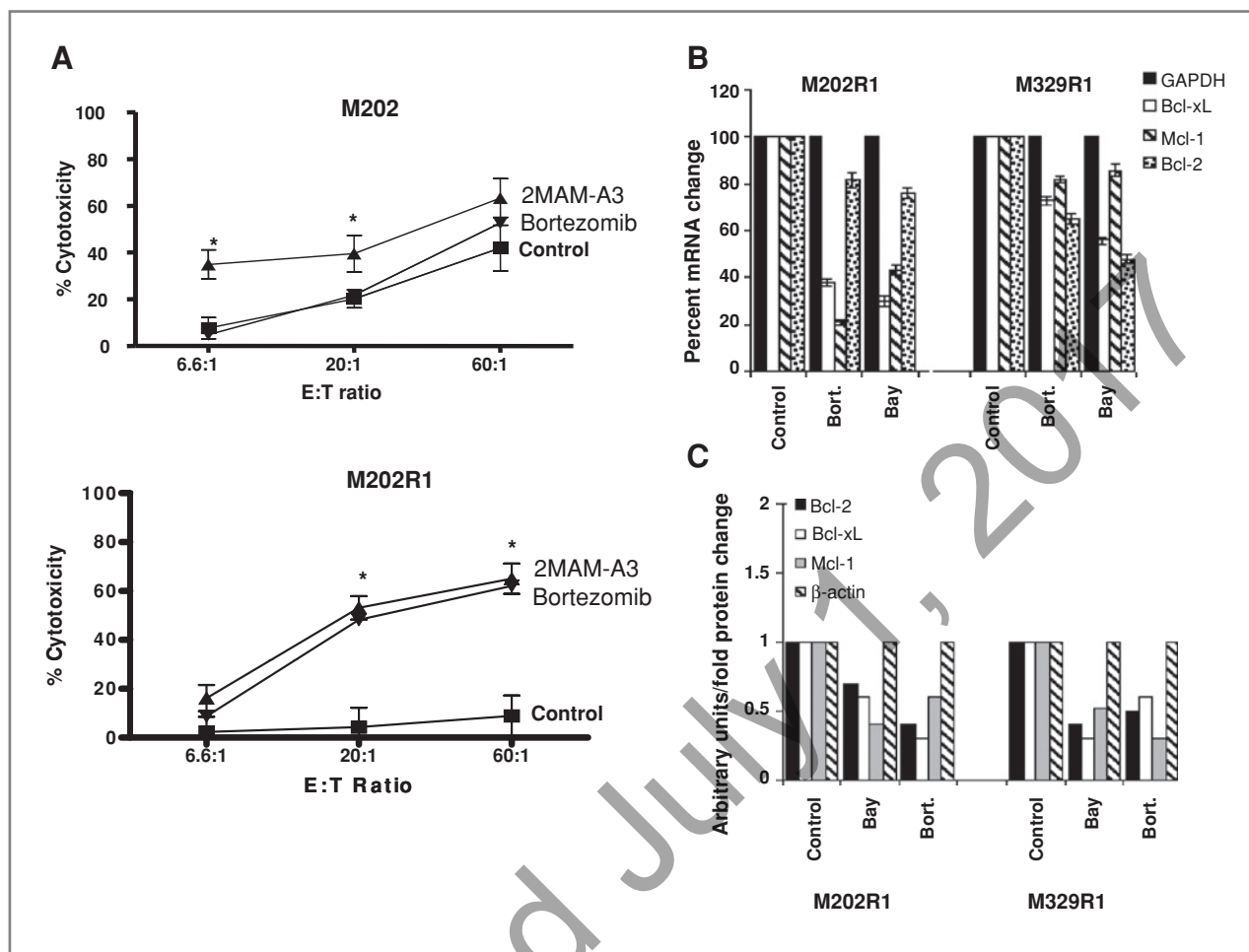
**Figure 4.** Hyperactivation of NF-κB survival pathway in R clones. After overnight growth in 1% FBS, R clones were washed and grown in complete medium. **A**, whole cell extracts (WCE) (40 μg) were subjected to immunoblot using phospho-specific Abs. **B**, IKK immune-complex kinase assay. **C**, 60 μg nuclear lysates (±bortezomib) were subjected to WB for levels of p-p65. **D**, quantification of NF-κB p65 activity in nuclear extracts (10 μg; ±bortezomib, Bay11-7085;  $n = 2$ ). High mobility group 1 (HMG1)- and TNF-α-treated Ramos were used as loading and positive controls, respectively.

activity in R clones, higher concentrations of inhibitors were required for immunosensitization of clones than those used for P cells; nontoxic effective concentrations of which were determined by pilot studies (data not shown). Cells were left either untreated or pretreated with Bay11-7085 and bortezomib. Escalating ratios of F5 CTLs were then added and percentage of cytotoxicity was measured. Bortezomib significantly augmented the cytotoxic effects of F5 CTLs in both P and M202R1 cells, although more dramatic effects were seen in the latter (Fig. 5A). Similar pattern of significant E:T-dependent sensitization of M329R1 was observed. Enhanced cytotoxicity by bortezomib was 5.4- to 7.2-fold and by Bay11-7085 was 3.9- to 5.1-fold, while PD098059 had no effect (Table 1). Similarly, inhibitors sensitized additional R clones, albeit to varying degrees (Supplementary Table 1). The ability of inhibitors to significantly sensitize the R1 clones (3.9- to 7.3-fold) suggests that inhibition of NF-κB pathway can reverse resistance in R clones to low numbers of F5 CTLs (Supplementary Fig. S5A).

Acquisition of resistance by melanoma lines to transgenic F5 CTLs and reversal of resistance by bortezomib was further confirmed using naturally occurring T cells expressing endogenous MART-1 TCR. We obtained naturally occurring MART-

1 specific CD8<sup>+</sup> CTLs from a metastatic melanoma patient. After overnight growth in 300 IU/mL IL-2, at 60:1 E:T ratio, these highly cytotoxic MART-1 specific CD8<sup>+</sup> cells (>95% purity of MART-1 tetramer<sup>+</sup>/CD8<sup>+</sup>) killed both parental M202 ( $38.6 \pm 3.6\%$ ) and M329 ( $57.2 \pm 3.8\%$ ) lines, while M202R1 and M329R1 were resistant ( $P < 0.05$ ). Exposure to bortezomib (600 nM for 6 hours) significantly ( $P < 0.05$ ) enhanced the sensitivity of both M202R1 ( $11.3 \pm 3.7 \rightarrow 42.3 \pm 4.1$ ) and M329R1 ( $12.9 \pm 3.3 \rightarrow 43.6 \pm 2.9$ ) to killing by MART-1 specific CTLs. Similar results were obtained at E:T ratio of 20:1. Also a comparable trend was observed in two independent experiments using bulk (unsorted) patient PBLs (data not shown).

The protective role of overexpressed Bcl-2, Bcl-xL, and Mcl-1 in clones was further confirmed by 2MAM-A3, a Bcl-2 family inhibitor (39) which sensitized them at levels comparable with those achieved in P cells. In M202, 2MAM-A3 augmented F5 CTL-killing by 1.51-fold ( $41.9 \pm 3.3\% \rightarrow 63.3 \pm 4.2\%$ ), whereas it was 7.3-fold ( $8.9 \pm 3.2\% \rightarrow 65 \pm 4.4\%$ ) in M202R1 (Fig. 5A). Similar patterns were observed in M329R1 and other R clones (Table 1, Supplementary Table 1). These findings support the protective role of overexpressed Bcl-2 members against F5



**Figure 5.** A, immunosensitization of P and R clones by inhibitors. Cells ( $10^6$ ) were left either untreated or pretreated with bortezomib (P: 300 nM, R1: 600 nmol/L for 6 hours), 2MAM-A3 (P: 15  $\mu$ g/mL, R1: 35  $\mu$ g/mL for 6 hours), used in  $^{51}$ Cr-release assay. Samples were set up in duplicates and results are represented as mean  $\pm$  SEM ( $n = 2$ ). \*,  $P$  values  $< 0.05$ . Inhibition of expression of antiapoptotic factors by inhibitors. R1 clones were left either untreated or treated with Bay11-7085 (20  $\mu$ g/mL), or bortezomib (600 nmol/L). B, 2.5  $\mu$ g cDNA was used in qPCR using specific primers. GAPDH was used for equal loading. Samples were set up in duplicates, results are represented as mean  $\pm$  SEM ( $n = 2$ ). C, cell lysates were subjected to immunoblotting. Levels of  $\beta$ -actin were used for equal loading. Results are representative of three independent experiments.

**Table 1.** Immunosenitization of human R1 melanoma clones by pharmacological inhibitors. Cells were treated under above conditions (Fig. 5); percentage cytotoxicity and fold potentiation of killing was measured by cytotoxicity assay

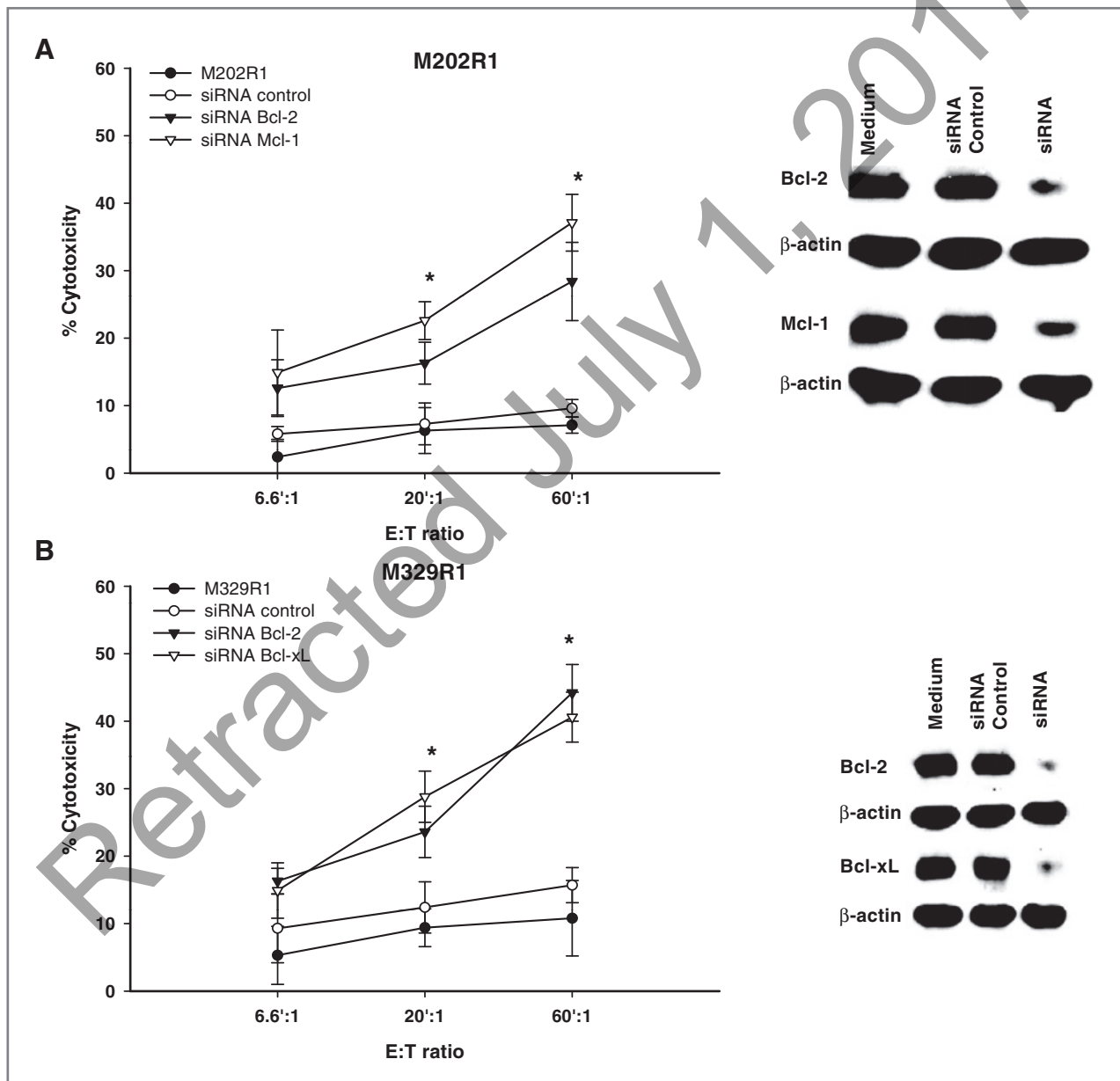
Cell line	Inhibitor	Medium	F5 CTL (%)	F5 CTL (fold)
M202R1	Control	4.9 $\pm$ 1.6	7.8 $\pm$ 3.6	
	Bortezomib	10.8 $\pm$ 2.9	56.1 $\pm$ 3.9	7.2
	Bay11-7085	9.3 $\pm$ 3.1	39.4 $\pm$ 4.3	5.1
	PD098059	5.3 $\pm$ 2.7	9.6 $\pm$ 3.7	0.6
	2MAM-A3	8.9 $\pm$ 3.2	65 $\pm$ 4.4	7.3
M329R1	Control	7.60 $\pm$ 4.4	12.2 $\pm$ 3.7	
	Bortezomib	12.4 $\pm$ 2.5	67.6 $\pm$ 5.5	5.4
	Bay11-7085	13.9 $\pm$ 4.7	47.4 $\pm$ 3.9	3.9
	PD098059	6.6 $\pm$ 3.4	10.7 $\pm$ 4.6	0.8
	2MAM-A3	10.7 $\pm$ 4.1	56.9 $\pm$ 6.3	4.7



CTL-cytotoxicity and that their functional impairment is critical for sensitization. Since inhibitors efficiently sensitized the R clones, we assessed their effect on expression of resistant-factors. As quantitated by qPCR, inhibitors reduced mRNA levels of Bcl-2, Bcl-xL, and Mcl-1 by 1.2- to 6.6-fold (Fig. 5B), and immunoblot confirmed 1.25- to 3.3-fold decrease in their protein levels (Fig. 5C) further supporting the involvement of NF- $\kappa$ B pathway in the expression of resistant-factors. The inhibitors also significantly reduced (48% to 62%) the proliferation rate of R clones (Supplementary Fig. S5B).

#### Direct role of the resistant factors in immune resistance of R1 clones

The direct role of Bcl-2, Bcl-xL, and Mcl-1 overexpression in sensitivity to F5 CTL-cytotoxicity was examined using gene-knockdown strategy. R1 cells were transfected with a pre-determined concentration of siRNA. Transfection efficiency was confirmed by immunoblot analysis, which showed significant decrease in protein levels of these gene products. Specificity of siRNA was established by transfection with siRNA control, which had no regulatory effect on gene



**Figure 6.** Direct role of antiapoptotic Bcl-2 members in immunosensitization. M202R1 (A) and M329R1 (B) cells at 50% confluency were transfected with various siRNAs. Thereafter, cells were harvested and divided into two portions. One portion was further subjected to immunoblot for confirmation of specific gene-silencing and second portion was immediately used in cytotoxicity assays. Samples were set up in duplicates, results are represented as mean  $\pm$  SEM. \*,  $P < 0.05$ .

expression. Next, siRNA transfected cells were used in a cytotoxicity assay. As depicted, specific gene-knockdown resulted in significant potentiation of F5 CTL-cytotoxicity (Fig. 6). Altogether these data support the direct involvement of antiapoptotic Bcl-2 members in CTL-resistance, whereby reduction in their expression levels can reverse the resistant phenotype.

## Discussion

This is the first report on the establishment of F5 CTL-resistant melanoma clones which exhibit a different genotypic profile compared to parental cells. Using various biochemical and functional assays, compared to P cells, R clones express similar levels of surface MART-1/HLA-A\*0201 complexes, yet proliferate at a faster rate, and exhibit significantly higher resistance to F5 CTL-cytotoxicity. The major survival pathway NF- $\kappa$ B is constitutively hyperactivated in the clones leading to overexpression of resistant-factors. Specific pharmacological inhibition of Bcl-2 family members, NF- $\kappa$ B, or gene-silencing of resistant-factors revert the resistant phenotype and clones exhibit sensitivity to F5 CTL-mediated apoptosis.

Successful elimination of tumors by tumor-reactive CTLs depends on temporal/spatial coordination of several processes including recognition and conjugate formation, unidirectional delivery of death signal, recycling of CTLs to kill additional tumors, and AICD. F5 CTLs specifically and efficiently induced apoptosis in melanomas (Fig. 1B) only when the tumors coexpress MART-1 and HLA-A\*0201 and failed to induce cytotoxicity when tumor cells (M328, M238) lack one or both of the recognition units (Fig. 1A). Results also validate the superior tumor reactivity of minimally activated F5 CTLs compared to nontransduced CD8<sup>+</sup> CTLs expressing endogenous TCR. Yet, fully functional F5 CTLs failed to kill R clones. Various approaches were utilized to investigate whether resistance is due to development of Ag-loss variants. Surface HLA-A2 and MART-1 expression analysis showed no differences in P and R clones (Supplementary Fig. S3A–C). Further, assessment of the ability of melanomas to trigger type-I cytokine release revealed that F5 CTLs release comparable levels of IFN- $\gamma$  and IL-2 upon coculture with P and R clones (Fig. 2B, Supplementary Fig. S3D) suggesting that fully functional F5 CTLs efficiently recognize and interact with both P and R clones to a similar extent. Thus, the tumors' recognition unit is intact and development of resistance is distinct from loss of Ag, MHC, and/or effector cell exhaustion.

Most ACT strategies seek to achieve a robust and long-lived CTL response; however, even in face of highly avid and specific CTLs a large percentage of patients does not respond to F5 CTL therapy (2,7–9), resulting in limited clinical responses. The vast majority of anticancer agents as well as CTLs eradicate tumor cells by apoptosis. Tumors, in turn, have adopted various mechanisms to resist apoptosis. Natural apoptosis-inhibitors protect tumors from apoptosis. Expression of these factors is regulated by several signal transduction pathways (NF- $\kappa$ B, MAPK, PI3/AKT) that are constitutively activated/deregulated in resistant tumors (26, 27).

Constitutive activation of NF- $\kappa$ B pathway is implicated in melanoma progression and resistance to therapy (22–27). NF- $\kappa$ B controls transcription of a wide array of target genes including those encoding potent cellular survival/antiapoptotic factors (19). Detailed biochemical analysis of clones revealed hyperactivation of the NF- $\kappa$ B pathway leading to overexpression of its downstream resistant-factors and higher immunoresistance concordant with the protective role of Bcl-2 members in melanoma (40–44). These data suggest that the selective pressure applied by prolonged F5 CTL treatment has coselected for cells (already present in the native culture) expressing higher levels of antiapoptotic proteins, which have lost the capacity to undergo apoptosis in response to F5 CTLs.

R clones exhibited hyperactivated NF- $\kappa$ B pathway. Thus, it was logical to speculate that constitutive hyperactivation of this pathway confers higher immune-resistance (19), hence, its inhibition could potentially avert the resistance; prompting us to evaluate the sensitizing effects of specific NF- $\kappa$ B inhibitor and bortezomib. The extracellular signal-regulated kinase1/2 (ERK1/2) pathway is deregulated in a subset of melanomas (45). To assess its involvement in the resistance we used PD098059, which specifically inhibits ERK1/2 activation (34). Bortezomib blocks the NF- $\kappa$ B pathway and increases treatment efficacy of melanoma *in vivo* and *in vitro* (37, 46, 47) and Bay11-7058 is an irreversible inhibitor of I $\kappa$ B $\alpha$  phosphorylation that inhibits NF- $\kappa$ B DBA (37,38). Bay11-7085 and bortezomib, but not PD098059, efficiently sensitized R clones to F5 CTL-killing (Table 1, Supplementary Table 1) suggesting involvement of NF- $\kappa$ B in immune-resistance. The inhibitors also reduced levels of resistant-factors further suggesting that deregulated signaling culminates in overexpression of antiapoptotic proteins in R clones leading to higher resistance. The protective role of Bcl-2 members was confirmed by using 2MAM-A3 that specifically impairs the function of Bcl-2, Bcl-x<sub>L</sub>, and Mcl-1 (39). Further, functional knockdown of genes encoding these resistant-factors was employed. Gene-silencing and 2MAM-A3 efficiently sensitized the clones, further attesting that higher expression of resistant-factors protects R clones from F5 CTL-induced apoptosis. Hence, aberrations in the normal dynamics of survival pathways upon continuous F5 CTL exposure contribute to acquired resistance while interruption of this pathway sensitizes the R clones. Alterations in gene expression and cell signaling dynamics account for resistance to specific CTL-killing in other models, where specific targeting of aberrant pathways or apoptosis-related proteins can overcome resistance to specific immune effector mechanisms (30–31, 48–50). Contrary to a recent report that NOXA induction by bortezomib accounts for enhanced sensitivity of tumors to CTL attack (50), we have observed no gene regulatory effect of bortezomib on NOXA. The discrepancy can be explained by the usage of different cell types, concentrations, and exposure time of bortezomib. Since Bcl-2, Bcl-x<sub>L</sub>, and Mcl-1 were the only genes whose expression levels were altered in R clones, our efforts were centered on their role in the resistance.

The possibility of pre-existence of resistant cells, with hyperactive signaling pathways, in native culture is not ruled out. In fact, by no criteria thus far has F5 CTL-induced

cytotoxicity in 100% of the cells. It is suspected that the resistant subclones in native culture will dominate sensitive population on long-term F5 CTL treatment as the sensitive cells will be eliminated over time. However, there is no unequivocal evidence, as yet, that this is the dominant mechanism *in vivo* as additional factors [e.g., tumor micro-environment, cytokines (TFG- $\beta$ , IL-10), regulatory T cells, etc.] may contribute to *in vivo* resistance.

The present findings are the first report on the establishment of an *in vitro* model of F5 CTL-resistant human melanoma clones which shows long-term F5 CTL exposure results in altered dynamics of cells to regulate molecular switches leading to constitutive hyperactivation of survival pathways, overexpression of resistant-factors, and increased apoptosis threshold. Accordingly, F5 CTLs fail to exert antimelanoma effects and R clones develop higher CTL-resistance, which may explain treatment-refractory and aggressive nature of clinical immune-resistant melanoma. R clones are still amenable to immunotherapy using specific molecular targeting of the components of deregulated pathway(s). Our data identify several such targets for potential molecular intervention in the treatment of immune resistant melanoma. Studies are underway to validate our *in vitro* findings with freshly derived F5 CTL-resistant specimens and to establish an R mouse model. Our studies also suggest that the development of resistance is

not commonly due to the outgrowth of peptide and/or MHC-loss variants and may be due to alterations in cell signaling dynamics.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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## Retraction: Molecular Mechanism of MART-1<sup>+</sup>/A\*0201<sup>+</sup> Human Melanoma Resistance to Specific CTL-Killing Despite Functional Tumor-CTL Interaction



The article titled "Molecular Mechanism of MART-1<sup>+</sup>/A\*0201<sup>+</sup> Human Melanoma Resistance to Specific CTL-Killing Despite Functional Tumor-CTL Interaction," which was published in the February 15, 2011, issue of *Cancer Research* (1), is being retracted at the request of the University of California, Los Angeles (Los Angeles, CA). Following an institutional review, it was determined that data used in some of the figures cannot be supported; specifically, the 3- and 6-hour bands in Fig. 4C are duplicates of the M329-actin bands in Fig. 4A. To ensure that the research record is correct, the institution has requested that the article be retracted. The authors have been made aware of this retraction.

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1. Jazirehi AR, Baritaki S, Koya RC, Bonavida B, Economou JS. Molecular mechanism of MART-1<sup>+</sup>/A\*0201<sup>+</sup> human melanoma resistance to specific CTL-killing despite functional tumor-CTL interaction. *Cancer Res* 2011;71:1406–17.

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# Cancer Research

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## Molecular Mechanism of MART-1<sup>+</sup>/A\*0201<sup>+</sup> Human Melanoma Resistance to Specific CTL-Killing Despite Functional Tumor–CTL Interaction

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