Proteasome inhibition blocks NF-κB and ERK1/2 pathways, restores antigen expression and sensitizes resistant human melanoma to TCR-engineered CTLs

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Short title: Immunosensitization of human melanoma by bortezomib

Key Words: Targeted therapy, Signal transduction, Melanoma, MART-1, Immunotherapy, NF-κB, ERK1/2 Apoptosis

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List of abbreviations

ACT: adoptive cell therapy
Bay 11-7085: [E-3- (4-butylphenyl sulfonyl) 2-propenentril]
Bcl-2: B cell lymphoma protein 2
Bcl-xL: Bcl-2 related gene (long alternatively spliced variant of Bcl-x gene)
CTL: cytotoxic T lymphocyte
ERK1/2 MAPK: extracellular signal-regulated kinase1/2 mitogen activated protein kinase
FACS: fluorescence activated cell sorter
HLA: human leukocyte antigen
HMG1: high mobility group 1
IFN-γ: Interferon gamma
IL-2: interleukin 2
JNK: cJun NH2-terminal kinase
MART-1: melanoma differentiation antigen recognized by T cells
Mcl-1: myeloid cell differentiation 1
2MAM-A3: 2-methoxyantimycin-A3
NK cells: Natural Killer cells
PBMC: peripheral blood mononuclear cell
RIPA: radioimmuno-precipitation assay
TCR: T cell receptor
Abstract
Adoptive cell transfer (ACT) of ex vivo engineered autologous lymphocytes encoding high-affinity MART-1/HLA-A*0201-specific T-cell receptor (TCR) α/β chains (F5 CTL), densely infiltrate into sites of metastatic disease, mediating dramatic but partial clinical responses in melanoma patients. We hypothesized that MART-1 down-modulation in addition to aberrant apoptotic/survival signaling could confer resistance to death signals delivered by transgenic CTLs. To explore this hypothesis, we established an in vitro model of resistant (R) lines from MART-1+/HLA-A*0201+ F5 CTL-sensitive parental (P) lines under serial F5 CTL-selective pressure. We have recently reported that several melanoma R lines, while retaining MART-1 expression, exhibited constitutive NF-κB activation and over-expression of NF-κB-dependent resistance factors. Another established melanoma cell line M244, otherwise sensitive to F5 CTL, yielded R lines after serial F5 CTL selective pressure which had both reduced MART-1 expression levels, thus, could not be recognized, and were resistant to CTL-delivered apoptotic death signals. The proteasome inhibitor bortezomib blocked NF-κB activity, decreased phospho-ERK1/2, increased phospho-JNK levels, reduced expression of resistance-factors, restored MART-1 expression to sufficient levels, which in combination allowed M244R lines be sensitized to F5 CTL-killing. These findings suggest that proteasome inhibition in immune resistant tumors can restore proapoptotic signaling and improve tumor antigen expression.
Introduction
Metastatic melanoma remains an aggressive malignancy with a poor clinical prognosis (1). Targeted therapies show increasing promise (2, 3), and immune based therapies – IL-2, CTLA-4 blockade, adoptive cell therapy – produce durable complete responses but only in a small percentage of patients (4). Adoptive cell transfer (ACT) of genetically engineered autologous T cells encoding high affinity TCRα/β chains for various melanoma TAAs including MART-1, NY-ESO-1 and gp-100 are being studied clinically (5-7). Despite dense infiltration of metastatic lesions by tumor-reactive CTLs, the clinical efficacy of this approach, while generally improved, is still limited. The acquisition of various resistance/survival mechanisms by tumors in the setting of an otherwise specific and robust anti-tumor immune response may be one limiting factor.

Various mechanisms have been implicated in tumor recurrence or resistance following ACT. These include effector cell exhaustion (8) and development of functional tolerance (9), down-regulation of molecules associated with Ag processing and presentation (10), qualitative (mutations) (11) or quantitative (complete loss) (12) changes of antigenic epitope. Most ACT strategies seek to achieve a robust and long-lived immune response. Given that the immune system kills tumor cells by apoptosis, a more fundamental property of tumors that may limit the effectiveness of immunotherapy – resistance to apoptosis – may also be a dominant mechanism. Several major survival pathways – ERK1/2 MAPK, PI-3/AKT, NF-κB – are frequently deregulated in melanoma enhancing the expression of Bcl-2, Bfl-1/A1, Mcl-1, Bcl-xL that confer apoptosis-resistance. Hence, their inhibition can potentiate melanoma sensitivity to apoptotic-stimuli (13-15).

Bortezomib (Velcade, PS-341) is the first FDA-approved proteasome inhibitor for cancer treatment. In addition to its well-established clinical efficacy in multiple myeloma (16) and mantle cell lymphoma (17), its use has been extended to other cancers (18). As single agent, bortezomib is ineffective in metastatic melanoma (19). Through various mechanisms, mainly inhibition of NF-κB (20) and apoptosis-resistance proteins (21-25), combined with other agents (26,27), bortezomib is effective against melanoma in vivo and in vitro. It also sensitizes melanoma cells to specific CTL attack via modulation of apoptotic machinery (28, 29). Given the constitutive activation of NF-κB and high expression of anti-apoptotic Bcl-2 members in melanoma and the ability of bortezomib to negatively regulate these survival mechanisms (20-29), the efficacy of bortezomib in combination with immunotherapy in the treatment of metastatic melanoma warrants further evaluation.

We have recently reported the establishment of MART TCR-engineered (F5 CTL)-resistant (R) melanoma clones from several MART-1+/A*0201+ lines. When compared to parental cells, R lines expressed comparable levels of surface MART-1+/A*0201 complex, but were resistant to F5 CTL. Targeted therapy using pharmacological inhibitors of NF-κB pathway (bortezomib, Bay11-7085, siRNA) sensitized these resistant cell lines to F5 CTL cytotoxicity (29). Other groups have reported alterations in gene expression and cell signaling dynamics, which account for resistance to specific CTL-killing, whereby targeting the aberrant pathways or apoptotic proteins can overcome resistance (30-32). Two resistant melanoma lines (M244R1, M244R2) generated under continuous F5 CTL exposure were found to have reduced levels of MART-1 antigen expression, in addition to aberrant apoptotic signaling. We investigated: 1) The functional properties of R lines (e.g., HLA-A*0201 and MART-1 expression, recognition and killing by F5 CTLs, status and functional role of NF-κB, ERK1/2, JNK1/2
signaling pathways and resistance-factors), 2) efficacy of bortezomib to sensitize the R lines to F5 CTLs, and MART-1 reactive patient-derived CTLs, and 3) molecular mechanism of sensitization. Our results indicate that during the course of acquisition of resistance, these R lines acquire constitutive NF-κB and ERK1/2 activation and reduced MART-1 expression. Boretzomib blocks these pathways, reduces the expression of anti-apoptotic proteins, and surprisingly restores MART-1 expression, thus, immunosensitizing the R lines.
Materials and Methods

Cell lines. M244 human melanoma cell line was established as described (33). For the generation of M244R lines, parental (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 4 weeks. 30-40% of cells survived the first selection cycle (20:1); their percentage drastically reduced during subsequent cycles until no further killing was observed. Remaining viable cells were subjected to limiting dilution analysis. Single cells were propagated, maintained in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) in excess (10:1) F5 CTLs, but were grown in F5 CTL-free medium at least one week prior to analysis (29). Cultures were incubated in controlled atmosphere at 37°C with saturated humidity at 0.5×10^6 cells/ml and were used at 50-70% confluency for all assays. Cell cultures were routinely authenticated and assayed for mycoplasma contamination.

Reagents. Phospho-IκB-α, p-IKKα/β and p-ERK1/2 Abs were obtained from Imgenex (San Diego, CA), and Cell Signaling (Beverley, MA), respectively. Other Abs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Bortezomib was diluted in DMSO. DMSO concentration did not exceed 0.1% in any experiment.

Transduction of CD8+ CTLs with F5 MART-1 TCRα/β retrovirus. Non-adherent population of healthy donor human PBMCs were cultured in AIM-V media supplemented with 5% human AB serum, αCD3 Ab (50ng/ml) and IL-2 (300IU/ml) for 48hr. CD3+CD8+ CTLs were 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 (5-7, 29). CD8+ CTLs with >95% MART-1 TCRα/β expression were used in all experiments.

Cytotoxicity assay. Melanoma cultures were trypsinized for 5min, washed once in cold PBS and labeled with 100µCi of Na_2^51CrO4 for 1hr (37°C/5%CO_2). After 3X washes, 10^4 cells were added to V-bottom 96-well plates and used immediately as described (29). Percentage of specific 51Cr-release was measured as: % Cytotoxicity = (experimental release-spontaneous release) / (total release-spontaneous release) x 100.

Immunoblot analysis. 10^7 cells were grown in complete medium (± inhibitors), lysed at 4°C in RIPA buffer [50mM Tris-HCl (pH 7.4), 1% NP-40, 0.25% sodium deoxycholate, 150mM NaCl] supplemented with protease inhibitor cocktail (Complete Mini; Roche) and subjected to immunoblot analysis as described (29). The relative intensity of bands, hence, relative alterations in expression was assessed by densitometric analysis of digitized images obtained from multiple independent blots.

MART-1_{26-35} peptide pulsing. 2X10^6/ml cells (in serum-free medium) were pulsed with MART-1_{26-35} (ELAGIGILTV) peptide (10μg/ml-1hr) at room temperature as described (34). Cells were then plated in 10cm petri-dishes at 0.5X10^5/ml in complete medium and used in assays as described in the text.

NF-κB transcription activity and cytokine release. Transcriptional activity of nuclear p65 and cytokine release were measured using TransAM (Active Motif, Carlsbad, CA) and ELISA assay kits (eBiosciences, San Deigo, CA), respectively, according to manufacturer’s instructions.
Quantitative real-time PCR (qPCR). Samples were analyzed in triplicate with iQ SYBR Green Supermix using iCycler Sequence Detection System (BioRad) as described (29).

RT-PCR analysis for MART-1 mRNA expression. Total RNA was extracted from ~10^7 cells with RNeasy mini kit (Qiagen), 3μg was reversed to first-stranded cDNA (1hr-42°C) with SuperScript II reverse transcriptase (200 units) and random hexamer primers (20 μM; Life Technologies, Bethesda, MD). Amplification of 2.5μl of these cDNAs by PCR was performed as described (29). Amplicons were resolved by 2% agarose gel electrophoresis and were of the expected size.

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 Student’s t-test with a confidence interval (CI) of 95% for determination of significance of differences between groups (P<0.05: significant). ANOVA was used to test significance among groups using InStat 2.01 software.
Results

Epitope-specific, MHC-restricted, apoptotic killing of human melanomas by F5 CTL. Human peripheral blood T lymphocytes, transduced to high efficiency (>95%) with a retroviral vector encoding the $\alpha/\beta$ chains of a MART TCR (F5 CTL), recognize the MART-1_{27-35} peptide epitope in the context of HLA A*0201 (29). Using standard $^{51}$Cr-release assay, F5 CTL efficiently and specifically kill MART-1$^+$/A*0201$^+$ M244 melanoma targets but not the M238 (MART-1$^-$/A*0201$^-$) melanoma cell line which lacks both MART-1_{27-35} expression and the proper MHC-I restricting element. Pretreatment with either MHC-I blocking mAb or pan caspase inhibitor zVAD-fmk significantly reduced the level of M244 killing (Figure 1A). Thus, F5 CTL, in an epitope- and MHC-restricted fashion, induce apoptosis in M244 human melanoma cell line.

We developed an in vitro model of F5 CTL-resistant (R) melanoma using the M244 line by continuous exposure of the parental (P) cells to F5 CTL for 4 weeks. Bulk cultures of surviving tumor cells were subjected to limiting dilution analysis (LDA) to acquire homogeneous populations. Through this selection process, two M244R lines were generated; both had higher growth rates compared to P line (R1: %114±4.3, R2: %127±5.1), were highly resistant to F5 CTL-killing (Figure 1B), and retained the resistant phenotype for at least two weeks in the absence of selective pressure (Figure 1C). Bortezomib reversed the resistant phenotype of the M244R lines (Figure 1D) as we have observed previously with other resistant melanoma cell lines (29) and slightly enhanced the sensitivity of the M244 parental line (Supplemental Figure 1A). In both instances, the enhanced F5 CTL sensitivity was MHC-I restricted (Supplemental Fig S1A, B).

M244R lines have reduced MART-1_{27-35} expression and resist F5 CTL killing. The M244P line triggers F5 CTL to secrete IFN-$\gamma$ and IL-2, while R lines fail to do so suggesting that M244R lines present levels of MART-1_{27-35}/A*0201 complex below the threshold to signal transgenic CTLs (Figure 2A). Flow cytometric analysis showed all three lines express relatively similar and high levels of surface HLA A2 (Figure 2B, top). However, MART-1 transcript levels were reduced in both R1 and R2 lines (Figure 2B, middle). Reduction in MART-1 mRNA expression was confirmed at the protein level (Figure 2B, bottom). MART-1_{26-35} peptide pulsing (10$\mu$g/ml-1hr) efficiently restored recognition of R cells by F5 CTLs, as shown by their ability to elicit type I cytokine release (Figure 2C). However, these MART-1_{26-35} peptide-loaded R cells, despite being recognized, retained insensitivity to killing (Figure 2D) suggesting that anti-apoptotic signaling pathways also contributes to resistance. As a control for antigen expression, we used the A375 (MART-1$^-$/A*0201$^+$) and A375 (MART-1) melanoma lines. As expected, the A375 (MART-1) line exhibited sensitivity to F5 CTLs, while A375 was resistant. MART-1_{26-35} loading sensitized the A375 line to F5 CTL-killing at levels comparable to A375 (MART-1) (Figure 2E). Peptide-specific killing of melanoma targets was further confirmed by Flu-specific CTLs. M244 line showed resistance to Flu-specific CTLs; loading the cells with Flu peptide reversed the resistance indicating peptide specific killing. In contrast M244R1 (±Flu peptide) were insensitive to Flu-specific CTLs suggesting that lack of killing of M244R1+Flu peptide is due to adoption of resistant mechanisms and can not be solely explained by defective recognition. Resistance to Flu-specific CTLs was reversed by bortezomib (Supplemental Fig. S2).
**Restoration of MART-1 expression by bortezomib.** M244R1 and R2 cells treated with bortezomib (600nM-6hr) were efficiently recognized by F5 CTL as shown by their ability to elicit F5 CTLs to release IFN-γ (Figures 3A). MART-126-35 loading plus bortezomib, but not MART-126-35 alone, also sensitized the M244R lines to F5 CTLs (Supplemental Fig. S3A, B).

RT-PCR analysis showed increased MART-1 mRNA expression levels in M244R1 and R2 upon bortezomib treatment. Real-time quantitative PCR analysis (qPCR) demonstrated reduced MART-1 expression levels in M244R1 (19.3±4.6%) and M244R2 (22.6±3.8%) (Figure 3B). Bortezomib enhanced the transcript levels of MART-1 in M244R1 and M244R2 lines by 2.06 and 2.4 fold, respectively. Immunoblot analysis further confirmed increased MART-1 protein levels (M244R1: 4.6 fold, M244R2: 5.3 fold) (Figure 3C) by bortezomib, while having no effect on the surface expression of HLA A2 in M244R lines (Figure 3D). These data suggest that bortezomib increases MART-1 expression levels apparently crossing the threshold of effective TCR engagement. To more specifically determine the threshold of MART-1 expression sufficient for efficient recognition and killing, MART-1 knock-down strategy was employed whereby approximately 70-90% reduction in MART-1 protein levels (0.5-1 μg/ml MART-1 siRNA; Supplemental Fig. S4A) reduced the killing and recognition of M244 line to levels comparable to M244R1 (Supplemental Fig. S4B, C).

**Altered anti-apoptotic/survival pathways in M244R lines and their regulation by bortezomib.** Guided by our recent report (29), we confirmed higher activity of NF-κB pathway in M244R lines compared to P line (Figure 4A) leading to over-expression of Bcl-2, Bcl-xL, and Mcl-1. Through NF-κB inhibition, bortezomib reduced the expression levels of these resistance factors. These events occurred concomitantly with an increase in MART-1 protein levels (Figure 4B). Specific pharmacological inhibitors of NF-κB (Bay11-7085), Bcl-2 members (2MAM-A3) or silencing of resistance-factors alone were insufficient to reverse the resistance of M244R lines. Boretzomib either alone or in combination with NF-κB inhibitors reversed the resistance of M244R lines (Supplemental Fig. S5, 6). These results suggest that M244R lines express higher levels of NF-κB–dependent anti-apoptotic factors, which renders them resistant to F5 CTL-killing and bortezomib can reduce their expression levels. These results also suggest that MART-1 restoration is NF-κB–independent as Bay11-7085 was unable to restore MART-1 expression (Figure 4B).

We next examined the regulation of additional survival pathways by bortezomib. We noted that M244R1 and M244R2 lines express relatively high levels of pERK1/2 and low levels of pJNK1/2. Bortezomib treatment reduced pERK1/2 levels (similar to specific ERK1/2 inhibitor PD098059) (35) while increasing pJNK1/2 concomitant with increased MART-1 levels (Figure 4C). While inhibition of ERK1/2 pathway restored recognition of M244R1 line (Figure 4D), it was insufficient to sensitize the M244R1 line to F5 CTL (Figure 4D). Bortezomib (± PD098059) efficiently sensitized M244R1 to F5 CTL-killing (Figure 4E).

Altogether, these results suggest that the activity of prosurvival pathways (NF-κB and ERK1/2) is high, while the activity of proapoptotic pathway (JNK) is low in M244R lines and by altering the dynamics of these
pathways, bortezomib imposes a pro-apoptotic phenotype. Thus, bortezomib sensitizes the M244R lines to F5 CTL-killing via at least two complementary mechanisms; by 1) increasing MART-1 expression (possibly through ERK1/2 deactivation and JNK activation), and 2) NF-κB inhibition and decreasing resistance-factors.

**Bortezomib restores the sensitivity of resistant cells to patient-derived MART-127-35-specific CTL.** Acquisition of F5 CTL-resistance by M244R lines and its reversal by bortezomib was also confirmed using three separate MART-1 specific CD8⁺ CTL populations obtained from melanoma patients. At 60:1 ratio, CTLs killed the P line (ranging between 36.8%-48.8%), while M244R1 was resistant ($p<0.05$). MART-1₂₆-₃₅ loading was unable to enhance M244R1 sensitivity to these CTLs. However, bortezomib pretreatment significantly ($p<0.05$) enhanced their sensitivity to patient-derived MART-1-specific CTL (#1: 13.5±4.1% $\rightarrow$ 43.6 ±4.2%, #2: 10.5±6.1% $\rightarrow$ 36.6±4.2%, #3: 15.5±6.1% $\rightarrow$38.9±5.6%). Bortezomib exposure followed by MART-1₂₆-₃₅ loading also increased the sensitivity of M244R1 to these CTL. The level of M244R1 killing was superior by bortezomib +MART-1 than bortezomib alone with patient MART-1 CTLs #2 and #3 but not with #1 (Figure 5A-C). This observation provides further support that the levels of antigenic epitope expression in the context of HLA A*0201 do not have to vary widely to be insufficient for TCR triggering. These results also suggest that selective pressure applied by F5 CTL exposure results in the generation of melanoma cells resistant to not only TCR transgenic CTLs but also CTLs expressing endogenous TCR. Bortezomib, either alone or combined with MART-1₂₆-₃₅ loading, enhances the sensitivity of M244R1 line to patient-derived MART-specific CTLs similar to transgenic F5 CTLs.
Discussion

Resistance to death signals delivered by TCR transgenic CTLs may prove to be a significant hurdle in ACT for cancer. To understand the mechanisms of resistance, we established an in vitro model of immune resistant melanoma lines. TCR transgenic CTLs, despite efficient recognition and interaction with these R lines (as they retain surface MART-1/A*0201 complex), were incapable of killing these targets. The R lines exhibited constitutive NF-κB activation and over-expressed NF-κB-dependent resistance factors. Pharmacological interruption of the NF-κB pathway rendered them sensitive to F5 CTL-killing (29). We identified additional R lines (M244R1, R2) which could be neither recognized nor killed by F5 CTLs since having down-regulated MART-1 epitope presentation. MART-126-35 peptide loading restored recognition but not killing of M244R lines indicating additional resistance mechanisms. Guided by our recent report (29), we evaluated the activation status and role of NF-κB. Pharmacological NF-κB inhibition and silencing of resistance factors alone were insufficient to restore immunosensitivity. However, bortezomib sensitized M244R lines to transgenic F5 CTLs and patient-derived MART-1-specific CTL lines through altering the dynamics of signaling pathways (inhibition of NF-κB, reduction of phospho-ERK1/2, and induction of phospho-JNK levels), reducing resistance factors and restoring adequate levels of MART-1 expression.

Postulated mechanisms of melanoma evasion from specific CTL attack include morphological changes and cytoskeletal reorganization due to overexpression of ephrin-A1 and scinderin (30), over-expression of ICAM-1 and interference with PTEN/AKT module (31, 32), and expression of anti-apoptotic XIAP and Bcl-2 members (28, 29, 36). Epigenetic modifications of regulatory elements such as MART-1 promoter silencing and low antigen expression due to the release of soluble factors (37) and selection of antigen-loss variants (38) also contribute to immune resistance.

F5 CTLs specifically and efficiently recognize and kill the M244 and A375(MART-1) melanoma lines expressing the relevant peptide in the context of the appropriate HLA restricting element (MART-127-35/A*0201). Consistent with previous reports (29, 32, 37), no changes in intensity of surface HLA A2.1 were observed in M244R lines. The M244R lines were incapable of triggering type I cytokine release from F5 CTLs suggesting that under selective pressure M244R lines express MART-1 levels below the threshold to fully engage F5 CTLs to release IFN-γ and IL-2. MART-1 down-regulation occurred at the transcription and translation levels. Lack of melanoma recognition following specific immune killing due to antigen down-modulation has been previously reported (37, 38). Upon reintroduction of surface MART-1 peptide, F5 CTLs efficiently recognized and interacted with M244R lines. However, this functional interaction led only to cytokine release and not killing of the tumor cells indicating that MART-1 restoration alone is insufficient to kill the R lines. M244R cells have acquired additional resistance mechanisms and the observed immune resistance could not solely be explained by MART-1 down-regulation.

Alterations in cell signaling, independent of MART-1 and A*0201 expression, account for differential sensitivity of primary versus metastatic melanoma to CTL lysis (32). Ample evidence confirms that aberrant antiapoptotic/survival signaling pathways such as MAPK, PI3K/AKT, NF-κB, which are frequently deregulated in melanomas
enhancing the expression of Bcl-2, Bfl-1/A1, Mcl-1, Bcl-xL, play a key role in determining melanoma response to apoptotic stimuli including CTLs (28, 29, 39, 40). Further support for the involvement of acquired or inherent properties of tumors in immunotherapy resistance was shown by melanoma progression despite the presence of significant numbers of antigen-specific CTLs in vivo (41). Biochemical analysis revealed constitutive activation of the NF-κB pathway and over-expression of anti-apoptotic Bcl-2, Bcl-xL and Mcl-1 in the R lines. Using specific inhibitors (Bay11-7085, 2MAM-A3; Figure 6) or specific gene silencing of resistance factors, we show that pharmacological inhibition of NF-κB or Bcl-2 members alone is inadequate to render the M244R lines recognizable by F5 CTLs or reverse their resistance. However, combining these strategies with MART-126-35 peptide loading reversed the resistance. These results plus the inability of Bay11-7085 to induce MART-1, suggest that MART-1 re-expression is independent of NF-κB activation status. However, brief pretreatment (6hr) of the M244R lines with low concentrations of bortezomib restored both the recognition and enhanced the sensitivity of M244R lines to CTL-lysis. Thus, bortezomib alters the resistant phenotype of the M244R lines via at least two complementary mechanisms; by a) inhibiting the NF-κB pathway, hence, reducing the expression of resistance factors, and b) restoring MART-1 expression. The possible involvement of other unidentified regulatory mechanisms triggered by bortezomib is not ruled out.

Interestingly, a relatively modest restoration in MART-1 transcript levels (2.06-2.4 fold) by bortezomib results in high protein expression levels (4.6-5.3 fold). This induction appears to be sufficient to exceed the threshold levels of surface MART-1/A*0201 complex to fully engage the F5 CTL. Further, gene knock-down studies more specifically revealed that approximately 70-90% decline in MART-1 protein levels reduces the killing and recognition of M244 line to levels comparable to M244R1 (Supplemental Fig. S4).

Contrary to a recent report that prolonged bortezomib treatment (20nM-18hr) sensitizes tumor cells to NK killing (42), we show that brief exposure of melanoma targets to bortezomib sensitizes them to specific CTL killing. Several lines of evidence support our observations: 1) We have used highly pure CD3+CD8+ CTL population as effectors, thereby ruling out the existence and involvement of NK cells, 2) bortezomib enhanced the sensitivity of both parental and resistant lines to F5 CTLs; the enhanced sensitivity in both cases was drastically reduced by MHC-I blockade (Supplemental Fig. S1). The discrepancy can further be explained by different experimental settings of bortezomib treatment (20nM-18hr versus 600nM-6hr).

Surprisingly, we observed MART-1 induction by bortezomib. Prolonged bortezomib exposure can impair proteasomal processing and presentation of tumor antigens (42, 43). In our model, MART-1 down-regulation occurred at both protein and transcriptional level implying the involvement of signal transduction pathways and transcriptional regulators. To investigate the underlying molecular mechanism of bortezomib-mediated MART-1 induction, we evaluated the effects of bortezomib on other signaling pathways operative in the R lines. In accordance with previous reports (44, 45), bortezomib reduced p-ERK1/2 levels in M244R lines (similar to PD098059) while inducing pJNK1/2 concomitant with increased MART-1 protein levels. Inhibition of phospho-ERK1/2 enhanced the recognition of M244R lines (cytokine release) but no their sensitivity to F5 CTL. These data suggest that blocking pERK1/2 enhances MART-1 expression. Since NF-κB, but not ERK1/2, regulates the expression of resistant factors, it would be expected that mere blockade of ERK1/2 cannot immuno-
sensitize the M244R lines. Bortezomib (±PD098059) enhanced both the recognition and sensitivity of M244R lines. Others have also reported that specific inhibition of the ERK1/2 pathway significantly increases the transcription of melanoma antigens (MART-1, gp-100, Tyrp-1, Tyrp-2) irrespective of BRAF mutational status (3). Altogether our data argue that the ERK1/2 pathway regulates MART-1 expression. Further investigation is warranted to determine whether bortezomib-mediated MART-1 induction is a direct effect of bortezomib on MAPK pathways as well identifying transcriptional regulators of MART-1 being modulated by bortezomib. Loss or down-modulation of antigenic epitope and amplification of survival pathways are potential mechanisms of melanoma resistance to immune-based therapies (32, 37, 38, 40). Therefore, approaches to derepress MART-1 expression while simultaneously altering the dynamics of aberrant survival pathways represent a novel treatment strategy, which can potentially be used in clinical immunotherapy trials. In this report, we provide evidence that brief exposure of immune resistant melanoma lines to low concentration of bortezomib will not directly induce apoptosis, but through altering gene expression profile (induction of MART-1 and inhibition of survival pathways) will predestine the tumor cells to die upon receipt of apoptotic death signals delivered by TCR transgenic CTLs.
Acknowledgements: The authors wish to acknowledge Drs. Mitchell Kronenberg (La Jolla Institute for Allergy and Immunology, CA), Benjamin Bonavida, Manuel Penichet and Antoni Ribas (UCLA, Jonsson Comprehensive Cancer Center) for the critical review of the manuscript. We also thank Dr. Steven Rosenberg (NCI, Surgery Branch) for the kind gift of MSCV-MART-1 F5 TCR vector, Drs. Bijay Mukherji, Arvind Chhabra, Nitya Chakraborty (University of Connecticut, School of Medicine), Begonya Comin-Anduix and Thinle Chodon (UCLA, Hematology/Oncology) for A375, A375(MART-1) lines and MART-1- and Flu-specific CTLs.

Grant support: This project was supported in part by the Melanoma Research Foundation Career Development Award (ARJ), Samuel Waxman Foundation, Joy and Jerry Monkarsh Fund, W. M. Keck Foundation, the National Institutes of Health through grants 1R21CA149938 (ARJ), R01CA129816, PO1 21B-1088934.
References


Figure Legends:

Figure 1. A. Epitope-specific and MHC-restricted apoptotic killing of M244 cells by F5 CTLs. M244 cells were left untreated or pretreated with MHC-I blocking mAb (20μg/ml-20min) or zVAD-fmk (1μM-18hr) and incubated with F5 CTLs at various E:T ratios in 6hr standard $^{51}$Cr-release assay. M238 (MART-1/A*0201) and non-transduced CD8+ cells were used as specificity and CTL controls, respectively. B. M244R lines exhibit resistance to F5 CTL-killing as measured by $^{51}$Cr-release assay. C. Persistence of the resistant phenotype of R lines. M244R lines were cultured for 1 and 2 weeks in the absence of F5 CTLs and used in cytotoxicity assay. D. Bortezomib (600nM-6hr) sensitizes the M244R lines to F5 CTLs. Results are represented as Mean± SEM of duplicate samples (n=2). *P values <0.05 significant.

Figure 2. A. M244P and M244R lines express different levels of surface MART-1/HLA-A*0201 complex. $10^6$ cells were coincubated overnight with F5 CTLs (various E:T ratios) and the amount of IFN-γ and IL-2 released was measured by ELISA. B. Top: Surface expression of HLA-A2. Cells were stained with either IgG isotype control or FITC-labeled anti-HLA-A2 mAb (filled histograms) and subjected to FACS analysis. Middle: MART-1 gene expression. RT-PCR analysis of MART-1 mRNA expression levels in M244 and M244R lines. Levels of GAPDH were used for equal loading (n=2). Bottom: MART-1 protein levels. Whole cell extracts (WCE; 40μg) of melanomas were subjected to immunoblot using MART-1 specific Ab. Levels of β-actin were used for equal loading (n=2). The A375 (MART-1+) and A375(MART-1) (stably transduced to express MART-1) melanoma lines were used as negative and positive controls, respectively. MART-126-35 peptide-pulsing restores recognition but not killing of R lines. R lines were pulsed with MART-126-35 peptide (10μg/ml-1hr) and used in C. recognition (IFN-γ and IL-2 ELISA assays), D. cytotoxicity assay ($^{51}$Cr-release). E. A375(MART-1/ A*0201) [±MART-126-35 (10μg/ml-1hr)], and A375(MART-1) lines were used as control. Results are represented as Mean±SEM of duplicate samples (n=3). *P values <0.05 significant.

Figure 3. Restoration of MART-1 expression by bortezomib. M244R1 and M244R2 cells (10^6/ml) [± bortezomib (600nM-6hr)] were used in: A. recognition (IFN-γ release) B. MART-1 mRNA expression analysis (RT-PCR and qPCR). The MART-1+ A375 and A375(MART-1) melanoma lines were used as negative and positive controls, respectively. qPCR values are normalized to GAPDH levels (n=2). C. Immunoblot analysis for MART-1 expression levels, D. Expression of surface HLA A2. Untreated (dashed black lines) or bortezomib treated (dashed gray lines) cells were stained with FITC-anti human A2 mAb and subjected to FACS analysis (n=2). *P values <0.05 significant.

Figure 4. Regulation of signaling pathways by bortezomib. P and R lines (± bortezomib, Bay11-7085, PD098059) were used for: A. Quantitation of NF-κB p65 transcriptional activity (10μg nuclear extracts). PD098059 was used as control. B. Expression analysis of apoptotic proteins. C. Regulation of pERK1/2, pJNK1/2 and MART-1. WCEs (40μg) were subjected to immunoblot analysis using specific Abs. Levels of HMG-1 and β-actin were used for equal loading (n=2). D. Recognition, and E. Immunosensitization and
recognition of M244R1 line by ERK1/2 inhibition. Cells [±PD98059 (25μg/ml-6hr), bortezomib (600nM-6hr), combination] were subjected to cytotoxicity and ELISA assay. Results are represented as Mean±SEM of duplicate samples (n=2). *P values <0.05 significant.

Figure 5. Bortezomib sensitizes M244R cells to patient-derived MART-1 specific CTLs. Three MART-1 specific patient-derived CTL lines (MART-1 tetramer+/CD8+) A-C (#1-3) were grown in AIM-V medium supplemented with 1000IU/ml IL-15+300IU/ml IL-2, 5% human AB serum (as effectors) were cocultured with melanoma targets (±bortezomib, MART-126-35, combination). Cytotoxicity results are presented as Mean±SEM of duplicate samples. *P values <0.05 significant.

Figure 6. Chemical structures of the reagents used in this study. A: Bortezomib, B: 2MAM-A3 (2-methoxyantimycin-A3), C: Bay11-7085 [E-3- (4-butylphenyl sulfonyl) 2-propenetril].
Figure 1

A. M244 (MART-1+/A2.1+)

- F5 CTL
- zVAD-fmk
- MHC blocking Ab
- Control CTL

% Cytotoxicity

E:T ratio

B. M238 (MART-1-/A2.1-)

- Control CTL
- F5 CTL

% Cytotoxicity

E:T ratio

C. Time course (weeks)

- M244
- M244R1 (1 W)
- M244R2 (1 W)
- M244R1 (2 W)
- M244R2 (2 W)

% Cytotoxicity

E:T ratio

D. Bortezomib

- M244R1
- M244R1 + bort.
- M244R2
- M244R2 + bort.

% Cytotoxicity

E:T ratio
Figure 2

A. IFN-γ

B. IL-2

C. MART-1

D. IL-2 (pg/ml/million cells)

E. Cytotoxicity

M244  M244R1  M244R2

IFN-γ (pg/ml/million cells)

RT-PCR

Immunoblot

M244  M244R1  M244R2

G-3-PDH

β-actin

A375 (A2.1/MART-1) + MART-1 peptide
Figure 3

A. RT-PCR

B. Real time-quantitative PCR

C. MART-1

D. Log Fluorescence Intensity

Figure 3

A. RT-PCR

B. Real time-quantitative PCR

C. MART-1

D. Log Fluorescence Intensity
Figure 5

A. MART-1 CTL #1

- M244
- M244R1
- M244R1 + MART-1
- M244R1 + bortz.
- M244R1 + bortz. + MART-1

B. MART-1 CTL #2

- M244
- M244R1
- M244R1 + MART-1
- M244R1 + bortz.
- M244R1 + bortz. + MART-1

C. MART-1 CTL #3

- M244
- M244R1
- M244R1 + MART-1
- M244R1 + bortz.
- M244R1 + bortz. + MART-1

% Cytotoxicity vs. E:T ratio

- 6:1
- 20:1
- 60:1

% Cytotoxicity

- 0
- 10
- 20
- 30
- 40
- 50
- 60
A. Bortezomib has the following chemical structure:

![Bortezomib Chemical Structure](image)

B. 2MAM-A3 has the following chemical structure:

![2MAM-A3 Chemical Structure](image)

C. Bay 11-7085 has the following chemical structure:

![Bay 11-7085 Chemical Structure](image)
Molecular Cancer Therapeutics

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Ali R. Jazirehi and James S. Economou

Mol Cancer Ther Published OnlineFirst April 24, 2012.

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