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Cancer Research

The Proteasome Inhibitor Bortezomib Sensitizes Melanoma Cells toward Adoptive CTL Attack

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Abstract

Adoptive transfer of tumor-specific cytolytic T lymphocytes (CTL) results in target cell lysis by activating the intrinsic apoptotic cell death program. Not surprisingly, deregulation of the apoptotic machinery is one of the central mechanisms by which tumor cells escape immune destruction despite specific CTL recognition. Here we show that treatment with the proteasome inhibitor bortezomib sensitizes previously resistant tumor cells for cytolytic T-cell attack. Human T cells were redirected toward melanoma cells by engineered expression of an immunoreceptor with binding specificity for high molecular weight–melanoma-associated antigen. Established melanoma cell lines as well as primary melanoma cells from tumor biopsies, which are notoriously resistant toward T-cell lysis, became sensitive upon bortezomib treatment. Detailed analysis of the underlying molecular mechanism revealed that bortezomib treatment induced mitochondrial accumulation of NOXA, which potentiated the release of mitochondrial second mitochondria-derived activator of caspase (SMAC) in response to CTL effector functions, including caspase-8 and granzyme B. Our data indicate that proteasome inhibition increases the sensitivity of tumor cells toward cytolytic T-cell attack by NOXA-mediated enhancement of mitochondrial SMAC release. *Cancer Res; 70(5); 1825–34. ©2010 AACR.*

Introduction

The aim of immunotherapeutic strategies using adoptive tumor-specific T-cell transfer is to mount a potent tumor cell–directed cytolytic T lymphocytes (CTL) response. Whereas recent strategies using *ex vivo* engineered T cells with redirected specificity showed promising results in some tumor entities, other tumors proved resistant to CTL attack. It is crucial, therefore, to understand the tumor cell intrinsic mechanisms responsible for CTL resistance if T cell–mediated immunotherapy against cancer is to be successful (1).

Various intrinsic mechanisms contribute to the resistance of tumor cells toward immune effector cell killing (2). Generally, CTLs execute target cell lysis by activating the apoptotic machinery (3); however, tumor cells often have impaired apoptotic pathways enabling their survival despite vigorous CTL attack. CTLs eliminate target cells either by engaging the death receptor CD95/Fas (4) or by calcium-dependent exocytosis of granules containing perforin and granzymes (3). As a result activated caspase-8 (casp-8) or cytoplasmic granzyme B (grzB) initiates the proteolytic activation of the effector (executioner) caspases, casp-3 and casp-7 (5) as well as mitochondrial outer membrane permeabilization (MOMP; ref. 6). The initial processing of executioner procaspases by casp-8 or grzB alone is insufficient to induce the level of executioner caspase activity required for target cell killing. To reach the critical level of executioner caspases, the release of additional proapoptotic factors such as second mitochondria-derived activator of caspase (SMAC) from the mitochondria is necessary to displace XIAP (X-linked inhibitor of apoptosis protein) from partially processed caspases, thus completing caspase autoactivation (7, 8). Elevated XIAP expression or dysfunctional mitochondrial apoptotic pathways in tumor cells are likely responsible for the inadequate caspase activation observed during failed CTL attacks (9, 10). The efficacy of adoptive CTL transfer may therefore be improved by restoring the defective mitochondrial apoptotic pathways in tumor cells.

A promising tool for breaking apoptosis resistance in tumor cells has recently become available in the form of pharmacologic proteasome inhibitors (11). The proteasome inhibitor bortezomib has selective antitumor activity and significant efficacy against a variety of malignancies when used as a single agent. In combination with other agents, bortezomib also sensitizes tumor cells toward chemotherapeutic drugs (12). Here we explored the effect of bortezomib on

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breaking tumor resistance to tumor-specific CTL attack. Human T cells from the peripheral blood were specifically redirected toward defined tumor antigens by expression of a recombinant receptor molecule (immunoreceptor, chimeric antigen receptor) composed of an extracellular single-chain antibody domain targeted at tumor cells and an intracellular CD28-CD3ζ signaling domain required for T-cell activation (13, 14). Upon antigen engagement of the engineered T cells, activation of the immunoreceptor leads to cytokine release, T-cell proliferation, and ultimately target cell lysis. Although tumor cells from various entities are effectively killed by redirected T cells, melanoma cells are notoriously resistant to T cell–driven cytolysis. Using the established melanoma cell line MeWo as well as primary melanoma cells isolated from different tumor biopsies, we here show that proteasome inhibition significantly enhances the efficacy of T cell–mediated killing of melanoma cells. Detailed analysis of the molecular mechanism shows that mitochondrial accumulation of NOXA restores the mitochondrial apoptotic pathway by potentiating the release of mitochondrial SMAC in response to CTL effector mechanisms, which results in enhanced caspase activation and ultimately increased tumor cell killing. The use of proteasome inhibitors to improve the sensitivity of tumor cells to adoptive T-cell attack represents a novel concept in the cellular immunotherapy of cancer.

Materials and Methods

Cells, antibodies, and reagents. MeWo [American Type Culture Collection (ATCC) HTB-65] is an established high molecular weight–melanoma-associated antigen (HMW-MAA) expressing melanoma cell line and LS174T (ATCC CCL 188), a carcinoembryonic antigen (CEA) expressing colon carcinoma cell line. Both were obtained from ATCC-LGC Standards GmbH, authenticated, and found to be free of contaminants. Cells were expanded, and a large number of frozen vials were systematically prepared from stocks after 10 passages to ensure consistent starting material for experiments. Cells were cultured in RPMI 1640 supplemented with 10% (v/v) FCS, 10 units/mL penicillin, and 200 units/mL streptomycin (Invitrogen) and regularly subjected to detection of melanoma and colon carcinoma–associated cell markers, including HMW-MAA and CEA. OKT3 antibody was affinity purified from hybridoma supernatants. The fluorescein (FITC)–conjugated anti-CD3, anti-tyrosinase monoclonal antibody (mAb) T311, anti-S100, and anti-melanA mAb A103 were purchased from DAKO GmbH, and the phycoerythrin (PE)–conjugated anti–HMW-MAA antibody EP-1 was from Miltenyi Biotec. Expression of the respective marker molecules was recorded by flow cytometry using the FACSCanto cytometer equipped with Diva software (Becton Dickinson).

Primary melanoma cells were isolated from brain (patients 1 and 4) and skin metastases (patients 2 and 3), respectively. To obtain melanoma cell suspensions, biopsies were disaggregated by incubation with tumor dissociation reagent (TDE, DCS) for 3 to 4 h at 37°C. Digested tissues were passed through a 70-μm cell strainer (FALCON), and melanoma cells were isolated as described previously (15). Isolated cells were cultured in RPMI 1640 supplemented with 10% (v/v) FCS, 10 units/mL penicillin, and 200 units/mL streptomycin. The study was approved by the local ethics committee in agreement with the Helsinki Declaration.

Immunohistochemistry. Paraffin sections for S100 detection were first deparaffinized by incubation in xylol and ethanol series and washed in TBS. Sections were blocked for 1 h with 10% (v/v) FCS in TBS before applying the primary antibody rabbit anti-S100 antibody (DAKO) for 16 h at 4°C. After three 15-min washes bound antibodies were detected with alkaline phosphatase–labeled antimouse/antirabbit polymer (DAKO) and neofuchsin as substrate. Nuclei were counterstained with hematoxylin solution for 1 min (Shandon).

Engineering of redirected T cells. The retroviral expression cassette for the CEA-specific immunoreceptor (16) and HMW-MAA–specific immunoreceptor (17, 18) were previously described. $CD3^+$ or $CD8^+$ T cells were isolated by magnetic cell sorting procedures (purity, >98%) using antihuman CD3 and CD8 microbeads (Miltenyi Biotec). Cells were retrovirally transduced as previously described (19). Briefly, T cells were preactivated by incubation with agonistic anti-CD3 (OKT3) and anti-CD28 (15E8) antibodies (100 ng/mL each) in the presence of interleukin-2 (200 units/mL) for 2 d. GALV pseudotyped retroviral particles were produced by 293T cells transfected with packaging plasmids and incubated with preactivated T cells for another 2 d. Immunoreceptor expression was monitored by flow cytometry using an antihuman IgG antibody, which binds to the common extracellular IgG1 chain of the immunoreceptor.

Specific cytotoxicity of redirected T cells was monitored by the 2,3-bis(2-methoxy-4-nitro-5-sulfonyl)-5[(phenyl-amino) carbonyl]-2H-tetrazolium hydroxide (XTT)–based colorimetric assay. Briefly, receptor-engineered T cells (10^4 per well) were coincubated with 2.5×10^4 tumor cells for 24 h, and viability of tumor cells was monitored using the Cell Proliferation Kit II (Roche Diagnostics). The reduction of XTT to formazan by viable tumor cells was monitored colorimetrically. Values were calculated as means of six wells containing tumor cells by subtracting the mean background level of wells containing RPMI 1640, 10% (v/v) FCS. Nonspecific formation of formazan due to the presence of effector cells was determined from triplicate wells containing effector cells in the same number as in the corresponding experimental wells. The number of viable tumor cells was calculated as follows: viability (%) = [absorbance (experimental wells – corresponding number of effector cells)] / [absorbance (tumor cells without effectors-medium)] × 100. Cytotoxicity (%) was defined as 100 – viability (%).

Sample preparation and immunoblotting. Whole-cell extracts were prepared by cell lysis in CHAPS buffer [10 mmol/L HEPES (pH 7.4), 150 mmol/L NaCl, 1% CHAPS, protease complete cocktail] on ice for 20 min. Cytosolic extracts were prepared in buffer A [20 mmol/L PIPES (pH 7.0), 50 mmol/L KCl, 2 mmol/L $MgCl_2$, 5 mmol/L EGTA, 1 mmol/L DTT]. Mitochondrial and postnuclear fractions were isolated as previously described (9, 20). Proteins were separated by SDS-PAGE. Rabbit polyclonal antisera specific for human casp-9, BAX, and BID and mouse anti-XIAP and anti–cytochrome *c* mAbs were

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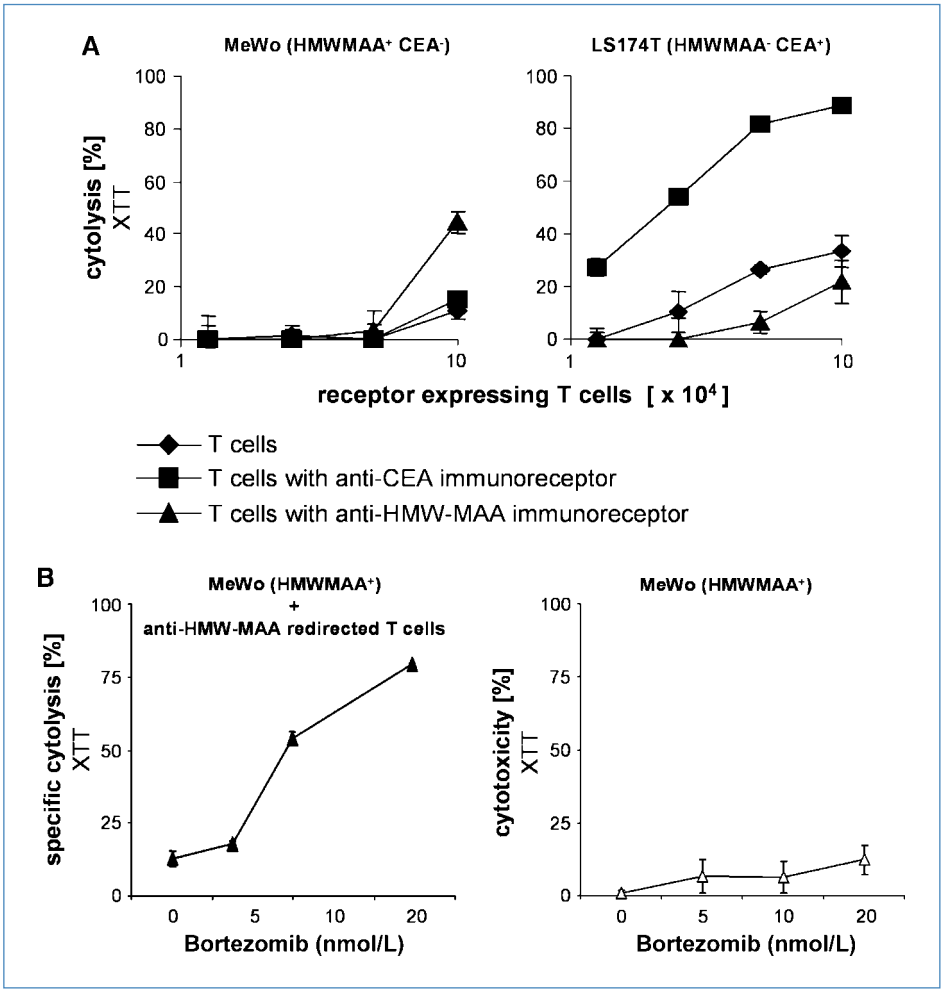

Figure 1. Bortezomib sensitizes melanoma cells for antigen-specific CTL attack. A, XTT-based viability assay of HMW-MAA$^+$ CEA$^-$ MeWo melanoma cells and HMW-MAA$^-$ CEA$^+$ LS174T colon carcinoma cells (4×10^4 per well) after coincubation with increasing numbers of engineered CD3$^+$ T cells with HMW-MAA–specific immunoreceptor and CEA-specific immunoreceptor, respectively. B, XTT-based viability assay of MeWo cells (4×10^4 per well) treated with increasing concentrations of bortezomib alone or together with engineered T cells with anti–HMW-MAA immunoreceptor (effector-target cell ratio, 1:1) for 24 h. Points, means of triplicates from at least three separate experiments; bars, SEM.

obtained from BD. Mouse anti-NOXA antibody was obtained from Calbiochem (Merck Chemicals), and rabbit polyclonal antisera specific for human BIM, BIK, BOK, Mcl-1, poly(ADP-ribose) polymerase and mouse mAb specific for SMAC were obtained from Cell Signaling. Polyclonal antiserum specific for PUMA was obtained from Oncogene Research Products. Mouse anti–complex II was obtained from Invitrogen.

Caspase activation and cytochrome c release. Mitochondria or postnuclear fractions (50 mg protein) were incubated with increasing amounts of NOXA (the inactivator NOXA BH3 peptide; refs. 21, 22), casp-8, or grzB (Axxora) for 30 min at 30°C. After centrifugation the release of cytochrome *c*/SMAC and the casp-3 activity were assessed in supernatants. To initiate caspase activation, 20 μl cytosolic extracts (10 μg protein/mL) were treated with either casp-8 or grzB with or without the SMAC NH_2 terminal peptide H-AVPIAQK-OH (10 μmol/L; Calbiochem) for 1 h at 30°C. Casp-3 activity was assayed using 100 μmol/L Ac-DEVD-AFC (Axxora) and presented as arbitrary fluorescence units (FU); 1 FU is equivalent to 0.65 pmol released 7-amino-4-trifluoromethylcoumarin (9, 23).

Small interfering RNA transfer. Double-stranded small interfering RNAs (siRNA) of SMAC, NOXA, and control (scrambled) were obtained from Ambion (Ambion Europe). Each siRNA (final concentration, 100 nmol/L) was transiently transfected into 5×10^6 cells using Lipofectamine (Invitrogen) according to the manufacturer's instructions. The most efficient NOXA and SMAC downregulating siRNAs were identified by transient transfection of 293HEK or MeWo cells and Western blot analysis after 72 h (data not shown). The recombinant lentiviral particles carrying XIAP-specific and scrambled sequences (pLenti6/V5DEST XIAP-siRNA and scr-siRNA expressing vectors, respectively) were generated as described before (9, 24) according to the instructions of the manufacturer (pLenti-Dest Gateway System; Invitrogen). The recombinant lentiviral constructs were transduced into the MeWo cells, and stable cell lines were generated using Blasticidine (Invitrogen) selection.

Results

Bortezomib enhances the susceptibility of established melanoma cells toward redirected cytolytic T-cell attack. To generate melanoma cell-specific T cells, human T cells from the peripheral blood were engineered to express immunoreceptors

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with specificity for HMW-MAA or CEA, respectively, using retroviral gene transfer as previously described (17). The extracellular domain of the immunoreceptors was composed of the anti–HMW-MAA or anti-CEA scFv for specific target antigen binding and intracellular signaling domain of the CD3ζ chain for T-cell activation upon antigen engagement. As shown in Fig. 1A, $CD3^+$ T cells equipped with an anti–HMW-MAA immunoreceptor specifically targeted and lysed the HMW-MAA^+ melanoma cell line MeWo. Cytolysis was antigen-specific, because HMW-MAA^- CEA^+ LS174T colon carcinoma cells were not lysed by anti–HMW-MAA immunoreceptor-engineered T cells, although they were targeted by T cells from the same donor, equipped with anti-CEA immunoreceptor. Conversely, T cells with an anti-CEA immunoreceptor did not lyse CEA^- MeWo cells showing the specificity of the engineered T cells.

Whereas the efficacy of anti–HMW-MAA T cell–mediated cytolysis of MeWo melanoma cells was poor, requiring large numbers of effector T cells (effector-to-target cell ratio = 10:1), cotreatment with the proteasome inhibitor bortezomib significantly enhanced the susceptibility of MeWo cells to redirected HMW-MAA–specific T-cell attack in a dose-dependent fashion (Fig. 1B). Significantly, improved tumor cell lysis was not due to direct cytotoxic effects of bortezomib (Fig. 1B), and alterations in T-cell functions including production and secretion of cytokines under these conditions were not observed (data not shown). These data suggest that bortezomib treatment increases the susceptibility of melanoma cells toward cytolytic effect of tumor-specific T cells.

Bortezomib treatment enhances MOMP and caspase activity in response to recombinant active casp-8 and grzB. Active casp-8 and cytosolic grzB are the major effector molecules of CTLs that initiate the proteolytic cascade leading to target cell death. A hallmark of casp-8–mediated and grzB-mediated cytolysis is the amplification of proapoptotic signals involving the release of mitochondrial proapoptotic factors, including cytochrome *c* and SMAC. We therefore tested whether bortezomib treatment facilitates mitochondrial release of the proapoptotic proteins SMAC and cytochrome *c* in response to casp-8 or grzB. In a cell-free system using postnuclear fractions comprising the cytosolic and mitochondrial fractions, recombinant active casp-8 and grzB induced the release of cytochrome *c* and SMAC (Fig. 2A). Strikingly, pretreatment of MeWo cells with subtoxic concentrations of bortezomib (50 nmol/L) significantly enhanced the casp-8–induced and grzB-induced release of SMAC and cytochrome *c*. Correspondingly, in response to casp-8 or grzB treatment increased casp-3 activity was detected in the postnuclear fractions of cells pretreated with bortezomib (Fig. 2B). These data suggest that bortezomib sensitizes melanoma cells to CTLs by enhancing the proapoptotic response of mitochondria to cytolytic effector functions, including casp-8 and grzB.

Mitochondrial NOXA accumulation upon proteasome inhibition enhances mitochondrial perturbation by casp-8 and grzB. Bortezomib has been shown to induce the mitochondrial apoptotic process in various tumor entities, predominantly by altering the expression levels of Bcl2 protein family members (25). To identify the molecular

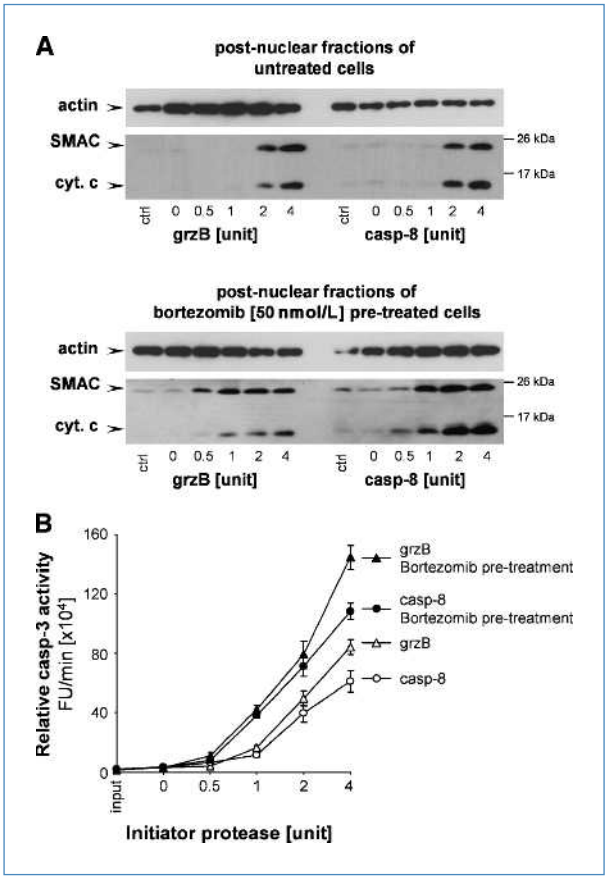

Figure 2. Bortezomib treatment enhances mitochondrial susceptibility to grzB and casp-8. Postnuclear fractions containing cytoplasm and mitochondria from untreated and bortezomib-pretreated (50 nmol/L for 24 h) MeWo cells (5×10^7) were prepared after incubation with increasing amounts of grzB or recombinant active casp-8 for 30 min. A, cytochrome *c* (cyt. c), SMAC, and actin were detected by Western blotting in supernatants (cytosolic fraction). Controls (ctrl) represent postnuclear fractions of bortezomib-pretreated or untreated MeWo cells, respectively, without incubation with grzB or casp-8. B, relative casp-3 activity was measured in supernatants from A and expressed as arbitrary fluorescence units. Points, means of triplicates from at least three separate experiments; bars, ±SEM.

targets of bortezomib action, we therefore examined the expression levels of Bcl2 protein family members in MeWo cells after treatment with increasing concentrations of bortezomib (up to 100 nmol/L). Bortezomib treatment resulted in the accumulation of Bcl2 member proteins, including Mcl-1, and BH3-only proteins PUMA, BID, and NOXA (Fig. 3A). Whereas increased expression of Mcl-1, PUMA, and BID was observed at concentrations associated with direct bortezomib cytotoxicity and casp-3 activation (100 nmol/L bortezomib), accumulation of NOXA occurred far below toxic bortezomib concentrations (between 25 and 50 nmol/L; Fig. 3A). NOXA lacks direct mitochondrial permeabilizing capacity but has been shown to increase MOMP in conjunction with other BH3-only proteins (26). Accordingly, cell fractionation analyses showed that bortezomib treatment (50 nmol/L)

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resulted in the mitochondrial accumulation of NOXA without inducing the release of mitochondrial cytochrome *c* or SMAC (Fig. 3B). Therefore no direct mitochondrial perturbation is initiated by mitochondrial NOXA accumulation and bortezomib treatment at concentrations of up to 50 nmol/L, in line with the lack of direct cytotoxicity of bortezomib under these conditions (Figs. 1 and 3).

To elucidate the effect of NOXA accumulation on casp-8–induced or grzB-induced mitochondrial perturbation, postnuclear fractions of MeWo cells were incubated with recombinant NOXA peptide containing the BH3 motif responsible for mitochondrial association and binding to antiapoptotic Bcl2 members (21, 22). In accordance with the current model of NOXA action (26), increasing amounts of NOXA alone did not result in the release of cytochrome *c* or SMAC from isolated mitochondria (Supplementary Fig. S1). However, casp-8 and grzB, in combination with NOXA, significantly increased SMAC and cytochrome *c* release in postnuclear fractions of MeWo cells (Fig. 4A). Similarly, addition of exogenous NOXA enhanced casp-8–induced or grzB-induced casp-3 activity (Fig. 4B).

These data suggest that NOXA is a key mediator of bortezomib-mediated mitochondrial susceptibility to casp-8 or grzB action. Therefore downregulation of NOXA expression in bortezomib-treated MeWo cells should abrogate the effects of bortezomib. To address this issue, we specifically downregulated NOXA expression using NOXA-specific siRNA. Whereas bortezomib treatment resulted in mitochondrial accumulation of NOXA both in untransfected MeWo and in MeWo cells transfected with nonspecific scrambled siRNA, efficient NOXA knockdown was shown in MeWo cells transfected with NOXA-specific siRNA. The lack of NOXA accumulation resulted in significantly reduced mitochondrial release of cytochrome *c*, SMAC, and casp-3 activity after grzB or casp-8 treatment, implicating NOXA as the central mediator of bortezomib-mediated sensitization of mitochondria to grzB and casp-8 action.

The central downstream event of NOXA action in enhancing the susceptibility to redirected CTL attack is to antagonize XIAP through mitochondrial release of SMAC. The effect of NOXA on CTL-mediated cytolysis was further examined by transient knockdown of NOXA in MeWo cells treated with bortezomib in conjunction with redirected CTLs (Fig. 5A; Supplementary Fig. S2). Bortezomib treatment enhanced cytolysis of scrambled siRNA–transfected MeWo cells by $CD8^+$ T cells equipped with anti–HMW-MAA, but not $CD8^+$ T cells

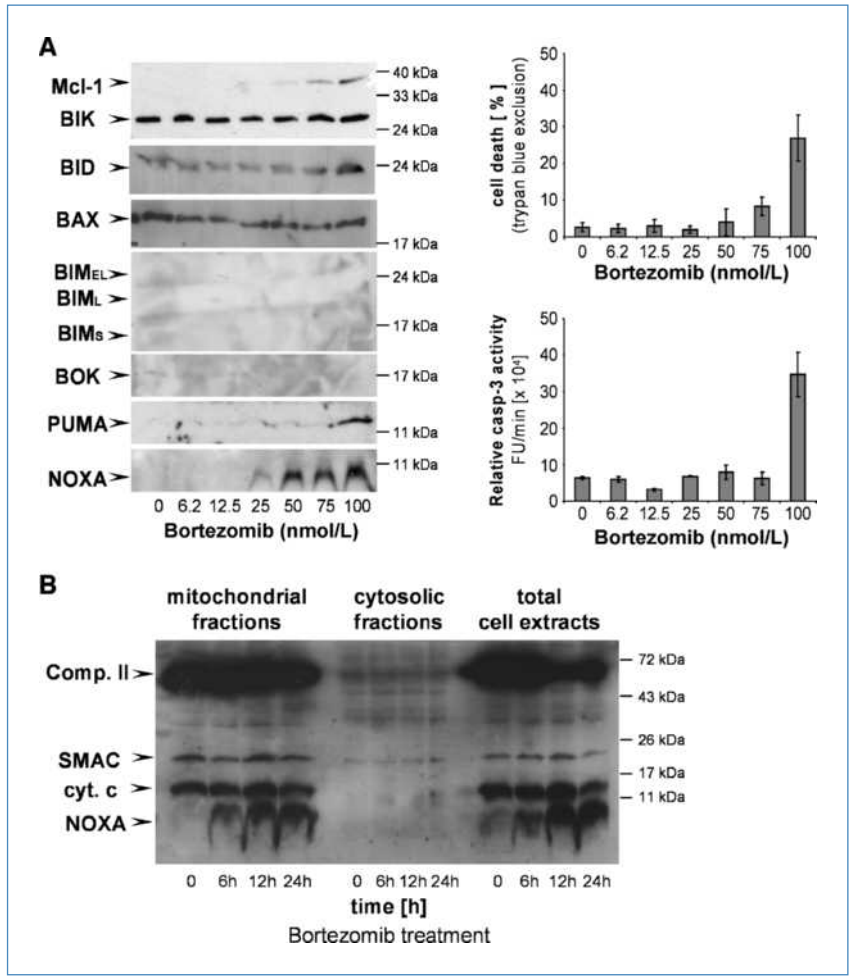

Figure 3. Bortezomib treatment results in mitochondrial accumulation of NOXA without initiating cytotoxic activity. A, Western blot analysis of Bcl2 protein family members Mcl-1, BIK, BID, BAX, BIM, BOK, PUMA, and NOXA detected in total cell extracts (10 μg protein per lane) of MeWo cells treated with increasing concentrations of bortezomib for 24 h. Cell death was determined by trypan blue exclusion. Relative casp-3 activity was measured in cytosolic extracts and expressed as arbitrary fluorescence units. The experimental values represent means of triplicates ± SEM from at least three separate experiments. B, Western blot analysis of SMAC, cytochrome *c* (cyt. c), and NOXA in mitochondrial (10 μg), cytosolic (10 μg), and total cell extracts (20 μg) of MeWo cells treated with bortezomib (50 nmol/L) for 6, 12, and 24 h as indicated. Reprobing with an antibody against complex II (Comp. II) of the mitochondrial electron transport complex (succinate dehydrogenase) served as a loading control.

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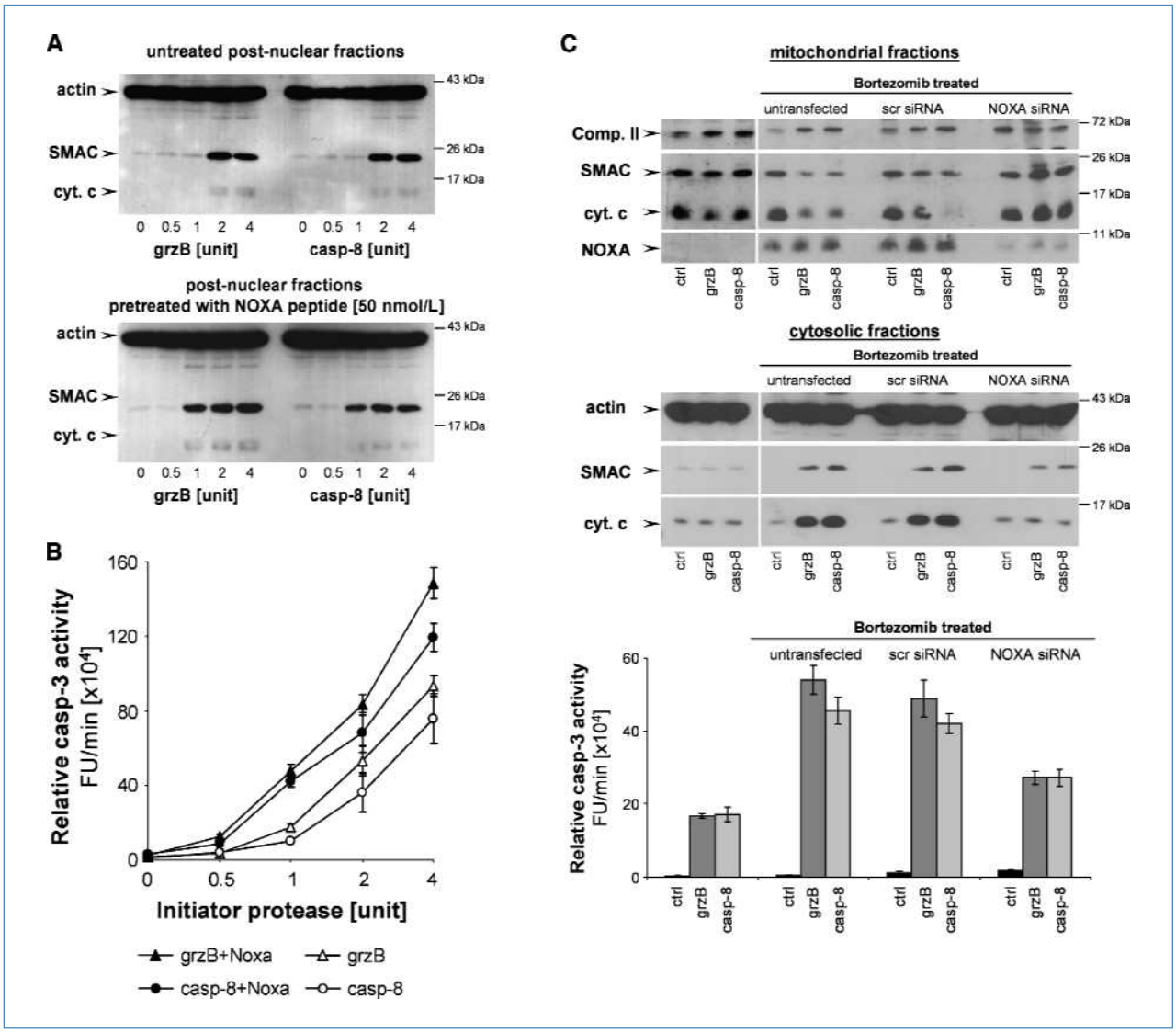

Figure 4. NOXA is the central mediator of the enhanced mitochondrial susceptibility to grzB and casp-8 induced by bortezomib. A, postnuclear fractions of MeWo cells (5×10^7) were prepared and treated with increasing amounts of grzB or recombinant active casp-8 in the absence or presence of NOXA peptide for 30 min. Cytochrome *c* (cyt. c), SMAC, and actin were detected by Western blotting. B, relative casp-3 activity was measured in supernatants from A as described. C, MeWo cells (5×10^6) were transfected with 100 nmol/L (final concentration) double-stranded siRNAs. After 48 h cells were treated with bortezomib (50 nmol/L for 24 h). Postnuclear fractions were prepared and treated with grzB or recombinant active casp-8 (1 unit) for 30 min. After centrifugation, cytochrome *c* (cyt. c), SMAC, NOXA, complex II (Comp. II), and actin were detected by Western blotting in supernatants (cytosolic fraction) or pellets (containing mitochondria). Relative casp-3 activity was measured in supernatants as described.

equipped with anti-CEA immunreceptor. Enhanced cytolysis was accompanied by increased casp-3 activity as the responsible executioner caspase in CTL-mediated cytolysis. NOXA downregulation, however, significantly reduced antigen-specific killing of bortezomib-treated MeWo cells by redirected CTLs. The reduced cytolytic activity of redirected CTLs was associated with a decline in casp-3 activity.

One of the central mitochondrial functions in response to CTL-mediated cytolysis is the release of SMAC, which by interacting with XIAP leads to the release of XIAP-mediated inhibition of casp-3. Accordingly, SMAC knockdown in MeWo cells completely diminished antigen-specific cytolysis and casp-3 activity in MeWo cells treated with bortezomib (Fig. 5A). Conversely, addition of the SMAC N7 peptide to cytosolic extracts of MeWo cells significantly increased casp-3 activity initiated by casp-8 or grzB (Fig. 5B). To further examine the role of XIAP/SMAC cross-talk, we specifically downregulated XIAP in stable MeWo cell derivatives expressing XIAP-specific small hairpin RNA (shRNA) following lentiviral gene transfer (Fig. 5C). Casp-8–induced and grzB-induced casp-3 activity was significantly increased in cytosolic extracts isolated from two independent MeWo clones with

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downregulated XIAP expression (C2 and C27 cell clones with 80% and 68% XIAP knockdown, respectively; Fig. 5B). Similarly, XIAP knockdown in MeWo cells enhanced their susceptibility to redirected T-cell attack (Fig. 5D), whereas control MeWo cells expressing scrambled shRNA (MeWo-shRNAscr) showed no enhanced cell lysis. These data indicate that XIAP is one of the major downstream targets of NOXA action.

Bortezomib treatment enhances redirected T-cell response in primary melanoma cells. Primary melanoma cells from four patients were short-term cultured *in vitro*, and the melanoma origin was confirmed by histologic and flow cytometric analyses for the melanoma-associated markers S-100, tyrosinase, and HMW-MAA (Supplementary Fig. S3; Fig. 6A). To assess the sensitivity of primary melanoma cells to specific T-cell attack, cells were treated with increasing concentrations of bortezomib and coincubated with HMW-MAA–redirected T cells. Although melanoma cells from one patient (patient 4) were apparently more sensitive to bortezomib treatment resulting in a substantial loss of viability in the presence of bortezomib alone, treatment with up to 20 nmol/L bortezomib

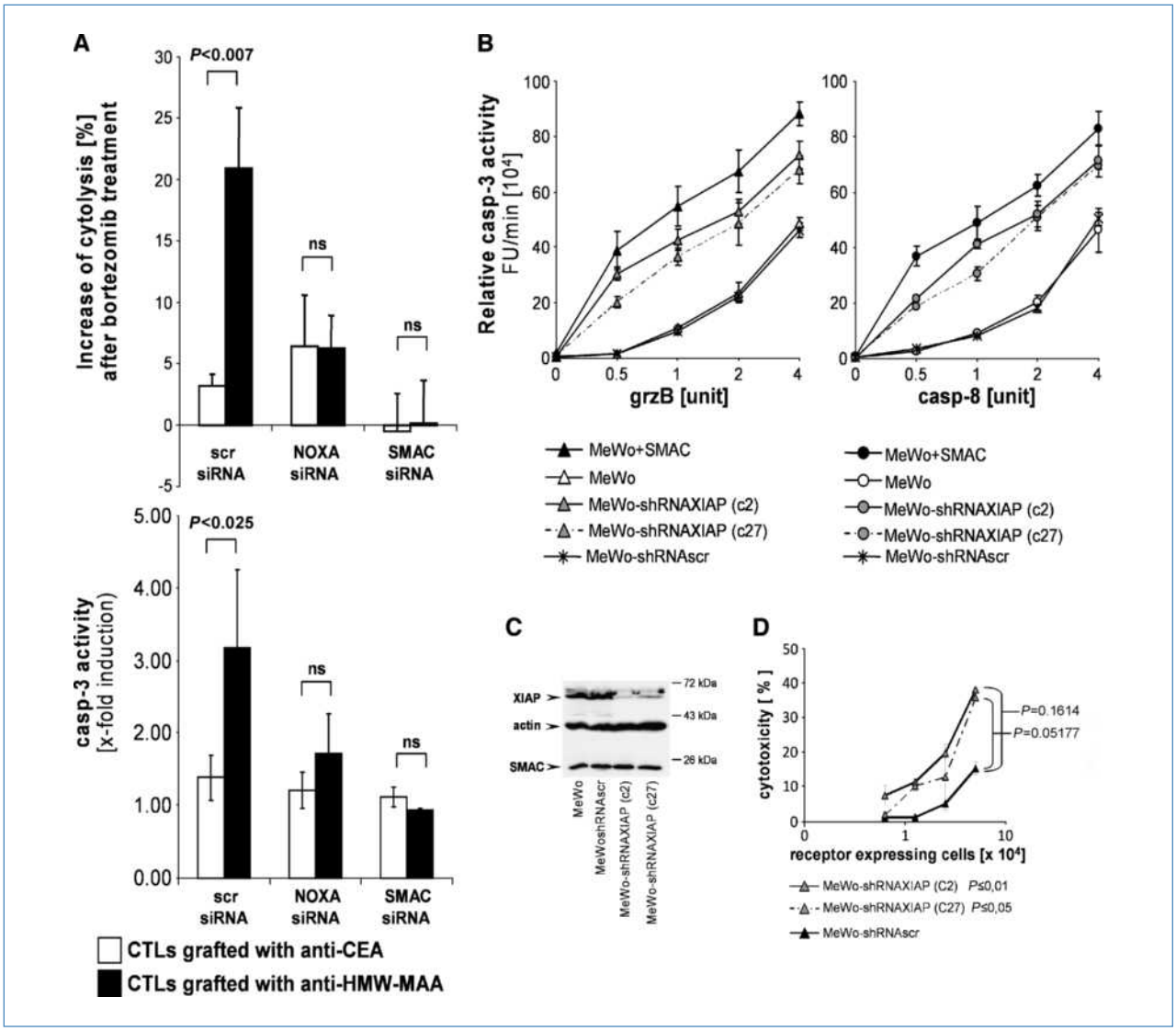

Figure 5. Mitochondrial release of SMAC enhances antigen-specific cytolysis by antagonizing XIAP. A, MeWo cells (5×10^6) were transfected with 100 nmol/L (final concentration) double-stranded siRNAs. After 48 h cells (5×10^4) were incubated for an additional 48 h with bortezomib together with engineered $CD8^+$ T cells with anti–HMW-MAA or anti-CEA immunoreceptor (effector-target cell ratio, 1:2). XTT-based viability and casp-3 activity assays were performed and expressed as increased cytolysis or fold induction compared with counterparts without bortezomib treatment. B, Casp-3 activity was measured in cytosolic extracts of MeWo, MeWoshRNAscr, MeWoshRNAXIAP (C2), and MeWoshRNAXIAP (C27) cells after incubation with increasing amounts of grzB or casp-8 in the absence or presence of SMAC N7 peptide (10 μmol/L). Protein content was adjusted to 10 μg/μL. C, Western blot analysis of XIAP, SMAC, and actin in total cell extracts of MeWo, MeWoshRNAscr (scrambled), MeWoshRNAXIAP (C2), and MeWoshRNAXIAP (C27) cells. D, XTT-based viability assay of MeWoshRNAscr and MeWoshRNAXIAP clones C2 and C27 (5×10^4 cells per well) incubated with increasing numbers of engineered T cells with HMW-MAA–specific immunoreceptor (up to 5×10^4 per well) after 24 h in triplicates ± SEM.

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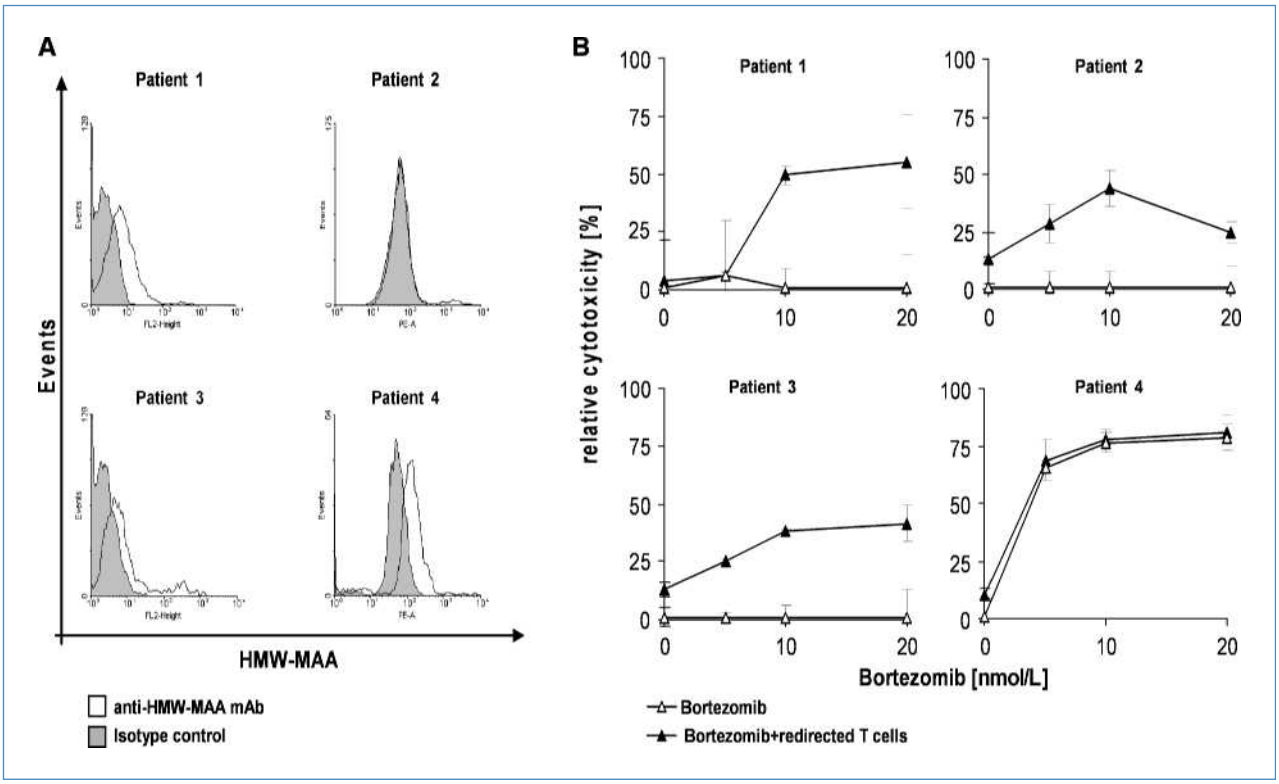

Figure 6. Bortezomib treatment enhances HMW-MAA–specific T cell–mediated cytolysis of primary melanoma cells. A, fluorescence-activated cell sorting analysis of isolated primary melanoma cells was performed using the PE-conjugated anti–HMW-MAA mAb EP-1. Histogram overlays against isotype control (PE-anti-mouseIgG1; gray). B, XTT-based viability assay of isolated primary melanoma cells (5×10^4 per well) treated with increasing concentrations of bortezomib alone or together with engineered T cells with anti–HMW-MAA immunoreceptor (effector-target cell ratio, 1:1) for 24 h in triplicates ± SEM.

produced virtually no toxicity in the primary melanoma cells of three further patients (Fig. 6B). In these patients (patients 1, 2, and 3) bortezomib significantly increased the sensitivity of primary melanoma cells for T cell–mediated cytolysis in a dose-dependent fashion (Fig. 6B). Significantly, the potency of bortezomib to enhance specific T cell–mediated cytolysis closely correlated with the degree of HMW-MAA expression by the tumor cells confirming the antigen-dependent effect of bortezomib on redirected T-cell killing of primary melanoma cells.

Discussion

Here we show that the proteasome inhibitor bortezomib sensitizes melanoma cells for redirected, antigen-specific T-cell attack. Mechanistically, this is based on the mitochondrial accumulation of NOXA, which potentiates the release of mitochondrial SMAC and ultimately results in enhanced caspase activation executing T cell–mediated cytolysis. Although melanoma cells are notoriously resistant to cytolytic T-cell attack, they do attract tumor-specific CTLs, resulting in massive lymphocyte infiltration during the early stages of the disease. Nevertheless adoptive transfer of cytolytically potent and tumor-specific tumor-infiltrating lymphocytes (TIL) resulted only in low therapeutic efficiency in the treatment of melanoma, whereas TILs in some other cancer entities proved more efficient (27). A number of mechanisms were proposed to explain the obvious therapeutic failure of specific T cells. Resistance to apoptosis, a hallmark of melanoma cells, has been considered as the major underlying mechanism by which melanoma cells escape immune destruction (2). As a consequence, successful immunotherapy of melanoma requires a combination of strategies aiming both at stimulation of specific T cells and restoration of melanoma cell intrinsic death programs.

In clinical trials proteasome inhibition has been successfully used to counter escape strategies of tumor cells toward chemotherapy (12). Here we show that proteasome inhibition sensitizes melanoma cells toward tumor-specific T cell–mediated cytolysis by enhancing mitochondrial apoptotic response. In general, damage signals involving mitochondria are believed to be transduced by BH3-only proteins that directly bind and block the antiapoptotic Bcl2 members or activate proapoptotic BAX and BAK. These interactions ultimately commit the cell to apoptosis by permeabilizing the outer mitochondrial membrane (28, 29). Mitochondrial perturbation induced by CTL effector mechanisms, including active casp-8 and grzB, is normally mediated by the BH3-only protein BID (3). Here we show that NOXA potentiates casp-8–mediated and grzB-mediated CTL action (Figs. 4

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and 5). The so-called "promiscuous binders BH3-only proteins" BID, BIM, and PUMA can directly bind and activate BAX and BAK, thus directly inducing cell death (30). NOXA, however, has been described as a "sensitizer," which initiates cell death only in conjunction with other BH3-only members (28, 30). Indeed, our data show that mitochondrial NOXA accumulation in response to bortezomib (Fig. 3) is not sufficient to induce cytotoxicity (Figs. 1–3). Equally, increasing concentrations of NOXA peptide alone were not capable of inducing MOMP in MeWo cells (Fig. 4; Supplementary Fig. S2). Yet, NOXA accumulation in response to bortezomib or NOXA peptide significantly enhanced casp-8–induced and grzB-induced mitochondrial perturbation inducing cell death. Because casp-8 and grzB are known to activate BID (by proteolytic truncation), it seems likely that NOXA sensitizes mitochondria by enhancing the effects of activated/truncated BID.

As a consequence of mitochondrial perturbation following CTL attack, SMAC released from the mitochondria is free to displace XIAP from partially processed executioner caspases to permit completion of caspase autoactivation, thereby inducing apoptosis (7–9). SMAC peptide or specific knockdown of XIAP significantly enhanced the cytolytic effect of CTLs in melanoma tumor cells (Fig. 5), mimicking the effects of bortezomib (Fig. 1). These data indicate that SMAC is a downstream effector molecule of NOXA (Fig. 5). Taken together our results provide a rationale for the use of bortezomib to sensitize tumor cells for CTL-mediated attack. Furthermore, our identification of NOXA and SMAC as the key players in sensitizing tumor cells toward CTL-mediated killing makes a strong case for the use of BH3-only (30) and SMAC mimetics (31) to further improve the cytolytic activity of tumor-specific CTLs in adoptive cellular immunotherapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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