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Sensitization of Tumor Cells to NK Cell-Mediated Killing by Proteasome Inhibition

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Bortezomib is a proteasome inhibitor that has direct antitumor effects. We and others have previously demonstrated that bortezomib could also sensitize tumor cells to killing via the death ligand, TRAIL. NK cells represent a potent antitumor effector cell. Therefore, we investigated whether bortezomib could sensitize tumor cells to NK cell-mediated killing. Preincubation of tumor cells with bortezomib had no effect on short-term NK cell killing or purified granule killing assays. Using a 24-h lysis assay, increases in tumor killing was only observed using perforin-deficient NK cells, and this increased killing was found to be dependent on both TRAIL and FasL, correlating with an increase in tumor Fas and DR5 expression. Long-term tumor outgrowth assays allowed for the detection of this increased tumor killing by activated NK cells following bortezomib treatment of the tumor. In a tumor purging assay, in which tumor:bone marrow cell mixtures were placed into lethally irradiated mice, only treatment of these mixtures with a combination of NK cells with bortezomib resulted in significant tumor-free survival of the recipients. These results demonstrate that bortezomib treatment can sensitize tumor cells to cellular effector pathways. These results suggest that the combination of proteasome inhibition with immune therapy may result in increased antitumor efficacy. The Journal of Immunology, 2008, 180: 163–170.

Molecular targeting of cancer represents a potential means to more specifically target the tumor and has great promise in cancer therapy. The proteasome represents a pivotal intracellular pathway involved in both protein degradation and cellular homeostasis. Proteasome inhibition has been shown to directly inhibit growth of various neoplastic cells as well as sensitizing them to various chemotherapeutics (1, 2). Bortezomib is a proteasome inhibitor recently approved for the treatment of multiple myeloma that is currently being investigated in other cancers as a single agent or in combination with chemotherapeutic regimens (3, 4). Due to numerous effects of proteasome inhibition on a variety of cellular pathways, the precise mechanism(s) by which bortezomib mediates its antitumor effects may vary depending on the particular tumor cell involved. It has been reported that bortezomib can also sensitize some tumor cells to various chemotherapeutics and radiation (2, 5). We have previously shown that bortezomib could sensitize tumor cells to TRAIL-mediated lysis, in part due to a reduction of the anti-apoptotic protein c-FLIP (6). TRAIL represents one cytotoxic pathway used by immune effector cells such as T and NK cells to lyse target cells. These immune effector cells have been shown to mediate potent anti-tumor effects both in vitro and in vivo (7–9). However, both T cells and NK cells have multiple mechanisms at their disposal to lyse tumor target cells. For NK cells, the major cytotoxic pathway is thought to be due to the release of perforin/granzymes (10). However, both TRAIL and Fas ligand (FasL)3 are also expressed on NK cells. Furthermore, these cells can produce cytostatic cytokines such as IFN-γ and TNF-α (11). When NK cell-mediated killing has been assessed through the use of short-term (4 h) lytic assays in vitro, perforin-mediated lysis has been predominantly observed (12). However, the use of longer-term (days) assays may reveal the importance of other, perhaps more physiologically meaningful, effector pathways. Because the killing of tumor cells by recombinant TRAIL could be increased with bortezomib, we investigated whether NK cell-mediated killing of tumor cells might also be augmented. We demonstrate in this study that pretreatment of tumor cells with bortezomib did not have an effect on NK cell-mediated killing when assessed by short-term assays. However, longer-term assays demonstrated marked augmentation of NK cell-mediated cytotoxicity due to enhanced TRAIL and FasL-mediated lysis. These results demonstrate that proteasome inhibition by bortezomib can sensitize tumor cells to immune effector killing pathways and this may be able to be exploited in cancer therapy.

Materials and Methods

Mice

C57BL/6 (H2b), BALB/c (H2d), CB.17 severe combined immunodeficient (scid/scid) (H2d, SCID), C57BL/6 perforin knockout (H2b; pfp−/−), C57BL/6 generalized lymphoproliferative disease mice (H2b; gld), and

Abbreviations used in this paper: FasL, Fas ligand; BMC, bone marrow cells; rh-IL2, recombinant human IL-2.

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B6-Ly5.2 (H2b) mice were purchased from the Animal Production Area (National Cancer Institute (NCI) at Frederick, Frederick, MD). Female mice were 2 to 4 mo of age at the initiation of experiments. Animals were maintained under specific-pathogen free conditions at the University of Nevada, Reno or the NCI facilities. Animal care was provided in accordance with the procedures outlined by the National Institutes of Health. Animal studies were performed at the University of Nevada, Reno animal facility according to approved protocols and in accordance with the Institutional Animal Care and Use Committees.

Cell culture

C1498 (H2b) murine leukemia was a gift from Dr. Bruce R Blazar (University of Minnesota, Twin Cities, MN) and murine renal carcinoma cell line (H2b; Renca) was provided by Dr. Robert Wiltrout (National Cancer Institute, Frederick, MD). For in vitro experiments using C1498, frozen stocks were thawed every month. For in vivo experiments using C1498, frozen stocks were thawed 7 days before in vivo administration and were grown in the log phase of growth until use.

Reagents

The proteasome inhibitor, bortezomib, was provided by Millennium Pharmaceuticals. Stock bortezomib solution (1 mg/ml) was prepared in Dulbecco’s PBS solution and stored at −70°C for up to 2 mo before use. Bortezomib solutions were protected from light at all times. The stock solutions were thawed and diluted to 1 μg/ml in RF10 complete medium and kept at 4°C for up to 2 wk.

Flow cytometry

Cells cultures were washed with blocking buffer (PBS with 1% FBS and 1% Human Ab Serum) and labeled with FITC-anti-H2K2 (AF6–88.5) and PE-anti-Fas (Jo2) (BD Biosciences). Anti-DR5 (MD5.1; a gift from Dr. Hideo Yagita, Juntendo University, Tokyo, Japan) expression was determined using anti-hamster IgG (G235–2356; BD Biosciences). All cellular labeling was performed for 15 min at 4°C. All flow cytometry was performed on a BD FACScan flow cytometer.

Generation of activated NK cells

To generate activated NK cells, splenocytes and bone marrow cells (BMC) from C57BL/6, B6-Ly5.2, pfp−/−, or gld mice were depleted of T cells by treatment with anti-Thy1.2 mAb (30H12) and rabbit-complement (Cedarlane Laboratories). T cell-depleted samples were cultured in RF10 complete medium (RPMI 1640 supplemented with 10% FBS (Gemini Bio-Products), 100 U/ml penicillin/streptomycin, 2 mM l-glutamine, 10 mM HEPES [N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid], 1 mM nonessential amino acids, 1 mM sodium pyruvate, and 2.5 × 10−5 M 2-ME containing 1000 IU/ml recombinant human IL-2 (rhIL-2; Developmental Therapeutics Program, NCI) at 1 to 2 × 106 cells/ml for 5 to 7 days at 37°C, 5% CO2. At day 3 or 4, nonadherent cells were transferred to new flasks, and all cells were fed with 50% conditioned medium, 50% new RF10 complete medium, and rhIL-2. Only the adherent cells were used for experiments. This was comprised of >95% NK1.1 CD3−, 3% to 5% NK1.1 CD3+ and <1% NK1.1 CD3+ cells. Purified NK cells were generated by negative selection using a MACS NK Cell Isolation Kit (Miltenyi Biotec).

Cytotoxicity assays

Tumor target cells C1498 or Renca were labeled with 111In-labeled Oxine or 111In Oxine (Med-Physics) as previously described (13). The cells were pretreated with 20 nM of bortezomib for 24 h before the addition of activated NK cells. After 24 h at 37°C, the release of isotope from target cells was calculated as previously described. Anti-FasL (MFL-1) and anti-TRAIL (N2B2) were used at 20 μg/ml in some assays (13). For some assays, C1498 cells were pretreated with indicated amounts of bortezomib and labeled with 53Cr (PerkinElmer) and used in a standard 4-h chromium release assay as described previously (13). The percent lysis was calculated as follows: (experimental lysis – spontaneous lysis)/(maximal lysis – spontaneous lysis) × 100. For granule killing assays, the rat NK cell tumor cell line RNK-16 was used to generate granules as previously described (14). In brief, RNK-16 cells were grown in ascites in F344 rats before cell disruption by nitrogen cavitation and 54% Percoll (Sigma-Aldrich) centrifugation. The granule preparation used in the assay demonstrated 5676.01 LU/ml. One lytic unit is the amount of perforin needed to mediate 50% lysis of the RBCs. Dilutions of isolated granules were incubated with control or bortezomib-treated C1498 that were treated with 5 mM 2-deoxy-D-glucose for 1 h at 37°C, 5% CO2 in round-bottom microtiter plates (BD Biosciences). A total of 1.5 mM NaN3, 1 mM KCN, and granules were then added and the assays were incubated for 4 h. The assay buffer contained 10 mM HEPES, 0.15 M NaCl, and 10 μg/ml BSA (A4503; Sigma-Aldrich) (pH 7.5). Plates were centrifuged and supernatants were analyzed using a Cobra II autot counter (PerkinElmer). Saponin (Sigma-Aldrich), 0.015% final concentration, was added to produce maximal target lysis.

Guava ViaCount kit

The Guava ViaCount kit was purchased from Guava Technologies and used in coordination with the Guava Personal Cytometry system. The Guava ViaCount kit allows for the identification of dead cells. Cells were added to Guava ViaCount Reagent and incubated for 5 min. Samples were run on a Guava PCA and analyzed using CytoSoft software (Guava Technologies).

Ex vivo purging of tumor: survival studies and tumor outgrowth

For in vivo studies, 4.5 × 106 BMCs from B6 mice and 4.5 × 106 C1498 cells were treated with 20 nM bortezomib for 4 h. BMC/tumor cultures were then washed and cultured with C57BL/6 or Ly5.2 activated NK cells at BMCC1498/activated NK cell ratio of 100:1 in RF10 complete medium with 1000 IU/ml rhIL-2 for 20 h. Cocultured cells were harvested, washed, and brought up in 10 ml RF10 complete medium. One hundred μl of each cell culture condition were plated in 96-well flat-bottom plates. Surviving tumor cells were analyzed at indicated days by adding 1 μCi of [3H]thymidine (Amersham Pharmacia Life Sciences) 16–18 h before harvesting, and thymidine incorporation was assessed in the presence of scintillation fluid on a 1450 Microbeta TRILUX Liquid scintillation luminescence counter (PerkinElmer). Three individual wells were analyzed per data point. Residual cells from cocultures were washed 2× in PBS then injected into B6 recipients irradiated with a single exposure of irradiation from a cesium 137 Cs source at 9.5 Gy (8 mice per group per experiment) at 4.5 × 105 BMCs, 4.5 × 105 NK cells, and 4.5 × 105 C1498 cells per mouse (i.v.) based on the cell numbers at the initiation of cocultures. Experiment was performed one time. Mice were euthanized when moribund in accordance with Institutional Animal Care and Use Committee guidelines.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 4 software (GraphPad). T-tests, one-way ANOVA, and two-way ANOVA were used where appropriate. A p value of <0.05 was considered significant. Means ± SDs are shown.

Results

Sensitization of tumor cells to NK cell-mediated lysis by perforin is not augmented by bortezomib

Because we and others have found that bortezomib could sensitize tumor cells in vitro to lytic pathways such as TRAIL (6, 15), we sought to determine whether NK cell-mediated killing was augmented by preincubation of tumor cells with bortezomib. To address this, we cultured the C1498 leukemia cell line with various concentrations of bortezomib for up to 24 h. The dose of 20 nM was used for the NK cell coculture studies as no significant cytotoxicity of the C1498 leukemia cells was observed at this dose alone (data not shown). When activated syngeneic NK cells were then cultured with labeled tumor cells at various E:T ratios for a 4-h release assay, it was observed that the activated NK cells were capable of mediating significant lysis of the C1498 targets (Fig. 1A). However, the prior incubation of the tumors cells with bortezomib did not result in any demonstrable increase in tumor lysis. Similar results were observed when a longer-term assay (24 h) using 111Indium release was used (Fig. 1B). Thus, prior incubation of tumor cells with bortezomib does not appear to result in increased NK cell-mediated lysis as determined by short-term (4–24 h) in vitro assays. These assays demonstrated that NK cell-mediated tumor killing did not increase in short-term assays, though it was uncertain whether these assays verified the effect of bortezomib on lytic killing pathways. To determine whether direct perforin-mediated killing could be augmented by bortezomib preincubation of target cells, purified granules from
the rat NK line RNK-16 were obtained. These granules, which are highly enriched for both perforin and granzymes, were incubated with bortezomib-treated C1498 cells. Treatment of the tumor cells with a poison (5 mM 2-deOG, 1.5 mM NaN₃, 1 mM KCN) to make them more sensitive to granule killing resulted in significant increase in lysis (Fig. 1C) (16). However, preincubation with bortezomib by itself or with the poison did not result in any increase in lytic capability of the granules. On the contrary, the preincubation with bortezomib and the poison resulted in significant protection from granule-mediated killing (p < 0.05; Fig. 1C). These results support the data with activated NK cells and the short-term assays, and suggest that bortezomib pretreatment does not predispose tumor cells to perforin/granzyme-mediated lysis.

Bortezomib pretreatment of leukemia cells results in the up-regulation of Fas and DR5 expression and results in increased natural killer cell-mediated killing when perforin-deficient natural killer cells are used as effectors

Pretreatment of C1498 leukemia cells to bortezomib resulted in marked up-regulation in surface expression of both Fas and DR5 (receptor for TRAIL) as determined by flow cytometric analysis (Fig. 2). These data are in contrast to bortezomib-mediated down-regulation of the MHC class I molecule H₂Kᵇ (Fig. 2A), which is in agreement with previously published results using other proteasome inhibitors (17). These bortezomib-induced increases in Fas and DR5 might potentiate NK cells lysis involving the death ligands FasL or TRAIL. Interestingly, the increase in expression of DR5 was maintained for >24 h after the removal of bortezomib by washing the tumor cells (Fig. 2F). Activated NK cells from wild-type mice could efficiently lyse labeled C1498 tumor cells, and pretreatment with bortezomib had no effect. In addition activated NK cells from another strain (BALB/c; H₂Kᵇ) efficiently lysed the syngeneic renal cell cancer line Renca (H₂Kᵇ) in the presence or absence of bortezomib pretreatment. Thus, this lack of an effect of bortezomib on NK killing capability by activated wild type NK cells was neither tumor nor NK cell strain-specific (Fig. 3). We then assessed the effects on NK cell killing using NK cells from mice deficient in perforin. Activated NK cells from perforin-deficient mice had markedly reduced cytotoxic potential compared with NK cells from wild-type mice in agreement with previous reports (Fig. 3) (18). However, preincubation of C1498 leukemia cells with bortezomib now resulted in significantly increased cytotoxicity of the targets when cocultured with the activated perforin-deficient NK cells using the 24 h ¹¹¹Indium release assay. Similar to WT mice, gld NK cells, which are deficient in FasL yet express perforin, did not have any increased cytotoxicity following bortezomib pretreatment of target cells (Fig. 3). To determine the pathways responsible for this increased cytotoxicity, neutralizing Abs to TRAIL and Fas ligand were used (Fig. 4). Both Abs could partially reduce the increased lysis of C1498 cells by perforin-deficient NK cells, with almost total abrogation being observed in the presence of both Abs (Fig. 4D). Thus, NK cell-mediated tumor cytotoxicity using FasL or TRAIL can be augmented by bortezomib and that can be detected even in short-term (up to 24 h) lytic assays in the absence of the perforin lytic pathway.

Resting and activated NK cells are sensitive to bortezomib

To examine the sensitivity of NK cells to bortezomib, activated NK cells were incubated for 24 h in culture medium containing 1000 IU/ml rhIL-2 and the indicated dose of bortezomib (Fig. 5A). As little as 1.3 nM bortezomib was significantly cytotoxic to activated NK cells (p < 0.05). We then examined the effect of bortezomib on resting NK cells. Freshly enriched NK cells were incubated in culture medium containing no IL-2 and indicated amounts of bortezomib (Fig. 5B). Resting NK cells were less sensitive to bortezomib than activated NK cells, though 10 nM bortezomib significantly decreased the viability of resting NK cells (p < 0.05). These data were similar to resting NK cells treated with bortezomib in the presence of 1000 IU/ml IL-2 (data not shown). This suggests cotreatment of tumor cells with bortezomib in the presence of NK cells would result in reduced NK cell-mediated cytotoxicity due to direct effects of bortezomib on NK cells. Therefore, superior NK cell-mediated killing needs to occur by...
pretreating tumor targets with bortezomib before the introduction of NK cells.

Bortezomib pretreatment of leukemia cells markedly enhances antitumor effects of natural killer cells, when determined by long-term clonal survival of tumor cells. The survival of even very small numbers of C1498 cells can result in significant lethality in vivo. We therefore used long-term tumor clonal survival assays in vitro to assess whether bortezomib pretreatment of C1498 cells could enhance the antitumor activity of WT NK cells. Activated NK cells were cocultured with C1498 leukemia cells, and after 7 days, the number of tumor cells was determined by MTT. We have previously used this assay to assess NK cell-mediated antitumor effects, and ascertained that only the leukemia cells survive and proliferate with long-term in vitro culture. The results demonstrate that a 20 h coculture of activated NK cells alone with tumor cells at a 1:1 E:T ratio results in significant antileukemic activity when assessed 3 days after coculture (Fig. 6B). Greater than 90% of the viable cells assessed by flow cytometric analysis were leukemia cells (data not shown). Interestingly, incubation of the leukemia cells alone with bortezomib had no effect on tumor growth using this exposure length and dose. When the combination of bortezomib and activated NK cells were used, after 3 days similar antitumor effects were obtained to those of NK cells alone. However, when the cell cultures were allowed to grow for a total of 10 days, interesting differences were observed. Only the group that had the tumor-BMC mixture purged with the combination of NK cells with bortezomib had no tumor outgrowth whereas the residual tumor cells in the group that were cultured with NK cells alone expanded significantly (Fig. 6C).

**FIGURE 2.** Bortezomib increases Fas and DR5 expression while reducing MHC class I expression. C1498 tumor cells were untreated (dashed lines) or pretreated for 24 h with 20 nM bortezomib (solid lines) before labeling with FITC-anti-H2Kb (A), PE-anti-Fas (B), or anti-DR5 and PE-anti-hamster IgG (C). Histograms representing mean fluorescence intensity for expression of H2Kb (D), Fas (E), or DR5 (F) are also shown (n = 3). C1498 cells were treated with 20 nM bortezomib for 4 h, at which time the cells were washed and DR5 expression was examined. G, DR5 expression before treatment (thick black line), immediately after 4 h of bortezomib (thick gray line), and 24 h after bortezomib has been removed (thin gray line) are shown. Data are representative of three individual experiments.

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Thus, the combination of activated NK cells with bortezomib results in the greatest antileukemic effects in long-term tumor outgrowth assays suggesting that lytic pathways other than perforin may indeed contribute to the antitumor effects, and these could be enhanced by bortezomib pretreatment of the tumor. It was then determined whether these effects seen in the long-term in vitro assays could be mirrored with in vivo tumor outgrowth (Fig. 6A). Lethally irradiated syngeneic mice received the tumor-BMC mixtures used in the previous experiments and were observed for effects on survival from the leukemia. Similar to the data obtained in the long-term tumor grow-out assays, only the mice receiving the tumor-BMC mixture that also were cultured with activated NK cells and bortezomib demonstrated significant increases in survival (Fig. 6D). No adverse effects on donor hemopoietic reconstitution

FIGURE 3. Bortezomib does not increase the killing of perforin-competent activated NK cells. A, C1498 cells were pretreated with 20 nM bortezomib for 24 h before the addition of C57BL/6 wt, pfp−/−, or gld activated NK cells for a 24 h Indium release assay. B, Renca cells were pretreated with 20 nM bortezomib for 24 h before the addition of wt BALB/c or C57BL/6 pfp−/− activated NK cells for a 24 h Indium release assay.

FIGURE 5. Activated and resting NK cells are sensitive to bortezomib. A, Day 5 activated NK cells were cultured in the presence of the indicated amounts of bortezomib and 1000 IU/ml rhIL-2 for 24 h. Cell proliferation was determined using [3H]thymidine incorporation. B, NK cells were negatively selected using a MACS NK cell isolation kit, and cultured for 24 h in the presence of indicated amounts of bortezomib. Cells were then analyzed for viability using Guava ViaCount and reported as fold increase in NK cell cytotoxicity. Statistical analysis was performed using a one-way ANOVA with Dunnett’s post test.
were observed using this regimen demonstrating that the combination of bortezomib and activated NK cells were preferentially destroying leukemic cells yet sparing normal hemopoietic cells in vitro (data not shown). Thus, the combination of bortezomib with activated NK cells results in marked increases in antitumor activity both in vitro and in vivo, which in part is due to increased susceptibility of the leukemia cells to NK cell lytic pathways.

Discussion

Molecular targeting in cancer offers great promise in selectively attacking tumor cells. Recent attention has focused on the ability of these agents to also predispose the cancer to other mechanisms of attack. In particular, augmenting immune attack is attractive in further allowing for selective killing of the tumor to occur. Proteasome inhibition with bortezomib has been shown to have potent and direct antitumor effects on many cancers, particularly multiple myeloma (19). We have shown that bortezomib could also sensitize tumor cells to TRAIL-mediated killing (6). We now show that this sensitization can be extended to cell-mediated killing pathways by activated NK cells. In this study, the sensitization correlated with tumor cell up-regulation of Fas and the TRAIL receptor, DR5. Bortezomib has been shown to markedly inhibit NF-κB, which has been shown to affect tumor cell survival and most likely contributes to the increased susceptibility to death receptor pathways. However, proteasome inhibition can affect numerous other cellular pathways, which may also play a role in the increased NK cell killing observed.

The predominant lytic pathway attributed to NK cells is through granule-mediated killing via perforin (10). This killing pathway is often reflected in short-term lysis assays. However, we observed no increase in tumor cell killing with bortezomib using these short-term killing assays. There is a fine line with the amount of bortezomib that the tumor cells can be exposed to due to the observations that bortezomib has potent direct inhibitory effects on many of these tumor lines (20, 21). Using a dose of bortezomib that was at the upper threshold but did not elicit direct cytotoxic effects on the tumor cell, no increase in killing was observed after coculture with NK cells using assays that ranged from 4 to 24 h in length. The data using short-term killing assays is also consistent with data using purified NK cell granules in which no increase in lysis was observed using targets incubated with bortezomib. Interestingly, bortezomib-treated targets were protected from granule-mediated lysis (Fig. 1C). It is possible that bortezomib pretreatment reduces uptake of the 2-deoxy-glucose and thus the cells are less poisoned. Previous studies show that chemotherapeutically treated cells have reduced 2-deoxy-glucose uptake of fluoro-2-deoxy-glucose following treatment with etoposide (22). This reduction in 2-deoxy-glucose may explain the
reduction in lysis by granule proteins following bortezomib treatment.

The only means by which increased killing was observed using NK cells and bortezomib was using the 24 h assay and using NK cells obtained from perforin-deficient mice, which significantly impaired the extent of short-term cytotoxicity observed. Under these conditions, increased cytotoxicity was observed using bortezomib-treated targets and this increase was completely abrogated when neutralizing Abs to both TRAIL and FasL were used in our experiments using perforin-deficient NK cells. Interestingly, the addition of blocking Abs to FasL or TRAIL to perforin-replete activated NK cells show no effect on lysis of either control or bortezomib-treated targets (data not shown). This finding is likely due to the redundant killing pathways of NK cells, in which perforin plays a dominant role in assays in which lysis is measured. It is predictable that in assays where target cells are surrounded by 10-fold more perforin-replete NK cells that significant effects of TRAIL- or FasL-mediated killing would not be observed.

The role of TRAIL and FasL was also supported by the data demonstrating increased expression of these target molecules by the tumor after bortezomib exposure, though the slight increase in DR5 and Fas expression is likely not as influential as the down-regulation of c-FLIP following bortezomib treatment (6). No increase in perforin-mediated killing was observed as the direct lytic capabilities of these molecules on the cellular membrane make it an unlikely target for proteasome inhibition compared with apoptotic pathways. Granule proteins consist of not only perforin but also granzymes, such as granzyme B, which can indirectly activate caspase 3 leading to apoptosis (23). However, these apoptotic events are not regulated by c-FLIP, which interacts with the intracellular portion of death receptors such as DR5 and has no known role in granzyme-mediated apoptosis. The observations by us and others showing the apoptotic pathways (i.e., c-FLIP) are particularly affected by bortezomib is also consistent with the data showing increases in TRAIL and Fas-mediated lysis by the NK cells after tumor cell treatment. It is possible that membrane-repair pathways could be affected by proteasome inhibition and the perforin pathway could still be affected albeit to a lesser extent.

Previous reports have demonstrated that TNF-α, but not perforin, FasL, or TRAIL, was responsible for the increased bortezomib-mediated sensitization of the B16 melanoma cell line (24). Using our perforin-deficient mice, we observed that sensitization occurred primarily through the apoptosis-inducing molecules FasL and TRAIL. This discrepancy may be due to the different cell lines used, as B16 has previously been shown to be insensitive to TRAIL (25). Furthermore, FasL-mediated apoptosis of B16 tumors has been shown to be dependant on the presence of IFN-γ (26). In the study performed by Schumacher et al. (24), agonist anti-Fas Abs were used as opposed to cellular effectors. The presence of activated NK cells in our study would provide IFN-γ that may be capable of engaging other death pathways.

A recent study by Lundqvist (15) has previously shown that pretreatment of tumors with either bortezomib or depsipeptide increases sensitivity to TRAIL-mediated NK cell killing, in agreement with our findings. However, while their study concluded that Fas-mediated cytoxicity was not affected, we find an increase in Fas expression correlating with increased sensitivity to FasL-mediated cytotoxicity. We also observed a decrease in MHC class I expression following bortezomib treatment (Fig. 2) in contrast to the studies by Lundqvist (15). These discrepancies may be attributed to differences in tumors or to dose of bortezomib used. Our studies used a higher dose of bortezomib (20 nM) than that used in the studies by Lundqvist (10 nM), which may be result in further alterations to the tumor.

Although marked increases in Fas and DR5 were observed, slight decreases in MHC expression were also observed after bortezomib treatment of the tumor cells. However, the extent of decrease was minor and this most likely does not represent a significant mechanism underlying the increase in NK cell killing. Previous studies demonstrate that the lack of MHC class I on target cells exposes these cells to NK cell-mediated killing in short-term, presumably granule-mediated killing (27, 28). As we did not see any increase in killing in short-term assays using perforin-competent NK cells, this would suggest that the decrease in MHC is not affecting granule-mediated lysis by NK cells. Other studies have shown that engagement of MHC by inhibitory molecules resulted in decreased expression of FasL on NK cells, suggesting that MHC down-regulation may also be involved in death ligand-mediated killing by NK cells (29). More studies assessing MHC modulation will be performed to determine the extent this decrease in MHC expression contributes to NK cell killing.

The use of longer term tumor outgrowth assays allowed for the detection of the increased killing using NK cells from normal mice. These assays may better represent the in vivo potential and antitumor capabilities of NK cells as the in vitro effects correlated with in vivo survival when the cells were transferred into lethally irradiated recipients. Interestingly, the survival of the mice receiving the tumor-BMC mixture correlated with the longer (10 day) tumor outgrowth data in which differences between the groups receiving NK cells alone vs bortezomib and NK cell purging was noted. The use of longer term assays most likely allow for the observance of various lytic pathways that the NK cell has in its arsenal and can use in tumor cell killing. We are currently examining whether bortezomib can augment the inhibition of tumor cell growth by cytokines produced by NK cells, notably IFN-γ and TNF. The data by Schumacher et al. (24) would suggest that these also may be augmented. These results would also suggest that the use of these longer-term assays may allow for a more complete picture and potential dissection of NK cell-mediated tumor cell inhibition. It is possible that perforin-mediated killing dominates early and then is followed by the use of death-receptor pathways in conjunction with cytokines (which also can up-regulate these same death receptor target molecules on the tumor cell), especially at lower E:T ratios. It is important to note that while perforin is still the dominant killing pathway used by NK cells in long-term, tumor outgrowth assays, that perforin-deficient NK cells can still mediate cytotoxic effects albeit at higher E:T ratios (data not shown).

An unfortunate consequence of bortezomib exposure was that activated NK cells were also inhibited. This is perhaps expected given the critical role of NF-κB in mediating immune cell activation and proliferation. This may also be a significant limitation in applying bortezomib with NK cells as an adoptive immunotherapy in vivo. Indeed, we have found attempts to coadminister bortezomib in combination with activated NK cells to tumor-bearing mice to be unsuccessful, even when bortezomib is administered before the NK cells by several days (data not shown). It may be possible to apply this approach, but the timing of bortezomib administration may be critical such that deleterious effects on the transferred immune cells does not predominate and negate any increases in tumor killing susceptibility. Alternatively, use of rest- and not activated NK cells may make these cells more resistant to these inhibitory effects of bortezomib. The lack of inhibitory effects on donor myeloid reconstitution would suggest that the antitumor effects of bortezomib outweigh the effects on normal cells. More studies will be needed to ascertain how long the increased susceptibility of tumor cells to lytic pathways can exist after cessation of exposure to bortezomib.
The results in this study demonstrate that murine NK-cell-mediated antitumor effects can be augmented by tumor exposure to proteasome inhibition. It is highly likely that similar results would be observed by other immune effector cells. Indeed, we have data that T-cell-mediated antitumor effects can also be augmented by tumor exposure to bortezomib (T. J. Sayers and A. Shanker, manuscript in preparation). This may represent another pathway in which bortezomib mediates antitumor effects after administration and potential venue that can be exploited with immune therapy. The advantage of cellular effectors in cancer therapy is their potential ability to seek out and kill disseminated tumor cells. These data therefore suggest that proteasome inhibition with bortezomib may be of use for augmentation of immune therapy in cancer involving these cell-types and/or their lytic pathways.

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Bortezomib increases NK cell killing