Bortezomib Improves Adoptive T-cell Therapy by Sensitizing Cancer Cells to FasL Cytotoxicity

Anil Shanker1,2,3, Samuel T. Pellom Jr.1,3,4, Duaafia F. Dudimah1, Menaka C. Thounaojam1, Rachel L. de Kluyver5, Alan D. Brooks5,6, Hideo Yagita7, Daniel W. McVica5, William J. Murphy8, Dan L. Longo9, and Thomas J. Sayers5,6

Abstract

Cancer immunotherapy shows great promise but many patients fail to show objective responses, including in cancers that can respond well, such as melanoma and renal adenocarcinoma. The proteasome inhibitor bortezomib sensitizes solid tumors to apoptosis in response to TNF-family death ligands. Because T cells provide multiple death ligands at the tumor site, we investigated the effects of bortezomib on T-cell responses in immunotherapy models involving low-avidity antigens. Bortezomib did not affect lymphocyte or tissue-resident CD11c+ CD8+ dendritic cell counts in tumor-bearing mice, did not inhibit dendritic cell expression of costimulatory molecules, and did not decrease MHC class I/II-associated antigen presentation to cognate T cells. Rather, bortezomib activated NF-kappaB in CD8+ T cells, stabilizing expression of T-cell receptor CD3ζ and IL2 receptor-α, while maintaining IFNγ secretion to improve FasL-mediated tumor lysis. Notably, bortezomib increased tumor cell surface expression of Fas in mice as well as human melanoma tissue from a responsive patient. In renal tumor-bearing immunodeficient Rag2−/− mice, bortezomib treatment after adoptive T-cell immunotherapy reduced lung metastases and enhanced host survival. Our findings highlight the potential of proteasome inhibitors to enhance antitumor T-cell function in the context of cancer immunotherapy.

Cancer Res; 75(24); 5260-72. ©2015 AACR.

Introduction

The proteasome is an essential component of the cellular protein degradation machinery. The greater dependence of cancer cells on the proteasome to remove aberrant proteins compared with nonmalignant cells, as well as the reliance of various tumors on the proteasome-dependent NF-kB activation pathway to maintain resistance to apoptosis, makes cancer cells selectively more susceptible to proteasome inhibitors (1). Bortezomib (Velcade/PS-341) is a dipetidyl boronate proteasome inhibitor that has been approved by the U.S. Food and Drug Administration for the treatment of multiple myeloma (2) and mantle cell lymphoma (3) and its use has been extended to advanced stage non-small-cell lung cancer (4). As shown by us and others, bortezomib sensitizes solid tumor cells to TRAIL or its receptor agonist mAb by amplifying tumor cell caspase-8 activation in the death-inducing signaling complex following death receptor ligation (5–8). However, as a single agent bortezomib is ineffective in most solid cancers, and there are concerns in combining bortezomib with adoptive T-cell therapy because of reports purporting immunosuppressive actions of bortezomib (9, 10). Indeed, concerns over the possible side effects of bortezomib on immune effector functions have been raised recently (11–15). On the other hand, there are a number of reports indicating that bortezomib either directly or indirectly can play a positive therapeutic role in amplifying immune antitumor effector functions (16–22). Nonetheless, to date there has been no systematic study of the effects of bortezomib on adoptive cellular immunotherapy (ACT) in mouse preclinical cancer models in vivo.

Studies using the influenza viral hemagglutinin (HA) expressed as a model tumor antigen (Ag) in a renal cell carcinoma (RencAHA), as well as a self-Ag in pancreatic islet β-cells in insulin-HA transgenic mice, showed that host T cells could react to a self low-avidity epitope of HA and reject HA-expressing tumor cells without inducing autoimmune diabetes (23). Taking advantage of this low-avidity model antigen and the availability of MHC class I and II–restricted HA-specific T-cell receptor (TCR)–transgenic Cln4 and 6.5+/+ mice, respectively (23, 24), we investigated the effects of bortezomib administration on T-cell responses in an adoptive T-cell transfer setting in mice with established renal RencAHA or mammary 4T1.2HA adenocarcinoma tumors. We compared the effects in two distinct in vivo protocols, one tumor...
therapeutic regimen of bortezomib (Bzb-T) standardized by us earlier (7) with another known suppressive regimen of bortezomib (Bzb-S) close to the maximal tolerated levels for this drug. The suppressive Bzb-S regimen was adapted from Sun and colleagues’ study, where bortezomib suppressed acute lethal graft-versus-host (GVHD) response in myeloablated mice following allogenic bone marrow transfer (10).

Surprisingly, adoptive T-cell transfer followed by bortezomib administration resulted in reduced numbers of lung metastatic tumor nodules and enhanced survival of tumor-bearing mice when compared with either treatment alone. The therapeutic regimen of bortezomib we used in this study had no major effect when compared with either treatment alone. The therapeutic regimen of bortezomib (Bzb-S) close to the maximal tolerated levels for this drug. 

Materials and Methods

Mice

BALB/c and recombination-activating-gene-2−/− (Rag2−/−) mice (7 to 12-week) were bred at the National Cancer Institute (NCI)-Frederick, or at Meharry Medical College. TCR transgenic Cln4 mice were provided by Linda A. Sherman, The Scripps Research Institute, La Jolla, CA. Cln4 mice were established on Rag2−/−, Pip−−, and gld/gld backgrounds. TCR transgenic 6.5+/+/− mice were provided by Hyam I. Levitsky, Johns Hopkins University, Baltimore, MD. TCR transgenic mice were genotyped according to the methods described earlier (23, 25). Mice were cared for in accordance with the procedures outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals. NCI-Frederick and Meharry Medical College are accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International and follow the Public Health Service Policy for the Care and Use of Laboratory Animals under pathogen-free conditions.

Cell lines

The RencaHA line (courtesy Hyam I. Levitsky), 4T1.2 (courtesy Suzanne Ostrand-Rosenberg, University of Maryland, Baltimore, MD), and C26 and A20 (ATCC) were maintained in FCS-supplemented standard RPMI-1640 culture medium. We regularly go back to reference stocks to ensure fidelity; routine sterility and mycoplasma testing are performed regularly. Low-passage (<5) tumor cell cultures were used for the experiments.

Human melanoma tissues

Paraffin-embedded melanoma tissue sections were provided by Ann Richmond and Jeffrey A. Sosman, Vanderbilt University Medical Center, Nashville, TN. They were collected in a phase I clinical trial (26) that included 19 histologically proven, advanced-stage (III or IV) metastatic melanoma patients (17 M, 10 elevated lactate dehydrogenase, 12 Eastern Cooperative Oncology Group (ECOG) performance status 1–2) enrolled onto escalating dose levels of temozolomide (50–75 mg/m²) daily, orally, for 6 of 9 weeks and bortezomib (0.75–1.5 mg/m²) by i.v. push on days 1, 4, 8, and 11 of every 21-d cycle. Twelve paired melanoma tissue samples collected on day 0 before treatment and on days 8 to 45 after treatment were analyzed for Fas immunostaining.

Tumor monitoring

Mice injected with tumor cells were monitored weekly for the relevant outcome (i.e., tumor metastasis or survival). Tumor metastatic nodules in mice intravenously injected with RencaHA cells were counted under a dissection microscope on the surgically removed lungs, fixed in Bouin’s fluid.

T-cell adoptive transfer

The naive HA-specific monoclonal CD8 T cells were obtained from the lymph node (LN) cells or red blood cell (RBC)-depleted splenocytes of Cln4 Rag2−/− mice. They were washed and injected intravenously in tumor-bearing Rag2−/− mice. In some experiments, a hamster neutralizing antibody to Fasl (M1L4) or a control isotype antibody (UC8-1B9; generated at Juntendo University, Japan) was injected i.v. at 0.5 mg/mouse on days 7 and 10 following injection of RencaHA cells.

Immunoblotting

CD8+ T cells were purified from splenocytes by incubating cells with anti-rat-mouse CD8 mAb, followed by positive selection of CD8+ cells with anti-rat IgG Microbeads (Miltenyi Biotec) and cell pellets were lysed in complete lysis buffer including protease and phosphatase inhibitors. Nuclear and cytoplasmic extracts were prepared using Ne-per nuclear and cytoplasmic extraction reagent (Life Technologies). Fifty micrograms of each protein sample was electrophoresed on NuPage 4% to 12% Bis-Tris gel (Novex Life Technologies) and transferred to polyvinylidene fluoride membranes using an iBlot Dry Blotting System (Life Technologies). The blots were then stripped and probed with HRP-conjugated anti-GAPDH antibody (Santa Cruz Biotechnology) at a dilution of 1:4,000 in 1X PBST, blots were incubated with goat anti-rabbit horseradish peroxidase (HRP; Santa Cruz Biotecnology) at a dilution of 1:4,000 in 1X PBST for 1.5 hours, with agitation. The blots were rinsed again in 1X PBST, and developed by using chemiluminescence reagent (Millipore) and a Kodak Image Station (Kodak). The blots were then stripped and probed with HRP-conjugated anti-GAPDH antibody (Cell Signaling Technology) to determine equivalent loading.

Immunohistochemistry

The immunohistochemical analyses of murine RencaHA solid tumors, thymus, or lungs were performed according to standard methods (27). Digital images of the specimens were captured using Spectrum image software (Aperio Technologies, Inc.). For human melanoma tissues embedded in paraffin, 6-μm-thick sections were cut, deparaffinized, and hydrated before treating for antigen retrieval with sodium citrate buffer for 20 minutes. Lab
Vision Autostainer360 was used for staining using Ultravision Quanto Detection System HRP DAB kit (#T-060-02HD). In brief, tissues were treated with peroxidase followed by UV block before treating with primary anti-human/mouse CD95/FAS (LS-B6851/33954) at 1:200 for 45 minutes. Tissues were then treated with labeled HRP and stained with 3,3′-diaminobenzidine for 5 minutes. Counter staining was done with hematoxin, dehydrated, and mounted in cytoseal. For negative control, tissues were stained with isotype IgG.

Proteasome inhibition assay
Red blood cell–depleted splenocytes, LN, lung, or excised tumor mass cells were prepared from mice at different time points after last bortezomib administration. Cells were plated in white 96-well plates at 1 × 10⁶ cells per well and assayed using the Proteasome-Glo Chymotrypsin-Like Cell-Based Assay (Promega) according to the manufacturer’s protocol.

Flow cytometry
Cells were stained for surface immunofluorescence staining with various antibodies or control isotypes (BD Biosciences or eBioscience Inc.) following FcR-blocking. Anti-hemagglutinin clone H28-E23 was obtained from Jonathan W. Yewdell (NIAID, Bethesda, MD) and was used with secondary sheep anti-mouse IgG clone H28-E23 was obtained from Jonathan W. Yewdell (NIAID, Bethesda, MD) and was used with secondary sheep anti-mouse IgG biotin and streptavidin-APC. IFNγ or CD3ζ intracellular staining was measured in T cells isolated from the tumor and the draining lymph nodes following ex vivo restimulation for 5 hours with 200 ng/ml ionomycin plus 10 ng/ml phorbol 12-myristate 13-acetate (Sigma) in the presence of GolgiStop protein transport inhibitor (BD Biosciences) as per the manufacturer’s protocol. Data were acquired on leukocyte gates as per forward and side scatters using a FACSCalibur or LSR-I flow cytometer (BD Biosciences) or Guava EasyCyte HT system (EMD Millipore) and analyzed using CellQuest (BD Biosciences) or FlowJo (Treestar Inc.) softwares.

T-cell proliferation
T-cell divisions were measured by flow cytometry using the intracytoplasmic stable carboxyfluorescein succinimidyl ester (CFSE) dye that exhibits sequential halving of intracellular fluorescence intensity at each division step. TCRHA CD8⁺ T cells were incubated for 10 minutes at 37°C with 5 μmol/L CFSE (Molecular Probes) and injected i.v. (2 to 5 × 10⁶ per mouse) in Rag2⁻⁻ mice or CD3ζ⁻⁻ mice expressing to irrelevant target cells was calculated to quantify specific target clearance.

Statistical analysis
Comparisons of mean values between the groups were analyzed using the GraphPad Instat software (GraphPad Prism). Statistical significance of the differences was analyzed by using unpaired Student t test or by analysis of variance (ANOVA). Comparisons of survival curves estimated by Kaplan–Meier plots using GraphPad Prism were performed by the log-rank (Mantel–Cox) test. All statistical tests were two-sided, with P values < 0.05 considered statistically significant.

Results
Effects of therapeutic and suppressive regimens of bortezomib on endogenous leukocyte numbers
We have previously reported that the proteasome inhibitor bortezomib (Velcade/PS-341) sensitizes solid tumors, such as renal or mammary adenocarcinoma cells to apoptosis in response to TNF-family death ligands or TRAIL receptor agonist mAb by amplifying caspase 8 activation in the death-inducing signaling complex, which translates into a survival benefit in tumor-bearing mice (7, 8). To address concerns over the possible side effects of bortezomib on immune functions, we tested two in vivo protocols...
with opposite effects on tumor burden and survival. The low-intensity tumor therapeutic regimen of bortezomib, Bzb-T, given twice a week (7) was compared with a previously described suppressive regimen of bortezomib, Bzb-S, given four times a week close to the maximal tolerated levels that suppressed acute lethal GVHD response in myeloablated mice following allogenic bone marrow transfer (10). Proteasome activity was determined following bortezomib administration as per the two regimens (Fig. 1A) in WT mice injected with RencaHA tumor cells subcutaneously. At 4 hours after last bortezomib administration, the cellular proteasome activity in spleen, tumor-draining LN, and tumor mass was reduced by 60% in the therapeutic protocol and 80% to 90% in the suppressive protocol (Fig. 1B). A similar level of proteasome inhibition was observed in the cell preparations of lungs at 4 hours after treatments, with no inhibition apparent in the tissues tested after 18 hours (data not shown). Furthermore, the Bzb-T regimen had no significant effects either on total leukocyte counts in control or tumor-draining LN, tumor-infiltrating lymphocytes, and spleen, or in subsets of CD4<sup>+</sup> T, CD8<sup>+</sup> T, and CD11c<sup>+</sup> cells (Fig. 1C–E) or in DX-5<sup>+</sup> NK cells (data not shown) analyzed in the tumor-draining LN on day 13 at 4 hours after last bortezomib injection. In contrast, the suppressive Bzb-S regimen resulted in a leukocytopenia (Fig. 1C–E), which may explain why bortezomib given at the time of allogeneic bone marrow transfer in mice attenuates acute GVHD (10).

**Effect of bortezomib treatment on antigen presentation in vitro and in vivo**

Because activity of the cellular proteasome is required to process antigens for presentation to T cells, we assessed whether
bortezomib might affect the ability of antigen-presenting cells to present antigen. We thus purified CD11c\(^+\) cells ex vivo from the spleen of mice (with no tumor injection) treated with either Bzb-T or Bzb-S regimens, and analyzed their capacity to process whole HA protein, pulsed by a noncovalent cationic Pro-Ject intracellular delivery reagent, and cross-present CD8\(^+\) T cells in vitro. Following a coculture of equal numbers of these purified CD11c\(^+\) cells from various groups of mice with a fixed number of TCRHA Cln4 CD8\(^+\) T cells for 3 days, we observed no differences in T-cell proliferation or IFN\(\gamma\) production. This suggested no reduction of antigen presentation on a per-cell basis by isolated splenic CD11c\(^+\) cells from the mice treated with the Bzb-T or Bzb-S regimens (Fig. 2). Thus, the cellular antigen presentation per se was not affected by bortezomib even though the total number of CD11c\(^+\) cells would be lower in mice treated with the suppressive Bzb-S regimen as per Fig. 1. Therefore, under these assay conditions, bortezomib has no major suppressive effect on T-cell function following HA antigen processing or presentation assessed in vitro.

Using the two experimental regimens of bortezomib administration, we also assessed HA antigen-specific proliferation of transferred CD4\(^+\) and CD8\(^+\) TCRHA T cells as a read out of the efficiency of class I and class II antigen presentation in an adoptive cell transfer setting in vivo. Lymphodecient Rag\(^{-/-}\) mice reconstituted with CFSE-labeled TCRHA 6.5\(^+\)/CD4\(^+\) or Cln4 CD8\(^+\) T cells were injected with RencaHA tumor cells and the Bzb-T and Bzb-S regimens were started on the same day (day 0) as shown in Fig. 2B. The HA-specific T-cell proliferation on day 4 in the LN draining to the site of injected tumor cells following therapeutic Bzb-T or suppressive Bzb-S regimens compared well with the untreated tumor-bearing mice (Fig. 2C) despite the 60% to 80% cellular proteasome inhibition (Fig. 1). We observed a decreased

**Figure 2.**

No diminution of antigen processing and cross-presentation by dendritic cells from bortezomib-treated mice. A, CD11c\(^+\) cells were harvested ex vivo at 4 hours after last bortezomib administration from the spleen of mice (with no tumor) treated according to the bortezomib regimens shown in Fig. 1A. They were CD11c-labeled magnetic bead-purified, irradiated, and pulsed for 2 hours with HA peptide (10\(^{-5}\) mol/L) or with HA protein (10\(^{-3}\) mol/L) along with Pro-Ject intracellular delivery reagent, and washed. Equal numbers of CD11c\(^+\) cells from various groups of mice were cocultured with CFSE-labeled Rag\(^{-/-}\) Cln4 TCRHA CD8\(^+\) T cells for 3 days. CFSE versus IFN\(\gamma\) staining is shown on gated CD8\(^+\) H2K\(^{b}\)-HA-tetramer\(^+\) cells. Numbers in dot plots depict the percentage of positive cells. Representative data are shown from five independent experiments. B, Rag\(^{-/-}\) mice were reconstituted with TCRHA 6.5\(^+\)/CD4\(^+\) or Rag\(^{-/-}\) Cln4 CD8\(^+\) T cells (5 \times 10\(^6\)) i.v. followed by injection of RencaHA tumor cells s.c. on the same day (day 0) when the bortezomib-therapeutic, Bzb-T, or suppressive, Bzb-S, regimens were also started according to the shown schedule. C, HA antigen-specific proliferation of CFSE-labeled CD4\(^+\) or CD8\(^+\) TCRHA T cells on day 4 in the tumor-draining LN is presented as readout of the efficiency of class I or class II antigen presentation. Numbers depict the percentage of undivided T cells. Italicized numbers depict total V\(\beta\)8.1/2 CD4\(^+\) or V\(\beta\)8.1/2 CD8\(^+\) cells, respectively. Representative data are shown from three (CD4\(^+\)) or four (CD8\(^+\)) independent experiments. D, expression of CD80, CD86, and CD40 on gated CD11c\(^+\) MHCII\(^+\) cells in the tumor-draining LN is shown. Numbers in dot plots and histograms depict the percentage of positive cells. Representative data are shown from three independent experiments. E, total cell counts of CD11c\(^+\) CD80\(^+\) CD86\(^+\) subset of DC in DLN expressing MHC II, CD80, CD86, or CD40 molecules. Data shown as mean values ± SD from three independent experiments. *P < 0.001 (ANOVA, two-sided) compared with saline or Bzb-T; n = 9, each group.
yield of Vβ8.1/2⁺CD4⁺ and Vβ8.1/2⁺CD8⁺ cells in the tumor-draining LN of mice treated with Bzb-S regimen, but not with Bzb-T regimen, with little effect on T cell proliferation per se. Even using an injection regimen where bortezomib was started one day before tumor injection, or using irradiated tumor cells instead of live tumor cells, no suppressive effect of bortezomib on subsequent T-cell proliferation was observed (data not shown). Moreover, there was no effect of bortezomib treatment on the expression of costimulatory molecules CD80, CD86, or CD40 by CD11c⁺MHCIi⁺ or costimulatory molecules CD80, CD86, and CD40 cells (Fig. 2E). It is important to note that even though the residual numbers of CD11c⁺MHCIi⁺DC are low following the suppressive Bzb-S regimen, they showed no decrease in the relative level of expression of CD80, CD86, and CD40 molecules (Fig. 2D), and no apparent decrease in CD4⁺ or CD8⁺ T-cell proliferation following the Bzb-S regimen (Fig. 2C). Thus, the effects of bortezomib appear merely quantitative and do not have a bearing on the maturation or antigen processing by CD11c⁺MHCIi⁺ tissue-resident antigen-presenting cells on a per-cell basis. Yet, if there is an excessive reduction in their numbers as it happens following the suppressive Bzb-S regimen, it may most likely compromise antitumor outcome. Indeed, treatment of mice with the Bzb-S (but not the Bzb-T) regimen immediately after injection of RencaHA cells substantially increased the number of metastases subsequently observed on day 14 (Supplementary Fig. S1). This immunosuppressive effect resulted in increases in the number of lung metastases following the injection of RencaHA cells even 4 or 7 days after treatment with the Bzb-S regimen (Supplementary Fig. S2). These data suggest that even though the suppressive Bzb-S regimen does not have a major effect on the maturation and antigen processing by individual DC per se, an excessive reduction in total DC numbers may still diminish antitumor immune effects in vivo. In contrast, the therapeutic Bzb-T regimen had no observable effects on lung metastases even when administered immediately after RencaHA injection (Supplementary Figs. S1 and S2). Moreover, we also observed that tumor cells treated with bortezomib in vitro exhibit higher expression of calreticulin (Supplementary Fig. S3). Increased cell surface calreticulin expression on tumor cells after bortezomib exposure could indicate “immunogenic cell death” so as to facilitate antigen uptake and presentation by DCs. These findings predict that the dose and regimen of bortezomib treatment is critical in deciding the outcome of antitumor immune response. Therefore, only the Bzb-T regimen was used in subsequent adoptive T-cell transfer experiments.

**Sustained T-cell function in vivo following bortezomib treatment**

We next investigated effects of bortezomib specifically on functional parameters of T-cell activation, proliferation, and effector function in vivo. Following the establishment of palpable subcutaneous RencaHA or 4T1.2HA tumors ranging in size from 0.5 to 0.8 cm³ in lymphodecient Rag−/− mice, naïve CFSE-labeled TCRHA CD8⁺ T cells were transferred on day 6, followed by the Bzb-T regimen, and analyzed at 4 hours or 24 hours after bortezomib treatment on day 9 as per the indicated scheme (Fig. 3A). Following Bzb-T regimen, there was no statistically significant change in the total yield of K⁺:HA tetramer⁺ CD8⁺ T cells from the tumor-draining LN, nor was there a decrease or delay in HA-specific CD8⁺ T-cell proliferation as analyzed on gated K⁺:HA tetramer⁺ T cells in these mice either at 4 hours or 24 hours after last bortezomib injection (Fig. 3B). A similar proliferation pattern was observed for HA-specific CD4⁺ T cells (data not shown). Rather, as analyzed on gated CD8⁺ Vβ8.1/2⁺ cells in the draining LN of RencaHA or 4T1.2HA tumor-bearing Rag−/− mice on day 10, we observed that Bzb-T regimen led to an accumulation of intracellular CD3⁺ CD25⁺ cells in activated CD25⁺ T-cell subset (Fig. 3C). Zebra plots demonstrate a pronounced increase in the event density of CD25⁺ T cells in the right quadrant when compared with untreated mice, suggesting
stabilization of T-cell receptor CD3ζ in activated T cells following Bzb-T regimen. We also investigated the influence of bortezomib regimen on the endogenous antitumor response in a polyclonal T-cell environment in the WT mice established with RencaHA or 4T1.2HA tumors and injected with Bzb-T regimen without any adoptive T-cell transfer as per the scheme shown in Fig. 3A. Results show that in gated CD8+Vβ8.1/2+ T cells (frequency in the range of 8% to 10%) from the tumor-draining LN of WT mice, there is an enhanced secretion of intracellular IFNγ and CD3ζ expression following administration of the therapeutic regimen of Bzb-T in both RencaHA as well as in the aggressive breast adenocarcinoma model 4T1.2HA (Fig. 4A). We also assessed immune cell viability by performing 7AAD staining along with CD3 or CD11c. Data reveal no increase in 7AAD+ cells either in CD3 or CD11c subsets, suggesting no loss of cell viability following the therapeutic Bzb-T regimen in the WT mice (Fig. 4B). These data suggest that there is no suppression of endogenous T-cell response either in the monoclonal or polyclonal T-cell environment by the tested therapeutic regimen of bortezomib. Rather, it is evident that proteasome inhibition by bortezomib stabilizes the T-cell receptor CD3ζ, and thereby sustains the T-cell activation signals.

To determine any effect of bortezomib on the cytotoxic function of activated T cells, we evaluated the ability of transferred TCRHA CD8+CTLs to kill HA-expressing target cells in presence of bortezomib using an in vivo CTL assay. No reduction in HA-specific lysis was observed by CTLs in bortezomib-administered mice (Fig. 4C). To further elaborate on the underlying mechanisms of the observed T-cell effects, we investigated the influence of bortezomib on NF-κB activation downstream of CD3ζ signaling. NF-κB is a “rapid-acting” primary transcription factor that does not require new protein synthesis in order to become activated and act as first responder in regulating various genes responsible for both the innate and adaptive immune cell proliferation and effector function. Purified CD8+ T cells treated in vitro with bortezomib (10 nmol/L) for 4 hours showed an increase in the phosphorylation of p65 in both nuclear and cytoplasmic fractions (Fig. 4D), suggesting that bortezomib promotes NF-κB activation in T cells. Taken together, these data show that bortezomib has no discernible immunosuppressive consequences on T-cell function.

Figure 4. Endogenous T-cell activity in vivo following the therapeutic regimen of bortezomib. Bortezomib regimen was started on day 6 in WT mice established with RencaHA (5 × 10^6) or 4T1.2HA (2 × 10^6) subcutaneous tumors as per the schedule shown in Fig. 3A. At 4 hours after last day 9 bortezomib injection, gated CD8+Vβ8.1/2+ cells in the tumor-draining LN were analyzed for intracellular IFNγ and CD3ζ (A), or total tumor-draining LN cells were analyzed for 7AAD versus surface CD3 or CD11c (B). Numbers in dot plots or histograms depict percentage of positive cells. C, HA-specific lysis of HA-labeled (CFSEhigh) or irrelevant NP-labeled (CFSElow) splenocytes in bortezomib-administered syngeneic WT mice 18 hours after transfer of in vitro-activated TCRHA CD8+ CTLs. D, total and phosphorylated forms of p65 are shown in the nuclear and cytoplasmic extracts of purified splenic CD8+ T cells treated with 10 nmol/L bortezomib for 4 hours. Representative data from three independent experiments are shown.
Adoptive T-cell transfer in combination with bortezomib administration results in reduced lung metastases and enhanced survival.

The adoptive transfer of T cells could provide a natural source of multiple death ligands (FasL, TRAIL, TNF, etc.) for cancer therapy at the tumor site. Recent studies have illustrated that adoptive T-cell transfer provides the maximal therapeutic benefit when a lymphocyte depleting preconditioning regimen is used and less differentiated naïve CD8⁺ T cells (Tᵥ) are transferred (29). Therefore, we investigated the effects of bortezomib administration combined with adoptive transfer of CD8 Tᵥ cells in lymphodecient Rag2⁻/⁻ mice with a large established (7 day) lung tumor burdens of RencaHA cells as per the indicated schemes (Fig. 5A and C). No difference in the immunohistologic staining for CD3 was seen in lung tumor nodules following Tᵥ cell transfer in the presence or absence of bortezomib treatment (Fig. 5B), suggesting that bortezomib had no noticeable detrimental effect on T-cell migration and infiltration of the lung tumor mass. Moreover, in tumor-bearing mice treated with bortezomib (1 mg/kg body weight, approximately 20 nmol/L) alone, there was little effect on the large number of tumor metastases with mice exhibiting numerous pulmonary tumor nodules (mean 442 ± 184 SD per mouse) close to the range of saline controls (753 ± 438) (Figs. 5A and 6A). TCRHA CD8 Tᵥ cell transfer reduced the nodule count to a mean of 144 ± 103 per mouse. The tumor nodule count was further significantly reduced to a mean of just 25 ± 34 when bortezomib was administered 3 days after Tᵥ cell transfer (Figs. 5A and 6A). Interestingly, a 3-week therapy regimen of bortezomib post Tᵥ cell transfer, similar to the combination regimen of bortezomib and TRAIL receptor agonist mAb we used previously (7), significantly enhanced survival with 40% of mice still alive after a 90-day observation period (Fig. 5C).

Immunohistochemical analyses of lungs from mice treated with bortezomib alone or saline showed numerous large tumor nodules with prominent proliferating cell nuclear antigen (PCNA) staining in contrast with lungs from tumor-bearing mice treated with Tᵥ cells or a combination of Tᵥ cells and bortezomib (Fig. 6A). Moreover, increased apoptosis as evaluated by TUNEL staining was observed in lungs of tumor-bearing mice treated with a combination of TN cells and bortezomib compared with other groups (Fig. 6B). We speculate that increased apoptosis at the site of tumor may increase...
the availability of tumor antigens, and thus enhance tumor control by T-cell therapy as observed in our combination regimen. We also tested whether the observed therapeutic effect could be ascribed to bortezomib’s influence on tumor vasculature and stromal cells as suggested elsewhere (30–32). We did not observe major change in numbers of vascular endothelial cells in bortezomib-treated mice when compared with untreated mice. However, based on staining pattern for the vascular endothelial cell marker CD34 in the lungs of tumor-bearing mice treated with bortezomib, the global architecture of the tumor looked different, suggesting some effects on tumor vasculature following bortezomib regimen (Fig. 6C).

Bortezomib improves T-cell therapy by sensitizing tumors to the FasL lytic pathway

Although we observed a stabilized expression of MHC molecules on RencaHA tumor cells in vivo from mice administered with the Bzb-T regimen, the expression of Fas receptor on MHC-I Kd+ tumor cells increased from 67% to 96% in mice treated with Bzb-T regimen when compared with untreated mice (Fig. 7A). We also noted that various tumor cells, including RencaHA, breast 4T1, colon C26, and lymphoma A20, that showed upregulated Fas expression following overnight treatment with bortezomib (20 nmol/L) in vitro (data not shown), all exhibited enhanced sensitivity to lysis by recombinant FasL molecules (Fig. 7B). Moreover, these tumor cells were more sensitive to death ligand-mediated lysis by T cells, as various bortezomib-treated cancer cells loaded with HA peptide (10−6 mol/L) were very efficiently killed by TCRHA Cln4 Pfp−/− CTLs, which utilize the FasL but not the perforin-dependent granule exocytosis lytic pathway (Fig. 7C). This FasL-mediated tumor cell lysis was necessary for the antitumor effects of adoptively transferred T cells in vivo as adoptive transfer of in vitro activated Cln4 gld.gld CTLs, which lacked functional FasL, to Rag2−/− mice injected with RencaHA tumor cells i.v. resulted in a significantly greater number of pulmonary tumor nodules when compared with mice receiving Cln4 Pfp−/− CTLs or Cln4 CTLs (Fig. 7D), even though different CTL produced similar amounts of IFNγ (data not shown). These results suggest that the FasL pathway is critical for tumor clearance following bortezomib administration in those tumors that express Fas. Indeed, therapeutic benefit of the combination of adoptive TCRHA Tc cell transfer and bortezomib was almost completely blocked by the administration of a FasL-neutralizing antibody in the RencaHA model (Fig. 7E). We also noted upregulated Fas expression in human melanoma tissue sections (Fig. 7F) from one responding advanced-stage (III or IV) metastatic melanoma patient, who showed a partial response of 8 months following treatment with a combination of an alkylating drug temozolomide and bortezomib in a phase I clinical trial (26).

Discussion

We investigated the effects of bortezomib administration on adoptive T-cell transfer in lymphodecient Rag2−/− mice with established RencaHA tumors and designed in vivo protocols to examine MHC class I and II-restricted antigen presentation, dendritic cell function, T-cell activation and proliferation, and lysis of specific targets. Adoptive T-cell transfer followed by a tested therapeutic regimen of bortezomib resulted in a reduction in lung tumor nodules and enhanced survival. Taken together, our data suggest that T-cell function is sustained in vivo following administration of
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Figure 7. Bortezomib augments T-cell therapy by sensitizing tumors to FasL. A, MHC Kβ versus Fas staining on gated HA+ tumor cells harvested from mice administered with Bzb-T regimen. Numbers in dot plots represent percentage of positive cells. Plots representative of two independent experiments. B, percent decrease in tumor nodules on day 14 in Rag2-/- mice injected with RencaHA tumor cells i.v. following adoptive transfer of naive Cln4 CD8 T cells and bortezomib along with the administration of FasL neutralizing or isotype control antibody. *P < 0.001 (two-sided unpaired t test) compared with untreated samples. Data are representative of three independent experiments. C, percent specific lysis of bortezomib-treated RencaHA, 4T1, C26, and A20 tumor cells loaded with HA peptide (10−5 mol/L) by TCRHA Cln4 or Cln4APC-/- CTLs. *P < 0.001 (two-sided unpaired t test) compared with untreated samples. Data are representative of two independent experiments. D, counts of pulmonary tumor nodules on day 14 in Rag2-/- mice injected with RencaHA tumor cells i.v. following adoptive transfer of 5 × 106 CTLs/mouse i.v. on day 10. Cln4 gld/gld CTLs were compared with Cln4 Pbl-/- CTLs or Cln4 CTLs. *P < 0.001 (ANOVA, two-sided), n = 9. Data are representative of three independent experiments. E, counts of pulmonary tumor nodules on day 14 in Rag2-/- mice injected with RencaHA tumor cells i.v. following transfer of naive Cln4 CD8 T cells and bortezomib along with the administration of Fasl neutralizing or isotype control antibody. *P < 0.001 (ANOVA, two-sided). The FasL neutralized group was compared with isotype IgG group, n = 10, from two independent experiments. F, Fas expression analyzed using human and mouse cross-reactive CD95 antibody on human melanoma sections from advanced-stage (III or IV) metastatic melanoma patients treated with a combination of temozolomide (50-75 mg/m²) daily, orally, for 6 of 9 weeks and bortezomib (0.75-1.5 mg/m²) by i.v. push on days 1, 4, 8, and 11 of every 21-day cycle. Paired pre (day 0)- and post (day 45)-treatment melanoma tissue samples from the sole responder patient are shown. Murine thymus is shown as a positive control for Fas staining. Magnification, ×20; Mu, murine; hu, human.

the therapeutic regimen of bortezomib when compared with a suppressive regimen close to the maximal tolerated levels for this drug. These findings provide evidence that, with appropriate timing and dosing of bortezomib, a significant level of therapeutic benefit can be achieved by combining the drug with adoptive T-cell therapy for the treatment of solid tumors.

Bortezomib can have multiple direct effects on tumor cells, including disruption of DNA damage repair pathways, increased calcium levels (33), endoplasmic reticulum stress, aggresome formation (34), and alterations in mitochondrial function (33). In addition to direct antitumor effects, bortezomib can also have immunostabilizing effects on host immune system. Chang and colleagues observed that treatment of ovarian tumor cells with bortezomib led to the upregulation of hsp60 and hsp90 on the cell surface and promoted their phagocytosis by dendritic cells. Expression of heat-shock proteins on ovarian tumor cells is an important determinant of whether bortezomib-treated tumor cells can generate tumor-specific CD8 T cells. In addition, dendritic cells that were treated with bortezomib in vitro showed increased maturation, which resulted in potent immune-mediated antitumor effects (18). It is also reported that bortezomib induces apoptosis in immature DCs, but to a much lesser extent in mature DCs (35). Bortezomib enhanced antigen-specific cytotoxic T-cell responses against immune-resistant cancer cells generated by STAT3-ablated dendritic cells (36). Also, bortezomib could restore MART-1 antigen expression on human melanoma cells to sensitize them to specific CTLs in vitro (37).

It is worth noting that bortezomib inhibits inducible NF-κB activity, but can activate constitutive NF-κB activity by triggering phosphorylation of IκB kinase and its upstream receptor-interacting protein 2, thereby enhancing cytotoxicity in multiple myeloma cells (38). Our data also suggest that bortezomib sustained Fasl-mediated T-cell cytotoxicity either by stabilizing expression of IL2 receptor-α chain and intracellular T-cell receptor CD3ζ and IFNγ in T cells of tumor-bearing mice via increased phosphorylation of nuclear and cytoplasmic NF-κB p65 levels in CD8+ T cells or by inhibiting their proteasomal degradation. In line with our observations, another study demonstrated differential effects of bortezomib on...
NF-κB activity depending on different cell types: bortezomib triggered NF-κB activity via the canonical pathway in peripheral blood mononuclear cells by enhancing nonproteosomal degradation of IκBε while NF-κB activation was inhibited in bone marrow-derived stromal cells (39).

Conversely, some studies report on bortezomib’s immunosuppressive effects. Bortezomib has been shown to reduce total numbers of dendritic cells and leukocytes in hosts (39), as also shown by the suppressive regimen of bortezomib we tested. Multiple administrations of bortezomib have also been found to limit antiviral responses in vivo (40). Nencioni and colleagues observed that exposure of dendritic cells to bortezomib reduces their phagocytic capacity. DCs treated with bortezomib showed skewed phenotypic maturation in response to stimuli of bacterial and endogenous sources, as well as reduced cytokine production and immunostimulatory capacity. DC migratory capabilities were also impaired following bortezomib treatment. Bortezomib was also seen to downregulate MyD88, toll-like receptor signaling, and to reduce IL12p40, IL12p70, IL15, and the chemokine RANTES (S.T. Pellom and A. Shanker; unpublished data) making it tempting to speculate that this enhanced inflammatory milieu may further prolong T-cell activation and sensitize tumor cells to cytolysis in vivo. Bortezomib can also maximize the apoptotic milieu of tumor cells on tumors (46). The sensitization of tumor cells to FasL pathway is particularly relevant in the context of immunoselection of antigen-deficient or low-tumor cells. We previously reported that FasL cytolytic pathway played a dominant role in the T-cell–mediated eradication of Fas-sensitive Renca tumors presenting low levels of antigens following adoptive T-cell therapy (47). Also more recently, FasL-mediated immune surveillance by T cells was found to be essential for the control of spontaneous B-cell lymphomas (48).

Second-generation proteasome inhibitors, such as carfilzomib (49), or the natural product withanolide E recently identified by us as a potent novel sensitizer of cancer cells to extrinsic apoptosis (50), which exhibit minimal toxic effects as single agents, might be even more therapeutically advantageous in combination with adoptive T-cell transfer. Preclinical studies testing these agents with adoptive T-cell transfer are currently underway. In summary, our study using the preclinical cancer models has provided both a molecular basis and a translationally relevant proof of principle that the therapeutic combination of adoptive T-cell therapy with agents that sensitize cancer cells to apoptosis may further improve responses to adoptive T-cell transfer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Authors’ Contributions

Conception and design: A. Shanker, A.D. Brooks, D.L. Longo, T.J. Sayers
Development of methodology: A. Shanker, D.F. Dudimah, D.A. Brooks, T.J. Sayers
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A. Shanker, A. Richmond, A.D. Brooks, D.L. Longo, T.J. Sayers
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A. Shanker, S.T. Pellom Jr, R.L. de Kuyver, D.W. McVicar, W.J. Murphy, D.L. Longo, T.J. Sayers
Writing, review, and/or revision of the manuscript: A. Shanker, A.D. Brooks, T.J. Sayers
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A. Shanker, A.D. Brooks, T.J. Sayers
Study supervision: A. Shanker, T.J. Sayers

Acknowledgments

The authors thank D. Haines, L. Sherman, H. Levitsky, J. Yewdell, S. Durum, A. Richmond, and D. Forbes for helpful feedback.
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Grant Support

This project has been funded in whole or in part with federal funds to T.J. Sayers from the NCI, NIH, under contracts NO1-CD-12400 and HHSN261200800015E. This work was also supported by grants to A. Shanker from the NIH grants U54 CA163609, SC1 CA182843, U54 MD007593, and R01 CA157530. S.T. Pellom was supported by NIH training grants T32 ST32HL007317 and R25 GM059934. The Myeloma Morphology and Flow Cytometry Cores are supported by NIH grants R01 MD007586, R24 DA036420, and S10 RR025497. This research was also supported (in part) by the Intramural Research Program of NIH, Frederick National Lab, Center for Cancer Research.

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Received March 23, 2015; revised September 1, 2015; accepted September 23, 2015; published OnlineFirst October 22, 2015.

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Anil Shanker, Samuel T. Pellom, Jr, Duafalia F. Dudimah, et al.

Cancer Res 2015;75:5260-5272. Published OnlineFirst October 22, 2015.