Immune Mechanism of the Antitumor Effects Generated by Bortezomib

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Bortezomib has emerged as a potent chemotherapeutic agent that can be used to treat advanced-stage cancer (for review, see Refs. 1 and 2). It is the first of a novel class of anticancer drugs, known as proteasome inhibitors, to be approved for clinical use. Since its discovery, bortezomib has been used to treat a variety of human tumors, such as multiple myeloma (3–8), lymphoma (9, 10), non-small cell lung cancer (11, 12), renal cancer (13, 14), prostate cancer (15–17), pancreatic cancer (18), melanoma (19), breast cancer (20, 21), and ovarian cancer (22). Bortezomib inhibits a key regulator of intracellular protein degradation, the 26S proteasome, which has the downstream effect of inducing apoptotic cancer cell death by means of multiple mechanisms. These mechanisms include the prevention of NF-κB activation, inhibition of cell growth via downregulation of cyclin D and IL-6 transcription, alteration of adhesion molecule expression, release of mitochondrial cytochrome c, induction of p53 and MDM2 protein expression, downregulation of gp130, and activation of caspase activity (23–25). These mechanisms exert immediate antitumor effects by inhibiting tumor cell proliferation or promoting tumor cell apoptosis.

The antitumor immune response that develops as a result of bortezomib treatment has yet to be fully explored. The bortezomib-induced tumor cell apoptosis provides an opportunity for generating tumor-specific immunity through cross-priming mechanisms (26). However, the precise manner by which bortezomib instigates tumor-specific immunity remains undetermined. It is known that the uptake and cross-presentation of tumor Ags by dendritic cells (DCs) are necessary to produce tumor-specific T cell immunity. Typically, apoptosis that occurs in the developmental stages is regarded as immunologically tolerizing and leads to T cells that are unresponsive to Ags from normal tissues. In contrast, bortezomib-induced apoptosis is regarded as immunostimulatory. It has been shown in vitro that the upregulation of maturation markers occurs in murine DCs that were fed bortezomib-treated cancer cells. However, the underlying mechanism(s) by which DC maturation occurs and whether these mature DCs can produce T cell immunity remains uninvestigated (27). What is known is that, for T cell immunity to be produced, there must be cell-to-cell contact between the DC and dying cell, and furthermore, the surface expression of heat shock protein (HSP) 90 by the dying cell may represent a distinct immunogenic stimulus (28, 29). These in vitro reports suggest bortezomib-mediated tumor cell apoptosis may be an immunogenic event.

In this study, we established an in vivo system to characterize the mechanisms of immune-mediated antitumor effects generated by bortezomib in a murine (C57BL/6×C3/He) F1) OV-HM ovarian cancer model (30). We observed the best antitumor effects in tumor-bearing mice that were treated with 0.5 mg/kg bortezomib.
Furthermore, these antitumor effects were completely abolished in nude mice, which is suggestive of immune involvement in the antitumor effects of bortezomib. To determine whether the development of a tumor cell-based immunotherapy vaccine is possible, we characterized the irradiated OV-HM tumor cells that were treated or untreated with bortezomib. Mice that were vaccinated with bortezomib-treated tumor cells developed tumor-specific CD8+ T cell immunity, thereby indicating the presence of immunologically significant differences between the treated and untreated tumor cells. We found bortezomib could induce the upregulation of chaperone proteins, HSP60 and HSP90, on the surface of bortezomib-treated tumor cells may play an important role in the immune-mediated antitumor effects generated by bortezomib.

Materials and Methods

Mouse and cell line

C57BL/6×C3He F1 origin mice and athymic nude mice were purchased from BioLASCO (Taipei, Taiwan). All animals were maintained under specific pathogen-free conditions. All procedures were performed in accordance to protocols and recommendations for the proper use and care of laboratory animals. The mouse ovarian cancer cell line HM-1 (C57BL/6×C3He F1, origin) was cultured in MEM (Life Technologies, Gaithersburg, MD) with 10% FBS (HyClone, Logan, UT), 100 U/ml penicillin (Life Technologies), and 100 pg/ml streptomycin (Sigma-Aldrich). All experiments were performed in a humidified atmosphere of 5% CO2/95% air at 37°C.

Cell culture and cell viability assay

Cell viability following bortezomib treatment in vitro (Millennium Pharmaceuticals, Cambridge, MA) was determined by MTT assay. HM-1 cells seeded in 96-well plates (5×103 HM-1 cells/well) were incubated for 24 h with different concentrations of bortezomib ranging from 0.1 to 1000 nM. HM-1 cells were washed to remove the bortezomib and cultured for another 24 h. MTT cell assay was performed in accordance to manufacturer (Sigma-Aldrich) protocol. The absorbance value of 570/630 nm for each well was determined by a microplate reader. Apoptotic and dead cells were detected by a Annexin V:FITC Apoptosis Detection kit (BD Biosciences). The absorbance value of 570/630 nm for each well was determined by a microplate reader. Apoptotic and dead cells were detected by a Annexin V:FITC Apoptosis Detection kit (BD Biosciences). Cell viability following bortezomib treatment in vitro (Millennium Pharmaceuticals, Cambridge, MA) was determined by MTT assay. HM-1 cells seeded in 96-well plates (5×103 HM-1 cells/well) were incubated for 24 h with different concentrations of bortezomib ranging from 0.1 to 1000 nM. HM-1 cells washed to remove the bortezomib and cultured for another 24 h. MTT cell assay was performed in accordance to manufacturer (Sigma-Aldrich) protocol. The absorbance value of 570/630 nm for each well was determined by a microplate reader. Apoptotic and dead cells were detected by a Annexin V:FITC Apoptosis Detection kit (BD Biosciences).

In vivo tumor growth following bortezomib treatment

(C57BL/6×C3He) F1 mice were s.c. inoculated in the left thigh with 1 million HM-1 cells on day 0. On day 5, mice received a single i.p. injection of bortezomib (0.5 mg/kg). One week before tumor challenge, lymphocyte subset depletion was initiated with the injection of 100 μg rat mAb GK1.5 (anti-CD4), 2.43 (anti-CD8), or PK136 (anti-NK1.1), which occurred every other day for the first week and then once per week afterward. One day after the fourth administration of Abs, depletion was confirmed by flow cytometry analysis of spleen cells stained with 2.43, GK1.5, or PK136. Depletion was maintained by weekly injections of Abs for the duration of the tumor growth follow-up.

Intracellular cytokine staining and flow cytometry analysis

Splenocytes from tumor-bearing mice were harvested 2 wk (day 19) after the bortezomib injection. For tumor-infiltrating lymphocyte analysis, HM-1 tumors from mice given 0.5 mg/kg bortezomib or control PBS were dissected, weighed, chopped into small pieces, and washed with HBSS. Tissues were incubated for 15 min at 37°C with a mixture of enzymes dissolved in HBSS. After digestion, single cells were harvested, and pooled splenocytes (5×107) from each group were cultured in vitro with 2×105 live HM-1 cells for 16 h with 2 μg Golgistop (BD Pharmingen, San Diego, CA). Cells were stained with allopachocyanin-conjugated monoclonal rat anti-mouse CD8α (1:100; ebiscience, San Diego, CA) for 20 min. Fixation with Cytofix/Cytoperm (BD Pharmingen) was followed by staining with FITC-conjugated rat anti-mouse IFN-γ (1:50; ebiscience) for 20 min. Flow analysis was performed on a BD Biosciences FACScan (BD FACSCalibur). Each group was analyzed in triplicates, and representative data were shown with mean ± SD in a numerical bar.

Immunohistochemistry

On day 19, tumor tissues were extracted from tumor-bearing mice. Dissected mouse tumor tissues were briefly paraffinized. Consecutive 5-μm paraffin-embedded tissue sections were deparaffinized and rehydrated. After antigen retrieval, blocking was performed by a blocking solution (DakoCytomation, Carpinteria, CA), followed by overnight incubation with rat anti-mouse CD8α mAb (1:100 dilution; clone 53-6.7; BioLegend, San Diego, CA) at 4°C then washed. Subsequently, secondary Ab amplification and visualization were achieved with a Rat-on-Mouse AP-Polymer kit (Biocare Medical, Concord, CA). The tissue sections were dehydrated and counterstained with Mayer’s hematoxylin. The primary Ab was replaced by appropriately diluted mouse IgG for negative control. For identification of proteins by 2D-PAGE, proteins were excised for further analysis to identify the immunologically relevant proteins.

Two-dimensional gel electrophoresis and image analysis

HM-1 cells were treated with either 10−8 or 10−7 M bortezomib and then harvested by trypsinization after 24 h of incubation. Cell surface proteins on these cells were separated using a Cell Surface Protein Isolation kit (Bio-Rad, Rockford, IL) in accordance with the manufacturer’s instructions. The eluted proteins were further purified and precipitated by a two-dimensional clean-up kit (Amersham Biosciences, Piscataway, NJ) and then resolubilized in lysis buffer containing 8 M urea and 4% CHAPS. After sonication, 80 μg protein was applied onto immobilized immobilized pH gradient strips (pH 3–10, linear, 13 cm; Amersham Biosciences, Arlington Heights, IL) for first-dimensional isoelectric focusing (IEF) using an Ettan IPGPhor System (GE Healthcare, Piscataway, NJ). The strips were rehydrated overnight with reduction and alkylaition steps (7 M urea, 2 M thiourea, 4% CHAPS, 45 mM DTT, and 0.5% immobilized pH gradient buffer). Subsequently, they were overlayed on 10% SDS polyacrylamide gels (18×13 cm) for two-dimensional protein separation using a Hoefer SE 600 gel electrophoresis unit (Hoefer, San Francisco, CA). Proteins were visualized by a Pierce Silver Stain kit (24612; Pierce). The stained two-dimensional gels were scanned with an ImageScanner I (GE Healthcare). The image analysis and two-dimensional gel proteome database management were performed with ImageMaster Two-Dimensional Platinum software 5.0 (GE Healthcare). For identification of proteins by mass spectrometry (MS), matching was done (by some landmark spots) between consecutively repeated analytical silver-stained gels, including preparative gel for each sample, to correlate the precise positions. Protein spots with differential intensity over area (ratio >2 or <0.5, p<0.01) were identified, upon which, 10 spots with the greatest statistical significance were selected and excised for further analysis to identify the immunologically relevant proteins.
In-gel digestion and liquid chromatography/tandem MS analysis

In-gel digestion of protein spots was performed on silver-stained gel. Selected spots were excised, cut, and washed twice with 25 mM ammonium bicarbonate buffer. The gel pieces were incubated in 100 μl 1% (v/v) 2-ME, followed by the addition of 5% (v/v) 4-vinylpyridine. The gel pieces were washed, soaked, dehydrated, and rehydrated in modified trypsin solution. Digested peptides were extracted and eluted with 0.1% formic acid and analyzed in a linear trap quadrupole-Orbitrap hybrid tandem mass spectrometer (ThermoFisher, Houston, TX) in-lined with an Agilent 1200 nanoflow HPLC system. The HPLC system was equipped with LC packing C18 PepMap100. Ten tandem MS (MS/MS) scans with linear trap quadrupole were collected following a full MS scan with Orbitrap. With TurboSequest, all MS/MS data were searched against a mouse protein database downloaded from National Center for Biotechnology Information. The criterion for positive protein identification is a minimum of three digested peptides whose Xcorr is over 2.5 and from the same protein.

Biotinylation of cell surface protein

Cell surface proteins of HM-1 cells were isolated using the Pierce Cell Surface Protein Isolation kit (Thermo Scientific), according to the manufacturer’s protocol. Protein concentration was determined by bicinchoninic acid assay. All cell lysates were applied to a NeutAvidin Agarose column, and biotinyl peptides were then eluted by 100 μl SDS-PAGE sample buffer with 50 mM DTT. The biotinyl proteins isolated from each 40 μg total proteins were analyzed by Western blots to determine the amount of cell surface Hsp90 and Hsp60 protein as described below. For total protein extraction, cells were lysed with protein extraction reagent (Pierce).

Western blotting

Equal amounts of proteins (25 μg surface or total) were loaded and separated by SDS-PAGE using a 10% polyacrylamide gel. The gels were electroblotted to a polyvinylidene difluoride membrane (Bio-Rad Laboratories). After blocking, membranes were probed with mouse anti-Hsp90 Abs (1:1,000; Abcam, Cambridge, U.K.) or mouse anti-β-actin Abs (1:10,000; Sigma-Aldrich) for 1 h and then washed and incubated with rabbit anti-mouse IgG conjugated to HRP (1:3,000; PerkinElmer Life Sciences, Santa Clara, CA). After incubation with the secondary Abs, the membranes were rinsed. Ab binding was detected with ECL kit (Amer sham Biosciences).

Chemical inhibition of Hsp90 and Hsp60 expression by HM-1 tumor cells treated with bortezomib in vitro

HM-1 cells were treated with bortezomib and the indicated inhibitors in vitro. Hsp90 inhibitor was geldanamycin (GA; Sigma-Aldrich), whereas the Hsp60 inhibitors were epoxatcaene (ETB; Wako Chemicals, Richmond, VA) and nonactin (Sigma-Aldrich). HM-1 cells were incubated with bortezomib for 24 h. Six hours after the initiation of bortezomib treatment, GA (2 μM) was added (18 h incubation). Twenty-two hours after the addition of bortezomib, ETB (11 μM) was added (2 h incubation). Nonactin (10 μg/ml) was added 24 h before bortezomib (48 h incubation). Treated HM-1 cells were used to infect immune mice or assess phagocytosis by DCs.

T cell proliferation assay

T lymphocytes isolated from the splenocytes of C57BL/6×C3H/He F1 mice by pan T cell isolation Kit II (MACs; Miltenyi Biotec, Auburn, CA) were stained with 5 × 10^7 M CFSE (Sigma-Aldrich) for 30 min at 37°C. These cells were washed, resuspended, and then seeded in 96-well plates (2 × 10^4 cells/well) in the presence of different doses of bortezomib for 24 h. After drug treatment, T cells were washed three times to remove the drug and cultured for another 72 h before stimulation with 2 μg/ml PHA (Sigma-Aldrich). T cells were then collected and subjected to flow cytometry analysis (BD FACS Calibur).

CD11c+ phagocytosis of bortezomib-treated HM-1 cells with or without cotreatment with HSP90 and/or HSP90 inhibitors

CD11c+ DCs from tumor-bearing mice were isolated using CD11c microbeads (MACs; Miltenyi Biotec) and resuspended in culture medium (5 × 10^6 cells/ml). HM-1 cells treated with bortezomib, as previously described, with or without HSP inhibitors were labeled with 5 × 10^7 M CFSE (Sigma-Aldrich) for 30 min at 37°C and then washed and resuspended in culture medium (1 × 10^5 cells/ml). Both CD11c+ DCs and HM-1 cells were seeded in 24-well plate (1 ml from each/well) and incubated in CO₂ incubator at 37°C for 6 h. Cells were stained with allopheococyanin-conjugated rat anti-mouse CD11c (MACs; Miltenyi Biotec) and subjected to flow cytometry analysis (BD FACS Calibur). In vitro effect of bortezomib on DC phagocytosis

Isolated CD11c+ DCs (2 × 10^6 cells/well) and HM-1 cells were treated with indicated doses of bortezomib in vitro for 24 h. After washing the CD11c+ DCs, either CFSE-labeled HM-1 cells or dextran–FITC (50 μg/ml) were incubated with the CD11c+ DCs (incubation times are 5 h for bortezomib-treated CD11c+ DCs and 1 h for dextran–FITC). DCs were stained with allopheococyanin-conjugated rat anti-mouse CD11c (MACs; Miltenyi Biotec) and subjected to flow cytometry analysis (BD FACS Calibur).

In vivo effect of bortezomib on DC maturation

Maturation assay of tumor-infiltrating DCs obtained from tumor-bearing mice was conducted in the following manner. HM-1 tumor-bearing mice were treated with 0.5 mg/kg bortezomib. A week later, tumors were dissected out and processed into single-cell preparations. Tumor-infiltrating CD11c+ cells were isolated from the tumor tissues using CD11c+ microbeads (MACs; Miltenyi Biotec) and measured for their expression of maturation markers. CD11c+ DCs were stained with FITC-conjugated rat anti-mouse CD40, CD80, CD83, or CD86 (eBioscience) and subjected to flow cytometry analysis.

Chemical inhibition of Hsp90 and Hsp60 expression by HM-1 tumor cells treated with bortezomib in vitro

HM-1 cells were treated with bortezomib and the indicated inhibitors in vitro. Hsp90 inhibitor was geldanamycin (GA; Sigma-Aldrich), whereas the Hsp60 inhibitors were epolaactaene (ETB; Wako Chemicals, Richmond, VA) and nonactin (Sigma-Aldrich). HM-1 cells were incubated with bortezomib for 24 h. Six hours after the initiation of bortezomib treatment, GA (2 μM) was added (18 h incubation). Twenty-two hours after the addition of bortezomib, ETB (11 μM) was added (2 h incubation). Nonactin (10 μg/ml) was added 24 h before bortezomib (48 h incubation). Treated HM-1 cells were used to infect immune mice or assess phagocytosis by DCs.

In vivo treatment with bortezomib led to inhibited tumor growth kinetics through CD8+ T cell-mediated immune effects

To characterize the therapeutic effect of bortezomib, we examined tumor growth in HM-1 tumor-bearing mice after bortezomib (0.5, 1, and 1.5 mg/kg) administration by i.p. injections. The greatest antitumor effect was observed after 0.5 mg/kg bortezomib (p = 0.0038; 0.5 mg/kg bortezomib versus control). These results imply the therapeutic effect is not dose dependent (Fig. 1A). To determine whether the observed antitumoral effect generated by bortezomib is immune mediated, we investigated bortezomib in tumor-bearing athymic mice. Bortezomib did not impede tumor growth in the immunodeficient mice, thereby suggesting host immunity is implicated in bortezomib-mediated tumor rejection (Fig. 1B). To identify the effector cell subset(s) involved in the bortezomib-mediated antitumoral effect, HM-1 tumor-bearing mice (C57BL/6×C3He F1) given 0.5 mg/kg bortezomib by i.p. injection were depleted of CD8+CD4+, or NK1.1+ lymphocyte subpopulations using monoclonal neutralizing Abs. The control bortezomib-treated mice were given rat IgG. The depletion of CD8+ T lymphocytes abolished the bortezomib-mediated therapeutic effect (p = 0.0038, anti-CD8 versus control; Fig. 1C), which suggests bortezomib exerts therapeutic effect through the CD8+ effectors cells. The depletion of CD4+ T cells had no immediate impact on the antitumoral effect elicited by bortezomib. However, long-term data indicate reduced production of memory cells when CD8+ T cells were primed in the absence of CD4+ T cells (Supplemental Fig. 1). Taken together, these data indicate systemic chemotherapy with 0.5 mg/kg bortezomib can elicit CD8+ T cell-dependent antitumoral response in tumor-bearing mice.

Treatment of tumor-bearing mice with bortezomib led to activated tumor-specific CD8+ T lymphocytes and tumor-infiltrating lymphocytes

Our initial observation of the mouse tumor model encouraged us to investigate whether the therapeutic effect of bortezomib is associated with immune responses. HM-1 tumor-bearing mice were given bortezomib (0.5, 1, or 1.5 mg/kg). Two weeks later,
One million ovarian cancer cells, HM-1, were s.c. transplanted into (C57BL/6×C3H/He) F1 mice on day 0. On day 5, mice were given 0.5 mg/kg bortezomib i.p. splenocytes from treated mice were isolated, cultured, and stimulated with irradiated HM-1 cells. Splenocytes were then analyzed by flow cytometry to characterize activated T lymphocytes, as indicated by IFN-γ secretion. Mice given 0.5 mg/kg bortezomib were found to have the highest numbers of CD8+IFN-γ+ cells (Fig. 2A). The immunologic activities somewhat correlated with the observed antitumor effect in the tumor-bearing mice. The tumors were dissected and examined for tumor-infiltrating CD8+ T lymphocytes. Immunohistochemical staining revealed treatment with bortezomib resulted in more recruitment of CD8+ T lymphocytes into the tumor (Fig. 2B). Single-cell suspensions prepared from the tumors were analyzed by flow cytometry to characterize the intratumoral immune cells. Tumor-bearing mice given 0.5 mg/kg bortezomib had significantly higher amounts of tumor-infiltrating CD8+IFN-γ+ and CD4+IFN-γ+ T lymphocytes (p = 0.001 and 0.05, respectively; Fig. 2C).

Mice immunized with HM-1 cells treated with bortezomib in vitro developed tumor-specific CD8+ T lymphocytes and protective antitumor effects

To investigate the underlying mechanisms driving the antitumor immunity, HM-1 cells were treated with 10^-8, 10^-7, or 10^-6 M bortezomib in vitro and examined for the development of immunogeneity. First, it was noted that bortezomib triggered HM-1 cell death at concentrations of 10^-8, 10^-7, and 10^-6 M at 48 h (~10, 50, and 75%, respectively; Fig. 3A, left). However the highest numbers of apoptotic (Annexin V positive) and dead (propidium iodide [PI] positive) tumor cells were observed 24 h after 10^-6 M bortezomib (p < 0.0001, ANOVA; Fig. 3A, right). Mice were then immunized twice (days 0 and 7) with cell-based vaccines produced from HM-1 tumor cells that were either irradiated or treated with bortezomib in vitro. On day 17, splenocytes were analyzed for tumor-specific CD8+ T cells. Although the highest amount of apoptosis occurred at 10^-6 M bortezomib, cell-based vaccine produced from HM-1 cells treated with 10^-7 M bortezomib elicited the greatest percentage of tumor-specific CD8+IFN-γ+ T lymphocytes in comparison with the control, irradiated HM-1, or HM-1 cells treated with 10^-8 and 10^-6 M bortezomib (p = 0.0002, 10^-7 versus 10^-8 M; p < 0.0001 10^-7 versus 10^-6 M; Fig. 3B). As we expected, the tumor growth curve shows that cell-based vaccine derived from HM-1 cells treated with 10^-7 M bortezomib in vitro induced the strongest antitumor effect (p = 0.02; Fig. 3C). The survival curve also demonstrated a similar trend in that mice immunized with HM-1 cells treated with 10^-7 M bortezomib had the longest survival and reduced tumor size (Fig. 3D).

Two-dimensional gel analysis revealed HM-1 cells treated with 10^-7 M bortezomib in vitro had increased expression of cell surface chaperone proteins, Hsp90 and Hsp60

To identify the molecules that may contribute to the immunogenicity of bortezomib-treated HM-1 cells, cell surface proteins of treated HM-1 cells were isolated through biotinylation, separated by two-dimensional gel electrophoresis, and analyzed by MS. The protein spots indicating differential expression between HM-1 cells treated with 10^-7 and 10^-8 M bortezomib were excised for further analysis. In total, 10 statistically significant protein spots were selected, of which, only 2 of the identified proteins were found to be immunologically significant. The immunologically significant and neutralizing Abs against CD8, CD4, NK1.1, or control rat IgG. Therapeutic effect of bortezomib was abolished by CD8+ T cells depletion (p = 0.0038; rat anti-mouse CD8 IgG versus control rat IgG), indicating therapeutic effect is CD8+ T cell dependent.
proteins were the chaperone proteins, Hsp90 and Hsp60 (Fig. 4A). The results were further validated by Western blot analysis, which showed that the HM-1 cells treated with 10^{-7} M bortezomib had greater amounts of cell surface Hsp90 and Hsp60. However, the total amounts of Hsp90 and Hsp60 in HM-1 cells treated with 10^{-8} or 10^{-7} M bortezomib were found to be similar (Fig. 4B). Flow cytometry analysis also confirmed HM-1 cells treated with 10^{-7} M bortezomib had higher levels of surface (membranous) Hsp90 and Hsp60 proteins in comparison with HM-1 cells treated with 10^{-8} M bortezomib (Fig. 4C). Thus, treating HM-1 cells with bortezomib can increase the amount of externalized Hsp60 and Hsp90.

Reduced expression of cell surface HSP60 and/or HSP90 greatly attenuates bortezomib-induced tumor cell immunogenicity and phagocytosis of tumor cells by DCs

HM-1 cells were treated with bortezomib in vitro in conjunction with the indicated inhibitors: Hsp60 inhibitors (nonactin and ETB) and Hsp90 inhibitor (GA). Bortezomib-treated HM-1 cells that were cotreated with Hsp60 and/or Hsp90 inhibitors elicited a smaller number of tumor-specific CD8^+ T lymphocytes in tumor-bearing mice (p \leq 0.0001). Control mice were given PBS only. (B) Representative photographs of immunohistochemical staining for tumor-infiltrating CD8^+ T lymphocytes. Tumors obtained from mice treated with bortezomib (0.5 mg/kg) or PBS (control) were stained for CD8^+ T cells (identified by brown staining). (C) Representative flow cytometry identification of intratumoral tumor-specific CD8^+ and CD4^+ T cells obtained from tumor-bearing mice given 0.5 mg/kg bortezomib. Bortezomib significantly increased the percentage of tumor-infiltrating CD8^+IFN-γ^+ T cells (1.6 versus 5.9%, control versus bortezomib treated; p = 0.001).

FIGURE 2. Characterization of tumor-specific CD8^+ T lymphocytes in tumor-bearing mice treated with bortezomib. (A) Activation of HM-1 tumor-specific CD8^+ T cells. A total of 1 \times 10^6 HM-1 were s.c. transplanted into (C57BL/6×C3/He) F1 mice on day 0. Tumor-bearing mice received different doses of bortezomib (0.5, 1, and 1.5 mg/kg, i.p. single dose) on day 5. On day 19, splenocytes from treated tumor-bearing mice were analyzed for tumor-specific CD8^+ T cells using intracellular cytokine staining for IFN-γ with irradiated HM-1 cells as the stimulator, as described in Materials and Methods. Left, Representative of flow cytometry data. Right, The bar graph depiction of the mean and SE derived from flow cytometric data. Significantly more tumor-specific CD8^+IFN-γ^+ T lymphocytes were produced in mice treated with 0.5 mg/kg bortezomib than mice treated with 1 or 1.5 mg/kg bortezomib (0.67 versus 0.38 and 0.36%; p < 0.0001 and 0.0003). Control mice were given PBS only. (B) Representative photographs of immunohistochemical staining for tumor-infiltrating CD8^+ T lymphocytes. Tumors obtained from mice treated with bortezomib (0.5 mg/kg) or PBS (control) were stained for CD8^+ T cells (identified by brown staining). Control: Top panels at magnifications \times 100 (left panels) and \times 400 (right). Bortezomib: Bottom panels at magnifications \times 100 (left panels) and \times 400 (right panels). More tumor-infiltrating CD8^+ T cells were found in mice given bortezomib. (C) Representative flow cytometry identification of intratumoral tumor-specific CD8^+ and CD4^+ T cells obtained from tumor-bearing mice given 0.5 mg/kg bortezomib. Bortezomib significantly increased the percentage of tumor-infiltrating CD8^+IFN-γ^+ T cells (1.6 versus 5.9%, control versus bortezomib treated; p = 0.001).
FIGURE 3. In vitro bortezomib-treated HM-1 cells induced tumor-specific CD8+ T lymphocyte production and antitumor protective immunity in HM-1 tumor-bearing mice. (A) MTT assay assessment of cell vitality and tumor cell apoptosis following bortezomib treatment for 48 and 96 h. Left, The bar graph indicating the proapoptotic effects of increasing concentrations of bortezomib on HM-1 in vitro. Right, Representative flow cytometric data indicating the percentage of tumor cell apoptosis (Annexin v positive) and cell death (PI positive) following bortezomib treatment for 24 h. Bortezomib induced HM-1 cell death at $10^{-6}$ M, $10^{-7}$, and $10^{-8}$ M after 48 and 96 h (~10, 50, and 75% at 48 h; 0, 15, and 35% at 96 h). Representative flow cytometric data indicate the highest number of apoptotic (Annexin V positive) and dead cells (PI positive) following 24 h of $10^{-6}$ M bortezomib treatment (3.12 versus 7.94 versus 21.75% of apoptotic cells; 1.84 versus 12.19 versus 18.92% of dead cells at $10^{-6}$, $10^{-7}$, and $10^{-8}$ M bortezomib; $p < 0.0001$; ANOVA). (B) Representative flow cytometric data indicating CD8+IFN-γ+ T cells from mice immunized with cell-based vaccine. Cell-based vaccines were produced from irradiated HM-1 cells or HM-1 cells treated with different concentrations of bortezomib in vitro. Mice were immunized with cell-based vaccine twice (days 0 and 7). Cell-based vaccine produced from HM-1 cells treated with $10^{-7}$ M bortezomib resulted in the most CD8+IFN-γ+ T cell activation (0.19, 0.25, 0.30, and 0.76% for unvaccinated control mice, vaccination with irradiated HM-1, and vaccination with HM-1 treated with $10^{-8}$ or $10^{-7}$ M bortezomib, respectively). However, the highest concentration of bortezomib ($10^{-6}$ M) significantly reduced the number of antitumor CD8+IFN-γ+ T lymphocytes (0.24%; $p < 0.0001$) $10^{-7}$ versus $10^{-6}$ M). (C) Tumor growth curve evaluation of cell-based vaccines derived from irradiated or HM-1 cells treated with bortezomib in vitro. Tumor growth curve indicates cell-based vaccine produced from HM-1 treated with $10^{-7}$ M bortezomib in vitro (Figure legend continues).
and $10^{-8}$ M bortezomib). However, the phagocytic activity of CD11c$^+$ cells was diminished when bortezomib-treated HM-1 cells were treated with Hsp60 inhibitors ($p = 0.0014$ by adding ETB and $p < 0.0001$ by adding nonactin) or Hsp90 inhibitor ($p = 0.036$ by adding GA). The inhibition of tumor cell surface Hsp60 had a greater impact on the immunogenicity of bortezomib-treated HM-1 cells ($p = 0.0002$) (Fig. 5B). Our data indicate that both Hsp60 and Hsp90 are important mediators of bortezomib-induced tumor immunogenicity and the resultant increase in the uptake of bortezomib-treated tumor cells by CD11c$^+$ DCs. However, it seems that Hsp60, more so than Hsp90, is responsible for the ability of bortezomib-treated tumor cells to be preferentially phagocytosed by CD11c$^+$ DCs and promote the generation of tumor-specific CD8$^+$ T lymphocytes. For flow histograms depicting CD8$^+$ T cells were treated with Hsp60 inhibitors ($p = 0.0001$, ANOVA; Fig. 6A). Bortezomib influenced both CD11c$^+$ DCs and HM-1 cells to promote phagocytosis. The phagocytic activities of bortezomib-treated DCs increased in a dose-dependent manner. In addition, the phagocytosis of bortezomib-treated CFSE-labeled HM-1 cells also increased in a dose-dependent manner. When both DCs and HM-1 cells were treated with $10^{-7}$ M bortezomib, treated HM-1 cells were phagocitized by the greatest percentage of treated DCs (Supplemental Fig. 3).

To examine the effect of bortezomib on DC maturation in vitro, DCs identified by a CD11c$^+$ marker were isolated from mouse splenocytes. After bortezomib treatment ($10^{-7}$ or $10^{-8}$ M), CD11c$^+$ DCs increased their expression of maturation markers (CD40, CD80, and CD86) with $10^{-7}$ M bortezomib, leading to greater expression of maturation markers ($p = 0.002$, $0.0014$, and 0.003, ANOVA; Fig. 6B). To understand the real in vivo effect of bortezomib on DC maturation, we studied the intratumoral CD11c$^+$ DCs obtained from tumor-bearing mice given bortezomib. In response to systemic drug treatment, tumor-infiltrating DCs upregulated their expression of CD40, CD80, CD83, and CD86 maturation markers ($p = 0.02$, 0.013, 0.006, and 0.019; Fig. 6C).

**Discussion**

The use of bortezomib in the treatment of cancer has benefits beyond the simple proapoptotic action on tumor cells, because in mice with intact host immunity, it also results in the promotion of antitumor immunity through several mechanisms. Tumor-specific CD8$^+$ IFN-γ$^+$ T lymphocytes were observed to be the effector cells by which bortezomib exerts its antitumoral action. In response to treatment with optimal bortezomib dose, the tumor-bearing mice were detected to have markedly higher numbers of CD8$^+$ tumor-infiltrating lymphocytes.

Other than the generation of tumor-specific activated CD8$^+$ T cells, bortezomib has the added capability of enhancing the vulnerability of tumor cells to the actions of immune cells. The systemic treatment of B16 melanoma with bortezomib sensitized B16 melanoma to DC-based immunotherapy, which generated activated CD8$^+$ IFN-γ$^+$ T lymphocytes (31). Previous research has also indicated that bortezomib may additionally enhance the susceptibility of a variety of formerly apoptosis-resistant tumor cells with dysfunctional mitochondrial apoptotic pathways to the cytolytic action of the treatment-generated CD8$^+$ T lymphocytes (31-34). A subtoxic concentration of bortezomib was able to drastically favor the mitochondrial apoptotic pathway of melanoma cells by enhancing the release of SMAC and cytochrome c in response to the CTL effector molecules of caspase-8 and cytosolic granzyme B (32). Furthermore, some cells respond to bortezomib with increased sensitivity to the cytotoxic downstream effects of TRAIL binding to its respective receptor on the tumor cell surface (34).

In an attempt to delineate the mechanism by which bortezomib was rendering HM-1 cells immunogenic, we discovered that the amplification of the cell surface expression of HSP60 and HSP90 proteins made bortezomib-treated HM-1 cells unique from the non-bortezomib-treated HM-1 cells. We reasoned that proteasome inhibition by bortezomib led to the augmented cell surface expression of HSP60 and HSP90 by HM-1 cells, because the evidence that suggests proteasome inhibition can bias gene expression in favor of various HSPs are plentiful (23, 35).

We attempted to identify the possible role(s) that HSP60 and HSP90 may have in immune signaling because the immunogenicity of HM-1 cells and their preferential uptake by CD11c$^+$ DCs, in conjunction with the subsequent potency of these tumor-loaded CD11c$^+$ DCs in the priming of HM-1–specific CD8$^+$ IFN-γ$^+$ effector cells, are directly correlated with the externalization of HSPs following bortezomib treatment. The present study demonstrates that bortezomib-treated HM-1 cells have greater susceptibility to phagocytosis and that the surface expression of Hsp60, rather than Hsp90, has the greater role in promoting CD8$^+$ T cell activation. Another report that highlights the importance of Hsp60 as an immune mediator demonstrates the ability of Hsp60 to enhance phagocytosis by monocyte U937 cells (36), consistent with the observation that DC phagocytosis of bortezomib-treated cancer cells, including human myeloma cells, results in mature DCs that are especially effective at eliciting tumor-specific CD8$^+$ IFN-γ$^+$ T lymphocytes (26).

Although the results of the current study emphasize the relationship between the externalization of Hsp60 by tumor cells and bortezomib-mediated generation of antitumor immunity, in truth, a variety of HSPs have been determined to modulate immune responses. APCs of several lineages have been found to express HSP receptors; one such is the CD91 receptor whose ligands are HSPs, such as calreticulin (CRT), gp96, hsp70, and hsp90 (37, 38). Moreover, the translocation of endogenous HSP90 to the cell surface has already been proven to be a part of the bortezomib-induced immunogenic cell death that induces antitumoral immunity (26).

Even though the nature of HSP60 and HSP90 as immune stimuli with respect to their role(s) in generating protective immunity against cancer is as yet undefined, the ability of HSP60 and HSP90 inhibitors to abrogate the bortezomib-induced HM-1 cell immu-
FIGURE 4. Two-dimensional gel analysis identification of upregulated cell surface chaperone proteins. (A) Two-dimensional gel electrophoresis comparison of differential expression levels of HM-1 cell surface proteins following treatment with either 10^-7 or 10^-8 M bortezomib. Left, The image of the two-dimensional gel containing separated tumor cell surface HSPs. Right, The synchronized three-dimensional view comparison of protein spots with differential intensity over area. Cell surface proteins of bortezomib-treated cells were isolated using biotinylation method as described in Materials and Methods. Surface proteins were run through two-dimensional gel electrophoresis, and selected spots were analyzed by MS. The differentially expressed protein spots (encircled by red line) between HM-1 cells treated with 10^-7 or 10^-8 M bortezomib were identified to be Hsp90 and Hsp60 proteins. (B) Western blot validation of two-dimensional gel results. Surface expressions of Hsp90 and Hsp60 proteins from HM-1 cells treated with 10^-7 M bortezomib were higher, whereas the total Hsp90 and Hsp60 protein expressions between the two treatment groups (10^-7 or 10^-8 M bortezomib) were similar. (C) Representative flow histogram depicting surface Hsp90 and Hsp60 expression levels by HM-1 cells following bortezomib treatment. HM-1 cells were treated with either 10^-7 or 10^-8 M bortezomib and then analyzed by flow cytometry to identify externalization of Hsp90 and Hsp60. Greater amounts of surface Hsp90 and Hsp60 were seen in HM-1 cells treated with 10^-7 M bortezomib (black solid line) and not in the cells treated with 10^-8 M bortezomib (gray solid line) or control cells (black dot line). The secondary Ab is represented by gray dot line.
nogenicity demonstrates that bortezomib-invoked antitumor immunity is HSP dependent. HSPs prepared from cancer cells can associate with endogenous tumor-specific antigenic peptide in vivo (39) and are effective immunogens capable of generating strong tumor-specific CD8+ T cell responses against syngeneic cancers (40, 41), which for this reason, have been used in multiple therapeutic cancer vaccine strategies (42). An important aspect of our study is the identification of HSP60 as a novel mediator of the protective antitumor immunity that is induced by bortezomib-treated tumor cells. Inhibition of HSP60 greatly reduces the phagocytosis of bortezomib-treated HM-1 cells by CD11c+ DCs and, in turn, would diminish the generation of tumor-specific CTLs. Although there is plenty of literature on the immunogenicity of Hsp90, our experimental results suggest the expression of Hsp60 on the tumor cell surface is even more essential than Hsp90 in the generation of tumor-specific CTLs.

FIGURE 5. Evaluation of bortezomib-induced HM-1 immunogenicity through chemical inhibition of Hsp90 and Hsp60 expressions. (A) Representative flow cytometric data of tumor-specific CD8+IFN-γ T lymphocytes following stimulation with bortezomib-treated HM-1 cells that were or were not cotreated with HSP90 and HSP60 inhibitors. Left, Representative flow cytometric data of tumor-specific CD8+ T cell activation (IFN-γ+) by HM-1 cells. Right, The bar graph of the mean and SE derived from flow cytometric data. HM-1 cells were treated with bortezomib and the indicated inhibitors (Hsp90 inhibitor, GA; Hsp60 inhibitors, ETB and nonactin) in vitro, as described in Materials and Methods. On day 0, mice were immunized once with HM-1 cells that were treated. On day 10, splenocytes from these mice were stimulated overnight by live HM-1 cells and analyzed for IFN-γ+ cells. Representative flow cytometric data indicates immunization of tumor-bearing mice with HM-1 cells treated in vivo with 10^{-7} M bortezomib leads to the highest numbers of tumor-specific CD8+IFN-γ+ T lymphocytes (p < 0.0001; 10^{-7} M versus irradiated and 10^{-8} M), but the coadministration of Hsp60 and/or Hsp90 inhibitors could abolish the immunogenicity of bortezomib-treated HM-1 cells (p = 0.0007 by adding GA, p = 0.0001 by adding ETB, and p < 0.0001 by adding nonactin). (B) Representative flow cytometric dot plot depicting the phagocytosis of bortezomib-treated HM-1 cells by CD11c+ DCs. Left, The flow cytometric data depicting the percentage of CD11c+ DCs that have engulfed HM-1 cells that were treated with different concentrations of bortezomib, with or without coadministration with HSP90 and HSP60 inhibitors. The controls are DCs only (DCs incubated in the absence of HM-1 cells) and PBS treatment (Tx) DC (DCs coincubated with PBS-treated HM-1 cells). Right, The bar graph depiction of the mean and SE of flow cytometric data. HM-1 cells were treated with bortezomib with or without Hsp90 and Hsp60 inhibitors in vitro. Treated HM-1 cells were labeled with CFSE and irradiated. CD11c+ cells isolated with microbeads were cocultured with treated HM-1 cells. DCs were then analyzed for CFSE staining, which represents phagocytosis of HM-1 cells. The greatest percentage of CD11c+ DCs phagocytized HM-1 cells treated with 10^{-7} M bortezomib (p = 0.002; 10^{-7} M bortezomib versus PBS and 10^{-8} M bortezomib). The coadministration of HSP90 inhibitors (p = 0.0014, ETB; p < 0.0001, nonactin) or Hsp90 inhibitor (p = 0.036, GA) or both inhibitors (p = 0.0002) during the in vitro treatment of HM-1 cells with bortezomib was able to diminish the phagocytic activities of CD11c+ cells.
The splenocyte isolated non–tumor-loaded CD11c+ DCs that were treated with bortezomib in vitro responded with enhanced phagocytosis of FITC-labeled dextran, the reason for which is unknown. In an attempt to define the effect of bortezomib on the living system, mice were treated with bortezomib in vivo, and the DC markers were characterized. The maturation level of DCs is highly correlated with their ability to prime T lymphocytes, which makes it interesting that bortezomib treatment in vivo led to the upregulated expression of costimulatory molecules by tumor-infiltrating CD11c+ DCs. We expect the in vivo effect that bortezomib has on tumor-infiltrating DCs is related to the maturation signals that are provided only when tumor cells undergo immunologically relevant death. It has been demonstrated that DCs acquire maturation signals via cell-to-cell contact with bortezomib-treated tumor cells expressing Hsp90 (26). When immature DCs were cocultured with bortezomib-treated apoptotic tumor cells, the mature DCs, identified by the upregulation of both CD40 and costimulatory molecules, were capable of generating protective antitumor immunity in mice (27).

Although the current study finds bortezomib to be beneficial in regard to the production of CTL-mediated immunity, other studies have suggested that bortezomib can restrain the production of tumor-reactive CD8+ T cells by acting on the DCs. It has been reported that bortezomib adversely affects monocyte-derived DCs by inhibiting proteasomal β5 subunit-located chymotrypsin-like peptidase, which is part of the ubiquitin–proteasome pathway that is crucial for the preservation of normal DC processes (43). Additional reports of unfavorable effects that bortezomib has on DCs include the reduction of monocyte-derived DC phagocytic activity (44) and the triggering of monocyte-derived DC apoptosis (45) with differentially greater apoptotic effect on immature than mature monocyte-derived DCs (46).

It is also a concern that bortezomib may cause immunosuppression by inhibiting the action and/or proliferation of T lymphocytes. Lundqvist et al. (47) demonstrated that bortezomib renders tumor cells resistant to Ag-specific CTLs by altering the proteasomal processing of the Ag for which CTLs have specificity. Another example of immunosuppression by bortezomib was illustrated in a study conducted by Sun et al. (48) in which they showed bortezomib could reduce graft-versus-host-disease following marrow transplantation via the dose-dependent antiproliferative effect that the drug has on T lymphocytes. Like many chemotherapeutic drugs, bortezomib leads to immunosuppression at high concentrations. Our results show that bortezomib positively influences the host immune system through the promotion of CTL-mediated antitumor immunity, with the caveat being at
a low dose that is still of sufficient amount to produce the desired response. Bortezomib does not compromise the proliferative abilities of T lymphocytes at most of the tested concentrations (10-14 to 10-6 M) and only at higher concentrations (10-7 and 10-6 M) does T cell proliferation become restricted (Supplementary Fig. 4).

Akin to bortezomib, doxorubicin stands out as a chemotherapeutic drug that is capable of modifying the manner in which cancer cells die such that their apoptosis is recognized as immunologically important. Apoptotic cancer cells that were treated with doxorubicin will translocate the HSP, CRT, to the cell surface where it is seen as a proapoptotic signal by DCs (49). Similarly, the surface expression of Hsp60 and Hsp90 by tumor cells following bortezomib treatment also stimulates DC phagocytosis. Other chemotherapeutic drugs that are capable of instigating the apoptosis of cancer cells, such as etoposide and mitomycin C, do not result in the development of antitumor immunity; and perhaps not coincidentally, the nonimmunogenic apoptosis coincides with a lack of cell surface expression of CRT (ecto-CRT) (50, 51), not coincidentally, the nonimmunogenic apoptosis coincides with the tumor-reactive CD8+ T lymphocytes that were generated by a low dose that is still of sufficient amount to produce the de-


References


