Bortezomib suppresses function and survival of plasmacytoid dendritic cells by targeting intracellular trafficking of Toll-like receptors and endoplasmic reticulum homeostasis

Running title Bortezomib suppresses plasmacytoid DC

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Abstract

Dendritic cells (DCs) play a pivotal role in the pathogenesis of inflammatory disorders. Thus, suppressing the activity of DCs is instrumental in treating such diseases. Here we show that a proteasome inhibitor bortezomib suppresses survival and immunostimulatory function of human plasmacytoid DCs (pDCs) by targeting two critical points, intracellular trafficking of nucleic acid-sensing Toll-like receptors (TLRs) and endoplasmic reticulum (ER) homeostasis. pDCs were most susceptible to the killing effect of bortezomib among immune cells in blood. This correlates with a decrease in the spliced form of a transcription factor XBP-1, which rescues cells from apoptosis by maintaining ER homeostasis. Bortezomib suppressed the production of interferon-α and interleukin-6 by pDCs activated with a TLR9-stimulating CpG DNA and a TLR7-stimulating influenza virus, which appears to be partially independent of apoptosis. Bortezomib inhibited translocation of TLR9 from the ER to endolysosomes but not of an ER membrane protein Unc93B1 that delivers TLR9 to endolysosomes. Thus, bortezomib suppresses the activity of pDCs (i) by inhibiting intracellular trafficking of TLRs through disrupting the coordinated translocation of TLRs and Unc93B1, and (ii) by disturbing ER homeostasis. This study suggests that proteasome inhibitors may alleviate inflammatory disorders that involve pDCs such as lupus and psoriasis.
Introduction

Dendritic cells (DCs) play a pivotal role in controlling immune responses by linking innate and adaptive arms of the immune system. Thus, DCs represent an important target for treatments of a variety of immune-related disorders.

In humans, DCs are composed of two subsets: plasmacytoid DCs (pDCs) and myeloid DCs (mDCs). These DCs express different sets of nucleic acid-sensing Toll-like receptors (TLRs); pDCs express TLR7 and TLR9, whereas mDCs express TLR3 and TLR8. pDCs are distinguished from other immune cells by their remarkable potency to produce interferon (IFN)-α in response to virus-derived single-stranded RNA or CpG DNA though TLR7 or TLR9, respectively. Owing to this distinctive capacity, on one hand, it has been assumed that pDCs play an important role in antiviral immune responses. On the other hand, it has been shown that, if the nucleic acids are derived from self tissues, pDCs cause inflammatory disorders such as systemic lupus erythematosus (SLE) and psoriasis. In SLE, immune complexes containing self DNA or RNA are incorporated into pDCs, and induce them to produce IFN-α via TLR9 or TLR7, respectively. It is proposed that such IFN-α production plays a key role in the pathogenesis of SLE. In psoriasis, aggregated particles composed of self DNA and RNA from damaged epithelial cells and the antimicrobial peptide LL37 are incorporated into pDCs, and induce them to produce IFN-α via TLR9 and TLR7, respectively, thus contributing to the pathogenesis. Therefore, suppressing IFN-α production by pDCs may represent a novel therapy for these inflammatory disorders in which pDCs are likely to play an important role.

A recent study has suggested that maintenance of endoplasmic reticulum (ER) homeostasis by the transcription factor XBP-1 is essential for development and survival of pDCs, thus representing a possible target for controlling the activity of pDCs. Proper functions of highly secretory cells like pDCs depend on the unfolded protein response (UPR), that is, coordinated handling of ER stress caused by a burden of unfolded proteins in the lumen of the ER. After sensing unfolded proteins, an ER-resident transmembrane endoribonuclease IRE1 exhibits unconventional splicing activity on XBP-1 mRNA, which results in the conversion of an inactive unspliced XBP-1 (XBP-1u) to an active spliced XBP-1 (XBP-1s) protein. XBP-1s induces transcription of a broad array of UPR genes that assist in protein synthesis and secretion. Development of XBP-1-deficient pDCs is reduced, likely due to their increased sensitivity to apoptosis induced by ER stress.

Another important step for physiological activity of pDCs is intracellular trafficking of nucleic acid-sensing TLRs from ER to endolysosomes. Recent studies have revealed that a multiple membrane-spanning protein Unc93B1 physically interacts with nucleic acid-sensing TLRs (TLR3,
-7, -9) in ER and delivers them to endolysosomes where the TLRs transmit an activating signal.\(^{19,20}\) Thus, the interaction between nucleic acid-sensing TLRs and Unc93B1 constitutes another target for controlling the activity of pDCs.

A selective inhibitor of the 26S proteasome, bortezomib, has been established as an effective drug for plasma cell myeloma.\(^{21}\) Although multiple mechanisms have been reported for the antitumor activity of bortezomib,\(^{21}\) growing evidence suggests that the selectivity of bortezomib for myeloma may be explained by increased susceptibility of myeloma cells to ER stress-induced apoptosis,\(^{22,23}\) which is consistent with a crucial role of XBP-1 in development of plasma cells\(^ {24,25}\) and in the pathogenesis of myeloma.\(^{26}\) Bortezomib is likely to disturb ER homeostasis of myeloma cells by targeting several points. For example, proteasome inhibitors have been shown to suppress the activity of IRE1 and to stabilize the dominant negative XBP-1\(u\) protein, resulting in the decrease in the activity of XBP-1\(s\) in myeloma cells.\(^ {22}\) Proteasome inhibitors also prevent retrograde translocation of misfolded proteins in the ER to the cytosol, resulting in the accumulation of a large amount of misfolded immunoglobulin in the ER in myeloma cells.\(^ {27}\) Such overloading of the ER might compromise physiological functions of the ER. Importantly, pDCs resemble plasma cells, in that both have the developed ER,\(^ {28}\) are highly secretory, and depend on ER homeostasis, particularly on XBP-1, for their development and survival.\(^ {14,24,25}\) Furthermore, coordinated trafficking of the two ER-resident proteins TLR and Unc93B1 is necessary for pDCs to respond to the TLR ligands.\(^ {20}\) Therefore, we hypothesized that bortezomib may suppress the activity of pDCs by targeting the two critical events in the ER: the UPR and the coordinated function of the ER-resident TLRs and Unc93B1.

Here we investigated the effects of bortezomib on human pDCs. We show that bortezomib inhibits the production of IFN-\(\alpha\) by blocking intracellular trafficking of nucleic acid-sensing TLRs at an early stage, and thereafter induces apoptosis of pDCs, which correlates the suppression of XBP-1 splicing. These results have significant implications for the physiological mechanisms of survival and activation of pDCs and for the application of proteasome inhibitors to inflammatory disorders in which pDCs play a key role.

**Methods**

*Culture media, reagents, and cell lines*

RPMI 1640 (Sigma-Aldrich, St. Louis, MO) supplemented with 10% heat-inactivated fetal calf serum (FCS) (ThermoTrace, Victoria, Australia), 2 mM L-glutamine, penicillin G, streptomycin (Gibco BRL, Carlsbad, CA), and 10 mM HEPES (Nacalai tesque, Kyoto, Japan) was used for cell
culture. Bortezomib (provided by Millennium Pharmaceuticals, Cambridge, MA) was dissolved in dimethylsulfoxide at 10 mM as a stock solution and was stored at -20 degrees Celsius. Influenza virus \([10^{5.3} \text{ tissue culture infectious dose 50 (TCID}_{50})/0.2 \text{ ml A/Niigata/05F254/2006}; \text{ a kind gift from Dr. Reiko Saito, Niigata University, Niigata, Japan}\) was inactivated at 56 degrees Celsius for 30 min, and was added at 0.1% vol/vol to cell culture. A cell line derived from blastic plasmacytoid dendritic cell neoplasm CAL-1 has been reported.\(^{29}\) A myeloma cell line RPMI 8226 was obtained from ATCC (Manassas, VA).

**Isolation of pDCs**

This study was approved by the Institutional Review Board at Graduate School of Medicine, Kyoto University, and abides by the tenets of the Declaration of Helsinki. PBMCs were obtained from healthy donors with written informed consent in accordance with the Declaration of Helsinki. pDCs were isolated as described.\(^{30}\) In brief, CD4^+CD11c^+lin^- cells were isolated as pDCs using FACS Aria™ cell sorter (BD Biosciences, San Jose, CA). Reanalysis of the sorted cells confirmed a purity of more than 98%.

**Cell viability assays**

PBMCs (2 x 10^6 cells per 2 ml in 12-well culture plates) were cultured with bortezomib for 6 hours or 24 hours. The cells were stained with the following combinations monoclonal antibodies (mAbs): FITC-conjugated anti-CD3 (BD Biosciences) and PE-conjugated anti-CD56 mAbs (Beckman Coulter, Miami, FL), FITC-conjugated CD19 (BD Biosciences) and PE-conjugated anti-BDCA-1 mAbs (Miltenyi Biotec, Bergisch Gladbach, Germany), or FITC-conjugated anti-BDCA-2 (Miltenyi Biotec) and PE-conjugated anti-CD14 mAbs (Beckman Coulter). T cells (CD3^+), NK cells (CD3^+CD56^+), B cells (CD19^+), mDCs (BDCA-1^+CD19^-), pDCs (BDCA-2^+), and monocytes (CD14^+) were identified by flow cytometry with FACSCalibur™ (BD Biosciences). Dead cells were excluded by staining with propidium iodide (PI). Viable cell numbers of each cell population were counted using Flow-Count™ Fluorospheres (Beckman Coulter) according to the manufacturer's instructions. Purified pDCs (4 x 10^4 cells per 200 μl in round-bottomed 96-well culture plates) were cultured with bortezomib for 3 hours, and were stimulated with 0.5 μM oligodeoxynucleotide (ODN)2216 (CpG-A)\(^{31}\) (Operon Biotecnologies, Tokyo, Japan) in the presence of bortezomib for 24 hours. The cells were stained with Annexin V (FITC-conjugated CELL LAB ApoScreen™ Annexin V, Beckman Coulter) and PI and were analyzed for viability by flow cytometry with FACSCalibur™. Alternatively, pDCs were treated with 50 μM pan-caspase inhibitor Z-VAD-FMK.
(R&D Systems, Minneapolis, MN) together with bortezomib and 0.5 μM ODN2216, stained with FITC-conjugated Annexin V, and analyzed by flow cytometry. The percentages of specific death induced by bortezomib were calculated as $100 \times (\text{experimental death %} - \text{spontaneous death %}) / (100 - \text{spontaneous death %})$, in which the percentages of experimental or spontaneous death were defined as the percentages of Annexin V-positive cells in the presence or absence of bortezomib, respectively.

**Reverse transcription and real-time PCR**

Cells were treated tunicamycin (Wako Pure Chemical Industries, Osaka, Japan) at 0.4 μg/ml for CAL-1, at 0.8 μg/ml for RPMI 8226, or at 5 μg/ml for pDCs and T cells to induce ER stress. Total RNA was isolated using QIAshredder and RNeasy Mini Kit (Qiagen, Valencia, CA). First-strand cDNA synthesis was performed with ReverTra Ace® qPCR RT Kit (TOYOBO, Osaka, Japan). Real-time PCR was performed on the Thermal Cycler Dice® Real Time System (TaKaRa, Shiga, Japan). XBP-1 was detected using SYBR® Premix Ex Taq™ (TakaRa) and gene-specific oligonucleotide primers as follows.

XBP-1u gene: 5’-CGAATGAGTGAGCTGGAACA-3’ (forward) and 5’-CTGCAGAGGTGCACGTAGTC-3’ (reverse)

XBP-1s gene: 5’-CGAATGAGTGAGCTGGAACA-3’ (forward) and 5’-CTGCACCGTGCACGTAGTC-3’ (reverse)

IFN-α1 and β-glucuronidase (GUS) were detected using TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA) and THUNDERBIRD™ Probe qPCR Mix (TOYOBO). Relative quantitation of mRNA expressions was performed by the ΔΔCt method. The mRNA expression levels of each gene were normalized to those of GUS.

**Analysis of cytokine production by ELISA**

Cytokines in the supernatants were analyzed by ELISA. The following reagents were used: Human IFN-alpha Module Set (Bender MedSystems, Burlingame, CA) and Human IL-6 ELISA MAX™ Standard Set (BioLegend, San Diego, CA).

**Retroviral transduction**

HEK293T cells were cotransfected with retroviral vectors (pMXpuro carrying a GFP-tagged mouse TLR9 gene32 or pMXneo carrying a GFP-tagged mouse UNC93B1 gene33) and retroviral packaging plasmids (pMLVg/p and pVSV-G) using CalPhos™ Mammalian Transfection Kit (Clontech,
Forty-eight hours after transfection, the supernatant was collected. A mouse B-cell line M12\(^{32}\) was infected with the virus suspension.

**Confocal analysis of intracellular trafficking of TLR9 and Unc93B1**

M12 cells expressing TLR9-GFP or Unc93B1-GFP were cultured in the absence or presence of 30 nM bortezomib for 1 hour, and were stimulated with 0.5 \(\mu\text{M}\) ODN1668\(^{34}\) (Operon Biotechnologies) in the absence or presence of 30 nM bortezomib for 2 hours. ER-Tracker\(^{\text{TM}}\) Red or Lysotracker\(^{\text{R}}\) Red DND-99 (Invitrogen, Carlsbad, CA) was added for 30 min before the harvest. After fixation with 4% paraformaldehyde for 15 min at 37 degrees Celsius, the cells were attached to poly-L-lysine-coated slide glass, and were examined with an LSM 510 confocal microscope with an \(\alpha\text{Plan-FLUAR} 100\times/1.45\) numeric aperture oil immersion objective (Carl Zeiss, Oberkochen, Germany). Data were acquired by the use of Laser Scanning Microscope LSM 510, Version 3.2 SP2 software (Carl Zeiss).

**Confocal analysis of nuclear translocation of IRF-7 and NF-\(\kappa\)B**

Purified pDCs were cultured in the absence or presence of 10 nM bortezomib for 3 hours, and were stimulated with 0.5 \(\mu\text{M}\) ODN2216 in the absence or presence of 10 nM bortezomib for 3 hours. After fixation with 2% paraformaldehyde for 15 min at 37 degrees Celsius and permeabilization with 100% methanol for 10 min at -20 degrees Celsius, the cells were stained with rabbit anti-IRF-7 or NF-\(\kappa\)Bp65 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and with Alexa Fluor\(^{\text{R}}\)488-conjugated goat-anti rabbit IgG (Invitrogen) as a secondary antibody. Nuclei were identified using TOTO-3 dye (Invitrogen). The cells were attached to slide glass by cytospin, and examined by confocal microscopy.

**Statistical analysis**

Data are presented as means plus standard error (SE). Statistical comparisons were performed using paired one-tailed t tests, with a P value below 0.05 taken to indicate significance.

**Results**

*\(p\)DCs were most susceptible to the killing effect of bortezomib among immune cells in blood*

We first examined the viability of each mononuclear cell population in peripheral blood after culture with bortezomib alone. PBMCs were cultured with bortezomib for 6 hours, washed to remove bortezomib, and then cultured for 18 hours. Alternatively, PBMCs were cultured with
bortezomib for 24 hours. Thereafter, the viability of each mononuclear cell population was examined by staining the cells with fluorochrome-conjugated mAbs (CD3+ for T cells, CD3 CD56+ for NK cells, CD19+ for B cells, CD14+ for monocytes, BDCA-1+CD19+ for mDCs and BDCA-2+ for pDCs) and PI. Bortezomib was added at 3 – 100 nM (1.15 – 38.5 ng/ml), which are clinically relevant concentrations observed in blood after administration.35,36 The 6-hour exposure to bortezomib mimics the situation in which concentrations are transiently elevated after administration. Resting T cells were most resistant to the killing effect of bortezomib (Figure 1A), consistent with a previous report.37 NK cells, B cells, and monocytes are more susceptible to the killing effect than T cells. Bortezomib killed mDCs more than these cells. Notably, pDCs were most susceptible to the killing effect, and even short term exposure to 10 nM greatly reduced viability after 24 hours in total.

We further examined whether bortezomib also reduces the viability of pDCs stimulated with a TLR9 ligand ODN2216 (CpG-A). Because pDCs cannot be unequivocally identified among PBMCs after stimulation with CpG ODN due to the change of their surface phenotype after stimulation,38 we purified pDCs, stimulated them with ODN2216 in the absence or presence of different concentrations of bortezomib for 24 hours, and analyzed the viability by Annexin V and PI staining (Figure 1B). Whereas the majority of pDCs underwent apoptosis without stimulation, as shown previously,28,39 stimulation with ODN2216 greatly improved the viability. Thirty nM or more bortezomib strongly induced apoptosis of ODN2216-stimulated pDCs.

These data indicate that bortezomib has the strongest killing effect on pDCs among different populations of PBMCs in a resting condition, and also induces apoptosis of pDCs stimulated with CpG ODN.

**Inhibition of XBP-1 splicing correlates with the induction of apoptosis of pDCs by bortezomib**

Because it has been shown that XBP-1 is essential for the development of plasma cells24,25 and pDCs,14 and that bortezomib disrupts the UPR by inhibiting the generation of the active form of XBP-1, spliced XBP-1 (XBP-1s), in plasma cells,22 we examined whether bortezomib also inhibits the generation of XBP-1s mRNA in pDCs. We cultured primary pDCs, a cell line derived from blastic plasmacytoid dendritic cell neoplasm CAL-1,29 a myeloma cell line RPMI 8226, and T cells in the absence or presence of bortezomib and tunicamycin, an inhibitor of N-linked glycosylation known to induce ER stress and the UPR. Then the amounts of spliced XBP-1 (XBP-1s) mRNA and unspliced XBP-1 (XBP-1u) mRNA were quantitated by real-time RT-PCR, and the ratios of XBP-1s to XBP-1u were calculated (Figure 2). The addition of tunicamycin increased the amounts of XBP-1s mRNA relative to those of XBP-1u mRNA in all the cell types. The addition of bortezomib

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suppressed the generation of XBP-1s mRNA induced by tunicamycin in pDCs, CAL-1, and RPMI 8226, whereas bortezomib did not decrease XBP-1s mRNA in T cells, which are resistant to the killing effect of bortezomib (Figure 1A). Thus, apoptosis of pDCs are correlated with the decrease in XBP-1s, suggesting that disruption of ER homeostasis by bortezomib results in apoptosis of pDCs as well as of plasma cells.22

Bortezomib suppresses cytokine production by pDCs stimulated with CpG ODN or influenza virus

Next we examined whether bortezomib suppresses cytokine production by pDCs stimulated with ODN2216 (a TLR9 ligand) or influenza virus (a TLR7 ligand). We pretreated PBMCs with different concentrations of bortezomib for 6 hours, washed the cells to remove bortezomib, and cultured them with the TLR ligands for 24 hours. Alternatively, we pretreated PBMCs with bortezomib for 6 hours, added the TLR ligands without removing bortezomib, and cultured the cells for 24 hours. Then concentrations of IFN-α in the supernatants were measured by ELISA. Because the absolute concentrations were variable depending on donors, the levels of cytokine concentrations were normalized to the maximum value obtained in the absence of bortezomib. Pretreatment with bortezomib suppressed the production of IFN-α induced by ODN2216 or influenza virus in a dose-dependent manner [Wash (+), Figure 3A]. The addition of bortezomib during the whole period of culture further suppressed the production of IFN-α [Wash (-), Figure 3A]. These data indicate that bortezomib strongly suppresses the production of IFN-α by pDCs stimulated with the TLR9 or TLR7 ligand in a dose- and time-dependent manner.

We also examined the effect of bortezomib on the production of IFN-α and IL-6 by purified pDCs stimulated with ODN2216. We pretreated purified pDCs with different concentrations of bortezomib for 3 hours, added ODN2216 without removing bortezomib, and cultured the cells for 24 hours. Then concentrations of IFN-α and IL-6 in the supernatants were measured by ELISA. Bortezomib suppressed the production of both IFN-α and IL-6 by purified pDCs in a dose-dependent manner (Figure 3B).

To examine whether the suppression of cytokine production is due to the induction of apoptosis or not, we stimulated pDCs with ODN2216 in the absence or presence of bortezomib for 4 hours. Then viability was measured by Annexin V/PI staining, and the amounts of IFN-α mRNA were measured by real-time RT-PCR. Whereas viability did not decrease during the culture, bortezomib strongly suppressed the transcription of IFN-α mRNA (Figure 4A). We also cultured pDCs with ODN2216 and different concentrations of bortezomib in the absence or presence of a pan-caspase inhibitor Z-VAD-FMK. Then the percentages of apoptosis specifically induced by bortezomib were calculated,
and concentrations of IFN-α in the supernatants were measured by ELISA. Although Z-VAD-FMK significantly suppressed apoptosis of pDCs induced by each concentration (3, 10, 100 nM) of bortezomib, the recovery of IFN-α production by Z-VAD-FMK was marginal; it was significant only at 10 nM bortezomib (Figure 4B). Based on these data, we concluded that bortezomib suppresses IFN-α production by pDCs in a partly apoptosis-independent manner.

**Bortezomib inhibits trafficking of TLR9 but not of Unc93B1 from ER to endolysosomes induced by CpG ODN**

We investigated the mechanisms by which bortezomib suppresses the cytokine production by pDCs. The earliest event leading to CpG ODN-induced IFN-α production is endocytosis of CpG ODN by pDCs. Thus, we examined whether bortezomib inhibits uptake of CpG ODN by pDCs. We pretreated pDCs with bortezomib for 3 hours, and added FITC-conjugated ODN2216. After 90 min, we examined the intracellular localization of ODN2216 by confocal microscopy (Supplementary Figure 1). Whereas pDCs kept on ice did not endocytose ODN2216, pDCs cultured at 37 degrees Celsius did so. Bortezomib did not inhibit the endocytosis of ODN2216. Thus, bortezomib targets further downstream signaling pathway(s).

Upon stimulation with CpG ODN, TLR9 rapidly moves from the ER to endolysosomes.18 Thus, we next examined whether bortezomib inhibits the intracellular trafficking of TLR9. We stimulated a mouse B-cell line M12 expressing TLR9-GFP with ODN1668 in the absence or presence of bortezomib, and examined whether TLR9 co-localizes with an ER marker (ER-Tracker) or a lysosomal marker (LysoTracker) by confocal microscopy. Whereas TLR9 was located in the ER (Figure 5A) but not in endolysosomes (Figure 5B) without stimulation, TLR9 left the ER (Figure 5A) and moved to endolysosomes (Figure 5B) after stimulation with CpG ODN. Importantly, in the presence of bortezomib, TLR9 remained in the ER (Figure 5A) and did not move to endolysosomes (Figure 5B) after stimulation. We also used a human pDC line CAL-1, and obtained similar results; bortezomib inhibited the trafficking of TLR9 from the ER to endolysosomes induced by ODN2216 (Supplementary Figure 2).

Recent studies have revealed that a multiple membrane-spanning protein Unc93B1 physically interacts with nucleic acid-sensing TLRs in the ER and delivers them to endolysosomes upon stimulation with a TLR ligand.19,20 Thus, we examined whether bortezomib inhibits the trafficking of TLR9 by inhibiting that of Unc93B1. We stimulated M12 expressing Unc93B1-GFP with ODN1668 in the absence or presence of bortezomib, and examined whether Unc93B1 co-localizes with an ER marker (ER-Tracker) or a lysosomal marker (LysoTracker) by confocal microscopy. Whereas
Unc93B1 was located in the ER (Figure 6A) but not in endolysosomes (Figure 6B) without stimulation. Unc93B1 left the ER (Figure 6A) and moved to endolysosomes (Figure 6B) after stimulation with CpG1668. Notably, the translocation of Unc93B1 from the ER to endolysosomes was not abrogated by bortezomib (Figure 6A, B).

Collectively, these data indicate that bortezomib inhibits the trafficking of TLR9 but not of Unc93B1 from the ER to endolysosomes upon stimulation with CpG ODN. This suggests that bortezomib suppresses the immunostimulatory functions of TLR-triggered pDCs by disrupting the coordinated trafficking of TLR and Unc93B1. In addition, as the inhibition of TLR9 trafficking was observed during the early period of culture (3 hours after adding bortezomib) when apoptosis had not ensued, bortezomib is likely to suppress the immunostimulatory function of pDCs independently of the induction of apoptosis at least in part.

**Bortezomib inhibits nuclear translocation of IRF-7 and NF-κB in pDCs stimulated with CpG ODN**

Stimulation of pDCs with CpG ODN induces nuclear translocation of two major transcription factors IRF-7 and NF-κB that induce the production of IFN-α and proinflammatory cytokines (TNF-α and IL-6), respectively, at the final step of the TLR signaling. Thus, we examined whether bortezomib inhibits the nuclear translocation of IRF-7 and NF-κB induced by ODN2216 in pDCs, consistently with the inhibition of intracellular trafficking of TLR9 (Figure 5) and the suppression of IFN-α and IL-6 production (Figure 3). We pretreated pDCs with bortezomib for 3 hours, and stimulated them with ODN2216 for 3 hours. Then we examined the localization of IRF-7 (Figure 7A) and NF-κB (Figure 7B) by confocal microscopy. Whereas IRF-7 and NF-κB are located in the cytoplasm in untreated pDCs, both of the transcription factors moved to the nucleus after the stimulation with ODN2216. Bortezomib inhibited the nuclear translocation of IRF-7 and NF-κB. These data indicate that the inhibition of TLR9 trafficking by bortezomib leads to the abrogation of the nuclear translocation of IRF-7 and NF-κB.

**Discussion**

Owing to the proposed involvement of pDCs in the pathogenesis of several inflammatory disorders, it is of great importance to find out reagents that modulate the functions of pDCs. Notably, pDCs and plasma cells share a distinctive property, in that both cells have highly developed ER and produce vast amounts of secretory proteins, i.e. IFN-α and immunoglobulin, respectively. Thus, we hypothesized that a proteasome inhibitor bortezomib, which kills myeloma cells partly due to its
effect on ER homeostasis, may affect the viability and function of pDCs. Here we showed two novel findings. First, bortezomib suppresses immunostimulatory activity of pDCs by disrupting the coordinated translocation of TLR9 and an ER-resident protein Unc93B1. Second, bortezomib induces apoptosis of pDCs apparently by disturbing ER homeostasis maintained through the activation of XBP-1. This is the first study showing pharmacological disruption of the coordinated translocation of nucleic acid-sensing TLRs and Unc93B1. This study sheds new light on the molecular mechanisms by which pDCs perform their immune functions and on the mechanisms by which bortezomib executes immunosuppressive activity.

We first examined the effect of bortezomib on the viability of pDCs using total PBMCs (Figure 1A) or purified pDCs (Figure 1B). Using total PBMCs, we found that pDCs were most prone to die among different immune cell types in blood even after 6-hour pretreatment with 10 nM bortezomib and subsequent culture without bortezomib for 18 hours. Because the majority of purified pDCs spontaneously underwent apoptosis after 24 hours, as shown previously, we examined the effect of bortezomib on the viability of purified pDCs (2 x 10^5/ml) under the stimulation with CpG ODN, and found that most of them died after exposure to 30 nM or more bortezomib. There have been conflicting reports concerning the effect of bortezomib on the viability of pDCs. Kukreja et al has reported that the majority of purified pDCs undergo apoptosis after culture either with 100 nM bortezomib for 48 hours or with 10 nM bortezomib for 24 hours. In contrast, Chauhan et al has reported that the majority of 1 x 10^6 purified pDCs are viable even after culture with 20 nM bortezomib for 24 hours. Although the reason for such differences in the viability of pDCs among the studies is not known, it appears that Chauhan et al cultured pDCs at a much higher cell concentration than we did in this study. Such difference in culture conditions might have resulted in the differences in the effect of bortezomib. In any event, our data using total PBMCs and purified pDCs clearly show that pDCs are prone to die after exposure to bortezomib.

Highly secretory cells such as plasma cells and exocrine gland acinar cells depend on the UPR for their development and survival, as evidenced in XBP-1-deficient conditions. It has been shown that proteasome inhibitors target XBP-1 through suppressing the activity of IRE1. Importantly, pDCs are also highly secretory, and depend on XBP-1 for their development and survival. Thus, we examined whether bortezomib suppresses the generation of XBP-1s in pDCs. We found that bortezomib strongly suppressed XBP-1 splicing in bortezomib-susceptible pDCs as well as in myeloma cells but not in bortezomib-resistant resting T cells. These data, together with the previous report, suggest that the suppression of XBP-1 splicing by bortezomib leads to the apoptosis of pDCs.
We next examined the effect of bortezomib on the cardinal feature of pDCs: the production of IFN-α in response to TLR7 and TLR9 ligands. We also examined the production of a proinflammatory cytokine IL-6, because IFN-α and IL-6 are induced by different transcription factors, IRF-7\(^{44}\) and NF-κB,\(^{40}\) respectively. Bortezomib suppressed the production of IFN-α and IL-6 by pDCs stimulated with a TLR9 ligand ODN2216 (CpG-A) and also the production of IFN-α induced by a TLR7 ligand influenza virus in a dose-dependent manner. Bortezomib appears to suppress the cytokine production by pDCs independently of the induction of apoptosis at least in part, because (i) bortezomib suppressed the trafficking of TLR9 and the induction of IFN-α mRNA at an early time point when apoptosis of pDCs had not ensued and (ii) a pan-caspase inhibitor Z-VAD-FMK rescued the IFN-α production only marginally, whereas the reagent significantly rescued pDCs from apoptosis. However, we cannot exclude the possibility that the diminution of cytokine production reflects early apoptotic signaling. For example, it has been shown that bortezomib induces the generation of reactive oxygen species\(^{45}\) and the activation of c-Jun NH\(_2\)-terminal kinase\(^{46}\) following ER stress, leading to the mitochondrial apoptotic pathway. Such early apoptotic signals might affect functions of pDCs.

Next we examined the mechanisms by which bortezomib suppresses the cytokine production by pDCs. The observation by confocal microscopy showed that bortezomib inhibits the intracellular trafficking of TLR9 from the ER to endolysosomes. Notably, however, bortezomib did not inhibit the trafficking of Unc93B1, which has been reported to deliver nucleic acid-sensing TLRs from the ER to endolysosomes.\(^{20}\) These data suggest that bortezomib inhibits the responses of pDCs to nucleic acids by disrupting the coordinated movement of TLRs and Unc93B1. We finally confirmed that such inhibition of the trafficking of TLR9 resulted in the abrogation of nuclear translocation of IRF-7 and NF-κB, which corresponds with the suppression of IFN-α\(^{44}\) and IL-6\(^{40}\) production, respectively.

As precise molecular mechanisms by which Unc93B1 delivers TLRs from the ER to endolysosomes remain to be known, the mechanism by which bortezomib disrupts the coordinated trafficking of TLR9 and Unc93B1 is not clear. But it is tempting to speculate that accumulation of misfolded proteins in the ER caused by bortezomib may disturb proper functions of ER-resident proteins responsible for the trafficking of TLR9 and Unc93B1. We have recently shown that wild type and D34A mutant Unc93B1 preferentially associate and translocate with TLR9 and TLR7, respectively,\(^{33}\) implying that as yet unidentified ER protein interacts with the N-terminal region of Unc93B1 and facilitates the trafficking of Unc93B1-TLR complexes to endolysosomes. Such a mechanism might be disarranged by overwhelming ER stress caused by bortezomib.
It has been proposed that IFN-α from pDCs stimulated with endogenous nucleic acids plays a key role in the pathogenesis of several autoimmune or inflammatory disorders. First, immune complexes composed of DNA/anti-DNA autoantibodies or ribonucleoprotein/anti-ribo-

ucleoprotein autoantibodies are incorporated into pDCs and stimulate them to produce IFN-α by triggering TLR9 and TLR7, respectively. Such IFN-α is implicated in the pathogenesis of SLE. Intriguingly, a study using the Unc93B1 3d mutant mice, in which Unc93B1 is incapable of binding to TLRs, has shown that nucleic acid-sensing TLRs are required for optimal production of autoantibodies in lupus-prone strains. Thus, the immune complexes and pDCs appear to constitute a positive feedback loop mediated by the TLR-Unc93B1 interaction. Proteasome inhibitors may disrupt such a vicious cycle by targeting pDCs, resulting in alleviation of SLE. Second, aggregates composed of the antimicrobial peptide LL37 and self DNA or RNA released from damaged cells are incorporated into pDCs and stimulate them to produce IFN-α by triggering TLR9 and TLR7, respectively. Such IFN-α is implicated in the pathogenesis of psoriasis. Bortezomib may alleviate psoriasis by targeting pDCs. Furthermore, pDCs may be involved in the pathogenesis of type 1 diabetes and chronic graft-versus-host disease. These findings suggest that proteasome inhibitors may be exploited for the treatment of these autoimmune or inflammatory disorders through its suppressive activity on pDCs. The second generation of proteasome inhibitors that has less neurotoxicity than bortezomib and is thus more suitable for long-term use may be applicable to such chronic inflammatory disorders.

In conclusion, this study suggests that bortezomib suppresses the activity of pDCs by disrupting the coordinated trafficking of nucleic acid-sensing TLRs and Unc93B1 from the ER to endolysosomes and by suppressing the UPR. Bortezomib or a next generation of proteasome inhibitors may therefore be instrumental in treating pDC-mediated, IFN-α-driven inflammatory disorders.
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Authorship Contributions
The specific contributions of all authors
M.H.: preformed research, wrote the manuscript
N.K.: designed research, analyzed and interpreted data, wrote the manuscript
T.K.: contributed vital experimental designs and techniques
H.F.: preformed research
A.T.-K.: contributed vital experimental designs and techniques
R.F.: contributed vital analytical tools
K.M.: contributed vital analytical tools
T.M.: contributed vital new reagents
S.K.: contributed vital new reagents
Y.M.: supervised the whole project
T.U.: supervised the whole project

Disclosures of Conflict of Interest
The authors declare no conflict of interests.
References


Figure 1. pDCs are susceptible to the killing effect of bortezomib.

(A) PBMCs (2 x 10^6 cells per 2 ml) were cultured without or with the indicated concentrations of bortezomib for 6 hours, washed to remove bortezomib, adjusted the cell concentration to 1 x 10^6/ml, and further cultured for 18 hours without bortezomib [Wash (+)]. Alternatively, PBMCs (2 x 10^6 cells per 2 ml) were cultured without or with bortezomib for 24 hours [Wash (-)]. After staining the PBMCs with mAbs to identify each cell population and with PI to exclude dead cells, viable cell numbers were counted using Flow-Count™ Fluorospheres by flow cytometry. Cell numbers in the presence of bortezomib relative to those in the absence of bortezomib were expressed by percentage. Mo: monocytes. (B) Purified pDCs (4 x 10^4 cells per 200 μl) were cultured without or with the indicated concentrations of bortezomib for 3 hours, and were stimulated with 0.5 μM ODN2216 in the presence of bortezomib for 24 hours. The cells were stained with Annexin V and PI, and were analyzed by flow cytometry. The numbers indicate the percentages of Annexin V- and PI-double negative viable cells. *, P < 0.05; **, P < 0.01; ***, P < 0.001. The data are shown as means + SE of 3 independent experiments. P values refer to the comparison between the data obtained without bortezomib and those obtained with each concentration of bortezomib.

Figure 2. Bortezomib inhibits XBP-1 splicing in pDCs.

Purified pDCs, a pDC tumor cell line CAL-1, a myeloma cell line RPMI 8226, or resting T cells were cultured without or with 100 nM bortezomib for 1 hour, and 5 μg/ml tunicamycin (Tm) was added 4 hours before harvest. The expression levels of XBP-1u and XBP-1s mRNA were measured by real-time RT-PCR, and were normalized to those of GUS. The XBP-1s/XBP-1u ratios were calculated. The data are normalized to the value obtained with tunicamycin in the absence of bortezomib. *, P < 0.05; **, P < 0.01. The data are shown as means + SE of 3 (pDC) or 4 (CAL-1, RPMI8226, T) independent experiments.

Figure 3. Bortezomib suppresses cytokine production by pDCs stimulated with ODN2216 or influenza virus.

(A) PBMCs (1 x 10^6/ml) were cultured without or with the indicated concentrations of bortezomib for 6 hours, washed to remove bortezomib, adjusted the cell concentration to 1 x 10^6/ml, and further cultured for 24 hours in the presence of 0.5 μM ODN2216 or influenza virus without bortezomib [Wash (+)]. Alternatively, PBMCs (1 x 10^6/ml) were cultured without or with bortezomib for 6 hours,
and cultured for 24 hours in the presence of ODN2216 or influenza virus without removing bortezomib [Wash (-)]. Concentrations of IFN-α in the supernatants were measured by ELISA. The data are normalized to the value obtained without bortezomib. *, P < 0.05; **, P < 0.01; ***, P < 0.001. The data are shown as means + SE of 3 independent experiments. P values refer to the comparison between the data obtained without bortezomib and those obtained with each concentration of bortezomib. The means and ranges of absolute concentrations are as follows: ODN2216, wash (-) 4292 pg/ml (1674-9455 pg/ml); influenza, wash (-) 1278 pg/ml (1008-1538 pg/ml). (B) Purified pDCs (2 x 10^5/ml) were cultured without or with the indicated concentrations of bortezomib for 3 hours, and cultured for 24 hours in the presence of 0.5 μM ODN2216 without removing bortezomib. Concentrations of IFN-α and IL-6 in the supernatants were measured by ELISA. **, P < 0.01; ***, P < 0.001. The data are shown as means + SE of 3 independent experiments. The means and ranges of absolute concentrations are as follows: IFN-α 74312 pg/ml (48118-92759 pg/ml); IL-6 3904 pg/ml (1970-6343 pg/ml).

**Figure 4. Relationship between viability and IFN-α production by pDCs.**

(A) Purified pDCs were cultured with 10 nM bortezomib for 2 hours, and 0.5 μM ODN2216 was added. The cells were harvested after 4 hours. The cells were stained with FITC-conjugated Annexin V and were analyzed for viability by flow cytometry (Upper panel). IFN-α mRNA was quantitated by real-time RT-PCR, and the expression levels were normalized to those of GUS (Lower panel). The data are normalized to the value obtained with ODN2216 in the absence of bortezomib. *, P < 0.05. The data are shown as means + SE of 3 independent experiments. (B) After purified pDCs (1 x 10^5/ml) were cultured without or with 50 μM Z-VAD-FMK for 1 hour, the indicated concentrations of bortezomib were added. After 6 hours, 0.5 μM ODN2216 was added, and the cells and supernatants were harvested 18 hours later. The cells were stained with FITC-conjugated Annexin V, and analyzed by flow cytometry. The percentages of cell death specifically induced by bortezomib were calculated (Upper panel). Concentrations of IFN-α in the supernatants were measured by ELISA (Lower panel). The data are normalized to the value obtained without bortezomib. *, P < 0.05; **, P < 0.01. NS: not significant. The data are shown as means + SE of 6 independent experiments.

**Figure 5. Bortezomib inhibits the trafficking of TLR9 from the ER to endolysosomes induced by CpG ODN.**

A mouse B-cell line M12 expressing TLR9-GFP was cultured in the absence or presence of 30 nM
bortezomib for 1 hour, and cultured with ODN1668 in the absence or presence of bortezomib for 2 hours. ER-Tracker (A) and LysoTracker (B) were added during the last 30 minutes. The cells were observed by confocal microscopy. The data are representative of four experiments.

Figure 6. Bortezomib does not inhibit the trafficking of Unc93B1 from the ER to endolysosomes induced by CpG ODN.

M12 expressing Unc93B1-GFP was cultured in the absence or presence of 30 nM bortezomib for 1 hour, and cultured with 0.5 μM ODN1668 in the absence or presence of bortezomib for 2 hours. ER-Tracker (A) and LysoTracker (B) were added during the last 30 minutes. The cells were observed by confocal microscopy. The data are representative of four experiments.

Figure 7. Bortezomib inhibits the nuclear translocation of IRF-7 and NF-κB in pDCs.
Purified pDCs were cultured in the absence or presence of 10 nM bortezomib for 3 hours, and were stimulated with 0.5 μM ODN2216 for 3 hours. The cells were stained with rabbit-anti-IRF-7 (A) or NF-κBp65 (B) and with Alexa Fluor® 488-conjugated goat anti-rabbit IgG as a secondary antibody. Nuclei were identified using TOTO-3 dye. The data are representative of three experiments.
Figure 1

A

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Figure 3

A

IFN-α (% of maximum)

CpG ODN

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Influenza virus

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B

IFN-α (% of maximum)

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IL-6 (% of maximum)

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* p < 0.05
** p < 0.01
*** p < 0.001
Figure 7

A

IRF-7

TOTO-3

Merge

CpG  -  +  +
Bortezomib  -  -  +

B

NF-κB

TOTO-3

Merge

CpG  -  +  +
Bortezomib  -  -  +
Bortezomib suppresses function and survival of plasmacytoid dendritic cells by targeting intracellular trafficking of Toll-like receptors and endoplasmic reticulum homeostasis

Makiko Hirai, Norimitsu Kadowaki, Toshio Kitawaki, Haruyuki Fujita, Akifumi Takaori-Kondo, Ryutaro Fukui, Kensuke Miyake, Takahiro Maeda, Shimeru Kamihira, Yoshiki Miyachi and Takashi Uchiyama