

Research article

Enhanced Dendritic Cell Development Through Long-Term Proteasome Inhibition

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Abstract

Bortezomib (BTZ) is a potent and reversible proteasome inhibitor which has shown efficacy in the treatment of multiple myeloma. BTZ also affects NF- κ B activity, however, the same mechanisms through which it curtails malignant cell proliferation may also compromise antitumor immunity. As dendritic cells (DC) play a vital role in the elicitation and maintenance of antitumor immunity, we herein studied the long-term effects of BTZ on human DC development and function. The CD34⁺ MUTZ-3 cell line, stably transduced with human telomerase reverse transcriptase (hTERT), was employed as a sustainable model to study human steady-state DC development and was grown in the presence of gradually increasing BTZ concentrations over a period of 4 months. The resultant BTZ-adapted cells were prospectively assessed for their ability to develop into mature Langerhans cells (LC). Long-term exposure to BTZ (>10 months) at a clinically relevant and apoptosis-inducing concentration (10 nM) provoked spontaneous differentiation in surviving precursors, evidenced by increased expression levels of CD14, TNF receptors and immunoproteasome subunits. Cytokine-dependent differentiation was also enhanced, resulting in increased numbers of mature DC with higher levels of co-stimulatory molecules and an increased capacity for T cell induction. Assessment of nuclear NF- κ B subunit levels provided evidence for a role of canonical RelB/p50 activation in the enhanced DC differentiation and maturation established through prolonged BTZ exposure. We conclude that long-term treatment with low dose BTZ is consistent with DC-dependent induction of antitumor immunity.

Keywords

Bortezomib; Proteasome inhibitor; Dendritic cell; Immunotherapy; Langerhans cells

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1. Introduction

The constitutive proteasome is part of the ubiquitin proteasome system (UPS). The constitutive proteasome is responsible for the degradation of cellular proteins and harbors the β 5 (PSMB5), β 1 (PSMB6) and β 2 (PSMB7) subunits, which cleave proteins after hydrophobic, acidic, and basic amino acid residues, respectively [1]. The immunoproteasome consists of the β 5i (LMP7), β 1i (LMP2), and β 2i (MECL1) subunits, which are expressed after stimulation with pro-inflammatory cytokines like IFN- γ and TNF- α and which replace the corresponding subunits of the constitutive proteasome. As opposed to constitutive proteasomes, which are expressed in most mammalian cells, immunoproteasomes are mainly expressed in cells of hematological origin. Although both types are important for antigen processing, immunoproteasome subunits are able to elicit distinct MHC class I epitopes [2].

Proteasomal degradation vitally controls intracellular levels of proteins involved in the regulation of key cellular processes such as differentiation, proliferation, stress, apoptosis, survival, immune activation and antigen presentation. A therapeutic window for pro-

teasome inhibitors as anticancer drugs has been established and attributed to higher expression levels of proteasome subunits, accompanied by increased proteasome activity, in malignant cells [3, 4]. Bortezomib (BTZ) is the first proteasome inhibitor that has shown clinical activity and is now the standard treatment for multiple myeloma (MM) and non-Hodgkin lymphoma [5]. Through a reversible interaction, mediated by a boron group, BTZ is able to inhibit both the constitutive proteasome and the immunoproteasome [5]. BTZ treatment of tumor cells leads to accumulation of pro-apoptotic proteins along with decreased levels of anti-apoptotic proteins [6] and interferes with NF- κ B activation, which besides apoptosis also controls the expression of growth factors and cytokines important for tumorigenesis and tumor metastasis [7].

BTZ has been reported to induce immunogenic cell death of malignant cells through exposure of heat shock proteins (HSP60 and HSP90), leading to uptake by and activation of antigen-presenting cells and subsequent activation of anti-tumor effector T cells [8]. As the most powerfully equipped antigen-presenting cells with the unique ability to induce naïve T cell activation and abrogate tumor-imposed immune tolerance, dendritic cells (DC) rely on the UPS

for antigen processing [9]. The effects of BTZ on DC have been studied in several preclinical studies with contradictory findings. Most studies have involved *in vitro* effects on monocyte-derived DC (MoDC) and have shown inhibition of DC development and function [10–14]. The effects reported included down-regulation of CD1a, DC-SIGN, HLA-DR and the co-stimulatory molecules CD40, CD80, and CD86. Furthermore, apoptosis was induced via up-regulation of the pro-apoptotic Bax protein [15]. The DC developmental stage appeared to determine susceptibility to BTZ, as mature MoDC displayed a relative resistance to both its inhibitory and pro-apoptotic effects [16]. In contrast to these *in vitro* studies, a more recent report showed improved *in vivo* DC maturation upon BTZ treatment with elevated frequencies of intratumoral DC and induction of effective T cell-mediated immune protection against tumor outgrowth [17]. Similarly, in a 4T1 mammary carcinoma mouse model, BTZ treatment increased IL-12 and IL-15 production by CD11c⁺ DC as well as IL-2 production by CD4⁺ T cells and granzyme-B and interferon- γ production by tumor-antigen specific CD8⁺ effector T cells, resulting in reduced metastatic spread. This reduction in metastatic spread was dependent on IL-12 and IL-15, since neutralization of these cytokines could negate the beneficial effects of BTZ therapy in this model [18]. Thus far, studies have concentrated on short-term exposure (5–7 days) to the drug. However, clinical efficacy requires long-term exposure to BTZ over the course of multiple treatment cycles [5]. Therefore, preclinical evaluation of the long-term effects of BTZ on DC is important, particularly in view of a possible immune component in the efficacy of BTZ [8, 17] and recent surges of interest in combining standard treatment schedules for MM with immunotherapy [19, 20].

The detailed preclinical assessment of the long-term effects of anticancer drugs on human DC development is hampered by the inability to culture primary DC progenitors over prolonged periods of time. Here, we studied the long-term effects of BTZ exposure using the human AML cell line MUTZ-3 as a DC developmental model [21]. This cell line model consists of CD34⁺ and CD14⁺ precursor cells, which, when exposed to appropriate cytokines, are able to differentiate and mature into interstitial DCs or Langerhans cells (LC) that phenotypically and functionally closely resemble their physiological equivalents [22, 23]. We previously successfully employed this model to assess the long-term effects of doxorubicin on DC development [21].

Over a period exceeding twelve months (>100 passages) MUTZ-3 cells were intermittently exposed to gradually increasing doses of BTZ, leading to low level resistance and spontaneous early DC differentiation (i.e. independent of differentiation-inducing cytokines). Accordingly, cytokine-induced LC differentiation of these BTZ-adapted precursors was enhanced and marked by higher levels of characteristic lineage and maturation markers and the induction of stronger allogeneic T cell responses. In an attempt to unravel the mechanism underlying these effects, we uncovered evidence for a possible role for BTZ-induced nuclear translocation of RelB/p50 NF- κ B complexes. Most importantly, our data support the notion that long-term treatment with BTZ is compatible with the induction of DC-dependent antitumor immunity.

2. Materials and Methods

2.1. hTERT-MUTZ3 progenitor culture and Langerhans Cell cultures

Maintainable MUTZ-3 DC precursors stably expressing human telomerase reverse transcriptase, i.e. the rate-limiting component of the telomerase complex (hereafter referred to as hTERT-MUTZ3), were derived through retroviral transduction of the AML-derived MUTZ-3 cell line, as described previously [21]. Equivalent LC/DC differentiation ability of the parent and hTERT-transduced cell line was demonstrated [21]. hTERT-MUTZ3 progenitor cells were cultured in MEM- α medium (Lonza, Vervier, Belgium) containing 20% fetal calf serum (FCS), 100IU/ml sodium-penicillin, 100 μ g/ml streptomycin, 2 mM L-glutamine, 50 μ M β -mercaptoethanol, and 10% conditioned medium of 5637 renal cell carcinoma cells (i.e. routine culture medium of MUTZ3 cells) in T75 flasks (BD Falcon) at a cell density of 0.2×10^6 cells/ml and were passaged twice weekly.

LC were cultured from hTERT-MUTZ3 progenitors with 10 ng/ml TGF- β (Biovision, Mountain View, CA), 1,000 IU/ml rhGM-CSF (Sagramostim, Berlex), and 125 IU/ml TNF- α (Miltenyi Biotec, Bergisch Gladbach, Germany) for 9 days to obtain immature MUTZ3-LC as described previously [22]. To study short-term effects of BTZ on MUTZ3-LC differentiation, BTZ (5 nM) was added to hTERT-MUTZ3 progenitors (i.e. BTZ unexposed) at the start of differentiation. Immature MUTZ3-LC were further matured by adding monocyte condition medium (MCM)—mimic consisting of 100 ng/ml IL-6 (R&D Systems, Abingdon, England), 1 μ g/ml prostaglandin-E2 (PGE2) (Sigma-Aldrich, Zwijndrecht, The Netherlands), 25 ng/ml IL-1 β (Miltenyi Biotec, Bergisch Gladbach, Germany) and 2,400 IU/ml TNF- α for 48 h.

2.2. Short- and long-term culture with bortezomib

Bortezomib (BTZ)/Velcade was provided by Millennium Pharmaceuticals (Cambridge, USA).

The bortezomib adaptation of hTERT-MUTZ3/10BTZ precursors was achieved by long-term exposure of hTERT-MUTZ3 progenitors to BTZ. The starting concentration (at passage 22) was 2 nM BTZ and was increased to 8 nM (passage 38) and 10 nM (passage 51). Both increases occurred after approximately 1 month. From passage 51 the drug was added intermittently (on and off every 2 passages). Of note, all analyses and experiments using the hTERT-MUTZ3/10BTZ were performed after the cells were exposed to the drug for at least one passage.

2.3. FACS analysis – immunophenotyping

hTERT-MUTZ3 and BTZ-adapted hTERT-MUTZ3/10BTZ cells were immunophenotyped using appropriate dilutions of FITC- or PE-conjugated monoclonal antibodies (mAbs): CD34 (PeriCluster 581) (Sanquin, Amsterdam, The Netherlands), CD14, CD1a, CD80, CD86, CD40, IL-3R, cKIT, TNF-RI, TNF-RII, HLA-ABC and HLA-DR (BD Biosciences, San Jose, CA), Langerin (Beckman Coulter, Woerden, The Netherlands). In short, 3×10^4 – 8×10^4 cells were washed with PBS supplemented with 0.1% BSA and 0.02% NaN₃, and incubated with specific or corresponding isotype-matched control mAbs for 30 minutes at 4°C. Cells were washed and analyzed with a FACSCalibur flow cytometer (Becton and Dickinson, San Jose, CA) using Cell Quest software. A live cell gate was used based on Forward and Side Scatter. Results were expressed as percentages

of positive cells and the mean fluorescence index was calculated based on mean fluorescence intensities.

2.4. Proteasome constitutive-immuno subunit ELISA assay (ProCISE)

The ProCISE assay for quantification of individual constitutive ($\beta 5$, $\beta 1$, $\beta 2$) and immunoproteasome ($\beta 5i$, $\beta 1i$, $\beta 2i$) subunits was performed as previously described [24, 25]. Expression of each constitutive 20 S and immunoproteasome 20 S subunit in hTERT-MUTZ3 and hTERT-MUTZ3/10BTZ cells was examined after cells were cultured in the absence of BTZ for one week. Pellets of $10\text{--}15 \times 10^6$ cells were washed three times with PBS, frozen on dry ice, thawed, and incubated in 100 μL of lysis buffer (20 mM Tris-HCl, pH 8.0, 5 mM EDTA) for 15 minutes on ice. Lysed pellets were microcentrifuged (15 min, 14,000 rpm, 4°C), the supernatant collected, and protein content was determined using the BCA assay (Pierce), utilizing BSA as a standard. Cell lysates were diluted in lysis buffer to 1 mg/ml and incubated with a proteasome active site probe (PABP; 5 μM) for 2 hours at 25°C. Samples were denatured with 8M guanidine hydrochloride (Fisher Scientific) and subunits bound to PABP were captured with streptavidin-conjugated sepharose beads (GE Healthcare) in 96-well, 0.65 μm porous filter plates (Millipore). Individual subunits were probed with subunit-specific primary antibodies, followed by secondary antibodies conjugated to horseradish peroxidase (HRP). The Super Signal ELISA Pico chemiluminescent substrate kit (Pierce) was utilized to generate luminescent signal associated with HRP binding. Luminescence after 5 minutes of signal development was monitored on a plate reader (Tecan Safire). Subunit content was expressed as ng of subunit per μg of total protein, as calculated from a standard curve of purified human constitutive or immunoproteasomes, assayed simultaneously with the cell lysate samples.

2.5. Mixed leukocyte reaction (MLR)

hTERT-MUTZ3 and hTERT-MUTZ3/10BTZ cells were irradiated with 5000 rad, washed, plated in a 96-well plate, and co-cultured with healthy donor peripheral blood lymphocytes (PBL), labeled with carboxyfluorescein succinimidyl ester (CFSE) (3 μM ; Sigma Aldrich, Zwijndrecht, The Netherlands), in a 1:10 ratio for 4 to 8 days in IMDM containing 10% human pooled serum (HPS) (Sanquin, Amsterdam, The Netherlands), 100 IU/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin and 2 mM L-glutamine. PBL proliferation was determined based on CFSE fluorescence intensity, which was determined by a FACSCalibur flow cytometer (Becton and Dickinson, San Jose, CA) using CellQuest analysis software.

2.6. Peptide kinase substrate array (PamGene analysis)

To evaluate changes in cell signaling based on tyrosine kinase activity, lysates of hTERT-MUTZ3 and hTERT-MUTZ3/10BTZ precursors were profiled employing a tyrosine kinase peptide substrate array (PamChip; PamGene International, 's-Hertogenbosch, The Netherlands). This substrate array comprises 144 peptides consisting of 13 aa including a tyrosine, corresponding to known or putative phosphorylation sites in a variety of human proteins. Precursors were either left untreated (CTR) or were incubated with the LC differentiation cytokine cocktail. The samples were lysed on ice using M-PER buffer containing phosphatase and protease inhibitors (Thermo Scientific, Rockford, IL, USA). Lysates were centrifuged

for 10 minutes at 20,000 \times g; the supernatant was stored at -80°C for further analysis. Forty μL of sample mix were applied to an array, consisting of 7.5 μg protein from lysates, with addition of reaction buffer containing PK buffer, 400 μM adenosine triphosphate, fluorescein-labeled antibody PY20, BSA & DTT (PTK reagent kit; PamGene International). After blocking the arrays with 2% BSA and subsequent loading of the sample mix onto the arrays, incubation (at 30°C) was started for 60 minutes utilizing a PamStation[®] 12 instrument (PamGene International), during which time the sample mix was pumped up and down through the array once per minute. Repeated fluorescence imaging of each array was performed with a 12-bit CCD camera every 5 minutes, and after a washing step, at the end of the run. Spot intensity was quantified for the post-wash data and corrected for local background using Bionavigator[®] software version 5.1 (PamGene International).

2.7. Analysis of NF- κ B subunit activation

hTERT-MUTZ3 and hTERT-MUTZ3/10BTZ precursor cells were either not stimulated (CTR) or stimulated with TGF- β (10 ng/ml), rhGM-CSF (1000 IU/ml) and TNF- α (120 IU/ml) for one hour. Immature LCs were incubated with maturation cytokines (MCM-mimic) for one hour. Nuclear pellets were made using Nuclear protein extraction kit (EMD Millipore, Massachusetts, USA) according to the manufactures' protocol. Activation of p50, p52, RelB, or RelA/p65 was determined using the TransAM NF- κ B Family kit (Active Motif Europe, Rixensart, Belgium) according to the manufacturer's instructions.

2.8. Statistical analysis

For comparisons between groups, ANOVA or Student's t-test were used. Differences were considered to be significant when $p < 0.05$. These tests, as well as linear regression analysis and plotting of 90% prediction band with the Pamchip data, were carried out using GraphPad Prism software, version 5.01.

3. Results

3.1. Long-term culture with BTZ induces "BTZ adaptation" in hTERT-MUTZ3 progenitor cells

To study the long-term effects of BTZ on human DC development, hTERT-MUTZ-3 progenitor cells were cultured in the presence of BTZ, starting at a concentration of 2 nM (from passage 22) and the concentration was gradually increased to 10 nM over the course of the next 30 passages, while maintaining stable expansion as shown in Fig. 1A. From passage 51 on, BTZ was given intermittently (2 passages on, 2 passages off), thus mimicking clinical bolus on/off periods in the BTZ treatment cycle. Concentrations higher than 10 nM BTZ were not tolerated (data not shown) and hTERT-MUTZ3/10BTZ progenitor cells were therefore kept at this concentration and intermittent exposure regimen for over 90 passages (i.e. > 45 weeks). These progenitors, termed "BTZ-adapted cells", were continuously cultured in parallel with unexposed parental hTERT-MUTZ3 cells and assessed at corresponding passages for their relative sensitivity to BTZ in a 3-day cytotoxicity assay. A significant reduction in survival of viable cells upon exposure to BTZ was apparent for the BTZ-adapted cells at concentrations of ≥ 7.5 nM (Fig. 1B) with the IC₅₀ value increasing from 5.9 ± 0.9 nM in hTERT-MUTZ3 cells to 9.1 ± 1.2 nM in hTERT-MUTZ3/10BTZ cells. As this rep-

resented a mere 1.54-fold resistance, acquired over a considerable period of time (4 months of culture on BTZ), we opted for the term BTZ-adapted cells to refer to the surviving hTERT-MUTZ3/10BTZ progenitor cells, rather than BTZ-resistant cells.

3.2. Long-term culture with BTZ induces differentiation of hTERT-MUTZ3 progenitor cells

MUTZ-3 cells consist of a predominant CD34⁺ proliferative fraction and a small (typically less than 10%) sub-population of CD14⁺ precursor cells, arising from CD34⁺ precursors, with the ability to rapidly differentiate to DC [23]. Over the course of long-term BTZ exposure CD14 expression was assessed (passages: 58, 62, 85 and 95, see Fig. 1C) and was consistently found to be increased in hTERT-MUTZ3/10BTZ as compared to unexposed progenitor cells, indicative of enhanced early differentiation events. Next, we also determined the expression of cytokine receptors involved in DC differentiation (TNF-RI and -RII) and DC precursor proliferation (cKIT/CD117 and IL-3R/CD123) [26, 27], under normal conditions or BTZ exposure at 10 nM, between passages 58 and 95. As shown in Fig. 1D, consistent and selective up-regulation of TNF-RI and -RII, both previously associated with DC differentiation ability [26], was in keeping with the observed increased differentiation propensity. Also an increase in immunoproteasome subunit content was observed, consistent with an elevated differentiation state [28], see Fig. 1E.

3.3. Enhanced LC differentiation and maturation of BTZ-adapted precursor cells

Next, the capacity of the BTZ adapted cells to develop into LC was assessed by cytokine-driven differentiation over a period of 9 days in the presence of GM-CSF, TGF β , and TNF α . Of note, hTERT-MUTZ3/10BTZ precursors were always cultured with BTZ during the passage preceding differentiation induction but were not exposed to the drug during differentiation. Short-term, immediate effects of BTZ on LC differentiation were also examined by adding the highest sub-cytotoxic concentration of 5 nM BTZ (>60% viable cell survival, see Fig. 1B) in combination with the differentiation inducing cytokines to previously unexposed parental hTERT-MUTZ3 cells. Fig. 2A shows the expression of the LC characterizing markers CD1a and Langerin at day 9 among hTERT-MUTZ3 and hTERT-MUTZ3/10BTZ cells from a representative LC differentiation experiment out of at least ten; whereas a decrease in CD1a⁺Langerin⁺ cells after short-term exposure to BTZ was observed, BTZ-adapted cells showed a clear increase in cells expressing these markers. This was confirmed by data shown in Fig. 2B, revealing selective and significant decreases in the expression of the progenitor- and precursor-associated markers CD34 and CD14 and a simultaneous increase in LC-associated CD1a, only in cultures of long-term (and not short-term) BTZ-exposed hTERT-MUTZ3 precursors. Similarly, significantly enhanced cell surface expression levels of the T cell (co-) stimulatory molecules CD80, CD86, CD40 and HLA-DR were observed (Fig. 2C-D). Collectively, these data demonstrate enhanced LC differentiation from CD34⁺ precursors upon their long-term exposure to BTZ. Of note, although we have focused on LC differentiation as a model for steady-state DC differentiation, long-term BTZ exposure also enhanced interstitial or dermal-like DC differentiation from the hTERT-MUTZ3 progenitors, denoted by increased CD1a and DC-SIGN expression after 7 days of culture in the presence of TNF α , GM-CSF and IL-4 (see Suppl. Fig. 1).

The immature LC arising in the different conditions were subsequently tested for their ability to mature in response to a cytokine cocktail consisting of IL-6, PGE2, IL-1 β and TNF α (see “Materials and Methods”). Fig. 3A is representative of at least 5 separate experiments and shows consistently enhanced up-regulation of all tested maturation markers, i.e. CD83, CD80, CD86, CD40, HLA-DR and HLA-ABC. A crucial function of mature LC is the induction of naive T cell activation. Consistent with their increased maturation state, mature LC derived from BTZ-adapted precursors induced higher proliferation rates in naïve allogeneic T cells (Fig. 3B).

3.4. Altered tyrosine kinase profile and NF- κ B signaling in LC derived from BTZ-adapted precursors

To further elucidate sub-cellular alterations accompanying enhanced LC development from long-term BTZ exposed precursors, we evaluated their tyrosine kinase activity profile employing the Tyrosine Kinase PamChip microarray. Lysates were tested from hTERT-MUTZ3 and hTERT-MUTZ3/10BTZ precursors that remained unstimulated or were stimulated for one hour by the LC differentiation inducing cytokine cocktail. Fig. 4A shows the ratios of stimulated over unstimulated kinase activities, based on evaluable phosphorylation of peptide substrates among a total of 144 substrates, in hTERT-MUTZ3 plotted against the corresponding ratios in hTERT-MUTZ3/10BTZ. The dotted line denotes the 90% prediction band, revealing very few outliers. These data show the observed enhanced LC differentiation not to be associated with major shifts or increased activity in signaling pathways previously reported to be involved in DC development, such as the JAK/STAT and MAPK pathways. Indeed, STAT3 and STAT5a activity, both known to be involved in DC/LC differentiation from CD34⁺ precursors [29, 30], was equally up-regulated in unexposed and BTZ-exposed precursors (at kinase activation ratios of 1.3 and 2.5, respectively). In contrast, Ephrin Type-A receptor 2 and 4 precursor kinase activities (both previously reported expressed in LC derived from CD34⁺ precursors and involved in adhesion and trafficking [31]) were up-regulated upon differentiation induction in unexposed precursors rather than in BTZ-adapted precursors (at ratios of 2.5 vs. 1.4 and 1.3 vs. 0.9, respectively), while Aminopeptidase A and Peripherin kinase activities were more selectively upregulated in BTZ-adapted cells (1.5 vs. 0.7 and 2.9 vs. 1.3, respectively). Whereas Aminopeptidase A activation may reflect a compensatory mechanism for protein breakdown due to proteasome inhibition, Peripherin is involved in dendrite formation, a hallmark of DC morphology [32].

NF- κ B signaling, in particular through complexes incorporating the non-canonical RelB subunit, has been identified as an important pathway in DC maturation. Activation of NF- κ B subunits belonging to the non-canonical (RelB and p52) as well as the canonical pathway (RelA and p50) was assessed by their presence in nuclear extracts of LC derived from hTERT-MUTZ3 and their BTZ-adapted counterparts. Immature LC were either left unstimulated or were stimulated for one hour with a maturation-inducing cocktail of cytokines (IL-1 β , TNF α , IL-6 and PGE2). Elevated levels of the canonical RelA (p65) and p50 subunits in LC derived from the BTZ-adapted precursors were observed both before and after (Fig. 4B) maturation induction. Most notably, RelB activation was induced in LC from BTZ-adapted precursors and virtually absent in their BTZ untreated counterparts. Although this BTZ-induced up-regulation of nuclear RelB levels did not reach significance due to variability, in all three independent experiments this increase was observed, varying from 2.6- to 20.6-fold.

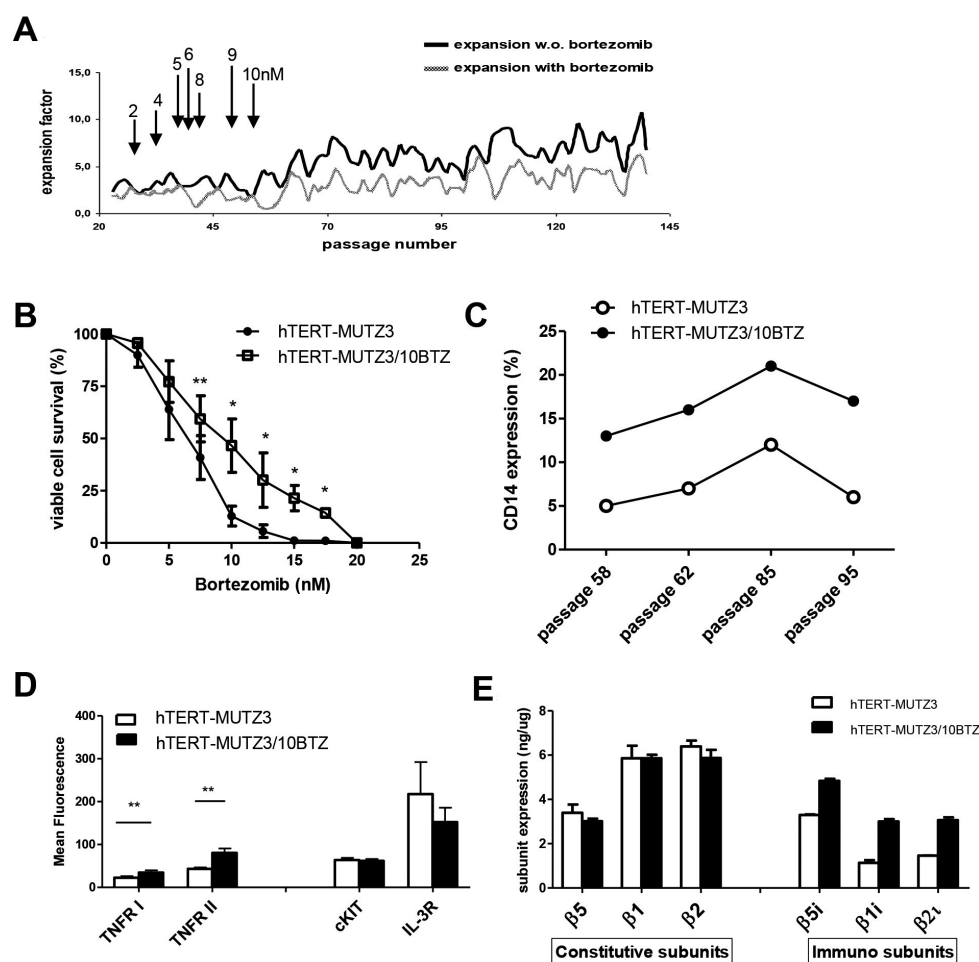


Fig. 1. The generation and properties of Bortezomib (BTZ) adapted hTERT-MUTZ3 progenitor cells. (A) Time table of adaptation to Bortezomib (BTZ) and growth of the BTZ adapted hTERT-MUTZ3 precursor cells. Starting concentration of BTZ inclusion at passage 22 was 2 nM BTZ and was gradually increased to 10 nM at passage 51. From then on, the drug was given intermittently (once every 2 passages). The cells were passaged twice-weekly. (B) Dose–response curve for BTZ-induced growth inhibition of hTERT-MUTZ3 and BTZ-adapted hTERT-MUTZ3/10BTZ progenitor cells following 72 hours' exposure to BTZ. Results show the percentage surviving cells and represent the mean \pm SD of 5 separate experiments ** $p < 0.01$, * $p < 0.05$ (C) Percentage of CD14⁺ hTERT-MUTZ3 and BTZ-adapted hTERT-MUTZ3/10BTZ progenitor cells over the course of culture from passage 58–95. (D) Expression of TNF-RI, TNF-RII, cKIT (CD117) and IL-3R (CD123) on hTERT-MUTZ3 and hTERT-MUTZ3/10BTZ adapted progenitor cells (passage 58–95). Results are depicted in mean fluorescence intensity (MFI) and represent the mean \pm SD of 6–8 separate experiments. (E) Protein expression levels of constitutive and immunoproteasome subunits in hTERT-MUTZ3 and BTZ-adapted cells hTERT-MUTZ3/10BTZ. Results depicted are means \pm SD of two separate experiments in hTERT-MUTZ3 and three separate experiments in hTERT-MUTZ3/10BTZ progenitors

Remarkably, activation of RelB's non-canonical heterodimer partner p52 was not observed under any of the tested conditions (Fig. 4B and C). These data indicate that apart from nuclear translocation of the canonical RelA/p50 heterodimer, translocation of non-classical RelB/p50 dimers in the BTZ-adapted precursors may account for the observed enhanced LC maturation.

4. Discussion

BTZ treatment can lead to immunogenic cell death in MM cells, resulting in uptake by, and contact-dependent activation of DC through, exposure of heat shock proteins [8]. Given that BTZ can enhance the efficacy of tumor vaccination [8, 33], these findings have prompted several groups to study the effects of BTZ on functional DC development and maturation. Thus far, the majority

of reports have described the effects of short-term exposure to BTZ on MoDC, an *in vitro* model of inflammatory DC development, and without exception, have shown deleterious effects on DC maturation, most notably through BTZ-mediated inhibition of proteasomal chymotrypsin activity [10–12]. Similarly, murine DC development and functionality were also inhibited by BTZ in short-term cultures, which coincided with reduced levels of nuclear NF- κ B [34]. As such, these studies point to the potential utility of BTZ as an immune-regulatory therapeutic agent for the treatment of chronic inflammatory and autoimmune disorders [35]. However, these data are in striking contrast with recent reports of the *in vivo* effects of BTZ on DC development and maturation [17, 18]. Chang *et al.*, showed increased DC maturation and tumor infiltration upon BTZ treatment, resulting in effective antitumor T cell-mediated immunity [17]. Pellom *et al.*, showed increased IL-12 and IL-15 production

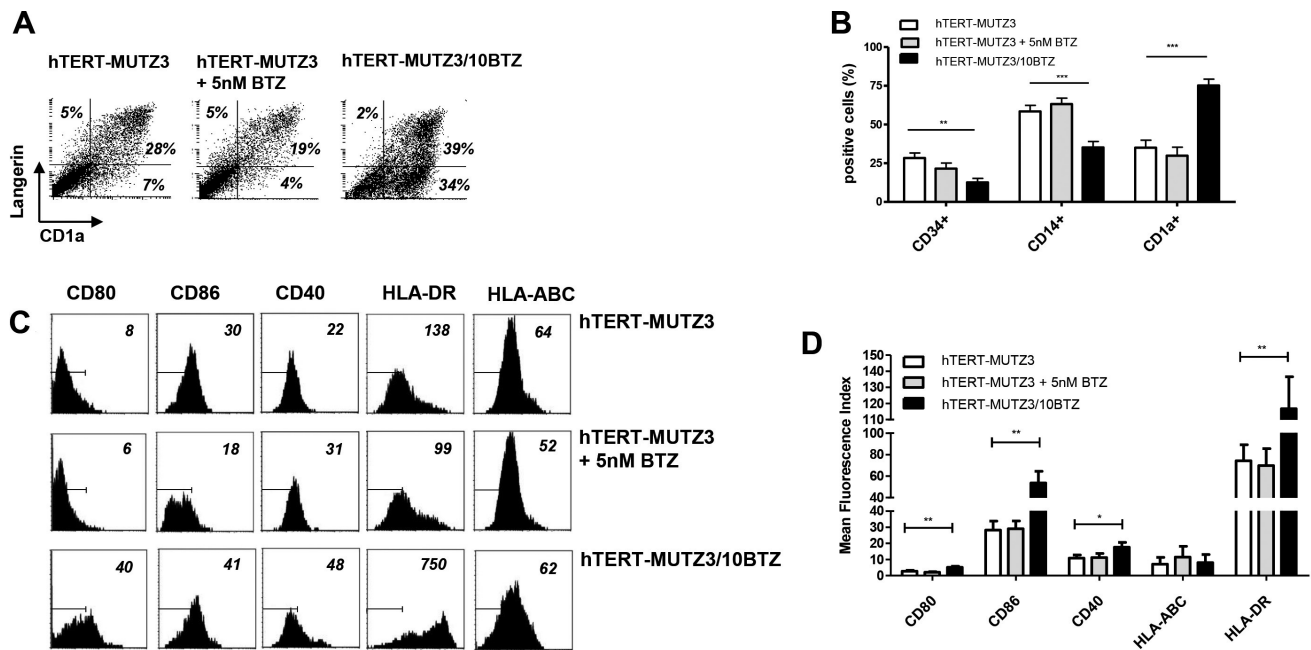


Fig. 2. Long-term but not short-term exposure to BTZ enhances the differentiation of Langerhans Cells (LC). (A) Langerin⁺ and CD1a⁺ LC, differentiated from hTERT-MUTZ3, hTERT-MUTZ3 in the presence of 5 nM BTZ and BTZ-adapted hTERT-MUTZ3/10BTZ cells. Data are representative of 10-15 independent experiments. (B) Expression of CD34, CD14, and CD1a on the surface of LC as percentage positive cells (mean \pm SD of 10-15 separate experiments). (C) Expression of T cell (co-)stimulatory molecules CD80, CD86, CD40, HLA-DR and HLA-ABC on LC differentiated in the absence or presence of 5 nM BTZ or differentiated from MUTZ-3 precursors upon their long-term exposure to BTZ (hTERT-MUTZ3/10BTZ). Results are representative of 10-15 separate experiments. (D) Expression on LC of CD80 and CD86, CD40 and HLA-ABC and HLA-DR in Mean Fluorescence Index (MFI) as mean \pm SD of 10-15 independent experiments for the indicated conditions

by CD11c⁺ DC and enhanced tumor reactivity upon BTZ treatment in a 4T1 breast cancer mouse model cell line [18]. These apparently contradictory results may be due to differential effects on inflammatory vs. steady-state DC differentiation as well as on short-term (<7 days) vs. more long-term (>21 days) effects of BTZ exposure. Current treatment schedules with BTZ consist of multiple cycles of drug exposure over prolonged periods of time [36]. Unfortunately, the study of long-term effects on human steady-state DC differentiation is hampered by the relative difficulty of culturing primary DC precursors over protracted periods of time. We therefore resorted to the use of the sustainable hTERT-MUTZ3 cell line model which we previously successfully applied to the assessment of the effects of chronic treatment with doxorubicin on human DC development from CD34⁺ precursors and CD14⁺ intermediates [21]. This model allowed us to study the effects of BTZ exposure on human CD34⁺ DC precursors and their CD14⁺ intermediates for over one year. We opted for an intermittent exposure schedule, which resembled the clinically relevant treatment regimen. The relatively low BTZ concentration that proved the highest tolerable level for the hTERT-MUTZ3 DC precursors was consistent with plasma levels of the drug found in clinical pharmacokinetics and pharmacodynamics studies [36]. From a clinical mechanistic drug resistance point of view, the 1.5-fold resistance level that was achieved in the current study is certainly a very low chemoresistance level, yet it might nevertheless bear clinical relevance [24, 25, 37]. Importantly, our findings in this human model are in agreement with the report of Chang *et al.*, [17] showing that long-term exposure to clinically relevant (i.e. low) concentrations of BTZ enhances DC maturation and is compatible with therapeutic strategies aimed at the DC-dependent induction of

antitumor T cell immunity.

Long-term exposure to BTZ was found to induce an adapted cellular state in hTERT-MUTZ3 progenitor cells, which facilitated a relative resistance to the induction of apoptosis and was accompanied by spontaneous early differentiation events and enhanced development and maturation of LC, with an increased ability to prime allogeneic T cells. The observed selective BTZ-induced increase in immunoproteasome subunits was consistent with this enhanced DC differentiation and maturation capacity [28, 38] as was the significant up-regulation of both TNF-RI and -RII levels on the BTZ-adapted progenitors [23, 26]. In particular, TNF-RI was previously shown to be essential for DC maturation and subsequent CD8⁺ T cell activation [39]. Of note, TNF-RI signaling involves the canonical NF- κ B pathway and is controlled at virtually all levels by ubiquitination and de-ubiquitination events [40]. It therefore stands to reason that proteasome inhibition would induce molecular modulation of TNF-RI-mediated NF- κ B signaling and thus affect DC development. BTZ adaptation may thus lead to favoring of the non-canonical RelB-mediated pathway as was reported in BTZ-treated Head and Neck squamous cell tumors, which might possibly account for the heterogeneous response to BTZ in patients harboring this tumor [41]. In DC, this phenomenon may be reflected by increased signaling through TNF-RII (up-regulated to higher levels on hTERT-MUTZ3/10BTZ), which involves signaling through the non-canonical RelB pathway [42]. However, our finding of nuclear RelB in the absence of p52 supports an alternative scenario in which RelB heterodimerizes with p50 for nuclear translocation. High levels of RelB is a characteristic of cells belonging to the DC lineage and may account for the recent observation of nuclear translocation of RelB/p50 dimers, thus com-

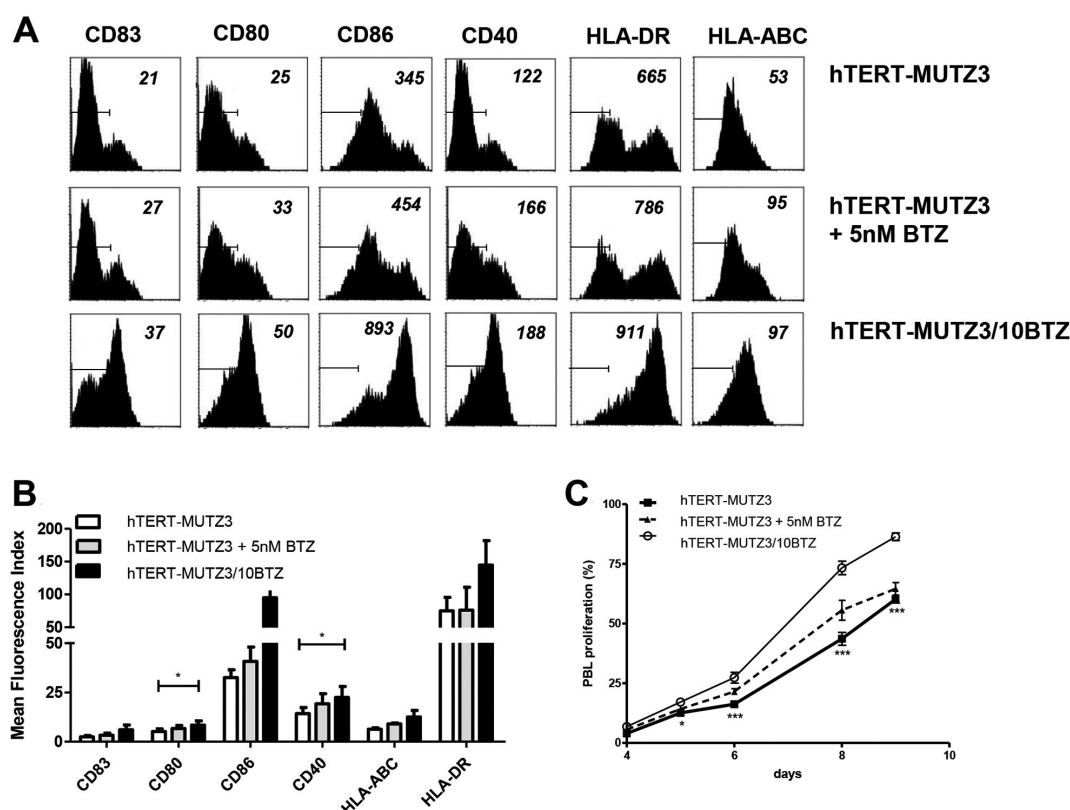


Fig. 3. Long-term but not short-term exposure to BTZ enhances the maturation of Langerhans Cells (LC). (A) Expression of CD83, the co-stimulatory molecules CD80 and CD86, CD40 and the MHC molecules HLA-ABC and HLA-DR after induction of maturation of LC differentiated from hTERT-MUTZ3 precursors in the absence or presence of 5 nM BTZ or from BTZ-adapted hTERT-MUTZ3/10BTZ cells. Results are representative of 5-10 separate experiments. (B) Expression of the same surface markers under the same conditions as Mean Fluorescence Index (MFI) \pm SD of 5-10 separate experiments. (C) Naïve T cell stimulatory capacity of the differentially matured LC in a Mixed Leukocyte Reaction. Proliferation using the CFSE dilution assay among monocyte-depleted PBMC (PBL) was determined on day 4 to day 9

binning canonical and non-canonical signaling elements in a molecular event that appears to be unique to DC activation [43]. Shih *et al.*, showed that RelB/p50 activation was induced by agents and receptors associated with the canonical NF- κ B pathway and was accordingly under the control of canonical signaling as evidenced by the binding of RelB/p50 dimers to IK- κ B in the cytoplasm [43, 44]. The lack of nuclear p52 activation together with the clear activation of p50 in our BTZ-adapted LC model, indicate that BTZ adaptation also induces this canonical activation, involving, besides the classical RelA/p50 pairing, the functional heterodimerization of RelB with p50. The higher basal activation level of RelB in the BTZ adapted LC is in accordance with previous findings that nuclear RelB is crucial for DC development, maturation and antigen-presenting and T cell stimulatory capacities [45–49]. Indeed, ablation of RelB endows DC with tolerating abilities [50, 51] and the absence of nuclear RelB in LC accounts for their reported ability for T cell tolerance induction [52]. Based on our findings, low-dose BTZ treatment may thus abolish DC/LC-mediated tolerization of effector T cells and support DC/LC-targeted tumor vaccination strategies.

Shih *et al.*, have proposed that the formation of RelB/p50 complexes, due to high levels of RelB during DC development, may represent a key mechanism leading to irreversible maturation [43, 44]. However, it remains unclear how RelA/p50 versus RelB/p50 may lead to activation of alternate signaling programs marking the on-

set of LC development and maturation. Indeed, as pointed out by Hayden [44], based on findings by Siggers and colleagues [53], very little difference in preference for DNA promoter-binding sites exists between p50 complexes encompassing different binding partners, which was born out by gene expression profiling studies undertaken by Shih *et al.* [43]. Similarly, we found very little difference in tyrosine kinase activity profiles between unexposed and long-term BTZ exposed hTERT-MUTZ3 precursors. In both, up-regulation of STAT kinase activity was observed upon differentiation induction (STAT3, STAT5, and STAT6—see Fig. 4A), in keeping with the control of DC development by the JAK/STAT signaling cascade, irrespective of their regulation by nuclear RelA/p50 or RelB/p50 complexes. In particular, STAT3 and STAT5 activation have previously been implicated in steady-state DC differentiation and maturation from CD34⁺ precursors [28, 29, 54]. Based on previous reports, some of the rare kinase activities we found to be differentially regulated between drug-free control cells and BTZ-exposed precursor cells, may actually signal functional differences. For instance, relatively lower induction of tyrosine kinase activity of the Ephrin Type-A receptor 2 in BTZ-exposed precursors may have contributed to the increased capacity of the resulting LC to stimulate naïve T cells [31]. Reciprocally, higher kinase activity of Peripherin, a neuronal intermediate filament protein [32], in BTZ adapted cells may be associated with a more dendritic morphology, consistent with enhanced LC development.

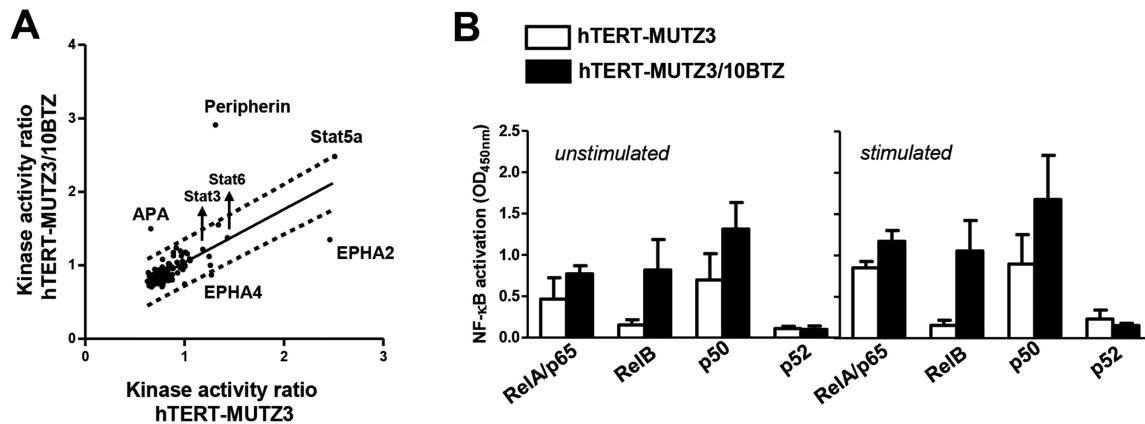


Fig. 4. Long-term exposure to BTZ: effects on tyrosine kinase activity and nuclear NF- κ B subunit profiles. (A) Activity of 144 Tyrosine kinases was determined in cell extracts of hTERT-MUTZ3 or BTZ-adapted hTERT-MUTZ3/10BTZ using the Tyrosine Kinase PamChip microarray. The precursor cells were either left unstimulated or were stimulated with LC differentiation-inducing cytokines (TNF α , TGF β 1, and GM-CSF) for one hour. Shown are the ratios of kinase activities of stimulated over unstimulated precursors, plotted for hTERT-MUTZ3 vs. BTZ-adapted hTERT-MUTZ3/10BTZ. Linear regression analysis was performed with the dotted lines indicating the 90% predictability intervals. Some (differentially) activated kinases are denoted. APA= Aminopeptidase A, EPHA = Ephrin Type-A Receptor. (B) NF κ B activation was measured in nuclear pellets of LC differentiated from hTERT-MUTZ3 (open bars) and BTZ-adapted hTERT-MUTZ3/10BTZ (closed bars). LC were either left unstimulated or stimulated for one hour with a maturation-inducing cytokine cocktail consisting of 2,400 IU/ml TNF α , 25 ng/ml IL-1 β , 1 μ g/ml PGE2 and 100 ng/ml IL-6. Activation (by nuclear levels) of the NF κ B subunits, RelA/p65, RelB, p50 and p52 is shown. Presented data are means \pm SD of 3 separate experiments

Rather than inducing major shifts in signaling programs and gene expression profiles, it was suggested that RelB/p50 dimers might be subject to different (in)activation mechanisms, leading to their preferred or prolonged activation and thus resulting in superior DC development and maturation [44].

Inhibitory effects of BTZ on human DC differentiation and maturation from previously unexposed precursors, as reported by others for MoDC [10–15], were not observed in our model, hence emphasizing differential effects on human DC development under inflammatory versus steady-state conditions. Thus, our findings do not negate the possible efficacy of BTZ as an anti-inflammatory agent, but rather confirm its compatibility with DC-dependent tumor immunotherapy, e.g. of MM. Coupled to previous observations that BTZ treatment can break detrimental clusters between leukemic tumor cells and immuno-compromised DC [10], induces immunogenic cell death [8, 55] and promotes effector molecule expression and effector functions of CD8⁺ tumor-reactive T cells and NK cells [18, 56–58], our finding of enhanced functional human DC development and maturation argues in favour of applying BTZ in combination with immunotherapy for cancer treatment.

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Conflict of Interest

All authors declare no conflict of interests.

Author contributions

RvdV developed and supplied the MUTZ3-hTERT cells and co-wrote the manuscript. SV performed experiments, interpreted the data and co-wrote the manuscript. MA and HD gave technical support, HMWV co-designed the project, JLA and ETC supplied reagents. BACD, WFL and RJS co-designed the project. GJ and TDdG designed the study, interpreted the data and co-wrote the manuscript.

References

- [1] Kisselev AF, Callard A, Goldberg AL. Importance of the different proteolytic sites of the proteasome and the efficacy of inhibitors varies with the protein substrate. *J Biol Chem*, 2006; 281(13): 8582–8590.
- [2] Groettrup M, Kirk CJ, Basler M. Proteasomes in immune cells: more than peptide producers? *Nat Rev Immunol*, 2010; 10(1): 73–78.
- [3] Busse A, Kraus M, Na I-K, Rietz A, Scheibenbogen C, Driessen C, et al. Sensitivity of tumor cells to proteasome inhibitors is associated with expression levels and composition of proteasome subunits. *Cancer-Am Cancer Soc*, 2008; 112(3): 659–670.
- [4] Edwards CM, Lwin ST, Fowler JA, Oyajobi BO, Zhuang J, Bates AL, et al. Myeloma cells exhibit an increase in proteasome activity and an enhanced response to proteasome inhibition in the bone marrow microenvironment in vivo. *Am J Hematol*, 2009; 84(5): 268–272.
- [5] Chen D, Frezza M, Schmitt S, Kanwar J, Dou QP. Bortezomib as the first proteasome inhibitor anticancer drug: current status and future perspectives. *Curr Cancer Drug Targets*, 11(3): 239–253.
- [6] Fennell DA, Chacko A, Mutti L. BCL-2 family regulation by the 20S proteasome inhibitor bortezomib. *Oncogene*, 2008; 27(9): 1189–1197.
- [7] Hideshima T, Anderson KC. Biologic impact of proteasome inhibition in multiple myeloma cells—from the aspects of preclinical studies. *Semin Hematol*, 2012; 49(3): 223–227.

- [8] Spisek R, Charalambous A, Mazumder A, Vesole DH, Jagannath S, Dhodapkar MV. Bortezomib enhances dendritic cell (DC)-mediated induction of immunity to human myeloma via exposure of cell surface heat shock protein 90 on dying tumor cells: therapeutic implications. *Blood*, 2007; 109(11): 4839–4845.
- [9] Goldberg AL, Cascio P, Saric T, Rock KL. The importance of the proteasome and subsequent proteolytic steps in the generation of antigenic peptides. *Mol Immunol*, 2002; 39(3): 147–164.
- [10] Kukreja A, Hutchinson A, Mazumder A, Vesole D, Angitapalli R, Jagannath S, et al. Bortezomib disrupts tumour-dendritic cell interactions in myeloma and lymphoma: therapeutic implications. *Br J Haematol*, 2007; 136(1): 106–110.
- [11] Hirai M, Kadowaki N, Kitawaki T, Fujita H, Takaori-Kondo A, Fukui R, et al. Bortezomib suppresses function and survival of plasmacytoid dendritic cells by targeting intracellular trafficking of Toll-like receptors and endoplasmic reticulum homeostasis. *Blood*, 2011; 117(2): 500–509.
- [12] Zinser E, Rösner S, Littmann L, Lütenegger D, Schubert U, Steinkasserer A. Inhibition of the proteasome influences murine and human dendritic cell development in vitro and in vivo. *Immunobiology*, 2009(9); 214: 843–851.
- [13] Nencioni A, Schwarzenberg K, Brauer KM, Schmidt SM, Ballestrero A, Gräbebach F, et al. Proteasome inhibitor bortezomib modulates TLR4-induced dendritic cell activation. *Blood*, 2006; 108(2): 551–558.
- [14] Straube C, Wehner R, Wendisch M, Bornhäser M, Bachmann M, Rieber EP, et al. Bortezomib significantly impairs the immunostimulatory capacity of human myeloid blood dendritic cells. *Leukemia*, 2007; 21(7): 1464–1471.
- [15] Nencioni A, Garuti A, Schwarzenberg K, Cirmena G, Dal Bello G, Rocco I, et al. Proteasome inhibitor-induced apoptosis in human monocyte-derived dendritic cells. *Eur J Immunol*, 2006; 36(3): 681–689.
- [16] Subklewe M, Sebelin-Wulf K, Beier C, Lietz A, Mathas S, Döken B, et al. Dendritic cell maturation stage determines susceptibility to the proteasome inhibitor bortezomib. *Hum Immunol*, 2007; 68(3): 147–155.
- [17] Chang C-L, Hsu Y-T, Wu C-C, Yang Y-C, Wang C, Wu TC, et al. Immune mechanism of the antitumor effects generated by bortezomib. *J Immunol*, 2012; 189(6): 3209–3220.
- [18] Pellom ST, Dudimah DF, Thounaojam MC, Uzhachenko RV, Singhal A, Richmond A, et al. Bortezomib augments lymphocyte stimulatory cytokine signaling in the tumor microenvironment to sustain CD8+T cell antitumor function. *Oncotarget*, 2017; 8(5): 8604–8621.
- [19] Ramakrishnan R, Huang C, Cho H-I, Lloyd M, Johnson J, Ren X, et al. Autophagy induced by conventional chemotherapy mediates tumor cell sensitivity to immunotherapy. *Cancer Res*. 2012; 72(21): 5483–5493.
- [20] de Carvalho F, Alves VLF, Braga WMT, Xavier CV, Colleoni GWB. MAGE-C1/CT7 and MAGE-C2/CT10 are frequently expressed in multiple myeloma and can be explored in combined immunotherapy for this malignancy. *Cancer Immunol Immunother*. 2013; 62(1): 191–195.
- [21] van de Ven R, Verbrugge SE, Reurs AW, Bontkes HJ, Hooijberg E, Jansen G, et al. High susceptibility of c-KIT+CD34+ precursors to prolonged doxorubicin exposure interferes with Langerhans cell differentiation in a human cell line model. *Cancer Immunol Immunother*, 2011; 60(7): 943–951.
- [22] Santegoets SJAM, Schreurs MWJ, Masterson AJ, Liu YP, Goletz S, Baumeister H, et al. In vitro priming of tumor-specific cytotoxic T lymphocytes using allogeneic dendritic cells derived from the human MUTZ-3 cell line. *Cancer Immunol Immunother*, 2006; 55(12): 1480–1490.
- [23] Santegoets SJAM, van den Eertwegh AJM, van de Loosdrecht AA, Scheper RJ, de Gruijl TD. Human dendritic cell line models for DC differentiation and clinical DC vaccination studies. *J Leukoc Biol*. 2008; 84(6): 1364–1373.
- [24] Verbrugge SE, Al M, Assaraf YG, Niewerth D, van Meerloo J, Cloos J, et al. Overcoming bortezomib resistance in human B cells by anti-CD20/rituximab-mediated complement-dependent cytotoxicity and epoxyketone-based irreversible proteasome inhibitors. *Exp Hematol Oncol*, 2013; 2(1): 2.
- [25] Verbrugge SE, Assaraf YG, Dijkmans BAC, Scheffer GL, Al M, Uyl den D, et al. Inactivating PSMB5 mutations and P-glycoprotein (multidrug resistance-associated protein/ATP-binding cassette B1) mediate resistance to proteasome inhibitors: ex vivo efficacy of (immuno)proteasome inhibitors in mononuclear blood cells from patients with rheumatoid arthritis. *J Pharmacol Exp Ther*, 2012; 34(1): 174–182.
- [26] Houtenbos I, Westers TM, de Gruijl TD, Scheper RJ, Ossenkoppele GJ, van de Loosdrecht AA. TNF-alpha receptor 1 expression on acute myeloid leukemic blasts predicts differentiation into leukemic dendritic cells. *Leukemia*, 2004; 18(6): 1149–1153.
- [27] Khoury E, Andre C, Pontvert-Delucq S, Drenou B, Baillou C, Guigon M, et al. Tumor necrosis factor alpha (TNF alpha) downregulates c-kit proto-oncogene product expression in normal and acute myeloid leukemia CD34+ cells via p55 TNF alpha receptors. *Blood*, 1994; 84(8): 2506–2514.
- [28] Li J, Schuler-Thurner B, Schuler G, Huber C, Seliger B. Bipartite regulation of different components of the MHC class I antigen-processing machinery during dendritic cell maturation. *Int Immunol*, 2001; 13(12): 1515–1523.
- [29] Cohen PA, Koski GK, Czerniecki BJ, Bunting KD, Fu X-Y, Wang Z, et al. STAT3- and STAT5-dependent pathways competitively regulate the pan-differentiation of CD34pos cells into tumor-competent dendritic cells. *Blood*, 2008; 112(5): 1832–1843.
- [30] van de Laar L, van den Bosch A, Wierenga ATJ, Janssen HLA, Coffey PJ, Woltman AM. Tight control of STAT5 activity determines human CD34-derived interstitial dendritic cell and langerhans cell development. *J Immunol*, 2011; 186(12): 7016–7024.
- [31] Munthe E, Finne EF, Aasheim H-C. Expression and functional effects of Eph receptor tyrosine kinase A family members on Langerhans like dendritic cells. *BMC Immunol*, 2004; 5(1): 9.
- [32] Yuan A, Sasaki T, Kumar A, Peterhoff CM, Rao MV, Liem RK, et al. Peripherin is a subunit of peripheral nerve neurofilaments: implications for differential vulnerability of CNS and peripheral nervous system axons. *J Neurosci*, 2012; 32(25): 8501–8508.
- [33] Kim J-E, Jin D-H, Lee WJ, Hur D, Wu TC, Kim D. Bortezomib enhances antigen-specific cytotoxic T cell responses against immune-resistant cancer cells generated by STAT3-ablated dendritic cells. *Pharmacol Res*, 2013; 71(5): 23–33.
- [34] Wang Y, Liang Y, Zhang Y, Wu D, Liu H. Bortezomib inhibits bone marrow-derived dendritic cells. *Int J Clin Exp Pathol*, 2015; 8(5): 4857–4862.
- [35] Verbrugge SE, Scheper RJ, Lems WF, de Gruijl TD, Jansen G. Proteasome inhibitors as experimental therapeutics of autoimmune diseases. *Arthritis Res Ther*, 2015; 17(1):17.

- [36] Moreau P, Richardson PG, Cavo M, Orlowski RZ, San Miguel JF, Palumbo A, et al. Proteasome inhibitors in multiple myeloma: 10 years later. *Blood*, 2012; 120(5): 947–959.
- [37] Oerlemans R, Franke NE, Assaraf YG, Cloos J, van Zantwijk I, Berkens CR, et al. Molecular basis of bortezomib resistance: proteasome subunit beta5 (PSMB5) gene mutation and overexpression of PSMB5 protein. *Blood*, 2008; 112(6): 2489–2499.
- [38] Tosello V, Zamarchi R, Merlo A, Gorza M, Piovon E, Mandruzzato S, et al. Differential expression of constitutive and inducible proteasome subunits in human monocyte-derived DC differentiated in the presence of IFN- α or IL-4. *Eur J Immunol*, 2009; 39(1): 56–66.
- [39] Ding X, Yang W, Shi X, Du P, Su L, Qin Z, et al. TNF receptor 1 mediates dendritic cell maturation and CD8 T cell response through two distinct mechanisms. *J Immunol*, 2011; 187(3): 1184–1191.
- [40] Wertz IE, Dixit VM. Ubiquitin-mediated regulation of TNFR1 signaling. *Cytokine and Growth Factor Reviews*, 2008; 19(3): 313–324.
- [41] Allen C, Saigal K, Nottingham L, Arun P, Chen Z, Van Waes C. Bortezomib-induced apoptosis with limited clinical response is accompanied by inhibition of canonical but not alternative nuclear factor- κ B subunits in head and neck cancer. *Clin Cancer Res*, 2008; 14(13): 4175–4185.
- [42] Rauert H, Wicovsky A, Müller N, Siegmund D, Spindler V, Waschke J, et al. Membrane tumor necrosis factor (TNF) induces p100 processing via TNF receptor-2 (TNFR2). *J Biol Chem*, 2010; 285(10): 7394–7404.
- [43] Shih VF-S, Davis-Turak J, Macal M, Huang JQ, Ponomarenko J, Kearns JD, et al. Control of RelB during dendritic cell activation integrates canonical and noncanonical NF- κ B pathways. *Nat Immunol*, 2012; 13(12): 1162–1170.
- [44] Hayden MS. A less-canonical, canonical NF- κ B pathway in DCs. *Nat Immunol*, 2012; 13(12): 1139–1141.
- [45] Neumann M, Fries H, Scheicher C, Keikavoussi P, Kolb-Märer A, Bröker E, et al. Differential expression of Rel/NF- κ B and octamer factors is a hallmark of the generation and maturation of dendritic cells. *Blood*, 2000; 95(1): 277–285.
- [46] Pettit AR, Quinn C, MacDonald KP, Cavanagh LL, Thomas G, Townsend W, et al. Nuclear localization of RelB is associated with effective antigen-presenting cell function. *J Immunol*, 1997; 159(8): 3681–3691.
- [47] Clark GJ, Gunningham S, Troy A, Vuckovic S, Hart DN. Expression of the RelB transcription factor correlates with the activation of human dendritic cells. *Immunology*, 1999; 98(2): 189–196.
- [48] O'Sullivan BJ, MacDonald KP, Pettit AR, Thomas R. RelB nuclear translocation regulates B cell MHC molecule, CD40 expression, and antigen-presenting cell function. *Proc Natl Acad Sci USA*, 2000; 97(21): 11421–11426.
- [49] Cejas PJ, Carlson LM, Kolonias D, Zhang J, Lindner I, Billadeau DD, et al. Regulation of RelB expression during the initiation of dendritic cell differentiation. *Mol Cell Biol*, 2005; 25(17): 7900–7916.
- [50] Zhu H-C, Qiu T, Liu X-H, Dong W-C, Weng X-D, Hu C-H, et al. Tolerogenic dendritic cells generated by RelB silencing using shRNA prevent acute rejection. *Cell Immunol*, 2012; 274(1): 12–18.
- [51] Luo L, Sun Z, Fang Q, Huang S, Bai X, Luo G. Effects of tolerogenic dendritic cells generated by siRNA-mediated RelB silencing on immune defense and surveillance functions of T cells. *Cell Immunol*, 2013(1): 282: 28–37.
- [52] Shklovskaya E, O'Sullivan BJ, Ng LG, Roediger B, Thomas R, Weninger W, et al. Langerhans cells are precommitted to immune tolerance induction. *Proceedings of the National Academy of Sciences*, 2011(44): 108: 18049–18054.
- [53] Siggers T, Chang AB, Teixeira A, Wong D, Williams KJ, Ahmed B, et al. Principles of dimer-specific gene regulation revealed by a comprehensive characterization of NF- κ B family DNA binding. *Nat Immunol*, 2011; 13(1): 95–102.
- [54] Li HS, Yang CY, Nallaparaju KC, Zhang H, Liu Y-J, Goldrath AW, et al. The signal transducers STAT5 and STAT3 control expression of Id2 and E2-2 during dendritic cell development. *Blood*, 2012; 120(22): 4363–4373.
- [55] Pol J, Vacchelli E, Aranda F, Castoldi F, Eggermont A, Cremer I, et al. Trial Watch: Immunogenic cell death inducers for anticancer chemotherapy. *Oncoimmunology*, 2015; 4(4): e1008866.
- [56] Thounaojam MC, Dudimah DF, Pellom ST, Uzhachenko RV, Carbone DP, Dikov MM, et al. Bortezomib enhances expression of effector molecules in anti-tumor CD8⁺ T lymphocytes by promoting Notch-nuclear factor- κ B crosstalk. *Oncotarget*, 2015; 6(32): 32439–32455.
- [57] Pellom ST, Dudimah DF, Thounaojam MC, Sayers TJ, Shanker A. Modulatory effects of bortezomib on host immune cell functions. *Immunotherapy-Uk*, 2015; 7(57): 1011–1022.
- [58] Ames E, Hallett WHD, Murphy WJ. Sensitization of human breast cancer cells to natural killer cell-mediated cytotoxicity by proteasome inhibition. *Clin Exp Immunol*, 2009; 155(3): 504–513.