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The Deubiquitinase Inhibitor b-AP15 and Its Effect on Phenotype and Function of Monocyte-Derived Dendritic Cells

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Abstract

The ubiquitin-proteasome system is elementary for cellular protein degradation and gained rising attention as a new target for cancer therapy due to promising clinical trials with bortezomib, the first-in class proteasome inhibitor meanwhile approved for multiple myeloma and mantle cell lymphoma. Both bortezomib and next-generation proteasome inhibitors mediate their effects by targeting the 20S core particle of the 26S proteasome. The novel small molecule inhibitor b-AP15 affects upstream elements of the ubiquitin-proteasome cascade by suppressing the deubiquitinase activity of both proteasomal regulatory 19S subunits and showed promising anticancer activity in preclinical models. Nonetheless, effects of inhibitors on the ubiquitin-proteasome system are not exclusively restricted to malignant cells: alteration of natural killer cell-mediated immune responses had already been described for drugs targeting either 19S or 20S proteasomal subunits. Moreover, it has been shown that bortezomib impairs dendritic cell (DC) phenotype and function at different levels. In the present study, we comparatively analyzed effects of bortezomib and b-AP15 on monocyte-derived DCs. In line with previous results, bortezomib exposure impaired maturation, antigen uptake, migration, cytokine secretion and immunostimulation, whereas treatment with b-AP15 had no compromising effects on these DC features. Our findings warrant the further investigation of b-AP15 as an alternative to clinically approved proteasome inhibitors in the therapy of malignancies, especially in the context of combinatorial treatment with DC-based immunotherapies.

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Abbreviations: CCL, C-C motif chemokine ligand; CCR, C-C motif chemokine receptor; DC, Dendritic cell; GPNMB, Glycoprotein non-metastatic b; HLA-DR, Human leukocyte antigen-D related; iDC, Immature dendritic cell; LPS, Lipopolysaccharide; mDC, Mature dendritic cell; PBMC, Peripheral blood mononuclear cell; SFI, Specific fluorescence intensity

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Introduction

The ubiquitin-proteasome system, physiologically striking a balance between protein synthesis and degradation, has been identified to play a crucial role not only in age-related diseases but also in many cancers [1,2]. Since tumor cells are often highly proliferative and show increased levels of protein turnover, a functioning ubiquitin-proteasome system is needed to maintain aberrant growth and resistance to apoptosis [3]. Thus, interrupting these complex interactions of enzymes has been proved to reduce tumor cell survival *in vitro* and achieved encouraging clinical effects in different tumor entities [4–8]. The first-in-class agent belonging to this group of anticancer drugs was bortezomib (Velcade®, PS-341), which is currently approved for the treatment of multiple myeloma and mantle cell lymphoma [9,10]. Due to the remarkable clinical benefit caused by the introduction of this substance into treatment algorithms, next-generation proteasome inhibitors were developed [11]. Its successor carfilzomib (Kyprolis®, PX-171-007) resulted in improved survival for patients suffering from relapsed multiple myeloma [12]. Nonetheless, bortezomib and others exhibit the same mode of action causing the proteasome's quiescence by blocking the chymotrypsin-like activity located in the 20S subunit of the proteasome bearing the risk of developing resistance [13]. Another promising target is the regulatory 19S subunit flanking the central part of the proteasome, whose selective inhibition is currently under investigation [14]. One of the novel drugs targeting these cap structures of the proteasome is b-AP15, provoking a blockage of the enzyme deubiquitinase *via* inhibiting both ubiquitin-specific peptidase 14 (USP14) and ubiquitin C-terminal hydrolase 5 (UCHL5) [15]. In contrast to conventional proteasome inhibitors, its mode of action prevents degradation *via* inhibition of access of poly-ubiquitinated proteins to the proteasome. This leads to an accumulation of flagged proteins within the cell which in consequence results in cell death [2]. Therefore, b-AP15 may serve as an innovative anticancer drug, driving both hematological and solid tumor cells into apoptosis [16–21].

However, effects of proteasome inhibitors are not restricted to tumor cells exclusively. All cell types may be affected, among those being cells of the immune system of particular interest. Impairment of immune responses due to decreased viability of natural killer cells had already been described [22,23].

In contrast, we and others recently showed that both bortezomib and b-AP15 enhance antitumor immunity mediated by natural killer cells [16,18,24].

Effects of bortezomib on DCs, another important immune subset, have already been identified [25–27]. Linking innate and adaptive immunity, DCs assume a key role in regulating immune responses [28]. Generally, DCs recognize mainly antigens derived from infectious or tumorous invasion [29]. Equipped with a wide repertoire of receptors enabling the identification of danger- and pathogen-associated molecular patterns, DCs mature in the presence of external stimuli in order to fulfill their main function as professional antigen-presenting cells [30]. For this purpose, they process and present ingested components followed by their migration to proximate lymphoid organs, where an initiation of antigen-specific immune responses occurs [31]. This requires, in particular, contact between DCs and T lymphocytes [32].

Various groups have previously demonstrated effects of bortezomib on DC phenotype and function on multiple levels by inhibition of DC maturation, impeding uptake of antigens through endocytosis and downmodulating DC responses to endogenous prostaglandins and inflammatory cytokines as well as the pathogen-derived product lipopolysaccharide (LPS) [25–27].

However, the impact of b-AP15 on DC phenotype and function is unknown so far. Thus, in the present study we contrast properties of DCs treated either with bortezomib or b-AP15 for a profound and comparative evaluation of the immunomodulatory capacity of this novel deubiquitinase inhibitor.

Materials and Methods

Cell Isolation, Generation and Treatment of DC

Adherent monocytes provided the basis for obtaining DCs *ex vivo* following a common approach as previously described [75,76]. Permission was obtained by the resident ethics committee (local institutional review board 344/2008BO2; Ethics Committee at the Medical Faculty and at the University Hospital Tübingen). After informed consent and in accordance with the Declaration of Helsinki buffy coat samples from healthy humans voluntarily giving blood at the blood bank of the University of Tübingen were processed. Isolating peripheral blood mononuclear cells (PBMC) using Ficoll/Biocoll (Biochrom AG, Berlin, Germany) density gradient centrifugation initiated this process. Then, isolated cells were added to serum-free X-VIVO 20 medium (Lonza, Basel, Switzerland) for up to two hours in order to facilitate their adherence in 75 cm^2 cell culture flasks (Corning Inc., New York, USA) under controlled conditions (37°C, 5% CO_2, 1 x 10^7 cells/mL). After washing, remaining adherent monocytes were subsequently cultured in RPMI 1640 medium [supplemented with 10% heat-inactivated FCS and 2% penicillin/streptomycin (Thermo Fisher Scientific, Waltham, USA)] for further 6 days with the addition of recombinant human GM-CSF (100 ng/mL; Sanofi-Aventis, Frankfurt, Germany) and IL-4 (20 ng/mL; R&D Systems, Wiesbaden, Germany) every other day leading to the generation of immature DCs (iDCs). To obtain mature DCs (mDCs), LPS (TLR4L, 100 ng/mL; Sigma, St. Louis, USA) was added to the existing iDC cell suspension for the last 16 hours of culture.

Bortezomib (10 ng/mL; Selleckchem, Munich, Germany) and b-AP15 (10 nM, 100 nM, 500 nM and 1000 nM; Active Biochem, Bonn, Germany), both dissolved in DMSO, were employed in accordance with previously applied concentrations *in vitro* and added to cell culture 24 hours before harvest [18,25,77]. DCs treated only with DMSO served as control. For light microscopy image acquisition a Zeiss microscope was used.

PCR

PCR was conducted as previously described [78]. Primers were 5′-GGCTTCAGCGCAGTATATTA-3′ and 5′-CAGATGAGGAG TCTGTCTCT-3′ for USP14 [79] and 5′-GAAGGACCGATT GATTTAGG-3′ and 5′-CCTTCACTGTAC TTTTGTATCC-3′ for UCHL5. For 18S RNA the QuantiTect primer assay (Qiagen, Hilden, Germany) was used.

Western Blot

Protein lysates were generated from cell pellets with Cell Lysis Buffer (Cell Signaling Technology, Frankfurt, Germany) containing Halt Protease and Phosphatase Inhibitor (Thermo Fisher) by incubating for 10 min on ice and 2 x 5 min in an ultrasonic-water bath. 40 μg of the protein lysate were used for a SDS-PAGE (4–12% gradient gel) *via* the Bolt Western Blot system and a subsequent western blot performed with the iBlot system (both Thermo Fisher), according to the manufacturers' protocols. Following the blotting,

blocking for 3 hours was conducted. The antibodies used afterwards were USP14 (A300-919A, rabbit polyclonal) (1:5000, Bethyl laboratories, Montgomery, Texas, USA), UCHL5 (H110, rabbit polyclonal) (1:400, Santa Cruz Biotechnology, Heidelberg, Germany) and cofilin (clone D3F9) (1:2000, Cell Signaling Technology). For detection in a LI-COR Odyssey imaging reader, anti-rabbit IRDye 680RD (1:20000) and IRDye 800CW (1:20000) secondary antibodies (both LI-COR Bioscience, Bad Homburg, Germany) were employed.

Determination of Cell Viability

Cells were stained using the FITC Annexin V Apoptosis Detection Kit (BD Biosciences, Heidelberg, Germany). In brief, DCs were harvested, washed and resuspended in binding buffer. After the addition of FITC-labeled Annexin V and 7-AAD (BioLegend, San Diego, USA), cells were incubated for 15 min at room temperature followed by FACS analysis for quantification of viable cells. DMSO and staurosporine (500 nM; Abcam, Cambridge, UK) served as control.

Immunostaining

Staining of harvested cells was performed using directly FITC-, PE-, PerCP- or APC-conjugated monoclonal murine anti-human antibodies against CD1a, CD14 (BD Biosciences), CD80, CD83 (eBioscience, San Diego, USA), CD86 (BD Biosciences), CD209 (R&D Systems), HLA-DR (eBioscience) and GPNMB (R&D Systems) as well as corresponding mouse IgG isotype controls (R&D Systems; BD Biosciences; eBioscience). Analysis of cells was performed on a FACSCalibur cytometer (BD Biosciences). Specific fluorescence intensities (SFIs), as ratios of mean fluorescence intensity reached by the antibody directed against a specific molecule of interest and its corresponding isotype, were assessed.

Analysis of Endocytic Competency

A total of 1×10^5 DCs were incubated with FITC-dextran (Sigma) for one hour at an incubation temperature of 37°C. Afterwards, cells were washed and dextran uptake was assessed on a FACSCalibur cytometer.

Measurement of DC Migration Towards a Chemokine Stimulus

Cultured and pretreated DCs (2×10^5) were transferred into transwell chambers (8 μm; Corning) inside 24-well plates (Corning) after harvest. Migratory pattern towards the chemoattractant CCL19 (100 ng/mL; R&D Systems) was subsequently examined after 16 hours of incubation at 37°C and 5% CO_2. FACSCalibur was used to determine the number of DCs arising in a preliminarily established gate within 1 minute of measurement.

Determination of Cytokine Levels

Drug- or DMSO-treated DCs were harvested and obtained supernatants were collected and stored at −20°C for joint analysis. Concentrations of cytokines were measured as described previously [80].

Evaluation of Immunostimulatory Capacity

Allogeneic PBMCs were cultured together with irradiated DCs generated and treated as previously described in 96-well flat-bottomed microplates (Greiner Bio-One, Frickenhausen, Germany) for 5 days. PBMCs, labeled with CFSE (Thermo Fisher Scientific) and 10 times more frequently present in culture than stimulator DCs, were harvested at day six of culture. Subsequent to several washing steps and surface staining with CD4-APC and CD8-PE (both Thermo Fisher Scientific), proliferating T cells indicating the immunostimulatory capacity of DCs could be identified by flow cytometry.

Statistics

Each experiment was conducted at least three times revealing similar results. Representative experiments as well as combined data are shown. If not declared otherwise, technical triplicates were performed leading to average value and enabling the calculation of a standard error of the mean. To prove statistical significance, a Student's t test was applied and P values $<.05$ were considered suggesting statistical significance between the treatment condition and DMSO control. For non-parametric values a Mann–Whitney U test was performed.

Results

b-AP15, in Contrast to Bortezomib, Does Not Interfere With Differentiation of Monocytes into Immature DCs

Initially and with the aim of legitimating our investigation towards DC properties in the presence of b-AP15, we studied the expression of USP14 and UCHL5, the molecules blocked by this deubiquitinase inhibitor, on a transcriptional as well as translational level. PCR analyses of total mRNA and western blot analyses of whole cell lysates derived from different donors could demonstrate the existence of both target molecules in immature human monocyte-derived DCs (iDCs) generated in our experimental setting (Figure 1*A* and *B*).

Morphological and phenotypical features of iDCs treated with bortezomib or b-AP15 were assessed using microscopy and flow cytometric measurements.

Microscopic images of iDCs from one representative experiment are exemplarily shown in Figure 1*C*. DC numbers in the presence of bortezomib seemed to be reduced compared to DCs treated with DMSO or b-AP15. Therefore, we performed Annexin V/7-AAD staining for determination of cell viability and found significantly reduced numbers of living DCs in the bortezomib group (Figure 1*D*).

Further, flow cytometric analyses revealed changes in bortezomib-treated DCs with regard to cell count, size and granularity illustrated by forward/side scatter gating compared to those pretreated with b-AP15 or vehicle control. In the presence of bortezomib both a discrete shrinking of the cells occurred and at the same time, granularity of analyzed DCs was decreased (Figure 1*E*). In contrast, the deubiquitinase inhibitor b-AP15 in therapeutic doses seemed not to affect iDC cellular characteristics [18].

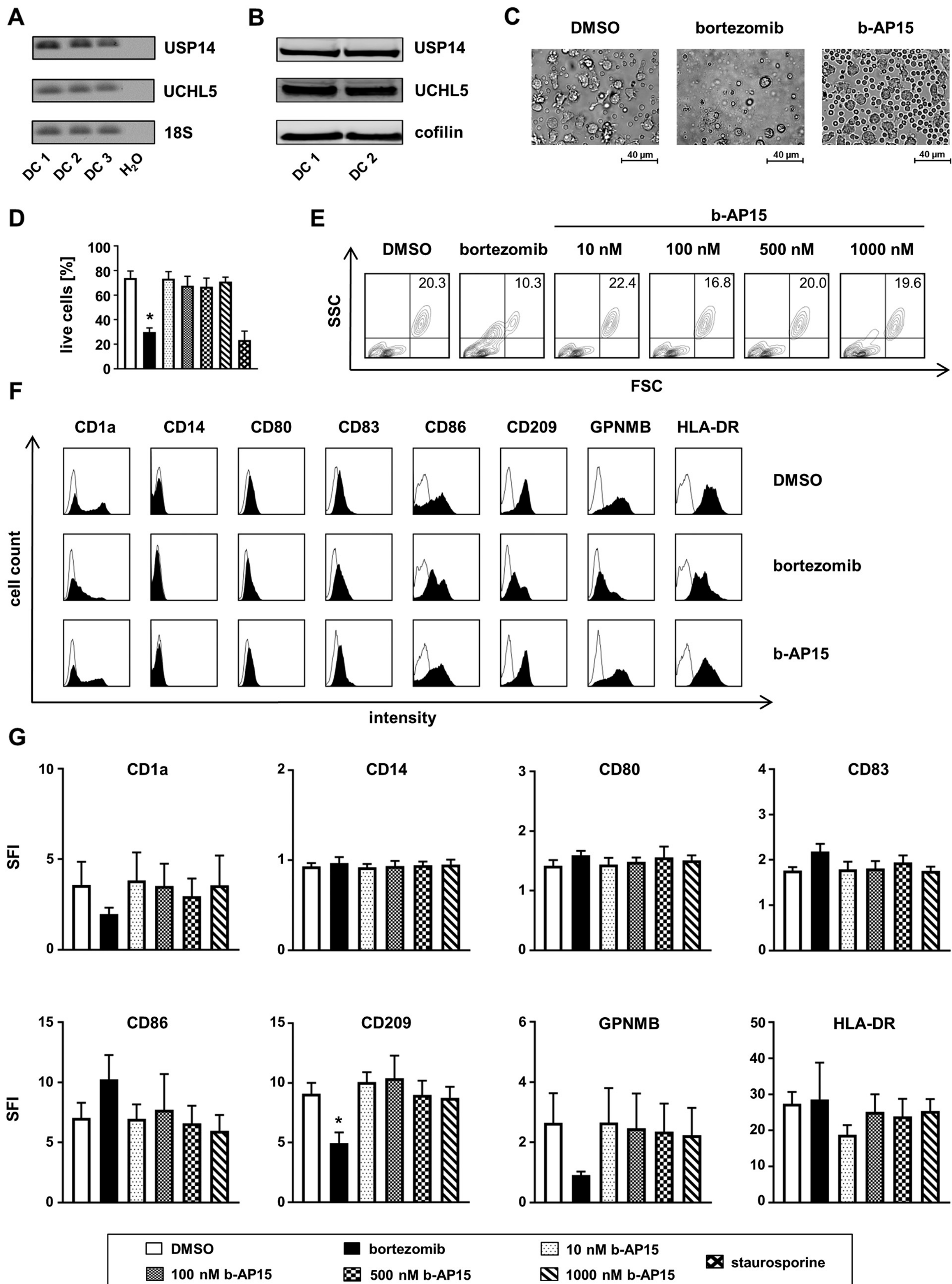
When monocytes differentiate into iDCs, a distinctive alteration of their surface marker signature occurs: CD14 gradually diminishes whereas CD1a becomes upregulated [33]. We observed that exposure to pharmacological concentrations of neither bortezomib nor b-AP15 had significant effects on CD1a, CD14, CD80, CD83, CD86, GPNMB and HLA-DR expression on iDCs. Interestingly, CD209, responsible for a stable interaction between DCs and T lymphocytes [34], was downregulated under the influence of bortezomib, but not by b-AP15. This implies that deubiquitininase inhibition does not result in changes of iDC phenotype whereas bortezomib in line with previous results shows no or only minor impact on iDC surface marker repertoire compared to DMSO control (Figure 1, *F* and *G*).

Morphologic and Phenotypical Properties of Monocyte-Derived DCs in Response to TLR4 Stimulation are Influenced by Bortezomib, But Not by b-AP15

Compliant with our previous experimental approach in the context of iDCs, the detection of both USP14 and UCHL5 by means of PCR

and western blot analyses in different healthy donors formed the basis for further studies of LPS-stimulated DCs (Figure 2, *A* and *B*). In line with our observations with iDCs, both microscopic images and Annexin V/7-AAD staining again suggested reduced mDC numbers with bortezomib treatment while b-AP15 and DMSO seemed to have no relevant effects (Figure 2, *C* and *D*).

Flow cytometry contour plots from one representative experiment are exemplarily shown in Figure 2*E*. Comparable to immature cells,



these analyses again revealed morphologic changes in mDCs after exposure to the proteasome inhibitor bortezomib, but not to those treated with b-AP15 or DMSO.

When expanding the analyses of Nencioni et al. [25], who already detected impaired upregulation of CD40, CD54, CD80, CD83, CD86 and CD209 on mDCs after bortezomib treatment, we could observe a significantly reduced expression of CD80, CD83, CD86, CD209 and HLA-DR in comparison to DMSO-treated controls. In contrast, b-AP15 treatment did not modify DC maturation response after addition of LPS. Interestingly, GPNMB, that has recently been characterized as a negative regulator of T cell activation [35–37], as well as CD1a and CD14 were not affected under treatment [38] (Figure 2, *F* and *G*).

In summary and concordant to our findings addressing morphological properties, investigation of immunophenotypical alterations in DCs encountering differential proteasome inhibition displayed no undesirable impact on DC activation and differentiation markers by the application of the deubiquitinase inhibitor b-AP15 in contrast to bortezomib.

Endocytic Activity of DCs Is Only Affected by Bortezomib-Mediated Blockade of Proteasomal 20S Subunit and Remains Preserved under Treatment with the Deubiquitinase Inhibitor b-AP15

Antigen uptake is essential for DC functionality. Therefore, we evaluated endocytic capacity in the presence of either bortezomib or b-AP15 with measurements of polysaccharide dextran incorporation. Accumulation of FITC-labeled molecules within DCs was determined by flow cytometry with or without LPS stimulation. DCs incubated at 4°C served as control (data not shown). Immature DCs, conventionally holding a higher activity with regard to endocytosis [39], showed nearly unchanged endocytic characteristics in the presence of b-AP15 compared to vehicle control (Figure 3*A*). The same result could be obtained when analyzing mature DCs (Figure 3*B*). In contrast to these observations, bortezomib caused a significant decrease in endocytic ability in both iDCs and mDCs (Figure 3, *A* and *B*).

Chemotaxis of DCs is Impaired by Bortezomib and Still Upheld in the Presence of b-AP15

Crucial for DC-mediated immune responses is their ability to migrate to secondary lymphoid tissues after the recognition and uptake of antigens. This process, also called “homing”, is fostered by certain chemokines, guiding the way for DCs [40]. One representative of these molecules is CCL19 (C-C motif chemokine ligand 19), which can predominantly be found in T cell enriched areas and is detected by its corresponding receptor CCR7 exclusively located on the surface of activated or mature DCs [41–45]. It has been previously shown that bortezomib exposure impaired the migratory capacity of LPS-activated DCs [25].

To assess the migratory capacity of both immature and mature DCs pretreated with bortezomib, b-AP15 or DMSO *in vitro*, a transwell migration assay using CCL19 as a stimulus was performed. As expected, in the status of immaturity DCs showed reduced chemotaxis compared to those stimulated with LPS before harvest and their migratory behavior was not significantly affected by proteasome inhibition (Figure 4*A*). Chemotaxis of mDCs towards CCL19 was not impaired in the presence of b-AP15 compared to cells cultivated with DMSO. However, bortezomib caused a significant reduction of migrated cells (Figure 4*B*). These findings suggest that the migration towards a chemokine stimulus appears untouched by blocking deubiquitinase of DCs.

Cytokine Release by mDCs is Differentially Affected by Bortezomib and b-AP15

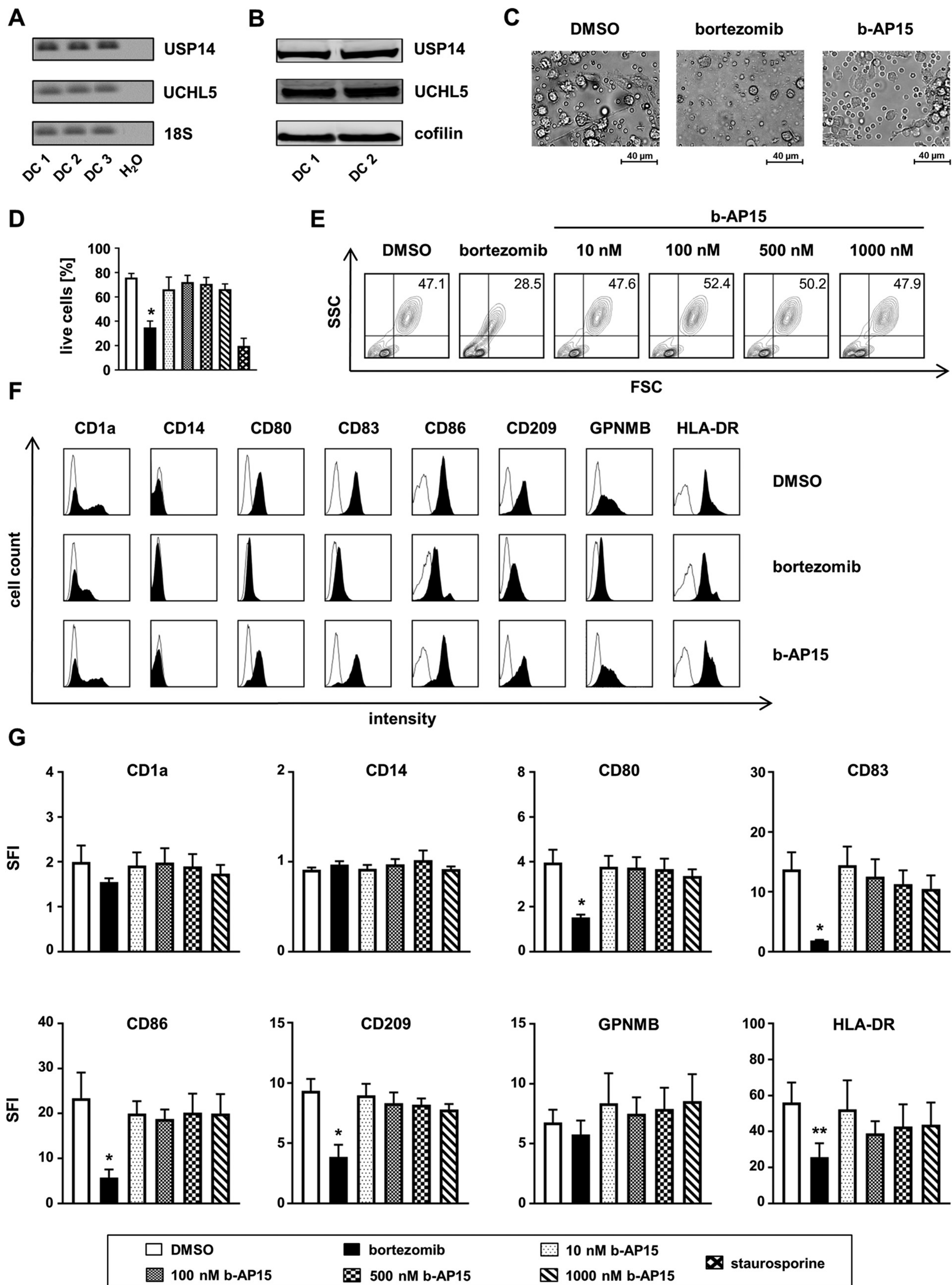
Since secretory function of DCs is pivotal for orchestrating the immune response and in turn DCs are influenced by cytokines in an autocrine or paracrine manner [46], comparative studies using standard ELISA were performed investigating the amount of IL-10, TNF and IL-6 released by DCs in the presence of bortezomib or b-AP15 after 6 days of culture. While cytokine release of resting iDCs was hardly detectable and neither affected by bortezomib nor by b-AP15 (data not shown), we were able to identify significantly lower levels of IL-10, TNF and IL-6 in the supernatant of mDCs cultured in the presence of bortezomib (Figure 5, *A*–*C*). Surprisingly, secretion of IL-10 was, in contrast to TNF and IL-6, clearly impaired after treatment of mDCs with doses of b-AP15 exceeding 500 nM (Figure 5*A*). Although the underlying mechanism responsible for this particular observation so far remains unclear, it is noteworthy that DC functionality can indeed be influenced by b-AP15.

Inhibition of Proteasomal Activity by b-AP15 Does Not Attenuate Immunostimulatory Capacity of DCs

The principal functional capability of DCs comprises T cell priming in secondary lymphoid tissues. Thus, this process paves the way for an antigen-specific response performed by the adaptive immunity. To address the question if T cell stimulation after DC encounter is affected by different proteasome inhibitors, we conducted CFSE proliferation assays with PHA serving as positive control. Immunostimulatory potency of

Figure 1. Effects of bortezomib and b-AP15 on morphology and phenotype of iDCs. Peripheral blood adhering monocytes were cultured with IL-4 and GM-CSF for six days and subsequently incubated with DMSO, bortezomib or b-AP15 for further 24 hours. Thereafter, cells were harvested and generated iDCs were analyzed using PCR, western blot, microscopy and flow cytometry. **(A, B)** Expression of USP14 and UCHL5 in monocyte-derived iDCs was determined by PCR analyses of total mRNA (derived from three different donors, using 18S RNA as control; **A**) and by means of western blot analyses of cell lysates (derived from two different donors using specific USP14 and UCHL5 antibodies, cofilin serving as loading control; **B**). **(C)** Photomicrograph depicts iDCs treated with DMSO (left), 10 ng/mL bortezomib (middle) and 1000 nM b-AP15 (right). Scale bar 40 μm. **(D)** iDCs were exposed to bortezomib and the indicated concentrations of b-AP15 for 24 h. DMSO and staurosporine served as control. The percentage of live (Annexin V−/7-AAD−) iDCs was determined by FACS with Annexin V/7-AAD staining. Combined data of five independent experiments with the indicated drug treatments are shown and compared to vehicle control DMSO (Mean ± S.E.M.). Student's *t* test was used to determine statistical significance (**P* < .05). **(E)** Effects of treatment on iDC size and granularity were examined and compared to the DMSO vehicle control. Exemplarily, one representative out of at least five similar experiments is depicted. **(F, G)** Expression of the indicated cell surface markers characterizing iDC phenotype was assessed after exposure to bortezomib or b-AP15 and contrasted to vehicle control DMSO. Levels of expression are shown as SFI. Results of single data (F) or pooled data consisting of at least five independent experiments (G) are shown. Matched isotype controls are presented as open histograms. (Student's *t* test, Mean ± S.E.M., **P* < .05).

DCs was determined using flow cytometry to detect proliferating CD4$^+$ and CD8$^+$ T cell subpopulations. In line with previously published data [25,27], we observed a restricted extent of both CD4$^+$ and CD8$^+$ T cell stimulation after treatment with bortezomib in mDCs (Figure 6, *B* and *D*). Further, we could identify this significant effect also in iDCs after treatment with bortezomib (Figure 6, *A* and *C*). Only mature DCs are potent in stimulating T cell responses by providing costimulatory signals and T cell adhesion molecules [47,48]. Accordingly, the fraction of



proliferating cells was, especially with regard to CD4$^+$ T cells, less pronounced when cultivated with iDCs. Both iDC and mDC capacity to prime naïve allogeneic T cells remained completely unaffected after the addition of b-AP15 (Figure 6). Exposure to PHA led to a considerably increase in T cell proliferation.

This insight, demonstrating no unfavorable effect of b-AP15 on this major DC feature, is in agreement with our investigations concerning morphologic and functional properties in response to this deubiquitinase inhibitor.

In summary, these findings make a contribution to appraise the impact of proteasome inhibition on DCs. Focusing on their central morphologic and functional qualities in the context of inducing adaptive immune responses, bortezomib, in accordance with previous work, seems to negatively affect DC function. Our results suggest the absence of adverse effects on DCs mediated by b-AP15 *in vitro*, providing an important rationale for further investigation.

Discussion

We have previously demonstrated that the proteasome inhibitor bortezomib influences DC functionality at different levels by reducing antigen uptake through endocytosis and by deteriorating DC response to endogenous and exogenous stimuli like LPS, prostaglandins and inflammatory cytokines. LPS-induced signaling in DCs *via* NF-κB, interferon regulatory factors (IRFs), and the mitogen-activated protein (MAP) kinase pathway was shown to be negatively affected by bortezomib [25]. Nevertheless, results on the effect of bortezomib on DC function are conflicting. On the one hand, studies identified bortezomib as a potent drug interfering with DC capacities at different levels [25,49]. On the other hand, there is evidence for an increase and facilitation of immune responses arising from DCs to different tumor entities after exposure to bortezomib [50,51]. Nonetheless, *in vivo* application of bortezomib suggests adverse effects on DC functionality enabling a controversial debate on the use of customary proteasome inhibitors as immunomodulating agents [52]. Additionally, there is growing evidence for clinical benefit of bortezomib treatment for patients suffering from Graft-*versus*-Host disease, possibly explainable by its modulating impact on DC functions and the potential to attenuate immune reactions [53,54].

In this study, we critically evaluated and contrasted our previous findings concerning conventional proteasome inhibition by bortezomib on DC function with a novel therapeutic substance, the deubiquitinase inhibitor b-AP15.

Anticancer effects by b-AP15 are mediated *via* acting upon the deubiquitination cascade resulting in caspase-dependent apoptosis of tumor cells derived from different malignancies as well as sensitizing various tumors to TRAIL-mediated apoptosis by natural killer and T cells [16–20,55]. Like bortezomib, b-AP15 seems to affect further, non-malignant cell types. As an example, PBMCs from healthy donors cultivated in the presence of b-AP15 demonstrate only a negligible reduction of their cell viability [16,17], whereas cytotoxic effects of b-AP15 become specifically apparent on natural killer cells. This cell type reveals a high susceptibility to this deubiquitinase inhibitor and as a result promptly tends to become apoptotic, while effects of bortezomib were less pronounced [23].

With sipuleucel-T (Provenge®, APC8015), personalized DC-based immunotherapy has already been approved for clinical use in advanced prostate cancer [56,57]. Further immunotherapeutic DC approaches have already showed promising effects in a multitude of cancers, including malignancies treated with the proteasome inhibitor bortezomib like multiple myeloma [58–62] and mantle cell lymphoma [63]. Interestingly, Spisek et al. could detect an improved immune response by tumor-specific DCs against primary multiple myeloma cells after the application of bortezomib [50]. Effects of b-AP15 on DCs are uninvestigated so far.

In line with previous results, our study indicates a considerably impaired differentiation from peripheral blood adherent monocytes into DCs caused by bortezomib. In contrast, after exposure to b-AP15 we could not identify significant alterations of both morphological and phenotypical features of neither iDCs nor mDCs after thorough investigation when DCs were subjected to the highest concentration level of b-AP15, shown to be way above the IC50 of different MCL cell lines *in vitro* [18].

In line with previous results, b-AP15 did not influence antigen uptake by either iDCs or mDCs whereas bortezomib caused a pronounced impairment of this important functional DC feature. The extent of reduction with regard to dextran ingestion apparently was more distinct in the case of bortezomib-treated iDCs, due to their *per se* higher endocytic activity compared to bortezomib-treated mDCs, probably caused by diminution of macropinocytosis and phagocytosis during processes of DC activation and maturation [64].

Antigen uptake and processing is followed by migration towards secondary lymphatic tissues, facilitated by a gradient of different signal molecules, in our setting represented by the chemokine CCL19 serving as a stimulus for chemotaxis. These movements were restricted when mDCs encountered bortezomib, but not b-AP15

Figure 2. Effects of bortezomib and b-AP15 on morphology and phenotype of mDCs. Peripheral blood adhering monocytes cultured with IL-4 and GM-CSF for six days were subsequently incubated with DMSO, bortezomib or b-AP15 according to the indicated concentrations for further 24 hours. LPS was added for the last 16 hours of incubation in order to induce maturation. Thereafter, cells were harvested and generated mDCs were analyzed using PCR, western blot, microscopy and flow cytometry. **(A, B)** Expression of USP14 and UCHL5 in monocyte-derived mDCs was determined PCR analyses of total mRNA (derived from three different donors, using 18S RNA as control; **A**) and by means of western blot analyses of cell lysates (derived from two different donors using specific USP14 and UCHL5 antibodies, cofilin serving as loading control; **B**). **(C)** Photomicrograph depicts mDCs treated with DMSO (left), 10 ng/mL bortezomib (middle) and 1000 nM b-AP15 (right). Scale bar 40 μm. **(D)** mDCs were exposed to bortezomib and the indicated concentrations of b-AP15 for 24 h. DMSO and staurosporine (Abcam) served as control. The percentage of live (Annexin V−/7-AAD−) mDCs was determined by FACS with Annexin V/PI staining. Combined data of five independent experiments with the indicated drug treatments are shown and compared to vehicle control DMSO (Mean ± S.E.M.). Student's *t* test was used to determine statistical significance (*$P < .05$). **(E)** Effects of treatment on mDC size and granularity were examined and compared to the DMSO vehicle control. Exemplarily, one representative out of at least five similar experiments is depicted. **(F, G)** Expression of the indicated cell surface markers characterizing mDC phenotype was assessed after exposure to bortezomib or b-AP15 and contrasted to vehicle control DMSO. Levels of expression are shown as SFI. Results of single data (F) or pooled data consisting of at least five independent experiments (G) are shown. Matched isotype controls are presented as open histograms. (Student's *t* test, Mann–Whitney *U* test, Mean ± S.E.M., **$P < .01$).

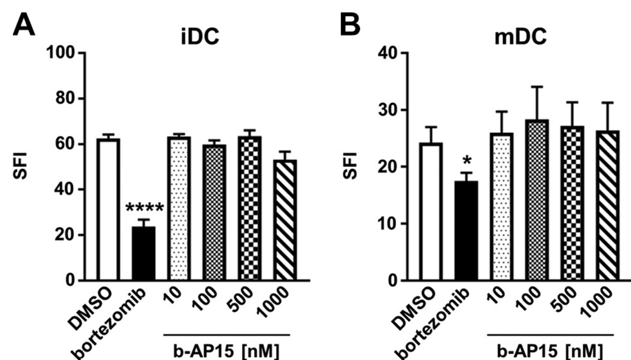


Figure 3. Effects of bortezomib and b-AP15 on endocytic activity of DCs. Bortezomib- or b-AP15-treated DCs were cultured according to the indicated concentrations in the absence **(A)** or presence **(B)** of LPS. Afterwards, DCs were incubated at physiological temperature after addition of FITC-dextran for one hour. Evaluation of endocytic capacity of both iDCs and mDCs was performed by determination of SFI levels by flow cytometry. Pooled data of at least five experiments are shown and compared to vehicle control DMSO. (Student's *t* test, Mean ± S.E.M., $^{****}P < .0001$).

which again did not seem to significantly affect migratory abilities of mDCs. CCL19 is recognized *via* its corresponding receptor CCR7, exclusively located on the surface of mDCs [45]. This fact could illustrate the general lower migratory activity of iDCs and the absent interference of bortezomib acting upon this subgroup of DCs.

Apart from these essential competencies, DCs display a specific signature of released cytokines needed for the establishment of inflammation, induction of immune response and self-stimulation [46]. This comprises, among others, molecules like IL-6, IL-12p70 or TNF. In line with previous findings, we observed significantly decreased concentrations of IL-10, TNF and IL-6 secreted by mDCs after treatment with bortezomib [25]. Interestingly and in contrast to our prior results, mDC functionality was directly affected by b-AP15 at concentration levels of at least 500 nM resulting in reduced IL-10 concentrations compared to DMSO control whereas no change in the release of TNF or IL-6 could be detected. IL-10 is known for limiting DC maturation and impairing the activation of Th1 cells by DCs [65,66]. Based on our observations, b-AP15 thus could contribute to overcome this restriction and facilitate a recovery of Th1-mediated immune responses against infections and malignancies. Additionally, these findings are of special interest since synthesis of certain cytokines, among those IL-12p70, TNF and IL-6 are the most evident, in DCs crucially depends on NF-κB signaling [67,68] which is known for being interrupted by b-AP15 at least in Waldenström macroglobulinaemia [17]. This could indicate the existence of further alternative signaling pathways governing cytokine release in DCs. Further investigation is needed to more precisely understand the exact mechanisms leading to these findings.

Finally, our investigations culminate in stable immunostimulatory properties of both iDCs and mDCs after the application of b-AP15 leading to the expansion of both allogeneic $CD4^+$ and $CD8^+$ T cells after co-culture with DCs. In opposition, bortezomib treatment resulted in impaired cell proliferation of allogeneic T cells in the presence of DCs as already observed by Naujokat et al. [27]. One explanation for this effect could be the differential expression of surface molecules that are required for DC-dependent T cell activation. These comprise mainly CD80, CD86 and of particular importance for $CD4^+$ T cells HLA-DR, all being reduced after exposure of mDCs to bortezomib, whereas b-AP15 did not seem to interfere with their expression levels.

Altogether, we could successfully reconfirm findings by Nencioni et al. and others [25–27], describing the critical interference between bortezomib and DCs and enabling a contrasting juxtaposition by the inclusion of the small molecule inhibitor b-AP15 into our experimental settings.

The key issue of this work was to determine whether DC functionality is affected by b-AP15. At this point, we can conclude that DCs under treatment with this deubiquitinase inhibitor mostly maintain their phenotype and function when exposed to therapeutically relevant concentrations in both hematological and solid tumor models *in vitro* [15–18,23,69]. DCs play a crucial role in regulating adaptive immune responses, especially mediated by T cells, which can be exploited for cancer therapeutic vaccines. However, immunostimulatory capacities of DCs seem to be compromised in different malignancies, for instance multiple myeloma [70,71]. By interfering with DC functionality, bortezomib, implemented in treatment algorithms for this hematologic disease, could even blunt the efficacy of such novel therapeutic approaches. In contrast, our findings showing no adverse effects of b-AP15 on different DC capabilities suggest further clinical investigation of this deubiquitinase inhibitor.

As the application of bortezomib is associated with reactivation of latent virus infections, Basler et al. could demonstrate restricted virus-specific T cell priming after the administration of this proteasome inhibitor in a mouse model of lymphocytic choriomeningitis virus (LCMV) [72–74]. Therefore, b-AP15 could represent a promising alternative possibly showing a more favorable spectrum of adverse effects compared to bortezomib which needs to be proved in further (pre)clinical trials.

Finally, an establishment of such innovative strategies may contribute to the abrogation of mechanisms responsible for a curtailed response to bortezomib, already proven for b-AP15 [16]. Nonetheless, prior to this the impact of b-AP15 on other subsets of

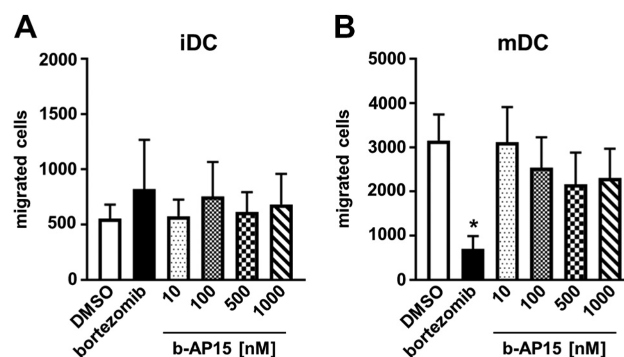


Figure 4. Migratory capacity of DCs towards CCL19 under treatment with bortezomib and b-AP15. Monocyte-derived DCs were exposed to DMSO, bortezomib and b-AP15 according to the indicated concentrations for 24 hours in the absence **(A)** or presence **(B)** of LPS as maturation stimulus during the last 16 hours of incubation. After harvest, transwell assays were performed and migratory capacity of both iDC and mDC towards CCL19 was assessed. Therefore, absolute counts of migrated cells were determined using flow cytometry. Pooled data consisting of at least five independent experiments are shown and compared to vehicle control DMSO. (Student's *t* test, Mean ± S.E.M., $^{*}P < .05$).

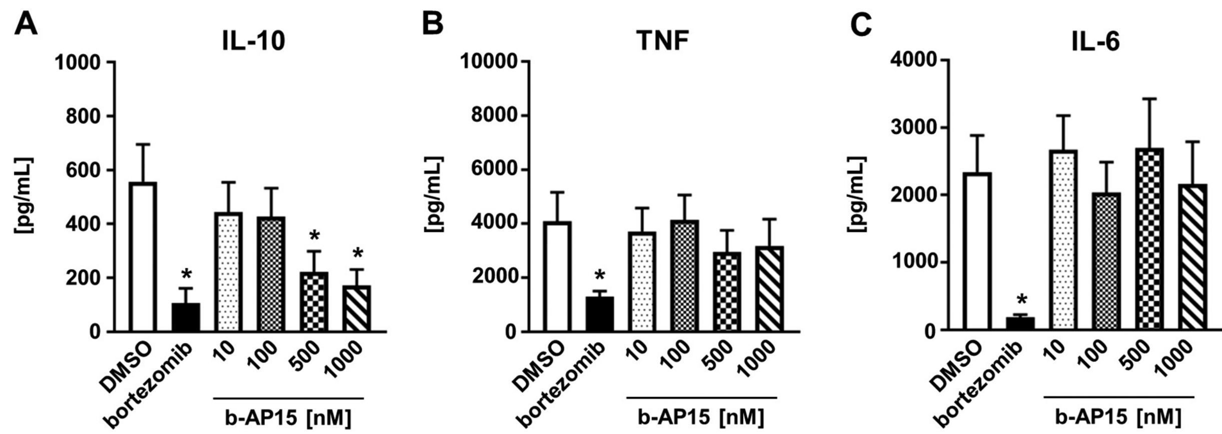


Figure 5. Modulation of cytokine release of DCs in the presence of bortezomib and b-AP15. DCs were generated by culturing adhering monocytes with IL-4 and GM-CSF for six days followed by incubation with DMSO, bortezomib or b-AP15 according to the indicated concentrations for further 24 hours in the presence of LPS as maturation stimulus during the last 16 hours of incubation. Afterwards, cells were harvested and supernatants were collected. Detection of IL-10, TNF and IL-6 released by mDCs was performed using standard ELISA measuring absolute cytokine concentrations **(A, B, C)**. Pooled data combining results out of at least five independent experiments is shown and compared to vehicle control DMSO. (Student's *t* test, Mean ± S.E.M., *$P < .05$).

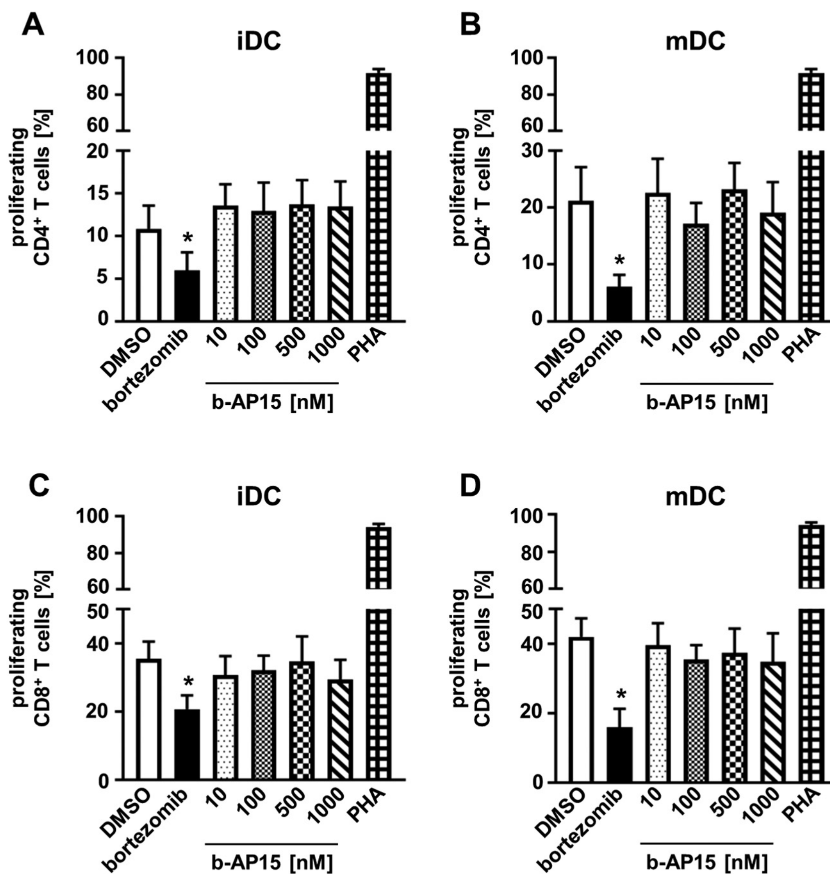


Figure 6. Impact of bortezomib and b-AP15 on immunostimulatory capacities of DCs. Culture of peripheral blood adhering monocytes in the presence of IL-4 and GM-CSF for six days led to the generation of DCs which were exposed for further 24 hours to bortezomib or b-AP15 according to the indicated concentrations without **(A, C)** or with LPS **(B, D)** followed by an analysis of their capacity to stimulate T cell responses *in vitro*. For this purpose, irradiated stimulator DCs were cultured together with previously CFSE-labeled allogeneic PBMCs for a total of five days. Subsequently, the resulting CFSE signal corresponding to a proliferating $CD4^+$ **(A, B)** or $CD8^+$ **(C, D)** T cell subpopulation was analyzed using flow cytometry as an indicator for the immunostimulatory capacity of either iDCs or mDCs. Pooled data of at least five experiments are shown and compared to vehicle control DMSO. (Student's *t* test, Mean ± S.E.M., *$P < .05$).

immune cells, like for example T cells, needs to be critically evaluated and clinical studies are required, additionally taking pharmacological aspects into account.

Author Contributions

M. S. analyzed and interpreted data and wrote the first draft of the manuscript. V. A., S. S. and A. R. F. performed the experiments and analyzed and interpreted data. K. N. K., S. M., M. R. M. and H. R. S. critically revised the manuscript and provided important advice. S. M. R. and F. G. contributed to the study design, provided important advice and critically revised the manuscript. D. D. designed the study, wrote the manuscript as lead author and supervised the study. All authors critically reviewed the manuscript and approved the final version.

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Conflict of interest disclosure

The authors declare no competing financial interests.

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