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The Proteasome Inhibitor Bortezomib Enhances the Susceptibility to Viral Infection

Michael Basler, Christoph Lauer, Ulrike Beck, and Marcus Groettrup

The proteasome, a multicatalytic protease, is responsible for the generation of most MHC class I ligands. Bortezomib, a proteasome inhibitor, is clinically approved for treatment of multiple myeloma and mantle cell myeloma. In the present study, we investigated the effect of bortezomib on viral infection. Infection of bortezomib-treated mice with the lymphocytic choriomeningitis virus (LCMV) led to a decreased cytotoxic T cell response to several LCMV-derived CD8+ T cell epitopes. Bortezomib treatment caused a reduced expansion of CD8+ T lymphocytes and increased viral titers in LCMV-infected mice. Administration of bortezomib during expansion of CD8+ T cells had no influence on the cytotoxic T cell response, suggesting that bortezomib interferes with priming of naive T cells. Indeed, determination of Ag load in spleen 4 days post infection, revealed a reduced presentation of LCMV-derived cytotoxic T cell epitopes on MHC class I molecules. In summary, we show that proteasome inhibition with bortezomib led to an increased susceptibility to viral infection, and demonstrate for the first time, that proteasome inhibitors can alter Ag processing in vivo. The Journal of Immunology, 2009, 183: 6145–6150.

Bortezomib (also named PS-341, or Velcade; Millennium Pharmaceuticals) is the first clinically approved proteasome inhibitor for relapsed and/or refractory myeloma and mantle cell lymphoma. The boronic acid dipeptide is a reversible proteasome inhibitor that selectively and specifically inhibits the chymotrypsin-like activity of the proteasome 20S subunit (13, 14).

Proteasome inhibitors were extensively used to study class-I Ag processing and presentation in vitro (15–19). Nevertheless, the influence of proteasome inhibition on Ag processing and the effect of proteasome suppression on a cytotoxic T cell response have not been investigated in vivo. To address this issue, we used the infection with lymphocytic choriomeningitis virus (LCMV) (3), a noncytopathic arenavirus, to study the characteristics of Ag processing and presentation in PS-341 treated mice. The cytotoxic immune response to LCMV is essential for the elimination of the virus from infected mice (20). In C57BL/6 mice, this response is shaped by CTLs specific for the dominant glycoprotein (GP)-derived epitopes GP33–41/Dd, GP34–41/Kb, GP276–286/Dd, and nucleoprotein (NP)-derived NP396–404/Dd, as well as the subdominant epitope NP205–212/Kb (21–23). Our results indicate that bortezomib reduces class-I presentation of virus-derived peptides, and thereby suppresses the cytotoxic T cell response, leading to augmented viral titers.

Materials and Methods

Mice, viruses, and medium

C57BL/6 mice (H-2b) were originally purchased from Charles River Laboratories. Mice were kept in a specific pathogen-free facility and used at 6–10 wk of age. Animal experiments were approved by the review board of Regierungspräsidium Freiburg. LCMV-WE was originally obtained from F. Lehmann-Grube (Heinrich-Pette-Institute, Hamburg, Germany) and propagated on the fibroblast line L929. LCMV was titered on MC57 cells as previously described (24). Mice were infected with 200 PFU LCMV-WE i.v. All medium were purchased from Invitrogen Life Technologies and contained GlutaMAX, 10% FCS, and 100U/ml penicillin/streptomycin.

Synthetic peptides

The synthetic peptides GP33–41 (KAVYNFATC), GP276–286 (SGVENPQYCL), NP396–404 (FQPQNGQFI), NP205–212 (YTVKYPNL), and GP35–40 (GLNGPDIYKVGGYFKSVEFD) were obtained from P. Henklein (Charité, Berlin, Germany).
Proteasome inhibitor

PS-341 (bortezomib, or Velcade; Millennium Pharmaceuticals) was purchased from a local pharmacy. The proteasome inhibitor was dissolved in DMSO at 1 mg/ml and stored at −80°C. For further applications, the inhibitor was diluted in medium or PBS. For animal experiments, PS-341 was administered to mice i.p. at 0.75 mg/kg in PBS.

Flow cytometry

Splenocytes from LCMV-infected C57BL/6 mice were incubated for 30 min with anti-CD4 (BD Biosciences), anti-CD8 (BD Biosciences), or anti-CD90.2 (Thy1.2)-coated microbeads (Miltenyi Biotec). Cells (4 × 10^6/well) were stimulated with plate-bound anti-CD3 (4 μg/ml, clone 145–2C11) and anti-CD28 (4 μg/ml, clone 37.51; BD Biosciences) in the presence of various PS-341 concentrations. Twenty-four hours later, dead cells were stained with propidium iodide (BD Biosciences) and analyzed by flow cytometry.

Intracellular cytokine staining (ICS) for IFN-γ

Analysis of T cell responses was performed as previously detailed (25). In brief, splenocytes were incubated in round-bottom 96-well plates with 10^6 M or 10^−9 M of the specific peptide in 100 μl IMDM 10% fetal calf serum. The staining, fixation, and permeabilization of the cells were performed exactly as detailed previously (26).

LacZ assay

MC57 (C57BL/6-derived methylcholanthrene-induced fibrosarcoma cell line) cells were infected with LCMV-WE (M.O.I. of 0.05). An acid wash procedure was performed to remove pre-existing peptide/MHC complexes from the surface. Cells were resuspended in acid wash buffer (131 mM citric acid and 66 mM disodium phosphate (pH 3.1)) for 3 min. Cells were washed three times with ice-cold medium. Removal of MHC was confirmed by staining for H-2D^d and analysis by flow cytometry. Cells were incubated for 6 h with indicated concentrations of PS-341. Cells were washed twice and incubated with GP276-specific T cell-hybridomas (18). Five × 10^4 T cell-hybridomas were cocultured overnight with 5 × 10^5 stimulator cells in 96-well plates overnight. The lacZ-based color reaction was performed and measured as detailed elsewhere (18).

Peptide-specific CTL-lines

LCMV-specific CTL-lines were generated exactly as previously described (19). An additional density centrifugation step was conducted 1–2 days before using CTL in Ag presentation experiments. CTL were used in ICS at an E:S ratio of 0.2 in the first dilution, and serial 3-fold dilution of stimulators were performed.

Statistical analysis

Statistical significance of differences was determined using Student’s t test. Differences were considered significant when p < 0.05.

Results

Bortezomib reduces Ag presentation in vitro

The proteasome plays a critical role in the generation of peptides from intracellular Ags that are presented to CTL by the class-I MHC (10). To investigate whether the proteasome inhibitor bortezomib is able to alter Ag presentation in vitro, the fibroblast line MC57 was infected with LCMV-WE for 24 h. Subsequently, the pre-existing epitopes from class-I molecules were removed by acid wash procedure and incubated for 8 h with bortezomib concentrations ranging from 12.5 to 2000 nM. The amount of LCMV-derived GP276–286 presented on MHC-I was detected by the help of GP276–286-specific T cell-hybridomas in lacZ assays (Fig. 1).

Increasing inhibitor concentrations led to a reduced GP276–286 presentation in a concentration-dependent manner, confirming the proteasome dependency of GP276–286 of an earlier study using epoxomicin and lactacystin to investigate Ag presentation (18). Hence, bortezomib is capable of inhibiting Ag presentation in vitro.

Reduced cytotoxic T cell response in bortezomib-treated mice

To analyze the impact of PS-341 on the cytotoxic T cell response, we used the well-characterized LCMV as model system for viral infection. C57BL/6 mice were infected with LCMV-WE (day 0) and treated three times with bortezomib (days −1, 1, and 3). Starting dose of bortezomib to treat multiple myeloma patients is currently 1.3 mg/m², which leads to an 80–80% inhibition of the proteasome’s activity in the blood 1 h post treatment (27, 28). To achieve a comparable inhibition in mice, we used a PS-341 dose of 0.75 mg/kg, which inhibits the proteasome to ~80% (29). Eight days post LCMV-WE infection, the cytotoxic T cell response in LCMV-infected PS-341-treated and untreated mice was assayed for their responses to four LCMV-derived epitopes by ICS for IFN-γ (Fig. 2A). CTL-responses to all epitopes were significantly reduced to ~50%. To control whether the LCMV-WE-specific Th cell response was similarly affected by bortezomib, GP61–80-specific CD4+ T cells were analyzed by ICS for IFN-γ (Fig. 2B). In contrast to the LCMV-specific CTL-response, no significant difference could be observed for the CD4 T cell epitope GP61–80. LCMV induces a massive cytotoxic T cell proliferation leading to huge numbers of LCMV-specific CTLs (30, 31). To investigate whether bortezomib treatment affects the LCMV-induced T cell expansion, CD4+ and CD8+ lymphocytes were analyzed in spleen by flow cytometry (Fig. 3). PS-341 led to a 30% reduction (from 45 to 30%) of the percentage of CD8+ of the total lymphocyte population, whereas CD4+ lymphocytes were even slightly increased. Taken together, bortezomib administration impairs the LCMV-WE specific cytotoxic T cell response.

Impaired viral clearance in bortezomib-treated mice

Viral load in LCMV-WE infected mice, peaking at day 4 post infection, is controlled by the CTLs response, leading to barely detectable virus titers on day 8 post infection (32). To investigate whether the impaired T cell response in inhibitor treated mice manifests in increased viral load, C57BL/6 mice were infected with LCMV-WE (day 0) and treated three times with bortezomib
(days −1, 1, and 3). On day 6 post infection, virus titers in spleen of LCMV-infected PS-341-treated and untreated mice were assayed (Fig. 4). Virus load in spleen was increased by ~10 times in PS-341-treated mice.

Bortezomib treatment during expansion phase is not affecting LCMV-specific CTL-response

Priming of LCMV-specific T cells takes place during the first days of infection leading to detectable LCMV-specific CTLs on day 5 post infection, followed by a massive expansion, which leads to an apex in the number of LCMV-specific T cells at day 8 post infection (31, 32). To investigate whether massively proliferating T cells are sensitive to bortezomib in vivo, mice were infected with LCMV-WE (day 0) and treated with PS-341 on days 4 and 6. The cytotoxic T cell response was assayed on day 8 post infection for responses to four defined LCMV-derived epitopes by intracellular cytokine staining for IFN-γ (Fig. 5). In contrast to the administration of PS-341 during the priming phase (Fig. 2), the cytotoxic T cell response was not reduced in this experimental setup. Unexpectedly, the T cell response was even slightly increased to all epitopes in PS-341-treated mice. Hence, administration of bortezomib does not hamper the expansion of CTLs during LCMV-WE infection if treatment commences after completion of the priming phase.

Reduced Ag presentation in PS-341-treated mice

Apart from impeded T cell activation of PS-341 treated T lymphocytes, proteasome inhibition in bortezomib-treated mice might negatively affect Ag presentation in vivo. The number of peptide-MHC-I-complexes required to optimally activate naive CD8+ T cells is increased in PS-341-treated mice.
cells can greatly limit the immunogenicity of such complexes (34, 35). Hence, administration of bortezomib during infection might inhibit the proteasome in APCs and thereby reduce the peptide supply to class-I complexes, which limits the activation of naive T cells. Numerous LCMV-derived class-I ligands are processed in a proteasome-dependent manner (17–19). As shown in Fig. 1, in vitro experiments demonstrated that PS-341 could reduce presentation of an LCMV-derived peptide. To determine the amount of Ag presented directly in vivo in bortezomib treated mice, C57BL/6 mice were infected with LCMV-WE (day 0) and treated with bortezomib on day 1 and 3. The status of GP33-41 and NP396-404 presentation was analyzed in the spleen on day 4 after infection. Splenocytes from bortezomib treated or untreated mice were used as stimulators for IFN-γ/H9253 production (ICS) by in vitro generated mono-specific CTL-lines specific for GP33-41 or NP396-404 (Fig. 7). To exclude that activated TCD8+ in infected spleens falsify the result, an intracellular IFN-γ staining (ICS) with infected splenocytes but without mono-specific CTLs was performed. No IFN-γ-producing CTLs were detected on day 4 after LCMV-WE infection in these spleens, thus indicating that they did not contribute to the number of IFN-γ+ cells (data not shown). As shown in Fig. 7, GP33-41 and NP396-404 are presented differently in spleens on day 4 post-LCMV-WE infection in treated compared with untreated mice, whereas splenocytes derived from uninfected mice were not able to activate mono-specific CTLs. APCs derived from PS-341 treated mice are hampered to produce and present GP33-41 and NP396-404 in similar amounts as splenocytes derived from untreated mice. Hence, the reduced T cell response in bortezomib treated mice seen in Fig. 2, is probably due to a reduced Ag presentation in proteasome inhibited APCs, which leads to an impaired activation of naive CTL.

Discussion
Bortezomib is a proteasome inhibitor with significant clinical activity in multiple myeloma and mantle cell myeloma. In this study, as stimulators for IFN-γ production (ICS) by in vitro generated mono-specific CTL-lines specific for GP33-41 or NP396-404 (Fig. 7). To exclude that activated TCD8+ in infected spleens falsify the result, an intracellular IFN-γ staining (ICS) with infected splenocytes but without mono-specific CTLs was performed. No IFN-γ-producing CTLs were detected on day 4 after LCMV-WE infection in these spleens, thus indicating that they did not contribute to the number of IFN-γ+ cells (data not shown). As shown in Fig. 7, GP33-41 and NP396-404 are presented differently in spleens on day 4 post-LCMV-WE infection in treated compared with untreated mice, whereas splenocytes derived from uninfected mice were not able to activate mono-specific CTLs. APCs derived from PS-341 treated mice are hampered to produce and present GP33-41 and NP396-404 in similar amounts as splenocytes derived from untreated mice. Hence, the reduced T cell response in bortezomib treated mice seen in Fig. 2, is probably due to a reduced Ag presentation in proteasome inhibited APCs, which leads to an impaired activation of naive CTL.

Discussion
Bortezomib is a proteasome inhibitor with significant clinical activity in multiple myeloma and mantle cell myeloma. In this study,
we investigated the impact of bortezomib on a virus-induced cytotoxic T cell response. CTL-responses to four different LCMV-derived class-I epitopes were markedly reduced in bortezomib-treated mice (Fig. 2A), whereas the CD4+ response to the class II epitope GP61-80 was not altered. Associated with the impaired CTL response was the reduced number of CD8+ T cells in spleen (Fig. 3). It has been reported that bortezomib, apart from direct anti-tumor activity, is also immunomodulatory with effects on the survival and function of lymphocytes and dendritic cells (DC) (36, 37). Exposure to the proteasome inhibitor bortezomib was found to promote apoptosis of human monocyte-derived DCs and to reduce the yield of viable DCs when given to monocytes early during differentiation to DCs (36, 38). Proteasome inhibition in mice drastically but reversibly impairs murine T lymphocyte development in the thymus (39). Additionally, bortezomib induces selective apoptosis and decreases Th1 responses among alloreactive T lymphocytes while barely affecting unstimulated T cells (33). The reduced T cell response (Fig. 2 and 3) manifested in increased viral load at day 6 post infection in bortezomib-treated mice (Fig. 4). It has been published that PS-341 can directly influence viral replication in vitro (40, 41). Whereas poliovirus replication was less sensitive to proteasome inhibition, bortezomib abrogated vesicular stomatitis virus replication (VSV). Proteasome targeting suppressed VSV protein synthesis and protected infected cells from toxic effect of VSV replication (40). Similar findings were observed for orthopoxviruses (41), where bortezomib treatment of cells had a marked effect on viral titers, severely blocking viral replication and propagation. In contrast, no diminished replication could be observed in vivo in our study with LCMV, where increased viral titers were observed most likely due to a restrained cytotoxic T cell response (Fig. 4).

Activation of LCMV-specific CTLs is followed by a massive expansion of primed T cells (31, 32). When bortezomib was applied to mice during this expansion phase, no reduction in the CTL-activity could be observed (Fig. 5), suggesting that bortezomib is not affecting proliferating T cells. Additionally, proliferation of adoptively transferred CFSE-labeled THY1.2+ P14 (TCR transgenic for the LCMV MHC-I epitope GP33-41) cells into PS-341 treated mice was not changed compared with untreated mice (data not shown). Hence, how is proteasome inhibition during the priming of T cells altering the immune response? Either T cells are directly affected by PS-341 or the priming of T cells by pAPC is impaired. In vitro, activation of T cells by plate bound CD3/CD28 is hampered by bortezomib (Fig. 6A), probably due to inhibition of the NF-κB pathway (33). In contrast to in vitro, the early activation marker CD69 expression on T cells was not altered in vivo (Fig. 6B). It has been observed, that the immunostimulatory capacity of bortezomib-treated human myeloid blood DC is significantly impaired (42). Apart from a reduced stimulation capacity of DC, the impact of Ag presentation on bortezomib-treated pAPC has not been investigated so far. The proteasome is the major protease in producing ligands for class-I presentation (43). Therefore, it seems obvious that proteasome inhibition alters Ag presentation.

In summary, the present study suggests a higher susceptibility of bortezomib-treated patients to viral infections. Indeed, several publications report on an increased incidence of varicella herpes zoster (46–49). Analysis of herpes zoster events among bortezomib-treated patients in the phase III APEX study revealed a significantly higher incidence of herpes zoster compared with dexamethasone treatment (49). During primary infection with the neurotropic human varicella zoster virus (VZV), viral replication in the skin or the respiratory mucosa is followed by a lifelong latent infection of neurons within sensory ganglia. VZV-specific T cells are important in controlling infection. CD8+ T cells that recognize the VZV encoded I662, which is critical for VZV replication, are present in immune individuals (50). In case such CD8+ T cells are involved in controlling VZV reactivation from latency, proteasome inhibition might reduce class-I presentation of VZV-derived peptides and thereby promote viral escape from the immune system.

Disclosures
The authors have no financial conflict of interest.

References
BORTezomib INCREASES VIRTUAL SUSCEPTIBILITY


