Anti-Inflammatory Effect of the Proteasome Inhibitor Bortezomib on Endotoxin-Induced Uveitis in Rats

Fang-Ting Chen,1,2 Yi-Chun Liu,2,3 Chung-May Yang,2,4 and Chang-Hao Yang2,4

PURPOSE. We evaluated the anti-inflammatory effect of bortezomib (Velcade), a proteasome inhibitor, on endotoxin-induced uveitis (EIU) in rats and lipopolysaccharide (LPS)-stimulated RAW 264.7 cells.

METHODS. EIU was induced by footpad injection of LPS into Lewis rats. MG-132 (10 mg/kg), or high-dose (0.2 mg/kg) or low-dose (0.05 mg/kg) bortezomib was given 30 minutes before LPS injection in each treatment group. The rats were sacrificed 24 hours later to observe the inflammatory response in tissues. The expression levels of fractalkine, MCP-1, ICAM-1, and iNOS were evaluated by PCR and Western blot analysis. Immunohistochemical (IHC) studies were used to demonstrate the expression of pro-inflammatory mediators and nuclear factor-kappa B (NF-κB) p65 in the iris and ciliary body. The DNA-binding activity of NF-κB was evaluated using an electrophoretic mobility shift assay (EMSA). An in vitro study using RAW 264.7 cells was performed to verify the results.

RESULTS. Pretreatment with high-dose bortezomib significantly attenuated the inflammatory response of EIU. Reduced expression of inflammatory mediators always was observed in the high-dose bortezomib and MG-132 groups, but invariably was not noted in the low-dose bortezomib group. Decreased DNA-binding activity of NF-κB was noted in those rats pretreated with high-dose bortezomib or MG-132. In vitro study demonstrated the dose-dependent anti-inflammatory effects of bortezomib in LPS-stimulated RAW cells, consistent with the results obtained in vivo.

CONCLUSIONS. Bortezomib inhibits EIU, probably by inhibiting the activation of NF-κB, which in turn, down-regulates the expression of the associated inflammatory genes. Proteasome inhibition may be a potential treatment strategy for uveitis. (Invest Ophthalmol Vis Sci. 2012;53:3682–3694) DOI: 10.1167/iovs.12-9505

Uveitis refers to inflammation of the uveal tissues and can cause significant ocular morbidities in individuals with protracted diseases.1 Autoimmune dysregulation, infection triggers, and genetic susceptibility all have been implicated as possible causes; however, the exact etiology is unknown in many conditions.2,3 Clinical courses refractory to conventional treatment with corticosteroids are not uncommon, and the adverse effects associated with steroids, such as cataracts and glaucoma, preclude its chronic use. Therefore, investigating the disease mechanisms and searching for alternative treatments for uveitis remain important issues.

Endotoxin-induced uveitis (EIU) in rats is considered to be a suitable animal model for studying human disease, because there may be an association between the cell wall products of some gram-negative bacteria and human leukocyte antigen (HLA)-B27-positive uveitis.4,5 After injecting lipopolysaccharide (LPS) through the footpad intraperitoneally, intravenously, or intravitreally, the breakdown of the blood-aqueous barrier bilaterally, and polymophonuclear leukocyte and mononuclear cell infiltration in the iris and ciliary body, and in the anterior chamber are noted within 24 hours. The intraocular inflammation subsides after 96 hours. Nuclear factor-kappa B (NF-κB) likely has a pivotal role in mediating uveal inflammation. Several experimental studies have noted that there is increased expression of activated NF-κB associated with EIU, and that the inhibition of NF-κB can halt inflammation.7–11 NF-κB is an ubiquitous transcription factor, and it regulates the transcription of a wide range of genes responsible for inflammation, such as cytokines, chemokines, cell-adhesion molecules, and stress-response proteins.12 Several inflammatory mediators, including interleukin (IL)-1, IL-6, tumor necrosis factor (TNF)-α, interferon (IFN)-γ, monocyte chemoattractant protein (MCP)-1, intercellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM)-1, inducible nitric oxide synthase (iNOS), and cyclooxygenase (COX)-2, are increased in animals with EIU and seem to be up-regulated by NF-κB.7–10,12–14–17

The ubiquitin-proteasome system is the major non-lysosomal system for intracellular protein degradation and is crucial in maintaining basic cellular functions, such as cell cycle progression and apoptosis. This system also is involved in many pathologic conditions, including cancer, ischemia-reperfusion injury, autoimmune disease, inflammation, and allograft rejection.18,19 In the canonical NF-κB pathway, the degradation of ubiquitinated IκB, the inhibitory factor for NF-κB, by the proteasome is necessary for the activation of NF-κB.20,21 The proteasome has been demonstrated to be important in regulating several LPS-responsive signaling pathways in macrophages, including NF-κB signaling.22 LPS binds to specific proteasome subunits and enhances its peptidase activity in vitro, and the use of proteasome inhibitors has been suggested as a potential treatment strategy for gram-negative sepsis.23,24 Clinically, proteasome inhibitors have been used in the treatment of hematologic cancers in humans, with tolerable side effects.25,26 The drugs also have been shown to be beneficial in animal models of experimental autoimmune disease, such as myasthenia gravis, psoriasis, arthritis, and autoimmune encephalomyelitis.27–30 The anti-inflammatory effect of proteasome inhibition may be attri-
Figure 1. (A) Quantification of infiltrating cells in the AqH. EIU rats pretreated with MG-132 or high-dose bortezomib (Vel H) showed significantly reduced cell numbers in the AqH. Pretreatment with low-dose bortezomib (Vel L) did not result in a significant difference in the number of cells in the AqH relative to no treatment. The AqH was pooled from one eye of five rats in each group. Data are expressed as the mean ± SD of three independent experiments (bar graph). *P < 0.05 compared to the LPS group. (B) Histological evaluation of cellular infiltration in the ICB. EIU rats showed increased leukocyte infiltration in the ICB and AqH, and the inflammation was attenuated by pretreatment with MG-132, or high-dose or low-dose bortezomib. Different eyes were used for assessing the histological changes in ICB and the leukocyte numbers in AqH. These images are representative of three rats in each group, and there was little disease variation between eyes in the animals within the same group. Original magnification 200×.
borted largely to the inhibition of NF-κB activation; however, the exact mechanisms still are unclear. In addition, little is known about the efficacy of proteasome inhibition in treating uveitis. We investigated the anti-inflammatory effect of bortezomib, a 26S proteasome inhibitor, in EIU rats and in LPS-stimulated RAW 264.7 cells. We demonstrated the inhibition of LPS-induced inflammation by bortezomib in vivo and in vitro.

**Figure 2.** The evaluation of mRNA expression of inflammatory mediators in the ICB by semi-quantitative PCR. Decreased expression of fractalkine (A), MCP-1 (B), ICAM-1 (C), and iNOS (D) relative to the expression in the LPS group was noted in the MG-132 and high-dose bortezomib (Vel [H]) groups but not in the low-dose bortezomib (Vel [L]) group. The Y scale represents the ratio of inflammatory mediator RNA to β-actin mRNA in each group. The sample was pooled from one eye of five rats in each group. Data are expressed as the mean ± SD of three independent experiments (bar graph). *P < 0.05 compared to the LPS group.
FiguRe 3. (3-1) Evaluation of the expression of pro-inflammatory proteins in EIU with Western blot analysis. Significantly decreased expression of fractalkine (A), MCP-1 (B), ICAM-1 (C), and iNOS (D) was noted in the MG-132, and high-dose (Vel [H]) and low-dose (Vel [L]) bortezomib groups compared to the LPS group. The Y scale represents the ratio of inflammatory mediator blot density to the β-actin blot density in each group. The sample was pooled from one eye of five rats in each group. Data are expressed as the mean ± SD of three independent experiments (bar graph). *P < 0.05 compared to the LPS group. (3-2) Evaluation of the sequential change of NF-κB p65 with Western blot analysis (n = 5 at each time point). The level of NF-κB p65 significantly increased after 12 hours of LPS stimulation. In contrast, there was no significant change in p65 levels in rats.
METHODS

Animal Preparation and Grouping

For the study, we used male Lewis rats that were 8 weeks old and weighed 200–250 g. All experiments were performed in compliance with a protocol approved by the Institutional Animal Care and Use Committee of National Taiwan University, and with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

For this study, 300 μg LPS from Salmonella typhimurium (Sigma Chemical, St. Louis, MO) in 0.1 mL sterile saline was injected through one footpad in each rat to induce EIU. The rats were injected with the drugs or vehicle intraperitoneally 30 minutes before the LPS administration. The rats were divided randomly into four groups (n = 20 in each group): (1) the LPS group (vehicle group), injected with 0.1 mL PBS before the induction of EIU; (2) the MG-132 group, injected with MG-132 (10 mg/kg, Sigma) before the induction of EIU; (3) the Velcade (H) group, injected with a high dose of bortezomib (0.2 mg/kg; Millennium Pharmaceuticals, Cambridge, MA) before the induction of EIU, and (4) the Velcade (L) group: injected a low dose of bortezomib (0.05 mg/kg) before the induction of EIU.

Most rats were euthanized at 24 hours after LPS injection. A small population of rats in the LPS and Velcade (H) groups were sacrificed earlier (at 3, 6, and 12 hours), and total protein in the ICB was extracted to determine the sequential change of NF-κB p65 levels. Under deep anesthesia, the animals were sacrificed by the intracardiac injection of phenobarbital. The eyeballs were extracted immediately and processed for further evaluation.

Histological Evaluation of the Anterior Segment and Quantification of the Infiltrating Cells in the Aqueous Humor (AqH)

AqH (2 μL) was obtained from each rat by anterior chamber paracentesis with a 30-gauge needle. The sample was stained with 0.4% trypan blue and then was observed under phase-contrast microscopy to calculate the number of leukocytes.

The eyeballs were enucleated and were immersed in 4% paraformaldehyde in 0.2 M phosphate buffer for 24 hours. After fixation, the eyes were dehydrated with alcohol and then embedded in paraffin. The specimens were cut into 5-μm sagittal sections near the optic nerve head, and were stained with hematoxylin and eosin (H&E) to evaluate the cellular infiltration in the iris and ciliary body (ICB).

Semi-Quantitative PCR

Total RNA was extracted from the ICB with TRIzol reagent (Invitrogen-Life Technologies, Inc., Gaithersburg, MD). Then, 1 μg of total RNA from each sample was annealed with 300 ng oligo(dT) (Promega, Madison, WI) for 5 minutes at 65°C, and reverse-transcribed to cDNA by using 80 U Moloney murine leukemia virus reverse transcriptase (MMLV-RT; Invitrogen-Gibco, Grand Island, NY) per 50-μL sample for 1 hour at 37°C. The reaction was stopped by increasing the temperature

**Figure 4.** Quantification of the levels of chemokines and NO in the AqH. Decreased expression of fractalkine (A), MCP-1 (B) and NO (C) was noted in the high-dose bortezomib (Vel [H]) group compared to the levels in the LPS group. In the MG-132 group, the levels of fractalkine and NO were reduced, but the MCP-1 concentration did not differ significantly in comparison with that of the LPS group. The levels of fractalkine, MCP-1, and NO were not decreased in those rats pretreated with low-dose bortezomib (Vel [L]). The AqH was pooled from one eye of five rats in each group. Data are expressed as the mean ± SD of three independent experiments (bar graph). *P < 0.05 compared to the LPS group.
to 90°C for 5 minutes. The resultant cDNA from each sample was subjected to PCR with specific primers (see Table). The 50 μL reaction mixture contained 5 μL cDNA, 1 μL sense and antisense primers, 200 μM of each deoxynucleotide, 5 μL of 10× Taq polymerase buffer, and 1.25 U Taq polymerase (Promega). Amplification was performed in a thermocycler (MJ Research, Waltham, MA) with a 1-minute denaturation at 94°C and a 3-minute extension at 72°C. The annealing temperature was between 62°C and 42°C, and the temperature was decreased in 1°C increments, followed by 21 cycles at 55°C. Finally, the temperature was elevated to 72°C for 10 minutes and then reduced to 4°C. We obtained a 10-μL sample of each PCR product to perform gel electrophoresis on a 2% agarose gel containing ethidium bromide (Sigma-Aldrich, St. Louis, MO), and the results were analyzed under ultraviolet light using DNA molecular length markers. The intensity was determined using image analysis software (Photoshop, version 7.0; Adobe Systems, San Jose, CA), and the results were standardized against the intensity of rat β-actin, a housekeeping gene. All experiments were repeated three times and yielded similar results.

**Western Blot Analysis**

Total protein was extracted from the ICB by lysing the sample in radioimmunoprecipitation assay (RIPA) buffer (0.5 M Tris-HCl [pH 7.4], 1.5 M NaCl, 2.5% deoxycholic acid, 10% NP-40, 10 mM EDTA and protease inhibitors [Complete Mini; Roche Diagnostics Corp., Indianapolis, IN]). The extract and Laemmli buffer were mixed at a 1:1 ratio, and the mixture was boiled for 5 minutes. Samples (100 μg) were separated on 10% SDS-polyacrylamide gels and then transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore Corp., Billerica, MA). The membranes were incubated with anti-fractalkine, anti-MCP-1, anti-ICAM-1, anti-iNOS, anti-NF-κB p65, and anti-β-actin antibodies. Then, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody and visualized by chemiluminescence (GE Healthcare, Buckinghamshire, UK). The density of blots was quantified using image-analysis software after scanning the image (Photoshop, version 7.0; Adobe Systems). The

![Figure 5. The evaluation of the expression levels of chemokines, ICAM-1, and iNOS in the ICB using IHC. The increased expression of fractalkine, MCP-1, ICAM-1, and iNOS was noted in the LPS group relative to the expression in the normal rats. Decreased expression of these inflammatory mediators was noted in EIU rats pretreated with MG-132 or a high dose of bortezomib (Vel [H]), but not in those treated with a low dose of bortezomib (Vel [L]). These images are representative of three rats in each group. There was little variation between eyes in the same group. Original magnification 200×.](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932981/)
Quantification of the Levels of Inflammatory Mediators and Nitric Oxide (NO) in AqH

The concentrations of fractalkine, MCP-1, and NO were determined using commercial ELISA kits (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. The samples were diluted to 50 μL for testing, and the optical density was determined at A450 (absorbance at 450 nm) with a microplate reader (Bio-Rad).

Immunohistochemical (IHC) Studies

The sections were obtained from the same paraffin blocks as used for histological evaluation. After deparaffinization with xylene solutions and rehydration with a graded series of ethanol in PBS, 0.3% hydrogen peroxidase was added to block the intrinsic peroxidase activity. The specimens were rinsed with 5% normal rat serum, and were incubated overnight with monoclonal antibodies against fractalkine, MCP-1, ICAM-1, iNOS (Santa Cruz Biotechnology, Santa Cruz, CA) and the p65 subunit of NF-κB (Chemicon, Temecula, CA) separately at 4°C. Then, a biotinylated secondary antibody against mouse IgG and an avidin-biotinylated peroxidase complex (Santa Cruz Biotechnology) were applied with 3,3′-diaminobenzidine as a peroxidase substrate. The sections were counterstained with hematoxylin, and then dehydrated and embedded. Specimens stained without the primary antibody were used as negative controls.

Nuclear Protein Extract and Electrophoretic Mobility Shift Assay of NF-κB (EMSA)

The ICB was minced in 0.5 mL of ice-cold buffer A containing 10 mM HEPES (pH 7.9), 1.5 mM KCl, 10 mM MgCl2, 1.0 mM dithiothreitol (DTT), and 1.0 mM phenylmethylsulfonyl fluoride (PMSF). The tissue was homogenized (Dounce; Bellco Glass Co., Vineland, NJ), followed by centrifugation at 5000 g at 4°C for 10 minutes. The sediment was suspended in 200 μL of buffer B containing 20 mM HEPES (pH 7.9), 25% glycerol, 1.5 mM MgCl2, 420 mM NaCl, 0.5 mM DTT, 0.2 mM EDTA, 0.5 mM PMSF, and 4 μM leupeptin, and the suspension was incubated on ice for 30 minutes. Then, the sample was centrifuged at
**Figure 7.** Evaluation of the IκB expression in LPS-stimulated RAW 264.7 cells by Western blot analysis. Reduced expression of IκB was noted in LPS-stimulated RAW cells relative to the expression in the normal state. Pretreatment with 10 nM bortezomib (Vel (L)) induced no significant effects, whereas pretreatment with 25 nM bortezomib (Vel (H)) increased the expression of IκB significantly relative to no treatment. However, the expression of IκB was decreased significantly relative to that in the normal state despite treatment with high-dose bortezomib. The Y scale represents the ratio of the IκB blot density to the β-actin blot density in each group. Data are expressed as the mean ± SD of three independent experiments (bar graph). *P < 0.05 compared to the normal state. #P < 0.05 compared to the LPS group. &P < 0.05.

12,000 g at 4°C for 30 minutes. The supernatant containing the nuclear proteins was collected and stored at −70°C until use. The protein concentration was determined with a bichinchoninic acid assay kit with BSA as the standard (Pierce Biotechnology, Rockford, IL). The EMSA was performed with an NF-κB DNA-binding protein-detection system (Pierce Biotechnology) according to the manufacturer’s instructions. A 10 μg nuclear protein aliquot was incubated with a biotin-labeled NF-κB consensus oligonucleotide probe (5'-AGTGGAGGGGACTTTCC-CAGGC-3') for 30 minutes in binding buffer. The specificity of the DNA/protein binding was determined by adding a 100-fold molar excess of unlabeled NF-κB oligonucleotide for competitive binding 10 minutes before adding the biotin-labeled probe.

RAW 264.7 Cell Culture and Evaluation of the Expression of Inflammatory Mediators

Mouse macrophage-like RAW 264.7 cells, purchased from the American Type Culture Collection (Manassas, VA), were cultured in RPMI-1640 medium at 37.8°C in a humidified incubator containing 5% CO₂. RAW cells were pretreated with PBS or different concentrations of bortezomib (10 nM and 25 nM) for 2 hours, and then stimulated with 10 ng/mL of LPS from Salmonella typhimurium (Sigma Chemical) for 30 minutes. Then, we extracted the cellular proteins with Laemmlı buffer, and the expression of IκB was determined by Western blot analysis.

Some cultured cells were maintained for 48 hours after LPS stimulation, and the levels of TNF-α, MCP-1, and NO in the culture supernatants were measured using ELISA kits (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions.

**Analysis of Apoptosis in RAW 264.7 Cells by Flow Cytometry**

The proportion of RAW cells undergoing apoptosis was determined at 48 hours by flow cytometry using annexin V and propidium iodide (PI) staining. The staining solution contained 5 μL annexin-V-FTTC in 250 μL binding buffer and 5 μL PI (Strong Biotech, Taipei, Taiwan). The 10⁶ cells were washed with PBS and centrifuged at 200 g for 5 minutes. Then, the cell pellet was resuspended in 100 μL of staining solution and incubated for 10 minutes at 20°C. Finally, the sample was added with 900 μL binding buffer and analyzed on FACScan cytometer (BD Bioscience, San Jose, CA).

**Analysis of Cell Apoptosis in ICB by In Situ TUNEL**

Eyeballs were harvested 24 hours after LPS injection and were cut into 5-μm sagittal sections near the optic nerve head to evaluate the apoptosis of cells in the ICB. The sections were stained by TUNEL-based kits (TdT FragEL; Oncogene, Darmstadt, Germany).

**Statistical Analysis**

Values are shown as the mean ± SD. The Mann-Whitney U-test was used for pairwise comparisons of the means of two independent groups. To compare data among three or more groups, one-way ANOVA followed by the Bonferroni test was used. P < 0.05 was considered statistically significant.

**RESULTS**

**Effects of Bortezomib on Cellular Infiltration in AqH and Histological Changes in ICB**

In the LPS group, the number of infiltrating cells in AqH was 125.5 ± 26.2 (10⁴/mL). The number of cells in the low-dose bortezomib group, although lower, was not significantly different from that in the LPS group (P = 0.083). In contrast, the number of cells was significantly lower in rats pretreated with MG-132 and high-dose bortezomib than in rats in the LPS group (P < 0.003 and 0.002, respectively, Fig. 1A).

Histologically, increased leukocyte infiltration in the AqH and ICB was noted after LPS injection. In addition, tissue swelling was noted in the ICB of LPS-stimulated rats. Only a few leukocytes were noted in the MG-132, low-dose bortezomib, and high-dose bortezomib groups, and there was no tissue swelling in these groups (Fig. 1B).

**Influence of Bortezomib on the mRNA Expression of Inflammatory Mediators in ICB**

The mRNA expression levels of fractalkine, MCP-1, ICAM-1, and iNOS determined by semi-quantitative PCR were reduced significantly in rats pretreated with MG-132 or high-dose bortezomib relative to the LPS group (P < 0.05 in all paired comparisons). The levels of these inflammatory mediators did not differ significantly between the LPS and low-dose bortezomib groups (Fig. 2).

**Western Blot Analysis for the Expression of Inflammatory Mediators and NF-κB**

Western blot analysis showed that the expression levels of fractalkine, MCP-1, ICAM-1, and iNOS were reduced significantly by the pretreatment with MG-132, or low-dose or high-
The concentrations of inflammatory mediators in the supernatant of RAW cell cultures as measured by ELISA. After LPS stimulation, the levels of TNF-α (A), MCP-1 (B), and NO (C) were increased relative to the levels in the normal state. Pretreatment with 10 nM (Vel [10]) and 25 nM (Vel [25]) bortezomib reduced the expression of these mediators significantly in LPS-stimulated RAW cells, especially those cells treated with 25 nM bortezomib (Vel [25]). Data are expressed as the mean ± SD of three independent experiments (bar graphs). *P < 0.05 compared to the normal group. #P < 0.05 compared with the LPS group. &P < 0.05. (8-2) Bortezomib inhibited the production of inflammatory mediators in LPS-stimulated RAW cells in addition to its pro-apoptotic effect on these cells. The reduction of cytokine level was greater than the decrease of viable cell numbers in every treated group. The solid line represents the ratio of cytokine levels in the treated group to the control group, and the dash line represents the ratio of cell numbers in the treated group to the control group.
Pretreatment with low-dose bortezomib did not decrease the expression of the p65 subunit of NF-κB, as determined by IHC. In contrast, MG-132 and high-dose bortezomib greatly reduced the expression of p65, indicating that the activation of NF-κB was suppressed (Fig. 6A).

The NF-κB/DNA binding activity was proportional to the density of the specific band on the EMSA gel. The increased activity of NF-κB/DNA binding after LPS stimulation was inhibited markedly by pretreatment with either MG-132 or high-dose bortezomib, but not by pretreatment with low-dose bortezomib. Adding a 100-fold molar excess of unlabeled NF-κB probe completely hindered the binding of the labeled probe to the NF-κB/DNA complex (Fig. 6B).

The level of NO was decreased significantly in the MG-132 and high-dose bortezomib groups (P < 0.001). Pretreatment with 10 nM bortezomib induced no obvious effect on iκB expression in LPS-stimulated RAW cells (P = 0.879), while pretreatment with 25 nM bortezomib increased the expression of iκB significantly in comparison with untreated cells (P = 0.013, Fig. 7).

An ELISA was used to measure the concentrations of inflammatory mediators, and showed increased expression of TNF-α, MCP-1, and NO after LPS stimulation relative to the expression in the normal state (P < 0.001). Pretreatment with bortezomib, either 10 or 25 nM, significantly reduced the levels of these mediators, especially in cells treated with 25 nM bortezomib (Fig. 8A). Taking the viable cell numbers at the end into account, these inhibitory effects still were prominent after normalization with cell numbers (Fig. 8B).

Pretreatment with 10 nM bortezomib did not decrease the expression of the p65 subunit of NF-κB, which is available for specific antibody binding only after dissociation from IκB, was increased after LPS stimulation. Pretreatment with low-dose bortezomib did not decrease the expression of the p65 subunit of NF-κB, as determined by IHC. In contrast, MG-132 and high-dose bortezomib greatly reduced the expression of p65, indicating that the activation of NF-κB was suppressed (Fig. 6A).

The NF-κB/DNA binding activity was proportional to the density of the specific band on the EMSA gel. The increased activity of NF-κB/DNA binding after LPS stimulation was inhibited markedly by pretreatment with either MG-132 or high-dose bortezomib, but not by pretreatment with low-dose bortezomib. Adding a 100-fold molar excess of unlabeled NF-κB probe completely hindered the binding of the labeled probe to the NF-κB/DNA complex (Fig. 6B).

**Effect of Bortezomib on Endotoxin-Induced Uveitis**

After 48 hours, the LPS-stimulated RAW 264.7 cells pretreated with 25 nM bortezomib exhibited a significant proportion of early apoptosis (annexin V positive, PI negative) or necrosis (annexin V negative, PI positive) to the LPS-stimulated cells (P = 0.004, Fig. 9). Few cells were undergoing late apoptosis (annexin V positive, PI positive) or necrosis (annexin V negative, PI positive) in all groups.

**Pro-Apoptotic Effects of Bortezomib on Cells in the ICB**

Some cells in the LPS group were labeled with TUNEL, and more cells were undergoing apoptosis in the low-dose bortezomib group compared to the LPS group. Almost no cells...
Figure 10. TUNEL labeling for evaluating the apoptosis of cells in ICB. Some cells were stained positively in the LPS group, and there were more TUNEL-positive cells in the low-dose bortezomib group than in the LPS group. Almost no cells stained positively in the high-dose bortezomib group. Ctrl: control group. LPS: EIU rats with no treatment. Vel-L: EIU rats pretreated with low-dose bortezomib. Vel-H: EIU rats pretreated with high-dose bortezomib. Images are representative of similar results in three experiments. Original magnification 200×.
stained positively in the high-dose bortezomib group and, notably, fewer cells infiltrated in the ICB in this group compared to the LPS and low-dose bortezomib groups (Fig. 10).

**DISCUSSION**

Our study demonstrated that bortezomib, a 26S proteasome inhibitor, could inhibit ocular inflammation induced by LPS in Lewis rats, as could MG-132, another proteasome inhibitor. Bortezomib was chosen for the study due to its high efficacy at minimal concentrations, with tolerable and manageable adverse effects in treating human hematologic diseases.\(^{31-33}\) Our study showed that a relatively high dose of bortezomib decreased NF-κB activation and reduced the expression of inflammatory mediators. Furthermore, an in vitro study with LPS-stimulated RAW 264.7 cells showed that pretreatment with bortezomib increased IκB expression, demonstrating the inhibition of LPS-induced NF-κB activation by bortezomib treatment.

There is emerging evidence that NF-κB has a pivotal role in EIU, as in other inflammatory conditions, and that the inhibition of NF-κB activation can reduce the levels of downstream inflammatory mediators and cell infiltration induced by LPS.\(^ {7-12}\) Proteasomal degradation of the inhibitory factor IκB is necessary for NF-κB activation, and the inhibition of proteasome function theoretically maintains NF-κB in the inactive state in the cytosol and prevents its nuclear translocation. Proteasome inhibition is effective in treating several animal models of autoimmune disease, and to our knowledge our study is the first to demonstrate its anti-inflammatory effect in EIU.\(^ {27-30}\) The systemic administration of LPS may induce ocular inflammation by stimulating the production of proinflammatory cytokines, such as IL-1 and TNF-α, by monocytes and activated macrophages. This cytokine production, in turn, provokes NF-κB activation and localized inflammation in the uvea.\(^ {3,9,34}\) In addition, LPS itself has been found to activate a toll-like receptor (TLR), an important pattern-recognition receptor for innate immunity, that is found on uvea-resident macrophages, and subsequently leads to the activation of NF-κB and downstream inflammatory cascades.\(^ {35-38}\) In our study, treatment with a proteasome inhibitor before LPS stimulation inhibited effectively the activation of NF-κB at a relatively early stage in the course of EIU (i.e., at 12 hours or earlier), and the expression of fractalkine, MCP-1, ICAM-1, and iNOS was decreased. Reduced expression of chemokines and cell adhesion molecules, and reduced NO production could explain the decreased numbers of inflammatory cells recruited to and infiltrating uveal tissues.

The mechanisms of the anti-inflammatory effects of proteasome inhibition are more complex than the mere inhibition of NF-κB activation. The proteasome seems to be the central regulator of LPS-induced inflammation and macrophage function. In vitro studies have revealed that LPS can bind to a specific subunit of the proteasome and augment its chymotrypsin-like peptidase activity.\(^ {23,24}\) Several LPS-inducible genes are modulated by the proteasome, including TNF-α, IL-6, iNOS, TLR-2, and TLR-4, and proteasome inhibitors reduce their expression.\(^ {22,24}\) In addition, the proteasome is important in processing antigens to be presented by major histocompatibility complex (MHC) class I molecules. Inhibition of the proteasome interferes with antigen presentation and the subsequent immune response.\(^ {18,39,40}\) Furthermore, proteasome inhibition leads to the accumulation of several regulators and signaling molecules in T cells, B cells, monocytes/macrophages, and dendritic cells, and prevents these cells from becoming fully activated.\(^ {18,41}\) Because the proteasome is essential for cell cycle progression and cell apoptosis, the inhibition of cell cycle progression and the promotion of target-cell apoptosis are expected after proteasome inhibition.\(^ {18,33}\) Proteasome inhibitors have been demonstrated to trigger the apoptosis of cancer cells and tumors, which contributes strongly to the anti-tumor effect.\(^ {42-47}\) However, whether the same mechanisms are activated in inflammatory cells remains unclear. Our study demonstrated a significant percentage of RAW 264.7 cells exhibited apoptosis under sufficient concentration of bortezomib, especially in LPS-stimulated RAW cells, and this result implies that bortezomib may promote selectively apoptosis in activated inflammatory cells. The observation that there were more TUNEL-positive cells in ICB of rats in the low-dose bortezomib group compared to the LPS group further supports the hypothesis. Almost no TUNEL-positive cells in the high-dose bortezomib group may be attributed to the strong anti-inflammatory effect of the drug resulting in sparse infiltrating cells in the ICB. Taken together, the proteasome is involved in several key steps necessary for activating the immune response, and proteasome inhibition has been suggested as an effective method to hamper inflammation.

In our study, the rats were treated with bortezomib before the induction of EIU to ensure the onset of the drug's effect during ocular inflammation. We have not evaluated the drug’s efficacy when it is applied after EIU is established, as always is the case in clinic. Concerning the wide range of biological functions regulated by the proteasome and its ubiquitous distribution, the systemic adverse effects associated with proteasome inhibitors deserve meticulous consideration. The evaluation of adverse effect is important especially in treating ocular inflammation diseases, because dosage that is greater than usual may be needed for adequate intraocular drug penetration. The use of proteasome inhibitors that preferentially block immunoproteasome subunits may be more feasible for the clinical treatment of uveitis.\(^ {48,49}\)

In conclusion, we demonstrated that bortezomib inhibited EIU in a dose-dependent manner. Reduced intraocular inflammation was associated with the inhibition of NF-κB activation, and decreased expression of chemokines and inflammatory mediators. Bortezomib also increased the expression of the inhibitory factor IκB in LPS-stimulated RAW 264.7 cells in vitro. Our data indicate that the proteasome modifies NF-κB activity and may have an important role in the pathogenesis of EIU. A drug targeting the proteasome, therefore, may be an effective treatment strategy for EIU.

**References**


