Prevention of Experimental Colitis by a Selective Inhibitor of the Immunoproteasome

Michael Basler, Maya Dajee, Carlo Moll, Marcus Groettrup and Christopher J. Kirk

J Immunol 2010; 185:634-641; Prepublished online 4 June 2010;
doi: 10.4049/jimmunol.0903182
http://www.jimmunol.org/content/185/1/634

Supplementary Material
http://www.jimmunol.org/content/suppl/2010/06/02/jimmunol.0903182.DC1

Why The JI?
• Rapid Reviews! 30 days* from submission to initial decision
• No Triage! Every submission reviewed by practicing scientists
• Speedy Publication! 4 weeks from acceptance to publication

*average

References
This article cites 48 articles, 17 of which you can access for free at:
http://www.jimmunol.org/content/185/1/634.full#ref-list-1

Subscription
Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Prevention of Experimental Colitis by a Selective Inhibitor of the Immunoproteasome

Michael Basler,*‡ Maya Dajee,§ Carlo Moll,§ Marcus Groettrup,*‡ and Christopher J. Kirk‡

The proteasome, a multicatalytic protease, is responsible for the degradation of intracellular proteins. Stimulation of cells with inflammatory cytokines, such as IFN-γ, leads to the replacement of the constitutive catalytic proteasome subunits by the inducible subunits low molecular mass polypeptide (LMP)2 (β1i), multicatalytic endopeptidase complex-like-1 (β2i), and LMP7 (β5i), which are required for the production of certain MHC class I-restricted T cell epitopes. In this study, we investigated the effect of immunoproteasomes on the development of dextran sulfate sodium-induced colitis. Colitis induction in LMP2-, LMP7-, and multicatalytic endopeptidase complex-like-1-deficient mice caused reduced weight loss compared with wild-type mice. Although colon lengths were shortened in wild-type mice, no reduction was observed in immunoproteasome-deficient mice. In accordance with this, proinflammatory cytokines, such as TNF-α and IL-1β, were not upregulated in these mice. Blockage of LMP7 by a novel LMP7-selective inhibitor (PR-957) strongly reduced pathological symptoms of dextran sulfate sodium-induced colitis. Production of numerous cytokines in PR-957–treated mice was suppressed, resulting in reduced inflammation and tissue destruction. Taken together, these results demonstrate that an immunoproteasome-specific inhibitor can be used to attenuate autoimmune diseases like colitis. The Journal of Immunology, 2010, 185: 634–641.

Inflammatory bowel disease (IBD) refers to a group of chronic inflammatory disorders of the gastrointestinal tract that affect ~1 million people in the United States and 2 million people in Europe (1). In humans, the two key forms of IBD are Crohn’s disease (CD) and ulcerative colitis (UC). The hallmark of IBD is chronic, uncontrolled inflammation that can affect any part of the gastrointestinal tract in CD and only the colon in UC (2). Both diseases are characterized by mucosal inflammation, diarrhea, hematochezia, weight loss, and abdominal pain. The pathogenesis of IBD involves the interplay of environmental, genetic, microbial, and immune factors that results in chronic intestinal inflammation. Current treatment options include 5-aminosalicylic acid, antibiotics, corticosteroids, nutritional therapy, as well as immunomodulators to induce and maintain remission (3–5). Although such treatments are effective in many cases of CD and UC, a large patient population suffers intolerable side effects, relapse, or fails to respond altogether. Therefore, further investigation of novel therapeutics is warranted. IBDs are hypothesized to result from abnormal immune responses to Ags derived from intestinal microbiota, which seem to contribute to the recruitment and activation of lamina propria macrophages and T cells (6, 7). Initiation and perpetuation of inflammation in UC and CD may be regulated, in part, by an increased secretion of proinflammatory cytokines, such as IL-1β and TNF-α, by these cells (8).

Multiple cellular processes, such as cell cycle regulation (9), apoptosis (10), cell adhesion (11), transcription (12), angiogenesis (13), and generation of peptides from intracellular Ags that are presented to cytotoxic T lymphocytes by MHC class I (14, 15), are controlled by the proteasome. It is the major cytosolic endoprotease in eukaryotes, consisting of α and β subunits that build a barrel-shaped complex of four rings with seven subunits each (called 20S proteasome) (16). The outer two rings consist of α subunits; the inner two rings consist of β subunits, which bear the proteolytically active centers. Three β subunits (β1, β2, and β5) are responsible for three catalytically distinct peptidase activities: caspase-like, trypsin-like, and chymotrypsin-like activities, respectively (17, 18). In the context of IFN-γ and TNF-α, the inducible proteolytically active subunits low molecular mass polypeptide (LMP) 2 (β1i), multicatalytic endopeptidase complex-like (MECL)-1 (β2i), and LMP7 (β5i) are incorporated into the so-called “immunoproteasome” (19). These immunosubunits play a role in class I ligand generation, in establishing the naïve CD8+ T cell repertoire, and in altering the cytotoxic T cell response (20, 21). In a recent study, a novel function of immunoproteasomes, independent of class I Ag presentation, was revealed. An LMP7-selective epoxyketone proteasome inhibitor (PR-957) blocked cytokine production and attenuated the progression of experimental arthritis (22, 23).

In this study, we investigated the role of immunoproteasomes in a murine model of colitis. Immunoproteasome-deficient mice developed only mild symptoms. Blockage of LMP7 by a selective inhibitor (PR-957) led to attenuated colitis manifested by reduced body weight loss and decreased cytokine production and tissue destruction. Our results suggest that selective inhibition of LMP7 by PR-957 has the potential to attenuate IBD in humans.

*Division of Immunology, Department of Biology, University of Constance, Konstanz, Germany; ‡Biotechnology Institute Thurgau, Constance University, Kreuzlingen, Institute of Pathology, Cantonal Hospital Münsterlingen, Münsterlingen, Switzerland; and ²ProteoX, Inc., South San Francisco, CA 94080

Received for publication September 29, 2009. Accepted for publication April 22, 2010.

This work was supported by German National Science Foundation Grant GR1517/4-2 and Swiss National Science Foundation Grant 31003A_119699.

Address correspondence and reprint requests to Dr. Michael Basler, Division of Immunology, Department of Biology, University of Constance, P1101 Universitätsstrasse 10, D-78457 Konstanz, Germany. E-mail address: michael.basler@uni-konstanz.de

The online version of this article contains supplemental material.

Abbreviations used in this paper: CD, Crohn’s disease; DSS, dextran sulfate sodium; IBD, inflammatory bowel disease; LMP, low molecular mass polypeptide; MECL, multicatalytic endopeptidase complex-like; UC, ulcerative colitis; WT, wild-type.
Materials and Methods

**Mice**

C57BL/6 mice (H-2b) were purchased from Charles River (Sulzfeld, Germany). MECL-1 (21), LMP2 (24), and LMP7 (25) gene-targeted mice were kindly provided by Dr. John J. Monaco (Department of Molecular Genetics, Cincinnati Medical Center, Cincinnati, OH). Gene knockout mice were backcrossed to C57BL/6 background for ≥10 generations. Female 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 or were done under protocols approved by an institutional animal care and use committee.

**Proteasome inhibition in mice**

PR-957 (22) was formulated in an aqueous solution of 10% (w/v) sulfobutyl ether-β-cyclodextrin and 10 mM sodium citrate (pH 6) and administered to mice as an s.c. or i.v. bolus dose of 6, 12, or 20 mg/kg (in a volume of 100 μL). PR-825 was dissolved in 2% ethanol in saline and administered as an s.c. bolus dose of 2 mg/kg (in a volume of 100 μL).

**Induction of colitis**

Colitis was induced in 8–10-wk-old mice by adding 2% dextran sulfate sodium (DSS) (m.w. 36,000–50,000; MP Biomedicals, Solon, OH) to the drinking water, beginning at day 0 for 5 d; thereafter, they were given regular drinking water. Body weight was measured daily throughout the experiment. In two sets of experiments (Figs. 1, 6A), 3% DSS was added to the drinking water for 7 d. Studies were carried out in both facilities in accordance with regulatory standards at each facility (University of Constance; Konstanz, Germany and Proteinex, Inc., South San Francisco, CA).

**CD4+ cell stimulation**

T cells from splenocytes of C57BL/6 or LMP7-deficient mice were isolated with antiCD4-conjugated microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). Cells were incubated with 200 nM PR-957 or 125 nM PR-825 for 1 h at 37°C. Washed cells (4 × 10^6/well) were stimulated with plate-bound anti-CD3 (4 μg/ml, clone17A2; eBioscience, San Diego, CA) for 2 d. IL-17 (eBioscience) and IFN-γ (BD Biosciences, San Jose, CA) in the supernatant were determined to be ELISA, according to the manufacturer’s protocol.

**Flow cytometry**

Flow cytometry was performed exactly as previously described (26). Abs to CD3, CD8, CD4, CD19, and CD11c were purchased from BD Biosciences. Annexin V/propidium iodide staining were performed according to the manufacturer’s protocol (Annexin V detection kit I; BD Biosciences).

**Proteasome active-site ELISA**

A proteasome active-site ELISA was used to monitor inhibition of constitutive and immunoproteasome active sites in mouse colon tissue, and it was performed as described previously (22).

**Quantitative real-time RT-PCR**

Real-time RT-PCR was used to quantify cytokine expression levels in mouse colons. Total RNA was extracted from colon of mice using a NucleoSpin RNA II extraction kit (Macherrey-Nagel, Düren, Germany). One microgram of total RNA was reverse transcribed using oligonucleotide (dT) primers and the reverse transcription system (Promega, Madison, WI). Quantitative PCR was performed with the LightCycler instrument, using the LightCycler Fast Start DNA Master SYBR Green I reaction mix (both from Roche Diagnostics, Mannheim, Germany), with the following primers: IL-1β–specific forward: 5′-GGG AGC CAC CCC AAA AGA TG-3′, IL-1β reverse primer: 5′-AGA AGG TGC TGC ATG CTC CA-3′, TNF-α–specific forward: 5′-AGT AGC ACA GGA AGC ATG ATC-3′, TNF-β reverse primer: 5′-TAC AGG CTT GTC ACT GCA ATT-3′, IL-23–specific forward: 5′-GCC CCG TAT CCA GTG TCA-3′, IL-23 reverse primer: 5′-GCT GCC ACT GCT GAC-3′, IL-17–specific forward: 5′-CTC CAG AAG GGC CTC AGA CTA C-3′, IL-17 reverse primer: 5′-GCT TTC CCT CCG TCA CAG AG-3′, IFN-γ–specific forward: 5′-ATG AAC GCT ACA CAG TGC ATC-3′, and IFN-γ reverse primer: 5′-CCA TTC TTT TTC CAG TCT CTC-3′. IL-6 primers were acquired from SABiosciences (Frederick, MD; PPM03015A). Mouse hypoxanthineguanine phosphoribosyltransferase was used as a reference gene with the following primers: 5′-TTG GCA GGA CTG AAA GAC TTG-3′ (forward) and 5′-CCA GCA GGT CAG CAA AGA ACT TA-3′ (reverse). In Fig. 6, RT-PCR analysis was performed by Gene Screen Technologies (Piscataway, NJ). Total RNA was extracted with an RNeasy extraction kit (Qiagen, Valencia, CA). After treatment with DNase I (Qiagen), cDNA was synthesized using random primers and SuperScript II as per the manufacturer’s protocol (Invitrogen, Carlsbad, CA). Gene expression was measured by TaqMan real-time PCR using target gene probes and primers, per the manufacturer’s protocol (Applied Biosystems, Foster City, CA). The experiments were performed on an ABI PRISM 7900 sequence detection system under the following conditions: 1 cycle of 50°C (2 min), followed by 95°C (10 min), 40 cycles of 95°C (15 s), and 60°C (1 min). All reactions were performed in triplicate, and the experiments were repeated three times. Cytokine mRNA levels were normalized to β-actin.

**Histological analysis**

Colons were fixed in 4% formalin in phosphate buffer (55 mM Na2HPO4, 12 mM NaH2PO4). The fixed tissues were dehydrated and embedded in paraffin. Seven-micrometer sections were stained with H&E and scored by a pathologist in a blind manner. The degree of inflammation and epithelial injury on microscopic cross-sections of the colon was graded semiquantitatively: 0, none; 1, minimal; 2, mild; 3, moderate; and 4, severe.

**Statistical analysis**

The statistical significance of the differences was determined using the Student t test or one-way ANOVA. For ANOVA, we used the Bonferroni post hoc analysis to compare treatment groups. All statistical analyses were performed using GraphPad Prism Software (version 4.03) (GraphPad, San Diego, CA). Statistical significance was achieved when p < 0.05.

**Results**

**DSS-induced colitis upregulates immunoproteasome subunits**

Compared with healthy humans, CD patients show a strong upregulation of the immunosubunits β1i and β2i in the inflamed mucosa (27). To investigate whether comparable results could be found in DSS-induced colitis, immunoproteasome content in colon of diseased mice was analyzed by a proteasome active-site ELISA. We compared the relative levels of proteasome active sites in colon tissue lysates from healthy mice with those challenged with DSS (3%) for 7 d, followed by a 7-d rest. Compared with healthy mice, LMP2, LMP7, and MECL-1 were strongly upregulated in mice with DSS-induced colitis (Fig. 1), demonstrating immunoproteasome induction in this model of colitis.

**Reduced severity of DSS-induced colitis in LMP7-deficient mice**

To analyze whether upregulation of immunosubunits in colon of diseased mice is due to an induction of immunosubunits by inflammatory cytokines produced by infiltrating immune cells or whether immunoproteasome are crucial, per se, in disease development, we induced colitis in LMP7-deficient mice by oral administration of DSS in drinking water for 5 d (Fig. 2). Body weight of the mice was recorded for 14 d. Although wild-type (WT) mice had lost ~20% of body weight by day 9, LMP7-deficient mice were fully protected from weight loss. Hence, we conclude that immunoproteasome upregulation in colitis is not only a side effect due to induction by cytokines produced by infiltrating immune cells, but also that LMP7 plays a crucial role in colitis development.

**Immunosubunit deficiency protects mice from DSS-induced colitis**

It was reported that LMP7-deficient mice have decreased incorporation of LMP2 and MECL-1 into proteasomes (28). Therefore, the decreased loss in body weight seen in Fig. 2 might originate from decreased LMP2 or MECL-1 expression. DSS-induced colitis was analyzed in LMP2- and MECL-1–deficient mice to determine whether they are protected similarly to LMP7−/− mice. LMP7−/−, LMP2−/−, and MECL-1−/− mice showed less weight loss compared with C57BL/6 WT mice (Fig. 3). Although LMP2−/− mice lost ~10% of weight, MECL-1−/− mice were fully protected. DSS-induced colitis is associated with reduced
colon length in diseased mice. To investigate whether the decreased body weight loss in LMP2-, LMP7-, and MECL-1–deficient mice is accompanied by an altered colon length, the latter parameter was measured in DSS-treated mice on day 9 postinduction (Fig. 4A). Colon length was reduced in DSS-treated C57BL/6 mice by ∼2 cm compared with untreated naive mice. MECL-1– and LMP7-deficient mice showed no significant alteration in colon length, whereas LMP2-deficient mice had a slight reduction in length. Nevertheless, the colon length of LMP2-deficient mice was still clearly increased compared with DSS-treated C57BL/6 WT mice.

IBD is accompanied by the accumulation of a variety of inflammatory cells and the release of several soluble mediators of inflammation, such as reactive oxygen species, lipid mediators, proteases, and cytokines. Among these, a number of proinflammatory cytokines were demonstrated to be elevated in colitis (29) and play an important role in the development of mucosal inflammation. To investigate whether inflammation is altered in immunoproteasome-deficient mice with colitis, TNF-α, IFN-γ, and IL-1β, -6, -17, and -23 expression was determined by real-time PCR in colon on day 9 after colitis induction (Fig. 4B). Cytokines were upregulated in WT mice, whereas MECL-1–, LMP2, and LMP7-deficient mice showed no significant increase compared with healthy untreated mice.
WT mice. The degree of inflammation and epithelial injury on microscopic cross-sections of the colon was graded semiquantitatively from 0 to 4 (Fig. 4C, 4D). In contrast to WT mice, immunoproteasome-deficient mice had only mild signs of inflammation and epithelial damage. Taken together, LMP2-, LMP7-, and MECL-1–deficient mice display a strongly attenuated form of DSS-induced colitis.

An LMP7-selective inhibitor reduces symptoms of DSS-induced colitis.

Recently, we demonstrated that selective inhibition of LMP7 by PR-957 blocked the production of IL-23 by activated monocytes and the production of IFN-γ and IL-2 by T cells (22). In mouse models of rheumatoid arthritis, PR-957 treatment reversed signs of disease and resulted in reductions of cellular infiltration, cytokine production, and autoantibody levels. Similar to rheumatoid arthritis, IBDs are characterized by infiltration of leukocytes and tissue destruction by immune mediators, like cytokines. PR-957 is an LMP7-selective peptide-ketoepoxide proteasome inhibitor that is 20- to 40-fold more selective for LMP7 over the next most sensitive sites, b5 or LMP2 (22). Selective inhibition of LMP7 by PR-957 occurs at doses ranging from 1–10 mg/kg body weight in blood and kidney. The maximum tolerated dose of PR-957 in mice...
is 30 mg/kg body weight, indicating that inhibition of LMP7 is well tolerated (22). To test whether PR-957 is able to block LMP7 in colon, the inhibitor was administered i.v. or s.c. at 6 or 20 mg/kg. The proteasome active-site selectivity of PR-957 was determined with a subunit-specific ELISA to quantify occupied proteasome active sites in colon (Fig. 5A). LMP7 activity was reduced to ~10% of untreated colon, independent of dose and injection route. β5 activity was reduced by ~50%. To investigate whether PR-957 can attenuate DSS-induced colitis, mice were treated daily with 12 mg/kg PR-957 for 9 d. Untreated mice lost ~25% of body weight between days 5 and 9 (Fig. 5B). In contrast, mice treated with the LMP7 selective inhibitor PR-957 showed only minor (~5%) body weight loss at day 9 postinduction. To test whether long-term treatment affects the viability of immune cells, mice were treated daily with PR-957 for 9 d. On day 10, T cells (CD3+, CD8+, and CD4+), B cells (CD19+), and dendritic cells (CD11c+) were analyzed by flow cytometry (Supplemental Fig. 1). The percentage of CD11c+ and CD19+ cells was decreased slightly in spleen of PR-957–treated mice, and a concomitant increase in CD3+ and CD8+ cells was observed. Additionally, Annexin V/propidium iodide staining 24 h after PR-957 treatment demonstrated that inhibitor treatment did not lead to increased cell death of CD4+, CD19+, or CD11c+ cells (Supplemental Fig. 2). Hence, the reduction in weight loss in PR-957–treated mice observed in Fig. 5B cannot be explained simply by the elimination of immune cells.

Targeting the proteasome by the proteasome inhibitor bortezomib reduced colitis development in a rat model (30). Because bortezomib mediates equipotent inhibition of β5 and LMP7, we investigated whether the selective β5 inhibitor PR-825 (22) could reduce the severity of colitis development. Mice were treated daily with 2 mg/kg PR-825 or vehicle for 21 d. Weight loss in PR-825 mice was similar to that in vehicle-treated mice (Fig. 5C). These results indicated that LMP7 inhibition underlies the reduced colitis development in bortezomib-treated mice.

Consistent with the marginal weight loss in PR-957–treated mice (Fig. 5B), colon length was only reduced in untreated mice, whereas inhibitor-treated mice displayed a colon length comparable to naive animals (Fig. 5D). Histological analysis of colon cross-sections revealed reduced inflammation and epithelial injury (Fig. 5E, 5F).

PR-957 attenuates cytokine production in colon of DSS-treated mice

We have shown that PR-957 blocks cytokine production in activated lymphocytes and monocytes (22). Among the tested cytokines, IL-23 was the most sensitive to PR-957 treatment in LPS-stimulated

---

**FIGURE 5.** Effect of PR-957 on DSS-induced colitis. A, Active-site ELISA analysis of the activity of LMP7 and β5 in colon (taken 1 h after dosing) from C57BL/6 mice administered PR-957 i.v. or s.c. at 6 or 20 mg/kg body weight. Data were normalized to the average activity of vehicle-treated mice and are presented as the average relative activity ± SEM (n = 3/dose level). B–F, Colitis was induced by oral administration of 2% DSS for 5 d. Mice were treated daily (s.c.) with PR-957 at 12 mg/kg (B, D–F) or PR-825 at 2 mg/kg (C). On day 9, colon length (D) and histological score (E) were analyzed. Naive mice were used as healthy controls. B and C, Body weight of individual mice was monitored daily. Percent weight loss (y-axis) is plotted versus time (x-axis). Data points represent mean ± SD of five mice. D, Colon length. Data represent mean ± SD of five mice. E, Quantitative histopathologic assessment of colitis. Data points represent mean ± SD of five mice. F, Representative histological colon sections (H&E, original magnification ×40 [upper panels] and ×100 [lower panels]). The experiments were performed twice, yielding similar results. **p < 0.01.
PBMCs from healthy donors and patients with rheumatoid arthritis. IL-23 has been implicated in intestinal inflammation (31, 32) and elicits numerous effector molecules, most notably IL-17 and TNF-α (33). Because Th17 differentiation is blocked by PR-957 (22), we assume that Th17-associated cytokine production is altered in PR-957–treated mice with DSS-induced colitis. Indeed, cytokine profiling of colon tissue by RT-PCR on day 7 showed that PR-957 treatment decreased the expression of multiple inflammatory mediators, including IL-1β and -23 (Fig. 6A). Inducible NO synthase and cyclooxygenase-2 are important enzymes that mediate inflammatory processes and have been associated with the pathogenesis of certain types of human cancers, as well as inflammatory disorders. Both enzymes were significantly reduced in PR-957–treated mice, indicating reduced leukocyte infiltration and inflammation in colon tissue. To test whether PR-957 and PR-825 directly inhibit cytokine secretion from activated WT or LMP7-deficient cells, CD4+ T cells were stimulated with plate-bound CD3. PR-957 blocked the production of IL-17 and IFN-γ in WT but not LMP7-deficient CD4+ T cells (Fig. 6B). The lack of effect in LMP7-deficient cells indicates that PR-957 affects cytokine production through specific inhibition of LMP7. The fact that LMP7-deficient and WT splenocytes did not differ in IL-17 or IFN-γ production may be the result of compensatory incorporation of β5 in proteasomes from LMP7-deficient cells. Indeed, treatment of LMP7-deficient splenocytes with the β5-selective inhibitor PR-825 reduced cytokine production only in LMP7-deficient cells. Taken together, selective inhibition of LMP7 by PR-957 reduced the symptoms of DSS-induced colitis, manifested by decreased weight loss, colon tissue destruction, inflammation, and cytokine production.

**Discussion**

Because of the role of proteasomes in the regulation of numerous cellular processes, inhibition of the proteasome is an attractive strategy for anticancer therapy. Bortezomib, which targets the constitutive proteasome and immunoproteasome, is the first clinically approved proteasome inhibitor for relapsed and/or refractory myeloma and mantle cell lymphoma (34). Recently, novel proteasome inhibitors preferentially targeting subunits of the immunoproteasome were described (22, 35–37). An LMP2-selective inhibitor attenuated proliferation in myeloma patient samples and other hematologic malignancies, although the chymotrypsin-like activity associated with the subunits β5 and β5i was also reduced at the concentrations used (37). Another study investigated the effect of an LMP7-selective inhibitor (PR-957) on the development of experimental arthritis in two mouse models (22). PR-957 treatment reversed signs of disease and resulted in reductions in cellular infiltration, cytokine production, and autoantibody levels. Additionally, selective inhibition of LMP7 by PR-957 blocked the production of IL-23 by activated monocytes and the production of IFN-γ and IL-2 by T cells, whereas the inhibition of β5 did not substantially affect cytokine release. Furthermore, LMP7 inhibition prevented the production of cytokines driving Th17 generation and IL-17 production in T cells cultured in the presence of polarizing cytokines. Because of a unique role for LMP7 in controlling cytokine production, it provides a therapeutic rationale for targeting LMP7 in autoimmune disorders. Therefore, the impact of immunoproteasomes in DSS-induced colitis was investigated. Induction of colitis upregulated the immunosubunits LMP2, LMP7, and MECL-1 (Fig. 1). Because colitis induction leads to an infiltration of immune cells into colon tissue, production of cytokines by these cells might lead to an induction of immunoproteasomes in colon by IFN-γ or TNF-α, as seen in Fig. 1. Alternatively, cells of hematopoietic origin, particularly lymphocytes and monocytes, constitutively express immunoproteasome subunits (38). Infiltration of such cells might contribute to the upregulation of LMP2, LMP7, and MECL-1 (Fig. 1). Comparable results were obtained from CD patients, who showed high LMP2 and MECL-1 expression in inflamed mucosa (27). Because LMP7 is already expressed in high amounts in healthy control patients, no upregulation of this subunit was observed in CD patients in two additional studies (39, 40). Induction of colitis by oral administration of DSS in the drinking water of LMP2-, LMP7-, and MECL-1–deficient mice revealed a nearly complete protection of these mice compared with WT mice (Figs. 2, 3). Colon inflammation, tissue destruction, and upregulation of proinflammatory cytokines were barely detectable in these mice (Fig. 4). Similar results were found in the LMP2 knockout mouse by another group (41), which demonstrated that LMP2 expression contributes to the pathogenesis of DSS-induced colitis in mice. In a model of murine autoimmune encephalomyelitis, it was demonstrated that the immunoproteasome is not required for the establishment of myelin oligodendrocyte glycoprotein-induced autoimmune encephalomyelitis (42), suggesting a contribution of the immunoproteasome in the etiology of IBD but not multiple sclerosis. Presumably, the prevention of DSS-induced colitis in immunoproteasome-deficient mice cannot be explained solely by an altered production of cytokines by T cells, because in vitro cytokine-secretion assays demonstrated that T cells derived from immunoproteasome-deficient mice produced similar amounts of cytokines as WT mice (Fig. 6B) (22). However, recent data suggest that immunoproteasomes might play a role in innate immunity. Production of IFN-α, IL-6 and -1β, and TNF-α was reduced in influenza A virus–infected bone marrow–derived dendritic cells derived from LMP2-deficient mice compared with those from WT mice (43). Challenge with DSS results in macrophage and granulocyte infiltration into intestinal submucosa and lamina propria (44). Therefore, altered activation or function of innate immune cells in immunoproteasome-deficient mice could explain the prevention of...
DSS-induced colitis in gene-deficient animals. However, the mu-
cosa is a complex environment with a physiology that is regulated
by a variety of cell types of nonhematopoietic origin. Experiments
using bone marrow chimeras of immunoproteasome-deficient and
WT mice could reveal whether immunoproteasome expression is
essential in immune cells or in cells of nonhematopoietic origin,
such as epithelial cells.

Instead of the gene-targeted immunoproteasome subunit, the re-
spective constitutive counterpart is incorporated into proteasomes
of LMP2-, LMP7-, or MECL-1-deficient mice. Furthermore, im-
nunoproteasomes from knockout mice express altered incorpora-
tion of the remaining 20Si subunits. Therefore, increased incor-
poration of the constitutive subunits, not the immunoproteasome
deficiency, might contribute to the reduced pathology in these
knockout mice (Figs. 2–4). An LMP7-selective inhibitor (PR-957)
was used to analyze the direct influence of LMP7 on the develop-
ment of colitis (Fig. 5). Administration of PR-957 attenuated
symptoms of DSS-induced colitis, as determined by weight loss
(Fig. 5B) and colon length (Fig. 5D). Inflammation in these mice
was reduced (Figs. 5E, 5F, 6A), and the production of numerous
cytokines was strongly impaired (Fig. 6A). Administration of a β5-
selective inhibitor had no effect on colitis severity. Taken together,
these data suggest that, despite the high basal expression of LMP7
in normal colon tissue (39, 40), LMP7 represents a targeted ap-
proach to the treatment of CD because of the unique role of the
immunoproteasome in regulatory inflammatory responses. Our
results indicated that LMP7 is directly involved in disease de-
velopment, presumably as the result of regulation of cytokine pro-
duction in inflammatory cells. Development and investigation of
inhibitors for LMP2, or MECL-1, are warranted to analyze whether
similar effects can be observed. Previous studies demonstrated a
requirement for IL-23 in driving intestinal inflammation mediated
by innate immune cells and T cells in murine colitis (31, 32, 45). A
genome-wide association study in humans found a highly significant
association between CD and the IL-23R genotype. The investiga-
tors suggested blockade of the IL-23–signaling pathway as a rational
therapeutic strategy for IBD (46). Because PR-957 blocked IL-23
production of human PBMCs (22) and IL-23 expression in inflamed
colon tissue (Fig. 6A), the LMP7-selective inhibitor would be an
extremely suitable candidate for such an approach. Th17 cytokines,
such as IL-17 and IL-22, in combination with IL-23, play crucial roles
in intestinal protection and homeostasis. TGF-β and IL-6, rather than
IL-23, were shown to be the key cytokines directing Th17 cell de-
velopment. It now seems that IL-23 is unnecessary in mice for the
initiation of Th17 differentiation, but it is required for expansion
and/or maintenance of the Th17 response (47). We speculate that
PR-957 affects the Th17 axis in our model of colitis by attenuated
cellularization of CD4+ cells to Th17 cells and reduced maintenance
of Th17 cells as the result of blocked IL-23 production. We dem-
onstrated that PR-957 alters cytokine production in WT, but not in
LMP7-deficient, CD4+ cells (Fig. 6B). In contrast, the selective
β5c inhibitor PR-825 reduced cytokine secretion in LMP7-
deficient CD4+ cells but not in WT cells. These data suggest that
proteasome composition in CD4+ cells mainly consists of the immu-
noproteasome type, because no inhibition of cytokine release by PR-
825 could be observed in WT cells.

Targeting the proteasome by the proteasome inhibitor bortezo-
mib recently led to success in multiple mouse models of auto-
immune disorders. Pharmacological inhibition with bortezomib
ameliorated glomerulonephritis and prolonged the survival of two
mouse strains with lupus-like disease (48), attenuated murine col-
gen-induced arthritis (49), reduced colitis development in a rat
model (30), and ameliorated autoimmune encephalomyelitis in
mice and rats in prophylactic and therapeutic treatment (50). It
is well established that bortezomib inhibits NF-κB activation by
blocking the degradation of the NF-κB inhibitor (IκB), leading to
reduced expression of proinflammatory factors, such as cytokines
and chemokines, and adhesion molecules on lymphoid cells. Hence,
the observed effects in multiple models of autoimmune diseases
might be due to an inhibition of the NF-κB pathway. In contrast,
PR-957 does not inhibit NF-κB activity in a reporter cell line at
selective concentrations, suggesting that LMP7 regulates cytokine
production via NF-κB–independent pathways that remain to be
eclucidated (22).

In this study, we investigated the effect of a novel LMP7-
selective inhibitor in a murine model of colitis. PR-957 prevented
disease development at far less than the maximal tolerated dose and
without notable toxic side effects; therefore, it holds great potential
for the modulation of immune responses and for the suppression of
autoimmune diseases like IBD.

Acknowledgments
We thank Ulrike Beck for excellent technical assistance.

Disclosures
M.D. and C.J.K. are (or were) employees of Proteolix, Inc. (now Onyx
Pharmaceuticals).

References
1. Loftus, E. V., Jr. 2004. Clinical epidemiology of inflammatory bowel disease:
Innidence, prevalence, and environmental influences. Gastroenterology 126:
1504–1517.
Dis. 8: 65–71.
429.
7. Macdonald, T. T., and G. Monteleone. 2005. Immunity, inflammation, and al-
8. Reinecker, H. C., M. Steffen, T. Witthoef, I. Pflueger, S. Schreiber,
factor alpha, IL-6, and IL-1 beta by isolated lamina propria mononuclear cells
10. Orlowski, R. Z. 1999. The role of the ubiquitin-proteasome pathway in apop-
11. Read, M. A., A. S. Neish, F. W. Luscinbas, T. Maniatis, and
T. Collins. 1995. The proteasome pathway is required for cytokine-
endothelial-leukocyte adhesion molecule expression. Immunity 2:
493–506.
1068.
15. Groettrup, M., A. Soza, U. Kuckelkorn, and P. M. Kloetzel. 1996. Peptide an-
gen production by the proteasome: complexity provides efficiency. Immunol.
Nature 386: 463–471.
inducible exchanges of 20S proteasome active site subunits: why? Biochimie
19. Griffim, T. A., D. Nandi, M. Cruz, H. J. Feihling, L. V. Kae, J. J. Monaco,
and R. A. Colbert. 1998. Immunoproteasome assembly: cooperative incorpora-
tion of interferon gamma (IFN-gamma)-inducible subunits. J. Exp. Med.
187: 97–104.
2004. Immunoproteasomes down-regulate presentation of a subdominant T cell