

# Expression of catalytic proteasome subunits in the gut of patients with Crohn's disease

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## Abstract

**Background and purpose** Activation of the transcription factor NF- $\kappa$ B by proteasomes and subsequent nuclear translocation of cytoplasmic complexes play a crucial role in the intestinal inflammation. Proteasomes have a pivotal function in NF- $\kappa$ B activation by mediating degradation of inhibitory I $\kappa$ B proteins and processing of NF- $\kappa$ B precursor proteins. This study aims to analyze the expression of the human proteasome subunits in colonic tissue of patients with Crohn's disease.

**Materials and methods** Thirteen patients with Crohn's disease and 12 control patients were studied. The expression of immunoproteasomes and constitutive proteasomes was examined by Western blot analysis, immunofluorescence and quantitative real-time PCR. For real-time PCR, AK2C was used as housekeeping gene.

**Results** The results indicate the influence of the intestinal inflammation on the expression of the proteasomes in Crohn's disease. Proteasomes from inflamed intestine of patients with Crohn's disease showed significantly increased expression of immunosubunits on both protein and mRNA levels. Especially, the replacement of the

constitutive proteasome subunit  $\beta$ 1 by inducible immunosubunit  $\beta$ 1i was observed in patients with active Crohn's disease. In contrast, relatively low abundance of immunoproteasomes was found in control tissue.

**Conclusions** Our data demonstrate that in contrast to normal colonic tissue, the expression of immunoproteasomes was evidently increased in the inflamed colonic mucosa of patients with Crohn's disease. Thus, the chronic intestinal inflammation process in Crohn's disease leads to significant alterations of proteasome subsets.

**Keywords** Crohn's disease · Inflammatory bowel disease · 26S proteasome · 20S proteasome · NF- $\kappa$ B

## Introduction

Although the etiology of Crohn's disease (CD) still remains partially unclear, substantial advances have been made recently in both understanding of the molecular pathogenesis and also in the development of new therapeutic strategies. The uncontrolled activation of proinflammatory effector CD4<sup>+</sup> T cells seems to be a pivotal pathogenic mechanism associated with initiation and perpetuation of the inflammatory process. Activation of Th1 CD4<sup>+</sup> T cells as well as of macrophages and dendritic cells leads to the increased production of proinflammatory cytokines in the inflamed intestinal mucosa in Crohn's disease [1–3]. The range of effector CD4<sup>+</sup> subsets has recently expanded with the description of Th17 cells, which produce cytokines IL-17, IL-17F, and IL-22 [4–6]. The proinflammatory cytokines IL-12, which is important for differentiation of Th1 cells, and recently described IL-23 have been shown to play a crucial role in the pathogenesis of CD. Novel studies in murine colitis models resembling human CD suggest that IL-23 is the

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dominant cytokine able to modulate Th17 immune response and to drive the regional intestinal inflammation [7, 8].

Studies in mice have shown that different members of NF- $\kappa$ B family play a pivotal role in defense of the host against the pathogens by regulating of proinflammatory gene expression [9]. Synthesis of most important proinflammatory cytokines and chemokines such as IL-12, IL-23, TNF- $\alpha$ , IL-6, IL-8, and IL-1 $\beta$  is mediated by NF- $\kappa$ B. Increased NF- $\kappa$ B activity with nuclear localization was observed in inflammatory bowel disease, especially in CD [10–12]. Proteasome is essential for IKK $\beta$ -induced NF- $\kappa$ B activation contributing directly to the activation of NF- $\kappa$ B by mediating degradation of inhibitory NF- $\kappa$ B proteins of I $\kappa$ B family and the processing of NF- $\kappa$ B precursor proteins p105 and p100 [13]. Therefore, the proteasome represents a potential target for strategies aimed at suppressing inflammation processes.

The eukaryotic 26S proteasome is the main protease in the cytoplasm and nucleus consisting of a proteolytic core, the 20S proteasome, and two 19S regulatory particles. The barrel-like 20S proteasome is composed of seven different  $\alpha$  and seven different  $\beta$  subunits which form four rings stacked on top of each other [14]. The subunits  $\beta$ 1,  $\beta$ 2, and  $\beta$ 5 bear the catalytic activity of the proteasome. In vertebrates, there exist three additional IFN- $\gamma$  inducible subunits, so-called immunosubunits  $\beta$ 1i,  $\beta$ 2i, and  $\beta$ 5i. IFN- $\gamma$  inducible substitution of proteasomal constitutive subunits by immunosubunits changes the proteolytic specificity of the proteasome and modulates the generation of the peptides used as ligands for MHC class I presentation [15, 16]. Even more importantly for inflammatory processes, the induction of proteasomal immunosubunits is associated with increased NF- $\kappa$ B activity [11, 17].

It has been shown that the constitutive proteasome is the predominant form in most tissues [18]. In the course of infection, IFN- $\gamma$  induces the expression of immunoproteasomes in affected tissues [19]. Since the levels of IFN- $\gamma$  are also elevated in CD, we decided to evaluate the possible effects of the inflammation on the expression of proteasomes in the intestine. In the present study, the relationship of proteasomal mRNA levels and abundance of encoded proteins in normal and inflamed colonic tissue have been analyzed. In particular, we compared the mRNA and protein expression of proteasomes of patients with CD with a control group of patients.

## Materials and methods

### Patients

Colonic mucosa specimens from patients with Crohn's disease were obtained from surgical resections after intestinal surgery performed at the Charité University Hospital, Berlin. Control specimens were obtained from patients undergoing surgery for colorectal cancer (macroscopically

and histologically non-affected normal colonic tissue). A total group of 13 patients with active CD and 12 control patients was investigated. In all cases, diagnosis of CD was confirmed histologically using scoring systems for CD: 0 = normal; 1 = mild edema and mild inflammation in the lamina propria; 2 = crypt abscesses and moderate inflammation of the lamina propria; 3 = more severe inflammation with destructive crypt abscesses ( $\pm$ granulomata); 4 = strong inflammation with ulcerations or fissures. Immediately after the surgery, intestinal mucosa was separated from the underlying tissue and used for the experiments. In all 13 CD patients, histology revealed severe inflammation with histological scores of 3 ( $n=7$ ) and 4 ( $n=6$ ), respectively. Informed consent was obtained from all patients, and the protocol was approved by the local ethics committee.

### Preparation of protein extracts from human colonic tissue

For the preparation of organ lysates, colonic mucosa was stripped from the submucosa, frozen in liquid nitrogen and stored at  $-196^{\circ}\text{C}$  until use. Colonic mucosa was homogenized with mortar and pestle followed with 20 strokes of the dauncer and subsequently immersed in ice-cold Lysis Buffer (20 mM Tris-HCl, pH 6.8, 50 mM NaCl, 1 mM EDTA pH 8, 1 mM NaN<sub>3</sub>, 1 mM DTT, 0.1% NP-40, 0.2 mM sodium vanadate, 10 mM sodium fluoride, 1 mM PMSF). 1 $\times$  complete protease inhibitor (Roche Applied Science, Mannheim, Germany) was added into lysis buffer. Samples were incubated on ice for 20 min and cell debris was sedimented by centrifugation at 13,000 $\times g$  for 10 min. Supernatants were used as cell lysates.

### Western blot analysis

For the detection of proteasomal proteins  $\beta$ 1 and  $\beta$ 1i, the color fluorescent Western blot analysis was performed. Briefly, for the cell lysates the protein concentration was determined using Micro BCA Protein Assay Kit (Pierce biotechnology, Rockford, IL, USA) and subsequently 20  $\mu\text{g}$  of total protein was denaturated in 4 $\times$  Laemmli Buffer and separated by 15 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Following SDS-PAGE, samples were transferred to Immobilon<sup>FL</sup> polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA, USA) at 100 V in transfer buffer (50 mM Tris, 40 mM glycine, 0.037% ( $w/v$ ) SDS, 20% ( $v/v$ ) methanol) for 60 min. Membranes were blocked for 30 min in an Odyssey Blocking Reagent (Licor Bioscience, Lincoln, NE, USA) and then incubated for 1 h with the appropriate primary antibody. As control for protein amounts a monoclonal anti-human  $\beta$ -actin antibody (Sigma-Aldrich, Munich, Germany) diluted 5,000-fold in Odyssey Blocking Reagent was used.  $\beta$ 1 and  $\beta$ 1i were detected using rabbit polyclonal anti-

human antibodies (Biomol, Hamburg, Germany) diluted 1,000-fold in the same buffer. Visualization was performed by using fluorescence-labeled secondary antibodies, goat-anti rabbit IRDye 800 (Rockland, Gilbertsville, PA, USA) and AlexaFluor 680 (Molecular Probes Eugene, OR, USA), respectively. The detection was performed using Odyssey infrared imaging system (Licor Biosciences, Lincoln, NE, USA).

#### Quantitative real-time PCR

Quantitative real-time PCR analysis for proteasomal subunits was performed by using an ABI Prism 7000 sequence detection system (Applied Biosystems, Forster city, CA, USA) according to manufacturer's instructions. Primers were manufactured by TIBMolbiol (Berlin, Germany). AK2C was used as a housekeeping gene for standardization. The analysis was performed as previously described [10]. Briefly, colonic mucosa was homogenized in Trizol (Invitrogen, Carlsbad, CA, USA) and total cellular RNA was extracted and treated with RNase-free DNase in order to remove contaminating genomic DNA from the sample. The determination of concentration and total RNA quality was analyzed with the 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). For the cDNA synthesis, 2 µg of total RNA was reverse transcribed and subsequently real-time PCR was performed using the SYBR Green Master Mix (Applied Biosystems, Forster city, CA, USA). During PCR reactions, the increase in SYBR Green fluorescence is directly proportional to the amount of double-stranded DNA generated; 5 µl cDNA template diluted 1:5 and 1:10, 10 pmol forward primer, 10 pm reverse primer and SYBR Green Mix ready-to-use solution (2×) in a 30 µl final reaction mixture were used. The evaluation of data was carried out by using SDS2.2.2. software (Applied Biosystems, Forster city, CA, USA).

#### Immunofluorescence microscopy

Immunofluorescence staining was performed on intestinal cryosections of 5 µm thickness. For intracellular staining of proteasome subunit β1i, the sections were washed in PBS for 5 min, fixed in acetone /methanol (1:1) and permeabilized with 0.1% (v/v) NP40 for 10 min. After permeabilization, normal goat serum was applied for binding nonspecific antigens. As primary antibody, polyclonal rabbit anti-β2i (Biomol, Hamburg, Germany) diluted 1:50 was used for 60 min. As negative control, sections were incubated with antibody diluent without primary antibody. β2i was detected using secondary Cy2-coupled anti-rabbit immunoglobulin G (DakoCytomation, Hamburg, Germany) for 60 min. The sections were analyzed on the fluorescence microscope Leica DMRB (Leica Microsystems, Wetzlar, Germany).

#### Statistical analysis

Real-time PCR data were compared using a two-tailed Student's *t*-test. A one-way ANOVA was used for analyzing multiple groups. Each set of data was expressed as SEM. *P* values ≤0.05 were regarded as statistically significant.

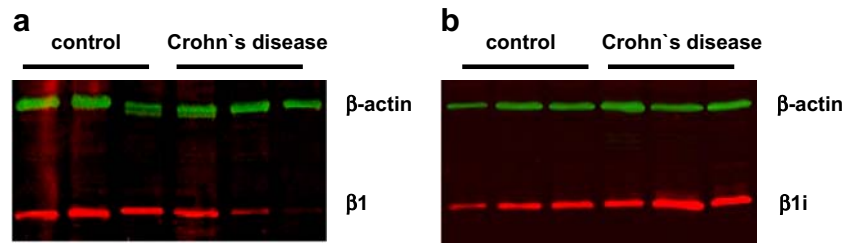
## Results

#### Preferential incorporation of proteasomal immunosubunit β1i in Crohn's disease

A previous study has shown that DSS treatment induced increased expression of β1i in the colon associated with histological damage in mice, whereas symptoms of DSS-induced colitis were much milder in β1i-deficient (LMP2<sup>-/-</sup>) mice lacking the β1i subunit [20]. To investigate the incorporation of distinct proteasomal catalytic subunits into proteasomes of CD patients, intestinal samples were examined by Western blotting and real-time PCR. In inflamed colon of patients with CD and non-inflamed colonic tissue of control patients, the abundance of the catalytic immunosubunit β1i was examined by Western blot analysis. By using specific antibody for β1i, a singular band for this protein was detected at approximately 25 kD. The increased protein expression of β1i was observed in patients with CD as compared to control patients (Fig. 1b). In order to analyze whether the increased incorporation of immunosubunit β1i into proteasomes in inflamed colonic mucosa of CD patients is the limiting factor for the expression of its counterpart protein β1, the whole cell lysates from colonic tissue of CD and control patients were also tested for β1. In all but one control patients, the protein levels of this constitutive proteasomal subunit were higher in control tissue than in inflamed colonic tissue of patients with CD (Fig. 1a). Thus, the increase of the β1i protein levels in CD was accompanied with a significantly decreased abundance of β1 (Fig. 1a and b).

#### Inflammation in Crohn's disease shifts the proteasome subunit composition towards immunoproteasomes

Since immunoproteasome subunits compete with their constitutive homologues for incorporation into the nascent proteasomes, we wondered whether mRNA levels of catalytic subunits β1i and β1 correlate with protein expression in CD. To study this, total RNA was extracted from colonic samples of CD patients and healthy controls and β1 and β1i mRNAs were quantified by quantitative real-time PCR. At the same time, we looked at the β1i/β1 ratio of their cellular mRNA levels in CD and controls. In the inflamed mucosa of CD patients, we observed an increase of β1i mRNA levels compared with normal



**Fig. 1** Protein expression of the proteasomal subunits  $\beta 1$  (a) and  $\beta 1i$  (b) in the inflamed mucosa of CD patients and normal colonic tissue (control,  $n=12$ ; CD,  $n=13$ ). Western blot analysis was performed using anti-human antibodies for  $\beta 1i$  and  $\beta 1$ .  $\beta$ -actin was used as

loading control. The results shown above are from three representative patients. Lanes 1–3, cell extracts of normal colonic mucosa; lane 4–6, cell extracts of inflamed colonic mucosa of CD patients

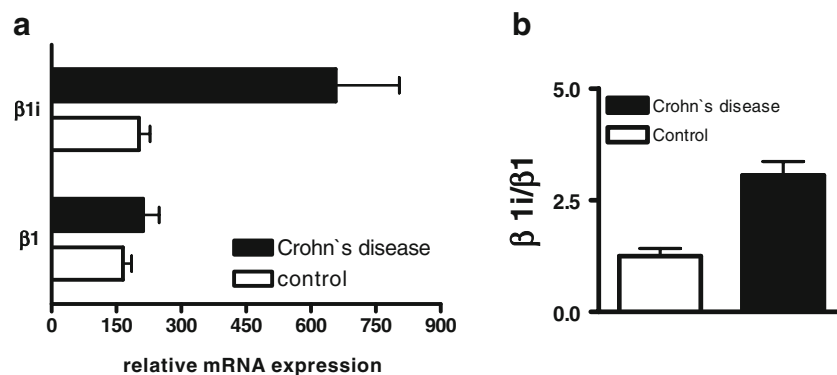
mucosa (Fig. 2a). By analyzing the ratio of immunosubunit  $\beta 1i$  mRNA to its counterpart  $\beta 1$  mRNA, we observed that in inflamed tissue of CD patients, threefold more  $\beta 1i$  mRNA was expressed relative to the  $\beta 1$  mRNA, whereas no significant difference in the expression of  $\beta 1i$  and  $\beta 1$  mRNA was observed in normal colonic tissue (Fig. 2b). These results indicate that due to inflammation significantly increased expression of  $\beta 1i$  mRNA leads to augmented amounts of  $\beta 1i$  protein and that induced overexpression of  $\beta 1i$  prevents incorporation of its constitutive counterpart  $\beta 1$  into 20S proteasomes in CD.

Interestingly, not only mRNA levels of  $\beta 1i$  but also of  $\beta 5i$  were enhanced in CD in comparison to control colonic tissue. However,  $\beta 5i$  mRNA expression was also very high in normal colonic tissue (Fig. 3a). By examining ratio of  $\beta 5i/\beta 5$  mRNA in patients with CD and control patients, we found that mRNA expression of  $\beta 5i$  was approximately fourfold higher than that of mRNA  $\beta 5$  in both groups (Fig. 3b). Previously, our proteasome analysis of distinct mouse and human organs by using two-dimensional gel electrophoresis and mass spectrometry has revealed high amounts of  $\beta 5i$  in normal human intestine [21]. In accordance with this study, we here detected high  $\beta 5i$

mRNA levels in both inflamed colon of CD and control non-inflamed colonic tissue, although mRNA expression of this immunosubunit was even higher in CD (Fig. 3a).

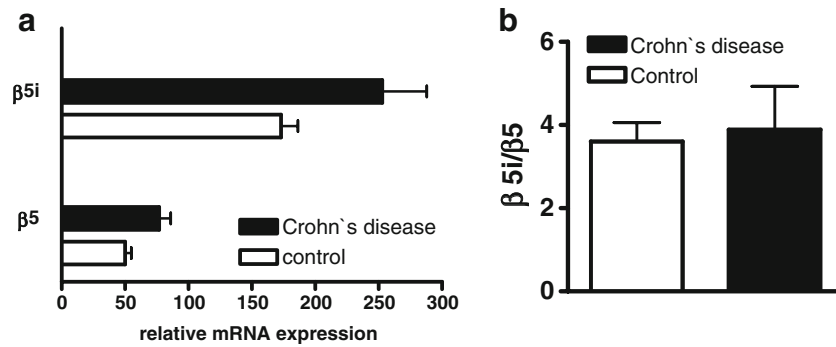
Expression of immunosubunit  $\beta 2i$  is increased in Crohn's disease

$\beta 2i$  is the third inducible catalytic subunit, which has been shown to be preferentially incorporated into the immunoproteasome [22]. To confirm that the immunoproteasome is predominant form of proteasomes in inflamed colonic mucosa of patients with CD we analyzed the protein expression of the immunosubunit  $\beta 2i$  using a polyclonal rabbit antibody detected by immunofluorescence staining. Analysis of colonic mucosa showed that the lamina propria cells as well as epithelial cells of the inflamed tissue in CD were positive after staining of  $\beta 2i$ . No  $\beta 2i$  expressing positive cells were visualized in normal colon (Fig. 4). Thus, we demonstrate here that all three inducible proteasomal immunosubunits are highly expressed in colonic mucosa of patients with CD but not in that of control patients. The  $\beta 5i$  was the only immunosubunit also abundant in normal colon, which indicates that also



**Fig. 2** a Relative mRNA expression of proteasomal subunits  $\beta 1i$  and  $\beta 1$  was analyzed in the colon of controls and CD patients by quantitative real-time PCR. Data represents means  $\pm$  SEM (control,  $n=12$ ; CD,  $n=13$ ). After extraction of total RNA, cDNA was

amplified with specific primers for  $\beta 1i$  and  $\beta 1$ . As housekeeping gene, AK2C was used. b Ratio of  $\beta 1i$  to  $\beta 1$  mRNA expression in colonic mucosa of controls and CD patients (control,  $n=12$ ; CD,  $n=13$ ). Data are presented as means  $\pm$  SEM



**Fig. 3** **a** Relative mRNA expression of proteasomal subunits  $\beta 5i$  and  $\beta 5$  measured in the colonic mucosa of control and CD patients (*control*,  $n=12$ ; *CD*,  $n=13$ ). The results are presented as mean  $\pm$  SEM. Quantitative real-time PCR was performed using specific primers for

$\beta 5i$  and  $\beta 5$ . As housekeeping gene, AK2C was used. **b**  $\beta 5i / \beta 5$  mRNA expression in inflamed colonic tissue of CD patients and control colon tissue (*control*,  $n=12$ ; *CD*,  $n=13$ ). Data are presented as means  $\pm$  SEM

different, more heterogeneous proteasome populations exist which may have different functions in vivo.

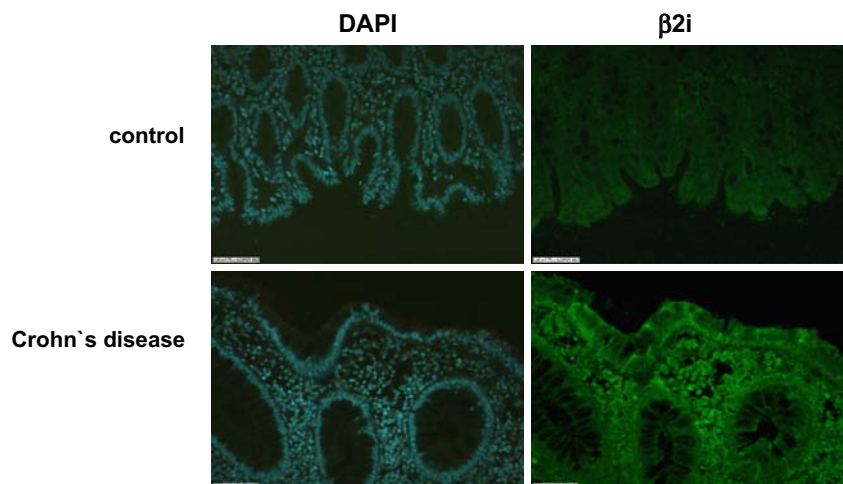
## Discussion

There is increasing evidence that primary dysregulation of the mucosal immune system is one of the most important factors in the development of CD [23]. Inappropriate secretion of proinflammatory cytokines and other inflammation mediators by intestinal macrophages, dendritic cells and T cells has been shown to be responsible for the immune disturbance in CD [24, 25]. The activation of the transcription factor NF- $\kappa$ B plays a pivotal role in responses to inflammatory signaling through Toll-like receptors, TNF receptor superfamily and the IL-1 receptor [26]. While some events triggering the induction of NF- $\kappa$ B activity remain still obscure, it is well-known that ubiquitin-proteasome system contributes directly to activation of this transcription factor—and thus to many aspects of inflammatory responses. The core particle of the enzymatically active 26S proteasome

is the 20S proteasome, a multicatalytic complex composed of 14 different subunits. The two inner rings are formed by the  $\beta$ -type subunits, of which  $\beta 1$ ,  $\beta 2$ , and  $\beta 5$  are catalytically active sites [16]. It has been shown that interferon- $\gamma$  (IFN- $\gamma$ ), the most important cytokine during viral infection, is the central molecule causing the alternation of the proteasomal subunit composition. Upon stimulation of cells with IFN- $\gamma$ , the constitutive catalytic subunits  $\beta 1$ ,  $\beta 2$  and  $\beta 5$  are replaced with  $\beta 1i$ ,  $\beta 2i$  and  $\beta 5i$ , respectively. This replacement of constitutive proteasomes with so-called immunoproteasomes is a way that can promote more efficient generation of epitopes from viral- and tumor-associated antigens suitable for binding to MHC class I molecules [27].

For many years IFN- $\gamma$  has been considered to be one of the most important cytokines involved in the pathogenesis of CD [25, 28]. The aim of this study was to investigate the expression of IFN- $\gamma$ -inducible proteasome immunosubunits both at the mRNA and protein level in the inflamed intestinal mucosa of CD patients. Despite the considerable variations between individual samples, we were able to find

**Fig. 4** Immunofluorescence analysis of proteasomal immunosubunit  $\beta 2i$  in the colon of CD patients and controls (*control*,  $n=12$ ; *CD*,  $n=13$ ). Representative images of staining for  $\beta 2i$ , magnification,  $\times 200$ . Increased expression of  $\beta 2i$  was observed in CD patients



significant changes in colonic mucosa of patients with CD when compared with control patients. Inflamed tissue of patient with CD showed markedly increased levels of immunoproteasomes. An important finding of this study is the pronouncedly high mRNA expression of all three immunosubunits  $\beta 1i$ ,  $\beta 2i$  and  $\beta 5i$  in CD. Interestingly, at the mRNA level, inflammation induces not only immunosubunits but to less extent also constitutive catalytic subunits. However, the induction of immunosubunit mRNAs was significantly stronger resulting in the replacement of constitutive proteasomal proteins by their homologous counterparts. Western blot analysis of the subunits  $\beta 1i$  and  $\beta 1$  clearly showed that the chronic inflammation in Crohn's disease shifts the proteasome subunit expression towards immunoproteasomes at the protein level. Since IFN- $\gamma$  has been shown to be the main inducer of immunoproteasomes in various murine and human cell lines and the IFN- $\gamma$  levels are highly elevated in inflamed intestine of CD patients, we suppose that induction of immunoproteasomes in CD is mediated by this cytokine. However, relatively high constitutive expression of mRNA  $\beta 5i$  was also observed in the control colonic tissue. In accordance with this finding, it has been previously demonstrated that beyond homogenous subtypes of constitutive and immunoproteasomes, also mixed proteasome forms exist in various mouse and human tissues [21, 29].

A major question is whether the difference in proteasome expression we observed in inflamed mucosa of CD patients has a strong functional implication *in vivo*. Many reports have shown that proteasomes are involved in regulation of various proteins important for distinct cellular functions [30, 31]. The replacement of constitutive proteasomes by immunoproteasomes has been reported to be crucial for the generation of active NF- $\kappa$ B subunits [17]. Although it has been demonstrated that the immunosubunits  $\beta 1i$  was essential for the processing of NF- $\kappa$ B precursor protein p105 and for the degradation of the inhibitory protein I $\kappa$ B $\alpha$  in human lymphocytes, little is known about the regulating NF- $\kappa$ B activity by the specific proteasomal subunits. Previously, we have shown that active 20S proteasomes purified from the inflamed intestine of CD patients efficiently degraded the inhibitory NF- $\kappa$ B protein I $\kappa$ B $\alpha$  [11]. This data, together with the present study, suggest that expression and activity of immunoproteasomes in the inflamed intestine of CD patients might be involved in the mechanisms underlying immunopathogenesis of this disease.

In conclusion, our results demonstrate that the development of chronic intestinal inflammation in CD leads to alternations in the subunit composition of 20S proteasomes. Inflamed colonic tissue of patients with CD showed a characteristic pattern of 20S proteasomes which was different from control tissue. Markedly increased mRNA

expression and high protein amounts of immunosubunits were found in CD as compared to control patients. Although this finding shows significant effects of Th1/Th17-mediated inflammation on the composition of proteasomes, further studies including induction of colitis in  $\beta 1i$ ,  $\beta 2i$ , and  $\beta 5i$  deficient mice are needed to determine physiological importance of this proteasomal shift. We suppose that immunoproteasome subunits might be involved in the complex inflammatory response during the chronic inflammation by increasing NF- $\kappa$ B activity.

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