

Original Paper

Digestion

Digestion 2012;86:34–47

DOI: [10.1159/000336353](https://doi.org/10.1159/000336353)

Received: June 9, 2011

Accepted: January 9, 2012

Published online: June 15, 2012

Downregulation of the Ubiquitin-Proteasome System in Normal Colonic Macrophages and Reinduction in Inflammatory Bowel Disease

A.M. Hetzenecker^a M.C. Seidl^a K. Kosovac^a H. Herfarth^b S. Kellermeier^c
 F. Obermeier^a W. Falk^a J. Schoelmerich^a M. Hausmann^c G. Rogler^c

^aDepartment of Internal Medicine I, University of Regensburg, Regensburg, Germany; ^bDivision of Gastroenterology and Hepatology, University of North Carolina (UNC) at Chapel Hill, Chapel Hill, N.C., USA;

^cDivision of Gastroenterology and Hepatology, Department of Internal Medicine, University Hospital of Zurich, Zurich, Switzerland

Key Words

Intestinal macrophages • Proteasome • Immunoproteasome • Ubiquitin • Tolerance

Abstract

Background: In normal mucosa, intestinal lamina propria macrophages (IMACs) maintain tolerance against food antigens and the commensal bacterial flora. Several mechanisms have been identified that mediate tolerance. The ubiquitin-proteasome system (UPS) is a large multiprotein complex that degrades cellular proteins. As the UPS may modulate immune functions of IMACs, we performed a detailed investigation of UPS expression and function under normal conditions and in cells derived from patients suffering from inflammatory bowel disease (IBD). **Methods:** IMACs were isolated from intestinal mucosa. mRNA expression of macrophages differentiated in vitro (i.v. MACs) and IMACs was compared by Affymetrix[®] oligonucleotide arrays. Quantitative Taqman-PCR was performed on five exemplary proteasomal and five ubiquitinylation genes each. Proteins were analyzed by immunohistochemistry and Western blotting. Proteasome function was assessed by a fluorimetric test. **Results:** Affymetrix analysis showed downregulation of mRNA expression of almost all represented proteasomal and of 22 ubiquitination-associated

genes in IMACs as compared to i.v. MACs and monocytes. By quantitative PCR, up to tenfold higher mRNA expression of 10 exemplary genes of the UPS (UBE2A, UBE2D2, UBE2L6, USP14, UBB and ATPase2, β 2, β 5, β 2i/MECL-1, β 5i/LMP7) was demonstrated in i.v. MACs as compared to IMACs. Immunohistochemistry and Western blots confirmed these findings in intestinal mucosa of controls and patients suffering from diverticulitis. In contrast, a significant increase in protein amounts was found in mucosa of patients with IBD. **Conclusion:** Reduced expression of subunits of the UPS in IMACs of normal mucosa supports the concept of the presence of a nonreactive, anergic macrophage phenotype in the gut under normal conditions. Reinduction in IMACs of IBD mucosa reflects activated IMACs which can present antigenic peptides and thus support inflammation.

Copyright © 2012 S. Karger AG, Basel

Introduction

The ubiquitin-proteasome system (UPS) represents the most important intracellular machinery for proteolysis and protein degradation [1–4]. Proteins destined for

A.M.H., M.C.S., M.H. and G.R. contributed equally to the paper.

KARGER

Fax +41 61 306 12 34
 E-Mail karger@karger.ch
www.karger.com

© 2012 S. Karger AG, Basel
 0012–2823/12/0861–0034\$38.00/0

Accessible online at:
www.karger.com/dig

Gerhard Rogler, MD, PhD
 Division of Gastroenterology and Hepatology
 University Hospital of Zurich
 Rämistrasse 100, CH–8091 Zurich (Switzerland)
 Tel. +41 44 255 9479, E-Mail gerhard.rogler@usz.ch

degradation are marked by polyubiquitination and digested by the proteasome [2–4]. Ubiquitin, a compact globular protein of 76 amino acids, is bound to cellular proteins that are going to be degraded.

Ubiquitin is activated by ‘ubiquitin-activating enzyme’ E1 in the presence of ATP, subsequently leading to the formation of ubiquitin-E1 thioester. The ubiquitin-E1 thioester is recognized by ‘ubiquitin-conjugating enzymes’ E2 and ubiquitin is transferred to E2 by a further thioester bond. For ubiquitylation, binding to the E2-ubiquitin complex and direct transfer of ubiquitin to the target protein are required. The ‘conventional’ enzymes E1, E2 and E3 add only a limited number of ubiquitin molecules to the substrate protein. As polyubiquitylation is necessary for the degradation signal, E4 enzymes support the formation of multiubiquitin conjugates.

Before degradation of the desired protein in the proteasome, ubiquitin will be released from the labeled protein. The classical deubiquitylation enzymes are members of the families of ubiquitin-processing enzymes (UBPs) and the ubiquitin carboxy terminal hydrolases (UBH). Normally, UBPs remove ubiquitin from polyubiquitinated proteins. UBHs remove small adducts from ubiquitin and form free monomeric ubiquitin.

The 26S proteasome degrades ubiquitinated proteins and mediates 70–90% of intracellular protein degradation [1, 4]. It is mainly located in the cytosol. It consists of the 20S proteasome and two 19S caps (19S regulator). The 20S proteasome is formed by four stapled heptametrical rings (α 1–7, β 1–7, β 1–7, α 1–7) [5, 6]. The proteolytic activity is found in the inner tube to prevent unspecific degradation of cytosolic proteins.

Besides this constitutive proteasome, a conditional proteasome can be found in humans: the immunoproteasome is mainly induced by microbial infections and/or by IFN- γ [7–11]. In addition to IFN- γ , IFN- β and TNF- α induce the expression of the three immunoproteasome subunits β 1i (LMP2), β 5i (LMP7) and β 2i (MECL-1), which substitute for the β 1, β 5 and β 2 subunits of the proteasome [10, 12]. In mice with a deletion of β 1i and β 5i, reduced expression of MHC class I molecules and reduced numbers of CD8+ T cells were found [13–15].

MHC class I molecules present peptides to CD8+ T cells. To make this possible, microbial protein must be degraded by the UPS. Inhibition of the UPS is followed by impaired function of MHC class I molecules. The UPS, therefore, has a central function for the induction of an adaptive immune response by antigen-presenting cells.

A targeted pharmacological inhibition of the proteasome system has been discussed in a number of diseases

[16–21]. However, before specifically targeting the whole proteasome or only specific (immuneoproteasome) components, such as β 5i/LMP7 or β 2i/MECL-1, it is necessary to investigate its normal tissue expression and function.

What role does the UPS play in intestinal inflammation? The UPS has been shown to degrade I κ B, the inhibitory protein for the proinflammatory transcription factor nuclear factor- κ B (NF- κ B). If there is very low proteasome activity, I κ B cannot be degraded and NF- κ B cannot be activated. In contrast, rapid I κ B degradation by the proteasome or immunoproteasome may trigger a proinflammatory cascade. In addition, antigen presentation is a prerequisite for T cell activation found in the mucosa of patients with inflammatory bowel disease (IBD). A downregulation or lack of the UPS will reduce the number of available antigens (e.g. derived from bacteria) that can be presented.

In a gene array expression study of macrophages differentiated *in vitro* (i.v. MACs) and intestinal lamina propria macrophages (IMACs) we found downregulation of mRNA expression of *virtually all* proteasome subunits in IMACs as compared to i.v. MACs and monocytes. Very similar findings were obtained for proteins of the E1, E2 and E3 groups. This was in line with earlier findings indicating that IMACs from normal mucosa are an anergic cell population mediating tolerance against food antigens and the commensal bacterial flora.

We therefore investigated the expression and function of proteins of the ubiquitylation machinery and the proteasomal degradation machinery during the differentiation of IMACs. We focused on several genes and proteins exemplary for the other genes found to be regulated in the array analysis.

Materials and Methods

Patients

For Affymetrix® arrays, mRNA from isolated IMACs was pooled from three mucosa specimens each (6 ‘controls’ in total) and 3 samples of i.v. MACs (6 in total). Additional tissue samples were obtained from intestinal mucosa of 18 patients with Crohn’s disease (CD), 10 patients with ulcerative colitis (UC), 22 controls with no intestinal inflammation, who underwent surgery for other reasons (e.g. colon cancer) and 5 ‘inflammatory controls’ with diverticulitis. For more details, see online supplementary table 1 (see www.karger.com/doi/10.1159/000336353 for all online suppl. material). The study was approved by the University of Regensburg Ethics Committee.

Isolation and Purification of IMACs

Surgical specimens from inflamed and normal mucosa were obtained by surgery (online suppl. table 1) after obtaining informed

consent. Control specimens were taken from patients who underwent surgery for other reasons (online suppl. table 1). Lamina propria mononuclear cells were isolated as published [22]. IMACs were labeled with anti-CD33 immunomagnetic MicroBeads and purified twice using type LS separation columns (Miltenyi Biotec, Bergisch Gladbach, Germany) [22]. The purity of the isolated macrophage populations was analyzed by flow cytometry after uncoupling of the anti-CD33 beads. Staining of the positive fraction demonstrated >90% purity of the isolated cells as described [22]. If purity was less, the cells were not used for further experiments.

Generation of *i.v.* MACs

Peripheral blood mononuclear cells were separated by leukapheresis of healthy donors. Monocytes were isolated by counter-current centrifugal elutriation in a J6M-E Beckmann centrifuge with a standard chamber and a JE-5 rotor at 2,500 rpm and a flow rate of 20 ml/min [22]. To induce monocyte to macrophage differentiation, purified monocytes were cultured in RPMI-1640 supplemented with 5×10^{-5} M mercaptoethanol, polyvitamins, antibiotics, pyruvate, nonessential amino acids and 2% human AB group serum (endotoxin <0.1 EU/ml as measured in the Limulus assay) on Teflon foils at 10^6 cells/ml (Biofolie 25, Heraeus, Hanau, Germany) for 7 or 14 days.

Affymetrix Oligonucleotide Array Analysis

Affymetrix GeneChip® analysis was performed with the help of the Kompetenzzentrum für fluoreszente Bioanalytik (KFB) in Regensburg. mRNA was isolated from IMACs from noninflamed mucosa and from *i.v.* MACs using polyT magnetic beads (Dyna, Oslo, Norway). In each case, RNA from 3 patients was pooled to avoid individual differences responsible for variation.

Double-stranded cDNA was synthesized by a Superscript II kit (Life Technologies, Karlsruhe, Germany) using T7-oligo(dT) as primer. Biotin-labeled cRNA was prepared by *in vitro* transcription reaction using an Enzo BioArray HighYield RNA Transcript Labeling Kit (Affymetrix, P/N 900182). The biotin-labeled cRNA was purified, fragmented and after addition of Control Oligonucleotide B2 (3 nM) and 20× Eukaryotic Hybridization Controls (bioB, bioC, bioD, cre) hybridized to Affymetrix HG-U133A GeneChips (Affymetrix, Santa Clara, Calif., USA), scanned on the Affymetrix array scanner and data analysis was performed using the Affymetrix statistical data analysis software, Affymetrix Microarray Suite (version 5.0).

Antibodies

The antibodies used in this work are summarized in online supplementary table 2. For the immunohistochemical identification of IMACs, mouse antihuman macrophage CD68 (clone KP1, DAKO, Hamburg, Germany) was applied. For immunofluorescence, an Alexa Fluor® 488 F(ab')₂ fragment of goat antimouse IgG (H + L) (Invitrogen, Karlsruhe, Germany) was used.

Western Blotting

After SDS-PAGE, the proteins were transferred to a nitrocellulose membrane (Optitran BA-S 83, Schleicher & Schuell). Transfer was performed in a semi-dry manner (Biometra Fastblott B44, Biometra, Göttingen, Germany) with 1.5 mA/cm² for 1.5 h. The membrane was incubated in 5% low-fat milk in PBS (blocking solution) for 1 h at room temperature. Incubation was performed at 4°C overnight. On the next day, the membrane was incubated

three times for 10 min in PBS/0.2% Tween 100 (washing buffer) and the HRP-conjugated secondary antibody (antirabbit, Amersham) was added at a dilution of 1:2,000 in blocking solution for 1 h at room temperature. After washing, detection with enhanced chemiluminescence (ECL) followed.

Immunohistochemistry

For peroxidase staining, frozen sections were cut (5 μm), air dried and fixed in acetone. Slides were rehydrated with PBS (pH 7.4) and incubated for 30 min with 0.3% H₂O₂ in PBS to quench endogenous peroxidase. Slides were washed with PBS and incubated for 60 min with 1% bovine serum albumin (Biomol, Hamburg, Germany) in PBS (blocking buffer) with goat serum (dilution 1:200, Dako, Denmark).

Microscopy was done with a Leitz DMRXE microscope with 400× magnification. Information about the magnification is given in the figure legends.

Real-Time PCR (Taqman®)

The primers used for quantitative PCR are given in online supplementary table 3.

A melting temperature of 60°C was chosen and the internal primers were labeled with 5'-FAM and 3'-TAMRA. DNA fragments of the 5 ubiquitinylation enzymes UBE2A, UBE2D2, UBE2L6, USP14 und UBB and the proteasome subunits ATPase 2, β2, β5, β2i and β5i were amplified with the respective primers. The reporter dye for the investigated mRNA was 6-Fam; for the housekeeping gene GAPDH, VIC for quenching TAMRA (6-carboxy-tetramethyl-rhodamine) was used. The primers were designed by Primer Express 1.5 Software (PE Applied Biosystems, Foster City, Calif., USA) and synthesized by MWG Biotech (Ebersberg, Germany). The primers for GAPDH and the Taqman-Master Mix were from PE Applied Biosystems. Reactions were carried out in a volume of 20 μl in triplicates. A dilution series was prepared for each primer set. Values were plotted and the slope of the graph was determined. The efficiency of the reaction was determined by calculating $E = 10^{-1/\text{Slope}}$. The error was determined by calculating $(2^n/E^n - 1) \times 100$ with $n = Ct$ and given in percent.

Proteasome Function

Proteasome activity was quantified with the Proteasome Activity Assay Kit (Chemicon®, Nurnberg, Germany) in biopsies from healthy and inflamed mucosa as indicated by the manufacturer. The test is based on the detection of the fluorophor 7-amino-4-methylcoumarin (AMC) after proteasomal cleavage from LLVY-AMC as a substrate and quantified in a fluorimeter at 380/460-nm wavelengths. For assay quantification, the AMC standard was used. For the AMC standard curve, a stepwise dilution with concentrations from 0.04 to 12.5 μM was performed. In addition, a positive control curve with the provided positive control was included in the assay with a stepwise dilution of the master solution with assay buffer from 1:4 to 1:256. The 96-well plate was incubated for 1–2 h.

Statistical Analysis

Data are expressed as means ± standard deviation if not otherwise stated. Statistical differences were evaluated using an unpaired Student's t test. $p < 0.05$ was considered statistically significant. All statistical testing was performed using SigmaPlot®.

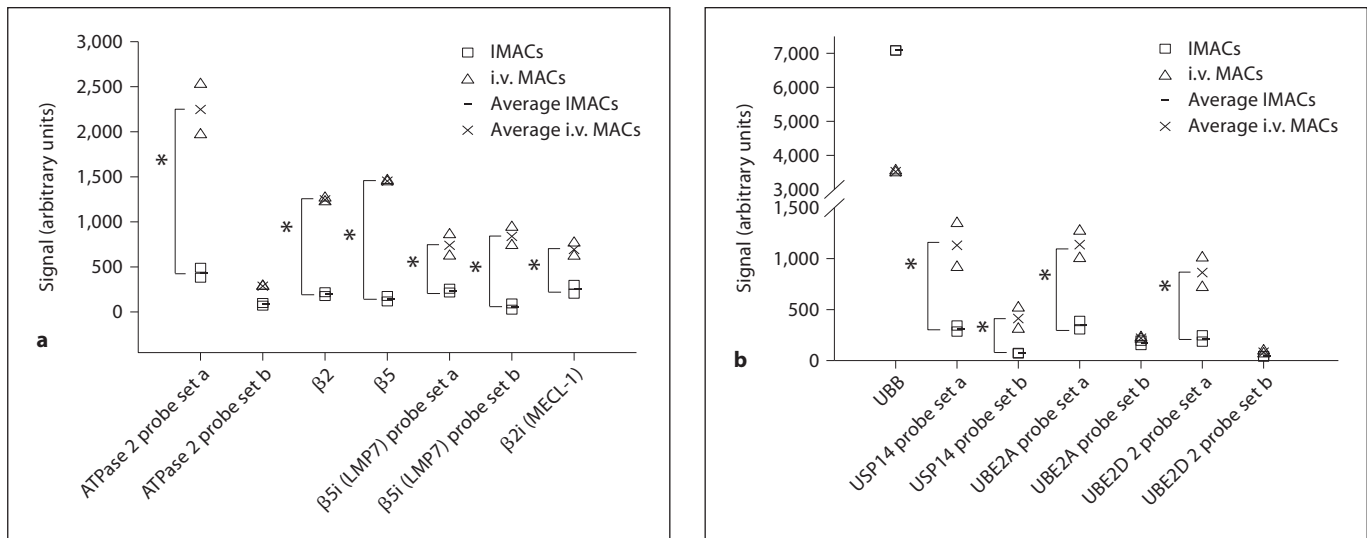


Fig. 1. Results of the Affymetrix genechip arrays in four selected proteasomal subunit genes (a) and four selected ubiquitinylation genes (b). * $p < 0.05$. For some mRNAs, only one probe set revealed a significant difference. Of note: the relation of UBB expression between IMACs and i.v. MACs was inverted as compared to all other genes.

Results

Affymetrix GeneChip Analysis

For Affymetrix GeneChip analysis, mRNA from 3 controls and 3 i.v. MAC donors was pooled and hybridized onto two chips. Affymetrix analysis showed a significant downregulation of virtually all proteasomal components as well as the ubiquitinylation machinery in IMACs (online suppl. tables 4 and 5).

There were 46 probe sets for proteasome subunits representing 35 different genes. For all probe sets, a higher expression in i.v. MACs as compared to IMACs was observed. The ratio $\text{signal}_{i.v. \text{ MACs}}/\text{signal}_{\text{IMACs}}$ was between 1.6 and 13.6 with an average of 4.3. Different probe sets for the same mRNA gave similar results (online suppl. table 4). This pointed to a lower mRNA expression of all proteasomal components in IMACs from normal mucosa as compared with i.v. MACs.

Similar observations were made for mRNA expression of genes of the ubiquitinylation machinery (online suppl. table 5): Out of 64 probe sets representing 42 genes, the majority had clearly a higher signal in i.v. MACs. This was not observed only for ubiquitins B and C, which showed a high signal in both i.v. MACs and IMACs. Ubiquitin D, ubiquitin carrier protein and some other probe sets showed very low signals, making the results unreliable. In general, mRNA of the proteasome and the ubiquitinyl-

ation machinery showed reduced expression in IMACs as compared to i.v. MACs.

To confirm these results by quantitative PCR, we chose five genes each. ATPase 2 was selected as it is an important functional component. The $\beta 2$ - and $\beta 5$ - as well as $\beta 2i$ - and $\beta 5i$ -subunits were chosen as they are exchanged against each other in the immunoproteasome. The specific results for all probe sets of the genes further analyzed are shown in figure 1. Ubiquitin B was further analyzed as it seemed to be regulated differently and highly expressed as well.

Quantification of mRNA Expression of Proteasome and Ubiquitinylation Genes in IMACs and i.v. MACs

ATPase2 expression was not found to be significantly higher in i.v. MACs as compared to IMACs (fig. 2a). For all four proteasomal subunits ($\beta 2$, $\beta 5$, $\beta 2i/\text{MECL-1}$ and $\beta 5i/\text{LMP7}$) we found higher expression in i.v. MACs as compared to IMACs from control mucosa (fig. 2a), thus confirming the array results.

Similar results were obtained for the ubiquitinylation-associated genes. However, a significant difference in mRNA expression between i.v. MACs and IMACs was only observed for UBE2A, UBE2D2, and surprisingly UBB (which was different to the gene chip analysis) (fig. 2b). No difference was observed for mRNA expression of UBE2L6 and USP14 (fig. 2b).

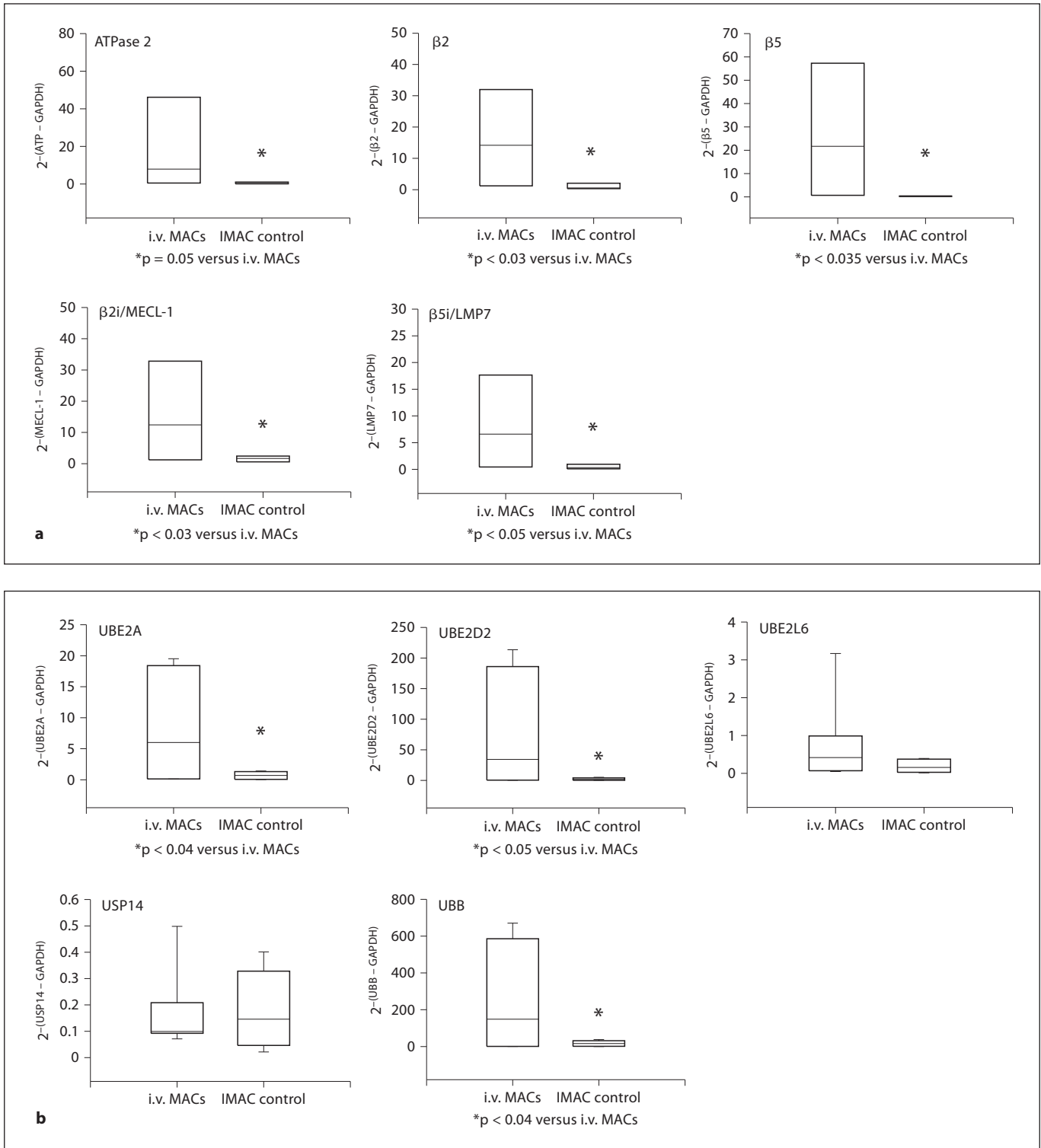


Fig. 2. Quantification of mRNA expression of five selected proteasomal subunit genes (**a**) and five selected ubiquitinylation genes (**b**) in IMACs and i.v. MACs. mRNA expression of the respective genes was determined by quantitative PCR in i.v. MACs and IMACs. **a** mRNA expression was higher in i.v. MACs in all

investigated proteasomal subunits. **b** A significant difference in ubiquitinylation genes was only found in UBE2A, UBE2D2 and UBB. No difference was observed in mRNA expression of UBE2L6 and USP14.

Quantification of mRNA Expression of Proteasome and Ubiquitination Genes in IBD and Diverticulitis Patients

mRNA expression of the respective genes was determined by quantitative PCR in IMACs isolated from patients with active and inactive CD or UC as well as diverticulitis. In general, mRNA expression of the respective genes in IMACs from diverticulitis mucosa did not differ significantly from control IMACs (fig. 3a, b). The situation was different in IBD-derived IMACs. In general, IMACs isolated from active UC mucosa showed a clear increase in the expression of proteasomal subunits (fig. 3a) which almost reached significance for ATPase2 ($p = 0.06$). In contrast, no differences were found if IMACs were isolated from mucosa of UC patients in remission. For CD, the situation was more complex. Only the expression of the $\beta 2$ subunit was significantly increased in IMACs isolated from active CD ($p = 0.016$, fig. 3a). For the expression of all other investigated proteasomal subunits, no significant difference was found. In addition IMACs from CD patients in remission showed no alterations of mRNA expression. Interestingly, the expression of the $\beta 2$ subunit was increased but not of that of the immunoproteasome subunits $\beta 2i/MECL-1$ and $\beta 5i/LMP7$. There was a trend towards an increased mRNA expression of these genes in IMACs from active CD as compared to IMACs from CD in remission and control IMACs (fig. 3a).

The situation found for the ubiquitination genes (fig. 3b) corresponded to the findings described above. No difference in mRNA expression of the respective genes was found for IMACs isolated from diverticulitis mucosa. A significantly increased mRNA expression in IMACs isolated from patients with active UC was found for UBE2D2, UBE2L6, USP14 and UBB (fig. 3b). No significant difference was found for UBE2A. Again, there was a trend towards an increased mRNA expression of these genes in IMACs from active CD as compared to IMACs from CD in remission and control IMACs (fig. 3b).

Comparison of mRNA Expression of Proteasome ($\beta 2$ and $\beta 5$) and Immunoproteasome Subunits ($\beta 2i/MECL1$ and $\beta 5i/LMP7$) in Normal and IBD Mucosa

As not only the comparison with control conditions but also the direct relation of $\beta 2$ and $\beta 5$ to $\beta 2i/MECL1$ and $\beta 5i/LMP7$ proteasomal subunits might be relevant, we directly compared the mRNA expression of these proteasomal subunits (fig. 4). Expressions of both proteasomal and immunoproteasomal subunits were low and

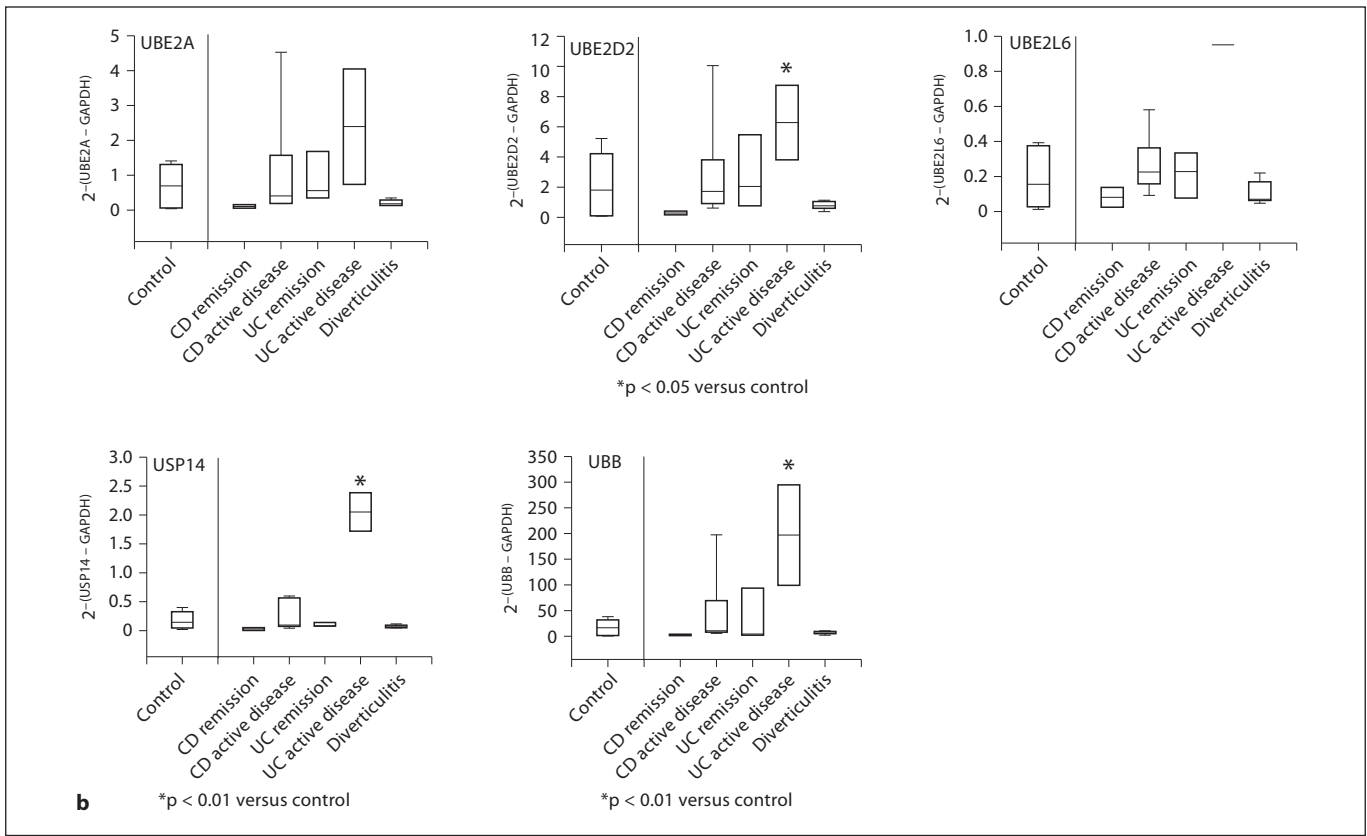
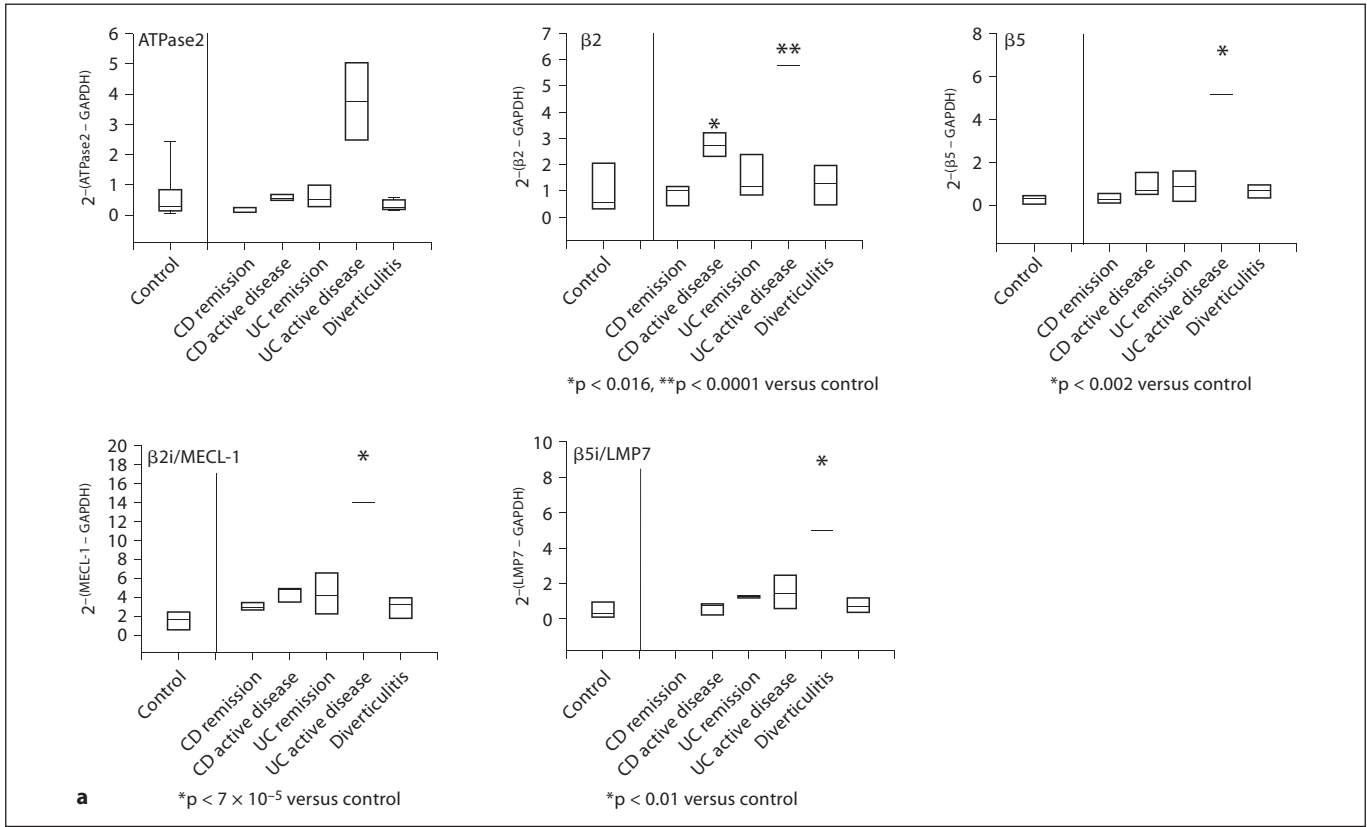
comparable in IMACs from normal mucosa. A clear trend towards an upregulation of the immunoproteasome subunits was observed in IMACs isolated from CD and UC patients in remission (fig. 4). In active disease, expression of $\beta 2$ and $\beta 5$ as well as $\beta 2i/MECL1$ and $\beta 5i/LMP7$ was further increased (fig. 4).

Quantification of mRNA Expression of Proteasome and Ubiquitination Genes in Circulating Monocytes Isolated from IBD Patients and Controls

To investigate whether our findings in IMACs reflect a functional difference in this specific cell population or may simply be attributed to invading monocytes, we quantified the expression of the five proteasome and ubiquitination genes in circulating monocytes from IBD patients (fig. 5). We further evaluated the impact of steroid treatment on mRNA expression. Surprisingly, the findings in circulating monocytes were completely different as compared to IMACs but very homogeneous for all investigated genes. In figure 5, six of the ten genes are shown. The mRNA expression of the investigated genes was comparable to that of IMACs for circulating monocytes from healthy controls. In contrast, the mRNA expression of all investigated genes was significantly downregulated in monocytes isolated from IBD patients irrespective of steroid treatment and disease activity (fig. 5). The data indicate that the upregulation of proteasomal subunits found in IBD IMACs is not caused by a contamination with infiltrating monocytes.

Differences in Protein Amounts of Proteasome and Ubiquitin Subunits in Control Mucosa and IBD Patients

To investigate whether the differences found for mRNA expression would be translated into differences in protein amounts, we performed Western blots, immunofluorescence and immunohistochemistry. As antibodies were not available for all techniques and for all investigated proteins, we analyzed each protein by at least one of these methods. In figure 6a, Western blots for specific proteasomal subunits are shown. In addition to the expected 50-kDa band for ATPase 2 in IBD patients, an additional 20-kDa band was detected which was most prominent in UC-derived tissue. The positive control clearly indicated that the 50-kDa band was specific. After densitometry, a significant upregulation of ATPase 2 protein in IBD-derived IMACs was observed. The same was true for $\beta 2i/MECL$ (CD vs. control: $p < 0.003$; UC vs. control: $p < 0.05$) and $\beta 5i/LMP7$ (CD vs. control and UC vs. control: $p < 0.02$ each) (fig. 6a). These findings confirmed



3

an upregulation of proteasomal subunits also on the protein level. Similar findings were obtained for the investigated ubiquitinylation proteins (fig. 6b): for UBE2A, one distinct band was detected at 28 kDa, which was lower than the estimated molecular size. Densitometry revealed a significant difference (IBD vs. control: $p < 0.01$). UBE2D2 protein was hardly detectable in controls at all and clearly induced in IBD (fig. 6b). UBE2L6 protein was similarly upregulated in IBD. Two bands were detected. The 17-kDa band corresponds to the expected protein

size. The 45-kDa band could reflect a trimer or a complex with another protein as it showed parallel differences to the 17-kDa band. The differences versus control were significant for both the 17-kDa band ($p < 0.01$) and the 45-kDa band ($p < 0.04$). As we found no antibody for Western blot detection of USP14, we next investigated UBB. Again, there was a significant increase of UBB (densitometry: $p < 0.005$). For all performed Western blots, the diverticulitis protein isolated showed results similar to controls.

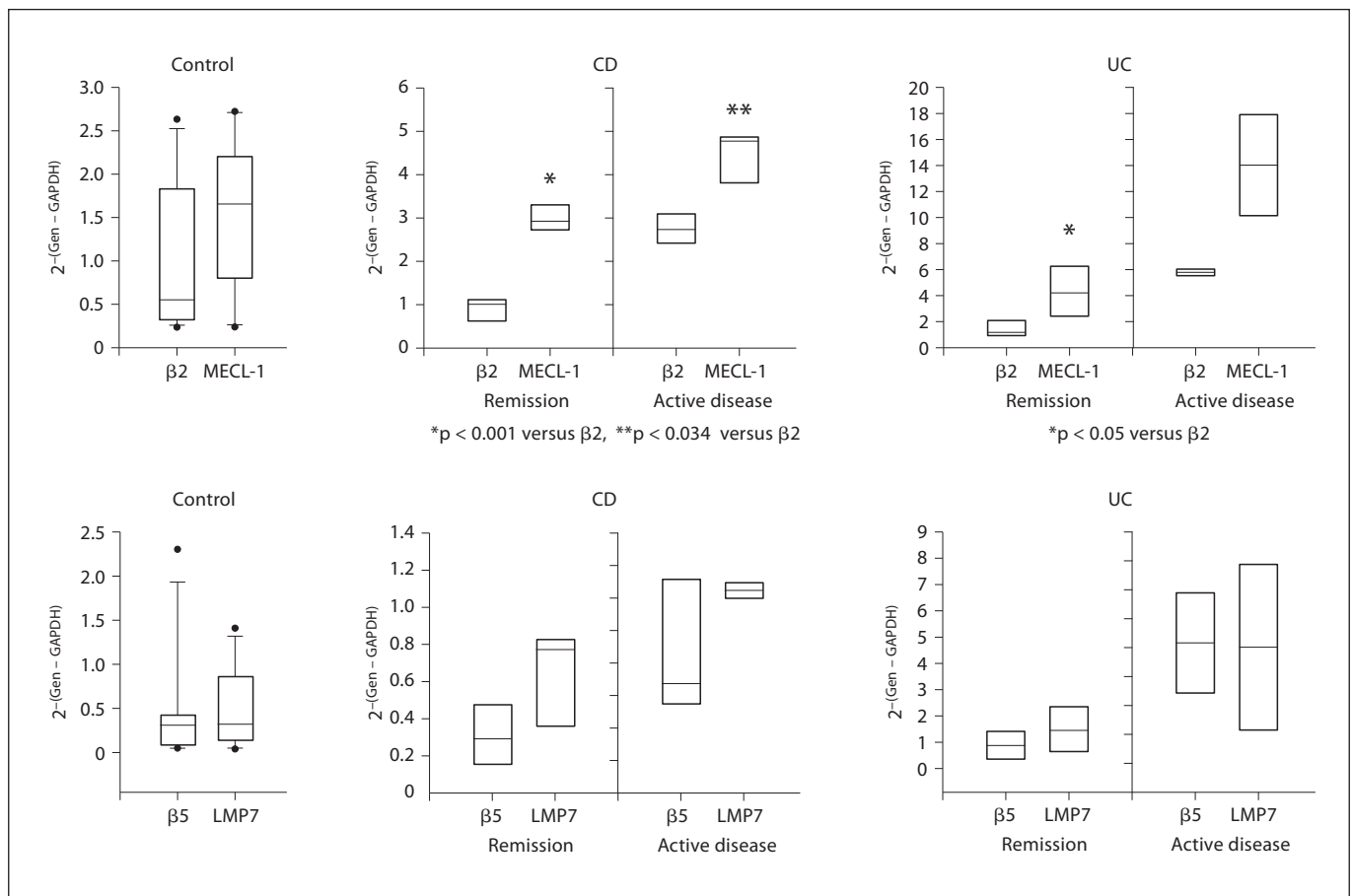


Fig. 3. Quantification of mRNA expression in five selected proteasomal subunit genes (a) and five selected ubiquitinylation genes (b) in IMACs isolated from control mucosa, CD in remission, active CD, UC in remission, active UC and diverticulitis mucosa. In general, mRNA expression in IMACs isolated from diverticulitis patients was not different from IMACs from normal mucosa. The same was true for IMACs isolated from patients with CD and UC in remission. In patients with active CD, there was increased expression, which was significantly higher in active UC. Obviously, not only immunoproteasome subunit expression was increased under the mentioned conditions.

Fig. 4. Quantification of mRNA expression of proteasome ($\beta 2$ and $\beta 5$) and immunoproteasome subunits ($\beta 2i$ /MECL1 and $\beta 5i$ /LMP7) in normal and IBD mucosa. **a** Comparison of $\beta 2$ and $\beta 2i$ /MECL1 mRNA expression in IMACs isolated from normal mucosa, CD in remission, active CD, UC in remission and active UC. **b** Comparison of $\beta 5$ and $\beta 5i$ /LMP7 mRNA expression in IMACs isolated from normal mucosa, CD in remission, active CD, UC in remission and active UC.

In control specimens, no positive cells could be detected in the lamina propria by immunohistochemistry (fig. 6c). Cells positive for $\beta 2i$ /MECL and $\beta 5i$ /LMP7 were detected in the lamina propria of CD and UC specimens. Very similar findings were obtained for UBE2L6, USP14 and UBB (fig. 6d). Again, cells staining positive for the respective proteins were only detected in CD and UC specimens. This was further confirmed by immunofluorescence (online suppl. material). To ensure that the cells expressing ubiquitination proteins in IBD patients were IMACs, we applied double labeling immunofluorescence techniques. For UBE2L6, USP14 and UBB, very similar staining patterns were observed: In specimens derived from normal mucosa, CD68+ macrophages were easily

detected. Only in mucosal specimens from CD and UC patients (but not in diverticulitis-derived tissue) was a green fluorescence (ubiquitinylation proteins) found that resulted in a yellow/orange overlay (online suppl. material). This clearly indicated that the expression of those proteins was induced in IMACs by chronic but not by acute inflammation.

Induction of Proteasome Function in IBD Patients

To study whether the observed induction of proteasome and ubiquitinylation gene mRNA and increased protein was also followed by increased proteasomal function, we applied a simple fluorimetric assay (fig. 7). There was a significant difference between control and IBD tis-

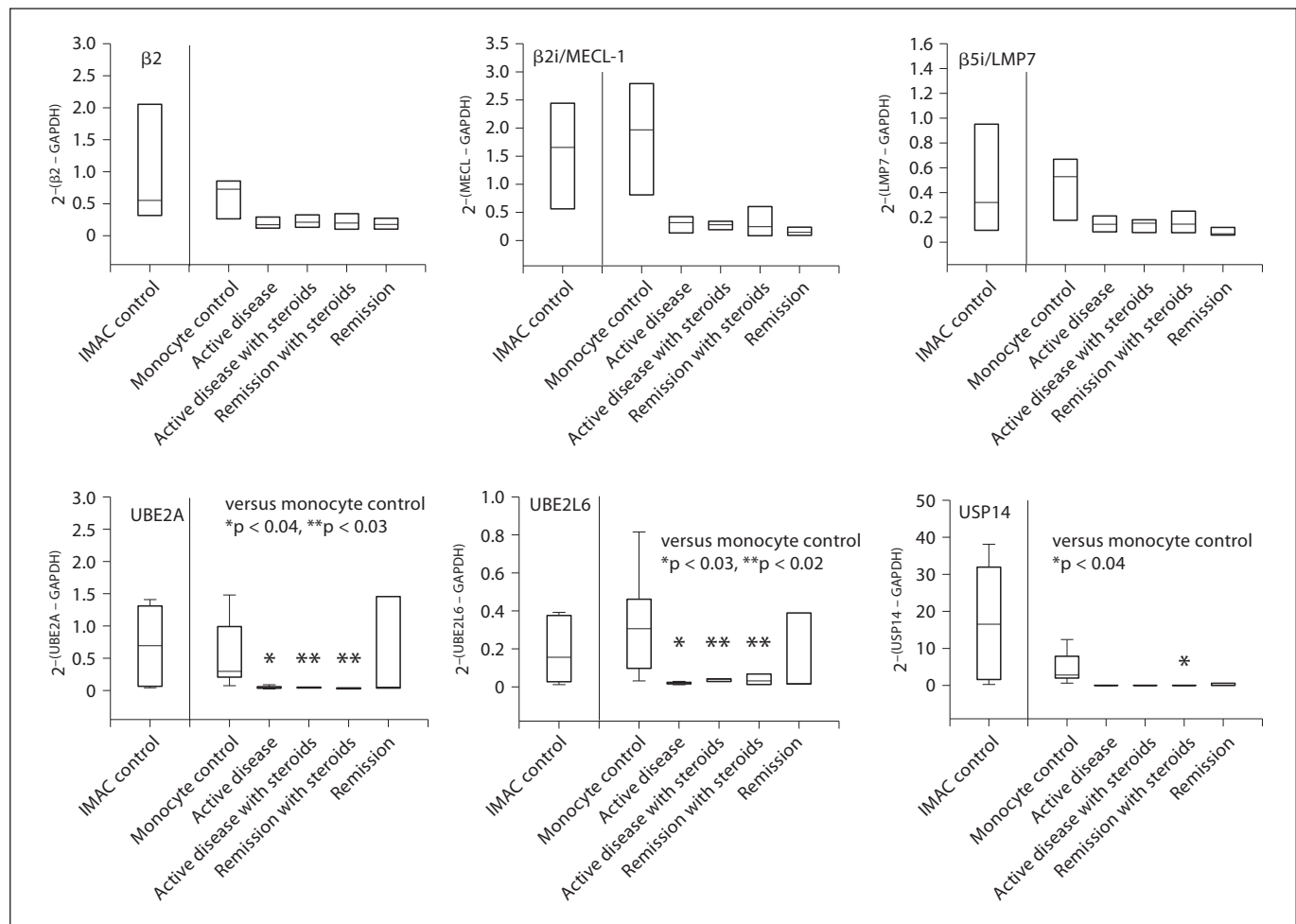


Fig. 5. Quantification of mRNA expression of proteasome and ubiquitination genes in circulating monocytes isolated from IBD patients and controls. The expression of the investigated mRNAs was comparable in IMACs from control mucosa and circulating

monocytes from healthy blood donors. In contrast, there was a clear downregulation of mRNA expression in monocytes of IBD patients, no matter how active the disease was and independent of the medication.

sue with respect to proteasomal function (fig. 7). As we analyzed proteasome function in protein lysates from biopsies, there is no parameter directly correlating proteasomal function to the amount of IMACs present in the tissue. Therefore, the evidence that proteasome and ubiquitinylation function is induced in IMACs from patients suffering from IBD is indirect at best.

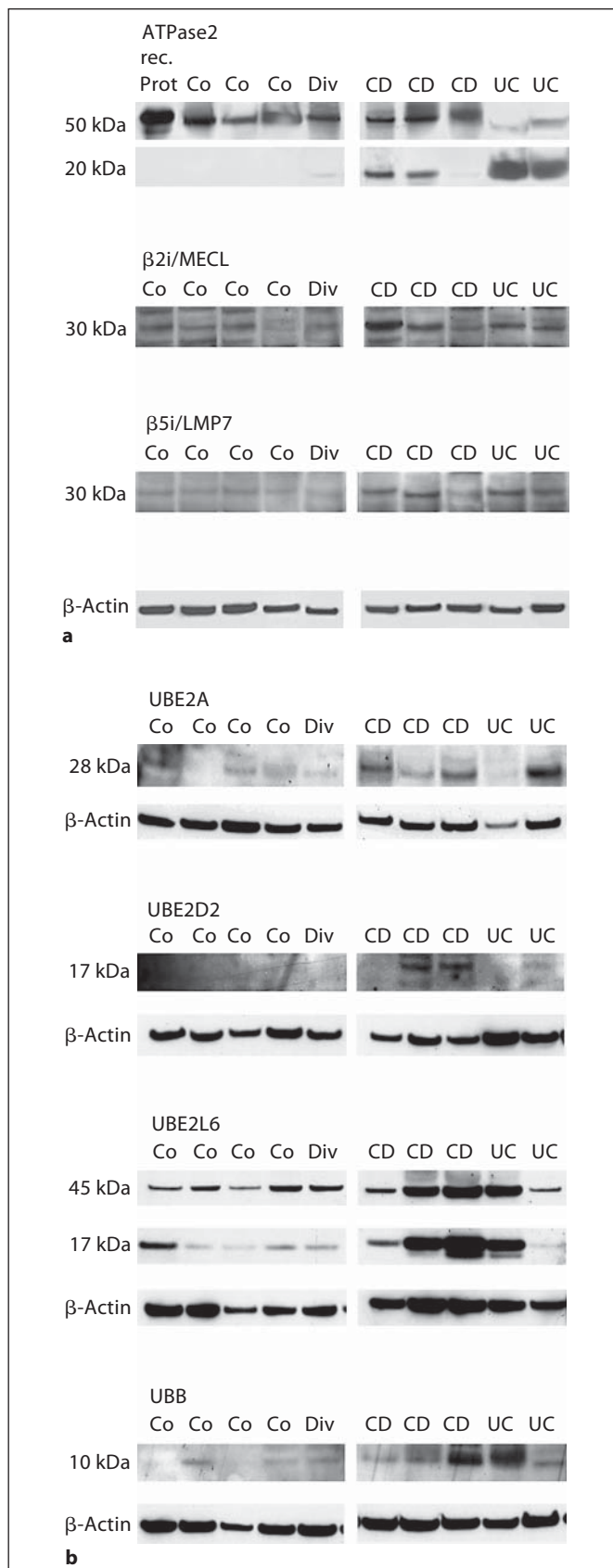
Discussion

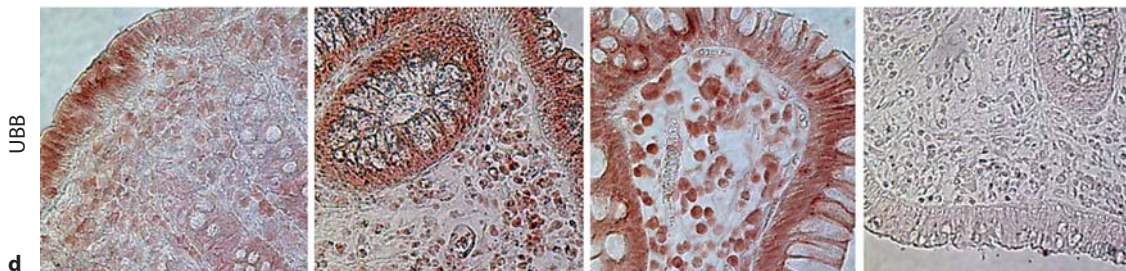
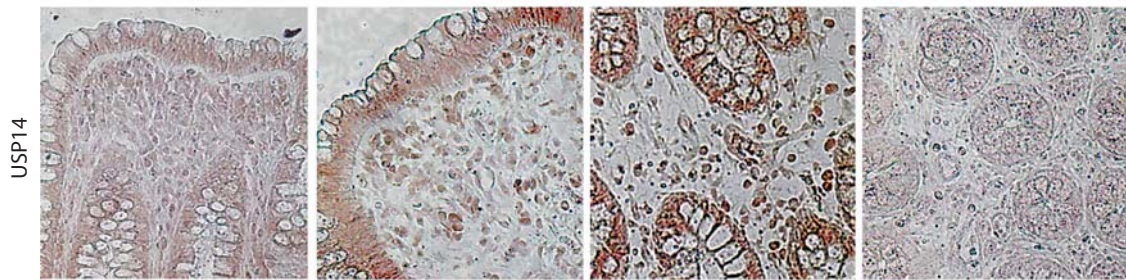
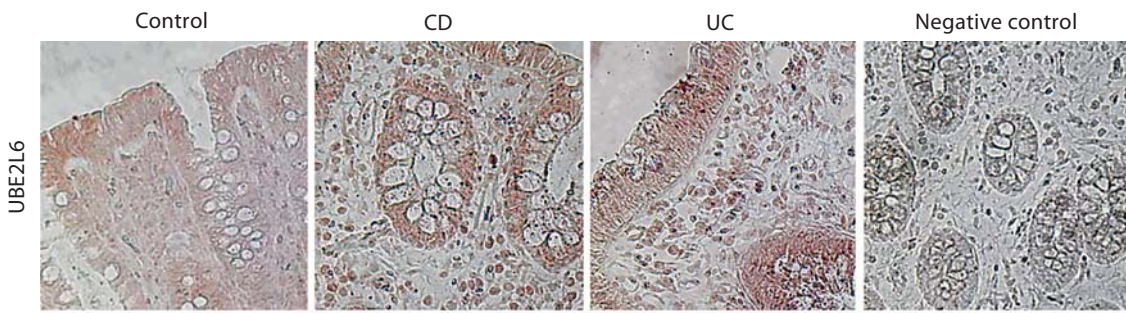
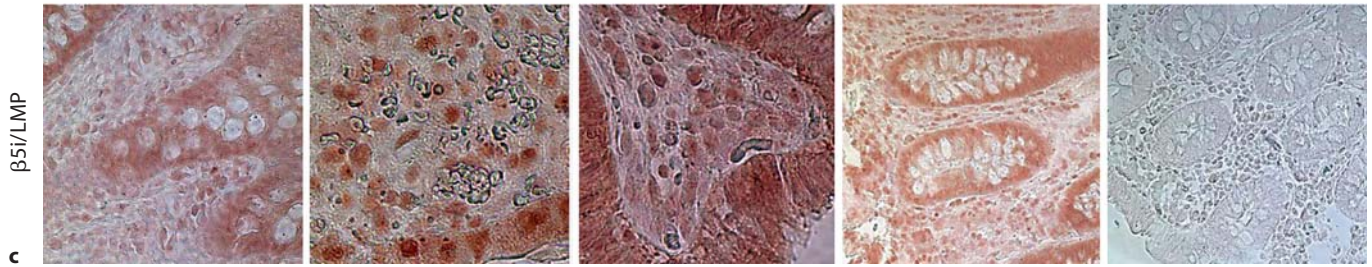
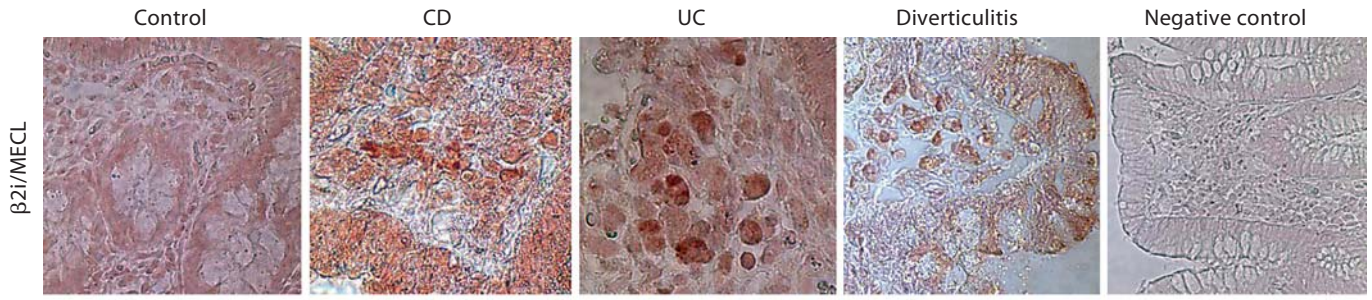
In this work, we demonstrated that the ubiquitinylation machinery and expression of proteasomal components is downregulated in IMACs as compared to i.v. MACs. This indicates that proteasomal function is decreased in IMACs. As proteasomal function, in addition to being associated with the intracellular degradation of proteins [23–25], is also important for antigen processing leading to antigen presentation [26], our data further explain why IMACs are ‘anergic’ and mediate tolerance. In this respect, the downregulation of the whole proteasomal machinery may be crucial for the prevention of mucosal inflammation.

This again sheds light on the specific function of IMACs and is in line with former reports demonstrating lower levels of MHC II molecules [22, 27–29] and costimulatory molecules [22, 30–33]. It is noteworthy that no specific part of the ubiquitinylation machinery or only the immune proteasome appears to be downregulated in IMACs. The reduced proteasomal activity affects all aspects as reflected by the homogeneous pattern in the array analysis that was confirmed by all subsequent mRNA and protein data as well as the functional assay.

In contrast, IMACs from IBD mucosa but not from diverticulitis mucosa showed mRNA and protein upregulation of most of the investigated components. This indicates that chronic but not acute inflammation alters the proteasomal function of IMACs. Our data further provide evidence that this functional difference is not only

Fig. 6. Protein expression of proteins involved in proteasome activity and ubiquitination in IMACs. **a** Protein expression of ATPase2, β 2i/MECL and β 5i/LMP was clearly higher in IMACs isolated from CD and UC mucosa as compared to controls. **b** Protein expression of UBE2A, UBE2D2, UBE2L6 and UBB was increased in IMACs derived from CD and UC mucosa as compared to control IMACs.





6 d

due to the influx of newly recruited monocytes which are attracted to the site of inflammation. In monocytes isolated from peripheral blood of IBD patients, the expression of ubiquitinylation gene expression and the proteasomal machinery were downregulated. Therefore, the differences found in the mucosa are likely to be local differences associated with IBD-specific events. As proinflammatory mediators also circulate in the bloodstream, this additionally indicates that the upregulation of the proteasomal machinery is not simply an unavoidable consequence of the presence of those soluble proinflammatory mediators.

In contrast to others [34], we did not find a striking difference in proteasome subunit composition in the inflamed mucosa of CD and UC patients. The induction of expression of the immune proteasome subunits $\beta 2i$ and $\beta 5i$ in IMACs from IBD mucosa was more pronounced as compared to $\beta 2$ and $\beta 5$ expression; however, the mRNA and protein of the latter also was increased in active IBD. It has been speculated that a difference in composition of the proteasome and a shift to the expression pattern of the immune proteasome may be followed by increased NF- κ B activation. Steinhoff's group suggested that increased processing of p105 and rapid degradation of $\text{I}\kappa\text{B}\alpha$ by immunoproteasomes in CD patients may be responsible for the enhanced expression of inflammatory genes regulated by p50/c-Rel and p50/p65 heterodimers [34]. In contrast, our data indicate that there is no selective induction of specific proteasome subunits but a more or less general induction of proteasomal function without major selectivity that not only induces degradation of p105 or $\text{I}\kappa\text{B}$ but also changes all proteasome-associated functions.

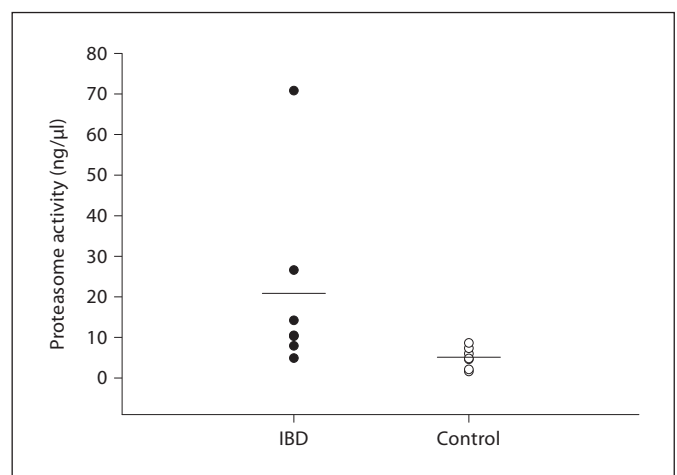
Fig. 6. Protein expression of proteins involved in proteasome activity and ubiquitination in IMACs. **c** $\beta 2i$ /MECL and $\beta 5i$ /LMP expression in the lamina propria as demonstrated by immunohistochemistry was low under noninflamed conditions. In contrast, in CD and UC specimens, there was highly positive immunohistochemical staining in the lamina propria probably in IMACs. **d** Similarly, protein expression of UBE2L6, USP14 and UBB was increased in lamina propria cells in the specimens derived from CD and UC mucosa.

Fig. 7. Proteasomal activity assay of tissue from IBD and normal mucosa. A clear difference between IBD mucosa and control tissue was found, indicating that proteasome and ubiquitinylation function might be induced in IMACs from patients suffering from IBD.

Indeed, NF- κ B activation is increased in IBD IMACs as our group has shown before [35]. However, this increased activation is associated with an impaired barrier function and NOD2 variants [36]. It would be of interest to further study whether the degree of induction of proteasomal functions is associated with bacterial translocation and NOD2 variants.

Clearly, the increased presence of proteasomal proteins was dependent on disease activity. Surprisingly, it was much less influenced by treatment. Therefore, it remains unclear whether induction of proteasomal function results from chronic inflammation and the presence of proinflammatory mediators [37, 38] or whether, vice versa, strongly increased proteasomal functions make patients more treatment refractory.

As striking evidence for the involvement of the commensal flora in the pathogenesis of IBD has emerged in recent years [39–41], the question arises how our findings could be related to these insights in disease pathophysiology. First, it may be speculated that downregulation of the proteasomal machinery prevents antigen presentation of nutritional proteins and antigens of commensal bacteria [42]. The increased expression in IBD mucosa is in line with findings of a broken tolerance to food antigens [43] or the own commensal flora that has been observed during active disease [44, 45]. In fact, the data of Duchmann et al. [44, 45] are frequently misinterpreted as a general intolerance against the own bacterial gut flora in CD patients. Those data show that intolerance only occurs in active disease, which would be in line with increased antigen presentation during this disease state.



As under normal, noninflamed conditions expression of proteasomal genes is very low, it may be speculated that an intestinal mucosa-specific inhibition of the proteasome could be a treatment option for IBD patients to restore IMAC tolerance or treatment response if it turns out that refractory patients have an especially high proteasomal activity. In contrast, our data clearly indicate that proteasomal inhibition as a treatment for the maintenance of remission will most likely not be useful.

Acknowledgements

This study was supported by Deutsche Forschungsgemeinschaft (SFB 585 Projekt A6 and Ro1236/14-1, 14-3) and BMBF Kompetenznetz CED. We thank the surgeons of the Department of Surgery of the University of Regensburg for their excellent cooperation.

Disclosure Statement

The authors have no conflict of interest to disclose.

References

- Demartino GN, Gillette TG: Proteasomes: machines for all reasons. *Cell* 2007;129:659–662.
- Segref A, Hoppe T: Think locally: control of ubiquitin-dependent protein degradation in neurons. *EMBO Rep* 2009;10:44–50.
- Hochstrasser M: Origin and function of ubiquitin-like proteins. *Nature* 2009;458:422–429.
- Hirsch C, Gauss R, Horn SC, Neuber O, Sommer T: The ubiquitylation machinery of the endoplasmic reticulum. *Nature* 2009;458:453–460.
- Murata S, Yashiroda H, Tanaka K: Molecular mechanisms of proteasome assembly. *Nat Rev Mol Cell Biol* 2009;10:104–115.
- Kruger E, Kuckelkorn U, Sijts A, Kloetzel PM: The components of the proteasome system and their role in MHC class I antigen processing. *Rev Physiol Biochem Pharmacol* 2003;148:81–104.
- Jung T, Catalgol B, Grune T: The proteasomal system. *Mol Aspects Med* 2009;30:191–296.
- Tu L, Moriya C, Imai T, Ishida H, Tetsutani K, Duan X, Murata S, Tanaka K, Shimokawa C, Hisaeda H, Himeno K: Critical role for the immunoproteasome subunit LMP7 in the resistance of mice to *Toxoplasma gondii* infection. *Eur J Immunol* 2009;39:3385–3394.
- Barton LF, Cruz M, Rangwala R, Deepe GS Jr, Monaco JJ: Regulation of immunoproteasome subunit expression in vivo following pathogenic fungal infection. *J Immunol* 2002;169:3046–3052.
- Groettrup M, Khan S, Schwarz K, Schmidtke G: Interferon- γ inducible exchanges of 20S proteasome active site subunits: why? *Biochimie* 2001;83:367–372.
- Frisan T, Levitsky V, Polack A, Masucci MG: Phenotype-dependent differences in proteasome subunit composition and cleavage specificity in B cell lines. *J Immunol* 1998;160:3281–3289.
- Hallermalm K, Seki K, Wei C, Castelli C, Rivoltini L, Kiessling R, Levitskaya J: Tumor necrosis factor- α induces coordinated changes in major histocompatibility class I presentation pathway, resulting in increased stability of class I complexes at the cell surface. *Blood* 2001;98:1108–1115.
- Stohwasser R, Kuckelkorn U, Kraft R, Kostka S, Kloetzel PM: 20S proteasome from LMP7 knock out mice reveals altered proteolytic activities and cleavage site preferences. *FEBS Lett* 1996;383:109–113.
- Basler M, Moebius J, Elenich L, Groettrup M, Monaco JJ: An altered T cell repertoire in MECL-1-deficient mice. *J Immunol* 2006;176:6665–6672.
- Caudill CM, Jayarapu K, Elenich L, Monaco JJ, Colbert RA, Griffin TA: T cells lacking immunoproteasome subunits MECL-1 and LMP7 hyperproliferate in response to polyclonal mitogens. *J Immunol* 2006;176:4075–4082.
- Yu X, Kem DC: Proteasome inhibition during myocardial infarction. *Cardiovasc Res* 2010;85:312–320.
- Testa U: Proteasome inhibitors in cancer therapy. *Curr Drug Targets* 2009;10:968–981.
- Koreth J, Alyea EP, Murphy WJ, Welniak LA: Proteasome inhibition and allogeneic hematopoietic stem cell transplantation: a review. *Biol Blood Marrow Transplant* 2009;15:1502–1512.
- Everly JJ, Walsh RC, Alloway RR, Woodle ES: Proteasome inhibition for antibody-mediated rejection. *Curr Opin Organ Transplant* 2009;14:662–666.
- Zavrski I, Kleeberg L, Kaiser M, Fleissner C, Heider U, Sterz J, Jakob C, Sezer O: Proteasome as an emerging therapeutic target in cancer. *Curr Pharm Des* 2007;13:471–485.
- Letoha T, Feher LZ, Pecze L, Somlai C, Varga I, Kaszaki J, Toth G, Vizler C, Tiszlavicz L, Takacs T: Therapeutic proteasome inhibition in experimental acute pancreatitis. *World J Gastroenterol* 2007;13:4452–4457.
- Rogler G, Hausmann M, Vogl D, Aschenbrenner E, Andus T, Falk W, Andreesen R, Scholmerich J, Gross V: Isolation and phenotypic characterization of colonic macrophages. *Clin Exp Immunol* 1998;112:205–215.
- Salomons FA, Acs K, Dantuma NP: Illuminating the ubiquitin/proteasome system. *Exp Cell Res* 2010;316:1289–1295.
- Sorokin AV, Kim ER, Ovchinnikov LP: Proteasome system of protein degradation and processing. *Biochemistry (Mosc)* 2009;74:1411–1442.
- Navon A, Ciechanover A: The 26 S proteasome: from basic mechanisms to drug targeting. *J Biol Chem* 2009;284:33713–33718.
- Loureiro J, Ploegh HL: Antigen presentation and the ubiquitin-proteasome system in host-pathogen interactions. *Adv Immunol* 2006;92:225–305.
- Makala LH, Nishikawa Y, Suzuki N, Nagasawa H: Immunology. Antigen-presenting cells in the gut. *J Biomed Sci* 2004;11:130–141.
- Pavli P, Maxwell L, Van de Pol E, Doe F: Distribution of human colonic dendritic cells and macrophages. *Clin Exp Immunol* 1996;104:124–132.
- Smythies LE, Sellers M, Clements RH, Mosteller-Barnum M, Meng G, Benjamin WH, Orenstein JM, Smith PD: Human intestinal macrophages display profound inflammatory anergy despite avid phagocytic and bacteriocidal activity. *J Clin Invest* 2005;115:66–75.
- Carlsen HS, Yamanaka T, Scott H, Rugtveit J, Brandtzaeg P: The proportion of CD40+ mucosal macrophages is increased in inflammatory bowel disease whereas CD40 ligand (CD154)+ T cells are relatively decreased, suggesting differential modulation of these costimulatory molecules in human gut lamina propria. *Inflamm Bowel Dis* 2006;12:1013–1024.

- 31 Spottl T, Hausmann M, Kreutz M, Peuker A, Vogl D, Scholmerich J, Falk W, Andreesen R, Andus T, Herfarth H, Rogler G: Monocyte differentiation in intestine-like macrophage phenotype induced by epithelial cells. *J Leukoc Biol* 2001;70:241–251.
- 32 Rugtveit J, Bakka A, Brandtzaeg P: Differential distribution of B7.1 (CD80) and B7.2 (CD86) costimulatory molecules on mucosal macrophage subsets in human inflammatory bowel disease (IBD). *Clin Exp Immunol* 1997;110:104–113.
- 33 Rogler G, Hausmann M, Spottl T, Vogl D, Aschenbrenner E, Andus T, Falk W, Scholmerich J, Gross V: T-cell co-stimulatory molecules are upregulated on intestinal macrophages from inflammatory bowel disease mucosa. *Eur J Gastroenterol Hepatol* 1999;11:1105–1111.
- 34 Visekruna A, Joeris T, Seidel D, Kroesen A, Loddenkemper C, Zeitz M, Kaufmann SH, Schmidt-Ullrich R, Steinhoff U: Proteasome-mediated degradation of I κ B α and processing of p105 in Crohn disease and ulcerative colitis. *J Clin Invest* 2006;116:3195–3203.
- 35 Rogler G, Brand K, Vogl D, Page S, Hofmeister R, Andus T, Knuechel R, Baeuerle PA, Scholmerich J, Gross V: Nuclear factor κ B is activated in macrophages and epithelial cells of inflamed intestinal mucosa. *Gastroenterology* 1998;115:357–369.
- 36 Kosovac K, Brenmoehl J, Holler E, Falk W, Schoelmerich J, Hausmann M, Rogler G: Association of the NOD2 genotype with bacterial translocation via altered cell-cell contacts in Crohn's disease patients. *Inflamm Bowel Dis* 2010.
- 37 Colpaert S, Vastraelen K, Liu Z, Maerten P, Shen C, Penninckx F, Geboes K, Rutgeerts P, Ceuppens JL: In vitro analysis of interferon gamma (IFN- γ) and interleukin-12 (IL-12) production and their effects in ileal Crohn's disease. *Eur Cytokine Netw* 2002;13:431–437.
- 38 Sasaki T, Hiwatashi N, Yamazaki H, Noguchi M, Toyota T: The role of interferon γ in the pathogenesis of Crohn's disease. *Gastroenterol Jpn* 1992;27:29–36.
- 39 Guarner F: What is the role of the enteric commensal flora in IBD? *Inflamm Bowel Dis* 2008;14(suppl 2):S83–S84.
- 40 Murphy SJ, Ullman TA, Abreu MT: Gut microbes in Crohn's disease: getting to know you better? *Am J Gastroenterol* 2008;103:397–398.
- 41 Conte MP, Schippa S, Zamboni I, Penta M, Chiarini F, Seganti L, Osborn J, Falconieri P, Borrelli O, Cucchiara S: Gut-associated bacterial microbiota in paediatric patients with inflammatory bowel disease. *Gut* 2006;55:1760–1767.
- 42 Tien MT, Girardin SE, Regnault B, Le Bourhis L, Dillies MA, Coppee JY, Bourdet-Sicard R, Sansonetti PJ, Pedron T: Anti-inflammatory effect of *Lactobacillus casei* on Shigella-infected human intestinal epithelial cells. *J Immunol* 2006;176:1228–1237.
- 43 Bentz S, Hausmann M, Piberger H, Kellermeier S, Paul S, Held L, Falk W, Obermeier F, Fried M, Scholmerich J, Rogler G: Clinical relevance of IgG antibodies against food antigens in Crohn's disease: a double-blind cross-over diet intervention study. *Digestion* 2010;81:252–264.
- 44 Duchmann R, Kaiser I, Hermann E, Mayet W, Ewe K, Meyer zum Buschenfelde KH: Tolerance exists towards resident intestinal flora but is broken in active inflammatory bowel disease (IBD). *Clin Exp Immunol* 1995;102:448–455.
- 45 Duchmann R, Schmitt E, Knolle P, Meyer zum Buschenfelde KH, Neurath M: Tolerance towards resident intestinal flora in mice is abrogated in experimental colitis and restored by treatment with interleukin-10 or antibodies to interleukin-12. *Eur J Immunol* 1996;26:934–938.