EXTENDED REPORT

# Immunoproteasome subunit LMP2 expression is deregulated in Sjögren's syndrome but not in other autoimmune disorders

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**Background:** The proteasome system has a pivotal role in the control of the immune response, which suggests that it might be involved in the pathogenesis of autoimmune disorders.
**Objective:** To investigate the expression profile of selected proteasomal genes in human peripheral blood mononuclear cells in patients with a variety of autoimmune diseases compared with healthy subjects.
**Methods:** Real time quantitative RT-PCR was used to analyse the mRNA expression pattern of the proteasome activator subunits PA28α and PA28β and of constitutive proteasome and interferon-γ-inducible immunoproteasome subunits in peripheral blood mononuclear cells. Simultaneously, protein expression of selected proteasome subunits was quantified by immunoblotting.
**Results:** Under systemic inflammatory conditions the proteasome subunits LMP2 (β1i), LMP7 (β5i), MECL1 (β2i), and PA28α were expressed abundantly at the protein level in the vast majority of systemic autoimmune disorders. However, simultaneous mRNA and protein quantification showed a characteristic proteasome expression signature in primary Sjögren's syndrome. At the transcript level, the interferon-γ-responsive subunits LMP2 (β1i), MECL1 (β2i), and the proteasome activator subunit PA28α were markedly up regulated. In contrast, LMP2 (β1i) deficiency was evident at the protein level, indicating deregulation of proteasome expression in Sjögren's syndrome.
**Conclusions:** These data provide evidence for a regulatory defect in the proteasome system in human autoimmune disorders, pointing to a unique role for LMP2 (β1i) in the pathogenesis of primary Sjögren's syndrome.

The proteasome is involved in the regulation of essential cellular processes such as transcription, cell cycle progression, differentiation, apoptosis, and, additionally, in the control of stress and immune responses.[1] The central role of the proteasome implies that it may have a primary or secondary role in the pathogenesis of different degenerative, metabolic, and autoimmune diseases. However, our knowledge about the structural and functional properties of the proteasome system under these conditions is limited.

The eukaryotic 26S proteasome is composed of the multisubunit and multicatalytic 20S core complex and two 19S regulator complexes that assist in binding and unfolding ubiquitinylated protein substrates.[2] The proteolytic activity resides in the β1, β2, and β5 subunits.[3] The proinflammatory cytokine interferon γ (IFNγ) induces expression of the proteasome activator PA28αβ[4] and three additional, alternative catalytic proteasome subunits, low molecular weight protein (LMP)2 (β1i), multicatalytic endopeptidase complex-like 1 (MECL1 (β2i)), and LMP7 (β5i). These subunits replace the constitutive catalytic subunits β1, β2, and β5 in newly formed immunoproteasomes[5 6] and may alter proteasomal protein processing qualitatively and quantitatively.[1 7] Whereas LMP2 (β1i) and LMP7 (β5i) are encoded in the major histocompatibility complex (MHC) class II region,[8 9] the gene for MECL1 (β2i) is localised on human chromosome 16 in a cluster of unrelated genes.[10] PA28 consists of two subunits PA28α and PA28β, probably forming an $\alpha_3\beta_4$ complex[11] that accelerates and enhances a subset of certain MHC class I epitopes in vitro and in vivo by binding to the outer α-rings of the 20S proteasome.[12]

The first evidence for the potential involvement of the proteasome in autoimmune disorders was provided by the detection of autoantibodies directed against different proteasome components.[13–16] Autoantibodies against the proteasome activator subunit PA28α were also found in systemic lupus erythematosus (SLE) and Sjögren's syndrome.[17] The proteasome was identified as a major autoantigen in multiple sclerosis[18] and in young patients with type 1 diabetes mellitus.[19] Although the presence of autoantibodies does not necessarily imply the involvement of the targeted antigen in the disease process and might represent a bystander phenomenon, circulating proteasomal components may be markers of cell damage and immunological activity in autoimmune diseases.[20]

Deregulation and aberration of proteasomal pathways contribute to the pathogenesis of human diseases such as malignant, degenerative, metabolic, and autoimmune disorders.[21] Regarding autoimmunity, the proteasome has a pivotal role in the MHC class I presentation pathway of endogenous antigens. However, detailed proteasomal gene and protein expression in autoimmune patients in comparison with healthy subjects has not been simultaneously monitored yet. In the present study, we have focused on the expression profiling of selected proteasomal genes in healthy and autoimmune conditions. The analysis at the

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**Abbreviations:** HPRT, hypoxanthine phosphoribosyl transferase; IFNγ, interferon γ; LMP, low molecular weight protein; MECL1, multicatalytic endopeptidase complex-like 1; MHC, major histocompatibility complex; NOD, non-obese diabetic; PM, polymyositis; RA, rheumatoid arthritis; PA, proteasome activator; pSS, primary Sjögren's syndrome; RT-PCR, reverse transcriptase-polymerase chain reaction; SLE, systemic lupus erythematosus; SSc, systemic sclerosis

transcriptional level as well as the protein expression level allowed us to identify distinct gene regulation mechanisms in individual autoimmune disorders.

## PATIENTS AND METHODS

### Patients

The study included 45 patients who were diagnosed at the Department of Rheumatology and Clinical Immunology, Charité-University Medicine Berlin from 1997 to 2000 with the following autoimmune disorders: 26 patients with SLE (23 female, 3 male, mean age at time of analysis 45.5 years, age range 25–70 years) meeting the revised American College of Rheumatology criteria,[22] six patients with primary Sjögren's syndrome (pSS) (female, mean age 56.3 years, age range 31–64 years) diagnosed according to the criteria of the European Study Group on Diagnostic Criteria for Sjögren's Syndrome,[23] four patients with systemic sclerosis (SSc) (three female, one male, mean age 57.5 years, age range 36–72 years) classified according to the preliminary criteria for the classification of SSc (scleroderma),[24] four patients with polymyositis (PM) (two female, two male, mean age 46.0 years, age range 41–53 years) meeting the criteria according to Bohan and Peter,[25 26] three patients with rheumatoid arthritis (RA) (one female, two male, mean age 61.0 years, age range 45–69 years) diagnosed according to the revised American College of Rheumatology criteria,[22] and two patients with ANCA associated primary vasculitis (female, mean age 49 years, 42 and 56 years) fulfilling the diagnostic criteria according to Jennette *et al*.[27]

At the time of blood sample collection, 15 patients with SLE received an immunosuppressive drug (azathioprine n = 10, cyclophosphamide n = 1, mycophenolate mofetil n = 1, hydroxychloroquine n = 3) in addition to corticoids (12 patients with low dose prednisolone <10 mg/day, two patients with prednisolone of <50 mg/day, and one patient with methylprednisolone 750 mg/day). The remaining 11 patients with SLE as well as all patients with pSS were treated with low dose corticoids. Three patients with SSc obtained low dose corticoids, one of them in combination with azathioprine. One patient with SSc had no immunosuppressant drugs. Two patients with PM received immunosuppressive treatment (one with azathioprine and one with cyclophosphamide) in addition to low dose prednisolone. All patients with RA received low dose corticosteroids. Patients with vasculitis were treated with corticoids and cyclophosphamide. Informed consent was obtained from all donors before peripheral blood was taken. The study was approved by the ethics committee of the Charité-University Medicine, Humboldt University, Berlin, Germany.

### Preparation of peripheral blood mononuclear cells (PBMCs)

PBMCs were isolated immediately after taking heparinised blood by density gradient centrifugation using Ficoll Separating Solution (Biochrom, Berlin, Germany). PBMCs from 22 healthy blood donors (Institute for Transfusion Medicine, Charité-University Medicine Berlin) were used as age and sex matched controls.

### Cell culture and IFNγ stimulation

JOSK-M cells derived from human PBMCs from a patient with chronic myeloid leukaemia were obtained from the German Collection of Micro-organisms and Cell Cultures, DSMZ, Braunschweig, No ACC 30.[28] Suspension cultures of JOSK-M cells were maintained in RPMI 1640 (Gibco BRL, Eggenstein, Germany) containing 10% fetal calf serum. For IFNγ treatment (Sigma, Deisenhofen, Germany), the medium was supplemented with 20 and 600 U/ml IFNγ respectively, for 72 hours.

**Table 1** Primers and probes for real time quantitative RT-PCR

| Protein/GeneBank accession No | Name | Length (bp) | Tm (°C) | GC (%) | Exon boundary | Sequence |
|---|---|---|---|---|---|---|
| LMP2 (β1i)/U01025 | 2F | 22 | 60 | 50 | | 5′-CGTTGTGATGGGTTCTGATTCC-3′ |
| | 2R | 23 | 59 | 48 | | 5′-GACAGCTTGTCAAACACTCGGTT-3′ |
| | 2P | 22 | 70 | 68 | Ex2/Ex3 | 5′-FAM-CACCGCCTC\|GCCTGCAGACACT-TAMRA-3′ |
| LMP7 (β5i)/U17496 | 7F | 18 | 60 | 67 | | 5′-GCAGTGGATTCTCGGGCC-3′ |
| | 7R | 24 | 60 | 50 | | 5′-GCCAAGCAGGTAAGGGTTAATCTC-3′ |
| | 7P | 30 | 69 | 53 | Ex2/Ex3 | 5′-FAM-AGCTGGGTCCTACATTA\|GTGCCTTACGGGT-TAMRA-3′ |
| MECL1 (β2i)/NM_002801 | MF | 19 | 60 | 63 | | 5′-GCTGCGGACACTGAGCTCA-3′ |
| | MR | 20 | 60 | 55 | | 5′-TGGTTCCAGGCACAAAGTGG-3′ |
| | MP | 25 | 70 | 64 | Ex7/Ex8 | 5′-FAM-CCACAGAGCCCGTGAAGAG\|GTCTGG-TAMRA-3′ |
| PA28α/U10360 | ALPHA-F | 19 | 59 | 53 | | 5′-TCCTTCTGCAGCGCTTGAA-3′ |
| | ALPHA-R | 22 | 60 | 55 | | 5′-CTCAATCCGAGGTATCTGCAGC-3′ |
| | ALPHA-P | 26 | 69 | 58 | Ex6/Ex7 | 5′-FAM-CAGCTCAACCTG\|GTCACCACCTGGTT-TAMRA-3′ |
| PA28β/D45248 | BETA-F | 15 | 60 | 80 | | 5′-GTGCGCCTGAGCGGG-3′ |
| | BETA-R | 23 | 61 | 48 | | 5′-TCAGCCTCCTGGAAAAGATTCTG-3′ |
| | BETA-P | 26 | 70 | 58 | Ex1/Ex2 | 5′-FAM-AAGCCCGCAAACAG\|GTGGAGGTCTTC-TAMRA-3′ |
| C3 (α2)/D00760 | 3F | 25 | 60 | 40 | | 5′-GATTGAATATGCTTTGGCTGCTGTA-3′ |
| | 3R | 29 | 60 | 31 | | 5′-GGATTTCTGTTTTTTCTCAGTTGCTAATA-3′ |
| | 3P | 28 | 70 | 46 | Ex2/Ex3 | 5′-FAM-TCCGTGGGAATTAAAG\|CTGCAAATGGTG-TAMRA-3′ |
| C9 (α3)/D00763 | 9F | 23 | 59 | 52 | | 5′-GTGAGCAGTTGGTTACAGCACTG-3′ |
| | 9R | 24 | 61 | 42 | | 5′-CAGCCAATGTACAGCAATGAAACA-3′ |
| | 9P | 33 | 69 | 42 | Ex1/Ex2 | 5′-FAM-CAAGCTTATACACAATTTGG\|AGGAAAACGTCCC-TAMRA-3′ |
| HPRT/NM_000194 | HPRT-F | 22 | 59 | 50 | | 5′-GACTTTGCTTTCCTTGGTCAGG-3′ |
| | HPRT-R | 25 | 59 | 44 | | 5′-AGTCTGGCTTATATCCAACACTTCG-3′ |
| | HPRT-P | 26 | 70 | 50 | Ex6/Ex7 | 5′-FAM-TTTCACCAGCAAG\|CTTGCGACCTTGA-TAMRA-3′ |

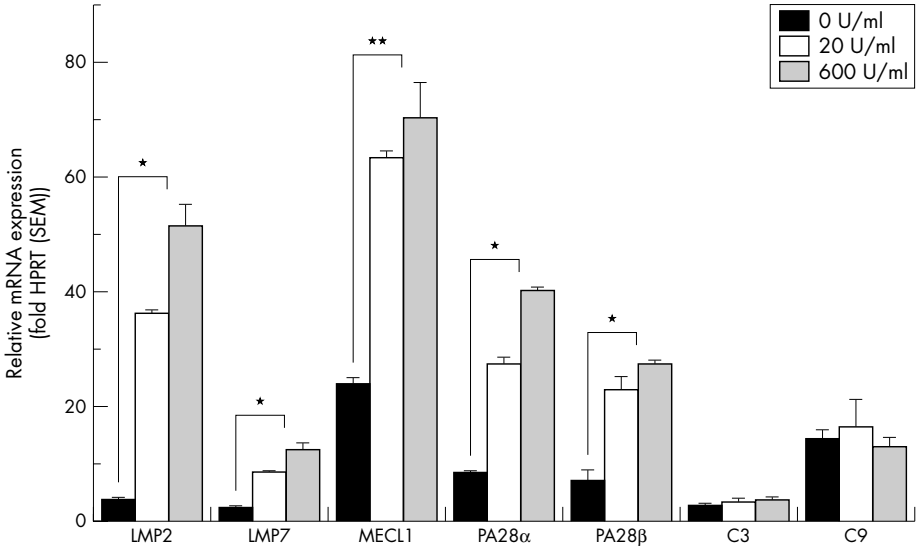


**Figure 1** Real time RT-PCR analysis of proteasome and proteasome activator subunit expression after IFNγ induction in vitro. JOSK-M cells were treated with IFNγ for 72 hours (20 U/ml, white bars; 600 U/ml grey bars). Untreated JOSK-M cells served as controls (black bars). The non-proteasomal housekeeping gene HPRT was used as a reference. Relative expression values were calculated based on the PCR amplification efficiencies *E* of the individual amplicons. The values ($E^{\Delta Ct}$) are given as relative mRNA expression. Values are given as means (SEM) Significance was assessed by two sided Student's *t* test (*p<0.05, **p<0.01).

### Real time quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted from PBMCs and JOSK-M cells by the TRIzol method (TRIzol reagent, PEQLAB, Erlangen, Germany). RNA (1 µg) was DNase-I digested (Roche, Basel, Switzerland) and reverse transcribed using Moloney murine leukaemia virus reverse transcriptase (Gibco). Real time quantitative RT-PCR analysis was performed using the ABI Prism 7700 Sequence Detection System (PE Applied Biosystems, Weiterstadt, Germany). Amplification reactions contained TaqMan Universal PCR Master Mix (PE Applied Biosystems), 50–900 nM forward and reverse primers in experimentally established optimal ratios, 200 nM 5′-FAM-3′-TAMRA labelled probe (BioSource Europe, Nivelles, Belgium), and about 250 ng of cDNA. Table 1 lists the primers (F, forward, R, reverse) and probes (P), which were designed using the PrimerExpress software (PE Applied Biosystems). Probes were chosen to span exon junctions of neighbouring exons, which are indicated by a vertical bar (|). The TaqMan PCR conditions for all amplifications were 40 cycles at 95℃ for 15 seconds and 60℃ for 1 minute, with initial steps of 2 minutes at 50℃ and 10 minutes at 95℃. Non-template controls for individual amplicons were included in each run. Specificity and quality of the PCR products were confirmed by agarose gel electrophoresis.

Standard curves were generated for each gene measured using cDNA from healthy controls. Expression of the target gene relative to the reference gene hypoxanthine phosphoribosyl transferase (HPRT) was calculated as the difference between threshold cycles of these two genes ($2^{-\Delta Ct}$) using the comparative Ct (threshold cycle) method.[29] Differences between duplicate Ct values were always $\leqslant 0.5$. The relative mRNA expression in autoimmune patients versus the expression in healthy control subjects was calculated according to the $2^{-\Delta\Delta Ct}$ method.[29]

### Western blot analysis

PBMCs were lysed on ice in 1/100 volume 10% Triton X-100 (final concentration 0.1%). Total protein (30 µg ) in each lane was fractionated by 15% sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred to nitrocellulose, which was incubated with the following primary antibodies (1:1000) for 1 hour at room temperature: polyclonal rabbit antisera against the proteasome subunits C3 (α2), C9 (α3), LMP2 (β1i), LMP7 (β5i), MECL1 (β2i), and PA28α.[30 31] The secondary antibody was a horseradish peroxidase conjugated, affinity purified goat antirabbit IgG Fc fragment (Dianova, Hamburg, Germany, 1: 20 000). Protein immunoreactivity was visualised by enhanced electrochemiluminescence (ECL; Amersham, Freiburg, Germany). Specific bands were quantified with the NIH image 1.61 software tool (http://rsb.info.nih.gov/nih-image/, accessed 18 May 2006) with integrated density scanning and normalised to the constitutive subunit C3 (α2).

### Evaluation of gene expression and statistical analysis

The relative expression software tool REST[31a] was used for group-wise comparison and statistical analysis of the real time quantitative RT-PCR results. REST analysis included an efficiency correction step and Ct values normalised to HPRT in order to compensate for variation between RT-PCR analyses or variation from sample to sample. For the analysis of protein expression levels, significance was assessed by a two sided Student's *t* test or Mann-Whitney U test where appropriate (*p<0.05, **p<0.01, ***p<0.001).

## RESULTS

### Real time RT-PCR analysis of selected immunoproteasome and proteasome activator subunit expression after IFNγ up regulation in vitro

A real time quantitative RT-PCR assay was established in order to investigate the gene expression of selected proteasome and proteasome activator subunits. Similar PCR amplification efficiencies of control and target genes were confirmed using serial dilutions of cDNA from human control PBMCs. These experiments were conducted to obtain standard curves as the prerequisite for the $2^{-\Delta\Delta Ct}$ method.[29]

When the quantitative RT-PCR system was established, the assay was verified in an in vitro IFNγ stimulation experiment. JOSK-M cells from a human chronic myeloid leukaemia cell line were stimulated for 72 hours with 20 and 600 U/ml

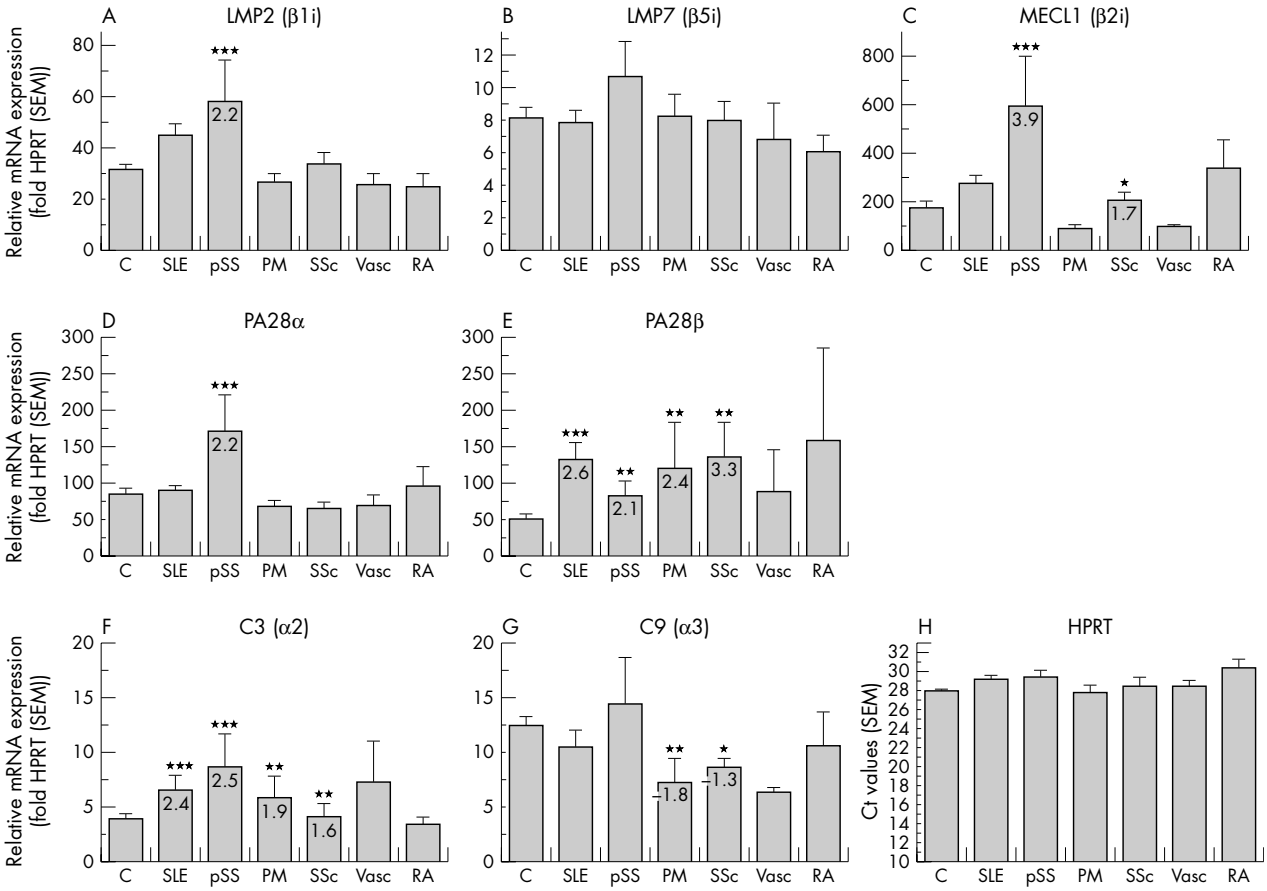


**Figure 2** Proteasomal gene expression profiling in autoimmune patients. Gene expression of IFNγ-inducible immunoproteasome subunits (A, B, C), proteasome activator subunits (D, E), and two selected constitutive proteasome subunits (F, G) was measured by real time quantitative RT-PCR in PBMCs from patients with SLE (n = 26), pSS (n = 6), PM (n = 4), SSc (n = 4), RA (n = 3), and primary vasculitis (Vasc; n = 2) showing clinically active disease. Healthy blood donors served as age and sex matched controls (C; n = 22). Only significant relative up regulation ⩾1.6-fold was discussed. Patients with vasculitis and RA were not included in the statistical evaluations owing to the small number of cases. Values are given as the mean (SEM) relative mRNA expression. If applicable, a two sided Mann-Whitney U test was used to verify statistical significance (*p<0.05, **p<0.01, ***p<0.001).

IFNγ, respectively (fig 1). Untreated JOSK-M cells served as controls for basal expression levels. The mRNA expression of LMP2, LMP7, MECL1, C3, C9, PA28α, and PA28β was determined. The expression of HPRT was measured as a control gene unrelated to the proteasome system.[32] After normalisation to the respective housekeeping gene, the individual data (ΔCt values) were converted to a linear form considering the PCR doubling efficiency *E* of each amplicon.

After IFNγ stimulation of JOSK-M cells, the relative mRNA expression of the inducible immunoproteasome subunits and the expression of the activator subunits PA28α and PA28β were increased significantly compared with untreated control cells (fig 1). The expression of the constitutive subunits C3 and C9 and the housekeeping gene HPRT remained stable. Normalisation to HPRT, C3 or C9 demonstrated significant up regulation of the immunoproteasome

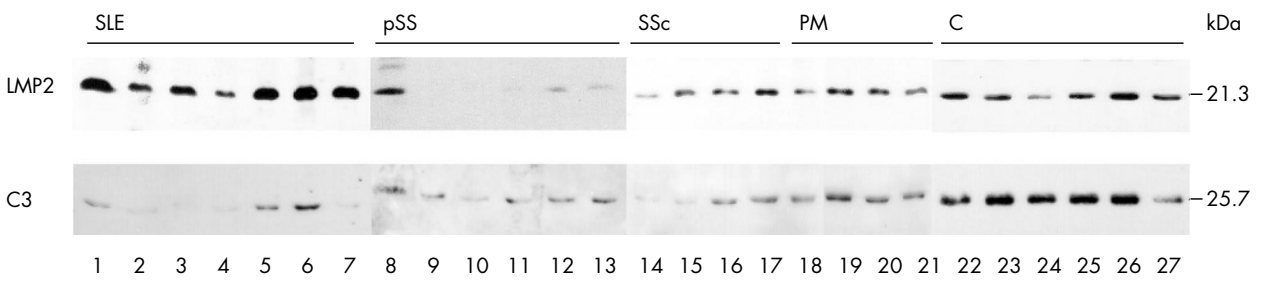


**Figure 3** Expression profile of LMP2 (β1i) in autoimmune disorders by immunoblot analysis of PBMC lysates. Representative data are shown for SLE (n = 7, lanes 1–7), pSS (n = 6, lanes 8–13), SSc (n = 4, lanes 14–17), PM (n = 4, lanes 18–21), and healthy controls (n = 6, lanes 22–27). Cell lysates were normalised for protein (30 μg total protein per lane) and analysed by immunoblotting with antibodies against LMP2 (β1i) and the constitutive human proteasome subunits C3 (α2). Note that lanes 8–13 were slightly overexposed in order to demonstrate reduced LMP2-specific signals in most patients with pSS.

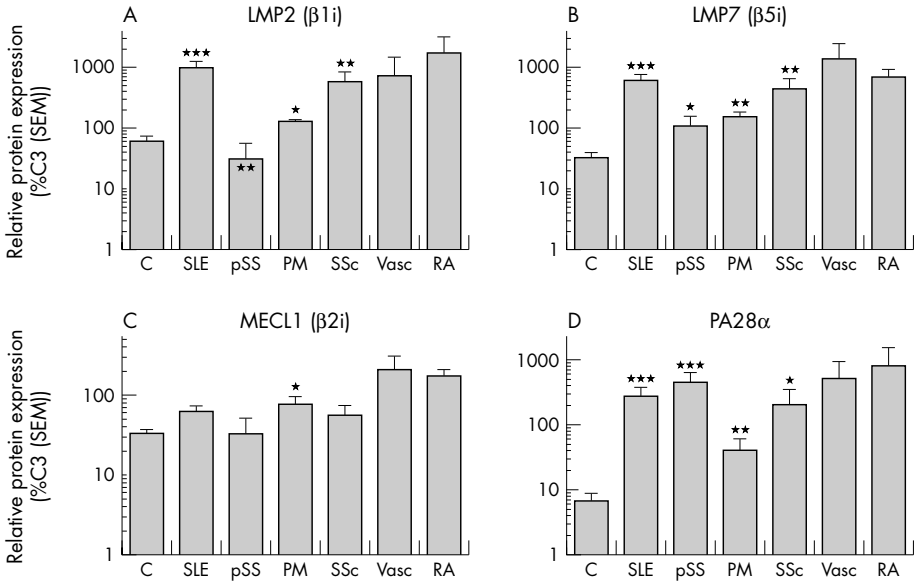


**Figure 4** Measurement of relative proteasomal protein expression in autoimmune disorders. PBMC lysates of all autoimmune patients and healthy controls (for numbers of cases see fig 2) were subjected to immunoblotting and densitometry. Mean protein expression is shown for LMP2 (β1i; A), LMP7 (β5i; B), MECL1 (β2i; C), and PA28α (D). Values are normalised to C3 (α2) and are given as relative protein expression in %C3 (α2) plotted on a logarithmic scale. If applicable, a two sided Mann-Whitney U test was used to assess significance (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

and the proteasome activator subunits after IFNγ stimulation (data not shown).

### mRNA expression of constitutive, immunoproteasome, and proteasome regulator subunits is differentially up regulated in autoimmune conditions

Gene expression was measured in PBMCs taken from patients with systemic autoimmune disorders such as SLE (n = 26), pSS (n = 6), PM (n = 4), RA (n = 3), SSc (n = 4), and primary vasculitis (n = 2) showing clinically active disease (fig 2). PBMCs from healthy blood donors served as controls (n = 22). Relative mRNA expression of the constitutive proteasome subunits C3 and C9, the immunoproteasome subunits LMP2, LMP7, and MECL1, the activator subunits PA28α and PA28β, and the housekeeping gene HPRT was measured. Figure 2 shows the mean (SEM) relative mRNA expression of each diagnosis group.

In pSS, a strong simultaneous up regulation of the IFNγ-inducible subunits LMP2 (fig 2A), MECL1 (fig 2C), and PA28α (fig 2D) was detectable at the mRNA level. As a common feature, PA28β was significantly up regulated in patients with connective tissue diseases (fig 2E). In RA, we observed that MECL1 was the only homogeneously up regulated, inducible subunit (fig 2C). Interestingly, the expression levels of the immunosubunit LMP7 were similar in controls and patients (fig 2B). An increased expression of the constitutive proteasomal subunit C3 was noted at the mRNA level in all autoimmune diseases (fig 2F), whereas the other constitutive subunit C9 was found in a similar or reduced range compared with controls (fig 2G). In vasculitis, the expression levels of the IFNγ-inducible subunits were not significantly altered. Except for RA (n = 3), plotting the mean Ct values for HPRT demonstrated largely stable expression of the reference gene (fig 2H).

In patients with SLE no significant differences were found for mRNA expression of any of the investigated proteasome subunits in PBMCs when patients with low dose corticoid treatment were compared with patients who received additive immunosuppressive treatment.

### Regulation of proteasomal protein expression in autoimmune disorders

In another set of experiments we investigated whether differential regulation of proteasomal expression at the transcript level was also reflected by the protein expression of constitutive or immunoproteasome subunits in PBMCs in autoimmune disorders. Protein expression was analysed in crude PBMC lysates (30 µg total protein) from all autoimmune patients whose mRNA expression had been quantified previously (compare fig 2). C3, C9, LMP2, LMP7, MECL1, and PA28α protein expression was analysed by western blotting. Representative immunoblots are shown for LMP2 protein expression (fig 3). The LMP2 signal was similar in all 22 healthy control subjects and 39 patients investigated except for the majority of patients with pSS (fig 3 and data not shown).

Specific protein bands were quantified by densitometry and normalised to C3 protein expression. C3 levels allow a good estimate of total functional proteasome in a cell. The mean protein expression was shown for LMP2 (fig 4A), LMP7 (fig 4B), MECL1 (fig 4C), and PA28α (fig 4D). The data indicate that LMP7, MECL1, and PA28α were abundantly expressed in PBMCs. In comparison with healthy controls, LMP2 protein levels were significantly decreased in the majority of patients with pSS (5 out of 6) but not in other autoimmune patients (n = 39) (fig 3, 4A, and data not shown). Individual treatment in patients with SLE (low dose corticoid monotherapy versus corticoid and add-on immunosuppressant drugs) had no significant effect on protein expression of LMP2, LMP7, MECL1, or PA28α in PBMCs.

## DISCUSSION

The present study provides evidence for a disturbed expression of components in the proteasome system at both the mRNA and the protein level in human autoimmune disorders. Interestingly, we found characteristic expression profiles for distinct autoimmune diseases. We substantiated the strong induction of IFNγ-inducible proteasomal subunits as well as of the proteasome activator in systemic autoimmune diseases at the mRNA level. However, increased

mRNA expression of certain proteasome subunits did not correspond directly with their respective protein expression.

The observed gene regulation patterns are in good agreement with established pathways of proteasome regulation, assembly, and maturation in vitro.[5 6] Conceivably, in autoimmune conditions components of the proteasomal system are induced by proinflammatory cytokines such as IFNγ. Moreover, LMP7 is expressed in lymphoid cells and tissues at high levels.[32a] Previously, it was demonstrated that pre-proteasomes containing LMP2 and MECL1 require LMP7 for efficient maturation.[5] In this context, LMP7 is up regulated to relatively constant levels in healthy controls as well as in autoimmune patients, which suggests individual gene regulation mechanisms other than for LMP2 and MECL1. This view is supported by the fact that the MECL1 gene is localised on another chromosome.[10] Alternatively, there may be a concerted regulation of both LMP2 and MECL1 involving the IFN regulatory factor IRF-1.[33]

The induction of the constitutive subunit C3 was described in human muscle tissue in septic patients.[34] In accordance with these data, C3 mRNA levels were up regulated in certain autoimmune disorders, including connective tissue diseases and systemic vasculitis. Therefore, we suggest that in autoimmune patients C3 underlies differential induction mechanisms as compared with C9. Raised C3 mRNA levels were also found after the exposure to glucocorticoids[35] probably owing to an impaired negative regulation of C3 expression by nuclear factor-kappaB (NF-κB).[36] These data imply an alternative induction mechanism of C3 mRNA because the vast majority of the autoimmune patients in this study obtained steroid immunosuppression. In this study, no difference was found between the respective immunosuppressive treatment and the expression of any of the investigated proteasome subunits at the mRNA level in patients with SLE.

Surprisingly, in contrast with the other autoimmune disorders, our data disclosed a significant up regulation of the IFNγ-inducible subunits LMP2, MECL1, and PA28α at the mRNA level in patients with pSS. On the other hand, the protein level of LMP2 was very low in most patients with pSS compared with healthy controls and other related autoimmune disorders. This intriguing finding was obtained in a small, but homogenous pSS study group. Increased mRNA levels of LMP2 may reflect (*a*) increased mRNA steady state levels, (*b*) increased mRNA stability, or (*c*) an increased transcription rate. Whereas increased mRNA levels in other autoimmune diseases correspond with the respective protein expression levels, this correlation is reversed in pSS. The relative lack of the subunit LMP2 might be due to raised C3 levels. However, the protein expression of C3 was not markedly increased in pSS. A decrease of LMP2 protein might result from (*a*) a post-transcriptional defect of the LMP2 mRNA, (*b*) an impaired translation process, or (*c*) reduced protein stability of LMP2. The proteasome system is important for T cell education and antigen presentation by MHC class I molecules.[37] Although experimental evidence has not been provided up to now, decreased LMP2 protein levels may contribute to an altered composition of the proteasome population in PBMCs of patients with pSS. As a consequence, the MHC class I peptide repertoire of antigen presenting cells in the periphery may be changed both qualitatively and quantitatively and might promote autoimmune pathogenesis.

A specific proteasome defect has been identified in non-obese diabetic (NOD) mice, an animal model of autoimmune diabetes, in lymphocytic and monocytic lineages, resulting in the down regulation of LMP2 expression.[38] It has been suggested that the molecular defect resides within the promoter-enhancer region of LMP2, which results in decreased mRNA levels of LMP2. This defect may prevent proteolytic proteasomal processing of NF-κB, affecting immune and inflammatory responses, as well as susceptibility to apoptosis induced by tumour necrosis factor α. Interestingly, NOD mice provide a suitable model of Sjögren's syndrome, in particular autoimmune sialadenitis exhibiting exocrine gland lymphocytic infiltration typical of the Sjögren's syndrome-like disease.[39]

Our findings cannot simply be explained by a transcriptional defect in the LMP2 gene in patients with pSS according to the NOD mouse model, because LMP2 mRNA was abundant in PBMCs. Previously, it had been shown that proteasomal subunits are up regulated at the transcriptional level upon treatment with proteasome inhibitors.[40] In addition, there is evidence for a regulatory feedback loop, not only in yeast but also in the mammalian proteasomal system.[41] Based on these results, we suggest that decreased protein levels of LMP2 in PBMCs in pSS may be followed by reactive up regulation of mRNA in the proteasomal system. This situation appears to be unique for pSS, because in SLE, SSc, PM, and RA transcript as well as protein levels of the respective subunits were regulated accordingly.

For the first time, expression patterns in the proteasome system at both the transcriptional and translational level were analysed simultaneously in active systemic rheumatoid disorders. Our observations showed that autoimmune diseases have distinct proteasomal gene and protein expression signatures. In this study we have provided new clues for a unique role of LMP2 in the pathogenesis of pSS, suggesting a regulatory molecular defect of LMP2 in pSS. Further genetic and biochemical studies should extend our understanding of the pathophysiology of the proteasome system, leading to new diagnostic and therapeutic strategies in human autoimmune disorders.

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The authors declare no competing financial interests.

The study was approved by the ethics committee of the Charité-University Medicine, Humboldt University, Berlin, Germany.

## REFERENCES

1 **Kloetzel PM**, Ossendorp F. Proteasome and peptidase function in MHC-class-I-mediated antigen presentation. *Curr Opin Immunol* 2004;**16**:76–81.
2 **Voges D**, Zwickl P, Baumeister W. The 26S proteasome: a molecular machine designed for controlled proteolysis. *Annu Rev Biochem* 1999;**68**:1015–68.
3 **Heinemeyer W**, Fischer M, Krimmer T, Stachon U, Wolf DH. The active sites of the eukaryotic 20 S proteasome and their involvement in subunit precursor processing. *J Biol Chem* 1997;**272**:25200–09.
4 **Macagno A**, Gilliet M, Sallusto F, Lanzavecchia A, Nestle FO, Groettrup M. Dendritic cells up-regulate immunoproteasomes and the proteasome regulator PA28 during maturation. *Eur J Immunol* 1999;**29**:4037–42.
5 **Griffin TA**, Nandi D, Cruz M, Fehling HJ, Kaer LV, Monaco JJ, *et al.* Immunoproteasome assembly: cooperative incorporation of interferon gamma (IFN-gamma)-inducible subunits. *J Exp Med* 1998;**187**:97–104.

6 **Groettrup M**, Standera S, Stohwasser R, Kloetzel PM. The subunits MECL-1 and LMP2 are mutually required for incorporation into the 20S proteasome. *Proc Natl Acad Sci USA* 1997;**94**:8970–5.
7 **Kloetzel PM**. Generation of major histocompatibility complex class I antigens: functional interplay between proteasomes and TPPII. *Nat Immunol* 2004;**5**:661–9.
8 **Brown MG**, Driscoll J, Monaco JJ. Structural and serological similarity of MHC-linked LMP and proteasome (multicatalytic proteinase) complexes. *Nature* 1991;**353**:355–7.
9 **Glynne R**, Powis SH, Beck S, Kelly A, Kerr LA, Trowsdale J. A proteasome-related gene between the two ABC transporter loci in the class II region of the human MHC. *Nature* 1991;**353**:357–60.
10 **Larsen F**, Solheim J, Kristensen T, Kolsto AB, Prydz H. A tight cluster of five unrelated human genes on chromosome 16q22.1. *Hum Mol Genet* 1993;**2**:1589–95.
11 **Knowlton JR**, Johnston SC, Whitby FG, Realini C, Zhang Z, Rechsteiner M, *et al*. Structure of the proteasome activator REGalpha (PA28alpha). *Nature* 1997;**390**:639–43.
12 **Rechsteiner M**, Hill CP. Mobilizing the proteolytic machine: cell biological roles of proteasome activators and inhibitors. *Trends Cell Biol* 2005;**15**:27–33.
13 **Feist E**, Dörner T, Kuckelkorn U, Schmidtke G, Micheel B, Hiepe F, *et al*. Proteasome alpha-type subunit C9 is a primary target of autoantibodies in sera of patients with myositis and systemic lupus erythematosus. *J Exp Med* 1996;**184**:1313–18.
14 **Feist E**, Dörner T, Kuckelkorn U, Scheffler S, Burmester G, Kloetzel P. Diagnostic importance of anti-proteasome antibodies. *Int Arch Allergy Immunol* 2000;**123**:92–7.
15 **Arribas J**, Luz Rodriguez M, Alvarez-Do Forno R, Castano JG. Autoantibodies against the multicatalytic proteinase in patients with systemic lupus erythematosus. *J Exp Med* 1991;**173**:423–7.
16 **Feist E**, Kuckelkorn U, Dörner T, Dönitz H, Scheffler S, Hiepe F, *et al*. Autoantibodies in primary Sjögren's syndrome are directed against proteasomal subunits of the alpha and beta type. *Arthritis Rheum* 1999;**42**:697–702.
17 **Matsushita M**, Takasaki Y, Takeuchi K, Yamada H, Matsudaira R, Hashimoto H. Autoimmune response to proteasome activator 28alpha in patients with connective tissue diseases. *J Rheumatol* 2004;**31**:252–9.
18 **Mayo I**, Arribas J, Villoslada P, Alvarez DoForno R, Rodriguez-Vilarino S, Montalban X, *et al*. The proteasome is a major autoantigen in multiple sclerosis. *Brain* 2002;**125**:2658–67.
19 **Kordonouri O**, Meyer K, Egerer K, Hartmann R, Scheffler S, Burmester GR, *et al*. Prevalence of 20S proteasome, anti-nuclear and thyroid antibodies in young patients at onset of type 1 diabetes mellitus and the risk of autoimmune thyroiditis. *J Pediatr Endocrinol Metab* 2004;**17**:975–81.
20 **Egerer K**, Kuckelkorn U, Rudolph PE, Ruckert JC, Dörner T, Burmester GR, *et al*. Circulating proteasomes are markers of cell damage and immunologic activity in autoimmune diseases. *J Rheumatol* 2002;**29**:2045–52.
21 **Ciechanover A**. The ubiquitin proteolytic system and pathogenesis of human diseases: a novel platform for mechanism-based drug targeting. *Biochem Soc Trans* 2003;**31**:474–81.
22 **Arnett FC**, Edworthy SM, Bloch DA, McShane DJ, Fries JF, Cooper NS, *et al*. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis Rheum* 1988;**31**:315–24.
23 **Vitali C**, Bombardieri S, Moutsopoulos HM, Coll J, Gerli R, Hatron PY, *et al*. Assessment of the European classification criteria for Sjögren's syndrome in a series of clinically defined cases: results of a prospective multicentre study. The European Study Group on Diagnostic Criteria for Sjögren's Syndrome. *Ann Rheum Dis* 1996;**55**:116–21.
24 **ARA**. Subcommittee for scleroderma criteria of the American Rheumatism Association Diagnostic and Therapeutic Criteria Committee. Preliminary criteria for the classification of systemic sclerosis (scleroderma). *Arthritis Rheum* 1980;**23**:581–90.
25 **Bohan A**, Peter JB. Polymyositis and dermatomyositis (first of two parts). *N Engl J Med* 1975;**292**:344–7.
26 **Bohan A**, Peter JB. Polymyositis and dermatomyositis (second of two parts). *N Engl J Med* 1975;**292**:403–7.
27 **Jennette JC**, Falk RJ, Andrassy K, Bacon PA, Churg J, Gross WL, *et al*. Nomenclature of systemic vasculitides. Proposal of an international consensus conference. *Arthritis Rheum* 1994;**37**:187–92.
28 **Ohta M**, Furukawa Y, Ide C, Akiyama N, Utakoji T, Miura Y, *et al*. Establishment and characterization of four human monocytoid leukemia cell lines (JOSK-I, -S, -M and -K) with capabilities of monocyte-macrophage lineage differentiation and constitutive production of interleukin 1. *Cancer Res* 1986;**46**:3067–74.
29 **Livak KJ**, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 2001;**25**:402–8.
30 **Frentzel S**, Pesold-Hurt B, Seelig A, Kloetzel PM. 20 S proteasomes are assembled via distinct precursor complexes. Processing of LMP2 and LMP7 proproteins takes place in 13–16 S preproteasome complexes. *J Mol Biol* 1994;**236**:975–81.
31 **Soza A**, Knuehl C, Groettrup M, Henklein P, Tanaka K, Kloetzel PM. Expression and subcellular localization of mouse 20S proteasome activator complex PA28. *FEBS Lett* 1997;**413**:27–34.
31a **Pfaffl MW**, Horgan GW, Dempfle L. Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Res* 2002;**30**:e36.
32 **Vandesompele J**, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, *et al*. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* 2002;**3**:1–10.
32a **Strehl B**, Seifert U, Kruger E, Heink S, Kuckelhorn U, Kloetzel PM. Interferon-gamma, the functional plasticity of the ubiquitin-proteasome system, and MHC class I antigen processing. *Immunol Rev* 2005;**207**:19–30.
33 **Foss GS**, Prydz H. Interferon regulatory factor 1 mediates the interferon-gamma induction of the human immunoproteasome subunit multicatalytic endopeptidase complex-like 1. *J Biol Chem* 1999;**274**:35196–202.
34 **Tiao G**, Hobler S, Wang JJ, Meyer TA, Luchette FA, Fischer JE, *et al*. Sepsis is associated with increased mRNAs of the ubiquitin-proteasome proteolytic pathways in human skeletal muscle. *J Clin Invest* 1997;**99**:163–8.
35 **Wang L**, Luo GJ, Wang JJ, Hasselgren PO. Dexamethasone stimulates proteasome- and calcium-dependent proteolysis in cultured L6 myotubes. *Shock* 1998;**10**:298–306.
36 **Du J**, Mitch WE, Wang X, Price SR. Glucocorticoids induce proteasome C3 subunit expression in L6 muscle cells by opposing the suppression of its transcription by NF-kB. *J Biol Chem* 2000;**275**:19661–6.
37 **Kloetzel PM**. The proteasome and MHC class I antigen processing. *Biochim Biophys Acta* 2004;**1695**:225–33.
38 **Hayashi T**, Faustman DL. Role of defective apoptosis in type 1 diabetes and other autoimmune diseases. *Recent Prog Horm Res* 2003;**58**:131–53.
39 **van Blokland SC**, Versnel MA. Pathogenesis of Sjögren's syndrome: characteristics of different mouse models for autoimmune exocrinopathy. *Clin Immunol* 2002;**103**:111–24.
40 **Meiners S**, Heyken D, Weller A, Ludwig A, Stangl K, Kloetzel PM, *et al*. Inhibition of proteasome activity induces concerted expression of proteasome genes and de novo formation of mammalian proteasomes. *J Biol Chem* 2003;**278**:21517–25.
41 **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.

# Immunoproteasome subunit LMP2 expression is deregulated in Sjögren's syndrome but not in other autoimmune disorders

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