

# Expression of Proteasomal Immunosubunit $\beta$ 1i Is Dysregulated in Inflammatory Infiltrates of Minor Salivary Glands in Sjögren's Syndrome

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**ABSTRACT. Objective.** Minor salivary gland specimens were analyzed to investigate dysregulation of the proteasome system in patients with Sjögren's syndrome (SS) and patients with sicca syndrome.

**Methods.** Labial biopsy specimens from 17 patients with SS and 11 patients with non-autoimmune sicca syndrome were analyzed by immunohistochemistry for expression of the inducible proteasomal subunits  $\beta$ 1i,  $\beta$ 2i, and  $\beta$ 5i. The infiltrating subsets of lymphocytes were characterized using immunofluorescence stainings against the cell-surface markers CD20 and CD27. Two-dimensional electrophoresis and immunoblotting were used for detection of the proteasomal subunits  $\beta$ 1 and  $\beta$ 1i in peripheral blood monocyte cells. Gene expression of the constitutive subunits  $\beta$ 1,  $\beta$ 2, and  $\beta$ 5 and the corresponding inducible subunits  $\beta$ 1i,  $\beta$ 2i, and  $\beta$ 5i was further investigated at the mRNA level in small lip biopsies using real-time polymerase chain reaction.

**Results.** The expression of  $\beta$ 1i in infiltrating and peripheral immune cells was altered in patients with SS compared to patients with non-autoimmune sicca syndrome and healthy controls. No significant differences were found in  $\beta$ 2i and  $\beta$ 5i expression between the same groups in small lip biopsies. Chisholm-Mason grade and  $\beta$ 1i expression were found to be inversely correlated (Spearman  $r = -0.461$ ,  $p = 0.014$ ). The phenotype and distribution of the lymphocytic infiltrate showed no differences between patients with primary and secondary SS regardless of  $\beta$ 1i expression.

**Conclusion.** The proteasomal  $\beta$ 1i subunit is dysregulated in peripheral white blood cells and in inflammatory infiltrates of minor salivary glands in patients with SS. (First Release Oct 15 2009; *J Rheumatol* 2009;36:2694–703; doi:10.3899/jrheum.081098)

## Key Indexing Terms:

PROTEASOME SYSTEM  
 $\beta$ 1i EXPRESSION

SJÖGREN'S SYNDROME  
SALIVARY GLANDS

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Sjögren's syndrome (SS) is a systemic autoimmune disorder characterized by dysfunction and inflammatory destruction of exocrine glands, leading to the manifestation of sicca symptoms<sup>1,2</sup>. Involvement of salivary glands represents a common feature of several autoimmune diseases, implicating a susceptibility of the exocrine epithelium for the development and maintenance of an autoimmune response. This phenomenon could be attributed to a preferred exposure to environmental factors such as recurrent viral infections or to intrinsic factors, e.g., altered antigen presentation.

In the minor salivary glands of patients with SS, infiltrating macrophages, B and T cells, and ductal epithelium release distinct chemokines and cytokines, including type 1 interferons<sup>3-5</sup>. This proinflammatory milieu can alter basic cellular functions and might also cause structural and functional changes in the ubiquitin-proteasome system. The proteasome is not only an essential degradation apparatus for cellular homeostasis, but is also involved in regulation of apoptosis and the cell cycle and immune response<sup>6-8</sup>. In

eukaryotic cells, the cylindrical 20S core complex consists of 7 different alpha-type subunits forming 2 outer rings and 7 beta-type subunits bearing 3 active sites in both inner rings<sup>9</sup>. However, under the influence of proinflammatory cytokines such as interferon- $\gamma$  or interferon- $\alpha$ , 3 different inducible proteolytic subunits,  $\beta$ 1i (LMP2),  $\beta$ 2i (MECL1), and  $\beta$ 5i (LMP7), are upregulated to replace their constitutive counterparts<sup>10-14</sup>. By favoring the generation of distinct antigenic peptides for the MHC class I pathway, the so-called immuno-proteasomes are critical for an effective immune response<sup>12,14-18</sup>. Thus, the proteasome is a multicatalytic protease with several active sites and regular structural modifications that have been shown to have a striking influence on the function of the entire complex.

Recently, a disease-specific dysregulation of the proteasome system was recognized in peripheral blood monocyte cells (PBMC) of patients with SS with altered expression of  $\beta$ 1i at the protein level<sup>19</sup>. The aim of our study was to investigate the expression of the inducible proteasome subunits in minor salivary glands of patients with SS and to clarify whether  $\beta$ 1i expression is also modified in the targeted tissue. In addition to standard histological examination of minor salivary gland biopsies by the Chisholm and Mason grading system, immunostaining of minor salivary glands and gene expression analysis were undertaken to compare the expression of the inducible proteasomal subunits at the protein and mRNA levels and to characterize the lymphocyte subsets<sup>20</sup>.

## MATERIALS AND METHODS

**Patients.** Biopsies from minor salivary glands were obtained from 28 consecutive patients with sicca symptoms. All patients were treated at the Department of Rheumatology and Clinical Immunology, Charité-Universitätsmedizin Berlin. The study was performed after approval by the local ethical committee, and informed consent was obtained from all donors before lip biopsies were taken.

Primary SS was diagnosed in 14 patients according to the US-European classification criteria<sup>21</sup> (all female, mean age 50.4 yrs, range 18–70 yrs). Secondary SS was diagnosed in 2 patients with primary biliary cirrhosis (PBC) and in one patient with undifferentiated connective tissue disease (UCTD) (all female, mean age 42.7 yrs, range 28–51 yrs). Sicca symptoms without an autoimmune sialadenitis were confirmed in 11 patients (9 female, 2 male, mean age 54.7 yrs, range 27–73 yrs; 7 with no autoimmune disorder, 2 with Hashimoto's thyroiditis, one with UCTD, one with PBC).

At the time of the biopsy, 4 of 14 patients with primary SS were receiving immunomodulating therapy (hydroxychloroquine,  $n = 2$ ; hydroxychloroquine plus prednisolone,  $n = 2$ ). One of the patients with sicca symptoms was taking prednisolone.

**Immunohistochemistry.** Paraffin-embedded sections (1–3  $\mu$ m) of minor labial gland biopsy tissues were deparaffinized and rehydrated. After antigen retrieval (pressure cooker, citrate buffer, pH 6) rabbit anti-human polyclonal antibodies anti- $\beta$ 1i (K463 and K464), anti- $\beta$ 2i, and anti- $\beta$ 5i<sup>22</sup> were incubated in a dilution of 1:1000. Staining was done using the labeled streptavidin-biotin method (LSAB-Kit; Dako, Glostrup, Denmark). Fuchsin (Dako) was used as a chromogen for the reaction with alkaline phosphatase. In a final step, nuclear counterstaining was performed with hematoxylin. Nonspecific binding of the secondary antibody was investigated by omitting the primary antibody.

Intensity of staining reactions was assessed on a scale from 0 (no stain-

ing) to 3 (strong reaction) by 2 independent pathologists blinded to the diagnosis.

**Immunofluorescence.** Paraffin-embedded sections of minor labial gland biopsy tissues (1–2  $\mu$ m) were deparaffinized and rehydrated. After antigen retrieval (pressure cooker, EDTA buffer, pH 8), proteins were blocked for 5 min in blocking solution (Zytomed Systems, Berlin, Germany). Next, rabbit polyclonal antibody anti-CD20 (dilution 1:50; Zytomed Systems) and the mouse monoclonal antibody anti-CD27 (clone 137B4, dilution 1:10; LabVision, Fremont, CA, USA) were incubated overnight at 4°C. The following steps were performed in darkness: Cy3-conjugated donkey anti-mouse antibody (dilution 1:200; Dianova, Hamburg, Germany) and Cy2-conjugated donkey anti-rabbit antibody (dilution 1:100; Dianova) were incubated for 30 min. Nuclear staining was performed using DAPI (dilution 1:2500; Fluka Chemie AG, Buchs, Switzerland). Slides were mounted with fluorescent mounting medium (Dako).

The slides were viewed on a Leitz DMRBE microscope (Leica, Wetzlar, Germany) with a fluorescent light application using filters for Cy2 (resulting in green fluorescent staining), Cy3 (red fluorescence), and DAPI (blue nuclear fluorescent staining). Slides were viewed under 50 $\times$ , 100 $\times$ , 200 $\times$ , and 400 $\times$  magnification; areas with inflammatory infiltrate were photographed successively using the 3 filters and an overlay was generated. Attention was given to potentially double-positive cells, which should appear yellow on the overlay pictures.

**Isolation of PBMC.** PBMC of 6 patients with SS and 3 healthy controls were isolated from 20 ml heparinized peripheral blood by density gradient centrifugation using Ficoll lymphocyte isolation solution (GE Healthcare Bio-Science, München, Germany). The cells' interphase containing the PBMC was harvested and washed with phosphate buffered saline (PBS). Remaining erythrocytes were lysed using erythrocyte lysis buffer (Qiagen, Hilden, Germany) according to the instructions of the manufacturer.

**Protein isolation.** For protein isolation, the PBMC pellet was lysed in 500  $\mu$ l solution of 9 M urea and 50 mM DTT by short sonication and incubation for 1 h at 30°C. After centrifugation, the supernatant was precipitated with TCA and redissolved in 100  $\mu$ l solution of 9 M urea, 50 mM DTT, 2% Ampholyte, pH 7–9, to get a higher protein concentration. The protein concentration was determined by the BCA method according to the manufacturer's instructions (Pierce, Thermo Fisher Scientific; Rockford, MA, USA).

**Two-dimensional electrophoresis.** Two-dimensional electrophoresis was performed on a mini-vertical gel electrophoresis unit SE 250 with SE 220 tube gel adapter (Hoefer Pharmacia Biotech, San Francisco, CA, USA).

For the first dimension, the tube gel used for isoelectric focusing was 1.5 mm  $\times$  70 mm. Samples from different individuals were normalized based on protein concentration. For each run, a volume of 10  $\mu$ l sample corresponding to 100  $\mu$ g of protein per gel tube was used.

For the second dimension, the gel used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was 60 mm  $\times$  80 mm  $\times$  1.5 mm. A 15% gel was used to allow appropriate separation of the proteasome subunits.

**Blotting and immune detection.** Immunoblotting using 10  $\mu$ g of total proteins derived from PBMC of 3 patients (SS) and 2 healthy donors as antigens was performed as described<sup>19</sup>. For detection of proteasomal subunit  $\beta$ 1i, 3 different polyclonal antibodies (K463, K464, and PW8345; Biomol, Hamburg, Germany) were used, each in a 1:1000 dilution.

K463 and K464 were generated against recombinant murine  $\beta$ 1i and PW8345 directed against a peptide sequence derived from human  $\beta$ 1i.

After two-dimensional PAGE, samples were transferred by semidry blotting to nitrocellulose membranes (Schleicher & Schüll, Dassel, Germany). All subsequent incubation and washing steps were performed at room temperature. Unspecific binding sites were blocked with 1% skim milk powder in PBS and 0.1% Tween 20 for 1 h. Membranes were then incubated with a mixture of polyclonal rabbit antibodies against proteasome subunits  $\beta$ 1 (K43) and  $\beta$ 1i (K463) all in 1:1000 dilution for 1 h.

Membranes were washed 5 times for 20 min each time before being

incubated with anti-rabbit immunoglobulin-POD conjugate for 30 min. Immunoreactive spots were visualized on photographic X-OMAT UV Plus film (Kodak, Stuttgart, Germany) by enhanced chemiluminescence using ECL reagent (Roth, Karlsruhe, Germany). An exposure time of 1 min was always applied.

**Salivary gland biopsies for isolation of RNA.** A total of 10 salivary gland biopsies were carried out for diagnostic purposes from consecutive patients presenting with sicca symptoms. From these, 4 patients were diagnosed as having primary SS. The remaining 6 patients showed no evidence of autoimmune sialadenitis and were used as controls. In order to avoid degradation of RNA, biopsies were stabilized in RNAlater (Qiagen). RNA was isolated using the NucleoSpin RNA XS kit (Macherey–Nagel, Düren, Germany) according to the manufacturer's instructions, including on-column DNA digestion. RNA was reverse-transcribed into cDNA using Superscript III reverse transcriptase (Invitrogen, Karlsruhe, Germany).

**Primer design for real-time polymerase chain reaction (PCR).** For analysis of gene expression of the 3 constitutive proteasomal catalytic subunits  $\beta 1$ ,  $\beta 2$ , and  $\beta 5$ , and the corresponding inducible immunosubunits  $\beta 1i$ ,  $\beta 2i$ , and  $\beta 5i$  in salivary gland biopsies, forward and reverse primers were selected based on the respective transcript sequences. Relative quantification of gene transcription was done in relation to the housekeeping gene  $\beta$ -actin.

Primers for PCR were stringently designed using the Primer 3 software (Version 0.4.0; Whitehead Institute for Biomedical Research, Cambridge, MA, USA)<sup>23</sup> with 19–21 bp length and an optimal annealing temperature of  $62^\circ \pm 2^\circ\text{C}$  to amplify cDNA fragments between 180 and 210 bp. Designed primer sequences are listed in Table 1.

Primers were synthesized by TIB MolBiol (Berlin, Germany) and were tested in conventional PCR using the human monocytic cell line U937. Amplification products were controlled by sequencing (DLMB, Rüdersdorf, Germany). Primer efficiency was determined according to the protocols of Applied Biosystems (Foster City, CA, USA).

**Relative quantification of gene expression.** Relative quantification of gene expression was performed with real-time PCR using SYBR Green PCR Master Mix (Applied Biosystems) and 10 pmol from each forward/reverse primer. Amplifications were run in triplicates for each sample/primer pair using the ABI Prism 5700 sequence detection system (Applied Biosystems), including an initial step of 2 min at  $50^\circ\text{C}$ , an initial denaturation step of 10 min at  $95^\circ\text{C}$ , and 40 cycles with  $95^\circ\text{C}$  over 15 s for denaturation and  $62^\circ\text{C}$  over 60 s for annealing and elongation. Unspecific amplifications were controlled by running a dissociation protocol starting at  $60^\circ\text{C}$  after each experiment, which showed the specific melting point of each PCR product.

**Measurement of gene expression.** Relative quantification of gene expression at the mRNA level was done by comparison with expression of the housekeeping gene  $\beta$ -actin. After checking the correct melting point of the PCR products, arithmetic means of CT values from triplicate samples were calculated. Differences in the expression of target genes were determined using the formula  $E^{-\text{Ct}_{\text{housekeeping gene}}/E^{\text{Ct}_{\text{target gene}}}}$ , as described<sup>11</sup>, where E represents the efficiency of the respective primers. Standard deviations of CT triplicates  $< 0.5$  were accepted for analysis.

**Statistical analysis.** Correlations between expression of proteasomal immunosubunits measured as the intensity of immunohistochemical staining (grade 0–3) and inflammatory infiltrates according to Chisholm-Mason grading were calculated using the Spearman r test.

To compare protein expression between different patient groups, non-parametric Mann-Whitney U-test and Fisher's exact test were applied. Differences in the relative expression of investigated genes were analyzed using the nonparametric Mann-Whitney t-test. P values  $< 0.05$  were considered significant.

## RESULTS

**Expression of proteasomal immunosubunits in minor salivary glands.** The expression pattern of proteasomal

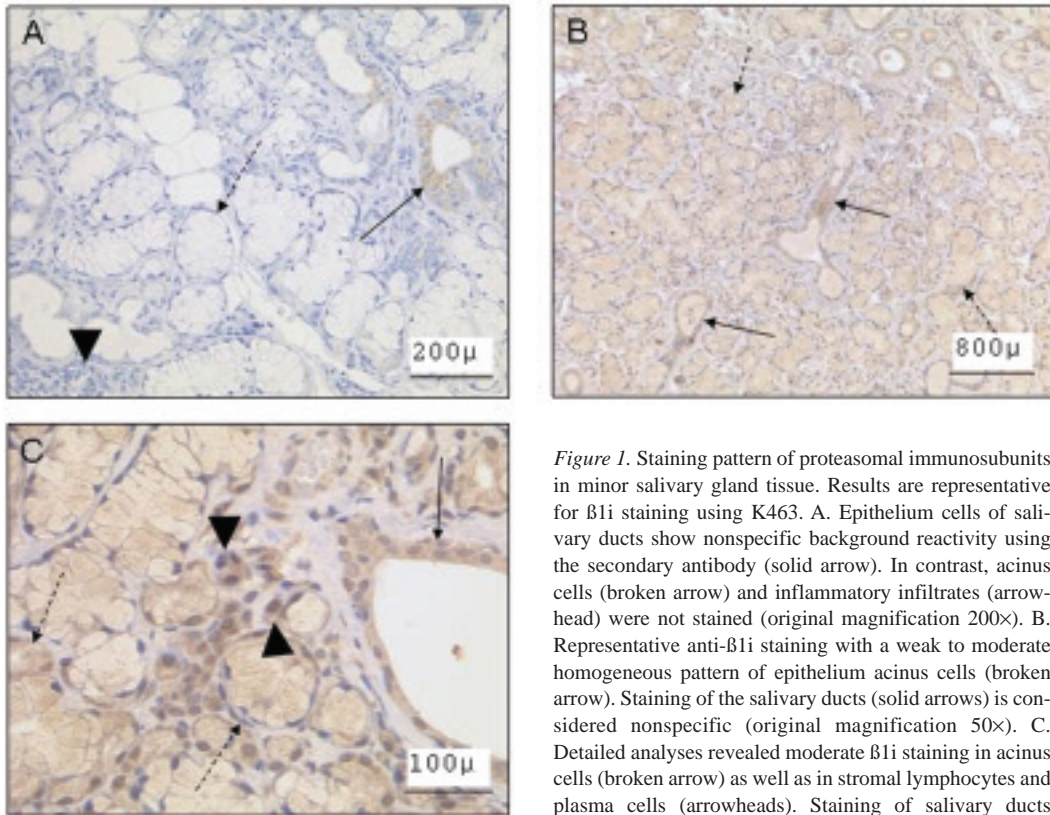
immunosubunits was investigated in minor salivary glands using immunohistochemistry and immunofluorescence. To exclude an unspecific reaction of the secondary antibody, staining was also performed by omitting the primary antibody. This showed positive reactivity in the epithelium of the salivary ducts, but not in the glandular acini or in the inflammatory infiltrate. Therefore, the duct staining was considered as nonspecific background reactivity in the subsequent analysis (Figure 1A).

Tissue-specific expression of inducible proteasomal subunits in minor salivary glands was identified in stromal lymphocytes and plasma cells as well as in the acinus epithelium by different primary antibodies against  $\beta 1i$  (K463 and K464),  $\beta 2i$ , and  $\beta 5i$ . Representative results for one patient with primary SS exhibiting moderate expression of  $\beta 1i$  using K463 are shown in Figure 1B and 1C.

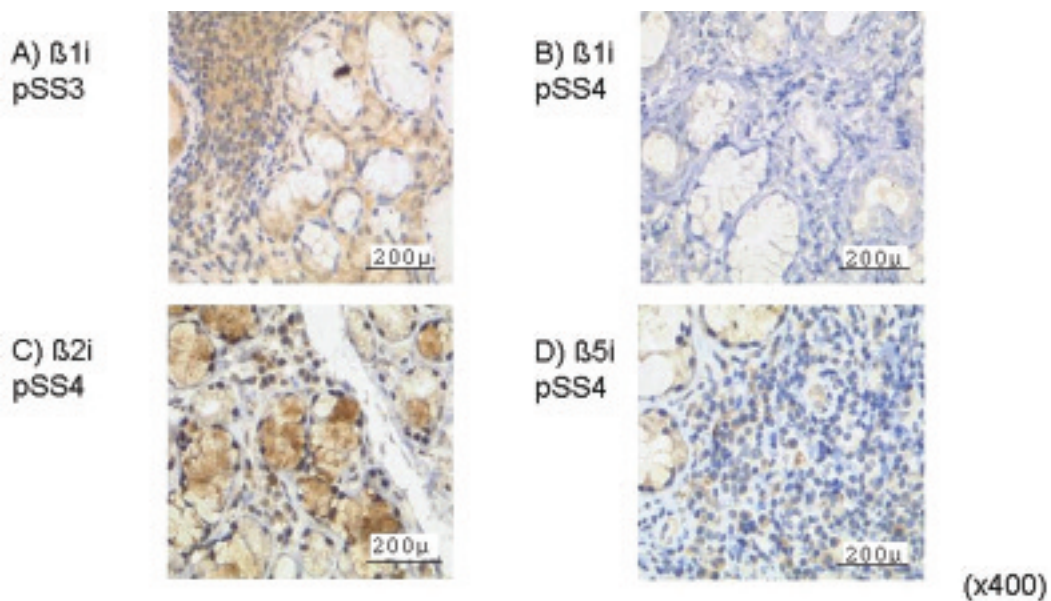
**Comparison of  $\beta 1i$ ,  $\beta 2i$ , and  $\beta 5i$  staining in salivary glands of patients with SS and controls using immunohistochemistry.** In all cases of positive staining, expression of the inducible subunits  $\beta 2i$  and  $\beta 5i$  was predominantly detectable in the inflammatory infiltrate, but also in acinus cells (Figure 2C, 2D). Importantly, staining of  $\beta 1i$  using K463 was significantly reduced in the majority of patients with SS (Figure 2B). Indeed, only 4 patients with SS showed detectable staining of  $\beta 1i$  using K463 in infiltrating immune cells and within serous and, to some extent, mucous acinus cells (Figure 2A). Using the polyclonal anti- $\beta 1i$  antibody K464, no clear differences were observed between the analyzed groups (data not shown).

Comparing expressions of the inducible proteasomal subunits, there were no differences in the immunohistochemical stainings of  $\beta 2i$  or of  $\beta 5i$  between patients with SS (primary and secondary SS) and patients with sicca symptoms (Figure 3C and 3D, respectively). Only a slight but nonsignificant increase of  $\beta 5i$  expression was observed in patients with primary and secondary SS. In contrast, staining of  $\beta 1i$  using K463 was significantly reduced in the infiltrating lymphocytes as well as in the acinus cells of patients with SS (Mann-Whitney test,  $p = 0.0487$ , Fisher exact test,  $p = 0.0189$ ; Figure 3A). This difference was even more pronounced when patients receiving hydroxychloroquine or prednisolone (SS,  $n = 4$ ; sicca symptoms,  $n = 1$ ) were excluded (Mann-Whitney test,  $p = 0.0122$ , Fisher exact test,  $p = 0.0059$ ; Figure 3B).

**Expression of proteasomal immunosubunits and Chisholm-Mason grading.** To investigate whether expression of proteasomal subunits was related to the magnitude of the inflammatory reaction in the salivary tissue, histological scoring was performed independently by 2 pathologists blinded for the diagnosis. Overall, staining for  $\beta 1i$  using K463 was found to be negatively correlated to Chisholm-Mason grade in patients with primary and secondary SS (Spearman  $r = -0.4611$ ,  $p = 0.0135$ ), but not in patients with sicca symptoms (Table 2). Further, no signifi-



*Figure 1.* Staining pattern of proteasomal immunosubunits in minor salivary gland tissue. Results are representative for  $\beta 1i$  staining using K463. A. Epithelium cells of salivary ducts show nonspecific background reactivity using the secondary antibody (solid arrow). In contrast, acinus cells (broken arrow) and inflammatory infiltrates (arrowhead) were not stained (original magnification 200 $\times$ ). B. Representative anti- $\beta 1i$  staining with a weak to moderate homogeneous pattern of epithelium acinus cells (broken arrow). Staining of the salivary ducts (solid arrows) is considered nonspecific (original magnification 50 $\times$ ). C. Detailed analyses revealed moderate  $\beta 1i$  staining in acinus cells (broken arrow) as well as in stromal lymphocytes and plasma cells (arrowheads). Staining of salivary ducts (solid arrow) considered nonspecific (400 $\times$ ).



*Figure 2.* Staining of proteasomal immunosubunits in minor labial salivary glands of patients with primary SS, determined by immunohistochemical techniques. A. Strong staining of  $\beta 1i$  using K463 in infiltrating immune cells and within serous and to some extent mucous acinus cells of one patient with primary SS (Patient 3). B-D. Representative stainings for proteasomal immunosubunits in one patient with primary SS (Patient 4). B. Negative signal for  $\beta 1i$  using K463. C and D. Staining for  $\beta 2i$  and  $\beta 5i$  showed expression within acinus cells, infiltrating lymphocytes, and plasma cells (original magnification).

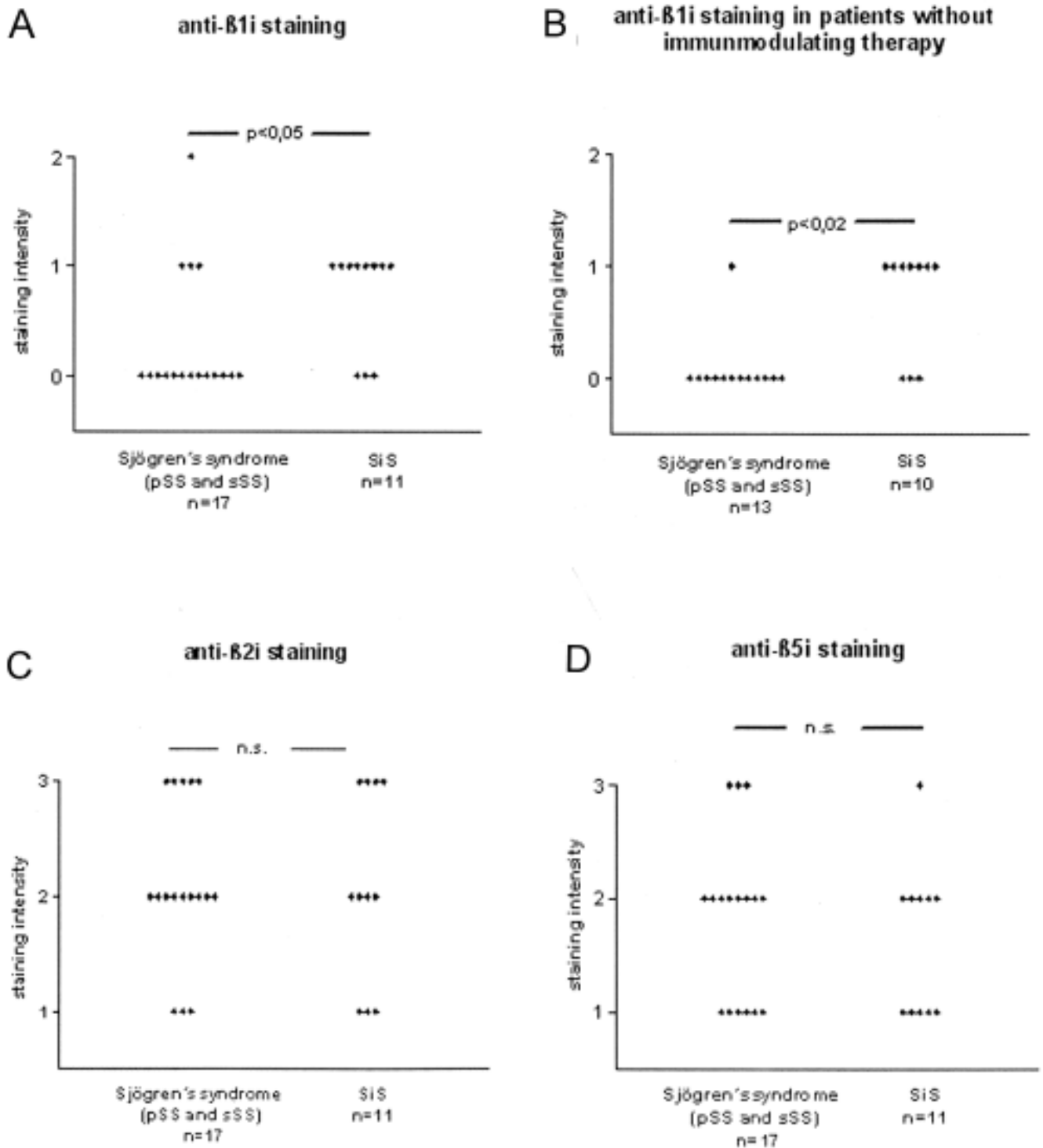


Figure 3. Statistical analysis of proteasomal immunosubunits in minor salivary glands after immunohistochemical staining. A. β1i staining (K463) was significantly decreased in patients with primary (pSS) and secondary SS (sSS) in comparison to patients with sicca symptoms (SiS) without autoimmune sialadenitis (Mann-Whitney test,  $p = 0.0487$ , Fisher exact test,  $p = 0.0189$ ). B. This difference was even more pronounced in patients receiving hydroxychloroquine or prednisolone (Mann-Whitney test,  $p = 0.0122$ , Fisher exact test,  $p = 0.0059$ ). C. β2i staining showed no significant differences between patients with primary and secondary SS and patients with sicca symptoms without autoimmune sialadenitis (Mann-Whitney test,  $p = 0.9811$ ). D. Staining of β5i was slightly but not significantly increased in patients with primary and secondary SS compared to patients with sicca symptoms without autoimmune background (Mann-Whitney test,  $p = 0.5520$ ). ns: nonsignificant.

Table 1. Sequences of selected polymerase chain reaction (PCR) primers.

Subunit/Gene Data Bank Acc. N	Primer sequence (5'–3')	Size of Expected PCR Product, bp
β1 (Delta) NM_002798	F: CAA GCT GAC ACC TAT TCA CGA C R: CGG TAT CGG TAA CAC ATC TCC T	177
β2 (Z) NM_002799	F: ATC GCT GGG GTG GTC TAT AAG R: AAG AAA TGA GCT GG TTG TCA T	176
β5 (MB1) NM_002797	F: GGC AAT GTC GAA TCT ATG AGC R: GTT CCC TTC ACT GTC CAC GTA	176
β1i (LMP2) U01025	F: CAA CGT GAA GGA GGT CAG GTA R: AGA GCA ATA GCG TCT GTG GTG	170
β2i (MECL1) Y13640	F: AAT GTG GAC GCA TGT GTG AT R: CAT AGC CTG CAC AGT TTC CTC	180
β5i (LMP7)/ U17497	F: CAC GGG TAG TGG GAA CAC TTA R: ACT TTC ACC CAA CCA TCT TCC	180
α3 (C9) NM_002789	F: GGA GCC AAT ACC TTG TGA GC R: CAT CCC CCG TAA TTT CCA C	171
Beta actin (ACTB) BC002409	F: CTG GAC TTC GAG CAA GAG ATG R: TGA AGG TAG TTT CGT GGA TGC	178

F: forward; R: reverse.

Table 2. Autoantibody profiles, histological scores, and β1i staining using K463 of different patient groups. Staining of β1i using K463 and Chisholm-Mason grade were negatively correlated (Spearman  $r = -0.4611$ ,  $p = 0.0135$ ). No correlation was found between Chisholm-Mason grade and β2i or β5i staining (data not shown). Anti-Ro and anti-La antibodies were determined using automated fluorescence immunoassays (EliA, UniCap 100, Phadia, Freiburg, Germany).

Diagnosis	Sex		Mean Age, yrs	Ro, no.	La, no.	Chisholm-Mason Grade				β1i Expression, no.		
	F, no.	M, no.				1	2	3	4	0	1	2
pSS	14	0	50.4	13	13	0	1	5	8	10	3	1
sSS	3	0	42.7	2	1	0	0	0	3	3	0	0
Sicca Symptoms	9	2	54.7	3	0	5	6	0	0	3	8	0

pSS: primary Sjögren's syndrome; sSS: secondary SS.

cant correlation was found between Chisholm-Mason grade and expression of the subunits β2i and β5i. Expression of proteasomal subunits was not related to other autoimmune manifestations or to autoantibodies against Ro and La antigens.

*Immunofluorescence comparison of lymphocytic infiltrates in salivary glands.* Lymphocytic inflammatory infiltrates were identified in the minor labial tissue biopsies. Phenotypic analysis was based on immunofluorescence stainings with anti-CD20 and anti-CD27 antibodies. In our study, CD20-positive, CD27-negative cells represent naive B lymphocytes, which should appear green, while CD20 and CD27 double-positive cells represent memory B lymphocytes and should appear yellow on the overlays. Red-stained cells are CD27-positive, representing specifically T lymphocytes. Thus, no differences were observed between patients with primary or secondary SS and between patients with strong or weak β1i expression of phenotype or distribution of infiltrating lymphocytes (Figure 4). Follicles could be identified with areas rich in CD27-naive B lymphocytes but with relatively few CD27-positive memory B cells. Areas with T lymphocytes could also be clearly distinguished.

*mRNA expression of β1i, β2i, and β5i in patients' salivary glands.* At the mRNA level, expression of all the inducible proteasome subunits β1i, β2i, and β5i was significantly higher in patients with SS compared to controls in PCR analyses (Figure 5). In contrast, no significant differences were found for levels of the respective constitutive subunits β1, β2, and β5. Since inducible subunits replace their constitutive counterpart to form the 20S proteasome, we calculated the ratio of the respective subunits to compare the expression of inducible subunits. Using this approach, β5i was identified as the subunit with the strongest expression (ratio of β5 to β5i = 0.88 in controls, β5:β5i = 0.16 in patients), followed by β2i (β2:β2i = 6.15 in controls, β2:β2i = 1.88 in patients) and β1i (β1:β1i = 20.03 in controls, β1:β1i = 1.70 in patients).

*Detection of β1 and β1i subunits in patients' PBMC using immunoblotting.* To analyze expression of β1i in the periph-

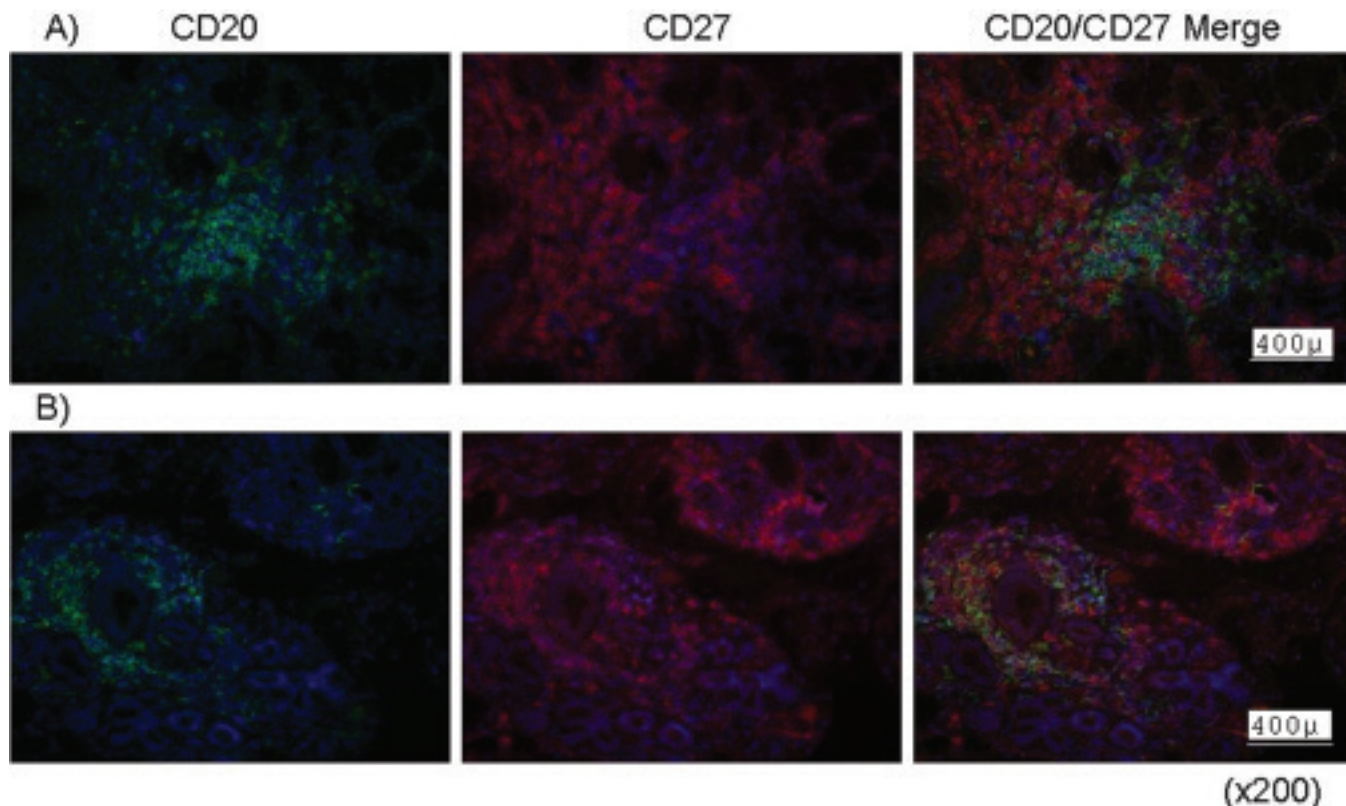


Figure 4. Representative immunofluorescence staining of lymphocyte subsets in infiltrates in labial biopsies from patients with primary SS (A) and secondary SS (B). CD20+/CD27- naive B cells (green), CD20+/CD27+ memory B cells (yellow), and CD27-positive T lymphocyte subsets (red) were detectable in both conditions with similar cellular distribution.

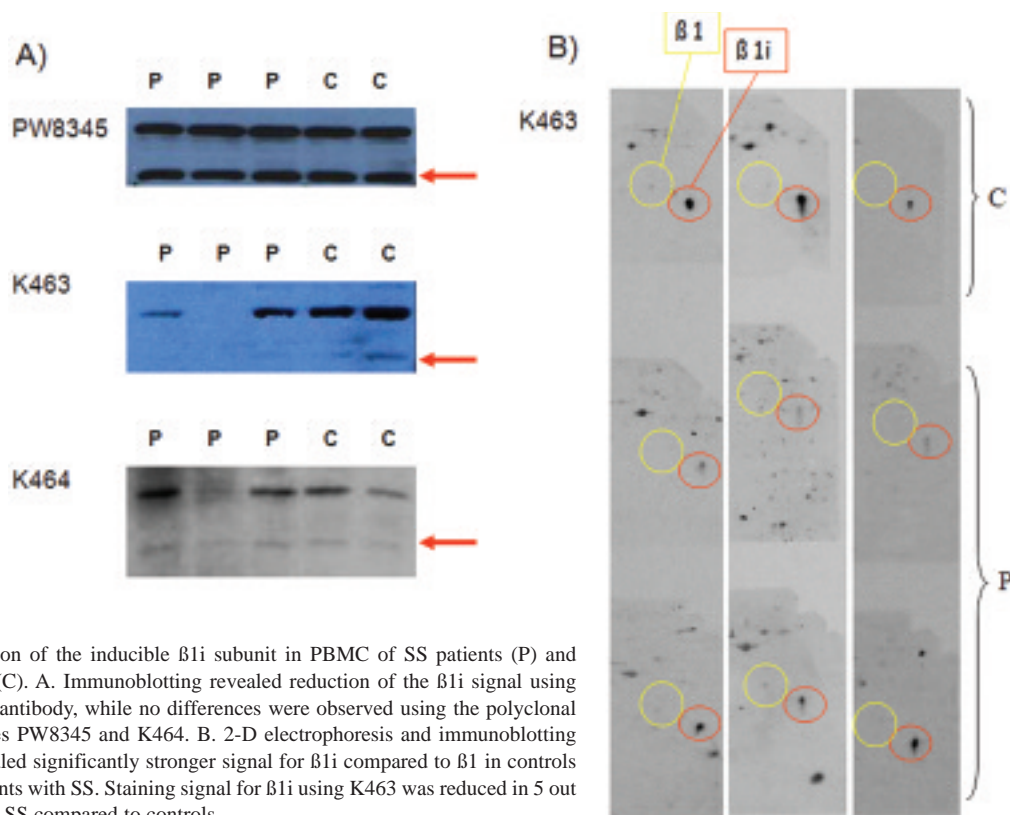


Figure 6. Detection of the inducible  $\beta 1i$  subunit in PBMC of SS patients (P) and healthy controls (C). A. Immunoblotting revealed reduction of the  $\beta 1i$  signal using K463 polyclonal antibody, while no differences were observed using the polyclonal anti- $\beta 1i$  antibodies PW8345 and K464. B. 2-D electrophoresis and immunoblotting using K463 revealed significantly stronger signal for  $\beta 1i$  compared to  $\beta 1$  in controls as well as in patients with SS. Staining signal for  $\beta 1i$  using K463 was reduced in 5 out of 6 patients with SS compared to controls.

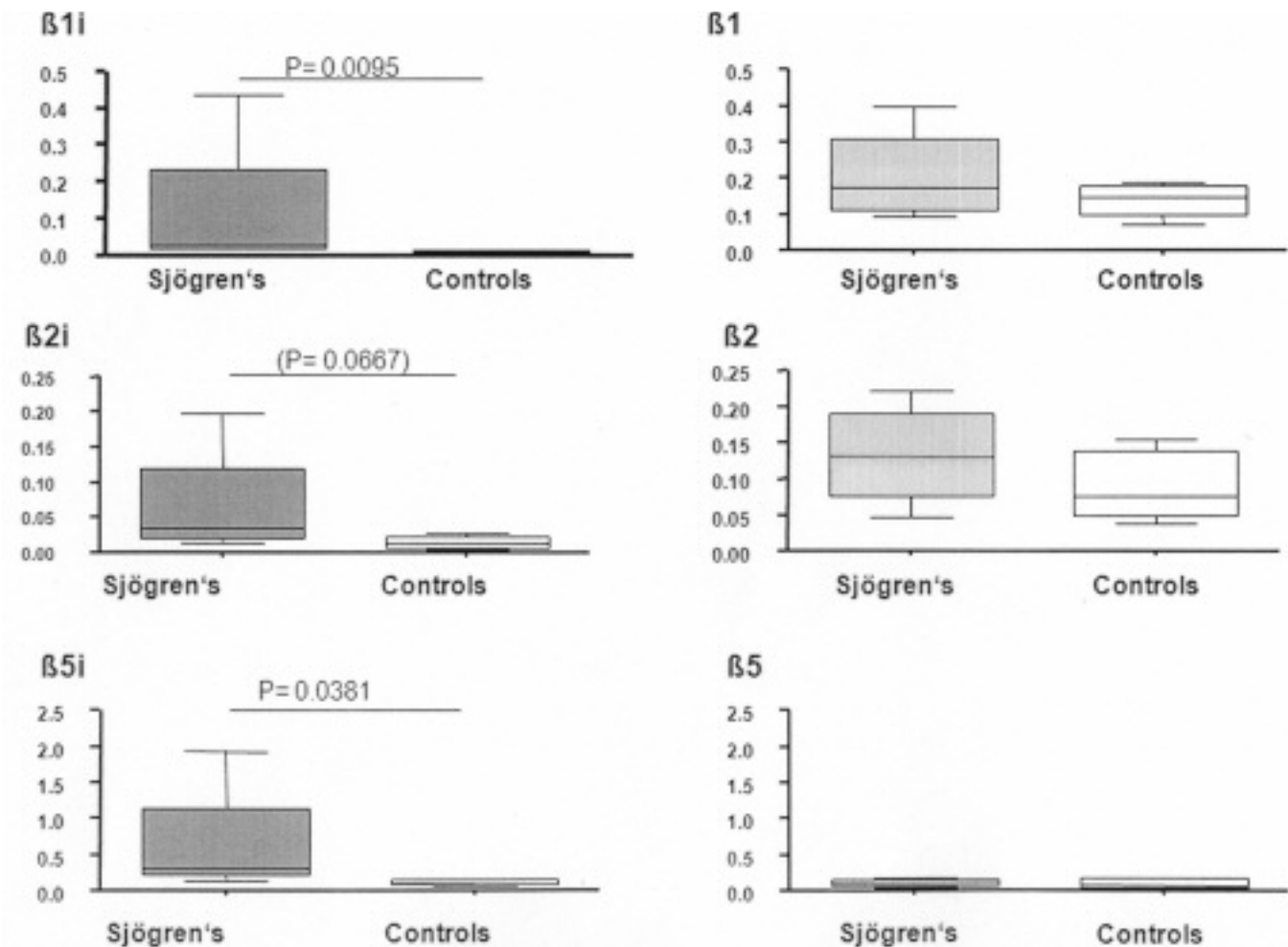


Figure 5. Comparisons of gene expression of inducible ( $\beta 1i$ ,  $\beta 2i$ , and  $\beta 5i$ ) and the corresponding constitutive ( $\beta 1$ ,  $\beta 2$ , and  $\beta 5$ ) proteasome subunits in salivary glands of SS patients ( $n = 4$ ) and controls ( $n = 6$ ). Nonparametric Mann-Whitney test with 95% confidence interval. For better appreciation of the magnitude of expression of each constitutive subunit compared with its inducible counterpart, the same scale was used for each subunit pair.

eral immune compartment, 3 different polyclonal anti- $\beta 1i$  antibodies (PW8345, K463, and K464) were used for detection of  $\beta 1i$  subunit expression in PBMC from patients with SS ( $n = 3$ ) compared to healthy controls ( $n = 2$ ). No clear differences were observed using PW8345 and K464, while results with K463 showed slightly weaker signals for  $\beta 1i$  subunits in patients with SS (Figure 6A). To confirm this result, we compared expression of  $\beta 1$  and  $\beta 1i$  in PBMC using identical amounts of protein in 2-D electrophoresis. Interestingly, the expression signal for  $\beta 1i$  was significantly stronger compared to the  $\beta 1$  subunit in controls as well as in patients with SS. In subsequent analyses, we used one healthy control as a standard in comparison to 6 patients with SS. In densitometric evaluation comparing the stained dots for  $\beta 1i$ , a clearly weaker signal for  $\beta 1i$  was detected in 5 out of 6 patients with SS (Figure 6B).

## DISCUSSION

We analyzed expression of the 3 inducible proteasomal sub-

units  $\beta 1i$ ,  $\beta 2i$ , and  $\beta 5i$  in minor salivary gland biopsy samples from patients with sicca symptoms. Patients with SS showed a characteristic dysregulation of the proteasomal immunosubunit  $\beta 1i$  in the targeted tissues, i.e., in the epithelium layer of acinus cells, compared to patients with sicca symptoms without autoimmune sialadenitis. The altered expression of  $\beta 1i$  in SS was visualized using polyclonal anti- $\beta 1i$  antibodies, showing a significantly reduced staining signal in minor salivary glands. Interestingly, the reduced  $\beta 1i$  signal was negatively correlated to the intensity of lymphocytic infiltrates according to Chisholm-Mason grading. According to CD20 and CD27 expression<sup>24</sup>, the phenotype and distribution of B and T lymphocytes were similar in the salivary glands of patients with SS regardless of staining signals for  $\beta 1i$ . In contrast to the reduced staining signal for  $\beta 1i$  using K463, the proteasome subunit  $\beta 1i$  was significantly upregulated at the mRNA level in the tissues of patients with SS. In accord with the observation of altered  $\beta 1i$  expression in the inflammatory glandular infiltrates, the



staining signal for  $\beta 1i$  was also reduced in PBMC of patients with SS compared to healthy controls using the same polyclonal anti- $\beta 1i$  antibody. These findings suggest that the proinflammatory signal of the infiltrating cells does upregulate  $\beta 1i$  expression in the targeted tissue of patients with SS at the mRNA level. Despite the fact that mRNA and protein expression do not necessarily correlate, the reduced staining intensity using a polyclonal antibody indicates the altered antigenic properties of  $\beta 1i$  in immune cells of SS patients compared to controls.

Of note, in our cohort 3 out of 4 patients undergoing immunomodulating therapy and with disease quiescence expressed detectable levels of  $\beta 1i$  in the salivary glands as well as in the infiltrating lymphocytes. In contrast, all but one untreated patient with SS had infiltrating lymphocytes that showed altered expression of the  $\beta 1i$  protein. Although it is not possible to draw conclusions from such a limited number of patients, this could potentially indicate that expression of  $\beta 1i$  at the inflammatory site is influenced by treatment and perhaps represents a marker of pathogenic cells. Interestingly, induction of the constitutive alpha-type C3 subunit was observed after exposure to glucocorticoid therapy<sup>25</sup>. However, it remains to be confirmed whether expression of  $\beta 1i$  or other proteasomal subunits is also influenced by antiinflammatory drugs. By interference with signaling pathways of transcriptional factors like nuclear factor- $\kappa B$  such medications might be immune modulating of the  $\beta 1i$ -deficient cells. Investigations in larger cohorts are necessary to clarify whether this expression profile could be useful for diagnostic purposes or whether altered expression of  $\beta 1i$  characterizes a distinct subset of patients with SS.

A molecular defect in the bidirectional promoter region of the  $\beta 1i$  gene has been described in the non-obese diabetic (NOD) mouse, a model of type I diabetes and SS<sup>26</sup>. In consequence, reduced levels of  $\beta 1i$  were reported at the mRNA and protein levels, with implications for development of autoimmune disease in that particular animal model of autoimmunity. In SS, mRNA levels of  $\beta 1i$  were found to be upregulated in PBMC, but no genetic defect was found in the promoter region (Krause, *et al*<sup>19</sup> and unpublished data). Therefore, observation of altered  $\beta 1i$  expression in the human disease remains obscure and could be explained by posttranscriptional alterations of  $\beta 1i$  turnover. Another possibility is a posttranslational modification of the  $\beta 1i$  protein, with a potential influence on antigenic properties, leading to altered antibody recognition. Thus, functional studies are required to confirm alterations in the proteasome system. Longterm observations of SS patients would be necessary to clarify the effects of  $\beta 1i$  dysregulation or modification on the disease course or outcome.

A polymorphism of  $\beta 1i$  that may be associated with different autoimmune diseases has been described<sup>27</sup>. This polymorphism in codon 60 (R/H) also resulted in reduced

recognition of the protein by antibodies, but the explanation for this remains unclear.

An intact proteasome system is crucial for cellular homeostasis, activation of several transcriptional factors, and control of the immune response<sup>28-30</sup>. In this regard, incorporation of immunoproteasome subunits into the catalytic complex is known to have a marked influence on the quality and quantity of the antigenic MHC I epitopes produced, including maintenance of a normal selection of self-peptides<sup>14,31-34</sup>. In this context, immunoproteasomes are also involved in effective clearance of, for example, viral infections<sup>13,35</sup>. However, altered expression of one singular proteasomal subunit could interfere with the assembly of the entire catalytic complex and alter the composition of certain immunoproteasome isoforms or hybrid proteasome complexes<sup>36</sup>. Given the influence of proteasomal subunit composition on the function of the proteasome system, our results suggest a possible role for the  $\beta 1i$  subunit in the pathogenesis of SS. Dysregulation of  $\beta 1i$  could potentially alter apoptosis of autoreactive T cells and lead to perpetuation of inflammatory responses. Further, proteasomes were also shown to be involved in the generation of intracellular peptides for inflammatory cell signaling<sup>37</sup>. Therefore, such altered proteasome expression could influence the initiation or maintenance of the inflammatory autoimmune response. Taken together, our data strongly encourage further investigations of the proteasome system in SS. Identifying tissue- or cell-specific markers for SS is crucial for our understanding of this complex disease, and could support early diagnosis and novel treatment strategies in clinical trials with targeted interventions of only disease-causing cells. The dysregulation of  $\beta 1i$  appears to be characteristic for SS, since it was rarely seen in patients with sicca symptoms who did not have autoimmune sialadenitis. The common dysregulation of  $\beta 1i$  in subsets of lymphoid cells of both SS-prone NOD mice and SS patients suggests that altered expression of  $\beta 1i$  protein could be a marker of pathogenic cells<sup>38,39</sup>.

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