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Upregulation of the immunoproteasome in peripheral blood mononuclear cells of patients with IgA nephropathy

Rosanna Coppo[1], Roberta Camilla[1], Alda Alfarano[2], Sabrina Balegno[1], Domenico Mancuso[1], Licia Peruzzi[1], Alessandro Amore[1], Antonio Dal Canton[3], Vincenzo Sepe[3] and Pierangelo Tovo[2]

[1]Nephrology, Dialysis and Transplantation Unit, Regina Margherita Children's Hospital, University of Turin, Turin, Italy; [2]Department of Pediatrics, University of Turin, Turin, Italy and [3]Nephrology, Dialysis and Transplantation Unit, Fondazione, IRCCS Policlinico San Matteo IRCCS, Pavia, Italy

In order to present an antigen to T-cells, the antigen must first be degraded by proteasomes. Following exposure to interferons, some proteasome subunits (ß1,ß2,ß5) are replaced by others (LMP2, LMP7, MECL-1) that have more optimal catalytic properties for peptide presentation; this more efficient organelle is termed the immuno-proteasome. Here we measured gene expression of various subunits in peripheral mononuclear cells of patients with IgA nephropathy, a disease with features of immune dysregulation. We used quantitative PCR to measure the expression of proteasomal subunit mRNA in mononuclear cells from IgA nephropathy patients, a group of proteinuric control patients with idiopathic nephrotic syndromes, and healthy controls. A significant switch in the expression of trypsin- and chymotrypsin-like proteasome subunits to corresponding immuno-proteasome subunits was found in patients as compared to healthy controls. Further, we found that nuclear translocation of NF-κB p50 and p65 was significantly greater in the IgA nephropathy patients, but this did not correlate with the switch to the immuno-proteasome phenotype. Patients with proteinuria greater than 0.5 g/1.73 m^2/day had a significant switch of the chymotryptic-like β5 protease to the LMP7 subunit, but this did not occur in patients with idiopathic nephrotic syndrome. The switch to an immuno-proteasome in peripheral blood mononuclear cells of patients with IgA nephropathy suggests an increased efficiency of antigen processing and presentation. This switch appears to be independent of a coincidental activation of the NF-κB pathway but is associated with high levels of proteinuria, a well known risk factor for progression of IgA nephropathy.

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Correspondence: *Rosanna Coppo, Nephrology, Dialysis and Transplantation Unit, Regina Margherita Children's Hospital, University of Turin, Piazza Polonia, Turin 10127, Italy. E-mail: rosanna.coppo@unito.it*

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Class A immunoglobulin (IgA) nephropathy (IgAN) is a chronic glomerular disease characterized by mesangial deposits of IgA likely due to an accumulation of IgA immune complexes.[1] IgAN is characterized by a dysregulation of the immune system, with an altered response to environmental antigens eventually leading to synthesis and mesangial deposition of aberrantly glycosylated polymeric IgA1.[2] Although the origin of deglycosylated IgA1 remains unknown, a function for a particular cytokine milieu has been suggested from experimental models.[3]

Among the recently identified new actors in the regulation of the immune response, the proteasome (PS) has been the focus of great interest, as it controls many crucial cell functions,[4] including the cell-cycle progression, the activation of transcriptional factors, such as nuclear factor-κB (NF-κB), and the production of cytokines and chemokines. Antigen processing and presentation onto major histocompatibility complex (MHC) class I molecules also are under proteasomal control.[5]

The PS is a 26S complex containing a 20S multicatalytic core and two 19S regulatory complexes. The 20S core is composed of 4 seven-member rings. The two outer rings are formed by α subunits, whereas the two inner rings contain β subunits; the β1, β2, and β5 subunits have caspase-like, trypsin-like, and chymotrypsin-like activities for cleavage of acidic, basic, and hydrophobic amino acids, respectively.[6] The interferons (IFN-γ or -α) induce a switch to a new PS form, the so called immunoproteasome (iPS), by replacing the β1 subunit with the low-molecular-weight protein 2 (LMP2), the β2 subunit with the endopeptidase complex-like-1 (MECL-1), and the β5 subunit with the LMP7.[7] The switch to iPS improves the catalytic proteasomal activities and leads to optimal MHC-I peptide presentation and T-cell response.[8]

The aim of this study was to investigate the PS and iPS system in patients with IgAN. We therefore evaluated mRNA encoding for the active subunits of PS and iPS in peripheral blood mononuclear cells (PBMCs) from patients with IgAN, taking into consideration also their clinical features, renal biopsy grading, and type of therapy.

RESULTS

We considered 55 patients in whom a diagnosis of IgAN was performed by renal biopsy eligible for the study (relevant data in Table 1). Histologic grading showed prevalence of moderate lesions (60%). All patients had a creatinine clearance >90 ml/min/1.73 m^2. A total of 22 patients had proteinuria >0.5 g/1.73 m^2/day. In the previous 3 months, 23 patients were treated with angiotensin inhibitors (either ACE inhibitors or angiotensin II receptors blockers), 7 with prednisone, whereas the other 26 had no treatment. None was assuming statins or other anti-inflammatory drugs. A disease control group of 30 patients with idiopathic nephrotic syndrome, as well as 50 age and sex-matched healthy controls (HCs) was also investigated (Table 1).

We detected a significant switch of the trypsin-like (MECL-1/β2) and chymotrypsin-like (LMP7/β5) catalytic PS subunits mRNA to the iPS corresponding subunits in patients with IgAN (MECL-1/β2: 1.49 ± 0.89 (s.d.) versus 1.02 ± 0.30 in HC, $P = 0.0044$; LMP7/β5: 1.73 ± 1.36 versus 1.07 ± 0.33 in HC, $P = 0.0083$) (Figure 1; Table 2). There was a highly significant correlation ($P < 0.0001$) of these two iPS/PS ratios in IgAN patients (Figure 2). The disease control group of children and adolescents with idiopathic nephrotic syndrome showed a switch limited to one of the three iPS (MECL-1/β2: 1.93 ± 2.25, $P = 0.0249$ versus HC). The other iPS subunits were similar to HC (LMP2/β1: 1.17 ± 0.66, $P = 0.0755$ versus HC; LMP7/β5: 1.12 ± 0.45, $P = 0.6678$ versus HC). No correlation was found among PS/iPS mRNA ratios in this diseased group and healthy controls.

The mean values of the switch of the caspase-like catalytic subunits (LMP2/β1) were similar in patients and nephrotic syndrome or healthy controls and not correlated with the trypsin-like and chymotrypsin-like catalytic subunits (Figure 1; Table 2).

In a control group of subjects with respiratory or gastrointestinal infections without urinary involvement, the switch was completely different from what observed in patients with IgAN, as MECL-1/β2 mRNA ratio, which was increased in IgAN, was found to be decreased (0.69 ± 0.36, $P < 0.02$ versus IgAN), whereas LMP2/β1, which was not affected in IgAN, was significantly increased (2.4 ± 1.1 in infectious controls, $P < 0.001$ versus both IgAN and healthy controls).

Patients with IgAN also had a significantly increased expression of the structural PS and iPS α2 subunit mRNA in comparison to HC ($P < 0.05$) (Figure 3). Neither linear nor nonlinear correlation was found between α2 mRNA levels in patients or in controls and the PS or iPS mRNA expression for either the catalytic subunit or the iPS/PS mRNA ratios.

Nuclear translocation of p50 and p65 active subunits of NF-κB was found to be significantly greater in PBMCs of patients with IgAN than in healthy controls (p50: 275.8 ± 341.7 versus 69.9 ± 59.8, $P = 0.0413$; p65: 287.3 ± 95.2 versus 110.9 ± 130.4, $P = 0.0001$) (Figure 4; Table 2). No significant correlation was found between NF-κB nuclear binding in PBMCs of patients with IgAN and the switch from PS to iPS mRNAs.

We sought correlations between the switch from PS to iPS mRNA and clinical data of the IgAN patients investigated. No correlation was found with age, sex, disease duration, or creatinine clearance. No correlation between histologic grading of the renal biopsy and PS/iPS switch was detected.

The switch of the chymotrypsin-like catalytic subunits (LMP7/β5) mRNA in IgAN with proteinuria >0.5 g/1.73 m^2/day was significantly higher than in patients with lower levels (2.25 ± 1.89 versus 1.34 ± 0.72, $P = 0.0182$) (Figure 5). No correlation with the therapy assumed, either angiotensin inhibitors or corticosteroids, was observed.

No correlation with proteinuria was found in the disease control group of idiopathic nephrotic syndromes.

Table 1 | Relevant clinical data of the 56 patients with IgAN and HCs investigated

	HC	IgAN
Gender: males/females	41/9	45/10
Age (years)	21.22 ± 6.18 (2.3–40.5)	20.5 ± 8.3 (4.3–39.5)
Time (years) from renal biopsy	—	4.4 ± 4.4 (0–18.4)
Proteinuria (g/day)	—	0.89 ± 0.86 (0–3.85)
Creatinine clearance (ml/min/1.72 m^2)	121.53 ± 10 (93–140)	122 ± 12 (92–140)
Histological classification	—	
Grade I mild		19/55 (35%)
Grade II moderate		33/55 (60%)
Grade II severe		4/55 (5%)

IgAN, IgA nephropathy; HC, healthy control.
Data are expressed as means ± standard deviations and ranges in brackets.

DISCUSSION

In patients with IgAN, several lymphomonocyte abnormalities have been reported, such as increased CD4 subset,[9] and a peculiar gene expression profile in cases with progressive renal disease.[10] This report highlights that PBMCs of patients with IgAN have a significant switch from PS to iPS mRNAs. The difference with healthy controls is mostly evident in the changes concerning the trypsin-like and chymotrypsin-like catalytic PS subunits, which were significantly correlated. The whole PS and iPS mass is likely to be increased too, as indicated by the increase in the mRNA encoding for the α2 subunits, which are structural components of both the PS and the iPS. A disease control group of children and adolescents with idiopathic nephrotic syndrome with various levels of proteinuria showed a switch limited to one of the three iPS (MECL-1/β2), without correlation with the other iPS and without correlation with the extent of proteinuria.

The switch from PS to iPS is thought to be aimed at improving the proteasomal catalytic properties for optimal MHC-I peptide presentation.[5] Indeed, the iPS provides a particular professional antigen processing, leading to improved peptide presentation to reactive T cells. This goal is reached by preparing the C terminus of the peptide residues to the high affinity bond with the MHC class I cleft. iPS is upregulated in dendritic cells when they migrate to the lymph

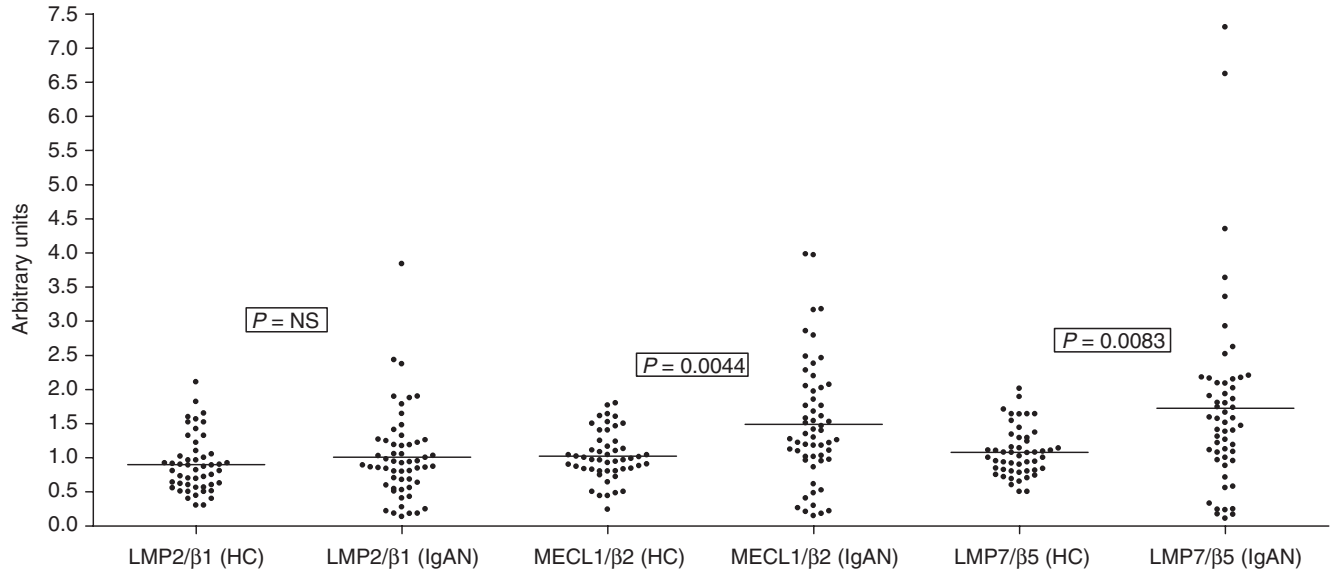

Figure 1 | Individual results of the switch from proteasome (PS) to immunoproteasome (iPS) detected as a ratio between each iPS catalytic unit (LMP2, LMP7, MECL-1) and the corresponding PS (β1, β2, β5) mRNA in patients with IgA nephropathy (IgAN) and healthy controls (HC).

Table 2 | Densitometric data (arbitrary units) of mean levels and standard deviations of the switch from PS to iPS as a ratio between each iPS catalytic subunit (LMP2, LMP7, MECL-1) mRNA and the corresponding PS subunits (*β*1, *β*2, *β*5); mRNA nuclear binding activity of the two active subunits of the transcription factor NF-κB p50 and p65; significance of the differences (*P*) between values in patients with IgAN and HCs

	HC	IgAN	*P*
LMP2/*β*1	0.91 ± 0.42	1.01 ± 0.65	NS
MECL-1/*β*2	1.02 ± 0.30	1.49 ± 0.89	0.0044
LMP7/*β*5	1.07 ± 0.33	1.73 ± 1.36	0.0083
NF-κB p50	69.9 ± 59.8	275.8 ± 341.7	0.0413
NF-κB p65	110.9 ± 130.4	287.3 ± 95.2	0.0001

IgAN, IgA nephropathy; HC, healthy control; NS: not significant; PS, proteasome; iPS, immunoproteasome; LMP2, low-molecular-weight protein 2; LMP7, low-molecular-weight protein 7; MECL-1, *β*2 subunit with the endopeptidase complex-like-1.

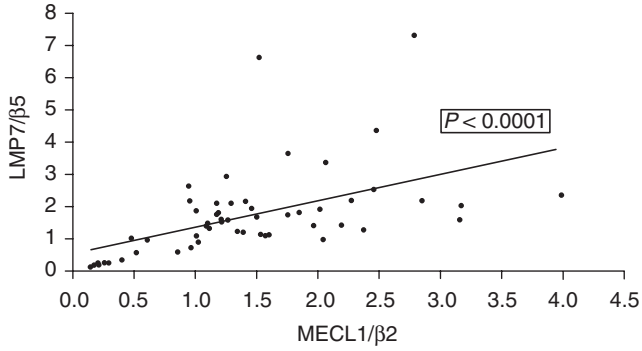

Figure 2 | A highly significant correlation was found between the two switches from PS to iPS mRNAs (MECL-1/β2 and LMP7/β5) in patients with IgA nephropathy. The correlation was not significant in patients with idiopathic nephrotic syndrome or in healthy controls (data not shown).

nodes to stimulate T cells.[11] Circulating monocytes are likely to be mostly involved in the switch to iPS found in our IgAN patients, as it was detected in some experiments on purified adherent blood cells (data not shown).

The novelty of our finding leaves the mechanism/s responsible for the upregulation of the iPS in IgAN to be elucidated. A switch from PS to iPS has been reported in some systemic autoimmune disorders,[12] and in IFN-induced antiviral states.[13,14] Indeed, IFNs are considered major actors in the switch from PS to iPS in immunocompetent cells.[15] We recently reported a significantly higher expression of iPS genes (LMP7/β5) in children with hepatitis C viral (HCV) infection, which was supposed to lead to an increased presentation of self-antigens onto MCH-I molecules, ultimately resulting in enhanced autoimmune responses.[16] A function for infectious agents in triggering IgAN, as suggested by the typical gross hematuria in coincidence with upper respiratory tract infections, has been researched for decades.[1] Our finding of a switch from PS to iPS, measured as a ratio between iPS/PS catalytic subunits mRNAs, in PBMCs of patients with IgAN may reflect a response to infectious challenges, for example viral infections, with an increased release of IFNs. This scenario fits with a Th1 lymphocyte prevalence and Th1 cytokine release in IgAN. This hypothesis is supported by recent observations which suggest a possible function for Th1 prevalence in experimental animal models.[17] A Th1/Th2 balance polarization toward Th1 has been reported in the ddY strain of mice with spontaneous IgAN, and it was particularly evident at the beginning of the disease.[17] The production of IFN-γ has also been suggested as influencing the risk of renal failure in mice with IgAN induced by Sendai virus.[18,19] Th2 cytokines have

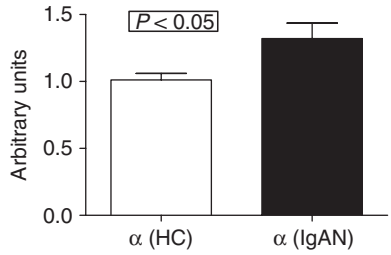

Figure 3 | PS and iPS α2 subunit mRNA values in patients with IgA nephropathy (IgAN) and in healthy controls (HCs).

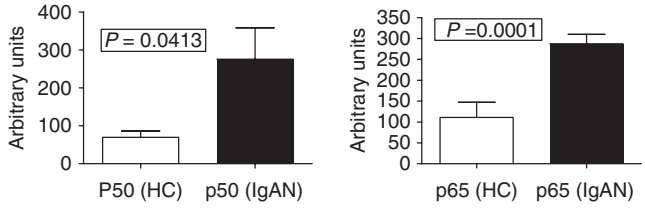

Figure 4 | Nuclear translocation of p50 and p65 subunits of NF-κB in patients with IgA nephropathy and in healthy controls.

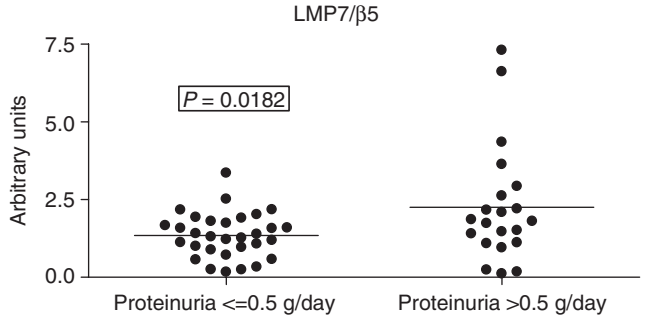

Figure 5 | LMP7/β5 mRNA ratio of iPS/PS subunits in patients with IgA nephropathy in remission (proteinuria $\leqslant$0.5 g/1.73 m^2 per day) and with proteinuria $>$1 g/1.73 m^2 per day.

rather supposed to have a function in aberrant glycosylation of serum IgA.[20]

It is of interest that the iPS switch we found in patients with IgAN does not seem to be a simple expression of a concomitant acute infectious disease, as in the control group of subjects with respiratory of intestinal acute febrile disease the iPR/PS pattern was completely different from what observed in IgAN.

An IFN-mediated switch from PS to iPS elicited by viral infections may result in NF-κB activation. In fact, the ubiquitin/PS pathway is a major regulator of NF-κB activation[4] being responsible for the degradation of NF-κB and of its inhibitory factor IκB. Hence, we investigated the relationship between NF-κB and the PS/iPS system in PBMCs in our patients with IgAN. We detected an increased nuclear translocation of the p50 and p65 active subunits of NF-κB, although this did not correlate with the switch from PS to iPS mRNAs. Therefore, a function for other mediators released during infections may be inferred. An increase in iPS, besides being possibly caused by viral induction of IFNs, has more recently been thought as being part of a response to oxidative stress, reflecting a compensatory, albeit insufficient, response to it, in age-related macular degeneration.[21] Notably, in IgAN there is an enhanced oxidative stress, leading to increased advanced oxidative stress protein products in progressive cases.[22]

The switch from PS to iPS of the chymotrypsin-like catalytic subunits (LMP7/β5) was significantly higher in IgAN patients with high proteinuria levels than in those in clinical remission. This association is of interest, as proteinuria is the most relevant risk factor for progression of IgAN.[23] No correlation with the therapy, either angiotensin antagonists or corticosteroids, was observed. No correlation with histologic grading was also observed, but the wide range of time elapsed since renal biopsy must be taken into account.

In conclusion, this is the first report of an upregulation of iPS PBMC of subjects with a renal disease, namely IgAN, suggesting an enhancement of the efficiency of the immune response to certain MHC-I antigens. The reports on switch from PS to iPS in human diseases are only a few (12, 14, 20) and the knowledge of the signals involved remains mostly speculative, like in our study. It is of note that in none of the few studies investigating iPS in clinical settings, all the three subunits are switched at the same time and the reasons are unknown. We do not know why in IgAN only two of the three iPS subunits are switched, one of them (LMP7/β5) being more correlated with proteinuria. It is of interest that the other iPS which failed to show a switch in patients with IgAN (LMP2/β2) was selectively increased in the control group of patients with febrile infectious disease without renal involvement.

It is presently impossible to say whether the switch from PS to iPS in PBMC of patients with IgAN reflects a response to infectious challenge. From the data detected in subjects with idiopathic nephrotic syndrome or with chronic viral infection, the switch from PS to iPS results not to be unique to IgAN, but the extent of subunits involved and the correlations with proteinuria we found in IgAN only, are relevant. It is of interest that genes involved in the iPS pathway have been found to be activated in IgAN during phases of clinical activity.[24] Further studies are needed to clarify whether the switch from PS to iPS that we detected in patients with IgAN has any function in pathogenesis and/or progression of the disease.

MATERIALS AND METHODS

Subjects

Patients with biopsy-proven diagnosis of IgAN, consecutively referred to the Nephrology, Dialysis and Transplant Unit of the Regina Margherita Children's Hospital, Turin, and of the Policlinico San Matteo, Pavia, from September 2004 to March 2007 were enrolled in the study. The histologic classification of IgAN in three grades of renal lesions (grade I: mild, grade II: moderate, grade III: severe) was performed according to a recent report.[25] Patients with Henoch-Schoenlein purpura nephritis,

lupus, celiac disease, chronic liver disease, or diabetes mellitus as well as those who expressed their unwillingness to blood sampling were excluded. As disease control population, we investigated 30 children and young subjects (mean age 8.8 ± 7.6) with idiopathic nephrotic syndrome (13 had renal biopsy showing focal segmental glomerulosclerosis, and 12 minimal change disease), consecutively presenting for visit or hospitalization in the Nephrology center in Turin. They were in various phase of renal activity, with mean 24 h proteinuria 7.12 ± 9.3 g/1.73 m^2 per day (range 0.1–15). A total of 9 were assuming steroids and 20 immunosuppressive drugs. Their CrCl was 111 ± 99 ml/min per 1.73 m^2. A group of 15 children and adolescents (mean age 6.7 ± 8.2) with respiratory or gastrointestinal infections without any detectable urinary involvement was investigated as well. They presented with acute febrile gastrointestinal diseases (stool culture negative for bacteria) (eight cases), viral pneumonia (two cases), febrile acute pharyngitis (four cases), and infectious mononucleosis (one case). As a healthy control population, a group of sex- and age-matched healthy subjects were randomly selected. All patients and controls were Italian Caucasians.

Creatinine clearance (CrCl) was calculated from blood and 24 h urine samples and corrected for the body surface area in both children and adults, in an effort to homogenize units of measurement in various ages. Similarly, 24 h proteinuria was corrected for 1.73 m^2, in order to have a common unit of measurement in children and adults. Urine collections ±7 days the date of serum sampling were available in 54 of 55 patients with IgAN. Proteinuria >0.5 g/1.73 m^2 per day was considered as sign of clinical activity of IgAN, as we did in a previous trial enrolling children and adults.[26]

The study was performed according to the usual guidelines and ethical principles for medical research, and informed consent was obtained from each participant. The study protocol conformed to the guidelines of the ethical committee of the two hospitals which enrolled the IgAN patients.

Peripheral blood mononuclear cells isolation

PBMC were separated by Fycoll-Hypaque density gradient (Sigma Co., St. Louis, MO, USA) according to standard protocols. More than 95% of cells were viable when tested by the Trypan blue exclusion test.

Reverse transcription

Total RNA was extracted from cells using the TRI-Reagent kit (Sigma) according to manufacturer's protocol.

Total RNA (1 μg) was reverse transcribed with 5 μl of PCR buffer II 10×, 11 μl of $MgCl_2$ 25 mM, 2 μl reverse-transcriptase MuLV 50 U/μl (murine Moloney leukemia virus), 1 μl of RNase inhibitor 20 U/μl, 5 μl random hexamers 50 μM (Applied Biosystems, Foster City, CA, USA), 1 μl mix dNTPs 100 mM (Amersham, Piscataway, NJ, USA), and dd-water in a final volume of 50 μl.

The reaction mix was carried out in a GeneAmp PCR system 9700 Thermal Cycle (Applied Biosystems) at the following conditions: 10 min at 20 °C, 45 min at 42 °C, and 5 min at 99 °C for the inactivation of enzyme; the cDNAs were stored at −80 °C.

Relative quantification by real-time PCR

Quantification of IP and PS mRNA expression was normalized to the expression of Abl, chosen as a reference gene and was achieved by means of the ABI PRISM 7700 Sequence Detection System (PE Applied Biosystems). We used a set of primers and a fluorogenic oligonucleotide probe designed to hybridize to the specific target sequence (Assay-on-Demand: α2Hs00541050_m1, βHs00382586_m1, β2Hs00160607_m1, β5Hs00655652_m1, LMP-2Hs00544762_m1, MECL-1Hs00160620_m1, LMP-7Hs00544758_m1, Abl Hs00245445 (Applied Biosystems). To obtain the quantification of IP (LMP-2, LMP-7, and MECL-1) and PS (β1, β2, and β5) we applied the ΔΔCt method. The ΔΔCt method is a relative quantification of target genes expression (IP and PS) in the patients compared with normal samples. The data obtained were analyzed using the comparative cycle threshold (Ct) method. To determine the quantity of IP and PS expression, Ct values were first normalized by subtracting the Ct value obtained from the Abl (reference gene, ΔCt = Ct IP/PS − Ct Abl). Relative IP and PS mRNA fold changes were calculated by subtracting the normalized Ct value of our patients from the controls (ΔΔCt = ΔCt patients − ΔCt controls) and the relative IP and PS fold changes were determined $2^{-\Delta\Delta Ct}$.

Western blotting

Nuclear extracts were prepared from PBMC as described previously. For the preparation of whole cell lysates, cells were lysed in a buffer containing 20 mmol/l HEPES pH 7.9, 1.5 mmol/l $MgCl_2$, 420 mmol/l NaCl, 0.2 mmol/l EDTA, 1 mmol/l DTT, 1 mmol/l PMSF, 25% glycerol. Protein concentration of protein lysates was determined using BCA assay (Pierce; Perbio Science, Bonn, Germany). For the detection of nuclear localized NF-κB family members, ~20 μg of nuclear extracts was separated on a 12% SDS–PAGE and transferred onto PVDF transfer membrane (GE Healthcare–Amersham, Fairfield, CT, USA). The blot was probed with antibodies against NF-κB p65 (SC-109, rabbit polyclonal; Santa Cruz Biotechnology, Santa Cruz, CA, USA), NF-κB p50 (SC-7178, rabbit polyclonal; Santa Cruz Biotechnology).

Incubation of all primary antibodies was followed by incubation with an appropriate horseradish peroxidase-conjugated secondary antibody. Bands were visualized by enhanced chemiluminescence staining and quantified by the Image Master Total lab 2.0 software (Amersham).

Statistical analysis

The normal distribution of variables was tested by means of the one-sample Kolmogorov–Smirnov test. Normally distributed variables were analyzed by means of the independent-sample *t*-test (for two groups). Nonnormally distributed variables were analyzed by means of the Mann–Whitney test. Data were expressed as mean ± standard deviation (s.d.). Pearson's test was used to correlate two series of normally distributed data. Statistics software SPSS 14.0 was used to elaborate data (SPSS Inc., Chicago, IL, USA).

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DISCLOSURE

All the authors declared no competing interests.

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