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SM Journal of Arthritis Research

Research Article

Quantification of Transcripts for Immunoproteasome Subunits PSMB8 and PSMB9 Discriminates Inflammatory from Non-Inflammatory Myopathies

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Article Information

Received date: Dec 06, 2017
Accepted date: Jan 31, 2018
Published date: Feb 05, 2018

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Keywords Housekeeping genes; Inflammatory Myopathies; Proteasome immunosubunits

Article DOI 10.36876/smjar.1005

Abstract

Objectives: Idiopathic inflammatory myopathies (IIM) are a group of muscle diseases characterized by inflammatory infiltrates in skeletal muscle. Associated pathways include increased antigen presentation with tissue specific inflammatory signatures could be related to the proteasome system. In this study we investigated the contribution of the proteasome in IIM.

Methods: Real time PCR was used for relative quantification of mRNA transcripts of proteasomal beta subunits in muscle biopsies from 21 patients with IIM in comparison to 13 patients with non-inflammatory myopathies (NI). Expression levels of the constitutive beta subunits (PSMB5, -6, -7), and the corresponding immunosubunits (PSMB8, -9, -10) were measured relative to different housekeeping genes (HKG). GeNorm, NormFinder and BestKeeper were used to evaluate the expression stability of HPRT1, ACTB and GAPDH as HKG.

Results: HPRT1 was identified as the most stable reference gene in the investigated biopsies. The analysis demonstrated that irrespective of the clinical disease classification, transcripts of PSMB8 and PSMB9 were upregulated in biopsies of all types of IIM and correlated with the intensity of inflammation. Furthermore, expression ratios of PSMB5:PSMB8 and PSMB6:PSMB9 differentiated clearly between IIM and NI.

Conclusions: The quantification of PSMB8 and PSMB9 could qualify for diagnostic scoring of inflammation in myopathy muscles.

Introduction

Idiopathic inflammatory myopathies (IIM) are a group of muscle diseases characterized by muscle weakness and the presence of inflammatory infiltrates in skeletal muscle [1]. Based on clinical, histopathological, immunological, and demographic features, the most clearly defined inflammatory myopathies include polymyositis (PM), dermatomyositis (DM), and inclusion body myositis (IBM) [2].

The immunopathology of dermatomyositis has been viewed as a complement mediated microangiopathy followed by perivascular inflammation which leads to perifascicular atrophy [3]. In polymyositis and inclusion body myositis, cytotoxic CD8+ T cells are the primary effector cells mediating muscle fiber injury via the perforin pathway. They also surround and invade major histocompatibility complex class I (MHC-I) antigen expressing, non-necrotic muscle fibers [4]. Furthermore, to confirm the diagnosis of PM and IBM and exclude disorders with secondary, non-specific inflammation, the CD8/MHC-I lesion is used as a relevant specific immunopathological marker [5].

Continuous upregulation of expression of MHC-I molecules on muscle fibers can cause an endoplasmic reticulum stress response leading to accumulation of misfolded glycoproteins and activation of nuclear factor kappa B (NF-κB) pathways. As a consequence, NF-κB can further stimulate the formation of CD8/MHC-I complexes, resulting in a self-sustaining inflammatory response [6].

The ubiquitin-proteasome system (UPS) is a 26S, multicatalytic and multi-subunit protease complex, which provides the main mechanism for a selective ubiquitin and ATP- dependent degradation of proteins in the cytosol and nucleus of all eukaryotic cells [7]. Of note, generation of epitopes for cytotoxic T lymphocytes begins with degradation of cellular proteins into peptides by the proteasome system. TAP molecules (transporters associated with antigen presentation 1

OPEN ACCESS
ISSN: 2637-8841

and 2) are subsequently responsible for the transfer of these peptides to the endoplasmic reticulum, where they are loaded onto MHC-I molecules for presentation at the plasma membrane [8]. Widespread over expression of MHC-I, and occasionally MHC II, is typically seen on the surface of muscle fibers in some inflammatory myopathies [9,10]. Thus, UPS could play an essential role for initiation and immunological surveillance of these diseases. In addition, activation of the transcription factor NF-κB, which is necessary for the development of inflammatory response, is also achieved via proteasomal degradation of the inhibitory protein IκB [11].

The 26S proteasome consists of a proteolytic core particle, the 20S proteasome, and is capped at one or both ends by 19S regulatory particles [12]. The 20S proteasome is a hollow cylindrical particle consisting of four stacked heptametrical rings. Each of the two outer rings is composed of seven different alpha subunits, while each inner ring is composed of seven different beta subunits [13]. Each beta ring harbours three different catalytic constitutive subunits with N-terminal threonines at the proteolytically active site, proteasome subunit beta type 5-PSMB5, type 6-PSMB6 and type 7-PSMB7 [14]. In addition, under the influence of pro-inflammatory cytokine IFN-γ, the structure and the catalytic properties of the proteasome are modified by substitution of the constitutive with three catalytic immunosubunits proteasome subunit beta type 8-PSMB8, type 9-PSMB9 and type 10-PSMB10, respectively, leading to the formation of the so-called immunoproteasome [15]. This process is considered to be strongly influence the production of peptides for antigen presentation by MHC-I as well as the immune response [16]. In this context, it was also found that the expression of both MHC-I and MHC-II on muscle cells is induced by interferon gamma IFN-γ [17]. The role of immunoproteasome subunits in the immune response was approved recently through a detectable mutation, which has been found in proteasome subunit PSMB8 and causes joint contractures, muscle atrophy, microcytic anemia, and panniculitis-induced lipodystrophy syndrome in addition to other autoinflammatory syndromes [18-21].

Previously we found that the levels of circulating proteasomes are increased in serum samples of myositis patients; and that the proteasome itself represents an autoantigen in patients with autoimmune myositis and other autoimmune disorders [22,23]. Additionally, we showed an altered expression of some proteasomal subunits in PBMCs of PM patients [24].

In our previous study, we found also increased expression levels of immunoproteasome subunits in inflammatory myopathies in muscle tissues classified only according to inflammatory status [25]. Therefore, the aim of this study was to clarify, if there is a difference in the expression of proteasomal subunits in myopathy muscle tissues according to underlying diseases or whether this only depends on the inflammation process. Hence, we used relative quantitative real time PCR to analyze the mRNA gene expression levels of proteasomal catalytic beta subunits in muscle biopsies taken from PM, DM and IBM patients, as well as, from non-inflammatory myopathies (NI). For this purpose, we validated different housekeeping genes to identify the most stable one in myopathy tissues that could be used in relative quantification PCR.

Patients and Methods

Patients

Investigation of gene expression of proteasome subunits was performed in muscle samples obtained from frozen biopsies collected at the Friedrich Baur Institute of the Ludwig Maximilians University (Munich, Germany), and from patients with manifestations of muscle pathology visiting the department of Rheumatology at the Charité University Hospital in Berlin, Germany. The study was approved by the ethic committee of the Charité – University Medicine Berlin and written informed consent was obtained from all patients before muscle biopsies were taken.

The study included 21 patients with idiopathic inflammatory myopathies (IIM) including 7 patients with PM, 9 patients with DM, and 5 patients with IBM (15 female, 6 male, mean age 57.9 years, age range 14-83 years). PM and DM patients were diagnosed according to the modified criteria proposed by Bohan and Peter [26,27]. IBM patients were diagnosed according to Griggs Criteria [28]. For comparison, another 13 non-inflammatory (NI) muscle biopsies were taken from patients with different clinical manifestations of muscle diseases (9 male, 4 female, mean age 55 years, age range 36-74).

Muscle biopsies for histological evaluation and mRNA expression

Histological evaluation of muscle biopsies was performed for diagnostic purpose. Patients were grouped according to the written reports, where inflammatory infiltrates were assigned as strong, weak or none.

For mRNA expression analysis, muscle tissues were stabilized in RNA later (Qiagen, Germany) to avoid RNA degradation, and were stored at -80°C.

Isolation of total RNA from muscle biopsies

Muscle tissues were triturated in a mortar with a pestle in the presence of liquid N_2 to protect RNA against digestion. Disrupted samples were then added to lysis buffer containing β-mercaptoethanol. Total RNA was isolated from lysed muscle tissues by NucleoSpin® RNA/Protein kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instruction. Integrity and quantity of isolated total RNA were controlled using the Bioanalyzer 2100 (Agilent Technologies, Böblingen, Germany).

Reverse transcription into complementary DNA (cDNA)

To synthesize first-strand cDNA from total RNA, SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen, Germany) was used according to the instructions of the manufacturer.

Real-time relative quantitative reverse transcriptase-polymerase chain reaction (real time RT-PCR)

For relative quantification of gene expression of catalytic proteasome subunits PSMB5, PSMB6, PSMB7, PSMB8, PSMB9 and PSMB10, forward and reverse primers for real time RT-PCR were designed as described elsewhere [29].

For relative quantification in muscle biopsies, primers were designed for three housekeeping genes (HKG), beta-actin (ACTB), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and hypoxanthine guanine phosphoribosyl transferase 1(HPRT1).

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https://dx.doi.org/10.36876/smjar.1005

Amplification reactions contained SYBR Green PCR Master Mix (Applied Biosystems), 200 nM forward and reverse primers for each gene and cDNA. Real-time PCR was performed in triplicates using the ABI prism 5700 Sequence Detection System (Applied Biosystems).

Expression of each target gene at the mRNA level was determined relatively to the housekeeping genes HPRT1, ACTB, and GAPDH and calculated as

$R = E^{Ct\ housekeeping\ gene} / E^{Ct\ target\ gene}$ [30], with E representing the amplification efficiency of the respective primer system. Mann-Whitney U-test was applied for group comparisons.

Identification of the most stable reference gene

To evaluate the expression stability of candidate reference genes (housekeeping genes), three different statistical algorithms: geNorm v3.5 [31], NormFinder v0.953 [32], and BestKeeper v1 [33] were used according to developer's recommendations. For each tissue sample, the mean Ct value for each reference gene was evaluated and used either directly to estimate its expression stability across all samples as in BestKeeper algorithm, or was first transformed to relative quantities using the comparative Ct-method as in geNorm and NormFinder [34].

GeNorm

The selection of reference genes in geNorm depends on the concept that the expression ratio of two ideal reference genes should be identical in all samples, despite the consequences of experiment or tissue type. This means that, increasing variations in the expression ratio of two housekeeping genes across samples reflect decreasing expression stability of one (or both) of the genes.

GeNorm identified the pair wise variation for each reference gene with all other reference genes as the standard deviation of the logarithmically transformed expression ratios, and determined the expression stability measure M as the mean of pair wise variation of that gene with all other tested reference genes. The lowest M values describe the most stable expression genes. Stepwise elimination of the least stable gene with the highest M value allows ranking of the tested genes according to their expression stability and results in the identification of the two most stable genes.

NormFinder

The NormFinder uses a model based approach to estimate not only the overall expression variation of the normalization genes but also the variation between sample subgroups of the sample set. NormFinder combines intragroup variation and intergroup variation into a stability value of control genes. It ranks the set of normalization genes according to their expression stability. The best gene has the lowest stability value.

Bestkeeper

Bestkeeper estimates inter-gene relations between each possible pair of housekeeping genes by making several pair-wise correlation analyses, within each correlation the pearson correlation coefficient (r) and the propability p value are calculated. All highly correlated Housekeeping genes are used to identify BestKeeper Index specific for the respective sample which is identified as the geometric mean of its candidate housekeeping genes Ct values.

The relationship between each housekeeping gene and the index is determined by calculating the Pearson correlation coefficient (r) and the p value. The most stable expression gene has the highest coefficient of correlation.

Results

Expression stability of candidate reference genes: HPRT1 was identified as the most stable gene by three algorithms

Expression levels of all investigated proteasomal subunits relative to the three HKG was quantified and medians of expression in different patient groups (PM, DM, IBM, and NI) were calculated. Comparison of results showed that in the investigated groups nearly similar expression profiles were obtained by using HPRT1 and ACTB in contrast to GAPDH (Figure 1).

Furthermore, according to analyses performed by using geNorm, NormFinder and BestKeeper software, HPRT1 was shown to represent the most stable reference gene, and therefore, was selected as housekeeping gene for relative quantifications. The respective ranking order of the 3 candidate reference genes using the three algorithms is shown in Table 1.

GeNorm analysis

GeNorm calculates the gene expression stability M. HPRT1 was characterized as the most stable expression gene with M = 1.107, and GAPDH as the least stable gene with M = 1.491. ACTB showed intermediate expression stability with M = 1.445. As seen, all our candidate reference genes achieved acceptable expression stability with M values below the default limit of 1.5 in the geNorm program. Elimination of the least stable gene with the highest M value led to the identification of HPRT1 and ACTB as the two most stable reference genes.

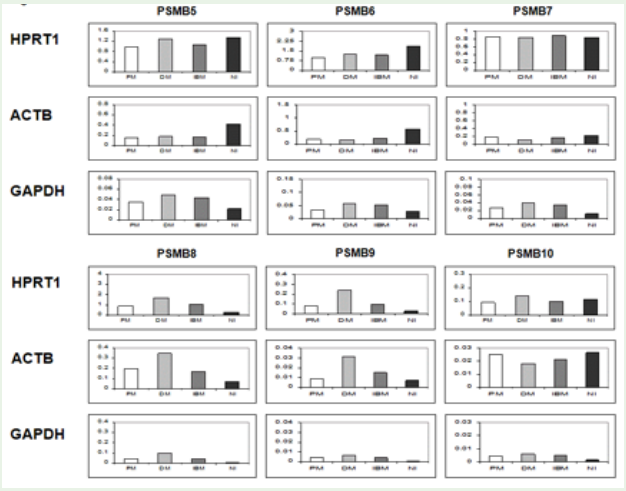

Figure 1: Expression of all catalytic proteasomal subunits in muscles relative to all housekeeping genes. mRNA expression of constitutive and immunoproteasome subunits was determined in different patient groups relative to the three housekeeping genes HPRT1, ACTB, and GAPDH. Median values were calculated for each subunit in each disease relative to the different housekeeping genes to illustrate similarities and differences depending on the housekeeping gene.

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Table 1: Ranking of the candidate housekeeping genes according to their expression stability values calculated by GeNorm, NormFinder and BestKeeper algorithms.

Ranking order	Gene symbol	GeNorm expression stability (M)	NormFinder stability value	BestKeeper Correlation coefficient (r)
1	HPRT1	1.017	0.186	0.97
2	ACTB	1.445	0.475	0.91
3	GAPDH	1.491	0.516	0.81

NormFinder analysis

Stability analysis of the gene expression of tested reference genes in the two subgroups IIM and NI group showed that HPRT1 is the most stable gene with the lowest stability value of 0.186. In the ranking order, the next stable genes were ACTB followed by GAPDH with stability values of 0.475 and 0.516, respectively. Further examination for genes with lowest intra group variations of IIM biopsies found also HPRT1 as the most stable gene followed by GAPDH and then ACTB with values of 0.031, 0.147 and 0.405, respectively. Intra group variations of control group showed ranking order with values of 0.204, 1.214, and 1.720 for HPRT1, ACTB, and GAPDH, respectively.

BestKeeper analysis

BestKeeper analysis revealed that the best correlation was obtained for HPRT1 (r = 0.97) followed by ACTB (r = 0.91) and GAPDH (r = 0.81) with a p value of 0.001.

Expression of immunoproteasome subunits is upregulated in inflammatory myopathy patients

The comparison analyses of the expression of constitutive and immunoproteasome catalytic beta subunits were performed in muscle biopsies of IIM patients including PM, DM, and IBM, as well as between IIM and NI biopsies (Figure 2). There were no significant differences in the levels of expression of the constitutive subunits PSMB5 and PSMB7. Only for PSMB6, a significantly increased expression was observed in NI compared to PM (p=0.0141).

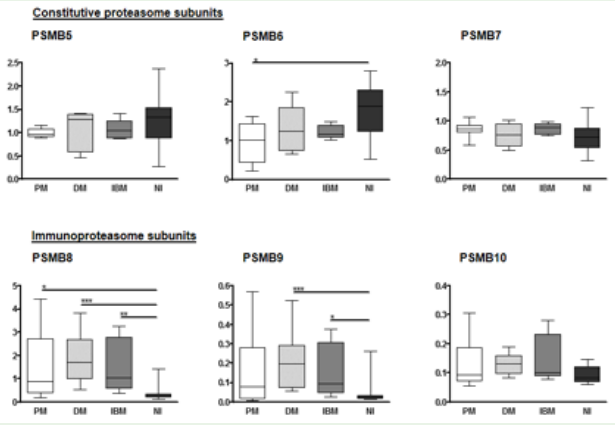

Figure 2: Expression of catalytic proteasomal subunits in muscle tissues classified according to underlying diseases. Expression of mRNA was quantified for constitutive and immunoproteasome subunits in muscle tissues of polymyositis (PM), Dermatomyositis (DM), inclusion body myositis (IBM) and non-inflammatory myopathy (NI) patients. Data are shown as relative expression normalized to HPRT1. Each box represents the 25th to 75th percentiles. The lines inside the boxes indicate the median. Horizontal bars outside the boxes display minimum and maximum.
*p<0.05, **p<0.01, ***p<0.001.

In Contrast, levels of expression of immunoproteasome subunits PSMB8 and PSMB9 were upregulated in IIM patients compared to the NI group. In detail, the immune subunit PSMB8 was significantly upregulated in all PM, DM, and IBM patients compared to NI (p=0.0175, p=0.0003, and p=0.0058, respectively). Expression of PSMB9 was significantly increased in DM and IBM compared to NI (p=0.0005 and p=0.018, respectively). The immune subunit PSMB10 was found increased but not significantly in all IIM groups when compared to NI. There were no differences detectable between IIM patient groups for expression levels of constitutive or immunoproteasome catalytic subunits.

Expression level of immunoproteasome subunits corresponds to the inflammatory status

In order to elucidate if inflammation itself has influence on the expression of proteasome subunits in muscle tissues independently of the underlying disease, we grouped biopsies according to the inflammatory status reported after histopathological examination into biopsies scored with strong, weak and non-inflammatory changes (Figure 3). Comparison of expression levels for the constitutive catalytic subunits PSMB5 and PSMB7 showed no differences between the three groups. In contrast, PSMB6 was significantly higher expressed in non-inflammatory compared to weak and strong inflammatory biopsies (p=0.0084 and p=0.0489, respectively). Expression of immunoproteasome catalytic beta subunits in different muscle tissues was increased gradually in parallel with increasing muscle infiltrates. In detail, PSMB8 expression was highly upregulated in strong inflammatory compared to weak and

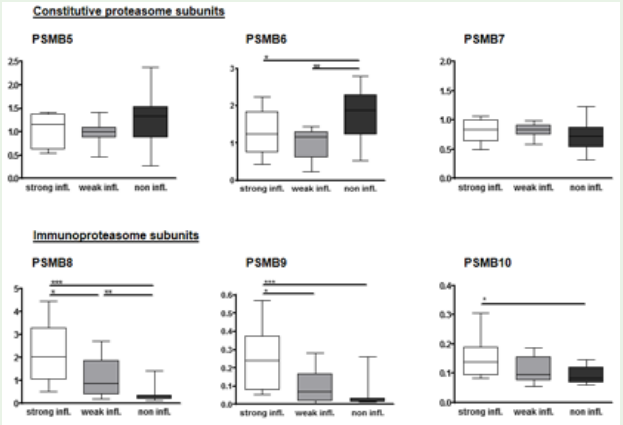

Figure 3: Expression of catalytic proteasomal subunits in muscle tissues classified according to inflammatory status. Expression of mRNA was quantified for constitutive and immunoproteasomal subunits in strong, weak and non-inflammatory biopsies. Data are shown as relative expression normalized to HPRT1. Each box represents the 25th to 75th percentiles. The lines inside the boxes indicate the median. Horizontal bars outside the boxes display minimum and maximum.
*p<0.05, **p<0.01, ***p<0.001.

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non-inflammatory biopsies (p=0.0318 and p=0.0001, respectively) and in weak inflammation compared to no inflammation (p=0.0048). Expression of PSMB9 was also significantly upregulated in strong inflammatory compared to both weak and non-inflammatory biopsies (p=0.0221 and p=0.0002, respectively). The immune subunit PSMB10 was significantly increased in strong inflammatory biopsies only when compared to non-inflammatory samples (p=0.0176). ROC and calculation of AUC revealed highest values for PSMB8 (0.92) followed by PSMB9 (=0.83) and PSMB10 (0.70).

Ratio of expression of constitutive vs. corresponding immunoproteasome catalytic subunits reflected the degree of tissue inflammation

Under inflammatory conditions, the constitutive catalytic proteasomal subunits PSMB5, PSMB6 and PSMB7 are substituted with their corresponding catalytic immunosubunits PSMB8, PSMB9 and PSMB10, respectively. Therefore, it was of interest if the ratio of constitutive subunits to their corresponding immunosubunits would reflect the degree of inflammation in muscle samples. For this analysis, patients were grouped according to the inflammatory status into the above described three groups and the ratios of constitutive vs. immunosubunits were calculated for each biopsy, mean values of ratios in each group were represented as pie charts (Figure 4).

The most remarkable differences were observed for the PSMB5:PSMB8 and PSMB6:PSMB9 calculated ratios. The ratios clearly correlated with the level of inflammation with approximately 10x-fold higher means of expression ratios in non-inflammatory than strong inflammatory biopsies. Therefore, the ratio of constitutive to immunosubunits was able to differentiate between non-inflammatory and inflammatory changes. In detail, the mean ratios were 5.6, 1.93 and 0.63 for PSMB5:PSMB8 and 85.6, 24.6 and 8.4 for PSMB6:PSMB9 in non- , weak and strong inflammatory biopsies, respectively. Using ROC, AUC reached values of 0.9 for both ratios. Ratios of PSMB7:PSMB10 showed no differences between the three investigated groups (AUC=0.65).

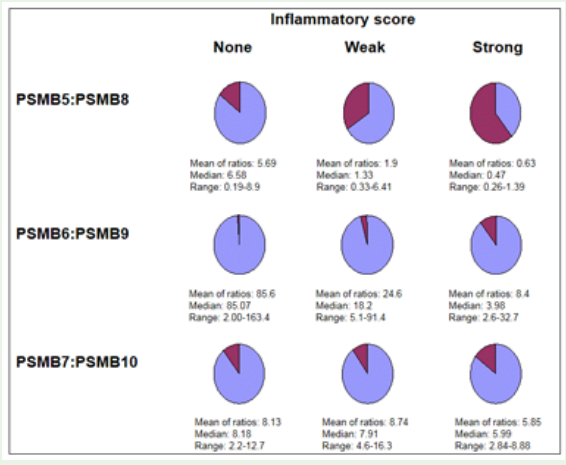

Figure 4: Ratios of expression of constitutive vs. immunoproteasome subunits in muscles classified according to inflammatory status. Mean values of ratios between constitutive and immunoproteasome subunits in muscle biopsies are displayed as relative areas for constitutive (blue) and immunoproteasome subunits (purple) in pie charts for the groups with none, weak and strong inflammation.

Discussion

In this analysis, we could demonstrate that real time RT-PCR is a valid method for diagnostic application and that HPRT1 is the most reliable housekeeping gene in myopathy muscle biopsies when compared to ACTB or GAPDH. This study also confirmed that irrespective of the clinical disease classification, immunoproteasome expression is involved in all types of inflammatory myopathies and is associated with the intensity of inflammation.

Real-time PCR is a standard method for investigating and quantifying changes in relative gene expression in different tissues. High interference of differences in number of cells per sample and efficiency of RNA extraction and reverse transcription methods with efforts of quantification [35,36] require appropriate methods of normalization. This can be achieved by quantification of target genes relative to the expression levels of constitutively expressed control genes called reference [37] or housekeeping genes [38].

Following previous studies, we tested the three most frequently applied housekeeping genes HPRT1, ACTB and GAPDH for their performance in myopathy biopsies. Three accepted statistical algorithms all identified HPRT1 as the most stably expressed housekeeping gene.

Analysis of catalytic constitutive and immunoproteasome subunits in muscle biopsies of patient with PM, DM, IBM and NI reflected significant upregulation of the two immunoproteasome subunits PSMB8 and PSMB9 in all IIM but not NI patients. In agreement to our results, immunoblots of proteasome subunits illustrated that these two immunosubunits were strongly expressed in IBM muscle biopsies, but were not detectable in control biopsies [39]. Additionally, serial consecutive sections processed for immunohistochemistry revealed co-localization of the three immunoproteasome subunits PSMB8, PSMB9 and PSMB10 in the same abnormal muscle fiber in myofibrillar myopathy and IBM [40]. In the same study, densitometric quantification of the immunoblots showed significantly elevated expression levels of PSMB9 in IBM biopsies as compared to controls. As mentioned above, under the influence of pro inflammatory cytokine IFN-γ, which is also involved in the immunopathogenesis of IIM [41], the three catalytic constitutive beta subunits can be substituted by their counterpart immunosubunits. The synthesis of this cytokine [42], as well as of PSMB9 and TAP1 [43] is mediated by NF-κB. In this context, activation of this transcription factor plays a central role in the development of inflammation in IIM [6] and also in endotheliopathy characterizing DM [44].

In the next step, we compared the expression levels of constitutive and immunoproteasome subunits between muscle biopsies with different inflammatory grades. The detected higher expression levels of all three immunosubunits PSMB8,-9,-10 in inflammatory biopsies relative to non-inflammatory samples could be explained depending on previous immunohistochemical studies that showed intense immunostaining for proteasomes in general in atrophic and necrotic fibers of PM and DM primarily in areas of mononuclear cell infiltrates [45,46]. Moreover, quantitative analysis revealed significantly more fibers positive for proteasomes in PM and DM biopsies than in control specimens [46]. These findings provide evidence that the proteasome content of muscle correlates with the intensity of inflammation. Beyond this general increase of proteasome

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content in inflamed muscle, this study confirms our previous finding of increased expression especially of immunosubunits of the proteasome. We could demonstrate recently that this increase is the result of an inflammation dependent active upregulation of immunoproteasome gene transcripts in myopathy biopsies and not only related to the infiltration of immune cells, which are expressing immunoproteasomes already under normal conditions while healthy muscle does not transcribe any of the immunoproteasome subunits [25]. The increased expression levels of constitutive subunit PSMB6 in our non-inflammatory samples as compared to strong and weak inflammatory samples could be due to substitution of the constitutive subunit with its corresponding immunosubunit PSMB9 under inflammatory conditions. Substitution of constitutive subunits with their corresponding immunosubunit according to the magnitude of inflammation was confirmed in our samples by comparing the ratios of constitutive to corresponding immunosubunits. Only PSMB5:PMB8 and PSMB6:PSMB9 calculated ratios were decreasing with increasing inflammation. This also confirmed our previous observation that the PSMB10 subunit seems to be related to the degree of infiltration but not further upregulated beyond the regular level of expression in immune cells [25]. Therefore, only in case of PSMB5 and PSMB6, the ratio of constitutive to immunosubunits could be used as a tool to differentiate between non-inflammatory and inflammatory changes. The catalytic subunit PSMB7 could require a stronger inflammatory signal to be substituted or follow different molecular mechanisms of gene regulation.

Conclusion

In conclusion, upregulation of immunoproteasome subunits was confirmed in all types of IIM muscle and correlated with the magnitude of inflammatory infiltrates in muscle biopsies. This indicates that quantification of immunoproteasome subunits PSMB8 and PSMB9 and their ratios with the corresponding constitutive subunits can qualify for diagnostic scoring of inflammatory activity in myopathy muscles. The role of immunoproteasomes in generating immunogenic epitopes for presentation by MHC-I to CD8+ lymphocytes also suggests a role of this protease complex in the pathogenesis of inflammatory myopathies.

Acknowledgements

We thank Toralf Kaiser and Jenny Kirsch from the Deutsches Rheuma-Forschungszentrum, Flow cytometry Core Facility in Berlin for technical assistance in cell sorting. The study was approved by the ethic committee of the Charité – University Medicine Berlin and written informed consent was obtained from all patients before muscle biopsies were taken.

Funding

German Research Foundation grant DFG FE470/3-1; German Research Foundation grant DFG GRK1631 (MyoGrad); EU IMI grant BeTheCure, contract no 115142-2; ArthroMark of the German Federal Ministry of Education and Research (BMBF). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Citation: Ghannam K, Gamboa LM, Fettke MK, Krause S, Häupl T, Bhattarai S, et al. Quantification of Transcripts for Immunoproteasome Subunits PSMB8 and PSMB9 Discriminates Inflammatory from Non-Inflammatory Myopathies. SM J Arthritis Res. 2018; 2(1): 1005.
https://dx.doi.org/10.36876/smjar.1005