The immunoproteasomes are key to regulate myokines and MHC class I expression in idiopathic inflammatory myopathies

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ABSTRACT

Idiopathic inflammatory myopathies (IIMs) are diseases with muscle weakness, morphologically characterized by inflammatory infiltration and increased expression of MHC class I molecule on myofibers. Immunoproteasome, as a proteolytic complex that shapes the repertoire of antigenic peptides, has been previously demonstrated to be over-expressed in IIMs at mRNA level. In this study, we investigated the expression and the function of the immunoproteasome in IIMs in more detail. As shown by immunofluorescence staining, expression of relevant players of the immunoproteasome was detectable in the inflamed skeletal muscle tissue from IIM patients. In fact, two subunits of the immunoproteasome, β1i or β5i, were upregulated in sporadic inclusion body myositis, immune-mediated necrotizing myopathies and dermatomyositis muscle biopsies and co-localized with the MHC class I expressing myofibers. Double immunofluorescence revealed that both myofibers and muscle infiltrating cells, including CD8+ T-cells and CD68+ macrophages in IIMs expressed β1i or β5i. In addition, we have also investigated the role of the immunoproteasome in myoblasts during in vitro inflammatory conditions. Using human primary myoblast cultures we found that pro-inflammatory cytokines, TNF-α or IFN-γ upregulate β1i or β5i. Selective inhibition or depletion of β5i amplified the TNF-α or IFN-γ mediated expression of cytokines/chemokines (myokines) in myoblasts. Furthermore, we demonstrated that specific inhibitors of β1i or β5i reduced the cell surface expression of MHC class I in myoblasts induced by IFN-γ. Taken together, our data suggest that the immunoproteasome is involved in pathologic MHC class I expression and maintenance of myokine production in IIMs. Thus, induction of the immunoproteasome was identified as a pathomechanism underlying inflammation in IIMs.

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1. Introduction

Idiopathic inflammatory myopathies (IIMs) are rare muscle diseases characterized by muscle weakness and specific inflammatory infiltrates in muscle. Based on distinct histopathological and clinical phenotypes they can be classified as polymyositis, sporadic inclusion body myositis (sIBM), dermatomyositis (DM) and immune-mediated necrotizing myopathies (IMNM) [1–3]. Upregulation of pro-inflammatory cytokines such as interferon (IFN)−γ, −α, −β, tumor necrosis factor (TNF)−α and IL-1, are common in IIMs [4–6]. With respect to histopathological features, infiltration of macrophages are present in all entities, while CD8+ T-cells are mainly involved in PM and sIBM, and CD4+ T-cells and B-cells in DM. The presence of rimmed vacuole structures in myofibers of sIBM [7], perifascicular atrophy in DM [8] and myo-phagocytosis in IMNM [1,6] are examples of their unique features.
Another important immunological feature of IIMs is the upregulation of MHC class I (MHC-I) and class II (MHC-II) [9] on the sarcolemma of myofibers, as integral part of the MHC-I/CD8+ T-cell, MHC-II/CD4+ T-cell complex [10,11]. Furthermore, continuous upregulation of MHC-I in myofibers can induce an endoplasmic reticulum stress response with the accumulation of misfolded glycoproteins and activation of NF-κB [12]. As a consequence, MHC-I/CD8+ T-cell complexes may form and maintain an autoinflammatory response. The resulting inflammatory myokine (muscle-derived cytokines and chemokines) induction in muscle is believed to have supportive effect in the diseases progression and prolongation in IIM [13].

As a relevant but not well studied player in this setting, the ubiquitin-proteasome system is responsible for degradation of proteins for MHC-I mediated antigen presentation as well as activation of NF-κB [14]. The core 20S proteasome complex interacts with one or two 19S regulatory particle that recognize ubiquitinylated clients for degradation. From the subunits of the two outer α-rings (α1-7) and two inner β-rings (β1-7)-subunit, only three active subunits (β1, β2, and β5) are responsible for distinct proteolytic activities: caspase-like (C-L), trypsin-like (T-L), and chymotrypsin-like (CT-L) activity, respectively [14]. Following the exposure to certain inflammatory cytokines, the constitutive catalytic subunits β1, β2, and β5 are replaced by three alternative inducible subunits β1i/LMP2, β2i/MECL-1, and β5i/LMP7, respectively. In contrast to constitutive catalytic subunits which are expressed in most bodily tissues, the so-called immunoproteasome subunits are mainly expressed in hematopoietic cells [14]. However, the expression of immunoproteasome in non-hematopoietic cells is associated with many pathologic conditions including cancer, neurodegenerative as well as inflammatory diseases [15,16]. Being an efficient MHC-I restricted peptide producer, it also contributes to maintenance of protein homeostasis and regulates cytokine production during inflammatory conditions [17,18]. Our group has previously explored the presence of the immunoproteasome subunit expression within IIMs muscle biopsies at the mRNA level [19]. However, the precise role of the immunoproteasome in the pathogenesis of IIMs is still unclear.

Here, we shed further light on the role of the immunoproteasome in the pathogenesis of IIMs. We have clearly demonstrated that both β1i and β5i subunits of the immunoproteasome are actively upregulated in myofibers and muscle infiltrating cells in IIMs. Furthermore, using human primary myoblasts we also investigated the pro-inflammatory cytokines responsible for upregulation of the immunoproteasome subunits. Interestingly, our data suggest that the immunoproteasome is responsible for maintenance of myokine production since its knockdown or inhibition using selective drugs enhanced myokine expression in myoblasts during inflammatory conditions. We also found that the surface expression of MHC-I in myoblasts under inflammatory conditions has direct relation to the expression and activity of the immunoproteasome. Taken together, we conclude that although the immunoproteasome is actively upregulated in myofibers and responsible for the expression of MHC-I in IIMs, it is required for maintenance of myokine in its setting.

2. Materials and methods

2.1. Patients and samples

In this study, muscle biopsy specimens from 45 individuals were analyzed, including sIBM, IMNM and DM containing 12 individuals each. Six biopsies were obtained from healthy control individuals and three were non-IIMs (nIIMs). The samples were stored either in the Departments of Neuropathology, Charité–Universitätsmedizin, Berlin, Germany or the Friedrich Baur Institute, Ludwig Maximilians University, Munich, Germany with the written informed consent of the patients according to the Declaration of Helsinki after approval by local ethics committee (No. EA1/204/11). All muscle biopsies were snap frozen after surgical removal and stored at −80 °C until analyzed. All patients had a distinct clinical and a morphological diagnosis based on the respective features (ENMC criteria) [20] (see Supplementary Table 1). Healthy controls in this studies included individual were biopsied for subjective myalgia, but for which no clinical, morphological, laboratory or electro-diagnostic abnormalities have been identified. The three nIIMs patients suffered from limb-girdle muscular dystrophy 2I (LGMD 2I), congenital myopathy and neurogenic disorder.

2.2. Cell culture

Human primary myoblasts were isolated as previously described [21]. In brief, after protease digestion of fresh muscle biopsies, cells were expanded at 37 °C in humidified atmosphere at 5% CO₂ in skeletal muscle growth medium (Provitro, Berlin, Germany) supplemented with 10% fetal calf serum (Biochrom, Germany), 1.5% GlutaMax (Gibco/Life, Darmstadt, Germany) and 40 µg/ml gentamycin (GIBCO/Invitrogen, Germany). Enriched myoblasts were prepared by immuno-magnetic cell sorting using anti-CD56/NCAM antibody coated magnetic beads (Miltenyi Biotech, Germany). Purity of the myoblasts preparation was confirmed by revealing more than 95% desmin-positive cells.

2.3. Cytokines and inhibitor treatment to cells

Myoblasts were grown till 50% of confluency and exposed to different recombinant human cytokines (all from PeproTech, Germany): IFN-γ (300 U/ml) and TNF-α (100 ng/ml) for the indicated time period. LU001i (β1i specific inhibitor) and LU015i (β5i specific inhibitor) were kindly provided from Prof. Hermen Overkleeft, Leiden University, The Netherlands. Both inhibitors were synthesized in the Leiden Institute of Chemistry using previously described protocol and demonstrated to block the human derived β1i and β5i immunoproteasome subunits in vitro irreversibly and specifically [22,23]. When required, various concentrations of both inhibitors were added to growth medium 2 h prior to cytokine treatment.

2.4. RNA isolation and real time quantitative polymerase chain reaction (RT-PCR)

Total RNA extraction and real time RT-PCR was performed as previously described [19]. RNA from cells was extracted with NucleoSpin® RNA/Protein Kit (Macherey-Nagel, USA), following manufacturer’s instruction. Briefly, cells were washed twice with cold PBS and lysed by cell scrapper in lysis buffer on ice. RNA was isolated in 30 µl of RNase free water and stored at −20 °C. After measuring RNA concentration using nanodrop, cDNA was prepared with the SuperScript III (Invitrogen, USA) reverse transcriptase, following the supplier’s instructions. The resulting cDNA product was stored at −20 °C. For real time RT-PCR, all the primers were designed as previously described [19] (Supplementary Table 4 for primer sequence). For amplification, 10 ng of cDNA was used in 20 µl of reaction volume prepared with SYBR Green PCR Master Mix (Applied Biosystems, USA) and 200 nM of each reverse and forward primers. Each sample was run in duplicate for PCR using ABI prism 5700 Sequence Detection System (Applied Biosystems, USA). The mRNA expression of the target gene relative to β-actin was determined by using △Ct method. The mean value of three independent experiments were obtained as the result.

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2.5. Immunofluorescence

For immunohistochemical analysis, transverse section (7 μM thick) of skeletal muscle specimen from sIBM (n = 6), IMNM (n = 6), DM (n = 6), nLIMs (n = 3) and healthy controls (n = 4) were obtained and stored at −80 °C until used. For staining, sections were fixed with acetone for 10 min at −20 °C and blocked with 5% normal goat serum (NGS, Sigma-Aldrich, Germany) for 1 h. Subsequently, sections were incubated overnight at 4 °C with the primary antibodies detailed in Supplementary Table 2. The primary antibodies were detected after 1 h incubation with the appropriate secondary antibodies detailed in Supplementary Table 3. Nuclei were counterstained with 4', 6-diamidino-2-phenylindole (DAPI) (1 μg/ml) for 10 min. After mounting on glass slides with aqueous fluorescent mounting medium (Dako, Denmark), images were captured with fluorescent microscope (KEYENCE, USA).

For immunofluorescence on cells, cells were grown on 8 well chamber slides. Cells were fixed with 4% paraformaldehyde (PFA) for 10 min, permeabilized with 0.1% Triton x-100 for 10 min and blocked with 5% NGS and processed further as mentioned above.

2.6. Western blot

Muscle lysate (30–50 of 10 μM thick muscle sections) from patients group (n = 9) and healthy controls (n = 6) and cell lysates were prepared in lysis buffer (1% Nonidet P-40, 20 mM Tris-HCl, pH 8, 4 mM sodium chloride, 40 mM sodium fluoride and protease inhibitors). Protein content was detected by Pierce BCA Protein Assay Kit (Thermo Scientific, USA). Equal amount (20–25 μg) of protein lysate was loaded on 12% SDS-polyacrylamide gels, and then transferred onto polyvinylidene fluoride membranes (Milibore, Germany). Except for the detection of ubiquitinated proteins and phospho-p38, in which membranes were blocked with 1% BSA, all membranes were blocked with 5% non-fat dried milk in TBS containing 0.1% Tween-20, and were then incubated overnight at 4 °C with the primary antibodies detailed in Supplementary Table 2. After washing, blots were then incubated with HRP-conjugated secondary antibodies. GAPDH was detected as a loading control. The intensity of the bands was quantified with ImageJ 1.49 (National Institutes of Health, USA), and the densitometry analysis is shown in arbitrary units normalized to the GAPDH.

2.7. Proteasome activity measurements

For protein lysate, 30–40 sections (10 μM thick) of muscle tissue or cells with 70% confluency in 60 mm plate were homogenized in lysis buffer (50 mM Tris HCl (pH 7.4), 150 mM NaCl, 5 mM MgCl2, 1 mM EDTA, 50 mM Sucrose, 0.1% Triton X-100 and 1 mM DTT). Protein (25 μg) were transferred to a Corning® 96 well opaque bottom plates (Sigma-Aldrich, Germany) and 100 μM of Z-GGL-AMC and Z-LLE-AMC (Enzo Life Sciences, Switzerland) fluorogenic substrates were used to measure CT-L and CL proteasome activity, respectively, in assay buffer (25 mM HEPES buffer (pH 7.4), 0.5 mM EDTA and 0.03% SDS) in 100 μl of total reaction mixture fluorescence (380 nm excitation, 460 nm emission) was monitored on a Synergy HT microplate reader (Biotek, Germany) after 2 h at 37 °C. Fluorescence of each lysate was determined by subtracting the background fluorescence of each lysate was determined by subtracting the fluorescence. Immunoblot (Fig. 1A) and its densitometric quantification (Fig. 1B) revealed that all muscle biopsies from sIBM patients (n = 9) displayed significant upregulation of both βi1 and βi5 subunits. Sixty-six percent (6/9) of βi1 and 51% (5/9) of βi5 subunits. Although all IMNM (n = 9) muscle biopsies demonstrated moderate expression of the immunoproteasome subunits, the expression was not significant compared to healthy controls. Interestingly, we could not detect the presence of immunoproteasome subunits in biopsies from healthy control biopsies (n = 6) (Fig. 1A, B). Compared to IMNM, sIBM showed significant increase in both βi1 and βi5. In contrast to immunoproteasome subunits, muscle biopsies of diseased group showed no significant changes in the expression level of the constitutive subunits β1 and β5 and non-catalytic subunit α5, compared to the healthy controls.

The detection of increased expression of the immunoproteasome subunits in IIMs muscle biopsies encouraged us to study
proteasome peptidase activities in the muscle lysates. The CT-L activity of proteasome was significantly higher in sIBM and DM (Fig. 1C), in agreement with the western blot results. Compared to the healthy controls (100% ± 8.1), there was almost a 3-fold increase in the activity in sIBM (305.6 ± 13.7) and DM (278.5 ± 37.3). Although a 1.7-fold increase of CT-L activity of proteasome was noticed in IMNM (178.5 ± 26.82), statistical analysis revealed no significant difference compared to healthy controls. However, in agreement with the expression of β1i and β5i, significant differences were observed between sIBM and IMNM. With respect to the C-L activity of proteasome, no significant differences between IIMs muscle tissue and healthy controls were observed (Fig. 1C). These results suggest that CT-L activity of proteasome is increased in IIMs muscle biopsies, reflecting the increase in expression of immunoproteasome subunits.

As muscle lysates of biopsies from patients are ‘contaminated’ with immune cells, which normally contain an abundant amount of immunoproteasome subunits, we next examined the expression pattern of immunoproteasome subunits in myofibers. For this purpose, immunofluorescence staining was performed on skeletal muscle sections derived from sIBM, IMNM and DM patients. We found that both β1i and β5i immunoproteasome were predominantly localized in the myofibers of sIBM, IMNM and DM (Fig. 1D). Noticeably, staining of β1i and β5i was more prominent in the perifascicular area in DM muscle. In addition, a positive staining result was also observed for β1i and β5i in infiltrating cells (Fig. 1D). In agreement with western blot results, healthy biopsies displayed no staining for these subunits. To study specificity of β1i and β5i staining in IIMs, we also stained biopsies from patients with LGMD 2I (n = 1), congenital myopathy (n = 1) and neurogenic disorder (n = 1) against β1i and β5i subunits. Interestingly, we found no staining of β1i and β5i in their muscles fibers (Supplementary Fig. 1A), suggesting specific expression of β1i and β5i in skeletal muscle of IIMs. The staining of constitutive subunits β1 and β5 for all patients’ group were comparable to the healthy controls (Supplementary Fig. 1B).

### 3.2. Muscle-infiltrating CD8⁺ T-cells and CD68⁺ macrophages show upregulated expression of β1i and β5i expression in IIMs

We previously showed by real time RT-PCR analysis that β1i and β5i expression is significantly higher in CD8⁺ T-cells and monocytes...

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**Fig. 1.** Quantification and localization of immunoproteasome subunits in sIBM, IMNM, DM and healthy muscle biopsies. (A) Western blot detection of immunoproteasome subunits (β1i and β5i), their constitutive subunits (β1 and β5) and non-catalytic proteasome subunit α5 in total protein lysate of healthy controls, sIBM, IMNM and DM patients muscle biopsies. Protein loading in each lane is confirmed by detection of GAPDH. (B) Densitometric evaluation of muscle biopsies from patients with sIBM (n = 9), IMNM (n = 9) and DM (n = 9) and compared with biopsies from healthy control (n = 6). Data are presented as mean ± SEM. One-way ANOVA was used to assess significance difference. ***P < 0.0005, **P < 0.005, *P < 0.05; (C) Increased proteasome catalytic activity in sIBM, IMNM and DM muscle biopsies as compared to healthy controls. Graphical representation of CT-L activity (upper panel) and CL activity (lower panel) of proteasome in muscle biopsies from healthy controls (n = 6), sIBM (n = 6), IMNM (n = 6) and DM (n = 4). The protein lysate was incubated with specific substrate for 2 h at 37 °C and fluorescent emission was measured. Data are expressed as percentage (mean ± SEM) of controls. One-way ANOVA was used to assess significance. ***P < 0.0005, **P < 0.005, *P < 0.05. (D) Fluorescent single-labeling immunohistochemistry in representative muscle biopsy specimens of sIBM, IMNM, DM patients (n = 6 for each group) and healthy controls (n = 4) using antibodies against β1i and β5i with Cy3 (red) anti rabbit secondary antibodies and counterstained with DAPI. Scale bar = 100 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
isolated from IIM patients derived PBMCs compared to healthy controls [19]. To further investigate the presence of the immunoproteasome subunits in infiltrating cells in IIMs, we co-stained β1i or β5i with antibodies for CD8+ T-cells in sIBM and IMNM or for CD68+ macrophages in muscle biopsies from sIBM, IMNM and DM. Both CD8+ T-cells and CD68+ macrophages expressed β1i or β5i in all observed muscle tissues (n = 6 for each group of IIMs) (Fig. 2A, B). The estimated percentage of CD8+ T-cells that express β1i or β5i in sIBM was 50–60% (Fig. 2C). However, not all CD8+ T-cells corexpressed β1i or β5i in a given biopsy. In contrast, more than 90% of CD8+ T-cells co-express β1i or β5i in IMNM. As CD68+ macrophages are the major professional antigen presenting cells, requiring expression of immunoproteasome subunits as a primary step for active generation of antigenic peptide [24], more than 95% of them were stained for β1i or β5i in all groups of IIMs (Fig. 2C), indicating the co-expression of β1i and β5i, which was further confirmed by triple staining (data not shown).

3.3. Expression of β1i and β5i frequently co-localize with MHC-I positive myofibers in IIMs

Since healthy myofibers do not express MHC-I, but sarcocellemal presence of MHC-I (although variable) is a hallmark of all IIMs [10,11], we next examined whether the expression of β1i and β5i co-localized with specific structure of myofibers expressing MHC-I. In fact, all of the fibers showing immunoreactivity to β1i and β5i also stained for sarcocellemal or sarcoplasmic MHC-I in the muscle biopsies of all three analyzed cases (Fig. 3). In IMNM, areas of muscle fibers not showing expression of β1i and β5i also did not show expression of MHC-I, as in healthy fibers (data not shown). In DM samples, the immunoproteasome subunits as well as MHC-I co-localized in perifascicular areas myofibers of muscle section, as shown in Fig. 3.

3.4. Immunoproteasome subunits are induced by pro-inflammatory cytokines in human primary myoblasts

To understand the role of cytokines leading to the possibility of the immunoproteasome expression during the pathogenesis of IIMs, we next investigated which cytokines are involved in the induction process of these subunits. For this purpose, myoblasts were exposed to pro-inflammatory cytokines TNF-α (100 ng/ml) and IFN-γ (300 U/ml). Under normal cell culture condition, myoblasts expressed very low levels of mRNA as well as protein of β1i and β5i (Fig. 4A–C). However, after 24 h of exposure with TNF-α or IFN-γ, there was a significant upregulation of the mRNA expression of β1i and β5i in the myoblasts (Fig. 4A). Next, to determine the expression of β1i and β5i at the protein level, we exposed cells from 6 up to 48 h with TNF-α or IFN-γ. As a result, it was confirmed that both TNF-α or IFN-γ induced β1i and β5i at protein level already after 24 h of exposure (Fig. 4B, C). Comparable to TNF-α, IFN-γ, which has been shown as a major cytokines evident in blood and muscle of different types of myositis [5,25,26], strongly upregulated β1i and β5i. Noticeably, the down-expression of constitutive proteasome subunits β1 and β5 after cytokine exposure was only detectable at protein level (Fig. 4A, B). Furthermore, a strong induction of the proteasome activator subunits, PA28γ (Fig. 4B) was also observed. As a control, no changes in the non-catalytic, non-inducible subunits, α5 upon treatment with TNF-α or IFN-γ was seen.

Since proteasome activity was found to be correlated with the expression of β1i and β5i in IIMs muscle biopsies (Fig. 1C), we were prompted to measure the CT-L activity and C-L activity of primary myoblasts under the exposure to TNF-α or IFN-γ. As shown in Fig. 4D, we observed a significant increase of CT-L activity of proteasome in primary myoblasts after 48 h of exposure to TNF-α or IFN-γ, however the effect was especially strong with IFN-γ treatment. In contrast, C-L activity of proteasome was significantly higher in TNF-α treated cells compared to IFN-γ treated cells (Fig. 4D), indicating the active presence of the catalytic constitutive subunits β1 even after 48 h treatment with TNF-α.

3.5. Selective inhibition of the immunoproteasome subunits impairs IFN-γ-induced cell surface expression of MHC-I in human primary myoblasts

The clear co-localization of β1i or β5i with MHC-I positive fibers in all groups of IIMs (Fig. 3) supported a crucial function of the immunoproteasome for antigen presentation via MHC-I. To prove the role of the immunoproteasomes for cell surface expression of MHC-I, we used the specific inhibitors for β1i and β5i catalytic activity, named LU0001i and LU015i, respectively. By FACS analysis, it was demonstrated that myoblasts under normal culture condition showed low surface expression of MHC-I. After IFN-γ treatment for 48 h an increased surface expression of MHC-I was evident as compared to TNF-α treatment (Fig. 5A, B). For functional analysis, myoblasts were treated with LU001i or LU015i at different (0.25–10 μM) concentration, for 2 h prior to IFN-γ exposure for 48 h. To probe the mechanism (detailed in Supplementary materials and methods), we visualized the decrease in the specific activity of β1i and β5i with an increase in the concentration of respective inhibitor, LU001i or LU005i (Supplementary Fig. 2). Thus, the surface expression of MHC-I in these cells were analyzed by FACS. As a result, LU001i as well as LU015i decreased the surface expression of MHC-I in myoblasts (Fig. 5C), LU001i at concentration ≥5 μM blocked the surface expression of MHC-I by only 25%. However, selective inhibition of β5i by LU015i at the same concentration inhibited the expression by approximately 50% (Fig. 5C). Furthermore, we also detected total MHC-I protein expression in myoblasts after the induction by IFN-γ alone or in combination with 5 μM of LU001i or LU015i using western blot. Hereby it was confirmed that there was no difference in IFN-γ induced total MHC-I expression in myoblasts treated or untreated with LU001i or LU015i (Fig. 5D). Also, the IFN-γ induced β1i and β5i expression in myoblast was unchanged by either of the inhibitor. These data indicates that myoblasts require specific catalytic activities of β1i or β5i for the stabilization of MHC-I expression in the cell membrane under inflammatory condition.

3.6. β5i inhibition increases myokine production in human primary myoblasts under inflammatory condition in vitro

It has been shown that exposure to proteasome inhibitors that target both β1i and β5i was able to block cytokines production in endotoxin-stimulated monocytes isolated from mice [27]. To evaluate the impact of β1i and β5i inhibition, we compared the myokines production in TNF-α or IFN-γ stimulated primary myoblasts after exposure to LU0001i or LU015i at 5 μM concentration. TNF-α treatment for 24 h increased the mRNA level of IL-6 and IL-1β, while treatment with IFN-γ increased CXCL9 and CXCL10 mRNA expression. However, upon exposure with LU001i or LU015i for 2 h prior to stimulation, surprisingly, a significant increase in the mRNA level of IL-6, IL-1ß, CXCL9 and CXCL10 was seen only in LU015i exposed cells (Fig. 6A, B). In contrast, LU001i did not significantly affect the expression of myokine. β5i inhibition doubled the TNF-α driven cytokines production. The effect was even much higher for the chemokine expression induced by IFN-γ, suggesting that β5i has a role in myokines regulation during the inflammatory condition in myoblasts. None of the inhibitors did induce IFN-γ mediated accumulation of oxidized or ubiquitinated proteins and showed no changes in phospho-p38 level, suggesting that increased
Both CD8+ T-cells and CD68+ macrophages co-express β1i and β5i in sIBM, IMNM and DM muscle biopsies. (A) Fluorescent double-labeling immunohistochemistry using antibodies against CD8+ T-cells (green, Alexa 488) co-expressed β1i (red, cy3) (left panel) and β5i (red, cy3) (right panel) in representative muscle biopsy specimens from sIBM (n = 6) and IMNM (n = 6). (C) Fluorescent double-labeling immunohistochemistry using antibodies against CD68+ macrophages (green, Alexa 488) co-expressed β1i (red, cy3) (left panel) and β5i (red, cy3) (right panel) in representative muscle biopsy specimens from sIBM (n = 6), IMNM (n = 6) and DM (n = 6). For both images (A and B), arrow denotes co-expression and arrowhead denotes no co-expression. Integrated small rectangular box is representative magnified image of co-expression indicated by one of the arrow. DAPI used to counterstain nuclei. Scale bar shown = 50 μm for sIBM and IMNM. For DM, scale bar = 100 μm. (C) Semi-quantitative analysis by manual photo-microscopy of sIBM (n = 6) and IMNM (n = 6), DM (n = 6) muscle specimen. Bar indicates mean ± SEM of percentage [(co-expressed cells/total cells present in the random field)*100%] of CD8+ T-cells or CD68+ macrophages that co-express β1i (left) or β5i (right). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 2. Both CD8+ T-cells and CD68+ macrophages co-express β1i and β5i in sIBM, IMNM and DM muscle biopsies. (A) Fluorescent double-labeling immunohistochemistry using antibodies against CD8+ T-cells (green, Alexa 488) co-expressed β1i (red, cy3) (left panel) and β5i (red, cy3) (right panel) in representative muscle biopsy specimens from sIBM (n = 6) and IMNM (n = 6). (C) Fluorescent double-labeling immunohistochemistry using antibodies against CD68+ macrophages (green, Alexa 488) co-expressed β1i (red, cy3) (left panel) and β5i (red, cy3) (right panel) in representative muscle biopsy specimens from sIBM (n = 6), IMNM (n = 6) and DM (n = 6). For both images (A and B), arrow denotes co-expression and arrowhead denotes no co-expression. Integrated small rectangular box is representative magnified image of co-expression indicated by one of the arrow. DAPI used to counterstain nuclei. Scale bar shown = 50 μm for sIBM and IMNM. For DM, scale bar = 100 μm. (C) Semi-quantitative analysis by manual photo-microscopy of sIBM (n = 6) and IMNM (n = 6), DM (n = 6) muscle specimen. Bar indicates mean ± SEM of percentage [(co-expressed cells/total cells present in the random field)*100%] of CD8+ T-cells or CD68+ macrophages that co-express β1i (left) or β5i (right). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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expression of myokines was not the result of ubiquitin or oxidative stress response. In addition, a marker of unfolded protein response, spliced form of XBP1, also did not alter upon treatment with cytokines and inhibitor, as observed by conventional PCR (Supplementary Fig. 3).

3.7. \( \beta \)5i knockdown increases myokines production in human primary myoblasts under inflammatory condition in vitro

To further verify the crucial role of \( \beta \)5i in myokines production in myoblasts during inflammatory condition, a knockdown strategy was implemented. In particular, primary myoblasts were stably transfected with shRNA pool targeted to \( \beta \)5i, and the impact on TNF-\( \alpha \) or IFN-\( \gamma \) driven production of myokines in myoblasts were examined. As shown in Fig. 7A, the shRNA pool targeted to \( \beta \)5i led to approximately 50% reduction in mRNA levels of \( \beta \)5i as determined by real time RT-PCR after 48 h treatment with IFN-\( \gamma \). Like LU015i treatment, knockdown of \( \beta \)5i also enhanced significantly the mRNA expression of myokines IL-6 and IL-1\( \beta \), CXCL9 and CXCL10 (Fig. 7A–D) under exposure to TNF-\( \alpha \) or IFN-\( \gamma \) compared to the myoblasts transfected with control shRNA. These results suggest that \( \beta \)5i controls the expression of myokines in myoblasts upon exposure to pro-inflammatory cytokines.

4. Discussion

In this study, we present first evidence for an involvement of the immunoproteasome in the inflammatory conditions of IIM. In contrast to healthy controls, the immunoproteasome subunits are highly upregulated in both sIBM, IMNM and DM myoblasts and infiltrating inflammatory cells. In sIBM and IMNM, the expression is strongly localized to infiltrated myofibers. However in DM, the increased expression of the immunoproteasome was predominant in perifascicular area. In all of the cases, the expression of the immunoproteasome subunits co-localized with the MHC-I expressing fibers. Under inflammatory conditions, purified human primary myoblasts increased expression of the immunoproteasome subunits. We assessed the function of the immunoproteasome subunits by two methods: using a selective inhibitor of \( \beta \)1i or \( \beta \)5i and next, knocking down the expression of \( \beta \)5i. By both approaches we were able to show that inhibition of \( \beta \)5i increased myokines production under inflammatory conditions. Taken together, we conclude that the immunoproteasome play a relevant role during pathogenesis of IIMs and are involved in MHC-I surface expression as well as regulation of pro-inflammatory cytokine response. Importantly, our data provide evidence that inhibition of the immunoproteasome function can aggravate the local inflammatory response, which would be in agreement with known proteasome-associated auto-inflammatory syndromes (PRAAS) [as reviewed in Ref. [28]].

We previously showed at mRNA level that the immunoproteasome subunits \( \beta \)1i and \( \beta \)5i are significantly upregulated in muscle biopsies of IIM [19]. Here, we confirmed and extended our previous work at protein level by western blot and immunofluorescence. In this context, we demonstrated that \( \beta \)1i and \( \beta \)5i are overexpressed in myofibers of sIBM, IMNM and DM (Fig. 1), which is in line with
other previous observations in sIBM [29,30]. For IMNM and DM, this is the first clear evidence of increased immunoproteasome expression at protein level. Since, we observed no staining of β1i and β5i in the myofibers of a comprehensive set of nIIMs, such as LGMD 2I, congenital myopathy and neurogenic disorder (Supplementary Fig. 1), the expression of the immunoproteasome subunits may serve as a potential biomarker for myositis. Proteasome dysfunction induce muscle growth defect and protein aggregation in mice [31]. Fratta and colleagues have reported decreased proteasome activity to explain the reason behind protein aggregation in sIBM [30]. In contrast, Ferrer and colleagues have shown increase in proteasome activity in sIBM [29]. Hence, we aimed at clarification of the activity of proteasomes in these entities. Here, we found that CT-L activity of the proteasome was significantly higher in sIBM and DM compared to healthy controls. However, no changes were observed in C-L activity of proteasome among the disease condition (Fig. 1). This suggests that the protein aggregation in sIBM is not caused by defective proteasome activity. Of note, we found less cellular infiltration and muscle necrosis in IMNM than in sIBM and DM. In line with this observation, significantly less expression of β1i and β5i and CT-L activity of the proteasome was observed in IMNM. This indicates that expression of the immunoproteasome subunits and CT-L activity in myofibers might depend on the percentage of cellular infiltration or MHC-I

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expression.

Myositis is essentially characterized by the presence of inflammatory infiltrates [10]. Since we observed upregulation of β1i and β5i in muscle infiltrating cells, we used cellular markers to identify these infiltrating cells in muscle biopsies. Only part of CD8+ T-cells expressed β1i or β5i in sIBM muscle biopsies. Although less CD8+ T-cells were detected in IMNM compared to sIBM, they were all positive for β1i or β5i (Fig. 2), indicating a phenotype of CD8+ T-cells based on the immunoproteasome subunits expression. Since lack of the immunoproteasome in T-cells was associated with their proliferative and inflammatory capacity in different mouse models [32,33], it would be interesting to further characterize CD8+ T-cells in sIBM and IMNM and study the functional response between these CD8+ T-cells clones. In contrast to CD8+ T-cells, we found that all of the CD68+ macrophages expressed β1i and β5i in all diseased groups (Fig. 2). This could be relevant since they are professional antigen presenting cells and require immunoproteasome to efficiently produce antigenic peptide [34,35] and maintain T-cell auto-reactivity [36].

While non-immune cells exclusively express constitutive proteasomes, pro-inflammatory cytokines are strong inducers of the immunoproteasome in these cells [37]. The existing data from studies of muscle biopsies provided evidence that pro-inflammatory cytokines, including TNF-α, and IFN-γ, are relevant in inflammatory myopathies [25]. Gene expression analysis of sIBM muscle fiber, isolated by laser microdissection revealed the increased production of immunoproteasome subunits mRNA with IFN-γ signaling cascade in the muscle fiber [38]. Our previous study in transcriptome data of myositis also suggest that IFN-γ strongly correlated with the expression of β1i or β5i [19]. In addition, recent study by Leoll et al. [39] in myositis demonstrated at the mRNA level that immunosuppressive treatment downregulate molecules from the interferon signaling pathway, including STAT1, IRF and immunoproteasome subunits β1i and β5i. We show here for the first time that, similar to other non-immune cell types such as parenchymal cells and hepatocytes [40,41], human primary myoblasts have the capability to induce mRNA and protein expression of β1i or β5i after exposure to the pro-inflammatory cytokines, TNF-α or IFN-γ. However, the expression was more pronounced under the influence of IFN-γ (Fig. 4). Since a presence or absence of IFN-α and its signaling molecule has been shown to be respectively correlated with the expression or downregulation of β1i and β5i in IIMs patients, these findings, together with our immunohistochemistry staining, collectively indicate that skeletal muscle fibers express immunoproteasome subunits under the influence of IFN-γ during inflammatory condition like IIMs. In a subsequent study, it would be interesting to prove this hypothesis by inhibiting IFN-γ signaling pathway in the myoblast isolated from patients to notice its effect in the expression of β1i and β5i.

While healthy myofibers do not over-express MHC-I molecules, a strong surface staining is known for myositis [29,42]. Several studies in mouse models have shown that induction of myositis

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Fig. 6. Selective inhibition of β5i amplifies the TNF-α and IFN-γ mediated expression of myokines. Expression of mRNA for (A) IL-6, (B) IL-1β, (C) CXCL9 and (D) CXCL10, as measured in primary myoblasts after treatment with 5 μM of LU001i or LU015i for 2 h prior to the induction by TNF-α or IFN-γ for 24 h. DMSO was used as a control for the inhibitor treatment. Cells were collected and analyzed by real time RT-PCR (relative to β-actin mRNA). Data are mean relative values ± SEM from three independent experiments. Unpaired student t-test was applied for determining significant difference between DMSO and inhibitor treatment. ***P < 0.0005, **P < 0.005, *P < 0.05.

Fig. 7. β5i knockdown in primary myoblasts amplifies the TNF-α and IFN-γ mediated expression of myokines. (A) Knockdown of β5i in primary myoblasts transfected with shRNA targeting β5i (β5i shRNA) when compared with myoblasts transfected with scrambled controls shRNA (cltrA shRNA) and un-transfected myoblasts. Cells were collected after exposure to IFN-γ for 48 h and mRNA expression for β5i was evaluated as per mentioned in Fig. 6. Data represent mean ± SEM from two independent experiments. Transfected myoblasts after exposure to TNF-α or IFN-γ for 24 h were analyzed via real time RT-PCR for mRNA expression of (B) IL-6, (C) IL-1β, (D) CXCL9 and (E) CXCL10. Data represent mean relative values ± SEM from three independent experiments. Unpaired student t-test was applied to detect significant difference between β5i shRNA and cltrA shRNA transfected myoblasts. ***P < 0.0005, **P < 0.005.
was accompanied by conditional overexpression of MHC-I in skeletal muscle [43,44]. The immunoproteasomes play a fundamental role in MHC-I antigen presentation. They are efficient producer of peptides that bind to MHC-I and are recognized by CD8⁺ T-cells on the cell surface. In sIBM, IMNM and DM, we found that the immunoproteasome expression was co-localized with the MHC-I positive fiber (Fig. 3). Importantly, fibers stained with the immunoproteasomes also brightly stained with MHC-I, suggesting a role of the immunoproteasome in the expression of MHC-I. We further confirmed this observation by using primary myoblast model in vitro. As human myoblasts express basal level of MHC-I in the normal culture conditions, the expression on the cell surface highly increased under the influence of IFN-γ after 48 h (Fig. 5). This is in line with previous studies, showing myoblasts as a conditional antigen presenter [45–47]. Here, we showed that treatment with LU0011 or LU0151, a selective inhibitor of βi1 or βi5 respectively, in a dose dependent manner reduced IFN-γ induced surface expression of MHC-I in primary myoblasts without affecting total intracellular MHC-I expression (Fig. 5). Although no studies have explored a direct role of the immunoproteasome in MHC-I expression in muscles so far, splenocytes derived from βi5-impaired mice showed a reduction in surface MHC-I expression on lymphocytes with a significant effect on the repertoire of class I-presented peptides [18,48]. Similarly, reduced MHC-I expression in myoblasts might be due to reduced peptide supply and inefficient conformation of MHC-I, enough to impair its trafficking to cell membrane after treatment with LU0011 or LU0151.

Recent investigation demonstrated that βi5 is required to produce several cytokines, including IL-6, IL-1β and TNF-α and play a role in the progression experimental arthritis as well as colitis. Production of cytokines in LPS induced splenocytes was blocked after treatment with a βi5 specific inhibitor [18,49]. In inflammatory disorder, muscle cells have been described as prolific secretors of several cytokines and chemokines [50,51]. In the present study, we investigate the role and input of the immunoproteasomes by using two approaches: chemical inhibitors and gene knockdown of the immunoproteasome function. Unexpectedly, both approaches enhanced the TNF-α and IFN-γ mediated expression of myokines in vitro (Figs. 6 and 7). Although several animal models have shown a role of βi5 in cytokines productions, none of these studies did investigate the effect on non-immune cells. In this context, recent studies showed that inhibition of the immunoproteasome intensifies the pathogenesis of systemic Candida albicans infection in mice with elevated number of neutrophils infiltration in kidneys and brains [52]. Furthermore, βi5 deficiency results in severe enterovirus myocarditis in mice [53]. In humans, increased serological concentration of cytokines such as IL-6 and TNF-α has been measured in protease related autoinflammatory diseases with mutations in βi5 immunoproteasome subunits [54,55]. Of note, an increased accumulation of ubiquitinated or oxidized protein was detected in the tissue extracted from these patients. In contrast to these observations, we were not able to detect the accumulation of both ubiquitinated and oxidized proteins, an increased level of phospho-p38 protein and spliced form of XBP1 in LU0011 or LU0151 treated myoblasts suggesting that the increase in cytokines is not caused as a response of cellular or endoplasmic reticulum stress. Therefore, the mechanism behind increased cytokines expression in βi5 defective myoblasts have to be clarified by subsequent studies. Collectively, our results suggest that βi5 plays a role in stabilizing the expression of cytokines in myoblasts under inflammatory condition.

5. Conclusion

In conclusion, our data disclose a general function of the immunoproteasomes during the pathogenesis of IIMs. The suppression of the immunoproteasome expression and activity could be an approach to reduce antigen presentation by skeletal muscle and consequently the cytotoxic effect of T-cells. However, the immunoproteasome is also known to serve as a regulatory machinery with non-immune function. Thus, the increased expression of immunoproteasome subunits in muscle fibers during the inflammatory condition might also be involved in degradation of substrates including mediators of the inflammatory response. Thus, expression of the immunoproteasome is also important to maintain the myokines homeostasis and myokines mediated attraction of immune cells in muscles fibers. Thus, the imbalance between these functions may have an impact on the disease phenotype or severity in IIMs. Taken together, our results do not support the approach to use immunoproteasome inhibitors as a monotherapy in IIMs.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jaut.2016.08.004.

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