Functional role of ubiquitin proteasome system in idiopathic inflammatory myopathies

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List of Abbreviation

ANOVA	Analysis of variance
APS	Ammonium per sulfate
BSA	Bovine serum albumin
CANDLE	Chronic atypical neutrophilic dermatosis with lipodystrophy and elevated temperature syndrome
CD	Cluster of differentiation
cDNA	Complementary DNA
CK	Creatine kinase
C-L	Caspase-like
cN1A	Cytoplasmic 50-nucleotidase 1A
COX	Cytochrome oxidase
CT-L	Chymotrypsin-like
CXCL10	C-X-C motif chemokine 10
CXCR3	Chemokine receptor 3
DAPI	4',6-diamidino-2-phenylindole
DM	Dermatomyositis
DMD	Duchene muscular dystrophy
DMSO	Dimethylsulfoxid
DNA	Deoxyribonucleic acid
DNPH	2,4-dinitrophenylhydrazone
DTT	Dithiothreitol
EDTA	Ethylene diamine tetra acetic acid
ER	Endoplasmic reticulum
g	Gram
h	hour
H&E	Haematoxylin and eosin
H_2O	Water
HCl	Hydrochloric acid
HLA	Human leukocyte antigen
HMGCR	3-hydroxy-3-methylglutaryl–coenzyme A reductase

IFN	Interferon
IIMs	Idiopathic inflammatory myopathies
IkB	Inhibitor of NF-kB
IL	Interleukin
ILD	Interstitial lung disease
IMNM	Immune mediated necrotizing myopathy
IRF-1	Interferon regulatory factor-1
kDa	Kilo Dalton
kg	Kilogram
LGMD	Limb-girdle muscular dystrophy 2I
LMP	Lower molecular weight protein
LPS	Lipopolysaccharide
LU	Leiden University
MAA	Myositis-associated autoantibodies
MAC	Membrane-attack-complex
MCP	Monocyte chemotactic protein
MDa	Mega Dalton
MDA5	Melanoma differentiation-associated gene-5
MECL-1	Multicatalytic endopeptidase complex subunit-1
MHC	Major histocompatibility complex I
min	Minute
MIP	Macrophage inflammatory protein
ml	Milliliter
mm	Millimetre
mM	Millimolar
mRNA	Messenger ribonucleic acid
MSA	Myositis-specific autoantibodies
NAM	Necrotizing autoimmune myopathy
NF-kB	Nuclear factor kappa B
NGS	Normal goat serum
n-IIMs	Non-IIMs

nm	Nanometer
nM	Nanomolar
NXP-2	Nuclear matrix protein-2
PA28	Proteasome activator 28
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PM	Polymyositis
PRAAS	Proteasome-associated auto-inflammatory syndromes
qPCR	Quantitative PCR
RNA	Ribonucleic acid
rpm	Round per minute
RT	Room temperature
SAE	SUMO-1-activating enzyme heterodimer
SDS	Sodium dodecyl sulfate
Sec	Second
SEM	Standard error mean
shRNA	Short hairpin RNA
sIBM	Sporadic inclusion body myositis
SRP	Signal recognition particle
STAT-1	Signal transducers and activators of transcription-1
TAP	Transporter associated with antigen processing
TBS	Tris buffer saline
TDP-48	TAR DNA-binding protein
TEMED	tetramethylendiamin
Th	T helper cell
TIF1-γ	Transcriptional intermediary factor-1γ
T-L	Trypsin-like
TNF-α	Tumor necrosis factor-α
U/ml	Units per ml
Ub	Ubiquitin

UPS	Ubiquitin proteasome system
µg	Microgram
µl	Microliter
µm	Micrometer
µM	Micromolar

1. Abstract

1.1. English

Idiopathic inflammatory myopathies (IIMs) are muscle diseases, characterized by inflammatory infiltration and increased expression of MHC-I molecules on myofibers. Proteasome is a multi-subunits complex that is expressed in all types of cells and shapes the repertoire of antigenic peptides. We have previously demonstrated the expression of different catalytic subunits of constitutive proteasomes and immunoproteasomes in the muscle tissue of IIMs at mRNA level. We disclosed that the expression of immunoproteasome catalytic subunits is significantly higher in IIMs muscle biopsies, compared to healthy controls and non-IIMs. However, it remains unclear whether this finding is related to the pathogenesis of IIMs. To extend our previous study, we analyzed the protein expression of proteasome catalytic subunits in the muscle fibers of IIMs and investigated their potential function in diseases pathogenesis.

Immunofluorescence staining revealed that constitutive catalytic subunits β1 and β5 were expressed in healthy controls as well as in IIMs and non-IIMs muscle fibers. However, immunoproteasome subunits β1i as well as β5i were upregulated only in sporadic inclusion body myositis (sIBM), immune-mediated necrotizing myopathy (IMNM) and dermatomyositis (DM) muscle biopsies. In addition, we confirmed that both muscle fibers and muscle infiltrating cells, including CD8+ T-cells and CD68+ macrophages in IIMs, expressed β1i or β5i. Furthermore, we have also investigated the role of the immunoproteasome under inflammatory conditions in human primary myoblasts *in vitro*. Under the influence of pro-inflammatory cytokines, such as TNF-α, IFN-α, IFN-β or IFN-γ, myoblast were capable to upregulate β1i or β5i. Of note, the expression of β1i or β5i co-localized with the MHC-I expressing myofibers in IIMs. Furthermore, the *in vitro* results showed that specific inhibition of either β1i or β5i reduced IFN-γ mediated cell surface expression of MHC-I on myoblasts. In addition, selective inhibition of β5i catalytic activity or gene knock-down of its gene amplified the TNF-α or IFN-γ mediated expression of cytokines and chemokines, such as IL-6, IL-1β, CXCL9 and CXCL10 in primary myoblasts. Taken together, these data suggest that the immunoproteasome is involved in pathologic MHC-I expression and maintenance of muscle derived

cytokines/chemokines production in IIMs. Thus, induction of the immunoproteasome was identified as hallmark of IIMs which provides strong evidence for its involvement in the underlying pathomechanism of inflammation.

1.2. Deutsch

Idiopathische inflammatorische Myopathien (IIMs) sind eine Gruppe von Muskelerkrankungen, die durch entzündliche Infiltrate in Muskelfasern mit erhöhter Expression von MHC-I Molekülen charakterisiert sind. Das Proteasom ist ein proteolytischer Komplex, der aus mehreren Untereinheiten besteht und in allen Körperzellen exprimiert wird. Eine wichtige Funktion übt es durch die Generierung von antigenen Peptiden für den MHC-Klasse I Präsentationsweg aus. In Vorarbeiten konnten wir eine Expression verschiedener katalytischer Untereinheiten des Proteasoms im Muskelgewebe von IIM Patienten auf mRNA Ebene zeigen. Es blieb jedoch unklar, ob diese Beobachtung im Zusammenhang zur Pathogenese von IIMs steht. In Weiterführung dieser Untersuchungen wurde die Expression von katalytischen proteasomalen Untereinheiten im Muskelgewebe von IIM Patienten sowie deren potentieller Beitrag zur Pathogenese untersucht. Die durchgeführten Immunofluoreszenzfärbungen zeigten, dass die konstitutiven katalytischen proteasomalen Untereinheiten β1 und β5 in gesunden Kontrollen sowie auch in IIM und nicht-IIM Muskelfasern exprimiert werden. Dem gegenüber wurden die Immunoproteasom Untereinheiten β1i sowie β5i nur in Muskelbiopsien bei sporadischer Einschlusskörpermyositis (sIBM), bei immun-mediierter nekrotisierender Myopathie (IMNM) und Dermatomyositis (DM) nachgewiesen. Weiterhin konnte bestätigt werden, dass sowohl Muskelfasern als auch infiltrierende Zellen, einschließlich CD8+ T-Zellen und CD68+ Makrophagen bei IIMs, β1i oder β5i exprimieren. Darüber hinaus wurde die Bedeutung des Immunoproteasoms unter inflammatorischen Bedingungen in humanen primären Myoblasten *in vitro* untersucht. Unter dem Einfluss von pro-inflammatorischen Zytokinen, wie TNF-α, IFN-α, IFN-β oder IFN-γ, waren Myoblasten in der Lage β1i sowie β5i hoch zu regulieren. Bemerkenswert war, dass die Expression von β1i sowie β5i mit der MHC-I Expression auf Muskelfasern bei IIM eine Co-Lokalization zeigte. Weiterhin konnten die *in vitro* Ergebnisse zeigen, dass eine spezifische Inhibition von β1i oder β5i zu einer reduzierten IFN-γ vermittelten Zelloberflächen-Expression von MHC-I auf Myoblasten führt. Eine selektive Inhibition der katalytischen Aktivität von β5i oder ein genetischer knock-down des Gens verstärkte eine TNF-α oder IFN-γ vermittelte Expression von Zytokinen und Chemokinen wie L-6, IL-1β, CXCL9 und CXCL10 in primären Myoblasten. Zusammengefasst belegen diese

Ergebnisse, dass das Immunoproteasom in die pathologische MHC-I Expression und die Produktion von muskulären Zytokinen und Chemokinen bei IIM einbezogen ist. Die Induktion des Immunoproteasoms konnte somit als ein Merkmal von IIM identifiziert werden, wodurch seine Einbeziehung in die zugrunde liegenden Pathomechanismen der Inflammation bestätigt wird.

2. Introduction

2.1. Skeletal muscle

Skeletal muscle is one of the largest single organ systems in mammals accounting for approximately 30-40% of total mass in human. It is mainly composed of bundle of highly specialized multinucleated myofibers, which are formed after the fusion of many muscle cells, called as myoblast. Individual muscles fiber is surrounded by connective tissue called the endomysium and the bundle of muscle fibers (known as fascicle) is surrounded by another connective tissue called perimysium (Figure 1) (Frontera & Ochala, 2015). Skeletal muscle, harbouring a remarkable properties of regeneration, basically plays major role in the body movement and stability. However, upon injury or inflammation, they can also be involved in the activation of immune cells and attracting them by secreting cytokines and chemokines (collectively known as myokines) to the site of inflammation (Pillon, Bilan, Fink, & Klip, 2013).

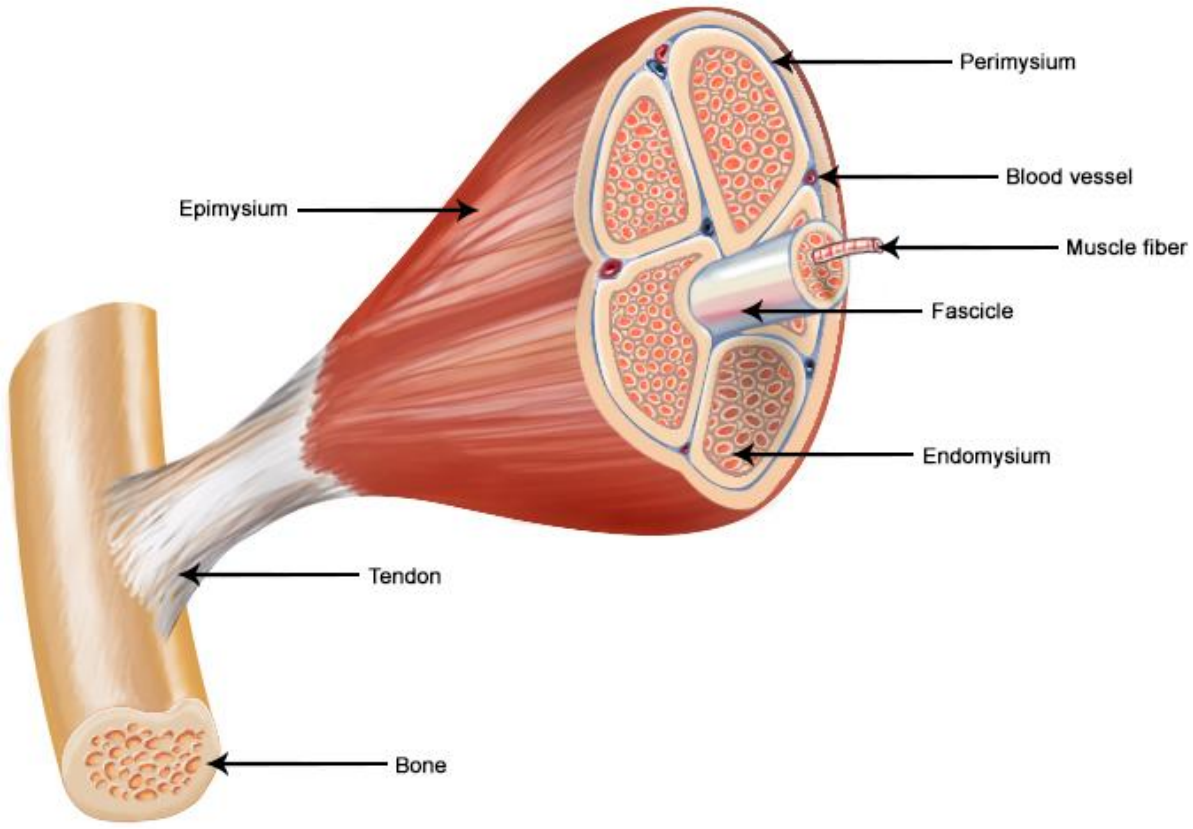


Figure 1: Structure of skeletal muscle. Figure adopted from National cancer Institute's Surveillance, Epidemiology and End Results (SEER), Training webpage (https://training.seer.cancer.gov/anatomy/muscular/structure.html).

2.2. Idiopathic inflammatory myopathies

Idiopathic inflammatory myopathies (IIMs), also known as myositis, are a heterogeneous group of muscle diseases classified as polymyositis (PM), sporadic inclusion body myositis (sIBM), dermatomyositis (DM) and immune-mediated necrotizing myopathies (IMNM). Although they share classical symptoms such as moderate to severe muscle weakness, inflammatory infiltrates and upregulation of major histocompatibility complex I (MHC-I) molecules in muscle tissue, they have distinct clinical and morphological features (Coley et al., 2012; Dalakas, 1991; M. C. Dalakas, 2011; Dimachkie, Barohn, & Amato, 2014; Rayavarapu, Coley, Kinder, & Nagaraju, 2013). However, the diagnosis of the disease is always accompanied with serological detection of level of creatine kinase (CK), myositis-associated (MAA) or myositis-specific autoantibodies (MSA). PM and sIBM is generally considered as a T cell dominated autoinflammatory myopathies and are characterized clinically by selective pattern of muscle weakness. However, the myofibers degeneration, presence of rimmed vacuole structures and abnormal protein aggregation in the myofibers makes sIBM more distinct from PM (Villanova et al., 1993). DM has been usually recognized to a humoral-driven microangiopathy with perifascicular atrophy of muscle fibers and increased evidence of $CD4^+$ T-cells and B-cells in the muscle infiltrating population (Pestronk, 2011). As a recently described form of inflammatory myopathy, the IMNM is a heterogeneous group of necrotizing myopathies which is characterized by myo-phagocytosis in the muscle fibers (Preuße et al., 2012) and is also associated with specific autoantibodies (Drouot et al., 2014). Although the disease activity of IIM is primarily connected with skeletal muscle, frequent involvement of other organs, such as lungs, skin and heart, make them a systemic and complex autoimmune diseases (Tieu, Lundberg, & Limaye, 2016).

The first line treatment of IIMs mostly includes glucocorticoids, which can lead to rapid normalization of serum muscle enzyme levels and improvement of muscle strength. To reduce long-term glucocorticoids exposure, immunosuppressive drugs and intravenous immunoglobulins are used. The efficacy of immunosuppressive treatment varies between the groups. In contrast to DM and PM, which show relatively good responsiveness to immunosuppressive therapies, sIBM is particularly resistant to the approaches (Gazeley & Cronin, 2011; Moghadam-Kia, Aggarwal, & Oddis, 2015). Since the molecular

mechanism of diseases pathogenesis between the groups is heterogeneous, the approaches designed for most of the treatment has no controlled and standard regimens. Therefore, more controlled clinical studies are needed to demonstrate the effectiveness of the different therapies in the treatment of IIMs.

2.2.1. Polymyositis (PM)

PM, as a specific entity, is rare and difficult to differentiate from sIBM, IMNM and inflammatory dystrophy, which can lead to misdiagnosis (M. C. Dalakas, 2011; Miller et al., 2004; M. F.G. van der Meulen et al., 2003). PM is best studied in adults as a myopathy with subacute proximal systemic weakness without skin involvement, family history of neuromuscular diseases and exposure to myotoxic drugs (such as statins, penicillamine and zidovudine). The diagnosis of PM is made when other IIMs, usually sIBM, are ruled out (Dalakas, 2015). Histopathological evidence of muscle biopsies showed endomysial or focal infiltration of inflammatory cells including high percentage of macrophages and effector CD8+ T cells with strong interaction with MHC-I molecules at the surface of muscle fiber (Figure 1). PM muscle biopsies shows the effects of massive cytotoxicity resulting from the differential release of perforin (N. Goebels et al., 1996). Similar to sIBM, CD8+ T cells but not CD4+ T cells undergo clonal expansion which is consistent with the autoantigen presented by the MHC-I molecules at the surface of muscle fibers (Müntzing, Lindberg, Moslemi, & Oldfors, 2003).

2.2.2. Sporadic inclusion body myositis (sIBM)

sIBM is the most common form of inflammatory myopathy that is more frequent in men and likely to affect person above the age of 50 years. It has all the inflammatory characteristics of PM, including the MHC-I/CD8+ T cells complex (Figure 1A). However, presence of rimmed vacuoles and abnormal aggregation of protein (Figure 1B), such as amyloid-β, phosphorylated tau, TDP-43, alpha B-crystallin, myotilin and p62, in the muscle fibers of most of the patients can differentiate it from PM. As a distinct phenotype, it also has large number of COX-negative fiber, indicating mitochondrial abnormalities, and the presence of intracellular congophilic deposits which is well-detectable by crystal violet staining. Compared to PM, it has chronic myopathic changes which include

variability in the fiber size and increases in connective tissue (Brady et al., 2014; Nogalska, Terracciano, D'Agostino, King Engel, & Askanas, 2009; Villanova et al., 1993) (Figure 1B). Electron microscopy reveal typical characteristics of filaments with 15-20 nm diameter next to the cytoplasmic inclusion bodies. However, about 30 % of sIBM patients do not show the presence of such filaments, which can lead to the misdiagnosis of PM (Chahin & Engel, 2008). Therefore, new biomarkers are urgently needed to improve diagnostic procedures in sIBM. Of note, antibodies against 43-kDa muscle autoantigen, cytoplasmic 50-nucleotidase 1A (cN1A), have recently described in the patients with sIBM (Benjamin Larman et al., 2013; Pluk et al., 2013). However, it seems that these autoantibodies are not highly specific for IBM since there were also detected in other systemic disease such as SLE (REF).

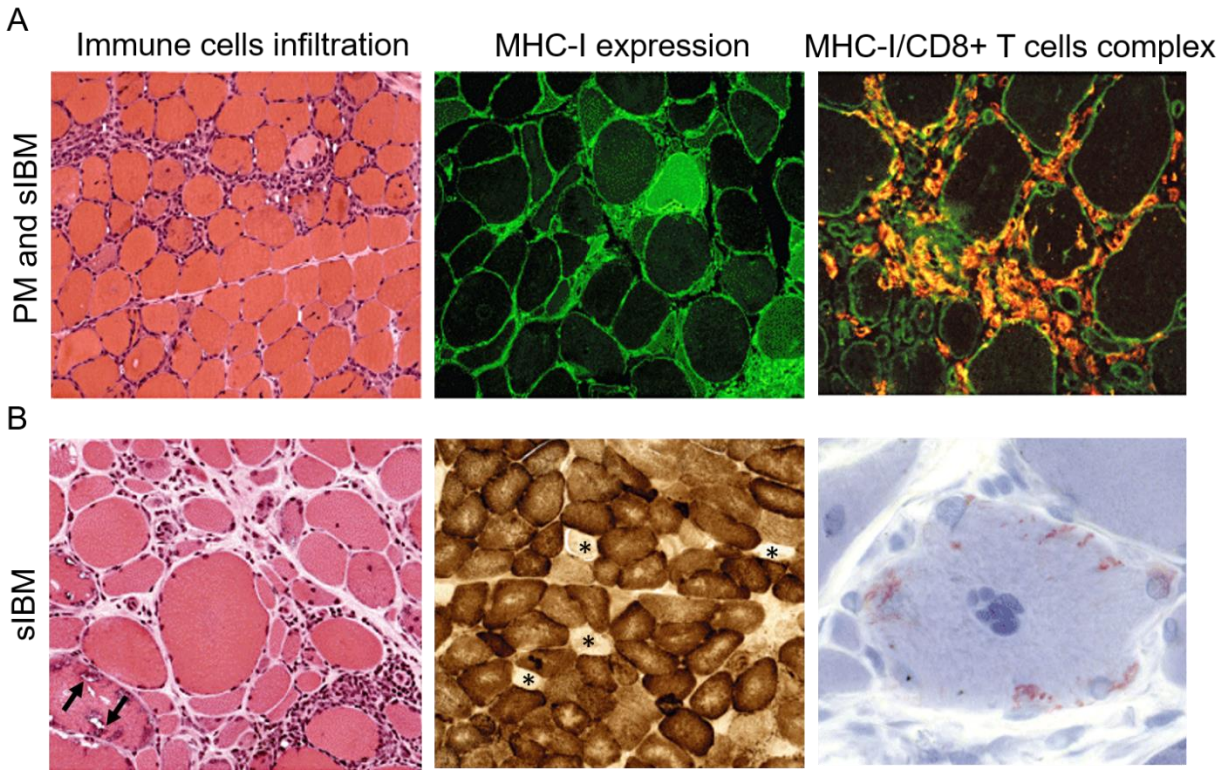


Figure 2: Histological features of PM and sIBM. (A) Common feature of PM and sIBM showing immune cells infiltration (left, H&E staining), MHC-I expression (indicated by green immunostaining, middle) and MHC-I/CD8+ T cell complex (indicated by red as T cells and green as MHC-I molecule, right). (B) Special feature of sIBM muscle histology showing increase cellular infiltration, rimmed vacuole, as indicated by bold arrow, and variation of fibers size (left, H&E staining), cox-negative fibers (middle, indicated by asterisk) and congophilic deposits (right, visualized by crystal violet staining). Images are adopted from (M. C. Dalakas, 2011).

2.2.3. Immune mediated necrotizing myopathy (IMNM)

IMNM, also known as necrotizing autoimmune myositis (NAM) is another frequently observed inflammatory myopathy with very high CK level (50 times more than upper limit of normal). In addition to myalgia and proximal weakness, patients with IMNM also present additional symptoms such as fever, weight loss, cardiac and pulmonary involvement. Initially proposed as a subset of PM, it is now classified as a distinct entity which is characterized by enhanced myofibers necrosis, myophagocytosis and a comparatively low level of inflammatory infiltration (Figure 2) (Liang & Needham, 2011). Cellular infiltration mainly comprises CD4+ T cells, CD8+ T cells and CD68+ macrophages with relatively few B cells (Preuße et al.). More morphological feature include the up-regulation of the membrane-attack-complex (MAC; C5b9) and sarcolemmal expression of MHC-I molecules in muscle biopsies (Preuße et al., 2012). Occurrence of IMNM is either alone or in association with other conditions that includes viral infection, cancer, scleroderma and statin medication (M. C. Dalakas, 2011). Autoantibodies can serve as useful markers in IMNM, since most of the patients with IMNM show the presence of antibodies against signal recognition particle (SRP), a ribonucleoprotein involved in the protein transport to the endoplasmic reticulum (ER) (Benveniste et al., 2011) or against 3-hydroxy-3-methylglutaryl–coenzyme A reductase (HMGCR) (Mammen et al., 2011).

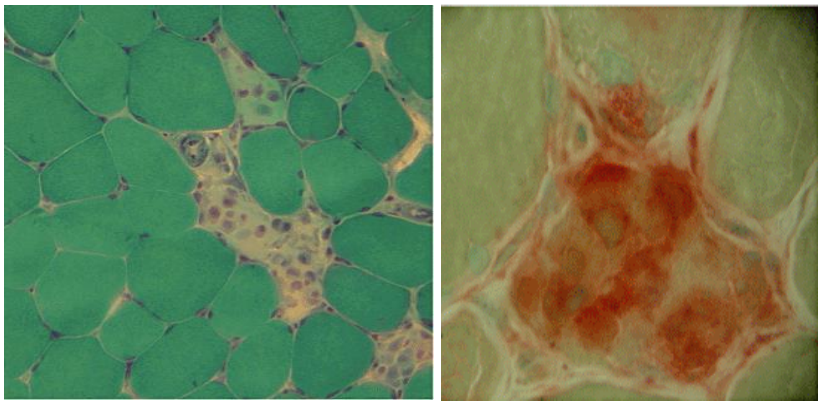


Figure 3: Histological representation of skeletal muscle from IMNM. Trichrome stain showing fiber necrosis (left) and acidic phosphatase staining showing myophagocytosis of muscle fibers (right). Images are adopted from (M. C. Dalakas, 2011).

2.2.4. Dermatomyositis (DM)

DM is an inflammatory myopathy that can occur in children as well as adults with predominance in females. DM is characterized by muscle pain and weakness accompanied with skin manifestation, which includes rash with edema; erythematous rash on the face, eye lid, elbows, knees, neck and back. However, these symptoms without muscle involvement is sub categorized as amyopathic DM or could be related to other systemic diseases such as systemic sclerosis or mixed connective-tissue disease (Dalakas, 1991; M. C. Dalakas, 2011). A prominent histological findings in DM muscle biopsies is often associated with perifascicular atrophy with increased infiltration of inflammatory cells at perivascular and perifascicular area (Figure 3). DM muscle biopsies also show increase expression of MHC-I molecule, however, intense and diffused expression is confined towards the muscle fibers in perifascular area (M. C. Dalakas, 2011). Cellular infiltration in DM comprises mostly CD68+ macrophages and lymphocytes, particularly B cells and CD4+ T cells with very few or no CD8+ T cells (Marinos C. Dalakas, 2011). Patients with DM shows the presence of specific antibodies including antibodies against Mi-2, transcriptional intermediary factor-1γ (TIF1-γ), melanoma differentiation-associated gene-5 (MDA5), nuclear matrix protein-2 (NXP-2) and SUMO-1-activating enzyme heterodimer (SAE). These antibodies are considered to be associated with several pathological outcomes including interstitial lung disease (ILD) and malignancies (Fujimoto, Watanabe, Ishitsuka, & Okiyama, 9000).

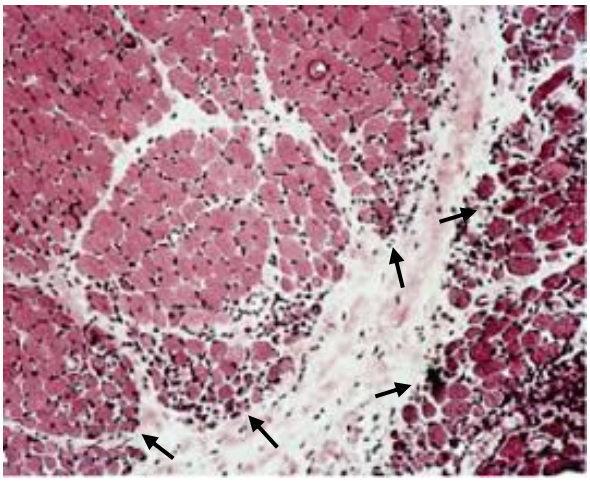


Figure 4: Typical H&E staining of DM muscle biopsy. Arrows indicate area of perifascicular atrophy, with inflammatory cells residing around perivascular areas. Image adopted from (M. C. Dalakas, 2011).

2.3. Pathologic mechanism of IIMs

IIMs are diseases with unknown aetiology, however, various proposed pathomechanism are under investigation.

2.3.1. PM and sIBM pathogenesis

Although no direct evidence for has been reported, it is believed that the inflammatory pathomechanism could be triggered by a viral infection in PM and sIBM. Furthermore, genetic risk factors involved in the regulation of the immune responses have also been proposed. sIBM has shown to be associated with HLA class I B8 and class II antigens DR3, DR52, and DQ2 (Badrising et al., 2004; Price et al., 2004).

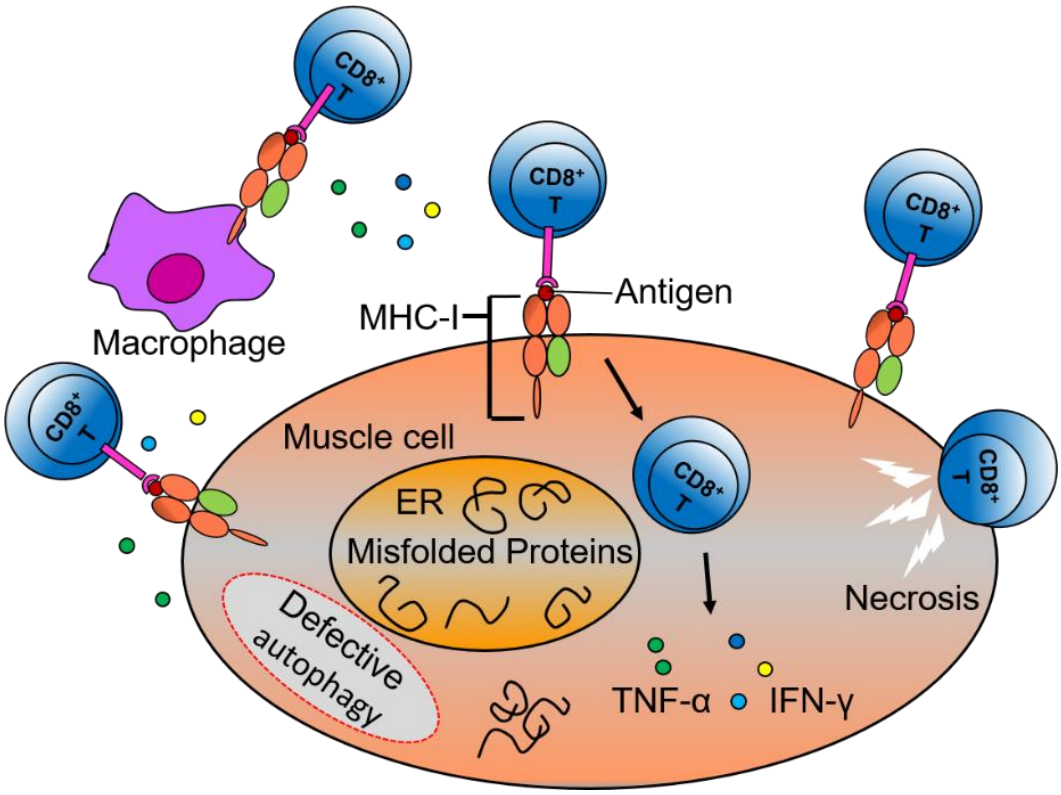


Figure 5: Molecular pathogenesis of sIBM. After exposure to autoantigen via antigen presenting cells, CD8+ T cells becomes activated and migrate towards muscle fiber. This results in association of activated CD8+ T cells with MHC-I expressed in the surface of muscle fibers. This event is further supported by the enhanced released of proinflammatory cytokines. As a result, the auto aggressive CD8+ T cell release perforin and induce muscle necrosis. Prolong expression of MHC-I in the muscle fibers cause ER stress response with more misfolded protein and might results in defective autophagy and exacerbates the disease pathogenesis. Self-modified model from (Dalakas, 2006).

PM and sIBM are mediated by cytotoxic effect of CD8+ T cells invading MHC-I expressing fibers (Emslie-Smith, Arahata, & Engel, 1989; Engel & Arahata, 1986). The perforin secreted by CD8+ T cells direct towards the invaded muscle fibers and cause myonecrosis. Studies showed that T cells receptor expressed by infiltrating CD8+ T cells is conserved in antigen binding region and undergo clonal expansion, suggesting that the T cell response is directed towards muscle antigens that are exposed through MHC-I molecule of the muscle fibers (Müntzing et al., 2003). Continuous upregulation of expression of MHC-I molecules in myofibers can induce an ER stress response with the accumulation of misfolded glycoproteins and activation of nuclear factor kappa B (NF-kB). As a consequence, MHC-I/CD8+ T-cells complexes may form and maintain an autoinflammatory response (K. Nagaraju et al., 2005) (Figure 4). This process is additionally sustained by the up-regulation of adhesion molecules and the expression of costimulatory molecules and, chemokines as well as cytokines. Several immune-cells-derived proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin (IL-1), IL-6 and interferons (IFNs), and chemokines, such as MCP-1, MIP-1a, CXCL10, MIG and its receptor CXCR3 are found to be differentially expressed in PM and sIBM (Confalonieri et al., 2000; Lundberg, Brengman, & Engel, 1995). In this context, the expression of cytokines and chemokines were significantly higher in muscle biopsies of sIBM compared to PM, as detected by qPCR (Schmidt et al., 2008). In addition, analysis of laser micro-dissected inflamed muscle fibers of sIBM patients revealed upregulation of IFN-γ signaling pathway (Ivanidze et al., 2011), indicating a role of IFN-γ in the pathogenesis of sIBM.

A distinct feature of IBM is the presence of rimmed vacuole and the abnormal accumulation of different protein within myofibers. This abnormal accumulation of protein is considered to be the cause of defective mechanism of intracellular protein clearance, called autophagy (Askanas, Engel, & Nogalska, 2012; Girolamo et al., 2013). The upregulation of autophagic markers p62 and LC3, that plays a key role in protein transportation during autophagy, were detected as a sensitive markers for sIBM. Furthermore, TDP-43, the protein aggregation marker, was also shown to be highly specific for sIBM (Hiniker, Daniels, Lee, & Margeta, 2013). In addition, the expression of IL-1β co-localized to β-amyloid depositions within myofibers of sIBM, which was not

observed in PM (Schmidt et al., 2008). This indicate the role of cytokines in protein accumulation in the muscle fibers of sIBM.

2.3.2. IMNM pathogenesis

The aetiology of IMNM has been found to be multifactorial. It has been associated with cancer (Allenbach et al., 2016), viral infection and drugs, such as statins (Needham et al., 2007). Furthermore, the presence of novel autoantibodies and complement deposit are associated specifically with IMNM suggesting that it could also be an antibody-mediated myopathy (Christopher-Stine et al., 2010). Like other conditions, upregulation of MHC-I molecule and cellular infiltration in the muscle fibers of IMNN has proven it to be immune mediated necrotizing myopathy (Preuße et al., 2012). In addition, IMNM has been found to be responsive towards immune mediated therapy.

2.3.3. DM pathogenesis

Similar to sIBM, DM pathogenesis is not fully understood. However, the early activation of complement system C5b9 has been noticed before the occurrence of muscle fiber damage in DM. C5b-9 mediated damage of endothelial cells leads to muscle necrosis, endomysial capillaries depletion, ischemia, and perifascicular pattern of muscle fibre atrophy (Dalakas, 1991; Kissel , Mendell , & Rammohan 1986). In addition, the activation of complement system leading to endofascicular hypoperfusion and perifascicular cellular stress (Marinos C. Dalakas, 2011) is presumably the cause of perifascicular atrophy. The activation of complement system further enhances the expression of adhesion molecules and release of proinflammatory cytokines and chemokines. Cytokines that are actively found to be upregulated in DM are interferons (IFN-α, IFN –β and IFN-γ) and TNF-α, indicating an active local inflammation caused by innate immune response. These cytokines further attract immune cells such as B cell, CD4+ T cells, macrophages and plasmacytoid dendritic cells towards the perimysial and endomysial space of muscle (Figure 5), supporting the role of humoral immunity (Greenberg et al., 2005). Under the influence of cytokines, MHC-I molecules expression in the perifascicular muscle fibers results in the prolongation the autoinflammatory response.

Although PM, sIBM, IMNM and DM have typical phenotypes and possibly a unique triggering factor as well as molecular pathogenesis, the very prominent common feature they share is the enhanced expression of MHC-I molecule in the muscle fibers of inflamed area. As a relevant but not well studied player in this setting, the proteasome system is responsible for degradation of antigenic proteins for MHC-I mediated antigen presentation (Cascio, Hilton, Kisselev, Rock, & Goldberg, 2001) as well as in the activation of NF-kB (Z. J. Chen & Maniatis, 1998).

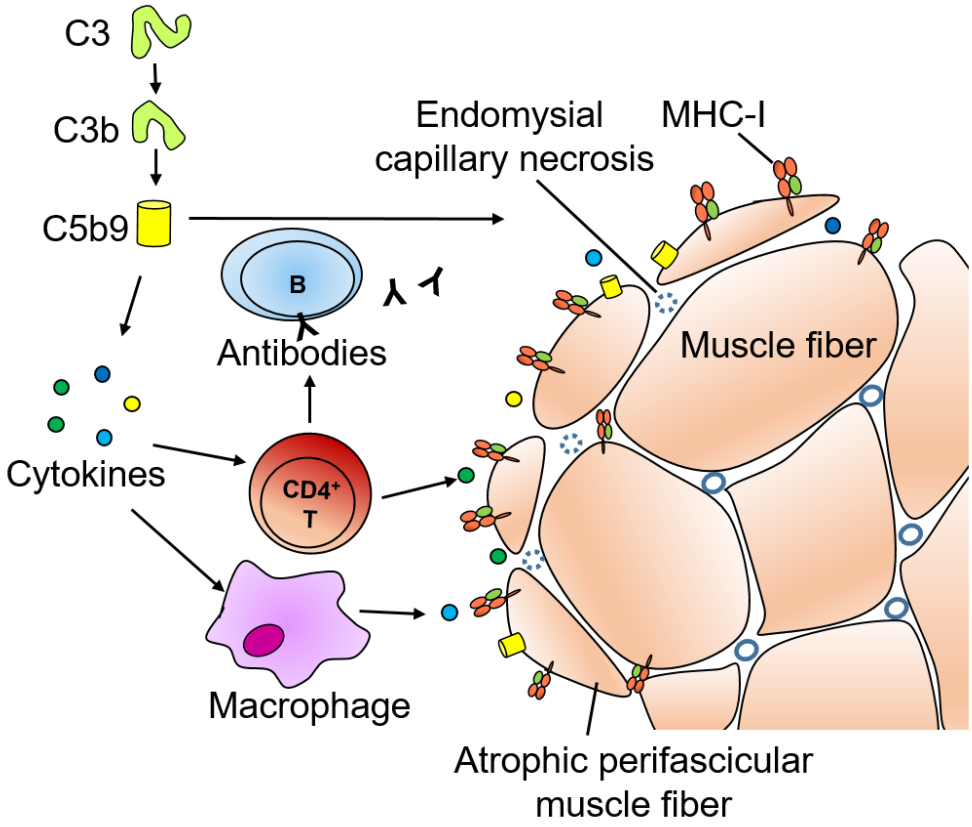


Figure 6: Schematic representative of pathogenesis of DM. The activation of complement pathway results in the formation of C5b9 (or MAC). C5b9 deposition leads to capillary and muscle necrosis in the periphery of fascicle. C5b9 also induces the expression of cytokines, which then attracts and activates immune cells such as macrophage, CD4+ T cell leading towards the activation of B cells for the production of antibody in the muscle fibers. Release of cytokines by migrated immune cells leads to enhance the expression MHC-I molecules in the muscle fiber and supports the prolongation of the disease pathogenesis. Image self-modified from (Dalakas, 2006).

2.4. The ubiquitin proteasome system (UPS)

2.4.1. Proteasome degradation pathway

The proteasome degradation pathway involves degradation of non-lysosomal proteins by the catalytic subunits of the proteasome. Proteasome can degrade protein in both ubiquitin dependent or independent fashion (Erales & Coffino, 2014). The attachment of ubiquitin group to the protein is the initial event in ubiquitin dependent degradation. As shown in figure 6, three enzymes, E1, E2 and E3, are involved in ubiquitin dependent degradation of protein. E1 activates ubiquitin group and transfer them to E2 forming Ub-E2 complex. Then Ub-E2 complex recruit another enzyme, E3, the ubiquitin-protein ligase enzyme. Finally, E3 mediates ubiquitin attachment to the target protein. This process undergoes for several times and forms a long ubiquitin chain attached to the substrate protein, which ultimately enter inside proteasome complex and get degraded into small peptides, releasing free ubiquitin group (Adams, 2003; Pickart, 2001).

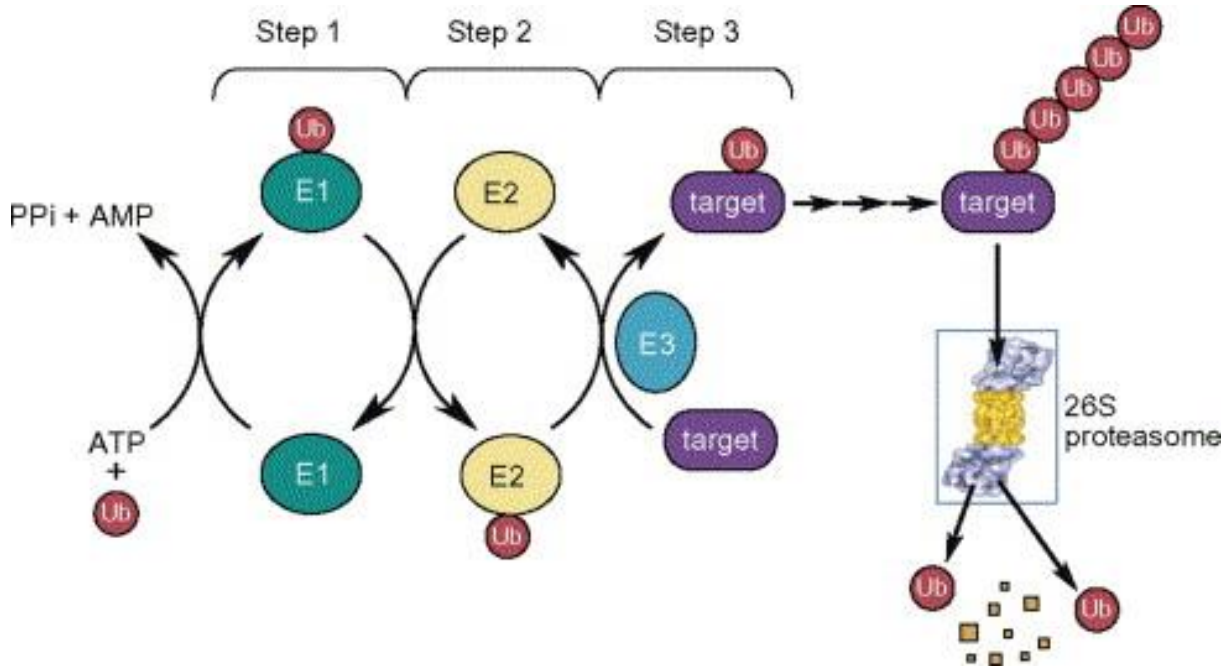


Figure 7: Ubiquitin dependent degradation of protein. The ubiquitin activating enzyme, E1 activates ubiquitin group and transfer it to another enzyme E2. With the help of ligase enzyme, E3, the ubiquitin group from E2 is carried and get attached to the target protein. Several processes attach a long chain of ubiquitin group to a target protein, which is finally degraded by 26S proteasome, releasing a free ubiquitin group. Image adapted from (Adams, 2003).

2.4.2. The Proteasome complex

The UPS proteasome system, also known as 26S proteasome, is a 2.5 MDa complex which is composed of two multisubunit complexes: a 20S proteasome complex as an inner core and two 19S regulatory particles as outer cap, forming 19S-20S-19S complex (Figure 7). The core 20S proteasome complex interacts with one or two 19S regulatory particle that recognize ubiquitinylated client for degradation. The core 20S proteasome complex is a cylindrical structure composed of 28 protein subunits ranges from 21-31 kDa molecular weight. The outer two rings of 20S proteasome, known as α-ring (α_{1-7}) as well as the inner two rings are β-rings (β_{1-7}) consists of seven subunits each (Figure 7) (Ferrington & Gregerson, 2012; Tanaka, 2009). Among these subunits of 20S proteasome, α-ring serves as a gate for substrate entry and three active constitutive β-subunits: β1 (PSMB6), β2 (PSMB7), and β5 (PSMB5) are responsible for distinct proteolytic activities. β1, β2 and β5 have caspase-like (C-L), trypsin-like (T-L), and chymotrypsin-like (CT-L) activity, respectively, which cleave proteins at the C terminal side of acidic, basic and hydrophobic amino-acid residues, respectively (Ferrington & Gregerson, 2012; Tanaka, 2009).

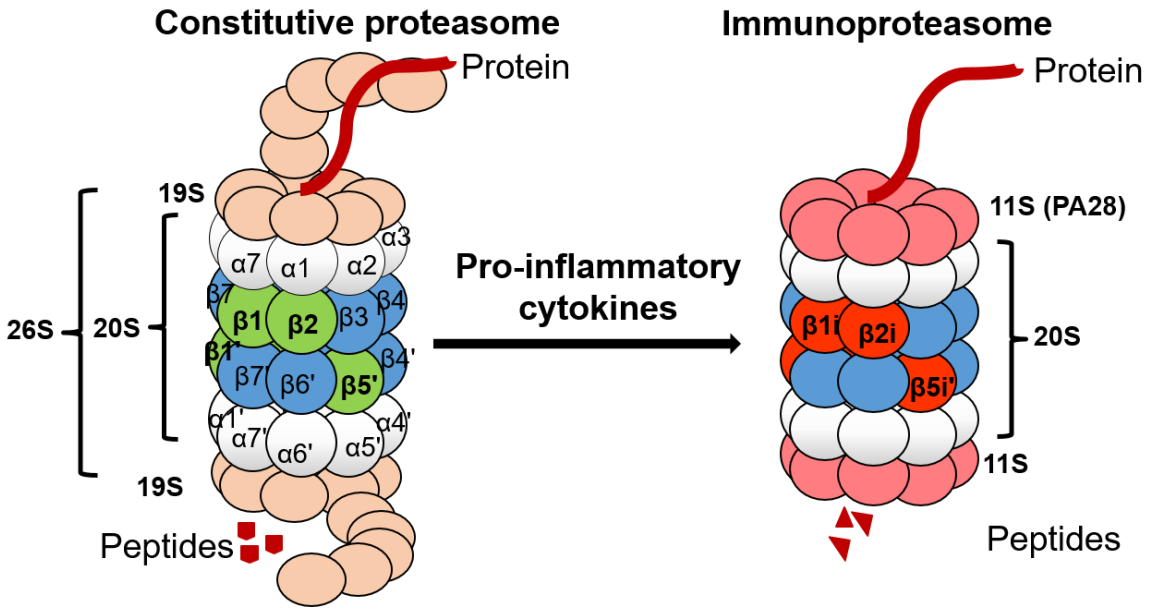


Figure 8: Cylindrical structure of the constitutive proteasomes and immunoproteasome. Under the influence of proinflammatory cytokines constitutive proteasome are modified as immunoproteasome.

Proteasome are ubiquitously expressed in all of the cell types. With the protein degradative properties, the proteasome has function to regulate various biological process including cellular homeostasis, cell cycle control, cell differentiation, apoptosis, signal transduction, DNA repairing, oxidative stress response, and immune response (Adams, 2003). However, the tissue specific expression of proteasome indicate their unique subunits composition and function. Proteasome expressed in thymus, called as thymoproteasome, facilitate positive selection of cytotoxic T cells in the thymus (Klein, Hinterberger, Wirnsberger, & Kyewski, 2009). Likewise, spermatoproteasome, a proteasome expressed in testis, plays key role during the process of spermatogenesis (Belote & Zhong, 2009). Proteasome that has “induced forms” of catalytic subunits, expressed prominently in immune cells, which plays a key role in immune response as immunoproteasome.

2.4.3. Immunoproteasome

Under the influence of proinflammatory cytokines, the constitutive proteasome is modified by substituting catalytic subunits β1, β2 and β5 with three alternative inducible catalytic subunits β1i (or lower molecular weight protein (LMP) 2), β2i (or multicatalytic endopeptidase complex subunit-1 (MECL-1)), and β5i (or LMP7), respectively. In addition, the regulatory particles 19S is also replaced by proteasome activator 11S (also known as PA28). These modified form of 20S proteasome is collectively known as immunoproteasome (Figure 7 and Table 1) (Ferrington & Gregerson, 2012; Tanaka, 2009). The gene responsible for encoding β1i and β5i is clustered with other genes like transporter of antigen presentation (TAP)-1 and TAP-2, required during antigen presentation, and located within the MHC class II region (Ortiz-Navarrete et al., 1991). However, the gene for β2i is located outside MHC class II region (Groettrup et al., 1996). The promoter regions of β1i and β5i genes have binding sites for multiple transcription factors, which includes signal transducers and activators of transcription-1 (STAT-1) (Chatterjee-Kishore, Wright, Ting, & Stark, 2000) and interferon regulatory factor-1 (IRF-1) (Chatterjee-Kishore et al., 2000) and NF-kB (Wright et al., 1995).

The immunoproteasome is predominantly found in immune cells. However, the expression is also induced in non-immune cells under the influence of proinflammatory

cytokines like IFN-γ and TNF-α (Kloetzel, 2001) during infection or autoinflammatory conditions or in response to unusual condition, such as oxidative stress. Immunoproteasome subunits are widely known to be induced by IFN-γ, which also induces other closely linked genes such as TAP-1 and TAP-2 and MHC-I molecules. The induction of immunoproteasome subunits by IFN-γ has been widely studied in both immune and non-immune cells such as neurons (Diaz-Hernandez, Martin-Aparicio, Avila, Hernandez, & Lucas, 2004), lungs (Keller et al., 2015) and salivary glands (Arellano-Garcia, Misuno, Tran, & Hu, 2014). Although not in the same extent, other cytokines such as TNF-α, IFN-α, IFN-β and lipopolysaccharide has also been shown to induce β1i, β2i and β5i expression (Hallermalm et al., 2001; Reis, Guan, et al., 2011; Shin et al.).

Table 1: Human immunoproteasome subunits, adapted from (Kimura, Caturegli, Takahashi, & Suzuki, 2015).

Subunits	Proteolytic activity	Molecular weight (kD)	Chromosome	Alternative name
β1i	CT-L	23.3	6p21.3	PSMB9, LMP2
β2i	T-L	28.9	16q22.1	PSMB10, LMP10, and MECL-1
β5i	CT-L	30.4	6p21.3	PSMB8, LMP7
PA28α	-	28.7	14q11.2	-
PA28β	-	27.4	14q11.2	-

CT-L= Chymotrypsin-like, T-L= Trypsin-like, kD= Kilo Dalton

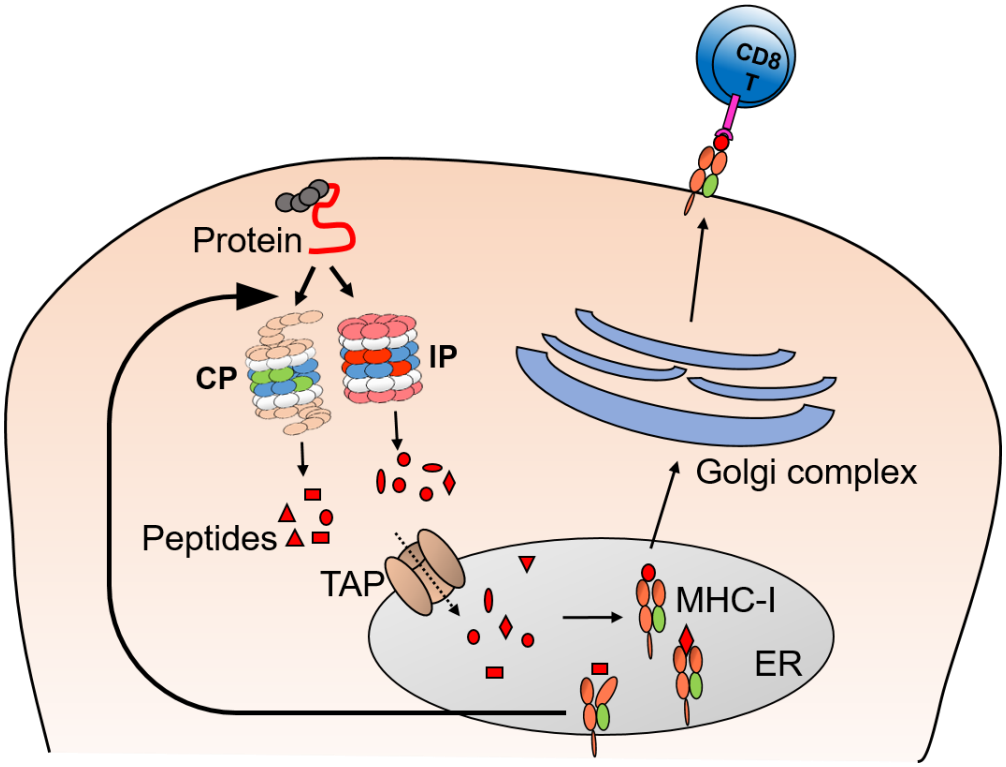


Figure 9: Graphical representation of MHC-I antigen presentation pathway. Immunoproteasome (IP) and constitutive proteasome (CP) receives protein and degrade into peptide. Through TAP protein, the peptides enter ER where they bind to the MHC-I. Peptides produced by IP binds with strong affinity to MHC-I. The MHC-I and peptide complex travel through golgi complex to the cell surface and get recognized by CD8+ T cells. MHC-I molecule that has low affinity to peptide move to cytosol and get cleared by proteasomal degradation.

2.4.4. Function of immunoproteasome

2.4.4.1. Antigen presentation

Almost all human cells can express MHC-I molecule. However, MHC-II expression is restricted mainly professional antigen cells, such as dendritic cells, B-cells and macrophages (Trombetta & Mellman, 2005). Expressing MHC-I molecule, almost all cell types behave like antigen presenting cells under certain conditions. They degrade antigenic protein to small peptide by the proteasome complex and can present antigen derived peptides via MHC-I molecule that is expressed on the cell surface. The MHC molecule and antigenic complex in the cell surface is recognized by the immune cells and therefore, the cell-mediated immune system is activated (Trombetta & Mellman, 2005).

The antigenic peptide can be either from external pathogens, during the infection or from autoantigen, such as during autoinflammatory disease conditions.

Compared to constitutive proteasome, immunoproteasome performs as an efficient, diverse and abundant producer of antigenic peptide. The replacement of β1 with β1i losses its caspase (C)-L activity and acquire CT-L activity of proteolysis which increases the overall CT-L activity of immunoproteasome. Consequently, it shows greater efficiency in governing the antigen presentation by generating quality peptides with strong affinity to MHC-I molecule, compared to the constitutive proteasome (Figure 8) (Gaczynska, Rock, & Goldberg, 1993; Groettrup et al., 1995; Rock & Goldberg, 1999). Indeed, immunoproteasome produced 2-4 times more ovalbumin peptide compared to constitutive proteasome (Cascio et al., 2001). Furthermore, it has been shown that several MHC restricted human tumor antigens can be more efficiently generated by immunoproteasome (Guillaume et al., 2012; Ma et al., 2011). Proteomic approach revealed that cells that are enriched in immunoproteasome subunits produced abundant and diverse MHC I-associated peptides (de Verteuil et al., 2010). Furthermore, PA28α/β-deficient mice showed impaired immunoproteasome assembly which leads to poor presentation of antigenic peptide and weak immune response (Murata et al., 2001). Functional analysis showed that mice completely lacking three immunoproteasome subunits have reduced peptide production and cell surface expression of MHC-I molecule. Data revealed that about 80% of the antigenic peptides, which were less efficiently presented by MHC-I, had undergone major changes in immune-subunit triple negative mice (Kincaid et al., 2012). While constitutive proteasome also shows preferential production of some antigenic peptide for antigen presentation, these peptide are not preferentially produced by immunoproteasome (Chapiro et al., 2006).

2.4.4.2. Regulation of immune cells function

Immunoproteasome has been reported to govern the function of different key player of immune system, including macrophages, CD4+ and CD+ T cells, during pathological condition. Study in mice revealed that immunoproteasome regulates LPS-induced nitric oxide production in macrophages by affecting the TLRs pathway (Reis, Hassan, et al., 2011). A recent study has disclosed that immunoproteasome dysfunction enhances the

IL-4 mediated alternative polarization of alveolar macrophages (S. Chen et al., 2016). Furthermore, immunoproteasome has also been reported to regulate the function of CD8+ T cells. CD8+ T cells deficient in immunoproteasome were shown to undergo hyper proliferation in response to mitogens (Caudill et al., 2006). Moreover, differential impact on virus specific CD8+ T response has been demonstrated to be regulated by immunoproteasome during viral infection (Pang et al., 2006). Upon infection of influenza virus in mice lacking either of three immunoproteasome catalytic subunits, the degree of CD8+ T cells response against virus varied and was fully determined by immunoproteasome subunits composition and consequently by peptide-specific T cells repertoire (Pang et al., 2006). As another player of immune cells, CD4+ T cells function has also been shown to depend on immunoproteasome. CD4+ T cells that lack immunoproteasome subunits showed enhanced inflammatory potential in mice model of induced ulcerative colitis (Rasid, Meulenbroeks, Gröne, Zaiss, & Sijts, 2014). Immunoproteasome also regulate Th cell lineage as inhibition of immunoproteasome leads to supressed Th1 and Th17 differentiation promoting the development of regulatory T cell in experimental colitis (Kalim, Basler, Kirk, & Groettrup, 2012).

2.4.4.3. Cytokines and chemokines production

The immunoproteasome was also identified to regulate the production of many inflammatory cytokines. Selective inhibition of β5i in human peripheral blood mononuclear cells (PBMCs) treated with PR-957 (also known as ONX-0914) showed reduced LPS induced production of IL-23, IL-6 and TNF-α (Muchamuel et al., 2009). Also, macrophages from β2i/β5i double knockout mice showed reduced production of LPS induced gene expression of IL-1 and IL-6 but reported no effect on TNF-α level (Reis, Hassan, et al., 2011), indicating the role of immunoproteasome in the transcription of selective cytokines. Furthermore, inhibition of β1i attenuates the inflammatory reaction of cytokines in a rat model of ischemic stroke by reducing the expression of TNF-α and IL-1β (X. Chen et al., 2015). Although these studies suggest that immunoproteasome enhances cytokines transcription and production, increased cytokine production has also been associated with defective immunoproteasome function in certain human diseases. Missense mutation in immunoproteasome subunit β5i gene has been well characterized

in a human Proteasome associated autoinflammatory syndrome (PRAAS) also named Chronic atypical neutrophilic dermatosis with lipodystrophy and elevated temperature (CANDLE) syndrome (Liu et al., 2012)or Nakajo-Nishimura syndrome (Arima et al., 2011) (Kitamura et al., 2011). Although, the exact pathogenesis of PRAAS is not clear, high level of cytokines and chemokines such as TNF-α, IL-6, IL-1β, IFN-γ and CXCL10 have been observed in the serum of these patients (Arima et al., 2011; Kitamura et al., 2011; Liu et al., 2012).

The mechanism behind a direct contribution of immunoproteasome in cytokine production is not fully understood. Proteasome is responsible for the activation of transcription factor NF-kB, which is a regulator of cytokines production during inflammatory condition. Immunoproteasome isolated from mucosa of Crohn's diseases, an intestinal inflammatory diseases accompanied by increased levels of IL-12, IFN-γ, and TNF-α, was found to enhanced activation of NF-κB with the degradation of IκBα, an inhibitor of NF-κB (Visekruna et al.). However, study in human embryonic kidney cell lines showed that selective inhibition of β5i reduced TNF-α and IL-6 production but showed no effect on the NF-kB activation, suggesting that immunoproteasome regulates cytokines production via NF-kB-independent pathway (Muchamuel et al., 2009). Induction of cytokines expression upon immunoproteasome dysfunction has been associated with increased oxidative stress response, accumulation of toxic ubiquitin-rich aggregates, and cytokine dysregulation (Arima et al., 2011; Opitz et al., 2011; Voigt, Rahnefeld, Kloetzel, & Kruger, 2013).

2.4.4.4. Non-immune function

Although immunoproteasomes expression is predominant in immune cells and tissues with important functions for immune responses, their constitutive expression is also found in non-immune cells, where they also serve with non-immune functions (Ebstein, Kloetzel, Krüger, & Seifert, 2012). The expression of immunoproteasome has been found to be crucial during the differentiation of mice myoblast cells as its inhibition or downregulation blocked myoblast (Cui, Hwang, & Gomes, 2014). Related to the β5i missense mutation in PRAAS, β5i has shown to directly regulate adipocyte differentiation (Arimochi, Sasaki, Kitamura, & Yasutomo, 2016). Arimochi et al. showed that inhibition of β5i mediated

proteasome activity impaired the differentiation of mature adipocytes. Same study revealed that β5i deficient mice had reduced adipocytes and its precursor cells showed a disturbed differentiation. Immunoproteasome deficiency has also been found to mediate loss of cardiac muscle and decrease in cardiac function in experimental diabetic mice (Zu et al., 2010). Moreover, the immunoproteasome is also well characterized to have non-immune function in the retina (Singh, Awasthi, Egwuagu, & Wagner, 2002), liver (M. Chen, Tabaczewski, Truscott, Van Kaer, & Stroynowski, 2005), heart muscle and brain (Piccinini et al., 2003) during both steady state or pathological condition. Functionally, immunoproteasome has been associated to have anti-oxidant and anti-protein aggregation properties in these cell types (Qunxing Ding, Sarah Martin, Edgardo Dimayuga, Annadora J. Bruce-Keller, & Jeffrey N. Keller, 2006; Hussong, Kapphahn, Phillips, Maldonado, & Ferrington, 2010; Seifert et al., 2010).

3. Background

The role of immunoproteasome in myoblast differentiation has been demonstrated under normal culture condition in-vitro (Cui et al., 2014). Furthermore, the expression of proteasome and immunoproteasome has also been studied under pathological condition of skeletal muscle focusing on Duchenne muscle dystrophy (DMD), Becker muscular dystrophies and PM (Kumamoto et al., 2000). In a mice model of DMD, it was also shown that the immunoproteasome content is higher in the dystrophic muscle compared to wild type mice despite of no difference in the total amount of proteasome(C.-n. J. Chen, Graber, Bratten, Ferrington, & Thompson, 2014). Furthermore, a recent study provided evidence to support a therapeutic targeting of immunoproteasome in mdx mice, a disease model for human muscular dystrophy. In detail, immunoproteasome subunit β5i was upregulated within dystrophic muscle fibers and treatment with a β5i inhibitor rescued dystrophin, a protein that is defective expressed in DMD muscle fibers (Farini et al., 2016). Moreover, the expression of immunoproteasomes in muscle fibres was also investigated in relation to age. Compared to the young rat, aged rat showed an altered composition of the proteasome complex with an up to 4-fold increase in immunoproteasome subunits β1i and β5i (Husom et al., 2004).

Expression and activation of proteasome has also been studied in inflammatory myopathies. In a myositis mice model, muscle fibres with an over expression of MHC-I showed also an upregulation of the ubiquitin proteasome pathway and treatment with the proteasome inhibitor Bortezomib improved muscle function and reduced muscle inflammation. In human disease, increased expression of different molecules of the UPS, such as immunoproteasome subunits β1i and β5i as well as the activator PA28-α and -β, has been confirmed in sIBM muscle fibres by immunohistochemistry (Ferrer et al., 2004; Fratta et al., 2005). The expression of immunoproteasome in the isolated sIBM muscles fibers has also been studied by laser microdissection technique at mRNA level (Ivanidze et al., 2011). As a result, immunoproteasome subunit β5i was found to be significantly upregulated in the sIBM muscle fibers compared to the normal muscle fibers. Within the sIBM muscle fiber, the immune attacked (or necrotic) muscle fibers showed significantly higher β5i expression compared to non-attacked muscle fibers.

Previously, our group has investigated the expression of all catalytic proteasome subunits in IIMs and non-inflammatory myopathies (NIM) at mRNA level to explore the role of proteasome in the pathogenesis of autoimmune myositis (Ghannam et al., 2014). Real-time PCR analysis on peripheral blood mononuclear cells (PBMCs) derived subset of immune cells from IIMs patient, including PM, DM and overlap myositis, revealed significantly increased expression of all immunoproteasome catalytic subunits β1i, β2i, and β5i as compared with that of NIM and age matched healthy donors. Analysis in the pooled muscle biopsies from different IIMs patients showed significant increase in the expression of β1i, and β5i, compared to that of NIM. However, the expression of another immunoproteasome catalytic subunits, β2i, was not altered significantly (Figure 9). Similarly, the expression of constitutive catalytic subunits β1, β2, and β5 did not altered significantly between IIMs and NIM. Compared to all PBMCs derived cellular sub-sets, the expression level of immunoproteasome subunits was higher in the muscle biopsies of IIMs patients. Furthermore, the transcriptome data from 78 myositis and 20 healthy muscle biopsies revealed high expressions of genes which were involved in chemotaxis, antigen presentation and processing including components of the UPS which was in line with the results of PCR. Moreover, the ratio of expression of immunosubunit in the muscle biopsies to that of the matched expression in PBMCs derived cells from both IIMs and healthy patients indicated that upregulation of β1i and β5i was not only related to the infiltrating cells within the muscle biopsies but also to the intrinsic muscle environment (Ghannam et al., 2014).

Taken together, preliminary evidence suggest a potential functional role of the immunoproteasome in the pathogenesis of IIMs.

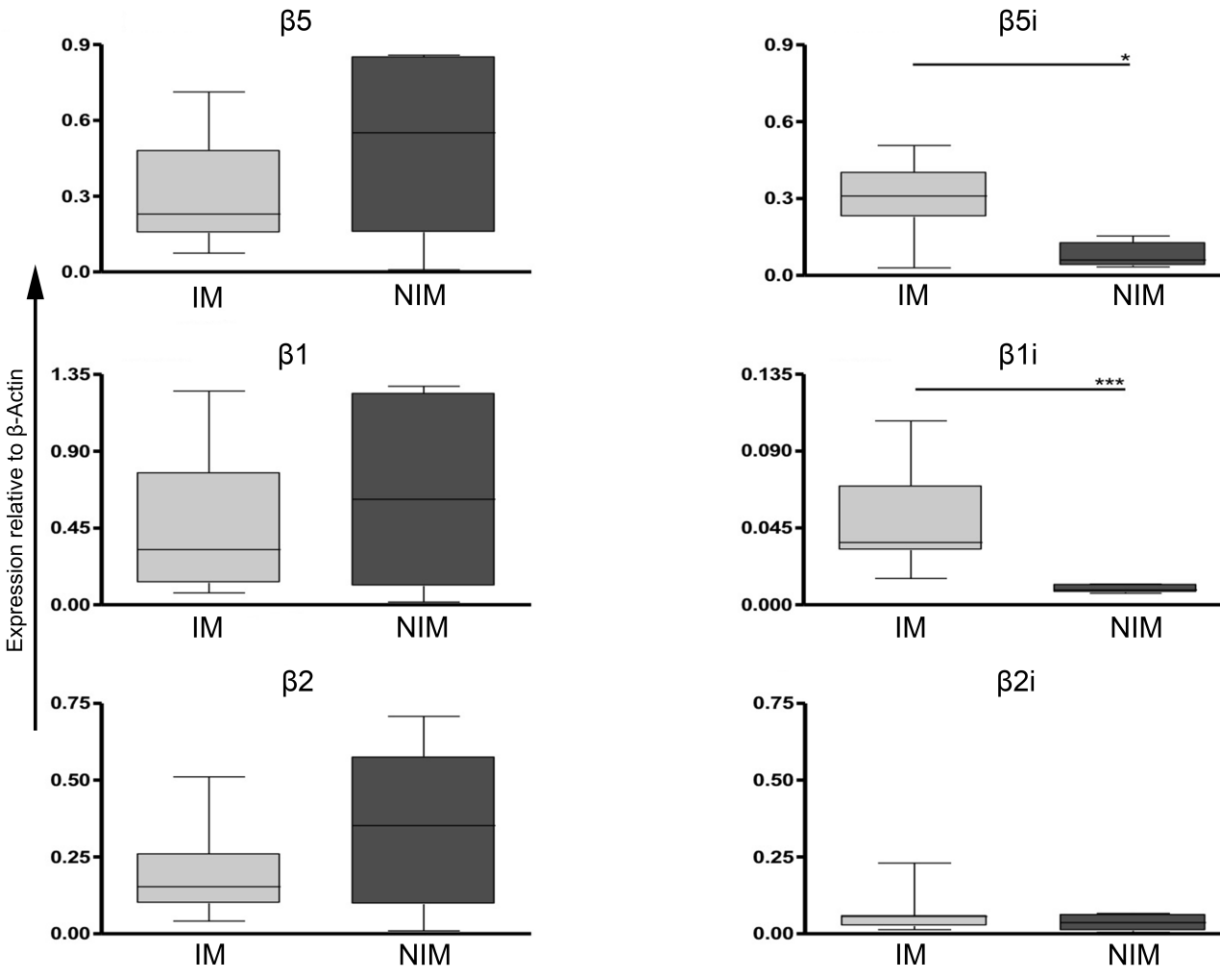


Figure 10: mRNA expression of catalytic subunits of proteasome in muscle biopsies from inflammatory myopathies (IM) and non-inflammatory myopathies (NIM). Data published in (Ghannam et al., 2014) and placed here with the same theme but with different nomenclature for proteasome subunits.

4. Aim

The proteasome system is involved in many crucial metabolic and immunologic processes and is also activated in IIM and (Ghannam et al., 2014). Therefore, it is of interest, whether it can contribute significantly to muscular and systemic pathology in disease conditions. The basic goal of this project was to characterize the role of the ubiquitin proteasome system in the pathogenesis of inflammatory myopathies. To clarify the question, whether the constitutive or immunoproteasome within the muscle is directly involved in the maintenance of the inflammatory condition in the IIM skeletal muscle. The specific aims of the project have been:

1. To analyze the protein level of each catalytic subunit from the muscle biopsies isolated from IIMs patients, including sIBM, IMNM and DM.
2. To measure the muscle biopsies proteasome catalytic properties, such as CT-L and C-L.
3. To visualize the tissue and cellular localization of immunoproteasome expression at the protein level in IIMs muscle biopsies.
4. To evaluate the effect of different cytokines in immunoproteasome induction in human primary myoblast.
5. To demonstrate the inflammatory state of primary myoblast upon reducing the activity of immunoproteasome by genetic and chemical inhibitor approaches.

5. Materials and methods

5.1. Antibodies

The following antibodies were used for detection of proteins by different immunoassays techniques

Antibodies against	Host	Company	Country	Catalogue number	Dilution	Use
β1i	Rabbit	Abcam	UK	ab3328	1:200	IF
					1:2000	WB
β5i	Rabbit	Abcam	UK	ab3329	1:300	IF
β1	Rabbit	Abcam	UK	ab3331	1:50	IF
β5	Rabbit	Abcam	UK	ab3330	1:50	IF
					1: 2000	WB
Desmin	Mouse	Dako	Denmark	M0760	1:50	IF
CD8	Mouse	Dako	Denmark	M7103	1:40	IF
CD68	Mouse	Dako	Denmark	M0718	1:100	IF
MHC-I	Mouse	Dako	Denmark	M0736	1:150	IF
Ubiquitin (FK2)	Mouse	Enzo Life Sciences	Switzerland	BML-PW8810	1:1000	WB
β5i	Mouse	Santa Cruz Biotechnology	Germany	sc-365699	1:1000	WB
β1	Mouse	Santa Cruz Biotechnology	Germany	sc-100455	1:1000	WB
α5	Mouse	Enzo Life Sciences	Switzerland	BML-PW8125	1:1000	WB
PA28α	Rabbit	Thermo Scientific	USA	PA5-17293	1:1000	WB
HLA Class 1 ABC	Mouse	Abcam	UK	ab70328	1: 5000	WB
Phospho-p38	Rabbit	Cell Signaling	Germany	#9211	1:1000	WB
Stat1 p84/p91	Rabbit	Santa Cruz Biotechnology	Germany	sc-592	1:1000	WB
Phospho-STAT1	Rabbit	Cell Signaling	Germany	#9171	1:1000	WB
GAPDH	Rabbit	Santa Cruz Biotechnology	Germany	sc-25778	1:1000	WB

Alexa 488 anti-mouse	Goat	Invitrogen	USA	A-11001	1:300	IF
Cy™3 anti-rabbit antibody	Donkey	Jackson Immuno Research	USA	711-165-152	1:200	IF
Anti-rabbit HRP antibody	Goat	Dako	Denmark	P0448	1:1000	WB
Anti-mouse HRP antibody	Rabbit	Dako	Denmark	P0206	1:1000	WB

IF: Immunofluorescence, WB: Western Blot

5.2. Buffers and solution

5.2.1. Western blot

Buffer	Composition
Tris buffer saline (TBS), pH 7.4	20 nM Tris base, 150 nM NaCl, and H_2O
TBS-Tween (TBST)	1X TBS, and 0.1% Tween-20
Lysis buffer for cell and tissue, pH 7.4	1% Nonidet P-40, 20 mM Tris-HCl, pH 8, 4 mM sodium chloride, 40 mM sodium fluoride, protease inhibitors, and H_2O
Sample buffer	250 mM Tris base (pH 6.8), 10% SDS, 30% Glycerol, 0.5 M DTT (MW: 154.2 g/L), 0.02% bromophenol blue, and H_2O
Running gel (12%, ~10 ml)	3.4 ml H_2O, 4 ml 30% Acryl-bis Acryl, 2.5 ml Tris-HCl (1.5 M pH 8.8), 100 µl 10% Sodium dodecyl sulfate (SDS),

	75 µl 10% ammonium per sulfate (APS), and 7.5 µl tetramethylendiamin (TEMED)
Stacking gel (5%, ~4 ml)	2.28 ml H2O, 660 µl 30% Acryl-bis Acryl, 1 ml Tris-HCl (0.5 M pH 6.8), 40 µl 10% Sodium dodecyl sulfate (SDS), 40 µl 10% ammonium per sulfate (APS), and 4 µl tetramethylendiamin (TEMED)
Running buffer, pH 8.3	25 mM Tris base, 190 mM glycine, 0.1% SDS, and H_2O
Cathod buffer, pH 8.3	25 mM Tris base, 150 mM Glycin, 10% Methanol, 0.1 % SDS, and H_2O
Anode buffer, pH 8.3	25 mM Tris base, 150 mM Glycin, 10% Methanol, and H_2O
Stripping buffer, 1L	15 g glycine, 1 g SDS, 10 ml Tween-20 , and H_2O

5.2.2. For Proteasome activity

Buffer	**Composition**
Lysis buffer for cell and tissue, pH 7.4	50 mM Tris HCL, pH 7.4, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 250 mM Sucrose, 0.1% Triton X-100, 1 mM DTT, and H_2O

Assay buffer	25 mM HEPES, pH 7.4, 0.5 mM EDTA, 0.03% SDS, and H_2O

5.2.3. Flow cytometry (FACS) buffer

Buffer	**Composition**
FACS buffer	1% Bovine serum albumin (BSA), 2 mM EDTA, and Phosphate buffer saline (PBS)

5.3. Primers sequence

Gene	**Forward (5'- 3')**	**Reverse (5'- 3')**
IL-6	agtcctgatccagttcctgca	tttgccgaagagccctcagg
IL-1β	acctgagctcgccagtgaaa	tcagcttgtccatggccaca
CXCL9	aagggtcgctgttcctgcat	ggctgacctgtttctcccact
CXCL10	ctgcaagccaattttgtcca	tctgtgtggtccatccttgg
β1	caagctgacacctattcacgac	cggtatcggtaacacatctcct
β1i	caacgtgaaggaggtcaggta	agagcaatagcgtctgtggtg
β5	ggcaatgtcgaatctatgagc	gttcccttcactgtccacgta
β5i	cacgggtagtgggaacactta	actttcacccaaccatcttcc
β-actin	ctggacttcgagcaagagatg	tgaaggtagtttcgtggatgc
XBP1	ccttgtagttgagaaccagg	ggggcttggtatatatgtgg

Company: TIB MOLBIOL, Germany

5.4. Patients and samples

A retrospective analysis from muscle tissue biopsies of all patients and normal controls were performed. All biopsies were obtained for diagnostic purpose and none of the patients were under relevant immunosuppressive medication. In total, 45 individual's muscle biopsies specimens were investigated. Out of 45 individuals, 12 individuals were included from the following diseased group: sIBM, IMNM and DM; 6 individuals were healthy controls and 3 were non-IIMs (nIIMs). All patients had a distinct clinical and a morphological diagnosis based on the respective features (ENMC criteria) (Hoogendijk et al., 2003) (see Supplementary Table 1 for detail clinical features). Healthy controls were defined as individuals with subjective myalgia but no clinical, histological, laboratory or electro-physiologic abnormalities. The three nIIMs patients were diagnosed with limb-girdle muscular dystrophy 2I (LGMD 2I), congenital myopathy and neurogenic disorder.
All muscle biopsies were collected in either Departments of Neuropathology, Charité–Universitätsmedizin, Berlin, Germany or Department of Neurology, Friedrich Baur Institute, Ludwig Maximilians University, Munich, Germany. The written informed consent of the patients were obtained after the approval by local ethics committee (No. EA1/204/11) according to the Declaration of Helsinki. All of the muscle biopsies were snap frozen and stored at -80 ^{o}C until analyzed.

5.5. Human Primary myoblast culture

Human primary myoblast were isolated as previously described (Schoewel et al., 2012), in the laboratory of Prof. Simone Spuler. Fresh muscle biopsy specimen were crushed and then enzymatic digested with solution comprising 254 U/ml Collagenase CLS II (Biochrom AG, Germany), 100 U/ml Dispase II (Roche, Germany) and trypsin/EDTA (GIBCO/Invitrogen, Germany) at 37 ^{o}C for 45 minutes. After the protease digestion, cells were collected and expanded at 37 ^{o}C in humidified atmosphere at 5 % CO_2 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). Subsequently, myoblasts were purified by immuno-magnetic cell sorting using anti-CD56/NCAM antibody coated magnetic beads (Miltenyi Biotech, Germany). Purity of the myoblast preparation was

confirmed by staining against desmin, revealing more than 95 % desmin-positive cells. Induction of myoblast differentiation was obtained after culturing myoblast in Optimem medium (GIBCO/Invitrogen, Germany) with 2% horse serum (GIBCO/Invitrogen, Germany) for 48 h.

5.6. Cytokines and inhibitor treatment to cells

For the cytokines and inhibitor treatment unless otherwise stated cells were maintained at proliferation condition.

Cells were cultured till they were 50 % of confluency before cytokines treatment. Cells were then exposed to different human recombinant cytokines (all from Peprotech, Germany): IFN-γ (300 U/ml), TNF-α (100 ng/ml), IFN-α (300 U/ml) and IFN-β (300 U/ml) for indicated time point and harvested for analysis.

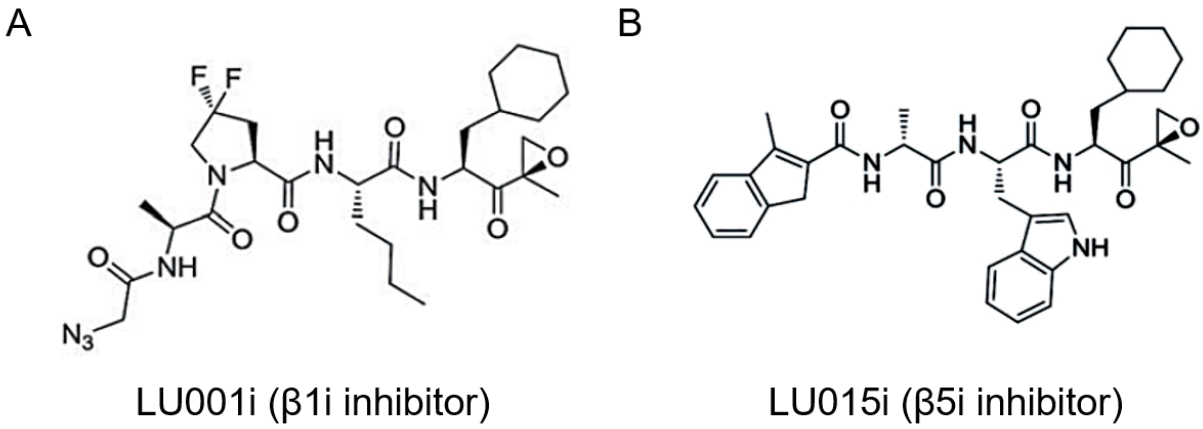


Figure 11: Chemical structure of LU001i (A, β1i specific inhibitor) and LU015i (B, β5i specific inhibitor). Image adopted from (de Bruin et al., 2014).

To inhibit immunoproteasome subunits, β1i and β5i specific inhibitors were used in human primary myoblast. The human immunoproteasome subunits specific inhibitors, named as LU001i and LU015i (where “LU” stands from Leiden University), respectively, were kindly provided from the laboratory of Prof. Hermen Overkleeft, Leiden University, The Netherlands. Both inhibitors were synthesize in Gorlaeus Laboratories, Leiden Institute of Chemistry using the structure-based design approach that has already been described (de Bruin et al., 2014; de Bruin et al., 2015). In addition, these inhibitors have showed cell-permeable capacity and as shown in figure 9, they have been structured to

block irreversibly and specifically the human derived β1i and β5i immunoproteasome subunits *in vitro* (de Bruin et al., 2014; de Bruin et al., 2015). Various concentration of inhibitors were added to the cell's growth medium 2 h prior to the cytokine treatment.

5.7. Immunohistochemistry (tissue)

Immunohistochemical analysis was performed on 7 µM thick cryosections obtained from muscle biopsies sectioning with Microm Cryo-Star HM 560' (Microm Laborgeräte, Germany). Cryosections were air-dried and stored at -80 ^{o}C until used. For staining, the sections were air-dried for 30 min and after washing three times with PBS, they were fixed with refrigerated acetone for 10 min at -20 ^{o}C. Sections were air-dried at room temperature (RT) to remove excess acetone. Subsequently after washing with PBS for three times, they were blocked with 5 % normal goat serum (NGS, Sigma-Aldrich, Germany) for 1 h followed by overnight incubation at 4 ^{o}C with primary antibodies (as indicated in section 4.1) in 1 % NGS. Following day, the sections were washed with PBS for three times. Then the primary antibodies were detected by 1 h incubation with respective fluorophore conjugated secondary antibody raised against the host of primary antibody. After washing with PBS, nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI, 1 µg/ml) for 10 min. Sections were then washed three times and mounted onto the glass slides with aqueous fluorescent mounting medium (Dako, Denmark). Finally, images were captured with fluorescent microscope (KEYENCE, USA) under 20X lens.

5.8. Immunofluorescence (Cells)

An equal number of 8000 cells were plated on each well of 8 well chamber slide (Thermo Scientific, USA). Cells were washed with cold PBS and fixed with 4 % paraformaldehyde (PFA) for 10 min at RT. To detect an intracellular protein, cell were permeabilized with 0.1 % triton x-100 for 10 min. Cell were then washed with PBS and blocked with 5 % NGS for 1 h at RT, and then incubated with respective primary antibody in 1% of NGS for overnight. Next day, cells were washed three times with PBS and primary antibodies were detected by 1 h incubation with respective fluorophore conjugated secondary antibody raised against the host of primary antibody. After washing with PBS, nuclei were

counterstained with 4',6-diamidino-2-phenylindole (DAPI, 1 µg/ml) for 10 min. Sections were then washed three times and mounted onto the glass slides with aqueous fluorescent mounting medium (Dako, Denmark). Images were captured with fluorescent microscope (KEYENCE, USA) under 20X lens.

5.9. Western Blot

Lysate from muscle biopsies were prepared from about 30-50 muscle sections of 10 µm thick. Muscle sections were homogenized with pestles in the lysis buffer for 1 h on ice. However, for cellular lysate, cultured cells were washed two times with cold PBS and scrapped with cell scrapper in lysis buffer followed by incubation on ice for 1 h. Cellular homogenate were centrifuged at 13000 rpm for 15min and pellet were discarded. Furthermore, to prepare equal amount of protein, protein concentration was detected by Bicinchoninic acid assay by Thermo Scientific Pierce BCA Protein Assay Kit. Equal amount (20 µg) of protein lysate was mixed and heated at 95 ^{o}C with 1X sample buffer. Prepared sample mix was cooled at RT and then loaded onto 12 % SDS-polyacrylamide gels, and allowed to run at 120 V for 1.5 h in running buffer. The gel, after equilibrating in cathode buffer was transferred onto polyvinylidene fluoride membranes, equilibrated in anode buffer, at 200 mA current for 2 h in semidry device placing gel on top of the membrane. Following the transfer, membranes were stained with Ponceau S staining solution (0.1 % (w/v) Ponceau S in 5 % (v/v) acetic acid) to visualized the transferred efficiency. Subsequently, membranes were washed for four times to remove the ponceau and then blocked with blocking buffer containing 5 % non-fat dried milk in TBST, except for the detection of mono/poly ubiquitinated proteins and signaling proteins, such as phospho-STAT1, phospho-p38, in which membranes were blocked with 1 % BSA in TBST. Membranes were then incubated overnight at 4 ^{o}C with the primary antibodies in 2.5 % non-fat dried milk in TBST for all antibodies except for Fk2 antibodies and for antibodies against phospho-protein, which is incubated in 1 % BSA in TBST. After washing with TBST for three times, blots were incubated with respective HRP-conjugated secondary antibodies. Next, blots were washed twice with TBST and one time with TBS before detection of HRP antibodies using the ECL system (ECL Plus; Thermo Scientific, USA). As a loading control, membranes were incubated with anti-GAPDH antibody

overnight. Membranes detected for ubiquitin group (in figure 23) and to detected different proteins with similar molecular weight (in figure 11A) with antibodies from different host were stripped with stripping buffer for 2-3 h at RT. Stripped membrane were washed twice with PBS and then with TBS, for 10 min, and re-blocked with 5 % non-fat dried milk in TBST. 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.

5.10. Oxyblot

Detection of oxidized protein follow the same principle of the western blot. However, the sample preparation is different. As indicated by manufacturer protocol in the OxyBlot kit (Millipore, Germany), cells were washed twice with cold PBS and lysed in the lysis buffer as described in section 4.8. After measuring protein concentration, 50 mM DTT was added to the sample to stop further oxidation of sample. 10 µM of protein was aliquoted and denatured by 6 % SDS. Subsequently, samples were derivatized to 2, 4-dinitrophenylhydrazone (DNPH) by reaction with 2, 4-dinitrophenylhydrazine for 15 min at RT. Next, the reaction was stopped by adding neutralizing solution and the samples were loaded to 12 % SDS-polyacrylamide gels and transferred as in section 4.8. The membranes were washed with PBS for 10 mins for three times each and blocked with 1 % BSA in PBS with 0.05 % tween-20 (PBST) for 1 h and incubated with anti-DNPH primary antibody to detect oxidized protein. Membranes were then washed with PBST for three times and incubated with HRP conjugated secondary antibody and analyzed as mentioned above in section 4.8. Membrane were stripped with membrane stripping buffer at RT for 2-3 h and washed and incubated with anti-GAPDH antibody overnight for the 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.

5.11. Proteasome activity measurements

Protein lysate were prepared from muscle biopsies or cells by using protocol mentioned in section 4.8. About 30-40 sections (10 µm thick) of muscle cryosections or cells with 70

% confluency in 60 mm plate were homogenized with proteasome activity lysis buffer (section 4.2.2). After measuring the protein concentration, 25 µg of protein of lysates were aliquoted to a Corning® 96 well opaque bottom plates (Sigma-Aldrich, Germany). Next, 100 µM of fluorogenic substrate was prepared and used with proteasome activity assay buffer (section 4.2.2) in 100 µl of total reaction mixture. To measure CT-L and C-L activity of proteasome Z-GGL-AMC and Z-LLE-AMC (Enzo Life Sciences, Switzerland) fluorogenic substrate, respectively, were used. After 2 h incubation of protein with the substrate at 37°C, fluorescence (380 nm excitation, 460 nm emission) was monitored on a Synergy HT microplate reader (Biotek, Germany). Fluorescence of each lysate wa4.2.4s calculated by subtracting the value from equal amount of same lysate incubated with specific inhibitor as a negative control. Here, Bortezomib (Enzo Life Sciences, Switzerland) at 50 nM and Z-Pro-Nle-Asp-CHO (Enzo Life Sciences, Switzerland) at 40 µM was used to inhibit CT-L and C-L activity of the proteasome, respectively.

5.12. Probe based competition assay in primary myoblast

For competition based assay, cell were exposed to inhibitors for 2 h at 37 °C prior to IFN-γ treatment for 48 h. Cells were trypsinized and washed twice with PBS. Cell pellets were treated with lysis buffer (50 µL, 50 mM Tris HCL pH 7.4, 150 mM NaCl, 2 mM ATP, 5 mM MgCl2, 1 mM EDTA, 250 mM Sucrose, 1 mM DTT and 0.1% Triton X-100) on ice for 1.5 h, followed by centrifugation at 13000 rpm for 15 min. Cells lysate (40 µg) were incubated with 1 µM of cocktail probe, containing Cy5-NC-001 (FL-Ala-Pro-Nle-Leu-EK) to probe β1, BODIPY(TMR)-NC-005 (MeTyr-Phe-Leu-VS) to probe β5 activity, for 3 h at 37 °C. These mixture were further boiled for 3 min with a reducing gel-loading buffer and fractionation on 12.5% SDS−PAGE. In-gel detection of residual proteasome activity was performed in the wet gel slabs directly on a ChemiDoc MP system using Cy3 settings to detect BODIPY-NC005 and Cy5 settings to detect BODIPYNC001. As a loading control, gels were stained by Coomassie Brilliant Blue.

5.13. RNA isolation, reverse transcription and real time quantitative polymerase chain reaction (RT-PCR)

Total RNA was extracted using NucleoSpin® RNA/Protein Kit (Marcherey-Nagel, USA), following manufacturer's instruction. In brief, cultured cells were washed twice with cold PBS and scrapped by cell scrapper in RP1 lysis buffer, provided with a kit, on ice. Lysate were collected and stored at -80 ^{o}C until processed further. Lysates were processed according to the kit's protocol and RNA was isolated in 30 µl of RNase free water and stored at -20 ^{o}C until further used.

After measuring RNA concentration using nanodrop, cDNA was prepared with the SuperScript III (Invitrogen, USA) reverse transcriptase, following the supplier's instruction. The resulting cDNA product was stored at -20 ^{o}C until used for RT-PCR.

The mRNA expression of genes were determined by RT-PCR as described previously (Ghannam et al., 2014). For amplification, 10 ng of cDNA was applied in 20 µl volume of reaction prepared with SYBR Green PCR Master Mix (Applied Biosystems, USA) and 200 nM of reverse and forward primer for each gene. The primer sequence for the genes are listed in section 4.3. For each gene duplicate reaction mixture were prepared. The prepared mixture was run for quantitative PCR using ABI prism 5700 Sequence Detection System (Applied Biosystems, USA) for 40 cycles under the conditions: 50 ^{o}C for 2 min and 95 ^{o}C for 10 min in holding stage; 95 ^{o}C for 15 sec, 63 ^{o}C for 30 sec and 72 ^{o}C for 30 sec in cycling stage; 95 ^{o}C for 15 sec, 60 ^{o}C for 1 min, 95 ^{o}C for 15 sec in melting curve stage. The mRNA expression of target gene was quantified using the $\triangle$c (t) method in relation to expression of *β-actin* mRNA. The mean value shown is a result of three independent experiments.

5.14. Transfection of cells with β5i/LMP7 shRNA

Human primary myoblast was transfected with LMP7 shRNA plasmid (Santa Cruz Biotechnology, Germany). As mentioned in the kit, the LMP7 shRNA plasmids (sc-35822-SH) is composed of a pool of three to five lentiviral vector plasmids each of which encodes for β5i/LMP7 gene specific 19-25 nt (plus hairpin) long shRNAs that is designed to knockdown the β5i gene expression. To yield higher number of transfected cells, human primary myoblast was allowed to be in proliferation state till 50 % confluency on

60 mm plate. Before transfection, cells were washed twice with Optimem medium (Life Technology, USA). The transfection was performed based on the protocol provided by manufacturer with few modifications. Briefly, transfection mixture was prepared with 3 µl of shRNA and 9 µl of transfection reagent (Santa Cruz Biotechnology, Germany) (in 1:3 ratio). Next, the mixture was incubated with the cells in Optimem transfection medium for 6 h at 37 ^{o}C in CO_2 incubator. After that the transfection media was replaced by normal growth medium and incubated for 48 h. Then, the untransfected cells were discarded by incubating cells in selection growth media consisting of 1 µg/ml of puromycin for 5 days. Since most of the cells were washed out while refreshing the media, the viability of remaining attached cells were increased by reducing the puromycin concentration to 0.5 µg/ml. As a negative control for shRNA transfection, control shRNA plasmid-A (sc-108060) (Santa Cruz Biotechnology, Germany) was used. Finally, the transfection efficiency was determined by RT-PCR and western blot.

5.15. Flow cytometry

Flow cytometry was used for the detection of cell surface expression of MHC-I in primary myoblast. For that, $1x10^6$ cells were detached with trypsinization and washed two times with FACS buffer (described in section 4.2.3). For staining, cells were resuspended in 100 µl of FACS buffer containing FITC labelled anti-HLA-ABC antibodies (Miltenyi Biotec, Germany) in 1:11 ratio. As isotype control for the antibody, cells were also incubated with REA control (S) antibodies (Miltenyi Biotec, Germany) in the same ratio. The staining mixture was allowed to incubate for 20 min at 4 ^{o}C under dark condition. Subsequently, cells were washed thrice with PBS and resuspended in 200 µl 1 % PFA prior to analysis. Cells were analyzed in FACScalibur (Becton Dickinson, Germany). Final analysis and graphical output were performed using FlowJo software, version 7.6.5.

5.16. Statistics

All statistical calculations were performed GraphPad Prism V5.0 (San Diego, CA, USA). P value less than 0.05 is considered as statistically significant. Error bars in the graph represent standard error of the mean (SEM). For determining statistical significance between two groups, Student's unpaired t-test was used. Significance levels for

comparisons between more than two groups with normal distribution were analyzed with One-way ANOVA followed by Turkey post-hoc test. Analysis of more than two groups with non-parametric values were analyzed by Kruskal-Wallis with Dunn's multiple comparison.

6. Results

The main results shown here were published by Bhattarai, et. al, J Autoimmunity, 2016.

6.1. General clinical analysis

Prevalence and severity of inflammatory myopathies has been shown to be associated with the patient's age and sex (Smoyer-Tomic, Amato, & Fernandes, 2012). The clinical data from healthy controls and diseased patients group was thus evaluated for the basic parameters, such as age and CK level. No significant difference in the average age between the diseased groups was found (Figure. 10A). However, the IBM group was the oldest among IMNM and DM group. The average age of healthy controls recruited was 52.8 ± 15.7.The male/female ratio for healthy, sIBM, IMNM and DM was about 50/50, 60/40, 40/60 and 50/50, respectively. In addition, we also compared the CK level between healthy controls and diseased group. As a result, CK level was found to be significantly higher in the patients with IMNM and DM compared to healthy controls. In IBM patients, the CK levels were also elevated, but statistically not significant different compared to healthy controls. In IMNM, we observed about 70 fold higher and in DM about 30 fold higher CK level than in healthy controls (Figure 10B).

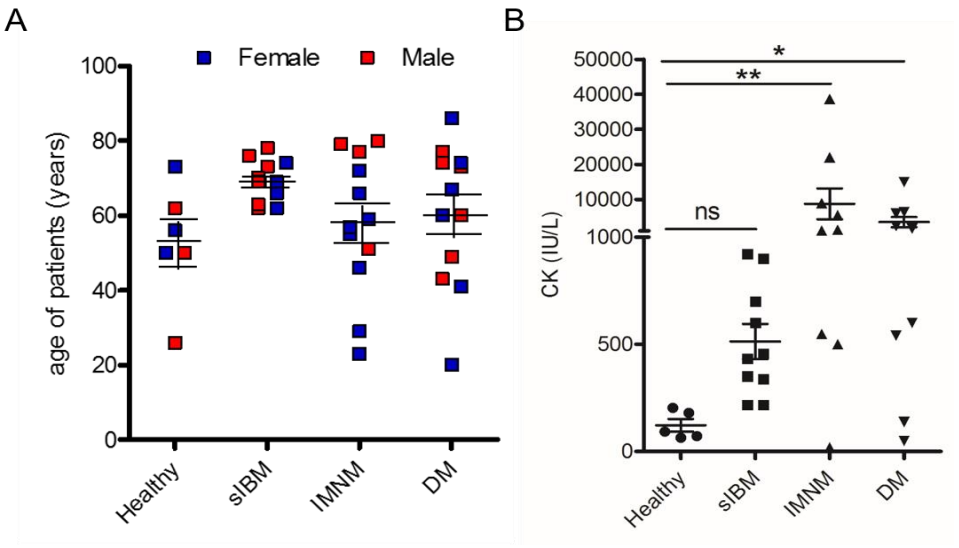


Figure 12: Basic parameter comparison between healthy and patients groups. (A) Age of healthy controls, sIBM, IMNM and DM patients enrolled in the study. Female and male are indicated by blue and red color, respectively. (B) Graphical representation of CK level of each patients enrolled as healthy control, sIBM, IMNM and DM. Compared to healthy

controls, significant difference in the CK level was observed only in the patients with IMNM and DM. Data shown are mean ± SEM. Significant difference was calculated by Kruskal-Wallis test. **P<0.005, *P<0.05. ns= not significant, CK= creatine kinase, IU/L= International unit/ liter.

6.2. Proteasome subunits expression in muscle biopsies of IIMs

Expression of catalytic and non-catalytic subunits of proteasome were analyzed at protein level in muscle biopsies from patients with sIBM, IMNM and DM and compared to those from healthy control by western blot. As shown in figure 11A, the expression of immunoproteasome subunits, both β1i and β5i, were not detectable in the healthy muscle biopsies (n=6) but increased in IIMs muscle biopsies. Densitometric analysis of western blot (Figure 11B-F) revealed that muscle biopsies from sIBM patients (8 out of 8) displayed significant upregulation of both β1i and β5i immunoproteasome subunits. In the case of DM, not all, but about 66 % (6 out of 9) patients showed increased expression of β1i and β5i in their muscle biopsies. Although all IMNM (9 out of 9) muscle biopsies demonstrated moderate expression of immunoproteasome subunits, the increased expression was not significant different compared to healthy controls. Within the disease groups, significant increase in β1i and β5i expression in the muscle biopsies of sIBM was seen as compared to IMNM. In addition, serum CK level of 65 % of sIBM and DM patients showed strong correlation with the muscle immunoproteasome subunits β1i and β5i expression (Supplementary Figure 1). However, serum CK level from IMNM patients did not show any correlation.

In contrast to immunoproteasome subunits, the proteasome constitutive catalytic subunits, β1 and β5, and non-catalytic subunits, α5, was detectable in healthy muscle biopsies. The densitometry analysis of muscle biopsies in disease groups showed no significant changes in the expression level of β1, β5 and α5, compared to the healthy controls. These result indicate that only immunoproteasome subunits are induced in sIBM, IMNM and DM.

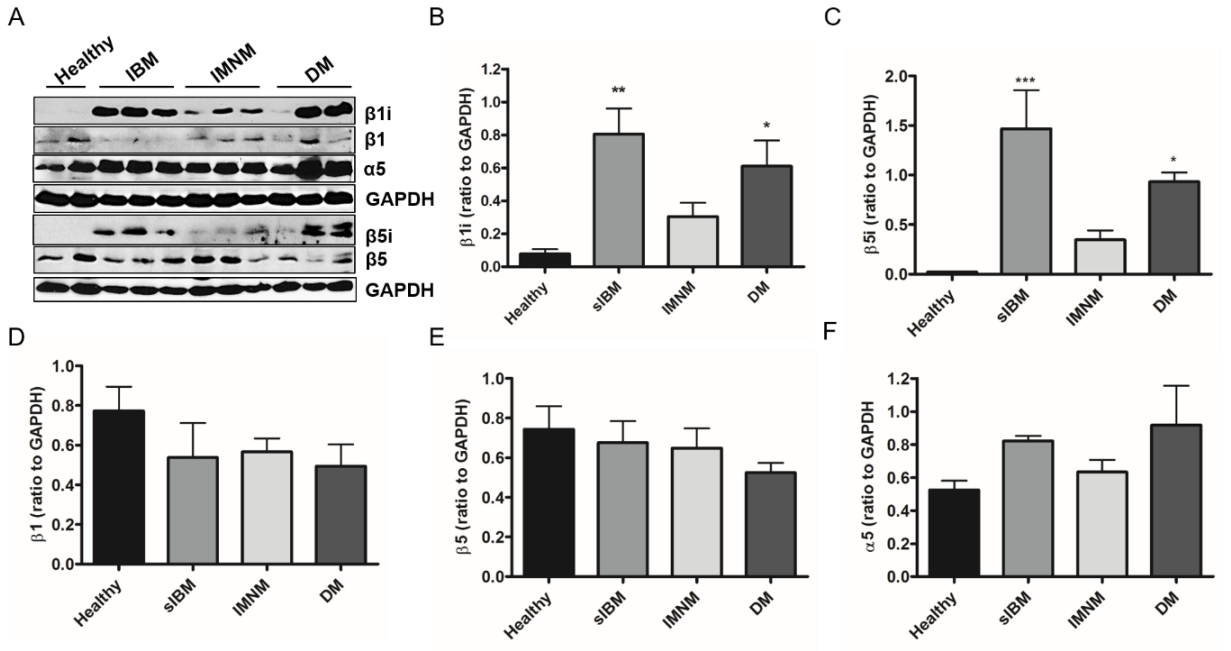


Figure 13: Expression and quantification of proteasome subunit in skeletal muscle biopsies of healthy controls, sIBM, IMNM and DM patients. (A) Representative western blot detection of immunoproteasome subunits (β1i and β5i), their constitutive subunits (β1 and β5) and non-catalytic proteasome subunit 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. Densitometric calculation of (B) β1i, (C) β5i, (D) β1, (E) β5, and (F) α5 expression in the 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.

6.3. Proteasome CT-L and C-L activities in IIMs muscle biopsies

Considering the alteration in the peptidase activities of proteasome with increased expression of immunoproteasome, the proteasome CT-L and C-L activities in the muscle lysates was further determined. In agreement with the western blot results, the muscle biopsies from sIBM and DM showed significantly higher CT-L activity of proteasome (Figure 12A). Compared to the healthy control (100 % ± 8.1), there was almost a 3 fold increase in CT-L activity in sIBM (305.6 ± 13.7) and DM (278.5 305.6 ± 37.3) muscles biopsies. 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 control. Compared with IMNM, corresponding to the expression of β1i and β5i, sIBM showed significantly higher CT-L activity of proteasome.

With respect to the C-L activity of proteasome, no significant differences between IIMs muscle tissue and healthy controls were observed (Figure 12B). These result suggest that CT-L activity of proteasome is increased in IIMs muscle biopsies with increase in the expression of immunoproteasome subunits.

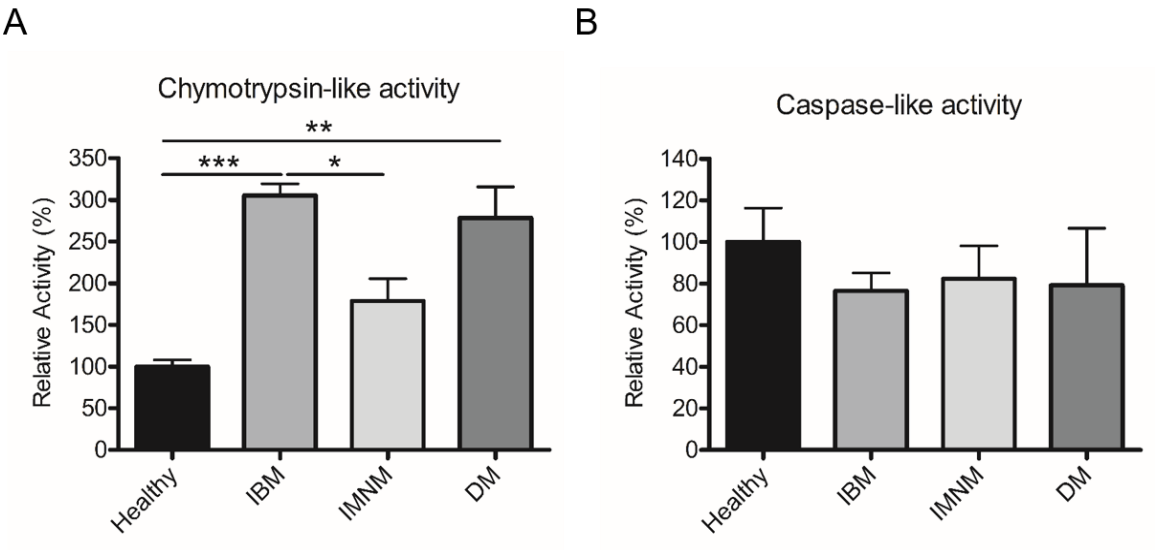


Figure 14: Proteasome catalytic activity in healthy controls, sIBM, IMNM and DM muscle biopsies. Graphical representation of (A) CT-L activity and (B) C-L activity of proteasome in muscle biopsies from healthy controls (n=6), sIBM (n=6), IMNM (n=6) and DM (n=4). The protein lysate was incubated with specific substrate for 2 h at 37oC 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.

6.4. Subcellular localization of proteasome subunits expression in IIMs muscle biopsies

Total muscle biopsies lysate from patients can contain also infiltrating immune cells, which under basal condition predominantly express an abundant amount of immunoproteasome subunits. Therefore, we investigated if the increased amount of (immuno) proteasome in the muscle lysate of patients is entirely attributed to infiltrating cells or at least partially derived from muscle fibers. For this purpose, immunofluorescence staining were performed on muscle section from the sIBM, IMNM and DM patients. Immunostaining revealed that constitutive subunits β1 and β5 were predominantly expressed in healthy muscle fibers. Their expression in all investigated

disease groups was comparable to the healthy controls, which was in agreement with the western blot results. Compared to the infiltrating cells, the staining of β1 and β5 were intense in the muscle fibers in all disease conditions (Figure 13).

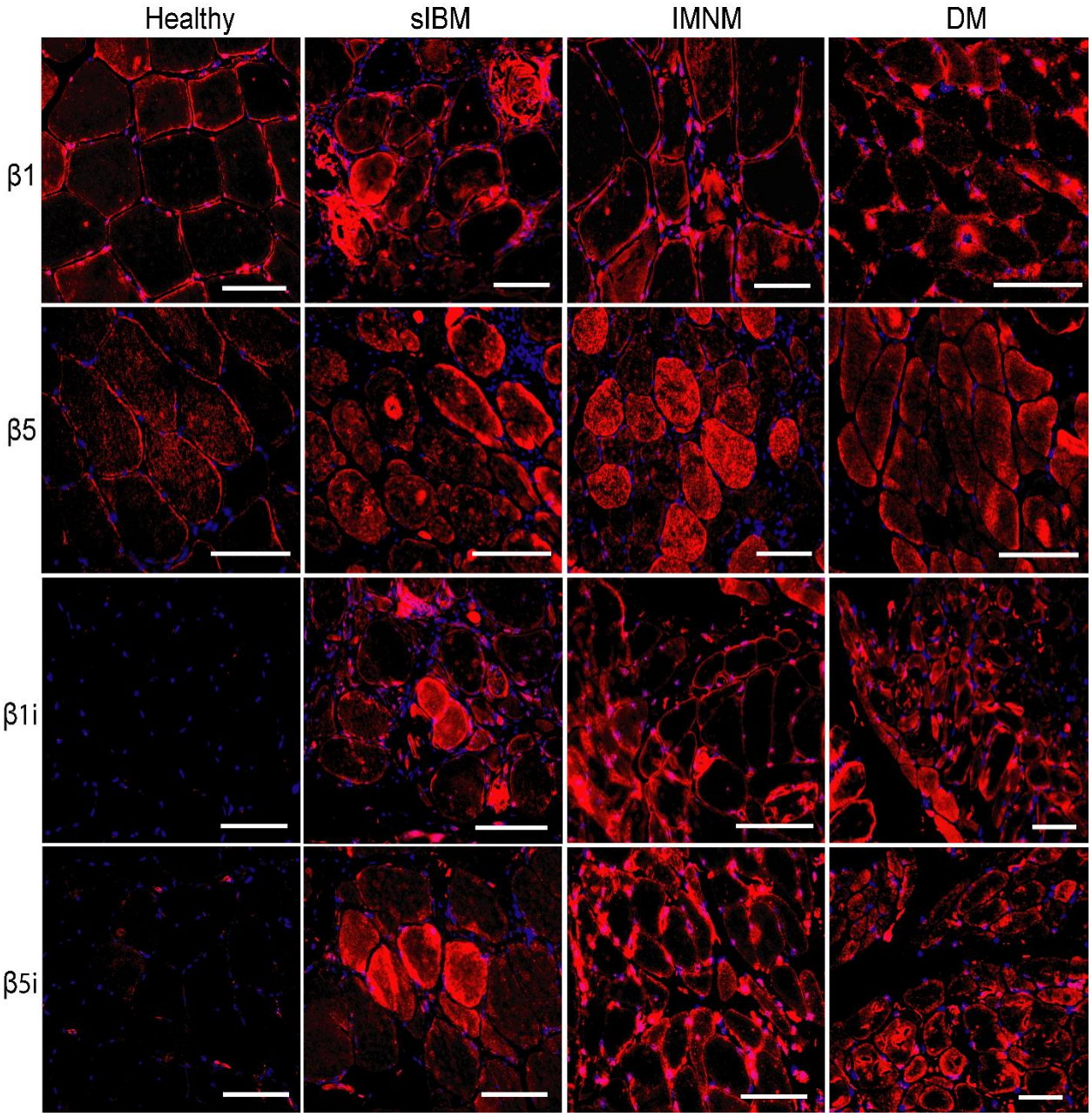


Figure 15: Localization of immunoproteasome subunits in sIBM, IMNM, DM and healthy muscle biopsies. Representative fluorescent single-labeling immunohistochemistry in muscle biopsy specimens of healthy controls (n=4), sIBM, IMNM, DM patients (n=6 for each group). Muscle fibers were stained with antibodies against β1, β5, β1i and β5i with Cy3 (red) anti rabbit secondary antibodies. Nuclei counterstained with DAPI (blue). Healthy muscle fibers

stained with of β1 and β5 but not with β1i and β5i. However, the diseased muscle fibers showed increased staining of β1i and β5i both in muscle fibers and infiltrating cells. Scale bar = 100 µm.

Staining of both immunoproteasome subunits β1i and β5i revealed that healthy muscle fibers shows no expression of immunoproteasome subunits (Figure 13). However, their expression was predominantly increased in the muscle fibers of sIBM, IMNM and DM. Noticeably, the diffused staining of β1i and β5i were observed in all disease entities within the muscle fibers. In DM, diffused expression of β1i and β5i was more prominent in the perifascicular area of muscle biopsies. In addition to the muscle fiber, a clear staining for β1i and β5i was also observed in infiltrating cells. To clarify whether the expression of β1i and β5i was specific for IIMs, the muscle biopsies from patients with limb girdle muscular dystrophy (LGMD, n=1), congenital myopathy (n=1) and neurological disorder (n=1) were stained against β1i and β5i subunits. Interestingly, no staining of β1i and β5i in their muscles fibers (Supplementary Figure 2) were noticed, suggesting a specific expression of β1i and β5i in skeletal muscle of IIMs.

6.5. Muscle-infiltrating CD8+ T-cells and CD68+ macrophages show increased expression of β1i and β5i in IIMs

Infiltrating cells showed intense staining of immunoproteasome subunits. Thus, to further investigate immunoproteasome expression in infiltrating cells in the muscle biopsies of IIMs, muscle biopsies were co-stained for immune cells marker and immunoproteasome subunits. In detail, CD8+ T-cells or CD68+ macrophages were co-stained with β1i or β5i by double immunofluorescence technique. As a result, both CD8+ T-cells as well as CD68+ macrophages expressed β1i and β5i in all observed muscle tissues (n=6 for each group of IIMs) (Figure 14 A and B).

Furthermore, the total percentage of each cell type co-stained for β1i or β5i was estimated. The estimated percentage of CD8+ T-cells that express β1i or β5i in sIBM was 50-60 % (Figure 14C). In contrast, muscle biopsies from IMNM showed more than 90 % of CD8+ T-cells that co-express β1i or β5i. As professional antigen presenting cells which require immunoproteasome subunits as a primary machinery for active generation of antigenic peptide (Meyer Zu Horste et al., 2010), more than 95 % of CD68+ macrophages were co-stained for β1i or β5i in all disease entities (Figure 14C). To prove the co-expression of both β1i and β5i in CD68+ macrophages, sIBM muscle section was

triple stained with antibodies against CD68+, β1i and β5i. Triple staining confirmed that CD68+ macrophages and also the muscle fibers in sIBM co-localize β1i and β5i (Supplementary Figure 4). As a typical finding in DM, muscle biopsies showed low number of infiltrating CD8+ T-cells.

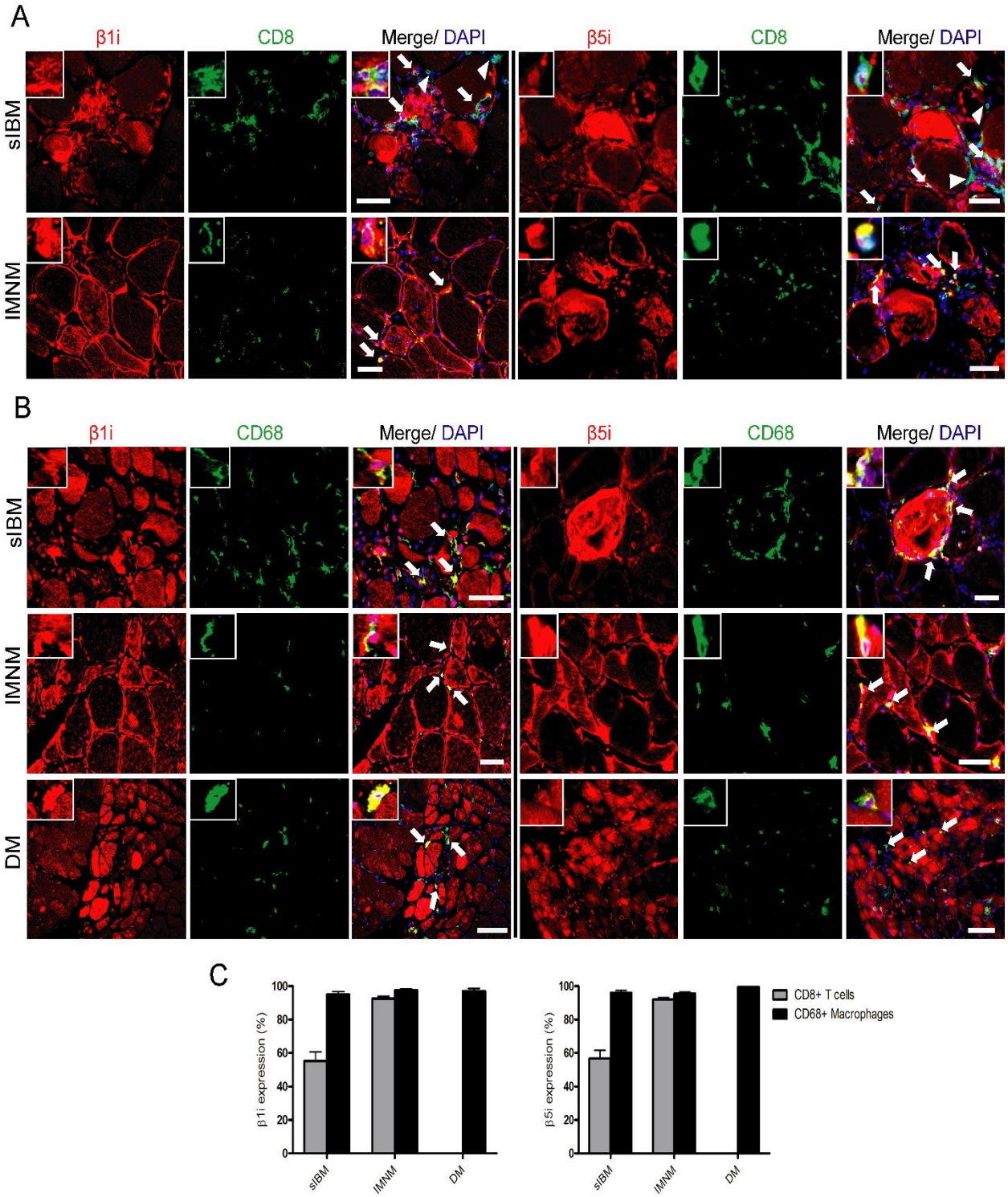


Figure 16: Muscle infiltrating CD8+ T-cells or CD68+ macrophages co-express β1i and β5i in IIMs. Double immunofluorescence using antibodies against (A) CD8+ T-cells (green, Alexa 488) co-expressed β1i (red, cy3) (left panel) and β5i (red, cy3) (right panel) in representative muscle biopsy specimens from sIBM (n=6) and IMNM (n=6), and against (B) 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). Arrow denotes co-expression and arrowhead denotes no co-expression. Integrated small rectangular box is representative 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).

6.6. β1i and β5i expression frequently co-localize with MHC-I positive muscle fibers in IIMs

Skeletal muscle fibers from healthy individuals shows no expression of MHC-I molecules. In contrast, the sarcolemmal presence of MHC-I is a hallmark of all IIMs (van der Pas, Hengstman, ter Laak, Borm, & van Engelen, 2004; Vattemi et al., 2014). As immunoproteasome shows greater efficiency in peptide production for MHC-I molecules (introduced in section 1.3.4.1), we next examined whether the expression of β1i and β5i co-localized with the specific structure of fibers expressing MHC-I in all diseased entities. As known, muscle fibers from healthy controls did not show increased expression of MHC-I molecules and neither of the immunoproteasome subunits. In fact, muscle fibers expressing β1i and β5i in the biopsies of all disease entities co-localized for MHC-I molecules (Figure 15). In other words, β1i or β5i expressing muscle fibers showed brighter co-staining for MHC-I molecules. In IMNM, some of the muscle fibers showed no expression of β1i and β5i, similar to healthy control. Staining of those muscle fibers against MHC-I molecule showed no expression of MHC-I, reflecting the characteristics of healthy fibers (data not shown). However, the immunoproteasome subunits as well as MHC-I expression co-localized in perifascicular area of muscle in DM, as shown in figure 15. These results indicate that immunoproteasome can be addressed as the primary source for antigens for presentation to cytotoxic T-cells in IIMs.

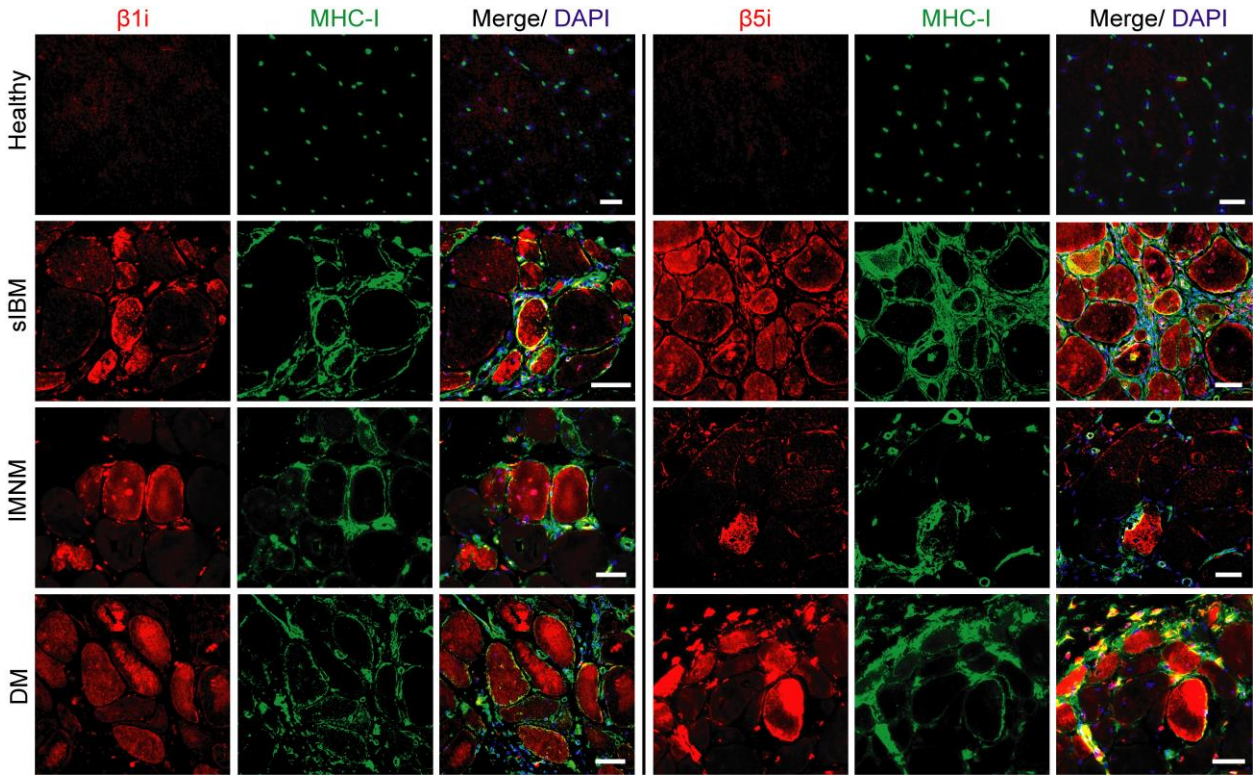


Figure 17: Co-staining of β1i and β5i and MHC-I in muscle biopsies of healthy controls and IIMs. Dual immunofluorescence of muscle sections against β1i (red, left) or β5i (red, right) along with MHC-I (Alexa 488, green). Healthy myofibers reveal negative staining for β1i, β5i and MHC-I. Myofibers in sIBM, IMNM and DM display co-localization of these proteins. Images are representative muscle biopsies from healthy controls (n=4) and patients with sIBM (n=6), IMNM (n=6) and DM (n=6). Nuclear counterstain with DAPI. Scale bar = 50 µm.

6.7. Pro-inflammatory cytokines upregulates immunoproteasome subunits expression in human primary myoblast

While the expression levels of immunoproteasomes subunits was shown to be low in the healthy muscle fibers, it was increased in immune cells and muscle fibers under inflammatory condition. Therefore, the role of pro-inflammatory cytokines responsible for immunoproteasomes induction during IIMs pathogenesis was further investigated. Since TNF-α, IFN-α, IFN-β, IFN-γ and IL-1β are the major cytokines that were found to be upregulated in the IIMs patients (Moran & Mastaglia, 2014), we asked if human primary myoblast exposed to these cytokines could upregulate immunoproteasome subunits expression at mRNA and protein levels.

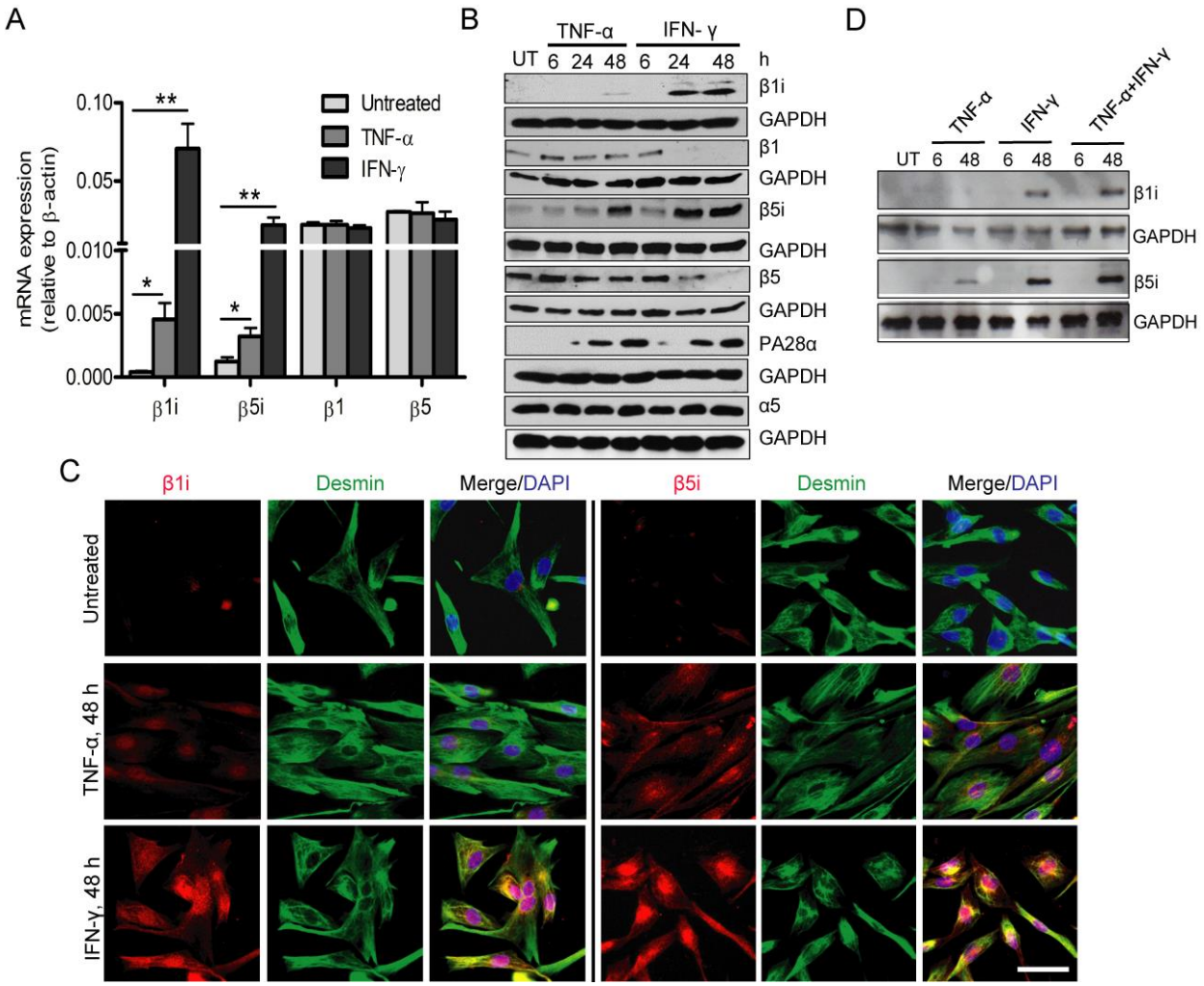


Figure 18: β1i and β5i expression is induced by pro-inflammatory cytokines in human primary myoblasts. (A) mRNA expression of the immunoproteasome subunits β1i and β5i and their constitutive one β1 and β5, respectively, detected by real time RT-PCR. Myoblasts were exposed to TNF-α (100 ng/ml) or IFN-γ (300 U/ml) for 24 h and real time RT-PCR were performed after extraction of total RNA. Data are depicted as mean ± SEM from three independent experiments, unpaired students t-test, **P < 0.005, *P<0.05 (B) Time course of expression of constitutive proteasome subunits (β1, β5), immunoproteasome subunits (β1i, β5i), proteasome activator subunits PA28α and non-catalytic proteasome subunit α5 in total protein lysates of myoblasts from 6 up to 48 h after TNF-α or IFN-γ treatment. Results are representative for three independent experiments. (C) Double immunofluorescence labeling against β1i (red, left panel), β5i (red, right panel) and desmin (green) in myoblasts incubated with TNF-α or IFN-γ for 48 h. Increased expression of β1i or β5i was observed in IFN-γ treated myoblasts. DAPI as nuclei counterstain. Scale bar= 50 µm. Western blot detection of β1i and β5i after treatment with (D) Synergic effect of TNF-α and IFN-γ on β1i and β5i protein expression at 6 and 48 h of incubation, as detected by western blot. No synergic effect was observed. For all western blot image, the expression GAPDH was used as a loading control. UT= untreated, APP=Amyloid precursor protein.

Similar to the muscle fiber in healthy controls, under normal cell culture condition, myoblast expressed very low levels of β1i and β5i at mRNA as well as protein level (Figure

16 A and B). However, after 24 h of exposure with TNF-α (100 ng/ml) or IFN-γ (300 U/ml), there was a significant upregulation of the mRNA expression of β1i and β5i in the myoblast (Figure 16A). Next, the expression of β1i and β5i at protein level was investigated by exposing cells with these cytokines for indicated time points. As a result, it was confirmed that pro-inflammatory cytokines TNF-α (100 ng/ml), IFN-α (300 U/ml), IFN-β (300 U/ml) and IFN-γ (300 U/ml) induced the protein level of β1i and β5i already after 24 h of exposure (Figure 16B, C and Supplementary Figure 3A). Although IL-1β at 20 ng/ml induced the expression of amyloid precursor protein (APP), an IL-1β-induced inflammatory marker for primary myoblast (Schmidt et al., 2008; Schmidt et al., 2012), it could not induce β1i and β5i expression in primary myoblast (Supplementary Figure 3B). Among all investigated cytokines, especially IFN- γ was a strong inducer of β1i and β5i already at 24 h of incubation. The induction of β1i and β5i by TNF-α, IFN-α or IFN-β was comparable and increased after 48 h.

Since TNF-α and IFN-γ have synergistic effects on various cellular functions and on the expression of several cytokines in myoblast and other cell types (De Rossi, Bernasconi, Baggi, de Waal Malefyt, & Mantegazza, 2000; Fish, Proujansky, & Reenstra, 1999; N Goebels, Michaelis, Wekerle, & Hohlfeld, 1992), their synergistic effect on immunoproteasome expression was further tested. Of note, upon incubating myoblast with TNF-α and IFN-γ, no synergic effect on β1i and β5i expression was observed (Figure 16E).

Noticeably, as shown in figure 16 A and B, the replacement of the constitutive proteasome subunits β1 and β5 by its immunoproteasome subunits after IFN- γ exposure was strongly detectable at protein level but not at mRNA level.

Furthermore, a strong induction of the proteasome activator subunits, PA28α was also observed. As a control, no changes in the non-catalytic, non-inducible subunit α5 upon treatment with TNF-α or IFN-γ was seen.

The subcellular localization of immunoproteasome was also determined by immunofluorescence approach in TNF-α and IFN-γ induced myoblast. As shown in figure 16C, the expression of immunoproteasome subunits was more prominent in the nuclear and perinuclear area. However, in the cytoplasm, immunoproteasome subunits were less prominent expressed. Since immunoproteasome expression in myofibers of IIMs were

located in sarcoplasm, differentiated myoblast, known as myotube, under IFN-γ for 48 h were also stained. As a result, along with nuclear expression, immunoproteasome was also located in the cytoplasm of myotubes (Supplementary Figure 5). This indicates that subcellular localization of immunoproteasome in myoblasts depends on its differentiation state.

6.8. Pro-inflammatory cytokines have an effect on catalytic activities of proteasome in human primary myoblast

CT-L activity of proteasome was significantly higher in IIMs diseases entities and was found to reflect the expression of β1i and β5i in IIMs muscle biopsies (Figure 12). Thus, the effect of pro-inflammatory cytokines TNF-α or IFN-γ on proteasome activity was measured in the primary myoblast after exposure to TNF-α or IFN-γ for 48 h. As shown in figure 17A, a significant increase in CT-L activity of proteasome in primary myoblast was observed with TNF-α or IFN-γ. Reflecting the effect of IFN-γ on β1i and β5i expression, the CT-L activity was higher with IFN-γ treatment as compared to that of TNF-α. In addition, C-L activity of proteasome was also significantly higher in both IFN-γ and TNF-α, treated myoblast. However, compared to the IFN-γ treated cells, the TNF-α effect on C-L activity was stronger. Since β1 is responsible for C-L activity of proteasome, these result indicate the active presence of the catalytic constitutive subunits β1 even after 48 h treatment with TNF-α.

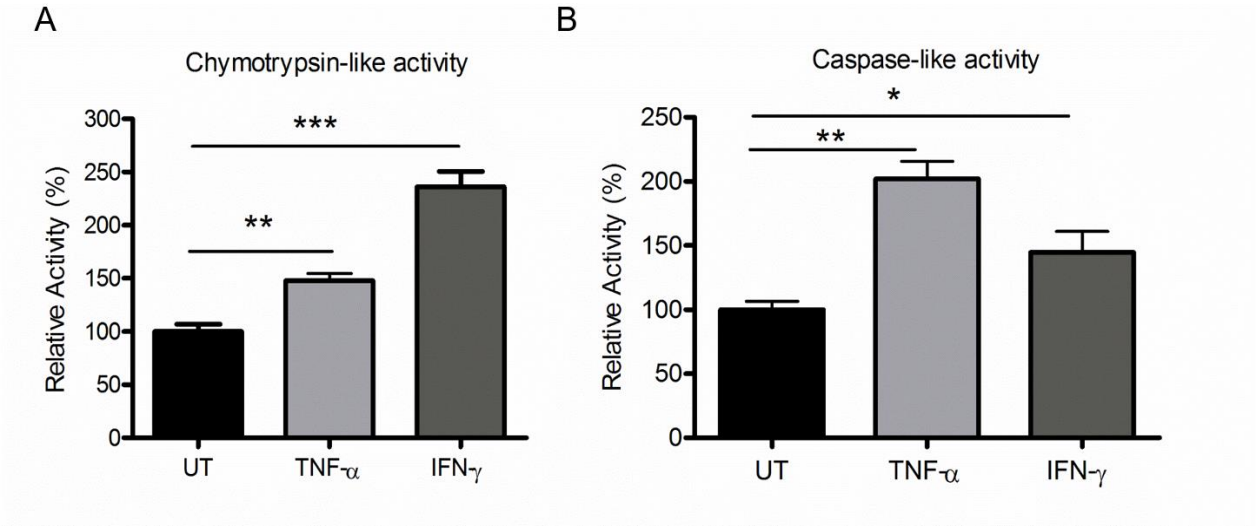


Figure 19: Proteasome activity in human primary myoblast after treatment with IFN-γ and TNF-α. Graphical representation of (A) chymotrypsin-like (CT-L) and (B) caspase-like (C-L) activities of proteasome in myoblasts treated with TNF-α (100 ng/ml) or IFN-γ (300 U/ml) for 48 h. The protein lysate was incubated with specific substrate for 2 h at 37oC and fluorescent was measured. Results are the mean +/− SEM of three experiments, unpaired students t-test, ***P < 0.0005, **P<0.005, *P<0.05. UT= untreated.

6.9. LU001i specifically inhibits β1i and LU015i specifically inhibits β5i activities of immunoproteasome in human primary myoblast

Since β5 is replaced by β5i under the influence of TNF-α and IFN-γ, the increased CT-L activity of immunoproteasome are mostly governed by β1i and β5i. To determine what functions β1i and β5i plays during the inflammatory conditions of myoblast, the specific activity of β1i and β5i was blocked using LU001i and LU015i inhibitor, respectively. Initially, the selectivity of LU001i and LU015i in myoblast was observed. For that, myoblast were incubated with different concentration of LU001i or LU015i (see section 4.6 for its detail) for 2 h. After subsequent incubation with IFN-γ for 48 h, the activity of catalytic subunits of proteasome was measured by probe based competition assay. Under the normal culture condition, the catalytic activity of constitutive subunits β1 and β5 was higher and the activity of immunoproteasome subunits β1i and β5i was not observed. However, under the influence of IFN-γ, the activity of β1 and β5 was replaced by the activity of β1i and β5i (Figure 18 A and B). As shown in Figure 18, LU001i specifically inhibited β1i activity, which decreased gradually with increased concentration of the inhibitor. In addition, the effect of LU001i on other catalytic subunits β1, β5 and β5i until the concentration of 5 μM was minimal. Likewise, LU015i was found to specifically inhibit the catalytic activity of β5i in a concentration dependent manner, while its effect on the activity of other subunits, such as β1, β1i and β5 was weak. These results shows that LU001i and LU015i has capacity to specifically block β1i and β5i subunits, respectively, in the primary myoblast under the influence of IFN-γ.

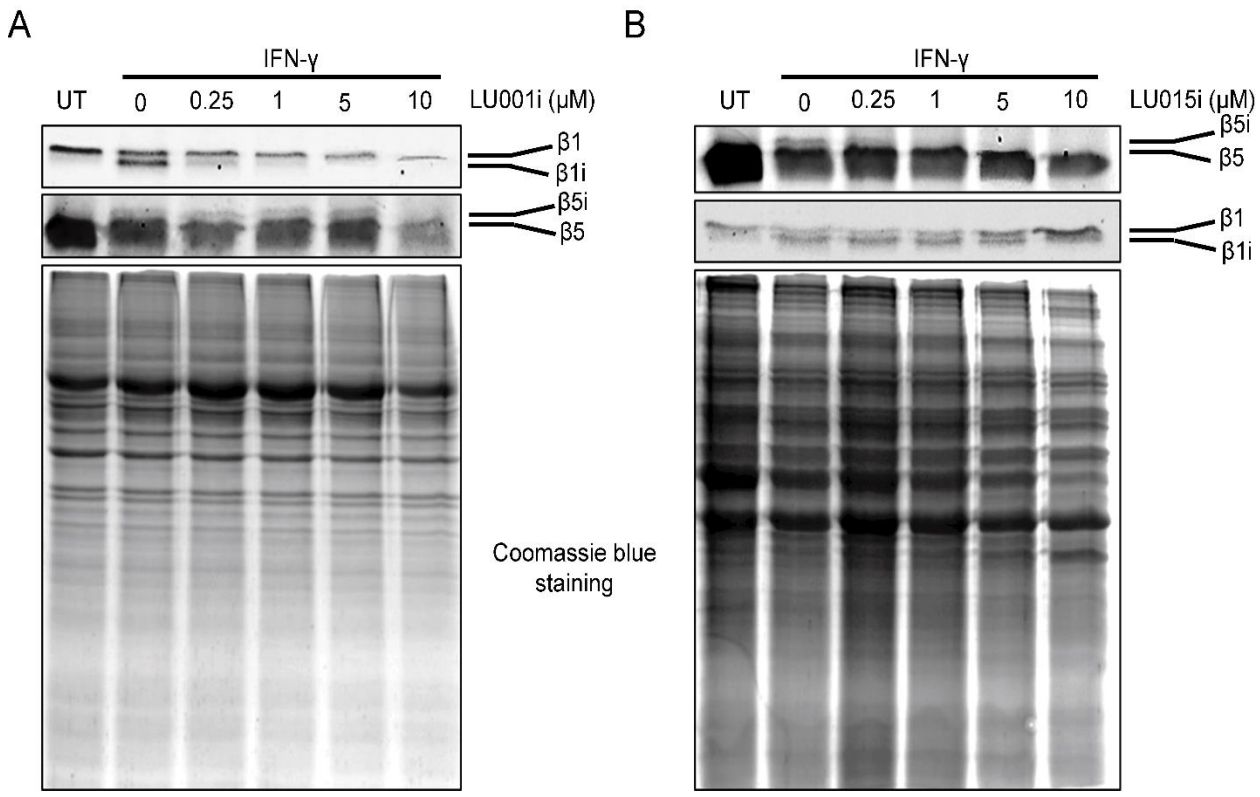


Figure 20: Probe based competition assay for LU001i and LU015i in IFN-γ induced myoblast. Inhibition profile of (A) β1i inhibitor, LU001i and (B) β5i inhibitor, LU015i in myoblast induced with IFN-γ. Cells were treated with the distinct compounds at indicated concentrations for 2 h, lysed, and the occupancy of the remaining active sites was probed with BODIPY NC001 (β1/β1i) and BODIPY NC005 (β5/β5i). The loading control was visualized by Coomassie blue staining UT means untreated.

6.10. LU001i and LU015i inhibits immunoproteasome specific IFN-γ-induced cell surface expression of MHC-I in human primary myoblast

The immunoproteasome generates both, endogenous and viral derived MHC-I–restricted antigens for the antigen presentation. Figure 15 also clearly demonstrate the co-expression of β1i or β5i with MHC-I positive muscle fibers in all groups of IIMs suggesting a function of immunoproteasome for antigen presentation via MHC-I. To prove the direct effect of immunoproteasomes subunits on MHC-I expression in primary myoblast, LU001i and LU015i were used under inflammatory condition. FACS analysis demonstrated that myoblast under normal culture condition showed low basal level of MHC-I surface expression. After treatment with only TNF-α and IFN-γ for 48 h an increased surface

expression of MHC-I was evident. Reflecting the expression of immunoproteasome subunits and proteasome CT-L activity, IFN-γ induced the MHC-I expression in myoblast in greater level than TNF- α (Figure 19 A and B).

Myoblast were treated with different concentration (0-10 µM) of LU001i or LU015i for 2 h prior to IFN-γ exposure for 48 h and surface expression of MHC-I was analyzed by FACS. As a result, LU001i as well as LU015i decreased the surface expression of MHC-I in myoblast (Figure 19C). 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 % (figure 5c). Furthermore, total MHC-I protein expression in myoblast after the induction by IFN-γ alone or in combination with 5 µM of LU001i or LU015i was investigated using western blot. As a result, no difference in IFN-γ-induced total MHC-I expression in myoblast treated or untreated with LU001i or LU015i (Figure 19D) was observed. These data indicates that myoblasts require β1i or β5i subunits for the stabilization of MHC-I expression in the primary myoblast under inflammatory condition.

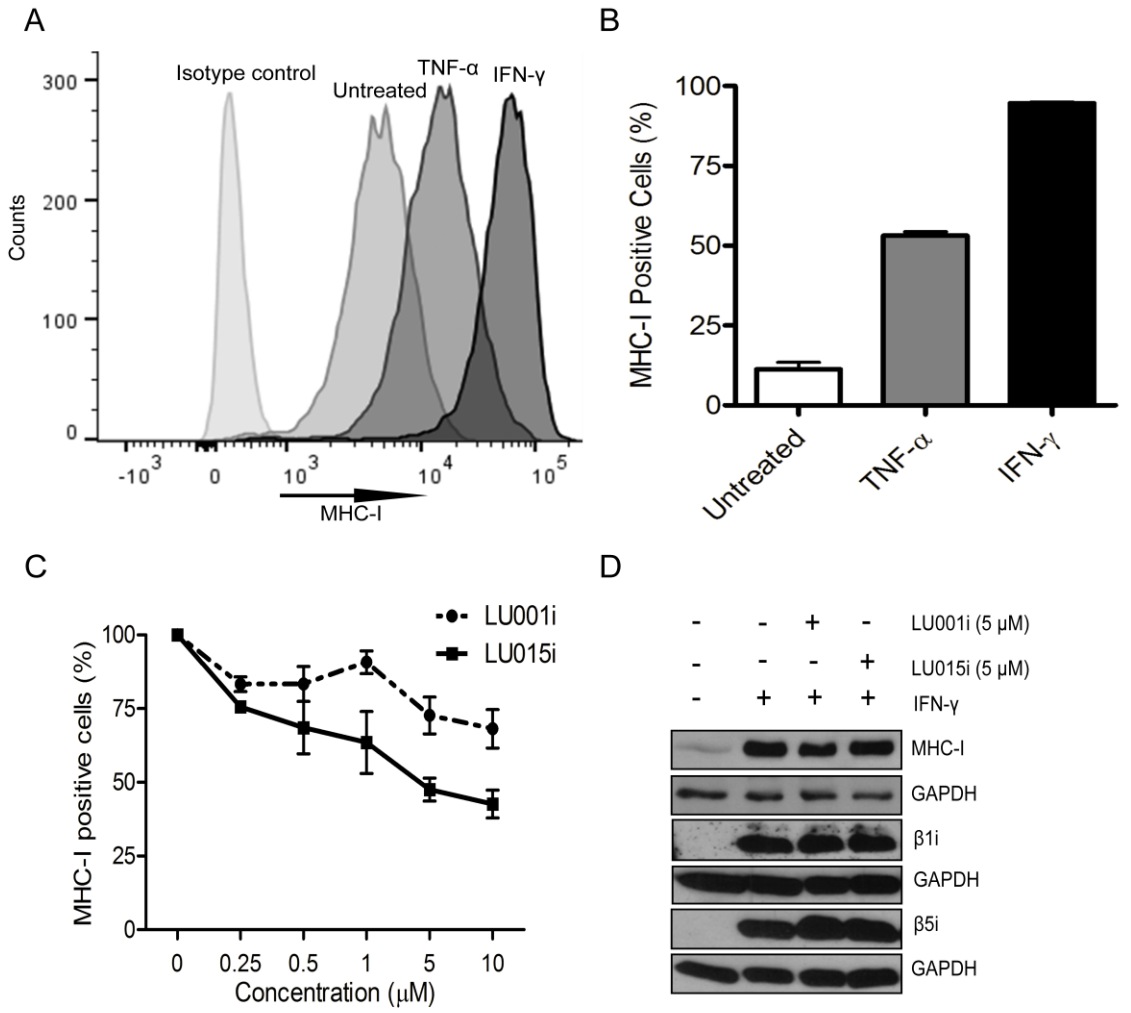


Figure 21: Both LU001i and LU015i reduce IFN-γ-induced membrane expression of MHC-I in human primary myoblasts. (A) Flow cytometric histogram showing labeling of membrane bound MHC-I on myoblasts following incubation of the cells with TNF-α (100 ng/ml) or IFN-γ (300 U/ml) for 48 h. Increased membrane expression of MHC-I was observed in the population incubated with IFN-γ, compared to that of TNF-α incubated cells. (B) Graph represents percentage of MHC-I positive cells incubated with TNF-α or IFN-γ compared with untreated. Values represent mean ± SEM from three independent experiments. (C) Graphical representation of inhibition of IFN-γ-induced membrane expression of MHC-I in myoblasts treated with LU001i or LU015i in concentration dependent manner (0-10 μM). Data are presented as percentage (mean ± SEM) of MHC-I positive cells from three independent experiments. (D) Western blot analysis of total MHC-I expression in myoblasts treated with IFN-γ alone or in combination with 5 μM of LU001i or LU015i.

6.11. Selective inhibition of β5i increases myokine production in human primary myoblast under inflammatory condition *in vitro*

The immunoproteasome is important for the inflammatory response as inhibitors that target both β1i and β5i were able to block cytokine production in endotoxin-stimulated monocytes isolated from mice (Qureshi et al., 2003). Cytokines are found to be overexpressed in IIMs muscle biopsies and possess a leading role for the pathogenesis of the disease. To demonstrate the impact of β1i and β5i inhibition on myokine production, we compared the myokine production in TNF-α or IFN-γ stimulated primary myoblast after exposure to LU001i or LU015i at 5 µM concentration. As shown in figure 20, myoblasts under normal culture condition expressed very low basal levels of myokines, such as IL-6, IL-1β, CXCL9 and CXCL10. However, TNF- α treatment for 24 h increased the mRNA level of IL-6 and IL-1β (Figure 20 A and B), while treatment with IFN-γ increased CXCL9 and CXCL10 mRNA expression (Figure 20 C and D).

Moreover, when myoblast were exposed to 5 µM of LU001i and LU015i for 2 h prior to the stimulation with TNF-α and IFN-γ, a significant increase in the mRNA level of TNF-α-induced IL-6 and IL-1β and IFN-γ-induced CXCL9 and CXCL10 was noticed only in LU015i exposed cells (Figure 20 A-D). In contrast, selective inhibition of β1i with LU001i did not significantly affect myokine expression. 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 immunoproteasome subunit β5i has a role in myokine regulation under inflammatory conditions in myoblast.

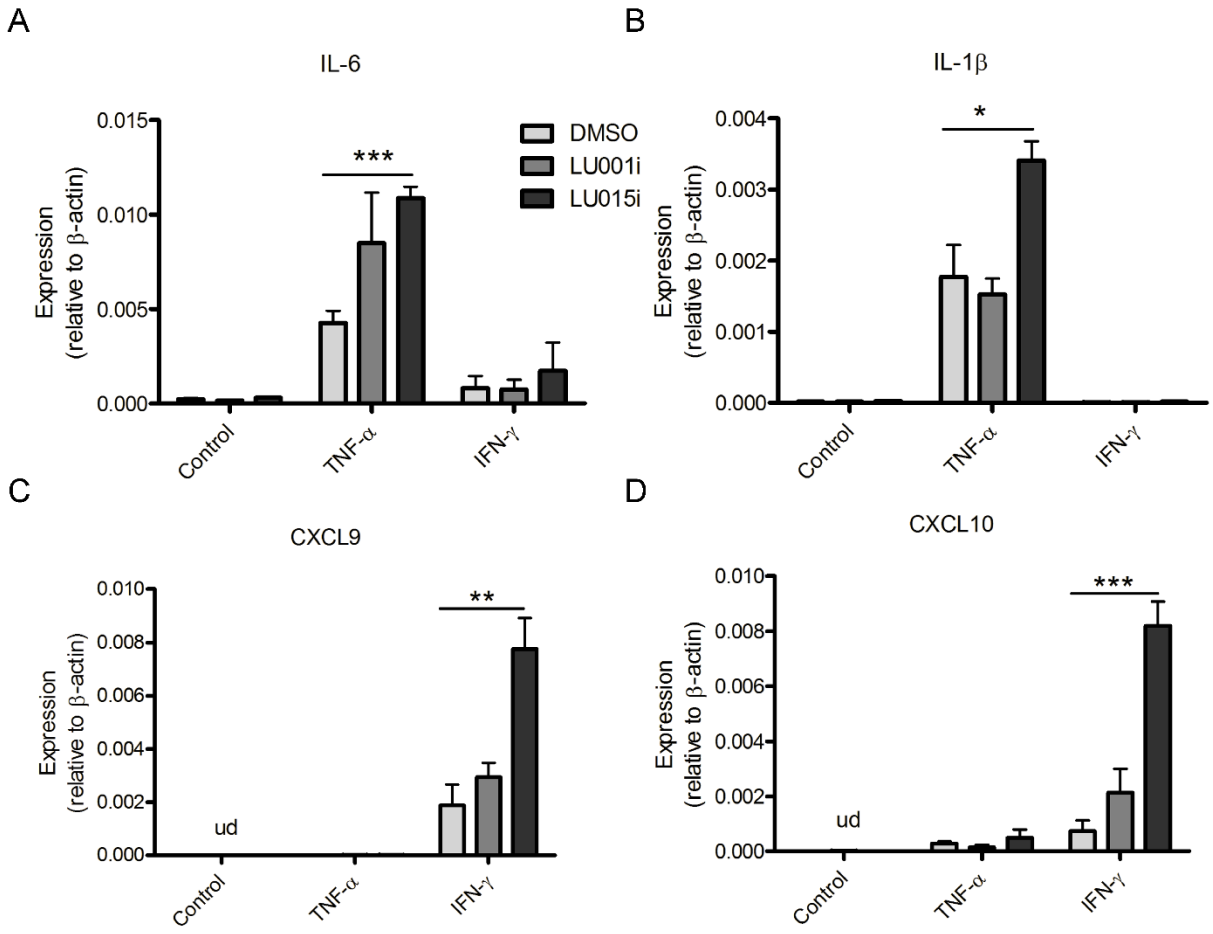


Figure 22: Selective inhibition of β5i amplifies the TNF-α and IFN-γ mediated expression of myokines. Expression of mRNA for (A) IL-6, (B) IL-1β, (C) CXCL9 and (D) CXCL10, as measured in primary myoblasts after treatment with 5 µM of LU001i or LU015i for 2 h prior to the induction by TNF-α or IFN-γ for 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. ud= undetermined.

6.12. β5i knockdown increases myokine production in human primary myoblast under inflammatory conditions *in vitro*

To further determine the role of β5i in myokine production under inflammatory conditions in myoblasts and to validate the above result, a genetic approach was implemented to knockdown the β5i gene. In particular, primary myoblast were stably transfected with shRNA pool targeted to β5i, and the impact on TNF-α or IFN-γ driven production of myokine in myoblast was examined. To estimate the β5i gene known down the stable transfected cells were incubated with IFN-γ for 48 h.

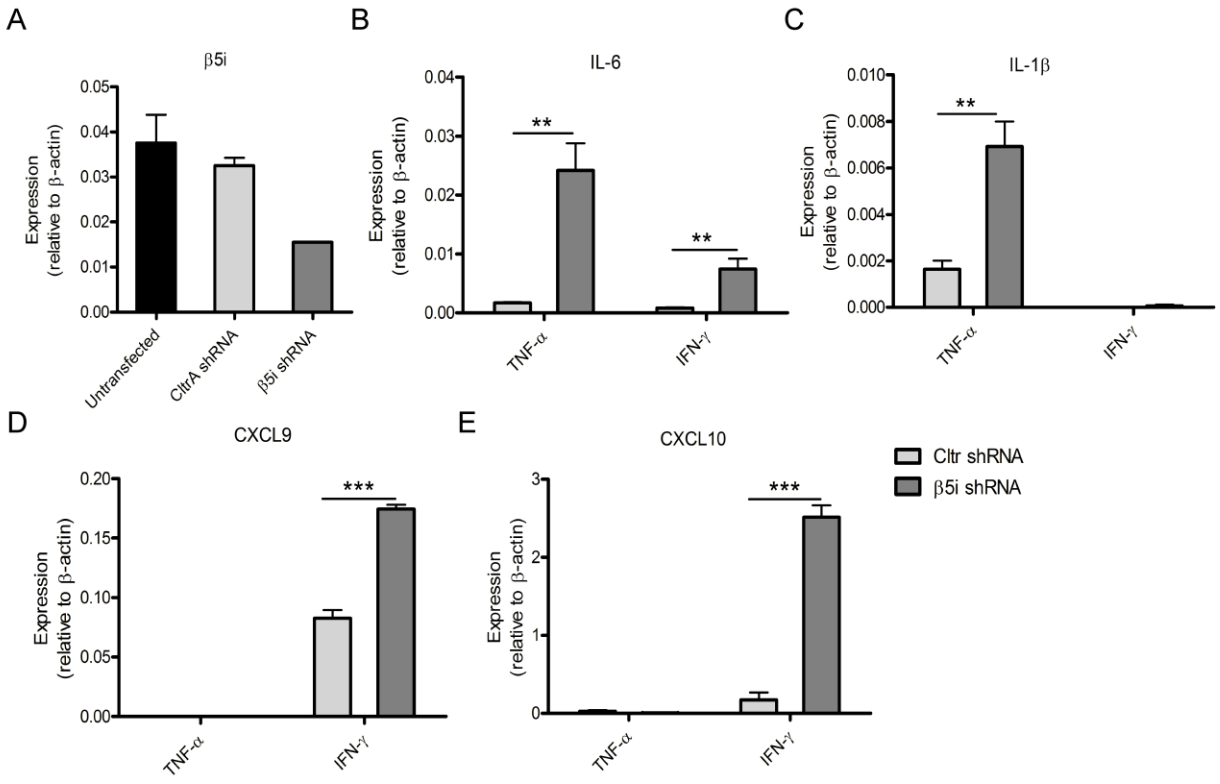


Figure 23: β5i knockdown in primary myoblasts amplifies the TNF-α and IFN-γ mediated expression of myokines. (A) Knockdown of β5i in primary myoblasts transfected with shRNA targeting β5i (β5i shRNA) when compared with myoblasts transfected with scrambled controls shRNA (cltrA shRNA) and un-transfected myoblasts. Cells were collected after exposure to IFN-γ for 48 h and mRNA expression for β5i was evaluated. 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.

As shown in Figure 21, the shRNA pool targeted to β5i gene directed to approximately 50% reduction in mRNA levels of β5i as determined by quantitative RT-PCR. Compared to the myokine production in control shRNA transfected myoblast, knockdown of β5i significantly amplified the TNF-α-induced mRNA expression of IL-6 and IFN-γ-induce mRNA expression of CXCL9 and CXCL10 in primary myoblast. These results suggest that β5i controls the expression of myokine in myoblast upon exposure to pro-inflammatory cytokines.

6.13. Selective inhibition of β5i, but not β1i increases STAT1 phosphorylation on human primary myoblast under inflammatory condition *in vitro*

As interferon regulated genes, the expression and phosphorylation of STAT1 and immunoproteasome subunits has been positively correlated in IIMs (Ghannam et al., 2014; Ivanidze et al., 2011). To further investigate the function of immunoproteasome in myoblast inflammatory conditions, the impact on LU001i and LU015i on STAT1 phosphorylation was investigated. In the normal culture condition of myoblast, the expression of STAT1 was detected but in un-phosphorylated state (Figure 22 A and B). However, the phosphorylation of STAT1 was increased after 24 h induction with either TNF-α or IFN-γ. Compare to TNF-α, the phosphorylation was stronger with IFN-γ induction.

Furthermore, prior treatment with or without LU015i for 2 h, the STAT1 phosphorylation was doubled as compared to that of inhibitor untreated myoblast. In contrast, LU001i treatment did not affect phosphorylation of STAT1. Although the expression of STAT1 was doubled with IFN-γ induction, the expression was not altered with LU001i or LU015i treatment (Figure 22 B and C).

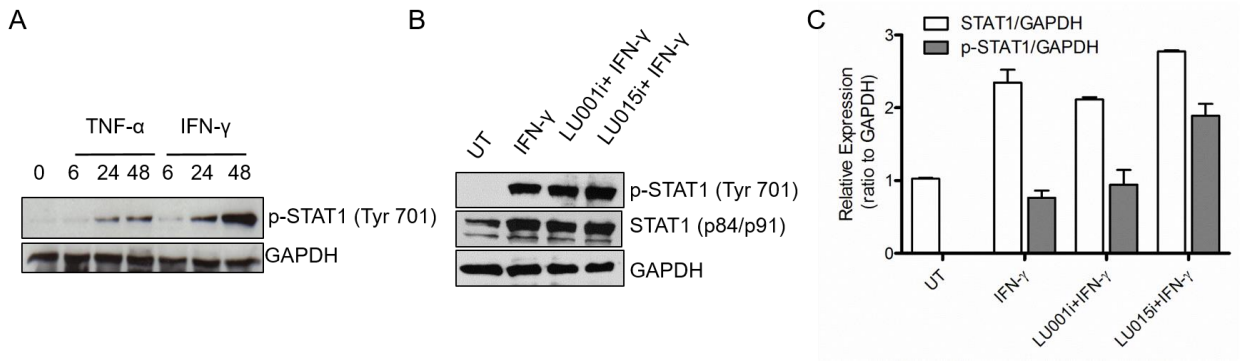


Figure 24: LU015i treatment increase STAT1 phosphorylation under inflammatory conditions. (A) Western blot analysis of primary myoblast against phospho-STAT1 (P-STAT1) after incubation with TNF-α or IFN-γ for different time point. (B) Western blot analysis of myoblast treated for 2 h in the presence or absence of LU001i and LU015i followed by 24 h incubation with IFN-γ. Blots were probed with STA1 and P-STAT1. For (A) and (B) GAPDH was used as a loading control. (C) Densitometry analysis of STAT-1 and p-STAT1 western blot with respect to GAPDH. Data represent mean relative values ± SEM from two independent experiments.

6.14. Immunoproteasome inhibition does not affect oxidized or ubiquitinated proteins on human primary myoblast under inflammatory conditions in vitro

As a non-immune function, immunoproteasome also preserves protein homeostasis during cellular stressed condition induced by inflammatory cytokines (Seifert et al., 2010; van Deventer & Neefjes, 2010). The role of immunoproteasome subunits in oxidative and ubiquitinated protein clearance was thus investigated. First, myoblast were incubated with IFN-γ for different time points to observe the IFN-γ induced kinetics of oxidized and ubiquitinated proteins. Immunoblot detection of oxidized and ubiquitinated proteins revealed no change in their level under the influence of IFN-γ until 48 h (Figure A and C). In addition, the function of immunoproteasome in maintaining oxidized and ubiquitinated protein during inflammatory condition was studied by treating cells with LU001i and LU015i. As a result, treatment with these inhibitors did not induce accumulation of oxidized or ubiquitinated proteins in the myoblast.

Furthermore, a cellular stress response marker, phospho-p38, was also determined by western blot and showed no difference in its level after inhibiting immunoproteasome (Figure 23E). Since increase cytokine expression could represent a response mechanism to endoplasmic reticulum stress, mRNA expression of spliced form of xbp1 was analyzed by RT-PCR. As a result, no changes in mRNA expression of spliced xbp1 was observed in LU001i or LU015i treated myoblast under inflammatory conditions (Figure 23F).

Together, these data suggest that immunoproteasome subunits are not involved in maintaining the oxidized and ubiquitinated protein in primary myoblast under the inflammatory condition and thus the increased expression of myokines was not the result of oxidative stress response or unfolded protein response.

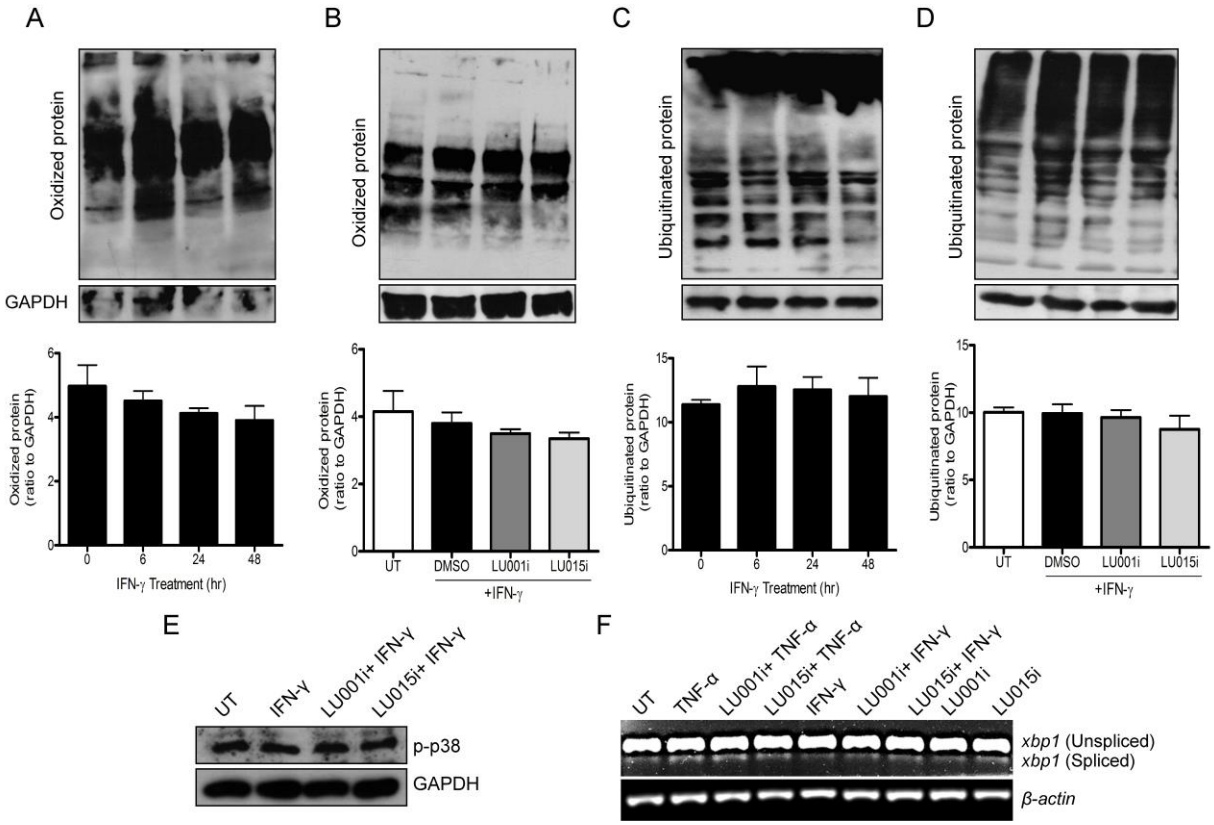


Figure 25: IFN-γ alone or prior treatment with LU001i or LU015i does not stress myoblasts with accumulation of ubiquitinated or oxidized proteins in primary myoblast. Myoblasts were induced with IFN-γ for 0, 6, 24, 48 h and oxidized protein (A) and ubiquitinated protein (C) were detected by oxyblot (as discussed in methods section) and western blot, respectively. Cells were treated with 5 µM of LU001i or LU015i for 2 h before induction with IFN-γ for 24 h and oxidized protein (B) and ubiquitinated protein (D) were detected. Graphical presentation of the means ± SEM of densitometric evaluation of respective immunoblots (top), demonstrating no significant accumulation of polyubiquitin conjugates or oxidized protein after IFN-γ stimulation with or without inhibitors (Student's t test). (E) Western blot detection of phospho-p38 (p-p38) after treatment with IFN-γ with or without inhibitors showed no difference in the activation of p38, indicating no stressed condition after the treatment.

7. Discussion

IIMs is rare muscle disease with infiltration of inflammatory immune cells in the muscle fibers. Although based on clinical and histological evidence they are categorized into distinct subgroups, they share many common immunological processes, including cytokines expression and antigen presentation. Part of these processes also includes the expression of proteasome subunits, which has been shown previously to be increased in IIMs at mRNA level, suggesting a possibility of its involvement in the diseases pathogenesis. This research work provides first evidence for an involvement of proteasome subunits in the inflammatory condition of IIMs, including sIBM, IMNM and DM. In contrast to healthy controls, immunoproteasome subunits are highly upregulated in both muscles fibers and infiltrating cells of patients with sIBM, IMNM and DM, whereas the expression of constitutive subunits was not significantly altered in the different diseased entity. In sIBM and IMNM, the expression of immunoproteasome subunits is predominantly localized in infiltrated muscle fibers. However, in DM the increased expression of immunoproteasome was predominant in the perifascicular area. In all of the cases, the expression of immunoproteasome subunits co-localized with MHC-I expressing fibers. Under inflammatory conditions, purified human primary myoblast showed an increased expression of immunoproteasome subunits. Furthermore, for the first time the function of immunoproteasome subunits in inflammatory conditions was studied by two methods: using a selective inhibitor of β1i or β5i and next, knocking down the expression of β5i. Both approaches showed that inhibition of β5i increased myokine production under inflammatory conditions. In addition, suppression of immunoproteasome subunits activity caused a decrease in MHC-I expression. Importantly, these data provide evidence that inhibition of immunoproteasome function can aggravate the local inflammatory response, which would be in agreement with known PRAAS (as reviewed in (Brehm & Krüger, 2015)).

Taken together, the results of this research work provide evidence immunoproteasome (a) may serve as a histological marker for inflammatory muscle disease, (b) may play a relevant role during pathogenesis of IIMs and (c) may or may not serve as a target for the therapy of IIMs.

Our group has previously showed that immunoproteasome subunits β1i and β5i are significantly upregulated in muscle biopsies of IIMs, including PM, DM, and overlap myositis at mRNA level (Ghannam et al., 2014). The present study has confirmed and extended the previous work at protein level by western blot and immunofluorescence. In this context, we demonstrated that β1i and β5i is overexpressed in muscle fibers of sIBM, IMNM and DM. Since the mRNA expression of proteasome subunits β2 and its immune subunits β2i was not found to be significantly different between healthy controls and diseased entities in our previous study, these subunits were not investigated in the present study.

Previous observation from other groups have shown an increased expression of all proteasome catalytic subunits β1, β5, β1i and β5i in sIBM at protein level (Ferrer et al., 2004; Fratta et al., 2005). Although the expression of β1i and β5i in sIBM was in line to these observation, in contrast to Fratta *et al.*, 2005, our densitometry analysis showed comparable expression of constitutive subunits β1 and β5 in sIBM and healthy controls muscle biopsies. 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 muscle fibers of non-inflammatory myopathies, such as LGMD, congenital myopathy and neurogenic disorder, the expression of immunoproteasome subunits in the muscle fibers may serve as a potential biomarker for IIMs.

Proteasome dysfunction induce muscle growth defect and protein aggregation in mice (Kitajima et al., 2014). Fratta and colleagues have reported decreased proteasome activity to explain the reason behind protein aggregation in sIBM (Fratta et al., 2005). They showed that muscle fibers overexpressing amyloid-β precursor protein, which is found to be aggregated in muscle fibers of sIBM, displayed reduced proteasome activities. In contrast, Ferrer and colleagues have shown increase in proteasome activity in sIBM muscle biopsies (Ferrer et al., 2004). Hence, the clarification of the activity of proteasomes in these diseased entities would be interesting. Here, we found that CT-L activity of proteasome was significantly higher in sIBM and DM compared to healthy. However, no changes was observed in C-L activity of proteasome among the disease condition. This suggest that the protein aggregation in sIBM is not caused by defective proteasome activity. As amyloid-β is a substrate of autophagy in sIBM (Lünemann et al.,

2007) the aggregation formed in sIBM might be the result of autophagy dysfunction (Askanas et al., 2012). Of note, we found less cellular infiltration in IMNM than in sIBM and DM. In line to this observation, significantly lower expression of β1i and β5i as well as a reduced CT-L activity of proteasome was observed in IMNM. This indicates that expression of immunoproteasome subunits and CT-L activity in muscle fibers might depends on the extent of cellular infiltration.

Increased proteasome activity has also been associated with muscle atrophy (Levine et al., 2011) and muscle wasting in the cancer patients (Bossola et al., 2003). Moreover, increased serum CK level is a key indication of muscle damage during the pathogenesis of IIMs. Here, muscle expression of immunoproteasome subunits β1i and β5i strongly correlated with serum CK level only in sIBM and DM but not in IMNM. This further supports the assumption that compared to sIBM and DM, muscle necrosis in IMNM might possibly be a non-immune mechanism, such as autophagy (Henriques-Pons & Nagaraju, 2009) but not a cytotoxic effect of infiltrating inflammatory. However, the possibilities of muscle protein breakdown leading towards atrophy by increased proteasome activity cannot be ignored in all diseased entities as a rat model treated with total proteasome activity inhibitor, Bortezomib, showed a reduced denervation-induced muscle atrophy (Beehler, Sleph, Benmassaoud, & Grover, 2006) and accelerated muscle regeneration in experimental autoimmune myositis model (Rayavarapu, Coley, Van der Meulen, et al., 2013).

The presence of inflammatory infiltrates in the muscle fibers is a hallmark of inflammatory myopathies (Vattemi et al., 2014). We observed upregulation of β1i and β5i also in muscle infiltrating cells. We therefore used cellular markers to identify infiltrating cells that express immunoproteasomes subunits in muscle biopsies. We analyzed for CD8+ T-cell and CD68+ macrophages and found that they express immunoproteasome in the muscle biopsies of all entities. CD8+ T-cells heterogeneity in the muscle lesion of sIBM has been reported in several studies (Bender, Behrens, Engel, & Hohlfeld, 1998; Fyhr, Moslemi, Lindberg, & Oldfors, 1998; M. F. G. van der Meulen et al., 2002). We were able report the heterogeneity of CD8+ T-cells based on immunoproteasome expression in sIBM muscle biopsies as about 55-60 % of CD8+ T-cells showed strong staining of β1i or β5i in the muscle biopsies. Although less CD8+ T-cells were detected in IMNM compared to sIBM,

they were all positive for β1i or β5i, indicating a unique phenotype of CD8+ T-cells in IMNM based on immunoproteasome subunits expression. Lack of immunoproteasome in T-cells was associated with their proliferative and inflammatory capacity in different mice model. CD8+ T-cells that lacks immunoproteasome have shown to hyper proliferate in response to polyclonal mitogen (Caudill et al., 2006). It could be that immunoproteasome-lacking CD8+ T-cells in sIBM is responsible for maintaining their number in the inflamed muscle environment for effective response, which might be later cytotoxic after adapting the immunoproteasome expression. In contrast, CD4+ T-cells lacking immunoproteasome had enhanced inflammatory potential in a mice model of colitis (Rasid et al., 2014). Thus, it would be interesting to further characterize CD8+ T-cells in sIBM and INMN and study the functional response between these CD8+ T-cells clones. In contrast to CD8+ T-cells in sIBM, we found that 100 % of the CD68+ macrophages expressed β1i and β5i in all disease groups. As professional antigen presenting cells, this finding was expected since they require immunoproteasome for the efficient degradation of peptide for antigen presentation (D'Souza et al., 2013; Naujokat et al., 2007). In addition, lipopolysaccharide induced over-expression of immunoproteasome subunit β5i in antigen presenting cells has been demonstrated to require the auto-reactivity of T-cells (Eleftheriadis, Pissas, Antoniadi, Liakopoulos, & Stefanidis, 2013). Therefore, in the pathogenesis of IIMs the expression of immunoproteasome in all CD68+ macrophages could be relevant for the maintenance of T- cell auto-reactivity in the pathogenesis of myositis.

Constitutive proteasome are exclusively express in non-immune cells. However, they are replaced by respective immunoproteasome under the influence of pro-inflammatory cytokines (McCarthy & Weinberg, 2015). The existing data from the muscle biopsies and serological analysis provided evidence that pro-inflammatory cytokines, including TNF-α, IFN-α, IFN-β, IFN-γ and IL-1β are highly relevant in inflammatory myopathies (Moran & Mastaglia, 2014). Although the expression of immunoproteasome has been assumed to be influenced by these cytokines in myositis, no data has proved this. In the present study, *in vitro* data show for the first time that human myoblast have the capability to express β1i or β5i after exposure to the pro-inflammatory cytokines TNF- α, IFN-α, IFN-β or IFN-γ. In contrast, IL-1β did not influence the expression. Among all cytokines,

especially IFN-γ had a pronounced effect on the expression of immunoproteasome. This effect in myoblast is in line to other non-immune cell types such as parenchymal cells and hepatocytes (Keller et al., 2015; Shin et al.). 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 (Ivanidze et al., 2011). Our previous study in transcriptome data of myositis suggest that IFN-γ correlated with the expression of β1i or β5i (Ghannam et al., 2014). In addition, recent study by Leoll et al. (Loell et al., 2016) 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. Since a presence or absence of proinflammatory cytokines, particularly IFN-γ 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 sIBM and IMNM. In a subsequent study, it would be interesting to further prove this hypothesis by inhibiting IFN-γ signaling pathway in the myoblast isolated from patients to observe its effect in the expression of β1i and β5i.

Our previous transcriptome study found no correlation of β1i or β5i expression with IFN-α expression in the muscle biopsies of IIMs patients (Ghannam et al., 2014). In contrast to this finding, out *in vitro* study in myoblast indicates that both type I interferons, IFN-α and IFN-β are potent inducers of immunoproteasome subunits in muscle. Since type 1 interferons are specifically elevated in DM muscle as compared to other groups of IIMs (Baechler, Bilgic, & Reed, 2011; Greenberg, 2010), the strong expression of immunoproteasome subunits in DM is relevant. Despite the fact that DM is a clinical heterogeneous disease (Peng et al., 2016; Wong et al., 2012), the reason for a missing upregulation of immunoproteasome subunits in some cases of DM muscle biopsies cannot be explained. It would be interesting to identify if immunoproteasome expression can be used as a marker to classify specific groups of DM with special clinical features or response to therapy.

Replacement of β1 with β1i also alters the C-L activity of proteasome to CT-L activity (Tanaka, 1998). Here, IFN-γ was capable to downregulate the expression of constitutive subunit β1. Western blot image show that TNF-α could not replace β1 with β1i as done by IFN-γ. This result was further confirmed by C-L like activity of proteasome which is increased in the myoblast stimulated with TNF-α as compared to IFN-γ.

Healthy muscle fiber do not over-express MHC-I molecules in contrast to the situation in IIM (Ferrer et al., 2004; Graça & Kouyoumdjian, 2015). Several studies in mice models have shown that induction of myositis was accompanied by conditional overexpression of MHC-I in skeletal muscle (Li et al., 2009; K. Nagaraju et al., 2000). Although it is well known that immunoproteasomes play a fundamental role in MHC-I antigen presentation, a direct relation between MHC-I expression and immunoproteasome expression and activity has not been studied so far in skeletal muscle. 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 immunoproteasome expression was co-localized with the MHC-I positive fiber. Importantly, fibers stained with immunoproteasomes showed brighter staining for MHC-I, suggesting a role of immunoproteasome in the expression of MHC-I. To further confirm this observation we used primary myoblast model *in vitro*. As human myoblast express low basal level of MHC-I in the normal culture conditions, the expression on the cell surface highly increased under the influence of pro-inflammatory cytokines, such as TNF-α or IFN-γ after 48 h. This is in line with previous studies, showing myoblast as a conditional antigen presenter (Dorothea Michaelis, 1993; N Goebels et al., 1992; Nagaraju et al., 1998). Here, we showed that treatment with LU001i or LU015i, a selective inhibitor of β1i or β5i respectively, in a dose dependent manner reduced IFN-γ induced surface expression of MHC-I in primary myoblast without affecting total intracellular MHC-I expression. Splenocytes derived from β5i-deficient mice or mice treated with β5i inhibitor showed also a reduction in surface MHC-I expression on lymphocytes (Fehling et al., 1994; Muchamuel et al., 2009). Reduced expression of MHC-I molecules in these splenocytes were linked with the insufficient production of repertoire of MHC-I restricted peptides that are generated by β5i activity. Similarly, reduced MHC-I expression in myoblast might be due to reduced peptide supply and inefficient conformation of MHC-I after treatment with LU001i or LU015i. In subsequent studies, it

would be interesting to clarify the effect of LU001i or LU015i on myoblast derived peptides under the influence of IFN-γ in comparison to muscle derived autoantigens in IIMs. In the context of myositis, by usingβ5i inhibitors, we propose to reduce the antigen presentation by the muscle fibers, as a result of which the T-cells auto-reactivity could be suppressed. Beside antigen presentation, immunoproteasome inhibitors has also been studied to have anti-inflammatory properties. Although non-selective inhibition of proteasome, with MG132 and Bortezomib, also has shown to have anti-inflammatory effect (Inoue et al., 2009; Sinn et al., 2007), they target both constitutive and immunosubunits and therefore effect basic proteasome function, which might be essential for cellular homeostasis. Recent investigation demonstrated that β5i activity is involved in the induction of several pro-inflammatory cytokines, including IL-6, IL-1β and TNF-α and plays a role in the progression of experimental arthritis as well as colitis. Production of cytokines in LPS induced splenocytes was blocked after treatment with a β5i specific inhibitor (Basler, Dajee, Moll, Groettrup, & Kirk, 2010; Muchamuel et al., 2009). Although several animal models have shown a role of β5i in cytokine productions, none of these studies did investigate the effect on non-immune cells. In this context, studies on mice showed that inhibition of the immunoproteasome intensifies the pathogenesis of systemic *Candida albicans* infection with increased neutrophils infiltration in kidneys and brains (Mundt, Basler, Buerger, Engler, & Groettrup, 2016). Furthermore, β5i deficiency results in severe enterovirus myocarditis in mice (Opitz et al., 2011). Increased serological concentration of cytokines such as IL-6 and TNF-α has been measured in PRAAS with mutations e.g. in the β5i immunoproteasome subunit (Arima et al., 2011; Kitamura et al., 2011). In inflammatory muscle disorders, muscle cells have been described as relevant source of several cytokines and chemokines (Kim et al., 2014; Podbregar, Lainscak, Prelovsek, & Mars, 2013). Therefore, in the present study, we investigate the role and input of immunoproteasomes by using two approaches: chemical inhibitors and gene knockdown of immunoproteasome function. As a result, both approaches enhanced the TNF-α and IFN-γ mediated expression of different myokines *in vitro*.

Recently, the inflammatory condition of skeletal muscle exposed to β5i specific inhibitor in muscular dystrophic mice has been studied (Farini et al., 2016). *In vivo* data showed that β5i specific inhibitor treatment reduced pro-inflammatory cytokines production and

the number of effector memory CD8+ T-cell in dystrophic muscle (Farini et al., 2016). In contrast to this study, where the inhibitor blocked the activity of β5i originated from both muscle and immune cells, our *in vitro* study only focused on the muscle mediated response upon β5i inhibition during inflammatory condition. In addition, in line with our *in vitro* result, the author also detected reduction in surface MHC-I expression in the muscles fibers, which might restrict antigen presentation to naive T-cell and transition towards CD8+ T-cells effector function, as reviewed in (Westermann, Ehlers, Exton, Kaiser, & Bode, 2001). Our *in vitro* analysis was done after 24 h exposure to the inhibitor, as longer exposure to the indicated concentration of inhibitor was toxic for the cells. It would be interesting also to measure the cytokine expression in these dystrophic muscle one week after treatment with inhibitor. Therefore, we acknowledge that such a study in a mice model of myositis may disclose different effects of β5i inhibitor on disease conditions. But based on our *in vitro* data, we can postulate that immunoproteasome inhibition might results in myokine release during inflammatory condition of muscle which attracts immune cells towards muscle.

IIMs are diseases governed by T helper 1 (Th1) type inflammatory response. Under the influence of TNF-α and IFN-γ, myoblast can be involved in the secretion of Th1-derivied chemokines, which is mediated by phosphorylation of STAT1 (Crescioli et al., 2012). This observation was further verified by the detection of increased phospho-STAT1 level in TNF-α or IFN-γ stimulated myoblast. Specific inhibition of β5i has shown to reduce the phosphorylation of STAT1 in naive CD4+ T-cells. In contrast to this observation, upon treatment with LU015i, phospho-STAT1 level is myoblast was increased, supporting the fact that human myoblast can also contribute towards to Th1 immune response and can activate an inflammatory loop as a result of immunoproteasome impairment during inflammatory condition.

Apart from immune function, immunoproteasome is also known to maintain protein homeostasis. Seifert *et al*. have revealed that immunoproteasome, compared to constitutive proteasome has greater ability to prevent IFN-γ induce harmful accumulation of ubiquitinated or oxidized protein (Seifert et al., 2010). In addition, increased level of ubiquitinated and oxidized protein accumulation has been demonstrated in heart and brain of immunoproteasome knockout mouse (Q. Ding, S. Martin, E. Dimayuga, A. J.

Bruce-Keller, & J. N. Keller, 2006; Opitz et al., 2011). In contrast, these findings were challenged by Nathan *et al*. who claimed that immunoproteasome did not showed increased ability to clear ubiquitinated protein accumulation (Nathan et al., 2013). In line to Nathan *et al.*, we were also not able to detect a significant changes in ubiquitinated or oxidized proteins in myoblast induced with IFN-γ until 48 h. Furthermore, we did not observe the accumulation of ubiquitinated or oxidized proteins in the myoblast treated with LU001i or LU05i. Although this effect might changes between the cell types, immunoproteasome does not seems to have increased ability to get rid of ubiquitinated or oxidized proteins in inflamed myoblast.

Release of cytokines by cells might be a response mechanism of stress condition e.g. of the ER (Baban, Liu, & Mozaffari, 2013; Carta et al., 2015). At present, upon treatment with immunoproteasome inhibitor, no increased level of cell stress marker, phospho-p38 protein, and ER stress marker, spliced form of *XBP1* was observed, suggesting that the increase in cytokines did not caused a stress response mechanism. Therefore, the mechanism behind increased cytokines expression in β5i defective myoblast have to be clarified by subsequent studies. Taken together, our results suggest that β5i plays a role in stabilizing the expression of cytokines in myoblast under inflammatory condition, which could be related to either immune mechanism or protein homeostasis.

8. Conclusion

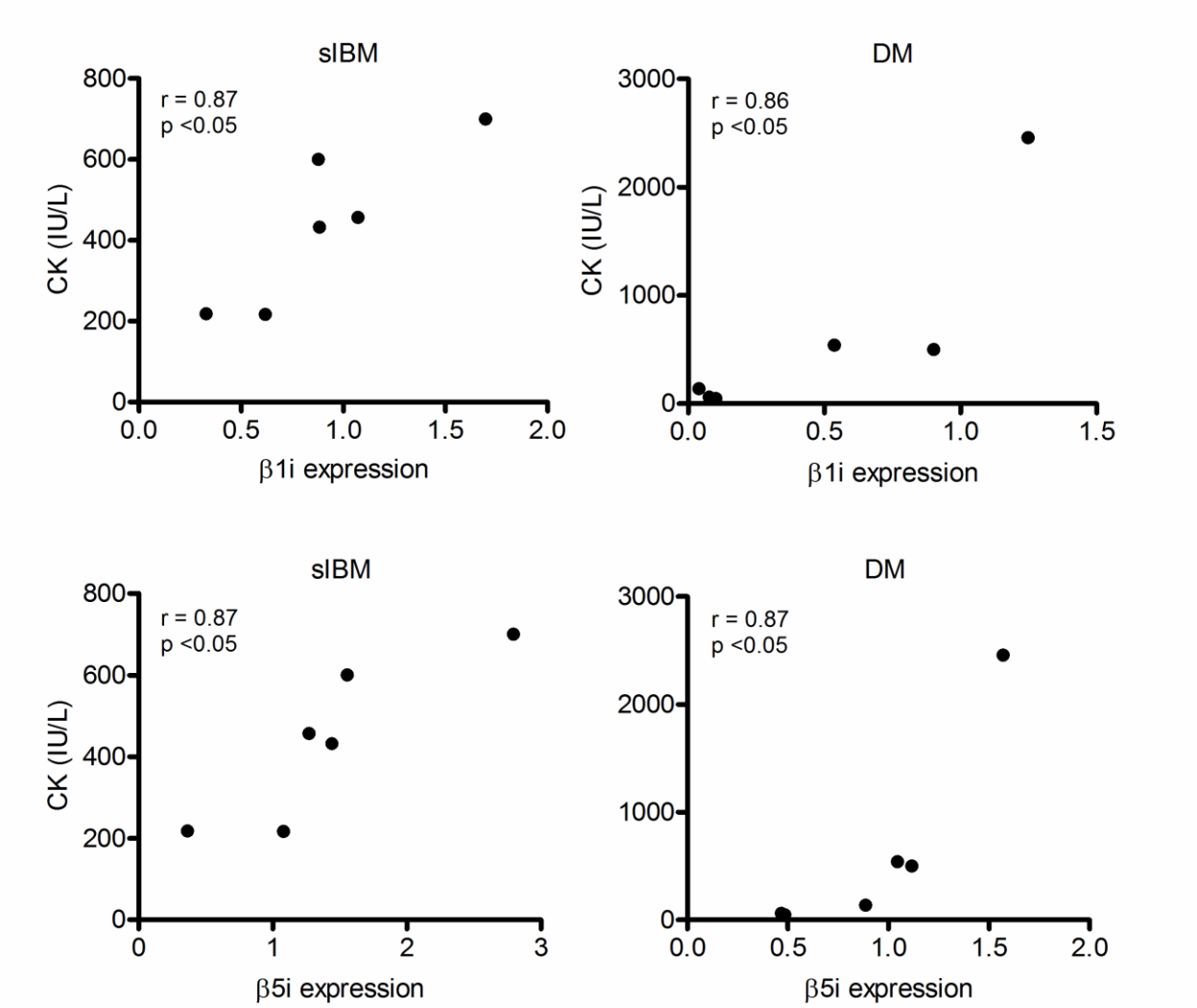
The results on expression and function of proteasome provide evidence that this proteolytic complex plays a role in the pathogenesis of IIMs, including sIBM, IMNM and DM. Although these diseases have distinct unknown etiology and pathological conditions, they might share an involvement of immunoproteasome in the molecular mechanism of antigen presentation and myokine production.

Since immunoproteasome plays a significant role in surface expression of MHC-I molecule in the muscle fibers, the immunoproteasome could be a potential therapeutic target to reduce the cytotoxic effect of T-cells that are incited by antigen bound to MHC-I molecule of muscle fibers.

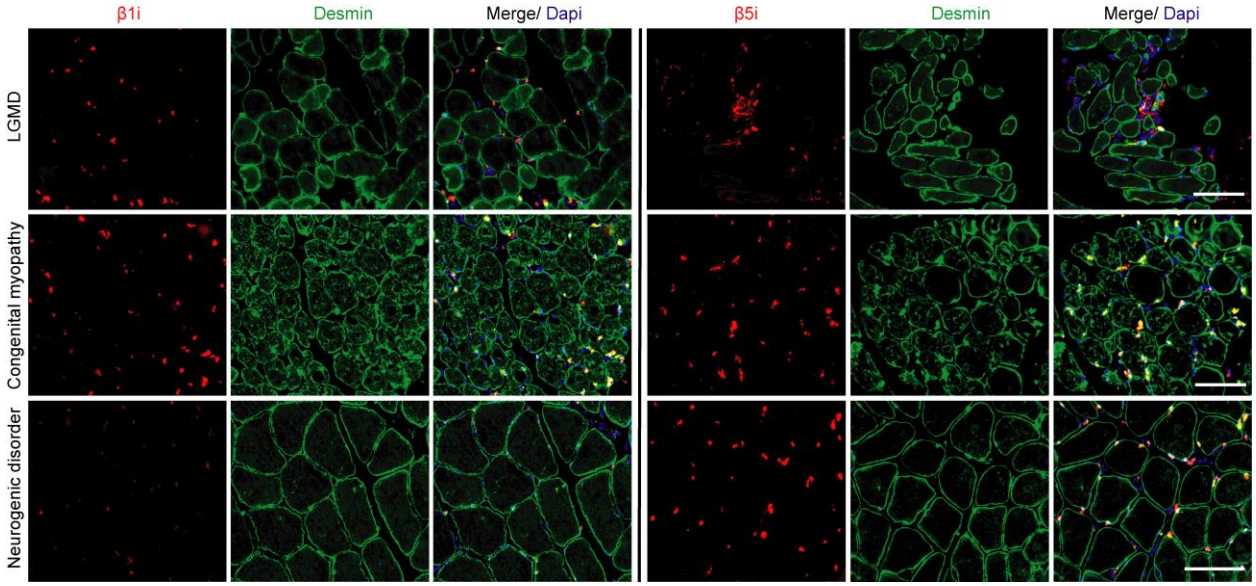
However, as non-immune function, the immunoproteasome is also known to serve as a regulatory machinery. The increased expression of immunoproteasome subunits in muscle fibers during the inflammatory condition might also be involved in degradation of substrates including mediators of the inflammatory response. Thus, expression of the immunoproteasome is also important to maintain the myokines homeostasis and myokines mediated attraction of immune cells in muscles fibers.

Taken together, the imbalance between these functions may have an impact on the disease phenotype or severity in IIMs. Therefore, our results do not support the approach to use immunoproteasome inhibitors as a monotherapy in IIMs but provides a new insight into mechanism underlying the pathogenesis of IIMs.

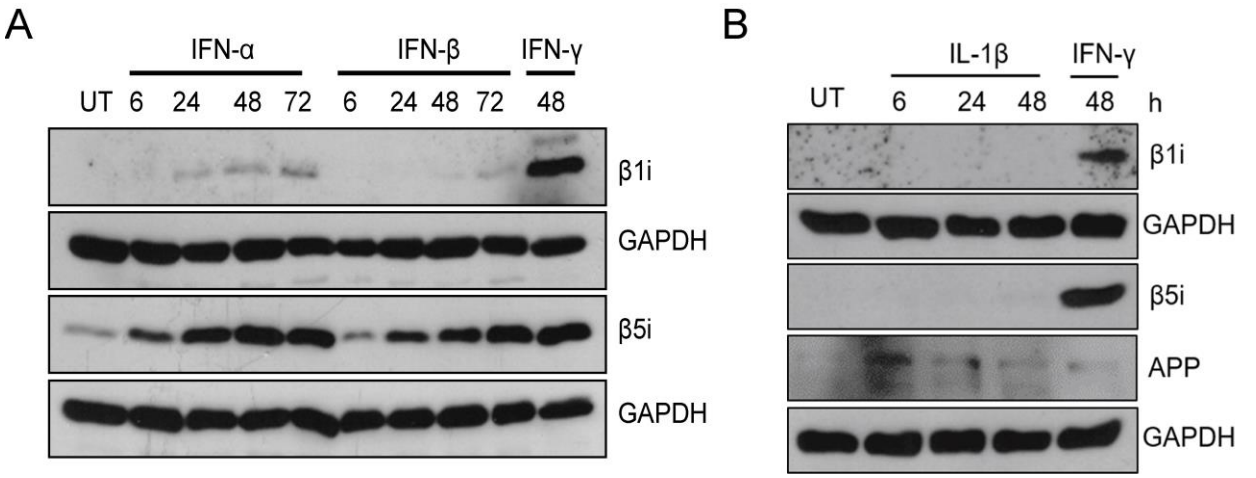
9. Supplementary Figures



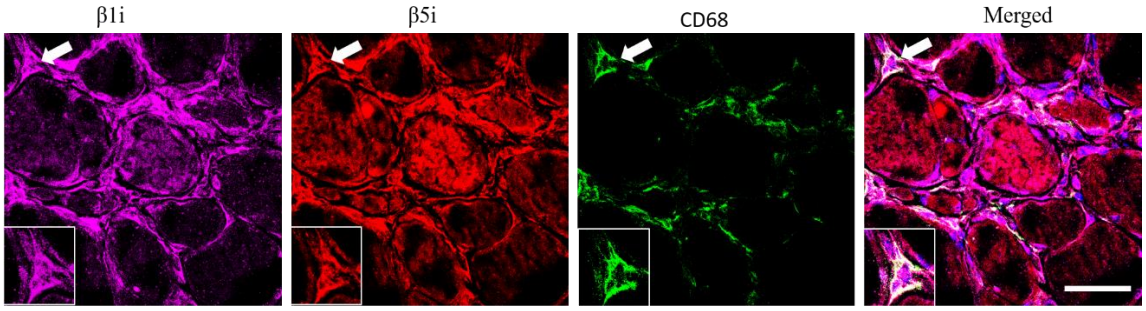
Supplementary Figure 1: β1i and β5i expression in muscle biopsies shows strong correlation with serum CK level. Graph shows correlation 6 out of 9 patients with sIBM or DM. r = Pearson correlation coefficient. IU/L= International unit per liter.



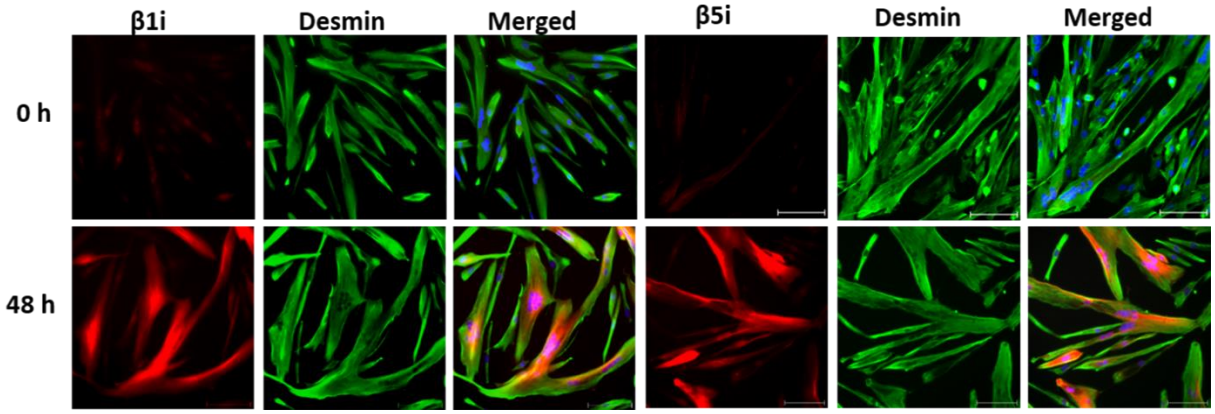
Supplementary Figure 2: Immunoproteasome subunits β1i and β5i expression in IIMs myofibers. (A) Fluorescent double-labeling immunohistochemistry in representative muscle biopsy specimens of LGMD 2I (n=1), Congenital myopathy (n=1) and neurogenic disorder (n=1), showing that myofibers are negatively stained for β1i (red, Cy3) (left panel) and β5i (red, Cy3) (right panel). Sections are co-stained by desmin (green, Alexa 488) to represent myofibers. Nuclei staining as DAPI (blue). Scale bar = 100 μm.



Supplementary Figure 3: IFN-α or IFN-β, but not IL-1β induces the expression of the immunoproteasome subunits in primary myoblasts. Myoblasts were treated with (A) IFN-α or IFN-β (B) IL-1β for indicated time points and the expression of β1i or β5i was analyzed by western blot. Both IFN-α and IFN-β showed induction of β1i or β5i but has comparatively less ability to induce the immunoproteasome than with IFN-γ for 48 h. However, IL-1β did not induce β1i or β5i, while it induced amyloid precursor protein (APP). GAPDH shows equal amount of sample loaded in each lane. Image shows results from two independent experiments. UT= untreated.



Supplementary Figure 4: Immunofluorescent image shows triple staining of sIBM muscle biopsy against CD68+ macrophages, β1i and β5i subunits of immunoproteasome. White arrow represent a population of CD68+ macrophages that co-express both β1i and β5i subunits. CD68 (green, Alexa 488), β1i (purple, Cy3) and β5i (red, Alexa 647). Embedded white box shows area pointed by white arrow in magnified form. Nucleus counterstained with DAPI. Scale bar is 50 µm.



Supplementary Figure 5: Immunofluorescent image of differentiated myotube showing cytoplasmic and nucleus localization of β1i and β5i. Myoblast were allowed to form differentiated myotube in differentiation media for 4 days, followed by the treatment with IFN-γ for 48 h, myotubes were stained against β1i and β5i (in red). Myotube was identified by desmin staining (in green). Nucleus counterstained with DAPI.

10. Supplementary Table 1

List of patients and their clinical information

Patients	Age	Sex	Diagnosis	CK level (U/L)	Paresis	Complain	Therapy
1	26	m	Healthy	203	-	-	-
2	50	f	Healthy	nr	-	-	-
3	62	m	Healthy	180	-	-	-
4	50	m	Healthy	72	-	-	-
5	73	f	Healthy	64	-	-	-
6	56	f	Healthy	92	-	-	-
7	76	m	sIBM	921	legs	Muscle atrophy	no
8	62	f	sIBM	900	arms	Muscle atrophy	no
9	69	f	sIBM	218	arms/legs	Muscle atrophy	no
10	73	m	sIBM	217	arms/legs	nr	40 mg * Prednisolon
11	62	m	sIBM	nr	arms/legs	Muscle atrophy	no
12	70	m	sIBM	457	arms/legs	Muscle atrophy	no
13	67	m	sIBM	350	nr	Muscle weakness and paresthesia	no
14	69	m	sIBM	600	arms/legs	Muscle weakness	no
15	78	m	sIBM	432	arms/legs	Muscle weakness	no
16	63	m	sIBM	338	legs	Muscle weakness	no
17	74	f	sIBM	nr	legs	Muscle weakness, muscle pain	no
18	66	f	sIBM	700	arms/legs	Chronic muscle pain	no
19	66	f	IMNM	5495	arms/legs	Muscle pain	no
20	29	f	IMNM	22000	arms/legs	Muscle pain/atrophy	no
21	46	f	IMNM	8928	arms/legs	Nr	no
22	59	f	IMNM	1300	arms/legs	Muscle atrophy	no
23	55	f	IMNM	1072	nr	Muscle pain	no

24	77	m	IMNM	3865 6	legs	Muscle pain	no
25	51	m	IMNM	nr	nr	Muscle pain	no
26	79	m	IMNM	nr	nr	Hepatocellular Carcinoma, Hemochromatosis, Muscle weakness	no
27	80	m	IMNM	19.1 8	arms/leg s	Muscle weakness	no
28	57	f	IMNM	548	nr	Muscle pain	no
29	23	f	IMNM	nr	arms/leg s	Muscle weakness, hypoesthesia and disturbance of pain perception of legs	no
30	72	f	IMNM	500	arms/leg s	Muscle weakness and atrophy	no
31	73	m	DM	138	arms/leg s	Muscle atrophy	no
32	67	f	DM	6412	arms/leg s	Muscle pain	no
33	60	m	DM	541	arms	Muscle pain	no
34	43	m	DM	1500 0	arms/leg s	Muscle pain	no
35	77	m	DM	2458	arms	Muscle pain	no
36	41	f	DM	6000	arms/leg s	Muscle pain	no
37	86	f	DM	nr	arms/leg s	Muscle weakness, dysphagia and erythema	no
38	49	m	DM	nr	legs	Muscle pain	no
39	74	f	DM	1700	arms	Erythema, dyspne and muscle weakness	no
40	74	m	DM	600	arms/leg s	Muscle weakness and pain	no
41	60	f	DM	2400	arms/leg s	Muscle weakness, erythema, dyspnea and weak reflexes	no
42	20	f	DM	49	arms/leg s	Muscle weakness	no
43	3	m	LGMD 2I	3740	nr	Proximal muscle weakness	no
44	4	f	Congenital myopathy	nr	arms/leg s	Muscle weakness	no

45	58	m	Neurogenic disorder	nr	nr	Suspected hereditary motor and sensory polyneuropathies	no

sIBM: Sporadic inclusion body myositis, IMNM: Immune mediated necrotizing myopathy, DM: dermatomyositis, LGMD: Limb girdle muscular dystrophy, m: male, f: female, nr: not reported, CK: Creatine kinase, * the drug was started 2 days before biopsies.

11. Publications

11.1. Peer reviewed publications

1. **Salyan Bhattarai**, Khetam Ghannam, Sabine Krause, Olivier Benveniste, Andreas Marg, Gerjan de Bruin, Bo-Tao Xin, Hermen S. Overkleeft, Simone Spuler, Werner Stenzel, Eugen Feist. The immunoproteasomes are key to regulate myokines and MHC class I expression in idiopathic inflammatory myopathies. J Autoimmunity. 2016; 75: 118–129. doi:10.1016/j.jaut.2016.08.004

2. Khetam Ghannam, Lorena Martinez-Gamboa, Lydia Spengler, Sabine Krause, Biljana Smiljanovic, Marc Bonin, **Salyan Bhattarai**, Andreas Grützkau, Gerd-R. Burmester, Thomas Häupl, Eugen Feist. Upregulation of Immunoproteasome Subunits in Myositis Indicates Active Inflammation with Involvement of Antigen Presenting Cells, CD8 T-Cells and IFNγ. PLoS ONE 9(8): e104048. doi:10.1371/journal.pone.0104048

3. Khetam Ghannam, Lorena Martinez Gamboa, Marie-Kristin Fettke, Sabine Krause, Thomas Häupl, **Salyan Bhattarai**, Gerd-Rüdiger Burmester, Eugen Feist. Quantification of transcripts for immunoproteasome subunits PSMB8 and PSMB9 discriminates inflammatory from non-inflammatory myopathies. (submitted)

11.2. Conference (abstract) publication

1. **Salyan Bhattarai**, Khetam Ghannam, Sabine Krause, Olivier Benveniste, Andreas Marg, Gerjan de Bruin, Bo-Tao Xin, Herman S Overkleeft, Simone Spuler, Werner Stenzel and Eugen Feist. The immunoproteasomes are essential for maintaining myokine production and MHC Class I expression in idiopathic inflammatory myopathies. ACR/ARMP Annual Meeting. 11th-16th November, 2013, Washington DC, USA.

2. **Salyan Bhattarai**, Khetam Ghannam, Werner Stenzel, Lorena Martinez Gamboa, Simone Spuler, Olivier Benveniste, Eugen Feist. Skeletal muscle fibers in myositis actively upregulate immunoproteasome subunits. European League Against Rheumatism (EULAR) Congress. 8th-11th June, 2016, London, UK.

3. **Salyan Bhattarai**, Khetam Ghannam, Werner Stenzel, Lorena Martinez Gamboa, Simone Spuler, Olivier Benveniste, Eugen Feist. Differential expression and functional activity of proteasomes in idiopathic inflammatory myopathies. 44th European Muscle Conference. 21st – 25th September, 2015, Warsaw, Poland

4. **Salyan Bhattarai**, Khetam Ghannam, Werner Stenzel, Lorena Martinez Gamboa, Simone Spuler, Olivier Benveniste, Eugen Feist. Proteasome immunosubunits in the pathogenesis of myopathies. 60th Annual Meeting of the German Society for Neuropathology and Neuroanatomy (DGNN). 26th – 28th August, 2015, Berlin, Germany.

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