Journal of Autoimmunity 75 (2016) 118–129

Contents lists available at ScienceDirect

Journal of Autoimmunity

journal homepage: www.elsevier.com/locate/jautimm

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

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ARTICLE INFO

Article history:
Received 27 May 2016
Received in revised form
26 July 2016
Accepted 3 August 2016
Available online 10 August 2016

Keywords:
Idiopathic inflammatory myopathies
Proteasome
Immunoproteasome subunits
Antigen presentation
Myokine

ABSTRACT

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

1. Introduction

Idiopathic inflammatory myopathies (IIMs) are rare muscle diseases characterized by muscle weakness and specific inflammatory infiltrates in muscle. Based on distinct histopathological and clinical phenotypes they can be classified as polymyositis, sporadic inclusion body myositis (sIBM), dermatomyositis (DM) and immune-mediated necrotizing myopathies (IMNM) [1–3]. Upregulation of pro-inflammatory cytokines such as interferon (IFN)-γ, -α, -β, tumor necrosis factor (TNF)-α and IL-1, are common in IIMs [4–6]. With respect to histopathological features, infiltration of macrophages are present in all entities, while $CD8^+$ T-cells are mainly involved in PM and sIBM, and $CD4^+$ T-cells and B-cells in DM. The presence of rimmed vacuole structures in myofibers of sIBM [7], perifascicular atrophy in DM [8] and myo-phagocytosis in IMNM [1,6] are examples of their unique features.

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http://dx.doi.org/10.1016/j.jaut.2016.08.004
0896-8411/

Another important immunological feature of IIMs is the upregulation of MHC class I (MHC-I) and class II (MHC-II) [9] on the sarcolemma of myofibers, as integral part of the MHC-I/CD8+ T-cell, MHC-II/CD4+ T-cell complex [10,11]. Furthermore, continuous upregulation of MHC-I in myofibers can induce an endoplasmic reticulum stress response with the accumulation of misfolded glycoproteins and activation of NF-kB [12]. As a consequence, MHC-I/CD8+ T-cells complexes may form and maintain an auto-inflammatory response. The resulting inflammatory myokine (muscle-derived cytokines and chemokines) induction in muscle is believed to have supportive effect in the diseases prolongation and progression in IIM [13].

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

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

2. Materials and methods

2.1. Patients and samples

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

2.2. Cell culture

Human primary myoblasts were isolated as previously described [21]. In brief, after protease digestion of fresh muscle biopsies, cells were expanded at 37 °C in humidified atmosphere at 5% 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). Enriched myoblasts were prepared by immuno-magnetic cell sorting using anti-CD56/NCAM antibody coated magnetic beads (Miltenyi Biotech, Germany). Purity of the myoblasts preparation was confirmed by revealing more than 95% desmin-positive cells.

2.3. Cytokines and inhibitor treatment to cells

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

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

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