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Profiling Proteasome Activities in Peripheral Blood – A Novel Biomarker Approach

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Abstract

The proteasome system in the cell degrades the majority of intracellular proteins. The broad nature of its substrates makes proteasome activity crucial for many cellular functions, such as protein quality control, transcription, apoptosis, immune responses, cell signaling and differentiation. The proteasome system is thus an effective therapeutic target for malignant and non-malignant diseases. In this mini-review, we would like to highlight that proteasome function has also the potential to serve as a biomarker for disease severity and response to treatment. This notion is based on the observation that the six catalytic sites of the proteasome are distinctly altered in peripheral immune cells of patients with chronic inflammatory and autoimmune diseases. We here propose that proteasome activity can be profiled using minimally invasive peripheral blood samples to model severity, progression, and exacerbation of chronic inflammatory diseases. Moreover, the differential alteration of the catalytic activities of the proteasome points towards the existence of multiple catalytic forms of the 20S proteasome that potentially have distinct functions in immune cells. Understanding the role of the distinct immunoproteasome and intermediate proteasome complexes for immune cell function will pave the way for the application of site-specific immunoproteasome inhibitors to specifically target unwanted immune cell functions.

Keywords: Activity-based Probes, Biomarker, Clinical immunology, PBMC, Proteasome

Introduction

The proteasome system is the main protein disposal system in the cell for targeted protein degradation. It mediates the hydrolysis of more than 80% of all cellular proteins, thereby regulating their function in the cell. Degradation products are used to recycle amino acids. A small subset is loaded onto major histocompatibility (MHC) class I molecules to communicate the intracellular protein composition to the immune system [1,2]. Due to the broad nature of substrates, the proteasome is involved in many essential cellular functions, such as protein quality control, transcription, apoptosis, immune responses, cell signaling and differentiation [3,4]. This function makes the proteasome an effective therapeutic target for cancer treatment but also in non-malignant diseases such as cardiovascular, neurodegenerative, lung and autoimmune disorders [5-9].

The proteasome is a multi-subunit protein complex that stands either alone as a 20S catalytic core complex or as a super-complex when bound to one or the other regulator or activator. The most abundant of these super-complexes in the cell is the 26S proteasome, assembled from the 20S core and one or two 19S regulatory particles that attach to one or both ends of the 20S catalytic core, respectively. 26S proteasome complexes enable ubiquitin- and ATP-dependent protein degradation [10]. Other regulators of the proteasome include the proteasome activators (PA) PA28αβ, PA28γ, and

PA200, which all mediate ubiquitin-independent substrate degradation [11,12], together with the putative proteasome inhibitor PI31 and proteasome interacting proteins ECM29 or VCP/p97 [13]. Each of these regulators can potentially bind to the 20S core to form singly capped proteasome complexes or doubly capped ones with the potential to assembly into hybrid proteasome complexes that contain two different regulators attached to each end of the 20S proteasome. This gives rise to various different proteasome complexes with the potential to degrade specific proteins at distinct subcellular sites or to modulate proteasome function in a defined manner [13,14].

The 20S proteasome consists of a barrel-like hollow structure composed of four stacked heptameric rings (**Figure 1**); α-subunits form the outer rings (α1-α7), and β-subunits form the inner rings β1-β7 [3,15]. Three of the seven β-subunits of each inner ring are catalytically active and confer the proteolytic capacity of the 20S proteasome that cleave substrates via an N-terminal threonine hydrolase-based mechanism.

The three proteolytic β-subunits determine the species of the 20S core particle: depending on the cell type, cytokine milieu, or activation state of the cell, different β-subunits are expressed and assembled into mature 20S (**Figure 1**). The standard 20S proteasome is expressed in every cell type and integrates the β1-, β2-, and β5-subunits, which cleave after acidic, basic, or hydrophobic amino acids, respectively, and thus harbor chymotrypsin-like (CT-L), caspase-like (C-L) and trypsin-like (T-L) activities [16]. In immune cells, however, three different β-subunits are constitutively expressed [1]: low molecular mass protein (LMP) 2, multicatalytic endopeptidase complex-like 1 (MECL1), and LMP7 (also called β1i, β2i, and β5i, respectively). In non-immune cells, these three so-called immunosubunits can be induced by IFN-γ or tumor necrosis factor (TNF)-α signaling [17,18] to build the immunoproteasome or induced proteasome. In addition, several other stimuli have been identified that upregulate immunosubunits, including retinoic acid [19], nitric oxide [20], cytokines such as IL-4 [21], Toll-like receptor agonists, type I interferons [22], and recently the mammalian target of rapamycin (mTOR) [23,24]. Importantly, not all immunosubunits are always assembled together to form a full immunoproteasome, but various intermediate proteasomes bearing a mix of immune and standard catalytic subunits exist (**Figure 1**). Formation of these intermediate complexes might be restricted by the rules of cooperative incorporation of immuno-subunits into nascent and newly assembled proteasomes [25,26]. Accordingly, intermediate complexes containing only a single LMP7/β5i or the two LMP2/β1 and LMP7/β5 immunosubunits (**Figure 1, I & II**) were identified in various cells and tissues including liver, heart and pancreatic β-cells [27-31]. Other types of intermediate proteasomes (**Figure 1, III & IV**) were found in experimental systems such as in mice or cells containing a single or combined immunoproteasome knockout. These data suggest that such complexes can be forced to form and that they are stable [17,25,32-37]. Of note, recent data that quantified all proteasome catalytic subunits in human peripheral immune cells using Activity-based Probes (ABPs) also suggested a stoichiometry of intermediate proteasomes other than only single and double immunoproteasomes which conflicts

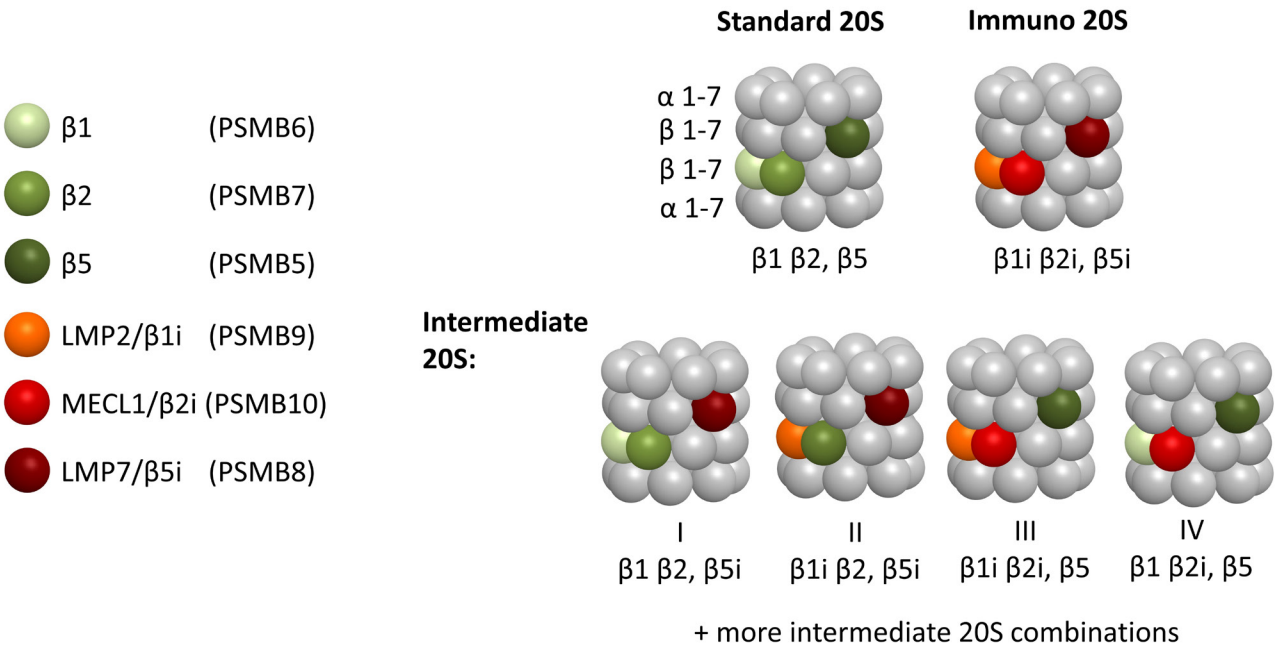

Figure 1. Diversity of 20S complexes. The 20S proteasome core consists of four heptameric rings, two outer α 1-7 rings, and two inner β 1-7 rings, each contain three catalytic subunits. 20S proteasome either contains only standard (β1, β2, β5) or immune (β1i, β2i, β5i) catalytic subunits, or mixed combinations that form intermediate 20S complexes. PSMB5-10 are the gene names for the respective subunits.

with the reported rules of cooperative incorporation [38,39]. To solve this ambiguity, it is crucial to develop new analytic methods for profiling the activity of each of the proteasome's six distinct catalytic active sites. This will allow for a comprehensive analysis of their cellular function and dysregulation in disease.

Proteasome Activity Profiling Methods

A set of methods have been developed in the last two decades to analyse the different proteasome activities and identify distinct proteasome complexes in a single sample parallel to the analysis of mRNA expression, Mass Spectrometry, ELISA or immunoblotting for protein subunit quantification. The Pros and Cons of each method are summarized in **Table 1**.

In-gel proteasome activity is used as an approach to dissect the different proteasome complexes, namely the 26S and the 20S proteasome complexes, upon separation on native gels according to their size. With this method, the separated proteasome complexes maintain their activity. They can be quantified by in-gel degradation of a fluorescently quenched substrate, e.g. for the chymotrypsin-like activity of the proteasome. Moreover, blotting of these gels then allows quantification of different proteasome complexes in the gel and to quantify the distinct activators (19S, PA28αβ, PA28γ, PA200, ECM29, VCP/p97) bound to active complexes. When determining both activity and abundance of the different complexes, their ratio determines the specific activity of the distinct proteasome complexes [39-41].

Fluorogenic or luminescent activity assays are *in vitro* assays to quantify the activity of each β-catalytic site, namely the chymotrypsin-like (CT-L), caspase-like (C-L) and trypsin-like (T-L) proteasome activity, using a specific fluorogenic or luminogenic substrate assay. Substrates consist of catalytic-site specific oligopeptides attached to a quenched reporter (either a fluorogenic or a luminogenic chemical group) which becomes activated upon cleavage. While several of these substrates do not discriminate between standard and immunoproteasomes, recent efforts have led to the development of more specific substrates that are preferentially degraded by either constitutive or immunosubunits [42-45].

Activity-based probes (ABPs) are another method to quantify the number of active sites in the cell. ABPs bind to the active-site threonine of the respective catalytic subunits and label them covalently with a fluorescent dye. The number of proteolytically active proteasome subunits is quantified by fluorescent detection using two distinct fluorescence

Table 1. Proteasome activity profiling methods - pros and cons.

Proteasome Activity Assay	Pros	Cons
In-gel proteasome activity Assay	- Quantification of activity in 20S and 26S complexes - Comparison of abundance of complexes between samples by immunoblotting - Determination of specific activity of each 20 or 26S complex: activity/abundance	- Long time - Large sample size needed - Not suitable for high throughput screening
Fluorogenic or luminescent activity assays	- Measures the activity of each specific β-catalytic active site in assembled proteasomes samples - High throughput activity quantification - Small sample size - Short time - Suitable for drug screening - Activity rates can be quantified	- Subcomplexes or subunit abundance cannot be quantified
Activity-based probes (ABPs)	- Quantifies the abundance of β-catalytic active sites in the cell. - The corresponding standard and immune proteasome active subunits can be quantified side-by-side. - Intermediate proteasomes can be detected - Intermediate time - Suitable for high throughput activity profiling of all active sites and the ratio of standard/immunosubunits	- Larger sample size needed - Subcomplexes cannot be quantified - Activity rate cannot be quantified

channels upon separation of labelled proteasome subunits by SDS-PAGE, enabling identification of the respective subunits according to their molecular weight [46-48] (**Figure 2**). MV151 is used to label all standard and immuno-subunits of the proteasome and to discriminate the β2 and MECL1 activities, while LW124 and MVB127 specifically bind to the LMP2/β1 and LMP7/β5 subunits, respectively (**Figure 2**) [39,49]. As the ABPs bind to the subunits with the same affinity, the standard versus immune proteasome activities can be directly compared and quantified. With this labelling approach, it is possible to obtain information on the total activity of the proteasome, the activity of the single catalytic subunits, and the ratio of the respective standard versus immune subunits in a single sample. Similar ABPs-based methods are also used to assess the proteasome activity in cell-based imaging procedures [45,50,51]. This profiling methods thereby allows the side-by-side quantification of the different active sites enabling the detection of intermediate proteasome complexes [38].

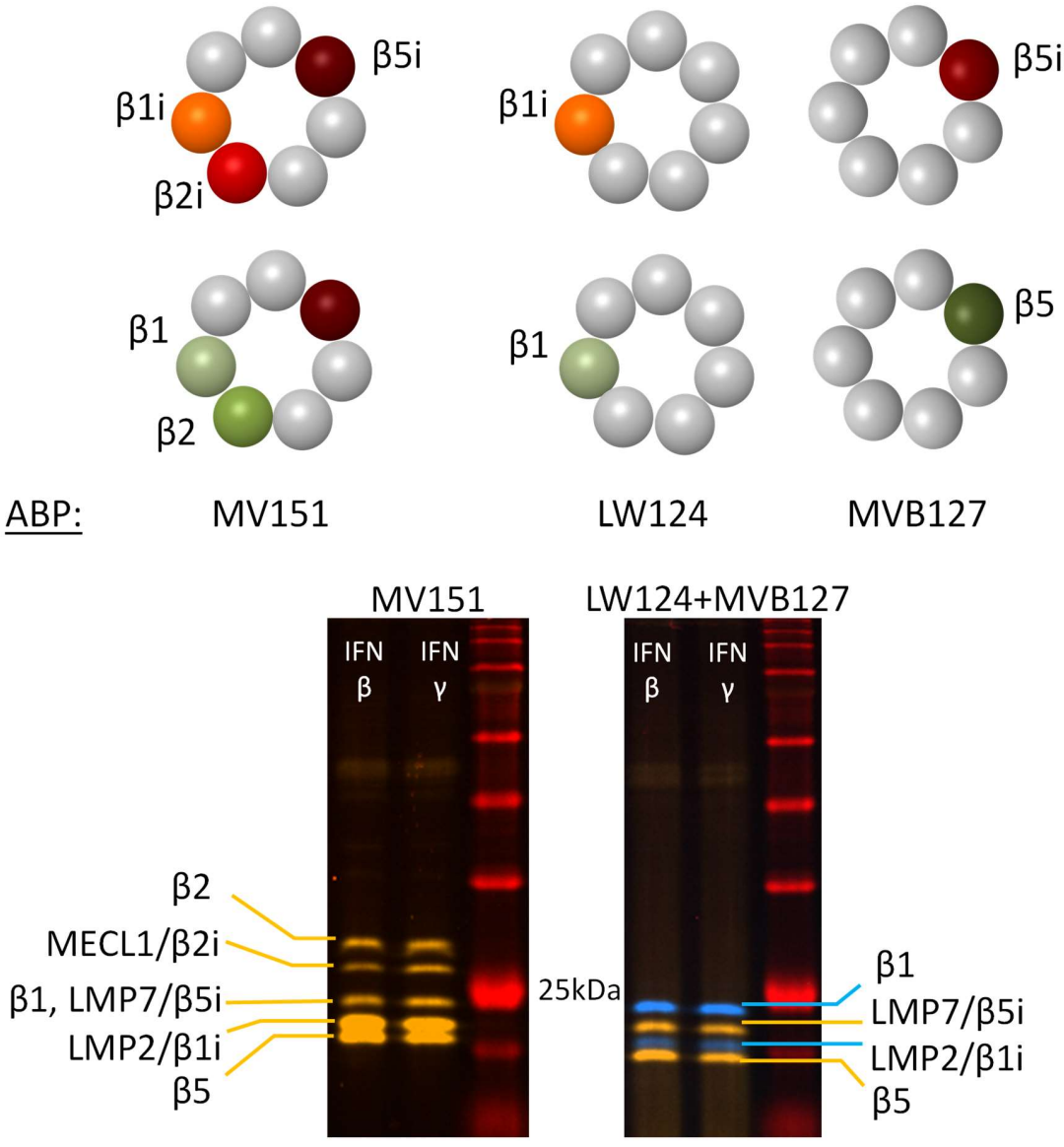

Figure 2. Activity-based probes separated by SDS gel. ABPs bind to the active-site of the respective catalytic subunits and label them covalently with a fluorescent dye. MV151 is used to label all standard and immuno-subunits of the proteasome and to discriminate the β2 and MECL1 activities, while LW124 and MVB127 specifically bind to the LMP2/β1 and LMP7/β5 subunits, respectively. The number of proteolytically active proteasome subunits is quantified by fluorescent detection using two distinct fluorescence channels upon separation of labelled proteasome subunits by SDS-PAGE, enabling identification of the respective subunits according to their molecular weight. Representative samples from neonatal fibroblasts treated with 100U/ml IFNβ or 75U/ml IFNγ are shown with 50 µg of native protein extracts loaded.

Standard- and Immunoproteasome Activity Profiles in Health and Disease

Until today, many studies have described dysregulation of the immunoproteasome as a feature of multiple chronic diseases, e.g. neurodegenerative, cardiovascular and age-related diseases, cancer and autoimmune disorders, by quantifying immunosubunit mRNA or protein expression using immunoblotting or ELISA analysis [5,13,52-55]. For example, only LMP7/β5i levels were found to be decreased in the gastrocnemius muscle while only MECL1/β2i was reduced in the tibialis anterior muscle of obese mice as measured by ELISA. Total proteasome in-gel activity in the obese muscles was reduced and inversely correlated with intramuscular accumulation of oxidatively damaged proteins [56]. Another example is human cirrhotic liver samples, where LMP7/β5i was increased both on the mRNA and protein level in response to chronic injury of liver endothelial cells that mediated pro-inflammatory cytokines expression [57]. Similarly, immunoproteasomal mRNA and protein levels were specifically upregulated in pancreatic islets and β-cells in type I diabetic patients and experimental models [29,58], while overall proteasome function was not altered suggesting a pathogenic role for the immunoproteasome [59-61]. This notion was supported experimentally in mice that developed a $CD8^+$ T cell-driven autoimmune form of type I diabetes upon deficiency of MECL1/β2i and LMP7/β5i in their bone-marrow-derived immune cells [62]. As an example of an autoimmune disorder, elevated mRNA levels of only LMP2/β1i were observed in lymphocytes of patients with Sjögren's Syndrome, pointing to a unique role for this subunit in this disease [63]. High mRNA and protein levels of LMP7/β5i and LMP2/β1i were detected in lymphocytes of patients with myositis [64], and in patients with fast progressive immunoglobulin A nephropathy [65].

As mRNA and protein expression of the proteasome subunits do not necessarily reflect the proteasome subunits' activity and do not provide any information on the existence of intermediate proteasome complexes, an increasing number of studies are assessing proteasome activities using the methods explained above along with examining proteasomal mRNA and protein expression to determine proteasome regulation in disease. Some studies analyzed blood plasma or bronchoalveolar lavage for an altered amount and activity of extracellular proteasomes as a potential biomarker for acute clinical conditions such as preeclampsia, burn injury, acute ischemic stroke, and acute respiratory stress syndrome [66-69]. Others monitored the blood or bone-marrow-derived samples of patients with multiple myeloma to determine response to treatment and development of resistance to approved proteasome inhibitors [70-72]. These data indicate that proteasome activity profiling is a valuable biomarker to determine response to treatment.

We and others have shown that proteasome activity profile and expression are affected by cigarette smoke and in patients with severe chronic obstructive pulmonary disease (COPD), a major tobacco smoke-related lung disease. While in the lungs, immunoproteasome expression is not altered, its activity is inhibited by cigarette smoke and in COPD [39,73-77]. Of note, immunoproteasome subunits expression was elevated and the activities of 20S and 26S proteasome complexes were altered in Peripheral Blood Mononuclear Cells (PBMCs) of COPD patients [39]. This led us to suggest that proteasome activity profiling in PBMCs might serve as a biomarker for inflammation and pathology severity. We corroborated this concept by examining the proteasome activity profile of PBMCs in the large population-based KORA F4 cohort of 924 subjects [38]. In this study, we demonstrated for the first time, that the proteasome activity profile in PBMCs has a strong sex dimorphism with significantly lower immunoproteasome activity in women. It also changes with age, as almost all catalytic activities of the proteasome were activated in aged women while maintained in ageing men. Moreover, we showed distinct sex-related activation patterns of standard and immunoproteasome active sites in chronic inflammatory diseases such as diabetes, asthma, cardiovascular or chronic obstructive pulmonary diseases [38]. In accordance with these data, a recent study has also suggested profiling the activity of PBMC's immunoproteasome as a sensitive biomarker for the disease activity of Systemic juvenile idiopathic arthritis (sJIA), enabling the detailed evaluation of response to treatment. This study also demonstrated that proteasome subunits expression, as measured by mass-spectrometry analysis, immediately increased upon inflammatory activation in sJIA [78].

Clinical Potential of Proteasome Activity Profiling as a Biomarker

The high clinical potential of proteasome activity profiling in peripheral blood cells as a biomarker is attributed to the distinct effects each combination of active proteasome subunit has on the cell, as reviewed above. Several studies demonstrated that loss of one of the immunoproteasome subunits changes the cleavage preferences of the 20S proteasome [79] and thereby differentially affects the generation of pathogen-derived MHC class I epitopes, and subsequent pathogen-specific $CD8^+$ T cell responses as reviewed by [1]. Moreover, differential immunoproteasome activity in lymphoid cells impacts their function through intracellular signaling. For instance, immunoproteasome activity, mainly LMP7/β5i activity, controls the balance of Th17/Treg differentiation through the potentiation of STAT3 activation thereby affecting downstream signaling and cytokine expression [80]. While inhibiting LMP7/β5i activity selectively reduced T cell receptor-induced ERK-phosphorylation in primary T cells, it did not affect NFκB signaling [81]. Of note, neither depletion of MECL1/β1i, LMP2/β2i, or LMP7/β5i impacted canonical

NFκB signaling suggesting that the immunoproteasome is not involved in NFκB signaling [82]. Moreover, LMP2/β1i has a major role in the maturation and survival of B and plasma cells and secretion of pro-inflammatory cytokines [35,36,80,81]. In addition, immunoproteasome function controls innate immune responses such as macrophage polarisation and dendritic cell programs [21,33]. Deficiency of LMP7/β5i but not of LMP2/β1i distorts the M2 profile of alveolar macrophages upon IL-4 stimulation [21]. Immunoproteasome activation also emerges as part of a broader response to stress, such as protein and oxidative stress [24,83,84]. Thus, any alteration in immunoproteasome function in lymphoid cells will broadly impact immune cell function, as lymphoid cells express immunoproteasomes or intermediate proteasomes with little standard proteasomes. This proves again that quantifying the distinct activities of the catalytic standard and immune subunits activity will reflect on the immune cell function.

Worth to mention that using proteasome activity as biomarker for disease would also extend to diseases caused by mutations and assembly defects of the proteasome that are increasingly being reported in the literature and that have been grouped into proteasome-associated autoinflammatory syndrome (PRAAS) [14,85]. For example, some mutations in PSMB8 (LMP7/β5) cause impaired assembly of immunoproteasomes resulting in autoinflammation and lipodystrophy, chronic atypical neutrophilic dermatosis, joint contractures, muscle atrophy, microcytic anemia, and autoinflammatory disorders [86-89].

Outlook

Current knowledge indicates differential activities and functions of distinct immunoproteasome complexes in health and disease. Together with the technological advances in activity profiling of the proteasome, this offers an exciting opportunity to apply proteasome activity profiling in peripheral blood cells as a biomarker approach for chronic inflammatory diseases. The use of activity-based probes (ABPs) enables high-throughput profiling and systematic monitoring of proteasome activities in clinical studies using large cohorts of patients or population-based samples, which can then be modelled with clinical data, disease severity and outcome to allow personalized patient stratification. ABP analysis also offers the potential of flow cytometry-based quantification, which can be combined with single subunit expression analysis for defined immune cell populations. In light of the unexpected sex-related regulation of immunoproteasome activity, we urgently need large study cohorts to stratify for sex but also age and BMI [38]. Understanding the role of the distinct immunoproteasome and intermediate proteasome complexes for immune cell function will also pave the way for the application of site-specific immunoproteasome inhibitors to specifically target unwanted immune cell functions, as exemplarily shown for the immunoproteasome specific inhibitor ONX0914 that is currently tested in clinical phase II for Lupus Erythematodes (www.clinicaltrials.gov) [52].

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