**Universidade de Lisboa**

**Faculdade de Farmácia**

# Evaluation of Immunoproteasome Inhibition in the Differentiation and Maturation of Dendritic Cells

**Raquel Alcarpe Coelho Gomes Rodrigues**

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# Final Report

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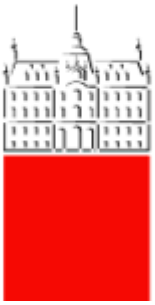


## Raquel Alcarpe Coelho Gomes Rodrigues

**Supervisor: Assist. Prof. Martina Gobec**


**2019**

# I – Resumo

Muitos processos fisiológicos e patológicos dependem do funcionamento do sistema ubiquitina-proteassoma (UPS). Este sistema é responsável pela degradação da maior parte das proteínas nas células eucarióticas. As proteínas são biomoléculas de nitrogénio de elevadas dimensões formadas por resíduos de aminoácidos e que possuem funções complexas que permitem o bom funcionamento do organismo. Deste modo, torna-se essencial manter a integridade celular das proteínas dentro de valores aceitáveis para garantir o seu correto funcionamento. Da incorreta conformação e agregação das proteínas podem surgir diversas doenças conhecidas como doenças conformacionais, entre as quais se destacam o cancro e doenças neurodegenerativas como Alzheimer e Parkinson. O proteassoma 26S é a forma constitutiva do proteassoma e é o local onde ocorre a degradação de proteínas marcadas com ubiquitina. Nos vertebrados podem ser encontradas três classes diferentes de proteassomas: timoproteassoma, imunoproteassoma e proteassoma constitutivo.

O imunoproteassoma é maioritariamente expresso nas células que fazem parte do sistema imunitário, podendo, no entanto, também ser induzida a sua expressão noutro tipo de células durante processos de inflamação e na presença de certas citoquinas. Consequentemente, este tipo de proteassoma está geralmente associado a doenças inflamatórias, autoimunes, neurodegenerativas e até mesmo cancerígenas. Assim, o uso de inibidores do proteassoma de forma geral e específicos para o imunoproteassoma parece ser uma estratégia potencial de auxílio na terapia deste tipo de doenças.

O objetivo principal desta tese de mestrado foi estudar o potencial efeito de inibidores do proteassoma e de diferentes subunidades do imunoproteassoma nos processos de diferenciação e ativação de células dendríticas primárias humanas. Para tal, utilizámos inibidores seletivos do (imuno)proteassoma: LU-001c, LU-001i, NC-001, LU-002c, LU-002i, LU-102, LU-025c e LU-015i, os quais foram fornecidos pelo Prof. Dr. H.S. Overkleeft (Leiden University, Netherlands).

O primeiro segmento experimental realizado teve o objetivo de investigar o potencial efeito dos inibidores no processo de diferenciação das células dendríticas a partir de monócitos. Observámos que a presença dos inibidores levava a uma menor expressão dos marcadores HLA-DR e DC-SIGN, enquanto nenhum efeito se registava nos marcadores

CD14, CD80 e CD86. Observámos também que este efeito era mais proeminente na presença de inibidores seletivos das subunidades catalíticas do imunoproteassoma. Tal indica que os inibidores do imunoproteassoma podem levar à supressão da diferenciação dos monócitos a células dendríticas imaturas e consequentemente a uma menor ativação das células T $CD4^+$. Determinámos também os níveis de secreção por parte das células dendríticas das citoquinas IL-2, IL-4, IL-6, IL-10, TNF-α e IFN-γ na presença ou ausência de inibidores seletivos do imunoproteassoma. No entanto, nenhum efeito significativo foi observado com os inibidores e citoquinas estudados. Concluímos que no futuro outras citoquinas que se relacionem mais com as células dendríticas devem ser igualmente estudadas e investigadas.

De seguida, realizámos duas experiências diferentes onde (i) tratámos os monócitos com inibidores apenas durante o seu processo de diferenciação a células dendríticas imaturas ou (ii) tratámos as células com inibidores durante os processos de diferenciação e maturação das células dendríticas. No primeiro caso, observámos resultados díspares na expressão dos marcadoes. Uma inibição das subunidades β5c e β5i simultãnea levou a uma diminuição notável da expressão dos marcadores CD80, CD86 e CD14, indicando uma menor maturação das células dendríticas. Contrariamente, perante um inibidor da subunidade β2i utilizado sozinho ou em conjunto com outros inibidores, observámos um ligeiro aumento da expressão do marcador DC-SIGN. Isto pode significar que diferentes subunidades catalíticas do imunoproteassoma estão envolvidas em vias intracelulares diferentes, o que explicar os diferentes fenótipos observados. Por outro lado, se os inibidores estavam presentes durante a diferenciação e maturação das células, foi possível observar um aumento significativo na expressão dos marcadores HLA-DR e DC-SIGN, enquanto CD80, CD86 e CD14 apenas foram afetados minimamente ou nada. Tal indica que a presença dos inibidores alterou as células dendríticas para um estado mais maduro. É de notar também, que de todos os inibidores utilizados durante este trabalho experimental, o inibidor da subunidade β5i, LU-015i, quando utilizado em combinação com outros inibidores parece ter um efeito inibidor sobre o imunoproteassoma mais potente. Deste modo, podemos afirmar que a modulação do (imuno)proteassoma parece ter um papel relevante no desenvolvimento das células dendríticas. No entanto, realçamos que são necessárias realizar mais repetições, modificações experimentais e diferentes testes no futuro de modo a melhor elucidar o papel do imunoproteassoma na diferenciação e maturação das células dendríticas.

**Palavras-chave:** Sistema ubiquitina-proteassoma (UPS), proteassoma, imunoproteassoma, doenças, cancro, inibidores, células dendríticas, monócitos, diferenciação, maturação.

# II – Abstract

Many key physiological and pathological processes are managed through the ubiquitin-proteasome system (UPS). UPS is the main system for the degradation of proteins in eukaryotic cells. The standard form of the proteasome is the 26S proteasome, where degradation with ubiquitin-labelled proteins takes place. In vertebrates there can be found three major classes of proteasomes: the thymoproteasome, the immunoproteasome and the constitutive proteasome.

The immunoproteasome is usually expressed in cells of the immune system. However, since its formation can be induced during inflammation and oxidative stress it is also found in other cell types. Studies show that it is involved in inflammatory, autoimmune and neurodegenerative diseases and also cancer. Thus, proteasome inhibitors seem to be a potential new strategy to use in the treatment of these pathologies.

The main goal of this master's thesis was to study the potential effect of the inhibition of different subunits of the (immuno)proteasome in the differentiation and activation process of primary human dendritic cells. For this purpose, we used (immuno)proteasome subunit selective inhibitors LU-001c, LU-001i, NC-001, LU-002c, LU-002i, LU-102, LU-025c, and LU-015i, which were kindly provided by Prof. Dr. H.S. Overkleeft (Leiden University, Netherlands).

In the first segment, we addressed the potential effect of the inhibitors on the differentiation process of DCs from monocytes. We observed that the inhibitors caused a lower expression of HLA-DR and DC-SIGN, while no effect could be observed on the CD14, CD80 and CD86 expression. These effects were more prominent if inhibitors of the catalytic subunits of the immunoproteasome were used. This indicates that the immunoproteasome inhibitors could lead to suppression of the differentiation of monocytes to iDC and subsequent lower activation of CD4+ T cells. We also determined the levels of iDC secretion of IL-2, IL-4, IL-6, IL-10, TNF-α and IFN-γ in the presence or absence of the immunoproteasome inhibitors. No effect could be observed if the inhibitors were present. In the future, other cytokines and chemokines, which are more related to DCs, should be investigated.

Next, we set up two experiments, where we either (i) treated the cells with inhibitors through the process of differentiation, but not through maturation or (ii) we treated the

cells through both processes. In the first case, we obtain contraductory results. A sumultaneous inhibition of the β5c and β5i subunits lead to a notable decrease of the expression of CD80, CD86 e CD14 markers, indiciating a lower maturation of DCs. However, inhibition of the β2i subunit alone of with others inhibitiors caused a slight increase of the expression of DC-SIGN marker. This could mean that different catalytic subunits of the immunoproteasome regulate distinct intracellular pathways, which lead to a different phenotype. On the other hand, if inhibitors were present during differentiation and maturation, we could observe a significant increase in expression HLA-DR and DC-SIGN, while the expression profile of CD80, CD86 and CD14 was only minor or not at all affected. This indicates that the presence of the inhibitors shifted the dendritic cell to a more mature type. Of all the inhibitors used during the experimental work, it seems that β5i inhibitor LU-015i is the most potent to inhibit the immunoproteasome when used in combination with other inhibitors. Taken together, it seems that modulation of (immuno)proteasome plays a role in the development of DCs. However, further repeats, modifications and experimental setups are needed to elucidate the role of immunoproteasome in the development of DCs.

**Key-words:** Ubiquitin-proteasome system (UPS), proteasome, immunoproteasome, diseases**,** cancer, inhibitors, dendritic cells, monocytes, differentiation, maturation.

# III – Acknowledgments

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This thesis is the end-point of five years of hard work, but mostly, it marks the starting point for new adventures in which I wish to apply everything that I have learned until this day. During these five years, I had the privilege to work and to be guided by people who inspired me.

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Lisbon, November 2019

Raquel Rodrigues

# IV – Acronyms

**Abs –** Conjugated antibodies

**APCs –** Antigen-presenting cells

**APF-1 –** ATP-dependent proteolysis factor 1

**ATCC –** American Type Culture Collection

**ATP –** Adenosine triphosphate

**CBA –** Cytometric bead array

**cDC –** Conventional DC

**CP –** Core Particle

**CTLs –** Cytotoxic T lymphocytes

**C-L –** Caspase-like

**CT-L** – Chymotrypsin-like

**DC –** Dendritic cell

**DC-SIGN –** Dendritic cell-specific ICAM-grabbing non-integrin

**DNA –** Deoxyribonucleic Acid

**DUBs –** Deubiquitylating Enzymes

**ECM –** ExtraCellular Mutant

**ER** – Endoplasmic reticulum

**FDA –** United States Food and Drug Administration

**FITC** – Fluorescein isothiocyanate

**Flt3L –** Fms-like tyrosine kinase 3 ligand

**GM-CSF –** Granulocyte-macrophage colony-stimulating factor

**IC50 –** Half maximal inhibitory concentration

**ICAM –** Intercellular adhesion molecule

**iDC –** Immature dendritic cell

**IFNs –** Type I interferons

**IFN-β –** Interferon-β

**IFN-γ –** Interferon-gamma

**IRF-1 –** Interferon regulatory factor-1

**KDa –** KiloDaltons

**LFA –** Lymphocyte function-associated antigen

**LMP –** Low molecular weight protein

**LMP2 –** Low molecular mass peptide 2

**LMP7 –** Low molecular mass peptide 7

**LPS –** Lipopolysaccharide

**mDC** – Mature dendritic cell

**MELC –** Multicatalytic endopeptidase-like-complex

**MELC1 –** Multicatalytic endopeptidase complex-like 1

**MHC-I –** Major histocompatibility complex class I

**NF-κB** – Nuclear factor-kappa of activated B cells

**Nm –** Nanometre

**NO –** Nitric oxide

**PA –** Post Acidic

**PA –** Proteasome Activator

**PBMCs –** Peripheral Blood Mononuclear Cells

**PBS –** Phosphate-buffered saline

**pDC –** Plasmacytoid DC

**PE –** Phycoerythrin

**PI –** Proteasome inhibitor

**POMP –** Proteasome Maturation Protein

**RBCs –** Red blood cells

**RP –** Regulatory Particle

**Rpn –** Regulatory particle of non-ATPase

**Rpt** – Regulatory particle of triple-ATPase

**TCR –** T cell receptor

**TGF-β –** Transforming growth factor-beta

**$T_H$17 –** T-helper 17 cell

**Thr –** Threonine

**T-L** – Trypsin-like

**TNF-α –** Tumor necrosis factor-alpha

**UPP –** Ubiquitin-Proteasome Pathway

**UPS –** Ubiquitin-Proteasome System

# Table Of Contents

# List of figures

# List of equations

# 1. Introduction

Higher living organisms possess tissues and structures of a great cellular complexity where its normal functioning and cellular homeostasis are dependend of several vigilance mechanisms and intercellular signaling. Many of these mechanism and pathways are protein-mediated. Proteins are large nitrogen-containing biomolecules formed by amino acid residues. They perform a vast array of functions within the organism, such as serving as the major structural component of muscle and other tissues in the body, enabling cell division and intercellular signaling and the correct function of the immune system. Besides, they can also be used as energy and to produce hormones, enzymes, and hemoglobin, catalyze metabolic reactions and DNA replication.(1)

Due to their importance, maintaining the integrity of the proteins seems to be essential for cell viability. It is known that proteins often misfold during the life of the cell, as a result of many factors such as disrupting mutations, stress conditions, cell aging or other specific metabolic challenges. Misfolded proteins can lack their important functions and activities and more importantly can engage in inappropriate interactions with other cellular components and subsequently accumulate in potentially toxic protein inclusions. The long-term health of the proteome depends upon the ability of the protein homeostasis or “proteostasis” networks to respond to the chronic expression of misfolded proteins. Thus, the cell has developed several mechanisms and elaborate strategies mainly through molecular chaperones, whose goal is to either refold, degrade or sequester misfolded proteins.(2)(3)(4)

Protein misfolding has been implicated as a basis for a large number of human diseases, commonly known as “conformational diseases”, which result from alterations in protein homeostasis. Intracellular accumulation of abnormal proteins, in the form of protein inclusions and aggregates, and dysfunction of the quality control mechanisms are common in all these disorders. These include a staggering range of pathologies, from lysosomal storage diseases, cancer, cystic fibrosis, to many neurodegenerative disorders such as Alzheimer’s, Parkinson’s, and Huntington’s diseases.(2)(3)

In eukaryotic cells, there are two main pathways responsible for intracellular protein degradation: the lysosomal pathway and the ubiquitin-proteasome pathway (UPP), also known as the ubiquitin-proteasome system (UPS). The UPS plays a crucial role in many

physiological and pathological processes since it is responsible for regulating a wide variety of cellular pathways.

## 1.1. Historical background

The identification of the proteasome as the proteolytic machine responsible for degrading cellular proteins *in vivo* tagged with ubiquitin was a key step in the scientific community. It allowed the discovery of a new regulatory principle of eukaryotic cells, now known as selective proteolysis via the ubiquitin-proteasome system (UPS).(5)

During the 70s scientists focused mostly on the translation of the genetic code into proteins neglecting for many decades the study of the mechanisms behind the removal of proteins, which was regarded as a nonspecific and unimportant end process. Later it was hypothesized that the mechanism was probably nonlysosomal, as lysosomal degradation of intracellular proteins could not explain certain observations related to the specificity of the process. It was also suggested that any cellular protease involved in this process should certainly be under control to prevent nonspecific destruction.(6)

Only until the 1980s, two complementary sets of discoveries were made and helped understand the mechanisms involved. Through the use of biochemical fractionation and enzymology, Avram Hershko alongside with his student Aaron Ciechanover from the Hershko's laboratory at the Technion (Haifa, Israel) discovered in 1978–1983 that some proteins added to a rabbit reticulocyte extract became covalently conjugated to a protein initially known as APF-1, later identified as ubiquitin, and that ubiquitylated proteins were progressively destroyed by an ATP-dependent protease in the extract. They observed that the reticulocyte "protease" contained at least two complementing fractions, the first one, the active component that was shown to be a small heat-stable protein, the APF-1 (ATP-dependent proteolysis factor 1), and a second one later explored and identified.(7)(8)

In 1977, the two scientists joined Irwin A. Rose at The Institute for Cancer Research at the Fox Chase Cancer Center and started working on elucidating the protein degradation system. Working together, Hershko, Ciechanover, and Rose reported that the second fraction of the reticulocyte lysate could be further subdivided into an ATP-stabilized protein of approximately 450 kDa. In 1980 Ciechanover and Hershko suggested that the

APF-1 served as a signal for a downstream protease and began dissecting the enzymology of APF-1 conjugation.(7) This set of three enzymes involved, termed E1 (ubiquitin-activating enzyme), E2 (ubiquitin carrier protein or ubiquitin-conjugating enzyme), and E3 (an accessory component that appeared to confer specificity on E2) were isolated and characterized later during 1981 and 1983 by Hershko and Ciechanover. Based on their observations, they hypothesized that E1 transferred ubiquitin to E2, which then transferred ubiquitin to the substrate in the presence of E3. They also prophesied that the specificity of the E3 component determined which proteins in the cell were marked for destruction, defining the high specificity of the system toward its potential numerous substrates. This marks the born of the "the multistep ubiquitin-tagging hypothesis".(8)

This ATP-dependent protease that mediates the destruction of ubiquitin-protein conjugates was further characterized by several laboratories later, in the 1990s, and is now known as the 26S proteasome.(7)(8)

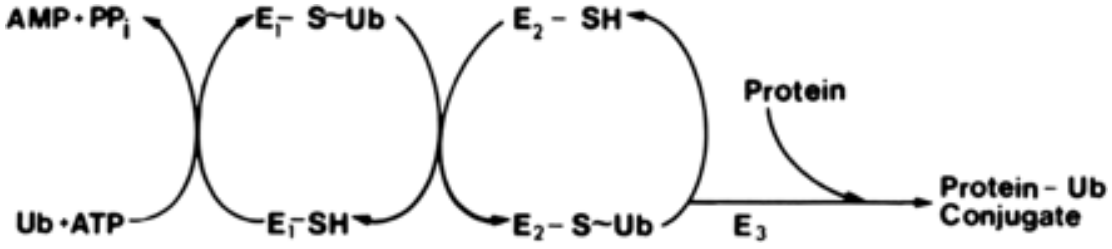


**Figure 1: Proposed sequence of events in the ubiquitin-protein ligase system. The ubiquitin protein suffers an activation by E1 ubiquitin-activating enzyme, which is dependent on ATP. E2 ubiquitin-conjugating enzymes catalyze the transfer of ubiquitin from E1 to the active site cysteine of E2. E3 ubiquitin ligases catalyze the final step of the ubiquitination cascade, creating a bond between a lysine of the target protein and the C-terminal glycine of ubiquitin. Thus E3 enzymes function as the substrate recognition modules of the system and are capable of interaction with both E2 and substrate.**(8)

In 1984–1990, Alexander Varshavsky and his colleagues at the California Institute of Technology, in Pasadena USA, continued the work of Hershko and his colleagues and discovered the first biological functions of the ubiquitin system, the source of its specificity and identified some of the system's fundamental attributes, such as the polyubiquitin chain and the subunit selectivity of protein degradation.(7)(8)

Through genetic, biochemical, and cell biological studies with mammalian cells and the yeast *Saccharomyces cerevisiae*, they discovered that the ubiquitin system was essential for protein degradation in living cells, was required for cell viability, and played major roles in the cell cycle and processes, including signal transduction, transcription, DNA repair, quality control, generation of antigenic peptides and stress responses.(7) In 1986, A. Bachmair, Dan Finley, and Varshavsky revealed the basis of the specificity of intracellular protein degradation by discovering the first degradation signals (degrons) in short-lived proteins that target them for ubiquitin conjugation and proteolysis.(7)

Not surprisingly, aberrations in this system have been since then implicated in the pathogenesis of many diseases, inflammatory and neurodegenerative disorders, and malignancies among them. Consequently, many mechanism-based drugs involving UPS proteolysis have been explored and developed.(6)(7)

## 1.2. Proteolysis

As explained before, the existence of a highly regulated turnover of cellular proteins contributes to maintaining cellular and protein homeostasis, a delicate balance between protein synthesis and protein degradation that determines the levels of proteins within. In this way, denatured proteins, damaged proteins or proteins that are no longer needed, are recognized and removed through proteolytic degradation, catalyzed by proteases that cleave peptide bonds. This degradation can also occur as a result of adverse cellular conditions such as extreme temperature, acidity, or salinity, which disrupts the molecules in the peptide bonds. Thus, the half-lives of proteins within cells vary widely, from minutes to several days, and differential rates of protein degradation are an important aspect of cell regulation. (9)(10)(11)

In eukaryotic cells more than 80% of cellular proteins are degraded through the UPS pathway, including those involved in the regulation of numerous cellular and physiological functions, such as cell cycle, apoptosis, transcription, DNA repair, protein quality control, and antigens.(9)(10)(12)

The UPS uses a small molecule called ubiquitin, which is made of 76-amino acids and targets cytosolic and nuclear proteins.(13) Proteins are marked for degradation by the attachment of the C-terminus of ubiquitin covalently to the amino group of the side chain of a lysine residue.(14) Additional ubiquitins are then added to form a multiubiquitin

chain and such polyubiquitinated proteins are recognized by a large multisubunit protease complex, called the proteasome, which degrades ubiquitinated proteins and recycles the ubiquitin for reuse.(9) Both the attachment of ubiquitin and the degradation of marked proteins require energy in the form of ATP.(10)

Since the attachment of ubiquitin marks proteins for rapid degradation, the stability of many proteins is determined by whether they become ubiquitinated or not.(10) Ubiquitination is a multistep process and the conjugation cascade of ubiquitin to substrates usually involves three steps that count with the activity of three different enzymes:(15)

1. Activation of ubiquitin: The cascade begins with an ubiquitin-activating enzyme, E1 that catalyzes the formation of a covalent thioester bond between the side chain of one of its own cysteine residues and the carboxyl group of the C-terminal glycine of ubiquitin. The human species has only two E1 enzymes for ubiquitin.(14)
2. Transfer of ubiquitin to an E2 enzyme: In the next step of the process the activated ubiquitin is transferred to a cysteine residue of an E2 or ubiquitin-conjugating (or carrier) enzyme by transesterification. Humans have approximately 35 E2 enzymes.(14)(15)
3. Ubiquitylation of target proteins: Finally, the E3 ubiquitin ligase acts as the recognition element and facilitate the transfer of ubiquitin from an E2-conjugate to the protein substrate, either directly or in two steps through an E3-ubiquitin intermediate.(14) This step is responsible for the selective recognition of appropriate substrate proteins. Most cells contain over 500 different E3 enzymes, providing exquisite substrate specificity to the UPS. Different E3 enzymes recognize different substrate proteins, and the specificity of these enzymes is what selectively targets cellular proteins for degradation by the ubiquitin-proteasome pathway.(10)(13)

The proteasome consists of a large protein complex responsible for the degradation of intracellular proteins, a process that requires metabolic energy. The polymerized ubiquitin chain acts as a signal that shuttles the target proteins to the proteasome, where the substrate is proteolytically broken down. Furthermore, it should be noted that ubiquitylation is a reversible reaction because many cysteine-protease and metalloprotease deubiquitylating enzymes (DUBs) are present in the cell.(16)

## 1.3. The Proteasome

The proteasome is a multicatalytic proteinase complex, critical for degradation of unwanted cellular proteins labelled by enzymatic conjugation with ubiquitin in the ubiquitin-proteasome system (UPS). In mammals, the rapid degradation of ubiquitinated proteins is catalyzed by the 26S proteasome, which name comes from its apparent sedimentation coefficient.(16) This structure is found in the nucleus and the cytosol of all cells and constitutes approximately 1 to 2% of cell mass.(9)(17)

### 1.3.1. Structure

The 26S proteasome has about 2500 kiloDaltons (kDa) of molecular mass and contains 31 principal subunits arranged in two distinct sub-complexes: a 20S core particle (CP) and one or two 19S regulatory particle(s) (RP). The 20S core particle is the key component of the UPS, containing several active centers (catalytic subunits) to degrade unneeded or damaged proteins, unfold protein-substrates and stimulate proteolytic activity.(9)(17)(18)(19)

The 20S CP is a well-organized protein complex with a sedimentation coefficient of 20S and a molecular mass of approximately 750 kDa. It is made up of 28 subunits and composed by four axially stacked heptameric rings (two outer α- and two inner β-rings), that form a barrel-shaped structure with a central pore. The outer α-rings contain seven similar, yet distinct α-subunits (α1-α7), and by forming a central pore, they function as a tightly regulated "gate" for the entrance of substrates and removal of degradation products from the complex. This "gate" which is made of the N-termini of a subset of α-subunits, blocks the unregulated entrance of substrates into the catalytic chamber, allowing α-rings to change the proteasome activity and specificity. This makes them responsible for substrate recognition and regulation of substrate access to the inner proteolytic chamber. The mechanism of the "gate" opening and proteasome activity are regulated by the interaction between proteasome regulators and the α rings, such as 19S RP, PA28, PA200, ECM29 and PI31.(13)(16)(19)

Similarly, each of the two inner β-rings consists of seven distinct β-subunits (β1-β7), which are flanked by the two outer α-rings. The β rings constitute the catalytic core of the proteasome. Three of the β-subunits, β1, β2 and β5, contain active sites with different

proteolytic specificities, based upon preference to cleave a peptide bond after a particular amino acid residue: the β1 subunit presents peptidyl-glutamyl-hydrolyzing, caspase-like (CL) or post acidic (PA) activity and cleaves peptide bonds after acidic amino acids; β2 subunit has trypsin-like (T-L) activity and cleaves peptides after basic amino acids and β5 subunit has chymotrypsin-like (CT-L) activity and acts after neutral or hydrophobic amino acids. Therefore, each mature eukaryotic proteasome has six proteolytic sites with three types of proteolytic activities.(13)(19)

Substrates gain access to the proteolytic chamber by binding to the 19S RP that is connected to one or both ends of the latent 20S proteasome, forming an enzymatically active proteasome. The 19S RP, also known as PA700, is a 700 kDa multifunctional complex which regulates proteasome function by identifying substrates and assuring the selectiveness of the process, unfolding the substrates for degradation, translocating them into the 20S catalytic particle, and possibly even influencing the nature of products generated by proteolysis.(20) The RP is further divided into two main structures: the "base" that binds to the α-rings of the 20S core proteasome and the "lid" that recognizes and binds polyubiquitinated proteins, thus regulating substrate entrance to the 20S proteasome.(21)

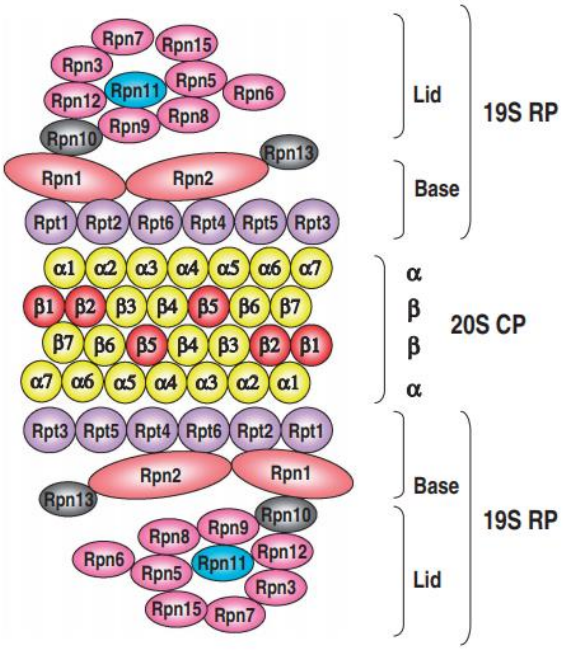


**Figure 2: Schematic drawing of the 26 S Proteasome subunit structure. The 20S Core Particle (20S Proteasome) in the center; two RP, 19S regulatory particles consisting of the base and lid sub complexes.**(16)

### 1.3.2 Types

The proteasome complex plays essentially the same proteolytic roles in all eukaryotes and the overall structures and functions of the individual subunits have been highly conserved among eukaryotic species during evolution. Substitutions and modifications of the core 26S proteasome may however affect its activity and/or specificity. In vertebrates there can be found three major classes of proteasomes: the thymoproteasome, exclusively found in epithelial cells of the thymus cortex and in which the β5 subunit is substituted for an alternate protein (β5t); the immunoproteasome which is normally expressed in monocytes and lymphocytes but its formation can also be induced during inflammation and presence of cytokines such as IFN-γ and TNF-α in other cells; and the constitutive proteasome, which is expressed in the majority of the other tissues.(22)

In addition to the constitutive proteasome (26S), it is also found in organisms the proteasome 20S in its free form, not coupled to the regulatory part 19S. The unbound 20S is the predominant proteasome form in most cells and may be the primary mechanism for the ATP-independent degradation of oxidized proteins following an oxidative insult.(23)(24)

## 1.4. Immunoproteasome

The UPS, besides having essential roles in cell growth regulation, metabolism and elimination of misfolded proteins, plays a critical role in the immune system. In higher vertebrates, the proteasome along with the lysosome is responsible for generating small antigenic peptides that are presented to the immune system and that enable a more efficient activation of immune responses.(17)(25)

A special inducible form of the 20S proteasome is called the immunoproteasome (i20S). This is the simplest evidence of proteasome plasticity.(23) Immune cells such as macrophages, B cells or dendritic cells constitutively express the immunoproteasome. More recently, basal expression has also been detected in non-immune cells such as medullary thymic epithelial cells that like many other cells under conditions of oxidative stress, inflammation, cytokine stimulation, viral or bacterial infection can assemble this type of proteasome.(26)

The i20S proteasome can be induced by inflammatory cytokines such as IFN-γ, that catalyze the replacement of the β1, β2, and β5 subunits for their inducible equivalents, β1i (LMP2 - low molecular mass peptide 2), β2i (MELC1 - multicatalytic endopeptidase complex-like 1) and β5i (LMP7 - low molecular mass peptide 7). Further inducers are IFN-α, IFN-β, TNF-α, and lipopolysaccharides. Hyperglycemia and high levels of endogenous NO are other conditions recently identified to regulate immunosubunit expression. In general, it has been proposed that this type of inducible proteasome should be called "immunoproteasome" to emphasize their specialized functions in immune responses and the three inducible β-subunits should be indicated by an additional "i". (21)(27)(28)

The inducible β-counterparts have been shown to exhibit differential cleavage preferences and efficiency to help diversify the antigenic peptide repertoire that may be more appropriate for the immunological processing of antigens. Like constitutively expressed catalytic β subunits, the β1i, β2i, and β5i subunits display caspase-like, trypsin-like and chymotrypsin-like proteolytic activities and exhibit preferential substrate cleavage after acidic, basic and hydrophobic amino acid residues, respectively. However, immunoproteasomes have distinct rates of proteolytic activities that generate a different spectrum of peptides from standard proteasomes to favor MHC class I antigen presentation. Compared with the standard proteasome, the immunoproteasome is characterized by enhanced chymotrypsin and trypsin-like activities and reduced caspase-like activity. These enzymatic properties lead to the generation of antigenic peptides with high affinity to the MHC class I molecules. The production of such MHC class I ligands is well-known to improve antigen presentation and subsequent cytotoxic T-lymphocyte (CTL) response. (16)(28)

The biogenesis of the immunoproteasome shares similar mechanisms to the standard proteasome. However, in cells that express both the inducible and constitutive β-subunits, the immunosubunits are preferentially incorporated. β5i subunit is particularly important: it is involved in the maturation of the β1i and β2i subunits and encodes the chymotryptic activity that improves binding to the MHC class I molecules and facilitates T cell recognition.(26) Thus it is incorporated into the α-ring earlier than β1 and is required for the subsequent incorporation of β2i.

The PA28 regulatory complex, also known as 11S, is also notably important for the immunoproteasome since its expression is upregulated by IFN-γ, suggesting a role in

regulating its function. When this regulatory molecule binds to the outer ring of the α-subunits, replaces the 19S regulatory complex and facilitates access of proteins to the catalytic core without the requirement for protein ubiquitinylation. As a result, assembly of this form of immunoproteasome facilitates protein degradation in general and most importantly of viral origin, and leads to a more efficient presentation of the corresponding antigens to cytotoxic T cells.(24)(26)(21)(28)

The transient and inducible feature of immunoproteasome biogenesis together with the shorter half-life and turnover of the immunoproteasome in comparison to the standard proteasome, indicates the existence of a tightly controlled mechanism for immunoproteasome regulation that allows a rapid response to environmental challenges and subsequent return to baseline levels that favor the standard proteasome subtype once the threat is gone. Alongside immune functions, the immunoproteasome has been demonstrated to possess broader biological functions that will be further addressed in this thesis.(24)(28)

### 1.4.1. Immune Functions

The recognition of peptides generated from the self and foreign proteins by T lymphocytes, mainly by the CD8 T cells, is essential for the efficacy and specificity of immune responses in an organism. These peptides are mainly provided by the proteolytic activity of proteasomes that act by processing them into small peptides required to fit into the groove of nascent MHC class I molecules in the ER.(24)

The major function of the immunoproteasome is the efficient production of specific short oligopeptide (8-10 amino acids) antigens that are transported to the ER, where they can bind easily to MHC class I molecules with high affinity for cell surface presentation to CTLs. The resulting complexes then relocate via the Golgi apparatus to the cell surface, where they are exposed. When antigenic peptides are displayed on the cell surface CD8 T cells bind strongly to them if their unique T cell receptor (TCR) sequence matches, then activate and release cytotoxins, initiating an immune response.(26) The increased proteolytic capacity and the high affinity of the MHC I-binding peptides generated by the immunoproteasome are what define its greater efficiency over the constitutive proteasome. It is likely that the acquisition of the immunoproteasomes during evolution

enabled organisms to produce MHC class I ligands and combat pathogens more efficiently.(16)(23)(28)

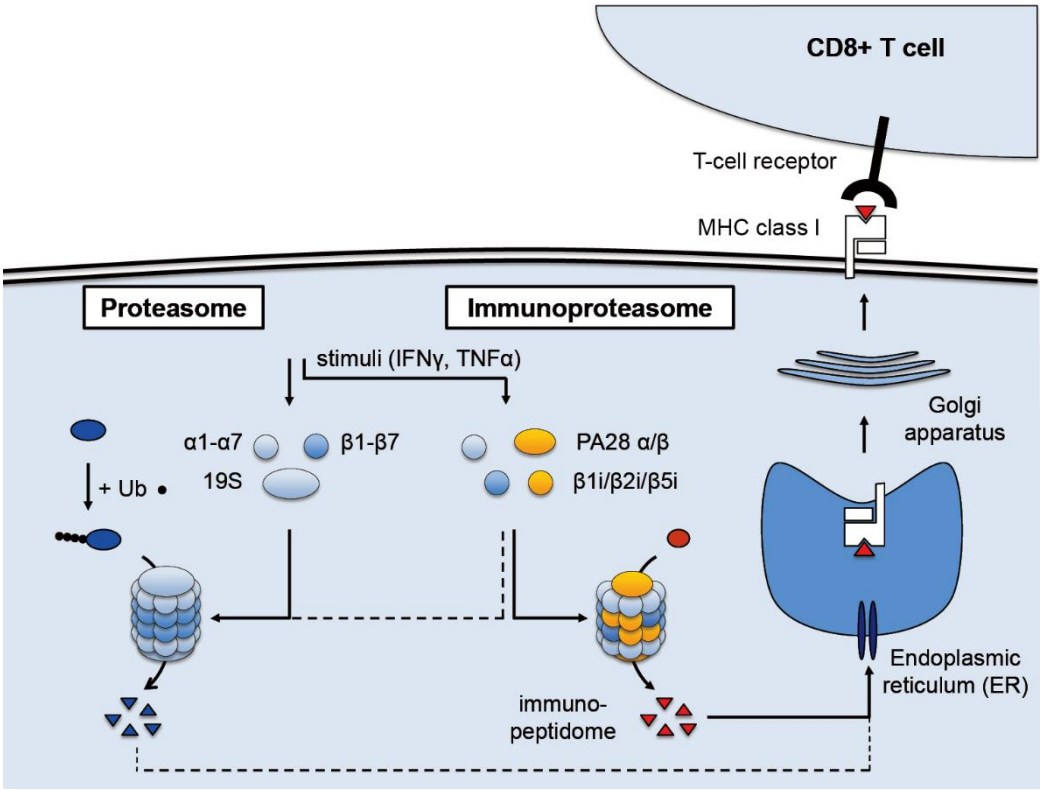


**Figure 3: Proteasome and immunoproteasome assembly. Signals inducing the expression of the immunoproteasome-specific subunits β1i, β2i, β5i and PA28 α/β result in the preferred assembly of the immunoproteasome over the regular proteasome. The resulting antigens processed bind more effectively to MHC class I molecules, and after processing in the endoplasmic reticulum (ER) and Golgi apparatus the individual peptides presented on the cell surface can be recognized by T-cell receptors on $CD8^+$ cells, initiating an immune response.**(26)

Despite the recognized role of the immunoproteasome in optimizing the presentation of certain MHC class I antigens, studies conducted in mice lacking immunosubunits suggested that antigen processing may not be the major biological function of the immunoproteasome.(28)

In fact, the immunoproteasome has also been implicated in other aspects of the regulation of immune responses, such as the regulation of cytokine production via the NF-κB pathway, T cell expansion and T helper cell differentiation.(26) Several studies have demonstrated a key role of the immunoproteasome in the regulation of cytokine production by immune cells. Studies conducted on bone marrow-derived dendritic cells from immunosubunits-deficient mice showed a considerable decrease of IFN-α, IL-1β,

IL-6 and TNF-α levels as compared to wild-type counterparts. This reduced ability to produce cytokines in these cells has been associated with compromised NF-κB signaling. It was also shown that selective inhibition of β5i, β1i and β2i subunits blocks production of IL-23 in monocytes, and TNF-α and IL-6 in T cells, indicating that the immunosubunits are involved in cytokine regulation.(28) The underlying mechanisms by which the immunoproteasome activates specific intracellular signaling pathways, such as the NF-κB signaling, more efficiently than the standard proteasome remain unclear.(28)

Additionally, studies have demonstrated the involvement of the immunoproteasome in T cell differentiation, survival, and proliferation. Administration of immunoproteasome inhibitors seems to prevent the induction of $CD4^+$ T cell differentiation into $T_H17$ cells, implying that the immunoproteasome may be required for the processing of certain differentiation factors of this type of cell. Moreover, β2i deficiency has been shown to increase the $CD4^+/CD8^+$ T cell ratio. Studies have also suggested a role for the immunoproteasome in T cell survival, as T cells transferred from immunosubunit-deficient mice into influenza or lymphocytic choriomeningitis virus-infected wild-type mice did not improve survival.(28)

## 1.4.2. Anti-oxidative stress function

Beyond its role in the immune system, recent studies have begun to unravel the non-immune functions of the immunoproteasome, especially in maintaining protein homeostasis. It has recently been demonstrated that the immunoproteasome plays a critical role in the clearance of oxidized proteins preventing protein aggregate formation. (29)

In an oxygen environment, protein oxidation constitutes a normal component of overall protein turnover. Young, healthy, mammalian cells can adapt to increases in oxidative stress by becoming temporarily more resistant to oxidative damage.(29)(30)

Oxidative stress is defined as an imbalance between pro-oxidants and antioxidants, resulting in increased release of free radicals and subsequent accumulation of damaged proteins. The pool of damaged proteins can accumulate rapidly under oxidative stress conditions, per example during an innate inflammatory response, to the extent that exceeds the proteolytic capacity of the standard proteasome, and subsequently instigates the formation of harmful protein aggregates, leading to cell apoptosis. One contributing

factor that causes protein oxidation is the increased generation of $H_2O_2$, that induces frank oxidative stress and increases immunoproteasome and 20S proteasome synthesis.(28)(29) High levels of nitric oxide (NO) are also many times involved in oxidative stress, leading in the same way to the upregulation of the immunoproteasome to help cope with the elevated protein damage.(29)

Due to the rapid induction properties under oxidative stress and the enhanced proteolytic activities compared to the constitutive proteasome, the immunoproteasome has been suggested to play an important role in efficiently removing the nascent, oxidatively-damaged proteins.(28)(29)

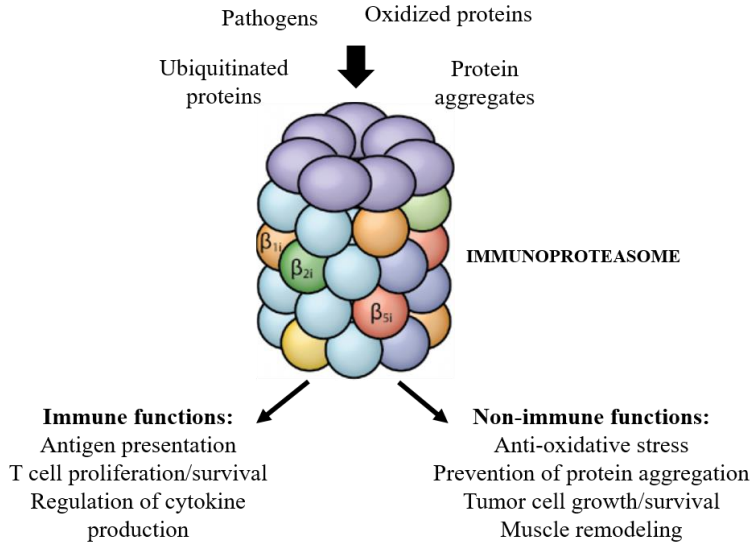


**Figure 4: Schematic resume of the biological functions of the immunoproteasome. The substrates of the immunoproteasome can be pathogens, ubiquitinated proteins, oxidatively-damaged proteins, and protein aggregates. This special form of inducible proteasome has immune and non-immune functions. In addition to the immune functions of the immunoproteasome in the regulation of MHC class I antigen presentation, the immunoproteasome is involved in cytokine production, and T cell proliferation and survival. Non-immune functions of the immunoproteasome include anti-oxidative stress, anti-protein aggregation, and regulation of muscle mass. Adapted from** (28)**.**

## 1.4.3. Clinical relevance

The therapeutic potential of intervention in the UPS has been already demonstrated by the development of proteasome inhibitors as an approach to the treatment of several diseases. Alterations in the expression, activity or function of the immunoproteasome have been linked to several diseases, causing either the pathology or a symptom of the

disease. Whereas increased proteasome concentrations are well known to be a general feature of tumor cells, abnormal immunoproteasome levels accompanied by increased concentrations of pro-inflammatory markers have also been associated with the development and progression of neurodegenerative diseases, autoimmune disorders, and certain types of cancers.(9)(13)(22)

Proteasome inhibitors were initially synthesized as *in vitro* probes to investigate initially the function of the proteasome's catalytic activity and later their potential as therapeutic agents in several diseases such as cancer, autoimmune diseases, inflammatory pathologies, organ transplants and infective diseases. While the initial studies established the potential of proteasome inhibitors as therapeutic agents, many of the compounds available were limited to laboratory studies due to a relative lack of potency, specificity or stability. This led to the design of new inhibitors with more potent and selective activity.(9)(13)

## *Cancer*

Included in the vast cellular functions of proteasomes, the regulation of cell cycle, apoptosis, cellular proliferation and activation of transcription of various genes seem to be closely connected to the development of cancer. The UPS and the immunoproteasome have been suggested to play an important role in regulating tumor development and are considered a promising target for cancer therapy.(31)

Several studies have shown that tumor cells can employ different techniques to manipulate the immunoproteasome function to escape immune surveillance. For instance, the downregulation of the immunoproteasome has been implicated to be an important immune evasion strategy for some tumor types.(28) Specifically, studies suggest that loss of the immunoproteasome subunits results in the development of tumors in mice and also leads to a lesser expression of INF-γ-induced IRF-1 expression, which is necessary for regulation of cell-cycle progression and reduced levels of MHC class I cell surface. (29)(31) However, given the crucial role of immunoproteasomes in the regulation of various pro-inflammatory mediators, it is also given a potential pro-carcinogenic role to immunoproteasomes during the progression of chronic inflammation. Recent studies have highlighted the mechanisms by which the cytokines secreted by inflammatory cells and regulated by the transcription factor NF-κB such as TNF-α, IL-6 and IL-17A stimulate tumor development and progression. Also, rapidly dividing malignant cells require the

proteasome to handle the accumulation of misfolded proteins. Thus, depending on the type of cancer, the presence of the immunoproteasome in cells may either be acting as a contributing factor in the development or progression or may only be a consequence of the disease.(28)(29)(31)

## *Autoimmune diseases*

Given the demonstrated function of the immunoproteasome in processing self-antigens, recent evidence suggests that the immunoproteasome may be involved in the development of autoimmune diseases and serve as a potential target for their treatment. Increased expression of the immunosubunits has been observed in several autoimmune diseases such as rheumatoid arthritis and inflammatory bowel disease.

Studies using mice with rheumatoid arthritis have shown that administration of a selective inhibitor of β5i results in reduced inflammatory infiltration, cytokine and autoantibody production, and attenuated disease symptoms.(28)(29)

## *Neurodegenerative diseases*

Increased expression of immunoproteasome has also been described in neurodegenerative diseases.(28) Alzheimer's disease is a neurodegenerative disorder characterized by its late start and progressive dementia resulting from a massive loss of neurons. Another particular factor of this disease is the presence and neurofibrillary knots and plaques formed by aggregates of tau proteins and amyloid β proteins respectively. The constitutive proteasome has been shown to be inhibited by these tau aggregate knots. Additionally, the accumulation of tau aggregates in astrocytes of Alzheimer's patients has also been linked to increased expression of the immunoproteasome. This suggests that cells try to cope by using the immunoproteasome, although unsuccessfully, to clear away protein aggregates.(29)

Another neurodegenerative disease characterized by protein aggregation is Huntington's disease. This is an autosomal dominant neurodegenerative disorder caused by a CAG repeat in which the expanded glutamine repeats induce the formation of neurotoxic huntingtin aggregates that can directly impair the function of the UPS and are thought to cause abnormal neuronal physiology and viability, possibly leading to an altered turnover of regulatory proteins and neuronal cell death. It has been shown that while there is no change in total proteasome content, there is an increase in immunoproteasome subunit

expression. It is possible that the increased chymotrypsin-like activity of the immunoproteasome is more important for the degradation of protein aggregates found in Huntington patients than is the caspase-like activity.(28)(29)

## 1.5. Proteasome Inhibitors

Over the past decades, extensive efforts have been made towards the identification and development of proteasome inhibitors that can be used both as molecular probes to investigate proteasome biology and as potential therapeutic agents to treat disease conditions. Thus, the proteasome 20S has been extensively explored as a drug target. The majority of proteasome inhibitors currently used in clinical settings or as research tools target both the constitutive proteasome and the immunoproteasome. With the growing importance of the immunoproteasome in many cellular processes, there have been many attempts to isolate the contribution of immunoproteasome catalytic subunits to the pharmacological activity and to discover the toxic effects associated with these unspecific inhibitors. This toxicity is mainly because standard proteasomes are constitutively expressed in all eukaryotic cells and its inhibition can easily lead to broader effects in the organism.(32)

Inhibition of the 20S proteasome results in the accumulation of misfolded proteins as well as reactive oxygen species, thereby giving rise to the induction of ER stress and the dysfunction of the cell homeostasis. Furthermore, proteasome inhibitors prevent the degradation of tumor suppressors and downregulate pro-inflammatory pathways, such as the NF-κB signaling cascade. Overexpression of the immunoproteasome positively correlates with chronic inflammation, dependent tumor pathogenesis, cardiovascular inflammation, and cytokine production. Thus, selective inhibition of the immunoproteasome represents a promising novel therapeutic strategy for these types of diseases since it primarily induces cell death.(13)(22)

Here I briefly summarize the inhibition profiles of clinically relevant proteasome inhibitors and its effects in constitutive proteasome and immunoproteasome.

## *Bortezomib*

Bortezomib (Velcade™, Millenium Pharmaceuticals) is a reversible dipeptide boronate proteasome inhibitor used for the treatment of relapsed and refractory multiple myeloma. Bortezomib was the first proteasome inhibitor for human use to be approved in 2003 by the U.S. Food and Drug Administration (FDA). Bortezomib is a dipeptidyl boronic acid that inhibits nonspecifically the chymotrypsin-like activity (β5 and β5i). Despite its success on the market, Bortezomib therapy presents several disadvantages since it can result in cytotoxicities, such as neuropathy, and the development of drug resistance. Notable side effects also include thrombocytopenia and neutropenia as well as gastrointestinal disorders mainly due to its off-target activity.(9)(13)(22)(32)

## *Carfilzomib*

The unwanted side effects of the Bortezomib encouraged the pharmaceutical industry to develop new but equipotent proteasome inhibitors with less off-target activity. In 2012, the FDA approved the inhibitor Carfilzomib (Kyprolis™, Onyx Pharmaceuticals) for the treatment of patients with multiple myeloma who have received at least two prior therapies and that revealed disease progression. Carfilzomib is a tetrapeptide derivative of the natural product epoxomicin, which targets the subunits β5c and β5i of the proteasome with much more subunit-selectivity than Bortezomib. Unlike Bortezomib, Carfilzomib binds irreversibly to the CT subunit, leading to a more sustained proteasome inhibition. Similar to Bortezomib, the dose-limiting effects in carfilzomib therapy are neutropenia and thrombocytopenia, but carfilzomib does not induce peripheral neurotoxicity due to its higher specificity for the proteasome and less off-target effects than Bortezomib.(9)(13)(22)

## *Marizomib*

A further promising proteasome inhibitor currently being tested in clinical phase studies is the natural compound Marizomib (Salinosporamide A, Nereus Pharmaceuticals). Marizomib is a secondary metabolite of the marine bacterium, actinomycetes *Salinispora tropica* and its unique chemical structure leads to a more sustained inhibition of the proteasome in an irreversible way, predominantly of the subunits β2 and β5. Even though

the oral and intravenous application of Marizomib is well-tolerated, its rather short half-life of fewer than five minutes and its ability to penetrate the blood-brain barrier may limit its therapeutic application.(22)(32)

### *Iksazomib*

Recently, in 2015, the FDA approved Ixazomib (Ninlaro™, Takeda Pharmaceutical Company Limited), a boronic acid derivative as the first orally administered, reversible proteasome inhibitor. Ixazomib, just like Bortezomib, inhibits particularly the β5 subunit of the 20S proteasome. Moreover, in higher concentrations, it can inhibit the β1 and β2 subunit and induce the accumulation of ubiquitinated proteins. It has improved pharmacokinetics and pharmacodynamics profiles.(9)(12)

### *Immunoproteasome inhibitors*

As mentioned before, the practical use of unspecific proteasome inhibitors is recurrently associated with toxicity, being the most severe peripheral neuropathy and gastrointestinal effects. This has encouraged scientists to search and develop more specific inhibitors of the individual subunits immunoproteasome complex.(33)

Most of these inhibitors contain the chemical structure of a peptide backbone and selectively target individual subunits of the immunoproteasome. Although there is a similarity in the substrate preferences of the constitutive proteasome and immunoproteasome subunits, distinct structural features and amino acid characteristics of the substrate-binding channels β1c and β1i as well as β5c and β5i could be identified and subsequently allowed for the development of specific inhibitors. On the other hand, the design of inhibitors targeting exclusively β2c or β2i however remains a challenge because of the high structural similarity between the trypsin-like active sites of these subunits.(13)(32)(34)

- β1i-Selective Compounds: UK-101, IPSI-001, YU-102 and LU-001i.(32)(35)
- β2i-Selective Compounds: LU-002i.(34)
- β5i-Selective Compounds: ONX 0914 (PR-957) and PR-924 (IPSI).(32)

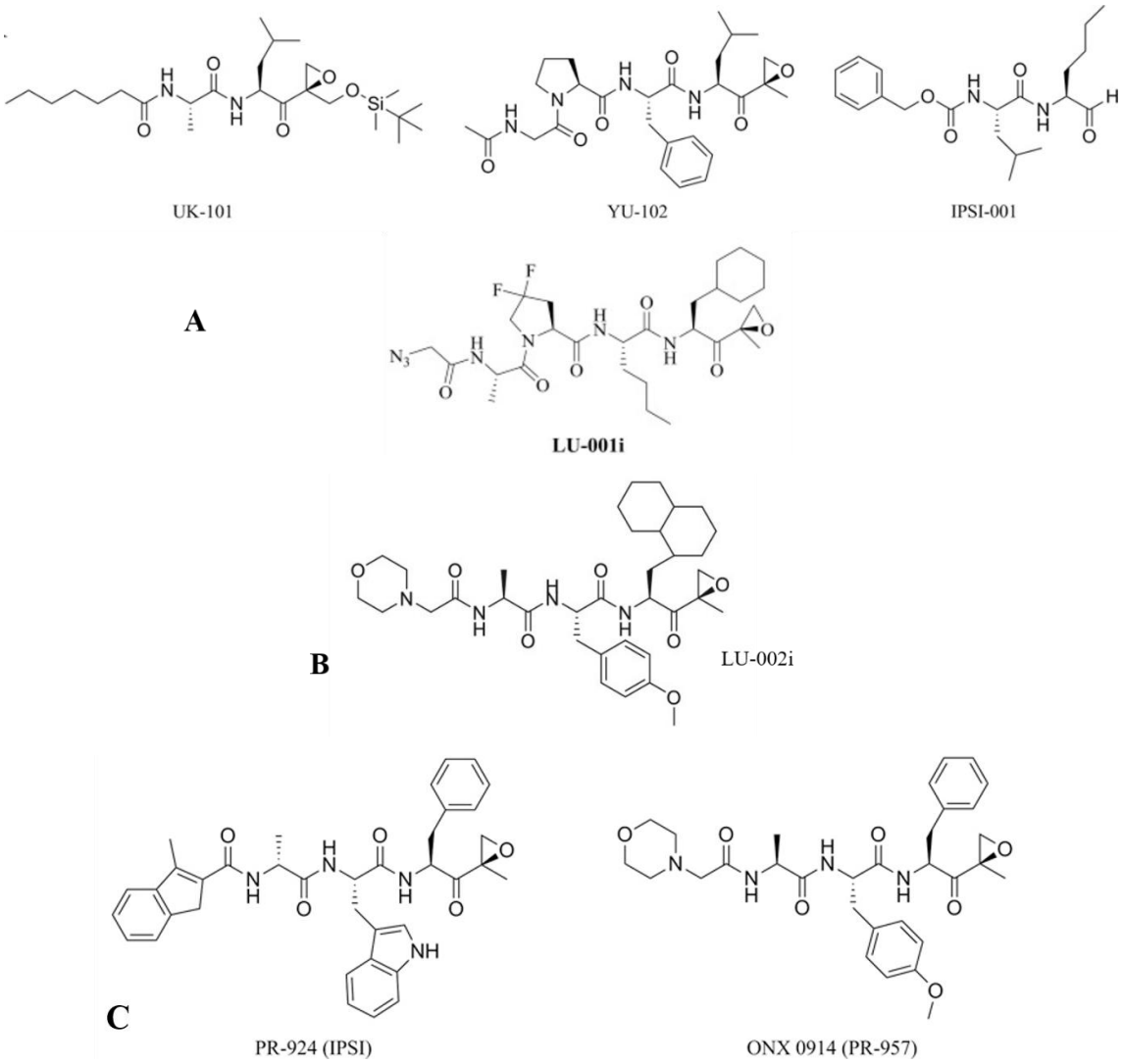


**Figure 5: Chemical structures of immunoproteasome-selective inhibitors targeting β1i (A), β2i (B) and β5i (C). Adapted from** (32)(35)(36)**.**

As the knowledge about the immunoproteasome advances, there is a crescent need to develop novel immunoproteasome inhibitors with higher potency and selectivity.

## 1.6. Dendritic cells

Dendritic cells (DCs) are highly specialized professional antigen-presenting cells with unique morphological and molecular properties enabling their most important function as "guards" of the immune system. These cells have the ability to induce primary immune responses necessary in innate immunity and adaptive immunity. DCs are derived from hematopoietic stem cells in the bone marrow and are originated from both myeloid and

lymphoid progenitors. Both subclasses, cDC and pDC, are derived from a common $CD34^+$ progenitor.(37)

DCs are found in an immature differentiation state (iDCs) in non-lymphoid peripheral tissues preferentially in the skin and mucosa that interface with the environment, detecting easily foreign antigens and microbial pathogens. (30)(38)(39)

Upon pathogen invasion, immature DCs become activated and are recruited to sites of inflammation in peripheral tissues. Internalization of foreign antigens can consequently trigger their maturation and migration from peripheral tissues to the T cell areas of the spleen and lymph nodes where they "communicate" naive T-lymphocytes to induct adaptive immune responses. During their migration, DCs evolve from immature, antigen-capturing cells to the most efficient antigen-presenting cells (APCs) by enhancing the cell surface expression of peptides, co-stimulatory molecules such as CD40, CD80 and CD86, as well as MHC class I and class II molecules to initiate an immune response through T-cell activation and generation. This process assures the communication of the molecular message obtained in the periphery to other cell varieties of both innate and adaptive immunity such as neutrophils, granulocytes, NKs, T cells, T- and B-lymphocytes. DCs present antigenic peptides complexed with MHC class I molecules to CD8-expressing T cells in order to generate cytotoxic cells. (30)(37)(38)(39)

Cell surface receptors help facilitate antigen uptake and also mediate physical contact between DCs and T cells. DC-specific intercellular adhesion molecule ICAM grabbing non-integrin (DC-SIGN), is a DC-specific ligand for ICAM-3 expressed on naive T cells. DC-SIGN can promote a transient clustering between a DC and T cell, thus allowing the DC to screen numerous T cells for an appropriately matched TCR.(39)

The cytokine profile secreted by DCs varies with the nature of the stimulus, stage of the DC maturation and the existing cytokine environment, influencing the different functions characteristic of immature and mature DCs. A wide variety of cytokines may be expressed by mature DCs including IL-12, IL-1 α, IL-1 β, IL-15, IL-18, IFN-α, IFN-β, IFN-γ, TNF-α, and MIF. The cytokine range expressed determines their Th1/Th2 differentiating capacities. Antigens that make DCs secrete IL-12 typically induce Th1 differentiation, whereas antigens that do not elicit or, on the contrary, inhibit IL-12 production promote Th2 differentiation.(39)

# 2. Research Aim

The immunoproteasome plays a very important role in cytokine production and regulation of several processes involved in cell differentiation. Hence, it seems expected to also influence the production of the cytokines and other chemical elements necessary for the maturation, proliferation and regulation of DCs, and their interaction with T cells. This master thesis aims to study the potential effect of selective (immune)proteasome inhibitors on the differentiation and activation of DCs.

To do so, we isolated monocytes from peripheral blood mononuclear cells (PMBCs) and induced their differentiation to dendritic cells. We treated them with the chosen inhibitors and observed their potential influence on the differentiation and maturation process to immature or mature DCs. We used selective proteasome and immunoproteasome inhibitors that target the following subunits: β1c (LU-001c), β1i (LU-001i), β1c/β1i (NC-001), β2c (LU-002c), β2i (LU-002i), β2c/β2i (LU-102), β5c (LU-025c), β5i (LU-015i), β5c + β5i, β5i + β2i and β5i + β1i.

The project was divided into three segments, each one with specific aim: the first was designed to study the effect of the (immune)proteasome inhibitors in the differentiation of monocytes to iDCs and their cytokine production; the second segment addressed the effect of the inhibitors on the maturation of iDC; in the last part, we tested how (immune)proteasome inhibition influences cell-mediated toxicity.

All experiments were conducted between February and April 2019.

# 3. Materials and Methods

## 3.1. Materials

### 3.1.1. Biological materials

| Biological Material | Acquired |
|---|---|
| Blood of healthy individuals | Healthy donors obtained at the Blood Transfusion Centre of Slovenia |

| | |
|---|---|
| Frozen PBMCs | Cell bank of the University of Ljubljana, Faculty of Pharmacy, Chair of Clinical Biochemistry |
| K562 cells | ATCC |

## 3.1.2. Media, Chemicals, Prepared Solutions and Reagents

| **Media and Chemicals** | **Company** |
|---|---|
| Bovine Serum Albumin (BSA) | Sigma-Aldrich |
| Ficoll Paque Plus | Sigma-Aldrich |
| Phosphate Buffer Saline (PBS) | Sigma-Aldrich |
| EDTA | Sigma-Aldrich |
| MojoSort™ Buffer (5X) (Cat. No. 480017) | BioLegend |
| MojoSort™ Human CD14+ Monocytes Isolation Kit | BioLegend |
| Recombinant human GM-CSF | BioLegend |
| Recombinant human IL-4 | BioLegend |
| Penicillin-Streptomycin Solution | Sigma-Aldrich |
| Recombinant human IL1β | Thermo Fischer |
| Recombinant human TNF-α | Thermo Fischer |
| Antibody anti-CD14 | Thermo Fischer |
| Antibody anti-CD80 | BioLegend |
| Antibody anti-CD86 | BioLegend |
| Antibody anti-DCSIGN | BioLegend |
| Antibody anti-HLADR | BioLegend |
| RPMI-1640 Medium for cell cultivation with additional 10% FBS, 1% Ab/Am and 1% L-Glu | Sigma-Aldrich |
| Carboxyfluorescein succinimidyl ester (CFSE) | Thermo Fischer |
| 1000 IU/mL GM-CSF, 1 µg/mL PGE-2, 200 IU/mL IL-1ß, 1000 IU/mL TNF-α and 1000 IU/mL IL-6 | |
| CBA Human Th1/Th2 cytokine kit II | BD Biosciences |
| Serum Enhancement Buffer | |

## 3.2. Equipment

### 3.2.1 Laboratory Equipment and Machines

| Name | Type | Company |
|---|---|---|
| Flow Cytometer | Attune NxT Acoustic Focusing Cytometer | Thermo Fisher |
| Centrifuge | Megafuge 16R | Thermo Fisher |
| $CO_2$ Incubator | MCO-18AIC(UV) | SANYO |
| Freezer | | Gorenje |
| Microscope | CK40 | OLYMPUS |
| Refrigerator | | Gorenje |
| Water bath | | Memmert |
| Microplate reader | Synergy HT | Biotek |
| Water purification system | | ELGA |
| Automatic pipettes | Research Plus | Eppendorf |
| Cell culture microplates | Tissue culture test | TPP |
| Centrifuge tubes | 15 mL and 50 mL | TPP |
| Counting chamber (Neubauer) | | Brand |
| Tubes | 0,5 mL; 1,5 mL | Eppendor |
| Multichannel pipette | Explorer | Eppendorf |
| Pipette controller | Midi Plus | BIOHit Midi Plus |
| Tubes for cytometry | 5 mL | FALCON |
| Vortex (mixer) | Bio Vortex B1 | BIOSAN |
| Serological pipette (2, 5, 10, 25, 50 mL) | | TPP |
| Plastic disposable transfer pipette | | |
| 70 µm filters | | BD Biosciences |
| EasySep Magnet | | StemCell Technologies |
| 6 and 24-well tissue culture plate | | TPP |

## 3.3. Experimental Procedures

### 3.3.1. Isolation of PBMCs from human blood by density gradient centrifugation

Density gradient centrifugation is the most common method for isolating PBMCs, specifically lymphocytes and monocytes. For this isolation procedure, PBMCs are centrifuged in the presence of a density gradient media, such as Ficoll. Each cell population exhibits a unique migration pattern through the medium that is related to the density of the cell, creating distinct layers of cell populations. This allows the cells to be isolated by extracting their respective layer.(40)

All the following steps were performed in a laminar flow hood:

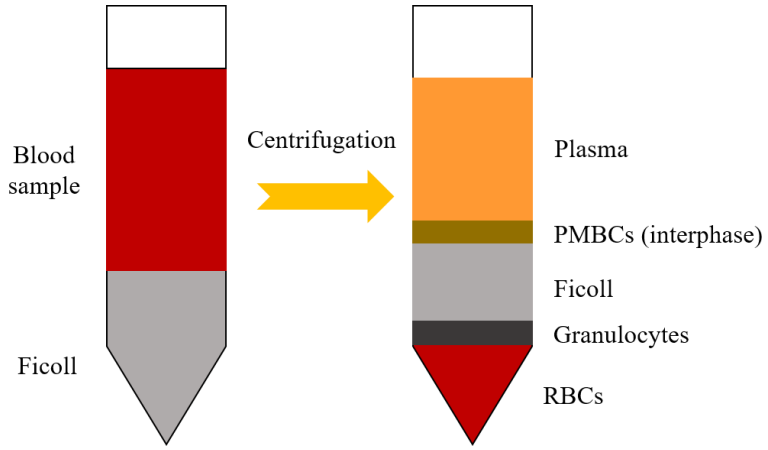


**Figure 6**: **Schematic presentation of PBMCs isolation from human blood by density gradient centrifugation. After centrifugation, five distinct bands are noted. Plasma has the lowest density and remains at the top, PBMCs forming an interphase, a Ficoll layer in-between, and Granulocytes and RBCs cells at the bottom of the tube. These last managed to pass through Ficoll due to their higher density. Adapted from** (47)**.**

1. We transferred 50 mL of blood concentrate in one 150 mL flask and diluted with sterile 1 x PBS in a V/V ratio 1:2, getting a total of 150 mL of diluted blood concentrate.

   Ficoll solution was left at room temperature for about 30 minutes and then divided into two 50 mL conical tubes, with 12,5 mL in each. 25 mL of diluted blood was carefully layered over each Ficoll solution in a V/V ratio of Ficoll : diluted blood = 1:2. The Ficoll solution is toxic to the blood cells so its addition to the blood should be handled with caution making sure that the blood and Ficoll do not mix.

2. Centrifugation at 2300 rpm for 17 minutes at 22ºC without break followed.
3. We aspired the upper layer (plasma) using a transfer pipette, leaving the mononuclear cell layer below undisturbed. Mononuclear cell layers from both conical tubes were carefully transferred to a new tube, using a new serological pipette.
4. We diluted the PBMCs with sterile 1 x PBS to 50 mL and centrifuged again at 2300 rpm for 7 minutes at room temperature with break on.
5. We completely removed the supernatant and resuspended the cell pellet in 10 mL of RPMI-1640 cell cultivation medium and counted the cells.
6. The cell suspension was again centrifuged under the same conditions as before and the supernatant aspired completely. We continued with the magnetic separation of obtained PBMCs.

### 3.3.2. Cell culture

Cell protocols were carried out in an aseptic environment keeping cells free from contamination. All cell procedures were performed in a laminar flow hood with the work surfaces and tools inside wiped with 70% ethanol before and after the procedure. Between uses, ultraviolet light was turned on to sterilize the air and surfaces under the hood. Outside containers, pipettes and other tools were also disinfected with 70% ethanol before they were placed under the hood.

#### 3.3.2.1 Manual cell counting using a haemocytometer

Manual cell counting using a haemocytometer is the simplest, most direct and cheapest method of counting cells in suspension. With the dye-exclusion method, it is also possible to determine the percentage of viable (intact) cells.

The haemocytometer is a device invented by the French anatomist Louis-Charles Malassez in the 19$^{th}$ century to perform blood cell counts. The most frequently used haemocytometer is the Neubauer chamber. It is a thick glass slide that bears two polished surfaces each of which displays a precisely ruled grid subdivided into nine primary squares, each measuring 1 mm$^2$, and limited by three closely spaced lines (2,5 µm apart),

which are used to determine if cells lie within or outside the grid. The central counting area of the haemocytometer contains 25 large squares and each square has 16 smaller squares. There is a depression at the outer edge of each polished surface, where cell suspension is added to be drawn across the grip by capillary action. Given the known parameters it is possible to count the number of cells in a specific volume of fluid and thereby calculate the concentration of cells in the fluid overall.

Dye-exclusion involves mixing the cell suspension with a volume of buffer or balanced isotonic saline containing water-soluble dye, such as trypan blue, which is visible when it diffuses into cells with damaged plasma membranes. Thus, it selectively penetrates cell membranes of dead cells, coloring them blue, whereas it is not absorbed by membranes of live cells, excluding live cells from staining. By counting the number of unstained (undamaged) and stained (damaged) cells, the viability percentage can be calculated.(41)

**Procedure**

1. Cells in the complete medium were mixed well to assure complete homogeneity. An aliquot of 10 µL was taken.
2. To the aliquot we added Trypan Blue and sterile PBS and mixed well again.
3. A clean coverslip was placed carefully on top of the haemocytometer.
4. The haemocytometer was loaded by expelling 10 µL of the suspension to the edge, which was drawn into by capillary action.
5. Under a light microscope, we counted the unstained (viable) cells.
6. The total cell concentration in the original suspension (cells/mL) was calculated using **Equation 1**:

$$\text{N of cells/ml} = \frac{N1 + N2 + N3 + N4}{4} \times 2(\text{dilution}) \times 10^4$$

N – Number of cells

**Equation 1 – Equation used to count the number of cells in a sample using a haemacytometer.**

## 3.3.3. Monocytes isolation by magnetic separation

Magnetic cell sorting is a common technique used to obtain a highly pure population of cells of interest from a mixed population of cells, making use of microbead conjugated antibodies (Abs) against the cell surface antigens. The microbeads used are suggested to be non-toxic, biodegradable carriers conjugated to various antibodies. This method is designed for the isolation of untouched monocytes from PBMCs. Monocytes express typical blood monocyte markers, such as CD14 and CD16. We used the MojoSort™ Human $CD14^+$ Monocytes Isolation Kit protocol. Using this protocol cell populations other than $CD14^+$ $CD16^-$ monocytes are depleted by incubating the sample with the biotin antibody cocktail followed by incubation with magnetic with magnetic Streptavidin Nanobeads. The magnetically labeled fraction is retained by the use of a magnetic separator. The untouched cells (cells of interest) are then collected. (42)(43)(44)(48)

**Separation Protocol**

1. After the final wash of isolated PBMC, we resuspended the cells in MojoSort™ Buffer by adding up to 4 mL in FACS tube. The MojoSort™ Buffer was kept on ice throughout the procedure.
2. We filtered the cells with a 70 µm cell strainer, centrifuged at 300 xg for 5 minutes, and resuspended in an appropriate volume of MojoSort™ Buffer. Counted and adjusted the cell concentration to 1 x 108 cells/mL.
3. We aliquoted 1 mL of cell suspension (108 cells) into a new tube. Added 50 µL of Human TruStain FcX™ (Fc Receptor Blocking Solution), mixed well and incubated at room temperature for 10 minutes.
4. In the following step, we proceeded to add 100 µL of the Biotin-Antibody Cocktail. Mixed well and incubated on ice for 15 minutes.
5. Resuspended the beads by vortexing, maximum speed, 5 touches. Added 100 µL of Streptavidin Nanobeads. Mixed well and incubated on ice for 15 minutes.
6. Washed the cells by adding MojoSort™ Buffer up to 4 mL. Centrifuged the cells at 300 xg for 5 minutes.
7. We discarded the supernatant.
8. Added 2,5 mL of MojoSort™ Buffer.
9. Placed the tube in the magnet for 5 minutes.

10. After the 5 minutes, we poured out and collected the liquid that contained our cells of interest.

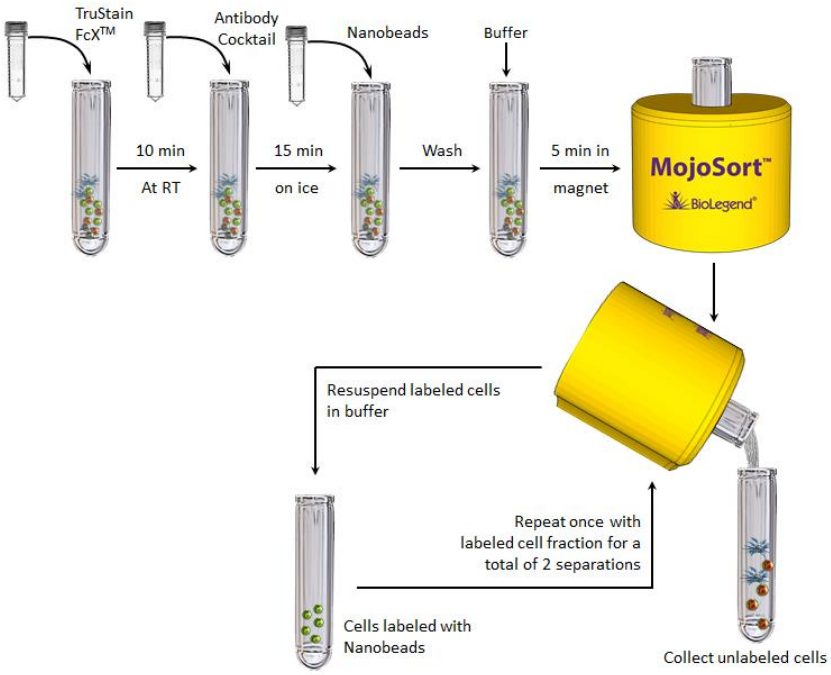


**Figure 7: Schematic representation of the protocol steps for the magnetic separation and isolation of the monocytes we used the MojoSort™ Human CD14$^+$ Monocytes Isolation Kit Protocol.**(48)

## 3.3.4. Monocytes differentiation to dendritic cells

An approach to study the biological functions of dendritic cells is to generate DC-like cells by culturing CD14$^+$ PBMCs in the presence of GM-CSF and IL-4. (45)

**Procedure**

**Day 0**

We started by re-suspending isolated monocytes at 0,8 x $10^6$ cells per mL in complete medium supplemented with 800 IU/mL GM-CSF and 400 IU/mL IL-4. 400 µL of monocytes suspension was transferred in a 48-well plate and treated with proteasome inhibitors at a concentration of 1 µM each. The rest of the monocytes were transferred to 6-well plate. These cells were incubated at 37 °C and 5% $CO_2$.

**Day 3:**

Both the 6-well plate and 48-well plate were centrifuged at 2000 rpm for 3 minutes. Half of the media was substituted with fresh complete medium supplemented with the same concentrations of GM-CSF, IL-4 and inhibitors.

**Day 6:**

We collected 0,8 x $10^5$ cells (100 µL) into FACS tube and added 0,7 µl of each antibody (anti-CD14, anti-CD80, anti-CD86, anti-DCSIGN, anti-HLADR) for 10 minutes at room temperature and then diluted with 100 µl of PBS and analyzed the samples on the flow cytometer. The immature human derived-DCs (iDC) were thus ready for further experimental use.

We took the cell suspension out of the 6-well plate and 48-well plate into a 15mL tube and 1,5 ml tubes and centrifuged at 1800 rpm for 10 minutes at 4 °C.

We discarded the supernatant and re-suspend cells at 1 x $10^6$ cells/ml in complete RPMI-1640 medium supplemented with 1000 IU/mL GM-CSF, 1 µg/mL PGE-2, 200 IU/mL IL-1ß, 1000 IU/mL TNF-α and 1000 IU/mL IL-6.

We then placed 0,2 x $10^6$ iDCs/well in a 48-well plate and incubated the iDCs in the presence or absence of proteasome inhibitors at the same concentration as before for 24 hours to generate mature DC (mDC).

**Day 7:**

Flow cytometry procedure:

- The 48-well plate was on ice for 10 minutes.
  Cells (mDC) were re-suspended and transferred to FACS tubes.
- 0,7 µl of each antibody (anti-CD14, anti-CD80, anti-CD86, anti-DCSIGN, anti-HLA-DR) was added and incubated for 10 minutes at room temperature. Afterward, samples were diluted with 100 µl of PBS and analyzed on the flow cytometer.
- Cells were gated according to their light-scattering properties to exclude cell debris and contaminating lymphocytes.

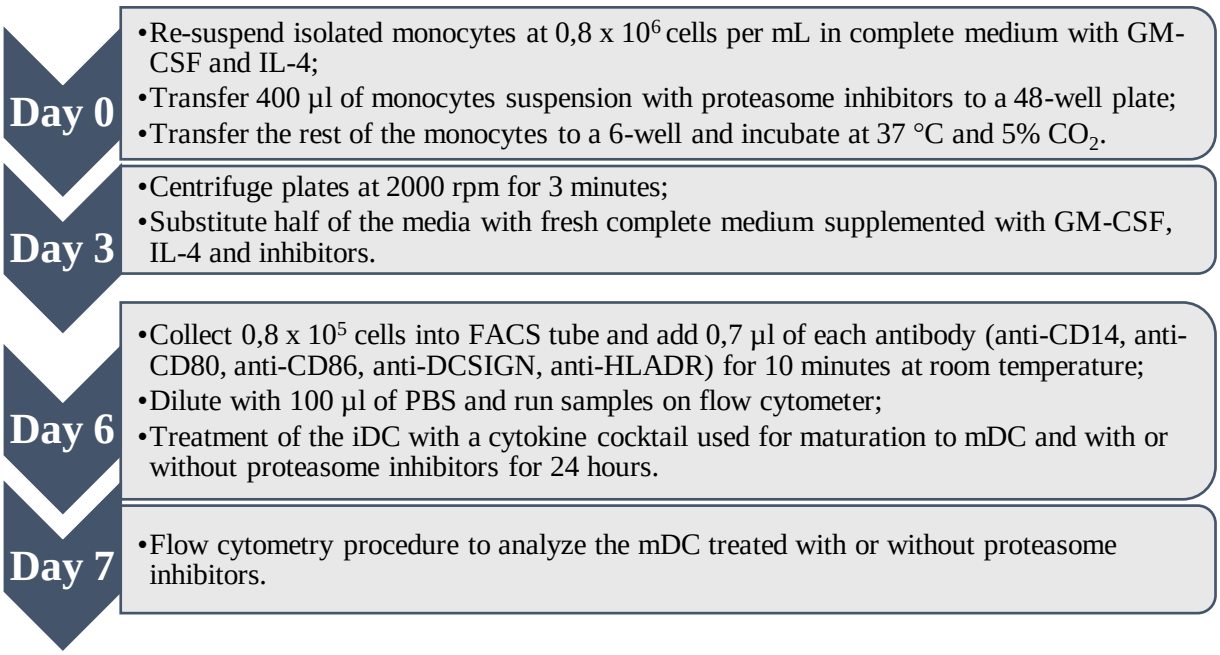


**Figure 8: Schematic representation of the protocol steps for isolated monocytes differentiation to immature dendritic cells (iDC).**

## 3.3.5. PBMC cytotoxicity towards K562 cells

In this experiment the parameter chosen to study was addition of proteasome inhibitors specific for the constitutive proteasome (β1c, β2c and β5c), specific for the immunoproteasome (β1i, β2i, β5i, β1i+β5i and β2i+β5i) and unspecific (β1c+β2i, β2c+ β2i and β5c+ β5i) to the PBMCs in the presence of K562 cells. We observed how the viability of the tumor cell line was altered.

1. The cell concentration of isolated PBMC was adjusted to 8 x $10^6$ cells/mL with complete RPMI-1640 medium and 50 µL of cell suspension was transferred in each well of 96-U-well plate.
2. Proteasome inhibitors were prepared in a 2-fold higher concentration (2 µM) in complete medium separately.
3. 50 µL of each inhibitors solution was transferred to PBMC cells in 96-well plate in duplicates.
4. PBMC cells were treated with inhibitors for 20 hours.
5. The next day, K562 cells were transferred from flask to 15 mL tube, counted and centrifuged at 1300 rpm for 5 minutes.

6. The supernatant was discarded and cells were re-suspended in 5 mL of 2,5 µM solution of CFSE in sterile 1 x PBS and mixed well.
7. Cell suspension in CFSE solution was placed in the incubator for 15 minutes, then centrifuged at 1800 rpm for 5 minutes.
8. The supernatant was discarded and cells were re-suspended in 10 mL of 1 x PBS and then centrifuged again at 1800 rpm for 5 minutes.
9. The supernatant was discarded and cells were re-suspended in complete RPMI-1640. Cell concentration was adjusted to 1 x $10^5$ cells/mL.
10. 100 µL of CFSE labelled K562 cells (10000 cells) were added to treated PBMC cells in 96-U-well plate. The effector cells-target cells was 40:1.
11. After 4 hours 0,8 µM Sytox Blue was added to each sample to label dead cells. Samples were analyzed on the flow cytometer. Cells were gated according to their light-scattering properties to exclude cell debris and gated on CFSE and Sytox Blue positive cells.

## 3.3.6. Cytokine assay

We used the BD™ cytometric bead array (CBA) human Th1/Th2 cytokine kit II to quantitatively measure IL-2, IL-4, IL-6, IL-10, TNF, IFN-γ protein levels in our sample. The inhibitors studied were immunoproteasome specific: β1i, β2i, β5i, β1i+β5i, β2i+β5i.

**Principle of CBA assays**

BD CBA assays allow the capture of a soluble analyte using beads of known size and fluorescence and detecting these analytes using flow cytometry. Each capture bead is conjugated with a specific antibody that connects with a specific cytokine. The detection reagent provided in the kit is a mixture of phycoerythrin (PE)-conjugated antibodies, which provides a fluorescent signal in proportion to the amount of bound analyte. When the capture beads and detector reagent are incubated with an unknown sample containing recognized analytes, sandwich complexes (capture bead + analyte + detection reagent) are formed. These complexes can be measured using flow cytometry to identify particles with fluorescence characteristics of both the bead and the detector.

The BD CBA Human Th1/Th2 Cytokine Kit II uses six bead populations with distinct fluorescence intensities covered with capture antibodies specific for IL-2, IL-4, IL-6, IL-10, TNF, and IFN-γ proteins.(46)

During the assay procedure, we mixed the cytokine capture beads with our sample treated with the chosen inhibitors and incubated them with the PE-conjugated detection antibodies to form sandwich complexes, according to the manufacturer's protocol steps. The intensity of PE fluorescence of each sandwich complex revealed the concentration of that cytokine. After acquiring samples on a flow cytometer, we used FlowJo software to generate results in graphical and tabular format.

### 3.3.7. Flow cytometry analysis

Flow cytometry is used for the analysis of multiple characteristics of the cells. It can measure cell size, cytoplasmic complexity, DNA or RNA content, and a wide range of membrane-bound and intracellular molecules. It is also used for measuring fluorescence intensity (FI) produced by fluorescent-labeled antibodies that bind to target proteins or ligands in the cell.

After the cells were stained with different antibodies, we analyzed them with a flow cytometer (Attune NxT from Thermo Fischer Scientific). The expression of different markers was determined by measuring the fluorescence of fluorophore-conjugated antibodies. The analysis was performed with FlowJo software.

# 4. Results and Discussion

As dendritic cells are antigen-presenting cells and play an important role in the induction of immune response, we aimed to investigate the role of the (immuno)proteasome in these cells. As previously mentioned, the proteasomes are multicatalytic enzyme complexes, which degrade proteins and regulate numerous intracellular processes. The later also includes the development and functionality of several immune cells. In this research, we addressed the potential role of (immuno)proteasome inhibition on the development and maturation of dendritic cells derived from PBMCs.

## 4.1. The role of (immuno)proteasome inhibition on the differentiation of monocytes to iDCs

The DCs present a crucial part in the presentation of antigens to other immune cells, which leads to the activation of the immune response. We wanted to address how the inhibition of individual catalytically active subunit of the (immuno)proteasome affects the development of iDC. For this purpose we treated monocytes that were differentiated to immature DCs with selective inhibitors of the (immuno)proteasome. Each cell type can be identified based on the set of markers it expresses (e.g. T cells with CD3, monocytes with CD14, B cells with CD19, etc). For DCs expression of adhesion molecules like CD50 (ICAM-2), CD54 (ICAM-1), CD58 (LFA-3), and CD102 (ICAM-3) is typical. Furthermore, DCs also represent costimulatory molecules including CD80, and CD86, which are upregulated through DC activation. CD86 is defined as a marker of primary DC maturation, while CD80 only increases in mature DC. This wide variety of molecular markers help identify the phenotype and function of dendritic cells.(37)

We analyzed the expression profile of relevant CD markers, which included CD14, CD80, CD86, DC-SIGN, HLA-DR. The initial step was isolating the monocytes from PBMCs as described in Chapter 3.3.1. of Experimental Procedures section. The differentiation of monocytes to immature DCs followed and was performed in the presence or absence of selective (immuno)proteasome inhibitors. After treatment, the cells were stained with antibodies against selected markers and analyzed by flow cytometry.

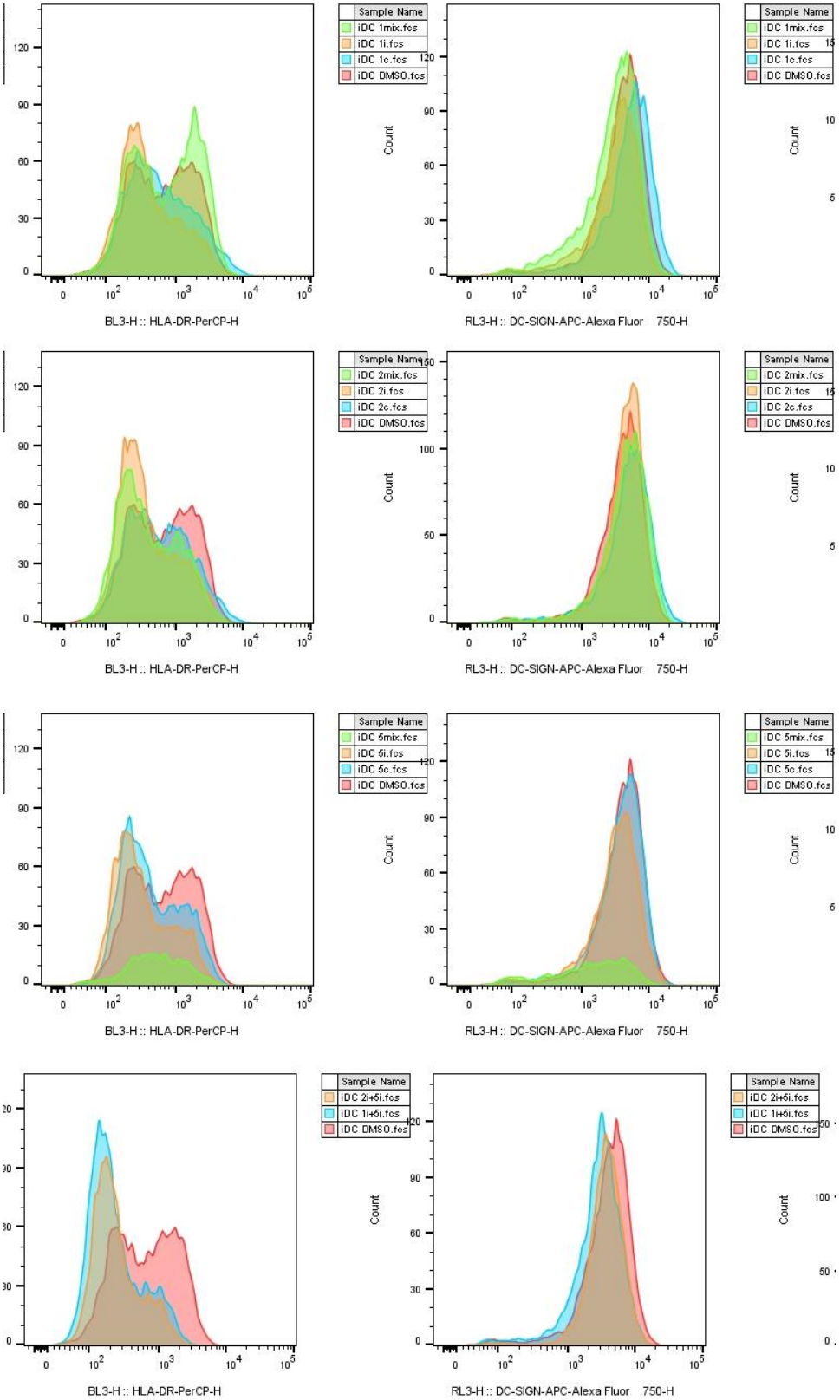


**Figure 9: Histogram overlays of the expression of HLA-DR and DC-SIGN markers in iDCs in the presence of (immuno)proteasome inhibitors.**

The analysis of the obtained data (Annex 1), shows that only the expression of HLA-DR and DC-SIGN was significantly affected in the presence of the inhibitors (Figure 8). We can observe a lower expression of both markers in practically every treatment with (immuno)proteasome inhibitors. However, we can observe that the inhibitors of the immunoproteasome's catalytic subunits possess a more prominent effect on the expression of HLA-DR and DC-SIGN than the inhibitors of the proteasome. Moreover, we observed a synergistic effect when the β5i inhibitor LU-015i was used in combination with inhibitors of the β5c, β2i or β1i subunits. The decrease of these markers indicates a limited interaction with $CD4^+$ T cells since HLA-DR presents antigenic peptides to naive $CD4^+$ T lymphocytes, while DC-SIGN leads to activation of $CD4^+$ T cells. This indicates that the immunoproteasome inhibitors could lead to suppression of the differentiation of monocytes to iDC and activation of $CD4^+$ T cells. This effect could be desirable in the treatment of T cell-mediated autoimmune pathologies. In these cases, the inhibition of the immunosubunits could reduce inflammatory infiltration, cytokine and autoantibody production, and thus attenuate disease symptoms. Further experiments with the β5i inhibitor should be addressed in the future to study its potentiality in disease therapy.

### 4.1.1. Inhibition of the Immunoproteasome had no effect on cytokine production of DCs

The cell-cell communication, which is pivotal for a proper immune response, is based on direct interactions of cells (e.g. through receptors) as well as indirect interactions, which are mediated through secretion of signaling molecules (e.g. cytokines). The proteasomes, especially the immunoproteasome, are known to be involved in the regulation of several signaling pathways that are shaping the cytokine synthesis and secretion. The most potent effect on iDC's expression on HLA-DR and DC-SIGN was observed when selective inhibitors of the immunoproteasome were used (individually or in combination). Therefore, we investigated only the potential effect of β1i, β2i, β5i, β1i+β5i and β2i+β5i inhibition on the secretion of selected cytokines from iDC. In order to study the role of the immunoproteasome in the regulation of cytokine production in iDCs we conducted a cytokine assay. We used the Bd™ cytometric bead array (CBA) human Th1/Th2 cytokine kit II, as explained in Chapter 3.3.6. of Experimental Procedures section.

After the analysis we concluded, that in this experimental settings, no relevant effect on the secretion of IL-2, IL-4, IL-10, IL-6, TNF-α and IFN-γ could be observed (Figure 9, Annex 2).

The analyzed cytokines were investigated due to reagent availability. These results are not expected and may have been due to the low concentration of the inhibitors used or due to the fact that the cytokines studied may have their secretion independent of the immunoproteasome. We conclude that the regulation of other relevant cytokines by immunoproteasome deserves further investigation (e.g TNF, IL-12).

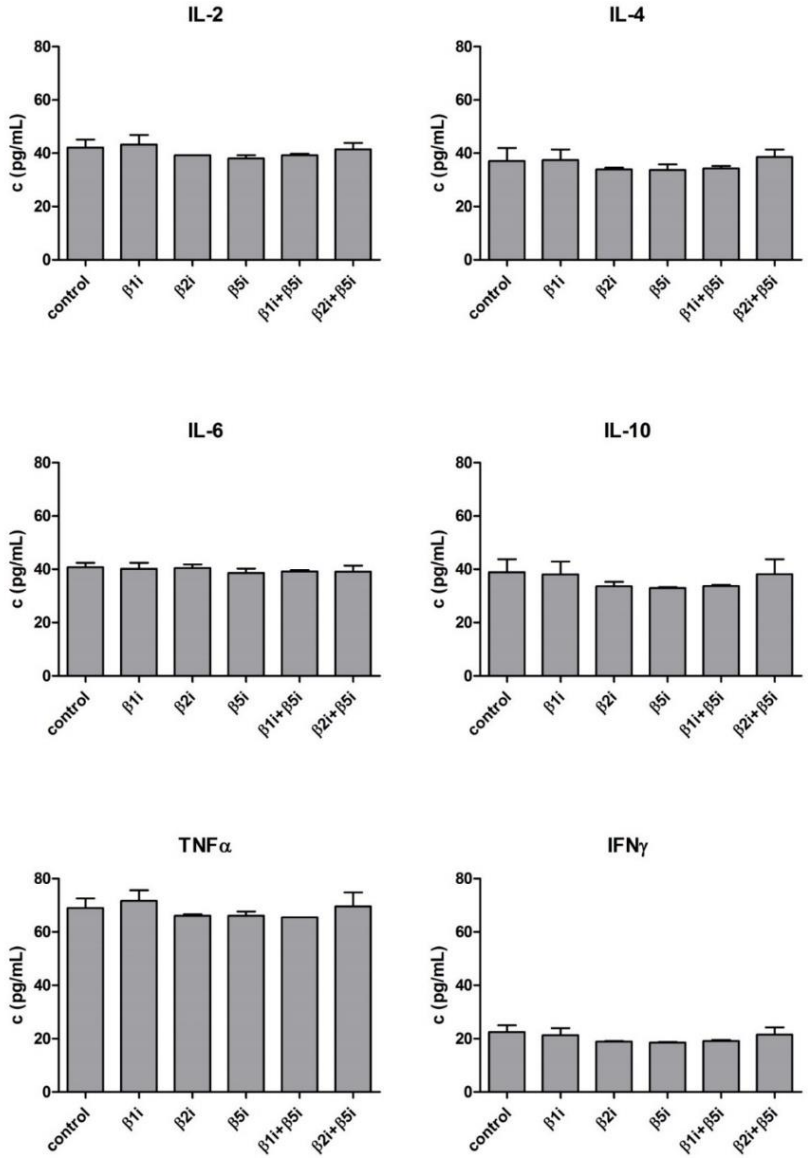


**Figure 10: Secretion of the cytokines IL-2, IL-4, IL-6, IL-10, TNF-α and IFN-γ by DCs differentiated in the presence of immunoproteasome inhibitors.**

## 4.2. The role of (immuno)proteasome inhibition on the maturation of iDC

As already mentioned, DCs are antigen-presenting cells that play an important role in connecting the innate and adaptive segments of the immune system. DCs can be present in immature and mature forms, which differ in phenotype and morphology and subsequently have different functional characteristics. Therefore, we also wanted to address, whether the inhibition of individual catalytically active subunit of the (immuno)proteasome affects the process of iDC maturation into mDCs. For this purpose we performed two different experimental settings:

- (Immuno)proteasome inhibitors present only during the differentiation, but not the maturation process;
- (Immuno)proteasome inhibitors present during the process of differentiation and maturation.

In both settings maturation of iDCs was governed by the addition of GM-CSF, PGE-2, IL-1β, TNF-α and IL-6. Next day analysis of relevant CD markers was performed and included, CD14, CD80, CD86, DC-SIGN and HLA-DR.

If the cells were treated with inhibitors through the process of differentiation, but not through maturation, almost no effect could be observed on the expression of the markers, except in the case of simultaneous inhibition of β5c and β5i subunit. This combination specifically led to a notable decrease in the expression of CD80, CD86 and CD14 markers, indicating a diminished maturation of the DCs. The only setting where an increase was observed was for the marker DC-SIGN if the inhibitor for β2i was used alone or in combination with β2c or β5i inhibitors (Annex 3). This could mean that different catalytic subunits of the immunoproteasome regulate distinct intracellular pathways, which lead to the different phenotype. On the other hand, if inhibitors were present during differentiation and maturation, we could observe a significant increase in expression in CD80, HLA-DR and DC-SIGN, while the expression profile of CD86 and CD14 was only minor or not at all affected (Annex 4). This indicates that the presence of the inhibitors shifted the dendritic cell to a more mature type. Taken together, it seems that modulation of (immuno)proteasome plays a role in the development of DCs. However, further repeats of the experiments and a more in depth investigation of the underlying pathways involved need to be performed in the future. The functionality of

the DCs treated with (immuno)proteasome inhibitors should be addressed to point out the relevance of the obtained results. For example, the phagocytosis level of DCs and their potential to trigger T cell activation, since these are some of the main roles of DCs in the *in vivo* system.

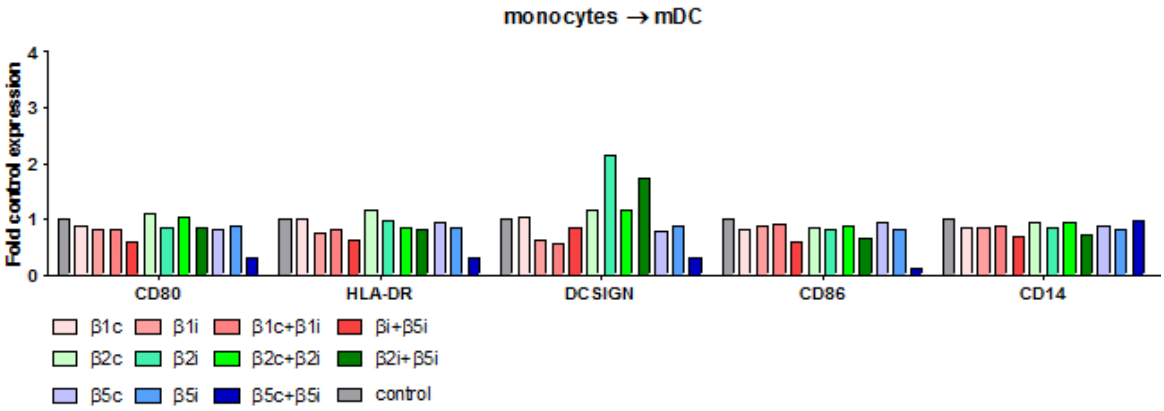


**Figure 11: Fold control expression of CD14, CD80, CD86, DC-SIGN and HLA-DR by mature DC after differentiation and maturation in the presence of the inhibitors.**

## 4.3. The role of (immuno)proteasome inhibition in the cell-mediated cytotoxicity

PBMCs originate from hematopoietic stem cells that reside in the bone marrow, through a process called hematopoiesis. PBMCs are blood cells with round nuclei that encompass a heterogeneous cell population comprising various types of lymphocytes (T cells, B cells, and NK cells), and monocytes. These cells are critical components of the innate and adaptive immune system that defend the body against viral, bacterial, and parasitic infection and destroys tumor cells and foreign substances.

Lastly, we addressed whether PBMCs mediated cell toxicity is affected by the immunoproteasome and proteasome. In this experimental setting, PBMCs were treated with the selective (immuno)proteasome inhibitors for 24 hours. Afterward, CFSE labeled K562 leukemic cells were added and their death in the presence of PBMCs was determined with a viability dye and flow cytometry.

Cells were gated according to their light-scattering properties to exclude cell debris and gated on CFSE and viability dye positive cells. The results are given as percentages of control (cell death of K526 in the presence of non-treated PBMCs).

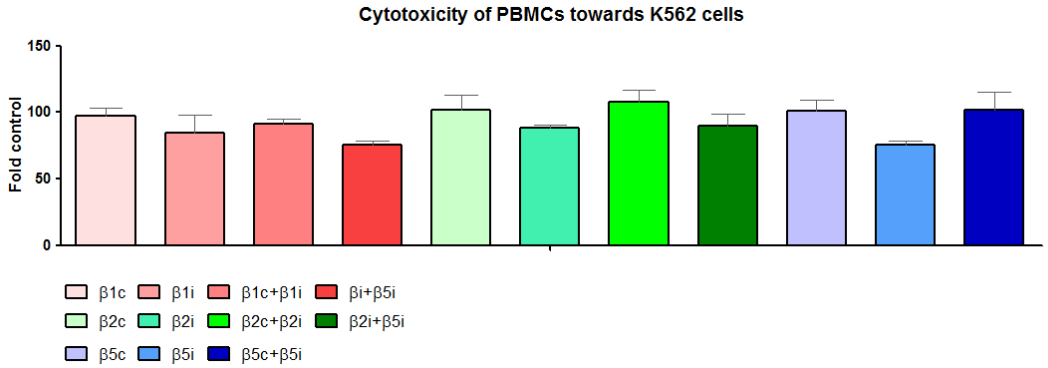


**Figure 12: Levels of PBMCs cytotoxicity in our sample against K562 cells when treated with different inhibitors. The results are a percentages of cytotoxicity with deducted spontaneous dead K562 cells normalized to control sample (DMSO).**

We can observe that, if compared to control (which is 100%), the inhibitors mostly do not affect the PBMC mediated cell toxicity (Annex 5). We noticed though a decreased death of K526 if β5i or β1i subunit inhibitors were present.

As mentioned before, the proteasome is central to proteostasis network functionality and its over-activation represents a hallmark of advanced tumors and therefore presents itself a valid anticancer target. However, the decrease of PBMC cytotoxicity, mainly CD8 T cells and NK cells, towards a tumor cell line indicates that immunoproteasome inhibitors in an *in vivo* setting may hamper the antitumor response of immune cells, which are pivotal in the effective fight against malignant cells. Thus their use in the pathology of cancer would be questionable. However, several additional studies would be needed to confirm this observation.

# 5. Conclusions

The results obtained from this master's thesis suggest that (immuno)proteasome inhibitors can influence the differentiation and maturation of DCs. When studying the effect of selective (immuno)proteasome inhibitors in the differentiation of monocytes to immature DCs, we observed that the immunoproteasome inhibitors could lead to suppression of the differentiation of monocytes to iDC. The cytokine assay conducted to study the role of the immunoproteasome in the secretion of selected cytokines from iDC showed no relevant results for the selected cytokines. We conclude that the regulation of other cytokines by immunoproteasome deserves further investigation.

We also addressed the way the inhibition of individual catalytically active subunit of the (immuno)proteasome affects the maturation of the iDCs to mDCs. It seems that when DCs were treated with inhibitors only during their differentiation and not maturation, these have little effects on the expression of the studied markers, except during simultaneous inhibition of β5c and β5i subunit, where a lowered maturation profile can be observed. An increase of DC-SIGN was noted which could mean that different catalytic subunits of the immunoproteasome regulate distinct intracellular pathways, which lead to the different phenotype. However, when DCs were treated with inhibitors through the process of differentiation and maturation, the results suggest that the presence of the inhibitors shifts the DCs maturation process toward a more mature profile.

Lastly, we observed almost no effect of (immuno)proteasome inhibitors on PBMC mediated cell toxicity towards the malignant K562 cell line. A slightly lowered cytotoxicity could be observed if the β5i and/or β1i inhibitors were used, indicating a diminished immune response to cancer cells.

Of all the inhibitors used during the experimental work, it seems that β5i catalytic subunit plays a role in the functionality of DCs, since the most prominent effects were observed if the β5i inhibitor LU-015i was used. However, further studies are needed, where more biological repeats are provided (we were not able to make more repeats due to limited time), the concentration of the inhibitors are further optimized and additional functionality assays are performed. Nonetheless, data indicates that the immunoproteasome plays a role in the differentiation and maturation process of DCs.

# 6. References


1. Hoffman JR, Falvo MJ. Protein - Which is Best? J Sports Sci Med [Internet]. 2004 Sep [cited 2019 Jun 1];3(3):118–30. Available from: http://www.ncbi.nlm.nih.gov/pubmed/24482589
2. Chen B, Retzlaff M, Roos T, Frydman J. Cellular strategies of protein quality control. Cold Spring Harb Perspect Biol [Internet]. 2011 Aug 1 [cited 2019 Jun 2];3(8):a004374. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21746797
3. Morimoto RI, Cuervo AM. Protein Homeostasis and Aging: Taking Care of Proteins From the Cradle to the Grave. Journals Gerontol Ser A Biol Sci Med Sci [Internet]. 2009 Feb 1 [cited 2019 Sep 1];64A(2):167–70. Available from: https://academic.oup.com/biomedgerontology/article-lookup/doi/10.1093/gerona/gln071
4. The Stress of Misfolded Proteins in Aging and Neurodegenerative Disease [Internet]. [cited 2019 Nov 13]. Available from: https://www.movementdisorders.org/MDS/News/Online-Web-Edition/Archived-Editions/The-Stress-of-Misfolded.htm
5. Wolf DH, Hilt W. The proteasome: a proteolytic nanomachine of cell regulation and waste disposal. 2004 [cited 2019 May 11]; Available from: http://www.elsevier.com/locate/bba
6. Ciechanover A. The Ubiquitin System Historical Perspective. Proc Am Thorac Soc [Internet]. 2010 [cited 2019 Feb 20];7. Available from: www.atsjournals.org
7. Varshavsky A. The early history of the ubiquitin field. Protein Sci [Internet]. 2006 [cited 2019 Feb 12];15. Available from: http://www.proteinscience.org/cgi/
8. Kresge N, Simoni RD, Hill RL. The Discovery of Ubiquitin-mediated Proteolysis by Aaron Ciechanover, Avram Hershko, and Irwin Rose. J Biol Chem [Internet]. 2006 [cited 2019 May 11];281(40):32. Available from: http://www.jbc.org/
9. Guedes RA, Serra P, Salvador JAR, Guedes RC. Computational approaches for the discovery of human proteasome inhibitors: An overview. Molecules. 2016;21(7):1–27.
10. Hausman RE, Geoffrey M.Cooper. Protein Degradation. In: Cooper, G The Cell: A Molecular Approach Tools of Cell Biology. 2007. p. 344.
11. Proteolysis - Definition, Structure and Function | Biology Dictionary [Internet]. [cited 2019 Nov 13]. Available from: https://biologydictionary.net/proteolysis/
12. Kubiczkova L, Pour L, Sedlarikova L, Hajek R, Sevcikova S. Proteasome inhibitors - molecular basis and current perspectives in multiple myeloma. J Cell Mol Med [Internet]. 2014 Jun [cited 2019 Jun 8];18(6):947–61. Available from: http://www.ncbi.nlm.nih.gov/pubmed/24712303
13. Crawford LJ, Walker B, Irvine AE. Proteasome inhibitors in cancer therapy. 2010 [cited 2019 May 23]; Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3088792/pdf/12079_2011_Article_121.pdf
14. Pollard T, William Earnshaw, Lippincott-Schwartz J, Johnson G. Processing and Degradation of Cellular Components. In: Cell Biology [Internet]. Elsevier; 2017 [cited 2019 May 22]. p. 393–405. Available from: https://www.sciencedirect.com/science/article/pii/B9780323341264000232

15. Pickart CM, Eddins MJ. Ubiquitin: structures, functions, mechanisms. 2004 [cited 2019 May 22]; Available from: http://www.elsevier.com/locate/bba

16. Tanaka K. The proteasome: Overview of structure and functions. 2009 [cited 2019 Mar 10];85. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3524306/pdf/pjab-85-12.pdf

17. Lecker SH, Goldberg AL, Mitch WE. Protein Degradation by the Ubiquitin-Proteasome Pathway in Normal and Disease States. J Am Soc Nephrol [Internet]. 2006 [cited 2019 May 23];17:1807–19. Available from: http://nobelprize.org/

18. Smalle J, Vierstra RD. The Ubiquitin 26S Proteasome Proteolytic Pathway. Annu Rev Plant Biol. 2004;

19. Livneh I, Cohen-Kaplan V, Cohen-Rosenzweig C, Avni N, Ciechanover A. The life cycle of the 26S proteasome: from birth, through regulation and function, and onto its death. Nat Publ Gr [Internet]. 2016 [cited 2019 May 26];26:869–85. Available from: www.nature.com/cr

20. Bar-Nun S, Glickman MH. Proteasomal AAA-ATPases: Structure and function. BBA - Mol Cell Res [Internet]. 2012 [cited 2019 May 27];1823:67–82. Available from: https://pdf.sciencedirectassets.com/271024/1-s2.0-S0167488911X00125/1-s2.0-S0167488911001984/main.pdf?x-amz-security-token=AgoJb3JpZ2luX2VjEBsaCXVzLWVhc3QtMSJHMEUCIQDYKvZvPiyp5GCDWGQ5JVqpiZtNQoq%2B2APdiCPYGHptYAIgX%2B4wpLJrJEUuFsOqMyfsWLrHcamzTknCdLK8pupO

21. Tobias Jung, Tilman Grune. Structure of The Proteasome. In: The Proteasomal System in Aging and Disease [Internet]. Elsevier Inc.; 2012 [cited 2019 May 27]. p. 1–26. Available from: https://books.google.pt/books?id=dp_9KV8GzvAC&pg=PA16&lpg=PA16&dq=The+lid+consists+of+nine+different+Rpn+subunits&source=bl&ots=wxeJ0nqZiS&sig=ACfU3U2S5cdEkPiKeYH5zipGpfYpyMG2jw&hl=pt-PT&sa=X&ved=2ahUKEwjF4OKZxLziAhWBShUIHR9yD9kQ6AEwBHoECAgQAQ#v=onepage&q

22. Huber EM, Groll M. Inhibitors for the immuno- and constitutive proteasome: Current and future trends in drug development. Angew Chemie - Int Ed. 2012;51(35):8708–20.

23. Raynes R, Pomatto LCD, Davies KJA. Degradation of oxidized proteins by the proteasome: Distinguishing between the 20S, 26S, and immunoproteasome proteolytic pathways. Mol Aspects Med [Internet]. 2016 [cited 2019 Jun 2];50:41–55. Available from: http://www.ncbi.nlm.nih.gov/pubmed/27155164

24. Ferrington DA, Gregerson DS. Immunoproteasomes: structure, function, and antigen presentation. Prog Mol Biol Transl Sci [Internet]. 2012 [cited 2019 Jul 22];109:75–112. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22727420

25. Lee SW, Dai G, Hu Z, Wang X, Du J, Mitch WE. Regulation of muscle protein degradation: coordinated control of apoptotic and ubiquitin-proteasome systems by phosphatidylinositol 3 kinase. J Am Soc Nephrol [Internet]. 2004 Jun 1 [cited 2019 Jul 9];15(6):1537–45. Available from: http://www.ncbi.nlm.nih.gov/pubmed/15153564

26. Kast J. Immunoproteasome deficiency in non-small cell lung cancer and its relevance to immunotherapy. J Thorac Dis [Internet]. 2016 Sep [cited 2019 Aug 21];8(9):E1082–6. Available from: http://www.ncbi.nlm.nih.gov/pubmed/27747070

27. Morozov A V., Karpov VL. Biological consequences of structural and functional proteasome diversity. Heliyon [Internet]. 2018 Oct [cited 2019 May 27];4(10):e00894. Available from: https://linkinghub.elsevier.com/retrieve/pii/S2405844018342981

28. Angeles A, Fung G, Luo H. Immune and non-immune functions of the

immunoproteasome. Front Biosci (Landmark Ed [Internet]. 2012 Jan 1 [cited 2019 Mar 19];17:1904–16. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22201844

29. Johnston-Carey HK, Pomatto LCD, Davies KJA. The Immunoproteasome in oxidative stress, aging, and disease. Crit Rev Biochem Mol Biol [Internet]. 2015 [cited 2019 Aug 24];51(4):268–81. Available from: http://www.ncbi.nlm.nih.gov/pubmed/27098648

30. Teoh CY, Davies KJA. Potential roles of protein oxidation and the immunoproteasome in MHC class I antigen presentation: The "PrOxI" hypothesis. Arch Biochem Biophys. 2004;423(1):88–96.

31. Vachharajani N, Joeris T, Luu M, Hartmann S, Pautz S, Jenike E, et al. Prevention of colitis-associated cancer by selective targeting of immunoproteasome subunit LMP7. Oncotarget [Internet]. 2017 Aug 1 [cited 2019 Sep 4];8(31):50447–59. Available from: http://www.oncotarget.com/fulltext/14579

32. Miller Z, Ao L, Kim KB, Lee W. Inhibitors of the immunoproteasome: current status and future directions. Curr Pharm Des [Internet]. 2013 [cited 2019 Jul 7];19(22):4140–51. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23181576

33. Basler M, Mundt S, Bitzer A, Schmidt C, Groettrup M. The immunoproteasome: A novel drug target for autoimmune diseases. Clin Exp Rheumatol [Internet]. 2015;33(1):74–9. Available from: http://www.embase.com/search/results?subaction=viewrecord&from=export&id=L608439967%0Ahttp://sfx.library.uu.nl/utrecht?sid=EMBASE&issn=1593098X&id=doi:&atitle=The+immunoproteasome%3A+A+novel+drug+target+for+autoimmune+diseases&stitle=Clin.+Exp.+Rheumatol.

34. Xin B-T, Huber EM, Bruin G de, Heinemeyer W, Maurits E, Espinal C, et al. Structure-BasedDesign of Inhibitors Selective forHuman Proteasome β2c or β2i Subunits. J Med Chem [Internet]. 2019 [cited 2019 Sep 5];62(3):1626. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6378654/

35. Kisselev AF, Groettrup M. Subunit specific inhibitors of proteasomes and their potential for immunomodulation. Curr Opin Chem Biol [Internet]. 2014 Dec [cited 2019 Sep 5];23:16–22. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25217863

36. Xi J, Zhuang R, Kong L, He R, Zhu H, Zhang J. Immunoproteasome-selective inhibitors: An overview of recent developments as potential drugs for hematologic malignancies and autoimmune diseases. Eur J Med Chem. 2019 Nov;182:111646.

37. Al-Ashmawy GMZ. Dendritic Cell Subsets, Maturation and Function. In: Dendritic Cells [Internet]. InTech; 2018 [cited 2019 Aug 31]. Available from: http://www.intechopen.com/books/dendritic-cells/dendritic-cell-subsets-maturation-and-function

38. Kawamura K, Iyonaga K, Ichiyasu H, Nagano J, Suga M, Sasaki Y. Differentiation, maturation, and survival of dendritic cells by osteopontin regulation. Clin Diagn Lab Immunol [Internet]. 2005 Jan 1 [cited 2019 Aug 29];12(1):206–12. Available from: http://www.ncbi.nlm.nih.gov/pubmed/15643009

39. R&D Systems. Dendritic Cell Maturation [Internet]. 2002 [cited 2019 Aug 31]. Available from: https://www.rndsystems.com/resources/articles/dendritic-cell-maturation

40. Doiron L. Steps for Isolating Peripheral Blood Mononuclear Cells from Whole Blood [Internet]. 2014 [cited 2019 Jun 10]. Available from: https://www.dls.com/biopharma/blog/what-is-the-typical-procedure-for-pbmc-isolation-from-whole-blood

41. Yevgeniy Grigoryev. Cell Counting with a Hemocytometer: Easy as 1, 2, 3 - Bitesize Bio [Internet]. [cited 2019 Sep 18]. Available from: https://bitesizebio.com/13687/cell-counting-with-a-hemocytometer-easy-as-1-2-3/

42. Bhattacharjee J, Das B, Mishra A, Sahay P, Upadhyay P. Monocytes isolated by positive and negative magnetic sorting techniques show different molecular characteristics and immunophenotypic behaviour. F1000Research [Internet]. 2017 [cited 2019 Jun 10];6:2045. Available from: http://www.ncbi.nlm.nih.gov/pubmed/29636897

43. Kapellos TS, Bonaguro L, Gemünd I, Reusch N, Saglam A, Hinkley ER, et al. Human Monocyte Subsets and Phenotypes in Major Chronic Inflammatory Diseases. Front Immunol. 2019 Aug 30;10.

44. Company B. MojoSort TM Human CD14+ Monocytes Isolation Kit Protocol [Internet]. [cited 2019 Jun 10]. Available from: https://www.biolegend.com/protocols/mojosort-human-cd14--monocytes-isolation-kit-protocol/4351/

45. Spadaro M, Montone M, Cavallo F. Generation and Maturation of Human Monocyte-derived DCs. BIO-PROTOCOL [Internet]. 2014 [cited 2019 Jun 12];4(15). Available from: https://bio-protocol.org/e1194

46. BD™ Cytometric Bead Array (CBA) Human Th1/Th2 Cytokine Kit II Instruction Manual [Internet]. 2014 [cited 2019 Jun 12]. Available from: http://www.bdbiosciences.com/ds/pm/others/23-12495.pdf

47. Buy Naive Cynomolgus Peripheral Blood Mononuclear cells (PBMCs), Cynomolgus Monkey – HumanCells Bio [Internet]. [cited 2019 Oct 18]. Available from: https://humancellsbio.com/products/naive-cynomolgus-peripheral-blood-mononuclear-cells-pbmcs

48. Protocol - MojoSort Human CD14+ Monocytes Isolation Kit Protocol [Internet]. [cited 2019 Oct 6]. Available from: https://www.biolegend.com/protocols/mojosort-human-cd14--monocytes-isolation-kit-protocol/4351/

# 7. Annexes

**Annex 1: The role of (immune)proteasome inhibition on the differentiation of monocytes to iDCs**

**Table 1: Expression of CD80, HLA-DR, DCSIGN, CD86 and CD14 markers by iDCs differentiated from monocytes treated with (immune)proteasome inhibitors.**

| Type of cell analysed: iDC | Sample: | CD80 | HLA-DR | DCSIGN | CD86 | CD14 |
|---|---|---|---|---|---|---|
| 1 | Non stained | 107 | 76 | 73 | 152 | 325 |
| 2 | Control | 230 | 666 | 4169 | 290 | 328 |
| 3 | β1c | 230 | 521 | 5440 | 168 | 261 |
| 4 | β1i | 205 | 336 | 3405 | 171 | 246 |
| 5 | β1c+β1i | 315 | 777 | 3270 | 278 | 285 |
| 6 | β1i+β5i | 165 | 193 | 3002 | 134 | 247 |
| 7 | β2c | 270 | 521 | 5176 | 206 | 314 |
| 8 | β2i | 188 | 313 | 4656 | 170 | 268 |
| 9 | β2c+β2i | 279 | 343 | 4751 | 187 | 318 |
| 10 | β2i+β5i | 162 | 211 | 3642 | 134 | 260 |
| 11 | β5c | 211 | 367 | 4123 | 179 | 279 |
| 12 | β5i | 197 | 276 | 3367 | 173 | 275 |
| 13 | β5c+β5i | 212 | 518 | 1502 | 261 | 396 |

**Annex 2: Inhibition of the Immunoproteasome decreases cytokine production in Dendritic Cells**

**Table 2: Secretion of the cytokines IL-2, IL-4, IL-6, IL-10, TNF-α and IFN-γ by DCs differentiated in the presence of immunoproteasome inhibitors.**

| Mean | Concentration (pg/uL) | | | | | |
|---|---|---|---|---|---|---|
| **Sample** | **IL-2** | **IL-4** | **IL-6** | **IL-10** | **TNF-α** | **IFN-γ** |
| **Control** | 42,1 | 37,1 | 40,8 | 38,9 | 69,0 | 22,5 |
| **β1i** | 43,3 | 37,4 | 40,3 | 38,1 | 71,7 | 21,3 |
| **β2i** | 39,2 | 34,0 | 40,5 | 33,6 | 66,1 | 18,9 |
| **β5i** | 43,0 | 37,4 | 42,1 | 39,1 | 70,6 | 21,3 |
| **β1i+β5i** | 39,2 | 34,3 | 39,2 | 33,7 | 65,5 | 19,1 |
| **β2i+β5i** | 41,5 | 38,6 | 39,2 | 38,2 | 69,6 | 21,5 |

**Annex 3: Cell culture of monocytes to immature DCs treated with inhibitors and differentiation to mature DCs without inhibitors**

**Table 3: Expression of CD80, HLA-DR, DCSIGN, CD86 and CD14 markers by mDCs differentiated from monocytes treated with (immune)proteasome inhibitors to iDCs. DCs activation to mature is without inhibitors.**

| Type of cell analysed: mDC | Sample: | CD80 | HLA-DR | DCSIGN | CD86 | CD14 |
|---|---|---|---|---|---|---|
| 1 | Non stained | 122 | 84 | 75 | 202 | 376 |
| 2 | Control | 792 | 1857 | 2158 | 3058 | 513 |
| 3 | β1c | 687 | 1845 | 2212 | 2492 | 442 |
| 4 | β1i | 650 | 1411 | 1359 | 2718 | 440 |
| 5 | β1c+β1i | 651 | 1492 | 1248 | 2804 | 453 |
| 6 | β1i+β5i | 482 | 1184 | 1816 | 1809 | 353 |
| 7 | β2c | 871 | 2168 | 2491 | 2611 | 483 |
| 8 | β2i | 682 | 1820 | 4666 | 2548 | 438 |
| 9 | β2c+β2i | 825 | 1550 | 2497 | 2658 | 491 |
| 10 | β2i+β5i | 670 | 1526 | 3725 | 2044 | 375 |
| 11 | β5c | 660 | 1729 | 1714 | 2854 | 453 |
| 12 | β5i | 693 | 1585 | 1908 | 2469 | 420 |
| 13 | β5c+β5i | 242 | 559 | 700 | 395 | 502 |

**Annex 4: Cell culture of iDC differentiating to mature DCs treated with inhibitors**

**Table 4: Expression of CD80, HLA-DR, DCSIGN, CD86 and CD14 markers by mDCs differentiated from monocytes treated with (immune)proteasome inhibitors.**

| Type of cell analysed: mDC | Sample: | CD80 | HLA-DR | DCSIGN | CD86 | CD14 |
|---|---|---|---|---|---|---|
| 1 | Non stained | 122 | 84 | 75 | 202 | 376 |
| 2 | Control | 792 | 1857 | 2158 | 3058 | 513 |
| 3 | β1c | 1130 | 3642 | 4332 | 2899 | 487 |
| 4 | β1i | 1125 | 3914 | 4677 | 2810 | 481 |
| 5 | β1c+β1i | 946 | 4461 | 4313 | 2670 | 447 |
| 6 | β1i+β5i | 1052 | 4511 | 4893 | 2514 | 462 |
| 7 | β2c | 965 | 5589 | 4532 | 2682 | 456 |
| 8 | β2i | 1173 | 4255 | 6863 | 2835 | 491 |
| 9 | β2c+β2i | 937 | 4645 | 4255 | 2652 | 455 |
| 10 | β2i+β5i | 935 | 6370 | 5847 | 2383 | 453 |
| 11 | β5c | 1112 | 4004 | 4625 | 2779 | 474 |
| 12 | β5i | 1013 | 3569 | 4401 | 2605 | 474 |
| 13 | β5c+β5i | 747 | 4151 | 4179 | 1375 | 410 |

**Annex 5: The role of (immune)proteasome inhibition in the cell-mediateded cytotoxicity**

**Table 5: Levels of PBMCs cytotoxicity in our sample against K562 cells when treated with different inhibitors. The results are percentages of cytotoxicity with deducted spontaneous dead K562 cells normalized to control sample (DMSO).**

| | Mean | SD | Replicats |
|---|---|---|---|
| **β1c** | 96,94987 | 10,97526 | 3 |
| **β1i** | 84,30684 | 23,24294 | 3 |
| **β1c+β1i** | 91,58953 | 5,683135 | 3 |
| **β1i+β5i** | 75,47873 | 4,817391 | 3 |
| **β2c** | 101,8136 | 18,9524 | 3 |
| **β2i** | 88,30586 | 4,329693 | 3 |
| **β2c+β2i** | 107,8261 | 15,00518 | 3 |
| **β2i+β5i** | 89,90141 | 15,78805 | 3 |
| **β5c** | 101,2402 | 13,93722 | 3 |
| **β5i** | 75,65102 | 4,458331 | 3 |
| **β5c+β5i** | 101,9873 | 23,35484 | 3 |