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Regulation and function of the proteasome in human platelets

Katharina Gründler

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Erklärung

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Table of contents

Abstract

Even though platelets are the smallest cells in circulating blood, they play an integral role in blood clotting where they are activated, adhere to the vessel wall, and contribute to hemostasis. But over the years it was discovered that those anucleate cells have more extended functions. They organize their cellular vitality similar to nucleated cells and have an active protein metabolism performing protein *de novo* synthesis as well as protein degradation. One of the main degradation systems in cells is the proteasome. Besides protein quality control, the proteasome is involved in important cellular processes like cell survival, transcription, development, selective elimination of abnormal proteins and antigen processing.
A dysregulation of this multicatalytic protein complex leads to various disease developments. Proteasome inhibitors, for instance, have been studied for treating cancer. Platelets like nucleated cells contain a proteasome. However, the impact of the proteasome on platelet functions remains poorly investigated until today. A better knowledge of signaling pathways in platelets aids in understanding how alterations in proteasome functions affect platelet-mediated processes and diseases.

This study confirms the existence of a functional proteasome in human platelets and illustrates an important role in platelet biology, as well as sepsis.
With this study the role of the proteasome in anucleate platelets is demonstrated in more detail and a signaling pathway regulating its activity was observed. Here, the proteasome in platelets is linked to platelet aggregation. First, proteasome inhibitors epoxomicin and bortezomib reduce ADP- and collagen-induced aggregation. Furthermore, the 26S chymotrypsin-like activity of the proteasome is enhanced when platelets are incubated with the platelet agonist collagen. Additionally, cytoskeletal proteins Filamin A and Talin-1, which are crucial for platelet activation, were identified as proteasome substrates and increased cleavage of these proteins occurs with proteasome activation.
To investigate possible mechanisms of regulating the proteasome, the signaling pathway related to NFκB was analyzed under platelet agonist treatment. The NFκB pathway, that mediates aggregation, is initiated when platelets are treated with collagen and the inhibitory protein of NFκB, IκBα, is degraded in collagen-stimulated platelets. More interestingly, NFκB inhibitors prevent collagen-stimulated enhancement of the proteasome activity. In return the connection of the proteasome and the NFκB pathway is further demonstrated as NFκB inhibitors restrict cleavage of the proteasome substrate Talin-1. These results propose a novel pathway that involves the proteasome and that is in return connected with non-genomic functions of NFκB in regulating platelet aggregation.

In a second part this work shows for the first time that mitochondrial membrane depolarization in platelets correlates with the disease course and disease severity in patients with sepsis. Additionally, during these studies increased proteasome activity was observed in sepsis patients compared to control patients and pathogenic bacteria intensified the 26S trypsin-like activity of human platelets. Therefore, molecular markers of platelet vitality may be valuable parameters to help evaluating the clinical outcome of sepsis patients.
In summary, the study confirms the existence of a functional proteasome in human platelets, contributes to our understanding how the proteasome affects platelet functions such as aggregation and how this may be regulated on a molecular basis. Furthermore, it allows for new insights in the disease course of sepsis and identifies new molecular markers for assessing the disease severity and clinical outcome of sepsis patients.

1 Introduction

1.1 Blood platelet: Crucial part of vascular integrity

Platelets are the smallest cells in circulating blood with a diameter of 2-4µm [1, 2]. A human platelet count lies between 150.000 and 300.000 platelets per µl blood. They derive from megakaryocytes in the bone marrow and have a physiological lifetime of about 7-10 days [3]. Platelets do not have a nucleus and are referred to as anucleate cells. It is well studied that they play an important role in vessel and wound repair.
Platelets prevent blood loss by forming thrombi. Under physiological conditions resting platelets circulate with the blood flow. They have a discoid shape and roll along the intact endothelium. Traumatic action on the vessel wall, such as a cut requires clot formation to stop bleeding. Platelets attach to the vascular lesion, adhere, and form a primary hemostatic thrombus while attracting more platelets. During adhesion platelets undergo shape change, spreading or rolling and activation. In a last step platelets aggregate with each other.

1.1.1 Resting and activated platelets

As for other cells, those anucleate cells have a cytoplasmic membrane with membrane proteins. The platelet plasma membrane expresses numerous integrated proteins that are receptors for soluble agonist (such as ADP, thrombin or thromboxane A2) or adhesion proteins (such as fibrinogen, collagen or von Willebrand factor) [4, 5]. The plateletal cytoskeleton is important to maintain the discoid shape of resting platelets and actively aids in the platelet shape change. The cytoskeleton consists mainly of actin (15-20% of total protein mass), microtubuli, and actin binding protein, myosin. Actin exists in a globular form, G-actin, and in a filament form, F-actin [6]. Upon activation when the intracellular calcium concentration reaches a specific threshold platelets undergo chape change and pseudopods are formed [7]. The platelet looses its discoid shape. Microtubuli enrich in the pseudopods and G-actin polymerizes to F-actin, which associates with other structural proteins. F-actin filaments have a connection to the cell organelles and reorganize them during this activation process. Well-known organelles of platelets are mitochondria, glycogen stores and the storage granules (dense granules, α-granules, and lysosomes) [8]. The lysosomes are similar to other cells and contain hydrolytic enzymes. The granules are characteristic for platelets and store proteins and other substances that are important for platelet function. The dense granules contain compounds, such as ADP, ATP, Ca^{2+}, and serotonin to promote aggregation

and α-granules carry proteins, such as P-selectin or fibrinogen that play differing roles in adhesion, aggregation, chemotaxis, proliferation and inflammation [9].

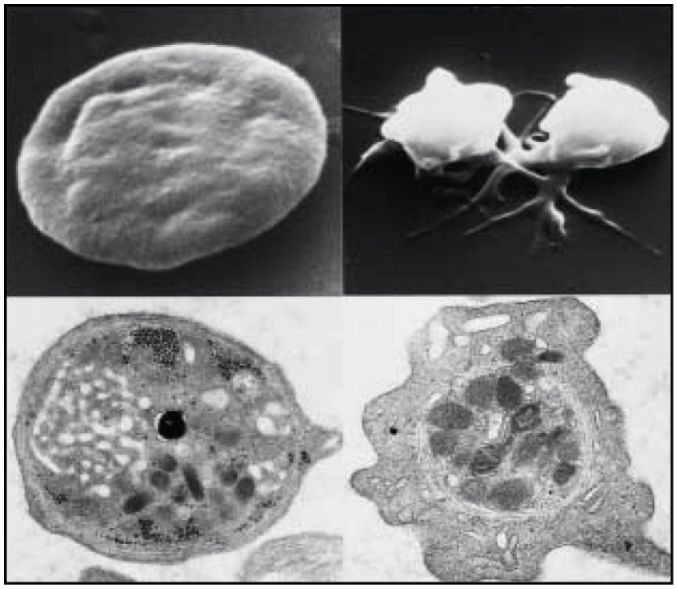


Figure 1: Morphology of resting and activated platelets. Upper panel shows electron micrographs of a resting platelet and an activated platelet (x20000 and x10000 respectively). The lower panel pictures transmission electron micrographs of cross-sections of a resting and an activated platelet (x21000 and x30000 respectively) [4].

1.1.2 Receptor pathways of platelet activation

Platelets have a number of different transmembrane receptors that interact with its physiological agonist to induce activation. Amongst others many integrins (such as αIIbβ3, α2β1), G-protein-coupled seven transmembrane receptors (protease-activated-receptor PAR-1 and PAR-4 thrombin receptors, P2Y1 and P2Y12 ADP receptors, TPa and TPb TxA2 receptors), proteins of the immunoglobulin superfamily (GPVI), and C-type lectin receptors (P-selectin) are found on platelets.
These platelet adhesion receptors are well understood and even though they differ in their functions and signaling pathways they have some similarities. First, there is the agonist/platelet receptor interaction followed by signaling pathways that promote secretion. Then released substances induce various platelet responses and cause further platelet activation as well as recruitment of other circulating platelets. The so-called integrin activation and outside-in signaling is started. In the following the main platelet receptors will be mentioned and Figure 2 gives a schematic representation of platelet adhesion and agonist receptors with their cellular pathways.

Fibrinogen receptor, integrin $\alpha_{IIb}\beta_3$

The most abundant and best-studied integrin in platelets is the $\alpha_{IIb}\beta_3$ (GPIIb/IIIa) integrin, the fibrinogen receptor. This receptor is inactive in resting platelets but upon

platelet activation it undergoes conformational changes becoming able to bind soluble plasma fibrinogen [10]. The outside-in signaling involves calcium mobilization, phosphorylation of proteins, activation of the phosphoinositide metabolism and cytoskeletal reorganization [11]. The $\alpha_{IIb}\beta_3$ integrin mediates the bridging between two platelets where fibrinogen connects two $\alpha_{IIb}\beta_3$ integrins with eachother. Integrin $\alpha_{IIb}\beta_3$ is the main adhesion molecule in platelet aggregation.

Thrombin (PAR) receptors

PAR receptors mediate platelet response to thrombin. PAR receptors are G-protein coupled receptors and on human platelets PAR-1 and PAR-4 are found. Both trigger PLC activation, Ca^{2+} mobilization, and PKC activation [12]. Thrombin stimulation results in platelet activation, shape change and the release of granules. PAR-1 and PAR-4 have different kinetics; to induce PAR-4 mediated signaling a higher thrombin concentration is necessary [13].

Thrombin as a protease acts on PAR receptors by binding to the extracellular domain and cleaving the receptor to form a new peptide ligand. Thrombin unmasks a specific ligand for the PAR receptors that then activates the receptor and induces transmembrane signaling [14].

ADP receptors, P2Y receptors

The agonist ADP binds to the ADP receptors $P2Y_1$ and $P2Y_{12}$, G-protein-coupled receptors [15, 16]. One receptor is coupled to $G\alpha_q$ G protein ($P2Y_1$) and the other is coupled to $G\alpha_i$ ($P2Y_{12}$). While $P2Y_1$ stimulates PLCβ enhancing cytosolic Ca^{2+}, that activates PKC and leads to platelet shape change [15], $P2Y_{12}$, which is coupled to $G\alpha_i$, goes another way. $P2Y_{12}$ inhibits the adenylate cyclase and activates phosphatidylinositol 3-kinase (PI3K) [17]. Activated PI3K promotes then AKT and Rap1B activation [18]. This stimulation of $G\alpha_q$ and $G\alpha_i$ signaling pathways is necessary for a fibrinogen receptor activation, thus for platelet-platelet adhesion.

Antiplatelet agents are effective in the treatment of arterial thrombosis. Those agents target different critical steps in thrombogenesis. But important antiplatelet agents are clopidogrel and prasugrel that target the ADP receptor $P2Y_{12}$ [19].

Thromboxane receptors

Thromboxane A2 (TXA2) is a prostaglandin with potent platelet activating characteristics. Thromboxane receptors (TPs) induce a cytosolic Ca^{2+} enhancement and an influx of extracellular Ca^{2+}, which activates the PLA2 resulting in arachidonic acid (AA) hydrolysis from membrane phopholipids and converting it into TXA2 [20]. TXA2 then acts on its receptor again [21]. Because TPs are coupled to $G\alpha_q$ but not $G\alpha_i$ family members they require a secretion of ADP for platelet aggregation. ADP inhibits the adenylate cyclase mediated through the $P2Y_{12}$ that is coupled to $G\alpha_i$.

Collagen receptors, integrin $\alpha_2\beta_1$ and GPVI

Following injury to the vascular wall, collagen is exposed on which amongst others platelets adhere rapidly. Collagen I and III are considered the most important collagens at the injured cite to induce platelet adhesion and platelets have many collagen receptors. Most collagen receptors are known but there might exist more due to the fact that there are receptors directly and indirectly binding collagen [22]. An indirect collagen receptor is the GPIb complex (CD42c) that interacts with von Willebrand factor, which then binds to various collagens in the subendothelium. The integrin $\alpha_2\beta_1$ and the Ig superfamily receptor GPVI are the major direct collagen receptors [22].

Integrin $\alpha_2\beta_1$ is known as GPIa/IIa or CD49b/CD29. As all integrins, $\alpha_2\beta_1$ is a large glycoprotein with extracellular domains, transmembrane domains and cytoplasmic domains. On resting platelets $\alpha_2\beta_1$ is expressed in a low-affinity state (similar to the inactive state of the fibrinogen receptor) and the affinity of $\alpha_2\beta_1$ to soluble collagen increases on platelet stimulation [23]. Thus integrin $\alpha_2\beta_1$ seems to require an earlier agonist-induced conformational change to bind to collagen [24]. Src and Syk family tyrosine kinases are activated by $\alpha_2\beta_1$ leading to the activation of PLCγ2 and the formation of lamellipodia [25]. There is a complex crosstalk between $\alpha_2\beta_1$ (collagen receptor) and $\alpha_{IIb}\beta_{b3}$ (fibrinogen receptor); $\alpha_2\beta_1$ promotes the activation of $\alpha_{IIb}\beta_{b3}$ and as a consequence induces fibrinogen binding to adherent platelets [26].

The collagen receptor GPVI is connected to the immunoreceptor tyrosine-based activation motif (ITAM) and its cytoplasmic domain is bound to the Src family kinases Fyn and Lyn [27, 28]. When collagen binds to GPVI, ITAM is tyrosine phosphorylated by those Src family kinases and initiates a complex signaling cascade activating a series of adapter and effector proteins [29]. The main enzyme activated is PLCγ2 that leads to the second messengers 1,2-diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP3). IP3 in turn triggers intracellular Ca^{2+} mobilization, protein phosphorylation and AA release. AA drives aggregation and secretion of TXA2 and ADP [30].

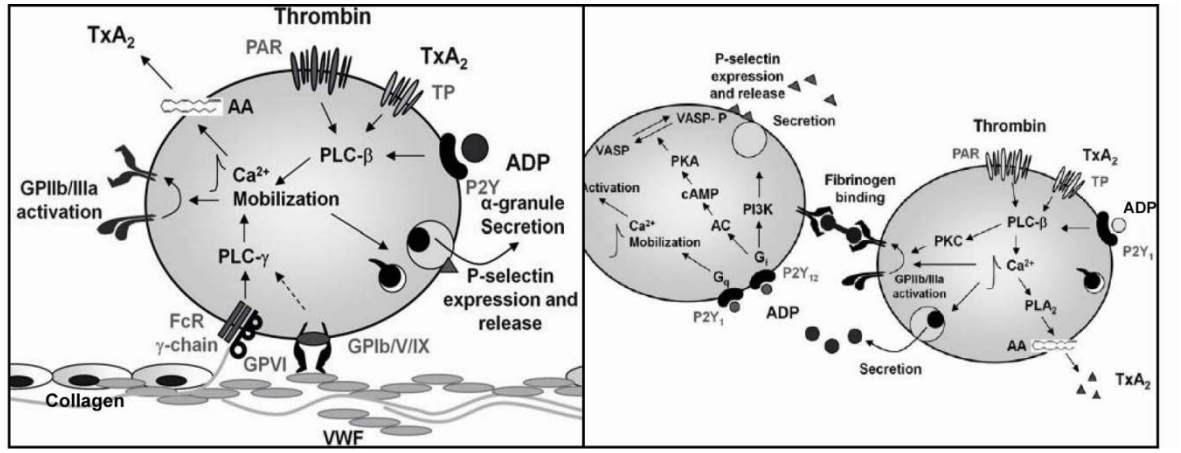


Figure 2: Schematic model of main platelet receptors and their cellular pathways [31]. The left panel shows adhesion as well as agonist receptors and the right panel shows major cellular pathways triggered by platelet agonists. The fibrinogen, thrombin, ADP, thromboxane A2 and collagen receptors are discussed above.

1.1.3 Aggregation

While platelets adhere they not only undergo activation but also aggregate. Aggregation is defined as the process of coadhesion of two platelets. Primary and secondary aggregation are told apart. Primary aggregation is reversible and platelets are only loosely connected by fibrinogen after activation. When they release their granule components secondary aggregation starts, the binding of fibrinogen becomes stronger and this process is irreversible [32]. Without fibrinogen or Ca^{2+} aggregation is not possible. Those two components are also stored in platelet granules to achieve high concentrations of both of them in a thrombus.

The measurement of aggregation is the most common method for the diagnosis of platelet function. Platelet aggregation can be detected amongst others by light transmission aggregometry, LTA, also called Born aggregometry or through impedance aggregometry, IA [33]. During IA electrical impedance in whole blood is measured with two sensor electrodes. When platelets aggregate they adhere on the metal wire and increase electrical resistance. During LTA light transmission is detected by a photocell that sits behind a cuvette. While platelets aggregate, less light is absorbed and the transmission increases. The aggregation curve measured by LTA, Born aggregometry, can follow a monophasic or biphasic aggregation depending on the agonist. After ADP stimulation platelets undergo a shape change that is recognized by a decrease in light transmission, followed by the primary aggregation (reversible) where the curve rises, and then the curve can either fall (deaggregation) or goes through a plateau into the second phase of aggregation (irreversible). Platelets activated with collagen show a delayed and longer decrease in light transmission (shape change) and have a monophasic curve.

1.1.4 Platelets in vascular inflammation and diseases

Next to inherited platelet disorders that affect, *e.g.*, platelet adhesion, activation or secretion, platelets play a crucial role in the involvement of various diseases.
Since platelets play a central role in stopping bleeding by clot formation they have a major part in pathophysiological thrombus formation as well. During atherosclerosis an interaction among platelets, endothelial cells, and leukocytes establishes a localized inflammatory response that accelerates atherosclerosis, thrombus formation and might result in a heart attack or stroke. Platelets normally interact with the endothelium to maintain their physiological function and to enhance leukocyte recruitment to sites of inflammation but when those physiological responses are exaggerated at atherosclerosis sites pathogenesis of this disease is stimulated [34].
Moreover, growing evidence shows that platelets contribute to cancer progression. Complex crosstalk between tumor cells and circulating platelets enhances tumor growth and platelet receptors as well as platelet agonist play a role in cancer metastasis [35].
Additionally, the exact role of platelets in sepsis with its underlying molecular mechanisms is still to be analyzed. But it is suggested that platelets promote inflammation during the early stages of infection thus helping prevent sepsis. Nevertheless, thrombocytopenia is a common finding in severe sepsis and it might result from platelets undergoing apoptosis [36, 37]. Although apoptotic proteolysis might contribute to thrombocytopenia in sepsis other mechanisms are involved. It is known that platelet activation and aggregation is regulated in sepsis through Toll-like receptors expressed by platelets and bacterial exotoxins [38]. Moreover, the role of patelet-leukocyte adhesion during sepsis coming from activated platelets needs to be analyzed. While activated platelets secrete key components of the coagulation and inflammatory cascade, there are only few studies on platelet function in sepsis.
Thrombocytopenia in general is found during many diseases. It might be drug-induced however it needs to be considered during the process of healing.

1.2 Organization of cellular vitality and protein metabolism in platelets

Platelets are known for their ability to stop bleeding and for many years that was thought to be their only function. But over the years platelets have shown more extended functions. They obsess many features that were believed, to be seen only in nucleated cells. It was discovered that those anucleate cells are not as simple as everyone believed. Those small particles are surprising. Platelets are able to migrate, proliferate, go into apoptosis, and present antigens. They even contain transcription factors and are able to perform protein *de novo* synthesis.

1.2.1 Migration and proliferation of platelets

For a long time platelets were believed to be static cells that do not leave the site of adhesion, even though there was some evidence that platelets *in vitro* are able to move through a Boyden chamber [39]. Years later platelets were found to migrate *in vivo* and more detailed analysis demonstrated that platelets are able to migrate in a SDF-1-mediated fashion even through an endothelium [40-42]. Further evidence for platelet migration is published continuously [43-45].
Yet, more astonishingly in 2010 Schwertz *et al.* discovered that platelets are able to produce functional progeny. There seems to exist a cell division that does not require a nucleus. Platelets form new cell bodies that contain mitochondria and α–granules. Moreover the new fragments adhere and spread normally, express P-selectin and annexin V in the typical way after stimulation [46].

1.2.2 Regulation of apoptosis

When apoptosis, programmed cell death, was observed it was exclusively assigned to nucleated cells [47]. However, apoptotic signs were discovered in platelets [48, 49]. Numerous chemical agents such as the calcium ionophore A23187 trigger platelet apoptosis but also the platelet activator thrombin is able to induce apoptotic events in platelets [50, 51]. Moreover, the process of apoptosis might be induced without stimulants in platelets, under pathological high shear stress or long-term incubation of platelets under blood banking conditions [50, 52].
While the intrinsic mitochondria-dependent pathway is well studied in anucleate platelets, the role of the extrinsic pathway remains unclear [53].
During apoptosis in platelets a depolarization of the mitochondrial membrane potential has been demonstrated. The mitochondrial membrane potential decreases in canine platelets after an estradiol treatment [54] or apoptotic stimulants A23187, thrombin or high shear stress induce a reduction of the mitochondrial membrane potential in human platelets [50, 51]. Degradation of the anti-apoptotic protein Bcl-xL after treating platelets with bacteria was shown and pro-apoptotic proteins Bax and Bak are higher expressed under, *e.g.*, thrombin-induced plateletal apoptosis [36, 51]. Pro-apoptotic proteins also show higher expression in *in vitro* aged platelets [55]. Induction of activation of caspases -3, -8 and -9 was found in human platelets as well [56]. Other apoptotic markers, such as phosphatidylserine translocation to the outer leaflet of the plasmamembrane, and chytochrome c release were analyzed in platelets [50, 57]. Furthermore, platelet shrinkage, membrane blebbing and microparticle shedding, visible by microscopy, can be found in plateletal apoptosis [50, 55]. All those apoptotic markers in platelets are summarized in a model for apoptosis in platelets (Figure 3).

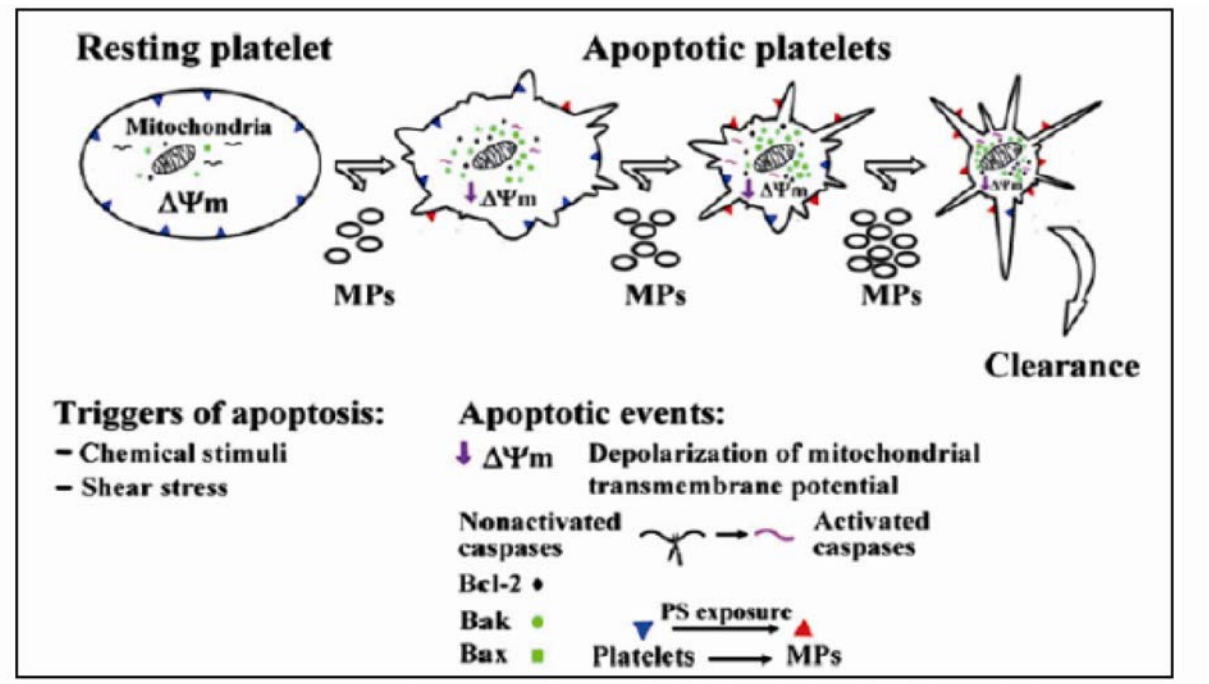


Figure 3: Model of apoptosis in platelets [53]. Shown are platelets with their apoptotic markers undergoing apoptosis when stimulated with chemical stimuli or high shear stress.

1.2.3 Platelets: Antigen presenting cells

A connection of platelets in processing and presenting antigens had long not been made. However, in 2012 Chapman *et al.* stated that platelets express MHC I (major histocompatibility complex class I) molecules and present antigens to naïve T cells. They not only measured antigen presenting molecules on platelets in different *in vitro*-incubations but also analyzed platelets isolated from infected and uninfected mice [58]. Additionally, it is known that platelets express many proteins or RNA transcripts, which are necessary for processing antigens and presenting them. Platelets contain the endoplasmatic reticulum and a Golgi, where peptides to be presented are processes and loaded on MHC I molecules. The proteasome that helps by cleaving peptides that are to be presented is found in platelets, as well as TAP (transporter associated with antigen processing), a protein that brings cytosolic peptides to the ER [59, 60]. Many more proteins (such as calnexin, calreticulin and Erp57) that facilitate correct folding of MHC I molecules and its association with β-microglobulin, were detected in platelets [61, 62].
Proteomic analysis of platelets has brought further evidence for the existence of MHC I molecules. The MHC I complex is found in α–granules of platelets [60] and the global proteome analysis from Klockenbusch *et al.* identified the MHC I maschinery as well [63]. Zufferey *et al.* provides a model of MHC I antigen-presenting pathway in human platelets with its identified proteins (Figure 4).

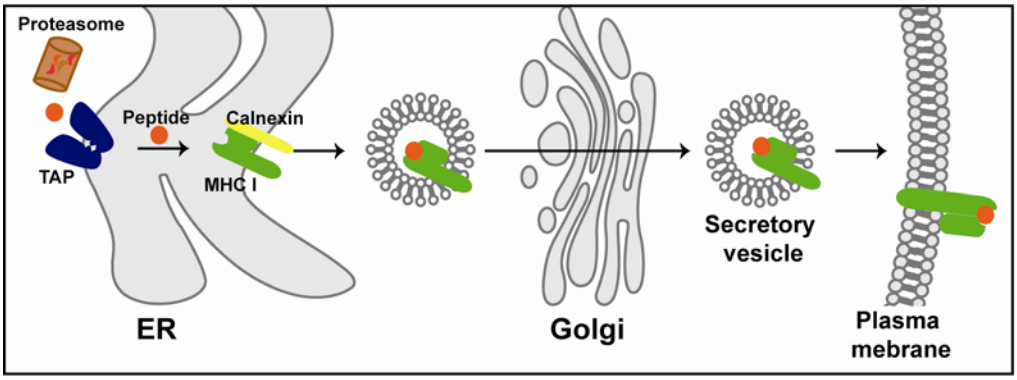


Figure 4: Schematic overview of the MHC I antigen-presenting pathway in human platelets. MHC I is loaded in the ER and brought to the plasma membrane over the Golgi in a secretory granule. Modified from Zufferey *et al.* [60].

1.2.4 Transcription factors in human platelets affect platelet functions

Although platelets are anucleate, recent publications show that platelets express transcription factors. Transcription factors including the steroid/nuclear receptors [64], peroxisome proliferator activated receptor (PPAR) β/δ and γ [65, 66], the glucocorticoid receptor (GR) [67], retinoid X receptors (RXR) [68] and nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) were found in platelets [69, 70] and they indeed influence platelet functions. For instance, the nuclear receptor estrogen β potentiates thrombin-stimulated platelet aggregation [71]. PPARγ prevents the release of TXB_2 and ATP after thrombin stimulation [66]. The glucocorticoid receptor bound to its ligand prednisolone seems to inhibit platelet aggregation [67] and the retinoid X receptors inhibit platelet aggregation through Rac inhibition and prevention of Ca^{2+} release [68].

Taken together, this suggests non-genomic functions of transcription factors in platelets. Also in erythrocytes, another anucleate cell type, transcription factors were found [72]. Thus, anucleate cells like platelets and erythrocytes seem to be the ideal human experimental model to study non-genomic functions of transcription factors. Since some transcription factors like nuclear receptors are already known to possess non-genomic functions in nucleated cells [73], it is not as surprising that they play a non-genomic role in platelets. But that other transcription factors might also have non-genomic functions is a whole new research field. Here I will focus on the transcription factor NFκB that had mostly been studied in nucleated cells and its genomic functions there are well known. Nevertheless, the recent identification of NFκB in anucleate platelets is fascinating and promises a better understanding of platelet biology.

NFκB signaling pathway

The NFκB pathway has diverse functions and in accordance with this the NFκB transcription factor family consists of 5 members (p50, p52, p65, RelB and c-Rel) [74, 75]. All of them contain a nuclear localization sequence (NLS) in their N-terminal domain and they are normally found in the cytoplasm associated to an inhibitory protein that masks their NLS [76]. The inhibitory proteins belong to the IκB family with its most common member IκBα. The NFκB transcription factor proteins can form homo- or heterodimers what again demonstrates the diverse function of this signaling pathway. NFκB bound to the inhibitory protein might be considered inactive.

There are several NFκB activation pathways but the most frequent one is the canonical. This pathway is activated in response to various inflammatory stimuli. Hereby the IκB kinase (IKK) complex is activated and phosphorylates the inhibitory protein IκBα at Ser32 and Ser36. This phosphorylation results in a rapid ubiquitination and degradation of IκBα by the proteasome [76]. Then the so-called active NFκB dimer with its liberated NLS sequence translocates to the nucleus where it selectively activates the transcription of various genes mainly involved in inflammation. The left panel of Figure 5 pictures this pathway.

As NFκB mainly activates inflammatory genes, it is found activated amongst others in rheumatoid arthritis, an inflammatory disease. In the joint tissue NFκB of resident macrophages provokes transcription of many pro-inflammatory cytokines and chemokines leading to a subsequent invasion of a large number of immune cells to the joint tissue. In an autocrine and paracrine manner cells are kept activated there [77].

NFκB in platelets

Over the last years evidence was regularly published that NFκB exists in anucleate platelets and exerts non-genomic functions. Already in 2002 Liu *et al.* showed the existence of some NFκB/IκB family members in human platelets and demonstrated a phosphorylation and degradation of IκB during platelet activation [69]. This was not as absurd because thrombin, a platelet activator, is able to induce the NFκB pathway in smooth muscle cells [78]. Malaver *et al.* analyzed further the functional significance of NFκB in human platelets. They confirmed the expression of p65, IκBα and its degradation. Moreover, they approached NFκB's function, which seems to mediate platelet aggregation [70]. NFκB inhibitors restricted platelet spreading, impaired aggregation and reduced ATP release, TXB_2 formation and P-selectin expression [70]. Just a little later further publications stating functional NFκB in platelets were published. Spinelli *et al.* used a different NFκB inhibitor and demonstrated reduced platelet spreading, as well as lamellapodia formation [79]. Gambaryan *et al.* described IKK activation after platelet activation in mice. However, they also showed an induction of aggregation by IKK inhibitors [80]. This seems as the exact opposite of findings from Malaver and Spinelli but it could just demonstrate the complexity of

the IKK complex, which is known to have multiple substrates (not only NFκB) in nucleated cells. The IKK complex might have multiple substrates in platelets as well [81].
Furthermore, additional evidence of NFκB's existence and non-genomic function in platelets is given from year to year [82-86]. There was a study on a substance that inhibits NFκB-mediated platelet aggregation [83] and the IKK seems to possess a non-genomic function in platelet secretion [85].
Taken together, NFκB pathway members are present in platelets and seem to function in a novel non-genomic way. The right panel of Figure 5 summarizes these findings of NFκB in platelets.

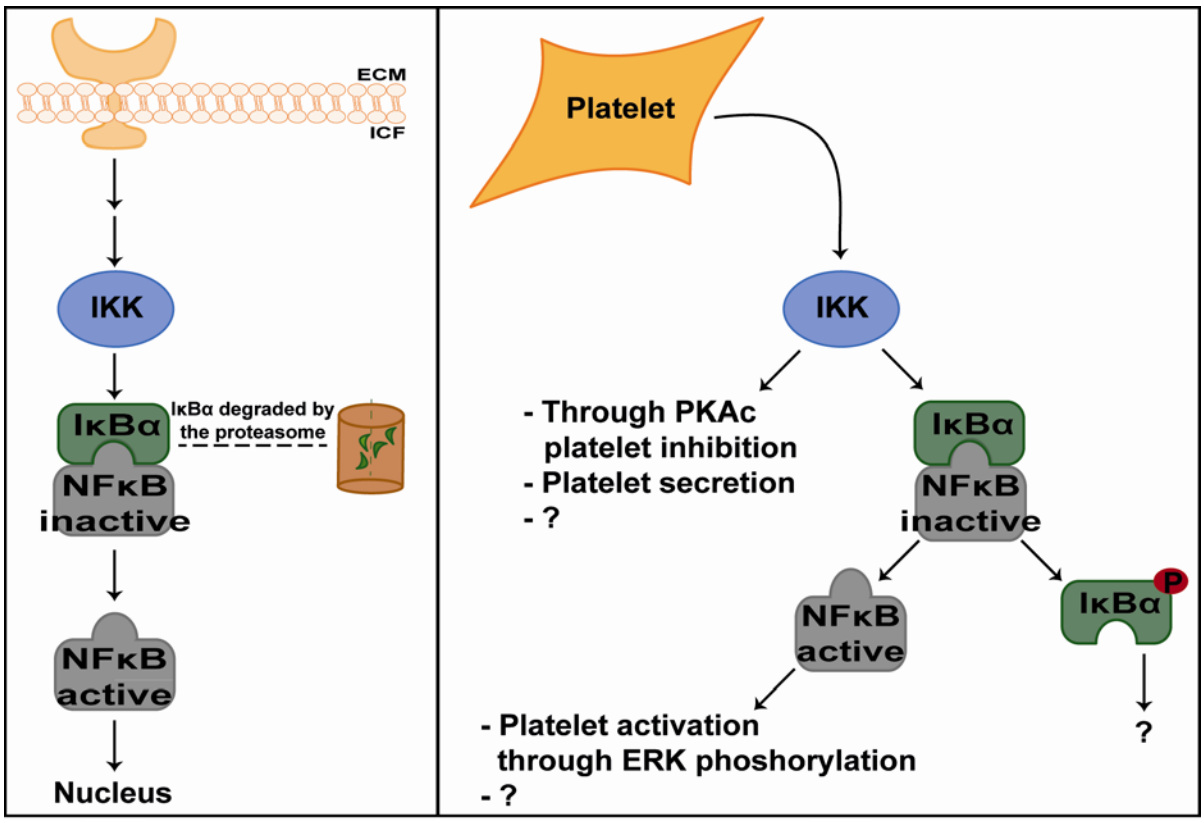


Figure 5: NFκB pathway in eukaryotic cells and its non-genomic functions in platelets. Left panel: Basic illustration of the canonical NFκB pathway in eukaryotic cells. ECM: extracellular matrix, ICF: intracellular fluid. **Right panel:** NFκB and its family members in platelets with possible non-genomic functions demonstrating the complexity of the NFκB pathway. This illustration is based on publications until today [70, 79, 80, 85, 87].

1.2.5 Protein *de novo* synthesis and protein degradation in platelets

Essential for nucleated cells is their ability to transcripe DNA to RNA and translate it in order to synthesize a protein. Platelets lack nuclei and as a consequence do not possess cellular DNA, but they have mitochondria with the mitochondrial genome [88, 89]. Nevertheless, they were considered incapable of regulating protein

synthesis [90]. In the 1960s it was published that platelets can absorb amino acids and might synthesize proteins but only in 1998 it was shown that platelets build a specific protein upon stimulation [91, 92]. Since then many proteins found in the platelet profile were found to be *de novo* synthesized in platelets [93]. Translation does not require a nucleus and is encountered in platelets giving them an alternative route for gene control [94]. Also an evolving area of research is the synthesis of proteins during storage of platelet concentrates.
More evidence for protein *de novo* synthesis in platelets is published every year. For instance, platelets indeed contain not only functional mRNA but also ribosomal RNAs and other protein components (such as Dicer or the spliceosome) to perform translation [93, 95].
Hand in hand with protein synthesis goes protein degradation. To maintain protein homeostasis, cells balance protein synthesis with degradation. Protein degradation in nucleated cells is well studied and is performed by two main pathways, the lysosomal and the ubiquitin proteasome system. Our current understanding of degradation pathways in platelets is not as detailed. Platelets contain the cysteine protease calpain which regulates many cellular processes with its proteolytic activity. Known substrates of calpain are cytoskeletal and membrane proteins. Therefore calpain regulates amongst others granule secretion and cell spreading [96]. Furthermore, platelets express many other proteases but essential here is that platelets possess the special protease, the proteasome, which is one of the main systems to degrade proteins [59, 97]. The proteasome is discussed in the following section.
All in all, anucleate platelets seem to have complex degradation systems [98].

1.3 The proteasome, a multicatalytic enzyme

Proteolysis is very important to regulate cellular protein levels and there exist two main pathways to degrade proteins in eurkaryotes. The lysosomal and the ubiquitin proteasome system are responsible for intracellular protein turnover [99, 100]. Since its discovery the ubiquitin proteasome system came into focus expanding the role of proteolysis from mere housekeeping to regulator of major cellular processes. It plays a crucial role in regulating the cell cycle, division, survival, oncogenesis, transcription, development, selective elimination of abnormal proteins, and antigen processing [101-104]. The protein to be degraded, the substrate, is tagged by a polyubiquitin chain to one of its lysine residues. This tagging is performed by a series of enzyme-mediated reactions. First, ubiquitin is activated by ubiquitin-activating enzyme (E1), the activated ubiquitin is brought to an ubiquitin-conjugating enzyme (E2) and finally ubiquitin is ligated to the lysine residue of the substrate through the action of an E3 ligase. Further activated ubiquitins are attached to internal lysine residues within the already attached ubiquitin to form polyubiquitin chains. The polyubiquitin tag is recognized by the 26S proteasome. The proteasome, just one single protease, has the central role of degrading the protein in the ubiquitin proteasome system [105,

106]. Indeed regulation of the proteasome occurs on muliple levels, is extraordinarily complex and not fully understood until today. The left panel of Figure 6 illustrates the ubiquitin proteasome system. Degradation products are short peptides that can be recycled to produce new proteins and the polyubiquitin tag can be hydrolyzed and reused as well.

1.3.1 Structure and complexity of the proteasome

The proteolytic component of the ubiquitin proteasome system is the 26S proteasome, which consists of two 19S (PA700) regulatory particles and a core particle, the 20S proteasome.

The 20S core particle

The 20S proteasome is a 700kDa, cylinder-shaped protease with four stacked heptameric rings. This cylinder contains 28 protein subunits that are arranged in a specific way, two outer α rings and the two inner β rings ($\alpha_7\beta_7\beta_7\alpha_7$) [107, 108]. The outer rings interact with the 19S particles and the inner rings harbor the proteolytic activites. In eurkaryotes three β-type subunits contain proteolytically active centers [107]. Their activity comes from their N-terminal threonine residue, which acts as a nucleophile. Therefore proteasomes are classified as N-terminal nucleophilic hydrolases/proteases [109]. The β5, β2, and β1 subunits contain the active centers and according to their distinct cleavage preferences they are termed chymotrypsin-like, trypsin-like, and caspase-like activities, respectively (CT-L, T-L, C-L) [110, 111]. The interior of the cylinder contains a cavity consisting of three contiguous chambers joint by narrow constrictions [107, 108]. The right panel of Figure 6 clarifies the structure of the 20S core particle of the proteasome. The structure indicates that substrates enter through a gated-channel [112]. The unfolded amino acid chains are brought through the particle in a continuous way and each active site can cleave the chain after specific amino acid residues. The CT-L activity (β5 subunit) cuts after hydrophobic amino acid residues [110], the T-L activity (β2 subunit) after basic amino acid residues [110] and the C-L activity (β1 subunit) cleaves peptide bonds after acidic and branched-chain amino acids [110, 111, 113, 114].

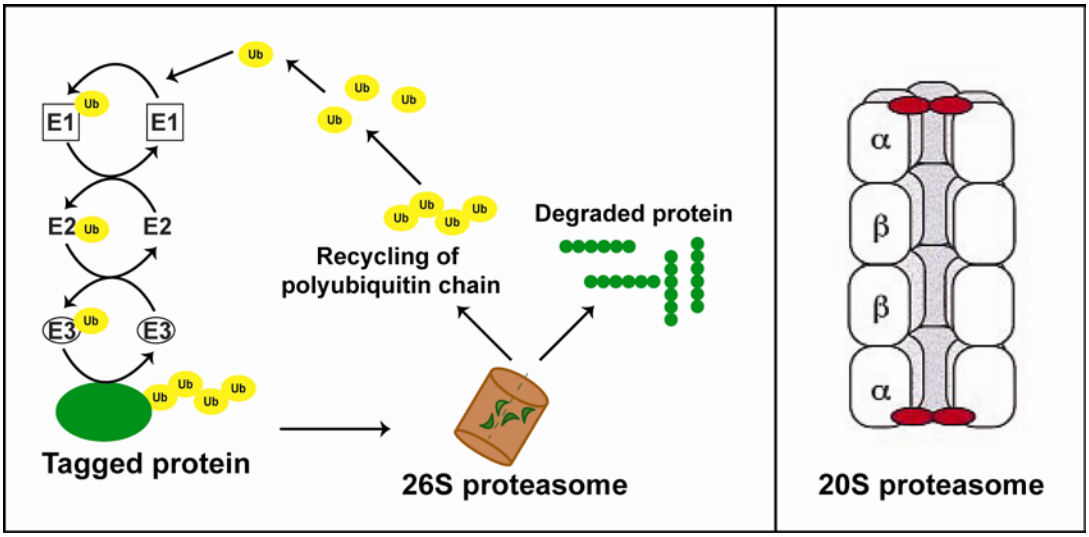


Figure 6: The ubiquitin proteasome system simplified with its central protein, the proteasome. The right panel pictures a schematic view of the ubiquitin proteasome system. Ub: ubiquitin, E1: ubiquitin-activating enzyme, E2: ubiquitin-conjugating enzyme, E3: a ligase. Left panel: The structure of a 20S core particle with its four heptameric rings stacked together [112].

Proteasome regulators and the formation of different complexes

There are various types of intracellular proteasomes because the catalytic core protein can be associated to several types of regulatory subunits (the 19S and the 11S particle) and it can be associated to just one or two regulatory subunits [115-117]. *In vitro*, the 20S core alone cannot degrade large proteins. They have to be denatured and the 20S core needs to be activated which happens with SDS, heat or specific ionic conditions [118, 119]. The exact mechanism for this is unclear. Under physiological conditions a regulatory particle can perform this activation of the 20S core.

The 19S regulatory particle is 700kDa and organized in a base and a lid where the base is attached to one of the terminal rings of the 20S core particle [120, 121]. The 19S aids in substrate recognition, untagging and unfolding [122-124]. Additionally, the 19S mediates conformational changes in the 20S core to let a substrate enter the core cavity. The 19S regulator consists of six ATPases and 15 additional subunits. Unfolding, allowing entrance to the 20S core and cutting off the polyubiquitin tag are ATP-dependent functions of the 19S [125]. Even though the 19S regulatory particle recognizes polyubiquitinated proteins that are to be degraded there is evidence that an ATP-dependent but ubiquitin-independent degradation by the 19S/20S particle of just a few proteins exists [126, 127].

The 11S (PA28) is another regulator that functions in an ATP- and ubiquitin-independent manner [128, 129]. It also associates with both or one of the terminal rings of the 20S core particle. The 11S is approximately 200kDa and opens the entrance for short peptides to the cavity of the 20S core [128, 129]. Those short peptides processed by the 11S-20S have been associated with antigen presentation on MHC I molecules [130, 131]. For its relevance in processing antigens for MHC I

presentation this 11S particle coupled to the 20S core is referred to as the immunoproteasome. The immunoproteasome is not only the 20S core particle with the 11S regulator but three of the subunits of the core are replaced by other subunits. In this way the cleavage specificity of peptides by the proteasome is altered in a manner that favors production of antigenic peptides [130].
Furthermore, hybrid complexes like 11S+20S+19S were discovered [115, 117] in eukaryotic cells but their functional role remains to be determined.
Figure 7 shows electron microscopy pictures of different proteasome complexes in an eukaryotic cell.

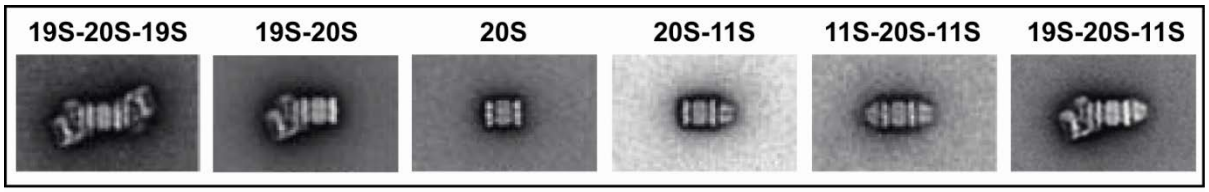


Figure 7: Electron micrographs of different proteasome complexes. 20S and 26S proteasomes were purified from rabbits and murine 11S proteasomes were recombinant expressed in *E. coli*. Modified from Cascio *et al.* [115].

1.3.2 Proteasome inhibitor classes

To understand an enzyme, the investigative tool of inhibiting its activity has been widely used. For many years the classes and mechanisms of proteasome inhibitors had been studied but only in 1995 with the ability to crystallize the huge protein complex, the proteasome, the exact biochemical mechanism of the proteasome could be suggested [108]. The proteasome can be classified as a threonine protease, a new family of proteases. Even though the three catalytic activities of the proteasome have distinct substrate specificities they share a common mechanism. An N-terminal threonine hydroxyl group at each active site serves as a nucleophil. With the help of one water molecule a peptide bond is cleaved [108, 132, 133].
There exist various natural and synthetic compounds that inhibit the proteasome. Most of them can be classified into groups but several new classes were and are identified through the synthesis of chemically modified versions of already existing inhibitors.
Synthetic reversible peptide aldehydes, such as leupeptin or MG132 are widely used to inhibit the proteasome *in vitro* and *in vivo*. Most of them inhibit primarily the CT-L activity but are capable of modifying all three activities at high concentrations [107]. Unfortunately, they interact with serine and cysteine proteases as well and therefore they are not selective for the inhibition of the proteasome [134]. Peptide boron acids do not cross-react with cysteine proteases but are still not highly specific for the proteasome. The dipeptide boronic acid bortezomib has been approved for treatment of multiple myeloma patients [135]. Bortezomib preferentially inhibits the CT-L acitivity, to a lesser extent the C-L activity but the T-L activity is left untouched [136,

137]. Only the crystallization of the 20S core with bortezomib gave an explanation for its different binding affinities to the three active sites [137]. The individual side chains of the inhibitor interact different with protein specificity pockets.
The natural compound lactacystin is a specific proteasome inhibitor that forms an intra-molecular lactone, which reacts then with the active site threonine [138]. Unfortunately lactacystin inhibits cathepsin A too [139]. In addtion, the synthesis is not as cheap and therefore its frequent use is limited.
Epoxomicin is another natural occurring highly specific proteasome inhibitor [140]. Epoxomicin belongs to the group of α',β' epoxyketones, where a highly stable six-member ring is formed. There is no cross-reactivity of epoxomicin known.

1.3.3 Regulation of the proteasome

Due to the diverse functions of the proteasome the regulation of the proteasome seems complex and is not fully understood. The proteasome does not only degrade proteins it plays an essential role in regulating the cell cycle, division and survival, oncogenesis, transcription, development, selective elimination of abnormal proteins, and antigen processing [101-104]. The regulation of the proteasome seems to result from proteins associating with the proteasome, from the different proteasome complexes itself, from an induction of specific proteasome subunits or posttranscriptional modifications of proteasome subunits.
It is known that the 11S regulator associated with the 20S core proteasome preferentially processes peptides for antigen presentation [130, 131]. The induction of specific proteasome subunits, as in the immunoproteasome, regulates also the function of the proteasome with a preference for processing antigens [141].
Associating partners of the proteasome are, for instance, PKA, and casein kinase II (CKII) [142, 143].
Subunits Rpt6 (PSMC5) and Rpt2 (PSMC1) of the 19S regulatory particle are known to undergo posttranscriptional modifications and influencing the proteasome activity in this way. The ATPase subunit Rpt6 is phosphorylated by PKA and based on this the proteasome is activated [144]. On the contrary, when the ATPase subunit Rpt2 is O-linked with N-acetylglucosamine, proteasome activity is suppressed [145]. Indeed, other subunits are posttranscriptional modified as well [146].
Additionally, recent studies demonstrated that the proteasome is able to cleave proteins and not degrade them. This proteasomal protein processing is a novel function of the proteasome. Some proteins are not completely degraded by the proteasome but degradation yields biologically active protein fragments. The transcription factors NFκB, and distant homologues of NFκB (Spt23p, Mga2p) are generated from precursors by the proteasome [147-149]. Recently, Gupta *et al.* indicated proteasomal cleavage of cytoskeletal proteins Filamin A and Talin-1 [150]. Some models of this novel mechanism of the proteasome have been discussed but the underlying process remains to be studied in detail [151].

1.3.4 Involvement of the proteasome in disease

Since most intracellular proteins are degraded by the proteasome and the proteasome plays an essential role in many cellular processes, an influence or abnormalities of the proteasome have been mentioned in many diseases. Proteolysis with the proteasome as one of its major initiators is crucial in nucleated as well as anucleate cells.
Formation of amyloid fibers in Alzheimer's disease could arise from proteasome defects [152]. In early stages of atherosclerosis the proteasome has been found to be involved [153]. As an example, in smoth muscle cells the proteasome favors a conversion from a contractile to a metabolic phenotype promoting atherosclerosis [154]. Moreover, Tisdale *et al.* demonstrates that muscle proteolysis comes from the increased activity and expression of the proteasome. Therefore to prevent muscle proteolysis, which is common in diseases such as sepsis, he suggests the ubiquitin proteasome pathway as a therapeutic target [155].
Already long ago proteasome inhibitors were identified to have antitumor effects and nowadays proteasome inhibitors as bortezomib or carflizomib are used as therapeutic agents [135, 156]. Bortezomib (Valcade®) was already approved in 2003 in multiple myeloma. In malignant cells a persistent proteasome inhibition results in growth arrest and apoptosis reducing tumor tissue [157]. Therefore improvement and synthesis of novel or second generation proteasome inhibitors are of huge relevance for cancer therapeutics [158].

1.3.5 The proteasome in anucleate platelets

Considering the highly conserved structures and functions of the proteasome in eukaryotic cells, the discovery of the proteasome in platelets seemed a logical finding. Already in 1991 Yukawa *et al.* purified the platelet proteasome [59] and in 1993 a proteasome activator in platelets was described [159]. Ostrowska *et al.* confirmed the identity of a proteasome in platelets and showed evidence of the 11S regulator in human platelets [160].
Even though the CT-L activity of the 20S proteasome from platelets was already measured by Yukawa *et al.* and Ostrowska *et al.*, detailed analysis of the regulation and function of the proteasome in platelets had not been provided for a long time.
Only when proteasome subunits were found to be downregulated in patients [161, 162], further studies followed. The importance of protein degradation and therefore the proteasome in anucleate platelets was implied in 2013 [98].
The 20S CT-L proteasome activity can be regulated. It was found to increase in response to agonist stimulation [163] but other activities had not been studied.
The 11S regulator was already mentioned in the past and there is data on individual proteasome subunits in platelets [60] but Klockenbusch *et al.* only recently published detailed proteomic analysis identifying nearly all subunits of the 26S proteasome in

human platelets [63]. They also discovered that platelets have an active immunoproteasome [63] which is of relevance since platelets present antigens as well (see section 1.2.3).
In megakaryocytes the proteasome is essential for platelet production [164]. But the importance of the proteasome in platelets has not been tested in detail. It is just mentioned that the proteasome might play a role in activation and aggregation [150]. Gupta *et al.* stated that low concentrations of thrombin reduced aggregation and thrombin and ADP stimulation suppressed microparticle shedding. They demonstrated that Filamin A and Talin-1, cytoskeletal proteins, are ubiquitinated and then cleaved by the proteasome in platelets. They hypothesize that the platelet proteasome modifies cytoskeletal proteins and promotes platelet activation. More in depth analysis is necessary to support this data and to find out the exact role of the proteasome in platelet activation.

1.4 Aim of the study

Platelets play an important role in vascular integrity and more extended functions of platelets have been described. Protein synthesis and degradation are essential processes in cells and although the last decade has shown that platelets also synthesize proteins *de novo*, the role of degradation in anucleate platelets is not fully understood. Thus, a more detailed knowledge of the proteolytic systems in platelets is necessary. In this context the proteasome as one of the major proteolytic systems has merely been studied in platelets.

Therefore we aimed to characterize the role of the proteasome in human platelets from a functional perspective. For this purpose a central function of platelets, aggregation was tested in regard to the role of the proteasome. Aggregation was analyzed under proteasome inhibitor treatment. Next, an activation of the proteasome including all proteolytic subunits by known platelet agonists, such as thrombin, ADP, and collagen was to be examined.
In order to explain the role of the proteasome in platelets, associated signaling pathways must be shown. Consequently, a connection of one signaling pathway with the proteasome under platelet agonist treatment was surveyed. The NFκB pathway has been identified in platelets and affects platelet aggregation. Since the inhibitory protein of NFκB, IκBα, is a proteasome substrate a connection of the proteasome and NFκB pathway in platelet aggregation was inspected. Moreover, shape change plays a crucial role during platelet aggregation and platelets contain a large amount of actin. Thus, it was important to investigate cytoskeletal protein processing as well. For that reason, cytoskeletal protein cleavage was viewed with proteasome inhibition.

In summary, this thesis has the intention to enlighten the role and function of the proteasome in human platelets with regard to aggregation, cytoskeletal protein regulation, connection to the NFκB pathway as well as potential underlying mechanisms (Figure 8).

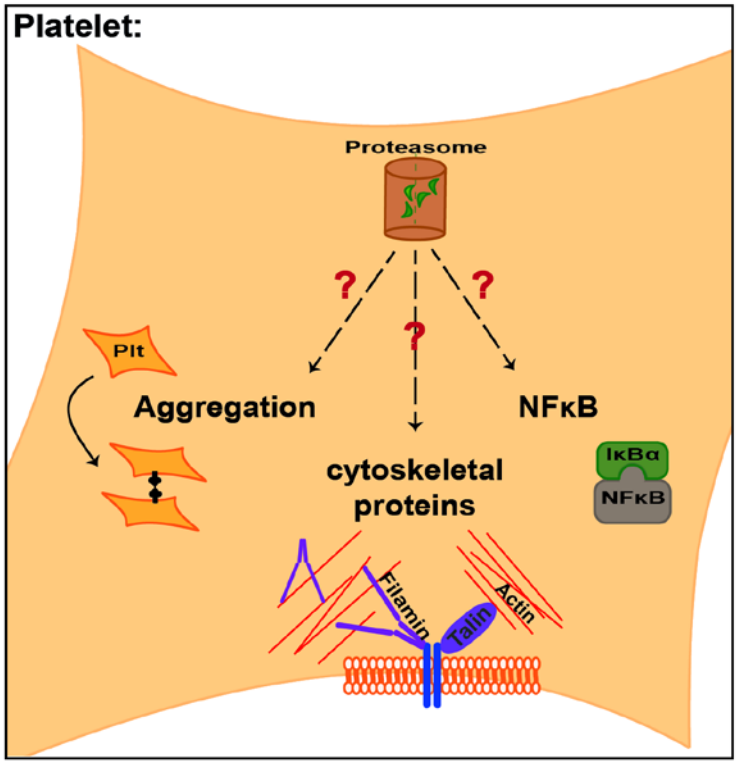


Figure 8: Aim of the study. What is the function of the proteasome in human platelets? Is there an impact of the proteasome on cytoskeletal proteins? Is there a connection of the NFκB pathway and the proteasome in platelets? Plt: platelet

2 Material and Methods

2.1 Material

2.1.1 Instruments

12-channel pipette 200µl	Eppendorf, Hamburg, Germany
Aggregometer 490-2D	Chrono-log Corporation, Havertown, USA
BD FACS Canto™ II	Becton Dickinson, Franklin Lakes, USA
Centrifuge 5810R	Eppendorf, Hamburg, Germany
Coulter® Ac·T diff™	Beckman Coulter, Krefeld, Germany
Digital CCD Camera Controller ORCA-ER	Hamamatsu Photonics, Hamamatsu, Japan
Fixed-angle rotor F-45-30-11	Eppendorf, Hamburg, Germany
Handystep® electronic	Brand, Wertheim, Germany
Heraeus Megafuge 1.0	Heraeus, Hanau, Germany
Horizontal table top autoclave Systec DE-65	Systec, Linden, Germany
Ice machine Scotsman AF 100	Scotsman, Vernon Hills, USA
Incubator Heraeus B 5042	Heraeus, Hanau, Germany
Incubator Heraeus Function Line	Heraeus, Hanau, Germany
Inverted microscope CKX41	Olympus, Shinjuku, Japan
Microcentrifuge 5815R	Eppendorf, Hamburg, Germany
Microflow biological safety cabinet	Nunc, Wiesbaden, Germany
Microplate reader FLUOstar	BMG Labtech, Worcester, USA
Microplate reader Tecan Spectra Fluor	Tecan Group, Männedorf, Switzerland
Multichannel pipette Transferpette S	Brand, Wertheim, Germany
Multipipette® puls	Eppendorf, Hamburg, Germany
Multiplate® Analyzer	Roche, Basel, Switzerland
Ocular lens 10x and 40x	Olympus, Shinjuku, Japan
PerfectBlue Dual Gel System Twin S	Peqlab, VWR, Erlangen, Germany
PerfectBlue Tank Electro Blotter Web S	Peqlab, VWR, Erlangen, Germany
Pipette aid accu-jet® pro	Brand, Wertheim, Germany
Pipettes Research® 2,5µl, 10µl, 100µl, 200µl, 1000µl, 5000µl	Eppendorf, Hamburg, Germany
Pipettes Research® Plus 20µl, 200µl, 1000µl	Eppendorf, Hamburg, Germany
PowerPac™ Basic	Bio-Rad Laboratories, Hercules, USA
Shaker Polymax 1040	Heidolph Instruments, Schwabach, Germany
Spectral photometer UV-1602	Shimadzu, Kyoto, Japan

Standard Power Pack P25	Biometra, Göttingen, Germany
Swing-bucket rotor A-4-62	Eppendorf, Hamburg, Germany
Tankblot Eco Mini Biometra	AnalytikJena, Jena, Germany
ThermoCell cooling heating block HB 202	Biozym Scientific, Hessisch Oldendorf, Germany
Vortexer	Kisker Biotech, Steinfurt, Germany
Waterbath WBN 10	Memmert, Schwabach, Germany
Weighing scale Acculab ALC 110.4	Sartorius, Göttingen, Germany

2.1.2 Glas and platic labware

Brand® cuvette semi-micro	Brand, Wertheim, Germany
Brandplates® microplates 96-wells black	Brand, Wertheim, Germany
Cell culture dish 100 x 20mm	Sarstedt, Nümbrecht, Germany
Cell culture flask 75cm^2	Greiner Bio-One, Kremsmünster, Austria
Cell scraper 2-position blade 25	Sarstedt, Nümbrecht, Germany
Corning® centrifuge tubes (15, 50ml)	Corning Incorporated, New York, USA
CryoPure cryo tube 1.6ml	Sarstedt, Nümbrecht, Germany
Fast-Read 102®	Biosigma, Cona, Italy
Gazin® gauze ball plum-size	Lohmann & Rauscher, Rengsdorf, Germany
Glass cuvettes 450µl	Probe & Go, Osburg, Germany
Injekt™ syringe 20ml	Braun, Melsungen, Germany
Micro tube 0.5ml, 1.5ml, 2ml	Sarstedt, Nümbrecht, Germany
Micro tube 1.5ml brown	Sarstedt, Nümbrecht, Germany
Micro tube 1.5ml low binding	Sarstedt, Nümbrecht, Germany
Microtest plate 96-well, flat base	Sarstedt, Nümbrecht, Germany
Multiply®-Pro cup 0.2ml	Sarstedt, Nümbrecht, Germany
Nalgene™ Rapid-Flow™Filter Unit, pore 0.2µm	Thermo Scientific, Waltham, USA
Parafilm M®	Pechiney Plastic Packaging, Chicago, USA
Pasteur pipettes GPP 1.0	Kisker Biotech, Steinfurt, Germany
Petri dish 92x16mm with cams	Sarstedt, Nümbrecht, Germany
Pipette tips (20µl, 200µl, 1ml, 5ml)	Sarstedt, Nümbrecht, Germany
Protective gloves Vasco® Basic	Braun, Melsungen, Germany
Safety-Multifly® needle 21G	Sarstedt, Nümbrecht, Germany
Serological pipettes nonsteril (10ml, 25ml)	Sarstedt, Nümbrecht, Germany
Serological pipettes steril (1ml, 2ml, 5ml, 10ml, 25ml)	Sarstedt, Nümbrecht, Germany
Single use filter unit, 0.2µm	Sartorius, Göttingen, Germany
S-Monovette®	Sarstedt, Nümbrecht, Germany
Stir bars siliconized	Probe & Go, Osburg, Germany
Tube 11.5ml	Sarstedt, Nümbrecht, Germany
Tube 5ml	Sarstedt, Nümbrecht, Germany
Various laboratory glassware	Brand, Wertheim, Germany

Various laboratory glassware	Duran Group, Mainz, Germany

2.1.3 Inhibitors, chemicals and reagents

Inhibitors

Bay 11-7082	NFκB inhibitor	Sigma-Aldrich, Seelze, Germany
Bortezomib	Proteasome inhibitor	Merck Millipore, Billerica, USA
Epoxomicin	Proteasome inhibitor	Merck Millipore, Billerica, USA
Lactacystin	Proteasome inhibitor	Enzo Biochem, Farmingdale, USA
MG132 (Z-Leu-Leu-Leu-al)	Proteasome inhibitor	Sigma-Aldrich, Seelze, Germany
Ro 106-9920	NFκB inhibitor	Tocris, Bristol, England
Z-Pro-Nle-Asp-CHO	Proteasome inhibitor	Enzo Biochem, Farmingdale, USA

Chemicals for platelet activation

Adenosine diphosphate	Sigma-Aldrich, Seelze, Germany
Adenosine diphosphate (ADPtest)	Roche, Basel, Switzerland
Collagen (COLtest)	Roche, Basel, Switzerland
Thrombin from bovine plasma	Sigma-Aldrich, Seelze, Germany
TRAP-6 (H-Ser-Phe-Leu-Leu-Arg-Asn-OH)	Bachem, Bubendorf, Switzerland

Chemicals and reagents

A23187	Merck Millipore, Billerica, USA
Acetic acid 100%	Merck, Darmstadt, Germany
Acrylamide	Panreac AppliChem, Cheshire, USA
Adenosine triphosphate	Sigma-Aldrich, Seelze, Germany
Agarose	Panreac AppliChem, Cheshire, USA
Aminomethylcoumarin	Biomol, Hamburg, Germany
Ammonium persulfate (APS)	Panreac AppliChem, Cheshire, USA
Bacto-Yeast Extract	Panreac AppliChem, Cheshire, USA
Bovine serum albumin (BSA), Fraction V	Panreac AppliChem, Cheshire, USA
Bromophenol blue	Sigma-Aldrich, Seelze, Germany
Calcium choride	Merck, Darmstadt, Deutschland
Cell Lysis Buffer 10x	Cell Signaling, Danvers, USA
Color Prestained Protein Standard, Broad Range	New England Biolabs, Ipswich, USA
cOmplete Mini Protease Inhibitor Cocktail Tablets	Roche, Basel, Switzerland
D-Glucose	Merck, Darmstadt, Deutschland
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich, Seelze, Germany
Disodium hydrogen phosphate	Sigma-Aldrich (former Fluka), Seelze, Germany
Dithiothreitol (DTT)	Panreac AppliChem, Cheshire, USA
DMEM	Sigma-Aldrich, Seelze, Germany
Endopan 3, Basal Medium for Endothelial Cells	PAN-Biotech, Aidenbach, Germany
Ethanol 70%	Panreac AppliChem, Cheshire, USA
Ethanol 99%	Panreac AppliChem, Cheshire, USA
Ethylenediaminetetraacetic acid (EDTA)	Merck, Darmstadt, Deutschland

Fetal Bovine Serum	Biochrom by Merck, Darmstadt, Deutschland
Glycerol	Panreac AppliChem, Cheshire, USA
Glycine	Panreac AppliChem, Cheshire, USA
HEPES	Panreac AppliChem, Cheshire, USA
Hydrogen peroxide	Panreac AppliChem, Cheshire, USA
IGEPAL® CA-630	Sigma-Aldrich, Seelze, Germany
Iloprost (Ilomedin®)	Bayer Schering Pharma AG, Berlin, Germany
Ionomycin	Cayman Chemical, Ann Arbor, USA
Isopropanol	Panreac AppliChem, Cheshire, USA
Luminol	Panreac AppliChem, Cheshire, USA
Magnesium chloride	Merck, Darmstadt, Deutschland
Medium 199	Sigma-Aldrich, Seelze, Germany
Methanol	Panreac AppliChem, Cheshire, USA
Monosodium phosphate	Merck, Darmstadt, Deutschland
Nonfat dried milk powder	Panreac AppliChem, Cheshire, USA
PageRuler Plus Prestained Protein Ladder	ThermoScientific, Waltham, USA
p-Coumaric acid	Sigma-Aldrich (former Fluka), Seelze, Germany
Penicillin-Streptomycin	Sigma-Aldrich, Seelze, Germany
Phenylmethylsulfonyl fluoride	Sigma-Aldrich, Seelze, Germany
Phosphatase Inhibitor Cocktail 2	Sigma-Aldrich, Seelze, Germany
Ponceau S solution	Sigma-Aldrich (former Fluka), Seelze, Germany
Potassium chloride	Panreac AppliChem, Cheshire, USA
Potassium dihydrogen phosphate	Merck, Darmstadt, Deutschland
Precise Tris-Glycine Gels 8-16%	ThermoScientific, Waltham, USA
purified human proteasome 20S	Enzo Biochem, Farmingdale, USA
RPMI 1640	Biochrom, Berlin, Germany
ServaGel™TG Prime™ 8-16%	Serva Electrophoresis, Heidelberg, Germany
Sodium bicarbonate	Sigma-Aldrich, Seelze, Germany
Sodium chloride	Panreac AppliChem, Cheshire, USA
Sodium dodecyl sulfate (SDS)	Panreac AppliChem, Cheshire, USA
Sodium hydroxide	Roth, Karlsruhe, Germany
ß-Mercaptoethanol	Sigma-Aldrich, Seelze, Germany
TEMED	Panreac AppliChem, Cheshire, USA
Tris (Tris(hydroxymethyl)-aminomethan)	Panreac AppliChem, Cheshire, USA
Trisodium citrate solution 0,11M	Waldeck, Münster, Germany
Triton-X 100	Sigma-Aldrich, Seelze, Germany
Trypan blue	Sigma-Aldrich, Seelze, Germany
Trypsin 10x	Sigma-Aldrich, Seelze, Germany
Tryptone CULTIMED	Panreac AppliChem, Cheshire, USA
Tween® 20	Panreac AppliChem, Cheshire, USA
Whatman filter paper	GE Healthcare, Munich, Germany

2.1.4 Bacteria strains

The uropathogenic *Escherichia coli* (UPEC) strain UTI89 was analyzed during this study, along with its generated knockouts for hlyA and cnf1. HlyA, α-hemolysin, is a pore-forming toxin and cnf1 is the cytotoxic necrotizing factor 1. The targeted knockouts were created using the lambda Red-mediated linear transformation system. Those strains were provided by M.A. Mulvey, University of Utah [165]. Form "Z" is on hand and approved.

UTI89
UTI89 ΔhlA::kan
UTI89 Δcnf-1::clm
UTI89 ΔhlA::kan Δcnf-1::clm

2.1.5 Kits

20S Proteasome Activity Assay	Merck Millipore, Billerica, Germany
BCA Protein Assay Kit	ThermoScientific, Waltham, USA
MitoPT™ JC-1 Assay Kit	ImmunoChemistry, Bloomington, USA
NFκB (p65) Transcription Factor Assay Kit	Abnova, Taipei, Taiwan

2.1.6 Gel preparations for SDS-PAGE home-made gels

Separating gels

	10%	***12%***
Acrylamide (30%)	15ml	18ml
1.5M Tris pH 8.8	11.335ml	11.335ml
10% SDS	450µl	450µl
Destilled H_2O	18.2ml	15.2ml
APS (10%)	250µl	250µl
TEMED	25µl	25µl

Stacking gel

	4%
Acrylamide (30%)	2.6ml
0.5M Tris pH 6.8	5ml
10% SDS	0.2ml
Destilled H_2O	12ml
APS (10%)	200µl
TEMED	40µl

2.1.7 Primary antibodies used for western blotting

Antibody	Host	Blocking, Dilution and buffer	Gel used	Amount of protein loaded	Company
Anti-Bcl-x	Mouse (MC)	5% milk, 1:330 in A	from ThermoScientific	20µg	Becton Dickinson, Franklin Lakes, USA
Anti-Filamin A	Rabbit (PC)	5% BSA, 1:1000 in TBS-T	Home-made 10% gel	20µg	Cell Signaling, Danvers, USA
Anti-Talin-1	Rabbit (MC)	5% milk, 1:1000 in B	from Serva	20µg	Cell Signaling, Danvers, USA
Anti-Ubiquitin (A-5)	Mouse (MC)	5% BSA, 1:500 in TBS-T	any	20µg	Santa Cruz, Dallas, USA
Anti-PSMA3	Rabbit (MC)	5% BSA, 1:1000 in TBS-T	Home-made 10% gel	20µg	Cell Signaling, Danvers, USA
Anti-PSMC5/TRIP1	Rabbit (PC)	5% BSA, 1:1000 in TBS-T	Home-made 10% gel	20µg	Cell Signaling, Danvers, USA
Anti-PA28α	Rabbit (MC)	5% BSA, 1:1000 in TBS-T	Home-made 10% gel	20µg	Cell Signaling, Danvers, USA
Anti-Human IκBα	Mouse (MC)	5% milk, 1:500 in B	From Serva	60µg	Becton Dickinson, Franklin Lakes, USA
Anti-β-Catenin	Rabbit (PC)	5% BSA, 1:1000 in TBS-T	Home-made 10% gel	60µg	Cell Signaling, Danvers, USA
Anti-phospho-IKKα(Ser176)/ IKKβ(Ser177)	Rabbit (MC)	5% milk, 1:1000 in B	Home-made 10% gel	60µg	Cell Signaling, Danvers, USA
Anti-β-Actin (13E5)	Rabbit (MC)	1:2000 in TBS-T	any	20µg-60µg	Cell Signaling, Danvers, USA

Table 1: Primary antibodies used for western blotting. MC: monoclonal; PC: polyclonal

Blocking occurred either in 5% milk or 5% BSA in TBS-T. TBS-T contains 50mM Tris, 150mM NaCl and 0.1% Tween. For buffer A 3% milk in TBS-T were prepared and buffer B consists of 5% BSA in TBS-T.

2.1.8 Secondary antibodies peroxidase conjugated used for western blotting

Antibody	Host	Dilution	Company
Anti-rabbit IgG	Goat	1:2000 in TBS-T	Merck Millipore, Billerica, USA
Anti-mouse IgG	Goat	1:2000 in TBS-T	Merck Millipore, Billerica, USA

Table 2: Secondary antibodies used for western blotting.

2.1.9 Substrates for proteasome acitivity analysis

Chymotrypsin-like activity	Suc-Leu-Leu-Val-Tyr-AMC	Bachem, Bubendorf, Switzerland
Trypsin-like activity	Boc-Leu-Ser-Thr-Arg-AMC	Bachem, Bubendorf, Switzerlad
Caspase-like activity	Z-Leu-Leu-Glu-AMC	Boston Biochem, Cambridge, USA

2.2 Methods

2.2.1 Cultivation and handling of used cell lines

Cell culture work was carried out under sterile conditions within a cell culture hood. The adherent human microvascular endothelial cells (HMEC) were provided by Ades *et al.* [166]. HMECs were cultivated in Dulbecco's Modified Eagle Medium (DMEM) enriched with 10% fetal calf serum (FCS), 10% endothelial growth medium and 1% penicillin/streptomycin at 37°C with 5% CO_2 and at 95% humidity. When cells reached confluency they were splitted, mostly twice a week 1:3, to avoid contact inhibition. For subculturing the adherent cells were gently washed with PBS (137mM NaCl, 2.7mM KCl, 8mM Na_2HPO_4 and 1.5mM KH_2PO_4, pH 7.4) and then treated with the serine protease trypsin until cells began to float. To stop the trypsin reaction and prevent cell damage the 2-fold amount of complete culture medium (compared to the trypsin solution) was added. One third of this cell suspension was given to a new cell culture dish with the adequate amount of cultivation medium.

The human leukaemia cells HL-60 (ACC-3) were obtained from German Resource Centre for Biological Material (Braunschweig, Germany). Those suspension cells are an acute myeloid leukemia cell line. They were established from the peripherial blood of a 35-year-old woman with acute myeloid leukemia (AML FAB M2) in 1976. They can be used for induction of differentiation studies. Here they were used as a nucleated control cell line. HL-60 cells were cultivated in RPMI 1640 medium enriched with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin at 37°C with

5% CO_2 and at 95% humidity. These suspension cells were cultivated in culture flasks 75cm^2 and splitted, mostly twice a week, to maintain them at 0.5-1 x 10^6 cells/ml. To split these suspension cells 1 x 10^6 cells were pelleted, washed with PBS and given to a new culture dish with the adequate amount of cultivation medium. Cells were counted using a Fast-Read 102® chamber and calculated accordingly. For the exclusion of dead cells trypan blue was added 1:5 to the cell suspension. Only viable (unstained) cells in 4 squares were counted.

$$\text{cells}/\text{ml} = \frac{\text{cell count}}{4\ (\text{squares})} \times 5\ (\text{dilution factor}) \times 10^4 (\text{chamber volume})$$

2.2.2 Thawing and freezing of cell lines

Cells were stored in a liquid nitrogen tank and when needed thawed rapidly at 37ºC. One vial HMEC cells was added to a culture dish with fresh cultivation medium. After 4-5 hours when all HMEC cells adhered new media was added. When confluent the cells were split normally. One vial of the suspension cells HL-60 was given to fresh cultivation medium and pelleted immediately to dispose the antifreezing agent DMSO. The pellet was given to a culture flask with fresh cultivation medium and cultivated as described under 2.2.1.
To freeze cells the pellet was resuspended in 90% FCS containing 10% antifreezing agent DMSO after a normal splitting step. HL-60 cells from a one culture flask were divided to three cryogenic vials. The pellet from one 10cm dish with confluent HMEC cells was frozen in one cryogenic vial. With an isopropanol filled freezing aid the vials were brought to -80°C (1°C/min). After 24h the cells were stored in a liquid nitrogen tank.

2.2.3 Platelet isolation

Blood samples were taken from healthy volunteers. Whole blood was drawn directly into plastic tubes containing sodium citrate (1:10). Sodium citrate is an effective anticoagulant which binds the calcium ions so that coagulation proteins cannot perform their tasks. After centrifugation of the whole blood without brake at 340g for 15 minutes at room temperature (RT), the platelet-rich plasma (PRP) was carefully removed and given to preheated platelet buffer (1:5) in the presence of 2ng/ml iloprost and pelleted at 600g for 10 minutes at RT. Platelet buffer contains 138mM NaCl, 2.7mM KCl, 12mM $NaHCO_3$, 0.4mM NaH_2PO_4, 1mM $MgCl_2$ x $6H_2O$, 5mM D-Glucose and 5mM HEPES, was adjusted to pH 7.35 and sterile filtered. Iloprost is an analogue of prostacyclin, a reversible platelet inhibitor [167]. Then the pellet was gently resuspended in platelet buffer. The platelet number was measured by a Beckman Coulter Ac-T Diff™ Analyser in a 1:10 diluted aliquot and platelet purity was

determined to be >99.5%. Contamination often was found to be at the lower limit of detection of the instrument. The Beckman Coulter Ac-T Diff™ Analyser is used in clinical laboratories to quantify automatically hematological values. In whole blood parameters, such as the leukocyte count, number of erythrocytes, hemoglobin concentration, hematocrit and number of platelets is analyzed using the Coulter technique. The Coulter principle is based on the change of impedance that occurs when particles pass a small capillary in an electrolyte. One pulse of impedance refers to the number of particles and the pulse height refers to the size of the particle [168]. The use of the Coulter Analyser to count platelets is described before [169].

2.2.4 Platelet stimulation

Washed platelets (up to $2x10^8$ cells/ml) were incubated in warm platelet buffer in 5ml plastic tubes at 37°C for indicated times with indicated stimuli. To pellet the cells they were transferred to 1.5ml micro tubes (protein low binding surface) and centrifuged at 600g for 2.5 minutes at RT. The pellet was lysed with the appropriate lysis buffer depending on the experiment.

2.2.5 Patient studies

Handling of blood from patients

Patients were recruited from the medical intensive care unit, intermediate care unit and emergency room of the University Medical Center Innenstadt of the University Hospital Munich. All patients were above 18 years and written informed consent was obtained from patients (control patients included) or next of kin. Blood was drawn within the first 48 hours of admission in all patients diagnosed with sepsis and controls. In the group of patients with severe sepsis, follow-up blood draws were taken according to the clinical follow-up assessment of the patient by the critical care team (see also Study population). Platelets were isolated as described under 2.2.3 but under security level S2.

Purification and resuspension of platelets in equal volumes and at equal concentrations guaranteed identical processing conditions and equalized the initial differences in platelet number among the clinical samples.

Study population

26 Patients with the diagnosis of sepsis or sever sepsis, including septic shock as previously defined were included in the study between January 2012 and January 2013 [170, 171]. Clinical disease severity was assessed with Acute Physiology and Chronic Health Evaluation II (APACHE II), Sequential Organ Failure Assessment (SOFA), and Simplified Acute Physiology Score II (SAPS II) on the days of blood draws. Severe sepsis was defined as sepsis complicated by organ failure [171]. We considered organ failure as a SOFA point score above 2, as previously used by

others [172-174]. Subsequently, the patient collective comprised nine patients with sepsis (nonsevere) and 17 patients with severe sepsis, including septic shock. In the group of patients with severe sepsis, follow-up blood draws were taken according to the clinical follow-up assessment of the patient by the critical care team, which was based on organ-failure score SOFA. Follow-up blood draws were initiated if the patient recovered to a SOFA score of less than 3 points or did not show improvement from initial SOFA score after 2 weeks. An earlier follow-up blood draw was initiated if the patient showed a rapid clinical deterioration. On average, follow-up reads were taken 7 days after admission. Seventeen control patients with noninfectious medical conditions (see also Table 5). Control patients had to have at least two permanent medical conditions requiring medical therapy and were matched by age to the severe-sepsis cohort. The study was approved by the local ethics committee of the University of Munich in accordance with the Declaration of Helsinki.

2.2.6 Platelet and bacterial interaction studies

The bacteria mentioned under 2.1.4 were used and analyzed. The bacteria were stored at -80°C in glycerol stocks (1:2). 24 hours prior to each study, 2µl of the bacteria stock were expanded on LB media plates overnight at 37°C until they reached a stationary growth phase. LB medium contained 0.5% yeast extract, 1% tryptone and 1% NaCl, was adjusted to pH 7 and autoclaved. The next day some colonies were resuspended in PBS and their concentration was determined spectrophotometrical at a wavelength of 600nm.

$$\mathrm{OD}_{600\mathrm{nm}} = \frac{1 \times 10^5 \mathrm{bacteria}}{\mu\mathrm{l}}$$

For each study, bacteria (4 x 10^6 total) were incubated in the presence of freshly-isolated platelets (1 x 10^8) for four hours in M199 culture media. This incubation period provided an environment for exponential growth for the bacteria. To lyse the cells the platelet/bacteria mixture was pelleted at 600g for 2.5 minutes at RT and further treated as described under 2.2.7 or 2.2.8.

2.2.7 Protein solubilisation for western blot analysis

HMEC protein solubilisation:

To obtain protein lysates for western blot analysis (wb) the HMEC were washed with cold PBS and ice-cold Cell Signaling lysis buffer containing an additional protease inhibitor 1mM phenylmethanesulfonylfluoride (PMSF) was added. The dishes were frozen for at least 20 minutes at -20°C and thawed on ice before scrapping off the cells. The cell suspension was transferred to a 1.5ml micro tube and for a mechanical

lysis the cells were passed through a small needle a few times. To lyse the cells completely they were incubated on ice for 20 minutes and vortexed every 10 minutes. To remove cellular debris the lysate was centrifuged at 10000g for 10 minutes at 4°C. The supernatant was given to a new micro tube and could be stored at -20°C or used directly for protein quantification.

HL-60 protein solubilisation:
To obtain protein lysates for wb the HL-60 cells were pelleted, washed with cold PBS and ice-cold Cell Signaling lysis buffer containing 1mM PMSF was added. The cell suspension was transferred to a 1.5ml micro tube and incubated for at least 20 minutes at -20°C before thawed on ice. For a mechanical lysis the cells were passed through a small needle a few times before lysing them completely on ice for 20 minutes and vortexing them every 10 minutes. To remove cellular debris the lysate was centrifuged at 10000g for 10 minutes at 4°C. The supernatant was given to a new micro tube and could be stored at -20°C or used directly for protein quantification.

Platelet protein solubilisation:
After adding the Cell Signaling lysis buffer containing 1mM PMSF the cell suspension was lysed on ice for 20 minutes and vortexed every 10 minutes. To remove cellular debris the lysate was centrifuged at 10000g for 5 minutes at 4°C. The supernatant was given to a new micro tube and could be stored at -20°C or used directly for protein quantification.

2.2.8 Protein solubilisation for proteasome activity measurements

HMEC protein solubilisation:
To obtain protein lysates for wb the HMEC were washed with cold PBS and freshly prepared and ice-cold proteasome activity lysis buffer containing 20mM HEPES (pH 7.5), 1mM MgCl2, 150mM NaCl, 0.5mM EDTA, 1mM DTT and 1% Phosphatase Inhibitor Cocktail 2 was added. The dishes were frozen for at least 20 minutes at -20°C and thawed on ice before scrapping off the cells. The cell suspension was transferred to a 1.5ml micro tube and for a mechanical lysis the cells were passed through a small needle a few times. To lyse the cells completely they were incubated on ice for 20 minutes and vortexed every 10 minutes. To measure the 26S proteasome activities the lysate had to be used right away. For measuring the 20S proteasome activities the lysate was used right away or stored at -80°C but could only be thawed once.

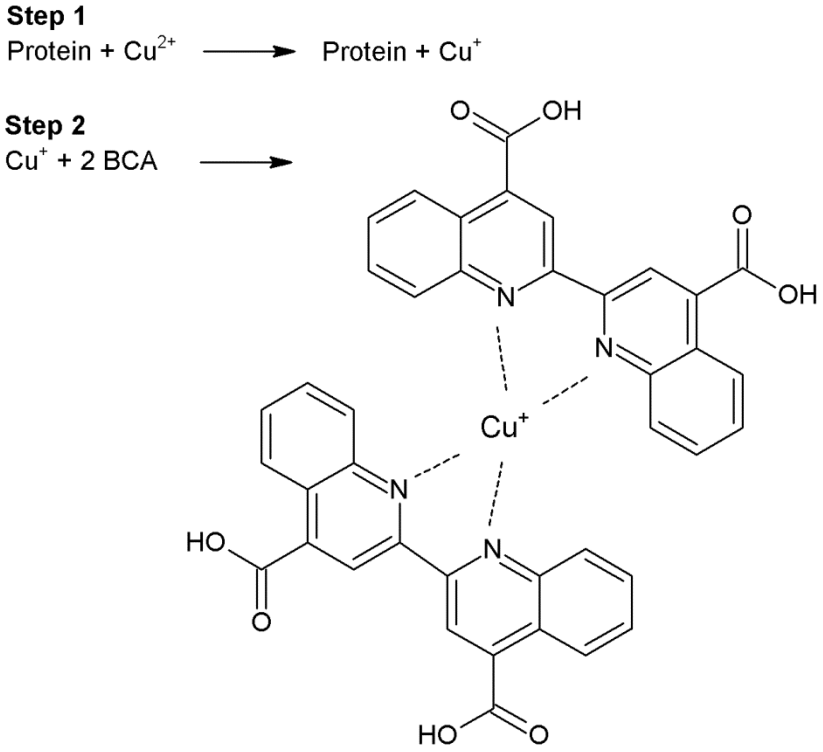
HL-60 protein solubilisation:
To obtain protein lysates for wb the HL-60 cells were pelleted, washed with cold PBS and ice-cold proteasome activity lysisbuffer was added. The cell suspension was

transferred to a 1.5ml micro tube and incubated for at least 20 minutes at -20°C before thawed on ice. For a mechanical lysis the cells were passed through a small needle a few times before lysing them completely on ice for 20 minutes and vortexing them every 10 minutes. To measure the 26S proteasome activities the lysate had to be used right away. For measuring the 20S proteasome activities the lysate was used right away or stored at -80°C but could only be thawed once.

Platelet protein solubilisation:
After adding the proteasome activity lysisbuffer the cell suspension was lysed on ice for 20 minutes and vortexed every 10 minutes. To measure the 26S proteasome activities the lysate had to be used right away. For measuring the 20S proteasome activities the lysate was used right away or stored at -80°C but could only be thawed once.

2.2.9 Protein quantification

Protein quantification was determined using the bicinchoninic acid assay (BCA) Kit. The BCA is a two-step colorimetric assay [175, 176]. The first step involves the reduction of Cu^{2+} to Cu^{1+} by protein in an alkaline medium. This is the well-known biuret reaction. In the second step a purple-colored reaction product results from the salt of the BCA chelating the reduced cuprous cation. The BCA/copper complex exhibits a strong linear absorbance at 562nm with increasing protein concentrations.



All cell lysates were analysed accordingly. The necessary amount of BCA reaction reagents A and B was mixed for one minute (50:1). In a 96-well plate 10µl of the protein standard albumin or samples were added in duplicates on ice. To each well 200µl of the mixed reaction reagents was added. The reaction took place while incubating the plate at 37°C for 30 minutes. The absorbance was measured at 550nm on a plate reader and the concentrations were calculated using a standard curve (serial dilution of albumin, BSA). Using the following equation x was dissolved to receive the amount of protein in µg.

$$y = mx + b$$

Where **y** is the measured absorbance, **m** is the gradient of the standard curve and **b** is the axis intercept of the standard curve.

2.2.10 Gel electrophoresis and western blot analysis

The western blot or immunoblot is a visualization of specific proteins. For this analytical method a protein solution needs to be separated first. Therefore proteins are separated according to their electrophoretic mobility, hence their molecular weight in SDS polyacrylamide gel electrophoresis [177]. This specific gel electrophoresis is called sodium dodecyl sulphate polyacrylamid gel electrophoresis (SDS-PAGE). Through ß-Mercaptoethanol and sodium dodecyl sulphate (SDS) proteins are denatured and applied with a negative charge. Denatured negatively-charged proteins migrate across the gel to the direction of the anode under an applied electric field.

Prepared or bought acrylamide gels were set in the electrophoresis apparatus which was filled up with running buffer consisting of 25mM Tris Base, 192mM glycine and 0.1% SDS. The self-prepared SDS gels consisted of a 10% or 12% separation gel and a 4% stacking gel. For the mixture of the gels see section 2.1.6, whereupon TEMED and APS were added immediately before pouring and the top of the separation gel was covered with isopropanol while polymerizing. 20µg up to 60µg protein, depending on the experiment and antibody (see section 2.1.7), were mixed with 4x loading buffer containing 0.25M Tris pH 6.8, 8% SDS, 40% glycerol, 0.02% bromophenol blue and 400mM ß-Mercaptoethanol and heated for 5min at 95°C. This denatured protein was loaded on the gel next to 5µl of a protein ladder. Through Bromophenol blue the loading front of the samples was visible in the gel.

Gel electrophoresis was performed at 100V in an electrophoresis apparatus by Peqlab for about 15 minutes until the loading front entered the separation gel. After the samples had passed the stacking gel electrophoresis was performed at 150V for about 40min (depending on the size of proteins).

To preserve the protein bands they were transferred to a polyvinylidene difluoride membrane (PVDF) before they could be detected using antibodies. The PVDF

membrane needed to be pre-wetted in methanol due to its hydrophobicity before it could be used with the aqueous freshly-prepared transfer buffer containing 25mM Tris Base, 200mM glycine and 20% methanol.
For blotting a wet electroblotting system was used. In between two sponges there were two sheets of Whatman filter paper, a membrane, the gel, and again two sheets of filter paper. This sandwich was prepared without any air bubbles and put in the transfer buffer-filled apparatus (the membrane facing the anode). In an electrical field for 2h at 55V the proteins were able to migrate to the membrane surface. All transferred protein bands could be visualized by staining with Ponceau S which binds reversible to amine groups of proteins. Staining occurred in 0.1% Ponceau S in 5% acetic acid for 10 minutes at RT and the stain was removed by continued washing in distilled water.
Finally specific proteins could be detected using specific primary and secondary antibodies (as indicated under sections 2.1.7 and 2.1.8) where the primary antibody binds to a specific protein on the membrane and the HRP-conjugated secondary antibody detects the heavy chain of the primary antibody. Addition of hydrogen peroxide and luminol or another chemiluminescent substrate gives a luminescence which is detected.
In the beginning the membrane was incubated for 1h in blocking solution (5% BSA or powdered milk in 1x TBS-T) at RT. TBST is a buffer consisting of 50mM Tris Base, 150mM NaCl and 0.1% Tween. Blocking excludes unspecific binding on protein free spots of the membrane. Incubation of the primary antibody was performed as composed in the antibody list under gentle shaking at 4°C overnight (2.1.7). After washing the membrane trice for 10min in TBS-T the incubation with the secondary antibody conjugated to horseradish peroxidase (HRP) took place for 1h at RT. Unbound secondary antibodies were removed by washing (3x). The protein bands were detected by adding a solution containing 0.1M Tris Base pH 8.5, 0.4mM p-coumaric acid, 2.5mM Luminol and 0.08% H_2O_2 (30%). Hereby is coumaric acid an enhancer of the reaction and H_2O_2 was added last because it starts the reaction. The membrane was developed for 20 up to 180 seconds in an imager from Hamamatsu (depending on the antibody).

$$2\ H_2O_2 + \text{Luminol} \xrightarrow{HRP} \text{3-aminophthalate} + \textbf{light} + N_2 + 2\ H_2O$$

More than one protein could be detected with one membrane. After an experiment the membrane was stored at -80°C. Density measurement was performed with the area analysis of Hokawo from Hamamatsu Photonics, Japan.

2.2.11 Proteasome activity measurements

Measurement of the chymotrypsin-like activity of the 20S proteasome using APT280 Millipore Kit

The Millipore Kit is based on the LLVY-AMC (7-amino-4-methylcoumarin) substrate that was used in many publications, such as Conconi *et al.* [178] or Pacifici *et al.* [179]. An intact proteasome is able to cleave the peptide LLVY from the fluorophor AMC with its chymotrypsin-like activity and the free AMC can be quantified using a fluorometer. Here AMC was excited at 360nm and emission at 460nm was detected.
The assay was performed as described by the manufacturer. $1x10^8$ washed platelets were lysed as described under 2.2.8 in a lysis buffer containing (150mM NaCl, 50mM HEPES (pH 7.5), 5mM EDTA, and 1%Triton-X 100). 10-20µl of lysed cell suspension was given to a black 96-well plate containing 1x assay buffer and 25µM of the substrate. Samples were measured in triplicates and standards in duplicates. Then either the kinetic for 2 hours every 15 minutes at 37°C was analyzed by detecting the emission at 460nm in a fluorometer or the end amount of AMC was detected after incubation in the dark at 37°C for 2h. Fluorescence units (FU) were detected and 20S chymotrypsin-like activitiy of the 20S proteasome is given as FU relative to the total amount of protein of each sample.

$$\mathrm{CT-L\ activity\ of\ the\ 20S\ proteasome} = \frac{\mathrm{FU}}{\mu\mathrm{g\ of\ protein}}$$

Therefore a protein quantification of each sample was performed for each experiment. To make sure the assay was functioning correctly a positive control provided by the manufacturer and an AMC standard curve was analyzed during each run.

Measurement of all six activities of the proteasome in cell lysates

As described under section 1.3.1 the multicatalytic enzyme, proteasome, possesses six activities; the chymotrypsin-like (CT-L), the trypsin-like (T-L), and the caspase-like (C-L) activity of the 26S proteasome and the same three of the 20S proteasome. With different peptides coupled to a flourophor it is possible to assess the CT-L, T-L or C-L activities and by changing the assay buffer one is able to measure either the activities of the 26S proteasome complex (dependent on ATP) or of the 20S proteasome complex (see also section 1.3.1). Activities were assayed according to modified published protocols [180, 181] and as described by Drews *et al.* 2010 [182].

Lysates were generated as described in section 2.2.8 with a freshly prepared proteasome lysis buffer containing 20mM HEPES (pH 7.5), 1mM $MgCl_2$, 150mM NaCl, 0.5mM EDTA, 1mM DTT, and 1% phosphatase inhibitor. Lysates were used directly after preparation to analyse all three activities of the 26S proteasome

complex, since this complex breaks down after freezing and then the activity will be significantly reduced.
For each 26S proteasome activity assay 10µg protein of each sample is used consequently a protein quantification was performed before measuring the proteasome activities. In order to always have the same sample amount for each assay each lysate was adjusted to a solution containing 1µg/µl protein. The assay was performed in a black 96-well plate with a total volume of 100µl per well.
All three 26S activities were measured in 20mM HEPES (pH 7.5), 1mM $MgCl_2$, 150mM NaCl, 0.5mM EDTA, 1mM DTT, and 50µM ATP [182]. Rapidly after adding 0.1mM of the appropriate peptide substrate the plate was analyzed for 2h at 37°C measuring the emission of free AMC every 15min at 460nm in a fluorometer. To measure the CT-L activity of the 26S proteasome the Suc-LLVY-AMC substrate was used; the T-L activity was measured by the Boc-LSTR-AMC; and the C-L activity was analyzed using the Z-LLE-AMC. All samples were analysed in triplicates for each substrate and standards were run in duplicates. To assure that each well could be compared the end volume of each well contained 2% of DMSO (since inhibitors and substrates were solubilised in DMSO). To calculate the rate of activity an AMC standard curve was run for each experiment. Also to assure correct functionality a positive control was analyzed each time containing a purified proteasome.
The measurement of the activities is given as enzyme turnover rate. The mean gradient of each sample was calculated and adjusted to the AMC produced per minute, and protein used. The rate is given in pmol x mg^{-1} x min^{-1}.

$$\text{Proteasome activity} = \frac{\text{gradient}}{\text{gradient of standard curve}} \times 100$$

The number 100 is based on 10µg of used protein converted to mg.
All three activities of the 20S proteasome complex were measured on one day but not necessarily the same day as the activities of the 26S proteasome complex were detected. The 20S proteasome complex is still intact after one freezing step. The measurement of the activities of the 20S proteasome complex was performed in the same way as the measurement of the activities of the 26S proteasome complex but the assay buffer varied. The CT-L activity of the 20S proteasome complex was detected in 25mM HEPES (pH 7.5), 0.5mM EDTA, and 0.03% SDS [182]. The T-L and C-L activities of the 20S proteasome complex were analyzed in 25mM HEPES (pH 7.5), 0.5mM EDTA, 0.05% NP-40, and 0.001% SDS [182]. Again samples were measured in triplicates for each substrate and the activities of the 20S proteasome complex were calculated in the same way as the activities of the 26S proteasome complex and are given in pmol x mg^{-1} x min^{-1}.
All six activities were compared and could have been compared when performed with the same lysates.

2.2.12 Fluorescence activated cell sorting

The Fluorescence Activated Cell Sorter (FACS) is able to carry out flow cytometry of cells at single cell level and cell sorting depending on the given model. For this cells are guided through a laser beam by hydrodynamic-focusing whereas parameters as forward scatter, side scatter and fluorescence can be detected. Each particle passing through the beam scatters the beam and fluorescent chemicals found in the cell or attached to the cell may be excited into emitting light. The forward scatter is a degree for the absorbed light and provides information about the cell dimensions; the side scatter indicates the reflected light and is therefore a measurement of the relative optical density which is proportional to the granularity of a cell. Through band-pass filters it is possible to detect emission spectra of various fluorescent dyes. Prior sorting the cells need to be treated with those dyes.
Analysis was performed using a FACS Canto II flow cytometer with two lasers. The data generated here is shown in histograms, in a single dimension, or in two-dimensional dot plots where every dot represents one cell. The histogram represents the cell count on the y-axis and the desired light intensity on the x-axis. To analyze the data in further detail the FACSDiva software was used during this work.
Here the FACS was used to measure the mitochondrial membrane potential in platelets.

To measure the mitochondrial membrane potential (Mmp) 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1 dye) was used. JC-1 is a lipophilic cationic dye that can penetrate living cells [183-185]. In healthy cells positively charged JC-1 can enter the negatively charged mitochondria and accumulates there [184]. In apoptotic cells when the Mmp drops JC-1 is located in the cytosol as monomers. JC-1 aggregates fluoresce red-orange and the monomeric form is green. Healthy cells are bright orange and green, whereas apoptotic cells lose their brightness and only have green fluorescence.
$5x10^6$ isolated platelets were incubated with 1x JC-1 in platelet buffer in a volume of 200µl for 20 minutes at RT in the dark in 5ml polysterene tubes. After adding 1ml of platelet buffer the samples were analyzed directly. They could not be stored.
Mmp was assessed as a ratio of the median red fluorescence and green fluorescence, as used in previous publications as well [186]. Here this ratio is called Mmp-Index. A decrease in the red-to-green fluorescence ratio (Mmp-Index) represents a loss in mitochondrial membrane potential (depolarization). Mmp-Index was calculated as the mean of triplicate readings for each patient or donor. To assure correct function of the assay in each experiment not only measurements of platelets from a healthy donor but also an internal positive control was used in each experiment. Therefore prior to JC-1 staining one sample from the healthy donor and one sample from the patient was stimulated with 10µM calcium ionophore A23187 for 10 minutes at 37°C which induced platelet apoptosis and rapid mitochondrial membrane depolarization [50].

$$\text{Mmp} - \text{Index} = \frac{\text{red fluorescence}}{\text{green fluorescence}}$$

2.2.13 Aggregometry

A classical platelet function test is aggregometry. Hereby the ability of various agonist on platelets to induce platelet activation and aggregation is analysed *in vitro*. Platelet aggregation can be assessed by light transmission aggregometry, LTA, also called Born aggregometry or through impedance aggregometry, IA [33]. During LTA light transmission is detected by a photocell that sits behind a cuvette. While platelets aggregate, less light is absorbed and the transmission increases. During IA electrical impedance in whole blood is measured with two sensor electrodes. When platelets aggregate they adhere on the metal wire and increase electrical resistance.
Born aggregometry was measured in PRP with the Aggregometer 490-2D. 400µl of freshly isolated PRP was given to a glas cuvette containing a small stirrer and hold on 37°C prior to use. PPP of the same donor was used as reference. The measurement was started by adding the agonist. Aggregation was measured 7-15 minutes depending on the agonist under constant stirring (1200rpm). Aggregation is shown here in percent change of light transmission whereas 100% refers to transmittance through reference, or aggregation is given in area under the curve whereas better aggregating samples had a higher area under the curve.
Aggregation in whole blood was performed using a Muliplate® Analyzer. Blood was drawn in tubes containing r-hirudin (0.045 mg/ml). 300µl whole blood was added to a test cell containing 300µl of saline. The measurement was started by adding an agonist and performed under 37°C for 6 minutes. Samples were measured in triplicates. The increase in impedance/aggregation is given in mean arbitrary units (AU).

2.2.14 p65 transcription factor assay kit

The transcription factor NFκB (p65) binds directly to DNA sequences when its inhibitor IκB is degraded. This active NFκB has been measured for many years. The p65 transcription factor assay from Abnova detects active NFκB by an enzyme-linked immunosorbent assay (ELISA). A 96-well plate is coated with a specific double stranded DNA (dsDNA) sequence containing the NFκB response element. Active NFκB in the samples binds to the dsDNA and is detected by a primary antibody directed against NFκB. Through a secondary antibody conjugated to HRP bound NFκB can be quantified photometrically. Normally 3,3',5,5'-tetramethylbenzidine (TMB) is used as a HRP substrate (but manufacturers do not state the exact

substrate used). The oxidised TMB is blue but not stable. By adding nitric acid a yellow product is generated which is stable and can be measured at 450nm.
Nucleated cells contain active NFκB in the nucleus and therefore an isolation of nuclei prior to lysis is necessary. The anucleate platelets not only express NFκB as well but it seems to be active [70, 79, 80].
Here 1 x 10^8 platelets were lysed according to section 2.2.7 but with a specific NFκB lysis buffer. A lysis buffer containing 200mM HEPES (pH 7.9), 400mM NaCl, 1mM EDTA, 1mM DTT, and 10% protease inhibitor cocktail was used to stabilze NFκB in the best way possible. 20µl of each platelet lysate were given to wells of a coated 96-well plate containing a specific assay buffer provided by manufacturer (100µl total volume in each well). Samples were detected in duplicates. To let binding occur the plate was incubated at 4°C over night without agitation. After washing the wells 5x with manufacturer's washing buffer the plate was incubated for 1h at RT with the primary antibody without agitation and in a second step with the secondary antibody. Prior to measuring the absorbance at 450nm photometrical the developing solution was added, the plate was incubated for 45min at RT and stopping solution was added. To assure the functionality of the assay a positive and negative control provided by manufacturer was analyzed in duplicates for each experiment.
Bound NFκB, meaning active NFκB, is given in activity/µg total protein. Therefore protein quantification was performed for each experiment.

2.2.15 MHC I peptide analysis

MHC I peptide analysis was done in cooperation with Professor Stefan Stevanovic, University of Tuebingen, Germany.
1 x 10^9 platelets were isolated and a pellet was send to Tuebingen. MHC I-presented peptides were obtained by standard immunoaffinity purification of MHC I molecules from platelets using the panHLA class I specific antibody W6/32 [187, 188]. HLA (human leukocyte antigen) ligands were analyzed by LC-MS/MS. Peptide samples were separated by reversed-phase liquid chromatography and subsequently analyzed in an on/line coupled LTQ Orbitrap-XL hybrid mass spectrometer. Eluting peptides were ionized by nanospray ionozation and analyzed in the mass spectrometer implementing a top 5 CID (collision induced dissociation) method. For data processing, the software Proteome Discoverer was used to integrate the search results of the Mascot search engine against the human proteome as comprised in the Swiss-Prot database [189].

2.2.16 Statistical analysis

Statistical analysis was performed with SigmaPlot 10.0. Data are presented as arithmetic means ±SEM. Statistical analysis was performed using a T-test for

unpaired data or ANOVAs when necessary. A p-value less than 0.05 is considered significant. For each result the specific test is named and the p-value is given under figure explanations.
Statistical analysis concerning results of patients was performed with SigmaPlot 10.0 along with SAS 9.3 for Unix and Linux. Patient characteristics, clinical scores, Mmp-Index, and proteasome acitivity values are given as median and interquartile range. Correlations of platelet Mmp-Index and clinical disease scores (APACHE II, SOFA, SAPS II) were calculated by using Spearman rank correlation coefficients. Here again for each result the specific T-test or ANOVA is named under figure explanations.

3 Results

3.1 Characterization of the proteasome in human platelets

For some years it is known that anucleate platelets express subunits of the proteasome [159-161]. Klockenbusch *et al.* [63] only recently (2014) identified all proteasome subunits in human platelets by a global proteome analysis. The proteasome has several catalytic activities [160] and can be regulated in response to an agonist stimulation [163]. But further research is necessary to have detailed knowledge of the proteasome with all its activities and its regulation in human platelets.

3.1.1 Human platelets express proteasome subunits

In order to confirm the expression of three subunits of the proteasome complex their protein levels in non-stimulated platelets was analyzed and quantitatively compared to those in nucleated cells. PSMA3, a subunit of the 20S core protein, PSMC5, a subunit of the 19S regulator, and PSME1, a subunit of the 11S regulator, are shown in Figure 9.

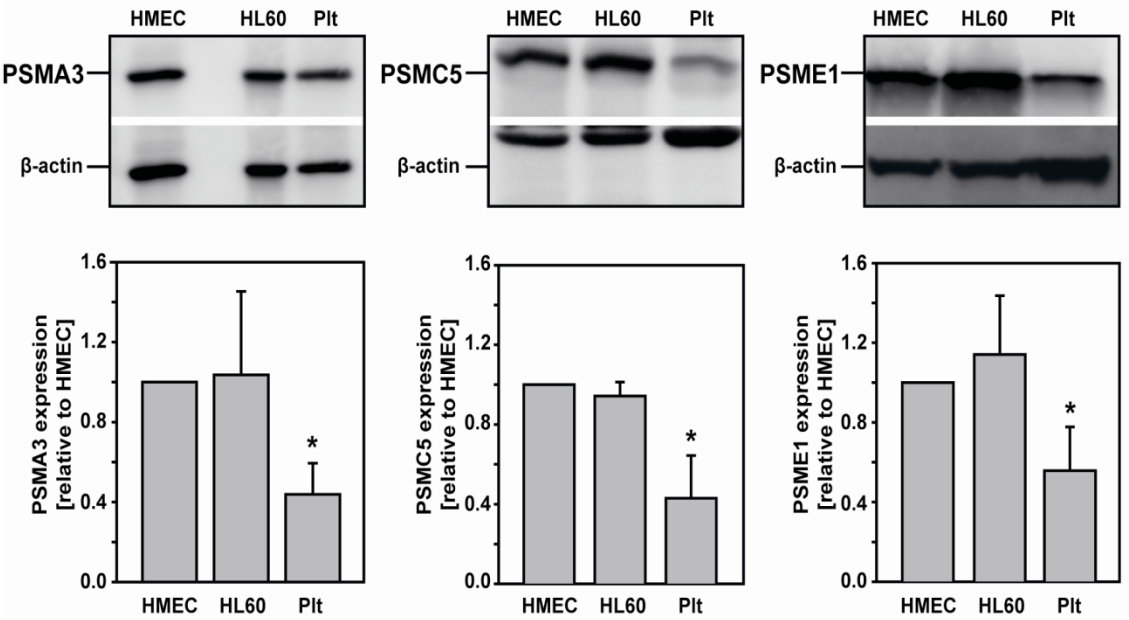


Figure 9: Expression of PSMA3, PSMC5 and PSME1 in human platelets compared to nucleated cells. Representative western blot images of the PSMA3, PSMC5 and PSME1 proteins and the loading control β-actin are illustrated of nucleated HMEC and HL-60 cells, as well as of platelets (plt). Lower panel: Proteasome subunit expression was quantified by band densitometry analysis with the help of a protein to actin ratio. Mean protein to actin ratio for the HMEC cells was set as 1 and the relative expression in HL-60 cells and platelets is seen (Kruskal-Wallis one-way analysis of variance; PSMA3: *p<0.005, n=6; PSMC5, PSME1: *p<0.003, n=5).

All three subunits of the proteasome, PSMA3, PSMC5, PSME1, are expressed in detectable levels in human platelets (Figure 9). Compared to the nucleated cells HL-60 and HMEC platelets express significantly less amount of these protein subunits. PSMA3 is 2.28±0.06-fold higher expressed in HMEC cells compared to platelets, PSMC5 is 2.33±0.10-fold higher expressed and PSME1 is 1.79±0.10-fold higher expressed in HMEC cells compared to platelets.

3.1.2 Human platelets contain an active proteasome

Further it is to be analyzed if the proteasome is active. Therefore the chymotrypsin-like (CT-L) activity of the 20S proteasome in human platelets was detected using the 20S Proteasome Activity Assay from Millipore. The kit is based on the fluorogenic peptide substrate LLVY-AMC (see section 2.2.11).

Detection and inhibition of the chymotrypsin-like activity of the 20S proteasome

After *in vitro* incubation of human platelets with the known proteasome inhibitors epoxomicin and lactacystin the 20S proteasome activity was tested. Epoxomicin belongs to the class of α',β'-epoxyketones and is highly specific for the proteasome [140]. The proteasome inhibitor lactacystin belongs to the class of β-lactones and is

less specific because it inhibits other proteases others than the proteasome as well [139] and it restrains the proteasome activity at a slower rate than epoxomicin [140].

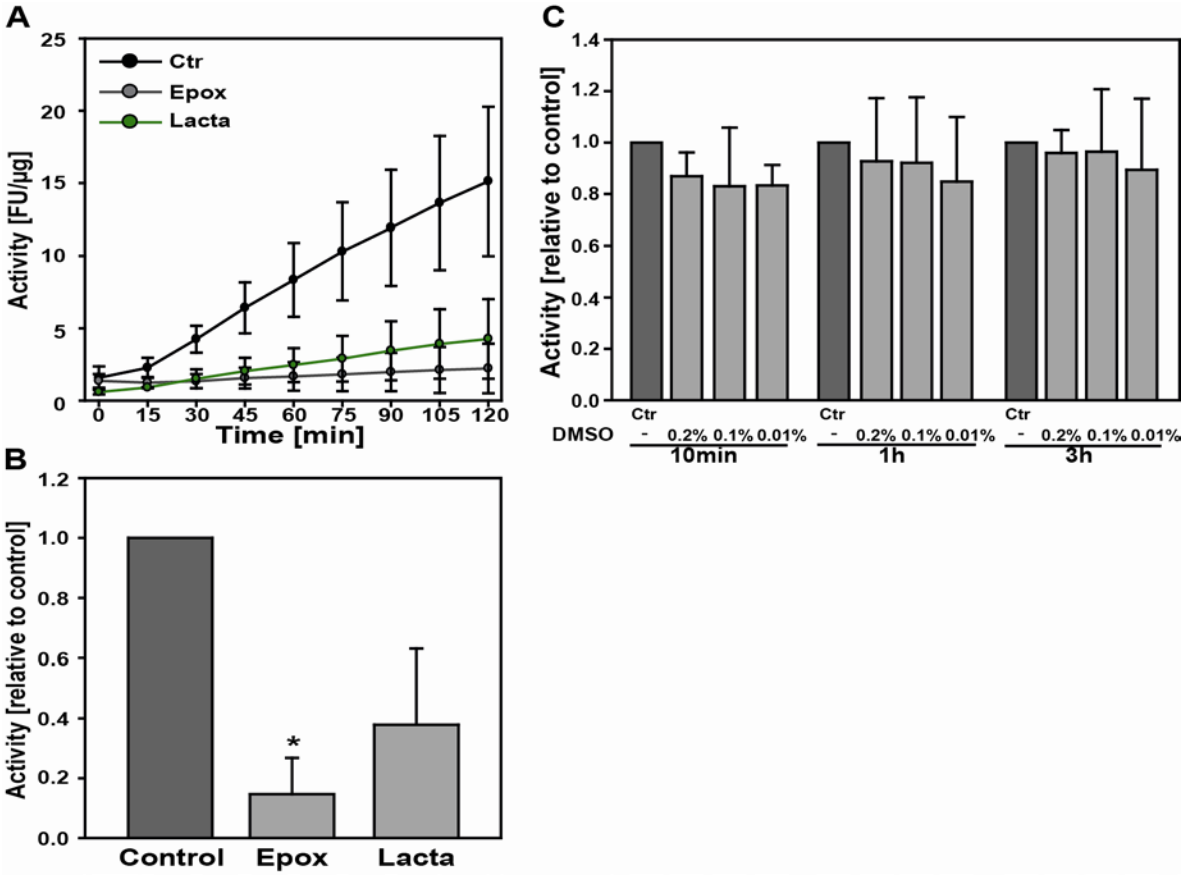


Figure 10: Chymotrypsin-like activity of the 20S proteasome in human platelets. 20S chymotrypsin-like (CT-L) activity was detected by a kit using LLVY-AMC as a substrate**. A:** The kinetic of the 20S CT-L activity in platelets was detected every 15min over a 2h time course. Resting platelets after 10min (ctr=control) and platelets treated with 1µM epoxomicin (epox) or 25µM lactacystin (lacta) for 10min are seen. **B:** Activity after 2h of treated lysates is shown relative to resting platelets after 10min (control) (Kruskal-Wallis one-way analysis of variance, *p<0.005, n=6, lacta: n=3). **C:** DMSO does not affect the 20S CT-L proteasome activity. Activity after 2h of DMSO treated cells is shown relative to resting platelets after indicated time points (ctr). Platelets were incubated with 0.2%, 0.1% or 0.01% dimethyl sulfoxide (DMSO) (Kruskal-Wallis one-way analysis of variance, n=4).

The flourogenic substrate was given to platelet lysates and fluorescence was detected every 15 minutes for 2 hours. The measured fluorescence units relative to the protein amount used are pictured (Figure 10A) or fluorescence units after 2 hours were normalized to resting platelets *in vitro* incubated for 10 minutes (here indicated as control, Figure 10B). Figure 10 shows that the 20S CT-L activity is detectable. Additionally the activity is significantly inhibited up to 85% (±5%) when platelets are incubated *in vitro* with 1µM of the specific proteasome inhibitor epoxomicin before lysis. In our experiments a reduction in the 20S CT-L activity by 62% (±18%) could be seen by incubation with lactacystin. This is not as effective as epoxomicin. Dimethyl sulfoxide (DMSO) is a common solvent to dissolve nonpolar and polar compounds

and does not have a significant influence on proteasome activity (Figure 10C). Concentrations of DMSO were tested at different time points (0.2%, 0.1% and 0.01% DMSO were given to platelets for either 10min, 1h or 3h).

Age dependency of 20S CT-L proteasome activity in platelets

In many tissues during ageing misfolded proteins are known to accumulate as a result of a declining proteasome activity [190]. Hence, aged platelets assessed by storing platelet concentrates for 8 days were analyzed according to their proteasome activity.

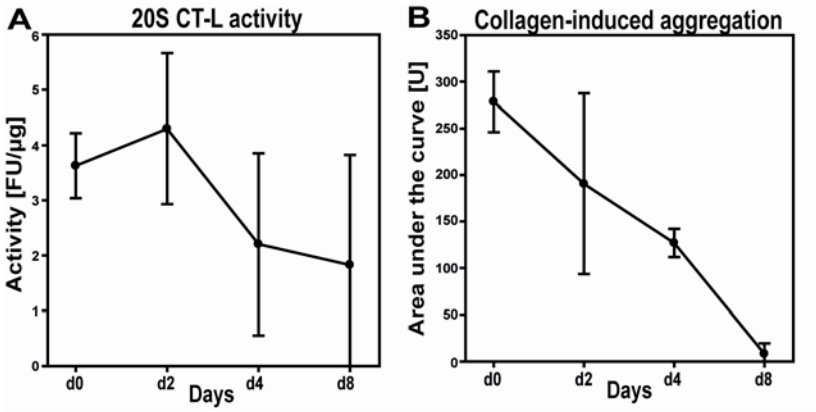


Figure 11: 20S CT-L activity in human platelet concentrates. A: Using the Millipore kit the 20S CT-L activity after 2h was measured. Platelet concentrates are depicted in FU/µg (n=2). **B:** Platelet aggregation of platelet concentrates was performed by Born aggregometry using 3µg/ml collagen for stimulation (n=2).

To analyze if the proteasome activity is age dependent we further tested the 20S CT-L activity in platelet concentrates over a time course of several days. As seen in Figure 11A platelet concentrates measured over 8 days show a decrease in 20S CT-L proteasome activity (3.63±0.42FU/µg day 0, 1.83±1.41FU/µg day 8). Furthermore a reduction in collagen-induced aggregation of platelet concentrates over 8 days is shown here (278.33±22.98Units day 0, 8.05±8.05Units day8, Figure 11B). Overall Figure 11 indicates age-dependent decrease in proteasome activity in human platelets. Supporting this data we observed a lower 20S CT-L activity in a small cohort of elderly donors with an average age of 63 years compared to young donors with an average age of 28 years.

3.1.3 26S and 20S proteasome complexes are active on all three catalytic activities

The kit used here only detects the 20S CT-L activity but the proteasome possesses three catalytic activities (as described in section 1.3.1 and 2.2.11). A method to measure all three catalytic activities of the 26S and 20S proteasome in platelets was

established in our laboratory. The 26S proteasome is ATP dependent and an addition of ATP to the assay buffer is obligatory. The three catalytic activities were measured with the fluorogenic peptide substrates mentioned under 2.1.9.

Comparison of all three catalytic 26S and 20S proteasome activities in human platelets and nucleated cells

Initially we looked at all 26S activities and 20S activities of the proteasome in platelets and at those activities of the proteasome in nucleated cells, the endothelial cell line HMEC and the leukaemia cell line HL-60.

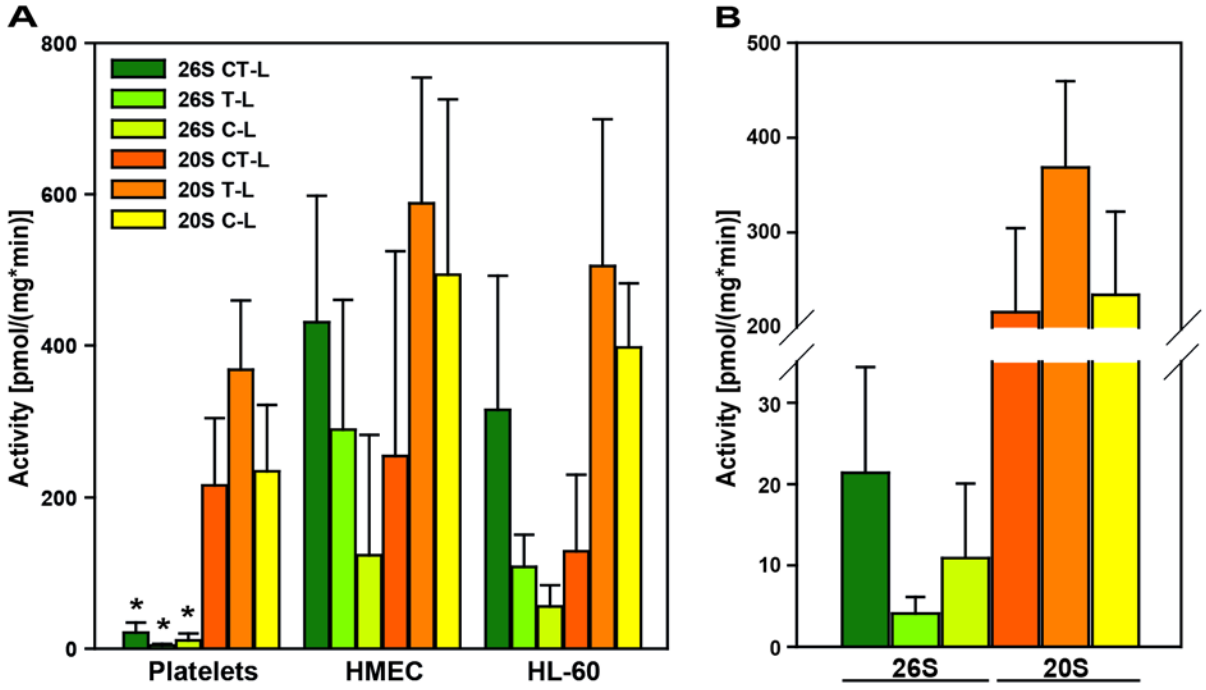


Figure 12: 26S and 20S proteasome activities of platelets, HMEC and HL-60 cells. A: The 26S and 20S chymotrypsin-like (CT-L), trypsin-like (T-L) and caspase-like (C-L) proteasome activities of platelets, HMEC and HL-60 cells were detected by fluorogenic substrates. **B:** A detailed view on the proteasome activities of platelets from panel A is given (Kruskal-Wallis one-way analysis of variance, *p<0.05 versus the respective activity of HMEC cells, n=6).

Activities are represented in pmol AMC produced relative to the amount of protein used over time. All activities of the 26S proteasome and 20S proteasome in human platelets were detectable levels but the 26S proteasome activities in platelets are significantly smaller compared to respective activities in nucleated cells (Figure 12A). The 26S CT-L activity in platelets is 21.38±4.93pmol/(mg*min) whereas the 26S CT-L activity in HMEC cells is 430.8 pmol/(mg*min). All exact activities are mentioned in Table 3. For a better resolution all proteasome activities of human platelets are pictured (Figure 12B). 26S proteasome activities in platelets show a rate of 21.38±4.93, 4.08±0.77 and 10.88±3.47pmol/(mg*min) (CT-L, T-L and C-L respectively) while 20S proteasome activities reach 215.99±33.54, 368.51±34.35 and 234.43±33.09pmol/(mg*min).

Cell type	26S proteasome activities [pmol/(mg*min)]		20S proteasome activities [pmol/(mg*min)]	
Platelets	CT-L	21.38±4.93	CT-L	215.99±33.54
	T-L	4.08±0.77	T-L	368.51±34.35
	C-L	10.88±3.47	C-L	234.43±33.09
HMEC	CT-L	430.76±68.24	CT-L	254.69±110.23
	T-L	289.52±69.69	T-L	588.00± 67.95
	C-L	123.37±71.18	C-L	493.58±103.68
HL-60	CT-L	315.89±71.96	CT-L	128.83±41.32
	T-L	108.07±17.36	T-L	505.03±79.25
	C-L	55.76±12.55	C-L	397.27±38.02

Table 3: 26S and 20S proteasome activities in platelets, HMEC ,and HL-60 cells.

Specificity of the established proteasome activity assay

From all proteases in platelets the proteasome is a unique type of protease and plays an important role not only for protein degradation but also for regulation of cellular processes (see section 1.3). The used peptide substrates are specific for measurements of proteasome activity and they are not cleaved by other proteases. Peptides are coupled to the fluorophor AMC which has a high fluorescence (emission at 460nm) and therefore is mostly used to measure proteasome activity [191]. Many peptide substrates exist but LLVY, LSTR, LLE were used here as described by others [112, 140, 159, 191]. The influence of platelet proteases others than the proteasome (*e.g.*, cathepsins, calpain, papain, trypsin, and chymotrypsin) on the fluorogenic peptide substrates was investigated by adding specific proteasome inhibitors to the assay (after lysis). 10µM epoxomicin was used to inhibit the CT-L and T-L proteasome activities and 30µM Z-Pro-Nle-Asp-CHO was used for the C-L proteasome activities [182]. Epoxomicin belongs to the class of α',β'-epoxyketones and is a highly specific proteasome inhibitor for the CT-L and T-L activities [140]. Epoxomicin most potently inhibits the 20S CT-L activity at a concentration of 1µM and the 20S T-L activity is inhibited at a concentration of 10µM [140]. Effects of inhibitors on the 26S proteasome (the 20S core particle with its 19S regulatory particle) has not been analyzed in detail. Z-Pro-Nle-Asp-CHO is an aldehyde based inhibitor and is known to have an inhibitory concentration of 30µM for the 20S C-L proteasome activity [182, 192], again its specificity for the 26S proteasome is not described in detail.

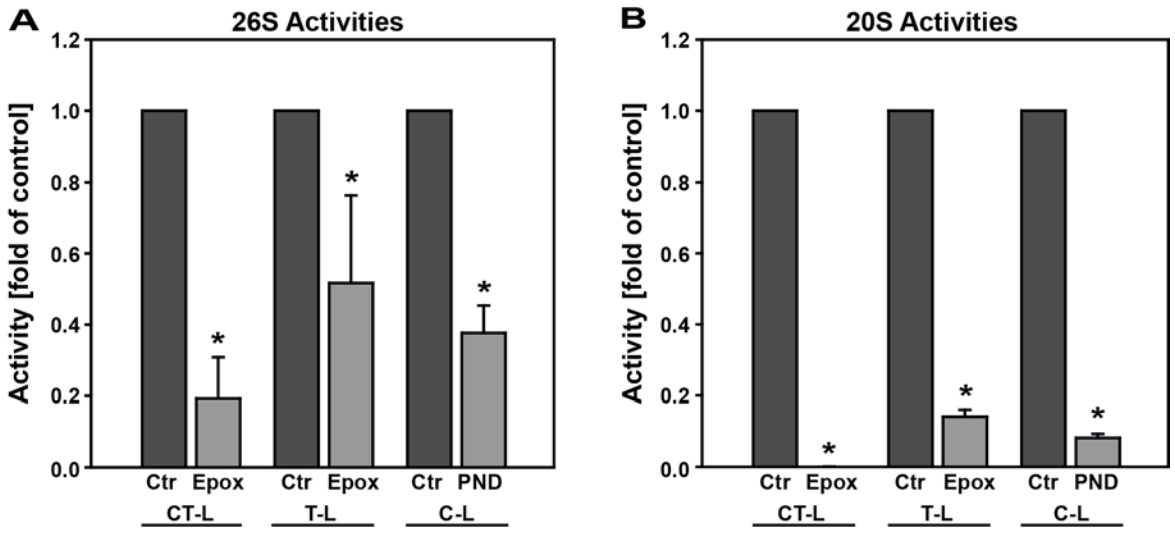


Figure 13: The established assay is specific to detect proteasome activity in platelets. The 26S (**A**) and 20S (**B**) chymotrypsin-like (CT-L), trypsin-like (T-L) and caspase-like (C-L) proteasome activities were analyzed and compared to lysates incubated with 10µM epoxomicin (epox) or 30µg Z-Pro-Nle-Asp-CHO (PND) for 15min (Mann-Whitney rank sum test, *p<0.03 versus control, n=5).

The CT-L, T-L, C-L activities are all effectively inhibited after incubating lysates with proteasome inhibitors compared to resting platelets (26S activities: 81±5% inhibition, 48±11% and 62±4%; 20S activities: 100±0.02% inhibition, 86±1%, 92±0.05%; Figure 13). Interestingly, Figure 13A shows that the 26S activities of platelets are effectively inhibited but not as effectively as the 20S activities. For that reason it can be concluded that the fluorogenic peptide substrates used here are highly specific for assessment of the 20S proteasome activities.

3.1.4 The proteasome is differentially regulated in human platelets

The discovery of the synthesis of the highly specific proteasome inhibitor epoxomicin, a natural product, was a breakthrough for proteasome inhibition. But until today the search for more sophisticated and effective inhibitors goes on. Here the effect of known proteasome inhibitors on all catalytic proteasome activities (CT-L, T-L, C-L) in human platelets is inspected. Furthermore, only few publications analyzed proteasome activation in human platelets. It is shown that the calcium ionophore A23187 stimulates the platelet 20S CT-L proteasome activity [163]. Here different potential agonists were tested in more detail on all activities of the 20S and 26S proteasome in human platelets. Regulations on all activities were not detectable. Therefore following graphs only show the enhanced activities by each agonist stimulation (Figure 15 and Figure 16).

Effects of known proteasome inhibitors on all 26S and 20S activities

Platelets treated with specific proteasome inhibitors before lysis were analyzed. Reduction of all proteasome activities of different inhibitors (epoxomicin, bortezomib and lactacystin) is demonstrated (Figure 14).

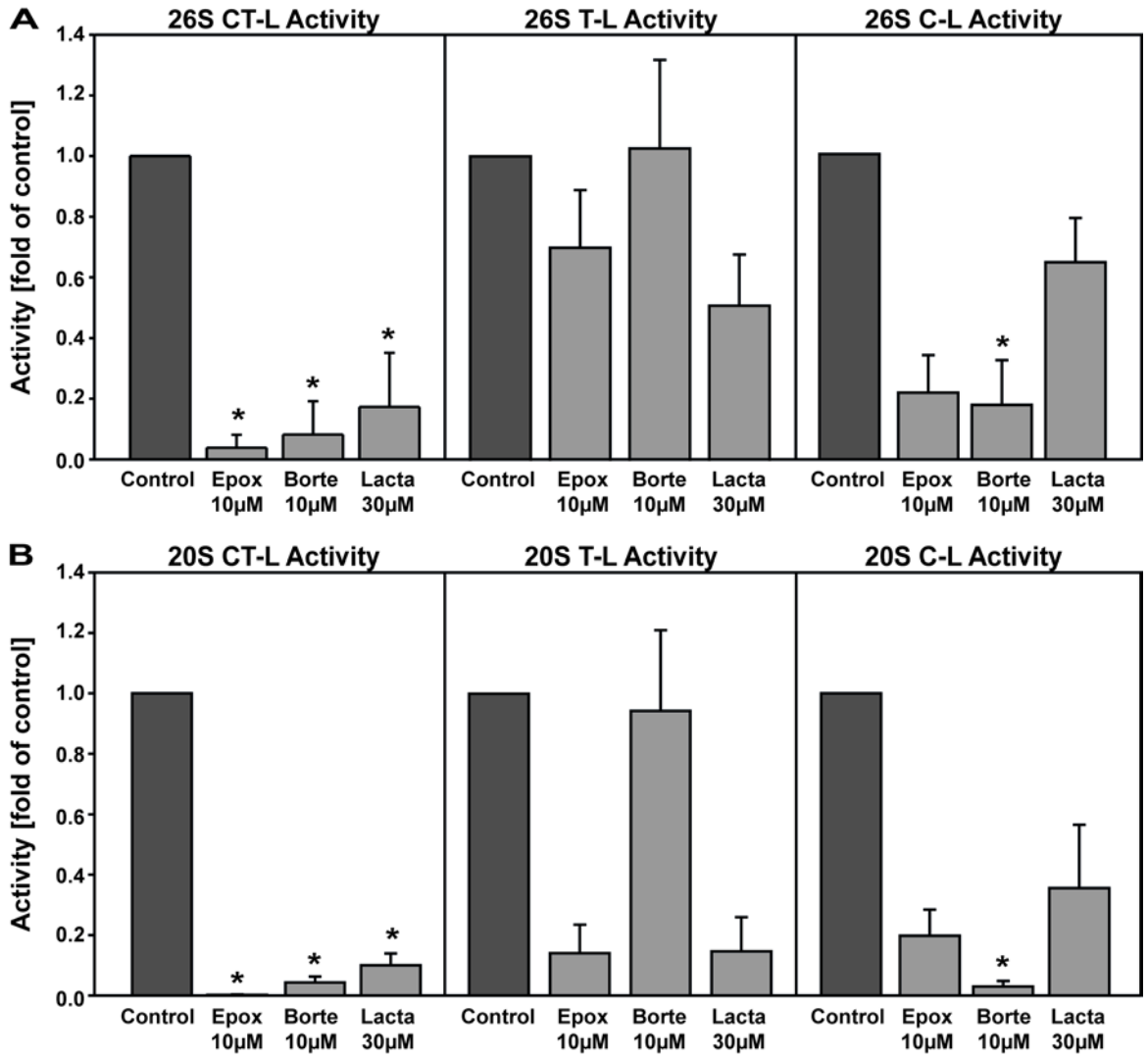


Figure 14: Inhibition of the 20S and 26S proteasome activities in human platelets. The 26S (**A**) and 20S (**B**) chymotrypsin-like (CT-L), trypsin-like (T-L) and caspase-like (C-L) proteasome activities of treated platelets are shown relative to resting platelets after 30min (control). Platelets were handled with 10µM epoxomicin (epox), 10µM bortezomib (borte) or 30µg lactacystin (lacta) for 30min (Kruskal-Wallis one-way analysis of variance, *p<0.03 versus control, n=4).

Figure 14A illustrates the 26S proteasome activities and Figure 14B the 20S proteasome activities. It is seen that both CT-L activities are significantly inhibited by 10µM epoxomocin, 10µM bortezomib and 30µM lactacystin (26S: with epoxomicin 96±2% reduction of activity, with bortezomib 92±6% reduction of activity, with lactacystin 83±9% reduction of activity; 20S: with epoxomicin 100±0.08% reduction of activity, with bortezomib 96±1% reduction of activity, with lactacystin 90±2% reduction of activity). The 26S C-L proteasome activity is restrained 78±6% by 10µM epoxomicin and significantly 82±7% by bortezomib, while the 20S C-L activity is

significantly inhibited by all three inhibitors (epoxomicin: 80±4%, bortezomib: 97±1%, lactacystin: 64±12%). The T-L activities are not significantly inhibited with epoxomicin or lactacystin and bortezomib does not restrict the T-L activities at all (Figure 14). These three specific proteasome inhibitors are known to have different inhibitory concentrations for each activity.

Calcium ionophores activate 26S proteasome activities

First the calcium ionophores A23187 and ionomycin were examined on all activities of the 20S and 26S proteasome complexes. Secondly, polyubiquitinated proteins were measured.

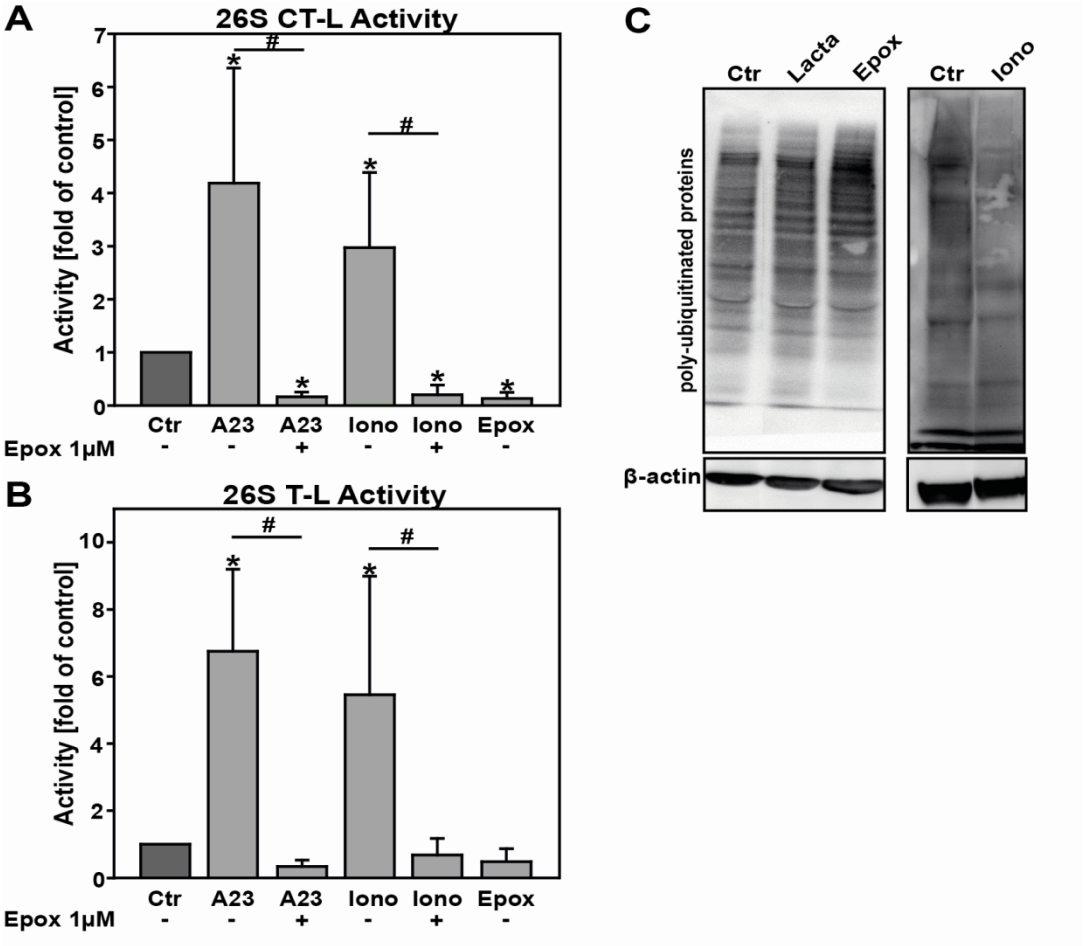


Figure 15: Calcium ionophores increase the 26S CT-L and T-L activities of the platelet proteasome leading to enhanced degradation of polyubiquitinated proteins. Measured 26S CT-L proteasome activity (**A**) and T-L proteasome activity (**B**) of treated platelets are illustrated relative to resting platelets (ctr). Platelets were either treated with 2µM A23187 (A23) or 0.5µM ionomycin (iono) alone or they were preincubated with 1µM epoxomicin (epox) before adding the stimulants (Kruskal-Wallis one-way analysis of variance, *p<0.004 versus control, #p<0.004 versus A23 or iono alone, n=7 CT-L, n=4 T-L). **C** Western blot analysis of polyubiquitinated proteins after an *in vitro* incubation with proteasome inhibitors 1µM epoxomicin (epox) and 30µM lactacystin (lacta) for 7h or the proteasome activator 0.5µM ionomycin (iono) for 10min (n=4).

A23187 or ionomycin stimulation of platelets enhances the 26S CT-L activity significantly by 4.19±0.82-fold and 2.97±0.53-fold respectively (Figure 15A). With Figure 15B an increase of the 26S T-L activity is observed for the first time. The 26S T-L activity is increased by A23187 and ionomycin to 6.75±1.22-fold and 5.46±1.77-fold respectively. Unique involvement of the proteasome complex was verified by preincubation with the proteasome inhibitor epoxomicin, which abrogated the upregulation of the activities. It comes to an inhibition similar to the one of epoxomicin alone (Figure 15A and B). Figure 15C shows the accumulation of polyubiquitinated proteins after proteasome inhibition whereas less polyubiquitinated proteins are detected after enhancing the proteasome activity by ionomycin.

The platelet agonist collagen enhances 26S CT-L proteasome activity

Proteasome activity is differentially regulated in platelets. Whether its activation plays a role in platelet activation/aggregation is not addressed in detail to date. As a consequence the influence of different platelet agonists on all proteasome activities was examined and collagen was found to regulate the proteasome activity. Figure 16 clarifies that the platelet agonist collagen enhances the 26S CT-L activity in human platelets.

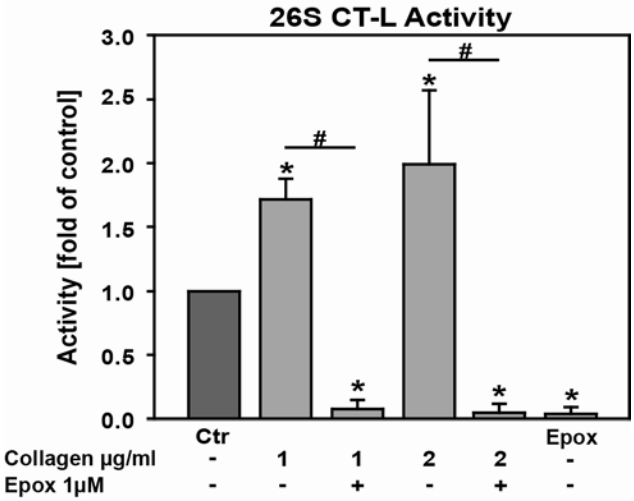


Figure 16: Collagen increases the 26S CT-L of the platelet proteasome. 26S CT-L proteasome activity was detected by a fluorogenic peptide substrate and is represented relative to resting platelets (ctr=control). Platelets were either incubated with 2µg/ml or 1µg/ml collagen alone for 7min or they were preincubated with 1µM epoxomicin (epox) for 15min before adding the stimulant (Kruskal-Wallis one-way analysis of variance, *p<0.001 versus control, #p<0.001 versus collagen alone, n=4).

As visualized in Figure 16 the platelet agonist collagen significantly stimulates the 26S CT-L activity of platelets to 1.72±0.08-fold when used in a concentration of 1µg/ml. 2µg/ml collagen activates the 26S CT-L activity even to 1.99±0.29-fold. A proteasome inhibition efficiently reverses this enhancement (0.08±0.04-fold 1µg/ml collagen, 0.05±0.04-fold 2µg/ml collagen), which is an inhibition similar to the one of epoxomicin alone (0.04±0.03-fold).

3.1.5 Proteasome substrates

The proteasome, as the major degradation system in eukaryotic cells, degrades proteins into peptides but over time the proteasome, more precisely the immunoproteasome, became known to generate peptides for cellular antigen presentation on major histocompatibility complex I (MHC I) [130, 131]. Additionally recent publications found out that the proteasome is able to cleave proteins into large functional fragments [150, 151]. The regulation and mechanism of this distinct proteolytic activity of the proteasome is not fully understood, yet. The following data illustrate the approach to identify proteasome substrates.

MHC I peptide analysis

In cooperation with Professor Stefan Stevanovic, University of Tübingen, MHC I complexes from platelet lysates were isolated and peptides presented on them were analyzed by mass spectrometry. Since 2012 it is known that platelets present antigens on MHC I complexes [58] and proteomic analysis already confirmed that anucleate platelets contain the protein machinery to process and present antigens to other cells [63]. Table 4 lists 10 representative proteins to which the peptides (HLA ligands) found on platelet MHC I belonged. The analysis was performed with one donor in two individual experiments and only peptides found in both experiments were considered. HLA ligands, the accession of the protein source, and descriptions are listed in Table 4. Ligands presented belong to proteins that are involved in translation or biochemical processes as glycolysis and lipid biosynthesis or signaling pathways as NO signaling. One quarter of ligands belong to cytoskeletal proteins. The cytoskeleton is indispensible for platelet morphology and function and the cytoskeletal proteins Filamin A and Talin-1 were not only identified as proteasome substrates in our study but recently Gupta *et al.* [150] mentioned them as proteasome substrates as well.

HLA ligand	Protein source	Accession	Description
ESAGGLIQTAR QAAGNAVKR KPKEADESL EVAPDVRLR RELETVREL SVALPAIMR LELLDHVLL RELETVRELL	Talin-1 cytoskeleton	Q9Y490	Cytoskeleton
GEITGEVRm GEITGEVRM QPASFAVSL	Filamin A cytoskeleton	P21333	Cytoskeleton
EAAAIIAQR	Drebrin	Q16643	Cytoskeleton
AAVAAVAAR AVAAVAARR VAAVAARR	Neutral alpha-glucosidase AB	Q14697	Glycoprotein formation
ETVKDFVAR ESFGVPKGR	Diacylglycerol kinase delta	Q16760	Lipid biosynthesis
ESTGSIAKR	Fructose-bisphosphate aldolase A	P04075	Glycolysis
GTSSVIVSR	F-box/WD repeat-containing protein 11	Q9UKB1	Ubiquitination
SAAGPVAAPR	Lysosomal amino acid transporter 1 homolog	Q6ZP29	Membrane transport
EVVTGVIGQR REFLQNLDAL RYDNVTILF	Guanylate cyclase soluble subunit beta-1	Q02153	NO signaling
GEVTNDFVmL KFIDTTSKF DVIGVTKGK	60S ribosomal protein L3	P39023	Translation

Table 4: HLA class I ligands from platelet lysates.

Cytoskeletal proteins are cleaved by the proteasome

The platelet cytoskeleton is indispensible for platelet morphology and function. Therefore the here found cytoskeletal proteasome substrates Filamin A and Talin-1 (through HLA ligand analysis, Table 4) were tested for cleavage under proteasome activation or inhibition. Protein expression was performed to confirm that they are proteasome substrates and to emphasize the importance of the proteasome.

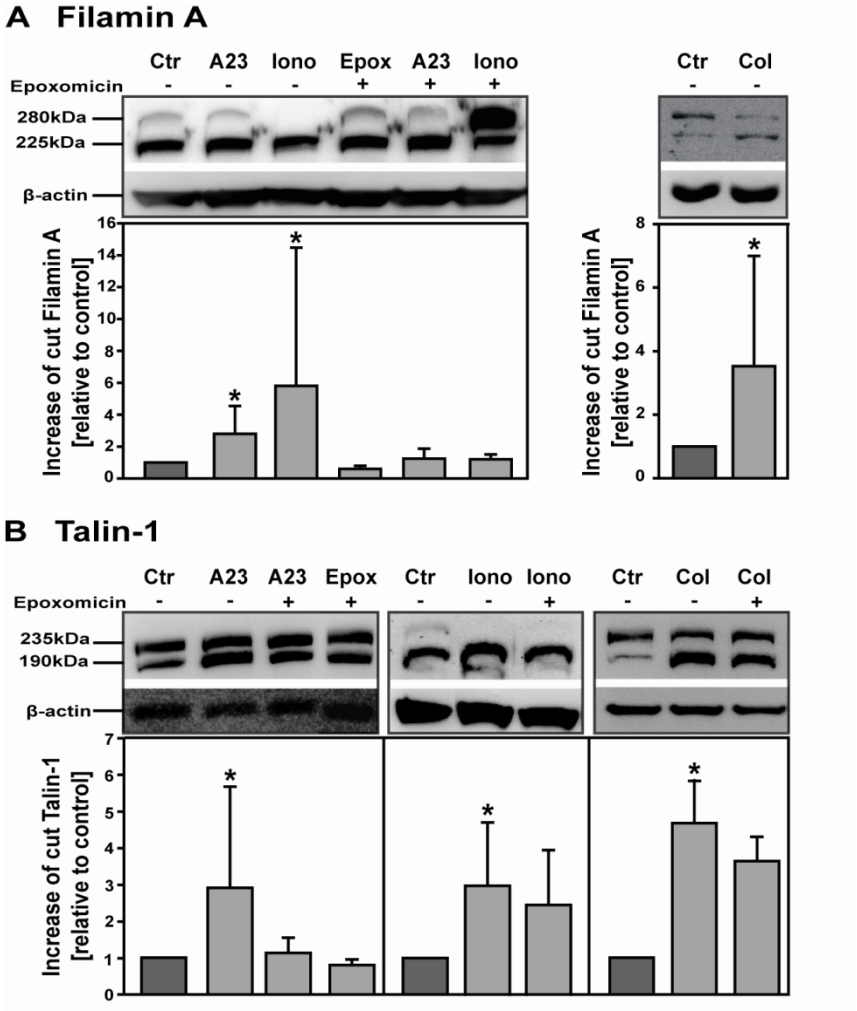


Figure 17: Proteasomal cleavage of Filamin A and Talin-1. Western blot analysis was performed after proteasome activation of platelets with 2µM A23187 (A23 n=11), 0.5µM ionomycin (iono n=11) and 7µg/ml collagen (col n=4) or plateletal proteasome was inhibited with 1µM epoxomicin for 10min (epox n=3). Expression of Filamin A (**A**) and Talin-1 (**B**) is demonstrated. ß-actin was used as a loading control. Band densitometry analysis is graphed of Filamin A and Talin-1 blots showing cleaved to full length protein ratios where resting platelets (control) were set as 1 (A A23, Iono: Kruskal-Wallis one-way analysis of variance, *p<0.001 versus control; others: Mann-Whitney rank sum test, *p<0.001 stimulus versus control).

Filamin A cleavage can be observed by detecting a 225kDa Filamin A fragment via western blot and Talin-1 cleavage is detected by expression of a 190kDa fragment. A proteasome activity enhancement with calcium ionophores (A23187 or ionomycin) and collagen yield significantly more cleaved Filamin A 225kDa (Figure 17A) and more cleaved Talin-1 190kDa (Figure 17B) respectively compared to resting platelets (control). Control was set as 1 and Filamin A cleavage is 2.80±0.50-fold increased by A23187, 5.81±2.40-fold by ionomycin, and 3.53±1.73-fold by collagen. Talin-1 cleavage is enhanced by 2.91±0.80-fold by A23187, 2.98±0.52-fold by ionomycin, and 4.68±0.67-fold by collagen. An inhibition of proteasome activity by epoxomicin shows less cleaved Filamin A (0.60±0.06-fold) and almost equally cleaved Talin-1 compared to resting platelets (0.80±0.09-fold). Preincubation with epoxomicin prior to

proteasome activation depresses the cleavage of the proteasome substrates Filamin A and Talin-1 a little. Since those substrates can be cleaved by other enzymes as well a complete suppression of cleavage was not detected.

3.2 Role of the proteasome in platelet function

Slowly the role of the proteasome in platelets is approached but until today it is not well studied. Only recently it was indicated for the first time that a proteasome inhibition by MG132 or bortezomib reduces thrombin-induced aggregation and microparticle shedding [150]. But how an inhibition of aggregation exactly works had not been analyzed yet. Therefore cellular signaling pathways involving the platelet proteasome are of interest.

3.2.1 Proteasome inhibition restrains platelet aggregation

The proteasome inhibitor epoxomicin itself is not known to possess antiplatelet or prothrombotic activity. But wether proteasomal inhibition by epoxomicin alters platelet functions after agonist stimulation is not well studied until today. Here we demonstrate that a proteasome inhibition by epoxomicin affects aggregation stimulated with ADP or low concentrations of collagen.

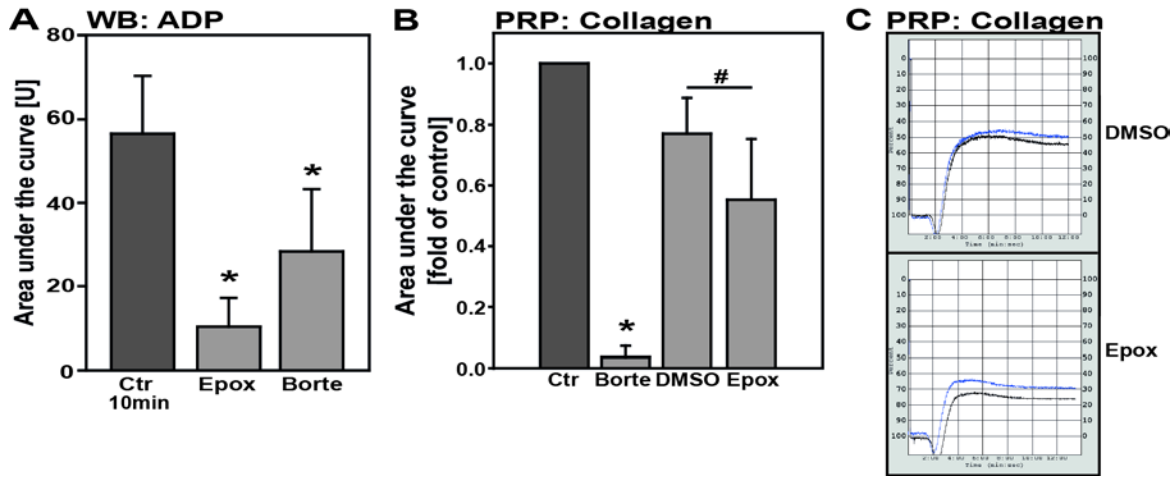


Figure 18: Aggregation stimulated with ADP or low concentrations of collagen is reduced by proteasome inhibition. A: Platelet aggregation in whole blood (WB) was performed by impedance aggregometry (Multiplate®) using 6.5µM ADP. Blood was either treated with 10µM epoxomicin (epox) or 10µM bortezomib (borte) for 10min (Kruskal-Wallis one-way analysis of variance, *p<0.001 versus control, n=5). **B:** Platelet aggregation in PRP was performed by Born aggregometry using 1-2.5µg/ml collagen. Illustrated is the area under the curve relative to a control. PRP was incubated with 200µM bortezomib (borte) and 5µM epoxomicin (epox) respectively for 10min (Kruskal-Wallis one-way analysis of variance, *p<0.001 versus control, #p<0.001 versus DMSO, n=8). **C:** Representative readings of the collagen-induced aggregation measurements (samples were analyzed in duplicates).

Figure 18A shows that proteasome inhibition decreases platelet aggregation stimulated with 6.5µM ADP in whole blood. Aggregation induced by low concentrations of collagen (1-2.5µg/ml) was measured in PRP. This way an effect of other blood cells can be excluded. Here aggregation is reduced as well by proteasome inhibitors (97±2% inhibition by 200µM bortezomib, and 45±7% inhibition by 5µM epoxomicin). Higher doses of agonists are able to overcome these reductions in aggregation. Representative aggregation traces measured by Born aggregometry stimulated with collagen are depicted in Figure 18C; decrease of aggregation by 5µM epoxomicin is illustrated.

3.2.2 NFκB as a regulator of the proteasome in platelets

The transcription factor NFκB is assumed to possess non-genomic functions. Analysis of non-genomic functions can elegantly be studied in anucleate platelets. In 2009 Malaver *et al.* [70] published data demonstrating the existence of the NFκB pathway in anucleate platelets and its effect on platelet aggregation was discovered. NFκB inhibitors were found to diminish platelet aggregation [70, 83], similar to proteasome inhibitors (Figure 18). Furthermore, IκBα, the inhibitory protein of NFκB, is degraded by the proteasome. In this study a connection of NFκB and the proteasome was investigated.

NFκB influences aggregation in human platelets

NFκB is found in platelets and influences platelet aggregation. Here ADP- and collagen- induced aggregation was inspected.

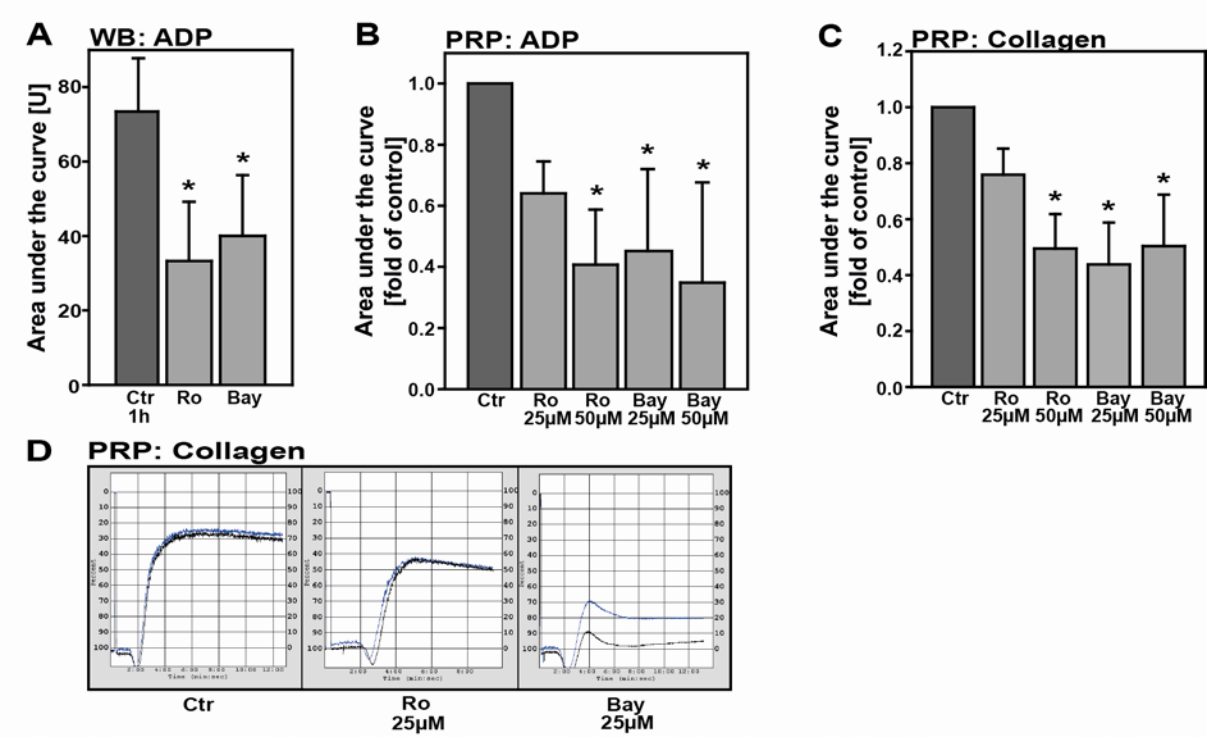


Figure 19: Reduction of ADP- and collagen-induced aggregation in human platelets by NFκB inhibitors. A: Platelet aggregation in whole blood (WB) was performed by impedance aggregometry (Multiplate®) using 6.5µM ADP. Blood was incubated with 10µM NFκB inhibitors Ro 106-9920 (Ro) and Bay 11-7082 (Bay) for 1h (Kruskal-Wallis one-way analysis of variance, *p<0.001 versus control, n=5). **B-D:** Platelet aggregation in PRP was performed by Born aggregometry using 6.5µM ADP (B) or 1-2.5µg/ml collagen (C,D). Illustrated is the area under the curve relative to a control. PRP was incubated as indicated with NFκB inhibitors Ro 106-9920 (Ro) and Bay 11-7082 (Bay) for 1h (Kruskal-Wallis one-way analysis of variance, *p<0.001 versus control, B: n=6, C: n=5). **D:** Representative readings of the collagen-induced aggregation measurements (samples were analyzed in duplicates).

Figure 19 confirms previous publications and pictures a reduction of ADP- and collagen-induced aggregation after inhibition of the NFκB pathway. Figure 19A shows that NFκB inhibition decreases platelet aggregation stimulated with ADP in whole blood. In PRP, to exclude an effect of other blood cells, aggregation is decreased as well (Figure 19B, 59±7% reduction in aggregation by 50µM Ro 106-9920, and 65±13% reduction in aggregation by 50µM Bay 11-7082). When aggregation is stimulated with low concentrations of collagen (1-2.5µg/ml) aggregation is reduced by NFκB inhibition (51±12% inhibition by 50µM Ro, and 50±19% inhibition by 50µM Bay). Higher doses of collagen are able to overcome these reductions in aggregation. Representative aggregation traces measured by Born aggregometry stimulated with collagen are depicted in Figure 19D; decrease of aggregation by 25µM Ro and Bay compared to a control).

NFκB inhibitors prevent collagen-stimulated proteasome activity enhancement

As demonstrated in Figure 16 collagen induces proteasome activity and collagen is a well studied platelet agonist leading to aggregation. Further the proteasome inhibitor

epoxomicin diminishes collagen-induced aggregation similar to NFκB inhibitors (Figure 18 and Figure 19). Thus to examine an interaction of the NFκB pathway and the proteasome on collagen-induced activation it was tested if not only epoxomicin reverses a collagen-stimulated increase of 26S CT-L activity but also NFκB inhibitors.

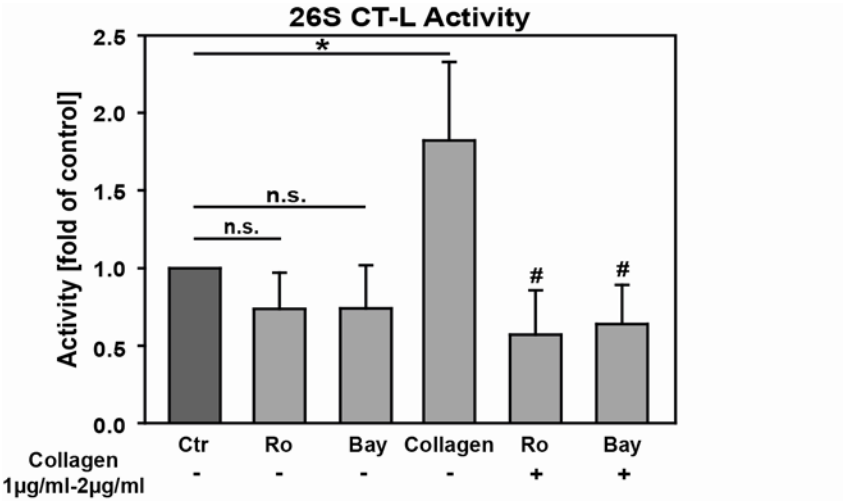


Figure 20: NFκB inhibitors prevent collagen-stimulated proteasome activation. Measured 26S CT-L proteasome activity of treated platelets is represented relative to resting platelets (ctr). Platelets were either treated with 1-2µg/ml collagen or NFκB inhibitors, 25µM Ro 106-9920 (Ro) and 25µM Bay 11-7082 (Bay), alone or they were preincubated with NFκB inhibitors before adding the stimulant collagen (Kruskal-Wallis one-way analysis of variance, *p<0.001 versus control, #p<0.001 versus collagen alone, n=8).

The collagen-induced boost in 26S CT-L activity is not seen by a preincubation with NFκB inhibitors (Figure 20). Platelets stimulated with collagen have a significant 1.82±0.11-fold higher proteasome activity compared to resting platelets. Platelets incubated with NFκB inhibitors Ro and Bay alone do not have a significantly altered activity (0.74±0.11-fold for both). A preincubation with NFκB inhibitors even prohibits a collagen-induced increase of the 26S CT-L activity (0.57±0.11-fold and 0.64±0.10-fold respectively significant versus 1.82-fold collagen). NFκB seems to play a role as a regulator of the proteasome in collagen-induced activation.

NFκB activity is increased by collagen

Since NFκB inhibitors prohibit collagen-induced aggregation, an activation of NFκB after collagen stimulation was studied. The proteasome inhibitor epoxomicin effectively inhibits NFκB activation as shown on HeLa cells [140]. NFκB is activated when its inhibitor IκBα is degraded leaving NFκB with its free nuclear localization signals (NLS). Those NLS can be detected even though non-genomic activity of NFκB is analyzed.

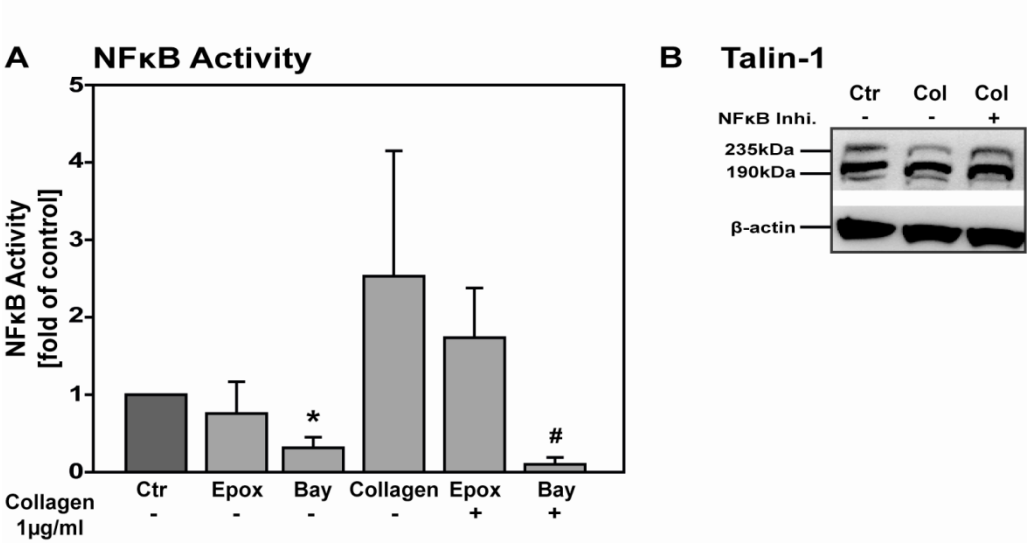


Figure 21: Collagen enhances NFκB activity in human platelets and increased proteasome substrate cleavage can be reversed by NFκB inhibitors. A: Through an NFκB transcription assay the NFκB activity is shown relative to resting platelets (ctr). Platelets were incubated with 25µM Bay 11-7082, 10µM epoxomicin (epox) or collagen alone or a preincubation with 25µM Bay 11-7082 or 10µM epoxomicin (epox) for 15min was performed before adding collagen for 7min (Mann-Whitney rank sum test, *p<0.02 versus control; Kruskal-Wallis one-way analysis of variance, #p<0.02 versus collagen alone, n=4). **B:** Platelets were incubated with collagen 7µg/ml or preincubated with NFκB inhibitors, 25µM Ro 106-9920 (Ro) or Bay 11-7082, for 10min (n=4). A representative western blot of the proteasome substrate Talin-1 is shown. ß-actin is used as a loading control.

Active NFκB was measured in human platelets and Figure 21 demonstrates that there is not only the obvious efficient inhibition of NFκB by its inhibitor Bay (69%±7%) but there is an increase in NFκB activity by collagen (2.53±0.72-fold). This induction by collagen might not only be reversed by the NFκB inhibitor but also by the proteasome inhibitor epoxomicin (0.10±0.04-fold, 1.74±0.32-fold respectively). The proteasome substrate Talin-1 is not only cleaved after proteasome activation with calcium ionophores or collagen (Figure 17) but this cleavage can be reversed by epoxomicin. Demonstrated here in Figure 21 the enhanced cleavage is partly prevented with NFκB inhibitors as well.

Collagen activates IκB kinase and promotes degradation of IκBα

To confirm NFκB as a regulator of the proteasome, activation of IκB kinase (IKK) by collagen was tested. IKK starts the NFκB pathway by phosphorylating the inhibitory IκBα protein [76]. Further IκBα degradation under a collagen stimulus was analyzed.

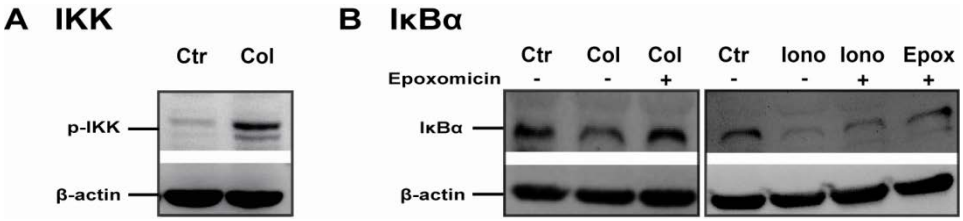


Figure 22: Collagen activates IKK and induces IκBα degradation. A: Expression of phosphorylated IκB kinase (p-IKK) after a 2µg/ml collagen stimulation for 7min (n=3). **B:** Illustrated are Western blots of platelet lysates after a 7µg/ml collagen treatment for 15min (col) or 0.5µM ionomycin (iono) for 10min (n=6). A preincubation with the specific proteasome inhibitor 1µM epoxomicin (epox) for 10min was used in comparison (before adding ionomycin n=5; before adding collagen n=2). Degradation of IκBα is indicated. ß-actin is used as a loading control.

As seen in Figure 22A stimulation of 2µg/ml collagen strongly induces phosphorylation of the IκB kinase (p-IKK). Expression of NFκB inhibitory protein IκBα is decreased in platelets after collagen stimulation compared to resting platelets meaning IκBα seems to be degraded in collagen-stimulated platelets. When the proteasome is inhibited IκBα degradation upon collagen stimulation seems to be prevented.
Taking all this data in account a connection of the NFκB pathway and the proteasome in collagen-induced aggregation of human platelets can be postulated.

3.3 Clinical investigation of mitochondrial function and proteolytic processes in platelets during sepsis

The ubiquitin proteasome system is known to be involved in many diseases because it is not only there to degrade proteins for intracellular protein turnover but regulates many cellular mechanisms. Proteolysis is essential for nucleated cells and the importance of proteolysis in anucleate platelets was also declared [98]. Therefore abnormalities of the ubiquitin proteasome system/proteasome can be found in diseases but in sepsis the proteasome has not been studied yet. Sepsis is still a leading cause of morbidity and mortality and platelet cell death leading to thrombocytopenia has been closely associated with it [36, 37, 193]. Apoptosis is a cellular process that is mainly regulated by caspases but the proteasome plays an important role as well, i.e. degrading pro-survival proteins [194]. Our group was able to show that bacteria can directly activate the apoptotic pathway in platelets [36] and Tisdale *et al.* [155] demonstrated that muscle proteolysis during sepsis is dependent on the proteasome. Therefore not only quantification of mitochondrial associated apoptotic markers in platelets was performed and a correlation of an apoptotic marker with the clinical disease severity in sepsis patients during disease course was analyzed but involvement of the proteasome during sepsis was also investigated.

3.3.1 Markers of platelet apoptosis and mitochondrial control of platelet apoptosis

Functions that traditionally are attributed to nucleated cells have been discovered in anucleate platelets as well. Platelets show signs of programmed cell death, called apoptosis [48, 49]. Platelet apoptosis is triggered by numerous chemical agents (such as the calcium ionophore A23187 [50]) and the intrinsic mitochondria-dependent pathway with, *e.g.*, depolarization of the mitochondrial membrane potential, degradation of the anti-apoptotic protein Bcl-xL, and activation of caspase-8 is well studied in platelets [36, 54, 56]. For analyzing the mitochondrial integrity in human platelets an assay detecting the mitochondrial membrane potential (Mmp) was established. It is assessed as a ratio of the mean red and mean green fluorescence of triplicates. A decrease in the ratio (Mmp-Index) represents a loss in mitochondrial membrane potential, which happens in apoptotic cells. The calcium ionophore A23187 that is known to induce cell death at a concentration of 10µM is used during this study to stimulate apoptosis.

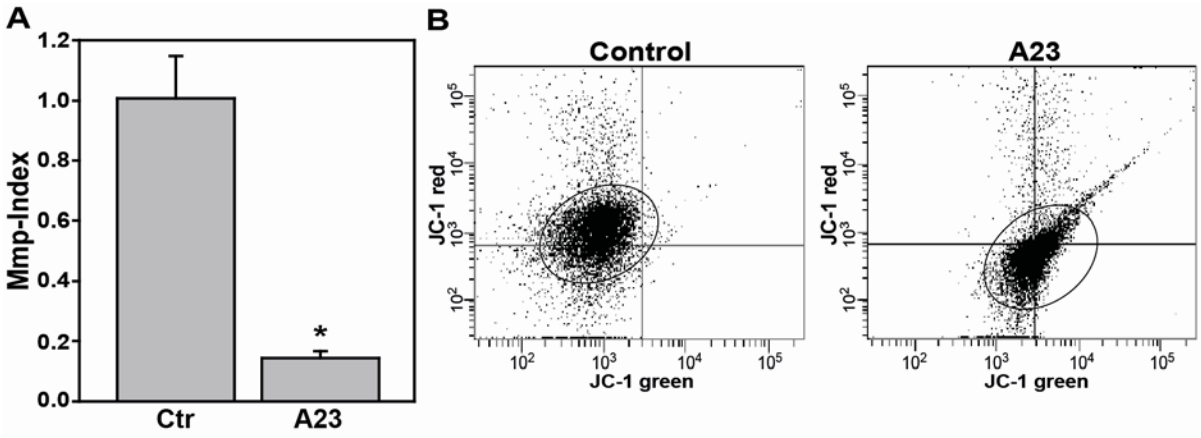


Figure 23: Depolarization of the mitochondrial membrane potential in human platelets. A: Mmp-Index was detected by JC-1 staining and flow cytometer analysis. Platelets from healthy donors were analyzed and those platelets were compared to apoptosis-induced cells (10µM A23187,A23, for 10min; Mann-Whitney rank sum test, n=10, *p<0.001). **B:** Representative flow cytometer measurements of one control and of an apoptosis-induced sample (10µM A23187=A23 for 10min) is pictured. Mitochondrial membrane depolarization is characterized by a reduction in median red fluorescence and an increase in median green fluorescence.

Figure 23 demonstrates the depolarization of the mitochondrial membrane potential in human platelets undergoing apoptosis. Apoptotic platelets lose their mitochondrial membrane potential shown by a significantly low Mmp-Index (0.14±0.01 versus control 1.01±0.04). A representative illustration of the decrease of Mmp-Index detected by JC-1 staining and FACS measurements is seen in Figure 23B. The control platelet population (circle) shows red fluorescence and the apoptotic platelet population (A23187) emits more green fluorescence.

3.3.2 Mitochondrial dysfunction of platelets correlates with clinical disease severity and outcome in sepsis

Sepsis is still a leading cause of morbidity and mortality and thrombocytopenia has been closely associated with it. Our group was able to show that bacteria can directly activate the apoptotic pathway in platelets [36]. Therefore the apoptotic marker, loss of mitochondrial membrane potential, was analyzed according to a correlation with clinical disease severity in sepsis patients during disease course and if it may be a valuable parameter to disease severity and clinical outcome.

Platelet mitochondrial membrane depolarization of sepsis patients correlates with clinical disease severity

First of all it was examined if the severity of sepsis is associated with the Mmp-Index of patients. Table 5 indicates the 26 sepsis patients and 17 control patients of our study. Sepsis patients were subclassified as severe sepsis (n=17) including septic shock and non-severe sepsis (n=9). Table 5 lists the site of infection in studied patients, platelet count and clinical disease scores Acute Physiology and Chronic Health Evaluation Score II (APACHE II), Sequential Organ Failure Assessment Score (SOFA) and Simplified Acute Physiology Score II (SAPS II). There was no statistical age difference among the groups.

	Sepsis	**Severe Sepsis/Septic shock**	**Controls**
Patient number	9	17	17
male/female	6/3	9/8	10/7
Age	57 [42 to 71]	69 [55 to 78]	74 [68 to 79]
Platelet counts (x 10^3/µl)	198 [161 to 210]	98 [79 to 159]	176 [145 to 230]
APACHE II score	9 [8 to 13]	23 [15 to 26]	
SOFA score	0 [0 to 1]	7 [5 to 11]	
SAPS II score	33 [25 to 35]	53 [40 to 58]	
Site of infection			**Admission cause**
pulmonary	4	7	cardiac: 3
urinary	0	2	pulmonary 2
abdominal	3	5	endocrine 3
endocarditis	0	1	rheumatic 1
soft tissue	0	1	GI 4
others	2	1	other 4

Table 5: Clinical patient characteristics. Numbers are given as median and [interquartile range] [37].

Comparision of Mmp-Index of patients with clinical scores APACHE II, SOFA and SAPS II was performed (Figure 24). Mmp of sepsis patients (n=26) correlated significantly with clinical disease severity scores (APACHE II: r=-0.867, SOFA: r=-0.857, SAPS II: r=-0.839). Accordingly lower Mmp-Index (apoptotic cells) are paralleled by higher score numbers (more severe disease status).

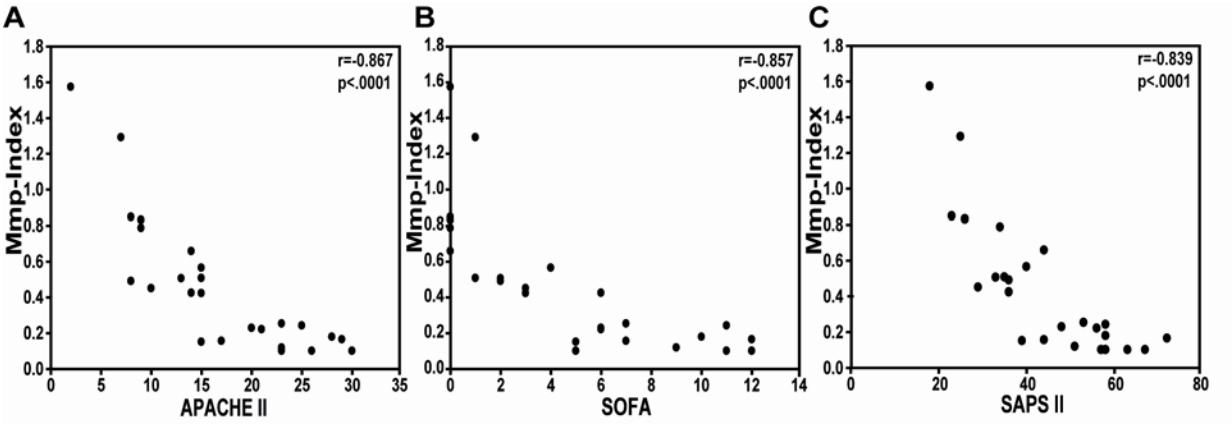


Figure 24: Correlation of platelet mitochondrial membrane potential with clinical scores. Dot plot correlation of the individual mean platelet Mmp-Index of each patient calculated from JC-1 measurements and the clinical disease severity scores APACHE II (**A**), SOFA (**B**), SAPS II (**C**) in 26 patients with sepsis are shown. Lower Mmp-Index values indicate a loss of mitochondrial membrane potential, whereas higher clinical disease scores indicate more severe illness. R = statistical correlation coefficient, p<0.0001 denotes statistically significant correlation of values [37].

Reduction of platelet mitochondrial membrane potential and pro-apoptotic Bcl-xL in patients with severe sepsis

To find out the level of apoptosis in patients with sepsis we detected the Mmp-Index in patients with severe sepsis (including septic shock), non-severe sepsis (sepsis without organ failure) and control patients. To strengthen previous data another apoptotic marker, expression of pro-survival protein Bcl-xL, was analyzed.

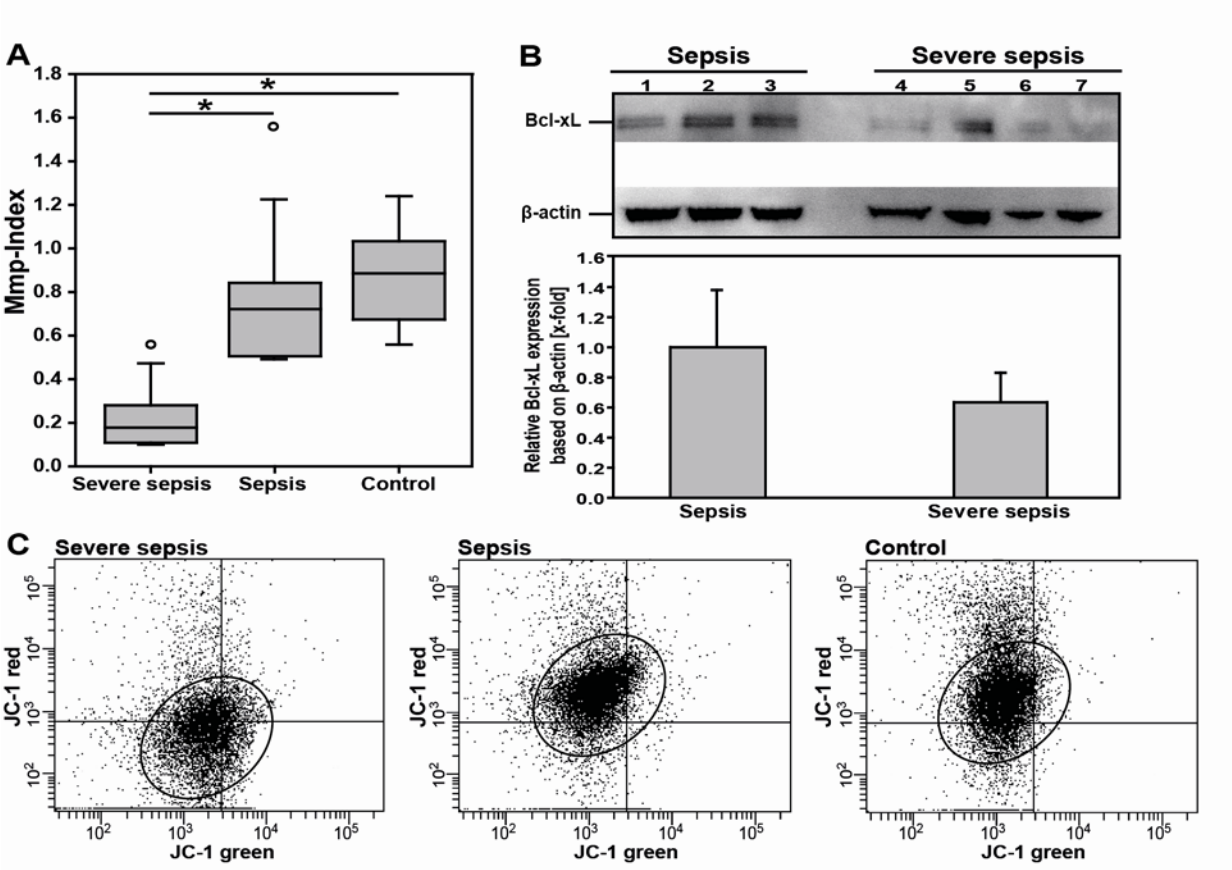


Figure 25: Comparison of platelet mitochondrial membrane potential of patients with sepsis, severe sepsis, and control patients. A: Box-and-whisker plots (with its median and lower/upper quartile) illustrate platelet Mmp-Index of patients with sepsis (non-severe without organ failure), severe sepsis including septic shock and control patients without infection (control n=14). Lower Mmp-Index values indicate a loss of mitochondrial membrane potential. (Kruskal-Wallis one-way analysis of variance, *p<0.001). **B:** Platelet Bcl-xL expression in patients with sepsis (lanes 1-3) and severe sepsis (lanes 4-7) was compared by immunoblotting. Bcl-xL expression was quantified by band densitometry analysis with the help of a Bcl-xL to actin ratio. Mean Bcl-xL to actin ratio for the sepsis group was set as 1. Relative Bcl-xL expression between groups based in actin loading is shown. **C:** Flow cytometry analysis of platelets from a patient with severe sepsis, sepsis and a control patient are shown. Mitochondrial membrane depolarization is characterized by a decrease in median red fluorescence and an increase in median green fluorescence. Adapted from Grundler *et al.* [37].

Platelet Mmp-Index is significantly reduced in patients with severe sepsis including septic shock compared to patients with sepsis without organ failure (non-severe) or control patients as seen in Figure 25A (median severe sepsis 0.18[0.12 to 0.25], sepsis 0.78[0.51 to 0.85], control patients 0.89[0.68 to 1.00]). Figure 25B demonstrates that the pro-survival protein Bcl-xL is less expressed in platelets of patients with severe sepsis (lanes 4 to 7) compared to the expression of patients with sepsis (lanes 1 to 3). Representatives of the flow cytometer analysis from each patient group are highlighted in Figure 25C, showing clearly the loss of red fluorescence and increase of green fluorescence in platelets of patients with severe sepsis.

Platelet mitochondrial membrane depolarization correlates with clinical disease outcome

Platelet mitochondrial membrane potential had not been investigated in a clinical follow-up design neither with regard to sepsis outcome before. Thus Mmp-Index on admission and follow-up in the group of sepsis survivors and non-survivors who died as a consequence of sepsis was detected.

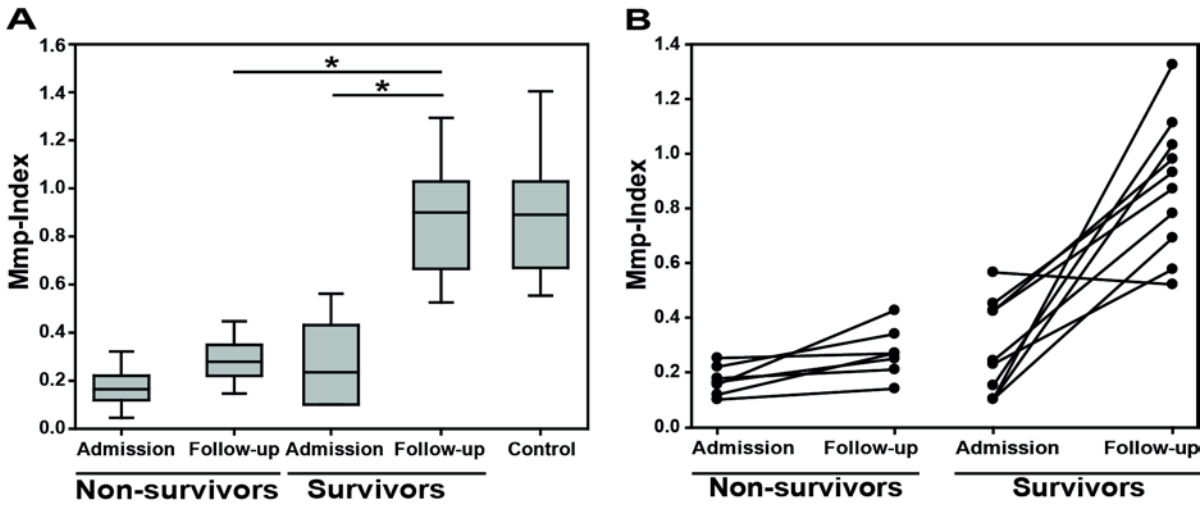


Figure 26: Comparison of platelet mitochondrial membrane potential of survivors and non-survivors of the severe sepsis group. A: Box-and whisker-plots (with its median and lower/upper quartile) show platelet Mmp-Index of survivors (n=10) and non-survivors (n=7) of the severe sepsis group on admission and during follow-up (control n=17). Lower Mmp-Index values indicate a loss of mitochondrial membrane potential. **B:** Individual Mmp-Index values of sepsis survivors and non-survivors on admission and during follow-up are shown (Kruskal-Wallis one-way analysis of variance, *p<0.001) [37].

As demonstrated in Figure 26 there was no significant difference in platelet Mmp-Index between survivors and non-survivors (Mmp-Index on admission of non-survivors 0.16[0.11 to 0.22], survivors 0.24[0.11 to 0.42]). During clinical course, significant recovery of Mmp-Index was observed in the group of survivors (Mmp-Index on follow-up of survivors 0.90[0.71 to 1.02]), this follow-up Mmp recovered to baseline levels of controls (Mmp of control 0.88[0.67 to 1.03]). But the Mmp values of non-survivors remained low (Mmp on follow-up of non-survivors 0.27[0.23 to 0.31]), that was non significant compared to the Mmp on admission but it is significantly reduced compared to the follow-up Mmp of survivors. Illustration of individual Mmp-values demonstrates the lack of recovery in non-survivors of the severe sepsis group which all remained below 0.5 Mmp-Index and the recovery of Mmp-Index values in survivors on follow-up (Figure 26B). This Mmp-Index might be a valuable adjunct parameter to help in the assessment of disease severity, risk stratification and clinical outcome.

3.3.3 Patients with severe sepsis show enhanced proteasome activity in human platelets

As demonstrated platelets of sepsis patients undergo signs of apoptosis (Figure 25). Since apoptosis is regulated through the proteasome amongst others by degrading proteins, such as pro-survival proteins, the platelet proteasome during sepsis was further analyzed. Here proteasome activity in platelets of sepsis patients was tested and platelets incubated with the pathogenic *E. coli* strain UTI89 which is known to induce Bcl-xL degradation [36] were examined.

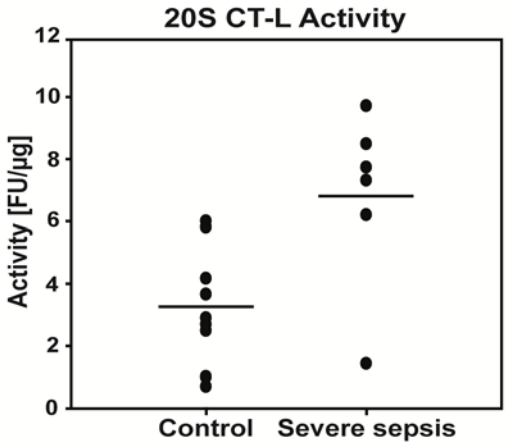


Figure 27: Proteasome activity is activated during sepsis. Plotted are individually control patients (n=9) and patients with severe sepsis including septic shock (n=6) illustrating platelet proteasome activity. The line represents the median.

Severe sepsis patients show a tendency to a higher 20S CT-L activity in platelets compared to control patients (median activity: 7.51±1.18FU/µg versus 4.40±0.52FU/µg, Figure 27). But a larger population should be studied.
Previous work demonstrated that under physiological conditions regulation of the 26S proteasome is more important than the 20S proteasome. Therefore we studied an uropathologic *E. coli* strain that might cause sepsis on its ability to enhance the 26S proteasome activity in platelets.

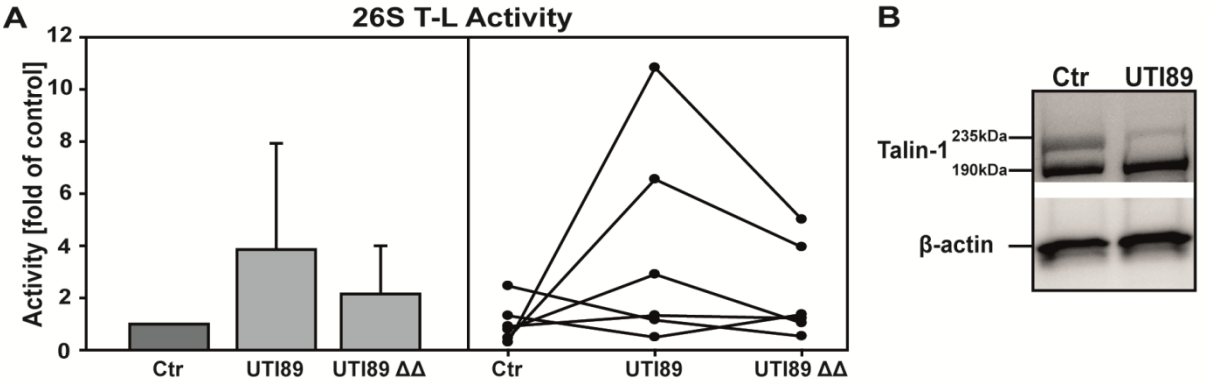


Figure 28: Proteasome activity is activated by *Escherichia coli* UTI89. A: 26S T-L activity of the platelet proteasome was analyzed and activity of treated platelets was calculated relative to resting platelets after 4h (ctr). Platelets were incubated with 4 x 10^6 bacteria/ 1 x 10^8 platelets for 4h. Next to the bars showing measured activities line plots indicate the activity from each individual donor (n=6). **B:** Western blot analysis of the proteasome substrate Talin-1 from control platelets after 4h (ctr) or UTI89 treated platelets for 4h. B-actin was used as a loading control (n=3).

UTI89 activates the 26S T-L activity to 3.86±1.66-fold and the strain without its toxins does not seem to activate the 26S activity to such a high extend (2.16±0.75, Figure 28A). The finding that the proteasome substrate Talin-1 is cleaved during an *E. coli* treatment supports this data (Figure 28B). Platelets incubated with UTI89 express more cleaved Talin-1 compared to resting platelets.

4 Discussion

4.1 Analysis of the proteasome in human platelets

As one of the major degradation systems the proteasome is an essential multicatalytic complex in eukaryotic cells. Since the crystallographic characterization of this huge particle we have a better knowledge of its structure but due to its complexity its regulation has not fully been understood until today.
A focus on anucleate platelets and its abilities to perform diverse cellular mechanism that were limited to nucleated cells turned up a couple of years ago. In this context little is known about the role of the proteasome in platelets although it has been identified in platelets. This study provides further evidence of the proteasome in platelets, which will be discussed in the following.

4.1.1 Anucleate platelets contain an active proteasome

The proteasome in anucleate platelets has been discovered earlier [59, 159, 160] and recently Klockenbusch *et al.* published a proteomic analysis of platelets identifying nearly all proteasome subunits [63]. Observations of Klockenbusch *et al.* [63] are in accordance with my data showing the expression of three representative subunits of the proteasome (Figure 9).
Furthermore, during this study the chymotrypsin-like (CT-L) activity of the 20S proteasome in human platelets was examined (Figure 10). The CT-L activity of the 20S proteasome was significantly inhibited by epoxomicin and lactacystin. Epoxomicin is shown to be the more effective inhibitor which is completely in accordance with literature [140]. Other work was able to show an active proteasome in platelets as well [159, 160] but up to date there are no measurements of all three proteolytic activities, namely the chymotrypsin-like, trypsin-like (T-L), and caspase-like (C-L) activities, of the 20S and 26S proteasomes. Figure 12 demonstrates that the CT-L, T-L and C-L activities of the 20S and 26S proteasome are detectable in human platelets. The 26S proteasome is considered more physiologically important than the 20S proteasome due to its role in degrading the majority of proteins. The role of the 20S core proteasome alone is not clear until today. A conformational change of the 20S proteasome is needed to let proteins/peptides enter its cavity [125] and this conformational change is physiologically only initiated through its regulators [125]. Furthermore, the 26S proteasome was differentially regulated by calcium ionophores (Figure 15) and collagen (Figure 16) whereas the 20S

proteasome hardly responded to those substances. This underlines the higher physiological role of the 26S proteasome.
Thus, the analysis presented (Figure 12) of all three activities of the 20S and 26S proteasome is a significant contribution providing a physiologically active proteasome in human platelets.

4.1.2 The proteasome is differentially regulated in human platelets

There are only few studies investigating the proteasome activity in platelets [159, 160, 163] and only few proteasome inhibitors were analyzed. This study demonstrates that established proteasome inhibitors epoxomicin, bortezomib and lactacystin restrain all catalytic proteasome activities in platelets (Figure 10 and Figure 14). The CT-L, T-L, and C-L activities of the 20S and 26S proteasome were inhibited to different extents (Figure 14). It is known that there are different inhibitory concentrations for those proteasome inhibitors. Here, Epoxomicin was found to be a more potent inhibitor for the CT-L and C-L activities than lactacystin which is in accordance with Meng *et al.* who detected a 4-fold faster inactivation rate of epoxomicin compared to lactacystin for the CT-L activity of the 20S proteasome, whereas the T-L activity was nearly identical [140]. Additionally, bortezomib hardly inhibited the T-L acitivities of the proteasome (Figure 14). This is demonstrated by various other groups as well [136, 137] and Groll *et al.* discovered that bortezomib has different binding affinities to the catalytic sites due to individual side chains of the inhibitor that interact distinctly with protein specificity pockets [137].
Nayak *et al.* were the first to describe a proteasome regulation upon chemical stimulation [163]. They treated platelets with a calcium ionophore and detected an increase of the 20S CT-L activity. The data communicated here show a regulation of the more physiological 26S proteasome for the first time (Figure 15 and Figure 16). A proteasome activity enhancement by calcium ionophors was measured in this context and the higher impact was observed for the 26S proteasome compared to the 20S proteasome (Figure 15). With their protocol Nayak *et al.* might have measured only the 20S proteasome activities and not the 26S proteasome activities and therefore only detected the activity enrichment in the 20S proteasome. Because the 26S proteasome is more relevant *in vivo*, it might be hypothesized that the proteasome *in vivo* in platelets is highly regulated by external factors.
Accordingly, Figure 16 illustrates a 26S CT-L proteasome activity increase for the platelet agonist collagen. Collagen is an established agonist for platelet aggregation, a fundamental function of platelets (see section 1.1.2). The observation that a platelet agonist is able to regulate proteasome activity emphasizes the significance of the proteasome in platelets for performing cellular mechanism. It needs to be considered that the proteasome plays a role in activation pathways of platelets.
Analysis of all catalytic activities of the 20S and 26S proteasome and especially the functionality of the proteasome in platelets, demonstrated by its ability to be

regulated, shows the importance of the platelet proteasome and its possible influence in cellular mechanisms.

4.1.3 Proteasome substrates

Through a comprehensive analysis of peptides bound to MHC I and processed by proteasome complexes cytoskeletal proteins such as Filamin A and Talin-1 were identified as proteasome substrates (Table 4 and Figure 17). The proteins Filamin A and Talin-1 have also been found by others to be proteasome substrates during the course of this study [150]. Cytoskeletal proteins are essential for the shape change of platelets upon activation and indeed, more than 50% of the platelets' total protein belong to the cytoskeleton. Cleaved Filamin A and Talin-1 fragments were found higher expressed when the proteasome activity was enhanced and preincubation with epoxomicin depresses the cleavage of the proteasome substrates Filamin A and Talin-1 (Figure 17). Those facts are an indication that the proteasome is involved in pathways that modify the cytoskeleton.
The data of Filamin A and Talin-1 presented here (Figure 17) suggest a novel function of proteasomes. Already in 2002 Rape discussed models of a novel mechanisms that might yield biologically active protein fragments [151]. The proteasome might not only degrade proteins but also cleave them into protein fragments. In nucleated cells proteins, such as the transcription factor NFκB are generated by the proteasome in that fashion [147-149]. It might be speculated that Filamin A and Talin-1 are proteins, which are processed into still active protein fragments in human platelets. This highlights the already suggested novel function of proteasomes.
In conclusion, cytoskeletal components were identified as proteasome substrates what demonstrates an impact of the proteasome on the cytoskeletal modifications.

4.2 Function of proteasomes in anucleate platelets

Not only the understanding of a protein structure and its active sites is crucial but the role of this protein or its purpose leads to the better knowledge of platelet biology. Protein degradation in anucleate platelets is an important system, which does not only degrade intracellular proteins for protein turnover but also regulates important cellular processes [98]. Yet how many and which proteins are degraded by the proteasome in platelets has not been analyzed until today.
Only when platelets were identified as antigen presenting cells [60] and since they contain the proteasome machinery [63], it was suggested that as in nucleated cells the proteasome (the immunoproteasome) has the role of processing antigens for antigen presentation on MHC I complexes. It is therefore very likely that the proteasome is involved in regulation of additional cellular mechanisms in platelets.

4.2.1 The proteasome affects platelet aggregation

In this study it was demonstrated that the proteasome affects platelet aggregation particularly in a collagen-induced aggregation pathway (Figure 18). During the course of this study Gupta *et al.* indicated a cellular process in platelets that depends on the proteasome [150]. They published data proposing an involvement of the proteasome in platelet activation. This is completely in accordance with data shown here, stating that proteasome inhibitors impair platelet aggregation (Figure 18). Gupta *et al.* stated that low concentrations of thrombin reduced aggregation and thrombin and ADP stimulation suppressed microparticle shedding. They demonstrated that Filamin A and Talin-1 are ubiquitinated and then cleaved by the proteasome in platelets. This let them to hypothesize that the platelet proteasome modifies cytoskeletal proteins and promotes platelet activation.
My data shows that aggregation induced by ADP is reduced when platelets are treated with proteasome inhibitors (Figure 18). Further findings propose that aggregation induced by low concentrations of collagen also weakens aggregation (Figure 18). This suggests that the proteasome is regulated by the platelet agonist collagen (Figure 16) and it can be concluded that the platelet proteasome modifies cytoskeletal proteins Filamin A and Talin-1 in this context (Figure 17).
This is supported by an observation of platelet concentrates, that show a reduction in their ability to aggregate over time (Figure 11) [195, 196] and they demonstrate a loss of proteasome activity as well (Figure 11).
Collagen, as stated here, activates the proteasome activity and then leads to aggregation (Figure 16 and Figure 18). Illustrated in this study is the function of the proteasome in platelet aggregation. However, the underlying signaling pathways have not been described yet. The next section will discuss a signaling pathway involving the platelet proteasome.

4.2.2 NFκB a regulator of the proteasome in platelets

NFκB is a known transcription factor in nucleated eukaryotic cells and it was shown to exist in anucleate platelets as well [69, 70, 79]. NFκB is not the only transcription factor discovered in anucleate platelets [64-68] and furthermore NFκB was demonstrated to influence platelet biology through a non-genomic function. Malaver *et al.* were the first to suggest this non-genomic function of NFκB in platelets [70]. They published amongst other results a reduction of aggregation by NFκB inhibitor treatment. Other groups confirmed functional NFκB in platelets [79, 80]. In accordance with this data, Figure 19 indicates a decrease of ADP- and collagen-stimulated aggregation by NFκB inhibitors. NFκB mediates aggregation in platelets similar to what is shown for the proteasome in Figure 18. This finding led to the assumption that NFκB inhibitors might reduce the collagen-induced proteasome activity induction similar to proteasome inhibitors. Figure 20 illustrates that NFκB

inhibitors reverse the proteasome activity increase with collagen. The 26S CT-L activity of the proteasome is increased by collagen but after a preincubation with NFκB inhibitors, Ro 106-9920 and Bay 11-7082, collagen did not result in an enhancement of the proteaome activity (Figure 20). Therefore inhibition of NFκB must reflect to proteasome activity and is connected to the collagen pathway.

Additionally, Figure 21 indicates an activation of NFκB activity in human platelets by collagen. NFκB is activated upon degradation of its inhibitor IκBα and subsequent release of its nuclear localization signals (NLS). Those freed NLS or the NFκB's response element can be detected even though non-genomic activity of NFκB is predicted. Gambaryan *et al.* measured NFκB activity in platelets in the same way and observed activation of the NFκB activity with another platelet agonist [80]. While they treated platelets with low concentrations of thrombin, evidence for an induction of NFκB activity upon treatment with low concentrations of collagen is provided in this study (Figure 21). As expected, the NFκB inhibitor Bay effectively inhibited the detected NFκB activity (Figure 21) and was therefore used as a negative control. Moreover, a reduction of NFκB activity by proteasome inhibitors might be expected as this was already shown in HeLa cells for epoxomicin [140]. The reduction of NFκB activity found in this study was only moderate (Figure 21) due to a relative small amount of replicates.

To study another read-out for proteasome activity the established proteasome substrate Talin-1 was tested. The full-length protein of the proteasome substrate Talin-1 is increasingly cleaved in platelets stimulated with collagen compared to resting platelets (Figure 21B). The cleaved fragment of Talin-1 is found at a higher expression level in collagen-stimulated platelets. This might result from the increased proteasome activity after stimulation of collagen. When proteasome activity enrichment is decreased by NFκB inhibitors, more full-length Talin-1 than cleaved Talin-1 is measured (Figure 21B). The change of these ratios indicates a connection of the NFκB pathway and the proteasome.

Subsequently, to further support an initiation of the NFκB pathway in platelets under collagen stimulation, activation of the IκB kinase (IKK), the activator of the NFκB pathway, was measured by assessing its phosphorylation state. IKK is phosphorylated when platelets are treated with collagen (Figure 22A) which has also previously been shown [83, 84].

Another important mode of NFκB regulation is through the degradation of the inhibitory protein of NFκB, IκBα. Figure 22B indicates a degradation of IκBα after stimulating platelets with collagen. In return, inhibition of the proteasome prevents IκBα degradation. In 2010 there was data published showing that IκBα is degraded and the NFκB pathway is activated when platelets are treated with thrombin, another platelet agonist [70, 80]. Figure 22B demonstrating the degradation of IκBα in the collagen pathway for the first time is an additional hint that the NFκB pathway is activated during platelet activation.

Taken together, an activation of the NFκB activity, phosphorylation of the IKK, and degradation of IκBα was observed in platelets in response to collagen stimulation. An activation of the NFκB pathway in activated platelets seems reasonable.
More importantly a potential connection of NFκB and the proteasome in collagen-stimulated platelets is implied (Figure 20 and Figure 21B). Not only is a collagen-induced proteasome activity enhancement prevented by NFκB inhibitors, but an increased cleavage of the proteasome substrate Talin-1 is also prevented by NFκB inhibitors. An inhibition of IKK by Bay does not show a collagen-induced enhancement of proteasome activity and reduces aggregation. The following picture summarizes the proposed activation pathway in platelets that connects NFκB and the proteasome (Figure 29).

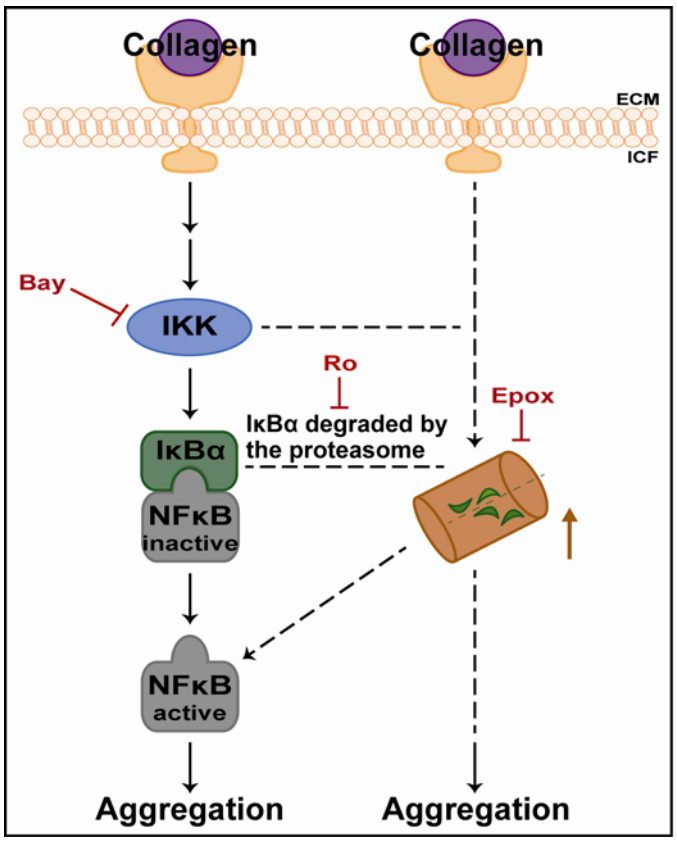


Figure 29: Hypothetical pathway of collagen-induced aggregation involving NFκB and the proteasome. Collagen stimulation leads to phosphorylation of IKK which further phosphorylates IκBα. This inhibitory protein is then degraded by the proteasome to transfer NFκB in an active state. Inhibition of IKK reduces NFκB activity and aggregation. As a side effect inhibition of IKK also restrains proteasome activity. An inhibition of the proteasome was shown to lower the ability of platelets to aggregate. In return, when the proteasome is inhibited, it no longer degrades the inhibitory IκBα protein of NFκB. The resulting inactivation of NFκB also reduces aggregation. Bay: Bay 11-7082, Ro: Ro 106-9920, Epox: epoxomicin, ECM: extracellular matrix, ICF: intracellular fluid

This pathway is a novel and significant contribution in understanding the platelet biology, proposing the involvement of the proteasome in platelet activation and stating a non-genomic role of NFκB in platelets. NFκB is involved in platelet activation by regulating the proteasome.

Further mechanisms for non-genomic functions of NFκB or more extended platelet pathways being regulated by the proteasome remain to be established. One must also keep in mind that NFκB inhibitors used here are not selectively inhibiting the NFκB pathway and other pathways in platelets might be affected to some extent [87]. Yet, more and more work on non-genomic functions of NFκB in platelet activation is being published regularly [82, 84-86]. NFκB might not only play a role in platelet activation and aggregation but also in granule secretion [82]. As mentioned in section 1.3.5, there has even been a study on IKK and its role in platelet secretion [85]. All these publications and performed experiments in this study with the identified novel pathway contribute to this functional aspect of the proteasome and NFκB in platelet biology. Platelet activation might start intracellular signaling by enhancing the proteasome activity and subsequently activating the NFκB pathway.
Collectively, inductors of platelet aggregation such as collagen activate both the proteasome and the NFκB pathway which are connected and mutually influence its activity on a molecular basis. Therefore inhibitors that affect the proteasome or the NFκB pathway also change the capacity of platelets to aggregate.

4.3 Sepsis as one of many clinical perspectives

The proteasome is one of two major degradation systems in nucleated cells and proteolysis is not only crucial for the total protein turnover but regulates important cellular processes, such as cell cycle, division, survival, oncogenesis, transcription, development, selective elimination of abnormal proteins, and antigen processing [101-104]. The importance of the proteasome as initiator of degradation has also been implied in anucleate platelets [98].
Previous work discovered that the proteasome can affect platelet vitality and apoptosis. Bacteria-induced apoptosis in platelets was shown [36]. Therefore analysis of platelets in a clinical setting was investigated. Since apoptosis is one of many cellular processes regulated by the proteasome (for instance, through degrading pro-survival proteins) [194] an involvement of the plateletal proteasome in sepsis was tested. It was already shown that proteolysis in smooth muscle cells of sepsis patients is depending on the proteasome [155, 197].

4.3.1 The mitochondrial membrane potential in platelets as a marker of sepsis

Sepsis is still a leading cause of morbidity and mortality and the link of thrombocytopenia as a predictor of clinical prognosis in sepsis patients has been well established, although investigations on a molecular level are still rare [193]. Some recent work states that bacteria isolated from sepsis patients directly activate the

apoptotic pathway in platelets [36] and other groups have described that bacterial factors of *S. aureus* induce mitochondrial membrane depolarization and apoptosis in platelets [198]. Thus, it is tempting to speculate that the loss of platelets in sepsis patients is a result of bacterial action or the septic milieu.
As shown in Figure 24 and Figure 25 the platelet mitochondrial membrane depolarization and decrease of pro-survival proteins correlate with clinical disease severity in patients with sepsis. This affirms previous work where the mitochondrial membrane depolarization also correlated with disease severity of a diverse group of sepsis patients [199]. However, this study is the first to demonstrate that the degree of platelet mitochondrial membrane depolarization correlates with disease severity during the disease course and outcome (Figure 26). Platelet mitochondrial membrane depolarization in a subgroup of patients with severe sepsis was compared on admission to a control group and they were compared during clinical disease course. Mmp-Index during disease course was studied in survivors and non-survivors. On admission, initial Mmp-Index values were significantly decreased but the patients who survived sepsis showed recovery of Mmp-Index on follow-up (Figure 26).
To conclude, mitochondrial membrane depolarization in platelets correlates with clinical disease severity in patients with sepsis during the disease course and may be a valuable adjunct parameter to aid in the assessment of disease severity, risk stratification, and clinical outcome. Results from this study and previous investigations seem to promise that markers of platelet apoptosis may assist in the evaluation of sepsis in the future.

4.3.2 Proteasome activity is enhanced in platelets during sepsis

This study emphasizes the event of apoptosis in platelets during sepsis. Apoptosis is one of many cellular processes regulated by the proteasome. Even though apoptosis is controlled through various caspases the proteasome degrades involved proteins, such as pro-survival proteins [194]. Bcl-xL protein degradation is increased when platelets are treated with pathogenic *E. coli* [36]. Hence, the involvement of the plateletal proteasome in sepsis should be investigated. It is already said that proteolysis in sepsis patients is depending on the proteasome [197] and this data was confirmed with a more detailed analysis on smooth muscle cells of sepsis patients [155].
Figure 27 indicates that the proteasome activity in platelets of sepsis patients might be enhanced. It is tempting to assume that platelets increase their proteasome activity and subsequently apoptosis is induced. Another indication might be the rising proteasome activity in platelets that were *in vitro* incubated with uropathogenic *E. coli* bacteria. Figure 28 illustrates a bacteria-induced enhancement of proteasome activity in platelets. In accordance with this, data exist demonstrating that lipopoly-saccharides (LPS), a membrane component of gram-negative bacteria, are able to

activate the proteasome activity [200]. Moreover, this study discusses that the proteasome substrate Talin-1 is cleaved more under a proteasome activity induction performed by calcium ionophores or collagen (Figure 17). Platelets treated with bacteria contain also more of the cleaved Talin-1 fragment. The full-length protein is found higher expressed in resting platelets than in bacteria incubated platelets (Figure 28B) suggesting an increased proteasome activity in bacteria treated platelets.
Those experiments are only preliminary. A larger study population should be analyzed according to their proteasome activity and the mechanism for an inclining proteasome activity during apoptosis should be clarified. Nevertheless, these findings argue an influence of the proteasome in platelets during sepsis. The proteasome plays an important role in many diseases and in platelets it is associated with the activation pathway. Here, data is provided suggesting a relevance of proteolysis in platelets during sepsis. This data is supported by a study by Qureshi *et al.* proposing the proteasome as an important target in gram-negative sepsis [200].

4.4 Conclusion and Outlook

This study confirms the existence of a functional 20S and 26S proteasome in human platelets and provides data that emphasize its significance in platelet biology.
Presented is a possible novel pathway during platelet activation that mutually connects the platelet proteasome during platelet activation with a non-genomic function of NFκB in platelets. Platelet activation might induce intracellular signaling by initiating the NFκB pathway that mediates proteasome activity. A better understanding of platelet biology and the exact role of the proteasome in platelets may offer the possibility to monitor side-effects of proteasome inhibitors on platelets but also study proteasome related disease processes that result from platelets.

This work further contributes data that illustrate how markers of mitochondrial function and proteasome activity could aid in the assessment of the disease severity and disease course of patients with sepsis.

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Appendix

Abbreviations

°C	Degree Celsius
AA	Arachidonic acid
ADP	Adenosine diphosphate
AMC	7-amino-4-methylcoumarin
AML	Acute myeloid leukemia
APACHE	Acute Physiology and Chronic Health Evaluation
APS	Ammonium persulfate
ATP	Adenosine triphosphate
AU	Arbitrary unit
Bay	Bay 11-7082
BCA	Bicinchoninic acid
borte	Bortezomib
BSA	Bovine serum albumin
CD	Cluster of differentiation
CID	Collision induced dissociation
CKII	Casein kinase II
C-L	Caspase-like
clm	Chloramphenicol
cnf1	cytotoxic necrotizing factor 1
col	Collagen
CT-L	Chymotrypsin-like
ctr	Control
DAG	Diacylglycerol
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
ds	Double stranded
DTT	Dithiothreitol
E. coli	*Escherichia coli*
e.g.	*Exempli gratia*
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
epox	Epoxomicin
et al.	And others (*et alii*)
FACS	Fluorescnece activated cell sorter
FCS	Fetal calf serum
FU	Fluorescence units
g	Acceleration due to gravity
GP	Glycoprotein
GR	Glucocorticoid receptor
h	Hour
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HLA	Human leukocyte antigen
hlyA	α-hemolysin
HMEC	Human microvascular endothelial cells
HRP	Horseradish peroxidase
ICF	Intracellular fluid
IKK	IκB kinase
IP3	1,4,5-triphosphate
ITAM	Immunoreceptor tyrosine-based activation motif
JC-1	Tetrachlorotetraethylbenzimidazolylcarbocyanine iodide
kan	Kanamycin
lacta	Lactacystin
LPS	Lipopolysaccharides
MC	Monoclonal
MHC I	Major histocompatibility complex class I
min	Minutes
Mmp	Mitochondrial membrane potential
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NLS	Nuclear localization sequence
p-	phosphorylated
PAGE	Polyacrylamid gel electrophoresis
PAR	Protease-activated-receptor
PBS	Phosphate buffered saline
PC	Polyclonal
pH	pH value (potentia hydrogenii)
PI3K	Phosphatidylinositol 3-kinase
plt	Platelets
PMSF	Phenylmethanesulfonylfluoride
PND	Z-Pro-Nle-Asp-CHO
PPAR	Peroxisome proliferator activated receptor
PRP	Platelet rich plasma
PVDF	Polyvinlylidene difluoride membrane
Ro	Ro 106-9920
rpm	Revolutions per minute
Rpt	Regulatory particle ATPases
RT	Room temperature
RXR	Retinoid X receptor
S. aureus	*Staphylococcus aureus*
SAPS	Simplified Acute Physiology Score
SDS	sodium dodecyl sulfate
SOFA	Sequential Organ Failure Assessment
TAP	Transporter associated with antigen processing
TEMED	Tetramethylethylenediamine
T-L	Trypsin-like
TMB	3,3',5,5'-tetramethylbenzidine
TP	Thromboxane receptor
TRAP	Thrombin receptor-activating peptide
TXA2	Thromboxane A2
Ub	Ubiquitin
USA	United States of America
WB	Whole blood
wb	Western blot

Publications

Gründler K, Angstwurm M, Hilge R, Baumann P, Annecke T, Crispin A, Sohn HY, Massberg S, Kraemer BF. *Platelet mitochondrial membrane depolarization reflects disease severity in patients with sepsis and correlates with clinical outcome.* Crit Care. 2014 Feb 12;18(1):R31.

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