Localisation and functionality of the inflammasome in neutrophils

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"There is no need to be a doctor or scientist
to wonder why the human body is capable of resisting
so many harmful agents in the course of everyday life."
I. Metschnikow, Russian immunologist, 1908

1. Introduction

1.1 Immune system

The immune system is a network of cells, tissues, and organs working together to defend biological organisms against foreign substances and microorganisms. The human system is divided into different, step-like sections. The two main subgroups are the innate, also called the non-specific immune system, and the acquired (or specific) system. The acquired immune system developed supplementary to the evolutionarily older defence strategy of the innate system and it is still supplemented and controlled by the phylogenetically older system.[40] This relationship is also reflected in the activity sequence during immune defence: the innate immune system is the first line of response against pathogenic agents. It offers a broad and instantaneous protection, also against previous unknown pathogens. The specific immune response is deployed in the subsequent phase of infection as well as in the formation of an immunological memory.[1] However, in contrast to the innate immune response it takes days to develop its maximum efficiency, but through the capability of the specific immune system to remember and recognize specific pathogens, it reacts faster and stronger in case of recurring infections.[40]

1.2 Innate immunity

The innate immunity is the dominant defensive weapon of multicellular organisms. The molecular components are evolutionary conserved and can also be found in fungi, plants, and animals.[3,55] Non-self substances and microorganisms are identified with a

limited number of germline-encoded pattern-recognition receptors (PRRs). The non-specific immune system represents the first instance of host defence and is composed of anatomical barriers, humoral factors, and special immune cells.

1.2.1 Anatomical barriers

During evolution, multifarious barriers were developed to make it difficult for pathogens to penetrate the body. The majority of infectious agents and environmental toxins are kept away from the host by these physical structures. The epithelial barrier of the skin, gastrointestinal, respiratory, as well as of the genitourinary tract separates the host from the external environment and forms an impermeable barrier for most of the pathogens. Additionally, infectious components are removed by the peristaltic of the gastrointestinal tract or the mucociliary apparatus of the lungs. Gastric acid and the physiological flora are other examples of how the body is protected from being colonized by microorganisms.

1.2.2 Humoral factors

The humoral defence of the innate immunity is composed of various substances, including complement system, lysozyme, the defensins, as well as cytokines. The complement system represents a cascade of 30 plasma proteins, which lead for example to opsonization and lysis of the pathogen cells. Lysozyme is a bactericidal enzyme, found in many secretes such as tears and saliva. Antimicrobial properties as well, are part of the polypeptide defensin, which is found for instance in the granules of neutrophil granulocytes. The group of cytokines is various; they include for example the interleukins.

1.2.3 Cells of the innate immune response

Natural killer cells, granulocytes, macrophages, and dendritic cells, as well as nonprofessional cells such as fibroblasts, epithelial and endothelial cells are numbered among the cells of the innate immune system.[55] The leukocytes of the innate immune system are able to identify and eliminate pathogens on their own, but they also cooperate closely with the acquired immune system. Dendritic cells, macrophages, and B lymphocytes are professional antigen-presenting cells. They present antigenic

peptides, bound to major histocompatibility complex class II molecules (MHC II), to T_h cells, which play an important role in the coordination of the adaptive immune response. Important strategies in the fight against pathogens are the phagocytosis and the respiratory burst. Professional phagocytes are monocytes, macrophages, neutrophil granulocytes, dendritic cells, as well as mast cells.[23] Neutrophils and macrophages are able to kill engulfed microorganisms through the generation of reactive oxygen species; a process called 'respiratory burst'.

1.3 Granulocytes

Granulocytes make up around 60 percent of the leukocytes. The nucleus of mature cells has a characteristic lobed appearance; therefore, these cells are also called the polymorphonuclear leukocytes (PMN). Three different types of granulocytes can be distinguished. They are named according to their dyeing characteristics: neutrophil, eosinophil, and basophil granulocytes. Neutrophil granulocytes comprise the largest population of the three granulocyte types and also the major fraction of all circulating immune cells.[56] These phagocytic and highly motile cells are the primary mediators of the innate immune response and the first line of defence against invading microorganisms.[44] They congregate quickly at the site of infection, not only to phagocyte and to digest the pathogen, but also in order to release cytotoxic, chemotactic, and inflammatory substances from their intracellular granules and vesicles. These mediators control the immediate immune response and attract other cells, including macrophages and dendritic cells, which play a significant role in the development of a long-term adaptive immunity.[22,42,47,60,64] Eosinophil granulocytes become crucial during parasitic infections and inflammatory processes.[13] The large granules of the basophil neutrophils contain histamine, heparin, serotonin, and other mediators. The typical allergy symptoms are caused by the release of histamine through activated basophils. For this reason, basophil granulocytes, along with mast cells, are considered to play the most crucial role in allergic diseases.[82]

1.4 Granulopoiesis

Like all cellular blood components, granulocytes derive from pluripotential hematopoietic stem cells in the bone marrow. During the complex process of

granulopoiesis, these early blood cell precursors divide, differentiate, and maturate to become functionally active granulocytes. Initially, the differentiation of the stem cell results in one of two hematopoietic lineages: either a multipotent lymphoid progenitor stem cell or a common myeloid progenitor cell. The myeloid progenitor is also called CFU-GEMM, which describes a colony forming unit that generates granulocytes, erythrocytes, monocytes, or megakaryocytes. CFU-GEMM gives rise to the CFU-GM sublineage. In early granulopoiesis, these granulocyte-macrophage progenitors further differentiate into granulocyte-colony-forming units, and these in turn lead to myeloblasts. The unipotent myeloblasts are the first morphologically distinguishable cells of the granulocytic lineage. As a result of their limited potentiality they are now fully restricted to the granulocytic cell line.[51] The next of five further development stages is the differentiation into promyelocytes. Consequently, three different precursors may arise: the neutrophilic, the basophilic or the eosinophilic myelocytes. Subsequent in the differentiation sequence are the metamyelocyte, band neutrophil and finally the mature granulocyte. The whole hematopoiesis is strictly controlled by cytokines, transcription, and growth factors and occurs in close contact to the stromal microenvironment of the bone marrow. For example, the cytokine G-CSF (granulocyte colony-stimulating factor) is a hormone-like glycoprotein, promoting proliferation and maturation. It is produced by monocytes, macrophages, fibroblasts, and endothelial cells in response to stimulation with lipopolysaccharides (LPS), Interleukin-1 (IL-1), or tumor necrosis factor alpha (TNFα).[51] All sections of the granulopoiesis are associated with the synthesis of stage-specific proteins and the granulocytic precursors undergo expressional changes and morphologic transformations during these stages. The resulting variety of innate immunity proteins is necessary for the highly complex role of polymorphonuclear leukocytes in phagocytosis, microbial defence, and the mediation of inflammatory processes.

1.5 Neutrophil granulocytes

1.5.1 Neutrophil granule heterogeneity

The wealth of the neutrophil proteins is not found freely dispersed through the cytoplasm, but is stored in granules and vesicles. Furthermore, their distribution in the cytoplasm is not random; you find them well-sorted according to a certain pattern. This

is motivated by a reason: not all stored proteins are needed at the same time and not all proteins can co-exist in the same granule, for example a precursor protein and its activating protease. Thus, each subset of granules has its characteristic protein profile and its specific condition of degranulation.[85] The granules are subdivided into primary granules (also known as peroxidase-positive or azurophil granules), secondary (or specific granules), and tertiary granules (also called gelatinase granules). The azurophilic granules contain the most harmful and toxic weapons of the phagocyte: serine proteases and peroxidase. The bactericide protein lactoferrin, for instance, is a component of the specific granules. Tertiary granules contain gelatinase to facilitate the migration through the extracellular matrix.

Table 1: Assortment of some neutrophil granule proteins.[8]

Azurophil granules	Specific granules	Gelatinase granules
α_1-antitrypsin, Cathepsins, Defensins, Elastase, Lysozyme, Myeloperoxidase, Proteinase-3, Sialidase	β_2-microglobulin, Collagenase, Histaminase, Lactoferrin, Lysozyme, NGAL, Sialidase	Acetyltransferase, Gelatinase, Lysozyme

Beside the liberation of proteins, granules and vesicles play an important role in the dynamic organisation of the cell membranes. Their endomembranes furnish the plasma membrane with important functional proteins, including phagocytosis and adherence mediating proteins, as well as receptors for chemoattractants or complement factors. The described diversity of granule composition can hardly arise through the process of protein targeting alone. However, the specific protein profile of granules can be explained if granules are formed continuously during granulopoiesis and concurrently filled with the proteins which are synthesised in the particular stage of development (see figure 1). This outlined mechanism is referred to as the 'targeting by timing hypothesis'.[10,11,30,85]

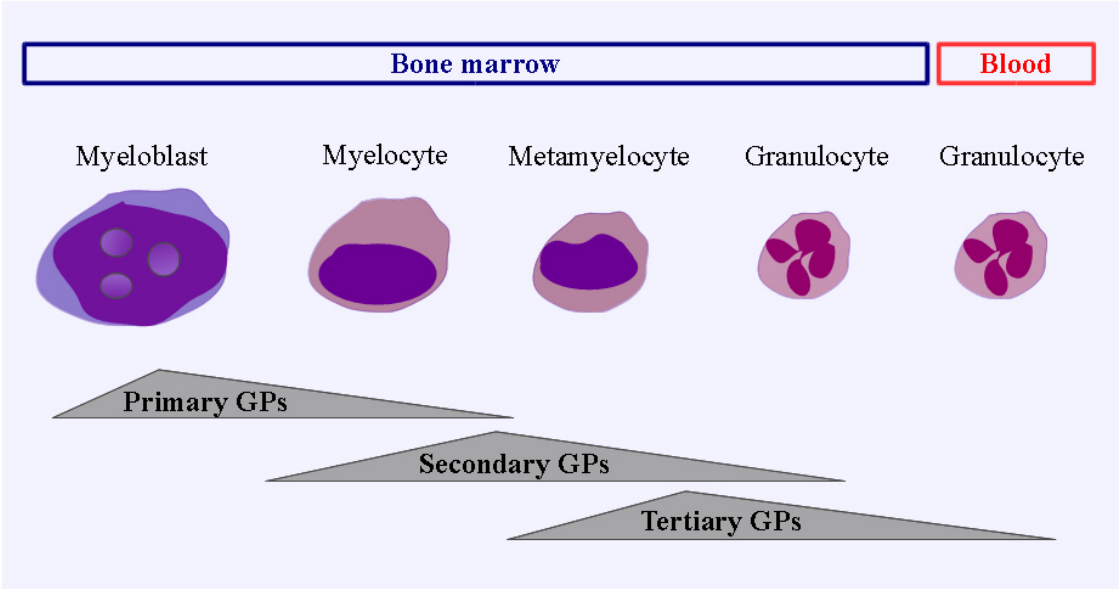

Figure 1: Targeting by timing hypothesis. GPs=Granular proteins. The granules of the granulocytes are formed during granulopoiesis and packed with the proteins synthesised in the particular stage of maturation.

1.5.2 Secretory vesicles

Another type of specialized cytoplasmic organelles, beside the described granules, are the secretory vesicles. These cellular components contain plasma proteins and are formed by endocytosis during the neutrophils' terminal maturation in the bone marrow. As a consequence, their importance does not lie in the storage of immunological or microbe harming substances. Instead, secretory vesicles store membrane proteins.[78] They represent the main source of receptors, and thus, contribute significantly to the instant responsiveness of neutrophils. Among the variety of receptors are: pathogen-recognition receptors, Fc receptors, interferon receptors, a diversity of chemokine receptors, and also receptors responsible for the mediation of diapedesis.[10,85]

1.5.3 Control of exocytosis

Neutrophils are unique in their capacity to mobilize granules from intracellular stores to the cell surface as well as fusing them with pathogen containing phagosomes. This flexibility enables the neutrophils to adapt rapidly to a changing microenvironment. Granules and vesicles are assigned to a hierarchy, according to their different roles during the immunity response. The importance of secretory vesicles in the regulation of neutrophils' interaction, especially in the early stages of inflammation, is accompanied

by a high propensity for degranulation. The propensity of degranulation is determined by the density of fusion proteins in the vesicle membrane. The increasing expression of those proteins during granulopoiesis entails that the secretory vesicles possess the highest and the azurophil granules the lowest quantity of fusion proteins. Accordingly, secretory vesicles are most sensitive to several stimuli and can be mobilized completely, whereas only a small amount of peroxidase positive granules are exocytosed at site of infection, leaving most of them retained ready for the digestion of phagocyted microorganisms.[8-10,85]

1.6 Pattern-recognition receptors

How do the phagocytes identify potential pathogens? Ingeniously, they make use of specific microbe components, for instance LPS, a cell wall component of gram-negative bacteria, or characteristic RNA/DNA motifs, which are not expressed in the host's system. As the structures are often essential for the microbes' survival, they are highly conserved and found in a wide group of microorganisms. These conserved and microbe specific components are known as microbe-associated-molecular patterns (MAMPs) or pathogen-associated molecular patterns (PAMPs). The receptors of the immune cells recognizing these motifs are called pathogen-recognition receptors (PRR). They are divided into several families, including transmembrane proteins as the Toll-like receptors (TLRs) and C-type lectin receptors (CLRs), secreted proteins like mannan binding lectin (MBL) as well as cytoplasmic proteins such as the retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) and NOD-like receptors (NLRs).[20,84] The recognition of microbial components triggers a cascade of cellular signals, which lead to the expression of inflammatory mediators. These mediators control the innate immune response but also the development of specific immunity cells.

In addition to PAMPS, PRRs can also be activated through DAMPs (damage-associated molecular patterns). DAMPS are endogenous danger signals released by damaged or stressed cells. They are intracellular (mostly nuclear or cytosolic) proteins and therefore hidden under normal physiological conditions. Examples of DAMPs are HMGB1, heat-shock proteins, or high extracellular concentrations of ATP.[59,71,72,74] The induced inflammatory response occurs in the absence of any pathogens and has therefore been

named ‘sterile inflammation'. Cell death can therefore be regarded as universal and potent trigger of inflammation, being essential for tissue repair and regeneration. Similar to the microbe-induced inflammation, sterile inflammation is characterized by the recruitment of leukocytes and the production of pro-inflammatory cytokines like TNF and interleukin-1.[16,71]

1.6.1 Toll-like receptors

Among the microbial-sensing receptors, the Toll-like receptors (TLRs) are the best studied ones.[83] So far, eleven different TLR are known in humans.[3] These transmembrane proteins recognise a wide range of microbial pathogens, including bacteria, viruses, fungi, and protozoa.[39] Activated TLRs initiate cellular signal transduction pathways, which trigger the activation of transcription factors, especially the activation of NF-κB.[21] NF-κB controls the expression of various genes involved in inflammation, for example proinflammatory cytokines, immune receptors, and cell surface adhesion molecules.[46] Each TLR recognises a distinct set of molecular patterns.[55] The Toll-like receptor 4 (TLR4) for instance, which is expressed by different types of immune cells including neutrophils and dendritic cells, detects small concentrations of LPS in the surrounding medium.[46,55,83]

1.6.2 NOD-like receptors

Unlike TLRs, which are membrane-bound molecules recognizing microbial components in the extracellular milieu or in endosomal compartments, NOD-like receptors (NLRs) sense cytosolic PAMPs and DAMPs. NLRs are characterized by C-terminal leucine-rich repeats (LRR domain), a central nucleotide-binding and oligomerization domain (NOD), and an N-terminal effector domain. The NLRs are divided, according to their N-terminal effector domain, into subfamilies. The majority of NLRs have a caspase recruitment domain (CARD) or a pyrin (PYD) domain here.[49] These, also named death-fold domains, possess the characteristic to form dimers or multimers with other members of the same subfamily. The LRR domain has a regulatory function and is responsible for sensing activating signals.[69] However, the precise sensing mechanisms are unclear, so it is not known, whether NOD proteins recognize pathogen products through direct interaction or if some other accessory molecules are required. In any

case, activation leads to a conformation change and to a nucleotide exchange on the NACHT domain, which is part of the NOD-domain. This structural rearrangement leads to an active state and the ability to oligomerize. The assembled protein oligomer is termed 'inflammasome'. These complexes serve as a molecular platform to activate NF-κB transcriptional pathways as well as pro-caspase-1. Active caspase-1 processes several precursors of interleukin-1 family cytokines into their biologically active forms. Furthermore, the processing of pro-caspase-1 is essential for the regulation of the inflammatory form of cell death, the pyroptosis.[12]

1.6.3 NALP3 inflammasome

The most thoroughly investigated inflammasome is NALP3, also known as cryopyrin. NALP3 contains an N-terminal PYD domain and activates pro-caspase-1 indirectly through the bridging molecule ASC, which is composed of a PYD and a CARD domain (see figure 2). It interacts with NLRP3 via homotypic PYD-PYD interaction and recruits on the other side pro-caspase-1 through a CARD-CARD interaction.[52] Mutations in the *NALP3* gene are linked to a series of hereditary autoinflammatory fever syndromes called CAPS (see chapter 1.9). Furthermore, a lot of infectious or sterile inflammatory conditions show a NALP3 activation. This is due to the broad variety of NALP3 activating substances, including DAMPS, microorganisms, as well as exogenous non-microbial substances like urate crystals. For instance, the microbial toxin and ionophore nigericin is a potent NALP3 inflammasome activator. This pore-forming toxin causes a net decrease in the intracellular potassium level in a pannexin-1-dependent manner. Similar to nigericin, ATP decreases also the intracellular potassium concentration. ATP binds to the purinergic receptor P2X7, which leads to the opening of ATP-gated cation channels and subsequent to the recruitment of the hemichannel protein pannexin-1. Pannexin-1 forms large pores in the cell membrane, through which microbial products like LPS can be delivered into the cytoplasm.[3,12] Nevertheless, the exact mechanisms of inflammasome activation are still poorly understood. Considering the differences in structure and function of the molecules reported to activate NLRP3 inflammasomes, a direct interaction is hardly probable. It seems, like the stimuli end up in a common pathway like the efflux of intracellular potassium. However, it is possible

that additional, so far unknown signals of cellular injury contribute to the assembly of NLRP3.

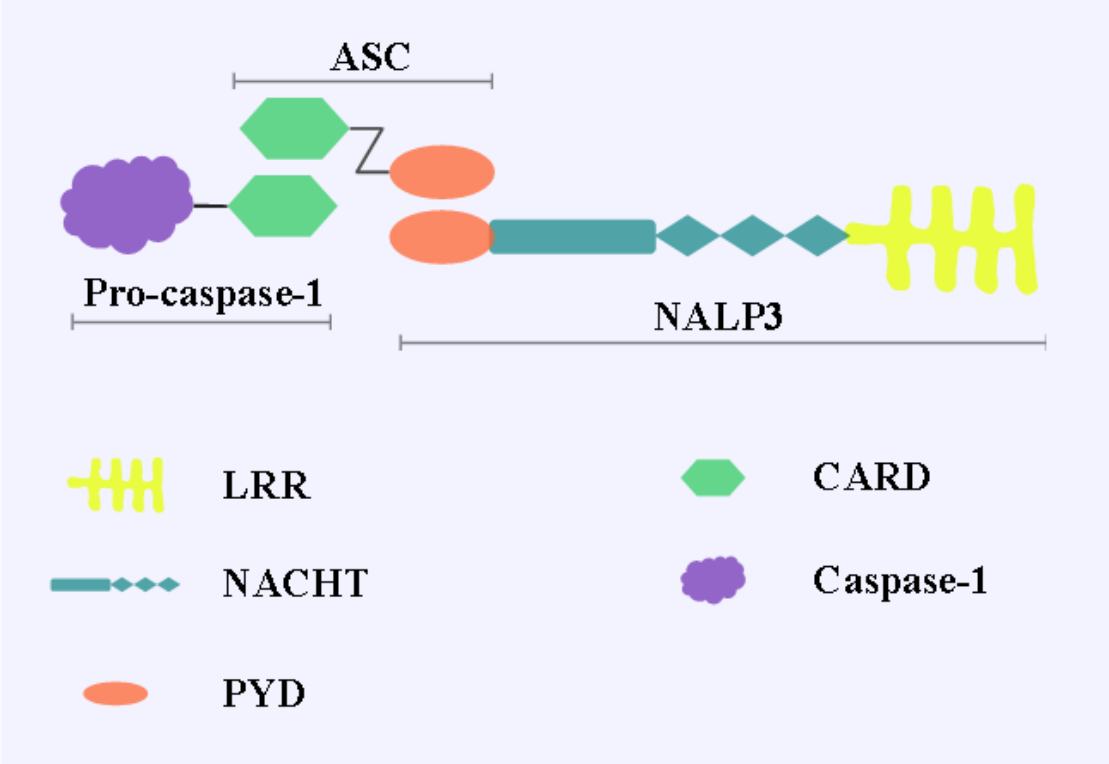

Figure 2: Structure of the NALP3 inflammasome. NALP3 is characterised by three functional domains, consisting of a C-terminal ligand-sensing leucine-rich repeat (LRR) domain, a central oligomerisation domain (NACHT), and an N-terminal pyrin (PYD) domain. The NALP3 inflammasome activates pro-caspase-1 through the bridging molecule ASC (apoptosis-associated specklike protein containing a caspase-recruitment domain (CARD)). ASC interacts with NALP3 via homotypic PYD-PYD interaction and recruits on the other side pro-caspase-1 through a CARD-CARD interaction.

1.7 Caspase-1

Caspase-1 is a member of the cysteine-aspartic acid protease (caspase) family and was previously termed interleukin-1ß-converting enzyme (ICE). However, it is not merely an ICE, it is also required for the proteolytic processing of pro-IL-18.[67] Like other caspases, caspase-1 is in turn synthesised as an inactive zymogen. It becomes autoproteolytically active after controlled dimerization through the inflammasomes. Beside cytokine activation, caspase-1 is capable of triggering pyroptosis. This form of programmed cell death is associated with the release of proinflammatory cellular contents and is therefore part of the defence mechanism.

1.8 IL-1α, IL-1ß, IL-18, and IL-33

The IL-1 family contains 11 members, notably IL-1α, IL-1ß, IL-18, and IL-33. These cytokines are all produced as inactive zymogens. Consequently, their activity is controlled in a posttranscriptional fashion in addition to their transcriptional regulation. To what extend the NLRP3/ASC/Caspase-1 axis is involved in their activation is not fully understood. In particular, the production mechanisms of those cytokines in neutrophil granulocytes are still relatively unclear.

1.8.1 IL-1ß

The proinflammatory cytokine IL-1ß has important local, but also systemic effects, for instance as endogenous pyrogen. It activates endotelium, T cells, and macrophages and is therefore an important mediator of the immune response during infection and inflammation. IL-1ß is involved in acute and chronic autoimmune diseases, diabetes, pain, as well as in neurological disorders like schizophrenia.[19,80] Furthermore, abnormal increased IL-1ß levels are a distinctive feature of autoinflammatory diseases. Various cell types are capable of releasing IL-1ß; among these are macrophages, keratinocytes, fibroblasts, mast cells, glial, as well as neuronal cells.[68,89] IL-1β is synthesised as a 33-kDa precursor protein, which lacks biologic activity and requires a processing into the mature 18-kDa protein. Pro-IL-1β can be proteolyticaly cleaved as a consequence of inflammasome activation and however, alternatively, in a caspase-1-independent fashion.[29,35,57] Proteases capable of cleaving the pro-IL-1β protein include, among others, the neutrophil serine proteases[7,35,37,76], bacterial cysteine proteases[36], granzyme A[34], and matrix metalloproteinases (MMPs)[75].

1.8.1.1 Regulation of IL-1ß secretion in PBMCs

Studies on the structure and function of inflammasomes in PBMCs resulted in a ‘two-hit theory’ concerning the secretion of IL-1ß (see figure 3). The reason behind is that the IL-1ß production seems to be the outcome of two distinct activating signals, acting through two different PRRs: the first signal induces, through a TLR, the transcription of pro-IL-1ß. After this 'cell priming', a second hit is required to activate the inflammasome, which cleaves the precursor protein into biologically active IL-1ß.

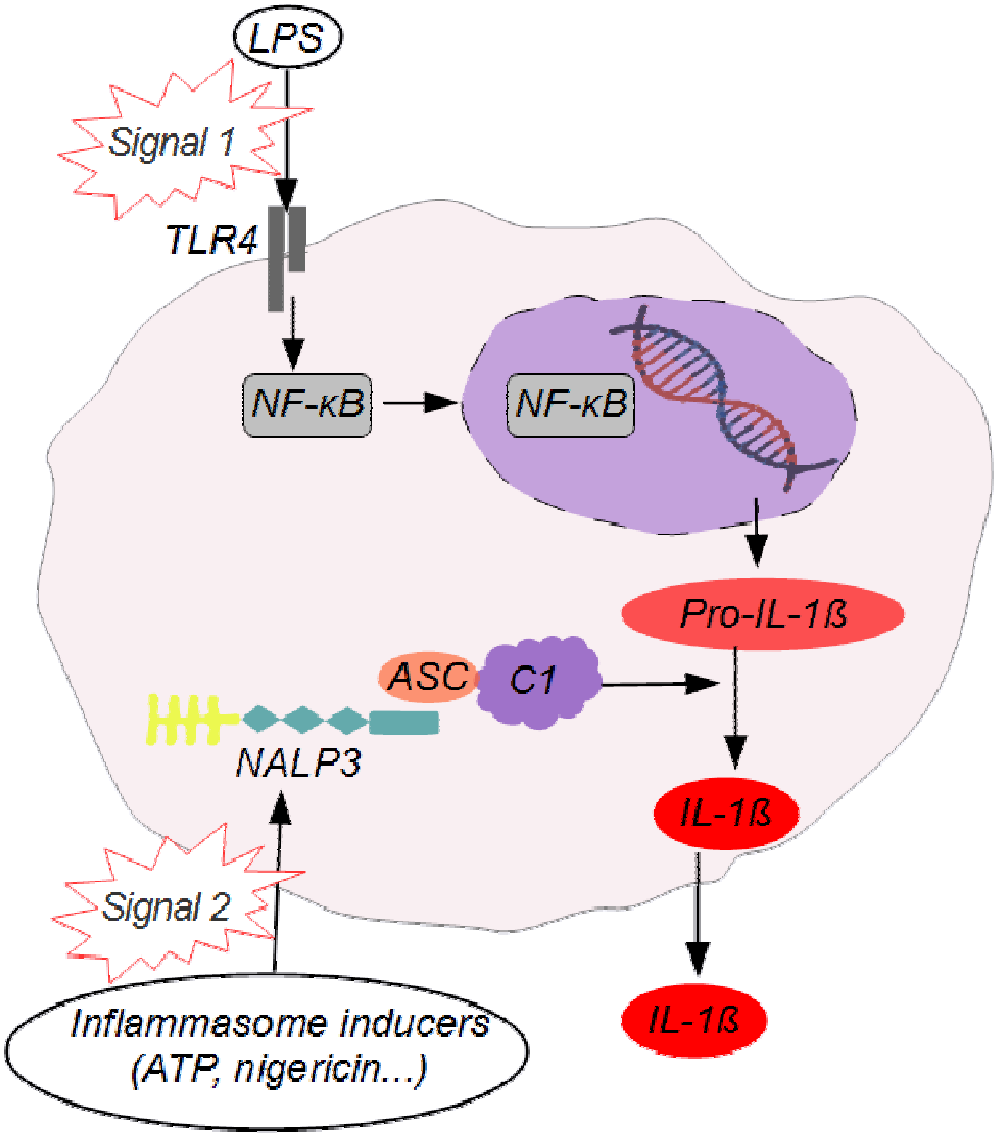

Figure 3: Two-hit theory of IL-1ß production in PBMCs. An initial priming step with LPS induces via TLR4 the NF-κB transcriptional pathways. The subsequently synthesised zymogen pro-IL-1ß is cleaved into its mature form through caspase-1 (C1). The second stimulus is required to generate the active form of caspase-1. ATP and nigericin induce NALP3 assembly and thereby the generation active caspase-1.

1.8.1.2 Regulation of IL-1ß secretion in neutrophil granulocytes

Several previous studies provided evidence that neutrophils represent a substantial source of IL-1β *in vitro* and *in vivo*, both in the murine as well as in the human system.[6,14,17,29,35,57] Although the regulation of IL-1ß processing and secretion has been thoroughly analysed in macrophages, the precise mode of IL-1ß generation by human neutrophils remains only partially understood. Different studies have drawn

controversial conclusions whether neutrophil derived IL-1ß stems mainly from inflammasome- or serine protease-dependent activities. This question is of particular relevance, as neutrophil granulocytes store the major source of serine proteases in their primary granules.[30,53] Previous studies pointed indeed to caspase-1-independent pathways of neutrophil IL-1ß processing: the investigations demonstrated that several neutrophil proteases, like proteinase 3 (Prtn3) and neutrophil elastase (Ela2), were able to cleave pro-IL-1ß into its biologically active form.[17,37,76] Furthermore, other studies indicated an IL-1ß production in caspase-1 deficient mice.[29,35,57] Recent studies refute, at least partially, inflammasome independent processing. In 2011, Guarda et al. detected NLRP3 expression in neutrophils, even under resting conditions, and the expression was highly augmented after exposure to pro-inflammatory stimuli.[28] This induced expression could suggest a functional role of NALP3 in activated neutrophils. Mankan et al. (2012) isolated neutrophils from *prtn3/ela2-, asc-, nalp3-,* and *caspase-1*-knockout mice and stimulated them according to the two-hit theory of inflammasome activation. In this study, no significant difference in the amount of released IL-1ß between WT and *prtn3/ela2*-knockout mice was found. The *prtn3¯/ela2¯* neutrophils were fully capable of secreting IL-1ß, whereas no IL-1ß was secreted by neutrophils from c*aspase-1, asc-,* or *nalp3*- knockout mice.[48] These findings imply that, at least in murine neutrophils, the NALP3/ASC/caspase-1 axis plays an essential role in the IL-1ß processing. The opposed data from different studies could argue for different types of IL-1ß driven inflammation, in which inflammasome-independent but also inflammasome-dependent processes exist. Finally, the contribution of NALP3 inflammasomes as well as the involvement of Prtn3 and Ela2 in the neutrophil IL-1ß production are not conclusively and need to be clarified by further investigations.

1.8.2 IL-1α

Pro-IL-1α is encoded in the same gene cluster like its close homologue pro-IL-1ß. These two members of the IL-1 family are structurally related and share the same cell surface receptor, the IL-1R. Through this common receptor, the biological function of IL-1ß and IL-1α should in principle be identical to a large extent. However, the immunological role of IL-1α is still not well defined and some studies suggest that IL-1α is less potent than IL-1ß.[63] The synthesis of pro-IL-1α is induced by Toll-like

receptor mediated NF-κB activation.[31] However, the exact factors leading to the processed form of IL-1α are not clear. Studies came to different conclusions regarding the inflammasome dependency of IL-1α activation. IL-1α was secreted in response to NLRP3 inflammasome activators but its secretion was not universally inflammasome dependent. Inflammasome-independent activation of IL-1α was observed through the calcium-activated protease calpain (CANP).[27,43,90]

1.8.3 IL-18

Since IL-18 induces an IFNγ production by T cells and NK cells, it was previously known as interferon-gamma-inducing factor (IGIF). It is known to be synthesised by a variety of cells including macrophages, dendritic cells, keratinocytes, osteoblasts, and synovial fibroblasts.[70] Pro-IL-18 is supposed to be constitutively expressed in PBMCs as well as in neutrophils. However, in the gene regulation, NF-κB seems to be involved as well.[24,50,65,70] As in the case of its related cytokine IL-1ß, IL-18 is synthesised as an inactive proenzyme requiring a proteolytic cleavage by caspase-1 to generate the bioactive molecule. Moreover, alternative caspase-1 independent mechanisms for IL-18 processing are discussed. These inflammasome independent pathways could consist of extracellular cleavage by neutrophil-derived serine proteases or by proteases released from cytotoxic T cells.[86]

1.9 CAPS

Cryopyrin-associated periodic syndromes (CAPS) are a group of rare hereditary inflammatory disorders characterized by recurrent periods of systemic inflammation, resulting in fever, rash, elevation of acute phase reactants, and joint pain. The group encompasses three, often overlapping, phenotypes varying in severity and the involvement of different physiological systems. The familial cold autoinflammatory syndrome (FCAS) is the mildest form; the other phenotypes are the Muckle-Wells syndrome (MWS) and the neonatal-onset multisystem inflammatory disease (NOMID), which represents the most severe form of the CAPS.[79] These monogenetic autoinflammatory disorders are often caused by a gain-of-function mutation in the *NLRP3* gene. The resulting spontaneous activation of NALP3 and overproduction of interleukin-1 represent the central pathophysiology.[33,38,61] The IL-1-mediated

symptoms flare and can be triggered by cold exposure, stress, or unknown causes. Symptom-free intervals are characterised by complete wellbeing and normalisation of the acute phase reactants. Due to the fact that IL-1ß plays a pivotal role in the pathogenesis, it represents an important therapeutic target. Currently, there are three drugs available: the interleukin-1 receptor antagonist Anakinra, the IL-1ß monoclonal antibody Canakinumab, and the interleukin-1 trap Rilonacept. The treatment targeting IL-1 has shown positive effects on patients' clinical symptoms and quality of life.[15,45,91]

1.10 Muckle-Wells syndrome

The Muckle-Wells syndrome (MWS) was first described in 1962 by Thomas Muckle and Michael Wells.[54] The autosomal dominant transmitted disorder occurs in a varying intra- and interfamilial degree of severity. Its exact prevalence is unknown, but with regard to Europe, MWS is listed in the Orphanet database of rare diseases. This presupposes that the Muckle-Wells syndrome and its subtypes affect less than 1 per 2,000 people. The age of MWS onset is variable, but the symptoms usually develop during childhood. Although genetic tests are feasible, the diagnosis is predominantly based on the clinical signs: MWS is characterized by urticarial or erythematous rashes, fever, musculoskeletal symptoms, conjunctivitis, and progressive sensorineural hearing loss. The severity resides in the inconstant appearance of a secondary systemic AA-amyloidosis. The levels of inflammation parameters are usually elevated in patients with MWS, particularly during the acute episodes.[32]

2. Aims of this study

The contribution of neutrophils, as dominant circulating human immune cell, to the production of IL-1 family cytokines remains poorly understood. Moreover, the modality of the neutrophil cytokine synthesis, processing, and secretion represents a controversial issue. Based on these open questions our project was focused in particular on the following subjects:

- To what extend are human neutrophils capable of secreting IL-1ß, IL-1α, IL-33, and IL-18? How high are the achieved interleukin concentrations in relation to the autologous PBMCs?
- Do the neutrophils produce cytokines in an inflammasome-dependent or serine protease-dependent manner?
- What are the effects of intrinsic inflammasome activation through *CIAS1* mutation on the cytokine production?
- Where is the intracellular localisation of the inflammasome in human neutrophils?

Since traditionally Ficoll density gradient isolated neutrophils are commonly contaminated with monocytes[62,73], we utilised negatively selected, highly purified neutrophils to assess their quantitative role in the generation of the investigated cytokines.

3. Materials

3.1 Chemicals, media, buffers, and reagents

Product name	Company; registered office
$ATPNa_2$	Sigma Aldrich Chemie; Munich, GER
Biocoll Separating Solution	Biochrom; Berlin, GER
BSA	Carl Roth, Karlsruhe; GER
Calcium Chloride	Sigma-Aldrich Chemie; Munich, GER
DNase I, RNase free (1 U/µl)	Biozym; Hessisch Oldendorf, GER
Dulbecco's PBS (1x)	PAA Laboratories; Cölbe, GER
EDTA	Sigma-Aldrich Chemie; Munich, GER
EGTA	Sigma-Aldrich Chemie; Munich, GER
FCS	Biochrom AG; Berlin, GER
GlutaMAX	Invitrogen; Karlsruhe, GER
Glycerol	Sigma-Aldrich Chemie; Munich, GER
Heparin-Natrium-25000	Ratiopharm; Ulm, GER
Hepes	Sigma-Aldrich Chemie; Munich, GER
Human Neutrophil Enrichment Kit	STEMCELL; Vancouver, CAN
Isopropanol	Merck; Darmstadt, GER
Magnesium chloride	Sigma-Aldrich Chemie; Munich, GER
Magnesium phosphate	Sigma-Aldrich Chemie; Munich, GER
Magnesium sulphate heptahydrate	Sigma-Aldrich Chemie; Munich, GER
NGS, heat inactivated	R&D Systems; Minneapolis, US
Penicillin-Streptomycin	Invitrogen; Karlsruhe, GER
Percoll	GE Healthcare; Buckinghamshire, UK
Phenylmethylsulfonyl fluoride	Sigma-Aldrich Chemie; Munich, GER
PIPES	Sigma-Aldrich Chemie; Munich, GER
Potassium chloride	Sigma-Aldrich Chemie; Munich, GER
Potassium dihydrogen phosphate	Sigma-Aldrich Chemie; Munich, GER
Protease Inhibitor Cocktail, Mini	Roche; Mannheim, GER
Roti-Nanoquant	Carl Roth; Karlsruhe, GER
RPMI 1640 medium	Biochrom; Berlin, GER

Product name	Company; registered office
Sodium bicarbonate	Sigma-Aldrich Chemie; Munich, GER
Sodium chloride	Sigma-Aldrich Chemie; Munich, GER
Sulphuric acid (2N)	Carl Roth, Karlsruhe; GER
Tween 20	Carl Roth; Karlsruhe, GER

3.2 Western blot

Product name	Company; registered office
Amersham Hyperfilm	GE Healthcare; Buckinghamshire, UK
ECL	GE Healthcare; Buckinghamshire, UK
iBlot 7-Minute Blotting System	Invitrogen; Karlsruhe; GER
iBlot Gel Transfer Stacks, PVDF	Invitrogen; Karlsruhe, GER
Novex Sharp Pre-stained Standard	Invitrogen; Karlsruhe, GER
NuPage 4 - 12 % Bis-Tris Gel	Invitrogen; Karlsruhe, GER
NuPage Antioxidant	Invitrogen; Karlsruhe, GER
NuPage LDS Sample Buffer	Invitrogen; Karlsruhe, GER
NuPage MES SDS Running Buffer	Invitrogen; Karlsruhe, GER
NuPage Reducing Agent	Invitrogen; Karlsruhe, GER
PowerEase 500	Invitrogen; Karlsruhe; GER
Slimfast Vanille	Allpharm; Messel, GER
xCell SureLock Electrophoresis cell	Invitrogen; Karlsruhe, GER

3.3 Antibodies

Product name	Company; registered office
Anti NALP3 (mouse, monoclonal)	Abcam; Cambridge, UK
Anti Caspase-1 (rabbit, polyclonal)	Abcam; Cambridge, UK
Rabbit anti Goat, HRP conjugate	Millipore; Massachusetts, US
Goat anti Rabbit, HRP conjugate	Dako; Glostrup, DK
Goat anti Mouse, HRP conjugate	Dako; Glostrup, DK
Anti ß2M (rabbit polyclonal)	Dako; Glostrup, DK
Anti HLA ABC(mouse, monoclonal)	Dako; Glostrup, DK
Anti MICA (rabbit, polyclonal)	Abcam; Cambridge, UK

3.4 ELISA

Product name	Company; registered office
Human Albumin ELISA Kit	ICL; Portland, US
Human Lipocalin-2/NGAL DuoSet	R&D Systems; Minneapolis, US
Human Myeloperoxidase DuoSet	R&D Systems; Minneapolis, US
Human IL-18 ELISA Kit	MBL Woburn; US
Human MMP-9 DuoSet	R&D Systems; Minneapolis, US
Human IL-1 beta/IL-1F2 DuoSet	R&D Systems; Minneapolis, US
Human IL-1α ELISA Set	Hologic; Massachusetts, US

3.5 Prepared solutions, buffers, and media

Solution/Buffer/Medium	Composition
cRPMI	RPMI 1640, 10 % FCS, 10 mM Penicillin-Streptomycin, 10 mM GlutaMax
Disruption buffer (10X)	100 mM KCL, 3 mM NaCl, 3,5 mM $MgCl_2$, 10 mM PIPES, 0,5 mM PMSF, 50 mM $ATPNa_2$ (pH 7,2)
EasySep recommended medium	PBS, 2 % FCS, 1 mM EDTA
ELISA reagent diluent	PBS, 1% BSA (0.2 µm filtered)
ELISA wash buffer	PBS, 0,05 % Tween 20
Krebs-Ringer solution	115 mM NaCl, 4,7 mM KCL, 2,56 mM $CaCl_2$, 1,2 mM KH_2PO_4, 1,2 mM $MgSO_4$ x 7 H_2O, 20 mM $NaHCO_3$, 16 mM Hepes (pH 7,2)

Solution/Buffer/Medium	Composition
Lysing buffer (Universitäts-Apotheke Tübingen)	1000 ml H_2O, 8,29 g NH_4Cl, 1 g $KHCO_3$, 0,0372 g Na_2-EDTA-$2H_2O$
PBS-T	PBS, 0,05 % Tween 20
Relaxation buffer	100 mM KCL, 3 mM NaCl, 3,5 mM $MgCl_2$, 12,5 mM EGTA, 10 mM Piperazine (pH 7,2)
Western blot blocking buffer	PBS-T, 3 % Slimfast Vanille

3.6 Stimulants and inhibitors

Product name	Company; registered office
ATP	Sigma Aldrich Chemie; Munich, GER
LPS	Sigma Aldrich Chemie; Munich, GER
Nigericin sodium salt (5 $^{mg}/_{ml}$)	Sigma Aldrich Chemie; Munich, GER
PMSF	Sigma Aldrich Chemie; Munich, GER
Z-VAD-FMK	Enzo Life Sciences; Lörrach, GER

3.7 Instrumental and electronic equipment

Product name	Company; registered office
Analytical balances BP121S	Sartorius; Göttingen, GER
Blockthermostat TCR 100	Roth; Karlsruhe, GER
Centrifuge 5430 R	Eppendorf; Hamburg, GER
Exposure cassette	GE Healthcare; Buckinghamshire, UK
FACS Calibur	BD Bioscience; New Jersey, US
Heraeus Megafuge 16R	Thermo Scientific; Massachusetts, US
Inkubator Hera Cell	Thermo Scientific; Massachusetts, US
Magnetic stirrer	Neolab; Heidelberg, GER
Microscope Olympus IX50	Olympus; Hamburg, GER

Product name	Company; registered office
Photometer Victor 1420	PerkinElmer; Rodgau, GER
Pressure chamber	PARR; Illinois, US
GraphPad Prism 5	Graph Pad Software
Roller Cat RM 5	Neolab; Heidelberg, GER
Safety-Multifly	Sarstedt; Nümbrecht, GER
Silver EasySep Magnet	STEMCELL; Vancouver, CAN
Sterile Filter 0,2 µm	Merck; Darmstadt, GER

4. Methods

4.1 Isolation of PBMCs and neutrophil granulocytes

4.1.1 Ficoll-Hypaque density gradient centrifugation

The density gradient centrifugation is a standard method to segregate different blood cells in a gentle and quick way. Therefore, a Ficoll separating solution is used, which conventionally contains a synthetic sucrose-epichlorhydrin copolymer with a specific density of 1.077 g/ml. The anticoagulated whole blood is carefully layered above the Ficoll and is separated during a centrifugation step. The blood cells with a higher density than 1.077 g/ml, such as granulocytes and erythrocytes, pass through the Ficoll layer and sediment on the bottom. The cells with a lower density cluster in the interphase above the Ficoll. These cells are called the peripheral blood mononuclear cells (PBMCs). The PBMC group consists of macrophages, monocytes, and lymphocytes. Plasma, which has the lowest density of all blood components, can be found at the top of all layers, above the PBMC-ring.

For cell isolation, one part of the Ficoll separating solution was given into a conical reaction tube and 1.5 parts of undiluted heparinized whole blood was slowly layered above. The tube was centrifuged at 400 x g for 35 minutes at 20 °C. To keep the components from intermixing, the acceleration and deceleration were turned down.

4.1.2 PBMC and plasma isolation

After the density gradient centrifugation, one millilitre of the plasma was collected and stored at -20 °C. The PBMC-ring was aspirated with a Pasteur pipette and transferred into a new tube. In order to remove remaining Ficoll solution, we washed the cells twice with RPMI medium. This was important for preventing cell toxic or cell stimulating effects of the Ficoll solution, resulting from prolonged cell exposure. The centrifugation steps were conducted for 10 minutes at 300 x g. Subsequently, the cells were resuspended and the cell count was determined.

4.1.3 Isolation of neutrophil granulocytes

4.1.3.1 Erythrocyte lysis

After the discharge of the Ficoll plaque the polymorphonuclear cells remained in the tube together with the erythrocytes. The erythrocytes were subjected to a cell lysis with an ammonium chloride-based reagent. The cells were resuspended with the precooled lysing buffer and incubated for 20 minutes at 4 °C. Each incubation was followed by a centrifugation step at 400 g for 10 minutes. Subsequently the PMNs could be resuspended and the cell count was determined.

4.1.3.2 Immunomagnetic separation of neutrophils with EasySep

The immunomagnetic cell separation method was used to get highly purified neutrophil granulocytes. In particular, we wanted to remove contaminating monocytes, as they would have falsified the subsequent experiments. Furthermore, the isolation method with EasySep was chosen since it is based on a negative selection. The advantage of a negative immunological separation, compared to a positive selection technique, is that the required cells are not antibody-labelled. Consequently, the exposure to potential activating agents is reduced and the natural behaviour of the cells is less influenced.[18]

The cells were adjusted in the recommended medium to a density of approximately 50 million cells per millilitre and 50 µL EasySep Enrichment Cocktail per ml cells were added. This is an antibody cocktail containing antibodies directed against cell surface markers of the unwanted cells: CD2, CD3, CD9, CD19, CD36, CD56, and glycophorin A. Thus, contaminating T and B cells, platelets, erythrocytes, monocytes, and NK-cells were tagged. Additionally, the Fc region of these antibodies bound specifically to dextran. This property was used in the next step, when 100 µl dextran encased ferrous particles per ml cells were added. After 10 minutes of incubation, the polystyrene tube, containing the tetrameric complexes composed of negative selected cell, antibody, dextran, and the ferrous particle, was places in the EasySep magnet. The magnetically labelled cells were attracted to the wall of the reaction vessel and held there by magnetic attraction. At this stage, the suspension containing the desired and still freely movable cells could be poured into a new tube. This separation in the magnet was repeated for a second time. Subsequently, we centrifuged the cell suspension at

350 x g for 10 minutes and resuspended the purified neutrophil granulocytes in the medium required for the next step.

4.1.4 Adjustment of cell densities

A small aliquot of the cell suspension was further diluted (e.g. 5 µl sample plus 995 µl RPMI medium) and 10 µl of this dilution were applied into a Neubauer chamber beneath the cover-slip. At least four large squares were counted and the mean value was calculated. The cell density was estimated with the following computational function:
A*verage count per large square* x *Dilution factor* x 10^4.

4.2 Subcellular fractionation of neutrophil granulocytes

(Method originally described by Borregaard, 1999[41])

The subcellular fractionation is a pivotal method in the investigation of neutrophils' subcellular organisation and granule composition. The granules (azurophilic/primary, specific/secondary, and gelatinase/tertiary), the secretory vesicles, the plasma membrane, as well as the cytosol can be separated by this method and the resulting single components can be further characterised. We worked with nitrogen cavitation followed by a three- and a four-layer Percoll density gradient. All steps were performed at 4 °C with precooled buffers and equipment.

4.2.1 Whole blood collection

500 ml blood was collected from healthy volunteers by antecubital venipuncture after informed consent and approval of the study by the ethics committee of the University of Tübingen. EDTA was used for anticoagulation. None of the donors had any known acute or chronic infections.

4.2.2 Nitrogen cavitation

For cell lysis we used the nitrogen cavitation method, which is an effective and indulgent tool for cell disruption. An important positive aspect is the preservation of the granule integrity during the whole process. Previous to the lysis, the highly purified neutrophils were suspended in 5 ml isotonic Krebs-Ringer-phosphate solution and incubated for 5 minutes. The supernatant was discharged after a 10 minute

centrifugation step at 200 x g and the pellet was resuspended in disruption buffer which additionally contained a mixture of different protease inhibitors. The resulting cell suspension was placed under high nitrogen pressure in an apparatus called 'cell bomb'. This pressure was released rapidly after a certain time. Whenever a three-layer Percoll density gradient was planned subsequent to nitrogen cavitation, the cells were kept at 380 psi for 10 minutes in the cell bomb. The cells intended to be used for a four-layer gradient were treated for 5 minutes at 600 psi. Different theories exist concerning the mechanism leading to the cell lysis: one explanation is that the nitrogen, dissolved in the cells' liquid phase under high pressure, bubbles out of solution and this results in cell disruption. Another possible explanation is that cell lysis is caused by shear stress during the release of the cell suspension through the cell bomb's outlet nozzle.[8] Probably both mechanisms contribute to the cell disruption. After nitrogen cavitation, we added EGTA (1.5 mM) to the homogenate to avoid aggregation of granules. Cell nuclei and non-disrupted cells were pelletized during a 15 minute centrifugation step at 400 x g. The resulting post-nuclear supernatant was used for the following fractionation on the Percoll density gradients.

4.2.3 Percoll density gradient

Percoll is a density separating medium, containing silica particles coated with PVP. An advantage of Percoll is its low viscosity, resulting in a good gradient stability and membrane integrity. Additionally, the osmolarity of Percoll is minimal and no shrinking effect of the organelles occurs. Beyond that, it is non-toxic towards cells or their constituents.[85]

We generated three- and four-layer gradients by layering progressively less dense Percoll solutions upon one another. The different cell components migrate down the gradient, until they reach a point where their density matches to that of the Percoll. After centrifugation, four distinct bands containing the different subcellular components, were visible: the lowest band contained azurophilic granules, followed by bands containing the specific granules, the gelatinase granules, the secretory vesicles, and the cytosol (see figure 4). The gain of a three-layer gradient was the presence of the cytosol as a single fraction. However, the plasma membrane and the secretory vesicles were found in one,

assorted fraction in the three-layer gradient. The secretory vesicles were dividable in the four-layer gradient, but the cytosol was mixed with the plasma membranes. One possibility to acquire every fraction separately is to combine the results of the two gradients. In order to simplify the procedure, we tried to separate the cytosol and the plasma membrane by measuring the MHC class I chain-related molecule A (MICA) in the upper regions of a four-layer gradient by western blot analyses.

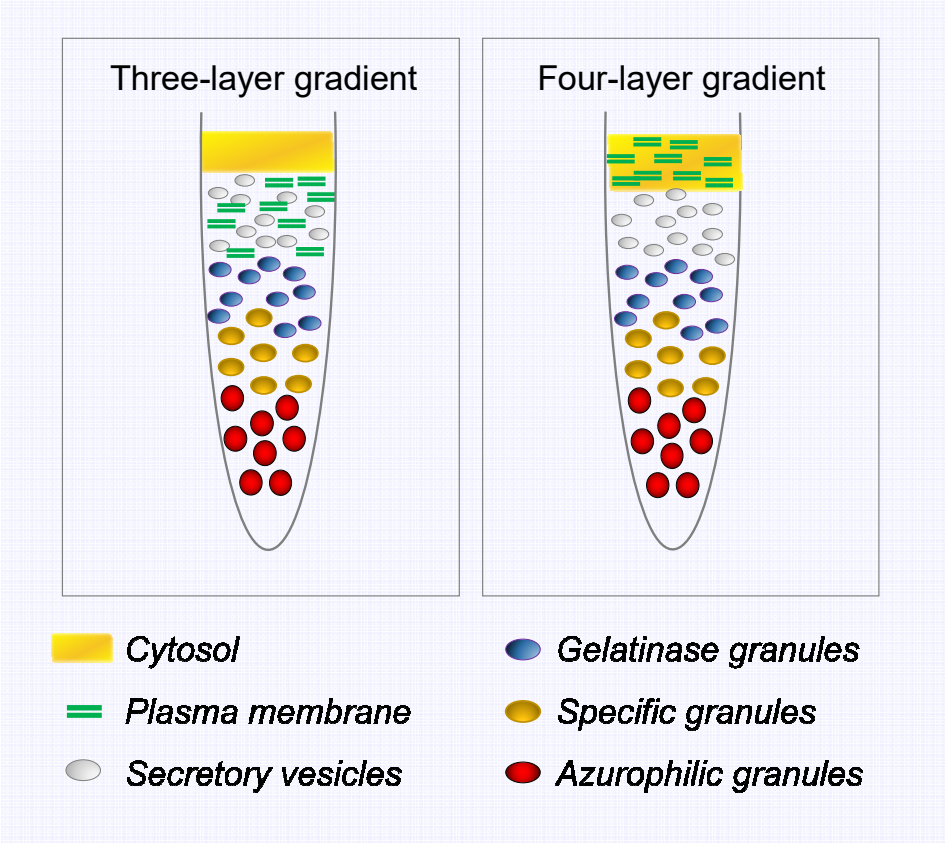

Figure 4: Three- and four-layer Percoll density gradients after centrifugation. The post-nuclear supernatant of nitrogen-cavitated neutrophils was layered on top of the density gradients. The subcellular components resolved into four distinct bands during the centrifugation step at 20,000 x g for 40 minutes. The lowermost band contained the azurophilic granules, followed by bands containing specific granules, gelatinase granules, secretory vesicles, and the cytosol. The three- and the four-layer gradients differed only in the localisation of the plasma membranes: within the three-layer gradient, the plasma membranes formed one fraction together with the secretory vesicles, whereas in the four-layer gradient they were colocalised with the cytosol. Therefore, the three-layer gradient was used to characterise cytosolic proteins, while the four-layer gradient was applied in order to discriminate secretory vesicle proteins from those in the plasma membrane.

4.2.3.1 Preparation of the Percoll solutions

The Percoll stock solution was mixed with 10-fold concentrated relaxing buffer and distilled water. The required volume of the Percoll stock solution was calculated using the following formula: $V_P = \frac{V_0 (D_0 - 1.006)}{(D_P - 1)}$.

V_P = Volume of the Percoll stock solution

V_o = Final volume of the Percoll solution

D_o = Desired density of the Percoll solution

D_P = Density of the Percoll stock solution

1.006 = Density of the 10-fold concentrated relaxing buffer in g/ml

4.2.3.2 Three-layer Percoll gradient

Three different Percoll density solutions, each with a volume of 30 ml and a pH of 7.0, were prepared as shown in table 2. Nine millilitres of the 1.050 g/ml solution were given into an ultracentrifuge tube. Through a lumbar puncture needle, nine millilitres of the 1.090 g/ml solution were gently layered under the 1.050 g/ml solution. Finally, nine millilitres of the 1.12 g/ml solution were brought to the bottommost position and ten millilitres of the post-nuclear supernatant were applied on the top of the Percoll gradient. After centrifugation at 20,000 x g for 40 minutes four bands, containing the different subcellular structures, were distinguishable (see figure 4).

Table 2: Preparation of Percoll solutions of different densities (three-layer)

Percoll stock solution [ml]	Relaxation buffer (10 X) [ml]	H_20 [ml]	Final density [g/ml]
10.27	3	16.73	1.050
19.58	3	7.42	1.090
26.55	3	0.45	1.12

4.2.3.3 Four-layer Percoll gradient

Four different density solutions, each with a volume of 10 ml and a pH of 7.0, were prepared as shown in table 3. The supernatant, resulting from the centrifugation step after nitrogen cavitation, was diluted 1 + 1 with the 1.11 g/ml Percoll solution. The

resulting mixture had a density of 1.055 g/ml. Nine millilitres of the 1.03 g/ml solution were given in the centrifugation tube and was underlayered with nine millilitres of the prepared sample-Percoll mixture. Again, nine millilitres of the 1.09 g/ml Percoll were given under the resulting liquid layers. In the last step, nine millilitres of the solution with the highest density were brought to the bottom of the ultracentrifuge tube. All underlying steps were performed with a lumbar puncture needle. Like the three-layer gradient, the centrifugation took 40 minutes at 20,000 x g by turned down deceleration and acceleration.

Table 3: Preparation of Percoll solutions of different densities (four-layer)

Percoll stock solution [ml]	Relaxation buffer (10 X) [ml]	H_20 [ml]	Final density [g/ml]
1.86	1	7.14	1.03
6.44	1	2.66	1.09
7.96	1	1.04	1.11
8.73	1	0.27	1.12

4.2.4 Fractionation and removal of Percoll

The fractions were collected millilitre by millilitre, whereby every millilitre represented a single fraction. In the end, 36 fractions were originated. Since the remaining Percoll caused a problem in western blotting, we removed it by ultracentrifugation. After this centrifugation step at 100,000 x g for 45 minutes, the Percoll formed a hard pellet at the bottom, whereas the fractions were found above the Percoll and could be collected. After the addition of 10 % glycerol, the fractions were stored at -20 °C.

4.3 Analysis of subcellular marker proteins

The 36 fractions had to be summarized to the final fractions, optimally containing only one single type of the desired cell organelles. Using the ELISA technique, we detected specific marker proteins to localise the organelles within the gradient. The exact borders had to be determined individually for every gradient, since they varied among different experiments and donors. Myeloperoxidase was used as a marker for azurophilic granules, neutrophil gelatinase-associated lipocalin (NGAL) for specific granules, HLA for the plasma membrane, and the gelatinase granules were defined by their high

content of matrix metalloproteinase 9 (MMP-9). The secretory vesicles were localized by the detection of albumin. The frozen fractions were thawed on ice and all ELISAs were run in duplicates.

4.4 Western blot

4.4.1 SDS-PAGE

The sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) makes it possible to separate proteins in an electric field according to their molecular mass. In order to eliminate charge differences, the proteins were boiled in sample loading buffer containing SDS. SDS is an anionic detergent, which binds uniformly along polypeptide chains and masks the proteins' intrinsic charge. This leads to an approximately constant net charge per unit mass. Additionally, we added a reducing reagent to the loading buffer to ensure a better unfolding of the proteins.[88] Beside its purpose in sample preparation, the loading buffer becomes important during the loading process: it weighs down the samples and prevents them from floating in the buffer solution. Secondly, the sample buffer contains a dye, which moves faster than the proteins and helps to stop the electrophoresis in time. We began the sample preparation with a DNase I treatment. Therefore, 5 µl of DNase I were added to a sample volume of 50 µl and the mixture was incubated for 30 minutes at room temperature. Afterwards, 6 µl of NuPage reducing agent and 24 µl NuPage sample buffer were added. The samples were kept for 20 minutes at 60 °C in the heating block. We loaded 20 µl samples and 8 µl prestained standard into the wells and run the electrophoresis at 200 volt for 35 minutes. We chose a discontinuous 4 - 12 % polyacrylamide gel for the separation. The gel's section with the lower percentage acts as a stacking gel. It has a large pore size and it is slightly acidic. The lower gel, called the separating or resolving gel, is more basic and has the higher polyacrylamide content. The purpose of the stacking gel is to concentrate the samples into a tight band during the first minutes of the electrophoresis. The glycine of the tris-bis buffer forms a zwitterion (trailing ion) in the low pH of the stacking gel. The chloride ion, which is mobile in both gels (leading ion), moves faster than the glycine and the electric field strength between glycine and chloride forces the SDS surrounded proteins towards the resolving gel. When the glycine molecules enter the alkaline resolving gel, their amphoteric character turns into anionic. This causes an increase of

mobility and the glycine overtakes the proteins. Now, the proteins are the main carrier of the electric current and get separated in the fine-meshed resolving gel according to their molecular mass.[87,88] The molecular weight of the proteins was estimated by comparison of their electrophoretic migration to a standard of known molecular weight.

4.4.2 Transfer of the proteins

After the length-dependent gel electrophoretic separation of the denatured proteins, we blotted (transferred) those from the polyacrylamide gel onto a PVDF membrane. We used the 'iBlot dry Blotting System', which works with an electric field and transfers the proteins within 6 minutes onto the membrane.

4.4.3 Blocking and detection

Non-specific binding sites were blocked by immersing the membrane in PBS-T supplemented with 5 % non-fat dried milk for three hours at room temperature. Afterwards, we washed three times for 5 minutes in PBS-T. The specific primary antibodies were diluted in PBS-T with 3 % non-fat dried milk and incubated overnight at 4 °C. In order to remove any unbound antibodies, the membrane was washed in PBS-T (4 x 10 minutes). In the second step of detection, a species-specific and HRP conjugated secondary antibody, diluted in PBS-T, was incubated for 2 hours. After four washing steps, we gave a luminol-containing ECL western blotting detection reagent on the membrane. HRP oxidises the luminol and brings it to an excited state. Luminol decays to ground state via light emitting. The light emission was detected by a photographic film. The exposure times varied from 30 seconds to 12 hours.

4.5 Inflammasome *in vitro* stimulation

4.5.1 Patients and controls

Blood was obtained after informed consent and approval of the study by the ethics committee of the University of Tübingen. Four male Muckle–Wells syndrome (MWS) patients (mean age: 31 years) were studied. All patients had a classic clinical presentation of MWS. Control blood samples were obtained from healthy volunteers (n=13, mean age: 27 years). Since substantial interdonor variabilities in the IL-1β production and effects of even mild infections and allergies were already observed in

previous experiments, we used control subjects without known infections, inflammatory disorders, allergies, or recent medication history. Fresh whole blood was drawn from both, patients and controls, by antecubital venipuncture in heparinized plastic tubes at a standardized daytime. Since the obtained cell amounts were limited, it was not possible to run all subsequent assays for each donor. As a consequence, the cytokine measurements varied concerning their number of analysed samples.

4.5.2 Stimulation of neutrophil granulocytes

The neutrophil granulocytes were isolated from venous whole blood in a standardised manner and with great caution to prevent unspecific activation. The experiments were performed according to a previously published protocol for inflammasome stimulation[26]. We stimulated highly purified neutrophils as well as traditional Ficoll isolated neutrophils. Initially, the suspension with the isolated cells was centrifuged at 350 x g for 10 minutes and the pellet was resuspended in cRPMI medium. The cells were cultivated in 1.5 ml Eppendorf vials at a density of 20 x 10^6 cells per millilitre. This cell density was chosen since correspondingly high concentrations reduce the constitutive apoptotic rate of neutrophils.[4] The neutrophils were stimulated with LPS (10 $^{ng}/_{ml}$, diluted in cRPMI) for 4 hours at 37 °C and 5 % CO_2. We always included a negative control to which we added pure cRPMI medium (see table 4). Subsequently, we stimulated the cells with ATP (5 mM) or nigericin (5 µM) for another 2 hours. In order to study the effects of the stimulants on their own, we also had approaches without the previous LPS treatment. Correspondingly, we performed stimulations only with LPS. After an altogether incubation time of 6 hours, the vials were centrifuged at 350 x g for 10 minutes at 4 °C. The cell free supernatants were frozen at -20 °C until required for further processing. To enhance protein stability, we added 10 % glycerol and a mixture of protease inhibitors to the samples.

Table 4: Stimulation procedure

	First stimulation (4 h)	Second stimulation (2 h)
Control	Medium	Medium
1.	LPS	ATP
2.	LPS	Nigericin
3.	Medium	LPS
4.	Medium	ATP
5.	Medium	Nigericin

4.5.3 Stimulation of PBMCs

PBMCs were separated by Ficoll density centrifugation and washed twice in RPMI medium. The pellet was resuspended in cRPMI and the cell count was determined. Unlike the neutrophils, the PBMCs were brought to a cell density of one million per millilitre. The subsequent stimulation procedure was similar to the one described for the neutrophil granulocytes (see table 4).

4.5.4 Stimulation under inhibited conditions

Since serine proteases are abundantly present in human neutrophils and have been shown to cleave some precursors of interleukin-1 family members[17,37,76,86], we analysed the contribution of the serine proteases to the generation of IL1ß, -1α, -18, and -33 in highly purified neutrophils compared to autologous PBMCs by adding the serine protease inhibitor PMSF. In parallel, we used the pan-caspase inhibitor Z-VAD-FMK to block the proteolytic activity especially of caspase-1. Neutrophils and PBMCs were prepared as described. We employed 50 µM Z-VAD-FMK and 1 mM PMSF and added the inhibitors prior to the LPS stimulation. To make the results better comparable we also stimulated simultaneously without inhibitors.

Table 5: Stimulation procedure with inhibitors

	First stimulation (4 h)	Second stimulation (2 h)
Control	Medium	Medium
1.	LPS	ATP
2.	LPS	Nigericin
4.	Z-VAD-FMK + LPS	ATP
5.	Z-VAD-FMK + LPS	Nigericin
7.	PMSF + LPS	ATP
8.	PMSF + LPS	Nigericin

4.5.5 Quantification of the interleukin production

The interleukin production of the neutrophils and PBMCs during the stimulations was quantified using ELISA technique. Therefore, we analysed the supernatants, which were separated from the cells directly after the six hours of stimulation.

4.6 ELISA

All our performed enzyme-linked immunosorbent assays (ELISAs) were based on the antibody sandwich technique. Here, the capture antibody, specific to the analyte of interest, is immobilized to the solid surface of the microtitre plate. The non-specific binding sites were blocked with a BSA-containing reagent. During sample incubation, the antigens were binding to the immobilized capture-antibodies. After any unbound analyte was removed, the biotinylated detection antibody completed the sandwich by binding to a different epitope of the measured protein. Following a further washing step, streptavidin-HRP was added. HRP, which bound via streptavidin-biotin interaction, converted a chromogenic substrate into a blue colour product. The progressive colour development was stopped by the addition of an acid, which turned the colour from blue to yellow. The intensity of the produced colour was directly proportional to the concentration of the measured protein.

The described principle was employed by the DuoSet ELISAs, which we most commonly used. The IL-18, IL-1α, and the albumin ELISA utilized the same

mechanisms but in slightly modified way. The degree of sample dilution (undiluted up to a dilution of 1:50,000) depended on the detected protein but also varied within different experiments. To prevent biased results, we applied samples, standards, and all reagents simultaneously with a multichannel pipette. Between each step, the ELISA plates were washed four times in wash buffer (4 x 400 µl). The buffer was poured out over a sink and the plates were tapped against paper towels to remove remaining drops. All our ELISAs worked with HRP and the visualising reagent TMB. Thus, optical density was measured at a wavelength of 450 nm. The wavelength correction was set to 570 nm. We averaged the duplicate readings for standards as well as for the samples and subtracted the average zero. A four parameter standard curve was generated for each set of samples. All steps were performed at room temperature.

4.6.1 DuoSet ELISAs: NGAL, MMP-9, MPO, IL-1ß, and IL-33

A 96-well microplate was coated during an overnight incubation with 100 µl capture antibody. After blocking with 300 µl reagent diluent, 100 µl sample or standard were given into the wells. After two hours of incubation unbound substances were removed. 100 µl of the diluted detection antibodies were incubated for another two hours. The streptavidin-HRP solution (100 µl) was incubated for 20 minutes. After a final washing step, 100 µl substrate solution were given on the plate. We stopped the reaction after 20 minutes with 50 µl stop solution.

4.6.2 Albumin and IL-18 ELISAs

The monoclonal capture antibody was pre-coated onto the provided microplate. 100 µl sample or standard were given respective the plate layout into the wells. After one hour of incubation the plate was washed. Subsequently, 100 µl of the HRP conjugated detection antibodies were given into each well. After the incubation time (albumin: 30 minutes; IL-18: 60 minutes) a wash step was performed. Bound HRP was assayed by addition of 100 µl TMB. We stopped the reaction after 10 minutes (albumin ELISA) or after 30 minutes (IL-18 ELISA) with 100 µl stop solution.

4.6.3 IL-1α ELISA

The IL-1α ELISA was similar to the performed procedure of the DuoSet ELISAs, only some incubation periods differed: the streptavidin-HRP solution was incubated for 30 minutes and the TMB substrate for 10 minutes.

4.6.4 HLA ELISA

The plate was coated with rabbit anti-human ß$_2$-microglobulin antibodies (dilution: 1:1000) during an overnight incubation step. Mouse anti-human HLA-ABC was used in a dilution of 1:750 as primary detection antibodies, anti-mouse HRP-labelled antibodies (dilution 1:1500) as secondary detection antibodies. We did not use standards for this ELISA; we analysed the optical density values instead. The remaining procedure was handled like described for the DuoSet ELISAs.

4.7 Determination of total protein concentrations

For the quantitative measurement of the fractions' total protein we used the Bradford method. Here, the dye Coomassie Brilliant Blue forms complexes with proteins, leading to a conversion from reddish to a blue colour. The absorption maximum shifts from 465 nm towards longer wavelengths. The increased absorbance at 595 nm is proportional to the amount of bound Coomassie blue and by association to the protein concentration in the solution. For every series of measurements, we created a new standard curve. It involved six points from blank up to 20 $^{\mu g}/_{ml}$ BSA. For the protein detection, we added 200 µl reagent to 80 µl of standard or diluted sample, mixed thoroughly, and incubated for 5 minutes at room temperature. The ratio of the absorbance, 595 nm over 450 nm, was calculated and the amount of protein was determined in relation to the standards.

4.8 Statistical analysis

Data are expressed as mean ± SEM. Statistical significance of differences was determined by one-way ANOVA followed by Bonferroni's post-hoc test. In all tests $P < 0.05$ was considered to be statistically significant. The significant levels were denoted with asterisks: $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (***).

5. Results

5.1 IL-1ß concentrations in cell culture supernatants

5.1.1 IL-1ß secreted by neutrophils from healthy donors

Blood was collected from healthy volunteers and the purified neutrophil granulocytes were stimulated according to the two-hit theory of inflammasome activation. These double stimulated cells increased their IL-1ß production highly significant in comparison to the negative, unstimulated controls (see figure 5). The average concentration of IL-1ß in supernatants derived from neutrophils treated with LPS + ATP was 14 $^{pg}/_{ml}$. LPS/nigericin treatment had the largest effect on the IL-1ß production: the supernatants obtained from these cells showed an average IL-1ß concentration of 22 $^{pg}/_{ml}$. Neither ATP, nor nigericin or LPS alone raised the IL-1ß production as observed in the double stimulations; the IL-1ß concentrations were considerably lower here. The smallest IL-1ß levels were secreted in response to the nigericin stimulation.

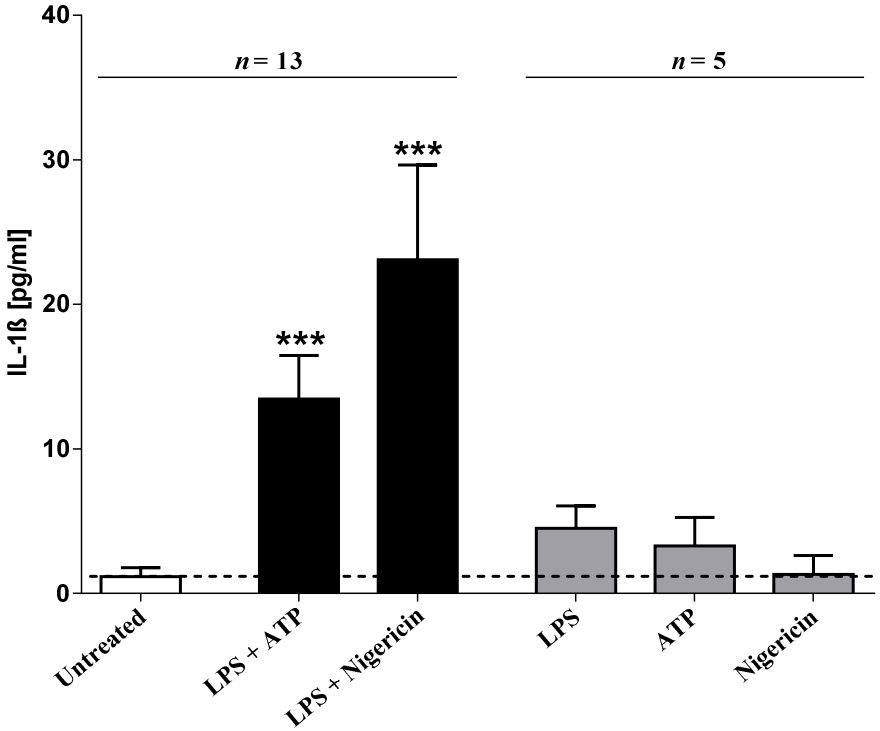

Figure 5: IL-1ß secreted by neutrophils from healthy donors. Neutrophil granulocytes were isolated from healthy donors and stimulated with LPS + ATP and with LPS + nigericin (black bars) as well as with LPS, ATP, or nigericin alone (grey bars). The amount of released IL-1ß after an altogether incubation time of 6 hours was determined using ELISA technique. Concentrations are given as $^{pg}/_{ml}$ (mean ± SEM).

5.1.2 IL-1ß secreted by healthy neutrophils under inhibited conditions

The study was performed to investigate the involvement of inflammasomes and serine proteases in the maturation of IL-1ß in neutrophils. To gain a better understanding about the mechanisms, we stimulated under addition of Z-VAD-FMK and PMSF. The blood was collected from three healthy donors. Again, as in experiment 5.1.1, the IL-1ß concentrations were significantly increased after stimulation with LPS + ATP or LPS + nigericin. Both inhibitors, PMSF as well as Z-VAD-FMK, affected the IL-1ß production (see figure 6). The LPS/ATP-induced IL-1ß secretion was reduces to one-third by Z-VAD-FMK; PMSF decreased the IL-1ß production to one quarter. The LPS/nigericin stimulated cells produced on average one-third less under serine protease inhibition, whereas the LPS/nigericin-induced IL-1ß secretion stopped almost completely under caspase inhibition.

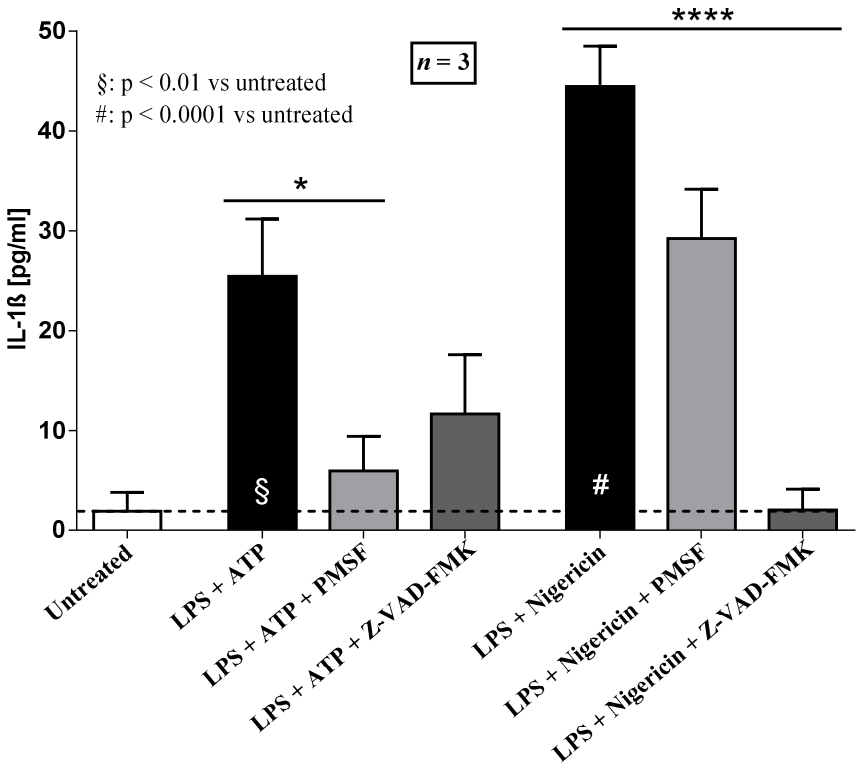

Figure 6: IL-1ß secreted by healthy neutrophils under inhibited conditions. Neutrophils were stimulated with LPS + ATP or LPS + nigericin (black bars) with or without pretreatment with a serine protease inhibitor (PMSF, 1 mM; light grey bars) or a caspase inhibitor (Z-VAD-FMK, 50 µM; dark grey bars). The amount of IL-1ß produced and released by the cells was measured using ELISA technique. Concentrations are given as $^{pg}/_{ml}$ (mean ± SEM).

5.1.3 IL-1ß secreted by neutrophils from MWS patients

To compare the IL-1ß production levels of healthy neutrophils with *CIAS1* mutated cells, we stimulated highly purified neutrophil granulocytes of 13 healthy donors and four MWS patients. Both groups increased the IL-1ß production in response to LPS/ATP stimulation as well as to the LPS/nigericin treatment. However, the *CIAS1* mutated cells secreted in total less IL-1ß upon stimulation than the healthy cells (see figure 7). Treatment with LPS/nigericin had the largest impact on the IL-1ß production in both batches: the MWS neutrophils produced on average 12 $^{pg}/_{ml}$ of IL-1ß (healthy control cells: 23 $^{pg}/_{ml}$). LPS/ATP showed lower effects: the average concentration achieved by MWS neutrophils was 7 $^{pg}/_{ml}$; the healthy cells produced 13 $^{pg}/_{ml}$. In contrast to these finding were the results found in the untreated controls of both groups: here, the MWS neutrophils produced about twice as much IL-1ß than the healthy cells did.

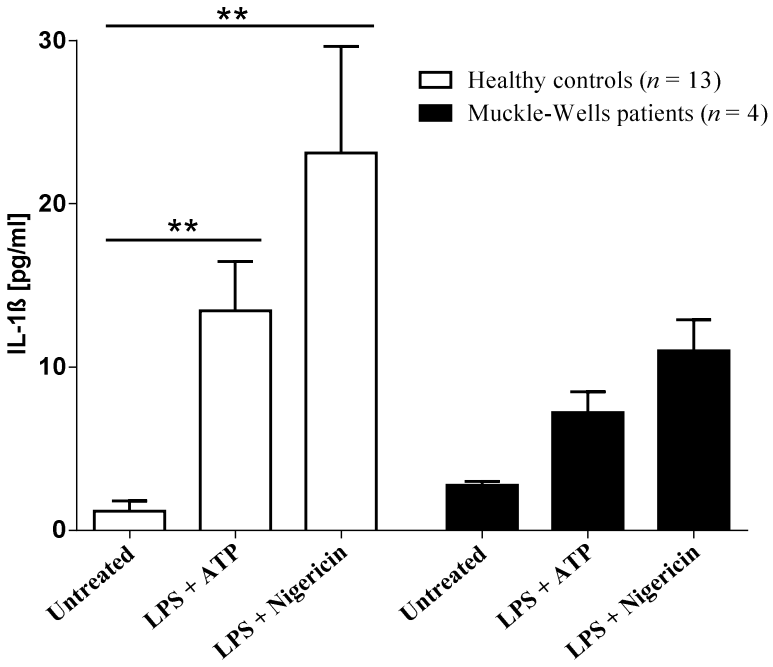

Figure 7: IL-1ß secreted by neutrophils from MWS patients. Neutrophils, isolated from 13 healthy donors (white bars) and from four MWS patients (black bars), were stimulated with LPS + ATP and with LPS + nigericin. The amount of released IL-1ß was determined using ELISA technique. Concentrations are given as $^{pg}/_{ml}$ (mean ± SEM).

5.1.4 IL-1ß secreted by PBMCs from healthy donors

We stimulated PBMCs of healthy donors with LPS followed by ATP or nigericin. These double stimulated cells increased their IL-1ß secretion highly significant in comparison to the negative, unstimulated controls. The average IL-1ß concentration in supernatants after LPS/ATP stimulation was 8,750 $^{pg}/_{ml}$. The supernatants obtained from cells stimulated with LPS + nigericin contained about 10,000 $^{pg}/_{ml}$ IL-1ß. Neither ATP, nor nigericin or LPS alone increased the IL-1ß production: the detected IL-1ß concentrations were comparable to the ones found in the supernatants of the unstimulated cells (see figure 8).

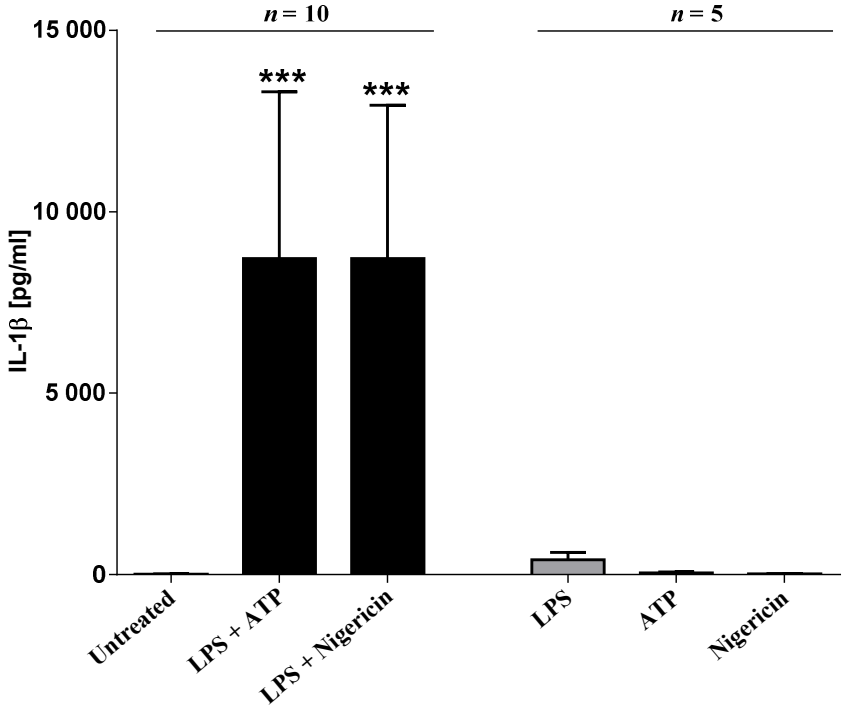

Figure 8: IL-1ß secreted by PBMCs from healthy donors. PBMCs were isolated from healthy donors and stimulated with LPS + ATP, or LPS + nigericin, (black bars) as well as with LPS, ATP, or nigericin alone (grey bars). The IL-1ß concentrations are given as $^{pg}/_{ml}$ *(mean ± SEM) and were determined by ELISA technique.*

5.1.5 IL-1ß secreted by healthy PBMCs under inhibited conditions

The PBMC fraction was isolated from three healthy donors and stimulated under different conditions. The cells increased their IL-1ß secretion in response to stimulation with LPS + ATP as well as to LPS + nigericin. The presence of the serine protease inhibitor PMSF had no inhibitory influence on the production; the concentrations were comparable to the ones found in the analogues stimulations without the inhibitor,

whereas the IL-1ß synthesis stopped completely under caspase inhibition (see figure 9). The addition of Z-VAD-FMK tended to result in even lower cytokine concentrations than the ones found in the supernatants of the untreated cells.

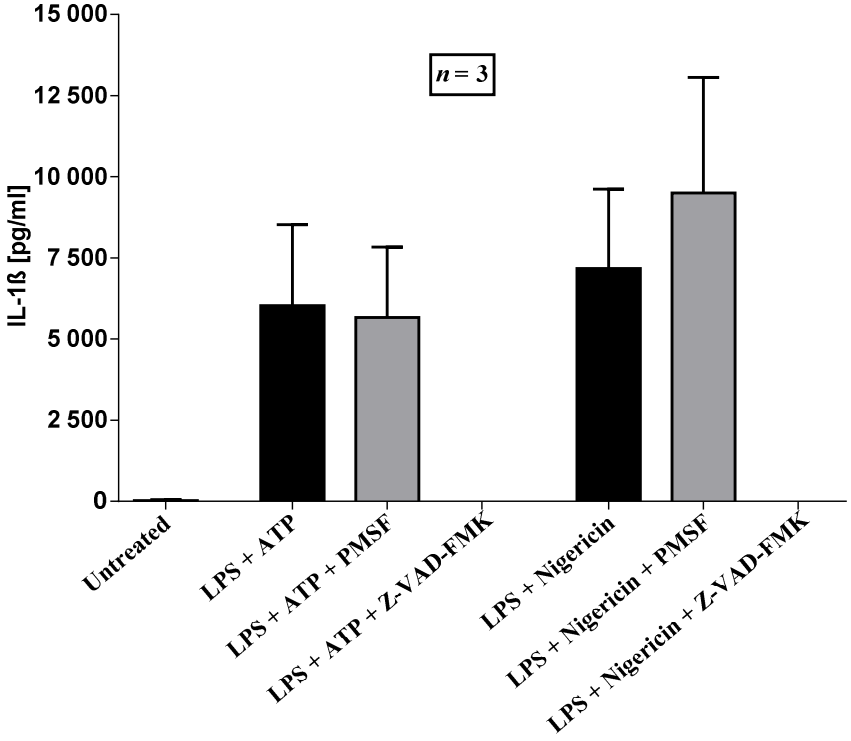

Figure 9: IL-1ß secreted by healthy PBMCs under inhibited conditions. PBMCs were isolated from three healthy donors and stimulated with LPS + ATP or LPS + nigericin (black bars) and with or without the pretreatment with a serine protease inhibitor (PMSF, 1 mM; light grey bars) or a caspase inhibitor (Z-VAD-FMK, 50 µM). The IL-1ß concentrations were measured by ELISA and are given as $^{pg}/_{ml}$ (mean ± SEM).

5.1.6 IL-1ß secreted by PBMCs from MWS patients

With the purpose to compare the IL-1ß production levels of healthy PBMCs with *CIAS1* mutated PBMCs, we collected blood from ten healthy donors and four MWS patients. Both groups increased the IL-1ß production significantly in response to the LPS/ATP stimulation as well as to the LPS/nigericin treatment. However, the *CIAS1* mutated cells produced in total less IL-1ß upon the stimulation compared to the values achieved by the healthy cells (see figure 10). In contrast to these finding were the results found in the unstimulated controls of both groups: here, the MWS PBMCs produced about twice as much IL-1ß than the healthy cells did.

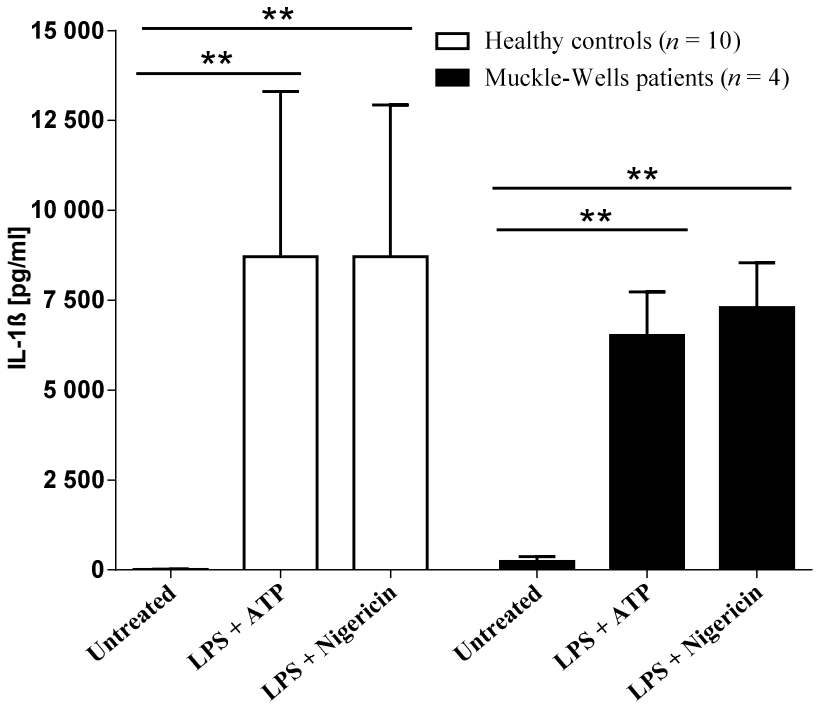

Figure 10: IL-1ß secreted by PBMCs from MWS patients. PBMCs were isolated from ten healthy donors (white bars) and four MWS patients (black bars). The cells were stimulated with LPS + ATP as well as with LPS combined with nigericin. The amount of released IL-1ß was measured using ELISA technique. Concentrations are given as $^{pg}/_{ml}$ *(mean ± SEM).*

5.2 IL-1α concentrations in cell culture supernatants

5.2.1 IL-1α secreted by healthy and MWS neutrophils

No detectable concentrations of IL-1α were found in the supernatants obtained from highly purified neutrophils, neither in the healthy nor in MWS group.

5.2.2 IL-1α secreted by PBMCs from healthy donors

We stimulated PBMCs of healthy donors with LPS followed by ATP or nigericin. These double stimulated cells increased their IL-1α secretion significantly in comparison to the negative, unstimulated controls. The average IL-1α concentration in the supernatants after LPS + ATP stimulation was 780 $^{pg}/_{ml}$. The supernatants obtained from cells, which were stimulated with LPS + nigericin had on average about 1,200 $^{pg}/_{ml}$ of IL-1α (see figure 11). Neither ATP, nor nigericin or LPS alone increased the IL-1α production as it was observed in cells stimulated with the stimulant combination. The IL-1α concentrations in these stimulations were comparable to the ones found in the supernatants of the untreated controls.

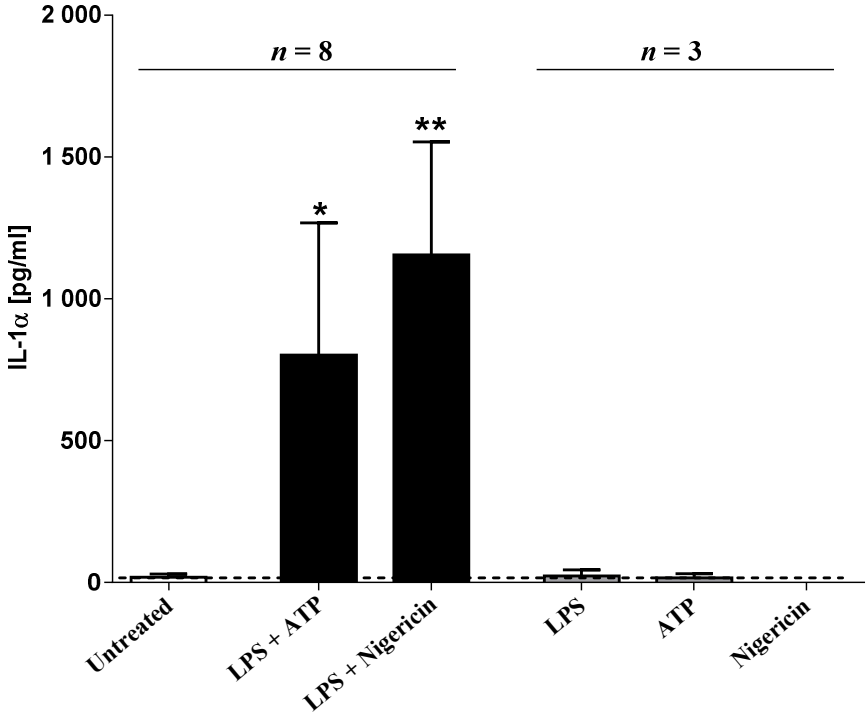

Figure 11: IL-1α secreted by PBMCs from healthy donors. PBMCs were stimulated with LPS + ATP or LPS + nigericin, (black bars) as well as with LPS, ATP, or nigericin alone (grey bars). IL-1α concentrations in the cell culture supernatants were determined by ELISA and are given as $^{pg}/_{ml}$ *(mean ± SEM).*

5.2.3 IL-1α secreted by healthy PBMCs under inhibited conditions

We collected whole blood from two healthy volunteers and isolated the PBMC fraction. The mononuclear cells were stimulated with a combination of LPS and either ATP or nigericin. To investigate to what extent caspase-1 is involved in the cleavage and activation of IL-1α, we stimulated also under addition of Z-VAD-FMK. The cells increased their IL-1α production significantly in response to LPS/ATP as well as to the LPS/nigericin stimulation. LPS/nigericin treatment resulted in an average IL-1ß concentration of 1,450 $^{pg}/_{ml}$. The LPS/ATP stimulated cells produced on average 950 $^{pg}/_{ml}$. In the untreated controls an averaged IL-1α concentration of 35 $^{pg}/_{ml}$ was found. The additional pretreatment with Z-VAD-FMK resulted in a stagnation of the IL-1α production (see figure 12).

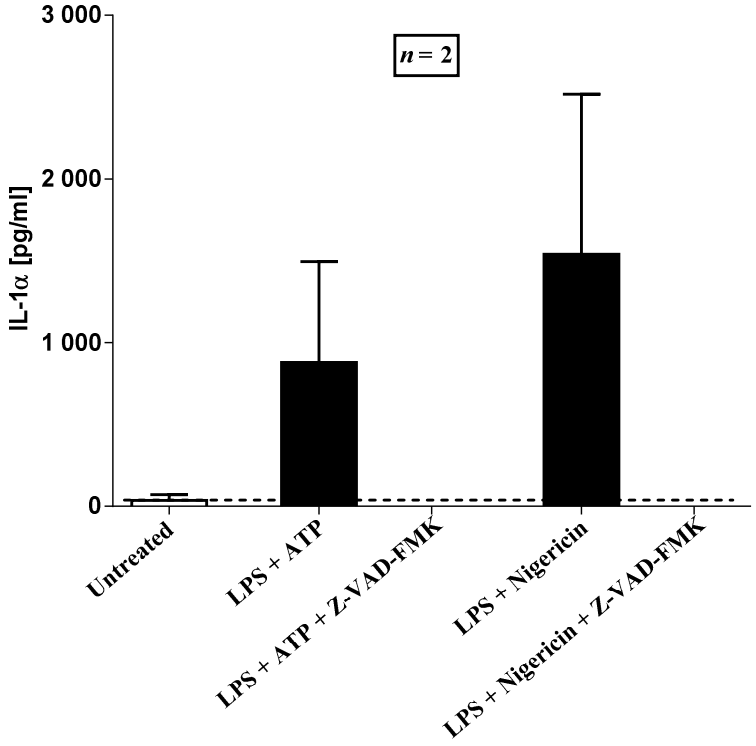

Figure 12: IL-1α secreted by healthy PBMCs under inhibited conditions. PBMCs were isolated from healthy donors and stimulated with LPS + ATP or LPS + nigericin (black bars). In order to investigate the influence of caspases in the maturation process of IL-1α we added Z-VAD-FMK (50 µM) to the stimulations. The amount of IL-1α secreted by the cells was measured by ELISA. Concentrations are given as pg/ml (mean ± SEM).

5.2.4 IL-1α secreted by PBMCs from MWS patients

This experiment was performed with the purpose to compare the IL-1α production levels of PBMCs from healthy donors and MWS patients. Therefore, whole blood was collected from three healthy volunteers and three MWS patients. The PBMC fraction was isolated and stimulated with LPS + ATP and LPS + nigericin. Both groups increased their IL-1α production in response to the stimulations. The cells carrying the *CIAS1* mutation produced on average greater amounts of the cytokine than the healthy PBMCs did. The IL-1α concentrations in supernatants derived from MWS PBMCs after LPS/ATP or LPS/nigericin treatment ranged around 610 pg/ml (see figure 13). The healthy control cells produced upon stimulation on average 320 pg/ml IL-1α. We also found higher IL-1α levels in the cell culture supernatants of the unstimulated MWS PBMCs compared to the healthy untreated cells.

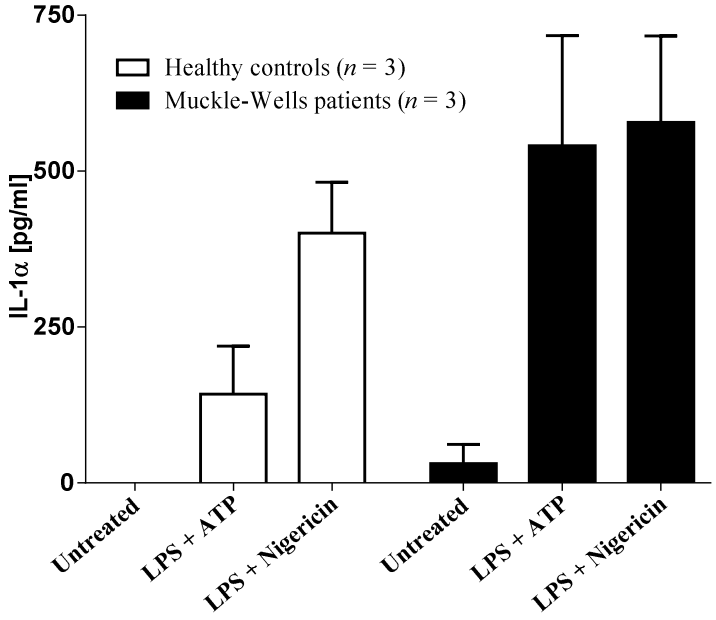

Figure 13: IL-1α secreted by PBMCs from MWS patients. PBMCs were isolated from three healthy donors (white bars) and three MWS patients (black bars) and stimulated with LPS + ATP or LPS + nigericin. The IL-1α concentrations in the cell culture supernatants were determined by ELISA and are given as pg/ml (mean ± SEM).

5.3 IL-18 concentrations in cell culture supernatants

5.3.1 IL-18 secreted by neutrophils from healthy donors

We stimulated highly purified neutrophils with LPS followed by ATP or nigericin. These double stimulated neutrophils produced around 350 pg IL-18 per millilitre (see figure 14). The IL-18 concentrations detected in the supernatants of the unstimulated control cells were on average 385 pg/ml. Thus, neither the stimulation with LPS + ATP nor with LPS + nigericin influenced the anyhow high IL-18 baseline production. The cells treated with LPS, ATP, or nigericin alone produced IL-18 concentrations ranging from 290 to 440 pg/ml.

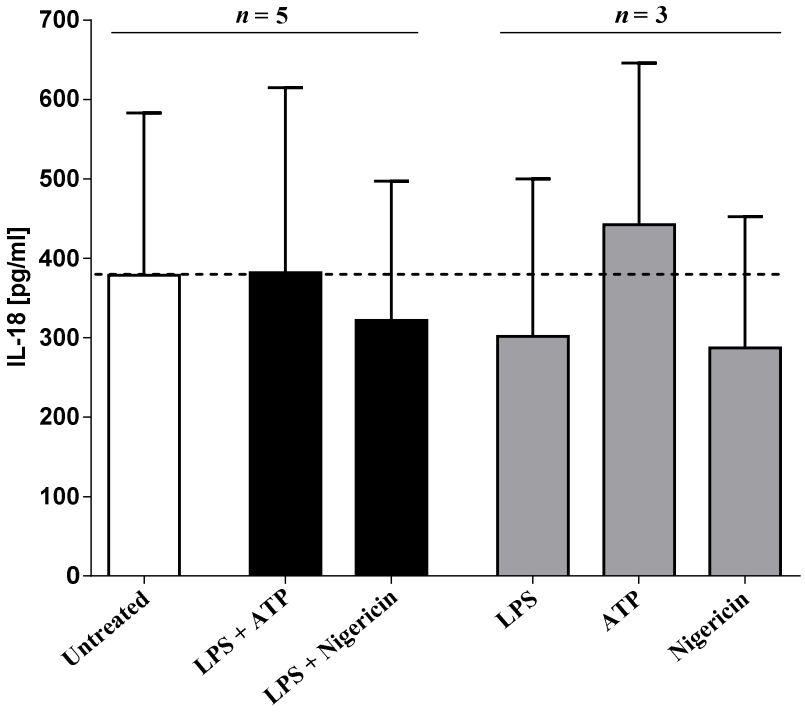

Figure 14: IL-18 secreted by neutrophils from healthy donors. Neutrophil granulocytes were highly purified and stimulated with LPS + ATP, or LPS + nigericin, (black bars) as well as with LPS, ATP, or nigericin alone (grey bars). IL-18 levels in the cell culture supernatants were determined using ELISA technique. Concentrations are given $^{pg}/_{ml}$ *(mean ± SEM).*

5.3.2 IL-18 secreted by neutrophils from MWS patients

This experiment was performed with the purpose to compare IL-18 production levels of healthy neutrophils with *CIAS1* mutated cells. We stimulated the highly purified neutrophils with LPS + ATP or with LPS + nigericin. Both groups, healthy and MWS, did not change their IL-18 production considerably upon stimulation. However, the IL-18 concentrations detected in supernatants removed from *CIAS1* mutated neutrophils were altogether on a lower level. Untreated healthy neutrophils produced in average 400 $^{pg}/_{ml}$ of IL-18; the stimulated MWS cells about 500 $^{pg}/_{ml}$ (see figure 15).

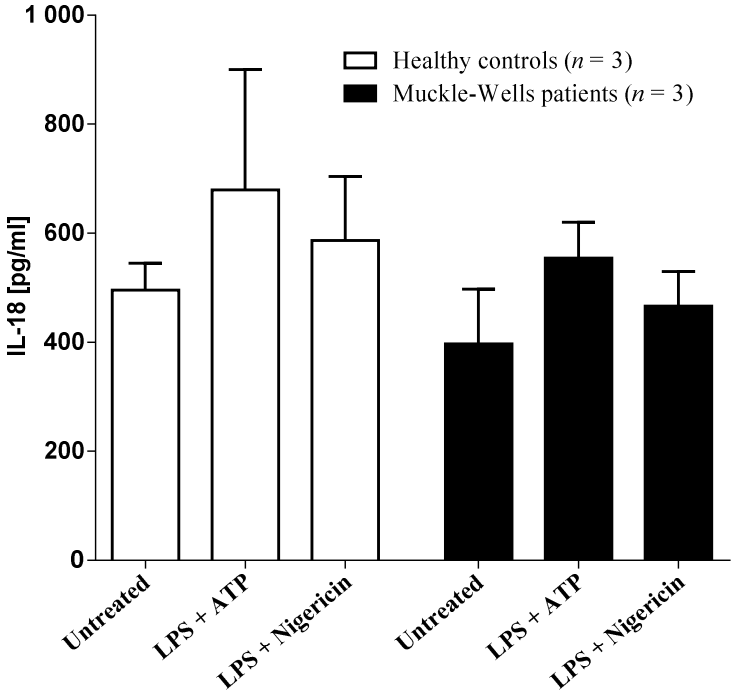

Figure 15: IL-18 secreted by neutrophils from MWS patients. Neutrophils were isolated from three healthy donors (white bars) and three MWS patients (black bars). The cells were highly purified and stimulated with LPS in combination with ATP or nigericin. The IL-18 concentrations in the cell culture supernatants were determined by ELISA and are given as pg/ml (mean ± SEM).

5.3.3 IL-18 secreted by PBMCs from healthy donors

We stimulated PBMCs with LPS followed by ATP or nigericin as well as with LPS, ATP and, nigericin alone. The IL-18 concentrations were significantly increased in the supernatants derived from these double stimulated cells. The average IL-18 concentration in supernatants after LPS/ATP stimulation was 195 pg/ml (see figure 16). The supernatants obtained from cells stimulated with LPS + nigericin had on average about 150 pg/ml of IL-18. Neither ATP nor nigericin or LPS alone raised the IL-18 production like it was observed in cells stimulated with the stimulant combination. The IL-18 concentrations released by PBMCs incubated with only one stimulant ranged from below detection limit up to 5 pg/ml (unstimulated controls: 3 pg/ml).

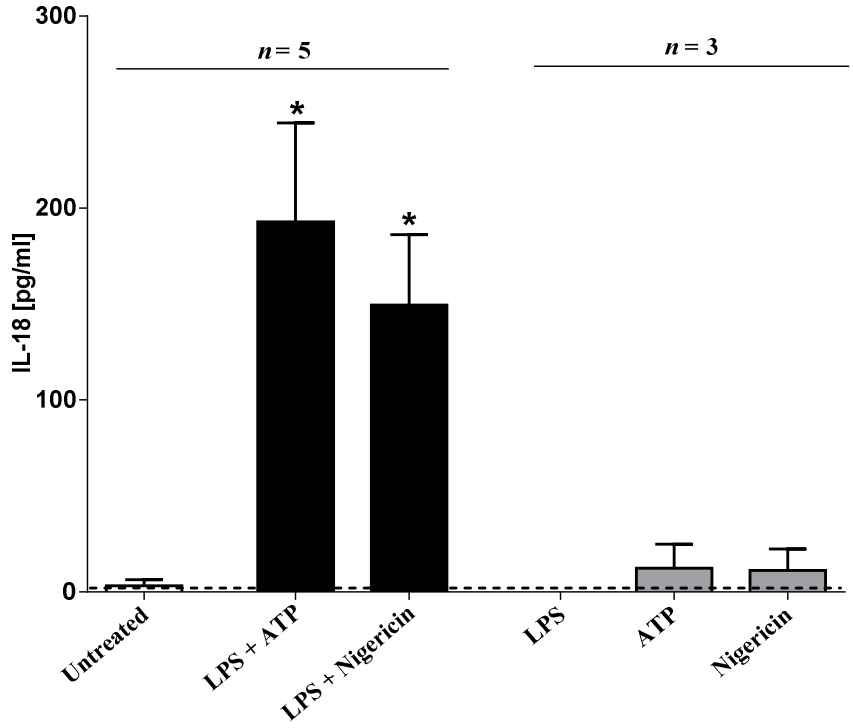

Figure 16: IL-18 secreted by PBMCs from healthy donors. PBMCs were stimulated with LPS + ATP or LPS + nigericin, (black bars) as well as with LPS, ATP, or nigericin alone (grey bars). The IL-18 concentrations in the cell culture supernatants were determined using ELISA technique and are given as $^{pg}/_{ml}$ *(mean ± SEM).*

5.3.4 IL-18 secreted by healthy PBMCs under inhibited conditions

We collected whole blood from healthy volunteers and isolated the PBMC fraction. The mononuclear cells were stimulated with a combination of LPS and either ATP or nigericin. To investigate to what extent caspase-1 is involved in the cleavage and activation of pro-IL-18, we stimulated also after Z-VAD-FMK pretreatment. In both stimulations, LPS/ATP and LPS/nigericin, the IL-18 concentrations were on average around 185 $^{pg}/_{ml}$ (see figure 17). The addition of Z-VAD-FMK resulted in an almost complete stagnation of the IL-18 production with concentrations ranging under 5 $^{pg}/_{ml}$ (untreated controls: 0 $^{pg}/_{ml}$).

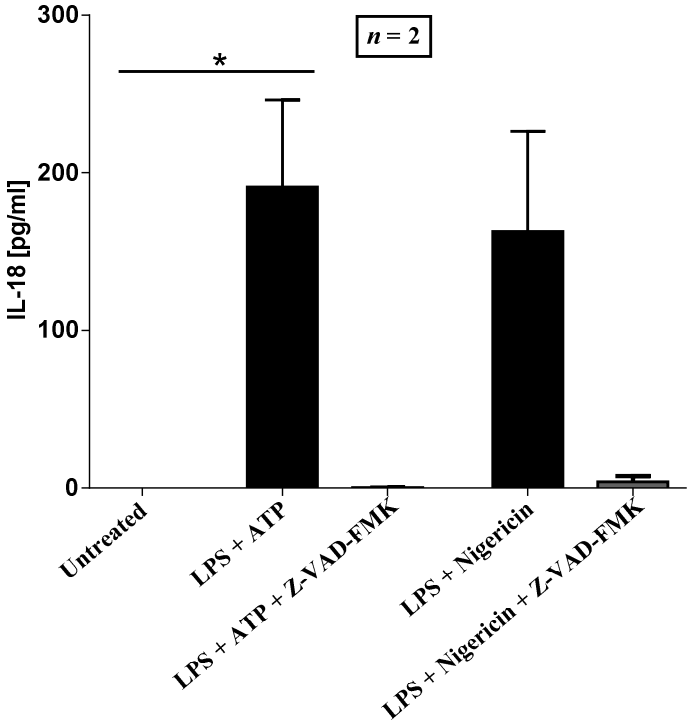

Figure 17: IL-18 secreted by healthy PBMCs under inhibited conditions. PBMCs were isolated from two healthy donors and stimulated with LPS + ATP as well as LPS + nigericin (black bars) and with or without pretreatment with a caspase inhibitor (Z-VAD-FMK, 50 µM; grey bars). The IL-18 concentrations were measured by ELISA and are given as $^{pg}/_{ml}$ (mean ± SEM).

5.3.5 IL-18 secreted by PBMCs from MWS patients

This experiment was performed with the purpose to compare IL-18 production levels of healthy PBMCs with *CIAS1* mutated cells. The PBMC fractions were isolated and stimulated with LPS + ATP and LPS + nigericin. The PBMCs obtained from MWS patients produced consistently higher levels of IL-18. The average IL-18 production of stimulated MWS PBMCs was about 400 $^{pg}/_{ml}$ and thus approximately twice as high as the one of the healthy cells (see figure 18). The untreated MWS PBMCs produced 45 $^{pg}/_{ml}$. No IL-18 was detectable in the supernatants of the unstimulated healthy cells.

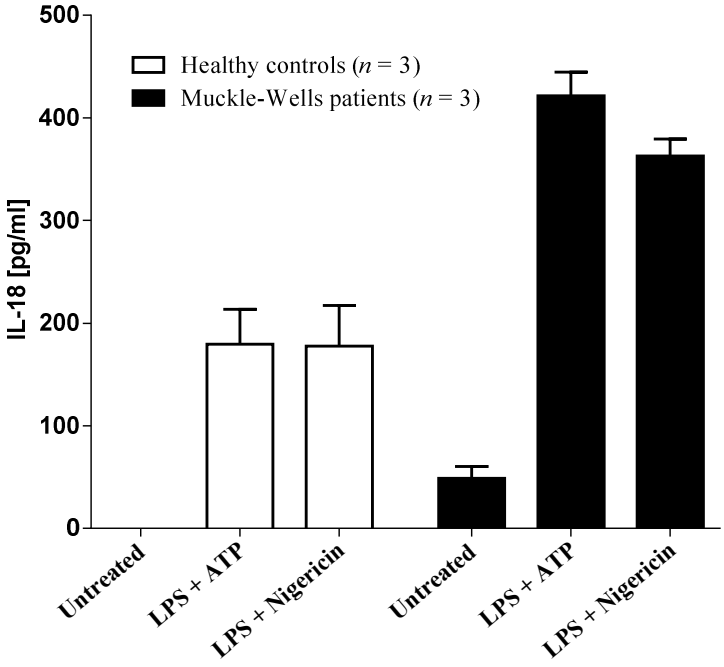

Figure 18: IL-18 secreted by PBMCs from MWS patients. PBMCs were isolated from three healthy donors (white bars) and three MWS patients (black bars). The cells were stimulated with a LPS/ATP combination as well as with LPS combined with nigericin. IL-18 concentrations were measured in the cell culture supernatants by ELISA technique and are given as $^{pg}/_{ml}$ (mean ± SEM).

5.4 IL-33 concentrations in cell culture supernatants

The supernatants obtained from neutrophils as well as from PBMCs showed in all cases IL-33 concentrations below or very close the assay's detection limit.

5.5 IL-1ß plasma levels in patients with MWS

We measured no differences between the IL-1ß plasma levels of MWS-patients (*n*=4) and healthy controls (*n*=10). Both groups achieved average concentrations of 4 $^{pg}/_{ml}$.

5.6 IL-1ß production by neutrophils of different purity degrees

We collected blood from healthy volunteers and isolated neutrophil granulocytes with the common Ficoll fractionation method. In four of 14 cases, we simulated the cells after this Ficoll separation. The neutrophils of the other 10 donors were undertaken an additional purifying step with EasySep. The purity of those neutrophils was > 98 %, as assessed by May-Grünwald-Giemsa stain and flow cytometry. Both groups were stimulated and the IL-1ß concentrations in the supernatants were measured. The IL-1β

protein levels released by the highly purified human neutrophils were substantially lower compared to the concentrations obtained by the partially purified neutrophils (see figure 19). The cells, which were only isolated with Ficoll, produced in average 300 $^{pg}/_{ml}$ of IL-1ß due to stimulation. The highly purified cells released under the same conditions 20 times less IL-1ß, on average 15 $^{pg}/_{ml}$.

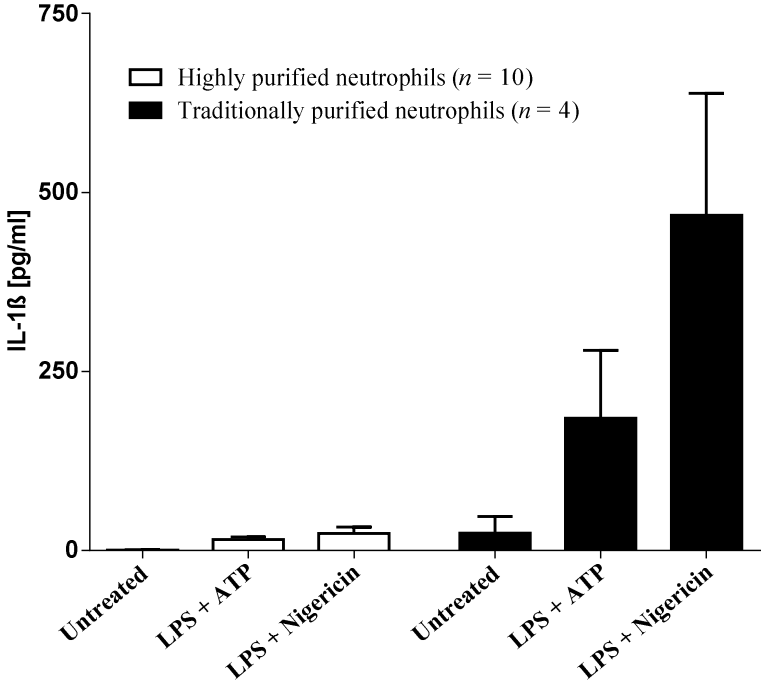

Figure 19: IL-1ß production by neutrophils of different purity levels. IL-1ß concentrations in cell culture supernatants after in vitro stimulation. Highly purified (white bars) and traditional Ficoll isolated neutrophils (black bars) were stimulated with LPS + ATP or LPS + nigericin. Concentrations are given as $^{pg}/_{ml}$ (mean ± SEM).

5.7 Neutrophil subcellular fractionation

5.7.1 Analysis of the subcellular marker protein MPO

Preliminary tests demonstrated that a sample dilution of 1:30,000 was most suitable for the quantification of the granule protein MPO. The highest MPO values were achieved in fraction 2 to 8 (see figure 20). Samples 12 to 36, which were obtained from upper regions of the gradient, showed MPO concentrations under detection limit.

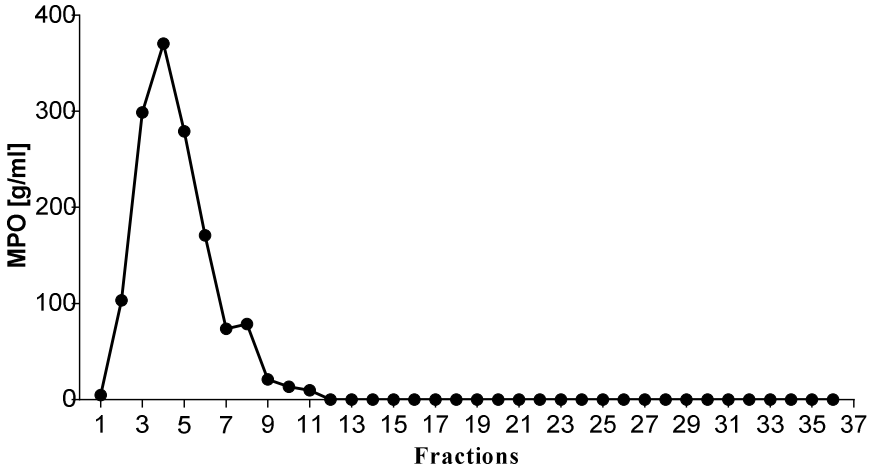

Figure 20: MPO distribution profile of the fractions. After the subcellular fractionation of highly purified neutrophil granulocytes on a four-layer Percoll density gradient, the gradient was divided into 36 fractions given on the x-axis. Each fraction was assayed for its content of MPO, the marker protein for the primary granules. Concentrations are given as $^{\mu g}/_{ml}$.

5.7.2 Analysis of the subcellular marker protein HLA

After initial test runs to estimate the dimension of the fractions' HLA concentration, we decided to perform the measurements in a sample dilution of 1:10,000. Since no suitable standard was available, we limited ourselves to interpret the optical densities of the assay. An increase of absorbance was observed from the 19th fraction onwards (see figure 21). These results suggested that the rare fractions, which were collected from the upper regions of the gradient, contained the plasma membrane.

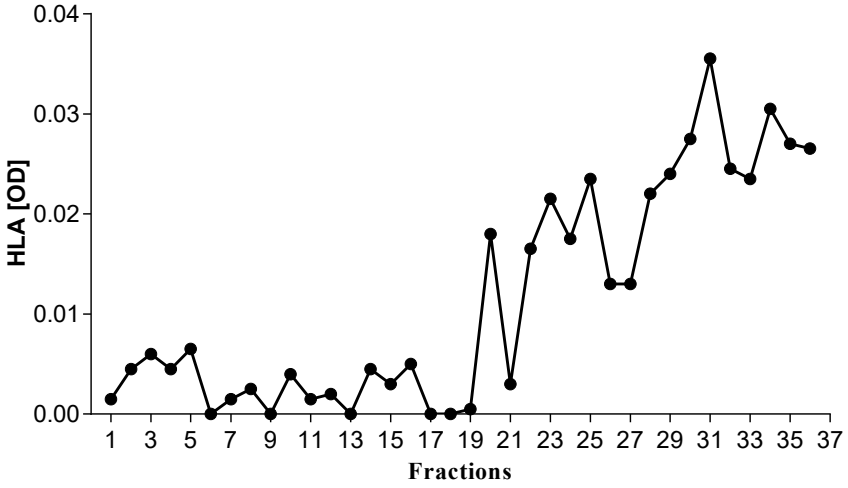

Figure 21: HLA distribution profile of the fractions. Highly purified neutrophil granulocytes were lysed through nitrogen cavitation. After the subcellular fractionation

on a four-layer Percoll density gradient, the HLA content of the fractions (dilution 1:10,000) was determined by ELISA. HLA was used as marker protein for detection of the plasma membrane. Values are given as optical density (OD).

5.7.3 Analysis of the subcellular marker protein MMP-9

The tertiary granules are characterized by their high content of gelatinase (MMP-9). Preliminary tests demonstrated that a sample dilution of 1:10,000 was most suitable for the quantification of this granule protein. The results showed that fraction 19 to 23 contained, with an average concentration of 22 µg/ml, the highest MMP-9 amounts (see figure 22). The remaining fractions showed MMP-9 levels under 6 µg/ml.

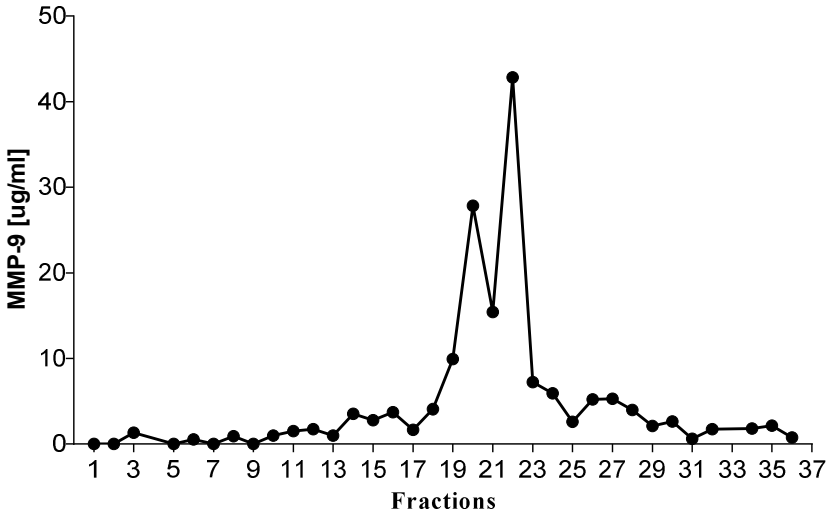

Figure 22: MMP-9 distribution profile of the fractions. After the subcellular fractionation of highly purified neutrophil granulocytes on a four-layer Percoll density gradient, the gradient was divided into 36 fractions given on the x-axis. Each fraction was assayed for its content of MMP-9, the marker protein for the tertiary granules. Concentrations are given as µg/ml.

5.7.4 Analysis of the subcellular marker protein NGAL

Secondary granules were characterized by their high content of NGAL. After the performance of test runs, we decided to quantitate the protein in a fraction dilution of 1:20,000. The results showed a clear tendency to higher NGAL concentrations in the fractions 10 to 17 (see figure 23). In these fractions the average NGAL concentration ranged around 29 µg/ml. The remaining fractions had NGAL levels under 10 µg/ml.

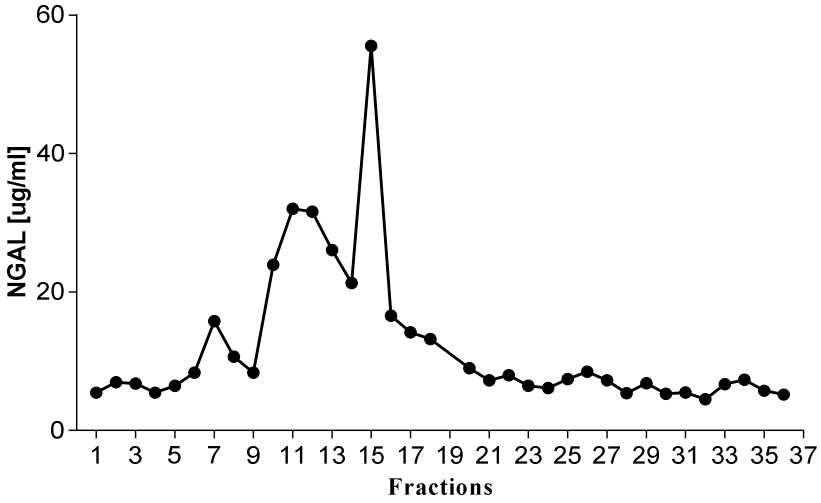

Figure 23: NGAL distribution profile of the fractions. Highly purified neutrophil granulocytes were lysed through nitrogen cavitation. After the subcellular fractionation on a four-layer Percoll density gradient, the 36 fractions were assayed for NGAL, the marker protein for secondary granules. Concentrations are given as $^{\mu g}/_{ml}$.

5.7.5 Analysis of the subcellular marker protein albumin

The secretory vesicles, which are generated by endocytosis, are detected by the measurement of the plasma protein albumin. The ELISA was run in a sample dilution of 1:200. The result showed an increased albumin concentration from fraction 17 onwards (see figure 24). The highest values were achieved in fraction 25 to 30 with average concentrations of 200 $^{\mu g}/_{ml}$.

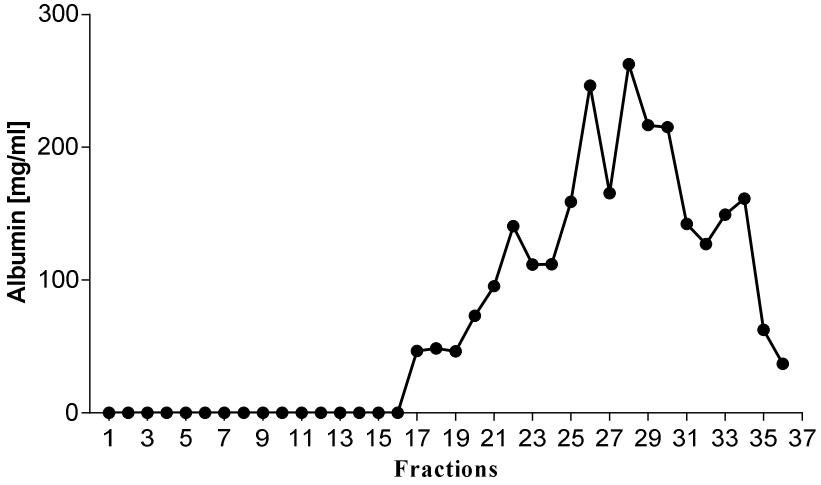

Figure 24: Albumin distribution profile of the fractions. After centrifugation, the four-layer Percoll density gradient was fractionated in 36 parts. Each fraction was assayed for albumin, the marker protein for secretory vesicles. Concentrations are given as $^{\mu g}/_{ml}$.

5.7.6 Definition of the final fractions

To allocate the fractions to a particular subcellular structure, we compared the results of the different markers proteins (see figure 25). The final fraction were defined as listed in table 6.

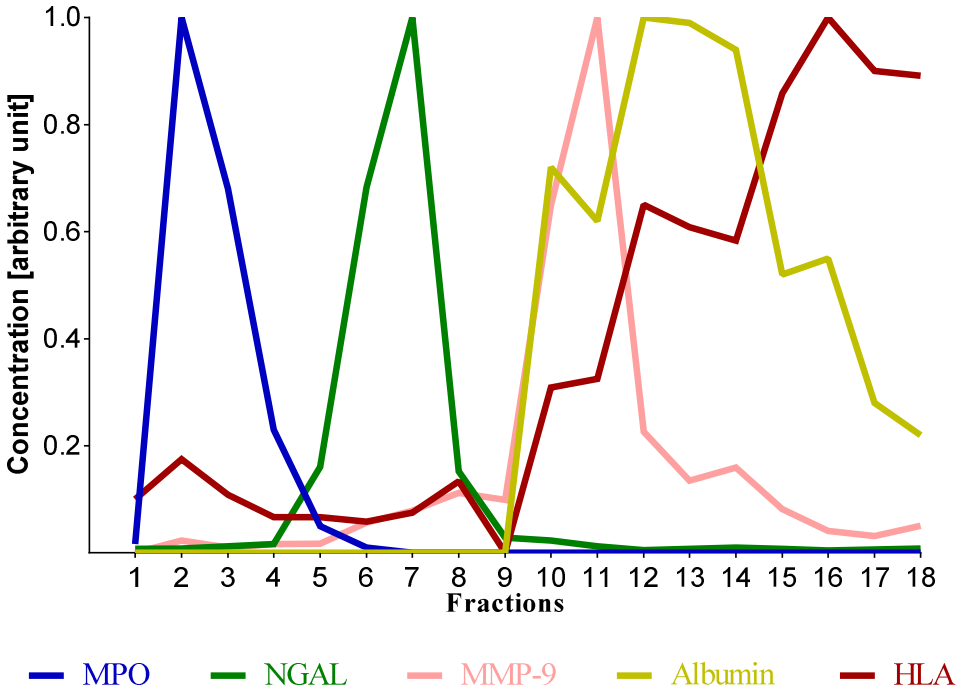

Figure 25: Distribution profiles of all marker proteins. For sake of clarity, the fractions were summarised to a total number of 18. The employed markers were: MPO for primary granules, NGAL for secondary granules, MMP-9 for tertiary granules, albumin for secretory vesicles, and HLA for the plasma membrane. The concentrations for each protein are given as measured concentration in the fraction relative to the maximal concentration of the marker protein.

Table 6: Definition of the final fractions

Subcellular structure:	Fraction
Primary granules (marker protein: MPO)	1–9
Secondary granules (marker protein: NGAL)	10–17
Tertiary granules (marker protein: MMP-9)	18–23
Secretory vesicles (marker protein: albumin)	24–29
Plasma membrane (marker protein: HLA)	30–36

5.7.7 Further subdivision of a four-layer Percoll gradient

To improve the efficiency of the subcellular fractionation procedure, we tried a demarcation of the plasma membrane from the cytosolic fraction within the four-layer Percoll gradient. For that reason, the last 7 fractions were analysed for their content of MICA, a component of the plasma membrane, using western blot analyses. However, MICA (molecular weight 43 kDa) was detectable in all analysed fractions with no observable concentration increase or decrease (see figure 26). Consequently, it did not succeed to separate the plasma membrane and the cytosolic fraction within the gradient.

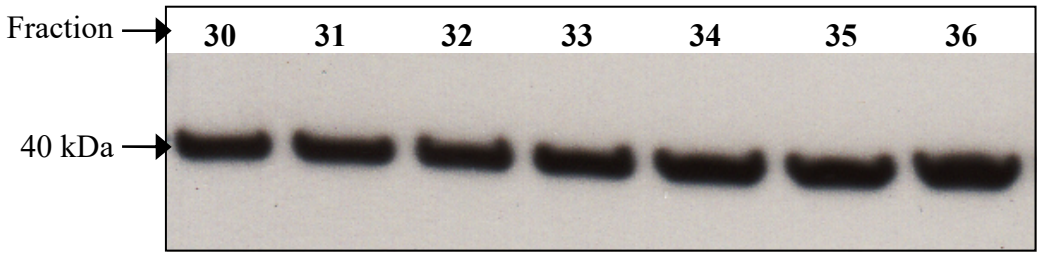

Figure 26: MICA immunoblot. The fractions, which were defined as plasma membrane containing fractions, were analysed for their content of MICA (43 kDa) in order to detect a possible shift from the plasma membrane to the cytosolic fractions.

5.7.8 Subcellular localisation of inflammasome components

To localise the inflammasome in neutrophils, the different subcellular components (cytosol, plasma membrane, secretory vesicles, and the three granules subtypes) were examined for the presence of ASC and caspase-1. The cytosol was obtained by a three-layer Percoll gradient; the other organelles were separated in a four-layer gradient. The studies demonstrated that the investigated inflammasome components were expressed in the cytoplasm and in the fraction reflecting the plasma membrane together with the cytosol, but also colocalised in secretory vesicles, and tertiary granules (see figure 27 and 28).

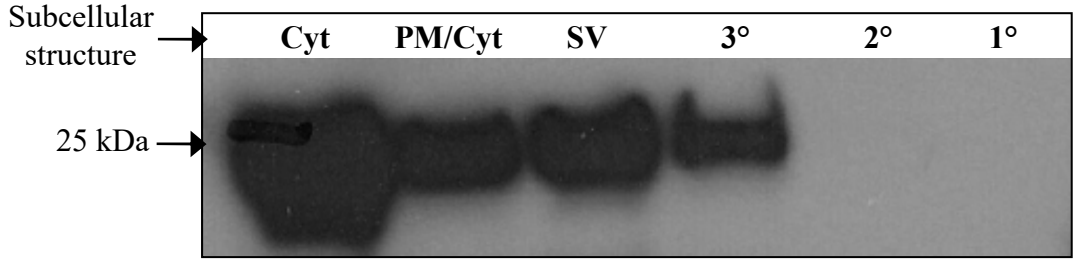

Figure 27: ASC immunoblot. Subcellular components of neutrophil granulocytes were analysed for their content of ASC (25 kDa), a protein of the inflammasomal multiprotein complex. ASC was detected in the cytosolic fraction (Cyt), in the fraction reflecting the plasma membrane together with the cytosol (PM/Cyt), in the secretory vesicles (SV), and in the tertiary granules (3°). No ASC could be detected in the secondary granules (2°) or in the fraction of the primary granules (1°).

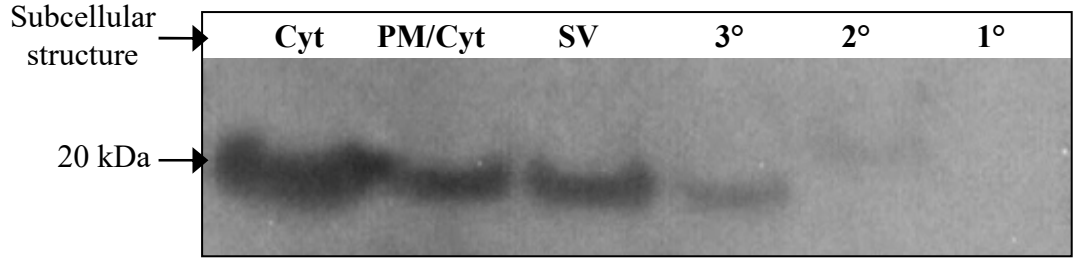

Figure 28: Caspase-1 immunoblot. Subcellular components of neutrophil granulocytes were analysed for their content of caspase-1 (20 kDa), a protein of the inflammasomal multiprotein complex. Caspase-1 was detected in the cytosolic fraction (Cyt), in the fraction reflecting the plasma membrane together with the cytosol (PM/Cyt), in the secretory vesicles (SV), and in the tertiary granules (3°). No caspase-1 could be detected in the secondary granules (2°) or in the fraction of the primary granules (1°).

6. Discussion

6.1 Neutrophil interleukin production

Studying the inflammasome-associated IL-1 family cytokines, we observed that highly purified human neutrophils, equally to the PBMCs, significantly increased their IL-1ß production when the stimulation included a previous LPS priming step. However, both cell groups differed concerning their behaviour under the influence of the chemical inhibitors. In PBMCS, the caspase inhibitor Z-VAD-FMK completely suppressed the IL-1ß generation, while the inhibition of serine proteases was found to have no effect on the IL-1β release. The neutrophils showed the greatest response upon stimulation with LPS combined with nigericin (nigericin was about 2-fold more potent in triggering IL-1β release than ATP). Those cells, stimulated with LPS/nigericin, behaved in addition similar to the mononuclear cells under inhibitory influence: Z-VAD-FMK abrogated almost completely the IL-1ß generation, whereas PMSF showed no comparable effects. The LPS/ATP triggered neutrophil IL-1ß secretion decreased under serine protease inhibition as well as after treatment with the caspase inhibitor. We concluded that serine proteases play a role in neutrophil-derived, but not in PBMC-derived IL-1β generation. The distinct involvement of serine proteases in the neutrophil IL-1ß production implies that the two inflammasome inducers, ATP and nigericin, differ substantially in their downstream mechanisms and hints at separate, stimulus depending, cytokine production pathways. These findings have a clear clinical relevance since they suggest that the therapeutic inhibition of caspases is not able to interfere with the whole spectrum of IL-1 production pathways. In inflammatory conditions in which neutrophils play a dominant role, especially in the acute phase of inflammation, a dual therapy with inhibition of caspase-1 and serine proteases could be more effective.

IL-18 protein was already secreted by neutrophils at baseline conditions and the production could not be further increased through any inflammasome stimulation. Pro-IL-18 is described to be constitutively expressed in PBMCs [50,58,65,66], whereas little is currently known about its expression profile in neutrophils. However, a recent study assumed also a constitutive expression of pro-IL-18 in human neutrophils.[70] The observed high IL-18 baseline production by neutrophils could be seen as a consequence of this constitutive protein expression. The incubation time of 6 hours and the isolation

procedure might have been sufficient to activate the constitutive expressed proenzyme. Furthermore, neutrophils possess, through their high content of proteinase 3, a potential additional option for IL-18 activation, which is not present in PBMCs. Another explanation for the consistent high IL-18 secretion might be the emergence of biologically inactive IL-18 forms. These peptides can be generated by caspase-3 or other proteases.[2,5] We are not sure if our used ELISA could differentiate between those IL-18 forms.

The neutrophils in our study did not secrete detectable amounts IL-1α or IL-33 proteins. This could also be reasoned in the intracellular or cell surface-associated localisation of those cytokines, what makes them undetectable in the supernatants.

6.2 PBMC interleukin production

Our inflammasome *in vitro* studies achieved plausible results and confirmed the present knowledge about the maturation of the studied interleukins in PBMCs. The levels of all examined cytokines, with the exception of IL-33, increased considerably after inflammasome stimulation. Furthermore, we could demonstrate that a second stimulus, after a previous LPS priming step, strongly enhanced the proteolytic maturation and secretion of IL-1ß, IL-1α, and IL-18. Various studies, including our own, showed that treatment with caspase inhibitors, such as Z-VAD-FMK, dramatically reduces the production of interleukin-1 family cytokines in PBMCs. Consequently, the secretions fully depended on caspases and were independent of serine protease activities.

6.3 Interleukin production by Muckle-Wells cells

We observed that neutrophils isolated from MWS patients released significantly lower IL-1β and IL-18 levels upon stimulation compared to the investigated age-matched healthy control cells. These results were contrary to expectations if one considers the underlying MWS pathology, which is characterised by a constitutively increased NLRP3 inflammasome activity. However, it has to be taken into account that the mechanisms of the neutrophil interleukin production remain largely unexplored. Furthermore, little is known to what extent the different cell lines are involved in the MWS pathology.

Speculative explanations for the observed phenomenon are:

- The mutated *NLRP3* gene leads to a constitutive activated inflammasome at baseline conditions, rendering neutrophils unresponsive towards exogenous inflammasome activators. Inside-out counteracting mechanisms and so far unappreciated auto-regulatory feed-back loops between intrinsic and extrinsic inflammasome activation might exist here. These could restrict the inflammasomal responsiveness when the inflammasome is already activated. A potential source of such feedback loops could involve a down-regulation of cell surface receptors, like TLR4 or ATP receptors.
- The constitutive over-activated inflammasome could simply lead to a consumption of pro-IL-1β, like a 'secretory exhaustion' of the neutrophils.
- The mutated cryopyrin could be freed from the requirement of classical inflammasome activators. There is the possibility for completely different activators *in vivo*.
- The stimulated neutrophils and PBMCs from MWS patients secreted lower IL-1ß levels than the simulated healthy controls but we observed a slightly higher IL-1ß baseline production in the *CIAS1* mutated cells. The clinical appearance of MWS, which underlies high IL-1ß levels, might be explained by even this high IL-1ß production of unstimulated cells. IL-1ß is a hormone-like cytokine with an effective concentration ranging from less than 1 nM to 1 pM.[1] It is potent to induce a broad spectrum of systemic changes. It is easy to imagine that even small imbalances are sufficient for triggering clinical symptoms.
- The number of experiments with MWS cells was severely limited. The reason behind was on the one hand the high blood quantity which was needed and on the other hand the rarity of the disease. We had four patients available and performed all MWS inflammasome stimulation experiments on a single day. The low interleukin production of MWS cells could also be due to a systematic error which occurred that day. Following errors are conceivable:
    - incorrect cell counts,
    - any cell interacting substance,
    - insufficient stimulation through decreased stimulant activity or too low concentrated stimulants,

- dilution effects through for instance the addition of too much glycerine or protease inhibitor.

To what extent the increased IL-18 production by MWS PBMCs has medical relevance in the MWS pathophysiology needs to be investigated in further studies.

6.4 Importance of neutrophil purity in inflammasome studies

We used negatively selected highly purified neutrophils, since traditionally density gradient isolated neutrophils contain a substantial proportion of contaminating monocytes.[62,73] Monocytes produced upon stimulation on average 8,000 pg IL-1ß per millilitre, whereas the equal number of neutrophils under the same conditions secreted only 1 $^{pg}/_{ml}$. It is easy to imagine, how even small amounts of contaminating monocytes could distort the results and have to be considered when interpreting quantitative IL-1β results from traditional density gradient derived neutrophils. Furthermore, monocytes were previously found to influence neutrophils' homeostasis as well as their responsiveness to inflammatory stimuli at several levels.[62,73] Monocyte-neutrophil interactions might amplify paracrine IL-1β production and modulate other cellular processes, an issue that requires further research.

6.5 Subcellular fractionation

Our western blot analysis demonstrated that highly purified human neutrophils expressed key components of the NLRP3 inflammasome machinery at protein level. Moreover, they showed that the inflammasomal components are stored, in addition to the already for a variety of cell types described cytoplasmic localisation[77], in secretory vesicles and tertiary granules. Secretory vesicles are uniquely sensitive for mobilisation and their special significance lies in providing a readily mobilisable reservoir of functional proteins. Our findings implicate that neutrophils are able to dynamically regulate their inflammasomes between intracellular stores and surface localisation. The subcellular mechanisms by which inflammasomes could interact with pro-IL-1β remain elusive, but given the role of secretory vesicles, it is tempting to speculate, that stimulated neutrophils translocate their inflammasome components to the cell surface, where the inflammasome could participate in pathogen recognition and uptake (see figure 30). This hypothesis is further supported by a recent study showing that NLRP3

inflammasomes control the phagosomal functionality of macrophages due to infection with gram-positive bacteria.[81]

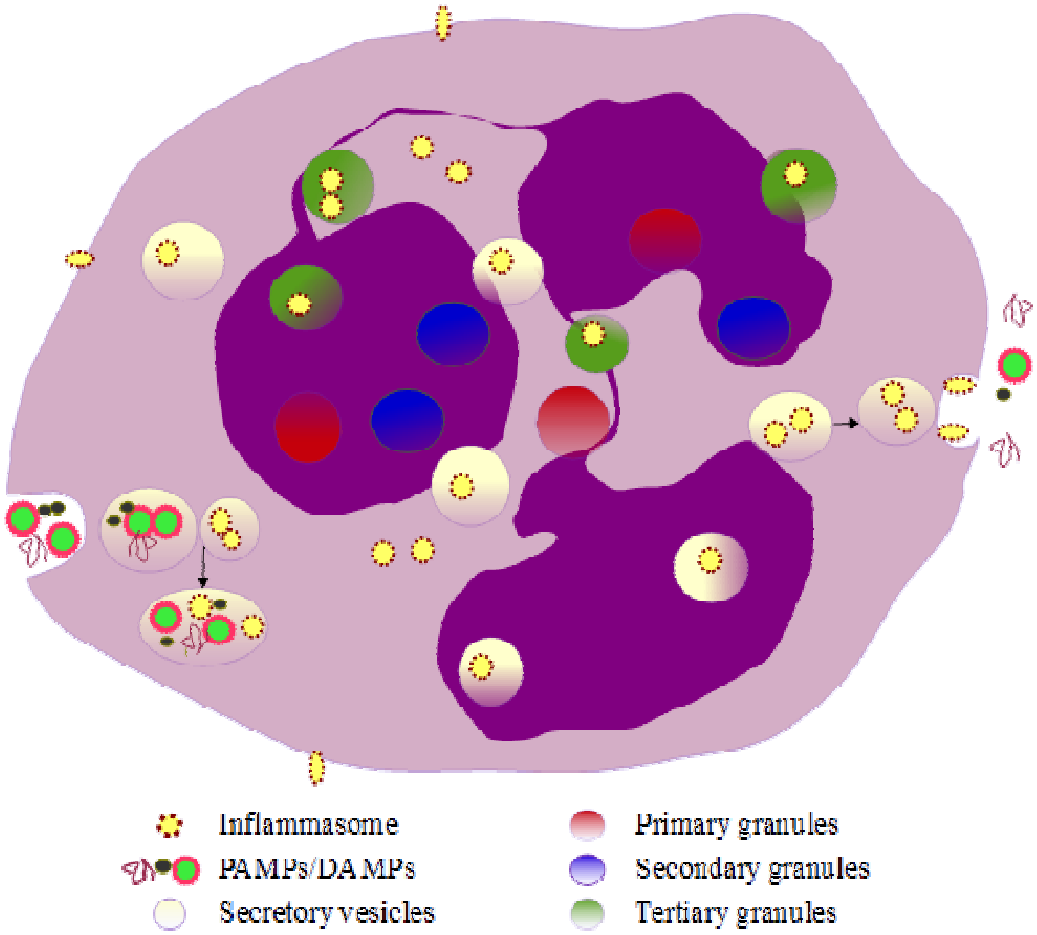

Figure 29: Subcellular localisation of the inflammasome. The results of our subcellular fractionation study confirmed the already for other cell types described cytoplasmic localisation of the inflammasome (1.). Moreover, our findings implicate a colocalisation of the inflammasome in secretory vesicles (2.) and tertiary granules (3.) in human neutrophils. Considering the function of secretory vesicles, it is conceivable that stimulated neutrophils are able to translocate their inflammasomes to the cell surface, where they could participate in pathogen recognition and uptake. Since inflammasomal components were also found in some granules, a function of the inflammasome in phagolysosomes is also feasible.

6.6 Perspectives

Clearly, many future studies are required to dissect the intracellular pathways that control the expression, processing, and secretion of the IL-1 family cytokines. Some possible further research directions and methodical optimisations are:

- Stimulation experiments with cells isolated from CINCA patients, the most severe form of CAPS, could provide clearer results concerning the intrinsic inflammasome activation.

- The examination if *CIAS1* mutated neutrophils show a down-regulation of activating cell surface receptors (LPS/TLR4 or ATP) could deliver interesting information about auto-regulatory feed-back loops.

- Possible strategies for a further clarification of the IL-18 expression mode in neutrophils: measurements at mRNA level by quantitative real-time polymerase chain reaction (RT-PCR) or at protein level by the detection of pro-IL-18 in lysed granulocytes.

- The general measurement of LDH in the supernatants could be useful to foreclose a role of cell lysis in the externalisation of the cytokines.

- AEBSF (4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride), a serine protease inhibitor that is stable in aqueous solutions, could be an alternative to PMSF, which is an irreversible inhibitor but very unstable in the presence of water.

7. Conclusions

- Neutrophils secreted IL-1β and IL-18, but not IL-1α or IL-33.
- Activation of neutrophils' inflammasome mediated IL-1β secretion, but not IL-1α, IL-18 or IL-33 release.
- Nigericin was about 2-fold more potent in triggering IL-1β release by both partially and highly purified neutrophils compared to ATP.
- Serine proteases and caspases were partially and differentially involved in the neutrophil IL-1ß production, depending on the stimulus. The LPS/nigericin-induced IL-1β generation seemed to depend stronger on caspases, the LPS/ATP-induced cytokine secretion was more strongly linked on serine protease activity.
- Inhibitors of caspase-1 are not able to interfere with the whole spectrum of IL-1ß production pathways.
- The IL-1β protein production by human neutrophils was substantially affected by the cell purity.
- The highly purified neutrophils secreted IL-1β and IL-1α in a substantially lower level compared to the PBMCs, whereas they produced nearly twice as much IL-18 as the autologous PBMCs.
- PBMCs secreted IL-1β, IL-18, and IL-1α in an inflammasome-dependent manner. IL-33 that was not detectable in the PBMC cell culture supernatants.
- Neutrophils from MWS patients released lower levels of IL-1β compared to healthy control neutrophils after extrinsic inflammasome stimulation. This suggests that constitutive intrinsic inflammasome activation limits the exogenous / ligand-induced inflammasome responsiveness.
- Production of IL-18 by MWS neutrophils appeared to be inflammasome independent and tended to be lower in the *CIAS1* mutated neutrophils than in the healthy control cells.

- ASC and caspase-1 were expressed at protein level by human neutrophils.
- Neutrophils stored NLRP3 inflammasome components in the cytoplasm as well as in mobilisable compartments (secretory vesicles and tertiary granules).

8. Summary

The '***inflammasome***', a multi-protein complex in monocytes and granulocytes, has emerged as playing a key role in innate immunity and inflammation. Regarding monocytes, definite data are available that the inflammasome, localised in the cytosol, controls the maturation of different interleukins via caspase-1 activation. Concerning the situation in ***neutrophil granulocytes***—which was the main focus of our research—little is currently known about the subcellular localisation and the function of the inflammasome. To precisely characterise the ***localisation*** of the inflammasome in neutrophils, we utilised subcellular fractionation and detected inflammasomal components not only in the ***cytoplasm*** but also in mobile ***vesicles*** and ***granules***. These findings may implicate that neutrophils are able to regulate their inflammasomes dynamically between intracellular stores and the cell surface, where they could participate in pathogen recognition and uptake. To gain a better understanding about the ***function*** of the inflammasomes in neutrophils, we stimulated highly purified cells with known inflammasome activators. The results of these experiments suggest that inflammasome activation in human neutrophils triggers the ***IL-1β, but not the IL-18, IL-1α, or IL-33*** protein secretion. Besides the inflammasome-mediated pathway, IL-1β was also produced in an inflammasome-independent fashion through the action of ***serine proteases***. IL-18 was already secreted at baseline conditions and we could not see a further increase after stimulation. In our experimental conditions, neutrophils were unable to release detectable amounts of IL-1α or IL-33. The isolated autologous ***monocytes*** produced IL-1ß, IL-1α, and IL-18 in an inflammasome-dependent manner, whereas IL-33 was not detectable in the cell culture supernatants of the monocytes. Methodologically, our studies raised the point that contaminating monocytes have to be considered when interpreting IL-1β quantification results from traditional density gradient derived neutrophils. To investigate the effect of intrinsic inflammasome activation on neutrophils, we studied patients with MWS (***Muckle-Wells syndrome***), a rare disorder characterised by constitutively increased inflammasome activity. The neutrophils from patients with MWS showed a tendency towards lower IL-1ß and IL-18 levels in the stimulation assays compared to healthy control cells. We hypothesised that the constitutive intrinsic activation of the inflammasome in MWS patients limits the exogenous inflammasome responsiveness.

9. Zusammenfassung

Eine Schlüsselrolle in der unspezifischen Immunantwort spielt das „***Inflammasom***“, ein Multiproteinkomplex in Monozyten und Granulozyten. Hierbei liegen in Bezug auf Monozyten eindeutige Daten vor, dass das im Zytosol lokalisierte Inflammasom über eine Caspase-1-Aktivierung die Generierung von verschiedenen Interleukinen steuert. Im Gegensatz dazu ist im Hinblick auf ***neutrophile Granulozyten*** - die im Fokus unserer Forschung standen - nur wenig über Funktion und subzelluläre Lokalisation des Inflammasoms bekannt. Zur genaueren ***Lokalisation*** des Inflammasoms fraktionierten wir die subzellulären Bestandteile von Neutrophilen und konnten inflammasomale Proteine nicht nur im ***Zytoplasma***, sondern auch in ***Vesikeln*** und ***Granula*** nachweisen. Dies gibt Anlass zur Spekulation, dass Neutrophile mit ihren Inflammasomen flexibel zwischen Zelloberfläche und intrazellulären Speichern operieren können. Um ein besseres Verständnis über die ***Funktion*** der Inflammasomen zu erlangen wurden hochaufgereinigte Neutrophile mit bekannten Inflammasomaktivatoren stimuliert. Die Ergebnisse dieser Experimente deuten darauf hin, dass das Inflammasom in Neutrophilen zwar die Bildung von ***IL-1ß, aber nicht die von IL 18, IL-1α oder IL-33*** triggert. Neben dem Inflammasom-abhängigen Produktionsweg wurde IL-1ß jedoch auch Inflammsom-unabhängig über ***Serinproteasen*** aktiviert. IL-18 wurde bereits durch unstimulierteNeutrophile sezerniert, ohne weitere Produktionssteigerung nach Stimulation. IL-1α und IL-33 konnten beide nicht in den Kulturüberständen der Neutrophilen nachgewiesen werden. Die autologen ***Monozyten*** produzierten die untersuchen Zytokine, mit der Ausnahme des nicht nachweisbaren IL-33, Inflammasom-abhängig. Bezüglich der methodischen Verfahrensweise konnten wir beim Vergleich von traditionell isolierten Neutrophilen mit speziell hochaufgereinigten Zellen zeigen, dass die Aussagekraft von Stimulationsexperimenten mit Neutrophilen in hohem Maße von kontaminierenden Monozyten abhängig ist. Um den Effekt von intrinsischer Inflammasomenaktivierung auf Neutrophile zu erforschen, untersuchten wir Patienten mit MWS (***Muckle-Wells-Syndrom***), einer seltenen, durch erhöhte Inflammasomaktivität gekennzeichnete, Erkrankung. Die MWS Neutrophile produzierten nach Stimulation weniger IL-1ß und IL-18 verglichen zu den gesunden Zellen. Wir vermuten, dass die in MWS-Patienten vorliegende konstitutive intrinsische Aktivierung die Bereitschaft für eine exogene Stimulation des Inflammasoms senkt.

10. Appendix

10.1 Abbreviations

AEBSF	4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride
ASC	apoptosis-associated speck-like protein containing a CARD
ATP	adenosine triphosphate
bis-tris	bis(2-hydroxyethyl)-amino-tris(hydroxymethyl)-methane
BSA	bovine serum albumin
CANP	calcium-activated neutral protease
CAPS	cryopyrin-associated periodic syndrome
CARD	caspase recruitment domain
CD	cluster of differentiation
CFU-GEMM	colony forming unit granulocyte, erythrocyte, macrophage, megakaryocyte
CLR	C-type lectin receptors
CO_2	carbon dioxide
cRPMI	complete Roswell Park Memorial Institute medium
DAMP	damage-associated molecular pattern
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
D-PBS	Dulbecco's phosphate buffered saline
dsDNA	double-stranded DNA
ECL	enhanced chemiluminescence
EGTA	ethylene glycol tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence-activated cell sorting
Fc region	fragment crystallisable region
FCS	fetal calf serum
GP	granular protein
H_20	water
HIN	H inversion
HLA	human leukocyte antigen
HMGB1	high mobility group 1
HRP	horseradish peroxidise

IgG	immunoglobulin G
IGIF	interferon-gamma inducing factor
IL-1R	IL-1 receptor
IL-1ß	interleukin-1 beta
LPS	lipopolysaccharide
LRR	leucine-rich repeat
MAMP	microbe-associated molecular pattern
MBL	mannan binding lectin
MES	morpholinoethansulphate
MHC	major histocompatibility complex
MICA	MHC class I chain-related molecule A
MMP-9	matrix metallopeptidase 9
MPO	myeloperoxidase
MWS	Muckle-Wells syndrome
NACHT	NAIP, CIITA, HET-E, TP-1
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NGAL	neutrophil gelatinase-associated lipocalin
NK-cell	natural killer cell
NLR	NOD-like receptor
NLRP3	NOD-like receptor family, pyrin domain containing protein 3
NOD	nucleotide-binding oligomerisation domain
PAMP	pathogen-associated molecular pattern
PBMC	peripheral blood mononuclear cell
PBS-T	phosphate buffered saline-Tween
PIPES	Piperazine-1,4-bis(2-ethanesulfonic acid)
PMN	polymorphonuclear leukocyte
PMSF	phenylmethylsulfonyl fluoride
PRR	pattern-recognition receptor
PVDF	polyvinylidenfluorid
PVP	polyvinylpyrrolidone
RIG	retinoic acid-inducible gene
RPMI	Roswell Park Memorial Institute medium
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis

ß2M	Beta-2 microglobulin
TLR	Toll-like receptor
TMB	3,3',5,5'-tetramethylbenzidine
TNFα	tumor necrosis factor alpha
WT	wild type
Z-VAD-FMK	carbobenzoxy-valyl-alanyl-aspartyl-(O-methyl)-fluoromethylketone

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10.5 Contribution statement

Professor D. Hartl and Dr. A. Hector designed the research (project conception, development of the overall research plan, study oversight, and experiment conception) and drafted the published paper.
Dipl.-Biol. M. Bakele conducted the cell bomb experiments.
Melanie Joos prepared and carried out the experiments, analysed the data, and wrote this thesis.

10.6 Publication

Bakele M.*, Joos M.*, Burdi S., Allgaier N., Pöschel S., Fehrenbacher B., Schaller M., Marcos V., Kümmerle-Deschner J., Rieber N., Borregaard N., Yazdi A., Hector A.*, and Hartl D.* 2014. Localization and functionality of the inflammasome in neutrophils. *The Journal of biological chemistry* 289: 5320-5329
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