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Final report

"Investigating the molecular mechanism of inflammasome activation using new camelid single – domain antibodies (VHHs)"

By

Raimond Stroe

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External Supervisor: Dr. Florian Schmidt

Internal Supervisor: Dr. Andreas Eger

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Abstract

Inflammasomes are macromolecular signalling platforms that assemble in myeloid cells and other cell types to interpret intracellular evidence of pathogen infection or cell damage, upon which they trigger an appropriate inflammatory response. Receptor activation by direct or indirect ligand binding triggers assembly of inflammasomes and licenses activation of the incorporated caspase-1 by autocatalytic cleavage. This, in turn, proteolytically cleaves cytokine precursors to yield IL-1β and IL-18, which are released to elicit an inflammatory response. Mechanistic aspects of inflammasome activation and cytokine release are poorly understood. Controlled activation of inflammasomes is of fundamental importance in the recruitment of other components of the immune system. Aberrant inflammasome activity can contribute to auto inflammatory diseases and to other important pathological conditions.

In this thesis I will summarize important aspect about the biology of inflammasomes and how to possibly study its mechanisms by means of so called nanobodies that can be found in the family of Camelidae.

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List of Abbreviations

AIM2 – Absent in melanoma 2

ALRs – AIM2-like receptors

ASC – Apoptosis-associated speck-like protein containing a CARD

ATP – Adenosine triphosphate

BIR – baculoviral inhibition of apoptosis protein repeat domain

CARD – Caspase activation and recruitment domain

CASR – calcium-sensing receptor

CGD – chronic granulomatous disease

CH – constant heavy chain domain

CIITA – MHC class 2 transcription activator

CL – constant light chain domain

DAMPs – Danger-associated molecular pattern

DNA - Deoxyribonucleic acid

EF – edema factor

FIIND – domain with function to find

Fv – variable fragment

HCAbs – heavy chain antibodies

HIN – haematopoietic interferon-inducible nuclear antigens with 200 amino-acid repeats

IFI16 – interferon induced protein 16

IgG – Immunoglobulin-γ

IP3R – Inositol-1,4,5-trisphosphate

IPAF – Interleukin 1β – converting enzyme protease-activating factor

kDa – kilo Dalton

KSHV – Kaposi's sarcoma-associated herpesvirus

LeTx – anthrax lethal toxin

LF – lethal factor

LFn – N-terminal domain of LF

LRR – Leucine rich repeat

MAPKKs – MAP kinase kinases

MAVS – mitochondrial antiviral signalling protein

NACHT or NBD – nucleotide-binding and oligomerization domain

NAIP – Neuronal apoptosis inhibitory protein

Ni-NTA – Nickel Nitrilotriacetic acid

NLRC – NLR family CARD domain containing protein

NLRP – NLR family PYRIN domain containing protein

NLRs – Nucleotide-binding domain and leucine-rich repeat containing protein

NOD – Nucleotide-binding and oligomerization domain

PA – protective antigen

PAMPs – Pathogen-associated molecular pattern

PRRs – Pattern recognition receptors

PYD – pyrin domain

PYHIN – pyrin and HIN domain-containing

ROS – reactive oxygen species

SDS-PAGE – Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SOCE – store-operated Ca^{2+} entry

T3SS – type 3 secretion system

TRX – thioredoxin

TXNIP – thioredoxin interacting protein

VACV – Vaccinia virus

VDAC – voltage dependent anion channel

VH – variable heavy chain domain

VHHs – Camelid single-chain antibodies

VL – variable light chain domain

1 Introduction

1.1 Inflammasomes

The innate immune system is dependent on conserved sets of receptors, present in many types of immune and non-immune cells to sense pathogens or other detrimental threats. This branch of the immune system can thus respond instantaneously to potentially dangerous insults. Once a pathogen is detected, a highly integrated network of cytokines and intracellular signalling pathways is activated in order to recruit immune cells to the site of pathogen invasion, and to impose an "armed" state on potential host cells capable of executing appropriate countermeasures (Murphy, Travers, & Walport, 2008).

Innate immune receptors sense pathogen-associated molecular patterns (PAMPs) or danger-associated molecular patterns (DAMPs) using specifically evolved protein families of pattern recognition receptors (PRRs) (Medzhitov & Janeway, 2000). In cells of the myeloid lineage, upon detection of exogenous or endogenous pathogens as well as perturbations of tissue homeostasis, some PRRs of the family of NOD-like receptors (NLRs) and AIM2-like receptors (ALRs) can assemble into large macromolecular complexes termed inflammasomes. These integrate intracellular inputs to elicit an inflammatory response in the respective tissues (Strowig, Henao-Mejia, Elinav, & Flavell, 2012).

NLR proteins contain an N-terminal protein-protein interaction domain, a NACHT domain of the AAA+ ATPase family, and a C-terminal LRR domain. NLRs are hypothesized to bind ligands through the LRR domain, although direct interaction has been shown only in the case of NAIP family members. NLR activation most likely involves dNTP binding by the NACHT domain, followed by dNTP hydrolysis and NACHT-domain mediated NLR oligomerization. The N-terminal effector domain of NLRs either directly recruits pro-caspase-1, or does so via the adaptor protein ASC. Oligomerization of pro-caspase-1 triggers autocatalytic cleavage and activation of caspase-1, which then catalyses proteolytic processing of pro-IL-1β and pro-IL-18 to produce active IL-1β and IL-18, respectively. Both cytokines, as

well as a number of other cytosolic proteins, are then secreted via a non-conventional pathway to assist in a strong immune response. In addition to cytokine secretion or as an alternative effector mechanism, certain types of inflammasomes also trigger pyroptosis (**Figure 1**). This form of programmed cell death has features of apoptosis, but unlike apoptotic cells causes a strong inflammatory signal (von Moltke, Ayres, Kofoed, Chavarría-Smith, & Vance, 2013).

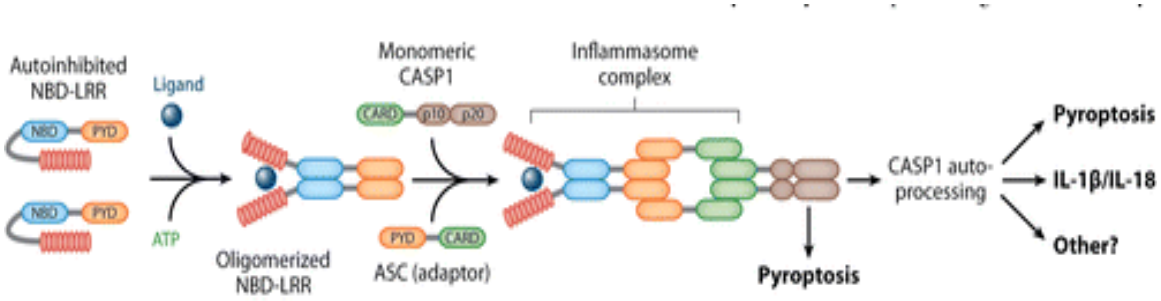

Figure 1 **Mechanism of inflammasome complex formation.** Under physiological conditions, NLR proteins are in an auto-inhibited state due to folding of the LRR domain onto the NACHT domain. This auto-inhibition is removed in the presence of pathogen-associated molecular patterns (PAMPs) from microorganisms or danger-associated molecular patterns (DAMPs) from endogenous damage signals. This subsequently leads to an opened confirmation and an exposure of the NACHT domain to its periphery. Thus, NLRs are able to oligomerize and recruit pro-caspase 1 either directly via an intrinsic CARD domain or via the apoptosis-associated speck-like protein (ASC) that also contains a CARD domain and serves as an adaptor protein. Caspase-1 is believed to be activated by proximity-induced auto-proteolysis (auto-processing), leading to downstream effector functions such as pyroptosis or the maturation and secretion of pro-inflammatory cytokines such as interleukin-1β (IL-1β) and IL-18 (von Moltke et al., 2013).

1.1.1 The NLR family and its domain architecture

The NLR family consists of 22 human genes and many more mouse genes. The family is characterized by a central nucleotide-binding and oligomerization domain, termed either NOD or NACHT, which is the only domain present in all NLRs. This central NACHT domain is flanked on one side usually by C-terminal Leucine-rich repeats (LRRs) and on the other side by an N-terminal protein–protein interaction domain necessary for downstream signalling, which can be either caspase recruitment (CARD) or pyrin (PYD) domains. Three subfamilies within the NLR family can be concluded based on the phylogenetic analysis of the central NACHT domain of different members of the family: The NOD subfamily with NOD 1-2, NOD3 = NLRC3, NOD4 = NLRC5, NOD5 = NLRX1, CIITA as members; the NLRP

subfamily with NLRP1-14, also called NALPs as members and the IPAF subfamily consisting of IPAF = NLRC4 and NAIP (**Figure 2**).

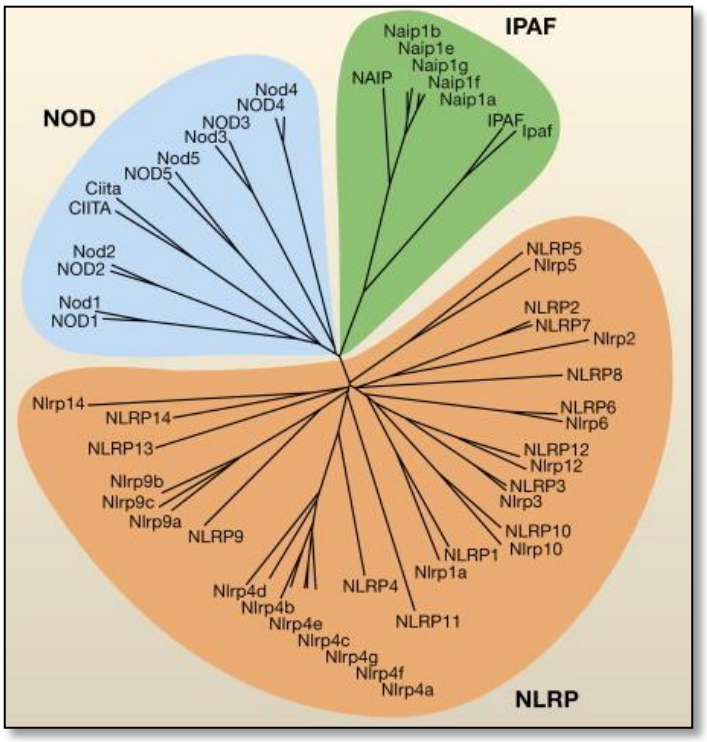

Figure 2 **Phylogenetic analysis of NACHT domains from each NOD-like receptor (NLR).** Three distinct subfamilies are determined within the NLRs: NOD, NLRP and IPAF subfamilies. Human NLRs are shown in capital letters, mouse NLRs in lowercase letters (Schroder & Tschopp, 2010).

The phylogenetic relationships between subfamily members are also strengthened by similarities in the domain architecture of different NLRs (**Figure 3**). All NLRPs contain PYD, NACHT and LRR domains, except NLRP10 that lacks Leucine-rich repeats. NLRCs contain typically CARD domains (Schroder & Tschopp, 2010). The human NLRP1 consists of both potential protein-protein interaction domains, the PYD domain and the CARD domain. Thus, it can recruit pro-caspase 1 either directly via the CARD domain or alternatively via the adaptor protein ASC. It is believed that downstream effector mechanisms are influenced by the different manner of recruitment of procaspase-1. NLRP1 is also the sole receptor with a FIIND domain making it a unique member of the NLR family. The function of the FIIND domain is yet to be determined. NAIP is the only NLR protein with a baculoviral inhibition of apoptosis protein repeat domain.

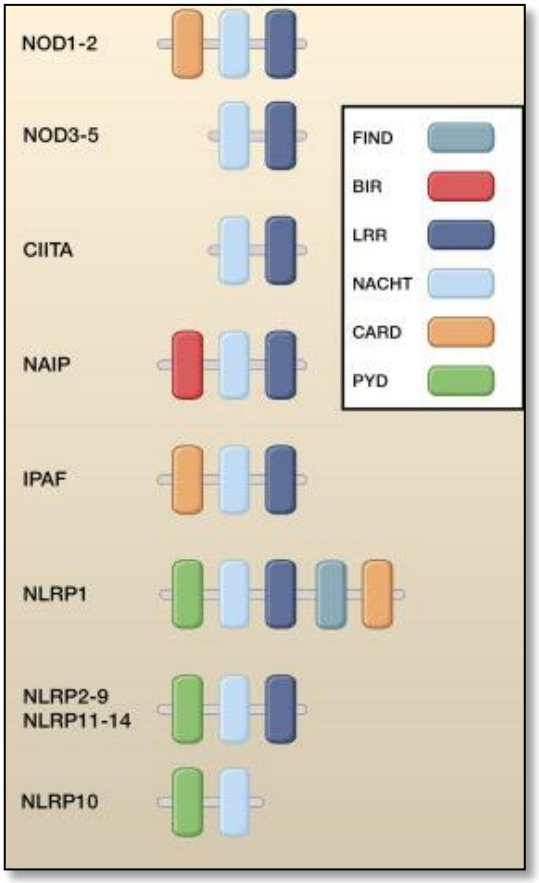

***Figure 3* Domain structure for human NLRs.** They all contain a nucleotide-binding and oligomerization domain (NBD or NACHT), carboxy-terminal leucine-rich repeat (LRR), and commonly a pyrin (PYD) domain or a caspase activation and recruitment domain (CARD), or both (Schroder & Tschopp, 2010).

1.1.2 The PYHIN family

Another family of inflammasome proteins next to the NLRs is the PYHIN family, which is relatively small compared to the NLR family. Members of this family are AIM2 and IFI16 proteins. AIM2 was described as the first non-NLR member to form an inflammasome scaffold. They are characterized by having additionally to a PYD, a HIN200 domain (**Figure 4**), which is involved in ligand binding (Strowig et al., 2012). The AIM2 inflammasome is composed of AIM2 itself, ASC and Caspase-1. By means of the PYD domain, AIM2 proteins can interact, as for NLRPs, via homotypic PYD-PYD interactions with ASC, allowing ASC further to recruit Procaspase-1.

Figure 4 **Domain architecture of the PYHIN family.** AIM2-like receptors (ALRs) consist of a PYD protein–protein interaction domain and a HIN200 domain that is acting as a sensor (Strowig et al., 2012).

1.1.3 Inflammatory caspases

Caspases are cysteine proteases that initiate or execute cellular programs that can finally lead to inflammation via proteolytic activation of cytokines or cell death via apoptosis. Caspases can be divided into two large groups, the effector versus the initiator caspases. Effector caspases cleave particular cellular substrates directly to produce apoptotic conditions and are often activated by initiator caspases. Effector caspases in humans are caspase-3, caspase-6 and caspase-7. They are commonly activated by initiator caspases such as caspase-8, caspase-10, caspase-2 and caspase-9. Other initiator caspases are caspase-1, caspase-4 and caspase-5 (Martinon & Tschopp, 2004).

Another differentiation of caspases can be made by distinguishing between apoptotic and inflammatory caspases. The initiator caspases -8, -9, -10 and the effector caspases -3, -6, -7 are assigned for apoptotic tasks while human caspases -1, -4 and -5 and mouse caspases -11 and -12 are regulating inflammatory processes in the cell (**Figure 5**).

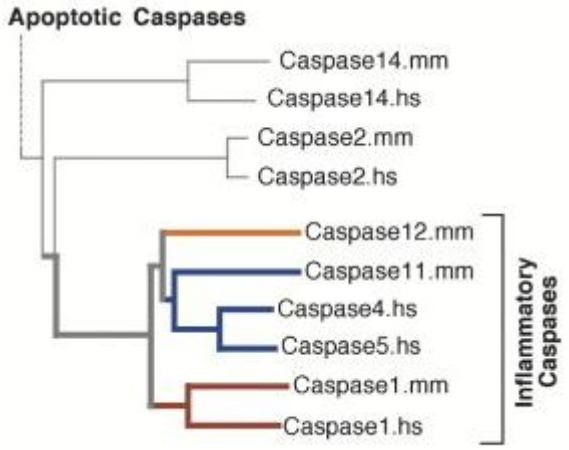

Figure 5 Classification of Caspases. Two major subfamilies of Caspases are the apoptotic caspases (caspase-8, -9, -10, -3, -6, -7) and the inflammatory caspases (caspase-1, -4, -5, -11, -12). Caspase 2 and caspase 14 do not belong to any group and form the own cluster (Martinon & Tschopp, 2004).

Of the inflammatory caspases, caspase-1 is the best characterized. Caspase-1 was originally identified as a result of attempts to purify the enzyme responsible for the processing of pro-IL-1β, previously known as IL-1β converting enzyme (ICE) (Thornberry et al., 1992). IL-1β mRNA encodes a cytosolic 33 kDa pro-IL-1β protein that requires proteolytic cleavage in order to be secreted and to function as an active protein. Cleavage gives rise to the 17 kDa C-terminal mature cytokine. The requirement for caspase-1 in IL-1β maturation was revealed by the generation of mice deficient in caspase-1. These mice have a defect in the maturation of pro-IL-1β and are resistant to the lethal effect of endotoxins.

Besides processing of pro-IL-1β, caspase-1 additionally activates another proinflammatory cytokine, IL-18.

Murine caspase-11 is also part of the inflammatory caspase subfamily. Caspase-11 deficient mice have a similar phenotype compared to caspase-1 deficient mice. Caspase-11 deficient mice show resistance to an endotoxic shock induced by bacterial lipopolysaccharide (LPS). They failed to produce mature IL-1β after LPS stimulation (Wang et al., 1998).

1.1.4 Canonical inflammasomes and their triggers

NLRs such as NLRP1b, NLRP3, and NLRC4, as well as the ALR AIM2 represent the best-characterized inflammasomes. NLRP3 is still the most intensively investigated NLR. It is activated by a vast majority of PAMPs and DAMPs. On the other hand, NLRP1 is activated by anthrax lethal toxin and NLRC4 has bacterial type III secretion system (T3SS) components and flagellin as its agonists. In contrast to the NLRs, AIM2 gets activated by sensing bacterial or viral double–stranded DNA in the cytoplasm.

1.1.4.1 The NLRP1 inflammasome

NLRP1b, a mouse inflammasome protein, is activated only by a single signal, the anthrax lethal toxin (LeTx), one of the main virulence factors of *Bacillus anthracis*. LeTx is composed of three proteins that are nontoxic on their own: protective antigen (PA), lethal factor (LF) and edema factor (EF). PA attaches to cell surface receptors where it forms a channel in the cell membrane facilitating the translocation of the other two proteins, LF, a zinc metalloprotease and EF which both disturb cellular processes once inside the cell. Proteolytic targets of LF inside the cell are MAP kinase kinases (MAPKKs) which after cleavage get inactivated, thus leading to an impairment especially of the immune system. LeTx can also lead to pyroptosis of macrophages in specific mice strains (von Moltke et al., 2013).

The mechanism of NLRP1b activation by LeTx is not fully understood yet but activation of NLRP1b is dependent on proteolytic activity of the metalloprotease LF since the recognition of the catalytic dead version of LF only does not induce pyroptosis. While the cleavage of MAPKKs is not believed to activate murine NLRP1b, it was shown that the cleavage of the rat NLRP1 allele is required for activation as mutations that made rat NLRP1 cleavage resistant, eliminated its function to activate caspase-1 (Levinsohn et al., 2012). It is likely that NLRP1 alleles of mouse and rat share a common activation mechanism although the cleavage site in rat does not appear to be conserved in any mouse. Thus, it remains unclear if mouse NLRP1b is also directly cleaved by LF and the exact mechanism is still to be discovered (**Figure 6**).

In contrast to the murine NLRP1b inflammasome, human NLRP1 does not respond to LeTx and human macrophages do not undergo pyroptosis in response to LeTx but human NLRP1 inflammasome can be activated by muramyl dipeptide, a bacterial cell wall component. However, the activation mechanism is not clear either (Faustin et al., 2007).

It was also shown that virus – encoded antagonists of NLRP1 can act as viral PAMPs and be recognized by NLRP1 inflammasomes. Kaposi's sarcoma-associated herpesvirus (KSHV) Orf63 is an open reading frame encoded in the genome of KSHV. The encoded protein, viral NLRP1 has been shown to be a viral homolog of human NLRP1 without the CARD and PYD effector domain. This suggests that Orf63 may function as an inhibitor of NLRP1. Additionally, although Orf63 did not show significant similarity to NLRP3, it also blocked NLRP3 activity. Inhibition of KSHV Orf63 expression also led to increased expression of IL-1β and IL-18 during the KSHV life cycle. Thus, Orf63 is capable of broad inhibition of NLR inflammasome responses such as caspase-1 activation (Gregory et al., 2011).

A Vaccinia virus (VACV) anti-apoptotic B-cell CLL/lymphoma 2 (Bcl-2) homolog, F1L, was demonstrated to bind and inhibit NLRP1 in vitro. Moreover, infection of macrophages in culture with virus lacking F1L (ΔF1L) caused increased caspase-1 activation and IL-1β secretion compared with wild-type virus. Virulence of ΔF1L virus was attenuated in vivo, causing altered febrile responses, increased proteolytic processing of caspase-1, and more rapid inflammation in lungs of infected mice without affecting cell death or virus replication. Cellular infection with wild-type F1L reconstituted virus-suppressed IL-1β production, whereas mutant F1L did not. In contrast, both wild-type and mutant versions of F1L equally suppressed apoptosis. In vivo, the NLR nonbinding F1L mutant virus exhibited an attenuated phenotype similar to ΔF1L virus, thus confirming the importance of F1L interactions with NLRP1 for viral pathogenicity in mice. Altogether, these findings reveal a unique viral mechanism for evading host innate immune responses (Gerlic et al., 2013).

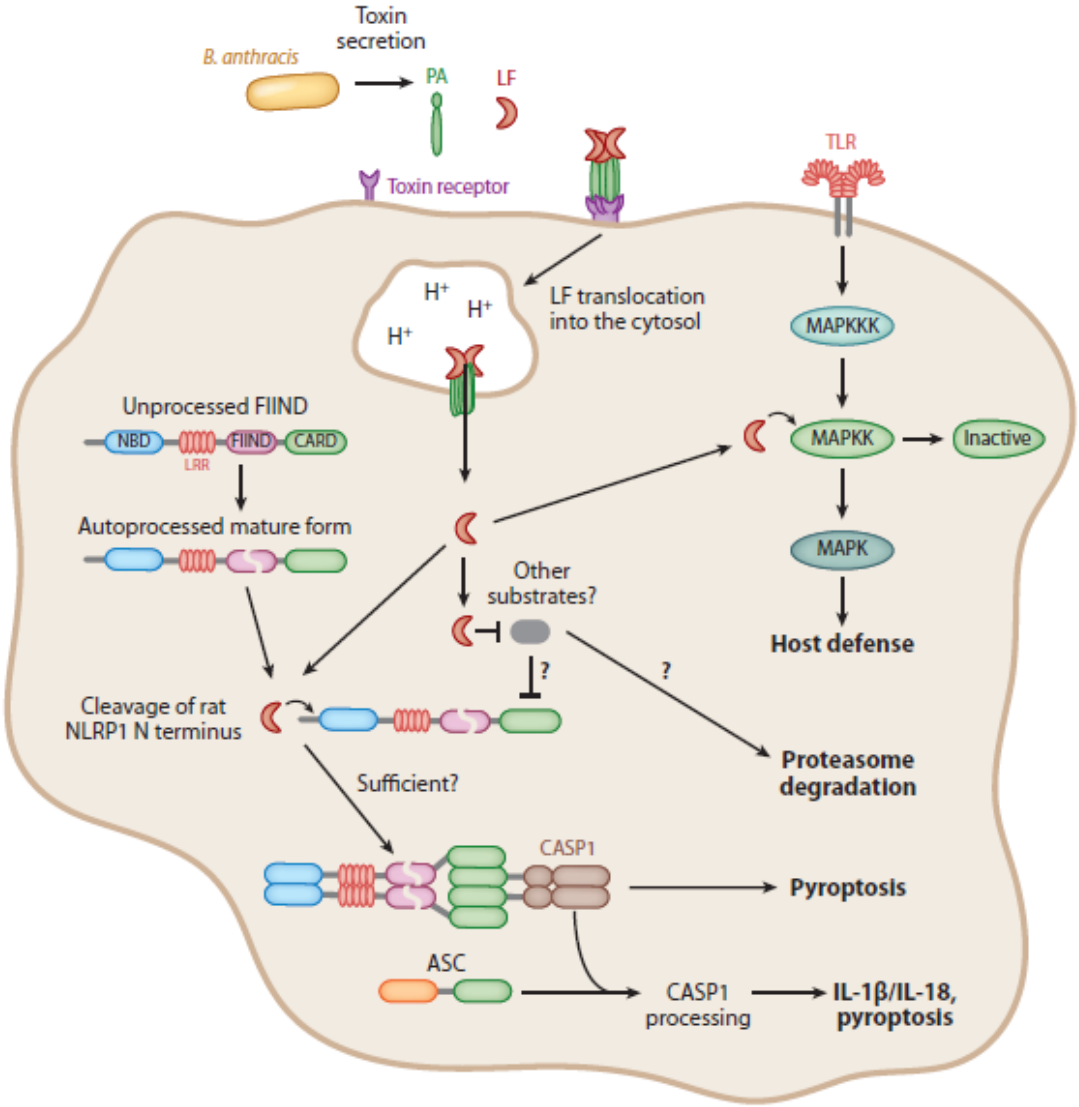

Figure 6 **Activation of the NLRP1b by anthrax lethal toxin.** *Bacillus anthracis* secretes the three constituents of Anthrax Lethal Toxin (LeTx). Protective antigen (PA) binds to the host cell's toxin receptor and starts to oligomerize and bind Lethal Factor (LF). LeTx is then endocytosed in an acidified endosome from where LF is translocated into the host cell's cytosol. LF attacks and cleaves MAP kinase kinases (MAPKKs), interfering with many host defense pathways. NLRP1 must experience autoproteolytic processing in the FIIND domain prior to LF activation. LF cleaves the N-terminus of sensitive rat NLRP1 alleles, but it is not certain that this cleavage is sufficient for activation. The activity of the proteasome is also needed for NLRP1 activation, thus LF may also cleave not yet defined substrates that become destabilized and degraded by the proteasome and cannot act as negative regulators of NLRP1 anymore. Once NLRP1b is activated, it can either recruit Caspase- 1 directly to induce pyroptosis or recruit it via the adaptor protein ASC to be also able to process the cytokines pro-IL-1β and pro-IL-18 to its active forms (von Moltke et al., 2013).

Another major difference between mouse and human NLRP1 is its domain structure. While human NLRP1 contains both protein-protein interaction domains, PYD and CARD, mouse NLRP1 lacks functional PYD domain and has only a CARD domain (**Figure 7**). Thus, murine NLRP1 is unable to interact with the adaptor protein ASC. Nevertheless, even without the recruitment of ASC, caspase-1 gets activated by NLRP1b in mouse macrophages. Human NLRP1 could also bypass the recruitment of ASC in inflammasome activation as it possesses a CARD domain but ASC inclusion in the inflammasome complex enhances human NLRP1 activity.

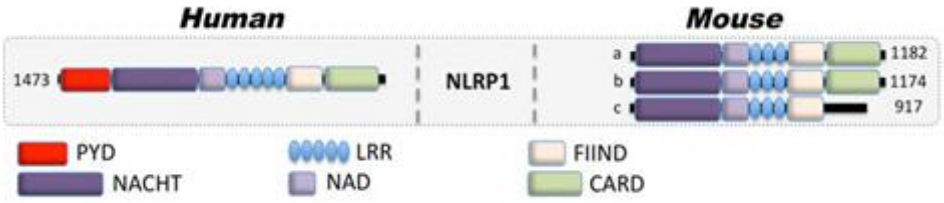

Figure 7 **Domain architecture of human NLRP1 and murine NLRP1.** Human NLRP1 contains PYD and CARD domains while the murine NLRP1 versions lack a PYD domain. Both NLRP1 proteins possess a FIIND domain (Ratsimandresey, Dorfleutner, & Stehlik, 2013).

The human versions as well as the mouse version of NLRP1 contain a unique domain termed FIIND (domain with function to find), which cannot be found in other NLRs. It resembles the ZU-5 and UPA domains which both have the ability to undergo autoproteolysis at a conserved Ser-Phe/Ser motif (where 'Phe/Ser' indicates either phenylalanine or serine). Similar intramolecular autoproteolysis was observed in the FIIND domain of murine and human NLRP1. Mutations that inactivate the ability for self-cleavage do not allow a response of NLRP1b to anthrax lethal toxin. Therefore, the autoproteolytic self-cleavage of NLRP1b within the FIIND domain seems to be a maturation step required for downstream signalling of NLRP1b.

1.1.4.2 The NLRP3 inflammasome

NLRP3, also known as Cryopyrin, NALP3, PYPAF1, CIAS1, CLR1.1, is the best-studied member of the NLRP family. It was initially discovered by positional cloning in the search for the genetic cause of a group of auto-inflammatory diseases, now referred to as Cryopyrinopathies or Cryopyrin-associated periodic syndromes

(CAPS). While initial overexpression studies suggested that NLRP3 affects NF-κB activation, NLRP3-deficient mice displayed defects restricted to inflammasome activation. In contrast to other NLRs, NLRP3 is activated by, and responds to a diverse set of stimuli originating from microbial pathogen-associated molecular patterns (PAMPs) or from environmental and endogenous danger signals danger-associated molecular patterns (DAMPs), which can be of either soluble or particulate matter. Microbial activators include various Gram-positive and -negative bacteria (*Listeria monocytogenes*, *Staphylococcus aureus*, *Vibrio cholera*, *Neisseria gonorrhoeae*, fungi (*Candida albicans*, *Saccharomyces cerevisiae*), RNA and DNA viruses (adenovirus, influenza virus, Sendai virus), as well as protozoa (*Plasmodium malariae*). The fact that NLRP3 also senses sterile environmental and endogenous stress signals, and promotes inflammatory responses further expands the repertoire of NLRP3 reactivity. Environmental triggers include the particulates alum, asbestos, silica, skin irritants (trinitrochlorobenzene, trinitrophenylchloride, and dinitrofluorobenzene), and even UV-B radiation. An increasing complexity of endogenous danger signals is now also known to activate NLRP3, since the discovery that monosodium urate crystals (MSU) and pyrophosphate dihydrate crystals are able to activate NLRP3. Other known NLRP3-inducing crystals are cholesterol, amyloid deposits, hydroxyapatite crystals, and hyaluronan. In addition to these crystalline danger signals, NLRP3 also senses non-crystalline stress signals, including ATP, high glucose, and saturated fatty acids. The mechanism that causes NLRP3 activation in response to so many different stimuli is still controversial and more discussed below.

In contrast to other NLR genes such as the gene responsible for the expression of NLRC4 and NLRP1, NLRP3 is not continuously expressed in most resting cells. Activation of the NLRP3 inflammasome requires therefore two signals. The first signal is the NF-κB dependent transcriptional induction of NLRP3 downstream of Toll-like receptors while the second signal is an agonist that is able to induce oligomerization. The first signal is called the transcriptional priming and represents a critical checkpoint: once primed, the NLRP3 inflammasome can be activated by the vast amount of stimuli mentioned before. Given the variety of NLRP3 agonists, it is accepted that they basically do not act as direct ligands of NLRP3 but instead

lead to one or more disruptions of the host cell's physiology that are sensed by the NLRP3 inflammasome. A unifying mechanism that leads to NLRP3 activation remains elusive but three main mechanisms have been proposed: (1) potassium efflux, (2) mitochondrial dysfunction and generation of mitochondria-derived reactive oxygen species (ROS), and (3) phagolysosomal destabilization in response to particulates.

(1) ATP is released into the extracellular space after tissue injury and cell death. The extracellular ATP then triggers the purogenic P2X7R, which is an ATP-gated K^+ ion channel that facilitates K^+ efflux, which activates the NLRP3 inflammasome. Although the interaction of P2X7R with the hemichannel protein pannexin-1 was initially proposed to allow influx of PAMPs/DAMPs into the cytosol through a 900 kDa pore, based on pannexin-1 blocking peptides. However, this scenario is not any longer considered to play any role in NLRP3 activation, since pannexin-1-deficient macrophages exhibit no defect in NLRP3 activation. Similarly, microbial pore-forming toxins (such as haemolysins) on the cell surface or on phagolysosomal membranes trigger K^+ efflux and NLRP3 activation. The precise mechanism by which low K^+ levels affect NLRP3 activation is not understood. While K^+ efflux in NLRP3 activation is well-established, Ca^{2+} mobilization and Ca^{2+}-mediated signalling has also been linked to NLRP3 activation, but this is controversial. ATP induced Ca^{2+} signalling is regulated by the calcium-sensing receptor (CASR), phospholipase C-mediated generation of inositol-1,4,5-trisphosphate, inositol-1,4,5-trisphosphate receptor (IP3R) mediated release of Ca^{2+} from the ER, and store-operated Ca^{2+} entry (SOCE) mediated influx of extracellular Ca^{2+}, which is important for NLRP3 inflammasome activation by extracellular ATP. Hence, caspase-1 and IL-1β processing and release are also controlled by phospholipase C, IP3R, and SOCE. In addition to ER stores, Ca^{2+} influx has also been proposed to occur through the plasma membrane channel TRPM2. However, the involvement of Ca^{2+} in NLRP3 activation has been recently disputed and linked to the precipitation of insoluble particulates, which then activates NLRP3 in a K^+ efflux-dependent manner.

(2) A second mechanism proposed to contribute to NLRP3 activation, involves mitochondria and generation of ROS. However, involvement of mitochondria and mitochondria-derived molecules, including mROS in NLRP3 inflammasome activation is controversial with arguments found for and against throughout the literature. ATP-mediated ROS production is necessary for caspase-1 activation and initial studies linked NADPH oxidase-produced ROS to NLRP3 activation. Interaction of NLRP3 with the thioredoxin (TRX)-interacting protein TXNIP through its LRR, has been proposed as a mechanism, since NLRP3 agonists caused ROS-dependent dissociation of TXNIP from TRX. However, subsequent studies in chronic granulomatous disease (CGD) patients disproved these earlier observations. CGD patients lack p22phox, which is essential for the proper function of the NADPH oxidase Nox1-4, but CGD macrophages showed either no defect in IL-1β release, or even an increased caspase-1 activity and IL-1β release. This is in agreement with the finding that ROS actually inhibit caspase-1 through reversible oxidation and glutathionylation of two redox-sensitive cysteine residues (C397 and C362), which is in contrast to an earlier study. Furthermore, the crystal structure of the NLRP3 PYD revealed that it is unique in containing a disulphide bond between C8 and C108, which could be important for redox potential-dependent regulation. Mitochondria are the other main source for ROS, and mitochondria have been linked to NLRP3 activation through mROS generation and as a platform for inflammasome assembly. While mROS are necessary for homeostasis, cellular stress including hypoxia, acidosis, changes in intracellular ionic milieu and membrane damage are known to promote release of mROS. It has also been proposed that all NLRP3-activating stimuli induce apoptosis in target cells, thereby causing opening of the voltage dependent anion channel (VDAC), decreasing the mitochondrial membrane potential ($\Delta\Psi$), generation of mROS, which in turn promotes mitochondrial permeability transition and cytosolic release of mitochondrial DNA leading to NRLP3 activation. Accordingly, inhibiting VDAC1 and 2, but not VDAC3 decreased NLRP3 activation. Furthermore, defect mitophagy or autophagy, and consequently, accumu-

lation of damaged mitochondria, causes NLRP3 activation and elevated IL-1β levels. However, autophagy is also involved in degrading ubiquitinated inflammasomes through recruiting the autophagic adaptor p62. Moreover, it has also been proposed that mitochondrial damage does not contribute to NLRP3 activation, but can occur in response to NLRP3-activating stimuli at later time points. Additional support for a significance of mitochondria as a platform facilitating NLRP3 activation is supported by studies showing that ER-localized NLRP3 is redistributed to mitochondria upon activation. This transport has been shown to occur by a dynein-mediated movement of mitochondria in response to reduced NAD^+ levels caused by defect mitochondria. This facilitates inactivation of sirtuin 2, an NAD^+-dependent α-tubulin deacetylase, and consequently, accumulation of acetylated α-tubulin necessary for mitochondrial movement. However, mitochondrial ASC and NLRP3 localization is also controversial. Yet another study proposed that the CARD-containing adaptor mitochondrial antiviral signalling protein (MAVS) is necessary for full NLRP3 inflammasome activation through targeting NLRP3 to mitochondria, which requires a short peptide within the PYD. However, MAVS appears to be only necessary for non-crystalline activators, suggesting that other adaptors might be involved in crystalline responses. However, this finding is controversial and has only been partially reproduced in the context of Sendai virus infection.

Altogether, there is widely conflicting information of the involvement of mitochondria and mROS to NLRP3 activation. Analyses of various mitochondria-targeted drugs suggested an involvement of mitochondria and mROS dependent and independent mechanisms. But a recent study suggested that, rather than acting on the signal 2 of NLRP3 inflammasome activation, ROS might only be necessary for inflammasome priming through NF-κ activation or deubiquitination. Yet, these studies have also been disputed and attributed to the use of high concentrations of ROS inhibitors and proposed that ROS do not play any role in signal 1 and 2.

(3) Reactive-oxygen species are also generated upon lysosomal rupture and leakage of lysosomal contents in the cytosol, as a consequence from the digestion

of particulate matter or infection. Phagolysosomal destabilization itself, rather than the absorbed particulate matter, seems to be perceived as the danger signal leading to NLRP3 activation. Abnormal release of H^+ into the cytosol, either from lysosomal rupture or from the activation of a proton-selective ion channel, such as the M2 channel upon infection with Influenza virus, activates NLRP3. The lysosomal-derived protease cathepsin B is one of the lysosomal factors that activate NLRP3. However, this finding was dependent on a chemical cathepsin B inhibitor, while cathepsin B−/− macrophages do not show defects in caspase-1 activation, suggesting off target effects of this inhibitor (Ratsimandresy et al., 2013)

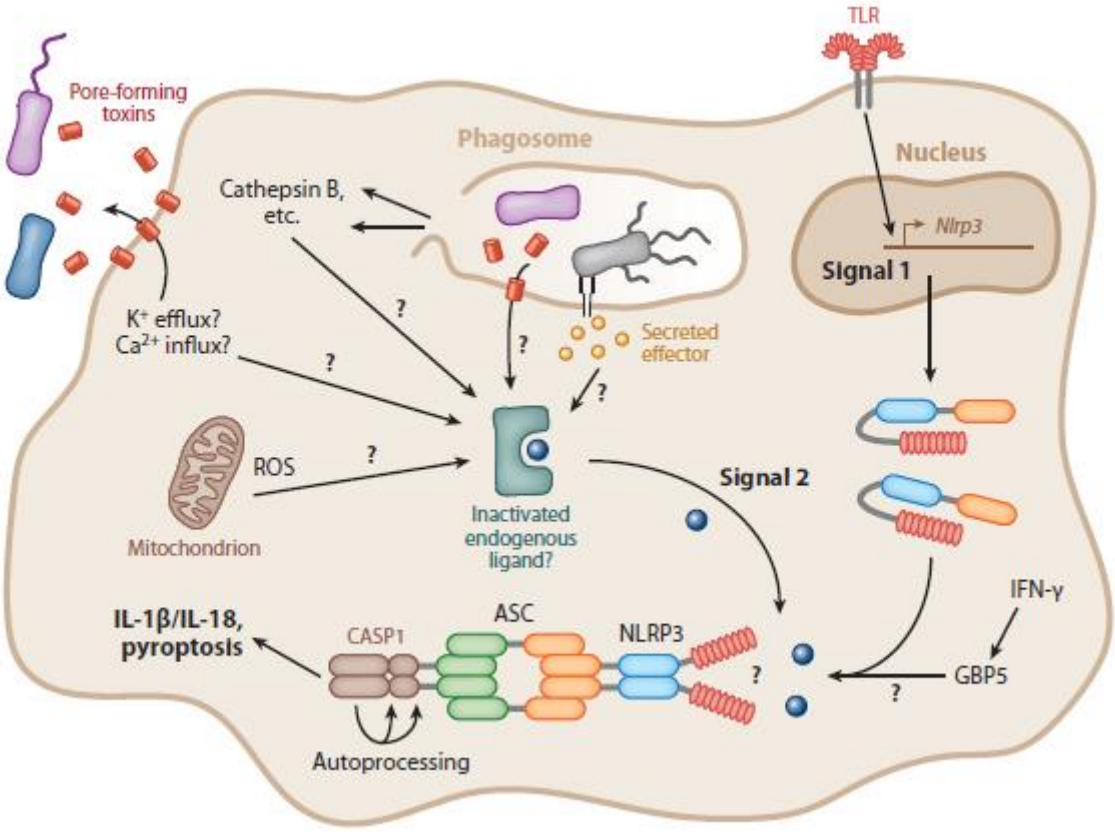

Figure 8 **Activation of the NLRP3 inflammasome.** Activation of the NLRP3 inflammasome requires two signals: Transcriptional induction of NLRP3 (Signal 1) and NLRP3 oligomerization driven by an agonist (Signal 2). The unifying mechanism of the second signal remains unresolved. The model shown here postulates an endogenous ligand for NLRP3 that is activated downstream of one or more disruptions of host cell physiology, including generation of reactive oxygen species (ROS), potassium (K^+) efflux, calcium (Ca^{2+}) influx, and/or phagolysosomal rupture or leakage (von Moltke et al., 2013).

1.1.4.3 The NLRC4 inflammasome

The NLRC4 inflammasome involves mixed oligomers of two distinct NLRs, NLRC4 and a NAIP family member. They are stimulated by type 3 secretion system (T3SS) needle proteins in human cells or by T3SS and T4SS components and flagellin in murine cells, all of which are directly bound by different members of the NAIP family. Inflammasome assembly can occur either directly via homotypic CARD-CARD interactions with Caspase-1 or via the adaptor ASC. In fact, ASC is not an absolute requirement of the NLRC4 inflammasome but when present, it substantially augments NLRC4-mediated inflammasome activation (Mariathasan et al., 2004).

The inflammasome structure formed by these proteins was unveiled only recently when two independent groups proposed a model for NAIP5/NLRC4 inflammasome assembly. Using the transfection of inflammasome components and microbial molecules in HEK 293T cells or followed by biochemical assays, the ability of flagellin from different bacterial species to bind NAIP5 was demonstrated (Kofoed & Vance, 2012; Zhao et al., 2011). This interaction was dependent on the three leucine residues of the C-terminal portion of flagellin. Furthermore, after the recognition of flagellin, a physical association between NAIP5 and NLRC4 was demonstrated, resulting in the formation of an oligomeric complex. Reconstitution experiments using truncated receptor variants showed that NAIPs are upstream of NLRC4 and suggest that they interact via the NBD domain. Notably, NAIP6 worked similarly to NAIP5, as it induced the oligomerization of NLRC4 in response to flagellin, and this could explain the response of $NAIP5^{-/-}$ cells to high concentrations of flagellin. NAIP1 and NAIP2 also recruit NLRC4 in response to the bacterial needle and rod proteins of T3SS, respectively. Therefore, NAIP proteins seem to be the universal sensors of cytosolic flagellin and secretory complex proteins, whereas NLRC4 acts as an adaptor molecule and is responsible for the recruitment and activation of caspase-1. It is noteworthy that there is only one functional NAIP found in humans, which is not activated by flagellin but is able to detect needle proteins of T3SS, similar to NAIP1.

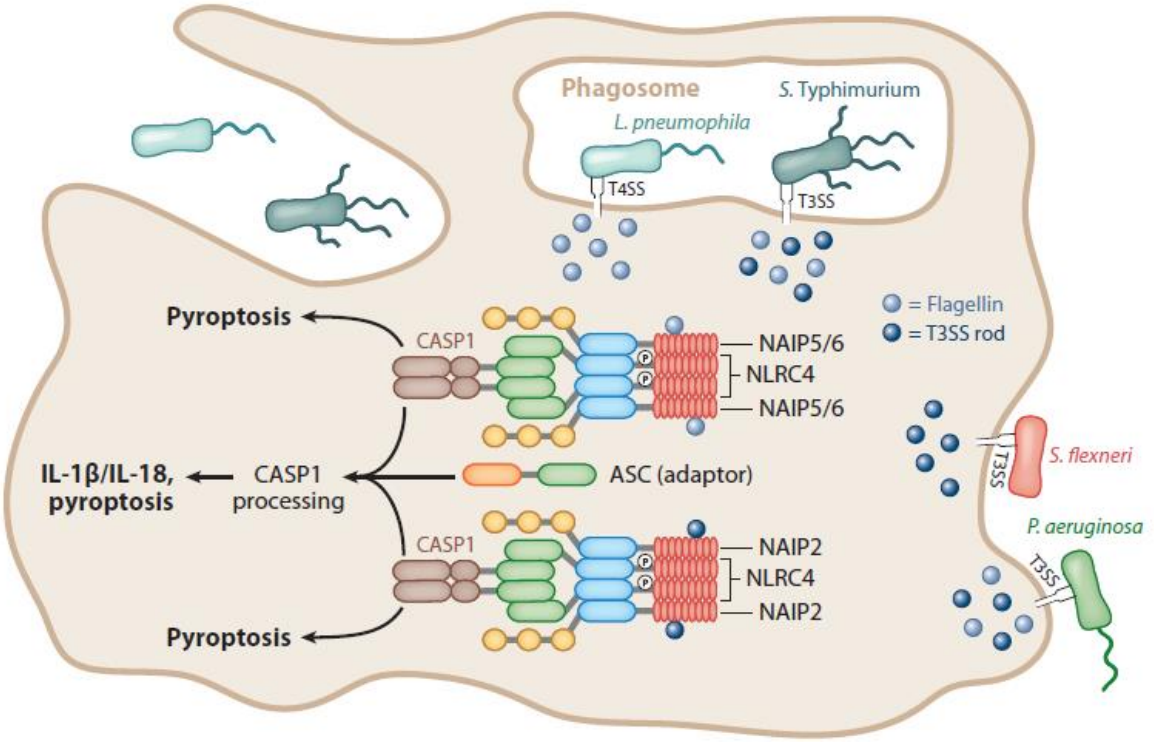

Figure 9 **Activation of NAIP/NLRC4 inflammasomes by bacteria.** NAIP/NLRC4 inflammasomes are activated following detection of flagellin or T3SS rod proteins secreted into the host cytosol during bacterial infection. Ligand specificity is determined by the NAIP proteins: NAIP5 and NAIP6 recognize flagellin, and NAIP2 recognizes the T3SS rod. The CARD-containing NLRC4 serves as an adaptor for recruitment of CASP1 downstream of the NAIPs. Phosphorylation of NLRC4 is required for NLRC4 function. NAIP, NLRC4, and CASP1 are sufficient to initiate pyroptosis, but processing of the IL-1β and IL-18 cytokines requires recruitment of the ASC adaptor into the complex (von Moltke et al., 2013).

1.2 Camelid single-chain antibodies (VHHs)

The overall structure of immunoglobulin-γ (IgG) antibodies assembled from two identical heavy chain and two identical light chain polypeptides is well established and highly conserved in mammals. The light chain of these immunoglobulins comprises two domains, whereas the heavy chain folds into four domains. The sequence of the N-terminal domain of the heavy and light polypeptide chains varies between antibodies, designated as variable domains VH and VL. The paired variable heavy chain and variable light chain domains constitute the variable fragment (Fv) that recognizes the antigen. The remaining heavy and light sequences are more conserved (abbreviated as CH and CL, respectively). The two last CH re-

gions are important for recruitment of immune cells (e.g., macrophages and natural killer cells) or for effector functions such as complement activation.

One notorious exception to this conventional mammalian IgG structure is found in sera of Camelidae. In addition to the conventional heterotetrameric antibodies, these sera possess special IgG antibodies also known as heavy-chain antibodies. They are devoid of the light chain polypeptide and are unique because they lack the first constant domain (CH1). At its N-terminal region, the heavy chain of the homodimeric protein contains a dedicated variable domain, referred to as VHH, which serves to associate with its cognate antigen. The VHH in a heavy chain antibody is the structural and functional equivalent of the antigen-binding fragment (Fab) of conventional antibodies (**Figure 10**). The biological family Camelidae comprises camels (one-humped Camelus dromedaries and two-humped Camelus bactrianus), llamas (Lama glama and Lama guanicoe), and vicugnas (Vicugna vicugna and Vicugna pacos).

Noteworthy, immunoglobulins lacking light chains and devoid of a conventional CH1 also occur in nurse shark, wobbegong, and maybe spotted ratfish. These Ig-NAR ancestral antibodies have a variable domain, known as V-NAR, for antigen recognition. Although the variable sequences of Ig-NAR and those of camel heavy chain antibodies are quite diverse, they show a surprising structural and functional convergent evolution.

To study different human inflammasome complexes, it was intended to generate a collection of VHHs against inflammasome components of interest. Selection of these VHHs that bind to desired proteins can be achieved with the help of phagemid vectors and phage display technology through multiple rounds of biopanning.

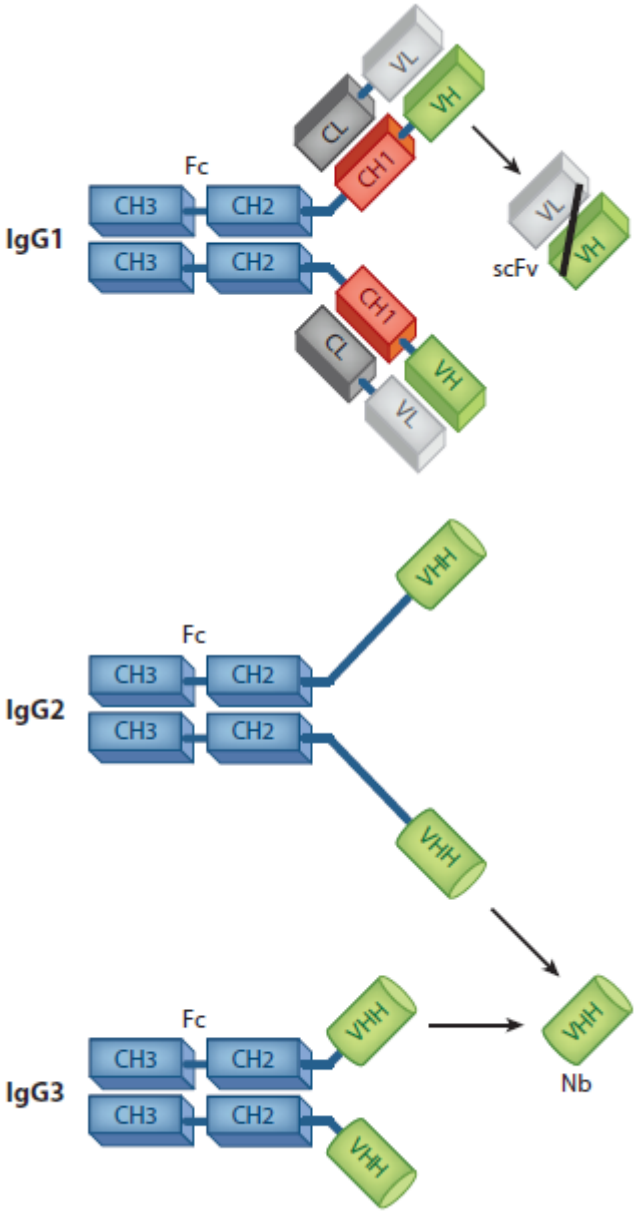

Figure 10 **Schematic representation of naturally occurring antibodies in sera of camelids:** Convential antibodies (IgG1) containing two light chains and two heavy chains and two types of homodimeric heavy chain antibodies (HCAbs), IgG2 and IgG3, which contain only heavy chains. The hinge of the IgG2 is longer than that of the IgG3. CH2 domains and CH3 domains form the constant part (Fc). In IgG1, the first two domains of the heavy chain and the light chain form the antibody-binding fragment (Fab). The smallest intact functional antigen-binding fragment that can be generated from conventional antibodies consists of a VH-VL pair linked by an oligopeptide and is known as single-chain variable fragment (scFv). The smallest intact functional antigen-binding fragment of heavy chain only antibodies is the single domain VHH, also known as Nanobody. (Muyldermans, 2013)

VHHs have many advantages for biotechnological applications, whereas the most important one is their high microbial production level (**Table 1**). Several advantages result from their single domain nature. VHH libraries generated from immunized camelids retain full functional diversity in contrast with the diminished diversity of conventional antibody libraries because of reshuffling of variable heavy chain and variable light chain domains during library construction. Thus, high affinity antigen-binding VHHs can be isolated directly by screening a limited number of clones from immune libraries without prior selection using phage display technologies (Frenken et al., 2000). In contrast to conventional antibodies, VHHs have been shown to have an increased thermostability. They remain functional at 90°C or after incubation at high temperatures (van der Linden et al., 1999). This high stability is mainly due to efficient refolding after chemical or thermal denaturation and to a lesser extent because of an increased resistance against denaturation. Furthermore, refolding of VHHs only requires domain refolding, whereas conventional antibodies also need association of the VL and VH domains.

Table 1 Advantages of camelid single-domain antibody fragments in contrast to conventional antibody fragments.

Advantage	**Molecular basis**
Easy genetic manipulation	Single-domain nature
Increased functional size of immune libraries	No decrease in library size because of reshuffling of VL and VH domains
Facile production of multivalent formats	More flexible linker design and no mispairing of VL and VH domain
Facile production of oligoclonal preparations from single cells	No mispairing of VL and VH domain
High physiochemical stability	Efficient refolding due to increased hydrophilicity and single-domain nature
High solubility	Increased hydrophilicity
Recognition of hidden antigenic sites	Small size and extended flexible CDR3
Rapid tissue penetration, fast clearance	Small size
Well expressed	Efficient folding due to increased hydrophilicity and single-domain nature

VHHs can also recognize antigenic sites that are generally not recognized by conventional antibodies. The ability to recognize these hidden antigenic sites results from their smaller size and the ability of the extended CDR3 loop to penetrate into such sites.

Because of their small size of approximately 15 kDa in contrast to a size of 150 kDa of a conventional antibody, VHHs can easily pass the renal filter, which has a cut-off of approximately 60 kDa, subsequently leading to their rapid blood clearance. Additionally, the small size also leads to faster and better tissue penetration. This can lead to essential advantages in different applications such as targeting tumours with VHHs coupled to toxic substances or in vivo diagnosis using imaging. On the other hand, for other therapeutic applications such as treatment of infectious diseases, the short serum half-life of about two hours is a disadvantage.

The unique properties of VHH would not only allow the generation of high-affinity binders against inflammasome components but also afford new possibilities to use VHHs to perturb protein functions. VHHs could be used to specifically prevent the association of distinct proteins with the inflammasome complex, while leaving the remainder of the complex intact or directly interact with particular domains of the NLRs.

2 Materials & Methods

2.1 Materials

2.1.1 Buffer solutions

2.1.1.1 Lysis buffer

50 mM NaH_2PO_4

300 mM NaCl

10 mM imidazole

pH 8.0

2.1.1.2 Washing buffer for Ni-NTA chromatography

50 mM NaH_2PO_4

300 mM NaCl

20 mM imidazole

pH 8.0

2.1.1.3 Elution buffer for Ni-NTA chromatography

50 mM NaH_2PO_4

300 mM NaCl

250 mM imidazole

pH 8.0

2.1.1.4 Gel filtration buffer for gel filtration chromatography

50 mM phosphate buffer

150 mM NaCl

10% glycerol

pH 7.4

Sterile filtered (0.2 µm)

2.1.2 Antibiotics

2.1.2.1 Ampicillin

2.1.2.2 Kanamycin

2.1.2.3 Tetracycline

2.1.3 Protease inhibitor tablets

2.1.4 Distilled water

2.1.5 Gels for SDS-PAGE

2.1.5.1 Separation gel (12%)

Protogel 30%	4 ml
4x resolving buffer	2.5 ml
Deionized water	3.39 ml
TEMED	10 µl
10% APS	100 µl

2.1.5.2 Stacking gel

Protogel 30%	1.3 ml
Stacking buffer	2.5 ml
Deionized water	6.1 ml
1% Bromophenol Blue	10 µl
TEMED	10 µl
10% APS	50 µl

2.2 Methods

2.2.1 Miniprep

According to E.Z.N.A. Plasmid Mini Kit I

2.2.2 Transformation

100 µl of E.coli DH5α cells were used when cells were needed for cloning while 100 µl of E.coli BL21 cells were used when transforming for protein expression. 1 µl of plasmid DNA was added to the cells and the mixture was then incubated for 10 minutes on ice. Incubation in a 42°C water bath followed for 1 min 30s and again the mixture was incubated 1 min on ice. 1 ml LB medium was added in a sterile manner followed by the recovery phase at 37°C for 20 min. The bacteria were then plated on agar plates or used to start a starter culture.

2.2.3 Primer design

Primers were designed using Invitrogen's online tool OligoPerfect™ Designer.

2.2.4 Molecular cloning

Molecular cloning was done either by Invitrogen's Gateway® Cloning technology or by classical cloning with restriction enzymes and ligase reactions.

2.2.5 Cell Lysis

Cells were lysed with the help of a French press.

2.2.6 Ni-NTA chromatography

Ni-NTA agarose beads were equilibrated with 25 ml lysis buffer. The lysate is spinned down at 20 000 rpm for 20 minutes at 4°C. The supernatant is transferred into the column with the Ni-NTA beads. The column is sealed with parafilm at its ends and incubated for 1 hour at 4°C with rotation. The column is unsealed and the flow through is collected and kept at 4°C. The beads are washed three times with 25 ml washing buffer. 1 ml elutions are collected with the elution buffer.

2.2.7 Gel filtration chromatography

The column is washed before and after every run with at least 100 ml of water. The injection loops are also flushed before every run with 120% of the total loop volume.

2.2.8 Magnetic phage display panning

The phagemid library is grown up until OD 0.7 in SOC medium and then infected with VCMS13 helper phage overnight, resulting in phages which display the VHH library on their surface as fusions to pIII. In parallel, an E.coli ER2738 culture is grown overnight in preparation for panning. This strain has a tet-inducible pili through which the phage infects later the cell. The culture is spinned down and the supernatant is transferred to a new bottle.10% of the total volume of a 20% PEG/2.5M NaCl solution which helps precipitating the phage. Incubate 2h at 4°C on ice and spin down the culture. Pour off the supernatant and resuspend the pellet in PEG solution again. Incubate again for 1 hour on ice and resuspend after that in PBS. Spin down again and keep the supernatant as the phage did not precipitate now.

Equilibrate 2x 100 µl of Streptavidin beads by washing 2x with PBS. Block beads in 2%BSA/PBS solution for 1h at 37°C.Wash beads and add to one part 20 µg pf

biotinylated proteins in 2%BSA/PBS solution and incubate at least 30 min at room temperature with inversion. To the other 100 µl of beads, add 200 µl of phage from the phage amplification step and 800 µl of 1% BSA in PBS solution in order to negatively select phages. Incubate 1 hour at room temperature with inversion. Collect the supernatant from the beads which is now your precleared phage stock. Add 200 µl of this precleared phage to the protein-biotin-streptavidin tubes with 800 µl of 1%BSA in PBS solution. Incubate 1 hour at room temperature with inversion. Wash beads 15 times with PBS-T (0.1% Tween 20). For final wash, add 500 µl PBS-T and incubate at 37°C with inversion. Add 500 µl of saturated ER2738 bacteria and incubate 15 min at 37°C. Remove supernatant (=cells) and store the "elution". Add 500 µl of 0.2M glycine, pH 2.2 and incubate 10 min with inversion. Remove glycine and add to 75µl of 1M Tris pH 9.1 to neutralize and store the second "elution". Pool elutions together and incubate for 15 min at 37°C. Plate the cells on agar plates and incubate overnight at 37°C.

Scrape down plates with SOC medium with Ampicillin, pool together, add glycerol to 15% final concentration and store at -80°C until second round of panning is started.

After the second round of panning, 96 individual colonies are picked from the agar plates and incubated into 96-well plates using 200 µl SOC medium + Ampicillin + Tetracycline per well. The 96-well plate is covered with an airpore sheet to allow oxygen exchange. The colonies are grown overnight at 37°C with agitation. This is the "Master plate".

The "soluble plate" is generated by incubation of 2 µl of the "Master plate" to a new 96-well plate with 180 µl of 2YT medium and ampicillin and tetracycline per well. Glycerol is added to the "Master Plate" to a final concentration of 15% and stored at -80°C. The "soluble plate" is covered with an airpore sheet and incubated for 4 hours at 37°C with agitation. 80 µl of 2YT with Tetracycline, Ampicillin and 10 mM IPTG are added to each well and incubated overnight at 30°C. An ELISA plate is also coated with antigen in PBS overnight at 4°C.

The following day, the antigen is removed from the ELISA plate and 200 µl PBS with 4% milk and 0.1% Tween20 per well is used block the plate for 2 hours at

37°C. The other “soluble” plate is spinned down at 2500 rpm at RT for 10 minutes. 50 µl of the supernatant (primary antibody) of this plate and 50 µl of blocking solution are added to the ELISA plate. Incubate the ELISA plate 1 hour at room temperature with rotation. The plate is washed 3 times in PBS-T and 3 times in PBS alone. 100 µl of anti E-tag HRP (secondary antibody) is added in blocking solution and incubated one hour at room temperature with rotation. The plate is washed again three times in PBS-T and three times in PBS. 100 µl of developing reagent is added and colour change is expected. The reaction must be stopped before background comes up by adding 100 µl of 1M HCl. The colour changes to yellow and absorbance is measured at 450 nm.

3 Results & Discussion

3.1 NLRP1 LRR

The C – terminal Leucine rich repeat domain of NLRs is believed to have two main functions. Firstly, it maintains NOD-like receptors in an autoinhibited state (**Figure 11**). Secondly, it is involved in initial inflammasome activation by sensing ligands, PAMPs and DAMPs, respectively (Jha & Ting, 2009). A VHH binding to the LRR domain of a particular inflammasome would be a natural binder and agonist of the complex, thus activating the inflammasome and allowing to study its mechanism.

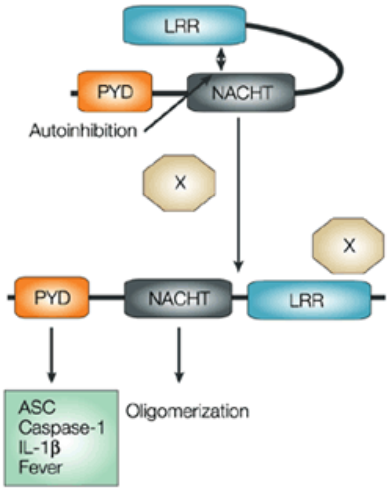

Figure 11 **Hypothetical mechanism of inflammasome autoinhibiton and activation.** The activity of NLRs in non-stimulated cells is inhibited by through binding of the leucine rich repeats (LRRs) to the NACHT domain, thereby inhibiting oligomerization and down-stream processing. This autoinhibtion is relieved through an agonist X to the LRR domain. This enables oligomerization of NLRs, which finally leads to caspase activation and inter-leukin-1β activation, leading to immune responses. (Tschopp, Martinon, & Burns, 2003)

3.1.1 Test expression of LRR domains

The LRR domains of human NLRP1, NLRP3 and NLRC4 were cloned into two different types of expression vectors, either with an N-terminal His-tag or a C-terminal one since differences in expression can occur dependent on the position of the tag. Test expression was performed to select for the best expressed and most soluble LRR domain. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) did not show strong bands, thus a Western Blot was performed (**Figure 12**). NLRP1 LRR was clearly the best expressed and most soluble LRR domain. NLRP3 LRR was well expressed but did not show any solubility.

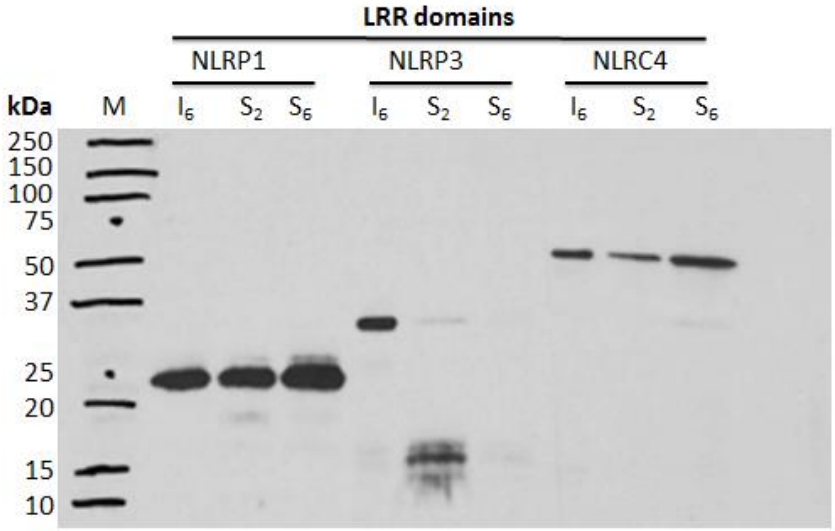

Figure 12 **Western Blot analysis of expressed LRR domains of different inflammasomes.** Test expression was performed at 37°C for 4h. Induction with 1 mM IPTG occurred at OD of 0.6. Primary antibody used was αHis-HRP. (M…Marker, I_6…sample taken after 6h induction, S_2…supernatant sample after 2h induction, S_6…supernatant sample after 6h induction)

3.1.2 Purification of NLRP1 LRR for biopanning

For several rounds of biopanning, NLRP1 LRR was expressed in big cultures of E.coli BL21 cells and further purified by Nickel Nitrilotriacetic acid (Ni-NTA) chromatography (**Figure 13**) and gel filtration chromatography (**Figure 14**, **Figure 15**).

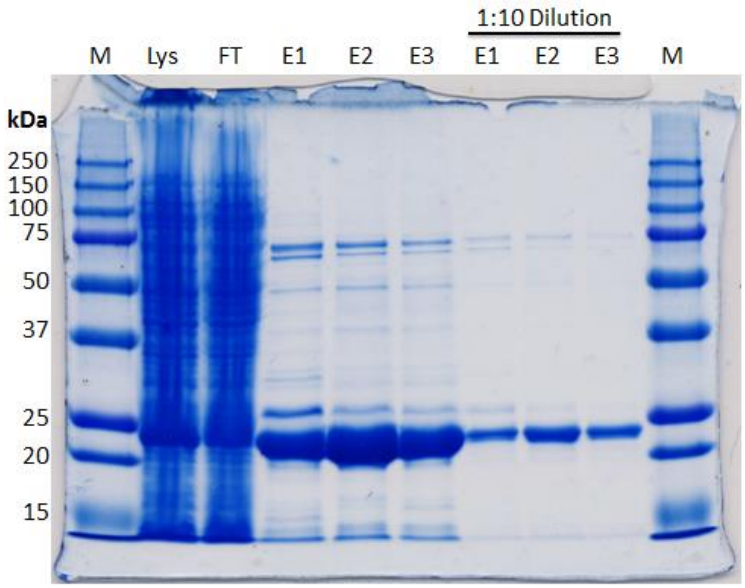

Figure 13 **Ni-NTA chromatography of NLRP1 LRR.** Protein was eluted with 2 ml of 250 mM imidazole solution. (M…marker, Lys…lysate, FT…flow through, E1…first elution, E1…second elution, E3…third elution)

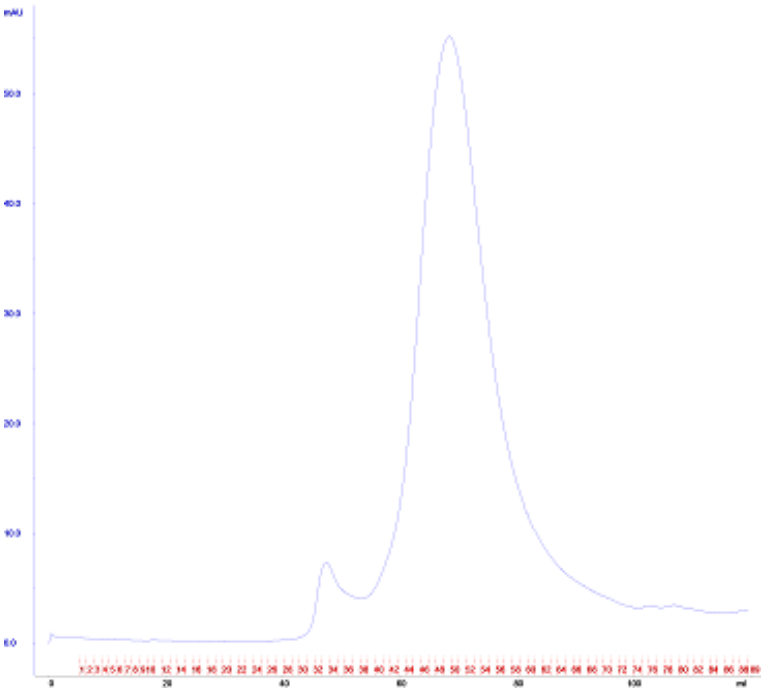

Figure 14 **Gel filtration chromatography of NLRP1 LRR.**

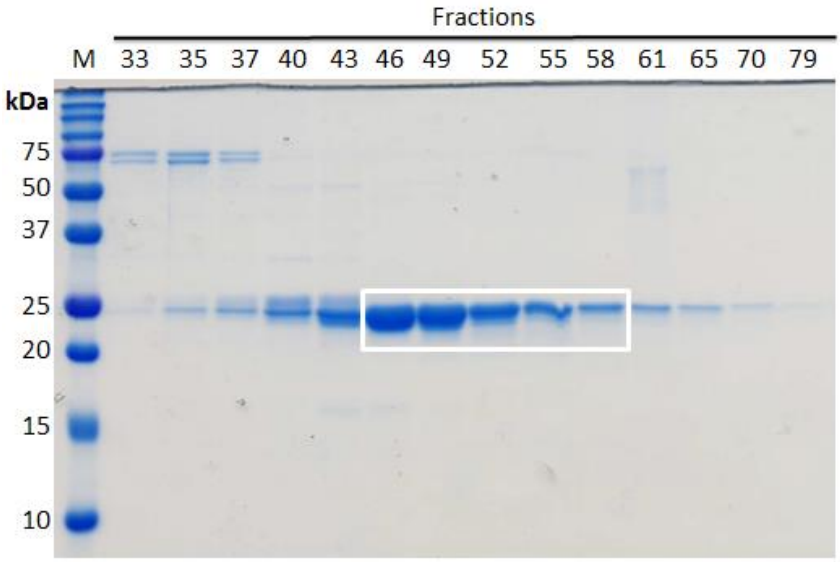

Figure 15 **Gel filtration chromatography fractions of NLRP1 LRR.** Fractions 46 to 58 have been pooled together and concentrated by Millipore Amicon Ultra centrifugal filters with a molecular cut-off of 10 kDa.

After successful purification of NLRP1 LRR, several rounds of biopanning have been started with two distinct VHH libraries. Both libraries have been established after immunization of alpacas. The alpaca Brutus was immunized with undefined inflammasome components while the alpaca Dezzi was immunized with a mouse spleen. No binder could be isolated from the two distinct libraries. Thus, more NLRP1 LRR was purified for immunizing another alpaca and generating a new VHH library.

3.2 NLRP1 PYD and NLRP1 CARD

NLRP1 is one of the most unique and interesting and also least understood inflammasomes. The major differences between mouse and human NLRP1 are the domain architecture (**Figure 7**) and its agonists. While mouse macrophages respond to LeTx, human macrophages do not. Human NLRP1 agonists are believed to be muramyl dipeptide, Kaposi's sarcoma-associated herpesvirus Orf63 and a Vaccinia virus anti-apoptotic B-cell CLL/lymphoma 2 homolog. Through immunization of an alpaca with NLRP1 LRR, further agonists may get known by subsequent experiments. As not only the known agonists are in low number but also the mechanism of NLRP1 activation and the difference in involvement or absence of

ASC in downstream processing is unknown, studies on NLRP1 and its domains are intended to be done.

3.2.1 Test expression NLRP1 domains

All NLRP1 domains were cloned into N-terminal His-tagged expression vectors. Test expression showed that NLRP1 PYD and NLRP1 CARD were clearly well expressed and soluble while the other domains were either not well expressed or insoluble (**Figure 16**).

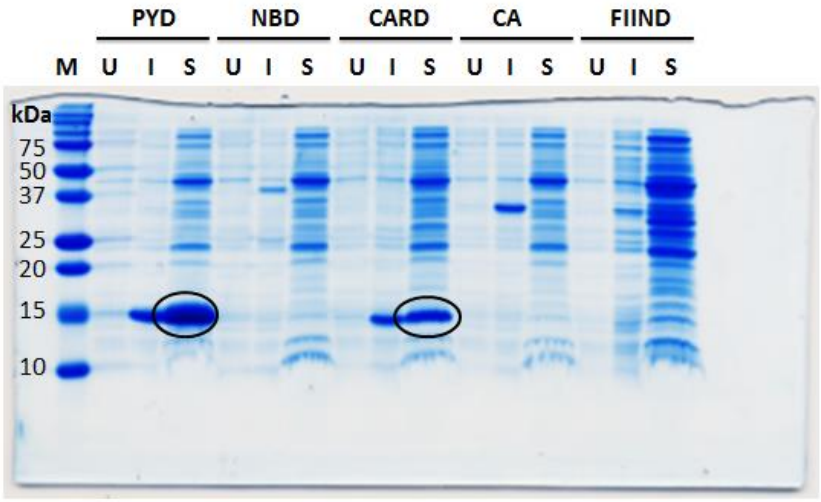

Figure 16 **Test expression NLRP1 domains.** Bacteria were grown until OD 0.8 at 37°C and protein expression was induced with 0.2 mM IPTG for 24h at 18°C. (U…uninduced sample, I…induced sample, S…sample of the supernatant)

3.2.2 Purification of NLRP1 PYD and NLRP1 CARD

For biopanning, NLRP1 PYD and NLRP1 CARD were expressed and further purified by Ni-NTA chromatography (**Figure 17**, **Figure 20**) and gel filtration chromatography (**Figure 18**, **Figure 21**). Fractions containing pure protein were pooled together and concentrated (**Figure 19**, **Figure 22**). Biopanning was performed again with the VHH library constructed from Brutus and Dezzi but no high-affinity binder could be detected during the final Enzyme Linked Immunosorbent Assay (ELISA). Thus, more NLRP1 PYD and NLRP1 CARD were purified for subsequent immunization of alpacas and generation of a new VHH library.

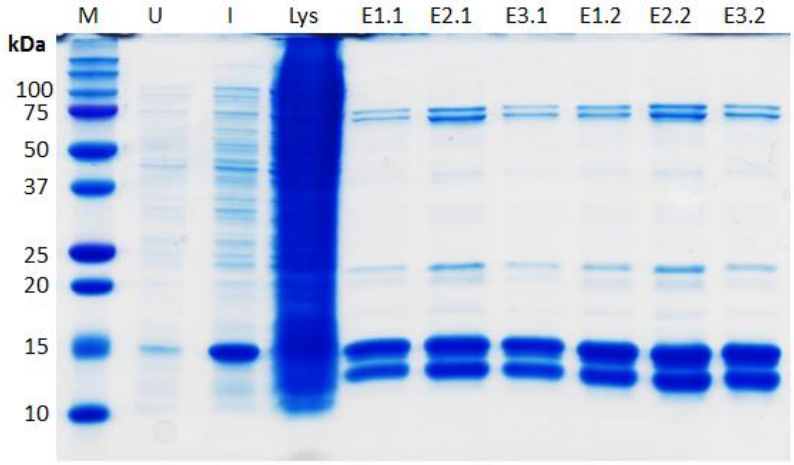

Figure 17 **Ni-NTA chromatography of NLRP1 PYD.** Protein was eluted with 1 ml elution buffer containing 250 mM imidazole. (M…marker, U…uninduced sample, I…induced sample, Lys…sample of lysate, E…sample of elution)

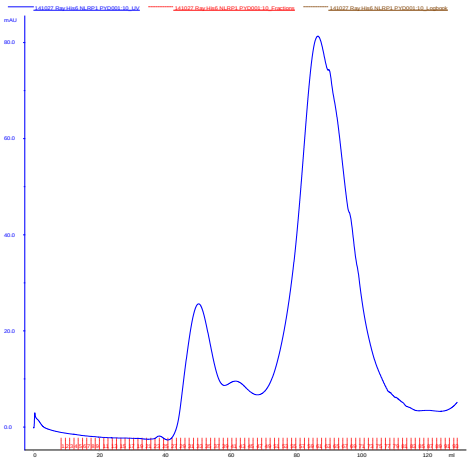

Figure 18 **Gel filtration chromatography of NLRP1 PYD.**

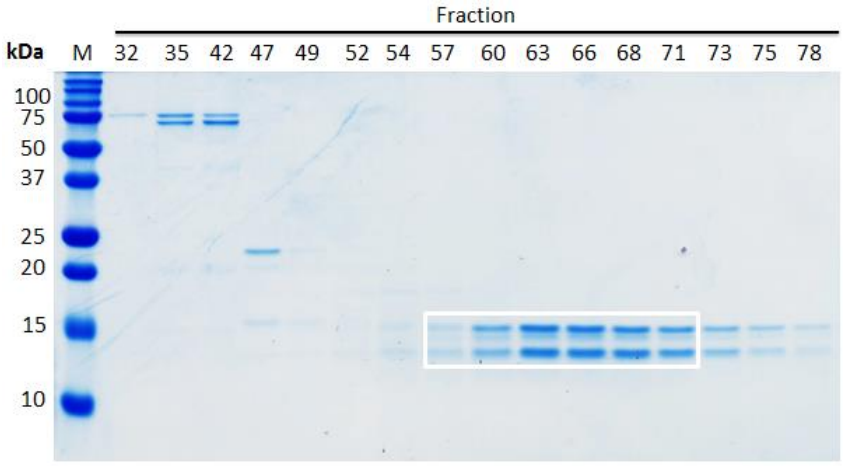

Figure 19 **Gel filtration chromatography fractions of NLRP1 PYD.** Fractions 57 to 71 were pooled together and concentrated by Millipore Amicon Ultra centrifugal filters with a molecular cut-off of 10 kDa.

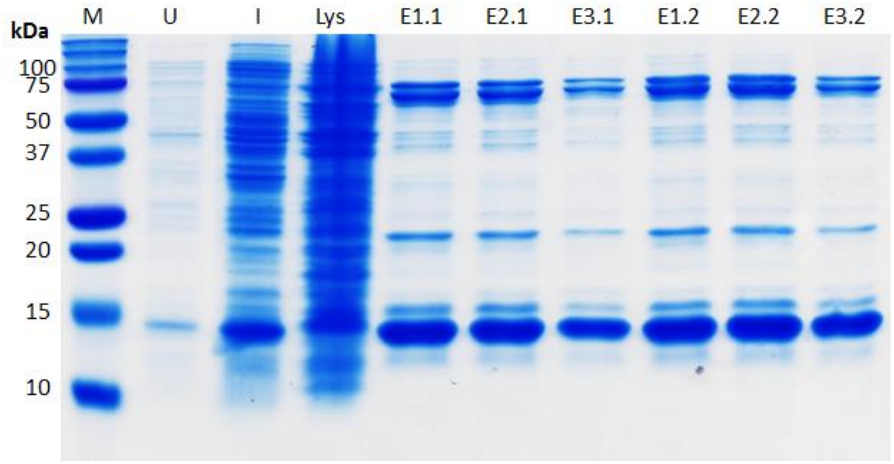

Figure 20 **Ni-NTA chromatograpy NLRP1 CARD**. Protein was eluted with 1 ml elution buffer containing 250 mM imidazole. (M…marker, U…uninduced sample, I…induced sample, Lys…sample of lysate, E…sample of elution)

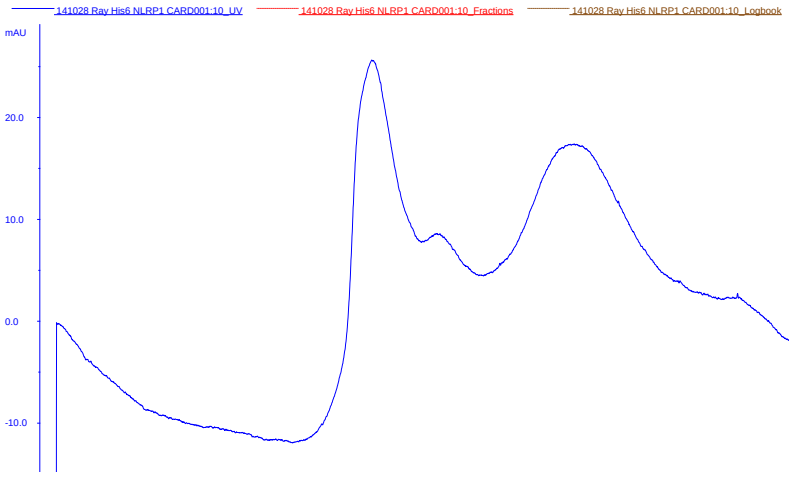

Figure 21 **Gel filtration chromatography NLRP1 CARD.**

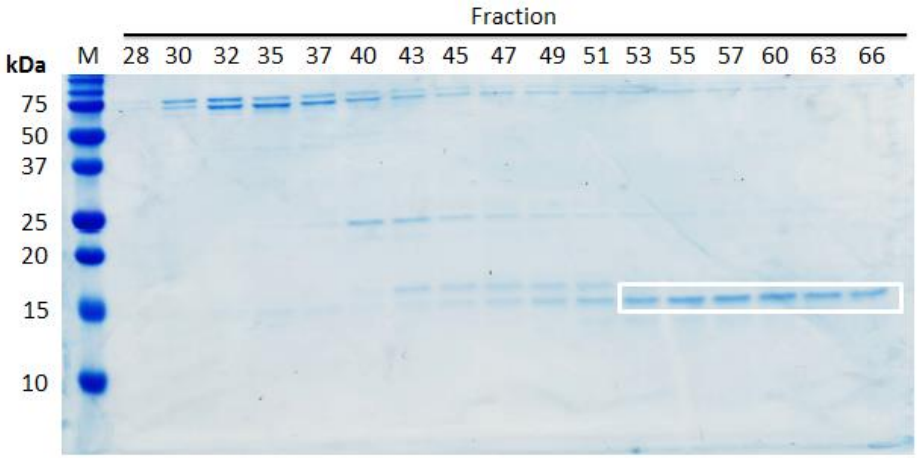

Figure 22 **Gel filtration chromatography fractions of NLRP1 CARD.** Fractions 53 – 66 were pooled together and concentrated by Millipore Amicon Ultra centrifugal filters with a molecular cut-off of 10 kDa.

3.3 Procaspase-1

Procaspase-1 belongs to the family of inflammatory caspases and is produced as an inactive zymogen. Only autoproteolytic processing of the zymogen leads to the conversion of pro-caspase 1 into its two active subunits: p10 + p20. This autoproteolytic process is needed in order to cleave pro-IL-1β and pro-IL-18 into its active form (Mariathasan et al., 2004). Moreover, it has been shown that caspase-1 plays an essential role in HIV infection. The phenotype of the virus is immunodeficiency because of the death of CD4 T-cells during infection. The key mechanism for CD4 T-cell loss was believed to be apoptosis of productively infected cells but

new studies show that the death pathway is actually linked to caspase-1 mediated pyroptosis of not even productively infected cells. By pyroptosis dying CD4 T-cells release inflammatory signals that attract even more cells to die, kind of suicidal. This cycle can be stopped by effective and safe caspase-1 inhibitors which could form a new anti-AIDS therapy for HIV patients (Doitsh et al., 2014). Thus, Caspase-1 would not only be an attractive player to study inflammasomes and its subsequent immune responses but also a potential therapeutic target for AIDS and other immune diseases.

3.3.1 Test expression of Procaspase 1

The mutant and inactive version of GST – tagged Procaspase 1 was test expressed at 18 different conditions, changing always one of three different parameters: the medium, the optical density at the moment of induction and the IPTG concentration when inducing protein expression (**Figure 23**). All 18 expression conditions were done 24h a18°C. The condition where GST-Procaspase-1 was best expressed was when bacteria were grown in TB medium and protein expression was induced at OD 0.8 with 0.2 mM IPTG.

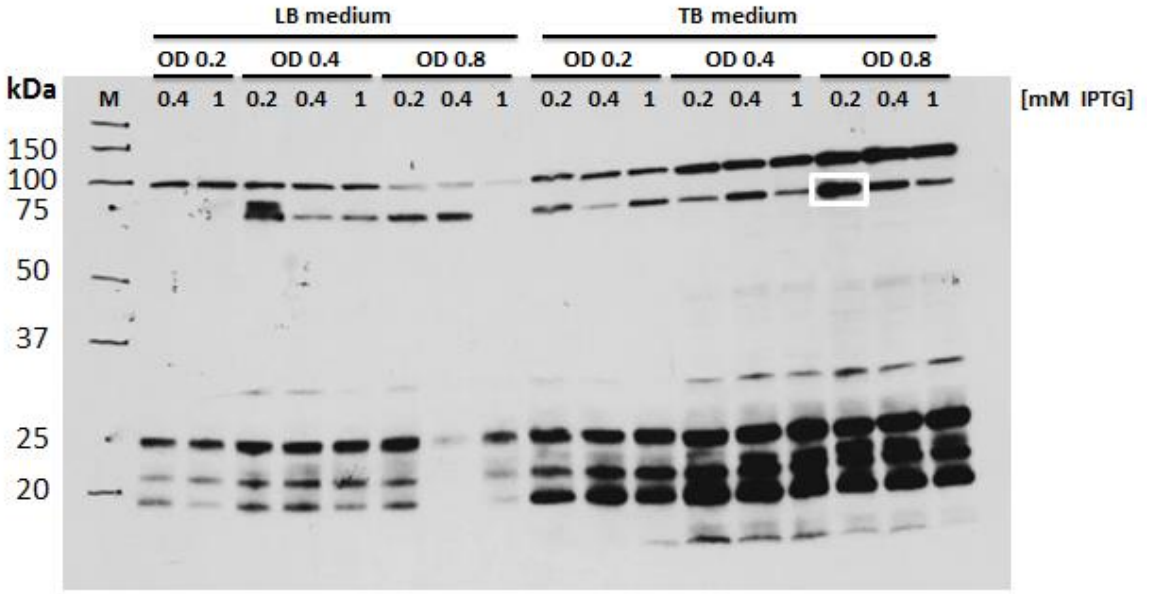

Figure 23 **Test expression of Procaspase-1.** 18 different conditions were used to select the best way to express Procaspase-1. αGST was used as the primary antibody while goat αrabbit HRP was the secondary antibody.

3.3.2 Purification of GST-procaspase-1

For biopanning, GST-procaspase-1 was expressed and further purified by affinity chromatography (**Figure 24**) and gel filtration chromatography (**Figure 25**). The fractions containing protein were pooled together and concentrated (**Figure 26**).

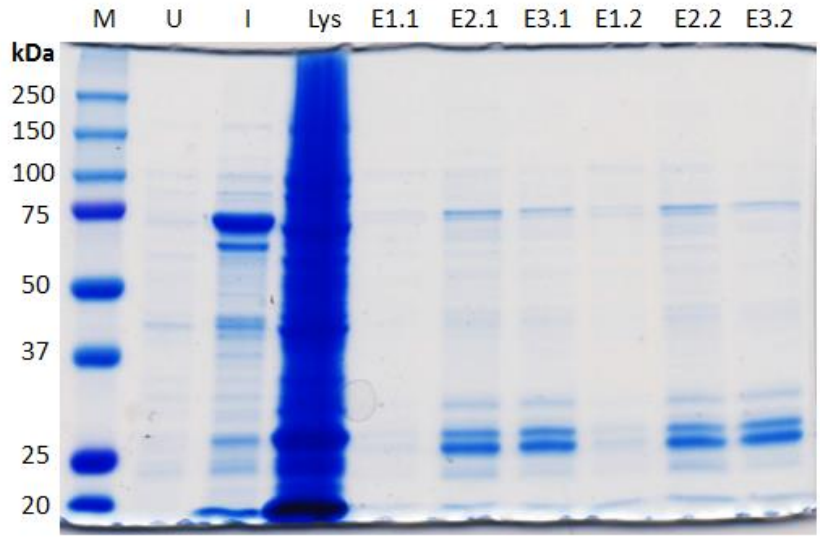

Figure 24 **Affinity chromatography of GST-Procaspase-1.** Elution of protein with 1 ml of reduced glutathione solution. (M…Marker, U…uninduced sample, I…induced sample, L…sample of lysate, E…elution)

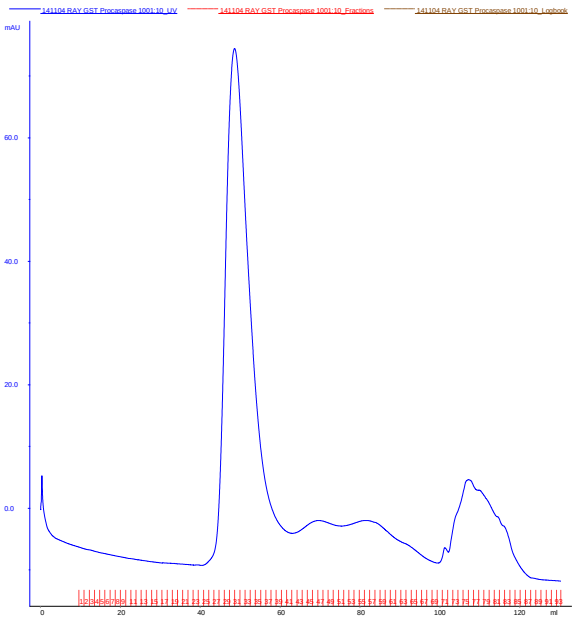

Figure 25 **Gel filtration chromatography of GST-Procaspase-1.**

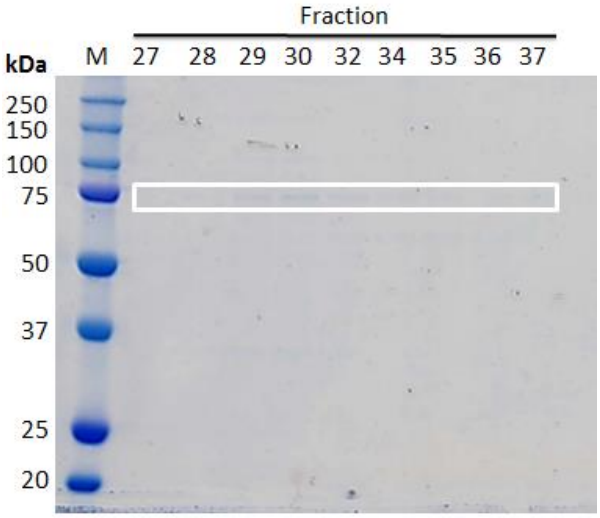

***Figure 26* Gel filtration chromatography fractions of GST-Procaspase-1.** Fractions 27 – 37 were pooled together and concentrated by Millipore Amicon Ultra centrifugal filters with a molecular cut-off of 10 kDa.

3.3.3 Biopanning against Procaspase-1

The yield of purified Procaspase-1 was too low for biopanning but another method of biopanning was established in order to screen against the VHH libraries. Since the GST-tagged Procaspase-1 binds to glutathione beads in preparation for affinity purification, we suggested not eluting Procaspase-1 from the beads but instead directly performing the biopanning against the protein. After the overnight lysate binding step, the beads were washed three times and precleared phage was added directly to the beads. To control, if Procaspase-1 bound to the beads and direct biopanning is possible, the beads were boiled in 1x SDS and the supernatant was analysed by removing the magnetic beads with help of the magnetic rack. A strong band of elution can be seen which indicates that a vast amount of Procaspase-1 bound to the glutathione beads and direct biopanning is possible (**Figure 27**). As no purification of protein is needed, this method is shorter and also more reliable. The differences of conventional biopanning and the used method for direct panning are listed in Table 2. Unfortunately, after screening both VHH libraries via this new biopanning method, no high-affinity binders could be detected.

Possible reasons of unsuccessful biopanning, beside the absence of high-affinity binders against the proteins of interest, can also be a weak immune response of

immunized alpacas after immunization, thus no generation of antibodies and VHHs or secondly, binding of the VHH to the antigen of interest in a very weakly manner, thus not resulting in a signal during the final ELISA.

Table 2 **Differences between conventional panning and used direct biopanning method.**

	Conventional Biopanning	**Used method**
Protein preparation	Purification of protein & Biotinylation of protein	Binding overnight to magnetic beads; no purification needed
Blocking	Blocking of streptavidin beads in 2% BSA in PBS	Blocking of magnetic glutathione beads in GST Lysate
Protein binding to beads	Addition of biotinylated protein to blocked beads	Addition of Procaspase 1 Lysate to magnetic glutathione beads
Preclearing of phage	Add phage to blocked streptavidin beads	Add phage to blocked magnetic glutathione beads
Phage binding to protein	Add precleared phage to protein-biotin-streptavidin tubes	Add precleared phage to Procaspase-1 bound beads

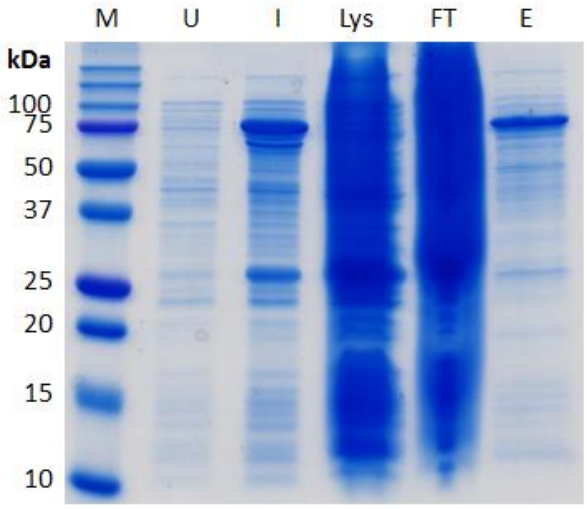

Figure 27 **Boiling glutathione beads bound to GST-Procaspase-1.** After overnight binding of the lysate at 4°C to the beads, they were washed three times. 100 µl 1xSDS was added and the beads with protein were cooked 5 minutes at 95°C. The supernatant without beads was collected by means of the magnetic rack and analysed = elution sample.

3.4 NLRC4 inflammasome activation assay

The NLRC4 inflammasome is known to get activated by bacterial PAMPs such as flagellin and T3SS components or T4SS components. In order to be able to activate the NLRC4 inflammasome in the THP-1, a human monocytic cell line, different recombinant bacterial agonists of the NLRC4 inflammasome were test expressed and the best one was used for treatment of the THP-1 cells (**Figure 28**). Needle proteins are the most critical components of a functional T3SS (Yang, Zhao, Shi, & Shao, 2013). Hence, the recombinant needle protein from *Shigella flexneri* (MxiH), *Salmonella typhimurium* (PrgI) and enterohemorrhagic *E. coli* (EHEC) (EprI) were used to choose from. Also the T3SS rod protein of *B. thailandensis* (BsaK) was used.

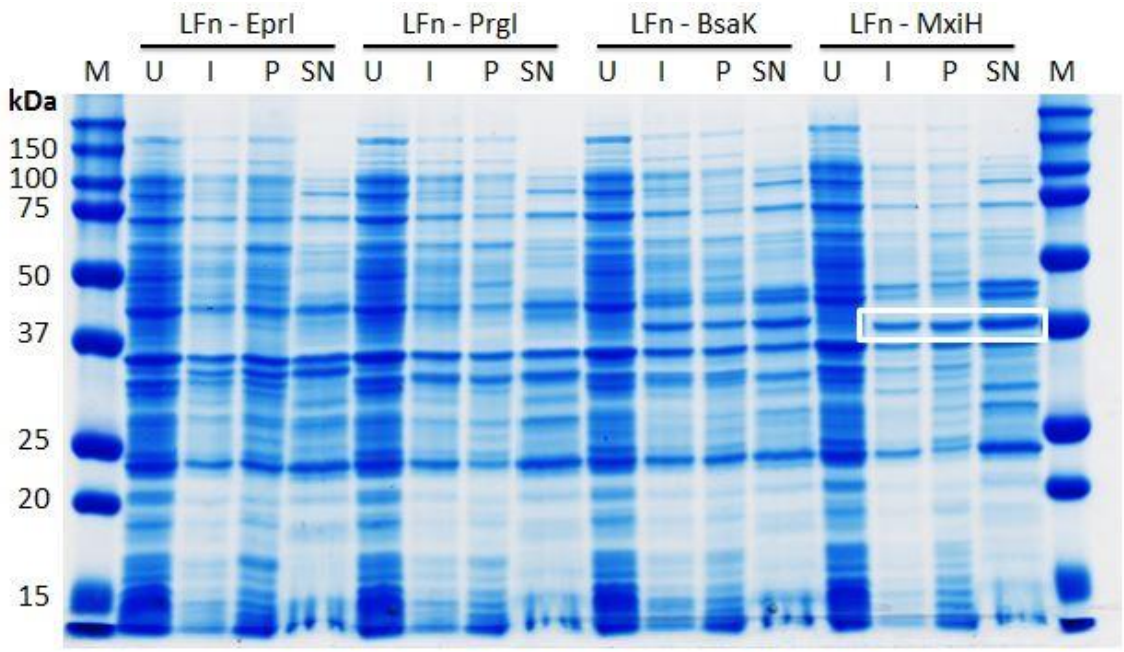

Figure 28 **Test expression of bacterial needle and rod proteins.** Bacteria were grown until OD reached 0.6. Protein expression was then induced with 1 mM IPTG overnight at 30°C. (M…Marker, U…uninduced sample, I…induced sample, P…sample of bacterial pellet, SN…sample of supernatant)

All tested proteins are fused to the N-terminal domain of LF (LFn) since this allows translocation of the protein into the cytosol when LFn is binding to PA at the cell surface (**Figure 29**). The best expressed bacterial protein which was used is the needle protein MxiH from *Shigella flexneri*. Thus, the protein was determined to be used as the potential agonist of NLRC4 activation.

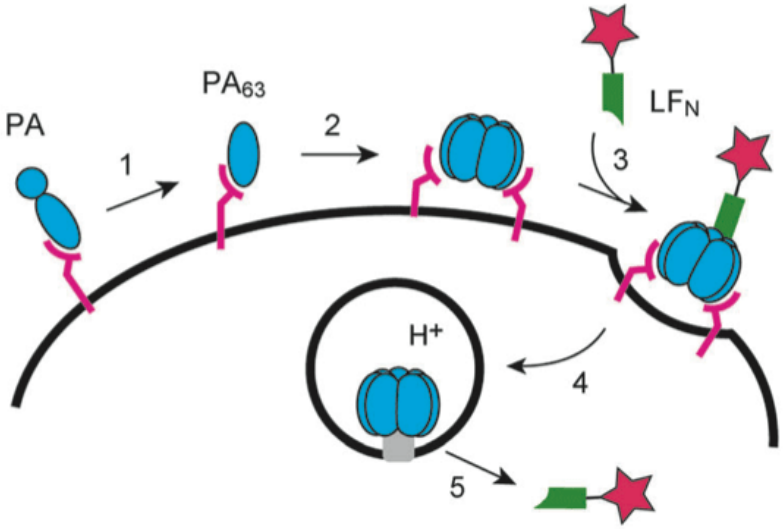

Figure 29 **Delivery of bacterial agonists into the host cell cytosol.** Protective antigen (PA), a component of anthrax toxin can efficiently transport flagellin, bacterial needle or rod proteins into the cytosol of mammalian cells when conjugated to the N-terminal domain of LF (LFn).

3.4.1 Purification of LFn-MxiH Wild type and mutant version

In order to perform the inflammasome activation assay, the catalytic active version LFn-MxiH Wild type (WT) as well as the catalytic inactive mutant version LFn-MxiH mutant (mut) was expressed and purified by Ni-NTA chromatography (**Figure 30**, **Figure 31**) and gel filtration chromatography (**Figure 32**, **Figure 33**).

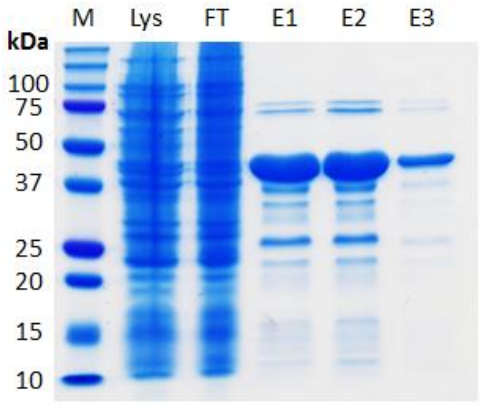

Figure 30 **Ni-NTA chromatography of LFN-MxiH WT.** 1 ml elutions were taken with 250 mM imidazole solution. (M…marker, Lys…lysate, FT…flow through, E…elution)

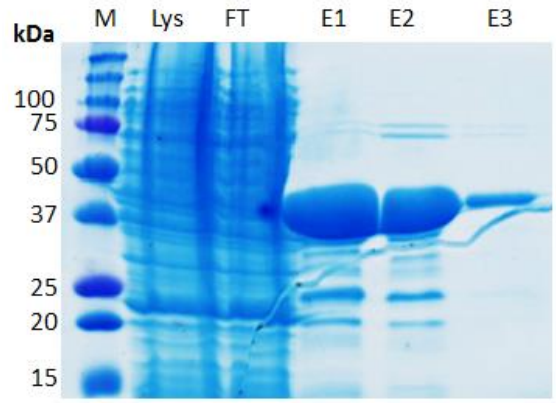

Figure 31 **Ni-NTA chromatography of LFN-MxiH mut.** 1 ml elutions were taken with 250 mM imidazole solution. (M…marker, Lys…lysate, FT…flows through, E…elution)

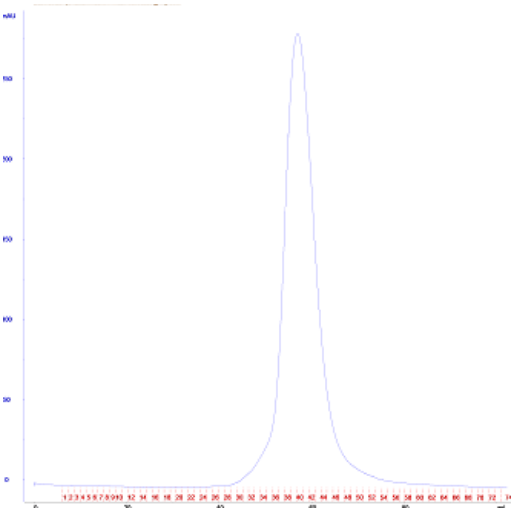

Figure 32 **Gel filtration chromatography of LFN-MxiH WT.**

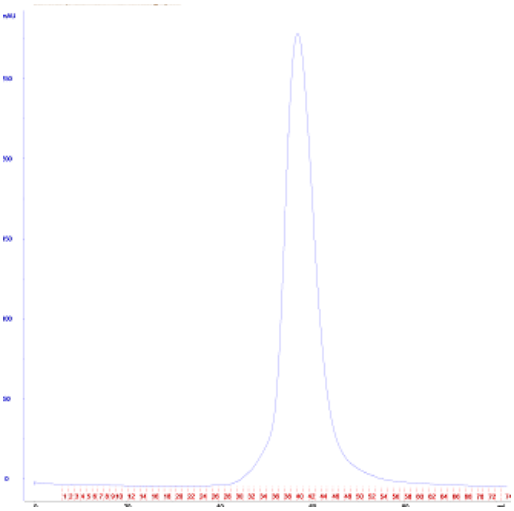

Figure 33 **Gel filtration chromatography of LFN-MxiH mut.**

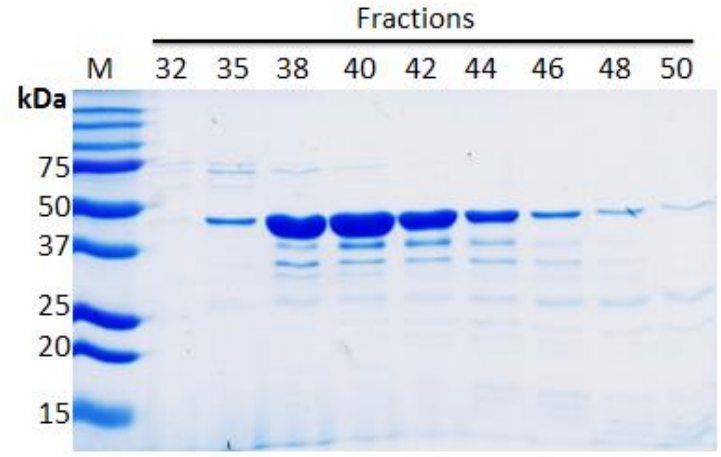

Figure 34 **Gel filtration chromatography fraction analysis of LFN-MxiH WT.** Fractions 38 – 44 were pooled together and concentrated by Millipore Amicon Ultra Centrifugal filters (MWCO 10 kDa).

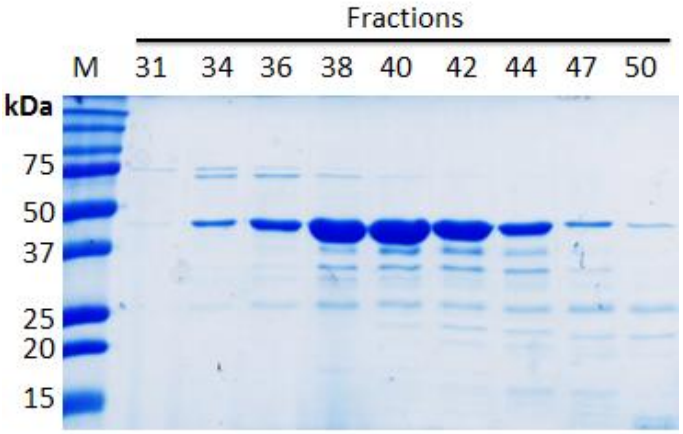

Figure 35 **Gel filtration chromatography fraction analysis of LFn-MxiH Mut.** Fractions 36 – 42 were pooled together and concentrated by Millipore Amicon Ultra Centrifugal filters (MWCO 10 kDa).

The fractions of the gel filtration chromatography were further analysed (**Figure 34**, **Figure 35**) and fractions containing protein were pooled together and used for the inflammasome activation assay.

3.4.2 Inflammasome activation assay

THP-1 cells were grown overnight and treated the following day with different concentrations of endotoxin-free LFn-MxiH WT, LFn-MxiH Mut and PA. The period of treatment with the different proteins was 16h at 37°C. After 16h, microscopy pictures were taken and the supernatant was collected for an ELISA.

3.4.2.1 Microscopy pictures

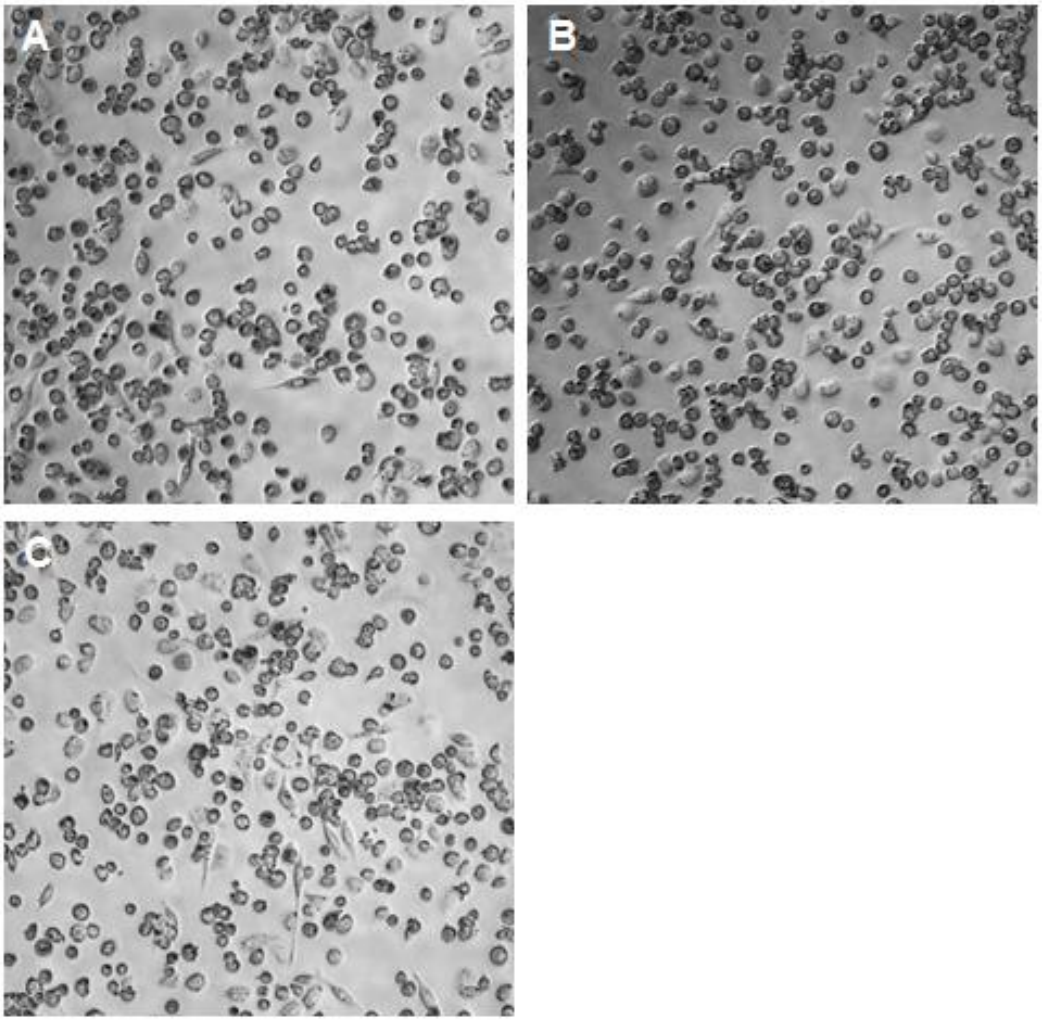

Figure 36 **THP-1 cells do not lead to pyroptosis when treated with bacterial needle protein LFn-MxiH alone.** (A) Untreated cells (B) cells treated only with PA (C) cells treated with LFn-MxiH WT

The wild type version of the needle protein MxiH from *Shigella flexneri* does not lead to NLRC4 inflammasome activation and pyroptosis on its own (**Figure 36**). Untreated cells as well as cells only treated with PA also show a clear, defined cell shape, thus no signs of pyroptosis. While cells treated with LFn-MxiH WT in combination with PA show a disturbed morphology, concluding inflammasome activation and cell death by pyroptosis. In contrast, the catalytic inactive mutant version of LFn-MxiH shows abolished inflammasome activity even in combination with PA (**Figure 37**).

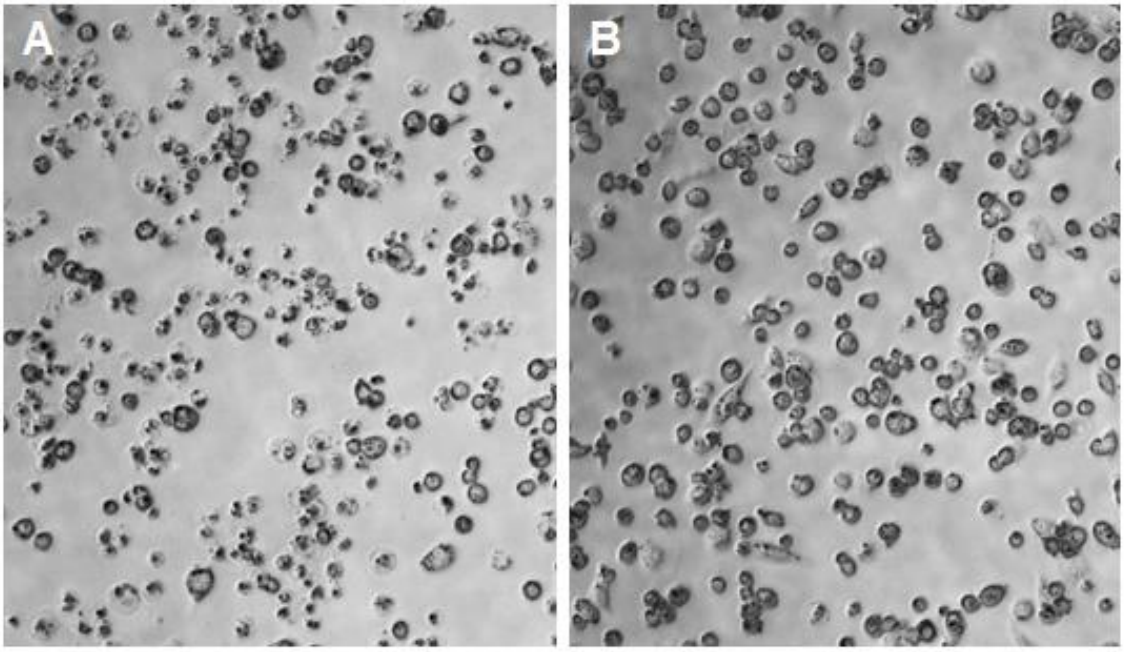

***Figure 37* THP-1 cells die via pyroptosis when treated with LFn-MxiH WT in combination with PA.** (A) Cells treated with the wild type version of LFn-MxiH in combination with PA (B) cells treated with the mutant version of LFn-MxiH in combination with PA

3.4.2.2 ELISA

The supernatant was collected after treatment of 16 hours and analysed by an ELISA. The assay confirms the previous results, suggesting that untreated THP-1 cells, cells treated only with LFn-MxiH WT, LFn-MxiH mutant or PA do not elicit an immune response whereas cells treated with PA in combination with the wild type version of LFn-MxiH show high IL-1β concentration in the supernatant concluding a strong immune response. Increasing the concentration of LFn-MxiH WT also leads to a stronger immune response and more IL-1β secretion by caspase-1 activation. The mutant version of LFn-MxiH in combination with PA does not lead to pyroptosis, as seen in **Figure 37**, but nevertheless, elicit a weak immune response by activating the NLRC4 inflammasome and processing and secreting IL-1β (**Figure 38**).

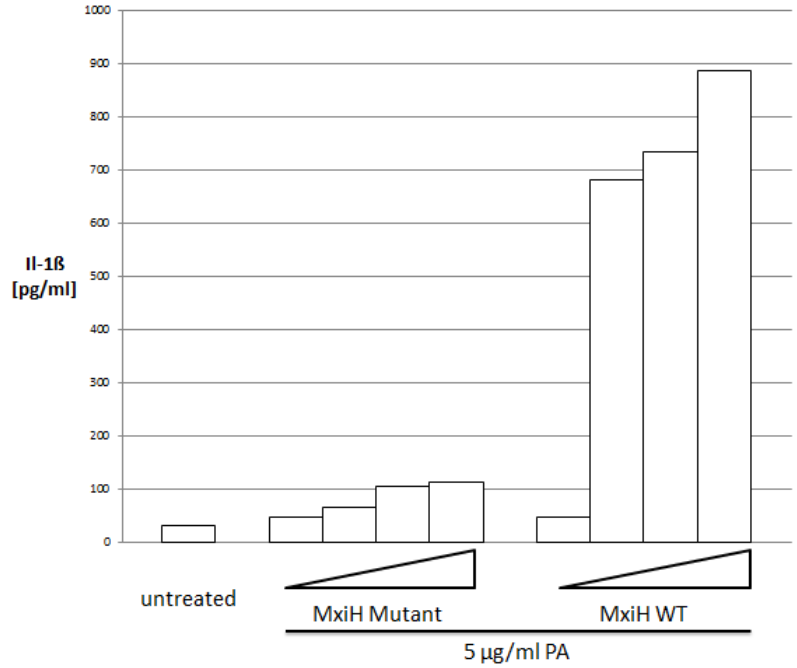

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Figure 38 **Increased concentration of bacterial needle protein MxiH in combination with PA leads to increased IL-1β secretion.** The PA concentration of 5 µg/ml was kept constant while the concentration of both MxiH versions was increased from 0 ng/ml to 100 ng/ml to 300 ng/ml to 900 ng/ml (from left to right bar)

4 Conclusion and future outlook

The discovery of the inflammasome has generated an exciting new field of immunology. Inflammasome activation is now recognized as being critical in the host response to microorganisms and damage-associated molecular patterns. However, many questions remain unanswered, including the nature of direct or indirect NLR ligands, mechanistic details of NLR activation, the role of ASC as well as inflammasome activators and inhibitors, the exact function of murine caspase-11/human caspase 4, and finally the secretion pathway of IL-1β and IL-18.These may be the focus of inflammasome research in coming years.

By means of VHHs, these aspects can be further studied. Thus, the setup of new VHH libraries from immunized alpacas should be promoted to isolate high-affinity binders against proteins of interest.

By means of the anthrax toxin delivery system, fusion of other proteins than flagellin or bacterial needle proteins to the anthrax toxin can lead to identification of new triggers while the ability to activate the inflammasome can be used to screen for VHHs that inhibit inflammasome activation by viability assays.

List of References

Doitsh, G., Galloway, N. L. K., Geng, X., Yang, Z., Monroe, K. M., Zepeda, O., … Greene, W. C. (2014). Cell death by pyroptosis drives CD4 T-cell depletion in HIV-1 infection. *Nature*, *505*, 509–14. doi:10.1038/nature12940

Faustin, B., Lartigue, L., Bruey, J. M., Luciano, F., Sergienko, E., Bailly-Maitre, B., … Reed, J. C. (2007). Reconstituted NALP1 Inflammasome Reveals Two-Step Mechanism of Caspase-1 Activation. *Molecular Cell*, *25*, 713–724. doi:10.1016/j.molcel.2007.01.032

Frenken, L. G. J., Van Der Linden, R. H. J., Hermans, P. W. J. J., Bos, J. W., Ruuls, R. C., De Geus, B., & Verrips, C. T. (2000). Isolation of antigen specific Llama V(HH) antibody fragments and their high level secretion by Saccharomyces cerevisiae. *Journal of Biotechnology*, *78*, 11–21. doi:10.1016/S0168-1656(99)00228-X

Gerlic, M., Faustin, B., Postigo, A., Yu, E. C.-W., Proell, M., Gombosuren, N., … Reed, J. C. (2013). Vaccinia virus F1L protein promotes virulence by inhibiting inflammasome activation. *Proceedings of the National Academy of Sciences of the United States of America*, *110*, 7808–13. doi:10.1073/pnas.1215995110

Gregory, S. M., Davis, B. K., West, J. A., Taxman, D. J., Matsuzawa, S., Reed, J. C., … Damania, B. (2011). Discovery of a viral NLR homolog that inhibits the inflammasome. *Science (New York, N.Y.)*, *331*, 330–334. doi:10.1126/science.1199478

Jha, S., & Ting, J. P.-Y. (2009). Inflammasome-associated nucleotide-binding domain, leucine-rich repeat proteins and inflammatory diseases. *Journal of Immunology (Baltimore, Md. : 1950)*, *183*, 7623–7629. doi:183/12/7623 [pii]\r10.4049/jimmunol.0902425

Kofoed, E. M., & Vance, R. E. (2012). NAIPs: building an innate immune barrier against bacterial pathogens. *Bioessays*, *34*, 589–598. doi:10.1002/bies.201200013

Levinsohn, J. L., Newman, Z. L., Hellmich, K. A., Fattah, R., Getz, M. A., Liu, S., … Moayeri, M. (2012). Anthrax lethal factor cleavage of Nlrp1 is required for activation of the inflammasome. *PLoS Pathogens*, *8*. doi:10.1371/journal.ppat.1002638

Mariathasan, S., Newton, K., Monack, D. M., Vucic, D., French, D. M., Lee, W. P., … Dixit, V. M. (2004). Differential activation of the inflammasome by caspase-1 adaptors ASC and Ipaf. *Nature*, *430*, 213–218. doi:10.1038/nature02664

Martinon, F., & Tschopp, J. (2004). Inflammatory caspases: Linking an intracellular innate immune system to autoinflammatory diseases. *Cell*. doi:10.1016/j.cell.2004.05.004

Medzhitov, R., & Janeway, C. (2000). Innate immune recognition: mechanisms and pathways. *Immunological Reviews*, *173*, 89–97. doi:10.1034/j.1600-065X.2000.917309.x

Murphy, K., Travers, P., & Walport, M. (2008). *Janeway's Immunobiology*. *Garland Science* (Vol. 7). doi:10.1086/596249

Muyldermans, S. (2013). Nanobodies: natural single-domain antibodies. *Annual Review of Biochemistry*, *82*, 775–97. doi:10.1146/annurev-biochem-063011-092449

Ratsimandresey, R. A., Dorfleutner, A., & Stehlik, C. (2013). An update on PYRIN domain-containing pattern recognition receptors: From immunity to pathology. *Frontiers in Immunology*. doi:10.3389/fimmu.2013.00440

Schroder, K., & Tschopp, J. (2010). The Inflammasomes. *Cell*. doi:10.1016/j.cell.2010.01.040

Strowig, T., Henao-Mejia, J., Elinav, E., & Flavell, R. (2012). Inflammasomes in health and disease. *Nature*. doi:10.1038/nature10759

Thornberry, N. A., Bull, H. G., Calaycay, J. R., Chapman, K. T., Howard, A. D., Kostura, M. J., … Aunins, J. (1992). A novel heterodimeric cysteine protease is required for interleukin-1 beta processing in monocytes. *Nature*, *356*, 768–774. doi:10.1038/356768a0

Tschopp, J., Martinon, F., & Burns, K. (2003). NALPs: a novel protein family involved in inflammation. *Nature Reviews. Molecular Cell Biology*, *4*, 95–104. doi:10.1038/nrm1019

Van der Linden, R. H., Frenken, L. G., de Geus, B., Harmsen, M. M., Ruuls, R. C., Stok, W., … Verrips, C. T. (1999). Comparison of physical chemical properties of llama VHH antibody fragments and mouse monoclonal antibodies. *Biochim Biophys Acta*, *1431*, 37–46. doi:S0167-4838(99)00030-8 [pii]

Von Moltke, J., Ayres, J. S., Kofoed, E. M., Chavarría-Smith, J., & Vance, R. E. (2013). *Recognition of bacteria by inflammasomes. Annual review of immunology* (Vol. 31). doi:10.1146/annurev-immunol-032712-095944

Wang, S., Miura, M., Jung, Y. K., Zhu, H., Li, E., & Yuan, J. (1998). Murine caspase-11, an ICE-interacting protease, is essential for the activation of ICE. *Cell*, *92*, 501–509. doi:10.1016/S0092-8674(00)80943-5

Yang, J., Zhao, Y., Shi, J., & Shao, F. (2013). Human NAIP and mouse NAIP1 recognize bacterial type III secretion needle protein for inflammasome activation. *Proceedings of the National Academy of Sciences of the United States of America*, *110*(35), 14408–13. doi:10.1073/pnas.1306376110

Zhao, Y., Yang, J., Shi, J., Gong, Y.-N., Lu, Q., Xu, H., … Shao, F. (2011). The NLRC4 inflammasome receptors for bacterial flagellin and type III secretion apparatus. *Nature*. doi:10.1038/nature10510