GENETIC VARIATIONS IN THE NLRP3 INFLAMMASOME
SUSCEPTIBILITY FACTOR FOR CHRONIC INFLAMMATION

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**ABSTRACT**

NLRP3 has been recognized as one of the key components of innate immunity. Upon activation, NLRP3 forms a multiprotein complex called as the ‘inflammasome’, which leads to the activation of pro-inflammatory caspase-1 which subsequently results in the formation of Interleukin (IL)-1β and IL-18. Mutations in the NLRP3 gene can lead to its constitutive activation resulting in an uncontrolled production of IL-1β. These mutations have been implicated in hereditary inflammatory diseases, often grouped under Cryopyrin-associated periodic syndromes (CAPS, cryopyrin being an alternative name for NLRP3).

Paper I in this thesis presents the case of a patient with a long history of arthritis and antibiotic resistant fever, but without the typical symptoms of CAPS. The patient was a heterozygous carrier of two common polymorphisms, Q705K in NLRP3 and C10X in CARD-8. Experimental studies indicated elevated activity of caspase-1 and IL-1β levels in the patient and a total clinical remission was achieved by IL-1β blockade. These two polymorphisms simultaneously occur in almost 4% of the control population, suggesting the possibility of a genetic predisposition for inflammation in these individuals. We, therefore, investigated a cohort of rheumatoid arthritis (RA) patients in paper II, and found that carrying the combined polymorphisms resulted in increased RA susceptibility and a more severe disease course. Hypothetically, this subgroup might benefit from IL-1β blockade. Paper III presents two patients: siblings, who did not fit into a typical CAPS phenotype. The inflammatory symptoms in both the patients appeared in adult life. A novel and functional M299V mutation in NLRP3 was detected in the siblings who neither had common symptoms nor the same disease severity. Consequent with inflammasome activation, abnormally elevated caspase-1 activity and IL-1β levels were seen. Patients in papers I and III highlight the risk of missing out such patients if attempting a very conventional diagnosis. Paper IV dissects the functional role of Q705K in NLRP3 using THP-1 cells in an in vitro model. Moderately elevated IL-1β and IL-18 levels could be observed in THP-1 cells expressing Q705K, as compared to the wild type expressing cells, indicating a gain-of-function. Due to the presence of this alteration in healthy individuals it can be classified as a low-penetrance alteration. Additional studies are warranted to elucidate the mechanistic details of this polymorphism.
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LIST OF ORIGINAL PAPERS

This thesis is based on the following four papers, which will be referred to in the text by their Roman numerals;

Paper I


Gene Polymorphisms in the NALP3 Inflammasome are Associated with Interleukin-1 Production and Severe Inflammation. Relation to Common Inflammatory Diseases?

Arthritis & Rheumatism 2008; 58(3): 888-894

Paper II

Kastbom A*, Verma D*, Eriksson P, Skogh T, Wingren G and Söderkvist P.

Genetic variation in proteins of the cryopyrin inflammasome influences susceptibility and severity of rheumatoid arthritis (the Swedish TIRA project).

Rheumatology 2008; 47:415-417

*These authors contributed equally to this work.

Paper III


Two Adult Siblings with Atypical Cryopyrin-Associated Periodic Syndrome Due to a Novel M299V Mutation in the NLRP3 Gene.

Arthritis & Rheumatism, 2010; 62(7): 2138-2143

Paper IV


The Q705K polymorphism in NLRP3 is a gain-of-function alteration leading to excessive Interleukin-1β and interleukin-18 production.

Submitted
ABBREVIATIONS

ACR  American College of Rheumatology
AD  Acidic activation domain
AS  Ankylosing spondylitis
ASC  Apoptosis-associated speck like protein containing a CARD
ATP  Adenosine triphosphate
BIR  Baculovirus inhibitor of apoptosis repeat
CAPS  Cryopyrin-associated periodic syndrome
CARD  Caspase recruitment domain
CD  Crohn’s disease
CIAS1  Cold-induced autoinflammatory syndrome-1
CINCA  Chronic infantile neurological cutaneous and articular syndrome
CPPD  Calcium pyrophosphate dihydrate
CRP  C-reactive protein
DAMP  Danger-associated molecular patterns
DMARD  Disease-modifying anti-rheumatic drugs
ESR  Erythrocyte sedimentation rate
FCAS  Familial cold-associated syndrome
FMF  familial Mediterranean fever
HPF  Hereditary periodic fevers
ICE  Interleukin converting enzyme
IL-1Ra  Interleukin-1 receptor antagonist
IL-1β  Interleukin-1β
LPS  Lipopolysaccharide
LRR  Leucin rich repeats
MDP  Muramyl dipeptide
MSU  Monosodium urate
MWS  Muckle Wells syndrome
NACHT: Domain present in Naip, CIITA, HET-E (plant het product involved in vegetative incompatibility) and TP-1 (telomerase-associated protein)

NAD: NACHT-associated domain

NALP: NACHT-LRR-PYD containing protein

NLR: Nucleotide-binding domain and leucine-rich repeat containing gene family (NOD-like receptor according to old nomenclature)

NLRP3: NLR family, pyrin-containing domain 3

NOD: Nucleotide-binding and oligomerization domain

NOMID: Neonatal onset multisystem inflammatory disease

PAMP: Pathogen-associated molecular patterns

PMA: Phorbol-12-myristate-13-acetate

PR3: Proteinase 3

PRR: Pattern recognition receptor

PYD: Pyrin domain

PYPAF: Pyrin-containing Apaf-1 like protein

RA: Rheumatoid arthritis

SE: Shared epitope

SNP: Single nucleotide polymorphism

TLR: Toll like receptors

TNF-α: Tumour necrosis factor-α

TRAPS: TNF-receptor associated periodic syndrome
Immune responses are traditionally classified as either innate or adaptive. This classification is primarily based upon the distinct sensing and effector mechanism of each type of response. Briefly, the innate immune system senses the pathogen directly at the site of infection without the need of any previous exposure of host cells to these pathogens. The adaptive or the ‘acquired’ immune system allows for elimination of pathogens in late infection stages and results in building up of an immunological memory of the pathogen/antigen that remains encoded in the immune cells.

For several decades, the immunological studies have been focused primarily on adaptive immunity as the key player in immune regulation. The underlying reason was that adaptive responses were considered to provide protection against a vast repertoire of pathogens in a highly specific manner, while innate responses were regarded as primitive, of importance only in invertebrates, and moreover unspecific. However, there was a shift in the paradigm of immune regulation in the late nineties, when the Toll protein, which previously was known for embryonal developmental function in *Drosophila melanogaster*, was shown to be essential for effective immune response against the fungi *Aspergillus fumigates* (Lemaitre, Nicolas et al. 1996). This finding led to the belief that the mammalian homologue of Toll would also have similar functions, and indeed, an induction of several genes and cytokines was observed, giving the idea that Toll-like Receptors (TLR) could link the innate immune recognition to adaptive immune responses (Medzhitov, Preston-Hurlburt et al. 1997). In 1998, TLR4 was cloned and shown to be the receptor for lipopolysaccharide (LPS), essential to mount an immune response against Gram-negative bacteria in a mouse model (Poltorak, He et al. 1998). Since then, there has been an explosion of knowledge about innate immunity, and its role in preceding and potentially modulating adaptive responses, has been appreciated.

**Pathogen recognition receptors**

Innate immunity relies on the host cell recognition of pathogens of certain conserved microbial motifs called pathogen-associated molecular patterns (PAMPs) e.g. peptidoglycan in all types of bacteria and lipopolysaccharide (LPS) in the Gram-negative bacteria
(Medzhitov and Janeway 1997), which are recognized by the hosts ‘Pattern recognition receptors’ (PRRs) (Janeway 1989).

TLRs are among the first and one of the most well studied PRRs and to date, 10 TLRs (TLR1-TLR10) have been identified in humans (Moresco, Lavine et al. 2011). The TLRs can have a cell surface presence (TLR 1, 2, 4, 5, 6) or an endosomal presence (TLR 3, 7, 8 and 9) (Moresco, Lavine et al. 2011), not much is known about TLR10. Each TLR is known to recognize a set of microbial motifs in a wide range of organisms including bacteria, fungi, helminthes, protozoa and viruses (Beutler 2004). The discovery of TLRs was a key step forward in understanding of host’s sensing of microbial pathogens and resulted in an accelerated understanding of innate responses. However, due to their superficial localization in the cell, they could not account for detection of the intracellular pathogens or the internal danger signals of host cells. Moreover, the TLR knockout mouse models confirmed that TLRs alone could not be responsible for the cytokine response of the innate system (Beutler 2004).

The issue of detection of intracellular pathogens was resolved a few years later with the discovery of cytoplasmically located Nod-like receptors (NLRs). Nod1 and Nod2 were the first NLRs to be cloned and intensely studied (Inohara, Koseki et al. 1999; Ogura, Inohara et al. 2001). The discovery of Nod1 was made when the intracellular, invasive pathogen Shigella flexneri, was shown to increase the activity of Nuclear factor-κB (NF-κB) in epithelial cells (Philpott, Yamaoka et al. 2000; Girardin, Tournebize et al. 2001). In the subsequent years, both Nod1 and Nod2 were demonstrated to detect bacterial peptidoglycan (PGN) structures: meso-diaminopimelic acid (meso-DAP) found in Gram-negative bacteria could be detected by Nod1 while muramyl dipeptide (MDP) found in both Gram-positive and Gram-negative bacteria could be detected by Nod2; both PGN structures being degradation products released from intracellular or phagocytosed bacteria (Chamaillard, Hashimoto et al. 2003; Girardin, Boneca et al. 2003; Girardin, Boneca et al. 2003; Inohara, Ogura et al. 2003).

It was known that endogenous danger signals could activate the innate immune system, although the mechanisms were not understood. Back in 1994, Polly Matzinger proposed a so-called ‘danger model’ (Matzinger 1994), challenging Janeway’s classical hypothesis (Janeway 1992) that host cells tolerate self and reject whatever is foreign or non-self. Matzinger’s alternate hypothesis was that host cells, rather than attacking the non-self (in which case an embryo would also be rejected), reacts to the danger signals released from
dying cells and tissues (Matzinger 1994; Matzinger 2002). As reviewed in (Bianchi 2007), the danger signals could arise from any trauma to host cells, for instance: exposure to UV or to extreme hot or cold temperatures, injury, infection and even certain drugs. The endogenous signals were said to constitute the danger-associated molecular patterns or ‘DAMPs’, more recently termed as ‘alarmins’, which have the property of directly activating the innate system and acting as a modulator of the adaptive system (Bianchi 2007).

The void in understanding of the innate systems potential sensors of ‘DAMPs’ or ‘alarmins’ was filled by the recent discovery of ‘inflammasome’-forming NLRs (Martinon, Burns et al. 2002). This subtype of NLRs are capable of forming a molecular scaffold which in addition to detecting microbial pathogens, also senses a vast repertoire of endogenous stimuli, through a common pathway leading to the activation of inflammatory caspase and formation of the pyrogenic cytokine, Interleukin-1β (IL-1β) (Martinon, Burns et al. 2002).

**Innate immune receptor: NLR**

**Classification and nomenclature**

Initially, the family members of the NLR family were referred to by a variety of names, like CATERPILLER, NACHT-LRR, NOD-LRR, CARD, NALP, NOD and PYPAF. Recently, in order to bring consistency in referring to members of the NLR family, a new nomenclature was agreed upon (Ting, Lovering et al. 2008) (Table I).

NLR earlier stood for NOD-like receptors but according to the recent nomenclature, it stands for ‘nucleotide-binding-domain-and-leucine-rich-repeat-containing-gene-family-of-receptors’. The NLR family has several subfamilies that are distinguishable by their N-terminal effector domains. The four distinct N-terminal domains are: The acidic transactivation domain (AD) with members designated as NLRA. The Pyrin domain (PYD) with members designated as NLRP, the Caspase-activation-and-recruitment-domain (CARD) with members designated as NLRC and baculovirus-inhibitor-of-apoptosis-repeat (BIR) domain with members designated as NLRB.

**NLRPs**

NLRPs form the largest family of pyrin-containing proteins comprising 14 members (NLRP1-NLRP14) (Tschopp, Martinon et al. 2003). As illustrated in Table 1, the basic structure of the NLRP family members comprises an N-terminal PYD domain, a central NACHT domain and...
a C-terminal LRR domain. The PYD domain, being a member of the death-domain superfamily, is implicated in pathways leading to apoptosis and inflammation. The NACHT domain is central to all NLRs and is essential for oligomerization leading to NLR activation. The NACHT domain shares structural similarities with the apoptotic protease-activating factor-1 (APAF-1) (Tschopp, Martinon et al. 2003), which is known to form the apoptosome. The ligand-sensing LRRs are highly conserved motifs of 20-30 amino acids, present in a variety of proteins involved in innate immunity, including the TLRs and the plant resistance proteins. The NLRP-LRRs are formed of exons comprising exactly 171 nucleotides, where each exon encodes one central LRR and two halves of the neighboring LRRs. It is postulated that this modular structure aids alternative splicing without affecting the three dimensional-fold of this region (Martinon, Gaide et al. 2007).

<table>
<thead>
<tr>
<th>NLR family</th>
<th>Symbol in Humans</th>
<th>Domain organization</th>
</tr>
</thead>
<tbody>
<tr>
<td>NLRA</td>
<td>CIITA</td>
<td>(CARD)-AD-NACHT-LRR</td>
</tr>
<tr>
<td>NLRB</td>
<td>NAIP</td>
<td>BIR3x-NACHT</td>
</tr>
<tr>
<td>NLRC</td>
<td>NOD1, NLRC3-5</td>
<td>CARD-NACHT-LRR</td>
</tr>
<tr>
<td></td>
<td>NOD2</td>
<td>CARD2x-NACHT-LRR</td>
</tr>
<tr>
<td>NLRP</td>
<td>NLRP1</td>
<td>PYD-NACHT-LRR-FIIND-CARD</td>
</tr>
<tr>
<td></td>
<td>NLRP2-9</td>
<td>PYD-NACHT-LRR</td>
</tr>
<tr>
<td></td>
<td>NLRP10</td>
<td>PYD-NACHT-PYD-NACHT-LRR</td>
</tr>
<tr>
<td></td>
<td>NLRP11-14</td>
<td></td>
</tr>
<tr>
<td>NLRX</td>
<td>NLRX1</td>
<td>X-NACHT-LRR</td>
</tr>
</tbody>
</table>

Table 1. Human NLR family members according to the recent nomenclature (Ting, Lovering et al. 2008). The following abbreviations are used: CARD, Card activation and recruitment domain; AD, acid activation domain; BIR, baculovirus inhibitor of apoptosis repeat (BIR) domain; LRR, leucine rich repeats; PYD, pyrin domain.
Discovered in 2001, NLRP1 is a structural exception in the NLRP family, since it additionally possesses a C-terminal tail, comprising a CARD domain and a domain with unknown function, designated function-to-find-domain (FIIND) (Figure 1) (Martinon, Hofmann et al. 2001). A year later, NLRP1 was demonstrated to bind to the adaptor protein ASC to form an ‘inflammasome’, which was involved in proinflammatory responses (Martinon, Burns et al. 2002). In the subsequent years, NLRP1 polymorphisms has been implicated in autoimmune diseases like vitiligo, Addison’s disease and type-1 diabetes (Jin, Birlea et al. 2007; Jin, Mailloux et al. 2007; Magitta, Boe Wolff et al. 2009; Jin, Riccardi et al. 2010; Zurawek, Fichna et al. 2010). However, the interest in NLRP1 has been limited, probably due to its not being conserved in mice, which instead harbors three paralogs of NLRP1 without a functional PYD domain (Martinon, Mayor et al. 2009).

**NLRP1 (CARD7/DEFCAP)**

In 2002, another member to the emerging NLRP family was added with the identification of NLRP3, then named as ‘Pyrin-containing-APAF-1-like-protein-1’ (PYPAF1) (Manji, Wang et al. 2002). Since the NLRPs were postulated to have an inflammatory function, a potential relation to NF-κB, a key mediator of inflammation, seemed inevitable. The authors investigated this relation and demonstrated that NLRP3 could not regulate the NF-κB reporter gene *per se*, but, this regulation was possible upon its association with low amounts of ASC (Manji, Wang et al. 2002). Incidentally, these studies coincided with the discovery of the gene encoding NLRP3 by another group, which alternatively denoted the protein as cryopyrin and called the gene *CIAS1* (Hoffman, Mueller et al. 2001). NLRP3, like NLRP1 was suggested to form an inflammasome upon binding to the adaptor proteins ASC and CARD-8 (Agostini, Martinon et al. 2004).

The NLRP3 inflammasome has been very intensely studied and the *NLRP3* gene has been implicated in a number of disorders, which is dealt with in the later sections. The gene and the protein names have at times been used interchangeably creating confusion. Following the recent nomenclature, in this thesis all the gene names would be in italics, e.g. *NLRP3, CARD-8* etc while protein names would be in the normal font, e.g. NLRP3, CARD-8 etc.
Adaptor CARD-8 (TUCAN/Cardinal)
Parallel with the discovery of NLRP1, another novel CARD-FIIND domain comprising protein was reported and named TUCAN (Tumour-Upregulated-CARD-containing-Antagonist-of-caspase-Nine, and also known as CARD-8 or Cardinal) (Pathan, Marusawa et al. 2001). CARD-8 was found to be over-expressed in colorectal cancer tissue and shown to suppress cytochrome c/procaspase-9 mediated apoptosis (Pathan, Marusawa et al. 2001). Since NLRP3 lacked a CARD-FIIND domain (Figure 1), an obvious role for CARD-8 as a binding partner in NLRP3 inflammasome was suggested (Agostini, Martinon et al. 2004), but is still a matter of debate (Allen, Scull et al. 2009). Since its discovery, two independent studies have demonstrated the physical interaction of CARD-8 with caspase-1 (Razmara, Srinivasula et al. 2002; Wagner, Proell et al. 2009) and a subsequent decrease in caspase-1 dependent IL-1β production in THP-1 cells (Razmara, Srinivasula et al. 2002). In addition, CARD-8 was shown bind to caspase-1 inhibitors, pseudo-ICE and ICEBERG (Razmara, Srinivasula et al. 2002). The other reported functions of CARD-8 include regulation of apoptosis and inhibition of NF-κB (Razmara, Srinivasula et al. 2002; Fontalba, Martinez-Taboada et al. 2007).

Adaptor ASC (CARD5/PYCARD/TMS1)
Apoptosis-associated-speck-like-protein-containing-a-CARD (ASC) is a 22-KDa protein composed of two death-domain family members: CARD and PYD (Figure 1), due to which it is postulated to have important roles in apoptosis and inflammation. The name comes from its formation of protein aggregates upon induction of apoptosis by anti-tumor drugs. (Martinon, Hofmann et al. 2001). ASC is an undisputed key adaptor molecule in the inflammasomes, and binds using homotypic PYD-PYD domain interaction with the NLRP while recruiting a caspase-1 with its CARD domain. Its essential role is evident by the ASC-deficient mouse models showing impaired IL-1β production in response to all the upstream inflammasome stimuli (Mariathasan 2007).
**Figure 1.** Domain organization of NLRP1, NLRP3, CARD-8 and ASC. NLRP1 possess a C-terminal function to find (FIIND)-CARD domain which is unique to the NLRP subfamily. CARD-8 comprises FIIND-CARD domains due to which it was suggested to be a binding partner of NLRP3. ASC is an adaptor which binds NLRP1 as well as NLRP3.

**NLRP expression**

For an optimal detection of pathogens, the NLRPs are distributed in a non-overlapping manner throughout the body. NLRP1 is primarily expressed in heart, spleen, thymus, kidney and liver and also in the epithelial cell lining of gastrointestinal tract and respiratory tract. NLRP3 expression is confined to the stratified non-keratinizing squamous epithelium of the oesophagus and ectocervix. NLRP1, NLRP3 and NLRP12 are abundantly expressed in the primary immune cells (Kummer, Broekhuizen et al. 2007).

Interestingly, in the recent years several NLRPs have been related to reproduction. Mutations in NLRP7 were associated with recurrent hydatidiform moles (Murdoch, Djuric et al. 2006; Qian, Deveault et al. 2007). NLRP4, NLRP5, NLRP8 and NLRP9 were demonstrated to be expressed in gametes and preimplantation embryos (Ponsuksili, Brunner et al. 2006). In a recent study, all 14 members of the NLRP family are reported to be expressed in gametes and preimplantation embryo at some stage, indicating a stage-dependent role of NLRP family during early human reproduction (Zhang, Dixon et al. 2008). Conceptually, since inflammation and infection can regulate fertility and abortions, this fascinating role of NLRPs beyond pathogen detection seems quite likely.
Innate immunity and disease associations

A greater understanding of the innate immune mechanisms came with the discovery of the genetic basis of hereditary periodic fever (HPF). This was a milestone which immensely aided the elucidation of pathogenic mechanisms at a molecular level and often times provided clues into the specific treatment options.

Patients suffering from HPF displayed a wide array of clinical manifestations posing a diagnostic challenge for the clinicians. A turn of events came with the identification of the MEFV gene underlying familial Mediterranean fever (FMF) (French FMF Consortium 1997; International FMF Consortium 1997), which was followed by the discovery of heterozygous mutations in the TNFRSF1A gene leading to TNF receptor 1-associated periodic fever syndrome (TRAPS) (McDermott, Aksentijevich et al. 1999). These two key discoveries led to the coining of the term ‘autoinflammatory diseases’ in the year 1999 (McDermott, Aksentijevich et al. 1999). Subsequently, at least 10 different autoinflammatory diseases showing mendelian inheritance were described (Goldbach-Mansky and Kastner 2009).

However, the major conceptual breakthrough came with the discovery of the NLRP3 gene (earlier known as CIAS1/NALP3), underlying Familial Cold-Associated Syndrome (FCAS) and Muckle Wells Syndrome (MWS) (Hoffman, Mueller et al. 2001). A few years later the protein NLRP3 was shown to form an IL-1β producing platform, called the ‘inflammasome’ (Agostini, Martinon et al. 2004), thereby explaining the fever symptoms. Mutations in NLRP3 were shown to produce unregulated amounts of IL-1β giving rise to inflammation, which could effectively be treated using a competitive inhibitor of IL-1β (Hawkins, Lachmann et al. 2003). Discovery of NLRP3 and its role in IL-1β production not only helped in the understanding of rare hereditary syndromes but also clarified the fundamental role of IL-1β in inflammation. The past few years have witnessed an astounding increase in IL-1β-dependent diseases, ranging from rare, to more common and more complex diseases.

Mutations in NLRP3

NLRP3 was discovered in 2001 by Hoffman and colleagues, while fine mapping the gene responsible for FCAS and MWS: two rare types of hereditary periodic fever syndromes (Hoffman, Mueller et al. 2001). FCAS was first described in 1944 and is characterized by fever, rash, arthralgia and conjunctivitis upon exposure to cold (Kile and Rusk 1940). MWS, first described in 1962, manifests in inflammatory episodes similar to FCAS, but the symptoms are not cold-induced (Muckle and Wells 1962). Additionally, MWS patients may
display a sensorineural hearing loss and systemic amyloidosis subsequently leading to renal failure (Muckle 1979).

FCAS and MWS were mapped to chromosome 1q44 (Cuisset, Drenth et al. 1999; Hoffmann and Romero 2000). Genetic screening of the critical 1Mb region on 1q44 revealed four distinct mutations in exon three of the \textit{NLRP3} gene (Hoffman, Mueller et al. 2001; Hoffman, Gregory et al. 2003), which comprised a total of nine exons. Exon three corresponds to the NACHT domain which is flanked by a pyrin (PYD) domain at amino-terminus and a LRR domain comprising seven LRR’s, at the C-terminus. At that time the protein was given the name cryopyrin (cryo: ice, pyrin: fire) (Hoffman, Mueller et al. 2001) but now commonly known as NLRP3. Certain similarities between NLRP3 and familial Mediterranean fever-causing MEFV proteins were observed: both possessed a PYD domain, and were predominantly expressed in blood leukocytes (Hoffman, Mueller et al. 2001), suggesting a role in inflammation and apoptosis (Bertin and DiStefano 2000; Hoffman, Mueller et al. 2001; Martinon, Hofmann et al. 2001).

A year after its discovery, mutations in \textit{NLRP3} were implicated in yet another disease, first described in the 1980’s (Prieur and Griscelli 1981) and given the name, chronic infantile neurological cutaneous articular syndrome (CINCA) also known as neonatal onset multiple inflammatory syndrome (NOMID) (Feldmann, Prieur et al. 2002). Patients suffering from NOMID displayed symptoms like in FCAS and MWS but with a much more severe inflammatory phenotype involving skin, joints and central nervous system. Non-inflammatory manifestations in the form of cartilage overgrowth and hearing loss could also be present.

\textbf{The clinical continuum of CAPS}

Owing to the common gene underlying FCAS, MWS and NOMID, these three diseases were broadly categorized under the name ‘Cryopyrin-associated periodic syndromes’ (CAPS) (Stojanov and Kastner 2005). While the above syndromes display certain distinct characteristics, they also share a number of overlapping symptoms, hence defining a perfect clinical continuum, with the mildest FCAS being on one end and the severest NOMID on the other, and MWS somewhere in between. The characteristic common symptoms of the CAPS patients are recurrent bouts of unprovoked fever, increased white blood cell count and elevated levels of the acute phase C-reactive protein (CRP), especially during the inflammatory attacks. Urticaria-like rashes characterized by increased neutrophil and
eosinophil infiltration with the absence of mast cells is observed in skin biopsies of infected areas. FCAS and NOMID patients typically have a neonatal to early childhood onset of disease symptoms, while MWS varies from childhood to an early adulthood-onset (Church, Savic et al. 2008; Hoffman and Simon 2009).

Prior to the discovery of NLRP3, the cause of excessive inflammation in these patients was not known. In the absence of a clinical diagnosis, futile attempts to treat the patients with anti-inflammatories, anti-histamine, immunosuppressants and TNF-blockers were done. The clinical investigations of these patients were limited to the demonstration of the absence of autoantibodies and rheumatoid factor. There were occasional fatalities in MWS patients, but mortality was up to 20% in the case of NOMID patients (Church, Savic et al. 2008).

CAPS are postulated to be monogenic diseases inherited in an autosomal dominant fashion. While some of the NLRP3 mutations are associated with a particular CAPS phenotype, others like V198M and R260W are associated with multiple CAPS phenotypes indicating a role of other non-NLRP3 modifier genes or environmental factors in orchestrating the symptoms. Absolutely no relationship between the position of mutation and disease severity is seen (Aksentijevich, Nowak et al. 2002; Masters, Simon et al. 2009).

**CAPS patients without detectable NLRP3 mutations**

The involvement of gain-of-function mutations in NLRP3 in CAPS is well established. However, about 40% of the patients displaying a classical CAPS phenotype do not reveal any mutations in the NLRP3 (Yu and Leslie 2011). There are several issues that can be discussed here; First of all, since almost all the disease-causing mutations are concentrated on the exon three of NLRP3, the most common practice is to sequence just this exon. However, the discovery of mutations in exons four and six prompt a screening of the entire coding region in the mutation-negative patients.

Secondly, it can be argued that, the regular screening methods miss the real, causative mutations in NLRP3, as in the special case of somatic mosaicism reported by Saito et al, where mutations in NLRP3 were detected in only a fraction of patient’s cells (Saito, Fujisawa et al. 2005; Saito, Nishikomori et al. 2008). A recently published multicenter study reported NLRP3 somatic mosaicism in 69% of mutation-negative NOMID patients (Tanaka, Izawa et al. 2011). Further, mutations could be present in yet-identified genes, that result in similar or modified symptoms, for example the truncating mutations discovered in the IL-1RN gene.
leading to unopposed production of IL-1 in the deficiency in interleukin-1 receptor antagonist syndrome (DIRA) (Aksentijevich, Masters et al. 2009); or mutations in NLRP12 leading to a CAPS-like phenotype (Jeru, Duquesnoy et al. 2008). Aksentijevich et al. performed a comprehensive genetic screening of other candidate genes in ten, mutation-negative patients, but nothing more than common polymorphisms could be detected (Aksentijevich, C et al. 2007).

Recent discoveries suggest that the gene expression levels of NLRP3 might be of importance in regulating its activity. Genetic alterations in the promoter region of NLRP3 have been associated with an increased in vitro NLRP3 expression (Anderson, Mueller et al. 2008). Further, it was demonstrated that a transcriptional upregulation of NLRP3, made possible through a series of cell-receptors converging in NF-κB upregulation, was indispensable for the NLRP3 inflammasome (described in later sections) activation (Bauernfeind, Horvath et al. 2009). It is interesting to speculate that the mutation-negative CAPS patients have an upregulated NLRP3 expression, which bypasses the need for mutations as an activating factor. Characterization of the NLRP3 promoter as well as NLRP3 expression defects in mutation-negative patients might be helpful in further elucidation of their roles in potential disease mechanisms.

**Low-penetrance alterations in NLRP3 and the atypical CAPS**

Certain missense alterations like V198M, R488Q and Q703K in NLRP3 can be detected in patients as well as healthy individuals, resulting in their being considered non-pathogenic. V198M and R488Q with allele frequencies close to 1% are classified as low-penetrance mutations, while Q703K with allele frequency exceeding 1% is classified as a polymorphism (Aksentijevich, C et al. 2007). The reports of patients possessing the above alterations show considerable clinical variability. For instance, typical or atypical CAPS features may be present, and likewise, while certain cases benefit from anakinra others do not (Arostegui, Aldea et al. 2004; Porksen, Lohse et al. 2004; Aksentijevich, C et al. 2007; Serrano, Ormazabal et al. 2009), making it hard to interpret their presence.

According to the conventional categorization, the CAPS patients typically display a neonatal to early childhood onset of symptoms. A general systemic inflammation is present in all the CAPS patients who display certain common symptoms like fever, arthralgia, headache, fatigue and conjunctivitis (Hoffman and Simon 2009). However a specific diagnosis is
achieved by the distinct clinical features like, cold-induced symptoms in FCAS, amyloidosis and progressive sensorineural effect in MWS, and central nervous system involvement, developmental delay and arthropathy in NOMID (Hoffman and Simon 2009; Yu and Leslie 2011).

A bigger challenge lies in the diagnosis of patients who display CAPS-like symptoms, but do not have the classical manifestations that characterize FCAS, MWS or NOMID. The atypical CAPS cases, upon genetic screening might reveal a mutation (Porksen, Lohse et al. 2004; Serrano, Ormazabal et al. 2009; Verma, Eriksson et al. 2010) or just common polymorphisms in the NLRP3 (Ting, Scalzi et al. 2007; Verma, Lerm et al. 2008). In such patients, a role for IL-1β in disease pathogenesis is suspected when they do not respond to other standard treatments that usually extend over a large period of time. A number of patients with atypical CAPS show a remarkable benefit to IL-1β blockade demonstrating a key role for this cytokine in disease pathogenesis (Aksentijevich, C et al. 2007; Ting, Scalzi et al. 2007; Verma, Lerm et al. 2008; Verma, Eriksson et al. 2010). This patient group suffers due to the lack of a diagnosable phenotype and genotype, preventing an early administration of IL-1β blockade.

The C10X in CARD-8

The C10X polymorphism results in a massive truncation of this normally 643 amino acid protein. Interestingly, this severe change is a common polymorphism that in the recent years has been associated with several inflammatory conditions, mainly related to disturbances in the NF-κB pathway (McGovern, Butler et al. 2006; Fontalba, Martinez-Taboada et al. 2007; Fontalba, Gutierrez et al. 2008). These associations are quite likely, since full length CARD-8 functions as an inhibitor of NF-κB potentially preventing a robust inflammatory response, whereas this critical function is abrogated in its truncated form (Fontalba, Martinez-Taboada et al. 2007). Owing to its similarity with NLRP1, CARD-8 was suggested to be a binding partner of NLRP3 in the inflammasome. Whether the CARD-8 functions exclusively through NF-κB inhibition or IL-1β/inflammasome, or both, remains to be elucidated.

The absence of CARD-8 in certain mammalian species (mouse, rat and cow) (Bagnall, Roberts et al. 2008) has contributed to doubts regarding its functional importance. Hypothetically, being a negative regulator of inflammatory response, some animal groups might have selected for a loss of CARD-8 during the adaptive evolution (Ko, Shukla et al.
2009). Several examples of the negative regulators of inflammation undergoing a loss of function during adaptive evolution exist. For instance, \textit{CASP12}, which is a negative regulator of caspase-1, has acquired a nonsense polymorphism (Saleh, Vaillancourt et al. 2004) and \textit{MAL/TIRAP}, a downstream regulator of TLRs 2 and 4 has acquired a missense polymorphism (Ferwerda, Alonso et al. 2009). \textit{CARD-8}, \textit{CASP12} and \textit{MAL/TIRAP} in their wild type forms are postulated to confer an increased risk for sepsis (Saleh, Vaillancourt et al. 2004; Ferwerda, Alonso et al. 2009; Ko, Shukla et al. 2009). In this disease condition the patients are found to have features consistent with immunosuppression (Hotchkiss and Karl 2003), and individual genetic variations (e.g. in \textit{TNF}, \textit{IL-1}, \textit{IL-6} etc) as an underlying basis are quite well studied (Villar, Maca-Meyer et al. 2004).

Selective alterations like the C10X could be an adaptive mechanism for providing better resistance to infections in a harsh environment, which, however comes with the by-product of chronic inflammatory consequences. As famously put by the immunologist Lewis Thomas: ‘Our arsenals for fighting off bacteria are so powerful, and involve so many different defense mechanisms, that we are in more danger from them than from the invaders’ (Thomas 1972).

\textbf{Targeting IL-1\textbeta in CAPS}

The recombinant human IL-1 receptor antagonist (IL-1Ra), anakinra (Kineret\textsuperscript{TM}), which initially was developed to treat sepsis patients (Fisher, Slotman et al. 1994), was administered in a clinical trial to two MWS patients. A dramatic cessation of inflammatory parameters within a few hours of its injection was observed in both the patients (Hawkins, Lachmann et al. 2003). Since then, anakinra has been the accepted treatment for CAPS patients with undisputed clinical results, demonstrating the effectiveness of targeting IL-1\textbeta (Goldbach-Mansky, Dailey et al. 2006; Leslie, Lachmann et al. 2006). Anakinra has a half life of four hours and therefore needs to be injected on a daily basis, causing discomfort to the patients. To overcome this problem and to provide CAPS patients with better alternatives, two new drugs for targeting IL-1\textbeta have been developed. One is canakinumab (ACS885), which is a monoclonal antibody against IL-1\textbeta, and can be injected every eight weeks and the other is the IL-1\textbeta trap, rilonacept (Arcalyst\textsuperscript{TM}), which can be injected on a weekly basis (Church, Savic et al. 2008; Church and McDermott 2009). Both canakinumab and rilonacept are now approved for treating CAPS. Ignoring the cost factor, the targeting of IL-1\textbeta has proved to be a blessing for the CAPS patients.
The Inflammasomes
With the discovery of mutations in \textit{NLRP3}, a gain-of-function leading to increased IL-1\(\beta\) production was postulated (Martinon, Burns et al. 2002). Evidence for this was soon provided by the demonstration of increased IL-1\(\beta\) levels in peripheral blood mononuclear cells of the CAPS patients (Aksentijevich, Nowak et al. 2002). NLRP3 protein was shown to form the ‘inflammasome’, which subsequently led to IL-1\(\beta\) production (Agostini, Martinon et al. 2004), thereby explaining the molecular basis of \textit{NLRP3} mutations.

In 2002, Tschopp and colleagues coined the term inflammasome to describe a multiprotein platform leading to the activation of caspase-1 which subsequently cleaved and produced the endogenous pyrogen, IL-1\(\beta\) (Martinon, Burns et al. 2002). NLRP1 was demonstrated to form a cytosolic complex, by recruiting the the adaptor protein ASC, which binds and activates caspase-1. The C-terminal extension, the FIIND-CARD domain was suggested to bind and activate caspase-5 (Martinon, Burns et al. 2002). The selection of the term inflammasome was motivated by its similarity to another caspase-activating complex termed apoptosome, which triggers apoptosis (Martinon, Burns et al. 2002).

In 2004, it was shown by Agoistini et al. that NLRP3 upon activation associates with ASC at the amino-terminus using the PYD-PYD interaction (Figure 2). The CARD domain of ASC recruits the CARD of an inactive caspase-1 (Agostini, Martinon et al. 2004). Authors postulated that the lack of FIIND-CARD domain in NLRP3 could be compensated by the structurally similar protein CARD-8, which would then recruit another caspase-1 (Agostini, Martinon et al. 2004). However, no evidence of binding of CARD-8 to the full length NLRP3 could be seen, removal of PYD or LRR domain or both were essential for its interaction with CARD-8, which created doubts about CARD-8 being a binding partner of NLRP3 in the inflammasome (Agostini, Martinon et al. 2004). Further, it was hypothesized that the oligomerization of the NACHT domain is a prerequisite for signal transduction upon stimulation through the PYD domain, which would recruit and activate caspase-1 through ASC (Martinon and Tschopp 2005).
Caspase-1

Figure 2. Structural organization of the NLRP3 inflammasome comprising NLRP3, ASC and probably CARD-8. It is postulated that upon sensing stimuli, NLRP3 oligomerizes (probably forming hexamers or heptamers) via NACHT domain interactions and recruits ASC via the PYD-PYD domain interactions. The CARD domain of ASC recruits caspase-1. The CARD-8 is postulated to bind to the NACHT domain through NACHT-FIIND interactions and recruit another caspase-1 via its CARD domain.

Caspases are mostly associated with apoptosis, but caspase-1, like caspase-5 is inflammatory in nature. Caspase-1 has formerly been known as Interleukin-converting enzyme (ICE) due to its ability to cleave the immature precursor of IL-1β to bioactive IL-1β. The two most well known substrates of caspase-1 are pro-IL-1β and pro-IL-18 (Fantuzzi and Dinarello 1999), pro-IL-33 has also been suggested as a caspase-1 substrate (Martinon, Mayor et al. 2009) but its role is not clearly understood. IL-18 is associated with the regulation of IFN-γ and T helper-1 responses and is also shown to be involved in maintaining intestinal homeostasis (Zaki, Lamkanfi et al. 2011). In a recent article (Walsh, Logue et al. 2011) it was demonstrated that when used in excess amounts, caspase-1 could cleave multiple substrates that are otherwise specific for the apoptotic caspses -3 and 7. However the specificity of caspase-1 towards the preferred substrates IL-1 and IL-18 is maintained through its rapid destabilization within minutes of its activation.

Inflammasome activators

Studies implicating a wide range of factors, including microbial PAMPs, exogenous non-microbial ligands as well as endogenous DAMPs, as possible inflammasome stimuli have been reported, which suggested that NLRP3 could be the long sought danger-sensing
receptor. Surprisingly, no evidence of direct ligand binding to NLRP3 was ever found, challenging its classification as a receptor. Now the common belief is that NLRP3, in line with Matzinger’s danger theory (Matzinger 2002) is sensitive to the changes in cellular milieu, rather than the stimuli itself. Several models in this regard have been proposed, but none of them alone can explain the vast array of stimuli that are sensed by the inflammasome.

One of the earliest models suggested that efflux of potassium ions (K\(^+\)), either through pore formation by bacterial toxins or through P2X7 receptors could activate the inflammasome (Mariathasan, Weiss et al. 2006). Another proposed mechanism of inflammasome activation is through lysosomal destabilization leading to cathepsin- B leakage which has been shown in the case of certain DAMPs (Halle, Hornung et al. 2008; Hornung, Bauernfeind et al. 2008). A recent observation is that all of the NLRP3 activators induce ROS production, and that the use of ROS scavengers could suppress inflammasome activation (Cruz, Rinna et al. 2007; Meissner, Molawi et al. 2008). It was also demonstrated that the thioredoxin-interacting protein (TXNIP) linked the ROS pathway to inflammasomes (Zhou, Yazdi et al. 2011). While the list of substances capable of inducing ROS is quite extensive not all of these can activate the inflammasome, suggesting that another, more specific indicator should exist.

None of the above mechanisms per se account for inflammasome activation in response to all the types of stimuli. Further research might disclose if these mechanisms act in concert, or if there is a yet unidentified mechanism(s). Looking at the complexity and specificity of the innate immune system it is likely that multiple pathways exist.

**PAMPs, DAMPs and exogenous substances**

As illustrated in Figure 3, a large number of factors are known to stimulate the inflammasome. These include a number of bacteria like *Staphylococcus aureus, Listeria monocytogenes, Escherichia coli, Neisseria gonorrhoe, Mycobacterium marinum, Candida albicans, Streptococcus pneumoniae* and also viruses like *Influenza A virus, Adenoviruses, Sendai virus* (Bauernfeind, Ablasser et al. 2011). Although the exact mechanisms are not known, the involvement of pore-forming toxins in case of bacterial stimuli (Gurcel, Abrami et al. 2006; Mariathasan, Weiss et al. 2006; Li, Willingham et al. 2008; Harder, Franchi et al. 2009), and M2 channel proteins in case of influenza viruses (Ichinohe, Pang et al. 2010) have been demonstrated.
The inflammasome can be activated by host-derived factors that can induce damage or cell-death. These include ATP, Monosodium urate (MSU), calcium pyrophosphate dehydrate (CPPD), aluminium hydroxide (alum), cholesterol crystals, β-amyloid and hyaluronan (Bauernfeind, Ablasser et al. 2011). Of these, ATP and MSU are normally present inside the cells and their extracellular presence indicating dying cells seems to activate NLRP3. Cholesterol, β-amyloid and hyaluronan are present extracellularly and their ingestion into the cells might activate NLRP3 (Davis, Wen et al. 2011).

In analogy to the endogenous particles, several environmental pollutants like silica and asbestos are demonstrated to trigger the NLRP3 inflammasome (Cassel, Eisenbarth et al. 2008; Dostert, Petrilli et al. 2008). The mechanism of activation of particulate or phagosomal matter is not clearly understood. One of the suggested mechanisms is that the large sized particles are not easily phagocytosed and remain trapped at the cell surface, resulting in a condition termed as ‘frustrated phagocytosis’, which leads to the formation of reactive oxygen species and causes inflammasome activation (Dostert, Petrilli et al. 2008).
Pathogen activators
Bacteria
*S. aureus, L. monocytogenes, E. coli, N. gonorrhoea*, etc.
Virus
Influenza A, Adenovirus, Sendai, etc.

Self activators:
ATP
Cholesterol
β-amyloid
Hyaluronan
etc.

Environmental activators
Asbestos
Silica
Alum
UV radiation
etc.

Disease causing genetic alterations

**Figure 3.** A list of stimuli known to activate the NLRP3 inflammasome. This includes pathogen activators, endogenous danger signals, environmental factors and the disease causing genetic factors.

**Mutations as activating factors**
Almost all the mutations in *NLRP3* are postulated to confer a gain of function, as evident by elevated IL-1β levels in the patients (Aksentijevich, Nowak et al. 2002; Goldbach-Mansky, Dailey et al. 2006), and further supported by the *in vitro* experiments using genetic constructs of mutated *NLRP3* (Dowds, Masumoto et al. 2004). It is not clear how mutations impact the inflammasome activity, but 3-D structural modeling predicts that mutations can lead to surface disruption, which might affect the subdomain interactions within the NACHT domain or in certain cases provoke interactions with the LRR domain (Aksentijevich, C et al. 2007). Owing to these disruptions, the structural stability of the hypothetically closed state of NLRP3 is disturbed and an opened state exposing the PYD domain and LRRs might be favored. The open state is probably the active one which results in the functional assembly of other components of inflammasome. (Aksentijevich, C et al. 2007).
So far, practically all mutations have been reported in exon three, corresponding to the NACHT domain, suggesting the presence of functionally important sites in this region. In total, more than one hundred missense alterations in NLRP3 gene have been reported, a detailed listing of the genetic alterations in the NLRP3 and its associated symptoms can be found at the ISSAID website (http://fmf.igh.cnrs.fr/ISSAID/infevers/). Only three more mutations lying outside of exon three have been identified, G755R, G755A in exon four and Y859C in exon 6, both these exons correspond to the LRR region.

**The Inflammasome and other IL-1β-mediated diseases**

After the exciting discovery of the role of inflammasomes in HPF, the list of inflammasome-dependent diseases have increased at an astounding pace. So far, the role of NLRP3 has been recognized in hypertension (Omi, Kumada et al. 2006), contact hypersensitivity (Watanabe, Gaide et al. 2007), gouty arthritis (So, De Smedt et al. 2007), asbestosis (Dostert, Petrilli et al. 2008), malaria (Dostert, Guarda et al. 2009; Shio, Eisenbarth et al. 2009), atherosclerosis (Duewell, Kono et al. 2010) and type-2 diabetes (Masters, Dunne et al. 2010). Other disease conditions that are known to benefit from targeting IL-1β, where a role of NLRP3 is not recognized are the deficiency of IL-1Ra (DIRA), systemic-onset juvenile idiopathic arthritis (SOJIA) and adult-onset Still’s disease (Lequerre, Quartier et al. 2008; Zeft, Hollister et al. 2009). Certain FMF patients refractory to the conventional colchicine treatment have been reported to benefit from anakinra (Belkhir, Moulonguet-Doleris et al. 2007). FMF arises from mutations in the MEFV gene that encodes pyrin, which is postulated to cause dysregulations in caspase-1 and IL-1β (Masters, Simon et al. 2009). Similar situations have been described in TRAPS (Simon, Bodar et al. 2004) and Hyper IgD syndrome (Caillez, Garaix et al. 2006) where patients refractory to the conventional therapies show remarkable benefit from anakinra. The above cases suggest IL-1β as the final, common pathway in inflammation.

**IL-1β regulatory mechanisms**

IL-1β is an extremely potent, pyrogenic cytokine essential to thwart pathogen attacks (Dinarello 1984), so its unsolicited production could be harmful, even fatal for the host. As illustrated in Figure 4, IL-1β levels are tightly regulated at the levels of expression, production and secretion (Dinarello 1988). Additionally, IL-1β can regulate its own production in an autocrine manner by activating MyD88 through IL-1 receptor (IL-1R) binding. The naturally occurring IL-1 receptor antagonist (IL-1Ra) can competitively bind to IL-1R and block the
downstream signaling (Dinarello 1997). These extensive regulatory mechanisms might reflect the remarkable potency of this cytokine.

Monocytes and macrophages are the main cell types that produce IL-1β (Dinarello 1988), this may be due to their ability to process the IL-1β precursor (pro-IL-1β) more efficiently than other cell types. The circulating monocytes lack a constitutive expression of the IL-1β mRNA. This can however be upregulated by a variety of factors, like adherence to surfaces, hypoxia or TLR stimuli, but due to the lack of a signal peptide it is not automatically converted to IL-1β protein (Dinarello 2009). The IL-1β mRNA has a short half-life and starts to degrade after 4 hrs. It has been shown that IL-1β mRNA upregulation begins within 15 minutes of LPS stimulation, but these levels start to decline after 4 hrs. A sustained IL-1β production, however, can be achieved with IL-1β used as stimuli to induce itself (Schindler, Ghezzi et al. 1990).

After the transcription of IL-1β mRNA, its translation to pro-IL-1β takes place in the cytosol (Dinarello 2009). Pro-IL-1β exists as an inert 31 kDa peptide, which requires cleavage to form the mature and bioactive 17 kDa IL-1β. Active caspase-1, produced by the inflammasomes, constitutes the rate-limiting, regulating factor at this step. Caspase-1 is a cysteine protease which specifically cleaves pro-IL-1β at the aspartic acid residue 116 to generate the 17 kDa active IL-1β fragment. To prevent an unwarranted production of caspase-1 from the inflammasomes, it is hypothesized that in a resting state, the LRR domain folds over the NACHT and PYD domains, thereby auto-inhibiting inflammasome activity (Tschopp, Martinon et al. 2003). Upon sensing a stimulus, the LRR domain folds back, NLRP3 oligomerizes and recruits the adaptor molecule ASC and probably CARD-8, to form the functionally active inflammasome complex which produces active caspase-1 (Kastner 2005; Mariathasan and Monack 2007). Thus, the IL-1β production in monocytes and macrophages requires two different signals: The first signal causes transcription and translation while the second signal causes IL-1β processing and secretion.

The above-mentioned mechanisms are important in monocytes and macrophages, which are the most well studied IL-1β producing cell-types. There are other endogenous inhibitors like serpin proteinase 9, which blocks the active site on caspase-1, preventing the processing of pro-IL-1β and IL-1β (Annand, Dahlen et al. 1999), similarly caspase-12 has a suppressive effect on caspase-1 catalysis (Saleh, Mathison et al. 2006). Pyrin is postulated to regulate the
inflammasomes through binding to either ASC or caspase-1, Pyrin-only proteins (POP), like POP-1&2 and CARD-only proteins (COP) like pseudoICE and ICEBERG are among the other endogenous regulators of the inflammasome (Stehlik and Dorfleutner 2007).

Figure 4. IL-1β production is regulated at the levels of transcription, translation and secretion. Upon stimulation through the TLR4 there is an increased transcription of IL-1β mRNA, which is then translated to the IL-1β precursor, pro-IL-1β. A disturbed cellular homeostasis causes the assembly of inflammasome components and leads to the formation of active caspase-1, which subsequently cleaves pro-IL-1β and produces mature IL-1β. Upon secretion, IL-1β can again bind to its receptor IL-1R, leading to a continuous production of IL-1β through a positive feedback loop. The naturally occurring antagonist IL-Rα can competitively bind to the receptor thereby preventing downstream signaling.
Alternative IL-1β production mechanisms

The post-inflammasome period has primarily been focused on caspase-1 dependent IL-1β release from monocytes and macrophages. Quite surprisingly, the role of other cells involved in inflammatory response, like neutrophils, mast cells, NK cells etc has not been clearly elucidated. The massive neutrophil infiltration observed in murine and human CAPS raises the possibility of inflammasome-independent cleavage of pro-IL-1β. Already in the 1990’s, the interesting discovery that a number of serine proteases could cleave and produce mature IL-1β was made. Some of these serine proteases are stored in the azurophil granules of neutrophils, mast cell and T cells (Hazuda, Strickler et al. 1990; Mizutani, Schechter et al. 1991; Coeshott, Ohnemus et al. 1999). In addition to serine proteases, the matrix metalloproteinase (MMP), trypsin, antitrypsin, S. aureus protease are also demonstrated to cleave pro-IL-1β, several of these are found in inflammatory lesions but whether or not they produce functional IL-1β fragments in vivo remains to be elucidated (Stehlik 2009).

The most well studied neutrophil ICE is the serine protease, proteinase-3 (PR3), which was demonstrated to produce bioactive IL-1β in a monocyte-neutrophil co-culture (Coeshott, Ohnemus et al. 1999). Recently, a demonstration of inflammasome-independent IL-1β production was done, authors showed that neutrophils, upon LPS stimulation could produce caspase-1 independent IL-1β, via the serine protease PR3 (Greten, Arkan et al. 2007). This was supported in another elegant study using experimental mouse models of acute and chronic arthritis. The authors showed that an increased IL-1β in acute arthritis came from a predominance of neutrophils, and is unaffected by caspase-1 inhibition. The chronic form of arthritis, on the other hand, displayed an abundance of macrophages, and caspase-1 inhibition in this case, resulted in the prevention of joint destruction (Joosten, Netea et al. 2009). It is possible that during acute inflammation, neutrophils are the major sources of pro-IL-1β processing via the PR3, whereas at chronic stages where monocytes and macrophages are dominant, this role is taken over by the inflammasomes and caspase-1.

After the pathbreaking discovery of the inflammasomes, the potential targeting of caspase-1 raised hopes of the drug companies which developed the caspase-1 inhibitor VX-765. The effectiveness of VX-765 in inhibiting IL-1β has been shown using murine models, in vitro and ex vivo studies (Stack, Beaumont et al. 2005; Wannamaker, Davies et al. 2007). Apart from that, no reports of caspase-1 blockade being effective in CAPS patients have come up and IL-1Ra continues to be the standard treatment.
The discovery of inflammasome dependent IL-1β production was no doubt a milestone, which revealed the role of IL-1β as a key cytokine involved in a vast array of diseases. However, considering caspase-1 as the sole ICE might be an oversimplification of the NLR family as well as of IL-1β regulation. The targeting of caspase-1 in CAPS, in that case, would have resulted in a tremendous success. Moreover, it should also be acknowledged that a dramatic response to IL-1β blockade is not an indication of the mechanism through which IL-1β was produced. It is logical that redundant mechanisms exist to make an essential cytokine like IL-1β rapidly available, when one fails. New sophisticated and rational therapies can be directed keeping this in mind.

Rheumatoid arthritis and Inflammasome

Rheumatoid arthritis (RA) is characterized by painful and swollen joints most commonly in hands and feet, fatigue and occasional fever.

The diagnosis of RA patients (in paper II) is based on the 1987 American College of Rheumatology (ACR) classification criteria (newer criteria were introduced in 2010), where at least four of the criteria given below need to be fulfilled: morning stiffness for at least 1 hr, swelling (arthritis) of three or more joint areas, arthritis of proximal phalangeal or wrist joints, formation of rheumatic nodules, presence of rheumatic factor and radiographic erosions (Arnett, Edworthy et al. 1988).

The 28-joint Disease-Activity-Score (DAS28) is commonly used for assessing momentary disease activity in patients. It may also aid the rheumatologists to titer the dose of the given treatment if the patient does not optimally respond (Vander Cruyssen, Van Looy et al. 2005). DAS28 comprises swollen joint count (SJC), tender joint count (TJC), erythrocyte sedimentation rate (ESR) and the patient’s self assessment of general health (Prevoo, van ’t Hof et al. 1995). A DAS28 >5.1 is classified as high disease activity, 3.2 – 5.1 is moderate and < 3.2 is low disease activity (Fransen, Creemers et al. 2004).

Several pro-inflammatory cytokines like TNF-α, IL-1β, IL-15 and IL-17 have been implicated in the pathophysiology of RA (Bertolini, Nedwin et al. 1986; Rooney, Symons et al. 1990; Brennan, Maini et al. 1992; Arend and Dayer 1995). Among these, TNF-α and IL-1β are characterized as having most significant roles, and their elevated levels have been detected in RA patients (Rooney, Symons et al. 1990; Chu, Field et al. 1991). In the recent years, an
essential role of IL-18 as an inducer of TNF-α and IL-1β, and as a mediator of angiogenesis in RA has also been suggested (Dai, Matsuno et al. 2004).

Owing to the elevated levels of IL-1β in RA patients, clinical trials using the IL-1Ra, anakinra, were performed in the late nineties. The results from these trials indicated significantly reduced joint damage in association with IL-1Ra treatment (Bresnihan, Alvaro-Gracia et al. 1998; Jiang, Genant et al. 2000). However, when compared to other biological treatments, IL-1Ra resulted only in a modest improvement in overall clinical inflammation (Bresnihan, Alvaro-Gracia et al. 1998). Low efficacy of IL-1Ra was indicated in a Dutch RA cohort, where only 14% of patients continued anakinra after 2 years (den Broeder, de Jong et al. 2006).

The NLRP3 inflammasome was recently discovered to be involved in the cleavage and secretion of IL-1β and IL-18, two important cytokines implicated in RA. NLRP3 is present on the locus 1q44 which is one of the reported susceptibility loci for RA (Jawaheer, Seldin et al. 2001). However, only a couple of studies investigating the connection between NLRP3 inflammasome and RA have so far been reported. The earliest study in this regard was done in 2005, where increased NLRP3 expression in synovial tissue from RA patients was demonstrated (Rosengren, Hoffman et al. 2005). In another study involving four generations of a family diagnosed as suffering from CAPS, joint destruction, bone and cartilage lesions leading to disability similar to RA was observed. Sequencing revealed a mutation (T348M) in the NLRP3 gene, which has been implicated in MWS. Joint destruction, being an irreversible damage remained, but other symptoms resolved upon treatment with anakinra (Lequerre, Vittecoq et al. 2007).

**Autoimmunity versus Autoinflammation**

Autoimmune diseases pertain to dysregulations in the adaptive immune system and involve the major histocompatibility complex (MHC) and autoantigens. The concept of autoimmunity was predominant in immunology, till, inspired by the discovery of the genetic basis of TRAPS and FMF, the term ‘autoinflammation’ was coined in 1999. Autoinflammation was characterized by self-directed inflammation and the involvement of neutrophils and macrophages resulting in local tissue damage, but without any evidence of autoantibodies and T and B cells. Examples of autoinflammatory manifestations are the cytokine imbalances in HPF and the aberrant bacterial sensing in Crohn’s disease (CD), causing site-specific
inflammation (McGonagle and McDermott 2006). The recognition of two separate classes of immunological diseases was paralleled by advances in a deeper understanding of the innate immune system. A lot of patients that previously were clinical enigmas could be explained, but, with the answers came the puzzling questions as well. A few of these issues, like the interpretation of polymorphisms in the monogenic diseases like CAPS, FMF and TRAPS, and the atypical CAPS patients where the details are too heterogeneous to permit classification, have been discussed in the earlier sections.

Another controversial issue is regarding the polygenic autoinflammatory diseases, defined-so due to symptoms that resemble HPF, and absence of MHC-associations or autoantibodies. CD is considered a polygenic autoinflammatory disease, where innate immune cells are detected at inflammation sites, but recently, the presence of autoantibodies has also been reported (Bizzaro 2008). Similarly, RA is a classical polygenic autoimmune disease, characterized by autoantibodies, but at the same time cytokine imbalances contribute to joint erosion, and benefit is achieved both by anticytokine and antilymphocyte therapies. It might be that certain elements of autoimmunity or autoinflammation can be found in such complex diseases, if carefully looked for. Adopting an immunological continuum, with autoimmunity and autoinflammatory at both ends of the spectrum, certainly helps defining the relative contributions of the innate and adaptive immune system in the inflammatory diseases (McGonagle and McDermott 2006). Awareness of the recently elucidated pathophysiological mechanisms can be of great help for practicing clinicians while dealing with the less well understood phenotypes.
AIMS

The general aim of the thesis was to elucidate the role of genetic alterations in *NLRP3* in chronic inflammatory conditions. The focus of each paper was as follows:

**Paper I**

The aim was to elucidate the role of NLRP3 inflammasome in chronic inflammatory symptoms of a patient. Specifically, genetic screening of the *NLRP3, ASC* and *CARD-8* genes were done. IL-1β and caspase-1 levels as indicators of inflammasome activity were determined in the patient monocytes.

**Paper II**

The aim was to explore the role of polymorphisms found in the above patient, *i.e.* Q705K in *NLRP3* and C10X in *CARD-8*, in RA susceptibility and severity.

**Paper III**

The aim was to investigate the presence of genetic alterations in *NLRP3* in two siblings with chronic inflammation. Inflammasome activity was elucidated by determining caspase-1 and IL-1β levels in patient’s and control’s monocytes. The functional role of the novel M299V mutation in *NLRP3* detected in the siblings was *in vitro* determined.

**Paper IV**

The aim was to elucidate the functional significance of the Q705K polymorphism in *NLRP3* using an *in vitro* model. Spontaneous and challenged cytokine levels were determined in the cells expressing mutated and wild type *NLRP3*. 
**MATERIAL AND METHODS**

Some of the methods used in this thesis are described here.

**Genotyping methods**

**DNA sequencing**

This was done by the Sanger chain termination method (Sanger, Nicklen et al. 1977). The presence of a free 3’-hydroxyl group on the DNA target sequence enables the DNA polymerase to continue incorporating the nucleotides. The reaction mixture contains the ordinary dioxynucleotide triphosphates (dNTPs) as well as the fluorescently-labeled dideoxynucleotides (ddNTPs), where the ddNTPs are used to terminate the chain. During the PCR reaction, since dNTPs are present in higher concentrations than the ddNTPs, the fragments continue to be extended until a ddNTP is randomly incorporated and the chain terminates. By the end of the reaction there will be a set of fragments that differ in length from each other by a single base. The generated fragments can be separated on a capillary gel electrophoresis system and different fluorescent labels for the four different ddNTPs help identify the terminating nucleotide. Capillary electrophoresis and detection in this thesis was performed with a MegaBACE™ 1000 DNA sequencing system (GE Healthcare).

The detection of the specific single nucleotide polymorphisms (SNPs) was done using two different methods: (a) MegaBACE™ SNuPe genotyping, which is based upon a single nucleotide base extension and (b) Real-time PCR based Taqman® genotyping assay, which is based upon hybridization of an allele-specific probe.

**SNuPe** (single nucleotide primer extension) is a Sanger-based minisequencing reaction, where a single base is sequenced. In this three step reaction, the first step is DNA amplification that is followed by treatment with Shrimp Alkaline Phosphatase and Exonuclease (EXO-SAP-IT) to remove the unincorporated dNTPs and primers. In the second step, unlabelled primers are synthesized to anneal directly upstream of the polymorphic site. The PCR product, primers and SNuPe premix comprising ddNTPs and DNA are thermally cycled and primer extension takes place by incorporating a single fluorescently labeled ddNTP at the specific site. The third step represents the electrophoretic separation and detection similar to the sequencing process. Using this method, up to 5 injections for 96 loci can be identified using the same matrix. The homozygous samples are seen as a single peak.
while heterozygous samples have two peaks. Even though this method is robust, it is time consuming and involves a number of steps.

**TaqMan allele discrimination**

This method is based upon hybridization by an allele-specific oligonucleotide probe. Two probes, each specific for one allele, are employed using conditions that prevent annealing of the probe with a mismatch. The 5’ end of each probe is labeled for detection with a fluorescent reporter (FAM or VIC), while the 3’ end is labeled to inhibit prolongation with a non-fluorescent quencher with a minor groove binder (MGB). Forward and reverse primers designed to anneal at sites flanking the SNP, and probe that hybridizes at the polymorphic site are used. This convenient one-step reaction involves addition of primers and probes, along with a polymerase containing mastermix, to the template DNA followed by thermal cycling on ABI 7500 detection system. During PCR the primers and the probes hybridize at target sites. During each amplification cycle, the probe, which anneals to the polymorphic site, is cleaved by the 5’ exonuclease activity of the AmpliTaqGold® DNA polymerase, resulting in an exponentially increasing fluorescent signal caused by the freeing of 5’ fluorophore from the 3’ quencher.

Taqman is practically a one-step reaction, making it very rapid and cost effective for analyzing a large number of samples.

**Cytokine determination**

*Enzyme Linked ImmunoSorbent Assay (ELISA)*

ELISA is one of the most commonly used techniques for cytokine determination. In sandwich ELISA, the specific antibody is coated on the surface followed by the addition of the antigen. A detection antibody containing an enzyme is added, which forms a specific antigen-antibody complex. In the final step, a substrate is added, which the enzyme can convert into a detectable signal. Absorbance (in case of IL-18 and TNF-α) or luminescence (in case of IL-1β) was determined and converted to a concentration unit, which was given by a standard curve that was run in every experiment.

All the samples were stored at -72° C prior to detection and were thawed on ice at the time of analysis. The standard protocol suggested by the manufacturer was followed in all the kits.
**LINCOplex**
This method relies upon a bead-based flow cytometry method, where the reaction takes place on the surface of specific polystyrene microbeads. Each bead has a specific spectral address based on its red/infrared content. A specific capture antibody is attached to each bead set, followed by the addition of the samples. This is followed by the addition of a fluorescent reporter tag, which helps in detection of the sample. The analysis is performed on Luminex 100, which is a dual laser flowcytometer. The bead ID and the reporter intensity are determined by the two separate lasers, standard curves are generated for each analyte and the values of samples are determined.

The advantages of this method are its broad range of detection (0.06-2,000 pg/ml) and requirement of relatively low sample volumes. An additional feature of the method is that it can be used for simultaneous detection of other cytokines of interest within the same sample. However, due to the unavailability of IL-18 detection beads in combination with IL-1β, we restricted our analysis to just IL-1β determination.

**Caspase-1 determination**
Caspase-1 activity was determined either by using a commercial fluorometric kit or a flow cytometry based fluorochrome inhibitor of caspases (FLICA) kit. In the first, a caspase-1 substrate that is conjugated to a fluorescent reporter molecule is added to the lysed monocytes. Upon encountering caspase-1 in the sample, the substrate molecule is cleaved and the fluorochrome is released. The level of caspase-1 activity in the cells is directly proportional to the fluorescence intensity. Data was obtained as relative fluorescence units and represented as percentage of controls.

In FLICA method, a green fluorescence marked inhibitor FAM-YVAD-FMK, which is specific for caspase-1 was used. This inhibitor is cell permeable and binds covalently to active caspase-1. While the surplus inhibitor is washed away, the remaining green fluorescence is an indication of cells with active caspase-1. An advantage of this method is that the cells can either be read immediately or after 24 hrs by preservation using a fixative. The evaluation of caspase-1 positive cells, which have increased fluorescence intensity, is done using flow cytometry. Also, by this method caspase-1 can be detected using whole blood (rather than blood monocytes) and requires very little sample volume.
**Transductions**

In order to achieve stable and efficient expression of NLRP3 we used a retroviral system. Two steps are involved: First, a replication-defective retroviral vector carrying the gene of interest is used to transfect the commonly used packaging cell line 293T. *NLRP3* cloned into the retroviral vector pHSPG was a kind gift from Dr. Jenny Ting. To enable production of retroviral particles, the plasmid is co-transfected with helper plasmids p-Gag-Pol, coding for reverse transcriptase and retroviral integrase and p-VSV-G coding for envelope proteins. This enables infection of the target cell population as well as stable integration of the provirus with the gene of interest into the host genome. The calcium phosphate precipitation method used for transfections is simple and inexpensive. It involves addition of calcium chloride and phosphate buffer to a mixture of retroviral vector and helper plasmids. The resulting precipitate is then added to the packaging cells. Basically, the single stranded viral RNA is injected into the host cell cytoplasm and after reverse transcription the resulting double stranded DNA is integrated into the host genome during cell division by retroviral integrase. The released viral particles from transfected 293T cells is collected and used for transducing the target cells THP-1 in the second step. In order to increase the transduction rate, polybrene, a positively charged polymer that increases attraction between virus particles and target cells, and spinoculation, comprising centrifugation for 1.5 hrs at 1200g are used. The presence of GFP enabled judging the efficiency of both the steps by using either a fluorescence microscope or a flow cytometer.
RESULTS AND DISCUSSION
Two single nucleotide polymorphisms (SNPs), Q705K (rs 35829419) and C10X (rs 2043211), in the genes NLRP3 (NCBI reference: NM_004895.3) and CARD-8 (NM_014959), respectively, and one novel mutation M299V (no rs ID yet assigned) in NLRP3 (NCBI reference: NM_004895.3) are described in this thesis. The SNP Q705K leads to an amino acid shift from glutamine (Q) to lysine (K) at codon 705, corresponding to exon three in the NLRP3. This SNP has previously been reported, although alternatively numbered as Q703K (Hoffman, Gregory et al. 2003) (http://fmf.igh.cnrs.fr/infevers). It is a common practice to count the first methionine after the stop codon as the first amino acid, in which case the first seven amino acids in NLRP3 are MKMASTR, and the Q to K change takes place at the codon 705 (http://www.ensembl.org and http://www.ncbi.nlm.nih.gov). This consensus coding sequence is conserved in primates, rat, dolphin, cow, cat, armadillo and elephant. The second methionine has been identified as an alternative start codon, which renders a protein shorter by two amino acids, i.e. instead of MKMASTR the first seven amino acids are MASTRCK. This sequence has a better kozak signal and is conserved in mice, which is commonly used as a model to study human disease, and thus often used as the start codon. This numbering system was followed in case of M299V (paper III), which otherwise would have been annotated M301V. There is however no experimental evidence to show which start codon is preferentially used in vivo.

Like the other mutations (with the exception of amino acids 198 and 662) in NLRP3 (Anderson, Mueller et al. 2004), both the amino acids: glutamine at position 705/703 and methionine at position 299/301 are conserved in mice. The C10X in CARD-8 leads to a change from cysteine (C) to a stop codon (X) at codon 10. Recent data using RNA from CD patients suggests the existence of multiple CARD-8 isoforms and hence different outcomes of the predicted stop codon, like C10X, C34X, F52I and F102I, exist. It is also shown that individuals, who are homozygous for the stop codon, still can express the immunoreactive protein, although in reduced amounts (Bagnall, Roberts et al. 2008). However, how the different isoforms affect the CARD-8 function is not known.
Both Q705K and C10X are quite prevalent in the healthy population and hence referred to as polymorphisms, while M299V is a mutation, which by definition is a rare variation (< 1%).

**Paper I**

The patient in this study showed strikingly elevated levels of CRP, ESR and white blood count, and suffered from frequent bouts of high fever in conjunction with skin rashes. He was HLA-B27 positive and lacked the characteristic symptoms of CAPS patients like short stature, urticaria, bony overgrowth of joints or neurological involvement. The patient’s symptoms appeared after a severe *Streptococcus* infection at the age of eight years. No clinical benefit was achieved by corticosteroids, DMARDs or TNF-α blockade, upon which a role of IL-1β in disease pathogenesis was suspected. Since inflammasome defects have previously been implicated in an increased IL-1β production, we genetically screened the entire coding regions of NLRP3, ASC and CARD-8 in this patient. Two commonly occurring polymorphisms, Q705K in exon three of NLRP3 and C10X in CARD-8 were detected.

Our experimental results are in line with our hypothesis that IL-1β could have a role in the symptoms of this patient and accordingly, elevated basal levels of both caspase-1 and IL-1β could be detected in the patient compared to the healthy volunteers. These data are in agreement with previous studies reporting elevated IL-1β levels in the CAPS patients possessing gain-of-function mutations (Aksentijevich, Nowak et al. 2002; Agostini, Martinon et al. 2004; Janssen, Verhard et al. 2004). In addition to above observations, the dramatic effect of anakinra on IL-1β levels, both *in vivo* (after anakinra administration) and *ex vivo* (using patient’s monocytes) provides evidence for IL-1β having a pivotal role in the pathogenesis of symptoms.

Elevated IL-1β secretion upon challenge in monocytes from CAPS patients has earlier been demonstrated (Janssen, Verhard et al. 2004; Stack, Beaumont et al. 2005; Gattorno, Tassi et al. 2007). In order to compare the IL-1β response upon challenge between the patients’ and control’s monocytes, we used *S. aureus*, which previously had been shown to stimulate the NLRP3 inflammasome (Mariathasan, Weiss et al. 2006). In aforementioned study, mouse macrophages were pretreated with LPS followed by *S.aureus*, which resulted in NLRP3-dependent IL-1β production through unidentified *S.aureus* factors. We did not use LPS pretreatment, but still detected a pronounced increase in IL-1β levels 24 and 34 hrs post anakinra withdrawal in response to *S.aureus* challenge. These observations were made despite
the fact that the patient was regularly administered corticosteroids. It was recently demonstrated that \textit{S.aureus} lipoproteins are capable of inducing pro-IL-1\(\beta\) expression as well as caspase-1 activation (Munoz-Planillo, Franchi et al. 2009), which can explain our results despite the lack of LPS prestimulation. \textit{S.aureus} is associated with multiple toxins and is demonstrated to be recognized by innate immune receptors like TLR2 (Takeuchi, Hoshino et al. 2000) and Nod2 (Deshmukh, Hamburger et al. 2009). It would have been of interest to elucidate whether the patient’s monocytes would have responded only to the second, caspase-1 activating stimuli like ATP or alum, as was done in paper III and paper IV.

The requirement of two different stimuli to activate the inflammasome was postulated by Gattorno et al. in 2007 (Gattorno, Tassi et al. 2007). It was suggested that a TLR agonist like LPS, serves as the first stimuli resulting in increased pro-IL-1\(\beta\), whereas a caspase-1-inducing second stimuli like ATP was needed for the processing and secretion of IL-1\(\beta\). Further, it was demonstrated in the same study that the requirement of ATP was bypassed in the CAPS patients who could produce copious amounts of IL-1\(\beta\) just upon LPS stimulation with almost no further increases upon addition of ATP, which was attributed to a secretory exhaustion of cells from such patients.

When we combined a prior TLR stimulation using LPS followed by \textit{S. aureus}, highly increased but comparable IL-1\(\beta\) levels in patient (at 34 hour post anakinra withdrawal) and controls could be seen. The patient had been administered anakinra for more than a year; anakinra is known to downregulate the expression of proinflammatory genes (Goldbach-Mansky, Dailey et al. 2006), which might be the reason behind the patient not responding more robustly than the controls. It is likely that performing these experiments upon a longer period of anakinra withdrawal would have resulted in much higher IL-1\(\beta\) levels in the patient. Alternatively, the results can be attributed to the ‘secretory exhaustion’ (Gattorno, Tassi et al. 2007) in response to the first stimuli, resulting in almost no further IL-1\(\beta\) increases in response to the second. Our healthy volunteers displayed wide variations in IL-1\(\beta\) levels after the two stimuli, a phenomenon that has previously been seen in control population (Gattorno, Tassi et al. 2007). Our results also highlight the effect of anakinra on spontaneous as well as induced IL-1\(\beta\) secretion in the patient’s monocytes depending upon the hours of anakinra withdrawal, which is in agreement with the complete clinical remission in the patient following treatment. While the decreased IL-1\(\beta\) secretion in response to bacterial challenge
might not have considerable implications on resistance to certain infections owing to the short half life of anakinra, this might be the case with the longer acting blockers of IL-1β.

A number of studies have been done to understand the cross-talk between NF-κB and NLRP3 with conflicting results, there are reports showing NLRP3 as an inhibitor as well as an activator of NF-κB (Manji, Wang et al. 2002; Stehlik, Fiorentino et al. 2002; O’Connor, Harton et al. 2003; Greten, Arkan et al. 2007). Later research using NLRP3-deficient mice has shown that NLRP3 deficiency did not affect NF-κB activation (Bodar, Drenth et al. 2009). NF-κB exists as a hetero- or homodimer composed of the following five subunits: RelA (p65), c-Rel, RelB, NF-κB1 (P50 and its precursor p105) and NF-κB2 (p52 and its precursor p100). The most abundant heterodimer is the p50/p65, which is associated with increased inflammation. In a resting cell, the NF-κB heterodimers are held in an inhibitory state by the Inhibitory (I)-κB family proteins, but upon activation the p50/p65 dimer is released from inhibition and translocated to the nucleus. We compared NF-κB activation in resting and LPS-stimulated monocytes from patient and controls by determining the translocation of the p65 subunit to the nucleus and expressing the result as a ratio of nuclear staining to cytoplasmic staining. No differences between the patient and controls could be detected and we concluded that the NF-κB and NLRP3 pathways apparently function independently of each other.

We also hypothesized in paper I that factors like Streptococcal infection during childhood and/or HLA-B27 might have been instrumental in triggering the inflammatory cascade in this patient possessing abnormal inflammasome components. No studies elucidating the effect of Streptococcus on the inflammasome had been reported at that time. However, recently, it was demonstrated that pneumolysin, the key virulence factor in Streptococcus, activates the NLRP3 inflammasome (McNeela, Burke et al. 2010), which provides support to our earlier hypothesis.

The widespread distribution of these two polymorphisms in the control population makes it logical to assume that carrying one of these **per se** should not lead to an inflammatory phenotype. However, it is tempting to speculate that either or both of these polymorphisms could act as a predisposing factor, which upon being triggered by a suitable PAMP or DAMP could contribute to dysregulation of IL-1β production.
After the publication of this paper, we even screened the coding regions of the other HPF genes (MEFV, TNFRS1A, MVK and NLRP12) in this patient to look for other potential variants contributing to his inflammatory condition, but no mutations could be detected.

**Paper II**

We next wanted to determine the roles of Q705K and C10X in common chronic conditions and hence investigated a well characterized cohort of RA patients with a follow-up of 5 years. RA is characterized by the presence of autoantibodies and T cells. However, the success of both antilymphocyte as well as anticytokine therapies confer an element of heterogeneity to this autoimmune disease.

Early diagnosis and treatment are regarded as key elements in controlling RA disease progression (Quinn, Conaghan et al. 2001). Owing to the insufficiency of DMARDs to treat RA, a number of other biological treatments targeting the pro-inflammatory cytokines have been proposed and their effects studied in the clinical trials. The outcomes using IL-1β directed therapies have not been very promising and as a consequence, anakinra is not used as the first line of treatment against RA. However, the clinical trials with anakinra indicate that a subset of patients show a clear improvement in symptoms (den Broeder, de Jong et al. 2006) and hence a timely intervention in this subset, if they could be identified, would be highly desirable.

The TIRA (Swedish acronym for 'early interventions in rheumatoid arthritis') cohort comprises RA patients enrolled according to the 1987 criteria, as described in Paper II. Investigations on this cohort revealed, that being a carrier of either of these polymorphisms individually did not confer any risk for RA. However, their combination constituted a significantly increased risk for RA (OR=2.2, P=0.04). This association was particularly evident in the patients that were SE/ anti-CCP positive (OR=3.5, P=0.005), both of which are markers of an aggressive disease course in RA (Berglin, Padyukov et al. 2004).

An interesting observation was that the fraction of patients (13%) with a severe disease activity, indicated by the prescription of TNF-blocking therapy, possessed an altered NLRP3 or CARD-8 genotype. Moreover, the probability of insufficiency of DMARDs, alternatively risk for receiving TNF-blockade, strikingly increased in individuals where both the genetic alterations were present. This raises the question whether IL-1β plays a central role in the
pathogenesis of this subgroup of individuals, and whether these individuals would have benefitted from IL-1β blockade.

The NLRP3 inflammasome, originally implicated in monogenic autoinflammatory disorders has lately shown associations with polygenic diseases like CD (Roberts, Topless et al.; Schoutz, Verma et al. 2009; Villani, Lemire et al. 2009), gout (Terkeltaub, Sundy et al. 2009), celiac disease and type-1 diabetes (Pontillo, Brandao et al. 2010). These associations are not unlikely considering the wide range of stimuli that activate the NLRP3. Moreover, IL-1β and IL-18 are known modulators of adaptive immune response. IL-1β is involved in the upregulation of IL-2 receptor, proliferation and increased antibody production by the B cells. Both IL-1β and IL-18 are involved in Th1 and Th17 differentiation and amplification (Eisenbarth and Flavell 2009). RA is a considerably heterogenous disease, where disease progression displays autoinflammatory components, which is in agreement with the Inflammatory Disease Continuum (IDC) proposed by McGonagle and McDermott (McGonagle and McDermott 2006).

**Paper III**

This paper describes two siblings suffering from unspecific inflammatory symptoms, debuting in middle age. The male patient presented with symptoms at 44 years of age and displayed episodes of moderate to high fever, pain, skin rashes and thoracic discomfort in combination with night sweats. CRP and erythrocyte sedimentation rate (ESR) values were elevated during the inflammatory episodes. A variety of medications were tried over a period of 12 years but had to be terminated due to unsatisfactory effect or adverse side-effects. IL-1β blockade using anakinra was attempted and resulted in a dramatic improvement in symptoms.

The sister of the above patient presented at the age of 52 years with milder symptoms. She displayed relapsing low-grade fever, headache and occasional pleural effusions. CRP, ESR and leukocyte count were mostly in the normal range. After trying several treatments she has successfully responded to prednisolone and NSAID and IL-1β blockade has not been attempted (this was the case when we submitted this manuscript - at present the patient is being treated with anakinra). None of the patients fulfilled the criteria for CAPS.

A previously unreported M299V alteration in exon three of NLRP3 was detected in both the siblings. This genetic variation could not be detected in one hundred healthy individuals. The male patient additionally possessed a C10X in CARD-8. No mutations in the coding regions
of genes for FMF, TRAPS or MVK were found. The clinical manifestations as well as the severity grade varied between the two patients despite the same genetic alteration in *NLRP3*. This might be explained by the additional, truncating alteration in *CARD-8* in the male patient which might have a synergistic effect with M299V in *NLRP3*. Also, the involvement of other modifier genetic loci/ environmental factors resulting in different phenotypes could be a possible explanation.

For our *ex vivo* studies we used monocytes from the patients, two age and gender-matched controls as well as five randomly chosen healthy controls. As in paper I, elevated basal levels of caspase-1 and IL-1β could be detected in the two patients compared to the healthy controls. In agreement with a previous report (Gattorno, Tassi et al. 2007), our patients did not show any further increase in caspase-1 upon ATP treatment, whereas the controls did, indicating that the M299V monocytes might already be producing caspase-1 at their maximum capacity which cannot be further increased by ATP. ATP treatment, probably due to potassium ion (K⁺) efflux causes caspase-1 activation (Perregaux and Gabel 1994).

We next incubated the monocytes overnight with LPS followed by a brief ATP treatment. LPS plus ATP are the most frequently used stimuli to demonstrate inflammasome activity (Mariathasan, Weiss et al. 2006; Sutterwala, Ogura et al. 2006; Gattorno, Tassi et al. 2007). LPS, a TLR ligand, results in increased transcription and translation to pro-IL-1β, which is then processed to mature IL-1β upon encountering the caspase-1 resulting from ATP stimulation. We observed enhanced caspase-1 and IL-1β levels upon LPS pretreatment *per se*, with almost no further increases upon adding ATP. Consistent with the ‘secretory exhaustion’ associated with the monocytes from CAPS patients (Gattorno, Tassi et al. 2007), we can explain our results upon LPS +ATP treatment. The lack of caspase-1 activity in the patients upon ATP treatment, but not upon LPS treatment, might indicate a role of pro-IL-1β as the rate limiting, regulatory factor. Owing to a constitutively active inflammasome in CAPS patients, this might be a mechanism to prevent the formation of copious amounts of active IL-1β, *i.e.* caspase-1 due to increased inflammasome activity might exist in such patients but due to a limited availability of pro-IL-1β, large amounts of IL-1β might not be formed. We, however, did not analyze IL-1β levels upon ATP stimulation, which might have provided some clues regarding this hypothesis.
The in vitro studies performed to elucidate the functional significance of M299V showed an increased IL-1β production by THP-1 cells expressing the M299V variant as compared to the wild type NLRP3. A previously known MWS-causing R260L was used as a positive control for comparison, which displayed the highest IL-1β levels. Lower transduction efficiency was seen in the mutant variants as compared to the wild type NLRP3 suggesting that the mutant forms might affect the viral uptake. An increased population of dead cells was observed in association with the mutant forms, but the percentage cell death or its mechanisms were not determined.

While a majority of NLRP3 mutations are associated with a particular CAPS phenotype, there exists a handful of mutations that show an overlapping phenotype (Masters, Simon et al. 2009). The exceptionally late onset of symptoms in the siblings and a comparatively milder phenotype in the female patient who responded well to anti-inflammatory treatment, might be indicative of M299V being a low-penetrance alteration. Our ethical permission did not allow for a genetic screen of the kindred of the respective families of the patients.

The above experimental and functional results demonstrate that the M299V in NLRP3 results in an increased IL-1β secretion. However the patients do not fulfill the CAPS criteria. The examples of these two patients obviously raise the concerns that are we missing out/misdiagnosing some patients if attempting a strictly conventional categorization? It can be emphasized that mutations in the NLRP3 gene and in other genes related to periodic fever syndromes may result in unspecific inflammatory illness with symptoms that are common for several periodic fever diseases. Moreover, the severity of inflammatory episodes may vary between the patients and could be triggered by a trivial stimuli getting converted into an exaggerated immune response. Specific genetic alterations may therefore only precipitate in an inflammatory phenotype depending on other host related genetic factors or exposures.

**Paper IV**

Based upon our results from papers I and II, we hypothesized that the Q705K in NLRP3 confers a gain of function. Following the publication of the above two papers, there were reports from our as well as other groups implicating Q705K in combination with C10X in diverse diseases like CD (Roberts, Topless et al.; Schoultz, Verma et al. 2009), aortic aneurysms (Roberts, Van Rij et al. 2011), while Q705K alone was implicated in celiac disease (Pontillo, Brandao et al. 2010; Pontillo, Vendramin et al. 2011) and melanoma (our
unpublished observation). Looking at the heterogeneity of the above diseases, we postulated an increased genetic predisposition leading to increased susceptibility for inflammatory conditions, the clinical phenotypes possibly being orchestrated by additional genetic or environmental factors.

We performed functional studies using an in vitro model similar to what was done in paper III. Higher spontaneous IL-1β levels were generated by THP-1 cells transduced with the NLRP3-Q705K as compared to those transduced with wild type NLRP3, indicating a gain-of-function associated with the polymorphic Q705K variant. The IL-1β levels were however lower as compared to the MWS-causing NLRP3-R260W, which fits into our hypothesis of this variant attributing towards a susceptible genetic background rather than a severe inflammatory phenotype.

The THP-1 cells per se possess negligible amount of endogenous pro-IL-1β, which can be upregulated by LPS or phorbol-12-myristate-13-acetate (PMA) treatment (Schwende, Fitzke et al. 1996; Martinon, Burns et al. 2002). This occurs in correlation with the activation of caspase-1 (Martinon, Burns et al. 2002; Netea, Nold-Petry et al. 2009) together with the release of small amounts of endogenous ATP (Netea, Nold-Petry et al. 2009). The elevated IL-1β secretion in THP-1 cells transduced with the polymorphic/mutated NLRP3 variants, in the absence of any stimulation or priming, suggests a gain-of-function leading to a spontaneously active inflammasome.

The stably transduced THP-1 cells were sorted based upon GFP expression and expanded for further experiments. Since the GFP expressing population gradually decreases when the cells are cultured for longer times, the transduced cells were re-sorted in order to have maximum number of cells expressing GFP. Prior to the stimulation experiments, THP-1 cells were treated with PMA, which results in the adherence of the cells accompanied by a loss-of-proliferation, increased phagocytic capability and upregulation of various cell-surface receptors (Schwende, Fitzke et al. 1996).

Alum, a well-known inflammasome activator (Eisenbarth, Colegio et al. 2008; Franchi and Nunez 2008; Li, Willingham et al. 2008), was used to stimulate the inflammasome. The practical convenience and the low-cost associated with alum contributed towards our choice of alum as stimuli. An increased IL-1β production could be observed in THP-1 cells
expressing the altered NLRP3 variants compared to the wild type NLRP3 expressing THP-1 cells, indicating an increased pro-inflammatory response in these cells.

Based upon our experiments in paper IV, we conclude that Q705K confers a gain of function, in terms of an increased spontaneous in vitro IL-1β and IL-18 secretion. The low-penetrance alterations are not only perturbing in CAPS, but also in other monogenic autoinflammatory conditions like FMF and TRAPS (Ravet, Rouaghe et al. 2006; Ustek, Ekmekci et al. 2008; Turanli, Beger et al. 2009). Further, digenic inheritance is evident in certain patients, who display coexistent common alterations in MEFV and NLRP3 (Singh-Grewal, Chaitow et al. 2007) or TRAPS and NLRP3 (Touitou, Perez et al. 2006). To further complicate things, associations of common polymorphisms in MEFV, TNFRS1 and NLRP3 have been reported in certain complex polygenic diseases like RA, CD and Multiple sclerosis (MS) (Ozen, Bakkaloglu et al. 2003; Rabinovich, Livneh et al. 2005; Kastbom, Verma et al. 2008; Schoultz, Verma et al. 2009; Goris, Fockaert et al. 2011) suggesting the role of other factors in orchestrating the symptoms.

Looking at the complexity of innate immune system, a plausible explanation is that these alterations favor inflammation and when such individuals encounter a suitable trigger in the form of infection or other environmental stimuli they manifest the inflammatory phenotype. This could be a lesson from the bedside to bench; Owing to the prevalence of these polymorphisms it is highly relevant to elucidate their pathophysiological mechanisms.

The potential shortcomings of paper IV were the lack of IL-1β data from a healthy control population, or even patients, representing different NLRP3-Q705K genotypes. IL-1β is hard to detect in plasma samples due to its low levels. Either using whole blood or blood-monocytes are the alternatives, which are time-consuming and require fresh samples. An attractive experiment would have been to stimulate the monocytes from above individuals with low quantities of alum, followed by IL-1β determination. Hypothetically, the individuals carrying the polymorphism should have a lower threshold of inflammation and hence would display a hyper-responsiveness, even at low doses as compared to those lacking the polymorphism.
CONCLUDING REMARKS

*NLRP3*, discovered almost exactly a decade back, was implicated in the rare, hereditary diseases grouped under the common name CAPS. The growing numbers of CAPS cases provide an evidence of this being a more common condition than originally thought. An increasing numbers of patients with atypical CAPS phenotype suggest that reviewing the diagnostic criteria as well as increasing awareness about the underlying pathogenic mechanisms might be helpful in an early diagnosis. Even though such undefined cases are quite common in clinical practice, these might rarely be reported owing to their vagueness, and hence continue to be mysteries. Such patients need to be closely clinically followed and genetic screening may be used to complement the clinical diagnosis.

A wide variety of stimuli have been shown to stimulate the NLRP3 inflammasomes. Recent papers have implicated the inflammasome complex and IL-1β in the pathogenesis of Alzheimer’s disease (AD), type 2 diabetes (T2D) and amyotrophic lateral sclerosis (ALS). In all these diseases, protein aggregates caused by misfolded proteins or inappropriate oligomerization are demonstrated to be sensed by the inflammasome (Halle, Hornung et al. 2008; Masters, Dunne et al. 2010; Meissner, Molawi et al. 2010). HLA-B27 allele found in ankylosing spondylitis (AS) is suggested to have a tendency to misfold and cause ER stress (Galocha and de Castro 2008). Whether HLA-B27 acts as an endogenous trigger of the multifaceted NLRP3 remains to be elucidated.

Polymorphisms as predisposing factors have been a much discussed subject not only in the context of HPF but also in other more common diseases like cancers (Serefoglou, Yapijakis et al. 2008; Persson, Canedo et al. 2011) and IBD (Khor, Gardet et al. 2011). However, elucidation of the functional roles of polymorphisms is still at its nascent stages. Although the polymorphisms cannot be used for predictive purposes they might identify the pathway to be targeted and hence are relevant to study. Q705K is one of the most commonly occurring missense polymorphisms in *NLRP3* and is suggested to be involved in a number of diseases. Functional studies performed to better understand its role indicate a moderate gain-of-function in terms of increased IL-1β production, which might be translated into a permissive genetic background in the carrier individuals. Such low penetrance alterations, distinct from the high penetrance variants only predispose the individuals to a higher risk but would not necessarily lead to a disease phenotype. Owing to the wide implications this polymorphisms might have,
several questions can be raised: What triggers the inflammatory loop in carrier individuals? Does this polymorphism affect the inflammation dosage threshold in such individuals? What determines the heterogeneity of diseases phenotype?

Recent years have witnessed tremendous advancements in the field of innate immunity, where NLRP3 has emerged as one of the key players. Disease associations of NLRP3 genetic variants and inflammasomes to diverse conditions provide evidence of a more fundamental role in inflammation. The recent success of IL-1β blockade in the clinical trials of gout (So, De Smedt et al. 2007; Singh and Huston 2009; Terkeltaub, Sundy et al. 2009), type 2 diabetes (Larsen, Faulenbach et al. 2007; Larsen, Faulenbach et al. 2009), heart failure (Abbate, Kontos et al. 2010) and several other diseases suggests a huge future potential for IL-1β blockade in clinical practice.
Vertebrates possess a sophisticated immune system that helps them not only to fight against a wide variety of microbes but also against non-microbial harmful substances. This ability partly exists from birth, denoted ‘innate immunity’ and is further acquired upon being exposed to the pathogens, in which case it is retained for life, and classified as adaptive immunity. Innate immunity has been intensely studied in the past few decades, and has led to a better understanding of the mechanisms behind recognition and response to pathogens using special host sensors.

Recently a special type of sensor termed as the ‘inflammasome’ was identified, which in addition to microbes, recognizes harmful particles in the environment as well as inside the body. The inflammasome, upon sensing danger responds by producing inflammatory substances, most important of which is Interleukin (IL)-1β. However, in certain cases the inflammasome complex can erroneously result in inflammation in the host by continuously producing IL-1β. An example of the above situation is the genetic alterations in the inflammasome which causes unsolicited IL-1β production, and thereby lead to serious inflammatory outcomes. Patients suffering from such defects are successfully treated with the blockade of IL-1β.

We report a patient who had been suffering from severe inflammation for 20 years. Genetic screening of the inflammasome components revealed two commonly occurring variants which may have contributed to his symptoms. Upon treatment with IL-1 blockade the patient showed remarkable recovery from the symptoms and the results from our experimental studies indicated an over-activity of the inflammasome. Since the above mentioned variants commonly occur in the normal population, it implies that the individuals carrying these may be more prone to develop inflammatory diseases. We, therefore, genetically investigated a group of patients with rheumatoid arthritis, which is a joint disease caused by long-term inflammation. We found that the patients, who carried both the genetic variants, were at increased risk for developing rheumatoid arthritis and presented with more aggressive form of disease. Our results suggest that such individuals could possibly benefit from an early genetic screening and a timely initiation of IL-1 blocking treatment. We further report two siblings with inflammatory symptoms, where we found a rare genetic alteration in the inflammasome. Consistent with previously reported cases in the literature, excessive IL-1β production was observed in these siblings whose symptoms were quite different from those of other reported
patients. Lastly, we provide experimental evidence for the commonly occurring genetic variant being involved in excessive IL-1β formation. Since this variant is detected in healthy individuals as well, we suggest that such individuals upon exposure to stimuli, like an infection, might have an increased susceptibility for long-term inflammation.
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