Mechanisms of inflammation in mevalonate kinase deficiency

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Mechanisms of inflammation in mevalonate kinase deficiency

Mechanismen van inflammatie in mevalonaat kinase deficiëntie
(met een samenvatting in het Nederlands)

Proefschrift

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"Times are bad. Children no longer obey their parents,
and everyone is writing a book."
Marcus Tullius Cicero,
statesman, orator and writer
(106-43 BC)

Voor mijn ouders

Commissie:
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Contents

Chapter *1*

General Introduction I

Episodic autoinflammatory disorders in children

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INTRODUCTION

The autoinflammatory diseases are a newly recognized and expanding class of inflammatory disorders that share many features with autoimmune diseases (1). They differ significantly, however, in that autoinflammatory syndromes are characterized by an absence, not only of pathogens, but also of high titre autoantibodies and pathogenic autoreactive T cells (2). Chronic autoinflammatory diseases in childhood include systemic onset juvenile idiopathic arthritis, sarcoidosis and Blau syndrome, which fall beyond the scope of this chapter. The intermittent autoinflammatory disorders, known as periodic fever syndromes, lead to recurrent episodes of fever alternating with more or less prolonged periods of disease remission. The fever episodes are usually accompanied by additional systemic and localized inflammatory symptoms involving joints, skin, eyes or abdomen (3).
Each of these disorders has unique symptoms, as well as a unique pathophysiology and treatment. Many are inherited, which has allowed for determination of the responsible genes. In the last decade, an increasing number of patients has been appropriately recognized, diagnosed and treated due to advances in the understanding of the clinical characteristics and molecular basis of these diseases. In this chapter the clinical presentation and recent progress in elucidating the underlying pathophysiology will be described for each of these disorders (4). The chapter will be concluded by a paragraph on diagnosis.

Familial Mediterranean Fever (FMF)

Prevalence/epidemiology
Familial Mediterranean Fever (FMF; MIM#249100) is the most prevalent of the hereditary autoinflammatory diseases, probably affecting more than 100,000 patients worldwide. It is an autosomal recessive disease, affecting mostly people from the Mediterranean area, including Armenians, Arabs, Turks and Sephardic Jews. By migration it has now also spread to Northern and Western Europe, Australia and the Americas.

Etiology/pathogenesis
The gene affected in FMF, *MEFV*, encodes the protein pyrin or marenostrin (5;6). Since its discovery in 1997, more than 100 exon mutations have been described for the *MEFV* gene (http://fmf.igh.cnrs.fr/infevers/). The most common mutations: M680I, M694V, M694I and V726A (7) are situated within exon 10, which encodes the C-terminal B30.2 domain of the protein. The functional role of this domain is unknown. The protein further consists of a B-Box-type zinc finger, a coiled-coil domain and an N-terminal PYRIN domain. Pyrin is mainly expressed as a cytoplasmic protein in mature neutrophils and monocytes (8), and it associates with actin (9). The exact role of pyrin in the clinical manifestations of FMF has not been elucidated thus far, but the N-terminal PYRIN domain was shown to interact with a protein called apoptosis-associated specklike protein containing a CARD (ASC), whereas the carboxyterminal B30.2 can bind to caspase-1. This interaction places

pyrin upstream in a pathway regulating caspase-1 and IL-1β processing, linking it to inflammation (10-12).

Clinical manifestations

Most patients suffer from recurrent fever attacks, with acute monoarthritis and/or serositis, affecting the peritoneum, pleura, pericardium or scrotum. Some patients display an erysipelas-like rash (Figure 1) and a few develop chronic erosive arthritis (13-17). However, in the rare case, recurrent abdominal pain during childhood can be the only manifestation of FMF (18).

Disease onset is usually in childhood, with 75-89% of patients having their first attack before the age of 20 years (14;19). Frequency of attacks can vary from several times per week to once every few months or even years. The attacks usually last 1-3 days.

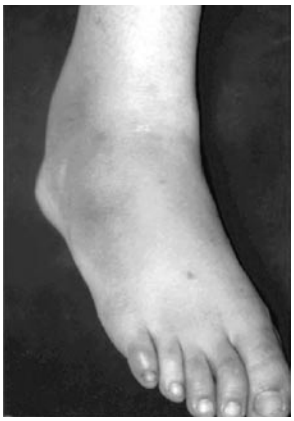

Figure 1: Characteristic erysipelas-like erythema in FMF.

Diagnostic investigations

During fever episodes serum markers of acute phase response: serum amyloid A (SAA) protein, C-reactive protein (CRP), complement and plasma fibrinogen are elevated and there is granulocytosis (20-22). Often, erythrocyte sedimentation rate (ESR) is increased. Between attacks patients are well, even though they may continue to have increased acute phase reactants. However, the prolonged elevation of SAA protein predisposes to AA systemic amyloidosis in which SAA deposition occurs in several organs leading to organ failure (16;23;24).

The diagnosis can be made on clinical grounds (Table I), provided that the patient is from a population with a high prevalence of FMF (25). The response to colchicine therapy is so characteristic that it is considered a major diagnostic criterion. Genetic testing may support the diagnosis, but in up to one-third of patients one or both *MEFV* alleles are normal (13;17). There are no clinical diagnostic criteria validated for populations with intermediate prevalence (Greeks, Italians, Spanish) and in these, genetic testing might offer an advantage. The value of *MEFV*-testing in populations with a low prevalence of FMF is limited at best (26).

Table I: Diagnostic criteria for FMF in a population with high prevalence of the disease (25)

Tel Hashomer criteria for diagnosis of Familial Mediterranean Fever	
Major criteria	Minor criteria
Recurrent fever with arthritis and/or serositis	Recurrent fever attacks
AA-amyloidosis in the absence of a predisposing illness	Erysipelas-like erythema
Favourable effect of colchicine	FMF in first degree relatives

Definite diagnosis: two major or one major and two minor criteria
Probable diagnosis: one major and one minor criterium

Treatment

The usual treatment of FMF is colchicine, which prevents inflammatory attacks in ~60% of the patients and significantly reduces the number of attacks in another 20-30% (27;28). The favourable response to colchicine is very characteristic for FMF, so much that it can be used as a diagnostic criterion. Its mode of action in FMF is poorly understood. The usual colchicine dose in young children is 0.5 mg/day, 1 mg/day in children 7-12 years of age and 1.5 mg (rarely 2 mg) in 2-3 doses in patients of 12 years and older. In order to minimize gastro-intestinal side effects, a 50% lower starting dose may be given. For pain relief during attacks nonsteroidal anti-inflammatory drugs (NSAIDS) can be used.

Long-term prognosis depends on the development of amyloidosis, which can result in organ damage, notably renal failure. The risk of amyloidosis is variable, depending on ethnic background, sex, *MEFV* mutation, disease modifying genes and especially on where the patient has lived (29). However, even in highrisk populations, colchicine treatment reduced the incidence from over 60% to less than 5% (30).

TNF Receptor-Associated Periodic Syndrome (TRAPS)

Prevalence/epidemiology

TNF receptor-associated periodic syndrome (TRAPS; MIM#142680) was first reported as Familial Hibernian Fever in 1982 in a large family of Irish and Scottish descent (31), but it has now been described in more than 20 families from a wide variety of ethnic groups (32;33). Although more than 100 patients with TRAPS have been described, the exact prevalence is unknown. TRAPS has an autosomal dominant inheritance mode. The mutations described to date have variable penetrance. Mutations with a low penetrance may lead to rather atypical inflammatory disorders, not diagnosed as TRAPS (32;34).

Etiology/pathogenesis

TRAPS results from mutations in the *TNFRSF1A* gene (35). It encodes TNFRSF1A, the 55 kDa receptor for tumour necrosis factor (TNF), also termed CD120a. The *TNFRSF1A* gene consists of 10 exons. Most mutations are present in exons 2, 3 and 4 (http://fmf.igh.cnrs.fr/infevers/), which

encode the extracellular cysteine-rich TNFR domains of the protein, thereby disrupting the tertiary structure. No mutations have been described for the intracellular domains. The mutations found in the extracellular TNFR domains can cause an impaired receptor shedding, leading to increased or prolonged signalling through the TNF receptor and to a reduced generation of soluble TNF receptor (sTNFRSF1A), the natural antagonist of TNF-α (35). However, not all patients show defective receptor shedding suggesting that there are additional mechanisms behind the fever attacks in TRAPS, such as altered receptor trafficking or impaired neutrophil apoptosis (36-39).

Clinical manifestations

The clinical presentation of TRAPS varies widely. The age of onset is between a few weeks and 53 years of age, but most patients have their first symptoms in childhood (33). Like FMF, TRAPS is characterized by periodic fever attacks, but they last days to weeks and recur two to six times a year. There is, however, a large variation between individuals. Fever attacks may start unprovoked, but can also be set off by emotional stress, minor infections or vigorous exercise.

Clinical features during attacks include conjunctivitis, pericarditis, migratory rash (Figure 2), prominent myalgias, monoarthritis, mostly of the lower limbs, abdominal pain and erythematous swelling of eyelids, limbs, fingers and/or ears. The abdominal pain may be due to serositis, which can also involve other serosal surfaces, including the testicular tunica vaginalis. Focal neurological signs are a rare feature of the disease (40;41).

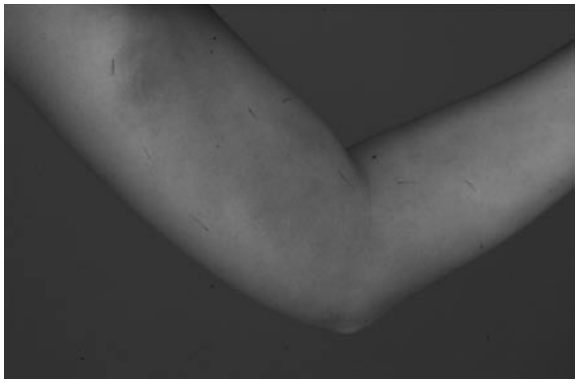

Figure 2: Migratory rash in TRAPS patient (Courtesy of Dr. E. Hoppenreys).

Diagnostic investigations

During episodes of fever there is a clear acute phase response: leukocytosis, elevation of CRP, SAA and ESR (40) and even in symptom-free intervals such inflammatory responses may be detected. Between attacks, many patients have reduced serum levels of sTNFRSF1A (35), indicative of impaired receptor trafficking or shedding. However, diagnosis depends on the identification of *TNFRSF1A* mutations on DNA-analysis.

Treatment
TRAPS can be treated with NSAIDs and glucocorticoids to alleviate the symptoms, but these drugs do not affect the frequency of attacks, or the development of amyloidosis. Clinical trials with etanercept, a fusion protein of TNFRSF1B with the Fc portion of human IgG1, have been more successful. Frequency, duration and/or severity of attacks were reduced in the majority of patients (33). The use of etanercept has even been shown to reverse a case of amyloidosis in one TRAPS patient (42). The usual dose of etanercept is 0.4 mg/kg (maximum 25 mg) twice weekly.
Development of amyloidosis is the main determinant for prognosis. Remarkably, this risk is associated with the type of mutation: up to 24% of patients with mutations affecting cysteine residues develop amyloidosis, versus 2% of patients with noncysteine mutations (33).

Hyper-IgD Syndrome (HIDS)

Prevalence/epidemiology
The Hyper-IgD Syndrome (HIDS; MIM#260920) is an autosomal recessive disease, mostly affecting people from Caucasian origin (43). It is a rare disorder; the International Hyper-IgD Syndrome Registry (http://www.hids.net) currently has clinical data on approximately 200 patients worldwide. The disease is relatively common in the Netherlands, where carrier frequency approaches 1:350 (44).

Etiology/pathogenesis
In 1999, mutations in the gene which codes for mevalonate kinase (*MVK*) were found to be the cause of HIDS (45;46). So far, approximately 70 mutations have been described that lead to documented HIDS or to the more severe phenotype of MVK deficiency, known as mevalonic aciduria (http://fmf.igh.cnrs.fr/infevers/). Two missense mutations are most prevalent: I268T and V377I, accounting for the vast majority of patients (47-49). Mevalonate kinase (MK) is an enzyme in the cholesterol biosynthesis pathway. Besides cholesterol, this pathway produces a number of other, nonsterol, end products such as farnesyl and geranylgeranyl groups, which can be covalently attached to specific proteins, including proteins of the Ras superfamily. Although patients with HIDS have only 1-7% residual activity of MK (45), plasma cholesterol levels in these patients are within the normal range. How the metabolic defect leads to the clinical manifestations of Hyper-IgD is unclear, but evidence is now emerging that the shortage of specific isoprenylated proteins can induce interleukin-1β-mediated inflammation (50).

Clinical manifestations
The recurrent fever attacks that are characteristic of HIDS usually return every 3-6 weeks and last for 3-5 days. They are almost always accompanied by painful cervical lymphadenopathy and often by abdominal pain, vomiting and diarrhoea (51;52). A variety of other symptoms including headache, skin rashes, mucosal ulcers, myalgia and arthralgia may also occur. The

profound MK deficiency, known as mevalonic aciduria, can, in addition, be accompanied by developmental delay, dysmorphic features, arthritis, tapetoretinal degeneration, ataxia, cerebellar atrophy and psychomotor retardation (Figures 3 and 4) (53;54). In reality, a phenotypic continuum exists between these extreme phenotypes (55).
Ninety percent of HIDS patients will experience their first fever attack within the first year of life, but the fever episodes tend to become less frequent and less severe with age. Fever episodes can be provoked by a variety of triggers, including vaccinations, infections or minor trauma, but most often such a trigger cannot be identified (51). When fever has subsided, malaise and arthritis may take days longer to resolve. Between attacks, patients are well and thrive normally.

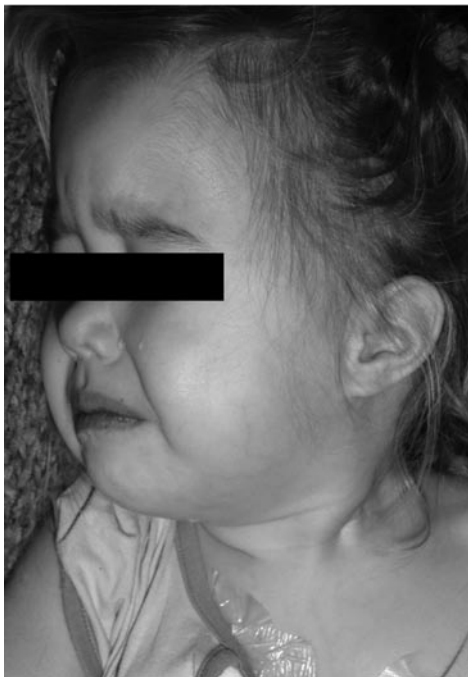

Figure 3: Facial dysmorphia in mevalonate kinase deficiency.

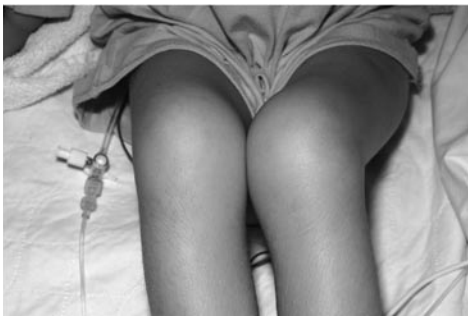

Figure 4: Monoarthritis in mevalonate kinase deficiency.

Diagnostic investigations
As the name implies, most patients have elevated serum immunoglobulin D levels, but how this relates to the clinical disease is poorly understood. Since some patients with *MVK* mutations have normal levels of IgD it is likely that elevated IgD levels are an epiphenomenon. Patients may also have elevated serum IgA (56). Urine mevalonic acid concentrations are consistently raised during fever episodes. In addition, patients exhibit an acute phase response with leukocytosis, high levels of SAA and CRP and the presence of pro-inflammatory cytokines (51;57).

Treatment

Therapy is problematic. Colchicine, thalidomide and immunosuppressive agents seem largely ineffective (51;58;59). Treatment with simvastatin may induce a modest improvement (60) and there have been case reports on successful treatment with etanercept (61). Studies with the recombinant interleukin-1 receptor antagonist, anakinra (Kineret®), are underway and look promising (62). Long-term prognosis of Hyper-IgD is usually good: amyloidosis occurs in less than 3% of patients and no excess mortality has been reported among the patients currently registered at the HIDS Registry.

Cryopyrin-Associated Periodic Syndromes (CAPS)

Prevalence/epidemiology

Cryopyrin-associated periodic inflammatory diseases include Familial Cold Autoinflammatory Syndrome (FCAS; MIM#120100), Muckle-Wells Syndrome (MWS; MIM#191900) and Neonatal Onset Multisystem Inflammatory Disease (NOMID; MIM#607115), also known as Chronic Infantile Onset Neurologic Cutaneous Articular (CINCA) Syndrome. They are all dominantly inherited. Previously considered as distinct disorders, they are now thought of as a spectrum of one systemic inflammatory disease with varying severity, FCAS being the mildest condition and CINCA/NOMID the most severe. The exact prevalence of these rare autoinflammatory diseases is unknown, but over 200 patients with FCAS live in North America, and more than 20 families with MWS and ~100 patients with CINCA/NOMID have been reported worldwide.

Etiology/pathogenesis

FCAS, MWS and CINCA/NOMID are all associated with missense mutation of the *CIAS1* gene (63-65). This gene encodes cryopyrin, also known as NALP3, PYPAF1, CATERPILLER1.1 or NLRP3. Its expression is limited to immune cells and chondrocytes. Cryopyrin contains an N-terminal PYRIN domain, a central NACHT domain with a nucleotide-binding site and a C-terminal leucine-rich repeat. The function of this protein is incompletely understood. Probably, it plays a central role in control of inflammation, cytokine processing and cell death (66;67). After activation, cryopyrin interacts with other molecules to form a macrocomplex called inflammasome. This interaction mediates procaspase-1 activation, thus caspase-1 processes proIL-1β to its pro-inflammatory active form, IL-1β. It has also been suggested that the interaction of cryopyrin with ASC mediates activation of NF-κB (68).

All but one of the mutations identified to date in FCAS, MWS and CINCA/NOMID are missense mutations. They are almost all localized in exon 3, irrespective of disease severity (64;69-71). This exon encodes for the NACHT domain and its flanking protein regions. Rare mutations have been described in the leucine-rich repeat region (72;73). Mutations are believed to confer a gain of function resulting in increased cytokine-mediated inflammation (74). There appears to be some genotype-phenotype correlation (71). Only 60% of patients with clinical findings of FCAS, MWS or CINCA/NOMID have mutations in the *CIAS1* gene. This suggests genetic heterogeneity.

Clinical manifestations

Cryopyrin-associated periodic inflammatory syndromes are all characterized by early onset of recurrent episodes of fever with remarkable systemic inflammation, a characteristic nonpruritic urticaria-like skin rash with perivascular polymorphonuclear cell infiltrates in skin biopsy and a broad spectrum of joint manifestations ranging from arthralgia in the mild forms to recurrent arthritis and permanent arthropathies in severe diseases. Sensorineural hearing loss can develop with increasing age in MWS and CINCA/NOMID. Neurological involvement is observed in CINCA/NOMID and is due to chronic aseptic meningitis with neutrophilic granulocytosis in the CSF.

Familial Cold Autoinflammatory Syndrome (FCAS)

Patients with FCAS, previously known as Familial Cold Urticaria (FCU), experience recurrent episodes of urticaria-like rash, fever and arthralgia precipitated by generalized cold exposure. Additional symptoms suffered during attacks include conjunctivitis, sweating, drowsiness, headache, extreme thirst and nausea. The symptoms usually develop 1-2 hours after cold exposure, peak approximately 6-8 hours later, and resolve in less than 24 hours. Most of the patients describe a correlation between the severity of the crisis and the intensity of cold exposure. Attacks are more frequent in winter, on damp and windy days. Relatively mild exposures such as air-conditioned rooms can precipitate episodes. Many patients have daily rash and fatigue that peak in the evening and resolve by morning, regardless of cold exposure. Disease onset is often at birth with neonatal rash and 95% of patients experience symptoms by six months of age. Amyloidosis is rare in FCAS (65).

Muckle-Wells Syndrome (MWS)

The course of the disease varies from typical recurrent attacks of inflammation very similar to those observed in FCAS but without a defined trigger to more permanent symptoms with periods of exacerbation. Attacks may be precipitated by cold exposure, but also heat, stress, exercise, tiredness. Fever is not always present. Joint manifestations can be mild with short episodes of arthralgia but recurrent episodes of synovitis affecting predominantly large joints can also be observed. Conjunctivitis is frequently noticed. Sensorineural deafness, one of the hallmark features of MWS, develops in up to two-thirds of patients in late childhood. AA amyloidosis, due to chronic inflammation, is the main complication and develops in adulthood in 20-40% of patients. Focal neurological involvement has not been reported in MWS. Headache and papilledema have been reported in some cases (75). Mild forms of MWS resemble FCAS and more severe phenotypes overlap with CINCA/NOMID. The age of onset is variable from childhood to adulthood (76).

CINCA/NOMID

CINCA/NOMID is the more severe phenotype in this spectrum of diseases. Urticaria (Figure 5) is usually present at birth or during the first months of life. Fever is intermittent,

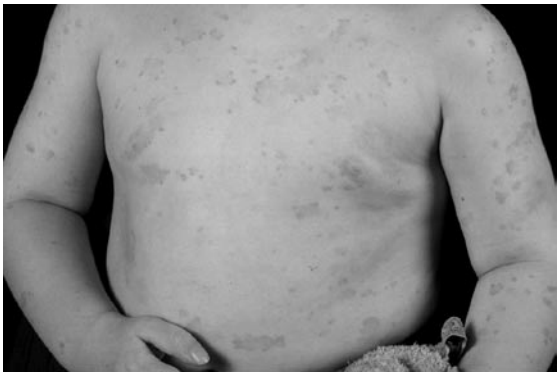

Figure 5: Urticaria-like rash in CINCA/NOMID patient.

can be absent or very mild in some cases. Bone and joint involvement vary in severity. In approximately two-thirds of the patients, joint manifestations are limited to arthralgia or transient non-erosive arthritis during flare-ups. In one-third, however, joint abnormalities are severe. The metaphyses and epiphyses of long bones are affected, resulting in marked bony overgrowth, deformity of the joints, chronic pain and loss of function. The knees, ankles, wrists and elbows are most commonly affected in a symmetric pattern. Abnormalities of the CNS are present in almost all patients and are due to chronic aseptic meningitis. Chronic headaches, sometimes with vomiting and papilledema are frequently noted. Spastic diplegia and epilepsia may develop. Progressive cognitive impairment occurs in severely affected patients. Chronic increased intracranial pressure often leads to late closure of the anterior fontanelle, macrocephaly, frontal bossing and saddle back nose. Ocular disease consists of anterior uveitis in half and posterior uveitis in another 20% of affected patients. Optic atrophy can develop. Ocular manifestations can progress to blindness (77). Perceptive deafness is frequently observed in older patients (78).

Diagnostic investigations

Patients suffering from the cryopyrin-associated periodic fever syndromes have elevated acute phase reactants and leukocytosis during attacks. In MWS and CINCA/NOMID patients acute phase reactants may be chronically elevated and anaemia of chronic illness is common. Cerebrospinal fluid examination in CINCA/NOMID usually reveals a raised opening pressure pleiocytosis and elevation of CSF protein. Radiological manifestations in CINCA/NOMID patients with hypertrophic arthropathies are distinctive with overgrowth and irregular ossification of metaphysis and epiphysis of long bones (Figure 6).

Treatment

Treatment, until recently, has been limited to avoidance of cold exposure and to nonsteroidal anti-inflammatory medications for FCAS patients, and high dose steroids for more severe FCAS,

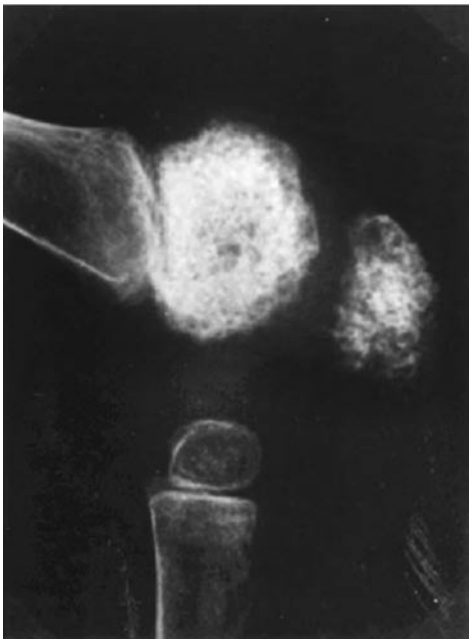

Figure 6: Radiographic appearance of arthropathy in CINCA/NOMID syndrome (Courtesy of Dr. A.M. Prieur)

MWS and CINCA/NOMID patients. Recently, numerous case reports have demonstrated the IL-1 receptor antagonist, anakinra (Kineret®), to be highly effective in all three diseases (73;75;79). The usual dose was 1 mg/kg subcutaneously once daily. These observations were confirmed in a large series by Goldbach-Mansky *et al.* (80). Drug withdrawal led to prompt relapse in all prospectively studied patients. Therefore, a placebo-controlled trial will not be ethically acceptable. Additional therapies targeting IL-1 are under investigation. Prognosis ranges from excellent for FCAS patients, except in rare instances where amyloidosis develops, to fair in MWS, where the risk of amyloidosis is up to 25-40%, to poor in untreated CINCA/NOMID patients with severe neurologic disease.

Pyogenic sterile Arthritis, Pyoderma gangrenosum and Acne syndrome (PAPA)

Prevalence/epidemiology

The PAPA Syndrome (MIM#604416), previously described as the Streaking Leukocyte Factor disease (81), was recognized and redescribed by Lindor *et al.* (82). Families described thus far are of European descent, but the number of documented cases is still very small. PAPA syndrome is an autosomal dominant disease, with a presumed complete penetrance.

Etiology/pathogenesis

The affected gene, *PSTPIP1*, encodes CD2 antigen-binding protein 1 (CD2BP1), also known

as proline/serine/threonine phosphatase-interacting protein 1, PSTPIP1 (83;84). This gene contains 15 exons, with only two mutations described thus far. The function of PSTPIP1 is still unclear, but it has been shown to bind to pyrin, the protein that is mutated in FMF, suggesting it may also be involved in IL-1β regulation (85).

Clinical manifestations
The PAPA syndrome is characterized by recurrent inflammation of joints, skin and muscle. During episodes of arthritis patients may present with fever. Ulcerative lesions (pyoderma gangrenosum) occur in the skin, often on the lower limbs or at sites of minor trauma or surgery. Patients also suffer from severe cystic acne starting in adolescence and persisting into adulthood, and from destructive arthritis (82;83;86). Onset of the disease is in early childhood, but additional symptoms, like insulin-dependent diabetes mellitus, can develop at older ages.

Diagnostic investigations
During episodes patients may have elevated white blood cell counts and ESR (86;87). Biopsies and joint aspirates usually show massive neutrophil influx.

Treatment
Treatment of the PAPA syndrome can consist of isotretinoin, for treatment of acne, usually in combination with steroids. Intra-articular steroids or surgical drainage of infiltrates in joints can be used to relieve severe arthritis. Alternative therapies include anti-TNF-α therapy with either infliximab or etanercept, which proved successful in several patients (87). Recently, several members of one affected kindred were successfully treated with the interleukin-1 receptor antagonist anakinra during attacks (88).
The PAPA syndrome can lead to pronounced disfigurement. In addition, the severe acne and the scarring resulting from it can cause psychological damage like anxiety and depression. Prognosis is mostly determined by the development of diabetes mellitus and/or renal failure (82).

Periodic Fever with Aphthous stomatitis, Pharyngitis and Adenitis (PFAPA)

Prevalence/epidemiology
Most children who present with recurrent fevers to paediatricians and specialists do not have one of the known hereditary autoinflammatory diseases discussed previously. A significant number of these children can be assigned a diagnosis of Periodic Fever with Aphthous stomatitis, Pharyngitis and Adenitis (PFAPA), an autoinflammatory disease characterized by recurrent episodes of fever for which no genetic cause has been identified thus far. The PFAPA syndrome, also known as Marshall's syndrome, was described in 1987 and is a relatively benign and common condition that has been reported in several areas of the world (89).

Etiology/pathogenesis
The pathophysiology is unknown.

Clinical manifestations
The PFAPA syndrome is usually accompanied by one or more inflammatory findings including pharyngitis, cervical adenitis or adenopathy and aphthous stomatitis. Additional symptoms are similar to those seen in many of the hereditary disorders, including headache, malaise, abdominal pain, arthralgia and myalgia. Onset is usually between 2 and 5 years of age and attacks last between 3 and 6 days. Episodes are often quite predictable occurring every 3-8 weeks and are separated by completely asymptomatic periods with normal growth and development. Unlike the hereditary disorders, this condition is self-limited and most children with PFAPA have a complete remission after 2-6 years of symptoms.

Diagnostic investigations
The only laboratory abnormalities are leukocytosis and increased acute phase proteins during attacks, and these abnormalities completely resolve between episodes. Increased serum levels of interferon-γ, TNF-α and IL-6 have been observed with fevers (90). It may be necessary to exclude other periodic fever syndromes, notably MK deficiency, by genetic analysis, before making the diagnosis of PFAPA syndrome.

Treatment
Symptoms are very sensitive to systemic corticosteroid therapy. Additional reported effective therapies include prophylactic use of cimetidine and tonsillectomy (91). Prognosis is good; symptoms tend to become less intense and less frequent with time. There is no known long-term morbidity associated with PFAPA.

DIFFERENTIAL DIAGNOSIS

Recurrent inflammation is the hallmark of the autoinflammatory syndromes. The first diagnostic step, therefore, is to examine the patient during an attack and to document an acute phase response (leukocytosis, ESR, CRP). Thus, factitious fever, autonomic dysregulation and most recurrent viral infections may be excluded. A careful evaluation should be performed for occult infection, such as urinary tract infections or otitis/sinusitis. A complete blood count with differential should be included to rule out cyclic neutropenia as the cause for periodic fever. Another characteristic of most of the autoinflammatory disorders is recovery between attacks. When the patient no longer fully recovers, chronic recurrent infections, autoimmune diseases and malignancies, notably lymphoma and leukaemia, must be ruled out.
If a patient has true autoinflammatory episodes, one of the periodic fever syndromes should

be considered. In some cases findings at history taking, such as age of onset, length of attacks, precipitating factors, associated symptoms and family history are adequate to distinguish a known periodic fever syndrome (Table II). This is true for many cases of CAPS and PFAPA. In patients with clinical findings consistent with HIDS, serum IgD and urine mevalonate during an episode may provide additional support for this diagnosis. However, in many patients there is no straightforward diagnostic work-up. Rather the combination of epidemiology, signs, symptoms and disease course should lead to a tentative diagnosis, which may then be supported by genetic testing (Figure 7). Appropriate genetic testing for these genes is available at several commercial laboratories internationally, but in some cases comprehensive testing of certain genes can only be performed in specialized research laboratories.

A major factor for diagnosis in many centres is ethnic background. In patients of Armenian, Turkish, Arab or Sephardic Jewish extraction, FMF is so prevalent, that a clinical diagnosis will suffice. When the presentation is not typical of FMF, initial *MEFV* testing may be

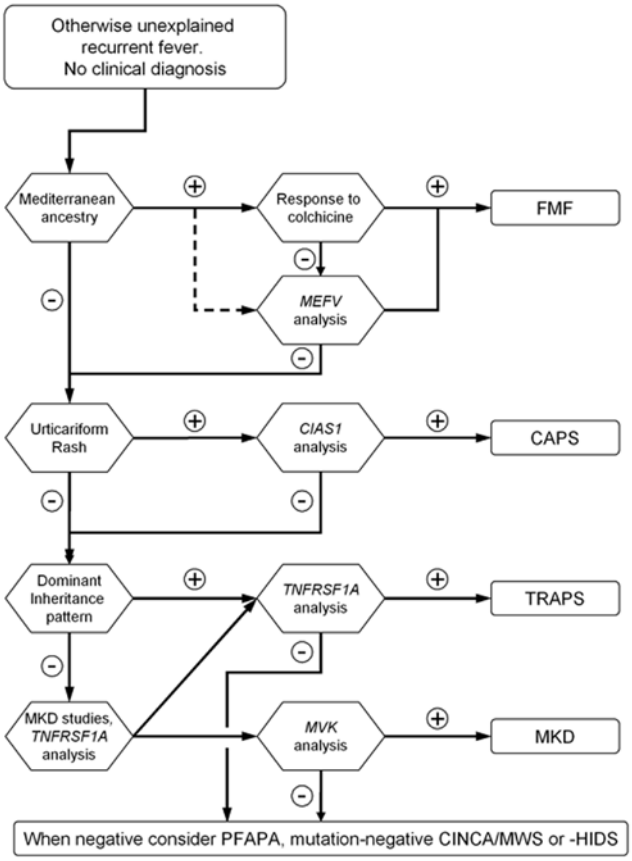

Figure 7: Proposed sequence of genetic analysis in patients with proven recurrent inflammation in whom no definitive clinical diagnosis could be established.

warranted. However, in Western Europeans and their descendants, disease-causing *MEFV* mutations are exceedingly rare and *MEFV* testing is of little benefit (26).

A problem arises when no tentative clinical diagnosis can be made, or when the tentative diagnosis is not supported by genetic testing. In such cases one might analyze other periodic fever genes. In cases where only the identified patient is affected, it is important to consider recessive as well as dominant (possibly *de novo*) diseases. If consecutive generations are affected, autosomal dominant disease genes should be tested initially. The yield of genetic testing, other than to confirm a tentative diagnosis, is usually very low (92). Unfortunately, there are also cases for each of the known disorders in which a patient has classic presentation, but exhaustive genetic screens are negative (between 20 and 50% of patients with CINCA/NOMID, FMF or HIDS). With the identification of targeted therapies for many of these disorders, a therapeutic trial might become another diagnostic approach for these patients.

Key points

- The autoinflammatory syndromes are a distinct group of disorders characterized by generalized inflammation in the absence of microbial pathogens, autoreactive T cells or autoantibodies.
- Clinically, these syndromes can be recognized by the presence of periodic fever episodes, accompanied by systemic inflammatory symptoms involving joints, skin, eyes or abdomen.
- Since many of the autoinflammatory syndromes are hereditary, genetic testing can aid toward the diagnosis. However, in many patients a genetic diagnosis cannot be established.
- To diagnose a patient presenting with periodic fever, a combination of clinical characteristics such as age of onset, length of attacks, precipitating factors, associated symptoms and family history should be documented as well as genetic screening of the appropriate genes.

Acknowledgements
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Table II: Characteristics of the auto-inflammatory syndromes
Symptoms encountered in the respective diseases are marked with +, or if very characteristic for the disorder with ++.
AD: autosomal dominant, AR: autosomal recessive.

	FMF	HIDS
Inheritance	AR	AR
Gene	*MEFV*	*MVK*
Protein	pyrin	mevalonate kinase
Function	Regulation of apoptosis and inflammation	Isoprenoid biosynthesis
Ethnic background	Armenian, Turkish, Jewish, Arabs	Dutch, French, German among others
Onset	Childhood (>90%)	Infancy
Duration of attacks	12-72 hr	3-5 days
Symptoms:		
vomiting		+
diarrhea		+
abdominal pain	++	+
peritonitis	++	
pleuritis	+	
acute scrotum	+	
rash	+ (erysipelas-like, Henoch-Schonlein purpura)	++ (pleiomorphic)
aphtosis		+
pharyngitis		+
eye signs		
sensorineural hearing loss		
joint symptoms	mainly monoarthritis	arthralgia, arthritis
headache		+
muscle aches	+	rare
lymphadenopathy		++
splenomegaly	+	+
amyloidosis	+	rare
Suggestive test results	favourable response to colchicine	raised serum IgD, IgA, elevated urinary mevalonic acid
Confirmation test	*MEFV* analysis	*MVK* analysis, MK activity

TRAPS	CAPS	PAPA	PFAPA
AD	AD	AD	unknown
TNFRSF1A	*CIAS1*	*PTSTPIP1*	
TNFRSF1A	cryopyrin	PTSTPIP1	
TNF-α receptor	Regulation of apoptosis and inflammation	Regulation of apoptosis and inflammation	
worldwide (more frequent in Scottish/Irish)	worldwide	worldwide	worldwide
Variable	Neonatal	Variable	2-5 years
Days to weeks	<24 hr		3-6 days
			+
+			+
++	+/-		+
++	+/-		
++			
++			
++ (migrating erythematous plaques)	++ (non-pruritic urticaria)	++ (acne conglobata, pyoderma gangrenosum)	
			++
			++
periorbital oedema, conjunctivitis	conjunctivitis, uveitis, papillitis		
	++		
arthralgia	varying from mild arthralgia to destructive arthropathy large joints	destructive purulent monoarthritis	arthralgia (rare)
++	++		+
++	+		
+	+		++
+	+		
+	+		
	CSF pleiocytosis, elevated CSF pressure	sterile prurulent joint aspirate	
TNFRSF1A analysis	*CIAS1* analysis	*PTSTPIP1* analysis	

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Chapter *1*

General Introduction II

Mevalonate Kinase Deficiency

The biochemical background of mevalonate kinase deficiency

In 1984, the first report was published of children with recurrent febrile attacks in the presence of an abnormally high serum IgD concentration (1). The first detailed description of this disorder was published in the same year and it was termed hyperimmunoglobulinemia D and periodic fever syndrome (HIDS)(2). In 1999, two separate reports described the genetic basis for HIDS: mutations in the gene *MVK* were found to cause a deficiency in the enzyme mevalonate kinase (3;4). This finding identified HIDS and the previously described syndrome mevalonic aciduria (5) as the mild and severe ends of a phenotypic spectrum, caused by a varying degree of mevalonate kinase enzyme activity. Consequently, both HIDS and mevalonic aciduria are now referred to as mevalonate kinase deficiency (MKD). Mutations in the *MVK* gene were shown to cause a decrease in mevalonate kinase protein levels (3), which is probably due to misfolding and hence rapid degradation of the protein. Residual activity of the enzyme in patients varies from ~7 to 20% in mildly affected patients to less than ~0.5% in mevalonic aciduria patients (6;7).

Mevalonate kinase is a critical enzyme in the isoprenoid biosynthesis pathway (Figure 1), which produces sterol and nonsterol isoprenoids. Cholesterol is the most important sterol end product of the pathway and functions mainly as a structural component of the plasma membrane and as a precursor for bile acids and steroid hormones. In addition, the isoprenoid pathway produces nonsterol compounds such as the isopentenyl groups of transfer RNA, involved in protein translation, the side-chain of ubiquinone-10 and heme A, both necessary for mitochondrial electron transport, dolichol, essential for N-linked protein glycosylation and farnesyl and geranylgeranyl groups used for prenylation of proteins of the Ras superfamily. Prenylation ensures that the proteins are correctly localized and, in addition, plays an important role in the regulation of activity of these types of proteins (8-10). Although patients with profoundly decreased mevalonate kinase activity exhibit reduced levels of ubiquinone-10 in plasma (11;12) and reduced biosynthesis of dolichol (13), squalene, cholesterol and bile acid levels are usually (near) normal (11;14). In addition to a decrease in isoprenoid biosynthesis, mevalonate kinase deficiency also results in an accumulation of mevalonic acid in patient's plasma. Because of this, some discussion was initially raised as to the fundamental cause of the inflammatory symptoms in mevalonate kinase deficiency. Inflammation might be caused by a reduced output of the biosynthesis pathway, or by a toxic effect of mevalonic acid. The use of statins in several models has now clarified this issue. Statins are synthetic compounds that inhibit 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase, the enzyme that catalyses the conversion of HMG-CoA into mevalonate (Figure 1). Incubation with statins causes reduced levels of downstream intermediates of the isoprenoid pathway, similar to the effect of mevalonate kinase deficiency. However, in contrast to MKD, incubation with statins will not lead to an accumulation, but a decrease in mevalonic acid levels. Several reports have established that incubation of peripheral blood mononuclear cells (PBMC) with statins results in increased production of inflammatory mediators and that this can be prevented by addition of mevalonate to the culture medium (15;16). These results indicate that a lack of downstream intermediates and not an accumulation of mevalonic acid is likely to be responsible for the inflammatory episodes characteristic of HIDS.

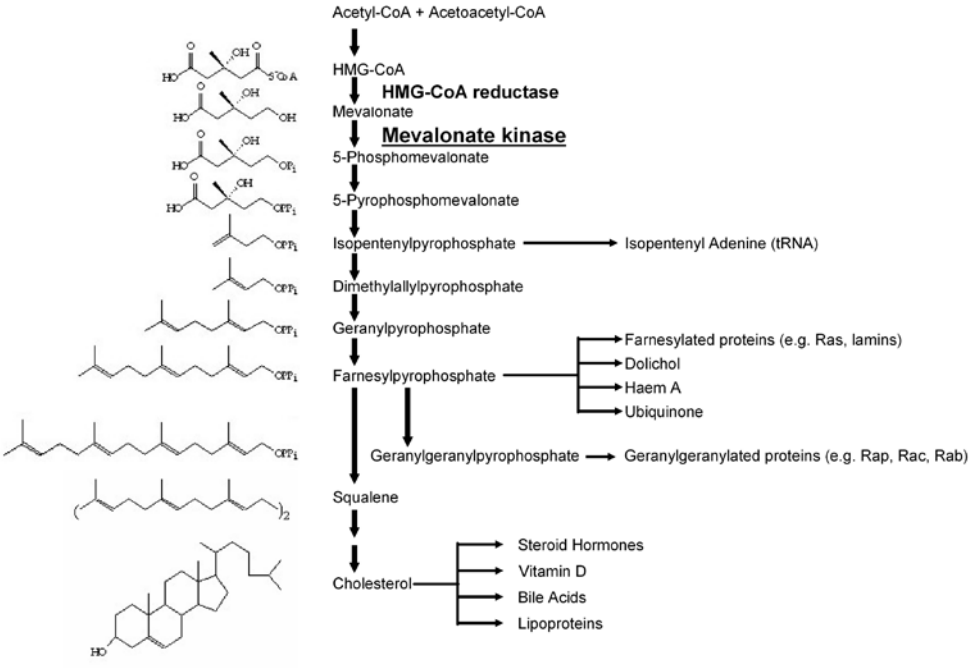

Figure 1: Schematic overview of the isoprenoid biosynthesis pathway.
Acetyl-CoA: acetyl-coenzyme A; HMG-CoA: 3-hydroxy-3-methylglutaryl-coenzyme A; tRNA: transfer RNA

The clinical characteristics of mevalonate kinase deficiency

HIDS, like all intermittent autoinflammatory syndromes, is mainly characterized by recurring episodes of fever. In HIDS, these episodes usually last 3 to 5 days and recur every 3 to 6 weeks. The fever episodes are very often accompanied by abdominal distress, vomiting, diarrhea and painful swelling of the cervical lymph nodes (6;17). Some patients also suffer from painful or inflamed joints and from skin rashes. The episodes are frequently triggered by immunological stimuli, such as childhood vaccinations, infections or minor trauma, however, such a trigger cannot always be identified. Between attacks, patients are usually well and thrive normally.

During attacks patients experience an acute phase response with leukocytosis, high levels of C-reactive protein and serum amyloid A and elevated erythrocyte sedimentation rate (17-19). Because fever and an acute phase response are considered to be initiated by the release of inflammatory mediators, research on MKD has focused on the release of inflammatory markers such as leukotriene E4, neopterin, soluble type-II phospholipase A2 (PLA_2) (18;20;21), but mostly on the release of inflammatory cytokines, such as interleukin (IL)-1α, IL-1β, IL-6, interferon (IFN)-γ and tumour necrosis factor (TNF)-α. Plasma concentrations of IFN-γ, IL-6 and TNF-α were found to be increased during attacks, whereas levels of IL-1α and IL-1β were not (18;21). Measurement of cytokine levels in a whole blood culture system demonstrated that there is an elevated basal level

of IL-1β and TNF-α production by immune cells, which can be further increased by stimulation with LPS (18). In addition, studies using PBMC isolated from HIDS patients between attacks show increased production of IL-1β, IL-6 and TNF-α by these cells (22). Taken together, elevated levels of IL-1β, IL-6 and TNF-α, together with the observed raise in urinary leukotriene E4 and neopterin excretion, suggests activation of macrophages at least during, but most likely also between attacks.

Research on models using statins demonstrated that fluvastatin-treated PBMC from healthy individuals produce large amounts of IL-1β, IFN-γ and IL-18 when stimulated with inactivated *M. tuberculosis* (16). In another study, statin-treated human monocytes were also shown to have increased production of IL-1β and TNF-α in response to LPS (23). Furthermore, PBMC from HIDS patients spontaneously secreted 9-fold more IL-1β than PBMC from healthy controls, a difference that increased another 2.4-fold in the presence of lovastatin (15).

In conclusion, previous research on mevalonate kinase deficiency points towards a role for spontaneous macrophage activation resulting in an acute phase response. In general, TNF-α and IL-1β are key mediators for initiating an acute phase response (24), of which IL-1β is considered the most potent pyrogen (fever-inducing agent). Macrophages are known to be a major source of IL-1β production *in vivo*. In addition, models using statins to mimic mevalonate kinase deficiency have demonstrated a great induction of IL-1β secretion by statin-treated immune cells when given an immunological trigger. Taken together, these data suggest an important role for IL-1β in the initiation of the inflammatory attacks observed in HIDS.

The role of interleukin-1β in mevalonate kinase deficiency

Unlike most cytokines, IL-1β is synthesized as an inactive precursor (proIL-1β), lacking a conventional secretory leader sequence (25). Instead of passing through the endoplasmic reticulum and the Golgi complex, proIL-1β is translated in the cytosol. There, the inactive proform requires processing by caspase-1, which cleaves proIL-1β directly after the aspartic acid residue at position 116 (26;27). Caspase-1 itself is synthesized as an inactive zymogen of ~45 kDa that, via induced proximity to another caspase-1 zymogen, can undergo autocleavage, creating 10 kDa and 20 kDa subunits. Two p10 and two p20 subunits form the fully functional heterodimeric enzyme. Caspase-1 auto-activates itself in a complex of proteins termed the inflammasome (Figure 2) (28). Caspase-1 contains an N-terminal caspase recruitment domain (CARD), which forms a homotypic interaction (CARD-CARD interaction) with either apoptosis-associated speck-like protein containing a CARD (ASC) or the protein CARDINAL. Via similar homotypic interactions these adaptor proteins interact with other members of the inflammasome, e.g. with NALP2 or NALP3 (29). Formation of this multiprotein complex enables autocleavage, thus activation, of caspase-1.

Active caspase-1 is thought to colocalize with proIL-1β in lysosomal compartments, in which processing of proIL-1β into mature IL-1β takes place (30). Through fusion of the lysosome with the plasma membrane, mature IL-1β and the active caspase-1 subunits are subsequently released into the extracellular environment.

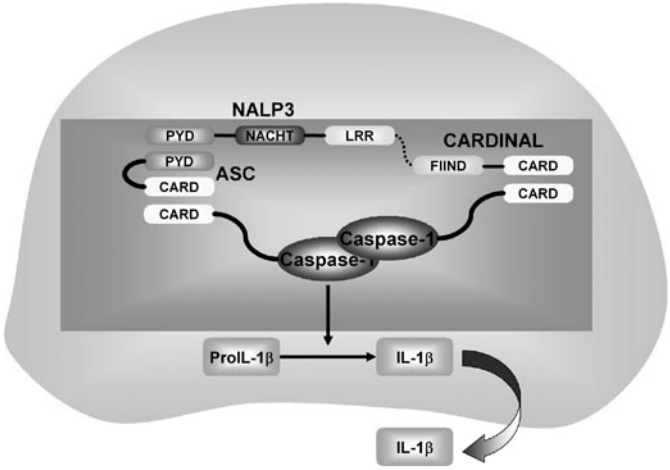

Figure 2: Schematic representation of the NALP3 containing inflammasome.
PYD: pyrin domain; CARD: caspase recruitment domain; LRR: leucin-rich repeat; FIIND: domain with function to find; NACHT: domain present in neuronal apoptosis inhibitor protein (NAIP), MHC class II transactivator (CIITA), HET-E and TP-1; ASC: apoptosis-associated speck-like protein containing a CARD; NALP: NACHT, leucine-rich repeat and pyrin containing protein.

Scope of this thesis

At the start of this research project the molecular mechanisms underlying the pathogenesis of mevalonate kinase deficiency were still unclear. Reduced levels of isoprenoid products appeared to be causing a spontaneous activation of macrophages resulting in release of the inflammatory mediator interleukin-1β. The molecular basis for activation of macrophages due to lower isoprenoid output was still lacking, as well as the regulatory mechanism behind enhanced IL-1β release by mevalonate kinase deficient macrophages. In addition, no conclusive evidence was available for the primary role of IL-1β in the initiation of fever attacks in MKD. This thesis provides answers to some of the remaining questions. In Chapter 2, enhanced IL-1β secretion by PBMC from MKD patients was demonstrated to be caused by a specific lack of geranylgeranylated proteins. Chapter 3 describes how impairment of the isoprenoid biosynthesis pathway results in activation of the enzyme caspase-1, explaining enhanced release of IL-1β and in Chapter 4 a molecular model is provided that clarifies how a reduced level of geranylgeranylpyrophosphate leads to activation of caspase-1. In Chapter 5 we have measured a broad panel of cytokines in culture supernatants of patient PBMC and in plasma of patients that were treated with the IL-1 receptor antagonist anakinra. As a result of these studies we were able to confirm the primary role for IL-1β in the initiation of inflammation in patients. Finally, Chapter 6 describes a case of Familial Mediterranean Fever that could also be successfully treated with anakinra. All results described in this thesis are summarized and discussed in Chapter 7.

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Chapter 2

A role for geranylgeranylation in interleukin-1β secretion

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ABSTRACT

Objective
Mevalonate kinase deficiency (MKD) is an autosomal-recessive disorder characterized by recurring episodes of inflammation. The enzyme mevalonate kinase (MK) catalyzes the phosphorylation of mevalonic acid, which is an early step in isoprenoid biosynthesis. The goal of our study was to determine whether a temporary shortage of certain isoprenoid end products or the accumulation of mevalonic acid is the cause of interleukin-1β (IL-1β) secretion in MKD.

Methods
We studied the effect of the addition of intermediate metabolites and inhibitors of the isoprenoid biosynthesis pathway on IL-1β secretion by peripheral blood mononuclear cells (PBMC) of patients with MKD and healthy controls.

Results
Inhibition of enzymes involved in geranylgeranyl pyrophosphate (GGPP) synthesis or geranylgeranylation of proteins led to a marked increase of lipopolysaccharide-stimulated IL-1β secretion in PBMC of control subjects. Furthermore, the increased IL-1β secretion by PBMC of patients with MKD was reversed by supplementation with GGPP as well as with mevalonic acid. IL-1β secretion was increased only when control PBMC were incubated with excessive amounts of mevalonic acid. Finally, a reduction in IL-1β secretion by MKD PBMC was also observed when sterol biosynthesis was inhibited, favouring nonsterol isoprenoid biosynthesis.

Conclusion
Our results indicate that a shortage of geranylgeranylated proteins, rather than an excess of mevalonate is likely to cause increased IL-1β secretion by PBMC of patients with MKD.

INTRODUCTION

Mevalonate kinase deficiency (MKD) is an autosomal-recessive autoinflammatory disorder characterized by recurring episodes of high fever associated with headache, arthritis, nausea, abdominal pain, diarrhea, and skin rash (1;2). Originally, two distinct syndromes had been defined, classic mevalonic aciduria (MA) (3) and hyperimmunoglobulinemia D with periodic fever syndrome (HIDS)(4), but after the discovery that both disorders are caused by a deficiency of the enzyme MK (5;6), they are now recognized as the severe and mild presentations of MKD. Patients with HIDS typically have recurrent episodes of fever with associated inflammatory symptoms (1), whereas patients with MA, in addition to these episodes, show developmental delay, dysmorphic features, ataxia, cerebellar atrophy, and psychomotor retardation and may die in early childhood (2). Cells of patients with HIDS show a residual MK enzyme activity of 1-8% (6-8), but in cells of patients with MA the enzyme activity is below detection level (2;9;10). This difference in residual enzyme activity is also reflected in the occurrence of high levels of mevalonic acid in the plasma and urine of patients with MA and low to moderate levels of mevalonic acid in patients with HIDS.

Blood analyses during the episodes of fever indicate an acute inflammatory state, with a marked rise in serum levels of proinflammatory cytokines, such as interleukin-6 (IL-6) and interferon-γ (11;12). Also, between attacks, isolated peripheral blood mononuclear cells (PBMC) of patients with MKD secrete increased amounts of proinflammatory cytokines, such as IL-1β (13;14). IL-1β is the prototypical proinflammatory cytokine and appears to play an important role in the pathogenesis of several autoinflammatory diseases, including MKD (15). This is supported by the beneficial effect of a recombinant form of IL-1 receptor antagonist, anakinra, used in the treatment of those autoinflammatory diseases (16-22).

MK catalyzes the ATP-dependent phosphorylation of mevalonate to produce 5-phosphomevalonate and is the first enzyme to follow the rate-limiting and highly regulated enzyme 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase in the isoprenoid biosynthesis pathway (Figure 1) (23). The isoprenoid biosynthesis pathway provides cells with several bioactive molecules, including isoprenyl groups, the polyprenyl chain of heme A, dolichol, and sterols. The isoprenyl groups come in two forms: the farnesyl groups (from farnesylpyrophosphate [FPP]) and the geranylgeranyl groups (from geranylgeranylpyrophosphate [GGPP]). Both can be attached to proteins of the Ras superfamily.

The precise molecular mechanism by which the depressed activity of MK leads to increased IL-1β secretion and fever episodes is still unknown. However, there are indications that it is due to a temporary shortage of certain isoprenylated proteins (24).

Recently, Simon *et al.* reported the outcome of simvastatin treatment of six patients with the HIDS phenotype, which led to shorter periods of fever in most patients (25). The rationale for testing simvastatin in these patients was based on the assumption that the elevated mevalonic acid levels were causing the inflammation. Since statins, such as simvastatin, are competitive inhibitors of HMG-CoA reductase, this treatment would lead to a lowering of mevalonate levels and thus was predicted to reduce inflammation.

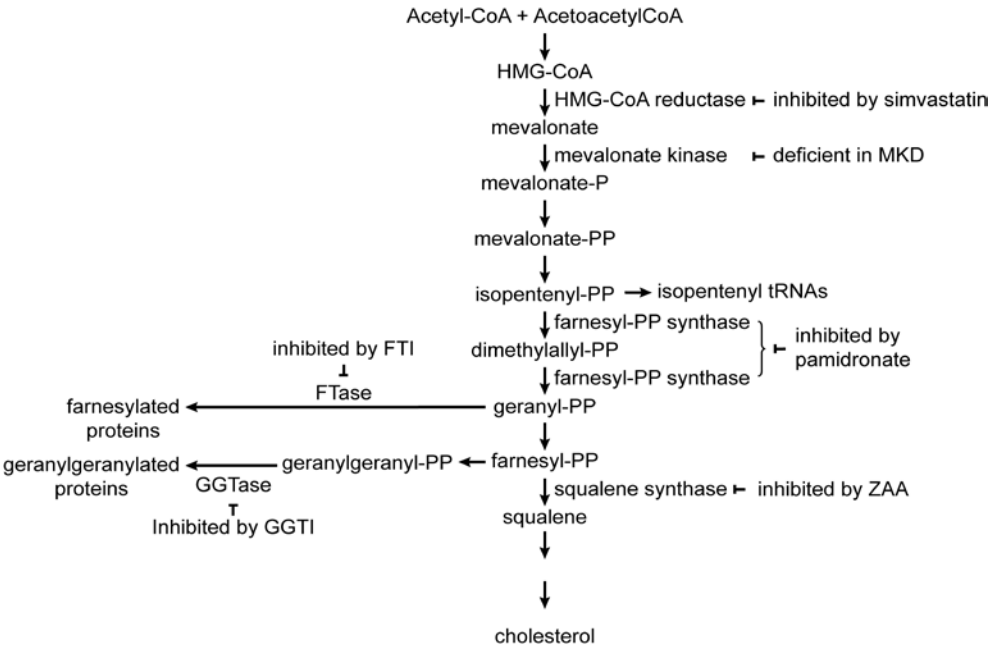

Figure 1: The isoprenoid biosynthesis pathway
Schematic overview of the isoprenoid biosynthesis pathway, indicating the various enzyme steps that were inhibited in this study. Acetyl-CoA = acetyl-coenzyme A; HMG-CoA = hydroxymethylglutaryl-coenzyme A; MKD = mevalonate kinase deficiency; P = phosphate; PP = pyrophosphate; tRNA = transfer RNA; FTI = farnesyl-transferase inhibitor; FTase = farnesyltransferase; GGTase = geranylgeranyltransferase; ZAA = zaragozic acid A; GGTI = geranylgeranyltransferase inhibitor

In contrast, we previously reported that it was not elevated mevalonic acid levels, but a shortage of isoprenoid end products, that contributed to the inflammation in MKD (14;24;26). In order to resolve this apparent discrepancy, we studied the inflammatory response of both MKD and control PBMC by measuring IL-1β secretion upon stimulation with lipopolysaccharide (LPS) after exposing the cells to a concentration range of mevalonate. Furthermore, we studied the effect of various enzyme inhibitors and intermediate metabolites of the isoprenoid biosynthesis pathway on IL-1β secretion by PBMC of patients with MKD and controls. Our results indicated that increased IL-1β secretion is correlated with a shortage of certain nonsterol isoprenoids rather than elevated mevalonic acid levels.

PATIENTS AND METHODS

Patients and samples

After approval by the ethics review board of the University Medical Centre and after written informed consent was obtained from their parents, blood was drawn from 4 patients with MKD

ranging in age from 3 to 14 years, by venipuncture using sterile, pyrogen-free heparinized tubes (Vacuette; Greiner, Alphen aan den Rijn, The Netherlands). Healthy volunteers served as controls. PBMC were isolated by density-gradient centrifugation using Lymphoprep according to the manufacturer's protocol (Axis-Shield, Oslo, Norway). Cells ($1x10^5$/well) were seeded in 96-well flat-bottomed microtiter plates in RPMI-1640 (Gibco Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated fetal calf serum (Gibco Invitrogen), 10 mM HEPES (Gibco Invitrogen) and 1% penicillin/streptomycin. Next, PBMC were incubated with culture medium that contained the indicated compound, at 37°C in a humidified atmosphere containing 5% CO_2 in air. After 18 hours of incubation, *Escherichia coli* 055:B5 LPS (final concentration of 100 ng/ml; Sigma, St. Louis, MO) or medium was added to the cultures followed by 24 hours of additional incubation. After this, supernatants were collected and stored at -20 °C until analysis.
Mevalonate was prepared by hydrolyzation of mevalonic acid lactone with 0.1 M NaOH, followed by neutralization and stabilization at pH 7.4 with 1 M HEPES and 0.1 M HCl. Simvastatin and Zaragozic acid A (ZAA) were prepared as previously described (27). Farnesyl transferase (FTase) inhibitor and geranylgeranyl transferase (GGTase) inhibitor (FTI-277 and GGTI-298; Calbiochem, La Jolla, CA) were also dissolved in DMSO (20 mM). Pamidronate (a gift from Novartis) was dissolved in distilled water (10 mM).

Cytokine Measurements
IL-1β concentrations were measured in duplicate in thawed supernatant samples, using commercially available enzyme-linked immunosorbent assays (ELISAs) (Pelikine-compact human IL-1β ELISA kit; Sanquin Reagents, Amsterdam, the Netherlands) according to the manufacturer's instructions.

Statistic analysis
All results are expressed as the mean and SEM. Statistical significance was calculated using Friedman's paired nonparametric analysis of variance followed by Dunn's multiple comparison test or a 2-tailed Wilcoxon's matched pairs test. P values less than 0.05 were considered significant.

RESULTS

Effect of upstream inhibition of MK and of mevalonic acid supplementation
The effect of inhibiting HMG-CoA reductase, which is the enzyme preceding MK, was studied by incubating PBMC from patients with MKD and control subjects with or without 5 μM simvastatin, after which they were stimulated with LPS. In the absence of simvastatin, stimulation of IL-1β secretion by LPS was greater in MKD PBMC than in control PBMC, whereas LPS-stimulated IL-1β secretion was markedly increased after incubation with simvastatin both in control and MKD PBMC (Figure 2A). These results showed that lowering the levels of mevalonate and/or downstream isoprenoids led to an increase in IL-1β secretion.

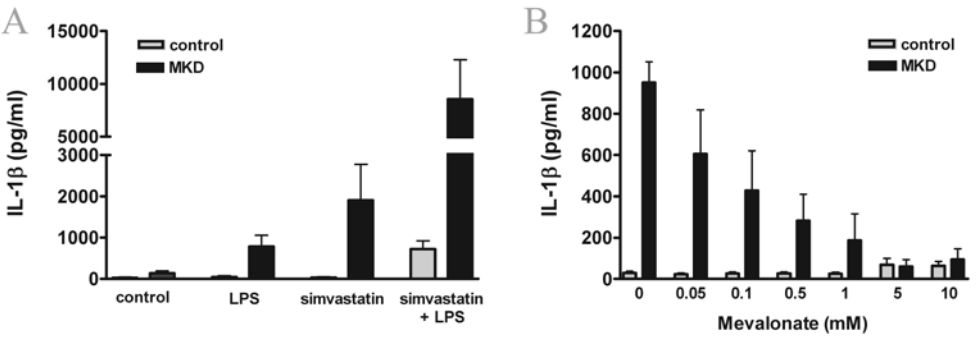

Figure 2: Effect of upstream inhibition of mevalonate kinase (MK) and mevalonic acid supplementation. A) Interleukin-1β (IL-1β) secretion by peripheral blood mononuclear cells (PBMC) from 4 controls and 4 patients with MK deficiency (MKD). Cells were incubated in the absence or presence of simvastatin for 18 hours and stimulated for 24 hours with lipopolysaccharide (LPS). B) The effect of mevalonate on IL-1β secretion by PBMC from controls and patients with MKD. The IL-1β concentrations determined in independent incubations using PBMC obtained from 7 different controls and 3 different patients with MKD are shown. Incubations with 10 mM mevalonate were performed in PBMC from 3 controls and 2 patients with MKD. Values are the mean and SEM.

To investigate whether elevated mevalonate levels also can be pro-inflammatory, we next determined LPS-induced IL-1β secretion by PBMC from control subjects and patients with MKD when they were exposed to increasing concentrations of mevalonate. Incubation of freshly isolated PBMC from control subjects with mevalonate concentrations ranging from 0.05 to 10 mM resulted in a noticeable increase of LPS-stimulated IL-1β secretion only at 5 and 10 mM (Figure 2B). When PBMC of patients with MKD were incubated with the same concentrations of mevalonate, there was a marked decrease in IL-1β secretion with increasing mevalonate concentrations (Figure 2B).

Effect of inhibition of enzymes located downstream of MK

The reduced IL-1β secretion observed after mevalonate supplementation supports the hypothesis that the LPS-induced IL-1β secretion by PBMC of patients with MKD is due to a shortage of one of the isoprenoid end products and not to elevated mevalonate levels. To further substantiate this we studied the effect of inhibiting different enzymes located downstream of MK in the isoprenoid biosynthesis pathway. To this end, we incubated control PBMC with increasing concentrations of pamidronate, which inhibits FPP synthase, the enzyme that catalyzes the formation of GGPP and FPP (Figure 1). This resulted in a significant elevation in LPS-stimulated IL-1β secretion (Figure 3A), which confirmed that a shortage of isoprenoid end products plays a role in elevated LPS-stimulated IL-1β secretion.

Both simvastatin and pamidronate inhibited the synthesis of sterol as well as nonsterol isoprenoid end products. Because our previous results indicated that the shortage underlying the symptoms in MK deficiency was probably related to nonsterol isoprenoid end products (14;24;26), we next studied the effect of increasing concentrations of ZAA on IL-1β secretion

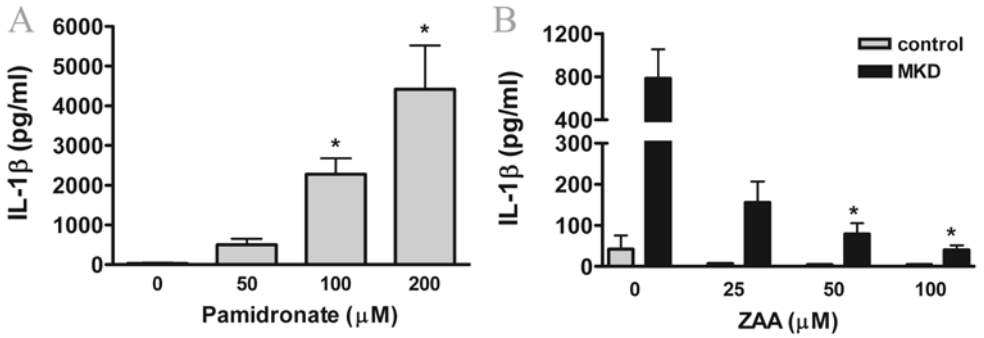

Figure 3: Effect of inhibiting enzymes downstream of MK
A) IL-1β secretion by LPS-stimulated PBMC in 4 controls in the absence or presence of pamidronate. * = p<0.05 versus concentrations determined in PBMC stimulated in the absence of pamidronate. B) IL-1β secretion by PBMC in 4 controls and 4 patients with MKD, stimulated with LPS in the absence or presence of zaragozic acid A (ZAA). * = p<0.05 versus concentrations determined in PBMC incubated in the absence of ZAA. Values are the mean and SEM. See Figure 2 for other definitions.

by control and MKD PBMC. ZAA is a specific inhibitor of squalene synthase, the first enzyme committed exclusively to sterol isoprenoid biosynthesis. Thus, ZAA inhibits the synthesis of sterol isoprenoids and, by doing so, promotes the synthesis of nonsterol isoprenoids (Figure 1). Moreover, the reduction in the synthesis of sterol end products leads to increased transcription of early isoprenoid biosynthetic genes (23). Consistent with this, the incubation of MKD PBMC with ZAA resulted in reduced LPS-stimulated IL-1β secretion (Figure 3B).

Role of isoprenylated proteins in IL-1β secretion

The previous experiment pointed to a role for nonsterol isoprenoid end products in IL-1β secretion. The most prominent nonsterol isoprenoids with a known biological function are the farnesyl and geranylgeranyl moieties, derived from FPP and GGPP, respectively. Both can be covalently attached to proteins in a process known as protein isoprenylation. To determine whether increased IL-1β secretion is due to a shortage of farnesyl or geranylgeranyl moieties, we tested the effect of incubating control PBMC with FTase inhibitor or GGTase inhibitor on LPS-stimulated IL-1β secretion. Results showed that the addition of GGTase inhibitor led to a marked increase in LPS-stimulated IL-1β secretion, whereas the addition of FTase inhibitor did not have a noticeable effect on the secretion of IL-1β (Figure 4A).

Consistent with these findings, we also found that the increased LPS-stimulated IL-1β secretion observed when control PBMC were incubated with simvastatin could be completely reversed when the cells were incubated with GGPP (Figure 4B). Also, the increase in LPS-stimulated IL-1β secretion in PBMC of patients with MKD could be reduced to control levels with GGPP (Figures 4C and D).

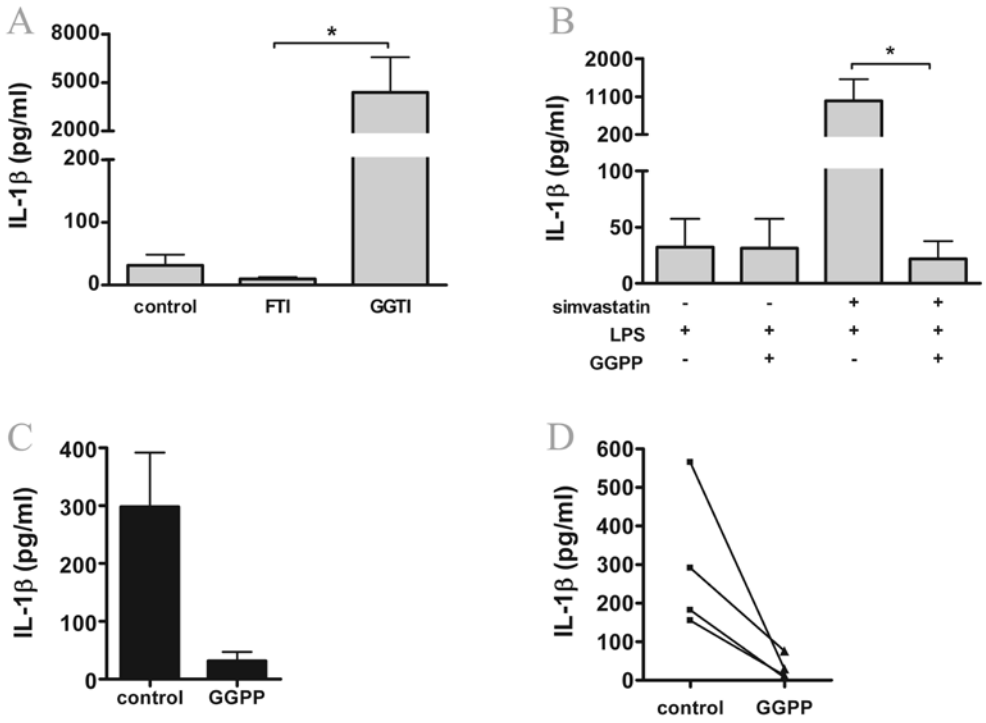

Figure 4: Role of isoprenylated proteins in IL-1β secretion
Shown are levels of IL-1β secretion by PBMC from controls (shaded bars) and patients with MKD (solid bars), incubated in the absence or presence of farnesyltransferase inhibitor (FTI), geranylgeranyltransferase inhibitor (GGTI), simvastatin, and geranylgeranylpyrophosphate (GGPP) for 18 hours, followed by stimulation with LPS. A) Control PBMC (n = 4) incubated with FTI and GGTI. * = $p<0.05$. B) Control PBMC (n = 3), stimulated with LPS after incubation with simvastatin and/or GGPP. * = $p<0.05$. C) MKD PBMC (n = 4) stimulated with LPS in the absence or presence of GGPP. D) Findings in paired samples (with and without GGPP) from individual patients, showing similar results in all 4. Values in A-C are the mean and SEM. See Figure 2 for other definitions.

DISCUSSION

Patients with the HIDS and the MA phenotypes experience similar inflammatory episodes despite marked differences in both residual MK activity and accumulating levels of mevalonic acid. This suggests that mevalonate itself has no major effect on the inflammatory response. This was confirmed in our study, in which only a small increase in LPS-stimulated IL-1β secretion was observed when control PBMC were incubated with 5 mM and 10 mM mevalonate but not at lower concentrations. Because 5 mM mevalonate is 10 times higher than the mevalonate levels found in the blood of MA patients and 1,000 times higher than that found in the blood of HIDS patients, it seems very unlikely that mevalonic acid is the main cause of the inflammatory response. In fact, the increase in LPS-stimulated IL-1β secretion by MKD PBMC is even reversed by the addition of mevalonate.

These data are consistent with previously reported results (26), which showed that MA and HIDS cells compensate for the reduced MK activity by elevating their intracellular mevalonate levels by increasing HMG-CoA reductase activity. This increased HMG-CoA reductase activity was also down-regulated when MKD cells were incubated with the isoprenoid precursors farnesol, geranylgeraniol, or mevalonate (26). Thus, in order to maintain the flux through the isoprenoid biosynthesis pathway, it seems important to increase the levels of intracellular mevalonic acid. Failure of this compensatory mechanism might even lead to increased IL-1β secretion and inflammation, as is suggested by the severe inflammatory crises provoked by treating two MA patients with lovastatin in an attempt to lower the mevalonic avid levels (2).

Other studies showed that incubation of control PBMC with lovastatin or fluvastatin increased IL-1β secretion (14;28;29) and that lovastatin was able to further increase the elevated IL-1β secretion in MKD PBMC (14). This was also observed in the present study, when simvastatin was added to PBMC from patients with MKD and controls. These observations confirm that mevalonate, by itself, is not the cause of the LPS-stimulated hypersecretion of IL-1β by PBMC of patients with MKD.

The results of our study strongly indicate that it probably is a shortage of a nonsterol isoprenoid end product, notably geranylgeranyl groups, that leads to increased IL-1β secretion in MKD. This is concluded from the fact that the inhibition of enzymes involved in GGPP synthesis or geranylgeranylation of proteins, i.e. HMG-CoA reductase, FPP synthase, and GGTase, all led to a marked increase in LPS-stimulated IL-1β secretion by control PBMC. Moreover, the increased IL-1β secretion in PBMC from patients with MKD could be reversed by supplementation with GGPP, whereas the inhibition of sterol synthesis with ZAA, which promotes the synthesis of nonsterol isoprenoids, also resulted in a reversal of the LPS-stimulated IL-1β secretion by MKD PBMC. Together, these findings clearly indicate an important role for nonsterol isoprenoids, notably geranylgeranyl groups, in the regulation of the inflammatory response.

There are currently no established treatments for MKD, although treatment with the recombinant form of the IL-1 receptor antagonist anakinra was shown to be effective in relieving the symptoms (19). Our results indicate that raising the availability of geranylgeranyl moieties in MKD, for example by increasing the pathway flux toward nonsterol isoprenoid biosynthesis, may provide another option in the treatment of MKD.

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Chapter 3

Statin synergizes with LPS to induce IL-1β release by THP-1 cells through activation of caspase-1

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ABSTRACT

Mevalonate kinase deficiency (MKD) is a hereditary syndrome characterized by recurring episodes of fever and inflammation. Peripheral blood mononuclear cells from MKD patients secrete high levels of interleukin (IL)-1β when stimulated with lipopolysaccharide (LPS), which is thought to be a primary cause of the inflammation. However, the link between a deficient mevalonate kinase and excessive IL-1β release remains unclear. To investigate this we made use of a model in which monocytic cells (THP-1) were treated with simvastatin. Statins are compounds that inhibit 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase and thereby artificially impair the isoprenoid biosynthesis pathway, mimicking mevalonate kinase deficiency.

Our study revealed that LPS-stimulated THP-1 cells treated with simvastatin had an increased caspase-1 mediated processing of proIL-1β. This increased processing was caused by enhanced autoprocessing of caspase-1, rather than enhanced transcription or translation of caspase-1 or proIL-1β. Simvastatin-induced activation of caspase-1 was caused by an impairment of non-sterol isoprenoid biosynthesis, as the isoprenyl intermediate GGPP could block activation of caspase-1 and mIL-1β release. In addition, inhibition of both farnesyl pyrophosphate synthase and geranylgeranyltransferase I also induce mIL-1β release.

Taken together, these results demonstrate that simvastatin augments LPS-induced IL-1β release post-translationally, by inducing caspase-1 activity. These findings suggest that MKD patients may have overactive caspase-1, causing enhanced IL-1β processing and subsequent inflammation in response to bacterial components.

INTRODUCTION

The Hyperimmunoglobulinemia D and periodic fever syndrome (HIDS; MIM#260920), is an autosomal recessive disorder, characterized by recurrent fever attacks and an elevated level of serum IgD (>100 IU/ml) (1). The febrile attacks are accompanied by painful cervical lymphadenopathy and often by abdominal pain, vomiting and diarrhea. A variety of other symptoms including headache, skin rashes, mucosal ulcers, myalgia and arthralgia may also occur (1-3). During the fever episodes an acute phase response is observed, with leukocytosis and elevated acute-phase reactants. Serum levels of proinflammatory cytokines, such as interleukin-6 (IL-6) and interferon-γ (IFN-γ), rise during fever attacks (4;5). Also, between attacks, isolated peripheral blood mononuclear cells (PBMC) from HIDS patients secrete large amounts of IL-1β (6), which further increases during fever (4).

In 1999, the genetic defect of HIDS was identified: patients were shown to have mutations in the gene *MVK*, which codes for the enzyme mevalonate kinase (7;8). Since this discovery, HIDS and its more severe allelic phenotype, mevalonic aciduria, are jointly referred to as mevalonate kinase deficiency (MKD). Mevalonate kinase is an important enzyme in the isoprenoid biosynthesis pathway (9). This pathway produces cholesterol and a number of nonsterol isoprenoids. The latter play a vital role in the prenylation of a variety of proteins, mostly of the Ras GTPase superfamily. Recently, it has become apparent that impairment of the isoprenoid pathway has widespread effects on immune function, both anti-inflammatory and pro-inflammatory (10-15). Several studies have shown that the secretion of IL-1β by activated PBMC was greatly augmented by the inhibition of isoprenoid biosynthesis using hydroxymethylglutaryl-CoA (HMG-CoA) reductase inhibitors, also known as statins. This increased cytokine release appeared to be specifically due to a lack of isoprenoids, since the addition of mevalonic acid, the product of HMG-CoA reductase, reduced cytokine production to control levels (15-17). In PBMC from patients suffering from mevalonate kinase deficiency it is the lack of isoprenoid products, specifically of geranylgeranylated proteins, that raises IL-1β production (18). This IL-1β production may be largely responsible for the inflammation and fever observed in MKD patients. However, it is not known how impaired isoprenoid biosynthesis leads to increased IL-1β release.

Unlike most cytokines, IL-1β is synthesized as an inactive precursor (proIL-1β), lacking a conventional leader sequence. Instead of passing through the endoplasmic reticulum and the Golgi complex, proIL-1β is translated in the cytosol. There, the inactive proform requires processing by caspase-1, which cleaves proIL-1β directly after the aspartic acid residue at position 116 (19;20). In addition to IL-1β, caspase-1 can also cleave interleukin-18 (21;22) and recently, interleukin-33 was also identified as a caspase-1 substrate (23). Caspase-1 itself is synthesized as an inactive zymogen of ~45 kDa that, via induced proximity to another caspase-1 zymogen, can undergo autocleavage, creating 10 kDa and 20 kDa subunits. Two p10 and two p20 subunits form the fully functional heterodimeric enzyme. In analogy to the apoptotic caspase-9 (24), caspase-1 auto-activates itself in a complex of proteins termed the inflammasome (25). Caspase-1 contains an N-terminal caspase recruitment domain (CARD), which forms a homotypic interaction (CARD-CARD interaction) with apoptosis-associated speck-like protein containing a CARD (ASC). This adaptor protein then recruits other

members of the inflammasome (26) via similar homotypic interactions, enabling oligomerization and autocleavage. Active caspase-1 can then process proIL-1β into mature IL-1β (mIL-1β), which is subsequently secreted. The exact export mechanism of mIL-1β remains unclear.
To investigate the regulation of increased mIL-1β production in mevalonate kinase deficiency, we studied the monocytic cell line THP-1 in which the isoprenoid biosynthesis pathway was artificially impaired using simvastatin. We examined the effect of this impairment on transcription and translation of (pro)caspase-1 and (pro)IL-1β and on caspase-1 enzyme activity. Our study revealed that simvastatin treatment induced an increase in caspase-1-mediated processing of proIL-1β by LPS-stimulated THP-1 cells. This increased processing was caused by enhanced autoprocessing of caspase-1, rather than enhanced transcription or translation of either caspase-1 or proIL-1β. The simvastatin-induced activation of caspase-1 was caused by an impairment of nonsterol isoprenoid biosynthesis, as the isoprenyl intermediate GGPP could completely block activation of caspase-1 and mIL-1β release and the inhibition of geranylgeranyltransferase I enhanced IL-1β release, similar to simvastatin.

MATERIALS AND METHODS

Reagents
Simvastatin, lipopolysaccharide (LPS; E. coli 0127:B8), geranylgeranylpyrophosphate (GGPP) and Actinomycin-D were purchased from Sigma-Aldrich. Simvastatin was prepared by dissolving the prodrug in ethanol, followed by hydrolysis of the lactone by adding NaOH. After neutralization with 1 M HEPES pH 7.4 and HCl the solution was sterilized by filtration through a 0.2 μm filter and stored as aliquots at -20°C. GGTI-298 was obtained from Calbiochem, benzyloxycarbonyl-Val-Ala-Asp fluoromethylketone (z-VAD-FMK) from R&D Systems. Pralnacasan (a specific caspase-1 inhibitor) was a kind gift from Vertex Pharmaceuticals (Boston, U.S.A.). Pamidronate (a specific farnesyl PP synthase inhibitor) was a kind gift from Novartis (Basel, Switzerland).

Cell Culture
THP-1 cells were routinely grown in RPMI-1640 (Life Technologies) containing 2 mM glutamine, 100 U/ml penicillin-streptomycin ($RPMI^{++}$) and 10% Fetal Calf Serum (FCS). Cells (at a density of $1x10^6$/ml) were cultured in 12-well microtiter plates in $RPMI^{++}$/5% FCS. Incubations were performed at 37°C in a humidified atmosphere containing 5% CO_2 in air, in the presence or absence of 5 μM simvastatin. After 24 hours of incubation, LPS at a final concentration of 200 ng/ml was added to the cells without any other change in the culture medium. The incubations were prolonged for an additional 4 hours after which supernatants were removed and either frozen at -20°C or assayed immediately. Cell pellets were stored at -20°C or snap frozen in liquid nitrogen and stored at -80°C for RT-PCR analysis. For the proIL-1β ELISA assay, cell pellets were thawed, taken up in lysis buffer (150 mM NaCl, 20 mM HEPES, 10 mM EDTA, 1% Triton X-100) containing protease inhibitors (Roche) and incubated on ice for 30 minutes. Cell lysates were centrifuged at 17,000g for 15 minutes and the supernatants were used for determination of proIL-β content.

Cytokine Measurements

Cytokine detection was carried out using commercially available ELISA kits: Pelikine-compact ™ human IL-1β and IL-6 ELISA kits (Sanquin, Amsterdam), human proIL-1β/IL-1F2 Quantikine ELISA Kit (R&D Systems), human IL-18 module set (Bender MedSystems). The assays were performed according to the manufacturer's instructions and all samples were tested in duplicate.

LDH assay

THP-1 cells were cultured for 24 hours in the presence or absence of simvastatin (5 μM) and stimulated for an additional 4 hours with 200 ng/ml LPS. Simvastatin-mediated effects were blocked by addition of 10 μM geranylgeranylpyrophosphate (GGPP) to the culture medium. After treatment the cells were thoroughly resuspended and 200 μl cell suspension was used for the CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega). The assays were performed according to the manufacturer's instructions and all samples were tested in duplicate.

Quantitative real-time RT-PCR analysis

The relative expression levels of IL-1β and caspase-1 to β-actin RNA were measured with the LightCycler® system (Roche, Mannheim, Germany). To this end, total RNA was isolated from THP-1 cells with trizol extraction. First strand cDNA was prepared using the first strand cDNA synthesis kit for RT-PCR (Roche) according to the manufacturer's instructions. The IL-1β fragment was amplified using primers: IL-1β Fw 5'-AGA AGA ACC TAT CTT CTT CGA C-3' and IL-1β Rev 5'-ACT CTC CAG CTG TAG AGT GG-3'. Caspase-1 primers were: Fw 5'-CTT CCT TTC CAG CTC CTC AG-3' and Rev 5'-CCT GTG ATG TCA ACC TCA GC-3'. The β-actin fragment was amplified using the following primer set: Fw 5'-GGC ACC AGG GCG TGA TGG-3' and Rev 5'- GTC TCA AAC ATG ATC TGG GTC-3'. Data were analyzed using LightCycler Software, version 3.5 (Roche) and the program LinRegPCR, version 7.5 (27) for analysis of real-time PCR data. To adjust for variations in the amount of input RNA, the IL-1β and caspase-1 mRNA levels were normalized against the mRNA levels of the housekeeping gene β-actin.

Measurement of caspase-1 cleavage

Caspase-1 p20 detection was carried out using a commercially available ELISA kit: Quantikine human caspase-1 immunoassay (R&D Systems). The assay was performed according to the manufacturer's instructions and all samples were tested in duplicate.

RESULTS

Simvastatin augments LPS-induced mIL-1β release in THP-1 cells

THP-1 cells were cultured for 24 hours in the presence or absence of simvastatin and stimulated for an additional 4 hours with LPS, after which supernatant was assayed for the presence of mIL-1β. THP-1 cells treated with a combination of simvastatin and LPS displayed dramatically increased mIL-1β release as compared to LPS stimulation alone (Figure 1A). Incubation with simvastatin

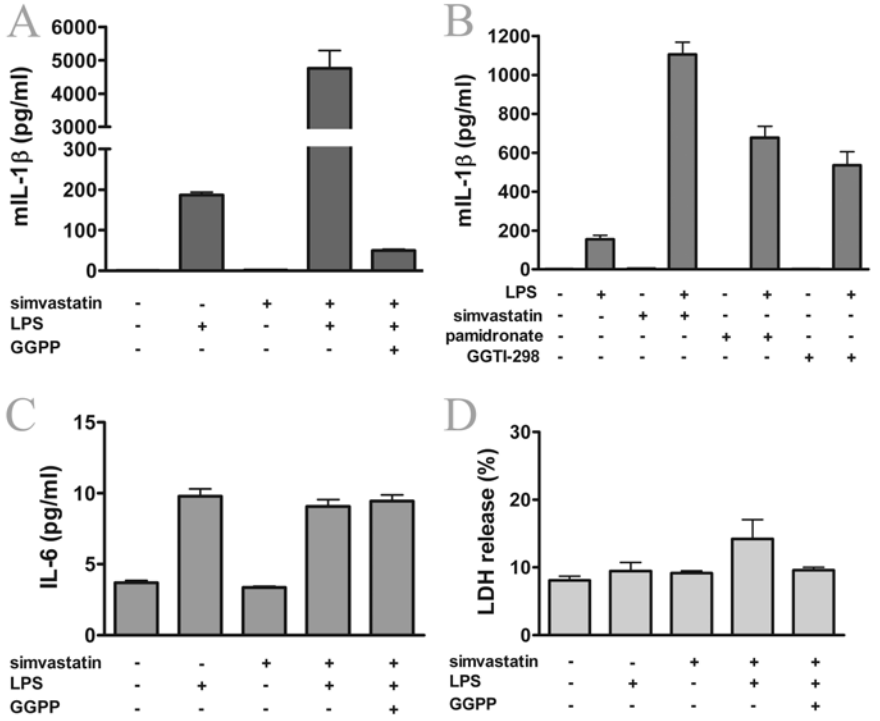

Figure 1: Simvastatin augments LPS-induced mIL-1β release in THP-1 cells.
THP-1 cells were cultured for 24 hours in the presence or absence of simvastatin (5 μM), pamidronate (100 μM) or GGTI-298 (50 μM) and stimulated for an additional 4 hours with 200 ng/ml LPS. Simvastatin-mediated effects were blocked by addition of 10 μM geranylgeranylpyrophosphate (GGPP) to the culture medium. Release of the pro-inflammatory cytokines IL-1β (A,B) and IL-6 (C) were determined by ELISA. Data are represented as means and SEM (n=4, n=3 and n=3 respectively). Cell viability was determined by an LDH assay (D). Data are represented as means and SEM of two independent experiments performed in duplicate.

alone did not induce detectable mIL-1β release. The addition of GGPP completely inhibited the simvastatin/LPS-induced mIL-1β release. In figure 1B inhibitors for two other enzymes of the isoprenoid biosynthesis pathway were included: geranylgeranyltransferase I (GGTase I) inhibitor GGTI-298 and farnesyl pyrophosphate synthase (FPP synthase) inhibitor pamidronate. GGTase I is the enzyme that attaches GGPP to the target protein and FPP synthase is the enzyme just prior to GGTase I in the pathway (9). When THP-1 cells were incubated with these inhibitors IL-1β release was enhanced, similar to simvastatin, indicating that increased IL-1β release was specifically caused by a shortage of geranylgeranylpyrophosphate. Simvastatin treatment affected only IL-1β release, since there was no effect on release of the proinflammatory cytokine IL-6 (Figure 1C). In addition, the enhanced IL-β release in the presence of simvastatin/LPS was not due to passive 'leakage' of IL-1β, since there were no major changes in lactate dehydrogenase release, indicating that the cell membrane remained intact during this treatment (Figure 1D).

Simvastatin-induced mIL-1β release is caspase-1 dependent

Since IL-1β is known to be a substrate of caspase-1 (19-22) it is likely that caspase-1 is actively involved in simvastatin-induced mIL-1β release. To test this hypothesis, THP-1 cells were cultured as before in the presence or absence of simvastatin. Prior to LPS stimulation either a general caspase inhibitor (z-VAD-FMK) or the specific caspase-1 inhibitor pralnacasan was added to the culture medium at the indicated concentrations. After 4 hours mIL-1β levels were determined. Both the general caspase inhibitor and the specific caspase-1 inhibitor inhibited mIL-1β release in a dose-dependent manner (Figure 2). Addition of 10 μM inhibitor reduced mIL-1β release to levels comparable to LPS stimulation alone. These data indicate that simvastatin-induced mIL-1β release requires caspase-1 activity.

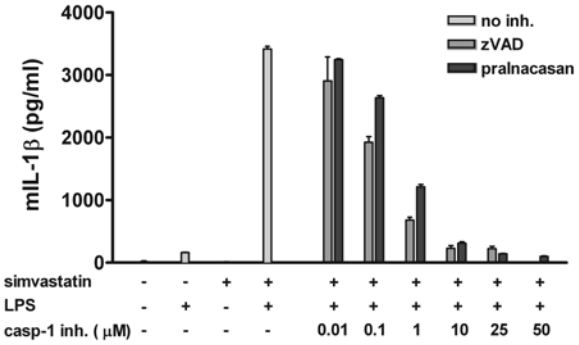

Figure 2: Simvastatin-induced mIL-1β release is caspase-1 dependent.
THP-1 cells were cultured for 24 hours in the presence or absence of simvastatin (5 μM) and stimulated for an additional 4 hours with 200 ng/ml LPS. Prior to LPS stimulation either a general caspase inhibitor (z-VAD-FMK) or the specific caspase-1 inhibitor pralnacasan was added to the culture medium at concentrations ranging from 0.01 to 50 μM. Data are represented as means and SEM (n=2).

Simvastatin synergizes with LPS to increase IL-1β mRNA levels

The increased caspase-1-mediated mIL-1β release could be due to increased availability of either proIL-1β or of caspase-1 or both. We therefore investigated whether simvastatin-induced mIL-1β release was due to an increase in transcription of either procaspase-1 or of proIL-1β or both. THP-1 cells were cultured as before with simvastatin and/or LPS and after stimulation mRNA levels for caspase-1 and proIL-1β were determined using quantitative real-time RT-PCR analysis. Statin/LPS treatment had no major effect on procaspase-1 mRNA levels (Figure 3A). For IL-1β mRNA levels, LPS alone induced a 120-fold increase compared to the untreated control cells (Figure 3B) (28). The combination of LPS and simvastatin had a modest synergistic effect (150-fold compared to untreated control cells) that was reversed by the isoprenylpyrophosphate GGPP. Thus, increased levels of IL-1β mRNA could, at least in part, be responsible for the increased mIL-1β release observed after statin/LPS treatment. However, the finding that LPS treatment alone does not lead to major IL-1β release despite inducing a dramatic increase in IL-1β mRNA levels, argues against transcriptional regulation as the underlying mechanism for our observations.

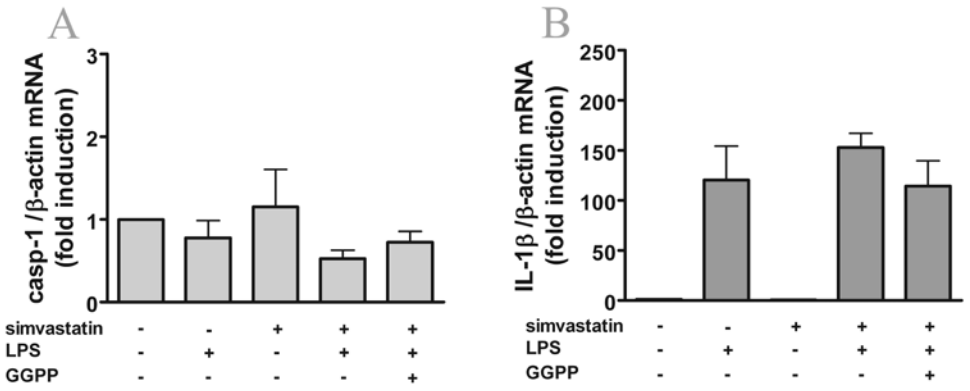

Figure 3: Simvastatin has no effect on caspase-1 transcription, but synergizes with LPS to increase IL-1β mRNA levels. THP-1 cells were cultured for 24 hours in the presence or absence of simvastatin (5 μM) and stimulated for an additional 4 hours with 200 ng/ml LPS. Simvastatin-mediated effects were blocked by addition of 10 μM geranylgeranylpyrophosphate (GGPP) to the culture medium. Data are represented as means and SEM (n=4).

Simvastatin treatment does not increase intracellular proIL-1β protein levels

We next wished to determine if the observed synergistic effect of simvastatin and LPS on proIL-1β transcription also leads to enhanced production of proIL-1β protein. THP-1 cells were cultured as before in presence or absence of LPS and/or simvastatin and after 4 hours of stimulation cells were harvested and cell extracts were prepared as previously described. ProIL-1β protein levels were determined by a specific proIL-1β ELISA, which does not recognize the mature processed form. In accordance with the dramatic rise in mRNA levels, LPS induced a large increase in proIL-1β protein levels (Figure 4A). This increase was a direct consequence of induced transcription as incubation with the transcription inhibitor Actinomycin-D (Act-D) reduced intracellular proIL-1β protein to an undetectable level. Furthermore, Act-D treatment reduced mIL-1β release to control levels in a dose-dependent manner (data not shown). The modest synergistic induction of transcription by additional simvastatin treatment (Figure 3B) did not result in increased levels of proIL-1β protein. The intracellular pool of proIL-1β actually decreased in the presence of both simvastatin and LPS as compared to LPS alone (Figure 4B), possibly due to increased processing and release of mIL-1β as demonstrated in Figure 1.

To determine whether this decrease was indeed due to a higher turnover of proIL-1β into the mature, secreted form, we incubated the cells, in addition to LPS and simvastatin, with the specific caspase-1 inhibitor pralnacasan at concentrations known to inhibit mIL-1β release efficiently (see Figure 2). Incubation with this inhibitor led to a restoration of intracellular proIL-1β protein levels in a dose-dependent manner (Figure 4B), suggesting that the observed decrease was indeed due to caspase-1 mediated proteolysis. However, maximum inhibition of caspase-1 activity did not result in an accumulation of proIL-1β protein, indicating that the synergistic induction of transcription by statin/LPS does not correlate with enhanced translation into proIL-1β protein. Taken together,

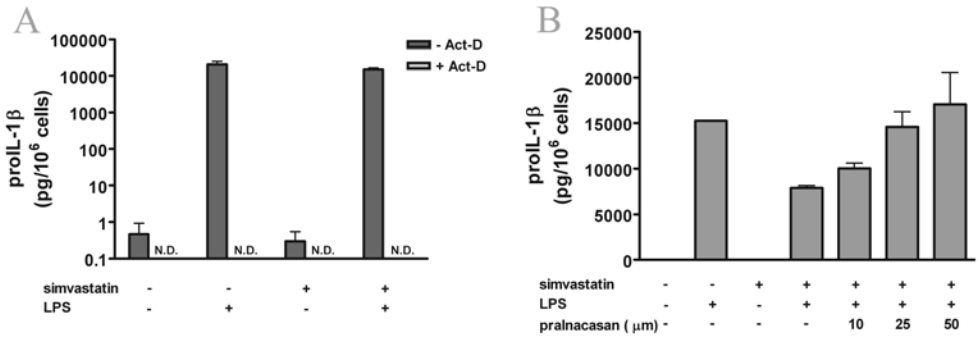

Figure 4: Simvastatin treatment does not increase intracellular proIL-1β protein levels.
THP-1 cells were cultured for 24 hours in the presence or absence of simvastatin (5 μM) and stimulated for an additional 4 hours with 200 ng/ml LPS. After stimulation proIL-1β protein levels were determined in cell extracts. A) 10 Minutes prior to LPS stimulation the transcription inhibitor Actinomycin-D was added to the culture medium at a concentration of 2 μg/ml. N.D. = non-detectable. Data are represented as means and SEM (n=3). B) Prior to LPS stimulation the specific caspase-1 inhibitor pralnacasan was added to the culture medium at the indicated concentrations. Data are represented as means and SEM (n=2).

these results further argue against an important role for enhanced transcription in increasing mIL-1β release.

Simvastatin augments LPS-induced mIL-1β release via activation of caspase-1

These data suggest that simvastatin-mediated mIL-1β release is regulated at a post-translational level. Therefore, we investigated whether simvastatin had an effect on activation of caspase-1 or on the export of IL-1β protein or both. If simvastatin specifically, and exclusively, induces export of IL-1β, without affecting caspase-1 activation, then inhibition of caspase-1 in the presence of simvastatin and LPS should lead to increased export of inactive proIL-1β. Studies by Thornberry *et al.* and our own unpublished observations support the concept that proIL-1β can be released independently of processing by caspase-1 (19). THP-1 cells cultured in the presence of the inhibitors z-VAD-FMK and pralnacasan did not show an increase in proIL-1β release (data not shown), suggesting that statin treatment does not exclusively target export of IL-1β. However, caspase-1 activation and subsequent export of the mIL-1β have been described to be very closely linked processes (29).

To further test the hypothesis that simvastatin induces mIL-1β release through activation of caspase-1 we examined autoprocessing of caspase-1. Since caspase-1 subunits have been shown to be readily secreted upon activation (30), autocleavage of caspase-1 was determined by measuring caspase-1 p20 in culture supernatant. Treatment with simvastatin resulted in a time-dependent (Figure 5A) and dose-dependent (Figure 5B) increase of caspase-1 p20 subunits in culture supernatants. Furthermore, LPS induced some caspase-1 p20 release on its own, but clearly synergized with simvastatin in the release of p20. GGPP completely blocked the statin-induced, but not the

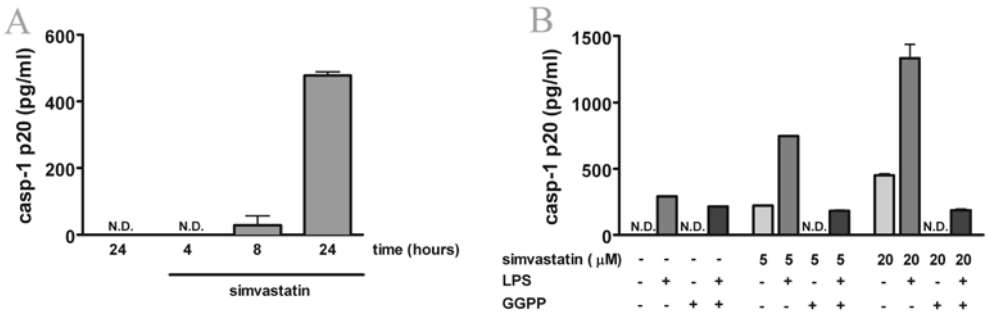

Figure 5: Simvastatin induces release of caspase-1 p20.
A) THP-1 cells were cultured in the presence or absence of simvastatin (20 μM) for the indicated time periods.
B) THP-1 cells were cultured in the presence or absence of GGPP (10 μM) and/or simvastatin (5 or 20 μM).
After 24 hours the cells were stimulated with 200 ng/ml LPS for 4 hours. N.D. = non-detectable.
Data are represented as means and SEM (n=2).

LPS-induced release of caspase-1 p20. Taken together, these data suggest that simvastatin alone or in combination with LPS can induce processing and activation of caspase-1.

Simvastatin induces IL-18 release

In addition to IL-1β, caspase-1 is also known to process IL-18. However, processing of IL-18 differs from IL-1β in that proIL-18 protein is already expressed without the need for LPS-induced transcription (31;32). To test the hypothesis that simvastatin primarily affects activation of caspase-1, we treated THP-1 cells as before in presence or absence of simvastatin for 24 hours and stimulated for an additional 4 hours with LPS, after which supernatant was assayed for the presence of IL-18. Similar to mIL-1β, LPS stimulation alone induced a moderate increase and coincubation of LPS and simvastatin a very strong increase in IL-18 levels in culture supernatant (Figure 6). However, in contrast to mIL-1β, treatment with simvastatin alone enhanced IL-18 release to the same extent as when cells were stimulated with LPS. Simvastatin-induced, but not LPS-induced IL-18 release could be completely reversed by coincubation with GGPP. These data support the conclusion that simvastatin acts by stimulating caspase-1 activity.

DISCUSSION

Mevalonate kinase deficiency is a metabolic disease, caused by a genetic defect in isoprenoid biosynthesis (7;8). However, clinically it is characterized by periodic fever accompanied by inflammation of joints, skin and serosa, suggesting an inflammatory disorder. Our previous studies have shown that inhibition of nonsterol isoprenoid biosynthesis can induce mIL-1β release by activated PBMC (18). This effect has also been reported by several other groups (15;33;34). In the current study we show that monocytic THP-1 cells pre-treated with simvastatin and subsequently stimulated with LPS also show augmented mIL-1β release (Figure 1). These results indicate that

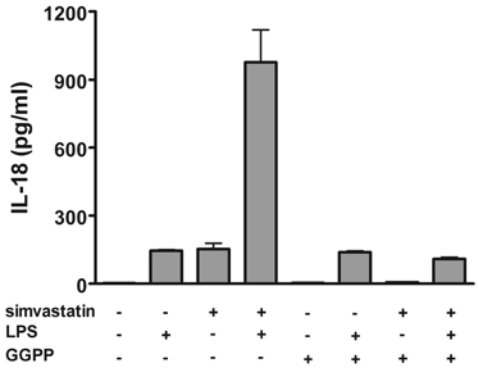

Figure 6: Simvastatin induces IL-18 release.
THP-1 cells were cultured for 24 hours in the presence or absence of simvastatin (5 μM) and/or GGPP (10 μM). Cells were subsequently stimulated for an additional 4 hours with 200 ng/ml LPS.
Data are represented as means and SEM (n=4).

IL-1β release in response to impairment of nonsterol isoprenoid biosynthesis is independent of T lymphocyte activation. The enhanced mIL-1β release could be abrogated by addition of geranylgeranylpyrophosphate (GGPP), a nonsterol intermediate of the pathway, which specifically restores one branch of nonsterol isoprenoid biosynthesis. In addition, specific inhibition of this branch by addition of GGTI-298 enhanced IL-1β release (Figure 1B). These data demonstrate that simvastatin-mediated IL-1β release is specifically due to a lack of geranylgeranylpyrophosphate. As expected, the simvastatin-enhanced mIL-1β release was mediated by caspase-1 (Figure 2).
Simvastatin-induced mIL-1β release could potentially be regulated at various levels: enhanced transcription or translation of either caspase-1 or of proIL-1β, increased proteolytic cleavage of proIL-1β or increased export of mIL-1β. We observed that simvastatin slightly enhanced LPS-induced transcription of IL-1β (Figure 3B). However, since LPS treatment alone led to a dramatic increase in mRNA levels without major effects on mIL-1β release, we thought it unlikely that the observed synergistic effect on transcription by simvastatin/LPS could account for the dramatic increase in mIL-1β release. On a translational level, we observed that after simvastatin/LPS treatment intracellular proIL-1β protein levels were not increased, but actually somewhat reduced compared to LPS treatment alone (Figure 4A). A similar decrease in cell-associated proIL-1β levels was observed by Sutterwala *et al.* after stimulation of LPS-primed macrophages (35). Blocking of caspase-1 activity resulted in a restoration of intracellular proIL-1β levels, but not to further accumulation (Figure 4A). All these data imply that the observed simvastatin-enhanced proIL-1β transcription does not result in increased levels of the proIL-1β protein and is therefore not responsible for increased release of mIL-1β. Therefore, simvastatin-induced mIL-1β release is most likely regulated at a post-translational level, either by regulating caspase-1 activity or by regulation of the export mechanism of mIL-1β. Since we did not find an increased release of proIL-1β in the presence of caspase-1 inhibitors, it is unlikely that simvastatin exclusively targets the

export mechanism of mIL-1β. Consequently, we continued by looking at the effect of simvastatin alone or in combination with LPS on autoprocessing of procaspase-1. Simvastatin treatment induced an increase in extracellular caspase-1 p20 in a time- and dose-dependent manner (Figure 5), suggesting that simvastatin can activate caspase-1. LPS again worked synergistically with simvastatin in inducing release of p20.

Taken together, our data suggest a "two-step" model where LPS stimulation is needed for efficient transcription of the IL-1β gene resulting in high levels of intracellular proIL-1β protein. In addition, inhibition of the isoprenoid biosynthesis pathway via simvastatin induces proteolytic activity of caspase-1. Active caspase-1 can then subsequently process proIL-1β protein into mIL-1β, which is secreted together with the caspase-1 subunits.

This model could also account for the finding that there is an increase in IL-18 release after simvastatin treatment. THP-1 cells are likely to express low levels of proIL-18 without the need for LPS priming, similar to monocytes and PBMC (31). Thus, statin-induced activation of caspase-1 would then indeed be sufficient for IL-18 processing and release.

Although little is known about the newly discovered caspase-1 substrate IL-33, it would be very interesting to investigate the effect of simvastatin on secretion of this new cytokine, which is thought to induce a T helper type 2 associated cytokine profile (23). An increased proteolytic activation of IL-33 could possibly help to explain symptoms like the skin rashes that are often observed in MKD patients.

Unfortunately, the exact mechanism behind simvastatin-induced caspase-1 activation remains unclear. However, the observations that addition of GGPP can completely counteract the effects induced by simvastatin and that both farnesyl pyrophosphate synthase inhibitor and geranylgeranyltransferase I inhibitor enhance IL-1β release clearly indicate that a shortage of one or more geranylgeranylated proteins is causing enhanced activation of caspase-1. This group of proteins includes the Rho subfamily of small GTPases and the gamma subunits of all heterotrimeric G proteins. Lack of geranylgeranylation of these proteins may cause mislocalization of the protein, since they lack the proper membrane localization anchor. In addition, it may cause an overactivity of the protein, since without the geranylgeranyl fatty acid tail, guanine nucleotide dissociation inhibitors (GDIs) can no longer bind and the protein can more easily change to its active GTP-bound state (36). Exactly which GTPases are involved and how they are connected to caspase-1 activation and inflammasome assembly remains to be investigated. Very interesting in this context are the findings by Basak *et al.* (37) who describe a role for Rac1/PAK1 signaling in activation of caspase-1. *H. pylori* LPS induced direct interaction between PAK1 and caspase-1, which was inhibited in cells transfected with dominant-negative Rac1. This would imply that in an environment where Rac1 is overactive, for example in statin-treated cells, there could be increased activation of caspase-1. Whether Rac1 and PAK1 are involved in statin-induced caspase-1 activation is currently being investigated.

Taken together, the current study provides evidence that simvastatin can activate caspase-1. These findings suggest that patients suffering from mevalonate kinase deficiency may have overactive

caspase-1, at least during fever episodes, causing cells of their immune system to more readily secrete mIL-1β in response to bacterial components like LPS. Although further investigations are necessary, our data suggest that therapies aimed at blocking the activity of caspase-1 or of its product mIL-1β may prove beneficial for MKD patients. Indeed, inhibition of IL-1 receptor function has been reported to be effective in several patients with MKD (38;39).

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Chapter 4

HMG-CoA reductase inhibition induces IL-1β release through Rac1/PI3K/PKB-dependent caspase-1 activation

Rac1 as a potential target for the treatment of MKD

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submitted

ABSTRACT

Mevalonate kinase deficiency (MKD) is an autoinflammatory disorder characterized by recurring fever episodes and results from disturbed isoprenoid biosynthesis. LPS-stimulated peripheral blood mononuclear cells (PBMC) from MKD patients secrete high levels of IL-1β, due to the presence of hyperactive caspase-1 and this has been proposed to be the primary cause of recurring inflammation. Here we show that inhibition of HMG-CoA reductase by simvastatin treatment, mimicking MKD, results in increased IL-1β secretion in a Rac1/PI3K-dependent manner. Simvastatin treatment was found to activate PKB/c-akt, a primary effector of PI3K, and ectopic expression of constitutively active PKB was sufficient to induce IL-1β release. The small GTPase Rac1 was also found to be activated by simvastatin and this was required for both PKB activation and IL-1β secretion. IL-1β release is dependent on processing by caspase-1, and simvastatin treatment resulted in increased caspase-1 activity in a Rac1/PI3K-dependent manner. These data suggest that in MKD, dysregulated isoprenoid biosynthesis activates Rac1/PI3K/PKB resulting in caspase-1 activation with increased IL-1β processing and release. Importantly, inhibition of Rac1 in PBMC isolated from MKD patients resulted in a dramatic reduction in IL-1β release. These data suggest that pharmacological inhibition of Rac1 could provide a novel therapeutic strategy for treatment of MKD.

INTRODUCTION

Mevalonate kinase deficiency (MKD; MIM#260920) is an autoinflammatory disorder characterized by spontaneous and recurrent fever attacks. The febrile attacks are accompanied by painful cervical lymphadenopathy and often by abdominal pain, vomiting and diarrhea. A variety of other symptoms including headache, skin rashes, mucosal ulcers, myalgia and arthralgia may also occur (1-3). MKD is inherited in an autosomal recessive manner and is caused by mutations in the gene *MVK*, which codes for the enzyme mevalonate kinase (4;5). Mevalonate kinase is a critical enzyme in the isoprenoid biosynthesis pathway, which produces cholesterol and a number of nonsterol isoprenoids (6;7). The latter includes geranylgeranylpyrophosphate, a fatty acid side-chain that is covalently attached to proteins of the Rho GTPase superfamily (8;9). The isoprenoid biosynthesis pathway can be perturbed by the use of statins, which strongly inhibit 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase, the enzyme that catalyzes the conversion of HMG-CoA into mevalonate (7). Recently, we and others have shown that impairment of the isoprenoid pathway by statins can augment IL-1β secretion by activated peripheral blood mononuclear cells (PBMC)(10-12). In PBMC from MKD patients it is the specific lack of geranylgeranylated proteins that results in increased IL-1β production (13). This IL-1β production may be largely responsible for the inflammation and fever observed in MKD patients, a notion that is strongly supported by the successful treatment of MKD patients with the IL-1 receptor antagonist anakinra (Kineret®) (14-16). However, it remains unclear how reduced levels of geranylgeranylated proteins can lead to increased IL-1β release.

To investigate the molecular mechanisms underlying increased IL-1β production in mevalonate kinase deficiency, we have utilised the monocytic cell line THP-1 in which the isoprenoid biosynthesis pathway is artificially impaired using simvastatin. In this model, simvastatin treatment results in increased IL-1β release by lipopolysaccharide-stimulated THP-1 cells. Previous research suggests a "two-step" model in which LPS stimulation is needed for efficient transcription of the IL-1β gene resulting in high levels of intracellular proIL-1β protein (17;18). The second step is provided by impairment of the isoprenoid biosynthesis pathway via simvastatin, which strongly enhances proteolytic activity of caspase-1 (19). Active caspase-1 can subsequently process the LPS-induced proIL-1β protein into mature IL-1β, which is then secreted together with the caspase-1 subunits.

To investigate the molecular pathways regulating simvastatin-induced activation of caspase-1, we have analysed the signal transduction routes involved in LPS/simvastatin-mediated IL-1β release. Taken together, our data demonstrate that simvastatin treatment results in caspase-1 activation through a Rac1/PI3K/PKB-dependent pathway and that inhibition of Rac1 can decrease the spontaneous IL-1β release by MKD PBMC. In addition we found that LPS augments IL-1β transcription via p38 MAPK-mediated regulation of the transcription factor NF-κB. These findings provide novel insights into the molecular mechanisms responsible for fever and inflammation in MKD patients and define Rac1 as a molecule for potential targeted therapy.

MATERIALS AND METHODS

Reagents

GSK-3 inhibitor SB216763, NF-κB inhibitor CAPE, simvastatin, mevalonate and lipopolysaccharide (LPS; E. coli 0127:B8) were purchased from Sigma (Sigma-Aldrich GmbH). Simvastatin, dissolved in ethanol, and mevalonate were prepared by hydrolyzation with 0.1M NaOH, followed by neutralization with 1 M HEPES pH 7.4 and 0.1M HCl. The solution was sterilized by filtration through a 0.2 μm filter and stored as aliquots at -20°C. Rac1 inhibitor, PKC_{ζ} pseudosubstrate, MEK1/2 inhibitor U0126 and NF-κB activation inhibitor II were obtained from Calbiochem. mTOR inhibitor rapamycin and PI3-kinase inhibitor LY294002 were from Biomol and p38 MAPK inhibitor SB203580 from Kordia Life Sciences (Leiden, The Netherlands). Primary antibodies to p-p38 MAPK, p-PKB, total PKB, total IκB, p-NF-kB, p-eIF4B, p-GSK-3 and p-S6 ribosomal protein were purchased from Cell Signaling. Anti-actin and anti-goat IgG were obtained from Santa Cruz.

Cell Culture

Cells were cultured in RPMI-1640 (Invitrogen Life Technologies) containing 2 mM glutamine, 100 U/ml penicillin-streptomycin and either 5% or 0.1% Fetal Calf Serum (FCS) at a density of $1x10^6$/ml. Incubations were performed at 37°C in a humidified atmosphere containing 5% CO_2, in the presence or absence of 10 μM simvastatin. After 24 hours of incubation, LPS at a final concentration of 200 ng/ml was added to the cells. When inhibitors were used, they were added either one hour prior to simvastatin or 1 hour prior to LPS treatment. After 4 hours of LPS stimulation supernatants were removed and frozen at -20°C. Cell pellets were snap frozen in liquid nitrogen and stored at -80°C for RT-PCR analysis. Cell lysates were prepared either by direct lysis in 4x sample buffer or by adding lysis buffer to the cell pellets (150 mM NaCl, 20 mM HEPES, 10 mM EDTA, 1% Triton X-100, protease inhibitors). Cell pellets were then lysed for 30 minutes on ice after which samples were centrifuged at 13,000 rpm for 15 minutes. Supernatants were transferred to fresh tubes. Protein levels were determined using the Biorad protein assay.

Cytokine Measurements

Cytokine detection was carried out using commercially available ELISA kits: Pelikine-compact™ human IL-1β ELISA kit (Sanquin, Amsterdam, The Netherlands), human proIL-1β/IL-1F2 and human caspase-1 Quantikine ELISA Kit (R&D Systems). The assays were performed according to the manufacturer's instructions and all samples were measured in duplicate.

Western Blot analysis

Cell lysates were resolved on SDS polyacrylamide gels and transferred onto PVDF membranes (Millipore). As a control for equal transfer of protein, the blots were stained reversibly with Ponceau S. Membranes were blocked in Tris buffered saline containing 0.3% Tween and 5% non-fat dry milk. Membranes were probed with a 1:1000 dilution of the primary antibody and with a 1:3000

dilution of horseradish peroxidase conjugated secondary antibody (DAKO). Chemiluminescence was detected with an ECL kit (Amersham Pharmacia Biotech).

Viral transduction of THP-1 cells

A bicistronic retroviral DNA construct was utilized, expressing PKBcaax and an Internal Ribosomal Entry Site (IRES) followed by the gene encoding eGFP (LZRS-eGFP). LZRS-eGFP retrovirus was produced by transient transfection as previously described (20). After transfection, viral supernatants were collected, filtered through a 0.2 μm filter and snap frozen in liquid nitrogen. THP-1 cells were transduced in 24-well dishes pre-coated overnight with 10 μg/cm^2 recombinant human fibronectin fragment CH-296 (RetroNectin; Takara, Japan) in PBS. Transduction was performed by addition of 0.5 ml viral supernatant to 0.5 ml culture medium containing 4 x 10^5 cells/ml. Approximately 16 hours after transduction, 0.7 ml medium was removed from the cells and 0.5 ml fresh virus supernatant was added together with 0.5 ml fresh medium. eGFP positive cells were sorted on a FACS Vantage and cultured in RPMI-1640 containing 50 μM β-mercaptoethanol, 40 U/ml penicillin, 40 μg/ml streptomycin and 10% fetal bovine serum.

GTPase pulldown assay

The activity of Rac1 was determined with the EZ-Detect™ Rac1 Activation Kit (Pierce). Briefly, a GST fusion protein containing the p21-binding domain of human Pak1 was used to specifically precipitate the active, GTP-bound, Rac1 (21). This binding domain was coupled to glutathion-S-transferase. Using glutathion beads, GTP-bound Rac1 was precipitated.

LDH assay

THP-1 cells were cultured for 24 hours in the presence or absence of simvastatin (20 μM) and stimulated for an additional 4 hours with 200 ng/ml LPS. After treatment the cells were thoroughly resuspended and 200 μl cell suspension was used for the CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega). The assays were performed according to the manufacturer's instructions and all samples were tested in duplicate.

Quantitative real-time RT-PCR analysis

The relative expression level of IL-1β to β-actin RNA was measured with the LightCycler® system (Roche). Total RNA was isolated from THP-1 cells with trizol extraction. First strand cDNA was prepared using the first strand cDNA synthesis kit for RT-PCR according to the manufacturer's instructions. The IL-1β and β-actin fragments were amplified using the following primer sets: IL-1β Fw 5'-AGA AGA ACC TAT CTT CTT CGA C-3', IL-1β Rev 5'-ACT CTC CAG CTG TAG AGT GG-3', β-actin Fw 5'-GGC ACC AGG GCG TGA TGG-3' and β-actin Rev 5'- GTC TCA AAC ATG ATC TGG GTC-3'. Data were analyzed using LightCycler Software, version 3.5 (Roche) and the program LinRegPCR, version 7.5 (22) for analysis of real-time PCR data. To adjust for variations in the amount of input RNA, the IL-1β mRNA levels were normalized against the mRNA levels of the housekeeping gene β-actin.

PBMC cell culture

Peripheral blood mononuclear cells were isolated by density gradient centrifugation using Ficoll-Paque (GE Healthcare). Isolated PBMC were cultured in RPMI-1640 (Invitrogen) containing 2 mM glutamine, 100 U/ml penicillin-streptomycin and 10% Fetal Calf Serum (FCS). Cells (at a density of $1x10^6$/ml) were seeded in 96-well microtiter plates in the presence or absence of 100 μM Rac1 inhibitor. Incubations were performed at 37°C in a humidified atmosphere containing 5% CO_2. After 24 hours of LPS stimulation supernatants were removed and stored at -20°C.

Statistics

All results are expressed as the mean and SEM. In Figure 1 and 6, statistical significance was calculated using a one sample t-test. According to the Bonferroni correction, significance level was set at 0.01. In Figure 5 the Wilcoxon signed-rank test was used with a significance level of 0.05. All other data were analysed using One-Way ANOVA, followed by Tukey's multiple comparison test, also with a significance level of 0.05.

RESULTS

PI3K and p38 MAPK regulate LPS/simvastatin-mediated IL-1β secretion

To induce IL-1β secretion THP-1 cells were cultured for 24 hours in the presence or absence of simvastatin and stimulated for an additional 4 hours with LPS. As described before for both PBMC and THP-1 cells (11;19), THP-1 cells stimulated with LPS in the presence of statin, to inhibit HMG-CoA reductase, displayed dramatically increased IL-1β release as compared to LPS stimulation alone (Figure 1A). Incubation with statin alone did not induce detectable IL-1β release. To investigate the potential role of intracellular signal transduction pathways in simvastatin-enhanced activation of IL-1β release, THP-1 cells were treated with a variety of kinase inhibitors prior to treatment with simvastatin. Inhibition of PI3K (LY294002) and p38 MAPK (SB203580) both inhibited IL-1β release when administered prior to simvastatin, whereas inhibition of MEK1/2 (U0126) had no significant effect (Figure 1B). To discern whether the observed inhibition was due to a simvastatin-mediated or an LPS-mediated effect, the inhibitors were also administered after statin treatment, prior to addition of LPS (Figure 1B). Inhibition of PI3K no longer had an effect on IL-1β release, whereas the effect of p38 MAPK inhibition was still evident. This suggests that while PI3K activity is crucial for statin-enhanced IL-1β release, p38 MAPK is required for LPS-mediated IL-1β release.

To further investigate the downstream mediators responsible for statin-mediated IL-1β release, several downstream targets of PI3K activation were also investigated. THP-1 cells were treated with inhibitors of mammalian target of rapamycin (mTOR; rapamycin), protein kinase C zeta (PKC_ζ pseudosubstrate) and glycogen synthase kinase 3 (GSK-3; SB216763) one hour prior to addition of simvastatin (Figure 1C and D). Since GSK-3 activity is inhibited rather than activated after PI3K activation, THP-1 cells were only stimulated with LPS to determine whether GSK-3 inhibition could mimic the effect of simvastatin treatment on IL-1β release. None of these inhibitors had an

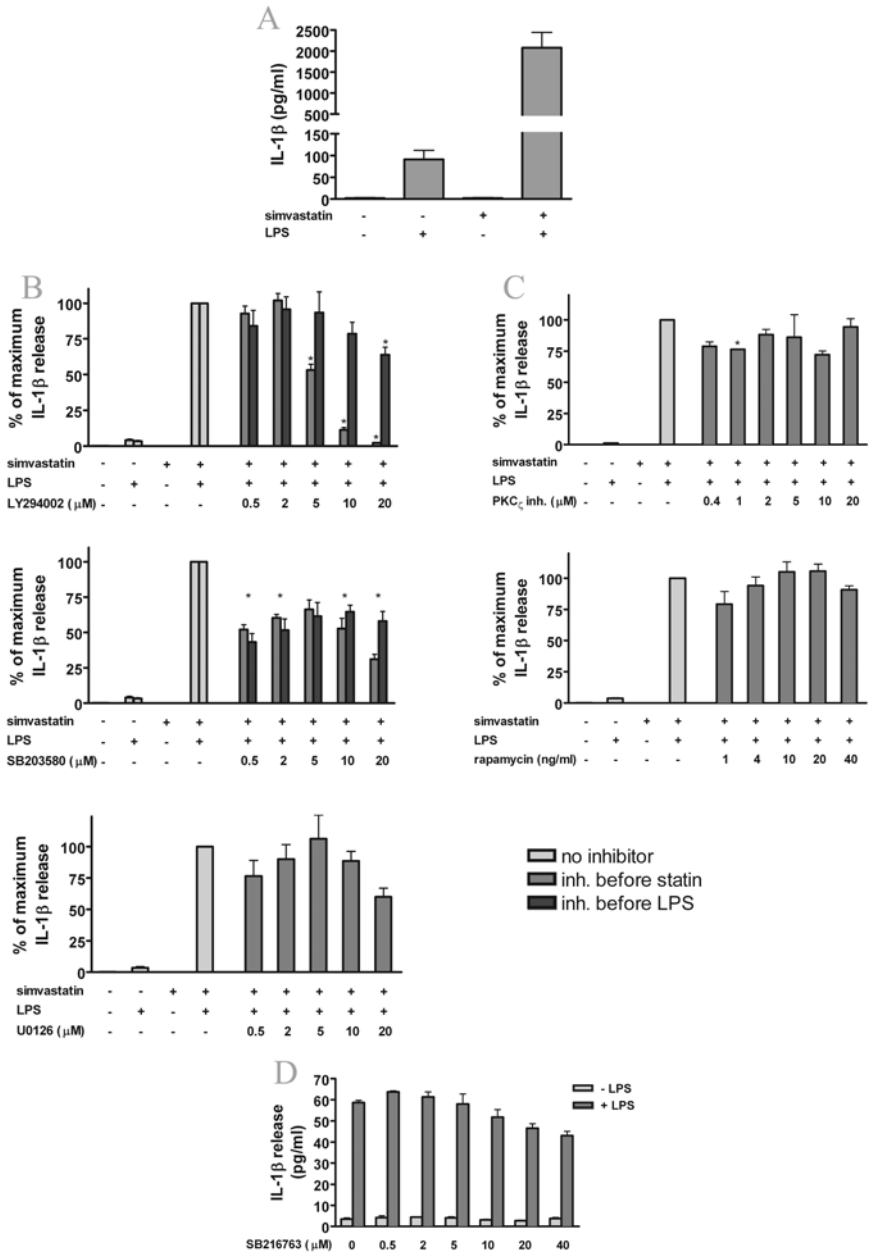

Figure 1: PI3K and p38 MAPK are involved in simvastatin-enhanced IL-1β secretion
THP-1 cells were cultured for 24 hours in the presence or absence of simvastatin (10 μM) and stimulated for an additional 4 hours with 200 ng/ml LPS. When inhibitors were used they were added either one hour prior to simvastatin or one hour prior to LPS treatment. Release of IL-1β was determined by ELISA; data are represented as means and SEM. A) Absolute levels of IL-1β secretion (n=8). B) Relative inhibition of IL-1β secretion by PI3K, p38 MAPK and MEK1/2 inhibitors (n=4, n=4 and n=3 respectively). C) Relative inhibition of IL-1β secretion by PKC_ζ and mTOR inhibitors (n=3 and n=4 respectively) D) Induction of LPS-induced IL-1β secretion by a GSK-3 inhibitor (n=2). * = $p<0.01$ compared to 100%

effect on IL-1β release, demonstrating that PI3K-mediated effects are independent of PKC_{ζ}, mTOR or GSK-3 activities.

Regulation of p38 MAPK and PKB activation by LPS and simvastatin

Inhibition of PI3K decreases simvastatin-mediated IL-1β release, whereas p38 MAPK inhibition perturbs IL-1β release in an LPS-dependent manner (Figure 1B). To determine whether indeed HMG-CoA reductase inhibition results in PI3K activation, we analysed the phosphorylation of protein kinase B (PKB/c-akt), which we have previously demonstrated is directly dependent on PI3K activity (23). Furthermore, we analysed whether LPS treatment could indeed enhance phosphorylation of p38 MAPK. THP-1 cells were serum-starved and subsequently stimulated with either LPS or with simvastatin. Cell extracts were analysed for phospho-p38 MAPK and phospho-PKB content (Figure 2). Incubation of THP-1 cells with simvastatin induced phosphorylation of PKB, whereas no change in the level of phospho-p38 MAPK was observed (Figure 2A). Simvastatin-induced PKB phosphorylation was inhibited by addition of the PI3K inhibitor LY294002 (Figure 2B). In contrast, stimulation of THP-1 cells with LPS induced a time-dependent increase in phospho-p38 MAPK, reaching a maximum at one hour, after which levels slowly declined. During this time, no change in phospho-PKB was observed (Figure 2C). These results further support the hypothesis that inhibition of HMG-CoA reductase by simvastatin treatment stimulates IL-1β secretion via a PI3K/PKB dependent pathway, whereas LPS enhances IL-1β secretion in a p38 MAPK dependent manner.

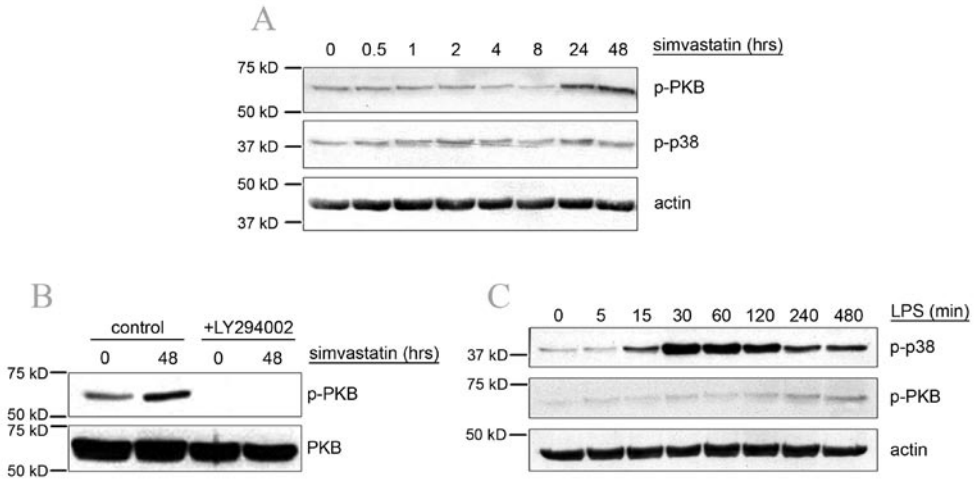

Figure 2: LPS induces p38 MAPK, simvastatin PKB phosphorylation
THP-1 cells were serum-starved overnight and subsequently cultured in presence of LPS (200 ng/ml) for up to 8 hours or simvastatin (10 μM) for up to 48 hours. Cells were lysed by adding hot sample buffer. Cell lysates were assayed for phospho-p38 MAPK and phospho-PKB content. Actin content served as control for equal protein loading. Shown are representative blots of at least four independent experiments.

p38 MAPK inhibition decreases proIL-1β transcription and translation

p38 MAPK inhibition was shown to reduce LPS-driven IL-1β secretion to approximately 60% of the maximum (Figure 1B). LPS has been described to increase the level of IL-1β transcription (18;24), although the exact molecular mechanism involved remains relatively undefined. To determine whether p38 MAPK is indeed required for LPS-induced IL-1β transcription in our model, IL-1β mRNA levels were determined. THP-1 cells were treated with LPS for up to 8 hours in presence or absence of p38 MAPK inhibitor SB203580. In parallel, levels of proIL-1β protein were determined using a specific proIL-1β ELISA assay (Figure 3). Inhibition of p38 MAPK resulted in a significant delay in IL-1β transcription as shown by the initial inhibition of total IL-1β mRNA levels followed by a recovery (Figure 3A). This pattern was similar for the proIL-1β protein levels (Figure 3B). These data indicate that p38 MAPK indeed plays a role in LPS-induced IL-1β transcription.

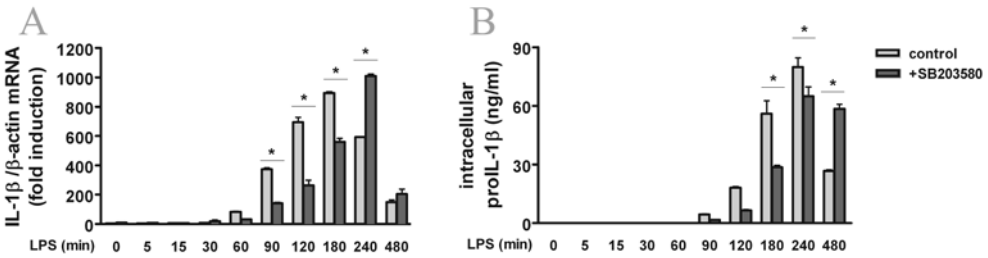

Figure 3: p38 MAPK inhibition decreases proIL-1β transcription and translation
THP-1 cells were cultured in presence of LPS (200 ng/ml) for up to 8 hours. p38 MAPK inhibitor was added one hour prior to LPS treatment. Cells were either snap frozen for determination of IL-1β mRNA content (A) or lysed by adding lysis buffer for determination of proIL-1β protein levels (B). Data are represented as means and SEM. Data shown are from one out of two experiments performed in duplicate. (* = $p<0.05$)

LPS-enhanced transcription of IL-1β is mediated via NF-κB

Several reports have shown that NF-κB can be regulated by p38 MAPK activity (25-27). NF-κB is present in the cytoplasm in an inactive complex containing IκB and two NF-κB subunits. When IκB is phosphorylated it becomes ubiquitinated and subsequently degraded (28). During or after phosphorylation of IκB, NF-κB is phosphorylated and translocates to the nucleus, where it enhances transcription of its target genes. We wanted to determine whether p38 MAPK might play a role in LPS-mediated IL-1β release through activation of NF-κB. Therefore, THP-1 cells were treated with LPS in presence or absence of p38 MAPK inhibitor SB203580. As a measure of NF-κB activation we analysed the levels of IκB (Figure 4A), where degradation of IκB is an indication of NF-κB activation. In the absence of SB203580, LPS induced IκB degradation and this degradation correlated with the increase in p38 MAPK phosphorylation observed earlier (Figure 2C), peaking after one hour of LPS stimulation. Inhibition of p38 MAPK prevented LPS-mediated IκB degradation, demonstrating that indeed p38 MAPK activation is required for

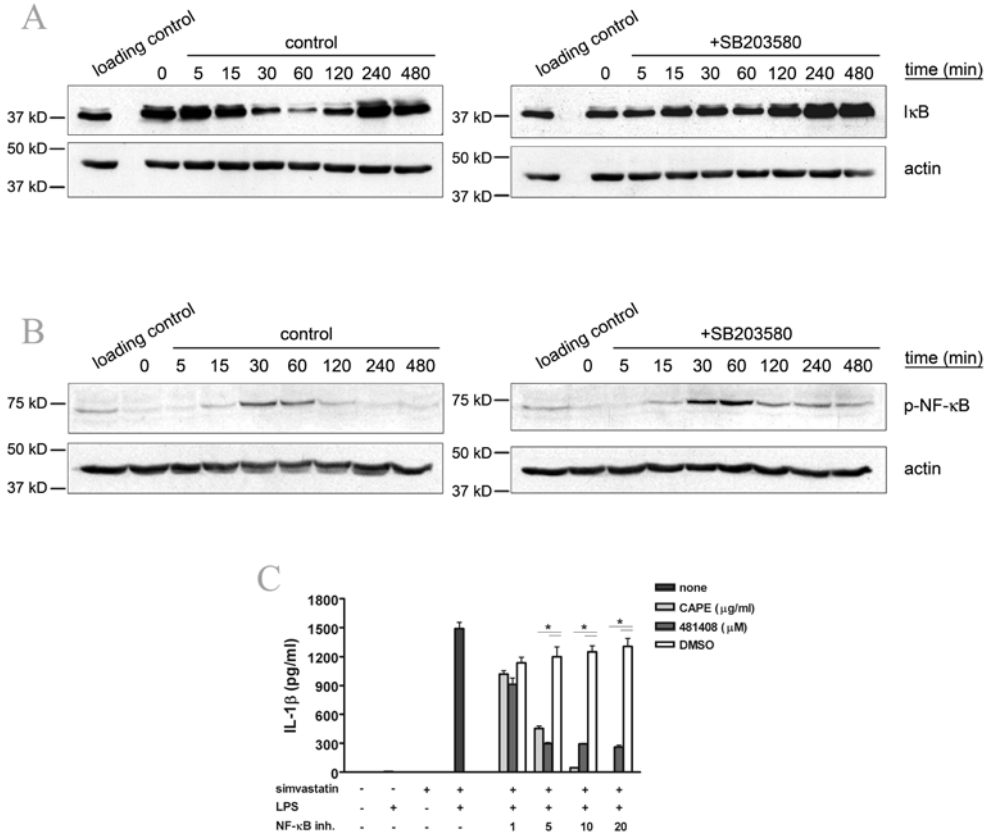

Figure 4: LPS-enhanced transcription of IL-1β is mediated via NF-κB
THP-1 cells were serum-starved overnight and subsequently cultured in presence of LPS (200 ng/ml) for up to 8 hours. p38 MAPK inhibitor was added one hour prior to LPS treatment. Cells were lysed by adding hot sample buffer (A) or by adding lysis buffer (B). Cell lysates were assayed for phospho-IκB (A) and phospho-NF-κB (B) content. Actin content served as control for equal protein loading. A loading control was prepared to compare between the separate blots. Shown are representative blots of at least 3 independent experiments. C) THP-1 cells were cultured for 24 hours in the presence or absence of simvastatin (10 μM) and stimulated for an additional 4 hours with 200 ng/ml LPS. The NF-κB inhibitors were added one hour prior to LPS treatment. Release of IL-1β was determined by ELISA; data are represented as means and SEM (n=3, * = $p<0.05$).

NF-κB activation. In addition, we wanted to establish whether p38 MAPK can also regulate NF-κB phosphorylation (Figure 4B). LPS induced phosphorylation of NF-κB, peaking after one hour of LPS stimulation. The phosphorylation of NF-κB was not inhibited by SB203580, indicating that phosphorylation of NF-κB is independent of p38 MAPK activity. To confirm that NF-κB activity is required for LPS-mediated IL-1β secretion, we incubated THP-1 cells with two disparate NF-κB inhibitors one hour prior to LPS stimulation and measured IL-1β secretion (Figure 4C). Both NF-κB inhibitors decreased IL-1β secretion in a dose-dependent manner, confirming that NF-κB activity is required for LPS-mediated IL-1β release.

Ectopic expression of constitutively active PKB enhances IL-1β secretion

In Figure 2A we show that PKB is phosphorylated in a time-dependent manner after treatment with simvastatin. To determine whether activation of PKB is sufficient to recapitulate the effects of statin treatment on IL-1β secretion, THP-1 cells were retrovirally transduced with a constitutively active form of PKB (PKBcaax) (29) or an eGFP control vector. Retroviral transduction resulted in approximately equal levels of PKBcaax compared to endogenous PKB (Figure 5A). Increased PKB activity was confirmed by analysis of phospho-PKB itself and of phosphorylated downstream PKB substrates, including eukaryotic initiation factor 4B (eIF4B), S6 ribosomal protein and GSK-3 (Figure 5A). Transduced THP-1 cells were incubated with simvastatin for 24 hours and subsequently stimulated with LPS, after which IL-1β secretion was measured. Ectopic expression of constitutively active PKB induced a significant, 2-fold, increase in IL-1β secretion by LPS-stimulated THP-1 cells. In the presence of both LPS and simvastatin, constitutively active PKB also induced a significant increase in IL-1β secretion (Figure 5B), which, considering the high levels of IL-1β being produced might approach a maximal level, being limited by the capacity of caspase-1 processing. These data suggest that PKB activity can indeed recapitulate simvastatin-enhanced activation of IL-1β secretion. The finding that active PKB significantly enhances IL-1β secretion is also in line with the hypothesis that PKB is required for simvastatin-mediated activation of caspase-1.

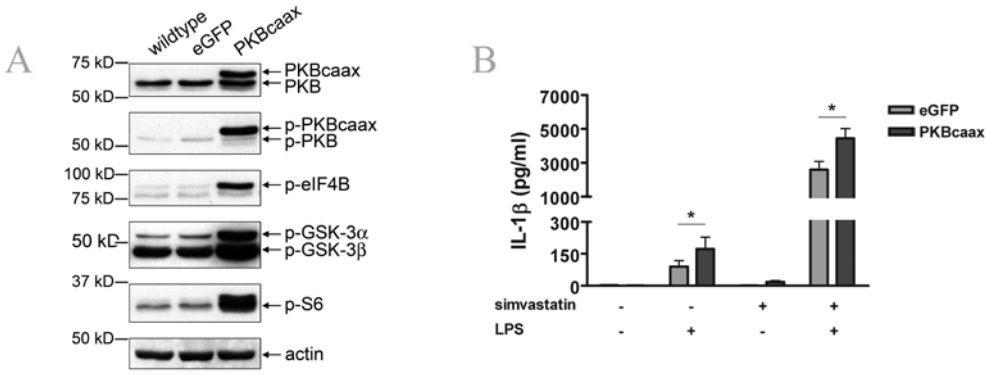

Figure 5: Expression of constitutively active PKB enhances IL-1β secretion
THP-1 cells were virally transduced with either a control vector (eGFP) or a constitutively active form of PKB (PKBcaax). A) Wildtype and transduced THP-1 cells were serum-starved overnight after which cell extracts were prepared by adding lysis buffer to the cell pellets. Cell lysates were assayed for phospho-PKB, phospho-eIF4B, phospho-GSK-3, phospho-S6, PKB and actin content. B) Transduced THP-1 cells were incubated with simvastatin for 24 hours, followed by a 4 hour stimulation with LPS, as described before. Release of IL-1β was determined by ELISA; data are represented as means and SEM (n=6; * = $p<0.05$)

The small GTPase Rac1 is activated by simvastatin treatment

In MKD patients, impairment of the isoprenoid biosynthesis pathway induces a shortage of geranylgeranylated proteins (13). The majority of geranylgeranylated proteins consists of small

GTPases, including proteins of the Rho family. Lack of geranylgeranylation of the small GTPases may cause mislocalization of the protein, since they lack the proper membrane localization anchor. Previously, it was assumed that geranylgeranylation is essential for the functioning of G proteins and lack of this modification would render them inactive (30). However, lack of geranylgeranylation may actually cause an overactivity of the protein, since without the geranylgeranyl fatty acid tail, guanine nucleotide dissociation inhibitors (GDIs) can no longer bind and the protein can more easily change to its active GTP-bound state (31;32). We wanted to investigate whether small GTPases are activated by statin treatment and may play a role in activating the PI3K/PKB signal transduction route. A likely candidate would be the small GTPase Rac1, since it was shown previously to be able to act upstream of PI3K (33;34). To determine if Rac1 activity is indeed directly affected by simvastatin treatment, we performed a pulldown assay to establish the amount of GTP-bound, and thus activated, Rac1 (Figure 6). THP-1 cells were cultured in presence or absence of simvastatin and cell extracts were prepared. The level of GTP-bound Rac1 was assessed by Western blot analysis

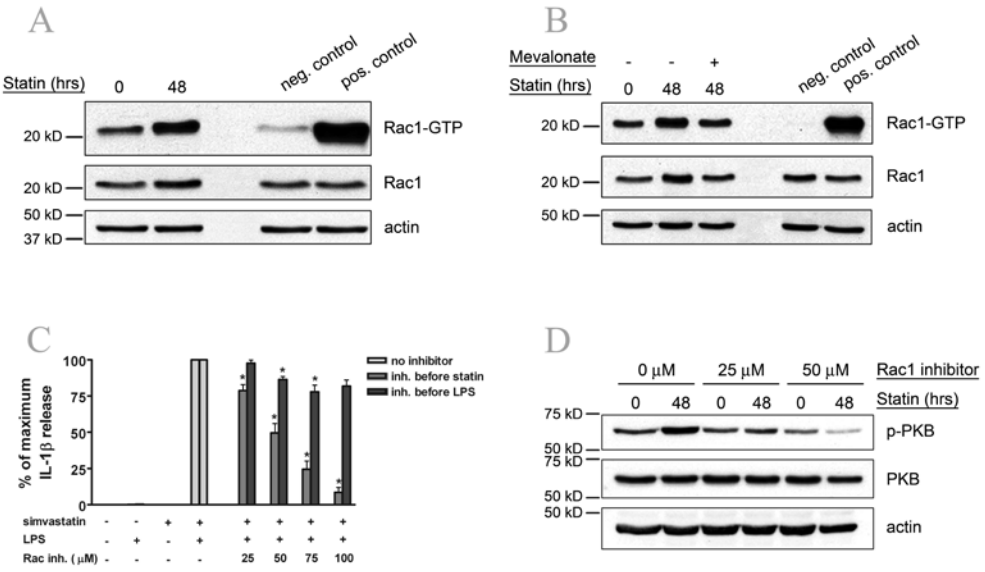

Figure 6: The small GTPase Rac1 is involved in simvastatin-enhanced IL-1β secretion
A) THP-1 cells were serum-starved overnight and subsequently cultured in presence or absence of simvastatin (10 μM) for 48 hours. GTPase pulldown fractions and cell lysates were assayed for Rac1 content. B) Mevalonate (1 mM) was added just prior to simvastatin. Actin content served as control for equal protein loading. Shown are representative blots of at least three independent experiments. C) THP-1 cells were cultured for 24 hours in the presence or absence of simvastatin (10 μM) and stimulated for an additional 4 hours with 200 ng/ml LPS. Rac1 inhibitor was added either one hour prior to simvastatin or one hour prior to LPS treatment. Release of IL-1β was determined by ELISA; data are represented as means and SEM (n=5; * = $p<0.05$). D) THP-1 cells were serum-starved overnight and subsequently cultured in presence or absence of simvastatin (10 μM) for 48 hours. Rac1 inhibitor was added one hour prior to simvastatin incubation. After the incubation, cells were lysed by adding lysis buffer and assayed for phospho-PKB and total PKB content. Actin content served as control for equal protein loading. Shown are representative blots of at least three independent experiments.

according to the manufacturer's protocol. THP-1 cells treated with simvastatin contained more GTP-bound, and thus active, Rac1 than untreated control cells (Figure 6A). To determine whether the increase in GTP-bound Rac1 was indeed due to an impairment of the isoprenoid biosynthesis pathway, mevalonate was added to simvastatin-treated cells. Mevalonate is the direct downstream product of HMG-CoA reductase and therefore relieves the inhibition imposed by simvastatin. Addition of mevalonate prevented the activation induced by simvastatin (Figure 6B). These results clearly indicate that impairment of the isoprenoid biosynthesis pathway using simvastatin activates the small GTPase Rac1.

The small GTPase Rac1 is required for simvastatin-mediated IL-1β secretion

To confirm the role of the small GTPase Rac1 in simvastatin-mediated IL-1β release, we incubated THP-1 cells with a specific inhibitor of Rac1 either prior to simvastatin or prior to LPS incubation (Figure 6C). Inhibition of Rac1 decreased IL-1β release in a dose-dependent manner when administered prior to simvastatin. This effect was abolished when the inhibitor was added after simvastatin, but before LPS treatment. These results demonstrate that Rac1 activity is required for statin-mediated enhancement of IL-1β release.

To determine whether Rac1 indeed functioned upstream of both PI3K and PKB, THP-1 cells were incubated with simvastatin in the presence or absence of Rac1 inhibitor and analysed for the phosphorylation status of PKB (Figure 6D). As observed previously, incubation with simvastatin induced an increase in phosphorylation of PKB, indicating activation. However, when cells were pretreated with Rac1 inhibitor, activation of PKB was inhibited in a dose-dependent manner, illustrating that Rac1 indeed functions upstream of PKB.

PI3K and Rac1 enhance IL-1β secretion through activation of caspase-1

Taken together, these data demonstrate that simvastatin treatment can directly activate Rac1 and that Rac1 is required for PKB activation and enhanced IL-1β release. To determine whether increased IL-1β release is mediated via activation of caspase-1, we measured caspase-1 p20 subunit levels in culture supernatants of the THP-1 cells as a marker for caspase-1 activation. Active caspase-1 has previously been shown to be localised in a lysosomal compartment within the cell together with proIL-1β. Upon activation, proIL-1β is cleaved in a caspase-1 dependent manner and both mature IL-1β and the active caspase-1 subunits are released extracellularly (35). In addition, we have previously shown that simvastatin treatment alone and in combination with LPS induces an increase in caspase-1 p20 subunit secretion in both a time and dose-dependent manner (19). THP-1 cells were cultured as before in the presence or absence of either the PI3K inhibitor LY294002 or the specific Rac1 inhibitor. Both caspase-1 p20 subunit and IL-1β levels in culture supernatant were determined (Figure 7). As demonstrated previously, secretion of IL-1β was only observed when both simvastatin and LPS were present (Figure 7A), whereas incubation with simvastatin alone already increased the release of caspase-1 p20 subunit, indicating activation. This release further increased when LPS was also present (Figure 7B). As observed previously (Figures 1B and 6C), inhibition of PI3K or Rac1 strongly decreased IL-1β secretion, coinciding

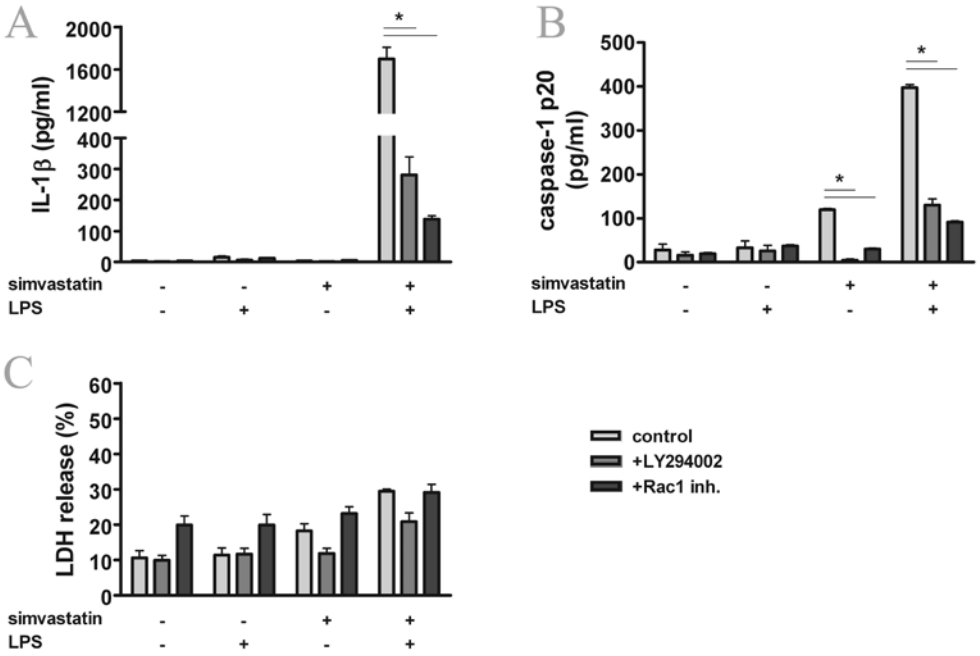

Figure 7: PI3K and Rac1 enhance IL-1β secretion through activation of caspase-1
THP-1 cells were cultured for 24 hours in the presence or absence of simvastatin (20 μM) and stimulated for an additional 4 hours with 200 ng/ml LPS. Rac1 inhibitor and PI3K inhibitor were added one hour prior to simvastatin treatment. Release of IL-1β (A) and caspase-1 p20 (B) were determined by ELISA; data are represented as means and SEM (n=6 for control and PI3K inhibitor-treated samples, n=3 for Rac1 inhibitor-treated samples, * = $p<0.05$). C) Cell viability was determined by an LDH assay (n=3; * = $p<0.05$).

with a decrease in caspase-1 p20 subunit, both in the simvastatin-only condition as well as the condition where both simvastatin and LPS are present (Figure 7B). The presence of caspase-1 p20 and IL-1β in the supernatant was not caused by a passive release due to cell death as confirmed by a lactate dehydrogenase assay for cell viability (Figure 7C). These results indicate that statin-mediated activation of caspase-1 is dependent on Rac1/PI3K activity.

Rac1 inhibition decreases spontaneous IL-1β secretion by MKD PBMC

To confirm our *in vitro* findings we wanted to establish whether administration of the Rac1 inhibitor would also be able to inhibit IL-1β secretion by PBMC from MKD patients. PBMC were isolated from two MKD patients and one healthy control. These cells were cultured for 24 hours in presence or absence of the specific Rac1 inhibitor (Figure 8). As expected, MKD PBMC secreted significantly higher amounts of IL-1β than the healthy control cells. Inhibition of Rac1 strongly reduced IL-1β release by MKD PBMC ($p=0.013$ and $p=0.096$ for patient 1 and 2 respectively), suggesting that Rac1 is a potential novel target for therapeutic intervention.

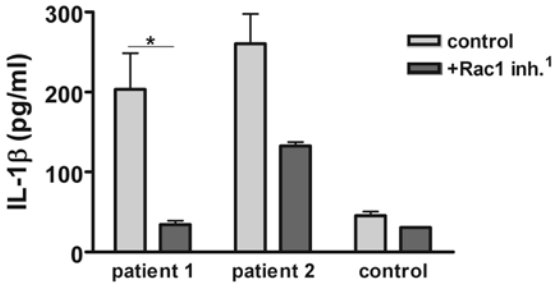

Figure 8: Rac1 inhibition decreases spontaneous IL-1β release by MKD PBMC
PBMC of two patients and one healthy control were cultured for 24 hours in the presence or absence of 100 μM Rac1 inhibitor. Release of IL-1β was determined by ELISA; data are represented as means and SEM (n=3, * = $p<0.05$). [1] n=2 for patient 2, n=1 for control

DISCUSSION

MKD is a metabolic disease, caused by a genetic defect in isoprenoid biosynthesis (4;5). Since the identification of mevalonate kinase as the affected enzyme, research has been directed towards elucidating the molecular mechanism underlying mevalonate kinase deficiency-induced inflammation. Previous studies have established that patient PBMC secrete very high levels of IL-1β upon endotoxin stimulation compared to healthy PBMC (12;13;36) and that this is thought to be caused by a lack of nonsterol isoprenoid products, specifically of geranylgeranylpyrophosphate (10-13). Indeed, we have recently shown that a shortage of geranylgeranylated proteins leads to activation of caspase-1, the enzyme responsible for processing proIL-1β into the active and secreted form (19). This suggests that an impaired isoprenoid biosynthesis pathway results in hyperactive caspase-1. Where healthy monocytic cells would respond to pattern associated molecular patterns (PAMPs), such as LPS, or damage-associated molecular patterns (DAMPs) by generating intracellular stores of proIL-1β protein (37;38), cells containing hyperactive caspase-1 would process the generated proIL-1β protein and release the pro-inflammatory, mature IL-1β in a dysregulated manner. This hypothesis provides a rationale for inflammation in mevalonate kinase deficiency: patients have hyperactive caspase-1 due to a shortage of geranylgeranylated proteins and will inappropriately secrete IL-1β whenever they come into contact with PAMPs or DAMPs resulting in inflammation and fever attacks.

In this study, we investigated the molecular mechanisms underlying simvastatin-mediated activation of caspase-1. Because of the extremely limited number of patients, we have previously described a model using THP-1 monocytic cells to study the disease mechanism in more detail (19). Using this model, we have now demonstrated that PI3K and Rac1 are required for simvastatin-mediated IL-1β secretion through regulation of caspase-1 activity (Figure 1B, 6C and 7B). One of the major intracellular mediators of PI3K activation is PKB (23;39). Indeed, we have shown

that simvastatin activates PKB (Figure 2) and that ectopic expression of constitutively active PKB significantly increases IL-1β secretion after LPS challenge in the presence as well as in the absence of HMG-CoA reductase activity (Figure 5). Furthermore, Rac1 activity was shown to increase upon simvastatin treatment and to act upstream of PKB (Figure 6). We therefore propose a molecular mechanism for IL-1β release, in which simvastatin activates Rac1, which subsequently activates a PI3K/PKB dependent pathway resulting in activation of caspase-1 (Figure 9).

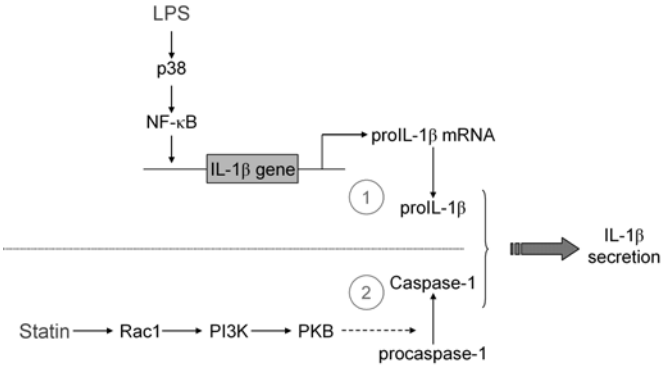

Figure 9: Schematic model of LPS/simvastatin-induced IL-1β secretion
LPS stimulation is needed for efficient transcription of the IL-1β gene resulting in high levels of intracellular proIL-1β protein. Inhibition of the isoprenoid biosynthesis pathway via simvastatin induces caspase-1 activation via a Rac1/PI3K/PKB-dependent signal transduction pathway. Active caspase-1 can subsequently process proIL-1β protein into mature IL-1β, which is secreted.

In addition to the simvastatin-mediated activation of caspase-1, we have demonstrated that p38 MAPK and NF-κB are required for LPS-induced IL-1β transcription (Figure 1B, 4A,C). However, inhibition of IL-1β release by the p38 MAPK inhibitor was not dose-dependent and p38 MAPK inhibition also resulted in a delay, rather then complete inhibition, of IL-1β transcription (Figure 3), indicating that additional factors might play a role. These data imply that IL-1β transcription is primarily regulated by p38 MAPK, but other factors possibly contribute, or substitute for p38 MAPK when it is inhibited. Exactly which other factors might be involved remains to be investigated.

Taken together, our data demonstrate that artificial inhibition of the isoprenoid biosynthesis pathway leads to activation of caspase-1 (Figure 9). This occurs via activation of the small GTPase Rac1. Exactly how simvastatin treatment induces activation of Rac1 is not entirely clear. However, as described above, impaired binding of guanine nucleotide dissociation inhibitors (GDIs) may result in more rapid switching between GDP- and GTP-binding by Rac1, leading to a net increase in activity. The importance of Rac1 in the processing and release of IL-1β is confirmed by the previous finding that Yersinia bacteria can prevent host

IL-1β release by targeting Rac1 (40). The Yersinia protein YopE is a GTPase-activating protein (GAP), which accelerates GTP hydrolysis, thereby switching off the small GTPases Rho, Rac and cdc42. Macrophages infected with a YopE knockout Yersinia *enterolitica* strain, so with increased GTPase activity compared to wildtype-infected cells, released significantly higher levels of IL-1β. Furthermore, overexpression of YopE, but not of a catalytically inactive mutant of YopE, inhibited autoprocessing of caspase-1 as well as processing of proIL-1β. These data correlate with our own findings that increased Rac1 activity enhances caspase-1 processing of proIL-1β.

Our data show that Rac1, PI3K and PKB mediate simvastatin-enhanced IL-1β secretion. PI3K has been described to serve as an activator as well as an effector of Rac1 (41-43). Lipid products derived from activated PI3K can activate guanine nucleotide exchange factors (GEFs) for Rac1, thereby activating Rac1, or active Rac1 can regulate the activation and localization of PI3K by binding to its p85 subunit. We demonstrated that Rac1 functions upstream of PKB (Figure 6D) and that PI3K is required for phosphorylation of PKB (Figure 2B), suggesting the sequential activation of Rac1, PI3K and PKB.

A major question that remains is how PKB activity can regulate caspase-1 autoprocessing. Caspase-1 is synthesized as an inactive zymogen of ~45 kDa that, via induced proximity to another caspase-1 zymogen, can undergo autocleavage, creating 10 kDa and 20 kDa subunits. Two p10 and two p20 subunits form the fully functional heterodimeric enzyme. Caspase-1 auto-activates itself in a complex of proteins termed the inflammasome (44). Caspase-1 contains an N-terminal caspase recruitment domain (CARD), which forms a homotypic interaction (CARD-CARD interaction) with apoptosis-associated speck-like protein containing a CARD (ASC). This adaptor protein interacts with other members of the inflammasome (45) via similar homotypic interactions, enabling oligomerization and autocleavage of caspase-1. Active caspase-1 is thought to colocalize with proIL-1β in lysosomal compartments, in which processing of proIL-1β into mature IL-1β takes place (35). Mature IL-1β and the active caspase-1 subunits are subsequently released into the extracellular environment. Because caspase-1 is activated in a multiprotein complex, PKB-enhanced activation of caspase-1 does not necessarily have to involve direct interactions with caspase-1, interactions with other members of the inflammasome could also be responsible for enhanced activation.

A possible connection between PKB and caspase-1 activation is the p21-activated kinase 1 (Pak1). This protein was shown to be activated by Rac1 (46) and can directly bind to and phosphorylate caspase-1 on serine 376 (47). Although the function of caspase-1 phosphorylation remains unclear, phosphorylation of this residue appears essential to caspase-1 activity, since caspase-1 activation was abrogated in LPS-stimulated THP-1 cells transfected with an S376A mutant. In addition, PI3K has been demonstrated to associate with the N-terminal regulatory domain of Pak1, leading to Pak1 activation (48) and PKB was found to phosphorylate Pak1 on serine 21, which enables Pak1 to bind to the adaptor protein Nck (49).

Possibly, PKB-mediated activation of caspase-1 involves cytoskeletal rearrangements. PKB has been described to bind directly to actin via its N-terminal PH domain (50). In addition,

Rac1-mediated inhibition of caspase-1 by the Yersinia protein YopE was found to be mediated by LIM kinase-1 (LIMK1) which targets the actin cytoskeleton (40). In fact, overexpression of constitutive-active LIMK1 promoted activation of caspase-1, whereas dominant-negative LIMK1 could abrogate Rac1-induced activation of caspase-1, suggesting that Rac1-mediated phosphorylation of the actin cytoskeleton is necessary for caspase-1 activation. A potential role for cytoskeletal rearrangements in activation of caspase-1 is also suggested by the molecular mechanism underlying Familial Mediterranean Fever and PAPA syndrome. These two autoinflammatory syndromes are strongly related to mevalonate kinase deficiency, with very similar symptoms and also thought to be caused by inappropriate release of IL-1β (51-53). PAPA syndrome is caused by mutations in the cytoskeleton-organizing protein PSTPIP1. PSTPIP1 can activate the actin binding protein pyrin, which is the protein affected in FMF. Mutations of both PSTPIP1 and pyrin were shown to facilitate the assembly of multiprotein complexes capable of activating caspase-1. However, a defined role for cytoskeletal rearrangements in activation of caspase-1 requires further research.

The data described in this study demonstrate that simvastatin-mediated IL-1β release is mediated via a Rac1/PI3K/PKB-dependent signal transduction pathway. Whether this signal transduction pathway is also involved in other (auto)inflammatory disorders remains to be investigated. However, the fact that spontaneous activation of caspase-1 seems to be a common mechanism of inflammation suggests that similar pathways may be involved. Currently, treatment of MKD consists mostly of nonsteroidal anti-inflammatory drugs (NSAIDS) and more recently cytokine-targeted therapies have been tried (anti-TNF-α and IL-1 receptor antagonist) with varying success. These therapies are, however, very costly and do not benefit all patients. The data presented in this study identify Rac1 as a possible new target for MKD therapy (Figure 8). Interestingly, Rac inhibitors are currently under investigation for the use in cancer therapy. Especially the development of a novel compound that targets a Rac specific GEF (NSC23766) appears promising as a Rac specific small molecule inhibitor. This compound was shown to suppress proliferation and the invasive phenotype of prostate tumor cells (54) and could effectively block Rac GTPase activity after intraperitoneal administration to laboratory mice (55). Other approaches for targeted inhibition of Rac1 include inhibition of Rac effector binding and stabilization of Rac-RhoGDI complexes (56;57).

In conclusion, our data demonstrate that inhibition of Rac1 can strongly reduce IL-1β release by both THP-1 cells and MKD PBMC through inhibition of caspase-1 activation. These data suggest that Rac1 inhibition may provide a novel therapeutic strategy for treatment of MKD.

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Chapter 5

Effects of IL-1 receptor antagonism in MKD patients

Loes M. Kuijk and Joost Frenkel

ABSTRACT

Mevalonate kinase deficiency (MKD) is an autosomal recessive auto-inflammatory disorder characterized by recurring episodes of fever. Inflammation is thought to be caused by an imbalance in pro-inflammatory cytokine production. Interleukin-1β (IL-1β) in particular is considered to play a major role in the initiation of inflammation in MKD. The importance of IL-1β in MKD has been confirmed by the successful treatment of several patients with the IL-1 receptor antagonist (IL-1ra) anakinra. However, the mechanism underlying the therapeutic action of anakinra remains unclear.

To study the role of IL-1β in MKD and to validate its significance in initiating inflammatory episodes in MKD, we studied the production of a broad panel of cytokines by LPS-stimulated peripheral blood mononuclear cells (PBMC) from MKD patients and healthy control subjects in the presence or absence of recombinant IL-1ra. In addition, we monitored the serum levels of these cytokines in the circulation of MKD patients that were being treated with anakinra.

Addition of IL-1ra to culture media of LPS-stimulated PBMC decreased the production of IL-1α, IL-1β, IL-18 and TNF-α by both patient and healthy control PBMC. Blocking IL-18 and TNF-α signalling using neutralizing antibodies had no effect, indicating that IL-1β indeed plays a primary role in LPS-induced inflammation. Treatment of MKD patients with anakinra resulted in lower levels of nearly all cytokines measured. In two out of three patients this correlated with clinical improvement.

In conclusion, blocking of IL-1β signalling indeed reduces cytokine production both *ex vivo* and *in vivo*, though this does not necessarily lead to clinical improvement.

INTRODUCTION

Mevalonate kinase deficiency (MKD) is a rare autosomal recessive disorder characterized by periodic occurrence of febrile attacks associated with headache, arthritis, nausea, abdominal pain, diarrhea and skin rash (1;2). Originally, two distinct syndromes were defined, classic mevalonic aciduria (MA) (3) and hyper-IgD and periodic fever syndrome (HIDS)(4). However, after the discovery that both disorders are usually caused by a strongly reduced activity of the enzyme mevalonate kinase (5;6), these syndromes are now recognized as the severe and mild presentation of MKD. Patients with the HIDS phenotype typically show recurrent episodes of fever with associated inflammatory symptoms (1), whereas patients with the MA phenotype, in addition to fever episodes and an inflammatory state, may present with developmental delay, dysmorphic features, ataxia, cerebellar atrophy and psychomotor retardation and they may die in early childhood (2). Cells from HIDS patients have a residual mevalonate kinase activity of 1 to 8% (6-8), whereas in cells of MA patients mevalonate kinase activity is often too low to detect (2;9). This difference in residual enzyme activity is also reflected in the occurrence of high levels of mevalonic acid in plasma and urine of patients with the MA phenotype and low to moderate levels of mevalonic acid in patients with the HIDS phenotype. As described in a report by Drenth *et al.*, blood analyses in patients during the episodes of fever indicated an acute inflammatory state, with high levels of C-reactive protein and a marked rise in serum levels of pro-inflammatory cytokines, such as interleukin-6 (IL-6) and interferon gamma (IFN-γ) (10;11). Also, between attacks, isolated PBMC from MKD patients secreted increased amounts of proinflammatory cytokines, such as IL-1β (12;13). Although the exact pathogenesis of MKD remains unclear, fever and an acute-phase response are generally considered to be mediated by cytokines such as IL-1β, IL-6 and tumour necrosis factor α (TNF-α). It can therefore be hypothesized that symptoms in MKD result from a disturbed cytokine secretion which may be responsible for some of its clinical manifestations. Of these pro-inflammatory cytokines IL-1β is the most potent pyrogen and appears to play an important role in the pathogenesis of several autoinflammatory diseases, including MKD (14). The importance of IL-1β is emphasized by the reported beneficial effect of a recombinant form of IL-1 receptor antagonist (IL-1ra), anakinra, in the treatment of MKD (15-17).
To verify the significance of IL-1β secretion in MKD patients, we studied the effect of blocking IL-1 receptor signalling using recombinant IL-1ra on the secretion of a broad panel of cytokines *ex vivo*, in cultured PBMC fractions. In addition, we studied cytokine profiles in sera and plasma samples from three MKD patients that were treated with anakinra.

PATIENTS AND METHODS

Anakinra-treated patients
Patient 1 is a 17-year-old boy who is compound heterozygote for *MVK* V377I and I268T, leading to a 1.8% residual mevalonate kinase activity. He has been affected by bouts of fever since the age of six months. Attacks were triggered by vaccinations and viral infections or

arose spontaneously. The episodes recurred at irregular intervals, averaging once every 2-4 weeks. Fever usually lasted between 3 and 5 days and was accompanied by chills, rash, painful lymphadenopathy, headache, abdominal pain, vomiting and diarrhea. When fever subsided, he developed a painful monoarthritis, affecting elbow, knee or ankle, lasting another 4-8 days. Malaise and anorexia persisted throughout the febrile and the arthritic phase of these attacks. During attacks his blood showed granulocytosis, thrombocytosis and strongly elevated C-reactive protein (CRP) levels and erythrocyte sedimentation rate (ESR). Treatment with steroids, indomethacin and the leukotriene receptor antagonist montelukast had been ineffective. Initially he recovered between attacks, but as these became more frequent and protracted it led to a 40% absence from school.
Patient 2 is an 8-year-old boy who is compound heterozygous for the *MVK* A334T and a 421insG frameshift mutations leading to less than 1% residual mevalonate kinase activity. He has suffered from inflammatory episodes since the age of 9 months. In addition to high fevers (up to 41°C), he suffered from skin rashes, arthralgia, painful lymphadenopathy, hepatosplenomegaly, headaches and abdominal pains. His disease course was remarkable for the development of retinitis pigmentosa (18), proximal myopathy, dysarthria and a mild developmental delay. By the age of 5 years his attacks had become very frequent (every two weeks) and so severe that he had to be admitted to hospital on several occasions to rule out bacterial sepsis. Treatment with nonsteroidal anti-inflammatory drugs had been ineffective.
Patient 3 is a 6-year-old girl who is compound heterozygote for *MVK* P36L and I268T leading to approximately 1% residual mevalonate kinase activity. She was born small for gestational age after a 36 week pregnancy complicated by polyhydramnios. From the second week of life she suffered from inflammatory episodes. Although some of these were related to documented bacterial and viral infections, most were not. Childhood vaccinations were consistently followed by such attacks. Fever lasted between 3 and 7 days, recurring every 2-4 weeks. Fever was accompanied by chills, irritability, vomiting, abdominal discomfort, diarrhea and stomatitis. During attacks, she had hepatosplenomegaly and lymphadenopathy. During each episode she developed granulocytosis and strongly elevated acute phase reactants. From the age of ten months she developed a painful non-erosive polyarthritis, which persisted between attacks. Her disease affected growth and both speech and motor development. Treatment with montelukast, acetaminophen and indomethacin had been ineffective.

Blood sampling
After approval by the ethical review board and written informed consent by the patient's parents, blood was drawn by venipuncture in either sterile pyrogen-free serum tubes or heparinised (plasma) tubes (Vacuette, Greiner Bio-one). Healthy volunteers served as controls. Serum tubes were centrifuged at 3000 rpm, plasma tubes at 1200 rpm, after which sera and plasma samples were immediately stored at -80°C until further analysis. Cerebrospinal fluid and bronchoalveolar lavage fluid were also immediately stored at -80°C. PBMC isolated from heparinised blood were used for *ex vivo* experiments (see below).

Cell Culture
Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation using Ficoll-Paque (GE Healthcare). Isolated PBMC were cultured in RPMI-1640 (Invitrogen) containing 2 mM glutamine, 100 U/ml penicillin-streptomycin and 5% Fetal Calf Serum (FCS). Cells (at a density of $2x10^6$/ml) were seeded in 48-well microtiter plates in the presence or absence of 500 ng/ml LPS. Incubations were performed at 37°C in a humidified atmosphere containing 5% CO_2 in air. After 24 hours supernatants were removed and stored at -20°C.

Multiplex bead analysis
Cytokine measurements (IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70, IL-13, IL-18, IFN-γ, TNF-α) were performed using multiplex bead analysis (Bio-Plex System, Biorad) according to the manufacturer's instructions. Control values for each cytokine were determined previously by analysing plasma levels in 20 healthy subjects (19).

Reagents
Lipopolysaccharide (LPS; E. coli 0127:B8) was purchased from Sigma-Aldrich, monoclonal anti-human IL-18 (125-2H) antibody was from MBL, monoclonal anti-human TNF-α antibody and recombinant human IL-1 receptor antagonist were from R&D Systems. Anakinra (Kineret®) was from Amgen Inc.

RESULTS

IL-1 receptor antagonist reduces TNF-α and IL-18 release by MKD PBMC
Freshly isolated peripheral blood mononuclear cells (PBMC) from 3 MKD patients and 2 healthy control subjects were incubated in presence or absence of 250 ng/ml IL-1ra and stimulated for 24 hours with 500 ng/ml LPS. Unstimulated PBMC from patients as well as from healthy donors were found to secrete a number of pro-inflammatory cytokines, including IL-1β, IL-6 and IL-8 and also low levels of IL-1α and TNF-α (Figure 1A). The secretion of these cytokines is most likely due to the isolation procedure which can be expected to cause minor activation of the cells. The only striking difference between patient and control cells was the lower level of IL-10 measured in supernatants of patient cells. When both patient and control PBMC were stimulated with LPS, increased levels of IL-1α, IL-1β, IL-6, IL-10 and TNF-α were observed. Cells from two out of three patients and both healthy controls also produced some IL-18 (figure 1B). T cells did not appear to be activated by LPS, since IL-2, IL-13 and IFN-γ, which are mainly produced by activated T cells, were not detected. Again, no major differences were observed between patient and control cells except for a modestly reduced level of IL-10 in patient cell supernatants. The presence of IL-1ra resulted in a reduction in TNF-α production by LPS-stimulated patient cells, but not by control cells (Figure 1B). Also, in patient cells producing IL-18 this production was completely blocked by the presence of IL-1ra, which was also not observed in the control cells. A slight inhibition of IL-1α and IL-β production could be observed in the presence of IL-1ra in both control and patients

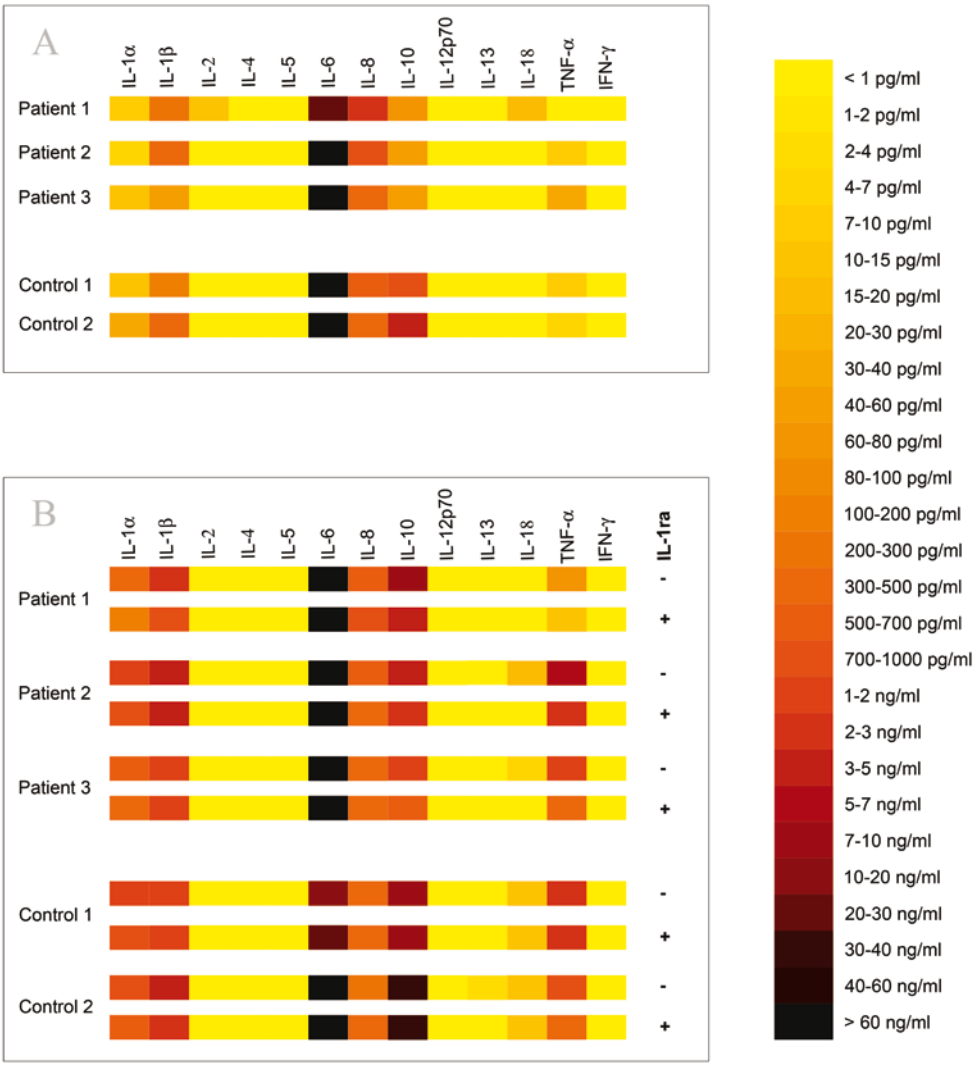

Figure 1: IL-1 receptor antagonist reduces TNF-α and IL-18 release by MKD PBMC
Isolated PBMC from 3 MKD patients and 2 healthy control subjects were cultured in the absence of stimuli (A) or stimulated with 500 ng/ml LPS (B) in the absence or presence of 250 ng/ml IL-1ra. After 24 hours culture supernatants were analysed for the presence of the indicated cytokines, using multiplex bead analysis.

cells (Table I). Unfortunately, the levels of IL-6 that were produced were too high to be accurately measured by the bioplex assay. From these data we conclude that both patient and control cells produce similar cytokines in response to LPS and to a comparable level, except for IL-10. IL-1ra treatment resulted in a small reduction in IL-1α and IL-β levels in both MKD and healthy control PBMC, while TNF-α and IL-18 were reduced in the patient cells, but not in the cells from healthy controls. This suggests that in MKD LPS-induced IL-18 and TNF-α secretion is, at least

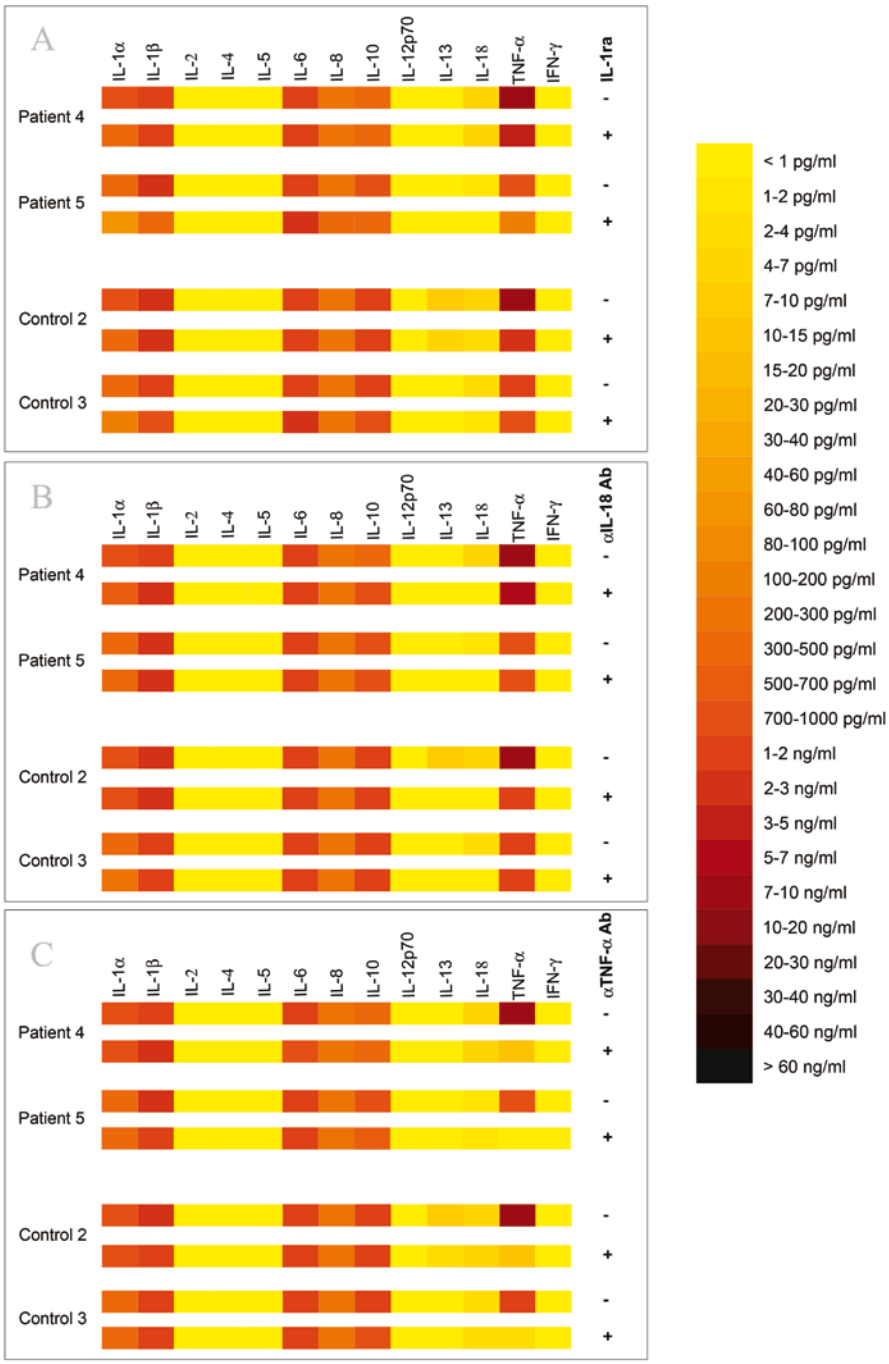

Figure 2: Blocking TNF-α or IL-18 function does not affect cytokine production by PBMC
Isolated PBMC from 2 MKD patients and 2 healthy control subjects were cultured in the presence of 500 ng/ml LPS, 250 ng/ml IL-1ra (A) or 500 ng/ml neutralizing antibodies against IL-18 (B) or TNF-α (C). After 24 hours culture supernatants were analysed for the presence of the indicated cytokines, using multiplex bead analysis.

in part, mediated by IL-1β and that IL-1β might therefore play a primary role in the initiation of inflammation in MKD.

Blocking TNF-α or IL-18 function does not affect cytokine production by PBMC
To confirm our data and to establish the specificity of IL-1ra in inhibiting the release of other cytokines, we repeated the experiment, also including blocking agents against IL-18 and TNF-α. We isolated PBMC from two different MKD patients, one identical and one additional healthy control subject. PBMC were again incubated in presence or absence of 250 ng/ml IL-1ra or 500 ng/ml neutralizing antibodies against human IL-18 or TNF-α and stimulated for 24 hours with 500 ng/ml LPS. LPS-stimulated PBMC from both patients and controls secreted IL-1α, IL-1β, IL-6, IL-8, IL-10, TNF-α and low levels of IL-18, similar to the previous experiment (Figure 2A). The lower cytokine production in this experiment probably reflects interassay- and intra-individual variation, since one control subject (control 2) was used in both experiments and the experimental set-up was identical in both experiments.
We were able to confirm the previous finding that IL-1ra inhibited TNF-α production by MKD PBMC. However, in this experiment the release of TNF-α was also inhibited in control PBMC (Figure 2A). When comparing the absolute values per cytokine we were able to detect an inhibitory effect of IL-1ra, not only on TNF-α, but also on IL-1α, IL-1β and IL-18 release by patient PBMC as well as control PBMC (Table II). There was no difference in IL-6 levels between patient and control cells and there was also no effect of IL-1ra. In addition, we again observed lower levels of IL-10 production by MKD PBMC compared to the control cells. Importantly, the addition of neutralizing antibodies against IL-18 or TNF-α had no effect on LPS-induced cytokine release by patient or control PBMC (Figure 2B and C), even when the concentration was raised to 2.0 μg/ml (Table II). Since neutralizing antibodies were used, IL-18 and TNF-α were low or absent in the samples where antibodies were present.
From these results we conclude that IL-1β is a primary cytokine in LPS-mediated inflammation and that blocking IL-1 receptor signalling inhibits the secretion of secondary proinflammatory cytokines, mainly IL-1α, IL-1β, IL-18 and TNF-α. In contrast, blocking functionality of TNF-α or IL-18 does not decrease cytokine production.

Anakinra treatment reduces cytokine production, but does not always improve clinical outcome
To confirm the primary role for IL-1β in initiating inflammatory processes in MKD, we followed the clinical response of three MKD patients to treatment with the IL-1 receptor antagonist anakinra. Of these patients, patient 1 was formerly classified as a HIDS patient and patient 2 and 3 were diagnosed as mevalonic aciduria patients. As a marker for inflammation, we determined CRP levels at each hospital visit. In addition, we determined the effect of blocking IL-1 receptor activation on the production of cytokines, by measuring serum/plasma levels of a broad panel of cytokines before, during and after anakinra treatment.
Patient 1 started maintenance treatment with 1 mg/kg/day of anakinra, injected subcutaneously, at the age of thirteen, on 6th April 2004, during disease remission when he was not experiencing a

fever episode and his CRP level was low (Figure 3A). Interestingly, at that point, plasma of this patient contained relatively high levels of most cytokines measured when compared to healthy control values (Figure 4 and Table III). IL-1α levels were raised to as much as ~50,000 times the average healthy value for this cytokine and also particularly high values were detected for IL-4. Within 48 hours after initiation of anakinra treatment, levels of all these cytokines started to decline, except for IL-8 which showed an initial increase and subsequently also declined. During treatment, cytokine levels remained low and this persisted even when treatment ceased (Figure 4 and Table III). Clinically, the treatment led to a complete remission for the first 7 months, with a 100% attendance at school. During this time CRP levels remained low (Figure 3A and Table III). The discomfort of daily anakinra injections led the patient to stop maintenance treatment. This was followed by two severe febrile attacks in the following month. After this, the patient started injecting anakinra (100 mg/day) only at the onset of a fever attack, which ameliorated his symptoms dramatically or even prevented the episodes altogether. Using this approach the patient has virtually remained without symptoms.

For patient 2, treatment with 1 mg/kg/day anakinra, injected subcutaneously, was initiated at the age of five, on 15th August 2005, during disease remission when he was not experiencing a fever episode and his CRP level was low (Figure 3B). At the start of treatment, cytokine levels were also low, except for IL-12 and IL-18 levels, which were slightly elevated (~2-fold and ~3-fold respectively, Figure 5 and Table IV). After initiation of treatment, the frequency of attacks remained unaltered, even after raising the daily dose to 2 mg/kg/day three months later. Surprisingly, his CRP values, which were measured during every hospital visit, remained consistently low during anakinra treatment (Figure 3B and Table IV), though they were likely to have been increased during his flares. During treatment, cytokine levels at the times of hospital visits remained low, except again for IL-12 levels which continued to be slightly elevated and for IL-18 levels, which briefly increased after 11 weeks of treatment, reaching levels of ~10-fold the healthy average. Interestingly, this peak coincided with a temporary increase in CRP values (figure 3B). After 7 months, treatment of attacks only, similar to patient 1, with 2.5 mg/kg/day of anakinra was tried, but proved ineffective (Figure 3B, dark grey bar). After another three months anakinra injections were discontinued. Sixteen months after discontinuation of anakinra, several cytokine levels had increased again, most notably IL-1α (Figure 5). There was also a sharp increase in IFN-γ levels, although the levels reached were still within the normal range (Table IV). This rise in cytokine levels was not paralleled by a change in CRP levels, which at that time were low. In conclusion, while blocking IL-1 receptor function in this patient was followed by an inhibition of cytokine production, at least between attacks, this did not prevent febrile episodes from recurring in this patient.

Patient 3 started anakinra treatment (1 mg/kg/day) at the age of 23 months, on 5th May 2004, just after the peak of a fever episode. The patient became afebrile within one day, CRP levels dropped to near-normal levels (Figure 3C and Table V) and arthritis went into complete

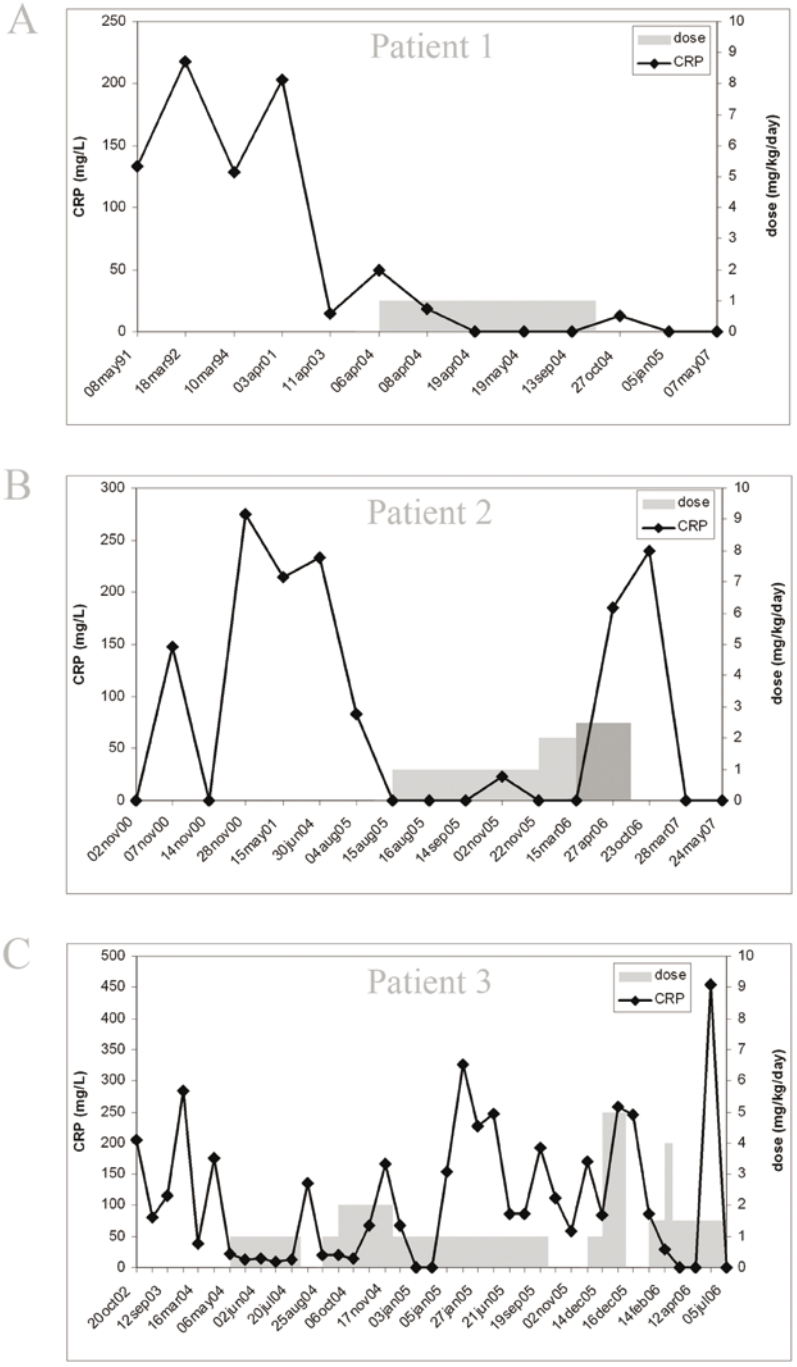

Figure 3: CRP levels of three MKD patients before, during and after anakinra treatment
C-reactive protein levels of one HIDS patient (A) and two MA patients (B and C) receiving anakinra treatment were determined at each hospital visit. The average CRP value for age-matched healthy children is ≤ 7.

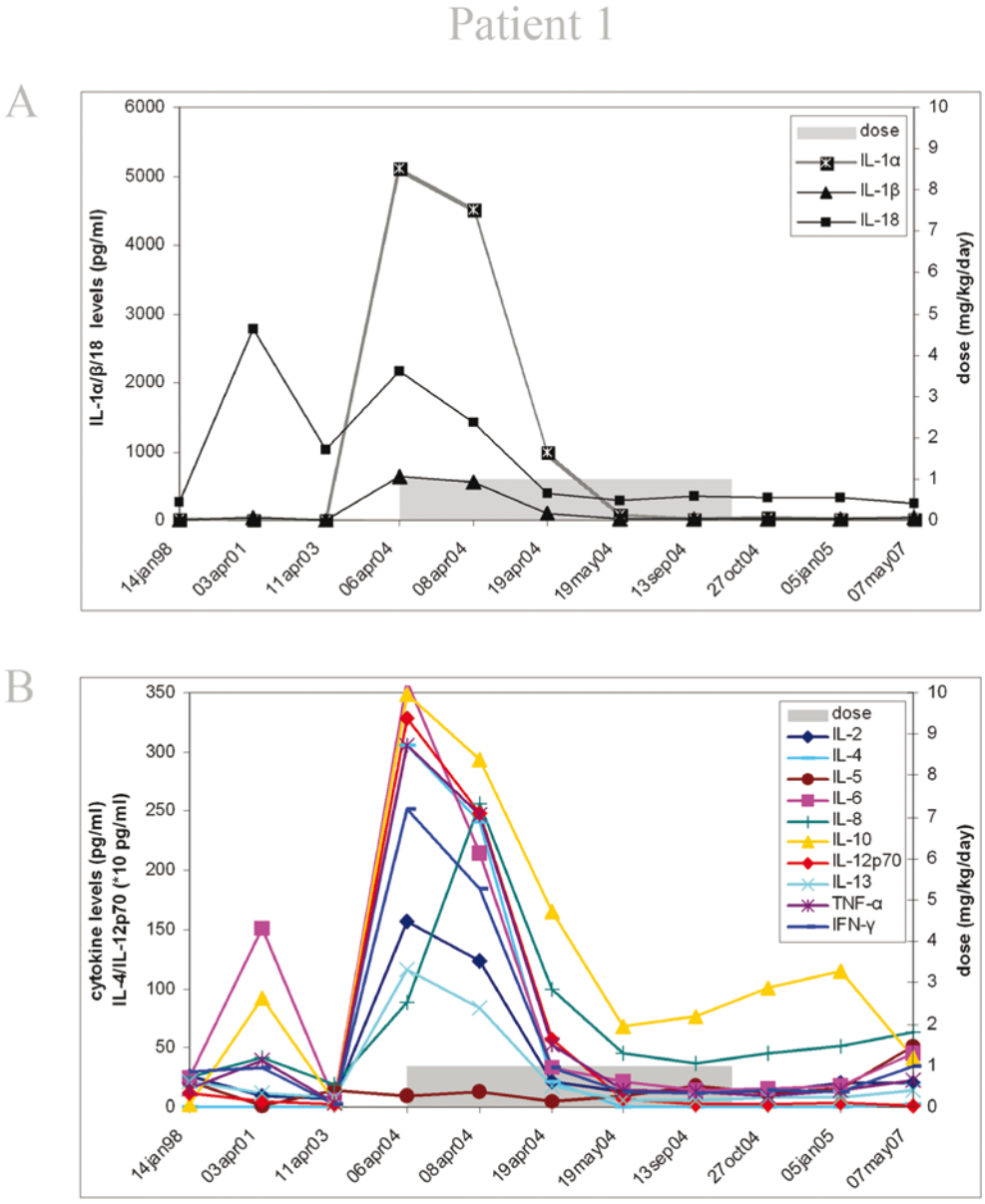

Figure 4: Cytokine levels of a HIDS patient before, during and after anakinra treatment
A) IL-1α, IL-1β and IL-18 levels and B) IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, IL-13, TNF-α and IFN-γ levels of patient 1 as determined in plasma or serum samples obtained during each hospital visit.

remission. In the next two months the patient experienced one two-day febrile episode. Unfortunately, we were only able to measure cytokine levels one day and fourteen days after initiation of treatment. Comparing cytokine levels one day before and at the two time points after initiation indicated a marginal decline in IL-1α, IL-2, IL-6, IL-10, IL-13 and IFN-γ levels (Figure 6 and Table V). In the second week of July 2004, the patient developed a pneumonia, clearly enhancing production of several cytokines, mainly IL-5, IL-6, IL-8, TNF-α and IFN-γ, as measured on 20th July (Figure 6 and Table V). Bronchoalveolar lavage revealed no other causative agent than rhinovirus. After twelve weeks, febrile attacks recurred, accompanied by headaches and diarrhea, but without reoccurrence of polyarthritis. Interruption of anakinra treatment at that point was immediately followed by high fever, increased CRP levels (Figure 3C) and a relapse of polyarthritis. At the end of August, treatment was resumed. Fever and polyarthritis again resolved which was accompanied with an acceleration of both growth and development and fairly low levels of circulating cytokines. When the daily dose of anakinra was raised to 2mg/kg, there was subjective improvement, but inflammatory episodes still recurred. Further elevation of the dose was not effective. Mid-October, the patient experienced a full-blown attack. Three days later she visited the hospital and at that time extremely high levels of most cytokines were measured, particularly IL-1α, IL-1β, IL-13 and IL-12. Anakinra treatment was interrupted for a couple of days. Upon re-initiation, the patient did not respond to the treatment as well as was observed after initial treatment. In the following eight months, she kept having attacks, as well as a variety of other problems, including pneumonia and peritonitis. Interestingly, during that time, her cytokine levels remained normal, except for IL-18, which was consistently high (Figure 6A) and one peak of IL-6 (Figure 6C), which corresponded with an episode of otitis. An attempt to add simvastatin to the regimen on 5th January 2005 was followed within hours by a severe febrile attack and increased CRP levels (Figure 3C). Replacing anakinra by the TNF-blocking agent etanercept was followed by relapse of fever and polyarthritis. Several attempts to control the disease with higher doses of anakinra also failed. Ultimately, from 15th March 2006 onwards anakinra treatment was combined with the oral use of prednisolone, which led to clinical improvement and decreased CRP levels. This treatment also further reduced cytokine levels, including IL-18 (Figure 6 and Table V). During this combination therapy, one sharp increase in IL-8 and CRP levels was observed, which coincided with another pneumonia attack. After three months the patient was still doing well. In conclusion, anakinra treatment in this patient generally induced a decrease in cytokine production, though this did not always correspond to clinical improvement.

Overall, anakinra treatment seemed to decrease cytokine production, though to a variable extent, in all three patients. This was paralleled by a good response in one, a partial response in a second and no response in the third patient. The extent of inhibition does not explain the difference in clinical response, since the non-responsive patient had the lowest cytokine levels during treatment, at least between attacks. Taken together, these results suggest that IL-1β might play an import role in initiating the inflammatory process in some, but not all, MKD patients.

DISCUSSION

IL-1β is thought to play a principle a role in the initiation of inflammatory episodes in MKD, which is supported by the observation that anakinra can be beneficial in treatment of MKD patients (15-17). However, precisely why anakinra treatment is effective is not entirely clear. IL-1β is known to affect transcription of several pro-inflammatory cytokines, such as IL-2, IL-6, TNF-α, IL-12, IL-8 and interferons (20). Possibly, decreased expression of these pro-inflammatory mediators contributes to the clinical improvement observed after anakinra treatment. However, extensive research on the effects of anakinra treatment on cytokine production is lacking. This study sought to elucidate some of the questions regarding the role of IL-1β and the mechanism behind anakinra-induced clinical improvement.

Ex vivo stimulation of PBMC from both MKD patients and healthy controls using LPS revealed that blocking of IL-1β signalling with recombinant IL-1 receptor antagonist (IL-1ra) decreased cytokine production. The effect was most pronounced on production of IL-1α, IL-1β, IL-18 and TNF-α (Figure 1 and 2). Importantly, blocking IL-18 or TNF-α did not result in inhibition of any of the other cytokines measured, supporting the primary role for IL-1β in inducing inflammation. From the experiments we could further conclude that PBMC from MKD patients and healthy controls seem to respond to the LPS stimulation in a similar manner and also blocking of IL-1β signalling seemed to have the same effect. This was contrary to a previous report by Drenth *et al.* (12), but can most likely be accounted for by the isolation procedure and the use of different reagents, such as LPS. The isolation procedure and the relatively high concentration of LPS used in our experiments are likely to activate MKD cells and healthy control cells alike, resulting in a similar inflammatory response, whereas *in vivo*, healthy control cells are likely to be more quiescent than MKD cells. However, we did observe that patient PBMC produce lower amounts of IL-10 than control PBMC. This might indicate that healthy PBMC are more efficient or more rapid in initiating anti-inflammatory feedback loops. Whether MKD patients have a fundamental defect in IL-10 production or whether they are simply slower in initiating an anti-inflammatory response is not clear, but studying IL-10 production in the course of time should elucidate this. Since IL-10 production is usually initiated in the later stages of inflammation, the difference in IL-10 production between patient and healthy control PBMC is unlikely to be causative for the inflammatory episodes in MKD.

In vivo, we found that anakinra treatment resulted in a reduction in circulating cytokine levels. However, decreased cytokine levels in peripheral blood of MKD patients was not always correlated with a good clinical response, as one patient showed consistently lower cytokine levels during treatment, but was still experiencing febrile episodes. The reason why a reduction of cytokine levels is associated with clinical improvement in one, but not the other patient remains unclear. Presumably, other factors influence the clinical outcome. In this study, we have only looked at a particular set of cytokines, for example not including endogenous IL-ra or chemokines. Also, various soluble factors, such as soluble IL-1 receptors or IL-18 binding protein are likely to affect IL-1β signalling efficiency and the subsequent immune activation status. Furthermore, differential

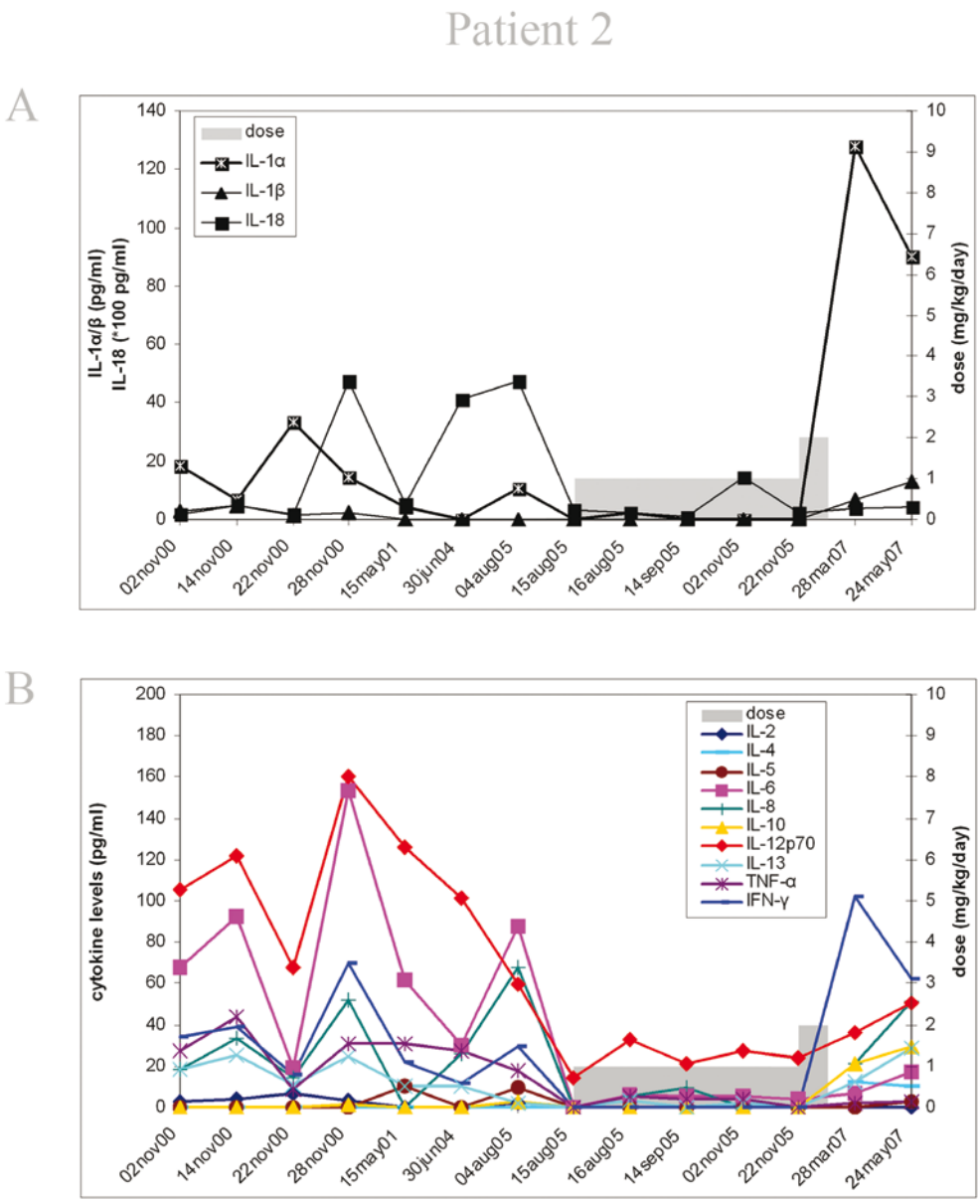

Figure 5: Cytokine levels of an MA patient before, during and after anakinra treatment
A) IL-1α, IL-1β and IL-18 levels and B) IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, IL-13, TNF-α and IFN-γ levels of patient 2 as determined in plasma or serum samples obtained during each hospital visit.

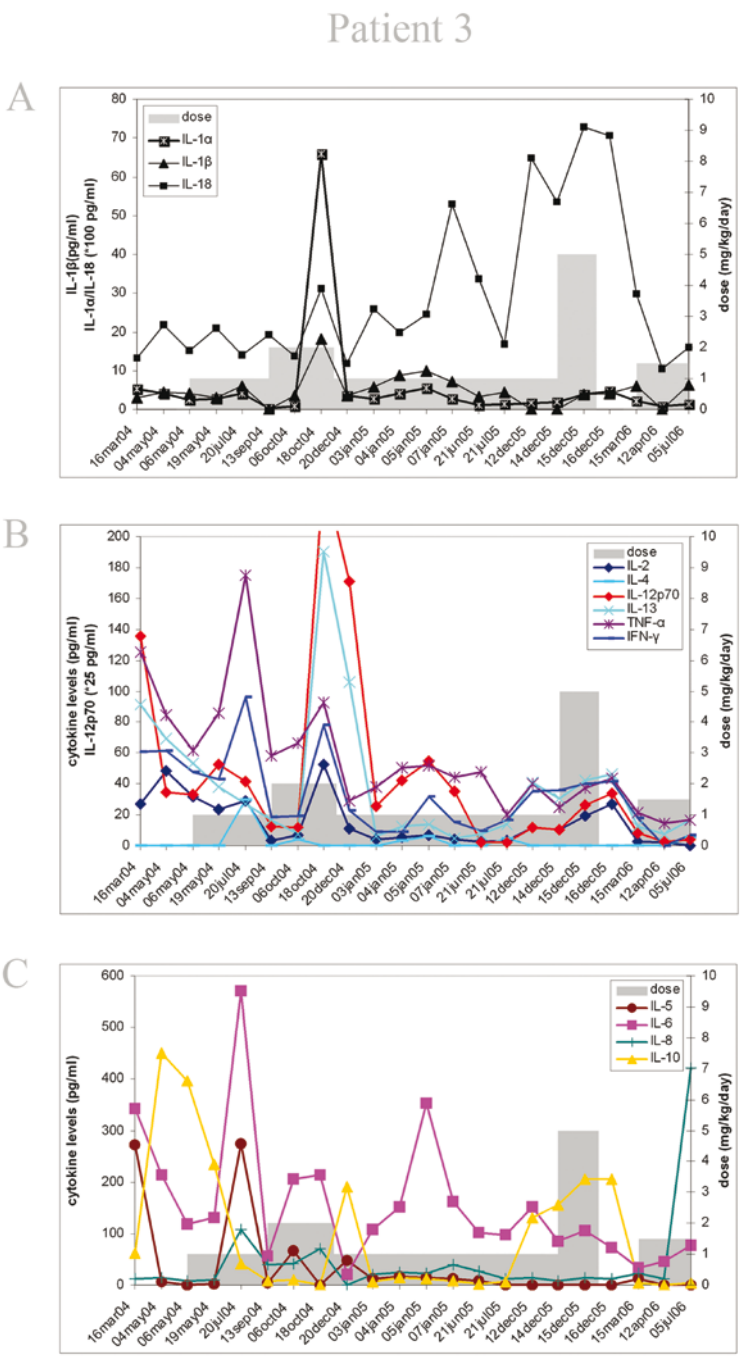

Figure 6: Cytokine levels of an MA patient before, during and after anakinra treatment
A) IL-1α, IL-1β and IL-18 levels, B) IL-2, IL-4, IL-12, IL-13, TNF-α and IFN-γ levels and C) IL-5, IL-6, IL-8 and IL-10 levels of patient 3 as determined in plasma or serum samples obtained during each hospital visit.

expression of various cytokine receptors could be of major influence on the clinical effect of anakinra treatment. For example, Goldbach-Mansky *et al*. have shown that patients suffering from neonatal-onset multisystem inflammatory disease (NOMID), one of the other auto-inflammatory disorders, have two- to three-fold higher expression levels of IL-1 receptor I and II and IL-ra and that these levels are downregulated upon successful treatment with anakinra (21). On the other hand, the difference in response might simply indicate that in some patients IL-1β plays a more dominant role than in others. Nevertheless, efficacy of anakinra treatment most likely depends on a wide set of factors, which may make it hard to predict which patients will respond and which ones will not.

One interesting finding resulting from the *in vivo* studies was the extremely high peaks in IL-18 concentration found in peripheral blood of MKD patients (note the different scale for IL-18 levels in two out of three MKD patients). These peaks very often coincided with high CRP levels (Table III to V). Moreover, IL-18 was one of only three cytokines found in the two cerebrospinal fluid (CSF) samples we had available of MKD patients, together with IL-12 and IL-8. From one MKD patient we also measured cytokines in bronchoalveolar lavage fluid, in which we also found IL-18 together with IL-8 and low levels of IL-12 and TNF-α (Table IV and V). CSF from four NOMID patients contained IL-18 as well, but here IL-6, IL-8 and IL-12 levels exceeded that of IL-18 (data not shown). Our previous research has demonstrated that enhanced release of IL-1β by MKD PBMC is most likely due to enhanced activation of the enzyme caspase-1 (22), which cleaves the inactive proIL-1β into the mature, secreted form. Besides IL-1β, caspase-1 also processes and activates IL-18 (23;24). Considering the major role for caspase-1 in the disease process, together with our interesting *in vivo* findings, more thorough investigation on IL-18 is warranted as it may contribute to MKD disease activity.

In conclusion, both our *ex vivo* and *in vivo* studies have demonstrated that blocking of IL-1β signalling can decrease the production of other cytokines, thereby dampening the activation status of the immune system. However, decreased production of the measured cytokines did not necessarily lead to improved clinical outcome, though two out of three MKD patients did have a beneficial response to treatment with anakinra. In order to better understand why certain patients respond well to anakinra treatment and others do not, we need to expand the set of studied parameters, including factors such as IL-ra, IL-1 receptors and also the newly identified caspase-1 substrate IL-33. Hopefully, this will increase our knowledge on the cytokine balance in MKD and ultimately result in an increased success rate of anakinra treatment.

Table I: Cytokine values (in pg/ml) corresponding to Figure 1.
The top row indicates the average plasma value for 20 healthy control samples in pg/ml.
<<: under detection limit; >>: exceeding standard curve; NM: not measured

			IL-1α	IL-1β	IL-2	IL-4	IL-5	IL-6	IL-8	IL-10	IL-12p70	IL-13	IL-18	TNF-α	IFN-γ
			0.1 ± 0.0	1.2 ± 0.6	0.1 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	6.0 ± 7.6	0.2 ± 0.1	0.3 ± 0.3	7.5 ± 2.4	1.9 ± 1.6	133 ± 29	1.1 ± 0.6	55 ± 104
	IL-1ra	LPS													
patient 1	-	-	9.2	230.0	11.9	<<	<<	20149	2360.3	66.2	<<	<<	19.0	<<	<<
		+	367.8	2099.2	<<	<<	<<	>>	670.5	>>	<<	<<	<<	61.3	<<
	+	-	<<	<<	7.1	<<	<<	26482	4547.3	50.0	<<	<<	<<		<<
		+	115.9	970.8	<<	<<	<<	>>	748.4	4666.9	<<	<<	<<	13.8	<<
patient 2	-	-	6.5	389.6	<<	<<	<<	81083	993.7	44.7	<<	<<	<<	7.5	<<
		+	1071.1	4733.0	<<	<<	<<	89121	514.6	3935.2	<<	<<	17.1	6111.5	<<
	+	-	NM	NM	NM	NM	NM	NM	NM	NM	NM	NM	NM	NM	NM
		+	999.9	4039.6	<<	<<	<<	84556	470.4	2093.3	<<	<<	<<	2926.4	<<
patient 3	-	-	11.8	54.9	<<	<<	<<	>>	472.9	52.1	<<	<<	<<	37.5	<<
		+	520.1	1194.9	<<	<<	<<	>>	308.3	1461.7	<<	<<	6.2	1255.4	<<
	+	-	10.9	55.5	<<	<<	<<	>>	758.5	<<	<<	<<	<<	<<	<<
		+	363.7	1115.4	<<	<<	<<	>>	311.9	617.7	<<	<<	<<	445.9	<<
control 1	-	-	13.5	139.3	<<	<<	<<	>>	675.6	801.4	<<	<<	<<	7.5	<<
		+	1400.9	1409.5	<<	<<	<<	19313	315.1	>>	<<	<<	14.1	2787.3	<<
	+	-	10.0	124.9	<<	<<	<<	40476	911.8	531.6	<<	<<	<<	<<	<<
		+	899.5	1555.0	<<	<<	<<	23546	422.5	9520.3	<<	<<	12.1	2189.6	<<
control 2	-	-	31.3	324.0	<<	<<	<<	>>	307.2	3093.6	<<	<<	<<	6.6	<<
		+	805.1	3165.1	<<	<<	<<	>>	286.4	34432	<<	2.7	13.4	705.4	<<
	+	-	21.3	155.4	<<	<<	<<	>>	486.9	514.7	<<	<<	<<	7.3	<<
		+	640.6	2800.3	<<	<<	<<	>>	329.8	35086	<<	<<	14.1	472.4	<<

Table II: Cytokine values (in pg/ml) corresponding to Figure 2.
The top row indicates the average plasma value for 20 healthy control samples in pg/ml.
<<: under detection limit; >>: exceeding standard curve

			IL-1α	IL-1β	IL-2	IL-4	IL-5	IL-6	IL-8	IL-10	IL-12p70	IL-13	IL-18	TNF-α	IFN-γ
			0.1 ± 0.0	1.2 ± 0.6	0.1 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	6.0 ± 7.6	0.2 ± 0.1	0.3 ± 0.3	7.5 ± 2.4	1.9 ± 1.6	133 ± 29	1.1 ± 0.6	55 ± 104
		LPS													
patient 4	-	-	0.0	3.6	<<	<<	<<	359.3	593.3	0.7	<<	<<	0.1	4.7	<<
		+	718.5	1688.8	<<	<<	<<	1192.5	221.1	443.2	<<	0.4	5.5	>>	<<
	IL-1ra	-	<<	0.6	<<	<<	<<	67.2	753.7	0.0	<<	<<	0.1	0.3	<<
		+	487.3	1426.2	<<	<<	<<	1830.3	244.8	398.5	<<	<<	4.2	4416.5	<<
	α-IL-18	-	0.0	5.0	<<	<<	<<	576.7	504.2	4.5	<<	<<	0.0	4.2	<<
		+	611.2	2406.6	<<	<<	<<	1216.9	239.3	836.4	<<	0.4	0.1	5453.5	<<
	α-TNF-α	-	0.0	3.5	<<	<<	<<	372.6	927.6	0.7	<<	<<	0.2	<<	<<
		+	799.5	2110.2	<<	<<	<<	987.8	221.1	443.8	<<	0.4	6.4	12.3	<<
patient 5	-	-	<<	0.7	<<	<<	<<	26.0	686.2	0.1	<<	<<	0.1	<<	<<
		+	355.1	2133.9	<<	<<	<<	1876.7	287.4	806.7	<<	<<	1.4	865.6	<<
	IL-1ra	-	<<	0.1	<<	<<	<<	6.4	1007.8	0.1	<<	<<	0.2	<<	<<
		+	64.8	381.6	<<	<<	<<	>>	323.6	355.7	<<	<<	0.5	119.7	<<
	α-IL-18	-	<<	0.6	<<	<<	<<	44.6	837.7	0.4	<<	<<	0.1	0.0	<<
		+	405.6	2576.4	<<	<<	<<	1793.8	294.0	862.9	<<	<<	0.1	961.9	<<
	α-TNF-α	-	<<	0.2	<<	<<	<<	12.1	871.2	0.0	<<	<<	0.1	<<	<<
		+	352.1	1160.2	<<	<<	<<	1647.4	276.7	534.9	<<	<<	1.4	0.0	<<
	α-IL-18 (2.0 μg/ml)	-	0.2	1.2	<<	<<	<<	283.0	617.6	7.3	<<	<<	0.1	1.2	<<
		+	205.0	1340.9	<<	<<	<<	>>	276.4	1890.8	<<	<<	0.1	1343.3	<<
	α-TNF-α (2.0 μg/ml)	-	<<	0.8	<<	<<	<<	80.9	791.9	2.8	<<	<<	0.2	<<	<<
		+	443.9	1901.7	<<	<<	<<	1547.5	251.6	879.8	<<	<<	2.8	0.4	<<

Table II: continued

			IL-1α	IL-1β	IL-2	IL-4	IL-5	IL-6	IL-8	IL-10	IL-12p70	IL-13	IL-18	TNF-α	IFN-γ
			0.1 ± 0.0	1.2 ± 0.6	0.1 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	6.0 ± 7.6	0.2 ± 0.1	0.3 ± 0.3	7.5 ± 2.4	1.9 ± 1.6	133 ± 29	1.1 ± 0.6	55 ± 104
		LPS													
control 2	-	-	3.1	11.5	<<	<<	<<	940.0	475.7	8.5	<<	<<	0.2	18.7	<<
		+	892.2	2229.5	<<	<<	<<	1126.7	238.0	1404.1	<<	9.4	5.6	>>	<<
	IL-1ra	-	0.3	2.7	<<	<<	<<	477.2	659.6	1.6	<<	<<	0.1	3.6	<<
		+	468.3	2048.8	<<	<<	<<	1389.8	268.6	1308.9	<<	4.2	3.0	2270.3	<<
	α-IL-18	-	2.1	11.4	<<	<<	<<	1690.2	447.4	20.9	<<	<<	0.0	17.6	<<
		+	913.8	2080.1	<<	<<	<<	1097.1	263.7	2262.0	<<	7.7	0.1	6908.8	<<
	α-TNF-α	-	2.4	8.4	<<	<<	<<	1036.3	704.2	6.1	<<	<<	0.2	<<	<<
		+	870.2	1964.3	<<	<<	<<	1181.8	261.5	1182.1	<<	2.4	4.7	14.4	<<
control 3	-	-	0.1	1.6	<<	<<	<<	186.4	664.2	2.2	<<	<<	0.1	0.8	<<
		+	434.7	1424.0	<<	<<	<<	1341.5	253.3	1459.6	<<	0.4	2.7	1602.2	<<
	IL-1ra	-	<<	0.3	<<	<<	<<	59.3	831.9	1.4	<<	<<	0.2	0.2	<<
		+	142.0	791.1	<<	<<	<<	>>	262.6	784.7	<<	<<	1.2	904.6	<<
	α-IL-18	-	0.2	1.8	<<	<<	<<	272.2	655.8	4.8	<<	<<	0.1	1.2	<<
		+	263.8	1168.6	<<	<<	<<	1991.4	260.5	1627.9	<<	<<	0.1	1571.3	<<
	α-TNF-α	-	<<	0.7	<<	<<	<<	103.5	935.1	3.0	<<	<<	0.1	<<	<<
		+	389.5	1211.6	<<	<<	<<	1336.3	260.9	959.0	<<	<<	2.5	3.3	<<
	α-IL-18 (2.0 μg/ml)	-	0.1	5.3	<<	<<	<<	647.8	494.7	12.0	<<	<<	0.0	2.8	<<
		+	396.0	2256.2	<<	<<	<<	1431.1	257.3	1533.6	<<	0.4	0.1	1953.9	<<
	α-TNF-α (2.0 μg/ml)	-	<<	1.9	<<	<<	<<	269.6	868.9	0.9	<<	<<	0.2	<<	<<
		+	833.1	1712.9	<<	<<	<<	1320.8	250.1	466.5	<<	2.4	6.0	2.8	<<

Table III: Date, dose (mg/kg/day), C-reactive protein levels (mg/L), sample type as used for multiplex bead analysis and cytokine values (pg/ml) for patient 1. The top row indicates the average plasma value for 20 healthy control samples in pg/ml. The average CRP value for age-matched healthy children is ≤ 7 mg/L.
s: serum; p: plasma; <<: under detection limit

date	dose	CRP	sample type	IL-1α	IL-1β	IL-2	IL-4	IL-5	IL-6	IL-8	IL-10	IL-12p70	IL-13	IL-18	TNF-α	IFN-γ
				0.1 ± 0.0	1.2 ± 0.6	0.1 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	6.0 ± 7.6	0.2 ± 0.1	0.3 ± 0.3	7.5 ± 2.4	1.9 ± 1.6	133 ± 29	1.1 ± 0.6	55 ± 104
08may91	0	133	s													
18mar92	0	218	s													
10mar94	0	129	s													
14jan98	0		s	16.2	2.6	26.0	2.4	21.1	25.5	24.8	1.9	118.6	21.1	275.2	16.1	29.8
03apr01	0	203	s	6.0	42.0	9.0	<<	1.0	151.5	42.0	92.0	45.0	11.5	2775.0	40.0	34.0
11apr03	0	15	s	5.2	0.3	7.8	<<	14.0	5.5	19.8	5.2	27.2	7.8	1039.9	3.53	2.0
06apr04	1	50	s	5113.3	636.3	157.3	3061.5	9.5	359.0	89.0	348.5	3287.8	116.8	2175.3	305.8	252.0
08apr04	1	18	s	4519.0	564.0	124.0	2410.0	13.0	215.0	256.0	293.5	2478.0	83.5	1419.5	247.0	184.0
19apr04	1	<7	s	998.5	101.0	22.0	210.0	5.0	34.0	99.5	166.0	578.5	18.5	397.0	53.0	34.0
19may04	1	<7	p	84.0	23.0	13.0	<<	9.0	22.0	45.0	68.0	68.0	6.5	283.0	14.0	13.0
13sep04	1	<7	p	12.0	19.5	14.0	<<	18.0	14.0	37.0	77.0	20.5	6.5	357.0	13.0	12.0
27oct04	0	13	s	41.0	17.0	13.0	<<	12.0	15.0	45.5	101.0	28.0	8.5	329.5	10.0	14.0
05jan05	0	<7	p	17.0	21.0	20.0	<<	15.0	18.5	52.0	115.0	31.0	8.5	335.0	14.0	13.5
07may07	0	<7	s	18.0	36.0	20.0	21.0	51.5	45.0	63.0	42.0	17.5	14.0	245.0	23.0	34.5

Table IV: Date, dose (mg/kg/day), C-reactive protein levels (mg/L), sample type as used for multiplex bead analysis and cytokine values (pg/ml) for patient 2. The top row indicates the average plasma value for 20 healthy control samples in pg/ml. The average CRP value for age-matched healthy children is ≤ 7 mg/L.
s: serum; p: plasma; CSF: cerebrospinal fluid; <<: under detection limit

date	dose	CRP	sample type	IL-1α	IL-1β	IL-2	IL-4	IL-5	IL-6	IL-8	IL-10	IL-12p70	IL-13	IL-18	TNF-α	IFN-γ
				0.1 ± 0.0	1.2 ± 0.6	0.1 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	6.0 ± 7.6	0.2 ± 0.1	0.3 ± 0.3	7.5 ± 2.4	1.9 ± 1.6	133 ± 29	1.1 ± 0.6	55 ± 104
02nov00	0	<7	s	18.6	3.0	2.8	<<	<<	67.8	18.4	<<	105.4	18.3	204.2	27.6	34.0
07nov00	0	148														
14nov00	0	<7	s	6.9	4.8	4.5	<<	<<	92.8	33.4	<<	121.8	25.2	467.8	43.6	39.1
22nov00	0		s	33.52	1.4	6.9	<<	<<	19.5	14.7	<<	67.9	11.0	211.1	8.8	15.7
28nov00	0	275	s	14.3	2.6	3.6	<<	<<	153.7	52.4	1.3	160.6	24.5	4724.9	30.8	69.6
15may01	0	215	s	4.3	<<	<<	<<	10.1	62.0	<<	<<	126.0	10.2	521.1	30.8	22.2
30jun04	0	233	s	<<	<<	<<	<<	<<	29.8	26.2	<<	101.6	10.5	4130.9	27.6	11.6
04aug05	0	83	p	10.9	<<	2.3	<<	9.3	87.7	68.2	2.4	59.8	1.8	4751.2	17.8	29.7
15aug05	1	<7	s	<<	<<	<<	<<	<<	<<	<<	<<	14.6	<<	328.4	<<	<<
16aug05	1	<7	s	2.4	<<	<<	<<	<<	6.3	5.3	<<	32.6	3.1	248.8	5.3	<<
14sep05	1	<7	s	<<	<<	<<	<<	<<	5.4	9.6	<<	21.4	<<	80.0	4.4	<<
02nov05	1	23	s	<<	<<	<<	<<	<<	5.4	<<	<<	27.5	2.2	1477.0	4.2	<<
22nov05	2	<7	s	<<	<<	<<	<<	<<	4.4	<<	<<	24.1	<<	227.6	<<	<<
15mar06	2.5	<7														
27apr06	2.5	185														
23oct06	0	240														
28mar07	0	<7	s	128.0	7.0	<<	12.0	<<	7.0	21.0	21.0	36.0	12.0	392.5	2.0	102.0
24may07	0	<7	s	90.0	13.0	<<	10.0	3.0	17.0	51.5	29.5	50.5	28.5	432.5	3.0	62.0
04aug05	0	83	CSF	<<	<<	<<	<<	<<	<<	38.4	<<	11.6	<<	6.0	<<	<<

Table V: Date, dose (mg/kg/day), C-reactive protein levels (mg/L), sample type as used for multiplex bead analysis and cytokine values (pg/ml) for patient 3. The top row indicates the average plasma value for 20 healthy control samples in pg/ml. The average CRP value for age-matched healthy children is ≤ 7 mg/L.
s: serum; p: plasma; CSF: cerebrospinal fluid; BAL: bronchoalveolar lavage fluid; <<: under detection limit; >>: exceeding standard curve

date	dose	CRP	sample type	IL-1α	IL-1β	IL-2	IL-4	IL-5	IL-6	IL-8	IL-10	IL-12p70	IL-13	IL-18	TNF-α	IFN-γ
				0.1 ± 0.0	1.2 ± 0.6	0.1 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	6.0 ± 7.6	0.2 ± 0.1	0.3 ± 0.3	7.5 ± 2.4	1.9 ± 1.6	133 ± 29	1.1 ± 0.6	55 ± 104
20oct02	0	205	s													
21jul03	0	80	s													
12sep03	0	116	s													
09feb04	0	284	s													
16mar04	0	38	s	528.0	3.0	27.3	<<	273.0	342.9	13.4	62.8	3391.9	91.5	1333.0	125.2	60.9
04may04	0	176	s	414.7	4.3	48.4	<<	5.5	213.0	13.6	450.0	860.7	69.5	2185.9	84.5	61.3
06may04	1	22	s	261.6	4.1	31.9	<<	<<	119.3	8.5	396.7	838.7	53.1	1522.7	61.6	48.0
19may04	1	12	s	285.7	3.1	23.7	<<	2.9	131.1	9.8	235.5	1308.6	38.0	2094.1	85.8	43.2
02jun04	1	14	s													
30jun04	1	10	s													
20jul04	1	12	p	402.0	6.0	29.3	29.6	275.1	570.5	107.7	40.8	1043.0	27.3	1406.9	174.9	96.4
29jul04	0	135	s													
25aug04	1	20	s													
13sep04	2	21	s	39.5	<<	3.7	<<	3.4	56.3	38.7	8.2	308.1	16.6	1944.1	58.0	18.8
06oct04	2	15	p	83.2	3.7	7.3	4.0	66.0	205.8	42.4	10.4	290.8	7.6	1371.8	66.3	19.6
18oct04	2	68	s	>>	18.1	52.7	<<	<<	214.4	71.0	<<	>>	190.4	3126.0	93.1	78.3
17nov04	2	167	s													
20dec04	1	68	s	363.8	3.5	11.1	<<	46.9	20.6	<<	190.0	4271.8	106.2	1178.2	29.2	22.7
03jan05	1	<7	p	284.0	5.7	4.3	<<	12.8	107.8	20.2	6.4	641.1	6.2	2584.7	38.0	9.2
04jan05	1	<7	p	402.9	8.7	5.8	3.2	16.2	152.3	25.6	13.6	1055.3	12.7	1982.1	50.3	8.8

Table V: continued

date	dose	CRP	sample type	IL-1α	IL-1β	IL-2	IL-4	IL-5	IL-6	IL-8	IL-10	IL-12p70	IL-13	IL-18	TNF-α	IFN-γ
				0.1 ± 0.0	1.2 ± 0.6	0.1 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	6.0 ± 7.6	0.2 ± 0.1	0.3 ± 0.3	7.5 ± 2.4	1.9 ± 1.6	133 ± 29	1.1 ± 0.6	55 ± 104
05jan05	1	153	p	565.2	9.9	6.8	6.5	14.5	353.9	22.9	12.5	1362.2	139.9	2457.5	52.1	31.6
07jan05	1	326	p	278.3	7.3	4.0	<<	12.8	162.8	38.6	8.9	882.4	4.8	5284.5	44.1	15.5
27jan05	1	227	s													
20apr05	1	247	s													
21jun05	1	86	p	110.5	3.4	2.8	<<	9.3	101.8	27.6	3.0	48.9	7.6	3363.2	47.7	9.9
21jul05	1	86	p	136.5	4.3	3.3	4.7	<<	96.6	11.8	6.4	48.9	13.9	1687.0	20.4	16.9
19sep05	1	193	s													
10oct05	0	112	s													
02nov05	0	59	s													
12dec05	1	171	s	165.5	<<	11.8	<<	<<	150.9	15.3	130.7	302.6	41.0	6489.4	39.8	35.4
14dec05	5	85	s	204.4	<<	10.7	<<	<<	85.3	8.3	156.2	252.9	30.7	5351.0	25.3	35.7
15dec05	5	258	s	394.8	3.7	19.7	<<	<<	106.7	13.6	205.9	655.3	42.4	7278.2	37.5	40.5
16dec05	0	245	s	482.6	4.2	26.9	<<	<<	72.2	12.8	205.4	846.4	46.7	7073.5	43.7	41.2
20dec05	1.5	86	s													
14feb06	4	30	s													
15mar06	1.5	<7	p	223.9	6.1	3.1	<<	12.8	33.4	22.9	4.2	207.9	12.7	2974.8	21.3	18.2
12apr06	1.5	<7	p	73.1	<<	2.3	<<	<<	45.82	13.2	<<	61.1	7.6	1053.3	14.7	<<
22apr06	1.5	455	s													
05jul06	1.5	<7	p	148.9	6.2	<<	5.3	<<	77.2	421.0	4.5	111.2	16.3	1599.6	16.9	7.1
05may04	1		CSF	<<	<<	<<	<<	<<	<<	<<	<<	21.9	<<	65.2	<<	<<
22jun05	1		BAL	<<	<<	<<	<<	<<	<<	397.6	<<	6.4	<<	54.0	4.2	<<

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Chapter 6

Effective treatment of a colchicine-resistant FMF patient with anakinra

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Familial Mediterranean fever (FMF) is an autoinflammatory disorder, characterized by periodic fever and serosal inflammation, often complicated by systemic amyloidosis. Maintenance treatment of FMF with colchicine can reduce disease activity and prevent amyloidosis. However, some patients fail colchicine therapy. Recently, multiple reports have been published concerning the effective use of a recombinant IL-1 receptor antagonist, anakinra, in several closely related disorders (1-4). These results prompted us to use anakinra in a 14-year-old FMF patient who was unresponsive to colchicine therapy (2mg/day). Diagnosis of FMF was confirmed by analysis of the *MEFV* gene (M694V, M694I).

The patient presented with febrile attacks accompanied by abdominal aches since the age of two years. With age, the attacks became frequent (every 3-10 days), prolonged (12-88 hours) and more severe. The episodes consisted of abruptly rising fever (>40°C) accompanied by chills, anorexia, abdominal and retrosternal pain, arthralgias and also arthritis. In addition, she suffered from frequent bitemporal headaches and depression. Blood tests showed a chronic microcytic anemia and elevated inflammatory markers, which increased even more during attacks.

The patient was admitted to hospital and after confirmation of colchicine-resistant FMF, patient and parents gave written informed consent for a trial of daily anakinra 100 mg subcutaneous. Colchicine was initially maintained at 2 mg daily.

After initiation of anakinra treatment fever did not recur (Figure 1), nor did the patient experience abdominal pain or arthralgias even after gradually reducing the daily colchicine dose to 1 mg. The patient was less depressed and much more active. A transient mild urticarial rash on the hands and face was reported, which could be controlled well with oral anti-histamines. Laboratory results showed a remarkable improvement of ESR and complete normalization of CRP. Furthermore, there was a reduction in serum IL-1α, IL-1β and IL-8 levels as measured by multiplex bead analysis (5) (Table I). Surprisingly, all other cytokines measured (IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, IL-18, IFN-γ and TNF-α) were either not elevated or did not change upon treatment with anakinra. Contrary to previous reports (2), we did not observe any changes in IL-6 levels after anakinra treatment.

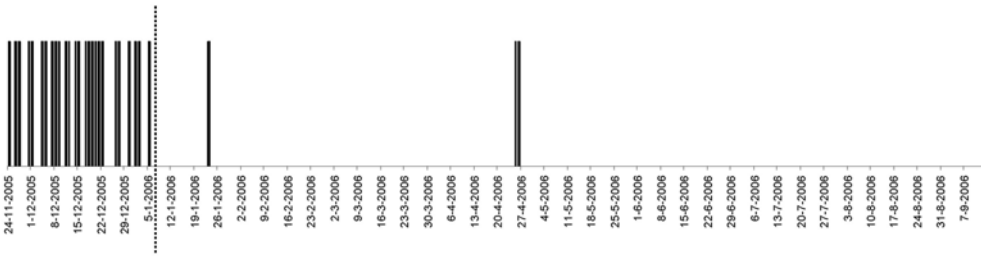

Figure 1: Disease activity before and during anakinra treatment.
The bars indicate days when the patient reported to be ill in her diary and/or her temperature exceeded 38°C.
The vertical dotted line indicates start of treatment.

Although favourable responses to anti-TNF-α agents (6-8) have been described, this is the first report of a child with colchicine-resistant FMF successfully treated with anakinra. Similar results were reported in one adult FMF patient (9). Serious side effects of anakinra are rare (10). It therefore seems to be a safe agent for treatment of auto-inflammatory disorders. However, increased susceptibility to infections may still pose a risk and patients obviously need to be monitored closely when anakinra treatment is initiated. We conclude that anti-IL-1 therapy in patients with colchicine-resistant FMF is potentially beneficial. Although none have been reported, treatment failures might occur in both therapies. Therefore, a prospective trial comparing anti-TNF-α and anti-IL-1 therapy in FMF is warranted.

Table I Inflammatory mediators before and after initiation of anakinra therapy

	normal values*	Before	After
CRP	≤ 7.0	44.4 ± 9.9	<7.0
IL-1α (pg/ml)	≤ 0.1	223.9	60.6
IL-β (pg/ml)	≤ 1.3	4.1	1.2
IL-8 (pg/ml)	≤ 0.3	238.7	3.8

* Average values for age-matched healthy children (CRP) or healthy adults (n=20, cytokines).

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Chapter 7

General Discussion

The role of cytokines in MKD

Since it was initially described by van der Meer *et al.* more than 20 years ago (1), much has been learned about the Hyper-IgD and periodic fever syndrome, currently also known as mevalonate kinase deficiency (MKD). It is now believed that a deficiency in the enzyme mevalonate kinase results in a reduced output of the isoprenoid biosynthesis pathway and that this causes inflammation, probably due to an imbalance in pro-inflammatory cytokine production. We and others have demonstrated an important role for the cytokine IL-1β in initiating inflammatory episodes in MKD (2;3, and this thesis), a notion that is supported by the successful treatment of MKD and other autoinflammatory syndromes with the IL-1 receptor antagonist anakinra (4-9). In Chapter 6 we describe that a case of Familial Mediterranean Fever (FMF) could also be successfully treated with this drug. Although anakinra treatment proved successful in several patients, exactly why it is effective remains unclear. IL-1β is known to affect transcription of several pro-inflammatory cytokines, such as IL-2, IL-6, TNF-α, IL-12, IL-8 and interferons (10). Possibly, decreased expression of these downstream inflammatory mediators accounts for the clinical improvement observed after anakinra treatment. However, extensive research on the effects of anakinra treatment on cytokine production is lacking. One report by Goldbach-Mansky *et al.* (8) describes effects of anakinra treatment on serum cytokine levels and their *ex vivo* production by PBMC from patients suffering from Neonatal-Onset Multisystem Inflammatory Disease (NOMID), also known as Chronic Infantile Onset Neurologic Cutaneous Articular syndrome (CINCA). These authors found strongly reduced IL-1β production by isolated PBMC after initiation of anakinra treatment. In addition, mRNA expression levels of cytokines including IL-1β, IL-6 and IFN-γ, and other IL-1β related molecules such as caspase-1, IL-1 receptor 1 and 2 and IL-1 receptor antagonist (IL-1ra) were significantly reduced after anakinra treatment. In addition to the results by Goldbach-Mansky *et al.*, our own observations show that treatment of MKD with anakinra indeed correlates with decreased production of IL-1α, IL-1β, IL-18 and TNF-α by MKD PBMC and decreased plasma levels of nearly all cytokines measured (Chapter 5). What can be concluded from this is that blocking IL-1β function can improve clinical outcome and that improvement indeed correlates with reduced levels of other inflammatory mediators. This suggests that IL-1β is a primary mediator, acting upstream of other inflammatory routes. However, in spite of the multiple published case reports in which anakinra treatment was beneficial, it is reasonable to assume that in many more cases treatment was not successful, as those patients are hardly ever described in medical literature, due to publication bias (11). Therefore, further studies on cytokine imbalance in auto-inflammatory syndromes are warranted. Perhaps the mechanism underlying the inflammatory response differs in each patient, with a major role for IL-1β in many, but not all patients. In that respect, IL-18 and the recently discovered IL-33 are of interest for further investigation, since both these cytokines, like IL-1β, are substrates for caspase-1 (12-14).

Although more direct evidence is needed, a major role for caspase-1 in the induction of inflammatory episodes in auto-inflammatory disorders is likely. Caspase-1 is required for processing of inactive proIL-1β into the mature and inflammatory form (15;16). In the cryopyrin-associated periodic

fever syndromes (FCAS, Muckle-Wells and CINCA) caspase-1 activation is thought to be mediated via spontaneous assembly of the NALP3 inflammasome, due to mutations in the oligomerization domain of NALP3 (17;18). In Familial Mediterranean Fever, the protein pyrin is affected, which is thought to enhance caspase-1 activity, either by direct binding to caspase-1 (19) or by enhanced binding of caspase-1 to the adaptor protein ASC (20;21), resulting in increased processing of IL-1β. In Chapter 3 we have shown that in MKD increased caspase-1 activity is also likely to underlie inflammation. When mimicking MKD by impairment of isoprenoid biosynthesis using simvastatin, we were able to demonstrate enhanced caspase-1 activation. If spontaneous activation of caspase-1 is indeed a unifying factor for autoinflammatory diseases, its substrates IL-18 and IL-33 may also significantly contribute to inflammation. Many inflammatory diseases, for example systemic lupus erythematosus, rheumatoid arthritis and Crohn's disease, are thought to be mediated in part by IL-18 (22-27). In addition, in Chapter 5 we describe that peaks of inflammation, as characterized by increased CRP levels, often coincided with peaks in plasma IL-18 levels and that IL-18 was one of only three cytokines found in the two cerebrospinal fluid (CSF) samples available from MKD patients. In one MKD patient we also measured cytokines in bronchoalveolar lavage fluid and also found IL-18 together with IL-8. CSF from four CINCA patients contained IL-18 as well, but here IL-6, IL-8 and IL-12 levels exceeded that of IL-18 (data not shown). These findings suggest that IL-18 may have an important role in auto-inflammation, which calls for more thorough investigation of this cytokine. The function of the recently discovered IL-33 remains largely unknown. However, binding of IL-33 to its receptor ST2 induces expression of IL-4, IL-5 and IL-13 *in vivo* and drives the production of these cytokines by *in vitro* polarized T_H2 cells (14;28), suggesting an involvement in allergy-related reactions. The effects of increased production of IL-33 due to enhanced caspase-1 activity may therefore also account for some of the symptoms in the intermittent autoinflammatory disorders. For example, since IL-5 is known to stimulate immunoglobulin production by B cells, IL-33-enhanced production of IL-5 could possibly provide an explanation for the strikingly high levels of IgA and IgD in MKD patients (29;30). Availability of recombinant IL-33, anti-IL-33 antibodies and IL-33 knockout mice will surely increase our understanding of this cytokine and the role it plays in inflammation. The fact that IL-33 was only very recently discovered also suggests that additional substrates for caspase-1 might still be identified. Indeed, a very recent study identified 41 putative caspase-1 substrates, including non-inflammatory proteins, such as enzymes of the glycolysis pathway (31). If and how these proteins play a role in MKD remains to be investigated.

Based on the findings in Chapter 5 and other unpublished results, IL-1α is another interesting cytokine in relation to auto-inflammatory disorders. We found very high plasma IL-1α levels in some, but not all, MKD and CINCA patients. IL-1α is not commonly found in the circulation except during severe disease, in which case it is released from dying cells or after calpain-mediated cleavage (32). Since IL-1α and IL-β bind to the same receptor, but IL-1β has a lower affinity (10), the increased release of IL-1α might reflect a compensatory mechanism to decrease IL-1β signalling. When IL-1α binds to the IL-1 receptor, the complex is rapidly internalized and translocates to the nucleus where it is thought to act as a transcription factor (33;34). However, the exact function

of IL-1α and its role in disease remains elusive. It is therefore important to keep in mind that the clinical use of anakinra will not only inhibit IL-1β signalling, but also that of IL-1α, and that the clinical effects observed after anakinra treatment might be attributed to blocking signalling of either one or the other, or both. Since caspase-1 is suspected to play a major role in auto-inflammatory syndromes and IL-1α is not a substrate for caspase-1, it is likely that at least in these types of diseases, it is the inhibition of IL-1β that leads to clinical improvement. This is supported by a study by Lachmann *et al.* who find excellent clinicial responses to anti-IL-1β monoclonal antibody treatment of four Muckle-Wells patients (35).

Identifying novel therapeutic targets for treatment of MKD

Although the use of anakinra has greatly benefited many patients suffering from auto-inflammatory diseases, not all patients respond to the treatment equally well, some not at all (Chapter 5). In addition, cytokine-targeted therapies are costly and some patients will require these drugs for life, which is of course a major disadvantage of this approach. Research should therefore continue to focus on new approaches to treat MKD and the other auto-inflammatory diseases. Figure 1 schematically depicts possible approaches for targeted therapy in MKD, as identified by the research described in this thesis.

The optimal therapy for MKD would be to overcome the direct consequence of mevalonate kinase deficiency, which is the shortage of nonsterol isoprenoids. In chapter 2 we describe that geranylgeranylpyrophosphate is the intermediate involved in increased IL-1β release by activated PBMC from MKD patients. We show this by interfering with the isoprenoid biosynthesis at various steps in the pathway. Unfortunately, simple administration of this isoprenoid intermediate is not feasible due to the instability of this compound. The use of upstream metabolites, such as farnesol or geraniol, which can be converted into farnesyl- and geranylpyrophosphate (36), could be of interest, since these compounds are much more stable (Figure 1, option 1). However, we have also shown that inhibiting the production of sterol isoprenoids with the squalene synthase inhibitor zaragozic acid can increase the flux towards the nonsterol end products and thereby decrease IL-1β release by activated MKD PBMC. These findings create a possibility for developing novel treatment regimes (Figure 1, option 2). Squalene synthase inhibitors are currently being investigated as cholesterol lowering agents (37) and might now turn out to be useful for the treatment of MKD as well. However, in MKD patients the metabolic balance in the isoprenoid pathway is fragile. Without disturbance, the pathway produces small (but adequate) amounts of its end products. Cholesterol levels, although within the normal range, are rather low and activity of HMG-CoA reductase, catalyzing the rate-limiting step of the pathway, reaches its maximum in these patients. The effects of further lowering cholesterol output using squalene synthase inhibitors in order to increase geranylgeranylpyrophosphate production might disturb the balance, the consequences of which will be hard to predict. Potential clinical trials with these compounds should therefore be very carefully monitored.

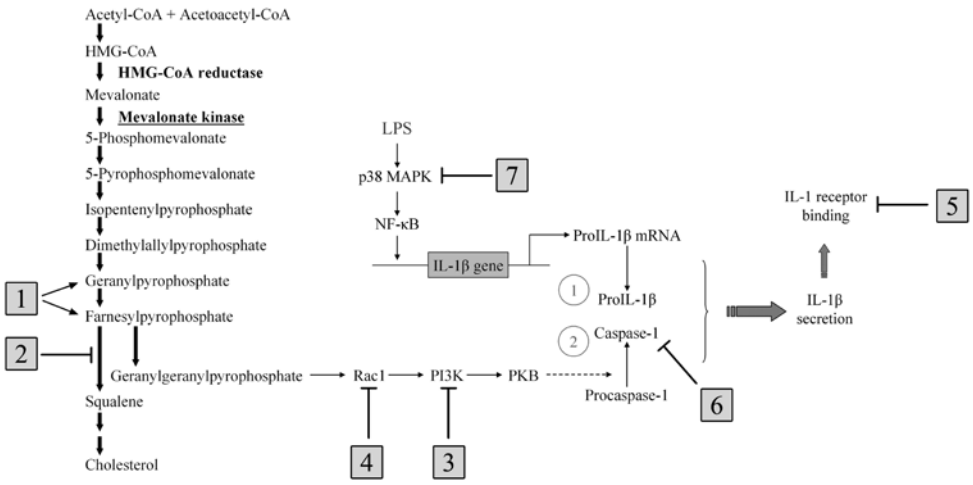

Figure 1: Possible approaches for targeted therapy in MKD.
The possible approaches for targeted therapy in MKD, as identified by the research described in this thesis, are schematically depicted. 1: administration of upstream metabolites farnesol (FOH) or geraniol (GOH) 2: inhibition of squalene synthase, 3: inhibition of PI3K isoforms, 4: inhibition of the small GTPase Rac1, 5: blocking IL-1 receptor signalling, 6: inhibition of caspase-1 proteolytic activity, 7: inhibition of p38 MAPK

In Chapter 4 we have succeeded in finding additional potential therapeutic targets for the treatment of MKD by identifying the molecular mechanism regulating simvastatin-mediated caspase-1 activation. Briefly, we found that impairment of isoprenoid biosynthesis in THP-1 monocytic cells causes an activation of the small GTPase Rac1, which in turn can activate phosphatidylinositol-3-kinase (PI3K) and protein kinase B (PKB/c-Akt), subsequently leading to activation of caspase-1. The finding that statin-enhanced activation of caspase-1 is mediated by PI3-kinase provides a rational basis for drug discovery (Figure 1, option 3), since chemical inhibitors for specific PI3K isoforms are already in phase I clinical trials, mostly for treatment of auto-immune disorders and various types of cancer (38-40). If further research can provide evidence that inhibition of PI3K can reduce caspase-1 activity in cells from MKD patients, as is expected from our data in Chapter 4, the PI3K inhibitors currently being evaluated might also prove to be effective for the use in mevalonate kinase deficiency. Moreover, chemical inhibitors have relatively low production costs and since these compounds are already in phase I clinical trials, a rapid introduction in the clinic is realistic. In addition, the findings described in Chapter 4 identify Rac1 as a target for intervention (Figure 1, option 4), particularly because in patient PBMC, inhibition of Rac1 was shown to strongly reduce LPS-induced IL-1β secretion. Rac inhibitors are also currently under investigation for the use in cancer therapy, although their use has not yet been developed to clinical trials.
The only largely successful targeted therapy for MKD, anakinra treatment, targets IL-1 receptor binding (Figure 1, option 5). As demonstrated in Figure 1, IL-1 receptor binding takes place at

a very late stage in the hypothesized order of events. One could therefore argue that anakinra treatment does not really target the cause of disease, but only diminishes symptoms, suggesting that anakinra treatment can never result in a complete cure. An alternative to blocking IL-1β signalling would be preventing the production and/or secretion of mature IL-1β. In some ways this approach would be an improvement over IL-1β receptor antagonism. Firstly, the IL-1 receptor binds not only to IL-1β, but also to IL-1α. In MKD, IL-1β is thought to be the primary cytokine involved in initiation of inflammation, so there would be no need to block signalling of other cytokines. In fact, the more cytokines that are blocked, the greater the chance of unwanted side effects. Secondly, instead of trying to block the effects of a circulating cytokine, prevention of mature IL-1β secretion requires targeting of the producer cells only, which may be easier to accomplish and is likely to have a lower impact on the patient, since fewer cells would be affected by such a drug. Prevention of IL-1β release has been investigated by means of an oral caspase-1 inhibitor, pralnacasan, as used in the experiments described in Chapter 3 (Figure 1, option 6). This compound was developed for the use in treatment of rheumatoid arthritis, but was predicted to be effective in MKD as well. Unfortunately, phase II clinical trials with pralnacasan were discontinued after animal toxicology reports demonstrated liver abnormalities after a nine-month exposure to high-dose pralnacasan (41). Currently, no other caspase-1 inhibitors have as yet been translated into successful clinical trials. Another method to decrease IL-1β secretion would be to prevent production of the inactive pro-form of IL-1β, for example by inhibition of p38 MAPK (Figure 1, option 7). Inhibition of p38 MAPK has been suggested before as an anti-inflammatory drug target (42). We have demonstrated in Chapter 4 that p38 MAPK is involved in LPS-mediated transcription of the IL-1β gene. However, p38 MAPK is known to be involved in a plethora of cellular processes, and the consequences of inhibition need to be more thoroughly studied. In addition, our experiments showed that, although p38 MAPK inhibition decreased IL-1β release, there seemed to be a delay in IL-1β transcription rather than a complete block. Therapeutic intervention aimed at p38 MAPK may therefore not be the best option.

Taken together, several new possibilities for targeted therapy have been identified. Of these possibilities, increasing the isoprenoid biosynthesis output is most likely to result in the best clinical response, as this would counter the direct consequence of mevalonate kinase deficiency. Unfortunately this is also a very difficult approach. Since trials with oral caspase-1 inhibitors have failed, the most promising targets now seem to be the signal transduction molecules involved in activation of caspase-1, Rac1 and PI3K. Until drugs targeting these newly identified targets have been developed, anakinra treatment remains the best treatment option for MKD.

Identifying the molecular mechanism underlying caspase-1 activation

Although several signalling modules have now been implicated in caspase-1 activation, many questions still remain concerning the regulatory mechanism underlying inflammasome assembly and caspase-1 activation. Further research into MKD and other auto-inflammatory syndromes might help elucidate more general mechanisms of caspase-1 activation. As described above, pyrin

(associated with FMF) and NALP3 (associated with the cryopyrin-associated periodic syndromes) are directly involved in the formation of caspase-1 activating multiprotein complexes, such as the inflammasome (Chapter 1) and the pyroptosome, which consists only of caspase-1 and ASC (43). Mutations in the genes encoding pyrin and NALP3 (*MEFV* and *CIAS1* respectively) were shown to enhance the assembly of these activating complexes (17-20). Mutated PSTPIP1, associated with the auto-inflammatory PAPA syndrome (Chapter 1), also enhances the formation of these complexes via its association with pyrin (21). Intensive research on these caspase-1 activating complexes has led to the identification of a large group of NALP3-related proteins, termed the CATERPILLAR family of proteins (44). This group now consists of 14 NALP proteins and also includes NOD1 and NOD2. Interestingly, mutations in the gene encoding NOD2 were found to be strongly associated with inflammatory conditions such as Blau syndrome and Crohn's disease (45-47). Mononuclear cells from Crohn's disease patients, carrying a NOD2 mutation, displayed enhanced IL-1β processing, most likely due to increased caspase-1 activity (48). These findings confirm that deregulation of caspase-1 activity is generally associated with a state of intermittent or chronic inflammation. Further research on the CATERPILLAR family of proteins and how they regulate caspase-1 activation will increase our knowledge on inflammatory processes and will hopefully also shed some light on how molecular signalling pathways regulate assembly of caspase-1 activating complexes. Currently, the precise molecular mechanism underlying caspase-1 activation remains unclear. One possible way to regulate its activation is through differential localization. Not much is known about the intracellular localization of NALPs, NODs and ASC, although recently NALP1 and 3 were shown to have a distinct subcellular distribution, NALP1 being mainly localized in the nucleus while NALP3 was predominantly cytoplasmic (49). Another important report describes the colocalization of caspase-1 and proIL-1β in a lysosomal compartment (50). However, the presence of caspase-1 does not necessarily infer that all components of the caspase-1 activating complexes are also present in that lysosomal compartment. Perhaps, inflammasome assembly takes place in the cytosol and caspase-1 dissociates from the complex after activation and translocates to, for example, lysosomes. Proteolytic cleavage of caspase-1 is known to occur between the CARD domain and the catalytic subunit, favouring the suggestion that active caspase-1 can dissociate from the inflammasome upon activation. Differential localization of caspase-1 may also explain some observations regarding its dual activity: processing of proIL-1β via assembly of the inflammasome and activation of NF-κB via binding of receptor interacting protein-2 (RIP2) (51;52). Lamkanfi *et al.* showed that caspase-1 mediated NF-κB activation is independent of proteolytic activity, therefore possibly also independent of inflammasome activity. In that way, uncleaved caspase-1 residing in, for example, the cytoplasm, might be responsible for NF-κB activation, whereas inflammasome-activated and thus cleaved caspase-1 may relocate to lysosomal compartments where it functions to process IL-1β. Indeed, Sarkar *et al.* were able to show that inhibition of inflammasome assembly with small interfering RNA to ASC decreased caspase-1 proteolytic activity, which was accompanied by an increase in NF-κB-inducing activity of caspase-1 (52). These data suggest that the caspase-1 activities might take place in different cellular compartments and when one of the activities of caspase-1 is inhibited, the other might

increase. If this is indeed the case, if differential localization is a regulatory mechanism for caspase-1 activity, could there be a role for phosphorylation of caspase-1? It is known that caspase-1 can be phosphorylated on serine 376 (53), but the effect or function of this phosphorylation remains unknown. Interestingly though, this phosphorylation is mediated by PAK1, which in its turn can be activated by Rac1. Given the finding that Rac1 is required for caspase-1 mediated IL-1β release by both THP-1 cells and MKD PBMC (Chapter 4), Rac1/PAK1-mediated phosphorylation of caspase-1 might prove to be a crucial step in assembly of functional inflammasomes.
Besides the Rac1/PI3K/PKB pathway, previous studies have implied a role for calcium-independent phospholipase A_2 ($iPLA_2$) in autoprocessing of caspase-1 (50). Specific inhibition of $iPLA_2$ resulted in a block in caspase-1 activation and IL-1β processing, whereas the secretion of procaspase-1, proIL-1β and the lysosomal marker cathepsin D was not affected. This suggests that $iPLA_2$ can somehow activate inflammasomes and/or pyroptosomes. Do these two signal transduction pathways overlap? Or is the PI3K/PKB pathway specific for mevalonate kinase deficiency-induced inflammation? If so, what signal transduction pathways are involved in the other auto-inflammatory syndromes? It will be interesting to see if common signal transduction pathways exist between the different auto-inflammatory conditions, since this would create possibilities to develop more broadly applicable therapies.

Future research

As demonstrated by the many remaining questions, the mechanism of caspase-1 activation is complex and its regulation appears to be tightly controlled. Further elucidation of the molecular and regulatory mechanisms and the signal transduction routes involved in caspase-1 activation will advance our knowledge on inflammation in general and on auto-inflammatory syndromes in particular. A useful tool in this research will be the development of disease models for the various auto-inflammatory syndromes. NALP3-deficient mice have already been generated. Research on these mice confirmed the requirement of NALP3 for caspase-1 mediated IL-1β release. Macrophages from $NALP3^{-/-}$ mice did not secrete IL-1β or IL-18 in response to a variety of stimuli due to defective caspase-1 activation (17;54-56). Not surprisingly, generation of *MVK* double knockout ($MVK^{-/-}$) mice has repeatedly failed in the past, most likely due to the fact that these mice can no longer produce cholesterol. Recently however, Hager *et al.* have described *MVK* single allele mice ($MVK^{+/-}$) that display significantly reduced liver mevalonate kinase activity (57). Cholesterol and isoprene end-products were normal, but mevalonate concentrations were increased in several organs. There was also a significant increase in serum IgD levels. These preliminary findings suggest that loss of a single *MVK* allele provides a relevant model for human MKD. However, further studies will need to clarify whether these mice also develop inflammatory symptoms when challenged with an immunological trigger. What is even more interesting, is the current use of knock-in techniques to generate a "true" mevalonate kinase deficient mouse, in which the wildtype *MVK* alleles are replaced by the V377I variant, the most commonly observed mutation in MKD. Instead of the ~50% residual activity found in the $MVK^{+/-}$ mice, these mice are

predicted to display less than 10% residual mevalonate kinase activity, more truly reflecting the physiological condition of human MKD. Hopefully, this mouse model will form an even better basis for studying the molecular and cellular mechanisms of mevalonate kinase deficiency than the *MVK*$^{+/-}$ mice. MKD mice will be useful to confirm the importance of *in vitro* findings, such as the molecular mechanisms involved in enhanced caspase-1 activation and the signal transduction routes involved. In addition, mouse models form the perfect platform to try new and experimental therapies before having to introduce them into the clinic. However, new findings resulting from these studies should still be very carefully interpreted. Mechanisms and regulatory systems can still differ between mice and men. Indeed, when it comes to caspase-1 activation, some important differences seem to exist between the two. Mouse caspase-1 does not appear to be secreted into the extracellular environment upon activation as it is by human cells, as demonstrated by the fact that active caspase-1 subunits are easily detectable in mouse cell extracts whereas human caspase-1 subunits are not (58). Unfortunately, not much is known of caspase-1 regulatory differences between the two species and more research is required before findings in human cells can be translated into mouse disease models and vice versa. Nonetheless, the generation of a disease model for MKD will surely result in great advances in MKD research.

Concluding remarks

MKD is an extremely rare disease, with approximately 200 diagnosed patients worldwide. The disease is usually not fatal and both severity and frequency of attacks gradually decline with age in the majority of patients, leaving them relatively healthy by the time they reach adulthood. So what is the added value of studying this disease in detail? As William Harvey already recognised in 1657, "...there is no better way to advance the proper practice of medicine than ... by careful investigation of cases of rarer forms of disease" (59). Studying rare diseases often results in important medical insights. An important reason for this is the fact that rare diseases are often the result of a single gene mutation producing a single defective protein, which creates a lot more clarity concerning defect versus consequence than is the case for more multi-factorial diseases. Studying MKD and other rare monogenetic auto-inflammatory disorders over the past few decades has greatly improved the knowledge on inflammation and the innate immune system. The identification of the CATERPILLAR family of proteins has given rise to a new "hype" within the field of innate immunity and its members will quite possibly prove to be involved in many incompletely resolved inflammatory disorders. Although the contribution that studying rare disorders can have for medical practise in general is an important motive, the suffering of patients, whether it is of a common or extremely rare disease, is reason enough for studying a disease in detail. Hopefully, the work described in this thesis will contribute to the MKD research field and will eventually result in the development of new therapies for those few, but very ill, MKD patients.

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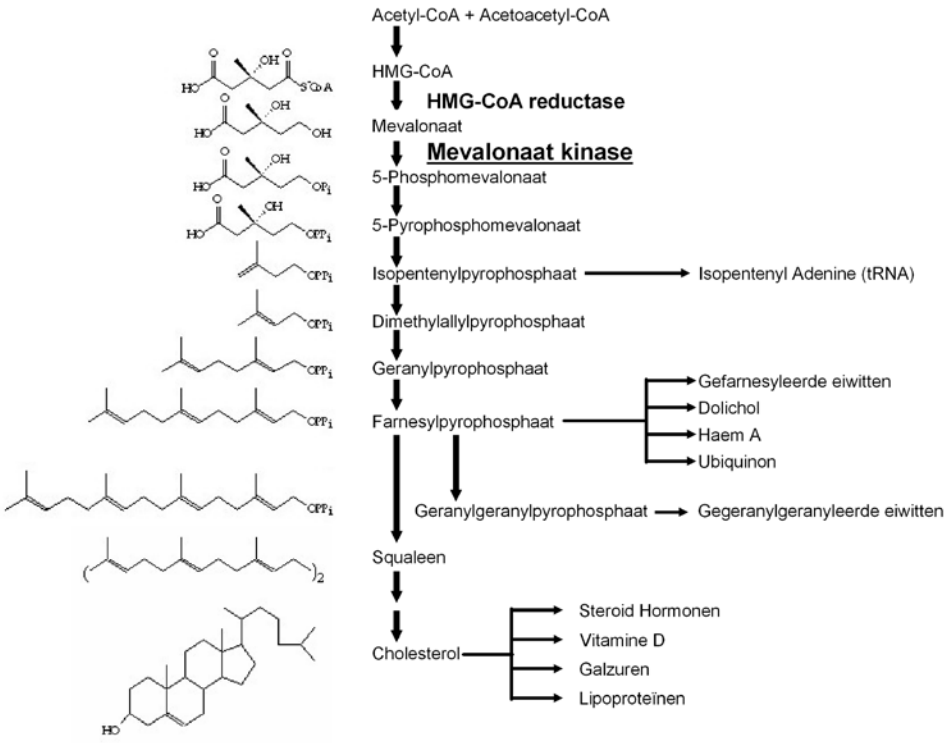
Nederlandse samenvatting

Het onderzoek beschreven in dit proefschrift, gaat over een aandoening genaamd hyperimmunoglobulinaemia D en periodiek koorts syndroom (HIDS), nu beter bekend onder de naam mevalonaat kinase deficiëntie (MKD). Dit is een genetisch overerfbare aandoening, wat wil zeggen dat het veroorzaakt wordt door een 'foutje' in het genetisch materiaal, het DNA. In 1999 hebben twee onderzoeksgroepen beschreven waar in het DNA dat foutje precies zit en dat dit stukje DNA codeert voor een enzym genaamd mevalonaat kinase. Dit enzym wordt door het foutje in de 'code' verkeerd in elkaar gezet, wordt daardoor erg instabiel en valt uit elkaar. Dit heeft tot gevolg dat de patiënten een tekort, een deficiëntie krijgen van dit enzym (vandaar de naam mevalonaat kinase deficiëntie).
Het enzym mevalonaat kinase speelt een belangrijke rol bij de aanmaak van cholesterol (zie Figuur 1). Cholesterol is vooral bekend door de relatie met hart- en vaatziekten en heeft zodoende een negatief imago, maar in feite is cholesterol een essentiële bouwstof van ons lichaam. Cholesterol houdt het celmembraan, het 'omhulsel' van cellen intact en is daarmee cruciaal voor het voortbestaan van de cel. Kort gezegd, zonder cholesterol ga je dood. Gelukkig hebben patiënten met MKD nog een beetje mevalonaat kinase over, het deel dat niet direct uit elkaar valt en dus nog wel functioneert. Deze zogenaamde restactiviteit van mevalonaat kinase in patiënten is ongeveer 1 tot 10% van wat een gezond persoon aan activiteit heeft. Al deze restactiviteit wordt in patiënten aangewend om cholesterol te maken, omdat dat zo belangrijk is voor het overleven van de cel. Patiënten met MKD hebben daarom wel een iets verlaagd cholesterolgehalte in hun bloed, maar niet abnormaal of ongezond laag. Zoals echter blijkt uit Figuur 1, wordt het enzym mevalonaat kinase niet alleen maar gebruikt voor de aanmaak van cholesterol. Er zijn nog een aantal andere eindproducten die met behulp van mevalonaat kinase worden gemaakt. Door de relatief normale cholesterolwaardes van patiënten is de hypothese ontstaan dat juist een tekort aan andere eindproducten er de oorzaak van is dat deze patiënten ziek worden. Welke eindproducten dat zijn en hoe een tekort daaraan dan kan leiden tot koortsaanvallen was nog onbekend. Het onderzoek beschreven in dit proefschrift heeft een antwoord proberen te geven op deze vragen.

Mevalonaat kinase deficiëntie (MKD) is een zogenaamde auto-inflammatoire aandoening. Auto-inflammatoire aandoeningen zijn verwant aan auto-immuunziekten, zoals reuma, de ziekte van Crohn en systemische lupus erythematosus (SLE). Bij die aandoeningen is het lichaam het vermogen verloren om het verschil te zien tussen vreemde indringers en lichaamseigen weefsels en cellen. Het immuunsysteem gaat eigen weefsels en cellen daarom aanvallen en afbreken. Dit heeft onder andere een chronische ontstekingsreactie tot gevolg. Auto-inflammatoire aandoeningen onderscheiden zich van auto-immuunziekten doordat bij deze ziektes een ontstekingsreactie ontstaat, niet ten gevolge van het aanvallen van lichaamseigen cellen en weefsels, maar spontaan, ogenschijnlijk zonder directe aanleiding.
Kinderen met MKD krijgen om de paar weken een heftige aanval van koorts, die vaak gepaard gaat met huiduitslag, pijnlijke gewrichten, opgezette lymfklieren, buikpijn, hoofdpijn en diarree. Bij de meer ernstig zieke patiënten kunnen zelfs symptomen als degeneratie van de kleine hersenen en het netvlies voorkomen en deze kinderen hebben vaak groei- en ontwikkelingsachterstanden.



Figuur 1: Schematische weergave van de cholesterol biosynthese

De aanvallen van koorts zijn het meest frequent en ook het meest ernstig in de eerste levensjaren, daarna wordt dat geleidelijk minder en de meerderheid van de patiënten is relatief gezond tegen de tijd dat ze de volwassen leeftijd bereiken. Vaak worden aanvallen uitgelokt door bijvoorbeeld vaccinaties of kleine infecties. Dit verschijnsel deed het vermoeden ontstaan dat kinderen met auto-inflammatoire aandoeningen moeite hebben om hun eigen ontstekingsreactie af te remmen. Gezonde mensen ondervinden na een vaccinatie meestal geen klachten, hoogstens wat opgezette lymfklieren, en pas bij een echte infectie met bijvoorbeeld een griepvirus zal een volledige ontstekingsreactie met koorts, pijnlijke spieren en soms braken of diarree opgestart worden. Daarentegen zetten mensen met auto-inflammatoire aandoeningen deze volledige ontstekingsreactie al bij de minste of geringste trigger, zoals een vaccinatie, in gang.

Bij gezonde mensen wordt een ontstekingsreactie in gang gezet door middel van signaalstoffen in het bloed. De immuuncellen van het lichaam, de witte bloedcellen, patrouilleren het lichaam en herkennen alles wat niet lichaamseigen is, zoals bacteriën en virussen. Op het moment dat ze een dergelijk potentieel gevaar tegenkomen zullen ze het lichaam hiertegen willen waarschuwen. Dit doen ze door het uitscheiden van signaalstoffen, genaamd cytokines. Eenmaal uitgescheiden circuleren deze cytokines ook in het bloed en kunnen daardoor alle cellen van het lichaam bereiken.

De andere cellen van het lichaam 'zien' de cytokines met behulp van receptoren aan de buitenkant van de cel, op het celmembraan, waar de cytokines aan kunnen binden. Als het cytokine bindt, dan weet de cel dat er gevaar dreigt en zal op zijn beurt weer andere cytokines gaan uitscheiden en zelf allerlei processen in gang zetten. Zo begint de ontstekingsreactie. De opeenvolging van alle processen zal uiteindelijk leiden tot temperatuurverhoging, aanmaak van meer witte bloedcellen, pijnsensaties, etc., kortom koorts en ontsteking.
Onderzoek bij MKD patiënten heeft uitgewezen dat tijdens de koortsaanvallen er in het bloed van deze patiënten verhoogde gehalten te vinden zijn van allerlei cytokines. Dit duidt erop dat (de cellen van) deze patiënten in een soort van verhoogde staat van paraatheid verkeren, dat de immuuncellen denken in gevaar te verkeren, terwijl er geen spoor is van virussen, bacteriën of andere potentiële bedreigingen in deze patiënten. Een belangrijke bevinding is dat witte bloedcellen van patiënten, zelfs buiten koortsaanvallen om, een belangrijk cytokine genaamd interleukine-1beta (IL-1β) produceren. Van IL-1β wordt gedacht dat het echt aan het begin staat van het ontstekingsproces. Het lijkt er dus op dat witte bloedcellen van patiënten met MKD te snel iets als gevaarlijk bestempelen, bijvoorbeeld een vaccinatie, waar een gezond persoon daar niet of nauwelijks op reageert. Het komt zelfs voor dat witte bloedcellen van patiënten zonder een enkele identificeerbare aanleiding spontaan IL-1β beginnen te maken en daarmee een ontstekingsreactie (inflammatie) in gang zetten. Vandaar dat MKD wordt geclassificeerd als een auto-inflammatoire aandoening.

Van mevalonaat kinase deficiëntie is dus bekend dat het wordt veroorzaakt door een tekort aan het enzym mevalonaat kinase en dat dit uiteindelijk leidt tot de spontane uitscheiding van IL-1β door witte bloedcellen van patiënten. De spontane uitscheiding van IL-1β heeft een ontstekingsreactie tot gevolg waardoor patiënten last krijgen van koortsaanvallen en andere symptomen als huiduitslag en pijnlijke gewrichten. Deze gegevens stelden onderzoekers voor een raadsel, gezien er niet eerder een verband was gelegd tussen de aanmaak van cholesterol en ontstekingsprocessen, een ogenschijnlijk onlogisch en onverklaarbaar verband. Het verrichtte onderzoek heeft getracht een oplossing te vinden voor dit raadsel.

In het eerste deel van **hoofdstuk 1** wordt een overzicht gegeven van de meest voorkomende auto-inflammatoire aandoeningen, waaronder mevalonaat kinase deficiëntie (MKD), met alle bijbehorende symptomen en karakteristieken. Er wordt aangegeven welke genetische oorzaak er aan elke ziekte ten grondslag ligt, hoe vaak en waar de ziekte voorkomt en wat de huidige behandelmethoden zijn. Verder wordt er besproken hoe een diagnose kan worden gesteld op basis van een aantal criteria.
In deel twee van hoofdstuk 1 wordt wat dieper ingegaan op MKD. De biochemische achtergrond, de klinische karakteristieken en de rol van IL-1β in de ziekte worden verder toegelicht.

Tijdens het onderzoek beschreven in **hoofdstuk 2** is uitgezocht aan welke eindproducten van het cholesterol biosynthese proces precies een tekort ontstaat in geval van mevalonaat kinase deficiëntie.

Dit is gedaan met behulp van een modelsysteem met gezonde cellen. Deze cellen werden behandeld met remmers van verschillende enzymen van de biosynthese route, bijvoorbeeld met de remmer simvastatine. Simvastatine remt het enzym HMG-CoA reductase (Figuur 1), het enzym dat net één stap voor mevalonaat kinase functioneert in het biosynthese proces. HMG-CoA reductase zorgt dus voor de aanmaak van mevalonaat, dat vervolgens door mevalonaat kinase kan worden omgezet in het volgende product: phospho-mevalonaat (Figuur 1). Door gezonde cellen te behandelen met een remmer van HMG-CoA reductase, simvastatine, kan een deficiëntie van mevalonaat kinase nagebootst worden, immers door HMG-CoA reductase te remmen ontstaat er een tekort aan eindproducten, net als bij patiënten met MKD. Gezonde cellen waarin MKD werd nagebootst met behulp van simvastatine, reageerden daarop met het uitscheiden van IL-1β, precies zoals in het bloed van MKD patiënten lijkt te gebeuren. Vervolgens werd gekeken naar de effecten van het remmen van de andere enzymen op de uitscheiding van IL-1β. Op deze manier kon achterhaald worden dat een tekort aan geranylgeranylpyrophosphaat (GGPP) er de oorzaak van was dat deze cellen IL-1β gingen uitscheiden en niet een tekort aan isopentenyl adenine, squaleen, dolichol, ubiquinon, haem A of gefarnesyleerde eiwitten (Figuur 1). Deze bevinding werd bevestigd toen de spontane uitscheiding van IL-1β door witte bloedcellen van MKD patiënten kon worden geremd door GGPP aan deze cellen toe te voegen.

Het onderzoek beschreven in **hoofdstuk 3** had tot doel te analyseren hoe de spontane uitscheiding van IL-1β door witte bloedcellen van MKD patiënten gereguleerd wordt. Het is bekend dat IL-1β, als het de witte bloedcel nog niet verlaten heeft, zich in een inactieve vorm bevindt. Op het moment dat een witte bloedcel een immuunreactie in gang wil zetten, wordt er als het ware een stukje van het inactieve IL-1β 'afgeknipt', waarna het actief wordt en de cel kan verlaten en andere cellen kan gaan waarschuwen. Dit 'knippen' gebeurt door het enzym caspase-1. Dit enzym bevindt zich zelf ook in een inactieve staat in de witte bloedcel, anders zou het voortdurend inactief IL-1β blijven knippen. Met behulp van het modelsysteem zoals beschreven in hoofdstuk 2 is aangetoond dat er twee stappen nodig zijn voordat IL-1β uiteindelijk de cel kan verlaten. De eerste stap is het aanmaken van inactief IL-1β. Dat gebeurt bijvoorbeeld na blootstelling aan bepaalde bestanddelen van bacteriën. Deze stap bereidt de cel als het ware voor op mogelijk gevaar, maar er wordt nog niet tot de aanval overgegaan. De tweede stap, waarbij daadwerkelijk de aanval wordt ingezet, bestaat uit de activatie van caspase-1, dat het inactieve IL-1β kan knippen, waarna het de cel verlaat. Die tweede stap wordt bij gezonde mensen niet zo snel genomen, pas als er echt gevaar dreigt. Onderzoek aan het modelsysteem voor MKD heeft uitgewezen dat behandeling met simvastatine, waarmee MKD nagebootst wordt, leidt tot activatie van het enzym caspase-1. Dit suggereert dat patiënten met MKD, net als gezonde mensen, reageren op een vaccinatie of kleine infectie door het aanmaken van inactief IL-1β, maar dat zij door hun deficiëntie, in tegenstelling tot gezonde mensen, ook al actief caspase-1 klaar hebben liggen, waardoor het aangemaakte inactieve IL-1β direct geknipt en actief wordt, uitgescheiden wordt, wat weer leidt tot koorts en ontsteking.

In **hoofdstuk 4** is getracht het verband te leggen tussen hoofdstuk 2 en hoofdstuk 3. Hoe leidt een tekort aan GGPP tot activatie van caspase-1? GGPP is een molecuul dat in de cel wordt vastgekoppeld aan een groep van eiwitten, genaamd GTPases, die hun functie uitoefenen in of vlak bij het celmembraan. Het celmembraan is een vettig omhulsel van de cel, wat de waterige inhoud van de cel scheidt van de eveneens waterige omgeving, immers vet en water mengen niet. GGPP is ook een vettige substantie en kan dus wel mengen met het celmembraan, maar niet zo goed met de waterige inhoud van de cel. Door een GGPP molecuul te koppelen aan een eiwit, wat zich juist weer liever in water bevindt, fungeert het GGPP molecuul als een soort anker waarmee het eiwit in het membraan wordt vastgelegd. Op die manier wordt die groep van eiwitten, de GTPases, vastgelegd op de plaats waar ze hun functie behoren uit te oefenen, namelijk bij het celmembraan. Bij een gebrek aan GGPP, bijvoorbeeld als gevolg van mevalonaat kinase deficiëntie, zullen de GTPases dus niet meer worden vastgelegd in het membraan, maar ergens anders in de cel blijven hangen. Bovendien hebben eerdere onderzoeken naar het functioneren van GTPases aangetoond dat een gebrek aan GGPP ook kan leiden tot spontane activatie van deze eiwitten. Kortom, gebrek aan GGPP heeft tot gevolg dat GTPases spontaan actief kunnen worden en bovendien op een verkeerde plek in de cel. Het onderzoek beschreven in hoofdstuk 4 laat zien dat behandeling van gezonde cellen met simvastatine inderdaad leidt tot de activatie van één specifiek GTPase, genaamd Rac1. Verder is gebleken dat de activatie van Rac1 een opeenvolging van activaties tot gevolg heeft, wat uiteindelijk resulteert in de activatie van caspase-1. Patiënten met MKD lijken dus als gevolg van een tekort aan GGPP een continu actief Rac1 in hun witte bloedcellen te hebben, waardoor Rac1 zorgt voor een aanhoudende activatie van een aantal andere eiwitten resulterend in activatie van caspase-1.

In het onderzoek beschreven in **hoofdstuk 5** is gekeken naar de rol van de verschillende cytokines en vooral naar de rol van IL-1β. Van IL-1β wordt sinds lange tijd vermoed dat het een grote rol speelt in MKD en ook in andere auto-inflammatoire aandoeningen. Dit vermoeden werd bevestigd door recente onderzoeken naar de effectiviteit van het medicijn anakinra bij de behandeling van auto-inflammatoire aandoeningen. Anakinra is een medicijn dat de werking van IL-1β tegengaat. Het bindt aan de receptoren van lichaamscellen, waardoor die het IL-1β niet meer kunnen 'zien' en dus ook niet meer reageren als dit cytokine in de bloedbaan verschijnt. Waarschijnlijk werkt dit medicijn zo goed, omdat IL-1β een zogenaamd 'primair' cytokine lijkt te zijn, dat aan het begin staat van de opeenvolging van reacties die uiteindelijk leidt tot inflammatie. IL-1β is eigenlijk als een sneeuwbal die van een besneeuwde berghelling wordt gegooid en uiteindelijk een lawine tot gevolg heeft. Het is gemakkelijk èn effectief om de sneeuwbal te stoppen in plaats van de lawine.
De vraag was of IL-1β inderdaad de sneeuwbal is en behandeling met anakinra daadwerkelijk een lawine kan voorkomen door de productie van andere cytokines te remmen. Daartoe is gekeken naar de productie van cytokines door witte bloedcellen van zowel patiënten als gezonde mensen en wat daarmee gebeurt in de aanwezigheid van anakinra. Bovendien zijn de cytokine concentraties in het bloed van patiënten gemeten vóór, tijdens en na behandeling met anakinra. Het onderzoek wees uit dat anakinra inderdaad de productie van andere cytokines door witte bloedcellen kan

remmen door simpelweg te voorkomen dat IL-1β bindt aan zijn receptoren. Behandeling van MKD patiënten met anakinra bracht bovendien een verlaging van cytokine concentraties in de bloedbaan teweeg. Dit ging regelmatig, maar helaas lang niet altijd, gepaard met een verbetering van de ziekteverschijnselen. Blijkbaar is IL-1β wel belangrijk, maar is het niet de enige factor die een rol speelt bij het in gang zetten van een koortsaanval.

Hoofdstuk 6 is een zogenaamd casus verslag, een korte beschrijving van een patiëntje met Familiaire Mediterrane Koorts, een andere auto-inflammatoire aandoening, dat niet reageerde op de standaard therapie voor deze ziekte. Omdat vermoed wordt dat IL-1β niet alleen bij MKD, maar ook bij andere auto-inflammatoire aandoeningen een grote rol speelt, werd na goedkeuring van patiënt en ouders een kuur met anakinra geprobeerd bij deze patiënt. Anakinra behandeling van deze patiënt leidde tot een bijna complete remissie van de koortsaanvallen. Waar de patiënt vóór behandeling gemiddeld een maal per week een koortsaanval kreeg, heeft ze na behandeling drie koortsaanvallen gehad in 26 maanden tijd. Ook andere verschijnselen als buikpijn en pijnlijke gewrichten zijn verdwenen na behandeling met anakinra. Deze resultaten onderstrepen de belangrijke rol van IL-1β en bevestigen de therapeutische mogelijkheden van anakinra in de behandeling van auto-inflammatoire aandoeningen.

In **hoofdstuk 7** worden de bevindingen, zoals beschreven in dit proefschrift, bediscussieerd en in de context van reeds bestaande literatuur geplaatst. Er wordt besproken welke andere factoren, naast IL-1β, een belangrijke rol zouden kunnen spelen en er worden tevens suggesties gedaan voor het ontwikkelen van nieuwe therapieën voor de behandeling van MKD.

Samenvattend is door middel van het uitgevoerde onderzoek duidelijk geworden dat het genetisch defect in patiënten met MKD leidt tot een verminderde functionaliteit van het enzym mevalonaat kinase. Dit heeft een tekort aan geranylgeranylpyrophosphaat (GGPP) tot gevolg, wat weer een hyperactivatie van het eiwit Rac1 veroorzaakt. Het overactieve Rac1 activeert op zijn beurt een aantal andere eiwitten, uiteindelijk resulterend in activatie van caspase-1. Het actieve caspase-1 zal het inactieve interleukine-1beta (IL-1β) gaan knippen zodra dat aangemaakt wordt, bijvoorbeeld na een vaccinatie of kleine infectie. Het spontaan uitgescheiden IL-1β veroorzaakt vervolgens koorts en ontsteking. Het verhinderen van het effect van IL-1β met behulp van anakinra heeft in veel, maar niet in alle gevallen een gunstige uitwerking op patiënten. De resultaten in dit proefschrift bieden verder nieuwe aanknopingspunten voor het ontwikkelen van additionele therapieën voor de behandeling van MKD en wellicht ook voor andere auto-inflammatoire aandoeningen.

Dankwoord

Curriculum Vitae

List of publications

Dankwoord

"The voyage of true discovery lies not in seeking new landscapes,
but in seeing with new eyes."
Marcel Proust

De reis is tot een einde gekomen, de taak is volbracht. Dat ik veel dingen met nieuwe ogen heb leren zien heb ik te danken aan heel veel mensen. Al die mensen wil ik hartelijk danken en aantal in het bijzonder.

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Loes

Curriculum Vitae

Loes Kuijk werd op 28 juni 1977 geboren te Roermond. Na het behalen van haar VWO diploma in 1995, aan de Stedelijke Scholengemeenschap te Roermond, begon zij aan de studie Biologie (Fundamentele Biomedische Wetenschappen) aan de Universiteit Utrecht. Als onderdeel van deze studie verrichtte zij in 1998, onder begeleiding van Dr. J.A. Post, zes maanden onderzoek bij de afdeling moleculaire celbiologie van de Universiteit Utrecht. Vervolgens voerde zij gedurende het collegejaar 1998/1999 een stage-onderzoek van 9 maanden uit bij de afdeling Pediatrische Immunologie in het Wilhelmina Kinderziekenhuis in Utrecht, onder begeleiding van Dr. ir. G.T. Rijkers. Na deze stage heeft zij nog een derde onderzoeksproject gedaan bij GlaxoWellcome Medical Research Centre in Stevenage, UK, onder begeleiding van Dr. J. Angier. Het doctoraalexamen werd behaald in 2001, waarna zij 9 maanden werkzaam was als moleculair bioloog in dienst van Yacht Technology. In 2003 werd zij aangenomen als assistent in opleiding bij de afdeling Kinderen in het Wilhelmina Kinderziekenhuis, onder begeleiding van Dr. J. Frenkel en Dr. ir. G.T. Rijkers. Na drie jaar is zij van het pediatrische immunologie lab verhuisd naar het moleculaire immunologie lab van Prof. dr. Paul Coffer, die ook een deel van de begeleiding op zich nam. De resultaten van het verrichte onderzoek zijn beschreven in dit proefschrift.

List of publications

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HMG-CoA reductase inhibition induces IL-1β release through Rac1/PI3K/PKB-dependent caspase-1 activation. Rac1 as a potential target for the treatment of MKD
Submitted

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