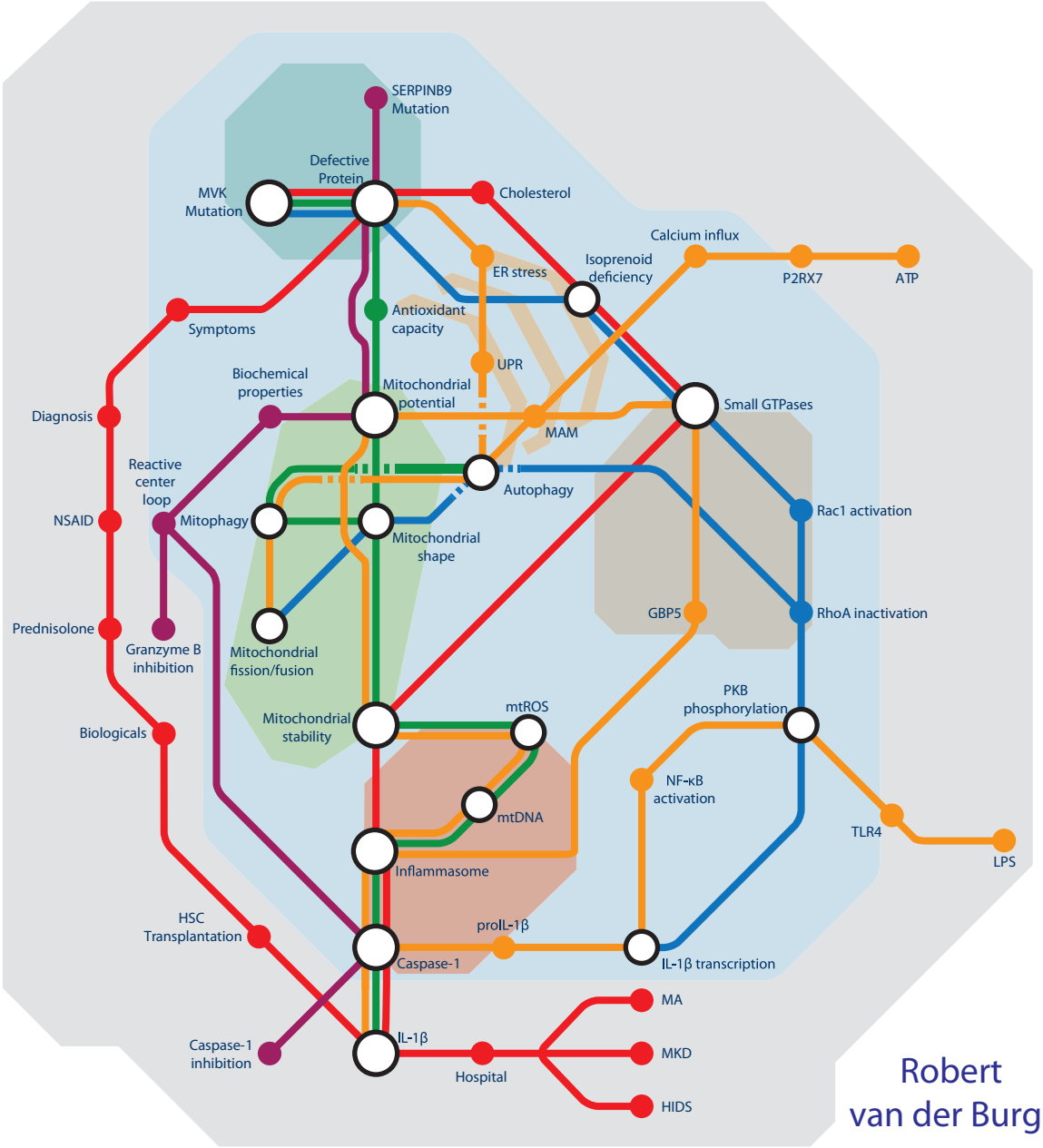
Mechanisms that Kindle Disease in Periodic Hereditary Diseases

How Isoprenoid Deficiency and SerpinB9 Mediate Autoinflammation



Robert van der Burgh

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Mechanisms that Kindle Disease in Periodic Hereditary Diseases

How Isoprenoid Deficiency and SerpinB9 Mediate Autoinflammation

Mechanismen die ziekte aanwakkeren in periodieke erfelijke ziekten

Hoe isoprenoïden deficiëntie en SerpinB9 autoinflammatie mediëren

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor

aan de Universiteit Utrecht

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Voor mijn ouders en de wetenschap

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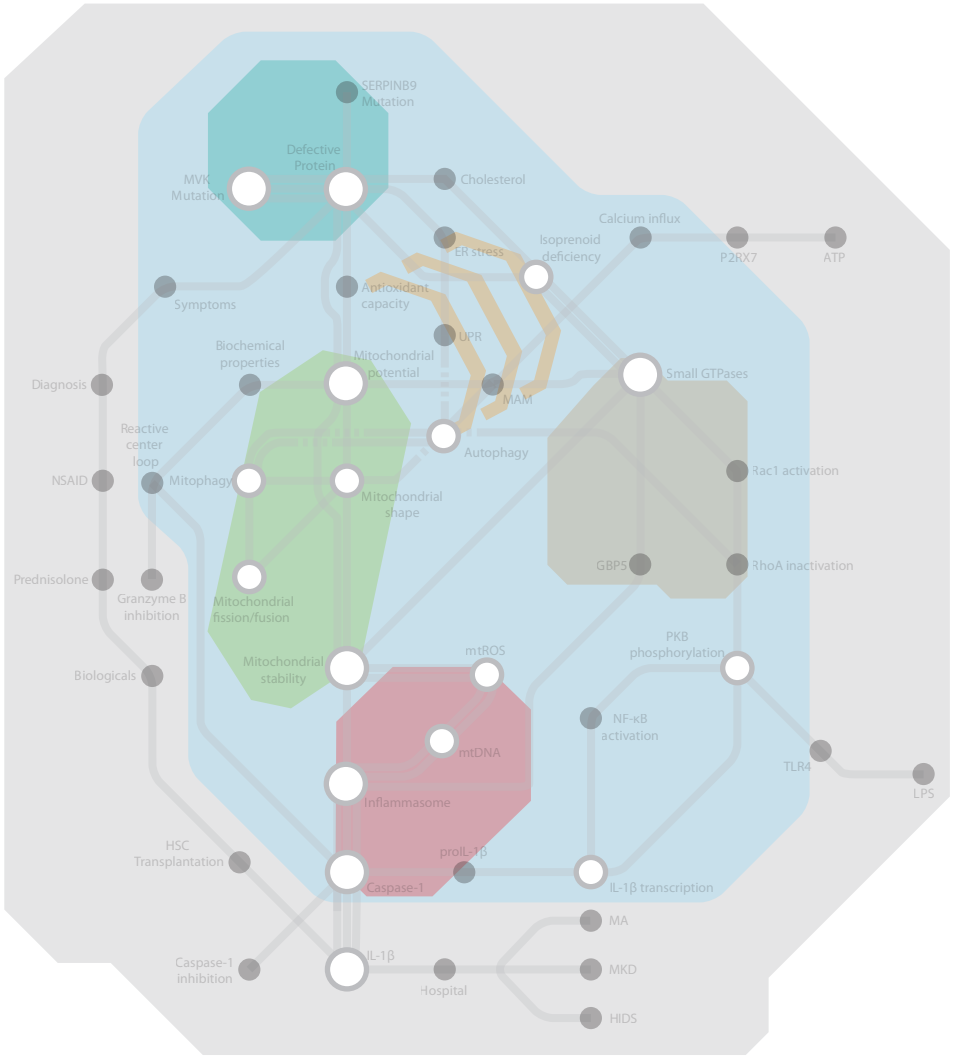
Contents


SERPINB9 Mutation
Defective Protein
MVK Mutation
Cholesterol
Calcium influx
Isoprenoid deficiency
P2RX7
ATP
ER stress
Antioxidant capacity
Symptoms
UPR
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Reactive center loop
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Rac1 activation
NSAID
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Mitochondrial shape
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MA
Caspase-1 inhibition
IL-1β
MKD
Hospital
HIDS

CHAPTER 1

General Introduction

1

The human body is equipped with an intricate system to protect itself from pathogens and foreign material. The innate defense provides the first line of protection. The detection of foreign material triggers two immediate reactions. First, it causes an enzymatic reaction called the complement cascade that opsonizes the material and stimulates its phagocytosis. More interesting perhaps, it simultaneously triggers a cellular reaction by which they release mediators that induce inflammation and prepare the site for the arrival of specialized immune cells. These cells also stimulate the clearance of the invading material. Foreign material is detected by the immune system's many pattern recognition receptors, which detect conserved molecular patterns that are usually not present in humans. When the foreign material is cleared, the recognition receptors are no longer triggered and the inflammation is slowly resolved. If the immune system is triggered without foreign agents being present, inflammation is still induced. In this case it is referred to as sterile inflammation. The signaling cascade leading to sterile inflammation is indistinguishable from pathogen-induced inflammation and can help with physiological processes such as wound healing(1). Inflammation is usually transient and resolved in a relatively short time span, however, if sterile inflammation occurs regularly and servers no particular purpose it proves to be dysfunctional and becomes known as autoinflammatory disease (AID). Patients suffering from AID have periodic fever episodes which can be accompanied by numerous other inflammation-related symptoms and discomforts(2). It is different from autoimmunity as the inflammation is not chronic; usually the inflammation is resolved, followed by a remission period until the next episode. Although AID can be diagnosed by symptoms alone, most are defined and characterized by underlying genetic mutations. It is generally recognized that not all AID are caused by a single mutation. On a cellular level AID originates from the circulating monocytes in the blood. Their activation leads to the release of the inflammatory cytokines: Interleukin (IL) 1β, IL-6, Tumor necrosis factor (TNF) and IL-18. These cytokines induce inflammation and IL-1β, in particular, leads to fever. The current treatment strategies are limited and involve general immunosuppression with steroids, or biologicals against IL-1β, IL-6 or TNF. Good clinical management of AID however requires better and faster recognition of the phenotype, as well as the identification of therapeutic intervention points which do not lead to general immune suppression and the related undesirable side effects.

In this thesis we explore the molecular mechanisms that lead to the release of IL-1β in the monogenetic AID mevalonate kinase deficiency in the hope of identifying common pathways, which are shared by multiple AID, to increase the chances of developing a therapeutic intervention.

Monocytes and the innate immune system

The immune system is divided into two branches, the innate and adaptive immune system. The adaptive immune system can modify its recognition receptors to fit nearly every molecular pattern of invading material, but this adaptation takes days to weeks to develop. The innate immune system instead uses a defined set of common molecular patterns to respond quickly to any foreign material or endogenous danger signals. This response is not very specific but initiates general inflammation to combat the invading pathogen. This innate immune response is dominant during the first days. In the meantime cells of the adaptive immune system are primed and recruited to the inflammatory site. After the initial innate phase the adaptive immune system takes over to remove the pathogen and hereafter the inflammation will resolve.

The innate system uses designated receptors to identify molecular patterns associated with pathogens, inert foreign material and endogenous danger signals. The best-studied class of

pattern recognition receptors (PRR) is the toll like receptors (TLRs). The TLRs are an evolutionary conserved class of receptors and they recognize general molecular patterns such as lipopolysaccharide, double stranded RNA and single stranded DNA. The TLRs are found on the plasma membrane and sense the extracellular environment or the inside of endocytic vesicles(3). The cytosolic environment is probed for pathogens or foreign material by a specialized class of receptors. Characteristic for these receptors are the NOD like receptors (NLRs) (4). Some of the recognized patterns are also present within the human body. The distribution of these receptors is therefore regulated to only detect the foreign or endogenous material in case of altered localization, for instance in cases of tissue damage.

PRR recognition receptors are not distributed equally across all cell types. The immune system utilizes specialized cells for swift and specific detection of foreign agents. Monocytes, macrophages and dendritic cells contain nearly all PRRs and are the first cells to respond to danger signals. The dendritic cells take up foreign matter to present to, and prime the cells of the adaptive immune system. Macrophages are distributed within tissues and in case of inflammation can phagocytize pathogens and dead cells. When damage occurs in the tissues the macrophages are amongst the first responders. Monocytes are the precursors of macrophages and are mostly found in the bloodstream. When monocytes leave the bloodstream and move into tissue, usually in response to inflammatory signals, they differentiate into macrophages. Monocytes are very sensitive and readily activated; they react to stimuli within hours.

The activation of monocytes and macrophages results in the release of inflammatory cytokines. These cytokines propagate the inflammatory signal to the surrounding cells. In normal situations, in which a pathogen comes into contact with macrophages, the induced inflammatory response remains localized. Immune cells are recruited from the blood to the location. The local tissue is prone to swelling and a slightly increased temperature. If the released cytokines reach the bloodstream in sufficient concentrations, or the cells in the bloodstream themselves are triggered, the inflammation can become systemic and usually results in fever. When the instigator of the inflammation is cleared, there is no more triggering of the innate immune system and the inflammation will resolve.

Auto inflammatory diseases

In the case of AID, monocytes are activated in absence of a known pathogenic trigger or are triggered by sub-threshold stimulation. The resulting immune activation leads to systemic inflammation, fever and periodic episodes. AIDs are quite rare, and the prevalence is unknown. Since most of the known AIDs are caused by genetic mutations (most are autosomal recessive disorders), certain ethnic groups have a much higher incidence than the global population. In the case of mevalonate kinase deficiency (MKD) for instance, the majority of patients are of Dutch or French origin, and the founder mutation most likely originates from the Netherlands(5, 6). The total number of registered patients in the world is around 300. For many other AIDs this number is of similar magnitude, but a few are more prevalent. For Familial Mediterranean Fever (FMF) it is estimated around 2.5 per 100.000 people in Western Europe(7), still a very rare disease. The diagnosis of AID is often a difficult and a lengthy process. As the occurring inflammation is indistinguishable from pathogenic inflammation and the inflammation resolves in a similar timespan to regular infections, it can take years before an AID is even considered. In addition, treatment to combat infections is mistakenly considered effective when the fever and symptoms of inflammation resolve, coincidently, after the administration of antibiotics. Diagnosis is established with certainty when known causative mutations are found. If no known mutation is present the subtype cannot be defined which

hampers the identification of optimal treatment.

For the molecular standpoint, the key molecular event in all AIDs is inflammasome activation. Although AIDs have different genetic origins, they converge on this point with inflammasome activation as a key step in the pathogenesis. The downstream effects are similar, resulting in the comparable phenotypes. When an immune cell is activated intracellular changes take place leading to inflammasome assembly. There are various types of inflammasomes, and not the same inflammasome is always activated. The most well studied inflammasome is the NLRP3 inflammasome. It consists of only three distinct subunits but each is present a number of times. The first subunit is the cytosolic sensor protein, NLRP3, which is the subunit that requires activation to trigger assembly of the inflammasome. The next subunit is ASC, which is an adaptor protein. ASC connects NLRP3 and caspase-1(8). The conformational changes induced by inflammasome assembly result in activation of caspase-1 and the subsequent maturation of IL-1β. As one of the first cytokines to be released, IL-1β, is usually already present in monocytes as an immature protein. In order to become bioactive a part of the protein, an inhibitor peptide, has to be cleaved. This is done by caspase-1, a zymogen. When caspase-1 is converted to its active form, it cleaves proIL-1β to yield the mature IL-1β.

The genetic origin of AID is quite diverse. Although not the subject of this thesis, we will briefly mention the more common AID and their genetic origin. There are excellent reviews on this subject with complete lists(7, 9, 10). Familial Mediterranean fever (FMF) is caused by mutations in MEFV(11, 12). As the name suggests, it is prevalent in certain ethnic groups in the Mediterranean area, in particular in Israel and Turkey. An interesting fact about FMF is that it is the only AID that can be treated with colchicine, which has no therapeutic benefit in other AID. The gene responsible was discovered over 15 years ago, but the function of the protein remained a mystery until a few months ago. MEFV was identified as an intracellular sensor for pathogenic glucosylation transferases, which inactivate RhoA(13). It is therefore similar to a PRR and important in the innate immune system. In TNF Receptor Associated Periodic Syndrome (TRAPS) there is a mutation in the TNF receptor 1 (TNFR1)(14). Many scientific breakthroughs in TRAPS were made in parallel with the research in this thesis, and helped to generate a more complete picture of the molecular pathways involved. In TRAPS the mutated TNFR1 is retained in the endoplasmic reticulum and resists autophagic degradation(15, 16). The resulting accumulation of protein leads to ER stress and inflammasome activation. In the Cryopyrin associated periodic syndrome, or CAPS, the pathogenic mutations are in the cytosolic sensor NLRP3(17). How these mutations yield altered activation of the inflammasome is unknown, but a relatively large number are in the nucleotide binding domain of NLRP3. The NLRP3 inflammasome is notorious for its promiscuity. There have been over 20 ligand described that can activate NLRP3, which has given rise to the idea that NLRP3 is actually activated by a signaling intermediate(8, 18, 19). There is no consensus yet on the both the existence and the identity of this intermediate. It is clear however, that NLRP3 is a very important intracellular PRR and a crucial link in the innate immune system.

Mevalonate kinase deficiency

In mevalonate kinase deficiency, the mevalonate pathway is affected. By definition it is caused by a defect in mevalonate kinase (MVK)(20, 21). Several mutations have been described with different implications leading to a broad phenotypical range. Some mutations lead to a truncated version of the protein, yielding a fully inactive protein. Several other substitution mutations lead to reduced stability, catalytic activity or both. On the molecular level this results in severely reduced activity of the enzyme (between 28% of healthy controls)(20, 22). The me-

valonate pathway is the biosynthesis route for cholesterol. Fortunately in the modern western world, cholesterol deficiency does not exist (rather the opposite) and cholesterol uptake from the diet means that cholesterol levels are normal in patients(23). However, the dysfunctional mevalonate pathway therefore also produces some branched unsaturated lipids, also named non-sterol isoprenoids. These pyrophosphorylated isoprenoids (farnesyl- and geranylgeranyl-pyrophosphate (FPP and GGPP)) are transferred to proteins containing a c-terminal prenylation motif(24, 25). This motif, the so-called CAAX-box, starts with a cysteine where a thio-ether is formed which links the isoprenoid to the protein. The next two residues can be any amino acid, but are usually aliphatic in nature (hence AA). The last amino acid can be any residue. It is believed that the type of residues can lead to geranylgeranylation versus farnesylation, although no clear cut consensus sequences have been identified yet. The addition of the isoprenoid to the protein acts as a localization signal, and can also act as a membrane anchor (in combination with other lipid modifications)(26, 27). In the case of MKD numerous proteins are not or only partially prenylated. How this leads to increased IL-1β secretion is unknown (and hence the subject of this thesis) but it involves defective prenylation. Supplementation of cells deficient in isoprenoids with GGPP leads to reversal of the phenotype(28–30).

Model systems

It is challenging to create a good model system for MKD. Attempts to knock-out MVK in mice have shown that deleting both alleles is embryonically lethal. Deletion of only one allele leads to some of the disease characteristics, such as elevated IgA and IgD. However, the amount of MVK enzymatic expression is very different between individual mice, making it a very inefficient model(31). The current standard for a mouse model is to treat mice with inhibitors of the mevalonate pathway. Two classes of inhibitors are used to inhibit the mevalonate pathway. The first class are the statins, which are used as cholesterol lowering drugs. Statins target HMG-CoA reductase, the enzyme directly before MVK in the mevalonate pathway. Lowering the amount of substrate available for MVK results in similar deficiencies as in MKD(32). The second class of inhibitors used are the bisphosphonates. Bisphosphonates resemble the pyrophosphate group found in substrates of the mevalonate pathway and become competitive inhibitors for farnesyl pyrophosphate synthase(33). It has to be said that the use of mouse models in MKD research is limited, most likely due to the lack of a true MKD model. For cellular systems the situation is similar. The optimal model would be the use of primary material from patients. Although available in small quantities, patients are simply too few in number to base fundamental research on. It is possible to culture material from patients for a longer period of time, such as fibroblasts(34). The downside is that these cells do not resemble monocytes in immune function. Monocytic cell lines are therefore frequently used. However, these cells lines have limitations of their own. Attaining the same level of MVK activity as seen in patients in is extremely challenging due to the low amount of residual activity that will still result in proper functioning of the pathway. It is very difficult to reach sufficient levels of knock down, since the estimated efficiency has to be over 95%. The matter is further complicated as some patients also suffer from an instable form of the protein, while the WT is much more stable(34). The current standard is therefore also, as with the mouse models, the use of mevalonate pathway inhibitors(28). In this thesis we use simvastatin treatment on THP-1 cells. The experimental use of simvastatin in mimicking MKD cellular features is well established and leads to reduced prenylation of proteins. The downside is that it does not lead to an accumulation of mevalonate, which happens in patients. THP-1 cells are of human origin and exhibit monocyte like characteristics. To our best knowledge this is the closest immortal cell line to human monocytes. Supplementing the growth medium with 10 μM simvastatin 24 hours before treatment results in depletion of the mevalonate pathway and cells (when stimulated)

accordingly show hyper-secretion of IL-1β(28, 35). Several important findings in my thesis I confirmed using primary monocytes from MKD patients (part of chapter 3).

Outline of this thesis:

In this thesis we explore the molecular mechanisms that lead to IL-1β secretion in MKD and we evaluate a mutation in the caspase-1 inhibitor SerpinB9 for potential to play a role in AID.

Chapter 2 is an extended review on MKD covering the current state of knowledge (at the time of publication) on MKD, including genetic origin, molecular mechanisms, phenotype, diagnosis and treatment(36).

In *chapter 3* we investigated the effect of isoprenoid depletion on the mitochondria and mitochondrial clearance and the downstream effect on IL-1β secretion. Using a monocytic MKD model we show that autophagy is less efficient in MKD and that there is an increased mitochondrial potential. The increased accumulation of damaged mitochondria and the presence of mitochondrial DNA in the cytosol lead to activation of the inflammasome and subsequent IL-1β secretion(37).

In *chapter 4* we researched the contribution of unprenylated RhoA in IL-1β hyper-secretion. We found that the localization and activation of RhoA is altered when the protein is not prenylated. This had no effect on mitochondrial potential or autophagy, but did increase the activation of Rac1(38).

Chapter 5 is a review on the role that mitochondria play in the pathogenesis and propagation of autoinflammatory disease. Although not directly affected in known AIDs, mitochondria are important mediators in the immune response and its signaling pathways could be a potential point for therapeutic intervention.

In *chapter 6* we explore the potential of a mutation in the caspase-1 inhibitor SerpinB9 for contributing to AID. The mutation was found using next generation sequencing on a small panel of inflammatory genes. We assessed the mutant on its ability to inhibit caspase-1 and its other main target, granzyme B. The mutant form is still capable of inhibiting granzymbe B, but seems to be less effective at inhibiting caspase-1.

In *chapter 7* we discuss the results of this thesis in the context of AID, briefly summarize the advancements made in the field and discuss the remaining outstanding questions in AID.

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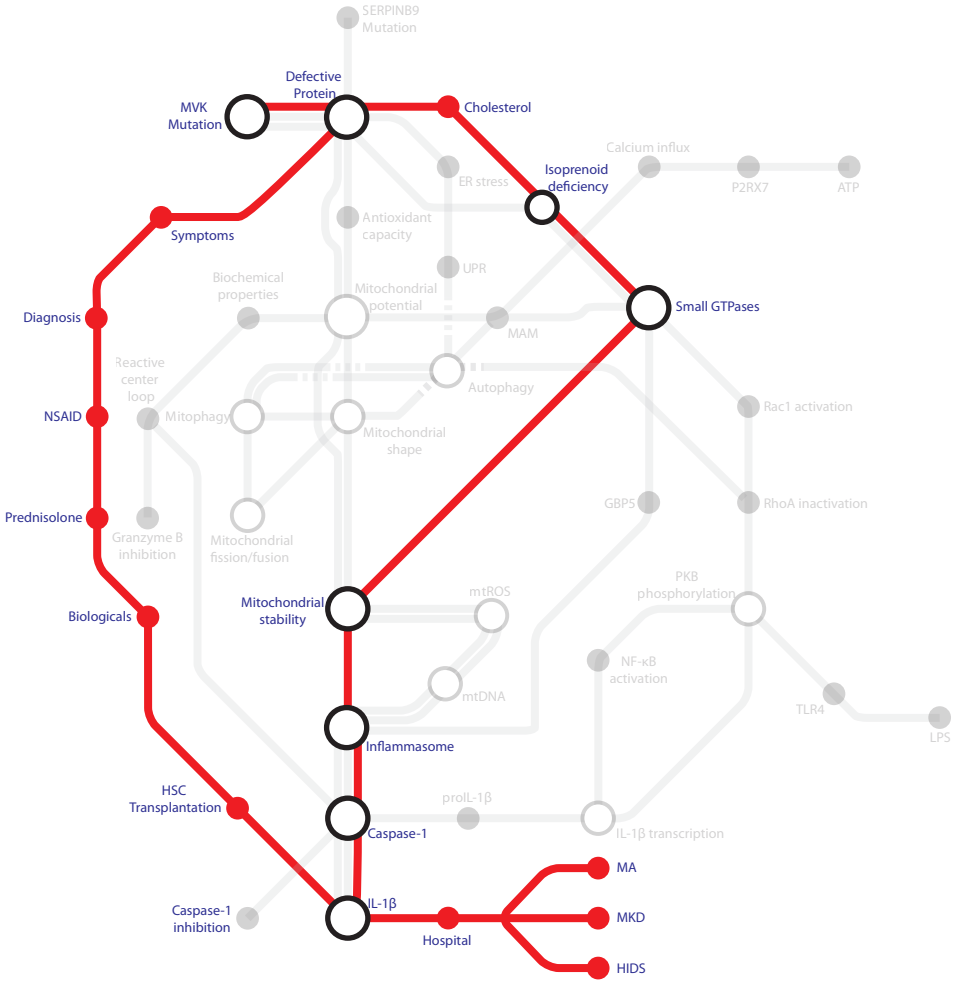
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LPS
Inflammasome
HSC Transplantation
proIL-1β
Caspase-1
IL-1β transcription
MA
Caspase-1 inhibition
IL-1β
MKD
Hospital
HIDS

CHAPTER 2

Mevalonate Kinase Deficiency, a Metabolic Autoinflammatory Disease

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Abstract

Mevalonate kinase deficiency is a rare autosomal recessive inborn error of metabolism that presents itself with an autoinflammatory phenotype. In this review we discuss its pathogenesis, clinical presentation and treatment. Mutations in both copies of the MVK-gene lead to a block in the mevalonate pathway. Interleukin-1beta (IL-1β) mediates the inflammatory phenotype. Shortage of a non-sterol isoprenoid product of the mevalonate pathway, Geranylgeranylpyrophosphate (GGPP) leads to aberrant activation of the small GTPase Rac1, and inflammasome activation. The clinical phenotype ranges widely, depending on the mutations and the inherent severity of the enzyme defect. All patients show recurrent fevers, lymphadenopathy and increased release of acute phase proteins, in severe cases supplemented with dysmorphic features, growth retardation, cognitive impairment and progressive cerebellar ataxia. Diagnosis relies on mutation analysis of the MVK-gene. To date, evidence- based therapy is not yet possible, while IL-1 blockade is usually effective. Severe cases require allogeneic stem cell transplantation. Here, we will discuss current treatment regimens as well as ongoing research on targeted therapies for mevalonate kinase deficiency patients.

1. Introduction

Mevalonate kinase deficiency (MKD) is an autosomal recessive inborn error of isoprenoid biosynthesis. In contrast to most inherited metabolic diseases, the main clinical features are those of an autoinflammatory disease: episodic fever and generalized inflammation. The associated phenotypes had been recognized nearly three decades ago as the Hyper ImmunoglobulinemiaD and periodic fever Syndrome (HIDS, MIM# 260920)(1, 2) and Mevalonic Aciduria (MA, MIM# 610377)(3). The latter was soon shown to be due to mevalonate kinase deficiency(4). While initially, MA and HIDS were considered unrelated diseases, at the turn of the century two Dutch groups independently identified mevalonate kinase deficiency, due to mutations in MVK, as the cause of HIDS(5, 6). The two disorders have similar inflammatory attacks with fever and a brisk acute phase response, accompanied by lymphadenopathy, hepatosplenomegaly, abdominal pain, vomiting diarrhea, arthralgia, myalgia, skin rash and mucosal ulcers. Elevation of serum IgD is a characteristic, though inconsistent finding. In addition to these features, the MA phenotype is characterized by dysmorphic features, pre- and postnatal growth retardation, ocular, and neurological involvement. Currently, the two presentations are considered the extremes of a phenotypic spectrum with a severe form, MA and a mild form, HIDS(7). In fact, the spectrum is probably even wider, ranging from embryonically lethal to apparently healthy(8). The pathogenesis is only partly understood and there is no evidence-based treatment available. In the following review, we will discuss current understanding of MKD pathogenesis, its clinical presentation and management.

2. Epidemiology

MKD is an autosomal recessive disease that occurs worldwide and affects both sexes equally(9, 10). A disproportionate number of HIDS patients have been reported from the Netherlands, probably due to a founder mutation (V377I) in the Dutch population(8, 11). The exact prevalence of MKD is unknown. Based on data from the international HIDS registry (www.hids.net) and the European Union sponsored Eurofever registry (http://www.printo.it/eurofever (10)) the number of known MKD patients worldwide is just over three hundred, most of whom have the HIDS phenotype. This is certainly an underestimate of the real disease prevalence, since many patients go undiagnosed, the median diagnostic delay being about 10 years(9, 10). Furthermore, not all of those who are correctly diagnosed will be reported to disease registries.

3. Pathophysiology

3.1. The Mevalonate Pathway

Mevalonate kinase (MK) is a key enzyme of the mevalonate pathway, a biosynthetic route that produces cholesterol and branched unsaturated lipid chains called non-sterol isoprenoids. The mevalonate pathway is highly regulated by several feedback mechanisms, the most important being the amount of free cholesterol available.(12) The pathway starts with the enzyme 3- Hydroxyl-3-MethylGlutaryl-Coenzyme A (HMG-CoA) reductase, which is the tightly regulated rate limiting enzyme of the mevalonate pathway. The widely prescribed class of cholesterol-lowering agents, statins, act by inhibiting HMG- CoA reductase. HMG-CoA reductase converts HMG-CoA to mevalonate. Mevalonate kinase subsequently phosphorylates mevalonate to 5 – phosphomevalonate, which is then phosphorylated to 5-pyrophosomevalonate. The addition of these polar phosphate groups supports the solubility of the metabolites in water as the hydrophobic domain of the isoprenoid increases in size. 5-Pyrophosphome-

valonate is converted to isopentenylpyrophosphate (IPP), which is the building block for all further products of the pathway (figure 1). The entire range of compounds produced by the mevalonate pathway passes through the first four critical enzymes, indicating that a defect in one of these enzymes could have far-reaching consequences(13).

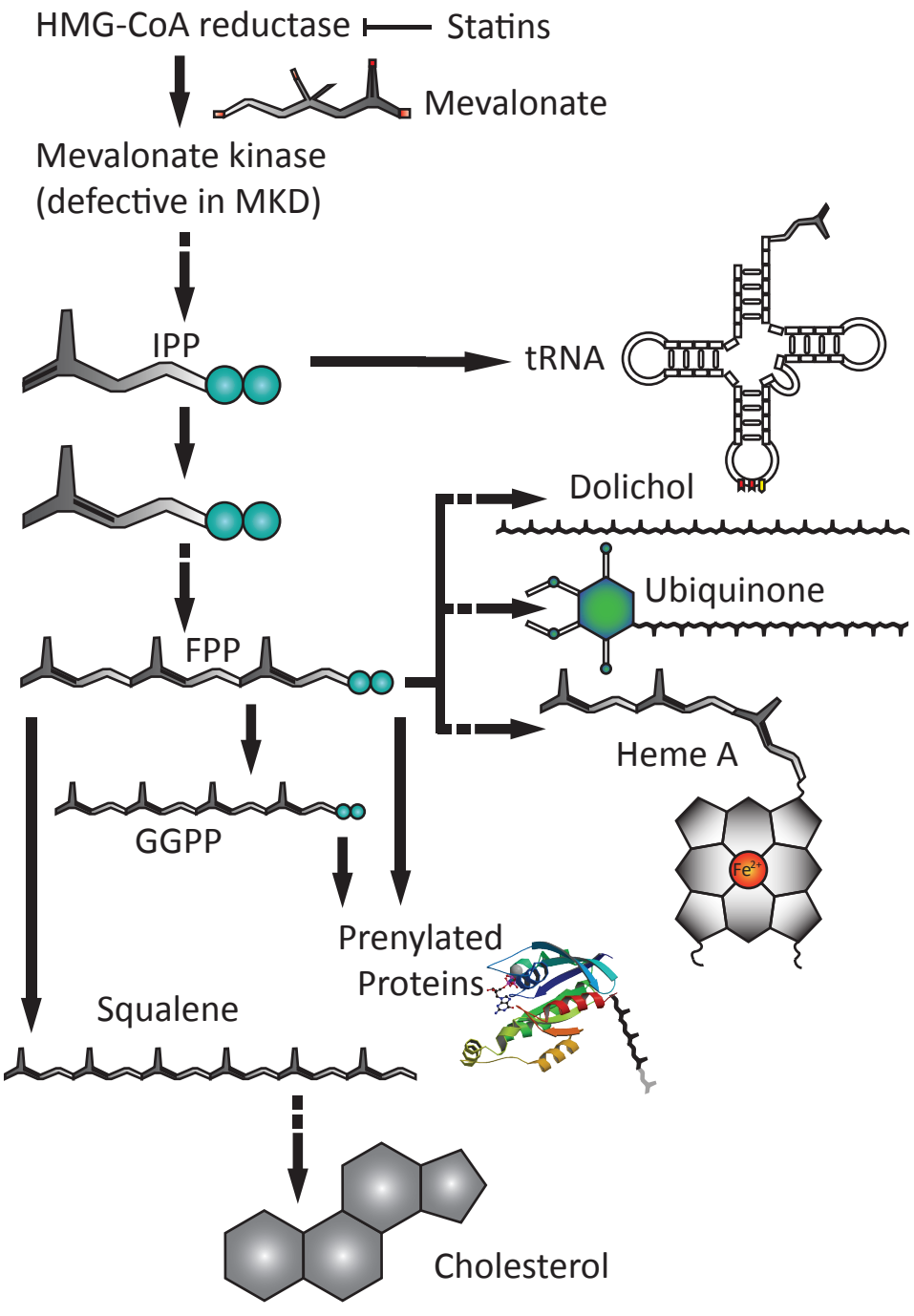
The mevalonate pathway produces a number of metabolites that are important for various functions in the cell. In this review we will focus on the role of prenylation, i.e. the post-translational modification of macromolecules by covalent attachment of isoprenoid lipid moieties. Protein prenylation can regulate the activation status of proteins, as is the case for some of the small GTPases of the Ras super family(14). These are key molecular switch proteins in multiple signal transduction routes. These signaling paths control vital processes such as cell migration, division, intracellular vesicle transport, and cytokine secretion. However, the production of these isoprenoids is compromised in MKD(15).

IPP is the source of all non sterol isoprenoids. In addition, IPP itself is required for isopentenylation which is an essential modification for the specialized tRNA that transfers the amino acid selenocysteine(16). This modified cysteine is an important amino acid for enzymes involved in redox control. For the biosynthesis of larger isoprenoids, multiple IPP units are coupled together to yield the 15-carbon farnesylpyrophosphate (FPP) and the 20-carbon geranylgeranylpyrophosphate (GGPP), the two isoprenoids that are used for protein prenylation. The prenyl transferase enzyme that couples the isoprenoid unit to the recipient protein requires a C-terminal consensus amino acid sequence(17). The transferase then adds a lipid tail to the C-terminus of the protein that can act as a membrane anchor, as is the case with the nuclear lamins. Absence of the prenylation domain on lamins results in their mis- localisation in the nucleus, instead of being incorporated in the nuclear lamina(18). There are also instances where the isoprenyl tail is part of a protein- protein interaction domain(14). The functional consequences of prenylation, be it subcellular localization or functional activity, may be different for each protein. FPP is the branch point metabolite in the mevalonate pathway from which several biosynthetic routes depart (figure 1), one of which leads to GGPP. Another important product of FPP is squalene which is the precursor for cholesterol. In addition, FPP can yield the polyprenyl chains of Heme A and ubiquinone, both necessary for optimal functioning of mitochondria(19–21) and dolichol, which is a cofactor in protein glycosylation(22). In MKD, it appears that reduced availability of non-sterol isoprenoids is central to the inflammatory phenotype, as will be discussed below.

Figure 1: Products of the mevalonate pathway. Schematic representation of the Mevalonate pathway. Solid arrows indicate single enzymatic steps, interrupted arrows indicate multiple enzymatic reactions, where intermediates have been omitted for sake of clarity. HMG-CoA reductase, the gatekeeper of isoprenoid biosynthesis and the target of statin drugs produces mevalonate. Mevalonate kinase produces isopentenyl pyrophosphate (IPP) which can be covalently attached to certain tRNA's. Three IPP units can be isomerized and coupled to yield Farnesyl Pyrophosphate (FPP), the branch point metabolite of the mevalonate pathway. Via squalene FPP can yield cholesterol. FPP is the substrate for the biosynthesis of the polyisoprenyl chains of dolichol, ubiquinone and heme A and of geranylgeranyl pyrophosphate (GGPP). Both GPPP and FPP are substrates for protein isoprenylation.

3.2. The molecular defect

In MKD-affected individuals, both MVK alleles carry mutations. The majority of these are missense mutations resulting in single amino acid change, the remainder being stop, frame shift, and splice mutants. Some mutants have a total lack of activity or are extremely unstable and therefore effectively inactive. Other mutations impair proper protein folding, compromising


HMG-CoA reductase
Statins
Mevalonate
Mevalonate kinase
(defective in MKD)
IPP
tRNA
Dolichol
Ubiquinone
FPP
Heme A
GGPP
Fe2+
Prenylated
Proteins
Squalene
Cholesterol

stability and hence activity. This folding defect worsens with increasing temperature, which could be clinically relevant in febrile patients(23). The effective residual activity in patient cells depends on the combination of both mutants. No patient has been reported to harbour two mutants resulting in complete enzyme deficiency. Probably, such a phenotype would cause lethality in utero. The residual enzyme activity in patients ranges from 1.8%-28% HIDS to below 0.5% in MA and is a fair indicator of the severity of the disease(5, 24).

In the majority of patients where both residual activity and protein expression have been measured, the total amount of MK protein is profoundly reduced compared to that in healthy controls(25). This indicates that protein stability is the main problem. Indeed, stable fusion proteins of most mutant MK enzymes have considerable residual enzyme activity(5).

Figure 1: Products of the mevalonate pathway. Schematic representation of the Mevalonate pathway. Solid arrows indicate single enzymatic steps, interrupted arrows indicate multiple enzymatic reactions, where intermediates have been omitted for sake of clarity. HMG-CoA reductase, the gatekeeper of isoprenoid biosynthesis and the target of statin drugs produces mevalonate. Mevalonate kinase produces isopentenyl pyrophosphate (IPP) which can be covalently attached to certain tRNA's. Three IPP units can be isomerized and coupled to yield Farnesyl Pyrophosphate (FPP), the branch point metabolite of the mevalonate pathway. Via squalene FPP can yield cholesterol. FPP is the substrate for the biosynthesis of the polyisoprenyl chains of dolichol, ubiquinone and heme A and of geranylgeranyl pyrophosphate (GGPP). Both GPPP and FPP are substrates for protein isoprenylation.

As of July 2012, 103 disease-associated mutations had been entered on the Infevers webpage (http://fmf.igh.cnrs.fr/ISSAID/infevers/). Considering these mutations, 63 had been reviewed in detail by Mandey et al. in 2006(25). The majority of reported patients has compound heterozygote mutations. Two mutations account for the majority of mutant alleles. The most common is the V377I mutation, which confers considerable residual enzyme activity and is exclusively associated with the HIDS phenotype. The other common mutation, I268T, is associated with a severe phenotype when the patient is homozygote, although compound heterozygotes may be less severely affected. The remaining mutations are found scattered over the entire length of the MVK-gene.

The defect causes an accumulation of the substrate of MK, mevalonate. The excess mevalonate is excreted via the kidneys in the urine. Indeed excessive urinary mevalonate levels led to the identification of the first case of MKD(4). The urinary mevalonate concentration is particularly high during fever attacks and is considerably lower in mildly affected (HIDS) patients than in those with severe enzyme deficiency(26). In addition to accumulation of mevalonate, affected individuals have reduced output of at least some of the branches of isoprenoid biosynthesis. Interestingly, not all metabolites are affected equally. Serum cholesterol usually remains within normal limits an on post mortem analysis the liver contains sufficient levels of cholesterol, squalene and dolichol(27). The initial hypothesis was that clinical symptoms were due to toxic levels of mevalonate. However, attempts to reduce mevalonate production using HMG-CoA reductase inhibitors were followed by disease exacerbation in MA patients(27). Recent ex-vivo data support the notion that the lack of FPP and GGPP mediates inflammation(28–30). Indeed, correction of this shortage with exogenous small molecular compounds like geraniol or farnesol can neutralize the pro-inflammatory phenotype ex vivo in patient cells and animal models(31, 32). It is our hope that this approach will yield small molecular drugs that specifically target the defect in MKD patients.

3.3. Inflammasome activation and Interleukin(IL)-1β secretion

The recurring fever attacks are accompanied by an acute phase response mediated by soluble

mediators that include acute phase proteins produced by the liver and by leukocyte-produced cytokines. Although it is unknown what triggers the attacks, the response is mediated to large extent by the release of cytokines. Peripheral blood mononuclear cells (PBMCs) from MKD patients produce larger amounts of pro-inflammatory cytokines like IL-1β, IL-6, IL-18 and tumor necrosis factor (TNF) α when stimulated with lipopolysaccharide (LPS)(28, 33). In addition, PBMCs from MKD patients have increased spontaneous release of IL-1β. Since IL-1β is a strong inducer of the acute phase response, IL-1β released from macrophages and blood monocytes, is likely to contribute to fever attacks in MKD. IL-1β is matured from pro-IL1β through cleavage by the cysteine protease caspase-1. Ex vivo, reduced production of non- sterol isoprenoids indeed leads to a caspase-1 mediated hypersecretion of IL-1β(30). The activation of caspase-1 is mediated by the formation of an inflammasome complex. Several types of inflammasomes are known, but murine data suggest that it is the NLRP3 inflammasome that activates caspase-1 when isoprenoids are low(34). The isoprenoids involved are GGPP and possibly FPP.(30, 32, 35) However, it is still unknown precisely how a shortage of FPP or GGPP activates the inflammasome. One likely factor is ectopic overactivity of small GTPases. Isoprenyl chains are required for both the proper localizationof these proteins as well as for their binding to Rho guanine nucleotide dissociation inhibitors (RhoGDI's)(36). When bound to small GTPases, the GDI proteins inhibit small GTPase activation by preventing the exchange of GDP for GTP(14). Conversely, inability to bind to GDI favors the GTP-bound active state of small GTPases. Only a few GTPases have been studied in the context of reduced isoprenoid output. Indeed, the small GTPases Rac1 and RhoA have altered activation and localization in MKD fibroblasts due to deficient prenylation. The balance between the GDP and GTP bound forms of both proteins is shifted towards the latter.(15) Reducing isoprenoid availability in the myeloid cell line THP1 increases both Rac1 activity and IL-1β secretion. The increased activity of Rac1 acts via phosphorylation of the serine/threonine- specific protein kinase B (PKB, also known as Akt). Inhibition of Rac1 leads to decreased levels of phosphorylated PKB. In addition, inhibition of either Rac1 or PKB reduces IL-1β secretion(37). However, other pathways may still be involved in the pathogenesis of inflammation in MKD. Indeed, numerous proteins from the small GTPase super family are prenylated and could have altered localization and/or activation when non-sterol isoprenoids are lacking.

It is not fully understood how the altered activity of small GTPases controls IL-1β secretion. Recent work suggests that one mechanism that is likely involved is autophagy. The formation of autophagosomes is controlled by several small GTPases(38). Autophagy in turn regulates the availability of pro-IL-1β and has also been reported to regulate the maturation and secretion of the mature IL-1β(39–42).

In addition to small GTPases, there are other proteins potentially affected by reduced non-sterol isoprenoids output. IPP is required for specific tRNA molecules, which introduce selenocysteine, essential in several antioxidant enzymes. Recent publications on related autoinflammatory diseases (i.e., CAPS and TRAPS) indicate that reactive oxygen species play an important role in inflammasome activation(12, 43). Whether reduced antioxidant capacity is involved in the pathogenesis of MKD is currently being investigated.

There is yet another process that is potentially involved in MKD and could affect inflammasome activation: the function of mitochondria(44). The mitochondrial respiratory chain , which supports much of the energy expenditure in cells, could be affected because it contains the isoprenylated compounds heme A and ubiquinone. It now appears that mitochondrial dysfunction can lead to the release of mitochondrial reactive oxygen species and other contents like mitochondrial DNA. Both have been shown capable to activate inflammasomes(45, 46).

Damaged mitochondria are normally cleared by autophagy. As pointed out above, autophagy may be impaired, potentiating these proinflammatory stimuli.

In addition to the above mechanisms, apoptosis of lymphocytes from HIDS patients has been shown to be impaired which might contribute to prolonged inflammatory signaling(47).

In summary, inflammation in MKD is, at least in part, IL-1β mediated. The increased IL-1β secretion is due to a lack of non-sterol isoprenoids, which in turn leads to ectopic activation of small GTPases. A role for impaired, antioxidant response and for impaired autophagy is plausible and currently under investigation. Hopefully study of the molecular mechanisms involved in MKD will yield novel, specifically targeted treatment approaches in the coming decade.

4. Clinical presentation

4.1. Hyper IgD Syndrome

The HIDS phenotype is characterized by early onset of febrile attacks, usually starting in infancy at a median age of 6 months and rarely after the age of 5 years(9, 48). The attacks recur at irregular intervals of 2-8 weeks. Attacks are often unprovoked, but sometimes triggered by emotional stress, trauma, infection or, quite characteristically, by vaccinations. Fever onset is abrupt, often with shaking chills. Temperature regularly exceeds 40ºC (104ºF). Unless antipyretics are taken, fever remains high for 3-7 days. Fever is accompanied by varying combinations symptoms (figure 2). Ninety percent of patients experience painful cervical lymphadenopathy(9). Abdominal pain, vomiting and/or diarrhea accompany attacks in most patients. Sterile peritonitis can mimic appendicitis and can lead to peritoneal adhesions, which in turn can cause acute intestinal obstruction. Joint pain occurs in the majority of patients and about half have arthritis, mainly affecting large joints. Arthritis is usually non- erosive, although contractures have been reported. Skin rash usually maculopapular but may be vasculitic with purpura or erythema nodosum. Aphtous ulcers affect the oral mucosa in half of he patients, but genital and rectal mucosa may be involved as well. For example, colonic involvement can result in bloody stools. Rare complications of MKD include proliferative glomerulonephritis, which may progress to end stage kidney failure(49). Systemic AA-amyloidosis occurs in less than 3% of HIDS patients and has not been reported in childhood.

When, in the course of an inflammatory attack, neutrophil and platelet counts become relatively low, this should alert the physician to the possible development of haemophagocytosis(48, 50). Serious bacterial infections, not related to immunosuppressive therapy, occur in up to 27% of MKD patients. These include otitis, septicemia, meningitis and pneumonia(48). Deaths from serious bacterial infection are extremely rare, but have been reported in all published series(9, 48, 51). A rare tumor, renal angiomyolipoma has been described in 6% of patients in one series(48).

The social impact of HIDS was studied by van der Hilst in 28 Dutch adults with HIDS. The disease had interfered with education in 45% of patients and was associated with a 6-fold increase in unemployment compared to the general population(9).

4.2. Mevalonic Aciduria

The MA phenotype is characterized by very prototypical inflammatory attacks. However these are often more severe than in HIDS and may prove fatal in infancy(27). Disease onset in MA

is early, often antenatal, as may be reflected by intrauterine growth retardation, stillbirth and congenital malformations(27, 52, 53). Congenital defects include cataracts, shortened limbs and dysmorphic craniofacial features such as dolichocephaly, microcephaly, a triangular-shaped face, down-slanting eyelids, and dysplastic ears(27, 52, 53).

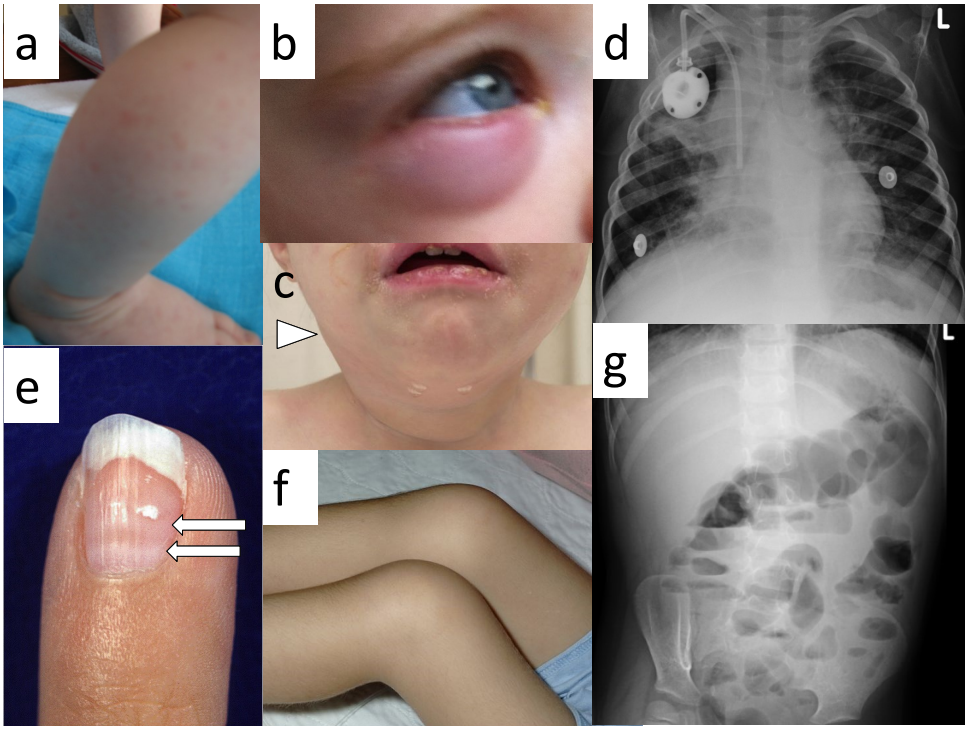


Figure 2: Clinical features of mevalonate kinase deficiency: a) maculopapular rash, b) periorbital erythema, c) cervical lymphadenopathy (arrowhead), d) bilateral pneumonia, e) transverse nail grooves (Beau lines) due to recent febrile episodes, f) arthritis of both knees, g) acute intestinal obstruction due to peritoneal adhesions.

Affected children are often born prematurely, fail to thrive as infants and usually remain short statured. Neurological involvement is common in MA including progressive cerebellar ataxia due to cerebellar atrophy and developmental delay which often is profound, but may be very mild(27, 52). Patients are often hypotonic either due to central causes or due to myopathy with elevated muscle enzymes. Ocular findings include cataracts and the development of tapetoretinal degeneration and uveitis(54).

Neonatal hepatitis leading to obstructive jaundice has been described. Dyserythropoietic anemia is a rare manifestation of MA(55). Rarely, extramedullary hematopoiesis presents neonatally as a blueberry muffin syndrome(56). The precise frequency of individual symptoms in MA is unclear, not only due to the rarity of the disorder, but primarily because there is no clear- cut distinction with HIDS and intermediate phenotypes.

5. Diagnosis

Clinical features that should prompt testing for MKD are fever episodes recurring during at least 6 months with an onset before the age of 5 years with either strongly elevated serum IgD, attacks triggered by vaccinations or at least three of the following: cervical lymphadenopathy, abdominal pain, vomiting, diarrhea, joint pain, aphtosis, or rash(9). Inflammatory attacks are always accompanied by a strong acute phase response, reflected by high Erythrocyte sedimentation rate (ESR), C-reactive Protein(CRP), or Serum Amyloid A protein (SAA). Frequent inflammation often leads to normocytic anemia. Continuous elevation of serum IgD used to define HIDS. Indeed, an strongly elevated serum IgD, supports the diagnosis of MKD, but this is an insensitive test: at least 20% of affected patients have entirely normal IgD values, probably even more so in pediatric patients(9, 51, 57). Serum IgA is elevated in about two thirds of patients. Other immunoglobulins may be elevated, but hypogammaglobulinemia and low B-cell counts have been reported in MKD as well(48, 58). Autoantibodies are usually absent in MKD, although their presence does not exclude the diagnosis(59).

The diagnosis of MKD rests on the identification of disease causing mutations in both alleles of MVK. All reported mutations can be found on the infevers website (http://fmf.igh.cnrs.fr/ISSAID/infevers/). If mutations of uncertain significance are found, measurement of MK activity in leukocytes or cultured skin fibroblasts may be required. Enzyme deficiency is profound in the MA phenotype, whereas patients with 1-28% residual MK activity usually present with the HIDS phenotype. However, this genotype-phenotype correlation is not perfect(48). Finding elevated levels of mevalonic acid in urine strongly supports the diagnosis. MA patients may have up to 10,000-fold raised urinary mevalonic acid levels. Yet, sensitive techniques are required, since, in the milder HIDS phenotype, urinary mevalonic acid is only slightly elevated during attacks and often normal in-between.

6. Management

6.1. Patient care

The goals of management in MKD are normal participation in activities of daily life, reduction of inflammatory symptoms, prevention of long-term sequelae, and supportive care for disabilities. There are no evidence-based management guidelines. Patients are followed on a regular basis to check for disease activity, complications and drug (side) effects. It has been shown in other inflammatory disorders that persistently elevated serum amyloid A protein levels are associated with progressive systemic amyloidosis(60). In order to prevent this rare but devastating complication in MKD, acute phase proteins should normalize entirely between the inflammatory attacks. The differentiation of autoinflammatory attacks from serious infections can be challenging. Patient or parents noting that an attack is different from others should raise suspicion of an infection. Although attacks are known to be precipitated by triggers such as vaccinations, MKD usually poses no contraindication to childhood immunizations, except for infants with life-threatening inflammatory attacks.

6.2. Therapeutic interventions

Treatment of MKD is challenging. There is no single therapy effective in all patients and the evidence base for most available treatment is slim. Given the wide spectrum of clinical severity, the physician has to strike an individual balance for each patient between perceived benefits versus risks and costs. None of the drugs discussed here have been registered for

this indication. Most patients use paracetamol during febrile attacks, which appears to confer limited benefit. However, this has never been studied in any formal sense. Non-steroidal anti-inflammatory drugs (NSAIDs), which are often used during inflammatory attacks, partially relieve symptoms in the majority of the reported patients(48, 61). Many patients with the HIDS phenotype benefit from corticosteroids, especially when given in high doses at the beginning of an attack(9, 61). Notably, in a recently described cohort one-third of the patients only used NSAIDs and/or corticosteroids to control the disease(61).

Based on the pathophysiology of MKD, statins were thought be beneficial. A small randomized controlled trial found that simvastatin decreased the attack frequency, but clinically the results were very modest(62). Moreover, in the majority of reported patients statins had no effect on the disease course (9, 48, 61) and statins triggered life threatening inflammatory crisis when tried in MA patients(27). In contrast to familial Mediterranean fever, colchicine fails to control the disease in MKD patients(9, 61).

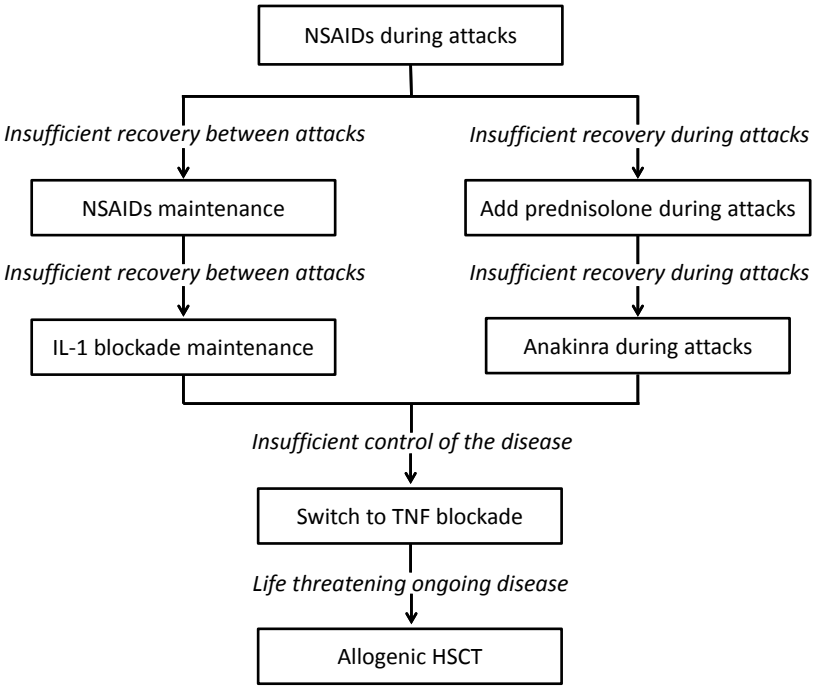


Figure 3: Therapeutic options in MKDThere is no registered or evidence based treatment for MKD. Physicians and patients should be aware that any drug prescribed for MKD implies its off label use. Therefore a step-wise approach is suggested, starting with well-known anti- inflammatory agents and only proceeding to cytokine blockade and ultimately stem cell transplantation if there is no other option left.

Anakinra, an IL-1 receptor antagonist, significantly decreased the attack duration and level of CRP in two small prospective trials(63, 64) and generally induced a favourable response in other reports(9, 48, 61). However, the response to anakinra is not as dramatic as compared to purely IL-1 driven diseases like the cryopyrin-associated periodic syndromes and several MKD patients have been described who failed to respond on anakinra. Patients who are

completely well between attacks can be managed with anakinra during febrile episodes only. Patients with very frequent disease episodes or incomplete recovery in-between merit maintenance therapy. Other anti-IL-1 agents as canakinumab and rilonacept have been successfully tried in some patients(48, 61, 64). Tumor- necrosis factor (TNF)-blockade by etanercept could be beneficial, but a completeresponse to this drug is not often seen(9, 48, 61). A small number of patients has been treated with infliximab and adalimumab with variable results(48, 61).

Very severely affected patients with the MA phenotype may fail all above therapies(27, 55, 63, 65–68). At least four such patients successfully underwent hematopoietic stem cell transplantation(65–67). This not only led to a sustained complete remission of systemic inflammation, but was also followed by improvement of the neurological symptoms. Given the inherent treatment related risks of transplantation, it should only be considered in very severely affected patients resistant to all other therapies.

Several authors have treated MA patients with ubiquinone (coenzyme Q10) alone or in combination with antioxidants like Vitamins E, C or A. Although there are no apparent risks involved, there is no evidence of clinical efficacy of this approach. The practice is based on the finding that plasma ubiquinone levels are slightly reduced in MA, although intracellular ubiquinone levels are in the normal range(27, 69, 70).

In view of these data, we would suggest a stepped approach if disease severity allows, as outlined in figure 3, starting with intermittent paracetamol and NSAIDs as first line of treatment. In patients with insufficient recovery between attacks, maintenance NSAIDs are warranted. If a patient does not have a satisfactory response or suffers from significant adverse effects, attacks may be treated with prednisolone. If this fails, IL-1 blockade could be initiated, again preferably during attacks only, but as maintenance if needed. If failure to IL-1 blockade is observed, the treatment could be switched to anti-TNF agents. The use of live attenuated vaccines is contraindicated during TNF blockade. Patients starting IL-1 blockade must have been fully vaccinated against S.pneumonia, H.influenzae and N.meningitidis and should be free of tuberculosis. Given the increased risk of staphylococcal skin infections under IL-1-blockade, we suggest eradication of nasal S.aureus carriage. Complications of mevalonate kinase deficiency should be treated with the appropriate interventions, e.g. aggressive immunosuppressive therapy in secondary hemophagocytosis. Allogeneic hematopoietic stem cell transplantation is a last resort and only warranted if the patient otherwise risks irreversible serious sequelae or death.

7. Prognosis

The prognosis of MKD depends on the severity of the defect. Most MKD patients have fewer attacks with age. Some HIDS patients even attain spontaneous complete disease remission. In contrast, severely affected (MA) patients risk death from a systemic inflammatory response syndrome early in life with up to 40% reportedly dying in infancy(27). With the advent of allogeneic stem cell transplantation for this population and improved supportive care, these figures are likely to improve. AA-amyloidosis occurs in about 3% of cases. Infectious complications are usually mild but can be fatal. Progressive cerebellar ataxia and dysarthria are common in MA patients who survive infancy. MA patients have a variable degree of cognitive impairment, ranging from minimal to profound(27).

8. Conclusion

Mevalonate Kinase Deficiency is a rare autoinflammatory disease due to an inborn error of metabolism. Depending on the specific genetic defect and inherent enzyme deficiency, the clinical expression ranges from stillbirth to apparent full health. Severely affected patients have neurological involvement in addition to inflammation. The pathogenesis of the inflammation in MKD is gradually being elucidated and interleukin-1β appears to be a major effector. There is no evidence based therapy, although IL-1 blockade and for the most severely affected patients allogeneic stem cell transplantation appear promising. However, understanding the cell biology of the disease should ultimately yield interventions targeted at the metabolic defect itself, providing quality of life without disproportionate risks and costs.

2

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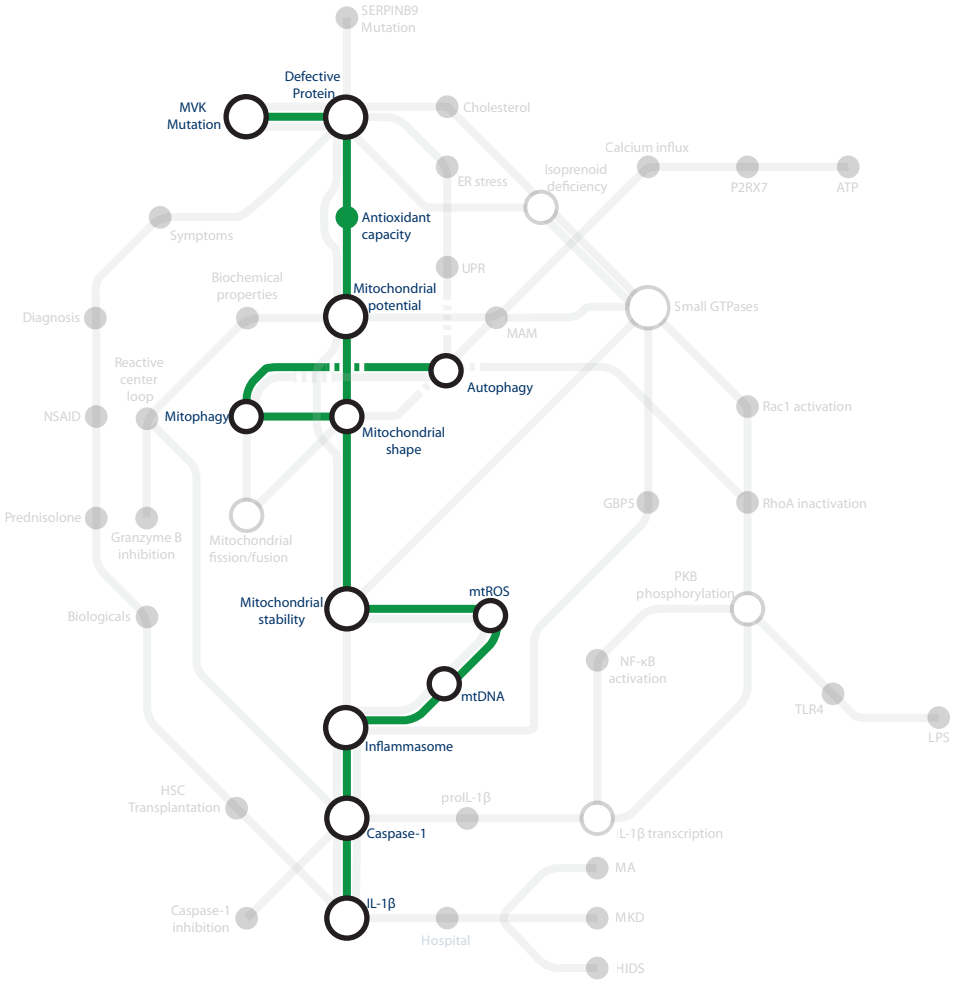
SERPINB9 Mutation
Defective Protein
MVK Mutation
Cholesterol
ER stress
Isoprenoid deficiency
Calcium influx
P2RX7
ATP
Antioxidant capacity
Symptoms
UPR
Biochemical properties
Mitochondrial potential
Diagnosis
MAM
Small GTPases
Reactive center loop
Autophagy
NSAID
Mitophagy
Rac1 activation
Mitochondrial shape
GBP5
RhoA inactivation
Prednisolone
Granzyme B inhibition
Mitochondrial fission/fusion
PKB phosphorylation
Biologicals
Mitochondrial stability
mtROS
NF-κB activation
mtDNA
TLR4
LPS
Inflammasome
HSC Transplantation
proIL-1β
Caspase-1
IL-1β transcription
MA
Caspase-1 inhibition
IL-1β
MKD
Hospital
HIDS

CHAPTER 3

Defects in Mitochondrial Clearance Predispose Human Monocytes to Interleukin-1β Hypersecretion

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Abstract

Most hereditary periodic fever syndromes are mediated by deregulated IL-1β secretion. The generation of mature IL-1β requires two signals: one that induces synthesis of inflammasome components and substrates, and a second that activates inflammasomes. The mechanisms that mediate autoinflammation in mevalonate kinase deficiency (MKD), a periodic fever disease characterized by a block in isoprenoid biosynthesis, are poorly understood. In studying the effects of isoprenoid shortage on IL-1β generation, we identified a new inflammasome activation signal that originates from defects in autophagy. We find that hyper-secretion of IL-1β and IL-18 requires reactive oxygen species (ROS) and is associated with an oxidized redox status of monocytes, but not lymphocytes. IL-1β hyper-secretion by monocytes involves decreased mitochondrial stability, release of mitochondrial content into the cytosol and attenuated autophagosomal degradation. Defective autophagy, as established by ATG7 knockdown, results in prolonged cytosolic retention of damaged mitochondria and increased IL-1β secretion. Finally, activation of autophagy in healthy but not MKD patient cells reduces IL-1β secretion. Together these results indicate that defective autophagy can prime monocytes for mitochondria-mediated NLRP3 inflammasome activation, thereby contributing to hyper-secretion of IL-1β in mevalonate kinase deficiency.

Introduction

Periodic fever syndromes are characterized by inflammation that occurs in the absence of apparent infection or high titer autoantibodies(1, 2). In most periodic fever syndromes the generalized inflammation is driven by interleukin (IL)-1β generated through proteolytic cleavage by the NLRP3 inflammasome. An additional feature associated with inflammation is the production of reactive oxygen species (ROS)(3, 4). During infection, ROS produced by nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase subunits at the plasma membrane are beneficial as ROS in phagosomes contribute to the killing of intracellular pathogens (5). In several inflammatory diseases, including periodic fever syndromes, ROS levels are increased in the cytosol and contribute to pathology(6). ROS play roles as intracellular second messengers and have immediate effects on the intracellular redox status. In the TNFR1-associated periodic syndrome (TRAPS), for example, mitochondria-derived ROS facilitate inflammatory cytokine production(7). Mitochondria are believed to be the main source of ROS in several autoinflammatory disorders(8).

ROS are normally generated within mitochondria as byproducts of oxidative phosphorylation, but liberated in the cytosol ROS can facilitate activation of the NLRP3 inflammasome(9–11). Normally, mitochondrial contents, including ROS and mitochondrial DNA (mtDNA) are prevented from reaching the cytosol because damaged mitochondria are swiftly neutralized by autophagy(12). During autophagy cytosolic constituents are enclosed within a double layered lipid membrane vesicle geared to fuse with lysosomes for degradation and recycling of the internal contents(12). Impaired autophagy may interfere with mitochondrial turnover and stimulate NLRP3 activation and IL-1β release, as was shown in murine sepsis models(11) 11, indicating the possibility of a link between defective mitochondrial clearance and autoinflammatory disease. Furthermore, in mice, mitochondria-derived ROS contributes to the activation of the NLRP3-inflammasome-mediated activation of caspase-1 by LPS and ATP(11).

We studied the potential contribution of disordered mitochondrial biology in to IL-1β mediated inflammation in the human monogenetic periodic fever disorder, mevalonate kinase deficiency (MKD), which gives rise to the hyper-IgD and periodic fever syndrome (HIDS). MKD is caused by loss of function of mevalonate kinase (MVK), an enzyme in the mevalonate pathway involved in cholesterol and non-sterol isoprenoid synthesis. The defect seriously impairs isoprenoid biosynthesis, resulting in reduced prenylation of proteins, particularly of some small GTPases. Individuals suffering from MKD experience recurring fever episodes that are to a large extent mediated by IL-1β(13). In this study, we aimed to clarify underlying mechanisms that cause hyper-secretion of IL-1β. We show that impaired isoprenoid biosynthesis interferes with autophagy, attenuates the activity of the mitochondrial respiratory chain and increases the release of mtDNA into the cytosol, thereby fostering an oxidized cytosolic milieu, which ultimately leads to hyper-secretion of IL-1β.

Materials and Methods

Reagents. Simvastatin, Geranylgeranylpyrophosphate, ATP, N-acetyl cysteine, Diphenylene iodonium, apocynin and Bafilomycin A1 were purchased from SIGMA-Aldrich. Mitotracker Green, Mitotracker Deep Red, MitoSOX, TMRM and H2DCFDA were purchased from invitrogen. Cyto-ID autophagy detection kit was purchased from Enzo life sciences. LPS (Escherichia coli EH 100) was obtained from Alexis Biochemical. MitoQ and decyltriphenylphosphonium (TPP) were synthesized as described 7. Simvastatin was hydrolyzed to its bioactive

form as previously described(14). ATP solution was made in RPMI 1640 and was buffered to a pH of 7,5.

Patient samples. Patients were children between the ages of 2 and 16 years with hyper IgD periodic fever Syndrome due to compound heterozygous mutations affecting both alleles of MVK. All had residual mevalonate kinase activities between 0.1% and 8.5% of healthy controls. At scheduled outpatient visits patients who were afebrile and well, underwent routine blood analysis. The ethical committee of the UMC Utrecht approved the use of residual material for this study. After informed consent was obtained from parents and from patients twelve years and older, residual material from routine blood tests was used to obtain peripheral blood mononuclear cells (PBMC). PBMC from patients and healthy donors were isolated using ficoll density gradient. PBMC fraction was washed twice in RPMI supplemented with 2% FBS and used immediately.

Cell cultures. THP-1 and HEK293T cells were both cultured in RPMI 1640 supplemented with 1% glutamine, antibiotics (penicillin, streptomycin) and 10% FBS. Simvastatin treatment of cells was 48 hours prior to the start of the experiment and at a concentration of 10 μM unless stated otherwise in the figure legends.

Mitochondrial damage, potential and superoxide measurements. Cells were washed once in PBS and resuspended in RPMI (w/o phenol red and w/o FBS) and appropriate probe. Staining concentrations: MitoTracker - 50 nM Mitotracker green and 50 nM Mitotracker deep red. TMRM - 20 nM. MitoSOX - 5 μM. Cells were incubated in the dark for 30 min at 37°C. Cells were centrifuged (500g 5 min), and suspended in RPMI w/o phenol red with 10% FBS. Cells were kept in the dark until measurement on FACS CANTO-II. Analysis was done with FACS Diva software.

Oxygen consumption and glycolysis measurements. Oxygen consumption rate and glycolysis were measured using the Seahorse XFe24 extracellular flux analyzer (Seahorse Biosciences) according to manufacturers' instructions. THP-1 cells were bound to the well using BD Cell-TAK coating. Coating of the wells was done according to manufacturer's instructions.

RNA isolation and quantification. RNA was isolated by dissolving cell pellets in TRIpure (RnD) and following manufacturers' protocols. Isolated RNA was converted to cDNA using iScript (Biorad) according to manufacturer's instructions. Detection was done with CF-96 (biorad) using SYBR green (biorad), 100 ng cDNA was used per reaction. Primers used: HO-1 Forward 5'-TCAGGCAGAGGGTGATAGAAG-3', reverse 5'- TTGGTGTCATGGGTCAGC-3'. ATG7 Forward 5'-CAGTTTGCCCCTTTTAGTAGTGC-3', reverse 5'- CCTTAATGTCCTTGGGAGCTTCA-3'. B2M Forward 5'-CCAGCAGAGAATGGAAAGTC-3', reverse 5'-GATGCTGCTTACATGTCTCG-3'. GAPDH Forward 5'-GTCGGAGTCAACGGATT-3', reverse 5'-AAGCTTCCCGTTCTCAG-3'

ATG7 shRNA knock down. Two different short hairpin RNA sequences (SIGMA-Aldrich) in a lentiviral vector (MISSION pLKO.1-puro) were used to make lentiviral particles. THP-1 cells were infected twice and then selected for puromycin resistance. ATG7 KD was tested with qPCR for ATG7. Hairpin sequences ATG7 KD1- 5'-CCGGGCCTGCTGAGGAGCTCTCCATCTCGAGATGGAGAGCTCCTCAGCAGGCTTTTT-3'. ATG7 KD2- 5'-CCGGGCTTTGGGATTTGACACATTTCTCGAGAAATGTGTCAAATCCCAAAGCTTTTT-3'. Control (scrambled) 5'-CCGGCAACAAGATGAAGAGCACCAACTCGAGTTGGTGCTCTTCATCTTGTTGTTTTT-3'. KD efficiency was determined by qPCR.

Cytosolic mitochondrial DNA measurement. Protocol was adapted slightly from(15). Cultured cells (1.5 * 107) were washed twice in ice cold PBS, then homogenized with a dounce homogenizer in ice cold 100 mM Tris-HCL (pH 7.4) containing 0.25 M Sucrose, 1 mM EDTA and protease inhibitor mix. Samples were centrifuged (700g, 4°C) for 10 min, the supernatant was collected and kept on ice. Protein content was determined by BCA protein assay. Samples were normalized to volume and protein concentration and centrifuged (10000g, 4°C) for 30 min. Supernatant was collected and 200 µl was used to isolate DNA with DNA blood and tissue kit (Qiagen). Mitochondrial DNA copy number was determined by qPCR for Cytochrome B. The following primers were used: CYTB forward: 5'-GCCTATATTACGGATCATTTCTCTACT-3'; and reverse: 5'-GCCTATGAAGGCTGTTGCTATAGT-3'.

Glutathione measurement. Glutathione and glutathione disulfide were colorimetrically measured as previously described 9. For glutathione disulfide 2.5 times more sample was used to achieve accurate readings. Ratio measurements were corrected accordingly.

Antioxidant capacity measurement. Cells were centrifuged and resuspended in staining mix (RPMI w/o phenol red with 10% FBS, 10 µM H2DCFCA) and incubated in the dark for 15 min at 37°C. Cells were immediately transferred to 6 FACS tubes and the fluorescence was measured. After the first fluorescent measurements hydrogen peroxide was added to the different tubes at a final concentration of 0, 5, 15, 45, 135 and 150 µM respectively. Tubes were briefly vortexed and incubated for 4 min covered from light and measured two more times in consecutive order. Analysis was performed by calculating the rate of fluorescence increase over time per sample (slope). The ratio of the fluorescence increase (slopes) at the different H2O2 concentrations over the background slope (without H2O2) provides a measure of antioxidant capacity (relative ratio). This allows the comparison of measurements by removing technical variation.

Cytokine measurements. Cells were centrifuged (500g, 5 min) and plated in 96 well plates in triplicates (2.0 * 105 cells/well in 200 µL). Inhibitors were added, followed by 1 hour incubation at 37°C. Next LPS (200 ng/mL) was added and supernatants were collected after 4 hours and stored at -80°C until measurement. Cytokine concentrations were determined by Mulitplex bead analysis.

Immunoblot analysis. Cells were washed twice in PBS and then resuspended in laemmli buffer and boiled for 10 min. Samples were then aliquoted and stored at -20°C until use. Protein content was determined with BCA assay and samples diluted to 1µg/µL. 5% v/v β-mercaptoethanol was added to the samples and they were separated on 12% SDS PAGE gel, followed by transfer to PVDF-FL membrane. 5% dried non-fat milk was used for blocking followed by primary antibody incubation (overnight 4°C, 0.5% milk in TBS-T), three washes and secondary antibody incubation (1 hr RT, 0.5% milk in TBS-T). Detection was done with enhanced chemiluminescence (ECL). Antibodies used: anti-p62 (Santa Cruz, sc-28359), anti-LC3 (Nanotools 0231-100/LC3-5F10), anti-actin (Santa Cruz, sc-1616) and anti-HSP90 (Cell Signaling Technology #4875S). We performed quantification of signal on blots using ImageJ [ref: Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997-2012]

Confocal analysis. HEK 293T cells were seeded in 24-well culture plates on 1,5mm glass coverslips pre-coated with poly-L-Lysine solution (0,1 w/v in H2O, SIGMA-Aldrich) for 30 min at 37°C. Plates washed twice in PBS, after which cells were plated at 40% confluency. After

24 hours Bafilomycin A1 was added (final conc. 10 nM, 4 hours at 37°C). The coverslips were washed twice in PBS and fixed (3.7% paraformaldehyde (Merck), 10 min at RT). Coverslips were mounted using Mowiol solution containing DAPI. Autophagosomes were counted in a semi-automated manner using Metamorph software (Molecular Devices). LC3 positive regions of interests were derived from binarized images obtained by thresholding immunofluorescence pictures, using the same threshold for all the samples. The number of cells was derived from the number of nuclei.
PBMC were plated on 8-well Lab-Tek® II Chamber Slides coated with Cell-Tak (BD biosciences) at a density of 1*106 cells per mL. After incubation with rapamycin, cells were washed twice with PBS and stained with Cyto-Id detection agent according to manufactures instructions. Next cells were washed twice with RPMI 1640 without phenol red, supplemented with 0.2% v/v Bovine Serum Albumin (BSA) (Roche) and 10 mM HEPES, and proceed for live cell imaging. Live cell imaging was performed on a Zeiss LSM710 confocal microscope equipped with a live-cell chamber device to maintain 37°C and 5% CO2 condition during experiments.
THP-1 cells were washed with PBS and stained in RPMI (w/o) phenol red with 100 nM Mitotracker green and 150 nM Mitotracker red for 30 min at 37°C. Cells were washed and plated on WillCo wells coated with Cell-Tak (BD biosciences) in RPMI (w/o) phenol red and 10% FBS. All images were obtained with 1.3x optical zoom using “Plan-Apochromat” 63x 1.40 oil DIC M27 objective on a Zeiss LSM710, and processed using Zen 2009 software (Zeiss Enhanced Navigation).

Statistics. Error bars shown represent SEM unless stated otherwise in the figure legends. Statistical test between two variables was done using the Mann-Whitney test. In figures one asterisks (*) indicates a p value < 0.05, two (**) indicate a p value of < 0.01.

Results

Altered redox state in model monocytes exhibiting IL-1β hyper secretion

Mouse studies support that mitochondrial (mt)ROS can mediate the activation of NRLP3 inflammasomes(16–18). We hypothesized that IL-1β hyper-secretion by human monocytic cells in analogy involves increased cytosolic levels of mtROS. To test this hypothesis, we used freshly isolated monocytes from healthy individuals or patients suffering from mevalonate kinase (MK) deficiency and THP-1 cells that were rendered similarly isoprenoid deficient due to 48-hour treatment with simvastatin. Simvastatin inhibits the HMG-CoA reductase, the rate-limiting enzyme in the mevalonate pathway, leading to a shortage of isoprenoids. The inclusion of simvastatin in LPS-treated monocytic cultures mimics the IL-1β hyper-secretion phenotype seen in the autoinflammatory syndrome MK deficiency (MKD)(19–23).

To verify the involvement of ROS in our model, we stimulated simvastatin-treated THP-1 cells with LPS for four hours in the presence of three different indiscriminate ROS inhibitors (1 hour pre-incubation): Apocynin, diphylene iodonium (DPI) and N-acetylcysteine (NAC). Generalized ROS inhibition normalized IL-1β and IL-18 secretion (fig. 1A). To next assess the involvement of altered cytosolic redox status in our model, we compared glutathione concentrations in reduced and oxidized form (SH/SS ratio), in simvastatin treated and control THP-1 monocytic cells. Simvastatin treatment lowers the SH/SS ratio by approximately 50%, reflecting a shift in redox balance towards a more oxidative state. Inclusion of the geranylgeranylpyrophosphate (GGPP) transferase inhibitor (GGTI) recapitulates this reduction, suggesting that the decrease in SH/SS ratio is caused by a lack of non-sterol isoprenoids (fig. 1B). Finally, oxidative stress causes the upregulation of heme oxygenase-1 (HO-1) mRNA levels(24). In-

deed, simvastatin treated cells express more HO-1 mRNA. This upregulation can be partly rescued by the addition of the generalized ROS scavenger N-acetyl cysteine (NAC) (fig. 1C).

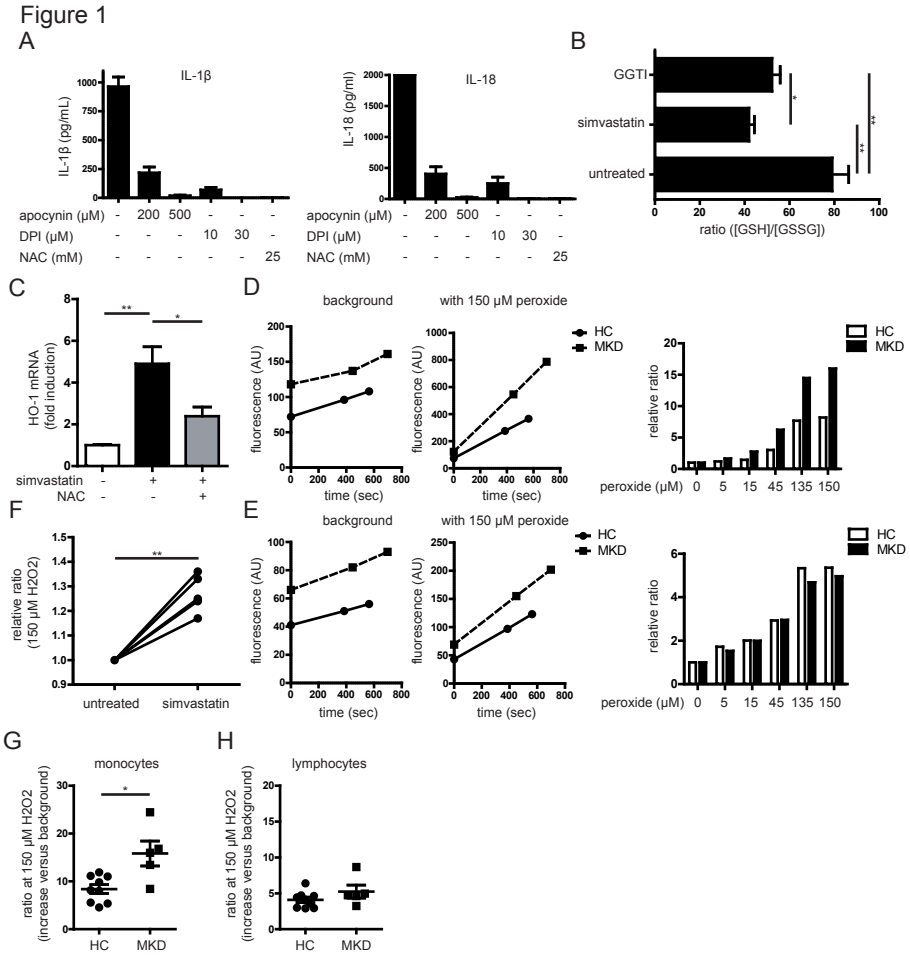

Figure 1: Altered redox state in periodic fever syndrome model cells and patient monocytes. (A) General ROS inhibition leads to decreased IL-1β (top) and IL-18 (bottom) secretion in simvastatin treated cells when stimulated with LPS. Representative of four independent experiments. (B) Ratio of reduced vs. oxidized glutathione in THP-1 cells. Simvastatin treatment leads to 50% reduction of ratio as does the GGPP transferase inhibitor GGTI, indicating a more oxidized intracellular environment. Average of five independent experiments. (C) Oxidative stress indicated by mRNA up regulation of HO-1 (normalized to β2M). Simvastatin treatment increases oxidative stress and up regulates HO-1. This can be partly rescued by co-incubation with the antioxidant NAC. Average of three independent experiments. (D) Example of antioxidant capacity measurements of a single patient with its coupled healthy control. Gating on monocytes (D) and lymphocytes (E) was done using size and granularity. On the background measurements the slopes are similar, indicating equal ROS production. (F) Simvastatin treatment leads to reduced antioxidant capacity in THP-1 cells. Shown is the ratio of the fluorescence increase with peroxide treatment divided by the background increase. A higher ratio is increased fluorescence and thus a lower capacity to prevent the probe from becoming oxidized. Each line represents an independent experiment. (G). Antioxidant capacity of blood monocytes from HC vs. MKD patients. The monocytes from patients have less capacity to clear

oxidative stress. (H) The difference in antioxidant capacity is specific for monocytes as MKD lymphocytes have a similar antioxidant capacity as HC.

To investigate if the increased oxidative burden is caused by increased ROS production or, alternatively, by decreased antioxidant capacity, we designed a new assay. In this assay we tested if lack of non-sterol isoprenoids impedes the ability to counter acute oxidative stress, in simvastatin treated and non-treated cells. We added the general ROS-sensitive fluorescent probe H2DCFDA to cells and exposed them to increasing concentrations of hydrogen peroxide (H2O2). Cells that have diminished capacity to clear oxidative stress exhibit a sharper increase in fluorescence in response to H2O2. The background measurements (which is essentially ROS production) show donor variation, but there is no indication that simvastatin treated cells have increased ROS production. The average slope of each sample is calculated and divided by the slope of the background (0 μM peroxide). This normalizes for the difference in ROS production between individuals (fig. 1D and E). Despite increased expression of HO-1, simvastatin-treated cells have impaired ability to clear acute oxidative stress, indicating diminished antioxidant capacity (fig. 1F). Together these data suggest that impaired non-sterol isoprenoid output is associated with a more oxidative cytosolic milieu due to diminishing antioxidant capacity and not due to increased ROS production.

To confirm these findings in actual patient cells, we isolated blood mononuclear cells (PBMC) from seven MKD patients that did not experience fever at the time of sampling, each paired with a healthy control, as well as two additional healthy controls. We stained fresh PBMC with the H2DCFDA probe, and gated on either monocytes or lymphocytes based on cell size and granularity features, using flow cytometry. MKD patient monocytes have a significantly reduced antioxidant capacity compared to monocytes from healthy control individuals, (fig. 1G). In contrast, lymphocytes from MKD patients and controls show comparable antioxidant responses (fig. 1H). We conclude that isoprenoid-deficient monocytic cells have reduced antioxidant capacity.

Isoprenoid deficiency raises mitochondrial membrane potential

Our data thus far suggests that isoprenoid deficiency causes prolonged exposure to oxidative stress. We hypothesized that mitochondria, a source of ROS, might have reduced integrity in MKD allowing leakage of ROS from the mitochondria. To address this possibility, we stained THP-1 cells with a fluorescent mitochondrial probe that reports the relative amount of mitochondria (MitoTracker Green) and, as a measure of mitochondrial damage, a mitochondrial probe that is sensitive to the mitochondrial inner transmembrane potential (MitoTracker Deep Red)(25). Damaged mitochondria lose membrane potential and lose MitoTracker Deep Red staining. Treatment with simvastatin resulted in an increase in transmembrane potential, as shown by increase in MitoTracker Deep Red staining, while the MitoTracker Green fluorescence was unchanged (fig. 2A-C, gating strategy fig. 2E). Thus, the lack of isoprenoids leads to increased mitochondrial potential. Earlier studies had shown that increase in mitochondrial transmembrane potential is associated with increased ROS production(26, 27). However, this work was done on isolated mitochondria. Other reports suggest that the regulation of mitochondrial potential can also be independent of ROS(28). To confirm the increase in mitochondrial potential in isoprenoid-deficient monocytes we used a different probe; tetramethyl rhodamine methyl ester (TMRM). Measurements with TMRM supported our MitoTracker results: simvastatin treatment increases mitochondrial potential. This is partially rescued by inclusion of GGPP, and therefore, increase in mitochondrial potential is mediated by the lack of non-sterol isoprenoids (fig. 2D).

Figure 2

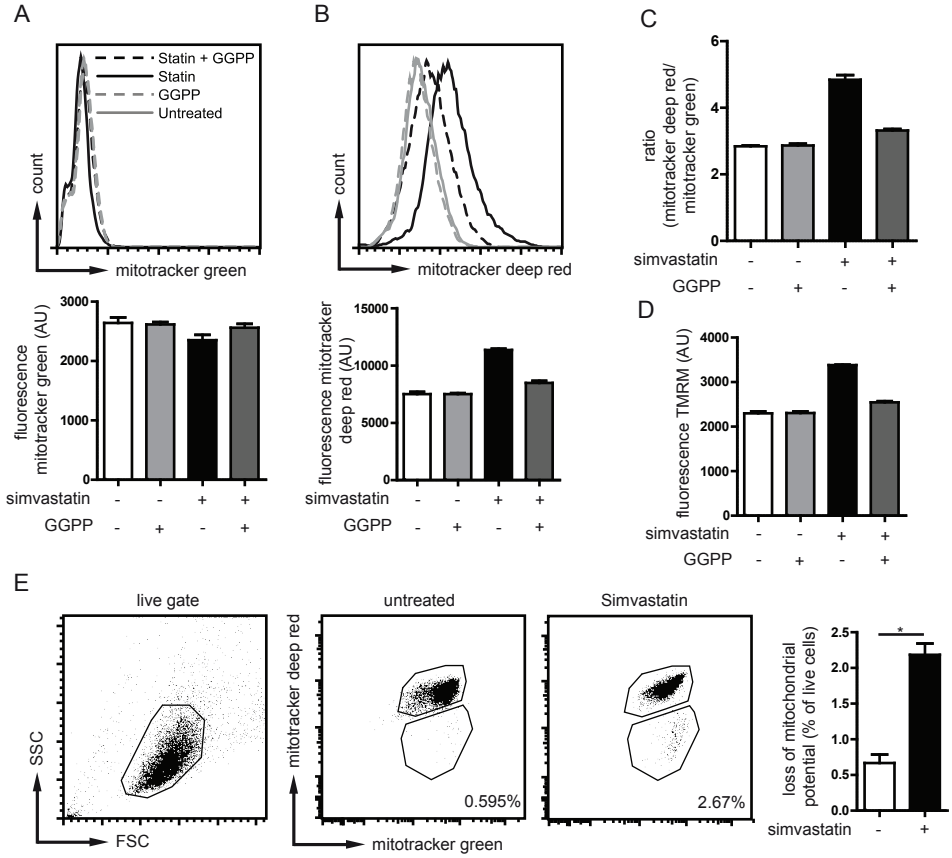

Figure 2: Mitochondrial potential is altered in isoprenoid deficient cells. (A) Above: total mitochondrial mass as determined with mitotracker green in THP-1 cells under different conditions. Below: bar graph representation of data. Gating was done as shown in 2E, only top gate was used for potential measurements. (B) Above: mitochondrial potential determined by mitotracker deep red staining. The potential is clearly increased in simvastatin treatment. Below: bar graph representation of data.(C). Ratio of mitochondrial potential over mitochondrial mass to more accurately determine the potential differences per unit of mitochondrial mass. Statin treatment leads to increased mitochondrial potential, which can be partially rescued by the addition of exogenous GGPP. Panel A, B and C show the data of a representative experiment out of five independent experiments, each consisting of triplicate measurements. (D) Staining of THP-1 cells with TMRM confirms mitotracker data; the increased potential of mitochondria with simvastatin treatment, and dependence on isoprenoid availability. Representative of three independent experiments shown. (E) An increasing amount of cells loses mitochondrial potential with simvastatin treatment as determined combined mirotracker green and deep red staining. The amount of cells (gated on live cells) losing mitochondrial potential is tripled by statin treatment. Representative plots shown of four independent experiments. Bar graph is shown as average and SEM of four independent experiments.

Although on average MitoTracker Deep Red fluorescence increased, the number of cells with intact MitoTracker Green fluorescence that lost MitoTracker Deep Red fluorescence altogether, increased 3-fold, from 0.7% in untreated cells to 2.2% of simvastatin-treated THP-1 cells (fig. 2E). This implies that the percentage of living cells that harbored damaged mi-

tochondria increased 3-fold, as. Together, these data suggest that inhibition of protein isoprenylation is associated with increased mitochondrial transmembrane potential and a decrease in mitochondrial stability.

Damaged mitochondria accumulate in the cytosol of isoprenoid deficient monocytes

We next investigated if the increase in mitochondrial transmembrane potential is due to an increase in mitochondrial energy metabolism. To this end we measured oxygen consumption and glycolysis rate. First basal respiration was measured, followed by addition of the ATPase inhibitor oligomycin to block respiration. Next, the uncoupler FCCP was added to induce maximum respiration, followed by the electron transport chain inhibitors antimycin A and rotenone (complex III and complex I respectively) to completely abolish mitochondrial respiration. Simvastatin treatment caused a lower basal energy metabolism than found in untreated cells, when assayed for either oxygen consumption or glycolysis rate (fig. 3A, left and right, respectively). However, the regulation of oxygen consumption was similar under all conditions.

To assay for additional changes in mitochondria that may associate with the high transmembrane potential, we next visualized mitochondria in MitoTracker-stained THP-1 cells using confocal microscopy. The majority of simvastatin-treated cells exhibited mitochondria that were elongated, while in untreated cells mitochondria appeared predominantly as small dots (fig. 3B). To confirm that the elongation is due to prenylation and not to of target effects of simvastatin, we repeated the experiment with the geranylgeranyl transferase inhititor. This resulted in mitochondrial elongation, although not to the same extend. Earlier work showed that elongated mitochondria are associated with induction of autophagy(29), and that elongation of mitochondria modulates metabolic efficiency(30). Furthermore, isoprenoid-deficiency caused by simvastatin treatment causes cells to proliferate more slowly. Taken all together, isoprenoid deficiency in THP-1 cells causes both an increase in mitochondrial potential as well as mitochondrial elongation. It is possible that the mitochondrial elongation is responsible for the increase in membrane potential, however this would need further investigation.

Mitochondrial components play an important role in priming immune responses(6, 7, 16). As we observed mitochondrial irregularity, and an increased proportion of cells harboring depolarized mitochondria, we hypothesized that isoprenoid deficient monocytes contain released internal mitochondrial constituents. We therefore investigated the presence of mtDNA into the cytosol, by isolation of cytosolic fractions of cells and measured in these fractions the content of mtDNA (by qPCR, assay for Cytochrome B for which the coding sequence is located exclusively on mtDNA). We consistently found higher levels of mtDNA in cytosolic fractions in simvastatin-treated cells, both before (fig. 3D) and after LPS/ATP stimulation (fig. 3E), although the latter was not significantly different. The effect of simvastatin was pronounced, inducing nearly half the amount of mtDNA release seen in cells treated with the potent mitochondrial toxin DPI, which was used as a positive control (fig. 3F). Raised mtDNA could be either actively released by the mitochondria, or alternatively be a consequence of defective autophagy-mediated clearance of damaged mitochondria. In mice, mtROS are necessary for the active release of mtDNA(11). To test whether the mtDNA is actively secreted by the mitochondria in human cells, we took a similar approach as Nakahira et al(11) and co-incubated untreated and simvastatin treated cells with the mitochondria-targeted antioxidant mitoQ, which is a ubiquinone targeted to the mitochondria by the triphenylphosphonium lipophillic cation. The non-antioxidant mitochondria targeting moiety decyl-triphenylphosphonium (TPP) was used as a control(31). Both compounds show similar and even increased release of mtDNA into the cytosol (fig. 3G), indicating that mtROS is not necessary to cause the mtDNA

release in our culture system.

Figure 3

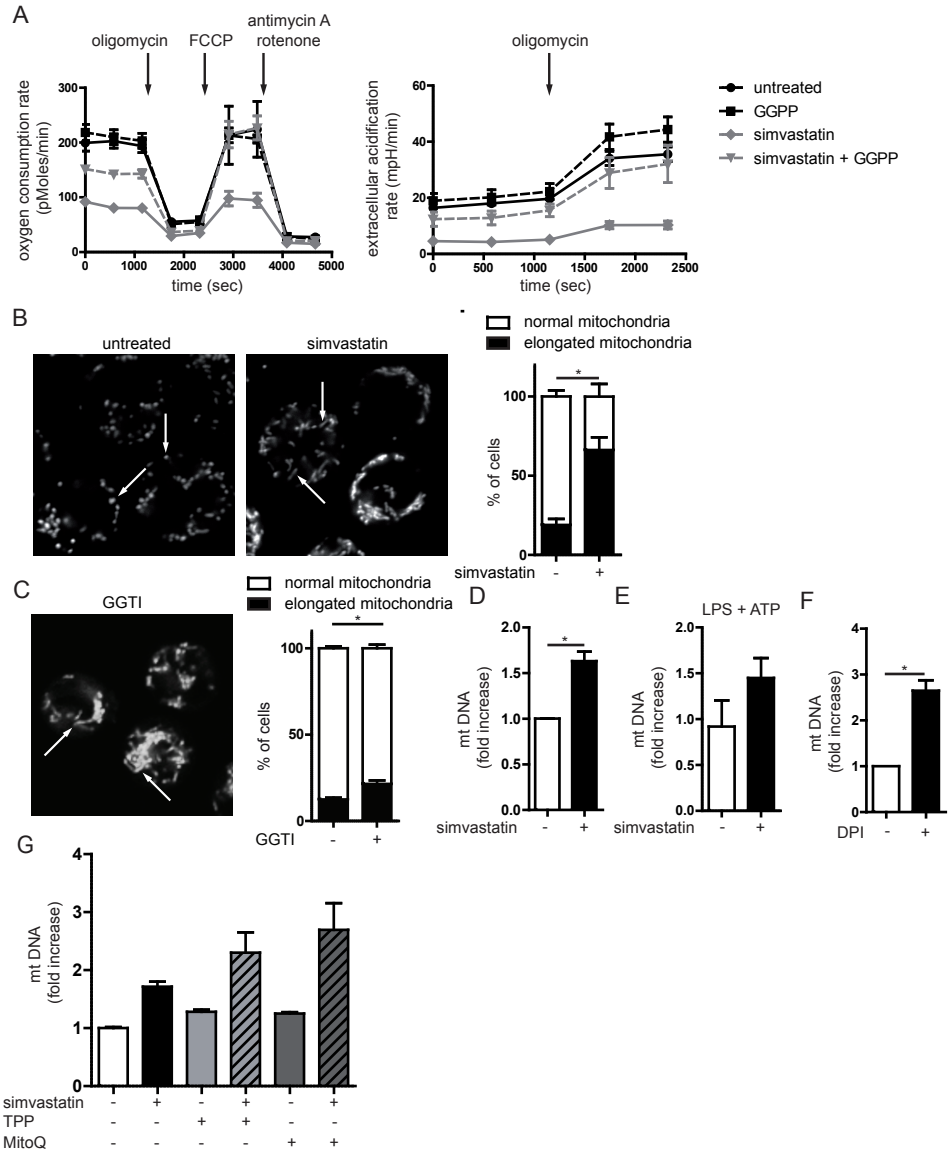

Figure 3: Altered metabolism and release of mitochondrial components in isoprenoid deficient cells. (A) Oxygen consumption and glycolysis. Simvastatin treated THP-1 cells have reduced metabolism, which is partly rescued by GGPP. Representative picture shown of three independent experiments. (B) THP-1 cells are stained with mitotracker green and deep red and checked for mitochondrial morphology with confocal microscopy. Mitochondria in simvastatin treated cells have a more elongated morphology. Representative pictures are shown. Bar graph consists of over 250 cells scored per condition; error bars indicate variance in scoring by three different observers. (C) To confirm that mitochondrial elongation is mediated by lack of isoprenylation THP-1 cells were treated with GGTI (10μM). This also leads to significant mitochondrial

elongation, although not as strong as with simvastatin. Bar graph consists of over 150 cells scored per condition; error bars indicate variance in scoring by three different observers. (D) Amount of mitochondrial DNA in the cytosolic fraction of THP-1 cells. Simvastatin treatment leads to an accumulation of mtDNA in the cytosol. Data of four independent experiments combined. (E) The accumulation of mitochondrial DNA is independent of cell activation. Average of two experiments shown. (F) Simvastatin does not induce the amount of cytosolic mtDNA as seen with DPI. Data of four independent experiments combined. (G) The release of mtDNA is not caused by ROS generated in the mitochondria. Co-incubation with mitochondrial ROS scavenger mitoQ cannot prevent the accumulation of mtDNA in the cytosol. TPP is used as control for mitochondrial targeting group in mitoQ. Average of two independent experiments shown.

Figure 4

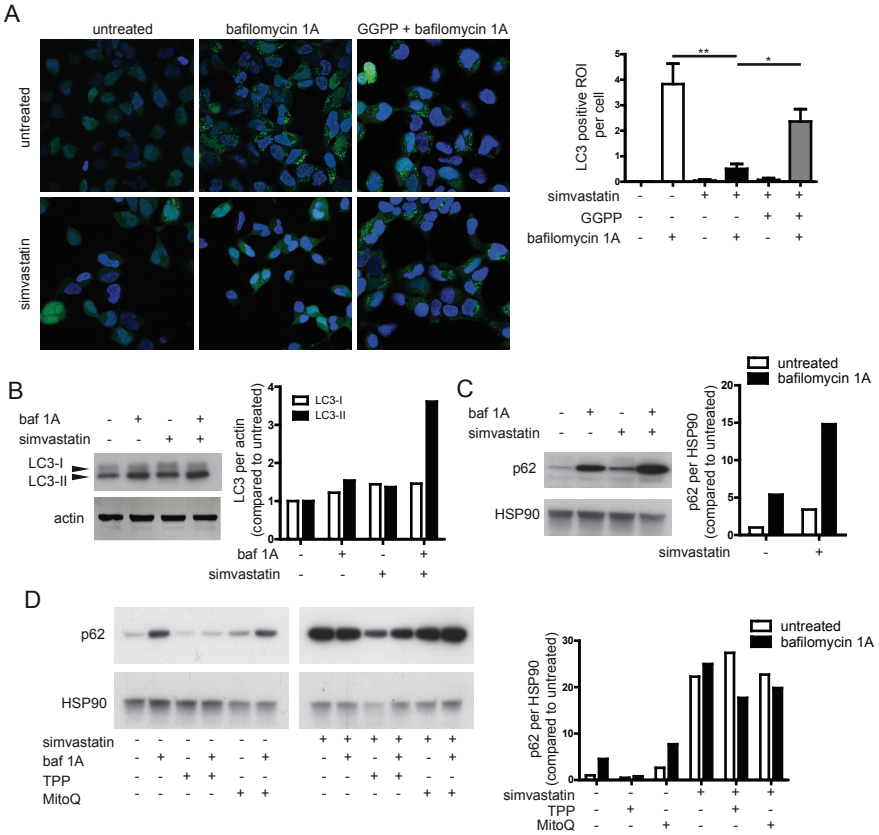

Figure 4: Isoprenoid shortage causes deficiency in autophagy. (A) In HEK293 LC3-GFP cells the lack of isoprenoids causes a clear reduction in the number of autophagosomes. This is almost completely dependent GGPP as addition of exogenous GGPP can rescue the phenotype. Representative pictures shown, bar graph is average of three experiments. (B) In THP-1 cells, the defect appears not to be in the induction of autophagy as no differences are observed in LC3 levels. Bar graphs contains quantification of shown blot, normalized first to loading control, then the untreated condition is set to 1; representative of 4 independent experiments (C) There is a late stage block in autophagy, as seen by the lack of p62 degradation upon simvastatin treatment. Bar graphs contains quantification of shown blot, treated as in (B), representative of 4 independent experiments shown. (D) The defect in autophagy is not mediated by mtROS. The p62 levels after treatment with scavenger MitoQ or control compound TPP could not rescue the phenotype of the statin treatment. Protein levels in TPP condition are lower as the concentration of TPP used approaches toxic levels. Bar graphs contains quantification of shown blot, normalized as in (B), representative of 2 independent experiments shown.

Accumulation of defective mitochondria is due to impaired autophagy

Can defects in autophagy of mitochondria predispose human monocytes to IL-1β hyper-secretion? Autophagy is controlled by multiple pathways; including those that are mediated by small GTPases. As small GTPases are isoprenylated proteins, defects in the mevalonate pathway can lead to unprenylated GTPases(19, 32). We proposed that defects in isoprenylation may cause impairment of autophagy. We tested if isoprenylation is required for generation of LC3+-mature autophagosomal membranes, using LC3-GFP fusion proteins expressed in HEK 293T cells(33). LC3 is a marker for autophagosomal membranes. Initially, it is an 18 kDa protein (LC3-I), which is matured to a 16 kDa form (LC3-II) when it is incorporated in the membranes. LC3-GFP cells were treated with simvastatin and/or the late stage autophagy inhibitor Bafilomycin A1 (Baf A1). Baf A1 single treatment causes an accumulation of autophagosomes, whereas addition of simvastatin treatment to Baf A1 counteracts Baf A1-mediated autophagosome buildup (fig. 4A). Thus, simvastatin treatment inhibits autophagosome formation as measured by LC3 autophagosomal membrane incorporation. This defect is caused specifically by defective isoprenylation as exogenously added GGPP rescues the phenotype.

To further analyze a possible role for autophagy in IL-1β hyper-secretion by isoprenoid deficient monocytes, we treated THP-1 cells with simvastatin (48 hours) and prepared whole cell lysates for LC3 and p62 protein analysis by Western blot. LC3-II levels represent the induction of autophagy, while p62 degradation is a marker for successful completion of autophagy. Simvastatin, Baf A1 and their combined treatment lead to increased levels of LC3-II in THP-1 cells (fig. 4B), confirming earlier data that statins can induce autophagy(34, 35), and suggesting that the induction of autophagy is not inhibited by simvastatin. Levels of p62 were induced by Baf A1 treatment, as expected, as Baf A1 inhibits autophagosomal turnover of p62 and p62-associated protein aggregates (fig. 4C). Statin treatment also caused an increase in p62, suggesting that statin inhibits autophagy at a stage between LC3-I/LC3-II conversion and successful completion of autophagy. Combined Baf A1 and simvastatin treatment resulted in a further increase in p62. To confirm that the increase in both p62 and LC3 is also isoprenoid dependent, we repeated these experiments in the presence of exogenously added GGPP, to rescue its deficiency caused by simvastatin treatment. We conclude that inhibition of protein isoprenylation causes defective autophagosomal degradation in THP-1 cells.

We demonstrated that mtDNA release is not mediated by mtROS, but wished to confirm that the mtROS released by defective mitochondria do not inhibit autophagy, supporting our hypothesis that lack of prenylation is the key factor in inhibited autophagy. Therefore, we specifically inhibited mtROS with the mitochondria-targeted antioxidant MitoQ, with TPP used as a control compound. We measured the protein levels of LC3 and p62 in monocytic cells. Again, MitoQ and TPP treatment did not change LC3 and p62 levels, negating a role for mtROS in the control of autophagy, with or without simvastatin pretreatment (fig. 4D). This was confirmed in HEK293T LC3-GFP cells where the addition of mitoQ had no effect autophagosme formation (not shown). Antioxidant capacity of the MitoQ reagent itself was intact (our unpublished results). We conclude the accumulation of damaged mitochondria originates from a defect in autophagy and leads to the release of mitochondrial components. Inhibition of mtROS cannot prevent the damage to mitochondria nor rescue the autophagy defect in these cells

IL-1β secretion involves both cytosolic release of mitochondrial components and defective autophagy

Is the release of mitochondrial components into the cytosol necessary for IL-1β release? The selective inhibition of mtROS using MitoQ counteracts proinflammatory cytokine secretion, in the autoinflammatory disease TRAPS (TNF receptor associated periodic syndrome)(7). We used MitoQ in simvastatin treated THP-1 monocytes to determine whether mtROS are required for LPS-induced IL-1β secretion. In cells treated with MitoQ, the IL-1β secretion upon LPS stimulation was unaffected (fig. 5A). We conclude that ROS, but not mitochondrial ROS, are involved in the IL-1β secretion in isoprenoid deficiency or MKD.

Figure 5

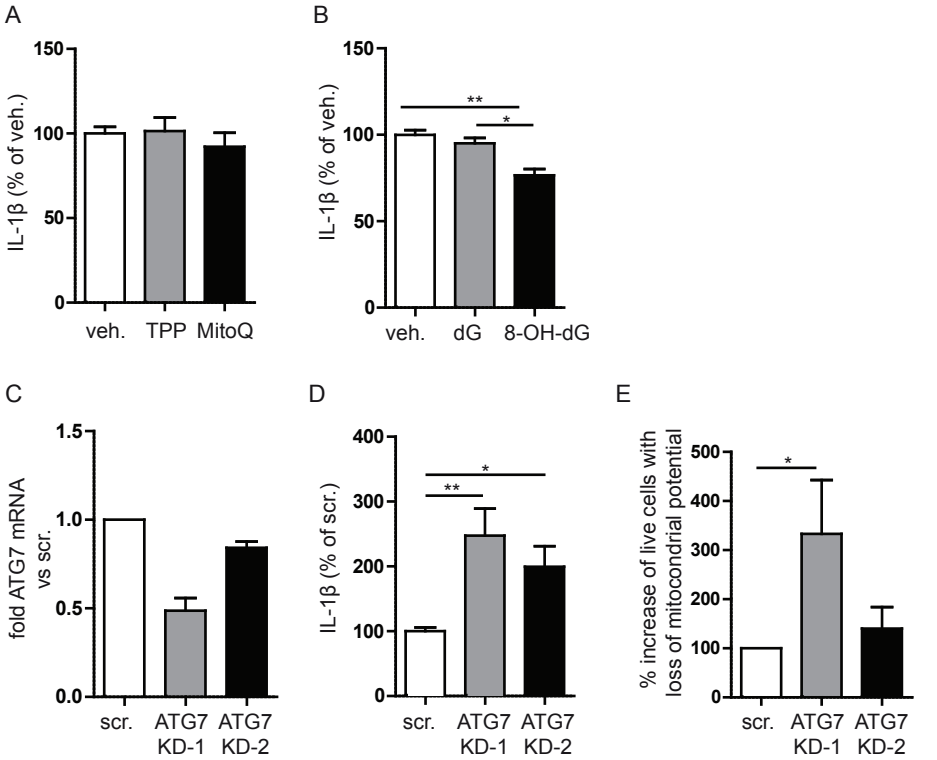

Figure 5: IL-1β secretion in isoprenoid deficient cells involves inflammasome activation by oxidized mitochondrial DNA and defective autophagy. (A) IL-1β secretion in MKD model is not mediated directly by mtROS. Despite MitoQ pretreatment, it did not ameliorate IL-1β secretion. (B) Inflammasome activation is mediated by oxidized mtDNA. Incubation of simvastatin treated cells with oxidized nucleotides prior to stimulation leads to decreased IL-1β secretion, while non-oxidized nucleotide has no effect. Average of three independent experiments shown. (C) Knock-down of ATG7 by two virally stably transduced shRNAs in THP-1 cells. The amount of mRNA for ATG7 was normalized to GAPDH and compared to scrambled control cells. (D) IL-1β secretion of ATG7 KD cells after stimulation with LPS and ATP. IL-1β secretion is clearly increased in cells with impaired autophagy. This was done without simvastatin pretreatment to isolate the effect of defective autophagy. Average of 3 experiments shown. (E) Defective autophagy leads to accumulation of damaged mitochondria. ATG7 KD cells were either treated with DPI or left untreated, followed by staining with mitotracker green and deep red. Cells were gated as done in fig. 2E. When autophagy is defective, induction of mitochondrial stress leads to accumulation of damaged mitochondria. Bar graph with average of 4 independent experiments shown.

Oxidized mtDNA serves as activating ligand for the NLRP3 inflammasome(16). Since we observed that isoprenoid depletion significantly increased mtDNA levels in the cytosol (Fig. 3C), we hypothesized that the mtDNA is oxidized and activates the NLRP3 inflammasome, thereby increasing IL-1β secretion. The binding of oxidized mtDNA to NLRP3 and subsequent inflammasome activation can be competitively inhibited by oxidized nucleotides that do not induce inflammasome activation(16). We incubated simvastatin-treated THP-1 cells for one hour with either oxidized deoxyguanosine (8-OH-dG) or regular deoxyguanosine (dG), followed by four hours of LPS stimulation. We found that 8-OH-dG significantly reduced the amount of secreted IL-1β compared to both vehicle and dG control (fig. 5B). Thus, mtDNA release contributes to the IL-1β hyper-secretion via the NLRP3 inflammasome. Having shown that isoprenoid shortage causes both IL-1β generation and impaired autophagy, we hypothesized that isoprenoid shortage causes IL-1β generation through impaired autophagy. We therefore transduced THP-1 monocytic cells with shRNA knock down (KD) constructs for the autophagy protein ATG7 or a scrambled control. We generated two different THP-1 lines with 50% and 20% KD efficiency (fig. 5C). Cells were stimulated with LPS and ATP and the level of secreted IL-1β was measured (fig. 5D). As expected, both KD lines secreted more IL-1β than the scrambled control, with the 50% KD secreting more than the 20% KD. This confirmed that impairment of autophagy enhances IL-β secretion, irrespective of isoprenoid biosynthesis. To finally confirm that the defective autophagy is responsible for the accumulation of damaged mitochondria, we induced mitochondrial damage in the ATG7 KD and control THP-1 cells as described (four hour DPI (15 μM) treatment(36)), and measured the fraction of cells that lose mitochondrial potential in ATG7 KD and control cells. Loss of potential was gauged by flow cytometry using cell staining with MitoTracker green and MitoTracker deep red. Using the same gating strategy as for figure 2E, we observed that ATG7 KD samples exhibit an increase in live cells that are losing mitochondrial potential (fig. 5E). The increase is evident in the 50% ATG7 KD cells, yet did not reach significance in the 20% ATG7 KD cells. Thus, defective autophagy results in the accumulation of damaged mitochondria in THP-1 cells.

Defective autophagy in MKD monocytes leads to increased IL-1β secretion

To confirm our findings of defective autophagy in isoprenoid deficient THP-1 cells we investigated autophagy in fresh MKD patient-derived monocytes, by visualization of autophagosomes and testing their ability to modulate levels of IL-1β secretion. We stained autophagosomes in PBMCs from both healthy controls and MKD patients. MKD patient monocytes display a partial block in autophagy, confirming our work in simvastatin-treated THP-1 cells. Autophagosomes are visible in both types of monocytes (fig. 6A). Treatment of these monocytes with the autophagy inducing agent rapamycin does not modify the number of cells with autophagosomes or autophagosomes per cell. However, there is a clear difference in the IL-1β secretion between healthy controls and patient cells treated with rapamycin. In healthy controls, pre-incubation of PBMCs with rapamycin (250 nM) one hour prior to LPS stimulation (4 hours) significantly reduces IL-1β secretion ($p<0.01$, $n=5$). In contrast, the reduction of IL-1β secretion in rapamycin-treated MKD PBMCs did not reach significance (NS, $n=3$; fig. 6B). This indicates that inducing autophagy counteracts IL-1β cytokine secretion in healthy controls. This is in line with previous studies done in mice, where rapamycin treated mice had lower IL-1β serum levels after LPS challenge(37). In MKD patients, where autophagy is already defective, inducing autophagy has limited effect. Taken all together, our data suggests that inflammasome activation in MKD, can involve damaged mitochondria in monocytes. Due to defects in autophagy these damaged mitochondria are not effectively cleared, resulting in accumulation of mitochondrial components in the cytosol. These components, left present in

the cytosol when autophagy is defective prime monocytes in MKD for IL-1β hyper-secretion. Figure 6

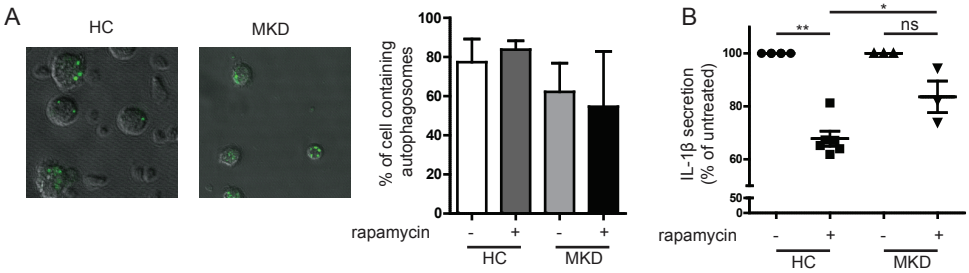

Figure 6: Primary MKD cells respond differently to autophagy stimulus. (A) Freshly isolated PBMCs from a healthy donor and MKD patient were stained with an autophagy probe. Samples were checked for number of cells with autophagosomes and autophagosomes per cell, but no significant differences were observed, largely due to significant variation in healthy controls. Representative pictures show autophagosomes in both HC and MKD patients. Bargraph shows quantification of % of monocytes containing 1 or more autophagosomes. HC n = 3, patients n = 2. (B) PBMCs from healthy donors and MKD patients were stimulated for one hour with rapamycin (250 nM) followed by 4 hours of LPS (200 ng/mL) stimulation. Inducing autophagy by rapamycin leads to a decrease of IL-1β secretion in healthy controls of approx. 40%, while in MKD patients the reduction is less than 20%. This indicates that inducing autophagy has less of an effect in IL-1β secretion in MKD patients.* = $p<0.05$, ** = $p<0.01$.

Discussion

We here addressed a role for autophagy in the development of autoinflammation in the periodic fever syndrome, mevalonate kinase deficiency (MKD). Autophagy is a cellular process for the turnover of damaged proteins and organelles, including mitochondria(12). It had been known that both insufficient and excessive autophagosomal degradation are harmful for the cell. Autophagy is therefore strictly regulated by several signaling pathways that are inhibitory (serine-threonine kinase mammalian target of rapamycin (mTOR) and class I phosphoinositide 3-kinases (PI3Ks)) or that activate autophagy (class III PI3Ks)(38). Isoprenylation can modulate the induction of autophagy as was suggested from experiments in which statin treatment induced autophagy(34, 39, 40). However, dissimilarities in statin dosage and treatment protocols complicate the interpretation of these studies. Also, the induction was not seen in all models, suggesting that statin induced autophagy is cell type specific. Our results here show a direct role for isoprenylation in the induction and completion of autophagy in monocytic cells. We show that autophagy controls IL-1β release through removal of mitochondrial components that otherwise stimulate NLRP3 inflammasome activation. A strong genetic link was recently described between the autophagy gene ATG16L1 and susceptibility to chronic inflammation in Crohn's disease(41, 42). ATG16L1 suppresses IL-1β signaling by promoting the degradation of autophagosomal p62(43). In addition, a recent study by Bachetti et al. showed that in TRAPS, a mutant version of TNF receptor 1 causes inhibition of autophagy, leading to autoinflammatory disease(22). In this current study we provide experimental proof that general autophagic dysfunction enhances IL-1β release.

While the effect of isoprenoid deficiency on autophagy is clear, there are still some unresolved issues. Despite our best efforts we have not been able to explain the increased mitochondrial potential associated with isoprenoid deficiency. This effect is highly reproducible, yet seems contradictory with the low oxygen metabolism. It is possible that the elongation of the mi-

tochondria, associated with increased efficiency, leads to a higher potential even with lower oxygen consumption rates. To clarify this issue, mitochondrial elongation should be induced without the starvation effect of simvastatin. Unfortunately we are unable to do so in our model system.

Recent work shows a role for ROS in the development of the IL-1β-driven autoinflammatory disorders(7, 17, 44). Initially, NADPH-oxidase-derived ROS production was also implicated in IL-1β release in the periodic fever CAPS (cryopyrin-associated periodic syndrome), but analyses of chronic granulomatous disease patients discounted a role for NADPH-oxidases in these disorders(17, 18). The source of ROS appeared mitochondrial (i.e., mtROS) rather than derived from the NADPH-oxidases at the plasma membrane, as was supported by work in cells from chronic granulomatous disease patients that have defective NADPH-dependent ROS due to mutations in p47-phox(17). A publication on TRAPS showed that specific inhibition of mtROS release alleviates cytokine secretion(7). Our own data here shows that monocytes of MKD patients exhibit defective antioxidant responses, whereas MKD lymphocytes responded similarly to healthy control cells when exposed to acute redox stress. While we can inhibit cytokine secretion using general ROS inhibitors, the specific inhibition of mtROS had no effect on the cytokine secretion. The question that remains is; what is the source of ROS that mediates the hyper-secretion? It is possible that the hyper-secretion of IL-1β is mediated by mtROS and that the (membrane-bound) MitoQ is unable to inhibit all ROS escaping from compromised mitochondria. However, additional research will be needed to confirm or discount this possibility. The notion that ROS play a role in statin-induced IL-1β generation has been recently supported by similar findings in simvastatin and fluvastatin treated human monocytes and murine macrophages(23).

Under conditions of oxidative stress, thioredoxin-interacting protein (TXNIP) associates to NLRP3, as was shown in human THP-1 cells. The presence of ROS in the cytosol was thereby directly linked to inflammasome activation and secretion of bioactive IL-1β(17). Our work supports the earlier reported requirement for cytosolic ROS in IL-1β release by monocytes(7, 9, 44), and adds that mitochondrial alterations and defects in autophagy increase mtDNA into the cytosol. Our data thereby place autophagy upstream of mtROS release and NLRP3 inflammasome activation. The defective autophagy cannot clear damaged mitochondria, which leads to release of mtROS and mtDNA in the cytosol, which in turn can activate the NLRP3 inflammasome. We observed increased IL-1β release is associated with reduced mitochondrial activity (i.e., attenuated respiratory chain activity), mtROS and mtDNA cytosolic release. The cytosolic presence of oxidized mtDNA release contributes directly to IL-1β generation, which supports earlier work showing that cytosolic mtDNA directly stimulates NLRP3 inflammasomes in mouse macrophages(18). Recent work shows the formation of autophagosomes at contact sites between mitochondria and endoplasmic reticulum, further corroborating a role for autophagosomes in clearance of mitochondrial constituents(45). Mouse macrophages that are deficient in autophagy proteins beclin-1 and LC3B secrete increased levels of IL-1β upon NLRP3 inflammasome activation using LPS/ATP combined treatment(18). Mouse macrophages that lack mtDNA due to culture in the presence ethidium bromide exhibit reduced caspase-1 activity and attenuation of respiratory chain activity and ROS production. The generation of mtROS was placed as an upstream mechanism in the release of IL-1β. However, NLRP3 inflammasome stimuli may differ in their subcellular source of ROS (i.e., crystalline particles such as asbestos and silica may induce NLRP3 inflammasome activation through ROS production by NADPH-oxidase(17, 46)). Accordingly, mito-TEMPO, which inhibits mtROS release, did not inhibit IL-1β secretion induced by LPS and monosodium urate(18). Our own findings in human cells support these data, as our block of mtROS release using MitoQ did

not inhibit IL-1β release.

Defective autophagy had earlier been linked to IL-1β release; depletion of autophagy proteins promotes NLRP3-mediated IL-1β release via the release of mtDNA and ROS in mouse macrophages(11). Moreover, Mycobacterium tuberculosis infected ATG7 KD macrophages secrete more IL-1β(47). In periodic fever disorders, a role for defective autophagy had not yet been established. We here show, for the first time, the complex interplay of autophagy and mitochondrial function, and how imbalance of this interplay leads to excessive IL-1β generation, in a well-established model of the autoinflammatory disease MKD. Furthermore, our data with fresh primary PBMC from MKD patients confirm the data found in the MKD model system. Together our results support that defective autophagy plays a role in the pathogenesis of this autoinflammatory disease and possibly that of other periodic fever syndromes as well.

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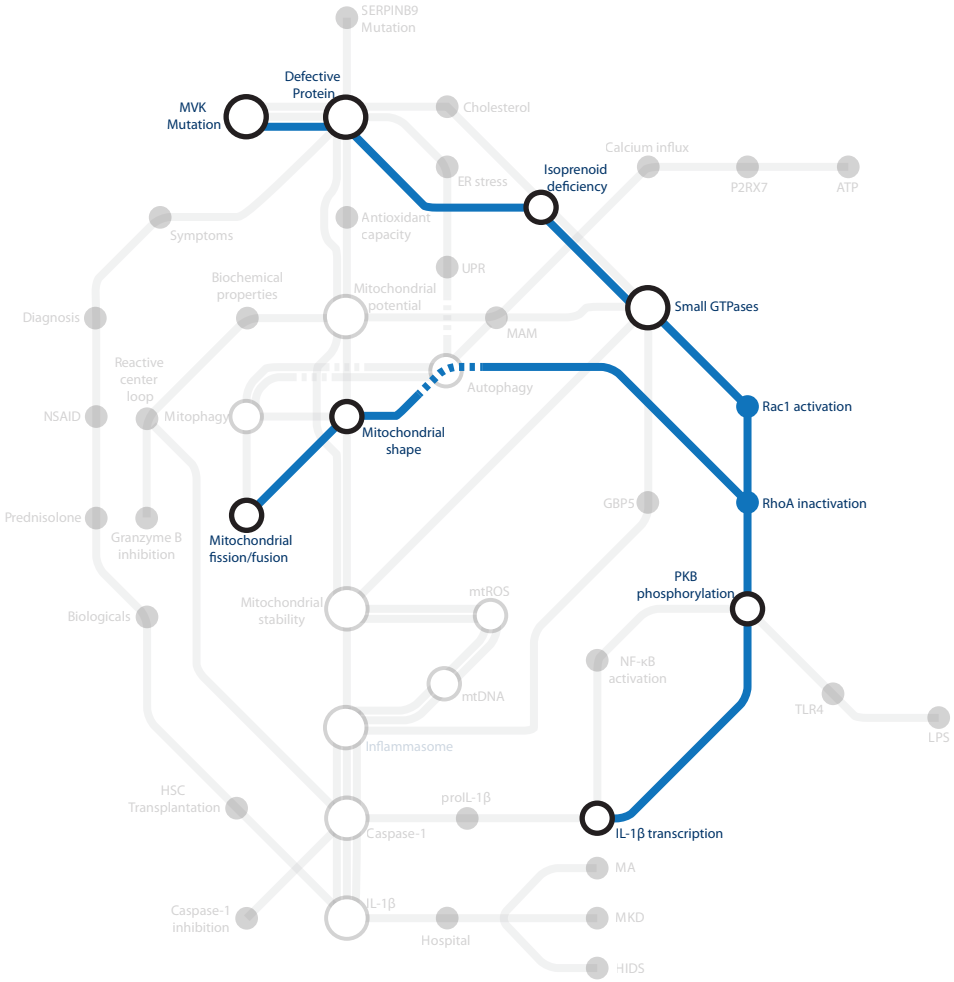
SERPINB9 Mutation
Defective Protein
MVK Mutation
Cholesterol
Calcium influx
ER stress
Isoprenoid deficiency
P2RX7
ATP
Symptoms
Antioxidant capacity
UPR
Biochemical properties
Mitochondrial potential
Small GTPases
Diagnosis
MAM
Reactive center loop
Autophagy
Rac1 activation
NSAID
Mitophagy
Mitochondrial shape
GBP5
RhoA inactivation
Prednisolone
Granzyme B inhibition
Mitochondrial fission/fusion
PKB phosphorylation
Biologicals
Mitochondrial stability
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NF-κB activation
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MKD
Hospital
HIDS

CHAPTER 4

Unprenylated RhoA Contributes to IL-1β Hypersecretion in Mevalonate Kinase Deficiency Model through Stimulation of Rac1 Activity

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Abstract

Protein prenylation is a post-translational modification whereby non-sterol isoprenoid lipid chains are added, thereby modifying the molecular partners with which proteins interact. The autoinflammatory disease mevalonate kinase deficiency (MKD) is characterized by a severe reduction in protein prenylation. A major class of proteins affected are small GTPases, including Rac1 and RhoA. It had not been understood how protein prenylation of small GTPases relates to GTP hydrolysis activity and downstream signaling. We here investigated the contribution of RhoA prenylation to the biochemical pathways that underlie MKD-associated IL-1β hyper secretion, using human cell cultures, Rac1 and RhoA protein variants and pharmacological inhibitors. We found that when unprenylated, the GTP-bound levels of RhoA decrease, causing a reduction in GTPase activity and increased Protein Kinase B (PKB) phosphorylation. Cells expressing unprenylated RhoA produce increased levels of interleukin 1β mRNA. Of other phenotypic cellular changes seen in MKD, increased mitochondrial potential and mitochondrial elongation, only mitochondrial elongation was observed. Finally, we show that pharmacological inactivation of RhoA boosts Rac1 activity, a small GTPase whose activity was earlier implied in MKD pathogenesis. Together our data show that RhoA plays a pivotal role in MKD pathogenesis, through Rac1/PKB signaling towards interleukin 1β production, and elucidate the effects of protein prenylation in monocytes.

Introduction

The functional consequence of protein prenylation to cell signaling is incompletely understood. Earlier work on prenylation of small GTPases provided experimental support that prenylation can be involved in subcellular protein localization and control of GTP hydrolysis. The prenylation modification can direct small GTPases to the membrane, or cover a nuclear localization signal, as is the case in Rac1 ((1)(2)). Small GTPases are heavily involved in cytoskeleton regulation and vesicular trafficking, where the prenylation moiety serves to localize GTPases to the membranes and regulate their activity.
The process of protein prenylation itself concerns the C-terminal post-translational modification of a protein with a non-sterol isoprenoid. Protein targets for prenylation express a short four amino acid sequence at the C-terminus, called a CAAX box, where the modification is attached at the C (or cysteine), while A stands for aliphatic, and X can be any residue. The prenylation is executed by the farnesyl- (C-15) or geranylgeranyl (C-20) transeferases. The prenylation moiety can have different functions. In case of the nuclear lamins for instance, the prenylation helps the cellular localization and provides a signal for further modification and stable integration in the membrane(3). Another major function of prenylation moieties is as a specific protein interaction domain(4). Prenylation is not always permanent. Although the thioether connecting the lipid to the protein is very stable, it has been described that the C-terminal residues can be removed, including the prenyl group, yielding an unprenylated but slightly shorter version of the protein. This is for instance mediated by the bacterial protease YopT (from Y.Pestis) to alter cellular shape and increase infection rate(5, 6).

The activity of small GTPases is regulated by an extensive network of Guanine-nucleotide exchange factors, or GEFs, and GTPase activating proteins (GAPs) When a small GTPase is bound to GDP it is generally considered inactive. GEFs can exchange the GDP for GTP, while GAPs increase the hydrolytic activity of GTPases, to catalyze the conversion of GTP back to GDP. This creates a network of molecular on-off switches(7). As an added layer of regulation there are proteins classified as GDP dissociation inhibitors (GDI). These proteins can prevent the binding of GEFs and GAPs thereby keeping the GTPase in its current state (8). Prenylation plays an important role in this process. One of the best studied GDIs, Rho GDI, uses the prenylation moiety on GTPases as an important part of its interaction surface with its targets, not only inhibiting GEF/GAP binding but controlling localization at the same time(4).

One obvious disease where protein prenylation is affected is Mevalonate Kinase Deficiency (MKD). MKD is caused by mutations in the mevalonate kinase protein(9, 10). This protein, as a key enzyme in the pathway that synthesizes cholesterol, produces an intermediate for isoprenoid synthesis. Previous studies have shown that MKD causes depletion and shortage of both farnesylpyrophosphate and geranylgeranylpyrophosphate, both substrates used for protein prenylation(11). Patients suffer from periodic fever episodes induced by uncontrolled release of Interleukin-1β (IL-1β). Although both isoprenoid versions are depleted, the inflammation related symptoms are caused specifically by a lack of geranylgeranylpyrophosphate(12). The exogenous addition of geranylgeranylpyrophosphate to patient cells or cell cultures restores the normal regulation of IL-1β secretion. The MKD phenotype can be mimicked in vitro by exposing cells to statins, which are compounds that inhibit HMG-Coa reductase, the enzyme directly upstream of mevalonate kinase. Inhibition of the enzyme geranylgeranyltransferase leads to a similar MKD phenotype(13).

In the context of MKD, the small GTPase Rac1 was identified as a mediator for the IL-1β hyper secretion. Rac1, with reduced prenylation due to isoprenoid shortage, was more active

in MKD cell culture models. Inhibition of Rac1 in THP-1 monocyte cultures leads to normalization of IL-1β levels(14). Yet there are a number of other biochemical hallmarks of MKD, including altered autophagy, mitochondrial potential and morphology and redox balance that cannot be explained by aberrant activity of Rac1 alone (15). Henneman et al (16) reported that RhoA, normally prenylated, activity was increased in MKD patient-derived fibroblasts, which however do not display the autoinflammation phenotype. We here asked what is the contribution of unprenylated RhoA to IL-1β-mediated autoinflammation in an MKD model.. We find that inhibiting prenylation in the monocytoid cell line THP-1, reduces, RhoA activity.. Reduced RhoA activity does not affect mitochondrial membrane potential or mitophagy, but does affect mitochondrial morphology. In addition, inactive RhoA leads to activation of Rac1 and PKB phosphorylation, thereby contributing to IL-1β gene transcription and the pathogenesis of MKD.

Experimental procedures

Reagents. Simvastatin and Bafilomycin A1 were purchased from SIGMA-Aldrich. Mitotracker Green, Mitotracker Deep Red and GGTI-298 were bought from Millipore. C3 Transferase (Rho inhibitor) was bought from Cytoskeleton. Simvastatin was hydrolyzed to its bioactive form as previously described(17).
Cell cultures. THP-1 cells were cultured in RPMI 1640 supplemented with 1% glutamine, antibiotics (penicillin, streptomycin) and 10% or 0.1% FBS. HEK293T cells were cultured in DMEM supplemented with 1% glutamine, antibiotics (penicillin, streptomycin) and 10% FBS cells Simvastatin treatment of cells was 48 hours prior to the start of the experiment and at a concentration of 10 μM unless stated otherwise in the figure legends.

Plasmids. Plasmids containing Rac1 and RhoA with and without CAAX box were made by amplifying cDNA isolated from human fibroblasts. The primers introduced a restriction site (KpnI forward, XhoI reverse) to allow further cloning. Primers: RhoA forward 5'-CGATA GGTACC ATG GCT GCC ATC CGG AAG AAA-3', RhoA reverse 5'-CGATA CTCGAG TCA CAA GAC AAG GCA CCC AGA TTT TTT CTT CC-3', RhoA (-CAAX) reverse 5'-CGATA CTCGAG TCA CCC AGA TTT TTT CTT CC-3', Rac1 forward 5'-CGATA GGTACC ATG CAG GCC ATC AAG TGT GTG-3', Rac1 reverse 5'-CGATA CTCGAG TTA CAA CAG CAG GCA TTT TCT C-3', Rac1 (-CAAX) reverse 5'-CGATA CTCGAG TTA TTT TCT CTT CCT CTT CTT CAC-3'. The amplicons were ligated into pGEM-T vector (Promega) and sequenced to ensure the correct sequences were amplified. The RhoA and Rac1 sequences were then removed from the pGEM-T vector with KpnI and XhoI and ligated into pcDNA3 vector (Invitrogen).

Activated RhoA and Rac1 immunoprecipitation assays. Activated RhoA and Rac1 assessment assays were performed as described in Henneman et al(16). Cultured THP-1 cells were washed three times with ice-cold PBS, lysed by scraping in the culture flask using lysis buffer [50 mM Tris pH 7.4, 200 mM NaCl, 10% glycerol, 1% tergitol-type NP-40 (NP-40), 2 mM magnesiumchloride (MgCl2), 0.1 mM phenylmethylsulfonylfluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM benzamidine, 1 mM dithiothreitol (DTT), 1 mM vanadate]. The lysates were then centrifuged (10 min, 12,000 g) and the supernatants collected. After determining protein concentration of the supernatants with a Bradford assay, 500 μg total protein in 500 ml was incubated (60 min, 4°C) with bacterially produced glutathione S-transferase Ras-binding domain (GST-RBD, Rhotekin) (Reid et al(18)) (for RhoA immunoprecipitations) or GST-p21-activated Ser/Thr kinase (PAK) (Sander et al(19)) (for Rac1 pulldowns) bound to glutathione-agarose beads (Sigma). The beads were washed three times with lysis buffer followed by centrifugation (10 s, 12,000 g). Bound proteins, i.e., active RhoA or Rac1, were

eluted by boiling in SDS-sample buffer and analyzed by immunoblot analysis.

Activated RhoA and Rac1 G-LISA assays. Rac1 and RhoA G-LISA kits (Cytoskeleton) were used to quantify the amount of GTP-bound Rac1 and RhoA according to manufacturers' instructions. Results were normalized by quantifying total Rac1 and RhoA by western blot. Analysis of band intensity was done with Gel Doc easy system and software (Biorad).

Transient transfection. THP-1 cells were transfected using the Amaxa Human Monocyte Nucleofector Kit (Lonza). Cells were pelleted and resuspended in 100 µl of Human Monocyte Nucleofector Solution and transferred into a cuvette together with 1 µg of DNA. A control plasmid containing GFP was cotransfected in 1:10 ratio to determine transfection efficiency. Transfection was done using a Nucleofector II (Lonza) using program Y-001. Following transfection, cells are resuspended in 500 µl of RPMI (2mM glutamine, antibiotics and 20% human serum) plated. Cells were analyzed 24 hours post transfection. Hek293T cells were seeded in 6-well culture plates at 50% confluence. Cells were transfected with 6 µg DNA using low toxicity (LTX) lipofectamine Kit (Invitrogen Life Technologies) according to the manufacturer's instructions. To determine the transfection efficiency, the pCMV-dsRed plasmid expressing the red fluorescent protein was used. Cells were analyzed 48 hours post transfection.

Western Blot analysis. Western blots were performed according to standard procedure. Equal amounts of proteins were separated by 12% SDS polyacrylamide gels and transferred polyvinylidene difluoride (PVDF-FL) membranes (Millipore). Membranes were blocked in Tris buffered saline containing 0,3% Tween and 5% non-fat dry milk (30 min, RT). Membranes were probed with the following antibodies: anti-RhoA (Santa Cruz Biotechnology sc-418), anti-Rac-1 (Santa Cruz Biotechnology sc-217), anti-phospho AKT (S473) (Cell Signaling Technology #4058S) and anti-HSP90 (Cell Signaling Technology #4875S)

Flow cytometry. Phospho-PKB levels were detected by flow cytometry by fixing cells with Fixation buffer (BD Cytofix) for 15 minutes at 37oC. Next cells were pelleted and resuspended in Perm Buffer III (BD Phosflow) and incubated for 30 minutes on ice. Cells were subsequently washed twice and stained with anti-phospho-AKT-PE (S473) (BD Phosflow #560378) for 30 minutes (RT, dark, shaking). Cells were washed twice and resuspended in flow cytometry buffer (PBS, 2% FCS) for acquisition.
For measurement of mitochondrial relative presence and mitochondrial membrane potential, cells were washed once in PBS, resuspended in staining mix containing RPMI without phenol red (Invitrogen Life Technologies), 50nM Mito Tracker Deep Green and 50nM Mito Tracker Deep Red and incubated (30 min, 37oC, dark). Cells were subsequently pelleted and resuspended in culture medium. Data were acquired with a FacsCanto II (BD bioscience) and were analyzed with FACS Diva or Flowjo analytical software.

Confocal microscopy. Hek293T cells were seeded in 24-well culture plates on 1,5mm glass coverslips pre-coated with 2% poly-L-Lysine solution (0,1 w/v in H20, SIGMA-Aldrich) for 30 min at room temperature. Plates were washed twice in PBS, after which cells were added on plates. After 24 hours incubation at 37oC, cells were treated with Bafilomycin A1 (10nM, 4 hours). The culture medium was removed and coverslips were washed twice in PBS. Cells were fixed in 3,7 % paraformaldehyde (MERC) for 10 min (RT) and washed twice with PBS. Residual PBS was carefully removed from coverslips and they were mounted on prolong containing DAPI. THP-1 cells were washed with PBS and stained in RPMI (w/o) phenol red with 100 nM Mitotracker green and 150 nM Mitotracker red for 30 min at 37°C. Cells were washed and plated on WillCo wells coated with Cell-Tak (BD biosciences) in RPMI (w/o) phenol red

and 10% FBS. All images were obtained with 1.3x optical zoom using “Plan-Apochromat” 63x 1.40 oil DIC M27 objective on a Zeiss LSM710, and processed using Zen 2009 software (Zeiss Enhanced Navigation). Images were analyzed with ImageJ software. Cell count was obtained by automated nuclei count in the DAPI channel. (threshold for brightness was set, followed by automated analysis with the following settings: size (square pixels) 75-infinity and circularity 0.05-1.00). Autophagosomes were quantified is the same way using the GFP channel (settings: size (square pixels) 5-75 and circularity 0.20-1.00).

RNA isolation and quantification. RNA was isolated by dissolving cell pellets in TRIpure (RnD) and following manufacturers’ protocols. Isolated RNA was converted to cDNA using iScript (Biorad) according to manufacturer’s instructions. Detection was done with CF-96 (biorad) using SYBR green (biorad), 100 ng cDNA was used per reaction. Primers used: GAPDH Forward 5’-GTCGGAGTCAACGGATT-3’, reverse 5’- AAGCTTCCCGTTCTCAG-3’, NLRP3 Forward 5’- CTTCCTTTCCAGTTTGCTGC-3’, reverse 5’- TCTCGCAGTCCACTTCCTTT-3’, CASP1 Forward 5’- ATAGCTGGGTTGTCCTGCAC-3’, reverse 5’- GCCAAATTTGCATCACATACA-3’, IL-1β Forward 5’- AGAAGAACCTATCTTCTTCGAC-3’, reverse 5’- ACTCTCCAGCTGTAGAGTGG-3’, IL-18 Forward 5’- TCCCCAGCTTGCTGAGCCCT-3’, reverse 5’-GTTGGCAGCCAGGAGGGCAA-3’

Statistics. Error bars shown represent SEM unless stated otherwise in the figure legends. Statistical test between two variables was done using the Mann-Whitney test. In figures one asterisks (*) indicates a p value < 0.05, two (**) indicate a p value of < 0.01.

4

Results

Prenylation-deficient RhoA has reduced GTP-binding activity in THP-1 cells

Prenylation status of small GTPases affects their activation to varying extent amongst small GTPase family members and the tissue or cell lines studied(20, 21). Activated Rac1 facilitated IL-1β-driven autoinflammation in a culture model of MKD (14). Rac1 and RhoA can be reciprocally regulated(22, 23), suggesting the possibility that deficiency in RhoA prenylation may cause Rac1 activation and thereby drive IL-1β-driven autoinflammation. We tested this hypothesis in a culture-based MKD model, where THP-1 monocytic cells were pretreated 24 to 48 hours with 10 μM simvastatin(13, 15). Simvastatin targets HMG-CoA reductase, the rate-limiting enzyme in the cholesterol synthesis cascade. Inhibition of HMG-CoA reductase is a well-established system to inhibit protein prenylation and mimic MKD. Simvastatin treated THP-1 cells were lysed and GTP-bound Rac1 or RhoA was immunoprecipitated from the lysate (fig. 1A and 1B, respectively). The total amount of Rac1 and RhoA protein was also quantified and β-actin included as a loading control. We found that Rac1 activity is indeed increased upon simvastatin treatment, however RhoA activity was significantly reduced. Of note, an opposite effect was seen by Henneman et al, where treatment of fibroblasts of both healthy individuals and MKD patients with low dose simvastatin caused increased GTP bound RhoA (16). This apparent disconcord may relate to the different cell types used. In support of our data, a study by Hiraoka et al. on monocyte motility found that pitavastatin treatment of THP-1 cells reduced RhoA activity(24), corroborating our finding of reduced RhoA activity in THP-1 cells.

What is the effect of the increased Rac1 or RhoA activity on downstream signaling? To this end, we measured the phosphorylation status of Protein Kinase B (PKB) in transientlytransfected THP-1 cells with either a plasmid with the wild-type protein, or a prenylation deficient

mutant that lacks the CAAX box. Indeed, Rac1 activation can increase pPKB without the need for additional stimuli(14). We found that transfection of both Rac1 and Rac1-caax led to increased Rac1 levels, but only the mutant increased phosphorylation of PKB (fig. 1C). The effect of the RhoA plasmids on the total amount of RhoA was small, however WT RhoA suppresses PKB phosphorylation. An effect that can be abrogated by rendering RhoA prenylation deficient (fig. 1D). As a second approach to address PKB phosphorylation, we analyzed the cells by flow cytometry (fig. 1E). Simvastatin treated cells were taken along as a positive control. While transfection with the wild-type Rac1 or RhoA proteins did not alter the phosphorylated PKB level, prenylation-deficient mutants did increase p-PKB levels although not to the same extent as simvastatin treatment. We therefore conclude that the increased phosphorylation of PKB is mediated by both Rac1 and RhoA-mediated signaling and that the latter effect is largely mediated via Rac1.

Figure 1

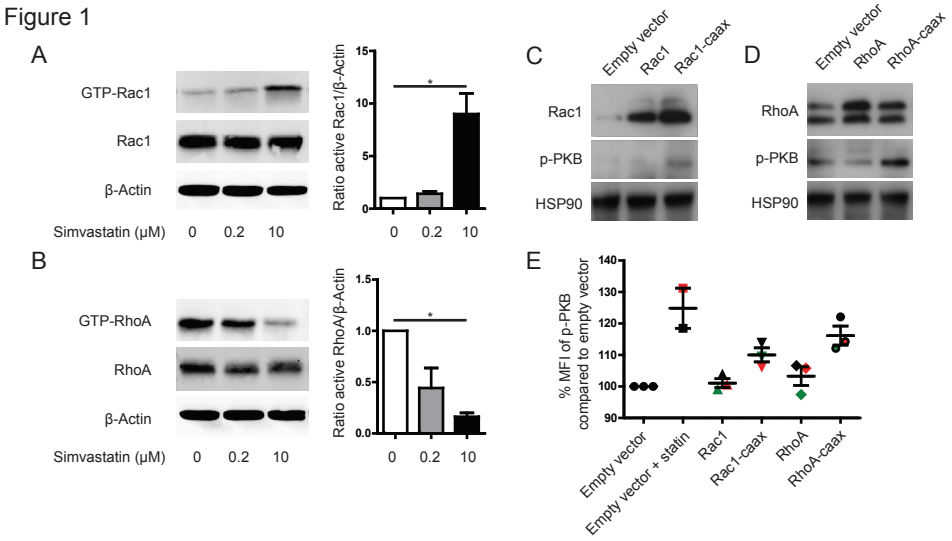

Figure 1: Lack of prenylation changes Rac1 and RhoA activation and signaling. (A) Pull down of GTP-bound Rac1 and RhoA (B) in THP-1 cells treated with various concentrations of simvastatin. Reducing the prenylation of both proteins changes their activation status. The amount of GTP-bound Rac1 is increased, while GTP-bound RhoA decreases with increasing simvastatin concentration. Left panels show representative blots of one experiment, right panels show bar graph of quantified blots as average of at least three independent experiments. (C) Transfection of Rac1 and RhoA (D) or its prenylation-dead mutants in THP-1 cells increases PKB signaling. Representative blots are shown of three independent experiments. HSP90 was used as loading control. (E) Unprenylated Rac1 and RhoA both contribute to increased p-PKB. THP- cells transfected with Rac1 and RhoA or its prenylation dead mutants are stained for p-PKB and analyzed with flow cytometry. Simvastatin treated cells were taken along as positive control.

Lack of prenylation on RhoA has no effect on autophagy

In a previous study we have shown that impaired mitophagy, i.e. autophagy of damaged mitochondria, contributes to MKD-associated IL-1β hypersecretion (15). The precise mechanism by which defective isoprenylation interferes with mitophagy remains elusive. We therefore investigated if RhoA prenylation modulates autophagosome formation. We used a HEK293T cell line where LC3 is N-terminally fused with GFP to enable direct visualization of autophagosomes. LC3 (or ATG8) is an essential protein for autophagosome formation. It is

recruited to autophagosomes upon their assembly and is incorporated into the autophagosomal membrane. It can therefore be used to identify autophagosomes.(25) We transfected the cells with RhoA or the prenylation-deficient RhoA mutant. Transfected cells were left untreated or treated with bafilomycin A1. Bafilomycin A1 impairs the acidification of lysosomes and indirectly inhibits autophagy (26), resulting in an accumulation of autophagosomes. We found that basal autophagy levels were unchanged in cells overexpressing either RhoA variant compared to controls (fig. 2A & 2B). Treatment with bafilomycin A1 resulted in equal accumulation of autophagosomes in all conditions (fig. 2A & 2B). We recently reported altered prenylation on autophagy by simvastatin treatment, where bafilomycin did not increase the amount of autophagosomes, suggesting a block in autophagosome development(15). Taken together, these data support that RhoA prenylation status does not alter or interfere with autophagy, surmising that simvastatin targets other molecules than RhoA alone. Of note, RhoA-caax mutant cells expressed a more stretched cell morphology than untreated, empty vector or wild-type RhoA-transfected HEK293T cells, confirming prior work on the regulatory role of RhoA on the cytoskeleton(27, 28). Thus, while RhoA-caax modulates cell morphology, most likely as a consequence of deregulating the RhoA signaling, unprenylated RhoA does not affect autophagy.

RhoA controls mitochondrial morphology, but not function

Another recently observed but poorly understood phenomenon is the increase of mitochondrial potential and the change of mitochondrial morphology with deficient isoprenylation.(15) Several studies have identified a role for RhoA and RhoA signaling in mitochondrial trafficking, protection and mitochondrial mediated apoptosis(29–31). We therefore investigated whether RhoA prenylation affects mitochondrial trans-membrane potential. To this end, we treated transfected HEK293T cells with mitotracker green and mitotracker deep red. Mitotracker green stains all mitochondria and provides a measure of the mitochondrial mass per cell. The affinity of mitotracker deep red for mitochondria increases with potential, thereby allowing comparison of mitochondrial potential by flow cytometry. Transfection of wild-type RhoA or RhoA-caax did not modulate either mitochondrial mass or potential (fig. 3A). When normalized for mitochondrial mass, through division of the deep red signal (potential) by the green signal (mass), the resulting calculated potentials again showed no difference (fig. 3B). RhoA may modulate mitochondrial morphology (32), considering that GTPases can control mitochondrial fusion of small vesicles into tubular structures and therefor might be involved in the observed change in mitochondrial morphology with impaired prenylation(15). THP-1 cells were therefore stained with the same mitotrackers and subsequently taken for live cell microscopy analysis, and enumeration of the percentage of cells expressing elongated mitochondria. We attained RhoA inactivation by culture of untransfected THP-1 cells in the presence of C3 transferase from Clostridium Botulinum linked to a cell penetrating moiety. In order to avoid electroporation or transfection-related cell damage, which might affect mitochondrial morphology unrelated to RhoA or RhoA-caax. Inactivation of RhoA led to a significant increase of cells with elongated mitochondria (fig 3C and 3D). This induction of tubular mitochondrial morphology we had previously observed as a consequence of simvastatin treatment, however simvastatin also led to increased mitochondrial potential. Thus, RhoA inactivation may account for elongation of mitochondria, but does not increase mitochondrial potential, which may require additional signals. It should be noted that the C3 transferase also inhibits RhoB and RhoC, and we therefore cannot unequivocally state that the effect are only mediated by RhoA. However is currently no literature describing signaling for either protein leading to mitochondrial morphology, potential or mitophagy. Furthermore, most reports seem to indicate that RhoB and RhoC become active (GTP bound) when unprenylated, while the C3 transferase leaves them inactive (although in different cell types) (33, 34). Considering that the same

Figure 2

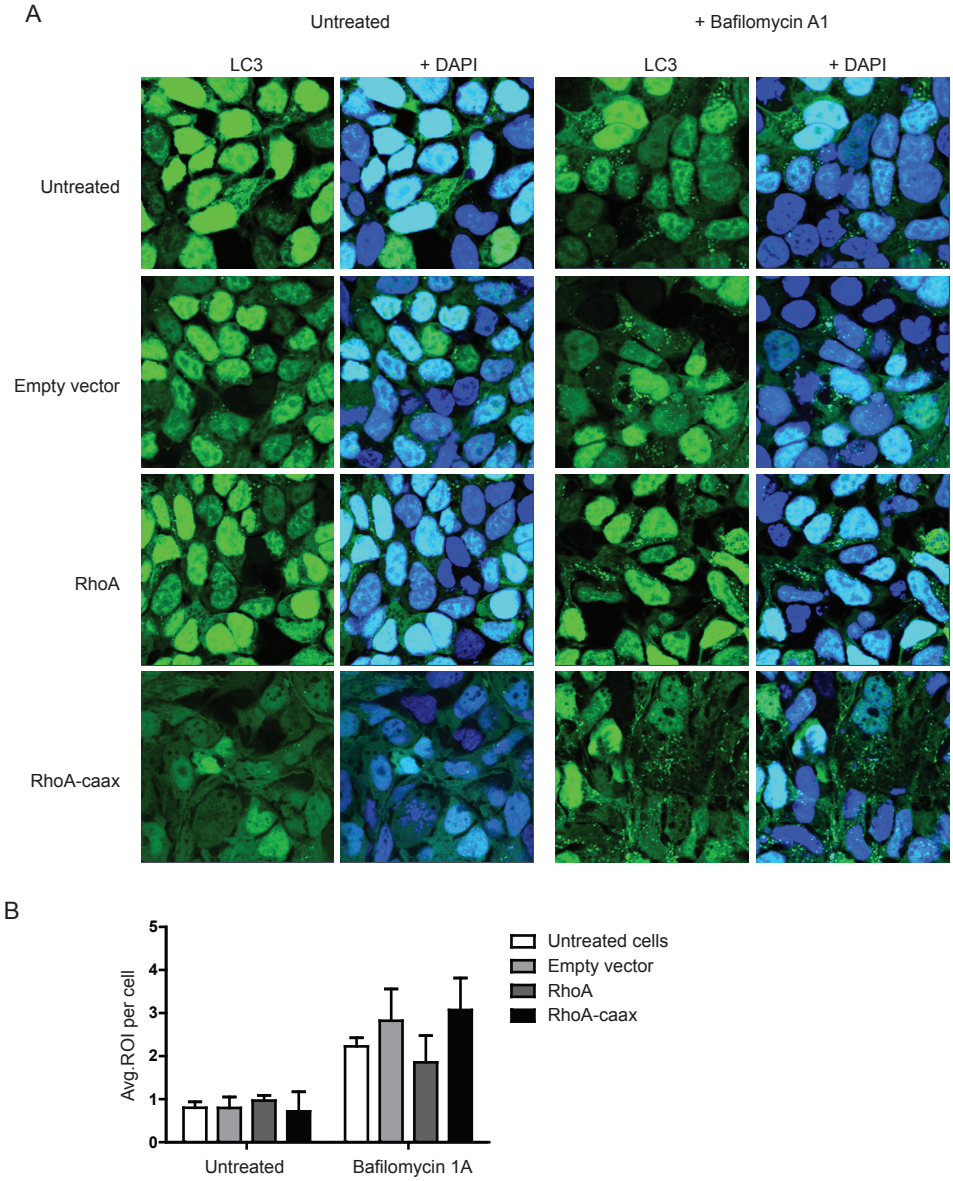

Figure 2: Lack of prenylation on RhoA does not affect autophagy. (A) HEK293T LC3-GFP cells are transfected with RhoA or the prenylation dead mutant. Following treatment with either vehicle or bafilomycin 1A cells are fixed and nuclei are counterstained with DAPI. During confocal microscopy the ability of the cells to form autophagosomes was assessed. Transfection both variants of RhoA does not hamper autophagosome formation, both with and without bafilomycin A1. The only notable difference is the altered cell morphology in the RhoA mutant. (B) Bar graph with the quantification of number of autophagosomes per cell. Each condition is based on at least 120 cells. No significant differences were observed between any of the conditions within the untreated or bafilomycin 1A groups.

change in mitochondrial morphology is seen with simvastatin treatment and Rho inhibition, where as far as we know the only common factor is RhoA inactivation, we propose that the mitochondrial elongation is a consequence or RhoA inactivation and RhoB and RhoC do not play an important role in the process.

Figure 3

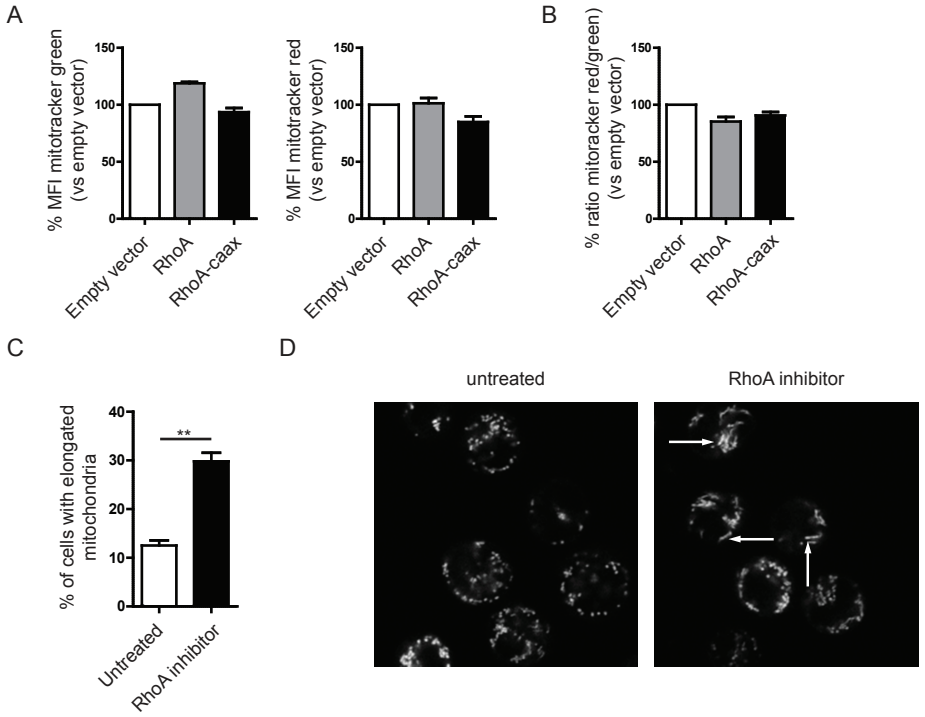

Figure 3: Mitochondrial morphology, but not potential, changes with reduced RhoA activity. (A) THP-1 cells transfected with RhoA and RhoA-caax are stained with mitotracker green and mitotracker deep red to determine mitochondrial mass and potential. Both do not significantly change with RhoA and RhoA-caax. Bar graphs represent the average of four independent experiments for mitotracker green (left panel) end mitotracker deep red (right panel). (B) mitochondrial potential is determined by the ratio of the deep red signal (potential) over the green signal (mitochondrial mass). No changes in potential are observed. Bar graph represents the average of four independent experiments. (C) Mitochondrial morphology is gauged by staining THP-1 cells with mitotracker green and deep red and live cell imaging. Treatment with the RhoA inhibitor leads to a significant increase of cells with elongated mitochondria. Bar graph shows average of three independent experiments, each condition contained at least 100 cells per experiment. Two representative pictures are shown (D).

RhoA inhibition primes cells for IL-1β secretion

One of the hallmarks of MKD is hyper secretion of the pro-inflammatory cytokine IL-1β. IL-1β is present in an inactive form (pro-IL-1β) in cells and requires proteolytic cleavage by caspase-1 to become bioactive. The caspase-1 required is, in immune mediated processes, part of a large protein complex called the inflammasome. Such inflammasome-mediated IL-1β secretion requires two signals, one that primes cells to form inflammasome components, and

one that activates the inflammasome (35). Previous studies have reported that decreased prenylation primes THP-1 cells for IL-1β hyper secretion(13, 15). We next asked how inactivation of RhoA, as happens in MKD models through reduced prenylation, may affect priming of THP-1 cells. We decided to use the C3 transferase as in figure 3c and 3d. Simvastatin treatment was taken along as comparison of the situation in MKD models. Inhibition of RhoA led to a strong increase of IL-1β mRNA, even more robust than achieved by simvastatin treatment (fig. 4A). However the levels of the closely-related IL-1β counterpart, IL-18, were unaltered (fig. 4B). Simvastatin treatment led to a slight up regulation of the inflammasome component NLRP3, while RhoA inactivation did not raise this significantly (fig. 4C). Messenger RNA of another inflammasome component and IL-1β maturation enzyme, caspase-1, were unaltered by both treatments (fig. 4D). The mere inhibition of RhoA unleashed the production of increased amounts of IL-1β mRNA.

Figure 4

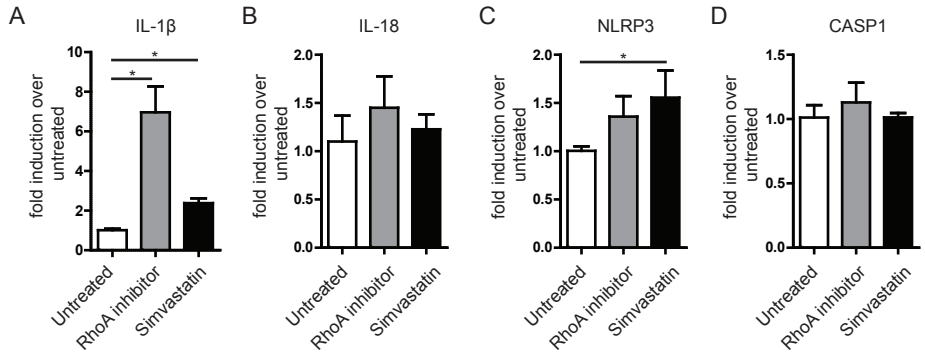

Figure 4: RhoA inhibiton provides a priming signal for IL-1β release. Messenger RNA of THP-1 cells treated with RhoA inhibitor is isolated and induction of four genes is determined in comparison to untreated control cells. Simvastatin treatment is taken along as broad-ranging prenylation inhibitor. Of the four genes, only IL-1β (A) is significantly up regulated. The related cytokine IL-18 (B) and the inflammasome components NLRP3 (C) and caspase-1 (D) show no difference. All genes are normalized to GAPDH, bar graps represent average of three independent experiments.

RhoA inactivation increases Rac1 activation

Having investigated some of the affected molecular pathways in MKD, we asked if RhoA and Rac1 may work in concert to cause increased IL-1β mRNA transcription, which may involve vice-versa control of each other's activity. We therefore studied the effects of RhoA inhibition on Rac1 activation. For control purposes we included the geranylgeranyl transferase inhibitor GGTI-298. We chose this inhibitor over simvastatin because simvastatin inhibits both farnesylation and geranylgeranylation, while GGTI-298 only inhibits the latter, thereby reducing off-target effects as Rac1 and RhoA are only geranylgeranylated. We chose to detect the GTP-bound fractions of both proteins using a G-LISA approach to complement our C3 transferase-based RhoA experiments. G-LISA moreover allowed us to measure both RhoA and Rac1 in the same sample. RhoA inhibition itself is enough to significantly raise the amount of GTP-bound Rac1 (fig. 5A). The level of GTP-bound Rac1 we detected surmounted the level observed using GGTI-298, our positive control. To ascertain that RhoA was actually inactivated, we measured GTP-bound RhoA in the same samples. We found that the reduction of GTP-bound RhoA was similar when C3 transferase was included as a RhoA inhibitor as with GGTI-298, confirming the validity of using C3 transferase in these experiments. Taken to-

gether, our data support a pivotal role for RhoA in MKD-associated autoinflammation through Rac1/PKB signaling towards interleukin 1β production.

Figure 5

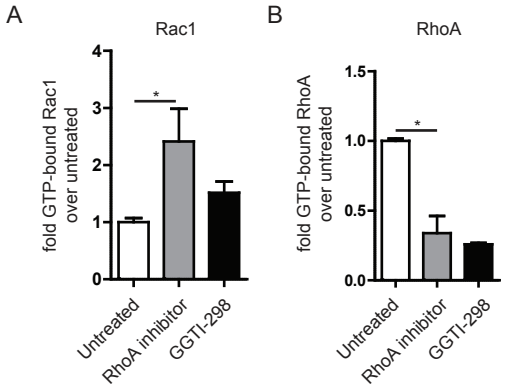

Figure 5: Inhibition of RhoA activity elicits Rac1 activation. (A) Inhibition of RhoA increases the GTP-bound portion of Rac1 in THP-1 cells. As a control a geranylgeranyl transferse inhibitor was taken along. GTP-bound RhoA (B) was measured for control purposes. The G-LISA signal was normalized for total Rac1 or RhoA in the samples. Average of three independent experiments shown.

Discussion

We here addressed the contribution of unprenylated RhoA to IL-1β hypersecretion as occurs in the autoinflammatory disease MKD. Patients suffering from MKD have severely reduced protein prenylation, but it is not fully understood how decreased prenylation is related to function of proteins that would normally be prenylated. As we and others have shown, lack of prenylation can affect activity of small GTPases (16, 24). However these effects can differ between cell types and tissues. Adding to the complexity are the large number of small GTPases, the differences in localization, and the numerous feedback and crosstalk pathways. In MKD the monocytes are the main affected cell type and responsible for the (biochemical) hallmark of the disease, IL-1β hyper secretion. Rac1 was earlier implicated in the MKD disease process. Moreover, Rac1 and RhoA have in multiple systems been shown to be reciprocally regulated (22, 23). We here investigated the effects loss of prenylation in a single protein, RhoA in IL-1β hypersecretion by monocytic cells, using THP-1 cells.

In a previous study we identified a defect in autophagy in MKD. Autopaghy is a complex process that requires numerous protein complexes, vesicle formation and transport. Due to the important role of RhoA in regulation of the actin cytoskeleton, we anticipated a detrimental effect of decreased RhoA activity on autophagy. Interestingly, autophagy was not affected at all. Vesicle transport mechanisms for autophagy most likely depend more on microtubules. Many other small GTPases, in particular of the Rab family have extensive roles in autophagy (36). It is therefore plausible that the RhoA plays a redundant role, if any, in this process.

The multiple and sometimes contradictory roles RhoA can play in different cellular systems becomes clear the literature on the association of RhoA with mitochondria. RhoA activation has been implicated in both the protection and generation of reactive oxygen species as well as the induction and protection of apoptosis. Lack of prenylation of RhoA, without an

additional signal has in our system no effect on mitochondrial potential, nor have we seen evidence of increased cell death. We did see clear differences in the shape of mitochondria upon inactivation of RhoA. Some studies have implicated RhoA in mitochondrial trafficking, but the elongation seems a different process. Its function, if any, is unclear. We had observed this phenotype, following simvastatin treatment(15). However using simvastatin to study mitochondrial elongation, would lead to numerous unprenylated proteins and affected pathways. This would make it very difficult to assess cause and consequence of mitochondrial elongation. Inhibition of RhoA could be a good approach to further elucidate the mechanism and function of mitochondrial elongation.

The inactivation of RhoA leads to increased priming of cells for IL-1β secretion. The levels of IL-1β mRNA are significantly increased. Several studies have implicated RhoA in the Il-1β secretion pathway, in particular the NF-κB signaling. It is reported that RhoA activation is necessary for the induction of G protein coupled receptor induced NF- κB activation(37). However we here report an opposite effect. This again emphasizes the cell type and activation signal specific roles of RhoA. In addition, we found it remarkable that simvastatin treatment of cells does not increase the mRNA level. This indicates that simvastatin can act on multiple pathways leading to IL-1β hyper-secretion, and that some feedback mechanisms can actually reduce the amount of IL-1β mRNA, or that the effect of RhoA inhibition on IL-1β mRNA is compensated in some way. Which protein or signal is responsible for the reduction of IL-1β mRNA in this system needs to be further investigated.

Finally we discovered that just by inhibiting RhoA activation, Rac1 is activated. It was already known that prenylation deficient mutants of Rac1 are also more activated. Therefore the lack of prenylation induced by MKD or simvastatin seems to have a synergistic role on Rac1 signaling. As Rac1 has been identified as one of the major players in IL-1β secretion, this could in part explain the hyper secretion of IL-1β in MKD.

Taken together, we have investigated how RhoA is affected by loss of prenylation in THP-1 monocytic cells. We found that RhoA is inactivated when prenylation is lacking. Despite the numerous cellular processes that RhoA is involved in, we only found effects on mitochondrial morphology and increased levels of IL-1β mRNA. These two features correspond in part with the phenotype seen in MKD monocytes and culture-based models. Furthermore we found that inactivated RhoA leads to increased Rac1 activity. Rac1/PKB signaling was earlier implicated in IL-1β hypersecretion in MKD, in an inflammasome/caspase-1-mediated route(14). Defective mitophagy also stimulates IL-1β hypersecretion through an inflammasome-mediated route(15). We here show that suppressed prenylation of RhoA primes monocytes to autoinflammation via Rac1 activation-induced IL-1β mRNA transcription. Together these results indicate a significant role for prenylation of RhoA and Rac1in MKD and help to clarify, in part, the complex molecular processes within monocytes that underlie IL-1β-driven autoinflammatory disorders.

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SERPINB9 Mutation
Defective Protein
MVK Mutation
Cholesterol
Calcium influx
ER stress
Isoprenoid deficiency
P2RX7
ATP
Symptoms
Antioxidant capacity
UPR
Biochemical properties
Mitochondrial potential
Small GTPases
Diagnosis
MAM
Reactive center loop
Autophagy
NSAID
Mitophagy
Mitochondrial shape
Rac1 activation
GBP5
Prednisolone
Granzyme B inhibition
RhoA inactivation
Mitochondrial fission/fusion
PKB phosphorylation
Mitochondrial stability
mtROS
Biologicals
NF-κB activation
mtDNA
TLR4
LPS
Inflammasome
HSC Transplantation
proIL-1β
Caspase-1
IL-1β transcription
MA
IL-1β
Caspase-1 inhibition
Hospital
MKD
HIDS

CHAPTER 5

Mitochondria in Autoinflammation: Cause, Mediator or Bystander?

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Abstract

People suffering from autoinflammatory disease (AID) have recurring sterile inflammation due to dysregulated inflammasome activation. Although certain genes have been associated with several AIDs, the molecular underpinnings of seemingly spontaneous inflammation are not well understood. Emerging data now suggest that mitochondrial reactive oxygen species (ROS), mitochondrial DNA (mtDNA), and autophagy might drive key signaling pathways towards activation of the inflammasome. In this review, we discuss recent findings and highlight common features between different AIDs and mitochondrial (dys)function. Although it is still early to identify clear therapeutic targets, the emerging paradigms in inflammation and mitochondrial biology show that mitochondria play an important role in AIDs, and understanding this interplay will be key in the development of new therapies.

Glossary

Autoinflammation: refers to the activation of the inflammation pathway without the presence of foreign or pathogenic triggers. The immune response is indistinguishable from a normally triggered response.

Autoinflammatory disease (AID): autoinflammatory disease is characterized by periodic fever and inflammatory symptoms followed by complete resolution of the inflammation. It is mostly caused by monogenetic hereditary mutations.

Inflammasomes: are large intracellular multiprotein complexes that play central role in innate immunity. They contain a cytosolic receptor for molecular patterns and, when triggered, activate caspase-1. Most known inflammasomes contain a member of the NOD-like receptor (NLR) family.

Mitochondria associated membrane (MAM): refers to the interface between mitochondria and the endoplasmic reticulum (ER). MAM are part of the ER and are reversibly tethered to mitochondria. This site is important for the exchange of metabolites, lipid biosynthesis, protein folding and calcium homeostasis.

Mitochondrial fission and fusion: Mitochondria undergo fission and fusion to ensure their proper function. This process is regulated by the master regulator proteins mitofusin 1 and 2 (MFN1 and MFN2), mitochondrial fission protein 1 (FIS1), dynamin-related protein 1 (DRP1), Optic atrophy 1 (OPA1), and the ER.

Reactive oxygen species (ROS): synthesis of ATP in mitochondria occurs through a process called respiration in which oxygen is used, and which simultaneously produces reactive byproducts. A foremost product is the superoxide radical O2, which can cause cellular damage if it is not neutralized. Under normal conditions such radicals are contained and neutralized in the mitochondria. There are other sources of ROS in the cell, but oxygen metabolism is the main contributor.

Mitophagy: mitochondria undergo continuous quality control to ascertain overall health of cellular mitochondria. Damaged mitochondria are, degraded by the cell by a specialized form of autophagy, called mitophagy.

NLR family, pyrin domain containing 3 (NLRP3): also called cryopyrin, is a Pyrin-like protein containing a Pyrin domain, a nucleotide-binding domain (NBD), and a leucine-rich repeat (LRR) motif. NLRP3 functions as cytosolic sensor and is activated by numerous stimuli. When activated it oligomerizes and forms a complex with the adaptor protein ASC and caspase-1. The mature complex is called an inflammasome and processes the inactive pro-forms of IL-1β and IL-18 into their mature bioactive forms.

Sterile inflammation: Inflammation that occurs without the presence of pathogens or foreign material. This can be triggered by endogenous danger signals, such as IL-1β, or by inappropriate activation of inflammasomes, as is the case in AID.

Importance of mitochondria in autoinflammatory disease

Mitochondria are best known for their role as the energy producers of the cell. In addition to supplying cellular energy, they play pivotal roles in various cellular processes including cell cycle progression, cellular differentiation and growth, and inflammation. Mitochondria are positioned near the endoplasmic reticulum (ER) [1], where they supply energy for protein production and ROS for disulfide-bond formation [2], and contribute to lipid biosynthesis [3]. They are also master regulators of apoptosis [4], and contribute to the activation of the inflammasome and thereby to directing immune responses [5] (Figure 1). The concept that mitochondria contribute to inflammasome activation is relatively new, with data gathered mostly during the last decade. Unsolicited inflammasome activation is now known to cause sterile inflammation [6]. Indeed, AID involves recurrent sterile inflammation due to inappropriate inflammasome activation [7]. In this review, we focus on the role of mitochondria in initiation and propagation of AID. We examine various aspects of mitochondrial signaling in the activation of the inflammasome and the possible consequences for AID development. Finally, we discuss possible mechanistic links between distinct AID types.

Autoinflammation and AID

The innate immune system is mobilized for activation upon sensing an external agent associated with pathogen infection. In autoinflammation (see Glossary), however, innate immune activation occurs upon perception of not external but internal cues [8]. A beneficial role for autoinflammation lies in responsiveness to injuries, supporting wound healing [9,10], through a process called sterile inflammation [6]. Autoinflammation can also turn pathological, presenting as AID. The signaling routes contributing to AID are not clear, although key molecules in immune activation pathways are deduced by clarification of genes that cause hereditary AID [11]. The result is the inadvertent triggering at subthreshold stimulation levels, for more or less periodic systemic activation of the innate immune response. Presentation of AID features includes: recurrent fever, joint inflammation, erythema, gastric inflammation, and mucosal inflammation [7]. Diagnosis of AID is often a long and difficult process. Because of the low incidence and the fact that activation of the inflammatory response in AID is indistinguishable from pathogen-induced immune activation, it usually takes multiple cycles before the possibility of autoinflammation is recognized. Final diagnosis of hereditary AID can only be made by DNA analysis, such as by next-generation sequencing [12].

We think it is relevant to emphasize here the mechanistic distinction between AID and autoimmune diseases. Autoimmune diseases are disorders of the adaptive, B and T lymphocyte-mediated branch of the immune system, notwithstanding that innate pathways also contribute. In autoimmunity, an important feature is chronic adaptive immune activation, while in autoinflammation, the innate immune system is activated in a recurrent manner that is followed by complete resolution of the inflammation until the next episode of activation. Clinically, autoinflammation is separated from autoimmunity throughthe lack of adaptive features, autoantibodies and auto-reactive T cells [13].

Inflammasomes

Inflammasomes commonly consist of three constituents: a nucleotide-binding oligomerization domain (NOD)-like receptor (NLR), the adaptor protein apoptosis-associated Speck like protein containing a CARD (ASC), and caspase-1. The NLR, which forms the core, determines the type of inflammasome. The exogenous signal that triggers activation determines which

type of inflammasome is formed, although the NLRP3 inflammasome responds to several stimuli (including even endogenous stimuli) [9,10], and is therefore special amongst NLRs. It has been suggested that NLRP3 inflammasomes may be activated by a single messenger or an intermediate one, but no consensus has been reached to date [14,15]. Interestingly, of the three general activators that have been suggested (potassium efflux, calcium ions, and ROS) (Figure 1), two are closely related to mitochondria [16–18]. The identification of a single conserved signal is complicated by the existence of a non-canonical pathway, which also activates NLRP3 via caspase-11 [19]. Multiple intermediate signaling pathways may therefore exist that all drive NLRP3 activation. Of note polymers of ASC resembling inflammasomes were recently described as being secreted mediators, thereby triggering the extracellular propagation of inflammation [20].

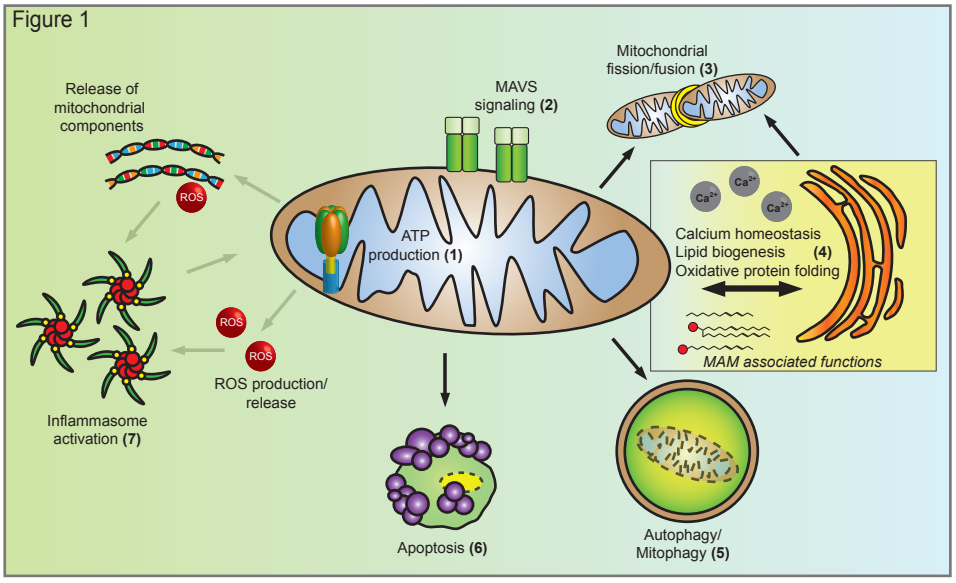

Figure 1. General overview of mitochondrial functions of the cell. The primary function of mitochondria is the generation of ATP (1). In addition, MAVS is expressed on the mitochondrial surface, and provides a signaling platform for immune activation, in particular for antiviral pathways (2). Mitochondrial fission and fusion can be adjusted to meet the demands of the cell or as response to mitochondrial damage (3). The MAM comprises the interface between the mitochondria and the endoplasmic reticulum. In this specialized compartment, mitochondria supply energy and metabolites for lipid synthesis and oxidative protein folding. In addition, calcium homeostasis is tightly regulated at the MAM (4). Released mitochondrial ROS can induce autophagy of cellular components, including mitochondria. Mitophagy degrades damaged or superfluous mitochondria (5). Release of mitochondrial components on large scale is a potent trigger for apoptosis (6). Mitochondria also play an important role in the induction of the inflammasome (7). There is no consensus on the exact order of events (gray arrows). Two main possibilities exist; either the mitochondria become damaged and release their content, leading to activation of the inflammasome. Or the inflammasome becomes activated and induces release of mitochondrial components, creating a feedback loop. Abbreviations: MAM, mitochondria-associated membrane; MAVS, mitochondria antiviral signaling; ROS, reactive oxygen species.

The process of inflammasome assembly is not fully understood [21]. It is however clear that the activation of the NLR can induce self-oligomerization via its NACHT domain; an evolutionarily conserved domain often found in proteins involved in apoptosis [22]. This domain

contains sites for protein–protein interaction and a nucleotide-binding domain (NBD). The induced conformational changes during oligomerization recruit ASC to the exposed Pyrin (PYR) domains. ASC, through its N-terminal PYR domain binds to the PYR domain of NLRP3. Consequently, the Cterminal region of ASC, containing a caspase recruiting domain (CARD), recruits caspase-1 to the inflammasome complex. The recruitment of several caspase-1 molecules results in self-dimerization and perpetuated self-activation of caspase-1. The activated caspase-1 cleaves the prointerleukin (IL)-1β to generate the mature inflammatory cytokine (Figure 2) [23].

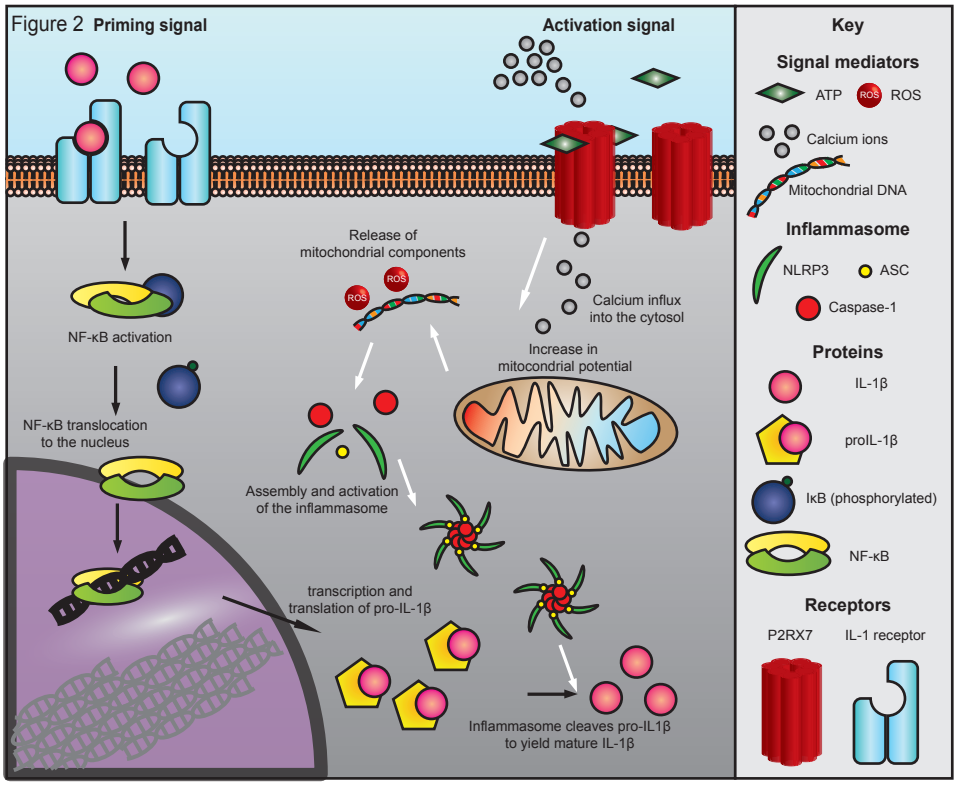

Figure 2. Two-signal system for activation of the cellular immune response. To evoke the cellular immune response two signals are required, a priming signal (black arrows) and an activation signal (white arrows). The priming signal is an initial danger signal like IL-1β, tumor necrosis factor, or a pathogen-associated molecule such as lipopolysaccharide. The priming signal leads to the activation of the NF-kB pathway and induces the production of inflammatory molecules like proIL-1β and proIL-18. The second signal causes the activation of the inflammasome. In this example this is ATP, but other signals (for instance type 1 interferons) can act as activation signal. ATP activates cellular receptors leading to an influx of calcium. The calcium damages the mitochondria, leading to the release of ROS and mitochondrial DNA into the cytosol. These mitochondrial components induce the assembly of the inflammasome and thereby activate caspase-1. The activated caspase-1 cleaves the proIL-1β and proIL-18 to yield their mature and bioactive forms. Abbreviations: IL, interleukin; NF, nuclear factor; ASC, apoptosis-associated speck-like protein containing a CARD; NLRP3, NACHT, LRR and PYD domains-containing protein 3; ROS, reactive oxygen species.

Although the crystal structure of the NLRP3 NACHT domain is yet to be resolved, sequence

alignments predict that the NACHT region is highly conserved throughout the NLR family [24]. The binding and hydrolysis of triphosphate nucleotides, which appears to be required for NLRP3 oligomerization, induces conformational changes, thereby facilitating protein oligomerization, to induce inflammasome activation [25].

One recurrent question is whether nucleotides are bound to the NACHT domain when NLRP3 is inactive. One possibility is that the NBD in resting cells is blocked, only to become available after activation, or alternatively, that the catalytic activity of the pocket is increased after activation. In resting cells, NLRP3 is ubiquitinated, and deubiquitination needs to take place for NLRP3 to become active [26]. The site of ubiquitination has not yet been identified and it is therefore possible that ubiquitin blocks the NBD. Another possibility was reported by Zhou and colleagues; they identified thioredoxin-interacting protein (TXNIP) as an activator of NLRP3, and showed that TXNIP interacts directly with NLRP3 [18]. TXNIP is normally bound to thioredoxin (TRX); a regulator of the redox potential in the cell. TRX can scavenge ROS directly or reduce oxidized proteins. Zhou and colleagues proposed that under increasing redox stress conditions (for example through increased ROS production by mitochondria) TXNIP is released from TRX, for activation of NLRP3 [18]. If the model is correct, mitochondria would be activated to produce increased amounts of mitochondrial ROS (mtROS) to facilitate NLRP3 oligomerization, placing inflammasome formation downstream of mitochondrial activation. The finding however was challenging to replicate [27]. Yet in 2014, several rodent-based studies reported involvement of TXNIP in NLRP3 activation [28–32], suggesting that at least in those organisms, but not necessarily humans, TXNIP may contribute to NLRP3inflammasome activation. In the next sections, we will discuss how inflammasome-mediated activation of caspase-1 is crucial to the ultimate secretion of IL-1β, and we will discuss its interactions with the mitochondria.

5

AIDs

Innate immune activation

Innate immune activation is considered the first step of the immune response, triggered by the detection of pathogens, foreign material, or endogenous danger signals by dedicated receptors. The cell responds with the release of proinflammatory cytokines such as IL-1β, IL-18 and tumor necrosis factor (TNF) a. The innate immune response is a potent and rapid defense system by virtue of the enormous variety of stimuli that can trigger a conserved set of cellular effector functions. However, the detection of foreign materials, that can abundantly express multiple innate stimuli, necessitates a safety guard to restrain unwarranted stimulation. This safety guard is built in, as cells require a combination of two signals for immune activation: first, a priming signal needs to be sensed that activates NF-kB and induces transcription of inflammasome components and proIL-1β. The second signal activates the inflammasome and mediates the maturation and release of bioactive IL-1β [15] (Figure 2). In AID, the activation of the innate response is altered and responds to subthreshold levels of activation, causing full-blown activation of the response even when the requirement of two signals is not fully fulfilled.

Known genetic associations

For several AIDs that are relatively common (although all with estimated incidences below 1 in 100-000), the affected proteins were identified allowing genetic tests to confirm the clinical diagnosis. The identification of disease-associated gene variants triggered investigations into underlying molecular mechanisms that converge into the activation of the inflammasome,

triggering sterile inflammation. We here discuss four examples, familial Mediterranean fever (FMF), mevalonate kinase deficiency (MKD), TNF receptor associated periodic syndrome (TRAPS), and cryopyrin-associated periodic syndrome (CAPS).

FMF is caused by mutations in the Mediterranean fever MEFV gene encoding the protein Pyrin [33,34]. Pyrin is a cytoskeleton-associated protein produced by immune cells and serves as an intracellular sensor for inactivation of Rho GTPases. Certain bacterial toxins can inactivate Rho GTPases by glucosylation. This leads to the activation of Pyrin, through an unknown mechanism. Pyrin accordingly acts as a pathogen recognition receptor (PRR) of the NLR family, and oligomerizes with ASC and caspase-1 to form an inflammasome [35].

Mutations in the mevalonate kinase (MVK) gene can lead to MKD [36]. Low activity of MVK blocks the cholesterol biosynthetic pathway, resulting in shortage of nonsterol isoprenoids and decreased post-translational isoprenylation of selected proteins [37], such as small GTPases. Lack of prenylation can cause dysregulation of small GTPase activity and subcellular localization to membranes [38–40], resulting in defective autophagy and priming of the IL-1β response.

Mutations of the TNF receptor 1 (TNFR1) gene can cause TRAPS [41]. The mutated receptor is retained in the ER, thus escaping clearance. This results in increased cellular stress and dysregulation of several processes including autophagy, as well as activation of the inflammasome [42,43].
CAPS is caused by mutations in the NLRP3 gene, many of which are found in the NDB domain [44]. Some mutations are predicted to modulate the hydrolytic activity of the inflammasome-associated NBD. Others are predicted to contribute to protein–protein interactions [24]. Regardless, these mutations presumably facilitate the oligomerization of NLRP3 inflammasomes spontaneously or at subthreshold levels of activation.

Mitochondria-derived signals

Mitochondrial damage

Induced mitochondrial damage can trigger apoptosis through the release of apoptotic factors; most notably cytochrome C [45]. Indirect damage to mitochondria can, for example, be triggered by cellular exposure to inert particles such as monosodium urate (MSU) crystals or silica. Such particles can temporarily be enclosed by endosomes and lysosomes followed by lysosomal rupture. Accordingly, cathepsin B activation and phagosomal acidification contribute to inflammasome activation via this route, with cathepsin B eventually damaging the mitochondrial membrane, leading to loss of potential and release of its content [46]. The mitochondrial content further stimulates activation of the inflammasome. Activation of the inflammasome through mitochondrial content is a major factor in AID and will be discussed in the next sections.

ROS

The release of ROS, and in particular, mitochondria-derived ROS (mtROS) can facilitate NLRP3 inflammasome activation. However, mtROS is a normal byproduct of mitochondrial respiration. Deviation of the mitochondrial biochemical status quo is therefore sensed as a trigger for the activation of the inflammasome [47]. One possibility of how mtROS may be made available for inflammasome activation pertains to mitochondrial damage, causing mtROS to

escape mitochondria and enter the cytosol. A second possibility is increasing mitochondrial metabolism, which would increase mtROS levels. The third option is a regulated release of mtROS, both in quantity and subcellular location [48]. How mtROS in AID is released is not always known, yet for both TRAPS and MKD, it has been shown that inhibition or neutralization of mtROS reduces inflammasome activity [43,49]. The release of mtROS is an important intermediate in inflammasome (Figure 1) activation and therefore in AID.

Disruption in mitophagy [50] also might lead to mtROS leaking into the cytosol. Cells deficient in autophagy components can increase secretion of IL-1β [51]. Such mechanisms can however work in opposite directions, as some autophagy proteins are also necessary for IL-1β release. Recently, a feedback loop was reported suggesting that inflammasome activation leads to mitochondrial damage and inhibition of mitophagy, further complicating the issue. It has also been reported that accumulation of damaged mitochondria can be responsible for increased IL-1β secretion [52] and defects in mitophagy have been suggested to play a role in MKD [49].

Finally, mtROS appearance in the cytosol may be consequential to increased mitochondrial activity. Mitochondrial activity is closely linked to calcium levels, and mitochondrial activity can be increased by calcium uptake [53,54]. High calcium levels can however damage and rupture the mitochondria [55]. The cytosolic levels of calcium are low, but high in the ER and the extracellular environment. Activation of 1,4,5-triphosphate-receptor (IP3R), a membrane glycoprotein complex acting as calcium channel on ER membranes, releases calcium into the cytosol that can now be taken up by mitochondria. Alternatively, lysosome rupture might cause calcium release into the cytosol, for entry into mitochondria [54]. This would have to occur in close proximity, as calcium is readily chelated in the cytosol [56]. Furthermore, binding of ATP to the transmembrane ionotropic receptor P2X purinoceptor 7 (P2RX7) can increase the intracellular calcium levels, causing a drop in mitochondrial potential and damaged mitochondria (Figure 2) [57]. Another and more regulated exchange of calcium with the mitochondria is direct contact with the ER via the mitochondriaassociated membrane (MAM). At the MAM, calcium levels can be altered and thereby adjust mitochondrial metabolism [58]. It is suggested that potassium efflux, as associated with inflammasome activation, is coupled to calcium influx to balance charges over the plasma membrane. Yet, it has been shown that they occur relatively independent from one another, with both being necessary for inflammasome activation [59]. The role of mtROS in AID is well accepted, but the mechanisms of mtROS release are not completely understood and might differ between AID types.

mtDNA

Mitochondria maintain their own genome, which under normal conditions is shielded from cellular receptors. Extensive cellular damage can however cause mtDNA to be released into the extracellular environment. Indeed, patients suffering from systemic inflammatory response syndrome (SIRS) (the stage leading up to sepsis) have increased levels of mtDNA in their blood plasma. Here, mtDNA provides a proinflammatory signal by binding the PRR Toll-like receptor (TLR)9 [60]. Release of mtDNA in the cytosol through loss of mitochondrial integrity [51], can also initiate inflammasome activation. Prior to, or during mitochondrial release, mtDNA is oxidized by ROS, transforming it into a direct ligand for NLRP3 (Figure 1) [61].

Cytosolic release of mtDNA may contribute to the pathogenesis or propagation of AID. In support, experiments using MKD as a disease model showed that increasing mitochondria with loss of membrane potential was associated with mtDNA accumulation in the cytosol

[49]. Similarly, in TRAPS, autophagy becomes defective when TNFR1 is retained for a prolonged period within the ER [42]. The authors detected elevated levels of mtROS, but unfortunately did not report mtDNA measurements. The study did not address whether damaged mitochondria contribute directly to pathogenesis of TRAPS, or are merely a consequence. Altogether, there is now credible support that mitochondrial components, mtROS, and mtDNA, are involved in inflammasome activation and subsequent immune responses. Although loss of mitochondrial membrane potential and integrity is a component of normal signaling pathways, we propose that dysregulation of mitochondrial integrity may tip the scale towards excess immune activation in AID.

MAMs

Calcium and ER stress

Mitochondria exist as separate cellular organelles, however, a fraction is associated with the ER [3]. At this interface, known as the MAM, mitochondria and the ER are in close proximity, which allows functional crosstalk [3,62]. At the MAM, the ER contributes to tight regulation of calcium levels, while vice versa, mitochondria provide the ER with the oxidizing environment required for oxidative protein folding [3]. In addition, several lipid-synthesizing enzymes are present at the MAM interface, which benefit from the exchange of metabolites between the ER and mitochondria (Figure 1) [3]. The physical connection between ER and mitochondria is vital for proper functioning of the cell and is involved in several immune signaling pathways [63].

The regulation of calcium exchange by calcium transporters is pivotal to the MAM, and essential for both ER and mitochondria [3]. Several chaperones depend on calcium and oxidation for their function in assisting nascent proteins to achieve their native folding [64,65]. Faulty chaperone function can cause accumulation of incorrectly folded proteins in the ER, and induction of the unfolded protein response (UPR) [66]. The UPR is a dedicated program to restore proper ER function, by reducing general protein translation, and increasing the expression of chaperone proteins [67]. In addition, UPR induces localized autophagy of the ER parts where the protein translation and folding is hampered [66,67]. The UPR is accompanied by calcium efflux from the ER, which is taken up by mitochondria. Prolonged duration of ER stress, however, can cause mitochondrial damage and even apoptosis through excessive calcium uptake by the mitochondria.

Most AIDs are caused by SNPs that result in amino acid substitutions affecting the protein structure. Such mutations may disrupt protein stability and folding efficiency. The mutation in TNFR1 in TRAPS causes retention of the receptor in the ER, thus inducing ER stress [42]. In the case of MKD, there is little MVK protein detectable in patient cells. Experiments aimed to increase MVK protein folding efficiency had limited success, even though higher activity of the protein was achieved [68]. This suggests that mutations might disturb protein folding and stability, thereby contributing to ER stress. ER stress can prime cells for the expression of pro-IL-1β via NF-kB activation, contributing to IL-1β secretion [69]. Thus, ER stress, induced by misfolding of mutant proteins, may contribute to the pathogenesis of AID. This example clarifies that AID may not always involve mutations in obvious protein suspects such as NLRP3 or caspase-1. ER stress can also be induced by mitochondrial dysfunction [70], so one could speculate that the protein folding problems seen in TRAPS and MKD could originate from mitochondrial dysfunction, rather that the ER. The maturation of IL-1β following ER stress is intact in ASC deficient cells. The maturation occurs through an alternative pathway and requires caspase-8 [71]. Thus, compensatory mechanisms do exist to ensure IL-1β production

upon stress, emphasizing the difficulty in separating cause from consequence in AID.

GTPases and scaffolds

The MAM is a highly organized membrane structure and many small GTPases are involved in the formation and regulation of membrane shape and protein contacts [72]. The small GTPase Rab32 can regulate important protein contacts in the MAM as well as influencing the calcium levels by disrupting calnexin retention at the ER. Calnexin, a marker for MAM, is a protein chaperone and a regulator of the sarcoendoplasmic reticulum Ca2+ ATPase (SERCA) 2b [73]. Perhaps more importantly, Rab32 has a predicted prenylation site, which would be affected in MKD, as here the biochemical synthesis route for prenylation motifs is diminished [74]. One may therefore deduce that dysregulated calcium homeostasis contributes to AID through induction of mitochondrial stress, as proposed in MKD.

The induced oligomerization of NLRP3 multiprotein complexes, via NACHT domains, is key to activation of the inflammasome. This process probably takes place at the MAM [47]. In CAPS, the mutations in NLRP3 are within the oligomerization domain [75]. Some affect the NBD, while others affect the predicted protein–protein interaction sites required for oligomerization. It is assumed that this interaction site is important for self-oligomerization, but it might also involve interaction with MAM-localized proteins. One such example, the MAMassociated protein mitochondria antiviral signaling (MAVS) is involved in antiviral signaling, and is mostly found on the outer mitochondrial membrane (Figure 1). Activated MAVS triggers the assembly of a macromolecular signaling complex that forms at the MAM and activates NF-kB [76]. A handful of studies describe the crosstalk between MAVS and NLRP3, with one report indicating that MAVS physically associates with NLRP3 and facilitates oligomerization [77]. There is no consensus on how exactly NLRP3 inflammasome activation relates to MAVS signaling, although MAVS signaling can also induce active caspase-1 without the need for NLRP3 [77–79].

A recent study identified another candidate protein that mediates activity of the NACHT domain, guanylate binding protein (GBP) 5. GBP proteins are induced by interferon (IFN)g and induce defenses against intracellular pathogens [80]. GBP5 binds NLRP3 and assists with oligomerization [81]. Here however, it was not reported if the NBD activity of NLRP3 was required for oligomerization during inflammasome activation, but other studies have supported this possibility [75,82]. Of note, the GTPase activity of GBP5 was not required [81]. A trigger for GBP5 activation is unknown, although it has been suggested that GBP5 dimerization is induced upon nucleotide binding. GBP5 itself is induced by (IFN)g [80], but such induction may be relevant to propagation of autoinflammation rather than initial immune activation. Although little is known about the cellular localization of GBP5, considering its role in NLRP3 oligomerization, localization to the MAM is a possibility. Finally, GBP5 shows interesting links to MKD. GBP5 contains a C-terminal motif that is normally prenylated with the lipid building moieties that MKD patients lack [37]. Dysregulation of small GTPases related to GBP5 has been seen in MKD, and could therefore contribute to the dysegulated IL-1β secretion in this AID.

Mitochondrial dynamics

Mitochondria are dynamic organelles. They continuously undergo fission, fusion, can migrate to different parts of the cell, respond to cellular signals, and become degraded (Figure 1). Although the study of fission and fusion has intensified recently, much remains unknown regarding mitochondrial morphology and how healthy mitochondria contribute to immune homeostasis [83,84].

Extensive stress or apoptosis causes mitochondria to disintegrate [85]. Proteins such as parkin, a component of the E3 ubiquitin ligase complex, protect against excessive fragmentation by the induction of mitophagy [86]. In addition, parkin protects against mitochondrial damage induced by ER stress. Parkin also provides protection against calcium transfer inhibitors, such as thapsigargin, that induce mitochondrial damage by disrupting calcium homeostasis between the ER and the mitochondria [87]. Caspase-1 can, however, cleave parkin and thereby diminish its protective properties. These findings suggest the existence of a possible positive feedback loop whereby ER stress or mitochondrial damage elicits caspase-1 activation, and caspase-1 activation in turn leads to more mitochondrial damage [52].

During mitophagy, inflammatory content such as mtROS, mtDNA, and cytochrome-C, are neutralized through engulfment within double-membrane vesicle[86]. Although some dedicated proteins regulate mitophagy, the majority are also involved in autophagy. Knockout models of autophagy have shown that proteins, such as Beclin-1, autophagy-related protein (ATG) 16L and microtubule-associated proteins 1A/1B light chain 3B (LC3B) play nonredundant roles in reducing inflammasome activation [51]. Indeed, experiments using cells deficient in these proteins showed significantly elevated IL-1β secretion upon stimulation, due to defective mitochondrial clearance. These findings further support the notion that mitochondrial stability is an important determinant in inflammasome activation [51].

Several examples are now reported in which deficiency in mitophagy is a feature of AID. It was recently shown that there is increased accumulation of defective mitochondria and a lower number of autophagosomes after induction of autophagy in MKD [49]. In TRAPS, defective autophagy fails to remove the mutant TNFR [42]. Coincidentally, an increase in mtROS is observed which promotes an increase in IL-1β [43]. Although the authors of the latter study argue that the mechanism is more general than the inhibition of mitophagy, a contributing role for mitophagy would be expected. The processes of mitochondrial fission and fusion rely heavily on small GTPases. Indeed, lack of prenylation in MKD can affect the localization and activity of GTPases. In addition, studies using an MKD model of monocytic cells have revealed cells containing mitochondria with altered shapes [49]. Experiments that will further dissect the mechanistic links that connect prenylation of small GTPases to mitochondrial fission or fusion and their contribution to AID pathogenesis is warranted.

Concluding remarks and future perspectives

The role of mitochondria in AID has turned out to be a surprising one thus far. None of the known mutations in the AID affect mitochondria directly. However, their involvement in the activation of inflammasomes is now recognized, and implies that mitochondrial malfunction may contribute to various AID subtypes, as we have discussed. Clarification of the effects of increased activity, reduced stability, altered shape or localization of mitochondria may thus provide important insights in the molecular pathways and the development and pathogenesis of AID. Another crucial issue for investigators is to untangle the sequence of events in inflammation and AID. Feedback loops exist and sometimes it is unclear whether reported findings are cause or consequence. We believe that combined research efforts of investigators in metabolic disease and in immunology are optimally geared to elucidate the roles played by mitochondria in AID development and propagation. Although they may not constitute a direct target for therapeutic intervention in AID, such understanding will help elucidate how mitochondria contribute to dysregulation of innate immunity (Box 1). Finally, such outcomes will help identify new avenues for the development of interventions, not only for AID but also other inflammation-related pathologies.

Box 1. Outstanding questions

- How does mitochondrial morphology influence inflammasome activation, and is mitochondrial
- turnover altered in AID?
- How does the MAM contribute to AID and is it crucial for the assembly of the inflammasome?
- Does a common mitochondria-centered pathway contribute to different AIDs that could be used as a potential therapeutic target?

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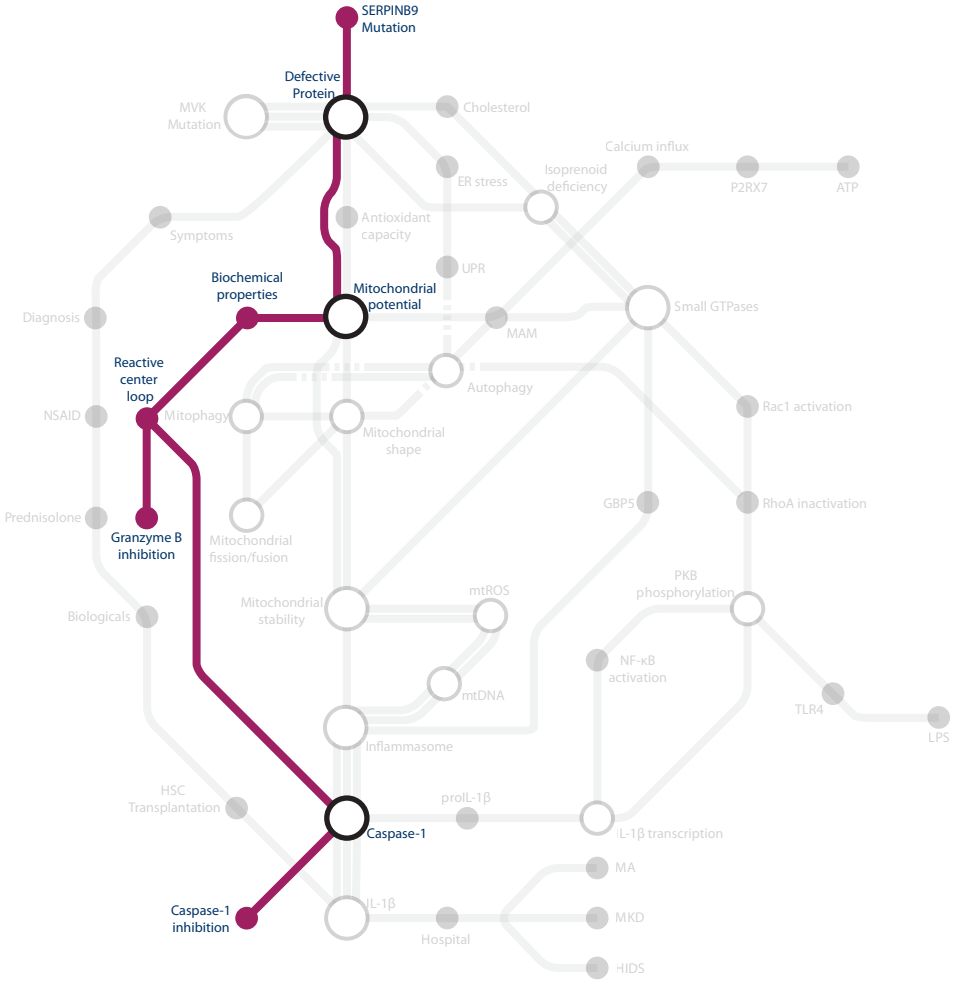
SERPINB9 Mutation
Defective Protein
MVK Mutation
Cholesterol
Calcium influx
ER stress
Isoprenoid deficiency
P2RX7
ATP
Symptoms
Antioxidant capacity
UPR
Biochemical properties
Mitochondrial potential
Small GTPases
Diagnosis
MAM
Reactive center loop
Autophagy
NSAID
Mitophagy
Rac1 activation
Mitochondrial shape
GBP5
RhoA inactivation
Prednisolone
Granzyme B inhibition
Mitochondrial fission/fusion
PKB phosphorylation
Mitochondrial stability
mtROS
Biologicals
NF-κB activation
mtDNA
TLR4
LPS
Inflammasome
HSC Transplantation
proIL-1β
Caspase-1
IL-1β transcription
MA
Caspase-1 inhibition
IL-1β
MKD
Hospital
HIDS

CHAPTER 6

Release of SerpinB9-mediated Caspase-1 Inhibition can Contribute to Autoinflammatory Disease

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Manuscript in preparation

Abstract

Serine protease inhibitor B9 (SerpinB9) is well known as inhibitor of Granzyme B (GrB) in cytotoxic T-cells. In addition it is the only endogenous inhibitor of the IL-1beta converting enzyme Caspase-1, most notably in antigen presenting cells. Untimely or hyper activation of monocytes leads to abnormal secretion of IL-1β, which is an important mediator of autoinflammatory diseases (AID). We have screened 96 patients AID for mutations in inflammation related genes. We identified one patient with a missense mutation in SerpinB9, located 12 amino acids preceding the reactive residue. To clarify possible functional consequences of this serpinB9 variant to AID, we cloned and overexpressed both the wild type (WT) and mutant serpinB9 in monocytic THP-1 cells. We found that the mutant is equal to the WT serpinB9 protein in stability and GrB inhibition. However the inhibition of caspase-1 was affected, which resulted in increased IL-1β secretion in cells harboring the infection. These data provide the first functional evidence that mutations in serpinB9 can contribute to AID, while retaining functional GrB inhibition.

Introduction

In patients suffering from autoinflammatory disease (AID), the regulation and release of interleukin (IL) 1β is often affected, yielding activation of the immune system in absence of pathogens and foreign material. Such so-called sterile inflammation leads to recurrent episodes characterized by fever and other inflammation-related symptoms. For a number of AIDs the underlying genetic causes have been identified, however, in many AID patients the causal gene defect has not been found(1). In AID, monocytes are primed or have a lower activation threshold, leading to assembly of the inflammasome complex. This complex mediates the activation of the IL-1β processing enzyme caspase-1(2). Here, caspase-1 cleaves the immature IL-1β into its bioactive form, allowing it to perform its pro-inflammatory function. In addition to IL-1β, caspase-1 also processes the IL-1 superfamily member IL-18 into its bioactive form(3).

Caspase-1 is highly expressed in monocytes but is also found in other cells. Because of its role in early immune activation, some pathogens have evolved proteins to inhibit its activity. The cowpox virus expresses a protein called Cytokine response modifier A (CrmA), which is a serine protease inhibitor (serpin). However it was also discovered that CmrA is also able to inhibit caspase-1 activity, even though caspase-1 is a cysteine protease(4), indicating that serpins can have a wider target scope. Serpins inhibit their target proteases by disrupting the catalytic site. They contain a specific inhibitory domain also called the serpin domain. The reactive center loop (RCL) in this domain is responsible for binding and inactivation of the protease. The inhibition is an irreversible 2-step process(5). The first step is the (reversible) binding of the RCL to the recognition and catalytic site of the protease. The RCL usually contains a residue (called P1) after which the protease disrupts the peptide binding. In the second step, the protease initiates the catalytic hydrolysis of the P1-P1' bond of the serpin. The P1 residue binds covalently to the catalytic serine and the bond between P1 and P1' is removed. This triggers a massive conformational change, in which the RCL folds back into the serpin protein. This movement disrupts the catalytic site and leaves both the serpin and protease inactivated (suppl. fig 1A)(6). Serpins have an inherent degree of instability that drives the conformational change that inactivates protease(7, 8). Although in cysteine proteases the link between serpin and protease is a less stable thioester bond, the conformational changes in both serpin and protease are suspected to be sufficient to leave both proteins inactive, although recovery from inhibition has been described in vitro(9).

Humans express an endogenous serpin that inhibits caspase-1. This serpin, serpinB9, is a 376 amino acid protein and is known for its function as the main inhibitor of GranzymeB (GrB) (10) which is highly expressed by cytotoxic (CD8+) T-cells and NK cells. When cytotoxic T-cells kill a target cell, they release perforin and GrB. Perforin creates pores in the target cell and GrB is delivered to the cytosol(11). GrB can activate caspases, inducing apoptosis pathways(12). In addition, GrB targets several mitochondria-related proteins, leading to the release of cytochrome C and activation of additional apoptosis pathways(13). SerpinB9 protects cells from the damaging effects of GrB. The cytosolic expression of serpinB9 increases the lifespan of leukocytes and dendritic cells(14). Moreover, there are reports of tumors that upregulate serpinB9 to escape cytotoxic T-cell mediated killing(15, 16). Later it was discovered that serpinB9 inhibits the activity of caspase-1. SerpinB9 is induced by NF-κB and IL-1β, and is part of a negative feedback loop for caspase-1 activation(9, 17, 18).

In this study we identified an A329S variant in the SerpinB9 gene in a patient with AID and assessed the capability of this mutant to inhibit both GrB and caspase-1. Whereas the serpinB9 A329S mutant fully retained its ability to block GrB, it failed to inhibit caspase-1 activity and

augmented pro-inflammatory IL1B secretion. These data provide the first functional evidence that mutations in serpinB9 can contribute to AID, while retaining functional GrB inhibition.

Materials and Methods

Reagents. Simvastatin was purchased from SIGMA-Aldrich. Mitotracker Green and Mitotracker Deep Red, were purchased from invitrogen. Caspase-1 colorimetric assay kit and recombinant human caspase-1 were purchased from Biovision. LPS (Escherichia coli EH 100) was obtained from Alexis Biochemical. iScript and iQ SYBRgreen supermix were purchased from Biorad laboratories. Simvastatin was hydrolyzed to its bioactive form as previously described(19).
Antibodies. FACS: CD3-APC, CD8-PerCP-Cy5 and CD14-PE (all BD biosciences, 555335, 341050 and 555398) CD4-PB (Biolegend, 300521). Western blot: Actin (Santa Cruz, sc-1616) and HSP90 (Cell Signaling Technology #4875S). Anti-FLAG (clone M2, Sigma Aldrich, F3165). Secondary antibodies for Odyssey: IRDye800 Donkey-anti-mouse and IRDye680 (Li-Cor, 925-32212 and 925-68073). Recombinant GrB was expressed and purified as we described previously(20, 21) and serpinB9 antibody (clone PI9-17) was made as described(22).

Patient samples. Patient was a 6 year old male with AID, recurrent episodes of high fever and generalized inflammation as reflected by elevated acute phase proteins in the absence of infection. However, in none of the genes known to cause AID could mutations be identified. The father was Dutch and reported healthy. The mother originated from Peru and did experience recurrent febrile illnesses as a child, it is unknown whether or not these had been related to infections. At scheduled outpatient visit patient was afebrile and well, underwent routine blood analysis. The ethical committee of the UMC Utrecht approved the use of residual material for this study. Residual material from routine blood tests was used to obtain peripheral blood mononuclear cells (PBMC). PBMC from patient, parents, and healthy donors were isolated using ficoll density gradient. PBMC fraction was washed twice in RPMI supplemented with 2% FBS and used immediately.

Sequencing of 120 inflammasome related genes. Barcoded whole genome fragment library were generated, and enriched for the coding regions of 120 inflammasome genes using a custom Agilent 1M microarrayThe enriched library was sequenced on the SOLiD4 sequencer as described previously (23, 24). The detected SerpinB9 variant was confirmed with Sanger sequencing. Primer sequences are available upon request.

Cell cultures. Freestyle 293F cells (Life Technologies) were cultured according to manufacturer's protocols. THP-1 cells were cultured in RPMI 1640 supplemented with 1% glutamine, antibiotics (penicillin, streptomycin) and 10% FBS. Simvastatin treatment of cells was 24 hours prior to the start of the experiment and at a concentration of 10 μM.

Cloning and mutagenesis. The mutant form of SerpinB9 was generated by site-specific mutagenesis of the wild type SerpinB9 (Source Bioscience, IRAUP969C028D). First, DNA from SerpinB9 was amplified for further cloning using primer serpinB9 fw and serpinB9 rev. The amplicon was ligated into the pGEM-T vector (Promega) and sequenced. The mutation was entered with a PCR reaction according to the guidelines of Phusion DNA polymerase (Finnzymes, F-5302S) adding 2 U/ 50ul reaction Phusion, 100ng template DNA and primer serpinB9 A329S fw and serpinB9 A329S rev. Template (methylated) DNA was degraded by adding 1 μL DpnI (NEB, R0176s) and incubated overnight at 37 °C. Wild type and mutant vector were transformed in commercial DH5a (NEB, C2987I) and the mutations were confirmed

by sequencing using primer serpinB9 seq. SerpinB9 WT and A329S were cloned with XhoI and NotI (NEB, R0146L and R0189L) into the retroviral vector pMSCV-puro from which the multiple cloning site was altered. The final constructs were confirmed with Sanger sequencing. Used primers: SerpinB9 fw 5' – CAC ACT CGA GGC CGC CAC CAT GGA AAC TCT TTC TAA TGC AAG – 3', SerpinB9 rv 5' – CAC AGC GGC CGC TTA TGG CGA TGA GAA CCT G – 3', SerpinB9 A329S fw 5' – GAA GGC ACC GAG TCA GCG GCA GC – 3', SerpinB9 A329S rv 5' – GCT GCC GCT GAC TCG GTG CCT TC – 3', SerpinB9 seq 5' – GAG AGA GAC CTG TGT CT – 3'

Transduction and selection of stable THP-1 serpinB9 lines. Wells of a 6 wells plate were coated for 2 hours at RT with 2 ml 50 ug/ml Retronectin (Takara Bio inc., T100B). Retronectin was removed and the plates were blocked with 2 ml 2% BSA/ PBS for 30 minutes at RT, followed by 2 washing steps with PBS. The retroviral constructs were transfected in Phoenix-ampho cells, producing the retrovirus. The retrovirus-containing sup was loaded on Retronectin-coated plates and spinned for 1,5 hour at 1800x g, 4oC. The supernatant was removed and 8*105 THP-1 cells were added with the addition of 8 µg/ml polybrene. The plate was briefly centrifuged and thereafter incubated overnight at 37oC. The day after the infection with retrovirus was repeated on the same cells. Overexpressing cells were selected with 0.5 µg/ml puromycin.

Flow cytometry and mitochondrial potential measurements. Isolated PBMCs were spinned and resuspended in FACS buffer and stained for CD3, CD4, CD8 and CD14. For the mitochondrial potential measurements, cells were washed once in PBS and resuspended in RPMI (w/o phenol red and w/o FBS) and probe. Staining concentrations: MitoTracker - 50 nM Mitotracker green and 50 nM Mitotracker deep red. Cells were incubated in the dark for 30 min at 37°C. Cells were centrifuged (500g 5 min), and suspended in RPMI w/o phenol red with 10% FBS. Cells were kept in the dark until measurement on FACS CANTO-II. Analysis was done with FACS Diva software.

RNA isolation and quantification. RNA was isolated by dissolving cell pellets in TRIpure (RnD) and following manufacturers' protocols. Isolated RNA was converted to cDNA using iScript according to manufacturer's instructions. Detection was done with CF-96 (biorad) using iQ SYBRgreen supermix, 100 ng cDNA was used per reaction. Primers used: GAPDH Forward 5'-GTC GGA GTC AAC GGA TT -3', reverse 5'- AAG CTT CCC GTT CTC AG -3', SERPINB9 Forward 5'- GAC ATG GAA TCT GTG CTT CGG -3', reverse 5'- CAC AAA ACT CTT GTG CAC GAA C-3'.

Cytokine measurements. Cells were centrifuged (500g, 5 min) and plated in 96 well plates in triplicates (2.0 * 105 cells/well in 200 µL), followed by 1 hour incubation at 37°C. Next LPS (200 ng/mL) was added and supernatants were collected after 4 hours and stored at -80°C until measurement. Cytokine concentrations were determined by Mulitplex bead analysis and normalized to the percentage of monocytes in the blood.

Immunoblot analysis. Cells were washed twice in PBS and then resuspended in laemmli buffer and boiled for 10 min. Samples were then distributed in small aliquots and stored at -20°C until use. For native blot freeze-thaw lysates were made in PBS (3 cycles), cleared by centrifugation (10000g, 10 min, 4°C) and stored at -20°C. Protein content was determined with BCA assay and samples diluted to 1µg/µL. 5% v/v β-mercaptoethanol was added to the samples and they were separated on 12% SDS PAGE gel, followed by transfer to PVDF-FL membrane. For native blot a 12% PAGE gel was used without SDS and β-mercaptoethanol but with coomassie G250. Loadingbuffer consisted of 20 mM Tris-Cl pH 6.8, 10% Glycerol and

1% coomassie G250. After separation, Protein was transferred to PVDF-FL membrane. 5% dried non-fat milk was used for blocking followed by primary antibody incubation (overnight 4°C, 0.5% milk in TBS-T), three washes and secondary antibody incubation (1 hr RT, 0.5% milk in TBS-T). Detection was done with enhanced chemiluminescence (ECL) on film or with Biorad Chemidoc MP. Some blots were visualized with labeled secondary antibodies and measured with Odyssey scanner.

Caspase-1 activity measurements. We used the colorimetric caspase-1 activity detection kit (biovision). THP-1 cells and THP-1 lines overexpressing SerpinB9 WT and mutant were lysed according to manufacturer's instructions. Protein content was determined with BCA assay of THP-1 lysate and THP-1 WT SerpinB9 lysate per well was used. The amount of mutant SerpinB9 THP-1 lysate was adapted to get equal SerpinB9 amounts based on WB protein amount determination. Assay was performed according to manufactures instructions with one addition. Recombinant human caspase-1 (Biovision, 1081-25) was added to the lysate (0.5 unit per well). Measurement was done with plate reader using a 405nm filter.

GranzymeB inhibition. Freestyle 293F cells were transfected according to manufacturer's protocol with either pBicDNA-FlagHA-SerpinB9 or pBicDNA-FlagHA-SerpinB9-A329S. Lysates were prepared 3 days post transfection. Cells were washed 3 times in cold PBS (5 min, 300g, 4°C) followed by 3 Freeze/Thaw cycles in PBS using liquid Nitrogen. Lysates were cleared by centrifugation (10000g, 10 min, 4°C) and stored at -20C. Protein concentration was determined by Bradford method. Lysate was pre-incubated with GrB for 2 hours at 37°C under mild shaking (BMGlabtech Thermostar, 150 rpm) in a volume of 50 µl per well (96 well plate). Different concentrations of SerpinB9 containing lysate were used; total lysate concentration was kept at 250 µg/ml by mixing with control 293F lysate. The GrB substrate (Ac-IEPD-pNA, ENZO Lifesciences, BML-P133-0005) was added to the wells in 10 µl solution (1.25 mM substrate, 100 mM Tris-Cl pH7.4, 200 mM NaCl, 0.01% Tween20). Final GrB concentration was 20 nM. Substrate conversion was measured by optical density at 405 nm with Anthos Zenith 340 rt plate reader at 37C in kinetic mode and the initial slope was determined.

Results

Identification and characteristics of a A329S mutation in serpinB9

As part of a research project to identify mutations in genes that contribute to hereditary autoinflammation, a panel of 120 candidate genes was screened for mutations that could play a role in the pathogenesis of autoinflammation. For the patient described in this study a mutation was discovered in one allele of SERPINB9 (NM 004155.4). No other potential diseases causing mutation in the other tested genes were identified. This serpinB9 mutation (Chr. 6: 2890543C>A, c.985G>T, fig. 1A) results in the substitution of the alanine at position 329 with a serine. This is highly conserved residue and predicted to be pathogenic by prediction programs SIFT, Mutation taster and PolyPhen-2 (fig. 1B.). The allele is not present in Europeans but has a minor allele frequency of 0.006% in the Latino population (http://exac.broadinstitute.org). Analysis of the parents showed that the mutation was inherited from the mother, who did experience recurrent febrile illnesses as a child (fig. 1C). We evaluated the cytokine release from stimulated PBMCs. Stimulation with LPS for four hours resulted in increased IL-1β, IL-6 and TNF secretion by the PBMC of individuals harboring the A329S serpinB9 mutation (fig. 1D). For comparison an unrelated healthy control was included. Next we evaluated the mitochondrial potential of the monocytes, as higher potential has been linked to AID (25–27). Interestingly, the potential seen in the patient is higher than the father, the mother that has the

variant and the unrelated control (fig. 1E). We next wanted to evaluate if the serpinB9 A329S mutant had any effect on the inhibition of its other main target, GrB. Due to the limited patient material available we decided to measure the relative contribution of CD8 T-cells in the peripheral blood. These cells contain significant amounts of GrB and if the inhibition would be negatively affected, the expectation is that CD8 T-cell numbers could be negatively affected. It has been reported that in spi6 (mouse homolog of serpinb9) KO mice, there are fewer antigen specific CD8 T-cells and CD8 memory T-cells, although the number of naive CD8 T-cells were similar(14, 28, 29). Flow cytometry on PBMCs with staining for CD3, CD4, CD8 and CD14 showed that the percentage of CD8 positive cells (gated on CD3, Suppl. fig. 1A) was 35%. These values fall within the normal range and nearly identical to a healthy control (fig. 1F). We also compared with an unrelated AID patient in case there would be any unexpected changes in the cell composition due to the disease. This control patient (Familial Mediterranean Fever) had 38.2% CD8 positive cells (Suppl. fig. 1A). Based on these results we decided to further investigate the characteristics of this serpinB9 mutation.

Figure 1

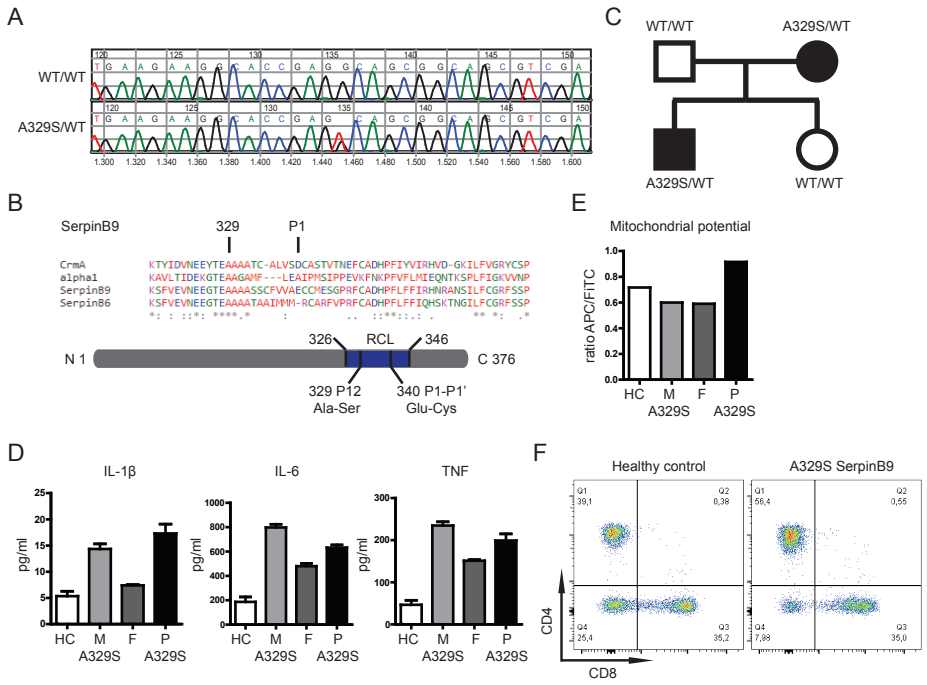


Figure 1: Identification and characterization of patient with the serpinB9 Mutant. (A) Sequencing results of the DNA with the mutation. Top results show WT alleles, bottom results show the mutation in the hetrozygote patient. (B) Top: Alignment of serpinB9 with a wide variety of serpin family members, CrmA, alpha-1-antitrypsin and serpinB6. Above the alignment shows the reactive P1 residue and the site of the mutation of serpinB9. The P12 alanine is highly conserved across serpins. Bottom: Schematic representation of the structure of serpinB9. The site of the P1 and P12 residues is shown, in respect to their place in the protein sequence and the reactive center loop. (C) Diagram of patient family tree. (D) Cytokine profile of the patient and parents compared to a healthy control after stimulation for 4 hours with LPS. IL-1β, IL-6 and TNF were measured. HC=Healthy control, M=Mother, F=Father, P=Patient. Secretion was normalized for percentage of monocytes in the sample. (E) Mitochondrial potential of monocytes from patient, parents and healthy control as determined flow cytometry with mitotracker staining. (F) Determi-

nation of CD8 T cell levels in healthy control and patient by flow cytometry. Shown is the distribution of CD4 and CD8, gated first on lymphocytes and followed by gating on CD3.

Creation of THP-1 cells overexpressing serpinB9

To investigate the properties of the serpinB9 mutant and its possible role in AID we performed a retro-viral transduction in THP-1 cells using WT serpinB9 and its A329S mutated version. After selection of transduced THP-1 cells based on puromycin resistance, we obtained two different cell lines overexpressing the serpinB9 variants, the WT and mutant versions. We first quantified the mRNA and protein levels in these cell lines (figs. 2A, 2B). Normal THP-1 cells express little serpinB9, which is evident at both the mRNA and protein level. Our newly generated cell lines overexpressed serpinB9 when compared to regular THP-1 cells. Furthermore, the A329S mutant expression is approximately 1.3 times higher than the WT. The cells were stimulated for four hours with LPS (200 ng/mL), after which the supernatant was assessed for IL-1β, IL-6 and IL-18 (fig. 2C-2E). Despite the overexpression of serpinB9, IL-1β is still detectable in all samples. THP-1 cells overexpressing the WT serpinB9 produced less IL-1β than the control cells. In contrast, the cells overexpressing the A329S serpinB9 that produced more IL-1β than the control cells. This suggests that serpinB9 inhibits caspase-I-mediated IL-1β production, a function that is disrupted by the A329S mutation. The IL-18 levels, however, are similar between all the cell lines, with only a slight increase in the mutant. IL-18 processing is also dependent on caspase-1, yet there seems to be no inhibitory effect of the serpinB9 overexpression. Finally, we observed decreased IL-6 levels in the overexpressing cell lines, which may relate to some feedback mechanism, or due to the insertion site of the serpinB9 in the THP-1 genome but is not likely a direct effect of serpinB9 inhibitory function.

SerpinB9 properties and GrB inhibition

It is well known that serpins can aggregate at an increased temperature, and some even at physiological temperatures as a regulatory mechanism. We evaluated whether the A329S mutation has an effect on the aggregation of serpinB9, especially because it is located in the relatively unstable reactive center loop (RCL). We made freeze-thaw lysate samples and incubated these at increasing temperatures for 10 minutes. The lysates were then separated on a native gel. The aggregates once formed are very large and serpinb9 in aggregated form no longer can enter the running gel. We found that for both the WT and mutant protein, serpinb9 aggregates formed above 50°C. The serpinB9 band disappears beyond this temperature in both variants, indicating that the thermal stability is not significantly altered by the A329S mutation (fig. 2F). SerpinB9 is described as the main inhibitor of GrB. The RCL has been intensely studied and most mutations in this loop (for all serpins) have a detrimental effect on the inhibition of the target(5, 30, 31). Considering that the alanine is highly conserved, we were expecting a difference in the binding to GrB. To this end, we titrated recombinant GrB into serpinB9 containing lysates of the THP-1 cells. After incubation, we separated the samples by SDS-PAGE (fig. 2G). The blot shows that there is complex formation of GrB with both the WT and the mutant forms of serpinB9 with equal kinetics. Next, we measured GrB activity in 293F lysates. To this end, we used different concentrations of serpinB9-containing lysate with 293F control lysate to ensure equal protein concentrations in all conditions. We confirmed with western blot that Serpin was present in different concentrations in the lysate (fig. 2H). GrB was added to the lysates and the residual activity of GrB was assessed with a GrB specific chromogenic substrate (fig 2I). There was no significant difference observed between the two lysates, indicating that, despite the mutation in the RCL, both serpinB9 protein variants inhibit GrB activity with similar kinetics.

Figure 2

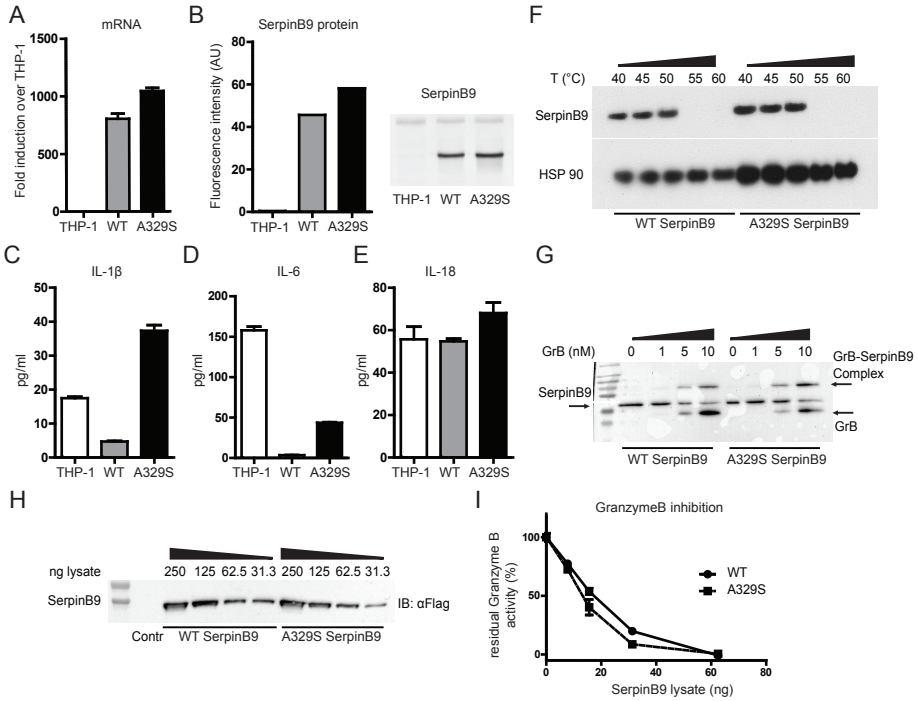


Figure 2: Characterization of THP-1 cell lines overexpressing serpinB9 and evaluation of GrB inhibition. (A) Measurement of serpinB9 mRNA in the generated THP-1 lines. Normalization was done against GAPDH. Representative picture shown of 4 independent experiments. (B) Western blot to determine protein levels of serpinB9. Bar graph is a quantification of shown blot. Representative picture shown of 4 independent experiments, WB detection with Odyssey scanner (C) Cytokine expression of serpinB9 containing THP-1 cells compared to WT THP-1 after 4 hours of stimulation with LPS. Levels shown for IL-1β (C), IL-6 (D) and IL-18 (E). Representative shown of 3 independent experiments. (F) Thermal stability of serpinB9. Lysate containing the mutant and WT serpinB9 was incubated for 10 min at indicated temperature, followed by a native protein gel. HSP90 was used as loading control. WB detection with film. (G) Titration of GrB into lysate with WT or mutant serpinB9. Both versions are able to form an SDS resistant complex with GrB. WB detection with Biorad Chemidoc MP (H) Western blot of control lysate with different amounts of serpinB9 lysate from 293-F cells. SerpinB9 is tagged with Flag, anti-flag was used for detection. WB detection with Biorad Chemidoc MP. (I) Inhibition of GrB activity with serpinB9 lysates from 293-F cells. GrB was added to the lysates and activity assessed with GrB specific colorimetric substrate.

Caspase-1 inhibition by serpinB9 variants

After assessing that the inhibition of GrB was not affected by the mutation, we investigated whether the inhibition of Caspase-1 was affected. If serpinB9 mutation would prohibit its function to suppress Caspase-1 processing of mature IL-1β, it could be a disease mechanism that partakes in AID through deregulation of IL-1β production. We considered that IL-1β secretion of THP-1 cells is relatively modest, and with the overexpression of a caspase-1 inhibitor we were unsure if we could properly evaluate the inhibition. The experiments in fig. 2C-E showed that the overexpression of serpinB9 does not fully inhibit the capacity of the cells to release proinflammatory cytokines. Therefore we decided to boost IL-1β secretion to unequivocally

measure the inhibition of the serpinB9 variants. Treatment of THP-1 cells with simvastatin primes them for hyper-secretion. In research on the AID mevalonate kinase deficiency we have successfully used simvastatin-treated THP-1 cells as a model system(25, 32). We therefore pretreated THP-1 cells and the THP-1 lines with serpinB9 overexpression with 10μM simvastatin for 24 hours, followed by 4 hours of LPS (200 ng/mL) stimulation. The control THP-1 cells responded with a greatly increased IL-1β secretion. The average secretion increased at least three-hundred fold, from 5 pg/mL to 1,5 ng/mL (suppl. fig. 1C). In contrast, the THP-1 cells overexpressing WT serpinB9 increased only about five to ten fold from 5 pg/mL to 40 pg/mL, supporting the inhibitory role of serpinB9 in suppressing the Caspase-I-mediated processing of bioactive IL-1β (figure 3A). The increase IL-1β production by in THP-1 cells overexpressing mutated serpinB9 was far greater than what was obtained in THP-1 cells overexpressing WT serpinB9, with levels that were comparable with the levels seen in control THP-1 cells expressing only little serpinB9 (figure 2B). Figure 3A shows the average IL-1β secretion which is much lower in the WT compared to the mutant. To confirm that the simvastatin and LPS treatment did not lead to altered serpinB9 levels, the cells were lysed after the stimulation and serpinB9 protein levels were assessed. Much like the results in fig. 2B, the serpinB9 the mutant is still present in higher amounts following the treatment (fig. 3A, right panel). These data together support that the mutant form of serpinB9 is less efficient in inhibiting caspase-1. To directly investigate if this is the case, we made freeze thaw lysates of the THP-1 cell lines and measured caspase-1 activity. Protein content was determined and calibrated for serpinB9 protein content (using 1.3-fold more WT lysate as serpinB9 content was 1.3-fold higher in the A329S overexpressing than the WT serpinB9 THP-1 cells). We then spiked the lysates with recombinant human caspase-1 and assayed the activity of caspase-1 in a colorimetric format. We found that the lysate of control THP-1 cells had some inhibitory effect on caspase-1 activity, compared to only reaction buffer with caspase-1 (fig. 3B). Lysate with the mutant serpinB9 showed similar activity for caspase-1, while the WT serpinB9 showed lower activity. The lower activity of caspase-1 is a consequence of the more efficient inhibition by the WT serpinB9, which was not obtained for the A329S serpinB9. Taken together, we conclude that the A329S serpinB9 mutant is less capable of inhibiting caspase-1, while its inhibitory capacity of GrB is unaltered. SerpinB9 can therefore be considered a protein that when mutated may contribute to AID.

Figure 3

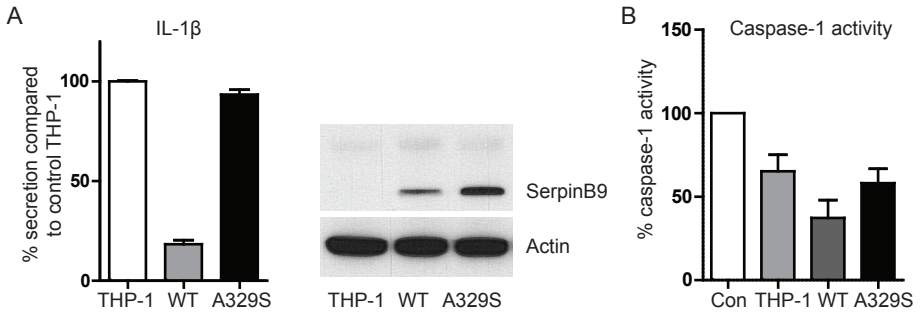


Figure 3: Assessment of caspase-1 inhibition by serpinB9. (A) IL-1β hyper-secretion of serpinB9 expressing THP-1 cells. Hyper-secretion (at least 60 fold increase over normal THP-1 secretion) was induced by 24 hour pretreatment of cells with simvastatin (10 μM) followed by 4 hours of stimulation with LPS. Average shown of 4 independent experiments. A western blot was done to assess that serpinB9 is still present during stimulation. Cells were separated from supernatant and lysed after the 4 hour stimulation. Representative blot shown of 3 independent experiments. WB detection with Odyssey scanner. (B) Colorimetric measurement of recombinant caspase-1 activity in lysates of THP-1 cells. Recombinant

caspase-1 was added to lysates, detection was with specific caspase-1 probe. Average of 4 independent experiments shown.

Discussion

Here we assessed the possible role of a mutant form of serpinB9 in the pathogenesis of AID. Our results demonstrate that the mutant is less efficient in the inhibition of caspase-1. Both the patient and the mother of the patient, who had self-reported symptoms during her childhood showed the mutation.. Because the variant is present at low frequency in the Latino populations (MAF 0.006%) the hypothesis that this mutation alone causes the AID in this patient is less likely. This would mean that ~1:100 Latino's would be affected with an AID. However non-penetrance is well-known for different mutations in different AID's (ref) and the patient we investigated has only one mutated allele; it would be interesting to see if people being homozygous for this mutation are affected with AID. Nonetheless, with the data we have generated on this mutant serpinB9 form, a contribution to the AID clinical symptoms is likely. Deregulation of IL-1β secretion leads to AID. Therefore if the feedback loop for limiting caspase-1 activity does not function correctly any initiation of immune activation would lead to a stronger response, compared to the normal situation. SerpinB9 is still the only endogenous inhibitor known for caspase-1(9, 17), and it is unlikely that there is another protein can (partly) take over the inhibitory function. The damage caused by this mutant would be much greater if the binding and inactivation of GrB was affected. Many cells of the immune system, such as dendritic cells, mast cells and cytotoxic T-cell, use serpinB9 as protection against accidental release of GrB(22, 28, 29, 33). Intracellular GrB activity can instigate apoptosis programs in minutes, as is the suspected mechanism in NK mediated cell death(34).

It is interesting that the WT and the mutant serpinB9 are equally effective in blocking GrB. The mutation is located at position P12 in a stretch of alanines, which is termed the hinge region, and is crucial in the conformation "flip" of the serpin to inactivate the protease. A study on mutations in the RCL reported that the activity of serpin was diminished with a mutations in this stretch of alanines, although the P12 position was not mutated(30). As our study has shown, this does not result in any detrimental effect on the inhibitory capacity of serpinB9. Perhaps the sidechain of serine offers the necessary molecular contacts that is needed in the P12 position, and therefore performs better than the glycine. Or perhaps the P12 is inserted into the beta sheets after the conformational changes and there is only limited space for a side chain. Yet the considerable level of conservation in this sequence amongst species and related proteins argues that there is not a lot of molecular and chemical space for alterations in this position. This is strengthened by the observation that mutations of the P12 position in other serpins leads to inactivation(35).

Another intriguing point is that the SerpinB9 variant affects the activity of caspase-1. The alanine in the P12 position is described as having an important function as a hinge residue, but was not mentioned to be very important for the substrate recognition. For most proteases some selectivity can already be generated with a small peptide of four residues, as is shown by the number of caspase-activity assay kits that are on the market. It has been reported that the residues in the P1'-P4' position can strongly influence the reaction efficiency. In this light it would seem likely that the P12 is too far away from the recognition site to majorly contribute to the selectivity. The interaction of caspase-1 and serpinB9 has not been mapped and it is not known on an atomic level how they interact. Based on our data, we suspect that in addition to the hinge-function, the P12 is important for the recognition and binding of caspase-1. It is reported that serpinB9 can also inhibit other caspases, such as 8 and 10(36). It would

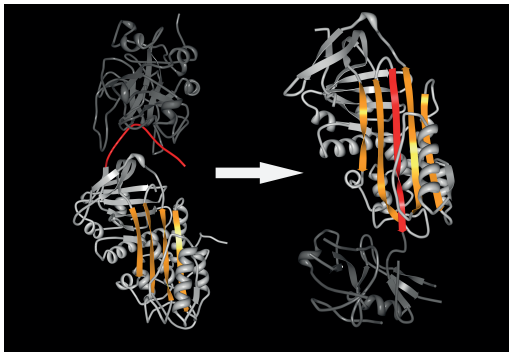
be interesting to compare the binding of the WT and mutant to these proteases. We did not assess the effect on the IL-18 secretion further although this can be an important contributing factor to AID.

Taken together, we have identified a mutant version of serpinB9 in a patient with AID. We demonstrated that the patient had slightly increased secretion of IL-1β. Furthermore we created a model cell system in which we overexpressed and evaluated the function of the mutant serpinB9 protein. Althought the GrB binding and inhibition was equivalent to the WT seprinB9 counterpart, the caspase-1 inhibition was affected in the mutant. It is therefore likely that the mutant serpinB9 does contribute to the pathogenesis of AID.

Supplemental figure 1: (A) mechanism of inhibition by serpins based on structures from alpha 1 anty-trypsin (light grey) and trypsin (dark grey) (PDB ID: 1EZX). The RCL (red, not completely shown) binds to the catalytic site of the protease. When the RCL is cut by the protease, it is briefly and covalently linked to the serpin. At this moment the RCL fold back and inserts into the beta sheets (orange) of the serpin. This induces massive conformational change in the protease and leaves both protease and serpin inactive. (B) Gating strategy for CD+ T-Cell determination. First gate was on lymphocytes based on size and granularity. The T-Cells were identified by CD3+ in the lymphocyte gate. The CD3+ cells were checked for CD4 and CD8. A serpin unrelated AID patient (Familial Mediterranean Fever) was taken along as additional control. (C) IL-1β secretion of THP-1 cells. Statin treatment enhances the IL-1β secretion more than 300 fold, but only when stimulated. Results are from 24 hour pretreatment with simvastatin and 4 hour stimulation with LPS.

Supplemental figure 1

A



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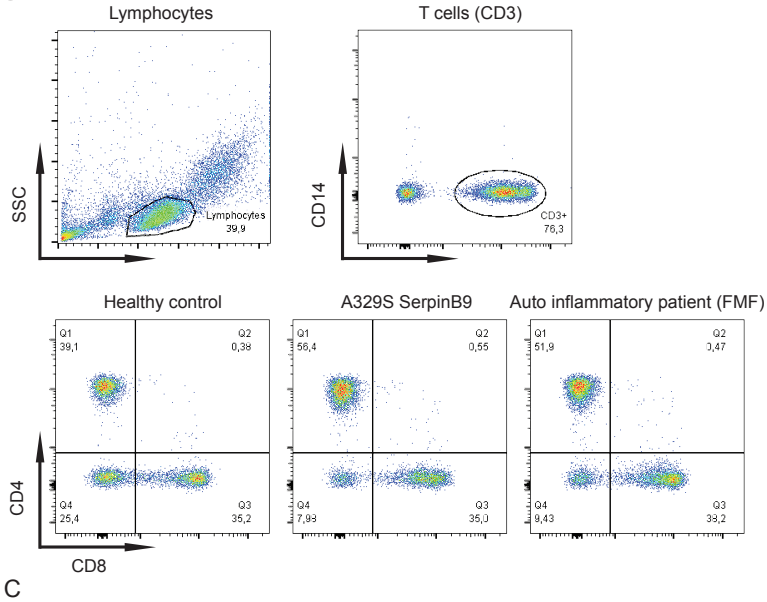

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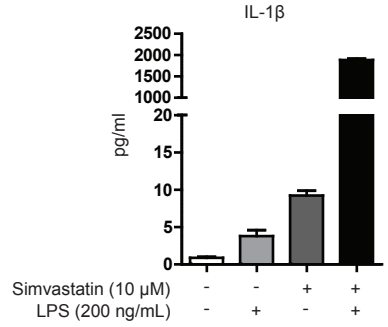

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SERPINB9 Mutation
Defective Protein
MVK Mutation
Cholesterol
Calcium influx
ER stress
Isoprenoid deficiency
P2RX7
ATP
Symptoms
Antioxidant capacity
UPR
Biochemical properties
Mitochondrial potential
Small GTPases
Diagnosis
MAM
Reactive center loop
Autophagy
NSAID
Mitophagy
Mitochondrial shape
Rac1 activation
GBP5
RhoA inactivation
Prednisolone
Granzyme B inhibition
Mitochondrial fission/fusion
PKB phosphorylation
Mitochondrial stability
mtROS
Biologicals
NF-κB activation
mtDNA
TLR4
LPS
Inflammasome
HSC Transplantation
proIL-1β
Caspase-1
IL-1β transcription
MA
Caspase-1 inhibition
IL-1β
MKD
Hospital
HIDS

CHAPTER 7

General Discussion

The knowledge in the field of immune activation and AID has expanded rapidly in recent years. In particular the mechanisms leading to activation of the inflammasome and ligands for NLRP3 were hot topics. This fundamental knowledge led to many new scientific leads to further investigate the molecular mechanisms underlying AID. The expanding knowledge on AID, to which we hope this thesis contributes, means there are many new potential targets for possible therapeutic intervention. Yet there are still challenging problems and mysteries to solve before we understand what is happening on the macroscopic and molecular level. Some of these challenges and gaps in our knowledge are discussed here.

After four years… it's complicated.

In this thesis I describe our considerable efforts in trying to identify the molecular mechanisms that lead from severely reduced MVK protein activity to inflammasome activation and IL-1β secretion. In chapter 3 we identified problems with the clearance of damaged mitochondria(1). The relationship between the release of mitochondrial components and the activation of the inflammasome has now been well established (2–4). However, some of the effects we observed in the mitochondria are still puzzling. We observed higher mitochondrial potential and altered mitochondrial shape; two things that can correspond with a higher efficiency, yet the oxygen consumption was reduced indicating a suppressed metabolic rate. We also noticed an accumulation of damaged mitochondria in statin-treated cells. Possibly there are fewer functional mitochondria in these cells and hence a lower oxygen consumption. The mitochondria that do still function might have a higher potential in order to reach the required levels of ATP for cell survival and may have an altered shape for increased efficiency. The elongated shape of the mitochondria does not contribute to higher potential, but seems to be a consequence of low RhoA activity (or increased Rac1 activity) as we show in chapter 4(5). Furthermore there is evidence that the elongated mitochondria are linked to induction of autophagy(6, 7), and might not have any functional link to the increased potential that we observed. Another link we have yet to identify is how the isoprenoid deficiency leads to increased potential in mitochondria. It could be a passive process as suggested in chapter 3, that the damaged mitochondria accumulate and this stresses the remaining mitochondria. We were unable to rule out that there also could be an active process that leads to this. A hypothetical link is, as described in chapter 5, the small GTPase Rab32. It has posttranslational modification and it sequence ends with two C-terminal cysteine residues, although not a classic CAAX-box. Rab32 is associated with mitochondria and the MAM(8). Disrupting this interaction might lead to altered calcium homeostasis and mitochondrial potential.

In chapter 3 we identified defective autophagy which was very clear in the model system, yet the proof of concept in patient material did not show a similar inhibition. The inhibition of the mevalonate pathway in our model is likely more stringent than that of patients experiencing a moderate phenotype. Autophagy and mitophagy are heavily regulated, like all vesicle-mediated processes, by small GTPases. It is therefore plausible to molecularly link MKD to these processes. Yet to identify a single protein, or a set of proteins responsible for this would be very difficult. It might be worthwhile to map the prenylation of small GTPases in a few MKD patients, to get a better idea of the abundance of prenylation and how it is altered compared to healthy controls. Such a comparison can provide crucial evidence if this pathway is affected, or whether this is a consequence of inducing a (too) stringent block of the mevalonate pathway.

Chapter 6 is a classic example of a 'side project' turning into a large and important one. The mutation in SerpinB9 was identified by genetic screening of autoinflammation patients, and at

the time was a novel mutation that was not present in the control cohort. Initial results were promising and the project became part of the main research performed in this thesis. The location of the mutation is in the reactive center loop, which is one of the most conserved residues in the protein. Moreover because of its location, it was expected that any mutation would detrimentally influence the function of the protein. With extensive experiments we were able to show that the inhibition of GranzymeB was not affected, while there are indications that caspase-1 inhibition is affected. However it needs to be said that the clearest difference was observed in THP-1 cells treated with statins. This was done to boost IL-1β levels and potentially overcome the inhibiting effect of the overexpressed SerpinB9. The true contribution of this mutant to AID features therefore remains somewhat unsure. It is safe to say there is some effect on the inhibition of caspase-1, but we cannot conclude it is physiologically relevant to AID. In addition, with more whole genome sequencing data emerging, the mutation has more recently been found more often in the general population (~73 per 100.000). This prevalence is much higher than the incidence of AID, meaning it is unlikely that this mutation on one allele would result in AID although it may contribute to disease development. A homozygote with the mutation might, but this has not been seen so far.

In conclusion, we have shed more light on the molecular mechanisms that lead to increased IL-1β secretion in MKD, by identifying the changes to mitochondria, Redox balance, mitophagy/autophagy and small GTPase localization and activity. In addition we have characterized a mutant of SerpinB9 that shows altered regulation of caspase-1, expanding the list of players that can contribute to AID. Hopefully these few stepping-stones help to increase the size of the ever-increasing pyramid of scientific knowledge.

Methods & limitations

Fundamental research regarding human diseases can be challenging due to ethical and practical limitations. The study of AID's is no different. Systemic inflammation and the resolution thereof are the result of a complex interplay between many cells of the immune system. For this reason it would be beneficial to use a mouse model in our research. However, as mentioned in chapter 1, none of the current mouse models are optimal. Furthermore, great care has to be taken, as with all mouse studies, with translation to the human setting. Primary material from patients simply remains the study material of choice, yet comes with significant drawbacks. Firstly, there are ethical considerations; can you request material from a (pediatric) patient for research and how much is reasonable? The practice of using left-over material from diagnostic procedures helps to limit the impact on patients, but also means the amount of material is limited and the processing sometimes makes material unsuitable for certain experiments. A second issue is the low number of AID patients in general. It is not feasible to base an entire laboratory study on this material, especially if taking AID subtypes into account. Inter-human variation is a factor that cannot be avoided, but contributes significantly to clouding of the results. Consequently, studies have to be powered properly to reach significance, which is not often possible with low patient numbers. Even if access to patient material would not be so limited, further difficulties are encountered. The collection of material is frequently limited by circumstances. A realistic time point to collect material from patients is during a requested consultation with a pediatrician. However, this increases the chance of fever at the time of sampling. Fever and elevated inflammation parameters was reason to exclude patient samples in this thesis. In chapter 3 we focus our investigations specifically on the activation of immune cells, a moment which has certainly been surpassed in samples from patients with symptoms such as fever. Finally, monocytes are a difficult cell type to work with. They are very easily activated (temperature changes, mechanical stress), which means that the act

of drawing a blood sample can already result in activation. Differentiation to macrophages starts within hours of isolation, so experiments have to be performed rapidly and directly after isolation. Furthermore, the average monocyte count in the blood is around 10% of the PBMC fraction, limiting the amount of cells that can be acquired from a sample. An appealing option is therefore immortalized cell lines from patients. This is common practice with fibroblasts and satisfies some of the needs for basic science studies(9), yet fibroblasts are very different form monocytes and therefore not very useful in our setting. There is light at the end of the tunnel though. The use of induced pluripotent stem cells means that primary cells can be turned into self-renewing stem cells(10). Protocols have been published that allow differentiation of monocytes from the stem cells, providing a nearly unlimited supply of pure monocytes(11). Unfortunately this development came too late to influence the methods used in this thesis. In this thesis we have used a monocytic cell line with chemical inhibition to mimic MKD. We believe this was the best option available to us at the time considering all of the mentioned factors. This model provides ample material and is very reproducible. There are of course limitations to consider. Although originating from monocytes, and having characteristics of monocytes, THP-1 cells are immortal which is an important difference. Furthermore, the simvastatin used to inhibit the mevalonate pathway in our experiments affects a different enzyme, meaning that the results would miss potentially unknown MVK functions independent of its synthesis role. It is important to acknowledge these limitations in our and other studies, as they can often explain seemingly conflicting results. The difference in cell type, model system, method of detection and timing are crucial factors in achieving reproducibility and effects that can be detected.

Autoinflammatory diseases

In the last few years, tremendous progress has been made in the understanding of AID. This is mainly due to increased understanding of the general inflammasome activation pathways. However, also for the individual AIDs specific progress has been made. Key papers have been published on the pathways in TRAPS(12, 13), the gene function of MEFV (affected in FMF)(14) has finally been identified and with this thesis we hope to have contributed to the understanding of MKD(1, 5). The spectrum of AID has also been widened by the recognition that some autoimmune diseases more closely resemble AID (such as systemic onset juvenile idiopathic arthritis(15, 16)). The increase in knowledge and the recognition of AID as a specific class of disease will help diagnosis and to identify and evaluate treatment options. However, there are still a number of unanswered questions regarding the pathophysiological mechanisms and pathology of AID.

There is just something about monocytes…

Despite the fact that most AIDs are caused by various genetic mutations, it seems that most affected cell type is the monocyte. In the case of FMF or CAPS, this is not necessarily surprising as the affected proteins are cytosolic sensor proteins. They are an important part of the activators of the innate immune system and are expressed in monocytes. This is in line with their general function as sentries of the immune system. However in the case of MKD or TRAPS, the genetic defect seems to have a much broader effect. Prenylated proteins are found in any cell type and the TNFR1 is expressed in numerous tissues. Yet the only obvious cells that are affected are the monocytes. The reason for this is still unknown. One possibility is that monocytes have an intrinsically lower threshold for activation, possibly to sense any changes to metabolism induced by pathogens, meaning that any insult leads to activation. Despite this fact, the processes that are affected on a molecular level by the mutations seem to be general. The answer is still unclear, but foreseeable answers have significant implica-

tions. Possibly, far more tissues are in fact affected but have no detrimental effects on one's health, or these effects are overshadowed by the inflammation. Another possibility is there is something specific to monocytes leading to their increased sensitivity. There is something to be said for both scenarios, but considering the role of monocytes in the immune system and that the majority of symptoms are all inflammation related, the latter option seems the most likely. This is strengthened by the observation that MKD can be "cured" by bone marrow transplantation, indicating that their disease state is directly related to cells of the hematopoietic lineage (17–19). Alternatively, it may be due to expansion of the diet. In support, it was reported in mice that the supplementation of the diet with isoprenoid precursors can reverse the phenotype induced by chemical inhibition of the mevalonate pathway.

It's all about the timing

Another issue that remains unresolved is the periodicity of AID. The synonym 'periodic fever' says it all, the disease presents in periodic episodes. Although there is great diversity amongst patients, some experience fever episodes that reoccur like clockwork. Why is the disease periodic? A logical explanation is that, once activated, the immune system cannot be activated again for some time. There is literature suggesting this(20), for instance on endotoxin tolerance. However, the monocyte is one of the shortest-lived cell types in the blood. What is it that prevents new monocytes from continuously becoming activated? Any soluble factor able to do this would have great benefit as potential treatment, and would work similarly to the current immunosuppressive therapies. There is a site where monocytes could have a longer lifespan. The spleen contains a reservoir of monocytes that are recruited when there is a significant injury(21). If the there is an accumulation of a molecular triggering signal that slowly accumulates over a period of weeks, than this efflux of monocytes can contribute to the triggering of an episode. Unfortunately the lifespan of monocytes is not known. Another possibility for monocytes to achieve longevity is to migrate into tissues and differentiate into dendritic cells or macrophages. It is possible that a molecular build-up takes place in these cells, yet it becomes more difficult for these cells to trigger inflammation that spreads throughout the body. The intracellular accumulation of a trigger is only one possible explanation to explain periodicity. Instead of activation it could also be a period of inactivity. Perhaps there is a distinct period of time in which monocytes are unable to be activated (approximately two weeks) and that new triggering is mostly determined by how sensitive the monocytes have become. Many other factors could play a role such as hormonal cycle, growth, or other infections.

How about the chicken and the egg…

When we consider the possibility of intervening in AID with a small-molecule drug, it is important to understand the molecular mechanisms that are being affected. As discussed extensively in this thesis, the sequence of events in the activation of the inflammasome is not yet fully determined. When trying to use a small molecule to limit the triggering of the inflammation, it is important to know when the inhibited step is initiated. Consider the increased potential in mitochondria; it is possible that the rise in potential leads to increased ROS generation and activation of the inflammasome. Protecting the mitochondrial potential could be very effective. Yet if the potential increases because ROS is leaking from the mitochondria, protecting the potential might not have the desired effect. Despite multiple studies trying to address this issue there is no consensus on the order of events around the mitochondria and the inflammasome. This indicates that there might not be a single pathway by which activation takes place. Possibly, there is one, or more, feedback loops involved or staged crosstalk required.

Considering the essential role of mitochondria in cells, it would be interesting to know when the damage to mitochondria crosses a critical threshold. Can the mitochondria release increased amounts of ROS and then go back to steady state? Or is the fate of this particular mitochondrion sealed upon ROS release? Can monocytes recover from the inflammasome activation or does the activation automatically lead to cell death via one route or another? Monocytes that migrate into tissue can differentiate into macrophages and dendritic cells, meaning that not all inflammasome triggering leads to cell death. Yet it is not known where the tipping point lies and how it is defined.

These are some of the fundamental questions that will require further research before accurate judgment can be made regarding the possibility of intervening in the mitochondrial signaling and the activation of the inflammasome.

On MKD and the importance of cholesterol

As mentioned before, cholesterol levels in MKD patients are usually not affected(22). Therefore, much research has been directed to the role of prenylation, and how lack of prenylation leads to increased IL-1β secretion(23). There is plenty of literature on the effects of cholesterol on the immune system, yet since cholesterol levels are normal in AID, this hasn't been of much interest in MKD research. However very recently a paper was published showing that an hydroxylated form of cholesterol can inhibit the transcription and secretion of IL-1β(24, 25). It is possible that the limited supply of cholesterol is preventing conversion into this immune-dampening form of cholesterol. This requires further investigation. The conversion is performed by a specific enzyme, which is induced with the inflammasome activating signal. There is no clear link to already known deficiencies in MKD to expression of this protein. So while this could clearly be a contributor to the pathology in MKD, there is much research that needs to be done in this field.

Again, why do we only notice the monocytes?

The monocytes are the main cell type to be affected in MKD. Yet most of the identified molecular mechanisms that play a role are not specific to monocytes at all. Kuijk et al identified the role of Rac1 activation(26), yet Rac1 is ubiquitously expressed throughout the body. In chapter 3 we report the effects on autophagy and mitophagy, while in chapter four the implications of RhoA are discussed. Neither of these proteins or processes is limited to monocytes. So one nagging question remains, why are only the monocytes notably affected? So many cellular processes require correct localization and activation of small GTPases, that it seems almost impossible that other cells are not somewhat affected in their functioning. Research done with fibroblasts shows that high mevalonate levels can mask reduced MVK activity, yet why isn't this the case in monocytes(27)? Possibly monocytes produce very little mevalonate, or perhaps there is an unknown function of MVK in monocytes. This is one of the mysteries that remains to be solved in the field of MKD. It is possible that other immune cells that are not circulating contribute significantly to AID, yet we have not identified if and how these cells contribute.

Small GTPases, big consequences?

Chapter 4 of this thesis focusses specifically on the effect that lack of prenylation has on RhoA, and to a lesser extent, Rac1. The differences are quite pronounced, so prenylation of these proteins seems to play a significant role,. Yet as previously mentioned, GTPases are impor-

tant in a huge variety of cellular processes, and a good portion of small GTPases is prenylated in the cell. However the effects seem to be quite limited in most tissues, possibly due to high mevalonate levels(27). It has been shown that complete absence of MVK activity causes embryonic lethality. The next step in the MKD spectrum is MA, with almost no detectable MVK activity (<1.0%)(28). Although this does come with significant problems (developmental delay and mental retardation), embryos with these mutations are viable. When there is a slightly greater percentage of MVK activity the main defect is AID. Still, fundamental cellular processes that are dependent on prenylation seem to function normally, and where a small insult can throw monocytes of balance, other tissues seem more robust. Prenylation is important for cellular localization and activity, yet is not the only lipid-like modification. Myristoylation is the attachment of myristoyl group, but this only happens on the N-terminal side of proteins(29). While there might be some redundancy between myristoylation and prenylation, it seems that this not very likely. It is on the wrong terminus of the protein. A more likely candidate for redundancy of prenylation is palmitoylation. Like prenylation, it can also take place on cysteine. However it produces a thioester bond, meaning it is reversible in the cellular environment(30). Possibly this modification can cover part of the functionality of prenylation, although there is little literature to support this. It has been shown that Rac1 carries a cysteine in its C-terminal stretch that can be palmitoylated(31) (RhoA does not carry an additional C-terminal cysteine). Analysis of the distribution of Rac1 and RhoA in the cell (water soluble vs detergent) shows that the majority in statin treated cells is unprenylated(32), supporting that myristoylation is not happening. The procedures that lead to this separation would most likely destroy a thioester bond meaning that palmitoylation would not be picked up. It is not known if these modifications cover for the reduced prenylation, yet it is interesting that the impact on such a global protein class results in only limited harmful consequences.

In conclusion

In this thesis we have attempted to elucidate some of the molecular mechanisms involved in MKD pathogenesis and have taken a side step to look at the inhibition of caspase-1 as a regulator for AID with a serpinB9 mutant. While major progress has been made in understanding the molecular mechanisms behind AID, important questions remain and especially in the MKD field. The initial steps in AID, how the mutation of a protein can affect the activation of the inflammasome, is becoming more and more clear. Yet what the following steps are and how these molecular steps finally result in the clinical phenotype of AID is still has largely unknown. Molecular explanations on the periodicity and resolving of inflammation would potentially be of great benefit for therapeutic intervention. The intervention at these stages should help reset the immune system or resolve inflammation faster, and thereby limit the disease impact on the patient. This would be of greater benefit then any attempt to prevent triggering of the inflammasome. Indeed, fullblown inflammasome inhibition will naturally lead to a generalized state of immunosuppression, which is what current treatments do, accompanied by undesirable side effects. Nevertheless the development of a small molecule that inhibits inflammasome activation would potentially be a great step forward in AID management as it would be far more cost efficient and less invasive for patients than the current treatment with biologicals.

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APPENDIX &

Summary & Samenvatting
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Introduction and summary

The immune system

The immune system plays an important role in the human body. It is a complex system that detects danger signals and protects against infections. There are two components, namely the innate and adaptive immune system. The later is mainly comprised of T-and B-cells. These cells are important because of their ability to adapt to almost all disease-causing microorganisms in order to neutralize and eliminate them from the body.

The activation and adaptation of T-and B-cells in the adaptive system takes time. During this time the body is protected by the innate system. This system acts as the first line of defense and reacts immediately to all kinds of microorganisms. It can distinguish between bacteria, viruses and parasites, however, it cannot distinguish between different kinds of viruses or bacteria. The response is therefore general and unspecific. The innate system is made up of macrophages, monocytes and dendritic cells. They are found in tissues such as the skin as well as in the blood. These cells are able to sense the presence of microorganisms and respond by releasing warning signals. One of the first signals is the release of the protein interleukin 1 beta (IL-1β). IL-1β instigates inflammation. It is therefore also an indicator for the strength of the inflammatory response; the more released the stronger the signal. Inflammation is characterized by fever, swelling, redness, pain and itchiness. It serves two main purposes; firstly to activate the adaptive immune system and secondly to deter the invading microorganism.

Inflammation and the immune response

Inflammation is a complicated process that can be compared to a warzone on a microscopic level. The cells of the innate immune system are like suicide bombers battling against pathogens. They do not aim specifically but rather fire in all directions and bombard the area, causing collateral damage. This damage is tolerated in order to isolate the enemy and is repaired once the danger is eliminated. Microorganisms are constantly trying to evade and escape the innate system by hiding within cells or moving to other areas of the body. It is an ongoing battle until the troops of the adaptive system arrive. They use specific antibodies as ammunition and can therefore specifically attack the invaders. They are able to recognize cells that have been invaded by microorganisms and are thereby able to gain the upper hand. The immune system then sets out to clean up and repair the overall damage. The adapted T-and B-cells are then also stored into memory for future use.

Autoinflammatory disease

Above is a brief illustration as to how the immune system responds to a disease-causing microorganism. The innate system can however make a mistake and release IL-1β without the presence of a foreign pathogen, leading to an unwarranted inflammatory response and the accompanying symptoms. After a few days the inflammation is resolved and the symptoms of fever, pain, rash and swelling disappear. In autoinflammatory disease this repeats itself in periodic episodes. These diseases are therefore also known as periodic fever syndromes. It is important to realize that autoinflammatory disease is not the same as autoimmune disease. Autoinflammation is an inappropriate initial response; the innate immune system is too sensitive and easily activated. The rest of the response is normal. In autoimmune disease the adaptive system makes the mistake of recognizing its own proteins as foreign and therefore specifically attacks them. The inflammation that occurs in this case is chronic, whereas the

inflammation in autoinflammation is periodic. This difference has important consequences for treatment and research into the underlying mechanisms.

What is it to have autoinflammatory disease?

There is great variation in the severity of inflammatory symptoms. In some cases fever will be present for several days while in more severe cases it can last up to two weeks with accompanying complaints of arthritis, intestinal discomfort and anemia. The cause is usually genetic as is therefore present from birth. It is difficult to diagnose as the symptoms mimic those of a general infection. It can therefore take years before the disease is recognized. In addition to the burden and inconvenience of frequent illness, hospital consultations and disruption in school, children experience developmental and growth delays due to the effects of frequent illness and the side effects of treatment. The disease tends to diminish towards adulthood but even then frequent episodes of illness can have a detrimental impact on ones carrier and social life.

What can be done?

There is currently no treatment available that specifically targets autoinflammation. Most of the diseases are treated with symptom reduction and immunosuppressive drugs such as glucocorticoids (prednisone) and biologicals (made of antibodies). For most patients these options are acceptable although they have substantial negative side effects. Steroids are detrimental to growing children and biologicals are administered by injection and are extremely expensive. The only curative treatment is bone marrow transplantation. The high risks associated with this, the difficulties in finding suitable donors and the high costs make it a rare option reserved only for extreme exceptions. Scientific research on the underlying mechanisms of autoinflammation is important for the development of improved treatment options and new medicines, something that I hope to have contributed towards in the work of this thesis.

Summary of this thesis

This thesis is the product of our research investigating the origin and underlying mechanism of the autoinflammatory disease mevalonate kinase deficiency (MKD). In MKD there is either an absolute deficiency of the enzyme mevalonte kinase, or a relative deficiency due to a less active form of the enzyme. Mevalonate kinase is involved in a series of enzymatic reactions that are important for the formation of cholesterol. As cholesterol is abundantly present in the modern diet a cholesterol deficiency in cells does not occur. However, in addition to cholesterol other compounds are affected. In MKD there is a shortage of two compounds that are normally coupled to, and important for the function of certain proteins. This coupling is called prenylation and it is lacking in MKD.

In *chapter two* we have performed a literature study on various aspects of MKD. The genetic basis of disease and the biochemical and molecular processes that are affected as a result of the enzyme deficiency are discussed. In the second half we focus on the clinical aspects of the disease such as the symptoms, diagnostic process, available treatment strategies as well the prognosis for patients.

In *chapter three* we investigated how a fault in the mevalonate kinase enzyme (and therefore a lack of protein prenylation) can induce inflammation. Prenylated proteins play an important role in general housekeeping. They are involved in the clearing of damaged and redundant cellular components. In the MKD model system we see that the cell is unable to sufficient-

ly perform this routine clearance, resulting in accumulation. In particular, the damaged mitochondria in monocytes that are not cleared leak their contents into the cell. This results in a premature release of IL-1β once they are activated. IL-1β release leads to inflammation and fever. A crucial step prior to the onset of inflammation in a cell is the formation of a protein complex known as inflammasome assembly and activation. In recent years research has placed increasing focus on inflammasome activation. An emerging finding is that mitochondria can be involved in this process. In chapter three and four we identify that damaged mitochondria in MKD can lead to easier inflammasome activation. This link has also been made to other autoinflammatory diseases.

In *chapter four* we chose an alternative approach. Due to the fact that prenylation is absent in MKD hundreds of proteins may be affected. In order to determine how this may affect protein function we studied two important proteins; Rac1 and RhoA. Both proteins are normally prenylated but not in MKD. It was already known that unprenylated Rac1 becomes more active, while unprenylated RhoA becomes less active. This alteration has consequences for the activation status of other proteins, for example for protein kinase B (PKB), which is known to cause greater IL-1β secretion.

Chapter five is once again a literature study, investigating the roll of mitochondria in various autoinflammatory diseases. We studied the similarities and relationships of mitochondria in various subtypes of disease in order to identify new targets for therapeutic intervention.

Some patients who have symptoms of autoinflammatory disease are unable to receive a diagnosis because of unknown mutations that probably cause the disease. In *chapter six* we describe a mutation that may play a roll in autoinflammation. The mutation was found in the protein SerpinB9. Up to now this is the only human protein known to actively inhibit caspase-1. Normally, caspase-1 activates IL-1β. When the inhibition of caspase-1 disappears there is increased IL-1β secretion from cells with this mutation. Although this mutation is rare, it is a newly identified mutation that may cause autoinflammation.

This thesis aims to describe the molecular mechanisms that play a role in MKD and possibly other autoinflammatory diseases. Although most mutations are due to simple gene mutations the consequences for the molecular and cellular housekeeping are rather complex. We have found that damaged mitochondria are involved in the disease process of MKD. Furthermore, we have gained insight on how the activation status of Rac1 and RhoA is dependent on prenylation and how this is altered in MKD. Lastly we have described a new mutation in SeprinB9, which may lead to autoinflammation. Improved molecular insights provide a greater understanding of the immune system at a cellular level and facilitate the development of safer and improved therapies for autoinflammation and other inflammatory-related diseases.

Introductie en samenvatting voor niet-vakgenoten

Het immuunsysteem

Het immuunsysteem speelt een belangrijke rol in het lichaam. Het is een complex systeem dat beschermt tegen infecties en allerlei gevaren signaleert. Het bestaat uit twee verschillende onderdelen: het verworven en het aangeboren immuunsysteem. Het verworven immuunsysteem wordt ook het adaptief immuunsysteem genoemd en bestaat voornamelijk uit T- en B-cellen. Deze cellen zijn belangrijk, omdat ze zich specifiek kunnen aanpassen aan bijna iedere ziekteverwekker zodat die onschadelijk gemaakt kan worden.

Het aanpassen van T- en B-cellen kost echter tijd, ongeveer een week. Gedurende deze tijd wordt het lichaam beschermd door het aangeboren immuunsysteem. Dit aangeboren immuunsysteem reageert vrijwel direct op ziekteverwekkers, maar in beperkte mate en met geringe specificiteit. Het kan wel onderscheid maken tussen ziekteverwekkers, zoals bacteriën, virussen of parasieten, maar bijvoorbeeld niet tussen een griepvirus en een verkoudheidsvirus. Cellen van dit systeem zijn onder andere macrofagen, monocyten en dendritische cellen. Ze zitten in weefsel, zoals de huid, en in het bloed. Als ze ziekteverwekkers detecteren, reageren ze door waarschuwingssignalen uit te zenden. Een van de eerste waarschuwingssignalen is het eiwit interleukine 1 beta (IL-1β). IL-1β is het signaal voor het lichaam om inflammatie (een ontstekingsreactie) te starten en is daarom ook een maatstaf van de sterkte van het alarmsignaal. Hoe meer IL-1β hoe sterker het signaal. Inflammatie heeft twee belangrijke functies, het aanzetten van het adaptief immuunsysteem en het vertragen van ziekteverwekkers. Inflammatie uit zich door koorts, zwelling, roodheid en pijn of jeuk.

Ontsteking en de immuunrespons

Inflammatie is een ingewikkeld proces dat iets weg heeft van oorlog op microscopische schaal. De cellen van het aangeboren immuunsysteem trekken ten strijde tegen de ziekteverwekkers en schieten wild om zich heen. Ze raken daarbij niet alleen de ziekteverwekkers, maar ook zichzelf en de omstanders. De schade die ontstaat wordt gezien als acceptabel, want het is belangrijker om de vijand te isoleren. De schade kan wel hersteld worden als de ziekteverwekkers zijn verslagen. De ziekteverwekkers slaan terug met hun eigen wapens en proberen zich te verbergen, ze gaan in de cellen van het lichaam zitten, of vluchten naar een ander lichaamsdeel, om zo het immuunsysteem te ontwijken. Het is een constante strijd die meestal gelijk opgaat tot de versterking van het adaptief immuunsysteem komt opdagen. De cellen van het adaptief immuunsysteem vallen specifiek de ziekteverwekkers aan, vuren op maat gemaakte antilichamen af die ziekteverwekkers neutraliseren en herkennen zelfs cellen van het lichaam waar ziekteverwekkers zich in verschuilen. Vanaf dat moment is het een ongelijke strijd en worden de laatste brandhaarden snel geblust. Als geen ziekteverwekkers meer gedetecteerd worden, schakelt het lichaam over naar het herstellen van de schade en het vastleggen van geheugen T- en B-cellen, voor het geval dat ze terugkomen.

Autoinflammatie ziekten

In de bovenstaande paragrafen is kort uitgelegd hoe het immuunsysteem in elkaar zit en hoe het reageert op ziekteverwekkers. Maar er gaat ook wel eens wat mis. Bij autoinflammatie ziekten wordt inflammatie gestart zonder dat er ziekteverwekkers zijn gedetecteerd. Het aangeboren immuunsysteem komt in actie, IL-1β komt vrij en er ontstaan zwellingen, koorts, roodheid en pijn. Na een paar dagen tot een week komt het lichaam tot de conclusie

dat er niets aan de hand is en de symptomen verdwijnen weer. Deze cyclus blijft zich herhalen bij autoinflammatie ziekten, waardoor ze ook wel periodieke koorts syndromen worden genoemd. Het is belangrijk om je te realiseren dat autoinflammatie niet hetzelfde is als auto-immuun ziekten. Bij autoinflammatie ziekte is sprake van een fout in het aanzetten van de immuun respons. Deze fout zet het immuunsysteem veel te snel aan, of op de verkeerde momenten. De rest van de immuunrespons is normaal. Bij auto-immuun ziekten herkent het adaptief immuunsysteem lichaamseigen stoffen als vijandig en valt ze aan. De ontsteking die hierdoor ontstaat is chronisch, terwijl bij autoinflammatie deze periodiek is. Het onderscheid is belangrijk omdat het consequenties heeft voor behandeling en onderzoek naar de oorzaken.

Wat betekent het om autoinflammatie te hebben?

Er zit veel variatie in de heftigheid van autoinflammatie. Soms is er sprake van koorts voor een paar dagen, terwijl in heftige gevallen de inflammatie twee weken kan duren. Naast de koorts kunnen ook gewichten ontstoken zijn en zijn er maag-darm problemen en bloedarmoede. Autoinflammatie heeft meestal een genetische oorzaak en is daarom al aanwezig vanaf de geboorte. Omdat de koortsaanvallen haast niet te onderscheiden zijn van normale bacteriele of virale infecties, duurt het vaak jaren voor de diagnose gesteld kan worden. Naast vaak ziek zijn, vele bezoeken aan de dokter of het ziekenhuis, lopen kinderen vaak een mentale en fysieke achterstand op. Ze missen veel school en de groei wordt geremd door de vele onstekingen. Als volwassenen neemt autoinflammatie vaak wat af in heftigheid, maar ook in deze levensfase zijn er nog regelmatige koortsaanvallen wat kan leiden tot veel ziekteverzuim, met alle professionele en sociale gevolgen van dien.

Wat is er aan te doen?

Er is geen specifieke therapie voor autoinflammatie beschikbaar. De meeste autoinflammatieziekten worden behandeld met immuunonderdrukkende medicijnen. Dat kan varieren van het alledaagse paracetamol tot steroiden als prednisolon en zelfs specifieke biologicals (antilichamen tegen lichaamseigen signaalstoffen). Voor een aanzienlijk deel van de patienten werken deze therapieen voldoende, al hebben ze alle ook behoorlijke nadelen. Zo zijn steroiden slecht voor kinderen in de groei, biologicals moeten ingespoten worden en zijn erg duur. De enige bekende therapie die voor genezing zorgt, is een beenmergtransplantatie. Door het hoge risico van een transplantatie, alsmede de moeilijkheden van het vinden van een donor en de hoge kosten, wordt dit alleen gedaan bij patienten waar geen andere opties meer voor zijn.
Verder onderzoek naar de mechanismen in autoinflammatie kan helpen bij de ontwikkeling voor specifiekere medicijnen, iets waarvan ik hoop dat dit proefschrift een bescheiden bijdrage aan kan leveren.

Samenvatting van dit proefschrift

In dit proefschrift hebben we onderzoek gedaan naar de oorzaak van een specifieke autoinflammatie ziekte, genaamd mevalonate kinase deficienty (MKD). In MKD is er een verminderde productie en geen of verminderde activiteit van het enzym mevalonate kinase. Mevalonate kinase is onderdeel van een serie enzymen die samen de basisblokken voor cholesterol maken. Aangezien cholesterol in zeer veel voedingmiddelen zit hebben de meeste cellen er geen tekort aan, maar de blokken worden ook voor andere stoffen gebruikt. In MKD is gevonden dat er specifiek een tekort onstaat aan twee stoffen die belangrijk zijn, omdat ze aan bepaalde eiwitten gekoppeld worden om deze eiwitten goed te laten functioneren. Deze koppeling heet

prenylatie en ontbreekt in MKD.

Hoofdstuk twee is een literatuurstudie over alle aspecten van MKD. In het eerste deel wordt de genetische oorzaak beschreven alsmede wat er bekend is over de biochemische en moleculaire processen die het gevolg zijn van het defect in het enzym. In het tweede deel wordt beschreven wat de klinische syptomen zijn van MKD, hoe diagnose plaatsvindt, wat de behandelstrategieën zijn en wat de vooruitzichten voor patienten.

In *hoofdstuk drie* hebben we onderzocht hoe slecht functioneren van het mevalonate kinase enzym (en daardoor gebrek aan prenylatie) kan leiden tot het aanzetten van inflammatie. Geprenyleerde eiwitten spelen een belangrijke rol om alledaagse beschadigde en overbodige componenten op te ruimen. In het MKD modelsysteem blijkt dit niet goed te werken. Het lijkt er op dat in MKD beschadigde onderdelen daarom ophopen en van binnenuit de immuun respons aanzetten. Om precies te zijn gaat het om beschadigde mitochondriën in monocyten, die hun inhoud in de cel lekken. Het gevolg is dat monocyten te snel en te veel IL-1β afgeven wanneer ze geactiveerd worden. Het vrijgekomen IL-1β leidt tot inflammatie en koorts.

Voor *hoofdstuk vier* hebben we gekozen voor een andere benadering. Doordat prenylatie voor een groot deel afwezig is in MKD zijn honderden eiwitten aangedaan. Om uit te kunnen zoeken welke functie prenylatie heeft voor individuele eiwitten hebben we gekeken naar twee belangrijke eiwitten voor het lichaam, Rac1 en RhoA. Beiden zijn normaal geprenyleerd, maar in MKD ontbreekt dit. Het was al bekend dat Rac1 actiever wordt zonder prenylatie. Voor RhoA blijkt het omgekeerde te gelden, en wordt het minder actief. Dit heeft gevolgen voor de activatie van andere eiwitten, bijvoorbeeld protein kinase B (PKB), waarvan al is aangetoond dat dit voor meer IL-1β afgifte zorgt. Rac1 en RhoA spelen daarom beide een belangrijke rol in de cellulaire signalering die leidt tot afgifte van IL-1β.

Om inflammatie in gang te zetten, moet eerst een aantal verschillende eiwitten samenkomen om geactiveerd te worden. Dit heet inflammasome activatie. In de laatste paar jaren is veel onderzoek gedaan naar de activatie van het inflammasome. Steeds vaker blijkt dat de mitochondriën daarin een belangrijke rol spelen. Uit het onderzoek in de hoofdstukken drie en vier is gebleken dat in MKD mitochondriën zijn aangedaan en ook in enkele andere autoinflammatie ziekten is een link met mitochondriën gevonden. *Hoofdstuk vijf* bevat een literatuurstudie naar de rol van mitochondriën in autoinflammatie, waarin we kijken welke verbanden er zijn tussen mitochondriën in verschillende autoinflammatie ziekten en of er mogelijke nieuwe opties zijn voor therapie of medicijnontwikkeling.

Er zijn nog steeds patiënten die alle symptomen hebben van autoinflammatie ziekten, maar niet met een variant gediagnostiseerd kunnen worden. Er zijn dus nog onbekende mutaties, of combinaties van mutaties, die autoinflammatie veroorzaken. *In hoofdstuk zes* beschrijven we een zeldzame mutatie die mogelijk een rol speelt in autoinflammatie. De mutatie zit in het eiwit SerpinB9. Dit is tot nu toe het enige bekende menselijk eiwit dat actief caspase-1 remt. Normaal activeert caspase-1 IL-1β. Als de remming van caspase-1 wegvalt door een mutatie in SerpinB9 ontstaat er een verhoogd IL-1β afgifte in cellen met deze mutatie. Deze mutatie, alhoewel zeldzaam, kan daarom een rol spelen bij het ontstaan van autoinflammatie.

Dit proefschrift beschrijft moleculaire mechanismen die een rol spelen in MKD en mogelijk andere autoinflammatie ziekten. Ondanks dat de meeste zijn terug te voeren op een enkele gen mutatie, zijn de gevolgen in de cellulaire huishouding en signalering zeer complex. We hebben gevonden dat in MKD mitochondriën zijn aangedaan en dit een bijdrage levert aan het

ziektebeeld. Verder hebben wij inzicht verkregen in hoe Rac1 en RhoA activiteit veranderd is in MKD en dat dit afhankelijk is van de prenylatie. Tevens beschrijven we een nieuwe mutatie in SerpinB9, die kan leiden tot autoinflammatie. Verbeterde moleculaire inzichten geven een beter begrip van het functioneren van het immuunsysteem op cellulaire schaal en leiden in de toekomst hopelijk tot betere en veiliger therapieën voor autoinflammatie en andere inflammatie gerelateerde ziekten.

Dankwoord

Een proefschrift schijven is een individuele aangelegenheid. Alleen achter de computer zuchten en steunen tot het eindelijk af is (in mijn geval tenminste). Onderzoek daarentegen is teamsport en er zijn dan ook heel veel mensen die een bijdrage hebben geleverd aan dit proefschrift. Ik wil die hier graag allemaal bedanken. Ik heb een fantastische tijd gehad en ben trots op de inhoud van dit proefschrift, zonder jullie was het nooit gelukt. Ontzettend bedankt!

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Dear Pier G, thank you for all your help with the mitochondrial work and for allowing me to use you XF analyzer. It made chapter three a much better story. I’ve enjoyed our collaboration and wish you all the best in the future!

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Dear Popi, it is said that good things come in small packages, which is especially true for you. Co-author on two papers, I didn´t think it was possible for someone to do so much work in a few months! You are an exceptional researcher and very organized (I have never seen anybody with a more organized lab journal, quite in contrast to my own), and I have no doubt that your PhD will be highly successful. All the best to you and your research in Heidelberg!

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Curriculum Vitae

Robert van der Burgh was born on the 15th of September 1982. In 2000 he completed his secondary education and went on to study geodesy at the technical university of Delft. After two successful years from a social point of view but less so from an academic perspective, he switched to the Utrecht University and started studying chemistry. In 2006 he received the Bachelor grade and continued at the Utrecht University with the combined chemistry/pharmaceutical sciences prestige master drug innovation. His interest in the origin of disease was not fully covered by curriculum, so as part of his master degree he joined the lab of Eyal Raz at UCSD for a research internship. This inspired him to continue with research and upon his return to Holland he graduated in 2009.

After graduation he applied for a PhD position in the laboratory of Berent Prakken, under the supervision of Dr. Marianne Boes en Dr. Joost Frenkel at the CMCI (currently LTI) at the UMC Utrecht. His research focused on molecular mechanisms in mevalonate kinase deficiency and autoinflammation. The results of that research are published in this thesis, with the exception of the most important finding; his soon-to-be wife Jennifer Speirs, who he met in the lab next door.

In 2013 he became a Marie Curie fellow in the EUTRAIN network focused on translation research in pediatric rheumatology. The network has allowed him to expand his horizons by attending the EUREKA course on translational research and the 64th Lindau Nobel laureate meeting. As part of Eutrain, he is currently based at Proteros Biostructures in Munich, Germany where he focusses on the development of screening assays and assesses the potential of new drug targets.

&

List of publications

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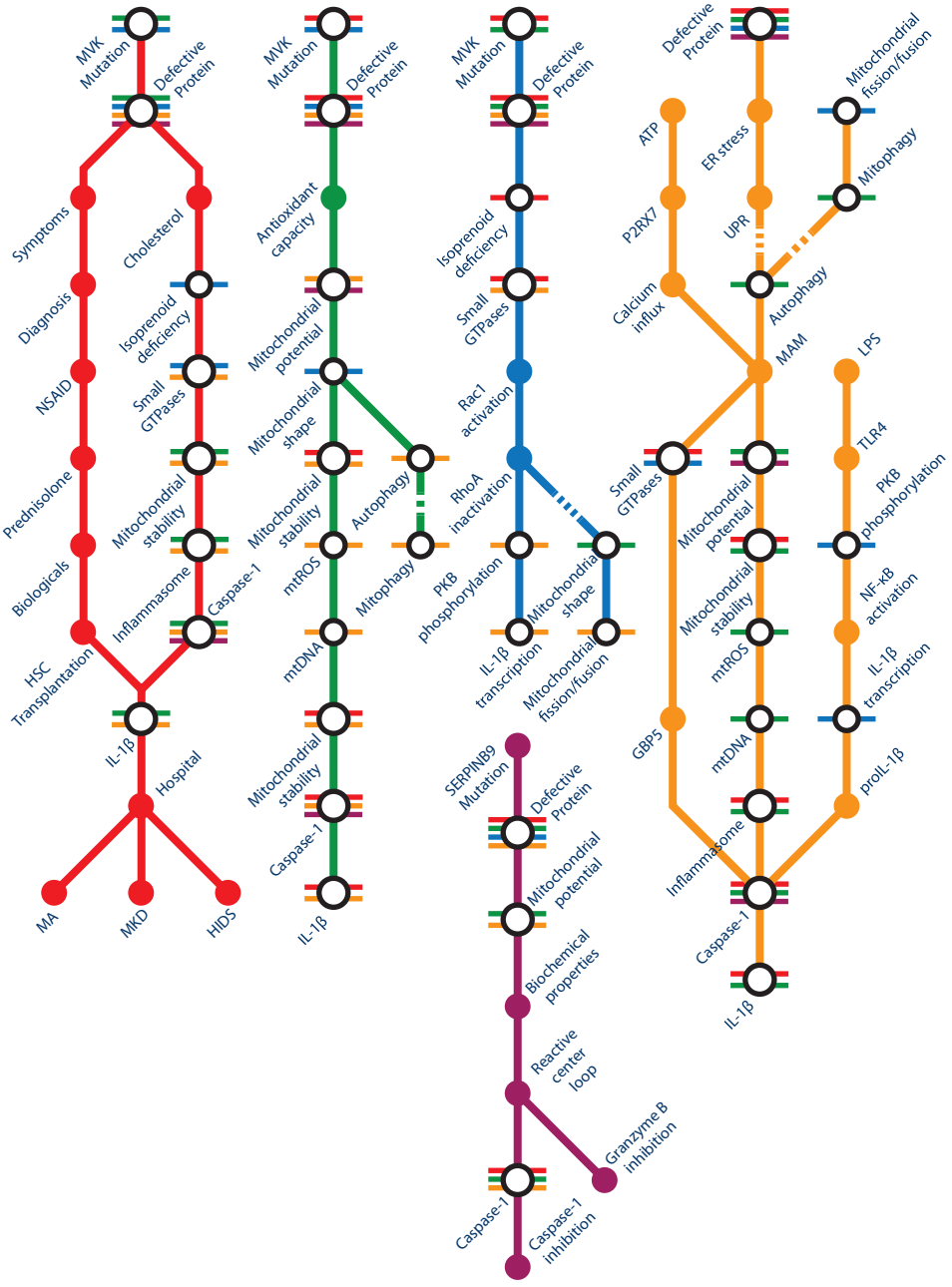
MVK Mutation
Defective Protein
Symptoms
Cholesterol
Diagnosis
Isoprenoid deficiency
NSAID
Small GTPases
Prednisolone
Mitochondrial stability
Biologicals
Inflammasome
Caspase-1
HSC Transplantation
IL-1β
Hospital
MA
MKD
HIDS
MVK Mutation
Defective Protein
Antioxidant capacity
Mitochondrial potential
Mitochondrial shape
Mitochondrial stability
Autophagy
mtROS
Mitophagy
mtDNA
Mitochondrial stability
Caspase-1
IL-1β
MVK Mutation
Defective Protein
Isoprenoid deficiency
Small GTPases
Rac1 activation
RhoA inactivation
PKB phosphorylation
Mitochondrial shape
IL-1β transcription
Mitochondrial fission/fusion
SERPINB9 Mutation
Defective Protein
Mitochondrial potential
Biochemical properties
Reactive center loop
Granzyme B inhibition
Caspase-1
Caspase-1 inhibition
Defective Protein
ATP
ER stress
Mitochondrial fission/fusion
P2RX7
UPR
Mitophagy
Calcium influx
Autophagy
MAM
LPS
Small GTPases
Mitochondrial potential
TLR4
PKB phosphorylation
Mitochondrial stability
mtROS
NF-κB activation
GBP5
mtDNA
IL-1β transcription
Inflammasome
proIL-1β
Caspase-1
IL-1β