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# The role of mitochondria in the development of autoinflammatory diseases

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# Abstract

Autoinflammatory diseases are hereditary inflammatory syndromes, which are characterized by unprovoked high activation of the innate immune response. The myeloid effector cells and the germline-encoded receptors of the innate immune system are implicated in the development of these diseases. Furthermore, in some autoinflammatory diseases the deregulated production of the proinflammatory cytokine IL-1β is reported. The processing of IL-1β requires the assembly of a macromolecular complex, the NLPR3 inflammasome. This complex activates caspase-1, which therefore cleaves the precursor of IL-1β. Compelling evidence shows the association of NLPR3 inflammasome activation with mitochondria. Increasing experimental evidence supports that the mitochondria regulate the innate immune response and they modulate inflammasome activation. Recent data also demonstrate that the impairment of mitochondrial integrity and homeostasis, accompanied by up-regulated production of ROS seems to be also involved in autoinflammatory diseases, such as TRAPS. The aim of the present thesis is to examine thoroughly all the research provided and try to shed light on the underlying mechanism by which mitochondria could induce the development of autoinflammatory syndromes.

# Chapter 1

## Introduction

Mitochondria participate in many vital cell processes and they regulate apoptosis, cell signaling and innate immunity. Compelling recent evidence indicates that they orchestrate an inflammatory response and they are associated with autoinflammatory diseases. The goal of this overview is to investigate the role of mitochondria in the development of these diseases. First, I will briefly give an overview of the autoinflammatory syndromes and mitochondria in innate immune response. In continuity, based on recent studies I will analyze extensively how mitochondria are implicated in the secretion of proinflammatory cytokines through the activation of the molecular complex NLPR3 inflammasome. In support of the latter mechanism, growing evidence demonstrate the potential participation of mitochondria in autoinflammation.

## Innate immune system

The innate immune system can detect plethora of microorganisms and stress stimuli through the pattern recognition receptors (PRRs). These are germline-encoded receptors that bind pathogen-associated molecular patterns (PAMPs) and danger-associated signals (DAMPs). PAMPs can be microbial cell wall components, nucleic acid, and proteins, and DAMPs are noninfectious host-derived signals such as uric acid, heat shock proteins, high-mobility group protein B1 (HMGB1) and extracellular ATP (Kanneganti, et al. 2007; Lamkanfi and Dixit 2012; Kono and Rock 2008). There are four different PRR families, which are classified into the membrane-associated receptors like Toll-like receptors (TLRs), C-type lectin receptors (CLRs), the cytosolic receptors such as retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs) and nucleotide-binding oligomerarization domain (NOD)-like receptors (NLRs) (Kawai and Akira 2009). The stimulation of PRRs triggers an immune response by the activation of signaling pathways, which lead to the production of proinflammatory cytokines, chemokines and growth factors (Lamkanfi and Dixit 2012). Thus, a robust antimicrobial environment is produced which eventually contributes to the activation of adaptive immunity. However, the innate immune

response should be tightly controlled so as to prevent excessive inflammation and tissue damage. The uncontrolled activation of the innate immune system is responsible for a variety of inflammatory diseases, including the autoinflammatory syndromes.

## Autoinflammatory diseases

Autoinflammatory diseases are hereditary monogenic diseases, which are characterized by abnormal activation and dysregulation of the innate immune system in the absence of high-titer autoantibodies or antigen-specific T cells (Kanazawa 2012; Masters, et al. 2009; Hashkes & Toker 2012). The myeloid effector cells and the PRRs of the innate immune system are implicated in the development of these diseases (Kastner, et al 2010). The autoinflammatory syndromes according to their pathogenesis can be classified to inflammasomopathies, NF-kB activation syndromes, protein misfolding disorders of the innate immune system, complement regulatory diseases, cytokine signaling disorders and macrophage activation disorders (Masters, et al. 2009). Herein, we focus on autoinflammatory diseases that have been associated with a deregulated production of interleukin (IL)-1β and they are refered as inflammasomopathies.

IL-1β is a pro-inflammatory cytokine responsible for an early immune response after an infection or tissue injury and it is a major mediator of fever and inflammation (Savic, et al. 2012; Kastner, et al 2010). The production of IL-1β requires two steps: first activation of TLRs by proinflammatory stimuli induces the synthesis of pro-IL-1β, and then a second step cleaves pro-IL-1β to an active form (Rubartelli 2012; Kanneganti, et al. 2007). The cleavage of pro-IL-1β is conducted by cysteine protease caspase-1 (Latz, 2010). Caspase-1 is an inactive precursor protein that belongs to inflammatory caspases and contains N-terminal caspase recruitment domain (CARD) (Martinon, et al.2002), a necessary domain for its interaction and recruitment with the macromolecular complexes, the inflammasomes. Inflammasomes are molecular platforms that induce caspase-1 activation which in turn proceed to IL-1β maturation.

However, secretion of IL-1β in large amount causes tissue damage, bone resorption, collagen deposition, neovasculatization and haemodynamic shock (Ozkurede and

Franchi 2011) and its exaggerated production is implicated in many autoinflammatory diseases. Some of the syndromes associated with deregulated secretion of IL-1β are the intristic inflammasomopathies which demonstrate mutations in the proteins of the inflammasome platform and they are represented by cryopirn-associated periodic syndromes (CAPS), which includes familial cold autoinflammatory syndrome (FCAS), Muckle-Wells syndrome (MWS) and neonatal-onset multisystem inflammatory disease (NOMID).

## Inflammasomes

Inflammasomes are multimeric procaspase-1 activating platform and assemble in response to pathogens and stress signals , which mediates activation of IL-1β and IL-18 and triggers pyroptosis to eradicate pathogens (Mankan, et al. 2011; Lamkanfi, et al 2011). Several NLR members upon activation trigger the assembly of the inflammasomes. NLRs are a large family of PRRs, which detect aberrant conditions in the cytosol by recognizing various pathogens and endogenous molecules (Tschopp 2011). NLRs have tripartite structural organization, which contains a N-terminal protein interaction domain, a central-nucleotide binding domain (NACHT) and a C-terminal leucine-rich repeat (LRR) (Kawai and Akira 2009). The N-terminal domain regulates protein-protein interaction and subcategorizes the NLR proteins (Latz 2010). This domain contains a protein-protein interaction domain a caspase recruitment domain (CARD), a pyrin domain (PYD) or bacilovirus inhibitor repeat (BIR) (Lamkanfi, et al. 2007). These motifs are necessary for the homotypic interaction of NLRs with adaptor proteins and effectors, like apoptosis-associated speck-like protein (ASC) and caspase-1 (Lamkanfi, et al. 2011). The NACHT domain, which binds to ribonucleotides (Latz 2010), contributes to the NLRs activation by self-oligomerization and is the core of the inflammasome complex (Mankan, et al 2011). The LRR domain is present in many proteins and contains a common motif of 20-30 aa rich in leucine, necessary for protein-protein interactions and ligand binding (Rubartelli 2012). LRR recognizes PAMPs and DAMPs and regulates NLR activity by conformational change of the LRR motif that triggers oligomerization (Lamkanfi, et al. 2007).

Five different inflammasome complexes have been identified, namely NLRP1, NLPR3, NLPR4, NLPR6 and AIM2. Different microbial or stress agents can activate each of these inflammasome platforms (Lamkanfi and Dixit 2012). However, the most extensive studied inflammasome is based on NLPR3, which has been associated with the development of disease, specifically with the progress of autoinflammatory diseases CAPS and recently with metabolic disorders like type 2 diabetes mellitus (T2DM).

NLPR3 protein is not abundant in resting conditions, so a priming step that initiates the increase of NLRP3 in order to allow its activation is required (Mankan, et al 2011). This first step can be mediated by an initial stimulus through NF-kB activation (Franchi, et al 2010). Then, a second signal, microbial or non-microbial, is necessary for the recruitment of the complex (Mankan, et al 2011). Upon activation, NLPR3 interacts with the adaptor protein apoptosis-associated speck-like protein (ASC), where the pyrin domain of ASC binds to the PYD domain of NLRP3 (Kanneganti, et al. 2007; Agostini, et al 2004). ASC is necessary to recruits procaspase-1, as it presents the CARD domain that interacts with procaspase-1 CARD (Schroder, et al. 2010). Then, procaspase-1 is activated via autoprocessing (Tschopp 2011). Thus, the NLPR3 inflammasome is consisted of NLPR3, ASC, and the caspase-1 (Agostini, et al 2004). The activation of this platform leads to caspase-1 maturation and therefore the secretion of IL-1β and IL-18 (Cassel, et al 2010).

The NLPR3 inflammasome is involved in the immune responses to several bacteria, viruses and fungi (Franchi, et al. 2010). Moreover, NLPR3 inflammasome activation is triggered by reactive oxygen species (ROS), potassium efflux, lysosomal disruption, extracellular ATP, monosodium urate crystals (MSU), silica, asbestos and aluminium particles (Mankan, et al. 2011; Martinon, et al. 2009). As it is unveiled, the NLPR3 inflammasome can be activated in response of either various pathogens or endogenous danger signals and it can be considered as a sensor for cellular stress (Cassel, et al 2010; Kawai and Akira 2009).

Three models, which are implicated in NLPR3 activation have been proposed (Fig.1). The first model (Fig.1.1), refered as the channel model, is linked with the concentration of cytosolic $K^+$ and the ATP-triggered activation. Extracellular ATP,

which is released from cells in conditions of cellular damage or stress, activates the P2X7R receptor and mediates $K^+$ efflux. Therefore, this ATP-mediated P2X7R stimulation contributes to pannexin-1 activation and pore formation, which mediates $K^+$ efflux and the cytoplasmic entry of NLPR3 activators (Tschopp and Schroder 2010; Martinon, et al. 2009; Perregaux and Gabbel 1994; Kanneganti; et al. 2007). However, this model has remained obscure since there is no clear evidence that all the NLPR3 activators could not enter through the pores and especially the large agonists, such as MSU and asbestos (Tschopp and Schroder 2010).

The lysosome rupture model (Fig.1.2) suggests that both the ingestion of particulate molecules and the sterile-non particulate by phagocytosis lead to lysosomal rupture. The latter triggers lysosomal leakage and release of cathepsin B, which in turn activates NLPR3 inflamassome (Hornung et al, 2008). However, Dostert et al. 2009 have demonstrated that in deficient cathepsin-B bone marrow derived macrophages (BMDMs) there is no alteration in NLRP3 activation. Moreover, Halle et al. 2008 have shown that the inhibition of cathepsin B leads to lower IL-1β secretion only after the stimulation of specific NLPR3 agonist (Aβ amyloid), whereas there is no difference in IL-1β release when cells where stimulated with ATP. There is no doubt that further studies need to be conducted to elucidate the exact mechanism of this model.

A third mechanism proposes the involvement of ROS in NLPR3 activation (Fig.1.3). ROS, which are produced by phagocytic effector cells, can attribute to immune responses by eliminating microbial pathogens through NAPDH oxidases activation (Bogdan, et al. 2000). It has been demonstrated that ATP stimulation triggers ROS production and therefore NLPR3 inflammasome activation via ROS-dependent the PI3K signaling pathway (Cruz, et al 2007). Moreover, asbestos can induce ROS generation, which in turns activates IL-1β production (Dostert, et al 2008). When ROS production is inhibited, NLPR3 inflammasome activation is blocked, even after the stimulation with MSU, asbestos and ATP (Dostert, et al 2008). However, van de Veerdonk et al 2010 reported that phagosomal ROS is not implicated in NLPR3 inflammasome activation, as patients with chronic granulomatous diseases (CGDs), who cannot generate NADPH-dependent ROS, demonstrate increased inflammasome activation and also high levels of IL-β. Furthermore, Bulua et al. 2011 show that

NAPDH oxidase (NOX2)-derived ROS is not necessary for inflammatory cytokine secretion. These conflicting results propose another source of ROS implicated in NLPR3 inflammasome activation. Mitochondrial oxidative phosphorylation is a major source of ROS. Indeed, a correlation between mitochondrial ROS (mtROS) and NLRP3 inflammasome activation is observed. More specifically, it has been demonstrated that mtROS is required for caspase-1 activation and active IL-1β secretion, whereas inhibition in ROS generation leads to impaired NLPR3 inflammasome activation (Zhou, et al. 2011; Nakahira, et al. 2011).

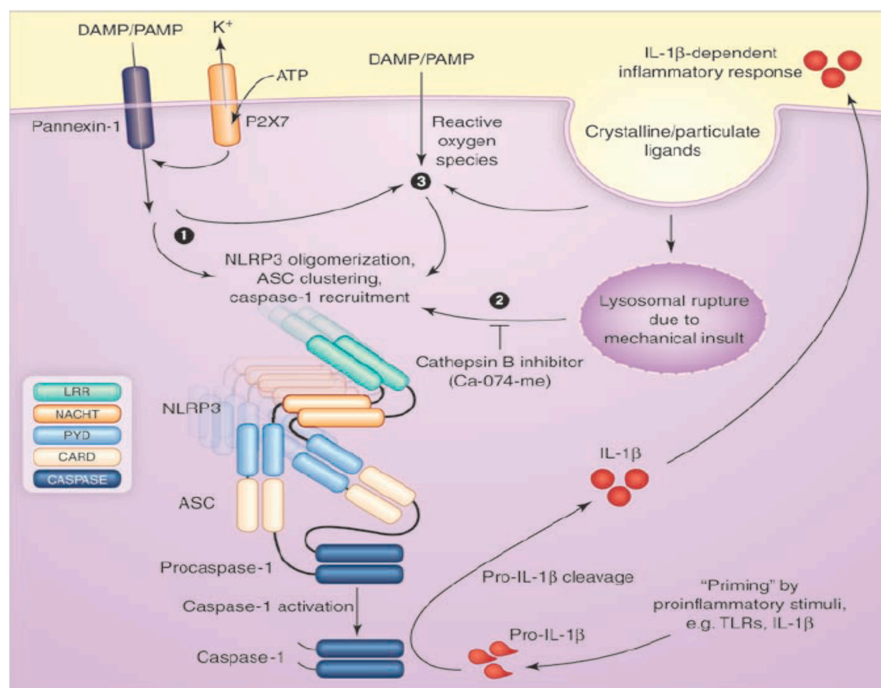


**Figure 1. Proposed models of NLPR3 inflammasome activation.** (1) Channel model. Extracellular ATP activates P2X7 receptor and induces $K^+$ influx. (2) The lysosome rupture model. Particulate molecules are phagocytosed and lead to lysosomal rupture with release of cathepsin B. (3) ROS model. All the NLPR3 activators induce either phagosomal ROS or mtROS in order to activate inflammasome. NLPR3 oligomerization and recruitment of ASC and procaspase-1 leads to IL-1β maturation. ASC: adaptor protein apoptosis-associated speck-like protein, CARD: caspase recruitment domain, PYD: a pyrin domain, LRR: leucine-rich repeat. *Schroder, et al. 2010*

As already mentioned the source of ROS generation is still debated, but also the exact mechanism by which ROS activates NLPR3 inflammasome is unclear (Martinon 2012). On the grounds that $K^+$ efflux is necessary for NLPR3 inflammasome activation (Dostert, et al. 2008), $K^+$ efflux should be implicated in ROS model for

caspase-1 activation. Indeed, a proposed mechanism by which $K^+$ efflux regulates NLPR3 inflammasome activation might be through the mitochondria, where the concentration of potassium through the $K^+$ channels in mitochondria can regulate mitochondrial respiration (Tschopp 2011; Heinen, et al. 2007). Furthermore, $K^+$ flux could alter the osmotic balance between the cytosol and mitochondrial matrix, providing potential mitochondria-derived danger signals that activate NLPR3 inflammasome (Martinon 2012). Nevertheless, this mechanism should be investigated further.

Moreover, recent evidences suggest that ROS induce thioredoxin-interacting protein (TXNIP) dissociation from thioredoxin (TRX), which in turn activates NLPR3 inflammasome (Zhou, et al. 2010). The TRX system has an antioxidant role, participating in cellular redox balance and functioning as a ROS scavenger (Lu, et al 2006; Schroder et al 2010). TXNIP, also known as vitamin $D_3$ up-regulated protein 1 (VDUP1), responds to stress stimuli and regulates oxidative state of the cells by binding to the catalytic center of TRX and inhibiting its activity (Nishiyama, et al. 1999; Junn, et al. 2000). Zhou et al. 2010 revealed that TXNIP dissociates from TRX in a ROS-dependent manner and binds to NLPR3 inflammasome, where it acts as NLPR3 activator and mediates IL-1β secretion. TXNIP ablation in mice has no effect on Ipaf and AIM2 inflammasomes, whereas it reduces IL-1β production suggesting that TXNIP regulates specifically the NLPR3 inflammasome activation (Zhou, et al. 2010). Moreover, it is shown that TXNIP redistributes to mitochondria dependent on ROS (Zhou, et al. 2011). However, Masters et al. (2010) demonstrate that TXNIP is not required for NLPR3 activation, as mice lacking TXNIP did not attenuate caspase-1 activation. These contradictory results address the necessity for further research on the role of TXNIP on NLPR3 inflammasome activation.

Further conflicting results show that superoxide dismutase 1 (SOD1), an anti-oxidant enzyme that provides vital defense against oxygen and superoxide toxicity (Fridovich 1975), is required for caspase-1 activation. More specifically, SOD1 inhibition leads to elevated ROS generation that diminishes the IL-1β secretion (Meissner, et al. 2008).

Therefore, although the role of ROS in the NLPR3 inflammasome activation seems to

be essential, there are still a lot of contradicting results. The source of ROS, the additional mechanisms that participate in the ROS model and the opposing studies about NLPR3 agonist TXNIP, are some examples of these discrepant observations. Moreover, the conflicting studies in the participation of the antioxidants in IL-1β secretion, whether they stimulate or inhibit it, demonstrate that there is no consensus yet and further research should be conducted to elucidate the underlying mechanism of the ROS model.

## Mitochondria in innate immunity

Mitochondria are double membrane organelles that they contain their own DNA genome (mtDNA), which is believed to derive from endosymbiosis of ancient bacteria with host pro-eukaryotes. Human mtDNA is a 16569 base pair (bp) double-stranded molecule, which encodes 2 rRNAs, 22 tRNAs and 13 structural proteins crucial for oxidative phosphorylation. However, the mitochondrion is dependent on nuclear DNA that encodes the rest of the proteins (~1000) for mitochondrial maintenance, replication and transcription. Mitochondria are dynamic organelles, which undergo fusion and fission, processes which regulate their shape, their size, their mtDNA maintenance, the number of mitochondria and their distribution.

Mitochondria are considered the "energy and cellular powerhouses" as they attribute to vital processes, including ATP synthesis via the process of oxidative phosphorylation (OXPHOS), the generation of reactive oxygen species (ROS), calcium homeostasis, β oxidation of fatty acids, and biosynthesis of steroids and heme. Additionally, mitochondria are also involved in various signaling pathways, such as cellular stress pathways and programmed cell death signaling (Tait and Green 2012; Rath and Haller 2012). Mitochondria have a key role in the induction of apoptosis following microbial infection (Arnoult, et al. 2009) and recent studies demonstrate that they also participate in innate immune signaling by controlling antiviral and antibacterial immunity (West, et al 2011; Tait and Green 2012). More specifically, viral RNA is detected by RIG-I and melanoma differentiation-associated gene 5 (MDA5) receptors (RLR family receptors) and then they interact with the mitochondrial antiviral signaling protein (MAVS). This interaction triggers the phosphorylation of interferon response factors (IRF)3 and IRF7 and NF-κB signaling,

which trigger the secretion of type I interferons (IFNs) and pro-inflammatory cytokines (Arnoult, et al. 2011; West, et al 2011). Besides MAVS, mitochondrial dynamics and mtROS are observed to participate in antiviral innate immunity (Arnoult, et al. 2011). The mitochondrial proteins mitofusins 1 and 2 (Mfn1, Mfn2), which participate in fusion, can also regulate antiviral immunity. More specifically, Mfn1 is a positive regulator of antiviral immunity, whereas Mfn2 protein is a negative mediator of antiviral immunity by binding to MAVS (Arnoult, et al. 2011; Yasukawa 2009). Moreover, the dissipation of the mitochondrial membrane potential by increasing the membrane permeability to protons corresponds to defective RLR-antiviral responses (Koshiba, et al. 2011). Furthermore, mtROS production initiated by DNA viruses, mediates innate immune response (Gonzalez-Dosal, et al. 2012).

Increasing experimental evidence shows that mitochondrial components behave as DAMPs triggering sterile inflammatory responses (West, et al 2011). As mitochondrial DAMPS (mtDAMPS) can be considered cytochrome c, formyl peptides, mtDNA and mtROS, and their function as DAMPS seems to be evolved so that mitochondrial damage can be detected (Arnoult, et al. 2011).

Moreover, compelling evidence demonstrate the implication of mitochondria in NLPR3 inflammasome activation. More specifically, it has been demonstrated that mtDNA triggers caspase-1 activation (Nakahira, et al. 2011). In addition, mtROS augments caspase-1 activation and IL-1β secretion (Zhou, et al. 2011; Nakahira, et al. 2011). Inhibition of autophagy induces the accumulation of dysfunctional mitochondria and mtROS production and enhances NLPR3 inflammasome activation (Zhou, et al. 2011). Furthermore, a connection between a mitochondrial apoptotic signaling and NLPR3 inflammasome activation was reported (Shimada et al. 2012).

A correlation between mitochondria and an autoinflammatory disease, namely tumor necrosis factor (TNF) receptor-associated periodic syndrome (TRAPS), was reported. TRAPS patients demonstrate augmented basal oxygen consumption, higher oxidative capacity and elevated mtROS production (Bulua, et al. 2011). This finding gives a new insight in the involvement of dysfunctional mitochondria in autoinflammatory diseases, where elevated ROS production leads to increased MAPK kinases activity, and therefore exaggerated inflammatory response (Bulua, et al. 2011).

As it is unveiled, mitochondria participate in the innate immunity and they can trigger several signaling pathways. They are crucial organelles that participate in the antiviral and antibacterial immunity and they play central role in the host innate immune defense. However, mitochondria have been associated with the progress of diseases, such as autoinflammatory syndromes. Herein, we will explore how mitochondria are implicated in those diseases. Moreover, we will give insight into their involvement in the macromolecular complex NLPR3 inflammasome, which is associated with the development of autoinflammatory syndromes, such as inflammasomopathies.

# Chapter 2

## Role of mitochondria in NLPR3 inflammasome

Compelling evidences demonstrate the implication of mitochondria in NLPR3 activation. To address the underlying mechanism by which the mitochondria induce caspase-1 activation, several studies were conducted on the role of dysfunctional mitochondria and mitochondrial components on NLPR3 activation.

There is growing evidence supporting that mtROS is implicated in NLPR3 inflammasome activation. Zhou et al. (2011) demonstrate that the inhibitors rotenone and antimycin A, which hinder the key enzymes of the respiratory chain complex I and III respectively and lead to potent ROS generation (Li, et al. 2003; Panduri, et al. 2004), activate NLPR3 inflammasome. This comes in an agreement with the Nakahira et al. (2011) study, where rotenone enhances IL-1β production in response to LPS and ATP. On the other hand, ROS inhibition attributes to the impairment of NLPR3 inflammasome activation (Zhou, et al. 2011), indicating the importance of ROS in caspase-1 activation. Also macrophages with mtDNA-deficient $p^0$ phenotype that generate reduced levels of ROS demonstrate diminished caspase-1 activation after the stimulation with rotenone, LPS and ATP (Nakahira, et al. 2011). Together, all these results support that mtROS is involved in the NLPR3 inflammasome activation and it seems to be a potent activator.

Furthermore, the voltage dependent anion channel (VDAC), which regulates mitochondrial function and is required for oxidative phosphorylation (Lemasters and Holmuhamedov 2006), was examined. VDAC is located in the outer mitochondrial membrane and is the major transporter for metabolites and ions (Manella 1998). By inhibiting the VDAC, a significant reduction in mtROS and therefore an impairment of NLPR3 inflammasome are observed (Zhou, et al. 2011). Mitochondrial ROS and calcium overload induce membrane permeability transition (MPT), rendering the inner mitochondrial membrane permeable to solutes about 1.5 kDa and resulting in membrane depolarization, uncoupling of oxidative phosphorylation and mitochondria swelling (Lemasters, et al. 2009). Inhibition of MPT with its potent inhibitor cyclosporine A reduces IL-1β secretion in response to NLPR3 activators (Nakahira, et

al. 2011). These data support that NLPR3 inflammasome activation is dependent on mtROS and MPT.

As ROS-damaged mitochondria are removed by mitophagy- a specialized form of autophagy (Novak 2012), further research was conducted on the role of autophagy in the inflammasome activation. Indeed, autophagy can modulate NLPR3 inflammasome activation through the regulation of mitochondrial homeostasis (Nakahira, et al. 2011). Autophagy is an eukaryotic homeostatic process, in which organelles are degraded and recycled (Klionsky and Emr 2000). Autophagy also removes mitochondria, which demonstrate altered MPT (Lemasters, et al. 1998). Moreover, recent studies report that autophagy regulates immune signaling and it serves as a negative regulator of the inflammasome activation (Levine, et al. 2011). In line with these observations, Zhou et al. (2011) demonstrate that autophagy clears mtROS and reduces NLPR3 inflammasome activation, whereas the inhibition of autophagy with 3-methyladenine (3-MA) accumulates mtROS and augments caspase-1 activation. This comes to an agreement with Nakahira et al. 2011, where the ablation of both autophagic proteins LC3B (microtubule-associated protein 1 light chain 3B- a dowstream of the autophagic pathway) and beclin 1 (an upstream of autophagy) in response to LPS and ATP lead to the mitochondrial dysfunction and augmented mtROS production which in turn activates NLPR3 inflammasome (Nakahira, et al 2011). On the other hand when an antioxidant (Mito-TEMPO) is administrated in macrophages depleted of LC3B and beclin 1, there is impaired NLPR3 inflammasome activation (Nakahira, et al. 2011). Thus, the depletion of autophagy results in the accumulation of damaged mitochondria and the excess of mtROS, which therefore provoke NLPR3 inflammasome activation.

One step further in order to investigate the implication of mtDNA in NLPR3 inflammasome activation is reported. It is shown that after stimulating the wild type BMDMs with LPS and ATP, the amount of cytosolic mtDNA is increased (Nakahira, et al. 2011). In autophagic deficient BMDMs treatment with LPS and ATP augments the cytosolic mtDNA twofold compared to the wild cells, indicating that the stronger activation of caspase-1 leads to higher amounts of cytosolic mtDNA (Nakahira, et al. 2011). Moreover, on the grounds that NLPR3 deficiency suppresses cytosolic mtDNA and hinders the loss of mitochondrial potential, it is suggested that NLPR3

inflammasome is necessary for MPT and the release of mtDNA in the cytosol (Nakahira, et al. 2011). However, Shimada et al. (2012) demonstrate that when mitochondria detect danger signals that initiate apoptosis, then oxidized mtDNA is released and binds to NLPR3, and therefore NLPR3 inflammasome activation is triggered. They show that the binding of oxidized mtDNA is prior to caspase-1 activation since in caspase-1 deficient macrophages oxidized DNA was pulled down with NLPR3, indicating that oxidized DNA might induce the assembly of the inflammasome (Shimada, et al. 2012). Shimada et al. (2012) gives a potential explanation about the contradictory results with Nakahira et al. (2011), suggesting that NLPR3 stabilizes mtDNA and this is a possible reason that mtDNA is not detected in NLPR3 deficient macrophages. Moreover, the Shimada et al. (2012) study stresses the role of apoptosis in NLPR3 inflammasome activation. Apoptosis seems to regulate inflammasome activation as overexpression of Bcl-2, an anti-apoptotic protein, show low levels of caspase-1 activation (Zhou, et al. 2011). More specifically, overexpression of Bcl-2 attributes to partial VDAC closure, which in turn attenuates mitochondrial $Ca^{2+}$ and ROS production (Zhou, et al. 2011). Nevertheless, apoptosis can mediate NLPR3 inflammasome activation only in the presence of a proinflammatory signal-1 and proapoptotic stimulus is sufficient to serve as a second signal for NLPR3 activation (Shimada, et al. 2012).

A further link between mitochondria and NLPR3 inflammasome is demonstrated by the position of the latter. In resting conditions NLPR3 is located in endoplasmic reticulum (ER), but when NLPR3 inflammasome is activated, NLPR3 re-locates into the perinuclear space and to the mitochondria associated ER membranes (MAMs) (Zhou, et al. 2011). MAMs are a connecting network between ER and mitochondria and attribute to lipid, calcium and metabolites exchange with mitochondria (Simmen, et al. 2010). ASC is detected in cytosol in non-activated state and it translocates to the MAMs and mitochondria in an NLPR3-dependent manner upon activation (Zhou, et al. 2011). These results could provide an indication that the position of NLPR3 inflammasome in the MAMs demonstrates a communication between the inflammasome and mitochondria, suggesting their potential regulation on the NLPR3 inflammasome activation.

One more observation indicates a potential role of mitochondria in NLPR3 activation through the calcium mobilization. More specifically, $Ca^{2+}$ mobilization can trigger NLPR3 activation through mitochondrial damage (Murakami, et al. 2012). Calcium is an important regulator of mitochondrial function. More specifically, $Ca^{2+}$ is essential for mitochondrial bioenergetics and can mediate ATP synthesis (Hayashi, et al. 2009), whereas the overload of $Ca^{2+}$ itself or accompanied with pathological stimulus, can modulate pathological effects and lead to ROS production, induce MPT onset and apoptosis (Brookes, et al. 2004; Lemasters, et al. 2009). Murakami et al. (2012) show that various NLPR3 activators, as ATP, MSU and nigericin, can induce mitochondrial $Ca^{2+}$ uptake from ER $Ca^{2+}$ release and extracellular $Ca^{2+}$ influx, which in turn might activate NLPR3 inflammasome. Interestingly, it has been proposed that $K^+$ efflux possibly by mediating $Ca^{2+}$ influx, and phagolysosomal rupture likely by triggering ER $Ca^{2+}$ release, contribute to NLPR3 inflammasome stimulation (Murakami, et al. 2012). However, the calcium mobilization by itself could not induce caspase-1 activation, indicating its secondary role in NLPR3 activation.

In conclusion, the aforementioned data could provide a rationale for a reconciling mechanism and a hypothetic model that links all the mitochondrial mechanisms could be proposed. Mitochondrial potassium transport, which is highly regulated to sense the alterations in the cytosol and mitochondria, is essential for maintaining mitochondria homeostasis and mediating ROS production for the activation of cell signaling (Garlid and Paucek 2003). Potassium senses the changes in membrane potential, which regulate mitochondrial $K^+$ cycle (Garlid and Paucek 2003), and promotes calcium mobilization. The modification in membrane potential also provides a marker for mitochondrial damage as it induces mtROS generation. Moreover, high membrane potential, high extracellular $K^+$ and inhibition of mitochondrial channels (VDAC) are shown to impair NLPR3 inflammasome activation. These observations demonstrate that a disruption in mitochondrial homeostasis is crucial for activating NLPR3 inflammasome.

In summary, a stress signal that induces loss of membrane potential and $K^+$ efflux, triggers mtROS production. mtROS can result in oxidized mitochondrial DNA, an apoptotic byproduct, and in combination with the $Ca^{2+}$ and the decrease in the $\Delta\Psi_m$ trigger the opening of MPT pore. The opening of the MPT pore in turn induces

apoptotic signals and promotes the mtDNA release in the cytosol. Then, the cytosolic oxidized mtDNA binds to NLPR3 and seems to recruit the assembly of the inflammasome (Fig.2).

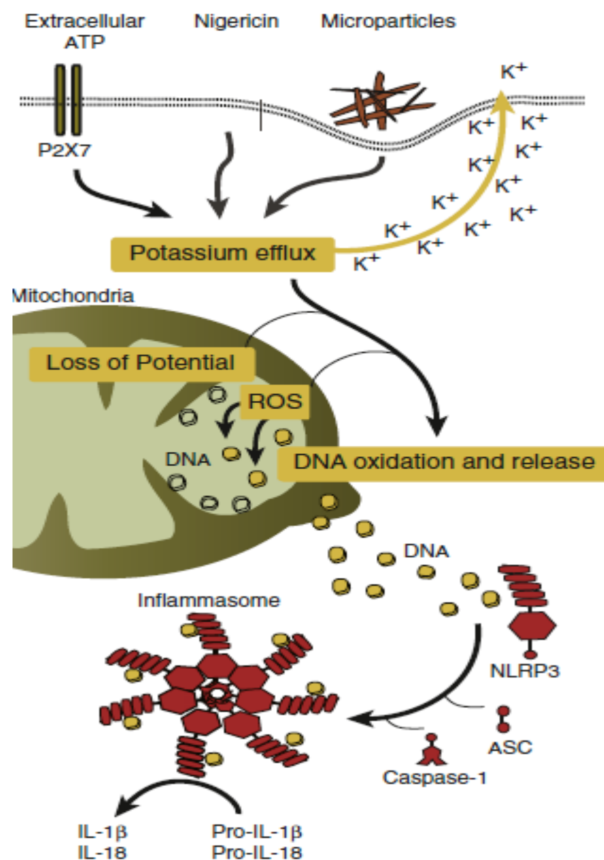


**Figure 2. Model of NLPR3 assembly and activation**. NLPR3 activators induce potassium efflux and loss of mitochondrial membrane potential, which induce mtROS production. As a result, oxidized mtDNA is reelased in the cytosol and binds to NLPR3. This promotes inflammasome assembyand activation, which leads to IL-1β secretion. *Martinon 2012*

However, there are still many questions posed about the underlying mechanism of NLPR3 inflammasome activation.  It is still obscure how NLPR3 can sense such a wide range of activators. Does it interact with this variety of stimuli directly or does it recognize a common signal that all the activators induce? Nevertheless, it seems unlikely that it could detect all those pathogens and stress signals separately and a common pathway that mediates its activation might exist. Moreover, it has been demonstrated that in resting conditions NLPR3 is localized in the ER. However, this position of NLPR3 could not explain how NLPR3 can detect the cytosol for danger signals. Is it possible that NLPR3 is located in the membrane of ER in a way that can detect the cytosol or is there an indirect pathway involved in the recognition of various stimuli and the priming of NLPR3? Further elucidation of the exact localization of NLPR3 in ER and the underlying mechanism by which detects all the signals, should be provided.

Furthermore, it is uncertain if oxidized DNA participates in NLPR3 priming or it initiates NLPR3 inflammasome activation. Further research into the participation of mtDNA will enhance the insight into its role on NLPR3 inflammasome. Moreover, the role of TXNIP in NLPR3 inflammasome activation is still debated. Could TXNIP be a co-activator factor that enhances the NLPR3 inflammation activation or it is necessarily needed for its activation? It is perceived the necessity of investigating further how the NLPR3 activators that bind to NLPR3, such as mtDNA and TXNIP, are implicated in the activation of NLPR3 inflammasome and in which step of the activation take place. To address these questions we need to examine more thoroughly all the mechanisms of inflammasome activation and further experiments in the presence of both oxidized mtDNA and TXNIP could be performed.

Moreover, a better understanding on the underlying mechanism by which mitochondria regulate apoptosis or the inflammatory response, is required. Although it is demonstrated clearly in Shimada et al. (2012) study that apoptosis induces NLPR3 inflammasome activation, it is crucial to shed light on the mechanistic link between cell death and IL-1β secretion. Moreover, it is important to understand the way that mitochondria distinct between those pathways. These findings could provide us a new insight on how mitochondria are involved in the inflammatory diseases and could provide a new therapeutic target.

We could briefly conclude that mtROS is a potent activator of NLPR3 inflammasome, as the inhibition of mtROS leads to inflammasome impairment. Robust generation of mtROS either by the inhibition of autophagy and mitochondrial channels or by the stimulation of the respiratory inhibitors or by the other pathways aforementioned, show ROS-dependent NLPR3 inflammasome activation. Thus, we could support that the ROS is necessary for caspase-1 activation and it could provide a useful therapeutic target in order to prevent excess inflammation.

In light of new data demonstrating that mitochondrial components and mitochondrial dysfunction are linked with inflammatory responses, it is of great interest to investigate further how mitochondria are involved in autoinflammatory diseases. Moreover, the implication of mitochondria in NLRP3 inflammasome activation

emerges the potential participation of mitochondria in autoinflammation. In the following chapter we will discuss further how mitochondria are involved in some autoinflammatory diseases.

# Chapter 3

## Role of mitochondria in autoinflammatory diseases

The aforementioned data support that NLPR3 inflammasome senses mitochondrial damage and stimulate an inflammatory response by secreting IL-1β. Moreover, compelling new evidence suggests that the disturbance of mitochondrial integrity and homeostasis, accompanied by the up-regulated production of ROS seems to be involved in autoinflammatory diseases. Herein, we will provide the potential involvement of mitochondria in the progress of the autoinflammatory syndromes CAPS, NLPR12 –associated periodic fever syndrome and TRAPS.

CAPS are autosomal autoinflammatory diseases, which include FCAS, MWS and NOMID/CINCA and are characterized by NLPR3 gene mutations (Park, et. al 2012). FCAS is characterized by cold-induced fever and urticarial-like rashes and is considered the less severe form of CAPS. MWS is more severe manifestation of the disease and patients suffer additional sensory neural hearing loss and arthritis. Last, in NOMID/CINCA the inflammatory symptoms are almost continuous. Moreover, the patients suffer also from chronic meningitis, hypertrophic bone and cartilaginous lesions in the epiphyses of the long bones (Ozkurede, et al. 2011; Park, et. al 2012). The gain-of function mutations are identified in the NACHT domain (Masters, et al. 2009; Ozkurede, et al. 2011). CAPS patients demonstrate higher levels of IL-1β with more rapid kinetics of IL-1β secretion than the healthy donors. However, they show the same pattern of pro-IL-1β as the healthy controls, indicating that the elevated levels are not based on early maturation of pro-IL-1β (Tassi, et al. 2010). In addition, high baseline levels of ROS and impaired antioxidant response are reported. More specifically, in unstimulated CAPS monocytes the level of antioxidants is elevated and is reduced after LPS treatment. On the contrary, in the case of healthy controls the antioxidants are increased after LPS stimulation (Tassi, et al. 2010). One step further is reported, where IL-1 receptor antagonist (IL-1Ra) and IL-6 production are observed. IL-1Ra is a natural antagonist of IL-1β and inhibits IL-1β signaling by binding to IL-1 receptor (IL-1R) with higher affinity (Kanazawa 2012). The absence of IL-1Ra leads to deregulated amount of IL-1β. The necessity of this antagonist is illustrated by the genetic deficiency of IL-1Ra which leads to the autoinflammatory

disease deficiency of IL-1 receptor antagonist (DIRA) (Aksentijevich, et al. 2009). Carta et al. (2012) report that in addition to the elevated ROS generation, an impairment production of IL-1Ra and IL-6 is observed. Their model proposes that the combination of increased ROS production with the deregulated antioxidant response in CAPS patients, leads to mitochondria damage and lower protein synthesis in CAPS monocytes. As a consequence, an attenuated production of IL-1Ra and IL-6 is also reported (Carta, et al. 2012). All these data support that redox alteration is implicated in the progress of this autoinflammatory syndrome. Both NLPR3 mutation and ROS production contribute to inflammasome activation and elevated IL-1β secretion. Moreover, IL-1Ra reduction as an outcome of the oxidative stress results in more robust IL-1β signaling.

Furthermore, an autoinflammatory disease that shows clinical manifestations of CAPS diseases but with molecular defects in NLPR12 and not in NLPR3 gene, namely NLPR12 –associated periodic fever syndrome, demonstrates an alteration on IL-1β kinetics associated with redox imbalance (Borghini, et al. 2011; Jéru, et al. 2008). The patients with NLPR12 mutations do not demonstrate increased IL-1β secretion in the absence of stimuli. However, the levels of ROS and antioxidants are high at baseline and after TLR stimulation IL-1β signalling is augmented rapidly (Borghini, et al. 2011). The strong redox alteration, which leads to high kinetics of IL-1β production, suggests the importance of ROS in this autoinflammatory disease as well. Nevertheless, in this study the source of ROS is not defined, so we cannot conclude that mitochondria participate in this autoinflammatory disease. Further research should be conducted to specify the source of ROS that participate in the progress of this syndrome.

Moreover, it has been demonstrated that mitochondrial reactive oxygen species are involved in TRAPS syndrome. TRAPS is an autosomal dominantly inherited autoinflammatory disease (Hull, et al. 2002). It is characterized by recurrent episodes of fever, myalgia, rash, abdominal pain, conjunctivitis and amyloidosis, as the most serious long-term effect (Hull, et al. 2002). TRAPS is caused by missense mutations of the tumor necrosis factor receptor (TNFR1, also known TNFRSF1A) (McDermott, et al. 1999). On the grounds that mitochondrial ROS can induce MAPK activation and that mtROS have been implicated in autoinflammatory diseases, Bulua et al. (2011)

studied the role of mtROS in TRAPS patients. The baseline levels of ROS were detected high due to an intrinsic feature of TRAPS and not as an outcome of the disease. Mutant TNFR1 cells report high mitochondrial respiration, which results in elevated ROS production. After the administration of scavenger of ROS, a decline in JNK and p38 phosphorylation is observed (Bulua, et al. 2011). Thus, these compelling data demonstrate that mitochondrial ROS is the source that induces the inflammatory response in TRAPS and that mitochondria participate in the development of this syndrome.

Although there is a lot of evidence supporting that mitochondria and redox alterations are implicated in autoinflammatory diseases, there are still many questions to be addressed. Firstly, even though it is shown that redox impairment participates in CAPS diseases and NLPR12 mutations, it is not clear if the source of augmented ROS is the mitochondria. Further research needs to be conducted in order to elucidate the participation of mitochondria in the pathophysiology of these diseases. However, the importance of redox homeostasis in the progress of these autoinflammatory diseases might demonstrate a potential involvement of mitochondria, as it is already been proved that mitochondria initiate inflammation and mtROS is a potent activator of IL-1β secretion.

Moreover, it is still obscure why these patients (CAPS, NLPR12- associated periodic fever syndrome and TRAPS) indicate high basal levels of ROS. It is important to understand the underlying mechanism by which ROS is elevated in order to understand the onset of the unprovoked inflammation on these patients. To our knowledge that ROS generation is involved in the progress of the specific autoinflammatory diseases, providing us with a new important therapeutic target.

Nevertheless, besides mitochondria we should also take into consideration the role of the ER on IL-1β secretion and generally on the development of autoinflammatory diseases. Misfolding proteins and metabolic stress can provoke uncontrolled ER stress and depending on the tissue and the disease can induce either cell death or inflammatory response (Park, et al. 2012). Moreover, in pathologic conditions calcium release during ER stress might induce mitochondrial damage, which activates NLPR3 inflammasome (Brookes, et al. 2004; Murakami, et al. 2012). Moreover,

NLPR3 in resting conditions localizes in the ER and the activation takes place in the MAMs, the part of the ER that communicates with mitochondria (Zhou, et al. 2011), indicating an involvement of the ER in NLPR3 inflammasome activation. Additionally, in the autoinflammatory syndrome TRAPS misfolded proteins are accumulated in the ER, which can stimulate inflammatory response of MAPK signaling (Bulua, et al. 2011). All these important observations indicate the involvement of the ER in inflammatory responses and autoinflammation through the communication with mitochondria. This striking evidence suggests that mitochondria might be the mediators and not the cause in the progress of autoinflammatory diseases. These data also propose that different signaling pathways might be involved in the autoinflammation by regulating mitochondrial function. It is crucial to study the exact role of the ER in autoinflammatory diseases more thoroughly. Moreover, it is essential to re-examine the function of mitochondria in autoinflammation, which for the last years are centrally positioned for IL-1β secretion.

On the grounds that the most of the autoinflammatory diseases have a high risk of developing amyloidosis, we should study how those amyloid deposits are involved in the pathogenesis of the syndromes. Compelling evidence demonstrate the involvement of amyloidosis in other diseases as well, such as in diabetes type 2 and Alzheimer disease (AD). More specifically, it is shown that amyloid deposits (islet amyloid poypeptide-IAPP) in the pancreas of T2DM, trigger NLPR3 inflammasome activation and IL-1β secretion and therefore pancreatic β cell death (Masters, et al. 2010). Amyloid accumulation (Amyloid beta-Aβ) is also shown in the neurodegenerative disease AD, where the amyloid precursor protein (APP) and amyloid deposits cause impaired mitochondrial dynamics by altering mitochondrial fusion/fission and lead to mitochondrial to mitochondrial damage (Wang, et al. 2008). NLPR3 inflammasome senses Aβ and triggers its activation through phagocytosis of the amyloids, which trigger lysosomal leakage and cathepsin B release (Halle, et al. 2008). Moreover, Leuner et al. (2012) have demonstrated that mitochondrial ROS is responsible for increased APP processing. Then, the overproduction of Aβ results in mitochondrial dysfunction and elevated ROS levels which in turn provoke inflammation and attribute to the development of AD (Leuner, et al. 2012). The involvement of amyloids in so many different diseases stresses its potent role in inflammatory responses. It is essential to understand how amyloid deposition

provokes mitochondrial impairment and NLPR3 inflammasome activation and how it is implicated in autoinflammatory diseases. In addition, the amyloids could provide a useful connection between autoinflammatory diseases, mitochondrial dysfunction and NLPR3 inflammasome activation without necessarily the presence of NLPR3 mutations. A better understanding in the underlying mechanism by which amyloids activate NLPR3 inflammasome and mitochondrial impairment, will provide us an answer in the progress of autoinflammatory diseases and could give a new insight on the underlying mechanism of severe autoinflammation. It will be crucial to make clear if amyloid participate in the onset of the disease or in the end stage when severe amyloidosis is usually expressed. If they start being phagocytized in the onset of the disease, then according to the model of Leuner et al. (2012) they might contribute to high levels of mtROS. Thus, we could explain the elevated mtROS that patients with those syndromes indicate. Amyloids as a therapeutic tool could prevent the severe form of autoinflammation.

In conclusion, according to all the data provided in this overview, an association between mitochondria, autoinflammatory syndromes and NLPR3 inflammasome activation is observed. Patients with elevated basal ROS levels after weak stimuli will demonstrate robust ROS production, which in turn will induce NLPR3 inflammasome activation and will trigger inflammatory response. This inflammatory response takes place concomitantly with the inflammatory cascade induced by the mutations of the specific autoinflammatory syndrome and leads to unprovoked strong inflammation.

It is unveiled that mitochondria exert their general role as “energy and cellular powerhouses” and they participate in the innate immunity. Disruption in the mitochondrial integrity could lead to the inflammatory responses. However, many questions still need to be clarified. For instance, are the mitochondria mediators or the source of NLPR3 inflammasome activation? How does NLPR3 sense such a wide range of pathogens and stress signals, and which is the exact mechanism that causes the activation? Why do the patients with autoinflammatory diseases demonstrate high basal ROS levels, which induce inflammation? Thus, further research in the underlying molecular mechanisms by which mitochondria ascribe to NLPR3 inflammasome activation and the progress of autoinflammatory diseases, is necessary

to be conducted for an in depth knowledge and full elucidation of mitochondrial-mediated autoinflammation.

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# Abbreviations

- Pattern recognition receptors (PRRs)
- Pathogen-associated molecular patterns (PAMPs)
- Danger-associated signals (DAMPs)
- High-mobility group protein B1 (HMGB1)
- Toll-like receptors (TLRs)
- C-type lectin receptors (CLRs)
- Retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs)
- Nucleotide-binding oligomerarization domain (NOD)-like receptors (NLRs)
- Interleukin (IL)-1β
- Cryopyrin-associated periodic syndromes (CAPS)
- Familial cold autoinflammatory syndrome (FCAS)
- Muckle-Wells syndrome (MWS)
- Neonatal-onset multisystem inflammatory disease (NOMID)
- Familiar Mediterranean Fever (FMF)
- Hyperinmmunoglobulinemia D with periodic fever syndrome (HIDS)
- Type 2 diabetes mellitus (T2DM)
- Central-nucleotide binding domain (NACHT)
- Caspase recruitment domain (CARD)
- Pyrin domain (PYD)
- Bacilovirus inhibitor repeat (BIR)
- Adaptor protein apoptosis-associated speck-like protein (ASC)
- Reactive oxygen species (ROS)
- Mitochondrial ROS (mtROS)
- Monosodium urate crystals (MSU)
- Bone marrow derived macrophages (BMDMs)
- Chronic granulomatous diseases (CGDs)
- Thioredoxin (TRX)
- TRX-interacting protein (TXNIP)
- Superoxide dismutase 1 (SOD1)
- Mitochondrial DAMPS (mtDAMPS)

- Mitochondrial DNA (mtDNA)
- Oxidative phosphorylation (OXPHOS)
- Tumour necrosis factor (TNF) receptor-associated periodic syndrome (TRAPS)
- Endoplasmic reticulum (ER)
- Mitochondria associated ER membranes (MAMs)
- Voltage dependent anion channel (VDAC)
- Mitochondrial permeability transition (MPT)
- 3-methyladenine (3-MA)
- LC3B (microtubule-associated protein 1 light chain 3B)
- Vitamin $D_3$ up-regulated protein 1 (VDPU1)
- Lysosomotropic peptide (Leu-Leu-OMe)
- Membrane potential ($\Delta\Psi_m$)
- Mitochondrial antiviral signaling protein (MAVS)
- Interferon response factors (IRF)
- Interferons (IFNs)
- Mitofusins (Mfn)
- *Chlamydia Pneumonia* (CP)
- IL-1 receptor (IL-1R)
- IL-1 receptor antagonist (IL-1Ra)
- Deficiency of IL-1 receptor antagonist (DIRA)
- Alzheimer disease (AD)
- Islet amyloid poypeptide (IAPP)
- Amyloid beta (Aβ)
- Amyloid precursor protein (APP)

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