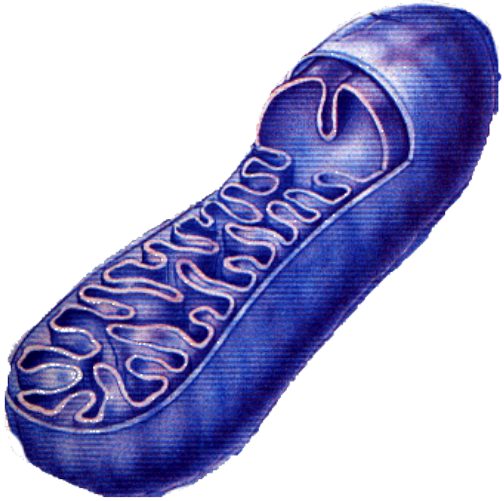
Thioredoxin(TRX) oxidation mediated by mitochondrial ROS is a key event in NLRP3 Inflammasome activation



$TRX\text{-}(SH)_2$

$TRX\text{-}S_2$

A review on the role of mitochondria and redox reactions in NLRP3 inflammasome activation.

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Abstract

The involvement of reactive oxygen species (ROS) in the NLRP3 inflammasome activation has sparked a lot of controversy in literature. This controversy can be explained by the usage of different experimental setup conditions due to the differing redox states of the most commonly used cell types. Mitochondria, ROS and the antioxidant response are key players in the activation cascade of the NLRP3 inflammasome. Thioredoxin (TRX) is of paramount importance in the regulation of the NLRP3 inflammasome and regulates the pro-IL-1β production as well as the activation of the inflammasome. Finally its reducing capacities also enable it to regulate the activity of caspase-1 by reducing oxidized cysteine residues. Literature data has been compiled to proposed a model for NLRP3 inflammasome activation in response to adinosinetriphosphate (ATP) and lipopolysaccaride (LPS). In this model the central role of TRX oxidation links mitochondrial ROS with the activation of the NLRP3 inflammasome. Finally this model has been used to better understand NLRP3-inflammasome-associated diseases. With this understanding several novel potential disease-causing genes have been identified.

Introduction

A complex multicellular organism like a human is at constant risk from endogenous and exogenous hazards. Pathogens, toxins, cellular damage from reactive oxygen metabolites (ROS) or ultraviolet radiation and many other factors can all pose a danger to survival for both the human individual and the species. During years of continuous exposure a regulatory mechanism evolved to keep these risk factors at bay. The human species has acquired a multitude of systems which increase our chances against the aforementioned endogenous and exogenous dangers. One of these systems is the innate immune system, often heralded as the first line of defense against exogenous pathogens. In addition to defending against pathogens the innate immune systems also functions as a general monitor system of cellular stress and damage. An important tool utilized by the innate immune system in recognition of hostile pathogens or endogenous danger signal are the germline encoded pattern recognition receptors (PRRs). These receptors recognize highly conserved microbial markers called pathogen-associated molecular patterns (PAMPs) and cellular danger signals called danger-associated molecular patterns (DAMPs). Important intracellular PRRs of the innate immune system are the NOD-like receptors (NLRs). The NLR protein family contains the proteins NLRP3, NLRP1, and NLRC4 amongst others. Another important PRR is absent in melanoma 2 (AIM2) The NLR proteins and AIM2 form a multi protein complex called the inflammasome in response to various PAMPs and DAMPs. This inflammasome is a very crucial and important protein complex. It enables the processing pro-caspase-1 into caspase-1. This caspase cleaves pro-Il-1β and pro-Il-18 into their biologically active forms. Il-1β is one of the most potent pro-inflammatory cytokines within the human body. A Different Inflammasome complex is formed depending on the PAMP or DAMP that triggered its formation. Each PRR reacts to different stimuli.

Inflammasome Structure

The NLR proteins share a basic structure. They contain an N-terminal protein-binding domain which can either be pyrin domain (PYD) in the case of NLRP1 and NLRP3 or a caspase recruiting domain (CARD) in the NLRC4. They also contain a nucleotide binding domain called NACHT and a C-terminal leucin-rich repeat domain (LRR), a domain frequently involved in protein-protein interactions (1). The AIM2 protein has a different structure. It contains a double stranded DNA binding domain called HIN-200 instead of the aforementioned LLR and NACHT domains (**Figure 1**).

In order to function properly NLRP3 and AIM2 rely on the adaptor protein, apoptosis associated speck-like protein containing a CARD (ASC). This ASC contains a CARD domain necessary for binding pro-caspase-1. Binding of pro-caspases-1 enables these pro-caspases to autoproteolytic cleave and become active caspase-1. NLRC4 and NLRP1 on the other hand already contains a CARD domain and therefore doesn't necessary need ASC. NLRP1 does have a PYR domain and can therefore still recruit ASC. NLRC4 is also unique in that it can activate pro-capase-1 without autoproteolytic cleavage and does not form a large cytosolic complex (**Figure 1**)(2).

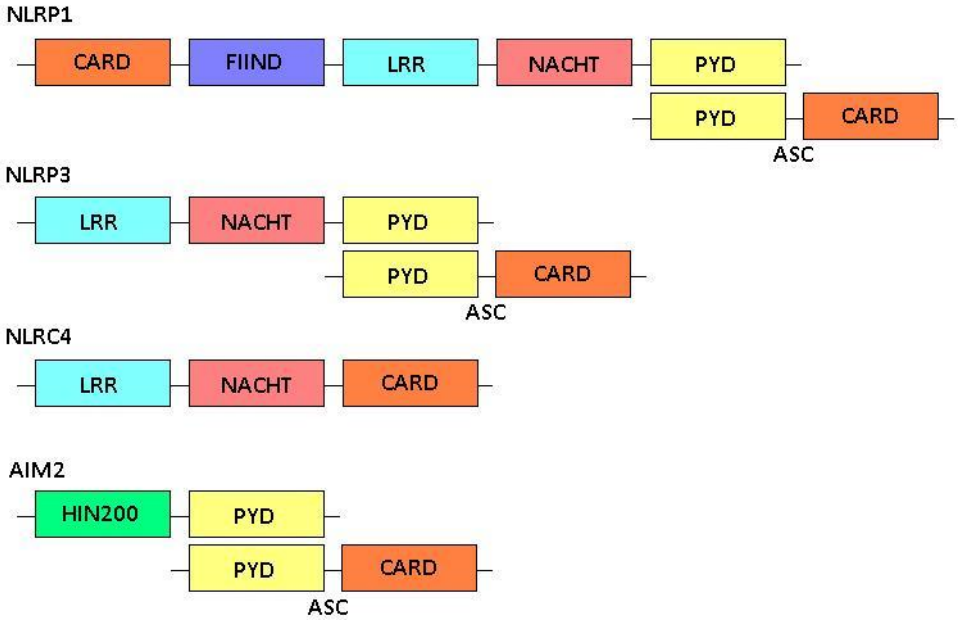


Figure 1. Different inflammasomes and their structural domains. NLRP3 forms a cytosolic multi-protein complex with ASC in order to contain a CARD. This CARD enables autoproteolytic cleavage of pro-caspase-1.

The NLRP3 Inflammasome

Of these different inflammasomes the NLRP3 inflammasome is the most widely studied. The NLRP3 inflammasome is associated with the cryopyrin-associated periodic syndromes (CAPS). CAPS are NLRP3-related periodic fever diseases. The most prominent CAPS are the familial cold autoinflammatory syndrome (FCAS), the Muckle-Wells Syndrome (MWS) and the neonatal onset multisystem inflammatory disease (NOMID). Each disease causes similar clinical manifestations however the severity of the symptoms varies.

Multiple genetic mutations associated with the NLRP3 inflammasome have been identified in these CAPS patients. Over 70 disease-associated mutations have been identified so far. However only 40% of patients with clinical symptoms of CAPS has a known mutation in the NLRP3 coding region (3). The remaining patients do show clinical signs of CAPS, but their underlying genetic mutations have not yet been discerned. Without a known genetic mutation conclusive diagnosis of these patients remains problematic. Thus finding novel mutations associated with NLRP3 regulation and activation can help with the proper diagnosis of CAPS.

CAPS patients' disease manifestations are probably caused by a lower inflammasome activation threshold (3). In order to discover novel disease-causing genes it is paramount to understand the regulation of inflammasome activation in molecular detail. Important players in the molecular mechanism of activation could potentially be mutated in CAPS patients and cause a lowered inflammasome activation threshold. Genes of these activation cascade components are therefore interesting screening targets for novel disease-causing genes.

The NLRP3 inflammasome is the most widely studied of the inflammasomes. We know a lot of about its role in disease and numerous mutations have been identified. Consequently studying the NLRP3 inflammasome is both clinically relevant and diagnostically promising. This review aims to provide a clear cut picture of recent advances in the molecular mechanism of the NLRP3 inflammasome activation. It will emphasize the role of redox signaling and the mitochondria in the NLRP3 activation cascade. Based on the elucidated activation cascade components a NLRP3 inflammasome activation model will be constructed. This model will then be used to identify potential candidates for the screening of novel disease-causing genes.

Molecular mechanism of the NLRP3 inflammasome

In order to gain a better understanding of the inflammasome it is important to understand its normal function and role within the human body. The NLRP3 inflammasome is foremost a molecular platform involved in the maturation of pro- Il-1β. Because Il-1β is such a potent pro-inflammatory cytokine its release requires two distinct signals for its production (4). The first signal is priming of the inflammasome and triggers the up regulation of pro- Il-1β and NLRP3. The second trigger is the activation of the inflammasome (5).

The NLRP3 Inflammasome has a wide array of activation triggers including but not limited to: Extracellular adenosinetriphosphate (ATP), lipopolysaccharide (LPS), nigericin, uric acid, asbestos, crystalline silica (6,7,8). The more in-depth molecular signaling mechanisms of NLRP3 activation after stimulation is relatively unknown. None of the aforementioned stimuli has been shown to directly associate with the NLRP3 inflammasome. So direct binding is excluded as a mode of action. Eventhough the stimuli are rather diverse it is probable that they at least share parts of a common pathway in activating the NLRP3 inflammasome.

The only discovered protein that directly interacts with the NLRP3 inflammasome is Thioredoxin Interacting Protein TXNIP (9). Furthermore oxidized mitochondrial DNA can bind the NLRP3 inflammasome (10). ROS and Potassium efflux are two other players that are essential for NLRP3 activation. High intracellular potassium inhibits inflammasome formation (11) and ROS blocker DPI inhibits the release of Il-1β (12). In addition it was discovered that the antioxidant response in reaction to ROS plays an important role (12).

Redox signaling

Because both ROS and antioxidants are involved in the activation, redox signaling might play an important factor in NLRP3 inflammasome regulation.
Redox signaling is a well recognized and important player within the cell and is involved in many intracellular signaling pathways. Important in ROS-signaling is the covalent modification of specific cysteine residues found within redox-sensitive target proteins. These cysteine residues can be oxidize, which can cause modification of activity (13).

One of the most important producers of intracellular ROS are the mitochondria, which produce superoxide anions at complex I and III of the cytochrome chain. There electrons derived from NADH or $FADH_2$ react with oxygen. However in addition to superoxide, hydrogen peroxide can also be directly generated by the mitochondria (14). Another important ROS producer in phagocytic cells is the NHDPH oxidase, which plays a key role during respiratory burst. There are also various other ROS producers, however their contribution to intracellular ROS production is relatively minor. It has been postulated that in inflammation especially mitochondrial ROS plays a very important function (15,16).

The Controversy of ROS.

Multiple studies (9,12,17) have emphasized the importance of ROS in NLRP3 inflammasome activation, however some controversy still remains. This controversy is partially caused by the difference in cell types used as a model during experiments. Diphenylene iodonium (DPI), a ROS inhibitor, used in several experiments is another reason for this controversy. Finally not only the involvement of ROS is disputed, but also the potential role of ROS regarding NLRP3 inflammasome activation. Another study (18) suggest that ROS plays an inhibitory role in the activation of NLRP3.

THP-1 vs freshly isolated primary monocytes.

Different cells types are used for NLRP3 inflammasome experiments. Two specific cell types are especially important because they are the most widely used and their basal redox state differs significantly. These are primary monocytes, isolated from healthy volunteers and THP-1 cells, a human acute monocytic leukemia cell line. Cell lines like THP-1 have a more reduced phenotype then primary monocytes (17). This has had major consequences on the results of several experiments. In these experiments the authors looked at the rate of IL-1β secretion after treatment with reducing agents and in response to thioredoxin (TRX) down modulation (8,9,12). In the primary monocytes treatment with an inhibitor of thioredoxin reductase or an inhibitor of superoxide dismutase 1 was found to inhibit IL-1β secretion (**Figure 2D**)(12). In THP-1 cells experiments with thioredoxin knock-down by shRNA and the addition of reductors showed opposite results (**Figure 2F**)(8,9).

This controversy can be explained by the different redox states of the cell types. First of all the more reducing environment of the THP-1 cells makes it harder for a PAMP to cause proper IL-1β release. The amount of intracellular pro-IL-1β after PAMP stimulation is 3-7 times higher in primary monocytes when compared with THP-1 cells (17). In addition IL-1β release by primary monocytes is also 15 to 40 times higher (**Figure 2A and 2C**)(17). Second both cell types respond differently to oxidative stress. At similar concentrations of H_2O_2 stimulation THP-1 cells showed an increase in IL-1β secretion, probably due to nullifying the reduced environment. Whereas primary monocytes showed a reduced IL-1β secretion, this effect is likely due to cytotoxicity (17). Third both cell types show a different anti-oxidant response to PAMP stimulation. TRX and SOD1, important anti-oxidants, are upregulated in PAMP activated primary monocytes. In contrast there is no upregulation in PAMP stimulated THP-1 cells. It is important to note that unstimulated THP-1 cells do already express very high TRX and SOD1 levels (17). In line with these results, cysteine release, an important component of the anti-oxidant response, was upregulated much stronger in primary monocytes then in THP-1 cells upon PAMP stimulation (17).

Similar to the THP-1 cells granulocyte-macrophage colony-stimulating factor (GM-CSF) cultured monocytes also show reduced IL-1β release in comparison to primary monocytes (19). These monocytes are primary monocytes cultured for 24-48 hours in GM-CSF. Further investigation revealed that these GM-CSF cultured monocytes also have a similar redox state to THP-1 cells (17). In response to PAMPs there was barely any increase in ROS levels and also the anti-oxidant response was lacking (17).

Inhibition of ROS by DPI.

Experiments, in which IL-1β release was inhibited by DPI have sparked a lot of controversy. Several researchers advocating the importance of ROS in NLRP3 inflammasome activation have attributed the IL-1β decrease to the reduction of ROS by DPI (12,17). However this is not widely accepted.

DPI is known for its effect as a NADPH oxidase inhibitor. Monocytes of patients with Chronic Granulomatous Disease (CGD) are incapable of NADPH–dependent ROS production due to a mutation in p46-phox (18). These CGD monocytes should therefore have a similar phenotype to DPI treated monocytes However it has been shown that these monocytes have normal caspase 1 activation and IL-1β secretion in response to PAMP stimulation. In addition these CGD primary monocytes showed a fourfold increase in IL-1β secretion in response to uric acid crystal stimulation when compared to primary monocytes from healthy volunteers (**Figure 2C and 2E**)(18). Finally DPI still reduces IL-1β secretion and caspase-1 activity in the CGD primary monocytes, even though these cells contain no active NHDPH oxidase (**Figure 2B**) Together these data suggest that DPI might have another means of suppressing IL-1β release independent of blocking NADPH oxidase.

DPI could affect pro- IL-1β levels, which in turn could lead to decreased IL-1β secretion in response to triggers. The extent to which this plays a role varies greatly amongst cells types. In the commonly used THP-1 cells DPI inhibits PAMP-induced pro- IL-1β synthesis. However in primary monocytes the synthesis reduction of pro IL-1β is only 20% (17).. Bis-chloroethylnitrosourea (BCNU) is a compound that blocks cysteine release and is a broad inhibitor of cellular oxidoreducatases (20). Experiments with BCNU have showed that IL-1β release can be inhibited without affecting pro- IL-1β levels in THP-1 cells (12). These findings suggest that there is an important role for ROS in IL-1β activation which is NADPH-independent. In support of this it has been shown that neutrophils in response to PAMPs can generate ROS in a NADPH-independent manner. The ROS generated in this experiment were subsequently identified as mitochondrial superoxides (21).

ROS generation is generally decreased in normal primary monocytes in response to DPI. This is due to the inhibition of NADPH. However in CGD primary monocytes DPI actually enhances superoxide and peroxide levels when compared to untreated CGD cells (**Figure 2B**)(21). This observation can be explained by the inhibition of mitochondrial complex I by DPI (22). Inhibition of the mitochondrial complex I by rotenone has shown a similar increase in mitochondrial superoxide generation (22).

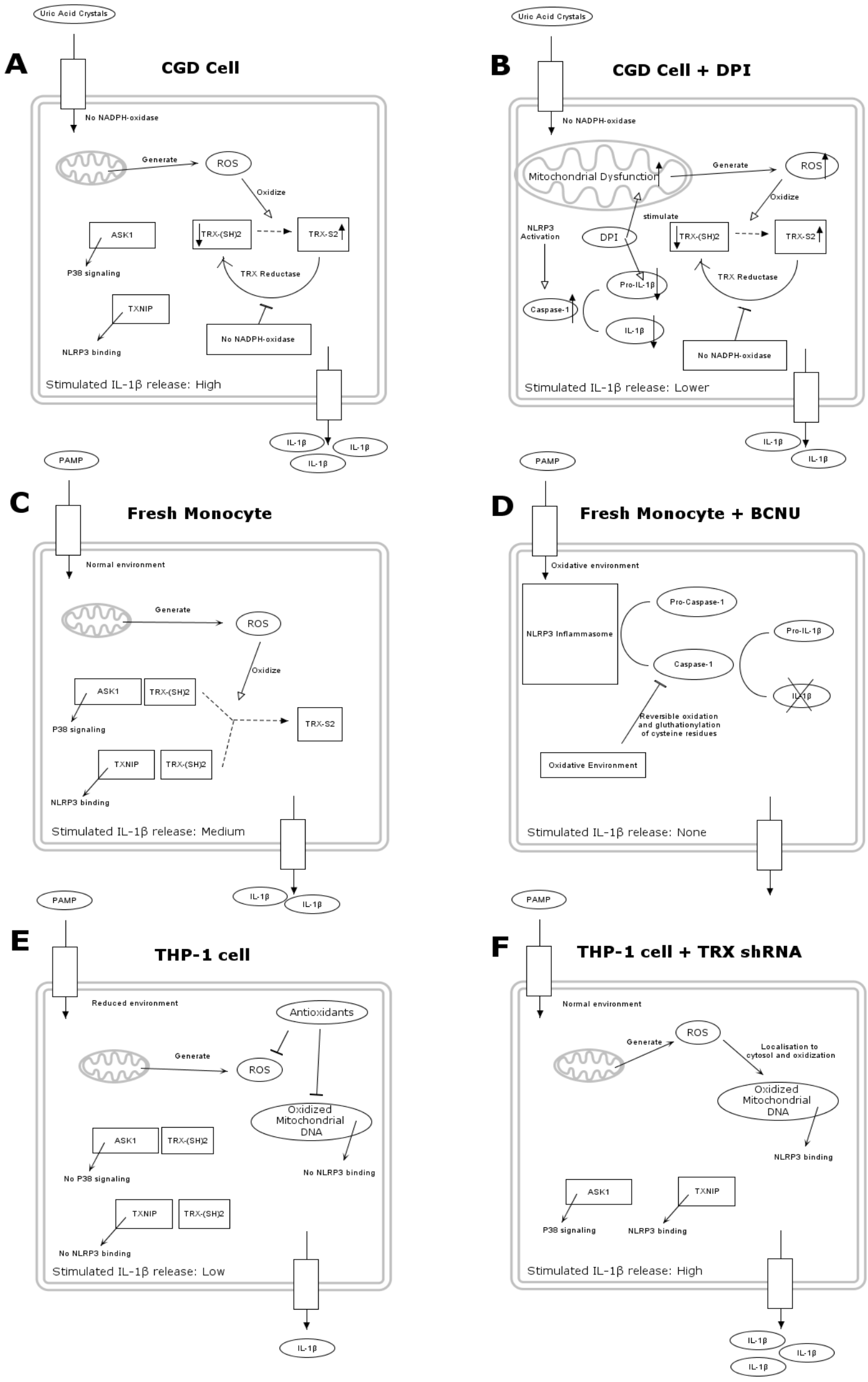
The role of ROS.

It has been postulated that ROS might actually decrease IL-1β release and inflammasome activation (18). The observation that CGD primary monocytes, which have less ROS production, have higher IL-1β release in response to uric acid stimulation further supports this statement (18). Furthermore SOD1 deficiency lowers PAMP stimulated IL-1β release (23). Because SOD1 reduces ROS, a SOD1 deficiency will result in superoxide over expression. Superoxide regulates the activation of caspase-1 by reversible oxidation of cysteine residues (23). ROS over expression leads to less active caspase-1 which subsequently leads to a decrease in IL-1β release. However this doesn't necessary exclude ROS as an activator of IL-1β. In the aforementioned CGD experiments caspase-1 activation and IL-1β maturation were normal in response to most PAMPs. Only in response to uric acid crystal stimulation was IL-1β release enhanced compared to

the controls (17). Interestingly enough in a human cell line model for CGD patients it was also observed that IL-1β release was enhanced in response to asbestos and silica crystals (8). Another experiment in a CGD mice model reported a contradicting observation (23). Macrophages of cytochrome b-deficient mice express a similar phenotype to those of CGD patients. The ROS production, caspase-1 activation and IL-1β maturation of Cytochrome b-deficient macrophages after PAMP stimulation is similar to wild type mouse macrophages (23). This indicates that NADPH-derived superoxide is not involved in IL-1β release and caspase-1 activation (23). The aforementioned IL-1β release after uric acid, asbestos and silica crystals stimulation could instead be a stimulus specific effect. In support of this all the stimuli share a crystalline structure. As even carbon nanotubes are capable of activating the NLRP3 inflammasome through a mechanism similar to asbestos (24). Meissner et al. (23) show the importance of SOD1 in the regulation of IL-1β release. However in additional experiments they observe that shifting the cellular redox potential towards oxidation similarly decreases the IL-1β release (**Figure 2C +2D**) in wild type macrophages. Furthermore the importance of reduction in the activation of the NLRP3 inflammasome has also been postulated by Tassi et al. (12), who observed that treatment with an inhibitor of TRX reductase inhibits IL-1β secretion. (**Figure 2D**)

There still remains a lot of controversy surrounding ROS' involvement in the NLRP3 inflammasome activation, however a lot of the discrepancies can be explain by the use of different model systems. Furthermore experiments concerning the use of DPI have controversial results, however similar experiments with other ROS inhibitors have confirmed the importance of ROS. Finally ROS is not only an activator of the NLRP3 inflammasome, but also a trigger for the subsequent anti-oxidant response. This anti-oxidant response is important for the proper activation of caspase-1.

Figure 2A. CGD cells have a heightened IL-1β release due to the lack of active NADPH-oxidase. TRX reductase is NADPH-dependant, and without NADPH oxidase TRX cannot be reduced. Upon stimulation this will cause a shift towards oxidized TRX. This results in more free TXNIP and ASK1 which leads to inflammasome activation and pro-IL-1β production. **2B.** DPI cause disruption of the respiratory complex I leading to increased mitochondrial oxide generation. This enhances the activation of the NLRP3 inflammasome leading to increased caspase-1 activation. DPI also affects pro- IL-1β production so due to lack of initial pro- IL-1β less IL-1β will be secreted. **2C.** Fresh monocytes have a relatively normal NLRP3 activation cascade. **2D.** Addition of BCNU to fresh monocytes inhibits a broad array of cellular reductases. This increases the cellular environment into an oxidative state. In this oxidative environment active caspase-1 becomes reversible oxidized and gluthathionylated which disables caspase-1's ability to process pro-IL-1β into IL-1β. **2E.** Normal THP-1 cells contain a more reducing environment. This environment makes it harder for PAMP stimulation to trigger a successful oxidative burst. Therefore IL-1β release is relatively low compared to fresh monocytes and CGD cells. **2F.** Inhibition of TRX frees TXNIP and ASK1, enabling their inflammasome activation and pro-IL-1β production mechanics. Furthermore the cellular environment is less reduced due to the TRX knockout. Mitochondrial DNA will locate to the cytosol and can become oxidized. This oxidized mitochondrial DNA will then bind and activate the inflammasome.


A
CGD Cell
Uric Acid Crystals
No NADPH-oxidase
Generate
ROS
Oxidize
ASK1
TRX-(SH)2
TRX-S2
P38 signaling
TRX Reductase
TXNIP
NLRP3 binding
No NADPH-oxidase
Stimulated IL-1β release: High
IL-1β
IL-1β
IL-1β
B
CGD Cell + DPI
Uric Acid Crystals
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Pro-IL-1β
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PAMP
Oxidative environment
NLRP3 Inflammasome
Pro-Caspase-1
Caspase-1
Pro-IL-1β
IL-1β
Reversible oxidation
and gluthationylation
of cysteine residues
Oxidative Environment
Stimulated IL-1β release: None
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Antioxidants
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ROS
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DNA
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TRX-(SH)2
No P38 signaling
No NLRP3 binding
TXNIP
TRX-(SH)2
No NLRP3 binding
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The Importance of mitochondrial DNA, Mitochondrial ROS and the subsequent Antioxidant response

Mitochondrial ROS and DNA

ROS derived from the mitochondria are an important activator in the inflammasome activation cascade (25). Nakahira et al. observed that mitophagy of ROS-generating mitochondria limits NLRP3 mediated caspase-1 activation in murine bone marrow derived macrophages (BMDM)(26). This shows that there is a link between the mitochondria and inflammasome activation. It has also been shown that mitophagy and autophagy blockade leads to the accumulation of ROS-generating mitochondria (16). These ROS-generating mitochondria in turn are capable of activating the NLRP3 inflammasome in THP-1 cells (16). Inactivated NLRP3 inflammasomes are normally located around the endoplasmic reticulum. When activated the NLRP3 inflammasomes and ACS traverse towards the perinuclear space and associate with mitochondria organelle clusters (16). Voltage-dependent anion channels (VDAC) play an important role in the production of mitochondrial ROS. Zhou et al. performed an experiment in which they down-regulated the expression of these VDAC by shRNA after which they stimulated the THP-1 cells with inflammasome activators. When VDAC1 and VDAC2 expression was reduced in these cells a reduction in IL-1β secretion and caspase-1 activation was observed. Another similar experiment which shows the relevance of mitochondrial ROS was performed by Nakahira at al. They inhibited mitochondrial ROS production with a specific mitochondria-targeted antioxidant, mito-TEMPO, in murine BMDM in response to ATP and LPS stimulation. This antioxidant causes a dose-dependent decrease in the release of IL-1β and IL-18 (26). These observations affirm that the mitochondrial ROS are an important component in the NLRP3 inflammasome activation cascade (16).

Not only mitochondrial ROS might be important in the activation of the NLRP3 inflammasome, but also mitochondrial DNA. Mitochondrial damage is often associated with various inflammatory diseases suggesting that there might be an important link to mitochondrial dysfunction and inflammation. Experiments have shown that mitochondrial DNA becomes translocated to the cytosol in response to ATP and LPS stimulation in bone-marrow derived macrophages (26). Furthermore this translocation is ROS and NLRP3 dependent (26). This cytosolic mitochondrial DNA enhances IL-1β and IL-18 release in response to ATP and LPS. In addition transfection of AIM2-deficient macrophages with mitochondrial DNA enhances IL-1β and IL-18 release in response to LPS and ATP stimulation (26). AIM2-deficient macrophages were used because AIM2 is another inflammasome that reacts in response to DNA and will subsequently produce IL-1β in an NLRP3 indepenent fassion. Because cytosolic mitochondrial DNA is a sign of mitochondrial dysfunction it is logical that it is involved in a danger sensing pathway such as the NLRP3 inflammasome. In support of this it has recently been established that oxidized mitochondrial DNA can bind the NLRP3 inflammasome (10).

The Antioxidant response

As noted in the introduction not only ROS is essential for NLRP3 inflammasome activation, but also the subsequent antioxidant response. Important components of the intracellular antioxidant response are SOD1, TRX/TRX reductase and glutathione (GSH) / GSH reductase amongst others. Another potent defense against oxidative stress is extracellular cystine uptake. Cystine is transported into the cell through the anionic amino acid transport system x_c^- (27). In order to diminish the oxidative stress a cell can take up additional extracellular cystine and reduce it intracellularly into cysteine. The cysteine is then released and causes a reduction of the extracellular medium.

Tassi et al. (12) have stimulated primary monocytes with NLRP3-specific PAMPs and studied the antioxidant response in reaction to ROS. They observed a noticeable increase in cysteine release after stimulation, whereas they barely noticed any changed in intracellular GSH levels. They also performed an experiment in which they inhibited cystine uptake through the x_c^- antiporter by adding L-glutamate to the culture. This inhibited the cysteine release by the monocytes, which in turn also decreased IL-1β secretion (12). This shows that cysteine release is an important mechanism part of the antioxidant response involved in the NLRP3 inflammasome activation.

Tassi et al. (12) also studied the role of TRX and TRX reductase in the antioxidant response. TRX was increased in response to NLRP3-specific PAMP stimulation in primary monocytes. They cultured PAMP-stimulated primary monocytes together with DNCB, a specific inhibitor of TRX reductase, strongly decreased IL-1β secretion and cysteine release (12). The experiment was repeated with BCNU, a broader inhibitor of intracellular oxidoreductases and this showed similar results (Figure 2D). In another experiment Tassi et al. reduced TRX expression by siRNA, this also resulted in a decrease in IL-1β release (12). These results show that TRX and TRX reductase play an important role in the cysteine release and IL-1β secretion from primary monocytes in response to NLRP3-specific PAMP stimulation. Interestingly the decrease in cysteine release can be rescued by adding extracellular Dithiothreitol (DTT), a reducing agent. The addition of exogenous DTT reverts the inhibition of IL-1β release by pharmacological compounds such as DNCB and BCNU (12). This experiment clarifies that the ability to reduce the extracellular environment is an essential component of NLRP3 inflammasome mediated IL-1β maturation.

Finally Tassi et al. (12) also elucidated the effects of SOD1 silencing in PAMP stimulated primary monocytes. Silencing of SOD1 by means of siRNA resulted in a remarkable decrease in IL-1β release. Meissner et al. (23) have shown that SOD1 plays a very important role in regulating caspase-1. In murine SOD1-deficient macrophages high superoxide levels cause reversible inhibition of caspase-1. This inhibition is due to reversible oxidation and gluthationylation of two redox-sensitive cysteine residues (23). Inhibition of caspase-1 by superoxide was reversible by both SOD1 and DDT. Because cysteine release has a similar reducing effect as DTT it could potentially also revert the oxidation and gluthationylation of cysteine residues on caspase-1.

Zhou et al. (9) have identified a binding partner of NLRP3. Using a yeast two-hybrid screen with the LRRs of the NLRP3 as a bait TXNIP was identified as a direct binding partner. The next section will elucidate how this protein and another binding partner, TRX, are involved and part of a key event in NLRP3 inflammasome activation.

TRX oxidation as a key factor in NLRP3 inflammasome activation.

Importance of the TRX oxidation

TRX and TXNIP play a very important role in NLRP3 inflammasome activation. TXNIP binds to the NLRP3 inflammasome in a ROS-dependent manner and is essential for IL-1β release (9). TXNIP also interacts with TRX, an essential reductor involved in IL-1β secretion. TRX is a complex protein, which has many other important cellular functions. Amongst its functions the regulation of the redox state (28) and inhibition of Apoptosis Signal-regulating Kinase 1 (ASK1) are the most relevant in regards to inflammation. In a reduced state TRX binds both TXNIP and ASK1 preventing the inflammasome activation cascade. However when TRX becomes oxidized by ROS the protein disjoins allowing TXNIP and ASK1 to become active. This is a mechanism through which ROS and the antioxidant response affect and regulate the NLRP3 inflammasome activation and subsequent IL-1β release.

TRX has a conserved catalytic site containing cysteine, this cysteine can become reversible oxidated to cysteine disulfide. This oxidized TRX (TRX-S_2) can subsequently be reduced back to reduced TRX (TRX-$(SH)_2$) by TRX reductase (29). The reduced form of TRX can catalyze the reduction of oxidized cellular proteins. The oxidation of reduced TRX takes depends on high superoxide concentrations. Because reduced TRX is capable of reducing oxidized cysteine residues it can also reduce the oxidized cysteine residues on caspase-1. Therefore TRX could play an important role in regulating caspase-1 activity. However binding of oxidized TXNIP to reduced TRX blocks the redox active site of TRX. I therefore hypothesize that TXNIP and TRX detachment in response to mitochondrial superoxide plays an important role in the activation of caspase-1

In addition TRX plays an important role in the regulation of the p38-MAPK-stress response pathway activity by binding to ASK1. In mice it has been shown that in response to LPS stimulation the TRAF6-ASK1-p38-MAPK is activated in a ROS-dependent manner (30). Furthermore it has been demonstrated in mice that mitochondrial generated ROS separated the TRX-ASK1 complex leading to p38 activation (31). P38 plays a very important role in the generation of pro-IL-1β (32). Furthermore in epithelial cells relocalisation of TRX to the nucleus occurs in response to superoxide, which in turn inhibits apoptosis (33). However binding of TXNIP to TRX in response to TNF-α translocates the complex to the plasma membrane and inhibits the anti-apoptotic function of TRX (34). It is interesting that in response to mitochondrial ROS both the interactions of TRX with ASK1 and with TXNIP are disrupted. In order to enable LPS induced p38 activation ASK1 needs to be activated; however this is not the case for the LPS induced activation of c-Jun N-terminal Kinase (JNK). With this in mind note that in response to LPS activation the p38 pathway plays a more important role than the JNK and ERK pathways in the regulation of pro-IL-1β and IL-1β (35). Regarding this data I postulate that TRX-ASK1 separation in response to mitochondrial ROS plays an essential role in the regulation of pro-IL-1β and IL-1β in response to LPS activation.

These mentioned observations suggest an interesting situation in which TRX and NLRP3 are competitors in regards to TXNIP binding. And TXNIP and ASK1 are competing for TRX binding. However in the later situation there is also additional competition from oxidized cellular proteins. When mitochondrial ROS levels rise more cellular protein cysteine residues will be oxidized. This in turn increases the chance of TRX exerting its antioxidant function instead of binding with TXNIP or

ASK1. This will free more ASK1, which should lead to an increase in p38 activation. Furthermore removing TRX as a competitor will ensue in more TXNIP and NLRP3 binding which activates the NLRP3 inflammasome. The activation of both p38 and NLRP3 should result in an increase in IL-1β release.

Upstream of the TRX oxidation

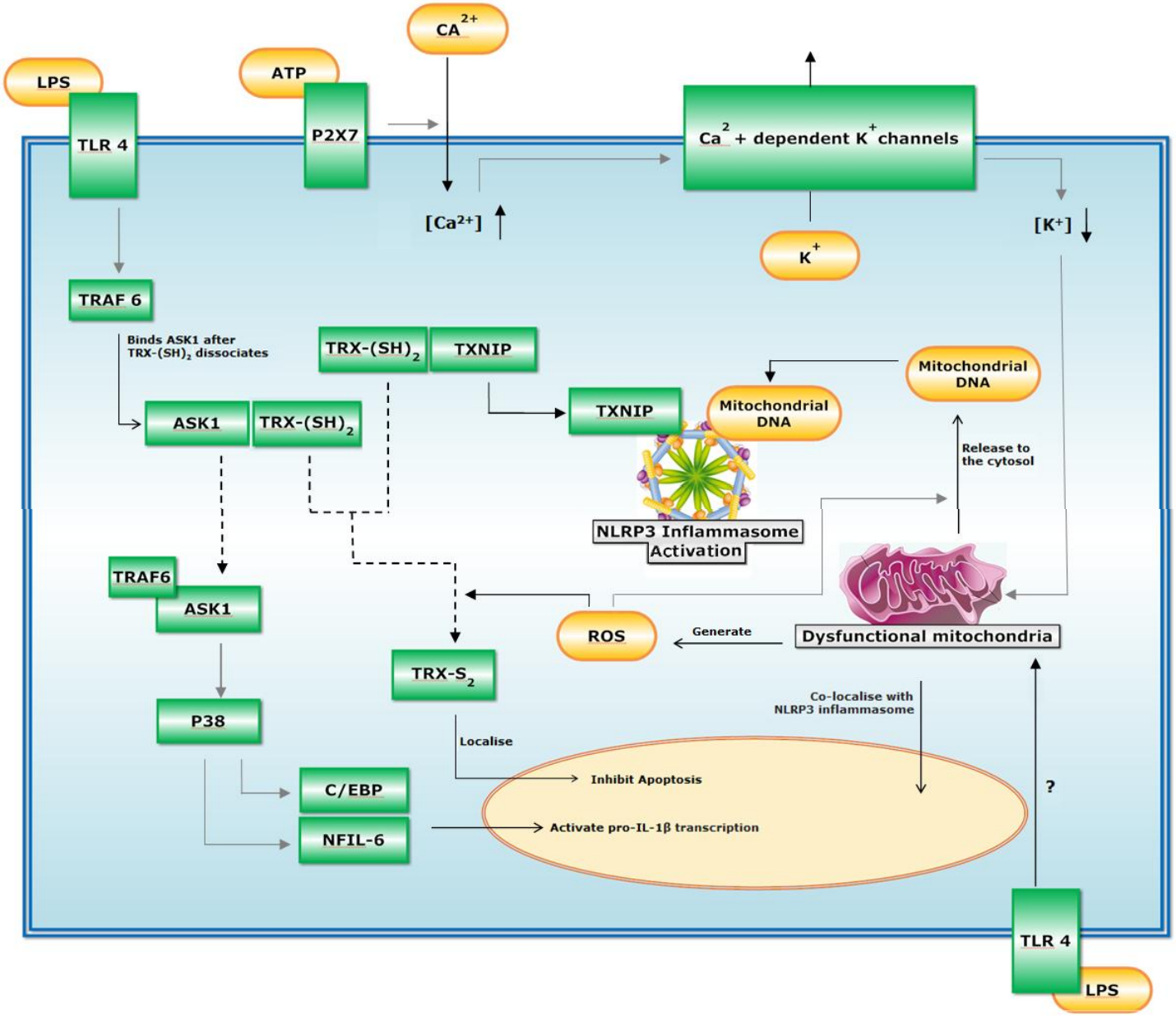
The importance of mitochondrial superoxide for NLRP3 activation is established. And one of the key downstream events is the oxidation of TRX. However it is also important to determine how mitochondrial ROS production is enhanced and regulated upstream of the TRX oxidation. As mentioned previously autophagy plays an important role in the regulation of mitochondrial ROS production. Dysfunctional mitochondria are removed via mitophagy, which results in less mitochondrial superoxide production and thus less IL-1β release. Depletion of autophagy proteins LCB3 and Beclin-1 increased the buildup of dysfunctional mitochondria in response to LPS and ATP stimulation (26). Abrogation of another autophagy protein, AL16L1, also confirmed that disabling autophagy enhances IL-1β release (36). Autophagy has also been suggested to play an important role downstream of NLRP3 activation by directly degrading pro-IL-1β. Autophagy is an important negative regulator of mitochondrial ROS production (37).

However in response to various PAMPs Mitochondrial ROS is upregulated. Challenge with the widely used NLRP3 associated PAMP, LPS, causes mitochondrial dysfunction and up-regulation of mitochondrial ROS production. Six hours after LPS stimulation in murine hepatocyte cells a remarkable decrease in respiratory complex 1 activity and ATP levels is observed. (38) Another finding regarding mitochondrial ROS production is that in neutrophils activation of the $Ca2^{+}$-activated potassium channels of small conductance (SK) and more specific SK3 results in enhanced mitochondrial ROS production and K^{+} efflux (21). I postulate that the K^{+} efflux caused by the SK3 channel is responsible for mitochondrial dysfunction, which in turn increases mitochondrial ROS production. In support of this it has been observed that inhibition of K^{+} efflux inhibits mitochondrial dysfunction and apoptosis in human neutrophils (39). Furthermore mitochondrial dysfunction caused by ATP can be abolished completely by the addition of extracellular potassium to compensate for the cellular potassium efflux (10).

The previous experiments focused on neutophils, however SK3 channels have also been found in rat microglial cells, immune cells of the central nervous system. In these cells SK3 channel mRNA is up-regulated in response to LPS stimulation and causes the microglia to undergo a change to a more neurotoxic state (40). It is also reported that these microglia release IL-1β upon LPS activation (41). At least in microglia LPS stimulation increases both SK channel mRNA and IL-1β release, potentially implying SK channels involvement in the regulation of IL-1β production. Further evidence linking SK channels and K^{+} efflux to inflammation is the observation that inhibition of both SK channels and their bigger equivalent Big Potassium channels (BK) completely abrogates LPS-induced mortality in mice (42). $Ca2^{+}$-activated potassium channels are not only restricted to neutrophils and microglia but are present in a plethora of immune cells amongst which also macrophages (43). The P2X7 receptor could be Upstream of these $Ca2^{+}$-activated potassium channels in the activation cascade. This receptor is involved in massive K^{+} efflux in response to ATP binding (44). Upon activation the P2X7 receptor causes a calcium influx. This calcium influx could subsequently trigger the $Ca2^{+}$-activated potassium channels, which in turn could cause the aforementioned massive K^{+} efflux. A similar interaction in which the P2X7 receptor activates SK3 channels has already been reported in cancer cells (45).

A model for LPS and ATP induced NLRP3 Activation

Based on the previously discussed data I would like to propose a model for NLRP3 inflammasome activation in response to ATP and LPS. LPS and ATP activate their respective receptors; TLR4 and P2X7. TLR activation leads to its downstream signaling cascade resulting in the activation of JNK, ERK and p38 and NF-κB. Especially p38 activation is important for the up regulation of pro-IL-1β. In addition this stress-signaling pathway will increase mitochondrial dysfunction and cause an increase in mitochondrial ROS production. P2X7 activation will lead to the influx of calcium. This will then activate the calcium-dependent potassium channels, such as SK3. K^+ efflux can then take place, which as explained before is necessary for proper NLRP3 inflammasome activation. The K^+ Efflux will also trigger mitochondrial dysfunction together with the initial LPS signaling. The NLRP3 inflammasome will co-localize around the perinuclear space near the dysfunctional mitochondria. The dysfunction will result in an increase in mitochondrial ROS production, which in turn enables mitochondrial DNA to be released into the cytosol. This oxidized mitochondrial DNA can then bind to the NLRP3 inflammasome. In addition the mitochondrial ROS release and subsequent anti-oxidant response will allow TRX-TXNIP and TRX-ASK1 to be separated by oxidizing the TRX. This Enables the activation of the ASK1-p38 pathway. TXNIP can now also bind to the NLRP3 inflammasome. These steps should activate the NLRP3 inflammasome (**Figure 3**). Moreover the reducing environment created by TRX, SOD1 and similar anti-oxidants will then also reduce the cysteine residues on caspase-1. This will enable its active form. Both active caspase-1 and the activated NLRP3 inflammasome will then enable pro-IL-1β to be processed into the active IL-1β.



Figuur 3. A model for LPS and ATP induced NLRP3 activation. Including K^+ efflux, NLRP3-TXNIP-binding, NLRP3-mitochondrial-DNA-binding and pro-IL-1β production. ATP leads to the activation of the P2X7 receptor. This subsequently leads to the activation of the Ca^{2+}-dependent K^+ channels. This in turn activates a K^+ efflux. This together with LPS stimulus causes mitochondrial dysfunction. Dysfunctional mitochondrial have increased ROS production. The released ROS triggers mitochondrial DNA leakage to the cytosol. Meanwhile reduced TRX reacts as an anti-oxidant to these ROS and oxidized cellular proteins and becomes oxidized TRX. Oxidized TRX separates from TXNIP and ASK1. The unbound ASK1 enables the LPS primed TRAF 6- ASK1-P38 pathway to activate. Which leads to the production of pro-IL-1β. Furthermore the freed TXNIP and cytosolic mitochondrial DNA will bind to the NLRP3 inflammasome and activate it. Finally the oxidized TRX translocates to the nucleus and inhibits apoptosis.

The CAPS monocytes IL-1β release kinectics differ from those of healthy monocytes. Therefore novel disease-causing genes screening targets need to evoke release kinectics similar to those in CAPS monocytes.

CAPS monocytes differ from healthy monocytes in their basic redox state and in their response to LPS stimulation.

There is important difference regarding CAPS monocytes and normal healthy monocytes in regards to their basal redox state. Unstimulated CAPS monocytes exhibit mild oxidative stress and elevated levels of ROS and anti-oxidants (46). More specifically, TRX was found up-regulated (46). In addition to the altered redox state also the unstimulated IL-1β secretion was different between healthy controls and CAPS monocytes. CAPS monocytes of patients not treated with IL-1β blockers were significantly higher than in healthy controls (46).

After 18hour stimulation with LPS, CAPS monocytes also exposed different behavior when compared with healthy controls. After stimulation the anti-oxidant response was greatly reduced in CAPS monocytes compared to unstimulated CAPS monocytes. Healthy monocytes on the other hand showed an enhanced anti-oxidant response when stimulated.

Another interesting find by Tassi et al. is that also the kinetics of the anti-oxidant response and the IL-1β secretion in response to LPS differ in CAPS monocytes (46). After approximately three hours of LPS stimulation CAPS monocytes already reach their maximum plateau of IL-1β secretion. Whereas healthy monocytes still show a steady increase around 18 hours post stimulation. The antioxidant response of CAPS monocytes also shows some interesting kinetics. After two hours of LPS stimulation CAPS monocytes express a high antioxidant response, however this quickly dwindles as time progresses (46). Contrary, healthy controls show once more a steady increase as time passes. Furthermore other inflammatory diseases such as systemic-onset Juvenile Idiopathic Arthritis (SoJIA) do not have the CAPS-typical accelerated IL-1β release (46). Although they do show a slight increase in basal anti-oxidant levels.

It is established that BCNU, an anti-oxidant inhibitor alters the IL-1β secretion kinetics of LPS-stimulated CAPS monocytes. IL-1β secretion was completely abolished in response to NRLP3 stimuli in BCNU-treated CAPS monocytes (46). This supports the hypothesis that also in CAPS monocytes a proper redox response is important for IL-1β secretion.

Tassi et al. (46) propose a model in which CAPS monocytes are continually expressing a heightened redox state. When LPS stimulation occurs an oxidative hit from the mitochondrial ROS takes place and the CAPS monocytes mount an antioxidant response. However due to the previously heightened redox state the response can't be sustained as long and thus the levels of TRX and other antioxidants will drop rapidly. The lack of TRX would mean that both TXNIP and ASK1 are unbound. Allowing NLRP3 inflammasome activation and pro-IL-1β production. Due to the strong initial anti-oxidant response caspase-1 can still be activated by reducing the oxidized cysteine residues.

An important facet of the rapid IL-1β release is the implication for the disease pathogenesis. A more rapid IL-1β release would mean that IL-1β secretion

reaches maximum level while control mechanisms might be barely activated (46). This could then cause clinical symptoms. Another possibility is that the heightened redox state allows for a weaker oxidative hit to trigger the NLRP3 cascade. There is already an increased amount of TRX present which could be oxidized by the mitochondrial ROS, which will cause the TRX to separate. In support of this weaker hit hypothesis is the observation that in CAPS monocytes IL-1β release is not upregulated in response to ATP and LPS stimulation compared to LPS stimulation only (47). Only the weaker single stimulation is needed for the CAPS monocytes to hit their maximum plateau IL-1β secretion. Yet the accelerated IL-1β kinetics don't correlate with disease severity or IL-1β secretion (46). There is also a third hypothesis: heightened redox state and oxidative stress causes more mitochondrial dysfunction, which in turn causes ROS more release and activates the inflammasome cascade.

A combination of these hypotheses would form an interesting model. Mutations in the NLRP3 inflammasome cause it to have a lower activation threshold. So a weak PAMP or DAMP could trigger the initial activation. The release of IL-1β would then signal autocrine, triggering mitochondrial dysfunction and subsequent ROS release. This would create a state in which the redox state is heightened. This heightened redox state then enhances the mitochondrial dysfunction once more. This idea is enforced by the observation that THP-1 cells transfected with disease-associated NLRP3 mutations, express mitochondrial damage in the majority of their cells, without expressing cleaved caspase-1 (48). This combined with the data from Tassi et al.(46) makes it creditable that CAPS monocytes are in a state in which there is a heightened redox state and mitochondrial dysfunction but not yet an enhanced NLRP3 inflammasome activation.

Because of the already high oxidative stress and mitochondrial dysfunction another external trigger such as LPS can easily trigger an accelerated IL-1β release due to the heightened redox environment and dysfunctional mitochondria. These together with the NLRP3 inflammasome can act in a positive feedback loop. This would result in small external triggers activating the inflammasome cascade in a stronger and more accelerated manner. The rapid IL-1β emission could then cause the clinical manifestations of CAPS.

Potential Screening targets for novel disease-causing genes

Using the model for CAPS monocytes and NLRP3 inflammasome activation as previously described. A couple of prerequisites for interesting targets emerge. First of all the mutation should either cause a heightened redox state or increased mitochondrial dysfunction. Either of those will suffice as one will result in the other. This enables the CAPS-typical rapid IL-1β release responsible for the clinical manifestations associated with CAPS. Second because the antioxidant response is still essential for proper caspase-1 activation the mutation should not disable the anti-oxidant response.

An interesting group of potential screening targets are the Ca^{2+}-dependent K^+ channels, such as SK1, SK2 and SK3. Gain-of-function mutations in these channels causing increased basal activation or mutations causing leakage can lead to K^+ efflux. This will subsequently result in increased mitochondrial dysfunction and mitochondrial ROS production.

Another potentially group are the redox proteins related to the TRX/TXNIP separation. Such as TRX reductase 1, 2 and 3, gluthatione reductase and TRX 2. However these proteins are involved in multiple important signaling pathways such as apoptosis. Thus mutations in these genes could possibly be embryonic lethal.

Another candidate for screening would be the VDAC in the mitochondria, VDAC-1 and VDAC-2. Gain-of-function mutations in these channels can lead to increased mitochondrial ROS production. Along this line of though other mitochondrial proteins involved in the respiratory chains might also be interesting targets for screening. As long as the mutations result in increased mitochondrial ROS production or mitochondrial dysfunction.

A final group of candidates are BCL-2 and auto- and mitophagy proteins. These proteins inversely regulate mitochondrial dysfunction. Therefore loss-of-function mutations in these proteins can increase mitochondrial dysfunction and cause a heightened redox state.

Screening these candidates could potentially reveal novel disease-causing genes. Especially the Ca^{2+}-dependent K^{+} channels deserve a mention. Not much is currently known about these channels in monocytes, however as mentioned in the model they probably play an important role in the regulation of the inflammasome.

Concluding remarks

This review has elucidated the literature controversy surrounding ROS and the NLRP3 inflammasome. Different experimental setups using different cell types have resulted in seemingly contradicting data and the interpretation of these experiments can differ based on one's perspective. Mitochondria, ROS and the antioxidant response play key roles in the activation of the NLRP3 inflammasome. TRX oxidation by mitochondrial ROS is a crucial event needed for NLRP3 activation and enhances both IL-1β secretion and pro- IL-1β production. Based on literature data I constructed a model for NLRP3 inflammasome activation in response to ATP and LPS stimulation. With the help of this model and data on CAPS monocyte IL-1β release kinetics (46). I have formulated an hypothesis on how CAPS can cause clinical symptoms and why CAPS monocytes exhibit an altered redox state. Finally I have used this CAPS monocyte hypothesis and my NLRP3 inflammasome activation model to identify potential targets for disease-causing genes.

As a final note I would like to propose the following experiment to confirm the key role of TRX oxidation in NLRP3 inflammasome activation. First CAPS patient monocytes will need to be isolated. These CAPS monocytes will then need to be altered by upregulating the TRX reductases expression or adding additional TRX reductase. This will cause the TRX-S_2 and TRX-$(SH)_2$ equlibrium to shift towards the reduced form. This should in turn increase the amount of TRX-bound ASK1 and TXNIP, which should raise the NLRP3 inflammasome activation threshold. After this modification these monocytes should be stimulated with LPS and a combination of LPS and ATP. The IL-1β release kinetics should subsequently be obtained in a similar fashion to the experiments preformed by Tassi et al. (46). In order to confirm the key role of TRX oxidation the altered CAPS monocytes should show much slower and more moderate IL-1β release kinetics then normal CAPS monocytes. Furthermore they should also show an increase in IL-1β release when comparing LPS and ATP stimulation with only LPS stimulation.

References

1. Kobe et al. (2001) The leucine-rich repeat as a protein recognition motif. Current Opinion in Structural Biology 11; 6:725-732.
2. Broz et al. (2010) Differential Requirement for Caspase-1 Autoproteolysis in Pathogen-Induced Cell Death and Cytokine Processing. Cell Host & Microbe 8; 6:471-483.
3. Aksentijevich et al. (2002) De novo CIAS1 mutations, cytokine activation, and evidence for genetic heterogeneity in patients with neonatal-onset multisystem inflammatory disease (NOMID): A new member of the expanding family of pyrin-associated autoinflammatory diseases. Arthritis Rheum 46:3340–3348.
4. Burns et al. (2003) New insights into the mechanism of IL-1β maturation. Current Opinion in Immunology 15; 1:26-30.
5. Bauernfeind et al. (2009) Cutting edge: NF-kappaB activating pattern recognition and cytokine receptors license NLRP3 inflammasome activation by regulating NLRP3 expression. Journal of Immunoly 183:787–791.
6. Mariathasan et al. (2006) Cryopyrin activates the inflammasome in response to toxins and ATP, Nature. 440(7081):228-32.
7.Martinon et al. (2006) Goutassociated uric acid crystals activate the NALP3 inflammasome. Nature 440:237-241.
8. Dostert et al. (2008) Innate immune activation through Nalp3 inflammasome sensing of asbestos and silica. Science 2008, 320:674-677.
9. Zhou et al. (2010) Thioredoxin-interacting protein links oxidative stress to inflammasome activation Nature Immunology 11:136–140.
10. Shimada et al. (2011) Oxidized Mitochondrial DNA Activates the NLRP3 Inflammasome during Apoptosis. Immunity 36; 3:401-414.
11. Dostert et al. (2009) Malarial hemozoin is a Nalp3 inflammasome activating danger signal. PLoS One 4:6510.
12.Tassi et al.(2009) Pathogen-induced Interleukin-1β Processing and secretion is regulated by a biphasic redox response, Journal of Immunology 183:1456-1462.
13. Finkel et al.(2011) Signal transduction by reactive oxigen species. Journal of Cell Biology 194:7-15.
14. Giorgio et al. (2005) Electron transfer between cytochrome c and p66Shc generates reactive oxygen species that trigger mitochondrial apoptosis. Cell 122:221–233.
15. Bulua et al.(2011) Mitochondrial reactive oxygen species promote production of proinflammatory cytokines and are elevated in TNFR1-associated periodic syndrome TRAPS. Journal of Experimental Medicine 208:519–533.
16. Zhou et al. (2010) A role for mitochondria in NLRP3 inflammasome activation. Nature 469:221-225.
17. Carta et al. (2011) The rate of IL-1βsecretion in different myeloid cells varies with the extent of redox response to toll-like receptor triggering. Journal of Biological Chemistry 5; 286:27069-27080
18. van der Veerdonk et al. (2010) Reactive oxygen species–independent activation of the IL-1β inflammasome in cells from patients with chronic granulomatous disease. PNAS 107: 3030-3033.
19. Neteaet al. (2009) Differential requirement for the activation of the inflammasome for processing and release of Il-1beta in monocytes and macrophages. Blood 113:2324–2335.
20. Gromer et al. (1997) The 58 kDa mouse seleno protein is a BCNU-thioredoxin sensitive reductase. FEBS Letters 412:318-320.
21. Fay et al. (2006) SK channels mediate NADPH oxidase-independent reactive oxygen species production and apoptosis in granulocytes. PNAS 103:17548-17553.
22. Hutchinson et al. (2007) Diphenylene iodonium stimulates glucose uptake in skeletal muscle cells through mitochondrial complex I inhibition and activation of AMP-activated protein kinase. Cell Signal 19:1610–1620
23. Meissner et al. (2008) Superoxide dismutase 1 regulates caspase-1 and endotoxic shock. Nature Immunology. 9:866-872.
24. Palomäki et al. (2011) Long,needle-like carbon nanotubes and asbestos activate the NLRP3 inflammasome through a similar mechanism. ASC Nano 5:6861-6870.
25. Sorbara and Girardin (2011) Mitochondrial ROS fuel the inflammasome. Cell Research 21:558-560
26. Nakahira et al. (2010) Autophagy proteins regulate innate immune responses by inhibiting the release of mitochondrial DNA mediated by the NALP3 inflammasome. Nature Immunology 12:222-230.
27. Bannai and Tateishi (1992) Role of membrane transport in metabolism and function of glutathione in mammals. Journal of Membrane Biology 89:1–8.
28. Yamawaki and Berk (2005) Thioredoxin: a multifunctional antioxidant enzyme in kidney, heart and vessels. Current Opinion in Nephrology and Hypertension. 14; 2 149-153
29. Lillig and Hoilgren (2007) Thioredoxin and related molecules—from biology to health and disease. Antioxid Redox signal 9:25-47
30. Matsuzawa et al. (2005) ROS-dependent activation of the TRAF6-ASK1-p38 pathway is selectively required for TLR4-mediated innate immunity. Nature Immunology. 6; 6:587-592
31. Hsieh and Papaconstantinou (2006) Thioredoxin-ASK1 complex levels regulate ROS-mediated p38 MAPK pathway activity in livers of aged and long-lived Snell dwarf mice. The FASEB Journal 20:259-268.

32. Baldassareet et al. (1999) The role of p38 Mitogen-activated Protein Kinase in IL-1β transcription. Journal of immunology. 162; 9:5367-5373.
33. Thioredoxin-Interacting Protein Mediates TRX1 Translocation to the Plasma Membrane in Response to Tumor Necrosis Factor-α: A Key Mechanism for Vascular Endothelial Growth Factor Receptor-2 Transactivation by Reactive Oxygen SpeciesArterioscler Thromb Vasc Biol.2011; 31:1890-1897
34. Schroeder et al. (2007) Nuclear Redox-Signaling Is Essential for Apoptosis Inhibition in Endothelial Cells– Important Role for Nuclear Thioredoxin-1. Arteriosclerosis, Thrombosis, and Vascular Biology. 27:2325-2331
35. Hsu and Wen (2002) Lipopolysaccharide-mediated Reactive Oxygen Species and Signal Transduction in the Regulation of Interleukin-1 Gene Expression. Journal of biologiscal chemistry. 277; 25:22131-22139
36. Tatsuya et al. (2008) Loss of the autophagy protein Atg16L1 enhances endotoxin-induced IL-1b production. Nature 456; 7219:264-268
37. Harris et al. (2011) Autophagy Controls IL-1 β Secretion by Targeting Pro-IL-1 β for Degradation. Journal of Biological Chemistry 286; 11:9587-9597
38. Choumar et al. (2011) Lipopolysaccharideinduced mitochondrial DNA depletion. Antioxidant Redox signaling. 15; 11:2837-2854
39. El kebir et al. (2006) Inhibition of K+ efflux prevents mitochondrial dysfunction and suppresses caspase-3-*apoptosis-inducing factor- and endonuclease G-mediated constitutive apoptosis in human neutrophils. Cellular signaling. 18; 12:2302-2313*
40. Slichter et al. (2010) The Ca2+ activated SK3 channel is expressed in microglia in the rat striatum and contributes to microglia-mediated neurotoxicity in vitro. Journal of neuroinflammation 7;4
41. Kim et al. (2006) IL-1b, an Immediate Early Protein Secreted by Activated Microglia, Induces iNOS/NO in C6 Astrocytoma Cells Through p38 MAPK and NF-kB Pathways. Journal of Neuroscience Research 84:1037–1046
42. Cauwels and Brouckaert (2008) Critical role for small and large conductance calcium-dependent potassium channels in endotoxemia and TNF toxicity. Shock. 29; 5:577-582
43. Gallin (1984) Calcium and voltage activated potassium channels in human macrophages. Biophysical journal 46:821-825.
44. Di Virgilio (2007) Liaisons dangereuses: $P2X_7$ and the inflammasome. Trends in pharmacological sciences 28; 9:465–472
45. Jelassi et al. (*2011) P2X7 receptor activation enhances SK3 channels- and cystein cathepsin-dependent cancer cells invasiveness. Oncogene 30; 18:2108–2122*
46. Tassi et al. (2010) *Altered redox state of monocytes from cryopyrin-associated periodic syndromes causes accelerated Il-1β secretion.* PNAS 107:9789-9794
47. Gattornoet al. Pattern of interleukin-beta secretion in response to lipopolysaccharide and ATP before and after interleukin-1beta blockade in patients with CIAS1 mutations. Arthritis Rheum. 46 3340-3348
48. Fujisawa et al. (2007) Disease-associated mutations in CIAS1 induce cathepsin B-dependent rapid cell death of human THP-1 monocytic cells. Blood 1; 109:2903-2911