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**REVIEW ARTICLE**

# NLRP3 inflammasome: its regulation and involvement in atherosclerosis†

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†This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: [10.1002/jcp.25930]

**Received 12 February 2017; Revised 21 March 2017; Accepted 22 March 2017**
**Journal of Cellular Physiology**
**This article is protected by copyright. All rights reserved**
**DOI 10.1002/jcp.25930**

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**Abstract**

Inflammasomes are intracellular complexes involved in the innate immunity that convert proIL-1β and proIL-18 to mature forms and initiate pyroptosis *via* cleaving procaspase-1. The most well-known inflammasome is NLRP3. Several studies have indicated a decisive and important role of NLRP3 inflammasome, IL-1β, IL-18, and pyroptosis in atherosclerosis. Modern hypotheses introduce atherosclerosis as an inflammatory/lipid-based disease and NLRP3 inflammasome has been considered as a link between lipid metabolism and inflammation because crystalline cholesterol and oxidized low-density lipoprotein (oxLDL) (two abundant components in atherosclerotic plaques) activate NLRP3 inflammasome. In addition, oxidative stress, mitochondrial dysfunction, endoplasmic reticulum (ER) stress, and lysosome rupture, which are implicated in inflammasome activation, have been discussed as important events in atherosclerosis. In spite of these clues, some studies have reported that NLRP3 inflammasome has no significant effect in atherogenesis. Our review reveals that some molecules such as JNK-1 and ASK-1 (upstream regulators of inflammasome activation) can reduce atherosclerosis through inducing apoptosis in macrophages. Notably, NLRP3 inflammasome can also cause apoptosis in macrophages, suggesting that NLRP3 inflammasome may mediate JNK-induced apoptosis, and the apoptotic function of NLRP3 inflammasome may be a reason for the conflicting results reported. The present review shows that the role of NLRP3 in atherogenesis can be significant. Here, the molecular pathways of NLRP3 inflammasome activation and the implications of this activation in atherosclerosis are explained. This article is protected by copyright. All rights reserved

**Keywords**: Inflammasome, NLRP3, atherosclerosis, signaling pathway

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## Introduction

Inflammation is a defense response aiming at eliminating the primary causes of cell damage. Although inflammation helps cleaning up infections and other toxic stimuli, inflammatory reactions can cause significant damages in such a way that inflammation is regarded as an underlying cause of some human diseases, especially diseases with a high rate of morbidly and mortality such as atherosclerosis. Atherosclerosis is a fundamental cause of cardiovascular diseases, accounting for about 50% of deaths worldwide. Atherosclerosis was conventionally believed to be caused by impaired lipid metabolism manifested mainly as increased levels of plasma low-density lipoprotein cholesterol (LDL-cholesterol) levels (Anderson et al., 1977; Brown and Goldstein, 1974; Brown and Goldstein, 1986; Brown et al., 1980). However, inflammation has also emerged as a causal factor in forming atherosclerotic plaques and, according to the newest hypotheses, inflammatory processes and lipid metabolism jointly contribute to the formation of atherosclerotic plaques in the arterial wall (Hansson and Hermansson, 2011; Libby and Hansson, 2015). Retention of atherogenic lipoproteins within the subendothelial space – which is due to the interaction of positively charged amino acids in apoB100 protein with negatively charged proteoglycans – plays a key role in triggering early steps of atherosclerosis (Gustafsson and Boren, 2004; Tabas et al., 2007). (Gustafsson and Boren, 2004).

Inflammasomes are components of the innate immune system and a considerable number of studies have reported an association of inflammasome with the development of several diseases such as atherosclerosis (Guo et al., 2015). Inflammasomes are multi-protein complexes in the cytoplasm that intensifyinflammation in response to a wide range of danger signals including pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs) (Bakker et al., 2014; Martinon et al., 2004; O'Connor et al., 2003; Petrilli and Martinon, 2007; Yin et al., 2013). NLRP3 inflammasome, the most well-known inflammasome, is essential for atherogenesis and its silence causes the stabilization of atherosclerotic plaque (Zheng et al., 2014).

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Cholesterol crystals and oxidized LDL (oxLDL), which are considerably present in atherosclerotic plaques, can activate inflammasomes. Consequently, inflammasomes induce inflammation by activating caspase-1 and subsequent maturation of inflammatory cytokines such as interleukin-1 beta (IL-1β) and interleukin-18 (IL-18), and initiating a type of inflammatory cell death called pyroptosis (Guo et al., 2015). However, some types of inflammasomes such as NLRP3 and AIM2 also activate caspase-8 and participate in apoptosis induction. The intensity of stimuli determines whether apoptosis or pyroptosis occurs (Sagulenko et al., 2013). Since inflammasomes serve as a link between inflammation and lipid metabolism, studying their role in atherosclerosis appears to be important.

Inflammasome has been shown to be up-regulated in the aortas of patients at a high risk of atherosclerosis such as diabetics, smokers, hypercholesterolemic subjects, and hypertensive subjects (Zheng et al., 2013). Almost all inflammasome activation mechanisms have been recognized as atherogenic mechanisms, even several years before the discovery of inflammasomes. These mechanisms are reactive oxygen species (ROS) overproduction, mitochondrial dysfunction (Ballinger et al., 2000; Victor et al., 2009), lysosome rupture (Yuan et al., 2000), and endoplasmic reticulum (ER) stress (Chistiakov et al., 2014; Outinen et al., 1999). In addition, a wide range of inflammasome activators are implicated in atherosclerosis (Table 1).

In the following parts, we explain inflammasome structure, mechanisms of NLRP3 inflammasome activation, and the role of NLRP3 inflammasome in atherosclerosis.

## Inflammasome structure

In 2002, inflammasome was introduced as an activator complex of caspase-1 which causes the maturation of proIL-1β. It was already clear that IL-1β is activated by caspase-1, but it was unknown how caspase-1 was activated. A landmark study revealed that formation of inflammasome complex is the activator of caspase-1 (Martinon et al., 2002). Thus far, two types of inflammasomes

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have been recognized: Nod-like receptor (NLR) inflammasomes, and Pyrin and HIN domain-containing protein (PYHIN) inflammasomes.

**NLR inflammasomes**

NLRs are a group of intracellular receptors of the immune system and a subset of them including NLRP1, NLRP2, NLRP3, NLRC4, NLRP6, NLRP7, NLRP12 act as components of inflammasome complexes with the sensory role. NLR proteins, except NLRP1 and NLRC4, are comprised of three domains: leucine-rich repeat (LRR), nucleotide-binding domain (NBD), and pyrin domain (PYD). NLRC4 consists of LRR, NBD and CARD, and NLRP1 has additional PYD and function-to-find domain (FIIND) domains (Finger et al., 2012; Harijith et al., 2014; Latz et al., 2013) (Fig 1, A-E). LRR and NBD domains, found in all types of NLRs, act as ligand-recognition domain and self-oligomerization domain, respectively.

Apoptosis-associated speck-like protein containing a C-terminal caspase recruitment domain (ASC) and procaspase-1 are other components of NLR inflammasomes (Stutz et al., 2009) (Figure 1). Sometimes, caspase-5 or caspase-8 also participates in forming inflammasome complex (Martinon et al., 2002; Sagulenko et al., 2013).

ASC contains PYD and caspase recruitment domain (CARD) and procaspase-1 contains CARD and caspase-1 domains. NLRs (except NLRC4 which has no PYD domain) bind to ASC through PYD-PYD interactions and, consequently, ASC binds to procaspase-1 (Sagulenko et al., 2013; Stutz et al., 2009) (Fig 1. A, E). NLRP1 and NLRC4 bind to procaspase-1 directly because of the interaction of positively charged amino acids in apoB100 protein with negatively charged proteoglycans *via* their CARD domains (Fig 1. B, D). However, it has been observed that NLRP1 and NLRC4 inflammasome complexes containing ASC have a higher capacity to produce IL-1β (Latz et al., 2013). Nevertheless, since NLRC4 lacks PYD domain, it is not exactly clear how ASC binds to NLRC4 and forms inflammasome complex. The attachment of NLRC4 to ASC through CARD domains has been observed in the mammalian two-hybrid analysis (Geddes et al., 2001). Therefore,

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it is possible that another ASC mediates the attachment of procaspase-1 to other complex components (Fig 1. C).

## PYHIN inflammasomes

In this type of inflammasome complexes, PYHIN acts as a sensory part and can interact with viral or bacterial dsDNA.

Absent in melanoma 2 (AIM2) and interferon, gamma-inducible protein 16 (IFI16) are two subsets of PYHIN family forming inflammasome complexes.

AIM2 has pyrin domain and DNA binding HIN domain. IFI16 in addition to these domains, has an extra HIN domain. In this type of inflammasomes, AIM2 or IFI16 attach to ASC through pyrin - pyrin interaction and ASC binds to the procaspase-(Bawadekar et al., 2015; Hornung et al., 2009; Kerur et al., 2011) (Figure 1. F, G).

## NLRP3 inflammasome activation

It has been showed that two signals are required for NLRP3 inflammasome activation: i) the first signal is NLRP3 up-regulation through the NF-κB activation (mainly considered a toll-like receptor-dependent pathway (TLR)) or BRCC3 activation (Abais et al., 2015). , ii) the second signal is NLRP3 activation and ASC phosphorylation, which lead to the formation of NLRP3 inflammasome complex (Figure 2). In addition, many mechanisms have been recognized for the inflammasome activation such as $K^+$ efflux, ROS overproduction, ER stress, mitochondrial dysfunction, $Ca^{2+}$ signaling, and lysosome rupture. These mechanisms could provide the second signal; however, as seen in the figure, these mechanisms also activate NF-κB (either directly or indirectly through ROS production) or participate in NLRP3 deubiquitination. Therefore, it seems that these mechanisms could have a role in providing the first signal. These mechanisms are also overlapped. TXNIP, NEK7, ox-mtDNA, PKR, and CB (cathepsin B) are downstream molecules of the second signal mechanisms. The second signal could activate JNK and SYK, which

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phosphorylate ASC and cause ASC speck formation. A notable point in the figure is the involvement of ROS in the first signal and in the upstream or downstream of all of the second signal mechanisms. All of the molecules in this picture except NEK7 have roles in atherosclerosis (Table 1) suggesting that the mechanism of the NLRP3 inflammasome activation and atherosclerosis share many similarities. In addition, positive feedback cycles are also notable in these pathways.

## 1) Signaling pathways affected by NLRP3

Several studies have indicated that some signaling pathways could be affected by NLRP3. NLRP3 regulation could occur at different levels: transcriptional, translational, and post-translational levels. In the below sections, we summarize a variety of signaling pathways and molecules that could be regulated by NLRP3.

### 1-1) NLRP3 and NF-κB

Among various types of molecules and signaling pathways that are affected by NLRP3 is nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). NF-κB is a nuclear transcription factor and is activated by various factors such as ROS, TNF-α, IL-1β, and LPS (Chandel et al., 2000; Luo et al., 2014). NF-κB up-regulation is observed in many inflammatory diseases and, seemingly, it is not easy to consider NF-κB activation as a protective or destructive event in inflammatory diseases.

It has been observed that NF-κB causes up-regulation of IL-1β and NLRP3 in a TLR-dependent pathway (Abais et al., 2015). One of the important components in TLR signaling pathway is protein kinase R (PKR). Lack of PKR in mice impaired the responsiveness to a variety of TLR ligands (Garcia et al., 2006). PKR also plays a role in NF-κB activation (Balachandran et al., 1998).

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Therefore, it is probable that at least a part of the TLR pathway in NF-κB activation is mediated through PKR. PKR is also activated by ROS (Li et al., 2010) (Figure 2). ROS can play a role in activating NLRP3 inflammasome through NF-κB activation (Luo et al., 2014). ER stress can also activate NF-κB (Tam et al., 2012) (Figs. 2 and 3). Several studies have suggested a decisive effect of ROS and ER stress as the second signal of inflammasome activation (Abais et al., 2015; Latz et al., 2013). More research on the role of ROS and ER stress in providing the first signal (NLRP3 and IL-1β up-regulation) is required. Additionally, viral double-strand RNA (dsRNA) can also activate NF-κB and NLRP3 *via* polymerizing mitochondrial antiviral-signaling protein (MAVS) in the mitochondrial membrane (van Kempen et al., 2015). Finally, NLRP3 could affect NF-κB and underlying signaling pathways which lead to the progression of atherosclerosis.

### 1-2) NLRP3 and microRNA

MicroRNAs down-regulate the expression of genes at the translational level (Ambros, 2004; Bartel, 2004; Bartel, 2009; Fabian et al., 2010; Fathullahzadeh et al., 2016; Mirzaei et al., 2017b; Mirzaei et al., 2016a; Mirzaei et al., 2016b; Mirzaei et al., 2016c; Mohammadi et al., 2016; Rashidi et al., 2016; Reza Mirzaei et al., 2016; Saadatpour et al., 2016; Salarinia et al., 2016; Simonian et al., 2017). MicroRNAs can degrade mRNA in cooperation with a protein complex called RNA-induced silencing complex (RISC) (Gholamin et al., 2017; Ha and Kim, 2014; Mirzaei et al., 2017a; Mirzaei et al., 2017b; Moridikia et al., 2017; Rashidi et al., 2017). MicroRNA-223 is one of the microRNAs which degrades NLRP3 mRNA leading to a reduced amount of NLRP3. MicroRNA-223 antagonists increase the amount of NLRP3 (Bauernfeind et al., 2012). MicroRNA-223 has also been shown to act as an important regulator of cholesterol (Vickers et al., 2014). Since microRNA-223 acts as a regulator of inflammasome activity and cholesterol metabolism, it is important to study its alterations in inflammatory/lipid-based diseases such as atherosclerosis.

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Few studies have assessed the effect of NLRP3 inflammasomes on the expression of microRNAs and have shown that inflammasomes could affect the expression profile of a variety of microRNAs. Hence, it seems that additional studies in this area are required.

**1-3) NLRP3 up-regulation at the post-translational level**

Deubiquitination/ubiquitination of proteins is a mechanism which can change the level of proteins within a few minutes. Ubiquitination causes protein degradation in the cellular proteasome whereas deubiquitination leads to the increased level of protein. Findings in mice and human macrophages suggest the presence of a basal level of ubiquitinated NLRP3. NLRP3 deubiquitination is required for increasing the available amount of NLRP3 (Juliana et al., 2012). TLR and ROS signaling can also increase the levels of NLRP3 through its deubiquitination. This deubiquitination is also potentiated by extracellular ATP (Juliana et al., 2012). It has been observed that BRCA1/BRCA2-Containing Complex Subunit 3 (BRCC3) is an enzyme which deubiquitinates the LRR domain of NLRP3 and this deubiquitination is necessary for NLRP3 activation (Py et al., 2013) (Figure 2).

**2) NLRP3 oligomerization and inflammasome complex formation**

NLRP3 is considered as the sensor of inflammasome complex. Various components such as Thioredoxin Interacting Protein (TXNIP) (Zhou et al., 2010), NIMA-related kinase 7 (NEK7) (He et al., 2016), oxidized mitochondrial DNA (ox-mtDNA) (Shimada et al., 2012), PKR (Lu et al., 2012), and cathepsin B can bind to NLRP3. These components are provided through different mechanisms including $K^+$ efflux, ROS generation, calcium signaling, ER stress, mitochondrial dysfunction, and lysosome rupture (Figure 2).

When NLRP3 it activated, it interacts with ASC. ASC, in the form of speck-like cytoplasmic aggregation in cooperation with NLRP3, causes procaspase-1 to be cleaved, resulting in caspase-1 activation (Compan et al., 2015; Neumann and Ruland, 2013).

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ASC phosphorylation is also required for the formation of speck. Spleen tyrosine kinase (SYK) and c-Jun N-terminal kinase (JNK) are two kinases which phosphorylate ASC. Inhibition of SYK or JNK interferes with the ASC speck formation but has no effect on binding of ASC to NLRP3 (Hara et al., 2013). ASC speck formation is required for inflammasome activation and the necessity of ASC phosphorylation for forming ASC speck suggests that the mechanisms proposed for the formation of inflammasome complex ($K^+$ efflux, ROS formation, $Ca^{2+}$ signaling, ER stress, mitochondrial dysfunction, and lysosome rupture) should lead not only to NLRP3 activation but also to ASC phosphorylation. These mechanisms are overlapping (Figure 2) and we explain them as four mechanisms: 1- $K^+$ efflux, 2- ROS formation, 3- ER stress-induced mitochondrial dysfunction- $Ca^{2+}$ signaling, and 4- lysosome rupture.

## $K^+$ efflux

$K^+$ efflux is one of the mechanisms for inflammasome activation. $K^+$ efflux is induced by many stimuli and is a common, necessary, and even sufficient signal for NLRP3 activation (Munoz-Planillo et al., 2013). ATP is an inflammasome activator (Mariathasan et al., 2006) and is able to induce $K^+$ efflux. ATP is a P2X7 agonist and binds to the P2X7 receptor (P2X7R) and activates it (Surprenant et al., 1996). Activated P2X7R causes $K^+$ efflux by creating some channels in the cell membrane (Pelegrin and Surprenant, 2006); the low level of intracellular $K^+$ induces NLRP3 inflammasome activation (Petrilli et al., 2007). P2X7R also phosphorylates and activates PKR (Peng et al., 2015) which is involved in the activation of NLRP1, NLRP3, NLRC4 and AIM2 inflammasomes by physical interaction with them (Lu et al., 2012).

NEK7, a member of the mammalian NIMA-related kinases family (NEK proteins), is a downstream protein of $K^+$ efflux signaling. NEK7 binds to the LRR domain of NLRP3, leading to the activation and oligomerization of NLRP3 (He et al., 2016).

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PKR also plays a role in the activation of JNK (Zhang et al., 2009), a kinase required for ASC phosphorylation. SYK activation is also observed downstream of the $K^+$ efflux (Yaron et al., 2016).

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**ROS**

ROS is among the most important factors affecting NLRP3 inflammasome activation. There are several sources for ROS production such as cyclooxygenase (COX), lipoxygenase (LPO), NADPH oxidase, mitochondrial dysfunction, and ER stress. Most of them participate in activating inflammasome or producing IL-1β (Figs. 2 and 3, table 1).

An *in vivo* study has shown that TXNIP is essential for NLRP3 inflammasome activation (Zhou et al., 2010). TXNIP is activated under the influence of ROS and induces ROS production. TXNIP is a protein linking the oxidative stress and the inflammasome activation (Harijith et al., 2014; Lane et al., 2013; Zhou et al., 2010). ROS production has been reported to be significantly decreased in TXNIP-deficient mice (Shah et al., 2013). It has also been shown that TXNIP reduction impairs NLRP3 inflammasome activation and reduces IL-1β production (Zhou et al., 2010).

TXNIP inhibits the redox activity of thioredoxin (TRX). Under the influence of ROS, TXNIP is translocated from the nucleus and binds to TRX1 in the cytosol and TRX2 in the mitochondria, leading to increased ROS generation (Harijith et al., 2014). TXNIP-TRX separation in a ROS-dependent pathway has also been reported (World et al., 2011). When TRX is oxidized, TXNIP is separated from TRX. Free TXNIP interacts physically with the LRR domain of NLRP3 (Zhou et al., 2010). It has been observed that inflammasome activators such as uric acid crystal induce the separation of TXNIP from TRX in a ROS-sensitive pathway (Zhou et al., 2010). Therefore, two groups of studies have mentioned the attachment and separation of TXNIP to/from TRX in a ROS-dependent pathway. It seems that this contradiction may be explained as follow:

Under the influence of ROS, TXNIP comes out of the nucleus, and inhibits the activity of TRX *via*binding to it, results in increased level of ROS. Increased amount of ROS can oxidize TRX1.

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TRX in the oxidized form is not able to bind to TXNIP and is separated from it. Now, free TXNIP can bind to NLRP3 leading to NLRP3 activation (Zhou et al., 2010) .

In unstressed cells, TRX is bound with apoptosis signal-regulating kinase (ASK) to block the ASC function. In stress conditions, oxidized TRX can also detach from ASK leading to ASK activation. Then activated ASK can stimulate JNK, a kinase that participates in forming ASC speck (Matsuzawa and Ichijo, 2008; Son et al., 2011) (Figure 2). In addition, TXNIP-TRX2 interaction increases the level of ROS in mitochondria (Harijith et al., 2014). Mitochondrial ROS activates NEK7, which is an inflammasome activator (He et al., 2016). Increased ROS also causes mtDNA to be oxidized (Ballinger et al., 2000). Observations suggest that NLRP3 can be activated by the entry of ox-mtDNA to the cytosol and binding to NLRP3 (Shimada et al., 2012). Therefore, TXNIP activates NLRP3 not only directly through binding to NLRP3, but also indirectly through producing ROS that leads to mtDNA oxidation and NEK7 activation (Figure 2).

PKR is another molecules activated by ROS (Li et al., 2010) and influences the activity of NLRP3. The role of PKR as the activator of NF-κB, JNK, and NLRP3 were mentioned earlier. Despite these observations, Yim et al. showed that PKR inhibits the activity of inflammasome through its kinase activity (Yim et al., 2016). More studies are needed to clarify the effects of PKR on inflammasome activity.

Mechanisms such as $K^+$ efflux (Tschopp and Schroder, 2010), ER stress (Malhotra and Kaufman, 2007), mitochondrial dysfunction, lysosome rupture, and cathepsin B (Freeman et al., 2013) also cause ROS production (Figure 2). It has been observed that many factors that are known as inflammasome activators are able to produce ROS. For example, a number of studies have reported the importance of NADPH oxidase-derived ROS in the activation of NLRP3 in response to ATP, asbestos, and silica (Cruz et al., 2007; Dostert et al., 2008; Hewinson et al., 2008). These findings indicate the importance of further investigations on ROS as an important mechanism for NLRP3 inflammasome activation. However, inflammasome activation in a ROS-independent pathway has

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also been reported. It has been observed that ROS production is not necessary for NLRP3 activation (van de Veerdonk et al., 2010).

Macrophages in which NADPH oxidase enzymes are genetically impaired have been shown to have normal level of NLRP3 inflammasome activity (van Bruggen et al., 2010). In addition, increased amounts of ROS result in the inhibition of caspase-1 *via* reversible oxidation and glutathionylation of the redox-sensitive cysteine residue Cys397 in SOD1-deficient macrophages (Meissner et al., 2008). Therefore, it seems that different concentrations of ROS have contrasting effects on inflammasome activation and this may explain the conflicting results reported.

**ER stress, mitochondrial dysfunction, and $Ca^{2+}$ signaling**

ER controls synthesis, changing, and folding of the secretory proteins. When protein synthesis exceeds the ER folding capacity, misfolded and unfolded proteins accumulate in the ER lumen. This accumulation leads to a condition known as ER stress. Various physiological and pathological factors can result in ER stress by disrupting ER homeostasis. ER stress has a role in the pathogenesis of many inflammatory diseases. When ER stress occurs, a compensatory system called unfolded protein response (UPR) is activated trying to turn the ER stress condition to the normal state. Nevertheless, in extreme or prolonged ER stress, the UPR system will impel the cell to death.

Inositol-requiring enzyme 1α (IRE1α), activating transcription factor 6 (ATF6) and protein kinase R-like endoplasmic reticulum kinase (PERK) are three ER stress transmembrane sensors which trigger UPR response in the presence of misfolded and unfolded proteins. In normal conditions, Binding immunoglobulin Protein (BiP, an ER chaperone, binds to the luminal domain of these three proteins, but during ER stress state, BiP is dissociated from the aforementioned proteins and attaches to the misfolded proteins. BiP separation from sensory proteins will activate these three sensor proteins.

ER stress activates the inflammasome (Mekahli et al., 2011; Ron and Walter, 2007; Schroder and Kaufman, 2005), although the exact molecular pathway of it has not been well understood. Studies

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have suggested that inflammasome activation occurs in a UPR-independent pathway (Menu et al., 2012); however, UPR sensor molecules such as IRE1α, ATF6 and PERK are involved in NF-κB activation and ROS production (Wang and Kaufman, 2014). Since NF-κB and ROS participate in the upregulation of NLRP3 and activation of the inflammasome, respectively, it seems that UPR may be able to activate inflammasome. This reveals the necessity for further studies on UPR response as an inflammasome activator.

PERK, IRE1α also increase the levels of TXNIP which is an NLRP3 activator. TXNIP reduction is observed in PERK knockdown mouse embryonic fibroblasts under the ER stress conditions (Oslowski et al., 2012). IRE1α, having kinase and endonuclease (RNAase) activities, degrades the microRNA-17 through its RNAase activity. MicroRNA-17 decreases the mRNA level of TXNIP. Therefore, IRE1α increases the level of TXNIP by reducing microRNA17 (Hassler et al., 2012). IRE1α also activates JNK, which is involved in the formation of ASC speck (Urano et al., 2000) (Fig 3).

ER also serves as a storage for $Ca^{2+}$ and can regulate the cytosolic $Ca^{2+}$ levels. When ER $Ca^{2+}$ level decreases, ER $Ca^{2+}$-dependent chaperone are deactivated, resulting in the accumulation of misfolded proteins in ER (Oslowski and Urano, 2011). There is an interaction between $Ca^{2+}$ level and ER stress, in such a way that IP3R, which regulates the $Ca^{2+}$ to flow out of the ER, serves as the fourth sensor of ER stress. At unstressed cell state, an appropriate and necessary amount of $Ca^{2+}$ leaves ER through IP3R. In the initial and adaptive phase of ER stress, the efflux of $Ca^{2+}$ is reduced by IP3R. However, in the prolonged ER stress which is beyond the adjustable level, IP3R pushes the cell toward apoptosis by increasing the outflow of $Ca^{2+}$ from the ER (Mekahli et al., 2011). Increased level of cytosolic $Ca^{2+}$ enters the mitochondria and causes ROS production. It mentioned that mitochondrial ROS causes mtDNA to be oxidized that leads to the inflammasome activation.

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$Ca^{2+}$ also activates JNK, a necessary kinase for ASC speck formation, by activating tat-associated kinase (TAK1)-JNK pathway (Okada et al., 2014). Increased extracellular $Ca^{2+}$ can also cause the activation of the inflammasome (Rossol et al., 2012) (Figure 3). The increased concentration of extracellular $Ca^{2+}$ at the site of infection (Kaslick et al., 1970) and chronic inflammation (Korff et al., 2006; Tzimas et al., 2004) is observed. Necrotic cells seem to be a resource for the increased extracellular $Ca^{2+}$ (Rossol et al., 2012). The increased extracellular $Ca^{2+}$ activates NLRP3 inflammasome through G protein-coupled calcium-sensing receptor in a inositol (1,4,5)-trisphosphate (PI3) / $Ca^{2+}$ pathway. PI3 binds to IP3R leading to the $Ca^{2+}$ to flow out the ER through IP3R (Lee et al., 2012; Rossol et al., 2012). G protein-coupled receptor (GPCR) signaling pathway also can reduce cyclic AMP (cAMP), which inhibits the activity of NLRP3 by binding to it. Hence, GPCR can activate NLRP3 by lowering the level of cAMP (Lee et al., 2012).

Increased amount of extracellular $Ca^{2+}$ in the site of infection or inflammation causes $Ca^{2+}$ to be released from ER in a G protein coupled receptor (GPCR) signaling pathway. $Ca^{2+}$ enters the mitochondria and increses ROS. Subsequently, ROS oxidize mtDNA leading to NLRP3 activation. Reduced $Ca^{2+}$of ER also causes ER stress followed by UPR response. IRE1α, ATF6, and PERK are three UPR sensor and cause NFkB, JNK, and TXNIP activation which all participate in inflammasome activation. G protein (GP), phospholipase C (PLC), 4,5-bisphosphate (PIP2), diacylglycerol ( DAG), inositol triphosphate (IP3), protein kinase C (PKC)

**Lysosome rupture:**

Lysosome rupture is another mechanism for NLRP3 inflammasome activation (Figure 2). During lysosome rupture, cathepsin B is released from the lysosome (Rajamaki et al., 2010). A recent study showed that cathepsin B binds to the LRR domain of NLRP3 and activates NLRP3 (Bruchard et al., 2013). Cathepsin B is also involved in the activation of mitogen-activated protein kinases (MAPKs), one of which is JNK (Figure 2). Inhibition of cathepsin B or cysteine proteases impairs the activation of MAPKs (Okada et al., 2014).

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Cholesterol crystals (Rajamaki et al., 2010) and ROS (Zdolsek et al., 1993) lead to lysosome rupture (Figure 2). The effects of cholesterol crystals in activating inflammasomes have led to an increasing number of studies on The joint inflammatory/lipid-based nature of atherosclerosis. Cluster of differentiation 36 (CD36) can also be considered as a link between lipid metabolism and inflammasome activation (Figure 2). CD36, a scavenger receptor on the cell surface, is involved in oxLDL internalization and its transformation into cholesterol crystals (Oury, 2014). In addition, CD36 interaction with TLR4 /TLR6 heterodimer is involved in NLRP3 and proIL-1β upregulation (Sheedy et al., 2013).

**Atherosclerosis and inflammasome:**

Atherosclerotic plaques are developed as a result of inflammatory processes and lipid metabolism abnormalities; these plaques are, in essence, accumulation of foam cells, immune cells, cholesterol crystals, and smooth muscle cells (SMCs) which are proliferated under the influence of inflammatory cytokines. Plaque formation is a slow but progressive process, starting in childhood and resulting in clinical symptoms in adulthood. There are several risk factors for atherosclerosis among which hypercholesterolemia is key one. In comparison to normal forms of cholesterol and LDL, crystalline cholesterol and oxLDL participate extensively in forming plaques. OxLDL binds with CD36 and is phagocytosed by macrophages. Unlike LDL, oxLDL is resistant to the lysosomal enzymes of macrophages, resulting in the oxLDL accumulation in macrophages and subsequent formation of foam cells (Hoff et al., 1993; Lougheed et al., 1991).

The exact mechanism of LDL oxidation is controversial and several oxidative sources such as ROS, metal ions, lipoxygenase, and myeloperoxidase have been suggested (Gaut and Heinecke, 2001; Parthasarathy et al., 2010; Yoshida and Kisugi, 2010). However, the presence of oxLDL in the subendothelial space and its impact on the development of atherosclerotic plaque has been suggested by a considerable number of studies.

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As mentioned earlier, cholesterol crystals and oxLDLs, found in abundance in atherosclerotic plaques, cause inflammasome activation. Thus, inflammasome links lipid metabolism and the inflammatory condition to each other. Several studies have shown the significant role of NLRP3 inflammasome in atherosclerosis. NLRP3 is correlated with the severity and prognosis of coronary atherosclerosis in patients with acute coronary syndrome (Afrasyab et al., 2015). It has also been observed that NLRP3 inflammasome is localized in the cytoplasm of foam cells and macrophages (Shi et al., 2015).

In the carotid atherosclerotic plaques of patients undergoing carotid endarterectomy, there is an up-regulation of NLRP3, ASC, caspase-1, IL-1β, and IL-18. These molecules are all more expressed in unstable plaques compared with stable ones (Shi et al., 2015).

Inhibition of NLRP3 signaling by lentivirus-mediated RNA interference inhibits pro-inflammatory cytokines in apolipoprotein E-deficient (apo E -/-) mice fed with a high-fat diet. This RNA interference also reduces macrophages and lipids, and increases SMCs and collagen of the plaque leading to plaque stabilization (Zheng et al., 2014).

Hypercholesterolemia, hypertension, diabetes, and smoking are other risk factors for atherosclerosis. Zheng et al. reported an overexpression of NLRP3 in the aorta of smoker patients with hypercholesterolemia, hypertension and diabetes. The expression of NLRP3 in the aorta of these patients was directly associated with total cholesterol, LDL-cholesterol, lipoprotein(a), and inversely associated with HDL-cholesterol levels (Zheng et al., 2013).

Oxidative stress, mitochondrial dysfunction (Victor et al., 2009), ER stress (Chistiakov et al., 2014), and lysosome rupture (Yuan et al., 2000) which are the mechanisms of inflammasome activation, are all observed in atherosclerosis. In addition, extracellular $Ca^{2+}$ is another inflammasome activator found in atherosclerotic plaques especially in necrotic parts. However, few studies have been conducted on these mechanisms in atherosclerosis.

IL-1β, IL-18, and pyroptosis are inflammasome activation products that have important roles in atherogenesis. In recent years, other mechanisms of IL-1β activation independent of the

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inflammasome and caspase-1 have been found (Mayer-Barber et al., 2010). However, the role of inflammasome in IL-1β activation is still impressive.

IL-1β production in endothelial cells and macrophages has been reported in the atherosclerotic coronary arteries (Galea et al., 1996). Around 30% reduction in the size of atherosclerotic plaques has been observed in apo E-/- and IL-1β-/- mice compared with the control group of apo E-/- mice (Kirii et al., 2003). In addition, monoclonal antibodies against IL-1β inhibit plaque formation in apo E-/- mice (Bhaskar et al., 2011). Decreased levels of inflammatory molecules such as VCAM-1, IL-6, IL-8, MCP-1, TNF-α, MMP-3 and MMP-9 have been reported to accompany reduced levels of IL-1β (Bhaskar et al., 2011; Kirii et al., 2003).

IL-18 is a pro-inflammatory and pro-atherogenic cytokine. Up-regulation of IL-18 and its receptor has been reported in macrophages, endothelial cells and SMCs (Gerdes et al., 2002). In addition, expression of IL-18 in unstable plaques is more than that in stable ones (Mallat et al., 2001). Studies in apo E-/- and IL-18-/- mice showed a reduction of IL-18 in plaque by 35%, compared with IL-18 competent littermates (Elhage et al., 2003).

Pyroptosis is an inflammatory cell death process that occurs during activation of caspase-1 by oxLDL in macrophages (Lin et al., 2013). It seems that pyroptosis is involved in the development of atherosclerotic plaque by intensifying the inflammation (Chang et al., 2013). Despite several observations regarding the role of NLRP3 inflammasome in the development of atherosclerosis, some reports have indicated no significant effect of NLRP3 inflammasome on the pathogenesis of atherosclerosis. It has been observed that atherosclerosis developed in apo E-/- mice is independent of NLRP3 inflammasome (Menu et al., 2011). It has also been suggested that NLRP1, not NLRP3, has a key role in shifting the endothelial cells to the proinflammatory state (Bleda et al., 2014). In addition, JNK-1 and ASK-1, which participate in inflammasome activation, attenuate plaque formation *via* inducing apoptosis in macrophages (Table 1). These are upstream molecules in caspase-8 activation which cause apoptosis (Bhattacharyya et al., 2003; Chinen et al., 2010). The interesting point is that NLRP3 inflammasome can also cause apoptosis *via* activating caspase-8 in

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macrophages.Whether NLRP3 inflammasome mediates the JNK and ASK-induced apoptosis, or if at the lower intensity of stimuli the NLRP3 inflammasome apoptotic function protects against atherosclerosis development are still unanswered questions (Figure 4).

NLRP3 inflammasome causes pyroptosis which is an inflammatory cell death. In addition, NLRP3 inflammasome-mediated production of IL-1β and IL-18 can intensify inflammation and plaque formation through up-regulation of some components. For example, IL-1β increases monocyte chemo-attractant protein-1 (MCP-1) and vascular cell adhesion molecule 1 (VCAM-1) which participate in leukocyte adhesion to endothelium, IL-8 which is a neutrophil chemotactic factor, and matrix metalloproteinases (MMPs) which degrade extracellular matrix such as collagen fibers. IL-1β also increases IL-8 and TNF-α. Unstable plaques are symptomatic ones with a lower amount of SMCs and collagens and more amount of lipids and macrophages, in comparison to stable plaques which are asymptomatic. NLRP3 inflammasome is more expressed in unstable plaques, decreases SMCs and collagen and increases lipids and macrophages. Therefore, NLRP3 inflammasome predisposes plaque to rupture which is followed by thrombus formation. Ultimately, NLRP3 inflammasome-induced plaque development and thrombus formation narrows the luminal space of the vessel and subsequently reduces the blood flow. On the other hand, NLRP3 inflammasome can also induce apoptosis in macrophages which may modulate plaque formation.

**Conclusion**

Inflammasomes are known as intracellular complexes which are able to convert pro-IL-1β and proIL-18 to mature forms and initiate pyroptosis through cleaving pro-caspase-1. NLRP3 is a well-know inflammasome which has central roles in atherosclerosis. It has been shown that NLRP3 inflammasome contributes to the progression of atherosclerosis *via* affecting a sequence of cellular and molecular targets such as STAT, MAPK, JNK, microRNA network, ROS and PKR. Some pathogenic events such as oxidative stress, mitochondrial dysfunction, ER stress, and lysosome

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rupture which are associated with atherosclerosis, could affect inflammasome activation. A broader understanding of biology and activation/inhibition mechanisms of inflammasomes is required to define the value of these complexes as potential therapeutic targets in atherosclerosis.

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Table 1. Role of some inflammasome activators in atherosclerosis.

| Molecule | Effect on inflammasome activation | Source of experimental evidence | Effect on atherosclerosis | Source of experimental evidence | Ref |
|---|---|---|---|---|---|
| **TLR** | • TLR agonists and LPS (TLR4 ligand) upregulate NLRP3 expression<br>TLR9 increases the expression of inflammasome components and the release of IL-1β in human but not mouse macrophages | • Murine macrophages<br>• Human macrophages | • TLR4 induces plaque formation<br>• TLR9 protects against atherosclerosis<br>TLR4 deficiency decreases plaque even in hypercholesterolemic state | • Apo E-/- mouse<br>• Apo E-/- mice genetic deficiency of TLR4 or MyD88 in atherosclerosis-prone Apo E-/- mice | (Qiao et al., 2012)<br>(Barlan et al., 2011)<br>(Koulis et al., 2014)<br>(Hollestelle et al., 2004) |
| **MicroRNA** | • MicroRNA-155 deficiency impairs the activation of the NLRP3 inflammasome<br>• MicroRNA-7 influences NLRP3 | • dendritic cells<br>• microglial in vitro<br>• THP1 cells derived from human lung adenocarcino | ? | - | (Chen et al., 2015a)<br>(Zhou et al., 2016)<br>(Bandyopadhyay et al., 2013) |

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| | gene and significantly reduced NLRP3 protein levels<br>• MicroRNA -133a-1 increases IL-1β release | ma | | | |
| **BRCC3** | • Increases the release of IL-1β by NLRP3 deubiquitination | • LPS-primed peritoneal macrophages of murine | **?** | **-** | (Py et al., 2013) |
| **ATP** | • Extracellular ATP activates NLRP3 inflammasome independent of P2X7R.<br>• NLRP3 inflammasome activation | • bovine monocytes<br>• bone marrow macrophages of murine | • Extracellular ATP promotes atherosclerosis via $P2Y_2$ | • in mice | (Hussen et al., 2012) (Xiang et al., 2013) (Stachon et al., 2016) |
| **P2X7R** | • P2X7R gene silencing prevent NLRP3 inflammasome to be activated<br>• The P2X7R | • in rat<br>• microglial and macrophage cells from P2X7R -/- mice | • Enhanced expression of P2X7R in atherosclerosis<br>• The progression of atherosclerosis is | • aortic sinus of cholesterol-fed Apo E -/- mice<br>• P2X7R knockdown Apo E -/- mice | (Feng et al., 2015) (Franceschini et al., 2015) (Peng et al., 2015) |

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| | directly interacts with the scaffold protein of NLRP3 inflammasome | | reduced by P2X7R siRNA | | |
| **NEK7** | • Is required for NLRP3 inflammasome activation<br>• NLRP3-NEK7 complex formation along with ASC speck | • NEK7 -/- or NLRP3 -/- hematopoietic cells of mouse in vivo | **?** | **-** | (He et al., 2016) |
| **TXNIP** | • participates in NLRP3 inflammasome complex activation<br>• knockdown of TXNIP decreases the activated amount of NLRP3<br>• TXNIP mediates the activation of endothelial NLRP3 | • Macrophages of TXNIP -/- mice<br>• endothelial cells<br>• high-fat diet rats | • TXNIP ablation reduces atherosclerotic lesion by 49% in the rout of aorta and 71% in the abdominal aorta | • Apo E -/- mice | (Zhou et al., 2010)<br>(Liu et al., 2014)<br>(Mohamed et al., 2014)<br>(Byon et al., 2015) |

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| | inflammasome and pushes cell toward death | | | | |
| **$Ca^{2+}$** | • Is essential for IL-1β release | • human macrophage in vitro | • $Ca^{2+}$ channel blockers would have atheroprotective roles<br>• $Ca^{2+}$ antagonists reduce the rate of atherogenesis | • in SMC in vitro and in vivo<br>• long-term clinical trials of $Ca^{2+}$ antagonists | (Rada et al., 2014) (Tulenko et al., 1997) (Hernandez et al., 2003) |
| **NO** | • Suppresses NLRP3 inflammasome-mediated IL-1β secretion and caspase-1 activation | • mouse macrophages | • Endothelial NO synthesis (eNOS ) enzyme protects from atherosclerosis | • carotid arteries of Apo E -/- and eNOS -/- mice | (Mao et al., 2013) (Ponnuswamy et al., 2012) |
| **PKR** | • Normal activated level of NLRP3 inflammasome<br>• Deficiency of PKR decreases the level of IL-1β | • PKR deficient bone marrow derived macrophages<br>• PKR -/- mice | • PKR deficiency significantly protected against atherogenesis | • PKR -/- mice | (Wu et al., 2013) (Stunden and Latz, 2013) (Lu et al., 2012) |
| **MAPK** | • HMGB1 promotes the | • THP-1 macrophages | • P38α MAPK -/- reduces the | • p38α MAPK -/- macrophage | (He et al., 2012) |

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| | synthesis of proIL-1β and proIL-18 in by the activation of p38 MAPK | | amounts of collagen and the fibrous cap<br>• P38α MAPK -/- did not affect the pathogenesis of atherosclerosis | in vivo<br>• P38α MAPK-/- endothelial cell and macrophage of ApoE-/- mice | (Seimon et al., 2009) (Kardakaris et al., 2011) |
| **NADPH oxidase** | • Knockdown of the common NADPH oxidase subunit ,p22phox, diminishes IL-1β secretion | • knockdown - p22 phox mice | • NADPH oxidase 4 prevented the endothelial dysfunction and atherosclerosis development<br>• 50% reduction of atherosclerotic lesion in NOX2 -/y and Apo E -/- mice | • NOX4 -/- LDL receptor -/- mice<br>• NOX2 -/y and Apo E -/- mice | (Dostert et al., 2008) (Langbein et al., 2016) (Judkins et al., 2010) |
| **COX-2** | • Activates NLRP3 inflammasome leading to the increased amount of IL-1β and the pyroptosis | • COX-2 -/- macrophages | • promotes the early stage of atherogenesis<br>• Macrophage COX-2 promoted early atherogenesis | • Apo E -/- mice<br>• LDL Receptor -/- Mice | (Hua et al., 2015) (Burleigh et al., 2005) (Burleigh et al., 2002) |

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| LPO | ? | - | • 15-lipoxygenase enzyme was active in human lesions | • in human in vivo | (Kuhn et al., 1997) |
|---|---|---|---|---|---|
| **Cathepsin B** | • Cathepsin B inhibitor abolishes LCWE-induced inflammasome activation in coronary arterial endothelium | • In mice | • Is upregulated in aortic lesions | • Apo E-/-mice | (Chen et al., 2015b) (Chen et al., 2002) |
| **Ox-mtDNA** | • Participates in inflammasome activation | • human monocytic cell line THP-1 | • Promoted atherosclerosis | • Apo E -/- mice | (Ding et al., 2014) (Yu et al., 2013) |
| **JNK** | • JNK inhibition causes reduced amount of IL-1β and decreased activation of caspase-1<br>• JNK1 and JNK2 participate in IL-1β cleavage | • Peritoneal macrophages<br>• JNK1 and JNK2 knockdown THP-1 macrophages | • JNK1 deficiency increased atherosclerosis through reducing macrophage apoptosis<br>• Loss of JNK2, but not JNK1, reduces plaque formation | • LDL receptor Null mice<br>• Apo E -/- and JNK1-/- mice. | (Hara et al., 2013) (Okada et al., 2014) (Babaev et al., 2016) (Ricci et al., 2004) |

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|---|---|---|---|---|---|
| **SYK** | • SYK inhibition reduces IL-1β secretion and caspase-1 activation<br>• not required for NLRP3 inflammasome | • peritoneal macrophages dendritic cells | • SYK inhibition reduces plaque development | • Apo E -/- mice | (Hara et al., 2013)<br>(Lindau et al., 2016) |
| **ASK-1** | • Activates p38MAPK which is an important factor for IL-1β production. | • ASK-/- macrophages<br>• In mice | • Suppresses hyperlipidemia-induced atherosclerosis via increased macrophage apoptosis | • ASK-/- and Apo E-/- mice | (Hsieh and Papaconstantinou, 2006)<br>(Yamada et al., 2011) |
| **ER stress** | • activates the inflammasome<br>• a UPR-independent pathway activated NLRP3 inflammasome<br>• sensors of ER stress inducing PERK and IRE1α | • In mice<br>• In mice<br>• livers from obese mice | • ER stress/UPR activation is observed in development of different stages of atherosclerotic lesion<br>• Improvement of ER chaperoning function inhibits | • Apo E-/- mice<br>• In macrophage | (Lebeaupin et al., 2015)<br>(Menu et al., 2012)<br>(Lebeaupin et al., 2015)<br>(Dickhout et al., 2007)<br>(Erbay et al., 2009) |

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| | | | | | |
|---|---|---|---|---|---|
| | increased the activity of the NLRP3 inflammasome | | plaque development induced by toxic lipids | | |

TLR: Toll-like receptor, BRCC3*:* BRCA1/BRCA2-Containing Complex Subunit 3, ATP : adenosine triphosphate, P2X7R: P2X7 receptor, *NEK7:* NIMA Related Kinase 7, Txnip*:* Thioredoxin-interacting protein, $Ca^{2+}$: Calcium ions, NO: Nitric oxide, PKR: protein kinase *R,* MAPK : mitogen-activated protein kinase, NADPH oxidase: nicotinamide adenine dinucleotide phosphate *oxidase*, COX-2: Cyclooxygenase 2, LPO: Lactoperoxidase, Ox-mtDNA: Oxidized mitochondrial DNA, JNK: c-Jun N-terminal kinase, SYK: Spleen tyrosine kinase, ASK1: Apoptosis signal-regulating kinase 1, ER: endoplasmic reticulum, PERK : PKR-like ER kinase and IRE1α: Inositol Requiring 1 alpha

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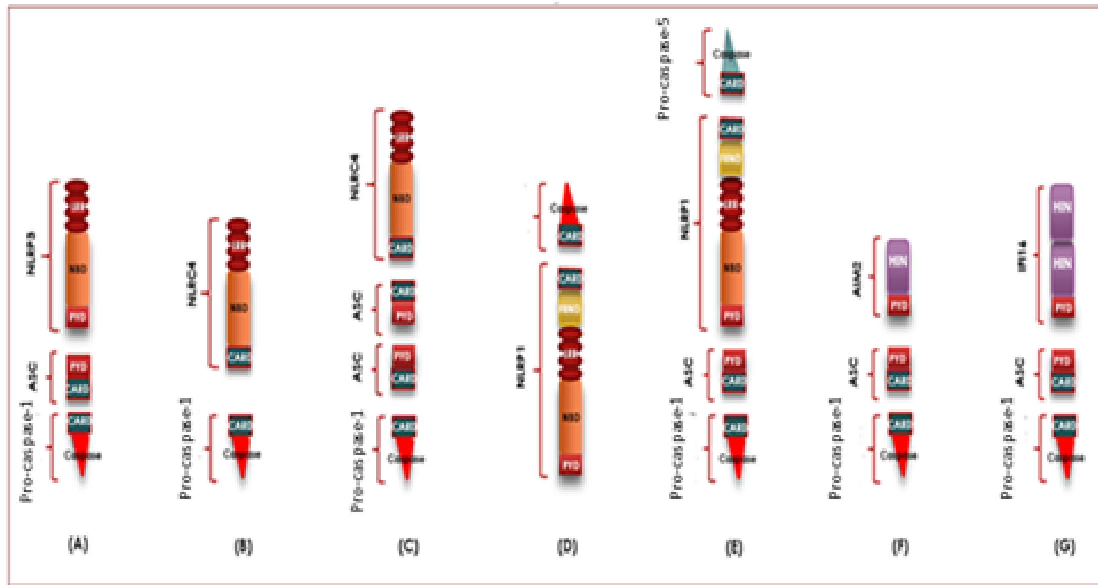

Figure 1: The interaction between the components of each inflammasome. A-E NLR, F and G PYHIN

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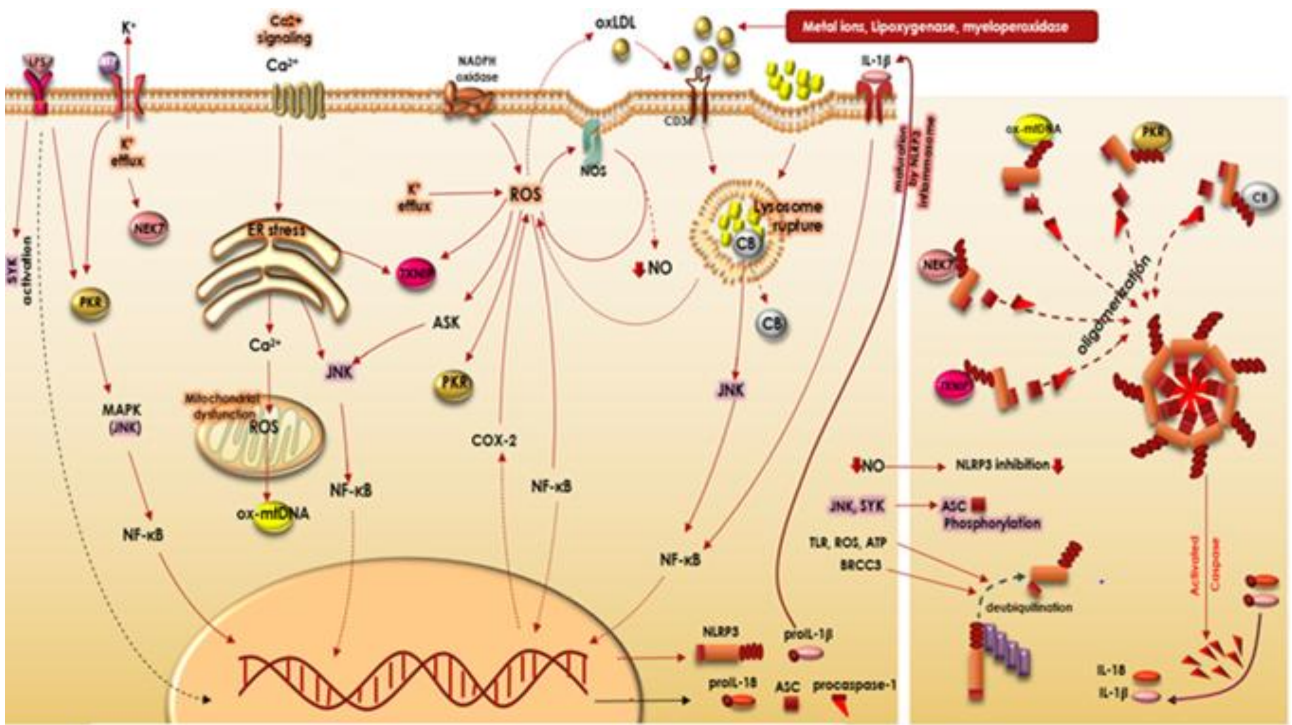


Figure 2: Activation of the NLRP3 inflammasome.

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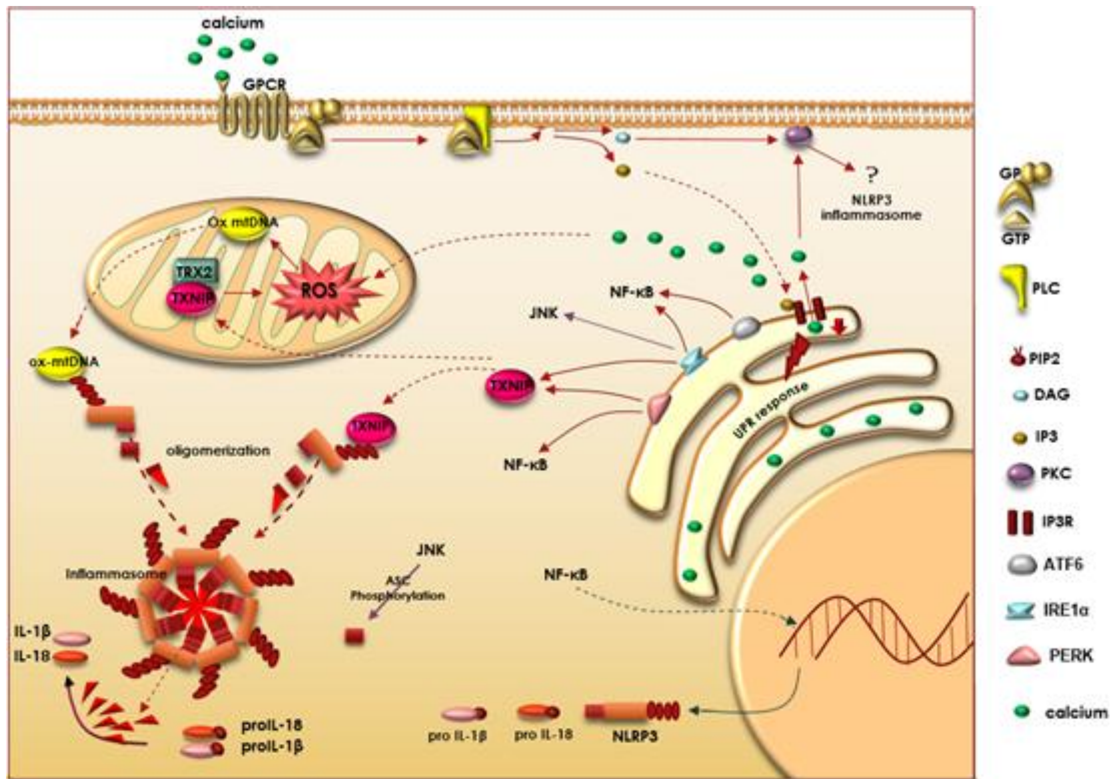


Figure 3: Role of ER stress, mitochondrial dysfunction, and Ca2+ signaling in NLRP3 inflammasome activation

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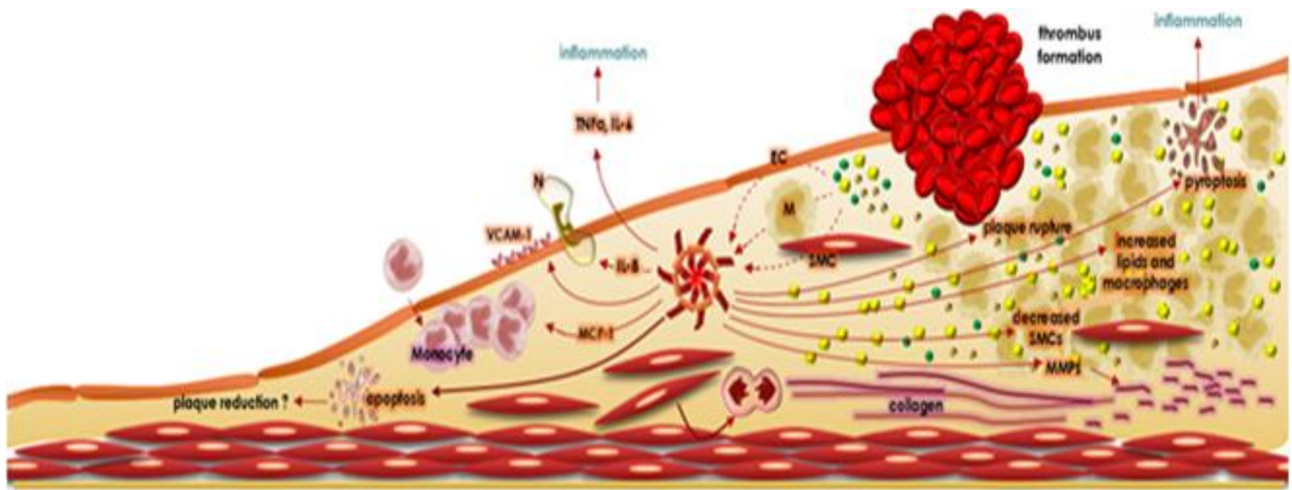


Figure 4: Role of NLRP3 inflammasome in plaque formation. OxLDL ( ), crystalline cholesterol ( ), and Ca2+ ( ) are found in abundant in atherosclerotic lesions which cause endothelial cells (EC), smooth muscle cells (SMC), and macrophages (M) to activate NLRP3 inflammasome within their cytoplasm

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