



# Role of NLRP3 Inflammasomes in Atherosclerosis

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Inflammation with macrophage infiltration is a key feature of atherosclerosis. Although the mechanisms had been unclear, emerging evidence unveiled that NLRP3 inflammasomes, which regulate caspase-1 activation and subsequent processing of pro-IL-1 $\beta$ , trigger vascular wall inflammatory responses and lead to progression of atherosclerosis. NLRP3 inflammasomes are activated by various danger signals, such as cholesterol crystals, calcium phosphate crystals, and oxidized low-density lipoprotein in macrophages, to initiate inflammatory responses in the atherosclerotic lesion. Recent studies have further clarified the regulatory mechanisms and the potential therapeutic agents that target NLRP3 inflammasomes. In this study, we reviewed the present state of knowledge on the role of NLRP3 inflammasomes in the pathogenesis of atherosclerosis and discussed the therapeutic approaches that target NLRP3 inflammasomes.

**Key words:** Cholesterol, Cytokines, Inflammation, Interleukin-1, Leukocytes

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## Introduction

Atherosclerosis is a chronic inflammatory disease characterized by lipid deposition, leukocyte infiltration, and proliferation of vascular smooth muscle cells (VSMCs)<sup>1,2</sup>. The inflammatory nature of atherosclerosis has been proven by several findings as follows: 1) the presence of inflammatory cells, mainly macrophages, which infiltrate into the atherosclerotic plaques and the correlation between the number of these cells and disease severity; 2) the presence of inflammatory cytokines and chemokines in all stages of atherosclerosis<sup>3</sup>; and 3) the widely accepted association between cardiovascular events and serum inflammatory markers, such as C-reactive protein and interleukin (IL)-6<sup>3-6</sup>. Until recently, however, the molecular basis of these inflammatory responses in atherosclerotic lesions has not been elucidated.

Inflammation is defined as the process by which the body responds to injury or infection and is mediated by components of the innate immune system, such as neutrophils and macrophages. The innate

immune system has been recognized as first-line defense against invading pathogens and plays a primary role in acute host defense; this concept was referred to as the “self–non-self theory” or the “stranger theory”<sup>7</sup>. In the 1990s, Polly Matzinger suggested a new concept called the “danger theory”<sup>8</sup>, which claimed that immune responses can be activated by danger signals, released from injured tissues or cells, as well as by the presence of pathogens. These danger signals can be recognized by pattern recognition receptors (PRRs) and stimulate immune responses similar to those triggered by pathogens. This danger theory has recently received considerable attention because it may explain activation of the innate immune system in the absence of pathogens. PRRs are evolutionary conserved and expressed in the innate immune system cells, such as macrophages, neutrophils, and dendritic cells, and can identify two types of molecular patterns: pathogen-associated molecular patterns (PAMPs) and damage/danger-associated molecular patterns (DAMPs). PRRs are classified into at least four groups: Toll-like receptors (TLR), nucleotide-binding oligomerization domain-like receptors (NLRs), retinoic acid-inducible gene (RIG)-I-like receptors, (RLRs), and C-type lectin receptors (CLRs)<sup>9</sup>. These clusters of PRRs exhibit distinct intracellular localization and recognize different PAMPs and DAMPs. In particular, NLR pyrin domain containing 3 (NLRP3), a member of the NLR family, recognizes various DAMPs to form an NLRP3

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Received: December 28, 2016

Accepted for publication: January 10, 2017

inflammasome molecular complex in the cytosol and leads to inflammatory responses. Indeed, recent studies demonstrated that NLRP3 inflammasome-driven inflammatory responses contributed to the progression of atherosclerosis<sup>10-13</sup>. This study highlighted the present state of knowledge on the role of NLRP3 inflammasomes in the pathogenesis of atherosclerosis.

## Inflammasomes

An inflammasome is a cytoplasmic complex containing multiple proteins, which is formed in response to DAMPs or PAMPs, and serves as a molecular platform for activation of the cysteine protease caspase-1<sup>14</sup>. It typically contains one NLR protein, an apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC), and the cysteine protease caspase-1. Since caspase-1 was originally identified as an IL-1 converting enzyme (ICE)<sup>15</sup>, inflammasome-mediated activation of caspase-1 promotes processing of the IL-1 $\beta$  precursor, a potent inflammatory cytokine, and induces mature IL-1 $\beta$  release to cause tissue inflammatory responses. Similar to IL-1 $\beta$ , caspase-1 can process pro-IL-18 into its mature form, which is another IL-1 family cytokine<sup>16</sup>. Several types of inflammasome complexes have been reported; among the NLRs, NLRP1, NLRP3, NLRC4, NLRP6, and NLRP12 can participate in the inflammasomes<sup>17</sup>. Similar to NLRs, the pyrin and HIN domain-containing protein (PYHIN) family, including absence in melanoma 2 (AIM2) and IFN- $\gamma$ -inducible protein 16 (IFI16), is also a component of inflammasomes. In general, inflammasomes are named according to the specific NLR that it contains; therefore, NLRP3 inflammasomes are composed of NLRP3, ASC, and caspase-1 (**Fig. 1**).

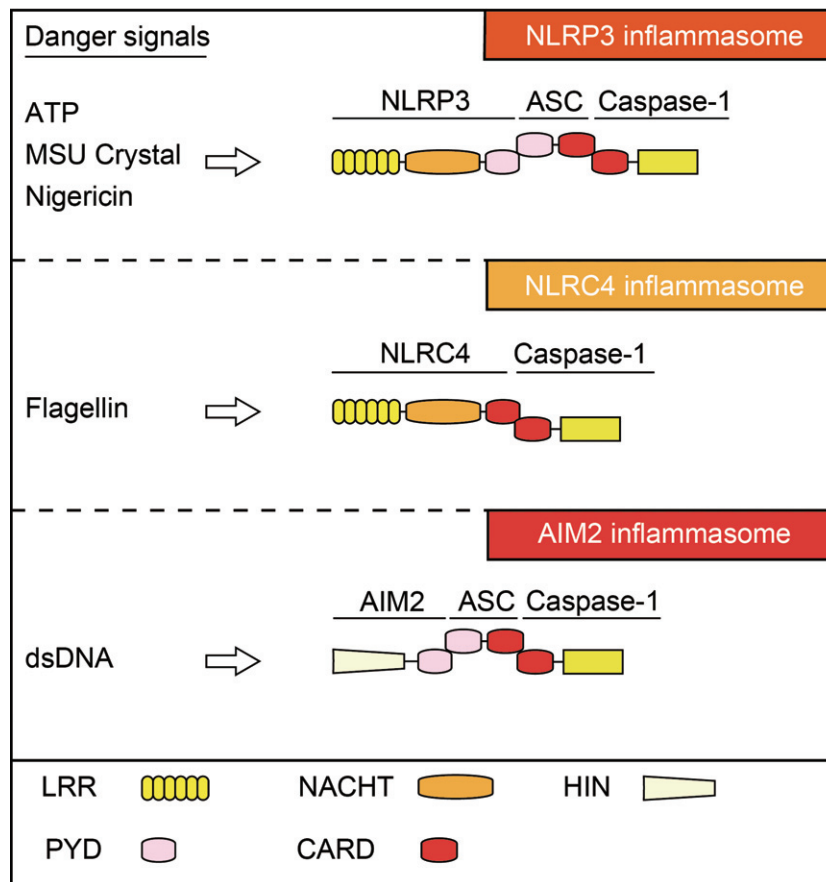
## NLRP3 Inflammasomes

Unlike other inflammasomes, NLRP3 inflammasomes are activated by endogenous or exogenous DAMPs and are involved in the process of sterile inflammation<sup>17</sup>. As described above, NLRP3 inflammasomes are composed of NLRP3, ASC, and caspase-1, which have conserved domains for homophilic interaction (**Fig. 1**). NLRP3 contains three domains: C-terminal leucine-rich repeats (LRRs), a central nucleotide domain termed as the NACHT domain, and an N-terminal effector domain (pyrin domain [PYD]). ASC contains an N-terminal PYD and a C-terminal caspase recruitment domain (CARD). Caspase-1 contains a CARD and catalytic domains (p10 and p20). When cells are stimulated by DAMPs, NLRP3 assembles by the NACHT domain to provide

a scaffold for ASC oligomerization by their interaction between PYDs. In turn, because NLRP3 lacks CARD and cannot recruit caspase-1 except in the presence of ASC, the oligomerized ASC interacts with caspase-1 via CARD homophilic interaction and induces caspase-1 auto-activation to exert pro-inflammatory effects by its proteolytic activity. As mentioned above, caspase-1 also processes pro-IL-18 to its bioactive mature form. In addition, recent studies showed that caspase-1 cleaves gasdermin D (GSDMD) to induce pyroptosis, which is an inflammatory programmed cell death accompanied by increased plasma membrane permeability<sup>18, 19</sup>. Although IL-1 $\beta$  has no signal sequence for exocytosis and the mechanism of its release is still unknown, this pyroptosis-mediated membrane permeabilization is critical for IL-1 $\beta$  release<sup>20</sup>.

Although various endogenous and exogenous danger signals, such as extracellular adenosine triphosphate (ATP), monosodium urate (MSU) crystal, and silica, are known to activate NLRP3 inflammasomes, the precise mechanism of NLRP3 recognition of DAMPs remains unclear<sup>21, 22</sup>. Nevertheless, several common upstream pathways, including potassium (K<sup>+</sup>) efflux, generation of mitochondrial reactive oxygen species (ROS), and lysosomal destabilization, have been reported to be important for activation of NLRP3 inflammasomes<sup>23</sup>. Of these, it has been well accepted that lysosomal destabilization and a subsequent cathepsin release is responsible for NLRP3 inflammasome activation by particulate matters. In addition, K<sup>+</sup> efflux inhibition prevents NLRP3 inflammasome activation in response to most or all NLRP3 stimuli, including particulate matters, suggesting K<sup>+</sup> efflux as a common trigger of its activation<sup>24, 25</sup>. The production and release of IL-1 $\beta$  is regulated in two-steps: Transcriptional synthesis of pro-IL-1 $\beta$  and proteolytic processing into a mature form by inflammasomes (**Fig. 2**). The transcriptional regulation of IL-1 $\beta$  mRNA is mediated by PRRs or cytokine receptors, including TLRs and IL-1 receptor (signal 1), and is known as priming. Apart from NF- $\kappa$ B-mediated mRNA induction of IL-1 $\beta$  and NLRP3, activation of these receptors has also been shown to prime the NLRP3 by posttranscriptional regulation, such as ubiquitination and deubiquitination<sup>26, 27</sup>. Thereafter, the accumulated pro-IL-1 $\beta$  in the cytosol is rapidly processed by activated caspase-1, which is activated by NLRP3 inflammasomes (signal 2). Thus, this two-step system is considered to be necessary for tight regulation of the potent inflammatory cytokine IL-1 $\beta$  to maintain inflammatory homeostasis.

At first, NLRP3, which is also known as *CIAS1* gene that encodes the cryopyrin protein, was identified by a gain-of-function mutation in a gene that was



**Fig. 1.** Representative inflammasomes and their components

Several PRRs that recognize distinct DAMPs form the inflammasome complex, which serves as the molecular platform to activate caspase-1. NLRP3 inflammasomes are composed of NLRP3, ASC, and caspase-1. NLRP3 binds ASC via PYD-PYD interaction. Subsequently, ASC binds caspase-1 via CARD-CARD interaction. NLRC4 inflammasomes are composed of NLRC4 and caspase-1, whereas AIM2 inflammasomes are composed of AIM2, ASC, and caspase-1. ATP, adenosine triphosphate; CARD, caspase recruitment domain; DAMPs, damage/danger-associated molecular patterns; HIN, hematopoietic interferon-inducible protein with a 200-amino-acid repeat; LRR, leucine-rich repeats; MSU, monosodium urate; NACHT, found in NAIP, CITA, HET-E, and TP1; NLR, nucleotide-binding oligomerization domain-like receptor; PRRs, pattern recognition receptors; PYD, pyrin domain.

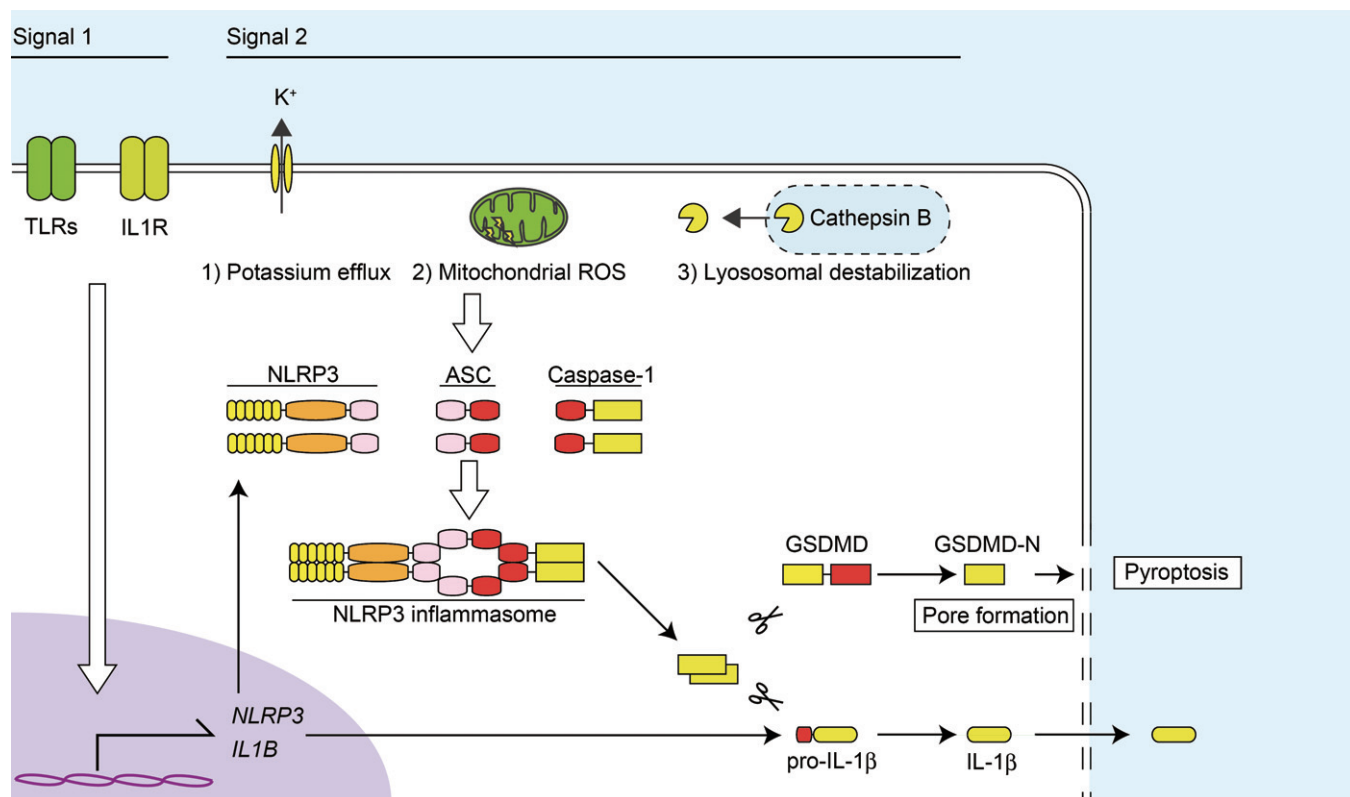
associated with autoinflammatory syndrome, cryopyrin-associated periodic syndrome (CAPS). CAPS includes three different conditions, including familial cold autoinflammatory syndrome, Muckle-Wells syndrome, and chronic infantile neurological cutaneous and articular syndromes<sup>28</sup>. Since CAPS is an extremely rare genetic disease, only a few studies on NLRP3 inflammasomes had been conducted. In 2006, however, uric acid crystals in gout were discovered to activate NLRP3 inflammasomes<sup>22</sup>. This discovery motivated further studies on the association between NLRP3 inflammasomes and sterile inflammatory diseases. Indeed, other research workers and we have revealed a pivotal role of NLRP3 inflammasome in

the pathophysiology of cardiovascular and renal diseases, including atherosclerosis, vascular injury, aortic aneurysm, myocardial infarction, chronic kidney disease, and acute kidney injury<sup>10, 11, 29-33</sup>. Furthermore, in the last decade, this premise was further supported by studies that described NLRP3 inflammasomes as a key mediator of other diseases associated with sterile inflammation, including type 2 diabetes and metabolic syndrome<sup>34, 35</sup>.

### NLRP3 Inflammasome in Atherosclerosis

#### Role of NLRP3 Inflammasomes in Atherosclerosis

Many clinical and experimental studies have



**Fig. 2.** Mechanisms of NLRP3 inflammasome-driven IL-1 $\beta$  release

IL-1 $\beta$  release is regulated in two-steps: Transcriptional synthesis of pro-IL-1 $\beta$  and proteolytic processing into its mature form by inflammasomes. The transcriptional regulation of IL-1 $\beta$  mRNA is mediated by TLRs and IL-1 receptor (signal 1), which also provides NLRP3 mRNA. Then, the NLRP3 inflammasome-activated caspase-1 processes accumulated pro-IL-1 $\beta$  and induces the release of IL-1 $\beta$  (signal 2). The common upstream pathways of NLRP3 inflammasomes include potassium efflux, generation of mitochondrial ROS, and lysosomal destabilization and leakage of cathepsin B. Activated caspase-1 also cleaves GSDMD, whose processed N-terminal fragment (GSDMD-N) increases plasma membrane permeability, resulting in pyroptosis. ROS, reactive oxygen species; TLRs, Toll-like receptors.

reported that IL-1 $\beta$  plays a crucial role in the progression of atherosclerosis and identified IL-1 $\beta$  as a pro-atherogenic cytokine<sup>36-38</sup>. These findings suggested that NLRP3 inflammasomes may contribute to the progression of atherosclerosis. In 2010, Duewell *et al.*<sup>10</sup> were the first to show the important role of NLRP3 inflammasomes in the progression of atherosclerosis. Using atherosclerosis-prone, low-density lipoprotein receptor-deficient (LDLR<sup>-/-</sup>) mice, they produced chimeric mice whose bone marrows were transplanted with NLRP3<sup>-/-</sup>, ASC<sup>-/-</sup>, or IL-1 $\alpha/\beta$ <sup>-/-</sup> bone marrow cells and showed that the lack of these inflammasome-related molecules in the bone marrow-derived inflammatory cells decreased atherosclerotic lesion development. Because cholesterol crystal formation is present in the early stages and can be detected in all stages of atherosclerosis, Duewell *et al.*<sup>10</sup> focused on cholesterol crystals as the candidate of DAMPs and found that these crystals strongly activated NLRP3 inflammasomes in macrophages. Interestingly, they further

showed that the presence of oxidized low-density lipoprotein (LDL), which exhibits highly atherogenic properties, can lead to cholesterol crystallization and activation of priming signals to induce NLRP3 and pro-IL-1 $\beta$  expressions; these indicated that oxidized LDL could sufficiently provide signal 1 and signal 2 to induce IL-1 $\beta$  release. According to a later study by Sheedy *et al.*<sup>39</sup>, incorporation of oxidized LDL via a scavenger receptor CD36 provoked intracellular cholesterol crystallization. Together with the findings that the lack of IL-1 $\beta$  decreased the severity of atherosclerosis in ApoE<sup>-/-</sup> mice<sup>36</sup>, which are frequently used as another atherosclerosis-prone model, these observations indicated that NLRP3 inflammasome-driven IL-1 $\beta$  release contributed to the progression of atherosclerosis. However, Menu *et al.*<sup>40</sup> reported that neither NLRP3 and ASC nor caspase-1 deficiency could prevent macrophage infiltration and atherosclerosis in ApoE<sup>-/-</sup> mice. Using a similar experimental model, we observed that caspase-1 deficiency clearly reduced mac-

**Table 1.** Effects of NLRP3 inflammasome deficiency on progression of atherosclerosis in mice

Reference	Model	Diet	Deficient gene	Lesion size	
Duewell <i>et al.</i>	2010 Ref. 8	Ldlr <sup>-/-</sup>	WTD (0.15% Cho)	Nlrp3, Asc (BMT)	decrease
Menu <i>et al.</i>	2011 Ref. 38	ApoE <sup>-/-</sup>	HFD (1.25% Cho)	Nlrp3, Asc, Casp1/11	no change
Usui <i>et al.</i>	2012 Ref. 9	ApoE <sup>-/-</sup>	WTD (0.15% Cho)	Casp1/11	decrease
Gage <i>et al.</i>	2012 Ref. 10	ApoE <sup>-/-</sup>	HFD (1.5% Cho)	Casp1/11	decrease
Hendrikx <i>et al.</i>	2015 Ref. 11	Ldlr <sup>-/-</sup>	HFD (0.2% Cho)	Casp1/11 (BMT)	decrease

Abbreviation: BMT, bone marrow transplant; Cho, cholesterol; HFD, high fat diet; WTD, western diet

rophage infiltration and development of atherosclerotic lesions in ApoE<sup>-/-</sup> mice<sup>11</sup>). The reason for this discrepancy is not entirely clear, but the more atherogenic diet used in that study, in comparison with our study, may have influenced the immune status and inflammatory responses. In addition to the studies mentioned above, several studies have described the role of NLRP3 inflammasomes in the progression of atherosclerosis (Table 1). It should be noted that the conventional caspase-1<sup>-/-</sup> mouse strain lacks both caspase-1 and caspase-11 because these genes were quite close in the genome to have been segregated.

### Mechanisms of NLRP3 Inflammasome Activation in Atherosclerosis

The mechanism of cholesterol crystal-induced NLRP3 inflammasome activation has been described. Since vascular wall macrophage infiltration is the hallmark of atherosclerosis<sup>4,41</sup>, studies have mainly focused on the role of NLRP3 inflammasomes in macrophages. Indeed, the components of NLRP3 inflammasomes are expressed by macrophages and are highly upregulated by priming signals (*e.g.*, TLR and IL-1R). Duewell *et al.*<sup>8</sup> found that cholesterol crystals were phagocytosed by macrophages, but induced phagolysosomal destabilization and rupture because phagocytosis cannot progress appropriately. This process has been known as frustrated phagocytosis wherein the phagocytosed crystals cannot be engulfed or digested. In such cases, lysosomal rupture induces leakage of the lysosomal enzyme cathepsin B, resulting in NLRP3 inflammasome activation (Fig. 3). Similar results were reported by Rajamaki *et al.*<sup>42</sup> on human macrophages. Furthermore, a recent study suggested that the complement system was involved in NLRP3 inflammasome activation by cholesterol crystals<sup>43</sup>. Calcium phosphate is also a particulate matter, which can accumulate in atherosclerotic lesions and is associated with vascular calcification. In this regard, other investigators and we showed that calcium phosphate crystals, including hydroxyl apatite and tricalcium phosphate, were also phagocytosed and activated NLRP3 inflammasomes through lysosomal rupture and cathepsin B<sup>11</sup>). At pres-

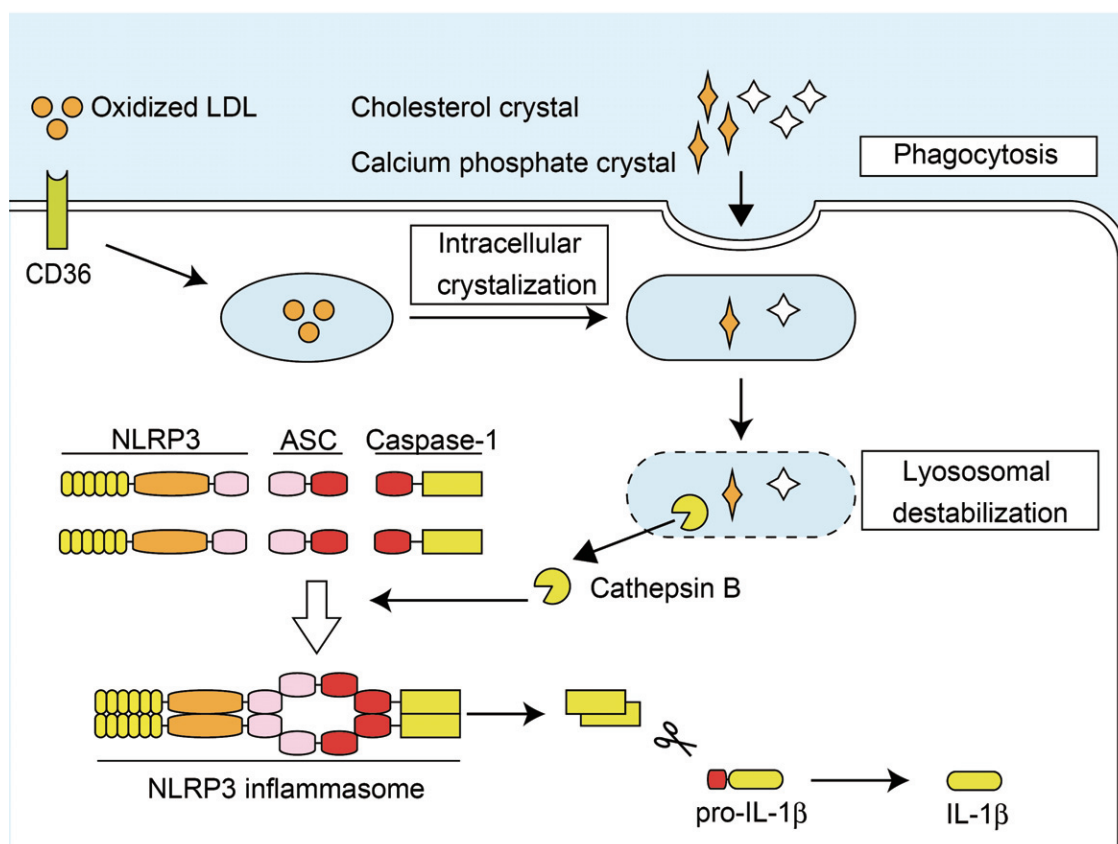
ent, however, the mechanism of NLRP3 inflammasome in sensing cathepsin B release by crystal-induced lysosomal destabilization remains undefined.

Recent studies uncovered an association between NLRP3 inflammasomes and metabolic disease-related lipid species, such as saturated fatty acids (SFAs), ceramide, and 7-ketocholesterol<sup>34, 35, 40</sup>. Palmitic acid and stearic acid, the major species of SFAs, activate NLRP3 inflammasomes via mitochondrial ROS production and lysosomal destabilization<sup>35, 44</sup>. An imbalance between SFAs and unsaturated fatty acids (USFAs) was assumed to play a causative role in this response because USFAs could inhibit SFA-induced activation of NLRP3 inflammasomes<sup>45, 46</sup>.

### Regulation of NLRP3 Inflammasomes in Atherosclerosis

The negative regulation of NLRP3 inflammasomes is a potential therapeutic target for atherosclerosis. Recent studies have shown that autophagy, an intracellular degradation system to maintain cellular homeostasis, negatively regulated NLRP3 inflammasome activation through several mechanisms<sup>47, 48</sup>. Because mitochondrial ROS is important for NLRP3 inflammasome activation in response to many stimuli, clearance of damaged mitochondria by autophagy (mitophagy) can inhibit its activation<sup>47</sup>. Another study showed that autophagy was able to capture and degrade the assembled complex of NLRP3 inflammasomes via its ubiquitination and modulate its activity<sup>48</sup>. Indeed, autophagy-defective Atg 5<sup>-/-</sup> mice developed accelerated atherosclerosis, accompanied by enhanced activation of NLRP3 inflammasomes<sup>49</sup>. Furthermore, because lysosome is an essential organelle for autolysosome formation and degradation of a particulate matter, activation of lysosome biogenesis by overexpression of transcription factor EB (TFEB) in macrophages has been shown to inhibit cholesterol crystal-induced NLRP3 inflammasome activation and attenuate the progression of atherosclerosis<sup>50</sup>.

Phosphorylation of protein kinase A (PKA) was reported to be a negative regulator of NLRP3 inflammasomes<sup>51</sup>. Transmembrane G protein-coupled recep-



**Fig. 3.** Proposed mechanism of NLRP3 inflammasome activation in atherosclerosis

Cholesterol crystals are incorporated into the lysosome by phagocytosis or formed intracellularly when oxidized LDL is incorporated with CD36. Overloaded crystals cause lysosomal destabilization and rupture, which induce leakage of the lysosomal enzyme cathepsin B, resulting in the activation of NLRP3 inflammasomes. Calcium phosphate crystals also activate NLRP3 inflammasomes through a similar mechanism. LDL, low-density lipoprotein.

tor 5 (TGR5) bile acid receptor-induced PKA activation leads to the ubiquitination of NLRP3, which is associated with the PKA-induced NLRP3 phosphorylation (Ser 291). Although TGR5 agonists prevent the progression of atherosclerosis by increased cholesterol efflux and alleviated inflammatory responses, anti-inflammatory mechanisms were not fully elucidated<sup>52</sup>. Therefore, further studies are necessary to clarify the precise mechanisms of NLRP3 inflammasome regulation in atherosclerosis.

### Concluding Remarks

Increasing evidence indicated the importance of NLRP3 inflammasomes in the pathophysiology of sterile inflammatory diseases. In the progression of atherosclerosis, cholesterol and/or calcium phosphate crystals activate NLRP3 inflammasomes and induce subsequent IL-1 $\beta$  release, leading to vascular inflammation. In a similar manner, using a wire-mediated vas-

cular injury mice model, we previously demonstrated that ASC was upregulated at the site of vascular injury and that its deficiency significantly attenuated IL-1 $\beta$  expression and neointimal formation after vascular injury<sup>29</sup>. In addition, inflammasome activation in bone marrow-derived inflammatory cells was demonstrated to be essential for neointimal formation in bone marrow chimeric mice. More recently, we found that NLRP3 inflammasome activation by mitochondrial oxidative stress on adventitial macrophages led to the development of abdominal aortic aneurysm in ApoE<sup>-/-</sup> mice infused with angiotensin II<sup>30</sup>. The importance of inflammasomes in adventitial macrophages was further supported by the finding that ASC was expressed in the cells of patients with abdominal aortic aneurysm. Therefore, these findings strongly suggested that NLRP3 inflammasomes were a key mediator of inflammation-related vascular diseases. A better understanding of the molecular mechanisms underlying NLRP3 inflammasome activation and development of specific

inhibitors will offer new therapeutic or preventive options and break new grounds for studying the role of inflammation in atherosclerosis as well as in other vascular diseases.

### Conflict of Interest

None.

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