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Review article

NLRP3 inflammasome pathways in atherosclerosis

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ABSTRACT

Atherosclerosis is the major cause of death and disability. Atherosclerotic plaques are characterized by a chronic sterile inflammation in the large blood vessels, where lipid-derived and damage-associated molecular patterns play important roles in inciting immune responses. Following the initial demonstration that NLR family Pyrin domain containing 3 (NLRP3) was important for atherogenesis, a substantial number of studies have emerged addressing the basic mechanisms of inflammasome activation and their relevance to atherosclerosis. In this review, we introduce the basic cellular and molecular mechanisms of NLRP3 inflammasome activation, and discuss the current findings and therapeutic strategies that target NLRP3 inflammasome activation during the development and progression of atherosclerosis.

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1. Introduction

1.1. Atherosclerosis

Atherosclerosis is the leading cause of death in the developed societies. Atherosclerosis is a progressive cardiovascular disease with lipid deposition in large arteries at areas of disturbed flow, resulting in the formation of atherosclerotic plaques. Plaque rupture or erosion can cause acute cardiovascular events, such as heart attack and stroke. Inflammation plays an important role, both in the development and complications of atherosclerosis [1,2]. In particular, inflammasome pathways have been extensively explored in the past decade and shown to be involved in regulating multiple inflammatory diseases, including atherosclerosis [3]. The rationale for the CANTOS trial testing the efficacy of IL-1β blockade in patients with coronary artery disease was largely based on the role of inflammasome-mediated activation of IL-1β in experimental models of atherosclerosis. Hence, the regulation of inflammasome activity in atherosclerosis has been largely explored at multiple levels.

1.2. NLRP3 inflammasome and its canonical activation

Inflammasomes are multiprotein signalling complexes. They play key roles in the mediation of innate inflammatory responses and are assembled in response to a wide range of stimuli including both PAMPs (Pathogen-Associated Molecular Patterns) and DAMPs (Damage-Associated Molecular Patterns).

Among all inflammasomes, NLRP3 has been more extensively studied and characterized, because of its crucial role in immunity and inflammation [4,5]. The key components of this signalling platform are NLRP3 protein, its adaptor protein ASC (apoptosis-associated speck-like protein containing a CARD), and Caspase-1, which is cleaved by this multiprotein complex. Cleavage of pro-Caspase-1 yields active Caspase-1, a proteolytic enzyme that in turn cleaves the pro-inflammatory cytokines IL-1β and IL-18 into their active forms, which induce inflammatory responses.

Apart from release of pro-inflammatory signals, canonical inflammasome activation leads to a special form of cell death called pyroptosis. Pyroptosis, or Caspase-1-dependent cell death, is a cellular program of self-destruction. Caspase 1 cleaves Gasdermin D, and active Gasdermin D oligomers form membrane pores leading to loss of cell integrity [6]. Pyroptosis is associated with the release of mature IL-1β and IL-18, and is characterized by cellular swelling and lysis [7]. Both cytokines can be released with different mechanisms, such as: (1) secretion through pores [7], (2) shedding in microvesicles [8,9] and (3) lysosome-mediated exocytosis [10]. Furthermore, with loss of cell integrity, more DAMPs are released

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from pyroptotic cells, including IL-1α, HMGB1 and ATP. These signals lead to further activation of the immune response.

NLRP3 inflammasome activation has been more extensively studied in macrophages, but it has been described in other cell types as well, including smooth muscle cells (SMC) [11], endothelial cells [12] and T cells [13–17]. Moreover, the roles of NLRP3 may extend beyond the regulation of the inflammasome pathway or innate immune responses. For example, NLRP3 is important for Th17 differentiation in a ASC-dependent manner [16,17] and NLRP3 can also behave as a transcriptional regulator, to drive inflammasome-independent Th2 differentiation [14]. In addition, NLRP3 activation in $CD4^+$ human T cells promotes autocrine Th1 differentiation with a mechanism that is dependent on activation of the intracellular C5a receptor 1 [18].

1.3. Molecular and cellular players involved in the regulation of canonical NLRP3 inflammasome priming and activation

In canonical NLRP3 inflammasome activation, two signals, a priming signal and an activation signal, are necessary for activation of NLRP3. This dual requirement acts as a safeguarding mechanism that tightly controls inflammatory cell activation.

NLRP3 is present at low levels in all myeloid cells but its expression increases when cells are primed in response to stimuli [19,20]. The priming signal induces the expression of NLRP3 and pro-IL-1β at the transcriptional level through activation of the NF-κB pathway [20]. The priming step can be modulated by both post-transcriptional and post-translational modifications of NLRP3.

Post-transcriptional modulation of NLRP3 expression can occur by regulatory RNA such as the negative regulator miR-223, a myeloid-specific microRNA that shows different expression levels across myeloid cell types. miR-223 suppresses NLRP3 expression by binding to a conserved region of its sequence [21]. Furthermore, recent evidence points to a role of the RNA-binding protein Tristetraprolin (TTP), which acts as a negative regulator of NLRP3 in human macrophages by binding to NLRP3 3′ UTR [22].

Post-translational modifications of NLRP3 protein play important roles in regulating its function. NLRP3 is deubiquitinated upon priming, leading to reduced proteasomal degradation and hence increased life span of the protein [23,24]. In mouse macrophages, deubiquitination is mediated by TLR4 through MyD88 [24]. Modification of NLRP3 by nitrosylation occurs upon activation of IFNγ receptor, and it has an inhibitory effect on NLRP3 protein, preventing its oligomerization [25]. The activity of NLRP3 is also regulated through phosphorylation of different serine sites, with phosphorylation at serine 5 having a particularly important role in NLRP3 inflammasome inhibition [26]. Phosphatase PP2A can dephosphorylate NLRP3 leading to its activation [26].

The activation step of NLRP3 inflammasome is under intense investigation. A wide range of stimuli converges on NLRP3 inflammasome complex for full activation, indicating that there are multiple pathways and players involved. A simple model of direct interaction of the different signals with NLRP3 protein is very unlikely, because of the diversity and variety of NLRP3 inflammasome activating stimuli [27,28].

Potassium efflux has emerged as a key step leading to inflammasome activation, but the precise link between the efflux of potassium ions and NLRP3 inflammasome assembly is not completely understood [29]. When potassium efflux is inhibited, NLRP3 inflammasome activation and IL-1β maturation are compromised [30,31]. Activating stimuli that are dependent on potassium ions efflux include: (1) extracellular ATP, by activation of the non-selective P2X7 cation channel, which results in localized K+ efflux from macrophages, in proximity to the site of activation [32,33]; (2) K+ efflux agonists such as the bacterial ionophore nigericin [34]; (3) bacterial pore-forming toxins [29]; (4) some antibiotics, including neomycin, polymyxin B, gramicidin and tyrothricin [35].

Tschopp and colleagues initially proposed a model in which different NLRP3-activating stimuli converge on mitochondria, resulting in excessive production of Reactive Oxygen Species (ROS), which then trigger inflammasome activation [36,37]. Stimuli that converge on ROS production include ATP [36], asbestos [38], silica [38], and endoplasmic reticulum (ER) stress [39]. Autophagy and mitophagy may regulate this pathway by removing damaged mitochondria, and impairment of mitophagy is suggested to trigger inflammasome activation through accumulation of ROS species [37,40]. However, production of ROS by mitochondria may not be a universal mechanism of inflammasome activation, but rather by-products of NLRP3 inflammasome activation [29,41].

Lysosome damage occurs upon cellular intake of crystals, such as cholesterol crystals [3,42], silica and monosodium urate (MSU) crystals [38,43]. Lysosome damage activates the NLRP3 inflammasome through the release of cathepsins [44], which may act as a converging mechanism in regulating particle-induced inflammasome activation. Other proposed regulatory mechanisms of NLRP3 inflammasome activation involve aberrant calcium signalling [45,46], and regulation of P2X7 receptor signalling [47].

Integrity of the cellular microtubule network is crucial for inflammasome activation [48,49]. Low NAD+ levels trigger inflammasome activation by preventing SIRT2 activation, leading to accumulation of acetylated α-tubulin, a mediator of mitochondrial transport [48]. More importantly, NLRP3 is transported along the microtubules to access mitochondria, then reaches the microtubule-organizing centre, where it forms the stereotypical inflammasome complex speck structure, which ensures an optimal inflammasome activation [49]. Shear stress is also known to affect the actin cytoskeleton in endothelial cells [50]. In particular, F-actin has an inhibitory role on NLRP3 inflammasome activation [51] and it is therefore likely that this protein is involved in licensing of the NLRP3 inflammasome upon shear stress. Consistent with this hypothesis, cell swelling, a process that leads to a decrease in F-actin levels [52], results in NLRP3 inflammasome activation [53].

A variety of novel players have been identified recently, that directly interact with NLRP3 and modulate its function: (1) Guanylate Binding Protein 5 (GBP5), a selective activator of NLRP3 inflammasome assembly, binds directly to NLRP3 to promote ASC assembly via a PYD/CARD interaction in response to soluble (but not crystalline) danger signals [54]; (2) the mitochondrial adaptor Mitochondrial Antiviral Signalling Protein (MAVS), which interacts with the N-terminal domain of NLRP3 and is crucial for correct mitochondrial localization and optimal inflammasome activation [55]; (3) NimA-related protein kinase 7 (NEK7), which acts downstream of the K+ efflux stimulus, interacts with NLRP3 and is essential for NLRP3 inflammasome assembly during interphase [56–58]; (4) Microtubule affinity regulating kinase 4 (MARK4) interacts with NLRP3, driving it to the microtubule organizing centre, and is crucial to ensure that one single speck complex is formed in each cell upon inflammasome activation. Interaction of NLRP3 with MARK4 enables correct subcellular localization of the inflammasome complex [49].

1.4. Non-canonical inflammasome activation

An alternative mechanism resulting in Caspase-1 cleavage is non-canonical inflammasome activation, which also leads to cell death (pyroptosis) and release of pro-inflammatory signals. Non-canonical activation occurs with a mechanism that targets Caspase-11 in mice or Caspase-4 and Caspase-5 in humans. It is triggered by a specific subset of activating stimuli, particularly intracellular LPS of Gram negative bacteria, such as *E. coli, C.*

rodentium and *V. cholerae* [59]. Activation of Caspase-11 triggers not only Caspase-1 independent pyroptosis but also Caspase-1-dependent release of the pro-inflammatory cytokines IL-1β and IL-18 [59]. Non-canonical inflammasome activation occurs independently of TLR4, because intracellular LPS binds directly to Caspase-11 (in mice) or Caspase 4/5 (in humans) [60,61]. The impact of non-canonical inflammasome activation on atherosclerosis is currently unknown.

2. NLRP3 inflammasome and atherosclerosis

2.1. Expression of NLRP3 inflammasome components in atherosclerotic arteries

NLRP3 inflammasome components are expressed in endothelial cells [62], smooth muscle cells (SMCs) [11], and immune cells such as dendritic cells, monocytes, macrophages [63,64] and T cells [13,14], although most of the work on atherosclerosis has focused on the role of inflammasome activation in monocytes/macrophages. In various rodent atherosclerosis models, high NLRP3 levels were observed in monocytes and macrophages, and were increased after LPS treatment [63,65]. Patients with coronary atherosclerosis show high levels of NLRP3 expression in the aorta, with NLRP3 expression correlating with the severity of coronary artery stenosis [66]. Presence of concomitant risk factors (smoking, hypertension, diabetes, high Lp(a), high total- or LDL-cholesterol, low HDL-cholesterol) also correlates with increased NLRP3 protein levels in the aorta of patients with coronary artery disease [66]. Furthermore, in expression studies comparing carotid atherosclerotic plaques with normal iliac or mesenteric arteries, mRNA and protein levels of the inflammasome components NLRP3, ASC and Caspase1, as well as IL-1β and IL-18 were found to be significantly increased in carotid plaques compared to healthy arteries [67,68]. Within the atherosclerotic plaques, NLRP3 and ASC co-stained in the CD68 positive macrophage population, particularly in association with cholesterol crystal clefts, and the association was also detected in a fraction of smooth muscle cells [67]. Finally, expression of NLRP3 in subcutaneous adipose tissue, which was mostly attributed to macrophages, positively correlated with body mass index and serum levels of uric acid, and showed independent association with the severity of coronary artery disease [69].

Zhao and colleagues failed to find significant associations between NLRP3 polymorphism and predisposition to coronary heart disease in pre-hypertensive Chinese patients [70]. However, the cohort examined in this study was very limited. In another epidemiological study, Zhou and colleagues observed a significant association between the NLRP3 rs10754558 polymorphism and the occurrence of coronary heart disease, which also corresponded to increased serum levels of IL-1β [71]. However, the authors did not explore the mechanism through which this polymorphism affects NLRP3 activation.

2.2. Loss of inflammasome components during atherosclerosis (Table 1)

Two murine models have been extensively applied by cardiovascular researchers to resolve the mechanisms of atherosclerosis development. These are Apolipoprotein E-deficient mice (*Apoe*$^{-/-}$) and LDL receptor deficient mice (*Ldlr*$^{-/-}$), both under a C57BL/6 background. *Apoe*$^{-/-}$ mice develop atherosclerosis on either a chow or high fat diet, whereas *Ldlr*$^{-/-}$ mice develop significant atherosclerosis only when fed a high fat diet. These two models show significant differences in the mechanisms that lead to high cholesterol diet-induced atherosclerosis and the choice of one over the other can significantly affect experimental observations [72]. In this context, it is interesting to note that the impact of NLRP3 inflammasome components on atherosclerosis substantially differed between the various atherosclerosis models.

In *Ldlr*$^{-/-}$ models, Duewell and colleagues found that irradiation and reconstitution with *Nlrp3*$^{-/-}$, *Asc*$^{-/-}$, or *Il1a*$^{-/-}$ *Il1b*$^{-/-}$ bone marrow significantly reduced the development of atherosclerotic lesions after 8 weeks of high fat diet in comparison with *Ldlr*$^{-/-}$ mice reconstituted with a control bone marrow [3]. Similarly, reconstitution of *Ldlr*$^{-/-}$ mice with Caspase-1-deficient bone marrow significantly decreased plaque size after 12 weeks of high fat diet in comparison with a control bone marrow [73]. Using a similar bone marrow transplantation model in *Ldlr*$^{-/-}$ mice put on high fat diet for 16 weeks, Freigang et al. confirmed the role of bone marrow-derived IL-1α in promoting atherosclerosis. Surprisingly, they found no impact of bone marrow-derived IL-1β on atherosclerosis [74]. However, a close look at their data indicates that mice with IL-1β deficiency developed smaller lesions compared to controls (although not reaching statistical significance), and the extent of lesion development did not significantly differ between mice with IL-1α and IL-1β deficiency. Thus, deletion of NLRP3 inflammasome components reduces atherosclerosis in *Ldlr*$^{-/-}$ mice, although the effect may be slightly attenuated with long periods of high fat diet.

Deletion of inflammasome components also reduces atherogenesis in the *Apoe*$^{-/-}$ background, when the mice are analysed under chow diet. Caspase-1 deletion reduces atherosclerosis [75]. Blockade of IL-18 activity or IL-18 deficiency in *Apoe*$^{-/-}$ mice significantly reduced lesion development compared to control animals [76]. Kirii and colleagues found that IL-1β deletion reduced lesion development by 30% compared to *Apoe*$^{-/-}$ controls [77]. Kamari and colleagues also reported a significant 32% reduction of plaque size for *Apoe*$^{-/-}$ *Il1b*$^{-/-}$ double knockouts and a 52% reduction for *Apoe*$^{-/-}$ *Il1a*$^{-/-}$ mice compared with *Apoe*$^{-/-}$ controls [78]. Similar observations were reported for irradiated *Apoe*$^{-/-}$ mice reconstituted with *Il1a*$^{-/-}$ or *Il1b*$^{-/-}$ bone marrow [78]. Interestingly, however, feeding *Apoe*$^{-/-}$ a high fat diet substantially alters the effects of inflammasome on atherosclerosis. Caspase-1 deletion still reduces lesion development after 8 weeks on high fat diet [75], but is unable to do so after 11 weeks on a similar diet [79]. Similarly, deletion of NLRP3 or ASC has no influence on lesion development in *Apoe*$^{-/-}$ after 11 weeks of high fat diet [79]. Consistent with the latter finding, deletion of *Il1r1* was unable to alter lesion size in *Apoe*$^{-/-}$ fed a high fat diet for 27–30 weeks [80]. Thus, it appears that prolonged high fat feeding tends to limit the impact of the inflammasome on atherosclerosis. The mechanisms behind this observation are unknown and may include reduced inflammasome activation (e.g., highly nitrosylated NLRP3), a redundant role of the inflammasome (hyperactivation of other innate immune pathways), or a dispensable role for some inflammasome targets. For example, IL-1 and IL-18 may substantially impact atherosclerosis through their roles in the adaptive immune response, and the latter is dispensable for the development of atherosclerosis under prolonged and severe hypercholesterolemia [81,82].

2.3. Priming stimuli of NLRP3 inflammasome and their roles in atherogenesis (Figs. 1 and 2)

TLRs are involved in the delivery of priming signal to enhance basal expression of NLRP3 and IL-1β in bone-marrow-derived macrophages (BMDMs) [83]. Most cells in the cardiovascular system express TLRs [84]. Different TLRs have different ligand specificities and therefore detect specific PAMPs and DAMPs. Increasing evidence suggest that TLRs are key orchestrators of atherosclerotic disease processes. Many TLRs are expressed in atherosclerotic

Table 1
Studies on the roles of NLRP3 inflammasome and related immune pathways in experimental atherosclerosis.

Molecules	Mouse models	Diet	Cells examined at the lesion	Impact on atherosclerosis	Ref.
NLRP3	*Ldlr*$^{-/-}$· irradiated and reconstituted with *Nlrp3*$^{-/-}$ bone marrow	HFD for 8 weeks	Macrophages	Decrease in early atherosclerosis and IL-18 levels	[3]
	Apoe$^{-/-}$, *NLRP3*$^{-/-}$	HFD for 11 weeks	Macrophages and SMCs	No impact on inflammation or atherosclerosis	[79]
IL-1β	*Ldlr*$^{-/-}$, irradiated and reconstituted with *Il1b*$^{-/-}$ bone marrow	HFD for 16 weeks	Macrophages	IL-1β drives inflammation but not atherosclerosis	[74]
	Ldlr−/−, irradiated and reconstituted with *Il1b*$^{-/-}$ *Il1a*$^{-/-}$ bone marrow	HFD for 8 weeks	Macrophages	Rate of atherosclerotic plaques development unaffected compared to wild type	[3]
	Apoe$^{-/-}$, *Il1b*$^{-/-}$	Normal diet for 12 weeks or 24 weeks	Unspecified	~30% reduction in atherosclerotic lesion size at 24 weeks	[77]
	Apoe$^{-/-}$, *Il1b*$^{-/-}$ and *Apoe*$^{-/-}$, irradiated and reconstituted with *Il1b*$^{-/-}$ bone marrow	Normal diet for 16 weeks or 32 weeks	Macrophages	~32% reduction in atherosclerotic lesion size	[78]
IL-1α	*Ldlr*$^{-/-}$, irradiated and reconstituted with *Il1a*$^{-/-}$ bone marrow	HFD for 8 weeks	Macrophages	Reduced development of atherosclerotic lesions	[3]
	Ldlr$^{-/-}$, irradiated and reconstituted with *Il1a* $^{-/-}$ bone marrow	HFD for 16 weeks	Macrophages	Reduced development of atherosclerotic lesions	[74]
	Apoe$^{-/-}$, *Il1a*$^{-/-}$ and *Apoe*$^{-/-}$, irradiated and reconstituted with *Il1a*$^{-/-}$ bone marrow	Normal diet for 16 weeks or 32 weeks	Macrophages	~50% reduction in atherosclerotic lesion size	[78]
IL-1r$^{-/-}$	*Apoe*$^{-/-}$, *Il1r*$^{-/-}$	HFD for 27–30 weeks	Macrophages and SMCs	Reduced plaque SMC content, reduced plaque size at the aortic root, but no difference in plaque size in brachiocephalic arteries, with greater plaque instability	[80]
	Apoe$^{+/-}$, *Il1r*$^{-/-}$ and *Apoe*$^{+/-}$, *Ilr*$^{+/-}$	HFD for 30 weeks	Whole lesion	Reduced progression of atherosclerotic plaques	[141]
Caspase-1	*Ldlr*$^{-/-}$, *caspase-1/11*$^{-/-}$	HFD for 12 weeks	Leukocytes and Macrophages	Reduced atherosclerotic plaque size	[73]
	Apoe$^{-/-}$, *Caspase1*$^{-/-}$	HFD for 11 weeks	Macrophages and SMCs	No impact on inflammation or atherosclerosis	[79]
	Apoe$^{-/-}$, *Caspase 1*$^{-/-}$	Saturated fat and cholesterol enriched diet for 8 weeks (compared to low fat diet for 26 weeks)	Whole lesion	Decrease in atherosclerosis in both low fat and high fat diet fed mice	[75]
ASC	*Ldlr*$^{-/-}$, irradiated and reconstituted with *Asc*$^{-/-}$ bone marrow	HFD for 8 weeks	Macrophages	Marked resistance to development of atherosclerosis	[3]
	Apoe$^{-/-}$, *Asc*$^{-/-}$	HFD for 11 weeks	Macrophages and SMCs	Reduced plaque layering and adventitial inflammation No impact on overall disease progression	[79]
IL-18	*Apoe* $^{-/-}$, *Il18*$^{-/-}$	Normal diet for 24 weeks	SMCs	Reduced atherosclerotic plaque size	[142]
	Apoe$^{-/-}$, electro-transferred with IL-18BP plasmid DNA	Normal diet for 23 weeks (IL-18BP injection at 14-week old for 9 weeks)	Macrophages, T cells, SMCs	Prevention of fatty streak in aorta and slower progression of atherosclerotic plaques in aortic sinus	[76]

plaques, and TLR (TLR2, TLR4, TLR6, and TLR9) polymorphisms have been associated with atherosclerosis [85–87]. Most of cell surface localized (extracellular) TLRs are pro-atherogenic whereas some endosomal (intracellular) TLRs may be atheroprotective (a description of the role of key TLRs involved in atherosclerosis is shown in Table 2).

MyD88 is a key downstream effector of IL1R/TLR signalling. Total deletion of MyD88 in *Apoe*$^{-/-}$ background significantly limits atherosclerotic lesion development compared to controls and is associated with lower circulating levels of pro-inflammatory cytokines [88,89]. However, Treg-mediated suppression of atherosclerosis requires MyD88 signalling in dendritic cells [55]. Other signalling adaptors downstream of TLRs also modulate atherosclerosis, with TRIF and TRAM being pro-atherogenic in hematopoietic cells [90].

Cholesterol crystals [91] can act as danger signals triggering neutrophils to release Neutrophil Extracellular Traps (NETs), which will prime macrophages for IL-1β and IL-18 cytokine release through activation of several TLRs [91,92]. The pro-inflammatory cytokines IL-1β and IL-18 promote further formation of NETs in a vicious circle [92].

Atherosclerotic plaques are also characterized by accumulation of oxidized low-density lipoproteins [93]. In this context, oxLDL is mostly enclosed in immune complexes containing antibodies directed against it [93]. Sheedy and colleagues showed that oxLDL both primed and activated the inflammasome in mouse BMDMs. They proposed a mechanism where the heterotrimeric CD36-TLR4-TLR6 complex is required for priming. CD36 also mediates uptake of oxLDL in macrophages, promoting the accumulation of intracellular crystals that activate NLRP3 inflammasome [94]. However, Rhoads et al. failed to replicate these findings in BMDMs, where they showed that only oxLDL immune complexes (and not oxLDL alone) can act as a priming signal for NLRP3, but confirmed the role of oxLDL in NLRP3 inflammasome priming in bone-marrow-derived dendritic cells [95]. Differences in experimental conditions and methods of oxLDL production may account for this inconsistency.

2.4. Activation stimuli of NLRP3 inflammasome and their roles in atherogenesis (Figs. 1 and 2, and Table 3)

One of the initial triggers of NLRP3 inflammasome activation in the context of atherosclerosis may be the oscillatory shear force acting on the endothelium to induce SREBP2 activation, which transcriptionally stimulates NLRP3 [12]. This stimulus acts both as a priming and an activating signal, resulting in a marked pro-inflammatory response in endothelial cells [12]. SREBP2 can also be activated in response to phospholipid oxidation products [96].

Besides, the presence of cholesterol crystals in the atherosclerotic region of the vessel is thought to be a major trigger of NLRP3 activation in macrophages [42,91]. Small crystals may already be present at the early stages of atherosclerotic lesions and become abundant in advanced lesions. They represent a major factor of plaque vulnerability and trigger inflammasome activation by inducing lysosomal damage [3]. This results in dose-dependent secretion of IL-1β [3]. Recent evidence suggests an important role for the complement system in cholesterol-mediated inflammasome activation, showing that, in a human whole blood model, cholesterol crystals activate the classical and alternative complement pathways, and that presence of C5 is necessary for Caspase-1 activation [97].

After its recognition as a danger and priming signal by scavenger receptor CD36 together with the TLR heterodimer formed by TLR4 and TLR6, oxLDL endocytosis through CD36 leads to its intracellular nucleation into cholesterol crystals, resulting in

Table 2
Key TLRs and TLR pathway players in atherosclerosis.

Molecules	Mouse model	Diet	Cells examined at the lesion	Impact on atherosclerosis	Phenotype	Ref.
TLR2	*Ldlr*$^{-/-}$, *Tlr2*$^{-/-}$ double knock-out and *Ldlr*$^{-/-}$, irradiated and reconstituted with *Tlr*$^{-/-}$ bone marrow	HFD for 4 weeks	Leukocytes and Endothelial cells	Proatherogenic, when expressed on non-bone marrow derived cells	Lack of TLR2 leads to reduction in atherosclerosis. Increased atherosclerosis is not rescued by expressing TLR2 on bone marrow-derived cells	[143]
	Apoe$^{-/-}$ single knock out, treated with synthetic Tlr2 ligand	HFD for 3 weeks	Vascular cells	Proatherogenic	Exogenous activation of TLR2 results in higher atherosclerotic plaque formation	[144]
TLR4	*Apoe*$^{-/-}$, *Tlr4*$^{-/-}$ double knock out and *Apoe*$^{-/-}$, *Myd88*$^{-/-}$ double knock out	HFD for 6 months	Leukocytes and Endothelial cells	Proatherogenic	Lack of TLR4 or MyD88 results in reduction of atherosclerotic lesion size	[89]
TLR4/TLR6 heterodimer	*Apoe*$^{-/-}$, *Tlr4*$^{-/-}$ double knock outs, *Apoe*$^{-/-}$, *Tlr6*$^{-/-}$ double knock outs	HFD for 12 weeks	Macrophages	Proinflammatory	oxLDL recognition by TLR4/TLR6 leads to inflammasome activation (together with CD36)	[94]
TLR7	*Apoe*$^{-/-}$, *Tlr7*$^{-/-}$ double knock out	Normal diet for up to 26 weeks	Macrophages	Atheroprotective	Loss of TLR7 leads to accelerated atherosclerotic lesion development and plaque instability	[145]
TLR9	*Apoe*$^{-/-}$, *Tlr9*$^{-/-}$ double knock out	HFD for 7 weeks	Macrophages and SMCs	Atheroprotective	Loss of function of TLR9 exacerbates atherosclerosis	[146]

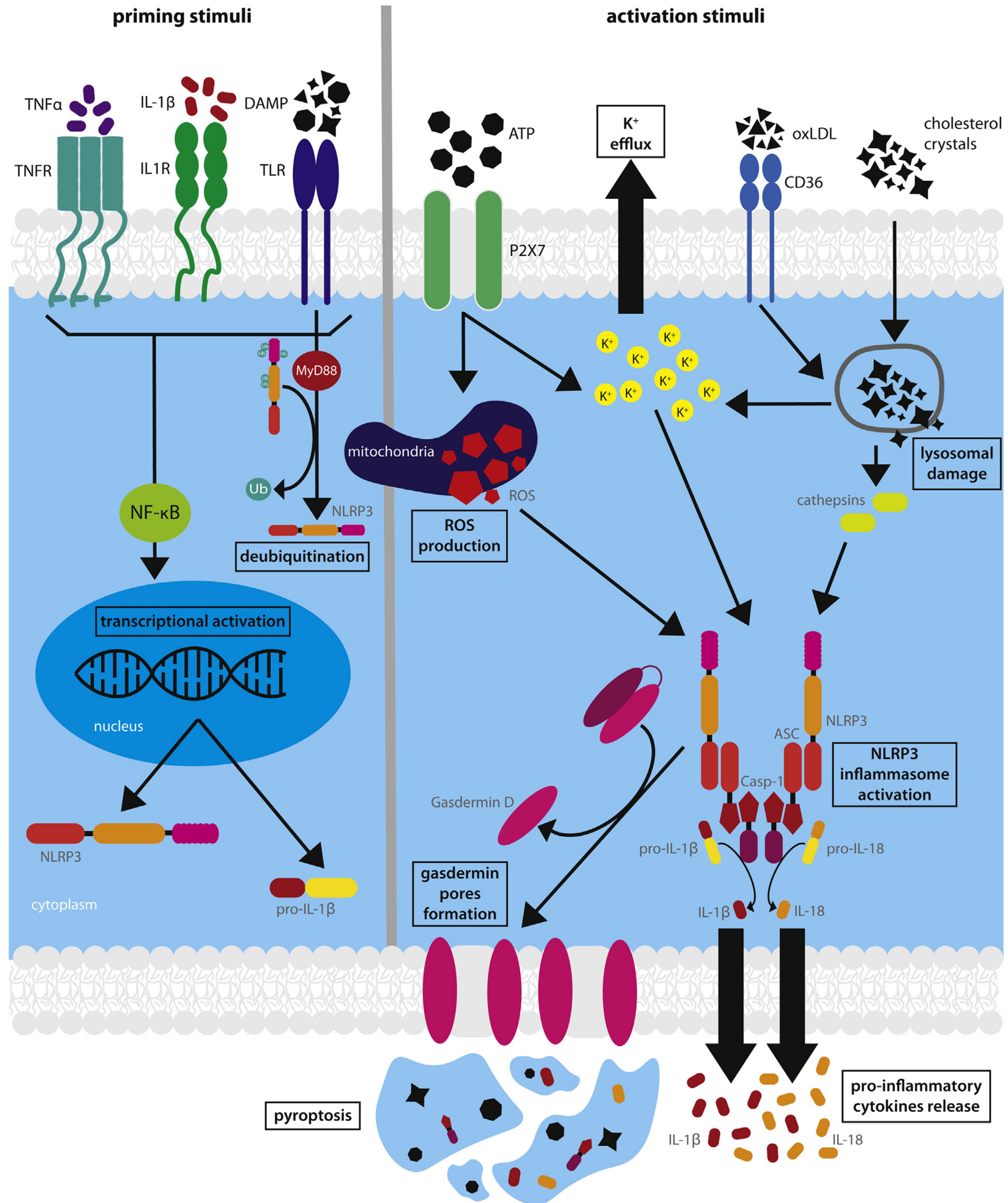

Fig. 1. Inflammasome priming and activation signals during atherogenesis.
Multiple stimuli may prime NLRP3 inflammasome in the context of atherosclerosis. Non-transcriptional priming is mediated by MyD88, which deubiquitinates NLRP3 upon TLR4 activation. Transcriptional priming occurs via the transcription factor NF-κB and results in an increase of the expression levels of NLRP3 and pro-IL-1β. NLRP3 activation is mediated mainly by ATP, oxLDL and cholesterol crystals. To date, the precise mechanisms that lead to activation of NLRP3 inflammasome in this context have not fully been defined. However, ROS production, potassium efflux and lysosomal damage are known key players in this process.

lysosomal damage and NLRP3 activation [94]. Additionally, CD36 expression is increased by cholesterol crystals via activation of the BTK-p300-STAT1-PPARγ signalling cascade [98], promoting further intake of oxLDL. Therefore, uptake of lipids and their derivatives substantially contributes to inflammasome activation and IL-1 release by the immune cells of the developing atherosclerotic lesions.

Metabolites such as extracellular ATP, a classical non-lipid danger stimulus, are released as a consequence of cell death. Therefore, they are also present in the atherosclerotic plaque environment, and are responsible for further activation of the immune response (immunogenic cell death) [99]. ATP mediates NLRP3 inflammasome activation in macrophages through activation of the purinergic receptor P2X7R, promoting pro-inflammatory cytokines release [33]. Knock-down of P2X7R in *Apoe*$^{-/-}$ mice [64] or its knock-out in *Ldlr*$^{-/-}$ mice [100] reduced inflammasome activation and the development of atherosclerosis.

High serum uric acid levels are associated with the presence of

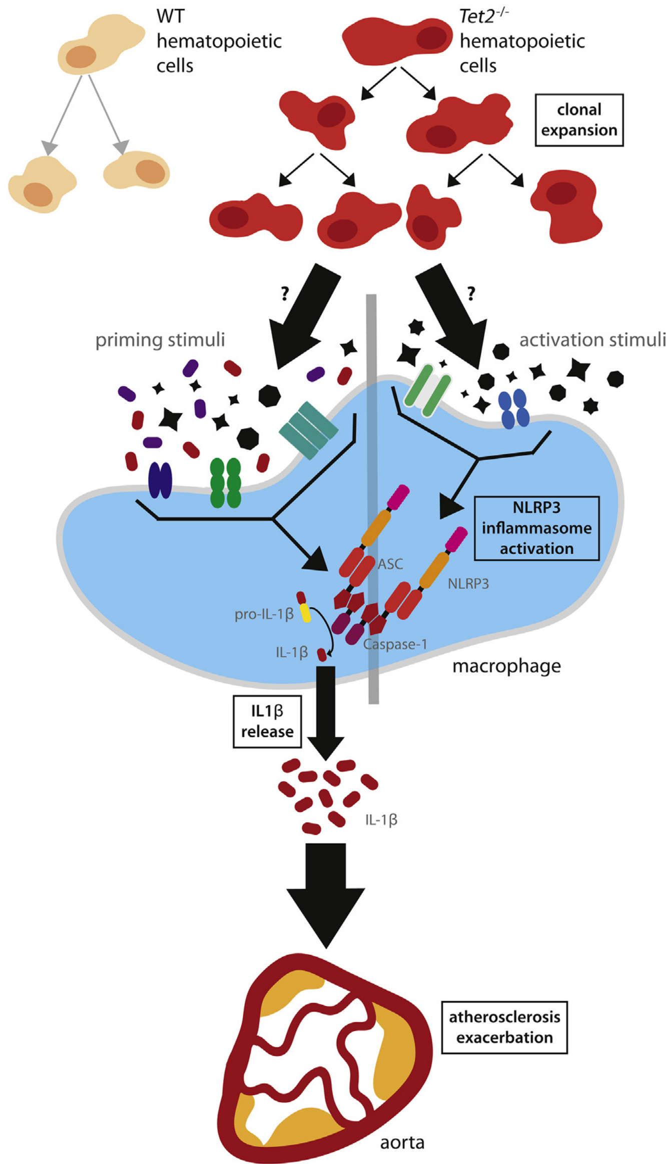

Fig. 2. Hematopoietic clonal expansion drives NLRP3 inflammasome priming and activation during atherosclerosis.
Loss of function TET2 mutations in hematopoietic cells can drive clonal expansion, and accelerate the development of atherosclerotic plaques in a NLRP3 inflammasome dependent manner. However, the mechanisms linking clonal expansion of myeloid cells to NLRP3 priming and activation are still unknown.

atherosclerotic plaques [101], and uric acid crystals MSU are known activators of NLRP3 inflammasome [43]. Recent evidence suggests that soluble uric acid can also trigger NLRP3-dependent IL-1β release in bone marrow-derived macrophages in a mitochondrial ROS-dependent mechanism [102].

Interestingly, activated macrophages release IL-1α and High-mobility Group Box Protein 1 (HMGB1) in a NLRP3-dependent mechanism that does not require Caspase-1 [74,103–105]. Both IL-1α and HMGB1 are alarmins, i.e. prototypical danger signals that induce pro-inflammatory cytokine release from macrophages, and affect atherosclerosis progression [106,74]. These results suggest that NLRP3 inflammasome might regulate atherogenesis through functions which are independent from IL-1β, IL-18 and Caspase-1.

Human studies have shown that aging is associated with

Table 3
Key NLRP3 activation stimuli in atherosclerosis.

Stimulus	Cell type affected	Evidence type	Mechanism of inflammasome activation
Oscillatory shear force	Endothelial cells	*In vitro*	SREBP2 activation leading to transcriptional activation of NLRP3 [12]
Cholesterol crystals	Macrophages	*In vitro* and *in vivo* (*Ldlr*$^{-/-}$ mouse)	Lysosomal damage [3,42]
	Neutrophils and macrophages	*In vitro* and *in vivo* (*Apoe*$^{-/-}$ mouse)	Triggering of neutrophils to release neutrophil extracellular traps (NETs) [91]
oxLDL	Macrophages	*In vitro* and *in vivo* (*Apoe*$^{-/-}$ mouse)	CD36-mediated intake followed by oxLDL nucleation into cholesterol crystals leading to lysosomal damage [94]
ATP	Macrophages	*In vitro* and *in vivo* (*Apoe*$^{-/-}$ [64] and *Ldlr*$^{-/-}$ mouse [100])	Activation of P2X7R receptor [64,100]
Hematopoietic stem cell mutations	Macrophages	*In vitro* and *in vivo* (*Ldlr*$^{-/-}$ mouse)	Yet to be investigated [110]

increased prevalence of hematopoietic stem cell mutations, which are linked with progressive and acquired clonal expansion, and are a risk factor for atherosclerotic disease [107–109]. Some of those somatic mutations lead to loss of function of TET2, an epigenetic modifier enzyme. Interestingly, clonal hematopoiesis associated with Tet2 deficiency accelerates the development of atherosclerosis in mice. *Tet2*$^{-/-}$ macrophages localizing in atherosclerotic lesions have increased inflammasome activation, associated with increased IL-1β release. Their presence is associated with greater atherosclerotic plaque size [110,109]. This effect is abrogated by administration of the NLRP3 inflammasome inhibitor MCC950 [110,111]. However, the molecular mechanisms linking TET2 or clonal expansion of myeloid cells to NLRP3 priming and activation are still unknown.

2.5. Molecular and cellular pathways involved in the regulation of NLRP3 activity during atherosclerosis

Cholesterol crystal-mediated NLRP3 activation has been shown to result in the production of ROS via a mechanism that requires NADPH and xanthine oxidase [98], leading to subsequent pro-inflammatory responses and increased CD36 expression. Increased CD36 expression may positively feedback to promote more intracellular nucleation of cholesterol crystals and NLRP3 activation [94]. Besides cholesterol crystal formation, cellular cholesterol and (phospho)lipid levels per se play an important role in the regulation of inflammasome activation. Indeed, low membrane cholesterol levels result in higher ion currents through P2X7 channels in macrophages [112]. Lipin-2 enzyme regulates lipid concentrations and is crucial to maintain correct currents through P2X7 channels. Lipin-2 also regulates pro-IL-1β levels upon priming of macrophages, with a mechanism that involves MAPKs [112]. A specific MAPK, p38δ, has been identified as an important regulator of inflammasome activation within atherosclerotic lesions [113].

Also, regardless of the presence of cholesterol crystals, mtDNA-induced mitochondrial dysfunction can drive atherosclerosis [114]. Recent evidence shows that *Ldlr*$^{-/-}$ mice depleted from OGG, an enzyme that removes damaged mtDNA from atherosclerotic plaques, show higher inflammasome activation and bigger plaques [115]. Lectin-like ox-LDL receptor-1 (LOX-1) is a major receptor for ox-LDL that plays a key role in several inflammatory disease states. LOX-1 activation following a pro-inflammatory signal leads to ROS generation and mtDNA damage and eventually to xanthine-oxidase-mediated NLRP3 inflammasome activation [116].

Furthermore, growing evidence supports the role of endoplasmic reticulum stress in atherosclerosis progression [117,118]. The conserved mediator of homeostasis in the unfolded protein response (UPR) IRE1 plays an important role in this process. The enzyme IRE1 regulates pro-atherogenic genes and is activated in macrophages upon exposure to excessive amounts of lipids, leading to activation of NLRP3 inflammasome through production of ROS [119,120]. Consistent with this notion, the use of the IRE1 inhibitor in a *Apoe*$^{-/-}$ mouse model challenged with a western diet, resulted in a reduction of the size of atherosclerotic plaques through reduced macrophage accumulation and activation [119]. Palmitoleate is a bioactive lipid that prevents lipid-induced activation of the inflammasome in macrophages through its role in ER membrane remodelling, which results in attenuation of ER stress *in vivo* in an *Apoe*$^{-/-}$ mouse model, leading to a reduction in atherosclerotic plaque size [39]. However, the evidence for a direct connection between ER stress and inflammasome activation during atherosclerosis needs to be explored further.

3. Control of NLRP3 activity during atherogenesis

Current drugs with potential impact on NLRP3 activation during atherogenesis include known drugs such as colchicine and statins. New drugs with capacity to regulate NLRP3 activity and disrupt cholesterol crystal formation have also been explored. However, most of these agents remain at the stage of either *in vitro* assays or *in vivo* experimentation in pre-clinical atherosclerosis models with limited data. Thus, further exploration is needed to confirm the effect of these agents on the development of atherosclerosis.

3.1. Therapies that may display inhibitory effects on NLRP3 inflammasome (Table 4)

Statins are structural analogues of HMG-CoA used in the pharmacological management of hyperlipidemia. They cause partial inhibition of HMG-CoA reductase, blocking the first committed step of the synthesis of endogenous sterols. Statins are most effective in reducing LDL levels through reduction of cholesterol synthesis and an increase in LDL receptor levels, leading to increased LDL clearance from plasma. Besides their effects on cholesterol levels, statins may exert direct immune modulatory effects. Atorvastatin, a commonly prescribed statin, inhibits TLR4/MyD88/NF-κB dependent NLRP3 expression in THP-1 cells *in vitro*, and consequently reduces IL-1β secretion [121].

The alkaloid colchicine is an established treatment for gout, and more recent studies also highlighted the potential of this compound in the management of atherosclerosis and the secondary prevention of cardiovascular events [122]. Colchicine disrupts inflammasome activation by preventing microtubule assembly [123]. Microtubule integrity is important for inflammasome activation, which requires precise spatial arrangement of specific subcellular compartments [48]. In cultured ATP-stimulated monocytes derived from acute coronary syndrome patients treated with colchicine, the drug was effective in reducing inflammasome-dependent inflammation. Moreover, colchicine acutely suppressed local cardiac production of IL-1β in patients with acute coronary syndromes [124].

Arglabin, a plant-derived sesquiterpene lactone tested for its anti-inflammatory and anti-tumour activities, has recently been shown to inhibit NLRP3 and reduce atherosclerotic lesion size in an ApoE2 knock-in mouse model [125].

3.2. Other agents that can suppress cholesterol crystal-induced NLRP3 inflammasome activation (Table 3)

Evidence in an *Apoe*$^{-/-}$ mouse model suggests that increasing cholesterol solubility with ursodeoxycholic acid leads to a reduction in atherosclerotic plaque size [126]. In the same mouse model, cyclodextrin reduces the formation of atherosclerotic plaques and promotes the regression of existing plaques [127]. Ethanol has been shown to reduce cholesterol crystal-induced NLRP3 inflammasome activation in primary human macrophages with consequent reduction of IL-1β secretion, by ameliorating lysosomal integrity [128]. Febuxostat, a xanthine oxidase inhibitor, protects against cholesterol crystal-induced ROS formation *in vitro*, and reduces atherosclerosis progression in an *Apoe*$^{-/-}$ mouse model [129]. Finally, HDL-cholesterol suppress cholesterol crystal-induced release of IL-1β in THP1 cells and in monocyte-derived macrophages [130]. HDL-cholesterol modulates the inflammasome by a combination of different mechanisms [130]: the main one consists in modulation of the expression of NLRP3 and pro-IL-1β by decreasing NF-κB signalling; moreover, HDL binds to cholesterol

Table 4
Main agents with inhibitory effect on NLRP3, tested in the context of atherosclerosis.

Agents	Inhibitory role	Impact on inflammation and atherosclerosis	Ref.
MCC950 (currently not for therapeutic use)	Selectively inhibits NLRP3 inflammasome activation	Attenuation of inflammation, by reduction of IL-1β and IL-18 levels	[111]
Arglabin (currently not for therapeutic use)	Selectively inhibits NLRP3 inflammasome activation	- Attenuation of inflammation by reduction of IL-1β and IL-18 levels - Activation of autophagy in primed macrophages	[125]
Atorvastatin	Inhibits HMG-CoA reductase	- Reduction of hyperlipidemia - decrease in oxidative stress - decrease in vascular inflammation	[147]
Colchicine	Prevents microtubule assembly, leading to inhibition of inflammasome assembly	Reduction of inflammasome-dependent inflammation	[123]
HDL (and rHDL)	- Decreases pro-IL-1β and NLRP3 expression upon inflammasome priming (main mechanism) - Blunts monocyte recruitment to site of inflammation - Reduces CC-mediated lysosomal damage	Anti-atherogenic and anti-inflammatory effect	[130]
Allopurinol and febuxostat	Inhibit xanthine oxidase	Reduction of atherosclerosis	[129,148]
Ethanol	Ameliorates lysosomal membrane integrity	Reduction of cholesterol crystals-induced NLRP3 inflammasome activation	[128]
Palmitoleate	Reduces lipid-induced NLRP3 inflammasome activation	Reduction of atherosclerosis	[39]
Ursodeoxycholic acid	Increases cholesterol solubility	Impaired atherosclerotic plaque development and regression of established vascular lesions	[126]
Cyclodextrin	- Dissolves extra and intracellular cholesterol crystals - Increases cholesterol metabolism and reverse transport - Promotes cellular transcriptional reprogramming	Prevention of plaque formation and regression of present atherosclerotic plaques	[127]

crystals, sequestering them and preserving lysosomal membrane integrity upon phagocytosis of cholesterol crystals; *in vivo*, HDL reduces monocyte recruitment to sites of inflammation.

4. Clinical trials targeting NLRP3 pathway in atherosclerotic patients

The discovery of the inflammasome has been quickly transferred from bench to bedside. In the cardiovascular research field, Abbate et al. conducted randomized double-blind pilot trials in acute myocardial infarction (MI) patients [131], and demonstrated that administration of IL-1 receptor antagonist (IL1RA) was safe and favourably affected left ventricular remodelling. However, short-term treatment (14 days) with IL-1RA in patients with non-ST elevation acute coronary syndromes did not result in sustained reduction of inflammatory markers and was even associated with significant excess of major adverse cardiovascular events after 1 year of follow-up [132]. A trial, named Canakinumab Anti-inflammatory Thrombosis Outcomes Study (CANTOS trial), evaluated the impact of IL-1β blockade on the occurrence/recurrence of cardiovascular events (myocardial infarction, stroke, and cardiovascular death) among 17,200 stable coronary artery disease patients who are at high vascular risk [133,134]. Canakinumab is a human monoclonal antibody that selectively neutralizes IL-1β. CANTOS evaluated three active doses of canakinumab in comparison to placebo. Preliminary data already supported the use of anti-IL-1β antibody as a potential therapeutic method as it significantly reduces inflammation [135], although it did not seem to affect vascular structure or function in those patients [136]. The results of CANTOS have been published recently. CANTOS met the primary endpoint, reducing the risk of major adverse cardiovascular events, a composite of cardiovascular death, non-fatal myocardial infarction and non-fatal stroke [134]. However, there was no difference in all-cause mortality, and canakinumab treatment was associated with a higher incidence of fatal infection compared to placebo. The trial expands our understanding of how the balance of innate immunity contributes to cardiovascular health, and provides critical safety and efficacy data on long-term inhibition of IL-1β dependent immunity.

5. Perspectives

In order to provide future reliable therapeutic strategies for atherosclerosis based on modulation of the inflammasome pathway, we still have to delineate a vast number of unknown mechanisms in NLRP3 activation, and how NLRP3 pathway entangles with other inflammatory pathways to contribute to atherosclerotic lesion development.

Compared with simplistic cell culture experiments based on sequential stimulation of TLRs and NLRP3 inflammasome *in vitro*, several ligands and stimuli for TLR and NLRP3 inflammasome pathways will co-exist *in vivo* during atherosclerosis development. The downstream effects of those signals would not be arranged in a sequential way as modelled by *in vitro* assays. Pro-inflammatory stimuli/mediators with activatory and inhibitory properties on NLRP3 inflammasome will co-exist. This is the case of IFNγ, a cytokine that displays pro-atherogenic activity [137] but is a potent inhibitor of NLRP3 inflammasome [25]. Therefore, caution and improved understanding of the modulators of NLRP3 inflammasome are needed for a better and safe targeting of the inflammatory response in atherosclerosis.

TLR signalling and NLRP3 inflammasome shape microbiota, and those pathways play key roles in the regulation of intestinal homeostasis [138,139]. In addition, infections and diets have been linked with the development of chronic and systemic inflammation

[140]. In turn, gut microbiota and metabolites of diets can influence atherosclerosis. Controversial findings regarding the role of NLRP3 in experimental atherosclerosis using $Ldlr^{-/-}$ or $Apoe^{-/-}$ models [3,79], and distinct contributions of IL-1α or IL-1β towards vascular inflammation [3,74] may result from differences in the basal status of microbiota, and the diets that can influence the response.

Furthermore, NLRP3 inflammasome is likely to play distinct roles in different cell types at various stages of lesion development. For example, work from Owen's lab demonstrates that IL-1 contributes to outward vessel remodelling and enhances features of plaque stability, which is beneficial for lesion repair [80], suggesting that IL-1 might play distinct and sometimes opposite roles by its effect on smooth muscle cells at late stage of atherosclerosis. Work from Kemper's lab shows that complement-driven NLRP3 activity in T cells leads to autocrine IL-1β dependent Th1 differentiation [15], whereas Ghiringelli and colleagues identified a requirement for nuclear NLRP3 in Th2 differentiation, independently of inflammasome activation [14]. Whether such mechanisms operate in T cells during atherosclerosis is not yet explored. Thus, more research on the effect of cell-type specific roles of NLRP3 during the different stages of atherosclerosis is required.

Current therapies targeting inflammasome activation are focusing directly on one of the end products of the pathway by blocking the function of IL-1. However, IL-1 may be generated downstream of many inflammasomes. Global interference with inflammasome-induced innate immunity, by direct and generic targeting of IL-1, may increase susceptibility to (opportunistic) infections. Thus, a better and more specific targeting of NLRP3 is needed.

Conflict of interest

The authors declared they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

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