Cardiovascular diseases, NLRP3 inflammasome, and western dietary patterns

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1. Introduction

Cardiovascular disease (CVD) is often thought to be a developed countries problem. In fact, as the leading cause of death worldwide, CVD now has a significant impact not only on developed countries but also on low and middle-income nations, where it accounts for nearly 30 percent of all deaths (Developing Countries et al. 2010). The increased prevalence of risk factors for CVD and related chronic diseases in developing countries is due to significant global changes in behavior and lifestyle, like smoking, acquiring unhealthy dietary habits, sedentariness. These changes contribute to an increase in blood lipids and the risk of suffering from hypertension, and, in turn, to the development of severe cardiovascular events.

Epidemiological studies suggest that CVD incidence in low and middle-income countries is directly associated with the increasing intake of energy-dense nutrients high in unhealthy fats, oils, sodium, and sugars (Hu 2008).

Inflammation has demonstrated to be a key factor in the progress of a variety of diseases including atherosclerosis (Ross 1999; Davis et al. 2011; Dostert et al. 2008). Obesity, the postprandial phase and chronic disease states such as the metabolic syndrome are associated with increased inflammation (Klop et al. 2012; Gregor & Hotamisligil 2011). It has been reported that postprandial inflammation, induced by specific dietary patterns, such as high in animal-derived products and refined carbohydrates, and deficient in whole grains, fruits, or vegetables (Hu 2008), may promote the onset of chronic metabolic diseases (Klop et al. 2012). Among these conditions are included atherosclerosis, type II diabetes mellitus, and obesity.

Despite markers of chronic inflammation such as C-reactive protein (CRP) are clearly predictive of clinical atherosclerosis (Ridker et al. 2003), the mechanisms originally causing inflammatory responses that lead to vascular disease are not entirely understood

(Abderrazak et al. 2015). Recent investigations suggest that the process of atherogenesis is characterized by a low-grade inflammation modifying the endothelium of the coronary arteries and is correlated with an increased level of markers of inflammation such as acute phase proteins and cytokines (Shrivastava et al. 2015).

Inflammation is a very complex process that protects our organism against others that try to destroy us and use as a source of energy. But in a pluricellular organism are very different cells, and we have bacteria, for instance in the gut, that are essential for our survival. Therefore, it is essential to recognize the harmful and detrimental organisms. The first line of defense against invading microbes are the pattern recognition receptors (PRRs), which are expressed in a variety of immune cells including macrophages, epithelial cells, dendritic cells, neutrophils, and adaptive immune cells. The Toll-like receptors (TLRs), a particular type of PRRs, are expressed on the cell surface and can be activated by external signals known as pathogen-associated molecular patterns (PAMPs). PRRs can also be triggered during sterile inflammatory diseases, suggesting a crucial role for danger signals that are host-derived, known as danger-associated molecular patterns (DAMPs). PAMPs and DAMPs can also trespass the cell's membrane where they can trigger intracellular innate immune receptors directly, for example via recognition of DNA or RNA in the cytoplasm. As a result of a loss of homeostasis, some cytosolic innate immune sensors can also be activated indirectly (Patel et al. 2017). These sensors include NOD-like receptors (NLRs) which, when assembled in the cytosol forming a complex with the adaptor ASC (Apoptosis-associated speck-like protein containing a caspase recruitment domain), are known as inflammasomes (Schroder & Tschopp 2010). These complex regulate the activation of the protease caspase-1, which in turn regulates the cleavage of cytokines interleukin-1beta (IL-1β) and interleukin-18 (IL-18) from their respective precursors (Thornberry et al. 1992). Many researchers have focused their studies on the NLRs: not only because of the variety of molecular patterns they can recognize and converge to incite inflammation, but also that, when dysregulated, inflammasome has been linked to the pathogenesis of various prevalent disorders including metabolic, inflammatory, and autoimmune diseases such as type 2 diabetes (Masters et al. 2010; Jourdan et al. 2013), neurodegenerative diseases (Heneka et al. 2013), gout (Martinon et al. 2006), Crohn's disease (Roberts et al. 2010), cancer (Zitvogel et al. 2012), irritable bowel disease (Sun et al. 2013), and atherosclerosis (Duewell et al. 2010).

Of all the NLR inflammasomes, NLRP3 (formerly cryopyrin, CIAS1, NALP3) is the most widely studied (Tschopp & Schroder 2010; Franchi et al. 2012). The NLRP3 inflammasome

is formed after indirect sensing of both non-sterile insults derived from pathogens (PAMPs) and sterile stressors (DAMPs). These range from bacterial products, mitochondrial DNA, viruses, and Adenosine triphosphate (ATP) to particulate matter such as crystals and amyloids (He et al. 2016).

Activation of the NLRP3 inflammasome requires a two-step process described as signal 1 (priming) and signal 2 (activation). Signal 1 can be initiated by numerous stimuli and importantly these can be both microbial and non-microbial in origin. The release of IL-1β/IL-18 requires the NLRP3 inflammasome, whereas pyroptosis is independent of it. In contrast to the NLRP3 inflammasome, this pathway is defined as a non-canonical inflammasome pathway because of the requirement for caspase-11 (Kayagaki et al. 2011).

In this review, we will briefly introduce the molecular mechanisms, regulation, and effects of inflammasome activation, and then, focus on discussing the current inflammasome studies on diet and cardiovascular disease research.

2. The NLRP3 Inflammasome

2.1. Activation

In vitro NLRP3 function requires cells to receive two distinct signals in succession. The first signal is a 'priming' signal that activates the transcription factor nuclear factor κB (NF-κB), which induces expression of cytokine-encoding genes. Microbial molecules (e.g., bacterial lipopolysaccharide (LPS)) or host cytokines (e.g., tumor necrosis factor (TNF), which is locally released at sites of tissue damage~~)~~ (Bauernfeind et al. 2009; Franchi et al. 2009) can function as signal 1, but most experimental protocols use LPS as a priming signal. Also, priming licenses NLRP3 by inducing its deubiquitination (Py et al. 2013; Juliana et al. 2012). Free fatty acids (FFA) are capable of priming the NLRP3 inflammasome by TLR2-TLR4 signaling. Oxidized low-density lipoprotein (oxLDL) primes the NLRP3 inflammasome through a CD36-TLR4-TLR6 signaling complex, as well. CD36 also facilitates the internalization of oxLDL and its intracellular conversion to cholesterol crystals, which disrupt the phagolysosome and activate the NLRP3 inflammasome through cathepsin release (Guo et al. 2015). The adaptor protein ASC must become linearly ubiquitinated and phosphorylated for inflammasome assembly to occur (Rodgers et al. 2014). Following priming, canonical NLRP3 inflammasome activation requires a second, different signal to activate NLRP3 and drive to the assembly of the NLRP3 inflammasome complex. The most

generally accepted activating stimuli for NLRP3 include relocalization of NLRP3 to the mitochondria, the detection of mitochondrial factors liberated into the cytosol (mitochondrial reactive oxygen species (ROS), mitochondrial DNA, or cardiolipin), potassium efflux through ion channels, and cathepsin release after destabilization of lysosomal membranes (Lamkanfi & Dixit 2014; Sutterwala et al. 2014; Vanaja et al. 2015). Recent studies have determined that activated NLRP3 nucleates ASC into prion-like filaments through PYD-PYD (PYRIN domain) interactions. Pro-caspase-1 filaments subsequently form off of the ASC filaments through CARD-CARD (caspase activation and recruitment) domain interactions, allowing autoproteolytic activation of pro-caspase-1 (Lu et al. 2014; Cai et al. 2014). The inset shows domain arrangement of the NLRP3 inflammasome components. (Guo et al. 2015)

2.2. Regulation

Several authors have reported various regulators of NLRP3 inflammasome activation, including double-stranded RNA-dependent protein kinase (PKR), guanylate-binding protein 5 (GBP5), and NIMA-related kinase 7 (Nek7) (Shi et al. 2016; He, Zeng, et al. 2016; Schmid-Burgk et al. 2016; Shenoy et al. 2012; Lu et al. 2012).

PKR regulates the activation of all known inflammasomes, including NLRP1, NLRP3, NLRC4, and AIM2 inflammasomes (Lu et al. 2012). Deletion or inhibition of PKR leads to diminished activation of caspase-1 and maturation of IL-1β and IL-18 in answer to a wide array of stimuli. Nevertheless, the role of PKR in inflammasome activation was not independently confirmed in another study with macrophages from two distinct PKR-deficient mice, including the mutant mice in the previous study (He et al. 2013). Therefore, additional investigations are required to clarify the role of PKR in NLRP3 inflammasome activation.

Similar to PKR, the part of GBP5 in NLRP3 inflammasome activation remains uncertain. GBP5 was proved to increase NLRP3 inflammasome activation in response to ATP, nigericin, and bacteria but not particulate matter (Shenoy et al. 2012). By contrast, another investigation reported normal activation of the NLRP3 inflammasome in macrophages from an independently generated GBP5-deficient mouse line (Meunier et al. 2014). It is unclear what accounts for the disparities in these investigations.

Unlike PKR and GBP5, the crucial role of Nek7 in NLRP3 inflammasome activation has been shown in three separate studies (Schmid-Burgk et al. 2016; He, Zeng, et al. 2016; Shi et al. 2016). Nek7 is part of the NIMA (never in mitosis gene a)-related expressed kinase

family, which controls the mitotic progression and DNA damage response (Fry et al. 2012). The lack of Nek7 in mice results in late death during embryogenesis and growth delay, proving a crucial role of Nek7 in development and survival (Salem et al. 2010). Nek7 is required for NLRP3 inflammasome activation induced by all NLRP3 stimuli tested, including ATP, nigericin, monosodium urate (MSU) crystals and alum (He, Zeng, et al. 2016; Shi et al. 2016). Nek7 interacts with NLRP3 by the catalytic domain of Nek7 and the leucine-rich repeat (LRR) domain of NLRP3, which is heightened by NLRP3 stimulation but is independent of Nek7 kinase activity (He, Zeng, et al. 2016; Shi et al. 2016). Moreover, the kinase activity of Nek7 is also not required for Nek7-mediated NLRP3 activation (He, Zeng, et al. 2016; Shi et al. 2016). Nek7 was shown to regulate NLRP3 oligomerization, ASC speck formation, and caspase-1 activation downstream of potassium efflux (He, Zeng, et al. 2016). Notably, neither Nek6, a similar paralog of Nek7, nor Nek9, an upstream regulator of Nek7 during mitosis, interacts with NLRP3, nor both are unnecessary for NLRP3 inflammasome activation (He, Zeng, et al. 2016). Moreover, Nek7 is also needed for NLRP3 activation in macrophages harboring an NLRP3-activating mutation (NLRP3 R258W) (He, Zeng, et al. 2016). The important role of Nek7 in NLRP3 activation has been further confirmed by three in vivo models in which Nek7 deficiency ends in reduced IL-1β secretion, lessened recruitment of immune cells, and reduced disease severity compared with wild-type mice (He, Zeng, et al. 2016; Shi et al. 2016). Consequently, these results reveal that Nek7 is a positive regulator of NLRP3 inflammasome activation.

The Nek7/NLRP3 interaction needs efflux as this interaction is inhibited in the presence of a high extracellular concentration of K^+. A decrease in intracellular K^+ is likely to cause conformational variations in NLRP3 that favor the coupling of Nek7 to NLRP3. In this line, a mutation in NLRP3 which permits K^+ efflux-independent activation still requires Nek7 for inflammasome activation (He, Zeng, et al. 2016). Understanding the signaling mechanism of Nek7 in response to NLRP3 activators will provide new comprehension into the mechanism of NLRP3 inflammasome activation (He, Hara, et al. 2016).

3. Inflammasome studies on diet and cardiovascular diseases

Many authors have investigated the function of the inflammasome complex proteins such as caspase-1 and ASC protein in the development of high-fat diet (HFD)-induced obesity. Lack of NLRP3 inflammasome and adaptor ASC protein in obese mice improves glucose tolerance and improves insulin signaling pathways (Youm et al. 2011). $Nlrp3^{-/-}$ mice fed with

an HFD for a long period of time are protected from pancreatic β-cells apoptosis as well as a substantial enlargement of Langerhans islets (Youm et al. 2011).

Another study implicated the oxidative stress-responsive transcription factor NF-E2- related 2 (Nrf2) in NRLP3 activation and atherosclerosis development in ApoE mice (Freigang et al. 2011).In this model, atherosclerotic lesion size was about 50% lower in $Nrf2^{-/-}/ApoE^{-/-}$ mice than in heterozygous $Nrf2^{+/-}/ApoE^{-/-}$ mice. Besides, expression of bioactive IL-1α and IL-1β proinflammatory cytokines was completely abrogated after exposure of Nrf2-deficient macrophages to cholesterol crystals. Cholesterol crystals act as an endogenous pro-atherogenic danger signal that triggers and sustains vascular inflammation in a Nrf2-dependent pathway. Induction of the Nrf2 signaling is required to initiate the atherogenic effects of IL-1β and IL-1α in an NLRP3/ caspase-1-dependent as well as -independent manner (Freigang et al. 2011).

It has been observed that, in addition to the large crystals that leave clefts in tissues, a wealth of much smaller cholesterol crystals are present in the extracellular space, as well as inside immune cells in atherosclerotic lesions. In vitro experiments have shown that primed macrophages secrete vast amounts of IL-1β in response to cholesterol crystals in an NLRP3 inflammasome-dependent manner in mouse and human cells (Rajamäki et al. 2010; Duewell et al. 2010). Notably, when bone marrow from mice lacking either NLRP3, ASC or IL-1α/β was transferred into irradiated atherosclerotic-prone low- density lipoprotein (LDL)-receptor-deficient mice, and fed with a high cholesterol diet, they exhibited considerably decreased aortic lesion area, as well as lower levels of circulating IL-18 when compared to mice transplanted with wild-type bone marrow (Duewell et al. 2010). This study suggests that inflammasome activation in bone-marrow-derived myeloid cells contributes to murine atherosclerosis.

In a recent study using the apolipoprotein E (ApoE)-deficient murine atherosclerosis model, however, no differences in lesions could be assessed when mice lacked NLRP3 inflammasome components (Menu et al. 2011). The different outcomes of the studies are not entirely unexpected. The speed and extent of the growth of the atherosclerosis might be influenced by the model and the choice of atherogenic diet. In the study using the ApoE-deficient atherosclerosis model (Menu et al. 2011), the atherogenic diet used had more than eightfold higher cholesterol than the diet used in the other study (Duewell et al. 2010) and by most other researchers.

Modified LDL has shown to enhance expression of pro-IL-1β in macrophages (Duewell et al. 2010; Kleemann et al. 2008). Inside the atherosclerotic lesion, oxidized-LDL also contributes to the activation of the NLRP3 inflammasome as it promotes the formation of cholesterol crystals (Duewell et al. 2010; Klinkner et al. 1995). The oxidation of LDL is dependent on ROS produced by macrophages and surrounding epithelial cells. Oxidized-LDL itself also provokes the production of ROS (Napoli et al. 2001) and causes lysosomal damage (Yuan et al. 1997), both of which are implicated in pathways of NLRP3 inflammasome activation. Moreover, the diminished secretion of IL-1β observed in cathepsin B, and L-deficient mouse macrophages stimulated with cholesterol crystals, suggests phagosomal leakage is required to activate the NLRP3 inflammasome in atherosclerosis (Duewell et al. 2010).

Mastrocola et al. investigated the balance between beneficial and harmful pathways inside the hearts of male mice fed a standard diet (SD) or a high-fat high-fructose diet (HFHF) for three months and exposed to cardiac ischemia/reperfusion (IR) injury performed ex vivo. When exposed to IR, HFHF mice hearts showed greater infarct size and lactic dehydrogenase release in comparison with SD mice. These effects were correlated with an increased overexpression of Nlrp3 inflammasome, ending in caspase-1 activation and a diminished activation of the cardioprotective RISK/HIF- 2 α pathways (Mastrocola et al. 2016).

On a study by Wang et al., the authors investigated the role of the activation of the NLRP3 inflammasome in vascular cells to free fatty acid-induced endothelial dysfunction and vascular injury in obesity. Eight-week-old male wild-type and NLRP3$^{-/-}$ mice were used. Mice were fed for six weeks either a standard diet or a high-fat diet. Their outcomes showed that high-fat diet produced impairment of vascular integrity and augmented vascular permeability in the myocardium were significantly attenuated by NLRP3 gene deletion and that the blockade of cathepsin B with Ca-074Me significantly inhibited palmitate-induced activation of Nlrp3 inflammasomes (Wang et al. 2016).

Chen et al. studied the role of endothelial cell inflammasome activation in mediating tight junction disruption, a crucial event of endothelial dysfunction leading to endothelial hyper permeability in diabetes. The authors observed that Nlrp3 ablation prevented inflammasome activation and tight junction disassembly in the coronary arterial endothelium of diabetic mice. Likewise, NLRP3 gene silencing prevented high glucose-induced downregulation of tight junction proteins on in vitro cultures of mouse vascular endothelial cells. They concluded that the ROS-dependent activation of endothelial NLRP3 inflammasomes by

hyperglycemia might be an important initiating mechanism to cause endothelial dysfunction. These effects could contribute to the early start of endothelial injury in diabetes (Chen et al. 2016).

The group of Wang et al., investigated whether the activation of NLRP3 inflammasomes contributes to hyperhomocysteinemia (HHcy)-induced inflammation and atherogenesis in apoE$^{-/-}$ mice. By silencing the NLRP3 gene, the researchers observed a considerably decreased NLRP3 inflammasome activation, diminished plasma levels of proinflammatory cytokines, reduced macrophage infiltration and improved HHcy-induced atherosclerosis. Their results suggest that the activation of NLRP3 inflammasomes contributes to HHcy-aggravated inflammation and atherosclerosis in apoE$^{-/-}$ mice (Wang et al. 2017).

Activation of the inflammasome in early atherosclerosis appears to be unique, in that the same sterile molecule indirectly mediates both signals 1 and 2, albeit by differing modes of action. Thus, although the NLRP3 inflammasome is a major source of the active form of IL-1β and IL-18, other NLRP3-independent pathways producing those cytokines have also been implicated in the pathogenesis of atherosclerosis (Menu et al. 2011).

So we can conclude that NLRP3 inflammasome could be an essential step in the inflammatory process that is behind atherosclerosis that takes place in the blood vessels.

4. Therapeutic Strategies

Therefore, one of the most promising therapeutic strategies should be to control the NLRP3 inflammasome. We know that the genetic removal of the NLRP3 inflammasome in mice guards against cardiovascular (Duewell et al. 2010), neurologic (Heneka et al. 2013) and metabolic diseases (Wen et al. 2011) and protects against age-related functional deterioration (Youm et al. 2013). Given these various ameliorative effects, investigators are actively searching for NLRP3 antagonists.

4.1. IL-1β Antagonists

The most widely used class of inflammasome-associated therapeutics to date involves anakinra, an interleukin 1 (IL1) receptor antagonist, and related compounds. These agents directly target IL-1β signaling to stop inflammasome-linked diseases (Dinarello & van der Meer 2013). IL-1β antagonists are already in clinical use to treat diseases such as gout and genetic syndromes such as Muckle-Wells syndrome (characterized by mutations in the

NLRP3 gene producing an abnormal inflammatory response). The Canakinumab Anti-inflammatory Thrombosis Outcomes Study (CANTOS) is meant to study the efficacy of canakinumab, an antibody that selectively inhibits IL-1β, to reduce plaque inflammation and the risk of cardiovascular diseases (Ridker et al. 2011). The study will provide indirect evidence of the benefits of attenuating inflammasome formation. Besides, since canakinumab does not affect lipid parameters, the study will provide proof of the benefits of targeting inflammation in coronary artery disease (Ridker et al. 2012). IL-1β antagonists, however, does not directly target the NLRP3 molecule or complex and, therefore, would not address IL-18 as the other inflammasome-activated cytokine.

4.2. Caspase 1 Inhibition

The antiatherosclerotic outcomes of caspase 1 inhibition in animal models are less clear. Menu et al. compared Apoe$^{-/-}$/Nlrp3$^{-/-}$, Apoe$^{-/-}$/Pycard$^{-/-}$, and Apoe$^{-/-}$/Casp1$^{-/-}$ double-deficient mice fed a high-fat diet for 11 weeks and afterwards determined atherosclerosis progression and plaque phenotype in comparison with Apoe $^{-/-}$ mice (Menu et al. 2011). The authors could not find any differences in atherosclerosis development, infiltration of plaques by macrophages, or plaque stability (Menu et al. 2011). As mentioned earlier these results might have been influenced by the model and the choice of atherogenic diet. However, Usui et al. demonstrated decreased vascular inflammation and atherosclerosis in ApoE/caspase 1 double-deficient mice (Usui et al. 2012). Similarly, Gage et al. observed a decrease in atherosclerosis despite comparable lipid levels in caspase 1/ApoE double-deficient mice compared with ApoE-deficient mice (Gage et al. 2012). The difference in these studies has been attributed to the difference in the composition of the diet (Abderrazak et al. 2015).

Although caspase 1 is primarily responsible for cleavage of pro-IL-1β intracellularly, other proteases (such as neutrophil elastase) can process the pro-IL forms into active cytokines (Hazuda et al. 1990; Alfaidi et al. 2015). Caspase 1-deficient mice have low, but comparable, quantities of circulating active IL-1β compared with wild-type controls after carotid ligation and do not present a statistically significant decrease in neointima development (Chamberlain et al. 2006). Thus the effect of caspase 1 inhibition on atherosclerosis is uncertain.

4.3. NLRP3 Inhibition

Preliminary animal data suggest that inhibiting NLRP3 itself may be potentially useful. Silencing of NLRP3 suppresses atherosclerosis and stabilizes plaques in ApoE-deficient mice (Zheng et al. 2014). Arglabin, an anti-inflammasome inhibitor, has been shown to reduce inflammation and atherosclerosis in ApoE-deficient mice (Abderrazak et al. 2015). Recently, MCC950 was identified as a direct inhibitor of the NLRP3 inflammasome (Coll et al. 2015). This compound was found to stop experimental diseases associated with NLRP3 inflammasome activation as well as ex vivo restraint of the inflammasome in PBMCs from patients with CAPS-associated mutations (Coll et al. 2015). Only one study has investigated the effect of MCC950 on atherosclerotic lesion development in apoE$^{-/-}$ mice. MCC950 administration significantly reduced the production of atherosclerotic lesions as determined by maximal stenosis, average plaque size, and plaque volume (van der Heijden et al. 2017). The neurotransmitter dopamine has also been found to repress the NLRP3 inflammasome via dopamine D1 receptor-mediated E3-ligase activation that results in proteasome-dependent degradation of NLRP3 (Yan et al. 2015). Cyclic adenosine monophosphate (cAMP) performs downstream of dopamine D1 receptor to exert its inhibitory outcomes (Yan et al. 2015), and notably, this metabolite has been associated with inflammasome inhibition in other studies (Mortimer et al. 2016; Sokolowska et al. 2015; Lee et al. 2013). A mechanism has been identified by Yan et al. (Yan et al. 2013) for how ω-3 fatty acids exert anti-inflammatory properties by inhibition of inflammasome activation. It is remarkable that ω-3FAs may inhibit this activation at several steps, by impeding both "signal 1" and "signal 2," because ω-3 FA inhibition of NF-κB signalling has also been reported (Glass & Olefsky 2012).

Conclusions

To improve the prevention and treatment of the cardiovascular disease is required to understand the involved inflammatory mechanisms. NLRP3 inflammasome is an essential step that is related to atherosclerotic diet and could give new clues to improve our prevention and treatment of the cardiovascular disease.

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