Review

Role of Pyroptosis in Cardiovascular Diseases and its Therapeutic Implications

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Abstract

Pyroptotic cell death or pyroptosis is characterized by caspase-1-dependent formation of plasma membrane pores, leading to the release of pro-inflammatory cytokines and cell lysis. Pyroptosis tightly controls the inflammatory responses and coordinates antimicrobial host defenses by releasing pro-inflammatory cellular contents, such as interleukin (IL)-1β and IL-18, and consequently expands or sustains inflammation. It is recognized as an important innate immune effector mechanism against intracellular pathogens. The induction of pyroptosis is closely associated with the activation of the NOD-like receptor 3 (NLRP3) inflammasome which has been linked to key cardiovascular risk factors including hyperlipidemia, diabetes, hypertension, obesity, and hyperhomocysteinemia. Emerging evidence has indicated pyroptosis as an important trigger and endogenous regulator of cardiovascular inflammation. Thus, pyroptosis may play an important role in the pathogenesis of cardiovascular diseases. Design of therapeutic strategies targeting the activation of NLRP3 inflammasome and pyroptosis holds promise for the treatment of cardiovascular diseases.

Key words: pyroptosis, inflammasome, NLRP3, caspase-1, inflammation, cardiovascular diseases

Introduction

Cell death is one of the ancient processes with fundamental biological importance in both normal physiology and pathological states. Recently, caspase-1 in both human and mouse, caspase-4/5 in human, and caspase-11 in mouse, are recognized to mediate a novel form of programmed cell death termed as pyroptosis. Pyroptosis is characterized by the formation of membrane pores, cell lysis and the release of pro-inflammatory cytokines and intracellular content. This event is predicted to be inherently inflammatory and coincides with the activation of inflammasome, a molecular platform resulting in caspase-1 activation and interleukin (IL)-1β and IL-18 secretion upon cellular infection or stress. The active caspase-1 triggered by inflammasome activation cleaves gasdermin D (GSDMD) to generate an N-terminal GSDMD fragment (GSDMD-NT), which further induces the formation of membrane pores and subsequent inflammatory responses. As an innate immune effector mechanism, pyroptosis has the ability to defense against infection. Emerging evidence has indicated that pyroptosis and related inflammasome activation play important roles in the progression of vascular inflammation and cardiovascular diseases. In this review, we summarize the molecular pathways of pyroptosis and the implications in cardiovascular research.

Pyroptosis: a form of pro-inflammatory programmed cell death.

In 1992, morphological features of pyroptosis were firstly observed in macrophages infected with the Gram-negative bacterial pathogen *Shigella flexneri*, which were mistakenly considered as morphological

changes of apoptosis at that time[1]. A similar phenotype was observed in *Salmonella*-infected macrophages in 1999[2]. Further studies revealed that both selective caspase-1 inhibitor and *caspase-1* knockdown efficiently blocked the *shigella flexneri*-induced cell death, whereas caspase-3 inhibitor did not, suggesting a novel form of cell death, depending on the activation of caspase-1, but not classical apoptotic caspase-3[3-5]. Until 2001, pyroptotic cell death (pyroptosis) was firstly defined as a novel form of caspase-1-dependent programmed cell death by Cookson BT and Brennan MA[6]. In addition to microbial signaling, endogenous contents released by cells in overwhelming stress were also identified to induce macrophage pyroptosis. Pyroptosis has been found not only in monocyte/macrophages, but also in other cells including dendritic cells[7], hepatic cells[8], endothelial cells[9] and myocardial cells[10].

Macrophages undergoing pyroptosis show several morphological features of apoptosis, and also exhibit some characters which are similar to necrosis. Indeed, pyroptosis is characterized by rapid formation of membrane pores with a diameter of 10-15 nm[11]. Cellular ionic gradients are dissipated by these pores, which allow water influx, cell swelling and osmotic lysis with the release of intracellular pro-inflammatory contents including IL-1β, IL-18, high mobility group box-1 protein (HMGB-1) and heat shock protein (HSP). These are similar to oncosis but remarkably opposite to apoptosis which is characterized with the formation and non-inflammatory phagocytic uptake of apoptotic bodies. Several features of pyroptotic cells seem to overlap with apoptotic cells. Both pyroptosis and apoptosis share the feature of chromatin condensation, but the nucleus remains intact and karyorrhexis does not occur in pyroptosis[12]. Another feature that is shared between pyroptosis and apoptosis is annexin V positive staining. During the early stages of apoptosis, phosphatidylserine is translocated to the outer leaflet, leading to positive cell surface staining with annexin V[13]. As cell membrane is ruptured during pyroptosis, annexin V is permited to enter the cell and stains the inner leaflet of the membrane. In contast, 7-aminoactinomycin or propidium iodide, as alive cell membrane impermeant dyes, can stain the nucleus of pyroptotic cell through the membrane pores, but not the early-stage apoptotic cell[14]. Thus, these dyes are applied to differentiate between apoptosis and pyroptosis. Moreover, pore formation causes cell swelling in pyroptotic cell, whereas cell shrinks in apoptosis[14]. Caspases play central role in initiating both apoptosis and pyroptosis. The characterized effectors of apoptosis are caspase-3, -5 and -7, whereas pyroptosis is induced by pro-inflammatory caspases (especially for caspase-1).

Mechanisms of Pyroptosis

The host can sense intracellular and extracellular 'danger' signals generated by invading microorganisms or by the host in response to tissue injury. The innate immune responses rely on specific host-receptors which are termed as pattern-recognition receptors (PRRs) to detect pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs)[15-18]. Based on their location, the PRRs can be differentiated into membrane-bound PRRs that recognize signals of infection in the cellular milieu or endosomes, such as Toll-like receptors (TLRs) and C-type lectin-like receptors, and cytoplasmic PRRs that recognize invasive pathogens, such as nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), retinoic acid-inducible gene I-like receptors, absent in melanoma 2 (AIM2)-like receptors (ALRs), cyclic GMP-AMP synthase, and stimulator of interferon gene[18-21]. Detection of PAMPs and DAMPs by NLRs and ALRs triggers the assembly of a caspase-1-activating complex that was firstly termed inflammasome by Tschopp *et al*[22]. Active caspase-1 further processes pro-inflammatory cytokines (IL-1β and IL-18), and initiates pyroptosis by disruption of plasma membrane integrity[18, 23]. In addition, mouse caspase-11 and human caspase-4 and -5 can directly bind to cytosolic lipopolysaccharide (LPS), leading to non-canonical inflammasome activation [18]. Non-canonical inflammasome can also induce pyroptosis, but cannot directly process pro-inflammatory IL-1β and IL-18. However, non-canonical inflammasome is capable of inducing the processing of pro-inflammatory cytokines indirectly by activating the canonical inflammasome through a not well-defined mechanism.

Pyroptotic pathway by caspase-1 dependent model

It is critical for pyroptosis to activate pro-inflammatory caspases. Thus, how inflammatory caspases are activated is a key question to elucidate the mechanism of pyroptosis. In 2002, inflammasome was firstly identified as the molecular platform triggering the activation of inflammatory caspases and consequently processing of pro-IL-1β and pro-IL-18 by Tschopp *et al*[22]. Inflammasome is multiprotein complex, composed of sensor, the adaptor protein ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain (CARD)) and caspase-1. To date, several types of inflammasomes have been identified, including NLRP1, NLRP3, NLRC4, NLRP6, and AIM2

inflammasomes. Among these different types of inflammasomes, the NLRP3 inflammasome has been extensively studied in a variety of mammalian cells, and has been linked with various autoimmune and inflammatory diseases. Therefore, the role of NLRP3 inflammasome and related pyroptosis in cardiovascular disease will be discussed in this review.

The NLR protein of NLRP3 inflammasome contains a conserved nucleotide-binding and oligomerization domain (NACHT domain), C-terminal leucine rich repeats and pyrin domain (PYD) for multimerization[24]. Upon the activation of NLRP3 inflammasome, the NLRs oligomerize via the NACHT domains, followed by the recruitment of adapter protein ASC through PYD-PYD interaction. ASC forms large speck-like structures and recruits pro-caspase-1 *via* CARD-CARD interaction. Pro-caspase-1 is subsequently autocatalytically cleaved into p10 and

p20 subunits that form the active caspase-1 p10/p20 tetramer, mediating the maturation and secretion of IL-1β and IL-18[25, 26]. Caspase-1 can also cleave GSDMD to generate GSDMD-NT, which forms plasma membrane pores to induce pyroptosis (**Figure 1**)[11, 27-29].

The canonical inflammasome activation has been proposed to be a two-step process involving priming followed by assembly[30]. The activation of NLRP3 inflammasome requires 'priming' with TLRs agonists (such as LPS) to initiate signaling cascades (primarily nuclear factor-κB (NF-κB)-dependent pathway) that ultimately promote a transcriptional response to up-regulate pro-inflammatory mediators (such as pro-IL-1β) (**Figure 1**). After this priming phase, inflammasome assembles in response to a diverse range of PAMPs or DAMPs to form a large molecular platform triggering activation of inflammatory caspases and processing of pro-IL-1β.

In addition, three models about the assembly of NLRP3 inflammasome have been proposed (**Figure 1**). Extracellular ATP stimulates the purinergic receptor P2X, ligand-gated ion channel 7 (P2X7) on cell membrane[31], triggering K^+ efflux and inducing recruitment of pannexin 1 to form large membrane pores[32]. Therefore, extracellular agonists are able to penetrate the cell membrane and activate NLRP3 inflammasome. A second model was presented for particulate or crystalline agonists, such as cholesterol crystal, monosodium urate, silica and asbestos. Uptake of such substances leads to lysosomal damage, resulting in the leakage of lysosomal cathepsins B and L that are sensed by the NLRP3 inflammasomes[33]. Under the third model, agonists trigger the generation of reactive oxygen species (ROS), which is the common pathway engaging the NLRP3 inflammasome. Intriguingly, all three models are not functionally independent but rather interactive.

The agonist of NLRP3 inflammasome serves as potential inducer for cell pyroptosis. The detailed molecular basis for pyroptosis was unclear until very recently. Several studies simultaneously identified GSDMD as a critical executor of pyroptosis.

GSDMD belongs to a gasdermin family that shares the pore-forming domain[34]. Both caspase-1 and caspase-4/5/11 can cleave GSDMD to release GSDMD-NT that perforates the plasma membrane to induce cell swelling and
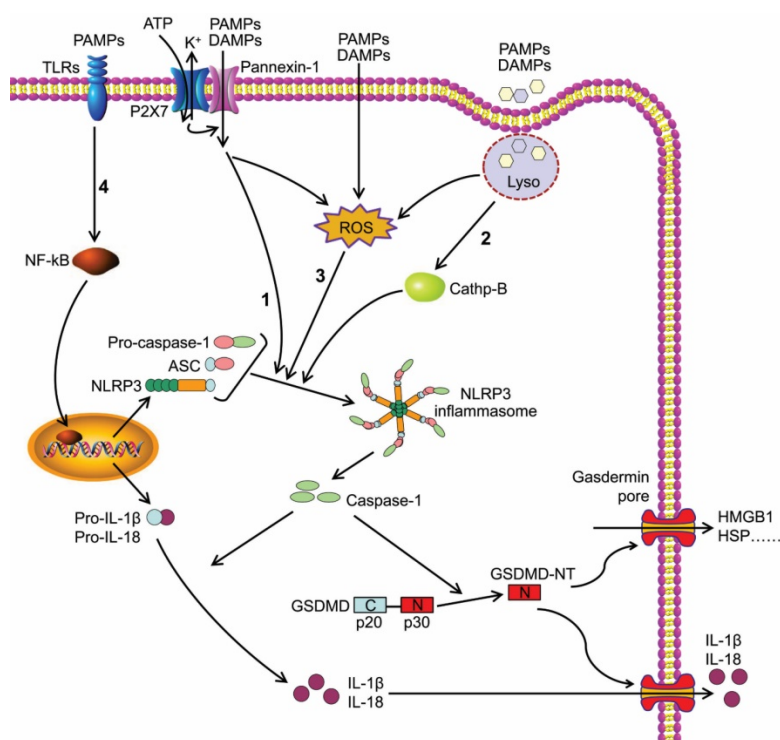
Figure 1. Caspase-1-dependent canonical pyroptotic cell death induced by NLRP3 inflammasome activation. The NLRP3 oligomerization and ASC recruitment trigger pro-caspase-1 autocleavage, leading to autocatalytic activation of caspase-1, which in turn converts inactive pro-IL-1β and pro-IL-18 into their bioactive and secreted forms (IL-1β and IL-18). The active caspase-1 also cleaves GSDMD to generate GSDMD-NT, which forms plasma membrane pores to induce pyroptosis. Three models about the assembly of NLRP3 inflammasome have been proposed: (1) The NLRP3 inflammasome is activated by extracellular ATP through one of the following mechanisms: the P2X7-activated pannexin-1 pore allows cytoplasmic entry of extracellular factors that are direct NLRP3 ligands, or NLRP3 senses either K^+ efflux or the loss of membrane integrity. (2) Crystalline or particulate agonists are phagocytosed, leading to the leakage of lysosomal cathepsins B and L, which are sensed by the NLRP3 inflammasome. (3) NLRP3 agonists including DAMPs and PAMPs trigger the production of ROS, which leads to the activation of the NLRP3 inflammasome. In general, the three models discussed above are not functionally independent but rather interactive. (4) Besides above three models, the activation of NLRP3 inflammasome also requires 'priming' with TLRs agonist (such as LPS).

osmotic lysis. Other gasdermin family members also show pore-forming activity, but they are not substrates of the inflammatory caspases[28]. GSDMD is cleaved at the conserved residue D276, which separates GSDMD into a GSDMD-NT domain (p30) and a GSDMD C-terminal domain (p20)[11]. GSDMD-NT can combine with lipid in the plasma membrane and form large oligomeric pores, leading to the disruption of cell membrane integrity, the increase of intracellular osmotic pressure, and the release of inflammatory intracellular contents, such as HMGB-1 and HSP (**Figure 1**)[11, 28, 35, 36]. This caspase-1-dependent GSDMD cleavage is defined as canonical pyroptotic pathway[37].

Pyroptotic pathway by caspase-1 independent model

The other inflammatory caspases, mouse caspase-11 and human caspase-4 and -5, are required for non-canonical inflammasome activation, thus are major mediators of non-canonical pyroptosis (**Figure 2**). In 2011, Kayagaki *et al* reported that gram-negative bacteria (*Escherichia coli*, *Vibrio cholerae*, and *Citrobacter rodentium*)-induced IL-1β maturation and secretion were blocked in caspase-11$^{-/-}$ macrophages rather than caspase-1$^{-/-}$ macrophages, whereas ATP and monosodium urate-induced canonical inflammasome activation was intact in caspase-11$^{-/-}$ macrophages,

indicating caspase-11 but not caspase-1 was required for non-canonical inflammasome activation and related cell death[38]. Follow-up studies identified intracellular LPS as the trigger of TLR4-independent non-canonical inflammasome[39, 40]. Recent study demonstrated that the high-affinity interaction between the lipid A component of LPS and the CARD domain of cytoplasmic caspase-11/4/5 led to direct binding of them, and resulted in inflammatory caspase oligomerization and activation[41, 42]. Active caspase-4/5/11 ultimately cleaved GSDMD to induce cell pyroptosis similar to canonical pathway (**Figure 2**)[41]. In addition, non-canonical inflammasome activation led to secondary activation of the canonical inflammasome and caspase-1, and subsequent maturation and secretion of proinflammatory cytokines[38], which may be an indirect consequence of GSDMD-NT[37]. Although a study reported that caspase-4 could cleave pro-IL-1β directly[43], the inability of caspase-4/5/11 to cleave the precursors of proinflammatory cytokines was widely accepted and considered as a major difference between canonical and non-canonical pyroptotic pathways.

In addition, gasdermin E (GSMDE) can shift caspase-3-mediated classical apoptosis induced by tumor necrosis factor alpha (TNFα) or chemotherapy drug to pyroptosis. GSDME is specifically cleaved by non-proinflammatory caspase-3, producing a GSDME N-terminal cleavage product that perforates membrane and thereby induces cell pyroptosis[44]. These findings reveal novel insights into mechanisms underlying pyroptosis.

Pyroptosis and Cardiovascular Disease

Pyroptosis and Atherosclerosis

Atherosclerosis is complicated by a number of events: endothelial dysfunction; accumulation and oxidation of low-density lipoprotein (LDL); recruitment of monocytes and lymphocytes; migration and proliferation of smooth muscle cells; activation of proinflammatory cytokines; adherence of platelets. In these processes, inflammatory reaction was recognized in the atherosclerotic lesions from the early stage of fatty streak to culmination into an acute cardiovascular event caused by plaque erosion or rupture. However, how the inflammation contributes to atherosclerosis is still largely unclear.

More and more studies revealed that
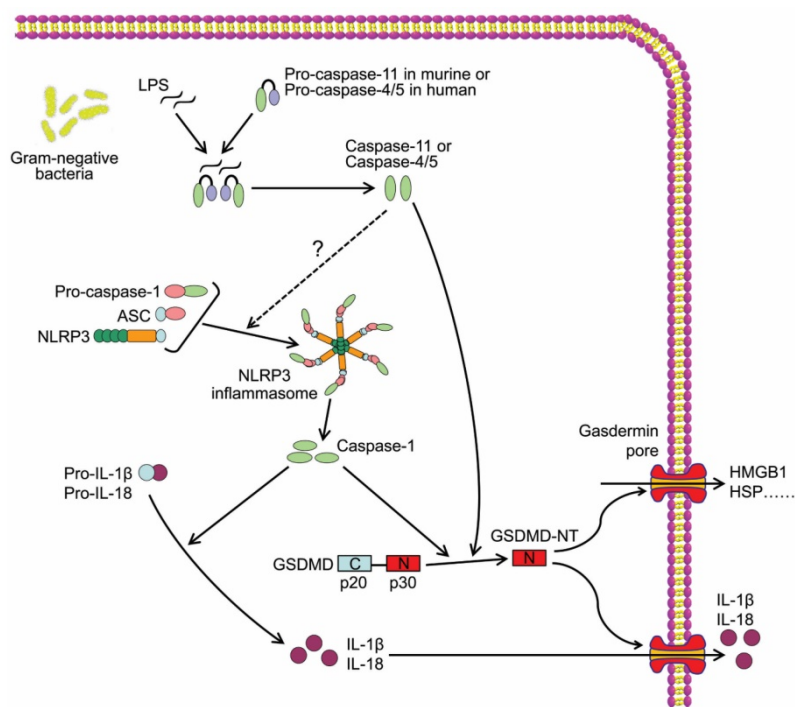
Figure 2. Caspase-11/4/5-dependent pyroptotic cell death. Cytosol LPS is sensed by proinflammatory caspases including caspase-11 in mouse and caspase-4/5 in human through a direct binding between LPS and the caspases, which induces the oligomerization and activation of the inflammatory caspases. The active caspase-11/4/5 subsequently cleaves GSDMD to generate GSDMD-NT and induces pyroptosis.

risk factors of atherosclerosis could activate NLRP3 inflammasomes in both endothelial cells and macrophages. In addition, NLRP3 inflammasome-mediated pyroptosis has been observed in the atherosclerotic plaques and is positively correlated with the plaque rupture and vascular inflammation, indicating that NLRP3 inflammasome and related pyroptosis play an important role in the progression of atherosclerosis.

Pyroptosis and Dyslipidemia

Cholesterol crystals have been identified as a potential biomarker for atherosclerosis and a major risk factor for plaque vulnerability[45]. Duewell *et al* firstly reported that cholesterol crystals activated NLRP3 inflammasomes by inducing lysosomal damage, and led to the release of IL-1β[46]. Combined confocal-reflection microscopy revealed that a large load of cholesterol crystals were deposited in the aortic wall and coincided with increased recruitment of macrophages in high cholesterol diet-fed low-density lipoprotein receptor-null (*LDLR*$^{-/-}$) mice. In contrast, a significant decrease of atherosclerotic lesion size was also observed at the aortic sinus of high fat diet-fed *LDLR*$^{-/-}$ mice reconstituted with *NLRP3-*, *ASC-* or *IL-1α/β*-knockout bone marrow cells[46]. Consistent with these *in vivo* findings, cholesterol crystals activated NLRP3 inflammasomes in human macrophages, which was suppressed by silencing of NLRP3[47]. These studies confirmed that NLRP3 inflammasomes were activated by cholesterol crystals and required for atherogenesis. Given that caspase-1 activation induced by NLRP3 inflammasome is a major mediator for pyroptosis, these work further suggested that pyroptotic macrophages might take part in the progression of atheroslerosis.

In addition, oxidized LDL (ox-LDL), a major risk factor for atherosclerosis, triggered the assembly of NLRP3 inflammasome, and subsqently activated caspase-1 and processed IL-1β in macrophages[46, 48]. Immunohistochemistry results showed that caspase-1 was activated in advanced atherosclerotic lesion and co-located with macrophages and TUNEL positive area, suggesting that pyroptosis was implicated in advanced plaque. Another *in vitro* experiment further confirmed that ox-LDL induced pyroptosis in macrophages[49], which was inhibited by the depletion of mitochondrial DNA[50].

In addition to ox-LDL, NLRP3 inflammasome was also activated by high level of triglyceride, a marker for several types of atherogenic lipoproteins [51, 52]. Yin *et al* also reported that early hyperlipidemia induced caspase-1 activation along with enhanced expression of intercellular adhension molecule, vascular cell adhension molecule and E-selectin in the aorta, and promoted monocyte recruitment in *ApoE*$^{-/-}$ mice, whereas the knockout of *caspase-1* obviously suppressed these effects and decreased the area of atheroclerotic plaque[53]. They further performed *in vitro* expriments and found that ox-LDL induced cell pyroptosis through ROS-dependent pathway in endothelial cells. The pyroptosis of vascular endothilal cells was firstly linked to atherosclerotic progression in this work.

As same as ox-LDL, palmitic acid, a saturated fatty acid, has been reported to induce pyroptosis in ROS-dependent manner in human umbilical venin endothelial cells (HUVECs), while pretreatment with dihydromyricetin inhibited palmitic acid-induced pyroptosis by activating Nrf2-antioxidant response element signaling pathway[54]. Another study also pointed out that cholesterol crystal obviously induced NLRP3 inflammasome-dependent pyroptosis in primary coronary endothelial cells[55], indicating that inflammasome-mediated pyroptosis might play an important role in cholesterol crystal-induced atherosclerosis. As endothelial cells cover the inner surface of vessel wall and are directly exposed to the atherosclerosis-related danger signals in the circulation, endothelial dysfunction is an initial event responsible for monocyte recruitment in athero-genesis[56]. It is very likely that cell pyroptosis can switch endothelial cells to the proinflammatory state which subsequently contributes to atherosclerosis.

Pyroptosis and Hyperhomocysteinemia

Hyperhomocysteinemia (HHcy) was defined as elevated plasma level of homocysteine (Hcy) (>15 µmol/L). HHcy is an important and independent risk factor involved in several disorders including atherosclerosis[57]. Earlier reports have shown that plasma levels of proinflammatory cytokines including IL-6 and TNFα were increased in HHcy individuals, which played a crucial role in endothelial damage and atherosclerosis[58]. Since Hcy can be autooxidized with anthor Hcy molecule to generate ROS and the disulfide[59], Hcy has the potential to activate NLRP3 inflammasome. Recently, a study from our laboratory demonstrated that HHcy-induced activation of NLRP3 inflammasome in macrophages contributed to vascular inflammation and atherosclerosis[60]. ROS scavenger N-acetyl-L-cysteine suppressed the activation of NLRP3 inflammasomes and alleviated atherosclerosis induced by HHcy[60]. In fact, there was an evidence showing that the conbination of Hcy and LPS synergistically induced endothelial cell pyroptosis *via* caspase-1-dependent inflammasome activation[61]. Further study found that intracellular ROS levels determined Hcy/LPS-induced death destiny in endothelial cells: relatively high ROS levels

directly induced apoptosis, while moderate ROS generation preferred to trigger pyroptosis[61]. These phenomena indicate that HHcy-aggravated atheroclerosis may be closely associated with not only apoptosis of endothelial cells but also the pyroptosis-mediated inflammation.

Evidence shows that certain heavy metals may regulate NLRP3 inflammasome activation. Cadmium (Cd), a toxic and nonessential heavy metal, has been linked to cardiovascular diseases including athero-sclerosis[62]. Chen *et al* recently found that Cd induced NLRP3 inflammasome activation and subsequent pyroptosis *via* mitochondrial ROS-dependent pathway in vascular endothelial cells[9]. Whether NLRP3 inflammasome and related pyropto-sis contribute to Cd-induced cardiovascular diseases needs further exploration in animal models.

Pyroptosis in Obesity and Diabetes: Reminding of NLRP3 Inflammasome

Obesity and related metabolic syndrome pose huge threats to human health. Active inflammation in obese adipose tissue is increasingly recognized as a key event underlying metabolic diseases. Obesity provides a proinflammatory condition which contri-butes to the increase of circulating proinflammatory cytokines[63]. Studies showed that the expression of caspase-1, ASC and NLRP3 was upregulated in high fat diet-induced obesity mice, while the knockout of *NLRP3* or *caspase-1* suppressed obesity-induced inflammasome activation in fat depots and liver[64]. Interestingly, obese *db/db* mice exhibited ultra-structural abnormalities similar to pyroptotic cells with ROS increment and caspase-1 activation in the subcutaneous and visceral hypertrophic adipocytes [65]. In addition, Konene *et al* found that the expression of caspase-1 in the abdominal adipose tissue was markedly higher than in the subcutaneous fat, indicating that pyroptotic cell death was probably presented in the abdominal adipose tissue[66].

A study showed that peripheral blood-derived macrophages from drug-naïve patients with type 2 diabetes displayed enhanced expression of NLRP3 and ASC together with caspase-1 activation and IL-1β maturation[67]. It was reported that high glucose stimulated IL-1β secretion in pancreatic β cells[68]. Further study demostrated that high glucose induced NLRP3 inflammasomes activation and IL-1β secretion, leading to pancreatic β cell death[68]. In normal condition, the thioredoxin (TRX)-interacting protein (TXNIP) was bound to TRX, however, TXNIP was dissociated from TRX to interact with and activate NLRP3 inflammasome in various types of cells when incubated with high glucose[68]. In contrast, γ-tocotrienol, known to exert potent anti-inflammatory function in various cell types[69], was shown to inhibit NLRP3 inflammasome activation and the recruitment of macrophages to adipose tissues, thus ameliorated insulin resistance [70]. In fact, γ-tocotrienol not only inhibited the tumor necrosis factor receptor-associated factor 6/NF-κB pathway, but also activated AMP-activated protein kinase/autophagy axis, leading to reduced cleavage of caspase-1. These results suggested that NLRP3 inflammasome and related pyroptosis might be potentially promising targets for new drug research in diabetes.

Pyroptosis in Hypertension: Reminding of NLRP3 Inflammasome

Hypertension is a well-known risk factor for atherogenesis-related cardiovascular and cerebro-vascular diseases. Recently, the role of inflammation in the pathologenesis of hypertension has attracted great interests. Although there was no direct proof that cell pyroptosis contributed to pathological process of hypertension, studies showed that circulating levels of IL-1β and IL-18 were elevated in hypertensive mice and rats[71, 72]. Dalekos *et al* found that serum level of IL-1β in patients with essential hypertension was higher than in normotensive controls[73]. Given that IL-1β maturation is dependent on caspase-1, there is a possibility that hypertension may be assiociated with the activation of caspase-1. Another study showed that mRNA expression of pro-caspase-1 in the aorta and renal artery was increased in spontaneously hypertensive rats, which indirectly supported this possibility[74]. Moreover, high salt increased the expression of NLRP3, ASC and pro-caspase-1, the activation of caspase-1, and the maturation of IL-1β in mouse kidney[71]. Interestingly, *NF-κB* knockdown was associated with reduced expression of NLRP3 inflammasome components in hypothalamic paraventricular nucleus, and thus delayed the progression of hypertension in Dahl salt-sensitive hypertensive rats[75]. Therefore, targeting NLRP3 inflammasome may be a therapeutic strategy to treat hypertension. Based on this hypothesis, Tang *et al* found that ellagic acid, a promising treatment candidate for pulmonary artery hypertension (PAH), inhibited NLRP3 inflammasomes activation primarily by exerting its antioxidant effect[76].

Pyroptosis and Myocardial Ischemia/Reperfusion Injury

Therapeutic strategies aimed at rescuing ischemic myocardium have been studied extensively. Reperfusion remains the definitive treatment for acute myocardial infarction (MI). However, restoring blood

flow carries the potential to exacerbate lethal tissue injury, a process termed as "ischemia/reperfusion injury". The major contributory factors of reperfusion injury include oxidative stress[34], calcium overload [41], opening of mitochondrial permeability transition pores[42], and in particular inflammation[18]. Myocardial ischemia/reperfusion injury (MIRI) may cause MI and cardiac arrhythmias as well as cardiac dysfunction[77]. MI is defined as myocardial cell death due to prolonged ischemia and usually caused by the sudden thrombotic occlusion of coronary artery [78]. Recent studies found that NLRP3 inflammasome and related pyroptosis were strongly associated with pathological process of MIRI and MI. As previously mentioned, NLRP3 inflammasome activation and pyroptosis contributed to plaque rupture, and the release of IL-1β was an early and prominent event of inflammatory responses in MI[49]. Investigators have also found that inflammatory responses and subsequent injuries, including myocardial dysfunction and fibrosis, were significantly alleviated in *ASC* or *caspase-1* deficient mice[79]. Further studies showed that NLRP3 inflammasome was activated in the ischemic heart as evidenced by increased NLRP3 expression and IL-1β and IL-18 production with caspase-1 activation, whereas NLRP3 siRNA, treated *via* intramyocardial injection, inhibited these effects and decreased TUNEL positive areas. Another study showed that pyroptosis was induced by ROS-dependent NLRP3 inflammasome activation, and inhibition of NLRP3 inflammasome activation alleviated MIRI in diabetic rats, suggesting that NLRP3 inflammasome-mediated pyroptosis played a critical role in inflammatory responses and MIRI in diabetic rats[80]. In addition, silence of cryopyrin (also known as NLRP3) and P2X7 prevented the formation of NLRP3 inflammasome, limited myocardial infarct size and ameliorated cardiac remodeling after acute MI[81]. These results indicated that NLRP3 inflammasome activation and related pyroptosis are potential novel therapeutic targets for MIRI.

Pyroptosis and Diabetic Cardiomyopathy

Diabetic cardiomyopathy (DCM) is a diabetes-induced cardiomyopathy that occurs in diabetic subjects without coronary artery disease and hypertension[82, 83]. DCM is characterized by functional and structural abnormalities, including myocardial dysfunction[84], myocardial cell death[85], myocardial fibroblast activation[86], and metabolic deregulation[87]. Among them, myocardial cell death is a key factor in the progression of DCM, leading to a loss of contractile units, conduction disturbances, compensatory hypertrophy of myo-

cardial cells, and myocardial fibrosis[88]. Apoptosis has been widely recognized in the pathogenesis of DCM. In addition, recent evidence showed pyroptosis could occur in DCM heart. Electron microscopy studies demonstrated that most of the dying cells had characteristics of swollen fibril and mitochondria similar to the features of pyroptosis in the myocardium of diabetic mice and rats[89-91]. Intriguingly, Luo *et al* reported that NLRP3 inflammasome-caspase-1-mediated pyroptosis was presented in myocardium of diabetic rats, while silencing of NLRP3 ameliorated cardiac inflammation and pyroptosis, and improved myocardial function[92].

Numerous studies have shown that microRNAs (miRNAs) are involved in the regulation of cardio-myocyte pyroptosis in DCM. For example, Li *et al* demonstrated that miR-30d was upregulated in high glucose-treated cardiomyocytes and streptozotocin (STZ)-induced diabetic rat hearts, which regulated cardiomyocyte pyroptosis in DCM[10]. In an effort to explore the pro-pyroptotic mechanisms of miR-30d, they found that miR-30d directly repressed the expression of Forkhead box O3 and its downstream factor, apoptosis repressor with caspase recruitment domain[10]. Moreover, miR-9 inhibited hypergly-cemia-induced pyroptosis in human ventricular cardiomyocytes[93], suggesting that miR-9 may have a protective effect on DCM.

Pyroptosis in Viral Myocarditis: Reminding of NLRP3 Inflammasome

About 20 common types of viruses have been related to myocarditis in human[94]. The dominant etiological agent of viral myocarditis is coxsackievirus B3 (CVB3)[95]. Wang *et al* firstly reported that the activation of NLRP3 inflammasome was involved in CVB3-induced myocarditis[96]. Both *in vivo* and *in vitro* results showed that CVB3 infection activated NLRP3 inflammasome. Conversely, once inflamma-some activation was inhibited, CVB3-induced myocarditis manifested by weight loss, increased serological indexes of creatine kinase and CK-MB activities, was obviously suppressed. Caspase-1 inhibitor also improved left ventricular ejection fraction and left ventricular fractional shortening in CVB3-induced myocarditis[96]. Further study showed that ROS generation and K$^+$ efflux were involved in the activation of NLRP3 inflammasome caused by CVB3 infection[96]. Recent study demonstrated that NOD2 mRNA expression was higher in endomyocardial biopsy tissue from CVB3-positive patients as compared with myocarditis patients without persistent CVB3 infection[97]. Interestingly, mesenchymal stromal cells suppressed NOD2 expression and NLRP3 inflammasome

activation, which was associated with improved expression of markers for cardiac contractility and fibrosis in the heart of CVB3-infected mice, suggesting that NOD2 might coordinate NLRP3 inflammasome activation in CVB3-infected myocardium[98]. In addition, Wang *et al.* found that CVB3-induced myocarditis was aggravated by cathepsin B-induced activation of NLRP3 inflammasome and pyroptosis [99]. It is still not clear whether other types of viruses regulate NLRP3 inflammasome activation in viral myocarditis.

Pyroptosis and Heart Failure

Heart failure (HF) means that the heart is unable to pump sufficiently to sustain blood flow to meet the needs of the body[100]. HF is the end stage of many cardiovascular diseases. Common causes of HF include myocarditis, MI, cardiomyopathy, hypertension, atrial fibrillation (AF), valvular heart disease, alcohol abuse, and infection. Recently, elevated mRNA of NLRP3 and enhanced cleavage of caspase-1 were observed along with cardiac hypertrophy and ventricular dilatation in calcineurin transgene (CNTg) mice, an established mouse model for chronic HF[101]. As expected, they also discovered that genetic ablation of *NLRP3* or administration of IL-1 receptor antagonist attenuated cardiac inflammation and systolic dysfunction[101]. Similarly, the expression of NLRP3 inflammasome components was significantly increased, and associated with elevated inflammatory mediators and profibrotic factors and impaired cardiac function in a mouse model of transverse aortic constriction (TAC)-induced ventricular hypertrophy[102] [103]. Interestingly, the inhibition of NLRP3 by triptolide[103] or pirfenidone[102] attenuated myocardial remodeling and improved cardiac function in TAC mice. In addition, the activation of NLRP3 inflammasome was observed in LPS-stimulated cardiac fibroblasts[104] and myofibroblasts[105], suggesting that NLRP3 inflammasome and related pyroptosis may contribute to myocardial dysfunction in sepsis.

AF, a common kind of arrhythmia, is associated with an increased risk of worsening HF[106, 107]. A strong inflammatory response is frequently associated with AF progression[108, 109]. Increased serum levels of IL-1β and IL-18 were positively correlated with AF progression from paroxysmal AF (pAF) to long-lasting persistent AF (perAF)[109, 110]. Recently, Yao *et al* firstly showed that NLRP3 inflammasome was activated in the atrial cardiomyocytes from patients with pAF and perAF[111]. Cardiomyocyte-specific activation of NLRP3 induced abnormal Ca^{2+} release from sarcoplasmic reticulum and promoted inducible AF, which was attenuated by MCC950, a specific NLRP3 inflammasome inhibitor, and genetic silencing of *NLRP3*[111]. Genetic silencing of *NLRP3* also prevented AF development in cAMP-responsive element modulator (CREM)-transgenic mice, a spontaneous AF mouse model[111].

Targeting of NLRP3 inflammasome or cell pyroptosis as a therapeutic strategy

Based on experimental data, inhibiting NLRP3 inflammasome or cell pyroptosis may supply a new therapeutic strategy for cardiovascular disease. The complicated signaling of NLRP3 inflammasome and related pyroptosis offers diverse targets for inhibiting their activation, including suppression of upstream signals of NLRP3 inflammasome, prevention of NLRP3 inflammasome assembly, inhibition of caspase-1 activation and GSDMD cleavage, and neutralizing antibodies targeting inflammasome-derived inflammatory cytokines. Preventing the assembly of NLRP3 inflammasome could be established by inhibition of K^+ efflux with glyburide[112] and β-hydroxybutyrate[113], blockage of P2X7 signaling with human cathelicidin LL-37[114], as well as the application of ROS scavenger[115]. It was reported that MCC950 disrupted the interaction between NLRP3 and ASC, and showed strong inhibitory effect on NLRP3 inflammasome and beneficial effect on inflammatory diseases[116]. In addition, parthenolide, BAY 11-7082, INF39, and 3,4-methylenedioxy-β-nitrostyrene (MNS) were reported to directly inhibit the ATPase activity of NLRP3, but these inhibitors have unspecific effects limiting their use[117-119]. Recently, Jiang *et al.* showed that CY-09 specifically blocked NLRP3 inflammasome activation by binding to the ATP-binding motif of NLRP3 NACHT domain and thus inhibiting the ATPase activity of NLRP3. Treatment with CY-09 showed therapeutic effects on cryopyrin-associated auto-inflammatory syndrome and type 2 diabetes in mouse models[120]. The suppression of NLRP3 inflammasome components could be achieved by genetic silencing of *NLRP3*, *ASC* or *caspase-1* using specific shRNAs[111] or clustered regularly interspaced short palindromic repeat (CRISPR)-associated system 9 (Cas9)[121]. Xu *et al* screened an optimized cationic lipid-assisted nanoparticle to deliver Cas9 mRNA and guide RNA targeting NLRP3 into macrophages, which improved insulin sensitivity and reduced adipose inflammation in high fat diet-induced diabetic mice[122]. Moreover, caspase-1 inhibitors are currently under development for inflammatory diseases[123, 124]. However, further studies are needed to determine whether these inhibitors are appropriate for cardiovascular diseases.

To date, most drug candidates targeting NLRP3

inflammasome have been studied only in animal models, and need to be further translated into clinical practices. The current available clinical treatments for NLRP3 inflammasome-related diseases are the agents that block IL-1β, including the recombinant IL-1 receptor antagonist anakinra, the IL-1β-neutralizing antibody canakinumab, and the soluble decoy IL-1β receptor rilonacept[125]. Interestingly, the recently completed large-scale clinical trial CANTOS showed that canakinumab targeting the IL-1β innate immunity pathway significantly decreased the rate of recurrent cardiovascular events[126], and reduced incident lung cancer and lung cancer mortality in patients with atherosclerosis[127]. However, IL-1β is not the only biological production of NLRP3 inflammasome and pyroptosis, other inflammatory cytokines, such as IL-18 and HMGB-1, also involved in the pathogenesis of cardiovascular diseases. Therefore, inhibition of IL-1β signaling may have limitations in clinical treatments.

Conclusion

Emerging evidence has demonstrated that NLRP3 inflammasome activation is a critical regulator for pyroptosis involved in the progression of various cardiovascular diseases (**Figure 3**). Pyroptosis has been observed in different types of cells and tissues in *in vivo* and *in vitro* models of cardiovascular diseases (**Table 1**). Despite of growing studies focused on the role of pyroptosis in cardiovascular diseases, the detailed molecular mechanisms remain elusive. Although to what extent does NLRP3 inflammasome activation lead to pyroptosis remains unclear, NLRP3 inflammasome activation indeed mediates pyroptosis in most situations. Therefore, blocking the inflammasome pathway has been predicated to have clinical benefits in delaying the progression of cardiovascular diseases. Several types of NLRP3 inflammasome inhibitors have been developed and validated in cell culture studies and animal models of inflammation-related diseases. However, prospective clinical trials are needed for their potential translation into clinical practice. Also other potential candidates are required for further development, such as autophagy-inducer, antioxidant and miRNA agents. Future studies are needed to answer many unresolved questions. For example, can drugs or antibodies targeting GSDMD pyroptotic pathway enhance the therapeutic potential of single NLRP3 inflammasome inhibitor on cardiovascular diseases? Further studies exploring novel signaling pathways of pyroptosis will be important for the development of new therapeutic drugs.
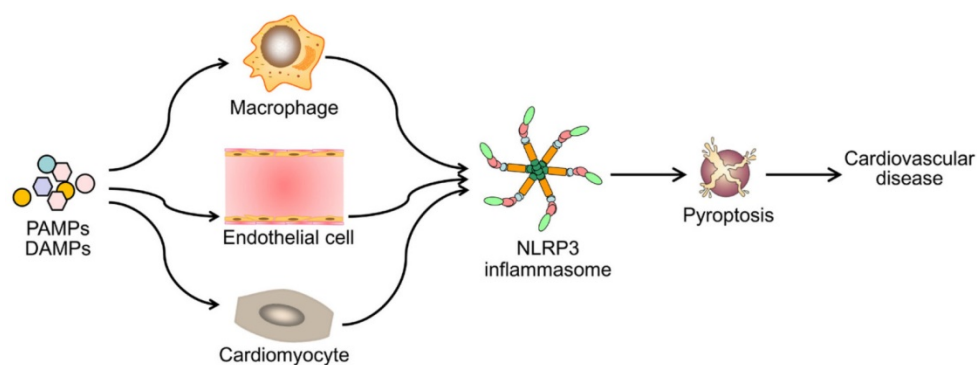
Figure 3. Diverse PAMPs and DAMPs induce cardiovascular disease by NLRP3 inflammasome-mediated pyroptosis. PAMPs and DAMPs trigger the activation of NLRP3 inflammasome and induce pyroptosis, which promotes vascular inflammation and heart remodeling, and ultimately lead to cardiovascular diseases.

Table 1. Types of cell and tissue injury subjected to cell pyroptosis in various cardiovascular diseases

Diseases	Cell types/Organ	Injury types	Effects	References
Atherosclerosis	Macrophages	Cholesterol crystals	NLRP3 inflammasome activation	Duewell *et al*.[46]
Atherosclerosis	Macrophages	Ox-LDL	Pyroptosis	Lin *et al.[49]*
Atherosclerosis	Macrophages	Triglyceride	Pyroptosis	Son *et al*.[52]
Atherosclerosis	Macrophages	Hcy	NLRP3 inflammasome activation	Wang *et al.[60]*
Atherosclerosis	HUVECs	Palmitic acid	Pyroptosis	Hu *et al*.[54]
Atherosclerosis /MI/ Hypertension	HUVECs	Cd	Pyroptosis	Chen *et al*.[9]
Atherosclerosis	Mouse primary aortic endothelial cells	Ox-LDL	Pyroptosis	Yin *et al*.[53]
Atherosclerosis	Mouse primary coronary arterial endothelial cells	Cholesterol crystals	NLRP3 inflammasome activation	Zhang *et al*.[55]
Atherosclerosis	Mouse primary aortic endothelium cells/HUVECs	Hcy+LPS	Pyroptosis	Xi *et al*.[61]

Diseases	Cell types/Organ	Injury types	Effects	References
Obesity	Subcutaneous and visceral hypertrophic adipocytes	High Fat	Ultrastructural abnormalities and caspase-1 activity	Giordano *et al.*[65]
Obesity	Human abdominal adipose tissue*		Caspase-1 expression	Koenen *et al.*[66]
Diabetes	Mouse primary β cells	High glucose	NLRP3 inflammasome activation	Zhou *et al.*[68]
Type 2 diabetes	Human monocyte-derived macrophages*		NLRP3 inflammasome activation	Lee *et al.*[67]
Hypertension	Rat aorta and renal artery	Spontaneity	mRNA expression of pro-caspase-1	Chen *et al.*[74]
Hypertension	Mouse kidney	Deoxycorticosterone acetate and salt or angiotensin II	NLRP3 inflammasome activation	Krishnan *et al.*[71]
Hypertension	Mouse myocardium	TAC	NLRP3 inflammasome activation	Wang *et al.*[102]
Hypertension	Rat hypothalamic paraventricular nucleus	Dahl salt	NLRP3 inflammasome activation	Qi *et al.*[128]
Hypertension (PAH)	Rat lung tissue	Monocrotaline	NLRP3 inflammasome activation	Tang *et al.*[76]
Hypertension	Human serum*		Increased serum levels of IL-1β and IL-18	Krishnan and Saito *et al.*[71, 72]
MI	Mouse cardiac microvascular endothelial cells	Coronary artery ligation	Pyroptosis	Liu *et al.*[115]
MI	Mouse ischemic heart	Coronary artery ligation	Pyroptosis	Mezzaroma *et al.*[81]
DCM	Rat myocardium/H9c2 cardiomyocytes	High glucose and STZ	Pyroptosis	Luo and Li *et al.*[10, 92]
Viral myocarditis	Mouse cardiomyocyte/Myocardial tissue	CVB3	NLRP3 inflammasome activation and pyroptosis	Wang *et al.*[96, 99]
Heart remodeling	Mouse myocardium	TAC	NLRP3 inflammasome activation	Wang *et al.*[102]
AF	Cardiomyocyte from human atrial biopsies* and myocardial tissue	CREM transgene	Pyroptosis	Yao *et al.*[111]
HF	Mouse myocardial tissue	CNTg	Cleaved caspase-1 expression	Bracey *et al.*[101]
Bacterial myocarditis	Mouse cardiac fibroblasts and myofibroblasts	LPS	NLRP3 inflammasome activation	Zhang *et al.[104]*

* indicated this study with cells or tissue from human.

Abbreviations

AF: atrial fibrillation; AIM2: absent in melanoma 2; ALRs: absent in melanoma 2-like receptors; ASC: apoptosis-associated speck-like protein; CANTOS: The Canakinumab Anti-inflammatory Thrombosis Outcomes Study; CARD: a caspase recruitment domain; Cd: cadmium; CK-MB: creatine kinase-MB; CNTg: calcineurin transgene; CREM: cAMP-responsive element modulator; CRISPR/Cas9: clustered regularly interspaced short palindromic repeat-associated system 9; CVB3: coxsackievirus B3; DAMPs: danger-associated molecular patterns; DCM: diabetic cardiomyopathy; GSDMD: gasdermin D; GSDMD-NT: gasdermin D N-terminal domain; Hcy: homocysteine; HF: heart failure; HHcy: hyperhomo-cysteinemia; HMGB-1: high mobility group box-1 protein; HSP: heat shock protein; HUVECs: human umbilical venin endothelial cells; IL: interleukin; LDL: low-density lipoprotein; LPS: lipopolysaccharide; MI: myocardial infarction; MIRI: myocardial ischemia/ reperfusion injury; miRNAs: microRNAs; NF-κB: nuclear factor-κB; NLRP: NOD-like receptor protein; NLRs: NOD-like receptors; NOD: nucleotide-binding oligomerization domain; Ox-LDL: oxidized low density lipoprotein; P2X7: purinergic receptor P2X, ligand-gated ion channel 7; pAF: paroxysmal AF; PAH: pulmonary artery hypertension; PAMPs: pathogen-associated molecular patterns; perAF: persistent AF; PRRs: pattern-recognition receptors; ROS: reactive oxygen species; STZ: streptozotocin; TAC: transverse aortic constriction; TLRs: toll-like receptors; TNFα: tumor necrosis factor alpha; TRX: thioredoxin; TXNIP: thioredoxin-interacting protein.

Competing Interests

The authors have declared that no competing interest exists.

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