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Inflammasomes, neutrophil extracellular traps, and cholesterol

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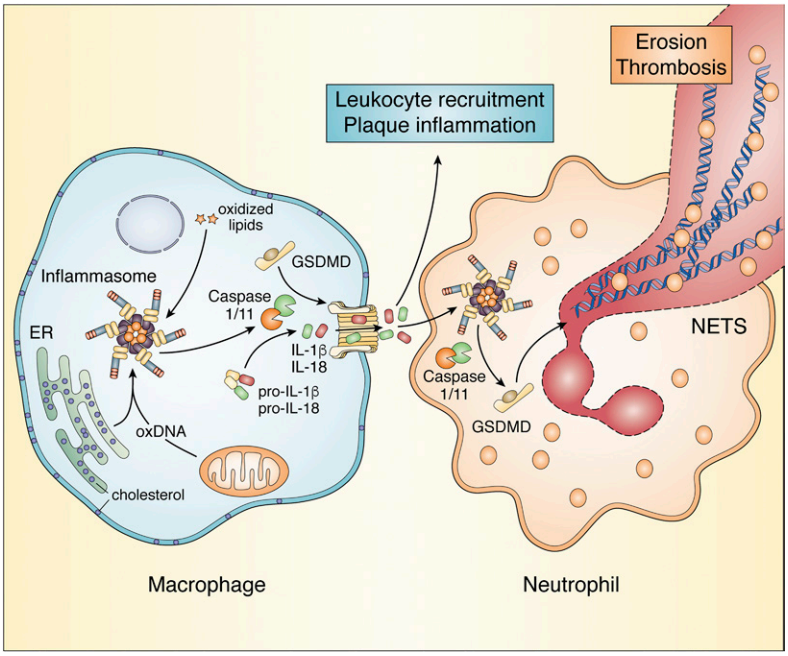
Abstract **Activation of macrophage inflammasomes leads to interleukin (IL)-1β and IL-18 secretion and promotes atherosclerosis and its complications in mice and humans. However, the specific role and underlying mechanisms of the inflammasome in atherogenesis are topics of active research. Several studies in hyperlipidemic mouse models found that the NOD-like receptor protein 3 (NLRP3) inflammasome contributes to atherosclerosis, but recent work suggests that a second hit, such as defective cholesterol efflux or accumulation of oxidized mitochondrial DNA, may be required for significant inflammasome activation. Cholesterol crystal uptake or formation in lysosomes may damage membranes and activate NLRP3 inflammasomes. Alternatively, plasma or ER membrane cholesterol accumulation may condition macrophages for inflammasome activation in the presence of danger-associated molecular patterns, such as oxidized LDL. Inflammasome activation in macrophages or neutrophils leads to gasdermin-D cleavage that induces membrane pore formation, releasing IL-1β and IL-18, and eventuating in pyroptosis or neutrophil extracellular trap formation (NETosis). In humans, inflammasome activation and NETosis may contribute to atherosclerotic plaque erosion and thrombosis, especially in patients with type 2 diabetes, chronic kidney disease, or clonal hematopoiesis. Suppression of the inflammasome by activation of cholesterol efflux or by direct inhibition of inflammasome components may benefit patients with CVD and underlying susceptibility to inflammasome activation.**—Tall, A. R., and M. Westerterp. **Inflammasomes, neutrophil extracellular traps, and cholesterol.** ***J. Lipid Res.*** **2019.** 60: **721–727.**



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Atherosclerotic CVD arises from a macrophage-driven inflammatory response to modified LDL in the arterial wall. This view has received strong support from the positive outcome of the CANTOS trial (Canakinumab Antiinflammatory Thrombosis Outcome Study), involving administration of an interleukin (IL)-1β antibody to patients with elevated levels of C-reactive protein (CRP) (1). IL-1β is a key inflammatory cytokine that promotes monocyte

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Abbreviations: AIM2, absent in melanoma 2; ASC, adaptor protein apoptosis-associated speck-like protein containing CARD; BM, bone marrow; GSDMD, gasdermin-D; IL, interleukin; LPS, lipopolysaccharide; NET, neutrophil extracellular trap; NETosis, neutrophil extracellular trap formation; NLRP3, NOD-like receptor protein 3; PAD4, peptidyl arginine deiminase 4; ROS, reactive oxygen species; TD, Tangier disease; TLR, Toll-like receptor; WTD, Western-type diet.

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and neutrophil entry into sites of inflammation. IL-1β is synthesized as a pro-form that undergoes proteolytic cleavage by CASPASE-1 in the inflammasome, a protein complex assembled in the cytosol of macrophages in response to pathogen-associated molecular patterns or danger-associated molecular patterns, leading to secretion of the active form of IL-1β. The pro-form of IL-18 is similarly processed by inflammasomes resulting in IL-18 secretion (2). Together with preclinical studies (3–7), CANTOS points to a role of inflammasomes in atherothrombotic disease.

This review will discuss the role of inflammasomes in atherosclerosis and the mechanisms underlying inflammasome activation in response to cholesterol accumulation in macrophages and neutrophils. Typical for an emerging area, several aspects of these studies are controversial. We will also discuss a potential link between inflammasomes and plaque neutrophil extracellular trap formation (NETosis). NETosis involves the release of chromatin and granule contents from neutrophils, giving rise to large extracellular webs containing DNA, proteases, and myeloperoxidase that help to trap and inactivate pathogens (8). NETosis has been implicated in atherothrombosis, notably in plaque erosion and thrombosis (9, 10), a process that may be increasingly important in acute coronary syndromes (11).

MECHANISMS OF INFLAMMASOME ACTIVATION

The NOD-like receptor protein 3 (NLRP3) inflammasome is activated by a wide variety of microbial and metabolic signals. This involves a priming step mediated by Toll-like receptors (TLRs) that leads to increased expression of *Il-1β*, and the inflammasome components, *Caspase-1* and *Nlrp3*, followed by an activation step in which the components of the inflammasome assemble in the cytoplasm and CASPASE-1 is cleaved (2). A variety of stimuli, including extracellular ATP, silica particles, uric acid crystals, or cholesterol crystals, can activate the NLRP3 inflammasome. Activation involves a sensor (NLRP3) that assembles with an adaptor [adaptor protein apoptosis-associated speck-like protein containing CARD (ASC)] and forms a filamentous structure that provides a platform for CASPASE-1 cleavage (12). The NLRP3 inflammasome seems to sense membrane damage (13), which may lead to K^+ efflux and mitochondrial reactive oxygen species (ROS) generation. Recent studies have shown that priming [in response to lipopolysaccharide (LPS)] involves the induction of mitochondrial DNA synthesis, including the enzyme mitochondrial deoxyribonucleotide kinase [uridine/cytidine monophosphate kinase 2 (UMP-CMPK2)], while activation in response to ATP or nigericin leads to the oxidation and release of mitochondrial DNA that binds and activates the NLRP3 inflammasome (14). However, it is not yet clear that this represents a universal mechanism of NLRP3 inflammasome activation. NLRP3 inflammasome activation can occur without evident mitochondrial ROS generation or lysosomal damage (7, 13), and, moreover, in some models, mitochondrial ROS generation is prevented by NLRP3 deficiency (15), suggesting that ROS can be generated downstream of inflammasome activation. Cytosolic double-stranded DNA introduced by microbes or formed in response to mitochondrial damage activates the NLRP3 inflammasome in human myeloid cells (16) and the absent in melanoma 2 (AIM2) inflammasome in mice (17). Deficiency of 25-hydroxycholesterol, a suppressor of cholesterol biosynthesis, triggers release of DNA from mitochondria, AIM2 inflammasome activation, and secretion of IL-1β and IL-18 (18). The noncanonical inflammasome is responsible for mortality during LPS-induced sepsis (13). This involves activation of cytosolic CASPASE-11, possibly in response to direct binding of LPS or oxidized lipids (19). Active CASPASE-11 can induce NLRP3-mediated CASPASE-1 cleavage (13), likely as a consequence of membrane damage and, thus, indirectly promote IL-1β and IL-18 cleavage.

INFLAMMASOMES, PYROPTOSIS, AND NETosis

Inflammasome activation can lead to pyroptosis, an inflammatory mode of cell death involving osmotic swelling, cell necrosis, and release of IL-1β and IL-18 as well as various danger-associated molecular patterns, such as IL-1α, high-mobility group box 1 (HMGB1) proteins, and ATP. Activated CASPASE-1 or CASPASE-11 cleave gasdermin-D (GSDMD), releasing an N-terminal fragment that forms membrane pores facilitating release of the aforementioned molecules, and likely as pores grow larger, eventuating in pyroptosis (20). Remarkably, a similar process in neutrophils, requiring inflammasome activation and GSDMD cleavage, leads to granule membrane dissolution, chromatin decondensation, plasma membrane leakiness, and expulsion of DNA and proteases, a process apparently identical to NETosis (21, 22).

INFLAMMASOMES AND ATHEROSCLEROSIS

The role of the NLRP3 inflammasome in atherosclerosis was first explored by Duewell et al. (3). They found a major impact of the NRLP3 inflammasome on early lesion area in Western-type diet (WTD)-fed $Ldlr^{-/-}$ mice that had been transplanted with bone marrow (BM) deficient in the key inflammasome components, *Asc* or *Nlrp3*. In contrast, Menu et al. (23) did not find any impact of whole-body deficiency of *Asc*, *Nlrp3*, or *Caspase-1/11* on the size of advanced atherosclerotic lesions in WTD-fed $Apoe^{-/-}$ mice. While some subsequent studies appeared to confirm the report of Duewell et al. (3) (see 4, 6, 24, 25), our own (7) and other studies (5) found no impact of deletion of the key inflammasome components, *Nlrp3* or *Caspase-1/11*, on the area or morphology of either early or advanced lesions in WTD-fed $Ldlr^{-/-}$ mice. The reason for the discrepant results is unknown; although unlike the studies by Duewell et al. (3), we did not find signs of inflammasome activation in $Ldlr^{-/-}$ mice (7). When additional mutations that caused macrophage inflammasome activation, such as myeloid deficiency of ABCA1 and ABCG1 or hematopoietic deficiency of 8-oxoguanine glycosylase, were introduced into $Ldlr^{-/-}$

mice, the NLRP3 inflammasome was clearly activated, as shown by increased CASPASE-1 cleavage, and did contribute to lesion area and macrophage content (5, 7). ABCA1 and ABCG1 are the principal transporters mediating cholesterol efflux from macrophages (26), indicating a role of defective cholesterol efflux in inflammasome activation. The 8-oxoguanine glycosylase is the main enzyme mediating repair of mitochondrial oxidized DNA that accumulates in atherosclerotic lesions and may directly activate the NLRP3 inflammasome (27). The AIM2 inflammasome may also promote atherogenesis: double-stranded DNA was found in lesional cells, and deficiency of hematopoietic AIM2 resulted in an increase in smooth muscle cells, collagen, and fibrous cap thickness, and a decrease in the necrotic area of advanced lesions in *Apoe*$^{-/-}$ mice (28).

Duewell et al. (3) showed that cholesterol crystals could induce macrophage NLRP3 inflammasome activation in vitro and related their in vivo findings to tiny cholesterol crystals detected in early foam cell lesions by confocal reflectance microscopy. However, prior studies by Small and Shipley (29) based on lipid phase behavior and observation of fresh plaques by polarized microscopy under temperature-controlled conditions suggested that early foam cell lesions did not contain cholesterol crystals. Rather, such lesions contained liquid or liquid crystalline cholesteryl esters that undergo artifactual crystal formation when cooled below body temperature (29). Thus, while cholesterol crystals in advanced lesions may be involved in inflammasome activation, in our opinion, a role of cholesterol crystals in early foam cell lesions is questionable.

NETosis AND ATHEROSCLEROSIS

The formation of neutrophil extracellular traps (NETs) promotes venous and arterial thrombosis in mice (30–33). NETs promote atherosclerosis and carotid thrombosis in *Apoe*$^{-/-}$ mice, as shown using chloramidine, a chemical inhibitor of NET formation (34). A recent study using mice with knockouts of peptidyl arginine deiminase 4 (PAD4), an essential enzyme in histone citrullination, suggested no impact of NETs on lesion area or macrophage content in early foam cell lesions in WTD-fed *Ldlr*$^{-/-}$ mice, even though NETs were detected in lesions (9). In the same study, deficiency of PAD4 led to decreased neutrophil adherence, arterial injury, and thrombosis in the setting of disturbed carotid arterial flow, consistent with a role of NETosis in plaque erosion (9). These findings may have relevance to humans because NETs have been associated with unstable human atherosclerotic plaques, especially in regions of superficial erosion (10). In contrast to these studies, myeloid PAD4 deficiency did have an impact on lesion area in *Apoe*$^{-/-}$ mice (35). Thus, like inflammasome activation, NETs seem to contribute to plaque development and complications under specific experimental conditions. As noted above, inflammasomes and NETs may be mechanistically interdependent. One study reported that cholesterol crystals could promote NET release that in turn promoted macrophage inflammasome activation (36). However, the conclusion that NETosis causes inflammasome activation has been questioned (33, 37). Our findings rather suggest that NETosis may be downstream of inflammasome activation in atherosclerosis (7). This conclusion is consistent with studies showing that inflammasome-dependent pyroptosis and NETosis are similar processes dependent on GSDMD (21, 22). We speculate that neutrophil inflammasome activation may induce pyroptosis/NETosis in murine atherosclerosis and perhaps contribute to plaque erosion and thrombosis in humans.

INFLAMMASOME ACTIVATION IN MICE WITH DEFECTIVE CHOLESTEROL EFFLUX PATHWAYS IN MYELOID OR DENDRITIC CELLS

To interrogate a potential role of defective cholesterol efflux pathways in macrophage inflammasome activation, we bred mice with myeloid knockout of *Abca1* and *Abcg1* using *LysM-Cre* transgenic mice. These *Myl*^{ABCDKO} mice were bred with *NLrp3*$^{-/-}$ or *Caspase1/11*$^{-/-}$ mice and BM was transplanted into *Ldlr*$^{-/-}$ recipients (7). *Myl*^{ABCDKO} BM-transplanted *Ldlr*$^{-/-}$ mice fed WTD showed prominently increased levels of plasma IL-18, a marker of inflammasome activation, increased caspase-1 cleavage, and IL-1β and IL-18 secretion by splenocytes. These findings were reversed by hematopoietic *Nlrp3* or *Caspase1/11* deficiency, indicating activation of the NLRP3 inflammasome in *Ldlr*$^{-/-}$ mice with myeloid *Abca1/Abcg1* deficiency (7). *Nlrp3* or *Caspase-1/11* deficiency decreased atherosclerotic lesion size in female *Myl*^{ABCDKO} BM-transplanted *Ldlr*$^{-/-}$ mice, particularly in early lesions (7).

Unexpectedly, there was marked neutrophil accumulation in early plaques of *Ldlr*$^{-/-}$ mice with myeloid *Abca1/Abcg1* deficiency and extensive NETosis (shown by coincident staining of neutrophil markers, myeloperoxidase, and citrullinated histones). Neutrophil accumulation and NETosis were reversed by hematopoietic *Nlrp3* or *Caspase-1/11* deficiency, indicating that inflammasome activation promotes neutrophil recruitment and NETosis in early atherosclerotic plaques. These findings are consistent with evidence that neutrophils contribute to early plaque formation (33, 38). The genetic dependence of plaque NETosis on the NLRP3 inflammasome and caspase-1/11 in *Myl*^{ABCDKO} mice might be due to inflammasome activation in neutrophils. Notably, *Abca1/Abcg1*-deficient neutrophils showed increased cholesterol content and increased cleavage of caspase-1 and caspase-11 (7), which could lead to GSDMD cleavage and pyroptosis/NETosis. We were not able to detect increased Caspase-1 or -11 cleavage in cells isolated from plaques of *Myl*^{ABCDKO} BM-transplanted *Ldlr*$^{-/-}$ mice (7). This may reflect technical limitations and the lack of authentic reagents for detection of inflammasome activation in tissues. Alternatively, the effects of the inflammasome could be mediated through systemic effects, for example by the impact of IL-1β derived from macrophages on neutrophil activation and entry into plaques (39).

In contrast to myeloid *Abca1/Abcg1* deficiency, we found that dendritic cell deficiency of *Abca1/Abcg1* (*DC*^{ABCDDKO}

mice) in normolipidemic chow-fed mice markedly promoted NLRP3 inflammasome activation, with a distinct phenotype of auto-immune inflammation and a lupus-like syndrome (40). Similar to cholesterol-25-hydroxylase-deficient mice that also show increased autoimmunity (41), $DC^{ABCDDKO}$ mice showed prominent induction of T_h17 cells (40), probably secondary to release of inflammatory cytokines from dendritic cells, including IL-1β. Thus, inflammasome activation due to disturbances of cholesterol homeostasis in dendritic cells can connect innate and acquired immune systems and promote auto-immunity.

MECHANISMS OF INFLAMMASOME ACTIVATION BY CHOLESTEROL

On a mechanistic level, myeloid *Abca1/Abcg1* deficiency led to increased expression of *Nlrp3*, *Caspase-1*, and *Il-1β* in $CD11b^+$ splenocytes, consistent with earlier findings showing plasma membrane cholesterol enrichment and increased TLR4 signaling in *Abca1/Abcg1*-deficient macrophages (42) and indicative of inflammasome priming. There was prominent accumulation of cholesterol in lysosomes and a small increase in refractile material inside these cells, as detected by confocal reflectance microscopy (7). Sheedy et al. (43) showed that CD36-mediated uptake of oxidized LDL by macrophages can lead to formation of cholesterol crystals in lysosomes leading to lysosomal damage and NLRP3 inflammasome activation. However, in Myl^{ABCDKO} macrophages, there was no evidence of lysosomal damage or increased mitochondrial ROS in splenic monocytes, macrophages, and neutrophils. Searching for alternative mechanisms to explain NLRP3 inflammasome activation, we discovered that myeloid *Abca1/Abcg1* deficiency also activated the noncanonical inflammasome (7). Moreover, there was increased susceptibility to LPS-induced death in Myl^{ABCDKO} mice, which was rescued by *Caspase-1/11* deficiency but not by *Nlrp3* deficiency, a signature of noncanonical inflammasome activation (7). Activation of the noncanonical inflammasome can lead to NLRP3 inflammasome activation (44), providing a potential mechanism to explain NLRP3 inflammasome activation. However, this is unlikely to be a complete explanation for the dramatic activation of the NLRP3 inflammasome in Myl^{ABCDKO} mice.

A NEW MODEL TO EXPLAIN STEROL-DEPENDENT INFLAMMASOME ACTIVATION

Recent studies have shown that macrophages from Niemann-Pick C1 (*Npc1*)-deficient mice, that display prominent lysosomal cholesterol accumulation similar to Myl^{ABCDKO} macrophages, are protected from NLRP3 inflammasome activation; these studies suggested that ER rather than lysosomal cholesterol accumulation promotes inflammasome activation (45) (**Fig. 1**). Our earlier studies in *Abca1/Abcg1*-deficient macrophages suggested that these cells have increased ER cholesterol content, as shown by reduced expression of the SREBP2 target genes, *Hmgcr* and *Ldlr* (46). Moreover, macrophages deficient in cholesterol-25-hydroxylase that likely have increased ER cholesterol content due to derepression of SREBP2 processing and increased cholesterol biosynthesis show increased NLRP3, AIM2, and NLRC4 inflammasome activation (41). Together these observations suggest that ER or plasma membrane cholesterol accumulation may promote the assembly of different inflammasome sensors with ASC leading to inflammasome formation. It is likely that an additional activation signal is required to produce NLRP3 inflammasome activation. We speculate that, in atherosclerosis, this may be dependent on the uptake or recognition of lipoprotein-derived oxidized phospholipids or oxysterols by macrophages.

HUMAN RELEVANCE

Whole-body *Abca1* deficiency induced NLRP3 inflammasome activation in $Ldlr^{-/-}$ mice, while myeloid deficiency of *Abca1* did not (7). Only the former is associated with low HDL levels (26), indicating that myeloid *Abca1* deficiency combined with low HDL levels is sufficient to induce inflammasome activation. This is presumably because the low HDL causes defective cholesterol efflux via non-ABCA1 pathways such as ABCG1. Tangier disease (TD) patients who are homozygous for a loss-of-function of the *ABCA1* gene displayed elevated IL-18 plasma levels, showing human relevance (7). This suggests that low HDL, defective apoA-1, and reduced expression of *ABCA1/ABCG1* in monocyte/macrophages may be sufficient to induce inflammasome activation in humans. Such changes occur commonly in patients with poorly controlled type 2 diabetes and chronic kidney disease and with ageing (47–53). TD patients sometimes present with premature atherosclerotic CVD (54); however, the more consistent phenotype among adult TD patients is peripheral neuropathy (55). A recent study has shown that defective myelin clearance due to microglial *Abca1/Abcg1* deficiency promotes inflammasome activation and limits remyelination following a neuronal injury in aged mice (56). Together with our findings, this suggests that macrophage inflammasome activation may be involved in the pathogenesis of peripheral neuropathy in TD and conceivably type 2 diabetes.

Clonal hematopoiesis involving variants in several genes that predispose to hematological malignancies, including loss-of-function epigenetic modifiers, such as *TET2*, or gain-of-function JAK/STAT signaling ($JAK2^{V617F}$), has recently emerged as a major risk factor for coronary heart disease, especially in the elderly (57, 58). Studies in mice with myeloid *Tet2* deficiency have shown macrophage inflammasome activation leading to increased IL-1β production and accelerated atherosclerosis (58, 59). NLRP3 inflammasome activation is also prominent in splenic myeloid cells in $JAK2^{V617F}$ BM-transplanted $Ldlr^{-/-}$ mice that have accelerated atherosclerosis with increased necrotic cores (60). NLRP3 inflammasome activation may promote atherosclerosis and thrombosis in $JAK2^{V617F}$ patients with clonal hematopoiesis or myeloproliferative neoplasms.

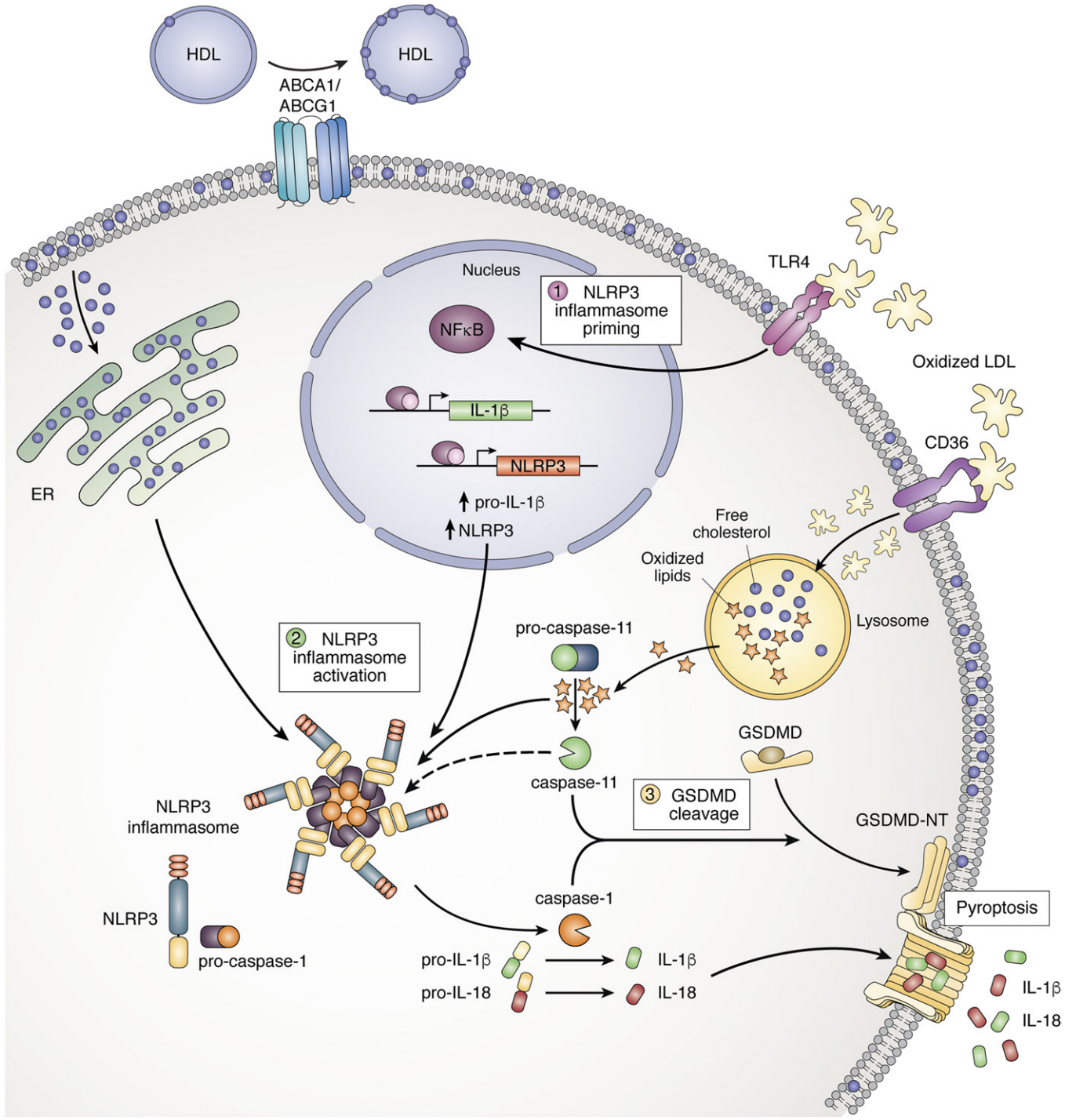

Fig. 1. Inflammasome activation by lipids in macrophages and neutrophils. Step 1: NLRP3 inflammasome priming. Oxidized LDL (oxLDL) activates the TLR4, leading to activation of NF-κB and transcription of NLRP3 and pro-IL-1β. Step 2: Inflammasome activation. OxLDL is taken up via the scavenger receptor CD36 and hydrolyzed in the lysosome. Oxidized lipids that enter the cytosol activate the noncanonical inflammasome resulting in caspase-11 cleavage. In the absence of Abca1 and Abcg1, cholesterol accumulates in the plasma membrane and is then transported to the ER. ER cholesterol accumulation activates the NLRP3 inflammasome, resulting in caspase-1 cleavage and subsequent cleavage of pro-IL-1β and pro-IL-18. Step 3: GSDMD cleavage. The active (cleaved) forms of caspase-1 and caspase-11 cleave GSDMD, and its N-terminal form (GSDMD-NT) stimulates membrane pore formation. The NLRP3 inflammasome is also activated downstream of caspase-11 cleavage as a result of membrane pore formation. In addition, pore formation leads to pyroptosis, NETosis, and IL-1β and IL-18 secretion.

PERSPECTIVES FOR FUTURE STUDIES

New mechanistic and genetic studies may help to clarify the upstream signals and molecules involved in inflammasome activation and their relevance in metabolic diseases. There is a need to develop sensitive authentic reagents for detection of inflammasome activation in mouse and human tissues. This may help to distinguish local versus systemic effects of inflammasomes in atherogenesis and to evaluate the role of inflammasome activation and pyroptosis/NETosis

in plaque erosion and atherothrombosis. That different underlying risk factors for CVD (such as type 2 diabetes, chronic kidney disease, and clonal hematopoiesis) may mechanistically link to atherothrombosis via inflammasome activation could be evaluated in human observational studies, using plasma IL-18 levels or tissue samples to measure inflammasome activation. While the CANTOS suggests a role for inflammasome-derived IL-1β in human CVD, the magnitude of the benefit was moderate, and there was an excess of infections associated with treatment (1), perhaps due to decreased neutrophil levels. It may be informative to determine whether elevated IL-18 levels or the presence of clonal hematopoiesis mutations help to define subgroups of patients who particularly benefitted from treatment. This may help to stratify patients in future clinical studies targeting NLRP3, noncanonical, or AIM2 inflammasomes. On a therapeutic level, removal of cholesterol from macrophages and neutrophils by infusion of reconstituted HDL particles, which are under clinical evaluation in a phase 3 clinical study (Aegis-II, NCT03473223), may alleviate inflammasome activation when administered after an acute coronary syndrome (61). LXR activators, perhaps targeted to myeloid cells in nanoparticles (62), could reduce inflammasome activation both by upregulating *ABCA1/ABCG1* and by direct suppression of *Il-1β* (63). Recent progress in inflammasome research suggests that molecules upstream of IL-1β secretion, such as NLRP3, caspase-1/11, or CMPK2, may provide additional therapeutic targets for preventing CVD in patients with evidence of underlying inflammasome activation.

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