REVIEW

The Role of Bacterial Extracellular Membrane Nanovesicles in Atherosclerosis: Unraveling a Potential Trigger

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Abstract

Purpose of Review In this review, we explore the intriguing and evolving connections between bacterial extracellular membrane nanovesicles (BEMNs) and atherosclerosis development, highlighting the evidence on molecular mechanisms by which BEMNs can promote the athero-inflammatory process that is central to the progression of atherosclerosis.

Recent Findings Atherosclerosis is a chronic inflammatory disease primarily driven by metabolic and lifestyle factors; however, some studies have suggested that bacterial infections may contribute to the development of both atherogenesis and inflammation in atherosclerotic lesions. In particular, the participation of BEMNs in atherosclerosis pathogenesis has attracted special attention.

Summary We provide some general insights into how the immune system responds to potential threats such as BEMNs during the development of atherosclerosis. A comprehensive understanding of contribution of BEMNs to atherosclerosis pathogenesis may lead to the development of targeted interventions for the prevention and treatment of the disease.

Keywords Atherosclerosis · Cardiovascular diseases · Inflammation · Bacterial extracellular membrane nanovesicles · Atherogenesis · Immune response

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Introduction

Atherosclerosis is a chronic inflammatory disease characterized by the accumulation of low-density lipoprotein (LDL) cholesterol, fibrous elements, and calcification in the walls of large and medium-sized arteries. It is the leading cause of many cardiovascular diseases (CVDs), including coronary heart disease, myocardial infarction, and stroke, which pose a huge burden to health and socioeconomic development [1]. The pathogenesis of atherosclerosis is a complex and multifaceted process that involves various cellular and molecular events related to endothelial dysfunction and sub-endothelial lipid build-up, resulting in chronic inflammation in the arterial intima [2]. Atherosclerosis is primarily driven by metabolic and lifestyle factors, like high levels of blood cholesterol, high blood pressure, smoking, and type 2 diabetes mellitus; however, there is some evidence suggesting that bacteria may contribute to the atherogenesis in atherosclerotic lesions. A direct link between atherosclerosis and atherosclerotic CVD (ASCVD) and infection has been established [3, 4••]. The presence of specific *Helicobacter pylori* (*H. pylori*) DNA in atherosclerotic lesions has been

demonstrated, providing biological evidence that this infection is a contributing factor to atherosclerosis pathophysiology [5]. Moreover, the contribution of bacterial extracellular membrane nanovesicles (BEMNs) to atherosclerosis pathogenesis attracted special attention. Highly invasive BEMNs are able to penetrate through the vascular endothelial cell (VEC) membrane, inducing inflammation and the development of atherosclerosis [6]. Notably, BEMNs can trigger an inflammatory response independently of maternal bacteria [7] and are able to up-regulate the expression of various cytokines and adhesion molecules, initiating a cascade of inflammatory reactions in the vascular wall [8]. In this review, we explore the intriguing and evolving connections between BEMNs and atherosclerosis development, highlighting the evidence on molecular mechanisms by which BEMNs can promote the athero-inflammatory process that is central to the progression of atherosclerosis. A comprehensive understanding of BEMNs` contribution to atherosclerosis pathogenesis may lead to the development of targeted interventions for the prevention and treatment of the disease.

Involvement of BEMNs in Atherosclerosis

BEMNs are nanosized (20–300 nm in diameter) spherical structures enclosed by a bilayer membrane formed from the mother cell envelope. They are secreted by almost all types of eukaryotic and prokaryotic cells, including both Gram-negative and Gram-positive bacteria [9]. They are produced during all stages of natural bacterial growth both in vivo and in vitro and the ubiquitous production of BEMNs by bacteria is nowadays recognized as a novel bacterial secretion system. Unlike bacterial cells, these membrane-bound nanostructures are incapable of independent replication [10]. BEMNs secreted by bacteria are exceedingly numerous with a cell-to-vesicle ratio of approximately 1:2000 [11]. Due to their small dimensions, BEMNs can carry and deliver a variety of compounds to host tissues in concentrated and protected forms. These compounds include outer membrane proteins, lipopolysaccharides (LPS), phospholipids, peptidoglycan, periplasmic, cytoplasmic and membrane-bound proteins, periplasmic components, nucleic acids (DNA, RNA), pathogen-associated molecular patterns (PAMPs), toxins, ion metabolites, and signaling molecules [12•, 13, 14]. The evidence indicating that BEMNs exert proteolytic damage to VEC membrane to overcome biological barriers and reach the sites not accessible to whole bacteria was reviewed [15]. A recent study has shown that BEMNs can also serve as vehicles for the delivery of virulence factors [9]. They deliver bacterial virulence factors deep into host tissues, promoting and dysregulating the immune response. Pathogenic BEMNs change host cell functions and trigger inflammatory reactions [16]. Therefore, BEMNs are perfect causative agents for a number of infectious and inflammatory

diseases, including periodontitis, endocarditis, peptic ulcer disease, cystic fibrosis, gastric cancer, and atherosclerosis among others [15]. Several specific contributions of BEMNs to atherosclerosis are presented in the next subsections. It is important to note that research on BEMNs` contribution to atherosclerosis is ongoing, and the specific contributions may vary depending on the bacterial species and individual host factors.

BEMNs Derived from *Helicobacter Pylori*

H. pylori bacterium colonizes the gastric mucosa of over 50% of the population around the world [17]. Its association with atherosclerosis represents one of the current paradigms of the disease pathogenesis [4••, 18]. Two most studied virulence factors of *H. pylori* such as cytotoxin-associated gene A (CagA) and vacuolating cytotoxin A (VacA) have been shown to influence the disease state [18, 19]. In particular, CagA-positive *H. pylori* infection contributes to the atherosclerosis progression by promoting atherogenesis and macrophage-derived foam cell formation via the release of CagA-containing BEMNs [18]. The mechanism of CagA action is associated with the inhibition the transcription of cholesterol removal transporters by suppressing the expression of transcription factors such as peroxisome proliferator-activated receptor gamma (PPAR-γ) and liver X receptor alpha (LXR-α). In this way, CagA-associated BEMNs enhance foam cell formation. Another study suggested that *H. pylori*-derived BEMNs containing CagA can accelerate atherosclerotic lesion formation through endothelium injury. It was demonstrated that these BEMNs activated reactive oxygen species (ROS)/nuclear factor κB (NF-κB) signaling pathway in human VECs, as well as promoted the expressions of proinflammatory cytokines such as interleukin-6 (IL)-6 and tumor necrosis factor-alpha (TNF-α) and, thus, impaired vascular endothelial function [6].

BEMNs Derived from *Escherichia Coli*

Escherichia coli(E. coli) BEMNs are able to stimulate the inflammatory response in the vascular wall and, thereby, may contribute to ASCVD development. It was shown that *E. coli* BEMNs enhanced the expression of adhesion molecules and pro-inflammatory cytokines and promoted the leukocyte binding on VECs in vitro [20]. Notably, *E. coli* BEMNs were much more effective in inducing both intercellular adhesion molecule-1 (ICAM-1) expression and leukocyte adhesion in comparison with free LPS and the Cytolysin A protein, a bacterial virulence factor. Besides, an in vivo study showed that BEMNs from *E. coli* can strongly upregulate functional cell adhesion molecules through the release of IL-8/C-X-C motif chemokine ligand 1 (CXCL1)

from endothelial cells involving NF-κB- and toll-like receptors 4 (TLR4)-dependent mechanisms [21].

BEMNs Derived from *Porphyromonas Gingivalis*

Pathogenic *Porphyromonas gingivalis (P. gingivalis)* is not only a dominating cause of periodontitis but it was also found to be implicated in the development other diseases, including atherosclerosis [22]. BEMNs derived from periodontal *P. gingivalis* have been associated with atherosclerosis [23]. In fact, both *P. gingivalis* and its derived BEMNs are able to upregulate the expression of chemoattractant proteins, including CXCL1, CXCL2, and CXCL8, and endothelial-leukocyte adhesion molecule such as E-selectin; however, BEMNs are more potent activators of the innate inflammatory response related to atherogenesis [23]. BEMNs of *P. gingivalis* released from areas of periodontitis into the circulation can deliver virulence factors to the arterial intima and induce or promote foam cell formation in macrophages, and contribute to the development of atherosclerotic plaque [24]. Moreover, noticeable effects of *P. gingivalis* BEMNs on macrophage inflammatory phenotype, mitochondrial function, inflammasome activation, and pyroptotic cell death have been reported [25]. They stimulated macrophages to release more of TNF-α, IL-1β, IL-18, IL-12p70, IL-6, IL-10, interferon beta (IFN-β), and nitric oxide (NO) compared to maternal bacteria, which released much less of these mediators. *P. gingivalis* BEMNs induced a metabolism change from oxidative phosphorylation (OXPHOS) to glycolysis in macrophages, decreased mitochondrial oxygen intake, and increased ROS production. Stimulated with *P. gingivalis* BEMNs, macrophages activate caspase-1, produce a large amounts of lactate dehydrogenase, and initiate a pyroptotic cell death. These effects may have potent implications for their roles in atherosclerosis. These BEMNs are able to stimulate VSMCs differentiation and calcification [26]. Calcification of VSMCs is linked to the atherosclerotic plaque rupture [27].

Furthermore, BEMNs derived from *P. gingivalis* can permeate into coronary vessels, providing the link between periodontitis and CVD [28]. A study reported that *P. gingivalis* BEMNs can mediate increased endothelial permeability via a gingipain-dependent mechanism that involves proteolytic cleavage of endothelial adhesion molecule PECAM-1, responsible for maintaining vascular integrity [29]. Gingipains are major virulence factors produced by *P. gingivalis*. This mechanism significantly drives the risk of CVD. Gingipains` activity can also initiate disturbance of the complement system. They break down the C3, C4, and C5 complement components, as well as exert proteolytic destruction of the CD46 complement regulator, reducing bacterial elimination and increasing inflammation

[30]. In particular, complement C5a has been found in atherosclerotic lesions, which acts as a proatherogenic moiety, promoting apoptosis of VECs and VSMCs and inducing the production of the metalloproteases MMP1 and MMP9 in human macrophages [31]. These metalloproteases play a major role in plaque destabilization and rupture. Gingipains, proteases, and LPS from *P. gingivalis*, which provoke the accumulation of C5a and antimicrobial peptides in atherosclerotic lesions, may putatively contribute to the progression of atherosclerosis [22].

Taken together, these data suggest a robust role that BEMNs play in the pathogenesis of atherosclerosis.

Molecular Attributes and Mechanisms Involved in the Innate Immune Response to BEMNs during the Development of Atherosclerosis

According to the current understanding, the innate immune system serves as the first barrier against microbial pathogens and executes its protection by non-specific immune defense and surveillance by innate immune cells. Innate immunity is essentially characterized by a variety of germline-encoded receptors, *i.e.*, pattern recognition receptors (PRRs) with high affinity to tightly conserved motifs in pathogens, which typically recognize PAMPs, including BEMNs. During atherogenesis, BEMNs admittedly furnish inflammatory stimuli, creating a pro-inflammatory milieu and stimulating immune responses at sites of atherosclerotic lesion formation. The scientific basis of inflammation in atherogenesis, demonstrating that inflammatory processes promote the initiation of atheroma in vascular walls, was discussed in the review of Libby, 2012 [32]. It was found that PRRs are involved in direct host-bacterial interactions through BEMNs [33]. A wide variety of PRRs, including TLRs, G-protein-coupled receptors (GPCRs), and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) are expressed on the surface of innate immune system cells, such as macrophages, monocytes, neutrophils, dendritic cells (DCs), natural killer (NK) cells, and mast cells. These cells play key roles in the early stages of atherosclerosis development [34]. The innate immune system exerts its antimicrobial defense function through other entities, including inflammasomes, nuclear factor κappa B (NF-κB), cytokines, chemokines, and mitochondria, which are also activated during the development of atherosclerosis in response to BEMNs. The schematic representation of the innate immune response to BEMNs in atherosclerotic lesions is shown in Fig. 1.

Fig. 1 Innate immune system signaling triggered by BEMNs during atherosclerosis development. Abbreviations: BEMNs, bacterial extracellular membrane nanovesicles; GPCRs, G-protein-coupled receptors; MyD88, myeloid differentiation factor 88; MtDys, mitochondrial dysfunction; NF-kB, nuclear factor kappa B; NLR, nucleotide-binding oligomerization domain (NOD)-like receptors; NLRP3, NOD-, LRR- and pyrin domain-containing protein 3; PRRs, pattern recognition receptors; TLR, toll-like receptors
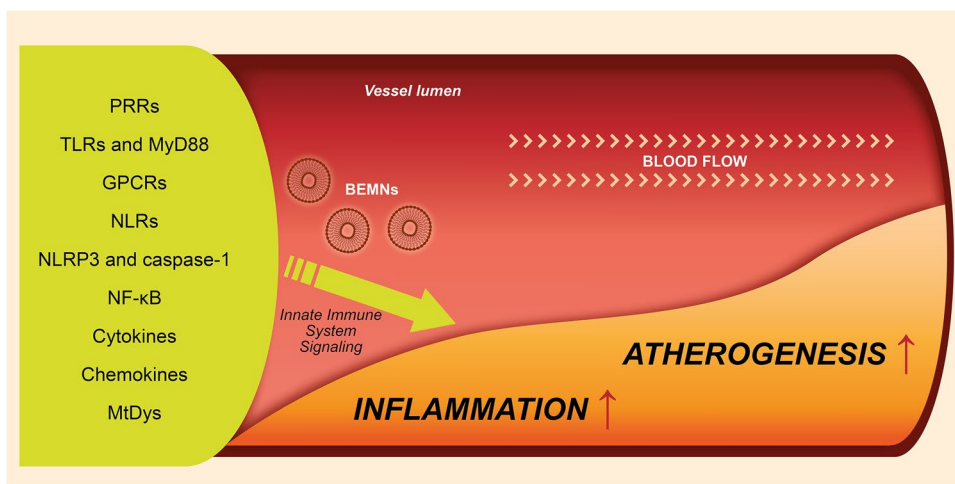
Toll-like Receptors

TLRs are the major component of the innate immune system, which plays a very important role in the development of the immune response [35]. These receptors are essentially characterized by an extracellular leucine-rich repeat domain, which mediates the recognition of PAMPs, a transmembrane domain in conjunction with its cytosolic or intracellular Toll/IL-1R-like domains necessary for downstream signaling pathways [35]. In accordance to their localization, TLRs are largely divided into two subfamilies, cell surface TLRs and intracellular TLRs. Cell surface TLRs recognize microbial membrane components, such as lipids, lipoproteins, and proteins. For example, TLR2, TLR1, and TLR6 recognize an array of PAMPs including lipoproteins, peptidoglycans, lipotechoic acids, zymosan, mannan, and tGPI-mucin [36]. Intracellular TLRs recognize nucleic acids derived from invading bacterial and viral pathogens [37]. TLR activation is accomplished via the myeloid differentiation factor 88 (MyD88) and the Toll/IL-1 receptor domain-related adaptor protein that induces interferon (TRIF) [38]. Experimental studies have established an important role of MyD88 and TRIF for TLR signaling, linking innate immunity and atherogenesis [39]. The effect of a TRIF deficiency on atherosclerosis in LDL receptor knockout mice showed that MyD88 participates in atherogenesis. Moreover, MyD88 deficiency leads to decreases in plaque size, LDL content, expression of proinflammatory genes, cytokines, and chemokines such as IL-12 and monocyte chemoattractant protein-1 [39]. Besides, MyD88 is involved in signaling of the IL-1 family of receptors and thus implicates TLRs in atherosclerosis. The IL-1 receptor family is the main regulator of both atherogenesis and inflammation, particularly through the activity of proatherogenic IL-1β and IL-18 [40]. In addition, MyD88 and TRIF regulate the stimulation of NOD-, LRR- and pyrin domain-containing protein 3 (NLRP3) and

IL-1β in response to TLR ligands. A study demonstrated that the innate immune system is activated via TLR2/4/MyD88/TRIF/mitogen-activated protein kinases (MAPK)/NF-κB and NLRP3/caspase-1 activation pathways [41].

G-Protein-Coupled Receptors

GPCRs are another diverse family of cell-surface receptors of the innate immune system, which deals with microbial intruders and regulates inflammation and immunity. In particular, GPCRs are responsible for the immune responses of macrophages toward extracellular pathogens [42]. Monocyte accumulation in the atherosclerotic plaque can be promoted by the functional activity *CX3CR1*, also known as the fractalkine receptor of the GPCR family [43]. Decreased atherosclerosis in *CX₃CR1* deficient mice confirms the pivotal role of this receptor during atherogenesis [44]. In atherogenesis, monocytes recruited to the site of atherosclerotic lesion formation turn into macrophages, accumulate lipids, and display morphological features of foam cells, which establish the foundation for plaque formation. The central role of macrophages in the initiation of atherosclerotic inflammation has been discussed [45]. Macrophages are professional phagocytes aimed at removing not only small particles and apoptotic cells, but also pathogens from the atherosclerotic tissue via phagocytosis. Moreover, the expression of C3a complement system receptors, members of the rhodopsin-like GPCR family, is significantly increased during inflammation, as demonstrated in human atherosclerotic coronary plaques [46]. The notion of complement as being mainly a host-defense system against microbial pathogens has been expanded markedly in the last decades [47]. An animal study showed that inhibition of C3a diminishes atherosclerosis, in particular, the C3a/C3a receptor axis mediates negative regulation of proinflammatory responses and macrophage polarization toward the anti-inflammatory phenotype [48].

Nucleotide-Binding Oligomerization Domain (NOD)-like Receptors

One class of PRRs, NLRs, are intracellular cytoplasmic sensors of PAMPs, including BEMN-containing PAMPs [49]. The most well-studied NLRs are *NOD1* and *NOD2*, which can detect bacterial peptidoglycans and BEMNs [50]. In particular, *NOD2* mediates bacterial clearance in the cytoplasm of myeloid cells and regulates innate immune responses via the detection of peptidoglycan derivatives diaminopimelic acid (DAP) through the recognition of bacterial cell wall fragment, the muramyl dipeptide (MDP) ligand, which is commonly distributed among both Gram positive and Gram negative bacteria [51]. It was demonstrated that *NOD2*-deficient mice are prone to bacterial infection, including *Staphylococcus aureus* [52], *Chlamydophila pneumonia* [53], *H. pylori* [54], and *Bacillus anthracis* [55]. This susceptibility is attributable to reduced proinflammatory cytokine production and leukocyte recruitment [55]. MDP of *NOD2* was found in the atherosclerotic plaque [56]. Therefore, *NOD2* may play some role as an immune regulator in atherosclerosis. Atherosclerosis development can be enhanced by the presence of *P. gingivalis* infection, and this enhancement is pronounced in ApoE and *NOD2* double-deficient mice to a greater extent [57]. When MDP is used to activate *NOD2*, levels of serum inflammatory cytokines are decreased [57]. The results of these studies suggest that *NOD2* exhibits anti-inflammatory and anti-atherogenic effects. Additionally, several, members of the NOD-receptor family, such as *NLRP1*, *NLRP3,* and *NLRC*4 among others, have been reported to operate via inflammasomes, which couple up microbial and endogenous danger signals to caspase-1 activation [58]. The caspase-1 pathway is a major inflammatory pathway. For example, NOD receptors have been described to synergize with NLRP3 sensors that detect a broad range of microbial motifs to activate the NLRP3 inflammasomes [59].

Inflammasomes

Inflammasomes are assembled by PRRs following the recognition of pathogenic microorganisms and BEMNs in the cytosol of host cells, and they activate inflammatory caspases to produce proinflammatory cytokines and generate pyroptosis, an inflammatory form of cell death [60]. The most studied inflammasome, the NLRP3 inflammasome, is the critical component of the innate immune system that governs the activation of the inflammatory caspase-1 pathway and the production of proinflammatory cytokines such as IL-1β and IL-18 in response to microbial infection [61]. A positive correlation of NLRP3 level with the severity of coronary atherosclerosis in patients with acute coronary disease was demonstrated [62]. In atherosclerosis, mature IL-1β induces an inflammatory response in ECs and promotes the accumulation of inflammatory cells in local vascular intima, which often occurs at the initiation of atherosclerosis [63]. Moreover, an experimental study showed that IL-1β plays a crucial role in the progression of bacteria-associated atherosclerosis and IL-1β was regarded as a proatherogenic cytokine [64]. Produced mainly by monocytes/macrophages, IL-18 is critical for the secretion of interferon gamma (INF-γ) and cytokines; it also enhances the cytolytic activity of NK cells, providing an important link between the innate and adaptive immune responses [65]. Several studies using transfected cell lines and NALP3 knockout mice showed that bacterial products, such as peptidoglycans, MDP, and bacterial toxins, can activate the NALP3 inflammasome [66, 67]. Additionally, BEMNs containing Gram-negative bacterial cell wall component LPS can activate caspase-11 with the aid of guanylate binding proteins that are the regulators of BEMN-mediated inflammation [68].

A two-signal model for the NLRP3 inflammasome activation was described [61]. The priming signal (signal 1) is delivered by microbial components or endogenous cytokines, leading to the activation of the NF-κB pathway and consequently to the upregulation of the NLRP3 inflammasome and pro-interleukin-1β (pro-IL-1β). Then, the NLRP3 inflammasome endures posttranslational modifications, which enable its activation. The activating signal (signal 2) is represented by various stimuli including extracellular adenosine triphosphate (ATP), pore-forming toxins, RNA viruses, and particulate matter. Signal 2 is able to directly activate inflammasome assembly. In this way, NLRP3 regulates the pathogen (including BEMNs)-induced inflammation and fundamentally impacts the progression of bacteria-enhanced atherosclerosis [69].

Nuclear Factor κB

Transcription factor NF-κB plays a crucial role in regulating the expression of genes involved in inflammation, immune responses, and cell survival [70]. Activation of NF-κB is a key component of the body's defense mechanisms against microbial infections. Microbial components, such as bacterial endotoxins such as LPS can activate NF-κB through the TLR signaling pathway [71]. At the early stages of atherosclerosis, the activation of NF-κB by microbial components can amplify the inflammatory response within the arterial walls, promoting the development and progression of atherosclerosis [70]. The activation of NF-κB by microbial components can have several effects on the vascular wall: (i) Inflammation: NF-κB activation leads to the production of pro-inflammatory cytokines such as TNF-α and IL-1. These cytokines promote inflammation within the arterial walls, contributing to the progression of atherosclerosis. (ii) Recruitment of immune cells: NF-κB activation also results in the expression of adhesion molecules on the surface of

endothelial cells lining the arteries. These molecules help recruit immune cells such as monocytes to the site of injury in the arterial wall. (iii) Foam cell formation: Activated monocytes can infiltrate the arterial wall and differentiate into macrophages. These macrophages can engulf oxidized LDL (oxLDL) and become foam cells, which are a hallmark of atherosclerotic plaques. (iv) Smooth muscle cell proliferation: NF-κB activation can stimulate the proliferation of vascular smooth muscle cells (VSMCs), contributing to the thickening of the arterial wall and the formation of atherosclerotic plaques.

Cytokines

Microbial components such as LPS from Gram-negative bacteria and other PAMPs can activate the innate immune system through monocytes and macrophages and trigger the release of various cytokines during atherosclerosis [72]. Cytokines are a subset of low-molecular weight proteins necessary for cell signaling. Involved in the immune cell activation, these proteins can further exacerbate inflammation and contribute to atherosclerosis [73]. Generally, cytokines can be classified as pro- or anti-atherogenic. Classical atherogenic cytokines include interferon gamma (IFN-γ) and TNF-α. IFN-γ promotes inflammatory response in macrophages, NK cells, and VSMCs and increases oxLDL accumulation and foam cell formation, as reviewed by Leon and Zuckerman, 2005 [74]. TNF-α promotes the expression of adhesion molecules and chemokines on VECs, which are critical in recruiting immune cells to the sites of inflammation within arterial walls [75]. Moreover, several mechanisms were described that support the proatherogenic effects of TNF-α on the endothelium, including its role in oxidative stress, decreasing NO bioavailability, and increasing vascular permeability to circulating blood components and cells [76]. Atherosclerosis progression is associated with the local increase in TNF-α expression in atherosclerotic lesions [77]. Besides, IL-1α and IL-1β are pro-inflammatory cytokines, produced by monocytes and macrophages. Majority of innate immune cells express either IL-1 family cytokines, their receptors, or both, and hence almost all innate immune cells are influenced by IL-1 signaling. The IL-1 family cytokines include a number of proinflammatory cytokines (IL-1α, IL-1β, IL-18, IL-33, IL-36α, IL-36β, and IL-36γ) and one anti-inflammatory cytokine (IL-37) [78]. They are profoundly involved in regulation of both, the innate and the adaptive immune responses. Some experiments in murine models proven proatherogenic properties of IL-1α and IL-1β that are involved into the upregulation of adhesion molecules expression by endothelial cells along with macrophage activation [79]. Together, IL-1RA possesses endogenous anti-inflammatory properties as a potent inhibitor of IL-1 signaling pathways [80]. In atherosclerosis, the production of IL-1β is determined by the activation of the NLRP3 inflammasome, as described above. Also, IL-1β can affect the proliferation and migration of VSMCs [81]. Cells known to express IL-18 include macrophages, VECs, and VSMCs [82]. IL-18 aggravates atherosclerosis by enhancement of an inflammatory response through an IFN-γ–related mechanism [82, 83]. In addition, IL-6, an early and key regulator of inflammation, has been long-identified in human atherosclerotic lesions [84]. It is synthesized by many cells of the arterial wall including macrophages, VSMCs, and VECs. The effects of IL-6 in atherosclerosis have been extensively reviewed [85]. In particular, IL-6, can promote LDL uptake in macrophages and stimulate VECs to secret adhesion molecules.

Chemokines

Microbial components can be recognized by TLR4 on immune cells, including macrophages and dendritic cells. This recognition is part of the innate immune system's response to pathogens. When microbial components activate TLR4, immune cells at the site of infection or injury produce chemokines. Chemokines are a group of small signaling proteins that play a crucial role in immune cell trafficking. Chemokines, which trigger directed chemotaxis of immune cells to the site of infection or tissue damage, include four subfamilies: CXC, CC, CX3C, and XC. When *E. coli*-derived BEMNs bind to TLR4, it initiates a signaling cascade leading to the activation of NF-κB, recruiting neutrophils via the release of IL-8/CXCL1 from endothelial cells [21]. In the context of atherosclerosis, this means that immune cells like monocytes are attracted to the arterial wall, promoting the inflammatory response. Animal studies demonstrated that CXCL1 drives the development of inflammation early in atherosclerosis. CXCL1 can recruit monocytes and neutrophils to the atherosclerotic plaque via its receptor CXCR2 [86]. CXCL1 supports the development of atherosclerosis by controlling the migration, diffusion, and differentiation of macrophages. Macrophage CXCR2 expression in atherosclerotic lesions is central in the progression of early atherosclerotic lesions, such as fatty streaks [87]. Distinct monocyte subsets also exploit *CX3CR1* and *CCR5* receptors to accumulate in atherosclerotic lesions [43]. Thus, chemokines and their receptors are the key factors in monocyte recruitment, which are able independently promote atherogenesis.

Mitochondria

The role of mitochondria as an innate immune signaling platform triggered by microbial components in atherosclerosis is an interesting and evolving area of research. The release of microbial components could

potentially engage mitochondrial DAMP-mediated immune responses. When cells are stressed or damaged, mitochondria can become dysfunctional, leading to the release of mitochondrial DNA (mtDNA) and mitochondrial reactive oxygen species (mtROS) [88]. MtDNA, can activate PRRs of the innate immune system, and inflammasomes. The actions of PRRs in the development of pathogen-associated atherosclerosis were described in previous subsections. MtDNA has been suggested to act as a mitochondrial danger signal. Such a signal triggers the activation of the NLRP3 inflammasome [89]. Mitochondrial ROS can induce the assembly and activation of the NLRP3 inflammasome [90]. The exposure to toxins delivered by BEMNs is a significant cause of mitochondrial disruption. These nanovesicles from *Neisseria gonorrhoeae*, *E. coli,* and *Pseudomonas aeruginosa* induce mitochondrial apoptosis and activation of the NLRP3 inflammasome [91]. This study showed that BEMN's toxins trigger inhibition of host protein synthesis, caspase-11-mediated pyroptosis, and activation and release of IL-1β. In atherosclerosis, the endothelial lining of blood vessels can become damaged, allowing the infiltration of lipids and immune cells. These immune cells, when exposed to mitochondrial DAMPs, can become activated, further promoting inflammation within the atherosclerotic plaque. The deterioration of mitochondria leads to a high ROS production, oxidative stress, chronic inflammation, and atherosclerosis progression [92]. Thus, mitochondria can serve as innate immune signaling platforms in atherosclerosis through the release of mtDNA, which can activate innate immune responses in response to microbial components, carried by BEMNs.

Atherosclerosis and Anti-Microbial Responses of Immune Cells(Fig. 2)

The vascular endothelium is a heterogeneous monolayer shaped by VECs, which lay the luminal side of all blood vessels, representing the first line of defense for molecules, cells, and pathogens circulating in the bloodstream [93]. It was found that BEMNs contribute the atherosclerotic plaque formation via endothelial damage [6]. At atherosclerotic lesion formation sites, damaged VECs release chemokines that trigger the inflammatory cascade and attract immune cells, such as monocytes. Monocytes are blood-derived mononuclear phagocytic cells that migrate around the body and can deliver rapid innate immune effector responses in response to microbial pathogen infections. Chemokines and their ligands as chemo-attractants that promote monocyte recruitment into arterial walls are explained above. Monocytes, followed by their penetration to the vascular wall, differentiate into macrophages or DCs according to the environmental signals [94]. Their study showed that monocytes are required to be stimulated by granulocyte–macrophage colony-stimulating factor (GM-CSF) together with IL-4, in order to differentiate into DCs. Besides, both IFN-γ and macrophage colony-stimulating factor (M-CSF) switch monocyte differentiation to CD64 + macrophages. Macrophages and DCs play different roles in the immune response. While DCs induce specific immune responses, macrophages and specifically IFN-γ–activated macrophages have powerful antibacterial and antitumoral activities [94]. As a part of the innate immune system, monocytes also influence adaptive immune responses [94].

Furthermore, DCs are the antigen presenting cells (APCs); their properties have been reviewed in detail [95]. Their main function is to deliver pathogen signals to naïve

Fig. 2 Cellular aspects of infection-enhanced atherosclerosis. Abbreviations: BEMNs, bacterial extracellular membrane nanovesicles; B Cell, B lymphocyte; CD4 + T cell, CD4 T (helper) lymphocyte; CD8 + T cell, CD8 T (cytotoxic) lymphocyte; IFN-γ, interferon gamma, IL, interleukin; M-CSF, macrophage colony-stimulating factor; TNF-α, tumor necrosis factor alpha; VECs, vascular endothelial cells
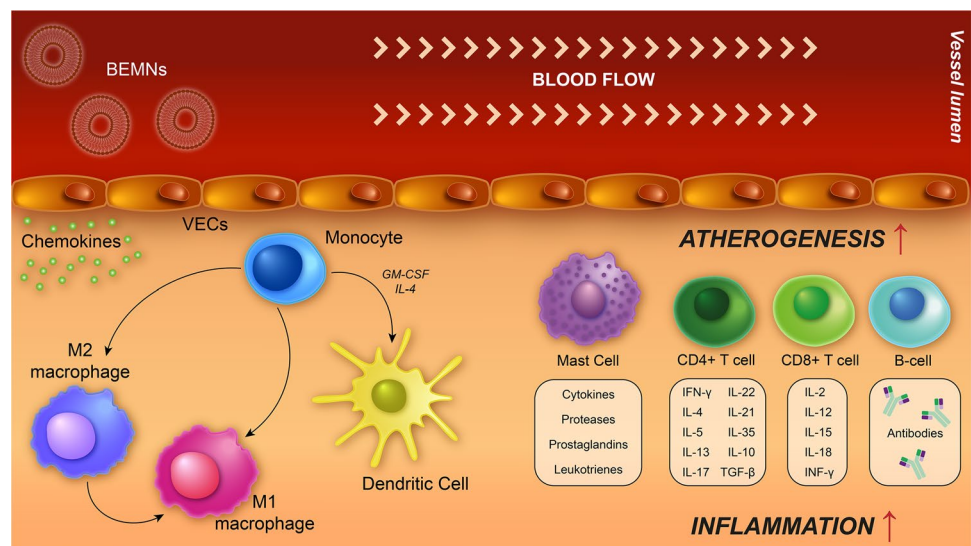
T cells. Besides, DCs have phagocytic activity as immature cells and a great ability to generate cytokines as mature cells. During infection, DCs recognize PAMPs via TLRs and transmit the signal via MyD88. After they associate with a presentable antigen, become activated, and interact with T and B cells to initiate the adaptive immune response. Therefore, DCs operate as intermediates between the innate and adaptive immune responses [96]. The role of vascular DCs in atherosclerosis was vigorously discussed [97].

Macrophages are the most significant cell type of innate immunity and the initial stages of atherosclerosis. In fact, they are multifunctional in atherogenesis [98]. First, macrophages are equipped with an extensive repertoire of PRRs that make them highly efficient at phagocytosis and the production of cytokines and growth factors. Second, macrophages can also function as APCs and orchestrate the immune response. Third, macrophages engulf LDL-cholesterol followed by foam cell formation and the development of atherosclerotic plaque. In the course of atherosclerosis progression, macrophages interact with VECs, VSMCs, T cells, and DCs [99]. Traditionally, activated macrophages have been divided into two groups: M1 and M2 macrophages. M1 are classically activated macrophages by T helper 1 (Th1) cytokines such as TNF-α and IFN-γ and/or by other stimuli such as microbial LPS, participating in the elimination of pathogens, activating the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase system, and, in turn, generating ROS [98, 100]. Aerobic glycolysis is also induced in M1 macrophages upon activation, which enhances their proinflammatory activity [101]. M1 macrophages are proinflammatory macrophages, which have been found both in human and mouse atherosclerotic plaques, and their production of the pro-inflammatory mediators is believed to maintain local inflammation and the degradation of extracellular matrix components, leading to atherosclerosis progression [102]. Pro-inflammatory M1 macrophages are usually activated through TLR4 or NF-κB pathways [94]. These are the mechanisms that M1 macrophages use for bacterial killing. The alternatively activated M2 macrophages are induced by Th2 cytokines, such as IL-4, IL-10, IL-13, or IL-1β [103]. They play a preventative role in the progression of the disease since they reduce inflammation and OXPHOS. M2 macrophages show increased ATP production by means of electron transport chain OXPHOS. Also, M2 macrophages have lower levels of glycolysis, higher levels of fatty acid oxidation, and increased arginine metabolism. These M2 mechanisms reduce inflammation, increase lipid degradation, inhibit foam cell formation, and therefore likely slow the progression of atherosclerosis [104].

Promoting pathogen elimination from the host, mast cells interact with various cell types, including T-cells [105], macrophages [106], neutrophils [107], DCs [108], SMCs [109], and ECs [110]. These cells mount innate immune responses and play critical roles in the initiation of atherosclerosis. Mast cells are able to sense pathogens through the expression of TLRs and specific Fc receptors. The stimulation of rodent mast cells by LPS via TLR4 induced cytokine production without their degranulation [111]. Also, mast cells can be activated through TLRs with subsequent degranulation. The stimulation through TLR2 by peptidoglycan lead to both degranulation and secretion of cytokines [111]. Owing to the upregulation of $Fc\gamma RI$ and $Fc\epsilon RI$ receptors, mast cells can bind both IgG and IgE immunoglobulins and become sensitized to host antigens [112]. It was shown that stimulation of TLRs and Fc receptors can have synergistic effects on production of inflammatory cytokines by mast cells, augmenting cytokine transcription via their joint action resulting in the increase in activity of MAPKs [113]. In the arterial intima, at the atherogenesis sites, mast cells are activated to degranulate, and in this way induced to release a large quantity of preformed inflammatory mediators, such as cytokines, chemokines, histamine, proteases, prostaglandins, and leukotrienes [114]. Mast cells are effector cells that initiate inflammatory responses and have a proatherogenic effect during atherogenesis [114].

T cells are the first cells among others recruited within the atheroma [115]. They are important participants in innate and adaptive immune defense mechanisms safeguarding against pathogens such as viruses and bacteria [116]. Atherosclerotic lesions encompass helper (CD4$^+$) and cytotoxic (CD8$^+$) T cells that respond to peptides from bacterial pathogens [117]. Pathogens such as human immunodeficiency virus and cytomegalovirus were accepted as candidate antigens relevant in atherosclerosis [118]. Such interactions result in the secretion of a large amount of cytokines [119]. CD8$^+$ T cells can control infections, by responding to the following cytokines: IL-2, IL-12, IL-15, and IL-18 that promote CD8$^+$ T cell responses to pathogens in an innate fashion and production of IFN-γ [120].

As a part of adaptive immune response, naïve CD8$^+$ T cells primarily interact through the T cell receptor (TCR) with a specific antigen presented via the major histocompatibility complex class I (MHC I) or human leukocyte antigen on the surface of APCs such as dendritic cells. Activated CD8$^+$ T cells undergo differentiation into effector T cells and clonal expansion. The activation and expansion of T cells is closely regulated to ensure an effective response to infection and preventing immunopathology [121]. Abundant evidence indicates that cytotoxic T cells play central roles in the pathology of atherosclerosis through cytotoxic mechanisms contributing to atheroma development and progression [122]. Atherosclerosis-associated viral antigens are able to activate, reactivate, and differentiate CD8$^+$ T cells to elaborate antigen-specific cytotoxic T cell-mediated responses [123].

In order to develop into effector populations that fight microbial infections, naive CD4$^+$ T cells require to recognize peptide antigens presented by MHC class II molecules on activated APCs. PRR-mediated signaling activates APCs to enhance their expression of MHC class II molecules, co-stimulatory molecules such as CD80 and CD86, and pro-inflammatory cytokines, such as type I interferon, TNF-α, IL-1, IL-6, and IL-12 [124]. Another activation pathway is carried out by means of B7 proteins, which are recognized by co-receptor such as CD28. The expression of B7 proteins on the surface of APCs is provoked by pathogens [125]. Activated CD4$^+$ T cells undergo differentiation into Th subsets. They are generated under the distinct influence of cytokine environment: Th1 (IFN-γ), Th2 (IL-4/IL-5/IL-13), Th17 (IL-17/IL-22), follicular (Tfh) (IL-21), and regulatory Treg (IL-10, TGF-β, IL-35) [126]. Helper function of CD4$^+$ T cells is to promote antibody production by B cells during pathogen exposure [124]. It is important to note that the subset of CD4$^+$ Th1 cells is pro-atherogenic. They promote atherosclerosis development by secretion of pro-inflammatory cytokines, such as IFN-γ, TNF-α, IL-2, IL-3, which sustains chronic inflammation and foam cells formation [127]. Recent review summarized the evidence on the molecular mechanisms by which Th1 cells contribute to atherosclerosis [128]. On the contrary, Treg cells display anti-atherogenic activities by inhibiting ongoing macrophage and T-cell-mediated proinflammatory responses [129], while the role of Th2 cells remains under discussion [117].

During the development of the atherosclerotic lesion, B cells can play both atheroprotective and pro-atherogenic roles by executing their main effector functions, such as antibody production, secretion of cytokines, and antigen presentation/interaction with T cells, as discussed in [130]. These functions depend on the B cell subset and their activation state. Naïve B cells become activated by detecting antigens via B cell receptors (BCRs), resulting in their differentiation into antibody-secreting plasma cells. This process is supported by Tfh cells and results in the release of various classes of immunoglobulins. Apparently, TLRs are also involved in B cell activation and differentiation. The involvement of both BCRs and TLRs leads to quicker induction of B cells. In atherosclerosis, dual TLR and BCR signaling allows B cells to be a part of the innate and adaptive immune responses [130, 131].

Perspective Therapeutic Concepts of Infection-Enhanced Atherosclerosis

Bacteria are known to aggravate atherosclerosis. In this regard, anti-atherosclerotic therapy can be aimed at the immune response caused by pathogenic BEMNs and the elimination of vascular inflammation. For this, several approaches can be suggested, which have been identified in pre-clinical settings, but the efficacy, side effects, and optimal dosage of potential drugs need to be verified in clinical trials.

The interruption of PRR signaling may be a reliable way to alleviate ASCVD. The suppression of TLR signaling using inhibitors can be an important target. For example, MyD88 inhibitors may be advantageous in this regard [132]. Because of the pivotal role of NF-κB dysregulation in atherosclerosis, this signaling pathway can become a highly potential therapeutic target for its treatment [133]. Also, the NLRP3-related pathway is a promising pharmacological target to attenuate ASCVD, reviewed in [134]. Several studies have emphasized potential pharmacological inhibitors in different sites of the NLRP3's complex signaling cascade, including 1) inhibition of upstream signaling, 2) impediment of the NLRP3 assembly, and 3) neutralization of the inflammatory cytokines that are released when the inflammasome is activated [135–137]. The notable role of the complement system in BEMNs-associated atherosclerosis development represents its potential for ASCVD treatment. It was shown that pexelizumab, a humanized monoclonal antibody against C5, significantly reduced rates of acute myocardial infarction [138]. A great deal of evidence suggests that targeting cytokines can be a part of atherosclerosis treatment [139]. For example, an early study using murine models of atherosclerosis proposed that blocking the activity of IL-1 and TNF-α and -β should be considered as a therapeutic option for the disease [140]. Numerous options for targeting chemokines have been investigated experimentally. An in vivo study using a hypercholesterolemic mouse model reported that blocking a chemokine pathway with the CC chemokine antagonist Met-RANTES can diminish the progression of atherosclerosis [141]. As an antagonist for the chemokine receptors CCR5 and CXCR3, TAK-779, can reduce the size of the atherosclerotic lesion and the content of Th1 cells in plaques [142]. Other potential anti-chemokine drugs, which showed anti-atherosclerotic effects, were also identified. Some examples of these drugs are listed in Table 1.

As for the cellular aspect of vascular inflammation, inhibition of macrophage activation can be an effective concept for ASCVD treatment. Animal studies suggested that the therapeutic strategies of switching macrophage phenotypes can aid in plaque regression [149]. In this study, the signal transducer and activator of transcription 3/6 (STAT3/6) altered macrophage M1 phenotype to M2 resulting in atherosclerotic plaque stabilization. Moreover, an independent group of researchers proposed that cholesterol-lowering drugs such as statins and proprotein convertase subtillisin/kexin type 9 (PCSK9) inhibitors are able to reduce atherosclerosis [150]. This might not only be because of the efficacy of these drugs in lipid-lowering but also, they might be playing a role in switching the macrophage phenotype

Table 1 Examples of anti-chemokine drugs with anti-atherosclerotic effects

Drug	Mechanisms of action	Anti-atherosclerotic effects	References
[^{44}AANA^{47}]-RANTES	Interference with chemokine oligomerization and chemokine/heparin interactions	Impaired leukocyte recruitment into plaques and reduced inflammation	[143]
Evasin-3	Binding and neutralization of CXCL1 and CXCL2	Reduced neutrophilic inflammation	[144]
Gremlin-1	Inhibition of macrophage MIF	Reduced macrophage formation	[145]
Maraviroc*	Inhibition of macrophage infiltration; downregulation of the local expression of adhesion molecules, such as VCAM-1, ICAM-1, MCP-1, and IL–17A; reduction of TNF-α	Reduced inflammatory cell recruitment and macrophage infiltration; reversed the proinflammatory profile and atherogenesis	[146]
Mozobil*	CXCR4 antagonist; mobilizing G-CSF	Increased circulating EPCs (moderate and long-term increase in EPC availability supported by G-CSF)	[147]
CKEY	Disruption of proinflammatory CCL5–CXCL4	Attenuated monocyte recruitment into plaques	[148]

*Approved by the United States Food and Drug Administration

RRRAbbreviations: G-CSF, granulocyte colony stimulating factor; CXCL1/2, chemokine (C-X-C motif) ligand 1/2; EPCs, endothelial progenitor cells; ICAM-1, intercellular adhesion molecule-1; IL-17A, Interleukin 17A; MCP-1, monocyte chemoattractant protein-1; MIF, macrophage migration inhibitory factor; TNF-α, tumor necrosis factor -alpha; VCAM-1, vascular cell adhesion molecule-1

and promoting plaque regression. This concept needs further exploration with advanced technologies under treated conditions. In addition, therapeutic suppression of Th1 cell activity can be a promising strategy to decrease atherosclerosis. This could be achieved by inhibiting the differentiation of naive T cells into Th1 cells or by inhibiting the effector functions of Th1 cells. Blocking the activity of IL-12 or IL-18 can limit the differentiation of Th1 cells [151].

Conclusion Remarks

The investigation into the role of BEMNs in atherosclerosis has shed light on a potential and previously overlooked trigger in its complex pathogenesis. Through our comprehensive analysis of the available literature, we have summarized compelling evidence supporting the involvement of these nanovesicles in the atherosclerotic process. The studies presented in this review underscore the intricate interplay between bacterial components such as BEMNs and the formation of atherosclerotic lesions. Several promising therapeutic concepts for infection-enhanced atherosclerosis have been suggested that can be tested in human clinical trials.

Future Perspectives

While the link between microbial components and atherosclerosis is not fully established, it is an interesting avenue for further exploration in understanding the disease's complex pathophysiology. In this regard, it is worth exploring whether pathogenic microbes that enter a cell are capable of activating the inflammasome not via PAMPs, but through modified cellular metabolism and mitochondrial activity. There is an example that indicates this possibility. An in vivo study revealed that the NLRP3 inflammasome mediates innate immunity to the influenza A virus by identifying its RNA [152]. This was established to be a result of the influenza virus *M2* protein action, a proton-selective ion channel, recognized to efficiently reduce the mitochondrial membrane potential [153]. Mitochondrial membrane potential is crucial for mitochondrial antiviral signaling (MAVS)-mediated antiviral signaling [154] and thus the physiological function of mitochondria is implicated in innate antiviral immunity. High levels of mtROS provoke oligomerization of MAVS with subsequent activation of NF-κB to regulate host defence and inflammation [155].

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Data Availability No datasets were generated or analysed during the current study.

Declarations

Competing Interests The authors declare no competing interests.

Conflict of Interest The authors declare that they have no conflict of interest.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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