Supplement submission

Evolving concepts in the pathophysiology of atherosclerosis: from endothelial dysfunction to thrombus formation through multiple shades of inflammation

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Atherosclerosis is the anatomo-pathological substrate of most cardio, cerebro and vascular diseases such as acute and chronic coronary syndromes, stroke and peripheral artery diseases. The pathophysiology of atherosclerotic plaque and its complications are under continuous investigation. In the last 2 decades our understanding on the formation, progression and complication of the atherosclerotic lesion has greatly improved and the role of immunity and inflammation is now well documented and accepted. The conventional risk factors modulate endothelial function determining the switch to a proatherosclerotic phenotype. From this point, lipid accumulation with an imbalance from cholesterol influx and efflux, foam cells formation, T-cell activation, cytokines release and matrix-degrading enzymes production occur. Lesions with high inflammatory rate become vulnerable and prone to rupture. Once complicated, the intraplaque thrombogenic material, such as the tissue factor, is exposed to the flowing blood, thus inducing coagulation cascade activation, platelets aggregation and finally intravascular thrombus formation that leads to clinical manifestations of this disease. Nonconventional risk factors, such as gut microbiome, are emerging novel markers of atherosclerosis. Several data indicate that gut microbiota may play a causative role in formation, progression and complication of atherosclerotic lesions. The gut dysbiosis-related inflammation and gut microbiota-derived metabolites have been proposed as the main working hypothesis in contributing to disease formation and progression. The current evidence suggest that the conventional and nonconventional risk factors may modulate the degree of inflammation of the atherosclerotic lesion, thus influencing its final fate. Based on this hypothesis, targeting inflammation seems to be a promising approach to further improve our management of atherosclerotic-related diseases.

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Introduction

Despite therapeutic strategies targeting the well known traditional risk factors underlying the development and progression of atherosclerosis having brought considerable improvements in life expectancy over the last decades, a plateau has been reached.[1] On the contrary, the latest promising treatments have shown disappointing results in clinical studies.[2,3] Consequently, the concept of an 'irreducible' residual cardiovascular risk has emerged.[4] Multiple experimental and clinical pieces of evidence point to inflammation as a key common element linking traditional and emerging risk factors and moderators.[5] Thus, modulation of inflammation may exert several antiatherosclerotic effects and bring down the detrimental residual risk.[6] This is a state of the art review summarizing the major pathophysiological partakers in atherosclerosis, underlying the most recent advances along with current gaps in knowledge and highlighting limitations of current treatments and discussing the potential of the most recent discoveries in the

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fields of cell biology, RNA therapeutics, and gene editing that might be game-changing to ignite the (r)evolution of clinical practice toward precision medicine.

Endothelial dysfunction: the starting point of inflammatory disorder

The homeostatic function of the vascular endothelium is regulated by a fine balance between relaxing factors (nitric oxide, prostacyclines) and contracting factors (endothelin-1 and constricting prostaglandins) of the endothelium.[7]

Endothelial dysfunction, a critical factor for cardiometabolic disorders and atherosclerotic vascular diseases, occurs when this balance is broken.[7] Inflammation and oxidative stress are the two predominant factors that cause endothelial dysfunction; therefore, they are the main objectives of intervention in patients with cardiovascular and metabolic diseases.[7] Endothelial activation is a proinflammatory and procoagulant state of endothelial cells, induced by cytokines secreted by tissues and organs under inflammatory conditions, and is characterized by the adhesion molecules expression and recruitment of inflammatory cells.[8,9] Oxidative stress plays a pivotal role in mediating the production and secretion of cytokines,[10,11] thus linking reactive oxygen species (ROS) with inflammation and endothelial activation and dysfunction. In endothelial cells, nitric oxide (NO) is majorly responsible for the maintenance of vascular homeostasis, thus, if its bioavailability is reduced (mainly by the action of the superoxide anion), endothelial dysfunction occurs.[12,13] Superoxide anion is generated by several enzymes, such as NADPH oxidase, xanthine oxidase and uncoupled endothelial nitric oxide synthase (eNOS). The regulation of endothelium-dependent contractions occurs through various mechanisms. Cyclooxygenases (COX) mediate the production of endothelium-derived contracting factors (EDCFs)-like prostaglandins derived from arachidonic acid in vascular endothelial cells.[14,15] Endothelial cells contain all the enzymes to synthesize arachidonic acid and form the five final products which are COX, PGD2, PGE2, PGF2α, prostaglandin I2 (PGI2) and TXA2.[16] Acetylcholine, bradykinin and substance P are intracellular agonists responsible for increasing calcium and activating the production and release of endothelial-derived NO. Moreover, the secretion of endothelium-derived contracting factors requires an increase in calcium ions in the endothelial cells. There is a significant increase in COX-2 activity in endothelial dysfunction that occurs through several regulatory mechanisms. All five prostaglandins are vasoactive, which causes vascular relaxation or contraction.[17] Healthy vascular individuals express a very low level of COX-2, but the presence of growth factors and proinflammatory cytokines increases its expression. Many substances that cause oxidative stress are involved in endothelial dysfunction and, consequently, in atherogenesis; they include bone morphogenetic protein (BMP), angiotensin II, saturated fatty acids and proinflammatory cytokines and shear stress. Between the BMPs represented in the cardiovascular system,[18] BMP4 is a protein expressed by vascular endothelial cells in response to shear stress. It causes apoptosis of endothelial cells through the activation of caspase-3 activity.[19] Furthermore, Ang II and COX-2 participate in vascular inflammation and remodeling. Ang II induces the expression of COX-2 through activation of protein kinase Cδ.[20] Although Ang II increases ROS generation, ROS inhibitors fail to affect Ang II-induced COX-2 expression in endothelial cells; this indicates that the role of oxidative stress in upregulation of COX-2 probably depends on the choice of a COX-2 inducer. Preclinical studies have shown that the upregulation of COX-2 causes endothelial dysfunction.[21] Although COX-2 is considered to be a proinflammatory enzyme that interconnects multiple inflammatory processes during disease progression,[22] conflicting clinical effects have been reported from COX-2 inhibition in the cardiovascular system.[23]

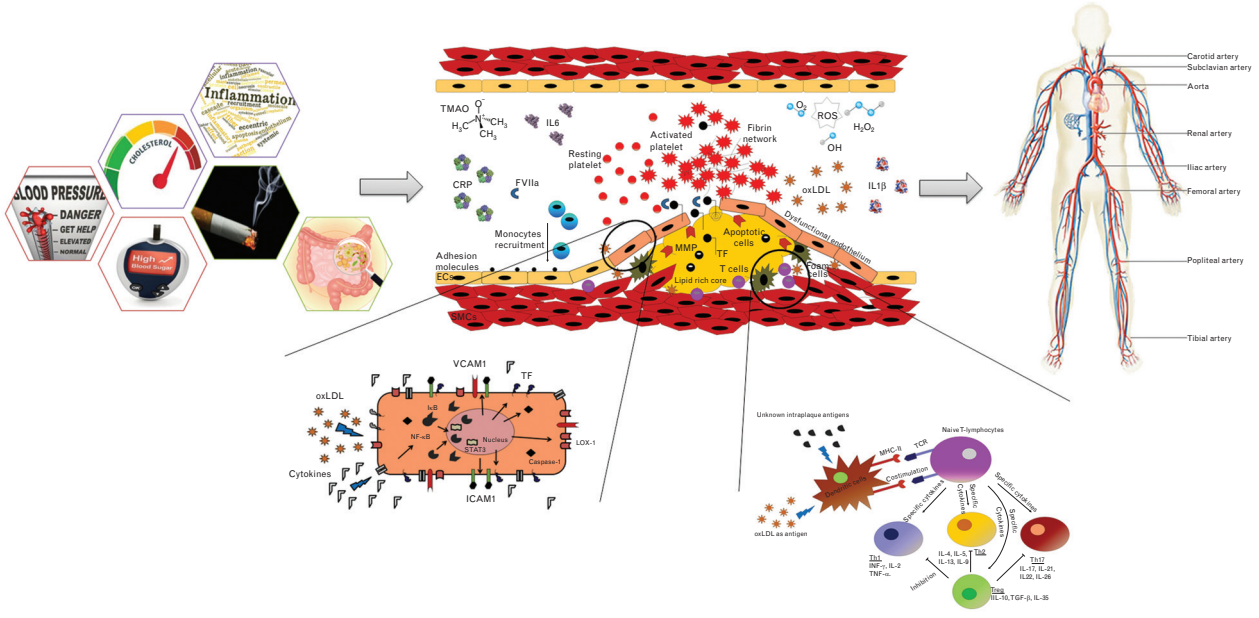
The cardiovascular risk factors such as hypertension, hyperglycemia, dyslipidemia, physical inactivity, estrogen deficiency and mental stress may induce oxidative stress, thus leading to the development of atherosclerotic vascular diseases.[24] Vascular endothelial cells respond rapidly to changes in hemodynamic forces and blood flow by interacting with circulating substances. Atherosclerotic plaques preferably develop around arterial curvature and bifurcations, where endothelial cells are constantly exposed to the bloodstream. These hemodynamic forces may have a profound impact on the endothelial cells homeostasis. Excessive ROS production chronically supports elevated COX-2 expression in endothelial cells.[7] Given the importance that oxidative stress and excessive ROS production assume in the progression of the main cardio-metabolic diseases, the recovery of endothelial function should delay the development of cardiovascular complications. Hence, by further analyzing the proaterogenic pathways it will be possible to know and develop more effective therapeutic strategies targeting the vascular endothelial system against atherosclerotic vascular diseases. A summary of endothelial dysfunction mechanisms is shown in Fig. 1.

Lipid deposition and lesion formation: how inflammation defines disease progression

Cholesterol accumulation and the vicious cycle of inflammation

More than 20 years have passed since Ross defined atherosclerosis as 'an inflammatory disease'.[25] Multiple studies have shown that cholesterol accumulation and immune system activation are partners in crime in atherosclerotic plaque formation.[26] The immune system that protects us every day from pathogens such as viruses and bacteria is unfortunately also involved in atherosclerosis pathogenesis.[26] In the model proposed by Ross of the

Fig. 1



Old and novel insights in the pathogenesis of atherosclerosis. Impact of conventional and less conventional risk factors on atherosclerotic plaque development. It is highlighted the effect on endothelial cells and the putative immune mechanisms involved.

'response to injury' at the beginning of the atherosclerotic process, this 'injury' underlying the formation of plaque remains unknown. Accumulated evidence considers modified low-density lipoproteins (from minimal oxidized LDLs to fully oxidized LDLs)[27,28] as well as lipoprotein(a)[29] as possible actors of this pathological condition. Several studies have shown that high LDL levels and Lp(a) correlate with a higher incidence of ischemic heart disease and mortality from cardiovascular causes.[29–31] LDLs are considered by our immune system as a 'self' molecule, since they contribute to many physiological processes, including cell membranes formation.[31] However, several experimental studies have shown that high levels of LDLs, associated with smoking, diabetes, and hypertension, contribute to endothelial dysfunction and the entry of LDLs into the vascular subendothelium.[32,33] Here, the lipoproteins are biochemically modified by oxidative stress enzymes, proteases, and lipases with the formation of oxidized LDLs (oxLDLs).[34] Becasue oxLDLs have a different biochemical conformation, they are recognized as 'not self' by our immune system.[35] These molecules begin the vicious circle in the subendothelium with inflammation that leads to atherosclerotic plaque formation.[36] The 'not self' oxLDLs as well as Lp(a) activate innate immunity and contribute to worsening the state of endothelial dysfunction, thus leading to the expression of the adhesion molecules VCAM-1 and ICAM-1, and the release of chemokines, necessary for monocytes recruitment.[36,37] Monocytes infiltrate the subendothelial space under the influence of local stimuli and cytokines, and differentiate into macrophages increasing the expression of scavenger receptors and receptors involved in the innate immune response, the Toll-like receptors (TLRs).[38] Scavenger receptors, including the oxLDL-specific receptor LOX-1, mediate the uptake of oxLDL particles by macrophages.[39] Within macrophages, modified LDLs may induce inflammasome NLRP3 priming and activation.[40] The NLRP3 inflammasome links the vascular deposition of lipids and lipoproteins to the inflammatory responses driving the atherosclerotic process.[41] However, when the ability of macrophages to degrade oxLDLs is impaired, they turn into foam cells: an event that contributes to the establishment of a chronic inflammatory response.[39] In turn, macrophages secrete proinflammatory cytokines and contribute to vascular remodeling. The onset of chronic inflammation, also due to the necrosis of foam cells, brings in another main actor: the dendritic cells.[42] These cells are highly potent professional antigen-presenting cells (APCs) responsible for capturing, processing, presenting antigen to T cells and thus priming primary and secondary immune responses. Dendritic cells are able to internalize oxLDLs, but unlike macrophages they do not turn into foam cells.[42] At this point, adaptive immunity is activated, with the arrival of T-lymphocytes, which is now considered a crucial point in atherosclerotic plaque.[42,43] A summary of atherosclerisis development is shown in Fig. 1.

The dramatic vicious circle between cholesterol and the immune system is supported by the constant increased levels of LDLs/oxLDLs in patients with high

cardiovascular risk and the consequent chronic related inflammation.[43] oxLDLs may be recognized by the TLRs, mainly the type 4.[44] Following this binding, the transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) is activated thus leading to inflammatory cytokines production of and the recall of T-lymphocytes. Furthermore, more recent studies have highlighted another harmful action of oxLDLs in atherosclerosis pathogenesis: the dysregulation of the immune system, namely the 'break of tolerance'.[45] It has been shown the macrophages, *in vivo* as well as *in vitro*, when exposed to a pathogen molecule such as bacterial lipopolysaccharide (LPS) may exert a 'reduced activation' to subsequent stimuli induced with the same antigen, after the first contact, developing an immunological 'tolerance'.[45] This is a physiological mechanism aimed at limiting and circumscribing the inflammatory process, as it reduces the production of TNF-α and IL-1β by macrophages.[45,46] More recent studies have shown that the interaction between macrophages and oxLDLs induces a 'break of tolerance', which leads to an uncontrolled inflammatory response to each stimulus with a dysregulation of the immune system and a rapid progression of atherosclerotic plaque.[45–47]

The dark side of immunity

As already reported above, despite the involvement of immune cells in atherosclerosis being good, a dark side is revealed.[32]

Monocytes

In the early stage of atherosclerosis, monocytes are the most representative immune cells.[48] They adhere to the damaged endothelium recruited in the area of injury by chemotactic stimuli, such as monocyte chemoattractant protein 1 and 3 (MCP-1 and -3), and migrate into the arterial wall.[49] This migration is facilitated by the L-selectin on the monocyte cell surface that interacts with the P and E selectin of the activated endothelium.[49] The major roles of monocytes in atherosclerosis progression are: first, initiation and formation of atherosclerotic plaque by traveling to the site of endothelial injury, migrating in the subendothelial space and differentiating into macrophages that ingest oxLDLs finally creating foam cells; second, generation and reinforcement of the acute inflammation that contributes to plaque destabilization and rupture via secretion of proteolytic enzymes such as matrix metalloproteinases (MMPs) and to acute thrombus formation via expression of tissue factor; third, handling the healing process, specifically in the myocardial tissue during the hypoxic phase, via various beneficial or detrimental inflammatory processes.[32,49]

Macrophages

Atherosclerotic lesions are enriched with macrophages, the activation of which defines the inflammatory environment within the plaque.[30] In the last decade, several studies have indicated that macrophages might be programmed in response to numerous stimuli, such as oxLDLs and several cytokines to M1 and M2.[50] The latest form are the precursors of foam cells.[51] The primary function of foam cells is to remove the cytotoxic and proinflammatory oxLDLs in the intima.[51] However, this process fails in the long term if oxLDL accumulation remains continuous.[52] As the foam cells accumulate in the plaque shoulders and necrotic core, MMPs are released thus facilitating plaque instability and disruption.[53] However, it has been shown that foam cells are not only of macrophages origin but are also smooth muscle cells-derived.[54] These two forms of foam cells are observed in different stages of human atherosclerosis.[54]

Neutrophils

Recently, increasing attention has been paid to neutrophils, the most abundant circulating white blood cells.[55] Based on epidemiological evidence, the counts of circulating neutrophils seems to predict the future of cardiovascular events in humans.[56] Some studies indicate that neutrophils localize at sites of plaque erosion,[57] their blood count correlates with lesion sizes, and their depletion reduces lesional macrophage accumulation[57] and prevents endothelial erosion.[55] This is in line with the working hypothesis that neutrophils might control macrophage migration and activates them via secretion of several proteins with chemotactic/stimulating activity.[55]

Lymphocytes

In the last 20 years, the increasing role of lymphocytes in atherosclerosis has been extensively studied and documented.[58] The inflammatory milieu generated by infiltrating and resident immune cells within the atherosclerotic lesion recruits more macrophages as well as B- and T-lymphocytes.[59,60] Despite T cells being a minority of the leukocytes infiltrating the plaques, accumulated evidence suggests their decisive role for the final fate of atherosclerotic lesions.[61] Specifically, T-lymphocytes instruct the more abundant monocytic cells[26,62] and amplify local inflammation via cytokines release. Moreover, based on the local inflammatory microenvironment and the specific activation by the APCs, T cells might be polarized in different subsets (Table 1).[63] This differentiation appears to be of great importance for plaque destiny.[64] It has been reported that some lineages exert more proinflammatory effects (Th-1 and Th-17 cells), while others may act as negative regulators of this inflammatory status (regulatory T cell (Treg) and Th-2 cells).[26,65] A first human study has shown that patients presenting with unstable angina displayed an increased number of T cells selectively recruited and producing large amounts of IFN-γ in peripheral blood, whereas patients with stable angina did not.[66] Direct evidence of specific oligoclonal intraplaque T-cells expansion has been reported in another human study that analyzed atherosclerotic plaques from living patients during an acute coronary event,[67] also showing that unstable

Table 1 T-cells subsets, cytokines released and effects on atherosclerotic plaque

T-lymphocytes	Cytokines	Effects on plaque
Th1	INF-g, TNF-a	Increased inflammation and vulnerability
Th2	IL-4, IL-5, IL-13	Reduction of elastin and increased susceptibility to aneurysms
Treg	IL-10, TGF-b	Reduction of inflammation and vulnerability
NKT	INF-g, TNF-a, IL-5	Increased inflammation and vulnerability

plaques are infiltrated by T cells with a greater T-cell receptor (TCR) rearrangement than stable lesions. These T cells might be also involved in thrombosis, since there is the possibility to express a functional tissue factor on their surface.[68] Moreover, the selection of T-cell repertoire within atherosclerotic lesions from acute coronary syndrome (ACS) patients has raised the issue of which intraplaque antigen might drive this process.[60] As already reported above oxLDLs are considered the major determinant of T-lymphocytes activation.[66,69,70] It has been postulated that oxLDLs might be processed within atherosclerotic lesions and then presented to naïve T cells by APCs,[32] thus inducing activation via a TLR-dependent as well as a TLR-independent manner.[71] Once activated, T-lymphocytes may differentiate into different subsets according the local microenvironment and the activating stimuli.[30] These subsets may have a different role in cardiovascular diseases (CVDs).[72] An imbalance between the Th-1/Th-2 ratio has been reported in patients with ACS,[73] while a higher number of Th-17 cells was found in the blood collected from the coronary sinuses of ACS patients presenting with a more severe disease at admission as demonstrated by the higher levels of troponin, as compared with patients with stable angina.[74] Th-22 cells, the fourth subpopulation of Th cells, identified in 2009[75] have been linked to myocarditis,[76] blood pressure[77] and atherosclerosis.[78]

The activity of Th cells is regulated by Treg lymphocytes.[79,80] This class of cells exerts a suppressing effect on Th-1, an enhancing function on Th-2 and inhibitory properties on the autoaggressive $CD4^{+}CD28^{null}$ cells. Thus, failure of these activities will result in a proinflammatory disequilibrium of the Th-1/Th-2 state[81] and expansion of $CD4^{+}CD28^{null}$ cells.[82] As the acute event exacerbates further the proinflammatory state with a cytokines storm: an uncontrolled immune response occurs with reduced Treg activity, finally leading to a worst outcome.[79] Thus, homeostasis of Treg becomes essential[80] as shown in Fig. 1.

Dendritic cells

Dendritic cells are APCs that present antigens to the T cells, initiating and maintaining an immune response or inhibiting the activation.[83] Their role is dependent on the kinds of cytokines that are released in the microenvironment.[83] It has been shown that dendritic cells are present at a very early stage of atherogenesis, in the areas at high shear stress that are prone to be atherosclerotic.[84] The shear-stress signal is believed to play a role in dendritic cells recruitment.[85] At this stage, dendritic cells intake lipoproteins and apoptotic material, thus becoming foam cells.[83] Then, by emigrating from the lesion into the draining lymph node they present antigens to naïve T cells, finally inducing their activation.[84] This emigration becomes defective in the advanced stage of atherogenesis, thus dendritic cells remain inside the plaque, enhancing local inflammation.[83] Further studies are needed to better understand the pathogenic role of dendritic cells in atherosclerosis.

Mast cells

Mast cells are part of the innate and adaptive immunity.[86] They express IgE receptors on their surface and possess granules full of proinflammatory cytokines, histamine and neuronal proteases.[86] Allergic/inflammatory reactions may trigger the release of the granular content thus inducing matrix degradation and apoptosis of the surrounding vessel wall.[86] In areas enriched with LDL particles, mast cells degranulation induces the conversion of macrophages into foam cells.[87] Once the atherosclerotic plaque grows, mast cells occur in the shoulder regions, and if degranulation occurs, they release some proteases, such as tryptase and chymase that may cause intraplaque hemorrhage, vascular leakage and macrophage and endothelial cells apoptosis.[87,88] The presence of the activated mast cells in atherosclerotic lesions at various stages has been confirmed.[88]

Plaque complication: the role of inflammation determining the final fate

Inflammation plays a crucial role in every stage of atherogenesis and is involved in plaque destabilization.[89] The lesions include macrophages and T cells, interspersed with acellular areas containing lipids and cellular material and enveloped in an extracellular matrix made of collagen fibers and other elements formed mainly by smooth muscle cells (SMCs).[90] The eventual rupture of endothelial integrity can facilitate plaque instability. Injury to the plaque layer may lead to thrombotic obstruction of the coronary artery. Plaque rupture and endothelial erosion are two types of surface damage that can lead to atherothrombosis and ACS in 30 and 70%, respectively.[91] Several pieces of evidence indicate a shift in the ratio, with more cases due to plaque erosion and fewer to plaque rupture.[92] Multiple factors can contribute to the instability of plaques. Disturbance of the equilibrium between thrombotic and fibrinolytic function on the plaque surface certainly plays a significant role in initiating the process. However, the exact order that occurs *in vivo* is not yet understood.[93] SMCs are crucial for plaque homeostasis, and their depletion is evident at the site of plaque rupture. SMCs are required for the repair and maintenance of the fibrous cap.[94] Cell death may potentially lead to plaque rupture through two mechanisms: efferocytosis (a form of

apoptosis with poor phagocytic clearance of apoptotic cells) and primary necrosis.[95] Inflammation and increased concentrations of proteolytic enzymes are essential for plaque rupture. Inflammatory events can stimulate macrophages, mast cells and T cells to secrete cytokines and enzymes, such as MMPs (mainly -2 and -9[96,97]), that reduce and break down the fibrous cap.[98]

Th-1 and NK cells play a crucial role in this scenario since they can suppress collagen production through INF-γ production, inducing plaque rupture. TGF-β produced by Treg counteracts the action of Th-1 cells.[99] This has a significant fibrogenic effect. It also reduces Th-1 and macrophage activity, which leads to a decrease in plaque inflammation. In addition, the Treg promote the degradation of LDLs, which leads to a reduction in lipid concentration.[100] Recently, studies have highlighted the role of Th-17 cells in tissue regeneration and fibrogenic activity. The Th-17 cytokine IL-17A promotes the development of collagen expression, protects against plaque rupture and increases stabilization.[101] In this regard, the TNF/TNF receptor superfamily members CD40 ligand (CD40L, CD154) and CD40 may be essential. The CD40/CD40L complex stimulates the production of tissue factor and MMPs; moreover, active platelets express CD40L and endothelial cells express CD40, providing various recombinant contacts that may cause atherothrombosis.[46] Other elements, such as prostaglandins, have distinct functions in the atherosclerotic arterial wall. The thrombogenic action of platelet-derived thromboxane A2 is balanced by endothelium-derived PGI2, which is essential for vascular homeostasis, while PGE2 generated by multiple cell types enhances vasodilation and macrophage activation, but also enhances IL-10 production.[102] According to clinical studies, infections are associated with ischemic atherothrombotic events such as myocardial infarction and stroke. Acute infections may increase inflammation via activation of systemic cytokines, leading to an increase in proinflammatory, proteolytic and prothrombotic activity, but definitive evidence for such a sequence of events is still lacking.[103]

The other mechanism that can lead to plaque instability is the erosion of the endothelium, the mechanism of which is not yet fully understood. Current research indicates, however, that innate immunity plays a significant role in this process.[104] Activation of TLR2 triggers apoptosis, a process that is further enhanced by leukocytes, which are highly represented at erosion sites. Consequently, both endogenous and viral stimuli can trigger atherothrombosis via this mechanism.[105] Experimental research implicating inflammation in atherosclerosis has generated much discussion regarding its clinical application to patients.[106] By reducing classic risk factors, the incidence of coronary artery disease has decreased by 40%.[31]

Understanding the causes of plaque destabilization may help identify individuals at high risk for thrombotic events. When mononuclear phagocytes and endothelial cells are activated by inflammation, their metabolism shifts into glycolytic pathways.[107] Atherosclerosis has also generated much attention in altered tryptophan metabolism. Cytokines decrease intracellular tryptophan reserves and increase the formation of kynurenine and its metabolites; this route may have a counter-regulatory role by reducing inflammation and the cellular immune response.[108] Recent clinical trials have demonstrated, however, that targeting inflammation can reduce cardiovascular events in patients who have already been treated with standard therapies.[5,109]

Inflammation and thrombus formation: is there a causal effect?

Thrombosis and inflammation are key partakers in atherosclerosis.[30,110] The term thrombo-inflammation has been suggested to underline the high degree of interdependency.[111] This interplay is at the core of several pathophysiological processes leveraging acute clinical complications of atherosclerosis.[112] Multiple noxae can exert direct or indirect effects on thrombo-inflammation, including infections, chronic autoimmune diseases, clonal haematopoiesis of indeterminate potential, or the derangement of host defense mechanisms.[113–115] Adverse effects include ischemic and thrombotic complications of both microvascular and macrovascular beds. The resulting vicious circle includes platelets, activation of innate immune cells, inflammation, the complement system and the coagulation cascade.[116,117] Platelets play a pivotal role in vascular inflammation and in atherosclerosis, representing a communication link for the horizontal transfer of biological information, including inflammatory cytokines and microRNAs, between cells, playing a key role in the pathophysiology of atherothrombosis.[118]

No clinical treatment is currently available to address the inflammatory component of thrombosis. However, it represents a promising target for the treatment of acute CVDs. Among coagulation factors, tissue factor is pivotal in atherothrombosis, closely linking inflammation, thrombosis and coagulation,[119] its expression in the vascular wall or in blood–borne cells being induced by inflammation.[119–121] Yet tissue factor is a promoter of tissue inflammation itself.[122] Similarly, platelets are both inducers[123] and targets of tissue factor.[124] More recently, tissue factor was found to be involved in neutrophil extracellular traps (NETs), a key host defense mechanism. Initially thought to mainly exert a bactericidal effect, it became increasingly clear that they largely contribute to thrombosis and atherosclerosis.[125,126] NETs are a key element within a complex machinery, including component factors, platelets, neutrophils and multiple cell adhesion proteins, that is able to induce a specific active cell death process in response to pathogen-induced cell activation patterns named NETosis. Recently, a

growing amount of experimental evidence has suggested the involvement of NETosis atherothrombosis.[127,128]

The recent advent of the COVID-19 pandemic has brought about a heavy burden of morbidity and mortality, particularly for patients with CVD at high cardiovascular risk.[129–131] In parallel, it emerged that coagulation and thrombosis are deeply involved in the clinical pathophysiology of COVID-19.[132] The thrombotic and procoagulation processes observed in COVID-19 share some similarities with atherothrombosis, strengthening the recent evidence suggesting a key role for purinergic receptors in atherothrombosis.[133]

Gut microbiome and atherosclerosis: a tale of inflammation?

The development of CVDs and, in particular, atherosclerosis have been also linked to infectious processes. Recently, the microbial ecosystems that live in different parts of the human body have been identified as the cause of metabolic and cardiovascular disorders through different mechanisms. First, infections, whether local or systemic, might cause an inflammatory response that may favor plaque progression and/or destabilization. Second, the intestinal microbiota, through the metabolism of its cholesterol and its lipids, may lead to atherosclerosis development. Third, dietary components metabolized by the gut microbiota could produce various effects on atherosclerosis.

Although the number of bacterial species varies between individuals, with higher taxonomic levels (like phyla), the microbiota is similar between individuals. There are two bacterial phyla that dominate the gut microbiota: bacteroidetes and firmicutes, which together make up more than 90% of all taxis in the human gut.[134,135] The cause of plaque development could be either a distant infection or an infection of the vessel wall cells; finding bacterial DNA in plaques supports this mechanism.[136,137] The bacteria found within the atherosclerotic plaques are also found in other body sites, in particular, in the intestine. In addition to bacterial infections in atherosclerotic plaques, the gut microbiota influences CVD through the regulation of host metabolism, including cholesterol and lipid metabolism. Patients with atherosclerosis have altered lipid metabolism, and bacterial taxa in the gut were found to correlate with plasma cholesterol levels.[136] Some species of microbiota produce specific enzymes[138] that allow the fermentation of indigestible carbohydrates into short-chain fatty acids (SCFAs).[139] These SCFAs may have anti-inflammatory effects. Bacteria regulate the permeability of the intestine. Some species can make the gut permeable so that metabolites associated with microbes leave the gut and enter the bloodstream. Our body produces cytokines and other mediators by triggering an inflammatory response.[140]

The persistence of this condition can lead to subacute or chronic inflammation, which can subsequently trigger the development of diseases such as inflammatory bowel disease, diabetes or CVD.[141] Different mechanisms link the intestinal microbiota to inflammation. In normal conditions, the intestinal barrier minimizes the passage of LPS from the intestine to the systemic circulation.[142] Disruption of intestinal impermeability by bacteria can lead to the passage of LPS through the intestinal barrier into the circulating blood. This loss of permeability means that macrophages can infiltrate the region and produce and activate inflammatory cytokines, which are leaders in local inflammation.[142] As stated above, gut bacteria possess the capability of metabolizing complex carbohydrates otherwise undigested by the host into SCFAs. SCFAs play a critical role in the interplay between diet, the gut microbiota and downstream activation or inhibition of inflammatory cascades. The intestinal microbiota allows the conversion of primary bile acids into secondary bile acids. Changes in the gut microbiota influence the types of secondary bile acids that are synthesized. Of particular relevance is that bile acids, via activating the farnesoid X receptor signaling pathways in enterocytes and adipocytes, cause inflammation.[143] Dietary choline, carnitine, and betaine, compounds present in red meat, fish, and another animal sources, are metabolized by gut microbes into trimethylamine, which is then converted into trimethylamine N-oxide (TMAO) by the actions of host hepatic flavin monooxygenases.[144,145]

When present in high serum levels, TMAO has been linked with deleterious effects, including endothelial dysfunction (Fig. 1), which in turn promotes vascular inflammation, atherosclerosis and other CVD risk factors.[144,146,147] Many clinical studies (conducted in both animals and humans) and meta-analysis[148] have indicated that there is a critical interaction between the gut microbiota and inflammation which could modify the therapeutic intervention for the treatment of these disorders.

Targeting inflammation as a novel therapeutical approach

Despite recent therapeutic advancements able even to promote coronary atherosclerosis regression by an intensive lipid-lowering strategy,[149] a residual cardiovascular risk remains. The role of inflammation in atherosclerosis and its impact on cardiovascular outcomes have been investigated in several experimental studies.[4,109] Beyond the known anti-inflammatory effects of statins and aspirin that are the cornerstone in the treatment of ischemic heart diseases,[150,151] in the last decade several additional anti-inflammatory approaches have been tested with different results.[4,109,152] Targeting oxLDLs, secretory phospholipases A2, lipoprotein-associated phospholipase A2, P-selectin, and p38 mitogen-activated protein kinase did not result in cardiovascular benefits.[153] Another trial targeting inflammation in atherosclerosis was the CANTOS in which patients with a recent myocardial infarction at least 1 month after the

event and with a high-sensitivity CRP (hsCRP) of at least 2 mg/l were enrolled to received guideline-directed medical therapy and the anti-IL-1β antibody canakinumab.[3] Administration of this anti-inflammatory agent resulted in a 15% reduction in major adverse cardiovascular events (MACEs), including myocardial infarction, stroke or cardiovascular death.[3] In addition, an exploratory analysis reveals that individuals treated with canakinumab had also a reduction in incident cancer and a striking decrease in mortality from lung cancer.[3] Moreover, a decrease in gout attacks, hospitalization or death from heart failure and incident anemia were reported in the treated arm. However, these benefits were overcome by a significant increase in infections, including fatal infections. Two smaller trials, MRC-ILA[154] and VCUART3 (Virginia Commonwealth University Anakinra Remodeling Trial 3),[155] have explored the potential benefits of both IL-1α and IL-1β inhibition by anakirna in ACS patients reporting a decrease in the inflammatory markers. However, the MRC-ILA trial reported an increase in MACEs at 1 year.[154]

Another anti-inflammatory trial has evaluated the use of low-dose weekly methotrexate in high risk cardiovascular patients. This study, the Cardiovascular Inflammation Reduction Trial (CIRT),[156] has been prematurely stopped because of safety concerns and futility. However, this study had a major bias of selection since the enrolled population had lower levels of hsCRP compared with CANTOS. Moreover, in CIRT a significant increase in cutaneous cancer was reported, thus raising another potential limitation for this approach.[156] A more reliable and convincing anti-inflammatory strategy comes from new evidence based on colchicine use.[157] Colchicine is a plant-derived product used for millennia for inflammatory diseases. In the CVDs, colchicine is recommended by current guidelines for management of acute and recurrent pericarditis. Two more trials have addressed the use of low-dose colchicine in the context of acute and chronic coronary syndrome. A first small study enrolled only 532 individuals (LoDoCo) with 3 years of follow-up reported a remarkable reduction of more than two-thirds in the primary end point of MACEs.[158] Later, the larger Colchicine Cardiovascular Outcomes Trial (COLCOT) enrolled nearly 5000 ACS patients within 30 days following the acute event who received low-dose colchicine (0.5 mg/die) or a placebo. After almost 2 years of follow-up, the colchicine treated arm showed a 23% reduction in the primary end point, driven primarily by a reduction in urgent hospitalizations for angina requiring revascularization.[159] More recently, the LoDoCo2 trial, which was of similar size to COLCOT, testing the same low dose, focused on stable patients (more than 6 months after the last coronary event or revascularization). This second large study even met its primary, event-driven end point, which included cardiovascular death, nonfatal ACS or nonfatal stroke with an acceptable tolerability.[160]

In Table 2 are summarized most of the available trials in this field. The discrepancies in the major results between them might have several explanations (study design, target population, index pathways).

However, some of the anti-inflammatory approaches tested are promising with data supporting a possible

Table 2 Clinical trials targeting inflammation in cardiovascular patients

Study	Target/Pathway	Drug	Population	Results	Side effects
ARISE 2008	oxLDL	Succinobocul	Post ACS 6144	No effect	Bleeding or anemia, new-onset AF
SELECT-ACS 2013	P-selectin	Inclacumab	ACS/PCI 544	No effect	No significant difference in adverse events
VCU-ART2 2013	IL-1 receptor	Anakinra	ACS 30	No effect	N/A
LoDoCo 2013	Neutrophil chemotaxis/ NLRP3 inflammasome	Colchicine	SCAD 532	Positive	Diarrhea and gastrointestinal AE
VISTA-16 2014	sPLA2	Varespladib	Post ACS 5145	No effect	Incresing risk of MI
STABILITY 2014	LpPLA2	Darapladib	SCAD 15 828	No effect	Asthma, anaphylaxis, diarrhea, and odor-related events
SOLID-TIMI 52 2014	LpPLA2	Darapladib	Post ACS 13 026	No effect	Odor-related concern
MRC-ILA 2015	IL-1	IL-1 receptor antagonist	ACS 182	No Effect	Increased MACE at 1 year
LATITUDE-TIMI 60 2016	P38 MAP kinase	Losmapimod	ACS 3503	No effect	No significant different in AE
CANTOS 2018	IL-1β	Canakinumab	SCAD 10 061	Positive	Fatal infections
CIRT 2019	IL-6, TNF	Methotrexate	SCAD + diabetes/MS 4786	No effect	Elevations in AST/ALT, reductions in leukocyte counts and hematocrit levels, higher incidence of non-basal-cell skin cancers
COLCOT 2019	Neutrophil chemotaxis/ NLRP3 inflammasome	Colchicine	Post ACS 4500	Positive	Diarrhea and gastrointestinal AE
VCU-ART3 2020	IL-1 receptor	Anakinra	ACS 99	Positive	Injection site reactions
LoDoCo2 2020	Neutrophil chemotaxis/ NLRP3 inflammasome	Colchicine	SCAD 5522	Positive	Diarrhea and gastrointestinal AE

ACS, acute coronary syndrome; AE, adverse events; AF, atrial fibrillation; CIRT, Cardiovascular Inflammation Reduction Trial; IL, interleukin; LpPLA2, lipoprotein-associated phospholipase A2; MAPK, mitogen-activated protein kinase; MI, myocardial infarction; MS, metabolic syndrome; NLRP3, nucleotide-binding oligomerization domain, leucine-rich repeat and pyrin domain containing protein 3; oxLDL, oxidized low-density lipoprotein; PCI, percutaneous coronary intervention; SCAD, stable coronary artery disease; sPLA2, secretory phospholipase A2.

use in clinical practice, such as colchicine. Despite these results, current guidelines still fail to recommend its use in daily practice in patients with ACS as well as chronic coronary syndrome. At the time of this review, more trials reinforcing the use of colchicine in CVDs are on the way. Beyond IL-1β and colchicine, anti-TNF therapy has been also suggested. However, a larger-scale randomized trial reported no benefit and safety concerns at higher doses.[109]

More recently, the COVID-19 pandemic has opened a new scenario about the possible role of IL-6 antagonism. Studies on the short-term administration of an IL-6 receptor (IL-6R) antagonist during acute myocardial infarction seems to encourage its use in cardiovascular patients. However, tocilizumab, a readily available drug that targets the IL-6R, consistently causes an increase in triglycerides. Larger clinical trials are needed to better address this issue.[161]

Moreover, targeting other cytokines or T-cell subsets may also be of potential interest. As stated in the 'Lipid deposition and lesion formation: how inflammation defines disease progression' section of this review, IL-17 and IL-23 may be promising targets to pursue for the coming years. Moreover, based on their regulatory mechanism, Treg modulation might be also of great importance. In this regard, an innovative clinical trial using low-dose IL-2 to skew the T-cell balance towards Treg cells is on the way.[162]

Conclusion

Atherosclerosis is a chronic immuno-inflammatory disease since the early stage. Modulation of both aspects seems to be promising to better manage its acute complications. However, in the era of evidence-based therapy, current guidelines should be better guide the clinician in this complex scenario.

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