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Innate and adaptive immunity in cardiovascular calcification

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Abstract

Despite the focus placed on cardiovascular research, the prevalence of vascular and valvular calcification is increasing and remains a leading contributor of cardiovascular morbidity and mortality. Accumulating studies provide evidence that cardiovascular calcification is an inflammatory disease in which innate immune signaling becomes sustained and/or excessive, shaping a deleterious adaptive response. The triggering immune factors and subsequent inflammatory events surrounding cardiovascular calcification remain poorly understood, despite sustained significant research interest and support in the field. Most studies on cardiovascular calcification focus on innate cells, particularly macrophages' ability to release calcification-prone extracellular vesicles and apoptotic bodies. Even though substantial evidence demonstrates that macrophages are key components in triggering cardiovascular calcification, the crosstalk between innate and adaptive immune cell components has not been adequately addressed. The only therapeutic options currently used are invasive procedures by surgery or transcatheter intervention. However, no approved drug has shown prophylactic or therapeutic effectiveness. Conventional diagnostic imaging is currently the best method for detecting, measuring, and assisting in the treatment of calcification. However, they are unable to detect microscopic changes in the early stage of disease; therefore, the vast majority of patients are diagnosed when macrocalcifications are already established. In this review, we unravel the current knowledge of how innate and adaptive immunity regulate cardiovascular calcification; and put forward differences and similarities between vascular and valvular disease. Additionally, we highlight potential immunomodulatory drugs with the potential to target calcification and propose avenues in need of further translational inquiry.

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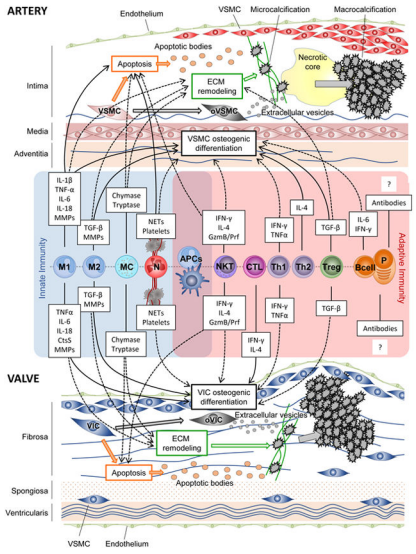
Conflict of interest

The authors declared they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

Graphical Abstract



INTRODUCTION

Inflammation drives calcification

Cardiovascular disease (CVD) has accounted for the greatest number of deaths in the United States over the past century [1]. Among CVD, atherosclerosis and calcific aortic valve disease (CAVD) remain the primary contributors to mortality worldwide. Atherosclerosis and aortic valve stenosis share epidemiological risk factors and may share similarities within their mechanisms.

Atherosclerosis is a chronic inflammatory disease of the arteries, which leads to mineralization in the intimal layer of the lesion. Atherosclerotic calcification is concomitant with the development of advanced plaque and a hallmark of disease progression [2]. In aortic valves, calcification leads to increased leaflet stiffness, resulting in eventual leaflet impairment. Coupled with reduced blood flow, this can cause heart failure and death [3]. Calcification has been shown to be an accurate predictor in the identification of future cardiovascular events and a key contributor in atherosclerosis and CAVD [4]. Currently, there is no therapy to prevent or treat calcification in CVD, beckoning the necessity for significant advancements in the field. Vascular calcification is a multifaceted disorder, as it can be initiated during many conditions, either through inflammation and/or hyperphosphatemia-associated pathways [5]. Extracellular vesicles (EVs), originating from vascular smooth muscle cells (VSMCs) and macrophages, share functional similarities to osteoblast-derived bone matrix vesicles and play an integral role in the genesis of cardiovascular calcification [6, 7]. Calcifying EVs derived from osteogenic macrophages, VSMCs or VICs, are enriched with pro-calcifying factors, including annexins and tissue non-specific alkaline phosphatase (TNAP). These factors facilitate ion entrance into the vesicle, providing all components needed for mineralization. The culmination of ions within the EV begins the mineralization process, while membrane enzymes and proteins drive the

localization [8]. Different degrees of calcium deposition are associated with progression and severity of CVD [9]. In the early stage of disease, microscopic changes are undetectable by conventional diagnostic imaging modalities. Therefore, most patients are diagnosed at advanced stage of disease, when macrocalcifications are already established.

Using molecular imaging techniques, Aikawa *et al.* showed an association between macrophage burden and osteogenesis in experimental early-stage atherosclerosis [10, 11]. Subsequently, an experimental model of CAVD, employing noninvasive near-infrared optical fluorescence molecular imaging, was developed. It detected inflammatory and osteogenic activity in early stage of disease, reinforcing the inflammation-dependent mechanisms of calcification [11, 12]. Additionally, this study identified the stages of disease progression. During initiation, activated macrophages release pro-osteogenic factors, promoting differentiation of valvular interstitial cells (VICs) into osteoblast-like cells. The propagation stage is characterized by excessive production of EVs that contribute to the formation of microcalcification [12, 13]. In the late stage, inflammatory activity is reduced and the morphological leaflet changes with progressive macrocalcification [11, 12]. Similar progressive stages occur during atherosclerotic lesion formation. These findings provide new insights into the biology of inflammation-triggered osteoblastic activity, especially during preclinical microcalcifications, and extends the paradigm that cardiovascular calcification is an inflammatory disease. Although knowledge surrounding CVD has been increasing, the triggering immune factors and subsequent inflammatory events remain poorly understood. This review covers insights into the inflammatory mechanisms in valvular and vascular calcification through an integrated combination of innate and adaptive immune response studies.

INNATE IMMUNE RESPONSE

The innate immune system is the first line of defense and is activated by pathogen- or host damage-associated molecular patterns (PAMPs or DAMPs). These patterns can be recognized by membrane receptors, Toll-like receptors (TLRs). These receptors are implicated in CVD because improper TLR activation either infiltrates immune cells or their presence in resident cells can lead to amplification of inflammation promoting chronic deleterious effects [14–16]. TLR activation initiates a cascade that results in the translocation of NF-κB into the nucleus, where it induces proinflammatory gene expression [17]. Innate components act through cell-dependent mechanisms or secreted factors, shaping the ensuing adaptive immune response [18].

In healthy heart valves, aortic valve interstitial cells (VICs), aortic valve endothelial cells (VECs), and tissue-resident innate immune cells are present [19]. Macrophages and dendritic cells exist in the heart at birth, and can increase 10–15% during postnatal valve development [19]. Innate and adaptive components reside in normal, non-inflamed mice aorta, indicating that constitutive trafficking regulates leukocytes within the vessel wall [20].

Most studies on cardiovascular calcification are focused on innate cells, their ability to release calcification-prone EV’s, and remodeling of the extracellular matrix (ECM) [6, 8]. Since most histopathological findings in human CVD are derived from explanted end-stage

disease tissue, it is difficult to extrapolate the role of innate resident immune cells in calcification.

Macrophages

Calcified aortic valve—Macrophages have been utilized to determine the degree of calcification because of their significant presence in calcified leaflets, making determination of their role in CAVD particularly important [21, 22]. Inflammatory cells enter into valvular tissue through neovessels or through trans-endothelial migration mediated by adhesion molecules, such as ICAM and VCAM [23, 24]. Proinflammatory macrophages (M1) produce pleiotropic cytokines, such as TNF-α and IL-6, and are the predominant macrophage subset present in CAVD [25, 26]. TNF-α and IL-6 have been associated with increased expression of osteogenic morphogenetic protein 2 (BMP2) [22, 27–29]. In addition, neutralization of TNF-α and IL-6 reduced osteogenic differentiation of VICs *in vitro* [25]. Furthermore, TNF-α strongly activates the canonical NF-κB pathway, leading to expression of several genes involved in the inflammatory response, while also influencing VIC mineralization [27, 28].

During macrophage polarization into a proinflammatory phenotype, arginine acts as a substrate for nitric oxide synthase (NOS); thereby, contributing to nitric oxide (NO) generation [30]. Increased oxidative stress leads to upregulation of Runx2 and receptor activator of nuclear factor κB ligand (RANKL), which mediates the crosstalk between calcifying VSMCs and the migration and differentiation of macrophages into osteoclast-like cells [31]. Additionally, high levels of superoxide and hydrogen peroxide are associated with CAVD [32]. Lipid retention is a common histological finding in the ECM in CAVD and may act as a potent agonist of the TLRs [33].

Oxidized-low density lipoproteins (ox-LDL) have been shown to stimulate TLR-2/4 and promote VIC mineralization [34]. Activated VICs negatively modulate the osteogenic response through TLR-2/4 activation by oxLDL. This can lead to down-regulation of IL-37, an anti-inflammatory cytokine, which suppresses NF-κB function and negatively modulates the osteogenic response [35–37]. Furthermore, biglycan expression is increased in CAVD and can participate in the retention of lipids within the valve tissue and promote osteogenic transition through TLR-2 in VICs [38].

Macrophage-derived proteinases, such as cathepsins or metalloproteinases (MMP-2 and MMP-9), lead to degradation of collagen and elastin in the valve matrix; thereby, disrupting valve architecture and promoting osteogenic differentiation of myofibroblasts [39]. Expression of MMPs is influenced by IL-1β expression in cells from CAVD, [40]. *In vivo* studies showed that inflammatory macrophages promote VEC and VIC calcification in a cathepsin S-dependent manner, especially during CAVD and aortic valve stenosis [21, 41]. *In vivo* proteolytic activity, measured via magneto fluorescent nanoparticle imaging probes, demonstrated that macrophages specifically target inflammatory processes, highlighting their importance during osteogenesis [21].

High-resolution microscopy revealed that macrophages release calcifying EVs that contribute to cardiovascular calcification in hyperphosphatemic milieu [6, 42]. Furthermore,

pro-inflammatory macrophage-derived EVs can deliver miR-214 to VICs, promoting calcification [25]. This data shows an additional mechanism of EVs in immune osteogenesis, as miR-214 is associated with an inflammatory response.

Atherosclerotic plaque—In atherosclerosis, macrophages play a central role by engulfing oxLDL, leading to foam cell formation and inducing the upregulation of TLR-4. This sequence promotes activation of the innate immune response and inflammatory response [43]. TLR signaling leads to release of proinflammatory cytokines, promoting cell adhesion and enhancing the release of MMPs by macrophages, which contribute to tissue injury. TLR-4 is upregulated and concentrated in the area most sensitive to undergoing plaque rupture [44]. Since infectious agents, such as *Chlamydia pneumoniae*, have been detected in atherosclerotic lesions, it is likely that there is a link between the development of atherosclerosis and the activation of proinflammatory TLR signaling [45, 46].

Macrophages secrete cytokines, proteases and other factors, driving the fate of plaque towards either an unstable or stable phenotype. Unstable plaque associates with a proinflammatory mechanism of promoting cell death and thinning of the fibrous cap. In contrast, stable plaque is linked to resolving macrophages [47]. Since calcification occurs concomitantly with inflammation, it remains difficult to unravel the specific effects of different macrophage subtypes. While it was suggested that unstable plaque, with a high content of proinflammatory macrophages, contains numerous macrocalcifications, several reports show a large prevalence of macrocalcification in stable plaque, which tend to be more fibrotic and rich in anti-inflammatory macrophages [48].

Pro-inflammatory macrophages release numerous cytokines in atherosclerotic plaque, including TNF-α, IL-1β and IL-6, which can be implicated in arterial calcification. TNF-α induces VSMC differentiation into an osteochondrogenic phenotype, releasing calcifying EVs. *In vitro*, TNF-α promotes osteochondrogenic differentiation of VSMCs through the Msx2/Wnt and cAMP/PKA pathways [49, 50]. Similarly, TNF-α upregulates endothelial cell production of BMPs [51]. Interestingly, treatment with TNFα-neutralizing antibody in $Ldlr^{-/-}$ mice under high fat diet reduces early calcium deposition in the aorta [50]. These observations highlight the importance of TNF-α in plaque calcification.

IL-1β pathway inhibition decreases the development of arterial calcification in $ApoE^{-/-}$ and $Ldlr^{-/-}$ mice atherosclerosis models [52, 53]. Nevertheless, blocking IL-1β does not reduce calcification content in advanced plaque of $ApoE^{-/-}$ mice [54]. Concurrently, Cereni *et al* showed that high serum levels of IL-1β are associated with elevated coronary calcium score, an independent predictor of cardiovascular death [53]. However, even with these insights, further studies are still required to unravel the direct effect of IL-1β on cardiovascular calcification.

IL-6, which can be secreted by macrophages, fibroblasts, VSMCs, T cells and B cells, has emerged as an important factor in bone homeostasis promoting osteoclast activity, in differentiation of T cell subsets (promoting T helper-17 and T follicular helper) and B cells (promoting plasma cell maturation) [55]. IL-6 demonstrated the ability to promote osteogenic differentiation and mineralization of VSMCs *in vitro* [56, 57]. Furthermore, IL-6

promotes VSMC differentiation through various pathways, including RANKL production, which serves an autocrine/paracrine function, BMP2, and STAT3/Runx2 [56–58]. In addition, in patients with rheumatoid arthritis or chronic kidney disease, coronary artery calcification (CAC) score is positively correlated with plasma levels of IL-6 [59, 60].

IL-18, which could be secreted by macrophages, showed a pro-calcifying function in the cardiovascular system. IL-18 promotes VSMC and VIC osteoblastic transition [61, 62]. Likewise, human serum analysis showed a positive correlation between IL-18 and CAC score [61].

MMPs play a critical role in the remodeling of atherosclerotic plaque. Previous studies observed co-localization between MMP-3, MMP-9, MMP-10 and vascular calcification in human and murine models [63–65]. The direct pro-calcifying role of MMPs is not clear, even if some data supports this hypothesis. For instance, MMP-10 deletion inhibits *in vitro* VSMC calcification and reduces mineralization of atherosclerotic plaque in conjunction with reduction of the plaque size [63].

Unexpectedly, anti-inflammatory macrophages, implicated in resolution of inflammation leading to plaque stabilization through promotion of fibrosis, can release pro-calcifying factors. More precisely, TGF-β was described as a direct promoter of VSMC-mediated calcification [66, 67]. However, the pro-fibrotic function of TGF-β can also support calcification, since the ECM supports EVs and crystal deposition [68].

Inflammatory milieu may direct disease outcome by driving macrophage polarization towards a mineral resorptive state (classically activated phenotype) or a mineral deposition state (alternatively activated phenotype) [69]. However, even though substantial evidence demonstrates that macrophages are key cellular components in cardiovascular calcification, the interaction between macrophages and other leukocyte populations needs further investigation. Identifying this connection will determine if it directs a protective or pathogenic immune response.

Dendritic cells

Dendritic cells (DCs) initiate an antigen-specific response through their antigen-presenting function to cells of the adaptive immune system. Found in areas of turbulent flow both in atherosclerotic vessels and calcified valves, few studies have addressed the role of DCs in cardiovascular calcification.

In atherosclerotic arteries, DCs are found in the intima layer and mainly localize in the shoulder of fragile plaque, and co-localize with clusters of T cells, expressing activation marker CD83 [70]. Remarkably, the highest number of DCs are found in vulnerable plaque, suggesting DC function is associated with plaque destabilization, which might occur through activation of T cells [70]. Studies from experimental atherosclerosis mice models did not provide evidence of direct involvement of DCs in calcification, but showed a strong implication of the T cell response through antigen-presenting cell (APC) activity, suggesting a possible indirect stimulation of arterial calcification [71].

In heart valves, DCs are situated below the aortic-side endothelium, a site exposed to turbulent flow [72]. The accumulation of reactive oxygen species in DCs under hypertensive conditions promote the formation of neo-antigens, which are presented to T $CD8^+$ cells, leading to IFN-γ and IL-17A secretion [73]. The hypertension stimuli can play a significant role in CAVD progression through APCs, including DCs, which is similar to atherosclerosis. However, the relationship between DCs and their role in cardiovascular calcification remains poorly understood.

Mast cells

Mast cells (MCs) are involved in first line defense against pathogens and are a good source of mediators, such as proteases and cytokines [74]. The main effector mechanism is mediated through the MC specific proteases, tryptase and chymase, released by degranulation [74].

Evidence exists that MC protease production associates with aortic stenosis (AS) and an increased number of MCs is linked with disease severity in patients with AS [75]. Additionally, activated MCs produce cathepsin G, which causes elastin degradation [21, 41]. Tryptase is another protease released by MCs that has been shown to degrade endostatin, an antiangiogenic molecule, in cells from AS patients [76]. Furthermore, these cells can produce vascular endothelial growth factor (VEGF), demonstrating an additional contribution to neovascularization [76]. Finally, chymase secretion by MCs may contribute to the conversion of angiotensin I to angiotensin II, which has been associated with the promotion of valvular thickening in mice [77]. Due to the significant role of MCs during valve tissue remodeling, they may also play an important role in calcification.

Throughout atherosclerosis progression, MCs are present in the intima and adventitia. MC tryptase staining is observed within atherosclerotic sites around small calcified deposits and microcalcification, but rarely around large calcification, suggesting a role in the initiation stage of calcification [78]. Even if no direct link has been established between MCs effector mechanisms and calcification, it is recognized that chymase induces VSMC apoptosis, which could be related to vascular calcification [79]. Tryptase and chymase are also implicated in ECM change within atherosclerotic plaque via activation of MMPs, which is linked to vascular calcification [80].

Even though no evidence exists as to the role of MCs in calcification processes, their role in ECM remodeling is relevant; therefore, they may contribute to cardiovascular calcification.

Neutrophils

Neutrophils are the most abundant leukocyte in circulation and are part of the innate immune response. Effector mechanisms involve release of granules, phagocytosis and formation of neutrophil extracellular traps (NETs). In the cardiovascular system, increased hemodynamic forces may activate neutrophils and stimulate the release of NETs [81]. These chromatin-based structures trigger blood coagulation by recruiting platelets, leading to thrombus formation [82].

Activated platelets might also directly promote valvular leaflet calcification during high shear stress by inducing osteogenic differentiation in VICs [83]. Furthermore, platelets express and release osteocalcin, reinforcing their involvement in mineralization. Moreover, neutrophilic proteins, such as myeloperoxidase (MPO) and neutrophil elastase (NE), stimulate macrophages to produce cytokines, including IL-1β and ROS, demonstrating another parallel to what occurs in atherosclerosis [84–86]. Additionally, Kopytek *et al.* evaluated the presence of NETs in stenotic valves and found a correlation with disease severity, suggesting the contribution of neutrophils in the pathogenic process [87]. Furthermore, neutrophil-to-lymphocyte ratio has been approached as a predictor of prognosis in CVD [88].

NETs are observed in atherosclerosis, where they can be induced by activation of TLR, oxLDL, or cholesterol crystals [89]. Components of NETs, such as cathepsin G and cathelicidins, exhibit monocyte-attracting activity in atherosclerotic plaques [90, 91] Neutrophils and NETs are shown to promote endothelial apoptosis and detachment in plaque erosion. Likewise, a recent report showed a direct pro-apoptotic effect on VSMCs through histone H4, which could be involved in calcification formation through the release of apoptotic bodies [89]. NETs strongly induce platelet adhesion, activation, and aggregation. Because of these functions, neutrophils may promote vascular calcification through activation of platelets, which then release pro-calcifying mediators, such as osteocalcin and TGF-β [92, 93]. In addition to these direct effects on calcification, neutrophils also drive lesion development, which can indirectly affect calcification [89].

Natural killer cells

Natural killer (NK) cells are potent immune cells acting through cytotoxic activity and can also display an immune-regulatory function. Even if they are present in plaque, the role of NK cells on atherosclerosis development is limited, as there are no studies addressing their role in CAVD [94, 95]. Once activated by stress signals, NK cells release cytotoxic molecules, granzymes and perforins, or act via death receptor ligands, such as FasL and TNF-related apoptosis-inducing ligand, leading to target cell apoptosis [96]. It is plausible that NK cells may contribute to cardiovascular calcification pathogenesis by promoting apoptosis, since cell death is related to CAC and CAVD at sites of microcalcification [97, 98]. On the other hand, an indirect function of NK cells could be hypothesized to work through the release of cytokines, such as IFN-γ, TNF or IL-10, potentially modulating the inflammatory state of atherosclerotic plaque within the aortic valve [96]. However, further studies are required to provide evidence of these hypotheses *in vitro* and *in vivo*.

ADAPTIVE IMMUNE RESPONSE

The processes by which the adaptive immune response acts directly on calcification processes are much less described when compared to innate immune response processes. However, it is very likely that the polarization of immune response by lymphocytes orchestrates the development of protective or pathogenic responses, interfering with disease onset.

Adaptive immunity recognizes non-self-antigens with a high specificity. Innate immunity-derived cells, termed APCs, determine the single recognition epitope. The specificity of recognition depends on the rearrangement of antigen receptors, such as T cell receptors (TCRs), B cell receptors (BCRs) and immunoglobulins, followed by clonal expansion of the specially activated cells [99]. In this context, macrophage polarization in the initial stages of disease influences the effector function of adaptive cellular components and may constitute a point of therapeutic interference. However, the direct mechanisms between adaptive response and calcium deposition are not yet fully understood.

Because of this cell education step, the adaptive response is slow and delayed compared to the innate immune response. Since CVDs are typically chronic diseases, adaptive components should play an important role in the disease progression over time, but are ineffective when mounting an immediate response.

Conversely to macrophages and DCs, there is no resident T cell or B cell in healthy arteries or valves. However, the presence of macrophages and DCs, both APCs, illustrate the immune surveillance leading to the adaptive immune response. In early to late stage atherosclerosis and in calcified aortic valves, studies show local infiltration of T cells and B cells [99–101].

T cells

T cells are the main regulators of the adaptive immune system, driven by specific antigen recognition. Depending on their function, T cells can be subdivided into T helpers (Th), as they help orchestrate activity of other cells; cytotoxic T cells (CTL), inducing death of damaged or infected cells; and T regulatory (Treg) cells, which serve a modulatory role in suppressing T cell activity and maintaining self-tolerance. Numerous studies have established the presence of T lymphocytes in calcified arteries and valves. In addition, infiltration of T cells in CAVD is correlated with an increased pressure gradient, an echocardiographic severity parameter of AS [102].

Prominent lymphocytic infiltrates have been observed in CAVD and are distributed in the vicinity of calcium deposits, which are close to the endothelial lining and regions of neoangiogenesis [103]. In a study conducted by Guauque-Olarte *et al.*, the RNA expression profile of calcified valves was evaluated by RNA sequencing. Enriched pathways analysis using highly expressed genes in CAVD, compared to normal aortic valves, showed several pathways involving T cell signaling, reinforcing the participation of the innate immune response in the pathogenesis of CAVD [104].

Cytotoxic T cells (CTL)—Comparison of T cell receptor-beta (TCR-β) with CAVD-infiltrated and circulating T cells demonstrated that infiltrating T CD4+ and CTL (CD8+) repertoire involve clonal expansion, signifying an antigen-specific immunological response in CAVD [105]. Accordingly, CTL consist of a clonal expanded population and play a memory function in blood and valve tissue [106]. Concurrently, higher CD4+ memory T cell levels were associated with higher CAC score [107].

Similarly, it was shown in human CAVD that cytotoxic T cell-derived IFN-γ impairs calcium resorption by having a direct effect on osteoclasts, supporting the notion that CTL promotes valvular calcification [24].

T helpers (Th1 and Th2)—T helper lymphocytes are responsible for modulating local inflammation. In atherosclerosis, Th1 cells are most represented in plaques and secrete cytokines, such as IFN-γ and TNF-α, which drive lesion progression. Th1 has not been directly connected to cardiovascular calcification, but its ability to promote inflammation may support the pro-calcifying process.

Conversely, the Th2 cell cytokine, IL-4, is negatively correlated with ischemic cardiovascular events, suggesting a positive effect on the stabilization of atherosclerosis [108]. Indeed, it is well established that Th2 lymphocytes are involved in tissue repair and remodeling, in part due to the promotion of an anti-inflammatory macrophage phenotype. Of note, IL-4 promotes osteoblast-mediated mineralization and inhibits osteoclast function in bone. Furthermore, IL-4 promotes calcification of VSMCs through a STAT6-dependant mechanism; inducing osteoprotegrin production and elevating osteoblastic transcription factor Runx2/Cbfa1, which drives osteoblast differentiation [109].

T regulatory cells (Treg)—T regulatory cells (Treg) are a specialized subpopulation of T cells involved in immune homeostasis, whose primary function is to reduce the immune response through production of anti-inflammatory cytokines, including TGF-β and IL-10 [110]. Studies in murine models demonstrate that Treg prevent atherosclerosis development and promote plaque stability through secretion of TGF-β and IL-10 [110]. *In vitro*, TGF-β induces VSMC and VIC mineralization [67, 111]. In addition, studies performed on mesenchymal stem cells revealed that TGF-β promotes VSMC differentiation, but reduces the expression of BMP2 under high phosphate conditions [112]. Patients with severe CAVD have higher circulating levels of Treg and the frequency of these cells decreases after surgical valve replacement [113]. Contrastingly, a decreased frequency of circulating Treg and low levels of IL-10 were found in vulnerable plaques of patients with acute coronary syndrome when compared to healthy controls [110]. However, no study has established a relationship between *in situ* Treg and calcification in arteries and aortic valves.

Natural killer T (NKT) cells—Natural killer T (NKT) cells can be activated by glycolipids or phospholipid antigens and secrete a large amount of cytokines, including IFN-γ, IL-4, IL-17, and cytotoxic proteins, such as perforin and granzyme B [114]. In CAVD, the amount of circulating and valve-infiltrated NKT cells was shown to correlate with echocardiographic parameters of AS severity [102]. As described above, lipid deposition is a common finding in CAVD and may be a potential antigen that triggers the NKT cell response. In atherosclerosis, the role of NKT cells remains unclear and could be associated with pro-atherogenic properties [114]. However, even if no correlation has been established with vascular calcification, one could hypothesize that secreted cytokines and cytotoxic proteins, such as IFN-γ and IL-4, modulate arterial mineralization. Finally, knowing that cell death is closely related to cardiovascular calcification, we can suggest that NKT cells promote cardiovascular calcification [98, 115].

B cells

B cells play a key role in adaptive immunity through secretion of antibodies, but can also function as an antigen-presenter and T cell regulator. In this manner, B cells can act systemically and locally through the release of antibodies and soluble factors [100].

Although they are a negligible part of the infiltrated lymphocytes inside the calcified aortic valves and around the atherosclerosis plaques, B cells have emerged as an important player in cardiovascular pathologies [100]. B cells are classically distinguished in 2 subtypes: B1 and B2. While B1 cells secrete germline-encoded IgM antibodies, generated without previous infection or immunization, B2 cells need to be activated by T cells in order to differentiate into IgG secreting plasma cells that target a specific antigen. In atherosclerotic plaques, B1-derived IgM is known to be atheroprotective and B2-derived IgG recognizing oxLDL or ApoB are pro-atherogenic [116]. Nevertheless, a direct link with cardiovascular calcification has not been clearly established.

Clinical studies demonstrated that higher plasma levels of autoimmune-disease-related antibodies correlate with higher coronary calcification levels. In a cohort of patients with rheumatoid arthritis, the level of anti-citrullinated peptide antibodies was correlated to CAC score [117]. In a healthy asymptomatic cohort, titers of anti-hsp65, but not anti-hsp60 antibodies, were higher in subjects with high CAC score (>150). As this was independent of CVD risk, but in correlation with *H pylori* infection, it may suggest a pathogen-triggered mechanism of atherosclerosis-related calcification [118]. Similarly, in patients with systemic lupus erythematosus, a strong correlation was observed between anti-cardiolipin and anti-β2-glycoprotein-I antibodies and the risk of coronary calcification [119].

The proteomic signature of CAVD shares protein-protein interaction networks with other autoimmune diseases associated with B cell disorders, such as systemic lupus erythematosus, dermatomyositis, vasculitis syndrome, and Takayasu arteritis [120]. The rate of local infiltration of B cells observed in CAVD was correlated with the degree of valve calcification and expression of B cell activating factor receptor (BAFFR) implicated in B2 cell maturation [121]. Moreover, the total number of B cells and BAFFR expressing B cells were associated with the number of macrophages, suggesting cooperation of both cell types in CAVD pathogenesis [121].

Therefore, it is possible that autoantibodies against vascular and valvular epitopes are an important part of the pathogenic mechanism of calcification. Thereby, demonstrating how B cell participation is a central component of the adaptive immune response in atherosclerosis and aortic valve disease. The identification of epitopes and subsequent antibodies directly related to calcification, whether locally within plaque and valves or systemically, remains to be explored.

In addition to antibody production, B cells can release several different cytokines with diverse functions. For instance, B regulatory cells are able to release IL-10, inducing immunosuppressive Treg. B effectors (Be) can exert pro-inflammatory functions through production of IL-6 and IFN-γ, both connected to pro-calcifying inflammation. Innate-response-activator-B cells release granulocyte-macrophage colony-stimulating factor, which

promotes Th1 differentiation, possibly related to calcification [116]. These functions of B cells may put forward some possible regulatory processes of cardiovascular calcification, which need to be further explored.

POTENTIAL DRUG TARGETS FOR IMMUNE RESPONSES IN CARDIOVASCULAR CALCIFICATION

Despite intensive research to understand the mechanisms of vascular and valvular calcification, no effective treatment exists to reduce, prevent or reverse ectopic tissue mineralization in patients, signifying the demand for further translational research.

Even if statins fail to reduce arterial calcification, a recent investigation on a small patient population showed that the combination of statins with PCSK-9 inhibition produces promising results by reducing the annual progression of coronary artery calcification by half compared to statins alone [122, 123]. Similarly, patients with genetic loss-of-function of PCSK-9 (R46L) showed low risk of CAVD [124]. Furthermore, PCSK-9-deficient mice are protected against calcium accumulation in their aortic valve compared to wild type mice. A similar tendency was observed in isolated VICs, since when placed under calcifying conditions, PCSK-9 knockout VICs develop lower calcification than wild type cells. This suggests a direct modulation of the VICs-dependent calcifying process [125]. Moreover, strong evidence has demonstrated a direct implication of PCSK-9 on inflammation in experimental and human studies. For instance, in mouse sepsis models, PCSK-9 inhibition or knockout reduces serum levels of cytokines implicated in cardiovascular mineralization (e.g., TNFα, IL-1β, IL-6) [126, 127]. Consistently, the clinical human data showed that PCSK-9 loss-of-function genetic variants were associated with a large decrease of pro-inflammatory factors, including TNF-α and IL-6, following experimental sepsis [128]. Whether the benefit of PCSK-9 inhibition on calcification is related to its anti-inflammatory effect remains unexplored.

As mentioned above, IL-1β can strongly promote vascular and valvular calcification, as well as osteochondrogenic differentiation of VSMCs and VICs. Furthermore, IL-1β inhibition reduces the early development stage of arterial calcification in experimental animal models of atherosclerosis, but failed to reduce calcification in advanced plaque [52–54]. In the CANTOS clinical trials using anti-IL-1β treatment (canakinumab), a significant reduction of arthritis prevalence occurred in the treated group compared to placebo, signifying an inhibition of the inflammatory-drive calcification process [129]. Moreover, Ridker *et al* showed that IL-1β blockade prevents cardiovascular events in chronic kidney disease patients without modulation of serum calcium, renal function, or phosphate levels [130]. Since vascular calcification is strongly related to cardiovascular mortality in CKD, it suggests a possible effect of IL-1β inhibition on the progression of calcification. However, no direct evidence has shown the impact of canakinumab treatment on cardiovascular calcification.

Immune checkpoint inhibitors (ICI) are a new class of treatment against cancer that intensify the T cell-mediated immune response by blocking inhibitory receptor (cytotoxic T-lymphocyte-associated protein 4, Programmed cell death-1). In parallel, the importance of T

cells in cardiovascular disease development is well established and questions the possible side effects on atherosclerosis and calcification. Emerging evidence indicates the development of serious immune-related adverse effects, like autoimmune reaction in the cardiovascular system [131, 132]. Mainly myocarditis and pericarditis, but some trials have also reported an increase in myocardial infarction, suggesting that stimulation of the adaptive immune system can destabilize atherosclerotic lesions and trigger acute cardiovascular events [131]. Furthermore, the question of long-term outcomes of ICI is still unanswered, including the effect on calcification in atherosclerotic plaques and valve disease.

Imaging of inflammation helps predict cardiovascular calcification

Currently in clinical use, imaging of cardiovascular calcification can be completed via non-invasive techniques, such as computed tomography (CT), positron emission tomography (PET) or magnetic resonance imaging (MRI), and invasive methods using catheter-based imaging, including intravascular ultrasound or optical coherence tomography.

The current paradigm of vascular calcification associates microcalcification (<50μm) with vulnerable plaque and macrocalcification (>50μm) with stable plaque [133]. Nonetheless, current CT detects larger calcification of >200μm and fails to identify microcalcification. The significant advance of multimodal imaging, merging CT and 18F-sodium fluoride (18F-NaF) PET imaging allows the detection of microcalcification [133]. Therefore, PET 18F-NaF has been recently proposed as a potential predictor of disease progression [134]. Functional imaging of inflammation burden revealed that inflammation is associated with and precedes the formation of calcification in arteries and valves [12]. In humans, superposition of morphological imaging with metabolic activity of the inflammation and calcification, through 18F-fluorodeoxyglucose (FDG) and 18F-NaF PET/CT respectively, improves characterization of disease. Indeed this multimodal/multiparametric approach provides promising tools, measuring both micro- and macro-calcification index with inflammation intensity and morphology, as was done previously in animal models of atherosclerosis and CAVD [10, 12, 135]. This method may bring important diagnosis and prognosis insight for monitoring cardiovascular risk linked to calcification and inflammation.

CONCLUSION

The lack of effective therapeutic or preventive strategies to fight cardiovascular calcification is an indicator of disease complexity. Despite the similarities in arterial and aortic valve calcification, differences between these two disorders could be associated to tissue structure, kinetic and progression of disease, or elicited immune responses.

Atherosclerosis is a chronic inflammatory disease triggered by plasma lipoprotein and is driven by both the innate and adaptive immune system. In this way, low-density-lipoprotein-cholesterol lowering treatment and anti-inflammatory strategy can prevent atherosclerosis progression. In contrast, in CAVD, cellular processes are not clearly understood and there is no therapy preventing or limiting the disease progression. However, the understanding of the

natural history of calcified nodule development remains unclear within both atherosclerosis and CAVD.

As demonstrated in this review, the development of pathogenic or protective clinical pathogenic outcomes might be related to the intricacy of the immune response. The implication of immune factors in calcification progression is mainly founded on an *in vitro* approach. Nevertheless, *in vivo*, the direct involvement of few immune mechanisms was correlated to the evolution of calcification. For instance, inflammatory states in atherosclerosis progression were related to different calcification stages. The development of spotted microcalcification is more prevalent in vulnerable inflamed plaque, as opposed to large macrocalcification, where stable and less inflamed plaque resides [97]. Inflammation related to aortic valve calcification is far less characterized (Figure 2).

This suggests a fundamental difference between development of microcalcification and macrocalcification. We can hypothesize that microcalcification development, or the initiation phase, is driven by the pro-inflammatory cytokines, IL-1β, IL-6, and TNF-α, enzymes, such as, MMPs, and cytotoxic factors. The evolution towards nodular macrocalcification seems more related to anti-inflammatory environment as the cytokine TGF-β (Figure 1). The switch from initiation phase to the end-stage macrocalcification results from a fine regulation of innate and adaptive immune system, doubtless different between arterial atherosclerosis and aortic valve stenosis.

Although knowledge surrounding calcification in cardiovascular disease has been improving, the triggering immune factors and subsequent inflammatory events remain poorly understood. Even though substantial evidence demonstrates that macrophages are key components in triggering cardiovascular calcification, the crosstalk between macrophages and adaptive immune cell components is much less described.

In summary, the innate and adaptive immune system clearly promotes calcification progression in CVD (Figure 1), but the modulation by the adaptive immune system has not been well characterized to date (Figure 2). Uncovering the individual contribution of cellular and soluble immune components, as well their interactions, particularly the components related to the adaptive immune system would improve our overall understanding and put new prophylactic and therapeutic treatments into practice.

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Highlights

- Vascular and valvular calcification are increasing in prevalence worldwide and remain a leading contributor of cardiovascular morbidity and mortality.
- Degrees of calcium deposition are linked to progression and severity of cardiovascular disease and associate with cardiovascular risk.
- The lack of effective therapeutic or preventative strategies in cardiovascular calcification is an indicator of disease complexity.
- Inflammatory mechanisms in valvular and vascular calcification act through an integrated combination of innate and adaptive immune responses. Immune responses in calcific aortic valve disease are largely understudied.
- Immunomodulatory drugs possess the potential to target and treat calcification.

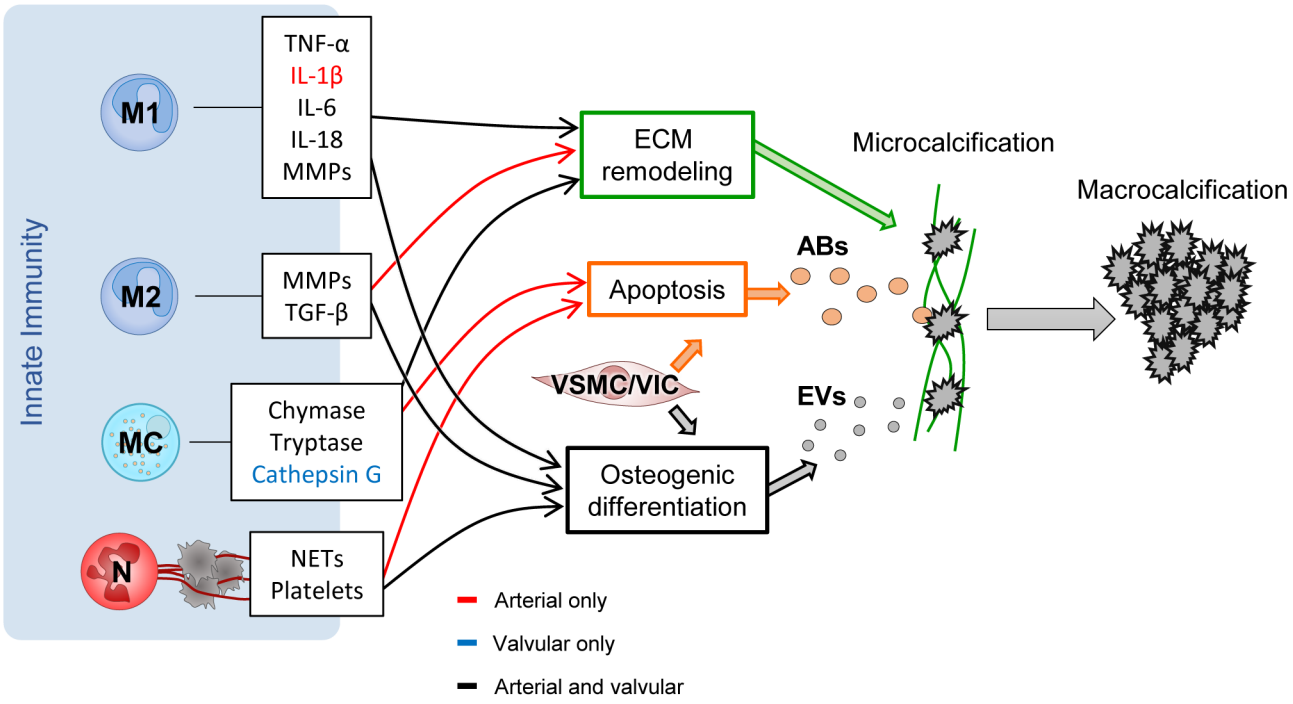

Figure 1: Innate immune system in cardiovascular calcification development.

Innate immune system promotes vascular and valvular calcification through stimulation of extracellular matrix (ECM) remodeling, as well as apoptosis and osteogenic differentiation of arterial vascular smooth muscle cells (VSMCs) and valvular interstitial cells (VICs). Macrophage M1 (pro-inflammatory) and M2 (anti-inflammatory) express soluble factors that might promote calcification by inducing osteogenic differentiation and matrix metalloproteinases (MMPs) able to induce ECM remodeling. Mast cell (MC) derived enzymes degrade ECM and could be associated with induction of VSMC apoptosis. Neutrophils release NETs, leading to VSMC apoptosis, as well as platelet activation and secretion of soluble factors, which in turn may promote calcification by inducing osteogenic differentiation.

ECM, Extracellular Matrix; VSMC, Vascular Smooth Muscle Cell; VIC, Valvular Interstitial Cell; ABs, Apoptotic Bodies; EVs, Extracellular Vesicles; M1, Macrophage 1; M2, Macrophage 2; MC, Mast Cell; N, Neutrophil; IL, interleukin; TNF-α, Tumor Necrosis Factor alpha; MMPs, Matrix Metalloproteinases; TGF-β, Transforming Growth Factor beta; NETs, Neutrophil Extracellular Traps; IFN-γ, Interferon gamma;

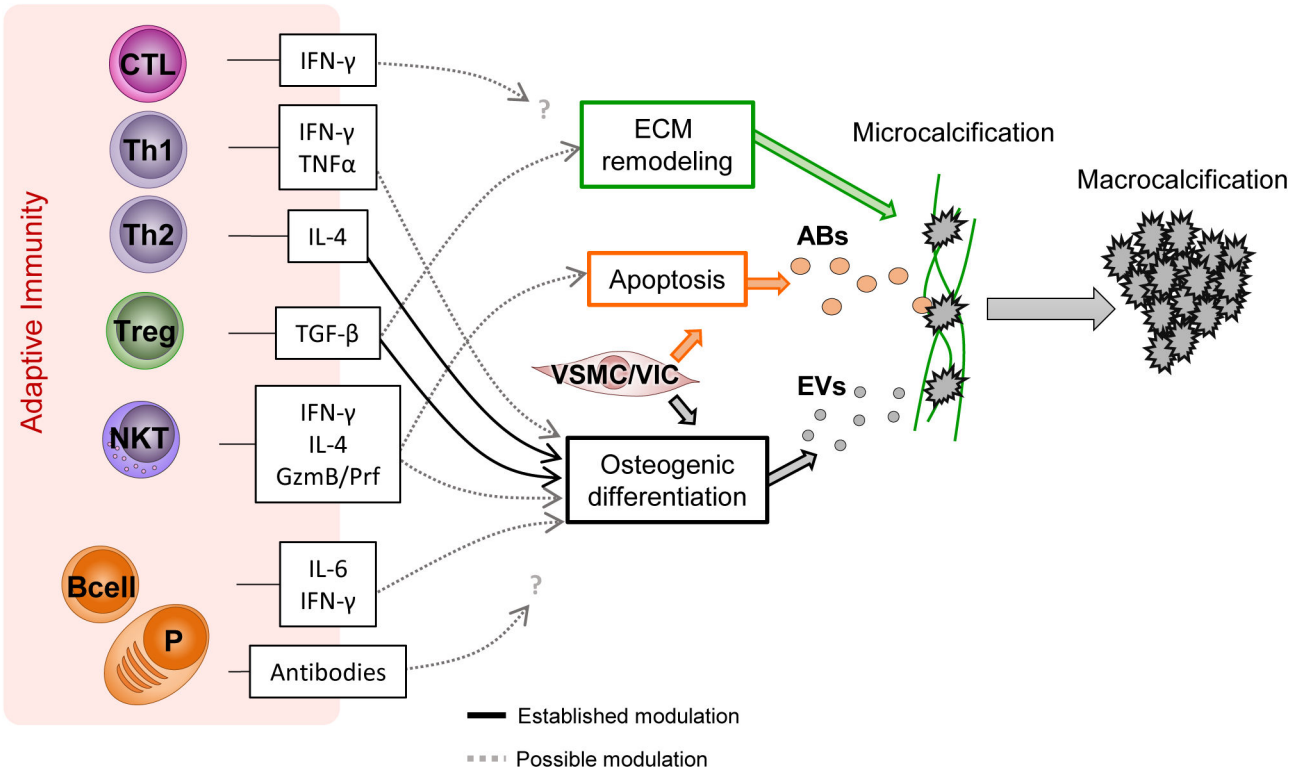

Figure 2: Adaptive immune system in cardiovascular calcification development.

Implication of the adaptive immune system in vascular and valvular calcification is not well established. However, Interleukin-4 (IL4) secreted by T-helpers 2 (Th2) promotes osteogenic differentiation of vascular smooth muscle cells (VSMCs). T regulatory cells (Treg) release Transforming Growth Factor-β (TGF-β) capable to induce VSMCs and valvular interstitial cells (VICs) mineralization. Other cytokines described here, produced by T cell subtypes, such as cytotoxic T cell (CTL), Th1, Natural killer T cells (NKT), as well as B cells, have been correlated with calcification without direct evidence. Similar indirect evidence, based on correlation, has been observed between antibodies and calcification, suggesting influence by B cells.

ECM, Extracellular Matrix; VSMC, Vascular Smooth Muscle Cell; VIC, Valvular Interstitial Cell; ABs, Apoptotic Bodies; EVs, Extracellular Vesicles; NKT, Natural Killer T cell; CTL, Cytotoxic T lymphocyte; Th, T helper; Treg, T regulatory cell; P, Plasmocyte; IL, interleukin; TGF-β, Transforming Growth Factor beta; IFN-γ, Interferon gamma; GzmB, Granzyme B; Prf, Perforin.