Book Chapter

The Dualistic Role of Macrophages in Aortic Valve Calcification

Nervana Issa[1], Alexandre Candellier[1,2], Cédric Boudot[1], Saïd Kamel[1,3] and Lucie Hénaut[1]*

[1]UR UPJV 7517 MP3CV, CURS, France
[2]Department of Nephrology, Amiens University Hospital, France
[3]Department of Biochemistry, Amiens University Hospital, France

***Corresponding Author:** Lucie Hénaut, UR UPJV 7517, MP3CV, CURS, Université de Picardie Jules Verne, Avenue René Laennec, 80054, Amiens, France, Tel: +33322825425; Email: lucie.henaut@u-picardie.fr

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Abstract

Calcific aortic valve stenosis (CAS) is the most common valvular heart disease worldwide, associated with cardiovascular morbidity and mortality. This pathology results from fibro-calcific degeneration of the aortic valve leaflets, causing major cardiovascular complications. To date, drug therapies have been ineffective in preventing the progression of CAS and aortic valve replacement remains the mainstay of management for patients with symptomatic CAS. Unfortunately, not all patients are eligible for this procedure, which is associated with a greater risk of mortality for subjects presenting comorbidities. A better understanding of the mechanisms responsible for CAS pathogenesis is therefore of crucial importance to develop new therapeutic strategies. Inflammation is a key driver of aortic valve fibrosis and calcification. Macrophages, which play critical roles in the induction and resolution of sterile inflammation, may therefore represent interesting therapeutic targets. Once infiltrated within the aortic leaflet, these dynamic cells can adopt a pro-inflammatory M1 phenotype or switch toward an alternatively activated M2 phenotype, resulting in wound healing and anti-inflammatory activities. This plasticity complicates the efforts to understand their role in the initiation and progression of CAS. This book chapter aims to summarize our current knowledge regarding the role played by macrophage subsets on aortic valve remodelling.

Keywords

Macrophages, Valvular Interstitial Cell, Inflammation, Calcific Aortic Valve Stenosis

Introduction

Calcific aortic valve stenosis (CAS) is the most prevalent valvular heart disease worldwide [1]. This pathology is characterized by slowly progressive fibro-calcific remodelling of the valve leaflets. Over the years, the disease evolves to severe valve calcification with impaired leaflet motion and vast blood flow obstruction, which leads to ventricular hypertrophy. Untreated, symptomatic CAS is associated with a dismal prognosis. Aortic valve replacement is the only treatment shown to improve survival for selected and eligible patients. Before symptoms occur, aortic stenosis is preceded by a silent, latent phase characterized by a slow progression at the molecular, cellular, and tissue levels. A better understanding of the pathophysiology of this latent phase of CAS is needed to develop new therapeutic strategies that would slow disease progression.

A growing body of evidence indicates that a close association exists between inflammation and CAS and that immune signalling, in particular that linked to infiltrated macrophages, may be a viable target for therapeutic intervention. Macrophages are present in healthy valves as a central component of the immune surveillance cell system. Once infiltrated, these dynamic cells play critical roles in the induction and resolution of sterile inflammation. Macrophages exhibit a considerable degree of plasticity depending on signals from the extracellular environment. Indeed, they can adopt a pro-inflammatory M1 phenotype in response to TH1 cytokines and switch toward an alternatively activated M2 phenotype when meeting Th2 cytokines, resulting in wound healing and anti-inflammatory activities. Macrophages' polarization is quickly reversible. Indeed, it takes less than 24 hours for macrophages cultured *in vitro* to switch from one phenotype to another in response to appropriate cytokines [2]. Infiltration of macrophages is enhanced in the human calcified aortic valves [3]. In this context, the recent observation that both pro-inflammatory M1 and anti-inflammatory M2 cytokines are upregulated in samples of calcified aortic valves compared with that in non-calcified valves, suggests that both M1 and M2 phenotypes may influence CAS. This book chapter aims to summarize our current

knowledge regarding the role played by macrophage subsets on aortic valve remodelling.

Pathophysiology of CAS

The human aortic valve is composed of three thin and flexible leaflets. Each leaflet is composed of three layers of extracellular matrix, named fibrosa, spongiosa, and ventricularis [4], covered by an outer layer of valvular endothelial cells (VECs) (Figure 1). The entire structure of each leaflet is less than 1 mm thick. The three layers composing the leaflets are principally populated with quiescent valvular interstitial cells (qVICs) [5]. The trilaminar structure of the leaflets determines the biomechanical properties of the aortic valve. Located on the aortic side of the valve, the lamina fibrosa is rich in circumferentially oriented type I and type III collagen fibrils. This composition helps to maintain structural integrity and transfer pressure load to the aortic root. The lamina ventricularis, which is located on the ventricular side of the leaflet, contains radially aligned collagen and elastin fibers. This composition provides more compliance, allowing the valve to expand under pressure [6]. The spongiosa, which has a high proteoglycan content, is located between the fibrosa and ventricularis [7].

During the cardiac cycle, the mechanical stresses applied to the aortic valve can disrupt the endothelial layer, allowing the infiltration of oxidized lipids (ox-LDL) and immune cells [8,9]. Monocytes and lymphocytes are the main cells that adhere and infiltrate the sub-endothelium. Once infiltrated, they differentiate into macrophages and activated T cells able to release growth factors and pro-inflammatory cytokines such as TGF-β, IL-1β, IL-6 and TNF-α. In response to TGFβ, qVICs differentiate into activated VICs (aVICs), displaying a myofibroblastic phenotype, characterized by the expression of α-smooth muscle actin (α-SMA). The concomitant exposure to pro-inflammatory cytokines promotes their proliferation and release of matrix metalloproteinases, inducing fibrosis, thickening and increased valvular stiffness [9-11]. This phenomenon is generally associated with a process of biomineralization during which aVICs differentiate toward osteoblast-like phenotype (obVICs).

During this process, aVICs downregulate their expression of α-SMA and acquire the capacity to express key osteogenic markers such as alkaline phosphatase (ALP), bone morphogenetic protein 2 (BMP2), Runt-related transcription factor 2 (RUNX2, a marker of terminal osteoblastic differentiation) and osteopontin (OPN) [12,13]**.** Through this phenomenon, they acquire the capacity to secrete a bone-like matrix able to calcify. The progressive calcification reduces the elasticity of aortic valve leaflets and over time narrows the aortic valve opening. Symptoms generally occur when the narrowing of the valve is severe.

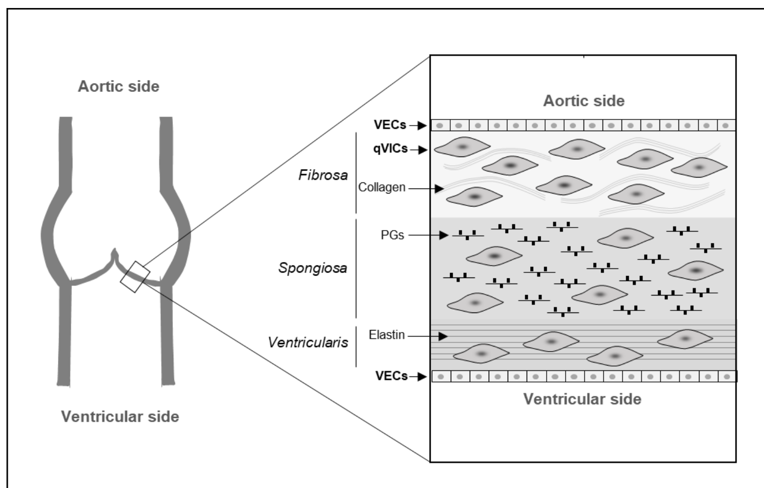

Figure 1: Structure and composition of aortic valve leaflets. PGs: proteoglycans, qVICs: quiescent valvular interstitial cells, VECs: valvular endothelial cells.

Macrophages Plasticity

Once infiltrated within the leaflet, macrophages can acquire distinct functional phenotypes as a reaction to specific micro-environmental stimuli. In response to Th1 cytokines, such as interferon-gamma (IFN-γ), they usually take a classically activated M1 phenotype. Macrophages with an M1 phenotype display cytotoxic and tissue-damaging pro-inflammatory functions after the release of pro-inflammatory mediators such as IL-1β, IL-6, TNFα or reactive oxygen species. Major markers for

the identification of the M1 phenotype are CD11c, CD80, CD86, CD64, CD16, CD32 and nitric oxide synthase (iNOS). In response to Th2 cytokines (e.g, IL-4 and IL-13) they can adopt an alternatively activated M2 phenotype. Macrophages with a M2 phenotype display the ability to secrete anti-inflammatory cytokines such as IL-1 receptor antagonist (IL1ra), IL-10, CCL22 or TGF-β1. Their activity is usually associated with wound healing and anti-inflammatory properties [14]. Major markers for the identification of the M2 phenotype are CD163 and CD206. The next chapter provides a summary of the most recent experimental data evaluating the influence of M1 and M2 macrophages on the calcification of the resident cells of the aortic valve (Figure 2).

M1 Macrophages and Aortic Valve Calcification

Inflammation is a potent driver of aortic valve calcification [15]. Indeed, the exposure *in vitro* to recombinant TNF-α, IL-6, IL-1β or IL-8 has been repeatedly reported to favour the osteogenic differentiation and calcification of primary human VICs (hVICs) [16-20]. These observations have led to a general hypothesis that M1 macrophages may promote VIC-to-osteoblast differentiation and subsequent valve calcification via paracrine pro-inflammatory signalling.

Confirming this hypothesis, in 2017 Li *et al.* reported that conditioned medium (CM) from M1 macrophages (M1-CM) enhances VICs expression of several osteoblastic markers, including BMP2, ALP, and OPN as compared to exposure to CM from unpolarised macrophages (M0-CM) [3]. The fact that neutralizing antibodies against TNF-α or IL-6 blocked hVICs osteogenic differentiation and mineralization, indicated that the procalcific effects of M1-CM are mediated, at least in part, by TNF-α and IL-6. These data were confirmed in 2020 when Grim *et al.* further described that TNF-α and IL-1β in M1-CM indeed deactivate aVIC, as evidenced by a robust reduction of αSMA expression and promote their proliferation, while IL-6 in M1-CM subsequently triggers their differentiation toward obVICs able to express RUNX2 and OPN [21]. These data indicate that

inflammatory M1 macrophages may be responsible for the switch from fibrosis to calcification during aortic valve stenosis progression through their ability to drive VICs myofibroblast-to-osteogenic transition.

Interestingly, M1-polarized macrophages not only communicate with VICs through the secretion of cytokines but also by releasing extracellular vesicles (EVs). In 2022, Xia *et al.* reported that the internalization of EVs produced by M1 macrophages (M1-EVs) increased calcium nodule formation and expression of osteogenesis-related genes in VICs, including RUNX2, BMP2 and OPN, compared with EVs from control macrophage [22]. In this study, the expression of α-SMA and collagen I in VICs was significantly increased in response to M1-EVs, suggesting that M1-EVs promote both the osteogenic and fibrotic processes of VICs. The authors identified that tsRNA-5006c, a novel type of noncoding RNA cleaved from tRNA (tsRNAs), was significantly up-regulated in M1-EVs and that its deletion reduced VICs osteogenic and fibrotic markers as well as nodule formation, indicating that M1-EVs promote VICs mineralization by delivering tsRNA-5006c. In addition, incubation of M1-EVs with tsRNA-5006c inhibitor led to a significant reduction in the expression of markers of autophagy/mitophagy activation, suggesting that the enhanced osteogenic differentiation capacity of M1-EVs tsRNA-5006c may be linked to autophagy/mitophagy. The data are in line with previous studies showing that excessive mitophagy/autophagy exacerbates CAS progression [23].

In 2016, Li et al reported that M1-polarization in CAS correlates with the upregulation of miR-214, a miRNA which expression is essential for M1-directed polarization [24]. Upregulation of miR-214 in aortic valve samples is generally associated with decreased expression of its target gene TWIST-1, a transcription factor that prevents hVICs osteoblastic differentiation by functionally antagonizing RUNX2 [25]. From this observation, Li et al. hypothesized that the release of miR-214 by macrophages may promote CAS development. They confirmed that the co-culture with M1 macrophages or M1-EVs decreased TWIST-1 expression in VICs and favoured their osteogenic

transition as evidenced by the elevation of ALP activity. In line with these data, TWIST1 expression was higher, while ALP activity was lower, in VICs exposed to EVs from miR-214-silenced M1 macrophages compared to those exposed to M1-EVs. These effects were abrogated in VICs silenced for TWIST1. Intravenous injections of a miR-214 inhibitor in hypercholesterolemic apoE-/- mice upregulated valvular TWIST-1 expression and reduced aortic valve calcification. These findings suggest that M1 macrophages' EVs can promote aortic valve calcification by delivering miR-214 to VICs.

It is interesting to note that physical interactions between macrophages and VICs promote the calcification process induced by macrophages' procalcific secretions [26]. Indeed, in a study published in 2020, Raddatz and colleagues showed that the direct co-culture with macrophages promoted VICs osteogenic transition (evidenced by the elevation of RUNX2 expression) as compared to a co-culture in transwell systems (no physical contact). In this study, the physical contact of macrophages with VICs was associated with a marked decrease in VICs expression of STAT3β, an alternative splice product of the STAT3 gene, displaying the ability to bind and inhibit RUNX2 activity [27]. Interestingly, treatment of VICs monoculture with an inhibitor of STAT3 phosphorylation increased RUNX2 transcription, suggesting that STAT3 mediates the connection between macrophage-secreted factors and RUNX2 expression. In line with these data, in calcified regions of diseased aortic valves, the elevation of RUNX2 expression negatively correlated with that of STAT3β. Further investigation of STAT3 and macrophage-driven inflammation as therapeutic targets in CAS is warranted.

M2 macrophages and aortic valve calcification

In 2023, Wu et al. reported that the exposure of qVICs to CM from M2 macrophages (M2-CM) promotes their differentiation toward myofibroblasts (aVICs) as evidenced by the elevation α-SMA expression but has no impact on VICs RUNX2 expression [28]. This observation suggests that although M2 macrophages secretions may promote myofibroblast activation, they may not directly contribute to osteogenesis induction. In line with these

data, areas staining positive for the M2 marker CD163 correlated with α-SMA expression in histological samples of CAS.

M2 macrophages are known to facilitate the resolution of inflammation and tissue reconstruction via secreting IL-10 and TGF-β [29,30]. Aortic valve inflammation and degeneration negatively correlate with plasma levels of IL-10 [31] and single nucleotide polymorphisms (SNPs) of IL-10 associated with CAS. Interestingly, cusps from CAS patients also contain higher levels of TGF-β1 than noncalcified normal cusps [32]. Transforming growth factor-β is a well-known fibrosis stimulative factor involved in tissue-repairing processes and immune homeostasis regulation [33]. *In vitro*, the exposure to recombinant TGF-β promotes qVICs differentiation toward myofibroblastic aVICs [34]**.** The addition of TGF-β1 to primary VICs also promotes VICs mobility, aggregation, formation of nodules enriched in ALP, and subsequent mineralization of these nodules [32]. In this model, blockade of TGF-β1-induced apoptosis with a caspase inhibitor significantly inhibits the calcification but has no effect on nodule formation. By contrast, the exposure to an actin-depolymerizing agent named cytochalasin D inhibits nodule formation but has no impact on calcification. Together these data indicate that strategies aiming at blocking M2-derived TGF-β activity may protect the valve from fibro-calcic remodelling.

In 2016, Villa-Bellosta and colleagues elegantly demonstrated that macrophages polarized *in vitro* toward a M2 phenotype are better able to trigger the synthesis of pyrophosphate (PPi) than M1 macrophages [2]. PPi is a calcification inhibitor that is produced after adenosine triphosphate (ATP) hydrolysis by ectoenzyme nucleotide pyrophosphatase/phosphodiesterase-1 (eNPP1) and is then degraded to Pi by tissue non-specific alkaline phosphatase. In Villa-Bellosta et al.’s study, alternatively activated M2 macrophages increased extracellular PPi levels *in vitro* through increased ATP release and eNPP1 overexpression. If this effect was shown to protect vascular smooth muscle cells cultured in the presence of M2 macrophages from calcification, its impact on VICs calcification remains to be demonstrated.

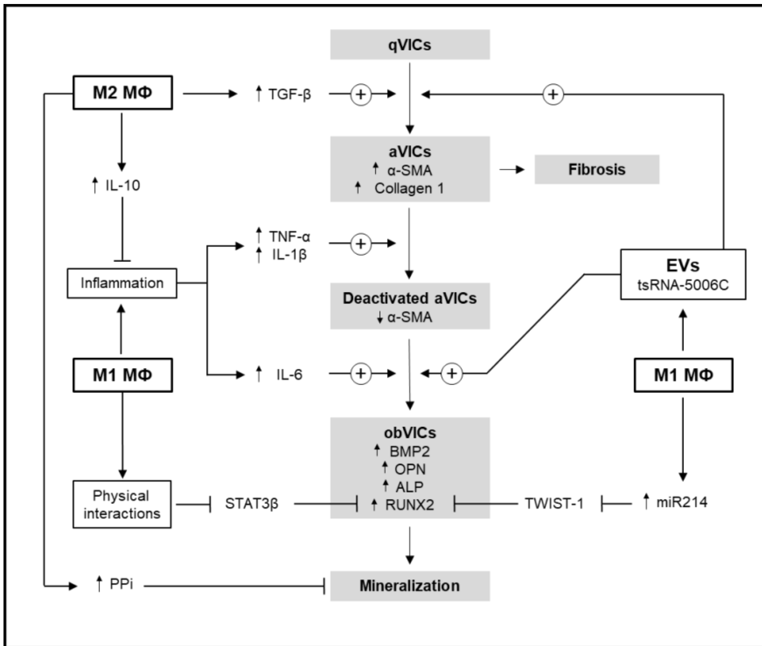

Figure 2: Dualistic role of macrophages in aortic valve calcification: A schematic representation. ALP: alkaline phosphatase, aVICs: activated VICs, α-SMA: α-smooth muscle actin, BMP2: bone morphogenetic protein 2, EVs: extracellular vesicles, IL-1β: interleukin 1β, IL-6: interleukin 6, IL-10: interleukin 10, M1 MΦ: classically-activated M1 macrophages, M2 MΦ: alternatively-activated M2 macrophages, obVICs: osteogenic VICs, OPN: osteopontin, PPi: pyrophosphate, qVICs: quiescent VICs, RUNX2: Runt-related transcription factor 2, TGF-β: transforming growth factor β, TNF-α: tumor necrosis factor α.

Targeting the M1/M2 Balance to prevent CAS

Over the last decade, our understanding of monocytes/macrophages's roles in CAS has improved considerably. In this regard, the therapeutic potential of approaches targeting macrophage recruitment and polarization to prevent the onset or progression of CAS is now being explored. Data from *in vitro* and *ex vivo* experiments have already identified several promising therapeutic targets, such as AFAP1-AS1 [35] and NLR family pyrin domain-containing 3 (NLRP3) [36].

AFAP1-AS1 is a long non-coding RNA (lncRNA) which overexpression in macrophages promotes the M1 but inhibits the M2 polarization [35]. *In vitro***,** exposure to CM from AFAP1-AS1-overexpressing macrophages, which display a M1-like phenotype, promotes VICs osteogenic transition and calcification**.** By contrast, exposure to conditioned medium from AFAP1-AS1-depleted macrophages, which display a M2-like phenotype, inhibits VICs osteogenic differentiation and calcification**.** Future studies should determine whether targeting AFAP1-AS1 is a feasible strategy to reduce CAS.

The pro-inflammatory NLRP3 inflammasome pathway is activated in circulating monocytes of CAS patients [37]. *In ApoE* $^{-/-}$ mice fed a high-fat diet, inhibition of NLRP3 activity thanks to an intraperitoneal injection of 2.5 mg/kg/day of the NLRP3 inhibitor CY-09 prevented the shift of macrophages towards the M1 phenotype, downregulated the levels of the pro-inflammatory factors IL-6 and TNF-α and reduced aortic valve calcification [36]. This study offers a proof-of-concept that pharmacological inhibition of the NLRP3 inflammasome is a feasible strategy for modulating macrophage polarization in order to alleviate CAS.

Conclusion

Over the last decade, it became clear that the diversity of macrophage subtypes complicates our understanding of the pathogenesis of CAS, a disorder for which effective treatments are still lacking. M1 macrophages' secretion of pro-inflammatory cytokines favours the onset and progression of both fibrosis and calcification, while the capacity of M2 macrophages to resolve inflammation may help to prevent CAS. These observations, together with the epidemiological data linking CAS to systemic inflammation, suggest that targeting inflammation may help to slow down CAS progression. However, despite the widespread use of biologicals targeting inflammatory cytokines for a variety of inflammatory conditions, there is yet no information available on these drugs on CAS in humans. In this regard, observational studies of CAS in patients treated with biologicals targeting inflammation for other reasons

may provide valuable information that may help to design an adequately powered concept clinical trial with CAS as a primary endpoint. For instance, it is estimated that over 2 million patients suffering from rheumatoid arthritis are treated every year with biologicals targeting TNF-α, and additional patients receive biologicals targeting IL-1β, and IL-6. The age range of these patients at initiation of therapy may allow studying the impact on CAS.

In recent years, an increasing number of experimental studies evaluated the therapeutic potential of approaches targeting macrophages recruitment and polarization to prevent the onset or progression of CAS. Data from these studies identified AFAP1-AS1 and NLRP3 as potential therapeutic targets able to reduce the pro-calcific M1 polarization. In this context, it is interesting to note that according to the most recent literature M2 macrophages may display dualistic actions on CAS. On the one hand, they may promote the fibrocalcic remodelling of the aortic valve by secreting high level of TGF-β, while on the other hand, their capacity to secrete high levels of IL-10 and PPi may help to prevent CAS. Therefore, to date, it is not clear whether increasing the M2 polarization should be a goal to achieve. Further studies are warranted to evaluate this aspect.

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