Original paper

High mobility group box 1 attenuates aortic stenosis by modulating macrophages to reduce valvular calcification

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Our previous study demonstrated that HMGB1 may suppress M1 macrophage polarisation and mitigate the progression of calcific aortic valve disease (CAVD). However, the role of HMGB1 in regulating macrophage-mediated valvular calcification remains to be further explored.

Serum samples from healthy individuals and CAVD patients with varying severity were collected and analysed by ELISA. Immunofluorescence staining of human heart tissue arrays assessed macrophage infiltration in calcified valves. A macrophage-aortic valve interstitial cell (haVIC) co-culture system was used to examine the effects of reHMGB1-treated macrophages. RUNX2 and osteopontin mRNA expression were measured by RT-qPCR, and alkaline phosphatase (ALP) staining was performed to evaluate calcification.

HMGB1 levels were significantly reduced in severe CAVD patients than controls. Immunofluorescence staining revealed increased CD68 expression in calcified valve samples, indicating macrophage infiltration. In the macrophage-haVIC co-culture system, macrophages pretreated with reHMGB1 led to reduced RUNX2 mRNA expression and lower ALP activity in haVICs, suggesting a potential inhibitory effect of HMGB1 on valvular calcification.

HMGB1 may have the potential to suppress inflammation and mitigate aortic valve calcification, making it a promising therapeutic target for preventing the progression of aortic stenosis.

Key words: HMGB1, valvular interstitial cells, calcific aortic valve disease, aortic stenosis, macrophage.

Introduction

Aortic stenosis (AS) is a degenerative valvular heart disease. It is the most common vascular heart condition among the elderly in developed countries [1]. It carries a potentially fatal risk, and once symptoms appear, the disease often progresses rapidly, with poor prognosis. Aortic stenosis is characterised by the narrowing of the aortic valve due to severe calcification, fibrosis, and lipid deposition, with calcification being the most common cause of AS. This narrowing increases the pressure and workload on the left ventricle [2]. Therefore, investigating the mechanisms of calcific aortic valve disease (CAVD) and developing therapeutic strategies for AS are of urgent importance.

The inflammatory process plays a pivotal role in the development and progression of CAVD [3]. It is driven by diverse factors such as tumour necrosis factor α (TNF-α), interleukin-6 (IL-6), IL-1β, and oxi-

dised low-density lipoprotein (ox-LDL), all of which are elevated and indicated to promote the calcification in aortic valves [2–5]. For example, increased TNF-α levels in AS patients [6, 7] can induce calcification in valvular interstitial cells (VICs) by upregulating alkaline phosphatase (ALP) activity, bone morphogenetic protein 2 (BMP-2) expression, and matrix mineralisation [7, 8]. These inflammatory stimuli promote the osteoblastic differentiation of VICs, contributing to CAVD progression.

Valvular interstitial cells are the predominant cell type in heart valves and are essential for maintaining valvular homeostasis. Under pathological conditions, VICs, stimulated by tumour growth factor β (TGF-β), can differentiate into myofibroblast-like or osteoblast-like cells, leading to aortic valvular fibrosis, calcification, and stenosis [3, 9–11]. This osteogenic differentiation involves the activation of transcription factors such as runt-related transcription factor 2 (RUNX2), nuclear factor of activated T cells 1 (NFATc1), and osterix, resulting in increased expression of osteopontin and BMPs [12, 13].

Inflammatory processes in CAVD involve macrophage infiltration into the aortic valve tissue. These macrophages can polarise into pro-inflammatory M1 or anti-inflammatory M2 phenotypes, each influencing disease outcomes differently. M1 macrophages secrete pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α to promote osteogenic differentiation of VICs. In contrast, M2 macrophages release anti-inflammatory cytokines like IL-10, contributing to tissue repair and potentially mitigating calcification [14–16]. An imbalance favouring M1 over M2 macrophages has been observed in calcified aortic valves [17], suggesting that modulating macrophage polarisation could be a therapeutic strategy in CAVD.

High mobility group box 1 (HMGB1) is a nuclear protein that, when released extracellularly, exhibits dual functions in modulating immune responses. In its pro-inflammatory role, HMGB1 acts as a damage-associated molecular pattern (DAMP) [18, 19], binding to receptors such as Toll-like receptors (TLRs) and the receptor for advanced glycation end products (RAGE), thereby promoting the release of pro-inflammatory cytokines like TNF-α, IL-1β, and IL-6 [20–23]. Conversely, HMGB1 can also exert anti-inflammatory properties [24] by influencing macrophage polarisation [25]. Specifically, it promotes the transition of macrophages from the M1 phenotype to the M2 phenotype, leading to increased production of cytokines such as IL-10 [26–28]. In the context of CAVD, this shift may mitigate disease progression by reducing inflammation and subsequent valvular calcification. Our previous research demonstrated that recombinant HMGB1 increased IL-10 expression and decreased inducible nitric oxide synthase (iNOS) expression in macrophages, indicating a promotion of M2 polarisation and a potential protective role against CAVD progression [29]. However, direct evidence supporting the role of HMGB1 in mitigating calcification remains limited. Therefore, this study aims to evaluate whether recombinant HMGB1-induced M2 macrophage polarisation can influence VICs by assessing the expression of RUNX2 and osteopontin mRNA, as well as ALP staining, to determine its potential impact on CAVD progression.

Material and methods

Cell culture and co-culture between human aortic valve interstitial cells and macrophage

THP-1 monocyte cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained in a concentration of 1×10^5 cells/ml. Cells were cultured in an RPMI-1640 medium (GIBCO, Carlsbad, CA, USA) supplemented with 10% foetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine at 37°C under 5% CO_2. For the differentiation processes, THP-1 monocytic cells at a density of 1×10^5 cells were induced to transform into M0 macrophages in a 6-well culture plate with the addition of 100 nM phorbol 12-myristate 13-acetate (PMA, Sigma, St. Louis, MO, USA). Following a 24-h incubation period, the medium was discarded and replaced with a RPMI/FBS medium devoid of PMA. Human aortic valve interstitial cells (haVIC) were obtained from iCell Bioscience (iCB, CA. USA, iCell-0084a). These cells were cultured under standard conditions in a medium recommended by the supplier (iCB, iCell-0084a-001b) at 37°C under 5% CO_2.

For the co-culture system establishment, THP-1 cells were seeded at a density of 2×10^5 cells per insert. After 4–6 h of attachment, cells were stimulated with 100 nM phorbol 12-myristate 13-acetate (PMA) for 24 h. Subsequently, the inserts were transferred to new 6-well plates containing fresh PMA-free RPMI/FBS medium. Following a 1-h equilibration period, the differentiated THP-1 cells were exposed to 500 ng/ml recombinant HMGB1 (MCE, Nanjing, China; HY-70570) for 24 h.

Concurrently, human aortic valve interstitial cells (haVICs) were seeded in separate 6-well plates at 2×10^5 cells per well. After 24 h, the THP-1 cell inserts were washed thrice with RPMI/FBS medium and transferred onto the haVIC cultures. The co-culture was maintained for 5 days. Following this period, haVIC cells were harvested for RNA extraction and subsequent RT-qPCR analysis or subjected to alkaline phosphatase (ALP) staining.

Table I. Primer sequences using in the real-time PCR

Gene	Primer	Sequences
RUNX2	Forward	ACAGTGACACCATGTCAGCA
	Reverse	TCGGCGATGATCTCCACCAT
Osteopontin	Forward	AGCAGAATCTCCTAGCCCCCA
	Reverse	TGGTCATGGCTTTCGTTGGA

Serum high mobility group box 1 quantification via ELISA

Serum samples from 6 patients with moderate calcific aortic valve disease (CAVD), 6 with severe CAVD, and 7 healthy controls were analysed for HMGB1 and IL-10 levels using a commercial ELISA kit (LSBio, Shanghai, China). The assay was performed following the manufacturer's protocol. In brief, serum samples were centrifuged (1,000 μg, 10 min) and incubated in antibody-coated wells for 60 min at 37°C. After aspiration, detection reagent A was applied for 60 min at 37°C, followed by 3 washes. Subsequently, detection reagent B was added and incubated for 30 min at 37°C. Following 5 washes, TMB (3,3',5,5'-tetramethylbenzidine) substrate was introduced, allowing colour development for 15 min. The reaction was terminated with stop solution, and absorbance was measured at 450 nm.

Immunohistochemistry of human heart tissue microarray

Human heart tissue microarray (BC30013) was procured from the TissueArray company (TissueArray.com, MD, USA). For immunohistochemistry, the slides were initially baked at 55°C for 40 minutes and subsequently deparaffinised using a xylene-ethanol protocol. Following deparaffinisation, antigen retrieval was performed in Tris-EDTA buffer (10 mM Tris Base, 1 mM EDTA, 0.05% Tween 20, pH 9.0) by heating the slides to 98°C for 40 minutes. Blocking was conducted using 1% bovine serum albumin (BSA) in PBS blocking buffer. The slides were then incubated with Alexa Fluor® 647-conjugated anti-CD68 (Abcam, Cambridge, UK), which was diluted in the blocking buffer. DNA was counterstained with 4',6-diamidino-2-phenylindole (DAPI). The stained samples were mounted with Vectashield mounting medium (Vector Laboratories) and visualised using the TissueFAXS Plus whole-slide tissue cytometry system.

Gene expression analysis by RT-qPCR

RUNX2 and osteopontin expression levels were assessed using RT-qPCR with SYBR Green chemistry. RNA isolation was performed using a TRIzol-based extraction kit (Life Technologies, MA, USA) as per the manufacturer's guidelines. Reverse transcription was carried out using the iScript cDNA synthesis kit (Bio-Rad, CA, USA). Quantitative PCR was conducted on a LightCycler 96 Real-Time PCR System using FastStart SYBR Green Master mix (Roche Applied Science, Mannheim, Germany). Primer sequences for target genes are provided in Table I. GAPDH served as the reference gene for normalisation.

Alkaline phosphatase staining

To analyse early osteogenic differentiation of valvular interstitial cells, we utilised an alkaline phosphatase (ALP) kit (Solarbio, Beijing, China, G1480) according to the manufacturer's protocol. Briefly, the culture medium was aspirated, and the cells were washed 3 times with 1 × PBS. Cells were then fixed using pre-chilled (4°C) ALP fixative for 1 minute, followed by another 3 washes with PBS. ALP staining solution was applied to cover the cells, and the samples were incubated at 37°C for 15 minutes. Subsequently, the cells were washed 3 more times with PBS. For counterstaining, the nuclei were stained with Nuclear Fast Red for 5 minutes, followed by 2 additional PBS washes. The slides were mounted with a sealing agent and observed under a fluorescence microscope (Nikon Eclipse C1). Blue light excitation (wavelength 330–380 nm, emission wavelength 420 nm) and red light excitation (wavelength 510–560 nm, emission wavelength 590 nm) were used. Regions of ALP activity appeared blue, while the nuclei were stained red.

Data analysis

Results are presented as mean ±standard error of the mean (SEM) for each group. Comparisons of serum HMGB1 concentrations between case and control groups were performed using unpaired 2-tailed *t*-tests, with $p < 0.05$ considered statistically significant. For serum IL-10 comparison, which was the non-normal distribution, the non-parametric Mann-Whitney test was performed. Data analysis was conducted using IBM SPSS Statistics version 22 for Windows (IBM Corp., Armonk, New York, USA).

Study design and ethical considerations

This study included participants aged ≥ 18 years, categorised into 3 groups: normal ($n = 7$), moderate CAVD ($n = 6$), and severe CAVD ($n = 6$). The Normal group comprised non-CAVD individuals, while the Moderate and Severe CAVD groups included patients diagnosed via echocardiography, with areas of maximal calcification severity < 2 mm and ≥ 2 mm, respectively. All participants had no prior aortic valve surgery. On the other hand, the person with acute myocardial infarction, pregnancy, acute or chronic infections, chronic inflammatory diseases (e.g., sepsis, autoimmune conditions, inflammatory bowel diseases), or malignancies was excluded from the study. For detailed criteria, see Fig. 1A.

This prospective observational study adhered to the principles outlined in the Declaration of Helsinki and received approval from the Internal Review Board of Shanghai Geriatric Medical Centre (Shanghai Municipal Centre for Aging Research) Ethics Committee (IRB No. B2023-009). Written informed consent was obtained from all participants prior to inclusion.

A

Inclusion			Exclusion
Normal (n = 7)	Moderate CAVD (n = 6)	Severe CAVD (n = 6)	1. Acute myocardial infarction
1. Age ≥ 18 years	1. Age ≥ 18 years	1. Age ≥ 18 years	2. Pregnant women or those likely to become pregnant
2. Non-CAVD patients	2. Patients with CAVD diagnosed via standard echocardiography	2. Patients with CAVD diagnosed via standard echocardiography	3. Acute or chronic infections
	3. AVCS: areas of maximal calcification severity < 2 mm	3. AVCS: areas of maximal calcification severity < 2 mm	4. Chronic inflammatory diseases (e.g., sepsis, autoimmune diseases, inflammatory bowl diseases)
	4. Patients without prior aortic valve surgery	4. Patients without prior aortic valve surgery	5. Malignancies

CAVD – calcific aortic valve disease; AVCS – aortic valve calcification score

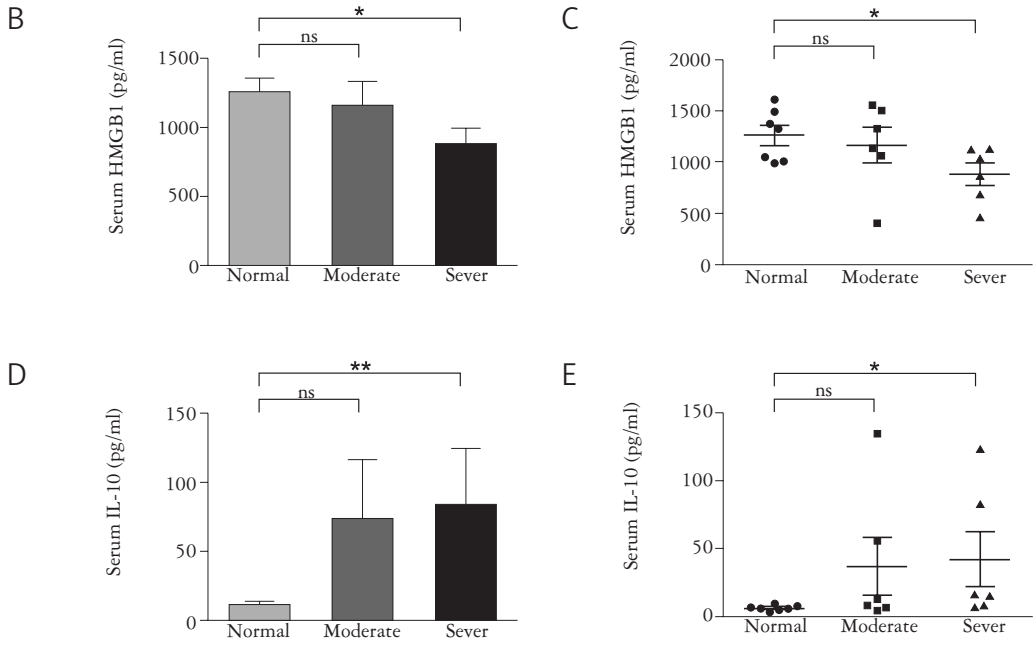

Fig. 1. Serum HMGB1 and IL-10 levels in relation to CAVD severity. **A)** Inclusion and exclusion criteria for study participants, detailing parameters for enrolling patients with moderate and severe calcific aortic valve disease (CAVD), as well as healthy controls. Histogram displaying mean serum HMGB1 (**B**) or IL-10 (**D**) concentrations among healthy individuals and patients with moderate and severe CAVD. Individual data distribution of serum HMGB1 (**C**) or IL-10 (**E**) levels within each group, which was derived from the same dataset (**B** and **D**, respectively). Each dot represents an individual sample. Data presented as mean ±SEM; * $p < 0.05$, ** $p < 0.01$; ns – not significant

Results

High mobility group box 1 and interleukin-10 levels in relation to calcific aortic valve disease severity

To compare serum HMGB1 levels across varying degrees of CAVD patients, we collected serum samples from individuals with moderate and severe CAVD, with inclusion and exclusion criteria detailed in Fig. 1A. The levels of serum HMGB1 and IL-10 were determined by ELISA assay. Our analysis revealed that serum HMGB1 levels were significantly reduced by approximately 30% in patients with severe calcific aortic valve disease (CAVD) compared to healthy controls (888.4 pg/ml vs. 1,266.8 pg/ml, respectively; Fig. 1B and C). Conversely, serum IL-10 levels were markedly elevated in CAVD patients, showing a more than sevenfold increase compared to healthy controls (11.7 pg/ml vs. 84.0 pg/ml, respectively; Fig. 1D, E).

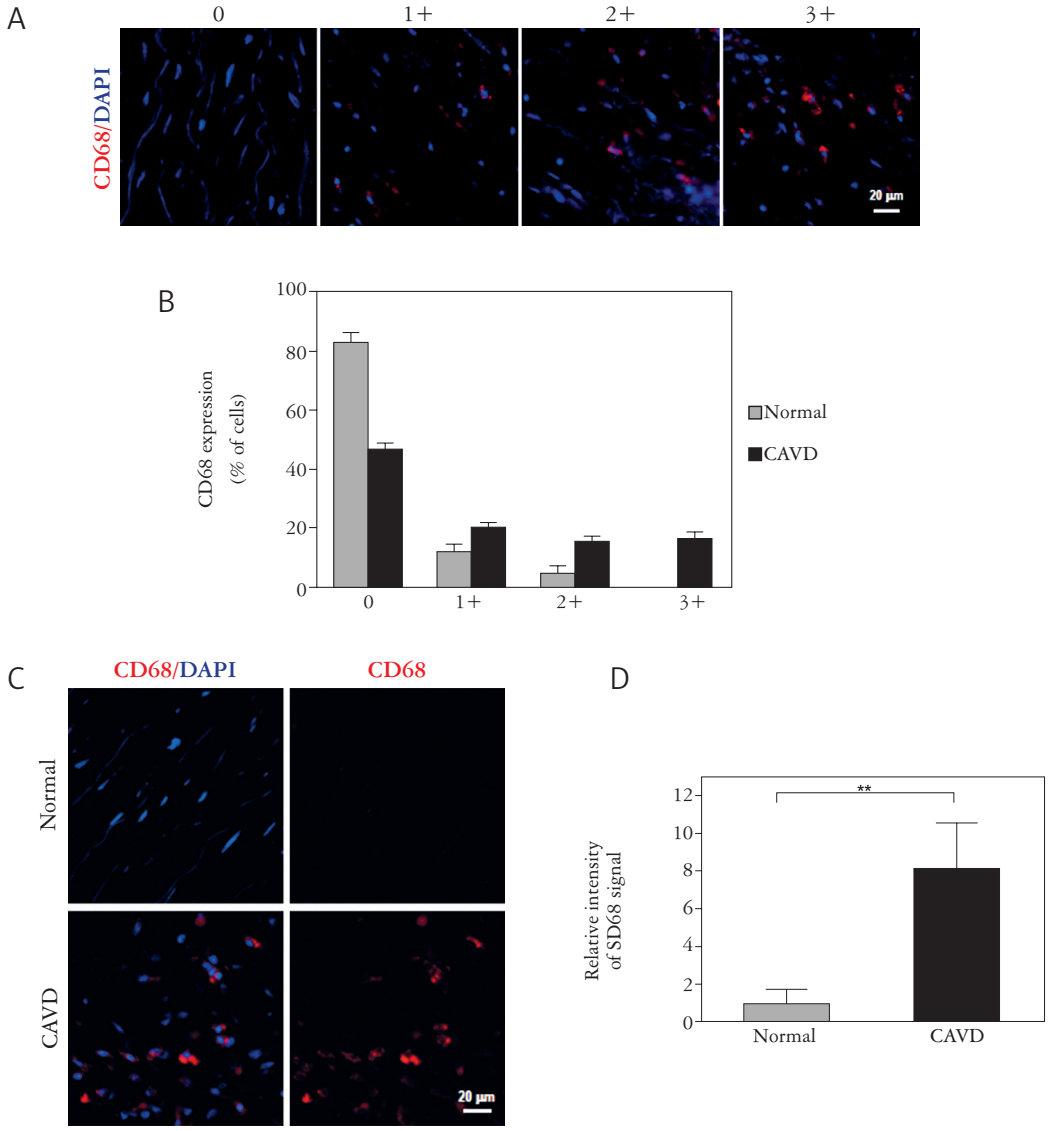

Fig. 2. Expression of CD68 in normal great artery tissues and tissues with chronic rheumatic valvular disease with calcification. **A)** Representative immunofluorescence staining for CD68 (red, macrophage marker) in normal and calcified valvular tissues from patients with chronic rheumatic valvular disease. DAPI (blue) was used to label nuclei. CD68 signals were localised within the cytoplasm. Scoring criteria were based on fluorescence intensity: Score 0, no detectable staining; Score 1+, weak staining in scattered cells; Score 2+, moderate staining in localised clusters; Score 3+, strong staining diffusely distributed across the tissue. **B)** Quantification of CD68-positive cells in normal and calcified valvular tissues. Data were expressed as the percentage of cells at each intensity grade (0, 1+, 2+, and 3+). **C)** Representative immunofluorescence images showing CD68 in normal and calcified valvular tissues. **D)** Quantification of CD68 expression intensity was performed on 5 randomly selected fields from each duplicate core per case in normal and calcified valvular tissues. Analysis was conducted using ImageJ software, and the results are presented as mean ±SEM; ** $p < 0.01$

This inverse relationship between HMGB1 and IL-10 levels suggests a shift in the inflammatory landscape of CAVD, where reduced HMGB1 expression may contribute to disease progression. Given the significant rise in IL-10, we hypothesised an ongoing inflammatory response and further investigated macrophage infiltration in calcified valvular tissue using CD68 immunostaining (Fig. 2).

Increased macrophage infiltration in calcified valvular tissue

To investigate inflammatory activity in calcified valvular tissue, we performed immunofluorescence staining on heart tissue arrays, comparing samples from patients with chronic rheumatic valvular disease with calcification to normal great arteries tissue. While degenerative calcific aortic stenosis remains the predominant pathology in the elderly, our study utilised human valvular tissues with chronic rheumatic calcification, which shares key features of immune-mediated calcific processes and provides a useful inflammatory model for exploratory investigations. Our analysis revealed a substantial increase in CD68-positive macrophage infiltration in calcified valve samples (Fig. 2), approximately sevenfold higher than in normal tissues (Fig. 2D). These findings further support the presence of a pronounced inflammatory response in CAVD, highlighting the involvement of immune cell recruitment in the disease process, which may contribute to disease progression.

High mobility group box 1 inhibited osteogenic differentiation in human aortic valve interstitial cells co-cultured with macrophages

Building on our earlier findings that severe CAVD patients exhibited decreased serum HMGB1 levels and increased macrophage infiltration, we hypothesised that HMGB1 may mitigate calcification in CAVD by modulating macrophage polarisation. Specifically, we propose that HMGB1 could promote the transition of macrophages toward the anti-inflammatory M2 phenotype, thereby alleviating osteogenic differentiation in the haVICs. To investigate this hypothesis, we employed a co-culture system of macrophages and haVICs, exploring the interplay between HMGB1 and macrophage-mediated mechanisms. Macrophages were pretreated with recombinant HMGB1 (reHMGB1) for 24 h and then co-cultured with haVIC for 5 days. RT-qPCR analysis revealed that RUNX2 mRNA expression in haVICs was reduced by 60% in the reHMGB1-treated group compared to the control group (1.0 vs. 0.4, Fig. 3A). Similarly, ALP staining showed a marked reduction in the percentage of ALP-positive haVICs, decreasing by approximately 75% in the reHMGB1 group compared to the control group (68.5% vs. 17.3%, Fig. 3C, D). In contrast, osteopontin mRNA levels remained largely unchanged between the HMGB1-treated and untreated groups (1.0 vs. 0.96, Fig. 3B). These findings suggest that HMGB1 appeared to mitigate osteogenic differentiation in haVICs through macrophage-mediated mechanisms.

Discussion

This study explored the role of HMGB1 in CAVD progression, particularly its impact on macrophage polarisation and VIC calcification. By analysing patient serum, tissue samples, and *in vitro* co-culture systems, our findings provide new insights into the relationship between inflammatory signalling, macrophage-mediated responses, and osteogenic differentiation in CAVD. While our *in vitro* co-culture model reveals a potential link between recombinant HMGB1 and reduced osteogenic activity in haVICs via macrophage modulation, the absence of in situ validation within native aortic valve tissue represents a limitation. Thus, the current data suggest an associative, rather than causative, role of HMGB1 in regulating valvular calcification. Moreover, the immunofluorescence analysis was based on tissue from chronic rheumatic valvular disease, which may not fully reflect the pathology of degenerative calcific AS. Therefore, the inflammatory landscape described herein should be interpreted with an awareness of this histological limitation. While previous studies have identified HMGB1 as a pro-inflammatory mediator in VICs via TLR4 signalling [30], our findings suggest that, in the context of macrophage-preconditioned co-cultures, HMGB1 may instead promote anti-inflammatory effects. This discrepancy highlights the importance of cellular context and microenvironment in HMGB1 function.

High mobility group box 1, interleukin-10, and macrophage polarisation in calcific aortic valve disease severity

Our results demonstrated that HMGB1 levels were significantly reduced in severe CAVD patients, while IL-10 levels were markedly elevated, and CD68-positive macrophage infiltration was increased in calcified valvular tissue. Given that IL-10 is a well-established anti-inflammatory cytokine, its upregulation could represent a compensatory mechanism in response to chronic inflammation in CAVD, limiting excessive immune responses and preventing tissue damage [31]. However, this paradoxical increase in IL-10 within a pro-inflammatory environment suggests a complex regulatory network underlying CAVD pathology.

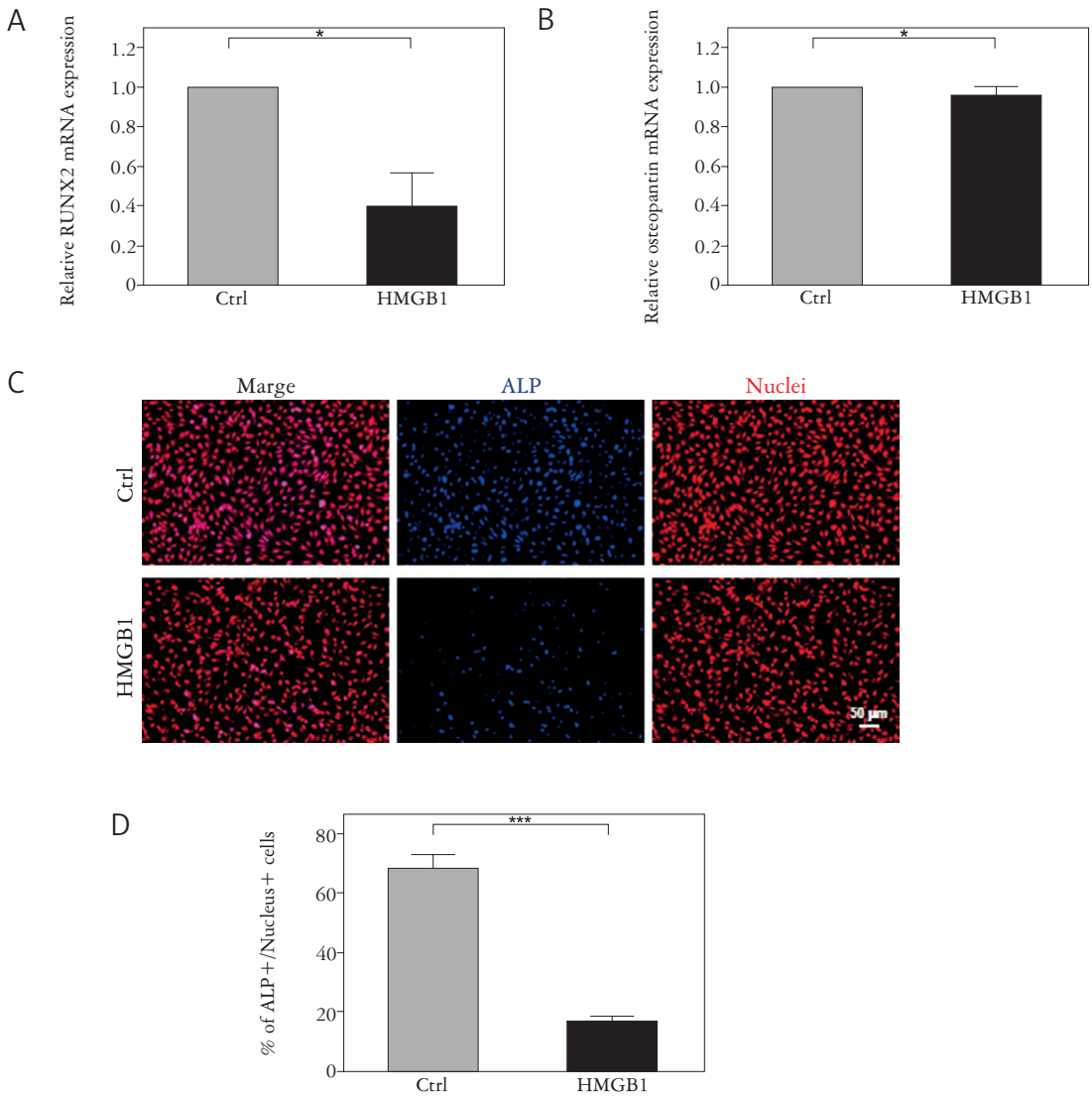

Fig. 3. Effects of recombinant HMGB1 on osteogenic markers in haVICs co-cultured with macrophages. Macrophages were treated with reHMGB1 (500 ng/ml) for 24 h, followed by co-culture with haVICs for 5 days. **A, B)** Relative mRNA expression levels of RUNX2 and osteopontin in haVICs, normalised to GAPDH. Each treatment was performed in triplicate ($n = 3$). **C)** Alkaline phosphatase (ALP) activity in haVICs. ALP activity in haVICs was assessed using histochemical staining, with ALP-positive areas appearing blue and nuclei counterstained in red. Quantification was performed on 3 randomly selected fields from each group by calculating the percentage of ALP-positive nuclei relative to the total nuclei **(D)**. Data are presented as mean ±SEM; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns – not significant

Nevertheless, given the limited cohort size and potential confounding variables such as age and comorbidities, the observed reduction in serum HMGB1 levels should be interpreted with caution. Further studies involving larger, stratified populations are needed to validate this association.

Macrophage polarisation plays a crucial role in inflammation-driven calcification

The pro-inflammatory M1 phenotype is associated with enhanced osteogenic signalling and the release of cytokines such as TNF-α, IL-6, and IL-1β, which promote VIC differentiation into osteoblast-like cells. In contrast, M2 macrophages, characterised by IL-10 secretion, are generally considered anti-inflammatory and are linked to tissue repair and resolution of inflammation [14–16, 32–34]. However, emerging evidence suggests that IL-10 signalling can also activate the mTOR pathway, which paradoxically inhibits autophagy and may contribute to disease progression in CAVD [35–37].

mTOR signalling, autophagy, and high mobility group box 1 in calcific aortic valve disease

The mTOR pathway plays a dual role in inflammation: it can either promote or suppress inflamma-

tion depending on the cellular context and environmental stimuli. Activation of mTOR can promote inflammatory responses and calcification by enhancing the production of pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α, and macrophage infiltration, facilitating immune cell proliferation and tissue remodeling [38–43]. Conversely, mTOR signalling can exert anti-inflammatory effects by modulating immune cell functions and promoting the polarisation of macrophages toward the anti-inflammatory M2 phenotype [44–49]. However, a key consequence of mTOR activation is the inhibition of autophagy, a process crucial for cellular homeostasis, stress adaptation, and degradation of calcification-promoting cellular debris [50–54]. Notably, autophagy has been reported to mitigate valvular calcification, as it reduces the accumulation of damaged organelles and apoptotic vesicles that serve as nucleation sites for calcium deposition [55, 56].

Given that HMGB1 is known to modulate mTOR signalling via TLRs and RAGE interactions, it is plausible that HMGB1 may counteract mTOR activation [57, 58], thereby promoting autophagy and reducing CAVD progression. This hypothesis aligns with our *in vitro* findings, where recombinant HMGB1 treatment led to a significant reduction in RUNX2 expression and ALP activity in co-cultured VICs. This raises the possibility that HMGB1 could influence mTOR-related signalling and promote autophagy, which in turn mitigates CAVD progression. However, direct validation of mTOR activity or autophagic flux in our model was not performed, and future work is needed to assess these pathways experimentally.

Distinct roles of RUNX2 and osteopontin in early- and late-stage calcification

Our findings also highlighted the differential regulation of RUNX2 and osteopontin, underscoring the complexity of calcification mechanisms in VICs. RUNX2, a key transcription factor for osteogenesis, is primarily involved in the early stages of calcification, driving the differentiation of VICs into osteoblast-like cells and promoting extracellular matrix mineralisation [59, 60]. In contrast, osteopontin plays a more prominent role in the later stages of calcification, regulating mineral deposition and matrix remodeling [61–64]. The fact that HMGB1 treatment suppressed RUNX2 and ALP activity but had no significant effect on osteopontin expression suggests that HMGB1 primarily interferes with early-stage osteogenic differentiation rather than the maturation of calcified nodules in CAVD. While our results are limited to mRNA-level changes, they provide a basis for future protein-level validation studies to determine the sustained functional relevance of HMGB1 in modulating VIC calcification.

Conclusions

Future studies could focus on delineating the precise interplay between HMGB1, mTOR, and autophagy in CAVD progression, specifically investigating whether HMGB1 directly inhibits mTOR signalling in macrophages could provide mechanistic insights into its role in CAVD pathology, and additionally exploring whether restoring autophagy via pharmacological mTOR inhibition can serve as a potential therapeutic strategy to mitigate CAVD progression.

Furthermore, it is worth examining the macrophage polarisation states in CAVD tissues and their correlation with mTOR activity, IL-10 signalling, and autophagy induction. Moreover, mechanistic studies should investigate whether HMGB1 influences key calcification-related pathways, such as Wnt/β-catenin, PI3K/Akt, or TGF-β signalling, in both macrophages and VICs.

Finally, given that HMGB1 has been implicated in various inflammatory and autophagic processes, its therapeutic potential in CAVD warrants further exploration, investigating whether HMGB1-based interventions can modulate macrophage polarisation and restore autophagy in preclinical CAVD models may pave the way for innovative treatment strategies aimed at preventing aortic valve calcification and stenosis.

Disclosures

1. Institutional review board statement: Not applicable.
2. Assistance with the article: None.
3. Financial support and sponsorship: None.
4. Conflicts of interest: None.

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