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Decreased serum HMGB1 associated with M2 macrophage polarization and patients with calcific aortic valve disease

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Abstract: Except for the standard aortic valve replacement, no effective medical treatment is available to prevent or delay calcific aortic valve disease (CAVD) progression. Recently, macrophages and high-mobility group box 1 (HMGB1) are the most intriguing candidates in various inflammatory disorders. However, the association between serum HMGB1, CAVD, and macrophage polarization remains unclear. Therefore, we examined whether the level of serum HMGB1 is clinically associated with aortic valve calcification and whether HMGB1 treatment can promote macrophage differentiation toward M1 or M2 phenotype. This experimental study included 19 CAVD patients and 20 healthy controls whose serum HMGB1 levels were examined by ELISA assay. THP-1 macrophage polarization system was established to test the polarization capability of HMGB1 treatment. The results showed that serum levels of HMGB1 were significantly reduced in patients with CAVD. HMGB1 treatment promoted M2 macrophage polarization but not M1 phenotype with increased IL-10 expression and reduced inducible nitric oxide synthase (iNOS) expression. Our findings suggest that serum HMGB1 is negatively associated with the development of aortic valve calcification, and HMGB1 treatment may facilitate M2 macrophage polarization for reducing aortic valve calcification.

Introduction

Calcific aortic valve disease (CAVD) is an inflammation-related disease and is the most common valvular heart disease in adults aged 65 years and older (Chen *et al.*, 2015). However, with the exception of standard aortic valve replacement and some potential drugs still under investigation, including statins, bisphosphonates, and angiotensin-converting enzyme inhibitors, no effective medical therapy is available currently to delay or prevent the progression of aortic valve calcification (Salas *et al.*, 2012). The pathological feature of CAVD is the large nodular calcification in the aortic valves. In the past, the disease was considered as a passive degenerative disease. However, it is recently accepted that the development of CAVD is an active process. Although the molecular mechanism of aortic valve calcification remains largely unclear, recent clinical data and in vitro experiments have suggested that the calcific processes involved inflammation-related macrophages (Li *et al.*, 2017; New and Aikawa, 2011; Li *et al.*, 2016) and osteogenesis-related factors (Miller *et al.*, 2011; New and Aikawa, 2011; Xu *et al.*, 2017).

During cardiovascular calcification, macrophages were found to abundantly infiltrate and accumulate in the aortic valvular lesions (Li *et al.*, 2017). Depending on the extant microenvironment, macrophages can differentiate toward a proinflammatory M1 or anti-inflammatory M2 macrophage (Motwani and Gilroy, 2015). Both M1 and M2 macrophages were found to coexist in the calcification and atherosclerotic regions and to transform each other based on microenvironmental stimuli (Li *et al.*, 2017; Liu *et al.*, 2014; Mantovani *et al.*, 2013). In M1 macrophage-directed polarization, M1 macrophage preferentially produces high-level IL-6, IL-1β, and upregulate the expression of inducible nitric oxide synthase (iNOS). In contrast, M2 macrophage polarization is accompanied by the production of cytokines such as IL-10. Although the balance of M1 and M2 macrophages' subtypes plays a crucial role in aortic valve calcification, the molecular mechanism of action remains unknown and controversial.

Recently, high-mobility group box 1 (HMGB1) has received increasing attention. HMGB1 is an important inflammation-associated protein that not only acts functionally as a cytokine and chemokine, but also participates in tumor progression and metastasis, autoimmune diseases, cell differentiation, DNA replication, gene transcription and ischemia-reperfusion injury (Andersson and Tracey, 2011; Andrassy *et al.*, 2008; Harris

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et al., 2014; Magna and Pisetsky, 2014). Increased expression of HMGB1 has been found in various acute and chronic inflammatory diseases and numerous cancers (Hou *et al.*, 2015; Lee *et al.*, 2012; Min *et al.*, 2015; Nativel *et al.*, 2013), including chronic rhinosinusitis, chronic asthma, type 2 diabetes, and colorectal cancer. In addition to localizing in the nucleus of all cells at baseline, HMGB1 can move rapidly to other sites within the cell, such as mitochondria and cytoplasm. In addition, HMGB1 can be secreted into the extracellular space in response to pathogenic factors or passively released by dying or injured cells (Andersson *et al.*, 2014). Secreted HMGB1 can interact with various receptors such as the receptor for advanced glycation end products (RAGE), toll-like receptor 2 (TLR2) and toll-like receptor 4 (TLR4) to elicit different signaling cascades within cells (Andersson and Tracey, 2011; Tang *et al.*, 2012). Several studies demonstrated that HMGB1 is involved in the pathogenesis of inflammatory disorders through its action on macrophages (Li *et al.*, 2015; Yamada and Maruyama, 2007). Three redox forms of HMGB1 include all-thiol-HMGB1 (fully reduced) with chemoattractant activity, disulfide-HMGB1 with cytokine-induced activity, and oxidized (sulfonyl) HMGB1 without immune activity (Kang *et al.*, 2014; Tang *et al.*, 2012; Venereau *et al.*, 2012). With a disulfide linkage between cysteines C23 and C45, disulfide HMGB1 promotes the release of proinflammatory cytokines and is involved in the inflammatory response (Kang *et al.*, 2014; Tang *et al.*, 2012). Myeloid differentiation factor 2 (MD-2) and toll-like receptor 4 (TLR4) complex bind specifically to this disulfide HMGB1 to stimulate inflammatory TNF release in macrophages (Yang *et al.*, 2015). However, the role of serum HMGB1 in CAVD patients and the association with macrophage polarization remains to be elucidated. Further, since the multifunction of HMGB1 depends on molecular localization (Andersson *et al.*, 2014), it is worthwhile to investigate whether serum HMGB1 is associated with CAVD.

Therefore, the purpose of the present study was to examine whether the level of serum HMGB1 is clinically associated with aortic valve calcification and whether HMGB1 treatment can promote macrophage differentiation toward M1 or M2 phenotype.

Materials and Methods

Study design and ethical considerations

This prospective observational research study complied with the Declaration of Helsinki and was approved by the Internal Review Board of Zhongshan Hospital. All included patients provided signed informed consent.

Cell culture

THP-1 monocyte cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in a concentration of 1×10^5 cells/mL. Cells were grown in an RPMI-1640 medium (GIBCO, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mM glutamine at 37°C under 5% CO_2. For differentiation experiments, THP-1 monocytic cells with a density of 1×10^5 cells were differentiated into M0 macrophage in a 6-well culture plate containing 100 nM phorbol 12-myristate 13-acetate (PMA, Sigma, St. Louis, MO, USA). After 24-h incubation, the medium was removed and replaced with a PMA-free RPMI medium for additional 24-h incubation. Before and after PMA treatment, the hallmarks of macrophages, such as cell adhesion and spreading, were examined under a microscope. For M1/M2 macrophage polarization, the PMA-stimulated THP-1 cells were then treated with 100 ng/mL LPS (Sigma, St. Louis, MO, USA) or 20 ng/mL IL-4 (Peprotech, Rocky Hill, NJ, USA) or 50–500 ng/mL carrier-free recombinant humanHMGB1 protein (R&D Systems, Minneapolis, MN, USA) for 18 h, and the cells were collected for subsequent RT-qPCR analysis.

Determination of serum HMGB1 levels by ELISA

The serum HMGB1 levels of 19 patients with CAVD and 20 healthy control were determined by HMGB1 ELISA assay kit (LSBio, Shanghai, Shanghai, China) according to the manufacturer's instructions. Briefly, each patient's serum was centrifuged at 1000 × *g* for 10 min and then added separately to the well for 60 min at 37°C to allow serum HMGB1 to bind specifically to the immobilized antibody. After aspirating the liquid of each well, 100 μL of detection reagent A was added to each well and incubated for 60 min at 37°C. After washing each well three times with 50 μL of washing buffer, 100 μL of detection reagent B was added to each well and incubated for 30 min at 37°C. After washing five times with washing buffer, 90 μL of TMB (3,3',5,5'-tetramethylbenzidine) substrate solution was added to each well, and the color was developed for 15 min. The reaction was stopped by adding 50 μL of stop solution, and the color intensity (OD value) of each well was measured at 450 nm.

Quantitative RT-PCR

The expression levels of iNOS, IL-10, IL-6, and IL-1β were determined by quantitative real-time PCR using the SYBR Green system. Total RNAs were extracted using Trizol total RNA extraction kit (Sangon Biotech, Shanghai, China) according to the manufacturer's instructions. Extracted RNAs were reverse-transcribed using Reverse Transcription Kit (Takara, Dalian, Liaoning, China). Quantitative real-time PCR was carried out using SYBR Green PCR kit (Takara, Dalian, Liaoning, China). The primers for iNOS, IL-10, IL-6, IL-1β, and β-actin are shown in Tab. 1. Herein, β-actin was used as an internal control probe.

Statistical analysis

Data are summarized as mean ± standard deviation (mean ± SD) by groups. The comparison of human serum HMGB1 concentration levels between the control group and the case group were analyzed using the independent *t*-test. THP-1 cell treatment experiments were compared using a one-way ANOVA test with a post-hoc pair-wise comparison with the least significant difference test (LSD). All statistical assessments were two-tailed, and $p < 0.05$ was considered statistically significant. All statistical analyses were carried out using IBM SPSS statistical software version 22 for Windows (IBM Corp., Armonk, New York, USA).

TABLE 1

Specific primer sequences used in real-time PCR

Gene	Primer	Sequences
iNOS	Forward	ATCCGCTATGCTGGCTACC
	Reverse	TCCCGAAACCACTCGTATTT
IL-1β	Forward	TGAAGCTGATGGCCCTAAAC
	Reverse	GCCCTTGCTGTAGTGGTGGT
IL-6	Forward	GCCACTCACCTCTTCAGAACG
	Reverse	GTGCCTCTTTGCTGCTTTCA
IL-10	Forward	CTGAGAACCAAGACCCAGACA
	Reverse	GCATTCTTCACCTGCTCCAC
β-actin	Forward	CTGGCACCACACCTTCTAC
	Reverse	CCAGAGGCGTACAGGGATAG

Results

Reduced serum levels of HMGB1 are associated with calcific aortic valve disease

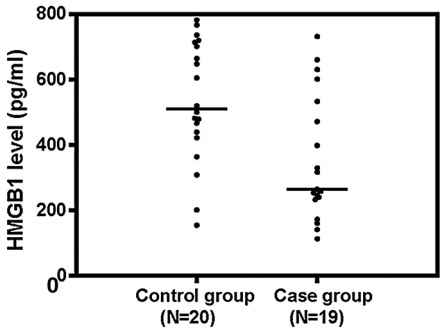
The serum HMGB1 levels of 19 patients with calcific aortic valves, and 20 healthy control, were determined by ELISA assay. The serum HMGB1 levels were significantly reduced in CAVD patients compared with those in the control group (p = 0.006, Fig. 1). The mean serum levels of HMGB1 were 533.60 ± 185.35 pg/mL and 355.76 ± 191.80 pg/mL in healthy individuals and CAVD patients, respectively. The median serum levels of HMGB1 were 499.7 pg/mL and 264.45 pg/mL in healthy individuals and CAVD patients, respectively. These clinical data suggest that serum HMGB1 may play a negative regulatory role in the development of CAVD.

HMGB1 treatment facilitates macrophage reprogramming towards a M2-like phenotype

To examine whether HMGB1 could affect the shit of macrophage toward M1 or M2 phenotype, we established a model of macrophage polarization, starting from THP-1 monocytes differentiated into macrophages using phorbol 12-myristate 13-acetate (PMA), and then treated THP-1 cells with 100 nM PMA for stable differentiation of M0 macrophage. As shown in Fig. 2A, undifferentiated THP-1 cells showed ultra-low attachment and displayed a round shape, while PMA-stimulated THP-1 cells were adherent with typical flat, elongated, amoeboid-like, and branched macrophage morphology. Once cells are differentiated after PMA stimulation, the M0 macrophages can be polarized into M1 or M2 macrophages. Thus, we next examined whether the M0 THP-1 macrophages were able to differentiate into M1 or M2 macrophages by treating with LPS or IL-4. The differentiation of M1/M2 macrophage was examined by measuring the expression of iNOS, which is a marker for the M1 phenotype. As shown in Fig. 2B, increased expression of iNOS was observed after M0 THP-1 macrophages were treated with 100 ng/mL LPS, indicating the shift of macrophage toward the M1 phenotype. Moreover, reduced expression of iNOS was observed when M0 THP-1 macrophages were incubated with 20 ng/mL IL-4, indicating the shift of macrophage toward the M2 phenotype.

Since we observed that serum HMGB1 levels were reduced in patients with CAVD (Fig. 1), we next examined whether HMGB1 could facilitate macrophage reprogramming

(A)



(B)

Summary of the raw data.

	N	HMGB1		p-value
		Mean	SD	
Group				0.006*
Control group	20	533.60	185.35	
Case group	19	355.76	191.80	

* Indicate significant difference between control group and case group

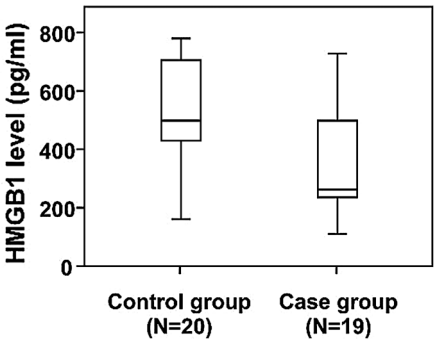


FIGURE 1. Serum levels of HMGB1 in healthy control and CAVD patients are shown. The serum levels of HMGB1 were remarkably reduced in patients with CAVD compared with those healthy controls (p = 0.006). The medians (499.7 pg/mL in healthy control and 264.45 pg/mL in CAVD patients) are indicated.

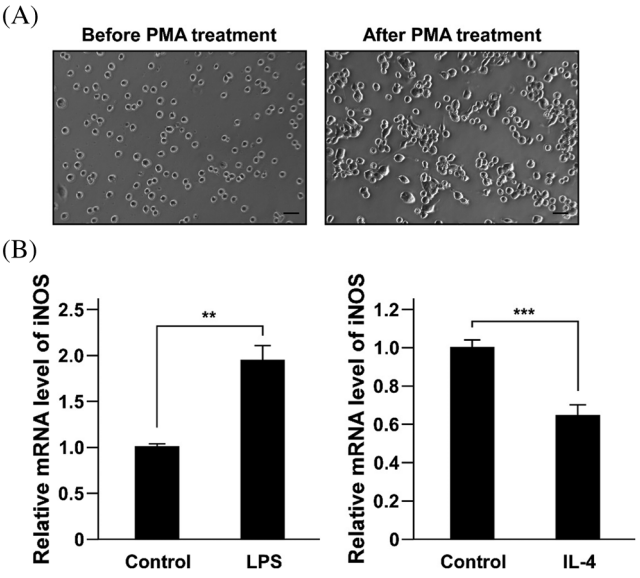


FIGURE 2. Effect of PMA in the differentiation of THP-1 cells (A) Representative images illustrated the morphologic change of THP-1 into macrophages. Increased cell size, enhanced granularity, and production of lamellipodia and filopodia were observed in PMA-treated THP-1 cells. (B) Effects of LPS and IL-4 in the polarization of PMA-treated THP-1 cells. PMA-treated THP-1 cells were treated with 100 ng/mL LPS or 20 ng/mL IL-4 for 18 h to differentiate into M1 and M2 phenotype, respectively. The expression levels of iNOS were determined by real-time PCR. Scale bar = 50 μm. Differences were found to be statistically significant at $^{**}p < 0.01$, and $^{***}p < 0.001$.

toward M2 macrophage. Thus, M0 THP-1 macrophages were treated with different doses of HMGB1 ranging from 50 to 500 ng/mL, and the expression of iNOS was examined. As shown in Fig. 3A, HMGB1 treatment reduced the expression of iNOS in a dose-dependent manner. A significant reduction of nearly 70% iNOS was observed when the cells were treated with 500 ng/mL HMGB1 ($p < 0.001$), suggesting that HMGB1 facilitated M0 THP-1 macrophages reprogramming to M2 macrophages. The reprogramming of M2 macrophages was further confirmed by examining the expression of IL-10. As shown in Fig. 3B, the level of IL-10 was significantly increased after HMGB1 treatments. In addition, HMGB1-mediated M2 reprogramming was confirmed by determining the expression of M1 macrophage markers, including IL-6 and IL-1β. When M0 macrophages were treated with HMGB1, the expression of IL-6 (Fig. 3C) and IL-1β (Fig. 3D) were not altered. Taken together, these results suggest that HMGB1 can promote the reprogramming of macrophage toward M2 macrophage. Fig. 4 is a schematic diagram that summarizes all the study results.

Discussion

In the past, CAVD was considered to be a passive degenerative disease in older adults, with calcium deposition in the vessel wall and aortic valve (Mohler, 2004). However, more recently, CAVD is no longer considered to be merely a passive process of calcium deposition. Instead, the development of aortic valve calcification is actively regulated and does not fully manifest as a degenerative process (Rajamannan *et al.*, 2011). Recently, differential macrophage programming in the aortic valve microenvironment was widely suggested to be the fundamental contributor in the development of CAVD. A number of M1 macrophages and activated T lymphocytes have been found to accumulate in the aortic stenotic valves (Olsson *et al.*, 1994; Wang *et al.*, 2014), suggesting that M1 macrophage polarization may affect the release of proinflammatory cytokines to trigger or regulate osteogenic differentiation and aortic valve calcification. However, it remains unclear which factors regulate the macrophage polarization for the development of CAVD. Do these factors also negatively regulate macrophage polarization in a healthy body? The results of this study suggest that serum HMGB1 may play an important role in the development of CAVD. Interestingly, we found that the level of serum HMGB1 was significantly reduced in CAVD patients compared to healthy individuals (Fig. 1, $p = 0.006$). In addition, HMGB1 is able to facilitate macrophage reprogramming toward an anti-inflammatory M2 macrophage (Fig. 3). This may indicate that, in healthy individuals, serum HMGB1promotes M2 macrophage polarization and creates a protected microenvironment in the aortic valve area. When the serum HMGB1 level is below the basal line, it may facilitate M1 macrophage polarization. Thus, the proportion of M1/M2 macrophages increased, and abundant infiltration and accumulation of M1 macrophages may occur in aortic valvular lesions, thereby creating an ideal microenvironment for valve calcification.

Once valvular calcification begins, the lesion of calcific aortic valves may create an inflammatory microenvironment to release more HMGB1 or to attract more M1 macrophages for valve calcification. This hypothesis is supported by the observation of increased infiltrated M1 macrophages and increased expression of HMGB1 in the tissues of calcific aortic valves (Passmore *et al.*, 2015; Shen *et al.*, 2017; Wang *et al.*, 2016). It is also likely that the multifunction of HMGB1 depends on cellular localization and post-translational modifications (Andersson *et al.*, 2014). Does serum HMGB1 and intracellular HMGB1 have different post-translational modifications that then may lead to different macrophage polarization? Alternatively, the different HMGB1 concentrations may result in different M1/M2 macrophage polarization. This warrants more in-depth investigations.

Oxidized low-density lipoproteins (ox-LDL) play a crucial role in the development of valvular aortic stenosis and valve calcification and are also considered as a marker of inflammation. Surgical pathological studies found that the level of ox-LDL was increased in calcified valves, with

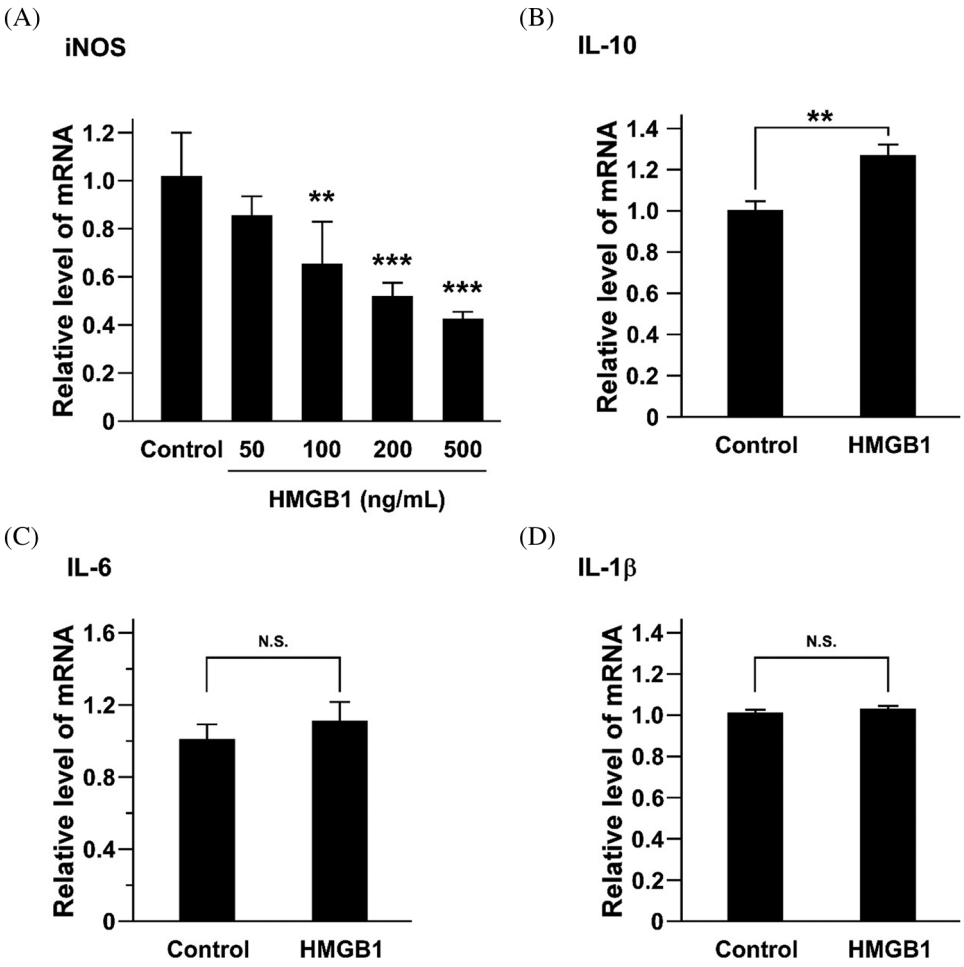


FIGURE 3. Effects of HMGB1 in the macrophage polarization. (A) HMGB1 treatment-induced iNOS expression in a concentration-dependent manner. PMA-treated THP-1 cells were treated with different doses of HMGB1 for 18 h, after which the expressions of iNOS were determined and quantified by real-time PCR. In addition, the expression levels of (B) IL-10, (C) IL-6, and (D) IL-1β, were determined by real-time PCR. Differences were found to be statistically significant at $^{**}p < 0.01$, and $^{***}p < 0.001$. Herein, N.S. denotes no significance.

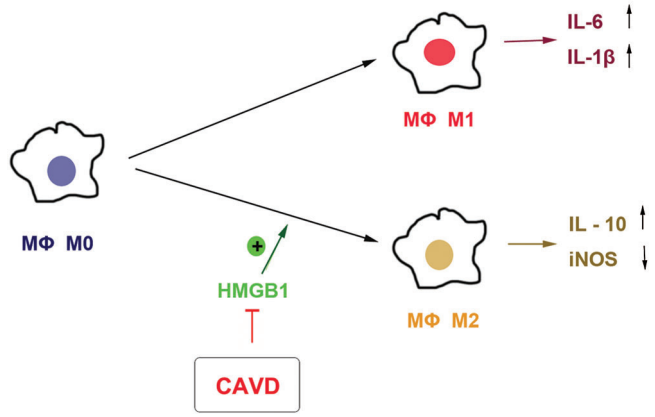


FIGURE 4. A schematic diagram displaying HMGB-mediated macrophage polarization in calcific aortic valve disease patients. Serum HMGB1 in healthy individuals may participate in the process by facilitating macrophage reprogramming toward the anti-inflammatory M2 phenotype. In patients with calcific aortic valve disease, serum HMGBI was significantly lower, and increased infiltrated numbers of M1 macrophages were observed. CAVD: calcific aortic valve disease, HMGB1: High-mobility group box 1, iNOS: inducible nitric oxide synthase, MΦ M0: unpolarized macrophages, MΦ M1: proinflammatory/classically macrophages, MΦ M2: anti-inflammatory/alternative macrophages.

distinct colocalization of ox-LDL and calcium deposits (Olsson *et al.*, 1999). On the other hand, low-density lipoproteins (LDL) are known to be involved in macrophage differentiation. Most recently, a study by Seo *et al.* (2015) showed that a high ox-LDL level could upregulate the expression of mannose receptor, monocyte chemoattractant protein-1, and IL-6, which is mostly consistent with the phenotype of M2 macrophages. Moreover, low ox-LDL treatment increased the level of TNF-α and CD86, indicating the phenotype of M1 macrophage. These studies implied that high ox-LDL may participate in the development of calcific valve through M2 macrophage, and that serum HMGB1, as in our study, may be negatively associated with the ox-LDL-mediated reprogramming of M2 macrophage during aortic valve calcification. However, a recent study observed different results in patients with ankylosing spondylitis whose ox-LDL/LDL ratio and serum HMGB1 were both increased (Hou *et al.*, 2018). When cells were treated with ox-LDL, mononuclear cells were facilitated to differentiate into osteoclasts by regulating cytoplasmic translocation and releasing of HMGB1, suggesting that in ankylosing spondylitis, circulating ox-LDL/LDL ratio is positively correlated with HMGB1. Does ox-LDL play a different role in the regulation of HMGB1 in CAVD? This aspect warrants further and more in-depth investigation.

In conclusion, this is the first study to demonstrate that serum HMGB1 is negatively associated with aortic valve calcification and that serum HMGB1 in healthy individuals may participate in the process by facilitating macrophage reprogramming toward the anti-inflammatory M2

phenotype. Serum HMGB1 may provide a protective effect in the development of aortic valve calcification. Therefore, further in-depth studies and approaches to modulate serum HMGB1 levels in vivo may lead to future pharmacological approaches to modulate M2 macrophage polarization for reducing aortic valve calcification.

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Availability of Data and Materials: The datasets analyzed during the current study are available from the corresponding author on reasonable request.

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Conflicts of Interest: All authors have no conflicts of interest to declare.

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