RESEARCH ARTICLE

Interleukin-32 plays an essential role in human calcified aortic valve cells

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ABSTRACT. Interleukin-32 (IL-32) is an inflammatory cytokine produced mainly by T, natural killer, and epithelial cells. Previous studies on IL-32 have primarily investigated its proinflammatory properties. The IL-32 also has been described as an activator of the p38 mitogen-activated protein kinase (MAPK) and NF-κB, and induces several cytokines. In this study, we hypothesized that the inflammatory regulators NF-κB, MAP kinase, STAT1, and STAT3 are associated with the expression of the IL-32 protein in human calcified aortic valve cells. This study comprised aortic valve sclerotic patients and control group patients without calcified aortic valve. Increased IL-32 expression in calcified aortic valvular tissue was shown by immunohistochemical staining and western blotting. There was an increase in NF-κB p65 level, p-ERK, p-JNK, and p-p38 MAPK activation underlying IL-32 expression in the study. The level of p-STAT3 but not p-STAT1 was found to be increased in calcified aortic valve tissue. In cultured primary human aortic valve interstitial cells, inhibition of NF-κB or MAPK kinase pathways results in a decrease of IL-32 expression. Treatment of recombinant IL-32 induced the levels of TNF-α, IL-6, IL-1β, and IL-8. Our findings demonstrate that IL-32 may be an important pro-inflammatory molecule involved in calcific aortic valve disease.

Key words: interleukin 32, MAPK, NF-κB, STAT3, calcific aortic valve disease

Calcific aortic valve disease (CAVD) is an age-related heart disease that predominantly occurs in persons over the age of 65 years and eventually leads to heart failure [1]. The progression of CAVD was thought to be "degenerative" in the past, but the mechanisms involved remain unclear. Chronic inflammation, angiogenesis, and fibrosis are all thought to play a role in the mechanisms of CAVD. Current evidence suggests that CAVD involves an active disease process related to chronic inflammation [2].

Interleukin-32 (IL-32), a proinflammatory cytokine, is produced mainly by T, natural killer, and epithelial cells. It has been described as an activator of p38 mitogen-activated protein kinase (MAPK) and NF-κB. Furthermore, it can induce tumor necrosis factor (TNF)-α, IL-6, and IL-1β, as well as chemokines such as IL-8, Th1-, and Th17-associated cytokines [3-5]. In clinical settings, IL-32 has been widely implicated in inflammatory/autoimmune diseases including chronic obstructive pulmonary disease, inflammatory bowel disease, psoriasis, allergic rhinitis, myasthenia gravis, and its levels are directly related to disease severity in rheumatoid arthritis [6, 7]. With regard to the pathologic process of cardiovascular disease, IL-32 is present in atherosclerosis of vessels [8].

The intracellular mitogen-activated protein kinase (MAPK) signaling cascades are thought to play an important role in the pathogenesis of cardiac and vascular diseases. It has been shown that MAPKs extracellular signal-regulated kinase (ERK), C-jun N-terminal kinase (JNK), and p38 MAPK individual signaling proteins play vital roles in the pathogenesis of various cardiovascular diseases. Activation of ERK1/2, JNK1/2, and p38 MAPK in vascular smooth muscle cells leads to the formation of neointima after vascular injury [9-12]. It is also strongly implicated in the development of atherosclerosis via inhibition of autophagy [13].

Nuclear factor kappa B (NF-κB), a transcription factor, is regarded as a proatherogenic factor and as a major therapeutic target for cardiovascular disease [14]. For example, the lipid lowering therapeutic agent simvastatin can reduce NF-κB activity and lead to decreased atherosclerosis [15].

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Signal transducer and activator of transcription (STAT) proteins are a family of latent cytoplasmic transcription factors involved in cytokine, hormone, and growth factor signal transduction [16-21]. In terms of their effects on cardiovascular pathology, all STAT proteins have been reported to be expressed in the myocardium, and have also shown altered expression or regulation in various heart diseases [22, 23]. STAT1 mediates pro-inflammatory responses and is essential for apoptosis in advanced atherosclerotic lesions *in vivo* [24, 25]. STAT3 plays a central role in cardiac pathophysiology and is a major mediator of pro-angiogenic signaling [26-29].

We hypothesized that the inflammatory regulators of NF-κB, MAP kinase, STAT1, and STAT3 are associated with the expression of the IL-32 protein in the progression of human calcified aortic valve cells.

METHODS

Patient profile

This cross-sectional study comprised patients with aortic valve sclerosis, who were admitted to the Cardiovascular Division of Taichung Veterans General Hospital (TCVGH), which is affiliated with the Department of Medical Laboratory Science and Biotechnology of China Medical University in Taichung, Taiwan. Between January 2013 and April 2017, aortic valve leaflets were collected from patients who underwent valve surgery at cardiovascular center, TCVGH, Taiwan. This study includes 58 patients with calcified aortic valve leaflets with functional stenosis (case group) and 29 patients with pure functional aortic valve regurgitation (control group). This study was approved by the China Medical University and Hospital Research Ethics committee, and informed consent was obtained from all individual participants included in the study.

Histology and immunohistochemistry

The tissues were fixed in 10% formalin and embedded in paraffin. Blocks were sectioned at 4 μm for each tissue. Calcium accumulation was analyzed histologically using Alizarin-Red Staining Solution (EMD Millipore). For immunohistochemistry, three sequential pieces of each specimen were stained in 1 batch, including one without and two with phosphorylation according to the standard protocol (Cell Signaling Technology, Inc., 3 Trask Lane, Danvers, MA). The immunostaining was assessed quantitatively by counting the total number of positively stained cytoplasma and nuclei of tissue per 10 high-power fields (×400 magnifications) microscopically from each specimen. A positive immunostain was defined as staining of ≧10% nuclei or cytoplasma of human aortic valve interstitial cells. The immunoreactivity expression was categorized as Level I (mean <10% nuclei or cytoplasma of human aortic valve interstitial cells stained), II (mean ≧10% to <25% nuclei or cytoplasma of human aortic valve interstitial cells), or III (mean ≧25% nuclei or cytoplasma of human aortic valve interstitial cells stained).

SYBR Green Real-Time PCR

IL-32 expression was analyzed by real-time quantitative reverse transcription polymerase chain reaction (RT–PCR). Total RNA was purified using the Trizol reagent (Invitrogen Life Technologies, Grand Island, NY, USA) according to the manufacturer's instructions. Total RNA (5 μg) was reverse-transcribed with Superscript II RNase H reverse transcriptase (Invitrogen Life Technologies) and an oligo (dT) primer. Synthesized cDNA was stored at -20°C until it was used. In brief, cDNA (2 μL) was subjected to real-time quantitative RT–PCR using the Opticon system with SYBR green I (Molecular Probes, Eugene, OR, USA) as a fluorescent reporter. IL-32 and β-actin cDNAs were amplified in separate reactions. The primers used for IL-32 were 5′-CGACTTCAAAGAGGGCTACC (forward) and 5′-GAGTGAGCTCTGGGTGCTG (reverse). The threshold cycle number of duplicate reactions was determined using the Opticon software, and levels of IL-32 mRNA expression were normalized to β-actin levels using the formula 2 (Rt–Et), where Rt is the mean threshold cycle for the reference gene (b-actin), and Et is the mean threshold cycle for the experimental gene. Data are expressed in arbitrary units. *P* values of less than 0.05 were considered significant.

Heart valve tissue protein extraction

Protein from heart valve tissue was extracted using T-PER Tissue Protein Extraction Reagent (Thermo Scientific), and protease inhibitors were added just before use. The tissue samples were weighed and a ratio of 1g of tissue to 20 mL T-PER Reagent was used. An appropriate amount of T-PER Reagent was added to the tissue sample and homogenized. The sample was centrifuged at 10,000 g for 5 min to form a pellet of cell/tissue debris, and the supernatant was then collected.

Western blot

Western blot analysis was performed according to a previously described method [30]. The process involved washing tissue cells with ice-cold PBS, followed by scraping and briefly centrifuging them. The cell pellet was then re-suspended in a hypotonic lysis buffer containing 10 mM of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1.5 mM MgCl2, 0.5 mM dithiothreitol (DTT), 0.5 μg/mL leupeptin, and 6.4% Nonidet P-40, followed by incubation on ice for 15 min. After another brief centrifugation, the nuclear pellet was gathered and suspended in a nuclear extraction buffer with 20 mM EDTA, 25% glycerol, 0.5mM DTT, 0.5 mM 4-(2-aminoethyl) benzenesulfonylfluoride, 5 μg/mL pepstatin A, and 5 μg/mL leupeptin. Following 30 min of incubation on ice, the nuclear extract was again gathered, boiled with 3 × sodiumdodecyl sulfate (SDS) sample buffer, and then subjected to SDS electrophoresis. A DC Protein Assay kit (Bio-Rad Laboratories) was used to evaluate the concentration of protein in the samples containing the p65. Sodium dodecyl sulfate/polyacrylamide gel electrophoresis was then used to size-fractionate equivalent quantities of protein before moving them onto a polyvinylidene difluoride membrane. Non-fat dry milk (5%) in Tris-buffered saline with 0.5% Tween-20 (TBST) was used to block the blot

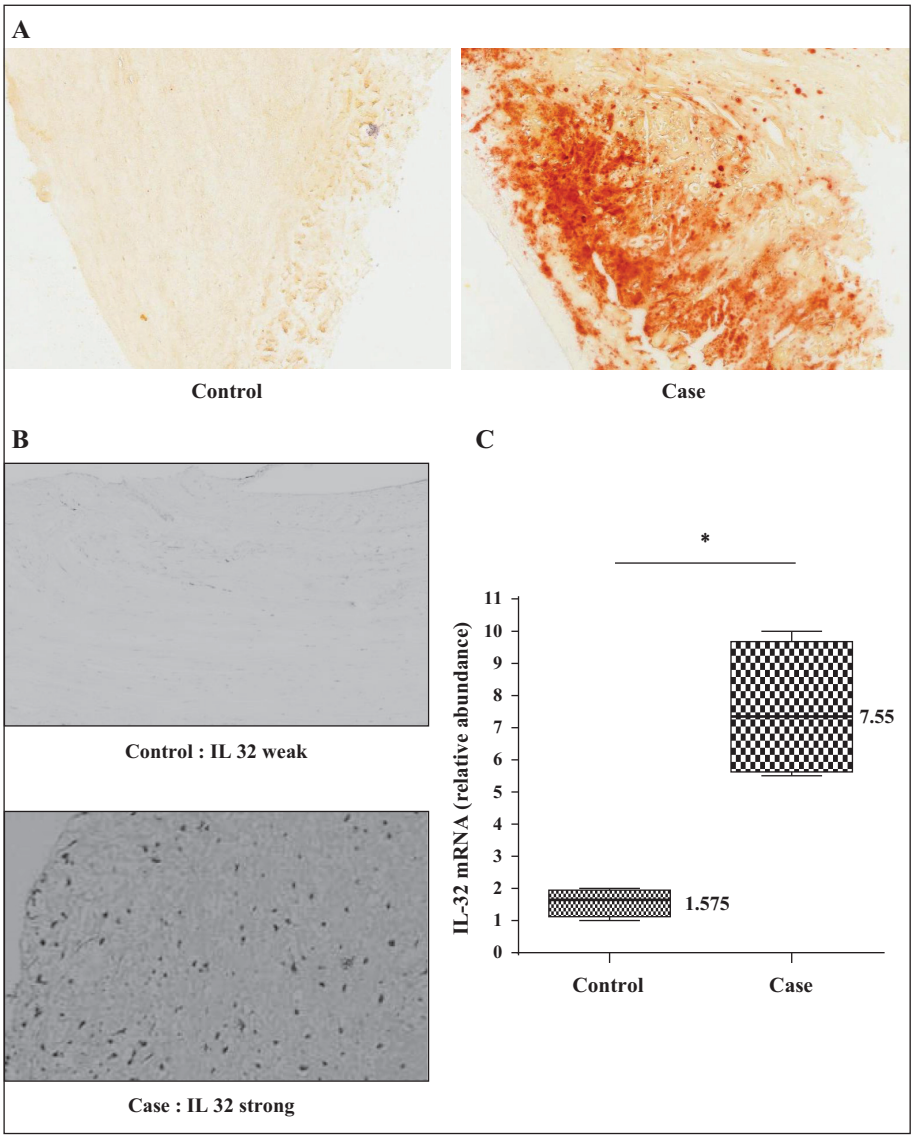

Figure 1
Increased IL-32 expression in human calcified aortic valve tissue. **(A)** Alizarin red staining of aortic valve tissue slides. Bright red staining shows calcification in calcified aortic valve cells. (10×) **(B)** IL-32 protein staining by immunohistochemistry in aortic valve tissue. **(C)** Relative expression level of IL-32 mRNA was significantly enhanced in calcified aortic valve tissue. The results are shown as mean relative abundance. SEM and quantified 4.79-fold change compared to control group. The data are expressed as mean ± SD. $*P < 0.05$ compared with control; $n = 8$.

overnight, which was then incubated with primary antibodies on a rocking platform at 4 °C for 24 h. The blots were washed in TBST and incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. After another wash, the membrane was subjected to film that had undergone chemiluminescence reagent treatment with ECL plus Western blotting reagents (Amersham). A densitometry method was used to calculate the bands. Afterwards, each blot was stripped and re-probed with anti-β actin antibodies to allow the normalization of expression between samples. This experiment was replicated 3 times in order to corroborate the results of this assay.

Human aortic valve interstitial cell isolation and culture

The human aortic valve interstitial cells were isolated following a modified method described elsewhere [31, 32]. Valve leaflets were rinsed in Earle's balanced salt solution (EBSS) and then digested in collagenase (2.5 mg/mL in M199 medium) for 30 mins at 37°C. After removing endothelial cells by vortex, the leaflets were further digested with a milder solution of collagenase medium (0.8 mg/mL) for 3 h at 37 °C. After vortexing and aspirating repeatedly to break up the tissue mass, the cell suspension was spun at 500 *g* for 2 mins to remove any remaining tissue. The supernatant was transferred into a fresh tube and spun again at 1100 *g* for 8 min at 4 °C. The cells were resuspended in full medium (M199 with penicillin G, streptomycin, amphotericin B, and 10% FBS), plated onto a 75-cm^2 flask, and cultured in a cell culture incubator supplied with 5% CO_2. When the cells reached 70–90% confluence, they were subcultured on plates and chamber slides. Cells from passages 4-6 were used for these experiments.

Enzyme-linked immunosorbent assay (ELISA)

RayBio® ELISA KIT was used to measure levels of cytokines (IL-8, IL-32, IL-6, TNF-α, and IL-1β). Capture antibodies of target cytokines were coated onto the wells

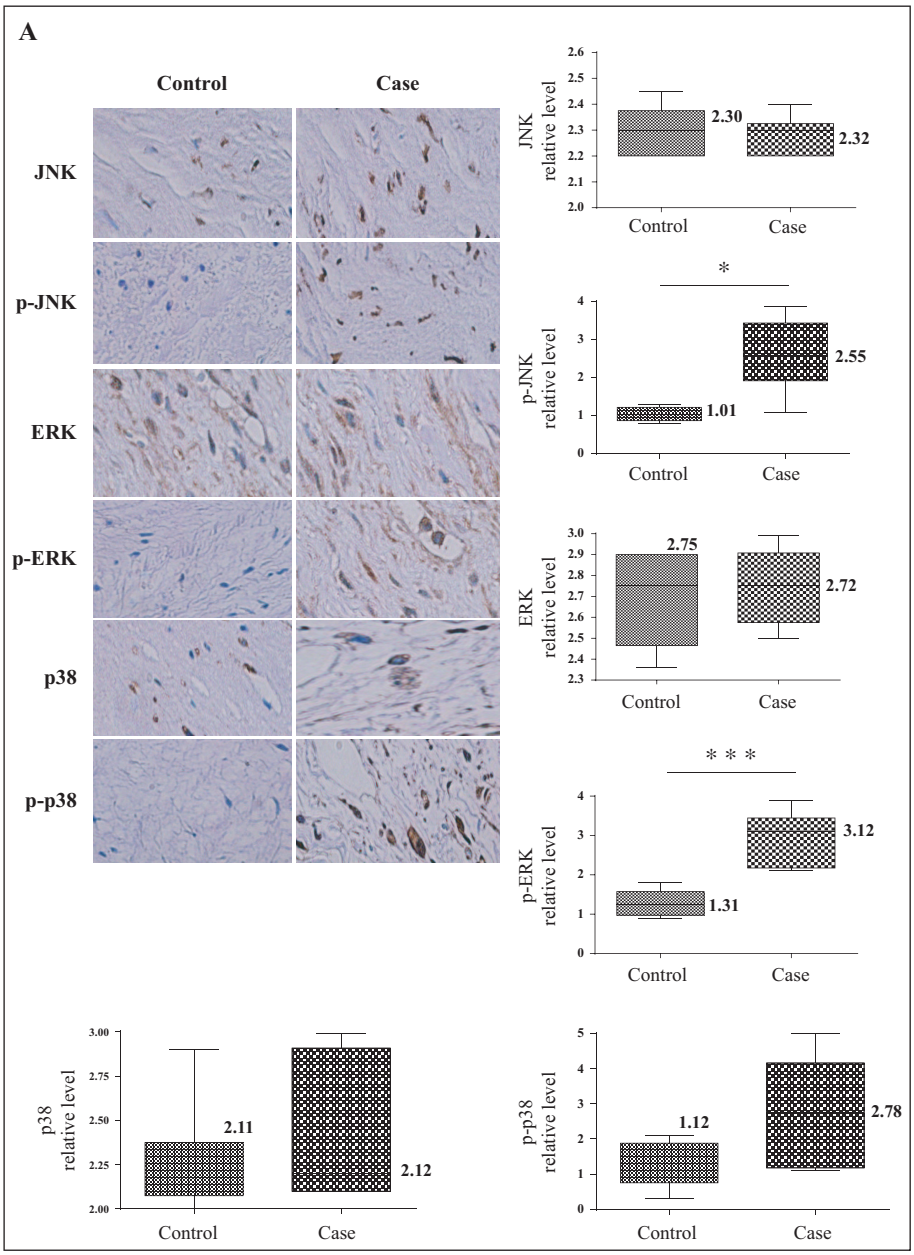

Figure 2

Increased MAPK pathway activity in calcified aortic valve tissue. **(A)** Immunohistochemical (IHC) staining of ERK, JNK, p38 and their phosphorylated forms in aortic valve tissue (400×); the quantification results are presented graphically. $*P < 0.05$, $***P < 0.001$; $n = 12$ **(B)** Western blotting of IL-32, ERK, JNK, p38 and their phosphorylated forms in aortic valve tissue. Quantification of protein levels was done by ImageJ, with the levels of each phosphor protein normalized to the relevant total protein and the level of IL-32 normalized to β-actin. $*P < 0.05$; $**P < 0.01$ and $***P < 0.001$; $n = 12$ **(C)** Immunohistochemical staining of NF-κB p65 in aortic valve tissue (400×); the quantification results are presented graphically; $n = 10$ **(D)** Western blotting of NF-κB p65 in nuclear extracts of aortic valve tissue.

of microplates. Samples, including standard (IL-32, IL-6, TNF-α and IL-1β), control specimens, and unknowns, were pipetted into these wells. During the first incubation, the protein antigen was bound to the capture antibody. After washing, a detection antibody was added to the wells and bound to the immobilized protein captured during the first incubation. After removal of excess detection antibody, an HRP conjugate (secondary antibody or streptavidin) was added. After a third incubation and washing to remove the excess HRP conjugate, a substrate solution was added and was converted by the enzyme to a detectable form (color signal). The intensity of this colored product was directly proportional to the concentration of antigen present in the original specimen.

Reagents and antibodies

SP600125, PD98059, and SB203580 as well as NF-kB activation inhibitor and IKK- inhibitor IV were purchased from EMD Chemicals (Gibbstown, NJ). Recombinant cytokine IL-32 was obtained from Peprotech (Rocky Hill, NJ). p38, JNK, ERK, phospho-p38, phospho-JNK, phospho-ERK, IL-32, NF-κB, and p65 antibodies were acquired from Cell Signaling (Beverly, MA).

Statistical analysis

Data are tested to determine whether it is normally distributed by Shipiro-Wilk test, then if there is homogeneity of variance by Levene's test to validate use of a parametric

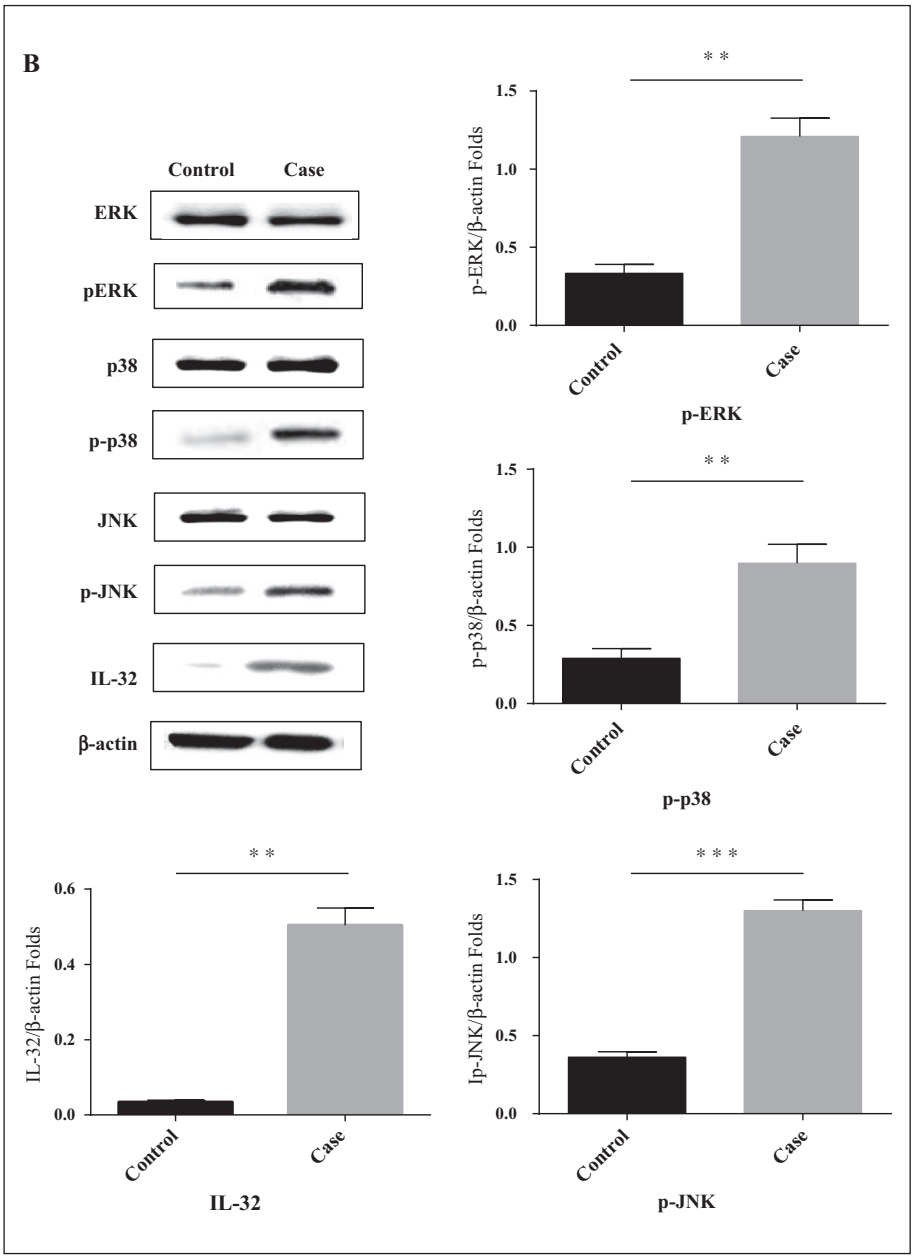

Figure 2

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or non-parametric test. Both patient data and immunohistochemistry data are use of a non-parametric test. Each experiment was performed in duplicate, and its average was used in the quantification. Data are expressed as the mean ± SD of averages from at least three experiments. Differences in means were assessed by the Student's *t*-test. A P value of <0.05 was considered statistically significant [33]. GraphPad Prism 6 statistical software was used for data analysis, and P values of less than 0.05 were considered significant.

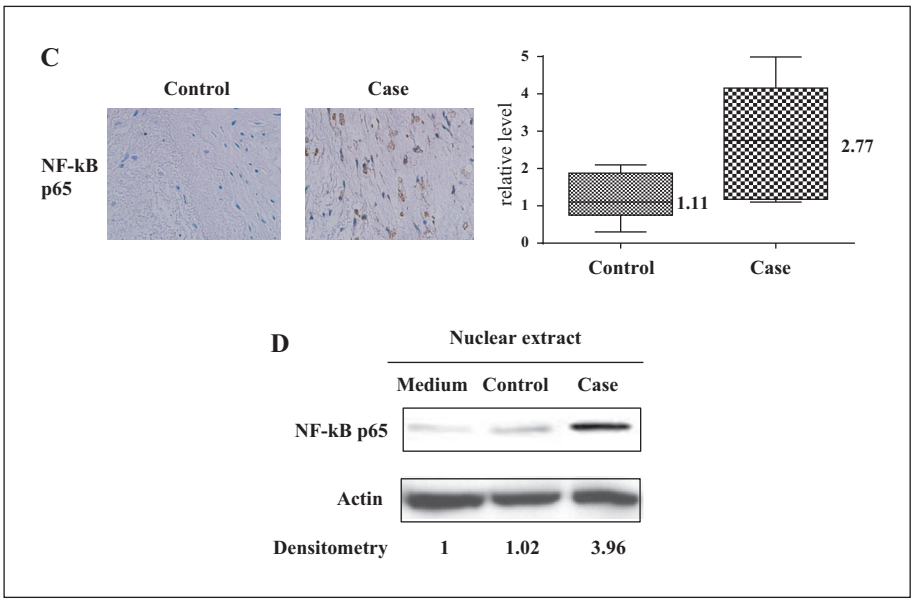
RESULTS

Increased IL-32 expression in human calcified aortic valve tissue

Human aortic valve specimens, calcified aortic valve tissue (case group) and non-calcified human aortic valve tissue (control group), were used for experiments throughout this study. As shown in *figure 1A*, the case group samples exhibited bright red staining, indicating calcium deposits. From tissue of 8 patients of each group, we analyzed expression of IL-32. Expression of IL-32 protein was detected by immunohistochemical staining using mAb raised against human IL-32. As shown in *figure 1B*, increased IL-32 expression was observed in interstitial cells of the case group as compared with the control group. Real-time PCR was applied to examine the expression level of IL-32 mRNA. In *figure 1C*, there was a 4.79-fold increase in IL-32 abundance in the case group as compared with the control group. These results suggest that IL-32 expression was upregulated in human calcified aortic valve tissue.

Increased IL-32 expression was associated with the MAPK pathway in calcified aortic valve tissue

We evaluated the MAPK pathway by immunohistochemical staining of ERK, p38, p-JNK, and their phosphorylated



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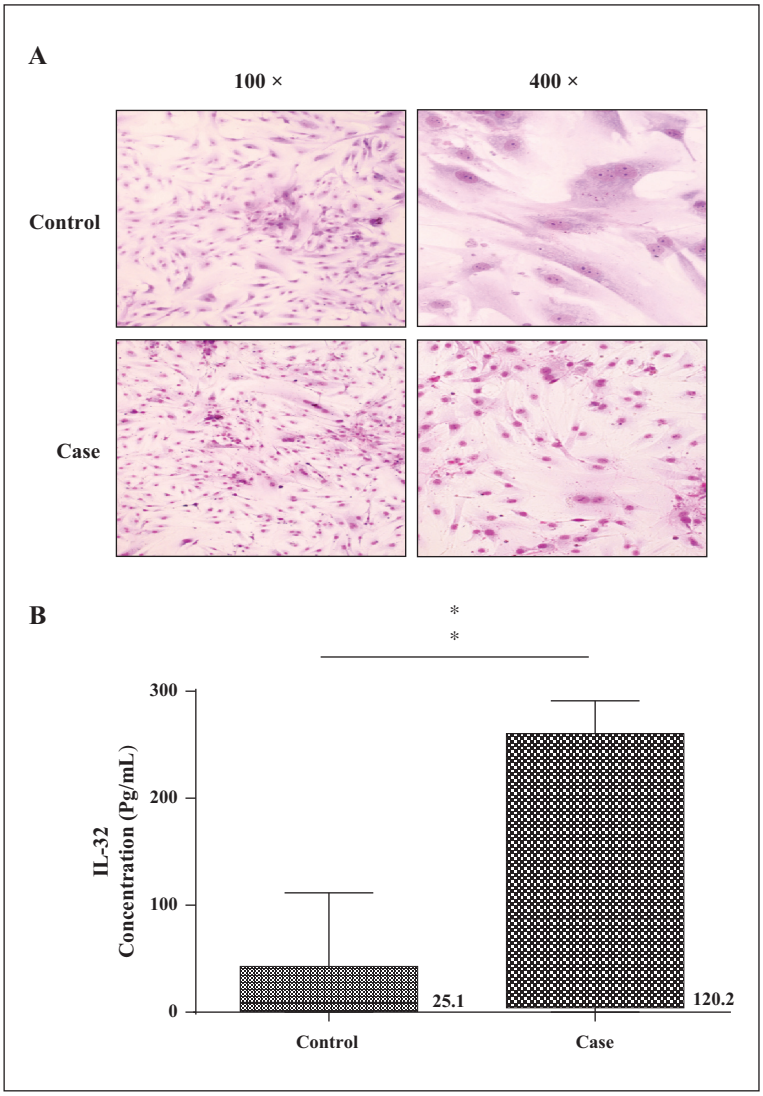

Figure 3

IL-32 is mainly induced via the MAPkinases/NF-κB Pathway. **(A)** Cell culture of human aortic valve interstitial cells in 10% FBS M199 medium, (100×, 400×) **(B)** IL-32 level determined by ELISA in primary cultured aortic valve interstitial cells; $n = 8$ **(C)** Effect of inhibitors of NF-κB, ERK, JNK, and p38 on IL-32 protein level (mean ± SEM); $n = 4$. Calcified human aortic valve interstitial cells were pretreated for 1 hour with various inhibitors as indicated (concentrations are given in 100 μM NF-κB inhibitor, 20 ng/mL ERK inhibitor, 1ng/mL JNK inhibitor, and 2.5 ng/mL p38 inhibitor). (*$P < 0.05$; **$P < 0.01$ and ***$P < 0.001$).

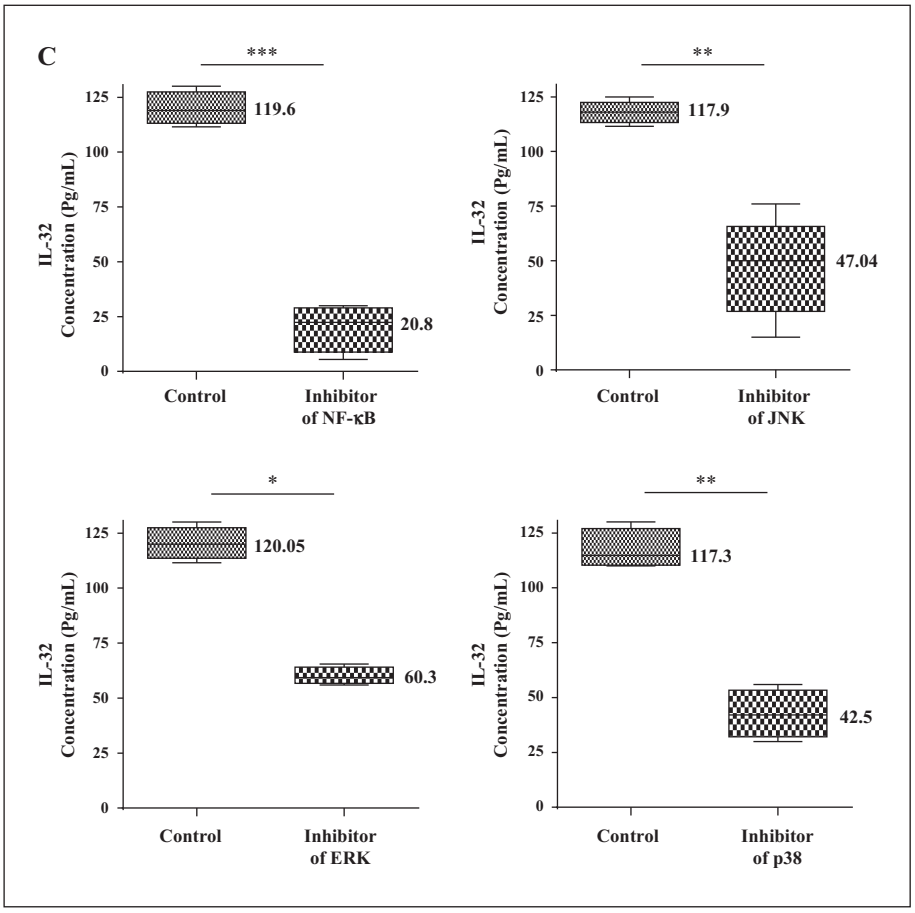

Figure 3

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forms in calcified aortic valve tissue. Valve tissue from 12 patients of each group was analyzed. As shown in *figure 2A*, staining of these proteins increased in the study group as compared with the control group. Increased levels of phosphorylated ERK, phosphorylated p38, phosphorylated JNK, and IL-32 in the study group as compared with the control group were also shown by Western blotting of these proteins (*figure 2B*). These results suggest that upregulation of IL-32 in calcified aortic valve tissue is associated with the activation of the MAPK pathway.

NF-κB activity was increased in human calcified aortic valve tissue

Next, we assessed NF-κB activity in calcified aortic valve tissue by immunohistochemical staining and Western blotting of nuclear extracts. Tissue from 10 patients from each group was analyzed. Results show that in the case group, presence of p65 in the nucleus is increased (*figure 2C, D*), suggesting that NF-kB pathway is more active in the calcified aortic valve tissue.

Increased IL-32 levels in cultured valvular interstitial cells

Human aortic valve interstitial cells isolation and culture are shown in (*figure 3A*). We analyzed cytokine levels in primary calcified aortic valve tissue cell cultures from 8 patients of each group by ELISA. Level of IL-32 was significantly increased in primary cultured valvular interstitial cells from human calcified aortic valve tissue with a fold change of 4.07 as compared with control (*figure 3B*).

IL-32 was mainly induced via the MAPkinases/NF-κB Pathway

To elucidate whether these pathways were involved in the induction of IL-32 in human calcified aortic valve interstitial cells, human calcified aortic valve interstitial cells cultures from 4 patients of each group were preincubated with inhibitors of NF-κB, JNK, ERK, and p38. For each inhibitor, we used a concentration close to its IC_{50}. As shown in *figure 3C*, IL-32 production was reduced by 82.6% in the presence of inhibitor of NF-κB. Whereas the JNK and the p38 MAPK inhibitors also inhibit IL-32 (reduced by 60.1% and 63.7%, respectively), there was a moderate but significant effect when the ERK inhibitor was applied (reduced by 49.5%).

Tissue phospho-activator of transcription 3 (p-STAT3) activity increased and was correlated with serum IL-32 expression in human calcified aortic valve tissue

Immunohistochemical staining of calcified and control aortic valve tissue revealed that while there was no difference in expression of p-STAT1, expression of p-STAT3 was increased in calcified aortic valve tissue (*figure 4A*). This suggests activation of the p-STAT3 pathway rather

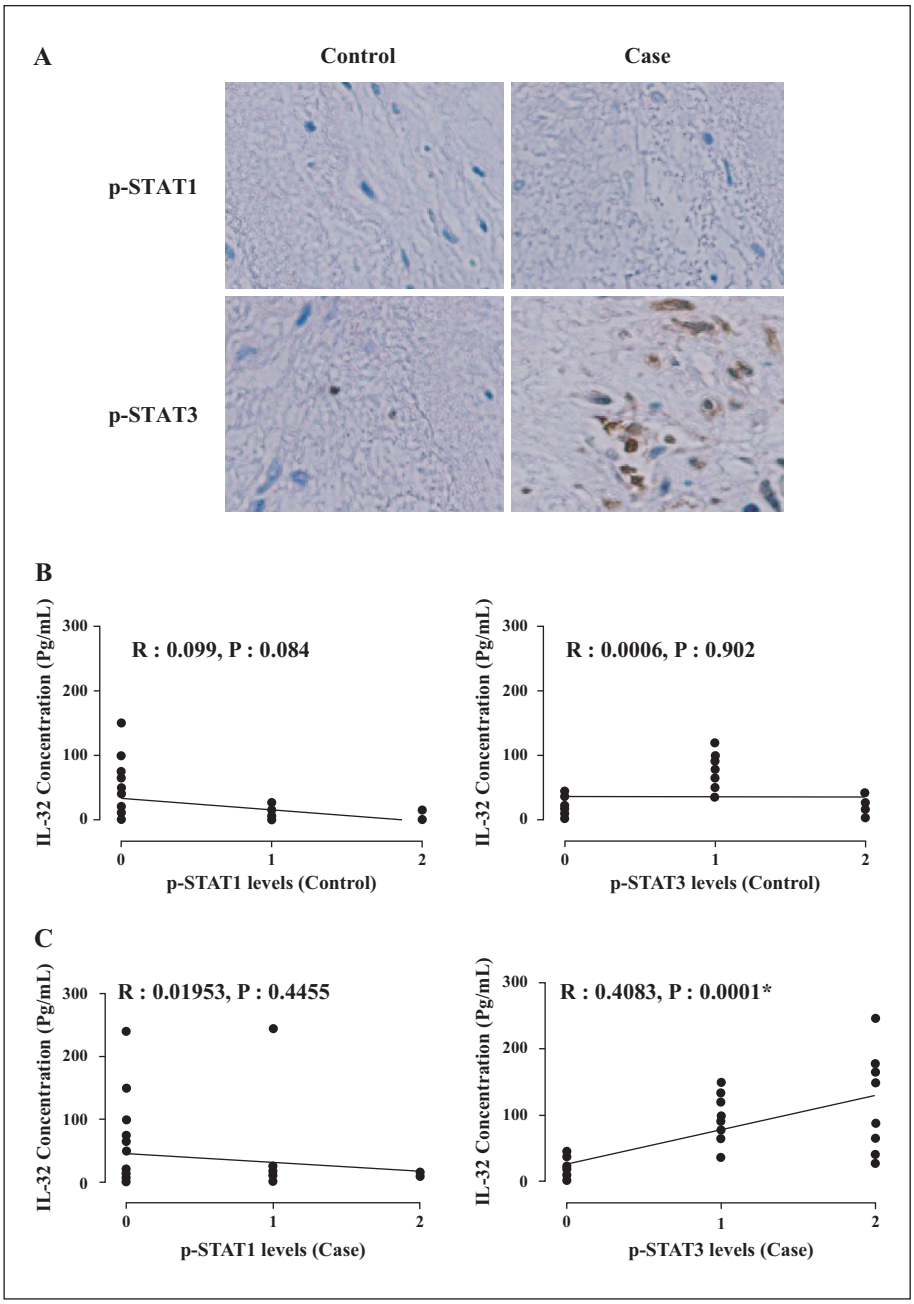

Figure 4

Signal transducer and phospho-activator of transcription 3 (p-STAT3)/IL-32 activity increased in human calcified aortic valve tissue. **(A)** Immunohistochemistry staining of p-STAT1 and p-STAT3 expression in aortic valve tissue (400×). **(B)** Immunoreactivity of p-STAT1 and p-STAT3 with over-expression of serum IL-32 in control (level 1: mean <10% nuclei or cytoplasma of human aortic valve interstitial cells stained, and level 2: mean ≧10% to <25% nuclei or cytoplasma of human aortic valve interstitial cells); $n = 5$ **(C)** Immunoreactivity of p-STAT1 and p-STAT3 with over-expression of serum IL-32 in calcified aortic valve tissue (level 1: mean <10% nuclei or cytoplasma of human aortic valve interstitial cells stained, and level 2: mean ≧10% to <25% nuclei or cytoplasma of human aortic valve interstitial cells); $n = 5$ (*$P < 0.05$).

than the p-STAT1 pathway in human calcified aortic valve tissue. From 5 patients of each group, immunoreactivity levels were determined by immunohistochemical staining (level 1: mean <10% nuclei or cytoplasma of human aortic valve interstitial cells stained, and level 2: mean ≧10% to <25% nuclei or cytoplasma of human aortic valve interstitial cells). Immunoreactivity levels of the control group for both p-STAT1 and p-STAT3 showed no correlation with overexpression of IL-32 (p-STAT1: $r = 0.099$, $P = 0.084$) (p-STAT3: $r = 0.0006$, $P = 0.902$) (*figure 4B*). However, expression of p-STAT3 in the study group showed a positive correlation with over-expression of IL-32 (p-STAT1: $r = 0.01953$, $P = 0.04455$) (p-STAT3: $r = 0.4083$, $P = 0.0001$*) (*figure 4C*).

Cultured valve cells treated with recombinant IL-32 induced downstream pro-inflammatory cytokines (TNF-α, IL-6, and IL-1β) and chemokine IL-8 expression

We further analyzed cytokines and chemokine levels in primary aortic valve tissue cell culture with recombinant IL-32 by ELISA. Cultures from 12 patients of each group were treated with recombinant IL-32 and those without treatment served as control. Levels of TNF-α, IL-6, IL-1β, and IL-8 were significantly increased in primary cultured cells treated with recombinant IL-32 as compared with control. Fold change in levels of IL-8, TNF-α, IL-6, and IL-1β were 9.7, 2.01, 2.47, and 2.24 as compared with control, respectively (*figure 5*).

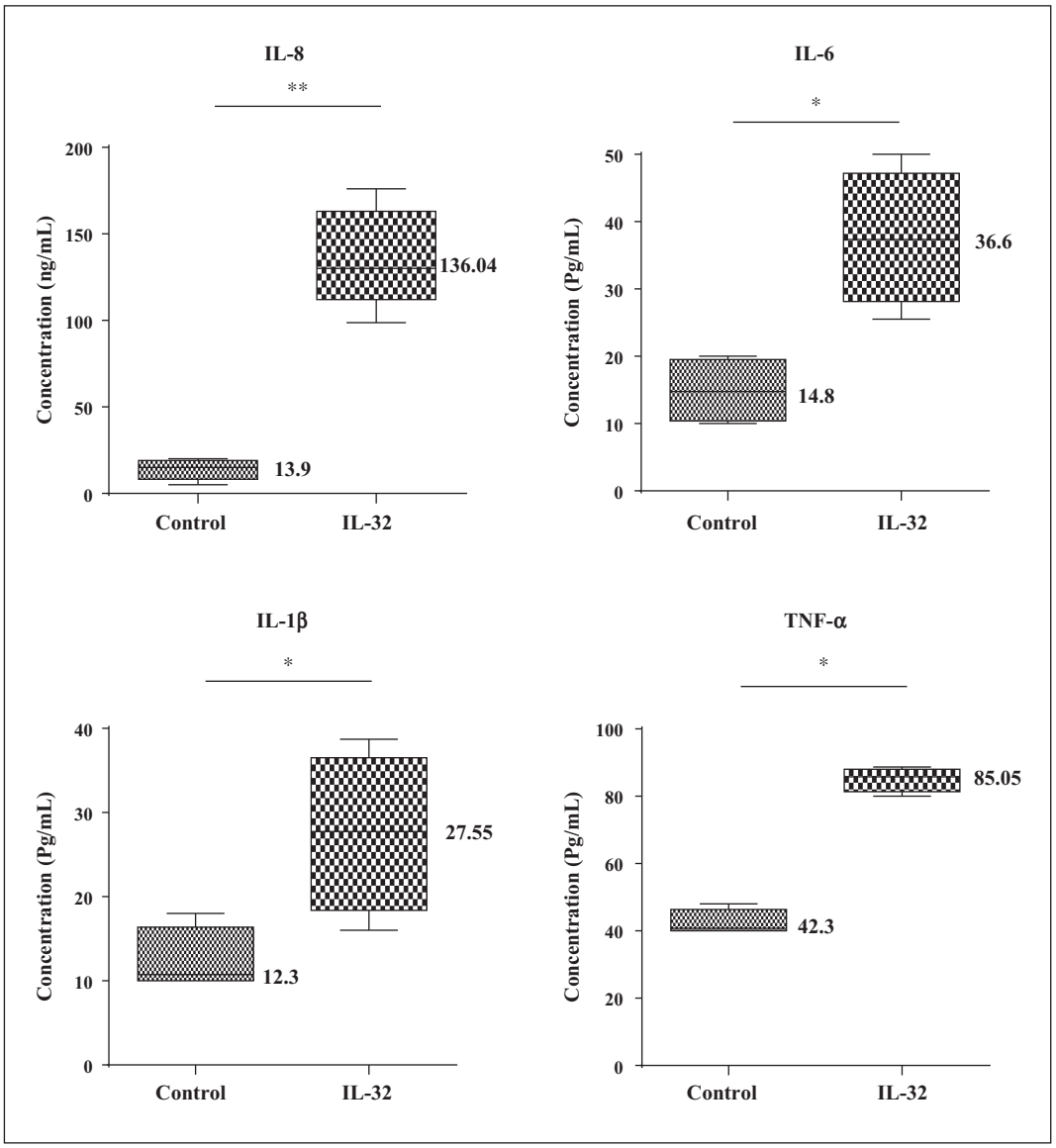

Figure 5
IL-32 induced expression of downstream cytokines. IL-8, TNF-α, IL-6, and IL-1β levels determined by ELISA in primary cultured aortic valve interstitial cells treated with (IL-32) or without (Control) recombinant IL-32; $n = 12$ (*$P < 0.05$; **$P < 0.005$).

DISCUSSION

Previous studies indicated that the progression of CAVD is an active disease process involving chronic inflammation leading to valve tissue calcification. However, the role of inflammatory cytokines and related protein kinases activity in the development of human aortic valve calcification process has not yet been fully elucidated.

The present study revealed a pro-inflammatory function of IL-32 in human calcified aortic valve cells. This is the first study to demonstrate that IL-32 protein expression was increased in human calcified aortic valve and that it is capable of inducing expression of various pro-inflammatory cytokines.

IL-32 is recognized as a "pro-inflammatory" mediator. In clinical settings, IL-32 has been widely implicated in inflammatory/autoimmune diseases, and its levels are directly related to disease severity in rheumatoid arthritis [6, 7]. In the pathology of cardiovascular disease, IL-32 can be present in vascular atherosclerotic plaques [8]. However, there are no published data on the activation and quantification of the IL-32 protein in human calcified aortic valve cells. In the present study, we found that IL-32 protein expression was significantly enhanced in calcified aortic valvular tissue compared with the control. Further, the quantified RT–PCR data revealed a good correlation, with a fold increase of 4.79.

In the treatment of clinical diseases, the therapeutic agent simvastatin can reduce NF-κB activity and downregulate atherosclerosis formation [15]. Another study also revealed an important role of the NF-κB pathway in mediating vascular cell osteogenic responses [34]. The potential of NF-κB to serve as a major therapeutic target in various cardiovascular disease was also reported in a previous study [14]. In the present study, we found that the level of NF-κB expression was strongly associated with human aortic valve calcification. Furthermore, we demonstrated an 82.6% decrease in IL-32 production in cells treated with inhibitor of NF-κB. This finding suggests NF-κB to be a potential attenuation regulator on IL-32 in valve cells.

The MAPKs play an important role in the pathogenesis of various types of cardiac and vascular diseases. The formation of neointima after injury also occurs through the

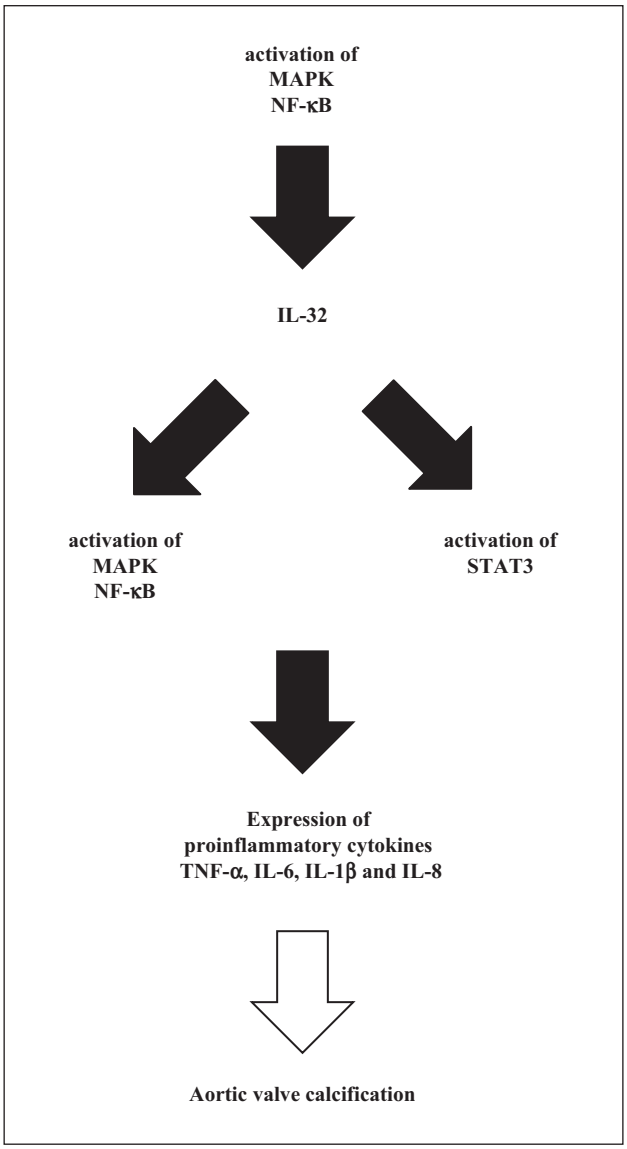

Figure 6
IL-32 plays an essential role in human calcified aortic valve via the regulation of the STAT3, MAPK, and NF-κB pathways.

activation of ERK1/2, JNK1/2, and p38 MAPK in vascular smooth muscle cells [9-12]. In this study, we revealed IL-32 upregulation was strongly associated with human aortic calcific valve cells via the MAPK pathway. p-ERK, p-JNK, and p-p38 had significantly higher expression in calcified aortic valve tissue. In human calcified aortic valve interstitial cell cultures inhibitors of JNK, ERK, and p38 mitigated the reduction of IL-32 production. Thus, in the present study, the results demonstrated that the pathway of IL-32 mainly involved the activity of MAPK signaling pathways in human calcified aortic valve interstitial cells.

STAT proteins are cytoplasmic transcription factors that are involved in various cytokine, hormone, and growth factor signal transduction pathways. In terms of the effects of STAT proteins on clinical cardiovascular condition, all of the STAT proteins have been reported to be strongly expressed in the myocardium, and they also augment the expression of other proteins in different types of heart diseases [22, 23]. STAT1 is essential for apoptosis in advanced atherosclerotic lesions *in vivo*. STAT3 plays a central role in cardiac pathophysiology and is a major mediator of pro-angiogenic signaling [26-29]. Currently, both STAT1 and STAT3 proteins have been implicated as having potential therapeutic value in the treatment of cardiovascular disease [35]. However, no studies have shown that STAT proteins are involved in the activity of human calcified aortic valve cells. Nonetheless, to further examine the relationship between tissue p-STAT proteins and IL-32 in human diseased aortic valves, we analyzed immunohistochemical expression of p-STAT1 and p-STAT3 proteins. Our data demonstrated that expression of p-STAT3 but not p-STAT1 was associated with overexpression of IL-32 in human aortic calcific valve cells (*figure 4B, C*). The p-STAT3 protein showed stronger positive staining compared to the control in the immunohistochemical study of calcified aortic valvular cells in the present study. Previous studies have noted that interleukins affect tumor progression through the p-STAT3 rather than p-STAT1 signaling pathway [36]. The present study is the first to demonstrate that STAT proteins play a role in human aortic valve calcification, and that STAT 3 has a greater effect on calcific aortic valve cells than on normal cells. The aforementioned evidence supports the notion that STAT proteins are expressed in human diseased aortic valve and have potential to suppress progression of valve calcification in an inflammatory milieu.

Several cytokines and molecules have been found to be associated with the calcification process. Interleukin-37 suppresses the osteogenic responses of human aortic valve interstitial cells *in vitro* and alleviates valve lesions in mice [37]. Galectin- 3 (Gal-3) is a proinflammatory molecule involved in vascular osteogenesis in atherosclerosis. Gal-3 was shown to mediate valve calcification in AS [38]. Progression of aortic valve stenosis is associated with TGF-β, which is present in calcified aortic valve cusps and promotes aortic valve interstitial cell calcification via apoptosis [39]. In the early stages of aortic stenosis, differentiation of myofibroblasts into osteoblasts appears to be coordinated by macrophages via the action of proinflammatory cytokines TNF-α, IL-6, IL-1β, and IL-8 [40]. TNF-α accelerates the calcification of human aortic valve interstitial cells via the BMP2-Dlx5 pathway [41], and promotes an osteoblast-like phenotype in human aortic valve myofibroblasts [42]. IL-6 production by VICs stimulates osteogenic transition and mineralization of the aortic valve through a BMP2 pathway [43]. IL-1β regulates remodeling of the extracellular matrix in calcific aortic valve stenosis [44] and induces production of IL-8 in human aortic valve interstitial cells [45]. Thus, we treated primary cultured cells with recombinant IL-32 to establish the effect of IL-32 on these cytokines in aortic valve cells. Levels of TNF-α, IL-6, IL-1β, and IL-8 were found to increase following recombinant IL-32 treatment (*figure 5*). These results suggest a role for IL-32 in regulating progression of aortic valve calcification.

In conclusion, our studied data demonstrate that IL-32 plays an essential role as an immune-regulatory cytokine in the tissue of calcific aortic valve cells. Here, we proposed a model of IL-32 action in calcified aortic valve cells based on our data and previous findings (*figure 6*). Because recombinant IL-32 induced downstream proinflammatory cytokines and chemokine expression in studied cultured valve cells, IL-32 could serve as a potential therapeutic target in diseased aortic valve calcific progression.

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