Simvastatin down-regulates osteogenic response in cultured human aortic valve interstitial cells

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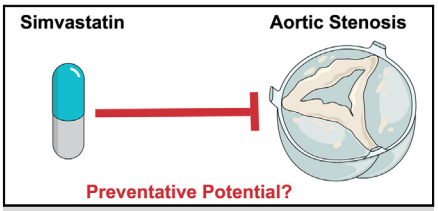
ABSTRACT

Background: Aortic valve interstitial cells have been implicated in the pathogenesis of aortic stenosis. In response to proinflammatory stimuli, aortic valve interstitial cells undergo an osteogenic phenotypic change. The purpose of this study was to determine whether the anti-inflammatory effects of statins prevent osteogenic activity in cultured aortic valve interstitial cells.

Methods: Human aortic valve interstitial cells were isolated from hearts explanted for cardiac transplantation. To test whether simvastatin down-regulates TLR4-induced osteogenic response, aortic valve interstitial cells were treated with simvastatin with and without TLR4 agonist lipopolysaccharide (LPS), and osteogenic markers were measured. Simvastatin's influence on in vitro calcium deposition was assessed by alizarin red staining. Knockdown of postreceptor signaling proteins (MyD88 and TRIF) was performed to determine which of 2 TLR4-associated pathways mediates the osteogenic response. Expression levels of TLR4-induced nuclear factor kappa light chain enhancer of activated B cells (NF-κB) and TLR4 expression were assessed after treatment with simvastatin. Statistical testing was done by analysis of variance ($P < .05$).

Results: Simvastatin decreased LPS-induced ALP and Runx2 expression and inhibited in vitro calcium deposition in aortic valve interstitial cells. Knockdown of MyD88 and TRIF attenuated the osteogenic response. Simvastatin attenuated TLR4-dependent NF-κB signaling and down-regulated TLR4 levels.

Conclusions: Simvastatin prevented TLR4-induced osteogenic phenotypic changes in isolated aortic valve interstitial cells via down-regulation of TLR4 and inhibition of NF-κB signaling. These data offer mechanistic insight into a possible therapeutic role for simvastatin in the prevention of aortic stenosis. (J Thorac Cardiovasc Surg 2021;161:e261-71)



Simvastatin may have preventative potential in aortic stenosis.

CENTRAL MESSAGE

Simvastatin prevents TLR4-induced osteogenic phenotypic changes in isolated human aortic valve interstitial cells. This finding has implications for prevention of aortic stenosis.

PERSPECTIVE

Aortic stenosis is a chronic inflammatory disease in which inflammation drives osteogenic phenotypic changes in aortic valve interstitial cells, resulting in the production of bone-forming proteins and calcium deposition. Simvastatin prevented TLR4-induced osteogenic phenotypic changes in isolated aortic valve interstitial cells, suggesting that statins may have a role in the prevention of aortic stenosis.

See Commentaries on pages e273 and e274.

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Video clip is available online.

As detailed in Video 1, which summarized the overall project design and findings, normal human aortic valve interstitial cells were grown in vitro and treated with lipopolysaccharide, an activator of Toll-like receptor 4 (TLR4). Osteogenic activity was elevated in response to

Abbreviations and Acronyms

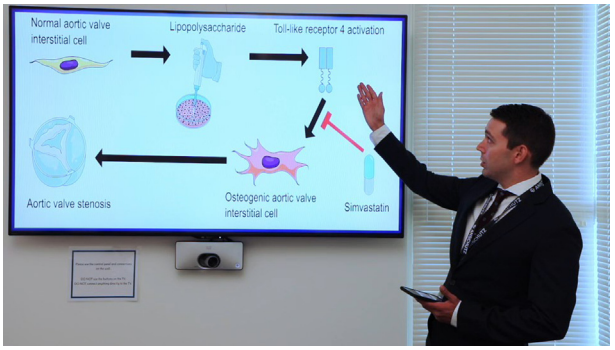
ALP = alkaline phosphatase
DAMP = damage-associated molecular pattern
DMSO = dimethyl sulfoxide
ELISA = enzyme-linked immunosorbent assay
FBS = fetal bovine serum
LPS = lipopolysaccharide
MyD88 = myeloid differentiation primary response 88
NF-kB = nuclear factor kappa light chain enhancer of activated B cells
PAMP = pathogen-associated molecular pattern
Runx2 = runt-related transcription factor 2
shRNA = short hairpin RNA
TLR4 = Toll-like receptor 4
TRIF = TIR-domain-containing adapter inducing interferon-β

TLR4 activation, and simvastatin abrogated this response. TLR4-induced osteogenic activity is believed to be a key pathobiological mechanism underlying the development of aortic stenosis.

Aortic stenosis is the third most prevalent cardiovascular disease worldwide, exceeded in prevalence only by hypertension and coronary artery disease. It is a disease of aging, and it is estimated that approximately 2% of the population age >70 years has moderate-to-severe aortic stenosis.[1] To date, there is no medical therapy for the disease.

Aortic valve interstitial cells represent the primary resident cell type within the aortic valve and have been implicated in the pathogenesis of aortic stenosis. In normal aortic valve leaflets, human aortic valve interstitial cells have a phenotype that is best characterized as a myofibroblast. However, in response to proinflammatory stimulation, isolated human aortic valve interstitial cells undergo phenotypic changes, first to that of an inflammatory cell and subsequently to an osteoblast-like cell.[2-4] The osteogenic phenotype is characterized by the expression of proteins necessary for skeletal bone formation, such as the osteogenic transcription factor runt-related transcription factor 2 (Runx2) and the enzyme alkaline phosphatase (ALP).[5,6] Thus, a growing body of evidence suggests that aortic stenosis is a chronic inflammatory disease.

Meng and colleagues have previously demonstrated that proinflammatory stimulation of Toll-like receptor 4 (TLR4) in aortic valve interstitial cells is an important mechanism by which the osteogenic phenotypic change may be induced.[3,7-9] TLR signaling is complex and may be mediated through 2 distinct intracellular pathways via unique adapter proteins: myeloid differentiation primary response 88 (MyD88) at the cell surface and TIR



VIDEO 1. A brief overview of the project, covering the importance of this topic, its relevance, the results of the project and the implications of findings. Toll-like receptor 4 (TLR4) activation by damage-associated molecular patterns (DAMPs) and pathogen- associated molecular patterns (PAMPs), such as lipopolysaccharide (LPS), results in activation of nuclear factor kappa light chain enhancer of activated B cells (NF-κB). This transcription factor influences the expression of 2 osteogenic factors, runt-related transcription factor 2 (Runx2) and alkaline phosphatase (ALP). This pathobiology is understood to underlie the development of aortic stenosis. TLR4 signals through 2 distinct pathways associated with unique adaptor proteins: myeloid differentiation primary response 88 (MyD88), associated with the cell membrane, and TIR-domain-containing adapter-inducing interferon-β (TRIF), which is found in the endosome. Simvastatin down-regulates TLR4 expression and inhibits TLR4-dependent osteogenic activity in aortic valve interstitial cells. Video available at: https://www.jtcvs.org/article/S0022-5223(19)32381-5/fulltext.

domain–containing adapter inducing interferon-β (TRIF), which is associated with the endosome (Figure 1). Each of these TLR4-associated signaling pathways activates a common transcription factor, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), which in turn modulates inflammatory and osteogenic responses in aortic valve interstitial cells.[2,10-13]

In addition to their antilipid properties, statins have anti-inflammatory properties.[14,15] We previously demonstrated that simvastatin down-regulates the TLR4-induced inflammatory response in isolated human aortic valve interstitial cells.[15] Therefore, we hypothesized that statins may prevent TLR4-induced osteogenic phenotypic changes in human aortic valve interstitial cells as well. The purpose of this study was to determine the effect of statins on TLR4-induced up-regulation of Runx2 and ALP in isolated human aortic valve interstitial cells. Our results demonstrate that these TLR4-induced osteogenic changes may be abrogated by statins.

MATERIALS AND METHODS

Cell Isolation and Culture

All patients gave informed consent for use of their aortic valves for this study, which was approved by the University of Colorado Denver's Institutional Review Board (IRB 11-1004).

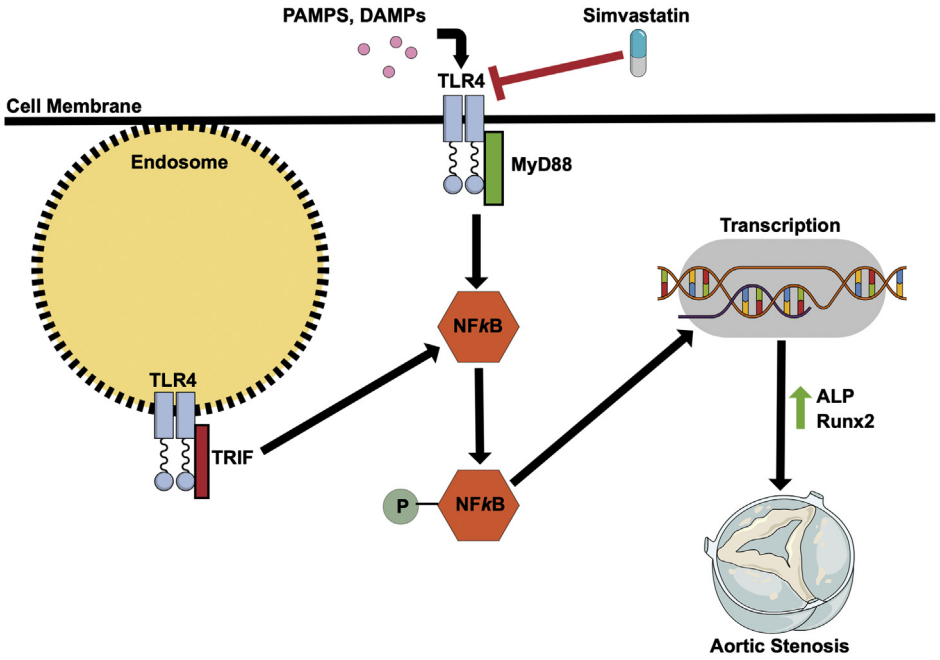


FIGURE 1. Relevant aspects of Toll-like receptor 4 (*TLR4*)-associated signaling pathways involved in valvular calcification mediated by aortic valve interstitial cells and the proposed mechanism of simvastatin's inhibition of TLR4-dependent signaling. Pathogen- associated molecular patterns (*PAMPs*) and damage-associated molecular patterns (*DAMPs*), such as lipopolysaccharide, activate TLR4 to activate the transcription factor, nuclear factor kappa light chain enhancer of activated B cells (*NF-κB*). TLR4 signals through 2 unique pathways associated with different adapter proteins: myeloid differentiation primary response 88 (*MyD88*) at the cell surface and TIR-domain-containing adapter-inducing interferon-β (*TRIF*) at the endosome. Both pathways converge onto NF-κB to phosphorylate this transcription factor, resulting in its nuclear translocation to increase the expression of alkaline phosphatase (*ALP*), an enzyme that facilitates valvular calcium deposition, and runt-related transcription factor 2 (*Runx2*), an osteogenic transcription factor. This represents the key pathobiology believed to underlie aortic stenosis.

Aortic valve interstitial cells were isolated and cultured as described previously,[16] with the following modifications: (1) valve leaflets were subjected to an initial digestion with a high concentration of collagenase (2.5 mg/mL) to remove endothelial cells (Sigma-Aldrich, S. Louis, Mo); (2) the remaining tissue was treated with a low concentration of collagenase (0.8 mg/mL) to free the interstitial cells; and (3) finally, cells were collected by centrifugation and cultured in M199 growth medium (Lonza, Walkersville, Md) containing penicillin G, streptomycin, amphotericin B, and 10% fetal bovine serum (FBS; Aleken Chemicals, Nash, Tex). Aortic valve interstitial cell isolates obtained by using this modified protocol are free of endothelial cells as verified by Von Willebrand factor staining.[16] Each isolate from a separate aortic valve was used as a cell line. Cells of passages 3 to 6 were used for this study and were treated when they reached 80% to 90% confluence.

Immunoblotting

Immunoblotting was applied to analyze levels of ALP and TLR4 (Santa Cruz Biotechnology, Dallas, Tex) and Runx2, MyD88, TRIF, total NF-κB, phosphorylated NF-κB, and β-actin (Cell Signaling, Danvers, Mass). Cells were lysed in sample buffer (100 mmol/L Tris·HCl pH 6.8, 2% SDS, 0.02% bromophenol blue, and 10% glycerol). Protein samples were separated on gradient (4%-20%) mini-gels and transferred onto nitrocellulose membranes (Bio-Rad, Hercules, Calif). The membranes were blocked with 5% skim milk solution for 1 hour at room temperature, incubated with a primary antibody against a protein of interest, and subsequently incubated with a peroxidase-linked secondary antibody specific to the primary antibody applied (Sigma-Aldrich). Chemiluminescent reagents (Thermo Fisher Scientific, Rockford, Ill) were used to expose protein bands in membranes, and ImageJ (Wayne Rasband, National Institutes of Health, Bethesda, Md) was used to analyze band density.

Simvastatin's Effect on TLR4-Induced Runx2 and ALP Levels in Aortic Valve Interstitial Cells

The concentrations of simvastatin (Sigma-Aldrich) used in this study were based on the precedent of a previous study.[15] Aortic valve interstitial cells were treated with 30, 40, and 50 μM simvastatin for 1 hour in cell culture medium (5% FBS), after which the medium was replaced with fresh cell culture medium (2.5% FBS). Cells were treated for 48 hours with TLR4 agonist lipopolysaccharide (LPS; 0.2 μg/mL; Sigma-Aldrich). Whole-cell lysates were analyzed for Runx2 and ALP by immunoblotting, and densitometry analysis was performed. LPS was dissolved in PBS and diluted in M199 culture medium. Dimethyl sulfoxide (DMSO) was used as a vehicle for simvastatin and thus served as a vehicle control.

Alizarin Red Staining

In vitro calcium deposit formation was analyzed by alizarin red staining after aortic valve interstitial cells were incubated with LPS (0.2 μg/mL) with or without pretreatment with simvastatin (50 μM for 1 hour, as described above) for 10 days in a conditioning medium (growth medium supplemented with 10 mmol/L β-glycerophosphate, 10 nmol/L vitamin

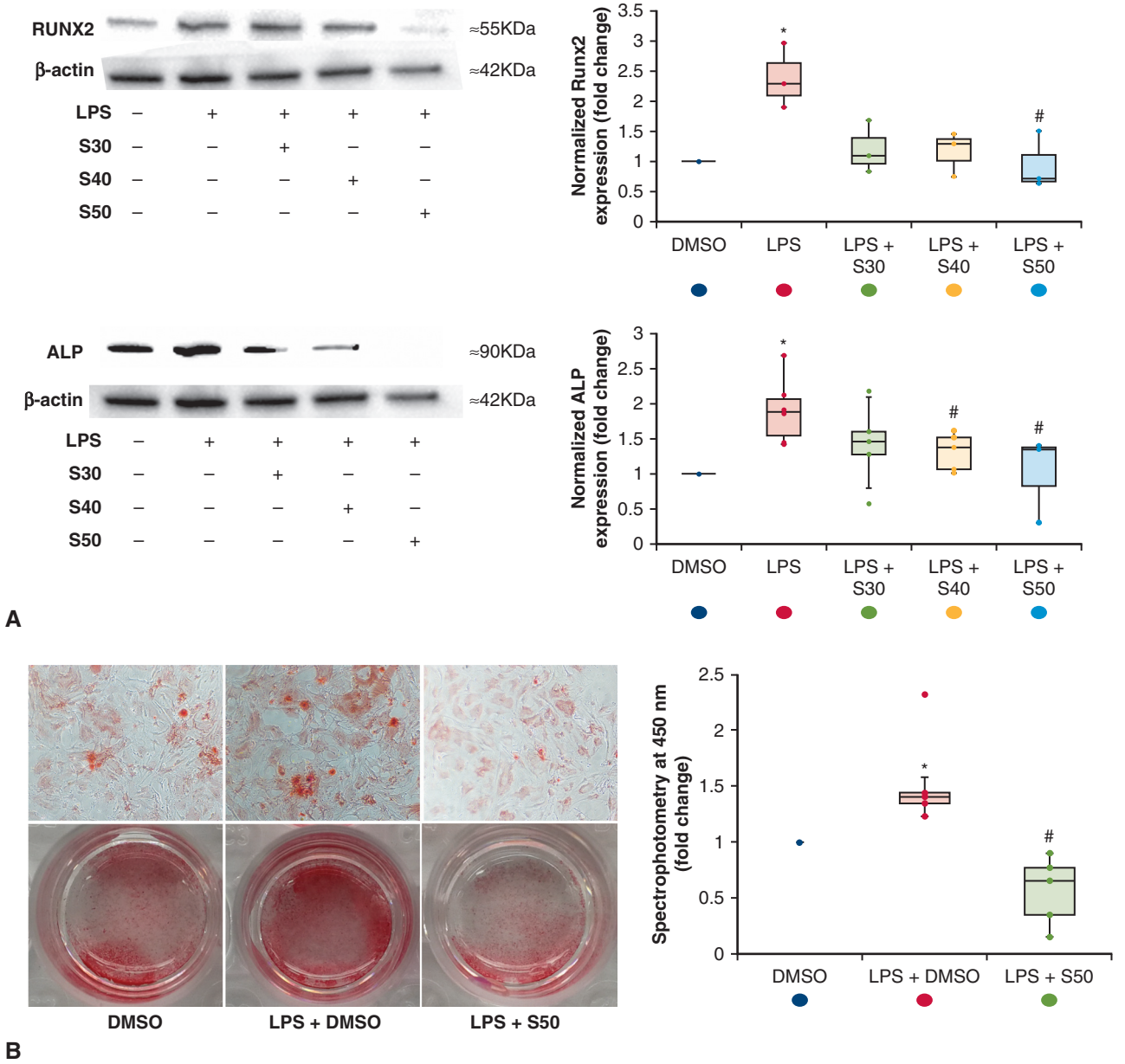


FIGURE 2. Simvastatin down-regulates runt-related transcription factor 2 (*Runx2*) and alkaline phosphatase (*ALP*) expression and in vitro calcium deposition. The *upper* and *lower borders* of each box represent the upper and lower quartiles, the *middle horizontal line* represents the median value, and the *upper* and *lower whiskers* represent the maximum and minimum values. (A) Normal human aortic valve interstitial cells were treated with the Toll-like receptor 4 (TLR4) activator lipopolysaccharide (*LPS*; 0.2 μg/mL) for 48 hours with and without pretreatment with simvastatin for 1 hour at concentrations of 30 μM, 40 μM, and 50 μM (designated s30, s40, and s50, respectively). Immunoblotting was used to assess levels of Runx2 and ALP. Simvastatin abolished the pro-osteogenic effects of LPS. *$P < .05$ vs dimethyl sulfoxide (*DMSO*) control; #$P < .05$ vs LPS + DMSO. (B) Normal human aortic valve interstitial cells were pretreated with simvastatin at a dose of 50 μM (*s50*) and subsequently treated with the TLR4 activator LPS (0.2 μg/mL) and calcium-supplemented conditioned medium for 10 days. Alizarin red staining was used to assess in vitro calcium deposition. Simvastatin abrogated the pro-calcific effects of LPS. *$P < .05$ vs DMSO control; #$P < .05$ vs LPS + DMSO.

D3, 10 nmol/L dexamethasone, and 8 mmol/L $CaCl_2$). Every 3 days, medium was removed and replaced with fresh conditioning medium. For aortic valve interstitial cells pretreated with simvastatin, a second 1-hour treatment with simvastatin (50 μM) was provided on day 4 of the 10-day incubation period. Day 4 was chosen because the cell culture medium is changed on this day according to our culture practices. After each treatment

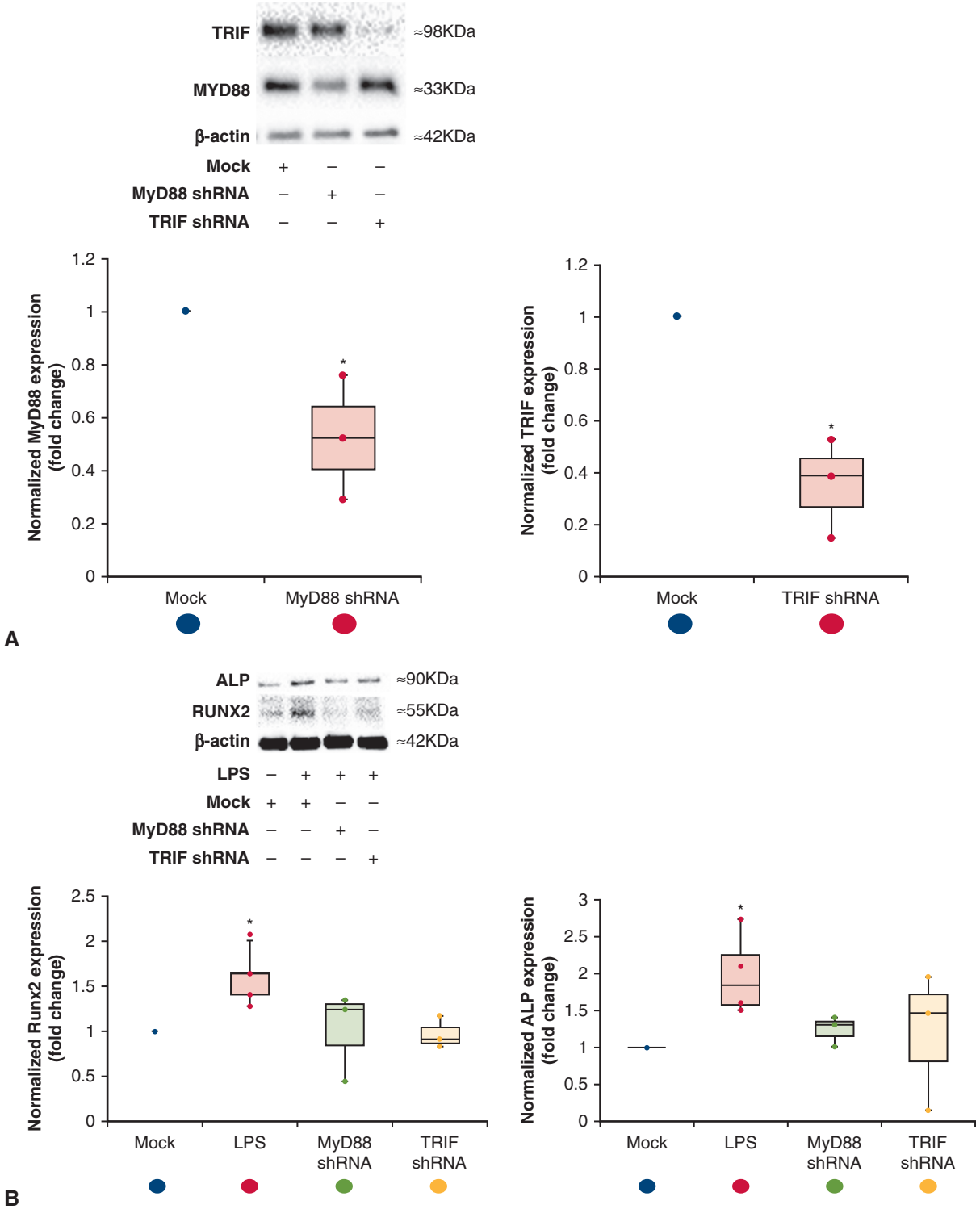


FIGURE 3. Knockdown of myeloid differentiation primary response 88 (*MyD88*) and TIR-domain-containing adapter-inducing interferon-β (*TRIF*) attenuates Toll-like receptor 4 (TLR4)-induced runt-related transcription factor 2 (*Runx2*) and alkaline phosphatase (*ALP*) expression. The *upper* and *lower borders* of each box represent the upper and lower quartiles, the *middle horizontal line* represents the median value, and the *upper* and *lower whiskers* represent the maximum and minimum values. (A) Diseased human aortic valve interstitial cells were treated with mock short hairpin RNA (*shRNA*) or shRNA for MyD88 or TRIF. Adequate knockdown of MyD88 and TRIF was achieved. *$P < .05$ vs mock control. (B) Normal human aortic valve interstitial cells were treated with the TLR4 activator lipopolysaccharide (*LPS*) for 48 hours after treatment with shRNA for MyD88 or TRIF and expression of 2 osteogenic factors, Runx2 and ALP, were assessed by immunoblotting. Knockdown of MyD88 and TRIF attenuated TLR4-induced Runx2 and ALP expression. *$P < .05$ vs mock + LPS. This indicates that both TLR4-associated adaptor proteins MyD88 and TRIF mediate the TLR4-dependent osteogenic response.

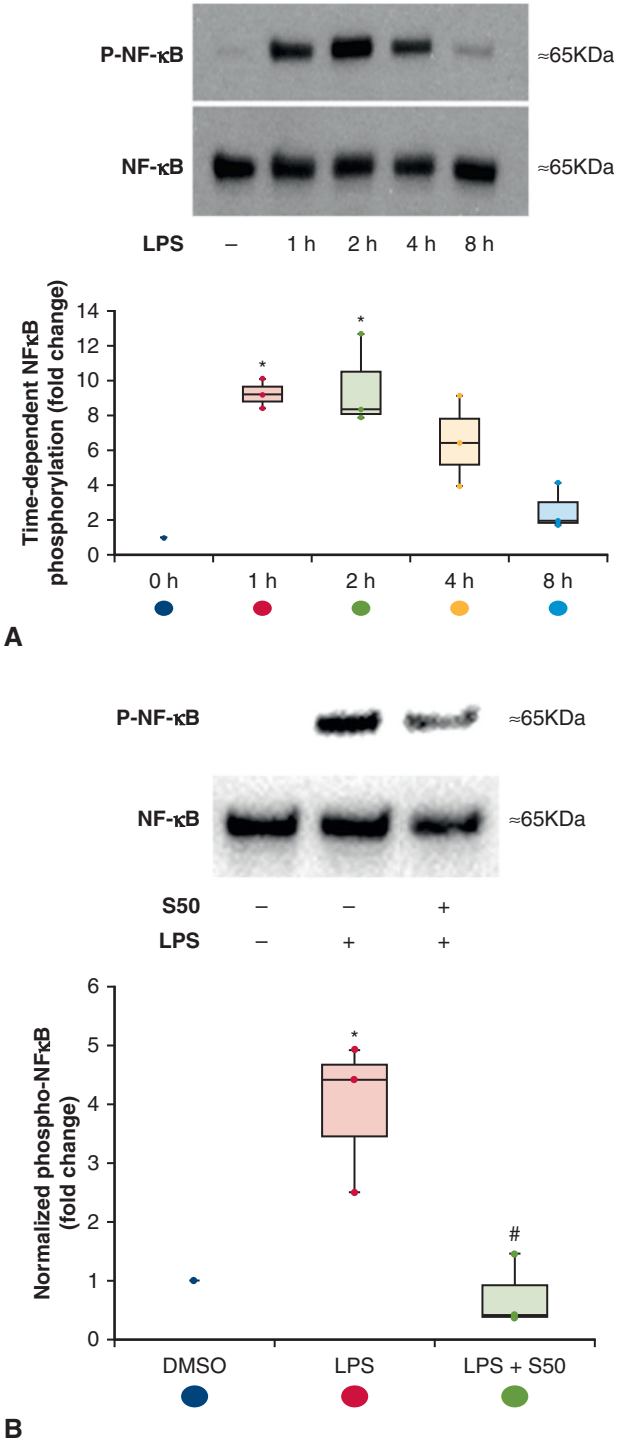


FIGURE 4. Peak activation of nuclear factor kappa light chain enhancer of activated B cells (*NF-κB*) following Toll-like receptor 4 (TLR4) activation occurs at 2 hours, and simvastatin attenuates this response. The *upper* and *lower borders* of each box represent the upper and lower quartiles, the *middle horizontal line* represents the median value, and the *upper* and *lower whiskers* represent the maximum and minimum values. (A) Normal human aortic valve interstitial cells were treated with TLR4 activator lipopolysaccharide (*LPS*; 0.2 μg/mL), and phosphorylation of the osteogenic transcription factor NF-κB was assessed at 1, 2, 4, and 8 hours. Phosphorylation of NF-κB was highest at 2 hours. *$P < .05$ vs control. (B) Normal human aortic valve interstitial cells were treated with LPS (0.2 μg/mL) for 2 hours with and without simvastatin pretreatment for 1 hour at a dose of 50 μM (*S50*). Simvastatin attenuated the pro-osteogenic activation of NF-κB mediated by TLR4 activation with LPS. *$P < .05$ vs control; #$P < .05$ vs LPS.

with simvastatin, medium was removed and replaced with fresh conditioning medium. LPS (0.2 μg/mL) was administered after each simvastatin treatment.

Alizarin red staining for calcium deposition was performed as described previously.[9] In brief, cell monolayers were washed twice with PBS and fixed for 15 min in 4% paraformaldehyde, followed by incubation with 0.2% alizarin red solution (pH 4.2) for 2 hours. Excessive dye was removed by washing with distilled water. Alizarin red staining was examined and photographed with a Nikon Eclipse TS100 microscope (Nikon, Tokyo, Japan). To quantitatively analyze alizarin red staining, wells were rinsed with distilled water, and alizarin red stains were bleached with 10% acetic acid at 85°C. Supernatant was analyzed spectrophotometrically at 450 nm.[17]

Modulation of NF-κB Activation by Simvastatin

The time of peak activation, or phosphorylation, of NF-κB was determined after treatment of aortic valve interstitial cells with LPS (0.2 μg/mL) and assessment of phospho-NF-κB levels by immunoblotting at 1, 2, 4, and 8 hours after application of LPS. Peak activation of NF-κB was found to occur at 2 hours after LPS treatment. The effect of simvastatin on activation of NF-κB was determined after pretreatment with simvastatin 50 μM for 1 hour in cell culture medium (5% FBS). The medium was then removed and replaced with fresh cell culture medium (2.5% FBS), and TLR4 stimulus (LPS, 0.2 μg/mL) was applied to cells for 2 hours. Cell lysates were analyzed by immunoblotting for phosphorylated and total NF-κB.

Lentiviral-Mediated Knockdown Experiments

Knockdown experiments were performed to decrease the expression of MyD88 or TRIF as described previously.[18] In brief, aortic valve interstitial cells were pretreated with polybrene for 30 minutes before infection. Then cells were infected with lentiviruses expressing mock short hairpin RNA (shRNA), MyD88 shRNA (TRCN0000008024), or TRIF shRNA (TRCN0000123200). After 3 days of infection, the effects of knockdown were validated by immunoblotting using specific antibodies. Levels of Runx2 and ALP in cell lysates were assessed by immunoblotting after treatment with TLR4 stimulus (LPS, 0.2 μg/mL) for 48 hours in cell culture medium (2.5% FBS).

Effect of Simvastatin Treatment on TLR4 Receptor Levels in Cell Lysates

Aortic valve interstitial cells were plated onto 24-well plates and treated with simvastatin (50 μM) for 1 hour in cell culture medium (5% FBS). The medium was then removed and replaced with fresh cell culture medium (10% FBS). Whole-cell lysates were harvested at various time points following treatment with simvastatin (1, 2, and 4 hours), and immunoblotting was used to measure TLR4 levels.

Enzyme-Linked Immunosorbent Assay for TLR4

Aortic valve interstitial cells were plated onto 24-well plates and treated with simvastatin (50 μM) for 1 hour in cell culture medium (5% FBS). The medium was then removed and replaced with fresh cell culture medium (10% FBS). Cell culture medium was collected at various time points (1, 2, and 4 hours) after simvastatin treatment for use in enzyme-linked immunosorbent assays (ELISAs) to assess TLR4 levels in the medium.

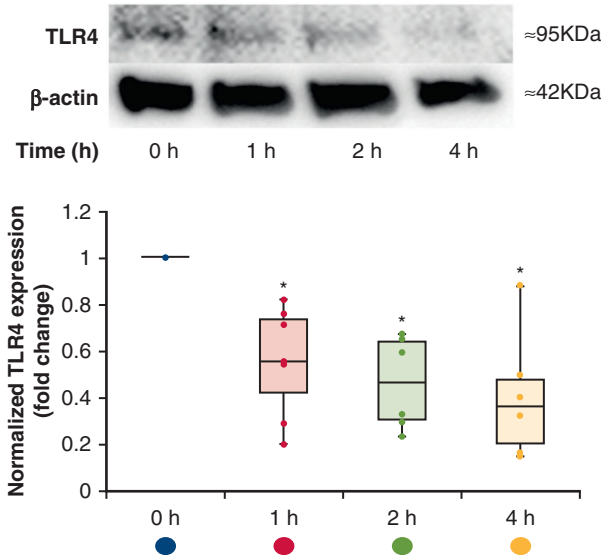


FIGURE 5. Simvastatin negatively regulates Toll-like receptor 4 (*TLR4*) in human aortic valve interstitial cells. The *upper* and *lower borders* of each box represent the upper and lower quartiles, the *middle horizontal line* represents the median value, and the *upper* and *lower whiskers* represent the maximum and minimum values. Normal human aortic valve interstitial cells were treated with simvastatin for 1 h at a concentration of 50 μM, and TLR4 levels in whole-cell lysates were assessed at 1, 2, and 4 hours. Simvastatin negatively regulated TLR4 at all time points. *$P < .05$ vs dimethyl sulfoxide control.

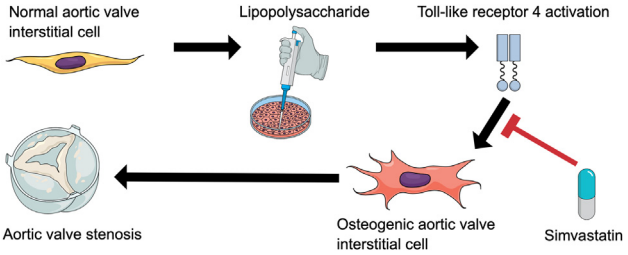


FIGURE 6. Normal human aortic valve interstitial cells were grown in vitro and treated with lipopolysaccharide (LPS), an activator of Toll-like receptor 4 (TLR4). Osteogenic activity was elevated in response to TLR4 activation, and pretreatment with simvastatin abrogated this response. TLR4-induced osteogenic activity is believed to be a key pathobiological mechanism underlying the development of aortic stenosis.

Before use in ELISA experiments, medium was hyperconcentrated (20×) via centrifugation in spin columns (Millipore Amicon Ultra 2-mL centrifugal filter; Ultracel 30-kDa filter, catalog no. UFC203024; Millipore Sigma, Burlington, Mass) according to the manufacturer's protocol. All samples were duplicated. The ELISA assay (Human TLR4 ELISA Kit, catalog no. RAB1088; Sigma-Aldrich) was performed according to the manufacturer's protocol. Optical densities were obtained with an Eon plate reader (Biotek, Winooski, Vt). Optical densities were averaged for each sample duplicate, and this value was used for analysis.

Statistical Analysis

Data were normalized to control groups and presented as box-and-whisker plots in which the upper and lower borders of each box represent the upper and lower quartiles, the middle horizontal line represents the median value, and the upper and lower whiskers represent the maximum and minimum values. Statistical analysis was performed using Prism (GraphPad Software, La Jolla, Calif). Analysis of variance with the post hoc Bonferroni/Dunn test was used to analyze differences between experimental groups, and Student's *t* test was applied to compare data between groups. Normal distribution of data was confirmed and if not met, the nonparametric Mann-Whitney *U* test was used instead. Statistical significance was defined as $P < .05$.

RESULTS

Donor Information

Normal aortic valve leaflets were obtained from 4 patients with the diagnosis of idiopathic dilated cardiomyopathy who underwent heart transplantation at the University of Colorado Hospital. At the time of excision, valve leaflets were grossly normal: thin, pliable, and with no evidence of calcification or fibrosis. Calcified aortic valve leaflets were obtained from 3 patients with severe aortic stenosis at the time of aortic valve replacement at the University of Colorado Hospital.

Simvastatin Inhibits TLR4-Induced Osteogenic Activity

Expression of Runx2 was up-regulated following TLR4 activation with LPS, and simvastatin abolished this effect at a dose of 50 μM ($P < .05$). ALP expression was enhanced after TLR4 activation, and simvastatin attenuated this response at doses of 40 μM and 50 μM ($P < .05$) (Figure 2). Simvastatin attenuated TLR4-induced in vitro calcium deposition by normal aortic valve interstitial cells. Macroscopic results are shown in Figure 2; greater calcium deposition was present in wells treated with LPS, whereas wells pretreated with simvastatin had lower calcium deposition than controls.

Lentiviral-Mediated Knockdown of MyD88 and TRIF

Diseased aortic valve interstitial cells were used to test various shRNAs to determine which provided the best knockdown results. Approximately 50% knockdown of MyD88 and 65% knockdown of TRIF was achieved with select shRNAs ($P < .05$) (Figure 3). TLR4 activation resulted in elevated Runx2 and ALP expression, and lentiviral-mediated knockdown of MyD88 and TRIF abolished this effect ($P < .05$) (Figure 3).

Modulation of NF-κB Activation by Simvastatin

Peak phosphorylation, or activation, of NF-κB was determined by treating aortic valve interstitial cells with LPS, and immunoblotting was used to assess phospho-

NF-κB at 1, 2, 4, and 8 hours after LPS treatment. Activation of NF-κB was found to be highest at 2 hours after LPS treatment. The effect of simvastatin on TLR4-mediated NF-κB phosphorylation at 2 hours after TLR4 activation was assessed. Simvastatin attenuated TLR4-induced NF-κB activation at a dose of 50 μM ($P < .05$) (Figure 4).

Effect of Simvastatin Treatment on TLR4

To determine the effect of simvastatin on TLR4, aortic valve interstitial cells were treated with simvastatin, and TLR4 levels were assessed by immunoblotting at various time points. Simvastatin negatively regulated TLR4 in whole-cell lysates of normal aortic valve interstitial cells at 1, 2, and 4 hours after treatment with simvastatin at a dose of 50 μM ($P < .05$) (Figure 5). On high-sensitivity ELISA of cell culture medium, TLR4 was undetectable in medium at 1, 2 and 4 hours after simvastatin treatment. ELISA was then repeated with hyperconcentrated medium (approximately 20×), and TLR4 levels were again undetectable in medium at 1, 2, and 4 hours after treatment with simvastatin.

DISCUSSION

This study demonstrates that statins may prevent TLR4-induced osteogenic phenotypic changes in aortic valve interstitial cells (Figure 6). Pretreatment with simvastatin decreased TLR4-induced expression of Runx2 and ALP and abrogated in vitro calcium deposition in isolated human aortic valve interstitial cells. Intracellular signaling by both MyD88- and TRIF-associated pathways appears to mediate TLR4-induced osteogenic changes, and statin pretreatment prevents TLR4-induced up-regulation of NF-kB. Our findings also suggest that anti-inflammatory actions of statins may be mediated in part by down-regulation of TLR4. The negative ELISA results suggest that receptor shedding is not responsible for this result. The ability of simvastatin to down-regulate TLR4 within 1 hour suggests that this likely occurs via increased protein degradation, rather than decreased gene expression, a process that is slower to affect protein levels. Overall, these data suggest that a pharmacologic agent, statins, may have a role in treating aortic stenosis.

The anti-inflammatory effects of statins have been demonstrated in a variety of clinical and experimental settings.[14,19,20] In isolated human aortic valve interstitial cells, Venardos and colleagues[15] previously demonstrated that statins blocked TLR4-mediated inflammation. In gastrointestinal malignancies, where immunoreceptor signaling has been found to be critical to disease initiation and propagation, the anti-inflammatory properties of statins have been shown to inhibit cellular pathobiology associated with oncogenesis.[21,22] Interestingly, TLR4-mediated inflammation has been specifically implicated in the pathogenesis of some of these cancers.[22] In the present study, statins prevented TLR4-induced osteogenic changes in isolated human aortic valve interstitial cells.

In addition, this study revealed MyD88 and TRIF-dependent TLR4 signaling modulates Runx2 and ALP expression. The pathobiology of aortic stenosis is complex and involves signaling through both pathways. The TLR4-MyD88 pathway is believed to be the predominant regulator of inflammation driven by TLR4 activation.[8] Interestingly, TRIF is an adaptor protein largely associated with the endosome, which suggests a mechanistic role for the endosome in regulating the pathophysiology responsible for valvular calcification.[10,17]

Clinical trials in which statins have been studied as a treatment for aortic stenosis have largely been negative.[23,24] In these trials, statins were initiated in patients who already had significant aortic stenosis; thus, it is possible that in such patients, the disease may have already advanced beyond a point where the actions of statins could demonstrate a benefit. However, the results of the present study and the study of Venardos and colleagues[15] suggests that treatment with statins earlier in the course of the disease may be preventive.

This study has several limitations. Foremost, this is an in vitro study of primary human aortic valve interstitial cells and as such cannot recapitulate the complex microenvironment of the aortic valve in vivo. Therefore, results from this study cannot be directly translated to clinical outcomes. In addition, patients from whom "normal" valve leaflets were harvested in this study suffered from idiopathic dilated cardiomyopathy. Although valves had no gross evidence of disease, it is possible that aortic valve interstitial cells isolated from these valves may differ from those in valves of normal hearts. Finally, whether the concentration of simvastatin used in this study truly approximates the concentration at the aortic valve in vivo is unknown. The statin concentrations studied were based on previous studies. Future studies may include in vivo animal or human studies in which subjects with risk factors for aortic stenosis, such as chronic kidney disease, are treated with statins as an attempt to prevent the development of aortic stenosis. It may be that statins have therapeutic efficacy to prevent aortic stenosis when used in this fashion.

In summary, the results of the present study demonstrate that simvastatin prevented TLR4-induced osteogenic changes in human aortic valve interstitial cells. The effects of simvastatin were accomplished through down-regulation of total TLR4 expression and/or modulation of NF-κB activation. Furthermore, this study highlights 2 mediators of the osteogenic response in aortic valve interstitial cells

downstream to TLR4: adaptor proteins MyD88 and TRIF. Simvastatin's ability to down-regulate TLR4 results in the inhibition of all downstream signaling pathways and ultimately attenuates the osteogenic response in aortic valve interstitial cells. These data offer mechanistic insight into a possible therapeutic role for simvastatin in the treatment and/or prevention of aortic stenosis.

Conflict of Interest Statement

Authors have nothing to disclose with regard to commercial support.

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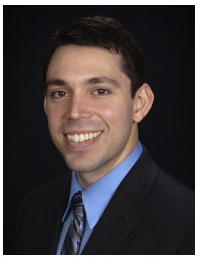
Key Words: aortic, valve, heart, statin, stenosis, calcification, cardiac

Discussion

Dr Joseph Woo *(Stanford, Calif)*. Good morning. I think we'll make our announcements first. On behalf of the WTSA, I welcome you to this morning's concurrent Adult Cardiac Surgery Forum. Evaluations for the annual meeting will be conducted through an online survey. The survey will be sent to all meeting attendees within a week of the conclusion of the annual meeting. Once you complete the brief survey, you will be able to retrieve and print out your CME certificate for your attendance at the annual meeting. The business meeting today at 12:00 pm is for active and senior members only. Immediately following the business meeting is a family luncheon on the Sun and Spa Deck at 12:30. If you have not already signed up for a table at this evening's President's Banquet, you must do so this morning. The seating chart and signup sheets are located in the registration area. All registered attendees must wear their badges to enter this event. This session will end at 8:15 sharp, and the General Session will begin at 8:30. Please keep presentation times to 5 minutes with a 3-minute discussion.

It is my privilege to introduce our first speaker, Michael Jarrett from the University of Colorado. His title is "Simvastatin down-regulates Toll-like receptor 4–induced osteogenic response in human aortic valve interstitial cells."



Dr Michael Jarrett *(Aurora, Colo)*. Dr Woo, members, and guests. Aortic stenosis is the third most common cardiovascular disease. It is exceeded in prevalence only by coronary artery disease and hypertension, yet there is no medical therapy available to treat or prevent this disease. Aortic valve interstitial cells are the predominant resident cell type in valvular leaflets. In response to inflammation, AVICs undergo phenotypic changes and transition from myofibroblasts to inflammatory cells and subsequently to an osteoblast-like cell. The osteogenic phenotype is characterized by increased expression of proteins important for skeletal bone formation such as Runx2, the transcription factor, and ALP, which is an enzyme. Proinflammatory stimulation of Toll-like receptor 4 is an important mechanism by which the osteogenic phenotypic change is induced. TLR4 signals through 2 pathways, MYD88 at the cell surface and TRIF at the endzone. Both pathways activate a common transcription factor, NF-κB that ultimately modulates inflammatory and osteogenic responses in AVICs.

In addition to antilipid properties, statins have anti-inflammatory properties. Previous work by Venardos and colleagues demonstrated that simvastatin inhibits Toll-like receptor 4-induced inflammatory responses in AVICs. We therefore hypothesize that simvastatin may inhibit the TLR4-induced osteogenic phenotypic change as well.

The first thing was to determine if simvastatin inhibits the osteogenic response that is mediated by TLR4. Here we had human primary AVICs and treated with LPS as TLR4 stimulus for a period of 48 hours and with or without simvastatin at 30, 40, and 50 μM concentrations, and then we assessed for expression of Runx2 and ALP. You can see here that both proteins were up-regulated after TLR4 stimulus, and that simvastatin attenuated that response.

We next sought to determine simvastatin's influence on in vitro calcium deposition. Here AVICs were treated with a condition media for a period of 10 days with LPS with or without simvastatin at a 50 μM dose. After that time, calcium deposition staining was performed, and results are shown here. TLR4 stimulus up-regulates in vitro calcium deposition, and simvastatin inhibited that response.

The second aim was to see if simvastatin inhibits NF-κB activation that is TLR4-dependent. Peak NF-κB phosphorylation was found to occur 2 hours after TLR4 stimulus was applied. Therefore, AVICs were treated with LPS with and without simvastatin and phosphorylation of NF-κB assessed. You can see here, simvastatin up-regulated or…excuse me, LPS up-regulated phosphorylation of NF-κB, and simvastatin abrogated that response.

We next sought to determine the role of the adapter proteins, MYD88 and TRIF in terms of mediating the TLR4-dependent osteogenic response. Here we used a lentiviral-mediated knockdown of both MYD88 and TRIF and then treated AVICs with LPS for a period of 48 hours and assessed for expression of Runx2 and ALP. Both proteins here were up-regulated by TLR4 stimulus, and knockdown of both MYD88 and TRIF attenuated that response.

A third and final aim was to see whether simvastatin modulates TLR4 levels in AVICs. Here AVICs were treated with simvastatin, and then TLR4 levels were measured at various time points afterward. Starting at 1 hour, TLR4 was down-regulated, and that response was maintained across all time points. We then followed up with a high- sensitivity ELISA of cell media to see if receptor shedding was occurring, and we were unable to detect TLR4 in cell culture media.

So in summary, simvastatin inhibits the TLR4-induced osteogenic responses in AVICs and attenuates the TLR4-mediated NF-κB activation. Both MYD88 and TRIF-dependent signaling modulate the osteogenic response, and simvastatin negatively regulates TLR4. In light of negative ELISA results, we can't attribute this to receptor shedding, so it is more likely due to increased protein degradation rather than decreased gene expression, which takes longer to affect protein levels.

So in conclusion, simvastatin may have a therapeutic potential in aortic stenosis. Thanks. I'm happy to answer any questions.

Unidentified speaker. So there have been clinical trials looking at statins in order to modify or reduce the calcium burden in aortic valve disease without positive results. Do you think it's just the time point in the progression of the disease, and that if you don't treat early enough, you're just not going to have an impact of pharmacologic therapy?

Dr Jarrett. Thank you for that question. It's a great point, and prior clinical studies have shown largely negative results as you have said. I do, you know, believe that that is the case, so all these patients in these clinical studies had significant valvular disease at the time that they were enrolled, and simvastatin was unable to reverse the disease that was already established, but I think treating a patient at risk for AS or with just mild clinically silent disease with a statin may have, you know, preventative potential, and I think that is where clinical trials should be focused in the future, absolutely.

Dr Tom Burdon *(Stanford, Calif).* Great talk, and congratulations to the Colorado group and David and the basic science pursuit of the inflammation of the aortic valve as a cause.

Dr Jarrett. Thank you.

Dr Burdon. So as statins are sort of swarming our culture and more and more literature comes out every day that everybody should be taking a statin, and a lot of people had been taking statins for a long time, is there any plan in Colorado to sort of somehow dovetail that in to what Richard says, into a clinical project because the time is right now? People have been taking statins for at least 15 years now, and an ever-increasing percentage of our population is taking them.

Dr Jarrett. That's a great question. At this point, we have not made plans to do that. I think it would be a great clinical study to follow up with, you know, in terms of our basic science findings. We have done some animal models that have shown that basically inhibition of inflammation has a positive result, but we haven't done that with statins yet, so I think that would be a great thing to do, we should get organized and try to do that.

Dr Woo. Last question.

Dr Burdon. One quick final question. Do you think that the mechanism of calcification that you've shown is the same in bioprosthetic valves?

Dr Jarrett. The same in what was that?

Dr Burdon. Bioprosthetic valves.

Dr Jarrett. Oh, that's a great question.

Dr Burdon. Should we take everyone and put them on a statin that we put a bioprosthesis in, which has also been tried clinically, but maybe the doses aren't right, or other issues.

Dr Jarrett. I see. So that's interesting, because the pathobiology of AS in a valve that's in vivo, you know, the normal valve is driven by living cells and in the prosthetic valve, those cells are absent or not alive. So I think that we do know that the pathobiology of aortic stenosis involves inflammation, it involves monocytes, it involves other inflammatory cells, and I think that some of these same transcription factors and enzymes are involved, and I think some of the inflammatory cells are involved, but I do think it has to be a different mechanism ultimately. I don't think it's the same, just due to the fact that the interstitial cells are not mediating the calcification in the prosthetic valve. It's the other cells, the inflammatory cells that are doing that.

Dr Woo. Great, well done.

Dr Jarrett. Thank you very much.