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Adult Aortic Valve Interstitial Cells Have Greater Responses to Toll-Like Receptor 4 Stimulation

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Abstract

Background—Aortic valve interstitial cells (AVICs) have been implicated in the pathogenesis of calcific aortic valve disease. Signal transducer and activator of transcription 3 (Stat3) possesses antiinflammatory effects. Given that calcification occurs in adult valves, we hypothesized that AVICs from adult valves more likely undergo a proosteogenic phenotypic change than those from pediatric valves and that may be related to different Stat3 activation in the response of those two age groups to toll-like receptor 4 (TLR4).

Methods—AVICs from healthy human aortic valve tissues were treated with TLR4 agonist lipopolysaccharide. Cellular levels of TLR4, intercellular adhesion molecule 1, bone morphogenetic protein 2, and alkaline phosphatase, as well as phosphorylation of p-38 mitogen-activated protein kinase (MAPK), nuclear factor- κ B (NF- κ B), and Stat3, were analyzed.

Results—Toll-like receptor 4 protein levels were comparable between adult and pediatric AVICs. Adult cells produce markedly higher levels of the above markers after TLR4 stimulation, which is negatively associated with phosphorylation of Stat3. Inhibition of Stat3 enhanced p-38 MAPK and NF- κ B phosphorylation and exaggerated the expression of the above markers in pediatric AVICs after TLR4 stimulation.

Conclusions—Adult AVICs exhibit greater inflammatory and osteogenic responses to TLR4 stimulation. The enhanced responses in adult AVICs are at least partly due to lower levels of Stat3 activation in response to TLR4 stimulation relative to pediatric cells. Stat3 functions as a negative regulator of the TLR4 responses in human AVICs. The results suggest that Stat3 activation (tyrosine phosphorylation) may be protective and that TLR4 inhibition could be targeted pharmacologically to treat calcific aortic valve disease.

Calcific aortic valve disease (CAVD) may be an active disease process. Mechanisms of inflammation and osteogenesis appear to play important roles in the pathogenesis of the disease [1–4]. For example, inflammation of heart valves due to chronic or recurrent oral bacterial infection [5] or rheumatic heart disease may cause calcific aortic valve disease [6]. Therefore, CAVD may be the result of pathologic inflammatory processes.

The aortic valve interstitial cells (AVICs) are the principal cell type within the aortic valve leaflet and have been implicated in the pathogenesis of CAVD [2, 7]. These cells are metabolically active and responsive to proinflammatory stimulation [2]. Under basal conditions, AVICs have a phenotype similar to myofibroblasts [2]. But proinflammatory stimulation by toll-like receptors 2 and 4 induces an inflammatory phenotype in adult AVICs characterized by the production of intercellular adhesion molecule 1 (ICAM-1) and bone morphogenetic protein 2 (BMP-2) [2]. In adult AVICs, toll-like receptor 4 (TLR4) stimulation activates protein kinases such as p38 mitogen-activated protein kinase (MAPK) and the transcription factor nuclear factor- κ B (NF- κ B) [2]. In turn, activation of NF- κ B in adult AVICs is associated with a proinflammatory and proosteogenic phenotype change. Characteristics of this proosteogenic phenotype include the production of bone-forming proteins, osteocalcin and osteopontin, bone-forming transcription factors, and enzymes necessary for bone formation (alkaline phosphatase [ALP]) [8]. Hence, there is a linkage between mechanisms of inflammation and osteogenesis in AVICs. Elucidation of this linkage will lead to a better understanding of the pathogenesis of CAVD.

Stat3 possess both proinflammatory and antiinflammatory characteristics. For example, Stat3 phosphorylation is important for proinflammatory cytokine release by macrophages after lipopolysaccharide (LPS) stimulation [9], but Stat3 also drives an antiinflammatory response in human macrophages [10]. In particular, interleukin-10, the best-studied antiinflammatory cytokine, is mediated through Stat3 activation [11, 12]. Furthermore, a recent study demonstrated that Stat3 seems to have an important role in the protection of inflammation-induced heart damage with advanced age [13]. Therefore, given that calcification occurs in adult valves, we speculated that adult AVICs have greater inflammatory and osteogenic responses to LPS stimulation than those of pediatric cells, and that this difference is due to lower Stat3 activation. Stat3 may function as a protective and antiinflammatory regulator of TLR4 response to the inflammatory stimulation in AVICs.

Material and Methods

Material

Antibodies against ICAM-1 and TLR4 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody against BMP-2 was purchased from ProSci (Poway, CA). Antibody against ALP was purchased from ABCAM (Cambridge, MA). Antibodies against phosphorylated p38 MAPK, total p38 MAPK, phosphorylated NF- κ B, total NF- κ B, phosphorylated Stat3(Y), phosphorylated Stat3(S), total Stat3, glyceraldehyde 3-phosphate dehydrogenase, and β -actin were purchased from Cell Signaling (Beverly, MA). Medium 199 was purchased from Lonza (Walkersville, MD). Lipopolysaccharide (*Escherichia coli* 0111:B4) and all other chemicals were from Sigma-Aldrich Chemical (St. Louis, MO).

Cell Isolation and Treatment

Aortic valve leaflets were collected from the explanted hearts with cardiomyopathy undergoing heart transplantation at the University of Colorado Hospital and Children's Hospital of Colorado (Table 1). All adult patients and children's parents gave informed

consent for the use of their own or their children's valves for this study, which was approved by the University of Colorado Denver Institutional Review Board.

The AVICs were isolated and cultured using a previously described method [14], with modification [2]. Briefly, valve leaflets were subjected to sequential digestions with collagenase, and cells were collected by centrifugation. Cells were cultured in M199 growth medium containing penicillin G, streptomycin, amphotericin B, and 10% fetal bovine serum. Cells from passages 2 to 6 were used for this study. Cells were treated when they reached 80% to 90% confluence. The AVICs were stimulated with LPS (200 ng/mL) for 24, 48, and 72 hours to assess changes in inflammatory and proosteogenic markers. Then, the AVICs were stimulated with LPS (200 ng/mL) for 5 to 120 minutes to evaluate NF- κ B and p-38 MAPK activation. The AVICs were also stimulated with LPS (200 ng/mL) for 5 to 30 minutes to study Stat3 activation. After pretreating pediatric AVICs with Stat3 inhibitor S3I-201 for 1 hour, LPS (200 ng/mL) was added for 1 hour to see NF- κ B and p-38 MAPK changes. Pediatric AVICs were treated with or without Stat3 inhibitor S3I-201 (5 μ m) in the presence of LPS (200 ng/mL) for 48 hours to determine inflammatory and proosteogenic marker changes.

Knockdown of Stat3 With Short Hairpin RNA

Pediatric AVICs were infected using a previously described method [15]. Briefly, cells were treated with control short hairpin RNA (shRNA) or Stat3 shRNA (shStat3) or combined with LPS (200 ng/mL) for 72 hours, and then collected and subjected to immunoblotting analysis.

Immunoblotting

The AVICs in culture were lysed. Cell lysates were resolved on sodium dodecyl sulfate polyacrylamide gel electrophoresis gels, and the proteins were transferred onto polyvinylidene difluoride membranes. After being blocked with 5% skim milk solution, membranes were incubated with primary antibodies, followed by peroxidase-linked secondary antibodies specific to the primary antibodies. Protein bands were revealed using the enhanced chemiluminescence system. Band density was analyzed using the National Institutes of Health Image J software (Wayne Rasband, National Institutes of Health, Bethesda, MD). Blots from specific antiphospho antibodies probed were stripped and reprobed with antibodies that recognize total analytes.

Statistical Analysis

Data are represented as mean $\pm$ SEM. An unpaired Student's *t* test was used for two-group comparisons. All *p* values less than 0.05 were considered significant.

Results

Markedly Higher Inflammatory and Osteogenic Responses in Adult Cells After TLR4 Stimulation

To determine the effect of TLR4 stimulation on the inflammatory and proosteogenic responses in AVICs of adult and pediatric valves, we analyzed cellular ICAM-1, BMP-2,

and ALP. Stimulation of cells with LPS induced ICAM-1, BMP-2, and ALP expression in both pediatric and adult cells. The ICAM-1 protein level was increased threefold in pediatric cells, but it was increased 4.6-fold in adult cells (Fig 1). In contrast, stimulation had a minimal effect on BMP-2 and ALP expression in pediatric AVICs. Therefore, adult AVICs have significantly greater inflammatory and osteogenic responses to TLR4 stimulation.

Comparable Levels of TLR4 in AVICs of Pediatric and Adult Aortic Valve

To examine whether the greater inflammatory and osteogenic responses to TLR4 stimulation are associated with increased levels of the receptor in adult AVICs, we analyzed cellular TLR4 levels among different age groups. Representative immunoblots in Figure 2A show that levels of TLR4 protein are comparable in pediatric donors of different ages. Furthermore, the cellular levels of TLR4 are comparable between pediatric cells and adult cells (Fig 2B). It appears that the greater inflammatory and osteogenic responses to TLR4 stimulation in adult cells are not due to alterations in the protein levels of this innate immune receptor.

Enhanced Activation by TLR4 of NF- κ B and p-38 MAPK in Adult AVICs

To understand the mechanism underlying the greater inflammatory and osteogenic responses to TLR4 stimulation in adult AVICs, we examined levels of NF- κ B and p-38 MAPK phosphorylation. Stimulation of TLR4 induced higher phosphorylation of NF- κ B p65 and p-38 MAPK in adult AVICs from 5 to 120 minutes (Fig 3A and 3B). The results show that the greater inflammatory and osteogenic responses to stimulation of TLR4 in adult AVICs are associated with postreceptor signaling. To elucidate the mechanism underlying the enhanced inflammatory and osteogenic responses to TLR4 stimulation in adult AVICs, we examined Stat3 phosphorylation. Lower levels of Stat3 phosphorylation were observed in adult cells, whereas higher levels of phosphorylation of Stat3 were observed in pediatric cells after stimulation of TLR4 from 5 to 30 minutes (Fig 4). The result showed that greater inflammatory and osteogenic responses to stimulation of TLR4 in adult AVICs may be due to lack of Stat3 activation.

Stat3 Inhibition-Induced NF- κ B and p-38 MAPK Activation and Higher ICAM-1, BMP-2, and ALP Expression in Pediatric AVICs

To test the hypothesis that a low level of Stat3 activation is responsible for greater inflammatory and osteogenic responses to TLR4 stimulation in adult AVICs, we examined the activation of NF- κ B and p-38 MAPK and the expression of ICAM-1, BMP-2, and ALP with Stat3 inhibition in pediatric cells. Inhibition of Stat3 enhanced NF- κ B and p-38 MAPK activation in pediatric AVICs (Fig 5A), and significantly increased expression of ICAM-1, BMP-2, and ALP (Fig 5B), which was further confirmed by knockdown of Stat3 (Fig 5C). Conversely, inhibition of Stat3 in adult cells did not induced significant increase of ICAM-1, BMP-2, and ALP. The results showed that Stat3 played an antiinflammatory and antiosteogenic role in AVICs upon stimulation of TLR4.

Comment

The results of the present study demonstrate age-related differences in the response of human AVICs to TLR4 stimulation. In response to TLR4 stimulation, adult AVICs produced higher levels of ICAM-1, BMP-2, and ALP compared with pediatric AVICs. These enhanced inflammatory and osteogenic responses in adult AVICs were characterized by higher activation of NF- κ B and p-38 MAPK and lower activation of Stat3 in response to TLR4 stimulation in the absence of altered cellular TLR4 level compared with pediatric AVICs. Further, inhibition of Stat3 in pediatric AVICs resulted in enhanced activation of NF- κ B and p-38 MAPK as well as greater expression of ICAM-1, BMP-2, and ALP. These data suggest that differential levels of Stat3 activation underlie the age-related difference in responses to TLR4 stimulation between adult and pediatric AVICs.

Mechanisms of inflammation have been implicated in the pathogenesis of CAVD. In response to proinflammatory stimulation, adult AVICs have been shown to undergo inflammatory phenotypic changes. In turn, these inflammatory changes lead adult AVICs to undergo changes to an osteogenic phenotype. Interestingly, the degree of inflammation and calcification can be measured by combined positron emission tomography and computed tomography in the valves of patients with CAVD [16]. That provides us with a reliable and reproducible method of measuring disease activity. Hence, mechanisms of inflammation and osteogenesis are implicated in the pathogenesis of CAVD.

In adults, the AVICs have been implicated in the pathogenesis of CAVD [2, 17]. Work from our laboratory has previously shown that when stimulated by mechanisms of inflammation acting through TLR4, adult AVICs demonstrate increased expression of proinflammatory genes and proteins [18]. In turn, we have also previously shown that such an inflammatory response is linked to mechanisms of osteogenesis [2, 3]; mechanisms of inflammation have been demonstrated to incite mechanisms of osteogenesis in human adult AVICs. Here, we present the similar finding that stimulation of TLR4 in human adult AVICs also induced the expression of proinflammatory and proosteogenic factors. More importantly, greater inflammatory and osteogenic responses were observed in adult cells compared with those of pediatric cells after stimulation of TLR4. The mechanisms underlying the increased inflammatory and osteogenic responses in adult AVICs could be due to a change in TLR4 distribution or induction of inflammatory and osteogenic responses. Intriguingly, our finding demonstrated that TLR4 protein baseline level in adult AVICs is not different from those of pediatric AVICs, either within different ages of pediatric donors or between pediatric and adult donors. The results indicated that increase of TLR4-mediated inflammatory and osteogenic responses in adult AVICs does not involve an increase in cellular level of TLR4. Next, we investigated the inflammatory and osteogenic responses in AVICs of those two age groups. As expected, TLR4 stimulation produced a significantly increased production of ICAM-1, BMP-2, and ALP in adult cells, which was associated with activation of p-38 MAPK and the transcription factor, NF- κ B. In pediatric cells, TLR4 stimulation produced significantly less ICAM-1, BMP-2, and ALP production, and was associated with significantly less activation of p-38 MAPK and NF- κ B.

Age-related differences in the cellular responses to inflammatory stimulation have been noted in human fibroblasts [19]. Levy and colleagues [20] have previously reported that monocytes from cord blood stimulated by agonists of TLR1 through 7 expressed significantly less proinflammatory cytokines than adult cells despite no differences in TLR expression [20]. Belderbos and colleagues [21] have previously demonstrated that TLR4 stimulation of pediatric monocytes produced a pronounced antiinflammatory response and a diminished proinflammatory response. Although such differences in the age-related responses to proinflammatory stimulation are poorly understood, data such as these suggest that such differences may be attributable to differences in downstream signaling.

The Stat3 proteins are latent cytoplasmic transcription factors. Seven members of the family have been identified (Stat1–7) [22]. Stat3 is central to many intracellular processes, including mechanisms of antiinflammation [10]. Although potentially activated by cytokines and growth factors, its intracellular actions have been shown to vary from one cell type to another [23]. For instance, Stat3 has been shown to inhibit NF- κ B in murine mesangial cells [24]. Conversely, Stat3 works with NF- κ B to mediate tumor-promoting inflammation in cancerous and inflammatory cells [25]. Strikingly, we found that activation of TLR4 produced significantly greater Stat3 activation in pediatric cells, which was associated with lower activation of p-38 MAPK and NF- κ B. After inhibition of Stat3 in pediatric AVICs, TLR4 stimulation led to significantly increased activation of p-38 MAPK and NF- κ B that was associated with increased production of ICAM-1, BMP-2, and ALP. In other words, after inhibition of Stat3, the responses to TLR4 stimulation in pediatric cells more closely resembled those of adult cells. These data imply that lack of TLR4-induced ICAM-1, BMP-2, and ALP expression in the pediatric cells was attributable to TLR4 activation of Stat3. Therefore, it is very likely that activation of Stat3 may play an important role in the age-related differences to TLR4 stimulation.

The present study has limitations. As with any study of isolated cells, the behavior of the cells in vitro may differ from the behavior of those in vivo. However, we have previously demonstrated that isolated human AVICs that have been grown through multiple passages in cell culture have functions comparable to those of freshly isolated cells [2]. Second, the adult valve specimens were grossly normal valves from patients with cardiomyopathy who were undergoing cardiac transplant. Responses of AVICs from these values have been demonstrated to be distinctively different from those of calcified aortic valves. Third, the pediatric valve specimens were from patients with congenital heart disease. The congenital heart disease that led to the need for heart transplant was varied in the pediatric specimens and may affect the response TLR4 stimulation; despite these differences among the pediatric cells, the responses to TLR4 stimulation among the pediatric cells in our study were internally consistent. Fourth, the underlying etiology of the cardiomyopathy would differ. Some pediatric patients may have had cyanotic heart disease. The duration of heart failure before transplantation may have differed. The proportion of female patients was different. The pressures that the aortic valve is subjected to would undoubtedly be different. The medications received by the patients would likely differ. The degree of exposure to prior inflammation could be different as well. Lastly, we do acknowledge that Stat3 activation could be occurring independently of TLR4 or in a TLR4-dependent fashion (Fig 6).

However, our study did not directly address this question. In either instance, Stat3 may function as a suppressor of the inflammatory and osteogenic process in AVICs.

In conclusion, the results of the present study demonstrated important age-related differences in the responses of adult and pediatric AVICs to TLR4 stimulation. Although there is comparable expression of TLR4 across age groups, TLR4 stimulation induced greater inflammatory and osteogenic responses in adult AVICs that is associated with the lack of Stat3 activation. After inhibition of Stat3, the responses to TLR4 stimulation of pediatric AVICs closely resembled those of adult cells. These data may help explain why CAVD is age related [26], and why there is a rare aortic valve inflammatory and osteogenic disease in children. Furthermore, the results suggest that Stat3 activation (tyrosine phosphorylation) may be protective against inflammatory and osteogenic stimulation in human AVICs. Because previous work has suggested that these mechanisms are part of this disease process, it is tempting to speculate that TLR4 inhibition could be targeted pharmacologically to treat CAVD.

Acknowledgments

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Abbreviations and Acronyms

ALP	alkaline phosphatase
AVICs	aortic valve interstitial cells
BMP-2	bone morphogenetic protein 2
CAVD	calcific aortic valve disease
ICAM-1	intercellular adhesion molecule 1
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinase
NF-κB	nuclear factor
shRNA	short hairpin RNA
Stat3	signal transducer and activator of transcription 3
TLR4	toll-like receptor 4

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Fig 1.

Adult cells had markedly higher inflammatory and osteogenic responses after toll-like receptor 4 (TLR4) stimulation. The lipopolysaccharide (LPS) stimulation induces higher intercellular adhesion molecule 1 (ICAM-1), bone morphogenetic protein 2 (BMP-2), and alkaline phosphatase (ALP) responses in aortic valve interstitial cells (AVICs) of adult cells (open bars) relative to pediatric cells (solid bars). The AVICs were stimulated with LPS, 200 ng/mL, for 24, 48, and 72 hours; ICAM-1, BMP-2, and ALP protein levels were analyzed by immunoblotting. Adult cells exhibited higher responses in ICAM-1, BMP-2, and ALP

levels. Results are expressed as mean $\pm$ SEM; n = 6; *p < 0.05 (adult versus pediatric); **p < 0.01 (adult versus pediatric).

Fig 2.

Comparable expression of toll-like receptor 4 (TLR4) in aortic valve interstitial cells (AVICs) of pediatric and adult cells. The AVICs from explanted hearts of heart transplant recipients were examined for TLR4 by immunoblotting. (A) Developmental expression of TLR4 in AVICs of children 2 months to 16 years old. (B) Equal expression of TLR4 in AVICs of pediatric and adult cells.

Fig 3.

Activation of nuclear factor-κB (NF-κB) and p-38 mitogen-activated protein kinase (MAPK) by toll-like receptor 4 (TLR4) is enhanced in adult aortic valve interstitial cells (AVICs). The AVICs were stimulated with lipopolysaccharide (LPS), 200 ng/mL, for 5 to 120 minutes. (A) Representative immunoblots show that stimulation of TLR4 induced greater NF-κB activation in adult AVICS (open bars) relative to pediatric cells (solid bars). Blots from specific antiphospho NF-κB antibody probed were stripped and reprobed with anti-NF-κB antibody. Results are expressed as mean ± SEM, n = 3. *p < 0.05 (adult versus

pediatric). $**p < 0.01$ (adult versus pediatric). (B) Representative immunoblots show that stimulation of TLR4 induced greater p-38 MAPK in adult AVICs (open bars) relative to pediatric cells (solid bars). Blots from specific antiphospho p-38 antibody probed were stripped and reprobed with anti-P-38 antibody. Results are expressed as mean $\pm$ SEM; $n = 3$; $*p < 0.05$ (adult versus pediatric); $**p < 0.01$ (adult versus pediatric).

Fig 4.

Activation of signal transducer and activator of transcription 3 (Stat3 [tyrosine]) by toll-like receptor 4 (TLR4) is augmented in pediatric aortic valve interstitial cells (AVICs), but not in adult cells. Pediatric AVICS (solid bars) and adult AVICs (open bars) were stimulated with lipopolysaccharide (LPS), 200 ng/mL, for 5 to 30 minutes. Immunoblots show that stimulation of TLR4 induced greater Stat3 activation in pediatric cells compared with adult cells. In the brackets, Y means tyrosine, S means serine. Blots from specific antiphospho Stat3 antibody probed were stripped and reprobed with anti-Stat3 antibody. Results are expressed as mean $\pm$ SEM; n = 3; *p < 0.05 (pediatric versus adult).

B

Fig 5.

Signal transducer and activator of transcription 3 (Stat3) demonstrated antiinflammatory and antiosteogenic effects to suppress the inflammatory and osteogenic responses in pediatric aortic valve interstitial cells (AVICs). (A) Pediatric AVICs were pretreated with Stat3 inhibitor S3I-201 for 1 hour, then with added lipopolysaccharide (LPS), 200 ng/mL, for 1 hour. Activation of p-38 mitogen-activated protein kinase (MAPK) and nuclear factor - κ B (NF- κ B) were examined by immunoblots. Representative immunoblots show that Stat3

inhibition induced p-38 MAPK and NF- κ B activation. Results are expressed as mean $\pm$ SEM; n = 3; **p < 0.01 versus control. (DMSO = dimethylsulfoxide.)

(B) Pediatric AVICs were treated with or without Stat3 inhibitor S3I-201 (5 μ m) in the presence of LPS (200 ng/mL) for 48 hours. Stat3 inhibition induced greater intercellular adhesion molecule 1 (ICAM-1), bone morphogenetic protein 2 (BMP-2), and alkaline phosphatase (ALP) responses in pediatric AVICs. Results are expressed as mean $\pm$ SEM; n = 3; **p < 0.01 versus control. (AVICs = aortic valve interstitial cells; DMSO = dimethylsulfoxide; LPS = lipopolysaccharide.)

(C) Pediatric AVICs were treated with ConshRNA (Con SH) or Stat3shRNA (shStat3) or combined with LPS (200 ng/mL) for 72 hours. Stat3 knockdown induced greater ICAM-1, BMP-2, and ALP responses in pediatric AVICs. Results are expressed as mean $\pm$ SEM; n = 3; *p < 0.05 versus control; **p < 0.01 versus vehicle control. (ALP = alkaline phosphatase; AVICs = aortic valve interstitial cells; BMP-2 = bone morphogenetic protein 2; ICAM-1 = intercellular adhesion molecule 1; LPS = lipopolysaccharide.)

(D) Adult AVICs were treated with or without Stat3 inhibitor S3I-201 (5 μ m) in the presence of LPS (200 ng/mL) for 48 hours. Stat3 inhibition induced greater ICAM-1, BMP-2, and ALP responses in pediatric AVICs. Results are expressed as mean $\pm$ SEM; n = 3; **p < 0.01 versus control. (ALP = alkaline phosphatase; AVICs = aortic valve interstitial cells; BMP-2 = bone morphogenetic protein 2; DMSO = dimethylsulfoxide; ICAM-1 = intercellular adhesion molecule 1; LPS = lipopolysaccharide.)

Fig 6.

Summary diagram of signal transducer and activator of transcription 3 (Stat3) modulation of the toll-like receptor 4 (TLR4) signaling pathway. The lipopolysaccharide (LPS) activates Stat3 either TLR4 independently or dependently. Activation of Stat3 inhibits inflammatory and osteogenic changes by decreasing the activity of both nuclear factor-κB (NF-κB) and p-38 mitogen-activated protein kinase (MAPK) in aortic valve interstitial cells. Activation and inhibition are indicated with arrows and blunt-ended arrows, respectively.

Table 1

Patient Demographics, Aortic Valve

Patients	Treatment	Age (years)	Sex	Diagnosis
Pediatric patients				
060107 ^a	Replacement	0.1	Female	Stenosis
031208	Transplant	0.5	Male	Cardiomyopathy
070307 ^a	Replacement	1	Female	Stenosis
111307	Transplant	3	Female	Cardiomyopathy
051508	Transplant	4	Female	Cardiomyopathy
050909	Transplant	4	Male	Cardiomyopathy
102307	Transplant	13	Male	Cardiomyopathy
120107	Transplant	16	Male	Cardiomyopathy
052308	Transplant	16	Male	Cardiomyopathy
Adult patients				
010709	Transplant	50	Male	Cardiomyopathy
051810	Transplant	60	Male	Cardiomyopathy
082410	Transplant	57	Female	Cardiomyopathy
040907	Transplant	51	Male	Cardiomyopathy
090707	Transplant	48	Male	Cardiomyopathy
022010	Transplant	47	Male	Cardiomyopathy
091106	Transplant	41	Male	Cardiomyopathy

^a Only used for toll-like receptor-4 distribution assay.