Viral and bacterial patterns induce TLR-mediated sustained inflammation and calcification in aortic valve interstitial cells

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Abstract

Background: Aortic stenosis shares some ethiopathological features with atherosclerosis and increasing evidence links Toll-like receptors (TLRs) to atherogenesis.

Methods: TLR-mediated inflammation and osteogenesis were investigated in human interstitial cells isolated from stenotic and non-stenotic aortic valves. TLR expression and signalling were evaluated by quantitative RT-PCR, flow cytometry, Western blot analysis, ELISA, and cytokine arrays. Osteogenesis was evaluated by measuring alkaline phosphatase activity.

Results: Interstitial cells from control valves express most TLRs, being TLR4 the most abundant, whereas cells from stenotic valves express higher TLR4 and TLR2 and lower TLR5 and TLR9 transcript levels. When pro-inflammatory pathways were analyzed, we observed that TLR4, TLR2 and TLR3 ligands induced an early activation of NF-κB and p38 MAPK activation in cells from control and stenotic valves. Strikingly, when TLRs sensing viral patterns were studied, a sustained TLR3-mediated activation of NF-κB, a κB-independent induction of catalytically active cyclooxigenase (COX)-2 and ICAM-1 expression, and induction of expression of several chemokines were observed. TLR4, but not TLR2, engagement produced a similar but NF-κB-dependent effect. Moreover, TLR3 and TLR4 agonists induced alkaline phosphatase expression and activity.

Conclusions: Exposure of aortic valve interstitial cells to viral and Gram-negative bacteria molecular patterns induces distinct and long-term TLR-mediated pro-inflammatory and pro-osteogenic responses that might be relevant to the pathogenesis of degenerative aortic stenosis.

Keywords: Aortic stenosis; heart valve; Toll-like receptor; inflammation; COX-2; ICAM-1

1. Introduction

Degenerative aortic stenosis, the most common cause of valve replacement in western countries, is a common disease in the elderly characterized by progressive calcification and increased valve thickness [1]. Valvular calcification is no longer considered a degenerative and passive process but an actively regulated process, the molecular mechanisms of which remain to be elucidated [1,2]. Many studies have shown the role of atherogenic risk factors in valve calcification. In fact aortic stenosis shares some ethiopathological features with the atherosclerosis, like inflammatory cell infiltration, oxidized lipid deposition, and cytokine expression, although there are some differentiating features [2,3]. At the molecular level, inflammatory mediators, growth factors, matrix metalloproteinases, the renin-angiotensin system, and pro-osteogenic pathways have been shown to participate in valve disease pathogenesis [1,2], although a pharmacological target valuable for the development of therapies is still to be defined.

Toll-like receptors (TLRs), key players in the innate and adaptive immunity, have been associated to the pathogenesis of inflammatory diseases such as atherosclerosis [4]. TLRs are a family of transmembrane receptors involved in the recognition of conserved patterns from microbes, as well as danger-associated endogenous molecules [5]. To date several TLRs, 11 in humans, have been identified [5]. Cell-surface receptors include TLR4 that recognizes lipopolysaccharide present in Gram-negative bacteria, TLR2 and its co-receptors TLR1/TLR6, which sense bacterial lipoproteins and lipoteichoic acid, TLR5 that recognizes bacterial flagellin, TLR11 that senses profiling-like molecules in parasites, and TLR10, the ligand of which remains unknown. Another subtype of receptors localized in the endosomal cell compartment include TLR3, that recognizes dsRNA, TLR9, that senses unmethylated CpG motifs present in viral and

bacterial genome and parasites, and TLR7, that binds ssDNA [5]. Upon stimulation, TLRs initiate intracellular signalling cascades that ultimately promote pro-inflammatory gene expression. All TLRs, but TLR3, induce MyD88-dependent activation of NF-κB and MAPK cascades, while a MyD88-independent pathway, involving the induction of IFN-inducible genes, have been described to be triggered upon TLR3 and TLR4 activation [5]. Accumulating evidence links TLR activation to atherogenesis, especially TLR2 and TLR4, although new evidences support the involvement of viral receptors [4,6]. Even more, the TLR4-D299G human polymorphism has been associated to lower risk of carotid atherosclerosis and increased risk of myocardial infarction [6]. More recently, a clinical study demonstrated that high expression of TLR2 on monocytes is a risk factor for atherogenesis, independent from other established risk factors [7].

Cardiovascular cells such as cardiomyocytes, vascular smooth muscle and endothelial cells are known to express TLRs [8]. Moreover, aortic valve interstitial cells (AVICs), a prominent cell type in the heart valve leaflets that can undergo a phenotypic differentiation to osteoblast-like cells and play a role in valve calcification [9,10], have been described to express TLR4 and TLR2 [11]. Given the connection between TLR and the atherosclerosis process, which shares some pathophysiological features with aortic stenosis, and to better understand the molecular mechanisms underlying the valve disease, we explored the role of TLR activation in human AVICs isolated from surgically explanted stenotic and non-stenotic aortic valves. For that purpose, we evaluated TLR expression in cells from control and calcified stenotic valves, as well as TLR-coupled signalling cascade, and induction of pro-inflammatory mediators and calcification markers.

2. Methods

2.1. Reagents

Collagenase type II and M199 medium were from Invitrogen (Carlsbad, CA). Bovine fetal serum and antibiotics were from Clonetics-Lonza (Walkersville, MD). *E. coli* LPS, bis-benzimide (DAPI), alkaline phosphatase (ALP) blue kit, and an anti-β-tubulin antibody were purchased from Sigma (St. Louis, MO). Pam_3CSK4, CpG-ODN, and polyinosinic-polycytidylic acid (poly (I:C)) were from InvivoGen (San Diego, CA). Antibodies against ERK, cyclooxigenase (COX)-2, and intercellular adhesion molecule (ICAM)-1 were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-JNK, anti-phospho-p38, and anti-phospho-Ser 536-pNF-κB p65 form antibodies were from Cell Signaling (Danvers, MA). Anti-phospho-ERK antibody was from Promega (Madison, WI). TRIzol was from Life Technologies (Grand Island, NY). PE-conjugated anti-human TLR4, IgG2a-PE isotype control, and goat anti-mouse IgG-FITC antibodies were from eBioscience (San Diego, CA). Enhanced chemiluminiscence detection system was from Pierce (Rockford, IL). NF-κB SN50 was from Calbiochem (Darmstadt, Germany). PGE_2 immunoassay kit was from GE-Amersham (Buckinghamshire, United Kingdom). Cytokine antibody array V was from RayBiotech, Inc. (Norcross, GA). ALP Fluorometric Assay Kit was from Abcam (Cambridge, UK).

2.2. Patient selection

The study included 10 explanted heart valves from patients with degenerative aortic stenosis and indication for valve replacement (6 males/4 females, mean age 72.0 ± 7.1

years). Aortic valve area was 0.7 ± 0.2 cm², peak gradient 88 ± 22 mmHg and mean gradient 56 ± 15 mmHg. Control group consisted of 10 aortic valves from transplanted patients with valve disease excluded by echocardiography (8 males/2 females, mean age 59.3 ± 6.2 years). Diagnosis of the aortic valve lesion and indications for valve replacement and heart transplant was performed following European guidelines. All patients gave informed consent and Hospital Clínico Universitario Review Board approved the study, which complies with the Declaration of Helsinki.

2.3. Isolation and culture of human AVICs

After surgery, the aortic valves were dissected to eliminate non-valvular tissue, and calcium deposits in the case of stenotic valves. Cells were isolated with a method based on sequential digestion of valve leaflets with 2.5 mg/ml collagenase for 30 min at 37 ºC, and 0.8 mg/ml collagenase for 3 h at 37 ºC [11]. Then, cells were cultured in M199 media supplemented with penicillin G, streptomycin, amphotericin B and 10% serum. For the experiments, AVICs from passages 3-6 were used. Before performing experiments, cells were cultured in low-serum media (M199 supplemented with antibiotics and 2% serum). α-SM-actin staining [12] revealed no apparent morphological differences in cells explanted from control and stenotic valves and confirmed that more than 95% cells were myofibroblast-like (data not shown). For calcification experiments, cells were cultured in conditioning medium (M199 supplemented with 10 mmol/l β-glycerophosphate, 10 nmol/l vitamin D_3, and 10 nmol/l dexamethasone) as previously described [13], and stimulated with TLR ligands or vehicle for 17-19 days.

2.4. Real-time RT-PCR analysis

RNA was isolated using TRIzol reagent and reverse transcription-PCR was performed using following sets of primers. TLR1 (sense 5´-GGGTCAGCTGGACTTCAGAG-3´, anti-sense 5´-AAAATCCAAATGCAGGAACG-3´); TLR2 (sense 5´-TCAGCCTCTCCAAGGAAGAA-3´, anti-sense 5´-AATGTTCAAGACTGCCCAGG-3´); TLR3 (sense 5´-AGCCTTCAACGACTGATGCT-3´, anti-sense 5´-TTTCCAGAGCCGTGCTAAGT-3´); TLR4 (sense 5´-TGAGCAGTCGTGCTGGTATC-3´, anti-sense 5´-CAGGGCTTTTCTGAGTCGTC-3); TLR5 (sense 5´-GGAACCAGCTCCTAGCTCCT-3´, anti-sense 5´-AAGAGGGAAACCCCAGAGAA-3´); TLR6 (sense 5´-CCCTTTAGGATAGCCACTGC-3´, anti-sense 5´-CTCACAATAGGATGGCAGGA-3´); TLR7 (sense 5´-CCTTGAGGCCAACAACATCT-3´, anti-sense 5´-GTAGGGACGGCTGTGACATT-3´); TLR8 (sense 5´-TCCTTCAGTCGTCAATGCTG-3´, anti-sense 5´-CGTTTGGGGAACTTCCTGTA-3´); TLR9 (sense 5´-GGACACTCCCAGCTCTGAAG-3´, and anti-sense 5´-TTG GCTGTGGATGTTGTTGT-3´). cDNA was amplified in a PTC-200 apparatus (BioRad) using SYBR Green I mix containing HotStart polymerase. β-actin was used as a housekeeping gene to assess the relative abundance of mRNA.

2.5. Flow cytometry analysis

TLR4 expression was analyzed by flow cytometry using cells permeabilized or not with 0.1% saponin prior to staining with PE-conjugated anti-TLR4 antibody (1:10

dilution in PBS, 2% serum, EDTA) for 30 min at 4 ºC. Positively stained cells were detected by comparison with the background fluorescence samples stained with a PE-conjugated isotype-specific control antibody. Flow cytometry analysis was performed in a Beckman Coulter device (Epics XL-MCL). Data was analyzed using WinMDI software.

2.6. Immunodetection of pNF-κB, MAP kinases, COX-2, and ICAM-1

TLR-activated cells were analyzed by Western blot as described earlier [14]. Briefly, cells were lysed with TNE buffer (20 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 5 mmol/L EDTA, 0.01% NP-40, and a cocktail of protease and phosphatase inhibitors) for 10 min on ice. 50 μg protein per lane was analyzed, and proteins were visualized using a chemiluminescence detection system. NF-κB activation was measured using an anti-phospho-NF-κB p65 antibody. MAP kinase activation was evaluated using phosphospecific antibodies for the phosphorylated forms of ERK, JNK, and p38 MAPK. Expression of pro-inflammatory molecules was detected with human COX-2 and ICAM-1 antibodies. Equal loading across the gel was confirmed using an anti-β-tubulin antibody.

2.7. ELISA for human prostaglandin (PG)-E_2

Supernatants from human AVICs stimulated with the corresponding TLR ligands for 8, 12 and 24 h were used to quantify PGE_2 secretion by an immunoassay kit following the manufacturer´s protocol. Absorbance was measured using a microplate reader Versamax (Molecular Devices, Sunnyvale, CA).

2.8. Analysis of cytokine expression levels by protein arrays

Supernatants from cells stimulated with corresponding ligand for 12 h at 37 ºC were analyzed with Human Cytokine Antibody Array V. Briefly, previously blocked membranes were incubated with supernatants for 2 h, later incubated with the primary biotin-conjugated antibody for 2 h, followed by incubation with HRP-conjugated streptavidin antibodies for 1 h. Chemiluminiscence detection was followed with exposure to x-ray film. Densitometry of cytokine spots was analyzed by Image Quant TL software (Amersham, Buckinghamshire, UK).

2.9. ALP activity assay and staining

To evaluate ALP expression, cells were fixed with p-formaldehyde, stained with the ALP blue kit following manufacturer´s protocol, and photographed. For measuring ALP activity, cells were lysed with 20 mmol/L Tris-HCl, pH 8, 150 mmol/L NaCl, 0.2% NP-40, and a cocktail of protease and phosphatase inhibitors, as previously described [13], the enzymatic activity was evaluated using a fluorometric assay kit, and results were expressed as pmol/min.h.

2.10. Statistical analysis

Results are expressed as mean ± SD. Data were analyzed by either unpaired t´ test using GraphPad Prism version 4 (San Diego, CA). Differences were considered statistically significant for a $p < 0.05$.

3. Results

3.1. Human AVICs from control valves express most of TLRs

To evaluate TLR gene expression in human AVICs, we quantified the TLR mRNA levels by real time RT-PCR. Experiments performed with n= 10 valves revealed that all TLRs, but TLR8, are expressed in human AVICs, the TLR4 receptor being the most abundant subtype, with the others expressed at lower levels (Fig. 1A). Cells express receptors such as TLR2 and TLR4 (Fig. 1A), consistent with a recent report [11], as well as TLR2 co-receptors TLR1 and TLR6, and TLR5. Interestingly, cells also express endosomal receptors involved in sensing nucleic acids like TLR3, and to a lower extent TLR7 and TLR9 (Fig. 1A).

3.2. Differential TLR mRNA expression in AVICs from stenotic and control valves

Next, we analyzed TLR gene expression in AVICs isolated from stenotic valves. Similar TLR expression profiles, but differences in transcript levels, were observed in AVICs from stenotic as compared to control valves (Fig. 1A). Quantitative analysis revealed an increase of TLR4 and TLR2 mRNA levels in stenotic as compared to control valves (TLR4: control 737.8 ± 122.9, stenotic 2163.0 ± 452.3; TLR2: control 53.8 ± 9.5, stenotic 101.2 ± 22.1), while no significant differences on TLR2 co-receptors TLR1 and TLR6 were observed. On the contrary, TLR5 levels were significantly diminished in stenotic valves (Fig. 1A). As for endosomal receptors, TLR9 showed a significant lower expression in stenotic valves, while no statistically significant differences in TLR3 and TLR7 levels were observed in control and diseased valves (Fig. 1A). Together, data show over-expression of TLR4 and TLR2, and down-

regulation of TLR5 and TLR9 in AVICs from stenotic valves when compared to those from control valves.

3.3. Total cellular but not cell-surface TLR4 is higher in AVICS from stenotic valves than in control valves

To assess TLR4 protein expression, AVICs labelled with a fluorescent anti-TLR4 antibody were analyzed by flow cytometry. In experiments performed with control AVICs permeabilized with saponin, a single peak of fluorescence was observed, while two peaks with low and higher fluorescence intensity were observed in non-permeabilized cells (Fig. 1B), suggesting the existence of two populations of TLR4 and that most receptors are not expressed on the cell-surface but are internalized or have not yet located into the membrane. Likewise, AVICs from stenotic valves showed two distinct TLR4 populations (Fig. 1C). When comparing the relative expression, the total cellular TLR4 was significantly higher in AVICs from stenotic as compared to control valves (Fig. 1D) consistent with quantitative RT-PCR results, while no significant differences in the number of cell-surface receptors were observed. Together, results suggest that most of TLR4 are not exposed to the cell-surface, being the total cellular, and not cell-surface, increased in AVICs from stenotic as compared to control valves.

3.4. TLR3, TLR2 and TLR4 agonists induce NF-κB and MAPK/p38 activation in AVICS from control and stenotic valves

Since TLR signalling promotes the activation of NF-κB and MAPK cascades [5], and AVICS express functional TLR2 and TLR4 [11], we assessed intracellular signalling of

TLRs involved in the recognition of nucleic acids and compared with TLRs that sense bacterial patterns. For this purpose, cells were either stimulated with a TLR3 agonist, poly (I:C), a synthetic polymer of inosine that resembles the RNA of infectious viruses and stimulates the production of interferon, or a TLR9 agonist, CpG-ODN, an unmethylated sequence present in bacterial and viral DNA. In AVICs from control and stenotic valves, NF-κB p65 phosphorylation was observed after 20-60 min stimulation with poly (I:C) but not with CpG-ODN (Fig. 2, panels A-B), consistent with the low expression of TLR9 in AVICs (Fig. 1A). As for bacterial patterns, LPS and Pam_3CSK4 stimulation induced NF-κB p65 phosphorylation in control AVICs (Fig. 2A), and similar results were observed with cells from diseased valves (Fig. 2B). Next, we evaluated the activation of MAPK cascades by using phosphospecific antibodies. Interestingly, a phosphorylated form of MAPK/p38, a kinase with an important role on inflammation, was detected 20-60 min upon stimulation with TLR3, TLR4, and TLR2 agonists (Fig. 2, panels A-B). We also evaluated the MAPK/ERK pathway, known to play a crucial role in osteoblast differentiation and mineralization [15], by measuring ERK1/ERK2 phosphorylation. Interestingly, AVICs from control and stenotic valves showed some degree of ERK phosphorylation in basal conditions (Fig. 2, panels A-B); being ERK2 the major phosphorylated form. Likewise, the MAPK/JNK cascade was already activated in resting cells (Fig. 2, panels A-B); being JNK2 the major phosphorylated form in AVICs. Together, results show that TLR3, TLR2, and TLR4 induce the activation of the NF-κB and MAPK/p38 pro-inflammatory pathways, in AVICs from both stenotic and non-stenotic valves.

3.5. TLR3 activation promotes NF-κB-independent induction of pro-inflammatory molecules in AVICs from control and stenotic valves

Next, we evaluated TLR signalling at late times and the induction of pro-inflammatory molecules. Interestingly, poly (I:C), but not CpG, induced sustained NF-κB activation, since a phosphorylated form of NF-κB p65 was detected 12-24 h after stimulation of AVICs from control and stenotic valves (Fig. 3A). Strikingly, poly (I:C) strongly induced the expression of the pro-inflammatory enzyme COX-2 in AVICs from control valves and stenotic valves (Fig. 3A). Consistent with these results, PGE_2, a major product of COX-2 activity, was detected in the supernatants of TLR3-activated AVICs from control and stenotic valves (Fig. 3, panels B-C). Later, we tested the expression of the adhesion molecule ICAM-1, previously described to be induced by LPS in AVICs [11]. TLR3-mediated induction of COX-2, PGE_2 and ICAM-1 expression was very robust and the kinetic showed a biphasic response (Fig. 3A). Moreover, the effect was not blocked by SN50, a NF-κB inhibitor that prevents NF-κB translocation into the nucleus (Fig. 3D), suggesting that NF-κB complexes containing p50 protein are not involved in the response, but a sequence initiated by TRIF/IRF3 activation might play a role. These results suggest that AVICs exposed to agonists mimicking viral patterns activate TLR3-mediated and NF-κB-independent pro-inflammatory responses.

3.5. Engagement of TLR4, but not TLR2, induces NF-κB-dependent expression of pro-inflammatory molecules in AVICs from control and stenotic valves

Next, we evaluated the induction of pro-inflammatory molecules by bacterial patterns. Notably, LPS-mediated NF-κB activation was sustained and detectable 12-24 h after stimulation in AVICs from control and stenotic valves (Fig. 4A), thus resembling

data observed upon TLR3-activation (Fig. 3A). On the contrary, after TLR2 stimulation with Pam_3CSK4, low or no activation was observed at late times (Fig. 4A). In parallel with NF-κB activation, a TLR4 ligand, but not a TLR2 agonist, induced the expression of COX-2 and ICAM-1 in AVICs from control valves and stenotic valves (Fig. 4A). Consistent with these results, PGE_2 was detected in the supernatants of TLR4-activated AVICs from control and stenotic valves (Fig. 4, panels B-C). Moreover, a NF-κB inhibitor blocked the TLR4-mediated induction of COX-2 and ICAM-1 (Fig. 4D), the gene of which is known to be regulated by NF-κB [16]. Together, data demonstrate that TLR4 activation promotes the long-lasting and NF-κB-dependent induction of COX-2 and ICAM-1 expression in human AVICs.

3.6. TLR3 and TLR4 engagement induce a similar profile of pro-inflammatory cytokines in AVICs

To compare the effect of TLR ligands on the production of other pro-inflammatory proteins, supernatants from cells activated for 12 h with TLR agonists were incubated with antibody arrays (Human Cytokine Antibody Array V). In resting conditions, human AVICs highly express the pro-inflamatory cytokine IL-6 and to a lower extent the chemokines IL-8, Gro, monocyte chemoattractant protein (MCP)-1 and RANTES, as well as cytokines such as osteoprotegerin (OPG), TIMP1 and 2 (Fig. 5A). Notably, poly (I:C) activation strongly induced the expression of several pro-inflammatory chemokines such as Gro, Gro-α, and IL-8, and to a lower extent of chemokines such as RANTES, macrophage inflammatory protein (MIP)-3α, and IP-10, and cytokines such as IL-6, VEGF, insulin-like growth factor binding protein-2 (IGFBP2), granulocyte chemotactic peptide (GCP)-2, and OPG (Fig. 5B). Also, LPS activation strongly

induced the expression of chemokines such as Gro, Gro-α and IL-8, and to a lower extent of cytokines such as IL-6, VEGF and OPG (Fig. 5C). Conversely, low induction of OPG and epidermal growth factor (EGF) was observed after Pam_3CSK4 stimulation (Fig. 5D), consistent with results from Fig. 4. Similar results were obtained in cells from diseased valves (data not shown). These results demonstrate that viral patterns can promote as strong pro-inflammatory phenotype as Gram-negative bacteria patterns in human AVICs.

3.7. TLR3 and TLR4 ligands induce the activity of the calcification biomarker ALP

Since it has recently been described that LPS promotes a pro-osteogenic phenotype via TLR4 activation [13], we tested whether a TLR3 ligand would induce ALP activity, a biomarker of early calcification, in human AVICs. Cells cultured with conditioning media showed some increase in ALP activity as compared with cells cultured in growth media, and LPS stimulation increased ALP activity further (Fig. 6, panels A-B), consistent with a previous study [13]. Strikingly, poly (I:C) increased ALP expression in staining experiments (Fig. 6A). Moreover, poly (I:C) significantly up-regulated ALP activity as compared to conditioning media (Fig. 6B), and also increased calcified nodule formation (data not shown). Together, these data suggest that TLR3 activation induces *in vitro* calcification of human AVICs that could be relevant to the pathogenesis of aortic stenosis.

4. Discussion

The present study demonstrates that human AVICs express most of TLRs, which are differentially expressed in cells from non-stenotic and stenotic valves. Moreover, cell exposure to viral as well as Gram-negative bacteria patterns promotes TLR-mediated sustained inflammatory and pro-osteogenic responses, which could be relevant to the pathophysiology of degenerative aortic stenosis.

Our data is the first demonstration that human AVICs can sense viral as well as bacterial patterns. Our study confirms the reported expression of *tlr4* and *tlr2* genes in human AVICs [11], and also demonstrates that these cells express all *tlr* genes, but *tlr8*, being *tlr4* the most abundant gene, followed by *tlr1*, *tlr6* and *tlr3*. The gene expression pattern is similar in AVICs from stenotic and control valve, although a differential expression was observed given the increase of TLR2 and TLR4 transcript levels, as recently described [13], and the decrease of TLR5, and TLR9 levels in diseased valves as compared to control. At the protein level, our data suggest the existence of two populations of TLR4, being the cell-surface receptor levels lower than the ones internalized, in agreement with a report showing the predominant TLR4 cytosolic expression [11]. Even more, the total cellular TLR4 protein is higher in diseased valves, consistent with mRNA expression data and a recent report [13], whereas the number of cell-surface receptors is similar in non-stenotic and stenotic valves. A plausible explanation is that internalized receptors may be ready to go to the cell surface, as part of a recycling process that could be up-regulated in diseased valves. Alternatively, internalized TLR4 could exert its function through the recognition of bacterial patterns inside the cells.

Our data disclose a potential role of viral infection in the inflammatory and osteogenic responses underlying aortic stenosis pathogenesis, since exposure to dsRNA found in some viruses promotes sustained and strong TLR3-mediated pro-inflammatory responses as well as *in vitro* calcification of human AVICs. Although the role of infective agents in aortic stenosis has been previously suggested based on reports showing the presence of *C. pneumoniae* in valves from both early and late stages of the disease [1,3], the association of viral agents and the disease has not previously been reported. Consistent with recent reports in human AVICs [11,13], LPS from Gram-negative bacteria promoted long-lasting pro-inflammatory responses but, in our hands, a lipopeptide mimicking lipoproteins from Gram-positive bacteria did not promote sustained responses, pointing to a specific role of Gram-negative bacteria motifs in promoting long-lasting inflammatory responses. Strikingly, the TLR-induced pro-inflammatory phenotype can be elicited in cells from control valves. One hypothesis to explain these findings would be that not a single but repeated exposure of AVICs to viral and/or Gram-negative bacteria infection could promote sustained inflammatory responses that in the long-term contribute to progressive and chronic inflammation underlying aortic stenosis, thus agreeing with the long-lasting clinical course of the disease. Although this view has not been tested experimentally, this theory would agree with both the induction of accumulative damage by repeated ligand challenge or with the induction of persistent epigenetic changes producing tissue remodelling and functional disturbances.

TLR3, TLR4 and TLR2 are functionally active in AVICs from control and stenotic valves, as their engagement activates several TLR-mediated pro-inflammatory signalling pathways like NF-κB and MAPK-p38. Our data demonstrate that TLR3 and TLR4 ligands promote long-term activation of NF-κB, a transcription factor involved in

pro-inflammatory gene regulation. Results are consistent with a report demonstrating TLR4-mediated NF-κB activation in AVICs [11], and further demonstrate the involvement of NF-κB p50/p65 proteins in the TLR4-mediated induction of the expression of COX-2 and ICAM-1, the promoters of which contain NF-κB binding sites [16,17]. Consistent with a specific role of TLR4 on long-lasting inflammatory responses, a specific ligand for TLR2/TLR1 induced NF-κB early activation, as previously reported [11], but did not activate long-term responses. These findings differ from recent reports demonstrating TLR2-mediated release of cytokines and low induction of ICAM-1 in human AVICs [11,13]. This discrepancy could be explained by the use of different TLR2 ligands, since peptidoglycan is a non-specific TLR2 ligand that can also bind to NOD receptors.

The present study is the first demonstration of a TLR-mediated induction of COX-2 in human AVICs exposed to some pathogen patterns. These findings would support the potential role of COX-2 inhibitors in preventing the development or delaying the progression of aortic stenosis. In this respect, the negative results of a randomized trial testing the effects of statins on aortic stenosis progression might be related to the fact that the same percentage of patients in the treated and the control groups were receiving aspirin, [18], given that aspirin might have blocked inflammation in both groups, thus masking the putative protective effect of statins on stenosis progression. Our data suggest that TLR4-, but not TLR3-mediated induction of COX-2 is NF-κB-dependent, pointing out to a different transcriptional regulation. Interestingly, COX-2 induction has also been described in functionally related cells in other tissues, such as intestinal myofibroblasts [19], and it has been associated to pulmonary fibrous disorders [20]. Furthermore, our data show TLR4-mediated secretion of the COX-2 product, PGE_2, a lipid mediator that could be involved in the inflammation underlying calcification of the

valves. In fact, PGE_2 has been reported to have potential to elevate expression of VEGF [21], a pro-angiogenic and pro-inflammatory mediator that have been associated to aortic stenosis pathogenesis [1]. The production of PGE_2 has also been described in myofibroblasts from rat lungs [22], and recent work suggests that LPS-induced PGE_2 secretion from intestinal myofibroblasts can exert paracrine effects on adjacent cells [19].

Our data demonstrate the TLR3- and TLR4-mediated induction of ICAM-1, an adhesion molecule typically expressed in endothelial cells. Our data agree with reports demonstrating TLR4-mediated ICAM-1 induction [11,23], and further show the involvement of NF-κB-p50 protein, a transcription factor known to regulate *icam-1* gene promoter [17]. Moreover, poly (I:C), which mimics a viral infection, strongly induces NF-κB-independent ICAM-1 expression in AVICs. Since poly (I:C) is known to induce adhesion molecules expression [24], it is important to mention that cell cultures were free from endothelial contaminants that could account for ICAM-1 expression (data not shown). Furthermore, ICAM-1 might play an important role on myofibroblast function, since it has been described to be expressed in myofibroblast-like cells like such as hepatic stellate cells [25]. Interestingly, in a recent multi-ethnic study of atherosclerosis, soluble ICAM-1 was associated with increased prevalence and severity of aortic valve calcification [26]. One might speculate that the source of sICAM-1 can not only be endothelial cells, but also TLR-activated interstitial cells.

Consistent with the reported characteristic signalling cascades [5], TLR4 activation promoted NF-κB-mediated pro-inflammatory responses, and TLR3 and TLR4 also induced NF-κB independent responses, most likely involving the TRIF/IRF-3 route as suggested by TLR3- and TLR4-mediated induction of IFN-β in AVICs (data not shown). This is consistent with recent reports stressing the involvement of the TRIF

route in COX-2 induction in dendritic cells stimulated with Gram-positive bacteria [27]. Our data also reveal the TLR3- and TLR4-mediated activation of the MAPK/p38, a pro-inflammatory kinase associated with both damaging and beneficial roles in the development of cardiovascular diseases [28]. Moreover, this kinase might be involved in the TLR-mediated inflammatory responses, since TLR3- and TLR4-mediated induction of pro-inflammatory molecules was blocked with a p38 inhibitor (data not shown). These findings are consistent with the reported role of p38 in the TLR4-mediated induction of COX-2 expression in murine intestinal myofibroblasts [19]. Interestingly, the MAPK/ERK pathway has been associated to calcification of osteoblast precursors [15], and AVICs from porcine origin [29]. Our data show ERK basal activation, suggesting that this pathway might be over-activated in human AVICs and poised to contribute to a pro-osteosteogenic phenotype. However, it cannot be ruled out that *in vitro* culture conditions might artificially activate ERK and JNK pathways, although under the same experimental conditions the stress MAPK/p38 is not basally activated.

Our data demonstrate that viral and bacterial patterns induce a strong pro-inflammatory phenotype in human AVICs. These cells constitutively express cytokines such IL-6 and IL-8, as described [30], and also chemokines such as Gro, IL-8, MCP-1, and RANTES, and molecules involved in osteogenesis regulation, like OPG, TIMP1 and 2. Consistent with TLR-signalling data, TLR4 engagement, but not TLR2, promoted the expression of several chemokines, i.e., strong induction of IL-8, as described [30], and also Gro and Groα, and a lower induction of MCP-1, and cytokines such as VEGF and OPG, known to be involved in osteogenesis regulation [2]. Strikingly, TLR3 activation promotes a similar cytokine profile, even induced the expression of more cytokines than LPS, i.e., chemokines such as RANTES, GCP-2, and

MIP-3α, and the cytokine IGFBP2. Altogether, these data argue in favour of an important role of viral patterns in the induction of a pro-inflammatory phenotype in human AVICs and, likely, in aortic stenosis pathogenesis.

Interestingly, our data show for the first time that *in vitro* exposure of human AVIC to viral patterns promote a pro-osteogenic phenotype, since a TLR3 ligand induces the up-regulation of ALP activity, which is essential to bone mineralization, and promotes nodule formation (not shown). These results argue in favour of a potential role of viral infection in aortic stenosis pathogenesis. Furthermore, these results are reminiscent of data from a recent report that demonstrated TLR4 and TLR2-mediated calcific phenotype in human AVIC [13], and support the role of TLRs in the calcification process underlying aortic stenosis.

Among the limitations of this study is the use of cultured AVICs, which might have changed some of their *in vivo* features, although it should be noted that TLR expression and signaling were similar in early and late passages. The study did not include analysis of TLR expression in patients nor in sections of valve tissue, although it should be taken into account that previous reports have shown TLR4 and TLR2 expression in the heart valve, and their increase in stenotic valves [11,13]. Further, experiments were performed *in vitro* by using agonists that artificially mimic TLR activation, and TLR-independent effects cannot be ruled out, although it should be noted that siRNA experiments strongly suggest that the effects are mostly mediated by TLR (not shown).

In summary, our study demonstrates that AVICs can sense a widespread range of bacterial and viral patterns through TLRs, which show differential expression in control and stenotic valve cells. Our results disclose a potential role of viral infection in the pathogenesis of aortic stenosis, and suggest the existence of selective pathogen-induced

pro-inflammatory and pro-osteogenic phenotypes in AVICs, which might be relevant to degenerative aortic stenosis pathogenesis.

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The authors of this manuscript have certified that they comply with the Principles of Ethical Publishing in the International Journal of Cardiology [31].

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Figure legends

Fig. 1. Differential TLR expression in AVICs from stenotic and control valves. (A) Total RNA was analyzed by quantitative RT-PCR as described in Methods. Graph includes results obtained with AVICs from n=10 control (white bars) and stenotic valves (black bars). Data represent mean ± SD of the relative mRNA levels normalized to results from the housekeeper actin. * Indicates $p < 0.05$ when comparing data from control and stenotic valves. AVICs from control (B) and stenotic valves (C) were permeabilized with saponin or left untreated and immunolabeled with a PE-anti-human TLR4 antibody. Isotype control antibody results are shown in gray. Graphs are representative from 5 independent experiments. (D) Data represent the ratio of the median of mean fluorescence intensity obtained with the anti-TLR4 and the control antibodies and the standard deviation.

Fig. 2. TLR4, TLR2, and TLR3 stimulation induces NF-κB and MAPK activation in AVICs from control and stenotic valves. Cells from control (A) and stenotic valves (B) were incubated with 1 μg/ml *E. coli* LPS, 100 ng/ml Pam_3CSK4, 10 μg/ml, poly (I:C),10 μg/ml CpG-ODN, or vehicle for the indicated times, and cells lysates were analyzed by Western blot with corresponding antibodies. Immunoblots are representative from at least 3 independent experiments.

Fig. 3. TLR3 but not TLR9 activation induces NF-κB-independent expression of COX-2, PGE_2 and ICAM-1 in AVICs. (A) Cells from control and stenotic valves were incubated with either 10 μg/ml poly (I:C) or 10 μg/ml CpG-ODN or vehicle for the indicated times, and cells lysates were analyzed by Western blot. Immunoblots are

representative from 3 independent experiments. Pam indicates Pam_3CSK4. (B, C) PGE_2 secretion was analyzed by ELISA in the supernatants of cells isolated from control (B) and stenotic valves (C), respectively. Image is representative from 2 independent experiments. (D) Cells were treated with 18 μM NF-κB SN50 before stimulation with poly (I:C) or vehicle. Images correspond to different lanes of the same gel; linear adjustment of contrast and brightness was applied equally to all lanes. Immunoblots are representative from at least 3 independent experiments.

Fig. 4. TLR4 but not TLR2 activation induces COX-2, PGE_2 and ICAM-1 expression in a NF-κB-dependent manner in AVICs. (A) Cells from control and stenotic valves were incubated with either 1 μg/ml *E. coli* LPS, or 100 ng/ml Pam_3CSK4, or vehicle for the indicated times, and cells lysates were analyzed as in Fig. 3. (B, C) PGE_2 secretion was measured in the supernatants of cells isolated from control (B) and stenotic valves (C), respectively. (D) Cells were treated with NF-κB SN50 before treatment with LPS or vehicle. Images correspond to different lanes of the same gel; linear adjustment of contrast and brightness was applied equally to all lanes.

Fig. 5. TLR3 activation induces a similar pro-inflammatory cytokine phenotype than TLR4 activation. Cells were incubated with vehicle, Poly (I:C), LPS or Pam_3CSK4 for 12 hours, and supernatants were analyzed for cytokine expression using an antibody array, as indicated in Material in Methods. Squares indicate positive controls, which were used to normalize results from different membranes. Arrows indicate constitutively expressed cytokines. Ovals indicate the cytokines unambiguously induced upon stimulation. Images are representative of two independent experiments.

Fig. 6. TLR3 and TLR4 activation induces alkaline phosphatase activity in human AVICs. Cells were treated for 17-19 days with growth media (M199) or conditioning media (CM) in the presence of 1 μg/ml *E. coli* LPS, or 10 μg/ml poly (I:C), or vehicle. (A) Cells were stained as indicated in methods. Image is representative of 3 experiments in duplicate. Arrows indicate areas with ALP blue staining (B) Cells were lysed and alkaline phosphatase activity was evaluated, as indicated in methods. Results are expressed as the increase fold as compared to the growth media. Data represent mean ± SD of 4 experiments in duplicate. * Indicates $p < 0.0001$ when comparing with conditioning media results.