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Ligation of ICAM-1 on human aortic valve interstitial cells induces the osteogenic response: A critical role of the Notch1-NF-κB pathway in BMP-2 expression

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ABSTRACT

Calcific aortic valve disease (CAVD) is a chronic inflammatory condition and affects a large number of elderly people. Aortic valve interstitial cells (AVICs) occupy an important role in valvular calcification and CAVD progression. While pro-inflammatory mechanisms are capable of inducing the osteogenic responses in AVICs, the molecular interaction between pro-inflammatory and pro-osteogenic mechanisms remains poorly understood. This study tested the hypothesis that intercellular adhesion molecule-1 (ICAM-1) plays a role in mediating pro-osteogenic factor expression in human AVICs. AVICs were isolated from normal human aortic valves and cultured in M199 medium. Treatment with leukocyte function-associated factor-1 (LFA-1, an ICAM-1 ligand) up-regulated the expression of bone morphogenetic protein-2 (BMP-2) and resulted in increased alkaline phosphatase activity and formation of calcification nodules. Pre-treatment with lipopolysaccharide (LPS, 0.05 μg/ml) increased ICAM-1 levels on cell surfaces and exaggerated the pro-osteogenic response to LFA-1, and neutralization of ICAM-1 suppressed this response. Further, ligation of ICAM-1 by antibody cross-linking also up-regulated BMP-2 expression. Interestingly, LFA-1 elicited Notch1 cleavage and NF-κB activation. Inhibition of NF-κB markedly reduced LFA-1-induced BMP-2 expression, and inhibition of Notch1 cleavage with a γ-secretase inhibitor suppressed LFA-1-induced NF-κB activation and BMP-2 expression. Ligation of ICAM-1 on human AVICs activates the Notch1 pathway. Notch1 up-regulates BMP-2 expression in human AVICs through activation of NF-κB. The results demonstrate a novel role of ICAM-1 in translating a pro-inflammatory signal into a pro-osteogenic response in human AVICs and suggest that ICAM-1 on the surfaces of AVICs contributes to the mechanism of aortic valve calcification.

1. Introduction

Calcific aortic valve disease (CAVD) is one of the leading cardiovascular diseases with a prevalence of 1% to 2% in people over the age of 65 years [1]. Similar to atherosclerosis, CAVD is a chronic inflammatory condition [2]. The "early lesions" associated with CAVD are characterized by inflammatory changes, including the accumulation of T lymphocytes and mononuclear cells in valvular tissue [3,4]. This is supported by the observation that explanted aortic valves from patients with CAVD exhibit abundant lymphocytes and macrophages. Aortic valve interstitial cells (AVICs) have been demonstrated to play an important role in the valvular inflammatory and osteogenic responses [5,6]. A number of studies indicate that bone morphogenetic protein-2 (BMP-2), a pro-osteogenic protein, is involved in vascular and valvular calcification [1,6], and several pro-inflammatory stimuli, including Toll-like receptor agonists, are capable of inducing BMP-2 expression in human AVICs [7–9]. Further, stimulation of human AVICs with BMP-2 results in osteogenic reprogramming [10]. Although leukocyte infiltration is evident in diseased human aortic valves, it remains unknown whether the interaction of infiltrated leukocytes with AVICs has an impact on AVIC osteogenic responses.

Bacterial products and pro-inflammatory cytokines evoke an osteogenic response in AVICs through Toll-like receptors or cytokine receptors [7,8,11]. There could be unique pro-inflammatory factors that exert effects on AVIC osteogenic response via novel signaling pathways. Intercellular adhesion molecule (ICAM)-1 may be one of such pro-inflammatory factors. ICAM-1 is an immunoglobulin (Ig)-like cell adhesion molecule constitutively expressed on cardiovascular cells [12]. Stimulation of cells with pro-inflammatory factors, such as interleukin (IL)-1, tumor

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necrosis factor (TNF) α and lipopolysaccharide (LPS) upregulates ICAM-1 expression in a variety of cell types. ICAM-1 is involved in leukocyte migration and adhesion in the sites of inflammation [13–16]. Therefore, it interacts directly with integrins on leukocyte surfaces. Interestingly, ICAM-1 is found to function as a receptor for leukocyte integrins to elicit intracellular signaling in cells that interact with leukocytes [17,18]. Indeed, the β2 integrins on leukocytes, i.e. lymphocyte function-associated molecule-1 (LFA-1, CD11a/CD18) and macrophage-1 antigen (MAC-1, CD11b/CD18), are found to be ligands for ICAM-1 on effector cells and induce ICAM-1-dependent cellular activation in a variety of effector cells, such as vascular endothelial cells [19–21].

The physiologic outcome of engagement of ICAM-1 by β2 integrins and the resultant cell activation depend, in part, upon the type of cells. Activation of ICAM-1 on endothelial cells might elicit increased production of cytokines, such as IL-8 and RANTES, or increased expression of adhesion molecules (including ICAM-1 and VCAM), leading to enhanced leukocyte trafficking [22]. Human AVICs express high levels of ICAM-1 in response to pro-inflammatory stimulation [7,23]. Elevated ICAM-1 expression by AVICs should play a critical role in mediating leukocyte infiltration to valvular tissue. It remains unknown, however, whether ligation of ICAM-1 on AVICs has an effect on cellular osteogenic response.

We hypothesize that ICAM-1 on cell surfaces of human AVICs modulates cellular osteogenic response. The purposes of this study were to determine the effect of ICAM-1 ligation on the expression of BMP-2 in human AVICs and to elucidate the signaling mechanism that mediates the effect.

2. Materials and methods

2.1. Materials

CD11a (LFA-1) peptide was purchased from Abcam, Inc. (Cambridge, MA). Monoclonal antibody against human ICAM-1 was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Polyclonal ICAM-1-neutralizing antibody was purchased from EMD Chemicals, Inc. (San Diego, CA). PE-conjugated human ICAM-1 antibody was purchased from eBioscience, Inc. (San Diego, CA). Antibodies against Notch1, NICD1, β-actin, phosphorylated NF-κB p65 and total NF-κB p65 were purchased from Cell Signaling, Inc. (Beverly, MA). The antibody against phosphorylated NF-κB p65 identifies the phosphorylation of Ser536. Antibody against BMP-2 was purchased from Prosci, Inc. (Poway, CA). Medium 199 was purchased from Lonza (Walkersville, MD). Recombinant Jagged1 and BMP-2 ELISA kit were purchased from R&D System (Minneapolis, MN). Jagged1 ELISA kit was purchased from Uscn Life Science Inc. (Germany). Kit for NF-κB DNA-binding assay was purchased from Active Motif (Carlsbad, CA). Cell lysis buffer was purchased from Thermo Fisher Scientific (Rockford, IL). LPS (E. coli 0111:B4) and all other chemicals and reagents were purchased from Sigma-Aldrich Chemical Co (St Louis, MO).

2.2. Cell isolation and treatment

Normal aortic valve leaflets were collected from the explanted hearts of 6 patients undergoing heart transplantation (4 males and 2 females, mean age 61 ± 7.2 years). All patients gave written consent for the use of their heart valves for this study. This study was approved by the Institutional Review Board of the University of Colorado Denver.

AVICs were isolated from each of the aortic valve leaflets, and individual isolate was cultured using a previously described method [7]. Briefly, valve leaflets were subjected to sequential digestions with collagenase to eliminate endothelial cells and release interstitial cells, and then interstitial cells were collected by centrifugation. Interstitial cells were cultured in M199 growth medium containing penicillin G, streptomycin, amphotericin B and 10% fetal bovine serum. When cell culture reached 80 to 90% confluence, cells were subcultured on plates and chamber slides for the experiments. Cells from passages 4 to 6 were used for this study.

Cells were stimulated with LPS (0.05 μg/ml) for 1 to 8 h to upregulate the levels of ICAM-1 on cell surfaces. Cells were treated with LFA-1 for 24 h, with or without prior to stimulation with LPS, to assess the effect of this ICAM-1 ligand on BMP-2 expression. To determine the role of ICAM-1 in the effect of LFA-1, cells were treated with ICAM-1-neutralizing antibody (10 μg/ml) prior to LFA-1 treatment.

Ligation of ICAM-1 by antibody cross-linking was performed to confirm the role of ICAM-1 in the induction of BMP-2 expression, using the method described previously [24–27]. Briefly, cells were incubated with 15 μg/ml of mouse monoclonal antibody against human ICAM-1 at 37 °C for 60 min, and control cells were incubated with 15 μg/ml of isotype-matching mouse IgG. Following incubation, cells were washed three times with medium and subsequently incubated with 50 μg/ml of anti-mouse IgG for 24 h.

Cells were treated with LFA-1 for 2 to 8 h to assess the effect of ICAM-1 ligation on Notch1 activation, Jagged1 release and NF-κB activation. Specific NF-κB inhibitor (SN50; 100 μmol/L) was added to culture medium 30 min before the addition of LFA-1 to determine the role of NF-κB in the induction of BMP-2 expression. To determine the role of Notch1 in NF-κB phosphorylation and BMP-2 expression, γ-secretase inhibitor DAPT (50 μmol/L) was added 30 min prior to addition of LFA-1.

2.3. Immunofluorescent staining

Immunofluorescent staining was applied to character cell surface ICAM-1 expression as described previously [28]. After permeabilization with a methanol/acetone mixture, cells on chamber slides were fixed in 4% paraformaldehyde, incubated with a rabbit polyclonal antibody against human ICAM-1 overnight at 4 °C. After washing with PBS, cells were incubated with Cy3-tagged secondary antibody against the primary antibody (imaged on the red channel). Nuclei were stained with bisbenzimide (4′,6-diamidino-2-phenylindole, imaged on the blue channel). Microscopy was performed with a Leica DMRXA digital microscope (Leica Mikroskopie und Systeme GmbH, Wetzlar, Germany) equipped with Slidebook software (I. I. I. Inc., Denver, CO).

2.4. Flow cytometry

For direct immunofluorescence staining, untreated or LPS-treated cells (2×10^6 cells/ml) were pre-incubated in 5% BSA to block non-specific binding. The cells were washed twice with PBS containing 1% BSA and 0.02% sodium azide, and then incubated with 10 μl of PE-conjugated monoclonal antibody against ICAM-1 or PE-conjugated non-immune IgG at 4 °C for 30 min, and analyzed by a flow cytometer (Beckman Coulter FC500, Becton Dickinson, Franklin Lakes, NJ, USA).

2.5. Immunoblotting

Immunoblotting was applied to analyze ICAM-1, BMP-2, NICD1, β-actin, phosphorylated NF-κB p65 and total NF-κB p65. Cells were lysed in a sample buffer (100 mM Tris-HCl, pH 6.8, 2% SDS, 0.02% bromophenol blue and 10% glycerol). Protein samples were separated on gradient (4–20%) minigels and transferred onto nitrocellulose membranes (Bio-Rad Laboratories, Hercules, California). The membranes were blocked with 5% non-fat dry milk solution for 1 h at room temperature. The blocked membranes were incubated with a primary antibody against the protein of interest. After washing with TPBS (PBS containing 0.05% Twen 20), the membranes were incubated with a peroxidase-linked secondary antibody specific to the primary antibody. Following further washes, membranes were treated with enhanced chemiluminescence reagents. Then the membrane was exposed on X-ray film. Image J was used to assess band density.

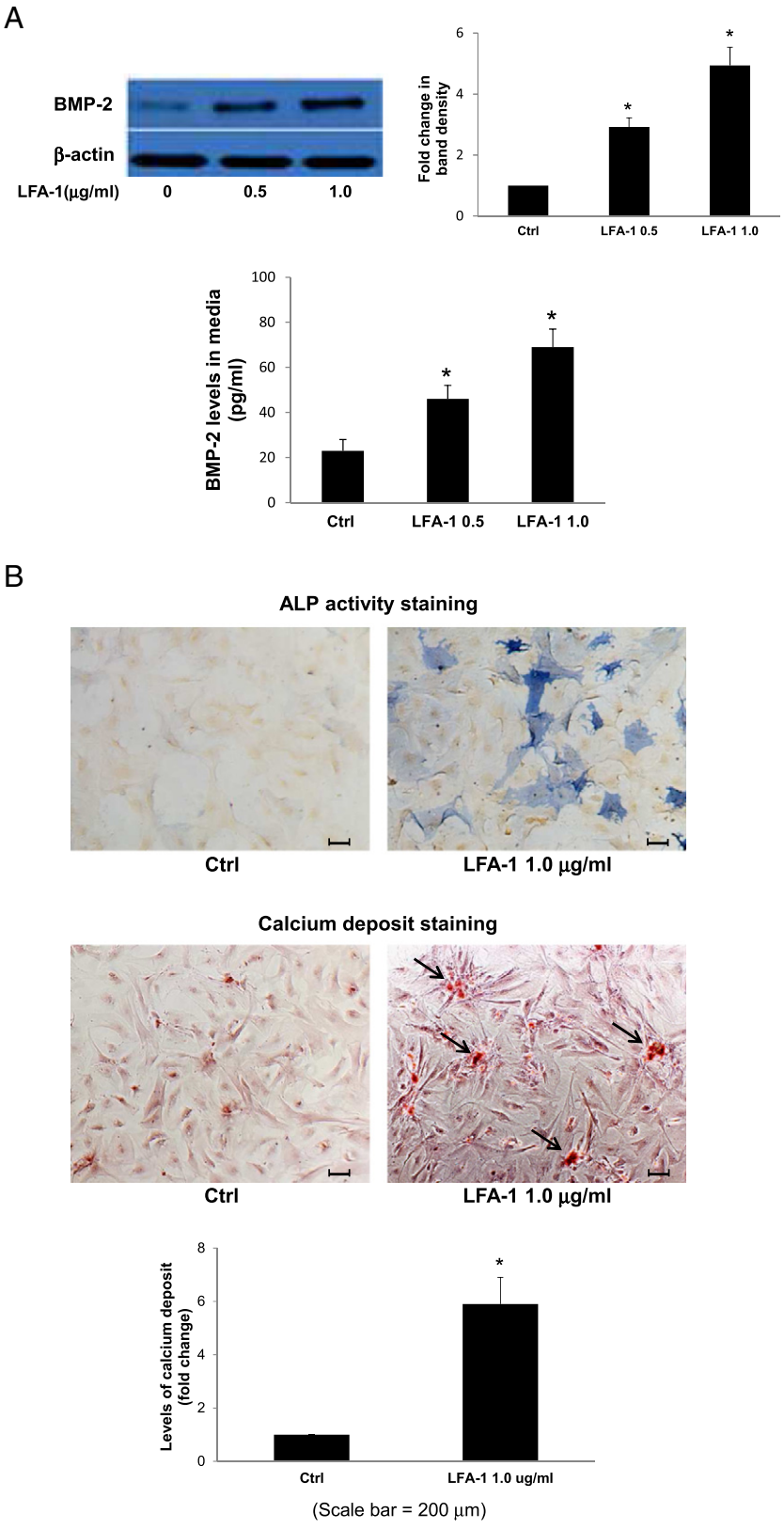
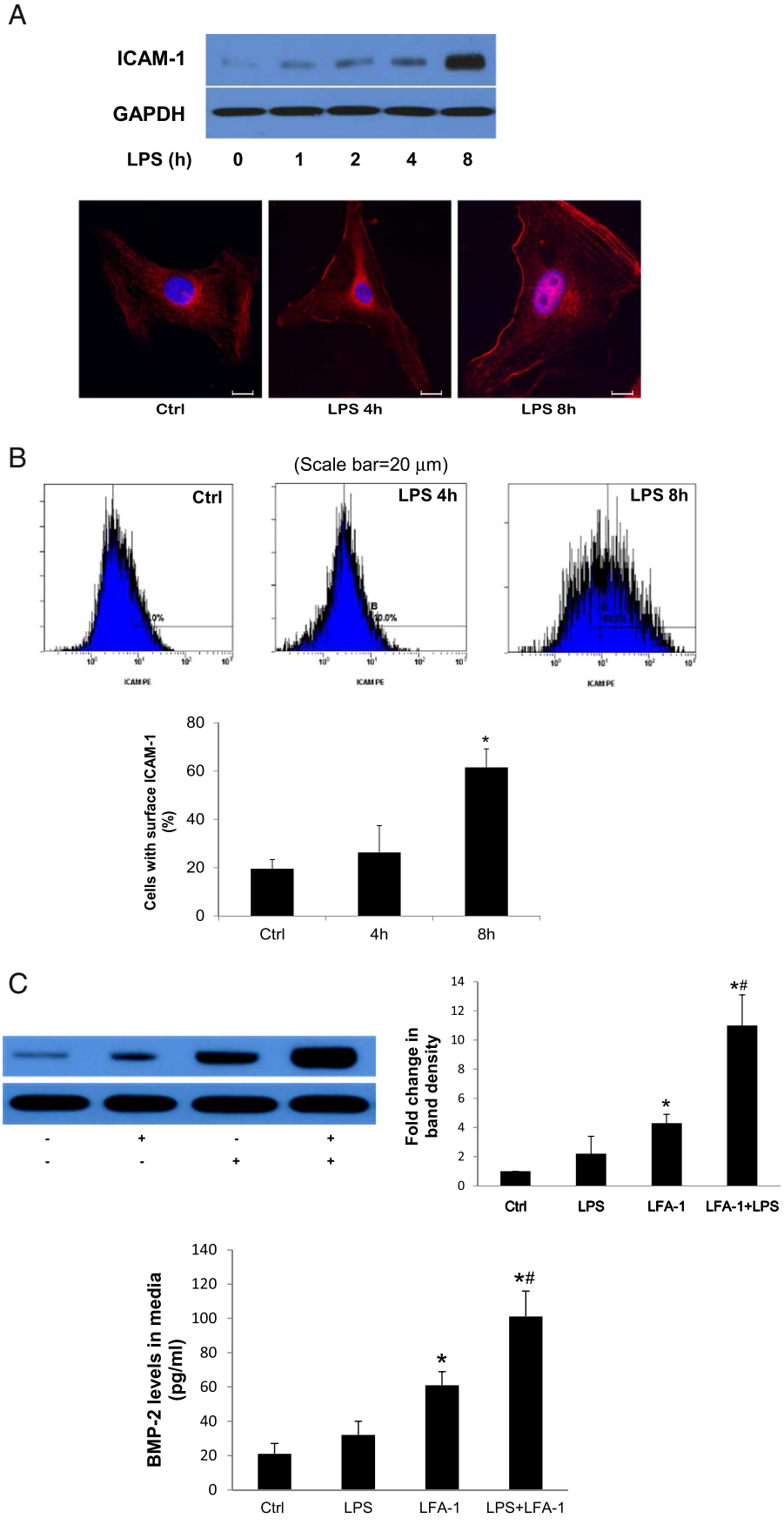


Fig. 1. LFA-1 induces BMP-2 expression in human AVICs. A. AVICs of normal aortic valves are treated with LFA-1 (0.5 or 1.0 μg/ml) for 24 h. A representative immunoblot, gel densitometric data and ELISA data show that treatment with LFA-1 increases BMP-2 levels in cell lysate and culture media. n = 6; *$P < 0.05$ vs. untreated controls (Ctrl). B. AVICs of normal aortic valves are treated with LFA-1 (1.0 μg/ml) for 7 or 21 days. Representative images (size bar = 200 μm) show that treatment with LFA-1 for 7 days up-regulates alkaline phosphatase activity and that treatment for 21 days in conditioning medium causes calcification nodule formation. Quantitative data (n = 6) confirms that treatment with LFA-1 for 21 days increases the levels of calcium deposits. *$P < 0.05$ vs. control cells (Ctrl) cultured in the conditioning medium.


A
ICAM-1
GAPDH
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Cells with surface ICAM-1 (%)
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20
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Fold change in band density
14
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140
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LPS
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LPS+LFA-1

2.6. NF-κB DNA-binding assay

NF-κB p65 DNA-binding activity in cell lysate was measured by an assay kit according to the manufacturer's instructions. The kit contains 96-well plates to which oligonucleotides containing the NF-κB binding site are immobilized. Activated NF-κB binds to the oligonucleotides and is detected with a specific antibody. The amount of active NF-κB was determined using a microplate reader.

2.7. ELISA for BMP-2 and Jagged1

Cell culture supernatants were collected. Levels of BMP-2 and Jagged1 were determined using ELISA kits as described previously [28].

2.8. ALP activity staining

Histochemical staining for alkaline phosphatase activity was performed as previously described [29]. Briefly, cell monolayers were washed with PBS and fixed for 10 min in 4% paraformaldehyde, followed by incubation at room temperature for 30 min with a mixture of 0.1 mg/ml of naphthol AS-MX phosphate, 0.5% N,N-dimethylformamide, 2 mM $MgCl_2$, and 0.6 mg/ml of fast blue BB salt in 0.1 M Tris–HCl, and pH8.5. Excessive dye was removed by washing with PBS. ALP activity staining was examined and photographed with a Nikon Eclipse TS100 microscope (Tokyo, Japan).

2.9. Alizarin red S staining

Alizarin red S staining for calcium deposits was performed as described previously [8,29,30]. Briefly, cell monolayers were washed three times with PBS and fixed for 10 min with 4% paraformaldehyde. Fixed cells were incubated with 0.2% alizarin red S solution (pH 4.2) for 30 min. Excessive dye was removed by washing with distilled water. Alizarin red S staining was examined and photographed with a Nikon Eclipse TS100 microscope (Tokyo, Japan). To quantitatively analyze Alizarin red stain, Alizarin red S stains were bleached with 10% acetic acid at 85 °C and assessed spectrophotometrically at 450 nm [31].

2.10. Statistical analysis

Data are presented as mean ± standard error (SE). Comparisons between groups were performed using StatView software (Abacus Concepts, Calabasas, CA) with one-way analysis of variance (ANOVA) with the post hoc Fisher test. A difference was considered significant at $P \leq 0.05$. Significant differences were confirmed with Mann–Whitney non-parametric test.

3. Results

3.1. Ligation of ICAM-1 with LFA-1 induces BMP-2 expression and osteogenic reprogramming in human AVICs

To determine the effect of ICAM-1 ligand on BMP-2 expression, we exposed AVICs of normal aortic valves to LFA-1 (0.5 or 1.0 μg/ml) for 24 h. As shown in Fig. 1A, treatment with LFA-1 resulted in a dose-dependent increase in BMP-2 protein levels in cell lysate and culture media. In cells exposed to 1.0 μg/ml of LFA-1, levels of cell associated BMP-2 increased by 4 folds ($P < 0.05$).

Our previous studies demonstrate that BMP-2 is capable of inducing osteogenic reprogramming in human AVICs, and this pro-osteogenic factor mediates human AVIC osteogenic reprogramming evoked by pro-inflammatory stimuli [8]. As ICAM-1 ligand LFA-1 up-regulates BMP-2 production, we tested the hypothesis that prolonged treatment with LFA-1 will result in osteogenic reprogramming (increased alkaline phosphatase activity and greater vulnerability to form calcification nodules) in human AVICs. As shown in Fig. 1B, treatment with LFA-1 (1.0 μg/ml) for 7 days increased the number of cells that are positive for alkaline phosphatase activity staining. In addition, treatment of with LFA-1 for 21 days resulted in the formation of calcification deposits in human AVICs cultured in conditioning medium (growth medium with 10 mmol/L β-glycerophosphate, 10 nmol/L vitamin D3 and 10 nmol/L dexamethasone).

3.2. LFA-1 induces BMP-2 expression in human AVICs in an ICAM-1-dependent fashion

Since BMP-2 plays an important role in vascular and valvular calcification [8,32], our further experiments concentrated on the mechanism by which LFA-1 induces BMP-2 expression.

We examined whether up-regulation of ICAM-1, using pretreatment with a low concentration of LPS as a tool, enhances the effect of LFA-1 on BMP-2 expression. As shown in Fig. 2A, treatment with a low concentration of LPS (0.05 μg/ml) increased ICAM-1 protein levels over time and resulted in a maximal increase at 8 h. Immunofluorescence images show apparent cell surface ICAM-1 after treatment with LPS for 8 h (Fig. 2B), and flow cytometry data show that ICAM-1 are detectable on cell surfaces in 60% of cells (Fig. 2B) at 8 h. The results in Fig. 2C show that LFA-1 induces a greater increase in BMP-2 levels in cell lysate and culture media if cells are pretreated with LPS for 8 h. Thus, up-regulation of ICAM-1 levels on cell surfaces exaggerates the effect of LFA-1 on BMP-2 expression. It appears that LFA-1 induces BMP-2 expression in human AVICs in an ICAM-1-dependent fashion. To confirm this, we examined whether blocking ICAM-1 with a neutralizing antibody may abrogate the effect of LFA-1 on BMP-2 expression. As shown in Fig. 3, blocking ICAM-1 abolished BMP-2 expression in cells treated with LFA-1 alone and markedly reduced BMP-2 levels in cells treated with LPS + LFA-1 while non-immune IgG had no effect. Thus, LFA-1 induces BMP-2 expression through an ICAM-1-dependent mechanism. As LPS alone can induce a slight increase in cellular BMP-2 levels (Fig. 3), the remaining low levels of BMP-2 in cells received LPS pretreatment before treatment with ICAM-1-neutralizing antibody and LFA-1 could be due to the ICAM-1 ligation-independent up-regulation of BMP-2 by LPS.

3.3. Ligation of ICAM-1 by antibody cross-linking also induces BMP-2 expression in human AVICs

To further validate that ICAM-1 ligation induces BMP-2 expression in human AVICs, we treated cells with cross-linking antibodies (monoclonal anti-CAM-1 followed by a secondary antibody) for 24 h and analyzed BMP-2 levels with immunoblotting. We found that ICAM-1 ligation with antibody cross-linking induces BMP-2 expression, especially in cells pretreated with LPS (Fig. 4). The results confirmed that ligation of ICAM-1 by different mechanisms up-regulates BMP-2 expression in human AVICs.

Fig. 2. Up-regulation of ICAM-1 levels augments the BMP-2 response to LFA-1. Human AVICs are treated with a low concentration of LPS (50 ng/ml) for 1–8 h. A. A representative immunoblot shows that cells exposed to LPS express ICAM-1 protein in a time-dependent fashion. B. Representative immunofluorescence images (size bar = 20 μm) show that ICAM-1 accumulates over time on the surfaces of cells treated with LPS, and flow cytometry data (n = 6) show increased numbers of cells with ICAM-1 on their surfaces after LPS stimulation for 8 h. *$P < 0.05$ vs. untreated controls (Ctrl). C. A representative immunoblot, gel densitometric data and ELISA data show that treatment with LFA-1 (1.0 μg/ml) markedly increases BMP-2 levels in cell lysate and culture media of cells pretreated with LPS for 8 h. n = 6; *$P < 0.05$ vs. untreated controls (Ctrl); #$P < 0.05$ vs. LFA-1 alone.

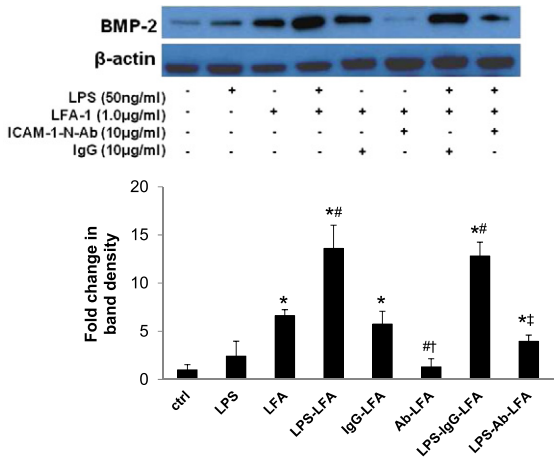


Fig. 3. Neutralization of ICAM-1 reduces the pro-osteogenic effect of LFA-1. Human AVICs are treated with ICAM-1-neutralizing antibody (10 μg/ml) for 1 h prior to treatment with LFA-1 (1.0 μg/ml). A representative immunoblot and densitometric data (n = 6) show that treatment with ICAM-1-neutralizing antibody reduces BMP-2 expression induced by LFA-1 or LFA-1 plus LPS pretreatment. *$P < 0.05$ vs. untreated controls (Ctrl); #$P < 0.05$ vs. LFA alone; †$P < 0.05$ vs. LFA + IgG; ‡$P < 0.05$ vs. LPS + LFA and LPS + IgG + LFA.

3.4. Inhibition of NF-κB suppresses the induction of BMP-2 expression by ICAM-1 ligation

It has been reported that ligation of ICAM-1 on endothelial cells can activate NF-κB [26]. We determined whether NF-κB plays a role in mediating the induction of BMP-2 expression by ICAM-1 ligation.

We examined NF-κB p65 phosphorylation in cells pretreated with LPS and then exposed to LFA-1 for 2 to 8 h. The low dose of LPS applied in this study induced detectable NF-κB p65 phosphorylation only at 2 h. Interestingly, NF-κB p65 phosphorylation was observed at 4 and 8 h after the exposure to LFA-1 (Fig. 5A). NF-κB DNA-binding activity was also increased at 4 and 8 h after the exposure to LFA-1 (Fig. 5B).

To evaluate the role of NF-κB in BMP-2 expression induced by ICAM-1 ligation, we treated cells with the specific NF-κB inhibitor SN-50 (100 μg/ml) or control reagent SN-50M prior to exposure to LFA-1.

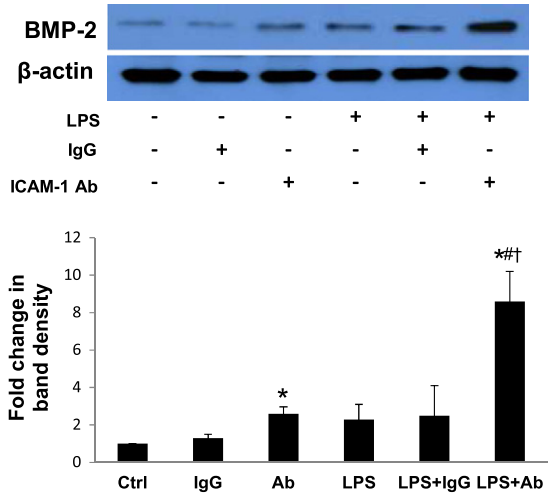


Fig. 4. ICAM-1 ligation by antibody cross-linking also induces BMP-2 expression. Human AVICs with LPS pretreatment were incubated with 15 μg/ml of monoclonal anti-ICAM-1 or isotype-matching non-immune mouse IgG for 60 min and subsequently incubated with 50 μg/ml of anti-mouse IgG for 24 h. A representative immunoblot and densitometric data (n = 6) show that cross-linking of ICAM-1 with antibodies up-regulates the expression of BMP-2. *$P < 0.05$ vs. untreated controls (Ctrl); #$P < 0.05$ vs. LPS alone and LPS + non-immune IgG, †$P < 0.05$ vs. cross-linking antibodies (Ab) in the absence of LPS pretreatment.

Results in Fig. 5C show that the NF-κB inhibitor SN50 reduces BMP-2 levels in cells exposed to LFA-1 alone or LPS + LFA-1. In contrast, the control reagent SN50M had no effect. To confirm the role of NF-κB, we applied BAY 11-7082, a specific IKK inhibitor, and observed that inhibition of NF-κB activation with BAY 11-7082 also reduces BMP-2 levels in cells exposed to LFA-1 alone or LPS + LFA-1 (Fig. 5C).

Together these results show that NF-κB activation occurs in human AVICs in response to ICAM-1 ligation and that NF-κB plays an important role in mediating BMP-2 expression induced by ICAM-1 ligation.

3.5. Notch1 has an essential role in BMP-2 expression induced by ICAM-1 ligation

We have observed that Notch1 modulates NF-κB activation in human AVICs [23]. In the present study, we treated cells with LFA-1 for 4 or 8 h to examine whether ICAM-1 ligation induces Notch1 activation in human AVICs. Notch1 intracellular domain (NICD1) generation was analyzed with immunoblotting, and cellular release of Notch1 ligand Jagged1 was examined by ELISA. We found that the low dose of LPS applied increases cellular levels of NICD1 slightly at 4 and 8 h. However, greater levels of NICD1 were observed in LPS-pretreated cells exposed to LFA-1 for 4 or 8 h (Fig. 6A). Further, we found that ICAM-1 ligation with LFA-1 caused the release of Jagged1. Jagged1 levels in culture media examined at 4 and 8 h were significantly elevated, especially in cells pretreated with LPS (Fig. 6B). Therefore, Notch1 activation induced by ICAM-1 ligation is associated with the secretion of Notch1 ligand Jagged1.

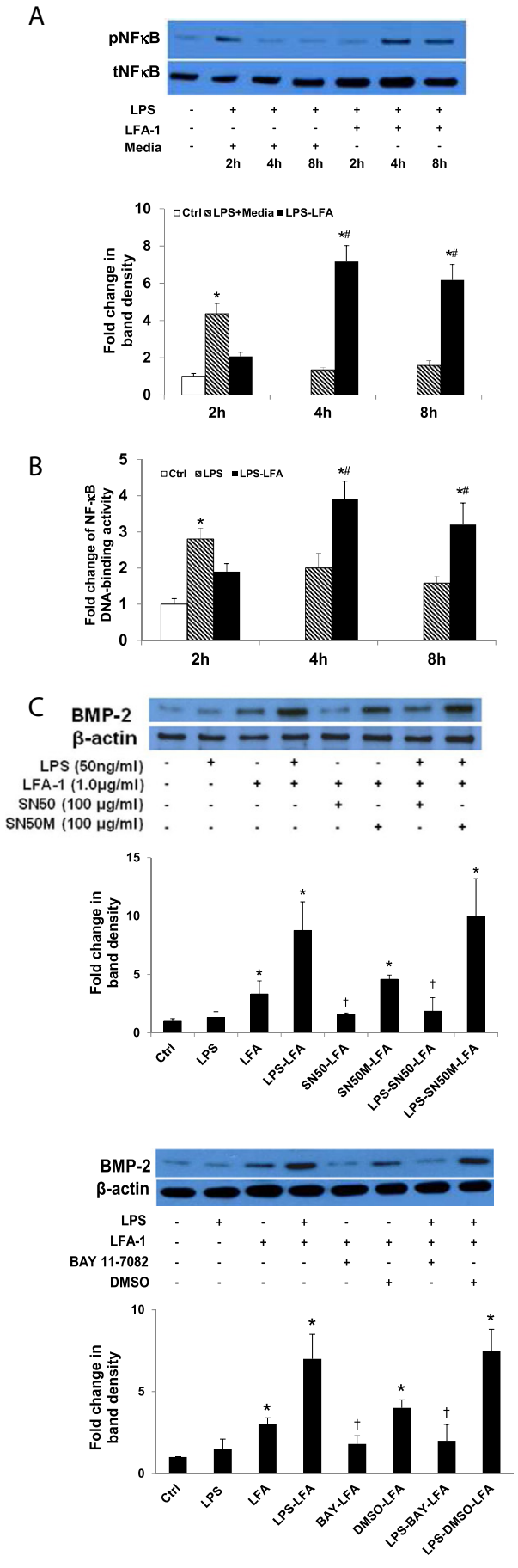
Notch1 is cleaved by γ-secretase to become active [33]. To determine the role of Notch1 activation in BMP-2 expression induced by ICAM-1 ligation, we applied DAPT, a specific γ-secretase inhibitor, to cells before LFA-1 treatment. Surprisingly, we found that treatment with DAPT reduced BMP-2 expression induced by LFA-1 alone or LPS + LFA-1 (Fig. 6C). It appears that the Notch1 pathway is required for the induction of BMP-2 expression by ICAM-1 ligation.

To understand whether activation of Notch1 modulates NF-κB activation, we examine the effect of DAPT treatment on NF-κB p65 phosphorylation and DNA-binding activity. The results in Fig. 6D show that the levels of NF-κB p65 phosphorylation and DNA-binding activity were markedly reduced by DAPT at 4 and 8 h in cells treated with LPS + LFA-1. Together, our results demonstrate that activation of Notch1 contributes to the mechanism underlying NF-κB activation and the NF-κB-mediated up-regulation of BMP-2 expression induced by ICAM-1 ligation.

4. Discussion

CAVD is a chronic inflammatory condition, and nodular calcification of the aortic valve leaflets is a significant manifestation of this disease [34]. Progressive aortic valve calcification results in the restriction of valvular opening and closing. Secondary left ventricular hypertrophy drives the development of heart failure. Currently, aortic valve replacement is the only available therapy. Understanding of the mechanism responsible for aortic valve calcification is important for identification of targets for pharmacological intervention.

AVICs are known to play an important role in valvular calcification and CAVD progression. In addition, AVICs are believed to have the ability to convert pro-inflammatory signals to a pro-osteogenic response. While pro-inflammatory mechanisms are capable of inducing the osteogenic responses in human AVICs, the molecular interaction between pro-inflammatory and pro-osteogenic mechanisms remains poorly understood. In the present study, we found that ligation of ICAM-1 on human AVICs by leukocyte β-integrin LFA-1 induces the expression and secretion of pro-osteogenic factor BMP-2 and that prolonged treatment with LFA-1 leads to AVIC osteogenic reprogramming (with elevated alkaline phosphatase activity and a greater vulnerability to form calcification deposits). It is known that secreted BMP-2 bind to its receptor in



neighboring cells to exert a pro-osteogenic effect, and this pro-osteogenic factor plays an important role in vascular and valvular calcification [8,32]. In the present study, we provided several lines of evidence to show that a Notch1-NF-κB axis plays a critical role in mediating the up-regulation of BMP-2 expression by ICAM-1 ligation in human AVICs. The results of this study support a novel concept that ICAM-1 senses an inflammatory signal to elaborate a pro-osteogenic response in human AVICs. This study highlights the potential role of ICAM-1 in mediating aortic valve calcification in an inflammatory milieu.

4.1. Ligation of ICAM-1 is potent to induce the expression of BMP-2 in human AVICs

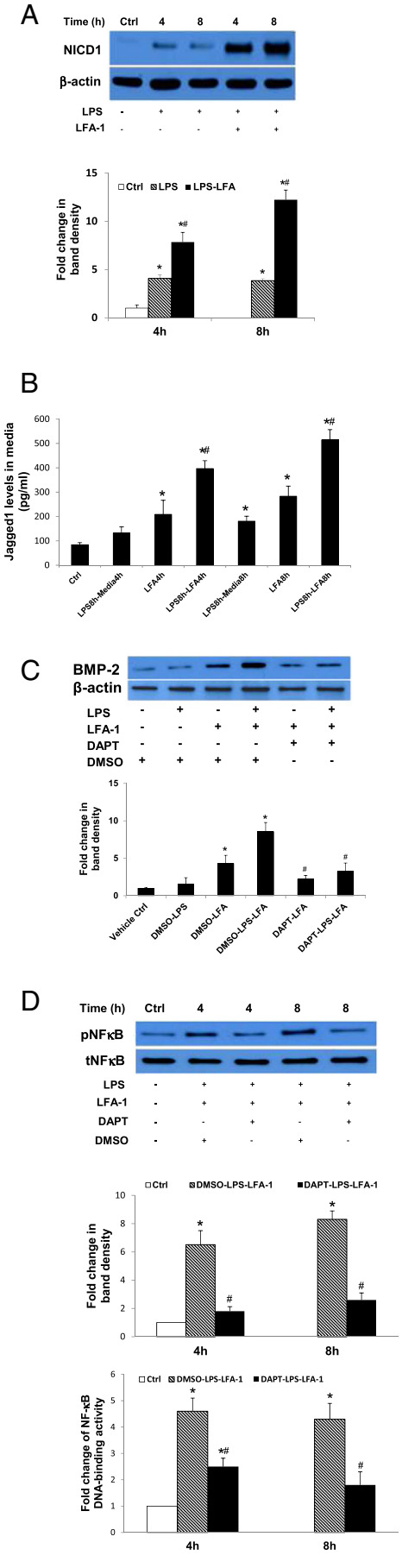
Several reports have demonstrated the presence of leukocytes in diseased aortic valves, suggesting that an active inflammatory process is involved in the development and/or progression of CAVD [1,3,4,35]. ICAM-1, the most extensively studied adhesion molecular of the five ICAM molecules, acts as an important portal of entry for inflammatory cell infiltration, and infiltrated monocytes and lymphocytes are known to exaggerate tissue inflammation [36]. Currently, few studies have examined ICAM-1 expression in AVICs [7,23]. Further, the impact of the interaction between leukocyte integrins and ICAM-1 on AVIC function is unknown.

LFA-1, a member of the β2-integrin family, is a potent ligand on the surfaces of leukocytes and mediates leukocyte adhesion through binding to ICAM-1 [37]. LFA-1 has αL- and β2-subunits, which are designated as CD11a and CD18, respectively. The αL-subunit of LFA-1 contains an I- (insert) domain that is essential for binding to ICAM-1 [38]. In this study we choose CD11a as a representative of LFA-1. It is well described that ligation of ICAM-1 by CD11a or LFA-1 is important for granulocyte extravasation, lymphocyte-mediated cytotoxicity and the development of specific leukocyte-mediated immunological responses [39].

The results of this study show that CD11a induces BMP-2 expression in human AVICs via ligation of ICAM-1. Three lines of evidence support this notion. First, up-regulation of cell surface ICAM-1 levels by LPS pretreatment augments the BMP-2 response to CD11a. Second, blockade of ICAM-1 using a neutralizing antibody prior to LFA-1 treatment abolishes the pro-osteogenic effect of CD11a. Lastly, we applied antibody cross-linking as a tool for ICAM-1 ligation and observed that this approach also up-regulates BMP-2 expression in human AVICs. Previous studies have shown that cross-linking of ICAM-1 with antibodies activates endothelial cells and mimics ligand/receptor interactions [20]. The present study reveals a novel role of ICAM-1 in human AVICs. In view of the fact that calcific aortic valve disease is a chronic inflammatory condition and involves valvular calcification, it is reasonable to postulate that ICAM-1 may function as a receptor in human AVICs that senses an inflammatory milieu and signals for the osteogenic response.

It should be noted that LPS induces the expression of multiple pro-inflammatory mediators in human AVICs [7,23], and some of these mediators may also contribute to the augmented BMP-2 expression observed in cells treated with LPS plus LFA-1. Indeed, TNF-α has been shown to induce BMP-2 expression in human AVICs [11]. However, we observed in a previous study that LPS at a higher concentration (200 ng/ml) fails to induce TNF-α in human AVICs [7]. Thus, TNF-α is unlikely to be an important factor in the robust BMP-2 expression induced by LPS plus LFA-1. Since ICAM-1-neutralization greatly reduces, but not abrogates, BMP-2 expression in cells exposed to LPS plus LFA-1,

Fig. 5. The NF-κB pathway mediates BMP-2 expression induced by ICAM-1 ligation. A. A representative immunoblot and densitometric data (n = 6) show that LFA-1 (1.0 μg/ml) induces NF-κB p65 phosphorylation in human AVICs pretreated with LPS. B. NF-κB DNA-binding assay confirms that LFA-1 increases NF-κB activity in LPS-pretreated cells. C. Representative immunoblots and densitometric data (n = 6) show that inhibition of NF-κB with SN50 or BAY 11-7082 suppresses BMP-2 expression induced by LFA-1 or LPS + LFA-1. *$P < 0.05$ vs. untreated controls (Ctrl); #$P < 0.05$ vs. LPS alone at the same time-point; †$P < 0.05$ vs. corresponding treatment + SN50M or DMSO.



ICAM-1-independent mechanism(s) activated by LPS may be operative and should be investigated in future studies.

4.2. The Notch1-NF-κB pathway is required for the induction of BMP-2 expression by ICAM-1 ligation in human AVICs

In the present study, we confirmed that ICAM-1 ligation can up-regulate NF-κB p65 phospholation and DNA-binding activity in human AVICs. More importantly, our results indicate that NF-κB plays an important role in mediating the effect of ICAM-1 ligation on BMP-2 expression since treatment of cells with either of the two NF-κB inhibitors applied (SN50 and BAY 11-7082) markedly suppresses BMP-2 expression following ICAM-1 ligation. Our previous studies on human AVICs suggest that the NF-κB pathway is critical for BMP-2 expression [7,9]. The results of the present study show that ligation of ICAM-1 activates this pathway to up-regulate BMP-2 expression.

It has been reported that ligation of ICAM-1 in endothelial cells activates PI3K that is linked to NF-κB [40]. However, human AVICs did not exhibit such a change in PI3K activity (not shown). Surprisingly, we found that ICAM-1 ligation causes Notch1 cleavage in human AVICs that is associated with the release of Notch1 ligand Jagged1. Several pro-inflammatory stimuli, particularly bacterial LPS, are found to activate Notch1 in monocytes or macrophages [41,42]. Our previous studies found Notch1 activation in human AVICs exposed to LPS [9,23]. This is the first report that ligation of ICAM-1 induces Notch1 cleavage. Thus, distinct pro-inflammatory signaling can lead to Notch1 activation in human AVICs. It appears that ICAM-1 signaling activates Notch1 in human AVICs via Jagged1 since ligation of ICAM-1 triggers the release of this Notch1 ligand.

Notch1 activation involves its cleavage by γ-secretase to generate the active segment NICD1, and we have found in a previous study that γ-secretase inhibitor DAPT reduces NICD1 generation in human AVICs [23]. To define the role of Notch1 activation in the pro-osteogenic response to ICAM-1 ligation, we determined the effect of DAPT on BMP-2 expression. Treatment with DAPT markedly reduces the production of BMP-2 following ICAM-1 ligation. It is noteworthy that inhibition of Notch1 and inhibition of NF-κB result in a comparable reduction in BMP2 expression. It is possible that Notch1 activation and NF-κB activation are linear signaling events in human AVICs following ICAM-1 ligation. In supporting this possibility, our previous work shows that Notch1 ligand enhances TLR4-mediated NF-κB activation in human AVICs [23]. We observed in the present study that inhibition of Notch1 activation with DAPT markedly reduces NF-κB activation in human AVICs following ICAM-1 ligation, resulting in lower levels of BMP-2 expression. Thus, Notch1 appears to be upstream of NF-κB in the induction of BMP-2 expression although the mechanism by which NICD1 activates NF-κB remains unclear from the present study. Nevertheless, Notch1 plays a role in NF-κB-mediated BMP-2 response induced by ICAM-1 ligation.

Our previous study found that NICD1 interacts with IKK and modulates TLR4-mediated NF-κB activation in human AVICs [23]. The results of the present study indicate that Notch1 modulates NF-κB activation induced by ICAM-1 ligation and thereby enhances BMP-2 expression. The finding supports the notion that Notch1 signaling plays a role in

Fig. 6. Notch1 plays a role in mediating NF-κB activation and BMP-2 expression induced by ICAM-1 ligation. A. A representative immunoblot and densitometric data (n = 6) show that LFA-1 (1.0 μg/ml) induces NICD1 generation in human AVICs pretreated with LPS. $^{*}P < 0.05$ vs. untreated controls (Ctrl); $\#P < 0.05$ vs. LPS alone. B. ELISA data (n = 6) show that LFA-1 (1.0 μg/ml) induces Jagged1 secretion in human AVICs with and without LPS pretreatment. $^{*}P < 0.05$ vs. untreated controls (Ctrl); $\#P < 0.05$ vs. LFA-1 alone or LPS alone. C. A representative immunoblot and densitometric data (n = 6) show that inhibition of Notch1 with DAPT reduces BMP-2 expression induced by ICAM-1 ligation. $^{*}P < 0.05$ vs. vehicle controls; $\#P < 0.05$ vs. corresponding treatment without DAPT. D. A representative immunoblot, densitometric data and DNA-binding assay show that inhibition of Notch1 with DAPT markedly reduces NF-κB p65 phosphorylation and DNA-binding activity in cells exposed to LPS plus LFA-1. n = 6; $^{*}P < 0.05$ vs. controls (Ctrl); $\#P < 0.05$ vs. LPS + LFA-1.

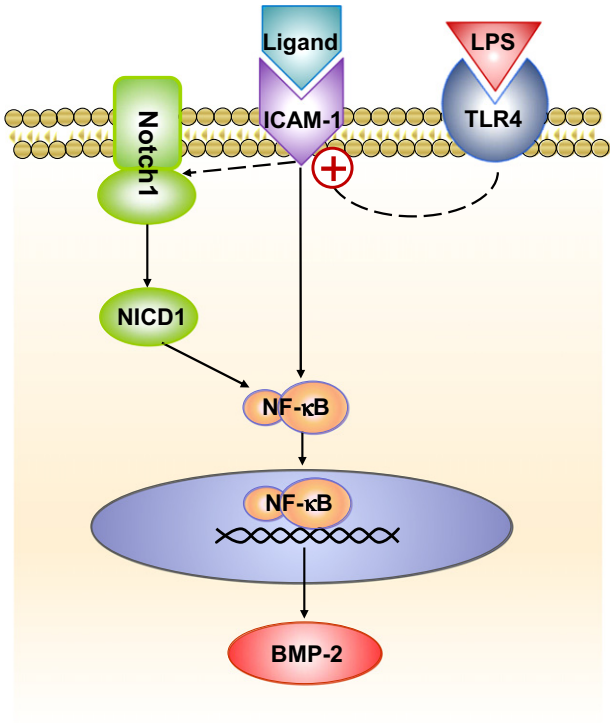


Fig. 7. Schematic diagram depicting the mechanism underlying the induction of BMP-2 in human AVICs by ICAM-1 ligation. The engagement of a ligand with ICAM-1 activates NF-κB through Notch1-dependent and -independent mechanisms. Activated NF-κB initiates BMP-2 expression to increase BMP-2 protein levels. A pre-exposure to LPS up-regulates ICAM-1 levels on cell surfaces and augments ICAM-1-mediated BMP-2 response.

mediating AVIC osteogenic response to pro-inflammatory stimuli [9] and is consistent with the reports that show an important role of Notch1 in vascular calcification. However, Notch is required for cardiac and valvular development [43,44], and genetic Notch1 deficiency is linked to aortic valve defects at birth and the development of calcific aortic valve disease [45]. In addition, several studies indicate that Notch1 has an anti-osteogenic effect in the aortic valve. In this regard, heterozygous Notch1 knockout in mice results in aortic valve calcification [46], and prolonged inhibition of Notch1 in AVICs induces osteogenic gene expression [46–48]. It should be noted, however, that the results of the present study and our previous findings [9] demonstrate a role for Notch1 signaling in modulating NF-κB-mediated BMP-2 expression in human AVICs exposed to an inflammatory setting. Our findings suggest that this signaling molecule plays differential roles in different settings even though in the same tissue or cells.

5. Conclusion

In summary, the results of this study demonstrate that ligation of ICAM-1 on human AVICs by leukocyte integrin LFA-1 induces BMP-2 expression via activation of the Notch1-NF-κB pathway (summarized in Fig. 7). Specifically, ligation of ICAM-1 activates Notch1 to modulate NF-κB activation and NF-κB-mediated BMP-2 expression. Thus, this study demonstrates a novel role of ICAM-1 in translation of an inflammatory signal into a pro-osteogenic response in human AVICs and suggests that ICAM-1 on the surfaces of AVICs may contribute to the mechanism of aortic valve calcification in an inflammatory milieu.

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Disclosures

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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