Interleukin-37 suppresses the osteogenic responses of human aortic valve interstitial cells in vitro and alleviates valve lesions in mice

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Calcific aortic valve disease is a chronic inflammatory process, and aortic valve interstitial cells (AVICs) from diseased aortic valves express greater levels of osteogenic factors in response to proinflammatory stimulation. Here, we report that lower cellular levels of IL-37 in AVICs of diseased human aortic valves likely account for augmented expression of bone morphogenetic protein-2 (BMP-2) and alkaline phosphatase (ALP) following stimulation of Toll-like receptor (TLR) 2 or 4. Treatment of diseased AVICs with recombinant human IL-37 suppresses the levels of BMP-2 and ALP as well as calcium deposit formation. In mice, aortic valve thickening is observed when exposed to a TLR4 agonist or a high fat diet for a prolonged period; however, mice expressing human IL-37 exhibit significantly lower BMP-2 levels and less aortic valve thickening when subjected to the same regimens. A high fat diet in mice results in oxidized low-density lipoprotein (oxLDL) deposition in aortic valve leaflets. Moreover, the osteogenic responses in human AVICs induced by oxLDL are suppressed by recombinant IL-37. Mechanistically, reduced osteogenic responses to oxLDL in human AVICs are associated with the ability of IL-37 to inhibit NF-κB and ERK1/2. These findings suggest that augmented expression of osteogenic factors in AVICs of diseased aortic valves from humans is at least partly due to a relative IL-37 deficiency. Because recombinant IL-37 suppresses the osteogenic responses in human AVICs and alleviates aortic valve lesions in mice exposed to high fat diet or a proinflammatory stimulus, IL-37 has therapeutic potential for progressive calcific aortic valve disease.

Toll-like receptors | inflammation | oxidized low-density lipoprotein | calcification | signal transduction

Calcific aortic valve disease (CAVD) is a leading cardiovascular disease in people over the age of 65. Progressive aortic valve calcification associated with CAVD often leads to heart failure and results in valve replacement, the second most common cardiovascular surgery performed (1). With greater lifespan, CAVD is becoming an increasingly important healthcare issue. Unfortunately, pharmacological intervention for slowing the progression of this disease is unavailable.

CAVD is recognized as a chronic inflammatory and osteogenic process (2). Inflammatory mediators promote valvular osteogenic responses and are believed to contribute to the mechanism for the pathogenesis of CAVD (2, 3). Aortic valve interstitial cells (AVICs), the dominant cellular components of aortic valve leaflets, play a critical role in aortic valve inflammation and calcification (4, 5). In this regard, proinflammatory mediators, such as tumor necrosis factor-α, have been shown to up-regulate the expression of osteogenic factor bone morphogenetic protein-2 (BMP-2) and early osteoblastic differentiation biomarker alkaline phosphatase (ALP) in AVICs (6, 7). We and others have observed that stimulation of either Toll-like receptor (TLR) 2 or TLR4 induces the osteogenic responses characterized by the expression of BMP-2 and ALP, among several other osteogenic biomarkers, in human AVICs (8–12), leading to the formation of calcification deposits in vitro (11–13). Moreover, bacteria associated with chronic periodontal infection and bacterial agents have been detected in diseased human aortic valves (14, 15), and inoculation of rabbits with oral bacteria induces aortic valve lesions (16). These findings suggest a link between TLRs and CAVD.

AVICs of diseased aortic valves have augmented inflammatory and osteogenic responses to TLR2/4 agonists (11, 12, 17). It appears that an imbalance between proinflammatory and antiinflammatory mechanisms results in the disruption of valvular homeostasis, and such an imbalance may contribute to the mechanism underlying the development and progression of CAVD. Thus, investigation of the proinflammatory signaling pathway responsible for the osteogenic responses and antiinflammatory mechanisms in AVICs of diseased aortic valves may provide important information for development of therapeutic limitation of inflammatory and osteogenic changes in aortic valves.

Epidemiologic and histological studies have also suggested a link between proatherogenic factors and CAVD (18, 19). CAVD is a frequent disease in North America, and proatherogenic factors that have been linked with CAVD are closely related to the North American lifestyle. However, a prospective trial using a statin was not effective (20). It is likely that hypercholesterolemia is one of

Significance

Calcific aortic valve disease (CAVD) is a chronic inflammatory and osteogenic condition with unknown underlying mechanism and unavailable pharmacological therapy. The present study shows that lower levels of IL-37 expression in aortic valve interstitial cells (AVICs) of diseased valves play a role in the elevated valvular osteogenic activity associated with CAVD. IL-37 inhibits NF-κB and ERK1/2 to suppress AVIC osteogenic responses, and recombinant IL-37 has a greater effect on AVICs of diseased valves. Moreover, expression of IL-37 in mice attenuates aortic valve thickening following a prolonged exposure to endotoxin or high fat diet. Thus, IL-37 is antiosteogenic in human AVICs and has the potential for limitation of CAVD progression.

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the multiple factors involved. Studies in laboratory animals have found that exposure to a "North American diet" consisting of high amounts of noncholesterol fats and carbohydrates could induce aortic valve abnormalities, including increased leaflet thickness and leaflet calcification if animals are fed with such a diet for a prolonged time (21, 22). Currently, the underlying mechanism of aortic valve lesions induced by high fat diet is incompletely understood. Low-density lipoprotein deposits in the diseased leaflets have been demonstrated (23–27). Interestingly, increased levels of oxidized low-density lipoprotein (oxLDL) in blood have been reported to correlate with aortic valve fibrosis and calcification (23). Further, oxLDL deposition in the vascular wall is known to provoke atherosclerotic calcification (28). We have reported that oxLDL induces BMP-2 expression in human coronary artery endothelial cells through a mechanism dependent on TLR2 and TLR4 (29).

Interleukin (IL)-37, previously known as IL-1 family member 7, is expressed in humans, but is absent in rodents (30, 31). IL-37 is an antiinflammatory member of the IL-1 family, which broadly inhibits innate and acquired immune responses in vitro and in vivo (31). It is unknown whether human AVICs express IL-37 and whether IL-37 plays a role in AVIC pathobiology associated with CAVD.

The objective of this study was to test the hypotheses that the proosteogenic phenotype of diseased AVICs is due to IL-37 deficiency and that IL-37 suppresses the osteogenic responses to prevent AVIC proosteogenic reprogramming. In this study, we examined the relationship of the augmented osteogenic responses of diseased human AVICs with cellular IL-37 levels, determined the role of endogenous IL-37 as a regulator of AVIC osteogenic responses, evaluated the effect of recombinant IL-37 on AVIC osteogenic responses, investigated the effect of expression of human IL-37 on aortic valve lesions in mice after prolonged exposure to a lipopolysaccharide (LPS) or high fat diet, and elucidated the molecular mechanism of IL-37 action in human AVICs.

Results

Endogenous IL-37 Negatively Modulates the Osteogenic Responses to Proinflammatory Stimuli in Human AVICs. We analyzed IL-37 protein and mRNA levels in AVICs from 20 human aortic valves, 10 normal and 10 diseased. As shown in Fig. 1*A*, AVICs from diseased aortic valves had markedly lower levels of IL-37 protein and mRNA compared with cells of normal aortic valves. AVICs of diseased aortic valves produced higher levels of BMP-2 and ALP after stimulation with TLR4 agonist LPS or TLR2 agonist Pam3CSK4 (PAM) (Fig. S1*A*). These data indicate that IL-37 deficiency contributes to the mechanism underlying the augmented osteogenic responses to proinflammatory stimulation in diseased AVICs.

To determine whether IL-37 modulates the osteogenic responses, we performed knockdown experiments in AVICs from normal aortic valves. Fig. 1*B* shows that IL-37 knockdown enhanced the BMP-2 and ALP responses to LPS and PAM in normal AVICs. Furthermore, IL-37 knockdown exaggerated calcification deposit formation induced by LPS and PAM (Fig. 1*C* and Fig. S1*B*). To confirm the role of IL-37 in modulation of the osteogenic responses, we examined BMP-2 and ALP production after stimulation with LPS and PAM in murine AVICs isolated from mice that constitutively express human IL-37 (IL-37 Tg mice). AVICs from IL-37 Tg mice displayed greatly reduced osteogenic responses to stimulation of TLR2 or TLR4 (Fig. S1*C*). Thus, IL-37 negatively modulates AVIC osteogenic responses to proinflammatory stimulation.

Recombinant IL-37 Suppresses the Osteogenic Responses in Diseased Human AVICs. To determine whether IL-37 has an effect on the osteogenic responses in diseased human AVICs, we treated AVICs from normal and diseased human aortic valves with recombinant IL-37 (0.1 and 1.0 ng/mL) 1 h before exposure to LPS or PAM. As shown in Fig. 2*A*, recombinant IL-37 markedly reduced the levels of BMP-2 and ALP in AVICs from either normal or diseased valves. It is noteworthy that treatment with recombinant IL-37 resulted in a greater reduction in the levels of osteogenic factors in diseased AVICs. Whereas diseased AVICs displayed elevated levels of BMP-2 in the baseline, recombinant IL-37 had no effect on BMP-2 levels in the absence of stimulation (Fig. S2*A*). Therefore, recombinant IL-37 suppresses human AVIC osteogenic responses to proinflammatory stimulation, and it has a greater effect on diseased AVICs that are IL-37 deficient. Further, we stimulated diseased AVICs with LPS or PAM for 3 wk in the presence and absence of recombinant IL-37 to determine the effect of IL-37 on in vitro osteogenic activity. Interestingly, recombinant IL-37 reduced calcification deposit formation in diseased AVICs (Fig. 2*B* and Fig. S2*B*). Therefore, IL-37 negatively modulates AVIC osteogenic responses and suppresses in vitro osteogenic activity in AVICs of diseased human aortic valves.

Expression of IL-37 in Mice Alleviates Aortic Valve Lesions in Vivo. To evaluate the effect of IL-37 in vivo, we developed a mouse model of aortic valve lesions by using prolonged treatment with LPS. WT mice and IL-37 Tg mice were treated with LPS for 12 wk with the aid of osmotic pumps. Histological images in Fig. 3, and echocardiographs and quantification data presented in Fig. S3*A* revealed that aortic valve leaflet thickness was increased in WT mice, and that the thickening was accompanied by elevated levels of BMP-2 in the valvular tissue (Fig. S3*B*). However, essentially normal valve thickness and greatly reduced BMP-2 levels were observed in IL-37 Tg mice (Fig. 3 and Fig. S3 *A* and *B*).

We also examined aortic valve thickness in WT and IL-37 Tg mice fed with a high fat diet (containing 24% fat and 41% carbohydrate) for 16 wk. This high fat diet resulted in accelerated weight gain, hyperglycemia, and a mild increase in plasma oxLDL levels, but no difference in these parameters between WT mice and IL-37 Tg was observed. Although WT mice exhibited aortic valve leaflet thickening accompanied by elevated BMP-2 levels in valvular tissue (Fig. 3 and Fig. S3*B*), aortic valve leaflets in IL-37 Tg mice were essentially normal at the end of the experiment (Fig. 3). These in vivo experiments demonstrate that IL-37 reduces early aortic valve lesions caused by specific stimuli.

We observed oxLDL deposits in the aortic valve tissue of both WT and IL-37 Tg mice fed with high fat diet (images of WT mice shown in Fig. S4*B*). However, the levels of oxLDL in aortic valves of IL-37 Tg mice were significantly lower compared with WT mice. In vitro human AVIC cultures showed that exposure of cells to oxLDL (40 μg/mL for 72 h) increases BMP-2 and ALP levels. However, neutralization of either TLR2 or TLR4, but not LOX-1, reduces the proosteogenic effect of oxLDL (Fig. S4*C*). Further, aortic valve thickening was absent in TLR2 KO mice and TLR4 mutant mice fed a high fat diet (Fig. 4*A* and Fig. S4*A*). Importantly, recombinant IL-37 suppressed oxLDL-induced osteogenic responses and in vitro osteogenic activity in human AVICs (Fig. 4*B*). Conversely, IL-37 knockdown enhanced oxLDL-induced BMP-2 and ALP expression in human AVICs (Fig. S4*D*). Therefore, IL-37 regulates AVIC osteogenic responses induced by pathogen-associated molecular pattern (PAMP) as well as danger-associated molecular pattern (DAMP).

IL-37 Suppresses AVIC Osteogenic Responses Through Inhibition of NF-κB and ERK1/2. To elucidate the mechanism by which IL-37 suppresses the osteogenic response, we examined the activation of NF-κB and ERK1/2, two important signaling molecules that regulate AVIC osteogenic responses. Human AVICs were stimulated with oxLDL for 0–8 h in the presence or absence of recombinant IL-37. Recombinant IL-37 markedly reduced NF-κB phosphorylation, NF-κB DNA-binding activity, and ERK1/2 phosphorylation following an exposure to oxLDL (Fig. 5*A*). Interestingly, oxLDL activates NF-κB and ERK1/2 in human AVICs through a mechanism depending on TLR2 and TLR4

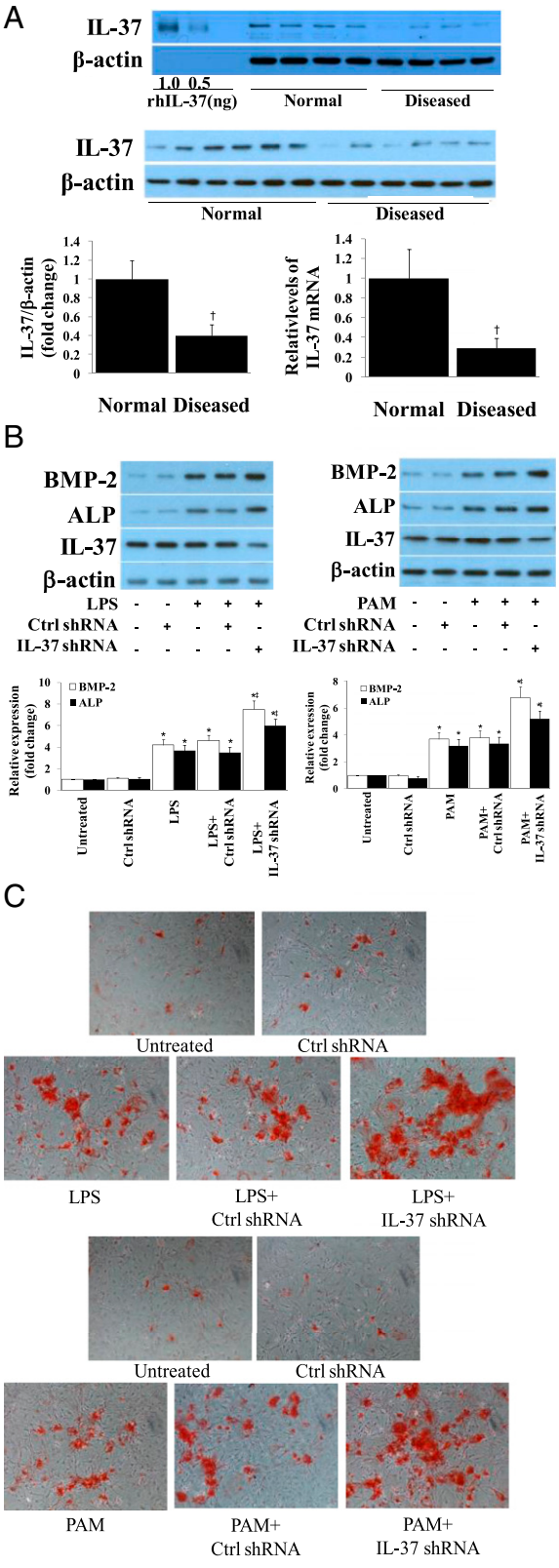

Fig. 1. Relative IL-37 deficiency in diseased human AVICs results in augmented osteogenic responses to proinflammatory stimulation. (*A*) Immunoblots, densitometric data, and PCR analysis show that diseased AVICs express lower levels of IL-37 protein and mRNA. $n = 10$ isolates from different donors; $^{\dagger}P < 0.05$ vs. cells of normal valves. (*B*) Normal AVICs were treated with IL-37 shRNA or scrambled shRNA, and then stimulated with LPS (0.2 μg/mL) or Pam3CSK4 (PAM, 0.1 μg/mL) for 3 d. Representative immunoblots and densitometric data ($n = 5$ separate experiments using different isolates) show that IL-37 knockdown in normal AVICs enhances BMP-2 and ALP expression. $^{*}P < 0.05$ vs. corresponding control; $^{\dagger}P < 0.05$ vs. stimulant alone and scrambled shRNA + stimulant. (*C*) Representative images ($n = 5$ separate experiments using distinct isolates) show that IL-37 knockdown augments calcification deposit formation after 21 d of stimulation in a conditioning medium. (Original magnification: 10×.)

(Fig. S5*A*). To confirm the role of NF-κB and ERK1/2 in mediating the osteogenic responses to oxLDL, we exposed AVICs to specific inhibitors before the exposure to oxLDL. Inhibition of NF-κB by Bay11-7082 or ERK1/2 by PD98059 markedly reduced the proosteogenic effects of oxLDL (Fig. 5*B*) or TLR2/4 agonists (Fig. S5*B*). Together, these observations reveal that NF-κB and ERK1/2 mediate the osteogenic responses to oxLDL in human AVICs and suggest that IL-37 inhibits NF-κB and ERK1/2 to suppress the osteogenic responses.

Discussion

Chronic inflammatory and osteogenic activities in the aortic valve tissue promote CAVD progression (2, 32). The present study uncovered an antiosteogenic function of IL-37 in human AVICs. IL-37 is an antiinflammatory cytokine expressed in humans, but not in rodents. Expression of human IL-37 in mice protects against models of systemic endotoxemia (33, 34), chemical colitis (35), spinal cord injury (36), sleep deprivation (37), myocardial infarction (38), and contact dermatitis (39). Recombinant IL-37 administered to wild-type mice has resulted in reduced severity of inflammatory injuries (31).

We and others have observed that stimulation of either TLR2 or TLR4 induces osteogenic responses in human AVICs (9–12, 17); moreover, AVICs from diseased aortic valves exhibit augmented osteogenic responses to proinflammatory stimulation (10, 11, 17). These studies suggest that innate immune receptors play a role in the mechanisms underlying the pathogenesis of CAVD.

We show that the augmented osteogenic responses to TLR2 and TLR4 agonists in AVICs of diseased aortic valves correlate to constitutively lower levels of cellular IL-37. Reducing IL-37 levels in AVICs of normal human aortic valves by gene knockdown enhances their osteogenic responses. Two observations support the role of IL-37: recombinant IL-37 suppresses the osteogenic responses in AVICs of diseased aortic valves; AVICs from IL-37 Tg mice are less sensitive to TLR2 and TLR4 agonists for BMP-2 and ALP expression. Further, IL-37 Tg mice exhibit reduced aortic valve thickening and BMP-2 levels after prolonged exposure to LPS or feeding with a high fat diet. Mechanistic data show that IL-37 suppresses the activation of NF-κB and ERK1/2, two important pathways that regulate BMP-2 and ALP expression in human AVICs (12, 13). It should be noted that diseased aortic valve tissue is heterogeneous. To minimize variability, we collect tissue from the areas where no apparent calcification is observed. Thus, the diseased AVICs used in this study represent those of valvular tissue in the relatively early stage of CAVD.

IL-37 Has an Antiosteogenic Effect in Human AVICs. Here, we demonstrated that AVICs of diseased human aortic valves have lower levels of IL-37 protein and mRNA and that this alteration in cellular IL-37 levels is associated with the augmented osteogenic response to TLR2/4 stimulation. IL-37 knockdown enhances the osteogenic responses and in vitro osteogenic activity (increased formation of calcification deposits) in normal AVICs. AVICs of IL-37 Tg mice exhibit markedly attenuated osteogenic responses to TLR2/4 stimulation. Recombinant IL-37 suppresses AVIC osteogenic responses to TLR2/4 agonists and oxLDL that up-regulates BMP-2 and ALP expression in human AVICs in a TLR2/4-dependent fashion. Our in vivo studies found that IL-37 Tg mice

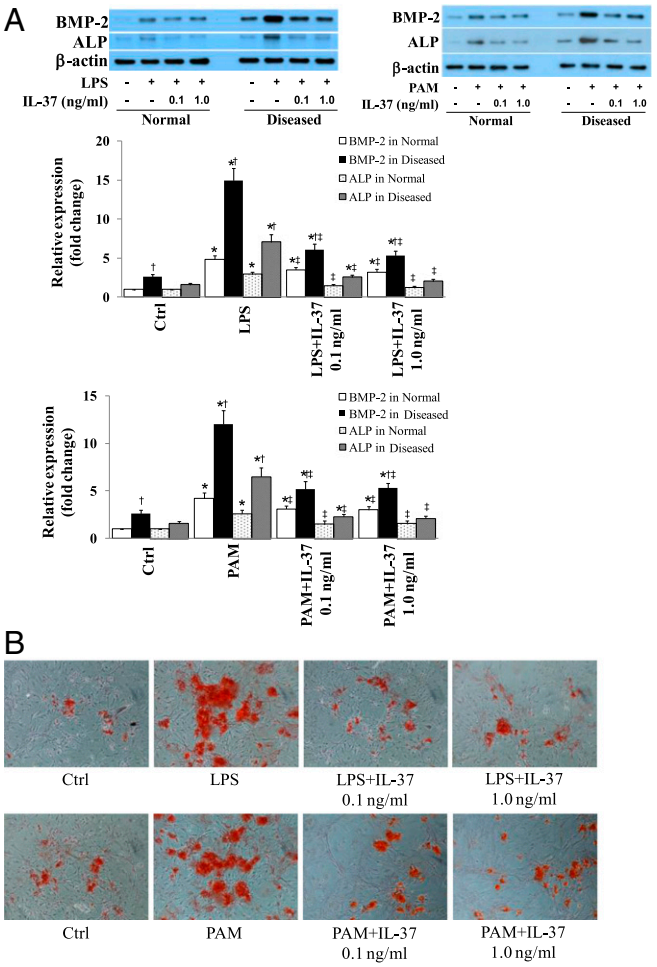

Fig. 2. Recombinant IL-37 suppresses the osteogenic responses in AVICs of diseased human aortic valves. AVICs of normal and diseased aortic valves were treated with recombinant IL-37 (0.1 and 1.0 ng/mL) 1 h before stimulation with LPS (0.2 μg/mL) or Pam3CSK4 (PAM, 0.1 μg/mL) for 3–21 d. (*A*) Representative immunoblots and densitometric data ($n = 6$ isolates from different donors) show that treatment with recombinant IL-37 results in greater reduction in BMP-2 and ALP levels at 3 d in AVICs of diseased aortic valves. (*B*) Representative images ($n = 5$ separate experiments using different isolates) show that recombinant IL-37 reduces calcium deposit formation in diseased cells after 21 d of stimulation in a conditioning medium. (Original magnification: 10×.) $^{*}P < 0.05$ vs. corresponding control; $^{\dagger}P < 0.05$ vs. normal cells receiving the same treatment; $^{\ddagger}P < 0.05$ vs. stimulant alone.

have markedly reduced BMP-2 levels in aortic valve tissue after treatment with LPS or high fat diet. Thus, these lines of evidence support the notion that endogenous IL-37 negatively regulates the osteogenic responses to proinflammatory stimulation in AVICs and suggest that relative IL-37 deficiency contributes to the mechanism underlying the augmented osteogenic responses in AVICs of diseased human aortic valves.

Low concentrations of recombinant IL-37 suppresses the osteogenic responses in human AVICs. Moreover, recombinant IL-37 has a greater effect on AVICs of diseased aortic valves and diminishes the differences in levels of BMP-2 and ALP between normal and diseased cells. In validating the effect of recombinant IL-37 on proosteogenic phenotypic changes in AVICs of diseased aortic valves, we observed that treatment with recombinant IL-37 reduces calcium deposit formation following prolonged stimulation with TLR2/4 agonists. These findings demonstrate that IL-37 suppresses in vitro osteogenic activity in diseased AVICs and further support our notion that relative IL-37 deficiency enhances the osteogenic responses in AVICs.

Our in vivo studies found that IL-37 Tg mice have markedly attenuated leaflet thickening after prolonged exposure to LPS or high fat diet. Similarly, aortic valve thickening is essentially absent in TLR2 KO mice and TLR4 mutant mice fed with a high fat diet, indicating an important role of TLR2/4 in mediating aortic valve lesions. It is interesting that oxLDL deposition is evident in the aortic valve tissue of mice fed with high fat diet because oxLDL has been reported to function as a DAMP (29, 40, 41). In this regard, we reported that oxLDL induces BMP-2 expression in human coronary artery endothelial cells through TLR2/4 (29). The in vitro murine AVIC culture experiments revealed that TLR2 KO or TLR4 mutation greatly reduces oxLDL-induced BMP-2 expression, providing a possible mechanism for the in vivo effect of high fat diet on aortic valves. It is particularly interesting that recombinant IL-37 markedly suppresses the osteogenic responses induced by oxLDL in human AVICs. This finding highlights the potential of IL-37 for the suppression of valvular osteogenic responses to both PAMP and DAMP. It should be noted that the attenuated aortic valve thickening in IL-37 Tg mice fed a high fat diet could be the combined effects of IL-37 on oxLDL accumulation and cellular osteogenic responses to oxLDL because oxLDL levels in the aortic valve leaflets of IL-37 Tg mice are lower than those in WT mice fed a high fat diet.

IL-37 Suppresses AVIC Osteogenic Responses Through Inhibition of NF-κB and ERK1/2. Several studies show an important role of the NF-κB pathway in mediating vascular cell osteogenic responses (42). Although we have reported that NF-κB and ERK1/2 mediate induction of BMP-2 and ALP in human AVICs (12), we now note that recombinant IL-37 suppresses NF-κB phosphorylation and DNA-binding activity and inhibits ERK1/2 phosphorylation in human AVICs exposed to LPS, Pam3CSK4, or oxLDL. A specific inhibitor of NF-κB or ERK1/2 reduces the levels of BMP-2 and ALP in cells exposed to these stimuli.

IL-37 binds to IL-18Rα, but the affinity of IL-37 to IL-18Rα is relatively lower compared with the affinity of IL-18 to IL-18Rα (43, 44). However, once IL-37 binds to the IL-18Rα, IL-1R8 (formerly SIGIRR) is recruited and the complex signals in the cell to suppress NF-κB function (33, 34). More importantly, recombinant IL-37 does not suppress inflammation in mice deficient in IL-1R8 (31). The signaling mechanism of IL-37 includes marked decreases in the activity of several signaling pathways, for example mTOR, but increases in the activity of antiinflammatory

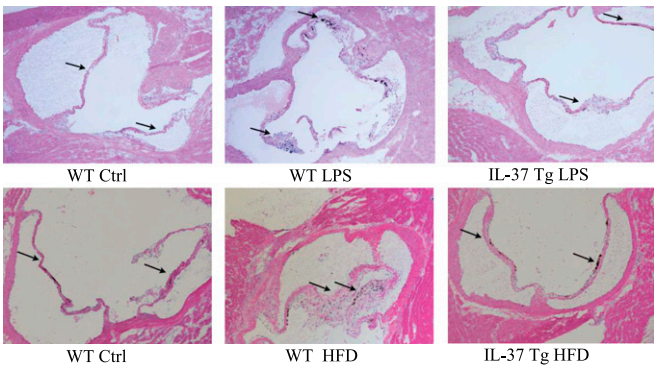

Fig. 3. IL-37 Tg mice display attenuated aortic valve thickening following a prolonged exposure to LPS or high fat diet. C57BL/6 (WT) mice and IL-37 transgenic (Tg) mice were treated with LPS (4.0 μg/d) for 12 wk by using osmotic pumps or fed with a high fat diet (HFD; 45% fat as in kcal%) for 16 wk. Representative histology images show that expression of IL-37 attenuates aortic valve thickening caused by LPS or HFD. $n = 10$ in each group. (Original magnification: 10×.)

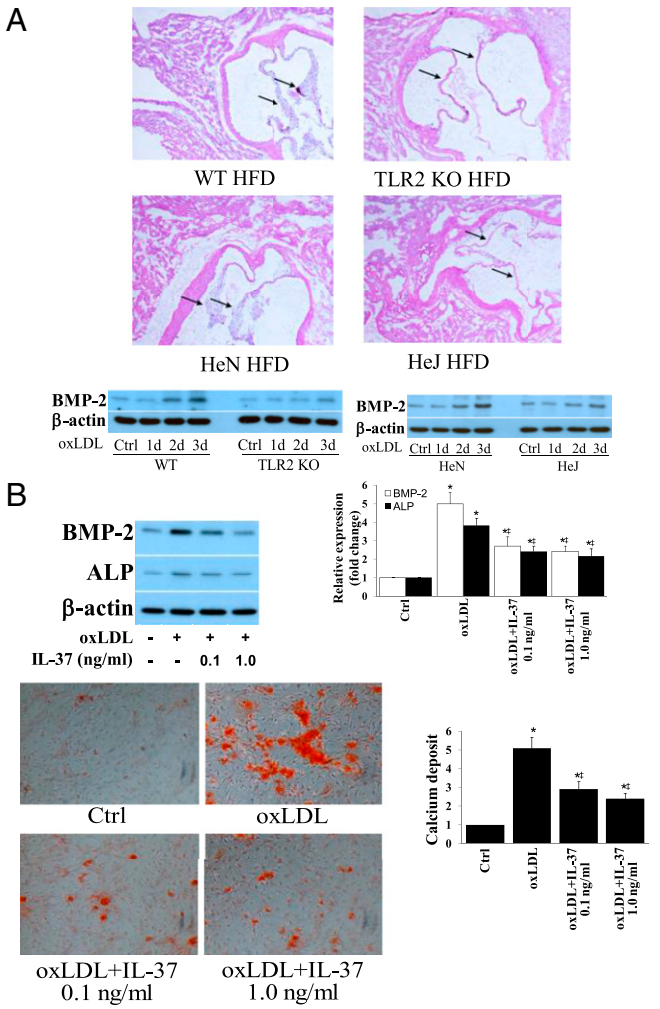

Fig. 4. Recombinant IL-37 suppresses AVIC osteogenic responses induced by oxLDL. (*A*) Representative histology images of 6 mice of each genotype show aortic valve thickening is essentially absent in TLR2 KO (C57BL/6 background) mice and TLR4 mutant (C3H/HeJ) mice fed with high fat diet for 12 wk. Representative immunoblots of four separate experiments show markedly reduced BMP-2 levels in murine AVICs from TLR2 KO mice and TLR4-mutant mice following treatment with oxLDL (40 μg/mL) for 1–3 d. (*B*) AVICs of normal human aortic valves were treated with oxLDL (40 μg/mL) for 3–21 d in the presence or absence of recombinant IL-37 (0.1 and 1.0 μg/mL). Representative immunoblots and densitometric data ($n = 5$ experiments using different isolates) show that recombinant IL-37 reduces BMP-2 and ALP levels in cells exposed to oxLDL for 3 d. Representative images and spectrophotometric data ($n = 5$ separate experiments using distinct cell isolates) show that reduced calcium deposit formation in IL-37–treated cells after 21 d of oxLDL stimulation in conditioning medium. (Original magnification: 10×.) $^{*}P < 0.05$ vs. corresponding control; $^{‡}P < 0.05$ vs. oxLDL alone.

pathways, such as PTEN and AMPK (31). In addition, there is an interaction of IL-37 with SMAD3 in LPS-stimulated macrophages (33). We assume that IL-37 may exert an influence on these signaling pathways in AVICs to modulate the osteogenic responses.

The present study shows that lower levels of IL-37 expression in AVICs of patients with CAVD are associated with augmented osteogenic responses to proinflammatory stimulation and that recombinant IL-37 suppresses the osteogenic responses of AVICs of diseased aortic valves. In mice, expression of IL-37 attenuates aortic valve thickening and reduces BMP-2 levels in valvular tissue following the exposure to LPS or high fat diet. Overall, these findings support the concept that IL-37 may have therapeutic potential for suppression of aortic valve osteogenic responses in an inflammatory milieu.

Methods

Isolation and Culture of Human AVICs. Normal human aortic valve leaflets were collected from the explanted hearts of 10 patients (10 males, mean age 59 ± 8.1 y) undergoing heart transplantation, and diseased aortic valve leaflets were obtained from 10 patients (10 males, mean age 63 ± 11.1 y) with CAVD and undergoing aortic valve replacement (patient demographics is displayed as Table S1). All patients gave informed consent for the use of their valves for this study. This study was approved by the Institutional Review Board of the University of Colorado Denver.

AVICs were isolated and cultured by using a described method (10). Briefly, valve leaflets were subjected to sequential digestions with collagenase. Cells were collected by centrifugation and cultured in M199 growth medium containing penicillin G, streptomycin, amphotericin B, and 10% (vol/vol) FBS. Cells from passages 3–6 were used for this study.

Cultures with ∼90% confluence were treated with LPS, Pam3CSK4, or oxLDL for 3 d. Cell lysate was used for the assessment of BMP-2 and ALP proteins. To

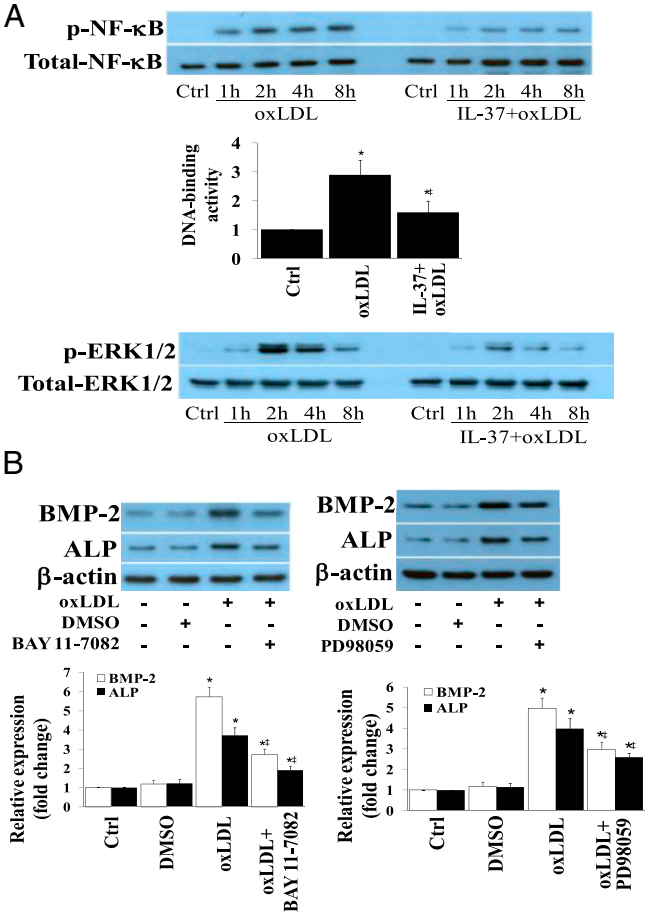

Fig. 5. The antiosteogenic effect of IL-37 is due to its impact on NF-κB and ERK1/2 activation. (*A*) AVICs of normal human aortic valves were treated with oxLDL (40 μg/mL) for 1–8 h in the presence or absence of recombinant IL-37 (0.1 ng/mL). Representative immunoblots and NF-κB DNA-binding data ($n = 5$ separate experiments using different isolates) show that recombinant IL-37 reduces NF-κB and ERK1/2 phosphorylation at each time point and suppresses NF-κB DNA-binding activity examined at 4 h. (*B*) AVICs of normal human aortic valves were treated with BAY 11-7082 (2.5 μM) or PD98059 (25 μM) 1 h before stimulation with oxLDL (40 μg/mL) for 3 d. Representative immunoblots and densitometric data ($n = 5$ separate experiments using different isolates) show that inhibition of NF-κB or ERK1/2 reduces BMP-2 and ALP expression after stimulation with oxLDL. $^{*}P < 0.05$ vs. corresponding control; $^{‡}P < 0.05$ vs. oxLDL alone. DMSO, dimethyl sulfoxide.

examine the phosphorylation of ERK1/2 and NF-κB p65, and NF-κB DNA-binding activity, cells were treated for 1–8 h. To examine the formation of calcium deposits, cells were treated with LPS, Pam3CSK4, or oxLDL for 21 d in a conditioning medium (growth medium supplemented with 10 mmol/L β-glycerophosphate, 10 nmol/L vitamin D_3, 10 nmol/L dexamethasone, and 8 mmol/L $CaCl_2$).

Animal Models of Aortic Valve Lesions. Twenty C57BL/6 male mice (WT, 3–5 mo old) and 20 IL-37 transgenic male mice (C57 BL/6 background, 3–4 mo old) were randomized, by drawing, to either vehicle (normal saline) or LPS group and were treated for 12 wk by using osmotic pumps. An additional 20 C57BL/6 mice and 20 IL-37 transgenic mice were randomized, by drawing, to regular diet or high fat diet (purchased from Research Diets; containing 24% fat, 41% carbohydrate, and 24% protein; 45% of kcal from fat) and observed for 16 wk. The sample size (10 animals in each group) was estimated with power analysis. Thicknesses of aortic valves were assessed by ultrasound, and then hematoxylin and eosin (H&E) staining at the end of the experiments. The tissue sections were examined by a blinded viewer. The experiments were approved by the Institutional Animal Care and Use Committee of the University of Colorado Denver, and this investigation conforms to the Guide for the Care and Use of Laboratory Animals (National Research Council, revised 1996).

Statistical Analysis. Data are presented as mean ± SE. Statistical analysis was performed by using StatView software (Abacus Concepts). ANOVA with the post hoc Bonferroni/Dunn test and *t* test were used to analyze differences between experimental groups, and differences were confirmed with Mann–Whitney *U* test. For time course data, two-way ANOVA was used to compare the difference between experimental groups at each time point. Statistical significance was defined as $P < 0.05$.

Materials and Additional Methods. Information and associated references are available in *SI Methods*.

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