

# Vinexin $\beta$ Ablation Inhibits Atherosclerosis in Apolipoprotein E–Deficient Mice by Inactivating the Akt–Nuclear Factor $\kappa$ B Inflammatory Axis

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**Background**—Vinexin  $\beta$  is a novel adaptor protein that regulates cellular adhesion, cytoskeletal reorganization, signal transduction, and transcription; however, the exact role that vinexin  $\beta$  plays in atherosclerosis remains unknown.

**Methods and Results**—Immunoblot analysis showed that vinexin  $\beta$  expression is upregulated in the atherosclerotic lesions of both patients with coronary heart disease and hyperlipemic apolipoprotein E–deficient mice and is primarily localized in macrophages indicated by immunofluorescence staining. The high-fat diet–induced double-knockout mice exhibited lower aortic plaque burdens than apolipoprotein E<sup>−/−</sup> littermates and decreased macrophage content. Vinexin  $\beta$  deficiency improved plaque stability by attenuating lipid accumulation and increasing smooth muscle cell content and collagen. Moreover, the bone marrow transplant experiment demonstrated that vinexin  $\beta$  deficiency exerts atheroprotective effects in hematopoietic cells. Consistent with these changes, the mRNA expression of proinflammatory cytokines were downregulated in vinexin  $\beta$ <sup>−/−</sup> apolipoprotein E<sup>−/−</sup> mice, whereas the anti-inflammatory M2 macrophage markers were upregulated. The immunohistochemical staining and in vitro experiments showed that deficiency of vinexin  $\beta$  inhibited the accumulation of monocytes and the migration of macrophages induced by tumor necrosis factor  $\alpha$ –stimulated human umbilical vein endothelial cells as well as macrophage proliferation. Finally, the inhibitory effects exerted by vinexin  $\beta$  deficiency on foam cell formation, nuclear factor  $\kappa$ B activation, and inflammatory cytokine expression were largely reversed by constitutive Akt activation, whereas the increased expression of the nuclear factor  $\kappa$ B subset promoted by adenoviral vinexin  $\beta$  was dramatically suppressed by inhibition of AKT.

**Conclusions**—Vinexin  $\beta$  deficiency attenuates atherogenesis primarily by suppressing vascular inflammation and inactivating Akt–nuclear factor  $\kappa$ B signaling. Our data suggest that vinexin  $\beta$  could be a therapeutic target for the treatment of atherosclerosis. (*J Am Heart Assoc.* 2017;6:e004585. DOI: 10.1161/JAHA.116.004585.)

**Key Words:** AKT • atherosclerosis • inflammation • vinexin  $\beta$

Cardiovascular disease is the leading cause of mortality in Western society.<sup>1,2</sup> The primary pathology underlying cardiovascular disease development is atherosclerosis, which is characterized by inflammation and lipid accumulation. Inflammation is a crucial factor in the initiation of atherogenesis and the eventual destabilization of

atherosclerotic lesions.<sup>3–5</sup> Accumulating evidence indicates that a network of inflammatory cytokines and signaling pathways are associated with atherosclerosis development; however, the precise molecular mechanisms that underlie atherosclerosis-related inflammation remain to be fully elucidated.

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An accompanying Figure S1 is available at <http://jaha.ahajournals.org/content/6/2/e004585/DC1/embed/inline-supplementary-material-1.pdf>

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Vinexin is a member of a novel adaptor protein family that includes vinexin, c-Cbl-associated protein (also known as CAP or ponsin) and Arg-binding protein 2.<sup>6</sup> Its molecular structure is highly conserved and features a sorbin homology domain at the N-terminus and 3 Src homology 3 domains at the C-terminus. Vinexin can be transcribed into 3 alternative splicing forms: vinexin  $\alpha$ ,  $\beta$ , and  $\gamma$ . Vinexin  $\beta$  contains only 3 Src homology 3 domains.<sup>7</sup> Vinexin  $\beta$  is ubiquitously expressed in the body and is most highly expressed in the heart. Vinexin  $\beta$ , localizing in the extracellular matrix and in cell-cell junctions, enhances actin cytoskeletal organization and cell spreading.<sup>8</sup> In addition, vinexin  $\beta$  regulates growth factor signal transduction via upstream interactions with specific receptors, resulting in activation of downstream signaling molecules.<sup>9–11</sup>

Based on its important role in cell adhesion, cytoskeletal reorganization, signal transduction and transcription regulation, we surmised that vinexin  $\beta$  plays an important role in cardiovascular disease development. In our previous studies, vinexin  $\beta$  was found to participate in cardiac hypertrophy and cardiac dysfunction after myocardial infarction through Akt signaling and inflammation regulation.<sup>12,13</sup> Moreover, proteomic studies have demonstrated that vinexin  $\beta$  is highly expressed in advanced atherosclerotic plaques, primarily in macrophages, vascular smooth muscle cells (VSMCs), and endothelial cells.<sup>14</sup> However, the exact role that vinexin  $\beta$  plays in atherosclerosis development remains unknown.

The present study showed significant vinexin  $\beta$  upregulation in macrophages of the atherosclerotic lesions of both patients with coronary heart disease and hyperlipemic ApoE-deficient mice. Vinexin  $\beta$  knockout mice exhibited decreased atheromatous lesion formation secondary to inactivation of the Akt and nuclear factor  $\kappa$ B (NF- $\kappa$ B) signaling pathway and alleviation of inflammation.

## Methods

### Human Specimens

All procedures involving human samples complied with the principles outlined in the Declaration of Helsinki and were approved by the Renmin Hospital of Wuhan University institutional review board in Wuhan, China. Written informed consent was obtained from relevant families. According to the definition and classification method for lesion grade,<sup>15</sup> the samples of atheromatous plaques in this study were collected from the coronary artery of coronary heart disease patients who undergoing transplantation and characterized as type V lesions, which showed several aspects of advanced atherosclerosis, such as fibrous thickening and cores of extracellular lipids. Meanwhile, the control samples were

obtained from the plaque-free coronary arteries of heart donors when the hearts were rejected for transplantation for noncardiac reasons.

### Mice and Diets

Vinexin  $\beta$ -deficient mice were constructed as described<sup>12</sup> and subsequently backcrossed onto the apolipoprotein E<sup>-/-</sup> (apo E<sup>-/-</sup>) mouse background. Vinexin  $\beta^{+/+}$ apo E<sup>-/-</sup> and vinexin  $\beta^{-/-}$ apo E<sup>-/-</sup> were obtained by breeding vinexin  $\beta^{+/+}$ apo E<sup>-/-</sup> pairs. Male vinexin  $\beta^{+/+}$ apo E<sup>-/-</sup> and vinexin  $\beta^{-/-}$ apo E<sup>-/-</sup> mice aged 8 weeks were maintained on a normal chow diet (n=10, each group) or a high-fat diet (HFD; n=27 or 28, respectively) for up to 28 weeks. Body weights and serum parameters were measured at the beginning of the experiment and when the animals were euthanized. All animal procedures were performed in accordance with protocols approved by the animal care and use committee of Renmin Hospital at Wuhan University.

### En Face Analysis of Atherosclerosis and Plaque Histology

For an en face atherosclerotic lesion analysis, the entire aorta, including the subclavian and right and left common carotid arteries, was removed and stained with Oil Red O, as described previously.<sup>16</sup> Lesion areas were quantified using Image-Pro Plus 6.0 (Media Cybernetics). Hearts were fixed in 4% paraformaldehyde and embedded in paraffin or optimal cutting temperature compound for histological analysis. Consecutive 5- $\mu$ m sections from the atrioventricular valve region of the heart were collected and stained with hematoxylin and eosin or picosirius red to evaluate morphology or collagen deposition.<sup>17</sup> For the morphometric analysis, lesion sizes were measured in 6 consecutive sections at 100- $\mu$ m intervals in 10 different littermates from each group.

### Quantitative Reverse Transcriptase Polymerase Chain Reaction and Western Blotting

Total mRNA was collected from the entire aorta and reverse transcribed with a Transcriptor First Strand cDNA Synthesis Kit (Roche).<sup>18</sup> Gene expression was evaluated via quantitative reverse transcriptase polymerase chain reaction (PCR) using LightCycler 480 SYBR Green 1 Master Mix (Roche) and a LightCycler 480 quantitative PCR system (Roche). The relative transcript was normalized against GAPDH. Proteins were extracted from the aorta and lysed in a RIPA buffer with protease and phosphatase cocktails (Roche). Protein extract (5  $\mu$ g of whole aorta) was used for SDS-PAGE. The proteins were then transferred to an Immobilon-FL transfer membrane

(Millipore) and probed with various primary antibodies overnight at 4°C. After incubation with secondary antibodies, the membranes were treated with electrochemiluminescence reagents (170-5061; Bio-Rad) before being visualized using a FluorChem E Imager (ProteinSimple). Protein expression was normalized against GAPDH protein expression.

## Immunofluorescence

Aortic sinus cross-sections were used for immunofluorescence. After deparaffinization or drying in a ventilation hood, slides were blocked in 10% goat serum diluted with PBS for 1 hour before being incubated overnight with the following primary antibodies: anti-vinexin  $\beta$ , anti-CD68, anti-smooth muscle actin, anti-CD31, anti-phosphorylated p65, anti-intercellular adhesion molecule 1 (anti-ICAM-1), anti-interleukin 6 (anti-IL-6), and anti-IL-10. After rewarming at 37°C for 1 hour, the sections were washed in PBS and incubated with the appropriate secondary antibody for 1 hour. The following secondary antibodies were used: Alexa Fluor 488 and 568 donkey anti-rabbit IgG (1:200 dilution; Invitrogen), Alexa Fluor 568 donkey anti-goat IgG (1:200 dilution; Invitrogen), and Alexa Fluor 568 donkey anti-rat IgG (1:200 dilution; Invitrogen). Nuclei were stained with DAPI (4',6-diamidino-2-phenylindole). Images were obtained using a fluorescence microscope (Olympus DX51) and DP2-BSW version 2.2 (Olympus) and analyzed with Image-Pro Plus 6.0.

## Cell Culture and Adenovirus Infection

Peritoneal macrophages were collected from male mice via peritoneal lavage 4 days after the intraperitoneal injection of 4% thioglycolate (1 mL). Cells were cultured in RPMI containing 10% fetal bovine serum and 1% penicillin-streptomycin.<sup>19</sup> The primary mouse peritoneal macrophages from apo E<sup>-/-</sup> and vinexin  $\beta$ <sup>-/-</sup>apo E<sup>-/-</sup> were infected with AdCaAKT (adenovirus expressing constitutively active AKT) or AdGFP (adenovirus expressing green fluorescent protein), whereas the peritoneal macrophages from apo E<sup>-/-</sup> were also coinfecting with AdGFP plus AdVinexin  $\beta$  (adenoviral vinexin  $\beta$ ) or AddnAKT (adenovirus expressing dominant-negative mutant of AKT) plus AdVinexin  $\beta$ . Next, the media was changed to serum-free RPMI for 15  $\mu$ g/mL of oxidized low-density lipoprotein (ox-LDL) treatment for 24 hours.

## Bone Marrow Transplantation Studies

Male apo E<sup>-/-</sup> recipient mice (n=10, each group) were irradiated with 2 doses of 5.5 Gy radiation each, for a total of 11 Gy, 4 hours apart. Bone marrow cells from both the

femurs and tibias of donor male mice (apo E<sup>-/-</sup>, vinexin  $\beta$ <sup>-/-</sup>apo E<sup>-/-</sup>) were harvested under sterile conditions. The bones were flushed with RPMI, and suspended bone marrow cells were washed and lysed in red blood cell lysing solution. For transplantation,  $5 \times 10^7$  bone marrow cells were injected into the retro-orbital venous plexus. After bone marrow reconstitution for 4 weeks or induced by HFD for 16 weeks, genomic DNA was extracted from circulating blood leukocytes and genotyped by PCR.

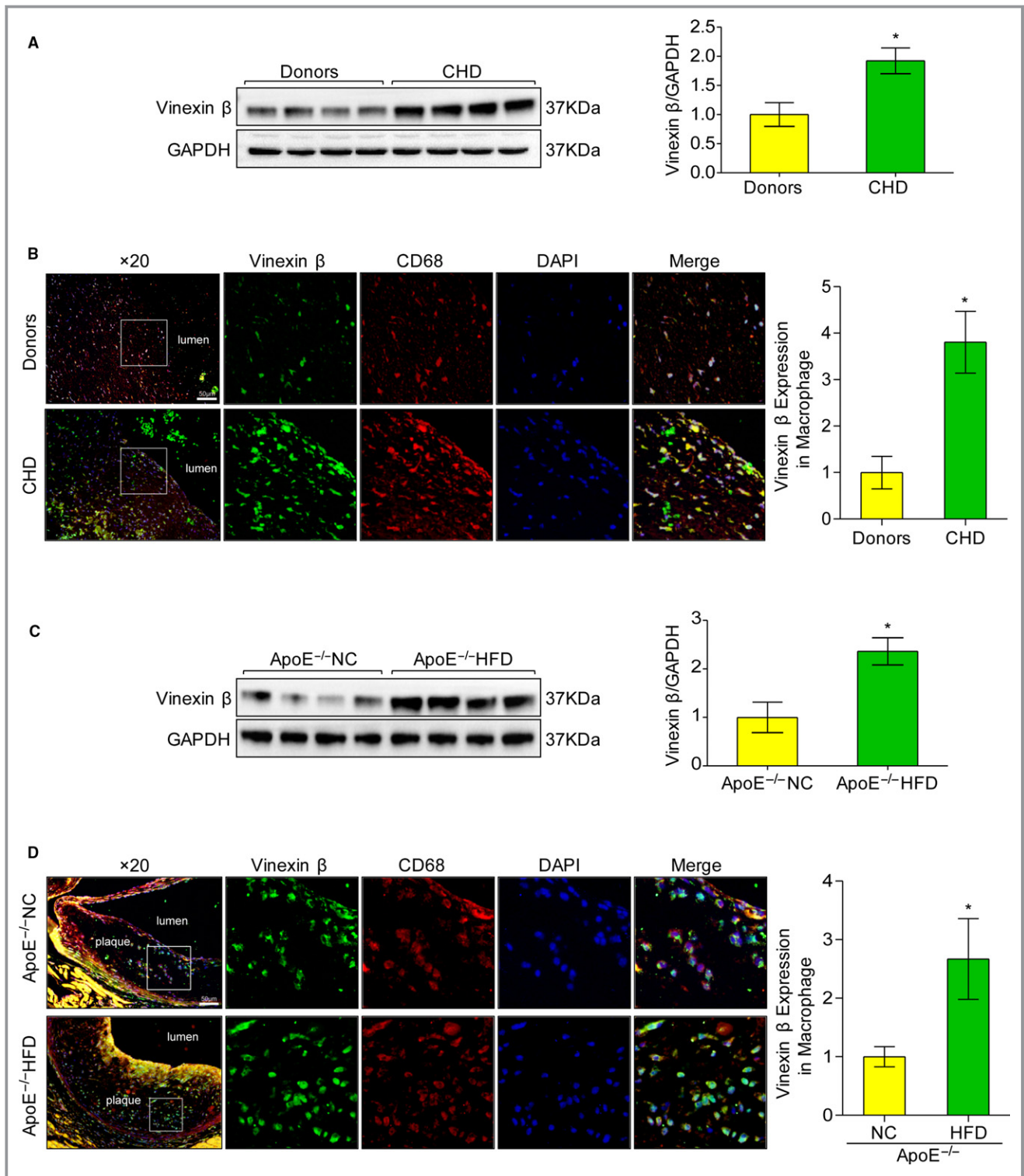
## Statistical Analysis

The data were analyzed using SPSS version 16.0 (IBM Corp) and presented as the mean with or without 2-sided 95% CIs. Between-group comparisons were analyzed using *t* tests or Wilcoxon tests depending on the distribution pattern of the data, whereas comparisons among the 4 groups were performed using 1-way ANOVA or the Kruskal-Wallis test.

## Results

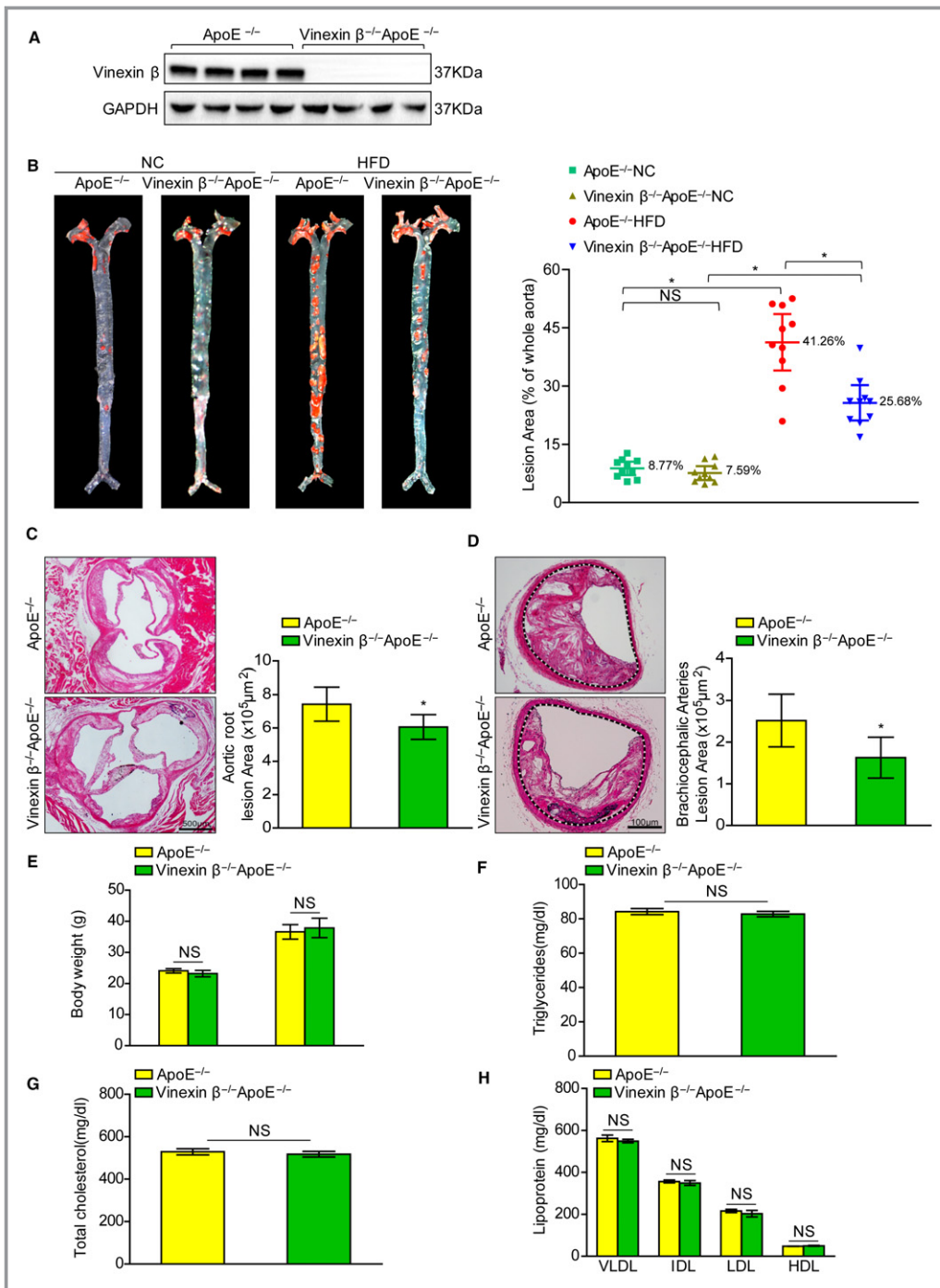
### Vinexin $\beta$ Expression Is Upregulated in Both Human and Mouse Atherosclerotic Plaques

To investigate the role of vinexin  $\beta$  in atherosclerosis development, we evaluated the expression level of vinexin  $\beta$  in atherosclerotic lesions. Because the expression of vinexin  $\beta$  has been reported in human atherosclerotic lesions, we compared the expression level of vinexin  $\beta$  in the right coronary arteries of patients with coronary heart disease with normal heart donors who were rejected for heart transplantation for noncardiac reasons. The Western blot results demonstrated that vinexin  $\beta$  expression levels were much higher in the coronary arteries of patients with coronary heart disease than in those of donors (Figure 1A). Double-immunofluorescence staining demonstrated that vinexin  $\beta$  was primarily colocalized with CD68<sup>+</sup> macrophages, representing the largest group in the lesions (Figure 1B). VSMCs and endothelial cells exhibited modest vinexin  $\beta$  expression (Figure S1A). Consistent with these results, HFDs induced higher vinexin  $\beta$  expression and more significant aortic root plaque deposition in atherosclerosis-prone apo E<sup>-/-</sup> mice than normal chow (Figure 1C). In addition, immunofluorescence staining showed that vinexin  $\beta$  expression was expressed primarily in macrophages (Figure 1D), similar to its expression pattern in human coronary arteries, whereas it was minimally expressed in VSMCs and endothelial cells, as well as in human umbilical vein endothelial cells stimulated by ox-LDL (Figure S1B and S1C). Taken together, these data indicate that vinexin  $\beta$  expression is upregulated in both mouse and human atherosclerotic plaques.



**Figure 1.** Vinexin  $\beta$  expression in the atherosclerotic plaques of human coronary arteries and apo E<sup>-/-</sup> mice. A, Representative images (left panel) and quantification (right panel) of Western blots of vinexin  $\beta$  in the coronary arteries of healthy donors and patients with CHD (n=4). B, Representative images showing double-immunofluorescence staining for vinexin  $\beta$  (green) and CD68 (macrophage, red) in the coronary arteries of normal donors and patients with CHD (n=3). Scale bar=50  $\mu$ m. C, Representative images (left panel) and quantification (right panel) of Western blots of vinexin  $\beta$  in the aortas of apo E<sup>-/-</sup> mice fed either NC or an HFD (n=4). D, Representative images of double-immunofluorescence staining for vinexin  $\beta$  (green) and CD68 (macrophage, red) in the aortic sinuses of apo E<sup>-/-</sup> mice fed either NC or an HFD (n=3). Scale bar=50  $\mu$ m. \*P<0.05. apo E indicates apolipoprotein E; CHD, coronary heart disease; DAPI, 4',6-diamidino-2-phenylindole; HFD, high-fat diet; NC, normal chow.





**Figure 2.** Vinexin  $\beta$  knockout protects mice from atherosclerosis. **A**, Vinexin  $\beta$  expression in vinexin  $\beta^{-/-}$  apo E<sup>-/-</sup> mice and apo E<sup>-/-</sup> littermates. **B**, Quantification of plaque occupation on entire aorta of vinexin  $\beta^{-/-}$  apo E<sup>-/-</sup> mice and apo E<sup>-/-</sup> littermates based on Oil Red O staining. Representative images are shown in the left panel (n=10). \**P*<0.01. **C**, Representative images (left panel) and plaque area quantification (right panel) of H&E-stained cross-sections of the aorta at sinus level. Data from 6 sections at 100- $\mu\text{m}$  intervals were averaged for each animal, and 5 hearts in each group were used for the analysis. Scale bar=500  $\mu\text{m}$ . \**P*<0.05. **D**, Representative images (left panel) and plaque area quantification (right panel) of H&E-stained cross-sections of the brachiocephalic arteries. Six slides from each animal and 5 different littermates in each group. Scale bar=100  $\mu\text{m}$ ; \**P*<0.05. **E** through **H**, Effect of vinexin  $\beta$  deficiency on the metabolic parameters of apo E-null mice: body weight (**E**), triglycerides (**F**), total cholesterol (**G**), and lipoprotein (**H**). apo E indicates apolipoprotein E; H&E, hematoxylin and eosin; HDL, high-density lipoprotein; HFD, high-fat diet; LDL, low-density lipoprotein; NC, normal chow; NS, not significant; VLDL, very low-density lipoprotein.

## Vinexin $\beta$ Deficiency Attenuates Atherosclerosis Development

Vinexin  $\beta^{-/-}$ -apo E $^{-/-}$  mice were created by crossing vinexin  $\beta^{-/-}$  mice with apo E $^{-/-}$  mice. Ablation of vinexin  $\beta$  was verified by Western blotting (Figure 2A). Oil Red O staining of the entire aortic surface indicated that vinexin  $\beta^{-/-}$ -apo E $^{-/-}$  mice exhibited a 40% reduction in total plaque area compared with apo E $^{-/-}$  control mice (Figure 2B). The aortic root lesions and brachiocephalic artery lesions were investigated further via a microscopic morphometric analysis. Consistent with the results of the en face analysis, the aortic root and brachiocephalic artery lesions were significantly reduced by 25% and 40%, respectively (Figure 2C and 2D). Collectively, these data indicate that vinexin  $\beta$  deficiency ameliorates atherosclerosis development. The body weight and the levels of total cholesterol, LDL, very LDL, high-density lipoprotein, and triglyceride remained the similar between apo E $^{-/-}$  mice and vinexin  $\beta^{-/-}$ -apo E $^{-/-}$  mice (Figure 2E through 2H).

## Vinexin $\beta$ Deficiency Improves Atherosclerotic Plaque Stability

Vulnerable plaques are typically composed of large necrotic/lipid cores covered by thin fibrous caps that exhibit low collagen content and severe macrophage infiltration. A morphological analysis based on picosirius red staining was performed to evaluate the necrotic cores of both the aortic root and the brachiocephalic artery. Necrotic core areas, indicated by the black circle, were significantly smaller in vinexin  $\beta^{-/-}$ -apo E $^{-/-}$  mice than in apo E $^{-/-}$  mice (Figure 3A and 3B). Collagen was more abundant in the plaques of vinexin  $\beta^{-/-}$ -apo E $^{-/-}$  mice than in those of apo E $^{-/-}$  mice (Figure 3C). Moreover, smooth muscle actin-positive area sizes were also significantly increased in vinexin  $\beta^{-/-}$ -apo E $^{-/-}$  mice compared with apo E $^{-/-}$  mice (Figure 3D). Vinexin  $\beta^{-/-}$ -apo E $^{-/-}$  mice exhibited less macrophage infiltration than apo E $^{-/-}$  mice (Figure 3E). Consistent with this finding, vinexin  $\beta^{-/-}$ -apo E $^{-/-}$  mice exhibited decreased plaque lipid content compared with control mice (Figure 3F). Collectively, these data indicate that vinexin  $\beta$  is strongly associated with plaque vulnerability.

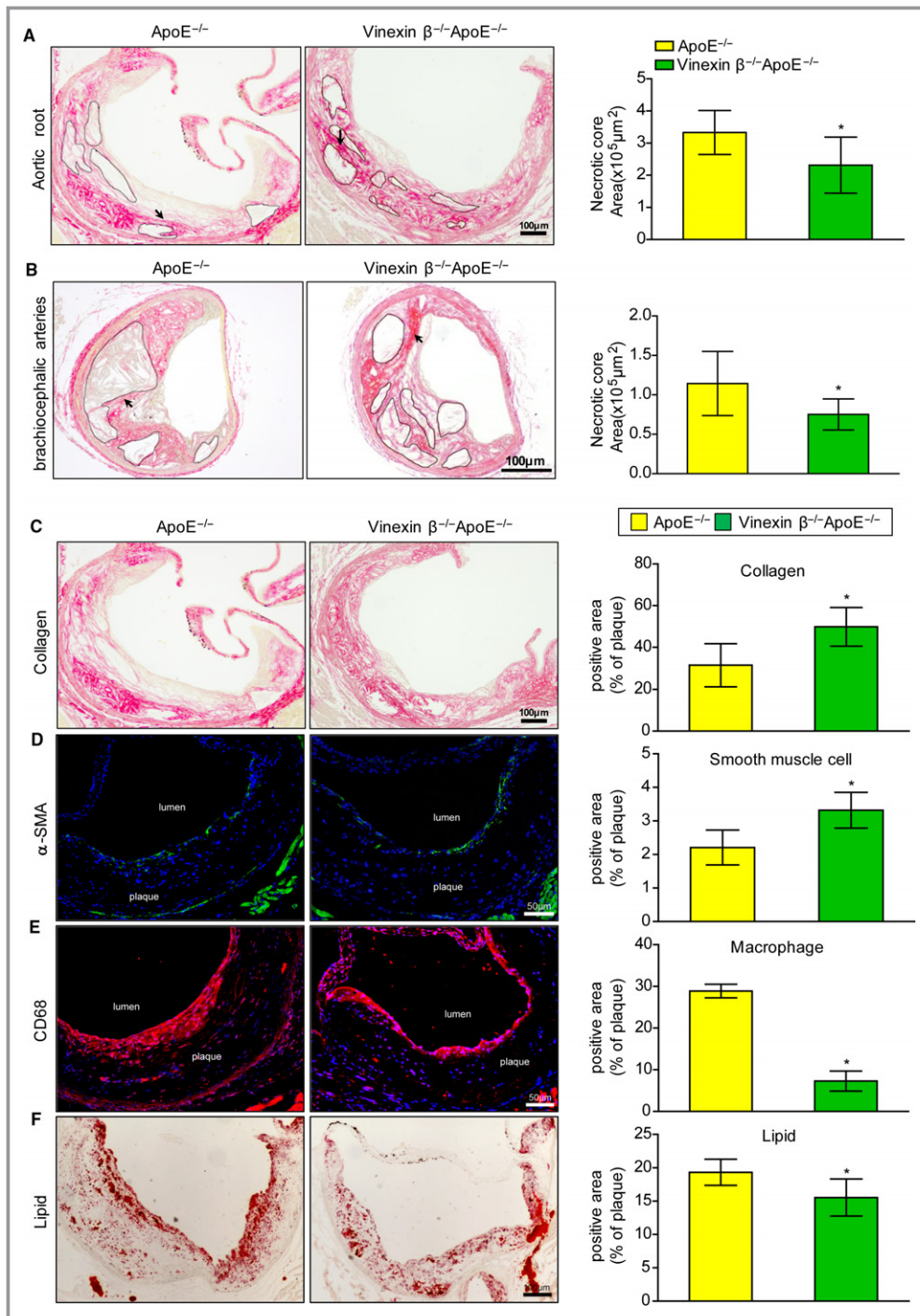
## The Absence of Vinexin $\beta$ in Marrow-Derived Cells Contributes to Atherosclerosis Development

Given that macrophages are the primary cells that express vinexin  $\beta$  in atherosclerotic plaques and usually play key roles in atherosclerosis, bone marrow transplantation was performed to determine the relative contributions of vinexin  $\beta$  in bone marrow-derived macrophages during atherogenesis. Bone marrow chimeras were produced by injecting irradiated

apo E $^{-/-}$  recipient mice with bone marrow cells from apo E $^{-/-}$  or vinexin  $\beta^{-/-}$ -apo E $^{-/-}$  mice, yielding apo E $^{-/-}$  mice with either apo E $^{-/-}$ →apo E $^{-/-}$  or vinexin  $\beta^{-/-}$ -apo E $^{-/-}$ →apo E $^{-/-}$  hematopoietic cells. Successful bone marrow reconstitution with donor bone marrow was verified using PCR for the deleted or wild-type vinexin  $\beta$  allele with genomic DNA isolated from chimera whole blood before or after HFD treatment (Figure 4A). The mice had been on HFD treatment for 16 weeks at the time of bleeding. Flow cytometry showed no significant difference in the monocyte numbers between the 2 groups (Figure 4B). The extent of atherosclerosis was examined via en face lesion area evaluations and Oil Red O staining, as described earlier. As expected, vinexin  $\beta^{-/-}$ -apo E $^{-/-}$ →apo E $^{-/-}$  mice exhibited significantly smaller aortic atherosclerotic lesions in the entire aorta than mice transplanted with apo E $^{-/-}$  cells (Figure 4C). Similarly, vinexin  $\beta^{-/-}$ -apo E $^{-/-}$ →apo E $^{-/-}$  mice exhibited smaller proximal aorta atherosclerotic lesions than mice transplanted with apo E $^{-/-}$  cells (Figure 4D). Taken together, these data suggest that hematopoietic cell vinexin  $\beta$  deficiency is sufficient to restrict atherosclerosis development.

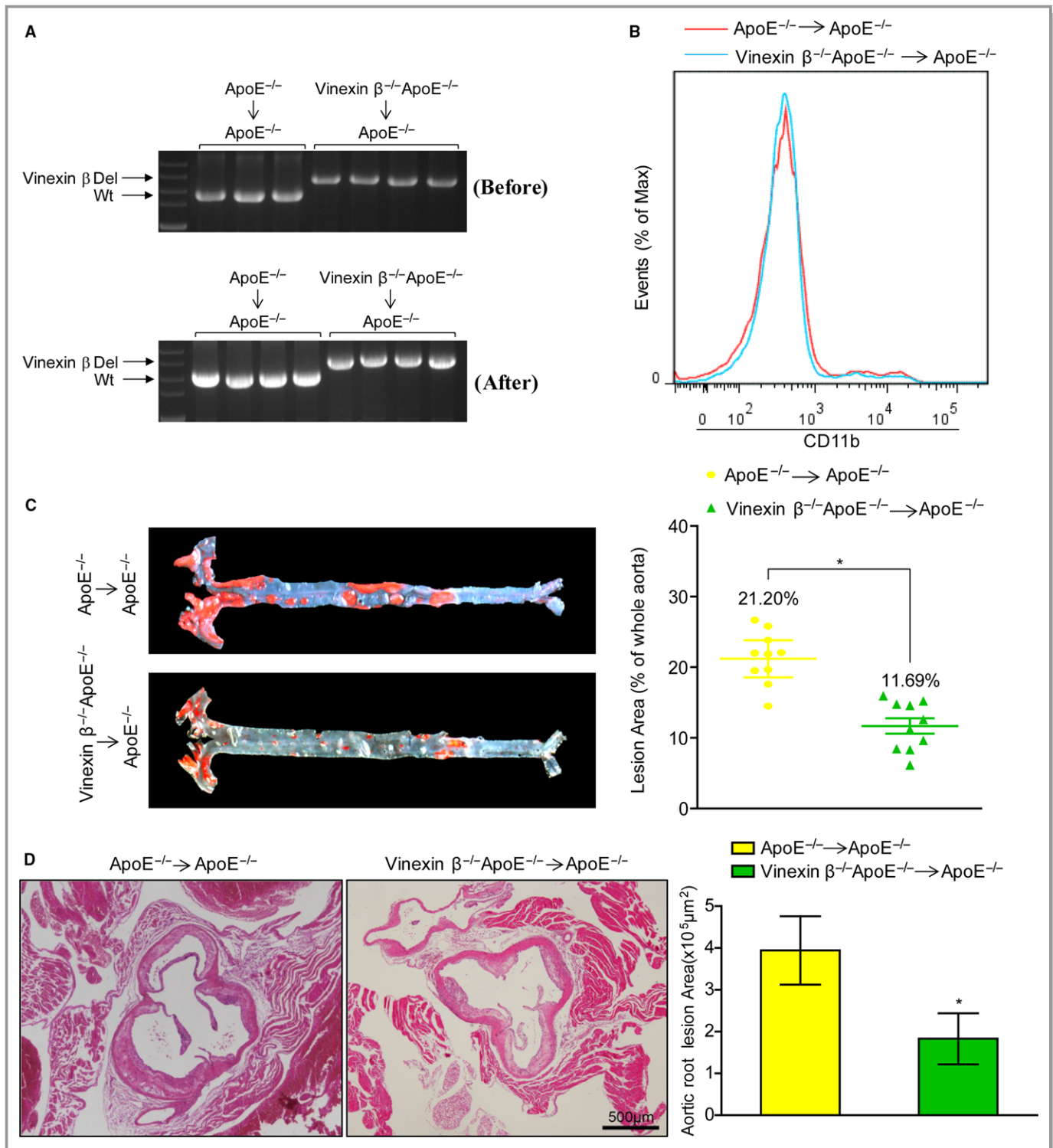
## Vinexin $\beta$ Deficiency Reduces Inflammation

Compelling evidence indicates that inflammation plays an important role during all stages of atherosclerosis, from initiation through progression to occurrence of complications. We quantified the expression levels of pro- and anti-inflammatory factors in atherosclerotic lesions. The mRNA expression levels of proinflammatory cytokines were downregulated in vinexin  $\beta^{-/-}$ -apo E $^{-/-}$  mice compared with apo E $^{-/-}$  mice, whereas the level of anti-inflammatory M2 macrophage markers was upregulated (Figure 5A). Serum levels of IL-6, IL-1 $\beta$ , tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ), and monocyte chemoattractant protein 1 were significantly decreased in vinexin  $\beta^{-/-}$ -apo E $^{-/-}$  mice compared with apo E $^{-/-}$  mice (Figure 5B). In addition, the intensity of ICAM-1 and IL-6 were decreased in the atherosclerotic lesions of vinexin  $\beta^{-/-}$ -apo E $^{-/-}$  mice, whereas the expression level of the anti-inflammatory factor IL-10 was increased (Figure 5C). Moreover, the immunoblot analysis showed that vinexin  $\beta$  ablation reduced ICAM-1 and IL-6 protein expression, whereas it increased the IL-10 expression level (Figure 5D). Taken together, these data indicate that vinexin  $\beta$  deficiency attenuates vascular and systemic inflammation. Previous studies demonstrated that the NF- $\kappa$ B signaling pathway is critically involved in vascular inflammation and atherosclerosis. I-Kappa-B kinase-beta (IKK $\beta$ ) is essential for rapid NF- $\kappa$ B activation via proinflammatory signaling cascades, and I $\kappa$ B $\alpha$  phosphorylation via IKK $\beta$  results in I-kappa-B-alpha (I $\kappa$ B $\alpha$ ) degradation and NF- $\kappa$ B release. IKK $\beta$  is also required for phosphorylation and the transactivation of the NF- $\kappa$ B p65 subunit.<sup>20</sup> Consequently, we



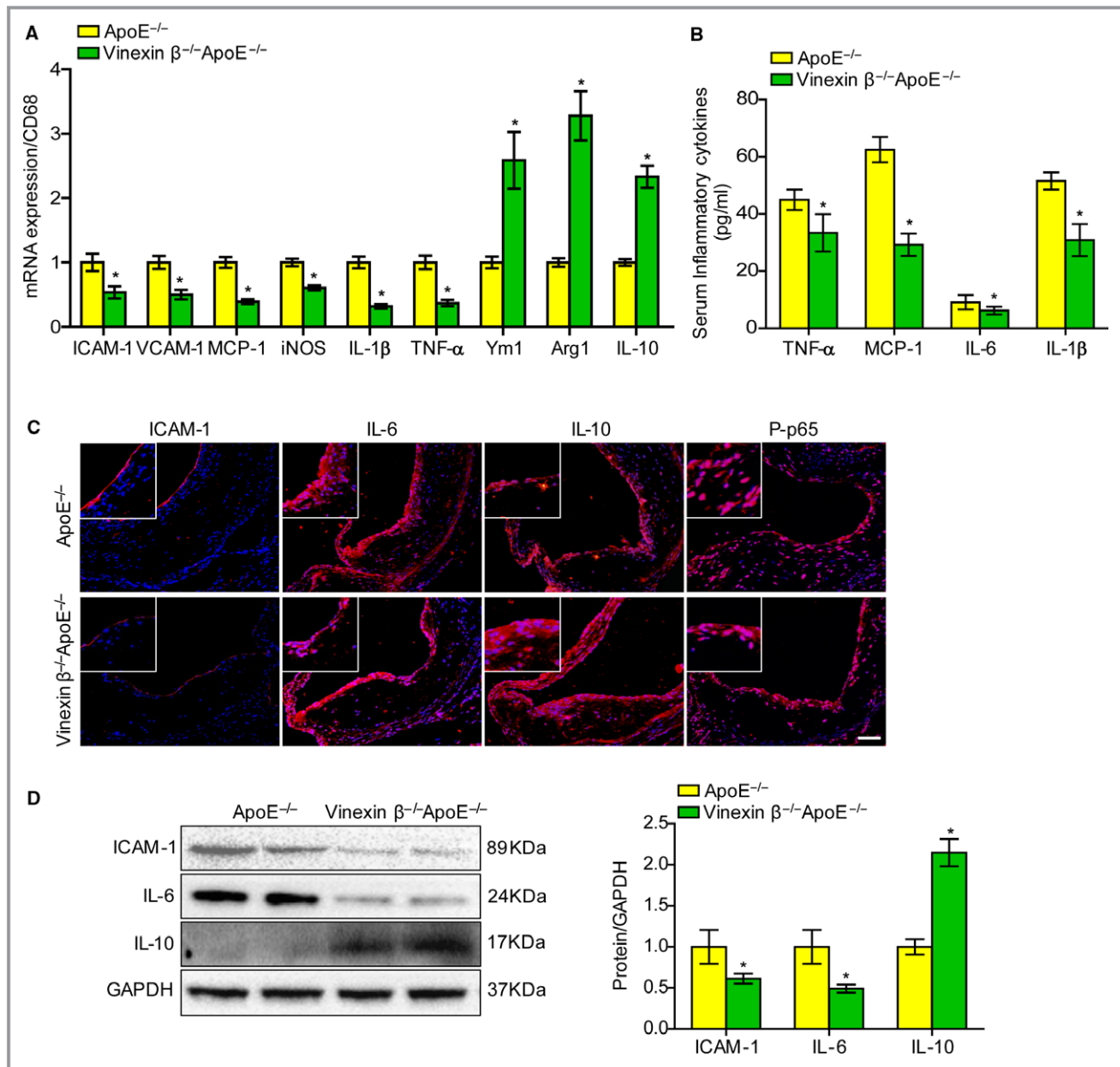
**Figure 3.** Vinixin  $\beta$  ablation improves atherosclerotic plaque stability. A and B, Necrosis analysis in aortic root or brachiocephalic artery lesions. Representative images showing H&E staining of aortic root (A) or brachiocephalic artery (B) sections from vinixin  $\beta^{-/-}$ apo E $^{-/-}$  mice and apo E $^{-/-}$  littermates (left panel). Quantitation of the percentages of necrotic areas in aortic roots (A) and brachiocephalic arteries (B) is shown in the right panel. Six slides from each animal and 5 different littermates in each group. Scale bar=100  $\mu$ m. C through F, Cross-sections of aortic sinus plaques were stained with picosirius red for collagen (C), scale bar=100  $\mu$ m;  $\alpha$ -SMA for smooth muscle cells (D), scale bar=50  $\mu$ m; CD68 for macrophages (E), scale bar=50  $\mu$ m; and Oil Red O for lipids (F), scale bar=100  $\mu$ m (left panel). Quantification of the positive staining area is shown in the right panel. Six slides from each animal and 3 to 4 different littermates in each group. \* $P$ <0.05.  $\alpha$ -SMA indicates  $\alpha$ -smooth muscle actin; apo E, apolipoprotein E; H&E, hematoxylin and eosin.





**Figure 4.** Absence of vinexin  $\beta$  in marrow-derived cells is sufficient to inhibit atherosclerosis. A, Recipient bone marrow reconstitution with donor bone marrow was verified using polymerase chain reaction involving genomic DNA isolated from whole blood. B, Representative flow plot of CD11b in serum of apo  $E^{-/-} \rightarrow apo E^{-/-}$  mice and vinexin  $\beta^{-/-}apo E^{-/-} \rightarrow apo E^{-/-}$  mice (n=3). C, Representative images of en face Oil Red O staining of aortas from apo  $E^{-/-} \rightarrow apo E^{-/-}$  mice and vinexin  $\beta^{-/-}apo E^{-/-} \rightarrow apo E^{-/-}$  mice (left panel). Plaque percentile was quantified and shown in the right panel (n=10); \* $P < 0.05$ . D, Representative images of hematoxylin and eosin staining of the aortic roots of apo  $E^{-/-} \rightarrow apo E^{-/-}$  mice and vinexin  $\beta^{-/-}apo E^{-/-} \rightarrow apo E^{-/-}$  mice (left panel). Lesion area was quantified and averaged on 6 slides from each animal, and 4 different littermates in each group were included (right panel). Scale bar=500  $\mu m$ ; \* $P < 0.05$ . apo E indicates apolipoprotein E. Del, deletion, Wt presents the intact gene without Vinexin deletion.





**Figure 5.** Vinexin  $\beta$  deficiency downregulates inflammatory monocyte/macrophage levels and cytokine expression. A, Quantitative polymerase chain reaction analysis of pro- and anti-inflammatory cytokine expression in the aorta. Target gene expression was normalized to mouse CD68 expression and compared with expression in apo E<sup>-/-</sup> mice (n=4). B, Measurement of serum MCP-1, TNF- $\alpha$ , IL-6, and IL-1 $\beta$  concentrations using enzyme-linked immunosorbent assay (n=5 to 6). C, Representative images showing immunofluorescence staining of cross-sections of the aortic sinus for ICAM-1, IL-6, IL-10, and P-p65 (n=3). Scale bar=50  $\mu$ m. D, The immunoblot analysis of ICAM-1, IL-6 and IL-10 expression in vinexin  $\beta^{-/-}$ apo E<sup>-/-</sup> mice and apo E<sup>-/-</sup> littermates (n=3). E, Detection of NF- $\kappa$ B pathway. Representative IKK $\beta$ , I $\kappa$ B $\alpha$ , and p65 phosphorylation and total protein expression in vinexin  $\beta^{-/-}$ apo E<sup>-/-</sup> mice and apo E<sup>-/-</sup> littermates (left panel). The expression of these proteins was quantified as relative expression after normalization to GAPDH expression (right panel) (n=3). \*P<0.05. apo E indicates apolipoprotein E; Arg1, Arginine 1; ICAM-1, intercellular adhesion molecule 1; I $\kappa$ B $\alpha$ , I-kappa-B-alpha; IKK $\beta$ , I-Kappa-B kinase-beta; IL, interleukin; iNOS, inducible nitric oxide synthase; MCP-1, monocyte chemoattractant protein 1; P, phosphorylated; T, Total; TNF- $\alpha$ , tumor necrosis factor  $\alpha$ ; VCAM-1, vascular cell adhesion molecule 1; Ym1, an alternative name of chitinase-like 3(Chi3l3).

examined the NF- $\kappa$ B signaling pathway activity. Phosphorylation of IKK $\beta$  was significantly attenuated in the aortas of vinexin  $\beta^{-/-}$ apo E<sup>-/-</sup> mice compared with apo E<sup>-/-</sup> mice. As a result, I $\kappa$ B $\alpha$  phosphorylation was significantly decreased, whereas total I $\kappa$ B $\alpha$  expression was increased in vinexin  $\beta^{-/-}$ apo E<sup>-/-</sup>

mice (Figure 5E). In parallel, phosphorylation of NF- $\kappa$ B p65 was also reduced in vinexin  $\beta^{-/-}$ apo E<sup>-/-</sup> mice (Figure 5C and 5E). These results indicate that vinexin  $\beta$  deficiency inhibits NF- $\kappa$ B signaling pathway activation and thus attenuates vascular inflammation.

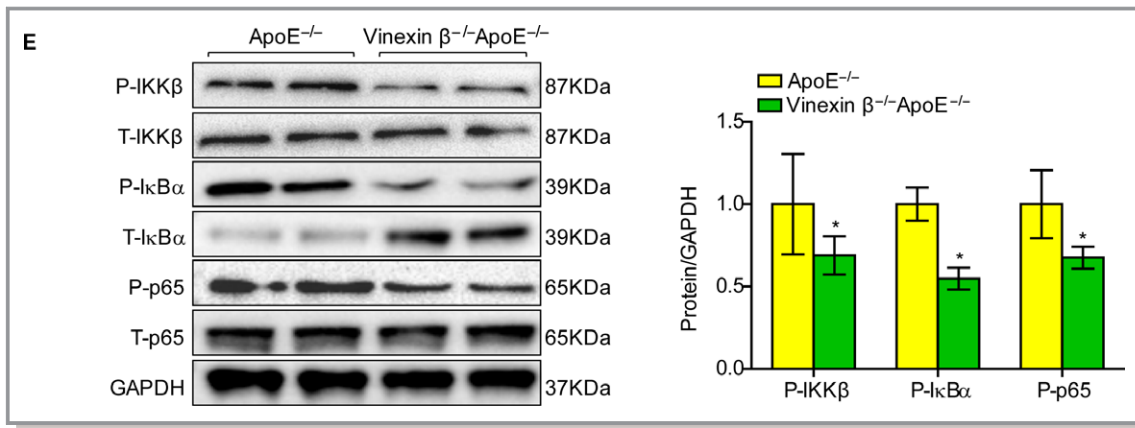


Figure 5. Continued.

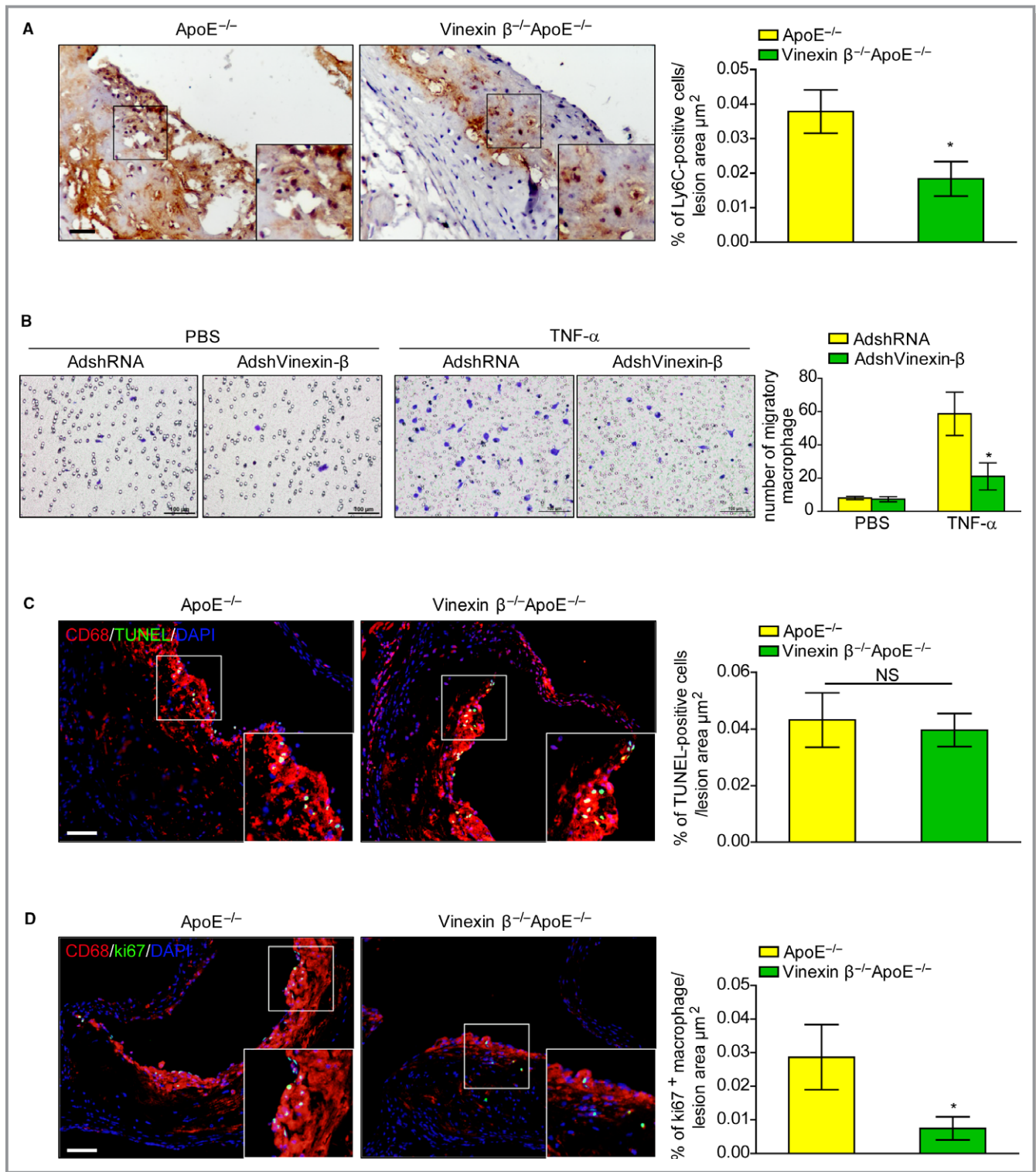
### Vinexin $\beta$ Deficiency Attenuates Monocyte/Macrophage Recruitment and Proliferation of Macrophage But Does Not Affect Macrophage Survival

We next tested whether vinexin  $\beta$  has an effect on binding of monocytes to the endothelium, based on the decreased expression of ICAM-1 and vascular cell adhesion molecule 1 in vinexin  $\beta$ <sup>-/-</sup>apo E<sup>-/-</sup> mice. Immunohistochemical staining showed that vinexin  $\beta$  ablation suppressed the abundance of monocyte in the atherosclerotic plaque (Figure 6A). We also observed that deficiency of vinexin  $\beta$  could inhibit the migration of macrophages induced by TNF- $\alpha$ -stimulated human umbilical vein endothelial cells (Figure 6B). Macrophage apoptosis and proliferation are important events in atherosclerosis plaque development.<sup>21,22</sup> We compared apoptosis of macrophages in atherosclerotic lesions of apo E<sup>-/-</sup> and vinexin  $\beta$ <sup>-/-</sup>apo E<sup>-/-</sup> mice. There was no significant difference in the percentage of TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling)-positive CD68<sup>+</sup> macrophages (Figure 6C). Double-immunofluorescence staining revealed a remarkable decrease of Ki67<sup>+</sup> macrophages in vinexin  $\beta$ <sup>-/-</sup>apo E<sup>-/-</sup> mice compared with the control group (Figure 6D). These findings suggest that vinexin  $\beta$  ablation limits the development of atherosclerosis by interfering with monocyte/macrophage activation and macrophage proliferation.

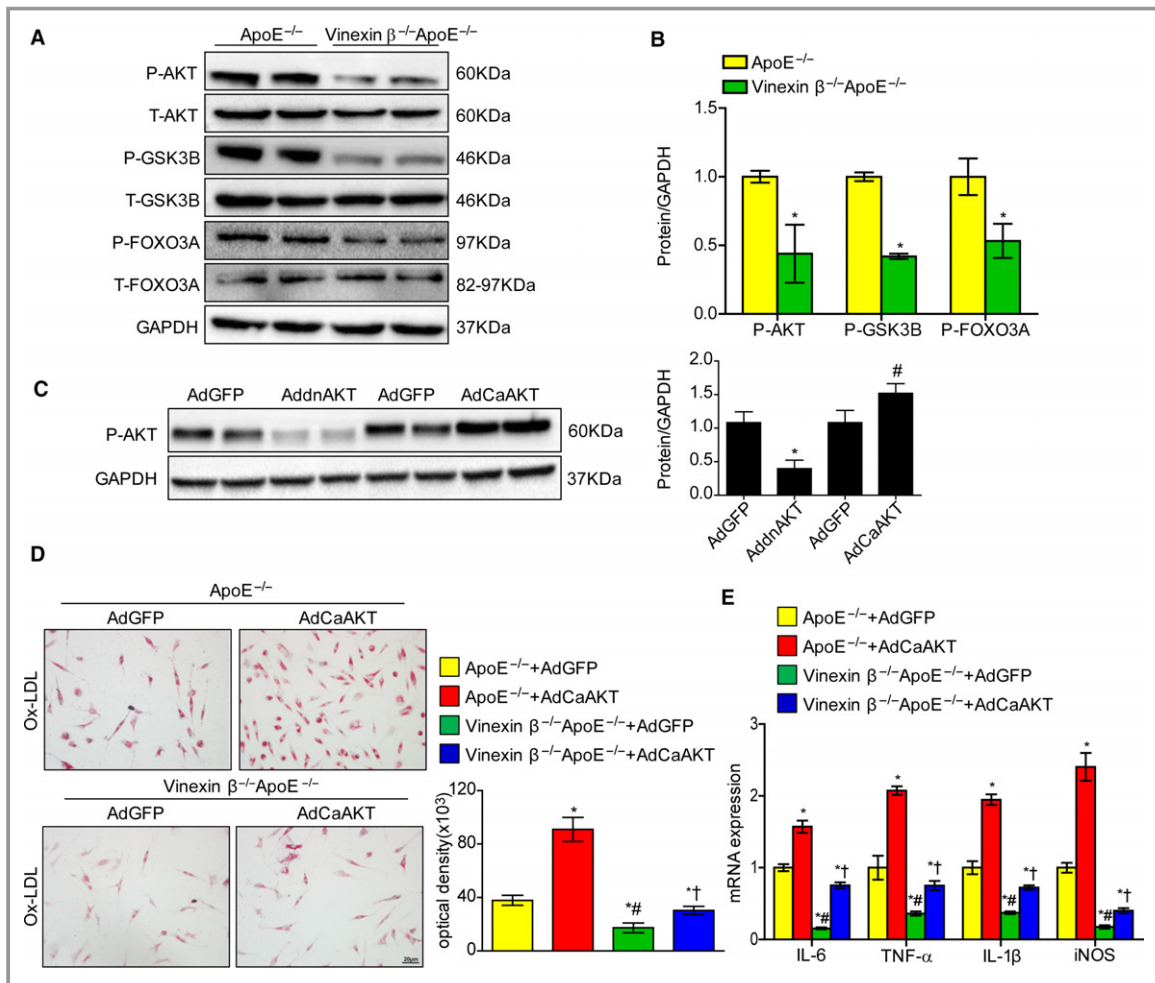
### The Loss of Vinexin $\beta$ Inhibits the Akt-NF- $\kappa$ B Signaling Pathway

We subsequently analyzed the molecular mechanisms of why vinexin  $\beta$  deficiency inhibits atherogenesis. We recently determined that vinexin  $\beta$  interacts with Akt and that vinexin  $\beta$  is associated with cardiac hypertrophy and post-myocardial infarction cardiac dysfunction by regulating the Akt signaling

pathway and the inflammatory response.<sup>12,13</sup> To determine whether the Akt signaling pathway is associated with the effects of vinexin  $\beta$  on atherosclerosis, the activation of Akt and its downstream targets, including GSK3 $\beta$  (glycogen synthase kinase 3 $\beta$ ) and FOXO3A (forkhead box O3), were examined in the aortic specimens of vinexin  $\beta$ <sup>-/-</sup>apo E<sup>-/-</sup> and apo E<sup>-/-</sup> mice. As shown in Figure 7A and 7B, Akt phosphorylation was significantly attenuated in the aortas of vinexin  $\beta$ <sup>-/-</sup>apo E<sup>-/-</sup> mice compared with those of apo E<sup>-/-</sup> mice. Consistent with this finding, GSK3 $\beta$  and FOXO3A phosphorylation was also attenuated. To confirm whether the role of vinexin  $\beta$  on the development of atherosclerosis depends on Akt activation, the peritoneal macrophages transfected with AddnAKT and AdCaAKT were utilized for further investigation (Figure 7C). The peritoneal macrophages from the apo E<sup>-/-</sup> and vinexin  $\beta$ <sup>-/-</sup>apo E<sup>-/-</sup> mice were coinfecting with AdCaAKT (constitutively active Akt) or AdGFP and then exposed to ox-LDL for 24 hours. Oil Red O staining was used to evaluate foam cell formation. The results showed that vinexin  $\beta$  deficiency in the peritoneal macrophages resulted in decreased Oil Red O-positive foam cell formation; however, foam cell formation suppression due to vinexin  $\beta$  deficiency could be reversed by constitutive Akt activation (Figure 7D). Next, we measured proinflammatory cytokine expression after ox-LDL stimulation. As shown in Figure 7E, vinexin  $\beta$  deficiency significantly reduced the expression of several proinflammatory cytokines in macrophages, including IL-6, TNF- $\alpha$ , IL-1 $\beta$ , and inducible nitric oxide synthase, on stimulation with ox-LDL; however, constitutive Akt activation facilitated cytokine expression restoration. Collectively, these data suggest that the regulatory role of vinexin  $\beta$  in pathological atherosclerosis at least partially depends on Akt inhibition. Previous studies have demonstrated that Akt stimulates IKK-mediated phosphorylation and the activation of the NF- $\kappa$ B p65 subunit.<sup>23-25</sup> We next examined whether NF- $\kappa$ B inactivation due to vinexin  $\beta$  deficiency depends on Akt



**Figure 6.** Decreased recruitment of monocyte-macrophage and reduced proliferation of macrophage in vinixin  $\beta$  ablation mice. A, Immunohistochemical staining of cross-sections from the aortic sinus to detect monocyte accumulation was marked by Ly6C (n=3). Scale bar=50  $\mu\text{m}$ . B, The migration of macrophage transfected with AdshRNA and AdshVinixin  $\beta$  on PBS or TNF- $\alpha$  stimulation. Scale bar=100  $\mu\text{m}$ . C, Double-immunofluorescence staining for TUNEL (green) and CD68 (red) in the aortic root of vinixin  $\beta^{-/-}$  apo E<sup>-/-</sup> mice and apo E<sup>-/-</sup> littermates (n=4). Scale bar=50  $\mu\text{m}$ . D, Representative photographs and quantification of aortic sinus sections stained with Ki67 (green) and CD68 (red) antibodies (n=4). Scale bar=50  $\mu\text{m}$ . \* $P$ <0.05. AdshRNA indicates adenoviral short hairpin RNA; AdshVinixin  $\beta$ , adenoviral short hairpin vinixin  $\beta$ ; apo E, apolipoprotein E; DAPI, 4',6-diamidino-2-phenylindole; NS, not significant; TNF- $\alpha$ , tumor necrosis factor  $\alpha$ ; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.



**Figure 7.** The regulatory effects of vinexin  $\beta$  on atherosclerosis depend on Akt signaling. A, Representative Akt and GSK3B phosphorylation expression and total protein expression in vinexin  $\beta^{-/-}$  apo E $^{-/-}$  mice and apo E $^{-/-}$  littermates (n=3). B, The expression of these proteins was quantified as relative expression after normalization to GAPDH expression. \* $P < 0.05$ . C, Immunoblot analysis of phosphorylation of AKT in macrophages transfected with AdGFP, AddnAKT, and AdCaAKT. \* $P < 0.05$ . D, Foam cell formation of vinexin  $\beta$ -intact vs vinexin  $\beta$ -null macrophages in the absence or presence of AdCaAKT after stimulation with ox-LDL. Scale bar=20  $\mu$ m. E, Proinflammatory cytokine expression in vinexin  $\beta$ -intact vs vinexin  $\beta$ -null macrophages in the absence or presence of AdCaAKT after stimulation with ox-LDL. F, NF- $\kappa$ B activation of vinexin  $\beta$ -intact vs vinexin  $\beta$ -null of macrophages in the absence or presence of AdCaAKT after stimulation with ox-LDL. G, NF- $\kappa$ B activation of vinexin  $\beta$ -intact and vinexin  $\beta$  overexpression of macrophages in the absence or presence of AddnAKT after stimulation with ox-LDL. \* $P < 0.01$  compared with apo E $^{-/-}$  plus AdGFP; # $P < 0.01$  compared with apo E $^{-/-}$  plus AdCaAKT or apo E $^{-/-}$  plus AddnAKT; † $P < 0.01$  compared with vinexin  $\beta^{-/-}$  apo E $^{-/-}$  plus AdGFP or apo E $^{-/-}$  plus AdGFP plus AdVinexin  $\beta$ . AdCaAKT, adenovirus expressing constitutively active AKT; AddnAKT, adenovirus expressing dominant-negative mutant of AKT; AdGFP, adenovirus expressing green fluorescent protein; AdVinexin  $\beta$ , adenoviral vinexin  $\beta$ ; apo E, apolipoprotein E; FOXO3A, forkhead box O3; GSK3B, glycogen synthase kinase 3 $\beta$ ; I $\kappa$ B $\alpha$ , I-kappa-B-alpha; IL, interleukin; iNOS, inducible nitric oxide synthase; NF- $\kappa$ B, nuclear factor  $\kappa$ B; ox-LDL, oxidized low-density lipoprotein; P, phosphorylated; T, Total; TNF- $\alpha$ , tumor necrosis factor  $\alpha$ .

signaling. Consistent with our in vivo results, vinexin  $\beta$  deficiency largely abolished ox-LDL-stimulated NF- $\kappa$ B activation in macrophages. Furthermore, NF- $\kappa$ B inhibition was reversed by constitutive Akt activation (Figure 7F). We also isolated the peritoneal macrophage from apo E-deficient mice and transfected with AdGFP or AddnAKT or coinfecting with AdGFP plus AdVinexin  $\beta$  or AddnAKT plus AdVinexin  $\beta$  and then treated the cells with ox-LDL for 24 hours. The results showed that the increased expression of the NF- $\kappa$ B subset

promoted by AdVinexin  $\beta$  was dramatically suppressed by inhibition of Akt (Figure 7G). These data suggest that vinexin  $\beta$ -mediated NF- $\kappa$ B activity depends on Akt signaling.

### Discussion

This study is the first to elucidate the regulatory effects of vinexin  $\beta$  on atherosclerosis development, identifying vinexin  $\beta$  as a proinflammatory mediator of atherosclerosis. This



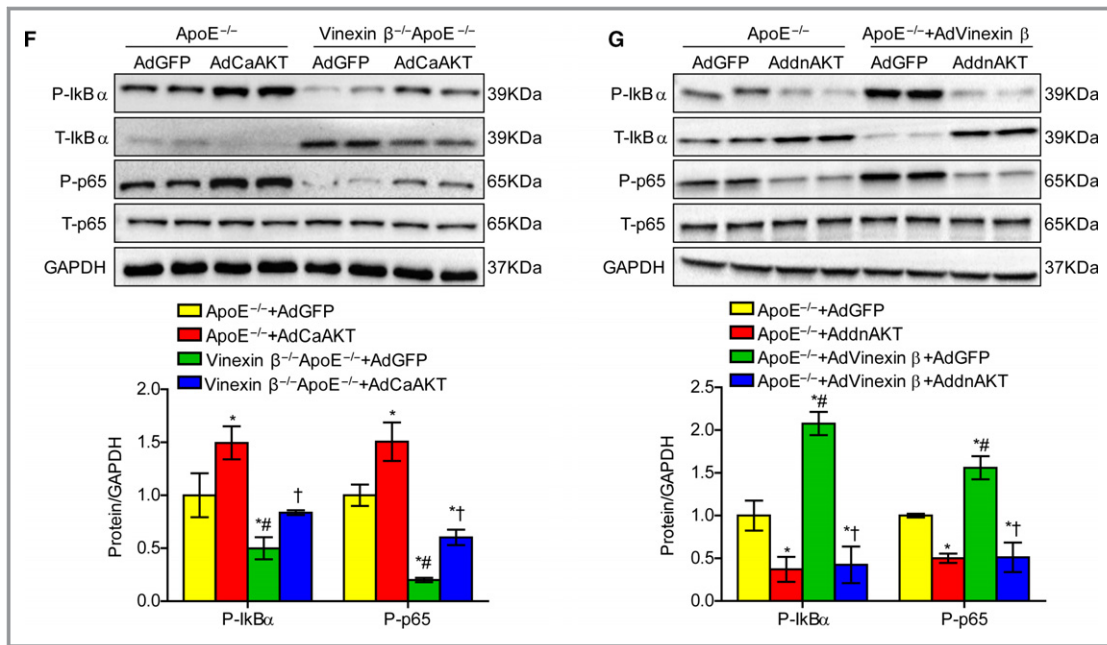


Figure 7. Continued.

study had several major findings. First, vinexin  $\beta$  expression was induced in atherosclerotic lesions. Second, vinexin  $\beta$  deficiency protected against atherosclerosis development in apo E<sup>-/-</sup> mice. Third, vinexin  $\beta$  deficiency inhibited monocyte/macrophage activation and macrophage proliferation and inflammatory response and increased atherosclerotic plaque stability. Finally, the absence of vinexin  $\beta$  resulted in Akt and NF- $\kappa$ B signaling pathway inhibition. These results suggest that vinexin  $\beta$  significantly contributes to atherosclerotic lesion formation.

In both the previous study<sup>14</sup> and our present study, vinexin  $\beta$  was highly expressed in advanced human atherosclerotic lesions. Moreover, we observed that vinexin  $\beta$  expression was induced in atheromas in apo E-deficient mice; however, whether vinexin  $\beta$  expression upregulation is part of a compensatory mechanism that limits atherosclerosis development or is proatherogenic per se remains unknown. A number of studies have demonstrated that with HFD treatment for 16 weeks, apo E deficiency can result in the formation of typical atherosclerotic plaques with macrophage infiltration and fibrous cap formation, and a more severe advanced plaque is exhibited with HFD for 28 weeks.<sup>26-30</sup> In this phase of our research, we observed atherosclerotic plaques formation at different times (8, 16, and 28 weeks) and found results that were consistent with previous studies.<sup>26-30</sup> Consequently, we used the HFD for 28 weeks in the first study of the double-knockout group and for 16 weeks in the later bone marrow transplant experiment. When induced for atherosclerosis with HFD, we found that vinexin  $\beta$ <sup>-/-</sup> apo E<sup>-/-</sup> mice developed smaller lesions than their control littermates at 3 different

locations: the aortic root, the brachiocephalic artery, and the en face aorta. These results suggest that a cause-and-effect relationship, not a compensatory relationship, exists between vinexin  $\beta$  and atherosclerosis.

Several risk factors, such as hyperlipidemia, obesity, and diabetes mellitus, have been implicated in the pathogenesis of atherosclerosis. In this study, circulating lipid levels and body weight did not differ statistically between vinexin  $\beta$ <sup>-/-</sup> apo E<sup>-/-</sup> mice and their littermates, suggesting that reductions in atherosclerosis severity caused by vinexin  $\beta$  deficiency are not due to alterations in lipid metabolism.

The inflammatory process has been implicated in all stages of atherosclerosis, from lesion initiation to progression and, ultimately, destabilization into a vulnerable plaque.<sup>3,4</sup> The most remarkable feature of atherogenesis in HFD-induced vinexin  $\beta$ <sup>-/-</sup> apo E<sup>-/-</sup> mice is reduced vascular inflammation, which is characterized by decreased infiltration of inflammatory cells and reduced proinflammatory signaling within lesions. Macrophages play critical roles in atherosclerosis by engulfing lipoprotein particles trapped in the arterial intima, activating the inflammatory response, and turning into foam cells.<sup>31</sup> Using bone marrow transplantation, we also observed significant atherosclerotic plaque formation, and the present results demonstrated that the absence of vinexin  $\beta$  in hematopoietic cells is sufficient to inhibit atherogenesis. Plaque macrophages, however, are dynamic because both the numbers of macrophages and the presence of an inflammatory phenotype can influence plaque fate.<sup>32</sup>

To elucidate the mechanisms that underlie the atheroprotective effects of vinexin  $\beta$  deficiency, we initially examined

atherosclerotic plaque macrophage content. An analysis of the aortic sinus plaques confirmed that vinexin  $\beta^{-/-}$  apo  $E^{-/-}$  mice exhibited fewer invading macrophages than apo  $E^{-/-}$  mice. Atherosclerotic plaque macrophage content is regulated by the following processes: adhesion, migration, differentiation, proliferation, and apoptosis.<sup>21,22,33</sup> A variety of proinflammatory cytokines and chemokines participate in these processes. We detected the vascular expression of monocyte chemoattractant protein 1, vascular cell adhesion molecule 1, and ICAM-1, which mediates monocyte adhesion and migration, via quantitative PCR and immunofluorescence and found that the levels of these cytokines were significantly reduced in the aortas of vinexin  $\beta^{-/-}$  apo  $E^{-/-}$  mice. Our results consistently exhibited remarkable reduction in monocyte accumulation and macrophage migration. In addition, we observed that there was no significant difference in the percentage of TUNEL-positive CD68<sup>+</sup> macrophages and a remarkable decrease of Ki67<sup>+</sup> macrophages in vinexin  $\beta^{-/-}$  apo  $E^{-/-}$  mice compared with the control group. This finding might be the result of and the reason for decreased macrophage influx into the aorta. In addition, less secretion of proinflammatory cytokines including TNF- $\alpha$ , IL-1 $\beta$ , IL-6, and inducible nitric oxide synthase by macrophages from vinexin  $\beta^{-/-}$  apo  $E^{-/-}$  mice was observed. Quantifying analysis with quantitative PCR also revealed declines in proinflammatory cytokine expression and increases in anti-inflammatory M2 macrophage markers in the aortas of vinexin  $\beta^{-/-}$  apo  $E^{-/-}$  mice. Our present study demonstrated that vinexin  $\beta$  has an effect on inflammatory response in atherogenesis mainly by regulating macrophage polarization; however, the detailed mechanism must be further investigated. All of these cytokines affect atheroma stability. Both TNF- $\alpha$  and IL-1 $\beta$ , for example, induce matrix-degrading metalloproteinase expression and promote tissue remodeling.<sup>34,35</sup> In addition, TNF- $\alpha$  facilitates increases in oxidative stress in VSMCs and facilitates VSMC apoptosis.<sup>36,37</sup> In contrast to these proinflammatory cytokines, IL-10 is a potent anti-inflammatory cytokine with the ability to deactivate macrophages.<sup>38</sup> Consistent with these studies, we found that vinexin  $\beta^{-/-}$  apo  $E^{-/-}$  mice exhibited more stable lesions characterized by diminished necrotic cores and increased collagen and VSMC content. These data suggest that vinexin  $\beta$  deficiency not only induces decreased monocyte/macrophage recruitment but also reduces cytokine expression, thereby inhibiting vascular inflammation and improving plaque stability, which might be the mechanism that underlies atherosclerotic lesion reduction and stabilization.

Multiple inflammatory pathways associated with the pathogenesis of atherosclerosis are regulated by the transcription factor NF- $\kappa$ B, which is a master regulator of innate and adaptive immunity.<sup>39</sup> Activated NF- $\kappa$ B has been identified in human atherosclerotic plaques, and it exhibits enhanced

expression in unstable coronary plaques.<sup>40,41</sup> NF- $\kappa$ B pathway activation regulates the transcription of inflammatory cytokines such as ICAM-1, vascular cell adhesion molecule 1, monocyte chemoattractant protein 1, and IL-6 as well as the expression of tissue factor and matrix metalloproteinases.<sup>42</sup> In addition, NF- $\kappa$ B activation facilitates polarization of macrophages toward the M1 phenotype.<sup>43</sup> I $\kappa$ B $\alpha$  phosphorylation via IKK $\beta$  results in I $\kappa$ B $\alpha$  degradation and NF- $\kappa$ B release.<sup>44</sup> IKK $\beta$  is a crucial component of the IKK-signalosome, which facilitates NF- $\kappa$ B activation in response to most inflammatory stimuli. We observed significant IKK $\beta$  activity attenuation in the aortas of vinexin  $\beta^{-/-}$  apo  $E^{-/-}$  mice compared with apo  $E^{-/-}$  mice. As a result, the level of phosphorylated I $\kappa$ B $\alpha$  was dramatically decreased, whereas the level of total I $\kappa$ B $\alpha$  was increased in vinexin  $\beta^{-/-}$  apo  $E^{-/-}$  mice. Moreover, NF- $\kappa$ B p65 phosphorylation was also decreased in vinexin  $\beta^{-/-}$  apo  $E^{-/-}$  mice. In conjunction with NF- $\kappa$ B signaling inhibition, the NF- $\kappa$ B-controlled genes that play important roles in inflammation, including ICAM-1, vascular cell adhesion molecule 1, monocyte chemoattractant protein 1, and IL-6, exhibited decreased expression in vinexin  $\beta^{-/-}$  apo  $E^{-/-}$  mice. These results indicate that vinexin  $\beta$  deficiency inhibits NF- $\kappa$ B signaling pathway activation and attenuates vascular inflammation.

We recently reported that Akt interacts with vinexin  $\beta$  in cardiac hypertrophy<sup>12</sup> and that disruptions in vinexin  $\beta$  expression result in pronounced Akt signaling pathway activation, whereas vinexin  $\beta$  expression inhibits Akt activation. These results suggest that the functional role of vinexin  $\beta$  in the heart depends on Akt activation. In atherogenesis, the absence of Akt2 in bone marrow-derived cells markedly reduces atherosclerotic plaque formation in mice.<sup>45</sup> Moreover, the Akt/GSK3 $\beta$  pathway plays a prominent role in monocyte activation and migration.<sup>46</sup> These findings raise the question of whether the Akt signaling pathway is associated with the effects of vinexin  $\beta$  on atherosclerosis. We compared the activity of Akt and its downstream targets, including GSK3B and FOXO3A, in the aortas of vinexin  $\beta^{-/-}$  apo  $E^{-/-}$  mice with the aortas of apo  $E^{-/-}$  mice. The results demonstrated that Akt, GSK3B, and FOXO3A phosphorylation was dramatically decreased in vinexin  $\beta^{-/-}$  apo  $E^{-/-}$  mice. In addition, phosphorylated Akt was visualized via costaining with CD68 and was less abundant in vinexin  $\beta^{-/-}$  apo  $E^{-/-}$  mice. More important, we found that foam cell formation and cytokine expression, which were inhibited by vinexin  $\beta$  deficiency, were largely restored by constitutive Akt activation in macrophages. Previous studies have demonstrated that Akt stimulates IKK-mediated phosphorylation and the activation of the NF- $\kappa$ B p65 subunit.<sup>23-25</sup> The present study found that constitutive Akt activation reversed NF- $\kappa$ B inhibition resulting from vinexin  $\beta$  deficiency, and the activation of NF- $\kappa$ B by vinexin  $\beta$  overexpression was suppressed by

inhibition of AKT. These results indicate that the effects of vinexin  $\beta$  on NF- $\kappa$ B activation are Akt dependent. Collectively, our results indicate that the Akt signaling pathway is critical for vinexin  $\beta$  with respect to regulating atherosclerosis development. We compared the results of this study with those of our previous study and found that the effects of vinexin  $\beta$  on the Akt signaling pathway in atherosclerosis were different from those of vinexin  $\beta$  on the Akt signaling pathway in cardiac hypertrophy. Different pathological processes and disparate cell types are associated with cardiac hypertrophy and atherosclerosis; therefore, the vinexin  $\beta$ -mediated regulation of Akt signaling pathway activation might be specific to cell type, stimulus, and disease. These results also suggest that vinexin  $\beta$  is a key regulator of Akt activation or inactivation in response to different stressors. By carrying out primary cell culture and a bone marrow transplant experiment, our study indicated that vinexin  $\beta$  functions mainly in macrophages and plays a critical role in atherogenesis by affecting the inflammation and Akt pathway. Future study using cell type-specific vinexin  $\beta$ -deficient mice will be useful to completely exclude the effect of vinexin  $\beta$  in the other cell types such as endothelial cells and VSMCs.

In conclusion, we demonstrated that vinexin  $\beta$  deficiency results in reduced atherosclerotic lesion formation and increased plaque stability in mice as well as decreased macrophage infiltration and production of proinflammatory cytokines. We attribute these changes to inhibition of the Akt-NF- $\kappa$ B signaling pathway, indicating that vinexin  $\beta$  plays a critical role in atherosclerosis. The genetic or pharmaceutical inhibition of vinexin  $\beta$  might be a therapeutic strategy to protect against atherosclerosis development.

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## Disclosures

None.

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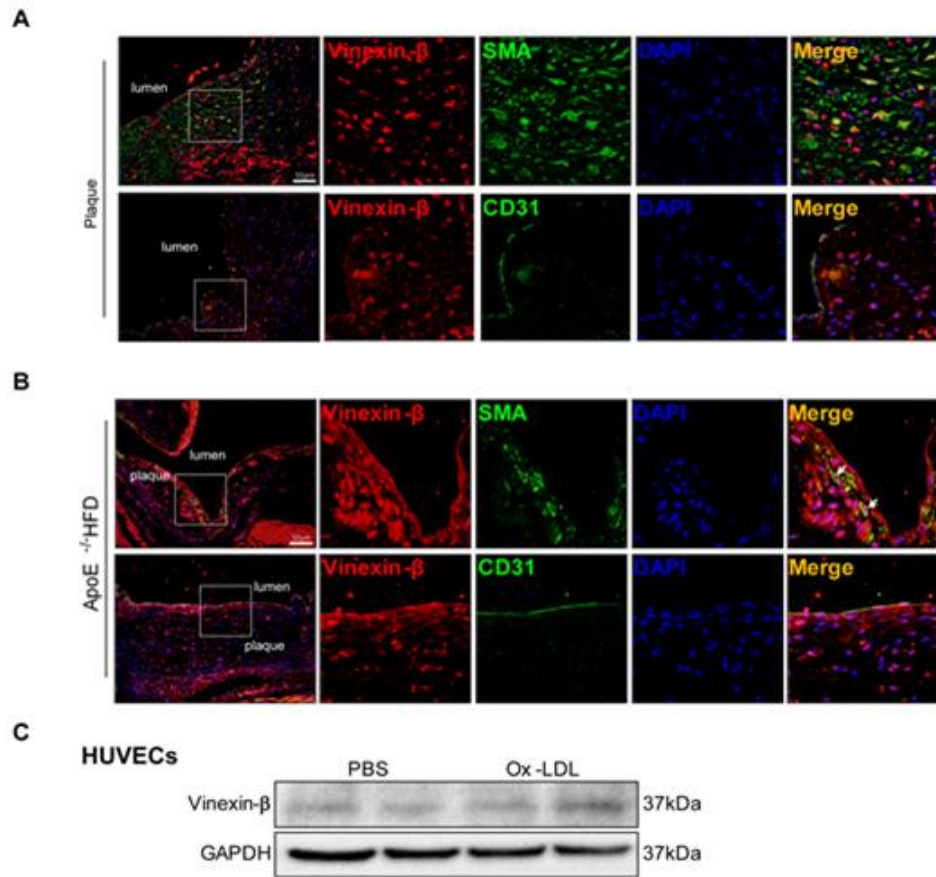
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# **Supplemental Material**

**Figure S1.** Expression of Vinexin- $\beta$  in endothelial cell and VSMCs.



**A.** Representative images showing double-immunofluorescence staining for Vinexin- $\beta$  (Red), SMA (smooth muscle cell, Green) or CD31 (endothelial cell, Green) in human plaques. **B.** Double-immunofluorescence staining for Vinexin- $\beta$  (Red), SMA (smooth muscle cell, Green) or CD31 (endothelial cell, Green) in mice plaques. **C.** Western blot analysis of Vinexin- $\beta$  expression in HUVECs upon Ox-LDL treatment.