ORIGINAL RESEARCH

Deficiency of Adipocyte IKKβ Affects Atherosclerotic Plaque Vulnerability in Obese LDLR Deficient Mice

Weiwei Lu, PhD; Se-Hyung Park, PhD; Zhaojie Meng, PhD; Fang Wang, PhD; Changcheng Zhou, PhD

***Background*—**Obesity-associated chronic inflammation has been known to contribute to atherosclerosis development, but the underlying mechanisms remain elusive. Recent studies have revealed novel functions of IKKβ (inhibitor of NF-κB [nuclear factor κB] kinase β), a key coordinator of inflammation through activation of NF-κB, in atherosclerosis and adipose tissue development. However, it is not clear whether IKKβ signaling in adipocytes can also affect atherogenesis. This study aims to investigate the impact of adipocyte IKKβ expression on atherosclerosis development in lean and obese LDLR (low-density lipoprotein receptor)–deficient ($LDLR^{-/-}$) mice.

***Methods and Results*—**To define the role of adipocyte IKKβ in atherogenesis, we generated adipocyte-specific IKKβ-deficient $LDLR^{-/-}$ ($IKK\beta^{\Delta Ad}LDLR^{-/-}$) mice. Targeted deletion of IKKβ in adipocytes did not affect adiposity and atherosclerosis in lean $LDLR^{-/-}$ mice when fed a low-fat diet. In response to high-fat feeding, however, $IKK\beta^{\Delta Ad}LDLR^{-/-}$ mice had defective adipose remodeling and increased adipose tissue and systemic inflammation. Deficiency of adipocyte IKKβ did not affect atherosclerotic lesion sizes but resulted in enhanced lesional inflammation and increased plaque vulnerability in obese $IKK\beta^{\Delta Ad}LDLR^{-/-}$ mice.

***Conclusions*—**These data demonstrate that adipocyte IKKβ signaling affects the evolution of atherosclerosis plaque vulnerability in obese $LDLR^{-/-}$ mice. This study suggests that the functions of IKKβ signaling in atherogenesis are complex, and IKKβ in different cell types or tissues may have different effects on atherosclerosis development. **(*J Am Heart Assoc*. 2019;8:e012009. DOI: 10.1161/JAHA.119.012009.)**

Key Words: adipocyte • arteriosclerosis • inflammation • nuclear factor-κB

Atherosclerosis is a chronic inflammatory disease characterized by accumulation of cholesterol, immune cells, and fibrous elements, leading to formation of atherosclerotic plaques in large and medium-sized arteries.[1–3] Atherosclerosis-related ischemic symptoms, including acute ischemic stroke and myocardial infarction, are generally thought to result from plaque rupture and thrombosis other than narrowing of the blood vessel lumen.[4–6] These critical events occur mostly in the presence of unstable atherosclerotic lesions or vulnerable plaques, whereas stable plaques are often asymptomatic throughout a patient's lifetime.[7] The progressive accumulation of inflammatory cells such as macrophages promotes plaque vulnerability by reducing smooth muscle cell (SMC) survival and collagen contents, leading to enlarged unstable plaques covered by thin fibrous caps.[5,6,8–10]

Obesity is a known risk factor for atherosclerotic cardiovascular disease, and obesity-associated chronic inflammation has been considered as a major contributor to atherosclerosis development.[11–13] Many inflammatory signaling pathways that contribute to the pathogenesis of obesity-associated atherosclerosis are regulated by the transcriptional factor NF-κB (nuclear factor κB), a master regulator of the innate and adaptive immune responses.[14–17] Activation of canonical or classical NF-κB signaling by inflammatory mediators requires IKKβ (inhibitor of NF-κB kinase β), the predominant catalytic subunit of the IKK complex that mediates the phosphorylation and degradation of the inhibitors of NF-κB.[17,18] Despite numerous studies suggesting the involvement

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Clinical Perspective

What Is New?

- IKKβ (inhibitor of NF-κB [nuclear factor κB] kinase β) is a key protein in regulating inflammatory responses.
- Genetic deletion of IKKβ in adipocytes did not affect atherosclerotic lesion sizes but increased atherosclerotic plaque vulnerability in obese hyperlipidemic LDLR (low-density lipoprotein receptor)–deficient mice.
- In response to feeding with a high-fat diet, IKKβ-deficient mice had defective adipose remodeling and increased adipose tissue and systemic inflammation, which likely contributed to the increased plaque inflammation and vulnerability in those mice.

What Are the Clinical Implications?

- Findings from this study suggest that the functions of IKKβ signaling in atherosclerosis are complex, and adipose tissue IKKβ may affect atherosclerotic plaque stability in an obese condition.
- Analyses of both plaque sizes and stability should be considered for early diagnosis of coronary syndromes in obese patients.

of NF-κB signaling in atherosclerosis development, the tissue or cell type–specific contribution of IKKβ-mediated NF-κB activation to atherogenesis has not been completely understood.

We and others have recently revealed important new functions of IKKβ in atherosclerosis and adipose tissue development.[16,17,19–22] Deficiency of myeloid IKKβ, for example, has been shown to reduce macrophage inflammatory responses and to decrease diet-induced atherosclerosis in LDLR (low-density lipoprotein receptor)–deficient ($LDLR^{-/-}$) mice.[16] Overexpression of IKKβ in liver can aggravate atherosclerosis development in APOE*3-Leiden mice.[19] Deletion of IKKβ in SMCs also protected $LDLR^{-/-}$ mice from diet-induced vascular inflammation and atherosclerosis.[17] Deficiency of SMC IKKβ has also been shown to affect vascular calcification in another study.[20] Interestingly, we found that many adipocyte precursor cells express SMC markers, and ablation of IKKβ in those cells inhibits adipocyte differentiation and renders mice resistant to diet-induced obesity.[17,21,23] In addition to adipose progenitors, we generated an adipocyte-specific IKKβ-deficient mouse model to investigate the impact of adipocyte IKKβ expression on adipose tissue development and metabolism.[22] Unexpectedly, adipocyte IKKβ-deficient mice have multiple histopathologies in visceral white adipose tissue (WAT) including increased adipocyte death, defective adaptive adipose remodeling, and elevated tissue inflammation when challenged with a high-fat diet (HFD).[22] Mechanistic studies revealed that IKKβ is a key adipocyte survival factor, and IKKβ can protect adipocytes from HFD-elicited adipocyte death in an obese condition.[22] These findings revealed the complex functions of IKKβ signaling in atherosclerosis and adipose tissue development. However, the impact of adipocyte IKKβ expression on atherosclerosis development in lean and obese animals has not been investigated.

In the current study, we generated an adipocyte-specific IKKβ-deficient $LDLR^{-/-}$ ($IKK\beta^{\Delta Ad}LDLR^{-/-}$) mouse model to evaluate the influence of adipocyte IKKβ expression on atherosclerosis development under lean and obese conditions. We demonstrate in this study that deficiency of adipocyte IKKβ increased atherosclerotic plaque vulnerability without affecting atherosclerotic lesion sizes in HFD-fed $LDLR^{-/-}$ mice, most likely due to increased systemic and lesional inflammation.

Methods

The authors declare that all supporting data are available within the article and its online supplementary files.

Animals

Adipocyte-specific IKKβ-knockout ($IKK\beta^{\Delta Ad}$) mice were previously generated by crossing mice carrying loxP-flanked IKKβ alleles ($IKK\beta^{F/F}$) with adiponectin (Adipoq)–Cre transgenic mice.[22] To increase susceptibility to atherosclerotic lesion development, $IKK\beta^{\Delta Ad}$ mice were then crossed with $LDLR^{-/-}$ mice to generate $IKK\beta^{F/F}LDLR^{-/-}$ and $IKK\beta^{\Delta Ad}LDLR^{-/-}$ mice. All mice used in this study had $IKK\beta^{F/F}LDLR^{-/-}$ double-mutant background, and $IKK\beta^{\Delta Ad}LDLR^{-/-}$ mice carried heterozygous knock-in for Adipoq-Cre. For atherosclerosis study, 4-week-old male $IKK\beta^{\Delta Ad}LDLR^{-/-}$ and $IKK\beta^{F/F}LDLR^{-/-}$ littermates (n=15 per group) were fed a modified semisynthetic low-fat AIN76 diet (4.2% fat and 0.02% cholesterol) or a Western-type HFD (21% fat and 0.2% cholesterol) for 12 weeks until they were euthanized at age 16 weeks.[16,17,24,25] All experimental mice used in this study were male; however, studying a single sex has limitations because sex differences have been widely reported in mouse atherosclerosis studies.[26] Body weight was measured weekly, and body composition was analyzed by EchoMRI (EchoMRI Corp) for measuring the fat and lean masses of mice. On the day of euthanasia, mice were fasted for 6 hours following the dark cycle (feeding cycle), and blood and tissues were then collected as described previously.[16,25] All animals were housed in an animal facility under a protocol approved by the University of Kentucky institutional animal care and use committee.

Blood Analysis

Plasma total cholesterol and triglyceride levels were determined enzymatically by a colorimetric method.[16,27] Lipoprotein fractions were isolated by centrifuging at 189 000*g* for 3 hours in a Beckman Optima TL-100 tabletop ultracentrifuge at its own density (1.006 g/mL). The infranatant was then adjusted to a density of 1.063 g/mL with solid potassium bromide to harvest the HDL (high-density lipoprotein) fraction by spinning at 189 000*g* for 18 hours. The cholesterol content of HDL infranatant was measured enzymatically. The cholesterol content of each supernatant and the final infranatant were measured and taken to be VLDL (very low-density lipoprotein; $d<1.006$ g/mL), LDL (low-density lipoprotein;$1.006\leq d\leq 1.063$ g/mL), and HDL ($d>1.063$ g/mL) cholesterol, respectively.[28] Cholesterol concentrations in all 3 fractions were then determined enzymatically by a colorimetric method. Plasma cytokine levels were also determined by a mouse cytokine multiplex assay kit and a BioPlex 200 system (Bio-Rad Laboratories), as described previously.[22,25]

Atherosclerotic Lesion Analysis

OCT (optimal cutting temperature compound)–embedded hearts and brachiocephalic arteries were sectioned and stained with Oil Red O, and atherosclerotic lesions were quantified, as described previously.[25,29,30]

Atherosclerotic Plaque Morphology Histomorphometric Analysis

Atherosclerotic plaques at the aortic root were sectioned, as described previously.[16,25] Plaque morphological histomorphometric characters were analyzed by hematoxylin and eosin staining.[25] Calcium deposits in aortic root sections were analyzed by alizarin red S staining, as described previously.[31] Plaque composition of lipid-rich cores, collagen, SMCs, and macrophage contents were analyzed by Oil Red O staining, trichrome staining, immunofluorescence staining for α-SMA (α-smooth muscle actin) and CD68, respectively. Plaque stability was evaluated by comparing the ratios of the plaque components mentioned with the entire plaques. The histological plaque stability score was also calculated, as described previously, following the following formula: (plaque stability score)=(SMC area+collagen area)/(macrophage area+lipid area).[32–34]

RNA Isolation and Quantitative Polymerase Chain Reaction Analysis

Total RNA was isolated from mouse tissues using TRIZOL Reagent (Life Technologies), and quantitative reverse transcription polymerase chain reaction was performed using gene-specific primers and the SYBR Green PCR Kit (Life Technologies), as described previously.[22,35,36] The sequences of the primer sets used in this study are listed in Table.

Table. Primer Sequences for Quantitative Polymerase Chain Reaction

Name	Sequence	
IKKβ	Forward	5′-GAGCTCAGCCCAAAGAACAG-3′
	Reverse	5′-AGGTTCTGCATCCCCTCTGG-3′
CD68	Forward	5′-CTTCCCACAGGCAGCACAG-3′
	Reverse	5′-AATGATGAGAGGCAGCAAGAGG-3′
F4/80	Forward	5′-CTTTGGCTATGGGCTTCCAGTC-3′
	Reverse	5′-GCAAGGAGGACAGAGTTTATCGTG-3′
IL-6	Forward	5′-TAGTCCTTCCTACCCCAATTTCC-3′
	Reverse	5′-TTGGTCCTTAGCCACTCCTTC-3′
MCP1	Forward	5′-TTAAAAACCTGGATCGGAACCAA-3′
	Reverse	5′-GCATTAGCTTCAGATTTACGGGT-3′
TNF-α	Forward	5′-CCCATATACCTGGGAGGAGTCTTC-3′
	Reverse	5′-CATTCCCTTCACAGAGCAATGAC-3′
IL-1β	Forward	5′-GCAACTGTTCCTGAACTCAACT-3′
	Reverse	5′-ATCTTTTGGGGTCCGTCAACT-3′
ICAM1	Forward	5′-GTGATCCCTGGGCCTGGTG-3′
	Reverse	5′-GGAAACGAATACACGGTGATGG-3′
VCAM1	Forward	5′-TACCAGCTCCCAAAATCCTG -3′
	Reverse	5′-TCTGCTAATTCCAGCCTCGT-3′
GAPDH	Forward	5′-AACTTTGGCATTGTGGAAGG-3′
	Reverse	5′-GGATGCAGGGATGATGTTCT-3′

ICAM1, intercellular adhesion molecule 1; IKKβ, inhibitor of NF-κB [nuclear factor κB] kinase β; IL, interleukin; MCP1, monocyte chemoattractant protein 1; TNF-α, tumor necrosis factor α; VCAM1, vascular cell adhesion molecule 1.

Immunostaining

Immunofluorescence staining of atherosclerotic lesions was performed on 12-μm sections of heart roots freshly embedded in OCT.[16,17] Sections were first fixed in 4% paraformaldehyde for 15 minutes and then washed with PBS for 20 minutes. Sections were permeabilized with 0.1% Triton X-100 in PBS for 10 minutes. Nonspecific binding was reduced by incubating slides in 10% rabbit sera diluted in PBS for 60 minutes at room temperature. Sections were then incubated with antibodies against CD68 (1:100; MCA1957, Bio-Rad AbD Serotec), rabbit MCP1 (monocyte chemoattractant protein 1; 1:100; ab7202, Abcam), IL-6 (interleukin 6; 1:100; MCA1490, Bio-Rad AbD Serotec), TNF-α (tumor necrosis factor α; 1:100; ab6671, Abcam) or α-SMA (1:100; ab5694, Abcam)

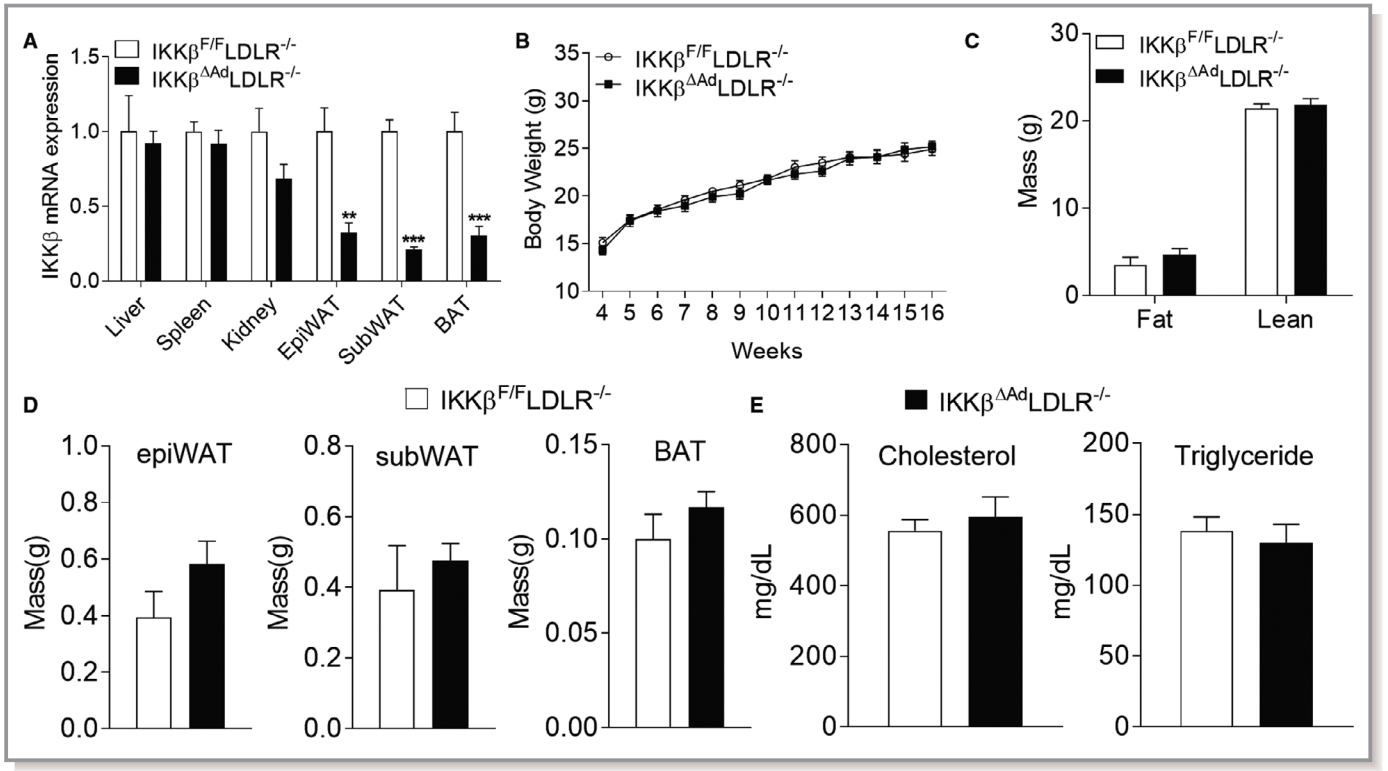

Figure 1. Deficiency of adipocyte IKKβ (inhibitor of NF-κB [nuclear factor κB] kinase β) does not affect adiposity and atherosclerosis at aortic root of lean LDLR (low-density lipoprotein receptor)–deficient ($LDLR^{-/-}$) mice fed a low-fat diet. **A**, Quantitative polymerase chain reaction analysis of IKKβ mRNA levels in major tissues of loxP-flanked IKKβ $LDLR^{-/-}$ ($IKK\beta^{F/F}LDLR^{-/-}$) and IKKβ-deficient $LDLR^{-/-}$ ($IKK\beta^{\Delta Ad}LDLR^{-/-}$) mice (n=5–7 each group; $^{**}P<0.01$ and $^{***}P<0.001$). **B**–**G**, Four-week-old male $IKK\beta^{F/F}LDLR^{-/-}$ and $IKK\beta^{\Delta Ad}LDLR^{-/-}$ littermates were fed a low-fat diet for 12 weeks. Growth curves (n=10 each group) (**B**), fat and lean mass (n=8 each group) (**C**), and fat pad weight (n=6–7 each group) (**D**) of lean $IKK\beta^{F/F}LDLR^{-/-}$ and $IKK\beta^{\Delta Ad}LDLR^{-/-}$ mice are shown. **E**, Plasma total cholesterol and triglyceride levels were measured (n=11–12 each group). **F**, Lipoprotein fractions (VLDL-C [very low-density lipoprotein cholesterol], LDL-C [low-density lipoprotein cholesterol], and HDL-C [high-density lipoprotein cholesterol]) were isolated, and the cholesterol levels of each fraction were measured (n=5 each group). **G**, Atherosclerotic lesion area at aortic root (n=9–10 each group) was also measured. Representative Oil Red O–stained sections from each genotype are displayed next to the quantification data (scale bars=500 μm). All values are mean±SEM. BAT indicates brown adipose tissue; epiWAT, epididymal white adipose tissue; subWAT, subcutaneous white adipose tissue.

at 4°C for 12 to 15 hours. Sections were rinsed with PBS and incubated with fluorescein-labeled secondary antibodies. The nuclei were stained by mounting the slides with DAPI (4′,6-diamidino-2-phenylindole) medium. Images were acquired with Nikon fluorescence microscopy. Immunohistochemical staining of adipose tissue macrophages were performed on 10-μm sections of adipose tissue embedded in paraffin. Rehydrated antigen-retrieved sections were incubated with antibodies against CD68 (1:100; MCA1957, Bio-Rad AbD Serotec) and visualized by the avidin–biotin complex method using the chromogen diaminobenzidine.

Statistical Analysis

Statistical comparisons and analysis were performed using an unpaired 2-tailed Student *t* test for data normally distributed and the Mann–Whitney test for data not normally distributed. $P<0.05$ was considered statistically significant. Data are expressed as mean±SEM (for *t* test) or median and interquartile range (for Mann–Whitney test). The differences of body weight between the 2 groups at different time points were assessed using an unpaired 2-tailed Student *t* test. Pearson correlation was used to test the interrelationships with body weight at each time point. All statistics were analyzed using GraphPad Prism v8.0 (GraphPad Software).

Results

Deficiency of Adipocyte IKKβ Does Not Affect Adiposity and Atherosclerosis in Lean $LDLR^{-/-}$ Mice When Fed a Low-Fat Diet

To investigate the role of adipocyte IKKβ signaling in atherosclerosis, we generated $LDLR^{-/-}$ mice with adipocyte-specific IKKβ deficiency by crossing $IKK\beta^{\Delta Ad}$ (Adipoq-

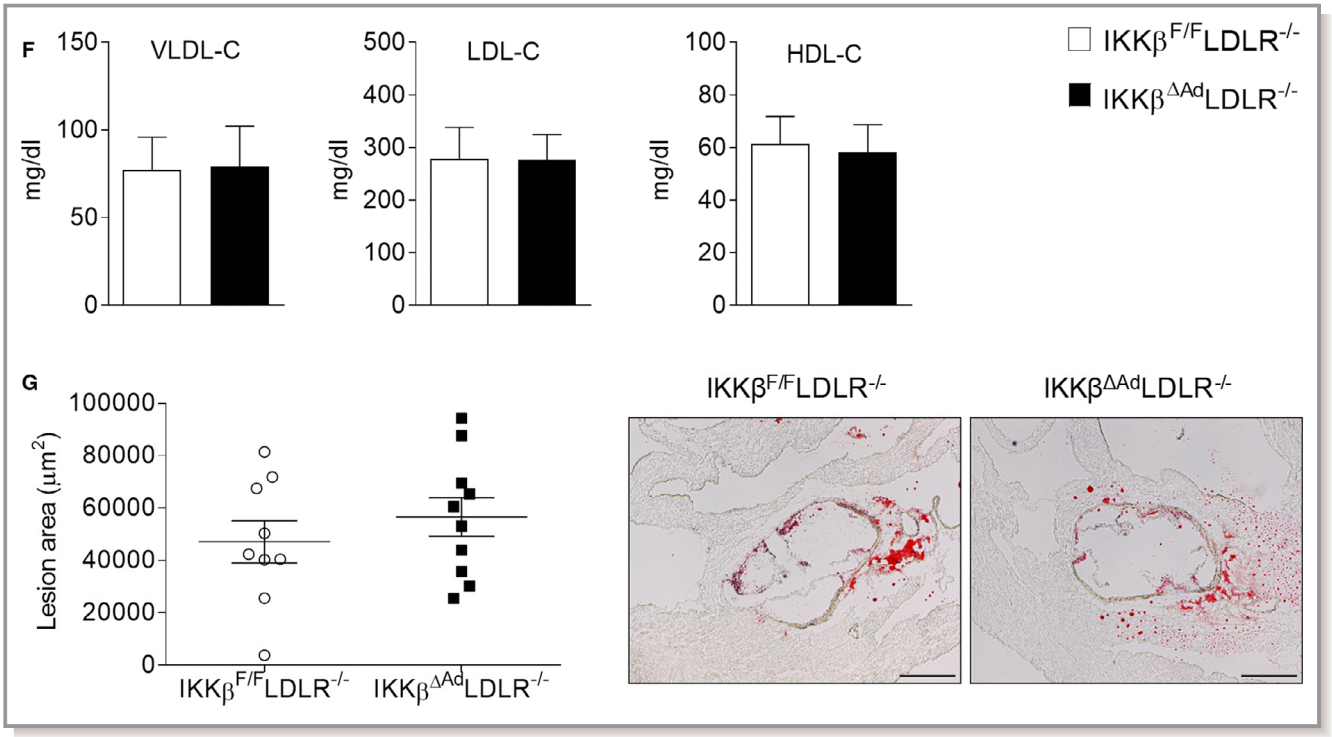

Figure 1. Continued

Cre/IKKβ$^{F/F}$) mice[22] with LDLR$^{-/-}$ mice. All mice used in this study had IKKβ$^{F/F}$LDLR$^{-/-}$ double-mutant background, and IKKβ$^{\Delta Ad}$ LDLR$^{-/-}$ mice carried heterozygous knock-in for Adipoq-Cre. Consistent with our previous report,[22] the mRNA levels of IKKβ were significantly decreased in adipose tissues, including subcutaneous WAT (subWAT), epididymal WAT (epiWAT), and brown adipose tissue (BAT), but not in other major tissues of IKKβ$^{\Delta Ad}$ LDLR$^{-/-}$ (Figure 1A).

To determine the role of adipocyte IKKβ in atherosclerosis development in a lean condition, 4-week-old male IKKβ$^{\Delta Ad}$ LDLR$^{-/-}$ and IKKβ$^{F/F}$ LDLR$^{-/-}$ littermates were fed a modified semisynthetic low-fat (4.3%) and low-cholesterol (0.02%) AIN76 diet.[37] The modified AIN76 diet has been used successfully in many studies by us and others to induce atherosclerosis in LDLR$^{-/-}$ or APOE (apolipoprotein E)–deficient mice without eliciting obesity and associated metabolic disorders.[28,29,37,38] Consistent with our previous studies on adipocyte IKKβ deficiency on C57BL/6 background,[22] deficiency of adipocyte IKKβ did not affect body weight, lean and fat mass, and the weight of subWAT, epiWAT, and BAT in lean LDLR$^{-/-}$ mice (Figure 1B–1D). In addition, deficiency of adipocyte IKKβ did not affect the plasma cholesterol and triglyceride levels in IKKβ$^{\Delta Ad}$LDLR$^{-/-}$ mice (Figure 1E). Lipoprotein fractions (VLDL, LDL, and HDL) were also isolated, and the cholesterol concentrations in all 3 fractions were measured.[27] The VLDL, LDL and HDL cholesterol levels of IKKβ$^{\Delta Ad}$LDLR$^{-/-}$ mice were also comparable with the control littermates (Figure 1F). To determine whether deletion of adipocyte IKKβ may affect systemic inflammation, we measured the plasma cytokine levels and found no difference for several important cytokines involved in atherogenesis including MCP1, TNF-α, and IL-1β between IKKβ$^{\Delta Ad}$LDLR$^{-/-}$ mice and IKKβ$^{F/F}$LDLR$^{-/-}$ littermates (Figure S1). Next, quantification of cross-sectional lesion areas at aortic root revealed that IKKβ$^{\Delta Ad}$LDLR$^{-/-}$ mice also had similar lesion size compared with IKKβ$^{F/F}$LDLR$^{-/-}$ littermates (Figure 1G). Taken together, deficiency of adipocyte IKKβ did not affect adiposity and atherosclerosis in lean LDLR$^{-/-}$ mice.

Deficiency of Adipocyte IKKβ Results in Defective Adipose Remodeling and Increased Adipose Tissue and Systemic Inflammation in Obese LDLR$^{-/-}$ Mice After HFD Feeding

We previously reported an important role of adipocyte IKKβ in regulating adaptive adipose remodeling and tissue inflammation in obese mice in responses to HFD feeding.[22] We next sought to determine whether deficiency of adipocyte IKKβ affects atherogenesis in obese LDLR$^{-/-}$ mice by feeding them a Western-type HFD for 12 weeks. Consistent with our previous report,[22] deficiency of adipocyte IKKβ did not affect total body weight but resulted in partial lipodystrophy and decreased fat mass in obese LDLR$^{-/-}$ mice (Figure 2A and

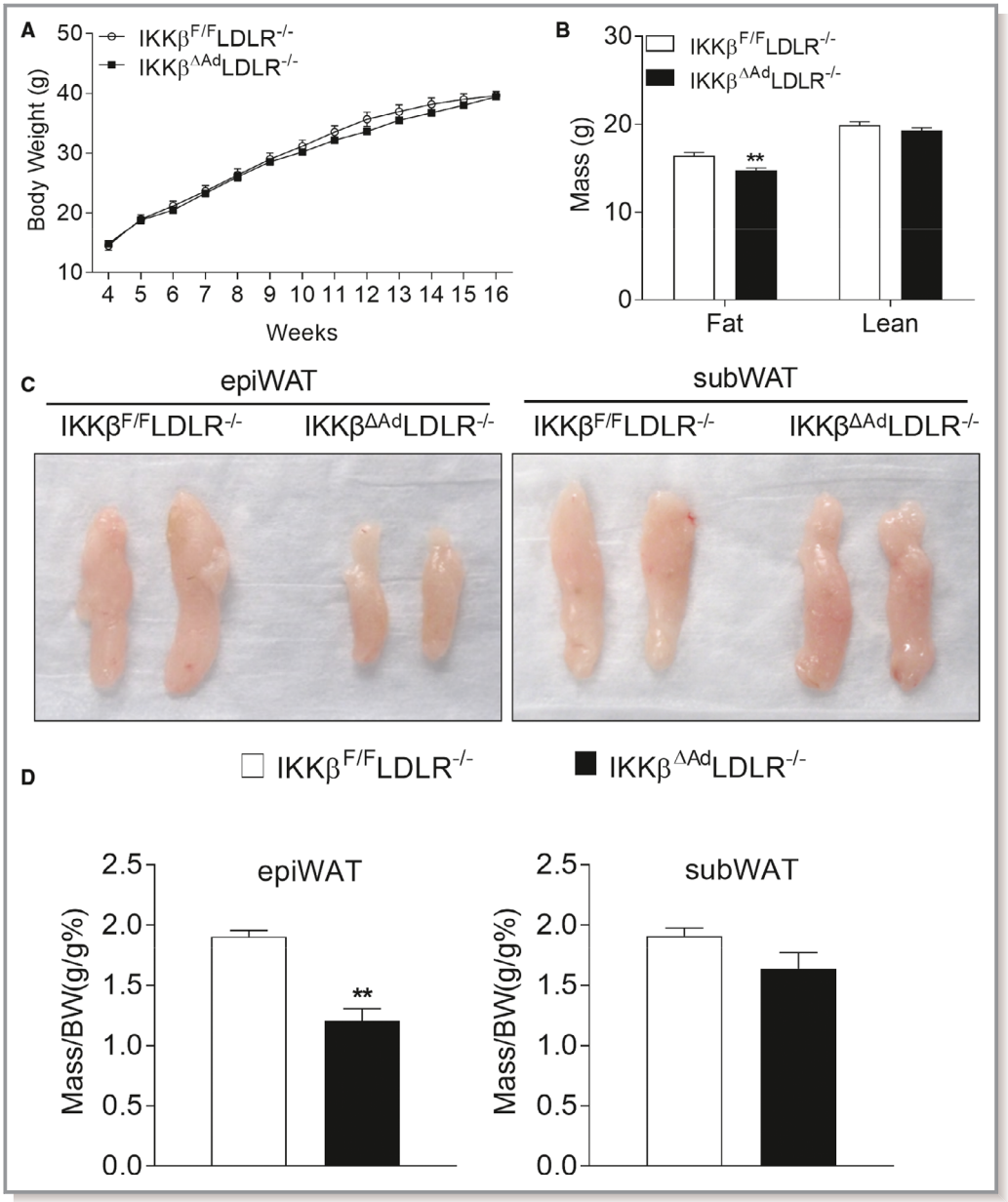

Figure 2. Visceral adipose tissue of IKKβ (inhibitor of NF-κB [nuclear factor κB] kinase β)–deficient LDLR (low-density lipoprotein receptor)–deficient ($IKK\beta^{\Delta Ad}LDLR^{-/-}$) mice fails to expand properly in response to high-fat diet (HFD) feeding. Four-week-old male loxP-flanked IKKβ $LDLR^{-/-}$ ($IKK\beta^{F/F}LDLR^{-/-}$) and $IKK\beta^{\Delta Ad}LDLR^{-/-}$ mice were fed an HFD for 12 weeks. Growth curves (n=10 each group) (**A**), fat and lean mass (n=7–11 each group; $^{**}P<0.01$) (**B**), representative photographs (**C**), and weight (n=7–10 each group; $^{**}P<0.01$) (**D**) of fat pads from obese $IKK\beta^{F/F}LDLR^{-/-}$ and $IKK\beta^{\Delta Ad}LDLR^{-/-}$ mice are shown. All values are mean±SEM. EpiWAT indicates epididymal white adipose tissue; subWAT, subcutaneous white adipose tissue.

2B). EpiWAT but not subWAT of $IKK\beta^{\Delta Ad}LDLR^{-/-}$ mice failed to expand properly in response to HFD feeding (Figure 2C and 2D). Histological examination revealed normal subWAT phenotype but severe degeneration of architecture and integrity in epiWAT of $IKK\beta^{\Delta Ad}$, indicative of defective adipose remodeling (Figure 3A). Furthermore, macrophage infiltration was substantially increased in epiWAT of $IKK\beta^{\Delta Ad}LDLR^{-/-}$ on HFD challenge (Figure 3B), suggesting increased adipose tissue inflammation under obese conditions. Indeed, the mRNA levels of macrophage markers, CD68 and F4/80, and several key proinflammatory genes including MCP1 and IL-6 were significantly elevated in epiWAT of $IKK\beta^{\Delta Ad}LDLR^{-/-}$ mice (Figure 3C). Consistent with normal morphology and histology of subWAT in $IKK\beta^{\Delta Ad}LDLR^{-/-}$ mice, deficiency of IKKβ did

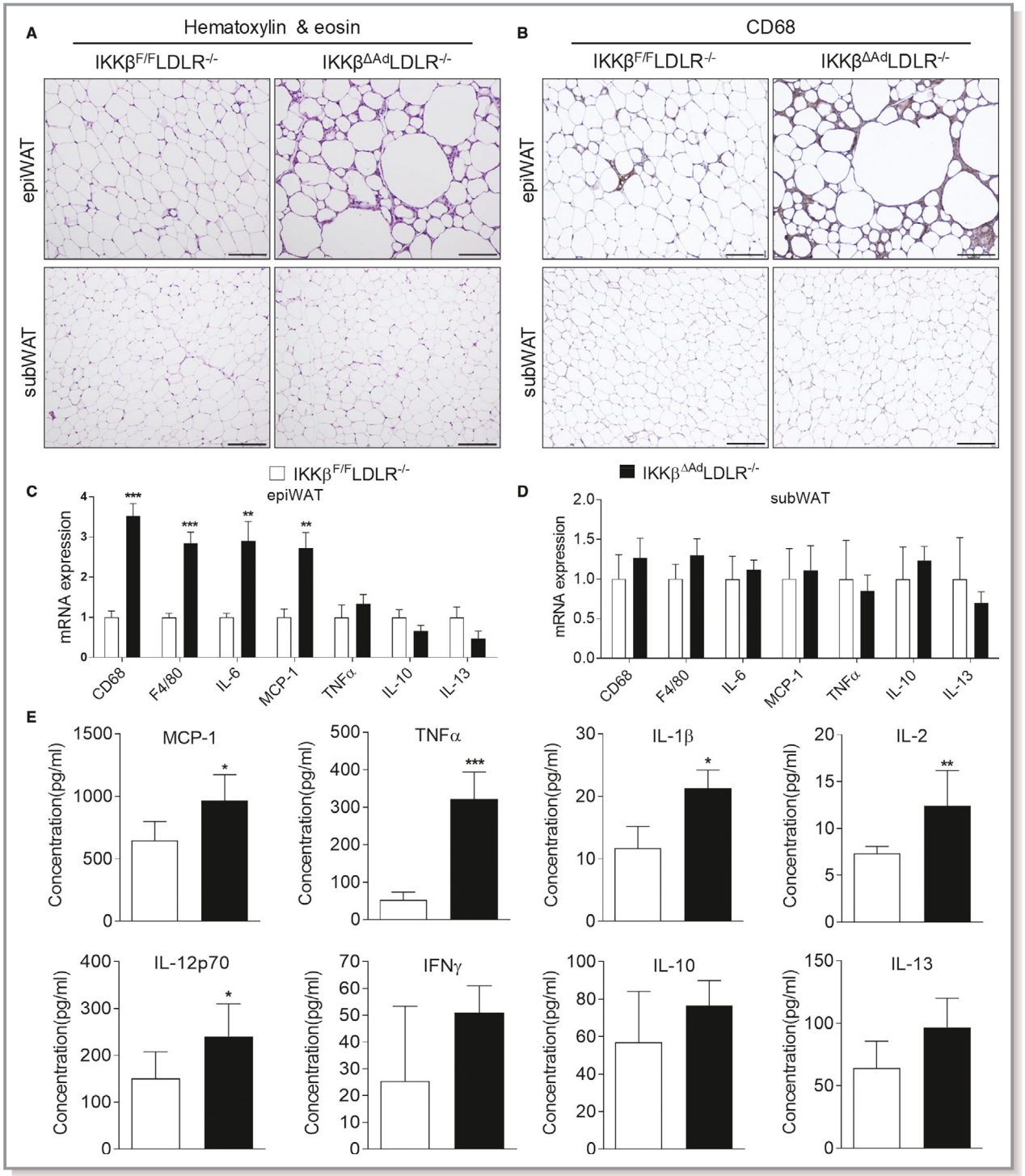

Figure 3. Deficiency of adipocyte IKKβ (inhibitor of NF-κB [nuclear factor κB] kinase β) leads to defective adipose remodeling and increased adipose tissue and systemic inflammation in obese LDLR (low-density lipoprotein receptor)–deficient ($LDLR^{-/-}$) mice. Four-week-old male loxP-flanked IKKβ $LDLR^{-/-}$ ($IKK\beta^{F/F}LDLR^{-/-}$) and IKKβ-deficient $LDLR^{-/-}$ ($IKK\beta^{\Delta Ad}LDLR^{-/-}$) mice were fed a high-fat diet for 12 weeks. Representative hematoxylin and eosin staining (**A**), immunohistochemistry for CD68 (**B**), and mRNA levels of macrophage markers and proinflammatory cytokines in epiWAT (n=5–7 each group; values are mean±SEM; $^{**}P<0.01$ and $^{***}P<0.001$) (**C**) and subWAT (n=5–6 each group; values are mean±SEM) (**D**) of obese $IKK\beta^{F/F}LDLR^{-/-}$ and $IKK\beta^{\Delta Ad}LDLR^{-/-}$ mice are shown. **E**, Plasma inflammatory cytokine levels were also measured (n=7–10 each group; values are median and interquartile range; $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$). Scale bars=200 μm. EpiWAT indicates epididymal white adipose tissue; IFN-γ, interferon γ; IL, interleukin; MCP1, monocyte chemoattractant protein 1; subWAT, subcutaneous white adipose tissue; TNF-α, tumor necrosis factor α.

not affect macrophage infiltration (Figure 3B) and inflammatory gene expression (Figure 3D) in subWAT of IKKβ$^{\Delta Ad}$LDLR$^{-/-}$ mice. In addition to subWAT and epiWAT, we measured the expression levels of several key cytokines and adipokines in periaortic adipose tissue and found that deficiency of IKKβ did not significantly affect the expression levels of these genes (Figure S2).

To determine whether deficiency of IKKβ may affect systemic inflammation in obese IKKβ$^{\Delta Ad}$LDLR$^{-/-}$ mice, we measured the plasma cytokine levels. As shown in Figure 3E, the plasma levels of several proinflammatory cytokines including MCP1 and TNF-α were also significantly increased in IKKβ$^{\Delta Ad}$LDLR$^{-/-}$ mice compared with IKKβ$^{F/F}$ LDLR$^{-/-}$ littermates (Figure 3E). Collectively, these results suggest that deficiency of adipocyte IKKβ may lead to defective adipose remodeling and accentuated inflammatory responses in obese LDLR$^{-/-}$ mice after HFD feeding.

Adipocyte IKKβ Deficiency Does Not Affect Atherosclerotic Lesion Size at Aortic Root and Brachiocephalic Artery of Obese LDLR$^{-/-}$ Mice

To determine the impact of adipocyte IKKβ deficiency on atherosclerosis in HFD-fed LDLR$^{-/-}$ mice, we analyzed the atherosclerotic lesion sizes at the aortic root and brachiocephalic artery of obese IKKβ$^{\Delta Ad}$LDLR$^{-/-}$ mice and IKKβ$^{F/F}$ LDLR$^{-/-}$ mice. Despite elevated systemic inflammation, IKKβ$^{\Delta Ad}$LDLR$^{-/-}$ mice had atherosclerotic lesion sizes similar to those of IKKβ$^{F/F}$ LDLR$^{-/-}$ mice (Figure 4A and 4B). In addition, deficiency of adipocyte IKKβ did not affect necrotic core areas in atherosclerotic lesions at the aortic root of obese LDLR$^{-/-}$ mice (Figure 4C). As expected, both IKKβ$^{\Delta Ad}$LDLR$^{-/-}$ and IKKβ$^{F/F}$LDLR$^{-/-}$ mice had diet-induced hyperlipidemia, and adipocyte IKKβ deletion did not alter plasma total cholesterol and triglyceride levels (Figure 4D) or VLDL, LDL, and HDL cholesterol levels (Figure 4E). Thus, deficiency of adipocyte IKKβ did not affect plasma lipid levels and atherosclerotic lesion sizes in obese LDLR$^{-/-}$ mice after HFD feeding.

Ablation of Adipocyte IKKβ Increases Atherosclerotic Lesional Inflammation and Plaque Vulnerability in Obese LDLR$^{-/-}$ Mice

Despite the unchanged atherosclerotic lesion size, the expression levels of several key proinflammatory proteins, including MCP1, TNF-α, and IL-6, were significantly increased in both atherosclerotic lesions and vessel walls at the aortic root of IKKβ$^{\Delta Ad}$ LDLR$^{-/-}$ mice, as indicated by immunofluorescent staining (Figure 5A–5C). This result is likely due to increased systemic inflammation. Consistently, gene expression analysis also demonstrated significantly elevated mRNA levels of proinflammatory cytokines and adhesion molecules, such as ICAM1 (intercellular adhesion molecule 1) and VCAM1 (vascular cell adhesion molecule 1), in aorta of IKKβ$^{\Delta Ad}$ LDLR$^{-/-}$ mice compared with control littermates (Figure 5D). We next sought to determine whether the enhanced inflammation affected atherosclerotic plaque stability. The phenotypic characteristics of vulnerable plaques include increased lipid-rich necrotic core size, decreased thickness of the fibrous cap, decreased plaque collagen and SMC contents, and increased macrophage contents, all of which have been used as indicators of plaque vulnerability.[5,32] Immunostaining for macrophage and SMC markers showed that macrophage contents were increased but SMC contents were decreased in the lesions of IKKβ$^{\Delta Ad}$LDLR$^{-/-}$ mice compared with IKKβ$^{F/F}$LDLR$^{-/-}$ mice (Figure 6A and 6B). Furthermore, trichrome staining showed that deficiency of adipocyte IKKβ decreased collagen contents in the atherosclerotic lesions of IKKβ$^{\Delta Ad}$LDLR$^{-/-}$ mice (Figure 6C). The contents of macrophages, SMCs, and collagens in the atherosclerotic lesions were also quantified, which confirmed the significant altered lesional contents in IKKβ$^{\Delta Ad}$LDLR$^{-/-}$ mice (Figure 6D). Based on these quantification data, the histological plaque stability scores were then calculated and confirmed that adipocyte IKKβ deficiency led to significantly decreased plaque stability scores in obese LDLR$^{-/-}$ mice (Figure 6E). In addition, we evaluated plaque calcification by alizarin red S staining, which has been associated with plaque vulnerability and an increased risk of rupture. Interestingly, deficiency of adipocyte IKKβ also resulted in increased calcification in the lesions of IKKβ$^{\Delta Ad}$LDLR$^{-/-}$ mice (Figure S3). Collectively, these results demonstrated increased atherosclerotic lesional inflammation and plaque vulnerability in obese IKKβ$^{\Delta Ad}$LDLR$^{-/-}$ mice.

Discussion

The role of IKKβ-mediated NF-κB signaling in the regulation of inflammation and immune responses has been well defined,[15,18,39,40] and IKKβ has been considered a key molecular link between obesity and metabolic diseases.[15,39,41–47] Recent studies by us and other groups have revealed complex functions of IKKβ in atherosclerosis, adipose tissue development, and metabolic disorders.[16,17,21,22,48–50] Nevertheless, it is unclear whether adipocyte IKKβ signaling may affect atherosclerosis development in response to different dietary challenges. In the current study, we generated an IKKβ$^{\Delta Ad}$LDLR$^{-/-}$ mouse model and found that deficiency of adipocyte IKKβ resulted in pathological defects in visceral adipose tissue of obese LDLR$^{-/-}$ mice. Consistent with the results from IKKβ$^{\Delta Ad}$

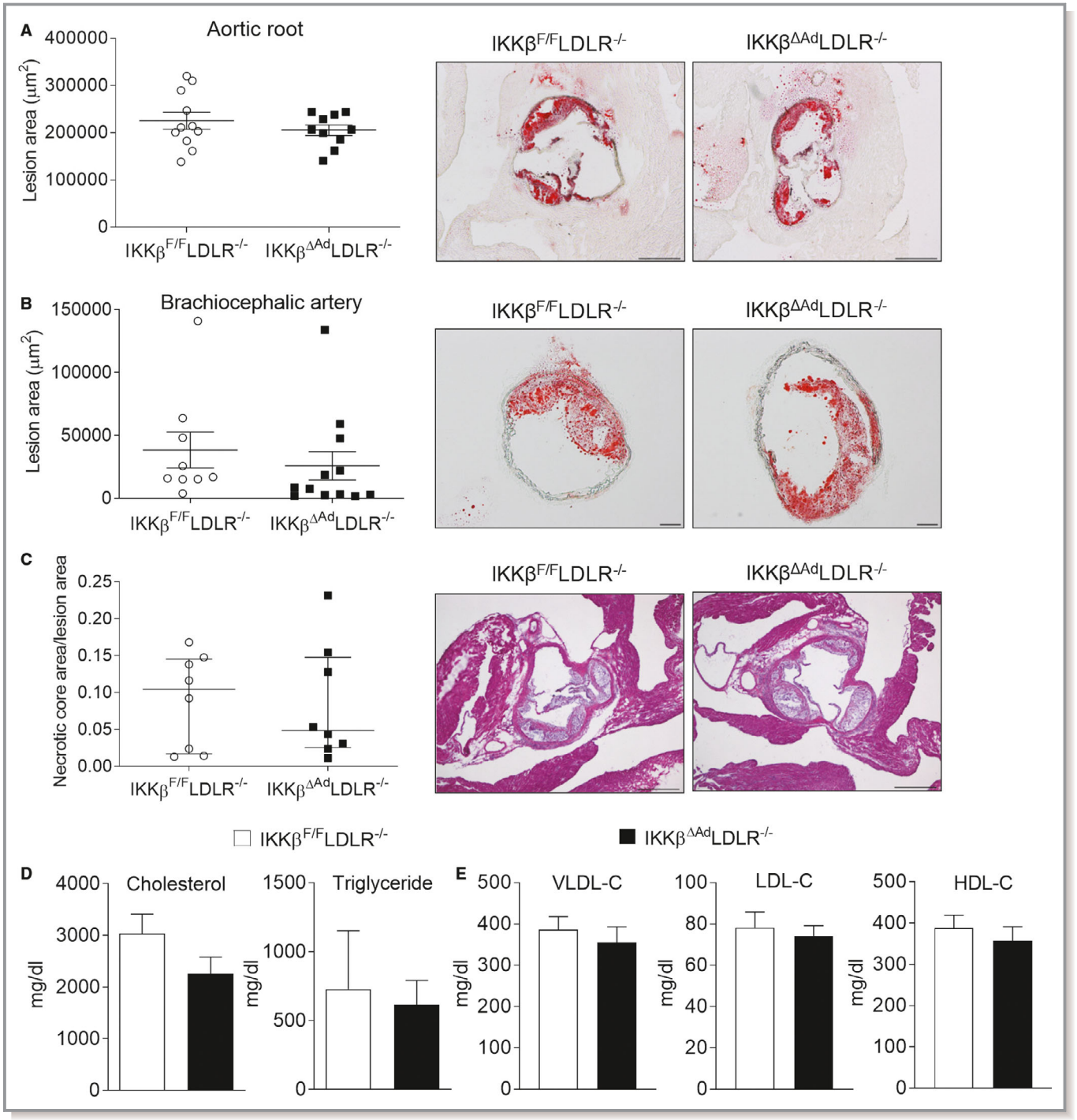

Figure 4. Adipocyte IKKβ (inhibitor of NF-κB [nuclear factor κB] kinase β) deficiency does not affect atherosclerotic lesion size and necrotic core formation at the aortic root or brachiocephalic artery of obese LDLR (low-density lipoprotein receptor)–deficient ($LDLR^{-/-}$) mice. Four-week-old male loxP-flanked IKKβ $LDLR^{-/-}$ ($IKK\beta^{F/F}LDLR^{-/-}$) and IKKβ-deficient $LDLR^{-/-}$ ($IKK\beta^{\Delta Ad}LDLR^{-/-}$) mice were fed a high-fat diet for 12 weeks. Quantification of atherosclerotic lesion area at aortic root (n=10–11 each group; values are mean±SEM; scale bars=500 μm) (**A**) and brachiocephalic artery (n=9–12 each group; values are mean±SEM; scale bars=100 μm) (**B**) of obese $IKK\beta^{F/F}LDLR^{-/-}$ and $IKK\beta^{\Delta Ad}LDLR^{-/-}$ mice are shown. Representative Oil Red O–stained sections from each genotype are displayed next to the quantification data. **C**, Quantitative analysis of necrotic core area at the aortic root of obese $IKK\beta^{F/F}LDLR^{-/-}$ and $IKK\beta^{\Delta Ad}LDLR^{-/-}$ mice (n=8 each group; values are median and interquartile range) are shown. Representative hematoxylin and eosin–stained sections from each genotype are displayed next to the quantification data (scale bars=500 μm). **D**, Plasma total cholesterol and triglyceride levels were measured (n=12–13 each group; values are median and interquartile range) are shown. **E**, Lipoprotein fractions (VLDL-C [very low-density lipoprotein cholesterol], LDL-C [low-density lipoprotein cholesterol], and HDL-C [high-density lipoprotein cholesterol]) were isolated, and the cholesterol levels of each fraction were measured (n=8 each group; values are mean±SEM).

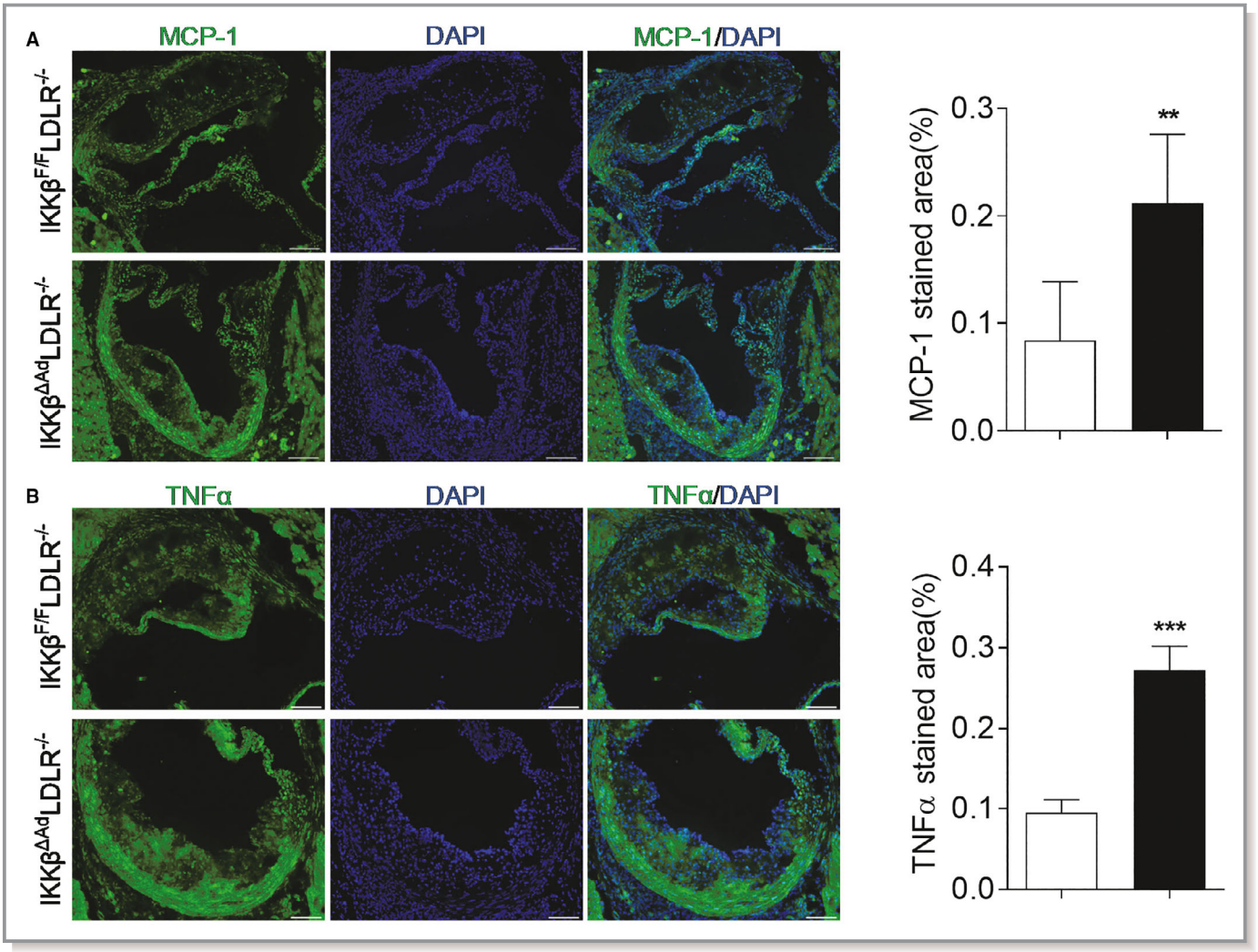

Figure 5. Ablation of adipocyte IKKβ (inhibitor of NF-κB [nuclear factor κB] kinase β) aggravates atherosclerotic lesional inflammation at aortic root of obese LDLR (low-density lipoprotein receptor)–deficient ($LDLR^{-/-}$) mice. Four-week-old male loxP-flanked IKKβ $LDLR^{-/-}$ ($IKK\beta^{F/F}LDLR^{-/-}$) and IKKβ-deficient $LDLR^{-/-}$ ($IKK\beta^{\Delta Ad}LDLR^{-/-}$) mice were fed a high-fat diet for 12 weeks. **A**–**C**, Representative images and quantification data are shown for immunofluorescence staining at the aortic root of obese $IKK\beta^{F/F}LDLR^{-/-}$ and $IKK\beta^{\Delta Ad}LDLR^{-/-}$ mice (scale bars=100 μm): (**A**) MCP1 (monocyte chemoattractant protein 1; n=5 each group; values are median and interquartile range; $^{**}P<0.01$); (**B**) TNF-α (tumor necrosis factor α; n=5 each group; values are mean±SEM; $^{***}P<0.001$); IL-6 (interleukin 6; n=5 each group; values are mean±SEM; $^{**}P<0.01$). **D**, Aortic proinflammatory gene expression was analyzed by quantitative polymerase chain reaction (n=5–6 each group; values are mean±SEM; $^{*}P<0.05$, $^{**}P<0.01$). DAPI indicates 4′,6-diamidino-2-phenylindole; ICAM1, intercellular adhesion molecule 1; VCAM1, vascular cell adhesion molecule 1.

mice,[22] obese $IKK\beta^{\Delta Ad}LDLR^{-/-}$ mice had defective adipose remodeling, increased adipose tissue, and systemic inflammation in response to HFD feeding. Although atherosclerotic lesion sizes were similar in $IKK\beta^{\Delta Ad}LDLR^{-/-}$ and control littermates, deficiency of IKKβ increased atherosclerotic lesional inflammation and plaque vulnerability in obese $IKK\beta^{\Delta Ad}LDLR^{-/-}$ mice.

Adipocytes are not only simple energy-storing cells but also secretory cells that produce proinflammatory cytokines and adipokines.[15] Many of these proinflammatory factors, including MCP1, IL-6, and TNF-α, which are regulated by IKKβ/NF-κB signaling, have been known to contribute to atherosclerosis development. As a central coordinator of inflammatory responses through activation of NF-κB, IKKβ has also been implicated in the pathogenesis of atherosclerosis in humans and animal models.[15,51] IKKβ-dependent NF-κB activation, for example, has been identified in human atherosclerotic plaques and was enhanced in unstable coronary plaques.[52,53] Animal studies have suggested that deficiency of IKKβ or inhibition of NF-κB activation in macrophages, SMCs, and endothelial cells resulted in decreased cellular inflammatory responses and atherosclerosis development.[16,17,54] However, the functions of adipose tissue IKKβ signaling in obesity and obesity-associated

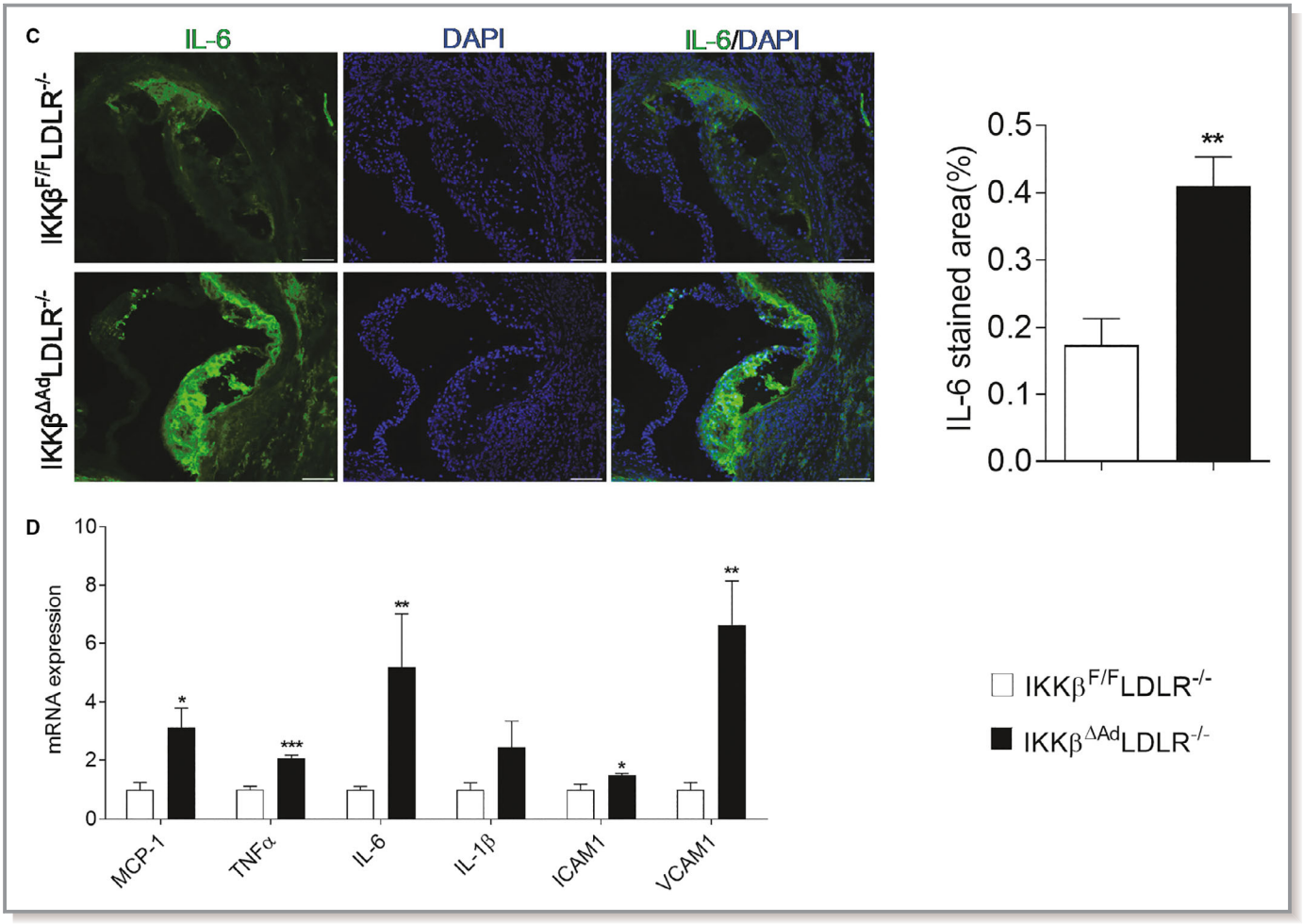

Figure 5. Continued

metabolic disorders are complex. Previous studies have investigated the role of IKKβ/NF-κB signaling in adipose tissue by overexpressing p65 or a constitutively active form of IKKβ in adipose tissue.[50,55] As expected, these transgenic mice had increased adipose tissue inflammation.[50,55] Paradoxically, these mice were also resistant to diet-induced obesity and insulin resistance.[50,55]

We recently demonstrated that IKKβ functions in both adipocyte precursor cells and mature adipocytes to regulate adipose tissue expansion and remodeling in diet-induced obesity.[17,21,22] Although IKKβ signaling in adipocyte precursor cells promotes the differentiation of those cells into mature adipocytes,[17,21,23] IKKβ is also required for mature adipocyte survival and proper adipose tissue remodeling in diet-induced obesity.[22] $IKK\beta^{\Delta Ad}$ mice that we previously generated had abnormal visceral adipose tissue characterized by marked heterogeneity in adipocyte sizes, increased adipocyte death, and accentuated inflammatory responses when challenged with an HFD.[22] Consistent with our results, another group demonstrated that deficiency of adipocyte IKKβ led to visceral adipose tissue inflammation under an HFD feeding condition.[49] Our mechanistic studies then revealed that IKKβ protects adipocytes from HFD-induced cell death; therefore, IKKβ is a key survival factor that protects adipocytes against cell death in response to HFD stress challenges.[22]

In the current study, lean $IKK\beta^{\Delta Ad}LDLR^{-/-}$ mice displayed no significant phenotype when fed a low-fat diet, and deficiency of adipocyte IKKβ did not affect atherosclerosis development in these mice. When challenged with an HFD, however, deficiency of IKKβ led to defective adipose tissue expansion, increased macrophage infiltration, and adipose tissue inflammation in obese $IKK\beta^{\Delta Ad}LDLR^{-/-}$ mice, which had phenotypes similar to those of obese $IKK\beta^{\Delta Ad}$ mice.[22] Furthermore, $IKK\beta^{\Delta Ad}LDLR^{-/-}$ mice also had elevated systemic inflammation with increased plasma levels of proatherogenic cytokines such as MCP1 and TNF-α. Expression levels of those proinflammatory cytokines were consistently increased in aorta and atherosclerotic lesions of $IKK\beta^{\Delta Ad}LDLR^{-/-}$ mice. However, we found the atherosclerotic lesion sizes at both the aortic root and brachiocephalic artery were comparable for $IKK\beta^{F/F}$ $LDLR^{-/-}$ and $IKK\beta^{\Delta Ad}LDLR^{-/-}$ littermates. It is plausible that the

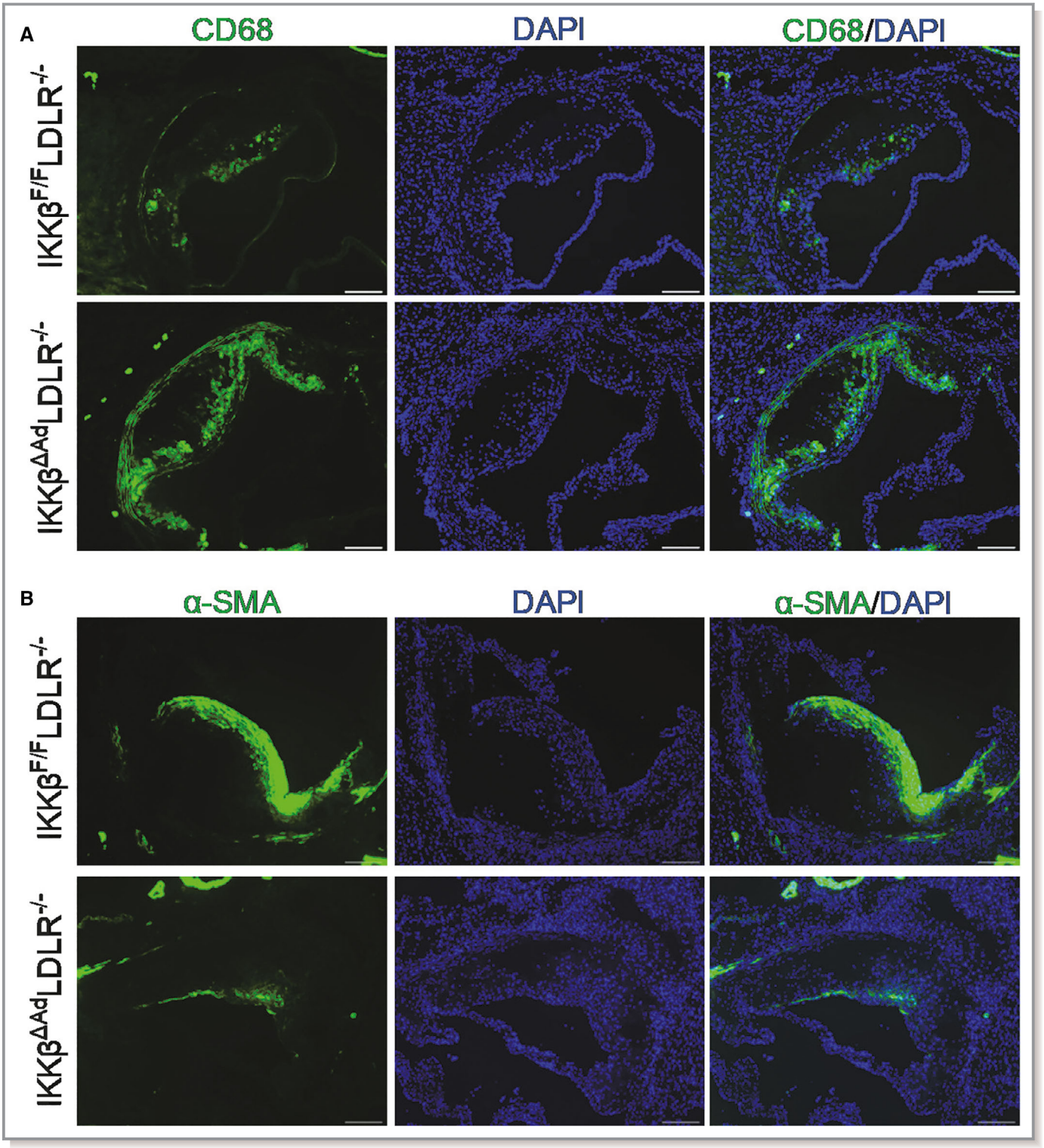

Figure 6. Adipocyte IKKβ (inhibitor of NF-κB [nuclear factor κB] kinase β) deficiency increases atherosclerotic plaque vulnerability at the aortic root of obese LDLR (low-density lipoprotein receptor)–deficient (LDLR$^{-/-}$) mice. Four-week-old male loxP-flanked IKKβ LDLR$^{-/-}$ (IKKβ$^{F/F}$LDLR$^{-/-}$) and IKKβ-deficient LDLR$^{-/-}$ (IKKβ$^{\Delta Ad}$LDLR$^{-/-}$) mice were fed a high-fat diet for 12 weeks. Representative images of immunofluorescence staining of CD68 (**A**) and α-SMA (α-smooth muscle actin) (**B**) and trichrome staining (**C**) at the aortic root of obese IKKβ$^{F/F}$LDLR$^{-/-}$ and IKKβ$^{\Delta Ad}$LDLR$^{-/-}$ mice (scale bars=100 μm). Quantitative analysis of lipid, collagen, smooth muscle cell (SMC), and macrophage contents (**D**) and plaque stability scores (**E**) at the aortic root of obese IKKβ$^{F/F}$LDLR$^{-/-}$ and IKKβ$^{\Delta Ad}$LDLR$^{-/-}$ mice (n=6–8 each group; values are mean±SEM; **P*<0.05 and ***P*<0.01). DAPI indicates 4′,6-diamidino-2-phenylindole.

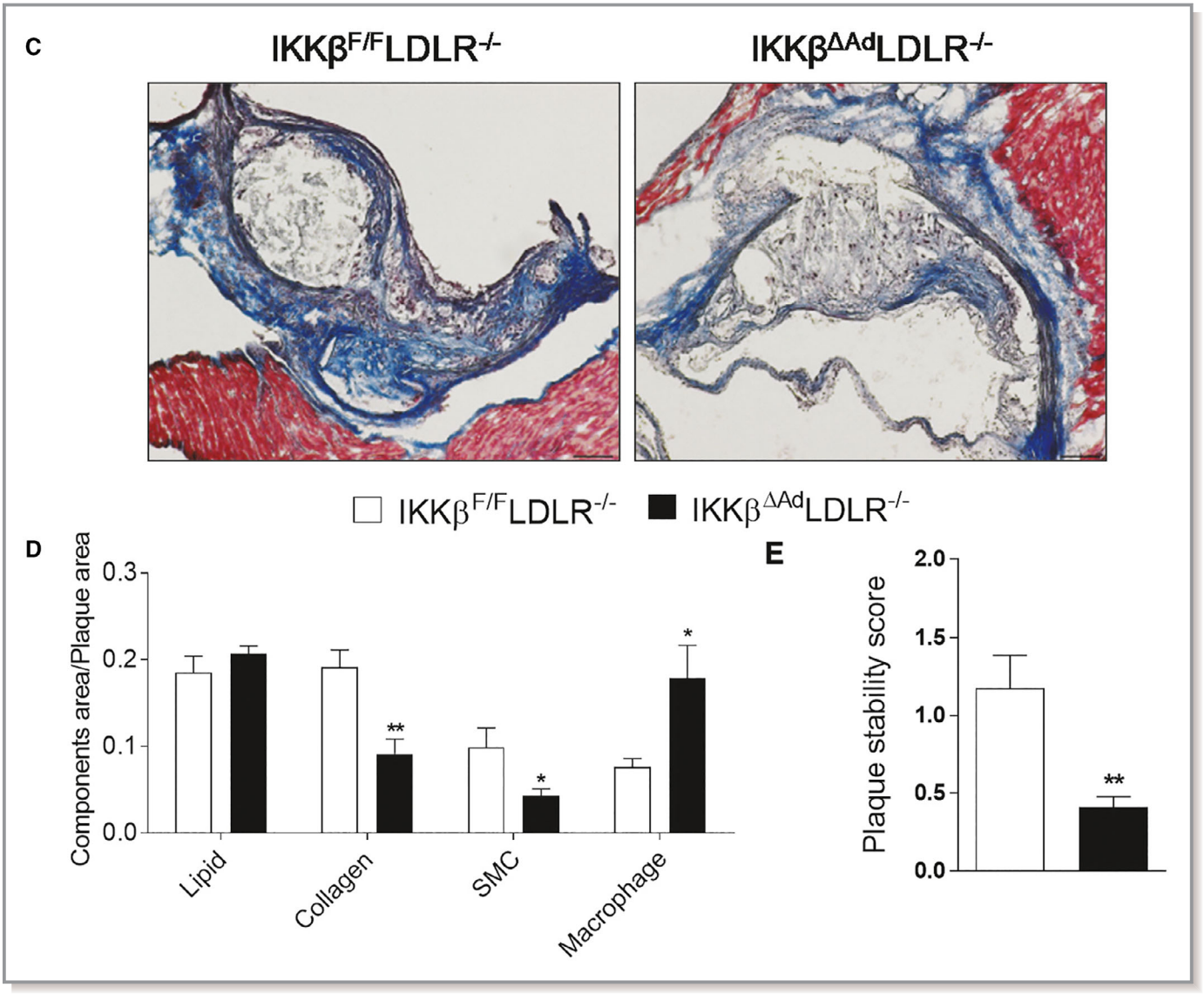

Figure 6. Continued

magnitude of the increased inflammation was not strong enough to constitute a major impact on the atherosclerotic lesion growth under our current feeding condition (12 weeks of HFD feeding for 4-week-old mice). It is also possible that a deficiency of adipocyte IKKβ may affect lesion sizes in advanced atherosclerosis in response to prolonged HFD feeding.

In addition to contributing to atherosclerotic lesion growth, inflammation may affect atherosclerotic plaque stability, plaque rupture, and thrombosis.[5,6,8,9,56] Accumulating evidence indicates that the major atherosclerosis complication is the rupture of atherosclerotic plaque,[57] and stabilizing plaque has become an important topic for atherosclerosis treatment. Atherosclerotic lesion composition rather than size has also been used to determine the plaque propensity that causes thrombotic complications.[58] Moreover, clinical studies have demonstrated that identification of high-risk unstable plaques (with morphological features of large necrotic core, higher macrophage count, positive remodeling, speckled calcium, and thin fibrous cap) on coronary imaging improves early diagnosis of acute coronary syndromes.[59,60] Inflammation can also promote the destabilization and rupture of atherosclerotic plaques by enhancing collagen degradation and macrophage recruitment and decreasing collagen synthesis.[9] Despite unchanged lesion sizes, further analysis of lesion composition demonstrated that IKKβ$^{\Delta Ad}$LDLR$^{-/-}$ mice had marked increased atherosclerotic lesional macrophage accumulation and significant reductions in lesional collagen and SMC contents—features of unstable plaques. Indeed, the histological plaque stability scores were significantly decreased in IKKβ$^{\Delta Ad}$LDLR$^{-/-}$ mice. In addition, IKKβ$^{\Delta Ad}$LDLR$^{-/-}$ mice had increased calcification in their

lesions. Accentuated inflammation has been known to increase calcium phosphate crystal formation and to initiate microcalcification, which is also associated with plaque vulnerability and an increased risk of rupture.[61] Collectively, these results demonstrated that obese IKKβ$^{\Delta Ad}$LDLR$^{-/-}$ mice had unstable atherosclerotic plaques compared with control littermates, likely due to increased inflammation in those mice.

In summary, the data in this study demonstrate that adipocyte IKKβ signaling affects the evolution of atherosclerotic plaque vulnerability in obese LDLR$^{-/-}$ mice. Deficiency of adipocyte IKKβ did not affect atherosclerotic lesion sizes but resulted in enhanced lesional inflammation and increased plaque vulnerability in HFD-fed IKKβ$^{\Delta Ad}$LDLR$^{-/-}$ mice. The results from previous studies and the current one suggest that the functions of IKKβ signaling in atherosclerosis are complex and that IKKβ in different tissues or cell types may have pro- and antiatherogenic effects in animal models when challenged with different diets. It is hoped that findings from the current study will stimulate further investigations of the contribution of cell-specific IKKβ to atherosclerosis development and plaque vulnerability and the detailed underlying mechanisms.

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This work was supported in part by National Institutes of Health (NIH) grants R01HL131925, R01HL123358, and R01ES023470 (to Zhou). The authors also acknowledge the core services (supported by NIH grant P30GM127211).

Disclosures

None.

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Supplemental Material

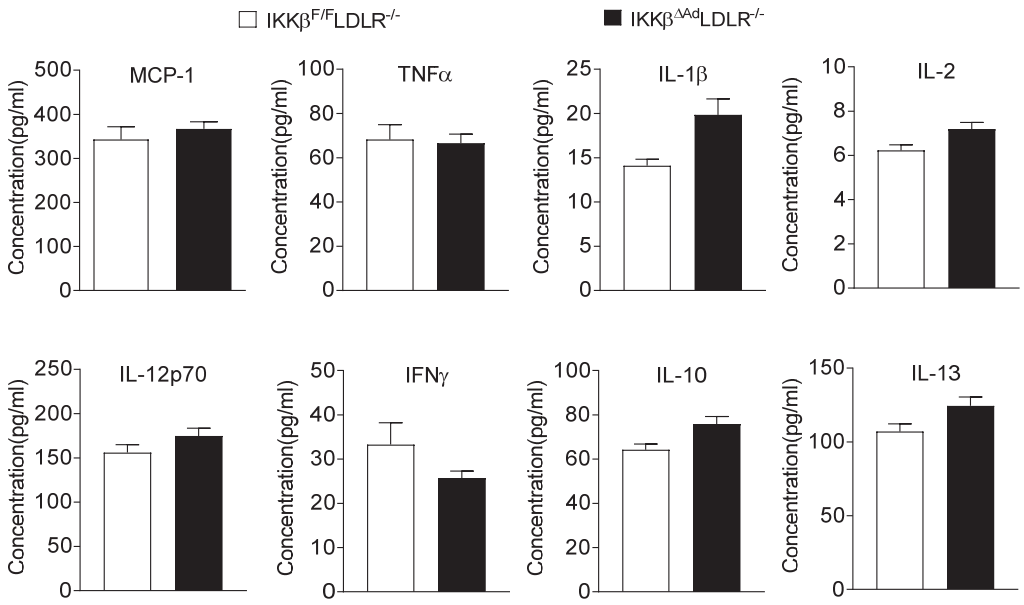

Figure S1. Deficiency of adipocyte IKKβ does not affect plasma cytokine levels in lean LDLR$^{-/-}$ mice fed a low-fat diet. Four-week-old male IKKβ$^{F/F}$LDLR$^{-/-}$ and IKKβ$^{\Delta Ad}$LDLR$^{-/-}$ littermates were fed a low-fat diet for 12 weeks and plasma cytokine levels were then measured (n=8 each group; values are mean ± SEM).

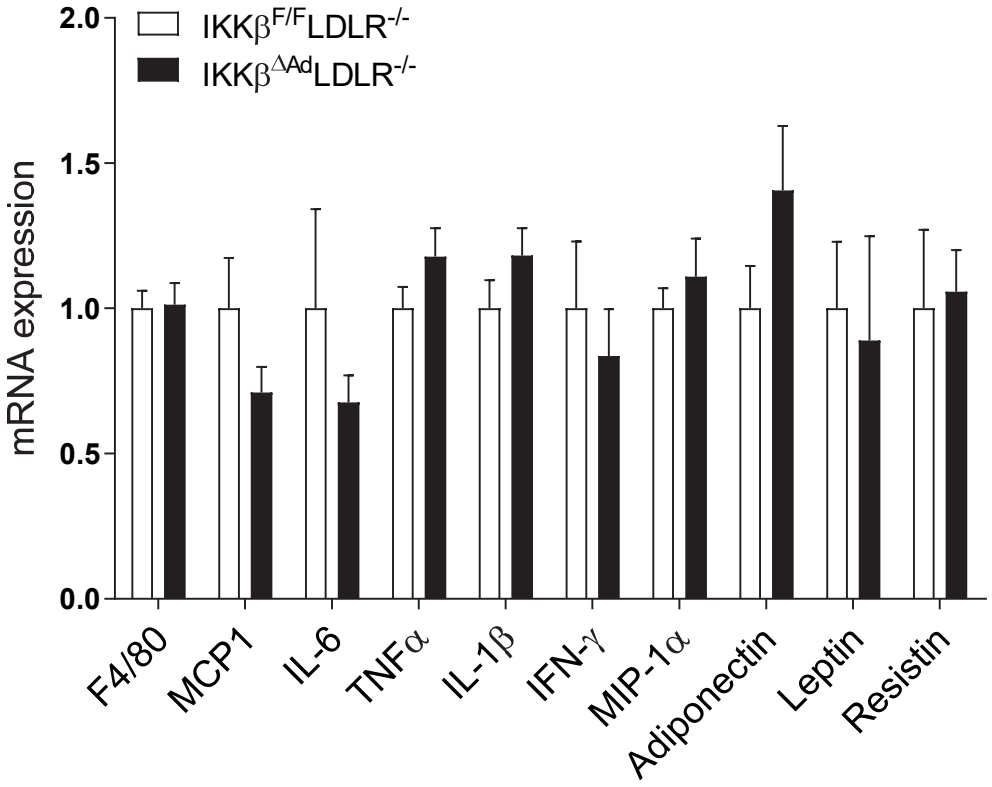

Figure S2. Deficiency of adipocyte IKKβ does not affect the expression of proinflammatory cytokines and adipokines in periaortic adipose tissue of obese LDLR$^{-/-}$ mice fed a high-fat diet.

Four-week-old male IKKβ$^{F/F}$LDLR$^{-/-}$ and IKKβ$^{\Delta Ad}$LDLR$^{-/-}$ mice were fed a high-fat diet for 12 weeks. Quantitative polymerase chain reaction analysis of mRNA levels of macrophage markers, proinflammatory cytokines, and adipokines in periaortic adipose tissue (n=6-8 each group; values are mean ± SEM.

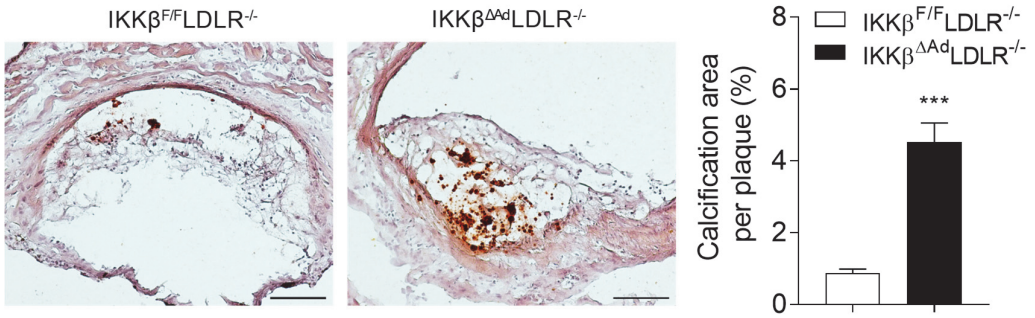

Figure S3. Adipocyte IKKβ deficiency increased calcification in the atherosclerotic lesions of aortic root of obese LDLR$^{-/-}$ mice fed a high-fat diet.

Four-week-old male IKKβ$^{F/F}$LDLR$^{-/-}$ and IKKβ$^{\Delta Ad}$LDLR$^{-/-}$ mice were fed a high-fat diet for 12 weeks. Representative images of Alizarin red S staining and quantification data of calcification area in the atherosclerotic lesions of aortic root were displayed next to the images (n=5 each group; values are mean ± SEM; ***P<.1.