Myeloid-Specific IκB Kinase β Deficiency Decreases Atherosclerosis in Low-Density Lipoprotein Receptor–Deficient Mice

Se-Hyung Park, Yipeng Sui, Florence Gizard, Jinxian Xu, Jennifer Rios-Pilier, Robert N. Helsley, Seong-Su Han, Changcheng Zhou

Objective—Inflammatory responses are the driving force of atherosclerosis development. IκB kinase β (IKKβ), a central coordinator in inflammation through regulation of nuclear factor-xB, has been implicated in the pathogenesis of atherosclerosis. Macrophages play an essential role in the initiation and progression of atherosclerosis, yet the role of macrophage IKKβ in atherosclerosis remains elusive and controversial. This study aims to investigate the impact of IKKβ expression on macrophage functions and to assess the effect of myeloid-specific IKKβ deletion on atherosclerosis development.

Methods and Results—To explore the issue of macrophage IKKβ involvement of atherogenesis, we generated myeloid-specific IKKβ-deficient low-density lipoprotein receptor–deficient mice (IKKββ–/–LDLR−/–). Deficiency of IKKβ in myeloid cells did not affect plasma lipid levels but significantly decreased diet-induced atherosclerotic lesion areas in the aortic root, brachiocephalic artery, and aortic arch of low-density lipoprotein receptor–deficient mice. Ablation of myeloid IKKβ attenuated macrophage inflammatory responses and decreased atherosclerotic lesional inflammation. Furthermore, deficiency of IKKβ decreased adhesion, migration, and lipid uptake in macrophages.

Conclusion—The present study demonstrates a pivotal role for myeloid IKKβ expression in atherosclerosis by modulating macrophage functions involved in atherogenesis. These results suggest that inhibiting nuclear factor-xB activation in macrophages may represent a feasible approach to combat atherosclerosis.

Key Words: atherosclerosis • inflammation • IκB kinase β • macrophage • nuclear factor-xB

Atherosclerosis is a chronic inflammatory disease. Many inflammatory pathways that contribute to the initiation and progression of atherosclerosis are regulated by the transcription factor nuclear factor (NF)-xB, a master regulator of innate and adaptive immune responses. The NF-xB family consists of 5 members: p65 (RelA), RelB, c-Rel, p100/p52, and p105/p50. NF-xB normally remains in the cytoplasm bound to the inhibitory protein IκB. Activating signals, such as proinflammatory cytokines, reactive oxygen species, and viral products, lead to activation of IκB kinase (IKK), which then phosphorylates IκB and promotes its degradation, allowing NF-xB to translocate to the nucleus where NF-xB can bind to the promoter regions of its target genes. IKK consists of 2 kinase subunits, IKKα and IKKβ, and a regulatory subunit NF-xB essential modulator or IKKγ. IKKβ is the predominant catalytic subunit of the IKK complex, which is required for canonical activation of NF-xB by inflammatory mediators. IKKβ-mediated NF-xB activation has been implicated in the pathogenesis of atherosclerosis. Activated NF-xB has been identified in human atherosclerotic plaques and was enhanced in unstable coronary plaques. NF-xB activation in human atherosclerosis was IKKβ-dependent and resulted in selective upregulation of major proinflammatory and prothrombotic mediators. Mapping atherosclerosis modifier loci on the apolipoprotein E–deficient (ApoE−/−) background had localized the negative regulator of NF-xB, A20 to a locus conferring sensitivity to atherosclerosis in ApoE−/− mice. Indeed, atherosclerosis was increased in A20 haploinsufficient and decreased in A20-overexpressing ApoE−/− mice. In addition, inhibiting NF-xB activity in endothelial cells (ECs) by the deletion of NF-xB essential modulator or expression of dominant-negative IκBα decreased vascular inflammation and atherosclerosis in ApoE−/− mice.

Macrophages are the major inflammatory cells involved in the progression of atherosclerosis, yet the role of macrophage IKKβ in atherosclerosis remains elusive and controversial. NF-xB–mediated inflammatory functions by macrophages have generally been considered to be proatherogenic. Interestingly, Kanter et al previously reported that low-density lipoprotein–deficient (LDLR−/−) mice transplanted with IKKβ-deficient macrophages had increased atherosclerosis. However, macrophage-specific inhibition of NF-xB activation...
by overexpressing transdominant, nondegradable forms of IκBα deletion promoted atherogenesis in LDLR−/− mice.14 Furthermore, LDLR−/− mice with hematopoietic cells deficient in the NF-κB subunit p50 also had decreased atherosclerotic lesion size.15 The inconsistent conclusions drawn from these studies suggest that further research is needed to define the role of macrophage IκKB signaling in atherosclerosis.

To explore the issue of macrophage IκKB involvement of atherogenesis, we generated myeloid-specific IκKB-deficient LDLR−/− mice (IκKBΔMye/LDLR−/−). Here, we report that myeloid IκKB deficiency protected LDLR−/− mice from diet-induced atherosclerosis, most likely because of modulated macrophage functions involved in atherogenesis.

Methods

Animals
Myeloid-specific IκKB knockout (IκKBΔMye) mice were generated by crossing mice carrying loxP-flanked IκKB alleles (IκKBΔF) with LysM-Cre transgenic mice,16 as previously described.17,18 IκKBΔF mice were kindly provided by Dr Michael Karin at the University of California, San Diego. The mice used in this study were backcrossed at least 7 additional generations onto the C57BL/6 background (>98% C57BL/6) using the marker-assisted Microsatellite Genotyping technique. To increase susceptibility to atherosclerotic lesion development, the IκKBΔMye mice were crossed to LDLR−/− mice (Jackson Laboratories) to generate IκKBΔMye/LDLR−/− and IκKBΔMye/LDLR−/+ mice. All mice used in this study had IκKBΔMye/LDLR−/+ double-mutant background, and IκKBΔMye/LDLR−/+ mice carried heterozygous knock-in for LysM-Cre. For atherosclerosis study, 4-week-old experimental male IκKBΔMye/LDLR−/− and IκKBΔMye/LDLR−/+ littermates were weaned and fed with a high-fat Western diet (21.2% fat, 0.2% cholesterol; TD 88137, Harlan Teklad) for 12 weeks until euthanization at 16 weeks of age. Body composition was measured by EchoMRI (Echo Medical System). All procedures were approved by the University of Kentucky Institutional Animal Care and Use Committee.

Blood Analysis
Plasma total cholesterol and triglyceride concentrations were determined enzymatically by a colorimetric method.19 Plasma from multiple mice (n=6) was pooled, and plasma lipoprotein cholesterol distributions were determined by fast-performance liquid chromatography.20

Atherosclerotic Lesion Analysis
Optimal cutting temperature compound–embedded hearts or brachiocephalic arteries were sectioned and stained with Oil-red-O and other atherosclerotic lesions were quantified as previously described.19 Aortas were harvested and fixed with 10% formalin, and en face analysis was performed as previously described.21

Macrophage Isolation and Function Assays
Macrophages were isolated as previously described.20,21 Bone marrow–derived macrophages (BMMs) were isolated from the femurs of mice and cultured in DMEM medium supplemented with 10% FBS, recombinant mouse macrophage colony–stimulating factor (Invitrogen) for 7 days before the experiment. Elicited peritoneal macrophages were harvested from each genotype by peritoneal lavage with PBS 3 days after intraperitoneal injection of 1 mL of 3% thioglycollate. For adhesion assay, calcium acetoxymethyl–labeled peritoneal macrophages were incubated with primary porcine ECs, and attached cells were fixed and counted.23 Migration assays were performed using transwells with 8.0-µm pore polycarbonate membrane inserts (Corning).24 Macrophages were seeded on the transwell filters, and the lower chambers were filled with either control media or media containing 500 ng/mL lipopolysaccharide (LPS). After 5 hours, cells were removed from the upper surface of the insert by scraping using Q-Tips. The membranes were fixed with 1% glutaraldehyde (Sigma), stained with hematoxylin (Leica) and mounted on the slides using glycerol gelatin. Hematoxylin-stained cells were counted under the microscope. For lipid uptake assay, macrophages were incubated with DMEM containing 100 µg/mL of oxidized LDL (Biomedical Technologies) for 24 hours, followed by washing with PBS and staining with Oil-red-O/hematoxylin.25

RNA Isolation and Quantitative Real-Time PCR Analysis
Total RNA was isolated from mouse tissues or cells using TRIzol Reagent (Life Technologies), and quantitative real-time polymerase chain reaction (PCR) was performed using gene-specific primers and the SYBR green PCR kit (Life Technologies) as previously described.19,20 The primer sets used in this study are listed in Table 1 in the online-only Data Supplement.

Statistical Analysis
All data are expressed as mean±SD. Statistically significant differences between 2 groups were analyzed by 2-tailed Student t test for data normally distributed and by the Mann-Whitney test for data not normally distributed using Prism software. A P value <0.05 was considered significant.

Results

Generation of LDLR−/− Mice With Myeloid-Specific IκKB Deficiency
To investigate the role of macrophage IκKB in atherosclerosis, we generated IκKBΔMye/LDLR−/− mice by crossing IκKBΔMye (LysM-Cre/IκKBΔF) mice with LDLR−/− mice. All mice used in this study had IκKBΔMye/LDLR−/− double-mutant background, and IκKBΔMye/LDLR−/+ mice also carried heterozygous knock-in for LysM-Cre. PCR analysis of genomic DNA indicates that the recombination was specific to the BMMs (Figure 1 in the online-only Data Supplement), consistent with previous reports that LysM-Cre–mediated gene excision almost exclusively occurs in macrophages and neutrophils.16,23 The mRNA levels of IκKB were significantly decreased in peritoneal macrophages and BMMs but not in other major tissues of IκKBΔMye/LDLR−/− mice compared with IκKBΔMye/LDLR−/+ mice (Figure 1A). Consistent with the quantitative real-time PCR results, protein levels IκKB were also substantially reduced in both peritoneal macrophages and BMMs of IκKBΔMye/LDLR−/+ mice (Figure 1B). The protein levels of IKKα were not affected in macrophages of IκKBΔMye/LDLR−/+ mice, indicating the specific deletion of IκKB (Figure 1B). To determine whether deficiency of IκKB inhibits NF-κB activity in macrophages, BMMs isolated from IκKBΔMye/LDLR−/+ and IκKBΔMye/LDLR−/+ mice were treated with NF-κB stimulator, tumor necrosis factor-α (TNFα). TNFα-induced NF-κB subunit p65 translocation from cytoplasm to nucleus was inhibited in BMMs of IκKBΔMye/LDLR−/+ mice (Figure 1C). Electrophoretic mobility shift assay confirmed that TNFα-induced DNA binding activity of NF-κB in macrophages was attenuated by IκKB deficiency (Figure 1D). It is worth noting that NF-κB activity was not completely blocked in macrophages of IκKBΔMye/LDLR−/+ mice, which may be because of the incomplete gene deletion induced by LysM-Cre/LoxP (Figure 1A and 1B). These results indicate that IκKB expression levels were significantly decreased in macrophages of IκKBΔMye/LDLR−/+ mice, and activation of NF-κB was inhibited in IκKBΔ-deficient macrophages.
Deficiency of IKKβ in Macrophages Does Not Affect Plasma Lipid Levels But Decreases Atherosclerosis in LDLR−/− Mice

To assess the impact of deficiency of macrophage IKKβ on atherosclerosis, 4-week-old male IKKβ<sup>Δ<sub>Mye</sub></sup>LDLR−/− and IKKβ<sup>F/L</sup>LDLR−/− littersmates were fed a high-fat Western diet for 12 weeks. At 16 weeks of age, no significant differences in body weight, lean mass, fat mass, and fasting glucose levels were observed in IKKβ<sup>Δ<sub>Mye</sub></sup>LDLR−/− and IKKβ<sup>F/L</sup>LDLR−/− mice (Figure II in the online-only Data Supplement). Both IKKβ<sup>Δ<sub>Mye</sub></sup>LDLR−/− and IKKβ<sup>F/L</sup>LDLR−/− mice had diet-induced hyperlipidemia, but myeloid IKKβ deficiency did not affect plasma total cholesterol and triglyceride levels (Figure 2A and 2B). Furthermore, fast-performance liquid chromatography analysis showed that IKKβ<sup>Δ<sub>Mye</sub></sup>LDLR−/− and IKKβ<sup>F/L</sup>LDLR−/− mice had similar plasma cholesterol distribution pattern (Figure 2C).

Quantification of cross-sectional lesion areas at the aortic root revealed that IKKβ<sup>Δ<sub>Mye</sub></sup>LDLR−/− mice had 25% decreased lesion area (310 900±28 310 μm<sup>2</sup>) compared with IKKβ<sup>F/L</sup>LDLR−/− littersmates (414 500±30 940 μm<sup>2</sup>; Figure 3A). In addition to aorta, deficiency of myeloid IKKβ also inhibited atherosclerosis development in the brachiocephalic artery, an artery prone to developing advanced lesions. Brachiocephalic artery cross-sectional lesion areas were decreased 51% in IKKβ<sup>Δ<sub>Mye</sub></sup>LDLR−/− mice (38 930±6571 μm<sup>2</sup>) compared with IKKβ<sup>F/L</sup>LDLR−/− mice (79 950±15 320 μm<sup>2</sup>; Figure 3B). Furthermore, en face analysis of the aortic arch also showed 32% decreased lesions in IKKβ<sup>Δ<sub>Mye</sub></sup>LDLR−/− mice (9.96±0.62% in IKKβ<sup>F/L</sup>LDLR−/− mice and 6.73±1.01% in IKKβ<sup>Δ<sub>Mye</sub></sup>LDLR−/− mice; Figure 3C). Taken together, deficiency of myeloid IKKβ significantly decreased diet-induced atherosclerosis in LDLR−/− mice.

Deficiency of IKKβ Attenuates Macrophage Inflammatory Responses and Reduces Lesional Inflammation

In atherosclerosis, macrophages participate in inflammatory responses that contribute to expansion of the subendothelial layer and lesion formation and progression. To determine
the role of IKKβ in the regulation of macrophage inflammatory responses, BMMs were isolated from IKKβ<sup>F/FLDLR<sup>−/−</sup> and IKKβ<sup>ΔMyeLDLR<sup>−/−</sup> mice and treated with endotoxin LPS. Gene expression analyses showed that the ability of LPS to induce expression of mRNAs encoding TNFα, monocyte chemotactic protein (MCP)-1, interleukin (IL)-1α, IL-1β, vascular cell adhesion molecule-1, and intercellular adhesion molecule-1 was attenuated in macrophages of IKKβ<sup>ΔMyeLDLR<sup>−/−</sup> mice (Figure 4A). These results suggest that ablation of IKKβ reduces NF-κB–regulated proinflammatory gene expression in macrophages. Indeed, gene profiling of peritoneal macrophages isolated from Western diet–fed IKKβ<sup>ΔMyeLDLR<sup>−/−</sup> mice showed downregulation of several proinflammatory molecules, including TNFα, IL-1α, IL-1β,
intercellular adhesion molecule-1, and vascular cell adhesion molecule-1 (Figure 4B). Interestingly, the expression levels of IL-10, an anti-inflammatory cytokine, were also decreased in peritoneal macrophages of IKKβΔMyeLDLR−/− mice. We also noted that the expression levels of another NF-κB target gene, CCR7,25 were significantly decreased by IKKβ deficiency. Consistent with in vitro gene expression analyses, immunofluorescence staining showed that the expression levels of several key inflammatory cytokines, including MCP-1, TNFα, and IL-1β, were decreased in the atherosclerotic lesions of IKKβΔMyeLDLR−/− mice (Figure 4C). Thus, deficiency of IKKβ attenuated macrophage inflammatory responses and reduced lesional inflammation.

Ablation of IKKβ Modulates Macrophage Functions Related to Atherosclerosis Development

One of the earliest events in atherogenesis is the entry of monocytes, the precursors of macrophages, into the arterial wall. Several NF-κB-regulated chemokines and adhesion molecules were downregulated in macrophages of IKKβΔMyeLDLR−/− mice (Figure 4). We next investigated the effects of IKKβ deficiency on macrophage adhesion and migration properties. Incubation of freshly isolated macrophages with primary ECs showed that ablation of IKKβ significantly decreased adhesion of macrophage to ECs (Figure 5A). We also examined the effects of IKKβ deficiency on macrophage migration by transwell assay. As shown in Figure 5B, IKKβ deficiency significantly decreased the migration of peritoneal macrophages. Furthermore, LPS stimulated migration of macrophages isolated from IKKβΔMyeLDLR−/− mice, and this induction was abolished in those from IKKβΔMyeLDLR−/− mice. Therefore, ablation of IKKβ decreases the macrophage adhesion and migration abilities.

Accumulation of lipid-loaded macrophages is a hallmark of atherosclerosis.26 Interestingly, oxidized LDL uptake and foam cell formation were substantially reduced in macrophages of IKKβΔMyeLDLR−/− mice compared with that of IKKβF/FLDLR−/− mice (Figure 5C and 5D), which may be attributed to the decreased type A scavenger receptor mRNA levels in IKKβ-deficient macrophages (Figure III in the online-only Data Supplement). Taken together, ablation of IKKβ decreased adhesion, migration, and lipid uptake in

![Image](https://example.com/image.png)
macrophages, which may coordinately contribute to decreased atherosclerosis in IKKβΔmyoLDLR−/− mice.

**Discussion**

Although the role of IKKβ/NF-κB pathway in inflammation and immune response has been extensively studied in the past 2 decades, the contribution of cell-type–specific NF-κB activation to atherosclerosis development is still poorly understood. In this study, we generated myeloid-specific IKKβ-deficient LDLR−/− mice to investigate the impact of macrophage IKKβ expression on atherosclerosis development. Deficiency of IKKβ in myeloid cells did not affect diet-induced hyperlipidemia but significantly decreased atherosclerosis in LDLR−/− mice (Figure 3). IKKβ deficiency decreased macrophage inflammatory responses and reduced LPS-induced proinflammatory gene expression (Figure 4). Many NF-κB-activated proinflammatory molecules were downregulated in IKKβΔmyoLDLR−/− macrophages. Furthermore, the protein levels of several proinflammatory cytokines, including MCP-1, TNFα, and IL-1β, were decreased in the atherosclerotic lesions of IKKβΔmyoLDLR−/− mice. Those molecules play important roles in atherosclerosis initiation and progression. For example, monocytes are attracted by MCP-1 to lesion-prone areas, which is considered a critical step in atherosclerosis initiation.1,2,6 Mice deficient in MCP-1 have significantly reduced atherosclerotic lesions.2 We also found that IKKβ-deficient macrophages adhered less robustly to ECs and showed impaired migratory responses compared with control macrophages (Figure 5). Therefore, the attenuated macrophage inflammatory responses and impaired adhesion and migration properties may coordinately contribute to the decreased atherosclerosis in IKKβΔmyoLDLR−/− mice.

In addition to decreased inflammatory responses, lipid uptake and foam cell formation were also substantially reduced in the macrophages of IKKβΔmyoLDLR−/− mice compared with that of IKKβFFLDLR−/− mice. The decreased lipid uptake in IKKβ-deficient macrophages may be attributed to the reduced expression levels of type A scavenger receptor that plays an important role in macrophage lipid uptake and foam cell formation.2,13 Our results are consistent with a previous report that inhibition of NF-κB activity in macrophages reduces foam cell formation.13 Deficiency of NF-κB subunit p50 was also reported to decrease type A scavenger receptor expression and reduce lipid uptake in activated macrophages.15 Whereas the in vivo effects of type A scavenger receptor deficiency on atherosclerosis development remain unclear and controversial,28–30 the decreased atherosclerosis in IKKβΔmyoLDLR−/− was very likely caused by the combined effects of decreased inflammation, adhesion, migration, and lipid uptake in macrophages.

IKKβ-dependent NF-κB activation has been implicated in vascular pathologies, and many studies have implicated that inhibition of NF-κB activation in macrophages have antatherogenic effects.2,13,14 However, Kantes et al12 previously reported that bone marrow transplantation of macrophages lacking IKKβ increased atherosclerotic lesion sizes in LDLR−/− mice. The discrepancy between our atherosclerosis results and those reported by Kantes et al12 may be attributed to differences in experimental design. Although the same LysM-Cre transgenic mice were used by both groups, Kantes et al12 used a different IKKβFF mouse model to generate IKKβΔmyo mice. In their study, 10-week-old irradiated LDLR−/− mice were transplanted with control or IKKβ-deficient macrophages from IKKβFF and IKKβΔmyo mice, respectively. After
4 weeks of recovery and 10 weeks on a high-fat diet (16% fat, 0.15% cholesterol), mice were euthanized, and the authors found that those 24-week-old LDLR−/− mice transplanted with IKKβ-deficient macrophages had increased atherosclerosis in the aortic root,12 the only location they measured athero-
sclerotic lesion size. The authors found that IKKβ deficiency inhibited LPS-induced production of IL-10, which has anti-
flammatory and antiatherogenic effects in mice. However, IKKβ-deficient macrophages also exhibited a strong reduc-
tion in TNFα production.12 Therefore, the definitive mecha-
nism for increased lesion area in LDLR−/− mice transplanted with IKKβ-deficient macrophages is unclear.

In this study, we used a different approach to explore the issue of macrophage IKKβ involvement of atherogenesis. Instead of bone marrow transplantation, we brought the well-characterized IKKβΔMβ mice17,18 onto a LDLR−/− background. Four-week-
old IKKβΔMβLDLR−/− mice and their control littersmates were fed a standard Western diet (21.2% fat, 0.2% chole-
terol) for 12 weeks. We found that IKKβΔMβLDLR−/− mice developed smaller lesions in 3 different locations, including aortic root, brachiocephalic artery, and aortic arch, compared with their control littersmates. Consistent with previous reports,17,18 IKKβ deficiency decreased macrophage inflammatory responses. Despite the decreased IL-10 expression levels, the expression levels of many NF-κB–regulated proinflammatory molecules were downregulated in IKKβΔMβLDLR−/− macrophages. Furthermore, IKKβΔMβLDLR−/− macrophages had impaired adhesion, migration, and lipid uptake properties, which may also contribute to decreased atherosclerosis. Whereas bone

marrow transplantation has been used extensively to study macrophage function in atherosclerosis, studies have found that bone marrow cells retain the potential to differentiate into a variety of nonhematopoietic cell lineages, including epithelial cells, hepatocytes, osteoblasts, chondrocytes, adipocytes, and perivascular cells.31–34 It is plausible that those progenitor cells in bone marrow of animals on different

background might affect the atherosclerosis development in LDLR−/− mice. Therefore, the methodological differences may account for the discrepant atherosclerosis outcomes between our and Kanters et al’s studies. In addition, the different diets (16% fat versus 21.2% fat), duration of high-fat feeding (10 weeks versus 12 weeks), and ages of animals (24 weeks old versus 16 weeks old) used in these studies may also contribute to the difference in atherosclerotic lesion development.

The role of IKKβ in the regulation of proinflammatory genes has been well documented. Interestingly, IKKβ-mediated NF-kB activation can also transcriptionally regulate anti-
inflammatory cytokines such as IL-10 (Figure 4B) and nega-
tively regulate IL-1β secretion in macrophages.35 For example, Greten et al35 used a high-dose LPS (30 mg/kg) to treat mice and found that mice with targeted IKKβ deletion in myeloid cells were more susceptible to endotoxin-induced shock. Consistent with our results (Figure 4A), LPS-mediated IL-1β mRNA expression levels were inhibited in IKKβ-deficient macrophages.35 However, NF-kB also controls genes such as PAI-2 and Bcl-XL, whose products inhibit caspase-1–mediated pro–IL-1β process in macrophages. Thus, NF-kB plays a dual role in the regulation of IL-1β secretion (positively regulate

IL-1β mRNA transcription and negatively regulate pro–IL-

1β process), and prolonged inhibition of IKKβ may enhance IL-1β secretion on endotoxin challenge. It is currently not clear whether IKKβ can negatively regulate IL-1β secretion in chronic inflammatory diseases such as atherosclerosis and arthritis. Our study showed that deficiency of IKKβ reduced protein levels of IL-1β and other key inflammatory mediators such as MCP-1 in atherosclerotic lesions of IKKβΔMβLDLR−/− mice (Figure 4C). Future studies are required to further understand the role of IKKβ in the regulation of IL-1β secretion under chronic inflammatory conditions.

Macrophage migration plays an essential role in atherosclerotic lesion initiation and progression. The down-

regulation of NF-kB–regulated chemokines and adhesion molecules such as CCR7, MCP-1, and intercellular adhesion molecule-1 may contribute to decreased adhesion and migration of IKKβ-deficient macrophages (Figure 5). Recent studies also demonstrated the possibility to decrease atherosclerosis by increasing macrophage emigration from atherosclerotic lesions.36,37 CCR7 has been identified to be an important chemokine for promoting the egress of macrophage from the arterial wall during atherosclerosis regression.36,37 It is possible that the decreased CCR7 expression in IKKβ-deficient macrophages may affect atherosclerosis regression. However, deficiency of CCR7 have been shown to decrease atherosclerotic plaque development in ApoE−/− mice38 and lead to suppressed monocyte recruitment and increased levels of circulating leukocytes in ApoE−/− mice during atherosclerosis regression.39 Very recently, Gils et al40 reported that other factors such as netrin-1 also regulate macrophage emigration, and targeted deletion of netrin-1 in macrophages promoted the emigration of macrophages from plaques and decreased atherosclerosis. Thus, the mechanisms that regulate atherosclerosis regression are not well understood, and it would be interesting to investigate the potential role of IKKβ in regulating macrophage emigration and atherosclerosis regression in the future.

In summary, we demonstrate that myeloid-specific IKKβ deficiency decreases atherosclerosis in LDLR−/− mice. IKKβ plays an important role in modulating macrophage func-
tions related to atherosclerosis. Ablation of IKKβ attenuated macrophage inflammatory responses and decreased macro-

phage adhesion, migration, and lipid uptake. These find-
ings suggest that inhibition of NF-kB activity in myeloid may represent a feasible therapeutic strategy to combat

atherosclerosis.

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Disclosures

None.

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Supplemental Material

Supplemental Methods

Tissue preparation
On the day of sacrifice, mice were fasted for 6 hours following the dark (feeding) cycle. Immediately prior to sacrifice the fasting plasma glucose was measured and mice were then anesthetized by intraperitoneal injection with ketamine/xylene. Mice were exsanguinated by left-ventricular puncture, and blood was collected into EDTA-containing syringes. Plasma was prepared by spinning at 16,000xg for 10 minutes. The circulation was flushed with PBS, the heart was removed and stored frozen in Tissue-Tek OCT compound as described previously. Aortas were harvested and fixed with 10% formalin for en face analysis.

Atherosclerotic lesion quantification
To quantify atherosclerosis at the aortic root, OCT-embedded hearts were sectioned and stained with Oil-red-O as described previously. The heart was oriented so that the three valves of the aortic root were in the same plane and 12 um sections were saved onto glass slides. Sections were stained with Oil-red-O. Lesion area was quantified in every fourth section, and the average was reported for five measurements. To quantify atherosclerosis at the brachiocephalic artery (BCA), the OCT-embedded BCAs were sectioned from distal to proximal at a thickness of 10 µm. Atherosclerotic lesions luminal to the internal elastic lamina were quantified in three equidistant Oil-red-O-stained sections 200, 400 and 600 µm proximal from the branching point of the BCA into the carotid and subclavian arteries. Aortas were harvested and fixed with 10% formalin and en face analysis was performed as described previously.

Western blotting
Proteins were isolated form peritoneal macrophages and BMMs by using RIPA buffer (Sigma-Aldrich) with complete mini protease inhibitors (Roche). Protein concentrations were determined by the Pierce BCA protein assay kit (Thermo scientific). 25 µg of protein was subjected to SDS-PAGE followed by electrotransfer onto nitrocellulose membrane. Anti-IKKβ, and anti-IKKα primary antibody was purchased from Cell Signaling and anti-GAPDH antibody was purchased Sigma-Aldrich. For the detection of antibody protein complexes, the SuperSignal West Pico (Pierce) was used according to the manufacturer's instructions. All blots were repeated in at least three different experiments.

Immunostaining
Immunocytochemistry was performed on cultured macrophages to measure p65 translocation. Briefly, macrophages harvested from IKKβΔMyeLDLR−/− mice and the control littermates were cultured in 8-chamber slides and treated with 20 ng/ml of TNFα or vehicle for 30 mines. Cells were then fixed with 100% acetone. Followed by washing with PBS, the cells blocked with 10% normal rabbit sera, probed with rabbit anti-mouse p65 antibody (Abcam), then detected with fluorescein-labeled secondary antibody. Immunohistochemical staining of atherosclerotic lesions were performed on 12 µm
sections of heart roots freshly embedded in OCT\textsuperscript{1,4}. Sections were first fixed in 100% ice-cold acetone for 15 min and then washed with PBS for 20 min. Sections were permeabilized with PBS + 0.1% Triton X100 (PBST) for 10 min. Nonspecific binding was reduced by incubating slides in 10% rabbit sera diluted in PBST for 20 min at room temperature. Sections were then incubated with antibodies against mouse MCP-1, TNF\(\alpha\) or IL-1\(\beta\) (Abcam) at 4°C for 12–15 h. Sections were rinsed with PBS and incubated with fluorescein-labeled secondary antibodies (Life Technologies). The nuclei were stained by mounting the slides with DAPI medium (Vector Laboratories). Images were acquired with a Nikon fluorescence microscopy (Nikon).

**Nuclear protein isolation and electrophoretic mobility shift assay (EMSA)**

Nuclear protein isolate and EMSA was performed as described before\textsuperscript{5}. Briefly, cells were treated with TNF\(\alpha\) (20 ng/ml) or vehicle for 30 min. Nuclear proteins were prepared by standard methods and aliquots were stored at –80 °C until use\textsuperscript{5}. Oligonucleotides containing consensus NF-\(\kappa\)B (Promega) was end-labeled to a specific activity of 10\textsuperscript{5} CPM with \(\gamma\)-\([\text{32P}]\)-ATP (GE Healthcare) using T4-polynucleotide kinase (Promega), followed by purification on a Nick column (GE Healthcare). The DNA-protein binding reactions were carried out in a final volume of 25 \(\mu\)l of buffer containing 50 mM Tris (pH 7.5), 500 mM NaCl, 5 mM DTT, 5 mM EDTA, 20% (w/v) glycerol, 0.4 mg/ml sonicated salmon sperm DNA, and 10 \(\mu\)g of nuclear extract on ice for 10 min. \(\text{32P}\)-labeled oligonucleotide (100,000 cpm) was then added, and the reaction was incubated at room temperature for 20 minutes. The binding complexes were subjected to electrophoresis in a 6% non-denaturing polyacrylamide gel containing 0.5 X TBE. The gels were dried and exposed to films.

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Supplemental Figure I. Myeloid-specific deletion of IKKβ. PCR analysis of genomic DNA from major organs and bone marrow-derived macrophage (BMM) of IKKβΔMyeLDLR−/− mice.
Supplemental Figure II. Four-week-old male IKKβ^{F/F}LDLR^{−/−} and IKKβ^{ΔMye}LDLR^{−/−} mice were fed WD for 12 weeks. Body weight (A), lean mass (B), fat mass (C), and fasting blood glucose levels (D) were measured in mice at 16 weeks old (n=13-15 per group).
Supplemental Figure III. Total RNAs were isolated from peritoneal macrophages of IKKβ<sup>F/F</sup>LDLR<sup>−/−</sup> and IKKβ<sup>ΔMye</sup>LDLR<sup>−/−</sup> mice. The expression levels of indicated genes were analyzed by QPCR (*P<0.05, n=5).
### Supplemental Table I

**Supplemental Table I. Primers used for Genomic PCR and QPCR.**

<table>
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<tr>
<th>Gene</th>
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<tr>
<td>IKKβ (G-PCR)</td>
<td>5'-TAGTCCAACTGGCAGCGAATAC-3'</td>
<td>IFNγ</td>
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