The Critical Role of IL-1 Receptor-Associated Kinase 4-Mediated NF-κB Activation in Modified Low-Density Lipoprotein-Induced Inflammatory Gene Expression and Atherosclerosis

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Exciting discoveries related to IL-1R/TLR signaling in the development of atherosclerosis plaque have triggered intense interest in the molecular mechanisms by which innate immune signaling modulates the onset and development of atherosclerosis. Previous studies have clearly shown the definitive role of proinflammatory cytokine IL-1 in the development of atherosclerosis. Recent studies have provided direct evidence supporting a link between innate immunity and atherogenesis. Although it is still controversial about whether infectious pathogens contribute to cardiovascular diseases, direct genetic evidence indicates the importance of IL-1R/TLR signaling in atherogenesis. In this study, we examined the role of IL-1R-associated kinase 4 (IRAK4) kinase activity in modified low-density lipoprotein (LDL)-mediated signaling using bone marrow-derived macrophage as well as an in vivo model of atherosclerosis. First, we found that the IRAK4 kinase activity was required for modified LDL-induced NF-κB activation and expression of a subset of proinflammatory genes but not for the activation of MAPKs in bone marrow-derived macrophage. IRAK4 kinase-inactive knockin (IRAK4KI) mice were bred onto ApoE−/− mice to generate IRAK4KI/ApoE−/− mice. Importantly, the aortic sinusus formation was impaired in IRAK4KI/ApoE−/− mice compared with that in ApoE−/− mice. Furthermore, proinflammatory cytokine production was reduced in the aortic sinusus region of IRAK4KI/ApoE−/− mice compared with that in ApoE−/− mice. Taken together, our results indicate that the IRAK4 kinase plays an important role in modified LDL-mediated signaling and the development of atherosclerosis, suggesting that pharmacological inhibition of IRAK4 kinase activity might be a feasible approach in the development of antiatherosclerosis drugs.

D evelopment of atherosclerosis, which is the leading cause of death in developed countries, is due to persistent chronic inflammation in the artery wall (1). Exciting discoveries related to IL-1R/TLR signaling in the development of atherosclerosis plaque have triggered intense interest in the molecular mechanisms by which innate immune signaling modulates the onset and the development of atherosclerosis. Previous studies have clearly shown the definitive role of proinflammatory cytokine IL-1 in the development of atherosclerosis. Removal of IL-1 or IL-1R reduced vascular inflammation and atherosclerosis in ApoE-deficient (ApoE−/−) mice (2, 3). Recent studies have provided direct evidence supporting a link between innate immunity and atherogenesis. Although it is still controversial about whether infectious pathogens contribute to cardiovascular diseases, direct genetic evidence indicates the importance of TLR/IL-1R signaling in atherogenesis (4). Elevated serum cholesterol is a major risk factor for the development of atherosclerosis in humans and in genetically altered mice. Macrophage scavenger receptors (SRs) that mediate the uptake of modified forms of low-density lipoproteins (LDLs) cross-talk with TLRs to modulate macrophage apoptosis, modulating inflammatory response, and atherosclerosis (5). A recent study demonstrated that modified LDL activates inflammatory signaling pathways through CD36-mediated activation of a new heterodimeric complex TLR4–TLR6 (6). TLR4 polymorphisms that map to extracellular domain of TLR4 and cause hyporesponsiveness to LPS is associated with reduced risk for carotid artery atherosclerosis (7). Importantly, TLR2-, TLR4-, and IL-1R-deficient mice displayed reduced atherosclerosis, linking elevated serum cholesterol levels to activation of IL-1R–TLR signaling pathways (4, 8, 9).

TLRs are used by the innate immune system to recognize conserved molecules associated with invading microorganisms, leading to inflammatory responses and linking to adaptive immunity (10–15). Upon binding the traditional TLR ligands (pathogens-associated molecular patterns), all of the TLRs, except TLR3, recruit the adaptor molecule MyD88 through the Toll/IL-1R domain, mediating MyD88-dependent pathways (16). MyD88
then recruits the serine–threonine kinases IL-1R–associated kinase (IRAK4) and IRAK1. IRAK4 phosphorylates IRAK1, which then mediates the recruitment of TNFR-associated factor 6 to the receptor complex (17). The IRAK1–TNFR-associated factor 6 complex then dissociates from the receptor to interact with and activate TGF-β–activated kinase 1 (TAK1) and MEK kinase 3 (MEKK3), members of the MAPK kinase kinase family (18). The activation of TAK1 and MEKK3 eventually leads to the activation of NF-κB and JNK (19), which in turn induce transcription of inflammatory cytokine and chemokine genes, such as those encoding TNF-α, IL-1β, IL-6, and IL-8. TLR3 and TLR4 also use a MyD88-independent pathway that uses TRIF to activate NF-κB and IFN regulatory factor 3. TLR4-mediated MyD88-independent activities are abolished in mice lacking TRIF-related adaptor molecule (TRAM), another adaptor in this pathway (20). Although recent studies have shown that different forms of modified LDLs can activate inflammatory signaling pathways through the activation of TLRs and SRs (6), the SR–TLR-mediated signaling pathways have not been well characterized.

IRAK4 has an essential role in TLR-mediated signaling (21, 22). IRAK4 kinase-inactive knockin (KI) mice were completely resistant to LPS- and CpG-induced shock because of impaired TLR-mediated induction of proinflammatory cytokines and chemokines (23–26). Although inactivation of IRAK4 kinase activity abolished TLR/IL-1R–mediated TAK1-dependent, but not MEKK3–dependent, NF-κB activation, a reduction of LPS-, R848-, and IL-1–mediated mRNA stability contributed to the reduced cytokine and chemokine production in bone marrow–derived macrophages (BMDMs) from IRAK4 kinase-inactive KI mice (24, 26). These in vivo studies indicate that IRAK4 kinase activity plays a critical role in TLR-dependent immune responses (27). Much effort has been devoted toward the search for IRAK4 inhibitors, with the hope of developing better anti-inflammatory therapies. Therefore, it is critical to determine the importance of IRAK4 kinase activity in different chronic inflammatory diseases. It has previously been shown that functional deficiency of IRAK4 inhibited formation of both early and advanced vascular lesions in a mouse model of physical vascular injury coupled with accelerated atherosclerosis in ApoE−/− mice (28). In the current study, we examined the importance of IRAK4 kinase activity in a mouse model of spontaneous atherosclerosis. We showed that functional deficiency of IRAK4 inhibited vascular lesion formation in ApoE−/− mice in this spontaneous atherosclerosis model. Importantly, the aortic sinus lesion formation was impaired in IRAK4KI/ApoE−/− mice compared with that in ApoE−/− mice. Furthermore, proinflammatory cytokine production was reduced in the aortic sinus region of IRAK4KI/ApoE−/− mice compared with that in ApoE−/− mice. We also began to investigate the mechanistic role of IRAK4 kinase activity in the development of atherosclerosis. We found that the IRAK4 kinase activity was required for modified LDL-induced IκB phosphorylation (NF-κB activation) and expression of a subset of proinflammatory genes but not for the activation of MAPKs in BMDMs. Interestingly, inactivation of IRAK4 kinase had no effect on modified LDL uptake and foam cell formation in BMDMs. Taken together, our results indicate that the IRAK4 kinase activity plays an important role in modified LDL-mediated signaling and the development of atherosclerosis, suggesting that pharmacological inhibition of IRAK4 kinase activity might be a feasible approach in the development of antiatherosclerosis drugs.

**Materials and Methods**

**Mice**

ApoE−/− mice (Taconic Farms) and IRAK4KI/ApoE−/− mice were described previously (28). Animals were fed a normal chow diet and maintained in a pathogen-free condition. All procedures were carried out with the approval of the Institutional Animal Care and Use Committee of Cleveland Clinic (Cleveland, OH).

**Biological reagents and cell culture**

Human LDL was isolated from the plasma of normolipidemic donors by sequential ultracentrifugation as a 1.019 g/ml fraction. Naive and modified LDL preparations were tested for possible endotoxin contamination using a Limulus amebocyte lysate kit (Cambrex, Walkersville, MD). We acetylated LDLs with sodium acetate and acetic anhydride and

![Figure 1](http://www.jimmunol.org/) Impaired acLDL-mediated gene expression in macrophages from IRAK4 kinase-inactive KI mice. A and B, BMDMs of WT and IRAK4 kinase-inactive KI mice were untreated or treated with acLDL (100 μg/ml) (A) or NO2-LDL (B) for 30 and 120 min. Total RNA was subjected to real-time PCR to measure the relative expression of TNF-α, MIP-2, and KC. Shown is the expression of TNF-α, MIP-2, and KC normalized to β-actin expression. The fold induction was calculated as compared with the expression of WT untreated cells. Data represent the mean ± SD of triplicate samples from a single experiment, and all results are representative of three independent experiments. *p < 0.05, **p < 0.01.
dialyzed and stored them at 4°C with antioxidant. NO2–LDL was prepared by incubating LDLs (0.2 mg protein/ml) at 37°C in 50 mM sodium phosphate (pH 7), 100 mM DTPA in the presence of 30 nM myeloperoxidase, 100 mg/ml glucose, 20 ng/ml glucose oxidase (grade II; Boehringer Mannheim Biochemicals, Indianapolis, IN), and 0.5 mM NaNO2 for 8 h unless otherwise specified. Preliminary studies demonstrated that under these conditions, a constant flux of H2O2 (0.18 mM/min) is generated by the GGOx system. Unless otherwise stated, oxidation reactions were terminated by addition of 40 mM butylated hydroxytoluene (from a 100 mM ethanolic stock) and 300 nM catalase to the reaction mixture. Abs against phosphorylated IkBα (Ser32/36), phosphorylated JNK, phosphorylated AKT (S473), and phosphorylated p38 were purchased from Cell Signaling Technology Abs against IkBα, and phosphorylated ERKs were purchased from Santa Cruz Biotechnology. Ab against β-actin was purchased from Sigma-Aldrich. Ab against IRAK2 was purchased from Abcam.

Cholesterol and lipoprotein analysis
Levels of total cholesterol were determined in the plasma of fasted mice by using chromatography assays (Sigma-Aldrich). A lipoprotein distribution assay was performed by fast protein liquid chromatography (FPLC). FPLC analysis of lipoproteins was done using pooled plasma from terminal bleeds.

Primary cell isolation
BMDMs were obtained from the bone marrow of tibia and femur by flushing with DMEM. The cells were cultured in DMEM supplemented with 20% FCS (FBS) and 30% L929 supernatant for 5 d.

Measurement of atherosclerotic lesions
Quantification of atherosclerotic lesions was done as described previously (28). Briefly, the hearts were stored in 10% buffered formalin for histology studies. Formaldehyde-fixed sections were stained with hematoxylin and oil red O. Atherosclerosis lesion areas were quantified using a Zeiss microscope and an image analysis system from six serial sections of each aorta. The investigator was blinded to the experimental conditions.

Illumina BeadChip microarray analysis
Two hundred fifty nanograms of RNA was reverse transcribed into cRNA and biotin-UTP labeled using the Illumina TotalPrep RNA Amplification Kit (Ambion, Austin, TX). cRNA was quantified using a nanodrop spectrophotometer, and the cRNA quality (size distribution) was further analyzed on a 1% agarose gel. cRNA was hybridized to the Illumina MouseRef8 version 1.1 Expression BeadChip using standard protocols (provided by Illumina, San Diego, CA). The microarray data were submitted to the Gene Expression Omnibus at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE24894).

FIGURE 2. Illumina mRNA expression profiling of IRAK4 kinase activity-dependent genes. A, Heat map of the genes that were induced only in WT BMDM, but not in the IRAK4 KI macrophage upon 24 h of acLDL (100 μg/ml) stimulation. B, Quantitative real-time PCR of selected genes from A. WT, IRAK4 kinase-inactive KI, and IRAK4-deficient macrophages were either untreated or stimulated with acLDL for 24 h. The fold change was calculated compared with the expression of untreated samples. Data represent the mean ± SD of triplicate samples from a single experiment, and all results are representative of two independent experiments.
Western blot analysis
Cells stimulated as indicated were harvested, washed once with PBS, and lysed for 30 min at 4°C in 1.0% Nonidet P-40, 100 mM Tris hydrochloride (pH 8), 20% glycerol, and 0.2 mM EDTA. Cellular debris was removed by centrifugation at 10,000 × g for 5 min. For immunoblotting, cell extracts were fractionated by NaDodSO₄-PAGE and transferred to Immobilon-P transfer membranes (Millipore, Bedford, MA), using a wet transfer apparatus (Bio-Rad, Hercules, CA). Immunoblot analysis was performed, and the bands were visualized with HRP-coupled goat anti-rabbit, goat anti-mouse, or donkey anti-goat Ig as appropriate (Rockland, Gilbertsville, PA), using the ECL chemiluminescence Western blotting detection system (Amersham Biosciences). Protein levels were equilibrated with the Protein Assay Reagent (Bio-Rad).

Quantitative real-time PCR
Total RNA was prepared from total aorta or BMDM of IRAK4KI and wild-type (WT) control mice with TRIZol reagent (Invitrogen). Three micrograms of total RNA was then used for reverse transcription reaction using SuperScript reverse transcriptase (Invitrogen). Quantitative PCR was performed in AB 7300 Real-Time PCR System, and the gene expression of mouse IL-6, IL-8, and β-actin was examined by SYBR GREEN PCR Master Mix (Applied Biosystems). PCR amplification was performed in triplicate, and water was used to replace cDNA in each run as a negative control. The reaction protocol included preincubation at 95°C to activate FastStart DNA polymerase for 10 min, amplification of 40 cycles that was set for 15 s at 95°C, and annealing for 60 s at 60°C. The results were normalized with the housekeeping gene mouse β-actin. Primer sequences were designed using online tools from GeneScript. The primer sequences used in this paper will be distributed upon request.

Statistical analysis
The difference between two groups was statistically analyzed by Student t test; p < 0.05 was considered significant.

Results
IRAK4 kinase activity is required for the induction of a subset of proinflammatory genes in response to modified LDLs
Our preliminary results indicated that the IRAK4 kinase activity is required for TLR/IL-1R–induced TAK1-dependent NF-κB activation and mRNA stability. Although IRAK4 kinase activity is required for TLR/IL-1R–mediated signaling, modified LDLs have been shown to activate inflammatory genes in macrophages through the coactivation of TLRs and SRs. Therefore, it is important to examine whether IRAK4 kinase activity is required for modified LDL-induced inflammatory response in macrophages.

To identify the acetylated (ac)LDL-induced–IRAK4-dependent target genes, we performed quantitative real-time PCR to measure the expression of a subset of inflammatory genes using total RNAs isolated from WT and IRAK4KI BMDMs with and without acLDL stimulation. Interestingly, acLDL-mediated proinflammatory gene expression was also greatly reduced in IRAK4 kinase-inactive KI macrophages compared with WT macrophages (Fig. 1B). To identify global changes in gene expression, we examined gene expression profiles of macrophages from WT and IRAK4

![Figure 1](http://www.jimmunol.org/DownloadedFrom)
kinase-inactive KI mice in response to acLDL stimulation using the Illumina microarray with probes for 23,000 transcripts. BMDMs from WT and IRAK4 kinase-inactive KI mice were treated with acLDL for 24 h. Importantly, we have identified a group of genes (91 genes) that were induced only in WT but not in IRAK4 kinase-inactive KI macrophages at 24 h of acLDL treatment, some of which were confirmed by real-time PCR (Fig. 2). For example, SAA1, matrix metalloproteinase (MMP)9, CXCL4 (as platelet factor 4 [PF4]), and sST2 (IL-1RL1) mRNAs were induced by acLDL stimulation in WT but not in IRAK4KI macrophages (Fig. 2). We also identified a set of IRAK4-independent genes that were similarly induced by 24 h of acLDL treatment (>1.5-fold of induction) in both WT and IRAK4 kinase-inactive KI macrophages (Fig. 3). For example, MMP8, a neutrophil collagenase, CDKN1c, a cyclin-dependent kinase inhibitor (p57), DUSP4, a dual-specific protein phosphatase, and MMP12, a macrophage metalloelastase mRNAs were similarly upregulated in WT and IRAK4 kinase-inactive KI macrophages.

TLR2 and TLR4 are partially required for acLDL-induced inflammatory gene expression

The fact that the impact of IRAK4 kinase activity on modified LDL-mediated inflammatory response is not due to the ability of the macrophages to uptake or accumulate lipid suggests acLDLs might directly activate the inflammatory signaling pathways through a specific receptor(s), which depends on IRAK4 kinase activity to signal. It has been shown that TLRs, including TLR2 and TLR4, are involved in the development of atherosclerotic plaques through the endogenous ligands, including modified LDLs. However, how TLRs participate or regulate the development of atherosclerosis remains unclear. Intriguingly, we indeed found that acLDL-mediated gene induction was substantially reduced in TLR2- or TLR4-deficient macrophages, indicating that TLR2 and/or TLR4 may play an important role in acLDL-mediated signaling pathway (Fig. 4A). Therefore, consistent with the in vivo evidence that TLRs contribute to the pathogenesis of atherosclerosis, TLRs, especially TLR2- and TLR4-mediated signaling by endogenous proteins...
ligands, such as a modified LDL, may indeed present a critical link between innate immune response and cardiovascular disease pathogenesis.

We then tested whether SRs SR-A and CD36 are required for acLDL-mediated induction in four inflammatory response genes in macrophages, because both receptors have been implicated as receptors for the uptake of acLDL. BMDMs from WT, SR-A−/−, or CD36-deficient mice were treated with acLDL. Whereas acLDL-mediated induction of these four inflammatory genes was substantially reduced in CD36-deficient macrophages, we observed partial reduction in SR-A−/−deficient macrophages compared with that in WT cells (Fig. 4B, 4C). These results suggest that CD36 is required for acLDL-induced inflammatory gene induction through cross-talk with TLR2 and/or TLR4, whereas SR-A might play a modulatory role in acLDL-mediated signaling.

**IRAK4 kinase activity is required for modified LDL-induced NF-κB activation but not for MAPK pathway activation**

To further determine the role of the kinase activity of IRAK4 in modified LDL-mediated signaling, we examined the activation of the NF-κB and MAPK pathways in WT and IRAK4 kinase-inactive KI BMDMs in response to acLDL stimulation. As shown in Fig. 5A and 5B, although acLDL-mediated phosphorylation of ERK, JNK, p38, and AKT were comparable, acLDL-induced IκBα phosphorylation was greatly reduced in BMDMs from IRAK4 kinase-inactive KI mice as compared with that in BMDMs from WT mice. acLDL-mediated NF-κB DNA-binding activity was greatly attenuated in macrophages from IRAK4 kinase-inactive KI mice as compared with that in WT mice, further supporting the importance of NF-κB activation in induction of subset of proinflammatory mediator genes (data not shown).

Moreover, CD36-specific ligand NO2−-LDL activation of the MAPK pathway, including JNK, p38, and ERK, were comparable; however, NO2−-LDL-mediated IκBα phosphorylation was greatly attenuated in macrophages from IRAK4 kinase-inactive KI as compared with WT mice (Fig. 5C, 5D). These results indicate that the kinase activity of IRAK4 is required for modified LDL-mediated NF-κB activation. Intriguingly, many of the IRAK4 kinase-dependent genes we identified above are well-known NF-κB–dependent genes (including SAA1, MMP9, sST2, and PF4), suggesting the critical role of modified LDL-induced IRAK4-dependent NF-κB activation in induction of these genes.

**Impaired aortic sinus lesion formation in IRAK4 kinase-inactive KI/ApoE−/− mice**

The above results indicate that the IRAK4 kinase activity is required for modified LDL-induced NF-κB activation and inflammatory gene expression. We then examined the role of IRAK4 kinase activity in the development and pathogenesis of atherosclerosis by using a spontaneous mouse model of atherosclerosis, the ApoE−/− mice, a well characterized mouse model of human atherosclerosis. ApoE is critical for normal metabolism of cholesterol-containing lipoproteins in mice and ApoE−/− mice spontaneously develop hypercholesterolemia and atherosclerosis, even when fed with a normal chow diet. To investigate the impact of IRAK4 kinase activity on atherosclerosis, IRAK4 kinase-inactive KI (IRAK4KI) mice were crossed with ApoE−/− mice to generate IRAK4KI/ApoE−/− mice. We first examined whether the inactivation of kinase activity of IRAK4 affects total plasma cholesterol level. The plasma cholesterol (Fig. 6A) and lipoprotein levels (Fig. 6B) were similar between female 16-wk-old IRAK4KI/ApoE−/− and ApoE−/− mice fed a normal chow diet, indicating that the disruption of IRAK4 kinase activity did not substantially affect lipid biosynthesis or metabolism. The impact of functional deficiency of IRAK4 on the development of spontaneous atherosclerosis was determined by comparing the severity of atherosclerosis of IRAK4KI/ApoE−/− with that of ApoE−/− mice at 18 wk of age on a chow diet. Atherosclerotic lesions were ~60% smaller in IRAK4KI/ApoE−/− compared with that of ApoE−/− mice (Fig. 7). The lesions, when present, consisted primarily of macrophages. Thus, functional deficiency of IRAK4 inhibited vascular lesion formation in IRAK4KI/ApoE−/− mice in a mouse model of spontaneous atherosclerosis.
Functional deficiency of IRAK4 leads to reduced proinflammatory mRNA expression in arterial tissue

ApoE<sup>−/−</sup> mice spontaneously develop hypercholesterolemia and atherosclerosis. Modified lipoproteins accumulate in the artery wall during hypercholesterolemia, setting off a cascade of proinflammatory events, particularly the expression of cytokines and chemokines that further amplify the inflammatory cascade, including recruitment and activation of macrophages. To examine the mechanism by which IRAK4 impacts on the development of spontaneous atherosclerosis in ApoE<sup>−/−</sup> mice, we analyzed the expression of proinflammatory cytokines using RNA isolated from the total aorta of ApoE<sup>−/−</sup> and IRAK4KI/ApoE<sup>−/−</sup> mice fed a normal chow diet for 20 wk. The expression of proinflammatory genes, such as MCP1, CCL4, IL-6, IFN-inducible protein 10 (IP-10), and IL-1β was indeed significantly increased in the aortic sinus region of these aged ApoE<sup>−/−</sup> mice compared with C57BL/6 WT mice (Fig. 8). Consistent with the reduced vascular lesion formation in IRAK4KI/ApoE<sup>−/−</sup> mice, the chemokine/cytokine production was impaired in the absence of IRAK4 kinase activity, suggesting IRAK4 kinase activity is required for the production of inflammatory genes (Fig. 8). Importantly, the mRNA expression of IL-1R, TLRs (TLR2, TLR1, TLR4, and TLR6) and SRs (SR-A and CD36) was similarly upregulated in both ApoE<sup>−/−</sup> and IRAK4KI/ApoE<sup>−/−</sup> aorta compared with that in C57BL/6 WT mice (Supplemental Fig. 1). Thus, the reduced inflammatory gene expression in the absence of IRAK4 kinase activity in this model is not due to downregulation of these innate immune receptors.

Inactivation of IRAK4 kinase activity has no effect on acLDL uptake and foam cell formation in macrophages

Endothelial cell injury and dysfunction is a characteristic of the formation of arterial lesions and is a prominent theory of atherosclerosis disease development. Circulating monocytes adhere to activated endothelium because of expression of adhesion molecules, where they differentiate into macrophages and accumulate lipoproteins, leading to their characteristic foam cell phenotype. Foam cells contribute to the growth and vulnerability of the atherosclerosis plaque by producing growth factors, cytokines, and MMPs and by interacting with surrounding endothelium, lymphocytes, and smooth muscle cells. Therefore, macrophage foam cell formation is the hallmark of early atherosclerosis. Because IRAK4 kinase activity is required for acLDL-induced inflammatory response in macrophages, it is important to determine whether the inactivation of IRAK4 kinase activity has any impact on acLDL uptake and foam cell formation in macrophages. BMDMs from IRAK4KI mice showed no difference in the number of acLDL-induced foam cells (detected by oil red O staining) compared with WT macrophages (data not shown), suggesting that

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**FIGURE 6.** Total cholesterol and plasma lipoprotein distribution in 16 wk-old apoE<sup>−/−</sup> or IRAK4KI/ApoE<sup>−/−</sup> mice. A, Total cholesterol in 16-wk-old ApoE<sup>−/−</sup> or IRAK4KI/ApoE<sup>−/−</sup> mice. B, FPLC elution profiles of cholesterol from ApoE<sup>−/−</sup> and IRAK4KI/ApoE<sup>−/−</sup> mice. Arrows indicate the elution peak for each lipoprotein class: very LDL (VLDL), LDL, and high-density lipoprotein (HDL).

**FIGURE 7.** Deficiency of IRAK4 kinase activity greatly reduced aortic root lesion area in ApoE<sup>−/−</sup> mice. A, Representative photographs of aortic sinus plaques from IRAK4KI/ApoE<sup>−/−</sup> (lower panel) compared with ApoE<sup>−/−</sup> (upper panel) mice. ApoE<sup>−/−</sup> and IRAK4KI/ApoE<sup>−/−</sup> mice were fed with normal chow diet for 18 wk after weaning. Cross-sections were stained with oil red O for neutral lipid (×4 objective lens). B, Lesion area in ApoE<sup>−/−</sup> and IRAK4KI/ApoE<sup>−/−</sup> mice (n = 15) on 18 wk of chow diet after weaning. Mean ± SD; p < 0.00005.
the kinase activity of IRAK4 is dispensable for acLDL-induced foam cell formation. We also measured cholesterol accumulation in macrophages upon acLDL stimulation. Consistent with foam cell formation results, BMDMs from IRAK4KI mice showed similar levels of cholesterol mass accumulation compared with that in WT cells (data not shown). These results are highly consistent with a previous study that MyD88 is required for early onset of atherosclerosis development but does not play a critical role in foam cell formation (9).

Discussion

The molecular mechanisms of atherosclerosis development are complex and still poorly understood. Recent studies revealed that TLR/IL-1R signaling is involved in the development of atherosclerotic plaques. In this study, we investigated the role of IRAK4 kinase activity in the development of atherosclerosis. NF-κB activation and NF-κB–dependent proinflammatory gene expression were abrogated in IRAK4KI macrophage upon addition of putative atherogenic ligands acLDL and NO2-LDL. IRAK4 protein, especially its kinase activity, is required for the aortic plaque formation as well as proinflammatory cytokine production important for the development of atherosclerosis. Although the total cholesterol and plasma lipoprotein distribution are comparable, IRAK4KI/ApoE−/− mice showed dramatic reduction in aortic sinus lesion size compared with that in ApoE−/− mice, indicating that the critical role of IRAK4 kinase activity in the development of atherosclerosis through the regulation of NF-κB–dependent target genes.

Emerging studies have indicated that in atherosclerosis, TLRs play a critical role in sensing deposition of modified LDLs and triggering a sterile inflammation. Removal of TLR2, TLR4, or adaptor MyD88 indeed reduced the development and pathogenesis of vascular inflammation and atherosclerosis in ApoE−/− or LDLR−/− mice (4, 8, 29). Consistent with these previous findings, we demonstrated in this study that IRAK4, a key kinase downstream of TLR–MyD88, plays an essential role in the development and pathogenesis of atherosclerosis through its kinase activity. The fact that IRAK4KI/ApoE−/− mice displayed substantial reduction in overall lesion area in the aortic root indicates that pharmacologic inhibitors of IRAK4 kinase activity might be effective in blocking atherogenesis. It has been suggested that SRs, including CD36 and SR-A, can form a complex with TLRs to uptake and mediate LDL signaling. Stewart et al. (6) recently showed that oxidized LDL, another form of modified LDL, can mediate receptor heteromerization among CD36, TLR2, and TLR4, and the IRAK4 kinase activity is required for acLDL-induced NF-κB activation and gene transcription (Supplemental Fig. 2). However, it is still not clear whether all these receptors form a huge complex or work independently. It will be very interesting to perform coimmunoprecipitation and/or colocalization experiments to show the heteromerization of these receptors if better Abs against TLRs become available in the future. Although it is clear that SR-A and CD36 are required for the uptake of modified LDLs and facilitate the activation and heterodimerization of TLRs (6), it is intriguing that CD36−/− SR-A−/−/ApoE−/− triple deficiency had minimum impact on overall lesion area in the aortic root as compared with that in ApoE−/− mice (30). However, these compound mutant mice had a substantial suppressive effect on inflammatory gene expression and plaque necrosis. These results implicate that other SRs (in addition to SR-A and CD36) might be involved in the detection of modified LDLs in vivo.
through their cross-talks with TLRs to trigger and participate in the initiation process of sterile inflammation in hyperlipidemic environment.

A previous study reported that functional deficiency of IRAK4 inhibited formation of both early and advanced vascular lesions in a mouse model of carotid ligation with accelerated atherosclerosis in ApoE−/− mice (28). This prior study combined injury with the atherogenic milieu in ApoE−/− mice, and the mechanistic role of IRAK4 kinase activity in the pathogenesis of vascular lesion formation was not addressed. In this study, we showed that functional deficiency of IRAK4 inhibited vascular lesion formation in ApoE−/− mice in a mouse model of spontaneous atherosclerosis. Importantly, we have clearly shown that IRAK4 is required for modified LDL-induced NF-κB but not MAPK activation. Consistent with this, we identified that a subset of genes regulated in an IRAK4 kinase activity-dependent manner in modified LDL-stimulated macrophages are mainly NF-κB target genes. This group of genes contains several known proteins that have a critical role in cardiovascular disease such as an atherosclerosis. For example, MMP9 is a critical regulator of macrophage migration and differentiation, whereas CXCL4, also known as PF4, is a known chemotactrant for monocytes and promotes their differentiation to uptake modified LDL or cholesterol. We also identified a group of genes regulated in an IRAK4-independent manner in aCL-LDL-stimulated macrophages. Although some of these genes might be induced through a TLR-dependent MyD88/IRAK4-independent manner, the IRAK4-independent genes might also be TLR-independent and are induced through the activation of SR-mediated signaling.

Two parallel IL-1–mediated signaling pathways have been uncovered for IL-1R/TLR–mediated NF-κB activation: TAK1 dependent and MEKK3 dependent, respectively. The TAK1-dependent pathway leads to IkB kinase (IKK)β phosphorylation and IKKβ activation, resulting in classical NF-κB activation through IkBα phosphorylation and degradation. The TAK1-independent MEKK3-dependent pathway involves IkB kinase (IKK)γ phosphorylation and IKKα activation, resulting in NF-κB activation through dissociation of phosphorylated IkBα from NF-κB without IkBα degradation. The IRAK4 kinase-inactive mutant failed to mediate IL-1R/TLR–induced TAK1-dependent NF-κB activation pathway but mediated IL-1–induced TAK1-independent NF-κB activation and retained the ability to activate substantial gene expression (31). One would predict that pharmacological blocking of IRAK4 kinase activity will leave intact some degree of host defense while reducing the levels and duration of inflammatory responses. It is intriguing that IRAK4 is required for modified LDL-induced NF-κB but not MAPK activation. However, we do not know whether the IRAK4-dependent signaling cascade induced by modified LDL uses TAK1 or MEKK3 as a central kinase to mediate NF-κB and MAPK activation. Our unpublished observations showed that the TAK1 inhibitor blocked modified LDL-induced NF-κB but not MAPK activation, which suggests that TAK1 might be important for modified LDL-induced NF-κB activation. In contrast, Yamazaki et al. (32) recently showed that the potential role of TAK1 and MEKK3 in temporally distinguished NF-κB activation pathways also points out the possibility of the requirement of both TAK1 and MEKK3 in modified LDL-mediated NF-κB activation. Because both TAK1- and MEKK3-deficient mice were embryonic lethal, cell-specific TAK1−/− and MEKK3−/− mice need to be used to determine the relative contribution of TAK1- and MEKK3-dependent signaling to the development of atherosclerosis in the future. Although the detailed signaling mechanism and physiological impact of the modified LDL-induced IRAK4-dependent or -independent pathway are still unclear, IRAK4 kinase activity is required for spontaneous development of atherosclerosis in ApoE−/− mice. Therefore, it is likely that pharmacological inhibition of IRAK4 kinase activity will be a promising strategy for antiatherosclerosis therapy.

Acknowledgments
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Disclosures
The authors have no financial conflicts of interest.

References


Supplemental Figure 1. Expression of TLRs in total aorta

At age 18 weeks, apoE-/- (n=6), IRAK4KI/apoE-/- (n=6), and control B6 mice (n=2) were sacrificed and aortas retrieved. Total RNA from aorta was analyzed by quantitative RT-PCR of specific primer pairs for TLR2, TLR1, TLR6, TLR4 and CD36.

Supplemental Figure 2. Hypothetical model of acLDL-mediated signaling pathway.

AcLDL signals through CD36, SR-A, TLR2 and TLR4 and the IRAK4 kinase activity is required for acLDL-induced NFκB activation and gene transcription, but not for the form cell formation.
Suppl Fig 2.

- AcLDL
  - TLR2/4
    - CD36 SR-A
      - MyD88
        - IRAK4
        - IRAK
          - MAPKs (JNK, ERK, p38, etc)
            - NFkB
              - MMPs
                - Cytokine/chemokines
                - Proapoptotic factors
              - Foam cell Formation