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 [1]. Aortas were harvested and fixed with 10% formalin for en face analysis [2].

Atherosclerotic lesion quantification

To quantify atherosclerosis at the aortic root, OCT-embedded hearts were sectioned and stained with Oil-red-O as described previously [1, 3]. 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 lumenal 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 [3]. Aortas were harvested and fixed with 10% formalin and en face analysis was performed as described previously [2].

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 IKK$\beta^{\Delta Mye}$LDLR$^{-/-}$ mice and the control littermates were cultured in 8-chember 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

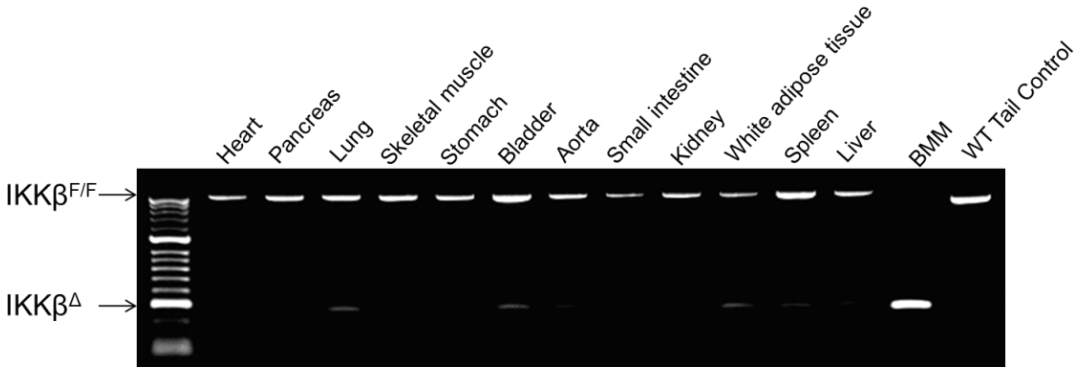
sections of heart roots freshly embedded in OCT [1, 4]. Sections were first fixed in 100% ice-cold acetone for 15 min and then washed with PBS for 20 min. Sections were permeabilized with PBS + 0.1% Triton X100 (PBST) for 10 min. Nonspecific binding was reduced by incubating slides in 10% rabbit sera diluted in PBST for 20 min at room temperature. Sections were then incubated with antibodies against mouse MCP-1, TNFα or IL-1β (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 [5]. Briefly, cells were treated with TNFα (20 ng/ml) or vehicle for 30 min. Nuclear proteins were prepared by standard methods and aliquots were stored at –80 ºC until use [5]. Oligonucleotides containing consensus NF-κB (Promega) was end-labeled to a specific activity of 10^5 CPM with γ-[^{32}P]-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 µl 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, and10 μg of nuclear extract on ice for 10 min. ^{32}P-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.

References

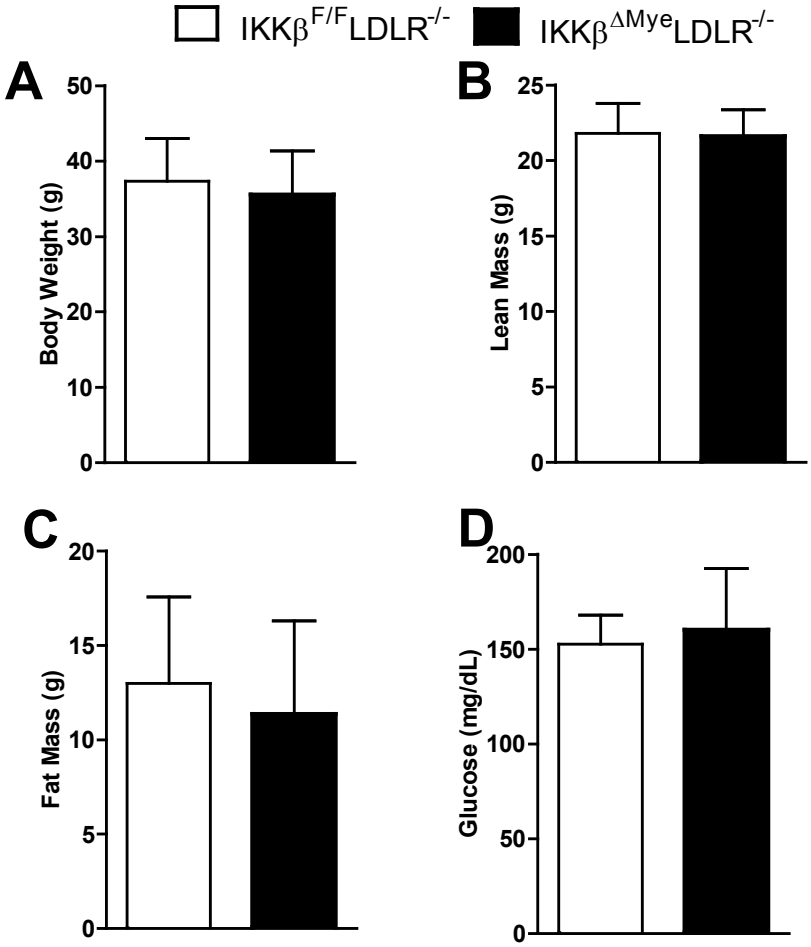
1. Zhou C, Pridgen B, King N, Xu J, Breslow JL. Hyperglycemic ins2akitaldlr-/- mice show severely elevated lipid levels and increased atherosclerosis: A model of type 1 diabetic macrovascular disease. *J Lipid Res*. 2011;52:1483-1493
2. Daugherty A, Whitman SC. Quantification of atherosclerosis in mice. *Methods Mol Biol*. 2003;209:293-309
3. Teupser D, Persky AD, Breslow JL. Induction of atherosclerosis by low-fat, semisynthetic diets in ldl receptor-deficient c57bl/6j and fvb/nj mice: Comparison of lesions of the aortic root, brachiocephalic artery, and whole aorta (en face measurement). *Arterioscler Thromb Vasc Biol*. 2003;23:1907-1913
4. Sui Y, Xu J, Rios-Pilier J, Zhou C. Deficiency of pxr decreases atherosclerosis in apoe-deficient mice. *J Lipid Res*. 2011;52:1652-1659
5. Zhou C, Tabb MM, Sadatrafiei A, Grun F, Sun A, Blumberg B. Hyperforin, the active component of st. John's wort, induces il-8 expression in human intestinal epithelial cells via a mapk-dependent, nf-kappab-independent pathway. *J Clin Immunol*. 2004;24:623-636

Supplemental Figure I



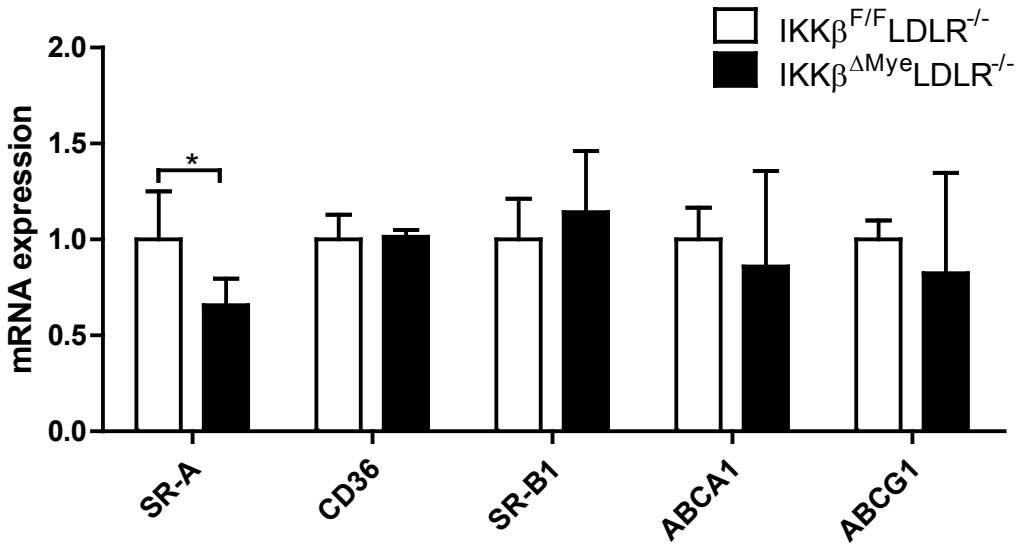
Supplemental Figure I. Myeloid-specific deletion of IKKβ. PCR analysis of genomic DNA from major organs and bone marrow-derived macrophage (BMM) of $IKK\beta^{\Delta Mye}LDLR^{-/-}$ mice.

Supplemental Figure II



Supplemental Figure II. Four-week-old male IKKβ$^{F/F}$LDLR$^{-/-}$ and IKKβ$^{\Delta Mye}$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



Supplemental Figure III. Total RNAs were isolated from peritoneal macrophages of $IKK\beta^{F/F}LDLR^{-/-}$ and $IKK\beta^{\Delta Mye}LDLR^{-/-}$ 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.

Gene	Primer sequence	Genes	Primer sequence
IKKβ	5'-TAGTCCAACTGGCAGCGAATAC-3'	IFNγ	5'-ATTGAAAGCCTAGAAAGTCTGAATAAC-3'
(G-PCR)	5'-CGCCTAGGTAAGATGGCTGTCT-3'		5'-TGGCTCTGCAGGATTTTCATG-3'
IKKβ	5'-GAGCTCAGCCCAAAGAACAG-3'	SR-A	5'- GGAGTGTAGGCGGATC-3'
	5'-AGGTTCTGCATCCCCTCTGG-3'		5'- GTCAATGGAGGCCCCA-3'
MCP-1	5'-TTAAAAACCTGGATCGGAACCAA-3'	CD36	5'- CAGTCGGAGACATGCT-3'
	5'-GCATTAGCTTCAGATTTACGGGT-3'		5'- CTCGGGGTCCTGAGTT-3'
IL-1α	5'-GCACCTTACACCTACCAGAGT-3'	SR-B1	5'- CTCATCAAGCAGCAGGTGCTCA-3'
	5'-TGCAGGTCATTTAACCAAGTGG-3'		5'- GAGGATTCGGGTGTCATGAA-3'
IL-1β	5'-GCAACTGTTCCTGAACTCAACT-3'	ABCA1	5'- CCGAGGAAGACGTGGACACCTTC-3'
	5'-ATCTTTTGGGGTCCGTCAACT-3'		5'- CCTCAGCCATGACCTGCCTTGTAG-3'
TNFα	5'-CCCATATACCTGGGAGGAGTCTTC-3'	ABCG1	5'- AGGTCTCAGCCTTCTAAAGTTCCTC-3'
	5'-CATTCCCTTCACAGAGCAATGAC-3'		5'- TCTCTCGAATGAAATTTATCG-3'
ICAM-1	5'-GTGATCCCTGGGCCTGGTG-3'	CCR7	5'- TGTACGAGTCGGTGTGCTTC-3'
	5'-GGAAACGAATACACGGTGATGG-3'		5'- GGTAGGTATCCGTCATGGTCTTG-3'
VCAM-1	5'-TACCAGCTCCCAAAATCCTG-3'	GAPDH	5'- AACTTTGGCATTGTGGAAGG-3'
	5'-TCTGCTAATTCCAGCCTCGT-3'		5'- GGATGCAGGGATGATGTTCT-3'