Supplemental Materials

Methods

Animals

Studies were done in accordance with the institutional guidelines at the University of Virginia. C57BL/6 (BL-6) mice were obtained from Jackson Laboratories. ApoE$^{-/-}$, ApoE$^{-/-}$/12/15LO$^{-/-}$(DKO), and 12/15LO-tg mice were described previously.[1-3] The12/15LO-tg/Id3$^{-/-}$ mouse was generated by crossing 12/15LO-tg and Id3$^{-/-}$ mice and the genotype of the mice was confirmed for 12/15LO overexpression by real-time RT-PCR and for 12/15LO and Id3 deficiency by PCR on genomic DNA (supplemental figure 1). Following weaning, mice were fed Chow diet (Harlan Taklad, 5.7% fat). All mice were fed a Western atherogenic diet containing 21% fat by weight (0.15% by weight cholesterol and 19.5% by weight casein without sodium cholate) from one week before injury and up to the time of euthanasia. In an attempt to minimize variation, bias and the number of animals used, all wire injury in our study was performed by a single experienced individual blinded to the genotype of the mice. Left common carotid artery (LCCA) wire injury was performed with a flexible 0.014 inch angioplasty guide wire as previously described.[4] At the time of wire injury, mice were anesthetized by intraperitoneal injection of ketamine (80 mg/kg body weight; Ketaset, Aveco, Inc.) and xylazine (8 mg/kg; AnaSed, Lloyd Laboratories). At the time of euthanasia (0 to 28 days after wire injury), animals were euthanized with an overdose of Ketamine/Xylazine. Blood samples were obtained at the time of euthanasia before induction of anesthetics via the tail vein for glucose and immediately after euthanasia from the right ventricle for lipid panel.

Real-Time RT-PCR

For total mRNA extraction, TRIzol (Invitrogen) reagent was used to digest the harvested carotids. The aqueous phase was transferred to Qiagen RNeasy Mini kit. cDNA was synthesized from mRNA using an Iscript cDNA synthesis kit (Bio-Rad). A BioRad MyIQ Single Color Real-Time PCR Detection system and IQ SYBR Green supermix were used. Data were normalized to cyclophilin as the internal control for each sample and standard curve method was used for analysis. The primers were: 12/15LO Forward: 5'–GAATCGGTACGTGGTGGGAATG–3', Reverse: 5'–ATCCTGAACAGCTTGGTCGGTCTT–3'. Cyclophilin: Forward:5'–TGGAGAGCACCAAGACAGACA–3', Reverse: 5'–TGCCGGAGTCGACAATGAT–3'. Id3: Forward: 5'–TGCTACGAGGCGGTGTGCTG–3', Reverse: 5'–TGTCGTCCAAGAGGCTAAGAGGCT–3'

Quantitative Histopathology

LCCAs were paraffin embedded. Beginning at a point 240μm proximal to the carotid bifurcation, 8 consecutive sections (each 120μm apart) were collected and stained using Russell's modified Movat method.[5] Every injury section in all groups was reviewed by a panel of scientists experienced in vascular injury who were blinded to the genotypes of the injured mice. Any animal with visible disruption of the internal elastic lamina was excluded prior to unblinding. As such, every section that is included in the analysis has intact elastic lamina. The number of animals that needed to be excluded was minimal and not significantly different among the groups. Image-Pro 3.0 software was used for quantitative histopathologic comparisons. The cell numbers in the neointimal lesion were also counted directly by two independent evaluators who were blinded to the study procedure.

Immunohistochemistry

Slide sections were selected at matching distances from the carotid bifurcation in all animals (2 sections for fibronectin staining, four sections for Ki-67 staining). The slides were rehydrated using a graded alcohol series. The sections were stained using the Vectastain Elite ABC kit (Vector Labs) as follows: Sections were blocked for 60 minutes in horse serum (Vector Labs) for Ki-67 staining and in goat serum (Atlanta Biologicals) for fibronectin staining. They were then incubated overnight at 4°C with a 1:100 dilution of a goat polycolonal anti-Ki-67 antibody (Santa Cruz Biotechnology) or a 1:100 rabbit polyclonal anti-fibronectin antibody (Sigma). Slides were washed twice with PBS and incubated with a 1:100 dilution of biotinylated anti-rabbit and anti-goat antibodies (Vector Labs) for 1 hour at room temperature. Slides were then incubated for 30 minutes with Vectastain Elite ABC Reagent at room temperature. Slides were washed twice with PBS and incubated with DAB peroxidase substrate (Sigma) for 5 minutes, then counterstained with hematoxylin, dehydrated through a graded alcohol series, and mounted using Cytoseal XYL (Richard-Allan Scientific). Expression of Fibronectin or Ki-67 was determined by microscopic observation of the diaminobenzidine reaction product on the analyzed sections. Images were digitized through an Olympus (BH-2) microprojection system with a Dage-MTI DC-330 color camera (Dage-MTI) and analyzed using Image-Pro software. The number of cells stained for Ki-67, and the area stained for fibronectin were determined by two reviewers who were blinded to the genotypes.

Cell Culture

Primary thoracic aortic VSMCs were isolated from BL-6, Id3$^{-/-}$, 12/15LO-tg, and 12/15LO-tg/Id3$^{-/-}$, ApoE$^{-/-}$, and DKO mice and grown in DMEM F12 containing 10% FBS. Cells were studied during passages 7 through 9. To determine cell proliferation, VSMCs from

the BL-6, Id3$^{-/-}$, 12/15LO-tg, 12/15LO-tg/Id3$^{-/-}$, ApoE$^{-/-}$, and DKO mice were plated on 6cm plates at equal densities. Twelve hours after plating (defined as the baseline), and then four times at 24-hour intervals, cells were detached from the plates by trypsinization and VSMCs were counted directly using a hemocytometer.

Chromatin Immunoprecipitation (ChIP)

ChIP assays were performed as previously described[6] using primary VSMCs isolated from aorta's of12/15LO-tg and 12/15LO-tg/Id3$^{-/-}$ mice. Assays were done on at least three independent experiments. 1 × 10^7 VSMCs were cross-linked with 1% formaldehyde per condition. Cell or tissue suspensions were sonicated and approximately 100µg of the clarified extracts were incubated overnight with either 5µg of antibody against ITF-2b (Abnova Corporation), RNA polymerase II (Abcam Inc.), or IgG control sera (Isotype). β-Galactosidase plasmid (β-gal) was added to the elution buffer to allow for correction of unequal precipitation efficiencies. Immunoprecipitated DNA fragments were analaysed by real-time RT-PCR using the threshold cycle methodology. Recovery of the mouse p21^{cip1} promoter was determined in triplicate using forward (5'-CTCCTACTTCTGTGGACATCA-3') and reverse (5'- CGGGTC ACTATGGAAACTAC-3') primer sequences.

p21^{cip1} Promoter-Reporter Analysis

The full length human ITF-2b was amplified by PCR and subcloned into the pEF4 expression vector. The human p21^{cip1} promoter containing -2.3 Kb of promoter sequence from the transcription start site was subcloned into pGL3 basic vector. In each well, 1X10^5 passage 7-9 primary VSMCs that were isolated from thoracic aorta's of BL-6 and Id3$^{-/-}$ mice were transfected with 0.5µg of pGL3-p21^{cip1} promoter-reporter construct and various concentrations (0, 0.25, and 0.5µg) of pEF4-ITF-2b expression

plasmid using Effectene Transfection Reagent (Qiagen). The total DNA concentration per well was brought up to 1µg by adding appropriate empty vectors. Twenty-four hours after transfection, cells were harvested for luciferase activity and protein. Luciferase was measured using the Luciferase Assay Kit (Promega) and protein was determined using the BCA Protein Assay Kit (Thermo Scientific) according to the manufacturers' instructions. Experiments were done three times in triplicate.

Statistical analysis

Statistical analyses were performed using PRISM 4 (GraphPad). Mann-Whitney U-test was used to compare continuous variables between groups and p-values <0.05 were considered significant. Analysis of Variance (ANOVA) was performed to evaluate the differences between multiple groups on continuous variables. Data is shown as Mean ± SD.

Supplemental References

1. Cyrus T, Witztum JL, Rader DJ, Tangirala R, Fazio S, Linton MF, Funk CD. Disruption of the 12/15-lipoxygenase gene diminishes atherosclerosis in apo E-deficient mice. *J Clin Invest.* 1999;103:1597-1604.
2. Pan L, Sato S, Frederick JP, Sun XH, Zhuang Y. Impaired immune responses and B-cell proliferation in mice lacking the Id3 gene. *Mol Cell Biol.* 1999;19:5969-5980.
3. Reilly KB, Srinivasan S, Hatley ME, Patricia MK, Lannigan J, Bolick DT, Vandenhoff G, Pei H, Natarajan R, Nadler JL, Hedrick CC. 12/15-Lipoxygenase activity mediates inflammatory monocyte/endothelial interactions and atherosclerosis in vivo. *J Biol Chem.* 2004;279:9440-9450.
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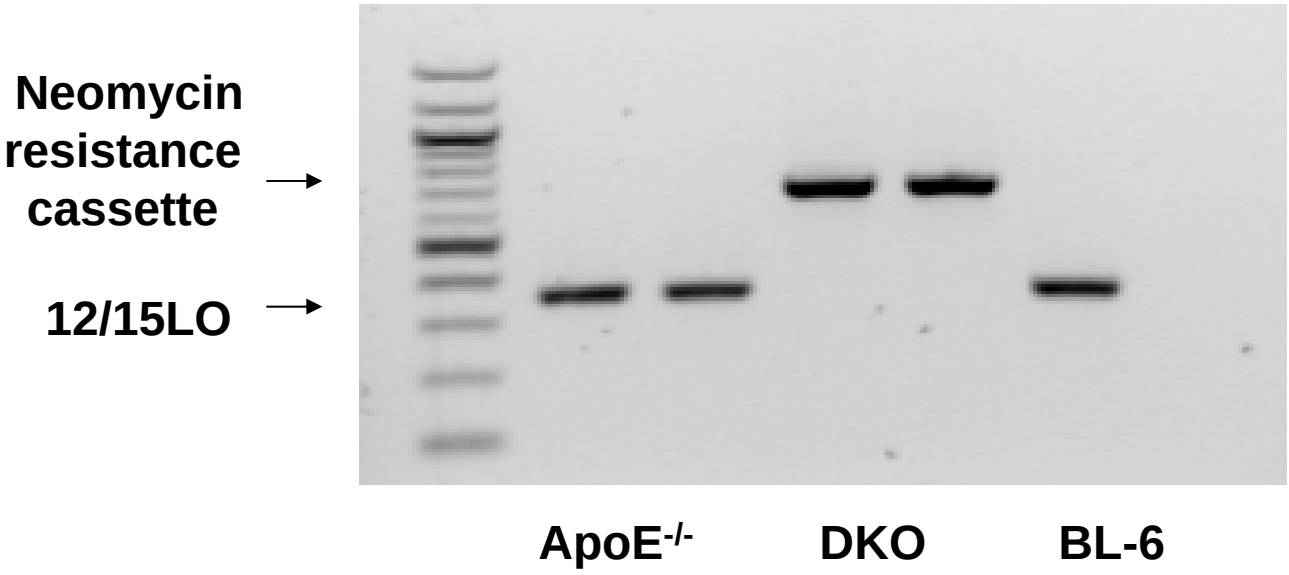
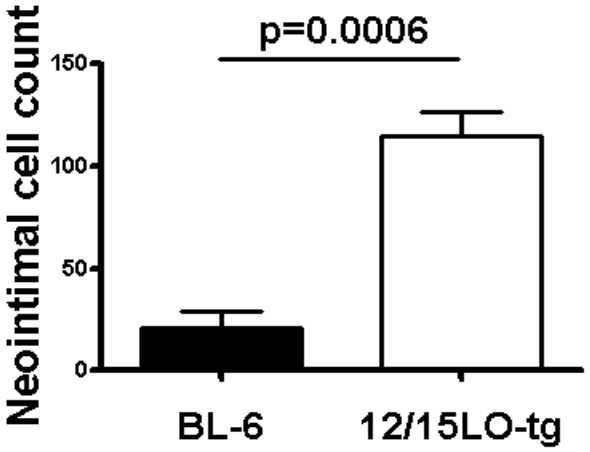
5. Movat HZ. Demonstration of all connective tissue elements in a single section; pentachrome stains. *AMA Arch Pathol.* 1955;60:289-295.
6. Doran AC, Meller N, Cutchins A, Deliri H, Slayton RP, Oldham SN, Kim JB, Keller SR, McNamara CA. The helix-loop-helix factors Id3 and E47 are novel regulators of adiponectin. *Circ Res.* 2008;103:624-634.

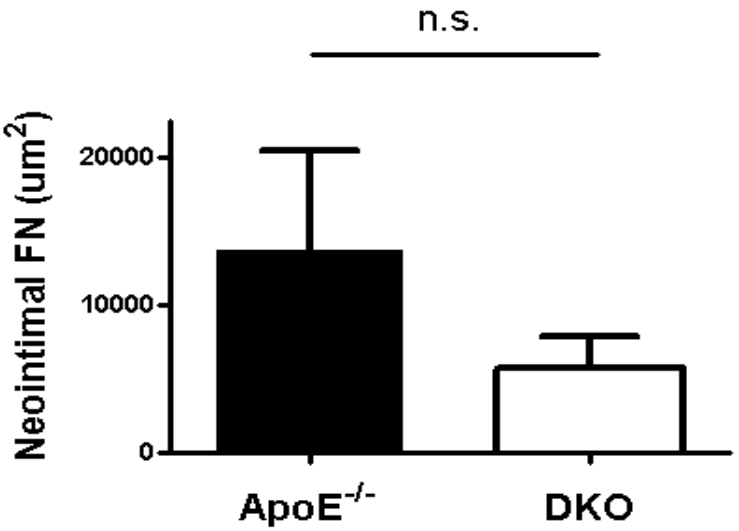
Supplemental Figure I



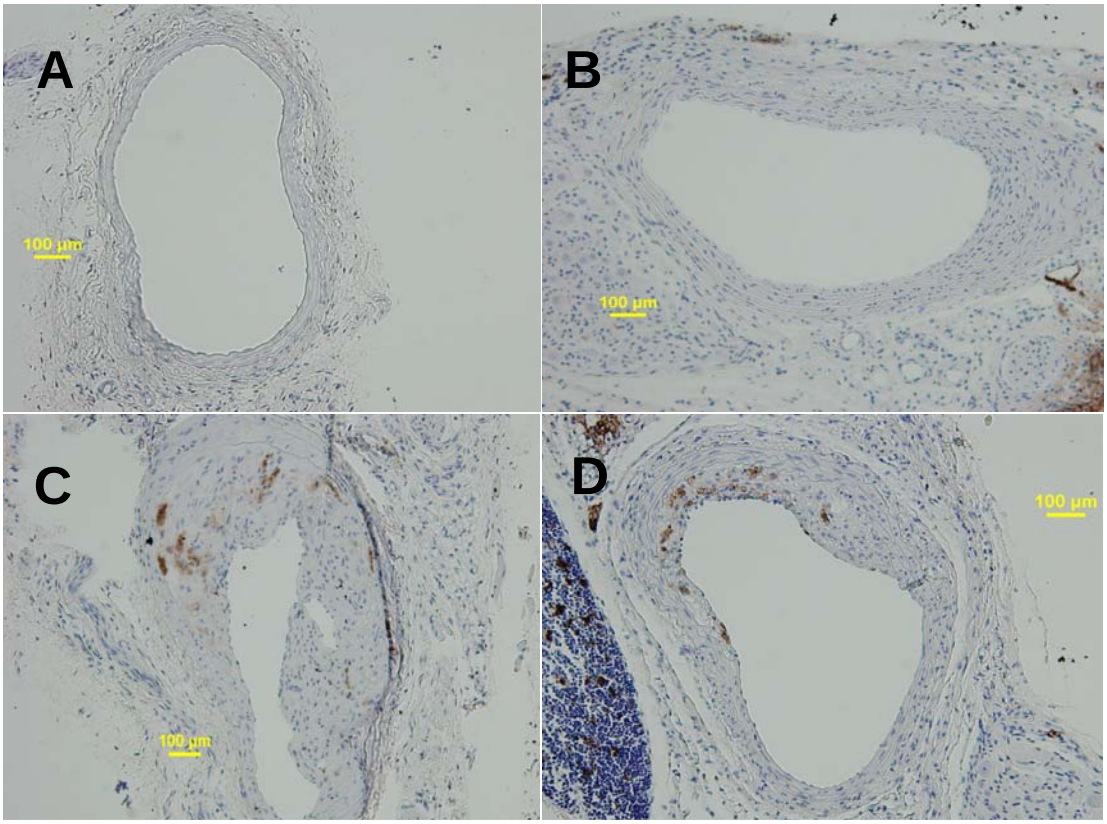
Supplemental Figure II



Supplemental Figure III



Supplemental Figure IV



Supplemental Figures

Figure I. Conventional PCR confirms loss of 12/15LO expression in LCCA of DKO mice. To confirm loss of 12/15LO expression in DKO mice, PCR was performed on genomic DNA from LCCAs harvested from ApoE$^{-/-}$, DKO , and BL-6 control mice. A representative gel is shown demonstrating lack of 12/15LO product (~350bp) and presence of neomycin band (~700bp) in DKO mice when compared to ApoE$^{-/-}$ and BL-6 control.

Figure II. There is greater neointimal cell number in 12/15LO-tg compared with BL-6 mice. Male BL-6,12/15LO-tg, and 12/15LO-tg/ Id3$^{-/-}$ mice were fed western diet starting at 10-12 weeks of age and underwent LCCA endothelial denudation one week later. LCCAs were harvested 28 days after the injury. Neointimal cell number was determined by direct counting of nuclei in BL-6 and 12/15LO-tg mice on day 28 after the injury. Data is mean ± SD.

Figure III. Neointimal fibronectin deposition (FN) in DKO compared with ApoE$^{-/-}$. We performed immunostaining for fibronectin in the 28D post-injury LCCA sections of the ApoE$^{-/-}$ and DKO mice. Mean of fibronectin-stained area of two LCCA sections obtained from equally distributed intervals from the carotid bifurcation of four animals in each group was determined. Data represented as mean ± SD.

Figure IV. Neointimal Mac-2-positive cell immunostaining in DKO compared with ApoE$^{-/-}$. We performed immunostaining for Mac-2 positive cells in the 28D post-injury LCCAs of the BL-6 **(A)**, 12/15LO-tg **(B)**, ApoE$^{-/}$**(C)**, and DKO **(D)** mice on sections obtained from equally distributed intervals from the carotid bifurcation of four animals in

each group. There was no statistically significant difference between BL-6 vs.12/15LO-tg or ApoE$^{-/-}$ vs. DKO groups.