Online Data Supplement

Materials and Methods

Immunostaining

Immunostaining of human atherosclerotic lesions was performed as described previously using primary antibodies against NOR1 (1:100 dilution; IMG-71915; Imgenex, Inc.) or von Willebrand Factor (vWF) (1:100 dilution; 115-01, SIGNET). [1, 2] For immunofluorescent co-localization studies, sections were incubated with primary antibodies against NOR1 (1:50 dilution; ab56340; Abcam) and vWF (1:100 dilution; 115-01, SIGNET). Sections were subsequently incubated with Alexa 488-conjugated goat anti-mouse antibody (1:1000 dilution, A11001, Invitrogen) and Alexa 594-conjugated goat anti-rabbit antibody (1:1000, A11012, Invitrogen), respectively. All studies on human tissues were performed with the approval of the University of Kentucky Institutional Review Board.

For immunohistochemical analysis of mouse aortic atherosclerotic lesions, the ascending aortae were embedded in OCT (Tissue-Tek; Miles Inc., Elkhart, Illinois, USA) and snap-frozen. Transverse cryosections (10 μm) were collected from the aortic arch, fixed in cold acetone, and immunostained using macrophage anti-sera (1:1000 dilution, AI-AD 31240, Accurate Chemicals) or a VCAM-1 antibody (BD 550547). All experiments on mice were approved by the University of Kentucky Institutional Animal Care and Use Committee.

Cell culture

Human umbilical vein endothelial cells (HUVEC, Lonza) and human aortic endothelial cells (HAEC, Cascade Biologics) were cultured as directed by the manufacturer. Human THP-1 monocytes (ATCC) were maintained in RPMI-1640 medium supplemented with 10% FBS. Murine WEHI-274.1 premyelocytic cells (ATCC) were cultured in DMEM supplemented with 10% FBS. Confluent endothelial cells were stimulated with different cytokines or proinflammatory factors as indicated in the Figure Legends. The reagents used in this study included human TNFα (R & D), human IL-1β (R & D), human IL-6 (R & D), human IFNγ (R & D), human oxidized low-density-lipoprotein (ox-LDL) (INTRACEL), mouse TNFα (R & D), mouse IL-1β (R & D) and LPS (Sigma). All experiments were performed with cells between passages 2 to 8, and each experiment was repeated at least three times with different preparations of cells.

Western blotting

Western blotting was performed as described using antibodies against NOR1 (PP-H7833, R & D Systems), human VCAM-1 (BD Pharmingen), human ICAM-1 (Abcam), mouse VCAM-1 (R & D Systems), mouse ICAM-1 (Abcam), β-actin (Sigma), and GAPDH (FL 335) (Santa Cruz). [1, 2]

Quantitative real-time RT-PCR

Total RNA was isolated using TRIzol® (Invitrogen) and reverse transcribed into cDNA using SuperScript II (Invitrogen). RNA expression levels of target genes were quantified using an iQ^{TM} SYBR Green Supermix (BioRad) and 5 pmol of the indicated primer pairs (Supplemental Table I). PCR reactions were performed on an iCycler (BioRad) using the following PCR cycles: 1 cycle of 95 °C 10 min; 40 cycles of 95 °C 30 sec, 55 °C 30 sec, 72 °C 30 sec; 1 cycle of a final extension at 72°C for 10 min. Each sample was analyzed in triplicate and normalized to expression values of the house-keeping gene TBP or TFIIB. Data were calculated using the $2^{-\Delta\Delta CT}$ method. [3]

Adenovirus-mediated overexpression in HUVEC

The adenovirus over-expressing the dominant-negative IκBα mutant (IκBα-S32A/S36A) (Ad-CMV-IκB(DN)) was purchased from Vector Biolabs. The adenovirus over-expressing human NOR1 (Ad-CMV-NOR1) was generously provided by Dr. Peter Tontonoz (University of California, Los Angeles, CA).

Subconfluent HUVEC were infected with 25 PFU Ad-CMV-IκB(DN) for 3 hours and 50 PFU Ad-CMV-NOR1 for 6 hours, respectively. Adenoviruses over-expressing GFP (Ad-CMV-GFP) or an empty vector (Ad-CMV-null) were used as controls.

Plasmids, Transient Transfections and Luciferase Assay

The human NOR1 promoter constructs have previously been described. [1] The NF-κB response elements located at -198bp to -190bp and -595bp to -496bp from the transcription initiation site were mutated from GGAGTTTCC to AGAGTTTAA and from GGGATTAGCC to ATGATTAGAA using the QuickChange II XL site–directed mutagenesis kit (Stratagene). HAEC were transiently transfected with NOR1 promoter constructs using promofectin (PromoKine). Following transfection, cells were recovered overnight and stimulated with TNFα. The human VCAM-1 promoter construct was commercially obtained from Epoch Biolabs Company. The NBRE site located at -2618 bp to -2611 bp from the transcription initiation site was mutated from TGACCTTT to TCGGAGTT. For overexpression of NOR1, HUVEC were infected with 50 PFU Ad-CMV-Null or Ad-CMV-NOR1 for 6 hours and recovered for 24 hours. Infected cells were subsequently transfected with luciferase reporter constructs driven by the VCAM-1 promoter. Following transfection, cells were recovered in growth media for 2 days. Luciferase activities were analyzed using a Dual Luciferase Reporter Assay (Promega). Transfection efficiency was normalized to renilla luciferase activities generated by cotransfection of 5 ng pRLCMV.

siRNA Experiments

siRNA experiments were performed using the SMARTpool technology (L003428, Dharmacon RNA Technologies), which provides a mix of four different proprietary siRNAs specific against human NOR1. HUVEC were seeded at a density of 1.2×10^5 cells/well in 6-well plates and transiently transfected for 3 h with 30 nM NOR1 siRNA or scrambled siRNA using promofectin (PromoKine). Following transfection, cells were recovered in complete growth media overnight and subsequently stimulated with TNFα for 6 h. Fluorescently labeled THP-1 monocytes were added onto the HUVEC monolayers and quantification of adhesion was performed after 30 min as indicated in the section "Adhesion assay".

Chromatin Immunoprecipitation (ChIP) Assays

ChIP assays were performed using the EZ-ChIP kit (Millipore) as described. [1, 2] Briefly, HUVEC were stimulated with TNFα and sheared chromatin was immunoprecipitated using 5 μg antibodies directed against NF-κB p65 (sc-372x, Santa Cruz) or NOR1 (PP-H7833, R & D Systems). Target DNA product was amplified by PCR using primer pairs covering the NF-κB binding sites in the NOR1 promoter or the NBRE site in the VCAM-1 promoter (Supplemental Table I).

Atherosclerosis quantification

Atherosclerosis was quantified as described and reviewed recently in detail. [4, 5] Briefly, after exsanguination aortic tissue was removed from the ascending aorta to the ileal bifurcation and fixed by perfusion with freshly prepared 4 % paraformaldehyde in PBS overnight at room temperature. After tissue fixation, the aorta was dissected from the adventitia. The intimal surface was exposed by a longitudinal cut through the inner curvature of the aortic arch that extended down the whole length of the aortic tree. To permit the arch region to be laid out flat, the greater curvature was cut down to the level of the subclavian artery. The tissue was laid out, and an image of the aorta was recorded. To quantify the extent of intimal surface covered by grossly discernible lesions, image analysis was performed with Image-Pro (Media Cybernetics). The extent of atherosclerotic lesions was quantified in the arch as defined from the ascending arch to 4 mm distal to the left subclavian artery. The data were presented as the percentage of lesion area on the aortic arch.

Quantification of macrophage accumulation

Macrophage content was quantified in a 2-mm segment as defined from the beginning of the ascending aortic arch using a modified technique described by Mach et al. [6] From this segment 180 serial transverse cryosections (10 μm) were collected and placed onto twenty slides per mouse. Slides were immunostained for macrophages as described above, and the macrophage content was quantified in nine sections 200 μm apart. Macrophage accumulation was determined by quantifying the total area positive for macrophage staining using computer-assisted image analysis (Image-Pro, Media Cybernetics). Each section was quantified by two observers blinded to the experimental design. The data were presented as mean area ± SEM positive for macrophage staining for each of the nine measurements and their cumulative sum.

Lipoprotein resolution and quantification

Lipoproteins were resolved using size exclusions chromatography as described. [4, 5] Briefly, serum samples were centrifuged and placed onto a single Sepharose 6 HR 10/30 column (300 x 10 mm, Pharmacia) with a mobile phase of saline/EDTA run at 0.5 ml/min. Fractions (0.5 ml) were collected and cholesterol concentrations were determined by placing 100 μl of each fraction into an equivalent volume of cholesterol reagent (Wako Chemical Company) that was diluted to half the manufacturer's instructions. Assays were performed in a 96 well format and absorbance was determined at 600 nm.

Statistical Analysis

Results were represented as means or medians depending on the distribution of data. Unpaired Student's t-test was utilized to compare the means between two independent groups on a single variable. One-way or Two-way ANOVA was used to compare groups. The effect of NOR1 on atherosclerosis was compared using the Kruskal-Wallis test followed by Dunn Test post-hoc analysis. P values < 0.05 were considered to be statistically significant.

References

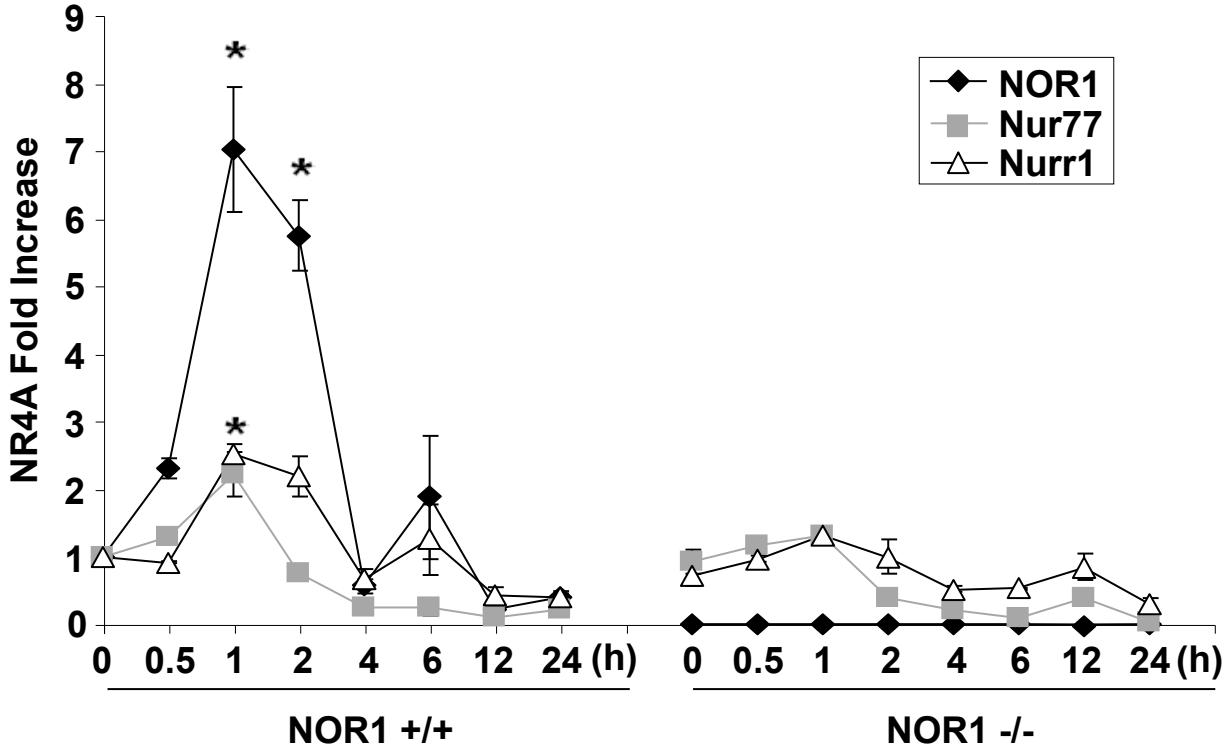
1. Nomiyama T, Nakamachi T, Gizard F, Heywood EB, Jones KL, Ohkura N, Kawamori R, Conneely OM, Bruemmer D. The NR4A orphan nuclear receptor NOR1 is induced by platelet-derived growth factor and mediates vascular smooth muscle cell proliferation. *J Biol Chem.* 2006;281:33467-33476.
2. Nomiyama T, Zhao Y, Gizard F, Findeisen HM, Heywood EB, Jones KL, Conneely OM, Bruemmer D. Deficiency of the NR4A neuron-derived orphan receptor-1 attenuates neointima formation after vascular injury. *Circulation.* 2009;119:577-586.
3. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods.* 2001;25:402-408.
4. Daugherty A, Whitman SC. Quantification of atherosclerosis in mice. *Methods Mol Biol.* 2003;209:293-309.
5. Daugherty A, Manning MW, Cassis LA. Angiotensin II promotes atherosclerotic lesions and aneurysms in apolipoprotein E-deficient mice. *J. Clin. Invest.* 2000;105:1605-1612.
6. Mach F, Schonbeck U, Sukhova GK, Atkinson E, Libby P. Reduction of atherosclerosis in mice by inhibition of CD40 signalling. *Nature.* 1998;394:200-203.

Supplemental Table I: Oligonucleotides used in this study.

-

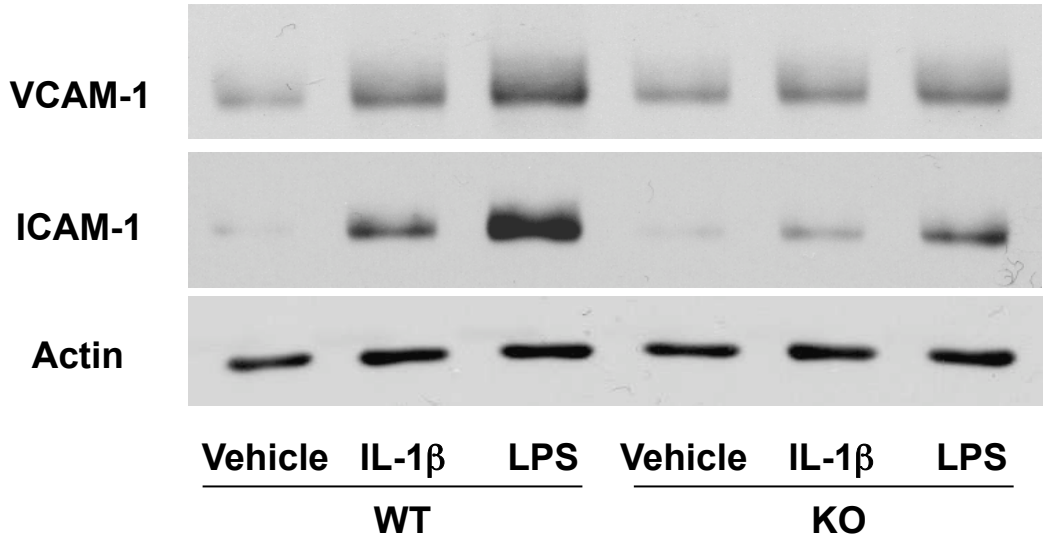
Name	Use	Sequence
human NOR1	Real-time RT-PCR	F : 5'-GGGCTTTTTCAAGAGAACAGTG-3' R: 5'-ATCTCTGGGTGTTGAGTCTGTT-3'
human VCAM-1	Real-time RT-PCR	F: 5'-TGAGGGGACCAATTCCAC-3' R: 5'-ATTCACGAGGCCACCACT-3'
human ICAM-1	Real-time RT-PCR	F: 5'-ACCGTGAATGTGCTCTCC-3' R: 5'-GGCTTGTGTGTTCGGTTT-3'
murine VCAM-1	Real-time RT-PCR	F: 5'-TCAAAGAAAGGGAGACTG-3' R: 5'-GCTGGAGAACTTCATTATC-3'
murine ICAM-1	Real-time RT-PCR	F: 5'-AGATCACATTCACGGTGCTG-3' R: 5'-CTTCAGAGGCAGGAAACAGG-3'
human TBP	Real-time RT-PCR	F: 5'-GGAGAGTTCTGGGATTGTACCGC-3' R: 5'-ATATTCGGCGTTTCGGGCAC-3'
murine TFIIB	Real-time RT-PCR	F: 5'-CTCTCCCAAGAGTCACATGTCC-3' R: 5'-CAATAACTCGGTCCCCTACAAC -3'
murine NOR1	Real-time RT-PCR	F: 5'-GGCCGCAGCTGCACTCAGTC -3' R: 5'-GCGGAGGGAAGGTCAGCGTG -3'
murine Nur77	Real-time RT-PCR	F: 5'-TTGATGTTCCCGCCTTTG-3' R: 5'-GGTAGCCATGTGCTCCTTC-3'
murine Nurr1	Real-time RT-PCR	F: 5'-TCACCTCCGGTGAGTCTGATC-3' R: 5'-TGCTGGATATGTTGGGTATCATCT-3'
hNOR1-NFKB	ChIP PCR	F: 5'-CCATCTGCATCCCTGTGT-3' R: 5'-GCTGCACTTTCCTCTTGC-3'
hVCAM-1-NOR1	ChIP PCR	F: 5'-CTGTACTCAAACATTGGAAACATT -3' R: 5'-CCTTAGAGATGAGAGAAGCAAGA-3'

Supplemental Figures

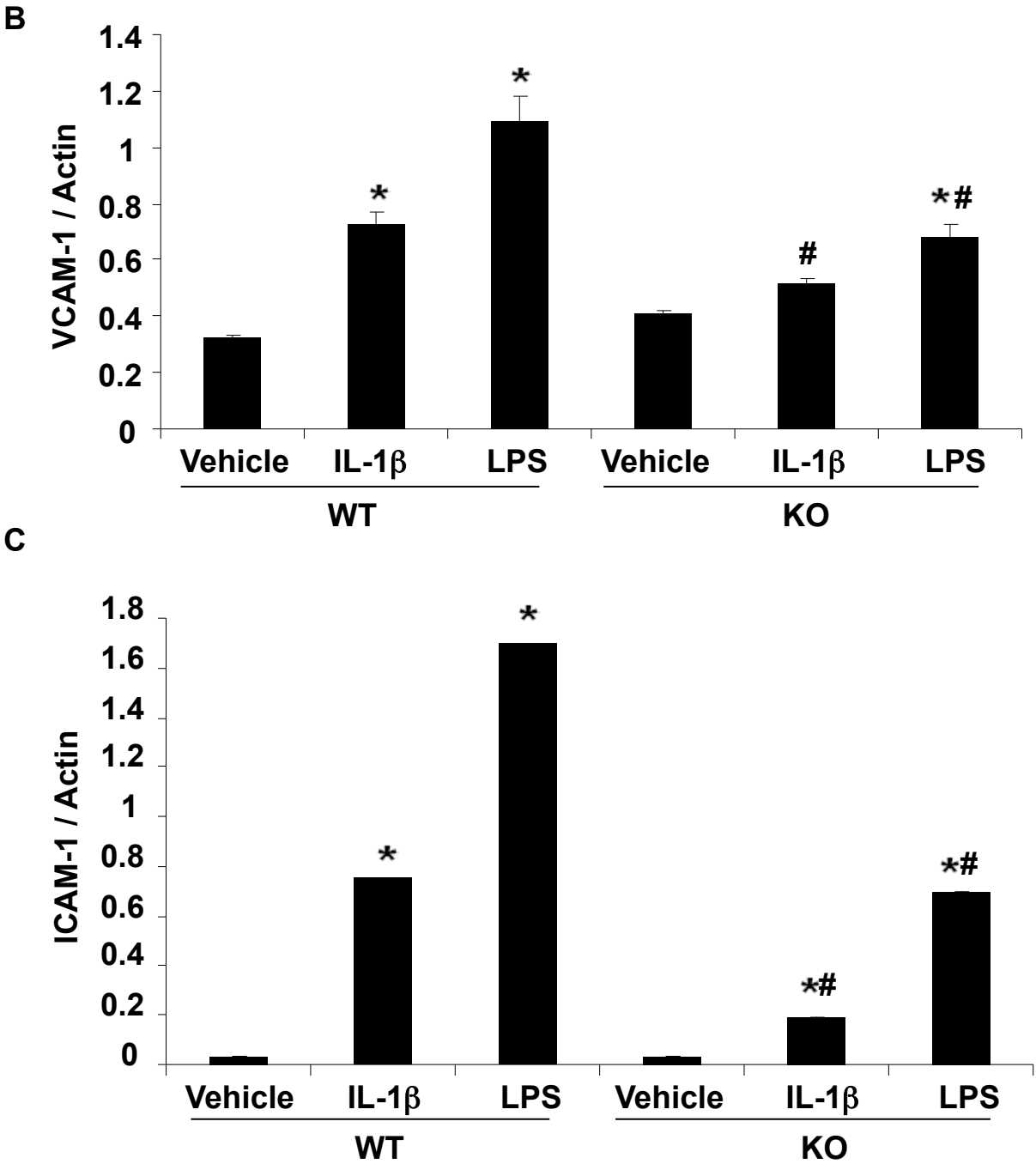


Supplemental Figure I. Expression of NR4A orphan nuclear receptors in endothelial cells. NOR1+/+ and NOR1-/- MAEC were incubated with TNFα (5 ng/ml) and mRNA expression of NOR1, Nur77 and Nurr1 was analyzed by real-time RT-PCR. Experiments were repeated at least three times in duplicates with different cell preparations. Results are presented as mean ± SEM fold increase over vehicle-treated wildtype cells (*P < 0.05 vs. vehicle).

A

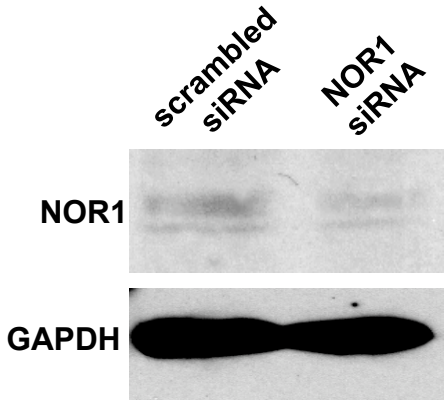


Supplemental Figure II. (A-C) NOR1+/+ and NOR1-/- MAEC were stimulated with vehicle, IL-1β (1 ng/ml), or LPS (50 ng/ml) for 6 hours. VCAM-1 and ICAM-1 protein expression were analyzed by Western blotting. Cohybridization for actin was performed to assess equal loading. (A) Autoradiograms are representative of three different experiments. (B and C) Densitometric quantification of VCAM-1 and ICAM-1 protein expression from three experiments with different cell preparations. Results are presented as mean ± SEM fold increase over vehicle-treated wildtype cells (*P < 0.05 vs. vehicle, # P < 0.05 vs. NOR1+/+ cells).



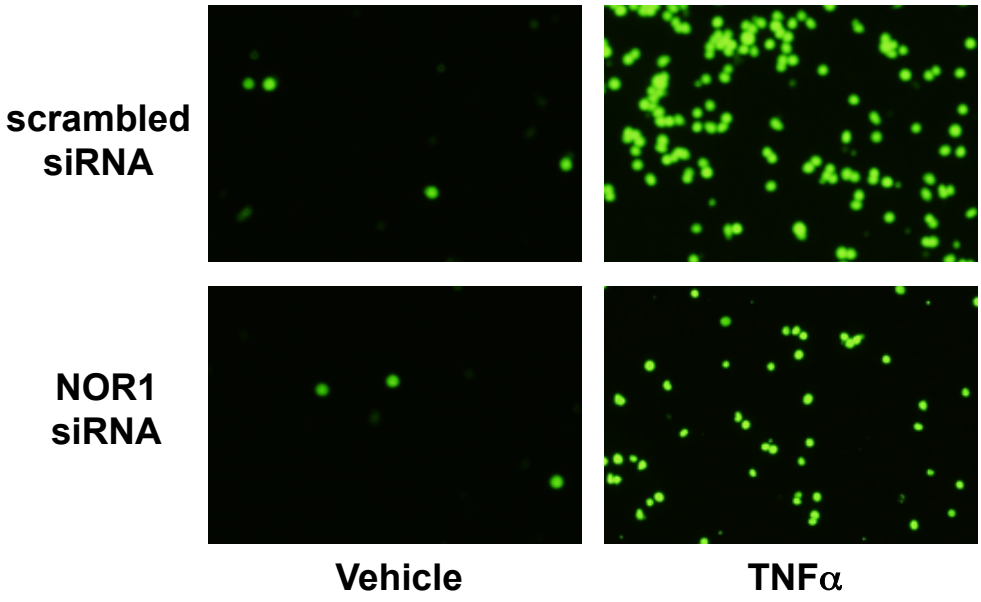
Supplemental Figure II. (A-C) NOR1+/+ and NOR1-/- MAEC were stimulated with vehicle, IL-1β (1 ng/ml), or LPS (50 ng/ml) for 6 hours. VCAM-1 and ICAM-1 protein expression were analyzed by Western blotting. Cohybridization for actin was performed to assess equal loading. (A) Autoradiograms are representative of three different experiments. (B and C) Densitometric quantification of VCAM-1 and ICAM-1 protein expression from three experiments with different cell preparations. Results are presented as mean ± SEM fold increase over vehicle-treated wildtype cells (*P < 0.05 vs. vehicle, # P < 0.05 vs. NOR1+/+ cells).

A

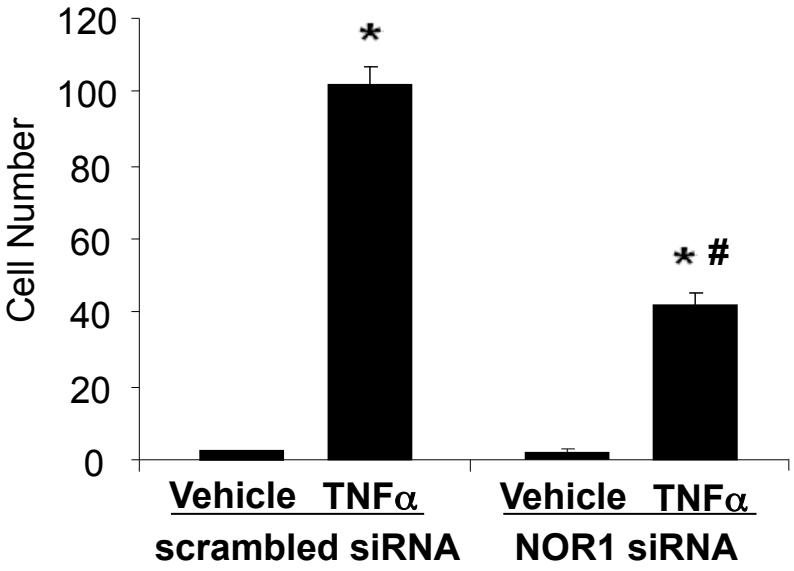


Supplemental Figure III. siRNA-mediated knock-down of NOR1 expression decreases monocyte adhesion. (A) Western Blotting for NOR1 in endothelial cells transfected with scrambled or NOR1 siRNA and treated with TNFα for 6 h. (B) HUVEC were transfected with scrambled or NOR1 siRNA, stimulated with TNFα (1 ng/ml), and analyzed for THP-1 monocyte adhesion as detailed in the Materials and Methods section. Representative images showing monocyte adhesion. (C) Quantification is presented as mean ± SEM from three independently performed experiments in duplicates (*$P < 0.05$ vs. vehicle, # $P < 0.05$ vs. scrambled siRNA).

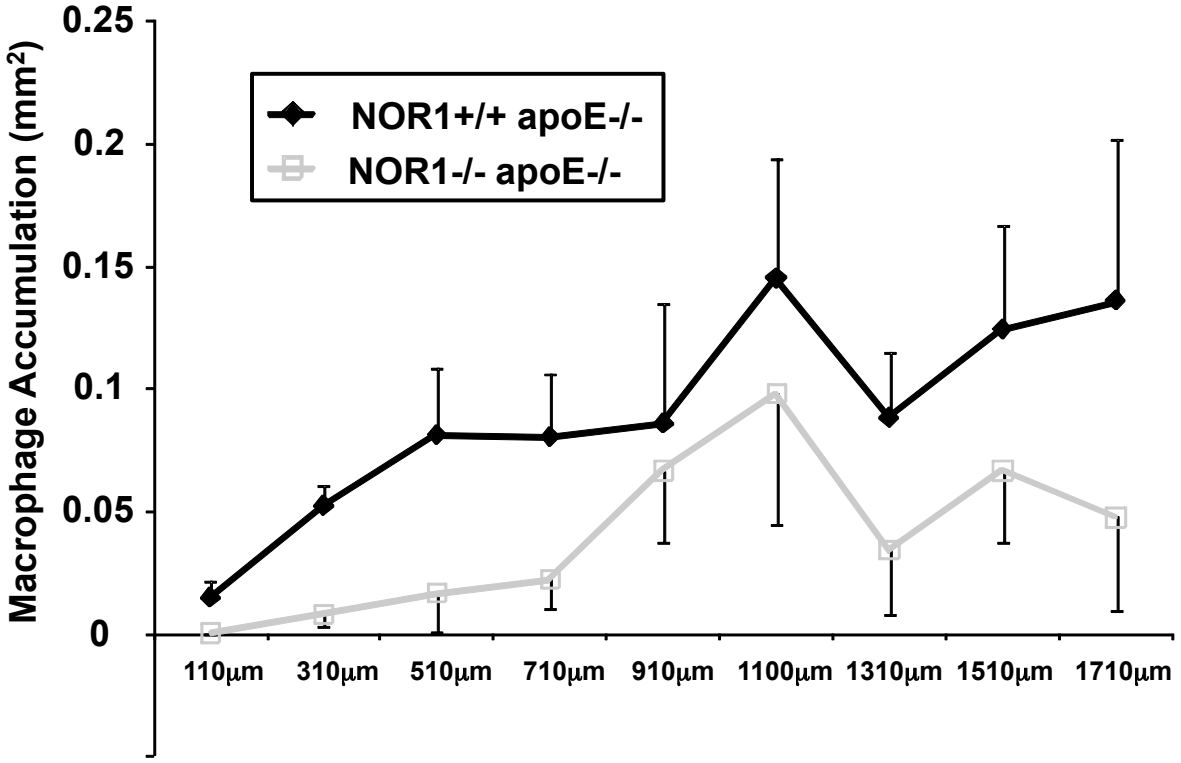
B



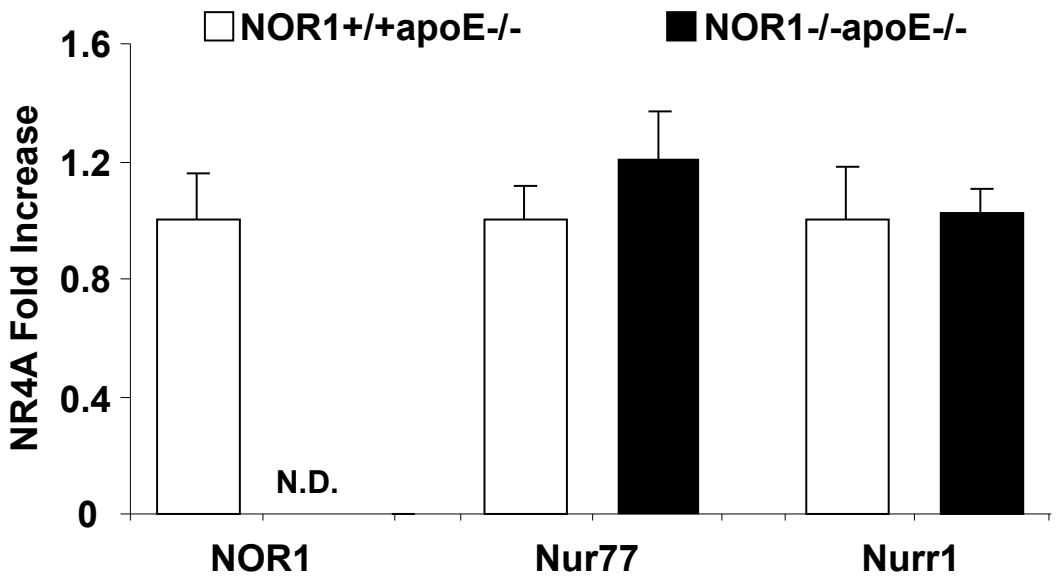
C



Supplemental Figure III. siRNA-mediated knock-down of NOR1 expression decreases monocyte adhesion. (A) Western Blotting for NOR1 in endothelial cells transfected with scrambled or NOR1 siRNA and treated with TNFα for 6 h. (B) HUVEC were transfected with scrambled or NOR1 siRNA, stimulated with TNFα (1 ng/ml), and analyzed for THP-1 monocyte adhesion as detailed in the Materials and Methods section. Representative images showing monocyte adhesion. (C) Quantification is presented as mean ± SEM from three independently performed experiments in duplicates (*$P < 0.05$ vs. vehicle, # $P < 0.05$ vs. scrambled siRNA).



Supplemental Figure IV. Macrophage accumulation in the aortic arch of NOR1+/+apoE-/- and NOR1-/-apoE-/- mice. Serial sections from a 2-mm segment beginning at the lesser curvature of the aortic arch were collected from NOR1+/+apoE-/- and NOR1-/-apoE-/- mice. Sections were immunostained for macrophages using antisera against macrophages or control IgG. Macrophage content was quantified in nine sections 200 μm apart using computer-assisted image analysis. Data are presented as mean area in mm^2 ± SEM. Statistical analysis was performed using factorial ANOVA test. No significant difference between single segments of the arch in NOR1+/+apoE-/- and NOR1-/-apoE-/- mice was detected.



Supplemental Figure V. NOR1+/+apoE-/- (n=5) and NOR1-/-apoE-/- (n=5) mice were fed a diet enriched in saturated fat for 2 weeks. Aortae were collected and mRNA expression of NOR1, Nur77 and Nurr1 was analyzed by real-time RT-PCR. Results are presented as mean ± SEM fold increase over NOR1+/+apoE-/- mice. Note, no NOR1 transcript was detected in NOR1-/-apoE-/- mice (N.D., not detectable).