Supplemental Data, Tables and Figures

Supplemental Data

Proliferation remains unchanged in aortic roots of mice lacking SPRR3

To determine whether changes in proliferation may play a role in reduced VSMC content, we performed immunohistochemistry for Ki67 in aortic root lesions of ApoE-null and DKO mice fed a high fat diet for 6 months. No significant difference was observed in %Ki67-positive cells between groups (Figure V in the online-only Data Supplement, p=0.33). Our data suggest, therefore, that loss of SPRR3 led to reduced VSMC survival in the atheroma and subsequent reduced lesion VSMC content.

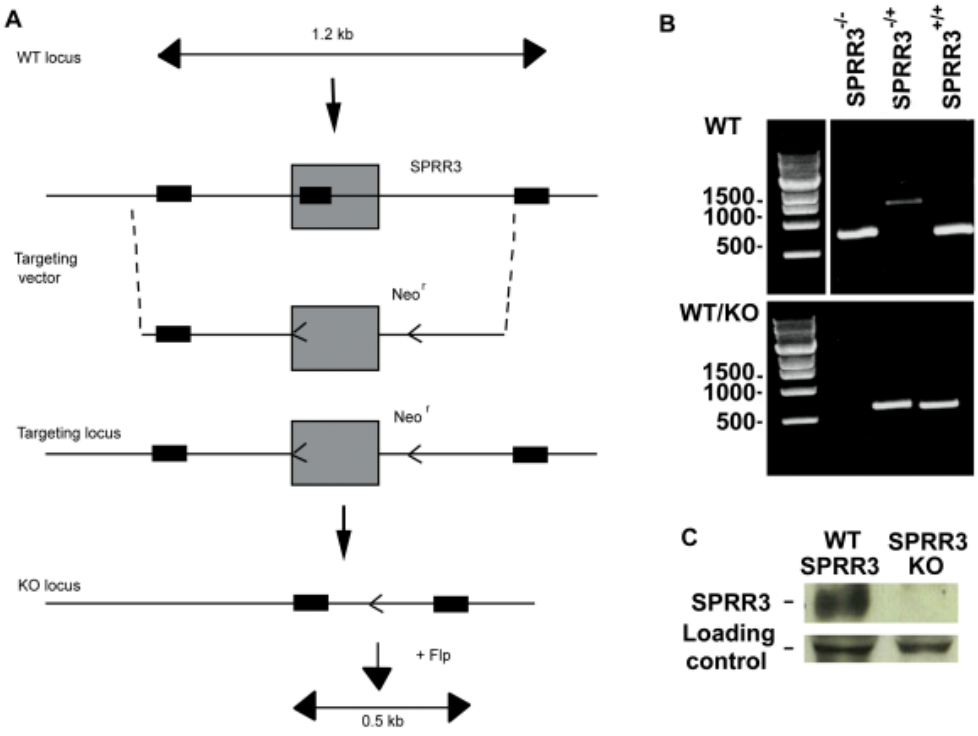
Supplemental Table

Supplemental Table I: Plasma cholesterol and triglyceride levels in SPRR3$^{-/-}$ApoE$^{-/-}$ mice are not significantly different from SPRR3$^{+/+}$ApoE$^{-/-}$

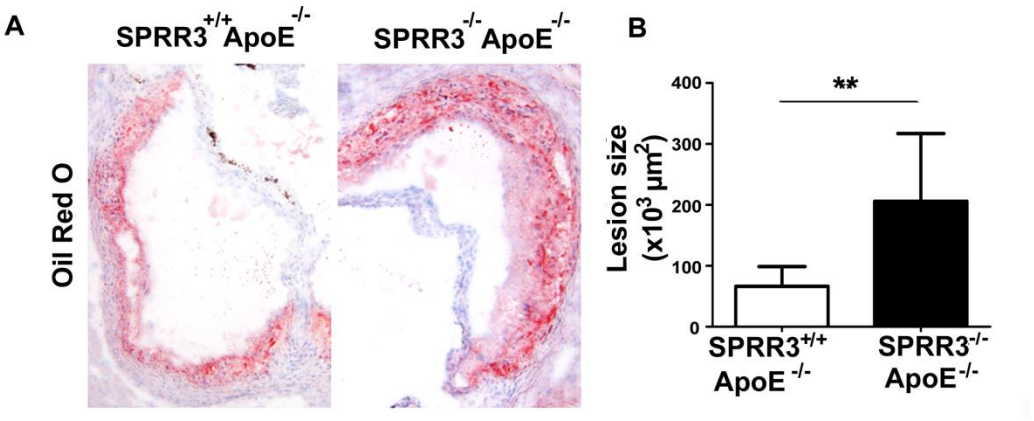
Genotype	n	Cholesterol (mg/dL)*	Triglycerides (mg/dL)*
C57Bl/6	5	78.0 ± 13.1	90.6 ± 4.3
SPRR3$^{-/-}$ApoE$^{+/+}$	6	101.4 ± 9.2	124.9 ± 39.9
SPRR3$^{+/+}$ApoE$^{-/-}$	6	412 ± 28.2$^{\|\|,\#}$	172.9 ± 29.7‡
SPRR3$^{-/-}$ApoE$^{-/-}$	6	455.4 ± 82.5$^{\|\|,\#}$	175 ± 31.6†,§

* Fasting levels of cholesterol and triglyceride levels were quantified in serum of 6 month old C57/Bl6, SPRR3$^{-/-}$ApoE$^{+/+}$, SPRR3$^{+/+}$ApoE$^{-/-}$, and SPRR3$^{-/-}$ApoE$^{-/-}$ mice fed normal chow. Values are represented as mean ± SD. † $p < 0.05$ vs C57/Bl6; $^{\ddagger}p < 0.01$ vs C57/Bl6 mice or §SPRR3$^{-/-}$ApoE$^{+/+}$ mice; $^{||}p< 0.0001$ vs SPRR3$^{-/-}$ApoE$^{+/+}$ mice or $^{\#}$C57/Bl6

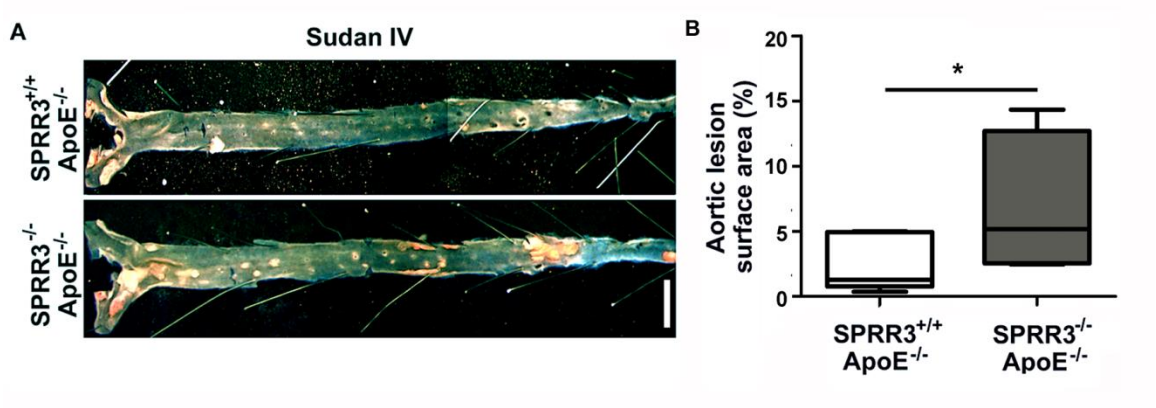
Supplemental Figures and Figure Legends



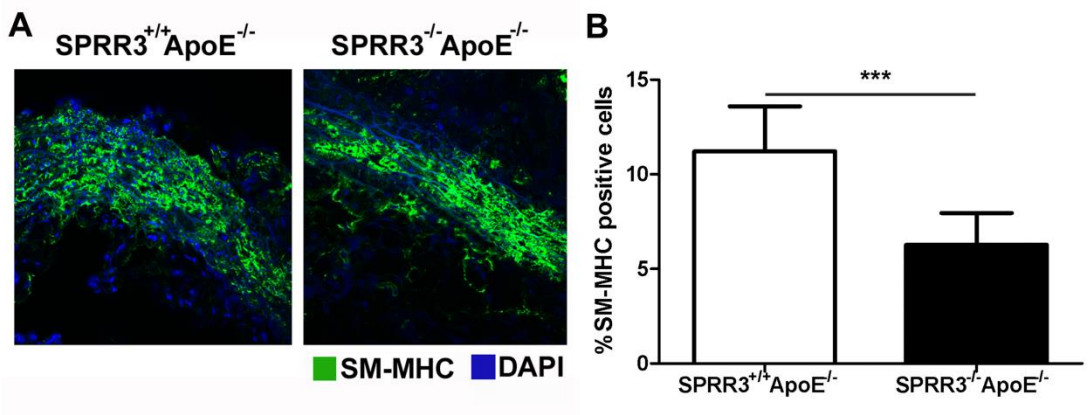
Supplemental Figure I: Generation of SPRR3$^{-/-}$ mice. (A) Targeting strategy used to disrupt the SPRR3 locus. A targeting vector with a Neo selection cassette was used to replace the SPRR3 gene by homologous recombination. SPRR3$^{-/-}$ mice were then crossed with Flp mice to remove the Neo cassette. Arrowhead = Frt site; Bar = forward and reverse primers. (B) SPRR3 knockout was confirmed with PCR using primers against flanking sequences (WT) and a sequence inside the target region (WT/KO). (C) Verification of SPRR3 knockout efficiency by Western against SPRR3 in wild type C57Bl/6 and SPRR3$^{-/-}$ forestomach, which expresses very high levels of SPRR3.



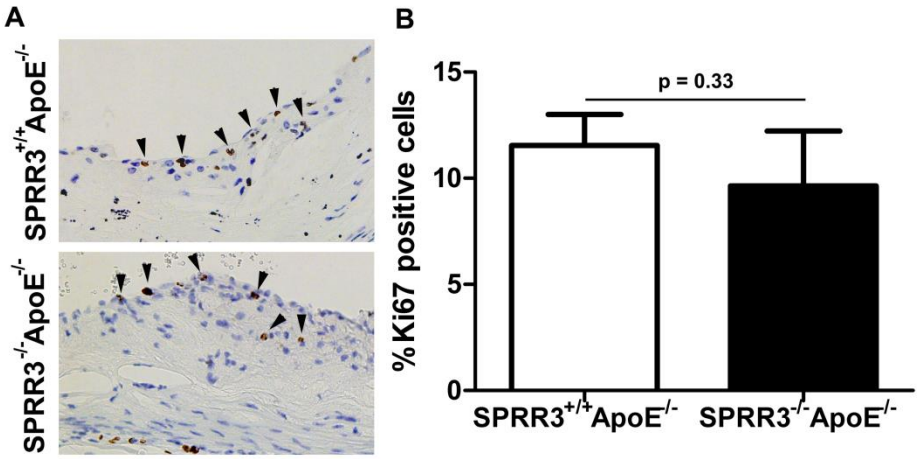
Supplemental Figure II: SPRR3-deficient mice have increased aortic root lesion size. (A) Representative photomicrographs of Oil Red O-stained aortic root sections from age-matched mice fed normal diet. (B) Quantification of Oil Red O staining. Bars represent mean ±SD.



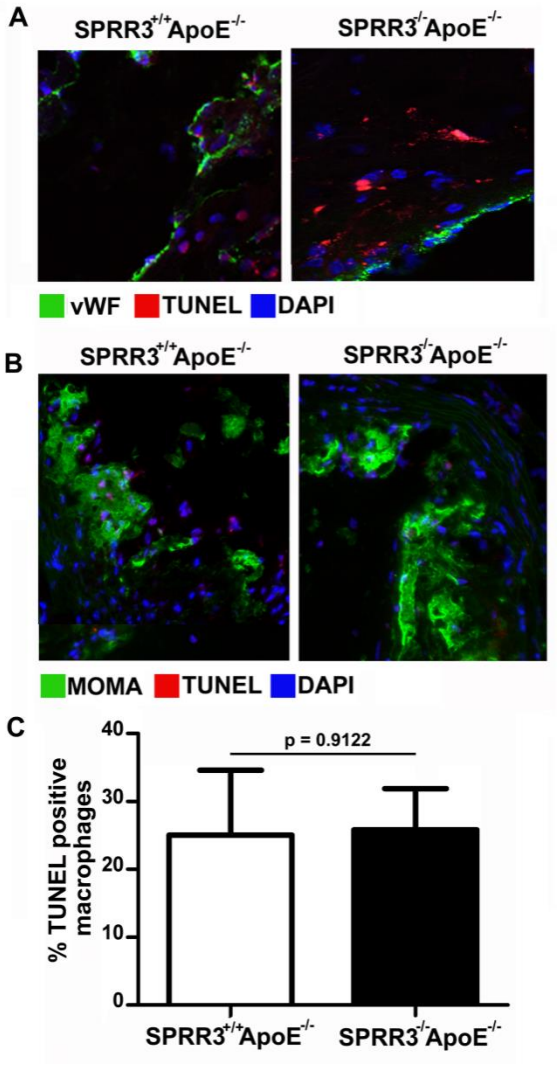
Supplemental Figure III: High fat diet leads to aggravated lesion burden in mice lacking SPRR3. (A) Representative photomicrographs were taken of aortas stained with Sudan IV for en face lesion analysis. Scale bar, 2mm. (B) Quantification shows surface area positive for Sudan IV staining in mice (N = 8 mice/group). Original magnification, x10 (A). * < 0.05, **<0.01.



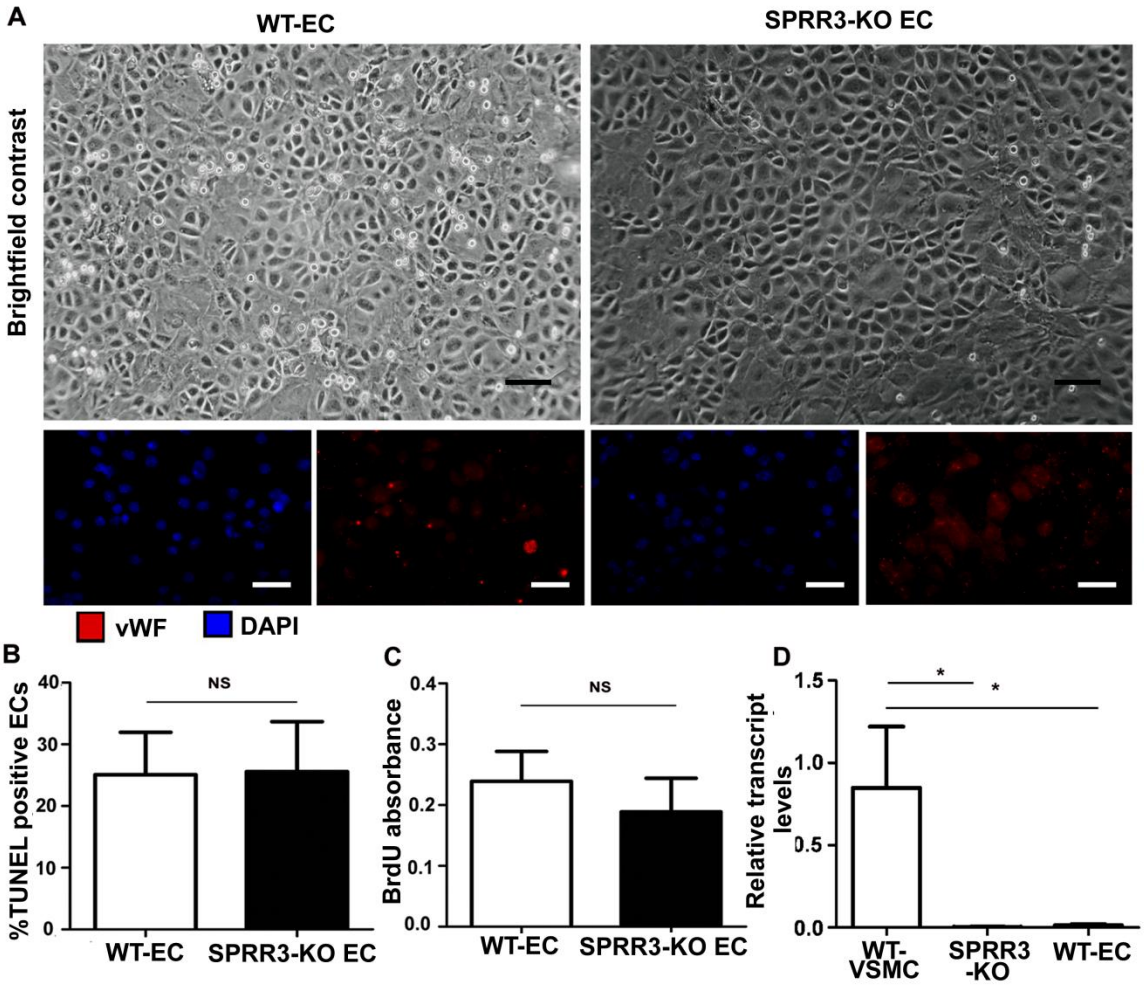
Supplemental Figure IV: SPRR3-deficient mice have reduced SM-MHC-positive VSMC content when compared with ApoE-null control. (A) Fluorescent images were acquired by confocal fluorescent microscopy with x40 lens. A representative Z plane shows a reduction in SM-MHC-positive cells (green) in aortic root lesions of 6 month normal diet-fed SPRR3$^{-/-}$ApoE$^{-/-}$ mice compared with control. (B) Quantification of %SM-MHC positive cells in aortic root sections from SPRR3$^{+/+}$ApoE$^{-/-}$ and SPRR3$^{-/-}$ApoE$^{-/-}$ mice; 35 HPF images evaluated from each cohort.



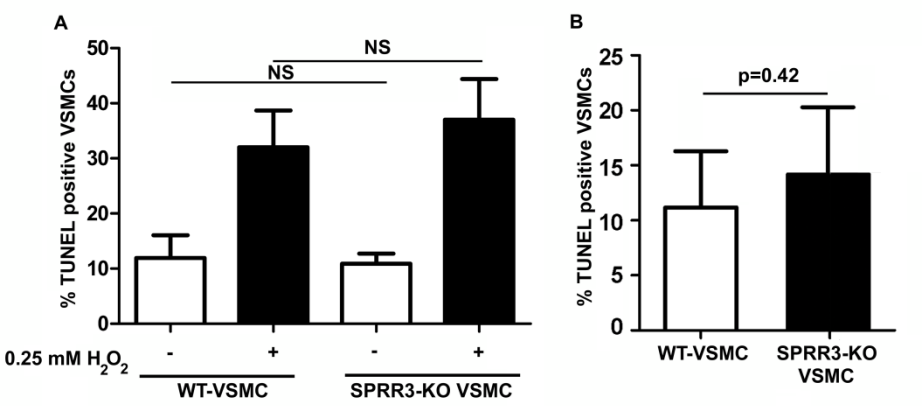
Supplemental Figure V: Loss of SPRR3 does not affect proliferation of lesion cells. (A) Immunohistochemical staining for Ki67 of aortic root sections from 6 month high fat diet-fed $SPRR3^{+/+}ApoE^{-/-}$ and $SPRR3^{-/-}ApoE^{-/-}$ mice. (B) Quantification of %Ki67+ cells from (A).



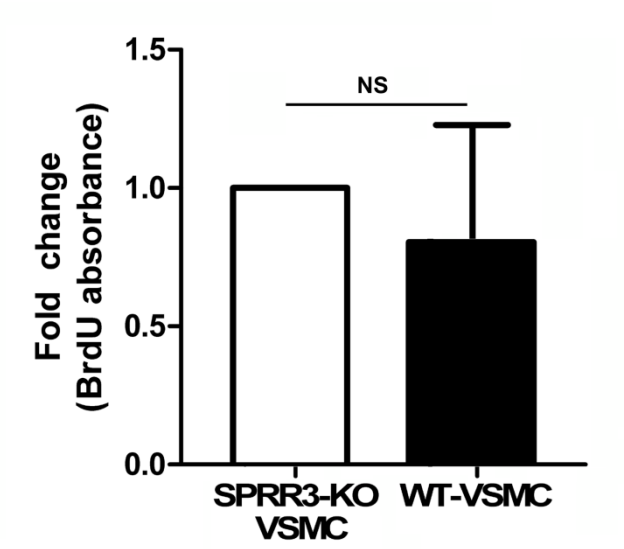
Supplemental Figure VI: Loss of SPRR3 does not affect cell death in endothelial cells or lesion macrophages. (A) Aortic root sections from 6 month old, normal diet-fed SPRR3$^{+/+}$ApoE$^{-/-}$ and SPRR3$^{-/-}$ApoE$^{-/-}$ mice were stained with vWF (green) and TUNEL (red). Fluorescent images were acquired by confocal fluorescent microscopy with x40 lens. A representative Z plane shows both groups lacked TUNEL-positive endothelial cells. (B) Serial sections from the aortic root of 6 month old, normal diet-fed SPRR3$^{+/+}$ApoE$^{-/-}$ and SPRR3$^{-/-}$ApoE$^{-/-}$ mice were stained with MOMA-2 (green) and TUNEL (red). (C) Quantification of images from (B) showed no significant difference in %TUNEL-positive macrophages between groups (n=4-5 per group).



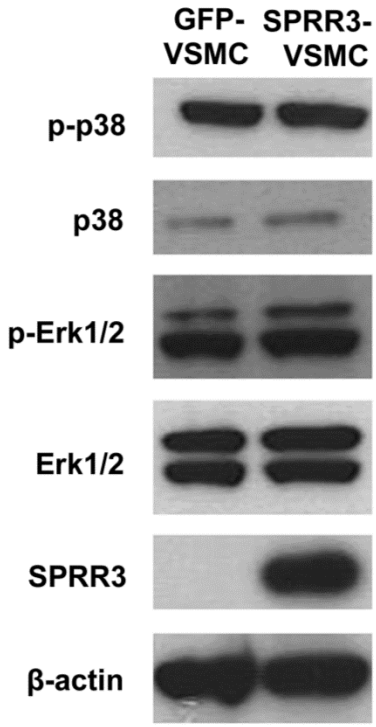
Supplemental Figure VII: Characterization of primary lung endothelial cells from wildtype and SPRR3 knockout mouse. (A) Primary endothelial cells in culture (brightfield contrast) and stained by DAPI (blue) and vWF antibody (red). Scale bar, 100 μm (brightfield), 50 μm (IF). (B) Loss of SPRR3 does not affect EC apoptosis. Cells were exposed to 0.5mM H_2O_2 for 2 hours prior to TUNEL assay. (C) Loss of SPRR3 does not affect EC proliferation as measured by BrdU staining. (D) Relative transcript levels of SPRR3 in WT compared to SPRR3-KO ECs as measured by real time RT-PCR. Bars represent the mean ±SD. * < 0.05.



Supplemental Figure VIII: SPRR3 loss in VSMCs does not confer protection from cell death in static conditions or in the absence of oxidative stress. (A) WT- and SPRR3-KO VSMCs were cultured under static conditions and treated with 0.25mM H_2O_2 for 4 hours before staining with TUNEL. TUNEL-positive VSMCs were quantified as a percent of total in >50 cells/treatment in 3 replicates. No difference in survival was observed with our without oxidative stress from WT cells (B) Primary VSMCs isolated from SPRR3$^{-/-}$ApoE$^{+/+}$ or WT mice exposed to cyclic strain were stained with TUNEL. %TUNEL-positive VSMCs were calculated among >50 cells/treatment in 3 replicates.



Supplemental Figure IX: Loss of SPRR3 does not affect VSMC proliferation *in vitro.* WT-VSMCs and VSMCs overexpressing SPRR3 were assessed for BrdU incorporation. Colorimetric measurements were collected for 4 experiments with 3 replicates/treatment.



Supplemental Figure X: SPRR3 overexpression in VSMCs does not affect activation of p38 or ERK1/2. Cell lysates from SPRR3-overexpressing VSMCs or GFP-expressing controls were subjected to immunoblot analysis using anti-p-p38 (~43 kDa), anti-p38 (~43 kDa), anti-pErk1/2 (~42/44 kDa), anti-ERK 1/2 (~42/44 kDa), anti-SPRR3 (~35 kDa), or loading control anti-β-actin (~42 kDa).

1. Babaev VR*, et al.* (2005) Conditional knockout of macrophage PPARgamma increases atherosclerosis in C57BL/6 and low-density lipoprotein receptor-deficient mice. *Arterioscler Thromb Vasc Biol* 25(8):1647-1653.