Supplemental Methods

Brachiocephalic artery vessel and lumen size analysis from atheroprone mice with and without MMP3 deficiency

Lumen size and vessel size (area within the IEL) of brachiocephalic arteries of male *Mmp3*$^{+/+}$*Apoe*$^{-/-}$ and *Mmp3*$^{-/-}$*Apoe*$^{-/-}$ mice after 8 weeks of feeding a high fat diet containing 21% (wt/wt) pork lard and 0.15% cholesterol was determined using digitized images of Miller's elastin/van Gieson-stained brachiocephalic arteries generated from a previous study (1). Given the relatively small size of the lesions after 8 weeks of high fat diet feeding and resulting infolding of the plaque-free vessel wall, the length of the internal elastic lamina (l) was used to calculate a circular area according to the formula $l^2/4\pi$ as described previously (2).

Smooth muscle cell culture

SMCs were isolated from 150-175 g male Sprague-Dawley rat thoracic aortas as described previously (3). Briefly, aortas were harvested, perivascular fat was removed, and aortas were digested in 1 g/L collagenase II (Worthington Biomedical), 0.25 g/L elastase (Worthington Biomedical), 1% penicillin-streptomycin (Gibco), and 1 g/L soybean trypsin inhibitor (Worthington Biomedical) in Hank's Balanced Salt Solution for 20 minutes. Following digestion the adventitia was carefully removed and the intimal surface was gently scraped with fine forceps. Aortas were cut into ~.5 mm pieces and placed in enzyme

solution again for 1 hour. Disaggregated medial SMCs were then grown and maintained in 10% serum-containing media (DMEM/F12 [Gibco], 10% fetal bovine serum [Hyclone], 100 U/mL penicillin/streptomycin [Gibco], 1.6 mmol/L L-glutamine [Gibco]). For some experiments as described, SMCs were switched to serum-free media (DMEM/F12 [Gibco], 1.6 mmol/L glutamine [Gibco], 0.2 mmol/L L-ascorbic acid [Gibco], 5 mg/L transferrin [Sigma], 2.8 mg/L recombinant human insulin [Gibco], 100 U/mL penicillin-streptomycin [Gibco], 6.25 µg/L selenium [Sigma]).

RNA Extraction and real-time RT-PCR

Total RNA was harvested from cells using Trizol reagent (Invitrogen) according to the manufacturer's instructions. cDNA was made using iScript cDNA synthesis kit (Bio-Rad). Real-time reverse transcription PCR was performed using iQ Sybergreen Supermix (Bio-Rad) and the following primers: MMP13 for: 5'-gccctgaatgggtatgacat, MMP13 rev: 5'-gcatgactctcacaatgcga; MMP9 for: 5'-agctgactacgacacagacagaa, MMP9 rev: 5'-ggccctcgaagatgaatggaaat; MMP3 for: 5'-gccaatgctgaagctttgatgtac, MMP3 rev: 5'-gggaggtccatagagggattgaat.

Affymetrix GeneChip Microarray Analysis

Total RNA extracted using Trizol reagent was labeled and hybridized with Affymetrix rat RAE230_2.0 GeneChips (n=2) at the University of Virginia Biomolecular Research Facility. Data were analyzed using the Affymetrix

Microarray Suite 5.0, Affymetrix Data Mining Tool 3.0, and the University of Virginia Gene Expression Open Source System (GEOSS). Significant differences were defined as fold changes greater than 2, differences in absolute signal intensity greater than 100, and p values from local pooled error testing less than 0.05 (4). Microarray results are deposited in the Gene Expression Omnibus as accession number GSE21403.

References

1. Johnson JL, George SJ, Newby AC, and Jackson CL. Divergent effects of matrix metalloproteinases 3, 7, 9, and 12 on atherosclerotic plaque stability in mouse brachiocephalic arteries. *Proc Natl Acad Sci U S A.* 2005;102(43):15575-15580.

2. Bonthu S, Heistad DD, Chappell DA, Lamping KG, and Faraci FM. Atherosclerosis, Vascular Remodeling, and Impairment of Endothelium-Dependent Relaxation in Genetically Altered Hyperlipidemic Mice. *Arterioscler Thromb Vasc Biol.* 1997;17(11):2333-2340.

3. Geisterfer AA, Peach MJ, and Owens GK. Angiotensin II induces hypertrophy, not hyperplasia, of cultured rat aortic smooth muscle cells. *Circ Res.* 1988;62(4):749-756.

4. Jain N, Thatte J, Braciale T, Ley K, O'Connell M, and Lee JK. Local-pooled-error test for identifying differentially expressed genes with a small number of replicated microarrays. *Bioinformatics.* 2003;19(15):1945-1951.

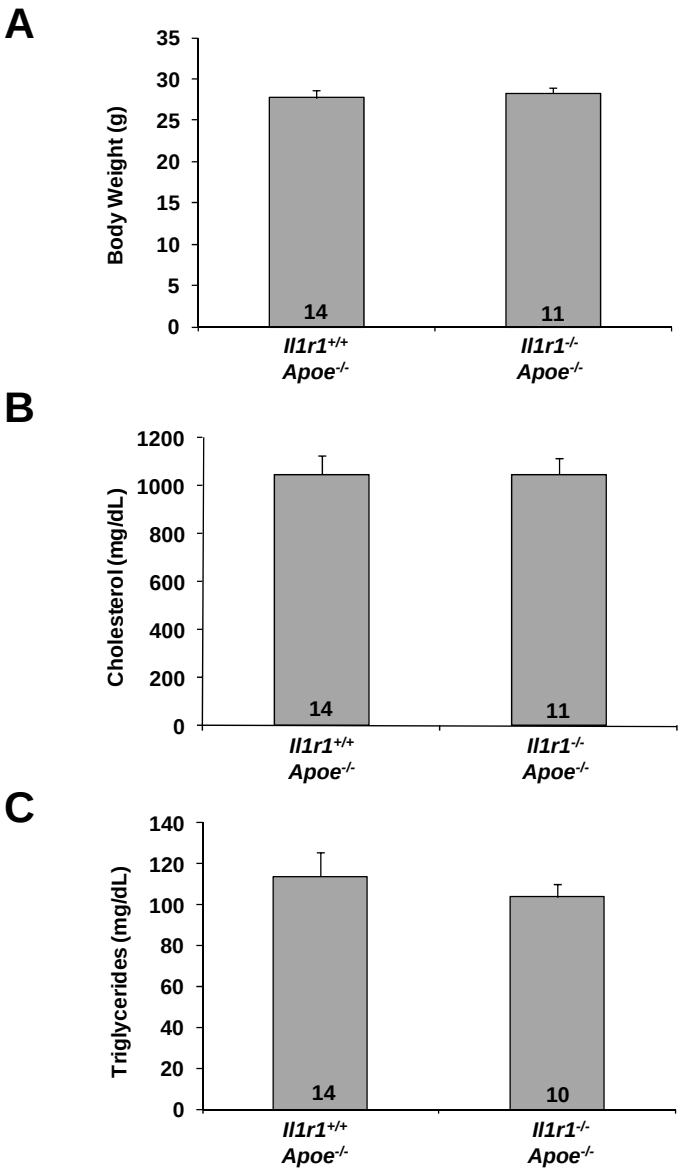
Supplemental Table 1
Matrix metalloproteinase gene expression in response to IL-1β treatment of cultured smooth muscle cells

Gene Title	Vehicle	IL-1β	Fold Change
Matrix metalloproteinase 3	5	1137	54.04
Matrix metalloproteinase 13	3	724	34.49
Matrix metalloproteinase 9	4	145	6.92
Matrix metalloproteinase 9	3	130	6.11
Matrix metalloproteinase 2	166	531	3.08
Matrix metalloproteinase 12	11	63	2.86
Matrix metalloproteinase 8	26	76	2.71
Matrix metalloproteinase 28	18	31	1.39
Matrix metalloproteinase 7	10	12	0.6
Matrix metalloproteinase 10	11	5	-0.54
Matrix metalloproteinase 23	151	159	-1
Matrix metalloproteinase 14	519	491	-1.1
Matrix metalloproteinase 24	24	15	-1.19
Matrix metalloproteinase 15	24	12	-1.2
Matrix metalloproteinase 14	3437	2884	-1.24
Matrix metalloproteinase 2	28	13	-1.37
Matrix metalloproteinase 19	180	134	-1.4
Matrix metalloproteinase 23	1648	978	-1.75
Matrix metalloproteinase 23	2551	1213	-2.18
Matrix metalloproteinase 16	83	35	-2.45
Matrix metalloproteinase 11	414	78	-5.59

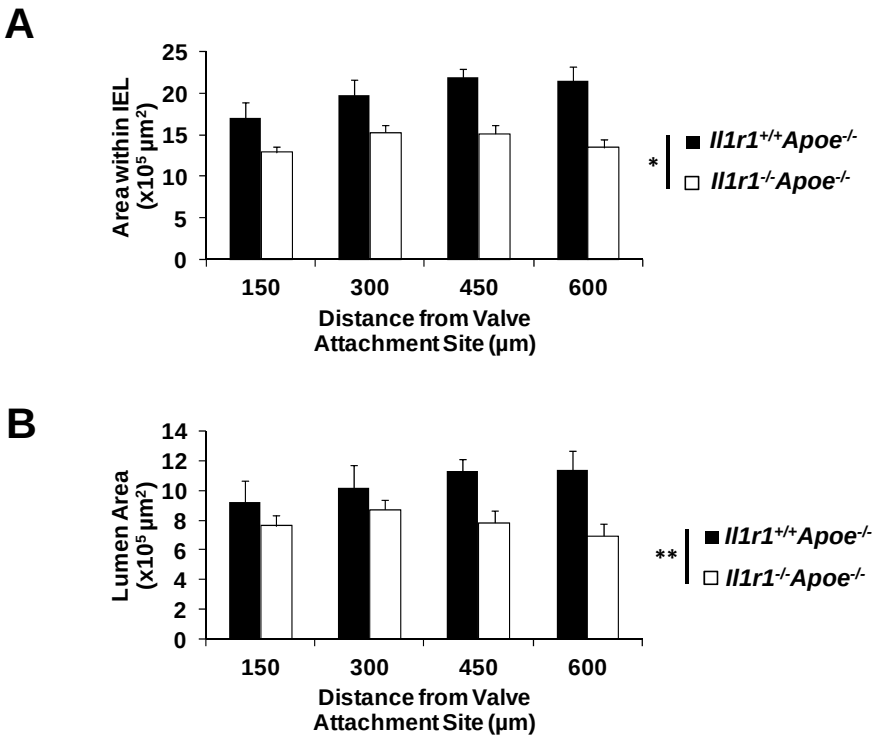
Data for vehicle and IL-1β (2.5 ng/mL)-treated SMCs represent mean values of signal intensity (n=2). Fold change of signal intensities of IL-1β treatment relative to vehicle calculated by Affymetrix Data Mining Tool. Genes in bold represent significant differences of IL-1β treatment relative to vehicle as described in Methods.

Supplemental Table 2
Data values for representative images

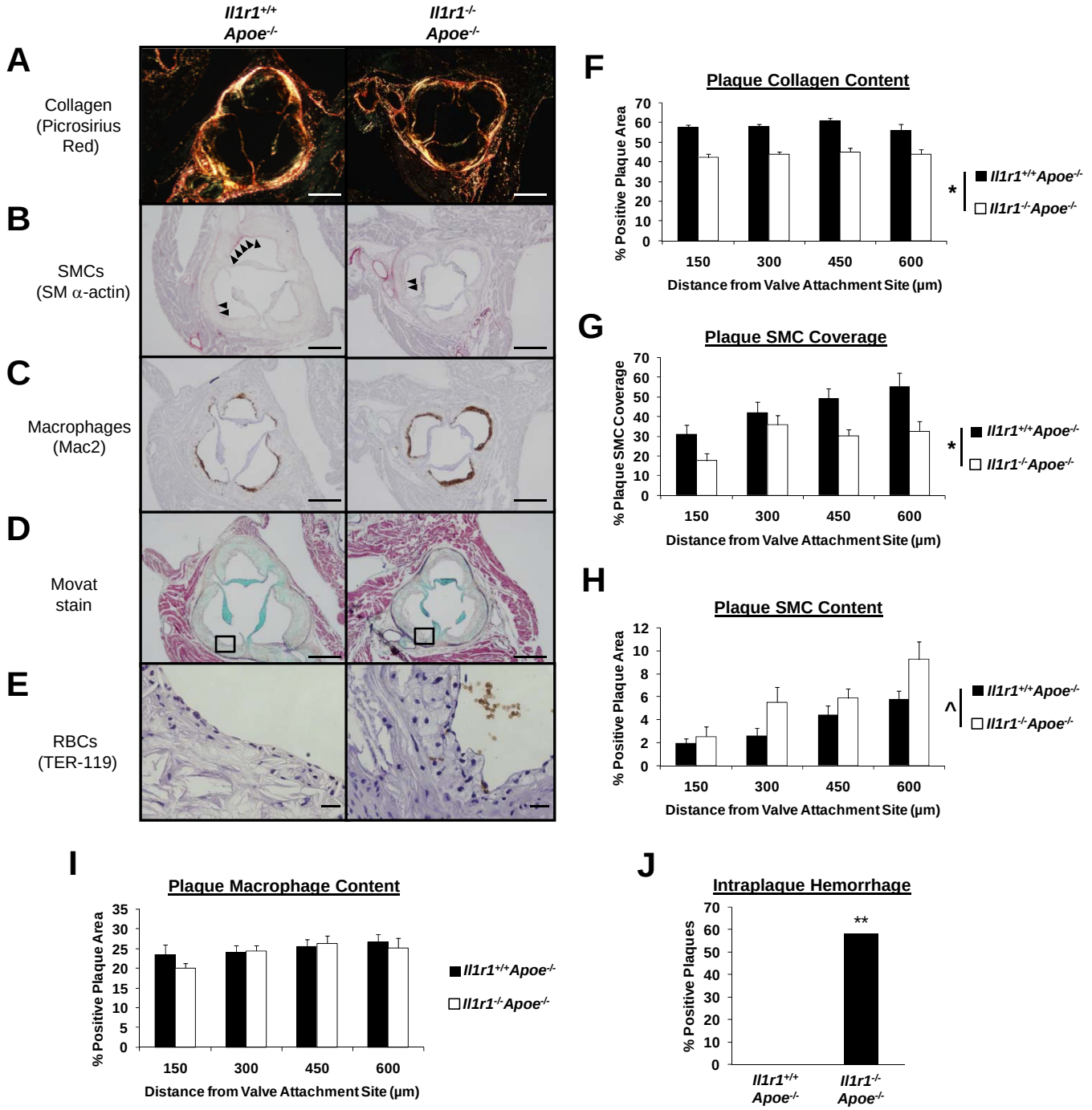
	Il1r1$^{+/+}$*Apoe*$^{-/-}$	*Il1r1*$^{-/-}$*Apoe*$^{-/-}$	Location	Figure
Plaque Area (μm^2)	1085839	564753	300 μm	1
Plaque Area (μm^2)	335802	347771	120 μm	2
Vessel Area (μm^2)	413216	371677	120 μm	2
Lumen Area (μm^2)	77413	23906	120 μm	2
Plaque Collagen (%)	45.3	11.3	360 μm	3
Plaque SMC Coverage (%)	67.3	18.7	360 μm	3
Plaque SMC Content (%)	0.29	0.10	360 μm	3
Plaque Macrophage Content (%)	9.2	10.2	360 μm	3
Plaque MMP3 (%)	27.924	0.004	0 μm	4
Medial MMP3 (%)	46.20	0.06	0 μm	4



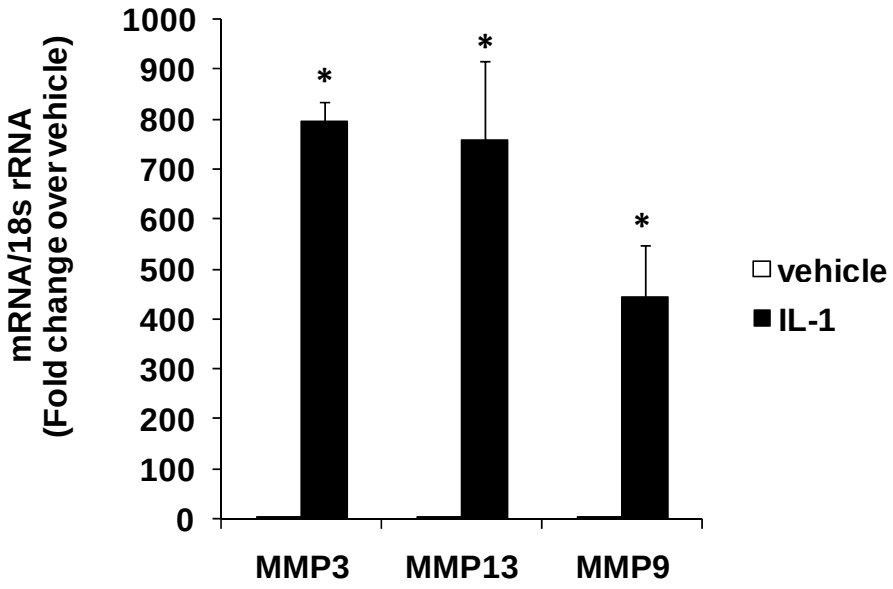
Supplemental Figure 1: IL1R1 deficiency does not significantly alter metabolic parameters. Body weight (**A**) and plasma levels of cholesterol (**B**) and triglycerides (**C**) were quantified from *Il1r1*$^{+/+}$*Apoe*$^{-/-}$ and *Il1r1*$^{-/-}$*Apoe*$^{-/-}$ mice after 27-30 weeks of high fat diet feeding. Data represents mean±SEM. The lower number in each bar represents sample size (n).



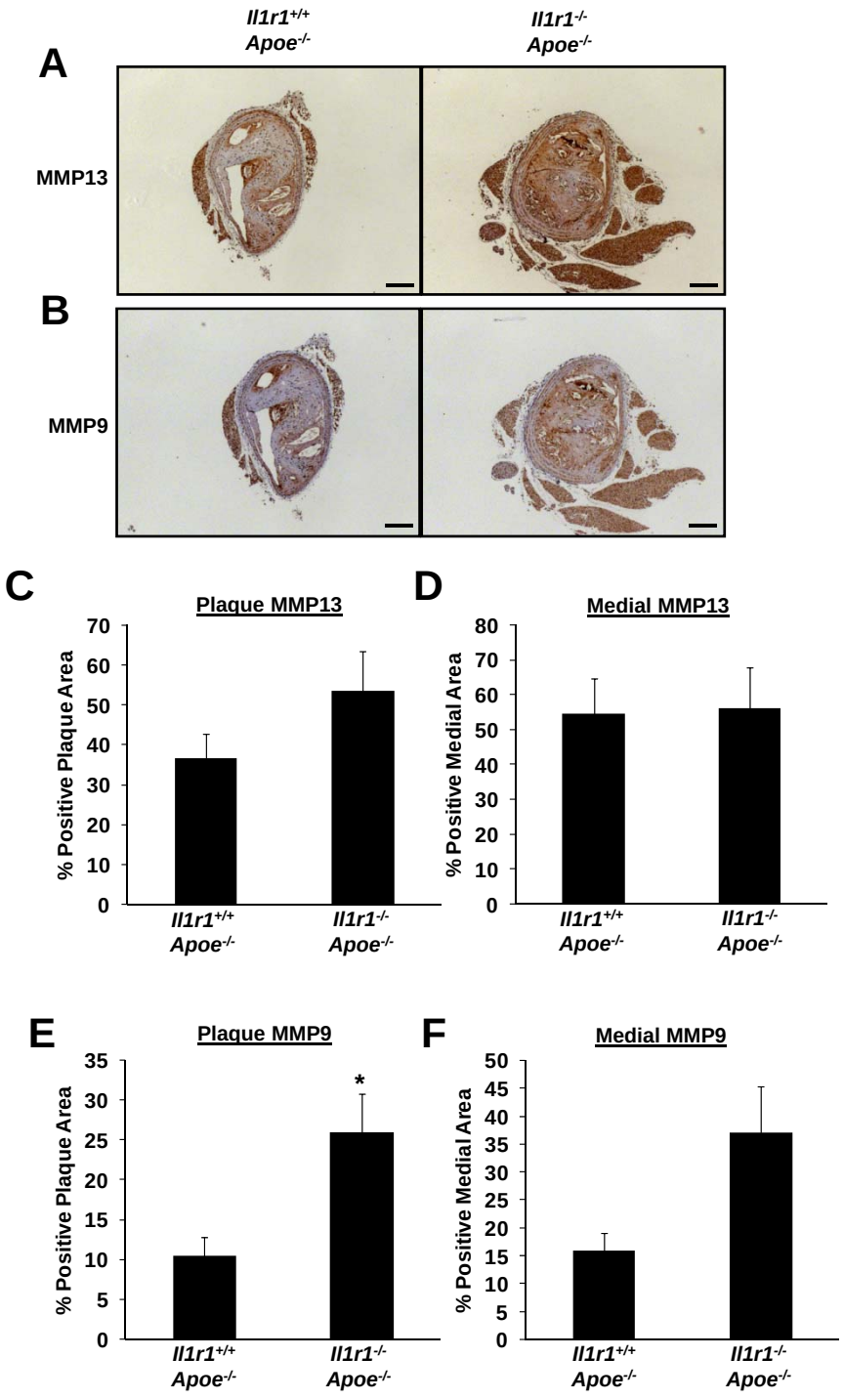
Supplemental Figure 2: IL1R1 deficiency reduces compensatory outward remodeling at the aortic root. Vessel area within the internal elastic lamina (IEL) **(A)** and lumen area **(B)** at 150 µm intervals from the aortic valve attachment site from *Il1r1*$^{-/-}$*Apoe*$^{-/-}$ and *Il1r1*$^{+/+}$*Apoe*$^{-/-}$ mice. (*p<0.001 for difference of genotype by two-way ANOVA; **p=0.001 for difference of genotype by Scheirer-Ray-Hare test). Data represent mean±SEM (n=13 *Il1r1*$^{+/+}$*Apoe*$^{-/-}$;n=12 *Il1r1*$^{-/-}$*Apoe*$^{-/-}$). Representative images from which these measurements are derived are found in Figure 1A.



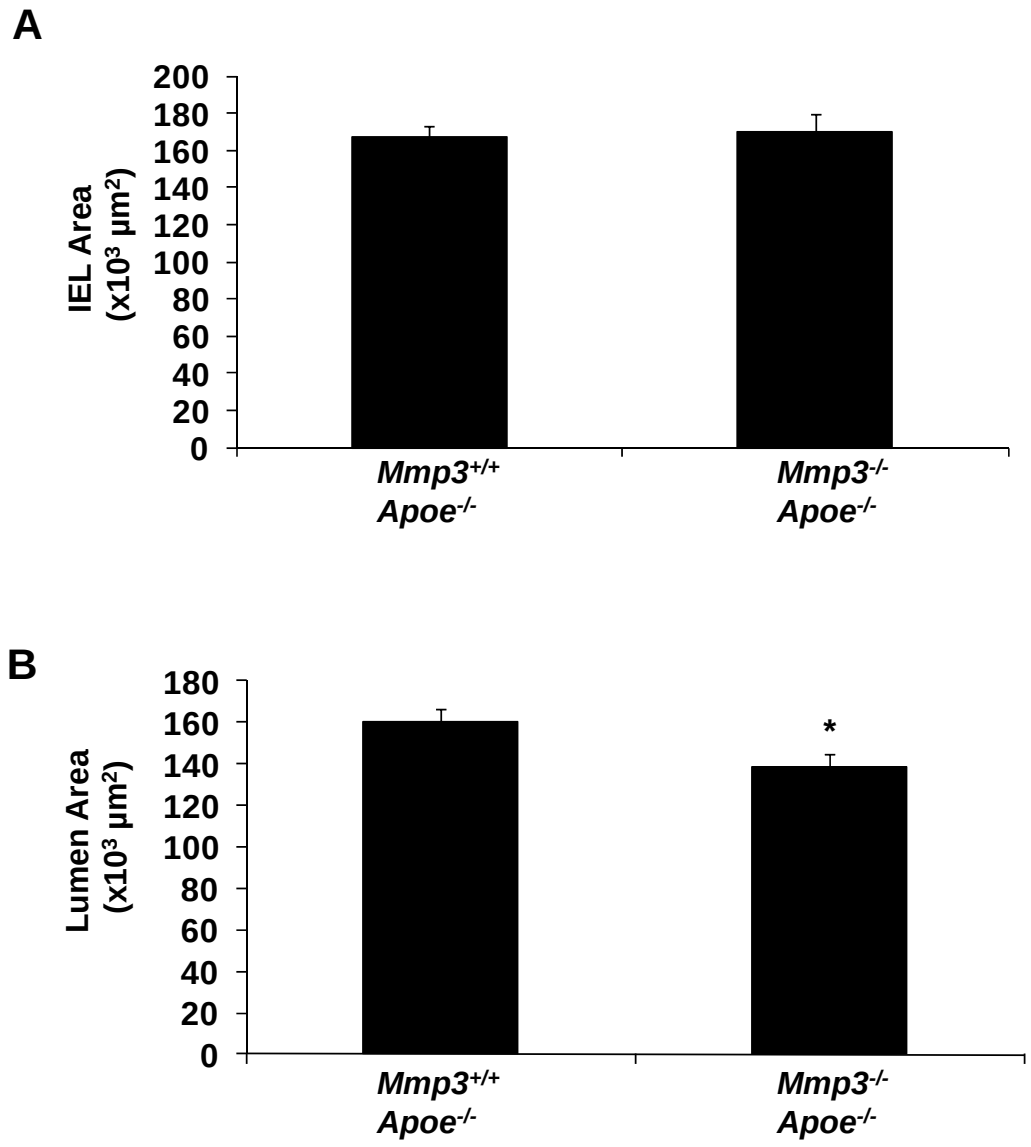
Supplemental Figure 3: IL1R1 deficiency increases features of atherosclerotic plaque instability at the aortic root. (**A-E**) Representative images from aortic root lesions of *Il1r1*$^{+/+}$*Apoe*$^{-/-}$ and *Il1r1*$^{-/-}$*Apoe*$^{-/-}$ mice with (**A**) picrosirius red staining and polarized light microscopy for collagen detection, (**B**) SM α–actin immunostaining for detecting SMCs on the plaque luminal surface (arrowheads) and total plaque SMC content, (**C**) Mac2 immunostaining for detection of plaque macrophages, (**D**) Movat staining for intraplaque RBCs, and (**E**) immunostaining for the RBC marker TER-119 (magnified from boxed area in **D**). (**F-J**) Quantification of (**F**) plaque collagen content based on picrosirius red staining, (**G**) plaque SMC coverage based on SM α-actin staining, (**H**) total plaque SMC content based on SM α–actin staining, (**I**) plaque macrophage content based on Mac2 staining, and (**J**) the percentage of brachiocephalic arteries exhibiting intraplaque hemorrhage based on Movat and TER-119 staining (*p<0.001 for difference of genotypes by two-way ANOVA, ^p=0.01 for difference of genotypes by two-way ANOVA after square root transformation, **p=0.002 by Fisher's Exact test). Data in (**F-I**) represent mean±SEM (n=13 *Il1r1*$^{+/+}$*Apoe*$^{-/-}$;n=12 *Il1r1*$^{-/-}$ *Apoe*$^{-/-}$). (**A-D**) Scale bar=500 μm; (**E**) Scale bar=20 μm.



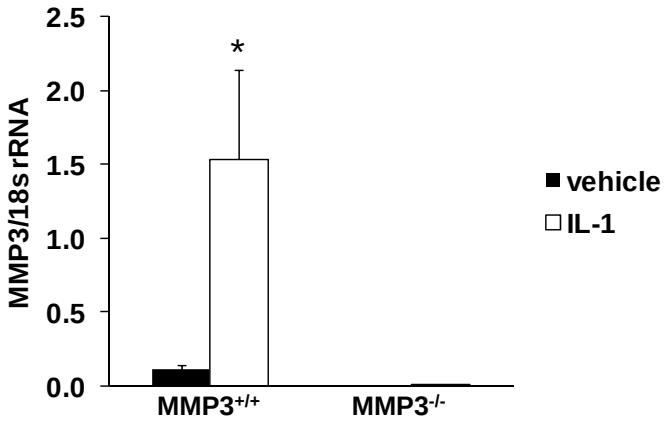
Supplemental Figure 4: IL-1β strongly induced expression of multiple matrix metalloproteinases in cultured SMCs. Real-time RT-PCR analysis of rat aortic SMCs grown to confluency in serum-containing media, followed by three days in serum-free media prior to treatment for 24 hours with vehicle or IL-1β (2.5 ng/mL) to determine mRNA levels of MMP3, MMP13, and MMP9. Data represent mean±SEM of 3 independent experiments. *p<0.001 versus vehicle by nested ANOVA.



Supplemental Figure 5: IL1R1 deficiency does not alter MMP13 levels within the plaque or vessel media but increases plaque MMP9 levels. (**A,B**) Representative images of MMP13 (**A**) and MMP9 (**B**) staining within atherosclerotic braciocephalic arteries just beyond the junction with the aortic arch from *Il1r1*$^{+/+}$*Apoe*$^{-/-}$ and *Il1r1*$^{-/-}$*Apoe*$^{-/-}$ mice. Scale bar=200 µm. (**C-F**) Quantification of the percent positive area within the plaque (**C,E**) and vessel media (**D,F**) for MMP13 (**C,D**) and MMP9 (**E,F**) from *Il1r1*$^{+/+}$*Apoe*$^{-/-}$ and *Il1r1*$^{-/-}$*Apoe*$^{-/-}$ mice (* $p<0.01$ by Student's t test). Data represent mean±SEM.



Supplemental Figure 6: MMP3 deficiency reduces outward remodeling of atherosclerotic brachiocephalic arteries. *Mmp3*$^{-/-}$*Apoe*$^{-/-}$ mice fed a high fat diet for 8 weeks did not demonstrate differences in IEL (internal elastic lamina) size (**A**) but exhibited reduced lumen size (**B**) within atherosclerotic brachiocephalic arteries relative to control *Mmp3*$^{+/+}$*Apoe*$^{-/-}$ mice (n=26, *p<0.01 by Mann-Whitney rank-sum test). Data represent mean±SEM.



Supplemental Figure 7: IL-1β induces MMP3 expression in MMP3$^{+/+}$ but not MMP3$^{-/-}$ SMCs. Real-time RT-PCR analysis of MMP3$^{+/+}$ and MMP3$^{-/-}$ SMCs grown to confluency in serum-containing media, then switched to serum-free media for two days followed by treatment for 24 hours with vehicle (0.1% BSA) or IL-1β (5 ng/mL) to match the conditions of the invasion assays (*$p<0.05$ versus vehicle by nested ANOVA). Data represent mean±SEM of 3 independent experiments.