SUPPLEMENTAL MATERIALS

Dichotomous roles of smooth muscle cell-derived MCP1 in development of atherosclerosis

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Major Resources Table

In order to allow validation and replication of experiments, all essential research materials listed in the Methods should be included in the Major Resources Table below. Authors are encouraged to use public repositories for protocols, data, code, and other materials and provide persistent identifiers and/or links to repositories when available. Authors may add or delete rows as needed.

Animals (in vivo studies)

Species	Vendor or Source	Background Strain	Sex	Persistent ID / URL
Myh11-Dre^{ERT2} Lgals3-Cre Rosa-tdTomato-eGFP ApoE$^{-/-}$ mice	Owens lab, described in Alencar et al Circulation 2020	C57/Bl6J	M/F	
Myh11-Cre^{ERT2} eYFP ApoE$^{-/-}$ Klf4$^{wt/wt}$ and Klf4$^{\Delta/\Delta}$	Owens lab, described in Shankman et al Nature Medicine 2015	C57/Bl6J	M	
MCP1-mCherry$^{flox/flox}$	The Jackson Laboratory	C57/Bl6J	M/F	016849
MCP1$^{flox/flox}$	The Jackson Laboratory	C57/Bl6J	M/F	023347

Genetically Modified Animals

		Species	Other Information
MCP1^{SMC}	**Parent - Male**	Myh11-Cre^{ERT2} eYFP ApoE$^{-/-}$ MCP1$^{wt/flox}$	
	Parent - Female	eYFP ApoE$^{-/-}$ MCP1$^{wt/flox}$	
MCP1$^{SMC\text{-}Lgals3}$	**Parent 1**	Myh11-Dre^{ERT2} Rosa-tdTomato-eGFP ApoE$^{-/-}$ MCP1$^{wt/flox}$	Because Myh11-Dre^{ERT2} is not on the Y chromosome, parents of both sexes and genotypes were used
	Parent 2	Lgals3-Cre Rosa-tdTomato-eGFP ApoE$^{-/-}$ MCP1$^{wt/flox}$	

Antibodies

Target antigen	Vendor or Source	Catalog #	Working concentration	Lot # (preferred but not required)	Persistent ID / URL
GFP	Abcam	ab6673	1:250		
tdTomato	Rockland	600-401-379	1:100		

Lgals3	Cedarlane	CL8942AP	1:500		
Acta2-Cy3	Sigma Aldrich	C6198	1:500 of 1.2 mg/mL		
Acta2-FITC	Sigma Aldrich	F3777	1:500 of 2.2 mg/mL		
Donkey anti-goat AF488	Invitrogen	A11055	1:250		
Donkey anti-goat AF647	Invitrogen	A21447	1:250		
Donkey anti-rabbit AF546	Invitrogen	A10040	1:250		
Donkey anti-rat DyLight 650	Abcam	Ab102263	1:250		
CD45-BV650	Biolegend	103151	0.156 uL per million cells		
CD11b-APC-eF780	Biolegend	101211	0.312 uL per million cells		
Ly6C-APC	Biolegend	128015	0.625 uL per million cells		
CD3e-PerCP	Biolegend	100325	0.625 uL per million cells		
CD4 –PE-Cy5.5	Biolegend	100410	1.25 uL per million cells		
CD8a-PE-Cy7	Biolegend	100722	0.625 uL per million cells		
CD44-BV711	Biolegend	103057	0.078 uL per million cells		
CD62L-BV605	Biolegend	104438	1.25 uL per million cells		
Ly6G-PE	Biolegend	127607	0.005 uL per million cells		
Ly6G-PerCP-Cy5.5	Biolegend	127615	0.312 uL per million cells		

Cultured Cells

Name	Vendor or Source	Sex (F, M, or unknown)	Persistent ID / URL
Mouse smooth muscle cells	8 week old Myh11-Cre^{ERT2} eYFP $ApoE^{-/-}$ SMC lineage tracing mice, sorted by flow cytometry for lineage tracing marker after tamoxifen injection	M	

Data & Code Availability

Description	Source / Repository	Persistent ID / URL
Single cell RNAseq data from 18 wk Western diet fed MCP1$^{SMC\text{-}Lgals3}$ mouse plaques	GEO	GSE204994

Other

Description	Source / Repository	Persistent ID / URL
RNAscope MCP1 probe, positive control probe, negative control probe	ACD bio	311791-C3 320871 323100

ARRIVE GUIDELINES

The ARRIVE guidelines (https://arriveguidelines.org/) are a checklist of recommendations to improve the reporting of research involving animals. Key elements of the study design should be included below to better enable readers to scrutinize the research adequately, evaluate its methodological rigor, and reproduce the methods or findings.

Study Design

Groups	Sex	Age	Number (prior to experiment)	Number (after termination)	Littermates (Yes/No)	Other description
MCP1$^{SMC\ wt/wt}$ Cohort 1 (BCA lesion morphometry, immunostaining, and flow cytometry)	M	26-28 wks	12	12	Yes	SMC lineage tracing of females not possible in this model
MCP1$^{SMC\ wt/fl}$ Cohort 1	M	26-28 wks	15	11	Yes	See above
MCP1$^{SMC\ fl/fl}$ Cohort 1	M	26-28 wks	11	9	Yes	See above
MCP1$^{SMC\ wt/wt}$ Cohort 2 (bead injection, frozen plaque samples)	M	26-28 wks	6	5	Yes	SMC lineage tracing and specific knockout of genes in females not possible in this model

MCP1$^{SMC\ wt/fl}$ Cohort 2	M	26-28 wks	17	15	Yes	See above
MCP1$^{SMC\ fl/fl}$ Cohort 2	M	26-28 wks	7	7	Yes	See above
MCP1$^{SMC\text{-}Lgals3\ wt/wt}$	M/F	26-28 wks	16	16	Yes	This model allows lineage tracing and knockout in males and females
MCP1$^{SMC\text{-}Lgals3\ wt/fl}$	M/F	26-28 wks	17	17	Yes	
MCP1$^{SMC\text{-}Lgals3\ fl/fl}$	M/F	26-28 wks	18	14	Yes	

Sample Size: Please explain how the sample size was decided. Please provide details of any a *prior* sample size calculation, if done.

Power analysis was done using R to determine sample sizes for each experiment, including an expected difference of 30%, variation of 5%, and 80% power level.

Inclusion Criteria

Viable mice born at UVA MR5 animal facility, able to be genotyped with tail snip

Exclusion Criteria

Suffers from common mouse congenital/genetic abnormality ie hydrocephalus, over-grooming >20% of body hair, malocclusion

For 18 wk Western diet experiments, blood cholesterol at harvest <200

Structural anomaly found at harvest (large aneurysm, atypical aortic arch anatomy)

Statistical outliers defined with ROUT criteria

Randomization

Mice are assigned a 6 digit identification number prior to first genotyping. Each experiment uses littermates breeding from heterozygous parents, so the expected ratio is 1:2:1 wt/het/knockout. After genotyping, mice are assigned to a cohort of animals for each experiment based on age. Their number identifies them for the duration of the experiment and analysis. At the end of analysis, animal numbers are re-correlated with the genotype.

Blinding

6 digit numbers are used during all steps of analysis (for example, fibrous cap quantification) so that the measuring scientist is not aware of the genotype when making measurements. At the end of the experiment, animal numbers are to re-correlated to the genotype/experimental group.

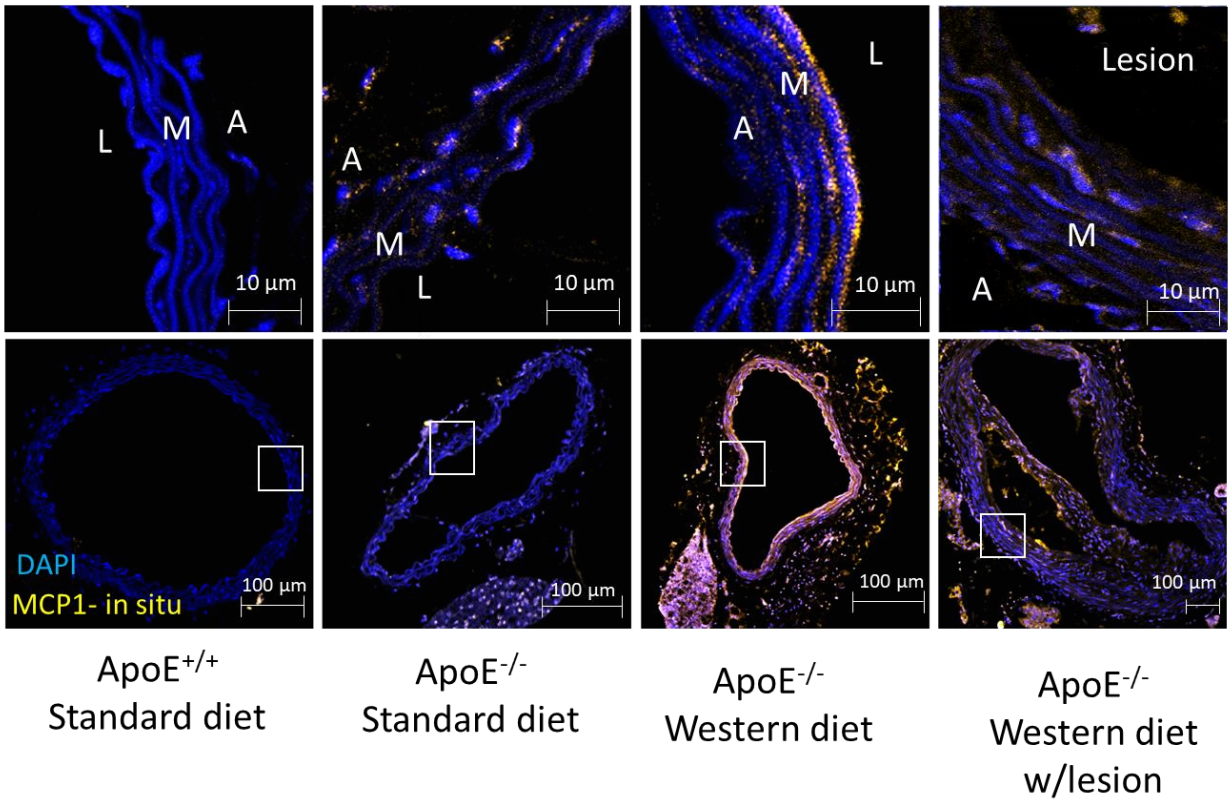
Supplemental Methods:

RNA hybridization

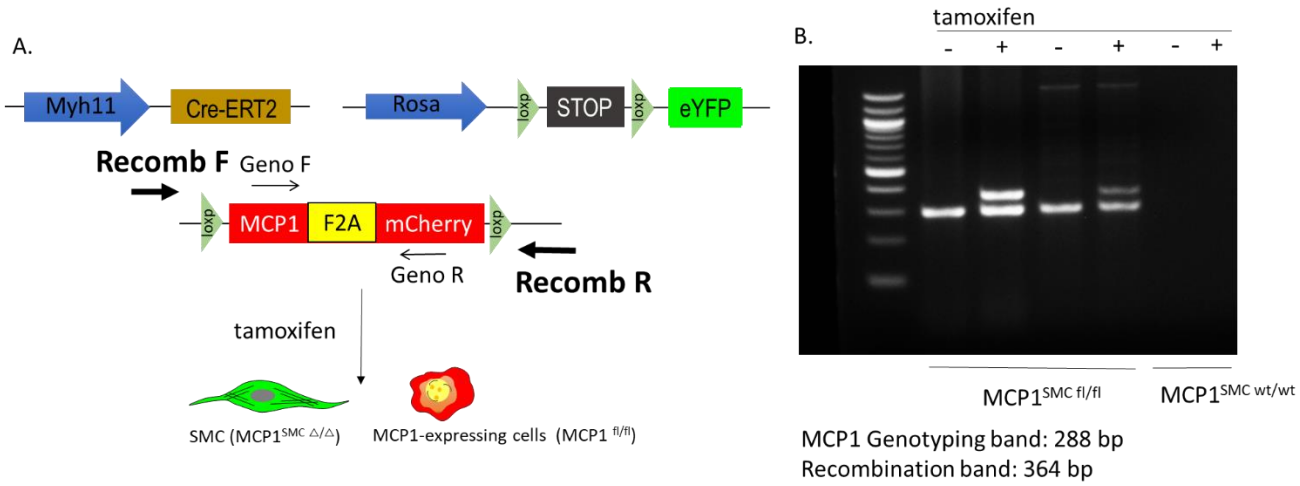
RNA hybridization probes for mouse MCP1/CCL2, negative control probes, and detection probes were purchased from the RNAscope system (ACDbio, #311791-C3, #320871, #323100). Paraffin sections of BCA from WT or ApoE-/- mice with or without 10 weeks of Western diet feeding were freshly cut using a microtome cleaned with RNAse Zap (ThermoFisher #AM9780). Sections were baked at 60 degrees Celsius for 1 hour and treated as per the manufacturer's instructions for deparaffinizing, pretreating, target retrieval, hybridization, and detection, and imaged using a Zeiss LSM700 confocal microscope.

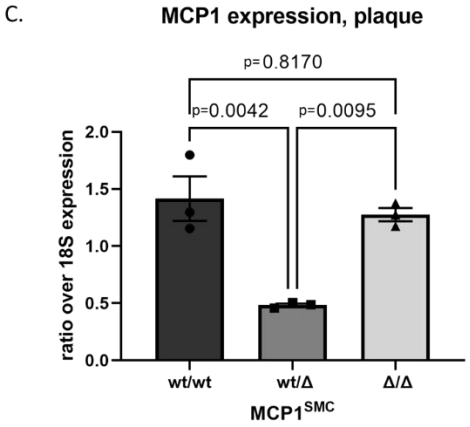
Cell culture and cholesterol loading

Primary mouse aortic SMCs were isolated using a previously described protocol[4] from 8-week old SMC-lineage tracing mice after a series of ten tamoxifen injections Cholesterol assays were performed on primary mouse aortic lineage-traced SMCs using water-soluble cholesterol from Sigma (C4951-30MG) as previously published. Cells were allowed to grow to ~70% confluency in DF10 (DMEM-F12 medium (Gibco) containing 10% FBS (Gibco), 100 U/ml penicillin/streptomycin (Gibco) and 1.6 mmol/liter l-glutamine (Gibco)) before being switched to 0, 20, 40, or 80 μg/ml cholesterol-containing medium. After 72 h, Total RNA was extracted from cultured cells using Trizol reagent (Invitrogen) according to the respective manufacturer's instructions. One microgram of RNA was used to perform a reverse transcription with iScript cDNA Synthesis Kit (Bio-Rad). A SensiFAST SYBR NO-ROX Mix (Bioline) was used to carry out RT-PCR on a C1000 Thermal Cycler CFX96TM (Bio-Rad).



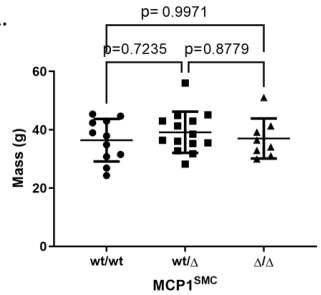
Supplemental Figure 1. Vascular smooth muscle cells express MCP1 in ApoE$^{(-/-)}$ mice.
Brachiocephalic arteries (BCA) of wild-type or ApoE$^{-/-}$ mice fed standard laboratory diet or Western diet for 10 weeks. In situ RNA hybridization (RNAscope) for MCP1 mRNA (yellow) was performed on 10 um paraffin sections, counterstained with DAPI, and imaged with high-resolution confocal microscopy at 20x, 0.5x zoom in z-stacks of 10 um thickness. Images are displayed as maximum intensity projections of the arterial wall (labeled L for lumen, M for media and A for adventitia), with zoomed in panels at top and full size images at bottom.





Supplemental Fig 2. MCP1 $^{SMC\ fl/fl}$ mice show efficient tamoxifen-dependent labeling and MCP1 knockout in SMC. A) Schematic of experimental mouse model of Myh11-Cre^{ERT2} eYFP ApoE$^{-/-}$ MCP1 SMC-specific lineage tracing MCP1 knockout model. (MCP1^{SMC}). B) Aortas from MCP1$^{SMC\ fl/fl}$ and MCP1$^{SMC\ wt/wt}$ mice with and without tamoxifen injection were flash frozen and extracted for DNA. PCR was done in combination for the MCP1 transgene (genotyping band at 288 bp) and a recombination product only able to be amplified in tamoxifen-induced knockout. C) Plaque samples were collected from MCP1^{SMC} wt/wt, wt/fl, and fl/fl mice after 18 weeks of Western diet and frozen for RNA expression analysis by qRTPCR. Values were corrected for total RNA content by standardizing starting quantities by 18S expression in each sample. Samples were then compared to the wild-type to produce the values in Supplemental Figure 2C. n=4 animals per group in two separate experiments.

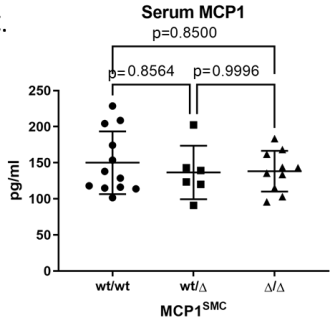
A.



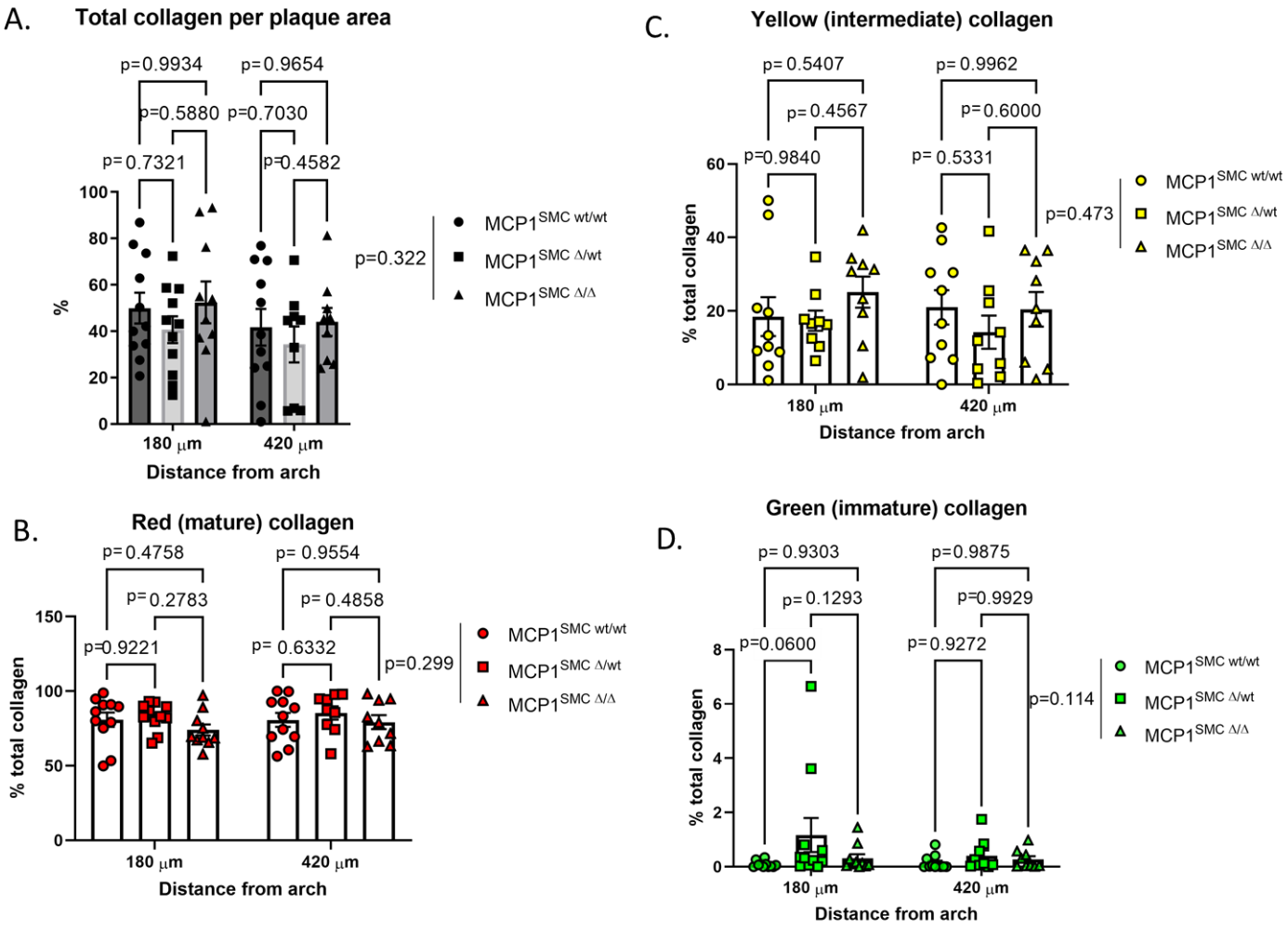
B.

	MCP1$^{SMC\ wt/wt}$	MCP1$^{SMC\ wt/\Delta}$	MCP1$^{SMC\ \Delta/\Delta}$
Mass (g)	36.42 +/- 2.198	39.14 +/- 1.889	37.01 +/- 2.433
Cholesterol(mg/dl)	698+/- 301	863+/- 297	951 +/- 513
Spleen	0.425 +/- 0.037	0.549 +/- 0.153	0.552 +/- 0.056
Liver	1.83 +/- 0.149	1.93 +/- 0.134	1.82 +/- 0.138
Lungs	0.283 +/- 0.028	0.279 +/- 0.026	0.335 +/- 0.054
Kidney	0.243 +/- 0.019	0.245 +/- 0.010	0.227 +/- 0.015
Heart	0.198 +/- 0.007	0.2 +/- 0.017	0.224 +/- 0.015
Adipose (sq)	0.369 +/- 0.101	0.460 +/- 0.064	0.392 +/- 0.079
Adipose (gon)	0.738 +/- 0.149	0.934 +/- 0.096	0.783 +/- 0.149
Number of animals	12	15	9

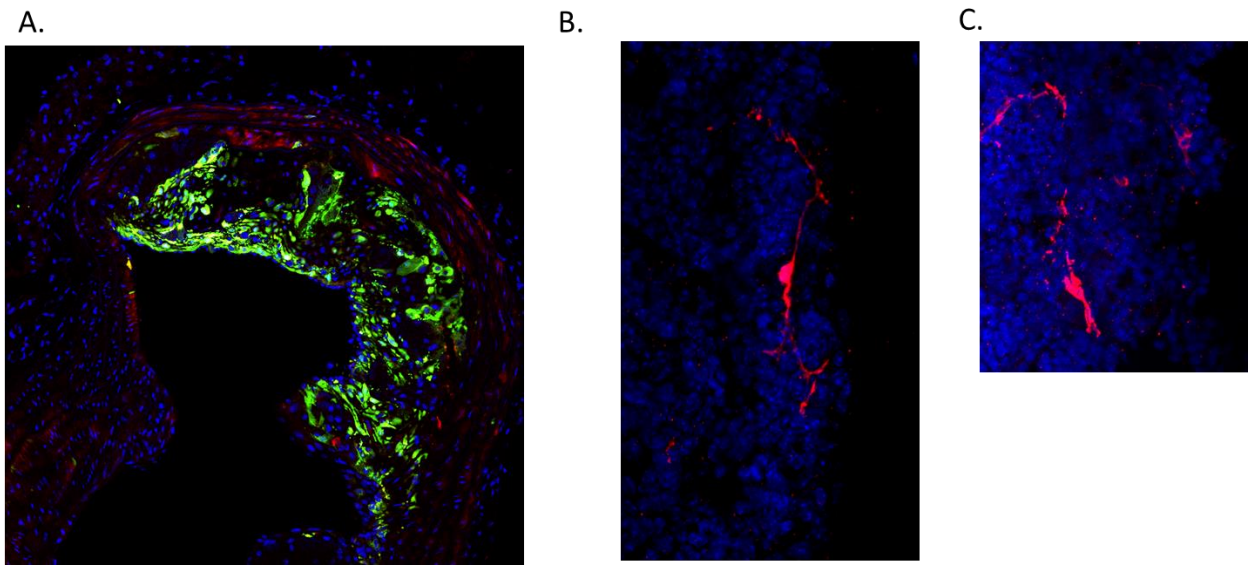
C.



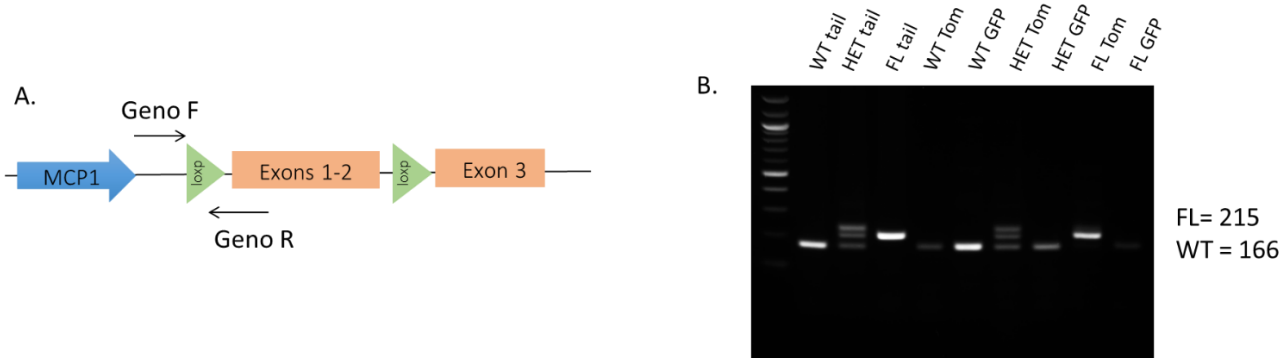
Supplemental Figure 3. There were no significant differences in body weight, organ weights, serum cholesterol, or serum MCP1 in MCP1$^{SMC\ wt/wt}$, MCP1$^{SMC\ wt/\Delta}$, and MCP1$^{SMC\ \Delta/\Delta}$ mice after 18 weeks of Western diet A) Mass of MCP1$^{SMC\ wt/wt}$, MCP1$^{SMC\ wt/\Delta}$, and MCP1$^{SMC\ \Delta/\Delta}$ animals after 18 weeks of Western diet. B) Additional harvest parameters including total mass, cholesterol level, and mass of various organs. C) Blood was taken from MCP1^{SMC} mice after 18 weeks of Western diet and ELISA for MCP1 was performed on serum.



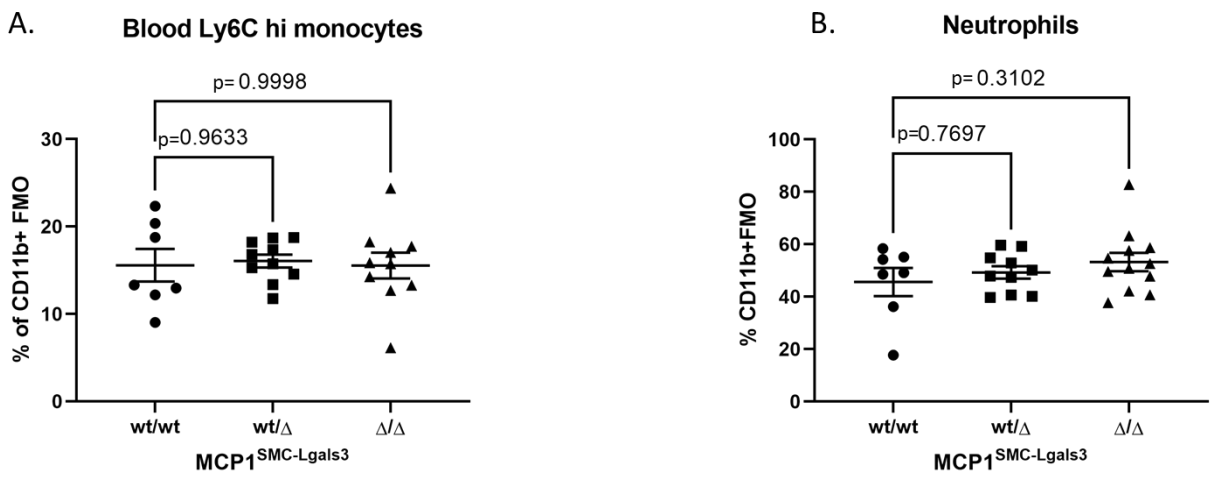
Supplemental Figure 4. There were no differences in BCA lesion collagen content in MCP1$^{SMC\ wt/wt}$, MCP1$^{SMC\ wt/\Delta}$ and MCP1$^{SMC\ \Delta/\Delta}$ mice. A) Total collagen per plaque area in BCA lesion sections from MCP1$^{SMC\ wt/wt}$, MCP1$^{SMC\ wt/\Delta}$ and MCP1$^{SMC\ \Delta/\Delta}$ mice was measured by Picosirus red staining and normalized to total plaque area. n= 9-15 mean +/- SEM B) Percent of collagen made up of red (mature) C yellow (intermediate) and D) green (immature) collagen is shown as percentage of total collagen in the lesion. No significant variation due to genotype were observed by 2 way ANOVA or post-hoc pairwise comparisons.



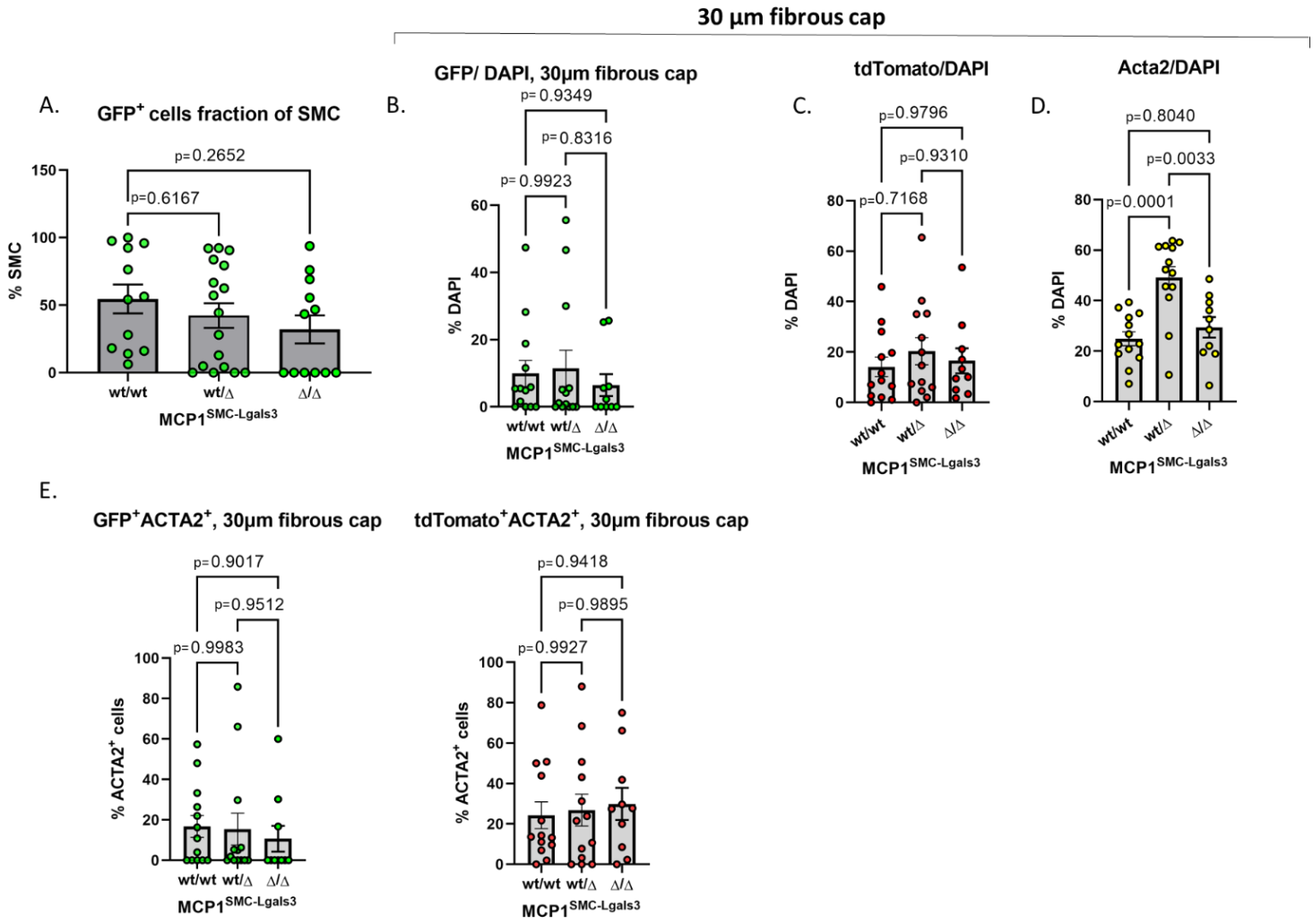
Supplemental Figure 5. Smooth muscle cells in perivascular bone marrow niche have not activated Lgals3. A) Image of Myh11-DreERT2 Lgals3-Cre Rosa-Tomato-GFP ApoE$^{-/-}$ mouse brachiocephalic artery (adapted from Alencar et al 2020) stained for GFP, tdTomato, and counterstained with DAPI, showing GFP^{+} cells in atherosclerotic lesions. B-C) Femur from Myh11-Dre^{ERT2} Lgals3-Cre Rosa-Tomato-GFP ApoE$^{-/-}$ mice were harvested, decalcified in EDTA for 12 hours, sectioned longitudinally to visualize bone marrow, stained for DAPI and and imaged for tdTomato and GFP fluorescence on a Leica LSM700 confocal microscope.



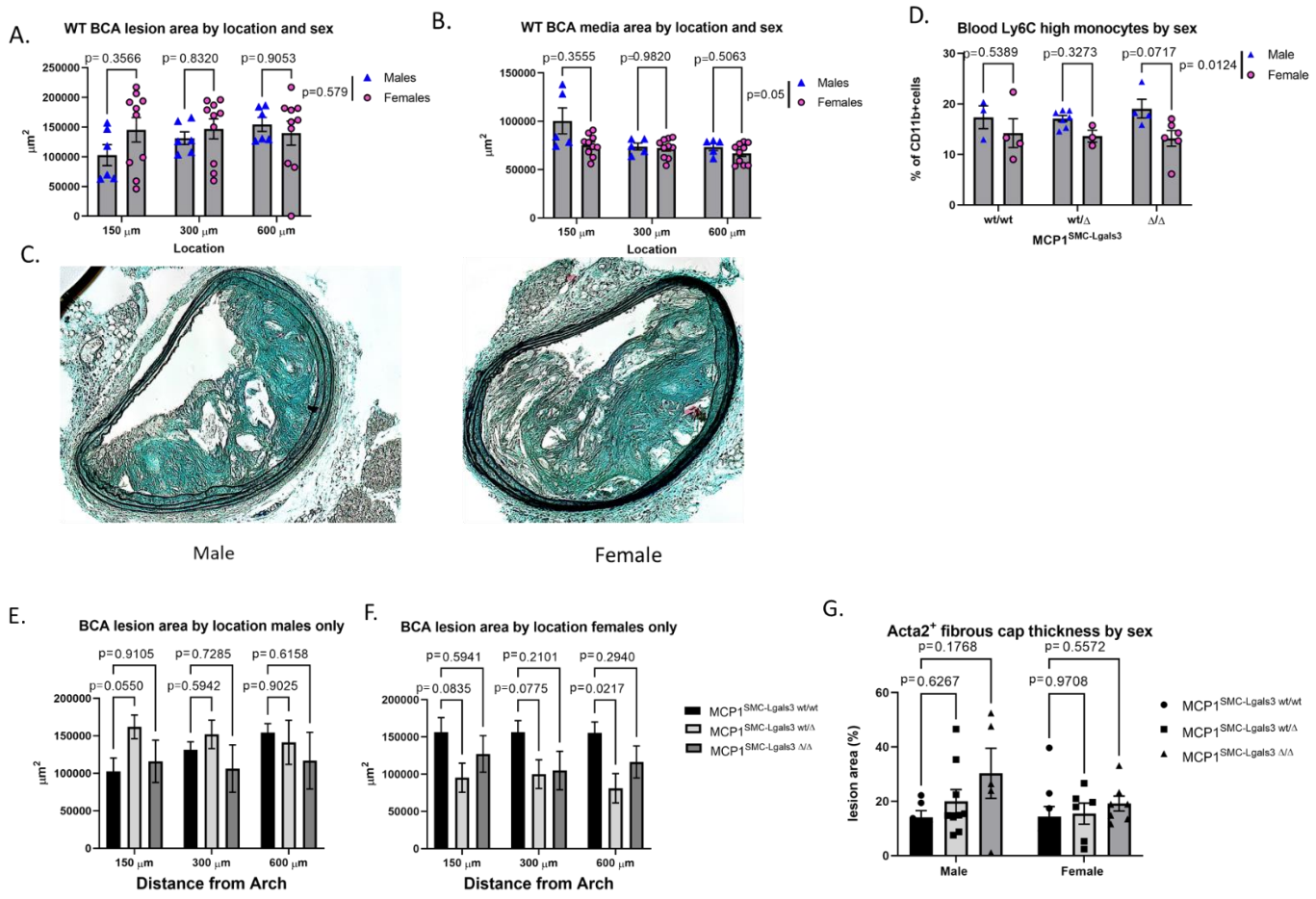
Supplemental Fig 6. MCP1$^{SMC\text{-}Lgals3}$ knockout validation. Atherosclerotic aortas from MCP1$^{SMC\text{-}Lgals3\ wt/wt}$, MCP1$^{SMC\text{-}Lgals3\ wt/\Delta}$, and MCP1$^{SMC\text{-}Lgals3\ \Delta/\Delta}$ mice were sorted for tdTomato^{+} and GFP^{+} cells. Tail biopsies were also taken from each animal for genotyping. A) Schematic of MCP1 flox allele locus and primers. B) amplification of tail DNA or DNA from tdTomato+ or GFP+ sorted cells with genotyping primers. The loss of the 215 bp band in the Het and Fl GFP sorted cells indicates recombination.



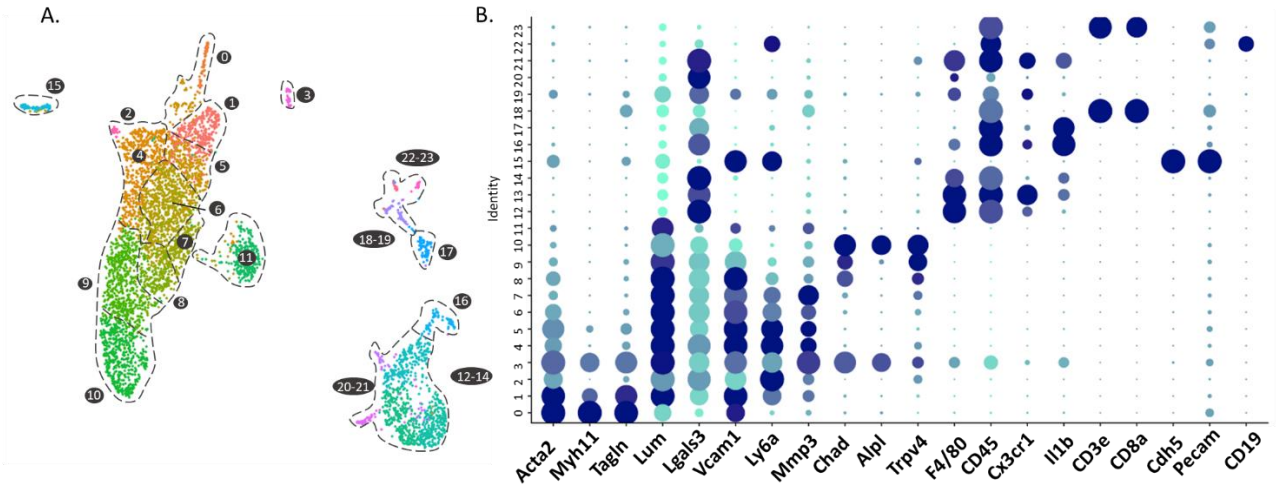
Supplemental Figure 7. MCP1 knockout in SMC transitioned through Lgals3 does not alter Ly6C^{hi} monocytes in blood after 18 weeks Western diet. A) Peripheral blood from MCP1$^{SMC\text{-}Lgals3\ wt/wt}$, MCP1$^{SMC\text{-}Lgals3\ wt/\Delta}$, and MCP1$^{SMC\text{-}Lgals3\ \Delta/\Delta}$ mice was collected at harvest, stained for lymphocyte markers, and analyzed by flow cytometry. Ly6C^{hi} cells were gated on live, single CD45+CD11b+ Ly6G- cells with fluorescence-minus-one controls. n=7-10, mean +/- SEM. B) Neutrophils gated on live, single CD45+CD11b+Ly6G+ cells with fluorescence-minus-one controls n=7-10, mean +/- SEM

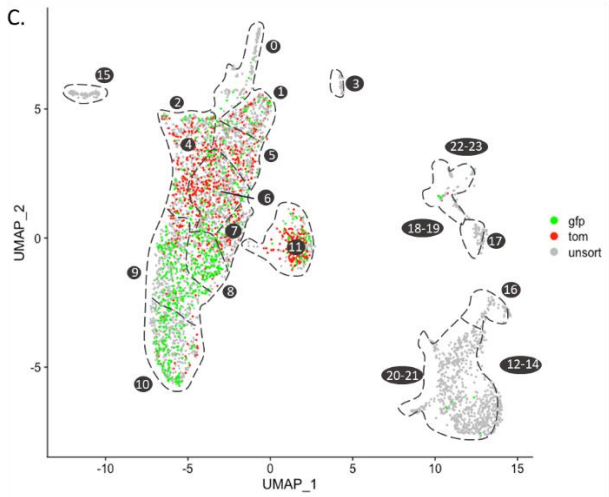


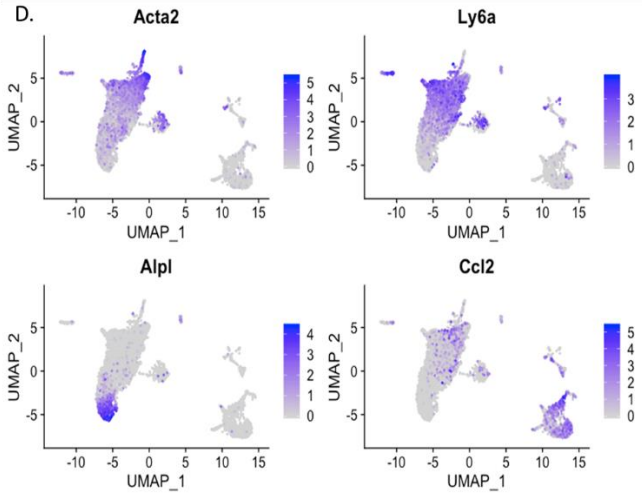
Supplemental Figure 8. High-resolution confocal analysis and quantification of eGFP$^+$ cells as a percentage of total lesion SMC, and quantification of cell types in the 30µm fibrous cap. A) Individual cells were quantified by single cell counting from confocal images from Figure 5 (MCP1$^{SMC\text{-}Lgals3}$ mouse BCA sections stained for GFP, tdTomato, Lgals3, and DAPI). GFP cells, when present, made up a majority of lesion SMCs, consistent with *Alencar et al* 2020. No differences were found to be significant by one way ANOVA. B-E) Individual cells were quantified from the 30 µm fibrous cap area in images from Figure 4B (MCP1$^{SMC\text{-}Lgals}$ mouse BCA sections stained for GFP, tdTomato, Acta2, and DAPI). In contrast to Figure 4B, which measures all layers of ACTA2+ cells in contact with the lumen, these analyses were conducted on a uniform area (30µm in contact with the lumen). B shows GFP cells in the 30µm cap, C shows tdTomato cells, and D shows ACTA2$^+$ cells. E. compares GFP$^+$ and tdTomato$^+$ prevalence among ACTA2$^+$ cells in the 30µm fibrous cap. Although differences were not significant between MCP1 genotypes, a greater fraction of ACTA2$^+$ cells in the cap were tdTomato (20-40%) compared to GFP (10-15%) (variation all GFP$^+$ ACTA2$^+$ vs tdTomato$^+$ ACTA2$^+$ cells 2 way ANOVA p=0.0355)



Supplemental Fig 9. Male and female MCP1$^{SMC\text{-}Lgals3\ wt/wt}$ **mice have similar atherosclerosis burden but may have different response to MCP1 deletion** A) BCA lesion sizes from the MCP1$^{SMC\text{-}Lgals3\ wt/wt}$ group are plotted by sex at three locations along the BCA. No differences due to sex were detected by overall two-way ANOVA or multiple comparison tests. B) Media sizes (measured as external elastic lamina to internal elastic lamina) from male and female MCP1$^{SMC\text{-}Lgals3\ wt/wt}$ animals were measured from sections in A and analyzed as above. C) Representative male and female BCA plaque sections from mice in A. D) Ly6C^{hi} monocytes in peripheral blood from MCP1$^{SMC\text{-}Lgals3\ wt/wt}$ animals in Supplemental Figure 7 were compared by sex. P value refers to variability due to sex across all genotypes by two-way ANOVA. No difference by genotype was observed within sexes. No significant differences due to sex were observed by pairwise comparison, although two-way ANOVA detected variability due to sex with a p value of 0.0124. E-G) BCA lesion area from Movat stained sections from Figure 4 were analyzed separately according to sex. There were no significant differences by 2-way ANOVA or pairwise comparisons in males. F) Differences in lesion size in females are nonsignificant by Sidak multiple comparison test except at the 600 μm location between MCP1$^{SMC\text{-}Lgals3\ wt/\Delta}$, and MCP1$^{SMC\text{-}Lgals3\ \Delta/\Delta}$ mice. No significant variation due to location was detected by two-way ANOVA, but given that it is detected in other experiments, these groups were not pooled for further analysis. H) Fibrous cap thickness from Figure 4B was analyzed separately by sex. No differences were observed by separate one way ANOVA.







E.

Differential expression in GFP⁺ MCP1^{Lgals3} plaque cells

Gene	Avg_logFC	p_val_adj
mt-Atp8	-0.4386247	7.37E-14
Gsn	-0.3621983	8.82E-05
Tmsb10	-0.3621455	0.00010995
Ccl2	-0.3583045	0.00014809
Atp5k	-0.3469608	0.00024955
Hsph1	-0.3456981	0.00030204
Pnrc1	-0.3284075	0.00424707
Cst3	-0.2927614	0.00533153
H3f3b	-0.2594122	0.00537817
Lars2	-0.2555257	0.00663388

F.

Differential expression in tdTomato⁺ MCP1^{Lgals3} plaque cells

Gene	Avg_logFC
Lcn2	-4.43898
Mt-Nd5	-4.225255
Cspg4	-4.053938
Ltbp2	-3.920478
Col6a1	-3.602730

Supplemental Figure 10. scRNAseq analysis of MCP1$^{SMC\text{-}Lgals3}$ **BCA plaque identifies several SMC-derived and immune cell subsets.** A) Cells from microdissected, advanced BCA lesions from MCP1$^{SMC\text{-}Lgals3\ wt/wt}$, MCP1$^{SMC\text{-}Lgals3\ wt/\triangle}$, and MCP1$^{SMC\text{-}Lgals3\ \triangle/\triangle}$ mice fed a Western diet for 18 weeks were collected for single cell RNAseq as unsorted sample or as GFP$^+$ or tdTomato$^+$ enriched libraries. 12 animals (4 per genotype) were assayed in 9 libraries, yielding 5,111 cells that passed quality control for analysis. A) shows a UMAP of all cells colored by cluster. B) Dot plot showing selected top markers for each UMAP cluster in A, including traditional markers such as Myh11, CD45, F4/80, CD4, and Pecam1 that identify SMC, macrophage and T cell immune subsets, and endothelial cells. Dark blue dots indicate increased expression level. Dot size represents portion of cells in each cluster with the given expression level. C) UMAP of cells colored by library of origin, revealing GFP$^+$ and tdTomato$^+$ SMC in diverse clusters expressing non-traditional markers, with GFP$^+$ cells dominating clusters positive for calcification markers and tdTomato$^+$ cells comprising the Acta2/Myh11$^+$ population but also present in stem and ECM-rich clusters. D) Feature plots representing lineage-traced SMC clusters positive for Acta2, Ly6a, Ccl2, and calcification marker Alpl. E-F) MAST analysis was conducted separately for GFP$^+$ cells (E) and tdTomato$^+$ (F) cells from MCP1$^{SMC\text{-}Lgals3\ wt/wt}$ and MCP1$^{SMC\text{-}Lgals3\ \triangle/\triangle}$ plaques. Several genes including *Ccl2* (*Mcp1*) were significantly affected by MCP1 genotype for GFP cells. No genes were significant for tdTomato cells (F), but a list of the highest fold-change genes are shown.