Online Appendix

Quantification of Histological Sections

Lesions within the aortic sinus were assessed by Oil Red O, a fat-soluble dye used to stain lipids, in frozen heart sections. Briefly, 8-μm-thick cryostat sections were cut and each tissue had about 40-50 sections. Sections that were 40 μm apart and covering 320 μm of the sinus were selected for staining with Oil red O to detect lesions. The area of lesion, which was defined as the red stain below the three leaflets of the aortic sinus, was quantitated with Image Pro Plus. For each mouse, lesion size (μm^2) was determined from the average of 5-7 cross sections.

For immunohistochemical analysis, manual tracing of the aorta or aortic sinus was performed in Image Pro Plus to determine the measurable area in each image, with the exclusion of the lumen (aorta) and lumen and leaflets (aortic sinus). Threshold between positive and negative staining was defined by using positive and negative control slides by a colour cube manual algorithm in Image Pro Plus with manual selection of positive stained colours. Each tissue was analysed using the same colour threshold. Values for positive staining were divided by the measurable area to calculate the percentage of positively stained tissue.

Online Appendix Table 1: Information for ELISA kits used in Section 2.5. and 2.7.

ELISA Kits	Company and Catalogue Number
Mouse IL-6 Quantikine ELISA Kit	R&D systems; M6000B
Mouse Caspase-1 ELISA kit (plasma)	Adipogen; AG-45B-0002-KI01
Urinary 8-isoprostanes	Oxford Biomedical Research; EA85
Mouse IL-1β ELISA (cell culture supernatants)	R&D systems; DY-401
Mouse caspase-1 ELISA (cell culture supernatants)	Adipogen; AG-46B-0003-KI01
Human IL-1β ELISA (cell culture supernatants)	R&D systems; DY-201

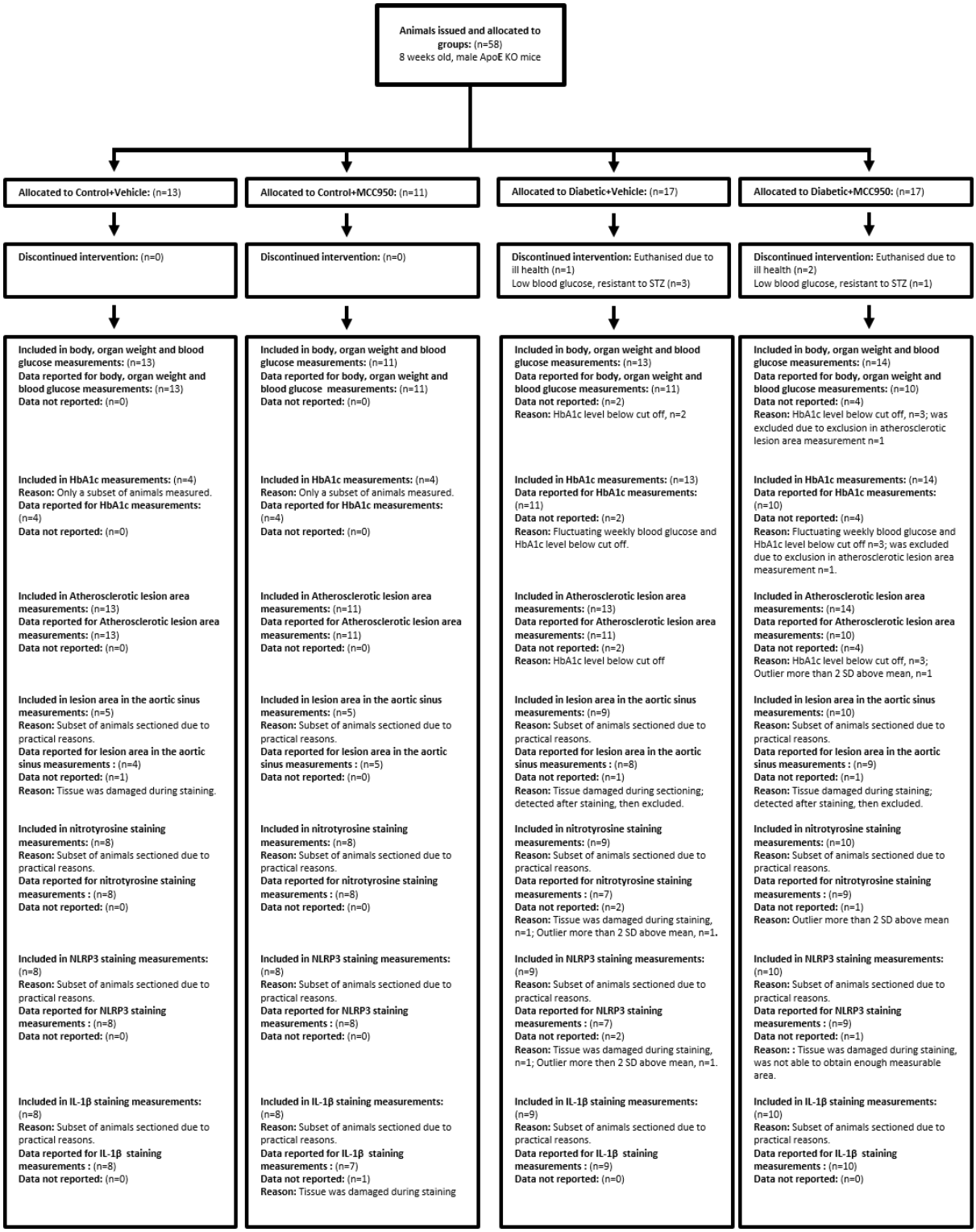
Online Appendix Table 2: Antibody information, catalogue numbers and dilutions used in Section 2.6.

Antibody/Reagent	Company and Catalogue Number	Dilution
Avidin and Biotin Blocking kit	Vector Labs; SP-2001	-
Rabbit polyclonal IL-1β (in aortas)	Abcam; Ab9722	1:100
Rabbit polyclonal NLRP3	Thermo Fisher; PA5-79740	1:100
Rat anti-mouse CD68	Biorad; MCA1957	1:200
Rabbit polyclonal Nitrotyrosine	Merck Millipore; AB5411	1:75
Rat anti-mouse Ly6C	Biorad clone ER-MP20; MCA2389GA	1:200
Rabbit polyclonal α-SMA	Abcam Ab5694	1:200
Polyclonal goat IL-1β (in aortic sinus)	R&D system; AB-401-NA	1:200
Biotinylated anti-rabbit secondary	Vector Labs; BA-1000	1:500
Biotinylated anti-rat secondary	Vector Labs; BA-4001	1:250
TRITC-conjugated goat anti-rat antibody	Jackson Immunoresearch; #112-025-167	1:200
Alexa Fluor 546 goat anti-rabbit	Invitrogen; A11010	1:200
Alexa Fluor 546 donkey anti-goat	Invitrogen; A11056	1:200
Prolong Gold Anti-fade mountant with DAPI	Invitrogen; #P36935	-

Online Appendix Table 3: Metabolic characteristics of control and diabetic ApoE$^{-/-}$ mice treated with vehicle of MCC950 (5mg/kg) at the end of the 18-week study. Plasma from diabetic ApoE$^{-/-}$ mice exhibited increased levels of cholesterol and LDL, which were unaffected by MCC950 treatment. Data expressed as mean ± SEM. *P<0.05 vs control ApoE$^{-/-}$ mice + Vehicle. A one-way ANOVA with Tukey's *post-hoc* test was used; n=9-11 per group.

	Control +Vehicle	Control + MCC950	Diabetic +Vehicle	Diabetic + MCC950
HbA1c (mmol/mol)	14.75 ± 1.75	22.25 ± 3.68	73.27 ± 10.54 *	73.00 ± 11.61 *
Cholesterol (mmol/L)	11.59 ± 0.98	11.58 ± 0.78	20.77 ± 3.86 *	16.39 ± 1.67
Triglycerides (mmol/L)	0.86 ± 0.14	1.09 ± 0.13	0.60 ± 0.09	0.66 ± 0.09
HDL (mmol/L)	0.61 ± 0.09	0.75 ± 0.09	0.98 ± 0.07	0.67 ± 0.06
LDL (mmol/L)	10.59 ± 0.86	10.32 ± 0.70	19.52 ± 3.84 *	15.41 ± 1.58
LH Ratio	37.70 ± 2.78	33.11 ± 6.68	56.66 ± 10.43	64.63 ± 7.28
Blood Pressure (mmHg)	96.9 ± 5.3	104.6 ± 4.7	104.8 ± 9.4	97.5 ± 4.3

Online Appendix Figure 1: Flow chart of animal use and n numbers for each experiment in the 18-week study.



Included in Ly6C staining measurements: (n=5)
Reason: Subset of animals sectioned due to practical reasons.
Data reported for Ly6C staining measurements : (n=5)
Data not reported: (n=0)

Included in CD68 staining measurements: (n=5)
Reason: Subset of animals sectioned due to practical reasons.
Data reported for CD68 staining measurements : (n=5)
Data not reported: (n=0)

Included in aortic sinus IL-1β staining measurements: (n=5)
Reason: Subset of animals sectioned due to practical reasons.
Data reported for aortic sinus IL-1β staining measurements: (n=5)
Data not reported: (n=0)

Included in aortic sinus SMA staining measurements: (n=5)
Reason: Subset of animals sectioned due to practical reasons.
Data reported for aortic sinus SMA staining measurements: (n=4)
Data not reported: (n=1)
Reason: Slide was damaged during staining

Included in picosirius red staining: (n=5)
Reason: Subset of animals sectioned due to practical reasons.
Data reported for aortic sinus SMA staining measurements: (n=5)
Data not reported: (n=0)

Included in H&E staining: (n=5)
Reason: Subset of animals sectioned due to practical reasons.
Data reported for aortic sinus SMA staining measurements: (n=5)
Data not reported: (n=0)

Included in urinary 8-isoprostane measurements: (n=12)
Reason: Could not locate sample.
Data reported for urinary 8-isoprostane measurements : (n=12)
Data not reported: (n=0)

Included in plasma caspase-1 ELISA: (n=9)
Reason: Subset of animals measured due to lack of samples.
Data reported for plasma caspase-1 ELISA: (n=9)
Data not reported: (n=0)

Included in Ly6C staining measurements: (n=5)
Reason: Subset of animals sectioned due to practical reasons.
Data reported for Ly6C staining measurements : (n=4)
Data not reported: (n=1)
Reason: Problem with tissue processing during staining.

Included in CD68 staining measurements: (n=5)
Reason: Subset of animals sectioned due to practical reasons.
Data reported for CD68 staining measurements : (n=4)
Data not reported: (n=1)
Reason: Sample was missing , n=1.

Included in aortic sinus IL-1β staining measurements: (n=5)
Reason: Subset of animals sectioned due to practical reasons.
Data reported for aortic sinus IL-1β staining measurements: (n=5)
Data not reported: (n=0)

Included in aortic sinus SMA staining measurements: (n=5)
Reason: Subset of animals sectioned due to practical reasons.
Data reported for aortic sinus SMA staining measurements: (n=5)
Data not reported: (n=0)

Included in picosirius red staining: (n=5)
Reason: Subset of animals sectioned due to practical reasons.
Data reported for aortic sinus SMA staining measurements: (n=5)
Data not reported: (n=0)

Included in H&E staining: (n=5)
Reason: Subset of animals sectioned due to practical reasons.
Data reported for aortic sinus SMA staining measurements: (n=5)
Data not reported: (n=0)

Included in urinary 8-isoprostane measurements: (n=11)
Data reported for urinary 8-isoprostane measurements : (n=11)
Data not reported: (n=0)

Included in plasma caspase-1 ELISA: (n=9)
Reason: Subset of animals measured due to lack of samples.
Data reported for plasma caspase-1 ELISA: (n=9)
Data not reported: (n=0)

Included in Ly6C staining measurements: (n=9)
Reason: Subset of animals sectioned due to practical reasons.
Data reported for Ly6C staining measurements : (n=9)
Data not reported: (n=0)

Included in CD68 staining measurements: (n=9)
Reason: Subset of animals sectioned due to practical reasons.
Data reported for CD68 staining measurements : (n=7)
Data not reported: (n=2)
Reason: Outlier more than 2 SD above mean, n=1; tissue was damaged during sectioning, n=1.

Included in aortic sinus IL-1β staining measurements: (n=9)
Reason: Subset of animals sectioned due to practical reasons.
Data reported for aortic sinus IL-1β staining measurements: (n=9)
Data not reported: (n=0)

Included in aortic sinus SMA staining measurements: (n=9)
Reason: Subset of animals sectioned due to practical reasons.
Data reported for aortic sinus SMA staining measurements: (n=9)
Data not reported: (n=0)

Included in picosirius red staining: (n=9)
Reason: Subset of animals sectioned due to practical reasons.
Data reported for aortic sinus SMA staining measurements: (n=9)
Data not reported: (n=0)

Included in H&E staining: (n=9)
Reason: Subset of animals sectioned due to practical reasons.
Data reported for aortic sinus SMA staining measurements: (n=8)
Data not reported: (n=1)
Reason: Tissue was damaged during staining

Included in urinary 8-isoprostane measurements: (n=11)
Reason: Subset of animals measured due to practical reasons.
Data reported for urinary 8-isoprostane measurements : (n=9)
Data not reported: (n=2)
Reason: 8-isoprostane level was below detection measure.

Included in plasma caspase-1 ELISA: (n=5)
Reason: Subset of animals measured due to lack of samples.
Data reported for plasma caspase-1 ELISA: (n=5)
Data not reported: (n=0)

Included in Ly6C staining measurements: (n=9)
Reason:
Data reported for Ly6C staining measurements : (n=9)
Data not reported: (n=0)

Included in CD68 staining measurements: (n=9)
Reason: Subset of animals sectioned due to practical reasons.
Data reported for CD68 staining measurements : (n=8)
Data not reported: (n=1)
Reason: Outlier more than 2 SD above mean.

Included in aortic sinus IL-1β staining measurements: (n=10)
Reason: Subset of animals sectioned due to practical reasons.
Data reported for aortic sinus IL-1β staining measurements: (n=10)
Data not reported: (n=0)

Included in aortic sinus SMA staining measurements: (n=10)
Reason: Subset of animals sectioned due to practical reasons.
Data reported for aortic sinus SMA staining measurements: (n=10)
Data not reported: (n=0)

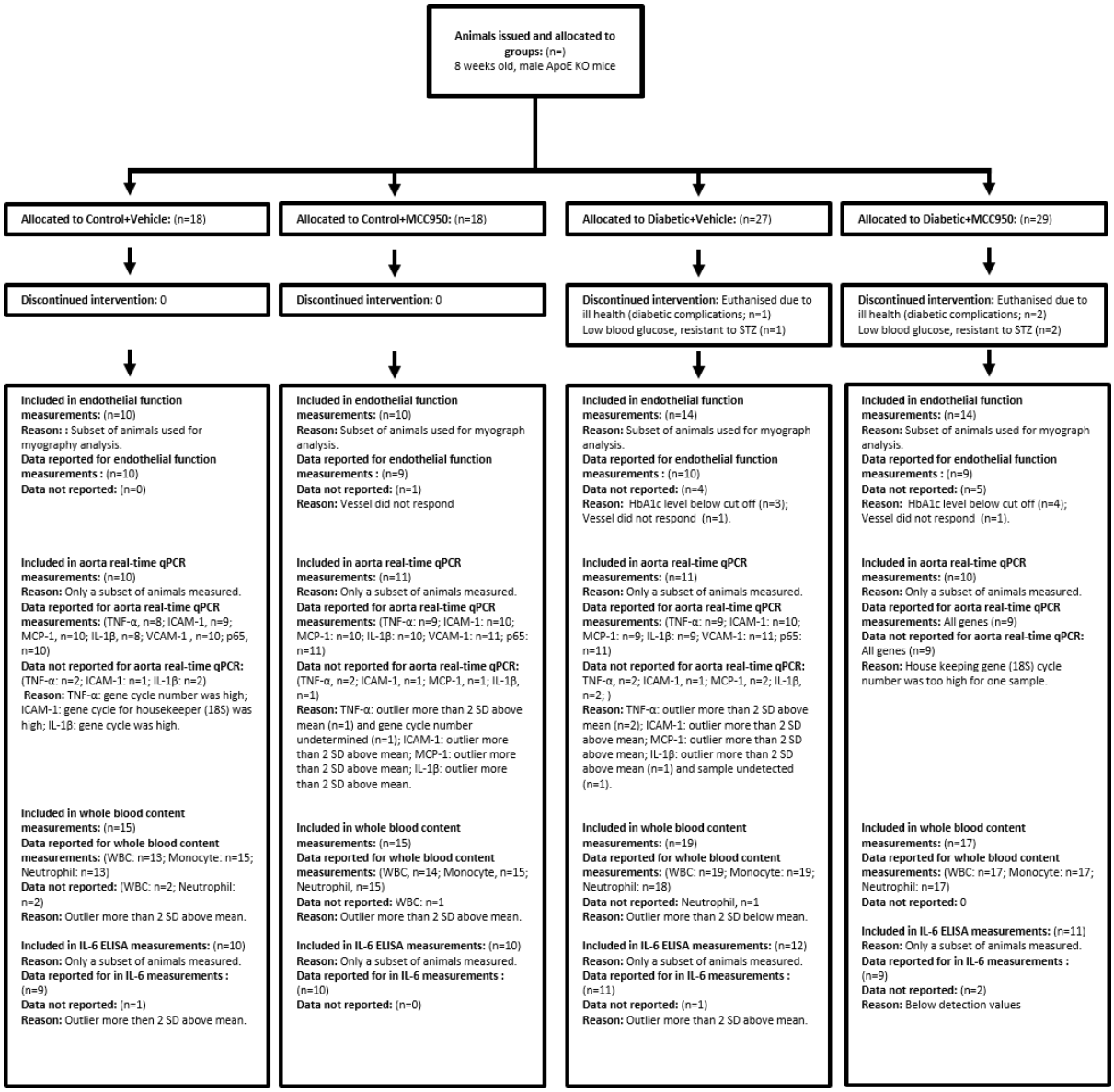
Included in picosirius red staining: (n=10)
Reason: Subset of animals sectioned due to practical reasons.
Data reported for aortic sinus SMA staining measurements: (n=9)
Data not reported: (n=1)
Reason: Tissue was damaged during staining,

Included in H&E staining: (n=10)
Reason: Subset of animals sectioned due to practical reasons.
Data reported for aortic sinus SMA staining measurements: (n=9)
Data not reported: (n=1)
Reason: Tissue was damaged during mounting.

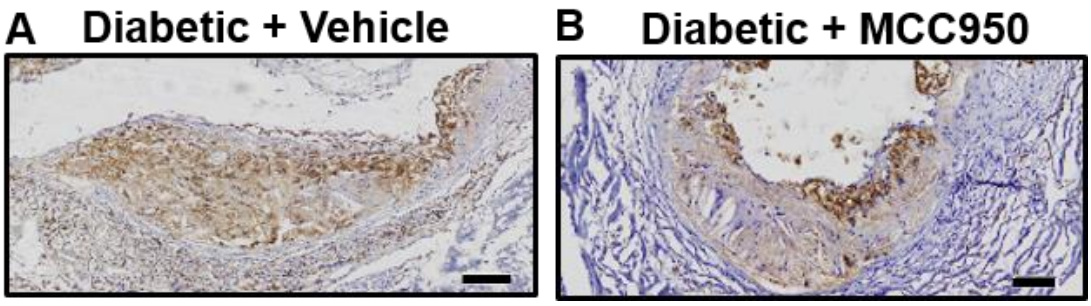
Included in urinary 8-isoprostane measurements: (n=10)
Reason: Subset of animals measured due to practical reasons.
Data reported for urinary 8-isoprostane measurements : (n=9)
Data not reported: (n=1)
Reason: Heavy sedimentation in sample giving an abnormal reading.

Included in plasma caspase-1 ELISA: (n=4)
Reason: Subset of animals measured due to lack of samples.
Data reported for plasma caspase-1 ELISA: (n=4)
Data not reported: (n=0)

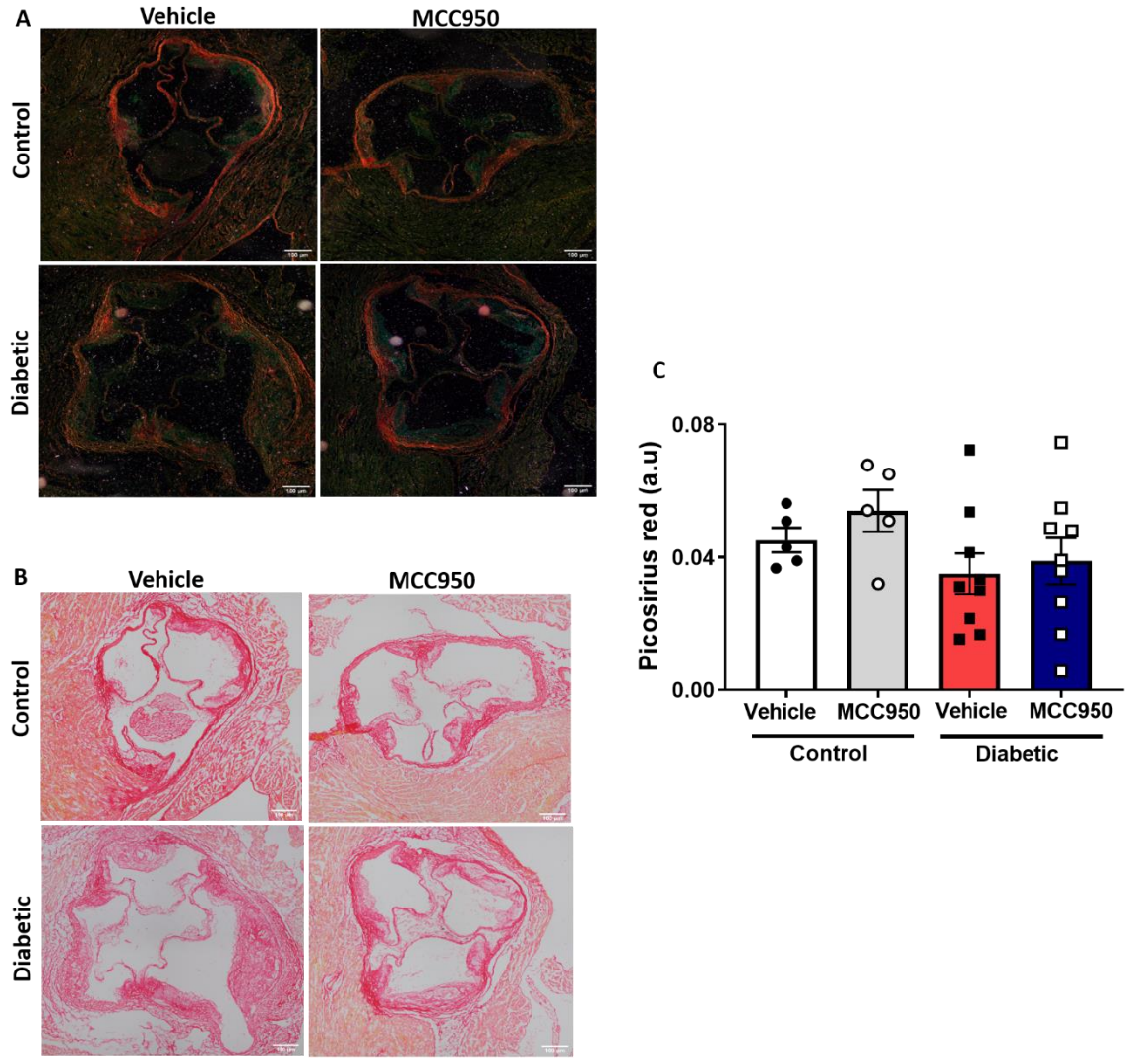
Online Appendix Figure 2: Flow chart of animal use and n numbers for each experiment in the 10-week study.



Online Appendix Figure 3: (A) and (B) are 40X images of plaque stained with CD68 to detect macrophages in diabetic ApoE$^{-/-}$ mice and diabetic ApoE$^{-/-}$ mice treated with MCC950 respectively. Diffuse staining is detected throughout the lesion in diabetic ApoE$^{-/-}$ mice whilst less staining is detected in lesions of mice treated with MCC950. Scale bars represent 50µm.

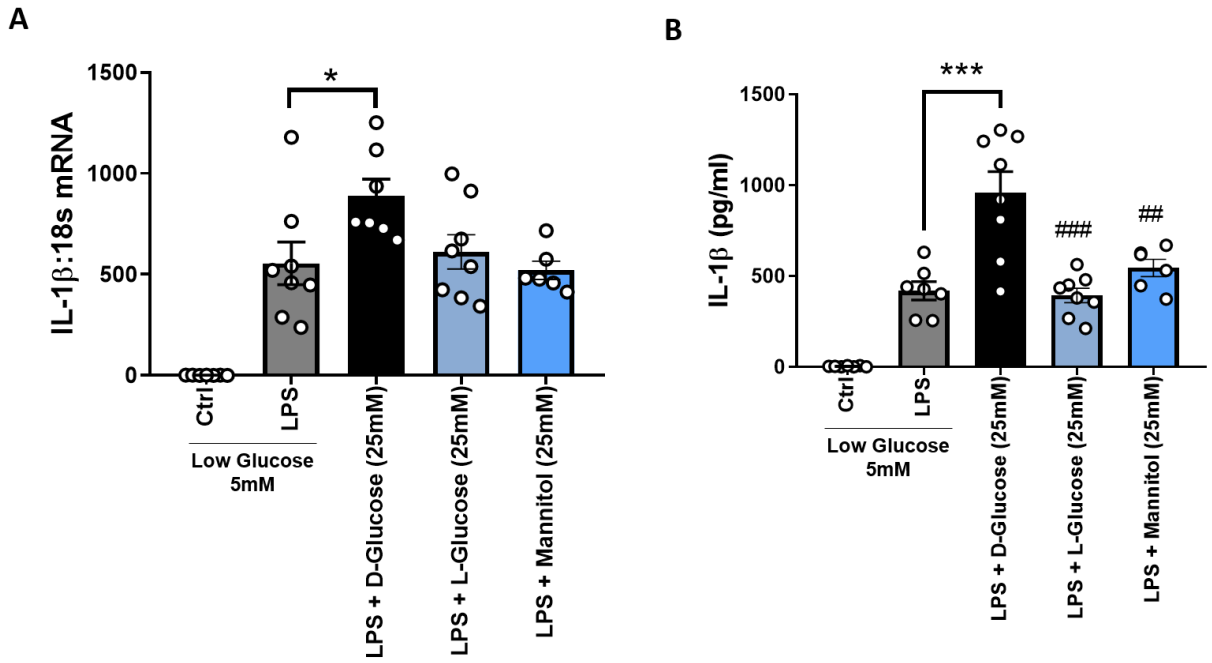


Online Appendix Figure 4: Representative images of picrosirius red staining in the aortic sinus region of control and diabetic ApoE$^{-/-}$ mice treated with either vehicle or MCC950 under (A) polarized light and (B) brightfield conditions. (C) Quantification of picrosirius red within the plaque in the aortic sinus. Scale bars represent 100µm. Bars represent mean ± SEM. A one-way ANOVA with Tukey's *post-hoc* test was used; n=5-9 per group with individual data points also shown.



Online Appendix Figure 5: D-glucose significantly increases IL-1β gene expression and protein secretion in supernatants, whereas, L-glucose and mannitol had no significant impact. BMDMs were grown in either low D-glucose (5mM), high D-glucose (25mM), high L-glucose (25mM) and mannitol (25mM) for 24h ± LPS (19h; 0.1µg/ml) and + ATP (4h; 1mM). (A) IL-1β gene expression and (B) secretion in supernatants was measured.

Bars represent mean ± SEM. ***P<0.001 as indicated; ##P<0.01 and ###P<0.001 vs LPS + D-Glucose (25mM). A one-way ANOVA with Tukey's *post-hoc* test was used; n=5-8 per group with individual data points also shown.

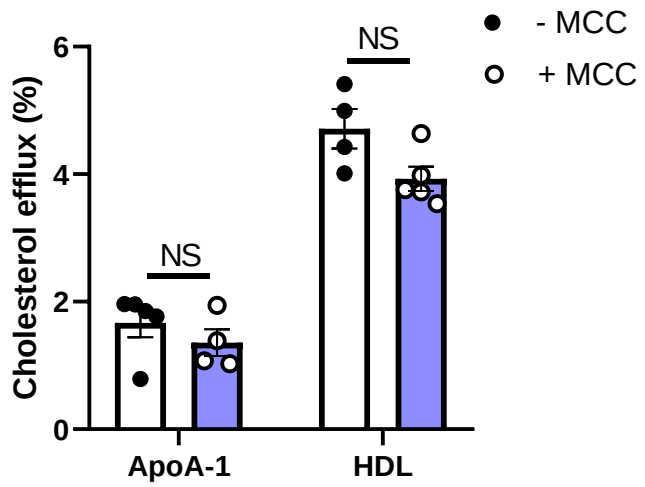


Online Appendix Figure 6: Cholesterol efflux of labelled (^{3}H)-cholesterol to lipid-free ApoA-I and to HDL, indicative of ABCA1 and ABCG1 activity respectively, was evaluated in the presence of MCC950 in murine BMDMs.

Rationale: MCC950 is known to directly interact with the Walker B motif within the NLRP3 NACHT domain thereby blocking ATP hydrolysis and inhibiting NLRP3 activation and inflammasome formation. Since both cholesterol transporters ABCA1 and ABCG1 also possess ATP binding Walker motifs, we tested the hypothesis that MCC950 might affect the ability of macrophages to traffic cholesterol, thereby influencing the results. ATP binding is essential for ABCA1 and ABCG1 trafficking and activity.

Method: To perform the assay, BMDM were labelled by incubation in serum-containing medium supplemented with [3H]cholesterol for 48 h. Cells were washed and incubated for 24h in serum-free medium in the presence of LPS (0.1µg/ml). Cells were washed again and treated with ± MCC950 (5h; 1µM) and ATP (4h; 1mM). Human ApoA-I and HDL was added to the final concentration of 20µg/ml and cells were incubated for 2 h at 37°C. The efflux was calculated as a proportion of radioactivity moved from cells to medium; non-specific efflux (i.e. the efflux to the medium without acceptor) was subtracted (Ref [30] from main text).

Results: Our results show that MCC950 treatment had no effect on cholesterol efflux of either cholesterol transporter, indicating that it does not interfere with ATP binding as required by these lipid transporters for trafficking and activity. Bars represent mean ± SEM with individual values plotted. An unpaired *t* test was used to compare ApoA-1 and HDL efflux in the presence of MCC950; n=4-5 per group. NS = Non-Significant.



Online Appendix Figure 7: Control and Diabetic HAoSMCs were primed with LPS (18h; 1µg/ml) and treated with MCC950 (5h; 1µM) and ± ATP (4h; 1mM) followed by incubation with fluorescently labelled THP-1 monocytic cells. Representative images of fluorescently labeled THP-1 cells in red (top panel) on HAoSMCs (bottom panel).