SUPPLEMENTAL MATERIALS

Wang Y *et al.*

Methods

Animal procedures. Male Myh11-Cre^{ERT2}, Rosa26$^{Rainbow/+}$,ApoE$^{-/-}$ mice (Rainbow mice) and Myh11-Cre^{ERT2}, Rosa26$^{tdTomato/tdTomato}$, ApoE$^{-/-}$ SMC lineage-tracing mice (Tomato mice) were provided by Stanford University. For Rainbow mice, all cells in these mice constitutively express green fluorescent protein (GFP), but upon tamoxifen injection, all cells of SMC lineage switch from GFP expression to stochastically express one of three other fluorescent proteins: mCherry, CFP, or mOrange, while non-SMCs continue to express GFP. A detailed construct of the Rainbow reporter has been described previously.[1] In brief, female Rosa26$^{Rainbow/+}$ mice were crossed with male Myh11-Cre^{ERT2}, ApoE$^{-/-}$ mice to generate male Myh11-Cre^{ERT2}, Rosa26$^{Rainbow/+}$, ApoE$^{+/-}$ mice, and back crossed with female ApoE$^{-/-}$ mice to obtain male Myh11-Cre^{ERT2}, Rosa26$^{Rainbow/+}$, ApoE$^{-/-}$ mice (Jackson Laboratory). A similar strategy were used to obtain Tomato mice using female B6.Cg-*Gt(ROSA)26Sor*$^{tm14(CAG-tdTomato)Hze}$/J (Jackson Laboratory) crossed with male Myh11-Cre^{ERT2} mice. For both lineage tracing mouse lines, Cre recombinase was activated with a series of 1-mg tamoxifen (Sigma) intraperitoneal injections at age 7 weeks, for 5 days. These studies were approved by the Stanford University Administrative Panel on Laboratory Animal Care (protocol 27279) and conform to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

To compare the results of foam cell estimation using lineage-tracing and regular ApoE$^{-/-}$ mice, Rainbow mice were fed a Western Diet (WD) for 6 weeks after tamoxifen injection, and aortas were harvested for flow cytometry. The ascending aorta and aortic arch (lesion-prone) and the descending thoracic aorta (lesion-resistant) were digested separately, and the cell suspensions collected following the same procedure described in the main methodology section. Resuspended vascular cells were stained with HCS LipidTOX™ Deep Red Neutral Lipid Stain (1:500 v/v, ThermoFisher Scientific) and BUV395 Rat Anti-Mouse CD45 (BD Biosciences) for 30 min at RT. After washing, cells were resuspended in PBS and analyzed by LSR II. UV (BD Biosciences).

Tomato mice were fed a WD for 12 weeks after tamoxifen injection to develop more advanced lesions. Individual lesions from the ascending aorta and brachiocephalic artery were cut into half upon dissection, with one half processed for FACS analysis to identify foam cells using BODIPY as described in the Methodology section of the main manuscript. The other mirrored half was embedded in OCT, sectioned and stained with BODIPY to image foam cells by confocal imaging. Tomato^{+}BODIPY^{+} (SMC-derived foam cells) and BODIPY+ (foam cells) were analyzed by FACS.

Cell lines. We have previously isolated mouse aortic smooth muscle cells (MASMCs)

from 4-week-old and 12-week-old Balb/c mice. MASMCs were grown in DMEM containing 10% fetal bovine serum (FBS) and used between the 3rd and 6th passage after re-establishing the culture. Within these passages the cells were still of a spindle shape and expressed SMA and myosin heavy chain. MASMCs from 4-week-old Balb/c mice turned to Oil Red O positive foam cells when treated with agLDL, whereas MASMCs from 12-week-old Balb/c mice are resistant to form foam cells upon agLDL incubation. Mouse monocyte/macrophage RAW 264.7 cells were cultured in RPMI 1640 supplemented with 10% FBS. All cell cultures were maintained at 37 ºC in a humidified incubator with 5% CO_2.

Immunostaining of CD45 by cultured MASMCs and RAW 264.7 macrophages. Cells were grown on cover slips, washed with PBS, fixed with 2% paraformaldehyde, and blocked with 5% goat serum before incubating with rat anti-mouse CD45 antibody (BD Pharmingen, 5 μg/ml). Goat anti-rat Alexa fluor633 was used as secondary antibody. Foam cells were stained by 10 µg/ml BODIPY 493/503 (Invitrogen) during incubation with secondary antibody. Images were captured by a Leica scanning confocal microscope.

RT-PCR of CD45. MASMCs from 4-week-old Balb/c mice and RAW 264.7 cells were incubated with either 2 mg/ml fatty acid-free albumin (FAFA) as control, or plus 100 µg/ml agLDL for 24 to 72 h. RNA was extracted from cultured cells using a standard procedure with Ribosol (Amresco). After reverse transcription, DNAs were amplified by a Biometra thermocyler. Annealing temperature was set at 58.5 ºC. Primer sequences were: CD45 forward 5'-ATGGTCCTCTGAATAAAGCCCA-3', reverse 5'-TCAGCACTATTGGTAGGCTCC-3'Amplified gene products were visualized by electrophoresis in a 1.2% agarose gel with SYBR safe DNA gel stain (Thermo Fisher Scientific).

Comparison of FACS method with collagen hydrogel co-culture. A stock solution of 10 mg/ml type 1 collagen in acetic acid (Corning) was diluted to 2 mg/ml and adjusted to pH 7, and added to cover the bottom of culture wells (24-well-plates). A collagen gel network was then formed by incubating plates at 37ºC for 30 min. MASMCs were labeled with 1 µmol/L CellTracker CM-DiI (Life Technologies) at 37ºC for 30 min and then seeded at 40,000 cells/well onto the collagen gel and allowed to settle for 2 h in DMEM/10% FBS. Another layer of neutralized collagen was then added to wrap the MASMCs in a collagen sandwich[2]. RAW 264.7 macrophages (10,000/well) were then seeded on top of the second collagen gel layer and co-cultures were incubated in DMEM with 10% FBS for 24 h. The medium was then changed to DMEM containing 2 mg/ml FAFA alone (control) or plus 100 µg/ml agLDL for 24h. To estimate the effect of the digestion and flow cytometry procedure on recovery of foam cells of different lineage, the MASMC/RAW 264.7 collagen co-cultures were digested and processed in the same way as the mouse tissues. Nucleated events released from the collagen gel were identified by Hoechst 33342 staining. Nucleated events from FAFA-treated collagen gels were used as negative controls to set the gate for BODIPY+ foam cells,

and the percentage of $CD45^+$ and $CD45^-$ foam cells among the total foam cells isolated from AgLDL-treated co-cultures were quantified. To compare the results to traditional microscopy, duplicate co-culture collagen gels were fixed in 2% paraformaldehyde at 37ºC for 30 min, immersed in 30% sucrose in PBS overnight at 4ºC, and snap frozen in OCT. Serial sections of 8 µm were cut vertically to obtain both macrophage and MASMC layers. Five sections 100µm apart from each other were selected to stain with 10 µg/ml BODIPY at room temperature for 30 min, and visualized under the confocal microscope. DAPI was used to stain nuclei and MASMCs were identified by CellTracker CM-DiI. FAFA-treated hydrogel sections were used to set the background staining of BODIPY. Cells that had more than 5 BODIPY-stained intracellular droplets were counted as foam cells. SMC (BODIPY+CellTracker+) and macrophage (BODIPY+CellTracker-) foam cells were counted by 2 independent observers, and the percentage of each in the total foam cells were calculated. At least 800 cells were counted in 3 independent experiments. There were no statistically-significant differences in estimates between the first and second observers.

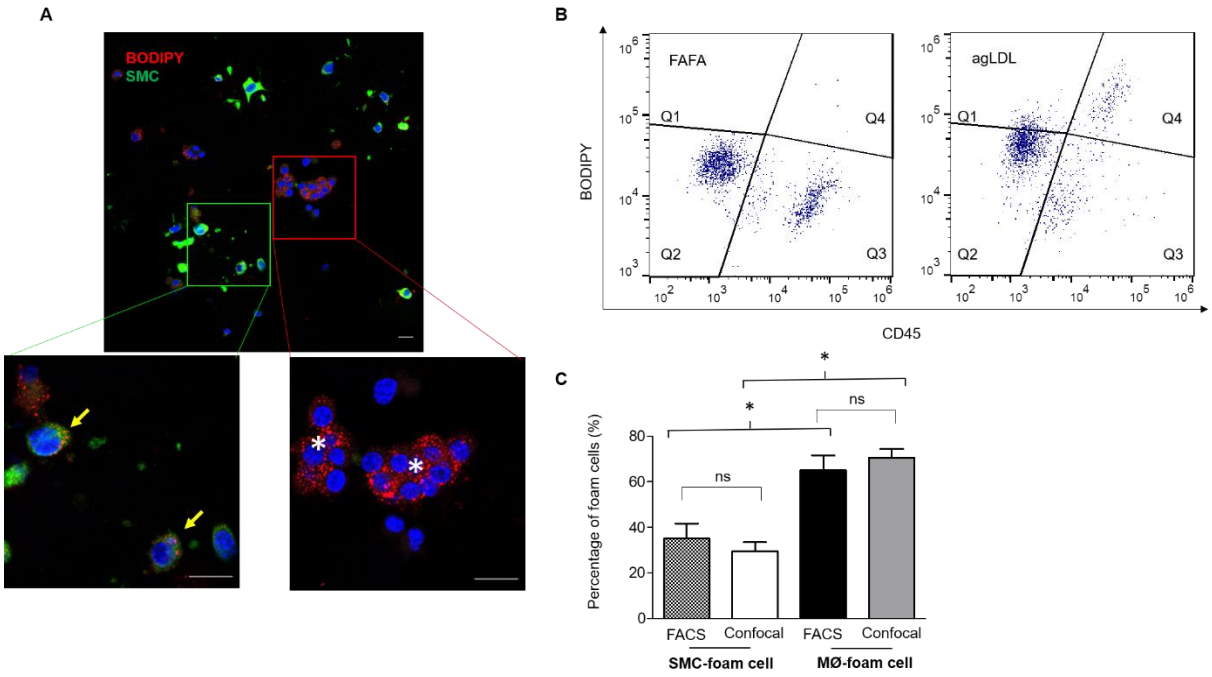
Real-time PCR of ABCA1. MASMCs and RAW 264.7 macrophages were treated with 100 µg/ml agLDL for 24 h and RNA extracted using standard procedure with Ribosol (Amresco). After reverse transcription to obtain cDNA, real-time PCR was conducted using PowerUp™ SYBR Green Master Mix with ViiA 7 system (Invitrogen). Annealing temperature was set at 58 ºC. Primer sequences were: ABCA1 forward 5'-AGTACCCCAGCCTGGAACTT-3', reverse 5'-TGGGTTTCCTTCCATACAGCG-3'; Cyclophilin A forward 5'-TCCAAAGACAGCAGAAAACTTTCG-3', reverse 5'-TCTTCTTGCTGGTCTTGCCATTCC-3'. ABCA1 mRNA levels were normalized to the house-keeping gene cyclophilin A before comparison.

Cholesterol mass analysis by LC/MS/MS. Cell populations from the aortic arch of $ApoE^{-/-}$ mice were sorted into Hank's balanced salt solution separately to extract total cholesterol for LC/MS/MS. Samples from 2 to 3 mice were pooled together when necessary in order to obtain sufficient instrument response. Cells isolated by flow cytometry were pelleted at 600g for 5 min. The aqueous layer was removed and cell pellets extracted using hexane:isopropanol (3:2).[3] Organic extracts were evaporated under a stream of air followed by the addition of 0.9 mL of ethanol and 100 µL of 250 ng/mL cholesterol-d7 internal standard (Avanti Polar Lipids). Saponification of cholesteryl esters for total cholesterol analysis was performed using ethanoic potassium hydroxide[4]. After removal of the organic solvent under a stream of air, extraction by the method of Folch, *et al*.[5] was performed. The organic solvent was then evaporated and the residues reconstituted with 200 µL of methanol and 30 µL was analyzed using a Shimadzu Prominence HPLC system coupled to an ABSCIEX 5500 triple quadrupole mass spectrometer. Cholesterol and cholesterol-d7 were detected by multiple reaction monitoring with modification to a previously reported method[6]. Total cholesterol was determined by the peak area ratio of cholesterol to cholesterol-d7 and a calibration curve ($r > 0.99$, range: 1293.2 to 517263.7 fmol) generated from extracted spiked standards. Total cholesterol was normalized to cell numbers counted by FACS

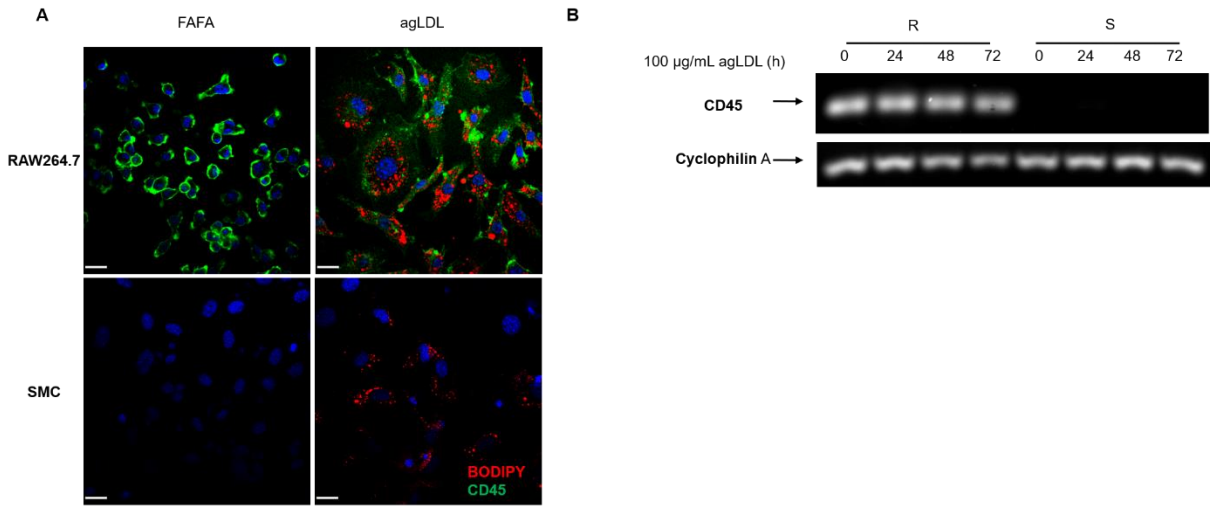
and reported at fmol/cell or as a percentage of total foam cell cholesterol.
Cholesterol efflux. RAW 264.7 and MASMCs were grown in 12-well plates to 70% confluence, treated with medium containing 10% lipoprotein-deficient serum (LPDS) for 24 hrs, and lipid loaded with medium containing 100 µg/mL agLDL and 2 mg/mL fatty acid free albumin (FAFA) for 48 hrs. Cells were then chased with serum free medium ± 10 µg/mL apoA-1 for 24 hrs in the presence of 0.3 mM 8Br-cAMP[7]. After extraction, apoAI-dependent efflux of cholesterol to the medium was determined by LC/MS/MS using cholesterol-d7 as an internal standard followed by normalization to cell proteins.

Co-culture of macrophages and SMCs. To determine the effects of macrophages on SMC foam cell formation, MASMCs from 12-week-old Balb/c mice were seeded on a 12-well-plate and grown to 70% confluence (lower chamber). RAW 264.7 macrophages were grown on cell culture inserts of 3µm pore size (Falcon) until 70% confluence, washed with PBS, and placed onto the SMC layers to be the upper chamber. For the control condition, cell culture inserts with no macrophages were placed onto the SMC layers. The co-culture system was then pre-incubated with DMEM supplemented with 2 mg/ml FAFA overnight and then switched to the same medium plus 100 µg/ml agLDL for 72 h. The upper chamber inserts were removed and MASMCs in the bottom chamber harvested, stained with Hoechst 33342, BODIPY493/503, and AlexaFluro 700 rat anti-mouse CD45(BD Pharmingen), and analyzed by flow cytometry to measure the ratio of SMC-foam cells ($CD45^-BODIPY^+$ nucleated events)/total SMCs ($CD45^-$ nucleated events).

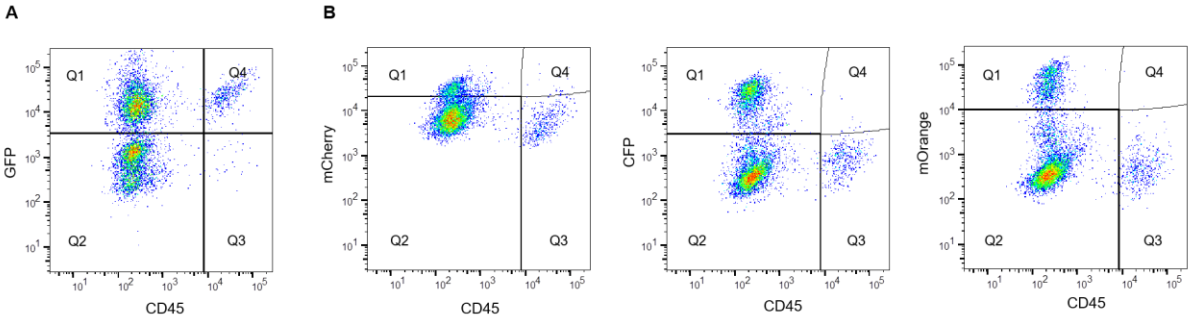
Supplemental Figures



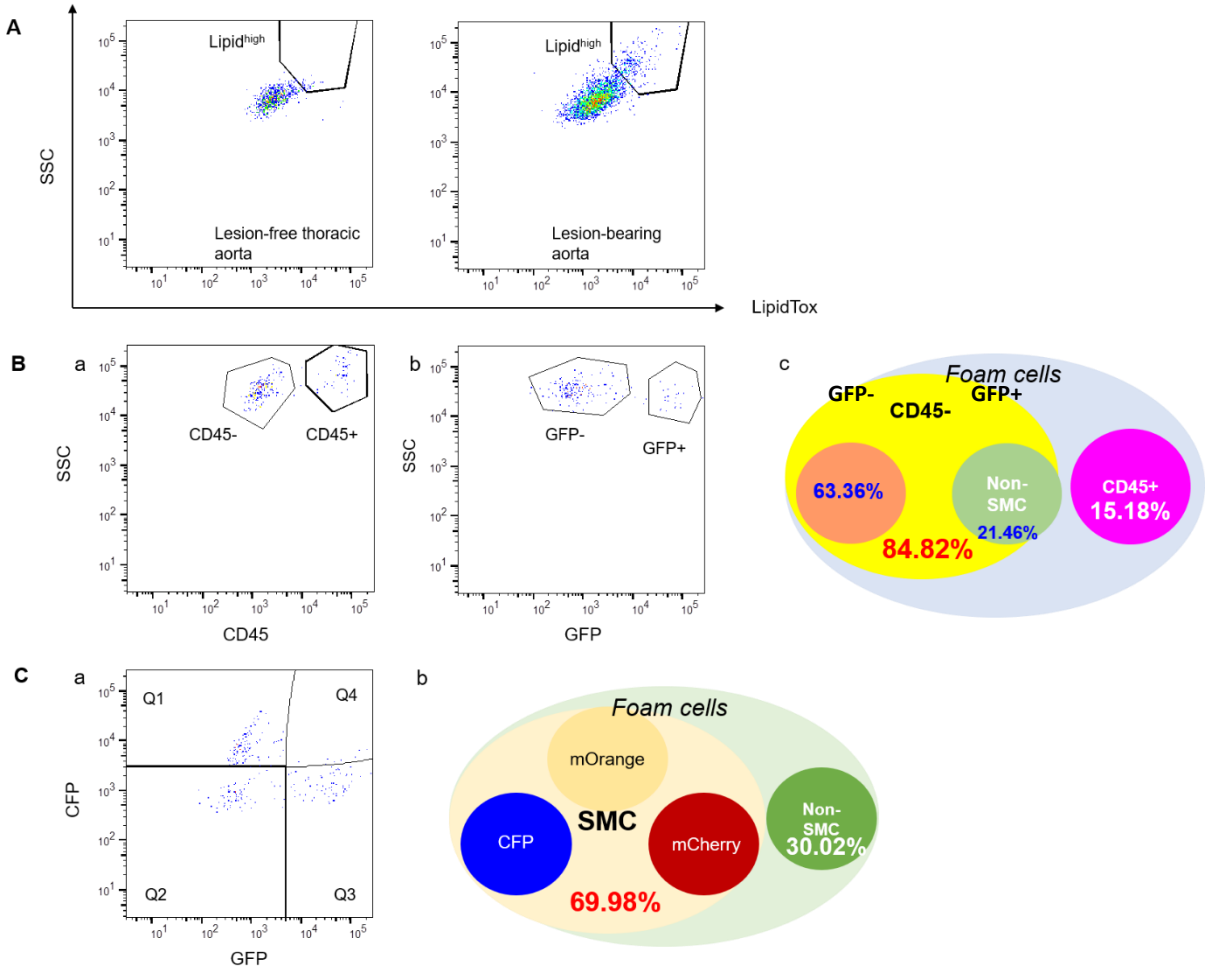
Supplemental Figure I. Comparison of foam cell estimation by flow cytometry and confocal microscopy using collagen hydrogel co-culture. (A) Confocal microscopy of a section of collagen gel with embedded macrophages and SMCs demonstrates SMCs labeled by Cell Tracker CM-Dil (green) and foam cells with intracellular lipid droplets (red). Yellow arrows point to SMC foam cells, and white asterisks indicate clusters of macrophage foam cells. Scale bar, 20 µm. (B) Representative histogram of co-cultured MASMCs and RAW264.7 following digestion of the collagen gel sandwich and analysis by FACS. Left panel shows the separation of SMC non-foam cells (Q2) and macrophage non-foam cells (Q3) isolated following digestion from FAFA-treated collagen gel, based on expression of leukocyte-specific CD45. Right panel shows SMC foam cells (Q1) and macrophage foam cells (Q4) isolated from collagen gels incubated with 100 µg/ml agLDL for 24 h. (C) Comparison of percentage of foam cells isolated following digestion and FACS or by confocal microscopy using the collagen gel co-culture system. N=3, ns: not significant using one-way ANOVA followed by Tukey's test.



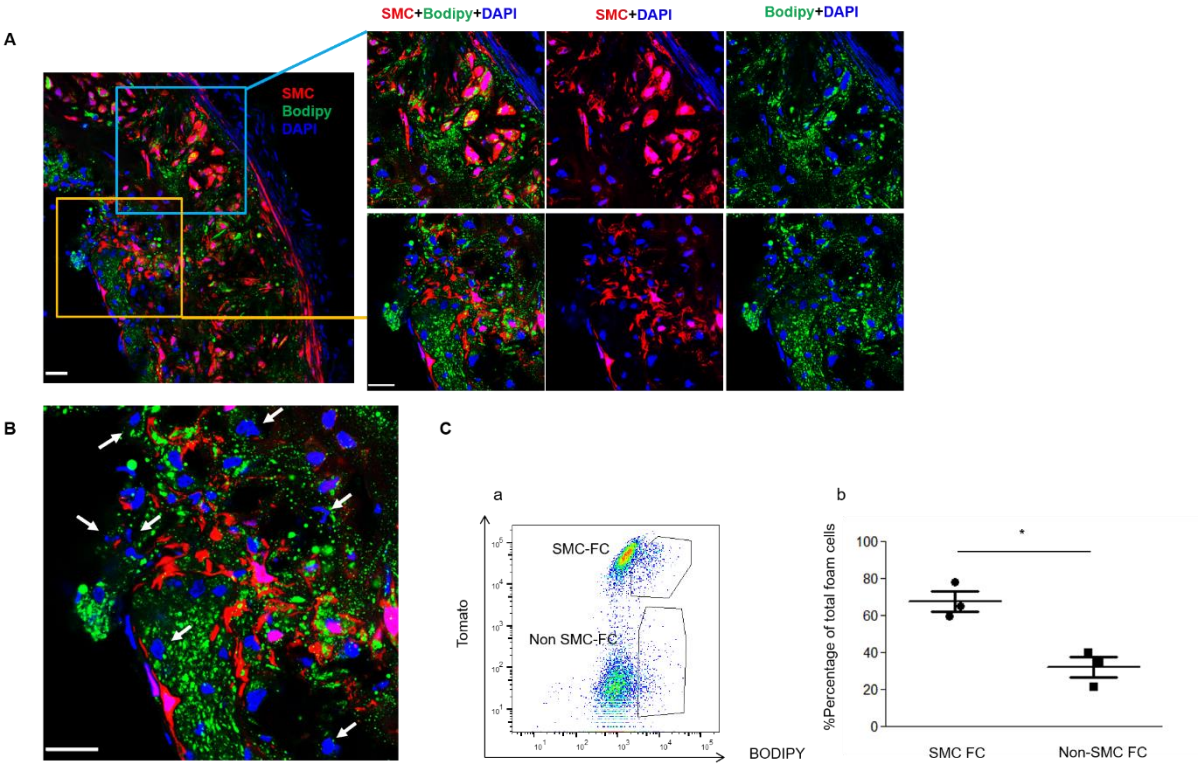
Supplemental Figure II. Absence of leukocyte marker CD45 expression by cultured arterial SMCs. (A) Confocal microscopy of intracellular lipid droplets (BODIPY, red) and CD45 (green) in RAW 264.7 macrophages and MASMCs treated with either FAFA alone or plus 100 µg/ml agLDL for 72 h. Note the absence of CD45 when SMCs become foam cells, and the decreased surface expression of CD45 on macrophage foam cells. (B) RT-PCR of CD45 in RAW 264.7 macrophages (R) and MASMCs (S) treated with 100 µg/ml agLDL for 0, 24, 48, and 72 h. CD45 mRNA was not observed in MASMCs in these conditions. The housekeeping gene cyclophilin A was measured (lower panel) to indicate equal total cDNA in samples.



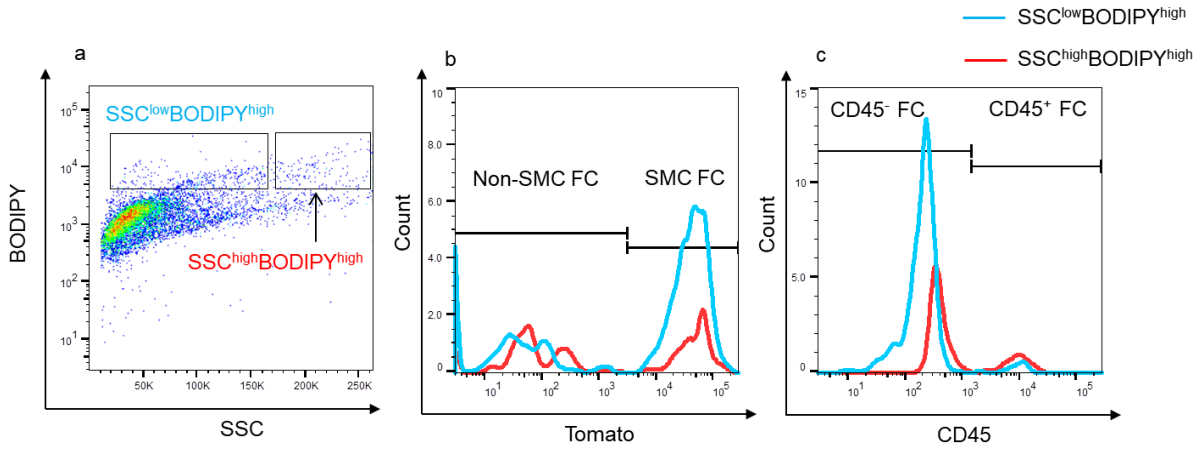
Supplemental Figure III. Expression of CD45 in the lesion area of SMC-lineage tracing mice. Representative histogram of cells in the aortic arch following 6 weeks of WD-fed Myh11-Cre^{ERT2}, Rosa26$^{Rainbow/+}$,ApoE$^{-/-}$mice separated by CD45 and the four SMC reporters. All the non-SMCs are GFP+ (Panel A, Gates Q1 and Q4), and SMCs will express one of the mCherry, CFP, or mOrange fluorescent proteins (Panel B, Q1 and Q4 for each color). Almost all the CD45^{+} cells are GFP+ (Panel A, gate Q4), and expression of CD45 by SMCs is minimal (Panel B, Q4 for each color). Experiments were repeated in 5 mice, with similar results seen in all animals.



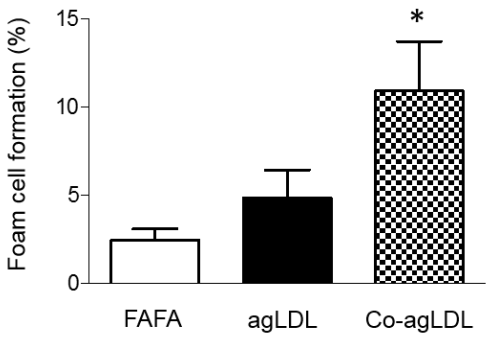
Supplemental Figure IV. Foam cell composition in SMC-lineage tracing mice. (A) Representative histogram identifying foam cells (Lipid^{high}) from the aortic arch (right panel) by setting up a lipid content threshold based on cells from lesion-free descending thoracic aortas of the same mice (left panel). (B) Lipid^{high} cells further separated based on CD45 expression (a), and within the CD45^{-} Lipid^{high} cells from panel (a) some GFP^{+} cells, indicating non-SMC origin (b). After subtracting the CD45^{-} GFP^{+} cells, the estimated contribution of SMCs to total foam cells is 63.36 ± 3.02% in 5 SMC-lineage tracing mice (c). (C) Using an alternative approach of foam cell quantification, SMC-derived foam cells in the same Lipid^{high} cell population were characterized instead by their intrinsic SMC lineage tracing fluorescence. A group of SMCs expressing CFP is shown within the Lipid^{high} cells as an example (gate Q1) (a). After combining all SMCs (CFP^{+}, mCherry^{+}. or mOrange^{+}), their contribution to total Lipid^{high} foam cells was 69.98 ± 4.49% (n=5 mice) (b).



Supplemental Figure V. Attempt at counting foam cells by confocal microscopy using SMC-lineage tracing mice. (A) Confocal microscopy of a lesion from Tomato mice fed with 12 weeks of WD (left panel) illustrating numerous red SMCs with green lipid droplets in both the deep intima (blue square, zoomed in on the right upper panel) and proximal intima (orange square, zoomed in on the right lower panel). Scale bar, 20 µm. (B) Examples of ambiguous areas where counting individual foam cells is not possible. White arrows point to nuclei surrounded by scattered BODIPY droplets without clear cell boundaries. (C) The same lesions were cut into half after harvesting, digested, stained with BODIPY and separated by FACS to count $Tomato^{+}BODIPY^{+}$ SMC foam cells (SMC-FC) and $Tomato^{-}BODIPY^{+}$ non-SMC foam cells (a). The percentage of SMC foam cells to total $BODIPY^{+}$ cells was 67.77 ± 5.47% (avg ± SEM, n=3) (b), which is significantly higher than non-SMC foam cells (32.23 ± 5.47%) * $P < 0.05$ using Wilcoxon signed rank test.



Supplemental Figure VI. SMC-derived foam cells exhibit lower granularity as determined by side scatter. The aortic arch from Tomato SMC mice fed for 12 weeks with WD was digested and cell suspension separated based on their BODIPY content and granularity as determined by side scatter (SSC) using FACS. Within the foam cell population showing high BODIPY content, cells were manually gated as either lower granularity ($SSC^{low}BODIPY^{high}$) or higher granularity ($SSC^{high}BODIPY^{high}$) (a). These two populations were further separated by SMC lineage tracing reporter "Tomato" (b) or macrophage-specific CD45 (c). There are more SMC-derived foam cells (SMC FC) within the $SSC^{low}BODIPY^{high}$ cell population (blue line) compared to the $SSC^{high}BODIPY^{high}$ cell population (red line), and more SMCs than macrophages in the total foam cell population (b). The $CD45^{+}$ macrophage foam cells show slightly more $SSC^{high}BODIPY^{high}$ than $SSC^{low}BODIPY^{high}$ cells, while the majority of foam cells are $CD45^{-}$ (c).



Supplemental Figure VII. Enhanced formation of MASMC foam cells during co-culture with macrophages. MASMCs from 12-week-old mice were treated with either FAFA alone or plus 100 µg/ml agLDL, in the absence (agLDL) or presence of RAW 264.7 macrophages in the upper chamber (Co-agLDL). Foam cell formation by MASMCs was determined by flow cytometry and expressed as % of foam cells out of total SMCs. Co-culture with macrophages significantly increased the number of MASMC foam cells. N=3, *$P < 0.05$ using Friedman test with Dunn's multiple comparisons versus MASMCs treated with FAFA alone.

References

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