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Characterization of Macrophage Polarization States Using Combined Measurement of 2-Deoxyglucose and Glutamine Accumulation: Implications for Imaging of Atherosclerosis

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Supplemental Material

Animals

C57BL/6J wild-type (N = 28 males and 10 females) and LDL-R$^{-/-}$ (N = 12 males and 5 females) mice were purchased from Jackson Laboratories and bred in-house. Animals were handled according to regulations of Animal Care and Use Committee of the University of Texas Health Science Center at San Antonio.

Cell Culture

Resident murine peritoneal cells were harvested from C57BL/6J wild-type mice through peritoneal lavage, as previously described [1]. Adherent macrophages were cultured for up to two days in RPMI-1640 (1:1 mix of Hyclone, Cat#: SH30096.02 and Cellgro, Cat#: 10-043-CV) supplemented with 10% fetal bovine serum (Gibco, Cat#: 10082), macrophage colony-stimulating factor (M-CSF, PeproTech, Cat#: 315-02, 2.5 ng/mL), sodium pyruvate (1 mM, Cellgro, Cat#: 25-000-C1), GlutaMAX (2 mM, Gibco, Cat#: 35050061), non-essential amino acids (Gibco, Cat#: 11140-050), and HEPES buffer (20 mM, Gibco, Cat#: 15630-080). To achieve a physiological concentration of glucose in the culture media, we mixed equal volumes of glucose-free and high-glucose RMPI-1640 medium (final glucose concentration: ~ 5.5 mM). Polarization was induced by incubating macrophages with the following stimuli: 1) $M\Phi_{IFN\text{-}\gamma+TNF\text{-}\alpha}$: interferon-γ (PeproTech, Cat#: 315-05, 50 ng/mL) and TNF-α (PeproTech, Cat#: 315-01, 10 ng/mL); 2) $M\Phi_{LPS}$: lipopolysaccharide (Calbiochem, Cat#: 437627, 10 ng/mL); and 3) $M\Phi_{IL\text{-}4}$: IL-4 (PeproTech, Cat#: 214-14, 10 ng/mL) [1]. Macrophages that did not receive any cytokines or lipopolysaccharide were considered non-activated ($M\Phi_0$).

^{3}H-2-Deoxyglucose and ^{3}H-Glutamine Accumulation

The accumulation of 2-deoxyglucose and glutamine was measured at three different time points (6, 24, and 48 h) after stimulation of macrophages. Because of their longer half-lives and convenience of use, we used ^{3}H-labeled 2-deoxyglucose and glutamine in cell culture experiments.

^{3}H-2-deoxyglucose accumulation assay: After a 15-min pre-incubation period, macrophages were incubated with 37 kBq/mL ^{3}H-2-deoxyglucose (American Radiolabeled Chemicals Inc. Cat#: ART0103A) for 30 min at 37°C in glucose and serum-free RPMI-1640 supplemented with pyruvate and non-essential amino acids, as described previously [1].

^{3}H-glutamine accumulation assay: To avoid competition of the labeled glutamine with the glutamine present in the culture media and fetal bovine serum, glutamine accumulation was measured in a glutamine-free buffer (NaCl: 140 mM, KCl: 5.4 mM, $CaCl_2$: 1.8 mM, $MgSO_4$: 0.8 mM, D-glucose: 5 mM, HEPES: 25 mM) [2]. Cells were pre-incubated for 15 min in the buffer, which was followed by a 15 min incubation with 148 kBq/mL ^{3}H-glutamine (Perkin-Elmer, Cat#: NET551250UC) at 37°C.

At the end of the incubation period, cells were washed with cold phosphate-buffered saline (PBS) and lysed with 0.1 mM NaOH. Accumulation values were then determined by liquid scintillation counting (Beckman LS 5000TD), normalized to the DNA content of the lysates, as determined by the PicoGreen reagent (Invitrogen, P7589) [1], and expressed relative to the values of $M\Phi_0$ at the same time point.

Gene Expression Assays

Gene expression assays were performed in cultured macrophages after 2-day incubation with polarizing stimuli. Total RNA extraction and reverse transcription was performed with TRIzol® reagent (Invitrogen, Cat#: 15596026) and QuantiTect® Reverse Transcription Kit (QIAGEN, Cat#: 205311). Quantitative PCR (RT-qPCR) was performed using TaqMan® gene expression assays (Invitrogen) and a high-throughput nanofluidic platform (Fluidigm, BioMark™), per manufacturer's protocol.

Gene expression assays were also performed in cultured macrophages from C57BL/6J wild-type mice after 6 hours of incubation with polarizing stimuli as well as in different segments of aorta harvested from LDL-$R^{-/-}$ mice fed with HFD for 3 months. For these experiments Taqman gene expression assays (Invitrogen) were performed using a real-time PCR system (model 7900HT; Applied Biosystems).

The expression level of each gene was normalized using the geometric mean of 18S ribosomal RNA (*Rn18s*) and hypoxanthine-guanine phosphoribosyltransferase (*Hprt*) which were most stably expressed across different polarization states. The complete list of primers is listed in SUPPLEMENTAL DATA (Supplemental Table 1).

Aortic Root Immunostaining

Fluorescent immunostaining of aortic root sections from LDL-$R^{-/-}$ mice, fed with a high-fat diet (HFD, fat: 21%, cholesterol: 0.15%, BioServe) for 3 months [3], was performed using commercially available antibodies (Supplemental Table 2). Slides were photographed using an EVOS® FL digital microscope.

***Ex Vivo* Autoradiography**

The accumulation of 2-deoxyglucose and glutamine was assessed in atherosclerotic aortas from LDL-$R^{-/-}$ mice (fed with HFD for 3 or 12 months) and control aortas from C57BL/6J wild-type mice (fed with a normal diet). Mice were euthanized and perfused with PBS through a left ventricular puncture. Aortas were dissected and excised from the root to the bifurcation, and then cleaned of the surrounding connective tissues under a dissection microscope. Subsequently, aortas were incubated for 30 min at 37 °C in a glucose and glutamine-free buffer (NaCl: 140 mM, KCl: 5.4 mM, $CaCl_2$: 1.8 mM, $MgSO_4$: 0.8 mM, HEPES: 25 mM) containing 5.92 MBq/mL of ^{18}F-FDG (Research Imaging Institute, San Antonio) and 0.296 MBq/mL of ^{14}C-glutamine (Perkin-Elmer). The specificity of ^{14}C-glutamine accumulation was determined in a subset of aortas from LDL-$R^{-/-}$ mice fed a HFD for 3 months through co-incubation with 20 mM unlabeled glutamine.

The use of a significantly higher activity of the short half-life ^{18}F-FDG relative to the long half-life ^{14}C-labeled glutamine allowed us to measure 2-deoxyglucose and glutamine accumulation in individual aortas using immediate and delayed exposures, respectively. We measured the ^{18}F-FDG accumulation by an immediate short-exposure (<15 min) of the aortas to a high-resolution phosphor screen (BAS-IP SR 0813 E Super Resolution, GE Healthcare). The phosphor screen was then scanned by a phosphor imager (Typhoon FLA 7000, GE Healthcare). The lack of significant contribution of ^{14}C-glutamine to this immediate autoradiography was confirmed through a series of known amount of ^{14}C-glutamine radioactivity (0-7.40 kBq) blotted on a paper, which yielded no detectable signal after this short exposure. For measurement of ^{14}C-glutamine accumulation, aortas were re-exposed to the phosphor screen for 48 h after at least 48 h delay (>25 half-life of ^{18}F) to allow for the complete decay of ^{18}F-FDG. Similar to the immediate autoradiography experiment, a series of known amount of ^{18}F-FDG radioactivity (0-740 kBq) was used to confirm the lack of detectable ^{18}F-FDG at the time of delayed autoradiography.

Standard curves were constructed using the known radioactivity blots, and the maximal accumulation in various segments of the aortas, i.e., ascending aorta, aortic arch, descending thoracic aorta and abdominal aorta, were quantified using ImageJ software. Quantitative autoradiography data in atherosclerotic aortas from LDL-$R^{-/-}$ mice are expressed as the maximal plaque accumulation relative to the activity present in the uptake buffer, indicating the capacity of lesions to internalize and concentrate the substrates. Considering the absence of atherosclerotic lesions in wild type mice, the mean uptake along the length of aorta was

quantified and compared to atherosclerotic aortas from LDL-$R^{-/-}$ mice. The autoradiography images were color-adjusted using the Fire Look-Up Table (Image J).

Statistical Analysis

Data are expressed as mean ± standard error of the mean. Excel add-in Multibase package (Numerical Dynamics, Japan) and Stata-12 were used for principal component analysis (PCA) of expression level of polarization markers. Analysis of variance, followed by the Fisher's Least Significant Difference test, was used to compare the mean values between the experimental groups (SigmaPlot software). $P<0.05$ was set as the statistical significance level.

References

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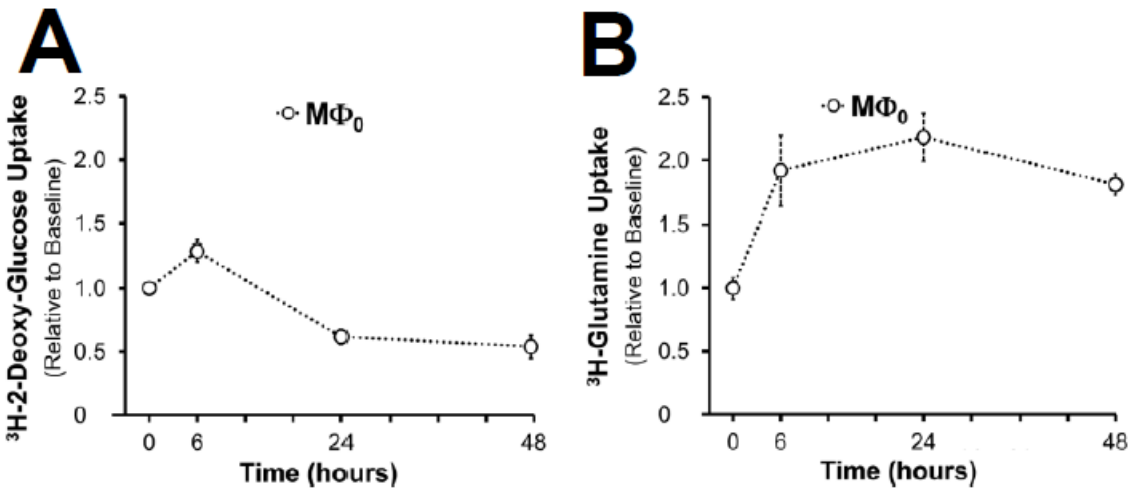
3. Zamora DA, Downs KP, Ullevig SL, Tavakoli S, Kim HS, Qiao M, Greaves DR, Asmis R. Glutaredoxin 2a overexpression in macrophages promotes mitochondrial dysfunction but has little or no effect on atherogenesis in ldl-receptor null mice. *Atherosclerosis*. 2015;241:69-78

SUPPLEMENTAL TABLE I. TaqMan® primers used for quantitative RT-PCR.

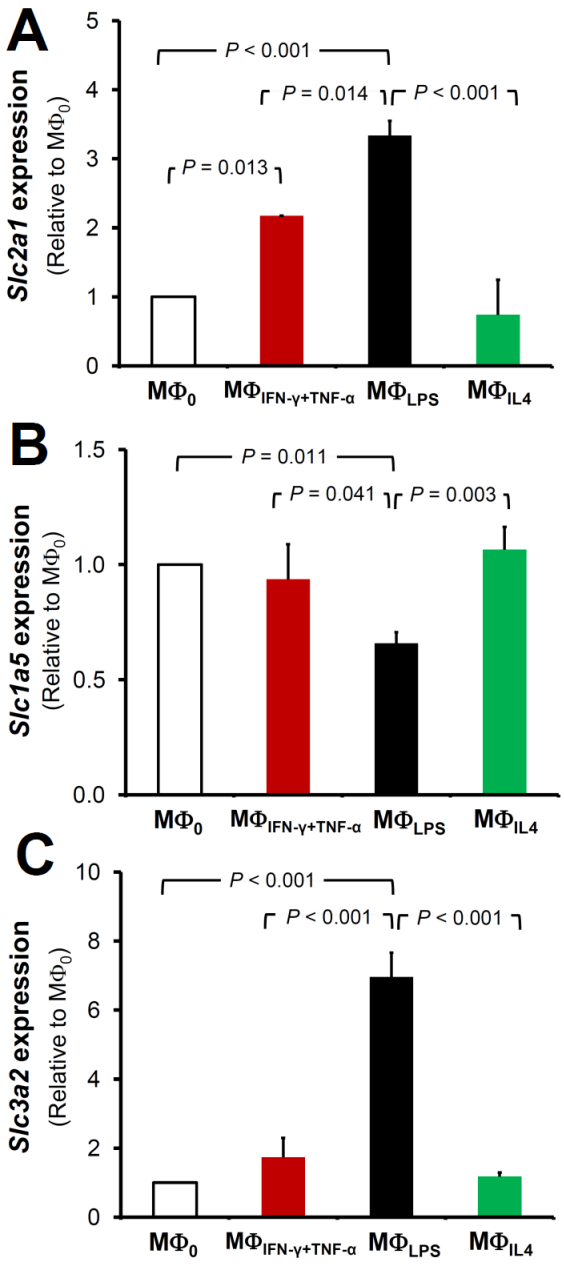
	Symbol	Gene Name	Assay ID
M1 Polarization Markers	*Cxcl9*	Chemokine (C-X-C motif) ligand 9	Mm00434946_m1
	Cxcl10	Chemokine (C-X-C motif) ligand 10	Mm00445235_m1
	Il1b	Interleukin 1 beta	Mm00434228_m1
	Il6	Interleukin 6	Mm00446190_m1
	Il12b	Interleukin 12b	Mm00434174_m1
	Nos2	Nitric oxide synthase-2	Mm00440502_m1
	Ptgs2	Prostaglandin-endoperoxide synthase 2	Mm00478374_m1
	Stat1	Signal transducer and activator of transcription 1	Mm00439531_m1
	Tnfa	Tumor necrosis factor	Mm00443258_m1
M2 Polarization Markers	*Arg1*	Arginase, liver	Mm00475988_m1
	Ccl9	Chemokine (C-C motif) ligand 9	Mm00441260_m1
	Ccl17	Chemokine (C-C motif) ligand 17	Mm00516136_m1
	CD36	CD36 antigen	Mm01135198_m1
	CD209a	CD209a antigen	Mm00460067_m1
	Chi3l3 (Ym1)	Chitinase-like 3	Mm00657889_mH
	Folr2	Folate receptor 2 (fetal)	Mm00433357_m1
	Igf1	Insulin-like growth factor 1	Mm00439560_m1
	Klf4	Kruppel-like factor 4	Mm00516104_m1
	Mgl2	Macrophage galactose N-acetyl-galactosamine specific lectin 2	Mm00460844_m1
	Mrc1 (CD206)	Mannose receptor, C type 1	Mm00485148_m1
	Retnla (Fizz1)	Resistin like alpha	Mm00445109_m1
	Tfrc	Transferrin receptor	Mm00441941_m1
	Tgfb1	Transforming growth factor, beta 1	Mm01178820_m1
Others	*Slc2a1* (*Glut1*)	Solute carrier family 2, member 1 (Glucose transporter-1)	Mm00441480_m1
	Slc1a5	Solute carrier family 1 (neutral amino acid transporter), member 5	Mm00436603_m1
	Slc3a2	Solute carrier family 3 (activators of dibasic and neutral amino acid transport), member 2	Mm00500521_m1
	Hprt	Hypoxanthine guanine phosphoribosyltransferase	Mm01545399_m1
	Rn18s	18S ribosomal RNA	Mm03928990_g1

SUPPLEMENTAL TABLE II. Reagents used for aortic root immunostaining.

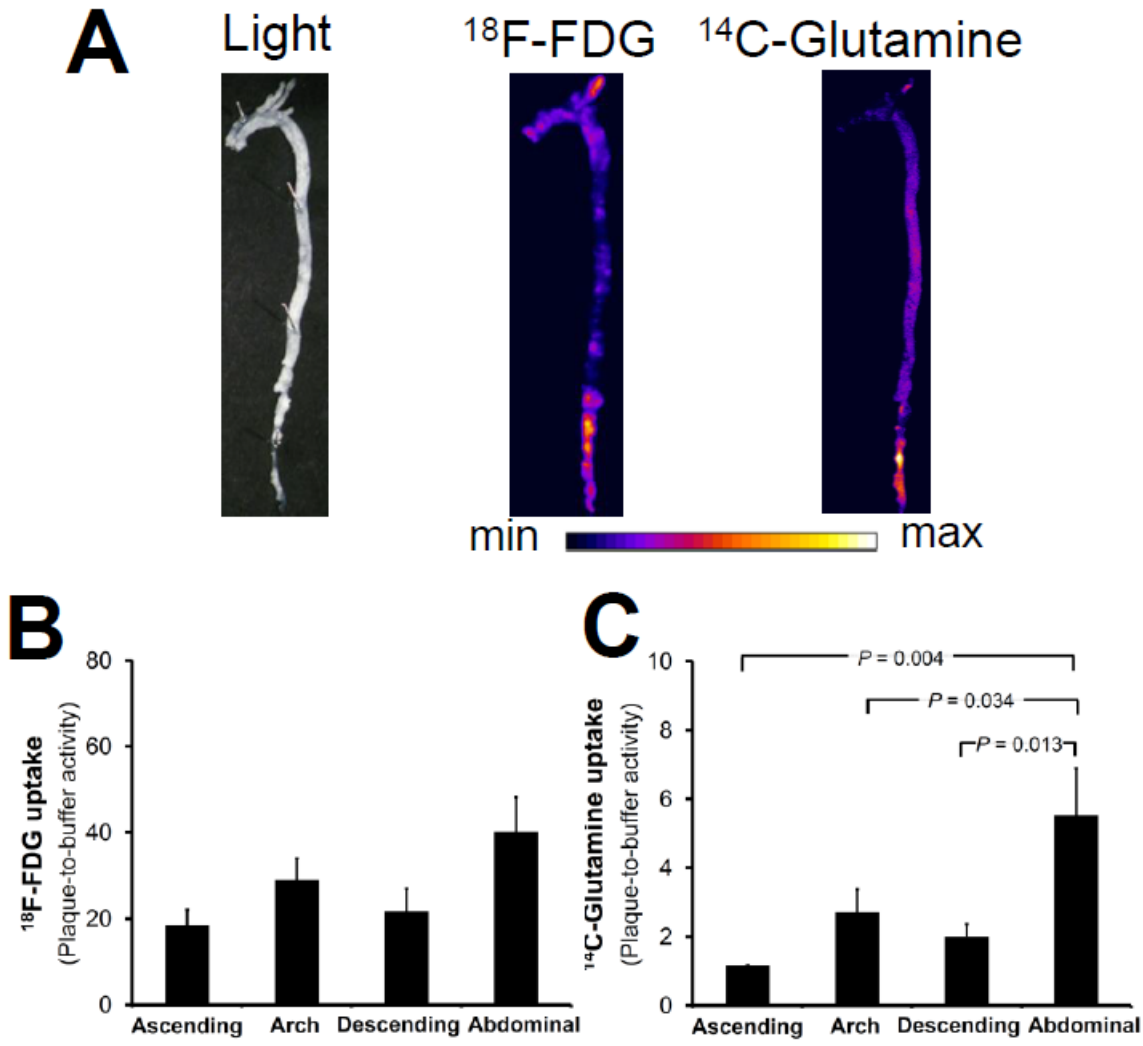
Reagent Name	**Source**
Biotin-labeled anti-CD68 (clone: FA-11)	AbD Serotec
Anti-GLUT1 (clone: PA1-46152)	Pierce
Anti-SLC3A2 (clone: S-16)	Santa Cruz Biotechnology
Anti-SLC1A5 (clone: A-14)	Santa Cruz Biotechnology
Cy2-labeled streptavidin (for CD68 detection)	Jackson ImmunoResearch
Cy3-labeled anti-rabbit antibody (for GLUT1 detection)	Jackson ImmunoResearch
Cy3-labeled anti-goat antibody (for SLC1A5 and SLC3A2)	Jackson ImmunoResearch
4′,6-diamidino-2-phenylindole (DAPI)	Invitrogen



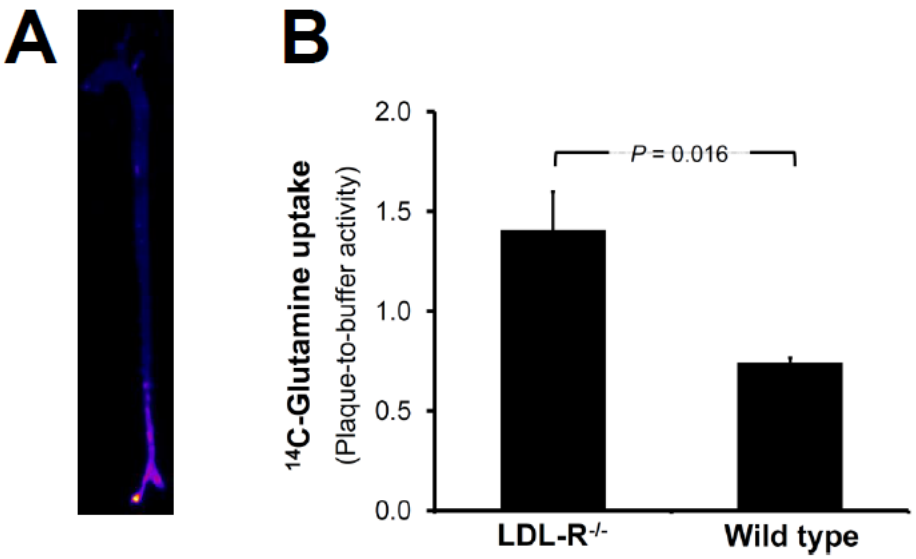
SUPPLEMENTAL FIGURE I. Divergent changes in ^{3}H-2-deoxyglucose and ^{3}H-glutamine accumulation in unstimulated macrophages (MΦ_0) as a function of time in culture. During the initial 24 h after plating, MΦ_0 showed a reduction in ^{3}H-2-deoxyglucose **(A)** and induction of ^{3}H-glutamine **(B)** accumulation in MΦ_0.



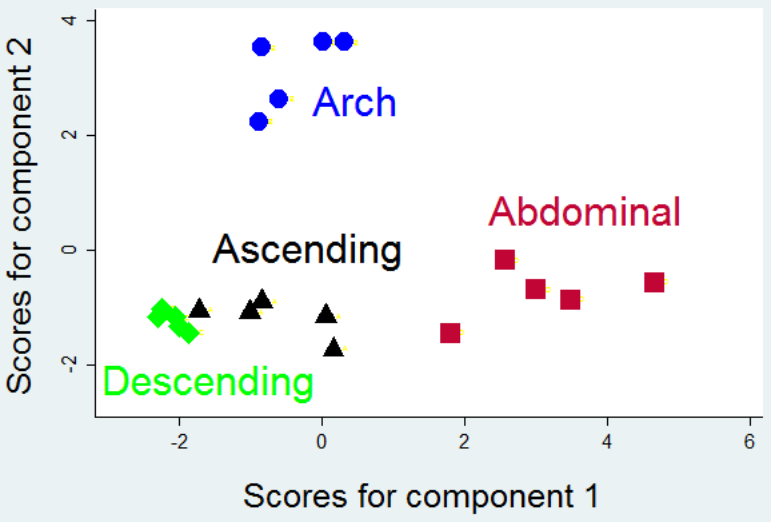
SUPPLEMENTAL FIGURE II. mRNA expression of glucose and glutamine transporters in macrophages. (A) RT-qPCR analysis of *Slc2a1* (*Glut1*) expression after 6 hours of polarization demonstrates increased expression of *Slc2a1* in $M\Phi_{LPS}$, consistent with their increased ^{3}H-2-deoxyglucose uptake. There is also a slight increase in the expression of *Slc2a1* after 6 hours of stimulation with IFN-γ and TNF-α, which precedes the modest and temporary increase in ^{3}H-2-deoxyglucose in $M\Phi_{IFN\text{-}\gamma+TNF\text{-}\alpha}$ at 24 hours. Coinciding with a temporary drop in glutamine uptake at 6 hours after stimulation with LPS, there is a significant decrease in expression of *Slc1a5* **(B)**, despite an increase in expression of *Slc3a2* **(C)**, in $M\Phi_{LPS}$ (*n* = 5).



SUPPLEMENTAL FIGURE III. Autoradiography of atherosclerotic aortas from LDL-R$^{-/-}$ mice fed with a HFD for 12 months. Representative *ex vivo* autoradiography images showed different patterns of ^{18}F-FDG and ^{14}C-glutamine accumulation along the aorta after 12 months feeding with a HFD, which is in concordance with our observation in 3 months HFD-fed mice **(A)**. The maximal ^{18}F-FDG accumulation was not statistically different among the various segments of the aorta **(B)**. However, consistent with our findings in mice fed a HFD for 3 months, ^{14}C-glutamine accumulation was significantly higher in abdominal aorta compared to the remaining segments **(C)**. (*n = 3*).



SUPPLEMENTAL FIGURE IV. Autoradiography of aortas from C57BL/6J wild-type mice fed with a normal diet. A Representative *ex vivo* autoradiography image demonstrates the low intensity of ^{14}C-glutamine accumulation in aorta of C57BL/6J wild-type mice fed with a normal diet **(A)**. Quantification of the mean accumulation along the length of aorta confirms a statistically significant lower accumulation in aortas from wild-type mice compared to LDL-R$^{-/-}$ mice fed with a HFD for 3 months **(B)**. (*n* = *4* for wild-type and *6* for LDL-R$^{-/-}$ mice).



SUPPLEMENTAL FIGURE V. Heterogeneous expression of polarization markers in different aortic segments. The PCA score plot using selected M1 (*Cxcl9*, *Il1b*, *Il6*, *Tnfa*, *Nos2*) and M2 (*Arg1*, *Ccl9*, *Cd36*, *Retnla*, *Mrc1*) polarization markers shows separate clustering of different aortic segments indicating the heterogeneity of polarization states along the length of aorta. Colored dots represent individual biological replicates (*n* = *5*).