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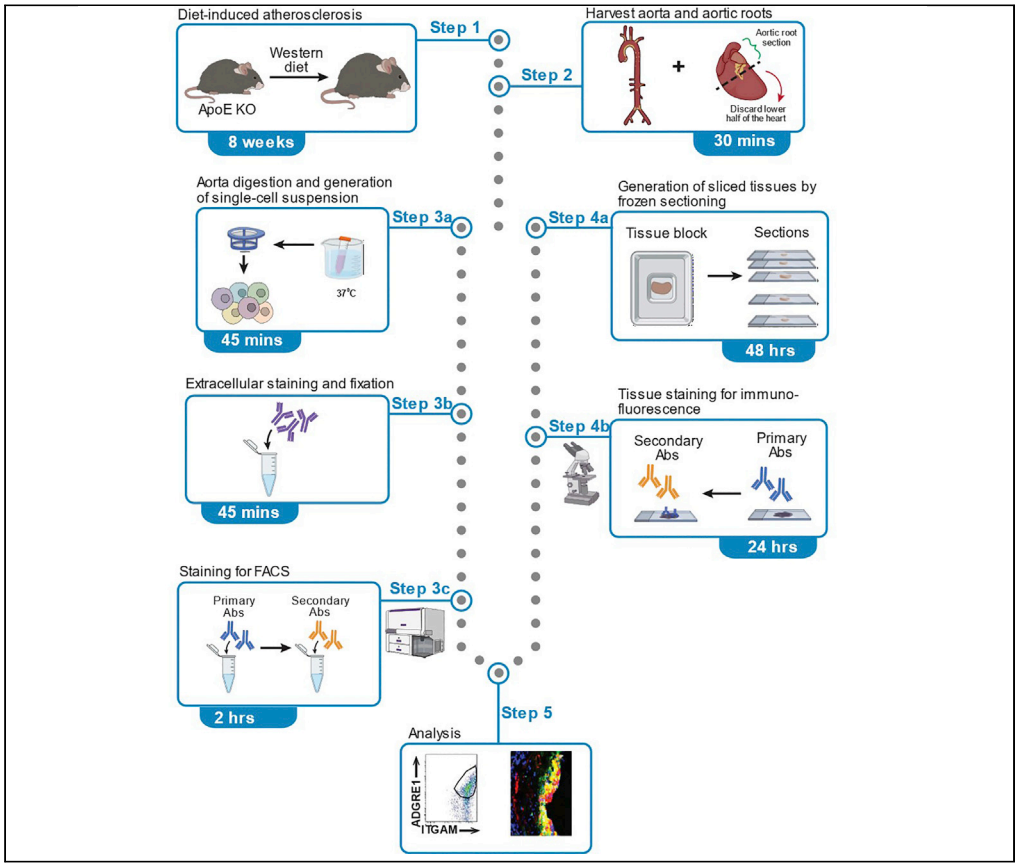
Evaluation of mTORC1 signaling in mouse atherosclerotic macrophages by flow cytometry and immunofluorescence



Previous studies have demonstrated that a high-protein diet leads to increased atherosclerosis in mice, and that this adverse effect is caused by activation of macrophage mTORC1 signaling. Here, we provide a detailed protocol for the evaluation of diet-induced mTORC1 signaling in plaque macrophages in atherosclerosis-prone apolipoprotein E (ApoE) knockout (KO) mice. This protocol includes flow cytometry and immunofluorescence analysis of atherosclerotic macrophages that can be used to study the atherogenic potential of a variety of mTORC1 modulators.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Procedure for the isolation and analysis of atherosclerotic macrophages

Protocols for evaluating macrophage mTORC1 signaling by FACS and tissue image

Description of a rapid and efficient technique on harvesting mouse aorta and aortic root

Zhang et al., STAR Protocols 4, 101665
December 16, 2022
https://doi.org/10.1016/j.xpro.2022.101665

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Evaluation of mTORC1 signaling in mouse atherosclerotic macrophages by flow cytometry and immunofluorescence

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https://doi.org/10.1016/j.xpro.2022.101665

SUMMARY

Previous studies have demonstrated that a high-protein diet leads to increased atherosclerosis in mice, and that this adverse effect is caused by activation of macrophage mTORC1 signaling. Here, we provide a detailed protocol for the evaluation of diet-induced mTORC1 signaling in plaque macrophages in atherosclerosis-prone apolipoprotein E (ApoE) knockout (KO) mice. This protocol includes flow cytometry and immunofluorescence analysis of atherosclerotic macrophages that can be used to study the atherogenic potential of a variety of mTORC1 modulators.

For complete details on the use and execution of this protocol, please refer to Zhang et al. (2020).

BEFORE YOU BEGIN

This protocol describes the evaluation of diet-induced mTORC1 signaling in plaque macrophages from atherosclerosis-prone ApoE KO mice fed either a standard or high-protein, low-carbohydrate Western diet for 8 weeks. However, this protocol has also been used for determining acute effects of high protein intake (e.g., gavage feeding) and other stimuli-induced mTORC1 signaling in atherosclerotic macrophages.

Institutional permissions

Animal handling and experimental procedures were approved by the Washington University Animal Studies Committee and all experiments conformed to Institutional Animal Care and Use Committee (IACUC) regulations, policies, and guidelines.

> *Note:* All mouse experiments must be approved by an Animal Care Committee in your research institution.

Dietary intervention and preparation of atherosclerosis-prone mouse models

Timing: 8 weeks

1. ApoE KO mice (or transgenic mice with ApoE KO background) housed in a specific pathogen–free barrier facility were weaned at 3 weeks of age to a standard mouse chow diet providing 6% calories as fat.
2. Mice were divided into two groups, ensuring similar or equal weights, then started at 8 weeks of age on Western-type diet, either:
   a. Standard (TD88137: 0.15% cholesterol, fat / protein / carbohydrate calories (kcal%) at 42% / 15% / 43% respectively).
   b. High-protein, low-carbohydrate (TD04524: similar cholesterol/fat content but with fat / protein / carbohydrate calories (kcal%) at 43% / 46% / 11%) Western-type diets (all from Harlan) (Foo et al., 2009).
3. Record body weight every week. Analysis of body composition can be measured with Echo-MRI at the end of 8 weeks.
4. Blood collection from mice at 0 weeks and 8 weeks of feeding on Western diet for measuring plasma or serum metabolic markers.
   a. Collect 150 μL blood from tail vein via capillary tube (in accordance with IACUC-approved protocol for blood collection).
   b. Allow the blood to clot for 15–30 min before centrifugation.
   c. Remove blood cells by centrifugation at 5000 rpm (2,400 × *g*) for 8 min at 4°C. Collect serum supernatant via pipette and store at −80°C.
5. Sacrifice mice and harvest aorta at the end of 8 weeks on either Western-type diet.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rat monoclonal CD45- Pacific Blue (clone 30-F11) - 1:100	BioLegend	Cat# 103126
Rat monoclonal ADGRE1/F4/80-APC (clone BM8) - 1:100	BioLegend	Cat# 123116
Rat monoclonal ITGAM/CD11b-PerCP-Cy5.5 (clone M1/70) - 1:100	BioLegend	Cat# 101228
Rabbit monoclonal phospho-RPS6/S6 (Ser235/236) (clone 2F9) - 1:100	Cell Signaling Technology	Cat# 4856
Goat anti-rat Alexa Fluor 594- 1:250	Invitrogen-Thermo Fisher Scientific	Cat# A11007
Goat anti-rabbit Alexa Fluor 488- 1:200	Invitrogen-Thermo Fisher Scientific	Cat# A11008
Chemicals, peptides, and recombinant proteins		
Liberase	Roche	Cat# 05401127001
DNase I	MilliporeSigma	Cat# D4527
Hyaluronidase	MilliporeSigma	Cat# H3506
DMEM	MilliporeSigma	Cat# D5796
Fetal Bovine Serum (FBS)	MilliporeSigma	Cat# F2242
Penicillin-Streptomycin (P/S)	Gibco	Cat# 15040-122
Sodium Pyruvate	Corning	Cat# 25-000-CI
Ammonium chloride (NH4Cl)	MilliporeSigma	Cat# A9434
Sodium bicarbonate (NaHCO3)	MilliporeSigma	Cat# S5761
EDTA	MilliporeSigma	Cat# ED25S
Anti-Mouse Fc Block	BD Biosciences	Cat# 553143
Saponin	MilliporeSigma	Cat# 84510
Tissue-Tek O.C.T.	Sakura	Cat# 4583
Bovine Serum Albumin (BSA)	MilliporeSigma	Cat# A7906
Milk (Blotting-Grade Blocker)	Bio-Rad	Cat# 170-6404
Triton-X 100	MilliporeSigma	Cat# X-100
Tris Base	MilliporeSigma	Cat# T6066

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Sodium chloride (NaCl)	MilliporeSigma	Cat# S3014
4′,6-diamidino-2-phenylindole (DAPI)	Invitrogen-Thermo Fisher Scientific	Cat# D1306
VECTASHIELD Hardset Mounting Medium	Vector Laboratories	Cat# H-1400
Phosphate buffered saline (PBS)	Corning	Cat# 21-040-CM
Experimental models: Organisms/strains		
Mouse: ApoE KO (male, 8 weeks)	The Jackson Laboratory	Cat# 002052
Software and algorithms		
FlowJo v10.7.1	FlowJo	https://www.flowjo.com/
BD LSRFortessa™ Cell Analyzer	BD Biosciences	https://www.bd.com/en-us/offerings/brands/lsrfortessa
ZEN microscope software	ZEISS	https://www.zeiss.com/
Zeiss LSM-700 confocal microscope	ZEISS	https://www.zeiss.com/
Other		
Standard Western diet (Adjusted calories diet (42% from fat)	Envigo	Cat# TD88137
High-protein, low-carbohydrate Western diet	Envigo	Cat# TD04524
70 mm Nylon Cell strainer	Corning	Cat# 431751
0.22 um CA Filter System	Corning	Cat# 0976140
0.45 um CA Filter System	Corning	Cat# 430514
Disposable base molds	Fisher Scientific	Cat# 22363553
5 mL Falcon Test tubes	Fisher Scientific	Cat# 14-959-5

MATERIALS AND EQUIPMENT

Liberase stock for digestion buffer

Add 10 mL DMEM medium into 5 mg Liberase powder to achieve a final concentration of 500 μg/mL, vortex until all powder fully dissolved. Separate the solution into 1.5 mL tube as 500 μL aliquots and store at −20°C for 12 months. Avoid repeated freeze/thaw cycles.

DNase I stock for digestion buffer

Add 10 mL double distilled water (DDW or DDH2O) into 100 mg DNase I powder to achieve a final concentration of 10 mg/mL, vortex until all powder fully dissolved. Separate the solution into 1.5 mL tube as 500 μL aliquots and store at −20°C for 12 months. Avoid repeated freeze/thaw cycles.

Hyaluronidase stock for digestion buffer

Add 1 mL DMEM medium into 10 mg hyaluronidase powder to achieve a final concentration of 10 mg/mL, vortex until all powder fully dissolved. Separate the solution into 1.5 mL tube as 1 mL aliquots and store at −20°C for 12 months. Avoid repeated freeze/thaw cycles.

Digestion buffer		
Reagent	Final concentration	Amount
DMEM	N/A	9 mL
Liberase (500 μg/mL)	2.5 μg/mL	50 μL
DNase I (10 mg/mL)	125 μg/mL	125 μL
Hyaluronidase (10 mg/mL)	0.8 mg/mL	800 μL
Total	**N/A**	**10 mL**

Prepare fresh and keep at 25°C for 1–2 h before digestion.

Red cell lysis buffer		
Reagent	Final concentration	Amount
DDW or DDH2O	N/A	499 mL
NH4Cl	155 mM	4.145 g
NaHCO3	12 mM	0.504 g
0.5 M EDTA	0.1 mM	0.1 mL
Total	**N/A**	**500 mL**
Prepare in a sterile environment and filter through 0.22 mm PES membrane. Store at 4°C for 6 months.		

DMEM complete medium		
Reagent	Final concentration	Amount
DMEM	N/A	440 mL
FBS (Inactivated)	10% (v/v)	50 mL
P/S (10,000 U/mL)	1% (v/v)	5 mL
Sodium pyruvate (100 mM)	1% (v/v)	5 mL
Total	**N/A**	**500 mL**
Prepare in a sterile environment and filter through 0.22 mm PES membrane. Store at 4°C for 6 months.		

FACS buffer		
Reagent	Final concentration	Amount
PBS	N/A	94 mL
FBS (Inactivated)	2%	2 mL
0.5 M EDTA	2 mM	4 mL
Total	**N/A**	**100 mL**
Prepare in a sterile environment and filter through 0.22 mm PES membrane. Store at 4°C for 6 months.		

Fc block		
Reagent	Final concentration	Amount
FACS Buffer	N/A	90 µL
Fc Block	1:10	10 µL
Total	**N/A**	**100 µL**
Prepare fresh and keep on ice before staining.		

Flow cytometry antibody cocktail		
Reagent	Final concentration	Amount
FACS Buffer	N/A	100 µL
Conjugated antibodies	1: 200	0.5 µL
Total	**N/A**	**100 µL**
Prepare fresh and keep on ice before staining.		

Permeabilization blocking buffer for intracellular staining		
Reagent	Final concentration	Amount
DDW or DDH2O	N/A	98 mL
FBS	2%	2 mL
Saponin	0.3%	300 µg
Total	**N/A**	**100 mL**
Prepare in a sterile environment and filter through 0.45 mm PES membrane. Store at 4°C for 1 month.		

Washing buffer for intracellular staining		
Reagent	Final concentration	Amount
DDW or DDH2O	N/A	98 mL
FBS	2%	2 mL
Saponin	0.1%	100 μg
Total	**N/A**	**100 mL**
Prepare in a sterile environment and filter through 0.45 mm PES membrane. Store at 4°C for 1 month.		

Roth buffer for immunofluorescence		
Reagent	Final concentration	Amount
DDW or DDH2O	N/A	92 mL
Milk	0.25%	0.25 g
BSA	0.5%	0.5 g
Triton-X 100	0.3%	300 μL
1 M Tris	50 mM	5 mL
5 M NaCl	150 mM	3 mL
Total	**N/A**	**100 mL**
Prepare in a sterile environment, adjust pH 7.4, and filter through 0.45 mm PES membrane. Store at 4°C for 1 month.		

STEP-BY-STEP METHOD DETAILS

Harvest whole aorta and aortic root

Timing: 20 min

1. Perfusion and exposure of aorta.
   a. Prepare 1.7 mL tubes containing pre-cooled DMEM medium, OCT medium and disposable base molds before dissection.
   b. Euthanize mouse with carbon dioxide chamber.
   c. Place mouse on a surgical board and sterilize the body using 70% ethanol.
   d. Open the skin and expose the chest cavity using tweezers and dissecting scissors, then cut through the ribcage to expose the heart.
   e. Take the syringe containing 30 mL PBS and insert the 21-g needle into the apex of the left ventricle.
   f. Cut the right atrium to let the blood flow out.
   g. Start injecting PBS into the lumen of ventricle slowly.

   Note: The liver should become pale after perfusion.

   h. Carefully remove all other organs/tissues from the coelom except the heart and aorta.
2. Prepare the aorta and heart samples.
   a. Rinse the coelom with PBS and remove extra liquid with sterile gauze, then transfer the mouse into the platform of dissection microscope.
   b. Separate the aorta from the dorsal surface of the coelom with sterile micro-scissors and micro-forceps.
   c. Carefully remove the perivascular adipose tissue along the aorta while avoid cutting the aortic wall (Figure 1).
   d. Cut the aorta above the base of heart and make sure keeping the intact of aortic arch, innominate artery, carotid arteries and subclavian arteries (Jeong et al., 2018).
   e. Transfer the aorta immediately into a 1.7 mL tube filled with pre-cooled DMEM medium and keep on ice before digestion.

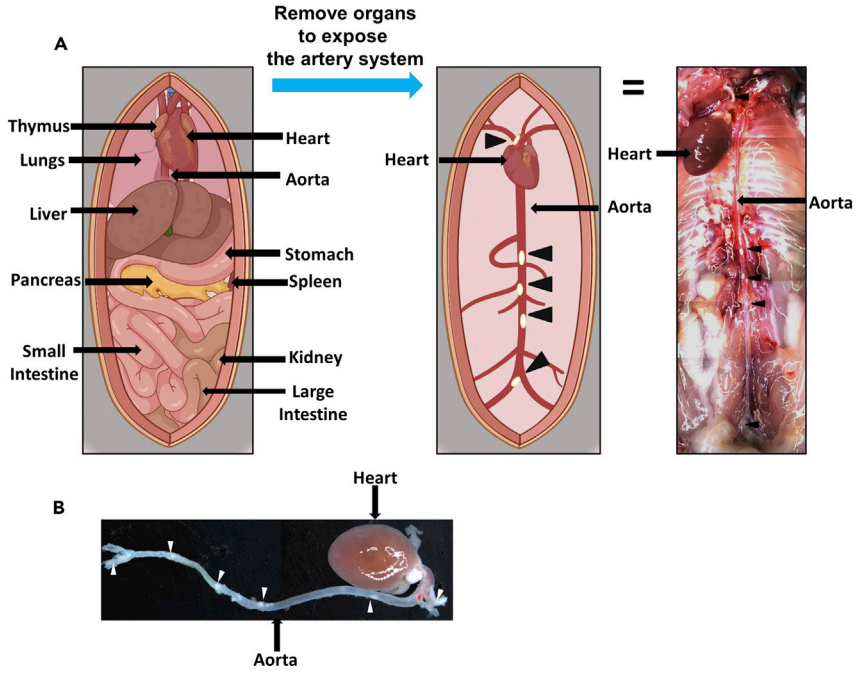

Figure 1. Representative pictures of exposing and isolating heart and aorta from ApoE KO mouse fed with Western diet for 2 months

(A) Exposure of heart and aorta after perfusion and removing adipose tissue. Organs are labeled in the schematic diagrams. Plaques inside the aorta are indicated by black arrows.

(B) Presentation of heart and aorta after removal from mouse. Plaques inside the aorta are indicated by white arrows.

f. Take the heart and cut it into halves using a razor. Put the half of heart into a base moldand make sure the heart lies vertically inside the chamber with the aortic root on the bottom. Add enough OCT medium to embed all tissues inside.

g. Put the chamber containing heart into −80°C freezer for frozen sectioning.

⚠ CRITICAL: Perivascular adipose tissue should be completely removed from the aorta to prevent contamination from adipose tissue macrophages (ATMs) (Figure 1B).

Enzymatic digestion of aorta and preparation of single-cell suspension

Timing: 45 min

3. Put the aorta into a sterile dish containing 0.5 mL digestion buffer and mince finely using a razor.
4. Transfer the aorta with a pipette into a 15 mL tube, centrifuge 700 rpm (50 × *g*) for 5 min at 4°C, discard the supernatant, and then add 3 mL pre-warmed digestion buffer to re-suspend the pellet.
5. Place the tube containing minced aorta on a shaker at 37°C to digest. Shake the tube every 10 min for a thorough digestion (may take 30–40 min).
6. Prepare a 50 mL tube containing 5 mL pre-cooled complete DMEM. Place a 70 μM cell strainer into this tube then add the mixture after digestion through the cell strainer. Wash the strainer once with 2 mL pre-chilled DMEM Complete Medium.

Note: A pellet should be visible after the washing step.

7. To stop the digestion process, add 500 μM EDTA into the strained mixture to a final concentration of 1 μM and mix well.

Table 1. Example staining panel for analysis by BD Fortessa flow cytometer

Reagent	Final concentration
CD45- Pacific Blue	1:100
ADGRE1- APC	1:100
ITGAM- PerCP-Cy5.5	1:100
Check key resources table for exact antibodies.	

8. Centrifuge the mixture at 700 rpm (50 × *g*) for 5 min at 4°C, remove the supernatant, and re-suspend the pellet with 1 mL red cell lysis buffer. Transfer to a 15 mL Eppendorf tube and incubate on ice for 5 min.
9. Add 5 mL cold FACS buffer and centrifuge cell suspension at 700 rpm (50 × *g*) for 5 min at 4°C, discard all the supernatant and keep the tube on ice.

⚠ CRITICAL: The digestion step should not exceed 45 min. Long-term enzymatic digestion can lead to increased cell death and generation of cellular debris.

Extracellular staining for flow cytometry

Timing: 45 min

10. Re-suspend the cell pellet with 10 μL Fc block solution, keep the tube on ice and incubate in the dark for 5 min.
11. Add 90 μL prepared antibody cocktail (Table 1) into the tube, mix well and incubate on ice in the dark for 30 min.
12. Add 500 μL FACS buffer into the mixture, wash cells twice by centrifuging at 2500 rpm (630 × *g*) for 5 min at 4°C.
13. Discard all the supernatant, add 500 μL 2% PFA to re-suspend cells while vortex the tube gently, then keep the tube in the dark at 4°C before intracellular staining.

Note: Protect samples from light for next step (intracellular staining).

Pause point: Samples can be kept at 4°C for 6–12 h until ready to proceed to the next step.

Intracellular staining for flow cytometry

Timing: 2 h

14. Centrifuge the fixed cells at 5000 rpm (2,400 × *g*) for 5 min at 25°C, remove all the supernatant and wash the pellets with 1 mL PBS.
15. Discard supernatant and add 500 μL permeabilization/blocking buffer to re-suspend the pellets, then incubate at 25°C in the dark for 30 min.
16. Centrifuge at 5000 rpm (2,400 × *g*) for 5 min at 25°C, remove all the supernatant and add 100 μL anti-mouse p-S6 (1:150 diluted in permeabilization/blocking buffer). Mix well and incubate at 25°C in the dark for 30 min.
17. Wash the cells by adding 500 μL washing buffer and centrifuge at 5000 rpm (2,400 × *g*) for 5 min at 25°C. Repeat once more.
18. Discard all the supernatant and add 100 μL Alexa Fluor 488-conjugated secondary antibody (1:200 diluted in permeabilization/blocking buffer). Mix well and incubate at 25°Cin the dark for 30 min.
19. Wash the cells twice with 500 μL washing buffer. Remove all liquids after washing and re-suspend the cells in 300 μL washing buffer.

20. Remove debris by filtering the cell suspension through a 70-mm cell strainer into a 50 mL tube, then transfer the liquid into a 5 mL FACS flow tube.
21. Analyze the samples with a flow cytometer.

Immunofluorescence staining of aortic roots

Timing: 1–2 days

22. Make frozen sections (10 μm) using a cryostat. Start collecting sections when plaque lesion appears and stop when most of lesion areas are gone. The slides can be kept at −80°C for 6 months.
23. Select the slide containing the sections with the largest lesion area. Embed this slide into 10 mL pre-chilled 4% PFA at 4°C for 15 min.
24. Wash the slide 3 times by adding pre-chilled (4°C) PBS onto the surface of the slides with a pipette, with subsequent gentle shaking every 1–2 min.
25. Block/permeabilize the sections in Roth Buffer at 25°C for 1 h.
26. Incubate the slide with the first primary antibody (Rat anti-mouse CD68 diluted at 1:250 in Roth buffer) at 25°C for 2 h.

Optional: The incubation with primary antibody can be alternatively done at 4°C for 12 h, then transfer the slides into 37°C for 30 min before moving to the next step.

27. Wash the slide with cold PBS for 5 min. Repeat the washing twice.
28. Incubate the slide with the correlating secondary antibody (Alexa Fluor 594 conjugated goat anti Rat IgG diluted at 1:250 in Roth buffer) at 25°C for 1 h.
29. Wash the slide with cold PBS for 5 min. Repeat the washing twice.
30. Incubate the slide with the second primary antibody (Rabbit anti-mouse phosphor-RPS6 diluted at 1:100 in Roth buffer) at 25°C for 2 h.

Optional: The incubation with primary antibody can be alternatively done at 4°C for 12 h, then transfer the slides into 37°C for 30 min before moving to the next step.

31. Wash the slide with 100 mL cold PBS for 5 min. Repeat the washing twice.
32. Incubate the slide with the correlating secondary antibody (Alexa Fluor 488 conjugated goat anti Rabbit IgG diluted at 1:200 in Roth buffer) at 25°C for 1 h.
33. Wash the slide with cold PBS for 5 min. Repeat the washing twice.
34. Stain the nucleus using 1× DAPI stock in PBS for 3 min at RT.
35. Wash the slide with 100 mL cold PBS for 5 min. Repeat the washing twice.
36. Remove all the liquid on the surface of sections, lay one drop of Vectashield® fluorescence mounting medium around the sections on slide, then slowly put the coverslip on the slide.
37. Dry the slide in the dark for 4 h, then it is ready for imaging with a confocal microscopy.

⚠ CRITICAL: Protect the samples on the slides from drying out during the staining process.

EXPECTED OUTCOMES

Related gating strategies have been described in the related publication. In short, forward versus side scatter (FSC vs SSC) gating was the initial gating utilized. Then, single cells were identified by plotting height versus width for forward scatter (FSC-H vs FSC-W) and width for side scatter versus height for forward scatter (SSC-W vs FSC-H). After the exclusion of doublets and debris, leukocytes were identified using the pan-hematopoietic marker CD45. Atherosclerotic macrophages were readily identified based on expression of ADGRE1 and ITGAM. The aortic macrophages were identified as ADGRE1^{hi}, ITGAM^{int}. Following our protocol and during FACS analysis, a specific

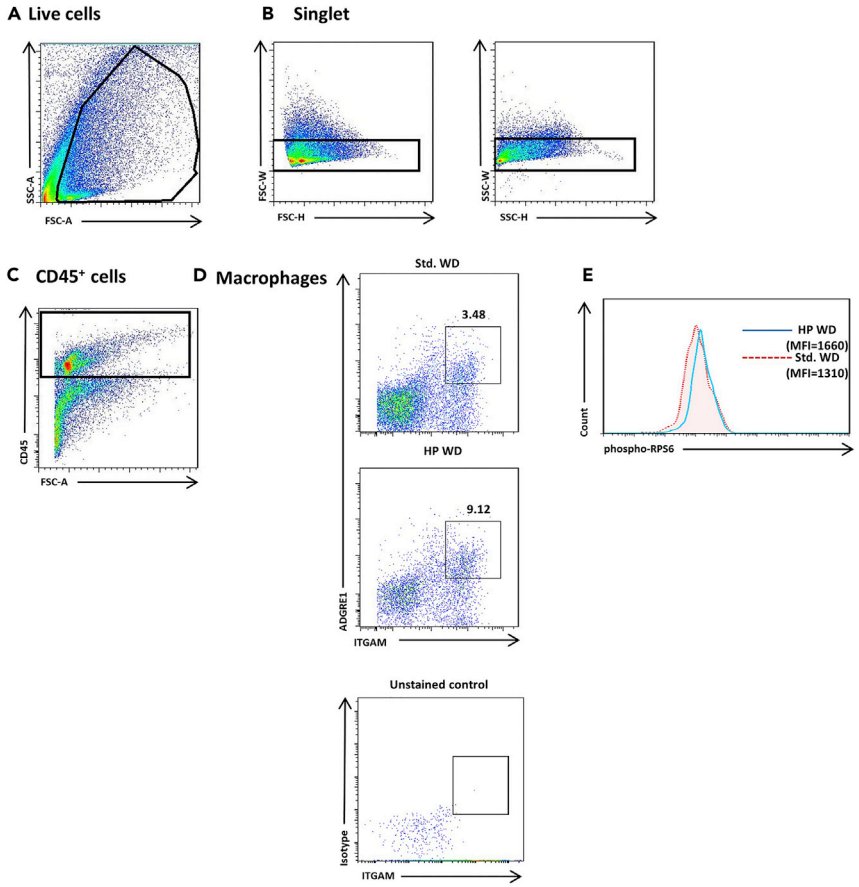

Figure 2. Example flow plots to evaluate mTORC1 activation in aortic macrophages

(A–C) Gating for live cells (A), singlet (B) and CD45$^+$ cells (C).

(D) Identify aortic macrophages as F4/80$^+$/CD11b$^+$ subset from CD45$^+$ cells.

(E)The phosphorylation level of S6 is significantly elevated in aortic macrophages from mice fed with high protein Western diet compared with controls fed with standard Western diet.

ADGRE1$^+$ITGAM$^+$ subpopulation should be found in CD45$^+$ cells from aorta (Figures 2A–2D), indicating the group of atherosclerotic macrophages. When gating is set around this population, a low FITC fluorescence signal is detected in only secondary antibody treated controls, while significant increase in fluorescence should be detected in samples incubated with phosphor-RPS6 antibody and secondary antibody. Compared with samples from mice fed a regular western diet, high-protein diet would induce an accumulation of macrophages inside the aorta (Figure 2D). Moreover, an obvious right-side movement of FITC fluorescence peak can be observed in the samples from high-protein diet group, implying increased activation of mTORC1 signaling in atherosclerotic macrophages (Figure 2E).

After immunofluorescence staining and confocal imaging, quantification of immunofluorescence can be performed using Zen software, whereby the threshold of red fluorescence is reset according to a non-staining control and gated for CD68$^+$ cells. Then, the intensity of green fluorescence (phospho-RPS6) in CD68$^+$ cells is quantified for the measurement of mTORC1 activation. Similar to the findings obtained from FACS analysis, increased CD68$^+$ cells should be detected inside the aortic root lesion area in the high-protein diet group. There will be elevated signal of green fluorescence shown in the lesion area from the same section, indicating increased mTORC1 activation. A moderate-to-high level of overlap between fluorescence from CD68$^+$ cells and pS6 will also be observed (Figure 3), which indicates high-protein diet induces significant mTORC1 activation in atherosclerotic macrophages (Zhang et al., 2020).

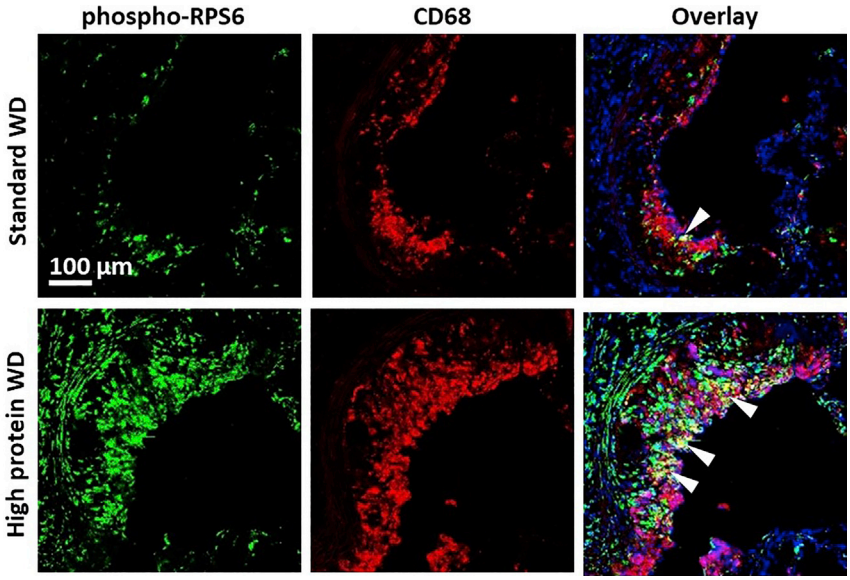

Figure 3. Example immunofluorescence images of evaluation of mTORC1 activation in macrophages from plaque lesion area of aortic roots

Co-localization of CD68 and pS6 are indicated with white arrows. Scale bar is 100 μM.

LIMITATIONS

The preparation of single cell suspension from the whole aorta for flow cytometry analysis involves tissue isolation and digestion, which may take as long as 1 h. Previous studies have stated mTORC1 signaling could be activated by multiple stimuli within this short period. Therefore, a prolonged preparation may lead to a reduction of mTORC1 signaling detection. The digestion process is at 37°C to maintain the activity of enzymes. This high temperature may also accelerate protein degradation and dephosphorylation, which will diminish mTORC1 signaling. Therefore, it is critical to make sure all samples are collected and treated at the same time for comparable results. In addition, analysis with immunofluorescence staining on aortic roots from the same mice is highly recommended. Since the sample preparation process is much more rapid than that for FACS analysis, mTORC1 signaling in aortic macrophages would remain intact and may work as a side-by-side quality control for data from FACS analysis.

TROUBLESHOOTING

Problem 1

Step 5. High ratio of cell death after aorta digestion.

Potential solution

Reduce the time spent on tissue isolation and digestion. If possible, increase Liberase concentration in the digestion buffer. Also, using a milder shaking speed could be helpful.

Problem 2

Steps 10–21. Low or minimal mTORC1 signaling detected by FACS analysis.

Potential solution

It is recommended to use ApoE KO mice fed with high-protein diet as positive controls. In addition, reducing the digestion time and keeping isolated cells on ice is also helpful to protect the diminishment of macrophage mTORC1 signaling. Also, for intracellular staining, it is better to use freshly prepared fixation buffer and permeabilization/blocking buffer. Finally, increasing both the

permeabilization time and the concentration of primary antibody may improve detection of mTORC1 signaling.

Problem 3

Steps 10–37. Autofluorescence in atherogenic macrophages during analyses with FACS or microscopy.

Potential solution

Long-term fixation in 4% PFA may lead to autofluorescence from cells, therefore, we recommend the fixation does not exceed 24 h at 4°C. Washing two times to remove any remaining PFA is needed before the next step. Most macrophages inside the aorta will take up lipids to become "foam cells" and may generate autofluorescence in the green channel. Therefore, non-stained control and only secondary antibody-stained control should be included during analyses with FACS or microscopy.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Babak Razani at brazani@wustl.edu.

Materials availability

No newly generated plasmids, mouse lines, or other unique materials are associated with this protocol.

Data and code availability

No datasets were generated or analyzed during this study.

ACKNOWLEDGMENTS

This work was supported by NIH grants R01 HL125838, R01 DK121560, P30 DK056341 (Washington University Nutrition and Obesity Research Center), and T32 HL134635 and grants from the American Heart Association (#897628), the Veterans' Administration (I01 BX003415 award), and the Longer Life Foundation. Cell sorting and flow cytometry data acquisition and analysis provided by the Flow Cytometry Core facility, Department of Pathology and Immunology, Washington University School of Medicine.

AUTHOR CONTRIBUTIONS

Conceptualization, investigation, and methodology, X.Z. and B.R.; writing – original draft, X.Z.; figures, X.Z., A.R.V., and S.J.J.; writing – review & editing, X.Z., J.S., A.R.V., S.J.J., A.P., Y.S.Y., D.K., and B.M.; resources, funding acquisition, and supervision, B.M. and B.R.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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