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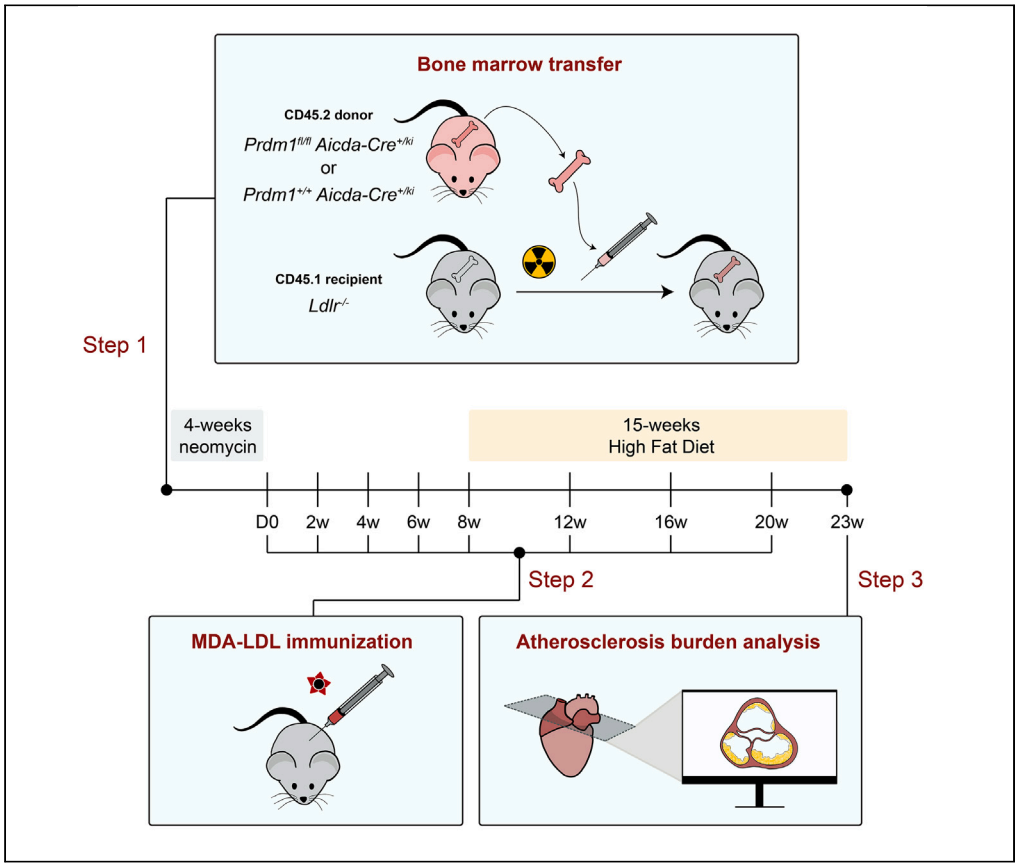
Assessing the impact of an antigen-specific antibody response on atherosclerosis development in mice



The antibody immune response plays a critical role in atherosclerosis. Here, we present a protocol for assessing the impact of an antigen-specific germinal center antibody response on atherosclerosis development, using a pro-atherogenic mouse model deficient for the production of germinal-center-derived antibodies. We describe steps for bone marrow transfer from donor mice into irradiated recipient mice. We then detail immunization of mouse chimeras with atheroprotective malondialdehyde low-density lipoprotein during high-fat diet feeding and atherosclerosis burden analysis.

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

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Highlights

Detailed protocol to generate $Ldlr^{-/-}$ $Prdm1^{fl/fl}$ $Aicda\text{-}Cre^{+/ki}$ bone marrow chimeras

Detailed instructions of MDA-LDL immunization during atherosclerosis induction

Extraction and processing of mouse hearts for the study of atherosclerosis

Quantification of atherosclerosis in MDA-LDL-immunized $Prdm1^{fl/fl}$ $Aicda\text{-}Cre^{+/ki}$ mice

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Assessing the impact of an antigen-specific antibody response on atherosclerosis development in mice

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SUMMARY

The antibody immune response plays a critical role in atherosclerosis. Here, we present a protocol for assessing the impact of an antigen-specific germinal center antibody response on atherosclerosis development, using a pro-atherogenic mouse model deficient for the production of germinal-center-derived antibodies. We describe steps for bone marrow transfer from donor mice into irradiated recipient mice. We then detail immunization of mouse chimeras with atheroprotective malondialdehyde low-density lipoprotein during high-fat diet feeding and atherosclerosis burden analysis.

For complete details on the use and execution of this protocol, please refer to Martos-Folgado et al. (2022).[1]

BEFORE YOU BEGIN

The steps below describe a protocol to transplant *Prdm1*$^{fl/fl}$ *Aicda-Cre*$^{+/ki}$ bone marrow (BM) to *Ldlr*$^{-/-}$ mice, followed by immunization with MDA-LDL while the mice develop atherosclerosis upon high fat diet (HFD) feeding. The objective of this protocol is assessing the role of germinal center-derived antibodies in the atheroprotection driven by MDA-LDL vaccination.

Institutional permissions

All animal procedures were approved by the CNIC Ethics Committee and the Madrid regional authorities (PROEX 377/15) and conformed to EU Directive 2010/63/EU and Recommendation 2007/526/EC regarding the protection of animals used for experimental and other scientific purposes, enforced in Spanish law under Real Decreto 1201/2005.

Mice generation

Timing: 10 weeks

1. Breed *Prdm1*$^{fl/+}$; *Aicda-Cre*$^{ki/ki}$ mice with *Prdm1*$^{fl/+}$; *Aicda-Cre*$^{+/+}$ mice to generate *Prdm1*$^{fl/fl}$; *Aicda-Cre*$^{+/ki}$ mice , and *Prdm1*$^{+/+}$; *Aicda-Cre*$^{+/ki}$ control mice.

   Note: Mice were generated at the expected Mendelian rate (25% *Prdm1*$^{fl/fl}$; *Aicda-Cre*$^{+/ki}$, 25% *Prdm1*$^{+/+}$; *Aicda-Cre*$^{+/ki}$).

2. Perform breeding monitoring genotyping.

Note: Behavior and physical condition are undistinguishable between genotypes. These strains are in a CD45.2 background. Genotyping protocols are specified at https://www.jax.org/strain/007770 for the *Aicda-Cre* mouse line and https://www.jax.org/strain/008100 for the Prdm1 mouse line.

3. Generate $Ldlr^{-/-}$ (CD45.1) mice by homozygous breeding of $Ldlr^{-/-}$ mice. Genotyping protocols are specified at https://www.jax.org/strain/002207.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-mouse CD45.1 (clone A20) (1/100)	BD Pharmingen	Cat#55870; RRID: AB_1645214
Anti-mouse CD45.2 (clone 104) (1/100)	BD Pharmingen	Cat#552950; RRID: AB_394528
Anti-mouse CD3 (clone 145-2C11) (1/100)	BD Biosciences	Cat#553062; RRID: AB_394595
Anti-mouse CD19 (clone 145-2C11) (1/200)	BD Pharmingen	Cat#557399; RRID: AB_396682
Anti-mouse GR-1 (clone RB6-8C5) (1/50)	BD Biosciences	Cat#553125; RRID: AB_394641
Mouse FC Block (Purified Rat Anti-Mouse CD16/CD32) (1/50)	BD Biosciences	Cat#553142; RRID: AB_394657
Streptavidin PE-Cy7 (1/200)	BD Pharmingen	Cat#557598
Chemicals, peptides, and recombinant proteins		
DAPI	Sigma-Aldrich	Cat#D9542
Neomycin trisulfate salt hydrate	Sigma-Aldrich	Cat#N1876
2-Propanol	Honeywell	Cat#24137-1L
Human LDL	Joan Carles Escolà-Gil	N/A
Malondialdehyde bis (dimethyl acetal)	Sigma-Aldrich	Cat#8207560005
Adjuvant Complete Freund's (CFA)	BD Biosciences	Cat#210485
Adjuvant Incomplete Freund's (IFA)	BD Biosciences	Cat#210486
Formaldehyde 4%	VWR	Cat#11699408
OCT compound	Sakura	Cat#4583
Mayer's hematoxylin	Bio-Optica	Cat#05-06002/L
Oil Red O	MERCK	Cat#S0389
Picrosirius	Riedel-de haën	Cat#4248
ACK lysis buffer	Lonza	Cat#BP10-548E
Aquatex	Merck	Cat#108562
Superfrost Plus slides	VWR	Cat#631-0108
Trypan blue	Hyclone	Cat#SV30084.01
HCL	Sigma-Aldrich	Cat#H1758
NaOH	MERCK	Cat#1064981000
PBS, pH 7.4	Thermo Fisher	Cat# 10010001
RPMI	Sigma-Aldrich	Cat#0030120086
Fetal bovine serum	Sigma-Aldrich	Cat#F7524
Critical commercial assays		
PD10 desalting columns	Sigma-Aldrich	Cat#GE17-0851-01
Amicon Ultra-15 PLTK Ultracel-PL membrane, 30 kDa	Sigma-Aldrich	Cat#Z740204
BCA Assay Kit	Thermo-Fisher	Cat# 23227
Experimental models: Organisms/strains		
Mouse: $Ldlr^{-/-}$, males, 8–28 weeks old (experimental details are described in the figure legends)	Jackson Laboratories[2]	Cat#002207
Mouse: *Aicda-Cre*$^{+/ki}$, male and female, 6–8 weeks old	Jackson Laboratories[3]	Cat#007770
Mouse: *Prdm1*$^{+/fl}$, male and female, 8–13 weeks old	Jackson Laboratories[4]	Cat#008100
Software and algorithms		
Image J	Schneider et al.[5]	https://imagej.nih.gov/ij/
FlowJo V10.4.2	BD Biosciences	https://www.flowjo.com/solutions/flowjo/

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Prism, version 8	GraphPad	https://www.graphpad.com/scientificsoftware/prism
Other		
Eppendorf PCR tubes	Eppendorf	Cat#0030124537
FACS tubes	Delta Lab	Cat#300800.1
Eppendorf Safe-Lock tubes	Eppendorf	Cat#21875-034
U-100 Insuline syringe	BD Micro-Fine	Cat#320926
21 G needle	BD Microlance 3	Cat#301156
23 G needle	BD Microlance 3	Cat#300800
25 G needle	BD Microlance 3	Cat#300600
Medipoint Goldenrod lancet 4 mm 1000/cs	Medipoint	Cat#GR-4MM1
Microvette® 100 K3 EDTA, 100 µL, cap red, flat base tubes	Sarstedt	Cat#20.1278
Falcon 15 mL tubes	Corning	Cat#352097
pH indicator strips PH 6.5-10.0	MColorpHast	Cat#1.09543.0001
Millex-GP 0.22 µm PES membrane	Millipore	Cat#SLMP025SS
HFD 21% of crude fat and 1% cholesterol*	SNIFF	Cat#EFD12079 mod. 1% chol
HFD 21% of crude fat and 1.25% cholesterol*	SNIFF	Cat#EFD12079 mod. 1.25% chol
1–68A irradiator	JL Shephed & Associates	N/A
Counting chamber Neubauer improved (BRAND)	BLAUBRAND	Cat#BR717805
Warming Cabinet Mini Thermacage	SCANBUR	N/A
LSRFortessa™ Cell Analyzer	BD Biosciences	N/A
Cryostat Leica CM1850	Leica	N/A
Microscope Leica DM2500	Leica	N/A

*High fat diets.

MATERIALS AND EQUIPMENT

Oil-red O Stock solution		
Reagent	Final concentration	Amount
Oil Red O (CI 26125)	0.5%	0.5 g
2-Propanol	60%	100 mL

Note: Dilute the reagent at 54°C for 24 h to facilitate the dilution.

Note: Oil-red O Stock solution can be stored at room temperature (RT, 20°C–25°C) for 6 months

STEP-BY-STEP METHOD DETAILS

Bone marrow transplant

Timing: 4 weeks

These steps describe the transfer of BM into a recipient mouse.

1. Irradiate *Ldlr*$^{-/-}$ (CD45.1) mice with 2 doses of 5.5 Gy (10 min each, at 37°C) separated by 5 h using a JL Shephed & Associates 1–68A irradiator with a source of 1000 Ci of Cs-137.2
2. Prepare donor BM cells (Figure 1).
   a. Euthanize *Prdm1*$^{fl/fl}$ *Aicda-Cre*$^{+/ki}$ and *Prdm1*$^{fl/fl}$ *Aicda-Cre*$^{+/+}$ mice by placing them into a CO_2 cage (>70% CO_2) until no breath movements are observed.
   b. Before proceeding, confirm the mouse is dead by pressing the footpad and detecting no movement.

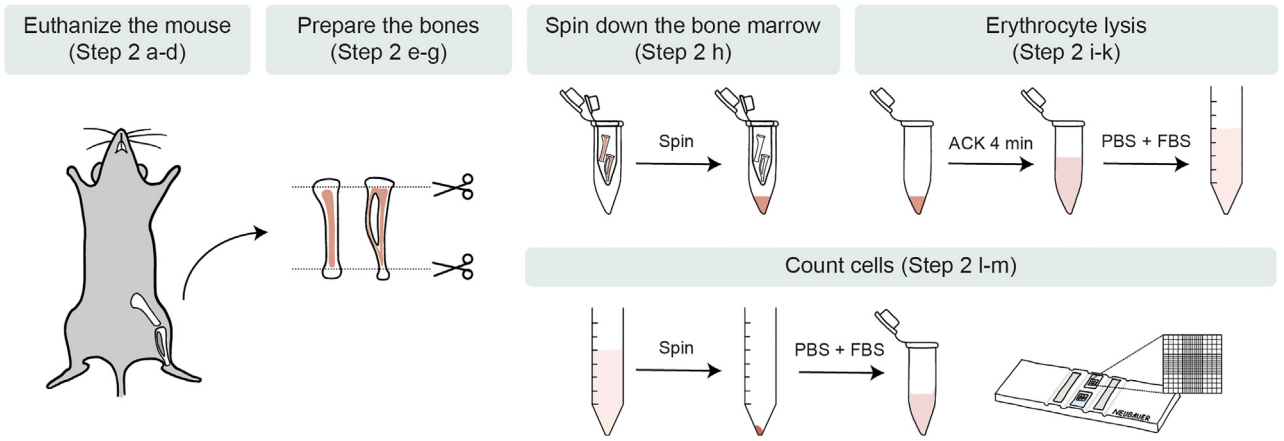

Figure 1. Procedure to harvest and count bone marrow cells

c. Clean the surgical material with EtOH 70% and prepare a sterile hood to perform the procedure inside.
d. Dislocate the hip joint by pulling from the mouse footpad.
e. Dissect the skin and the muscle surrounding the mouse leg to expose tibia and femur bones.
f. Cut over femur's head with scissors to separate the bones from the rest of the body.
g. Clean the bones by removing the surrounding muscle.
h. Carefully separate tibia and femur.
i. Cut the edges of tibia and femur and place them in an Eppendorf PCR tube with a hole in the bottom made with a 21 G needle.
j. Place the bone containing Eppendorf PCR tube into a 1.5 mL Eppendorf Safe-Lock tube and centrifuge 1 min at 16100 g at 4°C to spin down the BM into the 1.5 mL tube.
k. Resuspend the cell pellet with 1 mL of ACK Lysis Buffer by pipetting up and down and transfer the content to a 15 mL tube in order to lysate erythrocytes.
l. Incubate 4 min at RT (20°C–25°C).
m. Add 4 mL of sterile Phosphate Buffered Saline (PBS) 1× with 2% Fetal Bovine Serum (FBS) at 4°C.
n. Centrifuge at 450 g for 10 min and at 4°C, aspirate the supernatant and resuspend the cells in 500 µL of cold PBS 1×.
o. Count the cells.
    i. Dilute the cells 1/100 with PBS 1× to facilitate cell counting.
    ii. Mix the cells 1/10 with trypan blue 0.2% in PBS 1×.
    iii. Count the cells with a Neubauer counting chamber.

3. Transplant donor BM cells into the recipient mice.
    a. Pre-warm the recipient mice with a Warming Cabinet Mini Thermacage for 15 min at 40°C.
    b. Load the cells in a U-100 Insuline syringe.
    c. Inject 5 × 10^6 cells in 100 µL of PBS 1× per mouse through tail veins.
    d. Maintain recipient mice with 2 mg/mL neomycin in drinking water for 4 weeks.

⚠ CRITICAL: Neomycin could suffer degradation upon light exposure. Neomycin containing drinking water bottle must be covered by aluminum foil to protect it from the light.

4. Check transplant reconstitution (representative reconstitution result is shown in Figure 2).
    a. Immobilize the mouse and extract blood from the sub-maxillary plexus with a Medipoint Goldenrod lancet 4 mm 1000/cs.
    b. Place the blood in Microvette® 100 K3 EDTA tubes.
    c. Invert the EDTA tube gently up and down 6 times. to mix the EDTA with the blood.

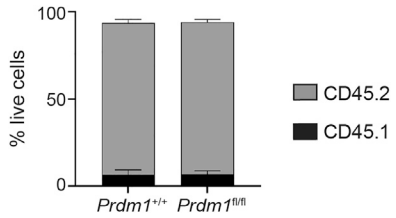

Figure 2. Representative result of flow cytometry analysis of donor (CD45.2) and receptor (CD45.1) cell in blood of *Prdm1*$^{+/+}$ *Aicda*-Cre$^{+/ki}$ *Ldlr*$^{-/-}$ and *Prdm1*$^{fl/fl}$ *Aicda*-Cre$^{+/ki}$ *Ldlr*$^{-/-}$ chimeras

d. Add 5 mL of ACK lysis buffer in 45 μL of blood and mix it in a 15 mL Falcon tube. Incubate 4 min at room temperature (RT).
e. Stop the lysis by adding 10 mL of RPMI with FBS 2%.
f. Centrifuge at 450 g 10 min at 4°C, aspirate the supernatant and resuspend the cells in 150 μL PBS 1× FBS 2%.
g. Add the cells into a 96-U-well plate.
h. Centrifuge the plate at 450 g 4 min at 4°C.
i. Resuspend the cells in 50 μL of mouse Fc block 1/50 diluted in PBS 1× FBS 2%.
j. Wash the wells with 200 μL of PBS 1× FBS 2%, centrifuge the plate at 450 g 4 min at 4°C and remove the supernatant.
k. Resuspend the cells in 50 μL of antibody mix solution containing: CD3 FITC (1/100), CD19 PE (1/200), CD45.1 APC (1/100), CD45.2 PerCP Cy5.5 (1/100), GR1 bio (1/50) diluted in PBS 1× FBS 2%.
l. Wash the wells with 200 μL of PBS 1× FBS 2%, centrifuge the plate at 450 g 4 min at 4°C and remove the supernatant.
m. Resuspend the cells in 50 μL of Streptavidin-PECy7 (1/200) diluted in PBS 1× FBS 2%.
n. Wash the wells with 200 μL of PBS 1× FBS 2%.
o. centrifuge the plaque at 450 g 4 min at 4°C and remove the supernatant.
p. Resuspend the cells in 150 μL of PBS 1× FBS 2% and put them into a FACS tube.
q. Add 1,5 μL of DAPI (1:1000) and incubate 5 min at 4°C.
r. Acquire the samples on a LSRFortessa and analyze them with FlowJo V10.4.2 software.

Note: BM reconstitution in blood is checked using the CD45.1 and CD45.2 haplotypes as markers to detect donor and recipient cells in all the major leukocyte subpopulations (Figure 2).

MDA-LDL preparation

Timing: 5 h

Here we describe the preparation of MDA-LDL.

5. Prepare MDA (0.54 M).
   a. Incubate 88 μL of malondialdehyde bis (dimethyl acetal) with 12 μL 4 N HCl and 400 μL distilled water at 37°C for 10 min with very soft agitation.
   b. Stop the reaction: add drops of NaOH 1 N until the solution pH is 7.4 (measure pH indicator strips).
   c. Increase the volume to 1 mL with distilled water.
6. MDA-LDL preparation.
   a. Incubate human LDL (low-density lipoprotein) with 0.54 M MDA for 3 h at 37°C at constant ratio of 100 μL/mg of LDL.
   b. Remove unbound MDA by gel filtration with PD-10 columns (Hansson, 2004).
   c. Concentrate the protein with Amicon Ultra-15, PLTK Ultracel-PL, 30 kDa membrane.

d. Check LDL concentration following the instructions of the BCA Assay Kit.
e. Filter the protein with a Millex-GP 0.22 μm polyethersulfone (PES) membrane filter using a 1 mL syringe.

Note: MDA-LDL solution can be stored for 3–6 months at −20°C.

Atherosclerosis induction and MDA-LDL immunization

Timing: 23 weeks

In this session we describe the immunization with MDA-LDL during the induction of atherosclerosis by high fat diet feeding.

7. Immunize bone marroe transfer (BMT) chimeras with MDA-LDL at weeks 0, 2, 4, 6, 8, 12, 16, 20.
   a. Mix 1:1 volume ratio MDA-LDL with Complete Freund's Adjuvant (CFA) by adding first the MDA-LDL and, subsequently the CFA.
   b. Maintain at 4°C until injection.
   c. Load MDA-LDL CFA or PBS-CFA mixture into 1 mL syringe and mix.
   d. At week 0, inject subcutaneously 50 μL of the MDA-LDL CFA mixture (corresponding to 50 μg of MDA-LDL) or CFA alone (25 μL in each foot pad).
   e. At weeks 2, 4, 6, 8, 12, 16 and 20 inject intraperitoneally 25 μL of the MDA-LDL CFA mixture (corresponding to 25 μg of MDA-LDL) or of CFA alone.

   Note: The recommended MDA-LDL concentration is 1 μg/μL. If necessary, dilute MDA-LDL with PBS 1× by adding first the MDA-LDL and next, the PBS 1×.

   Note: Mix the MDA-LDL CFA mixture in the injection syringe with a 23 G needle by pulling the plunger up and down until a whitish homogeneous mixture is observed.

8. High fat diet (HFD)
   a. Start feeding the mice with HFD 1% cholesterol at 8 weeks after the first immunization until week 10.
   b. Feed the mice with HFD 1.25% cholesterol at 10 weeks until week 23.

   Note: The specific HFD used in this protocol was chosen based on the protocol of atherosclerosis induction described by Freigang et al., 1998.[6] For different experiments, optimize your HFD protocol.

Atherosclerosis burden analysis

Timing: 2 weeks

In these steps we describe the quantification of atherosclerosis burden in the aortic root of the immunized BMT chimeras.

9. Extract mouse hearts.
   a. Euthanize immunized BMT chimeras by placing them into a CO_2 cage (>70% CO_2) until no breath movements are observed.
   b. Before proceeding, confirm the mouse is dead by pressing the footpad and detecting no movement.
   c. Expose the chest cavity.
   d. Cut the femoral artery and perfuse the mouse with 10 mL of PBS 1× through the left ventricle using a 10 mL syringe and a 25 G needle.

e. Remove the esophagus, the trachea and the lungs in order to expose the heart and the aorta.
f. Clean carefully the aorta using tweezers and separate the heart.

Optional: Save the aortas to analyze atherosclerotic burden in the aortic arch and the thoracic aorta.

10. Process the hearts for histology analysis.
    a. Fix the heart in formaldehyde 4% for 2 h at RT (20°C–25°C) in a 15 mL tube.
    b. Remove formaldehyde and incubate the hearts in sucrose 30% at 4°C for 12–16 h.

    Note: Properly included organ precipitates to the bottom of the 15 mL tube. Heart precipitation time depends on the size of the organ.

    c. Clean the heart briefly by embedding it in PBS 1× for 1 s.
    d. Cut the heart apex with a blade to facilitate inclusion.
    e. Inject OCT 33% in PBS 1× into the left ventricle until the liquid flows from the ascendant aorta.
    f. Place the heart in a Tissue-Tek Cryomold and include it in OCT 100%.
    g. Include the heart in a central orientation where you see the ascending aorta in the center of the cryomold.
    h. Once the heart is placed properly, start to freeze the OCT block by placing it on dry ice pellets.
    i. Wait until the OCT block is totally frozen, store it at −80°C.

    Note: Prepare the 33% OCT by adding the PBS 1× and, subsequently, the OCT. Mix well and centrifuge at RT 10 min at 450 g to remove the bubbles.

    ⚠ CRITICAL: OCT blocks must be stored at −80°C.They will be stable for several years at this temperature.

    ⚠ CRITICAL: formaldehyde is a harmful for humans. Handle formaldehyde inside a chemical fume hood.

11. Cut sections of the aortic root (scheme in Figure 3).
    a. Cool the cryostat Leica CM1850 to −20°C.
    b. Scrape sections from the block until the aortic root is reached.

    Note: This must be visually assessed under a light microscope Leica DM2500 (Figure 3, step 11).

    c. Cut eight 10 μm thick sections and place each section in an independent slide (slides #1–8).
    d. Repeat this process adding sequentially sections to slides #1–8 (Figure 3, step 11), and until the aortic root is not visible.

    Note: This ensures that every slide contains evenly spaced sections spanning the whole aortic root thus allowing representative staining across the root using a single slide.

    e. Let the sections dry for 1 h at RT (20°C–25°C).
    f. Preserve at −80°C.

    ⚠ CRITICAL: Be extremely careful with the cryostat blades. The wounds produced by this type of blades could be especially harmful.

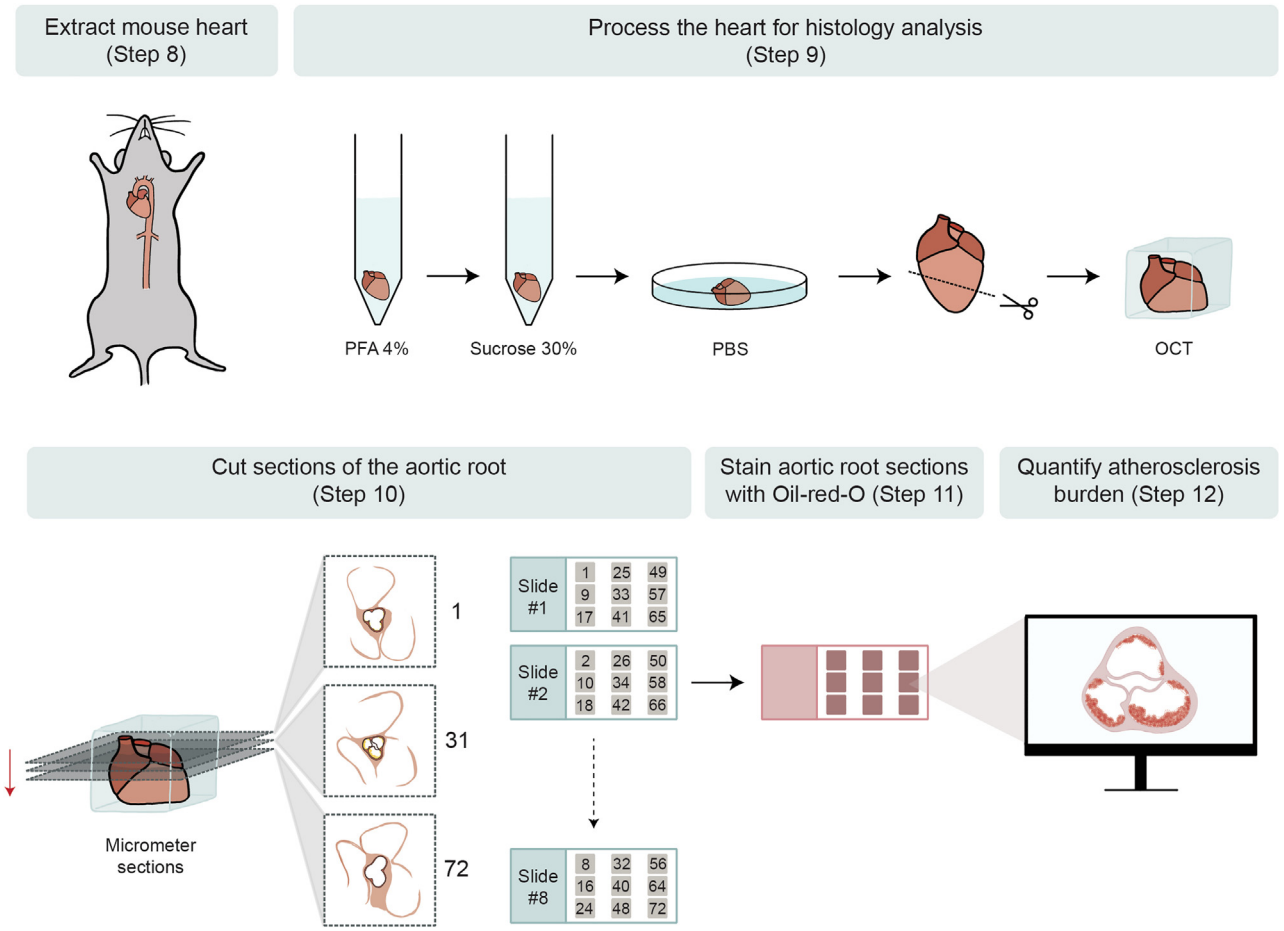

Figure 3. Preparation of the mouse heart for cryosections of the aortic root and subsequent atherosclerosis quantification

Note: OCT sections can be stored for several years at −80°C.

Note: Using Superfrost slides facilitates the attachment of the aortic root sections to the slide.

12. Stain aortic root sections with Oil-red-O.
    a. Thaw the slides with sections at RT for 1 h.
    b. Place the slides in a microscope slide rack.
    c. Prepare Oil-red O working solution: dilute Oil-red O stock solution 1:1.5 in distilled water and let it rest 10 min at RT.
    d. Filter the solution with a Millex-GP 0.22 μm PES membrane filter using a 1 mL syringe .
    e. Wash the slides in distilled water for 10 min by placing the slide rack in a bucket.
    f. Dip the slides 1 s 4 times in Isopropanol 60%.
    g. Incubate the slides with Oil-red-O working solution for 20 min.
    h. Wash the slides 10 min in cold distilled water.
    i. Dip the slides 1 s 5 times in Mayer's hematoxylin to counterstain the sections.
    j. Dip the slides in warm distilled water.
    k. Mount the samples with Aquatex.
13. Quantify atherosclerosis burden.
    a. Take digital images of the aortic root sections with a microscope Leica DM2500.
    b. Open the images in Image J.

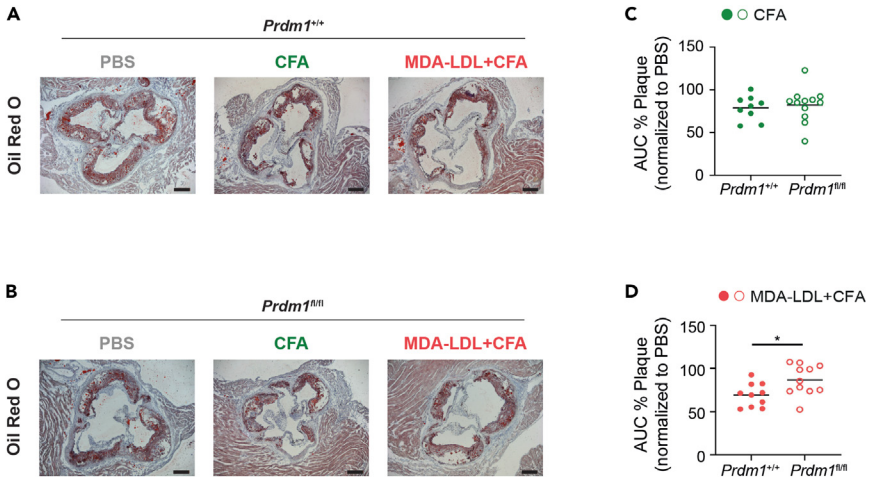

Figure 4. Representative results of atherosclerosis quantification in *Prdm1*$^{+/+}$ *Aicda*-Cre$^{+/ki}$ *Ldlr*$^{-/-}$ *Prdm1*$^{fl/fl}$ *Aicda*-Cre$^{+/ki}$ *Ldlr*$^{-/-}$ chimeras

(A and B) Representative oil red-O-stained aortic sinus cryosections of (A)*Prdm1*$^{+/+}$ *Aicda*-Cre$^{+/ki}$ *Ldlr*$^{-/-}$ chimeras and (B) *Prdm1*$^{fl/fl}$ *Aicda*-Cre$^{+/ki}$ *Ldlr*$^{-/-}$chimeras immunized with PBS, CFA, or MDA-LDL + CFA.

(C and D) AUC values of the atherosclerosis quantifications in five serial 80μm-spaced aortic sinus cryosections from *Prdm1*$^{+/+}$ *Aicda*-Cre$^{+/ki}$ *Ldlr*$^{-/-}$ and *Prdm1*$^{fl/fl}$ *Aicda*-Cre$^{+/ki}$ *Ldlr*$^{-/-}$ chimeras immunized with (C) CFA or (D) MDA-LDL + CFA. Atherosclerosis quantification of CFA- and MDA-LDL + CFA-immunized mice was normalized to the mean AUC value of their respective PBS-injected *Prdm1*$^{+/+}$ *Aicda*-Cre$^{+/ki}$ *Ldlr*$^{-/-}$ and *Prdm1*$^{fl/fl}$ *Aicda*-Cre$^{+/ki}$ *Ldlr*$^{-/-}$chimeras. All experiments were performed with 8- to 9-week-old male receptors.

Each dot in the graphs represents a biological replicate (individual mouse). Data are represented as mean ± SEM. Scale bars in images represent 200 μm. Data analyzed by unpaired t statistical test. *$p < 0.05$.

c. Use the selection tool to define the area of atherosclerotic plaque and the total aortic root area.
d. Add the defined areas to the ROI manager and quantify them.
e. Calculate the percentage of atheroma plaque out of the total area of aortic root in all the sections of the slide.
f. Calculate the area under the curve (AUC) using graphpad prism software.

EXPECTED OUTCOMES

This protocol describes a mouse model for the study of the role of germinal center derived antibody responses in the MDA-LDL immunization during atherosclerosis development. MDA-LDL is a major ox-LDL form and a prototypical atherosclerosis neoantigen which confers atheroprotection upon immunization.[7,8] Thus, our protocol was designed to specifically assess germinal center component MDA-LDL driven atheroprotection.[6,9,10]

This protocol combines conditional mouse models in immunology, the use of BMT, immunization and atherosclerosis mouse models in a single experiment. Thus, our protocol could provide experimental knowledge to study the role of different immune cell populations upon specific antigen immunization during atherosclerosis development.

We generate an atherosclerotic mouse model deficient for *Prdm1* in germinal center cells by the transfer of *Prdm1*$^{fl/fl}$ *Aicda-Cre*$^{+/ki}$ (CD45.2) bone marrow (BM) into irradiated *Ldlr*$^{-/-}$ (CD45.1). Moreover, we immunize the mice with MDA-LDL to induce a specific immune response against this atherosclerotic antigen. During the immunization process we feed the mice with HFD to induce atherosclerosis. By histopathology of the aortic root we are able to quantify atherosclerotic burden in the experimental mice. Using this protocol, we observed that both *Prdm1*$^{+/+}$ *Aicda*-Cre$^{+/ki}$ *Ldlr*$^{-/-}$ and *Prdm1*$^{fl/fl}$ *Aicda*-Cre$^{+/ki}$ *Ldlr*$^{-/-}$ chimeras treated with CFA alone developed atherosclerotic plaques of similar size (Figures 4A and 4C). On the contrary, *Prdm1*$^{fl/fl}$ *Aicda*-Cre$^{+/ki}$ *Ldlr*$^{-/-}$ chimeras

had larger aortic plaque lesions than *Prdm1*-proficient chimeras (Figures 4B and 4D) indicating that PCs and Abs derived from GC-experienced B cells drive MDA-LDL-mediated athero-protection.[1]

Alternative approaches could be used to assess the role of germinal center-derived antibodies in atherosclerosis. On the one hand, the BMT strategy could be avoided by breeding *Prdm1*$^{fl/fl}$ *Aicda*-Cre$^{+/ki}$ mice to *Ldlr*$^{-/-}$ mice . However, this breeding strategy is extremely time-consuming and involves the use of large number of mice, which conflicts with the application of the 3Rs (Reduce, Reuse and Recycle) recommendations. In this regard, it is important to note that the use of *ApoE*$^{-/-}$ mice as an alternative atherosclerosis prone model should only be used in BMT in donor mice with an *ApoE*$^{-/-}$ background and thus, involves comparable breeding problems to those for the plain *Prdm1*$^{fl/fl}$ *Aicda*-Cre$^{+/ki}$ *Ldlr*$^{-/-}$ approach or the *Prdm1*$^{fl/fl}$ *Aicda*-Cre$^{+/ki}$ *ApoE*$^{-/-}$ approach.[11] Finally, intravenous injection of adeno-associated viral (AAV) vectors encoding gain-of-function proprotein convertase subtilisin/kexin type 9 (PCSK9) mutants, followed by HFD feeding, would be an alternative atherosclerosis that avoids germline modification of the *Ldlr* or *ApoE* genes and thus simplifies breeding strategies.[12] In addition, we can speculate that alternative HFD feeding protocols (i.e., longer or shorter HFD feeding and the use of HFD with a different composition) should be able to reproduce the outcomes of our experiment. However, they should be empirically tested, given that both time and lipid content of HFD have a direct impact on the development of atherosclerotic plaques.

Regarding the use of *Aicda*-Cre driver as a germinal center conditional mouse model, alternative Cre strains have been developed that would presumably yield similar results to ours, specifically the *Cγ1*-Cre and the *S1PR2*-CreERT strains. We would expect that *Cγ1*-Cre-mediated deletion could be slightly earlier than that of *Aicda*-Cre, and would presumably occur in fewer germinal-center B cells (all cells where the gamma1 constant region is activated and a fraction of the cells where other constant regions are activated). Thus, a similar, but possibly milder result should be expected if the *Cγ1*-Cre strain were used.[13] Regarding *S1PR2*-CreERT mice, we anticipate it could yield very similar results to ours, provided that the Cre-ERT is activated by repeated injections of tamoxifen to allow germinal center-driven deletion for the whole duration of atherosclerosis induction.[14]

LIMITATIONS

A limitation of our protocol is the *Aicda-Cre*$^{+/ki}$ mouse model used to deplete *Prdm1*. The onset of AID expression shortly precedes the actual entering of the B cell into the follicle, and thus a minor fraction of non-GC B cells could be *Prdm1* depleted. However, this model provides a good approximation for GC studies because it is very efficient at depleting genes in GC B cells while having only a small effect on non-GC cells (see further details above on the use of alternative Cre strains).

The high variability in atherosclerosis experiments could be a source of inaccuracies and thus at least 9–10 mice per experimental group must be used.[1] Moreover, skin infections and dermatitis are not infrequent during the protocol[15] and could also affect the outcomes of the experiment; therefore, mice with dermatitis should not be included in the atherosclerosis assessment. To minimize those infections, mouse cages must be changed frequently. In addition, further analyses of the atherosclerosis burden, such as the quantification of atherosclerotic plaques in the thoracic aorta and the characterization of atherosclerotic plaque composition could also have been included in our study.

TROUBLESHOOTING

Problem 1

Low mouse litter productivity (step 1).

Potential solution

Check the age of your progenitor mice. If the mice are too old, fertility could be impaired, giving rise to productivity problems. This is particularly critical for females, which should be 6–30 weeks old to be in their optimal fertility.

Problem 2
Mouse feeders are usually empty during HFD feeding (step 8).

Potential solution
HFD pellets can easily disintegrate as mice gnaw it, which may result in uneven feeding across different cages or mice. To avoid this, quantity of pellets must be checked every 2 days.

Problem 3
Tissue separates from the OCT while cutting the cryosections (step 10).

Potential solution
Residual sucrose surrounding the tissue could affect OCT inclusion and lead the separation of the tissue from the OCT during the cut. To avoid this, make sure you remove excess sucrose around the tissue by PBS 1× washing and a brief OCT wash before the final inclusion.

Problem 4
Reduced number of cryosections as the aortic root is visible too early in the cryostat (step 10).

Potential solution
Be extremely careful when separating the heart from the aorta. Do not cut under the level of the aortic valves to ensure a proper grinding of the sample.

Problem 5
Presence of blod clots inside aortic root (step 13).

Potential solution
During heart extraction (step 9), PBS perfusion must be slow and continuous to prevent the accumulation of blod clots inside the aortic root.

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Almudena R. Ramiro (aramiro@cnic.es).

Materials availability
This protocol did not generate unique materials or reagents.

Data and code availability
This protocol did not generate datasets or code.

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AUTHOR CONTRIBUTIONS

A.D.M.M. wrote the manuscript; I.M.-F. designed, performed, and analyzed experiments; A.R-R. prepared figures and the graphical abstract; and A.R.R. conceived the project, designed and analyzed experiments, and wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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