Supplementary information, Materials and Methods, Figures

Chimeric antigen receptor (CAR) T cell therapy for atherosclerosis

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Materials and Methods

Mice

Male *Apoe*$^{-/-}$ mice (B6/JGpt-Apoeem1Cd82/Gpt), *Ldlr*$^{-/-}$ mice (B6/JGpt-Ldlrem1Cd82/Gpt), and C57BL/6JGpt mice aged 8 weeks were purchased from Gem Pharmatech Company. All mice were housed under specific pathogen-free conditions at the Laboratory Animal Center of Shenzhen Bay Laboratory. Animal facilities were maintained at 24°C with 40-70% relative humidity under a 12 h light/dark cycle, with free access to water and food. All animal procedures were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee at Shenzhen Bay Laboratory, Shenzhen, China.

Atherosclerosis studies

***Apoe*$^{-/-}$ and *Ldlr*$^{-/-}$ mouse models.** Eight-week-old male *Apoe*$^{-/-}$ and *Ldlr*$^{-/-}$ mice were fed a high-fat diet (HFD, Regular casein and 1.25% added Cholesterol; Research Diets, #D12108C) for a total of 16 weeks. After 12-week HFD feeding, mice were randomized to receive intravenous (IV) injection of 5×10^6 CAR T cells in 100 μL PBS or 5×10^6 untreated T cells in 100 μL PBS.

AAV8-PCSK9 mouse model. Eight-week-old male C57BL/6JGpt mice received a single tail vein injection of AAV8-PCSK9 (5×10^{11} viral genomes in 100 μL PBS; WZ Biosciences, #D377Y) and were then fed a HFD for a total of 20 weeks. After 16-week HFD feeding, mice were randomized to receive IV injection of 5×10^6 CAR T cells in 100 μL PBS or 5×10^6 untreated T cells in 100 μL PBS.

Single cell RNA-seq analysis

Raw sequencing data were obtained from the Gene Expression Omnibus (GEO). The Seurat package was used to generate integrated meta-datasets and conduct downstream analyses. Human arterial vessel datasets included GSE225650, GSE235436, GSE131778, GSE260657, GSE224273, GSE131778,

GSE159677, GSE224273, GSE179159, GSE247238, GSE155512, GSE234077, and GSE253903. Mouse aortic datasets included GSE264253, GSE252243, GSE248394, GSE225773, GSE209525, GSE205931, and GSE155513.

Standard quality-control filtering was applied to exclude low-quality and dead cells. Cells with mitochondrial gene content greater than 15% were removed. Principal component analysis (PCA) was conducted on the merged Seurat object, and uniform manifold approximation and projection (UMAP) was conducted using the first 25 principal components for two-dimensional visualization of cellular heterogeneity.

Identification of plaque macrophages and foamy macrophages

In the human arterial single-cell datasets, clustering was conducted using the FindNeighbors and FindClusters functions in Seurat at a resolution of 0.4, generating 21 distinct clusters. Based on expression of canonical macrophage markers, including *CD68*, *CSF1R*, and *CD14*, six clusters (2, 6, 7, 10, 17, and 19) were annotated as macrophages. These macrophages were subsequently subsetted and re-analyzed independently in Seurat, generating 10 subclusters. Among them, clusters 0 and 7 were annotated as foamy macrophages based on high expression of *PLAC8*, *ITGAX*, *MRC1*, *MMP12*, *GPNMB*, and *TREM2*.

In the mouse aortic samples, clustering was conducted at a resolution of 0.2, identifying 16 clusters in total. Macrophage populations were defined by the expression of *Spp1*, *Trem2*, *Ccl8*, *Cd63*, and *Ccl7*, corresponding to eight clusters (2, 4, 8, 9, 10, 12, 15, and 16). These macrophage clusters were then extracted and re-analyzed, generating 11 subclusters. Among them, subcluster 0 was annotated as foamy macrophage based on high expression of *Plac8*, *Itgax*, *Mrc1*, *Mmp12*, *Gpnmb*, and *Trem2*.

Construction of a Trem2 CAR lenti/retroviral vector

The Trem2 CAR lenti/retroviral vector were constructed with the following components arranged from 5' to 3': an Igκ leader sequence, Trem2 single-chain variable fragment (scFv), V5 tag, the co-stimulatory domain of human or mouse CD28, and intracellular signaling domain of human or mouse CD3ζ. The CAR1-TREM2 scFv (RS9.F6) sequence was obtained from the international patent application of Denali Therapeutics (#DNL919). The CAR2-TREM2 scFv (237920) sequence was obtained from the international patent application of R&D Systems (#MAB17291). The CAR3-TREM2 scFv (PI37012) sequence was obtained from the international patent application of Pionyr Immunotherapeutics (#PY314). The final construct was cloned into a Retro-EF1α-AmpR vector and packaged into viral particles using HEK293T cells.

Lenti/retrovirus packaging

Lentivirus and retrovirus were produced in HEK293T cells using similar procedures. For each 10-cm dish, 6×10^6 HEK293T cells were seeded in 10 mL complete DMEM (Gibco, #21063029) containing 10% FBS. After 18 h, cells were transiently transfected with polyethylenimine (PEI; Polysciences, #24765-1; prepared at 1 µg/µL in water).

For lentivirus production, each dish was transfected with 12 µg gene-of-interest plasmid, 6 µg pMD2.G envelope plasmid (Addgene, #12259), and 7.5 µg psPAX2 packaging plasmid (Addgene, #12260). For retrovirus production, each dish was transfected with 10 µg gene-of-interest plasmid and 10 µg pCL-Eco packaging plasmid (Addgene, #12371). Six hours after transfection, the medium was replaced with fresh DMEM containing 2% FBS for lentivirus production or fresh DMEM containing 10% FBS for retrovirus production.

Seventy-two hours later, virus-containing supernatants were collected and centrifugated at 400 × g for 10 min at 4 °C to remove cellular debris, and passed through a 0.45-μm PES filter (Biosharp, BS-PES-45). Viral particles were then concentrated by ultracentrifugation at 19,800 × g for 2 h at 4 °C. Pellets from each dish were resuspended in 500 μL RPMI 1640 medium and stored at −80 °C until use.

T cell isolation, activation, and viral transduction

Leukopaks from healthy donors were purchased from Milesbio. Peripheral blood mononuclear cells (PBMCs) were isolated by density-gradient centrifugation using Ficoll (Cytiva, #17544203). Briefly, whole blood was diluted 1:1 with PBS and centrifuged at 400 × g for 30 min at 18°C. The PBMC layer was collected and washed twice with PBS. Cells were either cryopreserved in CryoStor® CS10 at 2.5×10^7 cells/mL or used immediately for downstream experiments. For immunophenotyping, PBMCs were stained with FITC-conjugated anti-CD3 antibody and analyzed by flow cytometry to determine the percentage of CD3+ T cells.

Primary human T cells were activated using Dynabeads™ Human T-Expander CD3/CD28 (Invitrogen, #11141D) at a 1:1 bead-to-cell ratio. Cells were cultured in T cell medium containing 45% RPMI1640, 45% Click's medium (Sigma, #C5572), 10% fetal bovine serum (FBS; Sigma, #F8687), and 1% penicillin/streptomycin (Gibco, #15140122), supplemented with 100 U/mL IL-2 (Proteintech, #HZ-1015). After 48 h of activation, the beads were removed.

For lentiviral transduction, 24-well plates were coated with RetroNectin (Takara, #T100AC) for 4 h. Lentiviral supernatant was then added and centrifuged at 2,600 × g for 1.5 h at 4°C to facilitate viral binding. Activated human T cells were seeded at 0.5×10^6 cells per well and culture medium was replaced every two days.

Mouse T cells were isolated using the EasySep™ Mouse T Cell Isolation Kit (Stemcell Technologies, #19851), according to the manufacturer's instructions. Briefly, mice were euthanized by cervical dislocation under isoflurane anesthesia. Lymph nodes and spleen were collected, mechanically dissociated through a 40-μm cell strainer, and centrifuged at 600 × g for 5 min at 4°C. Red blood cells (RBC) were lysed using RBC lysis buffer for 1 min at room temperature, followed by centrifugation at 600 × g for 5 min at 4°C. The cell pellet was resuspended in 1 mL RPMI 1640, followed by the addition of 20 μL FcR blocker and 50 μL isolation cocktail. After mixing and incubation for 10 min at room temperature, 75 μL RapidSpheres™ were added and the mixture was incubated additional 2.5 min. After incubation on a magnet for 2.5 min, the supernatant containing total T cells was collected.

For activation, 1 x 10^6 mouse T cells were cultured in a 24-well plate in medium containing 45% RPMI 1640 and 45% Click's medium (Sigma, #C5572) supplemented with 10% FBS (Gibco, #A5669701), 1% Penicillin/Streptomycin (Solarbio, #P1400), L-glutamine (Gibco, #25030-081), β-mercaptoethanol (Sigma, #M6250), 1000U/mL human IL-2 (Proteintech, #HZ-1015-GMP), 0.25 ng/μL anti-CD3 antibody (Bio X Cell, #BE0002), and 1 ng/μL anti-CD28 antibody (Bio X Cell, #BE0015-1) for 48 h. Following activation, cells were transduced with retrovirus using the similar transduction protocol as described for human T cell transduction.

Cytokine secretion

THP1 cells cultured in RPMI 1640 supplemented with 10% FBS and 1% penicillin/streptomycin and RAW264.7 cells cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin were seeded separately in 96-well plates at a density of 1 × 10^4 cells per well in 100 μL the corresponding complete medium. After 4 h, CAR T cells were added at an effector (CAR T)-to-target (THP1 or RAW264.7) (E:T) ratio of 2:1. After 24 h co-culture, supernatants were collected and stored at −80°C. The concentrations of TNF-α and IFN-γ in the supernatants were measured using ELISA kits according

to the manufacturer's instructions. Human TNF-α and IFN-γ kits (Invitrogen, #88-7346-77 and #88-7316-86) were used for THP1 co-cultures, while mouse TNF-α and IFN-γ kits (Invitrogen, #88-7324-77 and #88-7314-88) were used for RAW264.7 co-cultures.

Flow cytometry-based cytotoxicity assay

THP1 and RAW264.7 cells were seeded in 24-well plates at a density of 1×10^5 cells per well and cultured as described above. After 4 h, CAR T cells were added at the E:T ratio of 1:20. Following a 4-day co-culture, all cells were collected and stained with CD3 APC-Cy7 anti-human (BD, #557832, 1:100) or CD3 APC anti-mouse (Gene Baygene 4Abio, FMA003-01, 1:100). Samples were analyzed by flow cytometry. Absolute cell counts were determined using counting beads (eBioscience, #01-1234-42). Target cell killing was calculated as:

Killing efficiency = 1 – (Target cell count in treated group / Target cell count in target-alone control)

Flow cytometry

Cells were centrifuged at 400 × g for 5 min at room temperature. The cell pellets were resuspended with 100 μL RPMI 1640 and incubated with the indicated antibodies for 30 min at room temperature. flow cytometric analysis was conducted on a BD LSRFortessa cytometer. Data were analyzed using FlowJo (Tree Star). The following antibodies were used: V5 tag-PE (Invitrogen, #12-6796-42, 1:100), Trem2 APC anti-mouse (R&D, #AF1729, 1:100), CD3 APC anti-mouse (Gene Baygene 4Abio, FMA003-01, 1:100), CD3 APC-Cy7 anti-human (BD, #557832, 1:100).

Generation of human blood vessel organoids

To generate human blood vessel organoids, U-bottom ultra-low attachment 96-well plates were coated with anti-adherence rinsing solution (Stemcell, #0-7010), according to the manufacturer's instructions. Human induced pluripotent stem cells (hiPSCs) were suspended in E8 medium (ThermoFisher Scientific,

#A1517001) supplemented with 10 μM ROCK inhibitor (Selleck, #S1049) at a concentration of 3,000 cells per 100 μL. A total of 100 μL of the cell suspension was seeded into each well. Plates were centrifugated at 300 × g for 3 min and then incubated at 37°C in a humidified incubator. After 24 h, the culture medium was changed to E8 medium. Vessel organoid differentiation was induced in a chemically defined medium (CDM). The CDM was prepared by mixing 245 mL of DMEM/F-12 (Gibco, #10565018), 245 mL of IMDM (Gibco, #C12440500BT), 5 mL of chemically defined lipid concentrate (Gibco, #11905031), 5 mL of GlutaMAX (Gibco, #35050-061), 500 μL of Insulin-Transferrin-Selenium (Abmole, #M19989), and 20 μL of α-monothioglycerol (Sigma-Aldrich, #M6145). hiPSCs were induced for vascular lineage cell differentiation by continuous treatments with 12 μM CHIR99021 (LC Laboratories, #C-6556) and 30 ng/mL BMP4 (PeproTech, #120-05ET) for three days. On day 3, medium was replaced by a cocktail of 100 ng/mL VEGF165 (PeproTech, #100-20), 100 ng/mL FGF2 (PeproTech, #100-18B), and 10 μM SB431542 (Selleck, #S1067) in CDM for two days. Vessel organoids were then embedded into a 4:1 mixture of Collagen I-Matrigel solution. The embedded organoids were then incubated with CDM medium supplemented with 100 ng/mL VEGF165 and 100 ng/mL FGF2. The medium was replaced every three days throughout the differentiation period until further use.

Serum cytokine and lipid analysis

Blood samples were collected and maintained at room temperature for 1 h and centrifuged at 10,000 × g for 10 min at 4°C. Serum was collected and used for measurements. Total cholesterol (TC), triglyceride (TG), and low-density lipoprotein cholesterol (LDL-C) were measured using colorimetric assay kits (Elabscience, #E-BC-K109-M, #E-BC-K261-M, and #E-BC-K205-M, respectively). Serum IFN-γ and TNF-α levels were measured using mouse ELISA kits (Invitrogen, #88-7314-88 and #88-7324-77) according to the manufacturers' protocols.

Aortic root imaging

Mice were euthanized by cervical dislocation under isoflurane anesthesia. The heart and aorta were exposed and perfused with PBS in situ. Aortic roots were harvested and embedded in Tissue-Tek® O.C.T. Compound (Sakura Finetek, #4583). Serial cryosections (8 μm) were prepared through the aortic sinus using a cryostat (Leica, #CM1950). Sections were fixed in 4% paraformaldehyde (PFA) for 5 min at room temperature. Hematoxylin–eosin (H&E), Masson's trichrome, and Oil Red O staining were conducted using commercial kits (Solarbio #G1120, Solarbio #G1340, and Beyotime #C0157S). Slides were scanned using an Olympus SLIDEVIEW VS200 system. Necrotic core area, lesion area and plaque area were quantified using ImageJ.

For immunofluorescence staining, sections were fixed by 4% PFA, permeabilized with PBS containing 0.5% Triton X-100 for 20 min, and blocked by PBS containing 10% FBS and 0.05% Triton X-100 for 2 h. Sections were then incubated with rat anti-mouse CD68 (BioLegend, #137002, 1:300) and rabbit anti-mouse Trem2 (HuaBio, #ER1918-04, 1:200). After washing with PBS, sections were stained with goat anti-rat Alexa Fluor 488 (Cell Signaling, #4416S) and goat anti-rabbit Alexa Fluor 555 (Cell Signaling, #4413S). Nuclei were counterstained with Hoechst 33342 (Sigma, #B2261, 1:5,000). Images were captured using a confocal microscope (Olympus FV3000) and analyzed using ImageJ.

Aortic plaque quantification

The entire aorta was dissected and fixed in 4% paraformaldehyde for 24 h. After removal of perivascular adipose tissue, the aorta was longitudinally opened. The aorta was briefly treated with 60% isopropanol for 3 s and then stained with Oil Red O solution (Servicebio, #G1015) at 37 °C for 1 h in a light-protected environment. The tissue was differentiated in 60% isopropanol until plaques appeared clearly red. Images were captured using a Canon 70D camera, and plaque area was quantified using ImageJ software.

Histological analysis of organs

Liver, brain, kidney, spleen, lung, intestine, pancreas, testicle, and skin were harvested and embedded in Tissue-Tek® O.C.T. Compound, sectioned stained by H&E, and imaged as described above.

Statistics

Statistical analyses and graph generation were performed using GraphPad Prism (version 8.4.3). Comparisons between two groups were analyzed using an unpaired two-tailed Student's *t*-test. Comparisons among multiple groups were performed using one-way analysis of variance (ANOVA). A P value < 0.05 was considered statistically significant. In the figures, statistical significance is indicated as follows: $P < 0.05$, $P < 0.01$, $P < 0.001$, and $P < 0.0001$.

Supplementary figure legends

Fig. S1. Immune cells in human and mouse atherosclerotic arteries. a and **c** UMAP visualization of single-cell RNA-seq profiles of human (**a**) and mouse (**c**) immune cells in normal and atherosclerotic arteries. **b** and **d** Proportion of monocyte/macrophage subclusters among total macrophages in human (**b**) and mouse (**d**) atherosclerotic arteries.

Fig. S2. Generation of human and mouse TREM2 CARs. a-b. Schematic representation of the human (**a**) and mouse (**b**) TREM2-CAR construct used in this study, with a V5 tag inserted between the TREM2 scFv and the human or mouse CD28 co-stimulatory domain. **c-d.** Flow cytometric analysis of the expression levels of Trem2 in human cells (NALM6 and THP1, **c**) and mouse cells (MC38 and RAW264.7, **d**). NALM6 and MC38 do not express Trem2.

Fig. S3. Trem2 CAR T cell treatment reduces plaque formation in *Apoe$^{-/-}$* mice. a. Quantification of the plaque area in aortic arch and descending aorta. **b-c.** Representative (**b**) and quantification (**c**) of H&E staining of aortic roots. $^{*}P < 0.05$, $^{**}P < 0.01$; n.s., not significant. Scale bar, 200 μm.

Fig. S4. Serum lipid and cytokine levels in *Apoe$^{-/-}$* mice. a–c. Serum levels of LDL-C (**a**), total TC (**b**), and TG (**c**) in HFD-fed *Apoe$^{-/-}$* mice treated with or without CAR T cells. **d-e.** Serum IFN-γ (**d**) and TNF-α (**e**) levels in HFD-fed *Apoe$^{-/-}$* mice treated with or without CAR T cells at different time points. $^{*}P < 0.05$; n.s., not significant.

Fig. S5. Trem2 CAR T cell treatment has no detectable effects on major organs' structure. Representative H&E staining of liver, brain, kidney, spleen, lung, intestine, pancreas, testis, and skin sections from HFD-fed mice treated with or without CAR T cells. Scale bars, 100 μm.

Fig. S6. Trem2 CAR T cell treatment reduces plaque formation in *Ldlr*$^{-/-}$ and AAV8-PCSK9 mouse models. a-b. Quantification of whole-aorta plaque area following ORO staining in *Ldlr*$^{-/-}$ (**a**) and AAV8-PCSK9 (**b**) mouse models. **c.** Representative H&E staining of aortic roots in *Ldlr*$^{-/-}$ (top) and AAV8-PCSK9 (bottom) mouse models. **d.** Quantification of aortic root plaque area based on H&E staining. $*P < 0.05$, $**P < 0.01$; n.s., not significant. Scale bar, 200 μm.