Adoptive transfer of metabolically reprogrammed macrophages for atherosclerosis treatment in diabetic ApoE\(^{-/-}\) mice

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ARTICLE INFO

A B S T R A C T

Atherosclerosis is characterized by inflammation in the arterial wall, which is known to be exacerbated by diabetes. Therapeutic repression of inflammation is a promising strategy for treating atherosclerosis. In this study, we showed that diabetes aggravated atherosclerosis in apolipoproteinE knockout (ApoE\(^{-/-}\)) mice, in which increased expression of long-chain acyl-CoA synthetase 1 (Acsl1) in macrophages played an important role. Knockdown of Acsl1 in macrophages (M\(^{\phi\text{-Acsl1}}\)) reprogrammed macrophages to an anti-inflammatory phenotype, especially under hyperglycemic conditions. Injection of M\(^{\phi\text{-Acsl1}}\) reprogrammed macrophages into streptozotocin (STZ)-induced diabetic ApoE\(^{-/-}\) mice alleviated inflammation locally in the plaque, liver and spleen. Consistent with the reduction in inflammation, plaques became smaller and more stable after the adoptive transfer of reprogrammed macrophages. Taken together, our findings indicate that increased Acsl1 expression in macrophages play a key role in aggravated atherosclerosis of diabetic mice, possibly by promoting inflammation. Adoptive transfer of Acsl1 silenced macrophages may serve as a potential therapeutic strategy for atherosclerosis.

1. Introduction

Atherosclerosis is characterized by inflammation in the arterial wall, which is known to be exacerbated by diabetes. Therapeutic repression of inflammation is a promising strategy for treating atherosclerosis. In this study, we showed that diabetes aggravated atherosclerosis in apolipoproteinE knockout (ApoE\(^{-/-}\)) mice, in which increased expression of long-chain acyl-CoA synthetase 1 (Acsl1) in macrophages played an important role. Knockdown of Acsl1 in macrophages (M\(^{\phi\text{-Acsl1}}\)) reprogrammed macrophages to an anti-inflammatory phenotype, especially under hyperglycemic conditions. Injection of M\(^{\phi\text{-Acsl1}}\) reprogrammed macrophages into streptozotocin (STZ)-induced diabetic ApoE\(^{-/-}\) mice alleviated inflammation locally in the plaque, liver and spleen. Consistent with the reduction in inflammation, plaques became smaller and more stable after the adoptive transfer of reprogrammed macrophages. Taken together, our findings indicate that increased Acsl1 expression in macrophages play a key role in aggravated atherosclerosis of diabetic mice, possibly by promoting inflammation. Adoptive transfer of Acsl1 silenced macrophages may serve as a potential therapeutic strategy for atherosclerosis.

Peer review under responsibility of KeAi Communications Co., Ltd.

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https://doi.org/10.1016/j.bioactmat.2022.02.002

Received 2 July 2021; Received in revised form 24 January 2022; Accepted 7 February 2022

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uptake of long-chain fatty acids and plays an important role in lipid metabolism [18,19]. Moreover, overexpression of Acsl1 in macrophages leads to decreased expression of ABCA1, suggesting an important role of ACsL1 in cholesterol transport [20]. In contrast, the protective role of myeloid Acsl1 deficiency has already been shown by transplanting bone marrow from mice with myeloid Acsl1-deficiency into virally induced Ldlr -/- mice [21,22].

In our study, we showed that diabetes aggravated atherosclerosis in ApoE -/- mice, mainly due to increased local and systemic inflammation. Moreover, increased expression of Acsl1 in macrophages from diabetic mice is responsible for exacerbated inflammation. Injection of the reprogrammed macrophages into ApoE -/-/ + STZ mice alleviated both local inflammation in the plaque and systemic inflammation in the liver and spleen. Together, our findings indicate that increased Acsl1 expression in macrophages is a key player in aggravated atherosclerosis, and adoptive transfer of shAcsl1 lentivirus-infected macrophages is a potential therapeutic strategy for atherosclerosis treatment.

2. Materials and methods

2.1. Diabetic mouse model

Green fluorescent protein (GFP +) transgenic mice (stock No: 003291) were obtained from the Jackson Laboratory, and ApoE +/ - mice (C57BL background) were obtained from the Model Animal Research Center of Nanjing University. All experimental procedures involving animals were approved by the Institutional Animal Care and Use Committee of the Fourth Military Medical University. For the diet-induced obesity study, 8-week-old male ApoE -/- mice and C57BL/6 mice were fed a 45% high-fat diet (HFD) for 12 weeks. Mice were then injected with streptozotocin (STZ) (50 mg/kg, Sigma) or citrate buffer for five consecutive days to induce diabetes or serve as a control. After four weeks, blood glucose was assayed using the One Touch Ultra Blood Glucose Monitoring System (ACCUCHEK Active, Roche 05144418).

2.2. Adoptive transfer of macrophages

ApoE -/- mice were fed with HFD. In the eighth week, diabetes was induced via STZ injection, as previously described. For prevention and therapy, the reprogrammed macrophages (5 × 10^6 cells) were injected into ApoE -/-/ + STZ via the tail vein at weeks 6, 11, and 14, respectively. At the end of the experiments, mice were euthanized with pentobarbital sodium (100 mg/kg), and aortas and other tissues of interest were collected for further analysis.

2.3. Serum biochemistry

Mice were deeply anesthetized via intraperitoneal injection of pentobarbital sodium (100 mg/kg). Blood samples were drawn into BD Vacutainer Blood Collection Tubes (BD, Bioscience) containing buffered sodium citrate as anticoagulant. Whole blood was placed at room temperature for 30 min and then centrifuged at 3000×g at 4 °C. After centrifugation, the serum was obtained for further analyses. The concentrations of alanine aminotransferase (ALT), aspartate aminotransferase (AST), plasma triglycerides, total cholesterol, high-density lipoprotein (HDL) cholesterol, and low-density lipoprotein (LDL) cholesterol were measured using Chemray 800 at Wuhan Servicebio Technology Co., Ltd.

2.4. Cell culture

The RAW264.7 cell line was obtained from ATCC. All cells were cultured in complete media containing high glucose Dulbecco’s modified Eagle medium (DMEM) (Hyclone, USA) supplemented with 10% fetal bovine serum (FBS; Exocell, China) and 1% antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin) from GIBCO at 37 °C with 5% CO2.

2.5. Isolation and culture of bone marrow-derived macrophages (BMDMs)

Femurs and tibias from GFP + transgenic mice and ApoE -/- mice were harvested, and bone marrow was flushed using DMEM to collect bone marrow cells. After centrifugation, the cell pellet was resuspended in ACK lysis buffer (Sangon Biotech) for 5 min to remove red blood cells. Cells were then incubated in tissue plates at 37 °C in 5% CO2 in the presence of M-CSF (20 ng/mL, Sino Biological Inc. Beijing) for six days in DMEM supplemented with 10% FBS and 1% antibiotics, with media being changed every two days. Anti-inflammatory macrophage or pro-inflammatory macrophage polarization was induced in cells using IL-4 (20 ng/mL, Peprotech, Rocky Hill, CT, USA) or IFN-γ (20 ng/mL, Peprotech Rocky Hill, CT) and lipopolysaccharide (LPS, 50 mg/mL, Sigma) in DMEM with 10% FBS for 12 h, respectively. To study the effects of glucose, macrophage differentiation and activation were performed in DMEM supplemented with either 5.5 (normal) or 25 mM (high) endotoxin-free d-glucose (Sigma Aldrich).

2.6. Virus infection and macrophage engineering

RAW264.7 cells or BMDMs were seeded into 6-well plates and incubated at 37 °C with 5% CO2 overnight. RAW264.7 cells or macrophages were infected with shAcsl1 lentiviruses (Gene Pharma) at MOI = 10. The sequences targeting the control or Acsl1 are listed in Table S1. The experimental procedure was conducted according to the manufacturer’s instructions. Briefly, the cells were infected with lentivirus containing 8 μg of polybrene (Sigma, St. Louis, USA). Cells then received fresh medium after infection for 12 h.

2.7. Flow cytometry

To measure the effect of shAcsl1 on the polarization of BMDMs, cells were exposed to vehicle or 1 × 10^3 TU/mL. shAcsl1 lentiviruses for three days. In some experiments, cells were treated with 5.5 (normal) or 25 mM (high) endotoxin-free d-glucose (Sigma Aldrich) before analysis. Briefly, cells were incubated with fluorescence-labeled antibodies (CD11b-APC, F4/80-Alexa Fluor 488, and CD86-PE or CD206-PE, Biolegend, USA) for 30 min. The cells were then washed three times with FACS buffer, followed by analysis using the BD FACS Calibur™. Flow cytometry data were analyzed using FlowJo software.

2.8. Macrophage trafficking

For in vivo tracking of macrophages in distribution analysis, BMDMs were obtained from C57BL/6-Tg (CAG-EGFP) 1Osbl/J mice and treated with shCtrl and shAcsl1 lentiviruses. Macrophages were injected into ApoE -/-/ + STZ mice via the tail vein. After 4 h or 72 h, ApoE -/-/ + STZ mice were anesthetized with pentobarbital sodium (50 mg/kg), and various organs were collected and stored in 4% paraformaldehyde (PFA; Thermo Fisher). For immunofluorescence staining, tissues were fixed in 4% PFA, mounted in paraffin blocks, and sectioned at 8 μm. Cell nuclei were counter-stained using Hoechst33342 (1:1000, Beyotime Biotechnology) for 15 min at room temperature in the dark. Images were captured using a laser scanning confocal microscope (Nikon A1R, Tokyo, Japan). We selected central veins as the origin and selected four quadrants. Each quadrant covered an area of 160,000 μm^2. The number of counted cells in more than 20 sections was averaged.

2.9. Histopathological staining and immunofluorescence analysis

Mice treated with the indicated treatments were euthanized with pentobarbital sodium (100 mg/kg). Tissues were fixed in 4% PFA, mounted in paraffin blocks, and sliced at 8 μm. Frozen aortic roots were
dissected for serial 8 μm cryo-sectioning that covered 500 μm of the root. Aortic root sections were stained with hematoxylin and eosin (H&E), Oil Red O (Sigma), Masson’s trichrome staining (Solarbio, China), and anti-ACSL1 (Abcam, ab177958) antibodies. Furthermore, quantitative analysis of atherosclerotic plaques was performed using Image-Pro Plus 6.0. For immunofluorescence analysis, mice were sacrificed, and tissues of interest were dissected. Tissues were fixed in 4% PFA, mounted in paraffin blocks, and sliced into 8 μm sections. CD68-iNOS+ and CD68 + ARG1+ cells in aortic roots were double stained with corresponding antibodies: CD68 (Abcam, ab53444), iNOS (Abcam, ab178945), and ARG1 (Abcam, ab91279), followed by incubation with specific fluorescence-labeled secondary antibodies for 2 h. Cell nuclei were counter-stained with Hoechst33342 for 15 min at room temperature in the dark. Images were captured using a laser scanning confocal microscope (Nikon A1R, Tokyo, Japan).

2.10. RNA isolation and qRT-PCR

Total RNA from the cells and tissues was extracted using TRIzol (Invitrogen, USA) as per the instructions provided by the manufacturer, and mRNA was reverse transcribed using a transcribed First-strand cDNA Synthesis Kit (Roche), following the manufacturer’s protocol. qPCR was performed using SYBR Green PCR Master Mix (Roche). Relative expression of mRNA was normalized to GAPDH and β-actin and calculated using the 2^–ΔΔCt method. The sequences of the PCR primers are listed in Supplementary Table 1.

2.11. Western blot analysis

Tissues and cells were harvested in 1 × RIPA buffer containing a protease inhibitor (Beijing, China). Samples were shaken on a rotator for 30 min and subsequently centrifuged at 12,000 rpm for 20 min at 4 °C. Protein concentration was determined using the Pierce BCA Protein Assay Kit (Thermo, USA). Protein samples were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane in an ice bath. Membrane was blocked with 5% bovine serum albumin for 1 h and incubated overnight with primary antibodies at 4 °C. The antibodies used were rabbit anti-ACSL1 (Abcam, ab177958) and rabbit anti-GAPDH (Abcam, ab181602). Membranes were then incubated with the corresponding secondary antibodies for 1 h at room temperature. The bands were visualized using ECL Prime western blotting Detection Reagent (GE Healthcare, Buckinghamshire, UK).

2.12. Statistical analysis

All experimental data are presented as mean ± S.E.M. Student’s t-test and one-way ANOVA were used to compare groups. Data were analyzed using GraphPad Prism 7 software. Statistical significance was set at P < 0.05.

3. Results

3.1. Diabetes exacerbates atherosclerosis in ApoE−/− mice

To induce atherosclerosis in diabetic mice, mice were fed a HFD for 12 weeks, then injected with streptozotocin to induce diabetes (Fig. 1 A). Diabetic ApoE−/− (ApoE−/− + STZ) mice exhibited elevated blood glucose levels compared to C57, C57 + STZ, and ApoE−/− mice (Fig. 1 B). In ApoE−/− + STZ mice, total cholesterol and LDL levels were higher than that observed in ApoE−/− mice (Fig. 1 C, E). In contrast, no significant differences in triglyceride and HDL were found among C57, C57 + STZ, ApoE−/−, and ApoE−/− + STZ mice (Fig. 1 D, F). Atherosclerotic plaque formation results from chronic inflammation and fibroproliferative remodeling of the vascular wall. Notably, the C57 and C57 + STZ mice did not show plaque growth. Furthermore, Oil Red O staining of both aortic tree and roots demonstrated that the atherosclerotic plaque burden was much higher in ApoE−/− + STZ mice than in ApoE−/− mice (Fig. 11, N, K, L, and Fig. S1B). Moreover, HE staining of the aortic root revealed that the necrotic core was much larger in ApoE−/− + STZ mice than in ApoE−/− mice (Fig. 1G and J, and Fig. S1A). Plaque rupture is thought to be caused by the loss of mechanical stability, often due to the reduced tensile strength of the collagen cap surrounding the plaque [23]. Therefore, plaques with reduced collagen content are believed to be more vulnerable than those with thick collagen caps [24]. Notably, Masson’s trichrome staining revealed reduced collagen content in the lesions of ApoE−/− + STZ mice compared to that in control ApoE−/− mice, suggesting that the plaque in the group may be unstable (Fig. 1H and M, Fig. S1C). Overall, these data indicated that diabetes exacerbates atherosclerosis in ApoE−/− mice.

3.2. Increased expression of Acsl1 in macrophages in mice

Diabetes accelerates the formation of atherosclerotic lesions by promoting macrophage accumulation in the susceptible arteries. To explore whether Acsl1 may contribute to atherosclerosis in ApoE−/− + STZ mice. ACSL1 expression in C57, C57 + STZ, ApoE−/−, and ApoE−/− + STZ mice were analyzed. Acsl1 was highly expressed in macrophages from ApoE−/− + STZ mice compared to C57, C57 + STZ, and ApoE−/− mice. The level of Acsl1 also increased in the ApoE control compared with the C57 control (Fig. 2A–D). In addition, we performed histchemistry analyses on atherosclerotic lesions using Acsl1 antibodies. The results showed that Acsl1 was highly expressed in the aortic root of ApoE−/− + STZ mice (Fig. S2A). Consistently, we also observed that ApoE−/− + STZ mice showed high expression of Acsl1 in the aortic root (Figs. S2B–D). Diabetes influences a wide spectrum of hepatic damage and often hastens the progression of steatosis through increased inflammation [25, 26]. To directly evaluate the effects of diabetes-induced liver injury, histopathological observations of the liver were performed. As shown in Fig. S3A, liver tissues were intact, hepatic lobules were clear, and hepatocytes were regularly arranged in C57 mice. Only a slight increase in the number of inflammatory cells and swelling in liver cells was observed in C57 + STZ mice compared with that in C57 mice. In ApoE−/− + STZ mice, it was found that the basic architecture of liver cells disappeared, and apparent steatosis and liver cell swelling were observed, with increased infiltration of inflammatory cells compared with ApoE−/− mice. Furthermore, Masson’s trichrome staining revealed significantly increased collagen content in ApoE−/− + STZ (Figs. S3B and C). To investigate whether hepatic damage is closely associated with the increased expression of Acsl1 in the liver, we performed immunohistochemical analysis of the liver using Acsl1 antibodies. Results showed that ACSL1 was highly expressed in ApoE−/− + STZ mice (Fig. S3C). Interestingly, Acsl1 gene levels were also increased in ApoE−/− + STZ mice (Fig. S3F). We also observed that ACSL1 protein levels were increased in ApoE−/− + STZ mice (Figs. S3D–E). We measured the expression of inflammatory factors, including Tnfα (Fig. S3H), Il-1β (Fig. S3I), Pges (Fig. S3J), and prostaglandin-endoperoxide synthase 2 (Pgs2) (Fig. S3K), using qPCR. The results showed that inflammatory factors were increased in ApoE−/− + STZ mice, along with increased ACSL1 expression.

Myeloid progenitor cells develop into circulating monocytes, and the spleen acts as a reservoir for monocytes infiltrating atherosclerotic lesions [27,28]. Next, we evaluated diabetes-induced spleen injury by observing changes to spleen tissue. In C57 mice, lymphocytes were arranged neatly, and the boundary between the white and red pith was clear compared to the C57 + STZ mice. Further analysis of ApoE−/− + STZ mice revealed disorganization of lymphocytes and a blurring of boundaries between the white and red pith compared with ApoE−/− mice (Fig. S4A). Consistently, we also observed that ApoE−/− + STZ mice displayed increased Acsl1 expression in the spleen (Figs. S4B–E). Furthermore, we extracted mRNA from the spleen tissue and observed an
Fig. 1. Diabetes accelerates atherosclerosis in ApoE<sup>−/−</sup> mice. A. A schematic diagram showing the experimental procedure. Apolipoprotein knockout (ApoE<sup>−/−</sup>) mice were fed a 45% high-fat diet (HFD) for 12 weeks and injected with streptozotocin (STZ) for five consecutive days during the eighth week to induce diabetes. B. Blood glucose levels measured in mice. C-F. Examination of the total cholesterol (C), total triglyceride (D), low-density lipoprotein (LDL) (E), and high-density lipoprotein (HDL) (F) levels. G-I. Representative images of the atherogenic lesion areas stained with hematoxylin and eosin (H&E) (G), Masson’s trichrome (H), and Oil Red O (I). Scale bar = 20 or 200 μm. J–K. Quantitative analysis of the necrotic core area, lesion areas relative to plaque area from G and H. L. Percentage analysis of the atherosclerosis region from N. M. Quantitative analysis of plaque collagen area relative to plaque area from H. All data are expressed as mean ± SD (n = 8 per group). *P < 0.05 and **P < 0.01, using one-way ANOVA; ns, no significance. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
increase in inflammatory mediators in ApoE−/−+STZ mice (Figs. S4F–I).

In summary, diabetes induces inflammatory changes in the mouse liver and spleen, which are associated with an increase in Acsl1 in macrophages.

3.3. Acsl1 knockdown induces anti-inflammatory macrophages

Our data demonstrated that Acsl1 plays a key role in promoting inflammation in diabetes-accelerated atherosclerosis. Therefore, we...
performed shRNA-mediated Acsl1 gene silencing in RAW 264.7 cells and found that the infection efficiency was approximately 30–40% (Fig. S5A). We then isolated macrophages from ApoE−/−+ STZ mice, performed shRNA-mediated Acsl1 gene silencing on the macrophages and observed a decrease in Acsl1 expression (Fig. 3A–E). These data indicate that the expression of Acsl1 in macrophages can be efficiently and persistently repressed. To evaluate whether shRNA-mediated Acsl1 gene silencing can induce macrophage polarization, the results showed that significantly increased the levels of several anti-inflammatory macrophage marker genes and induced significant mRNA changes in pro-inflammatory macrophage marker gene expression (Figs. S5B–C). In addition, Acsl1 knockdown increased the number of anti-inflammatory macrophages, as suggested by flow cytometry analysis of CD86 (a pro-inflammatory macrophage marker) and CD206 (an anti-inflammatory macrophage marker) (Fig. 3F). Taken together, these data show that shRNA mediated Acsl1 gene silencing can induce anti-macrophage polarization.

Hyperglycemia skews plaque macrophages toward an atherogenic pro-inflammatory phenotype instead of towards the atherosclerosis-resolving anti-inflammatory state [8]. We explored the potential function of Acsl1 knockdown under high-glucose conditions. Flow cytometry analysis further confirmed an increase in anti-inflammatory macrophages in the shAcsl1 group under high-glucose and low-glucose conditions (Fig. 4A–C). Interestingly, knockdown of Acsl1 in macrophages resulted in an anti-inflammatory phenotype, even under hyperglycemic conditions. (Fig. 4D–O, Fig. S5E–P).

ACSL1 is reported to localize both in mitochondria and endoplasmic reticulum [29]. When generated in the mitochondrial outer membrane by ACSL1, acyl-CoAs are destined for β-oxidation. Although the fate of acyl-CoA produced by ER-localized ACSL1 is not clearly understood, it is likely involved in lipid synthesis, since the other key lipid synthetic enzymes (GPAT and DGAT) are localized in ER [30,31]. We explored a correlation between lipid metabolism and Acsl1 in macrophages by analyzing its effects on accumulation of intracellular lipids in macrophages. The results showed that knocking down Acsl1 in macrophages decreased the intracellular lipid droplet accumulation under hyperglycemic conditions (Figs. S6A–B). These data indicated ACSL1 reduction in macrophage prevents lipid accumulation under hyperglycemic condition.

Increased release of PGE2 has been reported previously in several cell types in response to diabetes or elevated glucose concentrations [32], and it has been shown to be caused by increased activity of calcium-dependent phospholipase A2, which catalyzes the release of arachidonic acid from membrane phospholipids [33]. We thus analyzed mRNA levels of enzymes in the PGE2 synthesis pathway. Knockdown of Acsl1 reduced Ptg2 mRNA levels in pro-inflammatory macrophages (Fig. 4J). Similar results were obtained for microsomal Ptg2 mRNA (Fig. 4K). Thus, it is reasonable to deduce that knockdown of Acsl1 in macrophages may regulate the inflammation at least partially via lipid metabolism regulation under hyperglycemic conditions.

3.4 Adaptive transfer of shAcsl1 reprogrammed macrophages alleviates atherosclerosis in diabetic ApoE−/− mice

Anti-inflammatory macrophage polarization may provide a promising strategy for atherosclerosis treatment in diabetes. We systematically analyzed the therapeutic effects of MφshAcsl1. For in vivo tracking, macrophages obtained from GFP + mice were injected into ApoE−/− STZ mice via the tail vein. The results showed that more MφshAcsl1 were located in atherosclerosis plaque, liver, and spleen at 4 h in comparison with MφshCtrl +2 h post-injection (Fig. 5A–E, Fig. 7A–J). For treatment, approximately 5 × 10^6 reprogrammed macrophages were injected into diabetic ApoE−/− mice twice in one month (Fig. 6A). Blood glucose and lipid profiles did not significantly change in phosphate-buffered saline (PBS) mice compared to MφshAcsl1 mice (Fig. 6B–E). HDL-C significantly increased in MφshAcsl1 mice compared to MφshCtrl mice (Fig. 6F), suggesting that any treatment effects are lipid-independent. Furthermore, histological analysis of the aortic tree and roots demonstrated that the atherosclerotic plaque was significantly attenuated in mice receiving MφshAcsl1 treatment (Fig. 6G–N and Figs. S7A–B). Notably, Masson’s trichrome staining revealed that the collagen content in the plaque region also increased, suggesting that the plaque might become stable in the lesions of MφshAcsl1 mice (Fig. 6I, M and Fig. S7C). As mentioned previously, regressing plaques have reduced macrophage content, but have also shown a prevalence of both anti- and pro-inflammatory macrophages, INOS and arginase1 are commonly used as markers of pro-inflammatory macrophages and anti-inflammatory macrophages, respectively [34,35]. Thus, we evaluated the anti-inflammatory macrophage content in plaques and found that MφshAcsl1 treatment promoted the presence of anti-inflammatory macrophages over pro-inflammatory macrophages (Figs. S7D–E). Taken together, these data indicate that adoptive transfer of shAcsl1 reprogrammed macrophages alleviates atherosclerosis in diabetic ApoE−/− mice.

3.5. Adaptive transfer of reprogrammed macrophages reduced systemic inflammation in diabetic ApoE−/− mice

We further investigated the mechanism responsible for the in vivo treatment of atherosclerosis in these macrophages. First, we found that a higher number of GFP + macrophages were located in the liver and spleen 4 h after injection in comparison with MφshCtrl (Fig. 6A). After 72 h of injection, GFP + macrophages still appeared in the liver and spleen (Fig. 7A–I). For treatment, approximately 5 × 10^6 reprogrammed macrophages were injected into diabetic ApoE−/− mice twice in one month (Fig. 6A). In the PBS, MφshAcsl1 mice, it was found that the basic architecture of the liver cells disappeared (Fig. 8A). In contrast, liver tissues were intact, hepatic lobules were clear, and hepatocytes were regularly arranged after MφshAcsl1 treatment. MφshAcsl1 treatment significantly reduced the accumulation of lipid droplets in hepatocytes (Figs. S8B and D). Masson’s trichrome staining further confirmed that MφshAcsl1 treatment significantly reduced collagen content (Figs. S8A and C). We next assessed the effect of MφshAcsl1 treatment on liver function and observed that levels of serum biomarkers AST and ALT were significantly decreased in the MφshAcsl1 group (Fig. 8C and D). Moreover, qPCR analysis revealed that MφshAcsl1 treatment significantly reduced the expression of inflammatory genes (Fig. 8E–H). These data indicated that MφshAcsl1 treatment significantly decreased inflammation in the liver.

In the spleen, we observed disorganization of lymphocytes and a blurring of boundaries between the white and red pith in mice receiving PBS, Mφsh, and MφshAcsl1 treatments. In contrast, the lymphocytes were well arranged, and the boundary between the white and red pith was clear after MφshAcsl1 treatment (Fig. 8B). Moreover, qPCR analysis revealed that MφshAcsl1 treatment significantly reduced the expression of inflammatory genes in the spleen (Fig. 8I–L). In summary, these data showed that adoptive transfer of reprogrammed macrophages reduces systemic inflammation in diabetic ApoE−/− mice. In addition, we further tracked macrophage distribution in other organs, and the results showed that GFP + macrophages rarely appeared in the kidney, lung, and heart at either 4 h or 72 h post-injection (Figs. S9A–E, S10A–E). These data indicate that adoptive transfer of reprogrammed macrophages did not cause any noticeable toxic effects in these organs.

4. Discussion

In our study, we confirmed for the first time that increased expression of Acsl1 in macrophages is responsible for aggravating inflammation, and thus, exacerbating atherosclerosis. Knockdown of Acsl1 in macrophages reprograms the cells to an anti-inflammatory phenotype. Adoptive transfer of reprogrammed macrophages significantly repressed inflammation and alleviated atherosclerosis in diabetic ApoE−/− mice.

Significant advances in therapies to prevent and/or treat
A) Diagram showing the experimental protocol:
- ApoE\(^{-}\)+STZ
- M-CSF
- Lentivirus
- D-glucose
- PBS/LPS/IFN\(\gamma/IL-4\)
- Gene knockdown

B) Flow cytometry histograms showing the expression of CD86 and CD206 under different conditions:
- 5.5 mM D-glucose
- 25 mM D-glucose

C) Bar graphs showing the relative mRNA expression of various genes under different conditions:
- Tnf-\(\alpha\)
- Il1\(\beta\)
- Ptg2
- Ptges

D) Graphs showing the relative mRNA expression under different conditions:
- Trif-\(\alpha\)
- TGF-\(\beta\)-1
- Fizz1
- Mrc-1
- Arg1

(caption on next page)
Atherosclerosis have been achieved in clinics [36, 37]. For example, PCSK9 inhibitors lower plasma LDL-C levels and reduce the risk of major vascular events [38, 39]. Patients with diabetes mellitus remain at an increased risk of cardiovascular morbidity and mortality [40, 41]. Novel therapeutic strategies are urgently needed. Although it is believed that lesions of atherosclerosis from people with and without diabetes have no distinguishing morphological features, several mouse studies have suggested that there should be specific molecules involved in the mechanism how diabetes accelerated atherosclerosis. And ACSL1 might be such a candidate. In this study, we found that ACSL1 is expressed at higher levels in myeloid cells in mice with diabetes induced by STZ or virus [21, 22].

**Fig. 4.** Knockdown of Acyl coenzyme A synthetase-1 (Acsl1) reduces inflammatory cytokine secretion in macrophages under high-glucose conditions. A. Schematic diagram of the experimental procedure. Macrophages were differentiated with lipopolysaccharides (LPS), IFNγ, or IL4 in 5.5 (normal) or 25 mM (high) endotoxin-free d-glucose, respectively. B–C. Bone marrow-derived macrophages (BMDMs) were stained using CD86/CD206 and analyzed using flow cytometry. D–G. Expression of inflammatory genes (Tnf-α, Il1β, Ptg2, Pges) in macrophages treated with shctrl or shAcsl1. H–K. Expression of inflammatory cytokines (Tnf-α, Il1β, Ptg2, Pges) in BMDMs additionally treated with LPS. L–O. Expression of anti-inflammatory genes (Tgfβ-1, Arg1, Fizz 1, Mrc1) in macrophages additionally treated with IL-4. Gapdh served as an internal control. Data are expressed as mean ± S.E.M of five parallel experiments. *P < 0.05, **P < 0.01 using one-way ANOVA. ns, no significance.

**Fig. 5.** Distribution of injected macrophages in (ApoE−/−) + streptozotocin (STZ) mice within 4 h or 72 h. A. A schematic representation of the experimental procedure. Green fluorescent protein (GFP+) macrophages were injected into ApoE−/− + STZ mice for tracking 4 h or 72 h post-injection. B–C. Representative fluorescence images of the GFP+ -labeled macrophages in the aorta root (green). The nuclei were counter-stained with Hoechst (blue). Scale bar = 50 or 20 μm (n = 5 per group). D–E. Quantitative analysis of the number of GFP+ bone marrow-derived macrophages (BMDMs) per mm2. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
Fig. 6. Therapeutic effects of $\text{Mφ}^{\text{hAcDli}}$ in diabetic (ApoE$^{-/-}$) + streptozotocin (STZ) mice. A. Schematic showing the experimental procedure. For the prevention and therapy, the reprogrammed macrophages ($5 \times 10^6$ cells) were injected into ApoE$^{-/-} + $ STZ mice via the tail vein at weeks 6, 11, and 14, respectively. B. Blood glucose level test results in mice. C–F. Examination of total triglyceride (C), total cholesterol (D), low-density lipoproteins (LDL) (E), and high-density lipoproteins (HDL) (F) levels. G–I. Representative images of the atherogenic lesion areas stained with hematoxylin and eosin (H&E) (G), Oil Red O (H), and Masson’s trichrome (I). Scale bar = 200 µm. J–K. Quantitative analysis of the lesion areas relative to plaque area from G, H, L. Percentage analysis of the atherosclerosis region from N. M. Quantitative analysis of plaque collagen area relative to plaque area from I. Scale bar = 200 µm. All data are expressed as mean ± SD ($n=8$ per group). *P < 0.05 using one-way ANOVA. ns, no significance. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
Fig. 7. Distribution of macrophages in (ApoE<sup>−/−</sup>) + streptozotocin (STZ) mice within 4 h or 72 h. A. Schematic representation of the procedure. Green fluorescent protein (GFP<sup>+</sup>) macrophages were injected into ApoE<sup>−/−</sup> + STZ mice to enable tracking 4 h after injection. B–G. Representative fluorescence images of the GFP<sup>+</sup>-labeled macrophages in the liver and spleen (green). The nuclei were counter-stained with Hoechst (blue). Scale bar = 50 or 20 μm (n = 5 per group). D–I. Quantitative analysis of the number of GFP<sup>+</sup> bone marrow-derived macrophages (BMDMs) per mm<sup>2</sup>. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
monocytes of bone marrow, liver, and spleen of diabetic ApoE−/− mice. In addition, ACSL1 is localized in mitochondria, lipid droplets, endoplasmic reticulum and microsomes of liver [29,42,43]. Notably, ACSL1 is highly expressed by all parenchymal cells in ApoE−/−+ STZ mice. The key function of ACSL1 in the liver remains to be explored.

A dominant hypothesis in atherosclerosis is that excessive inflammation or failed inflammation resolution is a major contributor to plaque development and late-stage lesion destabilization [23]. Despite a growth in understanding the chronic inflammatory nature of atherosclerosis, a specific anti-inflammatory therapy is yet to be found. It has been revealed that Acsl1 induces the conversion of macrophages to the pro-inflammatory macrophage phenotype under diabetic conditions [44,45]. Another result provided evidence that TNFα-related inflammatory polarization in monocytes is an Acsl1-dependent process [46]. Furthermore, the aging macrophage significantly increases PGE2 expression [47]. Our data showed that ACSL1 deficiency inhibited the release of inflammatory factors, Ptges and Ptgs2, by macrophages even in hyperglycemic condition.

Atherosclerosis is a localized and systemic metabolic inflammatory disease [48]. It has become clear that diabetes-induced chronic tissue inflammation, particularly in the liver and spleen, can cause atherosclerosis [49,50]. The liver is a major organ that regulates whole-body cholesterol metabolism. Disrupted hepatic cholesterol hemostasis contributes to atherogenesis [51]. Hepatic macrophages have a central role
in restricting inflammation in the liver [52]. Thus, targeting hepatic macrophages to treat liver disease may provide a potential therapeutic strategy [53]. The spleen also serves as a critical reservoir of monocytes that regulate inflammation during ischemic myocardial injury [54,55]. Due to the bone marrow origin of these monocytes, it has been recently recognized that splenic hematopoietic stem and progenitor cells can be an extramedullary myelo poetic source of monocytes that are mobilized to inflammatory sites, including atherosclerotic plaques [56]. Splenectomy accelerates atherogenesis by modulating lipid changes, implying a protective effect of the spleen [57,58]. Considering that macrophages serve as a bridge between liver, spleen and arteriosclerosis, we engineered macrophages. As expected, adoptive transfer of the reprogrammed macrophages in diabetic ApoE−/− mice alleviated inflammation locally in the plaque and systemically in the liver and spleen.

Macrophages are the central cells in atherosclerosis, and the quantity and phenotype of these cells in plaques influence both disease progression and regression [59–62]. With these factors, bone marrow macrophage therapy has shown promising results as a novel therapeutic approach for atherosclerosis that complements existing therapies. For example, studies have shown that soluble ninjurin [60], deletion of macrophage LRP1 [63], and RIPK1 antisense oligonucleotides [59] are intensively studied strategies for the treatment of atherosclerosis. Recently, adoptive macrophage transfer has shown promising results an effective therapeutic option for bacterial sepsis [64], liver fibrosis [65], ovarian cancer [66], and high-grade gliomas [67]. Furthermore, studies have demonstrated that injection of exogenous reprogrammed macrophages hijacks existing macrophages, recruits signals, and activates the immune system to destroy the tumor [68,69]. Macrophages represent a readily available, autologous source of cells for cell therapy. Moreover, the major advantage of macrophage therapy is that there is no need for long-term immune suppression to preserve the transplanted cells. Our study further confirmed that adoptive transfer of reprogrammed macrophages significantly alleviated atherosclerosis in diabetic ApoE−/− mice. At the same time, macrophages are safe to treat patients, and there are no adverse clinical reactions during infusion or in the post-infusion period [70].

Regarding the engineering strategy, we used shRNA mediated AcsI1 gene silencing in BMDMs to induce anti-macrophages. There were more M0/AcsI1 in the liver and spleen, which could be attributed to altered viability or migration ability [71]. The reasons for this need to be explored further. Moreover, specifically engineered macrophages may have the ability to enter a lesion and migrate out of the damaged area. These potential treatment avenues need to be studied further.

5. Conclusion

In conclusion, our study provides evidence that increased expression of AcsI1 in macrophages of diabetic mice play a key role in aggravated arteriosclerosis of diabetic mice, possibly by promoting inflammation. Adoptive transfer of AcsI1 silenced macrophages is a potential therapeutic strategy for arteriosclerosis in patients with diabetes.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors are grateful to Dailing Si for her assistance with the Nikon microscope system. This work was funded by the National Natural Science Foundation of China (No. 81671910 to X Yang ), Shaxi Province Foundation of China (No. 2021SF-341 to X Yang).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioactmat.2022.02.002.

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