Comparison of adipose- and bone marrow-derived stem cells in protecting against ox-LDL-induced inflammation in M1-macrophage-derived foam cells

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Abstract. Adipose-derived stem cells (ADSCs) and bone marrow-derived stem cells (BMSCs) are considered to be prospective sources of mesenchymal stromal cells (MSCs), that can be used in cell therapy for atherosclerosis. The present study investigated whether ADSCs co-cultured with M1 foam macrophages via treatment with oxidized low-density lipoprotein (ox-LDL) would lead to similar or improved anti-inflammatory effects compared with BMSCs. ADSCs, peripheral blood monocytes, BMSCs and ox-LDL were isolated from ten coronary heart disease (CHD) patients. After three passages, the supernatants of the ADSCs and BMSCs were collected and systematically analysed by liquid chromatography-quadrupole time-of-flight-mass spectrometry (6530; Agilent Technologies, Inc., Santa Clara, CA, USA). Cis-9, trans-11 was deemed to be responsible for the potential differences in the metabolic characteristics of ADSCs and BMSCs. These peripheral blood monocytes were characterized using flow cytometry. Following peripheral blood monocytes differentiation into M1 macrophages, the formation of M1 foam macrophages was achieved through treatment with ox-LDL. Overall, $2x10^6$ ADSCs, BMSCs or BMSCs+cis-9, trans-11 were co-cultured with M1 foam macrophages. Anti-inflammatory capability, phagocytic activity, anti-apoptotic capability and cell viability assays were compared among these groups. It was demonstrated that the accumulation of lipid droplets decreased following ADSCs, BMSCs or BMSCs+cis-9, trans-11 treatment in M1 macrophages derived from foam cells. Consistently, ADSCs exhibited great advantageous anti-inflammatory capabilities, phagocytic activity, anti-apoptotic capability activity and cell viability over BMSCs or BMSCs+cis-9, trans-11. Additionally, BMSCs+cis-9, trans-11 also demonstrated marked improvement in anti-inflammatory capability, phagocytic activity, anti-apoptotic capability activity and cell viability in comparison with BMSCs. The present results indicated that ADSCs would be more appropriate for transplantation to treat atherosclerosis than BMSCs alone or BMSCs+cis-9, trans-11. This may be an important mechanism to regulate macrophage immune function.

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Introduction

Cardiovascular diseases are one of the leading causes of death worldwide, even though there has been great improvement in

medicine during the past few decades. Atherosclerosis (AS) is a major cause of cardiovascular diseases. Since it is a chronic inflammatory disease, multiple immune cell types are involved in the pathogenesis of AS (1-3). Much of the current evidence has indicated that macrophages participate in the pathogenesis of AS. Macrophages could respond rapidly to environmental signals through a multitude of receptors and develop a specific and optimized activation state. Under some conditions, monocytes form macrophage foam cells by taking up low-density lipoprotein (LDL) into the vascular intima. These lipid overloading foam cells are a sign of the initiation of AS. The development of atheromatous plaques eventually leads to serious cardiovascular diseases and the plaques are promoted through accumulation and necrosis or apoptosis of foam cells (4).

The diffuse involvement of arteries throughout the body and the appearance of multiple and simultaneous atherosclerotic lesions are the characteristic feature of atherosclerotic cardiovascular disease. Endovascular interventional procedures that are 'local' treatments, such as stenting and balloon angioplasty, of atherosclerotic arteries are the main treatments, yet they are not effective for treating the underlying cause of AS. Therefore, we need to find alternative therapies for atherosclerotic cardiovascular disease. Mesenchymal stem cell (MSCs) therapies have emerged as a promising tool for the treatment of atherosclerotic cardiovascular disease (5,6). MSCs, which mainly include adipose-derived stem cell (ADSCs) and bone marrow-derived stem cells (BMSCs), could be isolated from several tissues and expanded *in vitro*. MSCs could be intravenously transfused and could migrate to atherosclerotic arteries and differentiate into vascular smooth muscle cells, as well as circulate in the blood system of the whole body. It has been reported that intravenous infusion of MSCs for diffuse multiple atherosclerotic lesions contributes to remodelling of the vasculature (7,8). It is more important that anti-inflammatory properties of MSCs be confirmed (9-11), and it has also been reported that ADSCs and BMSCs could regulate the activation state of macrophages, which possess potent immunomodulatory abilities (12,13).

BMSCs are considered good candidates for stem cell therapy due to their accessibility and non-tumourigenic activity. However, their use has been limited by their low abundance, especially in elderly people. ADSCs with similar features to BMSCs can be obtained from subcutaneous adipose tissue from adult humans. Hundreds of millions of ADSCs could be extracted from 1 to 2 litres of adipose tissue. In addition, the separation process of ADSCs is an efficient and safe procedure (14). Additionally, whether ADSCs are better than BMSCs for altering macrophages has not been reported. In the present report, the difference in metabolomics between ADSCs and BMSCs from elderly people were explored, and conjugated linoleic acid (CLA) from the metabolites of ADSCs and BMSCs have marked anti-inflammatory effects through the regulation of macrophages. Specifically, we show that ADSCs are better suppressors of human macrophages compared to BMSCs when using CLA.

Patients and methods

Participants. The present study was approved by the Ethics Committee of The Second Affiliated Hospital of Harbin Medical University (Harbin, China). Ten patients with coronary atherosclerotic heart disease (CAD), aged 60-76 years old (age 67±7 years, 8 males, 2 females) were recruited into this research. The baseline characteristics of the donors are shown in Table I. Before cardiac surgery, written informed consent regarding this research were obtained from the patients.

Cell cultures. ADSCs were derived from thoracic subcutaneous fat of above-mentioned patients during operation under general anaesthesia, as previously described (15). The adipose tissues were then washed with phosphate-buffered saline (PBS) containing 1% penicillin and streptomycin and subsequently digested with collagenase type I (1 mg/ml; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). The conditions for digestion were maintained according to the instructions for collagenase at 37˚C for 60 min. Afterwards, we filtered the suspension using a 200-μm nylon mesh, and the floating adipocytes were removed after the suspension was centrifuged. The remaining cells were harvested and cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% foetal bovine serum (FBS) with 3.7 g/l sodium bicarbonate, and 1% penicillin and streptomycin at 37˚C with a 5% CO_2 humidified atmosphere. At passage 3, 10^5 cells in 2 ml cell culture medium were plated in 6-well plates. The supernatants were collected and frozen at -80˚C for subsequent analyses after 3 days.

Bone marrow was aspirated from the sternum of each participant and collected in heparinized tubes from upon cardiac surgery (16). DMEM with 10% FBS, 3.7 g/l sodium bicarbonate and 1% penicillin and streptomycin was used for culturing the isolated cells. Non-adherent cells were removed by washing the cultures with the PBS solution after 3 days. Again, 2 ml of cell culture medium of 10^5 cells were plated in 6-well plates at passage 3, and the supernatants were collected and frozen at -80˚C for subsequent analyses after 3 days.

Peripheral blood monocytes were isolated from 40 ml peripheral blood of the corresponding patient using human lymphocyte separation solution and differential gradient centrifugation with a Ficoll gradient (20 min, 800 x g) (17). Then, peripheral blood monocytes were cultured in RPMI-1640 supplemented with 10% FBS at 37˚C and a 5% CO_2 humidified atmosphere. The cells were plated and non-adherent cells were removed by gentle washing after 2 h. The remaining adherent cells were harvested and the cell type was confirmed by FSC-SSC parameters and surface expression of CD14 in flow cytometry (FACS Calibur; BD Biosciences, San Jose, CA, USA) (17,18). The identification results revealed that the purity of monocytes was 72%. After collection, the peripheral blood monocytes were cultured at 2x10^6 cells in six-well plates and incubated with 100 μg/ml ox-LDL for 48 h in serum-free RPMI-1640 to form foam cells.

Metabolomic profiling of supernatants of ADSCs and BMSCs. Metabolomic analyses of the supernatant samples from ADSCs and BMSCs were performed using the RRLC (6530 series; Agilent Technologies, Inc., Santa Clara, CA, USA). In the present report, 6 major metabolic compounds from various pathways were selected for metabolomics analysis.

Table I. Clinical characteristics of adipose-derived stem cells and bone marrow stem cells donors.

Characteristic	Total n, range
Number of subjects, n	10
Age, years (median, range)	67, 60-76
Weight, kg (median, range)	65, 56-80

Isolation and oxidation of LDL. Human LDL was isolated from the same participants as above and oxidized (ox) as described by Boullier *et al* (19). Briefly, LDL (density ¼ 1.019-1.063 g/ml) was isolated from plasma by sequential ultracentrifugation and incubated with 10 μmol/l CuSO4 for 18 h at 37˚C. To prevent further oxidation, 0.1 mmol/l ethylenediaminetetraacetic acid (EDTA) was added to collect the ox-LDL at a concentration of 1 mg/ml. The extent of LDL oxidation was assessed as described previously (20). In brief, ox-LDL preparations had thiobarbituric acid-reactive substances of 0.30 mmol/g protein and a relative mobility index on agarose gels of 2.0-2.5 compared to native LDL.

M1 macrophages and foam cell formation. Monocytes cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) with 10% FBS were differentiated into M1 macrophages with 20 ng/ml GM-CSF and stimulated with 20 ng/ml IFN-γ and 1 ng/ml LPS for 24 h. Afterwards, the treated macrophages were incubated with 100 μg/ml ox-LDL for another 24 h. The cells were fixed in 4% formaldehyde and stained with Oil Red O, and foam cells were counted by microscopy, as previously described (21,22).

Transwell co-culture assay. Next, $2x10^5$ macrophages or foam cells were seeded in the lower compartment of 6-well Transwell polyethylene terephthalate (PET) permeable supports in 24-mm polycarbonate Transwell inserts with a pore size of 8 μm (Corning, Inc., Corning, NY, USA) for 24 h. Serum-free medium was replaced 1 h before adding $2x10^6$ ADSCs, BMSCs or BMSCs +cis-9, trans-11 into the upper compartment of the Transwell inserts. In a humidified chamber at 37˚C, the co-cultures were incubated without a medium change for 24 h, and the supernatants, as well as the macrophages or foam cells at the bottom of the co-culture assay, were collected for Oil Red O staining, flow cytometry analysis and measurement of inflammatory factors in supernatants.

Measurement of intracellular lipid droplets using oil red O staining. The macrophages and foam cells after they were co-cultured with ADSCs, BMSCs or BMSCs +cis-9, trans-11 were washed with PBS and fixed with 4% paraformaldehyde solution for 20 min. Then, the cells were stained with 0.5% Oil Red O in isopropanol for 30 min and counterstained with haematoxylin for 5 min. The macrophages were observed with an inverted fluorescence Microscope (DMI4000B; Leica Microsystems GmbH, Wetzlar, Germany) and analysed using Image-Pro Plus software 6.0. The number of lipid droplets was presented as the mean value of integrated optical density (IOD).

Cell viability assay. Cell viability was measured using Cell Counting Assay kit-8 (CCK-8; CK04; Dojindo, Molecular Technologies, Inc., Kumamoto, Japan) according to the manufacturer's protocol. Briefly, following pre-incubation with ox-LDL (100 μg/ml) for 24 h, M1 macrophages pre-treated in HTS Transwell 96-well plates with or without ADSCs, BMSCs or BMSCs+ cis-9, trans-11 at a density of $1x10^4$ cells per well. Then, 10 μl CCK-8 reagent was added to the medium for 2 h, and the absorbance was measured using a microplate reader (Infinite M200 Pro; Tecan, Group, Ltd., Mannedorf, Switzerland) at 450 nm. Each experiment was performed in triplicate and was repeated at least three times.

Flow cytometry analysis. Before the analysis with flow cytometry, macrophages and foam cells were stained cells by using the Annexin V-FITC and PI Detection kit (BD Pharmingen; BD Biosciences). In brief, the cells were trypsinized and resuspended at a density of 10^6/ml. After centrifugation, the pellet was washed twice with ice-cold PBS and was suspended in binding buffer. The cells were maintained in the dark at room temperature for 15 min after incubation with FITC-labelled Annexin V and PI (Molecular Probes; Thermo Fisher Scientific, Inc.), and the cells were analysed using flow cytometry. More than $1x10^4$ cells per group were counted and each assessment was repeated three times to get a proper statistical analysis.

Measurement of inflammatory factors. The protein levels of TNF-α, IL-6, IL-8 and IL-10 in the culture supernatants of macrophages and foam cells were measured with ELISA kits (Sigma-Aldrich; Merck KGaA) according to the manufacturer's protocol.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from macrophages and foam cells using TRIzol Reagent (Invitrogen; Thermo Fisher Scientific, Inc.). First-strand complementary DNA (cDNA) was synthesized using Golden 1st cDNA Synthesis kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) from 1 mg of total RNA. Applied Biosystems synthesized the following primers: Hum P65 forward primer, 5'-CTGTATGGCACT GGCATTGTC-3' and reverse, 5'-GGCACTTGAGAAGAG GGAGAG-3' (length of 196 bp); Hum IL-6 forward, 5'-AGC CACTCACCTCTTCAGAAC-3' and reverse, 5'-GCAAGT CTCCTCATTGAATCCAG-3' (length of 200 bp); Hum TNF-α forward, 5'-GTCTGGGCAGGTCTACTTTGG-3' and reverse, 5'-GAGGTTGAGGGTGTCTGAAGG-3' (length of 119 bp); Hum GAPDH forward, 5'-CCACTCCTCCACCTT TGAC-3' and reverse, 5'-ACCCTGTTGCTGTAGCCA-3' (length of 102 bp). RT-qPCR was performed with a Bio-Rad Min-Opticon2 (Bio-Rad, Inc., Hercules, CA, USA) using SYBR-Green PCR master mix kits (Tian Gen Biotech, Beijing, China). Initial denaturation was performed at 95˚C for 15 min, followed by 40 cycles of denaturation at 95˚C for 10 sec, then annealing at 60˚C for 30 sec and extension at 60˚C for 30 sec. The data were analyzed using the Rotor-Gene Q software (version 1.7; Qiagen AB, Sollentuna, Sweden), and relative mRNA levels were calculated using the $2^{-\Delta\Delta Cq}$ method (23) and normalized against GAPDH. Each sample was analyzed in triplicate, and the experiment was repeated ten times.
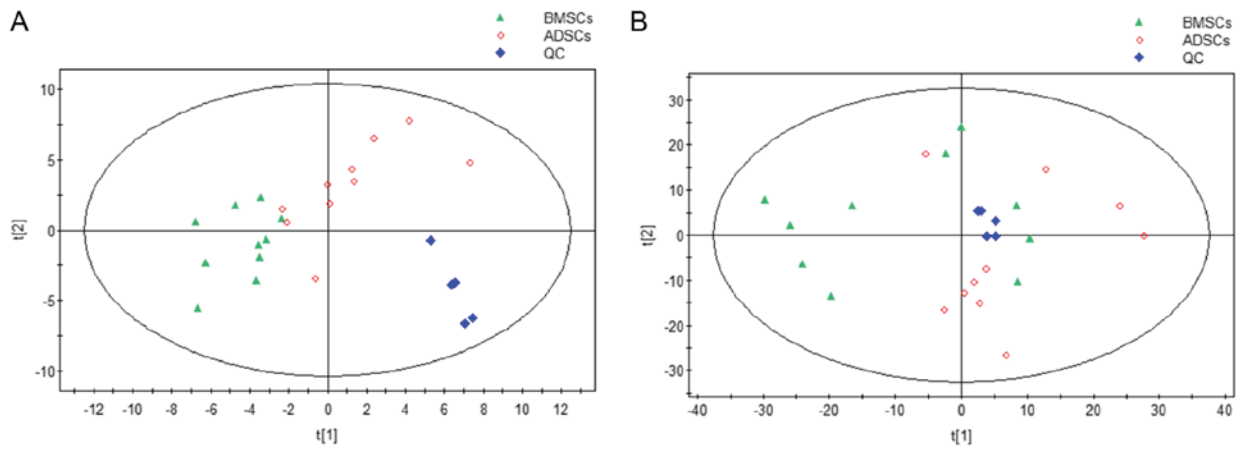
Figure 1. Principal component analysis score plots for discriminating BMSCs and ADSCs in (A) ESI⁺ and (B) ESI⁻ modes. BMSCs, bone marrow-derived stem cells; ADSCs, adipose-derived stem cells; ESI, electrospray ionization; QC, quality control.
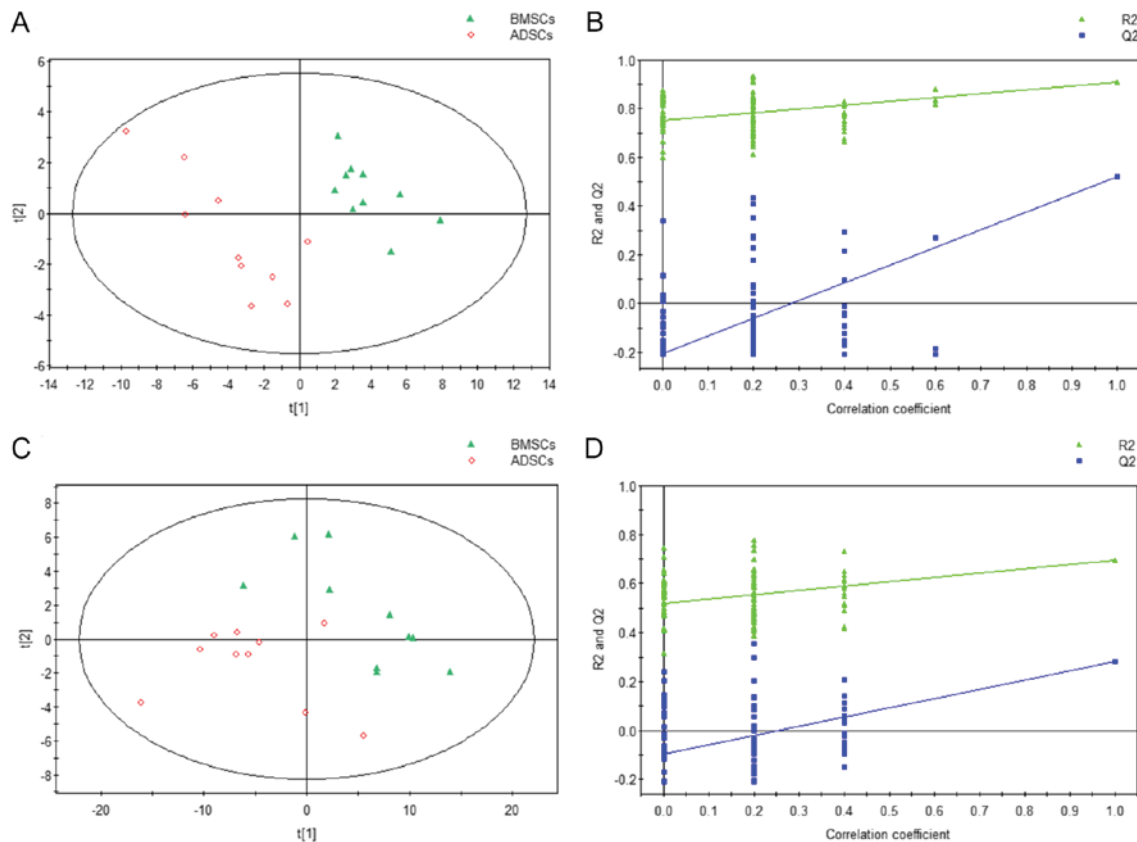

Figure 2. PLS-DA score plots and validation plots for discriminating ADSCs and BMSCs in ESI⁺ and ESI⁻ modes. (A) PLS-DA plot in ESI⁺ mode. (B) Validation plot in ESI⁺ mode. (C) PLS-DA plot in ESI⁻ mode. (D) Validation plot in ESI⁻ mode. PLS-DA, partial least squares discriminant analysis; BMSCs, bone marrow-derived stem cells; ADSCs, adipose-derived stem cells; ESI, electrospray ionization; Q2, second quartile; R2, coefficient of determination.

Western blot analysis. After the treatment described above, the protein expression levels of NF-κB p65 and phosphorylated NF-κB p65 in the lysates of cells harvested in RIPA sample buffer were detected by Western blot analysis (24). The samples were subjected to Western blot analyses using NF-κB p65, phosphorylated NF-κB p65 (both Boster Biological Technology, Pleasanton, CA, USA) and β-actin as a loading control. The protein content was quantified with a BCA kit (Genscript, Piscataway, NJ, USA), separated by 10% SDS-PAGE, and then transferred to polyvinylidene

fluoride (PVDF) membranes (EMD Millipore, Billerica, MA, USA). The membranes were blocked in 5% (w/v) non-fat milk diluted with TBST (Tris-HCl 20 mM, NaCl 150 mM, 0.1% Tween-20, pH 7.5) at room temperature for 1 h, incubated with a primary antibody for 1 h at room temperature or overnight at 4°C, and then the membranes were incubated with the appropriate secondary horseradish peroxidase-conjugated antibodies for 1 h at room temperature. The blot results were detected using Pierce ECL Plus Substrate (Genscript), as described by the manufacturer. The density of the target bands
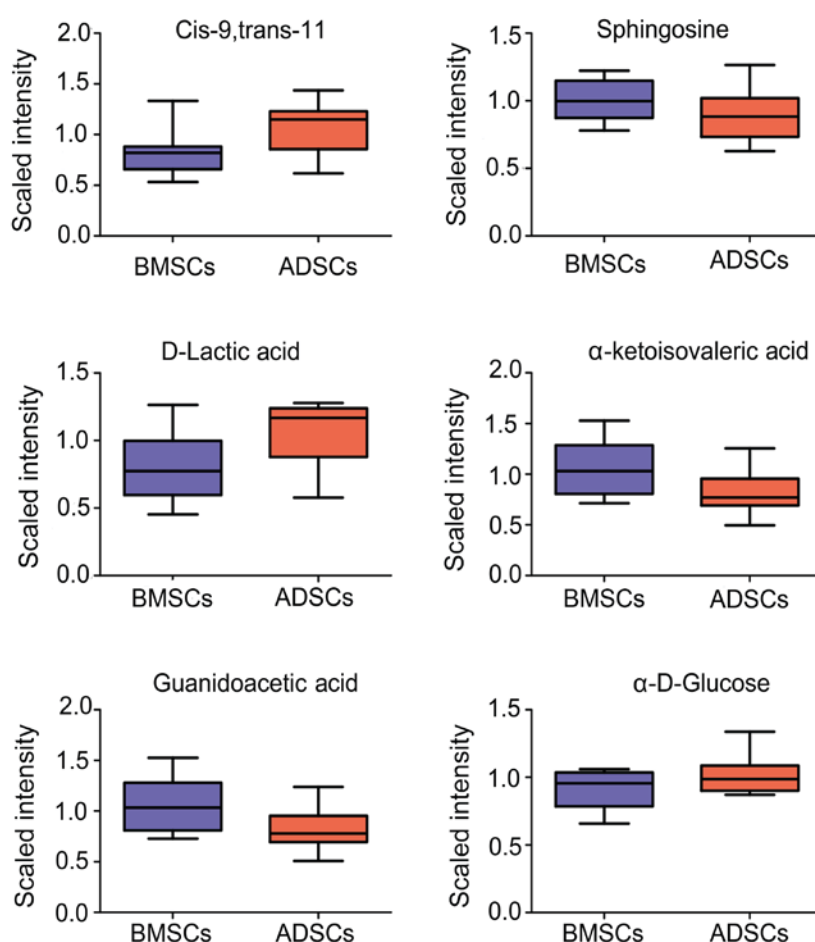
Figure 3. Metabolite profiles of potential biomarkers differing between epithelial BMSCs and ADSCs. The profiles are displayed as box & whiskers graphs (Min to Max). BMSCs, bone marrow-derived stem cells; ADSCs, adipose-derived stem cells.

was quantified with Image-Pro Plus 6.0 (Media Cybernetics, Inc., Rockville, MD, USA) software.

Statistical analysis. At first, the data on metabolic profiles of ADSCs or BMSCs were used in principal component analysis (PCA) to detect the group trends and outliers (25). Then, we applied a Wilcoxon rank sum test to determine the significance of each metabolite at $P<0.05$. To identify the differences in metabolites between ADSCs and BMSCs, a partial least squares discriminant analysis (PLS-DA) was used (25). We included permutation tests with 100 iterations to validate the supervised model and avoid overfitting (26). Based on the PLS-DA model, we calculated parameters that described the variable importance in the projection (VIP) for each metabolite. With thresholds of P-values and VIP values of 0.05 and 1, respectively, the metabolic biomarkers were detected. The Wilcoxon rank sum test was used with the R platform. The PCA and PLS-DA were performed using SIMCA-P (version 11.5; Umetrics, Malmo, Sweden).

The other statistical analyses and graphing were performed using GraphPad Prism software, version 5.00 (GraphPad Software, Inc., La Jolla, CA, USA) by setting the statistical significance at $P<0.05$. Two-group comparisons were analysed by the unpaired two-tailed t-test. Multiple comparisons were evaluated by analysis of variance with Tukey's or Dunnett's post hoc test, as appropriate.

Results

Metabolomics of supernatants from ADSCs and BMSCs. The results of the overall PCA based on all the samples suggested that the QC (quality control) samples were closely clustered in plots of PCA scores, which demonstrated that the results of our metabolic profiling platform were robust. Moreover, there were no outliers on the whole, and there were separation trends between BMSCs and ADSCs (Fig. 1). Additionally, there were no outliers overall, and there were separation trends between BMSCs and ADSCs (Fig. 2). Cis-9, trans-11, Sphingosine, D-Lactic acid, α-ketoisovaleric acid, Guanidinoacetic acid, and α-D-Glucose ester were observed to be elevated in the supernatant of ADSCs compared to BMSCs (Fig. 3).

Differential effects of inhibiting ox-LDL-induced lipid accumulation in macrophage foam cells. Many lipid droplets were present in macrophage foam cells after treatment with ox-LDL (100 mg/l) for 24 h through Oil Red O staining. However, the number of lipid droplets decreased after pre-incubation of cells with ADSCs, BMSCs or BMSCs+cis-9, trans-11 (Fig. 4A and B). The number of lipid droplets from pre-incubation of ADSCs and BMSCs+cis-9, trans-11 was less than those from pre-incubation with BMSCs. In addition, the number of lipid droplets from pre-incubation with ADSCs was less than those from pre-incubation with BMSCs+ cis-9, trans-11.
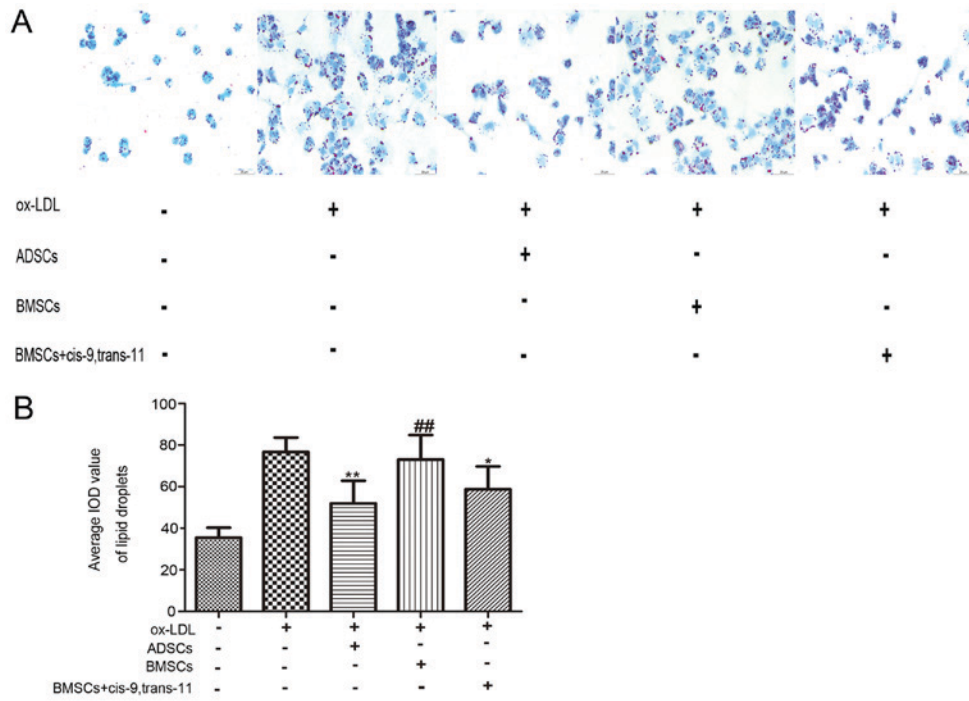
Figure 4. (A) Cells were treated with ox-LDL (100 mg/l) in the absence or presence of ADSCs, BMSCs or BMSCs+ cis-9, trans-11 for 24 h, and the intracellular lipid droplets were stained using Oil Red O. Representative lipid droplet staining images are shown. Scale bars, 25 μm. (B) The average IOD of lipid droplets stained with Oil Red O from differentiated macrophage foam cells was obtained by examining five fields in each condition (n=10). *P<0.05 and **P<0.01 vs. the ox-LDL group; ##P<0.01 vs. the ADSCs group. ox-LDL, oxidized low-density lipoprotein; BMSCs, bone marrow-derived stem cells; ADSCs, adipose-derived stem cells; IOD, integrated optical density.
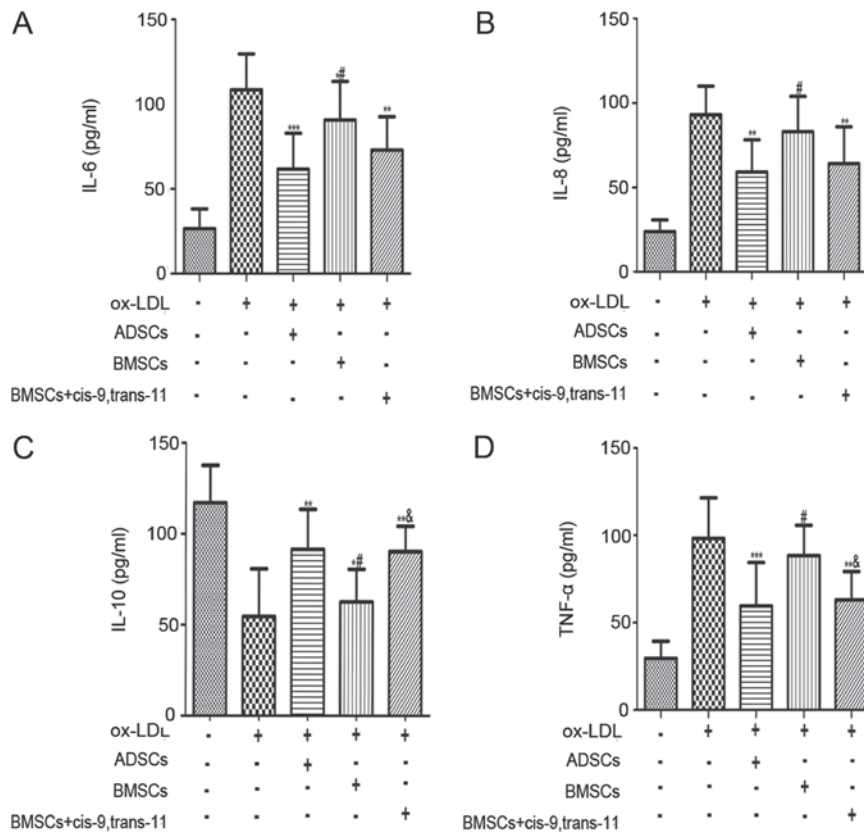

Figure 5. ADSCs, BMSCs and BMSCs+cis-9, trans-11 decreased the secretion of inflammatory factors (TNF-α and IL-6) and increased the secretion of inflammatory factors (IL-10) in ox-LDL-stimulated macrophages. Cells were pre-incubated with ADSCs, BMSCs and BMSCs+cis-9, trans-11 following treatment with 100 g/ml ox-LDL for 24 h. The concentrations of (A) IL-6, (B) IL-8, (C) IL-10 and (D) TNF-α secretion from the medium of macrophages were measured using an ELISA kit. The results are expressed as a percentage of the results obtained with a blank. Data are presented as the mean ± standard deviation (n=10). *P<0.05, **P<0.01 and ***P<0.001 vs. the ox-LDL group; #P<0.05 vs. the ADSCs group; &P<0.05 vs. the BMSCs group. BMSCs, bone marrow-derived stem cells; ADSCs, adipose-derived stem cells; TNF-α, tumour necrosis factor-α; IL-, interleukin-; ox-LDL, oxidized low-density lipoprotein.
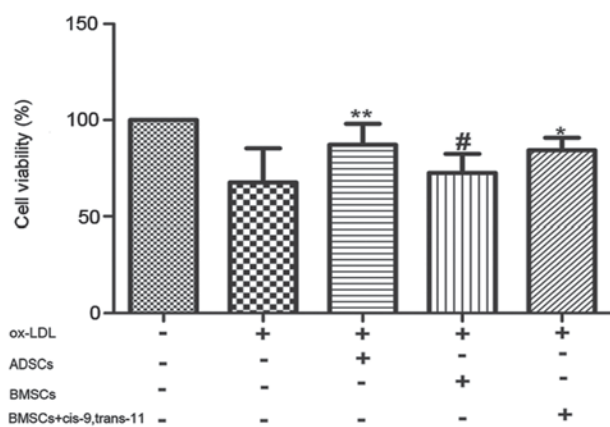
Figure 6. Effects of ADSCs, BMSCs and BMSCs+cis-9, trans-11 on the vitality of Mrc-5 cells. M1 macrophage foam cells were seeded in 96-well plates and co-cultured with ADSCs, BMSCs or BMSCs+cis-9, trans-11, and the cell viability of M1 macrophage foam cells was assessed at 24 h using a Cell Counting kit-8 assay (n=10). *P<0.05 and **P<0.01 vs. the ox-LDL group; #P<0.05 vs. the ADSCs group. BMSCs, bone marrow-derived stem cells; ADSCs, adipose-derived stem cells; ox-LDL, oxidized low-density lipoprotein.

Differential effects decreased the secretion of inflammatory cytokines (TNF-α, IL-6, IL-8 and IL-10) in ox-LDL-Stimulated macrophages. Lesional macrophages could secrete high amounts of cytokines and chemokines, which further promoted the development of AS by recruiting other immune cells into the vessel wall. TNF-α, IL-6, IL-8 and IL-10 are essential for initiation and progression of AS. As described in Fig. 5, TNF-α, IL-6 and IL-8 protein levels were markedly increased, and IL-10 had a marked decline in the medium of macrophages followed by pre-incubation with 100 g/ml ox-LDL for 24 h. These changes of the above inflammatory cytokines have decreased after treatment with ADSCs, BMSCs and BMSCs+cis-9, trans-11. Nevertheless, the inflammatory cytokines from pre-incubation with BMSCs showed smaller changes compared to ADSCs and BMSCs+cis-9, trans-11. In addition, pre-incubation with ADSCs significantly inhibited TNF-α, IL-6 and IL-8 protein expression and promoted IL-10 expression.

Effects of ADSCs, BMSCs and BMSCs+ cis-9, trans-11 on the viability of ox-LDL-stimulated macrophages. Regarding the viability of the macrophages followed 100 g/ml ox-LDL for 24 h, ADSCs, BMSCs or BMSCs+cis-9, trans-11 exerted an obvious positive effect on cell viability. Here, we showed that ADSCs significantly enhanced the viability of Macrophages stimulated by ox-LDL (Fig. 6). We also found that cis-9, trans-11 enhanced the proliferation of Macrophages stimulated by ox-LDL.

Different influence of attenuating ox-LDL-induced apoptosis of macrophages. Apoptosis of macrophages plays a key role in AS development. To determine the differences in ADSCs and BMSCs to protect against ox-LDL-induced apoptosis, the treated cells were subjected to Annexin V-FITC/PI double staining and analysed by flow cytometry. As shown in Fig. 7A and B, ox-LDL (100 g/ml) markedly increased apoptosis through the results of flow cytometry analysis. Following

pre-incubation with ADSCs, BMSCs or BMSCs+cis-9, trans-11, the results of flow cytometry analysis improved. Meanwhile, the effects of inhibited macrophage apoptosis from co-cultures with ADSCs were better than those with BMSCs and BMSCs+cis-9, trans-11, and BMSCs+cis-9, trans-11 had a better effect than BMSCs. These observations indicated that cis-9, trans-11 plays an important role in the macrophage apoptotic pathway.

Effects of ADSCs, BMSCs or BMSCs+cis-9, trans-11 expression on anti-inflammatory Gene mRNA expression. The mRNA levels of the genes related to pro-inflammatory factors were determined by RT-qPCR after macrophage pre-incubation with 100 g/ml ox-LDL for 24 h and after co-culture with ADSCs, BMSCs or BMSCs+cis-9, trans-11. As shown in Fig. 8, the pro-inflammatory genes TNF-α, CD36 and NF-κBp65 were increased after pre-incubation with 100 g/ml ox-LDL for 24 h, but ADSCs, BMSCs or cis-9, trans-11 all reduced the expression of inflammatory gene significantly. Comparatively, the decrease of expression of pro-inflammatory genes was most after co-culture with ADSCs, and BMSCs+cis-9, trans-11 also significantly inhibited these gene expression than BMSCs alone in this respect.

Effects of ADSCs, BMSCs and BMSCs+cis-9, trans-11 on inflammatory protein expression. The protein levels of NF-κBp65 (Fig. 9) and phosphorylated NF-κBp65 (Fig. 9) following macrophage co-culture with ADSCs, BMSCs and BMSCs+cis-9, trans-11 were detected using Western blot analysis as shown in Fig. 8A. The inflammatory protein NF-κBp65 and phosphorylated NF-κBp65 were decreased consistently by 0.6- (Non), 0.5- (Ox-LDL), and 0.7- (Non), 0.6- (Ox-LDL) fold.

Discussion

Macrophages are the key factor in the initiation and progression of AS. Macrophages demonstrate versatility and plasticity accompanied by environmental signals. Foam cells are a hallmark feature of atherosclerotic plaques that follow exposure to ox-LDL (27,28). As inflammation develops, necrotic lipid-filled cores are formed after the death of macrophages and foam cells. Substances that are toxic to cells are released from these cells and are thought to contribute to plaque destabilization. A clinical event, such as a heart attack or stroke, could be caused by this destabilization. Accordingly, targeting that improves the anti-inflammatory ability of macrophages may provide a therapeutic strategy for treating AS (27).

The immunomodulatory properties of MSCs are one factor that could lead to new strategies, but the mechanisms of action for these cells are complicated and not fully understood. The major finding of the metabolomics part of this study was that supernatant levels of Cis-9, trans-11 (Cis-9, trans-11) in ADSCs were significantly higher compared to BMSCs. Additionally, cis-9, trans-11 (Cis-9, trans-11) is one isomer of CLA that is present in human adipose tissue. CLA has anti-atherogenic properties that have been confirmed in animal models (29-31). Cis-9, trans-11 has been reported as the key isomer involved in the obstruction of AS development (32). Many studies have demonstrated that CLA dienes could modulate the function of immune system cells through some inflammatory pathways,
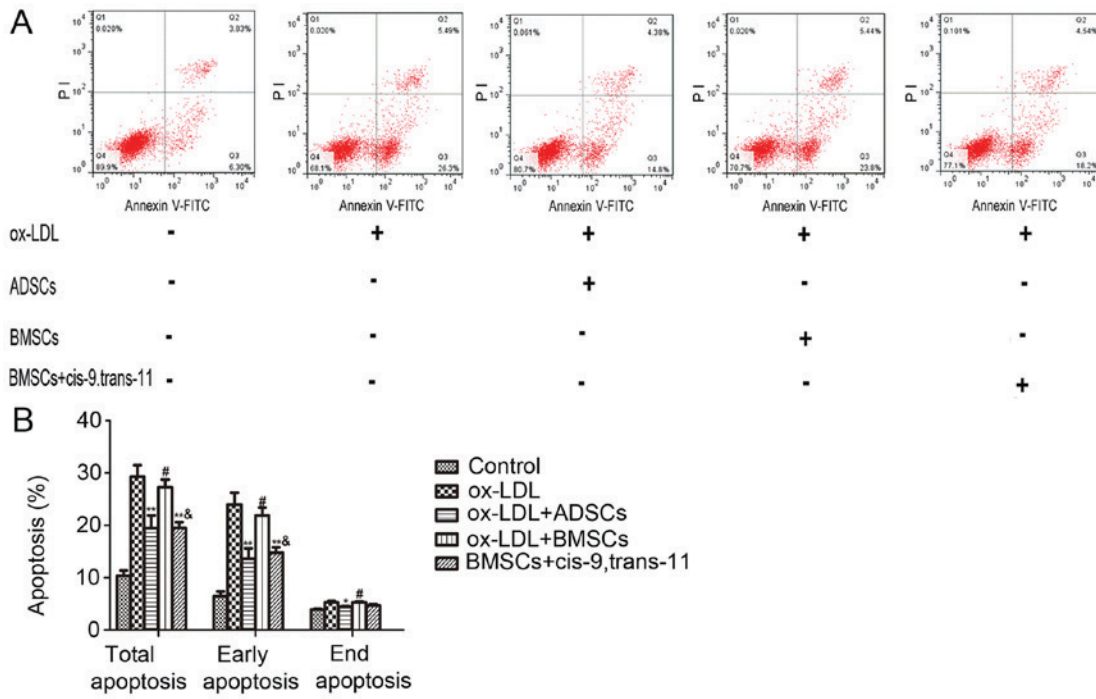
Figure 7. ADSCs, BMSCs and BMSCs+cis-9, trans-11 attenuated ox-LDL-induced apoptosis of M1 macrophages. Cells were pre-incubated with cystathionine-γ-lyase (ADSCs, BMSCs and BMSCs+cis-9, trans-11), followed by treatment with 100 g/ml ox-LDL for 24 h. (A) The representative data of apoptotic cells stained with Annexin V-FITC/PI which was detected by flow cytometry analysis. (B) The percentage of apoptotic cells under different treatments was measured and expressed as mean ± standard deviation of at least three independent experiments (n=10). *P<0.05 and **P<0.01 vs. the ox-LDL group; #P<0.05 vs. the ADSCs group; &P<0.05 vs. the BMSCs group. BMSCs, bone marrow-derived stem cells; ADSCs, adipose-derived stem cells; ox-LDL, oxidized low-density lipoprotein; FITC, fluorescein isothiocyanate; PI, propidium iodide.
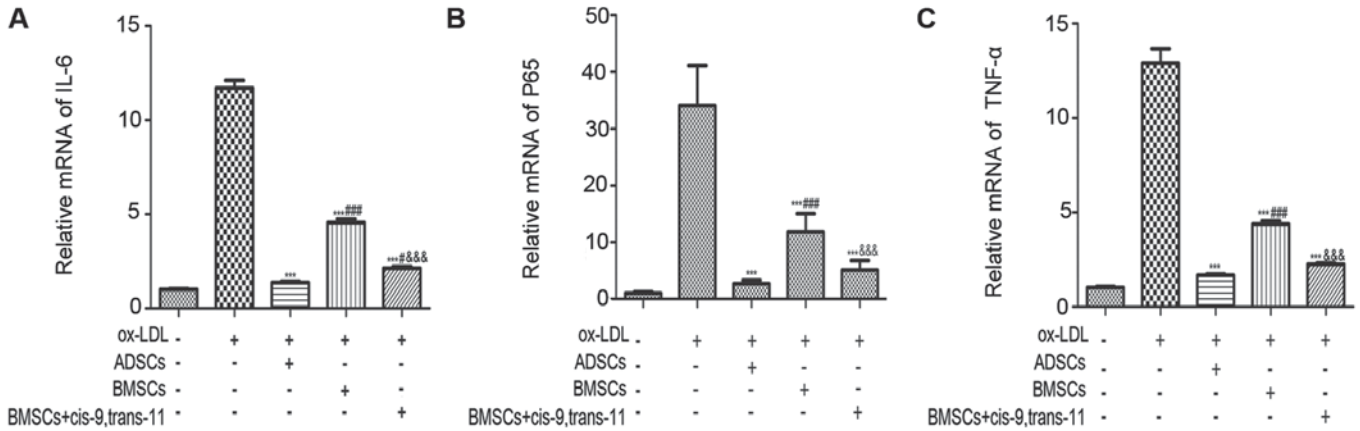

Figure 8. Effects of the inhibition of proinflammatory factors on oxLDL-induced NFκBp65, TNF-α and IL-6 upregulation. Reverse transcription-quantitative polymerase chain reaction analysis of (A) IL-6, (B) NFκBp65 and (C) TNF-α mRNA level in macrophages co-cultured with ADSCs, BMSCs or BMSCs+cis-9, trans-11. Data are expressed as the mean ± standard deviation (n=10). ***P<0.001 vs. the ox-LDL group; #P<0.05 and ###P<0.001 vs. the ADSCs group; &&&P<0.001 vs. the BMSCs group. NFκBp65, nuclear factor-κBp65; TNF-α, tumour necrosis factor-α; IL-, interleukin-; BMSCs, bone marrow-derived stem cells; ADSCs, adipose-derived stem cells; ox-LDL, oxidized low-density lipoprotein.

such as the PPARγ pathway, which is an important member of this nuclear receptor family (33-35).

A few recent reports have shown that MSCs may regulate the immunogenicity of macrophages (36-38). Indeed, it has been reported that the immunogenicity and phagocytic capacity of macrophages could be regulated through the release of cytokines by ADSCs (39). However, the effects of reducing the inflammatory reaction of macrophage by ADSCs was more obviously as compared to BMSCs, and whether they work through secretion of cis-9, trans-11 has yet to be

investigated. Unfortunately, the exact quantity of the secretion of cis-9, trans-11 by ADSCs and BMSCs via metabolomics has not been measured. Because whether slightly increased CLA is harmful to the human body has not been reported, 30 μM CLAs exogenous cis-9, trans-11 was added to the medium of BMSCs in our study (40).

The new findings of our present study suggest human MSCs, and particularly ADSCs, regulate immunogenicity of ox-LDL-stimulated M1 macrophages by secretion of cis-9, trans-11 (cis-9, trans-11). In this experiment, the culture
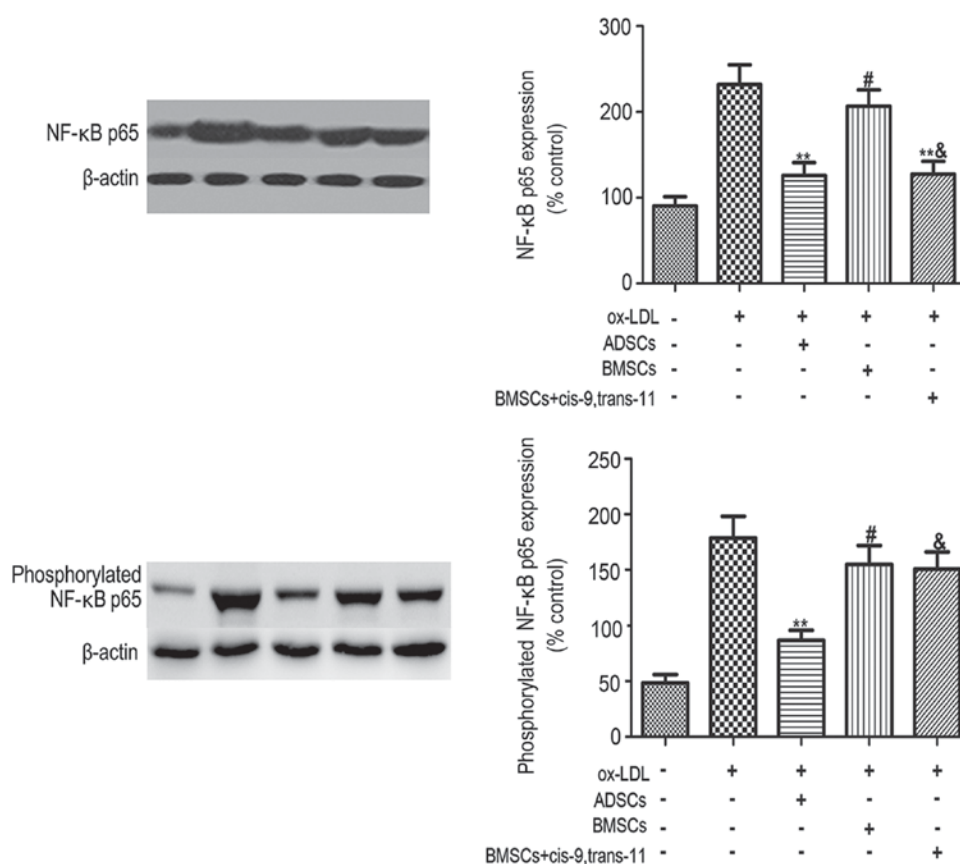
Figure 9. ADSCs, BMSCs and BMSCs+cis-9, trans-11 suppressed the expression of nuclear protein NF-κBp65 protein and phosphorylated NF-κBp65. Western blot analyses of NF-κBp65 protein and phosphorylated NF-κBp65 expression in ox-LDL-induced of macrophages co-cultured with ADSCs, BMSCs and BMSCs+ cis-9, trans-11. Representative blots from 5 different experiments are shown (n=5). **P<0.01 vs. the ox-LDL group; #P<0.05 vs. the ADSCs group; &P<0.05 vs. the BMSCs group. BMSCs, bone marrow-derived stem cells; ADSCs, adipose-derived stem cells; NFκBp65, nuclear factor-κBp65; ox-LDL, oxidized low-density lipoprotein.

of peripheral blood monocytes from the same donors in the presence of GM-CSF, IFN-γ and LPS triggered their differentiation and maturation led to the development of M1 macrophages, which was confirmed by FSC-SSC parameters and surface expression of CD14 by flow cytometry. However, co-culture of ox-LDL-stimulated M1 macrophages with ADSCs, BMSCs and BMSCs+cis-9, trans-11 caused significant changes. The findings demonstrated that ADSCs resulted in a greater increase in the secretion of anti-inflammatory cytokines, such as IL-10 and a greater decrease in the secretion of pro-inflammatory cytokines, such as TNF-, IL-6 and IL-8 of ox-LDL-stimulated M1 macrophages compared to BMSCs and BMSCs+bovinic acid. A similar effect was observed with mRNA expression of TNF-α, CD36 and NF-κBp65. Protein expression of NF-κBp65 and phosphorylated NF-κBp65 in ADSCs co-cultured with M1 macrophage foam cells were also significantly diminished. Additionally, we found that ADSCs increased macrophage foam cell phagocytosis and cell viability, as well as decreased macrophage foam cell apoptosis, compared to BMSCs. However, the anti-inflammatory effects, phagocytosis, cell viability and anti-apoptotic effects in BMSCs+cis-9, trans-11 co-cultured with M1 macrophage foam cells were more markedly enhanced compared to BMSCs, and the results were still weaker than ADSCs co-cultured with M1 macrophage foam cells. Overall, our data indicate that protection against AS of ADSCs acts on M1 macrophage

foam cells and that a major mechanism involves activation of an NF-κBp65-TNF-α pathway. These findings could provide further insight into a potential mechanism for ADSCs in AS.

MSCs have emerged as a promising tool for the treatment of AS due to their anti-inflammatory properties. As previously suggested for BMSCs, cyclo-oxygenase-2 (COX-2) activation seems to play an important role in anti-inflammatory properties (38). Although some previous studies have shown that ADSCs have the capacity to inhibit TNF-α-dependent inflammation and induce angiogenesis as well as enhance overall tissue repair (41), there are few studies that explain the underlying mechanism of inhibiting TNF-α by ADSCs. The results of our present study indicated decreases in TNF-α, IL-6 and IL-8 and an increase in IL-10 production following treatment with ADSCs, BMSCs and BMSCs+ cis-9, trans-11 in M1 macrophages, which coincides with previous studies exhibiting potent anti-inflammatory properties of cis-9, trans-11 in RAW macrophages (42,43). The decrease in TNF-α and IL-6 could impede the expression of NF-κBp65 and phosphorylated NF-κBp65, which are involved in systemic inflammation in the atherosclerotic process (44). Both RT-qPCR and Western blot analysis in this study have indicated that expression of NF-κB p65 and phosphorylated NF-κBp65 in nuclei of ADSCs had the strongest inhibitory effect on M1 macrophages. This outcome suggests the secretion of cis-9, trans-11 by ADSCs plays a major role in an anti-inflammatory capability that was better

than the capability of BMSCs. However, anti-inflammatory capability, phagocytic activity, anti-apoptotic capability and the cell viability assay in M1 macrophage foam cells of BMSCs+ cis-9, trans-11 was still weaker than ADSCs, which suggests that the mechanisms of cis-9, trans-11-TNF-α-dependent inflammation do not have a role in the anti-inflammatory processes of M1 macrophage foam cells. As shown in previous studies for ADSCs, soluble LIGHT (a lymphotoxin-related inducible ligand that competes for glycoprotein D binding to herpes virus entry mediator on T cells) also has a role in the immune reaction (45). Zhou et al (46) reported that the activated microglia/macrophage anti-inflammatory responses were more robust in Sprague-Dawley rats that underwent a transplant with ADSCs compared to Sprague-Dawley rats that underwent a transplant with BMSCs from the decreased numbers of ED1-positive macrophages (46).

At the same time, our experiments need to be improved. We considered that cis-9, trans-11 was the most abundant CLA isomer (over 75-80% of total CLA) suppressing inflammatory response, apoptosis and functioning at cholesterol homeostasis in macrophages, when we designed this experiment. Therefore, we have not examined the role of cis-9, trans-11 in ox-LDL-stimulated M1 macrophage without BMSCs. In the next experiment, we will design the experiment more reasonably.

In summary, our present study unmasked cis-9, trans-11 from ADSCs as an important mechanism to regulate macrophage immune function. The application of ADSC therapy can be further developed by studies of their specific mechanisms. Therefore, based on these results, ADSCs may be more suitable than BMSCs for stem cell therapy in AS, especially for older patients. The specific molecular mechanisms will be investigated more thoroughly in our future work.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

J-ZL, B-DX, HQ, J-CH, X-PL, KK and S-LJ were involved in the study design. J-ZL, T-HC, HQ, X-PL, X-LY, FZ, X-LL, HW and J-CH performed the experiments, data analysis and manuscript writing. J-ZL, KK, S-LJ and S-QJ were involved in the study design and revised the manuscript. All authors read and approved the manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethical Committee of the Second Affiliated Hospital of Harbin Medical University (approval no. KY-2017-076). All included patients were informed about the nature of the study and gave their written informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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