Research article

Adipose-derived stem cells regulate CD4⁺ T-cell-mediated macrophage polarization and fibrosis in fat grafting in a mouse model

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ABSTRACT

Autologous fat grafting is becoming increasingly common worldwide. However, the long-term retention of fat grafting is still unpredictable due to the inevitable fibrosis arising during tissue repair. Fibrosis may be regulated by T-cell immune responses that are influenced by adipose-derived stem cells (ASCs). Therefore, we hypothesized that overly abundant ASCs might promote fibrosis by promoting T-cell immune responses to adipose tissue. We performed 0.3 ml fat grafts with 10⁴/ml, 10⁶/ml and 10⁸/ml ASCs and control group in C57 BL/6 mice in vivo. We observed retention, fibrosis, T-cell immunity, and macrophage infiltration over 12 weeks. Besides, CD4⁺ T-helper 1 (Th1) cells and T-helper 2 (Th2) cells were co-cultured with ASCs or ASCs conditioned media (ASCs-CM) in vitro. We detected the ratio of Th2%/Th1%. Results showed that the retention rate was higher in 10⁴ group, while even lower in 10⁸ group with significantly increased inflammation and fibrosis than control group at week 12 in vivo. There was no significance between control group and 10⁶ group. Also, 10⁸ group increased the infiltration of M2 macrophages, CD4⁺ T-cells and Th2/Th1 ratio. In vitro, the ratio of Th2%/Th1% induced by ASCs-transwell group was higher than ASCs-CM group and showed concentration-dependent. Accordingly, high concentrations of ASCs in adipose tissue can promote Th1–Th2 shifting, and excessive Th2 cells might promote the persistence of M2 macrophages and increase the level of fibrosis which lead to a decrease in the long-term retention of fat grafts. Also, we found ASCs promoted Th1–Th2 shifting in vitro.

1. Introduction

Autologous fat grafting is becoming increasingly common around the world. Many studies have sought to improve the retention of fat grafts (Xing et al., 2016; Yoshimura et al., 2008). However, long-term retention remains unpredictable due to the inevitable tissue fibrosis that occurs following grafting (Cai et al., 2017).

Fibrosis arising during tissue repair may be regulated by the immune response (Gieseck et al., 2018), especially related with the infiltration of M2 macrophages (Wynn and Barron, 2010). In fact, the fibrosis and M2 macrophages level could be regulated by T-cells response (Martinez et al., 2009). The various types of CD4⁺ T-cells may play distinct roles in regulating tissue fibrosis. Th1 cells directly suppress fibroblast collagen synthesis by releasing interferon-γ (IFN-γ), whereas Th2 cells promote collagen deposition by releasing interleukin-4 (IL-4) (Wynn, 2004). Also,
tissue repair, this could increase M2 macrophages level and indeed the case that excessive ASCs induce high levels of Th2 cells during angiogenesis and adipogenesis by ASCs. However, there was no indication with pentobarbital sodium at 0.1 mg/100 g and shaved. The inguinal skin was incised, and the subcutaneous inguinal fat pad (~150 mg) was harvested and gently dissected into small pieces, similar to the aspirated fat used in clinic.

Three groups of ASCs-enriched fat grafts were generated: 1 × 10^{8}, 1 × 10^{9}, or 1 × 10^{10} ASCs added per 1 ml fat. Prior to transplantation, 0.3 ml of prepared C57 BL/6 fat was mixed with ASCs suspended in 100 μl phosphate-buffered saline (PBS; Gibco, Grand Island, NY, USA) (ASCs group). The control group received 0.3 ml fat supplemented with 100 μl PBS. The mixtures were injected into subcutaneous tissues of C57 BL/6 mice using a 1 ml syringe. At week 1, 4, 8, or 12 after grafting, the grafts were harvested and carefully separated from surrounding tissue, and their volumes were measured using the liquid overflow method as follows: grafts were added to phosphate-buffered saline in a graduated cylinder, and the difference in liquid volume before and after the addition of the grafts was calculated. This measurement was repeated 3 times and averaged. The retention rate of grafts is defined as follows: retention rate (%) = graft volume at harvest time/the injection fat volume (0.3 ml) × 100%.

2.4. Histological analysis

Full-thickness biopsies of the grafts were obtained at 1, 4, 8, and 12 weeks after grafting. Samples were fixed in 4% paraformaldehyde for 24 h, dehydrated, embedded in paraffin, and stained with hematoxylin-eosin and Masson’s trichrome. Staining was performed according to a standard protocol (Culling et al., 1985; Suvarna et al., 2013). Sections were sectioned and examined under an Olympus BX51 microscope. Images were acquired using an Olympus DP71 digital camera. Quantification of fibrosis area in Masson staining of all groups over time was calculated by the software Image J.

2.5. Western blot analysis

Samples at week 12 were prepared using M-PER Mammalian Protein Extraction Reagent (Thermo Fisher Scientific). Protein concentrations was estimated using the BCA protein assay (Thermo Fisher Scientific). Protein extracts were subjected SDS-PAGE using the NuPAGE electrophoresis system and then transferred to immobil polivinilidene difluorado membranes (Millipore, Billerica, MA, USA). Membranes were blocked in 5% milk and immunoblotted with anti-α-SMA antibody (1:500; Abcam, Cambridge, MA, USA). After incubation with secondary antibody, signals were detected using the WesternBreeze Chemiluminescent Detection Kit (Thermo Fisher Scientific). β-Actin served as an internal control.

2.6. Immunohistochemistry and immunofluorescence

Full-thickness biopsies of the grafts were obtained at 1, 4, 8, and 12 weeks after grafting. Tissue sections of 4 μm thickness were incubated with primary antibody at 4 °C overnight: rat anti-mouse CD4 (1:200; Abcam, Cambridge, MA, USA). Tissue sections were washed by PBS three times and then incubated with secondary antibody at 37 °C for an hour: biotin-labeled goat anti-rat IgG (1:200; Invitrogen). Signals were
observed using an avidin–biotin–horseradish peroxidase detection system. Slides were examined on an Olympus BX51 microscope. The number of CD4+ T-cell (indicated by red arrows in Figure 4A) in immunohistochemistry staining of all groups over time was counted and calculated in five randomly selected fields (400× magnification) per section by each of three professional researchers.

Immunofluorescence staining was performed with the following primary antibodies at 4 °C overnight: rat anti-mouse Mac2 (1:200; CL8942AP, Cedarlane Corp., Burlington, Ontario, Canada) and rabbit anti-mouse CD206 (1:300; ab64693, Abcam, Cambridge, UK). Tissue sections were washed by PBS three times and then incubated with secondary antibodies at 37 °C for an hour: rhodamine-conjugated goat anti-rat IgG (1:200; Invitrogen, North Ryde, NSW, Australia) and Alexa Fluor 488-conjugated chicken anti-rabbit IgG (1:200; Invitrogen). Nuclei were stained with DAPI (1:200; Sigma). Images were acquired and analyzed on a C1Si confocal laser scanning microscope (Nikon, Tokyo, Japan). We counted M1 macrophages as MAC2+/CD206− cells, and M2 macrophages as MAC2+/CD206+ cells.

2.7. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Fat tissue was excised, snap-frozen in liquid nitrogen and stored at −80 °C. Total RNA was extracted from 50 mg of tissue using the RNeasy Lipid Tissue Mini Kit (Qiagen, Hilden, Germany). cDNA was amplified for 40 cycles using the QuantTect Reverse Transcription Kit (Qiagen) and the Rotor-Gene 3000 Real-Time PCR Detection System (Corbett Research, Sydney, Australia). Expression levels were calculated by the 2−ΔΔCt method. The following primers were used: IL-6 (Invitrogen), forward 5'-GGTCCCTTCTGAGGACTAT-3', reverse 5'-TCTCCTGTGACCTCGTTCAA-3'; TNF-α (Invitrogen), forward 5'-GGCACAGAGGAGGGTGATCT-3'; IL-10 (Invitrogen), forward 5'-AACA-TACTCCTAACCACACT-3', reverse 5'-CAGTCGCTCTGCTTAT-3'; TGF-β (Invitrogen), forward 5'-GGAGGCTGCCCTTATATTG-3', reverse 5'-AGAGGCGCCAATCATGTG-3'; IFN-γ (Invitrogen), forward 5'-GCCGATGGGGAGGAGTCT-3', reverse 5'-TACCTCGGTTGTTAGTCA-CAGT-3'; IL-4 (Invitrogen), forward 5'-TCCTGTCATCAGGGCAGACAGA-3', reverse 5'-CTCTGGTGAGCAGCTTCA-3'; GAPDH (Invitrogen), forward 5'-AACCCTGATGAGGAGATTG-3', reverse 5'-CCCTGGTCTGATGCCG-TAT-3.

2.8. Collection of conditioned media

After the ASCs of passage 3–5 were 80% confluent in a culture dish with a diameter of 10 cm, we detected that the number of ASCs reached 1.6 × 10⁶. Then, the medium was replaced with 8 ml serum-free RPMI 1640 to obtain 2 × 10⁶/ml ASCs conditioned medium. After a 24 h culture, the medium was centrifuged at 1000 rpm for 5 min, and the supernatant was collected and passed through a syringe filter unit (0.22 μm) to yield 2 × 10⁵/ml ASCs-CM. Then, we diluted the above ASCs-CM 10 times and 100 times using serum-free RPMI 1640 to obtain 2 × 10⁴/ml and 2 × 10⁵/ml ASCs-CM. High concentrations of ASCs could produce more proteins/growth factors through paracrine action. In order to simulate the secretion level of ASCs at different concentrations (10⁴/ml, 10⁵/ml, 10⁶/ml in vitro), we set up ASCs-CM groups at different concentrations in vitro. Since the number of ASCs fusion in a cell culture dish is at most 1–2 × 10⁶, we set the ASCs-CM concentration gradients of 2 × 10⁴/ml and 2 × 10⁵/ml and 2 × 10⁶/ml. So, the 2 × 10⁴/ml ASCs-CM would have 10 times proteins/growth factors more than the 2 × 10⁵/ml ASCs-CM and 100 times more than 2 × 10⁶/ml ASCs-CM.

2.9. Co-culture in vitro

CD4+ T-cells from the spleens of C57 BL/6 mice were purified by negative selection using the CD4+ T-cell isolation kit MicroBeads (Miltenyi Biotec, Bergisch-Gladbach, Germany). Purified CD4+ T-cells were cultured in complete medium containing RPMI 1640 (Gibco) supplemented with 10% FBS (Gibco), 2 mM l-glutamine (Invitrogen), 100 U/ml penicillin (Gibco), and 100 μg/ml streptomycin (Gibco). In a 24-well plate, 2 × 10⁴ CD4+ T-cells were cultured for 24 h in 0.5 ml RPMI 1640 under Th1 or Th2 differentiation conditions. Th1 cells were differentiated with 10 ng/ml IL-12 (Peprotech, Rocky Hill, NJ, USA), 10 μg/ml anti-IL-4 antibody (eBiosciences, San Jose, CA, USA), 5 μg/ml anti-CD3 antibody (BD Biosciences, San Jose, CA, USA), 2 μg/ml anti-CD28 antibody (eBiosciences), 10 ng/ml IL-2 (Peprotech). Th2 cells were differentiated with 20 ng/ml IL-4 (Peprotech), 20 μg/ml anti-IFN-γ (eBioscience), 5 μg/ml anti-CD3 antibody (BD Biosciences), 2 μg/ml anti-CD28 antibody, 10 ng/ml IL-2 (Peprotech).

For the ASCs-transwell group, ASCs were preplanted at a ASCs:Th ratio of 1:1 (2 × 10⁴ ASCs), 1:10 (2 × 10⁴ ASCs) or 1:100 (2 × 10⁵ ASCs) in a 24-well plate, and Th1 or Th2 cells that had been activated for one day were transferred to the upper chamber of the ASCs-transwell. After the cells were in place, Th1 or Th2 differentiation medium was added to replenish total liquid volume to 1 ml in every well. For the ASCs-CM group, ASCs-CM at three different concentrations of 2 × 10⁶/ml (concentration 1:100), 2 × 10⁵/ml (concentration 1:10) or 2 × 10⁴/ml (concentration 1:1) were added with reagents according to Th1 or Th2 differentiation conditions. Then, the corresponding ASCs-CM was added into every well in which Th1 or Th2 cells were cultured to replenish total liquid volume to 1 ml.

2.10. Flow cytometry

After another 24 or 48 h of culture, T-cells were stimulated for 4 h with 20 ng/ml phorbol myristate acetate (PMA; Sigma-Aldrich) and 1 μg/ml ionomycin (Sigma-Aldrich) prior to addition of 10 μg/ml brefeldin A (BFA; eBiosciences). For the detection of surface markers, cells were stained with CD4-FITC (eBiosciences) and incubated for 15 min at 4 °C in the dark. After washing, intracellular staining for IFN-γ-PE (eBiosciences) and IL-4-PE (eBiosciences) was performed separately. For that purpose, cells were fixed and permeabilized using fixation buffer and permeabilization buffer (BD Biosciences). Acquisition was performed on a Coulter Epics-XL flow cytometer using System II software (Coulter Corporation, Brea, CA, USA). Analysis was performed using the FCS express software (De Novo Software, Los Angeles, CA, USA).

2.11. Statistical analysis

All data were analyzed using the IBM SPSS version 20.0 software (IBM Corp., Armonk, NY, USA). Data were expressed as mean ± SD. Two-way analysis of variance was used to compare groups at multiple time points. The independent Student’s t-test was used to compare two groups at a single time point. A two-tailed P-value less than 0.05 was considered statistically significant.

3. Results

3.1. High concentrations of ASCs decrease graft retention

At week 12, the grafts in the 10⁶ group were harder to the touch than those in the other groups; the textures of grafts in the 10⁴ and 10⁵ groups were similar to those in the control group. However, the shape and color of the grafts did not differ markedly among the four groups (Figure 1A). The graft retention rate was the lowest in the 10⁸ group at week 1 and decreased thereafter; consequently, the long-term retention rate (at week 12) was also the lowest in the 10⁶ group. The graft retention rate in the 10⁶ group never differed from that of the control group. The graft retention rate in the 10⁸ group was the highest among all groups at week 1 and decreased the most slowly until week 12. Consequently, the long-term retention rate was the highest in the 10⁴ group (Figure 1B).
3.2. High concentrations of ASCs increase graft fibrosis

Histological analyses at week 12 showed that the structure of adipose tissue in the $10^8$ group was abnormal, with a high level of fibrosis, whereas the $10^6$ group was similar to the control group, and the $10^4$ group had even better structure with more integrated fat structure, less inflammatory cell infiltration, less oil cyst formation and less fibrosis (Figure 2A).

Masson analyses at week 12 showed that the $10^8$ group exhibited a great deal of collagen deposition, which was less extensive in the other groups (Figure 2B). Quantification of collagen fibrosis area yielded similar results: fibrosis area was highest in the $10^8$ group, and lower in the $10^4$ group than in the control group at weeks 8 and 12 (Figure 2C).

Expression of $\alpha$-SMA at week 12 was highest in the $10^8$ group and did not differ significantly among the $10^6, 10^4$, and control groups (Figure 2D & E).

3.3. High concentrations of ASCs increase the level of M2 macrophage infiltration

Immunofluorescence revealed that in the control group, MAC2$^+$ macrophages had infiltrated adipose tissue at week 1, with additional MAC2$^+$/CD206$^+$ macrophages appearing at week 4. By contrast, in the $10^8$ group, obviously higher levels of MAC2$^+$ and M2 macrophages were observed from weeks 1 to 8, and could even be observed at week 12. The number of MAC2$^+$ and M2 macrophages in the $10^6$ group were more than that in the control group from weeks 1 to 8 and did not differ from that of the control group at week 12. By contrast, the number of MAC2$^+$ and M2 macrophages in the $10^4$ group increased and exceeded the control group at weeks 1 and 4, but decreased rapidly and became lower than in the control group at weeks 8 and 12 (Figure 3A & B). The M2/M1 ratio was higher in the $10^8$ group than in the control group from weeks 4 to 12. The ratio in the $10^6$ group was higher than in the control group only at week 8 and did not differ from that of the

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**Figure 1.** Appearance and changes of retention rate in grafts of ASCs-enriched groups and control group. (A) Appearance at week 12 after fat grafting in all groups (B) Retention rate in all groups at weeks 1, 4, 8, and 12. Data are expressed as the means ± standard deviation. *P < 0.05, $10^4$ vs. control; ^P < 0.05, $10^4$ vs. control ($n = 7$).

**Figure 2.** Comparison of fat grafts structure, fibrosis areas and fibrosis-related protein expression among ASCs-enriched groups and control group. (A) Hematoxylin/eosin staining of grafts in all groups at week 12 after fat grafting. Scale bar = 100 μm (B) Masson’s trichrome staining in grafts of all groups at week 12. Scale bar = 100 μm (C) Quantification of fibrosis area in Masson staining of all groups at weeks 1, 4, 8, and 12 after fat grafting. (D) Western blot analysis of $\alpha$-SMA at week 12 in all groups. The full versions of the western blot band were shown in Figure S2. (E) Quantification of band intensities in (D). Data were normalized to GAPDH expression and are presented as the mean relative quantity (compared with control). Data are expressed as the means ± standard deviation. *P < 0.05, $10^6$ vs. control; ^P < 0.05, $10^4$ vs. control ($n = 7$). HE, hematoxylin-eosin; $\alpha$-SMA, alpha-smooth muscle actin.
control group at week 12. The ratio was higher in the $10^4$ group than in the control group from weeks 1 to 4, but became lower than in the control group at week 8 (Figure 3C).

The relative expression of four inflammatory factors decreased after week 1 in all groups. Relative expression of IL-6 and TNF-α was higher in the $10^4$ group than in other groups from weeks 1 to 12, and lower in the $10^8$ group than in the other groups from weeks 1 to 4 (Figure 4A & B). In addition, the relative expression of IL-10 and TGF-β were highest in the $10^8$ group from weeks 1 to 12, but lowest in the $10^8$ group only at week 1 (Figure 4C & D).

**Figure 3.** Macrophage immunofluorescence staining and changes of M2/M1 macrophage ratio in grafts of ASCs-enriched groups and the control group over time. (A) High concentrations of ASCs increase the level of M2 macrophage infiltration. Macrophage infiltration into grafts of all groups at weeks 1, 4, 8, and 12. MAC2+ (red) indicates macrophages. We define M1 macrophages as MAC2+ CD206- cells (white arrows), and M2 macrophages as MAC2+ CD206+ cells (yellow arrows). The white square selection areas of immunofluorescence images at week 4 are magnified in the last row. Scale bar = 100 μm (B) Quantitative analysis of the number of total MAC2+ macrophages between groups over time. (C) Quantitative analysis of the ratio of M2 macrophage number to M1 macrophage number over time. Data are expressed as the means ± standard deviation. *P < 0.05, **P < 0.01, ***P < 0.001, $10^8$ vs. control; #P < 0.05, ##P < 0.01, $10^6$ vs. control; $\Delta$P < 0.05, $\Delta\Delta$P < 0.01, $10^4$ vs. control (n = 7). DAPI, 4’,6-diamidino-2-phenylindole; MAC2, macrophage surface antigen 2; CD206, cluster difference 206; M1, M1 macrophage; M2, M2 macrophage.
3.4. High concentrations of ASCs increase the number of CD4+ T-cells and the Th2/Th1 ratio in vivo

Immunohistochemistry at week 4 revealed that CD4+ area (red arrows) was greatest in the 108 group (Figure 5A); the count statistics of CD4+ T-cells confirmed this finding (Figure 5E). Relative expression of IFN-γ and IL-4 was higher in the 108, 106, and 104 groups than in the control group at week 1, but decreased thereafter (Figure 5B & C). However, the Th2/Th1 ratio followed a different pattern; in the 108 group, the ratio was higher than in the control group at weeks 4 and 12, whereas in the 104 group, the ratio was higher than in the control at week 1, but lower at weeks 4 and 12 (Figure 5D).

3.5. The Th2%/Th1% ratio is increased via the paracrine function of ASCs in vitro

Next, we measured the percentage of Th1 and Th2 cells (Th1% and Th2%) by flow cytometry over 48 h in vitro (Figure 6A–P). We standardized the Th2%/Th1% ratio of control group after 1 day activation as 1 and calculated the Th2%/Th1% ratio in each group at 24 and 48 h based on this standard (Figure 6Q). Quantification of the Th2%/Th1% ratio at 24 h showed that the ASCs-transwell group with concentration 1:100 was higher than the control group, but there was no statistical difference between the ASCs-CM group with concentration 1:100 and the control group. And, both ASCs-transwell and ASCs-CM groups with concentration 1:10 and 1:1 respectively were higher than the control group at 24 h. While the ratio in the ASCs-transwell group was higher than in the ASCs-CM group with each concentration at 24 h. However, the ratio in both ASCs-transwell and ASCs-CM groups with each concentration at 48 h were higher than the control group. There was no difference between the ASCs-transwell and ASCs-CM groups with any concentration at 48 h.

4. Discussion

In this study, we showed that high concentrations of ASCs in fat grafts promote fibrosis and decrease long-term retention. We also observed high levels of Th2 cells in the early stage and long-term persistence of M2 macrophages after fat grafting with excessive ASCs (Figure 7). In addition, we found that ASCs can promote Th1–Th2 shifting in vitro. The previous results showed that the best survival rate may require a seamless schedule for inflammatory cell behavior to match the fat graft survival pattern. This may require an appropriately elevated level of inflammatory cell (macrophage) infiltration at the early stage to initiate precursor cell proliferation and angiogenesis, followed by quick remission of inflammation at the late stage to allow hematopoietic stem cell differentiation and prevent fibrosis (Cai et al., 2017, 2018). However, the prolonged macrophage inflammation caused severe fibrosis, leading to impaired adipogenesis and poor fat graft survival (Cai et al., 2017, 2018). Based on the above, we proposed the potential mechanism by which excessive adipose-derived stem cells promote Th1–Th2 shifting and then the transformation of M1 to M2 macrophage to up-regulate fibrosis (Figure 8).

ASCs, which can be obtained from adipose tissue, have been experimentally shown to have angiogenic and adipogenic characteristics (Han et al., 2015). In light of these functions, and because graft retention is mainly due to tissue regeneration, many studies have focused on the long-term retention of ASCs-assisted lipotransfer (Hao et al., 2021; Mou...
et al., 2019; Tuin et al., 2016). Based on the previous report, the magnitude of 10^4 ASCs was considered to be the optimal cell concentration for improving fat graft retention (Zielins et al., 2017). However, the retention rate decreased gradually with the increase of cell concentration and was even lower than that of the control group when the concentration reached the magnitude of 10^7 cells (Paik et al., 2015; Kakudo et al., 2013). In order to explore the reason, we set three concentration gradients, in which the magnitude difference was 100 times, namely 10^4 ASCs/ml fat, 10^6 ASCs/ml fat and 10^8 ASCs/ml fat. Compared to previous studies, we elevate concentrations of ASCs to 10^8 cells/ml in the study. Our result showed that addition of a suitable concentration of ASCs (10^4 cells/ml) into adipose tissue significantly improved long-term retention, whereas excessive ASCs (≥10^7 cells/ml) not only did not improve long-term retention but also significantly decreased long-term retention by increasing inflammation and fibrosis in the 10^8 group than other groups. Moreover, the macrophages infiltration level of inflammatory cells in adipose tissue was significantly increased in the 10^8 group. This suggested that excessive ASCs in adipose tissue might lead to an increased macrophage inflammatory response that exacerbated fibrosis, thereby reducing the retention of fat grafts.

Macrophages play significant roles in tissue inflammation (Eming et al., 2017; Fujiwara and Kobayashi, 2005; Hamidzadeh et al., 2017; Li et al., 2018; Minutti et al., 2017). M1 (classically activated) macrophages mediate inflammatory responses, which are associated with high levels of pro-inflammatory cytokines (Juhas et al., 2015; Martinez et al., 2008). In fact, large numbers of macrophages infiltrated into the tissue after fat grafting. At the early stage of infiltration, mainly M1 macrophages occurred to clear necrotic tissues and cells (Kato et al., 2014). Then, macrophages gradually transform from M1 to M2 (alternatively activated), which is a necessary process after fat grafting (Mashiko and Yoshimura, 2015; Wang et al., 2020). And M2 macrophages could secrete anti-inflammatory factor and pro-angiogenic factor such as TGF-β and vascular endothelial growth factor (VEGF) to downregulate the level of inflammation and promote vascular growth into grafts which could recruit hematogenous stem cells to participate in the process of adipogenesis (Cai et al., 2018; Owen and Mohamadzadeh, 2013). Study

![Figure 5. CD4+ T-cell infiltration extent and the infiltrated level changes of Th1 and Th2 cells in the grafts of ASCs-enriched groups and the control group over time.](image-url)
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Th1

A

B

8.5%

13.6%

IFN-γ PE

IFN-γ PE

CD4 FITC

CD4 FITC

C

D

12.5%

12.1%

IFN-γ PE

IFN-γ PE

CD4 FITC

CD4 FITC

E

F

11.5%

11.0%

IFN-γ PE

IFN-γ PE

CD4 FITC

CD4 FITC

G

H

10.5%

9.9%

IFN-γ PE

IFN-γ PE

CD4 FITC

CD4 FITC

Th2

I

J

2.6%

6.5%

IL-4 PE

IL-4 PE

CD4 FITC

CD4 FITC

K

L

7.2%

7.5%

IL-4 PE

IL-4 PE

CD4 FITC

CD4 FITC

M

N

7.9%

8.2%

IL-4 PE

IL-4 PE

CD4 FITC

CD4 FITC

O

P

8.7%

9.0%

IL-4 PE

IL-4 PE

CD4 FITC

CD4 FITC

Q

control

ASCs-CM:T=1:100

ASCs-transwell:T=1:100

ASCs-CM:T=1:10

ASCs-transwell:T=1:10

ASCs-CM:T=1:1

ASCs-transwell:T=1:1

Th2% / Th1%

2.5

2.0

1.5

1.0

0.5

0

24 h

48 h

(caption on next page)
showed that when M2 macrophages were added into grafts appropriately, fat grafting could be promoted (Chappell et al., 2015). However, excess M2 macrophages can increase fibrosis inversely (Mashiko and Yoshimura, 2015; Mineda et al., 2014). M2 macrophages promote fibroblast proliferation and the expression of α-SMA, and α-SMA myofibroblast accumulation has been recognized as an early marker of tissue fibrosis (Yao et al., 2019). Indeed, M2 macrophages may be able to convert into fibroblasts (Wang et al., 2017). As have been reported, ASCs could promote fat grafts retention by upregulating polarization of M2 macrophages (Hao et al., 2021; Zhu et al., 2020). Our study observed that the total macrophages and M2/M1 ratio in the $10^4$ group obviously increased at early stage but decreased after weeks 4. The appropriate concentration of ASCs might exert suitable chemotaxis (Hong et al., 2013; Huayllani et al., 2020) and promoting M2-type transformation effect on macrophages so that moderately increased macrophages could arrive in the early stage and convert to M2 macrophages more rapidly. Then, M2 macrophages retreated earlier after they had worked, accounting for higher grafts retention and less fibrosis. However, prolonged infiltration by MAC2+/M2 macrophages and a high expression of α-SMA was observed in the $10^5$ group until weeks 12. The excessive concentration of ASCs might recruit a large number of macrophages in the early stage due to stronger chemotaxis (Hong et al., 2013; Huayllani et al., 2020), and the stronger paracrine and promoting phenotypic transformation effects of high concentrations of ASCs could induce more M2 macrophages. Then, the M2 macrophages sustained high levels of infiltration and declined slowly in the $10^5$ group. The long-term presence of M2 macrophages may have promoted inflammation and fibrosis (Mineda et al., 2014), perhaps explaining the higher level of tissue fibrosis and lower grafts retention in the $10^5$ group.

Interestingly, we found that the evolution of the Th2/Th1 ratio from weeks 1–12 was similar to that of the M2/M1 ratio from weeks 4–12 in all groups. This suggests that the Th1–Th2 shifting might promote the persistence of M2 macrophages after fat grafting. In fact, full macrophage activation requires two major signals in the context of the immune response, including the Th1 and Th2 responses (Romagnani, 2006). In naive T-helper cells, the IL-4 and IFN-γ genes are silent but can be activated to stimulate T-cells to begin to choose between the Th1 and Th2 cell fates (McKenzie et al., 1993; Mosmann and Coffman, 1989). IFN-γ and IL-4 are produced by mutually inhibitory CD4+ T-helper cells: Th1 and Th2, respectively (Mosmann and Coffman, 1989). In the Th1 response, innate IFN-γ induces the first wave of classical activation in M1 macrophages, stimulating IL-12 secretion, an important signal for Th1

Figure 6. The Th2%/Th1% ratio is increased via the paracrine function of ASCs in vitro. (A) Percentage of CD4+ Th1 cells activated after 1 day (B) Percentage of CD4+ Th1 cells cultured for 48 h after 1 day activation. Percentage of CD4+ Th1 cells cultured for 48 h after 1 day activation when ASCs-CM:T = 1:100 (C), ASCs-transwell:T = 1:100 (D), ASCs-CM:T = 1:10 (E), ASCs-transwell:T = 1:10 (F), ASCs-CM:T = 1:1 (G) and ASCs-transwell:T = 1:1 (H). (I) Percentage of CD4+ Th2 cells activated after 1 day (J) Percentage of CD4+ Th2 cells cultured for 48 h after 1 day activation. Percentage of CD4+ Th2 cells cultured for 48 h after 1 day activation when ASCs-CM:T = 1:100 (K), ASCs-transwell:T = 1:100 (L), ASCs-CM:T = 1:10 (M), ASCs-transwell:T = 1:10 (N), ASCs-CM:T = 1:1 (O), ASCs-transwell:T = 1:1 (P) (Q) Ratio of percentage of CD4+ Th2 cells to percentage of CD4+ Th1 cells in all groups including 24 and 48 h after 1 day activation. Data were normalized according to the Th2%/Th1% ratio of control group after 1 day activation and are presented as the mean relative ratio. Data are expressed as the means ± standard deviation. *P < 0.05, ASCs-CM or ASCs-transwell:T = 1:100 vs. control; #P < 0.05, ASCs-CM or ASCs-transwell:T = 1:10 vs. control; §P < 0.05, ASCs-CM or ASCs-transwell:T = 1:1 vs. control (n = 7). Th1, T helper 1; Th2, T helper 2; IFN-γ, interferon-γ; IL-4, interleukin-4; CD4, cluster difference 4; PE, phycoerythrin; FITC, fluorescein isothiocyanate; ASCs, adipose-derived stem cells; CM, conditioned media; h, hour.
activation. Upon Th1 activation, greater levels of IFN-γ induce long-lasting M1 macrophages; meanwhile, a full cytotoxic T-cell response is mounted. By contrast, in the Th2 response, IL-4 produced by Th2 cells induce a wave of alternative activation in M2 macrophages, which also provide signals that promote Th2 development (Martinez et al., 2009; Muraille et al., 2014). In addition, IL-10 secretion by M2 macrophages may also induce the development of repressor T-cells, which oppose Th1 activation (Rauh et al., 2005). A study published in SCIENCE pointed out a pro-regenerative response characterized by an mTOR/Rictor-dependent T helper 2 pathway that guides IL-4 dependent macrophage polarization is critical for tissue regeneration (Sadtler et al., 2016). Hence, the key process of M1 to M2 transformation of macrophages in fat grafting might be initiated by Th1–Th2 shifting. Th2 responses are essential for the control of extracellular parasites, including helminths, protozoa, and fungi, but they also contribute to allergy, increased susceptibility to other pathogens, and complications of infection such as fibrosis (Martinez et al., 2009). For instance, Th2 cells can promote the M2 macrophages by upregulating arginase activity and increase L-ornithine, L-proline and polyamine concentrations, which promotes fibroblast proliferation, collagen production and ultimately fibrosis (Wynn, 2004). Thus, the Th1–Th2 shifting might be necessary for adipose tissue regeneration, but the long-lasting high level of Th2/Th1 ratio might result in the long-term infiltration of M2 macrophage and fibrosis observed in the 10^6 group.

ASCs could regulate effector T-cell responses and have beneficial effects on various immune disorders (Bessout et al., 2015; Chow et al., 2017). Moreover, ASCs can down-regulate IFN-γ and up-regulate IL-4, which could stimulate T-cells to begin to choose between Th1 and Th2 cell fates (Baharlou et al., 2019). Li et al. showed that the amount of IFN-γ production by Th1 cells is reduced by treatment with ASCs (Li et al., 2010). In addition, Bassi et al. suggest that ASCs therapy could diminish the Th1 immune response (Bassi et al., 2012). ASCs also potentially promote a Th2 shift in another research (Baharlou et al., 2019). In addition, Fiorina et al. administered allogeneic ASCs to NOD mice and observed a shift in Th1/Th2 cell balance towards Th2 cells (Fiorina et al., 2009). That is to say, the immunoregulatory capacity of ASCs might be related to these cells’ ability to promote the Th1–Th2 shift. We used the ASCs-CM and ASCs-transwell models to explore the immunoregulatory capacity of ASCs and their ability to promote Th1–Th2 shifting in vitro, and the results also showed that when the concentrations of ASCs was up-regulated, the ratio of Th2%/Th1% increased. Moreover, at the same concentration, Th2%/Th1% was increased greater in the ASCs-transwell group than the ASCs-CM group after 24 h suggesting that not only can the ASCs-CM promote the shift from Th1 to Th2, but also the continuous paracrine interaction between ASCs and CD4+ T-cells in vivo can promote Th1–Th2 shifting more promptly. Above all, due to the immunoregulatory capacity of ASCs, high concentrations of ASCs in adipose tissue can promote Th1–Th2 shifting, and the resulting excess of Th2 cells might promote the persistence of M2 macrophages and increase the level of fibrosis. Likely due to these phenomena, the long-term retention of fat grafting decreased in the 10^6 group. However, the comparison of ASCs to ASCs-CM has yet some limitation in our experiment. The secretome of ASCs depends on cell density, passage number, status of the media used and several other unknown factors. The proportionate increases in protein release may not be guaranteed as the number of ASCs is increased. The proteins secreted and contained by different concentrations of ASCs and ASCs-CM need to be further determined.

By contrast, the ASCs in the 10^4 group played the opposite role. In these mice, long-term retention was higher than in the control group, and both fibrosis and the persistence of M2 macrophages were reduced. Further, α-SMA expression did not differ between the 10^4 group and control group. Hence, we postulated that the immunoregulatory capacity of ASCs differed between the 10^6 group and the 10^4 group. Given that the α-SMA level was the same as in the control group, the main function of ASCs in the 10^4 group might be to inhibit excessive secretion of extracellular matrix (ECM) proteins and promote degradation of ECM proteins (Wang et al., 2019). In fact, there are several possible mechanisms of ASCs antifibrotic effects, including the regulation of TGF-β/Smad axis, the paracrine mechanisms, the antioxidant effects of ASCs and so on (Borovikova et al., 2018). Consequently, fibrosis was reduced, and retention was higher in the 10^4 group.

However, the survival capability of ASCs after transplantation was still uncertain. Some studies indicate that ASCs might just survive for a period time after grafting (Han et al., 2015). By contrast, a tracing study revealed that intravenously injected ASCs, which were assumed to proliferate, were present in the graft until at least postoperative week 8 and mainly induced angiogenesis and adiopogenesis by paracrine action rather than direct differentiation (Hong et al., 2019). Anyway, although dead ASCs might affect their immunoregulatory function, it would appear after the death of ASCs, while the paracrine effect of ASCs in the early stage after grafting is extremely definite. Whether or not ASCs died in the late stage of transplantation, its paracrine effect has a regulatory effect on the death of ASCs, while the paracrine effect of ASCs in the early stage after grafting is extremely definite. Whether or not ASCs died in the late stage of transplantation, its paracrine effect has a regulatory effect on the death of ASCs, while the paracrine effect of ASCs in the early stage after grafting is extremely definite. Whether or not ASCs died in the late stage of transplantation, its paracrine effect has a regulatory effect on the death of ASCs, while the paracrine effect of ASCs in the early stage after grafting is extremely definite. Whether or not ASCs died in the late stage of transplantation, its paracrine effect has a regulatory effect on the death of ASCs, while the paracrine effect of ASCs in the early stage after grafting is extremely definite. Whether or not ASCs died in the late stage of transplantation, its paracrine effect has a regulatory effect on the death of ASCs, while the paracrine effect of ASCs in the early stage after grafting is extremely definite. Whether or not ASCs died in the late stage of transplantation, its paracrine effect has a regulatory effect on the death of ASCs, while the paracrine effect of ASCs in the early stage after grafting is extremely definite. Whether or not ASCs died in the late stage of transplantation, its paracrine effect has a regulatory effect on the death of ASCs, while the paracrine effect of ASCs in the early stage after grafting is extremely definite. Whether or not ASCs died in the late stage of transplantation, its paracrine effect has a regulatory effect on the death of ASCs, while the paracrine effect of ASCs in the early stage after grafting is extremely definite. Whether or not ASCs died in the late stage of transplantation, its paracrine effect has a regulatory effect on the death of ASCs, while the paracrine effect of ASCs in the early stage after grafting is extremely definite. Whether or not ASCs died in the late stage of transplantation, its paracrine effect has a regulatory effect on the death of ASCs, while the paracrine effect of ASCs in the early stage after grafting is extremely definite. Whether or not ASCs died in the late stage of transplantation, its paracrine effect has a regulatory effect on the death of ASCs, while the paracrine effect of ASCs in the early stage after grafting is extremely definite. Whether or not ASCs died in the late stage of transplantation, its paracrine effect has a regulatory effect on the death of ASCs, while the paracrine effect of ASCs in the early stage after grafting is extremely definite. Whether or not ASCs died in the late stage of transplantation, its paracrine effect has a regulatory effect on the death of ASCs, while the paracrine effect of ASCs in the early stage after grafting is extremely definite. Whether or not ASCs died in the late stage of transplantation, its paracrine effect has a regulatory effect on the death of ASCs, while the paracrine effect of ASCs in the early stage after grafting is extremely definite. Whether or not ASCs died in the late stage of transplantation, its paracrine effect has a regulatory effect on the death of ASCs, while the paracrine effect of ASCs in the early stage after grafting is extremely definite. Whether or not ASCs died in the late stage of transplantation, its paracrine effect has a regulatory effect on the death of ASCs, while the paracrine effect of ASCs in the early stage after grafting is extremely definite. Whether or not ASCs died in the late stage of transplantation, its paracrine effect has a regulatory effect on the death of ASCs, while the paracrine effect of ASCs in the early stage after grafting is extremely definitive.
Based on our findings, it seems reasonable to conclude that in a clinical context, it is important to pay attention to the concentrations of ASCs in fat grafts. A suitable concentration of ASCs could decrease fibrosis and increase long-term retention, whereas excessive ASCs could have the opposite effects (Li et al., 2019; James et al., 2018). Since ASCs can directly induce the phenotype of M2 macrophage, the transformation of macrophages may also affect the shifting process of CD4+ T-cells (Martínez et al., 2009; Sun et al., 2019). The immunoregulation effect of ASCs in fat grafting may be due to promote transformation of both CD4+ T-cells and macrophages at the same time (Babarliou et al., 2019; Fiorina et al., 2009; Lo Sicco et al., 2017). Thus, the transformation of CD4+ T-cells and macrophages might be a process of mutual promotion. However, in our study, it was still uncertain which key cytokines secreted by ASCs paracrine and related activated pathways might promote the transformation of CD4+ T cells and macrophages. We have now preliminarily examined and analysed the protein secretion profile of ASCs and will further clarify the above in the next experiment in progress. In addition, the immunoregulatory capacities of ASCs, i.e., inhibition of excessive ECM secretion, promotion of ECM degradation, and regulation of Th1–Th2 shifting, should be applied to the treatment of various fibrosis and inflammatory diseases in the future.

5. Conclusion

In conclusion, high concentrations of ASCs in adipose tissue can promote Th1–Th2 shifting, and the excess of Th2 cells might promote the persistence of M2 macrophages and increase the level of fibrosis which lead to a decrease in the long-term retention of fat grafts. In addition, we found that ASCs promoted Th1–Th2 shifting through paracrine function in vitro.

Declarations

Author contribution statement

Xinyao Chen: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.
Yunzi Chen; Zijie Wang: Performed the experiments; Analyzed and interpreted the data.
Ziqing Dong; Yao Yao; Lijun Hao; Sai Luo: Conceived and designed the experiments.
Ye Li; Feng Lu: Conceived and designed the experiments; Wrote the paper.
Qihuai Lai; Xinhui Wang; Ronggun Sun; Haoran Zhang; Ruoxue Bai: Contributed reagents, materials, analysis tools or data.
Jing Xia: Performed the experiments; Analyzed and interpreted the data.
Jingyan Guan: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

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Data availability statement

Data will be made available on request.

Declaration of interest’s statement

The authors declare no conflict of interest.

Additional information

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