Adipose-derived stem cells regulate fibrosis by altering CD4+ T-cell immune responses in fat grafting in a mouse model

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Research

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Abstract

Background

Autologous fat grafting is becoming increasingly common worldly. However, the long-term retention of fat grafting is still unpredictable due to the inevitable fibrosis that arises during tissue repair. Fibrosis may be regulated by T-cell immune responses that are influenced by adipose-derived stem cells (ASCs). Accordingly, we hypothesized that overly abundant ASCs might promote fibrosis by promoting T-cell immune responses to adipose tissue.

Methods

We performed 0.3 ml fat grafts with 10^4/ml, 10^6/ml and 10^8/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. In addition, CD4 + T-helper 1 (Th1) cells and T-helper 2 (Th2) cells were co-cultured with ASCs or ASCs conditioned media (CM) *in vitro*. We detected the ratio of Th2%/Th1% after 24 and 48 hours.

Results

In vivo, the retention rate was higher in the 10^4 group, while even lower in the 10^8 group with significantly increased inflammation and fibrosis than the control group at week 12. There was no significance between control group and the 10^6 group. Also, the 10^8 group increased infiltration of M2 macrophages, CD4 + T-cells and Th2/Th1 ratio. *In vitro*, the ratio of Th2%/Th1% induced by the ASCs-transwell group was higher than the ASCs-CM group and showed concentration-dependent.

Conclusions

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 *in vitro*.

Background

Autologous fat grafting is becoming increasingly common around the world. Many studies have sought to improve the retention of fat grafts [1, 2]. However, long-term retention remains unpredictable due to the inevitable tissue fibrosis that occurs following grafting [3].

Fibrosis arising during tissue repair may be regulated by the immune response [4], especially related with the infiltration of M2 macrophages [5]. In fact, the fibrosis and M2 macrophages level could be regulated

by T-cells response [6]. 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) [7]. Also, study showed that the shift of Th1-Th2 could increase the level of M2 macrophages infiltration [8]. Hence, the magnitude of fibrosis could be tightly regulated by the type of Th response that occurs during tissue repair [9, 10].

Moreover, ASCs can influence the Th response to tissue repair, inhibiting IFN-γ secretion from Th1 cells and increasing IL-4 secretion from Th2 cells [11]. In fat grafting, ASCs are present in the stromal vascular fraction (SVF) of adipose tissue, and the regeneration of early ischemic adipose tissue is dependent on tissue vascularization and adipogenic differentiation of ASCs [12]. In addition, ASCs can release many factors through their paracrine function and exert immunoregulatory function of up-regulating M2 macrophage level in adipose tissue, which can alter inflammatory microenvironment to influence adipose tissue repair [13, 14]. Thus, ASCs-assisted lipotransfer has been used in many studies to increase retention of fat grafts [15]. However, different concentrations of ASCs in adipose tissue could contribute to various outcomes. That is to say, appropriate concentrations of ASCs could improve retention by decreasing fibrosis whereas high concentrations of ASCs (1.5×10^6 to 5×10^7 per ml) could markedly decrease long-term retention by increasing fibrosis [16, 17]. Previous studies on the mechanism of ASCs assisted fat grafting mainly focused on the angiogenesis and adipogenesis by ASCs. However, there was no studies on the immunoregulatory effects of ASCs on grafts fibrosis. If it is indeed the case that excessive ASCs induce high levels of Th2 cells during tissue repair, this could increase M2 macrophages level and fibrosis and then decrease long-term retention.

To investigate this issue, we compared mice receiving fat grafts containing 0, 1×10^4, 1×10^6, or 1×10^8 ASCs per milliliter of fat tissue. We assessed the M2 macrophages level, fibrosis and long-term retention of the grafts, as well as the type of Th response that developed during the tissue repair process. In addition, we tested the effect of the concentrations of ASCs on Th1–Th2 shifting *in vitro*.

Materials And Methods

Animals

All experiments were approved by the Animal Committee of Harbin Medical University and conducted according to the guidelines of the National Health and Medical Research Council of China. A total of 260 C57 BL/6 mice were maintained under a 12 h day/night cycle under specific pathogen-free conditions, fed normal chow, and provided *ad libitum* access to water.

Animal model

6-week-old male C57 BL/6 mice were anesthetized by intraperitoneal injection of pentobarbital sodium (50 mg/kg). 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: 1×10^4, 1×10^6, or 1×10^8 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.

Isolation and expansion of C57 BL/6 ASCs

4-week-old C57 BL/6 mice were anesthetized by intraperitoneal injection with pentobarbital sodium at 0.1 mg/100 g and shaved. The inguinal fat pads were excised and extensively washed with PBS. They were then finely minced and rinsed three times in PBS for 5 min, followed by digestion with 0.075% type I collagenase (Sigma-Aldrich, St. Louis, MO, USA) in PBS and vigorous shaking for 40 min at 37 °C. The digested tissue was filtered to remove large debris and then centrifuged at 1000 rpm for 5 min. The cellular pellet (SVF) was resuspended in erythrocyte lysis buffer and centrifuged at 1000 rpm for 5 min. ASCs were plated and cultured in general culture medium and then identified by flow cytometry. Only cells from passages 3 to 5 were used.

Histological analysis

Samples were fixed in 4% paraformaldehyde, dehydrated, embedded in paraffin, and stained with hematoxylin-eosin and Masson's trichrome. Sections were sectioned and examined under an Olympus BX51 microscope. Images were acquired using an Olympus DP71 digital camera.

Immunohistochemistry and immunofluorescence

Tissue sections were incubated with primary antibody: rat anti-mouse CD4 (1:200; Abcam, Cambridge, MA, USA). Secondary antibody was 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.

Immunofluorescence staining was performed with the following primary antibodies: rat anti-mouse Mac2 (1:200; CL8942AP, Cedarlane Corp., Burlington, Ontario, Canada) and rabbit anti-mouse CD206 (1:300; ab64693, Abcam, Cambridge, UK). Secondary antibodies were 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).

Quantitative reverse transcription polymerase chain reaction

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 QuantiTect Reverse Transcription Kit (Qiagen) and the Rotor-Gene 3000 Real-Time

PCR Detection System (Corbett Research, Sydney, Australia). Expression levels were calculated by the $2^{-\Delta\Delta Ct}$ method. The following primers were used: IL-6, TNF-α, IL-10, TGF-β, IFN-γ and IL-4.

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 immobilon polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). Membranes were blocked in 5% milk and immunoblotted with anti–α-SMA antibody (1:60; 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.

Collection of conditioned media

After the ASCs of passage 3 to 5 were 80% confluent in 100 cm^2 culture dishes, the medium was replaced with 8 ml DMEM to obtain 2×10^5/ml ASCs-related 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 ASCs-CM.

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). To obtain activated Th1 or Th2 cells, 2×10^5 CD4 + T-cells were incubated for 24 h in 0.5 ml RPMI 1640 in every one of 24-well plates coated with 5 μg/ml anti-CD3 antibody (BD Biosciences, San Jose, CA, USA) 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), 2 μg/ml anti-CD28 (eBiosciences), 10 ng/ml IL-2 (Peprotech), 15% FBS (Gibco), and 1% penicillin–streptomycin (Gibco). Th2 cells were differentiated with 20 ng/ml IL-4 (Peprotech), 20 μg/ml anti-IFN-γ (eBioscience), 2 μg/ml anti-CD28, 10 ng/ml IL-2 (Peprotech), 15% FBS (Gibco), and 1% penicillin–streptomycin (Gibco).

For the ASCs-Transwell group, 2×10^3, 2×10^4, or 2×10^5 ASCs were seeded in every one of 24-well plates, and activated Th1 or Th2 cells were transferred to the upper chamber of the ASCs-Transwell. After the cells were in place, 1 ml of Th1 or Th2 differentiation medium was added. For the ASCs-CM group, Th1 and Th2 cells were added in 1 ml ASCs-CM at three different concentrations including 2×10^3 (concentration 1), 2×10^4 (concentration 2) and 2×10^5 (concentration 3), which were added with reagents according to Th1 or Th2 differentiation conditions.

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 the System II software (Coulter Corporation, Brea, CA, USA). Analysis was performed using the FCS express software (De Novo Software, Los Angeles, CA, USA).

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.

Results

High concentrations of ASCs decrease graft retention

At week 12, the grafts in the 10^8 group were harder to the touch than those in the other groups; the textures of grafts in the 10^6 and 10^4 groups were similar to those in the control group. However, the appearance of the grafts did not differ markedly among the four groups (Fig. 1A).

The graft retention rate was lowest in the 10^8 group at week 1 and decreased thereafter; consequently, the long-term retention rate (at week 12) was also the lowest in the 10^8 group. The graft retention rate in the 10^6 group never differed from that of the control group. The graft retention rate in the 10^4 group was 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 10^4 group (Fig. 1B).

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 (Fig. 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 (Fig. 2B). Quantification of collagen fibrosis area yielded similar results: fibrosis area was highest in the 10^8 group, and lower in the 10^4 than in the control group at weeks 8 and 12 (Fig. 2C).

Expression of α-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 (Fig. 2D&E).

High concentrations of ASCs increase the direction of M2 macrophage infiltration

Immunofluorescence revealed that in the control group, MAC2 + macrophages had infiltrated adipose tissue at week 1, with additional MAC2 + and M2 (MAC2+/CD206+) macrophages appearing at week 4. By contrast, in the 10^8 group, higher levels of MAC2 + and M2 macrophages were observed from weeks 1 to 8, and could even be observed at week 12. The MAC2 + and M2 macrophages in the 10^6 group were similar to those in the control group. By contrast, the MAC2 + and M2 macrophages in the 10^4 group were also observed at weeks 1 and 4, but decreased at weeks 8 and 12 (Fig S1A).

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 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 (Fig S1B).

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 10^8 group than in other groups from weeks 1 to 12, and lower in the 10^4 group than in the other groups from weeks 1 to 4 (Fig. 3A&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 10^4 group only at week 1 (Fig. 3C&D).

High concentrations of ASCs increase the number of CD4 + T-cells and the Th2/Th1 ratio

Immunohistochemistry at week 4 revealed that CD4 + area was greatest in the 10^8 group (Fig. 4A); quantification of the area ratio confirmed this finding (Fig. 4B). Relative expression of IFN-γ and IL-4 was higher in the 10^8, 10^6, and 10^4 groups than in the control group at week 1, but decreased thereafter (Fig. 4C&D). However, the Th2/Th1 ratio followed a different pattern; in the 10^8 group, the ratio was higher than in the control group at weeks 4 and 12, whereas in the 10^4 group, the ratio was higher than in the control at week 1, but lower at weeks 4 and 12 (Fig. 4E).

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* (Fig. 5A-P). Quantification of the Th2%/Th1% ratio at 24 h showed that only the ASCs-Transwell group with concentration 1 was higher than the control group, as both ASCs-Transwell and ASCs-CM groups with concentration 2 and 3 respectivly were higher than the control group. 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. (Fig. 5Q).

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 (Fig. 6). In addition, we found that ASCs can promote Th1–Th2 shifting *in vitro* (Fig. 7).

ASCs, which can be obtained from adipose tissue, have been experimentally shown to have angiogenic and adipogenic characteristics [18]. 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 [14, 19, 20]. However, consisitent with the results of Paik and Natsuko's research [16, 17], we also found that the concentrations of ASCs in adipose tissue influences the rate of retention. Also, 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 ($\geq 10^6$ 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 number of macrophages in inflammatory cells of 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 [21–25]. M1 (classically activated) macrophages mediate inflammatory responses, which are associated with high levels of pro-inflammatory cytokines [26, 27]. In fact, large numbers of macrophages infiltrated into the tissue after fat grafting. At the early stage of infiltration, mainly M1 macrophages occured to clear necrotic tissues and cells [28]. Then, macrophages gradually transform from M1 to M2 (alternatively activated), which is a necessary process after fat grafting [29]. While 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 [30, 31]. Study showed that when M2 macrophages were added into grafts appropriately, fat grafting could be promoted [32]. However, excess M2 macrophages can increase fibrosis inversely [29, 33]. 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 [34]. Indeed, M2 macrophages may be able to convert into fibroblasts [35]. In this study, we observed a high expression of α-SMA and prolonged infiltration by M2

macrophages in the 10^8 group. However, the long-term presence of M2 macrophages may have promoted fibrosis [33], perhaps explaining the higher level of tissue fibrosis in the 10^8 group.

Interestingly, we found that the evolution of the Th2/Th1 ratio from weeks 1 to 12 was similar to that of the M2/M1 ratio from weeks 4 to 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 [36]. 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 [37, 38]. IFN-γ and IL-4 are produced by mutually inhibitory CD4 + T-helper cells: Th1 and Th2, respectively [37]. 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 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 [6, 39]. In addition, IL-10 secretion by M2 macrophages may also induce the development of repressor T-cells, which oppose Th1 activation [40]. Meanwhile, 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 [41]. 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 [6]. 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 [7]. 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 10^8 group.

ASCs could regulate effector T-cell responses and have beneficial effects on various immune disorders [42, 43]. 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 [44]. Li et al. showed that the amount of IFN-γ production by Th1 cells is reduced by treatment with ASCs [45]. In addition, Bassi et al. suggest that ASCs therapy could diminish the Th1 immune response [46]. ASCs also potentially promote a Th2 shift in another research [44]. In addition, Fiorina et al. administered allogeneic ASCs to NOD mice and observed a shift in Th1/Th2 cell balance towards Th2 cells [47]. 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 hours 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 10^8 group.

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. However, 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 10^4 group and the 10^8 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 [48]. 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 [49]. Consequently, fibrosis was reduced, and retention was higher in the 10^4 group.

However, the surival capability of ASCs after transplantion was still uncertain. Some studies indicate that ASCs might just survive for a period time after grafting [18]. 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 adipogenesis by paracrine action rather than direct differentiation [50]. 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 CD4 + T-cell immune response, and both short-term and long-term paracrine effects of ASCs can promote Th1 to Th2 shifting. Similar results have been found in our experiments *in vitro*.

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 [51, 52]. Since ASCs can directly induce the phenotype of M2 macrophage, the transformation of macrophages may also affect the shifting process of CD4 + T-cells [6, 53]. 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 [44, 47, 54]. Thus, the transformation of CD4 + T-cells and macrophages might be a process of mutual promotion. We will further clarify the above in the next experiment. 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 diseases in the future.

Conclusions

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 *in vitro*.

Abbreviations

ASCs, Adipose-derived stem cells; BFA, Brefeldin A; CD, Cluster of differentiation; CM, Conditioned media; DAPI, Diamidino-phenyl-indole; DMEM, Dulbecco's modified Eagle medium; ECM, Extracellular matrix; ELISA, Enzyme-linked immunosorbent assay; FBS, Fetal bovine serum; FITC, Fluorescein Isothiocyanate; H&E, Hematoxylin and eosin; IFN-γ, Interferon-γ; IHC, Immunohistochemistry; IL-4, Interleukin-4; IL-6, Interleukin-6; IL-10, Interleukin-10; MT, Masson's trichrome; PBS, Phosphate-buffered saline; PMA, Phorbol myristate acetate; SVF, Stromal vascular fraction; Th1, T helper 1; Th2, T helper 2; TNF-α, Tumor necrosis factor
alpha; TGF-β, Transforming growth factor-beta.

Declarations

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Authors' contributions

Xinyao Chen, Yunzi Chen and Zijue Wang participated in research design, the performance of the research and the writing of the paper. Lijun Hao, Sai Luo, Feng Lu, Ziqing Dong, Yao Yao participated in research design, contributed new reagents or analytic tools and secured funding. Ye Li, Jing Xia participated in the writing of the paper and data analysis. Jingyan Guan, Xinhui Wang, Rongcun Sun assisted in conducting the experiments. All authors read and approved the final manuscript.

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Availability of data and materials

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

Ethics approval and consent to participate

All animal experiments were approved by the Animal Committee of Harbin Medical University and conducted according to the guidelines of the National Health and Medical Research Council of China.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

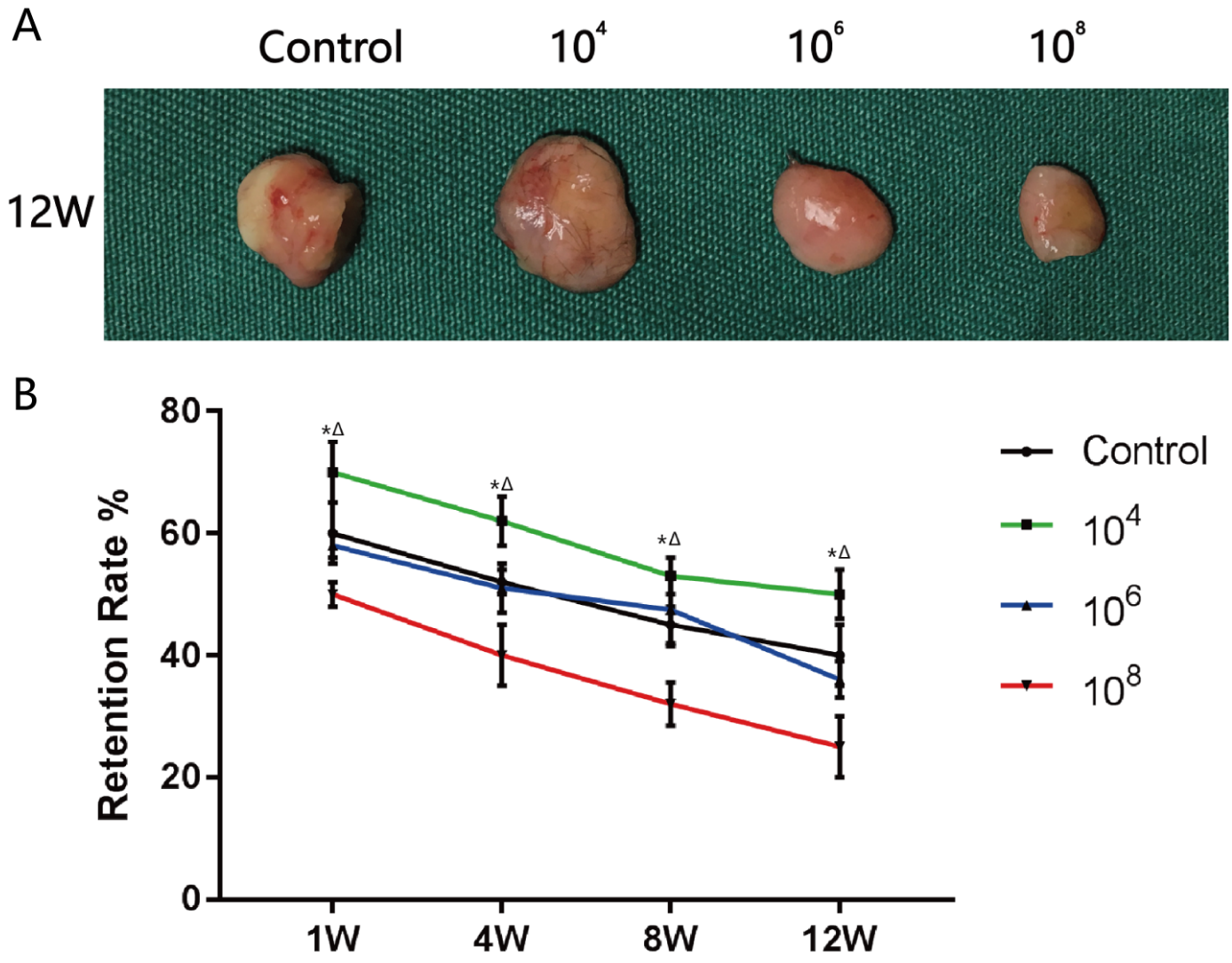

Figure 1

(A) Appearance at week 12 after fat grafting in all groups. (B) Retention in all groups at weeks 1, 4, 8, and 12. * 108 vs. control; Δ 104 vs. control; $P < 0.05$; $n = 7$.

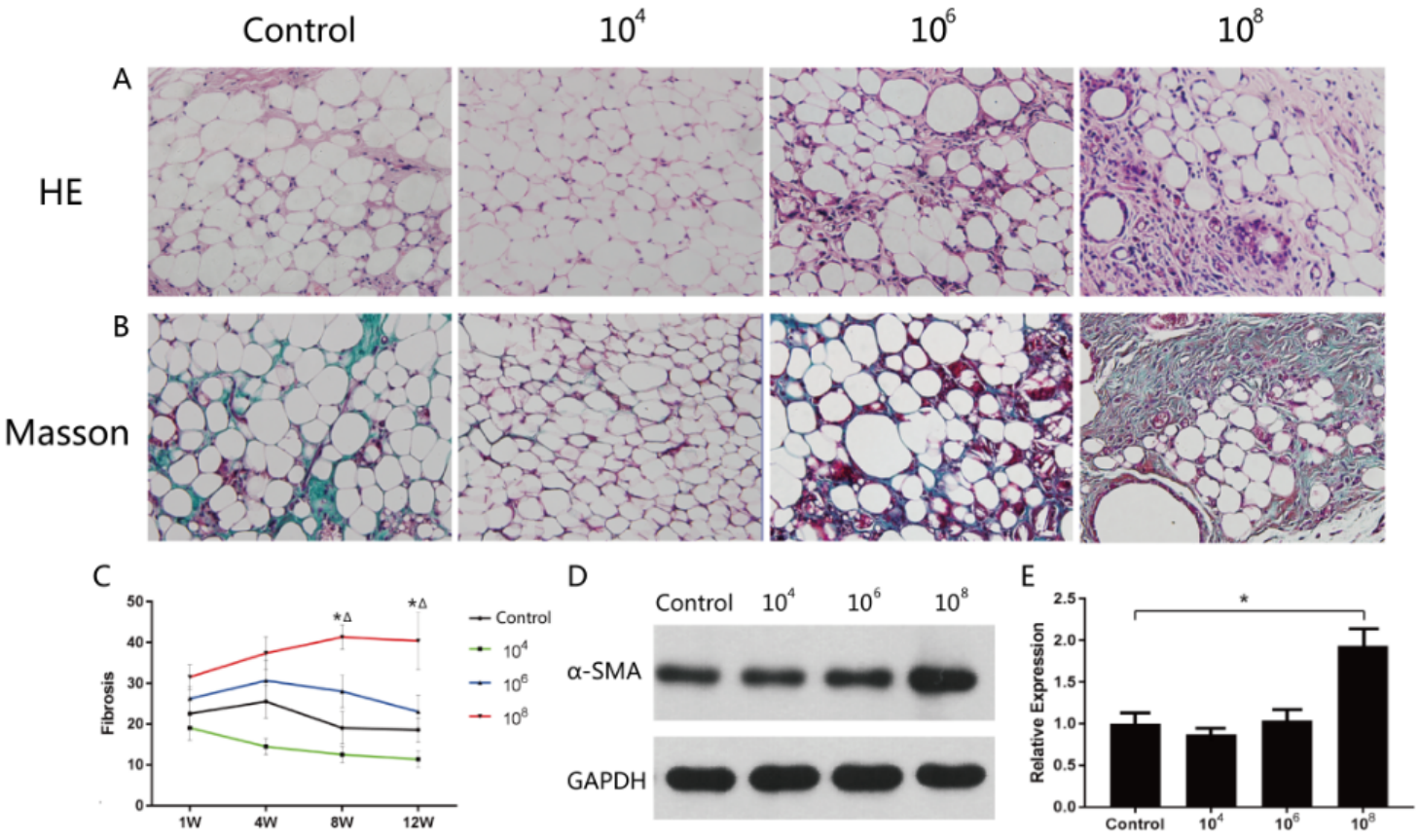

Figure 2

(A) Hematoxylin/eosin staining of grafts in all groups at week 12 after fat grafting. (B) Masson's trichrome staining in grafts of all groups at week 12. (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 α-SMA at week 12 in all groups. (E) Quantification of band intensities in (D). * 108 vs. control; Δ 104 vs. control; $P < 0.05$; $n = 7$.

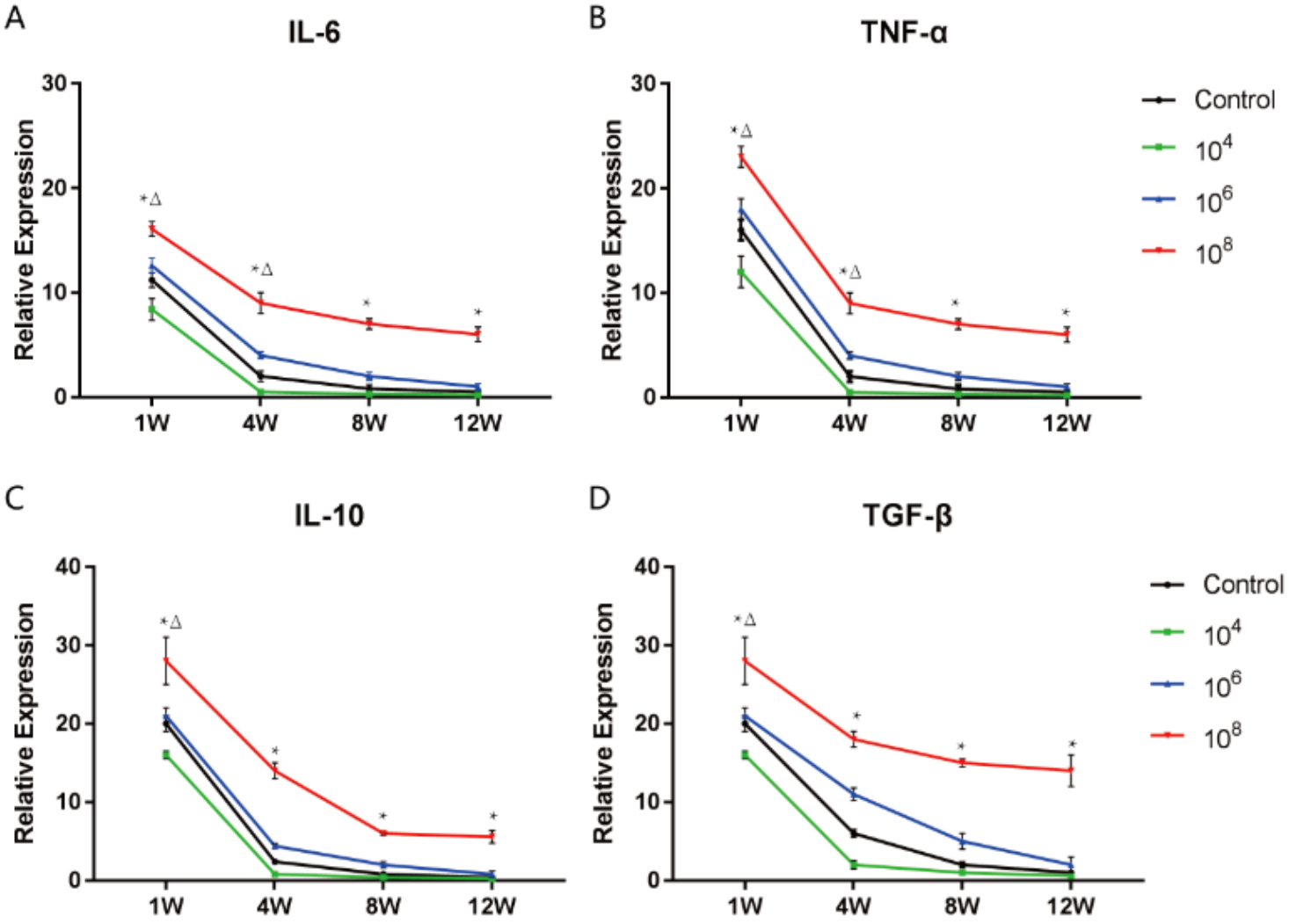

Figure 3

(A&B) qRT-PCR analysis of IL-6 and TNF-α mRNA relative expression in grafts of all groups at weeks 1, 4, 8, and 12. (C&D) qRT-PCR analysis of IL-10 and TGF-β mRNA relative expression in grafts of all groups at weeks 1, 4, 8, and 12. * 108 vs. control; Δ 104 vs. control; $P < 0.05$; $n = 7$.

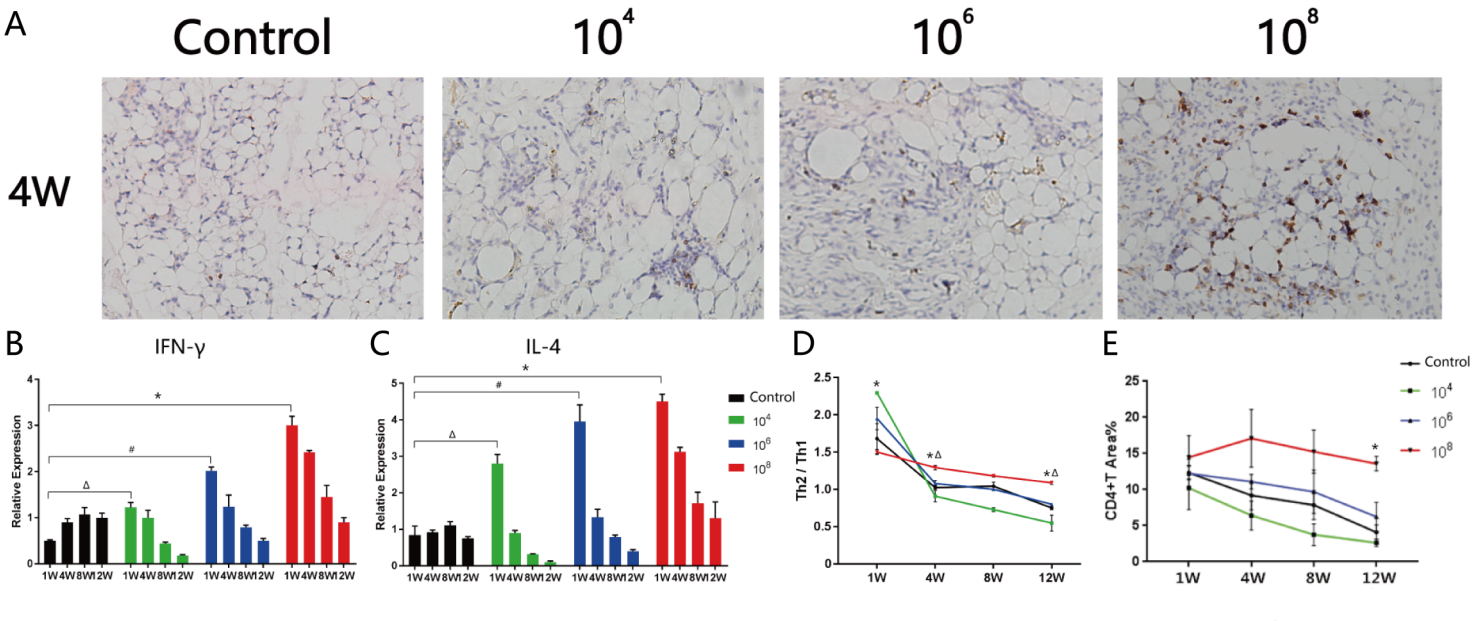

Figure 4

(A) CD4+ T-cell immunohistochemistry in grafts of all groups at week 4. (B) IFN-γ mRNA relative expression in grafts of all groups at weeks 1, 4, 8, and 12. (C) IL-4 mRNA relative expression in grafts of all groups at weeks 1, 4, 8, and 12. (D) Ratio of Th2/Th1 in grafts of all groups at weeks 1, 4, 8, and 12. (E) Quantification of CD4+ T-cell area in grafts of all groups at weeks 1, 4, 8, and 12. * 108 vs. control; # 106 vs. control; Δ 104 vs. control; $P < 0.05$; n = 7.

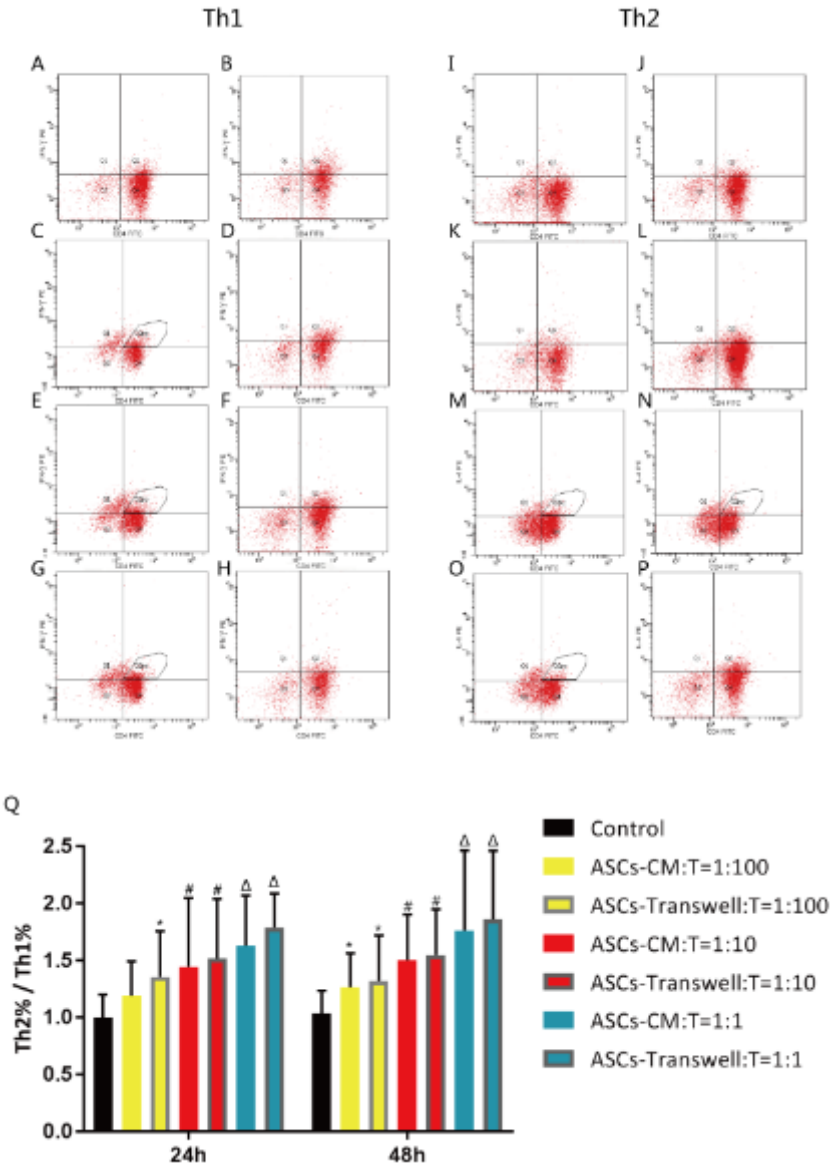

Figure 5

(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), 1:10 (E), or 1:1 (G). Percentage of CD4+ Th1 cells cultured for 48 h after 1 day activation when ASCs-Transwell:T = 1:100 (D), 1:10 (F), or 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), 1:10 (M), or 1:1 (O). Percentage of CD4+ Th2 cells cultured for 48 h after 1 day activation when ASCs-Transwell:T = 1:100 (L), 1:10 (N), or 1:1 (P). (Q) Ratio of percentage of CD4+ Th2 cells to percentage of CD4+ Th1 cells in all groups, 24 and 48 h after 1 day activation. * ASCs-CM or ASCs-Transwell:T = 1:100 vs. control; # ASCs-CM or ASCs-Transwell:T = 1:10 vs. control; Δ ASCs-CM:T or ASCs-Transwell:T = 1:1 vs. control; $P < 0.05$; $n = 7$.

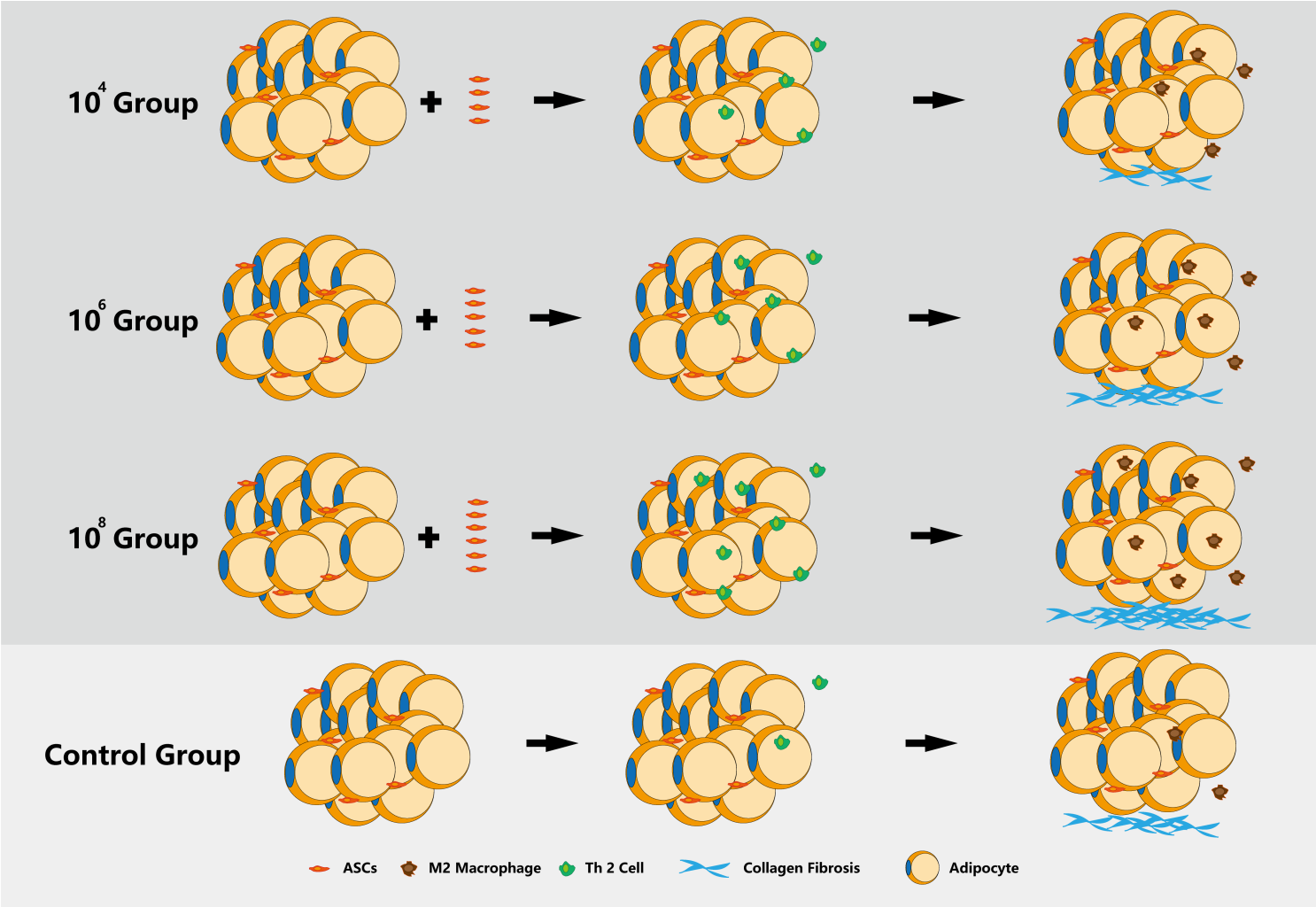

Figure 6

Potential mechanism by which different concentrations of adipose-derived stem cells regulate fibrosis through altering Th2 immune responses and M2 macrophages infiltration in vivo.

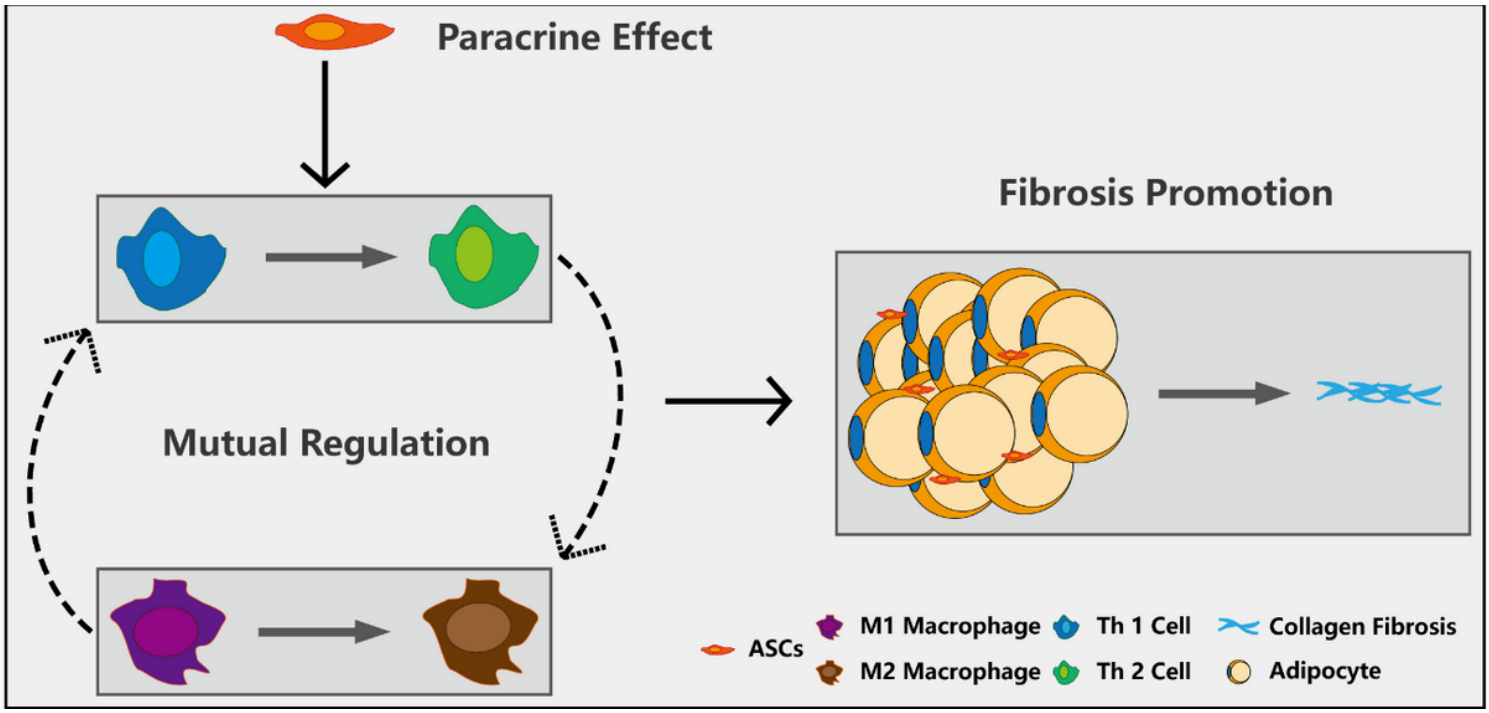

Figure 7

Potential mechanism by which adipose-derived stem cells promote Th1-Th2 shifting to up-regulate fibrosis, which might be caused by the transformation of M1 to M2 macrophage.

Supplementary Files

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