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ORIGINAL ARTICLE BASIC SCIENCE/EXPERIMENTAL

M1/M2 Macrophages Play Different Roles in Adipogenic Differentiation of PDGFRα$^+$ Preadipocytes In Vitro

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Abstract

Background PGGFRα$^+$ preadipocytes are the major subpopulations that can regenerate into adipocytes. Two different types of macrophages exist in the fat tissue: the classically activated macrophage (M1) and the alternatively activated macrophage (M2). In this study, we investigated whether M1/M2 macrophages play distinct roles in adipogenic differentiation of PDGFRα$^+$ preadipocytes.

Methods Mouse preadipocytes and macrophages were isolated from C57BL/6 male mice of 6–8 weeks. The culture supernate of M1 and M2 macrophages was collected and co-cultured with the PDGFRα$^+$ preadipocytes. After 3 days, Oil Red O staining was used to evaluate to adipogenic differentiation of PDGFRα$^+$ preadipocytes and the expression of adipogenic-related transcription factors (C/EBP-α, PPARγ) were also tested.

Results The results showed that when cultured in vitro, M1 macrophages could significantly suppress the adipogenesis of PDGFRα$^+$ preadipocytes as well as the C/EBP-α and PPARγ expression, but M2 macrophages did not have significant influence on the adipogenesis of PDGFRα$^+$ preadipocytes nor on C/EBP-α and PPARγ expression compared with the control group.

Conclusions M1 macrophages significantly suppress PDGFRα$^+$ preadipocyte adipogenesis which provides a possible way to improve adipogenesis and fat retention after fat-free grafting by mitigating acute inflammation and manipulating M1 macrophage levels.

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Keywords Macrophage · Preadipocyte · Adipogenic differentiation · Adipogenesis

Introduction

The inflammatory microenvironment such as macrophages plays a critical role in adipogenic differentiation of preadipocytes as well as tissue regeneration [1]. It has been reported that macrophages gathered around oil droplets in transplanted fat and are supposed to be closely related to fat survival via free oil clearance and dead cell phagocytosis [2]. Debels et al [3] reported that in the absence of macrophages, both angiogenesis and adipogenesis were dramatically inhibited. Cai et al [4] also proved that early depletion of macrophages resulted in incompetent angiogenesis, feeble Sca-1+/CD45+ stem cell recruitment and eventually a poor retention rate, whereas up-regulated macrophages allowed better angiogenesis and survival. However, macrophages undergo different types of polarization [5]. Generally two distinct subtypes exist in the fat graft: the classically activated macrophage (M1) and the alternatively activated macrophage (M2). M1 macrophages are associated with inflammatory responses and high levels

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of pro-inflammatory cytokines, whereas M2 macrophages are associated with tissue repair and producing anti-inflammatory cytokines [6]. Different phenotypes of macrophage may have different impacts on preadipocyte adipogenesis. Sorisky [7] and his colleagues have proposed the hypothesis that M1 macrophages inhibit the survival, proliferation and differentiation of preadipocytes, whereas M2 may promote them. However, as for now, no studies have verified this theory evidently.

In this study, we investigated whether M1 and M2 macrophages had different impacts on preadipocytes adipogenic differentiation in vitro.

Materials and Methods

Animals

All applicable institutional and/or national guidelines for the care and use of animals were followed. C57BL/6 male mice of 6–8 weeks were purchased from Beijing Vital River Laboratory Animal Technology Co. Ltd. Animals were slaughtered, and the inguinal fat tissues as well as bone marrow tissues were collected for further process.

The Isolation and Culturing of Mouse PGGFRα+ Preadipocytes

The preadipocytes were isolated and cultured using standard protocols [8]. Inguinal fat tissues of C57BL/6 mice were collected and digested with Collagenase I (Sigma, USA). Then, cells were cultured with MSCM medium containing 1% penicillin–streptomycin, 5% FBS and 1% mesenchymal stem cell growth supplement (ScienCell Research Laboratories, USA). All cells were cultured at 37 °C in 5% CO_2 humidified environment. The culture medium was replaced in the first 2 days and every 3 days thereafter. Flow cytometry was used to select Sca-1^+, $CD34^+$ and PGGFRα^+ preadipocytes (Fig. 1b, c). After cell sorting, the PGGFRα^+ preadipocytes were cultured with PAM medium containing 1% penicillin–streptomycin, 5% FBS and 1% preadipocyte growth supplement (ScienCell Research Laboratories, USA). After culturing up to passage three at an average cell number of 10^7, the PGGFRα^+ preadipocytes were used for further steps.

The Isolation and Culturing of Mouse Bone Marrow Cells

Bone marrow cells were harvested from mice bone marrows and were cultured in MaM medium containing 1% penicillin–streptomycin, 5% FBS and 1% macrophage growth supplement (ScienCell Research Laboratories, USA). All cells were cultured at 37 °C in 5% CO_2 humidified environment. The culture medium was replaced in the first 2 days and every 3 days thereafter. After culturing up to passage three at an average cell number of 10^6, the bone marrow cells were used for further activation.

The Induction and Activation of Macrophages In Vitro

The bone marrow cells were seeded in a 6-well plate in MaM medium. After overnight incubation, the culture medium was added with 50 ng/mg of GM-CSF (Thermo Fisher Scientific, USA) to induce bone marrow cells to differentiate into macrophages. The culture medium with 50 ng/mg of GM-CSF was replaced every 2 or 3 days for three times. Then, the cells were tested using flow cytometry to ensure that the bone marrow cells had differentiated into macrophages (Fig. 1a). The macrophages were added with 20 ng/ml of murine IFN-γ (Peprotech, USA) and 100 ng/ml of lipopolysaccharide (LPS) (Thermo Fisher Scientific, USA) for induction of M1 macrophages, or with 20 ng/ml of murine IL-4 (Peprotech, USA) for induction of M2 macrophages. The culture supernate of M1 and M2 macrophages was collected after 24 h and 48 h of stimulation, respectively. Inflammatory cytokine levels in the supernate were quantified by enzyme-linked immunosorbent assay.

Stimulation of PGGFRα+ Preadipocytes with M1 or M2 Macrophage Culture Supernates

PGGFRα^+ preadipocytes were seeded into 6-well plates (10^5 cells per well) and were cultured with PADM medium containing 1% penicillin–streptomycin, 5% FBS and 1% preadipocyte differentiation growth supplement (ScienCell Research Laboratories, USA). M1 or M2 macrophage supernates were added into the medium, respectively. A blank control group (with preadipocyte differentiation medium, but without supernate) was also set. The culture medium as well as the supernate was replaced every day for continuous 3 days.

Flow Cytometry Analysis

Surface markers of PGGFRα^+ preadipocytes were characterized by flow cytometry (BD FACS Aria, Germany) with monoclonal antibodies, including CD34, Sca-1 and PDGFRα (eBioscience, USA). Surface markers of macrophages were characterized with specific monoclonal antibodies, including F4/80 and CD11b (eBioscience, USA). After being resuspended with 200 μL buffer (PBS containing 0.1% bovine serum albumin) and incubated for 30 min on ice with the above antibodies, the cell

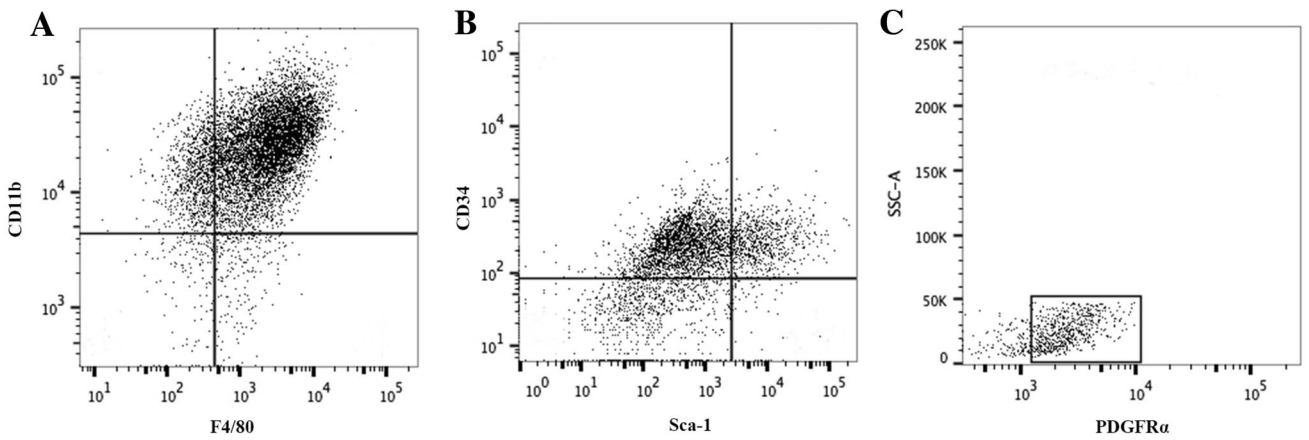

Fig. 1 **a** Most bone marrow cells differentiated into CD11b+ and F4/80+ macrophages. **b**, **c** Sca-1+, CD34+ and PDGFRα+ preadipocytes were sorted by flow cytometry

suspensions were tested. The results were analyzed by Flowjo software.

Real-Time Polymerase Chain Reaction (PCR)

Total RNA from cells was extracted using an ultrapure RNA kit (Cwbio, China). Quantitative PCR was carried out in triplicate in reactions consisting of 10 μl 2× SYBR Green PCR Mixture (Cwbio, China), 0.5 μl ROX, 2 μl cDNA, 20 μl nuclease-free water and 10 μM of each primer. Primer specifications are listed in Table 1. Amplifications were performed on a LineGene-3310 quantitative fluorescence detection system (BIOER) with the following parameters: activation at 95 °C for 10 min, 40 cycles of denaturation at 95 °C for 10 s, and annealing/ extension at 59 °C for 60 s. The threshold cycle (CT, the number of cycles to reach the threshold of detection) was determined for each reaction. GAPDH was used as the reference gene, and relative quantification was determined using the $2^{-\triangle\triangle CT}$ method.

Western Blotting

The cell lysates were prepared with RIPA lysis buffer of appropriate volume. Proteins were separated by 10% SDS-PAGE gel and transferred to PVDF membrane, following membrane blocking with 5% albumin from bovine serum (BSA). Then, the membranes were incubated in primary antibodies against targeted protein at 4 °C overnight, and after further incubation with HRP-conjugated secondary antibody, the bands were scanned. Antibodies used in the experiments included: anti-PPAR gamma (Abcam, USA) and anti-CEBP Alpha (Abcam, USA). All primary antibodies except for the internal control were used at 1:500 dilution. GAPDH (ZSGB-BIO, China) was used as the internal control at 1:1000 dilution.

Table 1 Primers used in real-time polymerase chain reaction

PPAR-γ forward	GCCCTTTGGTGACTTTATGGA
PPAR-γ reverse	GCAGCAGGTTGTCTTGGATG
C/EBP-α forward	AAACAACGCAACGTGGAGAC
C/EBP-α reverse	TGTCCAGTTCACGGCTCAG
GAPDH forward	GCCTTCCGTGTTCCTACC
GAPDH reverse	CTGCTTCACCACCTTCTTG

Oil Red O Staining

The PDGFRα^{+}preadipocytes were rinsed with PBS and fixed in 4% formalin for 30 min. Then, the cells were rinsed with PBS and stained with freshly prepared Oil Red O working solution for 30 min. Finally, the cells were rinsed with PBS and observed under microscope.

ELISA of Cytokines in Macrophage Supernate

Sandwich ELISA was performed to analyze TGF-β1, TNF-α, iNOS, IL-10 and IL-12 levels in macrophage supernates by commercially available ELISA kits (MultiSciences Biotech, China and Elabscience Biotechnology, China) according to the manufacturers' protocols.

Statistical Analysis

The data were analyzed using Student's test and one-way ANOVA test by Prism 6 software. Data were presented as mean ± SEM (standard error of the mean), and a p value less than 0.05 was considered statistically significant.

Results

M2 and M1 Macrophage Supernates Contain Different Levels of Inflammatory Cytokines

To evaluate the inflammatory cytokine levels secreted by M1 and M2 macrophages, 5 inflammatory cytokine levels (iNOS, TNF-α, TGFβ, IL-10 and IL-12) were tested. The ELISA results showed that the levels of iNOS, TNF-α and IL-12 were significantly higher in M1 supernate than those in M2 supernate ($p < 0.05$), whereas the levels of IL-10 and TGFβ were significantly higher in the M2 supernate than those in M1 supernate ($p < 0.05$) (Fig. 2). These results demonstrate that the M1 macrophage supernate contains more pro-inflammatory cytokines, whereas the M2 macrophage supernate contains more anti-inflammatory and pro-regeneration cytokines.

M1 Macrophage Supernate Inhibits PDGFRα$^+$ Preadipocyte Adipogenesis

The PDGFRα$^+$ preadipocytes were cultured with adipogenic differentiation medium. After co-cultured with M1 or M2 macrophage supernates for 3 days, the cells were stained with Oil Red O to evaluate the PDGFRα$^+$ preadipocyte adipogenesis. When cultured with M2 supernate, the preadipocytes showed normal adipogenesis as the control group (Fig. 3a, c). However, when cultured with M1 supernate, the preadipocytes showed obviously inhibited adipogenesis compared with the control group (Fig. 3b, c), which proved that the M1 supernate could inhibit PDGFRα$^+$ preadipocyte adipogenesis.

M1 Macrophage Supernate Inhibits Adipogenic Gene and Protein Expression in PDGFRα$^+$ Preadipocytes

C/EBP-α and PPARγ are two main transcription factors up-regulated during adipogenesis. When cultured in adipogenic differentiation medium, the levels of C/EBP-α and PPARγ mRNA expression in PDGFRα$^+$ preadipocytes were decreased after co-cultured with M1 macrophage supernate compared with the control group (Fig. 4). Similarly, the level of C/EBP-α and PPARγ protein expression in PDGFRα$^+$ preadipocytes was also decreased after co-cultured with M1 macrophage supernate compared with the control group ($p < 0.05$) (Fig. 5).

On the contrary, the level of C/EBP-α and PPARγ mRNA expression in PDGFRα$^+$ preadipocytes did not have statistical significance compared with the control group when co-cultured with M2 macrophage supernate ($p > 0.05$) (Fig. 4). Similarly, the level of C/EBP-α and PPARγ protein expression in PDGFRα+ preadipocytes did not have statistical significance compared with the control group when co-cultured with M2 macrophage supernate (Fig. 5).

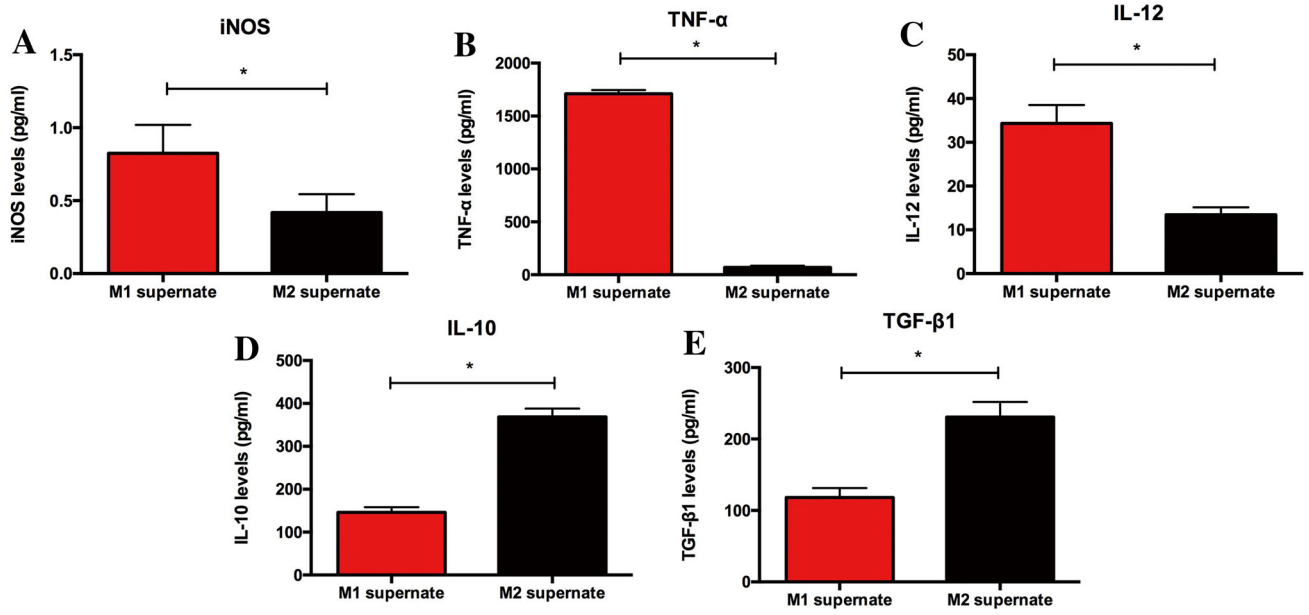

Fig. 2 M2 and M1 macrophage supernates contain different levels of inflammatory cytokines. M1 macrophage supernate contains more pro-inflammatory cytokines such as **a** iNOS, **b** TNF-α and **c** IL-12; M2 macrophage supernate contains more anti-inflammatory and pro-regeneration cytokines such as **d** IL-10 and **e** TGFβ. *$p < 0.05$

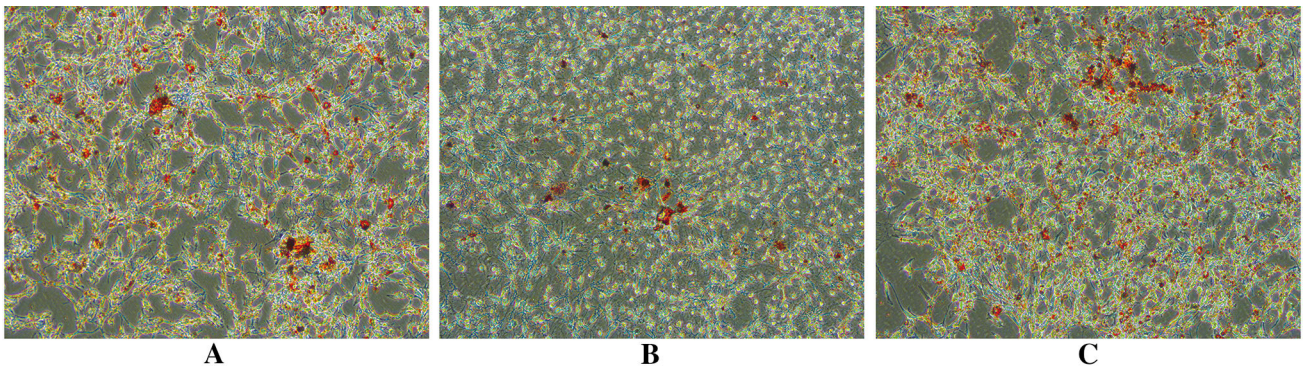

Fig. 3 PDGFRα$^+$ preadipocyte adipogenesis after stimulation by M1 or M2 macrophage supernates. **a** M2 macrophage supernate co-cultured PDGFRα$^+$ preadipocytes. **b** M1 macrophage supernate co-cultured PDGFRα$^+$ preadipocytes. **c** Control group. Magnification ×40

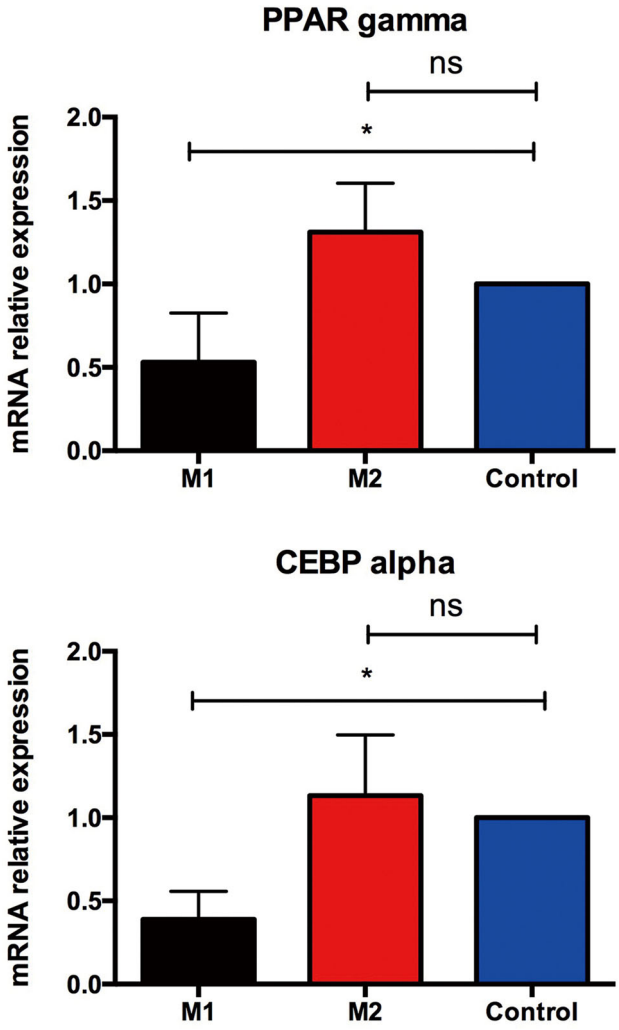

Fig. 4 Levels of C/EBP-α and PPARγ mRNA expression in PDGFRα+ preadipocytes were decreased after co-culturing with M1 macrophage supernate and without statistical significance after co-culturing with M2 macrophage supernate. *$p < 0.05$. Ns: without statistical significance

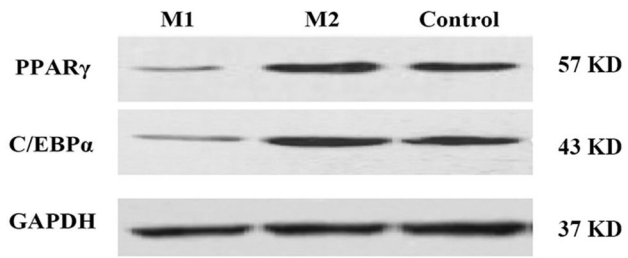

Fig. 5 Levels of C/EBP-α and PPARγ protein expression in PDGFRα$^+$ preadipocytes after stimulation by M1 or M2 macrophage supernates

Discussion

After transplantation, the adipose tissues including ADSC and the preadipocytes all face a complex microenvironment especially the inflammatory microenvironment. The adipogenic differentiation of preadipocytes is the main source of fat regeneration [9] and PGGFRα$^+$ preadipocytes expressing CD34$^+$ and Sca-1$^+$ are the major subpopulations that can regenerate into adipocytes [10, 11]. Thus, adipogenic differentiation of preadipocytes especially PGGFRα$^+$ preadipocytes is crucial for fat regeneration and retention of fat grafts. The surgical stimuli and the necrosis or apoptosis of adipocytes trigger the inflow of phagocytes including macrophages, neutrophils and NK cells to phagocytize cellular debris. By phagocytosis and secreting inflammatory cytokines, these inflammatory cells can have an impact on the function and fate of the preadipocytes.

Macrophages in the adipose tissue have a close relationship with the metabolism and regeneration of fat. Many previous studies [12–14] have reported that the number of macrophages is elevated in both subcutaneous and epididymal fat tissues of obese human subjects or rodents. Adipose tissue macrophages (ATMs) consist of at least two different phenotypes (i.e., classically activated M1 macrophages and alternatively activated M2 macrophages). M1 ATMs produce pro-inflammatory cytokines, such as tumor

necrosis factor (TNF)-α, interleukin (IL)-12 and monocyte chemoattractant protein (MCP)-1. On the other hand, M2 ATMs are reported to have a different gene expression profile, characterized by relatively high expression of CD206, arginase-1, MgII and IL-10, which are involved in the repair or remodeling of tissues [15–17]. Thus, the hypothesis has been proposed that M1 inhibit the survival, proliferation and differentiation of preadipocyte, whereas M2 may promote them [7].

Some previous studies have explored macrophages' influence on fat retention of fat grafts, but the results are inconsistent and inconclusive. Debels et al [3]. knocked out the local macrophages in the fat graft, and there was minimal new vascular and adipose tissue development. Cai et al [4]. found that early depletion of macrophages resulted in incompetent angiogenesis and a poor retention rate, whereas up-regulated macrophages allowed better angiogenesis and survival. These studies all demonstrated that the absence of macrophages was disadvantageous to fat survival. However, all of these studies have taken macrophages as a whole in the experiment, and none of them have studied the respective role of M1 and M2 macrophages in fat survival. Furthermore, these two studies have both focused on macrophages' impact on angiogenesis, while the impact on preadipocyte adipogenesis was not taken into consideration which is crucial for fat retention after transplantation. As far as we know, our study is the first to explore M1 and M2 macrophages' different influence on preadipocyte adipogenesis, respectively.

In this study, we proved that M1 and M2 macrophage supernates contained different levels of inflammatory cytokines. The M1 macrophage supernate contains more pro-inflammatory cytokines such as iNOS, TNF-α and IL-12. The M2 macrophage supernate contains more anti-inflammatory and pro-regeneration cytokines such as IL-10 and TGFβ. Additionally, we co-cultured PDGFRα$^+$ preadipocytes with M1 or M2 macrophage supernates, respectively. In adipogenic differentiation medium, we found that the M1 macrophage supernate could not only suppress preadipocytes adipogenesis, but also inhibited C/EBP-α and PPARγ mRNA and protein expression, which are two main transcription factors up-regulated during adipogenesis. On the contrary, the M2 macrophage supernate did not have a significant impact on preadipocyte adipogenesis nor on C/EBP-α or PPARγ mRNA and protein expression.

The classically activated M1-macrophages consist of immune effector cells with the acute inflammatory phenotype. They are highly aggressive against bacteria and produce large amounts of lymphokines. The alternatively activated, anti-inflammatory M2 macrophages have various different functions, including regulation of immunity, maintenance of tolerance and tissue repair/wound healing [18, 19]. As our study has revealed, the M1 macrophage supernate contains more pro-inflammatory cytokines, whereas the M2 macrophage supernate contains more anti-inflammatory and pro-regeneration cytokines. M1 macrophages are the majority of infiltration cells at the early stage of acute inflammation. As proved by our study that M1 macrophage suppresses preadipocytes adipogenesis, the pro-inflammatory microenvironment at the early stage of acute inflammation may have an adverse impact on preadipocyte adipogenesis. Especially after fat grafting, the adipose stem cell or preadipocytes would certainly be faced with an acute inflammatory microenvironment, which is disadvantageous to adipose stem cell or preadipocyte adipogenesis. In this sense, the extent of acute inflammation and the amount of M1 macrophages may play an important role on fat regeneration and fat retention rate after fat grafting. Thus, alleviating acute inflammation and minimizing the amount of M1 macrophages may be a possible way to improve fat retention rate after fat grafting. On the other hand, despite some studies suggesting that M2 macrophages could improve adipose stem cell or preadipocyte adipogenesis since M2 macrophages are involved in tissue repair and remodeling, our study did not find such phenomena.

Actually, fat retention is closely related to tissue angiogenesis as well as preadipocyte adipogenesis. According to previous studies [3, 4], macrophages as a whole are advantageous to tissue angiogenesis, however, our study proved that M1 macrophages are disadvantageous to preadipocyte adipogenesis. To get the full picture of macrophages' influence on fat retention, studies are needed in the future to verify whether M1 or M2 macrophages play different roles in tissue angiogenesis after fat grafting. Besides, some other limitations still exist. We only demonstrate our findings by experiment in vitro, an animal experiment will be needed in the future to support our point of view. Furthermore, the molecular mechanism behind our finding is also needed to be revealed by future studies. Macrophage supernates having an impact on preadipocyte adipogenesis demonstrate that macrophages play roles via secreting cytokines in the paracrine. However, whether macrophages act directly on preadipocytes by intercellular interaction is still unknown which needs future studies to investigate.

Conclusions

In conclusion, the inflammatory microenvironment plays an important role in adipogenesis after fat grafting. Our study proved that M1 macrophages inhibit PDGFRα$^+$ preadipocyte adipogenesis, which provides a possible way

to improve fat graft adipogenesis by mitigating acute inflammation and manipulating M1 macrophage levels.

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Author's Contribution SF, JL, DLM and HC conceived and designed the experiments; HC performed the experiments, interpreted the data and prepared the figures; HC wrote the manuscript; ZFL, JQ and QW helped perform the experiments; SF and JL helped in writing the manuscript. All authors read and approved the final manuscript.

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflicts of interest to disclose.

Human and Animal Rights All applicable institutional and/or national guidelines for the care and use of animal were followed.

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