

Salvianolic Acid B Improves the Effect of Fat Grafts by Inhibiting the NF- κ B Signalling Pathway in Macrophages

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Research

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Abstract

Background

Autologous fat grafting (AFG), although an appealing approach to repair soft tissue defects, has various complications. Excessive inflammation at the transplant site is one of the main reasons for the poor effect of fat transplantation and occurrence of complications. Our previous study proved that *Salvia miltiorrhiza* can enhance fat graft survival. Salvianolic acid B (Sal-B) is the most abundant and bioactive water-soluble compound in *Salvia miltiorrhiza* and has anti-inflammatory effects on other diseases. Therefore, we hypothesized that salvianolic acid B could improve the effect of fat grafts by inhibiting inflammation.

Methods

In vivo, 0.2 ml of Coleman fat was transplanted into nude mice with salvianolic acid B. The grafts were evaluated by HE and IF at 2, 4 and 12 weeks posttransplantation and by micro-CT at 4 weeks posttransplantation. *In vitro*, the proliferative and anti-inflammatory activities of salvianolic acid B were analyzed in cultured RAW264.7 cells to detect the mechanism by which salvianolic acid B affects graft survival by inhibiting inflammation.

Results

In vivo, the degree of adipose tissue fibrosis and inflammatory cell infiltration in the salvianolic acid B treatment group was lower, and the infiltration of M1 macrophages in fat grafts was also less than that in the control group. *In vitro*, salvianolic acid B inhibited the proliferation and activation of inflammatory pathways in RAW264.7 cells.

Conclusions

This study demonstrates the use of salvianolic acid B as a possible treatment to improve the effect of fat transplantation.

1. Background

Tissue defect repair is a key component of plastic and reconstructive surgery. In recent years, autologous fat grafting (AFG) has been an appealing approach to repair soft tissue defects because it is abundant, ubiquitous, biocompatible, and relatively simple to access[1]. AFG is used for various cosmetic or reconstructive indications, such as facial contour refinement[2], breast augmentation and reconstruction[3], posttraumatic deformity[4], congenital deformity[5] and other fields. According to the American Society of Plastic Surgeons[6], 24892 AFG procedures for breast reconstruction and

augmentation and 43177 AFG procedures for facial filling were performed in 2018. These values represent increases by 37.3% (breast) and 3% (face) compared with the number of AFG procedures performed in 2015.

Despite the advantages of AFG, the high absorption rate and various complications, such as oily cyst formation, fibrosis, calcification and fat necrosis, have influenced the filling effect. A systematic review[7] showed that the volume retention rate of AFG in facial filling varies from 26 to 83%. Another study reported that the overall rate of patient complications is 2.1% for breast cancer, 2.26% for facial filling and 10.5% for buttock filling[8]. Therefore, developing methods to improve the survival rate of fat transplantation and reduce complications is crucial. Some studies showed that lipid droplets and cell debris induce a tissue inflammatory response[9]. Excessive inflammation will further induce fat necrosis. Therefore, reducing the inflammation of fat grafts provides new treatment inspiration.

The anti-inflammatory role of traditional Chinese medicine is increasingly prominent. *Salvia miltiorrhiza* (SM) is one of the most widely used Chinese medicines in clinical practice. It has the functions of promoting blood circulation, removing blood stasis, anti-inflammatory effects, and antioxidative stress effects. It is widely used to treat artery diseases such as atherosclerosis[10]. Because *Salvia miltiorrhiza* is a mixed traditional Chinese medicine, different sources of production or different processing methods will affect the proportion of each component in *Salvia miltiorrhiza*, leading to inaccurate curative effects[11]. Salvianolic acid B (Sal-B, Fig. 1A) is the most abundant and bioactive water-soluble compound in *Salvia miltiorrhiza*[12]. Studies have shown that salvianolic acid B has anti-inflammatory and anti-immune effects[13, 14]. In this study, we attempted to investigate whether Sal-B improves the survival rate of fat transplantation through anti-inflammatory effects.

2. Materials And Methods

2.1 Animals

All the animal experiments were approved by Shanghai Ninth People's Hospital, Shanghai Jiao Tong University, School of Medicine, Shanghai, China. Female nude mice (aged 6 to 8 weeks) were housed in individual cages with a 12-hour light/dark cycle and provided with standard food and water ad libitum.

2.2 Fat Grafting Model and Treatments

The mice were randomly divided into three groups: saline, 10 $\mu\text{mol/L}$, and 50 $\mu\text{mol/L}$. Each mouse was injected subcutaneously on the back with 0.2 ml of Coleman fat using a 1 ml syringe with a blunt infiltration cannula (Supplementary material Fig. 1A). The grafts were injected into a spherical shape. The mice were locally injected with 0.2 ml of saline or salvianolic acid B (10 $\mu\text{mol/L}$, 50 $\mu\text{mol/L}$) once every two days. The mice were sacrificed after 2, 4, and 12 weeks ($n = 5$ per time point per group), the grafts were harvested and carefully separated from surrounding tissue, and their volumes and weights were measured. Each harvested sample was assessed histologically and immunohistochemically.

2.3 RAW264.7 Culture

RAW264.7 cells (ATCC, USA) were grown in culture medium, which was changed every 48 h. When the cells reached approximately 90% confluence, they were washed using high-sugar DMEM (Gibco, USA) with 10% foetal bovine serum (FBS; Gibco, USA). The cell suspension was further divided into three culture flasks containing growth medium.

2.4 Cell viability assays

The effects of salvianolic acid B on the viability of RAW264.7 cells were tested using Cell Counting Kit-8 (Beyotime, China) according to the manufacturer's instructions. Briefly, 5000 cells/well were seeded in a 96-well plate. The cells were cultured in growth medium with various concentrations of salvianolic acid B (0, 10, 25, 50, 75, 100 μ mol/L). At 72 h of culture, 10% CCK-8 reagent was mixed with medium and added to each well. The 96-well plate was incubated at 37 °C for 2 h. The relative number of cells was measured at an absorbance of 450 nm using a microplate reader (Thermo, USA).

2.5 2.6 5-Ethynyl-2'-deoxyuridine (EdU) proliferation assay

Cells were seeded in 24-well plates and incubated under standard conditions with various concentrations of salvianolic acid B (0, 10, 50, 100 μ mol/L). Twenty-four hours after incubation, cell proliferation was detected using the EdU Cell Proliferation Assay Kit (Invitrogen, USA) according to the manufacturer's protocol. Briefly, cells were incubated with 50 μ M EdU for 2 hours before fixation, permeabilization, and EdU staining. Next, cell nuclei were stained with Hoechst 33342 (Invitrogen, USA) for 30 minutes. The proportion of cells that incorporated EdU was determined by inverted fluorescence microscopy (Nikon, Japan). The cells were counted manually in each field, three fields were counted for each of the 3 experiments, and three technical replicates were performed in each of the 3 experiments. The proportion of Edu+ cells (%) = 100 * Number of Edu+ (Green) cells / Number of total cells (Hoechst33342+ cells, Blue). The significance of differences between the control and treated groups was set at P<0.05 and assessed by ANOVA with GraphPad Prism 8 (GraphPad Software, La Jolla, CA, USA).

2.6 Flow cytometry

After 3 days in culture with various concentrations of salvianolic acid B pretreatment, RAW264.7 cells were resuspended in PBS buffer according to the number of cells (5,000/ml). For Annexin V and propidium iodide staining, 195 μ L of cell suspension was mixed well with 5 μ L of Annexin V-FITC, followed by incubation at room temperature for 10 minutes. The cells were washed with PBS and resuspended in 190 μ L of deliquated binding buffer, and then 10 μ L of 20 μ g/ml propidium iodide was added. The samples were analyzed by flow cytometry using CytoFLEX LX (Beckman Coulter, USA). The data were analyzed using CytExpert (Beckman Coulter, USA).

2.7 Histological analysis and immunofluorescence staining

Tissues were fixed in paraformaldehyde overnight, embedded in paraffin, cut at a thickness of 5 µm and then stained with haematoxylin and eosin. We used the methods of Shoshani O[15] and Yu P[16] to evaluate histologic parameters, such as cell integrity, tissue inflammation, the presence of cysts/vacuoles, and the extent of fibrosis. Each parameter was scored as follows: 0 = absence, 1 = minimal presence, 2 = minimal to moderate presence, 3 = moderate presence, 4 = moderate to extensive presence, and 5 = extensive presence. The scoring was performed independently by 3 authors who were unaware of the grouping.

For immunofluorescent staining, tissue sections were incubated with the following primary antibodies: Perilipin (#15294-1-AP; 1:200; Proteintech, China), F4/80 (#27044-1-AP; 1:200; Proteintech, China), CD11c (ab11029; 1:200; Abcam, UK) and CD206 (#60143-1-Ig; 1:200; Proteintech, China) diluted in blocking solution overnight at 4 °C. After incubation with Alexa Fluor 488-conjugated goat anti-rabbit immunoglobulin G (#A-21206; 1:500; Invitrogen, USA), Alexa Fluor 555-conjugated goat anti-rabbit immunoglobulin G (#A-31572; 1:500; Invitrogen, USA) and Alexa Fluor 488-conjugated goat anti-mouse immunoglobulin G (#A-21202; 1:500; Invitrogen, USA), the nuclei were stained with 4',6-diamidino-2-phenylindole (Southern Biotech, USA).

For immunocytofluorescence, RAW264.7 cells were incubated with a primary antibody against p-p65 (#3033; 1:500; Cell Signaling Technology, USA) diluted in blocking solution overnight at 4 °C. After incubation with Alexa Fluor 488-conjugated goat anti-rabbit immunoglobulin G (#A-21206; 1:500; Invitrogen, USA), the nuclei were stained with 4',6-diamidino-2-phenylindole (Southern Biotech, USA).

ImageJ software was used for quantitative analysis. Image analysis was performed according to the website (<https://imagej.net/imaging/image-intensity-processing>) and the method of Keskin[17].

2.8 Micro-CT analysis

The fat grafts were scanned using micro-CT (PerkinElmer, USA) and analyzed by ProPlan CMF 3.0.

2.9 RNA Extraction and Real-time RT-PCR

To investigate the polarization level of RAW264.7 cells, RAW264.7 cells were incubated under standard conditions with various concentrations of salvianolic acid B (0, 10, 50, and 100 µmol/L). After 72 hours of culture, the cells were stimulated with ultrapure LPS (10 ng/ml; Sigma, USA), and then the transcriptional levels of iNOS and TNF-α in RAW264.7 cells were assessed by real-time PCR. Initially, the total RNA of RAW264.7 cells was extracted using a total RNA miniprep kit (Axygen, USA), and RT-qPCR was performed using an ABI 7900HT system and SYBR Premix (Takara, Japan) according to the manufacturer's instructions. mRNA quantification was performed using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) for normalization. The SYBR green primers for qRT-PCR are listed in Supplementary Table 1.

2.10 Western blotting

Cultured cells were lysed with RIPA buffer (Beyotime, China) supplemented with protease inhibitor (PMSF; Biosharp, China). Briefly, 20 µg of protein was resolved by 10% or 12% SDS-PAGE and electroblotted onto polyvinylidene difluoride membranes (Millipore Sigma, USA). The membranes were blocked with 5% nonfat milk at room temperature for 1 hour. The separated proteins were then immunoblotted and probed with the following primary antibodies: anti-glyceraldehyde 3-phosphate dehydrogenase (#10494-1-AP; 1:5,000; Proteintech, China), anti-NF-κB p65 (ab32536; 1:1,000; Abcam, UK), anti-NF-κB p65 (phospho S536) (ab76302; 1:1,000; Abcam, UK), anti-JNK1+JNK2+JNK3 (ab179461; 1:1,000; Abcam, UK) and anti-JNK1+JNK2+JNK3 (phospho T183+T183+T221) (ab124956; 1:1,000; Abcam, UK) at 4 °C overnight. The next day, the membranes were incubated with peroxidase-conjugated secondary antibody (ab205718; 1:10,000; Abcam, UK) at room temperature for 1 hour after washing with Tris-buffered saline with Tween 20 for 10 minutes three times. ImageJ software was used for quantitative analysis, which was conducted on immunoreactive bands. The number of experimental or technical replicates was three.

2.11 RNA-Seq analysis

RNA sequencing samples were acquired after the addition of LPS (10 ng/ml)+Sal-B (50 µmol/L), LPS (10 ng/ml) or solvent to RAW264.7 cells for 3 days in growth medium. The RNA quantity and quality were measured using the NanoDrop ND-1000 system. The cDNA library was constructed using the KAPA Stranded RNA-Seq Library Preparation Kit (Illumina) following the manufacturer's protocol. The final double-stranded cDNA samples were verified using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). After cluster generation (TruSeq SR Cluster Kit v3-cBot-HS; Illumina), sequencing was performed using an Illumina HiSeq 4000 sequencing platform. Image analysis, base calling, and error estimation were performed using Illumina/Solexa Pipeline (Off-Line Base Caller software, version 1.8). Quality control was checked on the raw sequence data using FastQC (https://en.wikipedia.org/wiki/FASTQ_format). Raw data were preprocessed using Solexa CHASTITY and Cutadapt to remove adaptor sequences, ribosomal RNA, and other contaminants that may interfere with clustering and assembly. The trimmed reads were mapped to the corresponding reference genome using HISAT2 (version 2.0.4) for RNA sequencing, and StringTie (version 1.2.3) was used to reconstruct the transcriptome. Ballgown software was applied to calculate the fragments per kilobase of exon per million fragments mapped in RNA sequencing data and analyze differentially expressed genes, with the fragments per kilobase of exon per million fragments mapped ≥ 0.5 (Cuffquant) considered for the analysis. The cut-off for defining which genes were differentially expressed was a fold change greater than 1.5. Gene Ontology functional and Kyoto Encyclopedia of Genes and Genomes pathway enrichment analyses were performed for differentially expressed genes using the Database for Annotation, Visualization and Integrated Discovery and Kyoto Encyclopedia of Genes and Genomes Orthology-Based Annotation System online tools (<http://www.geneontology.org> and <http://www.genome.jp/kegg>).

2.12 Molecular docking

The PDB file for the crystal structures of IKK β was obtained using the protein data bank code 4KIK. The molecular docking procedure was performed under the C-DOCKER protocol of Accelry's Discovery Studio

2019 software. For ligand preparation, the structure of Sal-B was constructed using ChemDraw Professional 17.0 software, saved in the SDF file format and minimized using Accelry's Discovery Studio 2019 software. The protein structure was cleaned and inspected for errors, hydrogens were added, and the water molecules were deleted. The IKK β proteins were defined as receptors, and the centroid of the binding site was defined based on the ligand in the cocrystal structure. Next, the original ligand was removed, and the molecule of Sal-B was placed in the sphere position to perform molecular docking. For energy minimization, the CHARMM force field was used within Accelry's Discovery Studio 2019 software. Finally, the types of interactions between the docked proteins and Sal-B were analyzed.

2.13 Statistical analysis

In the present study, all *in vitro* experiments were conducted 3 times. Single blinding was used for statistical analysis. Two blinded data analysts independently analyzed the data. The final data were consistent between the two analysts. The data were expressed as means \pm SD. The continuous variables between the groups were compared by the independent samples t-test. One-way ANOVA with Tukey's post hoc test was employed for pairwise comparisons among multiple groups. The significance of differences between the control and treated groups was set at P<0.05 and assessed using GraphPad Prism 8 (GraphPad Software, La Jolla, CA, USA).

3. Results

3.1 Tissues from the salvianolic acid B-treated group show higher retention rates and lower inflammation after transplantation

After 3 months of salvianolic acid B treatment, the fat graft retention in the treatment groups was higher (Fig. 1B). Additionally, by HE staining, the degree of adipose tissue fibrosis and inflammatory cell infiltration in the salvianolic acid B treatment groups was lower (Fig. 1C, D), and the structural integrity of adipose tissue and formation of oil cysts were lower (Supplementary material Fig. 1B). Furthermore, Perilipin staining further validated the above facts, and the salvianolic acid B treatment groups had more viable adipocytes (Fig. 2A, B). Interestingly, in the middle stage of fat transplantation, we analyzed the survival of subcutaneous fat transplantation and related complications, and the salvianolic acid B treatment groups exhibited more fat survival and less absorption (Fig. 2C). Thus, salvianolic acid B can improve the retention of transplanted fat, and the effect of a 50 μ mol/L concentration is better.

3.2 Salvianolic acid B reduces the activation ratio of M1-type macrophages in fat grafts

Macrophages play an essential role in mediating inflammation in fat transplantation. Studies[18] have shown that in the early period after fat transplantation, the immune cells of the graft are mainly M1 macrophages, which release a large amount of inflammatory factors. In the late period after fat transplantation, the immune cells of the graft are mainly M2 macrophages, which release a large amount of anti-inflammatory factors. Therefore, we further used immunofluorescence staining to determine whether salvianolic acid B affects macrophages. We defined M1 macrophages as F4/80- and CD11c-

positive and M2 macrophages as F4/80- and CD206-positive as described by J. Cai [19]. Consistent with other studies, in the control group, M1 macrophages infiltrated in the early stage after fat transplantation, while M2 macrophages infiltrated in the later stage of fat transplantation (Fig. 3A, 3B, 3C, 3D). Interestingly, in the experimental group, early M1 macrophage infiltration was reduced compared with that in the control group (Fig. 3A, 3B), further explaining the results observed in the previous HE staining. Additionally, the level of M2 macrophages also decreased at 12 weeks, but no significant difference was found (Fig. 3C, 3D). However, interestingly the ratio of M2/M1 increased at 4 and 12 weeks after salvianolic acid B treatment (Fig. 3E) compared with the control group. Thus, salvianolic acid B can reduce the infiltration of M1 macrophages in fat grafts.

3.3 Salvianolic acid B inhibits the proliferation of macrophages

In *in vitro* experiments, we used RAW264.7 cells as a macrophage model to clarify the effect of salvianolic acid B on macrophages. First, RAW264.7 cells were treated with different concentrations of salvianolic acid B. We found that when the concentration was greater than 10 µmol/L, the viability of RAW264.7 cells decreased (Fig. 4A). A decrease in proliferation or an increase in apoptosis can lead to a decrease in activity. Therefore, we used EdU staining and flow cytometry to detect the effect of salvianolic acid B on the proliferation and apoptosis of RAW264.7 cells. Interestingly, the proliferation level of RAW264.7 cells was inhibited at concentrations of 50 µmol/L and 100 µmol/L (Fig. 4B, 4D). At a concentration of 100 µmol/L, the apoptotic level of RAW264.7 cells increased (Fig. 4C, 4E). Therefore, we believe that a concentration of 50 µmol/L can inhibit the proliferation of macrophages without causing the apoptosis of macrophages.

3.4 RNA-Seq shows that salvianolic acid B reduces LPS-induced inflammation of macrophages and inhibits the activation of macrophages

To further clarify the effect of salvianolic acid B on the function of macrophages, we used the LPS-induced macrophage inflammation model. Through RNA-Seq analysis, compared with the control group, the expression of many genes in the LPS treatment group was changed (Fig. 5A, 5B). Through KEGG analysis, we found that many inflammatory pathways were upregulated, such as the IL-17 signalling pathway, TNF- α signalling pathway and NF- κ B signalling pathway (Fig. 5C). In the salvianolic acid B treatment group, the expression of many genes was also changed (Fig. 5A, 5B), and the abovementioned inflammatory pathways were downregulated (Fig. 5C). Additionally, by analyzing the biological process of macrophages in GO analysis, many biological processes of macrophages were changed (Fig. 5D). By merging the upregulated biological process after LPS treatment and the downregulated biological process after salvianolic acid B treatment, salvianolic acid B mainly affected biological processes related to macrophage activation (Fig. 5E). Thus, through RNA-Seq analysis, salvianolic acid B inhibits the activation of inflammatory pathways and macrophages.

3.5 Salvianolic acid B inhibits LPS-induced macrophage polarization to the M1 type by inhibiting the LPS signalling pathway

To verify the above sequencing results, the expression of iNOS and TNF- α was detected by PCR. Consistent with the above results, salvianolic acid B inhibited the expression of inflammatory genes (Fig. 6A). Furthermore, the activation of NF- κ B (p65) and JNK plays an important role in activating inflammatory macrophages. Therefore, the phosphorylation level of the abovementioned proteins was tested, and the phosphorylation of p-65 and JNK was inhibited by salvianolic acid B (Fig. 6B, 6C, 6D, 6E). Additionally, through computer simulation, salvianolic acid B might have potential binding sites with IKK- β (Supplementary material Fig. 1C).

Taken together, salvianolic acid B functions to inhibit the activation of inflammatory macrophages by inhibiting the NF- κ B signalling pathway.

4. Discussion

Autologous fat grafts have been widely used in reconstructive surgery and cosmetic surgery to solve soft tissue defects because of their convenient use, low cost, and good biocompatibility[20]. However, its unpredictable absorption rate, as well as subsequent complications, such as the formation of oil cysts, calcifications and nodules, all affect the final clinical effect[3]. Previous studies have attempted to solve the abovementioned problems by improving the blood supply or increasing adipose stem cells. For example, stromal vascular components (SVFs)[21], cell-assisted fat transplantation[22] and cytokines have been used [23]. Although substantial progress has been made, many complications persist, and the cumbersome operation is also a hindrance. Therefore, identifying a simpler and more effective method is required.

Salvia miltiorrhiza has been widely used as a traditional Chinese medicine in clinical practice [24]. Our previous studies have found that *Salvia miltiorrhiza* improved the survival rate of fat transplantation and promoted the proliferation and differentiation of adipose stem cells [25-27]. Because *Salvia miltiorrhiza* is a mixed traditional Chinese medicine, different sources of production or different processing methods will affect the proportion of each component in *Salvia miltiorrhiza*, leading to inaccurate curative effects [11]. Therefore, identifying the key small molecules in *Salvia miltiorrhiza* is critical. Salvianolic acid B (Sal-B) is the most abundant and bioactive water-soluble compound in *Salvia miltiorrhiza* [12]. Many studies have shown that salvianolic acid B has anti-inflammatory effects, particularly inhibiting inflammation induced by macrophages [28-30].

Macrophages play an important role in fat transplantation. Studies have shown that most macrophages are M1 polarized with a high level of TNF- α expression in the early stage of transplantation. However, in the middle and late stages of transplantation, M2 macrophages dominate the macrophage population and produce a high level of TGF- β , promoting tissue repair [19]. According to the results of the control group, we further confirmed the existence of this phenomenon. Over time, the proportion of M1 macrophages gradually decreased, while that of M2 macrophages gradually increased. M1 macrophages and M2 macrophages play different roles in the inflammatory response. Studies have reported that M1 macrophages release TNF- α to promote angiogenesis, but too many M1 macrophages will inhibit the

proliferation and differentiation of adipose stem cells, leading to more adipocyte necrosis and tissue fibrosis [9, 31]. M2 macrophages improve the blood supply by secreting large amounts of proangiogenic factors and recruiting more stem cells [19, 32]. Our findings showed that salvianolic acid B reduces the ratio of M1 macrophages in fat grafts. Additionally, *in vitro* salvianolic acid B inhibits the proliferation and inflammation of macrophages induced by LPS. Furthermore, salvianolic acid B does not affect the ratio of M2-type macrophages but can increase the M2/M1 ratio at 4 and 12 weeks.

In fat transplantation, because of the intervention of various surgical instruments, tissue trauma is inevitable, and the damaged tissue will induce an inflammatory response by activating the inflammation-related signalling pathways of macrophages, such as the TNF- α signalling pathway.

Therefore, many proinflammatory factors, such as TNF- α , iNOS, and IL-6, are released [33, 34]. In our RNA-Seq results, we observed that salvianolic acid B inhibits the activation of inflammatory signalling pathways, such as TNF- α , IL-17 and NF- κ B, after LPS treatment. The phosphorylation of NF- κ B (p65) and JNK is related to the activation of proinflammatory macrophages [35, 36], and we also detected the phosphorylation level of the abovementioned proteins in macrophages. Thus, salvianolic acid B can inhibit the phosphorylation of NF- κ B (p65) and JNK.

Our study revealed the mechanism of salvianolic acid B in regulating inflammation of autologous fat grafts and its potential value in translational applications. In summary, on the one hand, salvianolic acid B inhibits the activation of macrophage inflammatory signalling pathways by inhibiting the phosphorylation of NF- κ B and JNK. On the other hand, from *in vitro* experiments, salvianolic acid B inhibits the proliferation of macrophages (Fig7). The above two reasons lead to a decrease in the level of inflammation in fat grafts. Our study has shortcomings. First, this study adopted the method of local drug injection, the clinical safety and feasibility of which must be further evaluated. Additionally, our study only focused on the effect of salvianolic acid B on macrophages but not on the changes in other inflammatory cells, such as neutrophils, warranting further study.

This study suggests salvianolic acid B as a promising treatment to improve the effect of fat transplantation.

Conclusions

Our study reveals the mechanism of salvianolic acid B in regulating the inflammation of autologous fat grafts and its potential value in translational applications. We found that salvianolic acid B inhibits the activation of macrophage inflammatory signalling pathways by inhibiting the phosphorylation of NF- κ B and JNK. Additionally, salvianolic acid B inhibits the proliferation of macrophages. Thus, salvianolic acid B may be a promising agent to reduce the complications of fat transplantation.

Abbreviations

AFG: Autologous fat grafting; **CCK8:** Cell Counting Kit-8; **CT:** Computed tomography; **DMEM:** Dulbecco's modified Eagle medium; **ECM:** Extracellular matrix; **EdU:** 5-ethynyl-2'-deoxyuridine; **FBS:** Fetal bovine serum; **FITC:** Fluorescein isothiocyanate; **GSEA:** Gene Set Enrichment Analysis; **h-ADSC:** Human adipose stem cells; **HE:** Hematoxylin-eosin staining; **IF:** Immunofluorescence; **IKK β :** inhibitor of nuclear factor kappa B kinase subunit beta; **iNOS:** inducible nitric oxide synthase; **JNK:** c-Jun NH2-terminal kinase; **KEGG:** Kyoto Encyclopedia of Genes and Genomes; **LPS:** Lipopolysaccharide; **NF- κ B:** nuclear factor of kappa light polypeptide gene enhancer in B cells; **p-p65:** Phosphorylated p65; **PBS:** Phosphate-buffered saline; **PDB:** Protein Data Bank; **RNA:** Ribonucleic acid; **RNase:** Ribonuclease; **SM:** Salvia miltiorrhiza; **SVF:** Stromal vascular fraction; **TGF- β :** transforming growth factor beta; **TNF- α :** tumor necrosis factor a;

Declarations

Acknowledgements

Not applicable.

Authors' contributions

DZ, LY and YZ contributed to the conception, study design and data interpretation. JS, YG and CH conducted the study and performed the experimentation, manuscript writing and editing. CC and YL contributed to the data collection and analysis. JS and CH contributed to the sample collection and processing. All authors read and approved the final manuscript.

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Availability of data and materials

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

Ethics declarations

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Shanghai Ninth People's Hospital and complied with the principles of the Declaration of Helsinki. Consent to participate is not applicable.

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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Figures

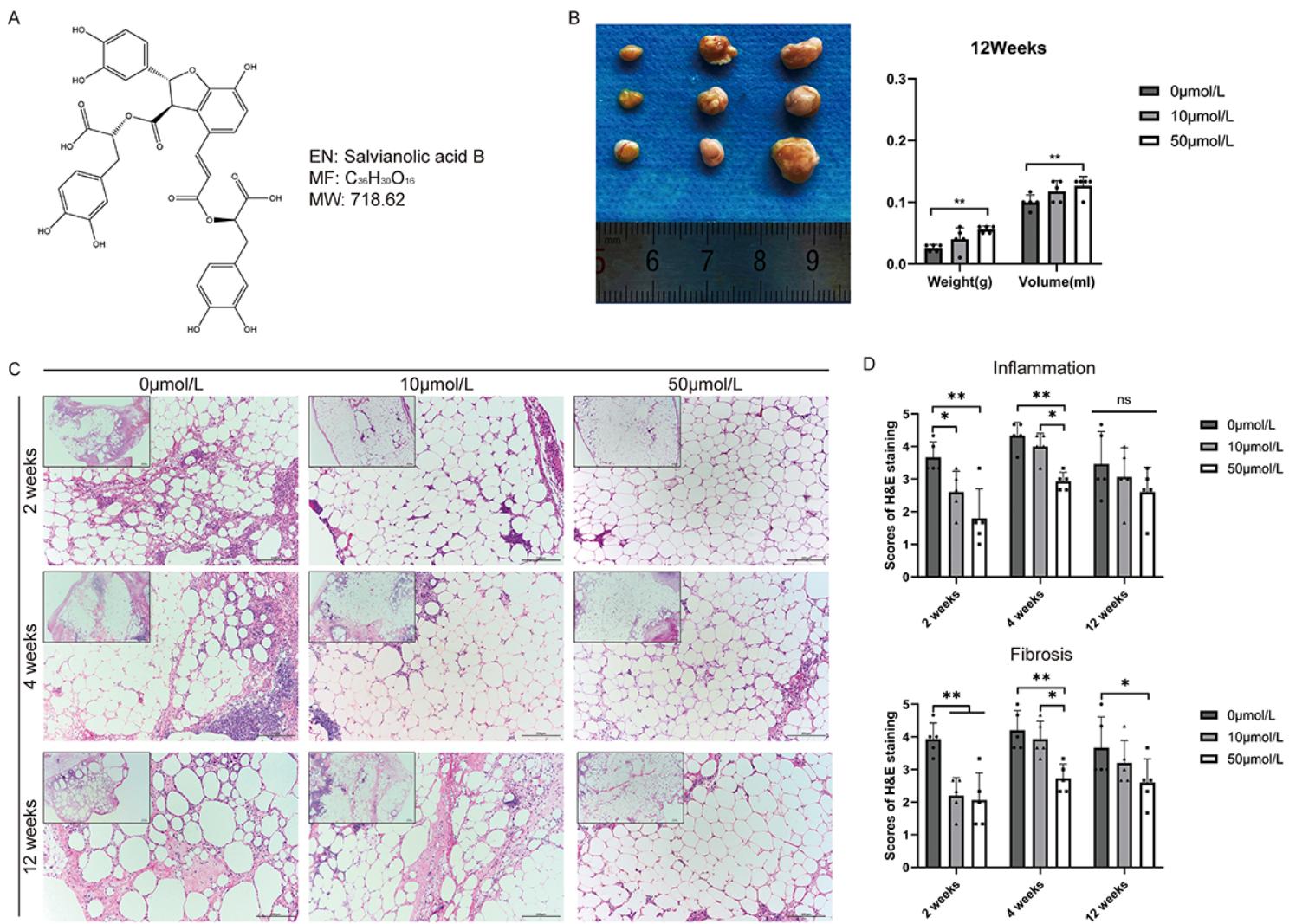


Figure 1

Salvianolic acid B reduces the level of inflammation and fibrosis in fat grafts. (A) Chemical structure of Salvianolic acid B (Sal-B). (B) Macroscopic views of harvested tissue in the control and Sal-B-treated groups at 12 weeks ($n = 5$ per group). (C) Images of H&E-stained tissue sections from the control group and Sal-B-treated group at different time points (scale bar = 200 μm). (D) Quantification of H&E staining: tissue inflammation and the extent of fibrosis. The data represent the means \pm SD. * $P < 0.05$, ** $P < 0.01$. SD, standard deviation.

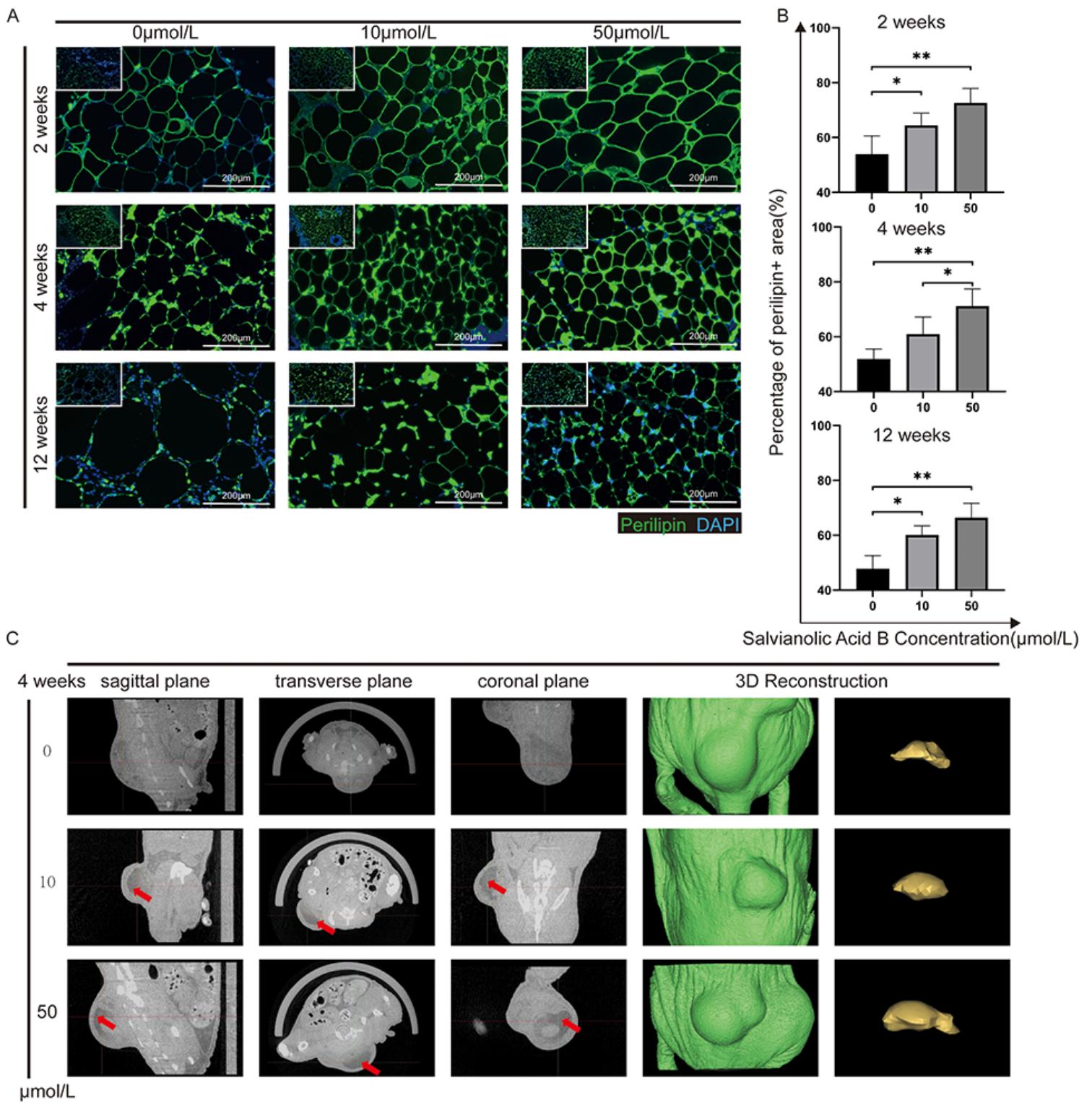


Figure 2

Salvianolic acid B promotes the survival rate of fat transplantation. (A) Restoration of subcutaneous fat tissue in the Sal-B-treated group was confirmed by immunofluorescence staining of perilipin (green). DAPI (blue) staining of nuclei (scale bar = 200 μm). (B) Quantification of immunofluorescence staining of perilipin. The level of living adipocytes was measured as the mean fluorescence intensity of perilipin (IOD/area). (C) Micro-CT analysis of subcutaneous transplantation in three sections (sagittal, transverse,

and coronal sections). The data are represented as means \pm SD. * P<0.05, ** P<0.01. ADSCs, adipose tissue-derived stromal cells; Micro-CT: microcomputed tomography program; SD, standard deviation.

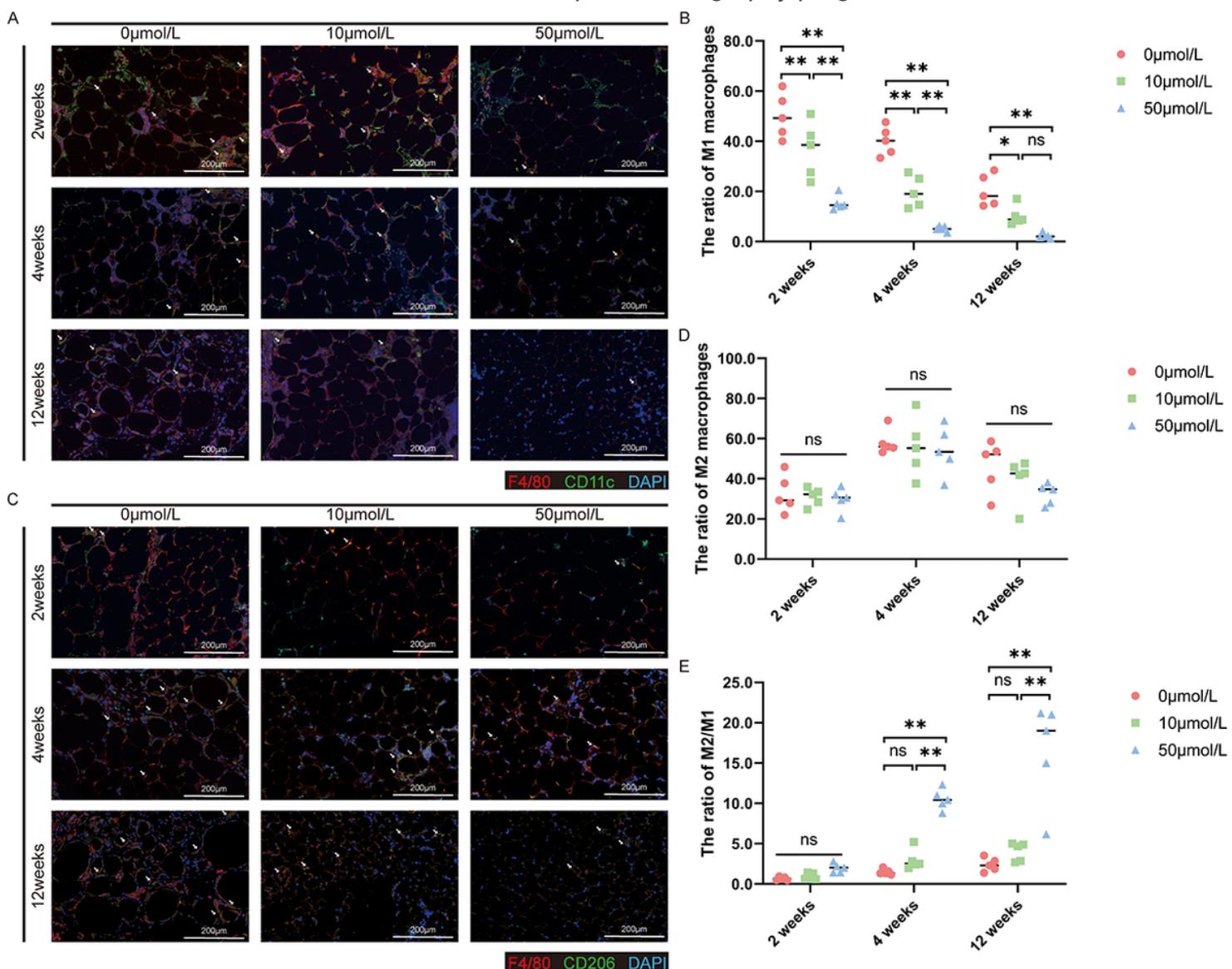
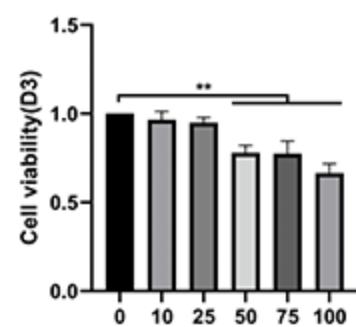


Figure 3

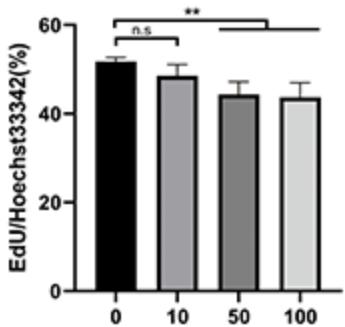
Salvianolic acid B reduces the activation of M1-type macrophages in fat grafts. (A) M1 macrophages in the control and Sal-B-treated groups at different time points were confirmed by immunofluorescence staining of F4/80 (red) and CD11c (green) (scale bar = 200 μm). (B) The number of M1 macrophages was counted manually in each field, and three fields were counted for each fat graft in the control and Sal-B-treated groups at different time points (n = 5 per time point). The proportion of M1 macrophages (%) = $100 \times \text{Number of M1 macrophages} / \text{Number of total macrophages (F4/80+ cells, red)}$. (C) The M2 macrophages in the control and Sal-B-treated groups at different time points were confirmed by immunofluorescence staining of F4/80 (red) and CD206 (green) (scale bar = 200 μm). (D) The number of M2 macrophages was counted manually in each field, and three fields were counted for each fat graft in the control and Sal-B-treated groups at different time points (n = 5 per time point). The proportion of M2 macrophages (%) = $100 \times \text{Number of M2 macrophages} / \text{Number of total macrophages (F4/80+ cells, red)}$. (E) The ratio of M2/M1.

(E) Ratio of M2/M1 in the control and Sal-B-treated groups at different time points. The data are represented as means \pm SD. * P<0.05, ** P<0.01. SD, standard deviation.

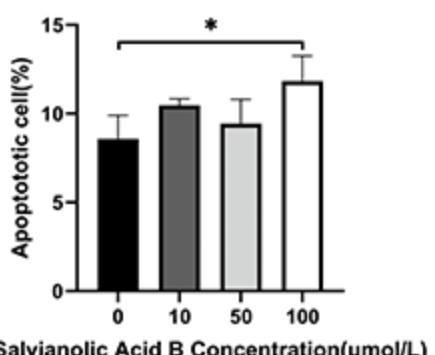
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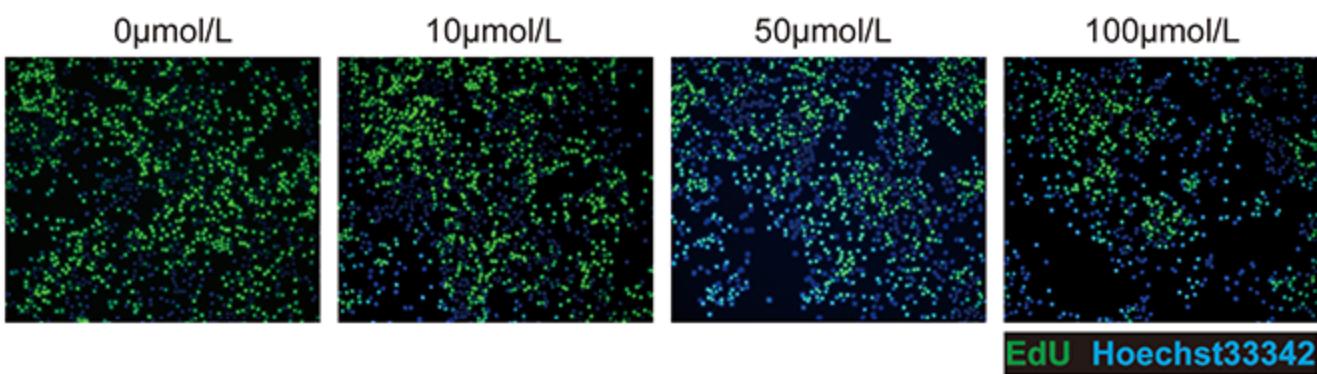
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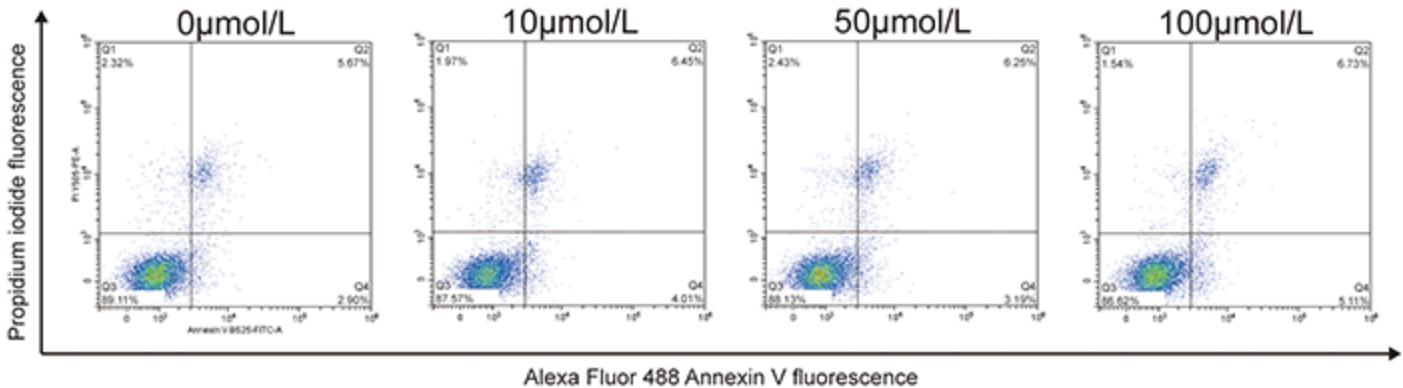


Figure 4

Salvianolic acid B inhibits the proliferation of macrophages. (A) Raw264.7 cell viability was determined using the CCK-8 reagent after Sal-B treatment for 72 h. (B) The EdU (green) proliferation assay was performed 24 hours after the addition of 10, 50 or 100 $\mu\text{mol/L}$ of Sal-B (PBS was added instead in the control group). Hoechst33342 (blue) staining of nuclei. Bar = 200 μm . (C) Apoptosis was detected by flow cytometry after treatment of RAW264.7 cells with Sal-B for 3 days. (D) The number of Edu+ cells was counted manually in each field, and three fields were counted for each of the 3 experiments in the control and Sal-B-treated groups. (E) Ratio of apoptotic cells (n=3). The data are represented as means \pm SD. * P<0.05, ** P<0.01. SD, standard deviation.

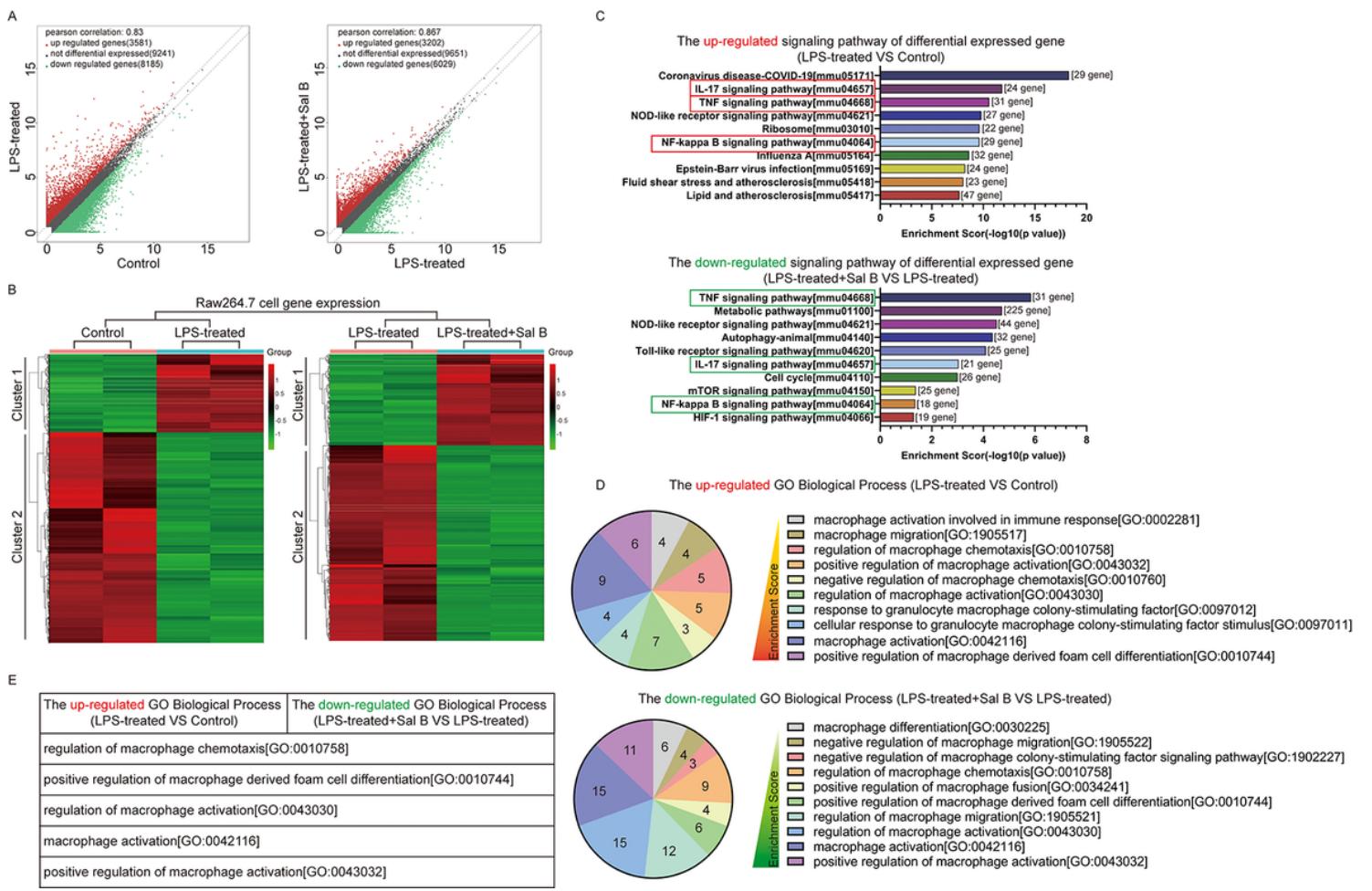


Figure 5

Transcriptomics data of RAW264.7 cells in the control group, LPS-treated group and Sal-B additional group. (A) Scatter diagram of differential expression analysis of RNA sequencing data from the control group, LPS-treated group and LPS-treated+Sal-B additional group (left: control group vs. LPS-treated group; right: LPS-treated vs. LPS-treated+Sal-B additional group). The cut-off for defining which genes were differentially expressed was a fold change greater than 1.5. (B) Heatmap of differential expression analysis of RNA sequencing data from the control group, LPS-treated group and LPS-treated+Sal-B additional group (left: control group vs. LPS-treated group; right: LPS-treated vs. LPS-treated+Sal-B additional group). (C) KEGG analysis of differentially expressed genes in the control group, LPS-treated group and LPS-treated+Sal-B additional group (Up: control group vs. LPS-treated group; Down: LPS-treated vs. LPS-treated+Sal-B additional group). (D). GO biological process of the differentially expressed genes in the control group, LPS-treated group and LPS-treated+Sal-B additional group (Up: control group vs. LPS-treated group; Down: LPS-treated group vs. LPS-treated+Sal-B additional group). (E) Overlap of GO biological processes between the 'control group vs. LPS-treated group' and the 'LPS-treated group vs. LPS-treated+Sal-B additional group'. KEGG, Kyoto Encyclopedia of Genes and Genomes;

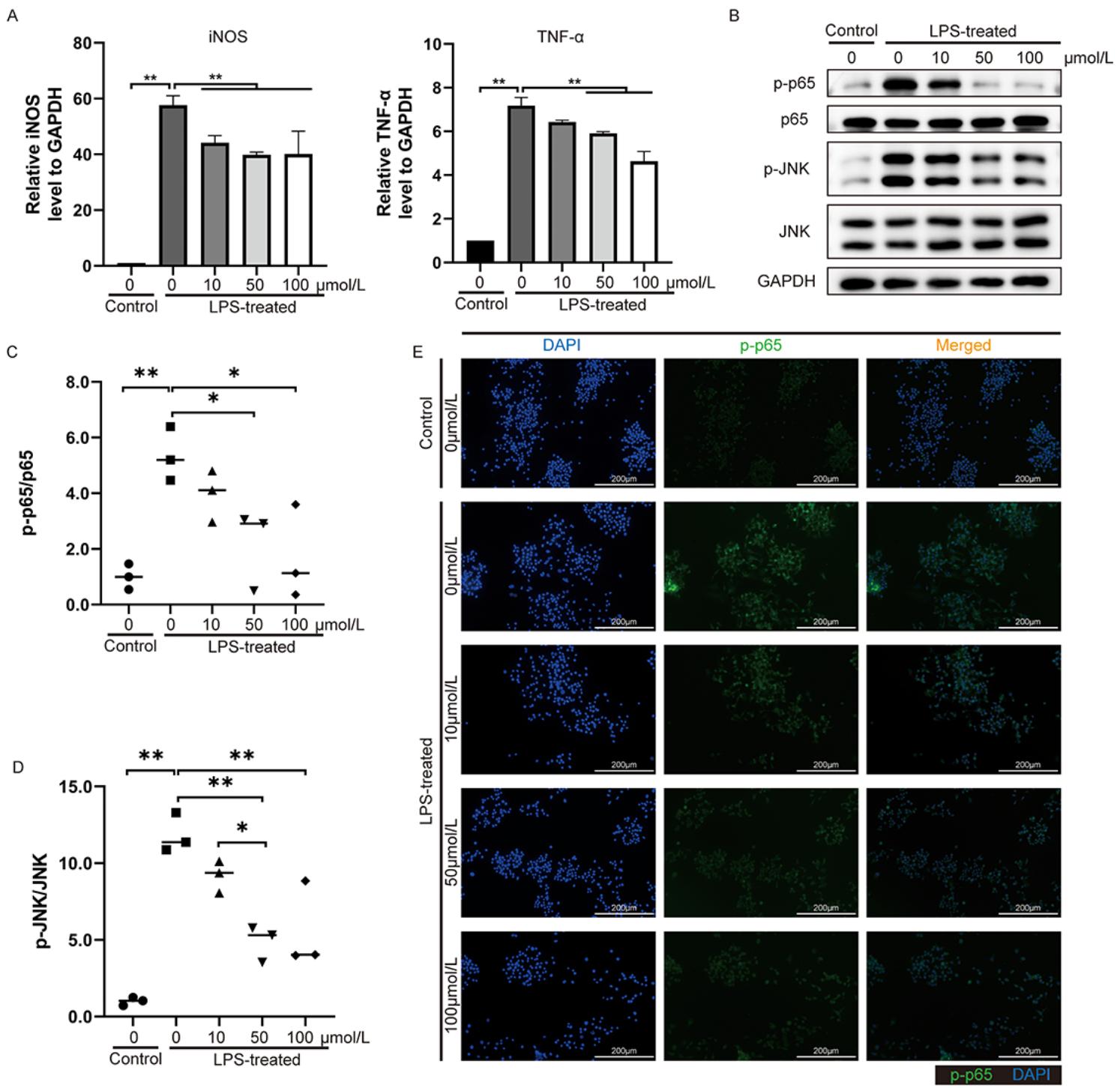


Figure 6

Salvianolic acid B inhibits LPS-induced macrophage polarization to the M1 type by inhibiting the LPS signalling pathway. (A) Relative mRNA expression of iNOS and TNF- α after Sal-B treatment in the LPS-treated group. (B) Relative protein expression of p-p65, p65, p-JNK, and JNK after Sal-B treatment in the LPS-treated group. (C) Expression of p-p65 relative to p65. (D) Expression of p-JNK relative to JNK. (E) Immunofluorescence assays were performed to determine the translocation of p-p65 in cells. The data are represented as means \pm SD. * P<0.05, ** P<0.01. SD, standard deviation.

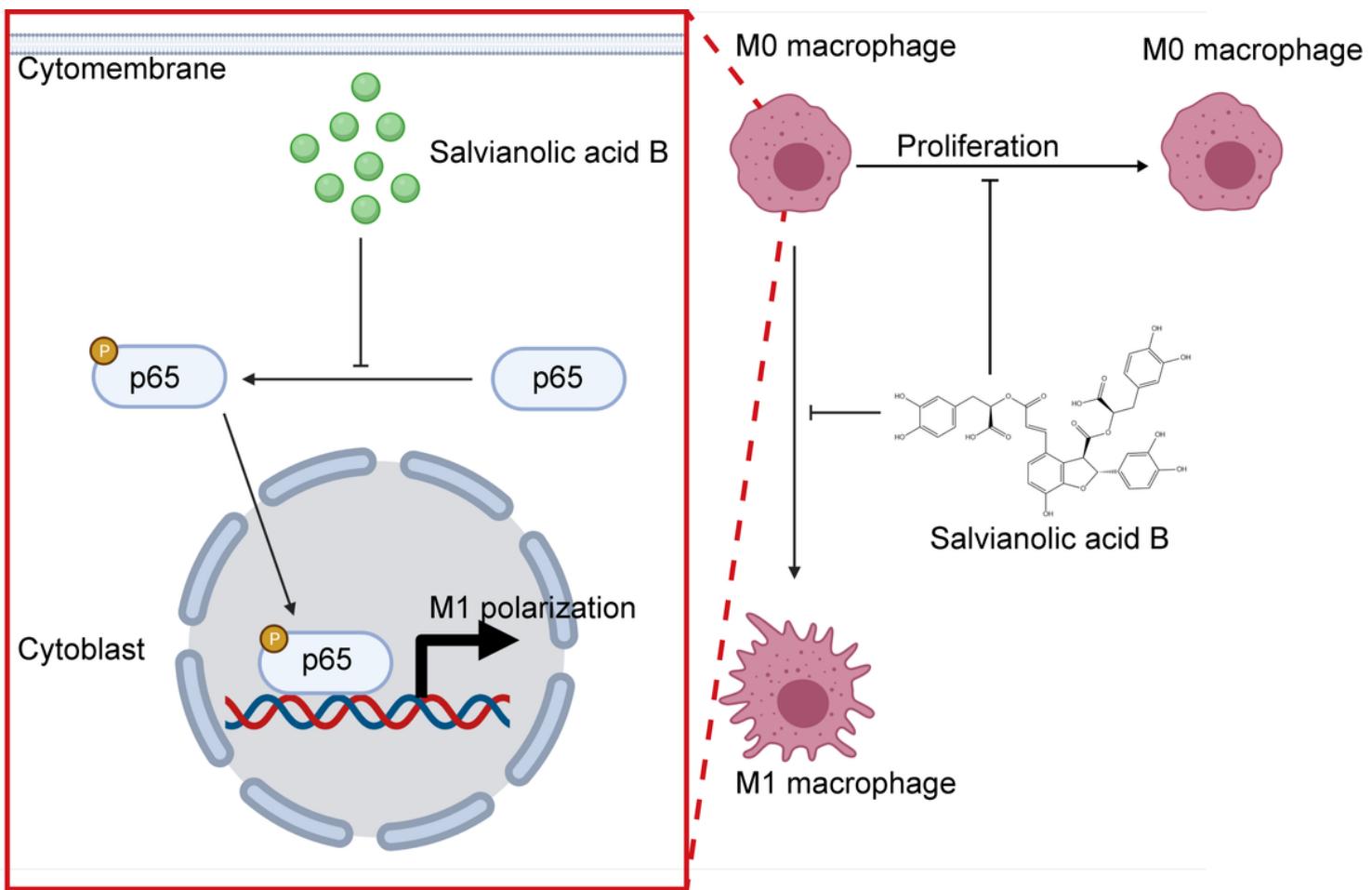


Figure 7

A schematic diagram of the proposed mechanism of salvianolic acid B improves graft survival. salvianolic acid B inhibits the activation of macrophage inflammatory signaling pathways by inhibiting the phosphorylation of p65. Meanwhile, salvianolic acid B can inhibit the proliferation of macrophages.

Supplementary Files

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- [SupplementaryTable1.docx](#)
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