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Interleukin-10 modified bone marrow mesenchymal stem cells prevent hypertrophic scar formation by inhibiting inflammation

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This study was performed to examine the effect of Interleukin-10 (IL-10) modified bone marrow mesenchymal stem cells (BMSCs) on hypertrophic scar formation on the rabbit ear hypertrophic scar model. Rabbit BMSCs were obtained by whole bone marrow adherence method and IL-10-modified BMSCs (IL-10-BMSCs) were established by transfecting BMSCs with an adenovirus. We treated the rabbit ear hypertrophic scar with BMSCs and IL-10-BMSCs, then evaluated the area and measured the height of the hypertrophic scar, and detected expression using real-time PCR and western blot. Compared with wild type BMSCs, the proliferative capability of IL-10 modified BMSCs was significantly reduced, but the expression of IL-10 in IL-10-BMSCs was significantly increased. After treating with a local injection of BMSCs or IL-10-BMSCs in the rabbit ear hypertrophic scar, we found that the time of wound healing, the area and height of scar were all significantly reduced in the IL-10-BMSCs group when compared to those in the BMSCs group. Moreover, the expression of Collagen-I, α-SMA, TNF-α , IL-6 and IL-1β mRNA, the number of CD45-positive cells, CD3-positive cells and ED-1-positive cells, and the expression of p-IKBα / IKBα , p-p65 / p65 , p-JNK / JNK and p-c-JUN / c-JUN in the scar of the IL-10-BMSCs group were significantly lower than those in BMSCs group. IL-10 modified BMSCs prevented hypertrophic scar formation in the rabbit ear hypertrophic scar model, and the results suggest this could be due to the inhibition of inflammation by IL-10 modified BMSCs through the JNK / NF-κB pathway.

1. Introduction

Skin wounds are affected by inflammation, cell proliferation and tissue remodeling during the healing process to form hypertrophic scars. Although the mechanism of hypertrophic scar formation has not currently been fully elucidated, it has been reported that inflammation (Eming et al. 2014; Wang et al. 2015) and immune responses (Enoch and Leaper 2008; Ogawa 2017) play important roles in the healing of scar wounds. Therefore, inhibition of excessive inflammation during wound healing may help prevent the formation of hypertrophic scars.

The results of preclinical/experimentation and clinical trials have shown that mesenchymal stem cells, such as bone marrow mesenchymal stem cells (BMSCs) (Kharaziha et al. 2009), umbilical cord mesenchymal stem cells (Zhang et al. 2012), and chorionic lining mesenchymal stem cells (Lee et al. 2010), adipose-derived mesenchymal stem cells (Cherubino et al. 2011), have the ability to promote scarless healing and inhibit tissue fibrosis in skin wounds. BMSCs have the ability to self-replication and continuous renewal, but also has certain plasticity (ie, multi-directional differentiation ability) and can differentiate into bone cells, nerve cells and skin epithelial cells, under specific culture conditions (Hempel et al. 2016; Woodbury et al. 2000). Compared with other adult stem cells, BMSCs have many advantages, such as high content of adult individuals, abundant sources, safe and convenient materials, simple *in vitro* culture methods, rapid proliferation, etc., and are recognized as the most ideal source of tissue engineering seed cells (Li et al. 2016). However, many previous studies have shown that although BMSCs can inhibit the formation of hypertrophic scars by regulating cell migration, proliferation, and accumulation of extracellular matrix *in vitro* (Fang et al. 2016), clinical trials have found that their effects are limited (Ohishi and Schipani 2010; Wu et al. 2007). In order to improve the efficacy of BMSCs transplantation in the prevention of scars, researchers have experimented with the injection site of BMSCs and combination with drugs and other methods, but there are few research reports on genetic engineering methods to transform BMSCs for the prevention of a scar. Interleukin-10 (IL-10) is a cytokine synthesis inhibitory factor secreted by murine Th2 cells, originally discovered by Mosmannand et al. (Moore et al. 1993). Currently, IL-10 has been found to be produced by a variety of cells, such as CD4+ T cells, CD8+ T cells, NK cells, and macrophages (Madan 2009; Sabat et al. 2010). Previous studies have shown that IL-10 is a cytokine with multi-directional regulation, such as immunosuppression and inflammation suppression. In fact, whether BMSCs treat scars or IL-10 anti-inflammatory, these have been studied by many scholars. However, the study on hypertrophic scar formation with the treatment of IL-10 modified BMSCs is innovation. The treatment of IL-10 modified BMSCs combines the advantages of BMSCs and IL-10 on inhibiting the formation of hypertrophic scar. Importantly, we not only introduced a potential method for preventing scar formation, but also revealed some molecular mechanisms which help to provide a reference for the development of drugs to treat scar formation. In this study, we established IL-10-modified BMSCs, which when injected locally prevented the formation of hypertrophic scars. We found that IL-10-BMSCs secreted higher levels of IL-10, and could significantly inhibit the formation of hypertrophic scars by inhibiting inflammation when compared to BMSCs.

2. Investigations and results

2.1. IL-10 modification promoted IL-10 expression in BMSCs

We isolated and cultured rabbit BMSCs, which were small spindle-shaped cells with relatively uniform morphology. The purity of BMSCs was analyzed by flow cytometry, and the BMSCs were determined to be negative for CD34 and CD45, and positive for CD44 and CD90. In addition, when compared to wild type BMSCs, the proliferative capability of IL-10 modified BMSCs was significantly reduced; however, the expression of IL-10 in the IL-10 modified BMSCs was significantly increased (Fig. 1).

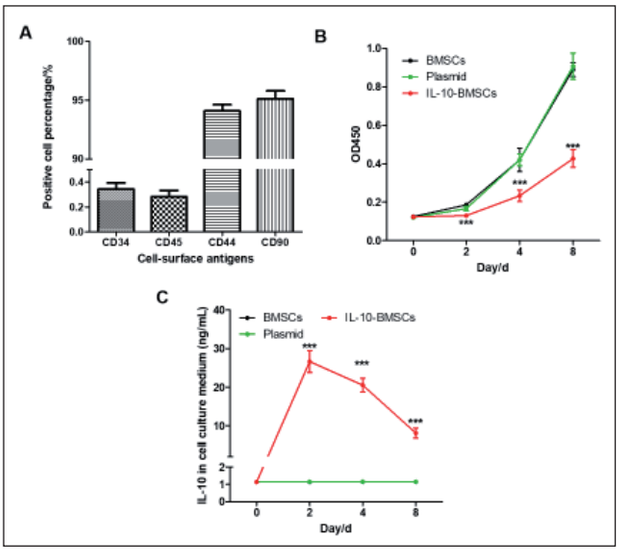

Fig. 1: IL-10 modified BMSCs inhibits BMSCs proliferation and promotes IL-10 secretion. A, Proportion of CD34, CD45, CD44 and CD90 positive cells using flow cytometry to identify BMSCs; B, CCk8 kit was used to detect the proliferation of BMSCs; C, Concentration of IL-10 in cell culture medium using elisa kit. 3 independent repetitions for each test, and *** was P<0.001 vs BMSCs group. BMSCs, Wild-type BMSCs; Plasmid, BMSCs transferred into plasmid; IL-10-BMSCs transfected with plasmids containing IL-10 expression sequences.

2.2. IL-10-BMSCs prevented the hypertrophic scar formation

The rabbit ear hypertrophic scar was treated by local injections of BMSCs or IL-10-BMSCs, and observed the healing of each wound every day. As shown in Fig. 2A, the time of wound healing in the BMSCs group was significantly shorter than that in the control group. Furthermore, the time of wound healing in the IL-10-BMSCs group was significantly less than that in BMSCs group (Fig. 2B). On postoperative day 28, we measured the scar area and height in all three groups, and found that the average scar areas and scar heights in the IL-10-BMSCs group were all the smallest among the three groups (Fig. 2C). Moreover, we found on postoperative day 7, the expression of collagen-I and α-SMA mRNA in scar tissues of IL-10-BMSCs group were significantly reduced when was compared to the BMSCs group. Also, the expression of Collagen-I and α-SMA mRNA in BMSCs group were also significantly lower than those in control group (Fig. 2C and 2D).

2.3. IL-10-BMSCs inhibited inflammation in the hypertrophic scar

Early inflammation is the main cause of hypertrophic scar formation, and cytokines, such as TNF-α, IL-6 and IL-1β, play a pro-inflammatory role in the early stage of scar formation. On postoperative day 3, the expression of TNF-α (Fig. 3A), IL-6 (Fig. 3B) and IL-1β (Fig. 3C) mRNA in the IL-10-BMSCs group were significantly lower to those in the BMSCs group. Similarly, the expression of TNF-α, IL-6

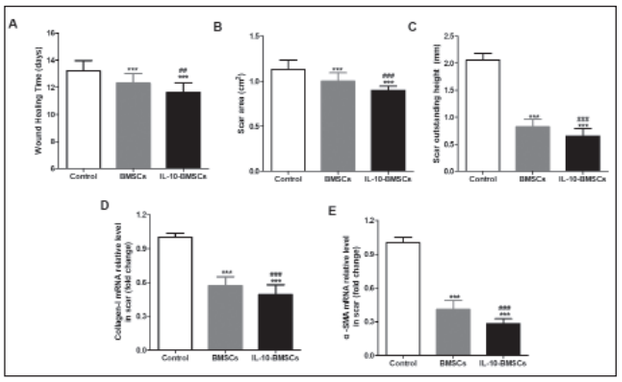

Fig. 2: Effect of IL-10 modified BMSCs on the scar formation in rabbit ears. A, The time of wound healing in rabbit ears in different group; B-C, The scar area (B) and scar outstanding height (C) on postoperative day 28 (n=12); D-E, The expression of Collagen-I (D) and α-SMA (E) mRNA in scar tissues on postoperative day 7 (n=8). *** was P<0.001 vs Control group, and ### was P<0.001 vs BMSCs group. Control, Wounds that have not received any treatment in rabbit ears; BMSCs, Wounds that have received the treatment of wild-type BMSCs in rabbit ears; IL-10-BMSCs, Wounds that have received the treatment of IL-10 modified BMSCs in rabbit ears.

and IL-1β mRNA in the BMSCs group was also lower than those in the control group. Immune cell infiltration, such as T cells, white blood cells and macrophages, is the initial stage of inflammation. We found that the number of CD45-positive cells, CD3-positive cells and ED-1-positive cells in the IL-10-BMSCs group were significantly lower than those in the BMSCs group. Compared with the control group, the number of CD45-positive cells, CD3-positive cells and ED-1-positive cells in the BMSCs group were also significantly reduced (Figs. 3D-F, respectively).

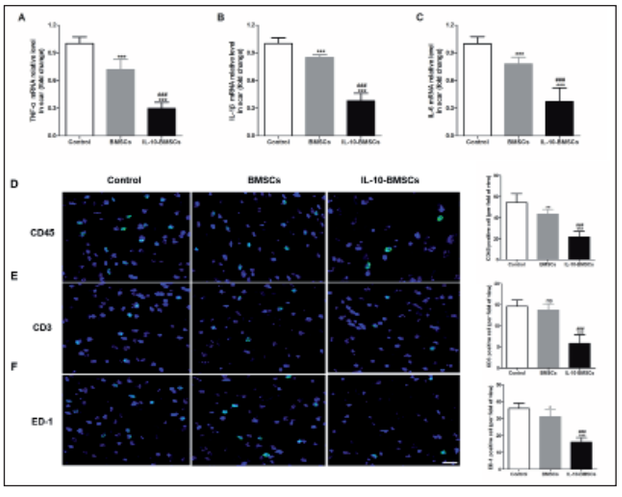

Fig. 3: Effect of IL-10 modified BMSCs on the inflammation in scar tissues. A-C, The expression of TNF-α (A), IL-1β (B) and IL-6 (C) mRNA in wound area tissues on postoperative day 3 (n=8); D-F, Local injection of BMSCs or IL-10-BMSCs prevented CD45-positive cell (D) , CD3-positive cell (E) and ED-1-positive cell (F) infiltration wound area tissues on postoperative day 3 (n=8). Scale bar=100 μm. ns was P>0.05, * was P<0.05, ** was P<0.01 and *** was P<0.001 vs Control group; ### was P<0.001 vs BMSCs group. Control, wounds that have not received any treatment in rabbit ears; BMSCs, wounds that have received the treatment of wild-type BMSCs in rabbit ears; IL-10-BMSCs, wounds that have received the treatment of IL-10 modified BMSCs in rabbit ears.

2.4. IL-10-BMSCs inhibit the JNK / NF-κB pathway in the hypertrophic scar

JNK/ NF-κB pathway is a signaling pathway closely related to inflammation, so we measured the expression of JNK/ NF-κB pathway in scar tissues on postoperative day 7. As shown in Fig. 4,

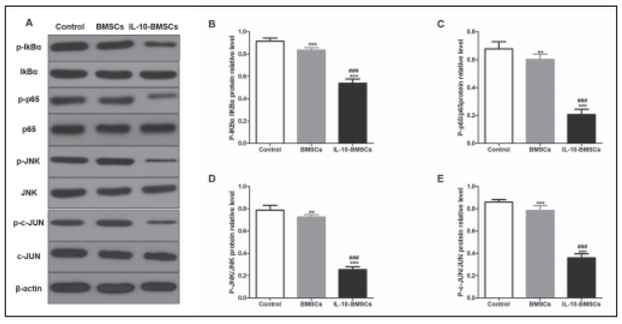

Fig. 4: Effect of IL-10 modified BMSCs on the JNK / NF-κB pathway in scar tissues. A-E, Representative images of protein brands (A), and the analysis of the expression of p-IKBα / IKBα (B), p-p65 / p65 (C), p-JNK / JNK (D) and p-c-JUN / c-JUN (E) in wound area tissues on postoperative day 3 (n=8). ** was P<0.01 and *** was P<0.001 vs Controlgroup; ### was P<0.001 vs BMSCs group. Control, Wounds that have not received any treatment in rabbit ears; BMSCs, Wounds that have received the treatment of wild-type BMSCs in rabbit ears; IL-10-BMSCs, Wounds that have received the treatment of IL-10 modified BMSCs in rabbit ears.

the expression of p-IKBα / IKBα, p-p65 / p65, p-JNK / JNK and p-c-JUN / c-JUN in the BMSCs group were significantly lower than those in Control group. Similarly, compared with the BMSCs group, those protein expressions were also significantly reduced in the IL-10-BMSCs group.

3. Discussion

IL-10 was first found to be synthesized and secreted by murine CD4+ Th2 cells and function in the inhabitation of the synthesis of IFN-γ by Th1-T cells. As studies progressed, the immunological characteristics of IL-10 were revealed. In terms of immunology, IL-10 has the ability to prevent the proliferation of antigen-specific T cells, inhibit the ability of the antigen-presenting cells to present, and inhibit the synthesis and expression of inflammatory cytokines and inflammatory mediators. All in all, IL-10 is a cytokine that inhibits the inflammatory response and immunosuppression of the body (Fujii and Lotze 2007). Therefore, IL-10 is widely used in the treatment of autoimmune diseases as a natural immunosuppressive agent (Dambuza et al. 2017; Groux and Cottrez 2003). Previous studies have also found that IL-10 could inhibit liver fibrosis (Guo et al. 2015; Mentink-Kane et al. 2011), promote healing (Leung et al. 2013; Peranteau et al. 2008) and be a possible indicator of wound vitality (Ohshima and Sato 1998). However, due to the short half-life of IL-10 and poor targeting, the effect of injection of IL-10 is poor. Therefore, in the present study, we established IL-10-modified BMSCs (IL-10-BMSCs) by transfecting BMSCs with an adenovirus. Here, we identified BMSCs by identifying cell surface markers, without conducting the experiments about differentiation analysis of BMSCs (adipogenesis, osteogenesis and chondrogenesis), as many previous studies did. We found that the proliferative capability of IL-10 modified BMSCs was significantly lower than that in BMSCs, and the expression of IL-10 in the IL-10 modified BMSCs was significantly higher compared to BMSCs. This might relate to the production of IL-10. Previous studies have found that IL-10 inhibits cell proliferation (Bejarano et al. 1992; Louis et al. 1998; Malefyt et al. 1991). Of course, these studies were done on BMSCs, but can at least explain that IL-10 may inhibit the proliferation of BMSCs. Therefore, IL-10 modified BMSCs secrete more IL-10 into the cell culture medium, which inhibits the proliferation of BMSCs. However, the levels of IL-10 expression in the IL-10-BMSCs gradually decreased over time after transfection, which may be related to the decrease of proliferation in the IL-10-BMSCs. All in all, we have successfully constructed BMSCs that show a high expression of IL-10.

We injected IL-10-BMSCs or BMSCs locally into the wounds, and found faster wound healing times, and smaller hypertrophic scar area and height after treatment with IL-10-BMSCs. Previous studies have shown that IL-10 promotes wound healing. Leung et al. (2013) found that lentiviral mediated IL-10 over-expression enhanced wound healing *via* STAT3 dependent increase in endothelial progenitor cells; Bodaan et al (2016) found that transfected IL-10 by off virus could reduce inflammation at the wound and promote wound healing. Furthermore, Wise et al. (2014) found that orf virus IL-10 accelerated wound healing while limiting inflammation and scarring; Kozin et al. (2004) found that adenovirus-transfected IL-10 can make scar-free repair of wounds by differentially regulating hyaluronan synthase 1. Combined with the results of the present study, it is suggested that IL-10-BMSCs promotes wound healing and prevention of the formation of hypertrophic scars, which might be related to the increased levels of IL-10 expression.

To further investigate the molecular mechanism of IL-10 in preventing scar formation, we examined the expression levels of inflammatory factors and immune cell infiltration around the wound three days after injection of BMSCs or IL-10-BMSCs, and found that the expression of TNF-α , IL-6 and IL-1β mRNA in the IL-10-BMSCs group was significantly reduced, and the CD45-positive cells, CD3-positive cells and ED-1-positive cells in IL-10-BMSCs group were significantly lower than those in BMSCs group. These results combined suggest that when compared with BMSCs treatment, local injection of IL-10-BMSCs could significantly reduce inflammation of the area around the wound tissue.

When adult skin tissue is damaged to a certain depth, a waterfall-like inflammatory reaction occurs early in the wound surface, and early fibrin clots release chemokines, causing a large number of neutrophils to migrate to the wound area. Then macrophages instead of neutrophils migrate to the wound surface and enter the advanced inflammatory reaction stage (Reinke and Sorg 2012). Macrophages have antibacterial activity, clear necrotic tissue, and simultaneously release certain amounts of cytokines. On one hand, inflammation acts as an immune barrier against infection; on the other hand, inflammation stimulates fibrous tissue formation to close the wound. Therefore, a moderate inflammatory response is beneficial to wound healing. However, the infiltration of a large number of inflammatory cells (such as neutrophils, macrophages, etc.) and the production of pro-inflammatory factors (such as IL-1β, TNF-α, IL-6, etc.) not only aggravate the inflammatory response, but also lead to excessive deposition of extracellular matrix and tissue fibrosis. Excessive deposition of extracellular matrix leads to the formation of hypertrophic scars. Previous studies also found that inflammatory cells could also produce some growth factors, such as TGF-β and PDGF, which promoted the proliferation and differentiation of fibroblasts and a large number of extracellular matrix components (such as collagen I, III and proteoglycan), inhibiting extracellular matrix degrading enzyme production, ultimately resulting in extracellular matrix unbalanced synthesis and degradation leads to the formation of pathological scars (Sun et al. 2014).

In addition, Kieran et al. (2013) found that IL-10 could promote wound healing and reduce scar formation by inhibiting the inflammatory reaction by comparing the skin soft tissue defect of IL-10 knockout mice and wild type mice. King et al. (2014) reported that IL-10 could inhibit IL-6, IL-8 and TGF-β synthesis, reduce scar formation, but also regulate extracellular matrix (especially promoting hyaluronic acid synthesis), endothelial progenitor cells, enhance fibroblast activity and is expected to promote skin regeneration. These indicated that IL-10-BMSCs could inhibit scar formation by regulating inflammatory responses and immune responses.

We also found that the expression of p-IKBα/IKBα, p-p65/p65, p-JNK/JNK and p-c-JUN / c-JUN was significantly reduced in the IL-10-BMSCs group. JNK/NF-κB pathway is a signaling pathway closely related to inflammation regulation. It was found that curcumin exerts anti-inflammatory effects by inhibiting the ERK/JNK/NF-kB signaling pathway (Jiang et al. 2016); Sadeghi et al. (2018) found that curcumin meliorated palmitate-induced inflammation in skeletal muscle cells by inhibiting the JNK/NF-kB pathway. At the same time, IL-10 has been found to inhibit the JNK/NF-kB pathway, and to attenuate TNF-α-induced NF-kB pathway activation and cardiomyocyte apoptosis. Overexpression of IL-10 in activated microglia inhibited neuroinflammation by inhibiting ERK1/2, JNK, and NF-κB.

In conclusion, IL-10 modified BMSCs prevented hypertrophic scar formation in the rabbit ear hypertrophic scar model. This decrease in scar formation may be the result of the inhibition of inflammation by IL-10 modified BMSCs through the JNK/NF-κB pathway. IL-10 modified BMSCs could be promising in scar-free wound healing, after further examination on large-scale animal experiments.

4. Experimental

4.1. Isolation, culture and identification of rabbit BMSCs

All animal experiments were in accordance with the Animal Ethics Committee of Plastic Surgery Hospital, Chinese Academy of Medical Sciences & Peking Union Medical College, and meet the NIH guidelines for the care and use of laboratory animals. The New Zealand white female rabbits (12 weeks old, 2800-3000 g) were anesthetized by intramuscular administration of 5% pentobarbitalum natricum (25 mg/kg), followed by euthanasia by ear vein air embolization. First, we monitored the anesthesia status by testing the rabbit's posture, eyeballs, and vomiting, and then determined the death of rabbit by observing the heartbeat and breathing of rabbit. Both ends of the humerus were removed and the bone marrow cavity was flushed and collected with pre-warmed DMEM medium (10569044, Gibco, USA) until the medullary cavity turned white. The collected DMEM was plated on a cell culture dish and placed in a cell incubator at 5% CO_2 and 37 °C. After 24 h, half of the medium was exchanged, which was then repeated every 3-4 days. At passage 3, BMSCs were harvested to obtain a single-cell suspension at a concentration of 1×10^6 cells/ml. 10 µl PE-CD34 (551387, BD Pharmingen, USA), PE-CD44 (563058, BD Pharmingen, USA), PE-CD45 (562452, BD Pharmingen, USA) and FITC-CD90 (553513, BD Pharmingen, USA) were incubated at 4 °C for 30 min. Fixed with 4% paraformaldehyde, then detected by flow cytometry (LSR II, BD Pharmingen, USA).

4.2. Establishment of IL-10 modified BMSCs

The rabbit IL-10 gene sequence (C_013684.1) was queried on the NCBI, and the ends of the IL-10 sequence were added the ends of the Sal I enzyme, then artificially synthesized (Sangon Biotech, Shanghai, China). We cloned the IL-10 with Sal 1 sites into the pDc316 recombinant plasmid, also digested with Sal I enzyme (NEB, CT, USA) with T4 DNA Ligase. The pDc316-IL10 was transfected into HEK293 cells (ATCC, VA, USA) according to Lipofectamine 2000 protocol and the adenovirus was harvested after repeated freezing and thawing. IL-10-modified BMSCs were established by transfecting BMSCs with the pDc316-IL10 adenovirus. We screened MSCs transfected with IL-10 by monoclonal technology. Briefly, to obtain a low concentration of MSC by dilution, we inoculated it into a 96-well cell culture plate. After one week, we observed each well and marked the culture wells with only one cell clone. Finally, we harvested the MSC in the monoclonal wells for further research.

4.3. Cell proliferation assay

CCK-8 cell proliferation assay kit was utilized to quantify the proliferation of BMSCs as described in the manufacturer's instructions. Briefly, a total of 2×10^3 cells/well were added to a 96-well culture plate, then 10 µl of CCK-8 reagent was added per well, and after 2 h of incubation at 37 °C with 5% CO_2, the absorbance was measured at a wavelength of 450 nm.

4.4. Rabbit ear hypertrophic scar model and treatment

A total of 24 the New Zealand white female rabbits (12 weeks old, 2800-3000 g) was used to establish rabbit ear hypertrophic scar model as previously described (Kloeters et al. 2007). Briefly, the rabbits were anesthetized by intramuscular administration of 5% pentobarbitalum natricum (50 mg / kg) and sumianxin (0.1 ml / kg), and then we used a 4 mm dermal biopsy punch (15110-40, PELCO, USA) to build 4 wounds on the ventral side of each ear (we defined this time as day 0). Here, we monitored the anesthesia status by testing the rabbit's posture, eyeballs, and vomiting. The wounds were randomly divided into 3 groups: control group, BMSCs group and IL-10-BMSCs group. 100 µl of phosphate buffer saline (PBS) containing nothing or 0.5×10^6 BMSCs or 0.5×10^6 IL-10-BMSCs were intradermally injected around each wound in control group, BMSCs group or IL-10-BMSCs group, respectively.

Table 1: Primers for RT-qPCR

Gene	Forward Sequences (5'-3')	Reverse Sequences (5'-3')
Collagen-I	CTGGCGGTTCAGGTCCAAT	TTCCAGGCAATCCACGAGC
α-SMA	TTCTGCATACGGTCAGCAAT	CATCCATGAAACCACCTACA
TNF-α	GAGGCCAAGCCCTGGTATG	CGGGCCGATTGATCTCAGC
IL-1β	GCAACTGTTCCTGAACTCAACT	ATCTTTTGGGGTCCGTCAACT
IL-6	CCTGAACCTTCCAAAGATGGC	TTCACCAGGCAAGTCTCCTCA
β-actin	GGCTGTATTCCCCTCCATCG	CCAGTTGGTAACAATGCCATGT

4.5. Real-time quantitative polymerase chain reaction (RT-qPCR)

An animal tissue total RNA extraction kit (DP431, Tian Gen, China) was used to exact the total RNA from the isolated scar tissues. cDNA was then synthesized from the total RNA using a reverse transcription kit (A3500, Promega, USA). As described in the manufacture's protocol for GoTaq qPCR (A6006A, Promega, USA), we used Xng of cDNA in a 20 µl RT-qPCR reaction. Primers for RT-qPCR was showed in the Table.

4.6. Immunofluorescence staining

The wound area tissues were harvested three days after treatment and examined for the presence of CD45, CD3 and ED-1 positive cells, which would reflect infiltrating cells into the wound. Frozen sections were processed and the slides were incubated with primary antibody overnight at 4 °C: CD45 antibody (GTX628507, 1:200, GeneTex, USA), CD3 antibody (GTX16669, 1:100, GeneTex, USA), and CD68 antibody (GTX41868, 1:200, GeneTex, USA). Secondary antibodies were incubated for 1 h at room temperature, then 5 µg/ml DAPI was used to stain the nuclei for 5 min at room temperature. Leica TCS SP5 microscope (Leica) with LAS AF Lite 4.0 image browser software (DMI3000, Leica, German) was used to analyze the fluorescence.

4.7. Western blot

One Step Animal Tissue Active Protein Extraction Kit (C500006-0020, Sangon Biotech, China) was used to extract the total protein from the scar tissues, and total protein concentrations were determined by BCA Protein Assay Kit (C503021-0500, Sangon Biotech, China). A total of 40 µg of protein was separated by a 10% SDS-PAGE. After the protein is transferred to a PVDF membrane, we used 5% skim milk to block at room temperature for 1 h. Primary antibody was incubated overnight at 4 °C: Anti-IKB alpha antibody [E130] (ab32518, 1:1000, Abcam, UK), Anti-IKB alpha (phospho S36) antibody [EPR6235(2)] (ab133462, 1:500, Abcam, UK), Anti-NF-kB p65 antibody (ab16502, 1:2000, Abcam, UK), Anti-NF-kB p65 (phospho S536) antibody (ab86299, 1:1000, Abcam, UK), Anti-JNK2 antibody [EP1595Y] (ab76125, 1:1500, Abcam, UK), Anti-JNK1 (phospho T183) antibody (ab47337, 1:1000, Abcam, UK), Anti-c-Jun antibody [EP693Y] (ab40766, 1:1000, Abcam, UK) and Anti-c-Jun (phospho S73) antibody (ab30620, 1:500, Abcam, UK). After washing three times with PBS-Tween 20, the membrane was incubated with secondary antibody at room temperature for 2 h: Goat Anti-Rabbit IgG H&L (HRP) (ab6721, 1:3000, Abcam, UK). After washing three times with PBS-Tween 20, ECL solution (WBKLS0100, Beijing Xinjingke Biotechnologies Co., Ltd, China) was added for detection.

4.8. Statistical analysis

Data in the present study were counted and analyzed in GraphPad Prism 5, and an unpaired t test was used to compared the difference between two groups, and one-way ANOVA with Duncan test as post hoc test to compare the difference between multiple groups. A p-value of less than 0.05 was considered significant.

Conflicts of interest: None declared

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