Clinical-grade hDPSCs Suppressed the Activation of Osteoarthritic Macrophages and Attenuated Cartilaginous Damage in a Rabbit OA Model

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Research Article

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Abstract

Background: Although increasing evidence has demonstrated that human dental pulp stem cells (hDPSCs) are efficacious for the clinical treatment of skeletal disorders, the underlying mechanisms remain incompletely understood. Osteoarthritis (OA) is one of the most common degenerative disorders in joints and is characterized by gradual and irreversible cartilaginous tissue damage. Notably, immune factors were newly identified to be closely related to OA development. In this study, we explored the modulatory effects of clinical-grade hDPSCs on osteoarthritic macrophages and their protective effects on cartilaginous tissues in OA joints.

Methods: The cell morphology, immunophenotype and inflammatory factor expression of osteoarthritic macrophages were explored. Additionally, the factors and signaling pathways that suppressed macrophage activation by hDPSCs were determined. Furthermore, hDPSCs were administered to a rabbit knee OA model via intra-articular injection. Macrophage activation in vivo and cartilaginous tissue damage were also evaluated. Statistical significance was analyzed using Student's t test. The one-way ANOVA was used in multiple group data analysis.

Results: We found that hDPSCs markedly inhibited osteoarthritic macrophage activation in vitro. The cell morphology, immunophenotype and inflammatory factor expression of osteoarthritic macrophages changed into less inflammatory status in the presence of hDPSCs. Mechanistically, we observed that hDPSC-derived HGF and TGF β 1 mediated the suppressive effects on osteoarthritic macrophages. Moreover, phosphorylation of MAPK pathway proteins contributed to osteoarthritic macrophage activation, and hDPSCs suppressed their activation by partially inactivating those pathways. Most importantly, injected hDPSCs inhibited macrophage activation in osteochondral tissues in a rabbit knee OA model in vivo. Further histological analysis showed that hDPSCs alleviated cartilaginous damage to knee joints.

Conclusions: In summary, our findings reveal a novel function for hDPSCs in suppressing osteoarthritic macrophages and suggest that macrophages are efficient cellular targets of hDPSCs for alleviation of cartilaginous damage in OA.

1. Introduction

Osteoarthritis (OA) is a disease of an entire synovial joint characterized by degradation of joint cartilaginous tissues, including articular cartilage and meniscus, and clinical symptoms, such as joint pain and disability [1, 2]. Although a number of therapeutic strategies have been developed to alleviate tissue damage in OA, including lifestyle improvement, pharmaceutical management, and arthroscopic debridement, currently, there are few curable treatments available until the end stage of the disease necessitates joint replacement [3, 4]. Accumulated studies prove that complex crosstalk of genetic, biochemical, biomechanical, and metabolic factors is involved in OA development [5]. Notably, increasing

evidence has demonstrated that activation of immune cells and subsequent inflammatory responses contribute to tissue injuries in OA [6–8].

Macrophages are hematopoietic stem cell-derived immune cells and have now been shown to play a prominent role in the progression of OA. Increasing evidence has demonstrated that overactivated macrophages promote the inflammatory microenvironment, increase the secretion of matrix metalloproteinases, inhibit the proliferation and viability of joint resident stem cells, and prevent cartilage repair [9–11]. Haraden et al. reported that osteoarthritic macrophages highly expressed costimulatory factors such as CD80 and CD86, which further initiated the adaptive immune response and induced expanded tissue injury [11, 12]. In addition, Fahy et al. stated that the osteoarthritic macrophage-associated cytokines interleukin-12 (IL-12) and tumor necrosis factor- α (TNF- α) induced destructive processes of articular cartilage by suppressing collagen type II and aggrecan synthesis [13]. Furthermore, osteoarthritic macrophages were shown to negatively affect the chondrogenesis of stem cells in OA [13, 14]. Thus, these data suggest the therapeutic potential of modulating macrophages to protect cartilaginous tissues in OA.

Human dental pulp stem cells (hDPSCs) are isolated from dental pulp and express a low level of costimulatory molecules, which indicates that they are at a low risk of triggering immune rejection against themselves [15, 16]. Under certain conditions, hDPSCs are capable of differentiating into multiple types of tissue cells that contribute to skeletal regeneration. Importantly, hDPSCs exhibit enhanced immunomodulatory properties compared with traditional mesenchymal stem cells (MSCs) by secreting high levels of immune factors such as hepatocyte growth factor (HGF) and transforming growth factor beta 1 (TGF β 1) [17–18]. Given their low immunogenicity, enhanced immunomodulatory properties, and strong tissue repair capacity, hDPSCs may be an effective tool for alleviating cartilaginous tissue injuries in OA by targeting macrophages.

In recent decades, we have been pursuing preclinical research and clinical applications of stem cell-based therapy for the treatment of severe diseases [19–29]. Notably, we explored the therapeutic effects of hDPSCs on several skeletal disorders, including rheumatoid arthritis, osteoporosis, and tooth loss [30–32]. These studies demonstrated that transfusing hDPSCs yields promising results in maintaining skeletal homeostasis. In addition, we launched clinical trials using clinical-grade hDPSCs to treat patients with severe COVID-19 [33].

In the current study, we explored the effects of clinical-grade hDPSCs on osteoarthritic macrophages in vitro and in vivo. Furthermore, the underlying cellular and molecular mechanisms regarding the capacity of hDPSCs to regulate osteoarthritic macrophages were investigated. Moreover, the protective effects of hDPSCs on articular cartilage in a knee OA model were evaluated.

2. Materials And Methods

Animals

Normal inbred male New Zealand White rabbits (weighing 3.0-3.5 kg, n = 21) were purchased from the Laboratory Animal Center of the Academy of Military Medical Sciences of China (Beijing). All of the animal experiments were performed in accordance with the Academy of Military Medical Sciences Guide for Laboratory Animals.

hDPSC preparation

Clinical-grade hDPSCs were obtained from Beijing SH Biotechnology (<http://www.bjshbio.com/>). The hDPSCs were prepared as previously described with written consent [31]. In brief, hDPSCs were isolated and cultured in a GMP-compliant facility following ISO 8 clean room standards. The pulp tissues were separated from the root and crown, digested by animal origin-free collagenase, and cultured by xenobiotic-free cell culture reagents. Passage 3–4 hDPSCs were used for in vivo and in vitro experiments unless otherwise described.

Human osteoarthritic macrophage generation

Synovial fluid from OA patients (OASF) is a potent inflammatory mediator that includes numerous inflammatory cytokines and has been reported to promote osteochondral lesions in osteoarthritis [21, 34, 35]. In the current study, OASF was used to mimic the joint microenvironments of OA and to cultivate osteoarthritic macrophages. Human OASF was harvested from the knee joints of 10 patients with OA with previous protocols [21] (Tab. S1). Ethics approval was obtained from the PLA General Hospital Research Ethics Committee, and participant consent was acquired prior to sample collection.

Human peripheral blood mononuclear cells were isolated by Ficoll-Paque (1.077 g/mL; Invitrogen) density gradient centrifugation. CD14⁺ monocytes were further harvested from PBMCs using the MACS Monocyte Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany). To generate osteoarthritic macrophages, 2×10^6 monocytes (more than 85% CD14⁺) were cultured in 3 ml of α -MEM supplemented with 10% fetal bovine serum in the presence of human recombinant macrophage-stimulating factor (M-CSF, 50 ng/ml) for 3 days and with the addition of 20% vol/vol human OASF for another 3 days.

To investigate the cellular mechanisms of hDPSCs on osteoarthritic macrophages, in some experiments, hDPSCs were cocultured with osteoarthritic macrophages with direct cell-cell contact or in a Transwell coculture system at graded cell ratios. In addition, anti-human HGF and anti-human TGF β 1 neutralizing antibody and specific inhibitors of the P38/MAPK, ERK/MAPK, and JNK/SAPK pathways were added to osteoarthritic macrophage culture systems to reveal the molecular mechanisms.

Cell morphology and ultrastructure of human osteoarthritic macrophages

A total of 2×10^6 osteoarthritic macrophages were cocultured with 2×10^6 hDPSCs in 6-well plates for 48 h. The cell morphology of the osteoarthritic macrophages or hDPSCs-osteoarthritic macrophages was observed under a phase contrast microscope (Nikon TE2000-U). For transmission electron microscopy ultrastructural observations (TEM, HITACHI, Tokyo, Japan), osteoarthritic macrophages or hDPSC-

osteoarthritic macrophages were collected and fixed for 4 h at 4 °C in 5% glutaraldehyde, washed 3 times in 0.1 mol/L phosphate-buffered saline (PBS), postfixed for 2 h at 4 °C in 2% osmium tetroxide, dehydrated in a graded series of ethanol, embedded in Epon 812, cut into ultrathin sections (75 nm), and then stained with uranyl acetate and lead citrate. The sections were then viewed and recorded with a HITACHI H-600 electron microscope at 80 kV.

Immunophenotyping and immunostaining of osteoarthritic macrophages

To detect the effect of hDPSCs on the osteoarthritic macrophage immunophenotype in vitro, osteoarthritic macrophages were cocultured with hDPSCs at different ratios for 48 h. The hDPSC: osteoarthritic macrophage ratios were 1:100, 1:50 and 1:10. The macrophages were stained with FITC-, PE- or allophycocyanin (APC)-conjugated monoclonal antibodies against human CD11b, CD86, and CD206 according to the manufacturer's protocol. Events were collected by flow cytometry with a FACSCalibur system (Bectone Dickinson). In addition, macrophages and hDPSC-macrophages were stained with CD11b and CD68 antibodies and fluorescent secondary antibodies and observed under a fluorescence microscope.

Immune factor determination by real-time PCR and ELISA

To assess the effect of hDPSCs on immune cytokine expression in macrophages in vitro, hDPSCs were cocultured with osteoarthritic macrophages at different ratios for 48 h. The hDPSC:macrophage ratios were 1:100, 1:50 and 1:10.

Total RNA was extracted with TRIzol reagent (Invitrogen) and reverse transcribed using the mRNA Selective PCR kit (TaKaRa). Human TNF- α , IL-10, and IL-12b cDNAs were amplified by real-time PCR using the SYBR Green PCR kit (Sigma). The primer sequences used for real-time PCR are shown in Table S2.

Meanwhile, the culture supernatants from the in vitro macrophage and hDPSC-macrophage culture systems were collected by centrifugation and filtered to deplete the cellular components. Human TNF- α , IL-10 and IL-12b concentrations in the supernatants were determined according to the reagent protocols of the quantitative determination kit (R&D Systems, Minneapolis).

Western blotting

hDPSC culture medium was added to the osteoarthritic macrophage culture system at a concentration of 10% (vol/vol). Osteoarthritic macrophages were collected at 0, 5, 10, 30, and 60 min. Protein lysis buffer (BioRad, Hercules, CA) was added, and the thawed lysates were vortexed and centrifuged. Proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. The membranes were blocked by incubation with 5% wt/vol nonfat dry milk. The membranes were then incubated with anti-p38, anti-phospho-38 (P-p38), anti-ERK, anti-phospho-ERK (P-ERK), anti-JNK, anti-phospho-JNK (P-JNK), and GADPH antibodies (Cell Signaling Technology) at the appropriate dilutions overnight at 4°C. After incubation, the membranes were washed in Tris-buffered

saline with Tween-20 (TBST). Horseradish peroxidase-conjugated secondary antibodies were added to the membranes in 5% nonfat dry milk in TBST.

Transplantation of hDPSCs to a rabbit knee OA model

In the current study, a rabbit OA model was induced in both knees of each of 21 rabbits by anterior cruciate ligament sectioning. All rabbits were randomly allocated to 3 groups, as shown in Table 1. Briefly, each rabbit was anesthetized with intramuscular injection of xylazine (5 mg/kg) and ketamine (35 mg/kg), and the surgery was performed under general anesthesia. After removing hair around the knees, the skin was disinfected with povidone-iodine and 70% ethanol. The medial skin of the knee joint, fascia, and joint capsule were cut. The anterior cruciate ligament (ACL) was exposed, and the medial parenchyma of the ACL and the meniscus ligament were completely cut. Then, the joint capsule, subcutaneous tissue and skin were sutured and disinfected with povidone-iodine. Intramuscular penicillin injections were administered to each rabbit to prevent infection. hDPSCs ((2×10^5 /knee joint and 1×10^6 /knee joint, respectively) were intraarticularly injected into the OA rabbit model 1 week post operation. All animals were sacrificed 10 weeks after surgery for further evaluation.

Table 1
Experimental groups of the *in vivo* study

Groups	Rabbit number	Treatment
OA group	7	anterior cruciate ligament sectioning
OA + hDPSC group (2×10^5 /knee joint)	7	anterior cruciate ligament sectioning + hDPSCs (2×10^5 /knee joint) intraarticular injection
OA + hDPSC group (1×10^6 /knee joint)	7	anterior cruciate ligament sectioning + hDPSCs (1×10^6 /knee joint) intraarticular injection
OA, osteoarthritis, hDPSC, human dental pulp stem cells		

Gross observation and pathological analysis

The dissected proximal parts of the tibia were evaluated following protocols recommended by OARSI for the rabbit OA models [36, 37]. To perform histopathological analysis, all specimens were fixed in 4% paraformaldehyde for 7 days, decalcified in 10% EDTA solution for 30 days, embedded in paraffin, sectioned into 6-mm slices and stained by HE staining. The cartilaginous matrix distribution was evaluated by Toluidine blue, Safranin-O/Fast Green, and immunohistochemical analysis. The CD11b⁺CD68⁺ macrophages in specimens were shown by in situ immunofluorescence.

Statistical analysis

Data are represented as the mean values with standard deviations. Statistical significance was analyzed using Student's t test. The one-way ANOVA was used in multiple group data analysis. P values less than 0.05 were considered to be significant.

3. Results

hDPSCs strongly inhibited the activation of osteoarthritic macrophages in vitro

To investigate whether hDPSCs affected macrophage morphology, osteoarthritic macrophages and hDPSC-treated osteoarthritic macrophages were observed with phase contrast and transmission electron microscopy. As shown in Fig. 1A, the osteoarthritic macrophages were large cells with an irregular outline. In addition, these cells generally displayed pseudopodia, vesicles, and membrane folding on the cell surface. Moreover, the predominant cytoplasmic organelles in osteoarthritic macrophages were lysosomes, secondary lysosomes and residual bodies. In contrast, the hDPSC-educated osteoarthritic macrophages were small, round, and lacked pseudopodia. Additionally, fewer lysosomes were observed in hDPSC-educated osteoarthritic macrophages.

Cell surface molecules and immune factors are indispensable for macrophages to initiate immune responses. Compared with osteoarthritic macrophages, hDPSC-educated osteoarthritic macrophages exhibited decreased CD11b and CD86 but increased CD206 expression (Fig. 1B and 1C). Importantly, the inhibitory effect was hDPSC dose-dependent. Notably, with a low hDPSC/macrophage ratio of 1:50, CD11b, CD86 and CD206 expression levels were all significantly changed (Fig. 1B and 1C. ***, $P < 0.001$; ****, $P < 0.0001$).

Further investigations showed that hDPSCs suppressed the expression of key immune factors in osteoarthritic macrophages. The real-time PCR results demonstrated that hDPSCs significantly suppressed the expression of IL-12b and TNF- α and increased the expression of IL-10 in osteoarthritic macrophages (Fig. 1D and 1E). Additionally, a low hDPSC/macrophage ratio (1:100) resulted in significant suppression of the transcription and secretion of TNF- α (Fig. 1D and 1E, **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$), implying that a small number of hDPSCs are capable of blocking osteoarthritic macrophage activation. Furthermore, the ELISA results showed that hDPSCs inhibited the secretion of IL-12b and TNF- α but induced that of IL-10 in osteoarthritic macrophages, which is consistent with the changes in mRNA levels (Fig. 1D and 1E). Moreover, immunofluorescence data demonstrated that hDPSCs downregulated the number of CD11b⁺CD68⁺ inflammatory macrophages in osteoarthritic macrophages in vitro (Fig. 1G and 1F).

hDPSCs suppress osteoarthritic macrophages without intercellular contact

To explore whether the inhibition of osteoarthritic macrophages by hDPSCs requires intercellular contact, we used a Transwell chamber system to separate hDPSCs (lower compartment) from macrophages

(upper compartment) (Fig. 2A). We found that hDPSCs markedly suppressed the expression of stimulatory molecules, including CD11b and CD86 (Fig. 2B and 2C, **, $P < 0.01$, ***, $P < 0.001$; ****, $P < 0.0001$), in the Transwell chamber system. In addition, hDPSCs significantly prevented the mRNA and secretion level of macrophage derived immune factors, including TNF- α and IL-12b, and promoted IL-10 secretion without direct cell-cell contact, indicating that soluble factors from hDPSCs were involved in the observed suppression (Fig. 2D and 2E, **, $P < 0.01$, ***, $P < 0.001$; ****, $P < 0.0001$). Importantly, cell contact-independent regulation was observed at a relatively low hDPSC/macrophage ratio (1:100).

hDPSCs suppress osteoarthritic macrophages partially by secreting HGF and TGF- β 1

We further defined which factors in the supernatant were involved in the inhibitory effects by utilizing neutralizing antibodies. TGF- β 1 and HGF were investigated because numerous studies have demonstrated that they are pivotal immune factors from hDPSCs and contribute to hDPSC-mediated immunoregulation. In the current study, the expression levels of CD11b, and CD86 were significantly reverted but the expression level of CD206 were remarkably abolished by direct supplementation with anti-TGF- β 1 (200 ng/ml) or anti-HGF (200 ng/ml) neutralizing antibodies (Fig. 3A, 3B and C, *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$; ****, $P < 0.0001$) in the presence of HDPSC (HDPSC:osteoarthritic macrophage, 1:10). In addition, the mRNA and secretion level of TNF- α and IL-12b increased and that of IL-10 decreased (Fig. 3D and 3E, *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$; ****, $P < 0.0001$). The data suggested that hDPSCs might maintain the observed inactivated macrophage phenotype via HGF and TGF- β 1 secretion.

hDPSCs suppress osteoarthritic macrophages partially by inactivating MAPK pathways

To examine the intracellular signaling cascades, we investigated MAPK pathway protein phosphorylation in osteoarthritic macrophages because the activation of MAPK signals is critical for macrophage action [38–40]. The WB results showed that the JNK/SAPK, ERK/MAPK, and p38/MAPK pathways in macrophages were markedly activated by OASF in a time-dependent manner (Fig. 4A). Most importantly, exposure of osteoarthritic macrophages to hDPSC conditional medium without any exogenous cytokine or chemical inhibitor addition caused significant inactivation of the JNK/SAPK, ERK/MAPK, and p38/MAPK pathways in macrophages (Fig. 4A).

To further clarify the association of osteoarthritic macrophages and the MAPK pathways, specific chemical pathway inhibitors, including SB203580 (for p38/MAPK), PD98059 (for ERK/MAPK), and JNK inhibitor II (for JNK/SAPK), were added to the arthritic macrophage culture system. As shown in Fig. 4B and 4C, hDPSC significantly suppressed the expression of the co-stimulatory molecules CD11b and CD86 but increased the expression of regulatory surface molecule CD206, while JNK inhibitor II, PD98059, and SB203580 showed a similar effects on osteoarthritic macrophages (**, $P < 0.01$, ***, $P < 0.001$, ****, $P < 0.0001$; #, $P < 0.05$, ##, $P < 0.01$, ###, $P < 0.001$). Additionally, hDPSC decreased the mRNA and secretion level of TNF- α and IL-12b, and increased that of IL-10 (Fig. 4D and 4E, **, $P < 0.01$, ***, $P < 0.001$). The changes of immune factors in the presence of the specific inhibitors were similar to that of hDPSC group. Notably, blockage of single pathway could not result in same suppression on osteoarthritic macrophage

as that of hDPSCs (Fig. 4B, 4C, 4D and 4E, *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$, ****, $P < 0.0001$; #, $P < 0.05$, ##, $P < 0.01$, ###, $P < 0.001$). Thus, we suggest that OASF activated macrophages partially by activating the JNK/SAPK, ERK/MAPK and p38/MAPK pathways, and hDPSCs might exhibit an inhibitory effect on osteoarthritic macrophages by blocking multiple pathways of MAPK signaling.

hDPSCs suppress osteoarthritic macrophage activation in a rabbit posttraumatic knee OA model

Although in vitro studies have shown that hDPSCs are capable of suppressing osteoarthritic macrophages, it remains unknown whether this is the case in vivo. Therefore, the phenotype of osteoarthritic macrophages was determined by immunofluorescence. Promisingly, we found that hDPSC infusion significantly reduced CD11b⁺CD68⁺ macrophages in the host articular osteochondral tissues (Fig. 5A and 5B) (***, $P < 0.001$). In addition, the numbers of CD11b⁺CD68⁺ macrophages in the articular osteochondral tissue of hDPSC-treated rabbits were markedly decreased with the increased number of infused hDPSCs (Fig. 5A and 5B). The suppressive effects of hDPSCs on osteoarthritic macrophages in an OA animal model in vivo are consistent with those observed in an in vitro cell model.

hDPSCs attenuated damage to the articular cartilage in vivo

At 10 weeks after hDPSC injection, the representative macroscopic observation displayed discriminatory regenerated tissue between the OA model group and the hDPSC treatment group. The articular surfaces in the untreated group were not as smooth as those in the hDPSC-treated group (Fig. 6A). Large osteophytes were observed at the periphery of the tibial plate. In contrast, no obvious osteochondral lesions in articular cartilage were observed in the hDPSC-treated groups (Fig. 6A). The histological evidence further supported the gross morphological findings. As shown in Fig. 6B, HE staining showed that hDPSC administration alleviated tissue erosion in OA knee joints. The toluidine blue, safranin-O/Fast Green staining, and immunochemistry of Col-II showed that hDPSCs significantly improved the histological findings associated with structure and proteoglycans in a cell dose-dependent manner (Fig. 6A). Quantitatively, the high-hDPSC group exhibited significantly lower pathological scores than the other 2 experimental groups (**, $P < 0.01$, ***, $P < 0.001$,) (Fig. 6B).

4. Discussion

In the current study, we demonstrated the suppressive effects of clinical-grade hDPSCs on the activation of osteoarthritic macrophages in vitro. Additionally, we found that hDPSC injection significantly reduced the formation of osteoarthritic macrophages in vivo and alleviated cartilaginous damage in knee OA.

The preparation and application of DPSCs is less invasive, more feasible and involves fewer ethical issues than their counterparts in other tissues. In addition, accumulated evidence has demonstrated that DPSCs share similar characteristics with BMSCs, which potentiates their application in treating intractable diseases, including Alzheimer's disease, cardiac ischemia-reperfusion injury, and diabetes [16, 41, 42]. Moreover, DPSCs have exhibited promising potential in the treatment of oral and skeletal disorders due to their high osteochondral capacity and immunoregulatory properties. Asutay et al.

reported that human DPSCs mixed with HA/TCP paste yield a high calcification rate and bone mineral density value in a rat calvarial defect model [43]. Further radiographic data showed that bone regeneration in the presence of DPSC-HA/TCP was greater than that without DPSCs, which suggests that DPSCs may be a suitable factor for skeletal tissue engineering [43]. Our previous study demonstrated that transplantation of hDPSCs markedly prevented bone loss in the early phase of ovariectomy-induced osteoporosis [31]. Moreover, HGF overexpression in hDPSCs significantly enhanced the protective effects against bone loss in vivo. In another independent study, hDPSCs exerted multifaceted benefits for treating experimental rheumatoid arthritis by secreting factors [44]. Furthermore, our recent works revealed that the regenerative properties of hDPSCs were controlled by HGF [32]. In the early phase of RA progression, HGF overexpression in hDPSCs inhibited pathological aggression by its immunosuppressive effects. In the late phase of RA, HGF promoted synovitis by activating fibroblast-like synoviocytes to produce pathogenic IL-6, which accelerated cell proliferation and induced apoptosis resistance. However, it remains largely unknown how hDPSCs influence OA progression and the corresponding cellular and molecular mechanisms.

Macrophages are hematopoietic stem cell-derived immune cells that have a pivotal role in innate and adaptive immune responses. By taking up innate or antigens or invading pathogens, activated macrophages highly express costimulatory molecules and secrete more immune cytokines, which are indispensable for the immune response and tissue regeneration [10]. However, recent studies have revealed that macrophage activation contributes to the pathological progression of OA. Xie et al. reported that activated macrophages enhance the progression of OA by increasing the secretion of matrix metalloproteinases [10]. Moreover, macrophages in the subchondral bone are not only involved in chondro-osteogenic remodeling but also cause joint pain by releasing inflammatory factors [45, 46]. Thus, a full understanding of the regulation of osteoarthritic macrophages will definitely shed light on OA therapy.

Macrophage activation is greatly dependent upon stromal microenvironments [47, 48]. Serbulea et al. reported that the adipose tissue microenvironment controls the macrophage phenotype either to maintain lean tissue homeostasis or to drive inflammation [47]. Li et al. found that the stromal niche of gastric cancer contributed to macrophage polarization within the gastric cancer niche through considerable secretion of IL-6 and IL-8 [48]. Additionally, our previous studies demonstrated that MSCs were capable of controlling the differentiation fate of monocytes, which are important progenitors of macrophages [21, 25]. Thus, we explored the possibility of osteoarthritic macrophage control by hDPSCs. We found that hDPSCs could suppress CD11b, CD86 and CD68 expression but increase CD206 expression. Functionally, hDPSCs inhibited the expression of IL-12b and TNF- α and promoted the production of IL-10. This observation indicates that hDPSCs suppressed the activation of osteoarthritic macrophages and further investigations are needed to explore whether osteoarthritic macrophages develop into a new type of macrophage [12, 45].

Previous reports have shown that direct cell-cell interactions of macrophages with neighboring stromal cells are required for MSC-mediated suppression [49–51]. However, in the current study, no remarkable

change of osteoarthritic macrophages generation in the Transwell chamber system was observed, which suggested that hDPSCs suppress osteoarthritic macrophages mainly by secreting soluble factors. Numerous reports have demonstrated that hDPSCs highly express HGF, and we previously found that HGF has a pivotal role in controlling the regenerative properties of hDPSCs [30]. Thus, we blocked hDPSC-derived HGF, and the suppressive effects on osteoarthritic macrophages were partially reverted, which revealed the novel role of HGF in the hDPSC-mediated regenerative capacity. In addition to HGF, we found that TGF- β 1 contributes to the inhibition of osteoarthritic macrophages by hDPSCs by using neutralization antibody. Collectively, these data suggested that soluble factors, including HGF and TGF- β 1, contribute to hDPSC-mediated suppression of OA macrophages, but we are aware that other unknown secreted factors may have a potential role in the suppressive effects.

To understand the molecular mechanism underlying the suppressive effect of hDPSCs on osteoarthritic macrophages, the MAPK pathway was chosen for investigation, as it was reported to be involved in macrophage activation in inflammatory microenvironments [29, 38–40]. OASF induced marked activation of the p38/MAPK, ERK/MAPK, and JNK/SAPK pathways in osteoarthritic macrophages. Notably, inhibition of these pathways with specific chemical inhibitors changed the expression of co-stimulatory molecules and the production of inflammatory factors in macrophages, indicating that the p38/MAPK, ERK/MAPK, and JNK/SAPK pathways are involved in osteoarthritic macrophage activation. Most importantly, hDPSCs significantly suppressed MAPK signal transduction in activated macrophages. However, a single pathway blockade could not completely suppress the activation of macrophages, indicating that some other mechanisms are also involved in OASF stimulation.

Notably, our data demonstrated that hDPSCs suppressed macrophage activation *in vivo*. Most importantly, the hDPSCs were capable of alleviating cartilaginous damage in OA knee joints, which implies the potential application of hDPSCs in the therapy of OA. Our findings showed marked infiltration of inflammatory macrophages (CD11b + CD68+) in the subchondral bones and articular cartilage of OA rabbits, which have been reported to be involved in the osteochondral remodeling of OA joints [12, 45, 52–55]. Promisingly, hDPSC infusion significantly reduced the number of inflammatory macrophages (CD11b + CD68+) in articular osteochondral tissue of OA rabbits. The change in the macrophages may contribute to the protective effects of hDPSCs in OA. Consistent with the macrophage alteration, further pathological analysis validated that hDPSCs contribute to the settlement of OA. The HE, toluidine blue staining, safranin-O/Fast Green, and immunochemistry of Col-II staining showed fewer lesions of the knee articular tissue post hDPSC infusion. However, we are aware that other mechanisms may contribute to the regenerative effects of hDPSCs.

Nevertheless, we must acknowledge several limitations in our study. First, our data are based on only one kind of OA animal model; studies from more OA models are required to further validate the findings. Second, to determine the cellular and molecular mechanisms that contribute to the therapeutic effects of hDPSCs on OA, high-throughput assays may be helpful. Third, the time point of hDPSC injection may influence the outcome of OA. Further investigations of the relevance of MSC treatment time and therapeutic results should be performed in the future.

5. Conclusion

In the present study, we reported the effect of hDPSCs on osteoarthritic macrophages and the effect of hDPSC injection on a rabbit OA model. Our study shows the suppressive effects of hDPSCs on osteoarthritic macrophages in vitro and in vivo and the protective role of hDPSCs on cartilaginous tissues in the OA knee joint, which suggests that hDPSCs may be potential candidates for stem cell-based OA therapy. However, a comprehensive analysis of therapeutic effects in more animal models and high-throughput assays is needed to further clarify the underlying mechanisms.

Abbreviations

hDPSC

human dental pulp stem cells; OA:osteoarthritis; IL-12:Interleukin 12; TNF- α :tumor necrosis factor α ; OASF:Synovial fluid from OA patients; MSCs:mesenchymal stem cells; HGF:hepatocyte growth factor; TGF β 1:transforming growth factor beta 1;PCR:polymerase chain reaction; M-CSF:microphage colony stimulating factor; IFN- γ :interferon γ ; FBS:fetal bovine serum; FTIC:Fluorescein isothiocyanate; PE:phycoerythrin; APC:allophycocyanin.

Declarations

Declarations

Ethics approval

All experiments in the current study were approved by the ethics and technological committees of the General Hospital of the Air Force (2016 – 0168).

Consent for publication

Not applicable.

Declarations

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

P-L L, Y-X W, Z-D Z, and Z-L L performed the experiments and wrote the manuscript supervised by Heng Z, C-T W, and Li D who edited the manuscript. J-W L, Qian W, B-F Y, R-C H, and M-Y H performed data interpretation and manuscript editing sup Heng Z, C-T W, and Li D. All authors read and approved the final manuscript.

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Figures

Figure 1

hDPSCs suppressed osteoarthritic macrophage activation in vitro hDPSCs suppressed the pseudopodia formation and reduced lysosomes in osteoarthritic macrophages (Fig.1A). In addition, hDPSC-educated osteoarthritic macrophages exhibited decreased CD11b and CD86 but increased CD206 expression in a hDPSCs dose-dependent way (Fig. 1B and 1C). Furthermore, hDPSCs significantly suppressed the mRNA expression and protein secretion of IL-12 and TNF- α and increased the expression of IL-10 in osteoarthritic macrophages (Fig. 1D and 1E). Moreover, hDPSCs downregulated the number of CD11b+CD68+ inflammatory macrophages in osteoarthritic macrophages in vitro (Fig. 1G). **, P < 0.01,***, P < 0.001; ****, P < 0.0001. Bars in Fig.1A upper row, Fig. 1A lower row, and Fig.1F represent 200 μ m, 20 μ m, and 200 μ m, respectively.

Figure 2

hDPSCs suppress osteoarthritic macrophages without intercellular contact In the current study, a Transwell chamber system was used to separate hDPSCs (lower compartment) from macrophages (upper compartment) (Fig. 2A). hDPSCs markedly suppressed the expression of stimulatory molecules, including CD11b and CD86 in the Transwell chamber system (Fig. 2B and 2C). In addition, hDPSCs significantly prevented the mRNA and secretion level of macrophage derived immune factors, including TNF- α and IL-12b, and promoted IL-10 expression without direct cell-cell contact (Fig. 2D and 2E). *, P < 0.05, **, P < 0.01,***, P < 0.001; ****, P < 0.0001.

Figure 3

hDPSCs suppress osteoarthritic macrophages partially by secreting HGF and TGF-β1. In the current study, the expression levels of CD11b and CD86 were significantly reverted but the expression level of CD206 were remarkably abolished by direct supplementation with anti-TGF-β1 (200 ng/ml) or anti-HGF (200 ng/ml) neutralizing antibodies in the presence of HDPSC (HDPSC:osteoarthritic macrophage,1:10) (Fig. 3A, 3B and C). In addition, the mRNA and secretion level of TNF-α and IL-12b increased and that of IL-10 decreased (Fig.3D and 3E), *, P < 0.05, **, P < 0.01,***, P < 0.001; ****, P < 0.0001.

Figure 4

hDPSCs suppress osteoarthritic macrophages partially by inactivating MAPK pathways. The JNK/SAPK, ERK/MAPK, and p38/MAPK pathways in macrophages were markedly activated by OASF in a time-dependent manner while exposure of osteoarthritic macrophages to hDPSC conditional medium suppressed the activation of these pathways in macrophages (Fig. 4A). Additionally, specific chemical pathway inhibitors, including SB203580 (for p38/MAPK), PD98059 (for ERK/MAPK), and JNK inhibitor II (for JNK/SAPK) showed a similar effect on the immunophenotype, and expression of TNF- α , IL-12b, and IL-10 of osteoarthritic macrophages to that of hDPSCs. Notably, blockage of single pathway could not result in same suppression on osteoarthritic macrophage as that of hDPSCs (Fig. 4B, 4C, 4D and 4E, *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$, ****, $P < 0.0001$, hDPSC group and inhibitor groups compared to osteoarthritic macrophage group). (Fig. 4B, 4C, 4D and 4E, #, $P < 0.05$, ##, $P < 0.01$, ###, $P < 0.001$, inhibitor groups compared to hDPSC group).

Figure 5

hDPSCs suppress osteoarthritic macrophage activation in a rabbit posttraumatic knee OA model hDPSC infusion significantly reduced CD11b+CD68+ macrophages in the host articular osteochondral tissues in an OA animal model at a hDPSC dose-dependent manner (Fig. 5A and 5B). ***, P < 0.001. Bars in Fig. 5A upper row and Fig. 5A lower row represent 5mm and 200µm, respectively.

Figure 6

hDPSCs attenuated damage to the articular cartilage in vivo The macroscopic characteristics of OA model group and the hDPSC treatment group were showed in Fig. 6A. In addition, the results of the HE, toluidine blue staining, safranin-O/Fast Green staining, and immunochemistry of Col-II showed that hDPSCs significantly improved the histological findings of osteochondral tissue in OA models (Fig.6A). Moreover, the high-hDPSC group exhibited significantly lower pathological scores than the other 2 experimental groups(Fig. 6B). **, $P < 0.01$, ***, $P < 0.001$.