Original Article

Dental pulp stem cells induce anti-inflammatory phenotypic transformation of macrophages to enhance osteogenic potential via IL-6/GP130/STAT3 signaling

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Background: Periodontitis is a major oral condition and current treatment outcomes can be unsatisfactory. Macrophages are essential to the regeneration process, so we investigated the influence of human dental pulp stem cells (hDPSCs) on macrophage differentiation and the microenvironment and the underlying mechanism.

Methods: hDPSCs were isolated from healthy third molars extracted from patients undergoing maxillofacial surgery. The surface antigens CD73, CD45, CD90 and CD11b of the hDPSCs were detected using flow cytometry. hDPSCs were induced for osteogenic and adipogenic differentiation, and the outcome was assessed by alizarin red staining or Oil Red O staining. The IL-6 level released by hDPSCs was measured by enzyme linked immunosorbent assay (ELISA). Tohoku Hospital Pediatrics-1 (THP-1) cells were cultured and induced into macrophages by phorbol-12-myristate-13-acetate. After coculture of THP-1-derived macrophages with hDPSCs, interleukin 6 (IL-6), Argininase-1 (Arg-1), Mannose receptor C-1 (Mrc-1), inducible nitric oxide synthase (iNOS), and tumor necrosis factor-α (TNF-α) levels in the medium were measured using ELISA and quantificational RT-PCR (qRT-PCR). The numbers of $CD80^+$ and $CD163^+$ macrophages were counted by immunofluorescence, and GP130/STAT3 signaling protein expression was detected. After coculturing the culture medium of hDPSCs with human bone marrow stem cells (BMSCs), scratch assays and transwell assays were performed to evaluate cell migration and invasion.

Results: Alkaline phosphatase (ALP) staining, alizarin red staining, and western blots were performed to assess osteoblast differentiation. The hDPSCs were positive for surface antigens CD73 and CD90 and negative for CD45 and CD11b expression. The level of IL-6 secreted by hDPSCs significantly increased the number of $CD80^+$ cells as well as the levels of Arg-1 and Mrc-1. It also promoted M2 macrophage polarization and activated GP130/STAT3 signaling. However, the medium cocultured with THP-1-derived macrophages by hDPSCs facilitated the migration, invasion, and osteogenic abilities of human bone marrow-derived stem cells (hBMSCs).

Conclusions: hDPSCs can regulate the periodontal microenvironment through IL-6 by inducing phenotypic transformation of M2 macrophages and stimulating osteogenic differentiation of BMSCs.

Keywords: Human dental pulp stem cells (hDPSCs); IL-6/GP130/STAT3 signaling; macrophages; osteogenic differentiation

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Introduction

The oral and maxillofacial region has multiple functions, such as mastication, swallowing, breathing, pronunciation, taste, and individual appearance, so the integrity and health of the soft and hard tissues, especially bone tissues as the structural basis, are greatly important to a person's quality of life and survival (1). However, oral conditions occur frequently and may severely affect the oral function and daily life of patients; of these, periodontitis affects 11.2% of the world's population, emerging as the sixth most common disease in humans (2). Currently, the main clinical treatment methods are drug therapy and surgical treatment. When periodontitis damages the supporting structure of the patient's oral cavity, which maintains tooth position, tooth displacement occurs and it may be necessary to correct or move the teeth through orthodontic treatment (ODT) (3). However, the therapeutic outcome may be unsatisfactory, which urges the need to find new treatments.

Mesenchymal stem cells (MSCs) have the potential for tissue regeneration with multilineage differentiation and are thus widely used in tissue engineering and stem cell therapy (4). However, the complexity involved in the structure and interaction of periodontal tissues is one of the reasons why periodontal tissues are difficult to regenerate. A previous study showed that human dental pulp stem cells (hDPSCs) are more appropriate for forming new cementum-like structures *in vivo* compared with other tissue-derived stem cells (5). hDPSCs have shown higher growth potential than human bone marrow-derived stem cells (hBMSCs). Meanwhile, hDPSCs have been shown to have potent immunomodulatory functions in treating systemic lupus erythematosus (SLE) in the SLE MRL/lpr mouse model (6). Yan *et al.* also indicated that hDPSCs could inhibit the progression of fibrosis in hypertrophic keloids (7). In addition, they are available from various tissue sources, including dental pulp stem cells, stem cells from exfoliated deciduous teeth, stem cells from apical papilla, periodontal ligament stem cells and dental follicle progenitor cells (8), and are highly available during tooth extraction, making them a reliable source for periodontal tissue regeneration techniques (9). DPSCs have emerged as a promising tool with a great potential for dental and oral tissue regeneration or engineering (10).

Cells can secrete different factors to regulate the surrounding microenvironment and change the characteristics of nearby cells by a phenomenon known as paracrine interaction (11). Several studies have demonstrated that hDPSCs can alter the biological behaviors of surrounding cells this way, including interleukin (IL)-6 secretion (12). Huang *et al.* found that exosomes from LPS (lipopolysaccharide)-stimulated hDPSCs activated the angiogenic potential of human umbilical vein endothelial cells (HUVECs) (13). Gervois *et al.* found by coculture that hDPSCs can significantly promote neuroblastoma cell (SH-SY5Y) migration and neural maturation (14). On the other hand, hDPSCs have an impressive immunomodulatory ability, and the interaction between local stem cells and immune cells in the periodontal microenvironment may alter the regenerative process (15). Immune cells are vital to the process of regeneration, in which macrophages can be induced into classically activated macrophages (M1 type) and alternating activated macrophages (M2 type), with the former having an inflammatory function and the latter an anti-inflammatory function (16). The proportion of M1 or M2 macrophages or the temporal sequence of their appearance is essential for tissue remodeling. Periodontal inflammation was proved to be associated with an enhancement of ratio of M1/M2 phenotypes of macrophages (17). In addition, increased M1/M2 ratio leads to alveolar bone resorption during orthodontics, while decreased M1/M2 ratio inhibits bone resorption (18). Therefore, the M1 and M2 status of macrophages are crucial in periodontitis.

Currently, the main limitation is that the regulatory effects of hDPSCs on macrophages and their mechanisms of action remain unknown. Thus, we aimed to investigate the

Highlight box

Key findings

- HDPSCs regulate the periodontal microenvironment through IL-6 by inducing phenotypic transformation of M2 macrophages and stimulating osteogenic differentiation of BMSCs.

What is known and what is new?

- Oral conditions occur frequently and can severely affect the oral function and daily life of patients. Periodontitis affects 11.2% of the world's population, emerging as the sixth most common disease in humans.
- We investigated the effects of hDPSCs on macrophages and osteogenic potential and the potential underlying mechanisms by observing the dynamic changes of macrophage polarization after hDPSCs transplantation.

What is the implication, and what should change now?

- Our work demonstrates that hDPSCs can promote M2 macrophage polarization by activating GP130/STAT3 signaling through IL-6.

effects of hDPSCs on macrophages and osteogenic potential and the potential underlying mechanisms by observing the dynamic changes of macrophage polarization after hDPSCs transplantation. We present the following article in accordance with the MDAR reporting checklist (available at https://atm.amegroups.com/article/view/10.21037/atm-22-6390/rc).

Methods

Clinical sample collection

Healthy third molars were extracted from 20 healthy patients without tooth decay, apical periodontitis, periodontitis or any systemic diseases who underwent orthodontic extraction during maxillofacial surgery. The hDPSCs were isolated from the obtained tooth samples. All patients signed an informed consent form. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by ethics committee of Jinling Hospital, Nanjing Medical University (General Hospital of the eastern theater of the Chinese People's Liberation Army, Nanjing, China) (No. 2001-164XD4E).

Isolation and culture of hDPSCs

Human DPSCs were isolated as previously reported (19). Briefly, the pulp tissue was gently separated from the crown and root of each third molar and then digested with collagenase type I (Invitrogen, USA) at 3 mg/mL and neutral protease at 2 mg/mL for 1 h at 37 ℃. The cells were passed through a 70-μm filter to obtain a single-cell suspension. Cells were subsequently cultured in DMEM (Gibco, USA) containing 10% fetal bovine serum (FBS; Gibco) and 1% penicillin-streptomycin at 37 ℃ in a 5% CO_2 humidified atmosphere. When the cell confluency reached 80–90%, they were digested with 0.25% trypsin as the first passage of hDPSCs cells. The third passage of cells was taken for subsequent assays.

Cell differentiation of hDPSCs

Osteogenic differentiation

hDPSCs at a density of 5×10^4 cells/well were seeded in 6-well plates. When the cells grew to 80% confluence, cells were cultured with DMEM (Gibco, USA) with 15% FBS, 0.1 μmol/L dexamethasone, 50 μmol/L vitamin C and 10 mmol/L β-phosphoglycerol (Sigma-Aldrich, USA). The medium was changed every 3 days and the cells were cultured for 14 days.

Adipogenic differentiation

hDPSCs were incubated in a culture medium for 2 days to reach cell confluence. When cell differentiation started, the first day of differentiation was designated as day 0. The cells were transferred to a medium supplemented with 10% FBS, 0.5 mM 3-isobutyl-1-methylxanthine, 1 μM dexamethasone, 10 nM rosiglitazone, and 10 μg/mL insulin (Sigma-Aldrich). On day 3, the medium was changed to one supplemented with 10% FBS and 10 μg/mL insulin. The growth medium was then changed every 2 days until mature lipid droplets were cultured after 8 days of induced differentiation.

Flow cytometry

The first generation of isolated and cultured hDPSCs cells was digested with 0.25% trypsin, and the cell density was adjusted to 1×10^6 cells/mL by counting. The antibodies to relevant antigens on the surface of the hDPSCs cells were diluted according to the manufacturers' instructions. Briefly, CD73, CD45, CD90 and CD11b (all from R&D Systems, USA) were added to the cells, which were incubated in the dark for 1 h, then rinsed three times with precooled phosphate-buffered saline (PBS). The cells were then loaded into a BD FACsCalibur flow cytometer (BD Biosciences, USA) for detection.

Coculture of THP-1-derived macrophages with hBMSCs

The human monocytic leukemia cell line (THP-1) was obtained from ATCC (VA, USA), and cultured in RPMI 1640 (Invitrogen) medium containing 10% FBS and 1% penicillin–streptomycin (Gibco). A cell suspension was added to the upper chamber of transwell inserts (BD Biosciences) at a dose of 2×10^5 cells and induced with 100 ng/mL phorbol-12-myristate-13-acetate (PMA) for 48 h to produce THP-1-derived macrophages. THP-1-derived macrophages cultured alone acted as the control group. IL-6 at the same concentration as secreted by hDPSCs was added to the THP-1-derived macrophage medium to form the IL-6 group. The hDPSC group was established with THP-1-derived macrophages seeded in the upper chamber and hDPSCs in the lower chamber. THP-1-derived macrophages treated with IL-6 were seeded in the upper chamber, and hDPSCs cultured in the medium containing

IL-6 inhibitors were seeded in the lower chamber as hDPSC + anti-IL-6 group.

Coculture of THP-1-derived macrophages with hBMSCs

BMSCs were purchased from SALIAI (Guangzhou, China) and cultured in α-MEM (Gibco) including 10% FBS. When cell confluence reached ≈90%, cells were divided into three groups: hDPSC-CM, BMSCs cultured in hDPSCs cultured medium; hDPSC + M (macrophage)-CM, BMSCs cultured in coculture medium of hDPSCs and THP-1-derived macrophages; HDPSC + M + anti-IL-6-CM, BMSCs cultured in hDPSCs cocultured with THP-1-derived macrophages in medium containing IL-6 inhibitor.

Scratch test

Cocultured hBMSCs were seeded into 6-well plates and a scratch vertical to the surface was made on each well with a 10 μL sterile tip. Cell debris and detached cells were removed by washing with PBS. Fresh serum-free medium was added, and after 24 h of culture, photographs were taken with an inverted microscope to record the experimental results. Image J software was used to calculate the scratch area.

Transwell assay

Using 100 μL hBMSCs cells from the hDPSC-CM group and hDPSC + M-CM group, hDPSC + M (macrophage) + anti-IL-6-CM were added to the upper chamber of the transwell, and 700 μL of culture medium containing 20% FBS to the lower chamber. The transwell inserts were removed after 12–24 h of culture at 37 ℃ in 5% CO_2. They were washed three times with PBS, fixed with 1% glutaraldehyde for 30 min, cleaned with PBS, then dried and impregnated with 0.1% crystal violet for 12 h. After washing with PBS and drying, the samples were viewed under an upright microscope. The number of positive cells in each field was recorded from 6–10 random fields. Three fields were randomly selected for photography and statistical analysis.

Alizarin red staining

On day 14 after osteogenic differentiation was induced, the culture medium of the cells was removed and rinsed three times with PBS at 37 ℃, followed by fixation of the cells with 4% paraformaldehyde for 15 min. Following removal of the fixative, the cells were washed three times with PBS, stained with 2% alizarin red staining solution (Sigma-Aldrich, USA) and incubated at room temperature for 15 min. The staining solution was then removed, and the cells were washed three times with PBS and placed under a microscope (BioTek, USA) to observe the formation of mineralized nodules.

Oil Red O staining

Adipogenic-induced mature cells were washed three times with PBS, fixed with 4% formaldehyde for 30 min at room temperature, and washed three times with PBS for 5 min each. They were then left for 1 h with Oil Red O staining (Sigma-Aldrich, China). The original Oil Red O solution was first diluted with water at a proportion of 3:2. The stained cells were washed three times with PBS for 5 min each, and finally the cell plate was photographed under a microscope to observe lipid droplet formation.

Alkaline phosphatase (ALP) staining

On the 14th day after osteogenic differentiation was induced, the culture medium of cells in each group was removed, and the cells were washed three times with PBS at 37 ℃. Then, the cells were fixed with 4% paraformaldehyde for 30 min. After removing the fixing solution, they were washed three times with PBS and placed in staining solution containing BCIP/NBT ALP staining reagent (Beyotime, China) in the dark for 30 min. The staining solution was removed, the cells were washed three times with PBS, and finally placed under a microscope to observe the staining.

Enzyme linked immunosorbent assay (ELISA)

The culture medium of hDPSCs being cultured for 48 h and the culture medium of coculture of cells in each group were centrifuged at 12,000 r/min and 4 ℃ for 10 min to remove cell debris. The supernatant was collected to detect the level of IL-6 in the culture medium of hDPSCs cultured for 48 h, as well as the levels of Argininase 1 (Arg-1), Mannose receptor 1 (Mrc-1), inducible macrophage-type nitric oxide synthase (iNOS) and tumor necrosis factor (TNF)-α in the culture medium of the cocultured cells in each group according to the requirements of the ELISA kit

Table 1 Quantitative primers

RNA	Sequences (5' to 3')
Arg-1	F: 5'-TCATCTGGGTGGATGCTCACAC
	R: 5'-GAGAATCCTGGCACATCGGGAA
Mrc-1	F: 5'-AGCCAACACCAGCTCCTCAAGA
	R: 5'-CAAAACGCTCGCGCATTGTCCA
iNOS	F: 5'-GCTCTACACCTCCAATGTGACC
	R: 5'-CTGCCGAGATTTGAGCCTCATG
TNF-α	F: 5'-CTCTTCTGCCTGCTGCACTTTG
	R: 5'-ATGGGCTACAGGCTTGTCACTC
GAPDH	F: 5'-GTCTCCTCTGACTTCAACAGCG
	R: 5'-ACCACCCTGTTGCTGTAGCCAA

(Nanjing Jiancheng Bioengineering Institute, China).

Quantitative real time polymerase chain reaction (qRT-PCR)

Total RNA from cells in each treatment group was extracted using TRizol (Invitrogen) and reverse transcribed to cDNA using a random primer reverse transcription kit (Thermo Fisher Scientific, USA). The concentration and purity of RNA were detected by NanoDrop (Thermo Fisher Scientific, USA). The expression levels of mRNA were detected according to the instructions of the SYBR GREEN kit (TaKaRa, Japan), with GAPDH used as an internal reference control and six replicates set up in the experiment. The experimental data obtained by qRT-PCR were used to calculate the relative expression of the target genes using the $2^{-\Delta\Delta Ct}$ method. The primer sequences for qRT-PCR are presented in *Table 1*.

Immunofluorescence detection

First, cells were fixed with 4% paraformaldehyde at room temperature for 30 min, then treated with 0.4% Triton X-100 for 10 min, and blocked with 2% bovine serum albumin for 30 min. After washing with PBS, the samples were incubated with primary antibodies against CD80 (ab225674, Abcam, UK) and CD163 (ab156769, Abcam, UK) overnight at 4 ℃ and probed with the corresponding protein secondary antibodies for 1 h. Following washing with PBS three times, the nuclei were stained with DAPI for 10 min, and the slides were placed under a fluorescence microscope to observe the numbers of CD80 and CD163 positive cells.

Western blot

Treated cells were rinsed twice with precooled sterile PBS. After removing PBS, 80 μL of lysis buffer (RIPA, Beyotime) was added and cells were lysed for 15 min at 4 ℃. The supernatant protein was collected by centrifugation at 12,000 r/min for 15 min at 4 ℃. The protein concentration was determined with a BCA kit (Beyotime), and 20 μg of protein was taken and denatured by boiling in 1× loading buffer. The proteins were resolved by 10% SDS-PAGE gels and transferred to PVDF membranes (Millipore, USA). The membranes were then blocked with 5% non-fat dry milk for 1 h and incubated with primary antibodies GP130 (ab259927, Abcam), p-JAK2 (ab32101, Abcam), JAK2 (ab108596, Abcam), p-STAT3 (ab76315, Abcam), STAT3 (ab119352, Abcam), and GAPDH (ab181602, Abcam) overnight at 4 ℃, as well as secondary antibodies for 1 h at room temperature. After washing with PBST (Phosphate Buffer Solution + Tween-20) three times, the membranes were visualized by electrochemiluminescence reagents, and images were collected in a gel imaging system. The gray levels of the protein bands were analyzed using Image J software, with GAPDH as an internal reference to calculate the relative protein expression.

Statistical analysis

One-way analysis of variance and independent samples *t*-test analysis were performed using GraphPad Prism version 8.0 software (GraphPad, USA). The results are expressed as mean ± standard deviation (SD). $P<0.05$ was used to indicate statistical significance.

Results

Isolation and identification of hDPSCs

To investigate their function, we isolated hDPSCs from collected tooth samples and detected their surface antigens by flow cytometry. The results showed that hDPSCs were positive for expression of surface antigens CD73 and CD90, but negative for CD45 and CD11b expression (*Figure 1A*). Microscopic images revealed the classical spindle morphology (*Figure 1B*), indicating that hDPSCs had been successfully isolated. Alizarin red staining and Oil Red O staining showed that hDPSCs were induced into osteogenesis (*Figure 1C*) and adipogenesis (*Figure 1D*).

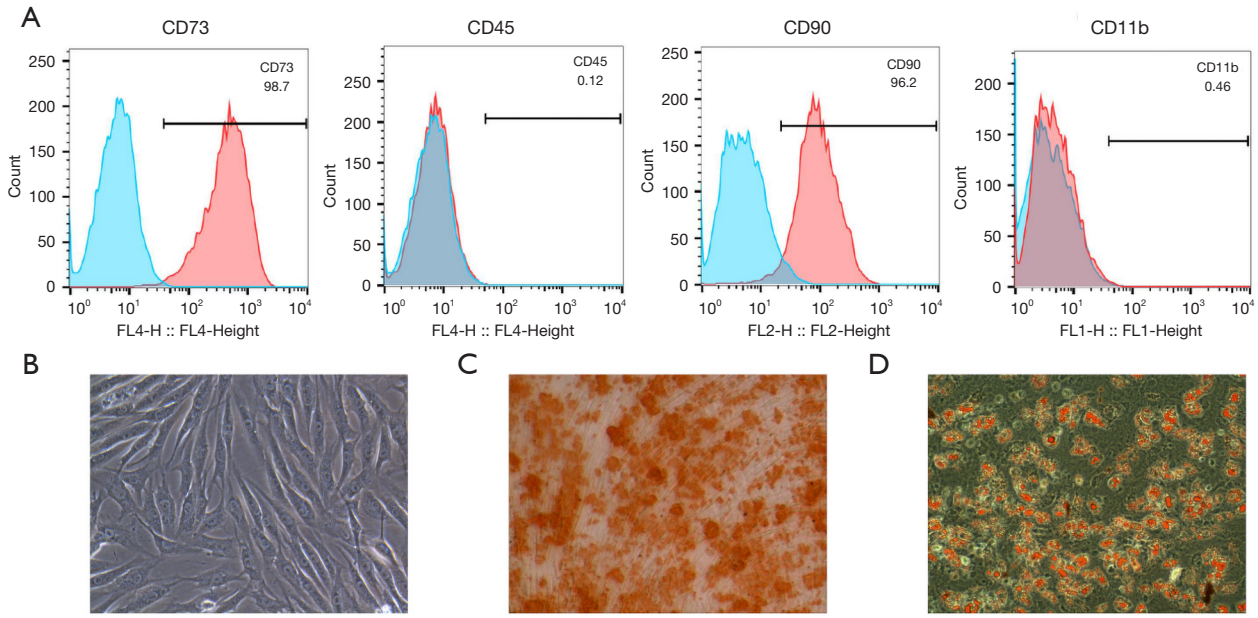

Figure 1 Isolation and identification of hDPSCs. (A) Surface antigens CD73, CD90, CD45 and CD11b of hDPSCs detected by flow cytometry, Blue is the same type of contrast, Red is the expression of CD surface antigen; (B) cell morphology of hDPSCs observed under a microscope; (C) ossification level of hDPSCs visualized by alizarin red staining; (D) adipogenic differentiation of hDPSC cells visualized by Oil Red O staining. Experiments were repeated three times. hDPSCs, human dental pulp stem cells. Magnification: ×200.

These findings suggested that we successfully isolated and induced hDPSCs.

hDPSCs promote M2 macrophage polarization by secreting IL-6

To investigate the effect of hDPSCs on macrophage polarization, we first examined the level of IL-6 secretion by the hDPSCs and found it was 13.07±1.70 ng/L. Next, we induced THP-1 cells using PMA for 48 h to differentiate them into macrophages (*Figure 2A*) that were subsequently cocultured with hDPSCs, and the phenotype of the cellular macrophages in each group was examined by immunofluorescence. Compared with the control group, the number of $CD80^+$ cells were significantly reduced, and that of $CD163^+$ cells increased in the IL-6 group and hDPSC group. However, compared with the hDPSC group, the hDPSC + anti-IL-6 group had significantly increased $CD80^+$ cells and fewer $CD163^+$ cells among the macrophages cultured (*Figure 2B*). In addition, ELISA and qRT-PCR identified an increase in Arg-1 and Mrc-1 levels in the cultured macrophages and a decrease in iNOS and TNF-α in the IL-6 and hDPSC groups. The addition of anti-IL-6 (hDPSC + anti-IL-6 group) significantly decreased Arg-1 and Mrc-1 levels and restored the levels of iNOS and TNF-α (*Figure 2C,2D*). These results suggested that hDPSCs promoted M2 macrophage polarization by secreting IL-6, and that the inhibition of IL-6 secretion also inhibits the effect of hDPSCs on macrophage polarization.

hDPSCs regulate macrophage polarization through the IL-6/GP130/STAT3 signaling pathway

It has been shown that IL-6 can regulate the macrophage phenotype by activating the GP130/STAT signaling pathway (20). Here, we examined the expression of proteins involved in the GP130/STAT3 signaling pathway in each group of cells by western blot to further explore the macrophage polarization mechanism of hDPSCs. The results showed that compared with the control group, the levels of proteins GP130, p-JAK2 and p-STAT3 were significantly elevated with increasing ratios of p-JAK2/JAK2 and p-STAT3/STAT3 in the IL-6 and hDPSC groups. However, inhibition of IL-6 in the hDPSC + anti-IL-6 group largely abrogated the effect of the hDPSCs, decreasing the protein levels of GP130, p-JAK2 and p-STAT3 as well as the ratios of p-JAK2/JAK2 and p-STAT3/STAT3 (*Figure 3*). These findings showed that

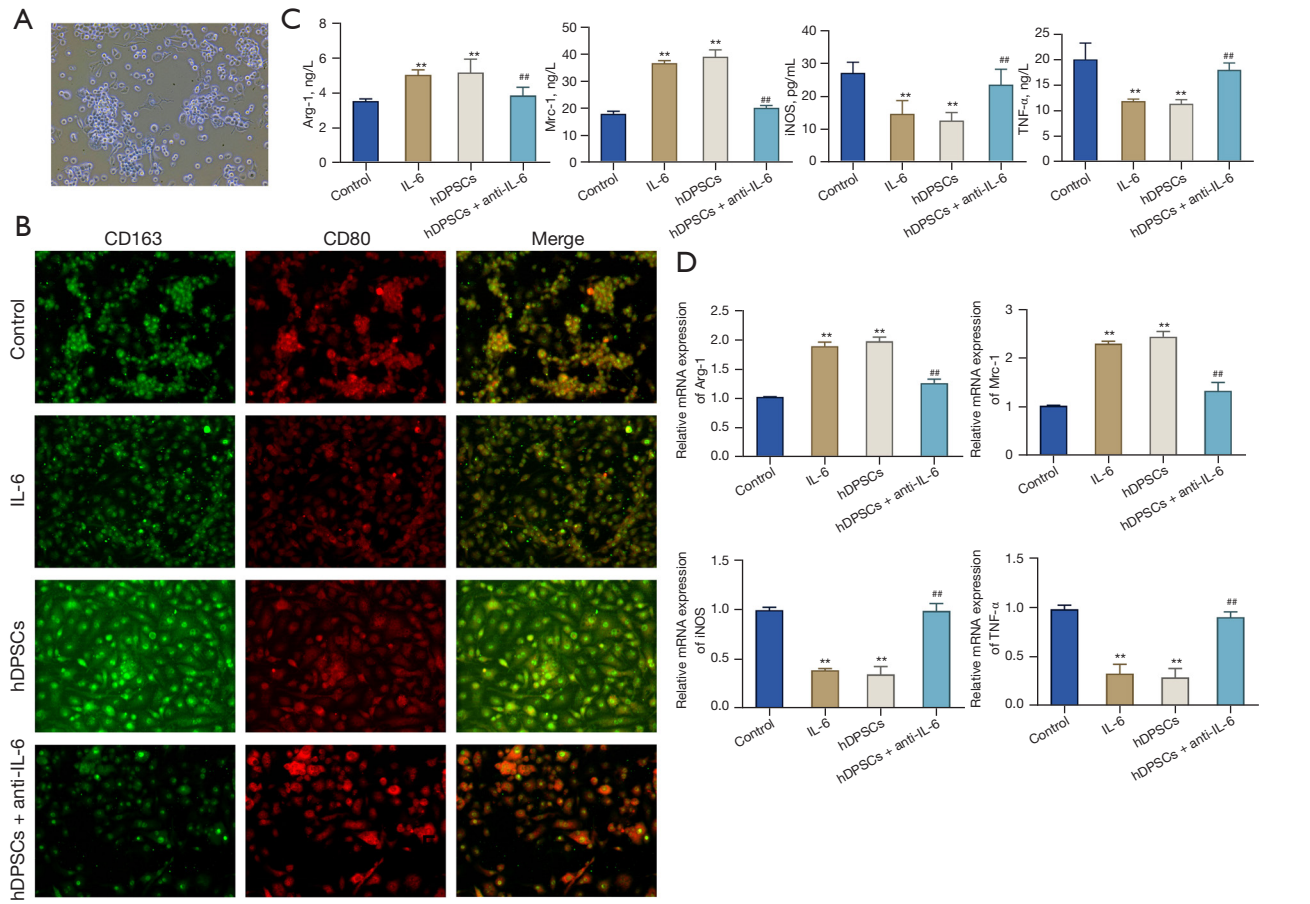

Figure 2 hDPSCs promote M2 macrophage polarization by secreting IL-6. (A) Differentiation of THP-1 cells observed microscopically, Dyeing method: PMA, Magnification: ×200; (B) expression of CD163 and CD80 in macrophages treated differently detected by immunofluorescence; (C) Arg-1, Mrc-1, iNOS, and TNF-α content in macrophages of each group detected by ELISA; (D) mRNA expression levels of Arg-1, Mrc-1, iNOS, and TNF-α in macrophages of each group detected by qRT-PCR. **, P<0.01 *vs.* Control; ##, P<0.01 *vs.* hDPSCs. Magnification: ×200. Representative data from three independent experiments are shown. hDPSCs, human dental pulp stem cells; IL-6, interleukin 6; THP-1, Tohoku Hospital Pediatrics-1; PMA, phorbol-12-myristate-13-acetate; Arg-1, Argininase-1; Mrc-1, Mannose receptor C-1; iNOS, inducible nitric oxide synthase; TNF-α, tumor necrosis factor-α; ELISA, enzyme linked immunosorbent assay; qRT-PCR, quantitative reverse transcription polymerase chain reaction.

hDPSCs significantly increased GP130/STAT3 signaling activity through IL-6.

Coculture of hDPSCs with THP-1-derived macrophages promotes migration and invasion of hBMSCs

Furthermore, to observe the effect of hDPSCs on hBMSCs after hDPSCs were cocultured with THP-1-derived macrophages, transwell and scratch assays were carried out to assess the invasion and migration abilities of hBMSCs. Both abilities of hBMSCs in the hDPSC + M-CM group were significantly increased compared with the hDPSC-CM group. However, for hBMSCs in the hDPSC + M + anti-IL-6-CM group, their abilities were significantly decreased compared with the hDPSC + M-CM group (*Figure 4A*,*4B*).

Coculture of hDPSCs with THP-1-derived macrophages enhances the osteogenic differentiation ability of hBMSCs

To observe the effects of hDPSCs on the osteogenic potential of hBMSCs cells after coculture with THP-1-derived macrophages, ALP and alizarin red staining were

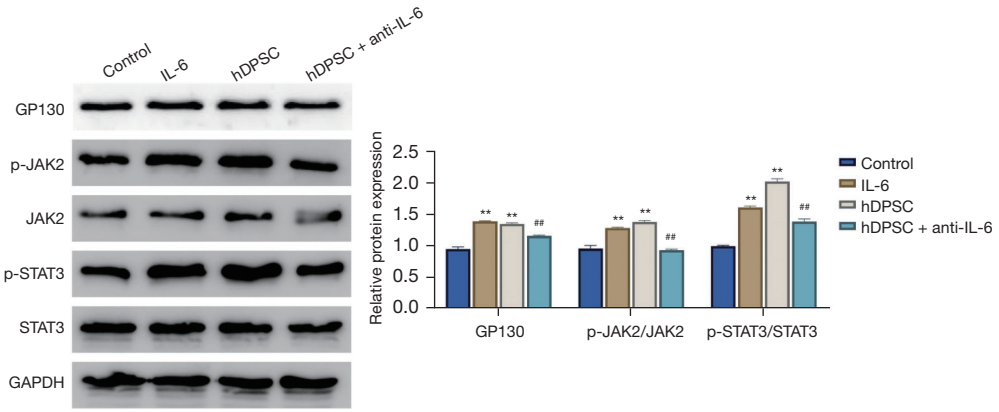

Figure 3 hDPSCs regulate macrophage polarization through IL-6/GP130/STAT3 signaling. Western blot analysis of the expression of GP130, p-JAK2, JAK2, p-STAT3, STAT3. **, P<0.01 *vs.* Control, ##, P<0.01 *vs.* hDPSCs. Representative data from three independent experiments are shown. hDPSCs, human dental pulp stem cells.

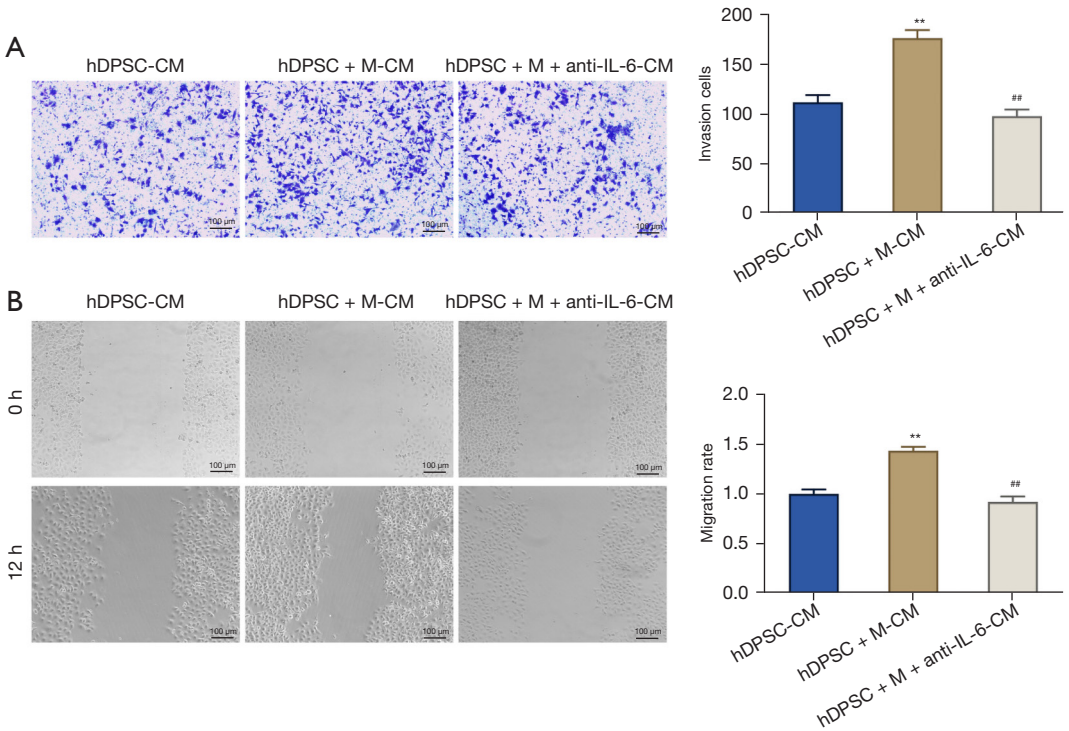

Figure 4 Coculture medium of hDPSCs and THP-1-derived macrophages promotes the migration and chemotaxis of hBMSCs. (A) Effect of coculture medium of hDPSCs and THP-1-derived macrophages on the invasion of hBMSCs detected by transwell assay, dyeing method: crystal violet staining solution; (B) effect of coculture medium of hDPSCs and THP-1-derived macrophages on the migration of hBMSCs detected by scratch assay. **, P<0.01 *vs.* hDPSC-CM group, ##, P<0.01 *vs.* hDPSC + M-CM group. Experiments were repeated three times. hDPSC-CM, BMSCs cultured in hDPSCs cultured medium; hDPSC + M-CM, BMSCs cultured in coculture medium of hDPSCs and THP-1-derived macrophages; hDPSC + M + anti-IL-6-CM, BMSCs cultured in hDPSCs cocultured with THP-1-derived macrophages in medium containing IL-6 inhibitor. hBMSCs, human bone marrow-derived stem cells; hDPSCs, human dental pulp stem cells.

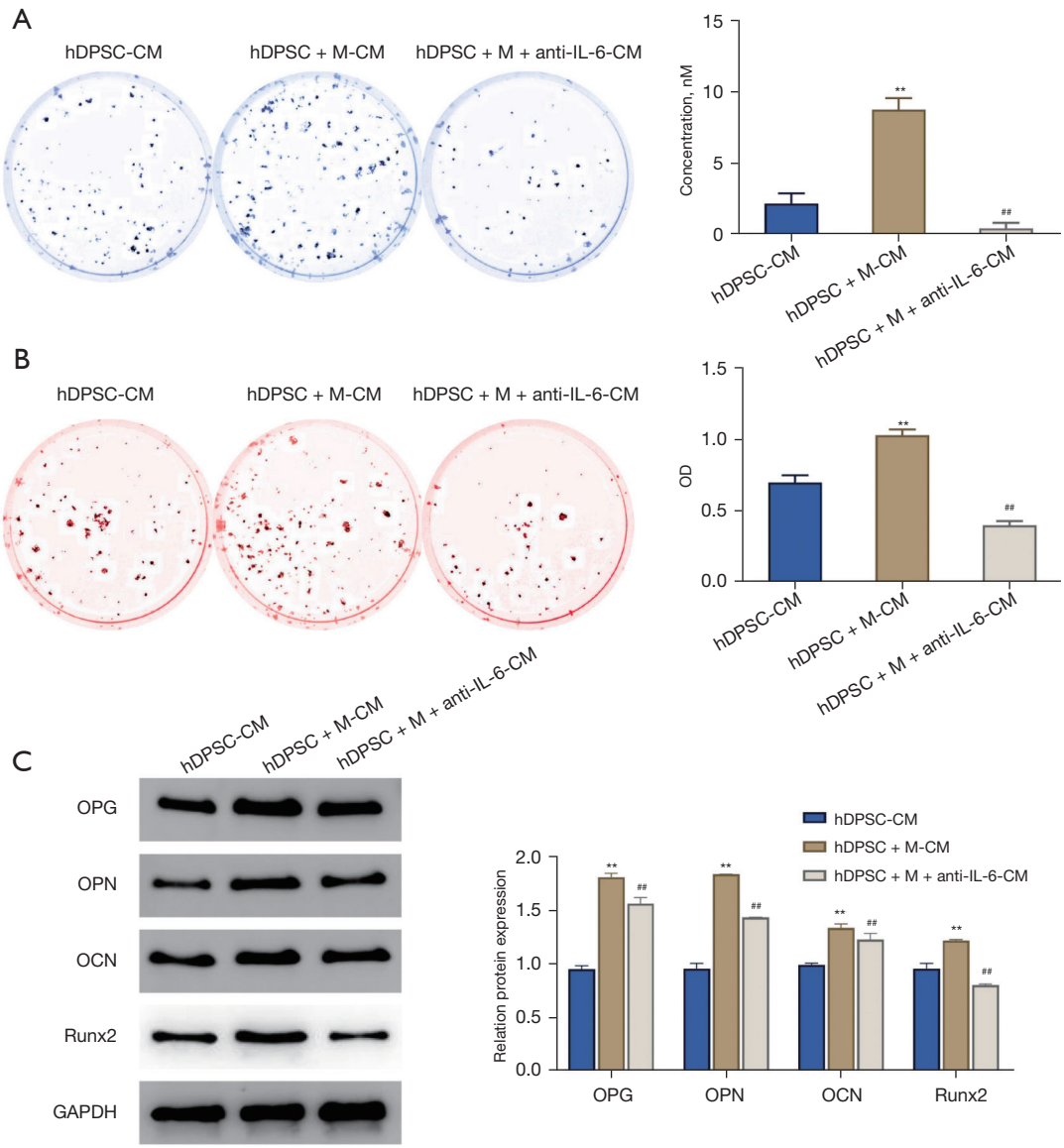

Figure 5 Osteogenic differentiation ability of hBMSCs promoted by coculture medium of hDPSCs and THP-1-derived macrophages. (A) ALP activity of hBMSCs in each group observed by staining, Dyeing method: ALP; (B) mineralized nodule formation of hBMSCs in each group determined by alizarin red staining; (C) OPG, OPN, OCN and Runx2 protein expression levels in hBMSCs in each group detected by western blot. **, P<0.01 *vs.* hDPSC-CM, ##, P<0.01 *vs.* hDPSC + M-CM. Experiments were repeated three times. hDPSCs, human dental pulp stem cells; OD, optical density; OPG, osteoprotegerin; OPN, osteopontin; OCN, osteocalcin; ALP, alkaline phosphatase; hBMSCs, human bone marrow-derived stem cells.

performed to evaluate the osteogenic potential of the cells. From the results, it was clear that the ALP activity and level of calcium deposition of hBMSCs in the hDPSC + M-CM group were significantly increased compared with the hDPSC-CM group. However, ALP activity and calcium deposition level of hBMSCs were suppressed in the presence of an IL-6 inhibitor (*Figure 5A,5B*). Subsequently, using western blot, we found the expression levels of cellular osteogenesis-related proteins osteoprotegerin, osteocalcin, osteopontin (OPN) and Runx2 in hBMSCs were increased in the hDPSC + M-CM group compared with the hDPSC-CM group. These protein levels were significantly decreased

in the hDPSC + M + anti-IL-6-CM group compared with the hDPSC + M-CM group (*Figure 5C*).

Discussion

Periodontitis is the common human chronic inflammatory disease. Studies have shown that inflammatory responses increase the secretion of receptor activator of nuclear factor kappa-B ligand (RANKL) from gingival fibroblasts or osteoblasts, which leads to the differentiation of osteoclasts (21). And the increase of osteoclast activity and inflammation in periodontitis can interfere with bone homeostasis and ultimately lead to osteolysis (22). In addition, the inflammation and ossification of periodontitis involve several signaling pathways, such as TLR4 /TRAF6 (23), PLCγ1-Ca^{2+}-NFATc1 (23), TLR4/MyD88/ NF-κB (24) and MAPK (25). In recent years, with the development of stem cell therapy and regenerative medicine, researchers have found that DPSCs have important application value in pulp and periodontal tissue regeneration and maxillofacial tissue defect repair. Meanwhile, researchers also found that the biological characteristics of DPSCs are affected by various factors, such as different environments (inflammatory microenvironment and scaffold material), different extraction and culture methods (oxygen content, medium and primary cell separation methods), different induction conditions, and inflammatory status of pulp tissue (26). The study of these influencing factors lays a solid foundation for the application of DPSCs in the field of regenerative medicine.

The hDPSCs are known to interact with various components of the innate and adaptive immune systems (e.g., T cells, natural killer cells, and macrophages, as well as the complement system). They can also inhibit the proliferation of allogeneic phytohemagglutinin-activated peripheral blood mononuclear cells in an independent manner through cell–cell contact (27). The hDPSCs have been found to stimulate M2 macrophage polarization (28), although the mechanism was not elucidated. Transplantation of hDPSCs into unilateral hindlimb skeletal muscle in a rat model of diabetes apparently triggered macrophage M2 polarization and thus inhibited sciatic nerve inflammation (29). IL-6 is generally regarded as a Th2 cytokine. To explore the mechanism by which hDPSCs regulate macrophages, their ability to secrete IL-6 was verified through coculture of hDPSCs and macrophages *in vitro*. IL-6 significantly upregulated the levels of Arg-1 and Mrc-1, downregulated the levels of NOS and TNF-α and upregulated $CD163^+$ cells in macrophages. This effect of hDPSCs was inhibited when IL-6 levels decreased, indicating that hDPSCs stimulate M2 polarization of macrophages through IL-6.

It has been demonstrated that IL-6 binds to GP130 to activate the phosphorylation of JAKs and STAT3, thereby activating the inflammatory cascade reaction and oncogenic pathways (30). STAT3 is a key regulator of inflammation, responsible for activating and regulating the growth of immune cells (31). Yin *et al.* found that the IL-6/STAT3 pathway mediates M1/M2 macrophage polarization during hepatocellular carcinoma development (32). Their finding has been verified in breast and gastric cancer (33). In our study, we found that the protein levels of GP130, p-JAK2, and p-STAT3 in macrophages were significantly increased with IL-6 treatment or coculture with hDPSCs. In summary, hDPSCs regulate GP130/STAT3 signaling and promote M2 macrophage polarization by releasing IL-6.

A wide range of cell–cell contact between macrophages and BMSCs occurs in injured bone, and is indispensable for macrophage apoptosis and cell endocytosis. This kind of juxtacrine interaction may cause juxtaposed cells to produce growth factors or pro-inflammatory cytokines such as transforming growth factor-β and IL-6 (34). It may also lead to chemotactic migration of nearby BMSCs (35). Of particular importance for the healing process of bone injury is the shift in macrophage phenotype from the more inflammatory M1 subtype to the anti-inflammatory M2 subtype, as nonunion is often attributed to a prolonged pro-inflammatory macrophage response (36). Studies in coculture systems and animal models have indicated that macrophage-derived chemokines (e.g., CCL2 and CXCL8) and osteoinductive factors (e.g., BMP2 and PGE2) can regulate the recruitment and osteogenic differentiation of BMSCs. However, the mechanism underlying this process has not been characterized (37). Recently, Zhao *et al.* stated that M2 macrophages could promote BMSC osteogenic differentiation by activating the PI3K/AKT/GSK3β/β-catenin pathway (38), and Li *et al.* discovered that exosomes derived from M2 macrophages promoted osteogenesis and reduced lipogenesis of BMSCs through the miR-690/IRS-1/TAZ axis (39). Hence, M2 macrophages can have a significant promoting osteogenic effect on BMSCs. We found that the hDPSCs culture medium had no influence on the migratory ability or chemotaxis of BMSCs, but the culture medium cocultured with hDPSCs and macrophages significantly increased migration ability and chemotaxis of BMSCs. Further, we

discovered that the hDPSC culture medium also rarely altered the osteogenic ability of BMSCs, but the addition of macrophages led to a significant increase in ALP activity, the calcium deposition level and the expression of osteogenesis-related proteins. When the IL-6 inhibitor was added, the connection between hDPSCs and macrophages was blocked, and the promoting effect on the osteogenesis of BMSCs was also inhibited. Thus, it can be seen that IL-6 is a key signaling molecule for hDPSCs to alter the cellular microenvironment.

Conclusions

Taken together, our work demonstrated that hDPSCs can promote M2 macrophage polarization by activating the GP130/STAT3 signaling through IL-6, as well as enhancing the migration, invasion and osteogenic differentiation of BMSCs. However, further studies are needed to elucidate the mechanism of macrophage polarization by hDPSCs and their immunomodulation of the periodontal microenvironment.

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Footnote

Reporting Checklist: The authors have completed the MDAR reporting checklist. Available at https://atm.amegroups.com/article/view/10.21037/atm-22-6390/rc

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://atm.amegroups.com/article/view/10.21037/atm-22-6390/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by ethics committee of Jinling Hospital, Nanjing Medical University (General Hospital of the eastern theater of the Chinese People's Liberation Army, Nanjing, China) (No. 2001-164XD4E) and informed consent was taken from all the patients.

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