Dental Pulp Mesenchymal Stem Cells Promote Polarization of M2 Macrophages and Accelerate Wound Healing by Secreting CCL2

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**Research Article**

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Abstract

Background

Mesenchymal stem cells (MSCs) are widely used in tissue engineering owing to their regenerative potential and immunomodulatory capacity. The crosstalk between MSCs and the host immune function plays a key role in the efficiency of tissue regeneration. However, the difference in immunological modulation and tissue regeneration function between MSCs from different sources remains unclear.

Methods

Human mesenchymal stem cells derived from bone marrow (BMMSCs), periodontal ligament (PDLSCs), adipose (ADSCs), and dental pulp (DPSCs) were obtained and induced to form cell sheets under the condition of 20 ug/ml vitamin C. The MSC cell sheets carried by hydroxyapatite/tricalciumphosphate (HA/TCP) particles were transplanted subcutaneously into C57BL6 mice for 8 weeks. Histological analyses were performed to detect the tissue regeneration potential and macrophages polarization in vivo. Then, THP-1 macrophages were co-cultured with MSCs and quantitative real-time polymerase chain reaction, immunofluorescent staining, western blotting, and enzyme-linked immunosorbent assay were used to investigate the function and mechanism of MSCs on macrophages in vitro. Finally, a wound healing model of the palatal mucosa was performed to confirm the effect of MSCs on macrophages and tissue healing efficiency.

Results

Compared to PDLSCs, BMMSCs, and ADSCs, DPSCs exhibited greater tissue regeneration potential, with greater tissue volume, higher Ki67 expression, and less apoptosis in the regenerated tissue of wild-type C57BL6 mice. In addition, DSPCs triggered more M2 macrophages in the regenerated tissue than other MSCs. Our data showed that DPSCs exhibited higher expression levels of C-C Motif Chemokine Ligand 2 (CCL2), and specific blocking of CCL2 by neutralising antibodies can significantly inhibit the DPSCs-induced polarization of M2 macrophages. Finally, DPSCs transplantation promoted wound healing of the palatal mucosa and M2 macrophages polarization in vivo, which could be significantly impaired by CCL2 neutralising antibody.

Conclusions

Our data indicate that DPSCs exert better tissue regeneration potential and immunoregulatory function by secreting CCL2. These results suggest that CCL2 application can enhance MSC-mediated tissue regeneration or wound healing.
Background

Mesenchymal stem cells (MSCs) are self-renewable and multipotent stromal cells which can differentiate into various cell types, such as osteoblasts, chondrocytes, myocytes, and adipocytes [1, 2]. In addition to their multiple differentiation potential, MSCs are capable of regulating various immune cells associated with both innate and adaptive immune systems [2, 3]. The differentiation and immunoregulatory capacity of MSCs make them suitable for wide clinical applications in various tissue regeneration, defect repair, and immunological diseases [2–5]. Evidence has shown that the tissue regeneration function is largely impaired when transplanted in wild-type mice compared to immune-deficient nude mice [6]. This indicated an intricate interaction between MSCs and the host immune function. To date, various functions and mechanisms have been identified between MSCs and host immune function.

On the one hand, MSCs regulate the phenotype and function of immune cells that participate in tissue repair and regeneration through a synergy of cell contact-dependent mechanisms and soluble factors [3–5]. MSCs express Fas ligand (FasL) to induce T-cell apoptosis through the FasL/Fas pathway [7, 8] and suppress B cell proliferation through programmed cell death protein 1 (PD-1) and programmed cell death 1 ligand (PD-L1) interaction in a cell-to-cell contact-dependent manner [9]. Meanwhile, the secretomes of MSCs, including transforming growth factor-β1 (TGF-β1) [10], indolamine 2,3-dioxygenase (IDO) [11], and prostaglandin E2 (PGE2) [12], play pivotal roles in immunomodulation and tissue repair [3–5]. On the other hand, pro-inflammatory T cells in the recipients inhibited bone marrow mesenchymal stem cells (BMMSCs)-mediated bone formation via T helper 1 (Th1) cytokine interferon (IFN)-γ and tumour necrosis factor (TNF)-α [6]. These results indicate that crosstalk between implanted donor MSCs and recipient immune cells may govern MSCs-mediated tissue regeneration; however, the different sources of MSCs-mediated immunomodulation during tissue regeneration need to be investigated.

In the present study, we chose human mesenchymal stem cells including BMMSCs, periodontal ligament stem cells (PDLSCs), adipose-derived stem cells (ADSCs), and dental pulp stem cells (DPSCs), which are widely used in clinical applications. The MSCs were induced to form cell sheets by vitamin C (Vc) and transplanted subcutaneously into C57BL/6 mice carried by hydroxyapatite/tricalciumphosphate (HA/TCP). Eight weeks after transplantation, the tissue regeneration potential and immune response were analysed, and the underlying molecular mechanisms of MSCs were investigated. The present study sheds light on the function and mechanism of intricate interactions between MSCs and the host immune system and provides strategies for enhancing the efficacy and ability of MSCs in tissue regeneration.

Methods

Mesenchymal Cells Cultures

Human BMMSCs, ADSCs, DPSCs and PDLSCs were isolated from iliac bone marrow, adipose tissue, dental pulp and periodontal tissue and obtained from the Department of Experimental Hematology, Beijing Institute of Radiation Medicine. All donors signed their informed consent form for their
participation in this study. The culture of four MSCs was performed as previously reported [13]. The same passage of BMMSCs, ADSCs, DPSCs and PDLSCs were used for each experiment.

Transplantation in C57BL6 mice

MSCs sheets were generated as described previously [14]. 1.5 × 10^5 PDLSCs, DPSCs, ADSCs and BMMSCs at passage 4 were subcultured in 60 mm dishes. 20 μg/ml Vc (Sigma-Aldrich Corp., St. Louis, MO, USA) was added to the culture medium. After 10-14 days, the cells at the edge of the dishes wrapped, which means that cell sheets had formed and could be detached. A complete Vc-induced DPSCs, PDLSCs, BMMSCs and ADSCs sheets were mixed with 40 mg of HA/TCP ceramic particles and transplanted subcutaneously into the dorsal surface of C57BL6 mice (n=5). At 8 weeks after transplantation, all animals were sacrificed, and the samples were harvested. Part of samples were grinded and the collect total RNA by RNAprep pure Tissue Kit (DP431, Tiangen Biotech, Beijing, China). The others fixed with 4% paraformaldehyde, decalcified with buffered 10% EDTA (pH 8.0), and then embedded in paraffin. 5 μm sections were obtained, deparaffinized, hydrated and stained with hematoxylin and eosin (HE) staining, TUNEL staining, immunohistochemistry and immunofluorescence.

THP-1 cell culture and stimulation

THP-1, a human monocyte cell line, was cultured in complete RPMI 1640 (Gibco; Thermo Fisher Scientific, Waltham, MA, USA) containing 10% fetal bovine serum (FBS, Gibco), 1% penicillin, streptomycin and L-glutamine (ThermoFisher Scientific) at 37 °C with 5% CO₂. Culture medium was changed every 2 - 3 days as necessary. For differentiation to naive macrophages, THP-1 were incubated with 100 ng/ml phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich) in 6-well plates for 24 h. Following differentiation, the plates were replaced by fresh medium, and cells were cultured for another 24 h.

Preparation of conditioned medium

Human DPSCs and BMMSCs, 70-80% confluent, were rinsed with phosphate-buffered saline (PBS), and fed serum-free Minimum Essential Medium α (α-MEM) (Gibco) containing 10% FBS (Gibco), 1% L-glutamine, penicillin and streptomycin (ThermoFisher Scientific). The medium was collected after 24 hours, centrifuged for 5 minutes at 1500 rpm and then for another 3 minutes at 3000 rpm to remove cells, filtered at 0.22 μm (Millex™ GP Filter Unit; Merck Millpore Ltd, Darmstadt, Germany), and stored at 4 °C within 2 weeks or at -80 °C within 1 month until use.

THP-1 cells cultured with conditioned medium (CM)

THP-1 macrophages (6 × 10^5) were seeded in 6-well plates. In M0 group, THP-1 cells were cultured with fresh complete medium (complete RPMI 1640 medium: complete MEM-α medium = 3: 2) for 3 d. In M0 + DPSCs-CM group, cells were cultured by mixture of 2/5 complete RPMI 1640 medium, 27/50 DPSCs-CM and 3/50 FBS. In M0 + BMMSCs-CM group, cells were cultured by mixture of 2/5 complete RPMI 1640 medium, 27/50 BMMSCs-CM and 3/50 FBS for 3 d.
THP-1 cells co-cultured with BMMSCs and DPSCs by transwell assay

THP-1 macrophages (6 × 10^5) were seeded in the lower chamber of the transwell, while DPSCs or BMMSCs (3 × 10^5) were loaded into the upper chamber of transwell insert (Corning Inc.) with each type of cell cultured alone as control. Under certain conditions, THP-1 macrophages and DPSCs or BMMSCs (2:1) of passage 4 and DPSCs of passage 8 were cocultured in a transwell system in the presence or absence of either 10 μg/ml C-C motif chemokine ligand 2 (CCL2) neutralising antibody (MAB679; R&D System) for 3 d. And recombinant human CCL2 (10 ng/ml or 15 ng/ml; Proteintech™, USA) were added for 3 d as positive control group. Then the culture media and whole cell lysates of THP-1 macrophages were prepared for quantitative real-time polymerase chain reaction (qRT-PCR), immunofluorescent staining, western blotting, and enzyme-linked immunosorbent assay (ELISA), respectively. All experiments were repeated at least three times with the similar results.

Bioinformatic analysis gene expression profile of BMMSCs and DPSCs

Gene expression datasets derived from Ma, et al [13], which was compared the gene expression between human BMMSCs and DPSCs. Those genes with an adjusted P-Value < 0.05 and absolute value of fold change (FC) > 2 were analyzed. Then differentially expressed genes related to immune were selected and verified by qRT-PCR.

qRT-PCR

Total RNA was extracted from macrophages with RNAprep Pure Cell/Bacteria Kit (DP430, Tiangen Biotech, Beijing, China) and were reversely transcribed to cDNA with FastKing RT Kit (KR116, Tiangen Biotech, Beijing, China) according to the manufacturer’s instructions. qRT-PCR was performed using an SuperReal PreMix Plus (SYBR Green) (FP205, Tiangen Biotech, Beijing, China). The PCR mixture comprised of 10 μl SYBR Grenn, 0.3 μM forward and reverse primers and 1 μl cDNA. After normalization of target gene expression, the data was quantified by the 2−ΔΔCt method. The genes and primer sequences are listed in Additional Table 1.

Western Blot

Cell extracts were prepared with whole cell lysis buffer (Applygen, Beijing, China) according to the manufacturer’s instructions. The protein concentrations were determined by the BCA Protein Assay Kit (PC0020, Solarbio, Beijing, China). Equal amounts of cellular proteins (20 μg) were boiled for 10 min at 98 °C. Equal amounts of protein extracts were loaded onto 10% polyacrylamide gels for electrophoresis and transferred to nitrocellulose membranes. Bands were detected immunologically with polyclonal antibodies (1: 1,000) against Arginase 1 (Abclonal, A4923). ACTB (Applygen, C1313) was used as a loading control. Immunoblot bands were visualized following the application of ECL detection system (ChemiDoc™ MP Imaging System, Biored).

Enzyme-linked immunosorbent assay (ELISA)
In vitro, cell free supernatants were collected and centrifuged at 14000 rpm for 30 min at 4 °C of each group and assayed for the concentration of TNF-α and IL-10 with ELISA kits (human TNF-α, 430204; human IL-10, 430604; Biolegend, Inc) following the manufacturer's instructions. The optical density was measured at 450 nm using a microplate reader with the correction wavelength set at 570 nm.

**Wound Healing Model and BMMSCs and DPSCs Treatment**

Male C57BL6 mice (n=5, body weights of 20 - 22 g) were anesthetized with 1% pentobarbital (30 mg/kg). For the wound healing model of palatal mucosa, the full-thickness palate ranging from the mesial edge of the first molar to the distal edge of the third molar, from gingival margin to palatal suture was removed. For treatment, mice were randomized to different treatment groups. Unless otherwise stated, 2.5 × 10^5 engineered DPSCs and BMMSCs at passage 5 suspended in PBS and recombinant human CCL2 (10 μg per mice) were directly injected into the surrounding mucosa in day 1. Neutralising antibodies to human CCL2 (R&D Systems) were given to mice by intraperitoneal injection in day 2 at a dose of 10 μg/mouse. The mice were sacrificed after 5 days, and the defect area in palates was observed by stereomicroscopy and assessed using ImageJ (NIH, MD, United States).

**Immunofluorescence staining**

THP-1 cells were adhered to the surface of glass cover slides were transferred to transwell systems or conditioned medium. After coculture, cells on the slides were fixed with 4% paraformaldehyde for 30 min, permeabilized with 0.3% Triton (MP Biomedicals) for 10 min and then blocked with 3% bovine serum albumin (BSA; MP Biomedicals) for 30 min at room temperature. Slides were incubated separately with antibodies against CD68 (1/100, ab955; Abcam), CD163 (1/100, A8383; Abclonal) and CD80 (1/100, A16039; Abclonal) at 4 °C overnight. The sections (5 μm) of paraffin-embedded tissue were stained with specific antibodies (1: 100) for Ki67 (Abcam, ab15580), CD68 (Abcam, ab955), CD206(CST, 24595S) and Arg1(Abclonal, A4923) overnight at 4 °C and subsequently incubated with FITC- or rhodamine-conjugated goat anti-mouse or anti-rabbit secondary antibodies (1: 1000) plus 4’,6-diamidino-2-phenylindole (DAPI) staining for nuclei. Double-stained samples were evaluated under a fluorescence microscope (BX61, Olympus).

**Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL) Assay**

Paraffinized samples were sectioned at 5 μm thickness, mounted on silane-coated glass slides. TUNEL staining was performed with a One Step TUNEL Apoptosis Assay Kit (C1090, Beyotime, Shanghai, China) according to the manufacturer’s instructions. Images were captured under a confocal microscope and the number of TUNEL-positive cells was calculated using Image J software (NIH, MD, United States).

**Statistical Analysis**

The results are presented as mean ± SDs. Among multiple groups, one-way ANOVA was used to determine significant differences. Student’s t-test was employed to compare difference between two
groups with normal distribution or Kruskal-Wallis test for others. \* \( P < 0.05 \) and \** \( P < 0.01 \). Statistical analysis was performed using SPSS 20.0.

**Results**

**DPSCs sheet exhibited higher tissue regeneration potential than PDLSCs, ADSCs and BMMSCs sheets in C57BL6 mice**

Human DPSCs, PDLSCs, BMMSCs, and ADSCs sheets with carrier HA/TCP particles were subcutaneously implanted into C57BL6 mice. Eight weeks after transplantation, the animals were sacrificed, and the grafts were harvested for histological analysis. HE staining and quantitative analysis results revealed that a more mineralised collagen-like matrix was regenerated in the DPSCs and PDLSCs group compared to the BMMSCs and ADSCs groups (\( P < 0.01 \)) (Fig. 1a, d). Furthermore, immunohistochemical staining and quantitative analysis showed that Ki67 was highly expressed in the DPSCs and ADSCs groups compared to the PDLSCs and BMMSCs groups (\( P < 0.01 \)) (Fig. 1b, e). Moreover, TUNEL staining indicated that the DPSCs group showed a lower positive percentage compared with the other three groups (\( P < 0.05, \ P < 0.01 \)) (Fig. 1c, f).

**DPSCs induces polarization of macrophages towards CD206\(^+\) M2 phenotype during tissue regeneration**

We next investigated the *in vivo* effects of human DPSCs, PDLSCs, BMMSCs, and ADSCs on the inflammatory cell response. Based on immunohistochemical staining, the ratio of CD68\(^+\) to CD206\(^+\) cells (M2 macrophages) was significantly increased in DPSCs and ADSCs treatment as compared to the PDLSCs and BMMSCs groups (\( P < 0.01 \)) (Fig. 2a, b). qRT-PCR analysis showed that the expression of CD68 did not differ between the different groups, but DPSCs induced the expression of CD206 and reduced the expression of CD80, TNF-\( \alpha \), IL-1\( \beta \), and IL-6, compared with the BMMSCs group (\( P < 0.05 \) or \( P < 0.01 \)) (Fig 2c).

**DPSCs-CM converted human THP-1 monocytes to M2 macrophages**

THP-1 to M0 macrophages were activated by pulsing with PMA (100 ng/ml). M0 macrophages were cultured with DPSCs-CM and BMMSCs-CM for 1 d and 3 d. Double immunofluorescence staining of CD68 and CD80 showed that DPSCs-CM and BMMSCs-CM could both significantly inhibit the polarization of M1 macrophages for 3 d (\( P < 0.01 \)) (Fig. 3a, b). Immunocytochemical staining of CD68 and CD163 showed that DPSCs-CM and BMMSCs-CM could both significantly elicit polarization of M2 macrophages for 3 d, but the ability of BMMSCs-CM was weaker than that of DPSCs (\( P < 0.05 \) or \( P < 0.01 \)) (Fig. 3b, d). For the expression of mRNA of pro-inflammatory and anti-inflammatory cytokines, qRT-PCR analysis showed that IL-6, TNF-\( \alpha \), IL-10, and Arg1 mRNA expression for 1 d were upregulated, whereas IL-6 and TNF-\( \alpha \) mRNA expression levels were downregulated and IL-10 and Arg1 mRNA expression levels were upregulated for 3 d. The immunomodulating function of DPSCs-CM was significantly better than that of BMMSCs-CM (\( P < 0.05, \ P < 0.01 \)) (Fig. 3e). Cultured by DPSCs-CM and BMMSCs-CM for 3 d, the secretion
of TNF-α of THP-1 macrophages was decreased and IL-10 was increased, and there was no significant difference between DPSCs-CM and BMMSCs-CM treatments ($P < 0.05$) (Fig. 3f).

**DPSCs elicit the polarization of M2 macrophages in transwell assay**

The M0 macrophages were co-cultured with DPSCs and BMMSCs at passage 3-5 at a ratio of 2:1 (macrophages: stem cells) cell density by transwell test for 3 d. Similar to MSCs-CM, DPSCs and BMMSCs could significantly inhibit the polarization of M1 macrophages and elicit polarization of M2 macrophages for 3 d by transwell with respect to immunofluorescence staining, the ability of BMMSCs was significantly weaker than that of DPSCs ($P < 0.05$ or $P < 0.01$) (Fig. 4a, b, c, d). qRT-PCR analysis showed that IL-1β and TNF-α mRNA expression for 1 d was upregulated, whereas IL-1β, IL-6, and TNF-α mRNA expression levels were downregulated after 3 d. The mRNA expression of Arg1 was upregulated for 1 d and 3 d, but the ability of BMMSCs was significantly weaker than that of DPSCs ($P < 0.05$, $P < 0.01$) (Fig. 4e). The secretion of TNF-α in THP-1 macrophages was decreased, and IL-10 was increased after co-culture with DPSCs and BMMSCs, and the concentration of IL-10 co-cultured with DPSCs was significantly higher than that of BMMSCs (Fig. 4f). This evidence showed that DPSCs and BMMSCs could elicit the polarization of M2 macrophages from M0 macrophages via soluble cytokines, and that DPSCs possess better immunomodulatory function than BMMSCs.

**DPSCs mediated induction of M2 macrophages by CCL2**

Gene expression datasets derived from Ma et al. [13] were used to compare gene expression between human BMMSCs and DPSCs. The top 20 differentially expressed genes related to immunity between DPSCs and BMMSCs were visualised (Fig. 5a). These results were confirmed by real-time RT-PCR analysis, demonstrating that the mRNA expression levels of CCL2, TGFBR3, C1S, IL1R1, IL10RB, IFNAR1, CD46, and CD302 were decreased in BMMSCs at passage three compared to those of DPSCs (Fig. 5b).

To confirm the function of CCL2 in this immunomodulatory process, macrophages were co-cultured with DPSCs and BMMSCs at passage 4 in transwell for 3 d in the presence or absence of specific neutralising antibodies for CCL2 (10 μg/ml) and CCL2 recombinant protein (15 ng/ml). With respect to mRNA expression, DPSCs and BMMSCs could downregulate the mRNA expression of IL-1β, IL-6, and TNF-α, and upregulate the mRNA expression of Arg1, and blocking CCL2 could inhibit the DPSCs-mediated decrease of pro-inflammatory cytokines ($P < 0.05$ or $P < 0.01$). However, the presence of neutralising antibodies against CCL2 did not influence the immunomodulatory function of BMMSCs (Fig. 6a, b, c, d). Meanwhile, DPSCs and BMMSCs could upregulate the concentration of IL-10 secreted and downregulate the concentration of TNF-α secreted and blocking CCL2 could inhibit DPSCs-mediated induction of M2, but not BMMSCs ($P < 0.05$ or $P < 0.01$) (Fig. 6e, f).

**Passage induced senescence of DPSCs still possess the ability to induce M2 macrophages polarization via CCL2**
MSCs cultured at passage 6 had a senescence phenotype, and we chose DPSCs at passage eight to examine their immunomodulatory function on macrophages of senescent DPSCs. DPSCs (P8) downregulated the mRNA expression of IL-1β, IL-6, and TNF-α and upregulated the expression of IL-10 for 3 d. Blocking CCL2 could inhibit DPSCs (P8)-mediated induction of M2 macrophages (Fig. 7a, b, c, d, g, h). Arg1 protein expression was upregulated by culturing with DPSCs (P8) in transwell for 3 d, and specific neutralising antibodies of CCL2 could weaken this ability of DPSCs (Fig. 7e, f). This evidence proved that CCL2 secreted by DPSCs plays an important role in the induction of M2 macrophages.

**DPSCs-based therapy enhanced mucosa wound healing in mice via CCL2**

Given the important roles of both MSCs and M2 macrophages in wound healing, we investigated the function of MSCs and CCL2 in wound healing models of palates in mice. First, we explored whether DPSCs and BMMSCs were capable of enhancing mucosal excisional wound repair. DPSCs and BMMSCs (2.5 × 10^5 per mouse) and recombinant human CCL2 (10 μg per mouse) were directly injected into the surrounding mucosa on day 1, and neutralising antibodies of human CCL2 were administered to mice by intraperitoneal injection on day 2 at a dose of 10 μg/mouse. Wound closure was carefully measured on day 5. Our results showed that mice receiving DPSCs and BMMSCs infusion displayed accelerated mucosal wound closure compared with the control mice without treatment, and the application of CCL2 neutralising antibodies inhibited DPSCs-mediated wound repair, but not BMMSCs (P < 0.05) (Fig. 8a, b). The usage of human CCL2 recombinant protein and neutralising antibodies did not differ from the control group [Additional Fig. 1a, b]. Histological analysis of wounds on day 5 showed a more organised granulation tissue at the excisional wound site in DPSCs-treated mice compared with other groups (Fig. 8c; [Additional Fig. 1c]).

Next, we investigated the *in vivo* effects of DPSCs and BMMSCs on the phenotype of macrophages in mucosal wounds. Dual-colour immunofluorescence assays were performed using specific antibodies CD68 and Arg1, which are well-known markers of M2 macrophages. DPSCs and BMMSCs treatment led to an increase in the number of CD68-and Arg1-positive macrophages compared with the control group, and the percentage of M2 macrophages was significantly higher in the DPSCs group than in the BMMSCs group (P < 0.05). Meanwhile, the application of neutralising antibody weakened this function of DPSCs, but not BMMSCs (Fig. 8d, e). The usage of CCL2 recombinant protein and neutralising antibodies did not differ from the control group [Additional Fig. 1d, e]. These findings suggest that DPSCs are capable of promoting the polarization of M2 macrophages and enhancing the healing of excisional mucosa in mice, and that CCL2 plays an essential role in the immunomodulatory activity of DPSCs.

**Discussion**

MSCs-based regenerative medicine is a promising strategy for tissue regeneration and wound healing [3]. MSCs display great properties of mineral tissue regeneration in nude mice [6, 15]. However, until now, the application of MSCs influenced by the cross-talk between MSCs and the immune system of recipients
was still unstable and limited in clinical trials. MSCs from different sources possess different characteristics associated with immunomodulation [16]. Dental-tissue-derived MSCs, such as DPSCs, PDLSCs, and SCAP, residing in human teeth and dental tissue, have been isolated and characterised, and exhibit different characteristics [1, 17]. Due to their neural crest origin, dental-derived MSCs have stronger stemness and neurogenic capacity [18]. Our previous study demonstrated that BMMSCs, ADSCs, DPSCs, and PDLSCs possess different capacities for tissue regeneration and varying immunomodulatory priority, providing evidence for selecting suitable MSCs based on different characteristics in clinical application. With greater proliferation and colony-forming properties and higher expression levels of the ALP gene, DSPP, and DMP-1 [19], DPSCs have a superior ability to maintain their stem cell properties during aging and have greater resistance under conditions of inflammation-induced senescence [13]. Local administration of allogeneic or autogenous BMMSCs, PDLSCs, and DPSCs could induce tissue regeneration in periodontal defects in vivo, and more hard tissue regeneration in the periodontium after DPSCs injection compared to BMMSCs injection in vivo [9, 12, 13, 20–23]. In the present study, DPSCs showed greater potential to enhance tissue regeneration with higher expression of proteins related to proliferation and less apoptosis compared to BMMSCs, PLDSCs, and ADSCs. In wound healing models, DPSCs possess better ability to accelerate wound closure compared to BMMSCs.

The interaction between implanted donor MSCs and recipient immune cells may play an essential role in MSC-mediated tissue regeneration [2–5]. Meanwhile, the immunomodulatory functions of MSCs from different sources varied in vitro and in vivo. BMMSCs, ADSCs, and MSCs of dental tissue can suppress the proliferation of T cells, B cells, NK cells, and other immune cells [16, 24]. This might partially be explained by the fact that MSCs from different sources and under different culture conditions express different surface markers and show varying cytokine secretion profiles. Macrophages, one of the major types of innate immune cells, play essential roles in inflammation, immunity, tissue regeneration, and tissue repair via classic (M1) and alternative (M2) polarization. M1 macrophages play important roles in inflammation by secreting pro-inflammatory mediators, including IL-1, IL-6, TNF-α, and IL-12. In contrast, M2 macrophages are characterised by the secretion of high levels of anti-inflammatory mediators, such as IL-10 and TGF-β [25–27]. In the present study, we found that macrophages, which partly govern tissue regeneration, showed different polarization patterns after translation of MSCs from different sources. Some studies have reported communication between MSCs and macrophages [28–30]. MSCs have been reported to increase macrophages phagocytic capacity [31]. MSCs such as BMMSCs [32], ADSCs [29], PDLSCs [33], and dental follicle stem cells (DFSCs) [34] can induce macrophages polarization into the M2 phenotype by stimulating Arginase (Arg)-1, CD163, and IL-10 and inhibiting TNF-α. Human gingiva-derived mesenchymal stem cells (hGMSCs) are capable of eliciting M2 polarization of macrophages, which might contribute to a marked acceleration of wound healing [30]. DPSCs can inhibit macrophages and elicit macrophage M2 polarization via the TNF-α/IDO axis [11, 24]. These studies showed different mechanisms of MSC interaction with macrophages.

Through bioinformatic analysis, we found that CCL2, a well-known macrophage chemoattractant [35], was more highly expressed in DPSCs than in BMMSCs. The use of neutralising anti-CCL2 antibody abolished the polarization of THP-1 macrophages mediated by DPSCs, but not BMMSCs, suggesting that
CCL2 could contribute to DPSCs-induced polarization of M2 macrophages. A previous study reported that CCL2 could modulate monocyte recruitment in multiple inflammatory diseases by interacting with its corresponding receptor, CCR2, which is present in monocytes [36]. CCL2 has been reported to mediate the polarization of macrophages that participate in tissue inflammation. Evidence has shown that MSC-derived CCL2 is necessary for the induction of IL-10 expression by macrophages. However, it needs to be combined with other cytokines, as CCL2 alone could not accelerate wound healing in the present study. In addition, CCL2 can promote the survival of human CD11b+ peripheral blood mononuclear cells and induce M2 macrophages polarization [37] and decrease TNF-α secretion in vitro [38], but the function is dependent on the MSC type [30, 39]. In addition, CCL2 may also play a role in the M1 macrophages. This showed that CCL2 induced bone-marrow-derived macrophages (BMDMs) to polarise to the M1 phenotype (proinflammatory phenotype) through the CCR2/RhoA axis [40]. Moreover, inhibition of CCL2 by oral administration of bindarit suppressed the infiltration of proinflammatory monocytes and altered the inflammatory properties of macrophages in the diabetic periodontium [41]. All these data indicated that CCL2 played an intricate function in the crosstalk between MSCs and macrophages, which needs to be further investigated.

**Conclusions**

In conclusion, this study demonstrated that DPSCs exert better tissue regeneration potential and immunoregulatory function by secreting CCL2. The results suggest that regulatory effects on macrophages with respect to phenotype and functions during tissue regeneration and wound healing via CCL2 application could enhance MSC-mediated tissue regeneration or wound healing, and an optimised stimulation to enhance DPSCs or other MSC immunomodulation needs to be further investigated.

**Abbreviations**

Adipose-derived stem cells (ADSCs)

Alkaline phosphatase (ALP)

Arginase-1 (Arg-1)

Bone marrow mesenchymal stem cells (BMMSCs)

C-C Motif Chemokine Ligand (CCL)

Cluster of differentiation (CD)

Conditioned medium (CM)

Dental follicle stem cells (DFSCs)

Dental pulp stem cells (DPSCs)
Dentin matrix acidic phosphoprotein 1 (DMP-1)
Dentin sialo phosphoprotein (DSPP)
Human gingiva-derived mesenchymal stem cells (hGMSCs)
Hyaluronic acid (HA)
Indolamine 2,3-dioxygenase (IDO)
Interferon (IFN)
Interleukin (IL)
Mesenchymal stem cells (MSCs)
Natural killer (NK)
Neutralising antibody (NTB)
Periodontal ligament stem cells (PDLSCs)
phorbol 12-myristate 13-acetate (PMA)
Programmed cell death 1 ligand (PD-L1)
Programmed cell death protein 1 (PD-1)
Prostaglandin E2 (PGE2)
Ras Homolog Family Member A (RhoA)
Real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR)
Stem Cells from the Apical Papilla (SCAP)
T helper 1 (Th1)
Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)
Transforming Growth Factor Beta Receptor 3 (TGFBR3)
Transforming growth factor-β1 (TGF-β1)
Tumour necrosis factor (TNF)
β-tricalcium phosphate (TCP)
Declarations

Ethics approval and consent to participate

The use of animals for cell isolation was approved by the Animal Use and Care Committee of Capital Medical University, and the experimental procedures were in accordance with the Intramural Animal Use and Care Committee of Capital Medical University. The human stem cells obtained from the Department of Experimental Hematology, Beijing Institute of Radiation Medicine and all donors signed their informed consent form for their participation in this study.

Consent for publication

Not applicable

Availability of data and materials

Original data are available upon request.

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Authors’ contributions

ZY and LH contributed to research design; experiment execution; data collection, assembly and analysis; and manuscript writing. LM, JW and CZ contributed to the experiment execution and data analysis and interpretation. CD contributed to the data collection, data analysis and interpretation. SW contributed to the study conception and design, financial support and manuscript writing. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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**Figures**

**Figure 1**

DPSCs sheet exhibited higher tissue regeneration potential in C57BL6 mice. a, d HE staining and quantitative analysis results revealed a more mineralised collagen-like matrix was regenerated in the DPSCs and PDLSCs group compared to the BMMSCs and ADSCs groups (scale bar: 200μm). b, e Immunohistochemical staining and quantitative analysis showed that Ki67 was highly expressed in the DPSCs and ADSCs groups compared to the PDLSCs and BMMSCs groups (scale bar: 50μm). c, f TUNEL staining indicated that the DPSCs group showed a lower positive percentage compared with the other three groups (scale bar: 100μm) (* P < 0.05, ** P < 0.01).

**Figure 2**

DPSCs induces polarization of macrophages towards CD206+ M2 phenotype during tissue regeneration. a Translation sections were dual-color immunostained with specific antibodies for CD68 (Green) and
CD206 (Red) (Scale bar: 50 μm). b The ratio of CD68+ to CD206+ cells (M2 macrophages) was significantly increased in DPSCs and ADSCs treatment as compared to the PDLSCs and BMMSCs groups. c Real-time PCR analysis revealed that the DPSCs could induce more M2 macrophage related genes in vivo compared with BMMSCs (* P < 0.05, ** P < 0.01).

**Figure 3**

DPSCs-CM converted human THP-1 monocytes to M2 macrophages. a, b Double immunofluorescence staining of CD68 and CD80 revealed that DPSCs-CM and BMMSCs-CM could both significantly inhibit the polarization of M1 macrophages for 3 d (Scale bar: 25μm). c, d Immunocytochemical staining of CD68 and CD163 showed that DPSCs-CM and BMMSCs-CM could both significantly elicit polarization of M2 macrophages for 3 d, but the ability of BMMSCs-CM was weaker than that of DPSCs (Scale bar: 50μm). e qRT-PCR analysis showed that IL-6, TNF-α, IL-10, and Arg1 mRNA expression for 1 d were upregulated, whereas IL-6 and TNF-α mRNA expression levels were downregulated and IL-10 and Arg1 mRNA expression levels were upregulated for 3 d. The immunomodulating ability of DPSCs-CM was significantly better than that of BMMSCs-CM. f After the coculture, the secretion of TNF-α of THP-1 macrophages was decreased and IL-10 was increased, and there was no significant difference between DPSCs-CM and BMMSCs-CM treatments. Data are presented as mean ± SD of three independent experiments (* P < 0.05, ** P < 0.01).

**Figure 4**

DPSCs elicit the polarization of M2 macrophages in transwell assay. a, b Immunocytochemistry staining of CD68 and CD80 showed that DPSCs and BMMSCs could both significantly inhibit the polarization of M1 macrophages for 3 d by transwell, and the ability of BMMSCs was significantly weaker than that of DPSCs (Scale bar: 50μm). c, d Immunocytochemistry staining of CD68 and CD163 showed that DPSCs and BMMSCs could both significantly elicit polarization of M2 macrophages for 3 d by transwell, but the ability of BMMSCs was weaker than DPSCs (Scale bar: 50μm). e qRT-PCR analysis showed that IL-1β and TNF-α mRNA expression for 1 d were upregulated, whereas IL-1β, IL-6 and TNF-α mRNA expression levels were downregulated for 3 d. The mRNA expression of Arg1 was upregulated for 1 d and 3 d, but the ability of BMMSCs was significantly weaker than that of DPSCs. f THP-1 cell lines cultured with DPSCs and BMMSCs could increase the concentration of IL-10 and decrease the concentration of TNF-α for 3 d, and the ability of BMMSCs was significantly weaker than DPSCs. Data are presented as mean ± SD of three independent experiments (* P < 0.05, ** P < 0.01).

**Figure 5**
Bioinformatic analysis gene expression profile of BMMSCs and DPSCs. a The top 20 differentially expressed genes related to immunity between DPSCs and BMMSCs were visualised. b qRT-PCR analysis demonstrated that the mRNA expression levels of CCL2, TGFB3, C1S, IL1R1, IL10RB, IFNAR1, CD46, and CD302 were decreased in BMMSCs at passage three compared to those of DPSCs. Values are means ± SDs. Student’s t-tests were used to determine statistical significance. Error bars represent SDs (n = 3) (** P < 0.01).

**Figure 6**

DPSCs mediated induction of M2 macrophages by CCL2. a, b, c, d DPSCs and BMMSCs could downregulate the mRNA expression of IL-1β, IL-6, and TNF-α, and upregulate the mRNA expression of Arg1, and blocking CCL2 could inhibit the DPSCs-mediated decrease of pro-inflammatory cytokines. The presence of neutralising antibodies against CCL2 did not influence the immunomodulatory function of BMMSCs. e, f DPSCs could increase the concentration of IL-10 and decrease the concentration of TNF-α for 3 d, and blocking CCL2 could inhibit DPSCs-mediated induction of M2 macrophages, but not BMMSCs (* P < 0.05, ** P < 0.01). NAB neutralising antibody

**Figure 7**

Passage induced senescence of DPSCs possess the ability to induce M2 macrophages polarization via CCL2. a, b, c, d DPSCs (P8) downregulated the mRNA expression of IL-1β, IL-6, and TNF-α and upregulated the expression of IL-10 for 3 d. Blocking CCL2 could inhibit DPSC (P8)-mediated induction of M2 macrophages. e, f DPSCs could increase the concentration of IL-10 and decrease the concentration of TNF-α for 3 d, and blocking CCL2 could inhibit DPSCs (P8)-mediated induction of M2 macrophages. g, h Arg1 protein expression were upregulated by cultured with DPSCs (P8) in transwell for 3 d, and specific neutralizing antibodies of CCL2 could weaken this ability of DPSCs (P8). Data are presented as mean ± SD of three independent experiments (* P < 0.05, ** P < 0.01). NAB neutralising antibody

**Figure 8**

DPSCs-based therapy enhanced mucosa wound healing in mice via CCL2. a, b Mice receiving DPSCs and BMMSCs infusion displayed accelerated mucosal wound closure compared with the control mice without treatment, and the application of CCL2 neutralising antibodies inhibited DPSCs-mediated wound repair, but not BMMSCs. c HE staining of wounds showed a more organised granulation tissue at the excisional wound site in DPSCs-treated mice compared with other groups. d, f Double immunofluorescence staining of CD68 and Arg1 revealed DPSCs and BMMSCs treatment led to an increase in the number of M2 macrophages compared with the control group, and the percentage of M2 macrophages was significantly
higher in the DPSCs group than in the BMMSCs group, and the application of neutralising antibody weakened this function of DPSCs, but not BMMSCs (* P < 0.05, ** P < 0.01). NAB neutralising antibody

**Supplementary Files**

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