Research Square

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Paeoniflorin drives immunomodulation of mesenchymal stem cells via regulating Th1/Th2 cytokines in oral lichen planus

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Research Article

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Abstract

Objective To explore the immunomodulatory role of paeoniflorin in mesenchymal stem cells (MSCs) and the involvement of Th1/Th2 cytokines in oral lichen planus.

Methods The proliferation of MSCs with different concentrations of paeoniflorin was detected by Cell Counting Kit-8 (CCK8). MSCs were induced by osteoblast, adipoblast and neuroblast followed by Alizarin red, oil red O, real-time PCR and immunofluorescence assays. The effects of paeoniflorin on the migration ability of MSCs was detected by Transwell assay.

Results Paeoniflorin promoted the proliferation, migration and multilineage differentiation of MSCs from OLP lesions (OLP-MSCs). OLP-MSCs pretreated with paeoniflorin inhibited the growth of peripheral blood mononuclear cell through G1 phase cell cycle arrest and S phase decrease. In addition, ELISA showed Th1 cytokines were decreased while Th2 cytokines were increased in T lymphocytes co-cultured OLP-MSCs. Furthermore, paeoniflorin pretreated OLP-MSCs exacerbated the secretory changes of these cytokines. OLP-MSCs prolonged the skin graft survival time and improved the graft rejection score and survival rates. Finally, paeoniflorin pretreated OLP-MSCs also adjusted the Th1/Th2 balance in the serum of allograft skin recipient mice.

Conclusions Paeoniflorin enhanced MSC immunomodulation and changed inflammatory microenvironment in T lymphocytes, providing a promising therapeutic target for OLP treatment through enhancing the function of OLP-MSCs.

Introduction

Lichen planus (LP) is a common chronic inflammatory disease, frequently involving the skin and mucous membranes. Oral lichen planus (OLP) mainly appears with oral mucosal reticular or ulcerative lesions [1], affecting up to 4% of the general population worldwide with malignant transformation about 5% [2-4]. The etiology of OLP is still unclear, however, immunological processes are believed to play critical roles [5]. It is well known that, cell-mediated immune responses, a lymphocyte–epithelium interaction has taken place directly against antigens of the basal epithelial keratinocytes in OLP [1, 6]. Specially, CD4+ cells were observed mainly in the lamina propria, with occasional close to basal keratinocytes T helper 1 (Th1) immune response that may promote CD8+ cytotoxic T-cell activity in OLP. IFN-γ, TNF-α, IL-6 and GM-SF released and provoked a local inflammatory response, thus further exasperating the tissue damage [7]. Furthermore, the imbalance of Th1/Th2 cytokines distribution is an important factor affecting the occurrence and pathological progression of OLP [8]. Therefore, to deeply explore the pathogenic factors of OLP and to take effective treatment for the etiology is of great value to the clinical and pathological contribution of the disease.

Mesenchymal stem cells (MSCs) are important members of the adult stem cell family and can be isolated from the bone marrow [9], cord blood [10], adipose tissue [11], placenta [12], dental pulp [13], skin [14] and tonsils [15]. MSCs have been demonstrated by the characteristics of multi-lineage differentiation

and immunomodulation capacity through recruition and migration into inflammatory or injury areas [16]. In addition, MSCs suppressed the activation, maturation and proliferation of innate or adaptive immune cells [17]. It also had been found that MSCs showed great potential to modulate chronic inflammation and enhance tissue regeneration in skin and other diseases [18, 19]. MSCs have been proved in the treatment of immune- and inflammation-mediated diseases, such as graft versus host disease (GVHD), osteoarthritis (OA), and inflammatory bowel disease (IBD) [20]. Lichen planus and scleroderma-like lesions are the most common manifestations of chronic GVHD. Given the considerable therapeutic cells of MSCs in the inflammatory response, the exact mechanisms underlying their immunoregulatory activities need further clarification. Hence, a better understanding of OLP-MSCs, extracted from inflammatory tissue, and exploring ways to improve the potential of MSCs, may offer effective therapeutic strategies to maintain their positive outcomes in inflammatory diseases.

Paeoniflorin is an anti-inflammatory and immuno-modulator compound, extracted from the roots of *Paeonia Lactiflora Pall* with a long history in traditional Chinese medicine [20]. It is the main bioactive component of the total glucosides of paeony (TGP), which is a safe and effective systemic treatment in OLP [21]. Recently, numerous studies have confirmed the immunomodulatory effects of paeoniflorin [22-24]. For example, in the chronic inflammatory process, paeoniflorin inhibits the proliferation of fibroblast-like synoviocytes by suppressing G-protein-coupled receptor kinase 2 [25]. Although MSCs from OLP tissues were first isolated in our previous study and participated in the anti-inflammatory effect [26], it is not verified whether and how paeoniflorin on the immunomodulatory function of MSCs from OLP.

In this study, we aim to demonstrate the pharmacological mechanisms underlying the therapeutic actions of paeoniflorin on MSCs, and to clarify and provide experimental evidence for its clinical applications. Results described here reveal that OLP-MSCs mediated immune responses may be related with regulating the balance of Th1 /Th2 cytokines in PHA-stimulated peripheral blood mononuclear cell (PBMC) and skin graft animal model. The effects of paeoniflorin on MSCs were due to the regulation of their immunomodulation via cytokines in inflammatory microenvironment.

Methods

Patients

The clinical and pathological diagnosis of OLP conforms to World Health Organization diagnostic criteria [27]. The summary of each participant information is shown in **Table 1**. The study was approved by the Peking University Third Hospital Medical Science Research Ethics Committee (M2020092), and every participant has written informed consent.

Animals

Male CD1 and BALB/c mice (8 -12 weeks) were obtained from animal laboratory center in a specific pathogen-free condition (Vitalriver, Beijing, China). The mice were housed under constant temperature (23±2 °C) and humidity (50±5%) and maintained on a 12 h/12 h light/dark cycle

with free access to food and water. All of the procedures were performed with approval from the Biomedical Ethics Committee for Animal Use and Protection of Peking University and in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Cell isolation and culture

OLP tissues were biopsied from the oral mucosa, and normal oral mucosal tissues were adjacent to benign mass, obtained from patients who underwent surgical resection (**Table 1**). The collected tissues were treated with 2 mg/ml dispase (Sigma–Aldrich) to separate the subepithelial lamina propria from the epithelial. The mesenchymal tissues were then minced into 1 mm^3 fragments and digested with type I collagenase (3 mg/ml) and dispase (4 mg/ml) for 1 h. The cell suspensions were filtered and plated on dishes in a-modified Eagle's minimum essential medium (a-MEM; Gibco) with 10% fetal bovine serum (FBS; Hyclone). The cells at passages 2 to 4 were used in subsequent experiments. All experiments were performed thrice and repeated at least twice.

Cell proliferation assay

MSCs from OLP lesions (OLP-MSCs) were seeded into the 96-well tissue culture plate at the density of 1 × 10^3 cells. Then, the cells were treated with various concentrations of paeoniflorin (0, 0.3, 3, 30, 300 μM) (Ningbo Liwah Pharmaceutical Co., Ltd.) for 11 days. The cell proliferation was assessed by a Cell Counting Kit-8 (CCK8; Dojindo Laboratory). The absorbance microplate reader (ELx808) was used to measure the optical density at 450 nm, and then the cell proliferation rates were calculated. To assess the potential immunomodulation role of paeoniflorin on MSCs, the allogeneic T lymphocytes were assessed [28, 29]. Human peripheral blood mononuclear cells (PBMC) were obtained from four healthy controls and extracted via Ficoll gradient separation. Different numbers of human OLP-MSCs (2.5 × 10^3/ml, 5 × 10^3/ml, 1 × 10^4/ml) were plated onto 96-well plates in 100 μl in triplicates and allowed to adhere to the plates overnight. PBMCs resuspended were added to wells at 2 × 10^5/ml containing or lacking OLP-MSCs in the presence or absence of 10 μg/ml PHA (Sigma-Aldrich). The OLP-MSCs and PF pretreated OLP-MSCs on PHA-stimulated PBMC were then set proportionally for 3 days. The proliferations of allogeneic T lymphocytes by MSCs were also assessed.

Transwell migration assay

MSCs (1.5 × 10^5/ml) without FBS were seeded in the upper chamber, and PHA-stimulated PBMC was placed into the lower well to induce cell migration in 24-well Transwell plates (Corning Costar). MSCs invaded into the lower chambers, containing PHA-stimulated PBMC with 10% FBS in RPMI-1640 after 24 h. Cells were fixed with methanol for 20 min at the room temperature and stained with 0.1% crystal violet counted at least five randomly fields by the microscope at 200 x magnification.

Multilineage differentiation in vitro

MSCs were cultured in osteogenic induction medium with 10 nM dexamethasone, 0.1 mM L-ascorbic acid-2-phosphate, 2 mM glutamine and 10 mM β-glycerophosphate. Cells were fixed by staining with 2% Alizarin Red S (Sigma-Aldrich) to visualize calcium deposition after 4 weeks,. MSCs were cultured in adipogenic induction medium supplemented with 1 μM dexamethasone, 60 mM indomethain, 0.5 mM 3-isobutyl-l-methylxanthine, 10 mg/ml insulin, and 2 mM glutamine. Cells were fixed for detecting lipid droplets by staining with 0.3% Oil Red O (Sigma-Aldrich) after 21 days. MSCs were induced with 100 μM $CoCl_2$ (Sigma-Aldrich) for neurogenic differentiation at least 3 days and fixed. They were incubated with primary antibody, rabbit polyclonal IgG for human MAP-2, and followed by FITC-labeled secondary antibody (Bioworld Technology, USA). Samples were observed with the confocal laser scanning microscope (LSM 5, Germany), according to previous studies [26].

Real-time qPCR

Total RNA in the plasma of each recipient mice was extracted using TRIzol reagent (Invitrogen Life Technologies). Revert Aid First Strand cDNA Synthesis Kit was used to perform reverse-transcription reactions (Fermentas) and cDNA was used as template for each PCR. The primers were described as follows: GAPDH forward primer, 5'-TGTGTCCGTCGTGGATCTGA-3' and reverse primer, 5'-TTGCTGTTGAAGTCGCAGGAG-3'; OPN forward primer, 5'- AGCCATGAGTCAAGTCAGCT -3' and reverse primer, 5'- ACTCGCCTGACTGTCGATAG -3'; and LPL forward primer, 5'- AGCTGACCAGTTATGGCACC-3' and reverse primer, 5'- ATCCTGACCCTCGTAGCCTT-3'. PCR was performed by 7500 Real-time PCR system (Applied Biosystems).

Flow Cytometry Analysis

PHA-stimulated PBMC treated with OLP-MSCs, or paeoniflorin pretreated MSCs and PHA-stimulated PBMC were subjected to a flow cytometry system (BeckmanCoulter, Fullerton, CA). Cell cycle distributions were analyzed in percentages of cells in G0, G1, S, and G2M phases.

Enzyme-Linked Immunosorbent Assay (ELISA)

OLP-MSCs, or paeoniflorin pretreated MSCs and MLR co-cultures were seeded into 96-well plates at ratios of 1:20 for 3 days. Then Th1 cytokines (TNF-α, IFN-γ, IL-1β and IL-2), Th2 cytokines (IL-4, IL-5, IL-10 and IL-13) released in the supernatants were collected for assessment by ELISA kits (Proteintech) according to the manufacturer's instructions.

EdU labeling of OLP-MSCs

EDU was used to label cells for transplantation *in vivo*. OLP-MSCs were seeded into a 10-cm dish in the medium with 20 μM EdU (Invitrogen, Carlsbad, CA) for labeling cells. 24 h later, cells were washed with PBS for 3 times, then added into the culture medium for further incubation and prepared for *in vivo* transplantation.

MSC transplantation *in vivo*

Male BALB/c (8 –12 weeks old) recipients were divided randomly into 4 groups (n=6 per group) and CD1 mice served as donors. Skin grafts (15 mm^2) obtained from the back of donors were transplanted into recipients and 2 × 10^6 MSCs were injected into the recipients. The groups were divided as: (1) Control group, non-transplantation group; (2) Skin graft group, naive group without any treatment; (3) OLP-MSCs group injection group; (4) Paeoniflorin pretreated OLP-MSCs injection group. The saline injection group was used as negative control.

The skin rejection was observed and recorded from the third day after the transplantation. Rejection was defined as eschar formation or the epidermal sloughing and served as the survival time. Survival data of mice were collected in each group after transplantation 3 weeks, and punch biopsies were fixed in neutral-buffered formalin for HE on day 14. Skin rejection after transplantation is classified and each slide was given a histological score ranging from 1 to 5 according to the previous parameters [30]. Serum of recipient mice was harvested before surgery. The expression level of Th1 cytokines (TNF-α, IFN-γ, IL-1β and IL-2) and Th2 cytokines (IL-4, IL-5, IL-10 and IL-13) were measured using ELISA.

Tracking of transplanted OLP-MSCs

After EDU labeled MSC transplantation, tissues were harvested, fixed and embedded in paraffin at day 1. Then, 4 mm sections were prepared and washed three times with PBS. Subsequently, the sections were incubated in 3% bovine serum albumin (BSA) in PBS, and followed by 0.5% Triton® X-100 in PBS for 20 min. Freshly prepared iClick reaction cocktails, which contained 1 × iClick reaction buffer, $CuSO_4$, Andy Flour 647 azide, and 1× reaction buffer additive (iClick™ EDU Andy Fluor 647 Imaging Kit, GeneCopoeia™) was used to incubate tissues for 30 min without light at room temperature. The reaction cocktail was removed and was washed with PBS. Nuclei were stained with DAPI for 5 min, washed twice with PBS, and imaged by fluorescence microscopy. At least three fields were randomly captured and the percentage of positive staining was measured with the Image J software.

Statistical analysis

Data analyses were performed by SPSS v.13.0 software and presented as Mean ± SEM of at least 3 independent experiments. One- or two-way analysis of variance (ANOVA) and the *post hoc* Bonferroni tests were used to compare differences among three or more groups. The survival curves were plotted by Kaplan-Meier method and log-rank analysis was used to compare the survival rates among each group. $P < 0.05$ was considered as statistically significant.

Results

Paeoniflorin promotes MSC proliferation and migration

To investigate the effects of paeoniflorin on the proliferation of MSCs, different concentrations of paeoniflorin were added to OLP-MSCs for 11 days. We found that the effects of paeoniflorin on the proliferation of MSCs were time-dependent and dose-dependent. The results showed that 30 μM

paeoniflorin could significantly promote the proliferation of OLP-MSCs since the 3rd day (Fig. 1A, *P < 0.05*). We next investigated the migration potential of OLP-MSCs after paeoniflorin pretreated by Transwell cultures. There is a significant difference in the number of transmigrated OLP-MSCs between groups absence or presence of paeoniflorin pretreated (109.67 ± 12.17% versus 211.33 ± 12.35% after paeoniflorin pretreated) at 24 h (Fig. 1B and C, *P < 0.01*). Moreover, the numbers of cells that migrated in paeoniflorin pretreated OLP-MSCs were significantly higher than OLP-MSCs group, and paeoniflorin pretreatment significantly improved the MSC migration.

Paeoniflorin promotes MSC multilineage differentiation

MSCs from OLP maintain multi-differentiation capacities. In osteogenic differentiation studies, we found that induced paeoniflorin pretreated OLP-MSC osteogenesis was improved compared with that of OLP-MSCs (Fig. 2A and D**,** $P < 0.05$). For the adipogenic differentiation analysis, there was no difference between induced paeoniflorin pretreated OLP-MSCs and OLP-MSCs (Fig. 2B and E). After neurogenic differentiation, the morphology formation of neuronal cells in induced paeoniflorin pretreated OLP-MSCs was improved compared with that of OLP-MSCs by expression of the MAP-2 antigen and indirect immunofluorescence (Fig. 2C and F, $P < 0.05$).

Paeoniflorin pretreated MSCs suppresses the proliferation of PBMC

Next, we sought to determine whether paeoniflorin influences MSC immunosuppressive effects on the proliferation of T lymphocytes *in vitro*. To this end, OLP-MSCs were co-cultured under cell-cell contact or Transwell systems with human PBMCs for 3 days. Our results showed that OLP-MSCs inhibited PHA-stimulated PBMC proliferation under both cell-cell contact and Transwell cultures in a cell density-dependent manner. OLP-MSCs mediated inhibition of T lymphocyte proliferation was more severe under cell-cell contact conditions than in Transwells (Fig. 3A and B). In addition, the proliferation inhibitions of T cell co-cultured with paeoniflorin pretreated OLP-MSCs were significantly enhanced under both cell-cell contact and Transwell conditions (Fig. 3C and D, *P < 0.05*). Meanwhile, our data also indicated that paeoniflorin pretreated OLP-MSCs mediated the proliferation inhibition of T lymphocyte was more severe under cell-cell contact conditions than in Transwells (Fig. 3C and D). These results suggest that direct cell-cell contact contributes, at least in part, to the mechanisms of paeoniflorin pretreated OLP-MSCs mediated immunosuppression via suppression of T lymphocytes proliferation. We furtherinvestigated the possible effects of paeoniflorin pretreated MSC on the regulation of cell cycle progression of PBMC. The data showed that a higher percentage of cells in G0 G1 phase (58.2% versus 47.5%) and a lower percentage in S phase (23.1% versus 41.4%) were detected in PHA-stimulated PBMC treated with OLP-MSCs compared with untreated cells (Fig. 3F, $P < 0.05$). In paeoniflorin pretreated OLP-MSCs -treated PBMC group, most cells remained in the G0 G1 phase (78%) and low S phase (10.1%). Combined with the CCK-8 test, the flow cytometry results suggested that PF pretreated OLP-MSCs inhibit the growth of PBMC through G1 phase cell cycle arrest and S phase decrease.

Paeoniflorin pretreated MSCs regulates cytokines in co-cultures

We next determined the role of soluble mediators in MSC mediated immunosuppressive suppression on T lymphocyte. To this purpose, we measured the cytokines secretion in MSCs and PBMC co-cultures under cell-cell contact condition. Th1 cytokines TNF-α (Fig. 4A, $P < 0.01$), IFN-γ (Fig. 4B, $P < 0.01$) and IL-1β IFN-γ (Fig. 4C, $P < 0.01$) were decreased, while IL-2 was not changed (Fig. 4D, $P > 0.05$). The Th2 cytokine IL-4 (Fig. 4E, $P < 0.01$), IL-10 (Fig. 4G, $P < 0.01$) and IL-13 (Fig. 4H, $P < 0.05$) but not IL-5 (Fig. 4F, $P > 0.05$) were increased in the supernatant by T lymphocyte co-cultured with OLP-MSCs. Paeoniflorin pretreated OLP-MSCs exacerbated the secretory changes of these cytokines.Taken together, these results suggest that paeoniflorin pretreated MSCs upon activation by T lymphocytes are capable of affecting the secretion of cytokines in the co-cultures.

Tracking of transplanted MSCs

To demonstrate that mesenchymal stem cells can migrate to sites of inflammation and injury *in vivo*, EdU-labeled OLP-MSCs and paeoniflorin pretreated OLP-MSCs were transplanted intravenously into mice that suffered skin rejection. On day 3 after transplantation, MSCs were visible in the skin graft by immunofluorescence staining of EDU (Fig. 5A, C). The numbers of EDU labeled positive MSCs in the skin per field at three time points were 12.51 ± 3.11 and 20.17 ± 5.33 in the group of OLP-MSCs and PF pretreated OLP-MSCs, respectively (Fig. 5B, $P < 0.01$).

Paeoniflorin pretreated MSC-based therapy in skin graft mice

To further explore the potential therapeutic effects of OLP-MSCs, or paeoniflorin pretreated OLP-MSCs, they were infused in an established murine model of skin graft to test whether they could reverse tissue injuries and harness inflammation. After skin transplantation, graft rejection was detected, similar to previous report [31]. While the group using MSCs had less rejection and paeoniflorin improved the ability of OLP-MSCs to alleviate graft rejection (Fig. 6A). Compared with the skin graft group, OLP-MSCs reduced the infiltration of inflammatory cells. The inhibitory effects of paeoniflorin pretreated OLP-MSCs on inflammatory infiltration achieved better immune regulation (Fig. 6A and E, $P < 0.05$).

All mice died 14 days after skin graft without MSC transplantation. We confirmed that the presence of sustained weight loss was in the group of skin graft rejection. OLP-MSCs application can alleviated this change and paeoniflorin pretreated MSCs groups were slightly less (Fig. 6B). Compared with the skin graft group, OLP-MSCs prolonged the skin graft survival time, and paeoniflorin pretreated OLP-MSCs improved the graft rejection score, which was superior to the untreated group (Fig.6C). The survival rates of OLP-MSCs, paeoniflorin pretreated OLP-MSCs were 53.44% and 66.17%, respectively (Fig. 6D).

Immunosuppressive activity via paeoniflorin pretreated MSCs in vivo

Th1 cytokines TNF-α (Fig. 7A, $P < 0.01$), IFN-γ (Fig. 7B, $P < 0.01$), IL-1β (Fig. 7C, $P < 0.01$) and IL-2 (Fig. 7D, $P < 0.05$) were decreased and Th2 cytokine IL-4 (Fig. 7E, $P < 0.05$), IL-5 (Fig. 7F, $P < 0.01$), IL-10 (Fig. 7G, $P < 0.05$) and IL-13 (Fig. 7H, $P < 0.05$) was increased with OLP-MSCs in the serum of allograft skin recipient mice. Paeoniflorin pretreated OLP-MSCs exacerbated the secretory change of these cytokines

than MSCs alone. These results suggest that paeoniflorin could enhance the anti-inflammatory properties of MSCs and reduce the immune-rejection in allogeneic skin graft mice.

Discussion

The results of the present study indicate that paeoniflorin enhanced MSC immunomodulation and regulated inflammatory microenvironment in T lymphocytes via Th1/Th2 cytokines. Specially, paeoniflorin pretreated OLP-MSCs exacerbated the secretory changes of these cytokines. OLP-MSCs prolonged the skin graft survival time and improved the graft rejection score and survival rates. Additionally, paeoniflorin pretreated OLP-MSCs also adjusted the Th1/Th2 balance in the serum of allograft skin recipient mice as well as prolonged skin graft survival and ameliorated inflammatory infiltration in experimental mice model.

The new population of precursor cells from human normal oral mucosa and OLP lesions have been isolated and characterized, termed OMMSCs and OLP-MSCs, which exhibit several unique stem cell-like properties as MSCs derived from bone marrow in our previous research [26]. MSCs are a population of adult multipotent stem or stromal cells, differentiation into osteocytes, adipocytes, neuroectodermal progenies, ability of proliferation and colony-forming ability *in vitro*, and expression of MSC surface markers [16, 32]. MSCs migrate and proliferate within damaged, inflamed and malignant tissues as part of the tissue regenerating process, also displaying immunomodulatory properties [33, 34]. These unique characteristics make MSCs attractive candidates for the cell-based therapeutic strategies in the repair and regeneration of inflammation or damaged tissues [35].

As previous studies have reported MSCs display chemotactic properties in response to inflammation and tissue insult, thus exhibiting tendency towards the site of inflammation [36, 37]. Pro-inflammatory cytokines can modulate MSC behaviors *in situ* of inflamed and injured tissues [38]. OLP is a chronic oral mucosal inflammatory disease mediated by T cells, meanwhile, simultaneous expression of Th1 and Th2 cytokines arise in OLP local lesion and tissue transudates [39, 40]. This evidence indicates that the function of MSCs may be affected by the inflammatory microenvironment in OLP.
Nevertheless, MSCs still have the chemotactic ability to inflammation, while paeoniflorin increased the migration ability of OLP-MSCs.

Our previous study showed the expression of inflammatory cytokines IFN-γ, IL-6, TNF-α, and IL-10 was increased in OLP, compared with normal sub-epithelial lamina tissues [26, 41]. Th1 cytokines IFN-γ/Th2 cytokines IL-4 ratio of OLP patients increased significantly, and Th1 cytokine predominance was proven [8]. Hence the balance of Th1/Th2 cytokines plays an important role in the pathogenesis and progression of OLP due to immunological regulation [42]. Here, we found that OLP-MSCs have anti-inflammatory functions via elevation of Th1 cytokines and reduction of Th2 cytokine through direct cell-cell contact in inflammation situation, which may be one of the key roles of MSCs playing the immunomodulatory effects in OLP. MSCs could induce co-cultured macrophages to produce increased IL-

10 and decreased TNF-α at higher temperatures [18]. Therefore, MSC mediated immune responses in OLP may be related with reversing the imbalance of Th1/Th2 and immunosuppressive factors.

The main treatment for OLP has been shown to be associated with the administration of topical or systemic drugs [43]. Total glycosides of peony (TGP) is a natural traditional Chinese drug and merits curative effects for OLP without toxicity on liver, kidney, or nerve system [44]. TGP inhibits Th1/Th17 cells via decreasing maturation and activation of dendritic cells in rheumatoid arthritis [45]. The levels of Th1 cytokine, IL-12, IFN-γ and TNF-α significantly decreased during TGP treatment in psoriatic arthritis [46]. TGP could inhibit LPS-induced production of IL-6 and TNF-α, simultaneously decreased the phosphorylation of IκBα and NF-κB p65 in HaCaT cells [47]. As the main component of TGP, paeoniflorin not only inhibits inflammation by regulating cytokines but also improves the inhibitory proliferation of T lymphocytes by OLP-MSCs. Paeoniflorin increased the proliferation and regulatory properties of OLP-MSCs, which may be helpful for further understanding the crucial mechanisms of TGP pharmacological treatment of action in OLP.

MSCs have exhibited early efficacy in attenuating the progression of several experimental inflammatory diseases. Recently, anti-inflammatory or immunomodulatory properties, trophic influence on tissue repair as well as homing to sites of inflammation produced by MSCs, have made them popular in the treatment of murine models and clinical trials [48]. The positive treatment effects of human OLP-MSCs on apparent lack of graft rejection model could be due to their inherent capabilities to regulate immune tolerance by increasing the production of anti-inflammatory cytokines and harness inflammatory infiltration, which are consistent with previous studies [49, 50]. Paeoniflorin has been used to protect against liver ischemia/reperfusion injury via inhibiting HMGB1-TLR4 signaling pathway [51]. Notably, paeoniflorin can significantly improve the anti-inflammatory effects of MSCs via regulating the proportion of cytokines, suggesting a potential pharmacological therapy for the application of MSCs.

Based on our observations, paeoniflorin enhanced MSC immunomodulation and regulated inflammatory microenvironment in T lymphocytes and skin graft mice. Moreover, as expected, the efficiency of paeoniflorin treated MSCs was improved, proposing that activated MSCs can be applied as a novel cell source in clinical cell-based treatment in immune-mediated inflammatory diseases.

The limitations of this study include that although we have demonstrated that paeoniflorin, the main component of TGP, plays an immune role by regulating the MSCs from OLP, the amount of which in tissue is limited and their regulatory mechanism is not completely clear. In addition, the further investigation conducted to establish a universally recognized animal model can reflect the precancerous immune inflammatory response of OLP. These clarifications will be helpful for the better understanding of the pathogenesis and the prevention of oral carcinoma.

Conclusion

Our study shows that paeoniflorin promoted the proliferation, migration and multilineage differentiation of MSCs from oral lichen planus lesions via regulation of the Th1/Th2 balance to prolong skin graft

survival and ameliorate inflammatory infiltration. Taken together, paeoniflorin enhances MSCs immunomodulation and regulates inflammatory microenvironment in T lymphocytes, providing a promising therapeutic target for oral lichen planus treatment through improvement of the function of MSCs.

Abbreviations

LP, Lichen planus; OLP, Oral lichen planus; MSCs, Mesenchymal stem cells; PBMC, peripheral blood mononuclear cell; PF, paeoniflorin; ELISA, Enzyme-Linked Immunosorbent Assay; OLP-MSCs, MSCs from OLP lesions.

Declarations

Author contributions

ZZ, ZW and WX conceived the study; ZY, ZZ, LP, ZJ, LH and CD performed the experiments; WW, HY, ZS, JX analysed the data; ZZ and ZW wrote the manuscript. All authors revised the manuscript critically and approved the final manuscript.

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Availability of data and materials

Please contact the corresponding author.

Conflict of interest

The authors declare no competing financial interests.

Consent for publication

Yes from all authors.

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Tables

Table 1. The characteristics of participants recruited in the experiment.

No.	Group	Gender	Age (year)	BMI	Education (year)	OLP or benign mass type	Sites of mucosal involvement
1	OLP	F	57	19.9	12	Erosion	Gingiva, Buccal mucosa, gingival buccal sulcus
2	OLP	M	62	26.4	12	Reticulate	Gingiva, Buccal mucosa
3	OLP	F	55	22.9	16	Erosion	Buccal mucosa, gingival buccal sulcus
4	OLP	M	40	21.6	16	Erosion	Lip, Buccal mucosa, Gingiva
5	OLP	F	51	19.8	12	Reticulate	Buccal mucosa, Gingiva
6	OLP	M	37	26.1	15	Reticulate	Buccal mucosa, Gingiva
7	OLP	F	43	20.5	16	Erosion	Buccal mucosa, Gingiva, Tongue
8	OLP	M	23	12.3	19	Reticulate	Lip, Tongue
9	OLP	F	21	26.8	15	Reticulate	Buccal mucosa, Lip
10	Control	F	41	19.5	15	Mucous retention cyst	Buccal mucosa
11	Control	M	65	27.7	15	Mucous retention cyst	Buccal mucosa
12	Control	F	45	23.4	12	Papilloma	Tongue
13	Control	M	43	25.8	19	Mucous retention cyst	Buccal mucosa
14	Control	F	44	23.4	9	Hemangioma	Buccal mucosa
15	Control	M	55	25.2	19	Mucous retention cyst	Lip
16	Control	F	43	24.8	15	Mucous retention cyst	Buccal mucosa
17	Control	M	20	23.3	12	Mucous retention cyst	Buccal mucosa
18	Control	F	31	19.4	19	Mucous retention cyst	Buccal mucosa
P value		> 0.05	> 0.05	> 0.05	> 0.05	/	/

BMI, Body Mass Index; OLP, oral lichen planus.

Figures

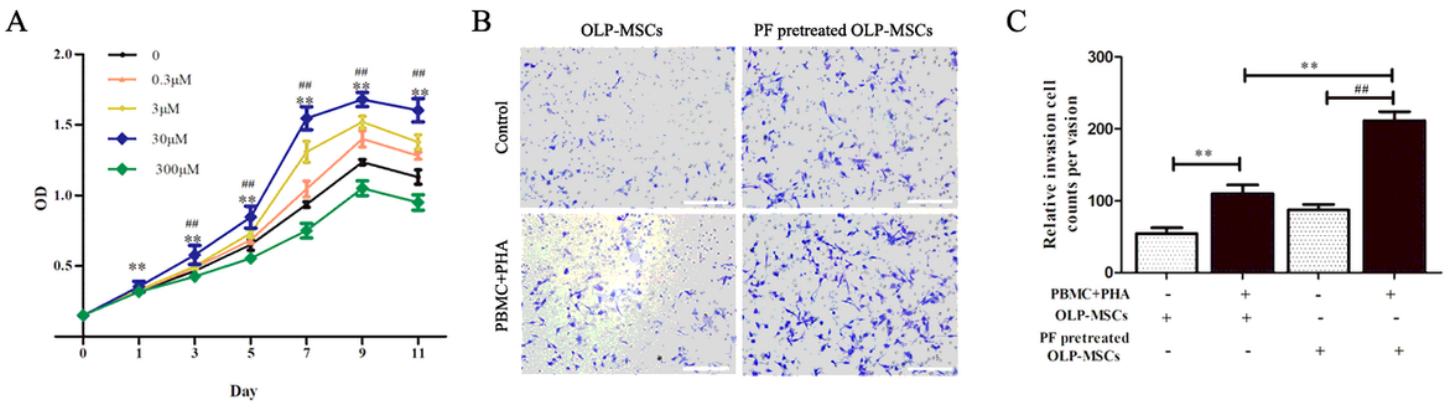

Figure 1

Characteristics of OLP-MSCs and paeoniflorin pretreated OLP-MSCs. (A) Comparison of cell proliferation capacities. Proliferation capacity of OLP-MSCs was up-regulated by PF. The invasion ability of MSCs to T lymphocytes was assessed by the migration assay. (B) Representative invasion images of paeoniflorin pretreated OLP-MSCs. (C) Relative invasion cells of paeoniflorin pretreated OLP-MSCs. Scale bars, 100 μm. All data are expressed as the means ± SEM. $^{**}P < 0.01$; $^{\#\#}P < 0.01$. PF, paeoniflorin.

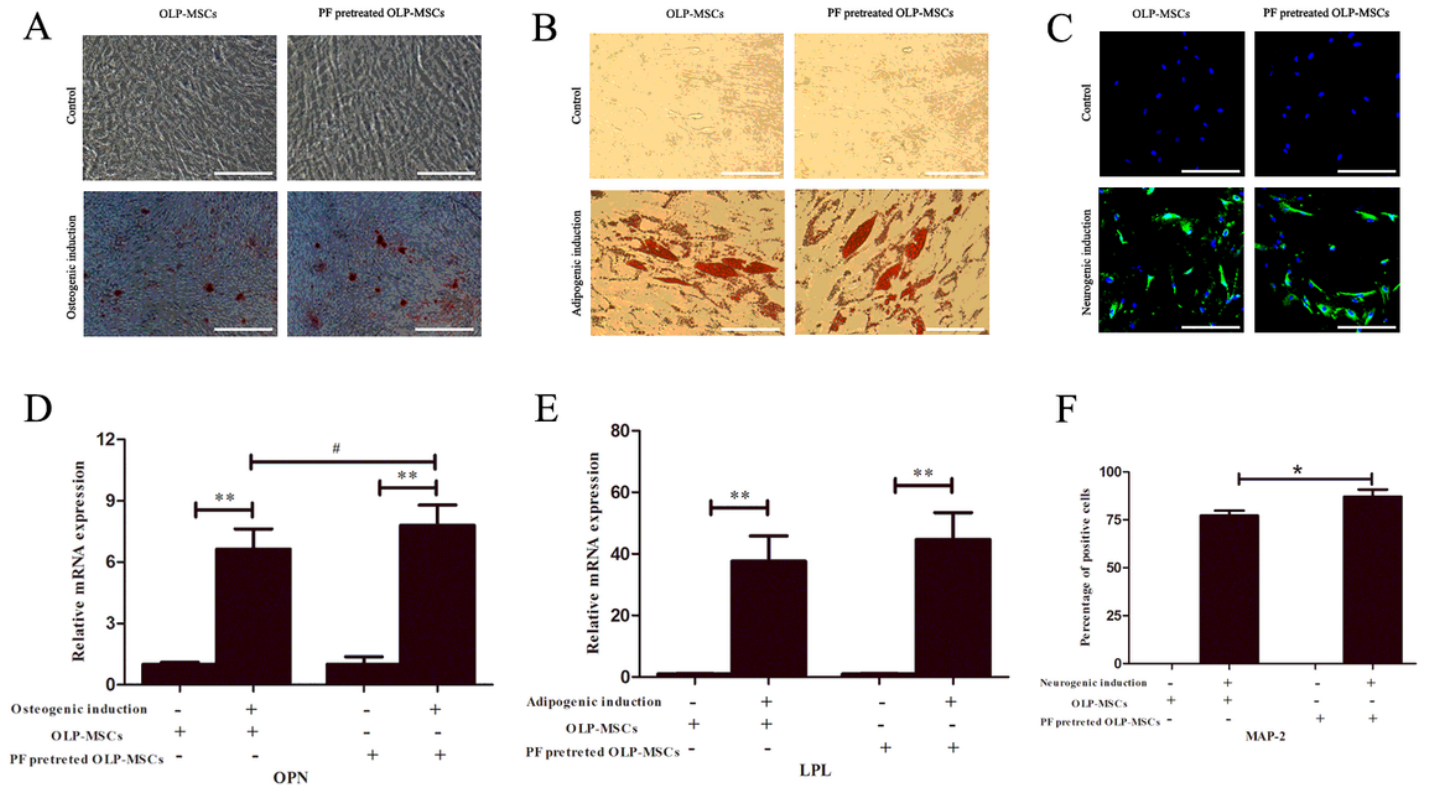

Figure 2

Multipotent differentiation of paeoniflorin pretreated OLP-MSCs. (A) Osteogenic differentiation potential of OLP-MSCs and PF pretreated OLP-MSCs. Mineralized nodules were in induced groups as assessed by alizarin red staining and were not formed in control groups. (B) Adipogenic differentiation potential of OLP-MSCs and PF pretreated OLP-MSCs. Oil red O staining of accumulated lipid droplets were in induced cells and were not formed in control groups. (C) Neurogenic differentiation potential of OLP-MSCs and PF pretreated OLP-MSCs. The morphology of MSC neural changes Immunofluorescently were stained with neuron-specific enolase (MAP-2) and not found in control groups. (D) Analysis of OPN mRNA expression during osteogenic differentiation as assessed by real-time PCR (n > 4). (E) Analysis of LPL mRNA expression during adipogenic differentiation as assessed by real-time PCR (n > 4). (F) Semi-quantification of MAP-2 positive cells after immunofluorescent staining (n > 4). Scale bar = 100 μm. All data are expressed as the means ± SEM. * $P < 0.05$, ** $P < 0.01$; # $P < 0.05$. PF, paeoniflorin.

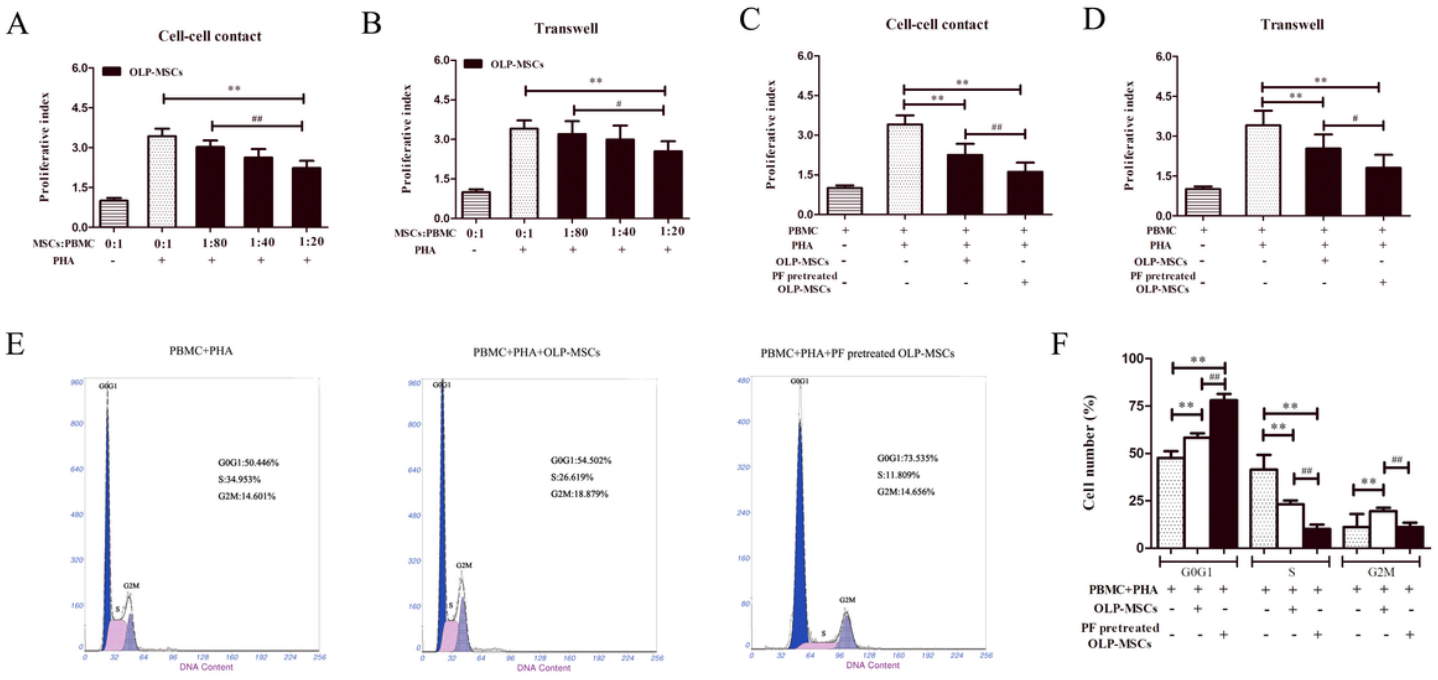

Figure 3

Inhibitory effects of OLP-MSCs and paeoniflorin pretreated OLP-MSCs on PHA-stimulated PBMC proliferation. PBMCs were cultured alone or cocultured with increasing numbers of OLP-MSCs or PF pretreated OLP-MSCs in the presence or absence of PHA for 72 h. Afterward, cell numbers were counted using a Cell Counting Kit-8. The most effective ratio was 1:20 for the suppression of allogeneic T cell proliferation under (A) cell-cell contact and (B) Transwell conditions. Analysis of T cell proliferation rate after paeoniflorin pretreated MSCs under (C) cell-cell contact and (D) Transwell conditions. The proliferation ability of MSCs on T lymphocytes was assessed by a Cell Counting Kit-8. (E) Effect of OLP-MSCs and PF pretreated OLP-MSCs on T lymphocyte cell cycle progression by flow cytometry. (F) Representative flow cytometry graphs in cells with OLP-MSCs and PF pretreated OLP-MSCs for 3 days (up). Mean values of cell cycle distribution in different groups for 3 days (n > 4, * $P < 0.05$, ** $P < 0.01$; # $P < 0.05$, ## $P < 0.01$). PF, paeoniflorin.

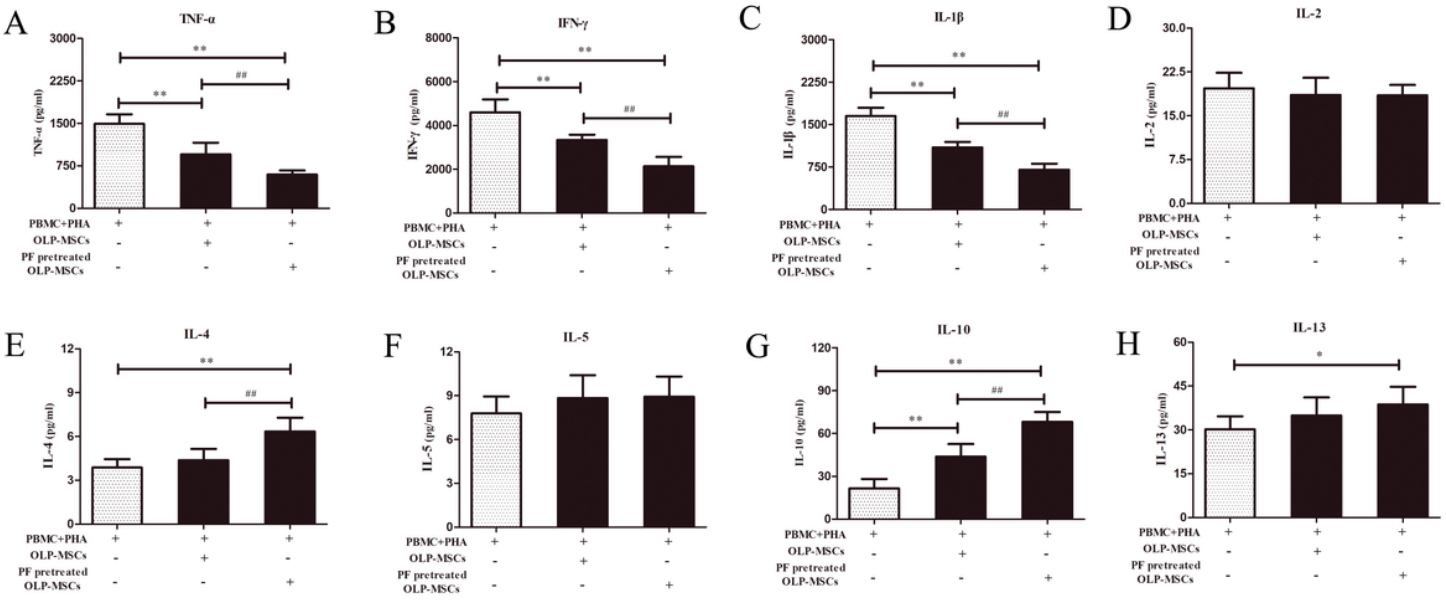

Figure 4

Production of immunomodulatory cytokines of T cells co-cultured with OLP-MSCs and paeoniflorin pretreated OLP-MSCs were determined. Low amounts of Th1 cytokines (A) TNF-α, (B) IFN-γ, (C) IL-1β and (D) IL-2 were produced, while high amounts of Th2 cytokine (E) IL-4, (F) IL-5, (G) IL-10 and (H) IL-13 produced by T cells co-cultured with OLP-MSCs or PF pretreated OLP-MSCs using ELISA. The regulation ability of paeoniflorin pretreated OLP-MSCs was stronger than that of the untreated group (*$P < 0.05$, **$P < 0.01$; #$P < 0.05$, ##$P < 0.01$). PF, paeoniflorin.

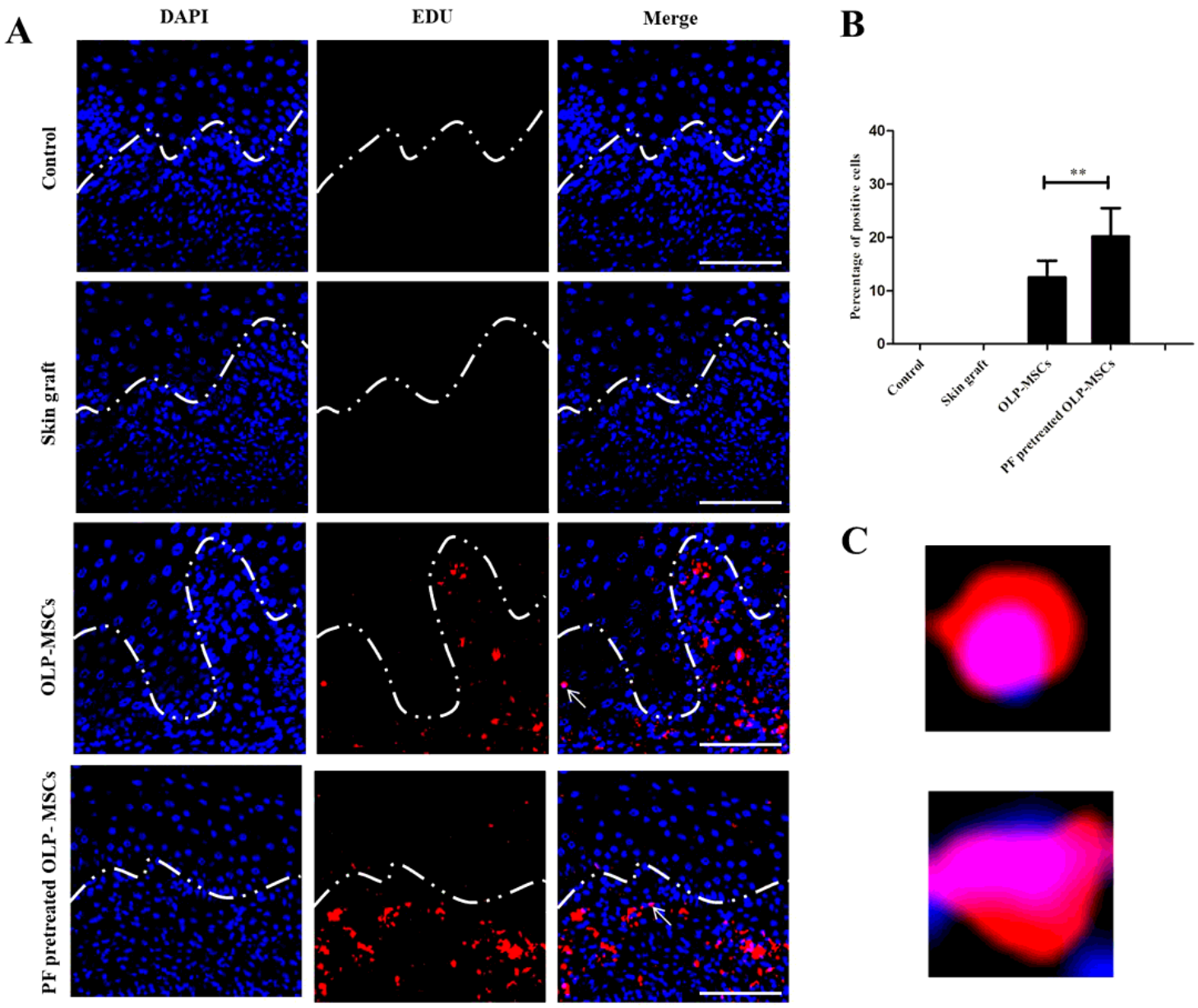

Figure 5

EdU labeled OLP-MSCs and paeoniflorin pretreated OLP-MSCs in skin tissues. Tissues were harvested at the 3rd and stained with EDU (red fluorescence) and DAPI (blue fluorescence). (A) The EDU and DAPI stained images were digitally merged. Four representative tissues at 400 × magnification. (B) Quantification of EdU labeled OLP-MSCs and PF pretreated OLP-MSCs. (C) Arrowheads in the A graphs point to nuclei that are shown as magnification. Scale bar = 50 μm. ** $P < 0.01$. PF, paeoniflorin.

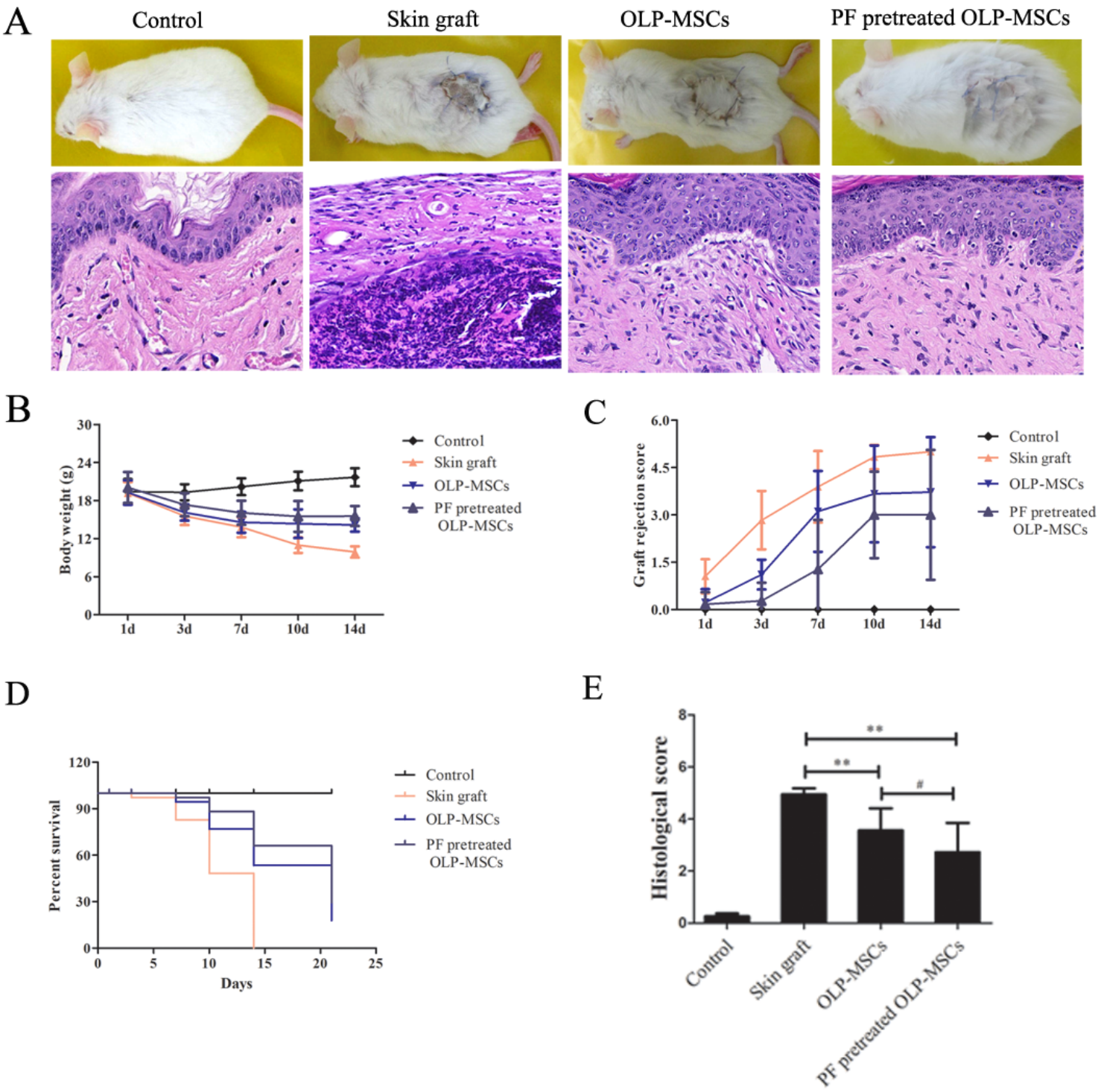

Figure 6

Treatment with paeoniflorin pretreated OLP-MSCs and PF pretreated OLP-MSCs ameliorated experimental skin graft rejection in mice. Paeoniflorin pretreated MSCs prolonged skin graft survival and reduced inflammatory response. 2×10^6 MSCs were injected into the recipients. (A) Skin graft changes and histological HE staining, (B) Body weight changes, (C) Graft rejection score, (D) Kaplan–Meier curve for the survival time and (E) Histological score, showed OLP-MSCs prolonged survival time of the skin graft

and inhibited the infiltration of lymphocytes. And PF pretreated OLP-MSCs improved their immunomodulatory function in skin graft animals (**$P < 0.01$; #$P < 0.05$). PF, paeoniflorin.

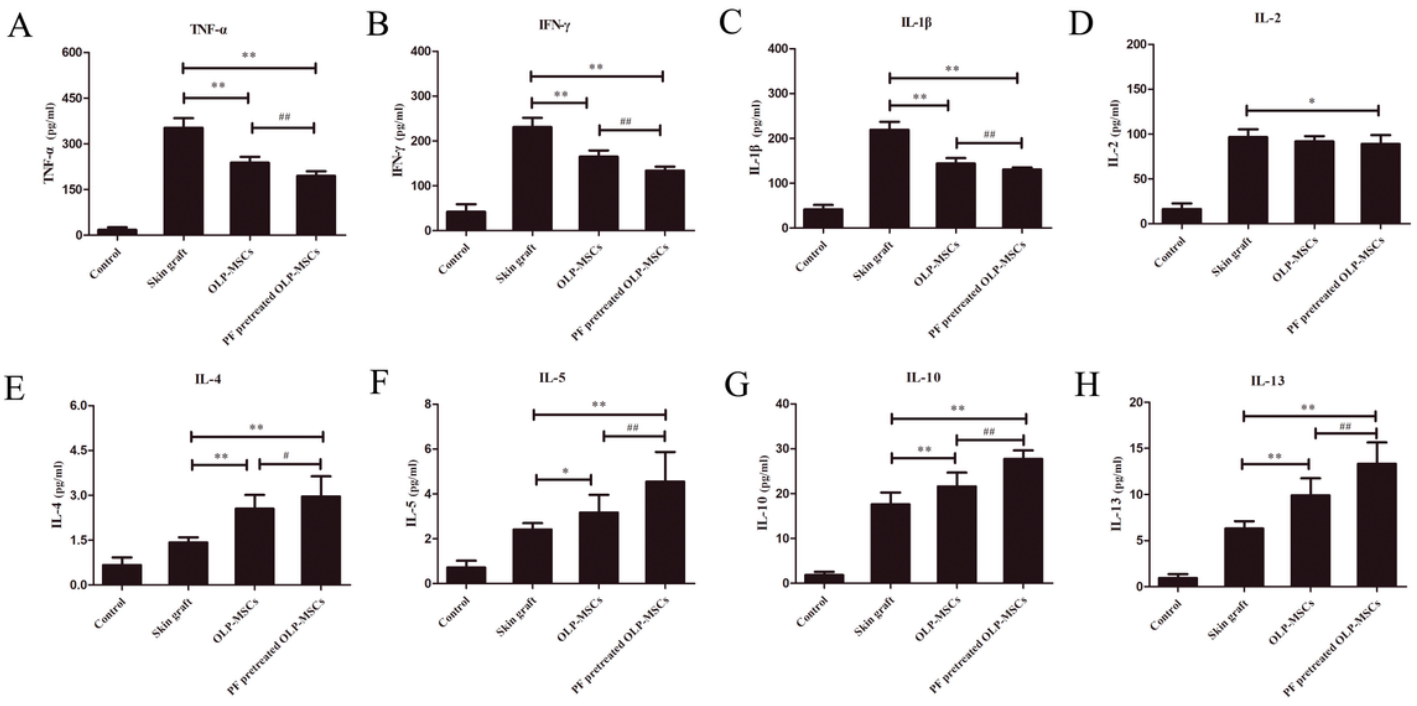

Figure 7

Serum cytokine levels in allograft skin recipient mice. Low amounts of Th1 cytokines (A) TNF-α, (B) IFN-γ, (C) IL-1β and (D) IL-2, while high amounts of Th2 cytokine (E) IL-4, (F) IL-5, (G) IL-10 and (H) IL-13 were produced in OLP-MSCs injection group by ELISA. Paeoniflorin pretreatment improved the anti-inflammatory ability of OLP-MSCs in skin transplantation mice (**$P < 0.01$; #$P < 0.05$, ##$P < 0.01$). PF, paeoniflorin.