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Study of the immunomodulatory effects of osteogenic differentiated human dental pulp stem cells

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ABSTRACT

Background and aims: Dental pulp stem cells (DPSC) are promising tools in regenerative medicine due to their differentiation potential and immunomodulatory properties. However, it is not clearly known whether or not DPSCs maintain their immunosuppressive effects after differentiation. In the present study, we examined the immunomodulatory effects of osteogenic differentiated DPSCs (OD-DPSCs).

Methods: OD-DPSCs and undifferentiated DPSCs were co-cultured with allogenic PBMCs in different ratios and the proliferation of the PBMCs was measured. The concentration of IL-10, TGF-β, PGE2, IL-6, and NO were then examined. Moreover, the expression of IDO, HLAG, and HGF genes were determined in undifferentiated and OD-DPSCs.

Findings: The results showed that OD-DPSCs could inhibit the proliferation of allogenic PBMCs. The levels of PGE2, IL-6, and TGF-β anti-inflammatory cytokines increased after the co-culture. Moreover, the levels of NO increased during the differentiation process and the expression of IDO, HLAG, and HGF genes remained unchanged after osteogenic differentiation.

Significance: Although, there were some differences between the OD-DPSCs and undifferentiated DPSCs in terms of their cytokine and NO production, undifferentiated DPSCs maintained their immunomodulatory activities upon differentiation.

1. Introduction

Human dental pulp stem cells (hDPSCs) are plastic-adherent, multipotent mesenchymal stem cells that arise from migrating neural crest progenitors and can be isolated from extracted wisdom teeth which are considered as medical waste [1]. They are characterized by their self-renewal and differentiation potential into several cell lineages, such as adipocyte, osteoblast, odontoblast, chondrocyte, endothelial cells, myocytes, and active neuron cells under proper conditioned media [1–5]. DPSCs express mesenchymal stem cell-specific surface molecules, such as CD105, CD73, and CD90; while they are negative for hematopoietic markers, including CD14, CD34, and CD45 [4].

DPSCs have some advantages when compared with other mesenchymal stem cells. DPSCs are easy to access; procedures to obtain them are low invasive and low cost with minimum ethical issues [6,7]. Furthermore, they possess higher number of stem cells and proliferation rate [8]. These features make them an ideal therapeutic tool in regenerative medicine and cell-based therapies, especially in bone tissue engineering [3,4,6–11].

It has been documented that regardless of mesenchymal stem cells source, they modulate immune system and suppress a wide range of immune cells, including T cells, B cells, macrophages, and natural killer cells [12]. DPSCs also exert immunosuppressive properties. They have low immunogenicity and do not express the major histocompatibility complex class II antigen [13,14]. DPSCs interact with T cells, inhibit their function, and induce T cell apoptosis. In addition, DPSCs inhibit

Abbreviations: hDPSC, human dental pulp stem cell; OD-DPSC, osteogenic differentiated dental pulp stem cell; PBMC, peripheral blood mononuclear cells; TGF-b, transforming growth factor beta; IL-6, interleukin-6; IL-10, interleukin-10; HGF, hepatocyte growth factor; PGE2, prostaglandin E2; HLA-G, human leukocyte antigen G; NO, nitric oxide; IDO, indole amine 2,3-dioxygenase

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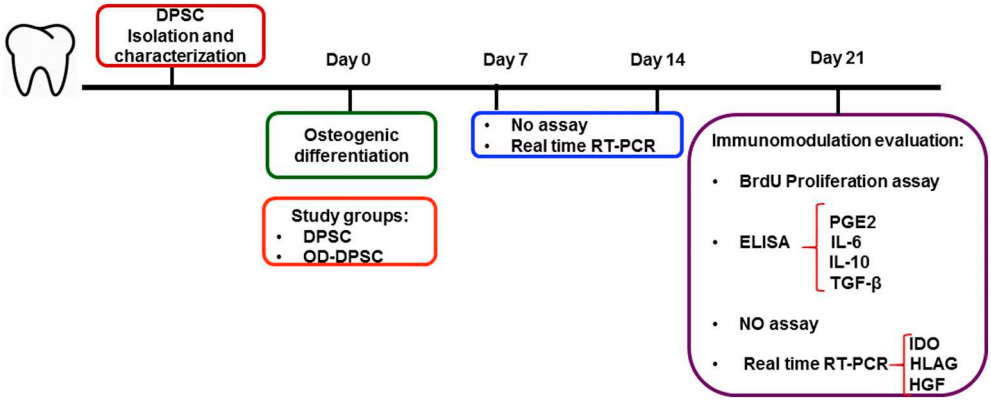

Fig. 1. The study design for evaluation of immunomodulatory effects of osteogenic differentiated hDPSCs.

proliferation of activated peripheral blood mononuclear cells (PBMCs) *in-vitro* [15]. DPSCs secret anti-inflammatory cytokines including transforming growth factor beta (TGF-b), interleukin-6 (IL-6), intrleukin-10 (IL-10), hepatocyte growth factor (HGF), prostaglandin E2 (PGE2), and human leukocyte antigen G (HLA-G). They also produce soluble mediators, such as nitric oxide (NO) and indole amine 2,3-dioxygenase (IDO) [15–17]. Furthermore, these cells have the ability to increase the number of regulatory T (T reg) cells [18,19].

However, the immunomodulatory effects of DPSCs after osteogenic differentiation are poorly understood. Since the autologous stem cell delivery is used to repair large bone defects and bone regeneration in clinical conditions, including fracture, trauma or congenital defects; if they maintain their immunosuppressive properties after differentiation into osteoblasts, they could be a perfect allogenic cell source candidate for oral and maxillofacial and orthopedic regeneration [20–22].

For a better undressing of the immunological characteristic of osteogenic differentiated dental pulp stem cells (OD-DPSCs), the immunosuppressive effects of hDPSCs after osteoblast differentiation *in-vitro* were investigated and compared (Fig. 1).

2. Materials and methods

2.1. DPSCs isolation and culture

The hDPSCs were obtained from normal human impacted third molars from six healthy adults (18–26 years of age), during routine extraction at Surgery Clinic of Dental School of Shahid Beheshti University of Medical Sciences under approved guidelines provided by the ethics committee (Ethics code: IR.SBMU.MSP.REC.1395.208). The surface of the teeth was disinfected with 70% ethanol. In order to reach the pulp chamber, sterile fissure diamond bur was used in a high speed hand piece to create access cavity, then coronal pulpal tissue was removed using sterile dental excavator. Minced dental pulp tissue was digested in a solution of 3 mg/ml collagenase type I (Sigma-Aldrich, St. Louis, MO, USA) for 1 h at 37 °C for releasing the cells from pulpal tissue. The cells were centrifuged at 12000 rpm for 5 min, then were resuspended in Dulbecco's Modified Eagle's Medium (DMEM)/Ham's F12 (Biosera, England), supplemented with 10% fetal bovine serum (GIBCO/BRL, Karlsruhe, Germany), 100 U/ml penicillin, and 100 g/ml streptomycin (Biosera, England). The cells were incubated at 37 °C in 5% CO_2. For precise analysis, all tests were performed using the cells at passage 3.

2.2. Flow cytometric analysis

The hDPSCs at passage 3 were used to detect mesenchymal stem cell markers. The expression of stem cell surface markers of hDPSCs was analyzed using flow cytometry. FITC and PE conjugated anti-human antibodies against cell surface molecules used for analysis, including anti-CD105, anti-CD73, anti-CD90, anti-CD45, anti-CD34, and anti-CD14; they were all obtained from Ebioscience. Experiments were performed six times.

2.3. Differentiation potential

hDPSCs were used to assess the ability to differentiate toward osteogenic and adipogenic lineages. DPSCs at passage 3 were seeded in 24-well plate (SPL) at the density of 4×10^3 cell/well and were cultured with Dulbecco's Modified Eagle's Medium (DMEM)/Ham's F12 supplemented with 10% fetal bovine serum (FBS, GIBCO/BRL, Karlsruhe, Germany), 100 U/ml penicillin and 100 g/ml streptomycin (Biosera, England). When the cells reached 80% confluency, they were exposed to the DMEM/F12 medium supplemented with 10% FBS, 100 U/ml penicillin and 100 g/ml streptomycin, glycerol phosphate (10 mM), dexamethasone, (10 mM) and ascorbic acid-2 phosphate (5 g/ml); they were all from Sigma-Aldrich, Germany. DPSCs were incubated at 37 °C and 5% CO_2 for 3 weeks. The osteogenic medium was exchanged every 3 to 4 days. After 21 days, osteogenic differentiated DPSCs were completely developed. The cells were stained with Alizarin Red S for the evaluation of mineralization. This osteogenic differentiation protocol was used for the rest of the experiments.

For adipogenic differentiation, 4×10^3 cell/well DPSCs (P3) were cultured. Confluent cells were incubated with the adipogenic induction medium which consists of 3-isobutyl-methylxanthine (0.5 mM), indomethacin (100 mM), insulin (5 mM), and dexamethasone (250 mM) (All from Sigma-Aldrich, Germany). After 3 weeks, cultured cells were stained with oil red-O for the examination of intracellular oil droplets.

2.4. Peripheral blood mononuclear cell (PBMC) isolation

The peripheral blood mononuclear cell (PBMC) was isolated from 6 healthy donors. The venous blood was collected in heparinized tube. The PBMC isolation was perfumed by density gradient centrifugation using Ficoll-Paque technique immediately after sample collection (Biosera, England). Freshly collected blood was diluted in phosphate-buffered saline (PBS; Sigma-Aldrich, Germany) and gently coated on the top of the Ficoll layer, then centrifuged at 2000 rpm for 20 min (break off). PBMCs were aspirated from interphase and washed with PBS (centrifuged at 200 *g* for 10 min). The cell pellet was resuspended in 1 ml 1640 rpm (Biosera, England) supplemented with 10% FBS, 100 iu/ml penicillin, and 100 µg/ml streptomycin.

2.5. Transwell co-culture assay

In order to study the immunomodulatory effects of undifferentiated DPSCs and differentiated hDPSC (21 days after osteogenic induction) on

proliferation of allogenic lymphocyte, a transwell co-culture system was established using transwell plate with a pore size of 0.4 μm (SPLInsert™ Hanging, SPL, Korea). Cells (4×10^4) were seeded in the lower compartment of 24-well plate. Then, 4×10^4 and 2×10^5 purified allogenic PBMCs were placed in the upper chamber of the plate (1:1 and 1:5 ratios). A group of PBMC was stimulated with 1.5% phytohaemagglutinin (PHA; Sigma-Aldrich, Germany). Co-culture duration was 72 h. PBMCs were cultured alone either with or without PHA as control groups. All experiments were repeated three times in duplicates.

2.6. 5-Bromo-2'deoxyuridine (BrdU) proliferation test

To investigate the effect of the undifferentiated and differentiated DPSCs on proliferation of allogenic PBMCs after 3 days of co-culture, PBMCs in different test and control groups were transferred into the 96-well plate (SPL, Korea). Then, proliferation was examined using the BrdU cell proliferation ELISA kit (Roche Diagnostics, Germany). Procedures were performed according to the manufacturer's instructions. Briefly, the cells were labeled with 10 μl/well BrdU labeling solution and were incubated overnight. After removing the labeling medium, the cells were fixed. BrdU incorporation was labeled using monoclonal anti-BrdU conjugated with peroxidase (Anti-BrdU-POD). Finally, substrate was added and absorbance of the samples was measured immediately at wavelength of 450 nm and a reference wavelength of 630 nm using microplate reader.

2.7. Cytokine production assay

The concentration of IL-10, IL-6, PGE2, and TGF-β in the differentiated DPSC: PBMC and undifferentiated DPSC: PBMC co-culture supernatant was quantitatively assayed using commercially available ELISA Kits IL-6, IL10, TGF-β (Invitrogen, Thermo Fisher Scientific, USA) and PGE2 (R&D Systems, UK). ELISA was performed according to the manufacturers' recommendations. The experiments were carried out in duplicate.

2.8. Nitric oxide production

To determine the levels of nitric oxide production in osteogenic differentiated DPSCs, the cells were differentiated into osteoblasts cell as described earlier; DPSCs were cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin at 37 °C and 5% CO_2. following 7, 14 and 21 days of culture, the supernatant of the cultures was collected and NO production was quantified using Griess assay kit (Cibbiotech, Iran). 100 μl of Griess reagent was added to each sample and optical density (OD) was measured at 550 nm.

2.9. Real-time RT-PCR

To evaluate the expression of the IDO, HLAG5, and HGF genes in osteogenic differentiated DPSCs, real-time polymerase chain reaction (Real-time RT-PCR) was used. For this purpose, total RNA was extracted from DPSCs and OD-DPSCs 7, 14 and 21 days after osteogenic induction by adding 800 μl Trizol reagent (Sinaclon, Iran). 200 μl chloroform was added and centrifuged (12,000 rpm, 15 min, 4 °C). Then, RNA was collected and precipitated using isopropanol. Finally, the RNA pellet was washed with 70% ethanol. The RNA samples were quantified by measuring 260/280 nm ratio using spectrophotometer. First strand DNA synthesis was carried out using First-Strand RT-PCR kit/Superscript III reverse transcriptase in final volume of 20 μl (Yekta Tajhiz Azma, Iran) according to the manufacturer's instructions. cDNA samples were stored in −20° until use. Real-time RT-PCR was performed using SYBR Green Master Mix (Takara, Japan). The assay was performed on ABI Step OnePlus Real-Time PCR System (Applied Biosystems, US). Primer sequences for human IDO, HLAG5, HGF, and GAPDH genes are listed in Table 1.

Table 1
Primers used for real-time RT-PCR.

Primer	Sequence (5′-3′)
HLA-G forward primer	CTGAGATGGAAGCAGTCTT
HLAG reverse primer	GCTCCCTCCTTTTCAATCT
IDO1 forward primer	CTGTTCCTTACTGCCAACT
IDO1 reverse primer	TCCATGTTCTCATAAGTCAGG
HGF forward primer	AGACCAATGTGCTAATAGATGTA
HGF reverse primer	GCAGTTTCTAATGTAGTCTTTGT
GAPDH forward primer	CGGATTTGGTCGTATIGG
GAPDH reverse primer	TCAAAGGTGGAGGAGTGG

2.10. Statistical analysis

Experiments were performed independently three times in two replicates. IBM SPSS Statistics Version 25.0 (SPSS Inc., Chicago, IL, USA) was used for all statistics. Mann-Withney *U*-test was used to compare the means. Values of $P < 0.05$ were classified as statistically significant. These values were expressed as mean ± standard deviation of the mean (SD).

3. Results

3.1. hDPSC surface marker profile

Flow cytometry analysis of all 6 (reported as mean ± SD values of percentage of cells) showed that the hDPSCs expressed high levels of CD105 (87.5 ± 5.8), CD73 (90.4 ± 10.1), and CD90 (91.35 ± 6.8) mesenchymal stem cell markers, whereas they expressed low levels of hematopoietic lineage markers including CD14 (4.8 ± 3.8), CD34 (16.5 ± 11.4), and CD45 (7.5 ± 4.9) (Fig. 2D). Thus, the DPSCs showed immunophenotype of human mesenchymal stem cells.

3.2. Differentiation into adipocytes and osteoblasts

After 21 days of culture in osteogenic inductive medium, the DPSCs were differentiated into osteoblast and produced extracellular mineralized nodules as confirmed by Alizarin Red S staining (Fig. 2B). Control group did not exhibit any calcium nodule. Moreover, after three weeks of culture under adipogenic medium, the cells were able to be differentiated into adipocytes and formed Oil red-O positive oil accumulation (Fig. 2C).

3.3. Effects of hDPSC on lymphocyte proliferation

Immunomodulatory properties of osteoblast-derived hDPSCs were studied using three days co-culture assay. The results showed that the osteogenic differentiated DPSCs (OD-DPSC) could significantly ($P < 0.05$) inhibit allogenic PBMCs in 1:1 and 1:5 ratios (OD-DPSC: PBMC) as compared to the control groups. PHA significantly ($P < 0.05$) stimulated proliferation of lymphocytes. The Br-du assay also revealed a significant ($P < 0.05$) decrease in the proliferation of mitogen induced PBMCs in both 1:1 and 1:5 ratios (OD-DPSC: PBMC) (Fig. 3).

The same results were observed in the co-culture of undifferentiated hDPSC: PBMCs in 1:1 and 1:5 ratios. The DPSCs could remarkably ($P < 0.05$) suppress lymphocyte proliferation in the presence and absence of PHA as compared to the control groups (Fig. 3). Both osteogenic differentiated hDPSCs and undifferentiated hDPSCs were able to impede proliferation of allogenic lymphocyte and no considerable difference ($P < 0.05$) was observed between these groups.

3.4. Cytokine quantification

The ELISA assay demonstrated that OD-DPSCs produced

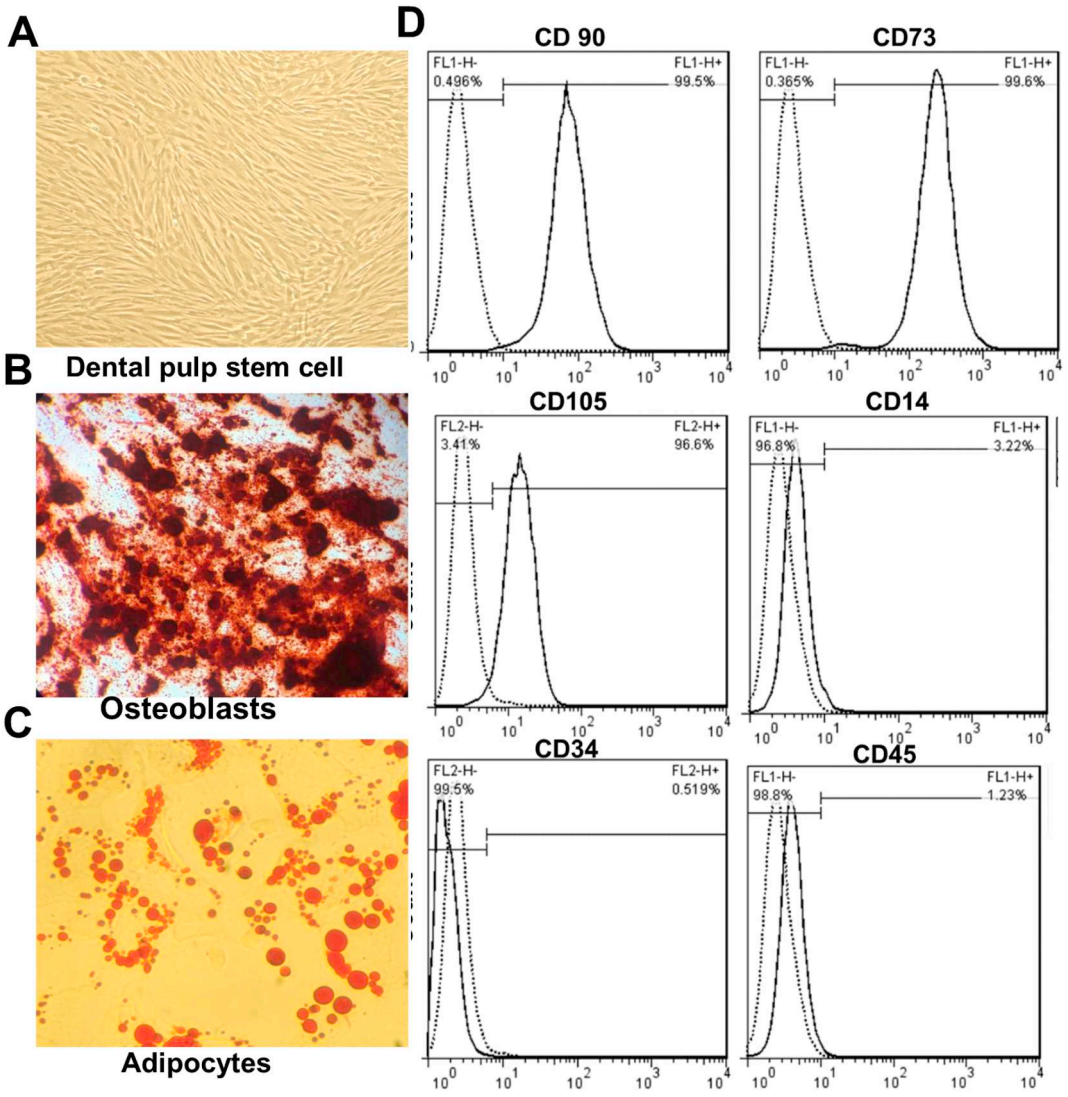

Fig. 2. Characterization of hDPSC: (A) morphology of hDPSCs, (B) osteogenic differentiated DPSCs, Alizarin-red staining, (C) adipogenic differentiated DPSCs, Oil red-O staining. (D) Representative flow cytometry analysis of DPSCs at passage three. hDPSC, Human dental pulp stem cell.

significantly higher ($P < 0.05$) levels of PGE2 and IL-6 when compared to undifferentiated DPSC. OD-DPSCs also secreted higher amount of TGF-β. IL-10 showed a significant decrease during osteogenesis ($P < 0.05$) (Fig. 4). The ELISA results showed that the expression levels of PGE2 was significantly increased in OD-DPSC: PBMC co-culture groups in both PHA-stimulated and non-stimulated PBMC when compared with the control groups ($P < 0.05$) (Fig. 5A). The results showed a significant increase in the levels of PGE2 in undifferentiated DPSC: PBMC co-culture group in both PHA positive and negative groups when compared with the control groups ($P < 0.05$). The concentration of IL-6 showed a remarkable ($P < 0.05$) increase in OD-DPSC: PBMC co-culture group when compared with the control. This increase was significantly higher in PHA induced group (Fig. 5A). DPSC: PBMC co-culture groups also showed a significant increase ($P < 0.05$) in the levels of IL-6 in both stimulated and non-stimulated groups when compared with the controls (Fig. 5B).

Following the OD-DPSC: PBMCs co-culture, the levels of TGF-β was elevated in PHA-induced and non-PHA-induced groups when compared with the control groups. But, this increase was not significant ($P < 0.05$). Undifferentiated DPSCs co-culture groups also showed a significant increase in the levels of TGF-β when compared with the control groups ($P < 0.05$) (Fig. 5C). Although the amount of IL-10 was significantly increased in co-culture groups in both PHA positive and PHA negative groups ($P < 0.05$), but there was a significant decrease in the levels of IL-10 ($P < 0.05$) when compared with the undifferentiated group. The amount of IL-10 in the undifferentiated DPSC co-culture groups was significantly increased in both PHA-induced and non-PHA-induced groups as compared to the control groups ($P < 0.05$) (Fig. 5D).

3.5. Nitric oxide production

The levels of NO stable metabolite, nitrite, were measured during osteogenic differentiation (0, 7, 14 and 21) and were compared to the undifferentiated hDPSCs. The osteoblast-derived DPSCs produced much higher levels ($P < 0.05$) of NO when compared with the undifferentiated DPSCs. When hDPSCs underwent osteogenic differentiation, levels of NO were remarkably ($P < 0.05$) increased during differentiation process. The amount of NO was increased until day 21, and considerably higher than the undifferentiated control group ($P < 0.05$) (Fig. 6).

3.6. Gene expression

The expression of the HLA-G, IDO, and HGF genes was normalized with GAPDH housekeeping gene. The levels of transcripts showed that

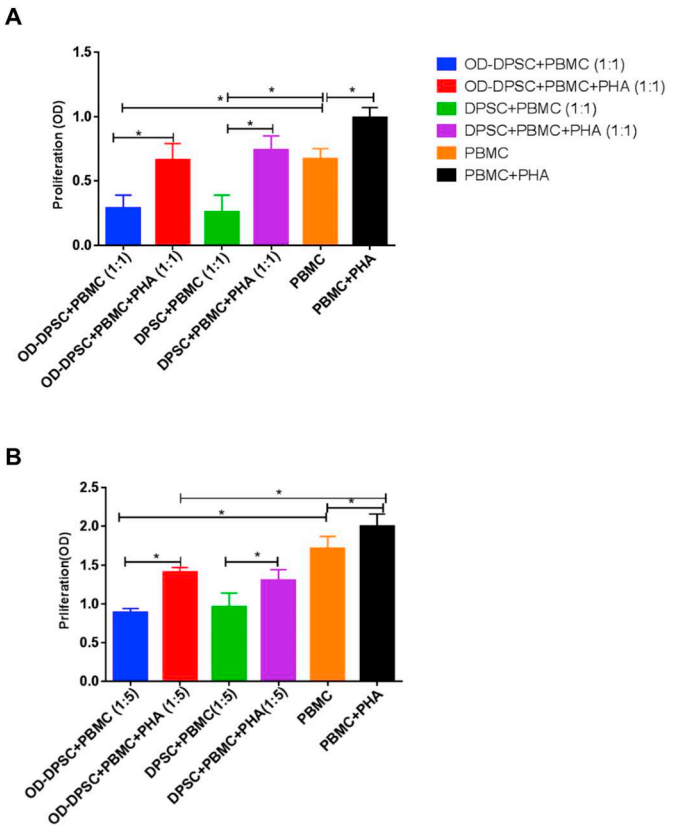

Fig. 3. Proliferation of stimulated and non-stimulated PBMCs after 72 h co-culture with osteoblast derived DPSC and DPSCs. (A) Co-culture of OD-DPSC and undifferentiated DPSC with PBMC (1:1). (B) Co-culture of OD-DPSC and undifferentiated DPSC with PBMC (1:5). The result is expressed as the mean ± SD of six independent experiments (*$P < 0.05$). PBMC, Peripheral blood mononuclear; DPSC, dental pulp stem cell; OD-DPSC, osteoblast derived DPSC; OD, optical density.

the expression of HLA-G, IDO, and HGF genes were not altered significantly ($P < 0.05$) after osteogenic differentiation when compared with the undifferentiated DPSCs at days 0, 7, 14 and 21 (Fig. 7).

4. Discussion

In the present study, different aspects of immune-regulating capabilities of osteogenic differentiated DPSCs to undifferentiated hDPSCs were investigated and compared. The osteogenic differentiated hDPSCs revealed immunomodulatory effects. The osteogenic differentiated DPSCs inhibited the proliferation of allogenic lymphocytes. These cells increased the levels of immune-regulating factors, such as PGE2, IL-6, TGF-β, and NO. In addition, the differentiated DPSCs were able to maintain the expression of IDO, HLAG, and HGF immunomodulatory molecules.

Results reported by Wada et al. and Sonoyama et al. showed that the DPSCs could inhibit proliferation and activation of lymphocytes. The present study showed that this inhibitory effect remained after differentiation into osteoblast and these cells could not prompt allogenic PBMCs. Osteogenic differentiated MSCs could also inhibit PHA-induced lymphocytes. These results were in line with the results of previous studies [23,24].

PGE2 is one of the arachidonic acid metabolites. PGE2 produce from the MSCs and osteoblasts is one of the important inducers of bone remodeling [25–27]. It is suggested that PGE2 has immunosuppressive effects on lymphocyte proliferation and inflammatory cytokine reduction [28–30]. The amount of PGE2 up-regulated significantly in the osteoblast-derived DPSC: PBMC co-culture. Tang et al. also showed that

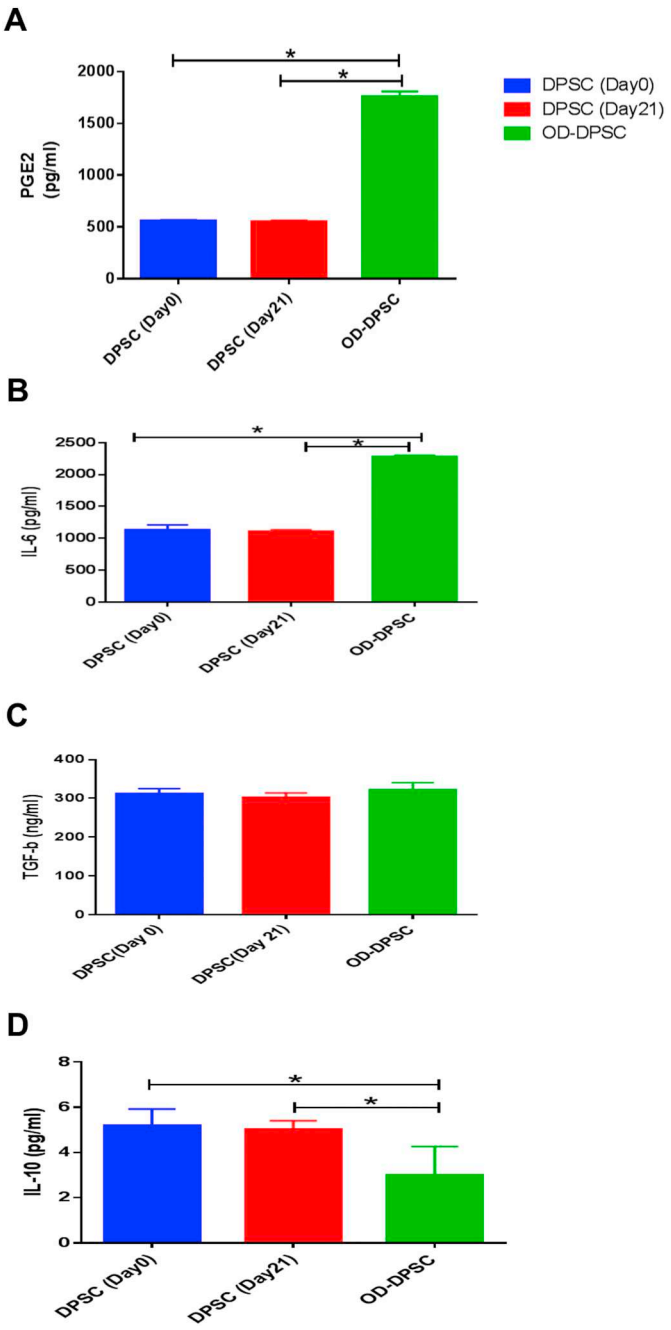

Fig. 4. Production of PGE2, IL-6, TGF-β and IL-10 cytokines by DPSCs (day 0 and 21) and OD-DPSCs (A-D). The data is expressed as the mean ± SD of three independent experiments (*$P < 0.05$). DPSC, dental pulp stem cell; OD-DPSC, osteogenic differentiated dental pulp stem cell; PGE2, prostaglandin E2; IL-6, interleukine-6; TGF-β, transforming growth factor beta; IL-10, interleukine-10.

the PGE2 played an important role in immune-regulatory effects of differentiated and undifferentiated PDLSCs.

IL-6 was described as a binary cytokine that exerts both pro-inflammatory and anti-inflammatory effects [26,31,32]. In this experiment, IL-6 was elevated significantly in differentiated DPSCs groups. It has been reported that immunosuppressive factors collaborate with each other to exert their regulatory activities. It has been described that PGE2 could upregulate IL-6 and through this pathway they exhibit their suppressive properties [33,34]. The present study also showed that these two cytokines increased together in different groups.

Omi et al. reported that IL-10 production by DPSCs could reduce pro-inflammatory cytokines and increased M2 macrophages in diabetes

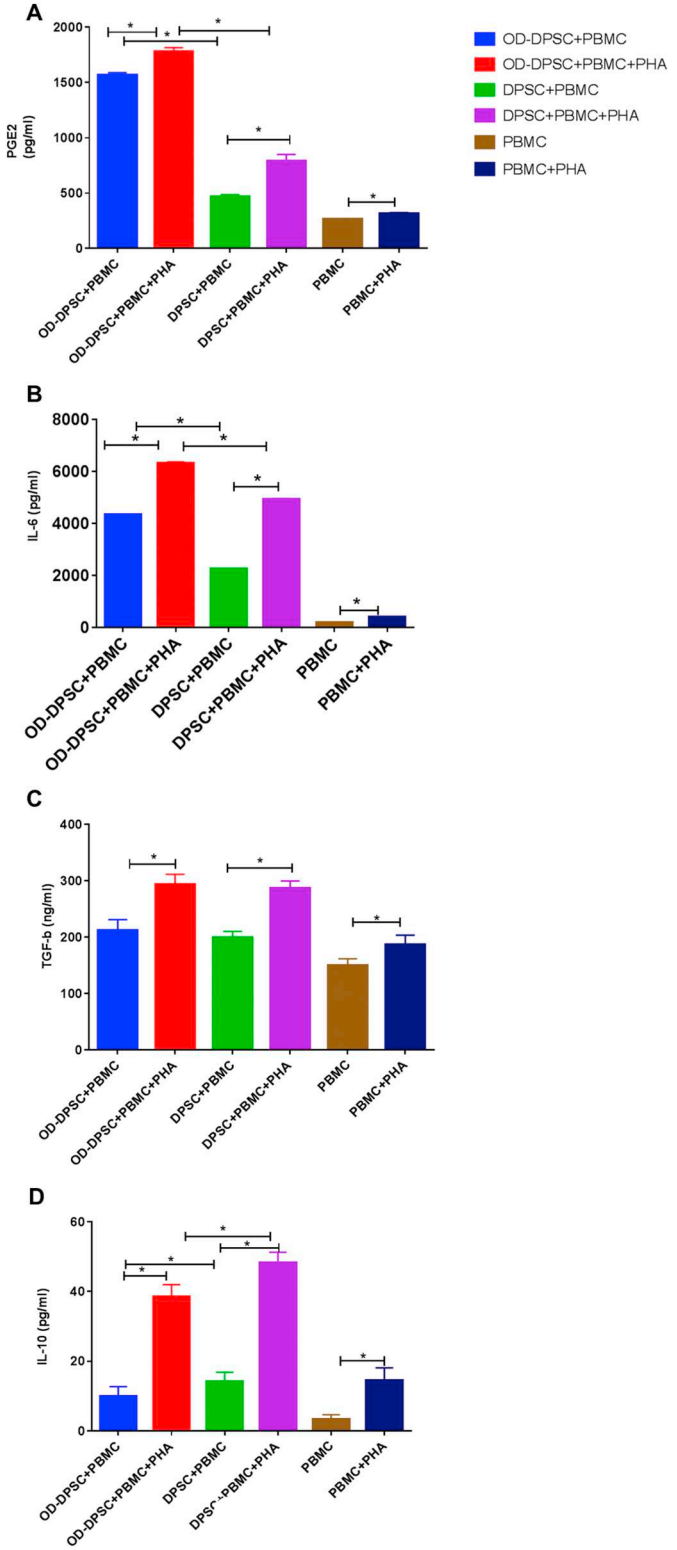

Fig. 5. Production of PGE2, IL-6, TGF-β and IL-10 cytokines by PBMCs after 72 h co-culture with OD-DPSCs and DPSCs (A-D). The data is expressed as the mean ± SD of three independent experiments (*P < 0.05). DPSC, dental pulp stem cell; OD-DPSC, osteogenic differentiated dental pulp stem cell; PGE2, prostaglandin E2; IL-6, interleukine-6; TGF-β, transforming growth factor beta; IL-10, interleukine-10.

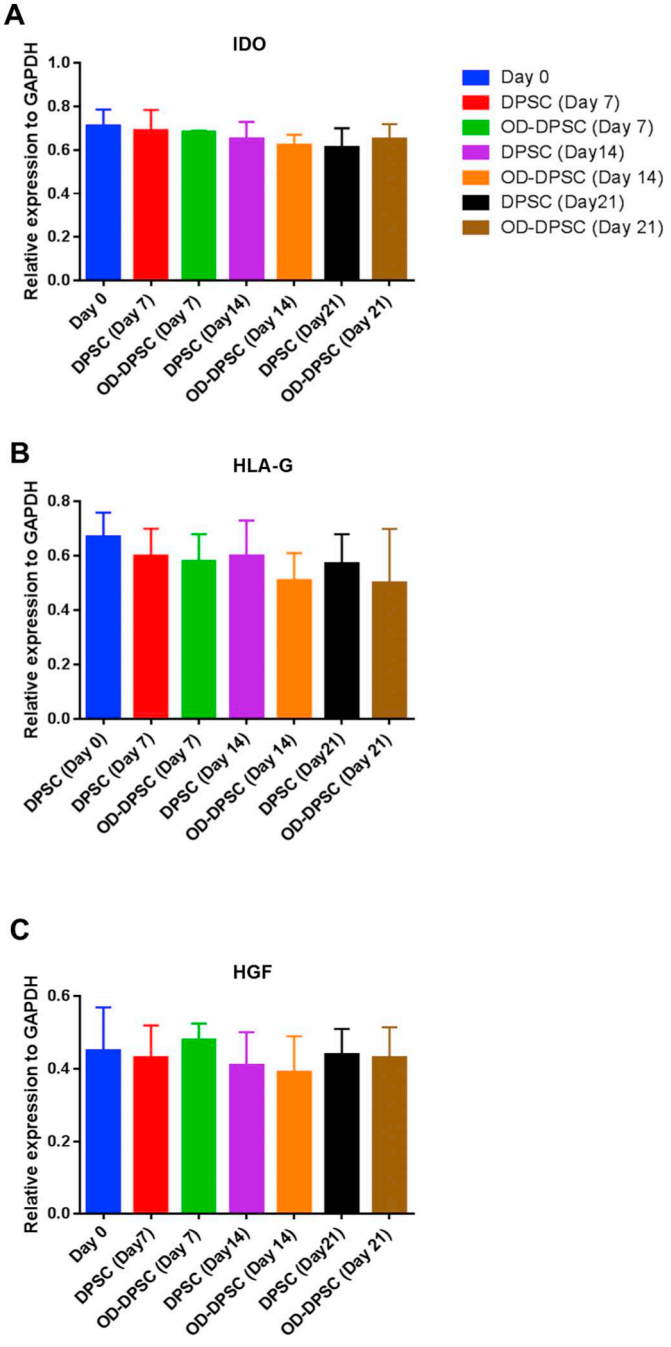

Fig. 6. Nitric oxide concentration during osteogenic differentiation of hDPSC. The level of significance was considered < 0.05 (*P < 0.05) and the data is expressed as the mean ± SD of three independent experiments. NO, nitric oxide; DPSC, dental pulp stem cell.

model. Rasmusson et al. suggested that MSCs alone do not secret IL-10; however, increased in co-culture. The present study is in agreement with this conclusion [28]. The results of the present study showed that IL-10 was significantly increased during co-culture of DPSCs and PHA induced PBMCs which is in line with the results of Ding et al. [35]. Unlike the previous study reported by Liu et al. which showed that the levels of IL-10 increased in osteoblast differentiated rabbit BM-MSC, the present results showed that the levels of IL-10 decreased after osteoblastic differentiation, but the amount of this cytokine increased significantly after co-culture with allogenic PBMCs when compared with the control group. This difference could be due to the different source of MSC, different isolation, and culture methods.

It has been proven that in addition to immunosuppressive effects, TGF-β is one of the important factors for osteogenic and chondrogenic differentiation and is essential for maintenance and proliferation of

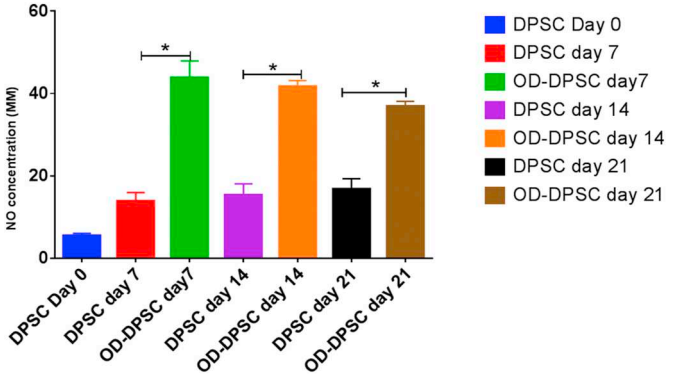

Fig. 7. Expression of HLAG, IDO, and HGF genes. The data is expressed as the mean ± SD of three independent experiments (*P < 0.05). HLA-G, human leukocyte antigen G; IDO, indole amine 2,3-dioxygenase; HGF, hepatocyte growth factor.

osteoblasts [36–40]. It was found that TGF-β increased during osteogenesis, but this increase was not significant. Lui et al. have also reported that the levels of TGF-β were decreased in differentiated MSCs. English et al. reported that TGF-β and PGE2 play a non-redundant part in the induction of FoxP3+ CD4 + CD25+ T cells by MSCs. Another study by Ding et al. reported that the inhibitory effect of DPSCs is mediated by the secretion of NO, IDO, TGF-β1, and PGE2.

One of the important soluble factors produced by MSCs is NO. NO is a gaseous signaling molecule that exerts several physiological activities, including regulating proliferation and bone homeostasis [41]. The present results showed that NO production increased significantly during osteogenic differentiation and osteoblasts produced much higher amount of NO when compared with undifferentiated DPSCs since it was reported that NO is produced from MSCs under osteogenic condition and involved in proliferation and maintenance of osteoblasts. NO modulates immune responses and plays an important role in suppressing T lymphocytes by inhibiting Stat5 phosphorylation [42]. The results showed that the amount of NO was significantly increased at days 7, 14 and 21 when compared to undifferentiated DPSC. Another important finding in this study was the suppressive capacity of NO. Several studies have suggested that NO plays an important role in murine MSC immunosuppression, whereas indicated an essential role for IDO in human MSCs [43,44]. The present study showed that although NO does not have an influence in immunosuppressive effect of undifferentiated DPSCs, but it may have a great impact in immunosuppression mediated by osteogenic differentiated DPSCs due to its high concentration after osteogenic induction, whereas the expression levels of IDO gene was not altered after differentiation.

Niemeyer et al. suggested that the bone marrow mesenchymal stem cells (BM-MSCs) and adipose derived mesenchymal stem cells (AD-MSCs) retained their immunomodulatory effects after osteogenic differentiation [45]. Similarly, Blanc et al. observed that differentiated MSC was able to suppress immune response. In contrast to these studies, a recent investigation revealed that the chondrocyte differentiated MSCs lost their ability to inhibit proliferation of allogenic T cells [46]. Another study by Chen and colleague also showed that the immunogenicity of chondrogenic differentiated MSCs increased when compared with undifferentiated MSCs [47].

It was assumed that the suppression of allogenic PBMCs was mediated by soluble factors and exosomes released by differentiated DPSCs. However, the content and impact of the exosomes and the effect of cell-cell contact in differentiated MSCs immunosuppressive potential should be explored.

In conclusion, the levels of PGE2, IL-6, and TGF-β increased in the OD-DPSCs compared to the DPSCs while IL-10 decreased during osteogenesis. Moreover, NO production increased in the OD-DPSCs. The expression of HGF, HLA-G and IDO genes did not altered during osteogenic differentiation. Our results demonstrated that the differentiation stage of OD-DPSCs does not influence their immunomodulation characteristics in terms of gene expression and NO production. The OD-DPSCs maintained their immunomodulatory effects. Since, the suppressive component of the osteogenic differentiated DPSCs has been proven, further studies are needed to investigate these observations *in-vivo*.

5. Conclusion

In conclusion, the osteogenic differentiated hDPSCs exhibited immunomodulatory effects. It seems that the factors involved in osteogenic differentiation play a major part in immunomodulation of differentiated DPSCs. These soluble mediators have benefit function for both suppressing immune response and bone remodeling.

Conflicts of interest

The authors declare no conflict of interest.

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