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Osteogenically differentiated mesenchymal stem cells induced by hydrolyzed fish collagen maintain their immunomodulatory effects

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ABSTRACT

Aims: The reciprocity between stem cells and biomaterials is an essential topic in bone tissue engineering. Bone marrow mesenchymal stromal cells (BMSCs) have attracted considerable attention in regenerative medicine owing to their ability to self-renew and differentiate into osteoblasts, and more importantly, their immunomodulatory effects on the immune response. Ideal biomaterials should be osteo-inductive, environmentally sustainable, and economical. Our previous study showed that hydrolyzed fish collagen (HFC) can meet each of the above requirements. However, it is still unclear whether BMSCs maintain their immunomodulatory properties after osteogenic differentiation induced by HFC.

Main methods: Non-commercial sources of BMSCs were isolated from Sprague-Dawley (SD) rats. Osteogenically differentiated BMSCs induced by HFC and undifferentiated BMSCs were co-cultured with PBMC or NR 8383 macrophages, respectively. Cell proliferation of PBMC was examined using a BrdU uptake assay. In addition, the IL-6, TGF-β1, IL-10, PGE2, and nitric oxide levels were determined. The expressions of TSG-6 (TNF-stimulated gene 6) and IDO (indoleamine 2, 3-dioxygenase) genes were analyzed using qRT-PCR.

Key findings: The results revealed that HFC-induced BMSCs suppressed the proliferation of PBMC. The expression levels of anti-inflammatory mediators including IL-6, TGF-β1, and PGE2 significantly increased after 48 h of co-culture. Moreover, the nitric oxide production increased during osteogenesis induced by HFC, whereas the level of TSG-6 and IDO remained unchanged after osteogenic differentiation. HFC-BMSCs inhibited the inflammatory mediator production (IL-1β, TNF-α) in LPS-stimulated macrophages.

Significance: Taken together, these findings suggest that the immunomodulation ability is still retained in osteogenically differentiated BMSCs induced by HFC.

1. Introduction

Osteo-inductive materials are indispensable in bone tissue engineering because they can drive stem cells into an osteogenic lineage [1]. Some biomaterials have been found to exhibit osteo-inductive properties; however, certain issues relevant to the side effects with detrimental immunological responses, high expenditure, and limited material availability should be addressed in a comprehensive manner [2].

Biologically active materials or compounds from various marine organisms have received significant attention owing to their sustainability and low cost. Among the "blue biomaterials," hydrolyzed fish collagen (HFC) have been studied extensively and applied in a wide range of fields such as pharmaceutical products [3] and cosmetics [4]. Interestingly, it has been documented that HFC exhibits an osteo-inductive activity, which drives the differentiation of bone marrow mesenchymal stem cells toward the osteoblast phenotype [5], beyond which HFC can induce osteogenic differentiation of human periodontal ligament cells [6], and together with its high accessibility and easy processability, represents a considerable potential for use as an active ingredient in bone tissue engineering.

Bone marrow mesenchymal stem cells (MSCs) are plastic-adherent, multipotent, nonhematopoietic progenitor cells that can differentiate into several cell lineages, such as osteoblasts, neurons, adipocytes, and chondrocytes. MSCs have been used to repair damaged tissues, including but not limited to bone, neuronal, cartilage, or myocardial tissues [7–9]. Mesenchymal stem cells (MSCs) are ideal candidates for cell-based therapy because they act on varieties of mechanisms of protection and repair, improving the structural and functional integrity [10]. For stem cell-based treatments, one important aspect that needs to be addressed is the interaction between the donor transplantation and the immune system of the host. It has been shown that mesenchymal

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stem cells can modulate the immune system and suppress a variety of immune cells, including macrophages, T cells, B cells, and natural killer cells [11]. A substantial amount of evidence suggests that the mesenchymal stem-cell-mediated immunosuppression of macrophages is crucial for an inflammatory response and tissue regeneration [12]. For example, MSCs exert their immunomodulatory function through the inhibition of $CD4^+$ and $CD8^+$ T-cell proliferation [13]. MSCs further exert their immunosuppressive effects on B cells, significantly suppressing their proliferation [14].

Thus far, it has been shown that HFC possesses an excellent immunomodulatory property [15,16]. However, it is still unclear whether BMSCs maintain their immunomodulatory properties after osteogenic differentiation induced by HFC. Because cell-therapy based on advanced biomaterials is a promising strategy for bone regeneration, if BMSCs retain their immunomodulation properties after osteogenic differentiation induced by HFC, they can be an innovative combination for bone tissue regenerative applications.

2. Materials and methods

2.1. Materials

HFC used in this study was supplied by the Shanghai Fisheries Research Institute (Shanghai, China).

2.2. Isolation of bone marrow mesenchymal stem cells

BMSCs were obtained from the femurs and tibias of Sprague Dawley rats by flushing out the bone marrow tissue with a 1 cc syringe. The isolated BMSCs were cultured in DMEM supplied with 10% fetal bovine serum, 100 U mL^{-1} of penicillin, and 100 μg mL^{-1} of streptomycin at 37 °C under a 5% CO_2 atmosphere. Cells were harvested at 80% confluence using 0.25% trypsin and 0.02% EDTA and plated in 12-well culture plates (5.0×10^4 cells/cm^2) or 96-well plates (1×10^4 cells/well). BMSCs of passage 3 were used for all experiments. The experiment was approved by the Ethics Committee of Shanghai Ninth People's Hospital, affiliated with the School of Medicine, Shanghai Jiao Tong University.

2.3. Peripheral blood mononuclear cell (PBMC) isolation

The peripheral blood mononuclear cells (PBMCs) from donor SD rats were isolated from heparinized peripheral blood using density gradient centrifugation, and freshly isolated blood was diluted with PBS. The diluted blood was then gently layered on top of the Ficoll. The sample was centrifuged for 20 min at 2,000 rpm in a microcentrifuge at room temperature. PBMCs were collected from the plasma–Ficoll interphase and washed twice with PBS. The cell pellets were then resuspended in a DMEM medium containing 5% FBS, 100 U/mL of penicillin, and 100 μg/mL of streptomycin. The experiment was approved by the Ethics Committee of Shanghai Ninth People's Hospital, affiliated with the School of Medicine, Shanghai Jiao Tong University.

2.4. Cell culture of NR8383 rat alveolar macrophages

The NR8383 rat alveolar macrophage cell line was obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China), and was grown in DMEM supplemented with 15% heat-inactivated fetal bovine serum, 100 U/mL of penicillin, and 100 g/mL of streptomycin at 37 °C in a 5% CO_2 atmosphere. The medium was changed every 3 days.

2.5. Co-culture system

To systematically study the immunomodulatory effects of differentiated BMSCs induced by HFC on lymphocyte proliferation, a Transwell co-culture system was employed (0.4 μm pore size, BD Biosciences). The BMSCs were seeded in the lower compartment of a 24-well plate at a density of 1×10^4/mL. When the growth reached 80% confluency, the cells were treated with a DMEM medium containing 0.2 mg/mL of HFC for 7 days. This medium was changed each day. Next, 1×10^4 PBMCs or 5×10^4 NR8383 rat macrophages were loaded into the upper chamber of the plate. A group of peripheral blood mononuclear cells were treated with 10 μg/mL of phytohemagglutinin (PHA, Sigma-Aldrich, USA), NR8383 cells were pre-treated with 10 μg/mL of LPS for 4 h. The cells were then maintained in a co-culture for 48 h. PBMC or NR8383 cells incubated in a DMEM medium with or without PHA/LPS served as the control groups.

2.6. Cell proliferation evaluation

After 2 days of co-culturing, PBMCs in the different experimental and control groups were transferred into a 96-well plate (SPL, Korea). Then, Cell Proliferation ELISA, using a BrdU (colorimetric) kit (Roche Diagnostics), was used to measure the PBMC proliferation, following the manufacturer's instructions. Briefly, BrdU (20 μl/well, 10 μM) was added into each well and the plate was incubated overnight at 37 °C. After removing the labeling medium, 200 μl of the FixDent solution was loaded into each well and the plate was incubated at room temperature for 30 min. Thereafter, an antibody conjugate (anti-BrdU-POD solution, 100 μL/well) was added, and the plate was further incubated at room temperature for 1 h. Finally, the substrate solution was added, and the absorbance of each well was measured immediately by a plate reader at a wavelength of 450 nm.

2.7. Alkaline phosphatase (ALP) activity measurements

Alkaline phosphatase (ALP) is considered a relatively early indicator of osteogenic differentiation, and to assess the osteogenic differentiation of the BMSCs induced by HFC, the ALP activity was measured. After 7 days of incubation in the HFC medium, the BMSCs were washed three times with PBS and then lysed with a RIPA buffer (Sigma Aldrich, St Louis, MO, USA) for 20 min on ice. The cell lysate was centrifuged at 4 °C 16,000 × *g* for 10 min. The total protein quantification was conducted using a BCA assay kit (Pierce, Rockford, IL, USA) according to the manufacturer's instructions, and the ALP activity was measured using an ALP activity assay kit (Jiancheng, Nanjing, China) according to the instructions. The ALP levels were normalized to the total protein content, and each sample was repeated in triplicate.

2.8. Measurements of cytokine and PGE2 levels

The concentrations of IL-10, IL-6, TGF-β1, and PGE2 in the differentiated BMSCs; the PBMC and undifferentiated BMSCs; the PBMC co-culture supernatant and the content of IL-1β in the differentiated BMSCs; NR8383 macrophages and undifferentiated BMSCs; and NR8383 macrophages in a co-culture supernatant were measured using an enzyme-linked immunosorbent assay kit (R&D, Minneapolis, USA), following the manufacturer's instructions.

2.9. Nitric oxide quantification

Following 1-, 3-, and 7-day treatments with HFC, the supernatant of differentiated BMSCs was collected and stored at −20 °C. The NO levels in the cell supernatants were measured using a commercially available Promega Griess reagent system kit (Promega, WI, USA). An assay was performed following the manufacturer's instructions. Briefly, 100 μl of the supernatant was mixed with an equal volume of Griess reagent and incubated in the dark for 10 min. The absorbance was measured using a microplate reader at a wavelength of 540 nm. A computation of the nitrite concentration was conducted with reference to a standard curve established using $NaNO_2$. The results were normalized by the cell number.

Table 1
Primers used in this study.

Target gene	Forward primer Sequence(5′-3′)	Reverse primer Sequence(5′-3′)
IDO	GGGCTTTGCTCTACCACATC	GCTTCCCATTCTCAATCAGC
TSG-6	AGGCTGTTTGGCTGACTATGT	TTTCCTGTGCTGATGATGTCTT
GAPDH	GCTCTCTGCTCCTCCCTGTTCTAG	TGGTAACCAGGCGTCCGAT

2.10. Real-time RT-PCR

After 7 days in the HFC osteogenic induction medium, the total RNA was isolated from the BMSCs and osteogenically differentiated BMSCs using a Trizol reagent (Takara, Japan). The total RNA was then reverse transcribed into the cDNA using a reverse transcriptase synthesis kit (Takara, Japan) according to the manufacturer's instructions. Quantitative RT-PCR (qRT-PCR) was performed using SYBR Green (TaKaRa, Japan). The $2^{-\Delta\Delta Ct}$ method was used to determine the relative expression levels for each gene, and the relative expression of the target genes was calculated after normalization to the housekeeping gene, GAPDH. The primers used in this experiment are listed in Table 1.

2.11. Western blotting

BMSCs treated with or without HFC for 7 days, and NR8383 macrophages in the co-cultured system, were lysed using a RIPA buffer supplemented with a protease inhibitor cocktail (Roche, Indianapolis, IN, USA). The concentration of proteins was determined using the BCA protein kit (Pierce, Rockford, IL, USA). A total of 40 μg of each sample was loaded onto a 10% polyacrylamide SDS-PAGE gel for electrophoresis and then transferred to a polyvinylidene difluoride (PVDF) membrane. The blots were blocked with a blocking buffer (5% nonfat dry milk) for 1 h at room temperature. The membranes were then incubated with primary antibodies (GAPDH, OPN, TNF-α) at 4 °C overnight. After three washes for 10 min in PBST, the horseradish peroxidase (HRP) conjugated secondary antibody was applied at room temperature for 1 h followed by detection using an enhanced chemiluminescence (ECL) detection kit (Pierce, Rockford, IL, USA).

2.12. Statistical analysis

All experiments were conducted three times and all data in this study were presented as the mean ± standard deviation (SD). A Student's t-test was used for comparison between the two groups. The differences between groups at *$p < 0.05$ were considered statistically significant.

3. Results

3.1. Osteogenic differentiation of BMSCs induced by HFC

It was found that the ALP activity increased more significantly in the HFC group than in the control group ($p < 0.05$, Fig. 1A). The Western blot results showed that the HFC increased the protein expressions of OPN in the BMSCs (Fig. 1B). In addition, ALP staining was conducted to demonstrate the osteogenic differentiation of BMSCs found in our previous study, where the BMSCs cultured with HFC displayed stronger ALP staining compared to the control groups [5].

3.2. Effects of HFC-induced BMSCs on lymphocyte proliferation

PBMC proliferation was significantly stimulated by PHA. The BMSCs significantly ($p < 0.05$) inhibited the PBMC proliferation in the absence or presence of PHA. Similar results were obtained in the osteogenically differentiated BMSCs induced by HFC, and no statistically significant difference was shown in terms of suppressing the PBMC proliferation between the osteogenically differentiated and undifferentiated groups (Fig. 2 and Supplementary Fig. 1).

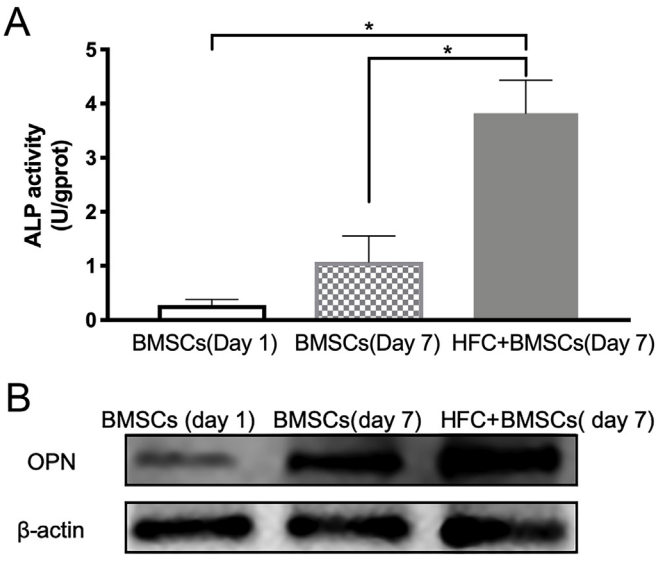

Fig. 1. HFC promoting the osteogenic differentiation of BMSCs. (A) ALP activity of BMSCs when cultured with HFC. (B) Protein expression of OPN in BMSCs measured using Western blotting. *$p < 0.05$.

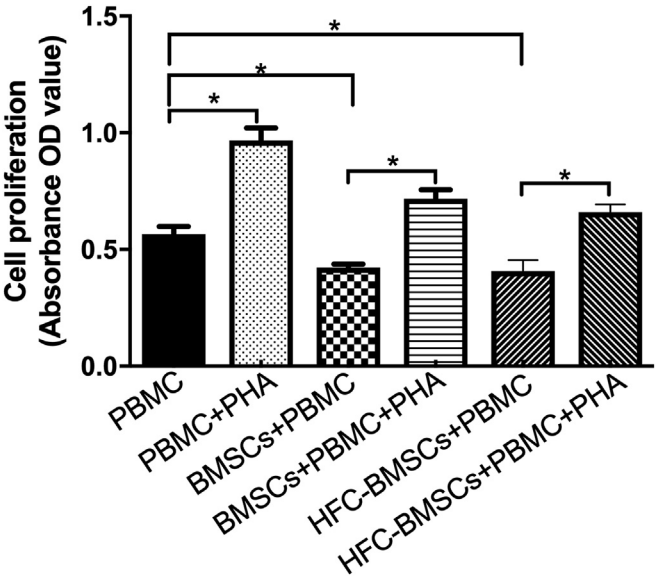

Fig. 2. Proliferation of stimulated and non-stimulated PBMCs after coculture with BMSCs and HFC-BMSCs for 48 h. PBMCs, peripheral blood mononuclear cells; BMSCs, bone marrow mesenchymal stem cells; HFC-BMSCs, bone marrow mesenchymal stem cells treated with HFC for 7 days; OD, optical density (*$p < 0.05$).

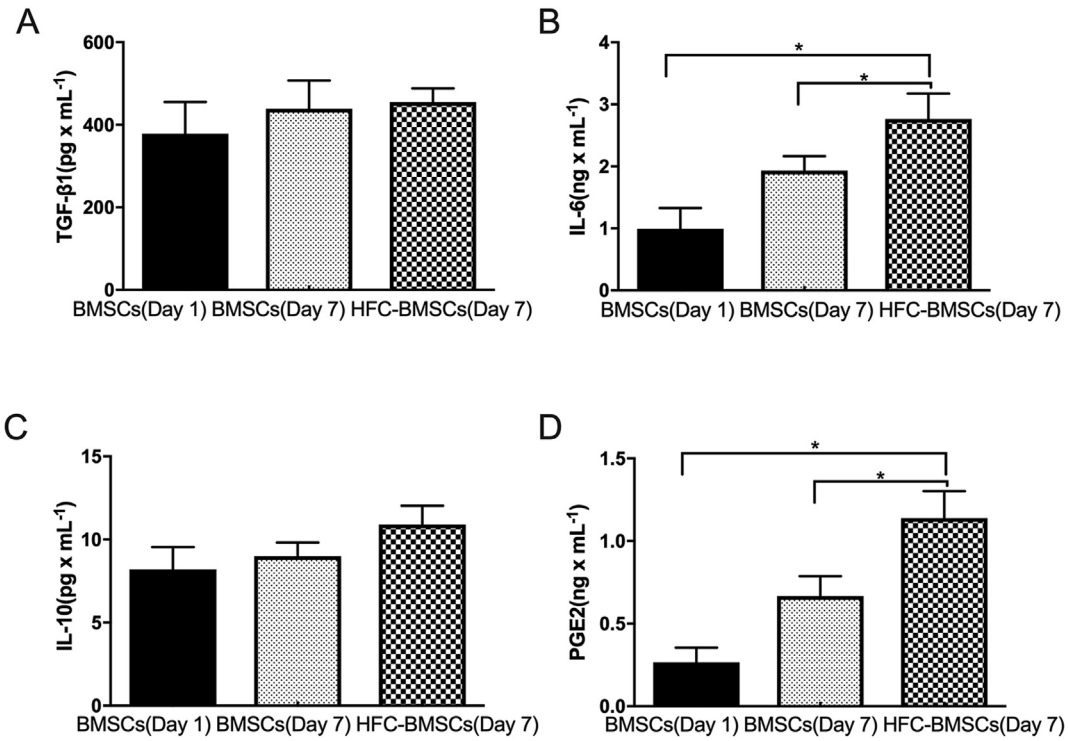

Fig. 3. Secretion of TGF-β1, IL-6, IL-10, and PGE2 by BMSCs and HFC-BMSCs (A–D). BMSCs, bone marrow mesenchymal stem cells; HFC-BMSCs, bone marrow mesenchymal stem cells treated with HFC for 7 days (*$p < 0.05$).

3.3. Production of cytokines by BMSCs and HFC-induced BMSCs

The ELISA results revealed that after HFC treatment, the IL-6 (Fig. 3B) and PGE2 (Fig. 3D) content in the osteogenically differentiated BMSCs were significantly higher than in the undifferentiated BMSCs. There were no significant differences between the two groups in terms of TGF-β1 (Fig. 3A), whereas the HFC exposure resulted in a slight, but non-significant, increase in the level of IL-10 (Fig. 3C).

3.4. Production of cytokines and PGE2 by PBMC

Following a co-culture with HFC-induced BMSCs, the levels of TGF-β1 were increased in both PHA-stimulated and non-PHA-stimulated groups when compared with the PBMC alone. The results of the elevated TGF-β1 were also shown in the undifferentiated BMSC groups ($p < 0.05$) (Fig. 4A). Regardless of the absence or presence of PHA, PBMC produced a higher amount of IL-6 when co-cultured with osteogenically differentiated BMSCs; by contrast, the IL-6 contents were also increased significantly in the co-culture system of the PBMCs and undifferentiated BMSCs (Fig. 4B). Similar results were observed for PGE2 secretion, and in both the PHA-stimulated and non-PHA-stimulated groups, the PGE2 levels were significantly higher in the co-culture systems than in the PBMC alone (Fig. 4D). Compared with the PBMC alone, the IL-10 level in all other groups was significantly higher. In the co-culture groups with undifferentiated BMSCs, regardless of whether PHA was added, the IL-10 production was remarkably increased compared to the PBMC alone. Most interestingly, the level of IL-10 in the HFC-induced BMSC co-culture group was higher than that in the undifferentiated BMSC co-culture group (Fig. 4C).

3.5. Nitric oxide production

The concentrations of nitric oxide (NO) in the cell culture media were detected in all groups. HFC-induced BMSCs significantly enhanced the NO levels compared to those of the undifferentiated BMSCs. The NO concentration was increased in a time-dependent manner during osteogenesis in the HFC-induced BMSCs (Fig. 5).

3.6. Gene expression

To better understand the mechanism of the immunomodulation of HFC-induced BMSCs, the transcriptional expression levels of some immunomodulation-related genes, IDO and TSG-6, were determined. No statistically significant differences were found in the gene expressions of IDO and TSG-6 between the HFC-induced BMSCs and undifferentiated BMSCs at days 1, 3, and 7 (Fig. 6A and B).

3.7. IL-1β production and TNF-α protein expression in macrophages

We next attempted to examine the immunomodulatory effects of HFC-BMSCs on the macrophages. As shown in Fig. 7A, the IL-1β secretion significantly increased upon the administration of LPS in the NR8383 macrophages. Following a co-culture with HFC-induced BMSCs, the levels of IL-1β were significantly inhibited compared with the LPS + NR8383 macrophages group (Fig. 7A). Western blotting was used to determine the effects of the HFC-BMSCs on the TNF-α protein expressions in the macrophages. Fig. 7B shows that the TNF-α protein expressions increased in the LPS-activated macrophages. Compared to the LPS + NR8383 macrophages, both BMSCs and HFC-BMSCs significantly suppressed the TNF-α expression.

4. Discussion

Immunomodulatory properties and a multi-differentiation potential are essential mechanisms in MSC-based cell therapy. Numerous experimental and clinical studies revealed that MSCs regulate the immune response, both acquired and innate, with possible involvement in the management of inflammatory disorders and transplantation [17]. In the present study, the immunomodulatory effects of osteogenically differentiated BMSCs induced by HFC were fully elucidated. Our results showed that HFC-induced BMSCs inhibited proliferation of PBMC, which resulted in increased levels of immunomodulation-related

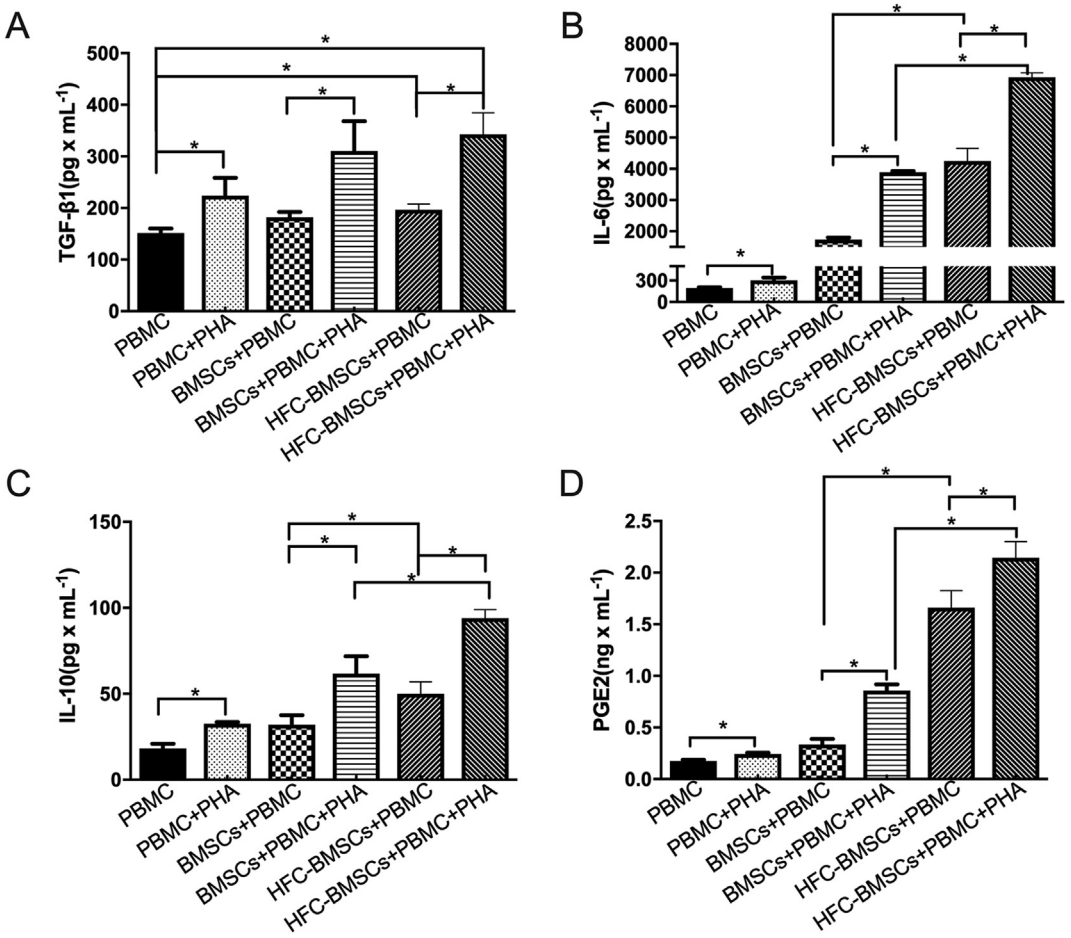

Fig. 4. Detection of TGF-β1, IL-6, IL-10, and PGE2 in co-culture supernatant (A–D). PBMCs, peripheral blood mononuclear cells; BMSCs, bone marrow mesenchymal stem cells; HFC-BMSCs, bone marrow mesenchymal stem cells treated with HFC for 7 days (*p < 0.05).

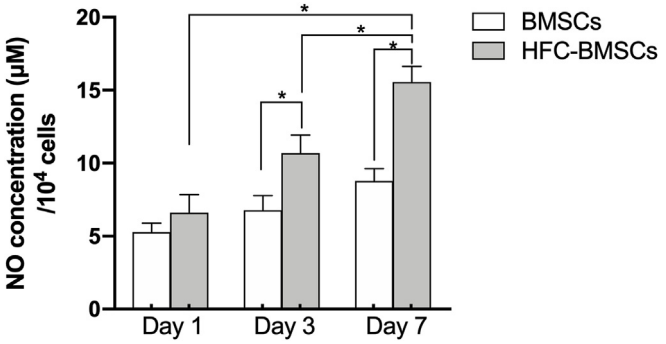

Fig. 5. Nitric oxide production examined during osteogenic differentiation of BMSCs. BMSCs, bone marrow mesenchymal stem cells; HFC-BMSCs, bone marrow mesenchymal stem cells treated with HFC for 1, 3, and 7 days (*p < 0.05).

factors, such as TGF-β1, IL-6, PGE2, and NO. Beyond that, HFC-treated BMSCs showed the same immune-regulating gene expression level compared to undifferentiated BMSCs. Our results provide direct evidence that HFC-treated MSCs maintain the immunomodulatory features.

During osteogenic differentiation, many genes, enzymes, or proteins present different alteration profiles, which were regarded as osteogenic markers to evaluate cell osteogenic differentiation [18]. Among them, alkaline phosphatase (ALP) has been well known as an early-stage osteogenic marker of bone formation. ALP can hydrolyze many types of phosphates under alkaline conditions to enhance the cell maturation and calcification [19], and it has been shown that ALP increases the local content of inorganic phosphate and promotes cell mineralization during osteogenic differentiation [20]. Therefore, an ALP activity measurement is a practical verification of osteogenic differentiation. On the other hand, OPN, also known as secreted phosphoprotein 1 (SPP1), is a well-documented marker during osteogenic differentiation [21]. In the present study, the ALP activity and OPN expression level were significantly increased in the HFC group as compared to the control group, indicating that HFC induces the osteogenic differentiation of BMSCs.

It has been shown that mesenchymal stem cells inhibit the proliferation of lymphocytes [22]. The results of this study confirm that HFC-induced BMSCs still possess this inhibitory effect on lymphocytes *in vitro*, and therefore cannot activate the PBMCs (Fig. 2). In addition, HFC-induced MSCs can also inhibit PHA-stimulated lymphocytes proliferation.

It is well known that TGF-β1 is not only responsible for immunosuppressive effects, it is also involved in osteogenic differentiation and is critical for the proliferation of osteoblasts [23,24]. In the present study, the data indicated that there remains a high level of TGF-β1 in HFC-treated BMSCs (Fig. 3A). MSC-derived TGF-β1 and PGE2 have been shown to have a non-redundant role in the induction of $CD4^+CD25^+FoxP3^+$ T-cells [25]. In addition, a monoclonal antibody against TGF-β1 restored the T-cell proliferation inhibited by dental pulp stem cells [26].

Interleukin-6 (IL-6) has dual roles serving as an anti-inflammatory or inflammatory cytokine [27]. In the present study, the IL-6 level was significantly elevated in HFC-induced BMSCs (Fig. 3B). It has been

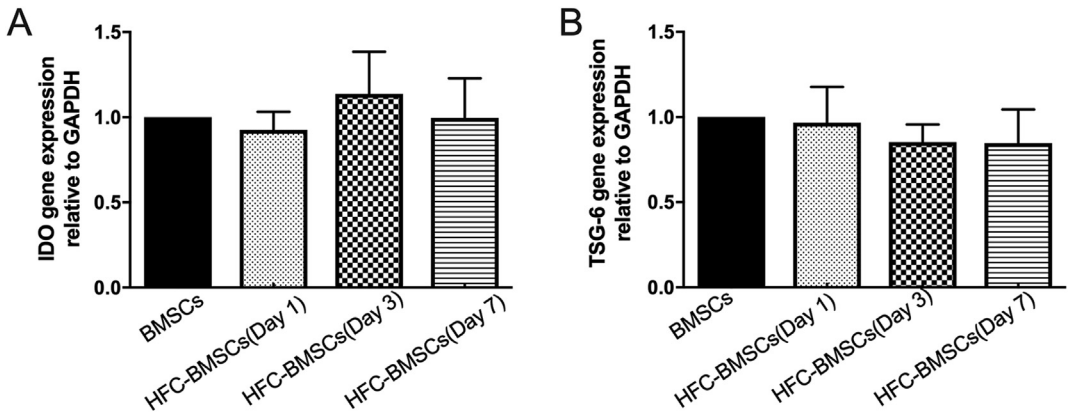

Fig. 6. Quantitative RT-PCR analyses of IDO(A) and TSG-6(B) gene expression. BMSCs, bone marrow mesenchymal stem cells; HFC-BMSCs, bone marrow mesenchymal stem cells treated with HFC for 1, 3, and 7 days (*p < 0.05).

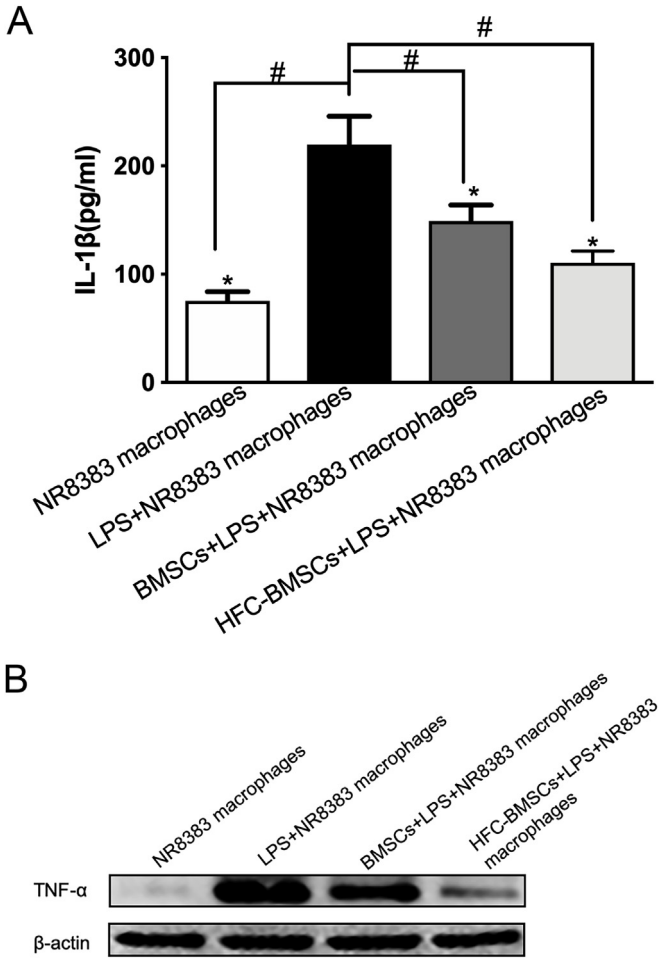

Fig. 7. HFC inhibited inflammatory mediators in LPS-stimulated macrophages. (A) HFC reduced the production of IL-1β in LPS-stimulated macrophages. (B) Effects of HFC on protein levels of TNF-α in LPS-stimulated macrophages. *p < 0.05 vs. NR8383 macrophages treated with LPS alone. #p < 0.05.

suggested that immune-related cytokines interact with each other to enhance their regulatory effects. Bouffi et al. showed that the secretion of PGE2 was enhanced proportionally to the expression levels of IL-6 [28]. The current study also showed that PGE2 and IL-6 increased together in different groups (Fig. 4B and D).

PGE2 is a primary effector molecule responsible for the immunoregulatory capacity of MSCs. PGE2 produced by MSCs exert regulatory effects on the proliferation, differentiation, and function of immune cells from an innate and adaptive immune system [29]. In the present study, the PGE2 level in the co-culture system of HFC-treated BMSCs and PBMCs significantly increased (Fig. 4D). Shang et al. have also found that PGE2 has a major role in immune-regulatory activities of differentiated MSCs [30].

Earlier research indicated that IL-10 is increased in a co-cultured system with MSCs [31]. Our data are in line with their findings. A slight increase in the level of IL-10 was observed after osteogenic differentiation, but was remarkably up-regulated after a co-culture with PBMCs as compared with the control group (Figs. 3C and 4C), which is consistent with a previous study showing that IL-10 increases in osteogenically differentiated MSCs [32]. It has been suggested that MSCs modify the differentiation of the macrophage populations toward anti-inflammatory or M2 phenotype by increasing the interleukin-10 production [33].

NO plays a pivotal role in several physiological processes including osteogenesis and stem cell differentiation [34]. The production of NO was significantly increased during osteogenesis in the present study, and the osteoblasts generated much higher concentrations of NO when compared with BMSCs without treatment with HFC (Fig. 4). Sato et al. found that NO is involved in the suppression of Stat5 phosphorylation and T-cell proliferation [35]. The concentration of NO in the HFC-BMSCs group was significantly higher at days 3 and 7 than in the undifferentiated BMSC group (Fig. 5). Some studies have suggested that NO mediates the immunosuppression of mesenchymal stromal cells [36,37]. In the present study, NO may have some influence on the immunosuppression mediated by HFC-induced MSCs owing to its high concentration after osteogenic induction.

It is intriguing that the expression of the IDO and TSG-6 genes did not change upon osteogenic induction by HFC (Fig. 6), which indicates that the osteogenic-differentiated BMSCs retain their immunomodulatory capacity at the transcriptional level. It has been reported that BMSCs maintain their immunomodulatory effects by using a traditional osteogenic medium [38], and conversely, Ryan et al. showed that chondrogenically differentiated MSCs lose the ability to inhibit T-cell proliferation *in vitro* [39]. We speculated that the inhibition of PBMCs is mediated by exosomes or soluble factors produced by HFC-induced BMSCs, although the exact mechanism is not completely understood and requires further study.

Finally, the immunomodulatory effect of HFC-BMSCs was verified by using macrophages. It has been shown that the exposure of macrophages to LPS activates pro-inflammatory signals, enhancing the secretion of various cytokines including IL-1β and TNF-α [40]. IL-1β, produced predominantly by macrophages, is involved in cytotoxic T-cell differentiation and Th1 inflammation by activating dendritic cells to produce IL-12 [41]. TNF-α is also produced primarily by

macrophages, which can activate T-cells, promote the production of Th1 type cytokines, and enhance the DC cell (dendritic cell) antigen presentation [42]. Both IL-1β and TNF-α are important indicators for pro-inflammatory M1 macrophages. All cytokines and inflammatory mediators mentioned above are intimately related, participating in various inflammatory signaling pathways. It has been suggested that MSCs inhibit the function of pro-inflammatory M1-like macrophages, and in the present study, the inhibition of IL-1β and TNF-α by BMSCs are consistent with such findings [43,44]. More importantly, our results confirmed that HFC-BMSCs maintain their immunoregulatory effects toward the macrophages.

Overall, the contents of TGF-β1, IL-6, and PGE2 are significantly increased in the HFC-induced BMSCs compared to the BMSCs, whereas IL-10 showed a slight increase during osteogenic differentiation. In addition, compared to BMSCs without HFC treatment, HFC-induced BMSCs significantly increased the NO production. The mRNA levels of IDO and TSG-6 did not change during osteogenesis. The present study suggested that the state of differentiation of mesenchymal stem cells in the bone marrow does not necessarily result in an alteration of their immunoregulatory capacities with regard to immunomodulation-related gene expression and NO production. The immunomodulation was still retained in the osteogenically differentiated BMSCs induced by HFC. In the future, we will attempt to study this effect *in vivo*.

5. Conclusion

In conclusion, osteogenically differentiated BMSCs induced by HFC exhibited immunoregulatory effects. Some factors related to osteogenic differentiation exert a potent effect on the immunoregulation of HFC-induced BMSCs, which will be a beneficial for both bone regeneration and immune suppression.

Declaration of competing interest

The authors declare that they have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2019.116970.

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