Modulation of the secretion of mesenchymal stem cell immunoregulatory factors by hydrolyzed fish collagen

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Abstract. The aim of the present study was to investigate the possible immunomodulatory effects of osteogenically differentiated bone marrow mesenchymal stem cells induced by hydrolyzed fish collagen. Marine biomaterials have attracted significant attention for their environmental friendliness and renewability. Hydrolyzed fish collagen (HFC) has been discovered to induce the osteoblastic differentiation of stem cells, which underlies the foundation for its application in tissue engineering. Stem cells and their biomaterial carriers face acute immune rejection mediated by host macrophages. A potential strategy for combatting rejection in stem cell therapy is to modify the polarization of macrophages. However, whether HFC-induced mesenchymal stem cells maintain their immunomodulatory ability remains to be determined. To understand this phenomenon, a co-culture model of direct contact was established between bone marrow mesenchymal stem cells (BMSCs) and RAW264.7 macrophages, where the secretion of nitrous oxide from macrophages was measured using Griess colorimetric assay. ELISAs were performed to measure the secretion of interleukin (IL)-1β, IL-6, transforming growth factor (TGF)-β and IL-10, whilst reverse transcription-quantitative PCR was used to assess the expression levels of IL-1β, IL-6, CD206, resistin-like molecule α (FIZZ1) and prostaglandin E2 receptor 4 (EP4). In addition, the expression levels of relevant proteins in the phosphorylated-cyclic AMP-responsive element-binding protein-CCAAT/enhancer-binding protein β (EBPβ) pathway were investigated using western blotting. HFC-induced BMSCs were found to suppress the expression levels of IL-1β and IL-6, whilst increasing the expression levels of CD206 and FIZZ1 in RAW264.7 macrophages. HFC-induced BMSCs also inhibited the secretion of IL-1β and IL-6, whilst promoting the secretion of TGF-β and IL-10 secretion from RAW264.7 macrophages. Mechanistic studies using western blotting discovered that HFC stimulated the secretion of prostaglandin E2 from BMSCs, which subsequently increased the expression of EP4 on the macrophages. EP4 then increased the expression levels of C/EBPβ and arginase 1 further. In conclusion, results from the present study suggested that following induction with HFC, BMSCs maintain their immunomodulatory activity.

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Introduction

With the technological advancement of stem cell and tissue engineering, regenerative medicine has become a hot topic in the field of biological medicine (1). The self-renewal and multi-directional differentiation of stem cells enables them to serve as seed cells for tissue engineering, facilitating the repair of damaged tissues or organs (2). In tissue engineering, ≥2 conditions are required to optimize the application of stem cells, including the presence of an effective inducer and tolerance to immunological assault (3). A number of biomaterials have been previously found to induce the osteogenic differentiation of bone marrow mesenchymal stem cells (BMSCs) (4), such as hydrolyzed fish collagen (HFC) (5). Since it is readily available and accessible, HFC is a material that warrants further investigations in this field.

The biological activity of HFC has become a notable focus of research. Blanco *et al* (6) revealed that HFC possessed significant antioxidant properties, whilst Liu and Sun (7) observed that HFC induced the osteogenic differentiation of human periodontal ligament cells in another study. HFC was found to induce adipose-derived stromal cell chondrogenesis as effectively as transforming growth factor (TGF)-β1 (8), whereas the anti-inflammatory properties of HFC has also been previously documented (9). Collectively, these findings suggested that HFC possesses a number of biological activities with the potential for future clinical application.

Immunological rejection remains to be the primary limitation for the transplantation of allogenic stem cells and their derivatives (10). Stem cells and their associated scaffolds are known to face acute immune rejection mediated by host macrophages, hindering the migration of reparative cells and weakening the ability of new tissues to propagate to their surroundings, in turn leading to failure in tissue

regeneration (11). Although previous research has largely focused on inhibiting the activation of host immunity (12,13), macrophages have also attracted increased attention for their substantial plasticity and phenotype-switching capacity (14). Owing to their prominent phenotypic plasticity, macrophages have been discovered to mediate both proinflammatory rejection and anti-inflammatory tissue remodeling (15). The exposure of M1 macrophages to M2 signals and vice versa, has been discovered to induce the re-polarization of differentiated macrophages, which demonstrates their high functional plasticity and potential therapeutic use (16). Therefore, modulating macrophage plasticity may provide a novel strategy for combating immune rejection in tissue engineering.

Mesenchymal stem cells (MSCs) possess unique immunoregulatory properties. A previous study reported that MSC transplantation modulated the immune response against allografts and alleviated transplant rejection, prolonging allograft survival (17). In addition, macrophages co-cultured with MSCs were found to consistently express high levels of CD206, a marker of alternatively-activated macrophages (18). In addition, the secretion levels of interleukin (IL)-10 and IL-12 were found to be increased and reduced, respectively, which is characteristic of alternatively-activated macrophages (19). In another previous study, macrophages co-cultured with MSCs were revealed to express lower levels of tumor necrosis factor α (TNF-α) compared with macrophages cultured alone, suggesting that MSCs modulate the inflammatory response by inducing M2 macrophage differentiation (20,21). Although HFC has been revealed to induce the osteogenic differentiation of MSCs as aforementioned, the effects of HFC on the immunomodulatory functions of MSCs remain unknown.

In the present study, a cell-cell contact co-culture model between BMSCs and macrophages was established to determine the regulatory effects of HFC-induced BMSCs on the crucial inflammatory factors associated with macrophages. Additionally, the immunomodulatory mechanism of BMSCs was investigated, providing a foundation for the application of HFC and BMSCs in tissue engineering.

Materials and methods

Materials. HFC was supplied by the Shanghai Fisheries Research Institute (Shanghai, China).

RAW264.7 cell culture. The murine macrophage RAW264.7 cell line was obtained from The Cell Bank of Type Culture Collection of the Chinese Academy of Sciences. The macrophages were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.), 50 μg/ml streptomycin and 100 IU/ml penicillin, which were maintained at 37˚C (5% CO_2) in a humidified atmosphere.

Isolation and in vitro culture of BMSCs. The present study was approved by the Ethics Committee of Shanghai Ninth People's Hospital, affiliated with the School of Medicine, Shanghai Jiao Tong University (Shanghai, China). The BMSCs originated from bone marrow mononuclear cells, which have the potential to differentiate into a number of different cell types, including osteoblasts, adipocytes, chondrocytes and neural cells (22). BMSCs have garnered considerable research attention due to their simplicity of preparation, ethical considerations, accessibility and low immunogenicity (23). In the present study, 10 male Sprague Dawley rats (age at sacrifice, 4 weeks; weight, 62.3±2.5 g) were sacrificed by cervical dislocation and the body was soaked in 75% ethanol for 5 min at room temperature. All rats were housed in a temperature-controlled room (21±2˚C) with relative air humidity of 40-60%, under a 12-h light/dark cycle, with free access to food and water. The tibia and femur of the rats were then obtained under sterile conditions at room temperature, where a 5 ml syringe and a 25-gauge needle was used to flush the bone marrow from the femur and tibia of rats by injecting 0.5 ml DMEM supplemented with 10% FBS, 100 IU/ml penicillin and 50 μg/ml streptomycin, into the bone marrow cavity three times. The washing fluid was collected and centrifuged at 200 x g at room temperature for 10 min, following which the supernatant was discarded. Subsequently, the pelleted cells were dispersed and centrifuged at 200 x g at room temperature for 10 min again and the supernatant was discarded.

BMSCs were subsequently cultured in low glucose DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) at 37˚C in 5% CO2. thereafter, with the medium being changed every day for the first 3 days. At 80% confluence, the cells were passaged into fresh plates by trypsinization, where third generation BMSCs were collected for follow-up experiments. The morphology of the primary BMSCs was observed under a phase contrast microscope (Magnification, x10; Nikon Corporation).

For osteogenic and adipogenic differentiation, $1x10^5$ BMSCs/ml were seeded into six-well plates in the low glucose DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.). After reaching 80% confluence, the cells were treated with osteogenic differentiation medium (DMEM supplemented with 10% FBS, 50 ng/ml ascorbic acid, 100 μmol/l dexamethasone and 10 mmol/l β-glycerophosphate (All from Sigma-Aldrich; Merck KGaA) or adipogenic differentiation medium [DMEM supplemented with 10% FBS, 0.5 mM 3-isobutyl-1-methylxanthine (Sigma-Aldrich; Merck KGaA), 50 μM indomethacin (Sigma-Aldrich; Merck KGaA), 10 μM dexamethasone Sigma-Aldrich; Merck KGaA) and 10 μg/ml insulin Sigma-Aldrich; Merck KGaA)] for 14 days at 37 ˚C, where the culture medium was changed every 3 days.

Establishment of the cell co-culture system. BMSCs were treated with either 0.2 mg/ml HFC for 7 days at 37˚C or with 0.2 mg/ml HFC for 7 days followed by 10 μM NS-398 (Tocris Bioscience), a specific cyclooxygenase 2 (COX-2) inhibitor, for 1 day at 37˚C. The cells were then plated into 24-well plates at a density of $5x10^4$/ml. Following 24 h of incubation at 37˚C, RAW264.7 macrophages were pre-stimulated with lipopolysaccharide (1 μg/ml; Sigma-Aldrich; Merck KGaA) for 30 min at 37˚C and then added to the plates containing the BMSCs at a density of $1x10^4$ cells/ml. RAW264.7 macrophages cultured alone were used as a control. The cells and supernatants were collected following incubation for 24 h at 37˚C in 5% CO_2.

MTT assay. At 24 h after co-culture initiation, cell viability was determined for all experimental groups using an MTT

Table I. Sequences of primers used for reverse transcription-quantitative PCR.

Gene	Primer sequence (5'→3')
Runt-related transcription factor 2	F: GCCGGGAATGATGAGAACTA
	R: GGACCGTCCACTGTCACTTT
Osteocalcin	F: TGCATTCTGCCTCTCTGACC
	R: ACCACCTTACTGCCCTCCTG
Alkaline phosphatase	F: AAGGCTTCTTCTTGCTGGTG
	R: GCCTTACCCTCATGATGTCC
Peroxisome proliferator-activated receptor γ	F: CCAAGTGACTCTGCTCAAGTATGG
	R: CATGAATCCTTGTCCCTCTGATATG
Lipoprotein lipase	F: TGAAGACACAGCTGAGGACA
	R: GATCACCACAAAGGTTTTGC
Adipose differentiation related protein	F: ATTCTGGACCGTGCCGATT
	R: CTGCTACTGATGCCATTTTTCCT
IL-1β	F: GGACAGAATATCAACCAACAAGTGATA
	R: GTGTGCCCGTCTTTCATTACACAG
IL-6	F: CCAGAAACCGCTATGAAGTTCCT
	R: CACCAGCATCAGTCCCAAGA
CD206	F: GTCTGAGTGTACGCAGTGGTTGG
	R: TCTGATGATGGACTTCCTGGTAGCC
Resistin-like α	F: TGCTGGGATGACTGCTACTG
	R: TGCTGGGATGACTGCTACTG
Prostaglandin E2 receptor 4	F: TCTACTTGCTCCCAGTGGACATAGATGG
	R:GAACAGACTCCTGAACTGGGTATGGTTC
GAPDH	F: AGGTGAAGGTCGGAGTCAACG
	R: CCTGGAAGATGGTGATGGGAT

IL, interleukin; F, forward; R, reverse.

assay. Briefly, 400 μl MTT solution was added to each well and incubated for 4 h at 37˚C. Following the removal of medium, 200 μl DMSO was added to each well and incubated for a further 10 min at room temperature. The optical density value of each well was measured using a plate reader at 570 nm.

Determination of nitrous oxide (NO) concentration in cell supernatants. The supernatants from each group were collected, where the concentration of NO in the supernatant was determined using Griess' method (cat. no. S0021; Beyotime Institute of Biotechnology), according to the manufacturer's protocol.

ELISAs. The concentrations of IL-1β (cat. no. SMLB00C), IL-6 (cat. no. SM6000B), TGF-β (cat. no. DY1679) and IL-10 (cat. no. SM1000B) in the co-culture medium were measured in the supernatants using corresponding ELISA kits (R&D Systems, Inc.) according to the manufacturer's protocols. To analyze the concentration of prostaglandin E2 (PGE2), the supernatants of the treated BMSCs were collected before the RAW264.7 macrophages were added, where the concentration was determined using an ELISA kit (cat. no. MBS262150; MyBioSource, Inc.), according to the manufacturer's protocol.

Reverse transcription-quantitative PCR (RT-qPCR). The cells were separated using magnetic beads as previously described (24). CD34 (10 μg; cat. no. ab187282; Abcam), CD45 (10 μg; cat. no. ab25078; Abcam) and Dynabeads™ Goat Anti-Mouse IgG beads (cat. no. 11033; Invitrogen; Thermo Fisher Scientific, Inc.) were used according to the manufacturer's protocol. In RAW264.7 macrophages, the expression levels of IL-1β, IL-6, CD206, resistin-like α (FIZZ1) and prostaglandin E2 receptor 4 (EP4) were analyzed. In BMSCs, IL-1β, IL-6, CD206, FIZZ1, runt-related transcription factor 2 (RUNX2), osteocalcin (OCN), alkaline phosphatase (ALP), lipoprotein lipase (LPL), adipose differentiation related protein (ADRP) and peroxisome proliferator-activated receptor γ (PPARγ) expression levels were assessed. Total RNA was extracted from RAW264.7 macrophages or BMSCs using RNAeasy™ Animal RNA Isolation Kit (cat. no. R0024FT; Beyotime Institute of Biotechnology) according to the manufacturer's protocol, the total RNA (10 μg) was then reverse transcribed into cDNA using the PrimeScript™ RT Reagent kit (Takara Bio, Inc.), according to the manufacturer's protocol, the reverse transcription reaction condition was as follows: 37˚C for 15 min, 85˚C for 5 sec and 4˚C for 2 min. qPCR was subsequently performed using the Power SYBR™ Green Master Mix according to manufacturer's protocol (cat. no. 4368577; Thermo Fisher Scientific, Inc.), The thermal cycling conditions consisted of an initial denaturation at 95˚C for 3 min, followed by 40 cycles of denaturation at 95˚C, 15 sec, annealing 60˚C for

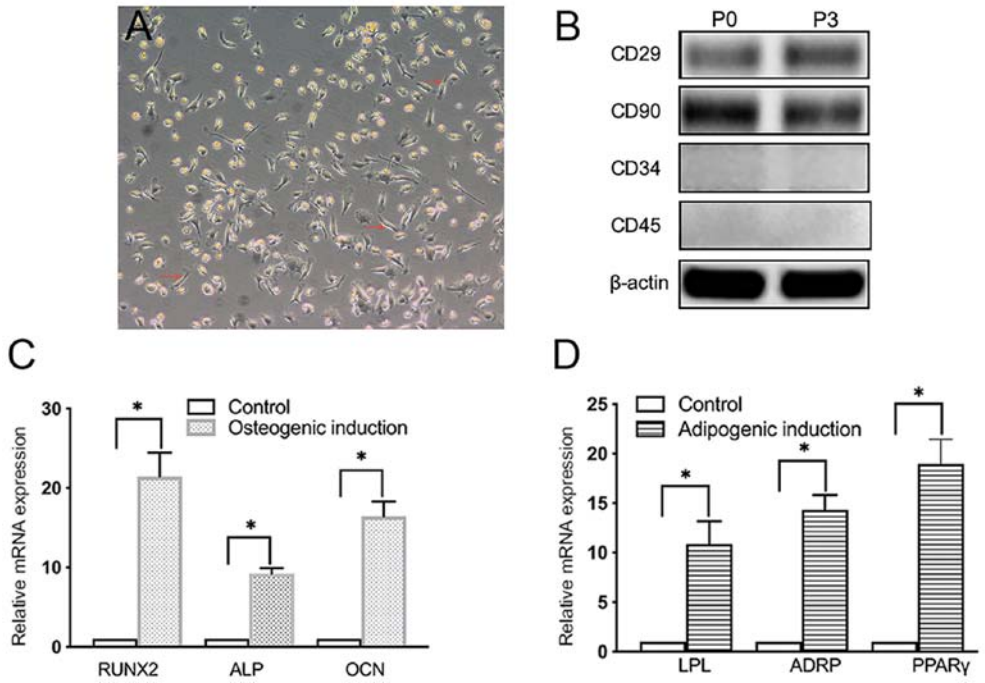

Figure 1. Presentation of data of morphology, identification, osteogenic and adipogenic differentiation of BMSCs. (A) Morphology of primary BMSCs. Magnification, x10. Red arrows indicate the location of exemplary BMSCs. (B) Western blot analysis of the expression levels of CD29, CD90, CD34 and CD45 in primary BMSCs at passages 0 and 3. (C) RT-qPCR analysis of the expression levels of osteogenic markers RUNX2, ALP and OCN in BMSCs 14 days after osteogenic induction. (D) RT-qPCR analysis of the expression levels of adipogenic markers LPL, ADRP and PPARγ in BMSCs 14 days after adipogenic induction. *P<0.05. BMSCs, bone marrow mesenchymal stem cells; RUNX2, runt-related transcription factor 2; ALP, alkaline phosphatase; OCN, osteocalcin; LPL, lipoprotein lipase; ADRP, adipose differentiation related protein; PPARγ, peroxisome proliferator-activated receptor γ; P, passage; RT-qPCR, reverse transcription-quantitative PCR.

30 sec and elongation 72˚C for 30 sec. The primer sequences used for qPCR are listed in Table I. Target gene expression was quantified using the $2^{-\Delta\Delta Cq}$ method and normalized to that of the GAPDH gene (25).

Western blotting. The cells were separated using the magnetic beads method as aforementioned and previously described (24). Total protein was extracted from RAW264.7 macrophages or BMSCs using RIPA lysis buffer (Beyotime Institute of Biotechnology) with a protease inhibitor cocktail (Roche Diagnostics). Total protein was quantified using a bicinchoninic acid assay kit (Pierce; Thermo Fisher Scientific, Inc.) and 20 μg protein/lane was separated via 12% SDS-PAGE. The separated proteins were subsequently transferred onto a polyvinylidene fluoride membrane and blocked with 5% skim milk for 2 h at room temperature. The membranes were further incubated overnight at 4˚C with the following mouse primary antibodies for the RAW264.7 protein samples: Anti-cyclic AMP-responsive element-binding protein (CREB) (1:1,000; cat. no. ab31387; Abcam), anti-phosphorylated (p)-CREB (1:500; cat. no. ab32096; Abcam), anti-CCAAT/enhancer-binding protein (C/EBP) (1:1,000; cat. no. ab40764; Abcam), anti-arginase 1 (Arg-1; 1:500; cat. no. 93668; Cell Signaling Technology, Inc.), anti-inducible nitric oxide synthase (iNOS; 1:1,000; cat. no. ab178945; Abcam), and anti-β-actin (1:1,000; cat. no. ab8227; Abcam). The membranes were incubated with the following rat primary antibodies for the BMSC protein samples at room temperature for 1 h: Anti-CD29 (1:500; cat. no. AF2405, R&D Systems, Inc.), anti-CD90 (1:1,000; cat. no. ab92574; Abcam), anti-CD34 (1:1,000 dilution; cat. no. AF4117; R&D Systems, Inc.), anti-CD45 (1:1,000; cat. no. ab10558; Abcam) and anti-β-actin (1:1,000; cat. no. ab8227; Abcam). Following the primary antibody incubation, the membranes were washed with 0.1% TBS-Tween 3 times for 10 min each prior to incubation with a horseradish peroxidase-conjugated secondary antibodies at room temperature for 1 h [(cat. no. ab6802; 1:5,000; Abcam) or (cat. no. ab6885; 1:5,000; Abcam)]. The protein bands were visualized using ECL reagents (EMD Millipore) and quantified using ImageJ software (version 1.48; National Institutes of Health).

Statistical analysis. All experiments were repeated three times and all data in this study were presented as the mean ± SD. Statistical analysis was performed using SPSS 22.0 software (IBM Corp.). Statistical comparisons were made using one-way ANOVA and a Tukey's post hoc test for multiple comparisons. P<0.05 was considered to indicate a statistically significant difference.

Results

Characterization of BMSCs. The isolated cells were confirmed to be BMSCs based on their spindle-shaped morphology and adherence properties (Fig. 1A). In addition, western blotting confirmed the expression of CD29 and CD90 and the lack of CD34 and CD45 expression of BMSCs (Fig. 1B), consistent with the previous studies that BMSCs express CD29 and CD90, but not CD34 and CD45 (26,27). The expression levels of osteogenic (RUNX2, ALP and OCN) and adipogenic (LPL,

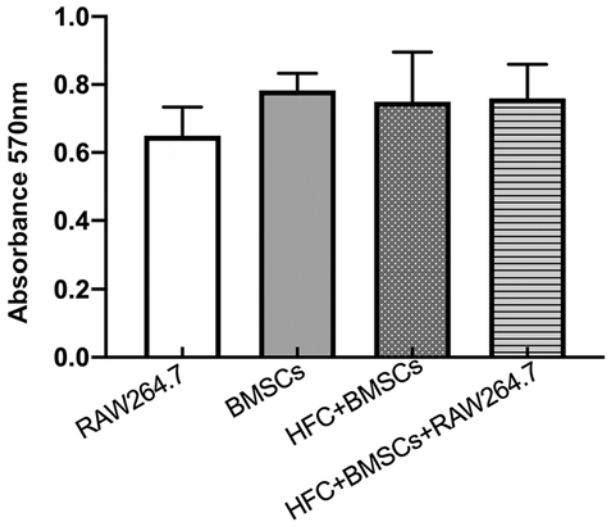

Figure 2. Measurement of cell viability in the monoculture and co-culture systems using MTT assay. Data are expressed as the mean ± SD from three independent experiments. BMSCs, bone marrow mesenchymal stem cells; HFC, hydrolyzed fish collagen.

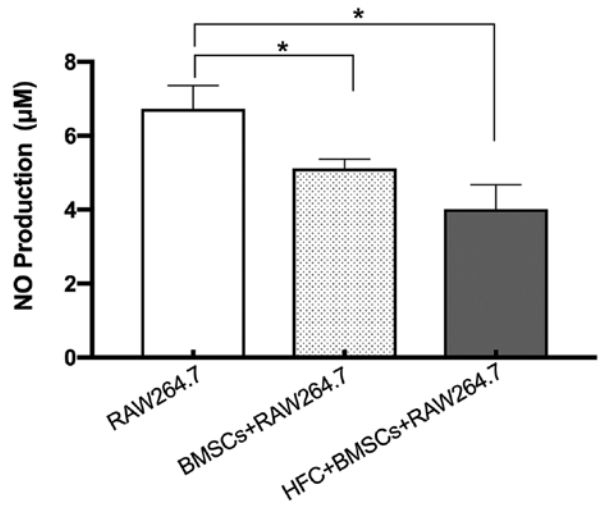

Figure 3. Measurement of NO production by the RAW264.7 macrophage monoculture, co-culture consisting of untreated BMSCs + RAW264.7 macrophages and HFC-induced BMSCs + RAW264.7 macrophages. Data are expressed as the mean ± SD. *P<0.05. NO, nitrous oxide; BMSCs, bone marrow mesenchymal stem cells; HFC, hydrolyzed fish collagen.

ADRP and PPARγ) markers were also significantly increased in osteogenic- or adipogenic-induced BMSCs in vitro compared with those in unstimulated BMSCs (Fig. 1C and D).

Cell viability. Viability in the co-culture system was found to be comparable compared with that observed for RAW264.7 macrophages or BMSCs when either were cultured alone, since no significant differences were observed in cell viability between the co-culture and the monocultures (Fig. 2), suggesting that co-culturing or HFC does not negatively influence the viability of RAW264.7 macrophages and BMSCs.

NO production. Co-culturing with HFC-induced BMSCs was revealed to significantly reduce NO production by RAW264.7 macrophages compared with that by RAW264.7 macrophages when cultured alone (Fig. 3). NO production in the co-culture system consisting of untreated BMSCs and RAW264.7 macrophages was also found to be significantly decreased compared with that by RAW264.7 macrophages alone (Fig. 3). However, no significant difference was observed between the RAW264.7 co-cultured with unstimulated BMSCs and RAW264.7 co-cultured with HFC-induced BMSCs (Fig. 3).

Cytokine secretion. The concentrations of IL-1β, IL-6, TGF-β and IL-10 in the RAW264.7 macrophage supernatants were measured using ELISAs. The secretion of IL-1β and IL-6 from the RAW264.7 macrophages co-cultured with HFC-induced BMSCs was found to be significantly decreased compared with that from the RAW264.7 group when cultured alone (Fig. 4A and B). By contrast, the concentrations of TGF-β and IL-10 in the supernatants of RAW264.7 macrophages co-cultured with HFC-induced BMSCs were significantly increased compared with those found in those of the RAW264.7 monoculture (Fig. 4C and D). Notably, the concentration of IL-10 in the supernatants of the HFC-induced BMSC + RAW264.7 group was also significantly increased compared with that in the untreated BMSC + RAW264.7 group (Fig. 4D). However, a significant difference in the concentrations of IL-1β, IL-6 or TGF-β between the untreated or HFC-induced BMSC + RAW264.7 groups was not observed.

Expression of genes associated with inflammation in RAW264.7 macrophages and BMSCs. IL-1β and IL-6 are important M1-type macrophage markers (28). RT-qPCR results demonstrated that HFC-induced BMSCs significantly reduced the expression levels of IL-1β and IL-6 mRNA in RAW264.7 macrophages compared with those in the RAW264.7 macrophage monoculture. In addition, there were no significant differences in RAW264.7 macrophages co-cultured with either HFC-induced BMSCs or untreated BMSCs (Fig. 5A and B). Conversely, HFC-induced BMSCs increased the expression levels of CD206 and FIZZ1 mRNA in RAW264.7 macrophages compared with those in the RAW264.7 macrophages alone (Fig. 5C and D), which are important M2-type macrophage markers (29). Notably, the mRNA expression levels of IL-1β and IL-6 in BMSCs alone were found to be significantly decreased compared with those in RAW264.7 macrophages alone (Fig. 5A and B). No CD206 and FIZZ1 mRNA expression could be detected in BMSCs (Fig. 5C and D).

Measurement of PGE2 secretion by BMSCs and EP4 expression in RAW264.7 macrophages. ELISA results revealed that HFC treatment significantly increased the secretion of PGE2 in BMSCs compared with that by untreated BMSCs, which was antagonized by NS-398, a specific inhibitor of COX-2, which is responsible for PGE2 production (Fig. 6A) (30). EP4 is the receptor for PGE2, an important transmembrane G protein-coupled receptor in macrophages (31). The results of the RT-qPCR analysis demonstrated that HFC-induced BMSCs significantly increased the expression levels of EP4 mRNA compared with RAW264.7 alone. The stimulatory effect of increased EP4 expression following co-culture with

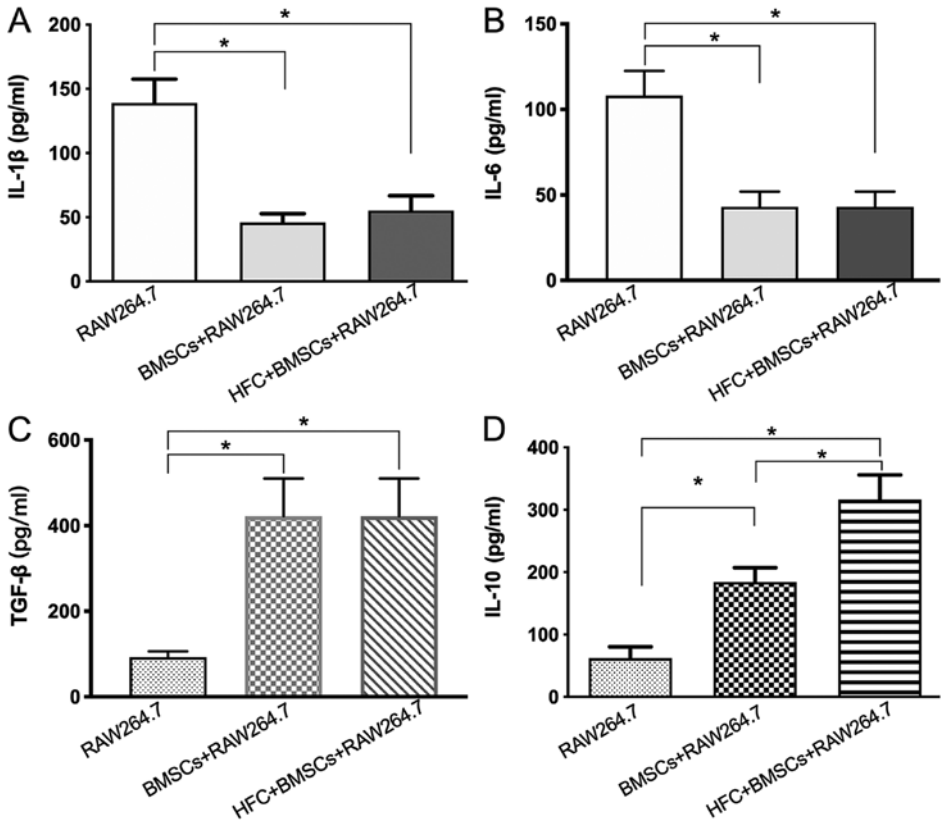

Figure 4. Measurement of immunomodulatory cytokine secretion. Concentrations of (A) IL-1β, (B) IL-6, (C) TGF-β and (D) IL-10 in the cell supernatants from the respective mono- and co-culture systems were detected using the respective ELISA kits. Data are expressed as the mean ± SD. *P<0.05. IL, interleukin; TGF-β, transforming growth factor β; BMSCs, bone marrow mesenchymal stem cells; HFC, hydrolyzed fish collagen.

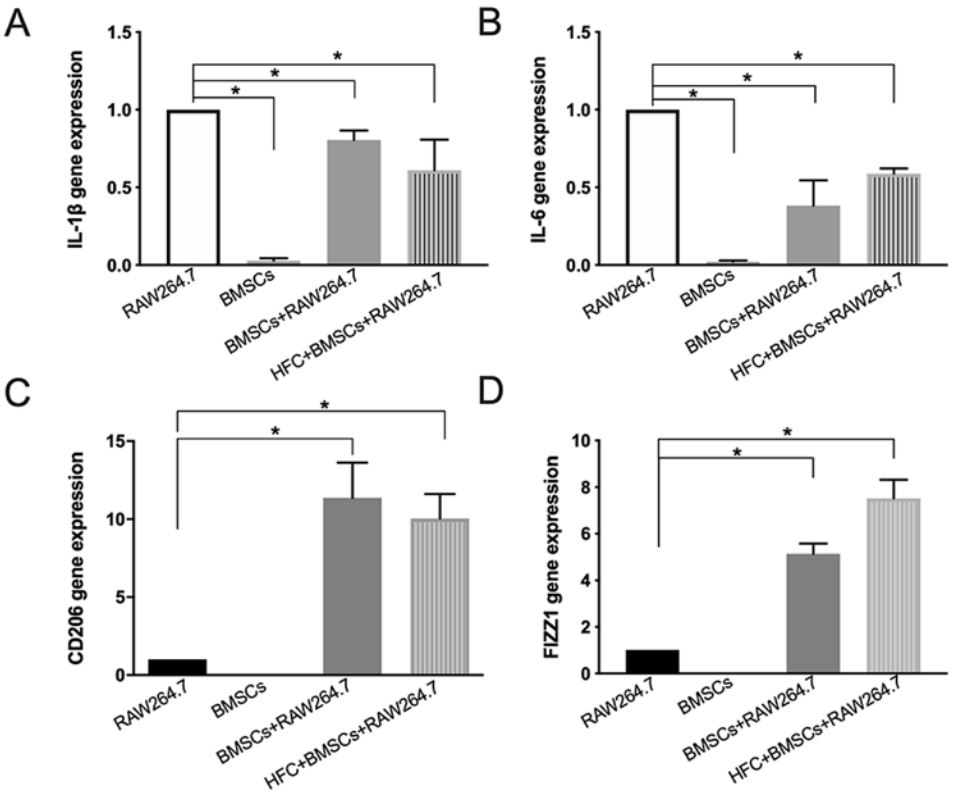

Figure 5. Expression of genes associated with inflammation in RAW264.7 macrophage and BMSC monocultures and co-culture systems. (A) IL-1β, (B) IL-6, (C) CD206 and (D) FIZZ1 in RAW264.7 macrophage and BMSCs monocultures or co-culture systems, with or without HFC induction. Data are expressed as the mean ± SD. *P<0.05. IL, interleukin; FIZZ1, resistin-like α; BMSCs, bone marrow mesenchymal stem cells; HFC, hydrolyzed fish collagen.

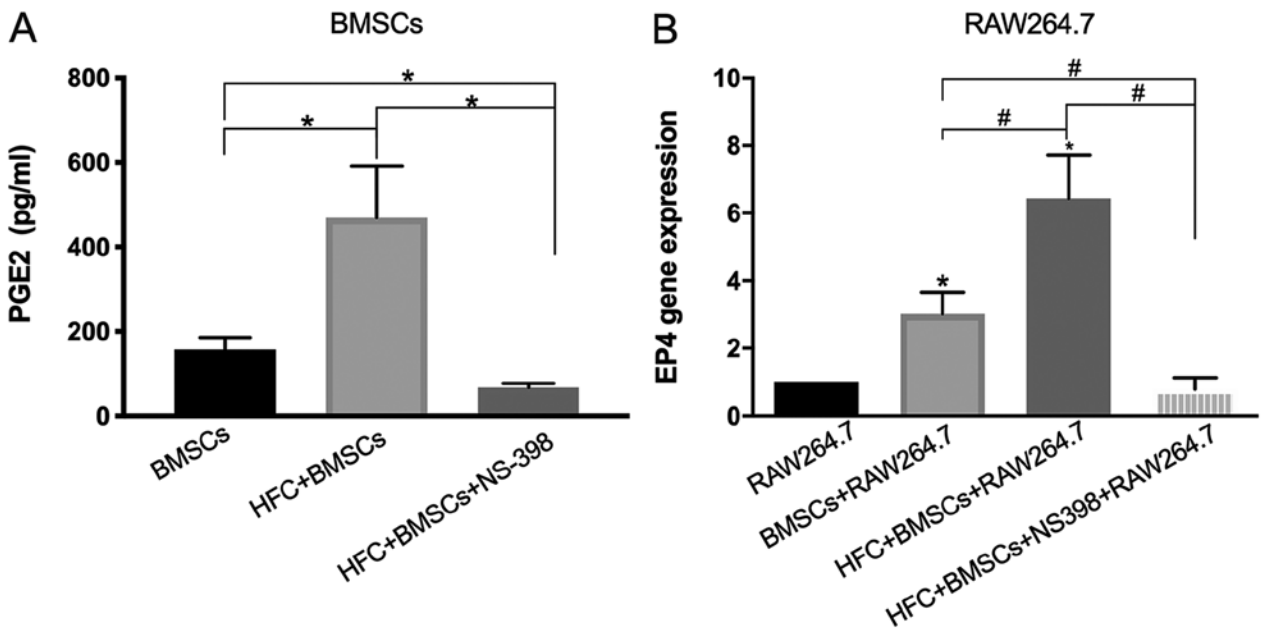

Figure 6. PGE2-EP4 signaling is involved in the regulation of effects exerted by HFC-induced BMSCs. (A) PGE2 production by BMSCs with or without HFC treatment was measured using ELISA. *P<0.05. (B) EP4 mRNA expression levels in RAW264.7 macrophages following co-culture with were analyzed using reverse transcription-quantitative PCR. *P<0.05 vs. RAW264.7. #P<0.05. Data are expressed as the mean ± SD. BMSCs, bone marrow mesenchymal stem cells; PGE2, prostaglandin E2; EP4, prostaglandin E2 receptor 4; HFC, hydrolyzed fish collagen.

HFC-induced BMSCs was significantly greater compared with those in the co-culture consisting of macrophages and untreated BMSCs (Fig. 6B). Notably, the effects induced by HFC-induced BMSCs were significantly reversed in the presence of NS-398 (Fig. 6B).

Effect of HFC-induced BMSCs on the expression levels of proteins associated with the CREB pathway in RAW264.7 macrophages. Western blotting data suggested that HFC-induced BMSCs significantly increased CREB phosphorylation in the macrophages compared with that in all other groups, especially when compared with that in the BMSCs + RAW264.7 group (Fig. 7A and B), resulting in the increased expression levels of the C/EBPβ protein (Fig. 7A and C), upregulation of the Arg-1 protein and the inhibition of iNOS expression compared with those in all other groups, especially when compared with those in the BMSCs + RAW264.7 group (Fig. 7A and D). These aforementioned effects were all found to be significantly reversed following the application of NS-398 (Fig. 7).

Discussion

In close proximity, cells communicate via paracrine signaling or cell-cell contact. Direct-contact co-culture systems cover both of these aspects; therefore, they can be considered to be more representative of the cellular microenvironment in vivo compared with monoculture systems, which can be used to study cell-cell interactions effectively *in vitro* (32). To date, studies on the immunomodulatory effects of BMSCs have primarily focused on interactions with T and B lymphocytes, natural killer and dendritic cells (33-36), but those on macrophages remain insufficient.

Since NO is considered the most sensitive and efficient indicator of inflammatory macrophages (37), it was analyzed in the present study. HFC-induced BMSCs were found to significantly inhibit the production of NO in the co-culture system, with this effect was comparable with the untreated BMSCs, suggesting that the HFC-induced BMSCs had retained their immunomodulatory functions.

Macrophages serve an important role in immunomodulation by secreting inflammatory factors, where the polarization state of the macrophages can be identified by changes to the cytokine profile (38). For example, M1 macrophages primarily secrete proinflammatory cytokines IL-1β, IL-6 and TNF-α (39), whilst M2 macrophages secrete vascular endothelial growth factor, TGF-β, endothelial growth factor and IL-10, all of which are involved in anti-inflammatory responses and tissue regeneration (16). To confirm the effect of HFC-induced BMSCs on macrophage polarization in the present study, the secretion of the relevant inflammatory factors was also analyzed. The results revealed that HFC-induced BMSCs inhibited the secretion of IL-1β and IL-6, whilst promoting the secretion of TGF-β and IL-10 by RAW246.7 macrophages. IL-1β and IL-6 are well-recognized proinflammatory cytokines and important markers of M1 macrophages. IL-1β is derived from macrophages and serve as the primary regulator of innate immune and inflammatory responses (40). By contrast, IL-6, which is predominantly secreted by T cells and macrophages, is an important member of the inflammatory network (41). As a multifunctional cytokine, IL-6 has been discovered to regulate cellular immune responses, inflammation and hematopoiesis (41). Since macrophages are one of the main sources of TGF-β, they serve a broader role in cellular proliferation, differentiation and immune functioning (42). TGF-β inhibits the proliferation of immune

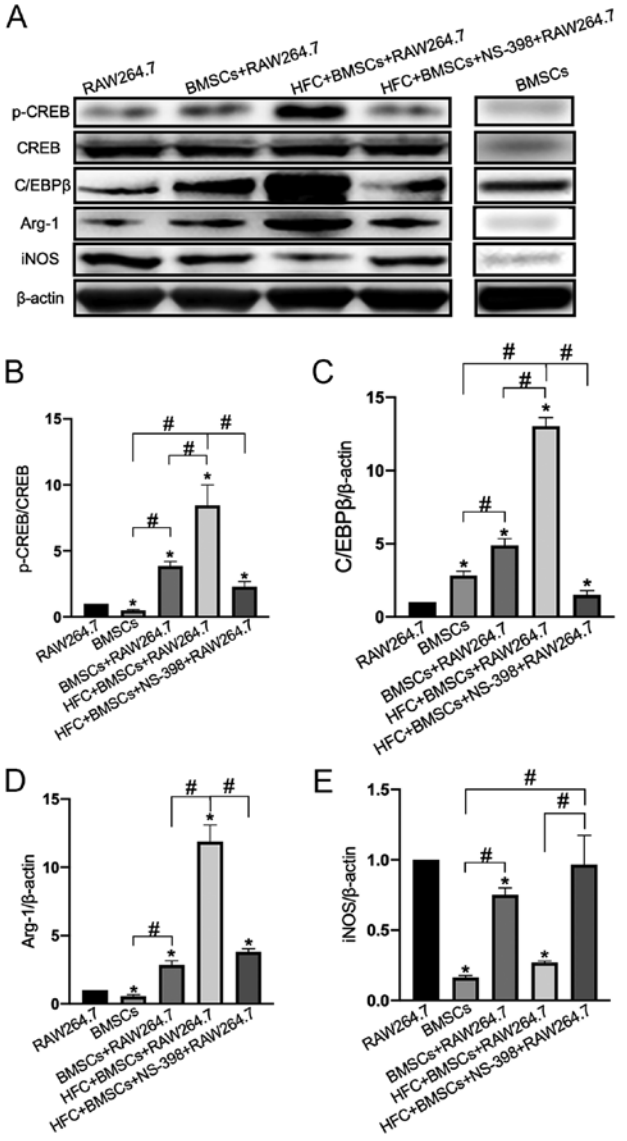

Figure 7. CREB/C/EBPβ signaling pathway mediates the immunomodulatory role of HFC-induced BMSCs. (A) Levels of CREB phosphorylation, in addition to C/EBPβ, Arg-1 and iNOS protein expression were determined using western blotting. Densitometric analysis of (B) p-CREB, (C) C/EBPβ, (D) Arg-1 and (E) iNOS expression levels. Data are presented as the mean ± SD from three independent experiments. *P<0.05 vs. RAW264.7; #P<0.05. CREB, cyclic AMP-responsive element-binding protein; p-, phosphorylated; C/EBPβ, CCAAT/enhancer-binding protein β; Arg-1, arginase 1; iNOS, inducible nitric oxide synthase; BMSCs, bone marrow mesenchymal stem cells; HFC, hydrolyzed fish collagen; NS-398, a specific cyclooxygenase 2 inhibitor.

effector cells and the generation of cytotoxic lymphocytes from CD8+ cells, in addition to directly inhibiting T-helper cell differentiation (43). IL-10 is an anti-inflammatory cytokine that can directly inhibit the activation of inflammatory cells, thereby reducing the production of inflammatory cytokines (44). The timely and moderate production of IL-10 has also been discovered to relieve inflammation and protect normal tissues from inflammatory injuries (45). In the present study, HFC-induced BMSCs were found to inhibit the secretion of IL-1β and IL-6, whilst increasing the levels of TGF-β and IL-10 secretion, suggesting that HFC treatment did not impair the immunomodulatory functions of BMSCs.

The immunomodulatory role of HFC-induced BMSCs was subsequently investigated on genetic level. HFC-induced BMSCs inhibited the expression levels of IL-1β and IL-6 mRNA, whilst increasing the expression levels of CD206 and FIZZ1 in the macrophages. Both CD206 and FIZZ1 are M2 macrophage markers (46), therefore, these results indicated that HFC-induced BMSCs may regulate macrophage polarization on transcriptional level.

A previous study demonstrated that PGE2 also regulated macrophage polarization (47). To verify whether HFC-induced BMSCs exerted their activity via PGE2, its secretion by BMSCs was investigated in the present study. HFC treatment was found to induce the production of PGE2 in BMSCs, which was reversed by the specific PGE2 inhibitor NS-398. It has been suggested that the regulatory role of PGE2 is mediated via the EP4 receptor on macrophages (48). Consistent with these findings, results of the current study revealed that the expression levels of EP4 were increased in macrophages following co-culture with HFC-induced BMSCs, which were reversed by the presence of NS-398. These results suggested that the immunomodulatory role of HFC-induced BMSCs may be mediated through PGE2.

After identifying the important immunoregulatory role of PGE2 in BMSCs, the question of how PGE2 inhibited M1-polarization whilst promoting M2 polarization was raised. To clarify the underlying mechanism of PGE2, potential pathways were studied using western blotting. The results discovered that the phosphorylation levels of CREB and the protein expression levels of C/EBPβ were significantly increased in the presence of HFC-induced BMSCs, which ultimately increased the expression of Arg-1 and inhibited the production of iNOS. By contrast, in the presence of NS-398, the expression levels of C/EBPβ, Arg-1 and CREB phosphorylation were inhibited, whereas the expression levels of iNOS were increased. These findings indicated that HFC may induce the production of PGE2 by BMSCs, which may subsequently activate the transcription of Arg-1 in macrophages by activating the p-CREB/C/EBPβ pathway, resulting in polarization towards the M2 subtype.

It is important to differentiate the two different cell types in the co-culture system, as both BMSCs and RAW264.7 macrophages produce NO and express a number of inflammation-associated genes, including IL-1β and IL-6. In the present study, a sufficient and suitable control group was used to achieve this. The BMSC + RAW264.7 co-culture group was used as a control for NO production, whereas the BMSC + RAW264.7 co-culture and BMSC monoculture were used for mRNA expression, which confirmed that HFC-induced BMSCs were influencing these effects.

It should be noted that BMSCs are the most widely used cells in bone tissue engineering applications. The focus of the present study was on bone tissue engineering, which was the reason for BMSCs being used. In fact, co-culture of RAW264.7 macrophages with other types of cells, including hematopoietic stem cells or embryonic stem cells, would serve as an excellent control for comparative purposes to the effects of BMSCs, which will be investigated in future studies.

In conclusion, the findings of the present study suggested that HFC-induced MSCs may significantly inhibit the

expression of M1-macrophage markers and promote the expression of M2 markers, indicating that HFC-induced BMSCs may exert significant immunomodulatory activities. Mechanistically, the findings indicated that HFC may promote the secretion of PGE2 from BMSCs, which may activate the p-CREB/C/EBPβ pathway through binding to the EP4 receptor on the macrophages, resulting in an increase in Arg-1 expression and a reduction in iNOS expression. The biological functions of HFC were clarified, which may facilitate further development of HFC-based biomaterials.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

CL performed all the experiments. JS interpreted and analyzed the data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Shanghai Ninth People's Hospital, affiliated with the School of Medicine, Shanghai Jiao Tong University (approval no. 31600760; Shanghai, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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