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Original Article

Low-inflammatory macrophage-BMSC crosstalk enhances osteogenesis with attenuation of oxidative stress

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Abstract: Aim: Macrophage-mesenchymal stem cell (MSC) interactions critically influence bone regeneration during periodontitis resolution. While macrophage polarization under strong inflammatory conditions has been well studied, the functional role of macrophages exposed to low-grade inflammatory stimulation and their impact on MSC osteogenesis remain poorly understood. Methods: RAW264.7 macrophages were stimulated with reduced doses of classical M1 inducers to generate a low-inflammatory transitional phenotype ($M1^{semi}$). Macrophage polarization and inflammatory cytokine expression were assessed by quantitative real-time PCR. $M1^{semi}$ macrophages were co-cultured with bone marrow mesenchymal stem cells (BMSCs) using conditioned medium, Transwell indirect co-culture, or direct cell-cell contact, with or without oxidative stress induction by hydrogen peroxide. Intracellular reactive oxygen species (ROS) levels in BMSCs were evaluated by fluorescence staining, and osteogenic differentiation was assessed by alkaline phosphatase (ALP) staining, ALP activity, and osteogenic gene expression. Results: Compared with classically activated M1 macrophages, $M1^{semi}$ macrophages exhibited reduced expression of pro-inflammatory cytokines and a polarization profile intermediate between M1 and M2 phenotypes. Among the co-culture systems tested, only direct contact with $M1^{semi}$ macrophages significantly enhanced osteogenic and angiogenic marker expression and increased ALP activity in BMSCs. This osteogenic enhancement was accompanied by a marked reduction in intracellular ROS levels. Importantly, exogenous induction of oxidative stress attenuated both ROS suppression and the osteogenic effects observed in direct co-culture. Conclusion: Macrophages exposed to low-grade inflammatory stimulation acquire a transitional phenotype with distinct immunomodulatory properties. Direct interaction with these macrophages enhances BMSC osteogenic differentiation, which is associated with reduced intracellular ROS accumulation. These findings suggest that modulation of oxidative stress contributes to macrophage-mediated osteogenesis in low-inflammatory environments, providing insight into immune-regulated bone regeneration during periodontal healing.

Keywords: Macrophages, low inflammatory environment, co-culture, BMSCs, ROS

Introduction

The management of periodontal diseases and bone regeneration remains a major challenges in dentistry and regenerative medicine. It is found that only 20% of the periodontal tissue damage can be directly attributed to bacteria, so the process of periodontitis is just like a “tragic civil war” of the autoimmune system [1, 2]. Notably, the immune cells that initially arrive at the periodontal tissue are macrophages, which remove invading bacteria and regulate the local immune response simultaneously [3, 4]. Previous studies found that macrophages, as highly plastic immune cells, can be polarized into different functional phenotypes in response to various microenvironmental stimuli [5]. Classically activated M1 macrophages are characterized by the secretion of pro-inflammatory cytokines, including tumor necrosis factor-α (TNFα), interleukin-1 (IL-1), and interleukin-6 (IL-6), which strongly exacerbate inflammatory

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responses [6, 7]. In contrast, alternatively activated M2 macrophages primarily produce anti-inflammatory cytokines and play a key role in tissue repair and angiogenesis [8, 9]. The dynamic equilibrium between M1 and M2 macrophage subsets is crucial for maintaining tissue homeostasis. It is worth noting that during the development of periodontitis, this equilibrium is often broken, which affects the repair and regeneration of bone tissue.

Bone regeneration is a highly coordinated biological cascade reaction, involving multiple cell groups, especially macrophages and bone marrow mesenchymal stem cells (BMSCs) [10, 11]. Macrophages regulate the osteogenic capacity of BMSCs through two mechanisms: direct cell-cell crosstalk and the paracrine secretion of bioactive cytokines and growth factors [12-14]. Understanding the underlying mechanisms of the interaction between macrophages and BMSCs in the damaged area of periodontal tissue is crucial for formulating effective treatment strategies to promote bone regeneration during periodontal treatment.

Although previous studies have emphasized the regulatory functions of macrophages in bone regeneration and immunomodulation, the effects of low-grade inflammatory stimuli on the phenotype of macrophage, especially the transitional M1 phenotype ($M1^{semi}$), are still lacking in characteristics [15-18]. In addition, the mechanism of macrophages regulating the osteogenic differentiation of BMSCs in a low inflammatory microenvironment is still unclear [19-21]. Therefore, the purpose of this study aims to investigate the regulatory role of $M1^{semi}$ macrophages in both inflammation and osteogenic differentiation of BMSCs, and clarify its potential mechanisms.

Material and methods

Macrophage culture and polarization induction

The RAW264.7 murine macrophage cell line was obtained from Zhongqiaoxinzhou Biotech (Shanghai, China). Cells were cultured as a clonal population in MEM-α medium (Gibco, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, Australia), and maintained at 37°C in a humidified atmosphere with 5% CO_2. The culture medium was refreshed every other day, as per the researcher's previous studies [22, 23].

Interleukin-4 (IL-4) and interferon-gamma (IFN-γ) were purchased from Peprotech (China), and Escherichia coli 055: B5 LPS was from Solarbio (China). To induce the in vitro differentiation of RAW264.7 cells into M1 or M2 macrophages, LPS (100 ng/mL)/IFNγ (20 ng/mL) or IL-4 (20 ng/mL) was added to the culture medium respectively. Non-activated macrophages were designated as M0 macrophages [23]. In order to simulate macrophages with low inflammatory stimulation in the process of tissue repair after periodontal treatment, RAW264.7 cells were treated with LPS (50 ng/mL)/IFNγ (10 ng/mL) for 12 hours, and $M1^{semi}$ was produced. After incubation at 37°C for 24 h, all cells were harvested and RNA was extracted for subsequent quantitative real-time PCR assays. Triplicate repeats were used in this assay.

Co-culture of $M1^{semi}$ macrophages with BMSCs

BMSCs of C57BL/6 mice were obtained from Cyagen Biosciences (Guangzhou, China). The 6th-8th generation of cells were used in the experimental model. To investigate the interaction between $M1^{semi}$ macrophages and BMSCs, the following four methods were used to carry out co-culture experiments.

(1) Conditioned Medium group (CM): RAW 264.7 cells were treated with LPS (50 ng/mL) and IFNγ (10 ng/mL) at a cell density of 5 × 10^5 cells/well for 12 hours. They were then washed twice with phosphate-buffered saline (PBS) and then starved for another 12 hours with serum-free MEM to collect the conditioned medium (CM). After centrifugation, osteogenic medium was added to the conditioned medium, resulting in a final concentration of 50 μg/mL vitamin C, 10 nM dexamethasone, and 10 mM β-glycerophosphate (all sourced from Sigma, USA). This conditioned medium, the current osteogenic induction medium, replaced the basic medium used for BMSCs culture. BMSCs were incubated for another 24 hours before further analysis was conducted (**Figure 1A**).

(2) Indirect Co-culture group (IC): An indirect co-culture system was established by using hanging cell culture insert of polyethylene terephthalate (PET) membranes (Millipore, USA) with a pore size of 0.4 μm. $M1^{semi}$ macrophages were inoculated into the upper compartments of the inserts, while BMSCs were plated into six-well plates (**Figure 1B**). Both cell types were

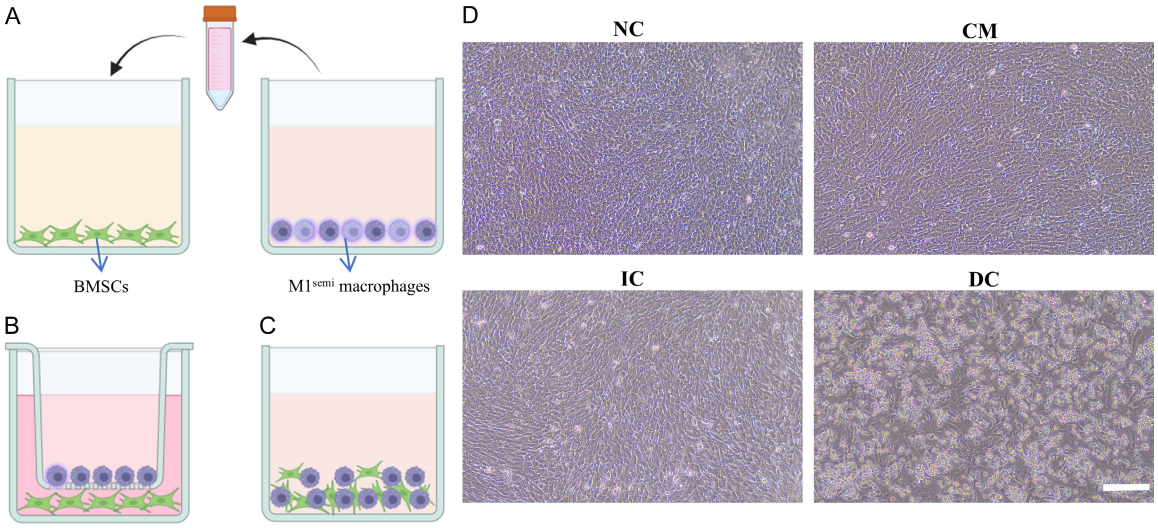

Figure 1. Schematic of co-culture systems. A-C. A schematic representation of BMSCs co-cultured with $M1^{semi}$ macrophages using different methods: conditioned medium generated by $M1^{semi}$ macrophages (CM), indirect culture via Transwell with $M1^{semi}$ macrophages (IC), and direct culture with $M1^{semi}$ macrophages (DC). D. Cell morphology after 7 days of culture (Scale Bar: 200 μm).

seeded at a density of 5×10^5 cells per well and allowed to adhere in standard culture medium for 12 h. Subsequently, the inserts were transferred into the corresponding wells to establish a physically separated co-culture system mediated by soluble factors ($M1^{semi}$ macrophage: BMSC ratio = 1:1). The culture medium was then replaced with the osteogenic medium described above. After 24 h of co-culture, BMSCs in the lower chamber were harvested for subsequent analysis.

(3) Direct Co-culture group (DC): $M1^{semi}$ macrophages and BMSCs were co-inoculated directly into the same six-well plates to achieve a physical co-culture of the two cell types, with a cell density of 5×10^5 cells per well for each ($M1^{semi}$ macrophages: BMSCs = 1:1) (**Figure 1C**). The cultures were maintained in double the standard volume of osteogenic medium. After 24 hours, the co-cultured cells were collected for analysis.

(4) Control group (NC): BMSCs were cultured alone in six-well plates at a density of 5×10^5 cells per well in osteogenic medium, without $M1^{semi}$ macrophage co-culture. These served as the negative control group (**Figure 1D**).

Osteogenic differentiation

BMSCs from various co-culture groups were seeded into 6-well plates at a density of 5 × 10^5 cells per well and cultured until they reached 70% confluence. Then, osteogenic induction medium was used to replace the basic culture medium. After 7 days of osteogenic induction, the cell culture supernatant was collected and centrifuged at 1000 rpm for 15 minutes to remove cell debris and other impurities. Alkaline phosphatase (ALP) activity was subsequently determined using an ALP activity detection kit (Nanjing Jiancheng, China) following the manufacturer's instructions. The absorbance of the red product at a wavelength of 530 nm is directly proportional to ALP activity when measured with a microplate reader (BioTek, Swindon, UK). Next, the ALP staining experiment was conducted using a BCIP/NBT alkaline phosphatase chromogenic kit (C3206, Beyotime, China). Briefly, cells were fixed with 4% paraformaldehyde for 15 minutes and then co-incubated with the prepared working solution for 0.5 to 1 hour at room temperature. The reaction was terminated by rinsing the cells three times with ddH_2O, as per the manufacturer's protocol. Triplicate repeats were performed in these assays.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from cells using the EZ-Press RNA Purification Kit (B0004D, EZBioscience, USA). The RNA concentration and purity were evaluated with a NanoDrop

Table 1. Primers for qRT-PCR

Gene symbol	Sequence 5'-3'	Bp
M-spp1 (OCN)	F: AGAGCGGTGAGTCTAAGGAGT	21
	R: TGCCCTTTCCGTTGTTGTCC	20
M-OSM	F: CCCGGCACAATATCCTCGG	19
	R: TCTGGTGTTGTAGTGGACCGT	21
M-VEGF	F: GCACATAGAGAGAATGAGCTTCC	23
	R: CTCCGCTCTGAACAAGGCT	19
M-Wnt6	F: TAGCACACCTTCTGTTCTTT	20
	R: GGGTGTGTTGTTTGGGGTGT	20
M-TNFα	F: CGACGTGGAACTGGCAGAAG	20
	R: GCCACAAGCAGGAATGAGAAGAG	23
M-IL6	F: CTGCAAGAGACTTCCATCCAG	21
	R: CAGGTCTGTTGGGAGTGGTATC	22
M-IL-1β	F: CAGCAGCACATCAACAAGAGC	21
	R: CCACGGGAAAGACACAGGTAG	21
M-iNOS	F: TGCCACGGACGAGACGGATAG	21
	R: CTCTTCAAGCACCTCCAGGAACG	23
M-Arg1	F: CATATCTGCCAAAGACATCGTG	22
	R: GACATCAAAGCTCAGGTGAATC	22
M-GAPDH	F: AGGTCGGTGTGAACGGATTTG	21
	R: TGTAGACCATGTAGTTGAGGTCA	23

spectrophotometer (Thermo Fisher, USA). Subsequently, 1 μg of total RNA was reverse transcribed using the Color Reverse Transcription Kit (A0010GQ, EZBioscience, USA) in an Applied Biosystems Veriti™ Thermal Cycler (Thermo Fisher, USA). Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed with the SYBR-Green PCR Kit (EZBioscience, USA), following the manufacturer's protocol, on a CFX96 Touch Deep Well™ Real-Time PCR Detection System (Bio-Rad, USA). Three independent experiments were conducted, and the ΔΔCt method was used for data analysis. The primer sequences for *iNOS, Arg1, TNFα, Il-6*, *Il-1β, OCN, OSM, VEGF, Wnt6*, and *Gapdh* are listed in **Table 1**. Each sample was analyzed in triplicate to ensure statistical reliability.

Detection of intracellular reactive oxygen species (ROS)

Intracellular ROS levels were determined using an ROS Assay Kit (Genxion, China) according to the manufacturer's instructions. BMSCs of different groups were seeded in 6-well plates at a density of 5×10^5 cells per well. After routine culture for 24 h, the basal medium was replaced with serum-free osteogenic induction medium. After 24 h of dark culture, the induced solution was removed, and 2',7'-dichlorodihydrofluorescin diacetate (DCFH2-DA, Genxion) with a final concentration of 10 μM was added. The control group was added with 100 mM Rosup (NC). The cells cultured in basic culture medium without osteogenic-inducing medium were used as the untreated group (UT), and 0.5 mM hydrogen peroxide was added to the culture medium as DC+H_2O_2 group. After incubation in the dark for 30 minutes, add serum-free cell culture medium and wash it for three times to ensure that DCFH-DA entering cells is fully removed. FITC filter was used to observe the expression of intracellular fluorescence signal under fluorescence microscope (Zeiss, Germany). Quantification of fluorescence staining was performed by measuring the area covered by the green fluorescence intensity using ImageJ software (version 1.43u) and presented as a percent (%) of the total area. Triplicate individual views of each group were chosen and analyzed in this assay.

Statistical analysis

All data were derived from at least three independent experiments. The results are presented as means ± standard deviation. Statistical analyses were conducted using an independent samples t-test for comparisons between two groups or a one-way ANOVA with Tukey's post hoc test for comparisons among multiple groups. Multiple comparisons were performed using two-way ANOVA, followed by the Holm-Sidak post hoc test. A *P*-value of less than 0.05 was considered statistically significant. The data were analyzed, and graphs were generated using GraphPad Prism 9.3.1.

Results

Macrophages with a low level of inflammatory stimulation tended to alleviate inflammation

During the process of inflammation, macrophages undergo a phenotypic transformation

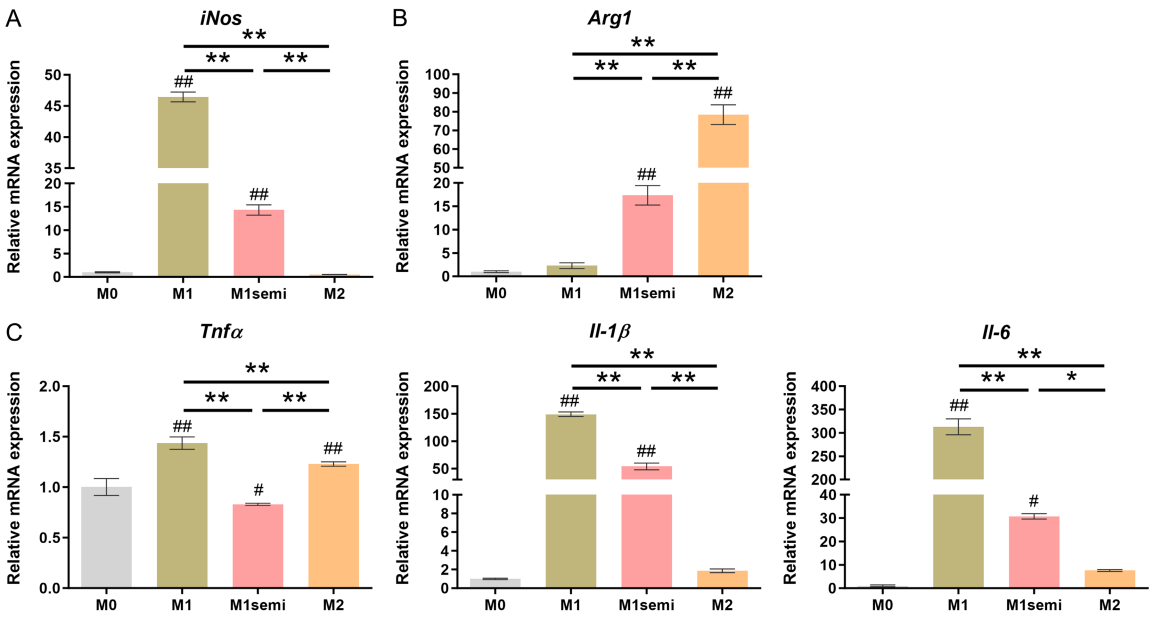

Figure 2. M1^{semi} regulates macrophage phenotype and reduces inflammation. A, B. Relative mRNA expression of M1 marker iNOS and M2 marker Arg1. In M1^{semi} macrophages, the M2 macrophage marker Arg1 increased, while the M1 macrophage marker iNOS decreased, indicating that M1^{semi} macrophages exhibit intermediate polarization. C. qRT-PCR results showing decreased expression of inflammatory cytokines *TNFα, Il-1β*, and *Il-6* under low-level inflammatory stimulation (The data is presented as mean ± SD. $^{\#}P < 0.05$ vs M0, $^{\#\#}P < 0.01$ vs M0. $^{*}P < 0.05$, $^{**}P < 0.01$).

from the M1 to the M2 state, which was characterized by a gradual loss of the M1 phenotype. Our previous study found that the transformation of M1-type macrophages into M2-type macrophages is beneficial for periodontal tissue repair [22]. We used a lower inflammatory dose than that usually used for M1 to stimulate semi-stimulated macrophages in order to compare the effects of transitional M1 and typical M1 on inflammation [16]. The characteristics of M0, M1, M1^{semi}, and M2 were then compared. Compared to M0 macrophages, classical M1 macrophages displayed strong expression of iNOS (a canonical M1 marker), while the expression of Arg1 (a signature M2 marker) was little. Conversely, M2 macrophages exhibited high expression of Arg1 and low iNOS levels. Compared with M2 cells, M1^{semi} macrophages showed a slight increase in the mRNA expression of iNOS, while the expression of Arg1 decreased slightly. It is noteworthy that, compared with M1 cells, M1^{semi} macrophages exhibited a significant decrease in the mRNA expression of iNOS, while the expression of Arg1 was significantly increased ($P < 0.01$) (**Figure 2A**, **2B**). This indicates that M1^{semi} macrophages occupy an intermediate phenotypic state, with the expression levels of iNOS and Arg1 acting as a bridge between those of M1 and M2 populations. Further investigation into inflammatory factor expression under low-intensity inflammatory stimulation revealed that qRT-PCR analysis consistently demonstrated significantly downregulated expression of pro-inflammatory cytokines (*TNFα, Il-1β*, and *Il-6*) in M1^{semi} macrophages ($P < 0.01$) (**Figure 2C**). The results indicate that in a low-inflammatory microenvironment, macrophages progressively transition from an M1 to an M2 phenotype, accompanied by a reduction in the production of inflammatory mediators. The identification of this transitional M1^{semi} phenotype highlights its potential therapeutic relevance for managing periodontal diseases by modulating macrophage polarization towards a less pro-inflammatory state.

Low-inflammatory macrophages enhanced the early-stage osteogenic differentiation of rBMSCs

In the context of periodontitis treatment, more M1^{semi} macrophage infiltrating around alveolar bone, which participate in bone tissue metabolism through immunomodulation. To further clarify the interaction between macrophages

and BMSCs under low-inflammatory stimulation, we employed both indirect and direct co-culture methods to evaluate the effects of transitional M1 macrophages on osteogenesis. For indirect co-culture, we used two approaches: CM and IC. The results showed that compared with the control group, as well as the CM and IC groups, the DC group had significantly higher expression levels of osteogenic and angiogenic factors such as *OCN, Wnt6, OSM,* and *VEGF* ($P < 0.01$) (**Figure 3A**). It is puzzling that the mRNA expression of *Wnt6, OSM*, and *VEGF* in the CM or IC groups was significantly lower than that in the control group ($P < 0.01$) (**Figure 3A**), whereas the expression of *OCN* in the CM group was slightly lower than that in the control group, with no statistical difference ($P > 0.05$) (**Figure 3A** *OCN*). This differential expression pattern suggests that the semi-stimulated state of M1 could serve as a functional bridge between anti-inflammatory responses and comprehensive tissue repair mechanisms.

Subsequently, to explore the osteogenic potential of various co-culture methods involving semi-stimulated macrophages and BMSCs, we conducted ALP staining and ALP activity assays. Throughout the induction culture, all groups exhibited osteogenic differentiation potential in the early stage. The ALP staining results indicated that the number of ALP-positive cells in the DC group was higher than in the control, IC, and CM groups (**Figure 3B**). Consistent with this, the ALP activity in the DC group was significantly higher than that in the control group ($P < 0.01$) (**Figure 3C**). Although the ALP activity in the IC and CM groups increased slightly compared with the control group, the increase was more obvious in the DC group ($P < 0.01$) ($P < 0.05$) (**Figure 3C**). The findings suggest that the direct co-culture method is more conducive to the expression of osteogenic and angiogenic factors, and is accompanied by a significant increase in ALP activity. This implies that direct contact between semi-stimulated macrophages and BMSCs may promote osteogenic and angiogenic differentiation in the early stage.

The co-culture of low-inflammatory macrophages with BMSCs may promote osteogenesis with attenuation of reactive oxygen species (ROS)

To make a preliminary exploration of the mechanism by which macrophages stimulate osteogenic differentiation under low-inflammatory conditions, we initially assessed the intracellular ROS levels in BMSCs after a 24-hour co-culture with these macrophages. This is a semi-quantitative analysis. Compared to the basal medium (UT group), the intracellular ROS level induced by osteogenic induction medium (NC group) was markedly elevated ($P < 0.01$) (**Figure 4A**). Notably, co-culturing with macrophages significantly suppressed the increase in ROS levels during osteogenic induction, with the DC group exhibiting the most pronounced regulation of ROS levels ($P < 0.01$) ($P < 0.05$) (**Figure 4B**). Although cell-cell direct contact induces the least ROS production, DC co-culture therapy can effectively promote osteogenic differentiation of BMSCs. Therefore, the DC co-culture method was employed to the next experiment.

Next step, we introduced 0.5 mM hydrogen peroxide (H_2O_2) into the osteoblast differentiation medium, establishing a co-culture system (DC+H_2O_2 group). This system included macrophages under low-inflammatory stimulation and BMSCs in an oxidative stress induction environment. The co-cultures were incubated for 24 hours to quantify ROS levels, and for 7 days to evaluate osteogenic differentiation by molecular analyses. qRT-PCR analysis showed that compared with the control group, the mRNA expression of bone angiogenesis-related factors such as *OCN, OSM, Wnt6*, and *VEGF* in DC group was significantly up-regulated ($P < 0.01$) (**Figure 5A**). Notably, compared with the control group, the expressions of *OSM* and *Wnt6* increased slightly in the DC+H_2O_2 group, while the expression of *OCN* and *VEGF* was opposite ($P > 0.05$) (**Figure 5A** *VEGF*). The results showed that the promoting effect of DC group on the expression of osteogenesis and angiogenesis factors was significantly weakened. In line with this, the regulation of DC co-culture on ROS was reversed after adding hydrogen peroxide ($P < 0.05$) (**Figure 5B**, **5C**). The results suggest that direct interaction with these macrophages enhances the osteogenic differentiation of BMSCs, which is accompanied by the decrease of ROS. In this process, direct cell-cell contact appears to play a more effective regulatory role.

Discussion

In this report, we demonstrated that macrophages gradually lose their M1 phenotype

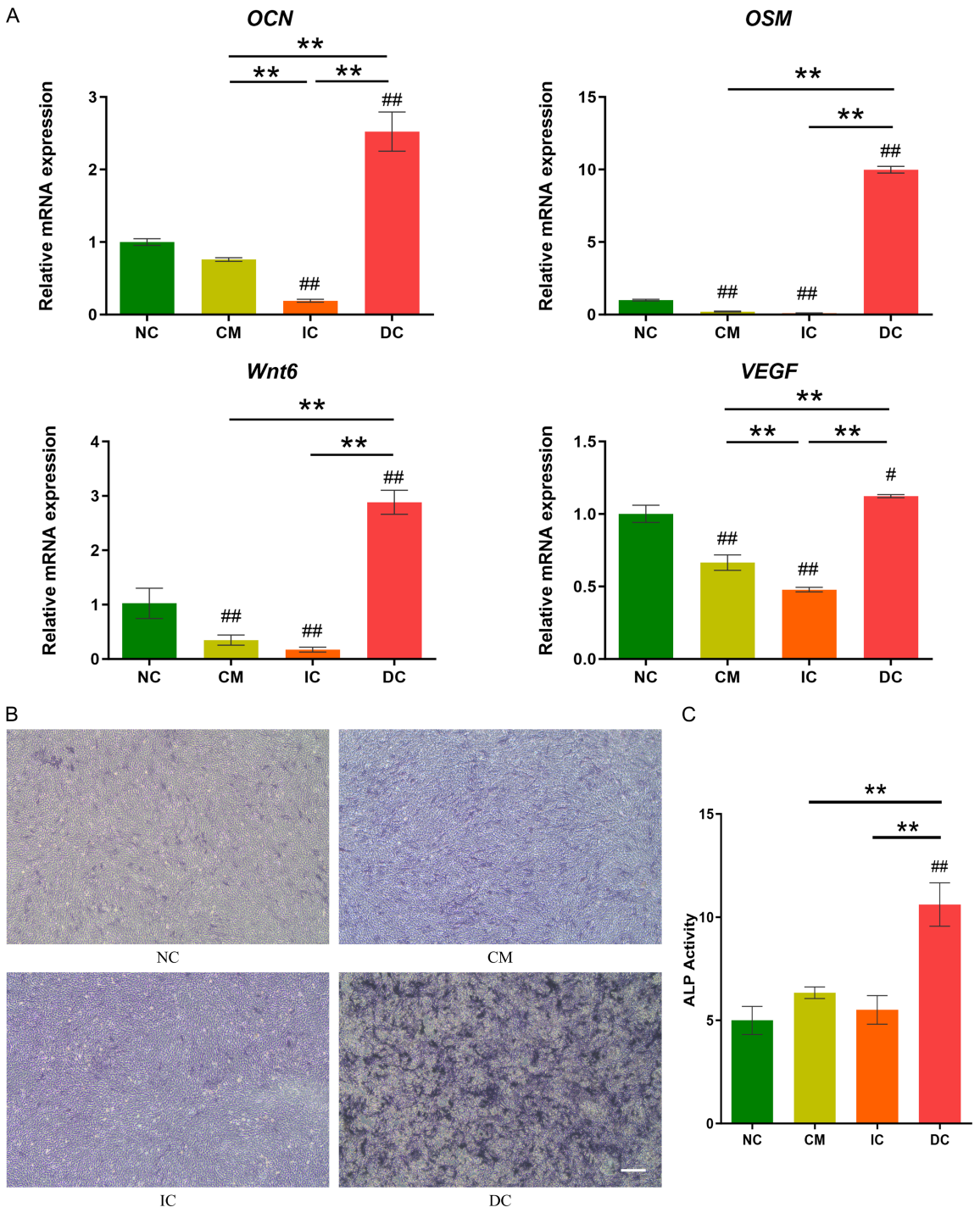

Figure 3. Low-inflammatory macrophages promote early-stage osteogenic differentiation of BMSCs. A. Co-culture systems: indirect co-culture by Transwell (IC) and conditioned medium (CM), and direct co-culture (DC). Relative mRNA expression of osteogenic/angiogenic markers, *OCN, OSM, Wnt6*, and *VEGF*, significantly elevated in DC group. B. ALP staining result of BMSCs applied with CM from $M1^{semi}$, transwell indirect co-culture with $M1^{semi}$, and direct co-culture with $M1^{semi}$ macrophages during osteogenic differentiation for 7 days. C. Quantitative analysis of ALP activity (The data is presented as mean ± SD. $^{\#}P < 0.05$ vs NC, $^{\#\#}P < 0.01$ vs NC. $^{*}P < 0.05$, $^{**}P < 0.01$. Scale Bar: 200 μm).

when transitioning from M1-type to M2-type states in low inflammatory microenvironment. Macrophages in this microenvironment have stronger ability to regulate the expression of inflammatory factors. Notably, direct interaction with $M1^{semi}$ macrophages enhances BMSC

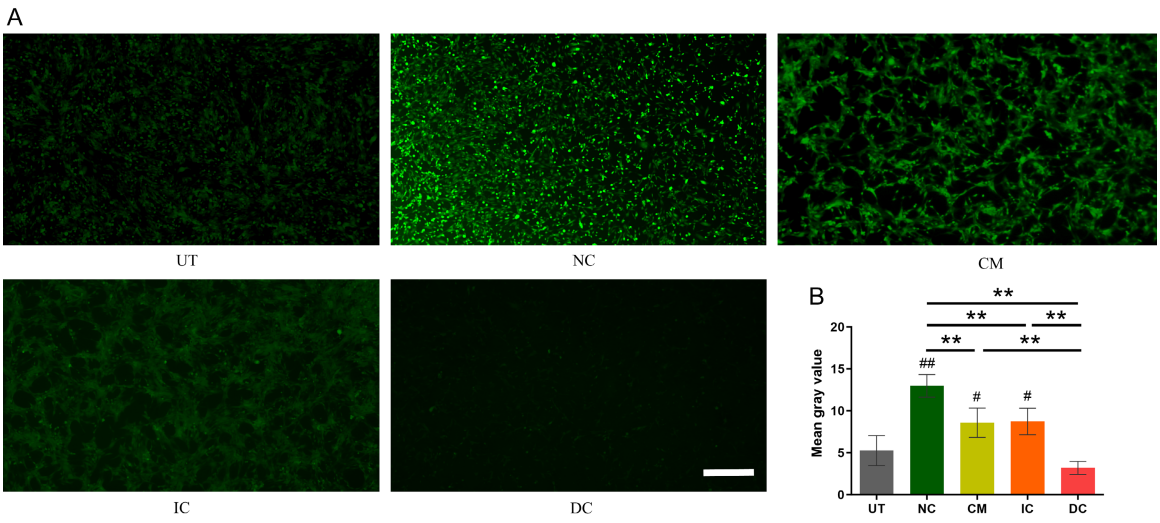

Figure 4. ROS production during osteogenic induction co-culture of M1^{semi} macrophages with BMSCs. A. The fluorescence of intracellular ROS is detected by a Reactive oxygen species assay kit. BMSCs cultured in basic culture medium were used as the untreated group (UT). B. Quantitative analysis of ROS (The data is presented as mean ± SD. $^{\#}P < 0.05$ vs UT, $^{\#\#}P < 0.01$ vs UT. $^{*}P < 0.05$, $^{**}P < 0.01$. Scale Bar: 200 μm).

early-stage osteogenic differentiation, which is accompanied by the decrease of ROS.

The emergence of the macrophage subset, particularly those resembling the M1-like macrophages, is a pivotal aspect of the diverse array of macrophage phenotypes. Previous studies have observed the transition from the early M1 phenotype to the late M2 phenotype, and these macrophages in low-inflammatory state have played an active role in promoting osteogenesis [13, 24, 25]. In this study, we simulated the immune microenvironment during the process of tissue repair after periodontal treatment. By exposing macrophages to inflammatory stimuli, the intensity was lower than that of inducing conventional M1 macrophages, thus replicating the transitional cells expressing M1 and M2 markers. Similar to previous studies, our findings indicate that compared to M1 macrophages, the expression of iNOS in macrophages significantly decreases, while the expression of Arg1 is increased. The activation of M1 macrophages and the secretion of inflammatory factors accelerate the progression of inflammation and tissue destruction. However, the effect of macrophages with low inflammatory stimuli on the expression of inflammatory factors remains unclear. In the present study, we demonstrated that the expression of pro-inflammatory markers, such as *Il-1β, TNF-α*, and *Il-6,* in M1^{semi} macrophages is significantly reduced. The down-regulation of this inflammatory factor has biologically significant, indicating that M1^{semi} may possess a unique form of immune regulation. Macrophages regulate the inflammatory environment and affect the osteogenic potential of mesenchymal stem cells. The impact on the osteogenic differentiation of MSCs, whether it is positive or negative, is largely dependent on the dose of inflammatory factors. Studies have indicated that low concentrations of IL-1β and TNF-α can promote the osteogenic differentiation of MSCs in vitro. Conversely, high concentrations of IL-1β and TNF-α will inhibit the positive regulatory factors involved in the process of osteogenesis. Rojasawasthien et al. found that excessive IL-1β disrupts mesenchymal stem cell (MSC) differentiation into osteoblasts by interfering with core osteogenic signaling pathways [26]. In this study, it is interesting to find that the expression level of TNF-a in M1^{semi} is even lower than that in M2 macrophages. We all know that TNFα is generally regarded as a negative factor of MSCs' osteogenic differentiation. Qin et al. found that overexpression of TNF-α activated Wnt/β-catenin signaling pathway, and inhibited the mineralization of MSCs [27]. It was also discovered that TNF-α inhibits osteogenic differentiation through NF-κB signaling pathway or upregulats Smurf1 and Smurf2 [28, 29]. Additionally, TNF-α can activate p38 and inhibit the expression of c-fos and bone matrix deposition in rat BMSCs [29]. Therefore, TNF-α related inflammatory signals

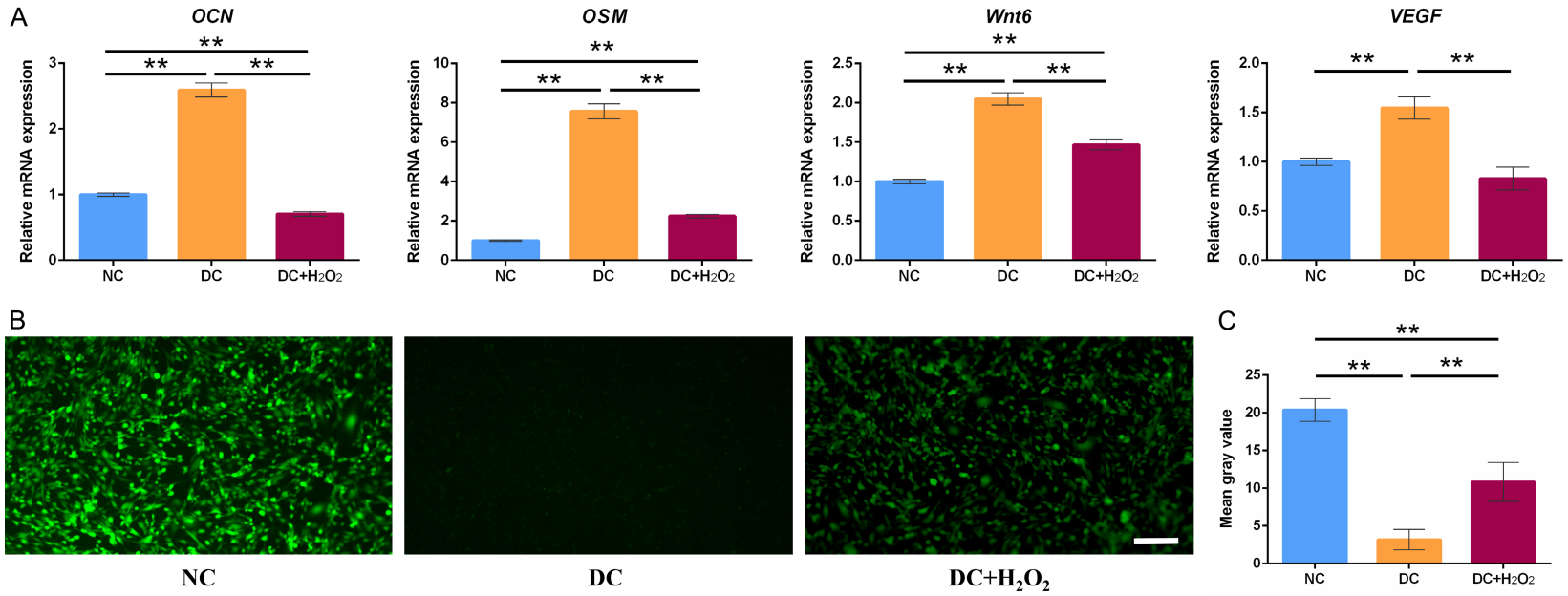

Figure 5. Co-culture of M1^{semi} macrophages with BMSCs promotes osteogenesis by reducing intracellular ROS levels. A. Relative mRNA expression of osteogenesis-related genes (*OCN, OSM, Wnt6*, and *VEGF*) in Rosup-induced direct co-culture, detected by qRT-PCR. Rosup is an active oxygen species-inducing agent. B. Intracellular ROS fluorescence staining images of BMSCs cultured under osteogenic induction culture, direct co-culture with M1^{semi}, and direct co-culture with M1^{semi} plus Rosup application. C. Quantitative analysis of ROS (The data is presented as mean ± SD. *$P < 0.05$, **$P < 0.01$. Scale Bar: 200 μm).

play an important role in the osteogenic differentiation of mesenchymal progenitor cells, and macrophages with low inflammatory stimulation may have a unique regulatory effect on TNF-α expression. The purpose of this study is to explore the effect of low-level inflammatory stimulation on macrophage polarization and clarify the role of regulating macrophage-related immune response in the process of tissue repair after periodontal treatment.

Because the periodontal tissue is in a low-inflammatory microenvironment for a long time after periodontitis treatment, it is necessary to pay special attention to the interaction between macrophages and BMSCs in a low-inflammatory state [30]. The co-culture methods of integrating macrophages and MSCs, including IC, DC, and CM, have obvious effects on osteogenesis. Luo et al. reported that all three co-culture approaches can promote the osteogenic differentiation of BMSCs, with their efficacies ranked as DC > IC > CM [31]. Nicolaidou et al. showed that oncostatin M (OSM) can be directly transferred from macrophages to MSCs, activating the STAT3 signaling pathway in MSCs, which is crucial for osteogenic differentiation [32]. That is to say, direct contact may be beneficial to regulate the exchange of ions and small molecules in MSCs, thus promoting osteogenesis. In order to study the influence of macrophages with low inflammatory stimulation on BMSCs, we chose direct co-culture and indirect co-culture methods. The results showed that the DC method of direct physical contact between macrophages and BMSCs showed the most significant enhancement of the expression of osteoblasts and antigen factor and ALP activity. This finding is consistent with previous research. The direct cell-to-cell contact in the DC method may facilitate the transfer of key signaling molecules. IC method uses Transwell system to physically separate two cell types, and at the same time allows the exchange of soluble factors, which really enhances the osteogenic activities to some extent. However, our results indicated that the mRNA expression levels of osteogenic and angiogenic factors in the IC group were lower than those in the DC group, and even lower than those in the control group. This discrepancy can primarily be attributed to the physical separation feature of the IC method, which limits the overall interaction between the two cell types. Although soluble factors can pass through the membrane, the absence of direct cell-to-cell contact may impede the transmission of some critical signals crucial for optimal osteogenic differentiation. For instance, some membrane-bound proteins and extracellular matrix components are crucial for intercellular communication and osteogenesis induction, and may not be transferred efficiently by IC system [33]. The CM method, which entails culturing MSCs in macrophage-conditioned medium, has been shown to enhance osteogenesis, albeit to a lesser extent than DC. CM derived from macrophage is rich in various cytokines and growth factors, including bone morphogenetic protein-2 (BMP-2), which binds to specific receptors on MSCs, activates Smad signaling pathway, and promote osteogenic differentiation [34]. However, there are critical differences between CM and direct co-culture systems in factor concentration, composition and cell interaction kinetics. The CM method lacks direct cell-cell contact, which may limit synergistic effects that arise from the physical proximity between macrophages and MSCs, such as the amplification of paracrine signaling or interactions mediated by the extracellular matrix. These mechanistic differences may explain in the observed disparity in osteogenic potency between the two culture systems.

The interaction between ROS and peroxidation plays a multifaceted and pivotal role in the osteogenic process following the interaction between MSCs. The increase of ROS level is harmful to bone formation. Excessive ROS induces oxidative stress, potentially damaging cell components in MSCs, such as DNA, proteins and lipids. This kind of damage can destroy the normal cell functions, including the process of osteogenic differentiation. For instance, ROS can inhibit the activity of key osteogenic transcription factors, such as Runx2. Runx2 is the main regulator of osteoblast differentiation, and its activity is crucial for the expression of bone formation related genes, including OCN and ALP. High levels of ROS can oxidize and inactivate Runx2, thus impairing osteogenic differentiation [35]. Nguyen et al. found that macrophages can activate the Nrf2-ARE pathway in MSCs to regulate ROS production, which is the key pathway of antioxidant gene expression [36]. Canton et al. reported that macrophages can release enzymes such

as superoxide dismutase (SOD), catalase, or glutathione peroxidase, which are instrumental in breaking down ROS and mitigating oxidative stress [37]. In most studies, macrophages can enhance the osteogenic potential by downregulating ROS levels. However, it remains to be clarified whether macrophages can promote the osteogenesis of stem cells in a low inflammatory environment with the decrease of ROS expression level. Our experimental results suggest that MSCs tend to display elevated levels of ROS during osteogenic differentiation. Furthermore, when co-cultured with macrophages, especially in the presence of $M1^{semi}$ macrophages, the expression of osteogenic and angiogenic factors is up-regulated and the expression of ROS is down-regulated. Subsequently, after treating BMSCs with 0.5 mM hydrogen peroxide, the inhibition of ROS accumulation in the DC group was significantly diminished, and the promotion of osteogenesis and angiogenesis factor expression was also significantly reduced. These findings emphasize the crucial importance of maintaining an optimal level of ROS in a low-inflammatory microenvironment to enhance the osteogenesis of MSCs mediated by macrophages.

However, our in vitro cell experiments have primarily focused on investigating the effects of a low inflammatory microenvironment on the polarization and expression of inflammatory factors by macrophages. We have also sought to explore the impact and molecular mechanisms of macrophages experiencing low inflammatory stimulation on the osteogenic differentiation of BMSCs through co-culture experiments. Moving forward, future studies should analyze the antioxidant pathway (Nrf2, SOD, catalase) and the expression of genes related to oxidative stress. Secondly, to address the concern that the 24-hour evaluation period in this study might not fully capture the complete dynamics of $M1^{semi}$ macrophage-BMSC interactions, we will design additional experiments with extended time-course studies to fully elucidate the influence of ROS pathway activation on the interaction between macrophages and BMSCs under low inflammatory stimulation.

Conclusion

In conclusion, we simulated the low-inflammatory microenvironment in the process of tissue repair after periodontal treatment, and found that macrophages within this microenvironment have unique inflammatory regulation. Furthermore, the co-culture experiments showed that the interaction between cells significantly promoted the osteogenic differentiation of BMSCs only under direct contact, which was accompanied by the decrease of ROS in cells. These findings not only deepen our understanding of the macrophage-related immune response mechanism in a low-inflammatory microenvironment but also provide new insights into understanding the tissue repair process following periodontitis treatment.

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Disclosure of conflict of interest

None.

Abbreviations

BMSCs, marrow mesenchymal stem cells; qRT-PCR, quantitative real-time PCR; ROS, reactive oxygen species; ALP, alkaline phosphatase; iNOS, inducible nitric oxide synthase; Arg1, Arginase 1; TNF-α, tumor necrosis factor-α; IL-1, interleukin-1; IL-6, interleukin-6; FBS, fetal bovine serum; IL-4, Interleukin-4; IFN-γ, interferon-gamma; CM, Conditioned medium Group; IC, Indirect Co-culture Group; PET, polyethylene terephthalate; DC, Direct Co-culture Group; Con, Control Group; ALP, alkaline phosphatase; ROS, oxygen species; DCFH2-DA, 2',7'-dichlorodihydrofluorescin diacetate; UT, untreated group; H_2O_2, hydrogen peroxide; OSM, oncostatin M; BMP-2, bone morphogenetic protein-2; SOD, superoxide dismutase; MSC, macrophage-mesenchymal stem cell.

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