ORIGINAL ARTICLE

Basic Study

Effects of interleukin-10 treated macrophages on bone marrow mesenchymal stem cells *via* signal transducer and activator of transcription 3 pathway

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Abstract

BACKGROUND
Alveolar bone defects caused by inflammation are an urgent issue in oral implant surgery that must be solved. Regulating the various phenotypes of macrophages to enhance the inflammatory environment can significantly affect the progression of diseases and tissue engineering repair process.

AIM
To assess the influence of interleukin-10 (IL-10) on the osteogenic differentiation of bone marrow mesenchymal stem cells (BMSCs) following their interaction with macrophages in an inflammatory environment.

METHODS
IL-10 modulates the differentiation of peritoneal macrophages in Wistar rats in an

inflammatory environment. In this study, we investigated its impact on the proliferation, migration, and osteogenesis of BMSCs. The expression levels of signal transducer and activator of transcription 3 (STAT3) and its activated form, phos-phorylated-STAT3, were examined in IL-10-stimulated macrophages. Subsequently, a specific STAT3 signaling inhibitor was used to impede STAT3 signal activation to further investigate the role of STAT3 signaling.

RESULTS

IL-10-stimulated macrophages underwent polarization to the M2 type through substitution, and these M2 macrophages actively facilitated the osteogenic differentiation of BMSCs. Mechanistically, STAT3 signaling plays a crucial role in the process by which IL-10 influences macrophages. Specifically, IL-10 stimulated the activation of the STAT3 signaling pathway and reduced the macrophage inflammatory response, as evidenced by its diminished impact on the osteogenic differentiation of BMSCs.

CONCLUSION

Stimulating macrophages with IL-10 proved effective in improving the inflammatory environment and promoting the osteogenic differentiation of BMSCs. The IL-10/STAT3 signaling pathway has emerged as a key regulator in the macrophage-mediated control of BMSCs' osteogenic differentiation.

Key Words: Macrophages; Interleukin-10; Bone marrow mesenchymal stem cells; Signal transducer and activator of transcription 3; Inflammatory response

Core Tip: This study investigated the mechanism of interleukin-10 (IL-10) affecting macrophages in inflammatory environments, observed the effects of different macrophages on the biological behavior and osteogenic differentiation of bone marrow mesenchymal stem cells, and found that IL-10/signal transducer and activator of transcription 3 signaling plays a crucial role in promoting bone formation by affecting macrophages. This study provides a new strategy for solving the problem of poor osteogenesis in bone defect repair caused by an excessive inflammatory response in clinical work.

INTRODUCTION

Long-term tooth defects or loss is frequently accompanied by bone loss in the edentulous area. Substantial bone augmentation is often necessary to achieve optimal conditions for implant placement before proceeding with implant surgery [1]. Augmentation typically involves the implantation of bone-replacement materials to restore the original alveolar ridge shape and ensure favorable conditions for implant placement in clinical settings[2]. However, trauma associated with implant surgery and post-implantation immune reactions can impede effective bone augmentation, hindering the successful restoration of implants.

Previous studies on osteogenesis primarily focused on the biological behavior of osteoblasts, osteoclasts, or fibroblasts, explaining osteogenesis by investigating the behavioral phenotypes of these cells[3]. The concept of "osteoimmunology" was introduced in 2000, which prompted scholars to explore osseointegration and bone loss from an immunological perspective. This approach aims to gain insight into the healing mechanisms of the interactions between bone graft materials and hosts[4]. The "osteoimmunology" concept underscores both the host's immune response to the graft during osteogenesis and the pivotal regulatory role of immune cells in the bone repair process[5]. Immune and osteogenesis-associated cells collaborate during osteogenesis to establish a microenvironment conducive to bone healing[6].

Macrophages are vital components of the intrinsic immune system and play a crucial role in various immune responses. They are frequently employed as common cellular models for assessing immune responses and as targets for regulating immune responses[7]. During the acute inflammatory phase, macrophages engulf invading microorganisms and tissue debris, releasing pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), and interleukin-6 (IL-6). In the subsequent repair response phase, macrophages secrete anti-inflammatory cytokines [IL-1 receptor (IL-1R) antagonist, Arginase 1 (Arg1)] to dampen the inflammatory response[8]. The functional diversity exhibited by macrophages is dictated by their distinctly differentiated phenotypes, categorized into the classically activated M1 type and the alternatively activated M2 type, a phenomenon commonly referred to as macrophage polarization. M1-type macrophages predominantly play a proinflammatory role, while M2-type macrophages contribute to tissue repair[9]. Mesenchymal stem cells (MSCs) are stromal cells derived from mesenchymal tissues, such as bone marrow or other tissues, such as adipose and dental pulp. They exhibit *in vitro* proliferative and multidirectional differen-

tiation potentials. Consequently, MSCs have widespread applications in tissue engineering, stem cell transplantation, and other research areas aimed at promoting functional tissue regeneration and repair[10]. Bone marrow MSCs (BMSCs), primarily sourced from the bone marrow, are commonly used in experimental osteogenic studies because of their abundant availability, ease of accessibility, high proliferation capacity, and potential for multidirectional differentiation during proliferation. Moreover, immune response can influence the osteogenic differentiation of MSCs[11]. In the acute inflammatory microenvironment, immune cells release cytokines and chemokines, attracting various cell types to the bone defect area, including BMSCs, which are then recruited to the bone tissue to regulate their biological behavior.

The effect of IL-4 on the biological behavior and function of BMSCs following the induction of macrophage M2 polarization has been previously studied[12]. However, the effects of the induced macrophages on the biological behavior and function of BMSCs in an inflammatory environment remain unknown. IL-10, recognized as a potent anti-inflammatory cytokine that plays a crucial role in the immune response to external substances[13,14]. IL-10 is the founding member of a family of cytokines that includes IL-19, IL-20, IL-22, IL-24, IL-26, IL-28A, IL-28B, and IL-29[15]. The cytokine IL-10 is a key mediator that ensures the protection of a host from over-exuberant responses to pathogens and microbiota while playing important roles in other settings, such as sterile wound healing, autoimmunity, cancer, and homeostasis [16]. Type 2 helper T cells were the first identified cellular source of IL-10[13], more and more cells were subsequently confirmed to produce IL-10, such as CD4/CD8 T cells, B cells, macrophages, monocytes, dendritic cells, neutrophils, mast cells, eosinophils, and natural killer (NK) cells[17]. In addition to being produced by different cell types, IL-10 targets different cells and exerts a wide range of anti-inflammatory activities. Macrophages express high levels of the IL-10R on their surface, IL-10 have been shown to trigger strong immunosuppressive responses, primarily through transcriptional inhibition of cytokines and chemokines, as well as major histocompatibility complex class II[18]. IL-10 exerts regulatory effects on various cells *via* the IL-10R. After stimulation with IL-10, the intracellular phosphorylation of IL-10R related Janus kinase 1 and tyrosine kinase 2 occurs[19]. These kinases further phosphorylate tyrosine residues within IL-10R cells, thereby recruiting the signal transducer and activator of transcription 3 (STAT3). After phosphorylation of STAT3, it enters the nucleus and initiates a specific transcriptional program that largely defines the IL-10-mediated anti-inflammatory response[20,21].

This study aimed to investigate the mechanism by which IL-10 influences macrophages in an inflammatory environment and observe the diverse effects of different macrophages on the biological behavior and osteogenic differentiation of BMSCs. This study aimed to provide insights that could address the issue of poor osteogenesis in bone-defect repair attributed to an excessive inflammatory response.

MATERIALS AND METHODS

Macrophage culture and induction
Primary peritoneal macrophages were obtained from Wistar rats by intraperitoneal lavage, followed by cultivation (37 °C, 5% CO_2, saturated humidity) in Dulbecco's modified Eagle medium (DMEM) (Gibco, United States) containing 10% fetal bovine serum (Gibco, United States) and 1% penicillin (Gibco, United States). After 24 h, the cells were allowed to adhere to the well walls of sterile 6-well plates. Macrophages were detached using sterile phosphate buffered saline (HyClone, Logan, UT, United States) and seeded into 6-well plates at a density of 1×10^6/well. Following cell adhesion, lipopolysaccharide (LPS, Sigma, Japan) at a concentration of 1 μg/mL was added to induce macrophages for 12 h. Subsequently, IL-10 (100 ng/mL) (R&D, United States) was introduced for an additional 12 h of culture. The LPS (1 μg/mL) alone group and blank group served as controls. Upon completion of cell culture, the cells were collected for subsequent experiments.

Real-time fluorescence quantitative polymerase chain reaction for macrophage expression detection
Total RNA was extracted from macrophages using TRIzol reagent, and RNA purity and concentration were assessed using a spectrophotometer. Real-time fluorescence quantitative polymerase chain reaction (RT-qPCR) was conducted according to the SYBR Green Real-time PCR reagent instructions (Kangwei Century Biotechnology Co., Ltd., China). The reaction time and temperature were determined through preliminary experiments. The RT-qPCR primer sequences (Shanghai Biotech, China) are shown (Table 1).

BMSCs culture and non-contact co-culture
Femoral and tibial BMSCs from Wistar rats were extracted using the whole bone marrow apposition method and cultured (37 °C, 5% CO_2, saturated humidity) in MSCM (ScienCell, United States) containing 10% fetal bovine serum (Gibco, United States) and 1% cyanostatin (Gibco, United States) by volume. The 3rd generation BMSCs were obtained by digestion with 0.25% trypsin (Gibco, United States) when cell growth reached 75%-85% confluence. After culturing each group of macrophages for 24 h using the method described above, the culture medium was changed to normal DMEM, and the cells were incubated for another 24 h. The supernatants from each group of macrophage cultures were collected for non-contact co-culture with BMSCs. Non-contact co-culture persisted throughout the osteogenic differentiation of BMSCs. Differences in the biological behavior of BMSCs were observed, and the osteogenic differentiation and mineralization of BMSCs were examined after switching to an osteogenic induction medium (Cyagen, China).

Proliferation, migration, differentiation, and mineralization of BMSCs
Proliferation of BMSCs: The 3rd generation BMSCs were uniformly seeded into 24-well plates at a density of 1×10^4/well.

Table 1 The real-time fluorescence quantitative polymerase chain reaction primer sequences for macrophage expression detection

Primer	Sequences
Actb	Forward: 5'-GGAGATTACTGCCCTGGCTCCTAGC-3'
	Reverse: 5'-GGCCGGACTCATCGTACTCCTGCTT-3'
Gapdh	Forward: 5'-TATGACTCTACCCACGGCAA-3'
	Reverse: 5'-ATACTCAGCACCAGCATCACC-3'
Arg1	Forward: 5'-AGTGTGGTGCTGGGTGGAGAC-3'
	Reverse: 5'-GCGGAGTGTTGATGTCAGTGTGAG-3'
Il10	Forward: 5'-CGCATCCAGACACACACAGACTAG-3'
	Reverse: 5'-GCCCAGAGACAGACAAGCAAGAG-3'
Inos	Forward: 5'-GAGACGCACAGGCAGAGGTTG-3'
	Reverse: 5'-AGCAGGCACACGCAATGATG G-3'
Tnfa	Forward: 5'-ATGGGCTCCCTCTCATCAGT-3'
	Reverse: 5'-GCTTGGTGGTTTGCTACGAC-3'

Relative mRNA expression levels were analyzed using the $2^{-\Delta\Delta Ct}$ method with glyceraldehyde-3-phosphate dehydrogenase and β-actin as internal references. *Arg1*: Arginase 1; *Il10*: Interleukin-10; *Inos*: Inducible nitric oxide synthase (gene); *Tnfa*: Tumor necrosis factor-α; *Gapdh*: Glyceraldehyde-3-phosphate dehydrogenase; *Actb*: β-actin.

The culture supernatant of the macrophages after different treatments was added for non-contact co-culture. The proliferation of BMSCs was assessed on days 0, 1st, 3rd, 5th, and 7th d using the Cell Counting Kit-8 (CCK-8, DOJINDO, Japan). A CCK-8 reaction solution (CCK-8 solution: Culture medium = 1:10) was prepared and added to each well containing BMSCs. After incubation for 3 h in the dark, the absorbance (OD) was measured at 495 nm using an enzyme marker (Molecular Devices, China).

Migration of BMSCs: The 3rd generation BMSCs were uniformly seeded in 6-well plates at a density of $3 × 10^5$/well. Once the cells adhered to the well surface, a straight-line scratch was created along the midline of the well bottom using a 1 mL pipette tip. Subsequently, the culture medium was aspirated and serum-free medium along with the macrophage culture supernatant from each group was added. The migration ability of the BMSCs was then observed.

Differentiation of BMSCs: The 3rd generation BMSCs were uniformly seeded in 6-well plates at a density of $3 × 10^5$/well. Upon cell adhesion, the medium was switched to osteogenic induction medium (Cyagen, China) and macrophage culture supernatants from different groups were added for non-contact co-culture. Total RNA from BMSCs after 3 d of osteogenic induction was extracted using a previously described method and subjected to RT-qPCR. The PCR primer sequences (Shanghai Biotech, China) are listed (Table 2).

Mineralization of BMSCs: Alizarin red staining was conducted for each group of BMSCs following a 14-d osteogenic induction culture to observe cell mineralization. The cells were fixed with 4% paraformaldehyde for 15 min. Subsequently, 1 mL of 1% alizarin red staining solution was added to each well and incubated for 15 min at room temperature, followed by gentle rinsing with deionized water three times. The mineralized red nodules were observed under a microscope. After allowing them to dry naturally, a scanner was used to photograph and record the mineralization of each well.

Detection of STAT3 and phosphorylated-STAT3 expression in macrophages by protein blotting (western blot)

Macrophages were extracted and cultured using the same method as previously described. They were then treated differently and grouped as follows: IL-10 (100 ng/mL) was added to macrophages in the experimental group, while Tyrphostin AG490 (10 μM), a specific inhibitor of STAT3 phosphorylation (MCE, United States), was added to macrophages in another group in addition to IL-10. A blank control was used in this study. Total cellular protein was extracted after 24 h of culture.

Proteins were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrotransferred onto a polyvinylidene fluoride membrane. The membranes were blocked with 5% skim milk at room temperature for 1 h. Primary antibodies phosphorylated-STAT3 (p-STAT3) and STAT3 (Abcam, United States) were added and incubated overnight at 4 °C. After washing the membrane the next day, a goat anti-rabbit secondary antibody (Abclonal, China) was applied and incubated at room temperature for one hour. After further washing, the image was developed and the imaging system was used to capture images to assess p-STAT3 and STAT3 expression. Supernatants from macrophage cultures in each of the aforementioned groups were collected and co-cultured with BMSCs. The proliferation, differentiation, and mineralization of BMSCs were evaluated using the method described as above.

Table 2 The real-time fluorescence quantitative polymerase chain reaction primer sequences for differentiation of bone marrow mesenchymal stem cells detection	
Primer	**Sequences**
Gapdh	Forward: 5'-TATGACTCTACCCACGGCAA-3'
	Reverse: 5'-ATACTCAGCACCAGCATCACC-3'
Alp	Forward: 5'-AACGTGGCCAAGAACATCATCA-3'
	Reverse: 5'-TGTCCATCTCCAGCCGTGTC-3'
Ocn	Forward: 5'-AAAGCCCAGCGACTCT-3'
	Reverse: 5'-CTAAACGGTGGTGCCATAGAT-3'
*Runx*2	Forward: 5'-GCTTCTCCAACCCACGAATG-3'
	Reverse: 5'-GAACTGATAGGACGCTGACGA-3'

Relative mRNA expression levels were analyzed using the $2^{-\Delta\Delta Ct}$ method with glyceraldehyde-3-phosphate dehydrogenase as an internal reference. *Alp*: Alkaline phosphatase; *Ocn*: Osteocalcin; *Runx2*: Recombinant runt related transcription factor 2; *Gapdh*: Glyceraldehyde-3-phosphate dehydrogenase.

Statistical analysis

Cell migration images were processed using the ImageJ software. One-way analysis of variance and paired sample *T*-test was used for statistical analyses between different groups. Data were statistically analyzed using SPSS 26.0, with the significance level set at $P < 0.05$. GraphPad Prism software (version 8.0) was used to generate graphs.

RESULTS

IL-10 promotes macrophage polarization towards M2 type

In this study, the optimal concentration of IL-10 was determined using a gradient of 0, 50, 100, and 200 ng/mL. The expression of *Arg1* and *Il10* in each group of macrophages was assessed *via* RT-PCR, revealing the highest expression of *Arg1* and *Il10* at an active concentration of 100 ng/mL (Figure 1). Therefore, 100 ng/mL was selected as the optimal concentration of IL-10 for subsequent experiments.

To investigate the impact of IL-10 on macrophages in an inflammatory environment, we induced a macrophage inflammatory response model using LPS (1 μg/mL) for 12 h. Subsequently, IL-10 (100 ng/mL) was added, and the macrophages were cultured for an additional 12 h before collection. The expression of the M1 markers inducible isoform of nitric oxide synthase (*Inos*) and *Tnfa*, as well as the M2 markers *Arg1* and *Il10*, in macrophages was simultaneously assessed by RT-PCR. Under LPS-stimulated conditions, an elevation was noted in the expression of the inflammatory factors *Inos* and *Tnfa*, indicating polarization toward the M1 type. Conversely, after the addition of IL-10, there was a decrease in the expression of the inflammatory factors *Inos* and *Tnfa* and an increase in the expression of the anti-inflammatory factors *Arg1* and *Il10* (Figure 2).

Macrophages express no effect on the proliferation of BMSCs

To explore the effects of different types of macrophages on BMSCs' proliferation, we co-cultured macrophages and BMSCs. The OD values of the 3rd generation BMSCs were measured and recorded at days 1, 3, 5, and 7 using the CCK-8 method, and proliferation curves were plotted. The results indicated that the proliferation curves of BMSCs in each group exhibited exponential growth in the first 3 d and leveled off by the 5th d, with no significant differences among the groups. Additionally, no significant variation was observed in the proliferation rate of BMSCs in each group. Hence, macrophages in each group had no discernible effect on the proliferation of BMSCs (Figure 3A).

IL-10 treated macrophages promote the migration of BMSCs

To assess the impact of different macrophages on BMSC migration, we created a straight-line scratch along the midline of the bottom of the well and recorded the scratch width after 24 h, 48 h, and 72 h. Among the various BMSC groups, the BMSCs in the M0 + LPS + IL-10 group exhibited the fastest migration rate after exposure to the supernatant, with scratches healing the earliest. In contrast, the scratches in the M0 + LPS group healed the latest, and the cells at the scratches were sparsely arranged. These results indicated that the migration rate of BMSCs was slowed in the inflammatory environment, and IL-10 treatment promoted BMSC migration, accelerating scratch healing (Figure 3B). The residual area of the scratches was quantitatively analyzed using ImageJ software (Figure 3C).

IL-10 treated macrophages promote BMSCs differentiation into osteoblasts

In the RT-PCR analysis of osteogenic markers [alkaline phosphatase (*Alp*), osteocalcin (*Ocn*), and related transcription factor 2 (*Runx2*)] in each BMSC group on the 3rd d after post-osteogenic induction, we observed a reduction in the
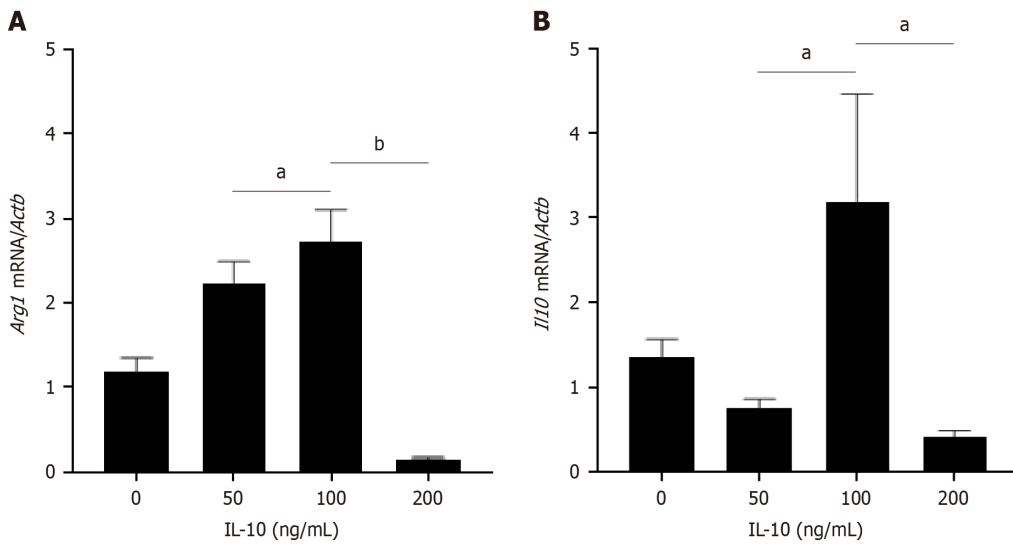
Figure 1 Expression of Arginase 1 and interleukin-10 in macrophages after stimulated by different concentrations of interleukin-10. A: Real-time polymerase chain reaction (PCR) analysis of mRNA level of Arginase 1 (*Arg1*) in macrophages after stimulated by different concentrations of interleukin-10 (IL-10) (0, 50, 100, 200 ng/uL) for 24 h; B: Real-time PCR analysis of mRNA level of *Il10* in macrophages (groups as above). The expression levels were normalized to the expression of β-actin (*Actb*). n = 3 biological replicates. Data are represented as mean ± SD, statistically significant difference at the levels as [a]P < 0.05 and [b]P < 0.01. Arg1: Arginase 1; IL-10: Interleukin-10; Actb: β-actin.
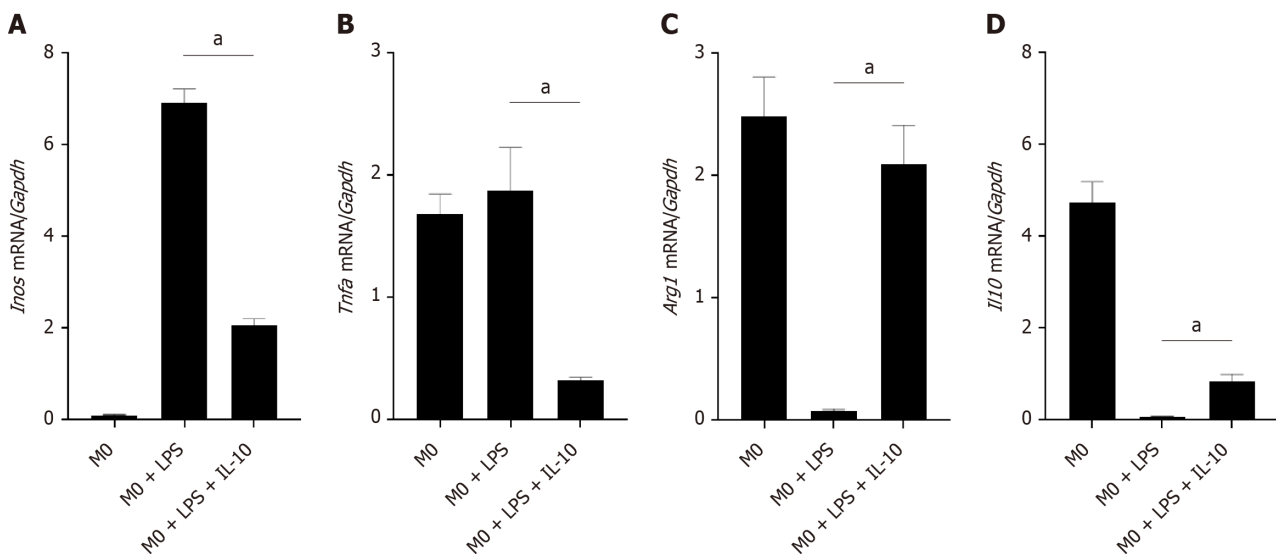
Figure 2 The effect of interleukin-10 acting on the macrophages on the inflammatory reaction environment. A: Real-time polymerase chain reaction (PCR) analysis for inducible nitric oxide synthase mRNA level in different groups of macrophages; B: Real-time PCR analysis for tumor necrosis factor-α mRNA level in different groups of macrophages; C: Real-time PCR analysis for Arginase 1 mRNA level in different groups of macrophages; D: Real-time PCR analysis for interleukin-10 mRNA level in different groups of macrophages. M0 group was stimulated without any special treatment. M0 + lipopolysaccharide (LPS) group was stimulated with LPS (1 μg/mL) for 12 h, then cultured the cells in normal medium for another 12 h. M0 + LPS + interleukin-10 group was stimulated with LPS (1 μg/mL) for 12 h, then stimulated with interleukin-10 (100 ng/mL) for another 12 h. The expression levels were normalized to the expression of glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*). n = 3 biological replicates. Data are represented as mean ± SD, statistically significant difference at the levels as [a]P < 0.05 and [b]P < 0.01. *Arg1*: Arginase 1; *Il10*: Interleukin-10; *Inos*: Inducible nitric oxide synthase (gene); *Tnfa*: Tumor necrosis factor-α; *Gapdh*: Glyceraldehyde-3-phosphate dehydrogenase; LPS: Lipopolysaccharide.

inhibitory effect of the inflammatory state of macrophages on LPS-induced BMSC osteogenesis due to LPS. Additionally, the expression of osteoblast-related genes increased after the addition of IL-10 (Figure 4A-C).

ALP served as an early marker in osteoblast differentiation. To assess BMSC differentiation, BMSCs were cocultured with macrophages and treated with osteogenic liquids. ALP expression was measured on the 1st, 3rd, 5th, and 7th after the post-osteogenic induction. ALP expression in all BMSC groups peaked on the 3rd d. The M0 + LPS coculture group exhibited the lowest ALP expression, whereas BMSCs in the M0 + LPS + IL-10 group showed elevated ALP expression, indicating improved osteogenic differentiation in the M0 + LPS + IL-10 group (Figure 4D).
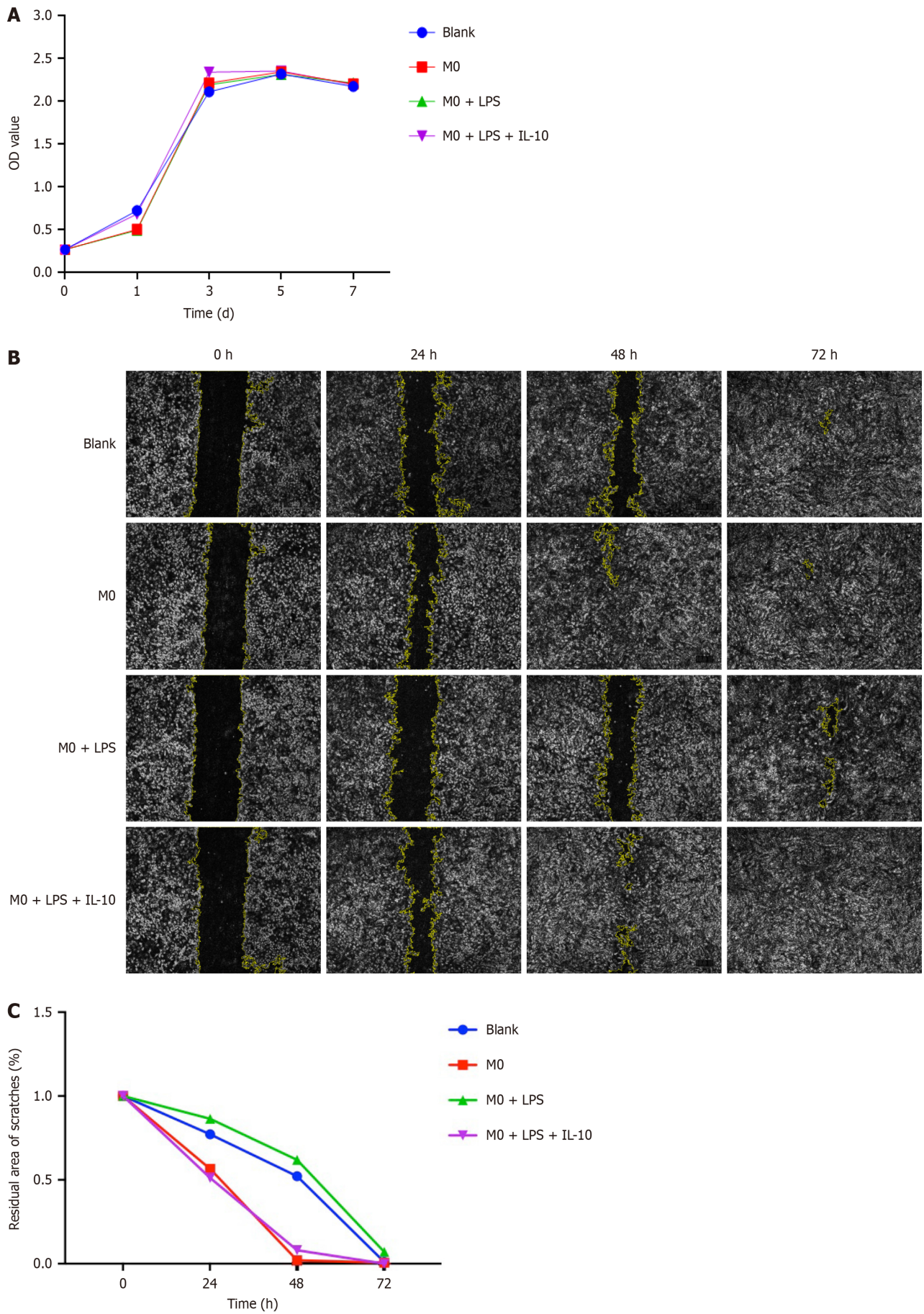
A

B

C

Figure 3 The effect of macrophages on the proliferation and migration of bone marrow mesenchymal stem cells. A: Cell Counting Kit-8 assay

of the 3rd generation of bone marrow mesenchymal stem cells (BMSCs) proliferation after co-cultured with different groups of macrophages: M0, M0 + lipopolysaccharide (LPS), M0 + LPS + interleukin-10; compared with the control group for 1, 3, 5, and 7 d; B: Migration analysis of BMSCs and those co-cultured with different groups of macrophages (groups as above). The Image J software was adopted to process the pictures; C: Quantitative analysis of residual scratch area to initial scratch area percentage. Macrophages influenced the migration but not the proliferation of bone marrow mesenchymal stem cells. IL-10: Interleukin-10; LPS: Lipopolysaccharide.

Subsequently, BMSCs were subjected to osteoblast induction for 14 d. Each group was stained with alizarin red to observe cell mineralization. Red-stained Ca²⁺ nodules were observed in all the groups. Photographs revealed that The M0 + LPS + IL-10 group exhibited the deepest staining, whereas the M0 + LPS group showed poor osteogenic effect. These results indicate that under inflammatory conditions, macrophages weaken the osteogenic effects of BMSCs. However, this phenomenon was ameliorated by IL-10 treatment, which restored osteogenic effects (Figure 4E).

IL-10 promotes macrophages via STAT3 signaling pathway

To further explore the signaling pathway of IL-10 in macrophages, we used a STAT3 phosphorylation-specific inhibitor (AG490) to elucidate its function. The effect of the STAT3 inhibitor on macrophage activity was assessed using a CCK-8 assay. Macrophage activity was not significantly affected in the STAT3 inhibitor group compared to the other groups, and the cytotoxicity of the inhibitor AG490 was negligible (Figure 5). Inhibition of STAT3 phosphorylation was evaluated by western blotting, which revealed a significant increase in p-STAT3 expression levels in macrophages after IL-10 stimulation. However, AG490 effectively inhibited STAT3 phosphorylation (Figure 6).

To confirm the role of STAT3 in IL-10-treated macrophages, BMSCs were co-cultured with macrophages in which p-STAT3 expression was inhibited. The proliferation of 3rd-generation BMSCs was assessed using the CCK-8 assay, and proliferation curves were plotted. The results indicated no significant difference in the proliferation rate of BMSCs between the groups. Furthermore, macrophages did not have any discernible effects on the proliferation of BMSCs in any group (Figure 7A).

RT-PCR was conducted on osteogenic markers (*Alp*, *Ocn*, and *Runx2*) in each group of BMSCs at 3 d post-osteogenic induction. The expression of osteogenic markers in the co-cultured BMSCs decreased after p-STAT3 inhibition in macrophages, thereby diminishing the osteogenesis-promoting effects of macrophages (Figure 7B-D).

After 14 d of osteogenic induction culture for alizarin red staining, the degree of cell mineralization was reduced after the addition of AG490 to inhibit p-STAT3 compared to the M0 + IL-10 group. These results suggested that the inhibition of STAT3 phosphorylation in macrophages weakened their ability to promote osteogenesis in BMSCs (Figure 7E).

DISCUSSION

The discovery of receptor activator of nuclear factor-κ B ligand (RANKL) has drawn attention to the relationship between the immune system and bone, leading to the emergence of a field known as "osteoimmunology". The concept of "osteoimmunology" has significantly expanded our understanding of the role of immune cells in bone repair process [22]. BMSCs were crucial engineered cells in bone tissue engineering and represented the primary cells influenced by relevant immune cells within the framework of "osteoimmunology"[23]. During the induction of new bone formation in areas with bone defects, BMSCs initially migrated, divided, and proliferated towards the bone defect area. They differentiate into osteoblasts in response to environmental stimuli. Osteoblasts secrete collagen fibers that envelop the cells, and with the gradual deposition of calcium on these collagen fibers, osteoblasts undergo further transformation into bone cells.

Throughout this process, various types of immune cells accumulate in the defect area alongside the BMSCs. Macrophages are essential components of intrinsic immune cells and not only aid the host in defending against external pathogens but also promote tissue repair following injury[24]. Preclinical studies have shown that macrophages can influence bone cells *via* either paracrine signaling or direct cell-cell contact. M1-type macrophages secreted inflammatory factors (such as, TNF-α, IL-1β, IL-6, and IL-8), chemokines [such as, C-C motif ligand (CCL)15, CCL20, and C-X-C motif ligand (CXCL)-13], reactive oxygen species, and nitrogen intermediates (iNOS), displaying a robust antigen-killing capacity. In contrast, M2-type macrophages, through the release of anti-inflammatory cytokines (IL-4, IL-10), chemokines (CCL17, CCL22, and CCL24), scavenger receptors (CD163, mannose receptors), matrix metalloproteinases, and Arg 1, inhibit the continued development of inflammation in the middle and late stages of the inflammatory response and maintain tissue homeostasis[25]. All of these factors directly influence bone cells. In conclusion, macrophages may act differently on bone cells depending on their polarization profiles and secreted paracrine factors. This is related to the microenvironment in which the macrophages are located, as changes in the surroundings can also cause corresponding changes in the polarization state of the macrophages.

Numerous studies have indicated a close relationship between osteogenic differentiation of BMSCs and the inflammatory state of macrophages. When macrophages exhibit an imbalance in polarization, they secrete more inflammatory factors, thereby maintaining the body in a constant inflammatory state. Influenced by inflammatory factors, MSCs can alter their differentiation direction, resulting in poor bone regeneration and repair or even failure[26]. Yuan *et al*[27] discovered that the expression levels of osteogenic-related genes, such as *Ocn*, *Alp*, and *Runx2* decreased during osteogenesis under the influence of inflammatory factors, such as TNF-α[27,28]. Inhibition of the proliferation and differentiation ability of BMSCs led to a decrease in the number of osteoblasts and a poor osteogenic effect. Besides the effects
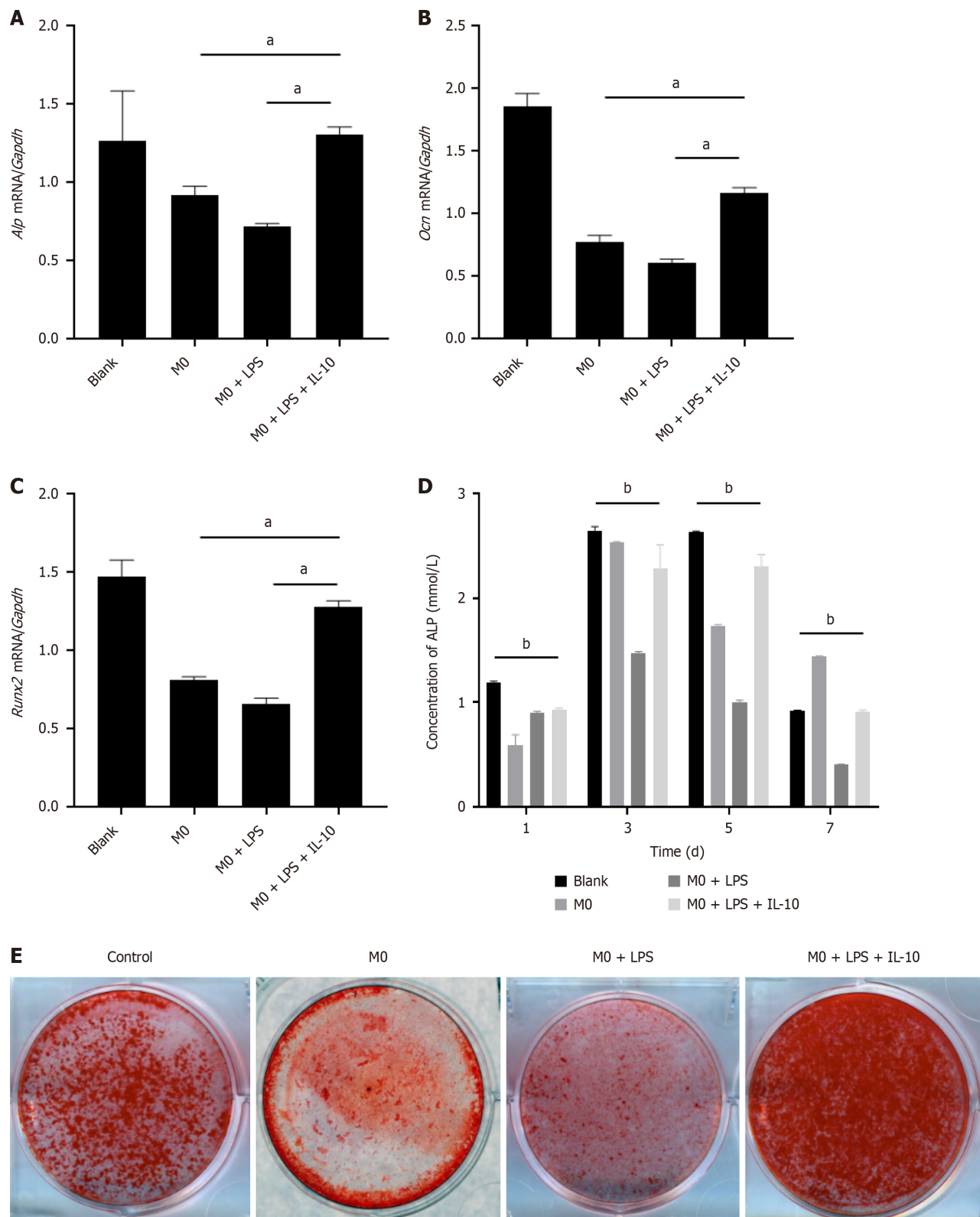
Figure 4 The effect of macrophages on osteogenic differentiation of bone marrow mesenchymal stem cells. A: Real-time polymerase chain reaction (PCR) analysis for Alkaline phosphatase (*Alp*) mRNA level in bone marrow mesenchymal stem cells (BMSCs) at the 3rd d after osteogenic induction, which were co cultured with different groups of macrophages: M0, M0 + lipopolysaccharide (LPS), M0 + LPS + interleukin-10 (IL-10); compared with the control group; B: Real-time PCR analysis for osteocalcin mRNA level in BMSCs (groups as above); C: Real-time PCR analysis for Recombinant runt related transcription factor 2 mRNA level in BMSCs (groups as above). The expression levels were normalized to the expression of glyceraldehyde-3-phosphate dehydrogenase; D: Analysis of ALP activity of BMSCs following range of co-cultured with different groups of macrophages at 1, 3, 5, 7 d, compared with the control group; E: At the 14th d after osteogenic induction of different BMSCs, quantitative measurement of calcium mineral deposition was stained by Alizarin Red S staining. *n* = 3 biological replicates. Data are represented as mean ± SD, statistically significant difference at the levels as [a]$P < 0.05$ and [b]$P < 0.01$. *Alp*: Alkaline phosphatase; *Ocn*: Osteocalcin; *Runx2*: Recombinant runt related transcription factor 2; *Gapdh*: Glyceraldehyde-3-phosphate dehydrogenase; IL-10: Interleukin-10; LPS: Lipopolysaccharide.
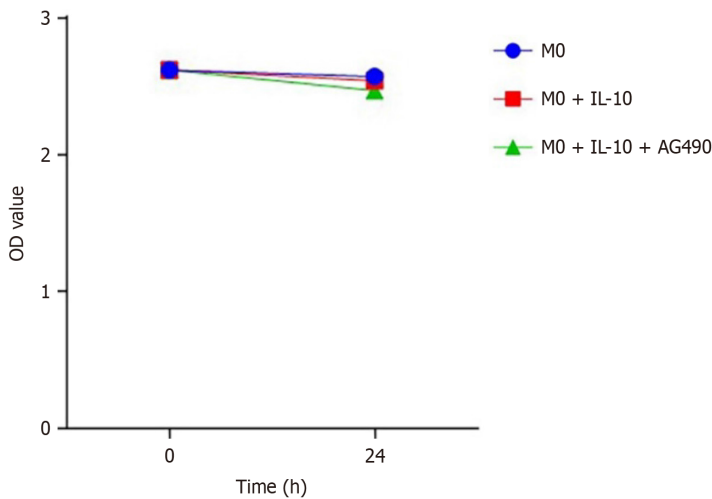
Figure 5 The cytotoxicity of the special inhibitor Tyrphostin AG490. Analysis of the macrophage activity showed no significantly influences in the group with phosphorylated signal transducer and activator of transcription 3 inhibitor Tyrphostin AG490 compared with the rest of the groups by using Cell Counting Kit-8. IL-10: Interleukin-10.
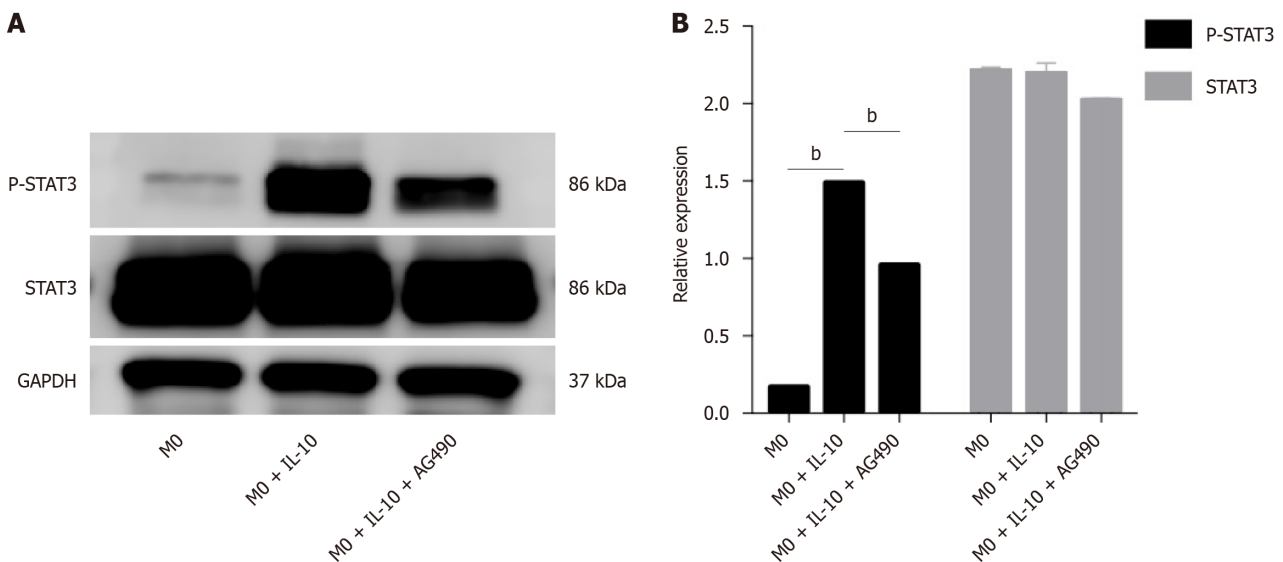

Figure 6 The effect of interleukin-10 treatment on the activation of signal transducer and activator of transcription 3 in macrophages. A: Western analysis of signal transducer and activator of transcription 3 (STAT3) and phosphorylated STAT3 protein in macrophages treated with/without interleukin-10. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a loading control. Tyrphostin AG490 was used at 10 μM concentration in culture medium as a phosphorylation specific inhibitor of STAT3. Data are representative of three independent experiments; B: Density values were measured by using Image J software for the representative blot shown. *n* = 3 biological replicates. Data are represented as mean ± SD, statistically significant difference at the levels as [a]$P < 0.05$ and [b]$P < 0.01$. IL-10: Interleukin-10; p-STAT3: Phosphorylated signal transducer and activator of transcription 3.

of immune cells on bone cells, there are also reciprocal effects of osteoclasts and osteoblasts on the cells of the immune system. During inflammatory conditions, the so-called "inflammatory osteoclasts" originating from dendritic cells influenced CD4^{+} T lymphocytes in an antigen-dependent manner and altered their TNFα production[29]. However, osteoblasts are an important source of activated complement proteins under inflammatory conditions[30], thereby activating immune system[31]. With increasing in-depth research, this field of osteoimmunology has convinced the scientific community that a two-way communication exists between the bone and immune system.

In the present study, an inflammatory environment was created by the LPS-induced differentiation of macrophages into the M1 type, followed by non-contact co-culture of the induced macrophages with BMSCs. The results also demonstrated a decrease in the expression of genes, such as *Ocn*, *Alp*, and *Runx2* during osteogenesis in BMSCs, indicating that macrophages in an inflammatory environment exert an inhibitory effect on osteogenesis. Moreover, implantation of a material containing the cytokine bone morphogenetic protein-4 regulates the polarization of the mouse macrophage line RAW264.7 towards an anti-inflammatory type, ultimately promoting BMSC osteogenesis[25]. These results suggested that improving the inflammatory microenvironment can accelerate bone repair.
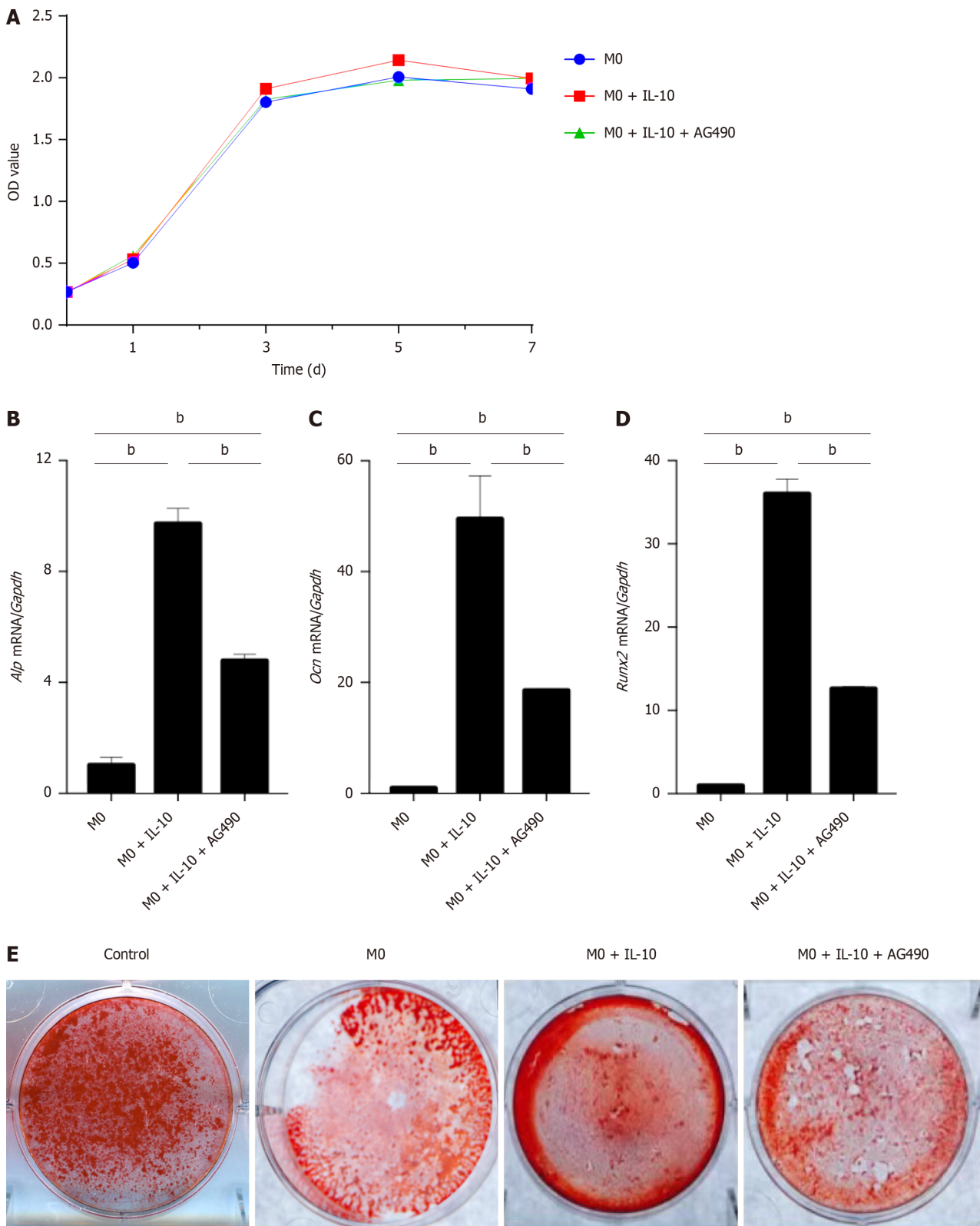
Figure 7 The effect of interleukin-10 acting on macrophages after the inhibition of signal transducer and activator of transcription 3. A: Cell Counting Kit-8 assay of the bone marrow mesenchymal stem cells (BMSCs) proliferation after co-cultured with different groups of macrophages: M0, M0 + interleukin-10 (IL-10), M0 + IL-10 + Tyrphostin AG490 for 1, 3, 5, and 7 d; B: Real-time polymerase chain reaction (PCR) analysis for Alkaline phosphatase mRNA level in different groups of BMSCs (groups as above); C: Real-time PCR analysis for osteocalcin mRNA level in different groups of BMSCs (groups as above); D: Real-time PCR analysis for recombinant runt related transcription factor 2 mRNA level in different groups of BMSCs (groups as above). The expression levels were normalized to the expression of glyceraldehyde-3-phosphate dehydrogenase; E: Quantitative measurement of calcium mineral deposition was stained by Alizarin Red S staining after 14 d of osteogenic induction on BMSCs. *n* = 3 biological replicates. Data are represented as mean ± SD, statistically significant difference at the levels as [a]$P < 0.05$ and [b]$P < 0.01$. *Alp*: Alkaline phosphatase; *Ocn*: Osteocalcin; *Runx2*: Recombinant runt related transcription factor 2; *Gapdh*: Glyceraldehyde-3-phosphate dehydrogenase; IL-10: Interleukin-10.

Previous studies have confirmed that cytokines in the osteogenic environment are crucial for bone regeneration. However, in an *in vivo* environment, the bone is a very specific microenvironment, and bone remodeling is a continuous dynamic equilibrium process, while repair and regeneration are ongoing. The innate immune system is the main source of various cytokines, providing an immediate response to infection and injury and initiating tissue repair[32]. Immune cells, such as macrophages utilize pattern recognition receptors (PRR) to recognize local "danger signals" such as damage-associated molecular patterns released by tissue damage caused by infectious organisms. The activation of PRRs can also lead these cells to produce inflammatory cytokines such as TNFα, IL-1, IL-6, and IFN-γ. In the bone microenvironment, these inflammatory cytokines have direct effects on both osteoclasts and osteoblasts as well as indirect effects on osteoclasts *via* osteoblast upregulation of RANKL[33,34]. Therefore, studying the molecular mechanisms underlying the cross-relationship between immune cells and MSCs is important to clarify the role of the immune system in recruiting MSCs during bone regeneration and regulating their differentiation. This paves the way for exploring new therapeutic methods to enhance bone regeneration by leveraging interactions between immune cells and MSCs[35].

IL-10 is a potent anti-inflammatory cytokine that participates in various cellular activities and regulates cell growth and differentiation. Although IL-10 is primarily produced by mononuclear macrophages and T cells, it can also be synthesized by immune cells, such as dendritic cells, B cells, NK cells, and neutrophils *in vivo*[36,37]. In one study, IL-10 was found to reduce NO production in LPS-stimulated mouse RAW 264.7, leading to a significant decrease in the expression of iNOS and cyclooxygenase 2, as well as other related proteins, resulting in a reduced inflammatory response[38]. However, although IL-10 has a strong inhibitory effect on the inflammatory response, its effect on BMSCs is two-sided. IL-10 can promote the osteogenic differentiation of BMSCs at low concentrations, while high concentrations demonstrated an inhibitory effect on the osteogenic differentiation of BMSCs. Another study discovered that a certain concentration of IL-10 enhanced the osteogenic differentiation of dental pulp stem cells (DPSCs) by regulating DPSC metabolism[27].

In this study, we focused on the role of IL-10 in the inflammatory response. Therefore, we created an inflammatory environment by stimulating macrophage differentiation into the M1 type using LPS and then added IL-10 to macrophages in an inflammatory state to investigate the effect of IL-10 on macrophages in such an environment. The results revealed a decrease in the expression of inflammatory factors and an increase in the expression of anti-inflammatory factors in macrophages after addition of IL-10, indicating that IL-10 weakened the inflammatory response. Further studies have demonstrated that the addition of IL-10 can regulate the inflammatory environment during the non-contact co-culture of macrophages and BMSCs, leading to the promotion of the expression of genes, such as *Alp*, *Ocn*, and *Runx2,* in the osteogenesis of BMSCs. This suggested that the osteogenic inhibitory effect of macrophages on BMSCs was diminished, highlighting the powerful anti-inflammatory effects of IL-10.

Most anti-inflammatory factors regulate cell expression by binding to cell surface receptors and subsequently activating various signaling pathways[39]. This is also true of the anti-inflammatory mechanisms of IL-10. STAT3 signaling plays a pivotal role in the anti-inflammatory action of IL-10 on macrophages. A previous study used culture supernatants from prostate cancer cells to induce an immunophenotypic shift in macrophages from pro-inflammatory to anti-inflammatory and demonstrated a significant increase in p-STAT3 signaling[28]. IL-10 exerts its effects in close association with STAT3 signaling. Degboé *et al*[40] explored the pathogenesis of macrophages and rheumatoid arthritis and found that anti-cytokine biotherapeutic agents activated alternative functions of macrophages under inflammatory conditions. Increased production of IL-10 in the early stages can induce negative feedback control of inflammation, which is closely related to STAT3. Western blotting results showed an elevated expression level of p-STAT3 in macrophages after IL-10 induction. The promotion of osteogenesis in BMSCs was greater than that in macrophages without IL-10. After the addition of AG490 as a specific inhibitor, effectively inhibiting STAT3 phosphorylation, the osteogenesis-promoting effect of IL-10-activated macrophages on BMSCs was also diminished. These results demonstrate that the inhibitory effect of IL-10 on inflammation is closely related to STAT3 signaling, and that the IL-10/STAT3 axis is critical for the promotion of osteogenesis in BMSCs by macrophages.

Returning to the inception of our research, it holds significance not only within the realm of oral alveolar bone regeneration and repair but also in the broader field of tissue regeneration engineering. The findings outlined in this manuscript present novel avenues for enhancing the immune response process triggered by allogeneic materials. Recent literature increasingly underscores the convergence of biomaterials, macrophage phenotypes, and tissue regeneration, with numerous studies exploring the modulation of inflammatory responses through modifications to implanted material characteristics. For instance, in guided bone regeneration, the collagen membrane, a commonly utilized material, demonstrates the capacity to influence macrophage phenotype, thereby shaping the healing environment[41]. Augmenting such materials with bioactive substances possessing immunomodulatory properties has proven to enhance biocompatibility and bolster the potential for bone regeneration, offering insights and directions for future applications [42,43]. Notably, IL-10 emerges as a regulator of macrophage phenotype. In further research in the future, we also hope to improve the properties of allogeneic materials by applying IL-10, enabling sustained release of IL-10 in the *in vivo* environment and regulating local inflammatory status, promoting new bone regeneration and remodeling around the material. The outcomes of this study present a promising approach to mitigating the challenges posed by inflammatory environments in bone defect repair, while also paving the way for innovative clinical applications in oral implantology and beyond.

CONCLUSION

IL-10 can improve the inflammatory response of macrophages. However, macrophages in different states did not induce changes in the proliferation ability of BMSCs. Microenvironments constructed by macrophages in different states can

affect osteogenic differentiation of BMSCs. Macrophages treated with IL-10 enhanced the osteogenic differentiation of BMSCs. The IL-10/STAT3 signal played a crucial role in promoting bone formation by influencing macrophages.

FOOTNOTES

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