M2 macrophage-conditioned medium inhibits intervertebral disc degeneration in a tumor necrosis factor-α-rich environment

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Abstract
Inflammation is the primary pathological phenomenon associated with disc degeneration; the inflammatory cytokine tumor necrosis factor (TNF-α) plays a crucial role in this pathology. The anti-inflammatory and regenerative effects of M2 macrophages on nucleus pulposus cells (NPCs) in intervertebral disc degeneration (IDD) progression remain unknown. Here, M2 conditioned medium (M2CM) was harvested and purified from human acute monocytic leukaemia cell line (THP-1) cells and mouse peritoneal macrophages, respectively; it was used for culturing human NPCs and a mouse intervertebral disc (IVD) organ culture model. NPCs and IVD organ models were divided into three groups: group 1 treated with 10% fetal bovine serum (control); group 2 treated with 10 ng/ml TNF-α; and group 3 treated with 10 ng/ml TNF-α and M2CM (coculture group). After 2–14 days, cell proliferation, extracellular matrix synthesis, apoptosis, and NPC senescence were assessed. Cell proliferation was reduced in TNF-α-treated NPCs and inhibited in the M2CM co-culture treatment. Moreover, TNF-α treatment enhanced apoptosis, senescence, and expression of inflammatory factor-related genes, including interleukin-6, MMP-13, ADAMTS-4, and ADAMTS-5, whereas M2CM coculture significantly reversed these effects. In addition, co-culture with M2CM promoted aggrecan and collagen II synthesis, but reduced collagen Iα1 levels in TNF-α treatment groups. Using our established three-dimensional murine IVD organ culture model, we show that M2CM suppressed the inhibitory effect of TNF-α-rich environment. Therefore, co-culture with M2CM promotes cell proliferation and extracellular matrix synthesis and inhibits inflammation, apoptosis, and NPC senescence. This study highlights the therapeutic potential of M2CM for IDD.

KEYWORDS
inflammation, intervertebral disc, M2, macrophage, tissue regeneration
1 | INTRODUCTION

Intervertebral disc degeneration (IDD) is the pathological basis of lower back pain, which reduces the quality of life and generates a massive economic burden. Surgical treatment is the accepted solution for IDD patients with end-stage degeneration; however, it is associated with surgical complications, function loss, and poor clinical effects. In patients with early-stage IDD, current therapies are aimed at mitigating symptoms rather than treating the pathological condition. Therefore, biological therapy to restore disc morphology and function and inhibit IDD progression is highly significant.

Immune cells such as T and B cells, mast cells, and macrophages are implicated in IDD. The roles of different macrophage phenotypes such as proinflammatory M1, remodeling M2c, and antiinflammatory M2a in IDD are increasingly recognized. M0 and M1 macrophages and nucleus pulposus cells (NPCs) interact under either pathological or physiological culture conditions. Studies in animal models indicate an increased number of infiltrating macrophages with a high level of inflammatory factors in IDD. A cadaver study indicated the differential distribution of macrophages in different parts of the intervertebral disc (IVD); M2 macrophages accumulate more around the granulation tissue, indicating their remodeling potential in IDD. Macrophages maintain homeostasis and regulate the immune system, serving as the body’s first line of defense against external damage. These cells display plasticity predominantly through the proinflammatory state of M1 polarization in the early stage of tissue injury and through the antiinflammation and healing states of M2 polarization in the later stages; the transition state is described in various tissue injuries. The human acute monocytic leukaemia cell line (THP-1) is a monocyte cell line with the ability to differentiate into macrophages. It is widely used to study the biological characteristics of macrophage by inducing them into M1/M2 in vitro.

Several studies have focused on the roles of M0 or M1 macrophages and disc cells in IDD; however, the role of M2 cells remains unclear. M2 macrophages help maintain tissue homeostasis in the heart, lungs, muscles, and spinal cord. Conditioned medium (CM) contains a mixture of different factors secreted by the cells, including growth factors, cytokines, enzymes, nucleic acids, and bioactive lipids, suggesting a new class of therapeutics with wide applications in injury and disease treatment. M2 macrophage-CM (M2CM) inhibits inflammation and apoptosis in chondrocytes, through generating a prochondrogenic environment by producing immunoregulatory factors, such as tumor growth factor-beta (TGF-β), and M2-related cytokines such as interleukin (IL)-4 and IL-10. The antiinflammatory and remodeling potential of M2CM in alleviating IDD progression is unclear.

IDD is primarily associated with inflammation; therefore, the proinflammatory cytokine tumor necrosis factor-alpha (TNF-α) is widely used to mimic the degeneration conditions in IDD. We hypothesized that M2 macrophages attenuate the harmful effects of TNF-α, including the effects on the proliferation, senescence, apoptosis, and inflammation of NPCs. To test this possibility and provide a foundation for the potential therapeutic applications of M2CM in IDD, we investigated the antiinflammatory, antiapoptotic, and antisenescence effects of M2CM in TNF-α-treated NPCs.

2 | MATERIALS AND METHODS

2.1 | Ethics statement

The study was performed according to the amended declaration of Helsinki; human nucleus pulposus (NP) tissues were isolated from eight patients (Table 1). The experiment was approved by the Committee of Gaozhou People’s Hospital (No. 2018-012). Informed consent was obtained from all participants.

2.2 | Human NP isolation and human NPC culture

NPCs were isolated and harvested as previously reported. Briefly, NP samples were collected and cut into small pieces (<1 mm³), digested with 2 mg/ml collagenase II (Sigma), and the cells were cultured in culture medium consisting of F12 Dulbecco’s modified Eagle medium (DMEM) (HyClone), 10% fetal calf serum (Gibco), and 1% penicillin/streptomycin (Gibco) in 25 cm² cell culture flasks at a density of 1 × 10⁵ cells/ml in a humidified incubator at 37°C under 5% CO₂. After 3 days, the suspended cells and medium were removed, the adherent cells were cultured, and the medium was replaced every 2–3 days. When the cells reached 70%–80% confluence, the primary cells were harvested with 0.25% trypsin-ethylenediaminetetraacetic acid (Sigma) for 1 min, and the harvested passage 1 (P1) NPCs were subcultured at a ratio of 1:3.

2.3 | Isolation and culture of IVD organ culture models

Forty-five 8-week-old male C57 mice were obtained from the experimental animal center of Southern Medical University. The mice were sacrificed using inhalation anesthesia. Detailed characteristics of the enrolled patients are shown in Table 1.

| Case No. | Sex | Age (years) | Diagnosis | Disc level | Pfirrmann grade |
|----------|-----|-------------|-----------|------------|----------------|
| Case 1   | Male| 20          | LDH       | L4-L5      | III            |
| Case 2   | Male| 26          | LDH       | L4-L5      | III            |
| Case 3   | Male| 39          | LDH       | L5-S1      | III            |
| Case 4   | Male| 27          | LDH       | L4-L5      | III            |
| Case 5   | Male| 31          | LDH       | L4-L5      | III            |
| Case 6   | Male| 30          | LDH       | L5-S1      | III            |
| Case 7   | Male| 42          | LDH       | L5-S1      | III            |
| Case 8   | Male| 25          | LDH       | L4-L5      | III            |

Abbreviation: LDH, lumbar disk herniation.
were euthanized, and the tails were cut from the base segment. After soaking in iodophor for 5 min, the tail skin was removed, and tail spines were harvested under sterile condition. The Co7/8 and Co8/9 IVD from the same mouse were harvested under sterile conditions, rinsed in sterile phosphate-buffered saline (PBS), and immediately placed in culture medium as mouse IVD organ models. Each group comprised five IVDs from different mice that were cultured in 25 cm² cell culture bottles with 15 ml culture medium containing F12 DMEM (HyClone), 10% fetal calf serum (Gibco), and 1% penicillin/streptomycin (Gibco). Samples were cultured in a humidified incubator at 37°C under 5% CO₂, and the culture medium was replaced every 3 days. All the experiments were performed three times for each group.

2.4 Harvest of human M2CM and treatment of samples

THP-1 cells were obtained from the cell bank of type culture collection of the Chinese Academy of Sciences, seeded at a density of 2 × 10⁶ in 175-cm² cell culture bottle for 24 h, and then treated with PMA (100 ng/ml; RD) for 24 h. Thereafter, they were changed into serum-free media and treated with or without IL-4 (100 ng/ml; RD) for another 24 h. The supernatant medium was replaced with serum-free medium and cultured for additional 24 h. Finally, the corresponding supernatant CM was obtained and centrifuged for 15 min at 4°C at 1500 g to remove cellular debris; the procedure was repeated at 1500 g. The cells were collected for identification, and the harvested supernatant CM was defined as M2CM (Figure 1A). The NPCs or IVD organ models were cultured in the medium for 24 h. They were divided into three groups: group 1, treated with 10% fetal bovine serum (FBS) culture medium (control); group 2, treated with 10 ng/ml TNF-α (Recombinant TNF-α, peptoprotein company, Number: 300-01); and group 3, treated with 10 ng/ml TNF-α and 30% M2CM (coculture group). After 2–14 days, the cells or IVD culture models were harvested for analysis (Figure S1A).

2.5 Cell proliferation assay

Cell Counting Kit-8 (CCK-8; Dojindo Laboratories) was used as previously described to measure cell proliferation.²⁸ In Brief, NPCs were seeded in 96-well plates (2 × 10⁵ cells/well), and different groups were incubated for 24, 48, and 96 h. After removing the culture medium and M2CM, 10 μl of CCK-8 solution was added to 100 μl of fresh medium, and the mixture was incubated at 37°C for 1 h. Finally, the samples were added to 96-well plates for final measurements. The absorbance of the solution was measured at 450 nm using a microplate reader (Bio-Rad). A blank 96-well plate was used as the control. In addition, cell numbers in six-well plates (1 × 10⁶ cells/well) were calculated using a cell counter after culturing in different groups for 24, 48, and 96 h. All experiments were performed four times for each sample.

2.6 Cell apoptotic rate determination using the TUNEL assay

Cell apoptosis was confirmed using the TUNEL assay (Promega) according to the manufacturer’s instructions and previous reports.²⁸ Briefly, the cells were washed with PBS thrice and fixed in 4% paraformaldehyde for 30 min. The cells were washed with PBS thrice and incubated with TdT-mediated dUTP for 1 h in the dark. The cells with the entire nuclear area labeled red were defined as apoptotic cells. The cell nuclei were stained with DAPI solution (Recombinant TNF-α, peptoprotein company, Number: 300-01); and group 3, treated with 10 ng/ml TNF-α and 30% M2CM (coculture group). After 2–14 days, the cells or IVD culture models were harvested for analysis (Figure 1A).

Primary mouse peritoneal macrophages were obtained from the peritoneal exudates of 4–6-week-old mice. The peritoneal exudate cells were washed twice with PBS and adjusted to 1 × 10⁶ cells/ml in six-well plates with DMEM. They were cultured for 3–4 h at 37°C and 5% CO₂. The nonadherent cells were removed by washing with warm PBS. Subsequently, macrophages were incubated continually with 20 ng/ml of mouse recombinant IL-4 (100 ng/ml; RD) for 24 h to generate M2 macrophages. The supernatant medium was replaced with serum-free medium and the cells cultured for an additional 24 h. Finally, the corresponding supernatant CM was obtained and centrifuged for 15 min at 4°C at 1500 g. The cells were collected for identification, and the harvested supernatant CM was defined as M2CM (Figure S1A). The IVD organ models were cultured in the medium for 24 h. They were divided into three groups: group 1, treated with 10% FBS culture medium (control); group 2, treated with 10 ng/ml TNF-α (Recombinant TNF-α, peptoprotein company, Number: 300-01); and group 3, treated with 10 ng/ml TNF-α and 30% M2CM (coculture group). After 2–14 days, the cells or IVD culture models were harvested for analysis (Figure S1A).

2.7 Senescence-associated β-galactosidase (SA-β-gal) staining

After 5 days of incubation, NPCs were analyzed using a Senescence β-Galactosidase Staining Kit (Beyotime Institute of Biotechnology). Briefly, the cells were washed with PBS, fixed in the SA-β-gal fixative solution for 15 min at room temperature, rinsed thrice with PBS, and incubated in SA-β-gal working solution (Reagents A, B, C, and X-Gal) overnight at 37°C under atmospheric conditions. Quantiﬁcation was performed by counting the number of SA-β-gal-positive cells and the total number of cells from three randomly selected areas for each sample.
2.8 | Immunofluorescence microscopy

NPCs were plated in flat-bottomed 24-well plates (1 × 10^4 cells/well) and fixed with 4% paraformaldehyde for 30 min, permeabilized with 0.2% triton X-100 in PBS (PBS-T) for 10 min, blocked with PBS containing 5% FBS, and incubated with antibodies against collagen II (1:100; Abcam) at 4°C overnight. For the negative control, the cells were incubated with antibody diluents without antibodies under similar conditions. After washing, the cells were incubated with anti-rabbit secondary antibody (Jackson) at a dilution of 1:100 for 1 h at room temperature. The cells were washed thrice, and the cell nuclei were stained with DAPI solution (1:1000; Invitrogen) for 2 min at room temperature. The sample images were recorded using a fluorescence microscope (FV-1000; Olympus). Image-Pro Plus software (Version 5.1; Media Cybernetics, Inc.) was used for quantitative analysis. The positive staining of NPCs was verified, and the sections were randomly coded and scored by two blinded observers for three sections per sample.

2.9 | Real-time quantitative PCR (qPCR) analysis

After incubation under different conditions for 2 days, approximately 3 × 10^5 cells and 20–50 mg of IVD samples were used for total RNA extraction using TRIzol (Invitrogen) according to the manufacturer’s instructions. The RNA concentration was determined through spectrophotometry and samples with an optical density (OD)_{260/280} of 1.8–2.0,
and OD_{260/230} > 1.8 was used. RNA was reverse-transcribed using the PrimeScript™ RT Master Mix (Takara). The qPCR was performed in triplicate in 96-well plates, using the SYBR Premix Ex Taq Kit; the final volume of the reaction mixture was 20 μl. All primers were obtained from Sangon (Table S1). qPCR was performed using the One Step SYBR® PrimerScript RT-PCR Kit (Takara). Glyceraldehyde 3-phosphate dehydrogenase was used for normalization. The relative quantification was carried out using the 2^\(-\Delta\Delta C_{t}\) method.

### 2.10 Total protein isolation and Western blotting

Western blotting was performed as previously described.²⁹ Approximately 3 × 10⁵ cells were lysed in the RIPA buffer (Beyotime) with phenylmethylsulfonyl fluoride (Sigma-Aldrich). The total protein concentration was determined using a BCA protein assay kit (Beyotime). Protein samples (30 μg each) were separated through sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The proteins were transferred onto polyvinylidene fluoride membranes (Perkin Elmer). The membranes were blocked with 1× Tris-buffered saline and Tween 20 (TBST) containing 5% blotting-grade blocker nonfat dry milk (Bio-Rad). They were incubated overnight with appropriate primary antibodies at 4°C followed by incubation with the horseradish peroxidase-conjugated secondary antibody (1:1000; Cell Signaling Technology). Subsequently, the protein bands on the blots were visualized using the Clarity Western ECL Kit (Bio-Rad) and imaged using ChemiDoc Touch Imaging System (Bio-Rad). The primary antibodies against Sox9 (82630), Bax (2774), Bcl-2 (4223), PCNA (13110), cyclin D1 (55506), and β-tubulin (2146) were purchased from Cell Signaling Technology and used at 1:1000 dilution. The primary antibodies against collagen I (ab138492, 1:1000) and aggrecan (ab3773, 1:100) were obtained from Abcam. Quantification through densitometry of protein bands was performed using Image-Pro Plus software (Version 5.1; Media Cybernetics, Inc.). The experiment was performed at least in triplicate.

### 2.11 Statistical analysis

The results are presented as mean ± standard deviation. Mann–Whitney U test, a nonparametric test, was used to determine the statistical difference between groups. All statistical analyses were carried out using SPSS software (V11.0; SPSS, Inc.). Differences were considered statistically significant at p < 0.05. All quantitative results were calculated from a minimum of three biological replicates.

## 3 RESULTS

### 3.1 Identification of polarized M2 macrophages

THP-1 cells were cultured in a cell culture bottle in suspension medium and treated with PMA for 24 h; the cells gradually adhered to the bottom of the culture bottle as M0 macrophages (Figure 1B). Thereafter, the cells differentiated into M2 macrophages in the presence of IL-4 for another 24 h in serum-free medium, exhibiting a homogeneous elongated spindle-like morphology with slender tentacles (Figure 1B). The expression of M2 macrophage markers CD206 and IL-10 was significantly upregulated in M2-induced macrophages compared with that in M0 macrophages (both p < 0.01, Figure 1C,D). In addition, the primary mouse peritoneal macrophages were also identified by testing the expression of M2 macrophage markers CD206 and IL-10. Both genes were significantly upregulated in M2-induced macrophages compared with that in the control group (both p < 0.01, Figure S1B,C).

### 3.2 M2CM promoted the proliferation of TNF-α-treated NPCs

The CCK-8 assay was performed to evaluate the proliferative potential of NPCs. The OD of TNF-α-treated NPCs was significantly lower than that of the control group at 24, 48, and 96 h (all p < 0.05; Figure 2A). Interestingly, in the TNF-α-treated groups, cells cocultured with M2CM presented higher OD values than those treated with only TNF-α (all p < 0.05; Figure 2A). A significantly higher number of NPCs was detected in the M2CM coculture group compared to that in the TNF-α-treated groups at 24, 48, and 96 h (p < 0.05, p < 0.05, and p < 0.01, respectively; Figure 2B). In contrast, in the TNF-α-treated NPCs, a higher cell number was observed in the M2CM coculture group, with a significant difference at all time points (all p < 0.05; Figure 2B). In western blotting analysis, the cell proliferation markers, PCNA and cyclin D1 were significantly upregulated in M2CM (both p < 0.05; Figure 2C–E).

### 3.3 M2CM protected TNF-α-treated NPCs from apoptosis

The effect of M2CM on TNF-α-induced apoptosis of NPCs was assessed using the TUNEL assay. Following treatment with TNF-α for 3 days, the apoptotic rate gradually increased in NPCs compared with that in the control group (p < 0.001), whereas coculture with M2CM significantly mitigated this effect (p < 0.001; Figure 3A,B). In western blotting analysis, the expression of the proapoptotic protein Bax was upregulated, while that of the antiapoptotic protein Bcl-2 was downregulated in TNF-α-treated NPCs (p < 0.01 and p < 0.05, respectively); these effects were reversed upon coculturing with M2CM (both p < 0.05; Figure 3C–E).
3.4 | M2CM decreased senescence and inflammatory factor levels in TNF-α-treated NPCs

Cell senescence was analyzed via SA-β-gal staining and senescence-associated gene expression. The number of SA-β-gal-positive NPCs increased upon TNF-α treatment compared with that in the control group, after 7 days (p < 0.01; Figure 4A,B). However, when coculturing TNF-α-treated cells with M2CM, the number of SA-β-gal-positive cells significantly decreased (p < 0.05; Figure 4A,B). TNF-α significantly upregulated the expression of cell senescence-related genes, such as p16, p21, and p53 compared to that in the control group (p < 0.001, p < 0.01, and p < 0.05, respectively; Figure 4C–E); however, M2CM downregulated the expression of these markers following TNF-α treatment (all p < 0.05; Figure 4C–E).

To evaluate the effect of M2CM on ECM biosynthesis, immunofluorescence staining of collagen II in NPCs was performed (Figure 5A). After treatment with TNF-α for 3 days, the OD value significantly decreased (p < 0.01; Figure 5B); however, this effect was considerably inhibited in the coculture group (p < 0.05; Figure 5B). Western blotting indicated the downregulation of aggrecan and Sox-9 in the TNF-α-treated group (p < 0.05; Figure 5C–E); however, they were upregulated in the coculture group (p < 0.05; Figure 5D,E). Collagen I expression exhibited the reverse pattern (both p < 0.05; Figure 5F). We investigated ECM-related gene expression by evaluating the expression of genes encoding collagen type Iα1, collagen type IIα1, Sox-9, and aggrecan. The ECM-related genes (aggrecan, collagen Iα1, and Sox-9) were downregulated in TNF-α-treated NPCs (p < 0.01, p < 0.05, and p < 0.01, respectively; Figure 5G–I); M2CM significantly eliminated this inhibitory effect (both p < 0.05; Figure 5G–I). Conversely, the expression of collagen type Iα1 was further upregulated in the TNF-α-treated group (p < 0.05; Figure 5J); coculturing with M2CM significantly inhibited this increase (p < 0.05; Figure 5J). Therefore, M2CM potentially promotes matrix synthesis in TNF-α-treated NPCs.

3.5 | M2CM upregulated extracellular matrix (ECM) components in TNF-α-treated NPCs


3.6 M2CM decreased IVD degeneration in TNF-α-treated IVD organ cultures

To evaluate the effect of M2CM on ECM biosynthesis, HE staining of mouse IVD organ models was performed. After 2 weeks of culture, the NP tissue in the control group displayed a spherical morphology and accounted for 50% of the disc area; the NPCs displayed a stellar or spherical morphology. In contrast, in the TNF-α-treated group, the NP constituted 25%–50% of the disc area, and large, spherical NPCs were separated by dense areas of a proteoglycan matrix (Figure 6A). However, these degeneration effects were reversed in both NP and NPCs (Figure 6A). We analyzed the annulus fibrosus (AF) and annulus fibrosus cells (AFCs); the ruptured or serpentine fibers constituted <25% of the AF. Fibroblast-like cells comprised >75%–90% of the cells in the control group. Ruptured or serpentine fibers constituted >50% of the AF with chondrocyte-like cells comprising >75% of the cells in the TNF-α-treated group (Figure 6A). Coculture in M2CM inhibited the degeneration of both AF and AFCs (Figure 6A). The border between the NP and AF resulted from minimal, moderate, and severe interruption in the control, coculture, and TNF-α-treated groups, respectively (Figure 6A). The modified histological grading system of IVD was used for quantitative analysis (Table S2). The TNF-α-treated group displayed significant degeneration-related morphological changes with higher scores (p < 0.01, Figure 6B), whereas the coculture group displayed significant decrease with lower scores (p < 0.05, Figure 6B), compared to that in the control group. We investigated ECM synthesis by evaluating the expression of genes encoding collagen type II α1 and aggrecan. The expression of ECM proteins (aggrecan and collagen II α1) was reduced in the TNF-α-treated mouse IVD model (both p < 0.01, Figure 6C,D); however, M2CM significantly abolished this inhibitory effect (both p < 0.05, Figure 6C,D). In the cell apoptotic process, the proapoptotic protein Bax and antiapoptotic protein Bcl-2 are key regulators. Bax was upregulated and Bcl-2 was downregulated in TNF-α-treated mouse IVD model (Figure 6E); this trend was reversed with the coculture in M2CM. In the Bax/Bcl-2 ratio assay, the TNF-α-treated IVDs cultured with TNF-α displayed a significantly higher ratio (p < 0.01; Figure 6E), whereas this aberrant ratio was significantly attenuated by the M2CM treatment (p < 0.05; Figure 6E). Cell senescence was analyzed through senescence-related gene analysis; TNF-α significantly upregulated the expression of p16 and p21 compared to that in the control group (both p < 0.05; Figure 6E,F). However, M2CM
treatment decreased the expression of these markers following the TNF-α treatment (both *p < 0.05; Figure 6E,F).

3.7 | Influence of M2CM derived from mouse primary macrophages on IDD in TNF-α-treated IVD organ culture models

To further test the biological effects of M2CM derived from mouse primary macrophages, we repeated the IVD organ culture experiments by qPCR analysis. After 2 weeks of culture, the cell proliferation marker, PCNA was significantly downregulated in TNF-α-treated NPCs compared to that in the control group (*p < 0.01; Figure 7A); however, coculture of TNF-α-treated NPCs with M2CM upregulated the expression of PCNA (both *p < 0.05, **p < 0.01; Figure 7A). Bax was upregulated and Bcl-2 was downregulated in TNF-α-treated models (both *p < 0.05, **p < 0.01, ***p < 0.001). Statistical significance was determined by Mann–Whitney U test, and all data are shown as mean ± standard deviation. IL, interleukin; MMP, matrix metalloproteinase; mRNA, messenger RNA; NPC, nucleus pulposus cell; TNF, tumor necrosis factor.
M2CM treatment decreased the expression of these markers following TNF-α treatment ($p < 0.01; p < 0.05$; Figure 7F,G). The effect of TNF-α on the secretion of inflammatory factors, such as IL-6 and extracellular proteases, such as MMP-13 was assessed. These genes were significantly upregulated in the NPCs compared to that in the control group (both $p < 0.001$; Figure 7H,I); however, M2CM significantly downregulated these markers following TNF-α treatment (both $p < 0.01$; Figure 7H,I). Therefore, M2CM derived from mouse

**FIGURE 5** Effects of macrophage-conditioned medium (M2CM) on the expression of matrix macromolecules in TNF-α-treated NPCs. Representative images of immunofluorescence staining (A) and quantification analysis of collagen II protein expression (B) in the three groups after 3 days of culture; $n = 3$ donors, *$p < 0.05$, **$p < 0.01$. Western blot analysis (C) and densitometric quantification of aggrecan (D), Sox-9 (E), and collagen I (F) proteins in the three groups; $n = 3$ donors, *$p < 0.05$. The mRNA expression levels of aggrecan (G), collagen IIα1 (H), Sox-9 (I), and collagen Iα1 (J) in NPCs were compared among different groups after 3 days of culture. $n = 3$ donors, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Statistical significance was determined by Mann–Whitney $U$ test, and all data are shown as mean ± standard deviation. mRNA, messenger RNA; NPC, nucleus pulposus cell; TNF, tumor necrosis factor.
primary macrophages displayed promising effects against IDD in a TNF-α treated environment.

4 | DISCUSSION

M2 macrophages down-regulate the expression of proinflammatory cytokines and promote tissue remodeling by producing antiinflammatory cytokines, such as TGF-β, vascular endothelial growth factor, IL-10, and insulin-like growth factor. M2CM can regenerate cartilage tissue, which have similarities with IVD tissue. However, whether it exerts similar effects in NPCs during IDD progression has remained unknown. Therefore, we compared these effects in cells with or without TNF-α treatment and cultured in M2CM. The protective ability of M2CM in IDD was mediated through the promotion of cell proliferation; synthesis of ECM; and inhibition of inflammation, apoptosis, and NPC senescence, thereby, indicating a potential in alleviating IDD. Our results provide fundamental evidence for the clinical application of M2CM therapies.
Macrophage reprogramming, characterized by the transformation of M1 to M2 macrophages, is an effective alternative treatment for osteoarthritis.19 An imbalance in the proportion of M1/M2 is observed in both patients and mouse models, suggesting that it could contribute to IDD pathophysiology.9,13 In this study, we successfully induced the differentiation of THP-1 cells and mouse primary macrophages to M2 macrophages, as previously described.10,11 The change in cell morphology and significant upregulation of the M2 surface markers, CD206 and IL-10 in both cells indicated that polarization was successful. In the conditioning medium, we used 30% M2CM, as reported previously.36,37 We successfully induced the generation of M2 macrophages and harvested M2CM by supplementing THP-1 cells with IL-4.

Disc degeneration results in a concomitant reduction in the number of NPCs, which are responsible for the production of NP matrix components.38 In this study, NPC proliferation decreased in the TNF-α-treated group; however, it was restored upon M2CM coculture. The increase in cell number was attributed to an increase in cell proliferation rather than the inhibition of cell death. These data are critical for the development of potential treatments aimed at inhibiting the reduction in cell number observed during IDD.38 This pro-proliferative effect can be potentially attributed to several cytokines, such as TGF-β1, ARG-1, and chemokine ligand 18 (CCL-18), which reportedly promote cell proliferation.39,40 The coculture with M2CM decreased the incidence of NPC apoptosis. Excessive disc cell apoptosis, leading to decreased cell density and ECM catabolism, exerts a detrimental effect in IDD.41,42 The TUNEL assay showed that the increased apoptotic rate of NPCs treated with TNF-α was significantly reversed upon coculturing with M2CM. Furthermore, the expression of the proapoptotic protein Bax and the antiapoptotic

FIGURE 7 Influence of macrophage-conditioned medium (M2CM) derived from mouse primary macrophages on IDD in TNF-α-treated IVD organ culture models. The mRNA expression levels of cell proliferation markers PCNA (A), cell apoptotic related gene Bax (B) and Bcl-2 (C), cell senescence gene markers P16 (F) and P21 (G), the expression of genes encoding collagen type IIα1 (E) and aggrecan (F), as well as the inflammatory factors IL-6 (H) and extracellular proteases MMP-13 (I) in IVDs were compared among different groups after 14 days of culture. n = 5, *p < 0.05, **p < 0.01, ***p < 0.001. Statistical significance was determined by Mann–Whitney U test, and all data are shown as mean ± standard deviation. IDD, intervertebral disc degeneration; IL, interleukin; mRNA, messenger RNA; TNF, tumor necrosis factor
protein Bcl-2 was significantly upregulated and downregulated, respectively, in TNF-α-treated NPCs. These changes were significantly inhibited upon coculturing with M2CM; a similar trend was observed in the IVD culture model. Aberrant apoptosis of NPCs is considered a major cellular phenomenon associated with IDD.43 Our results are further supported by that from Dai et al; increasing M2 macrophage polarization reduces cell apoptosis in degenerative osteoarthritis, and the increasing TGF-β probably contributes to this.44 Therefore, the apoptotic rate of TNF-α-treated NPCs was reduced upon coculturing with M2CM.

The gradual increase in NPC senescence during IDD has a detrimental effect by decreasing the number of functional cells45; therefore, the inhibition of NPC senescence is an important strategy for IDD treatment. SA-β-gal is a senescent cell biomarker.40 In this study, a significantly lower percentage of SA-β-gal-positive cells were observed in the coculture group than that in the TNF-α group, indicating a protective role of M2CM on NPC senescence; the same trend was detected in the IVD culture model. Therefore, M2CM could attenuate the premature senescence of NPCs in an inflammatory microenvironment. The telomere-based p53–p21–pRb pathway and the stress-based p16–pRb pathway are the predominant pathways in IDD.46 Although our results suggest that both pathways mediate the effect of TNF-α on NPCs in vitro, the extrinsic factor p16 probably plays a particularly crucial role in the exogenous TNF-α treatment process. Moreover, coculture with M2CM downregulated the proinflammatory cytokines (IL-6) and extracellular proteases (MMP-13, ADAMTS-4, and ADAMTS-5), which is a widely accepted risk factor in disc degeneration.

The balance between ECM anabolism and catabolism by disc cells is disrupted by proinflammatory cytokines during IDD.47 In this study, we used exogenous TNF-α stimulation to mimic the degeneration environment, and we found that coculture with M2CM increases ECM synthesis by promoting both gene and protein expression of aggrecan and collagen IIα1. Conversely, the increasing expression of collagen Iα1 was characterized as the fibrotic change. Therefore, the downregulation of collagen Iα1 expression suggests that the inhibitory effect of M2CM during fibrosis further facilitates IVD regeneration. This result was in accordance with that from previous studies; M2 macrophages attenuate fibrosis progression by secreting multiple cytokines in wound healing process.48 Taken together, these results indicate that coculture with M2CM promotes ECM synthesis in TNF-α-treated NPCs. M2 macrophages contribute to tissue repair by producing antiinflammatory cytokines such as TGF-β, IL-10, and angiogenic vascular endothelial growth factor. In addition, M2CM exhibit prochondrogenic effects by inducing the secretion of cytokines, such as TGF-β, ARG-1, and CCL-18.19 Therefore, we speculate that these factors may be key regulators in the protective effect of M2CM on TNF-α-treated NPCs. However, further studies are required to further characterize these factors for clinical application.

Finally, three-dimensional mouse organ culture model for IVD is widely used to investigate the effects of specific treatment strategies in a controlled environment.49–51 However, the use of mouse models has certain limitations; they do not reflect the nutritional deficiencies, collagen disorganization, or cell death, which are often seen in chronic human IDD.52 In addition, disc size, biomechanical stress (less axial compression or torsion), and cell composition (primarily notochordal cells) could be very different from that in human IDD.53 Last, the mouse tail is non weight-bearing; therefore, the biomechanics of the vertebra-disc-vertebra motion segment differ from that of the human lumbar spine.54 In spite of the small number of previous mouse IDD models, the tail injury method has certain advantages, such as easy access to the tail disc, low morbidity, and a reproducible course of degeneration. One main advantage of the mouse model is the ability to perform genetic manipulations during IDD and the possibility to test a large sample. Mice have been used successfully for knee OA studies; therefore, they are promising for IDD research, considering the surgical precision.53,55,56 We used the mouse IVD organ culture model to assess the biological effect of M2CM in TNF-α-supplemented medium. A modified histological scoring system was used to analyze the protective effect of M2CM.57 Treatment with only TNF-α significantly increased the score in the IVD organ culture model, whereas coculturing with M2CM inhibited this effect. Whether the M2CM originating from THP-1 or primary cell, both kinds of M2CM considerably attenuated the downregulation of collagen Iα1 and ACAN expression, demonstrating the regeneration potential of M2CM in the three-dimensional model. Bax was downregulated and Bcl-2 was upregulated in M2CM derived primary cells, and the Bax/Bcl-2 ratio was significantly attenuated by the M2CM derived from THP-1. Cell senescence increased with TNF-α treatment and decreased with both types of M2CM treatment. Finally, the two kinds of M2CM treatment decreased the expression of inflammatory markers following TNF-α treatment. These findings further strengthen our conclusion that M2CM exhibits protective effects against IDD through the promotion of cell proliferation; synthesis of ECM; and inhibition of inflammation, apoptosis, and NPC senescence.

This study has certain limitations. First, the microenvironment regulated by M1 and M2 macrophages usually exhibits a dynamic balance between the pro- and antiinflammatory effects57; therefore, it is difficult to completely mimic this process in the TNF-α treated environment. Second, the present conclusion is based on evidence obtained from in vitro and IVD culture models; the therapeutic effects on disc degeneration in vivo need to be assessed in an animal model. In addition, only samples from male patients were selected in the study. Finally, the precise cytokines and the mechanism of action of M2CM remain unclear and warrant further investigation; our future work will focus on elucidating the underlying mechanisms.

5 | CONCLUSIONS

M2CM positively influences NPCs and IVD culture models in an antiinflammatory microenvironment by increasing cell proliferation, decreasing cellular senescence and apoptosis, and promoting ECM accumulation. This study provides novel insights into the therapeutic
potential of M2CM in IDD. Further studies are warranted to provide adequate evidence for the therapeutic potential of inducing resident IVD cells to produce sufficient ECM for IDD treatment.

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CONFLICT OF INTERESTS
The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS
Xiao-Chuan Li, Mao-Sheng Wang, and Chun-Ming Huang: conceived and designed the experiments; Xiao-Chuan Li, Shao-Jian Luo, Tian-Li Zhou, Wen Chen, and Wu Fan: performed, analyzed, and interpreted the data; and Xiao-Chuan Li and Mao-Sheng Wang: provided reagents and reviewed the manuscript. Xiao-Chuan Li and Mao-Sheng Wang: provided reagents and reviewed the manuscript for intellectual content. All authors have read and approved the final submitted manuscript.

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REFERENCES
1. Deyo RA, Mirza SK. Clinical practice. Herniated Lumbar Intervertebral Disk. N Engl J Med. 2016;374:1763-1772.
2. Bailey CS, Rasoulinejad P, Taylor D, et al. Surgery versus conservative care for persistent sciatica lasting 4 to 12 months. N Engl J Med. 2020;382:1093-1102.
3. Ramaswami R, Ghogawala Z, Weinstein JN. Management of Sciatica. N Engl J Med. 2017;376(12):1175-1177.
4. Liu ZH, Sun Z, Wang HQ, et al. FasL expression on human nucleus pulposus cells contributes to the immune privilege of intervertebral disc by interacting with immunocytes. Int J Med Sci. 2013;10:1053-1060.
5. Sun Z, Liu B, Luo ZJ. The immune privilege of the intervertebral disc: implications for intervertebral disc degeneration treatment. Int J Med Sci. 2017;13:995-1001.
6. Kawakubo A, Uchida K, Miyagi M, et al. 2020. Investigation of resident and recruited macrophages following disc injury in mice. J Orthop Res 38:1703-1709.
7. Yang C, Cao P, Gao Y, et al. Differential expression of p38 MAPK alpha, beta, gamma, delta isoforms in nucleus pulposus modulates macrophage polarization in intervertebral disc degeneration. Sci Rep. 2016;6:22182.
8. Wiet MG, Picioneri A, Khan SN, Ballinger MN, Hoyland JA, Purmessur D, Mast Cell-Intervertebral disc cell interactions regulate inflammation, catabolism and angiogenesis in Discogenic Back Pain. Sci Rep. 2017;7:12492.
9. Nakazawa KR, Walter BA, Lauzier DM, et al. Accumulation and localization of macrophage phenotypes with human intervertebral disc degeneration. Spine J. 2017;18:343-356.
10. Li L, Wei K, Ding Y, et al. M2a macrophage-secreted CHI3L1 promotes extracellular matrix metabolic imbalances via activation of IL-13Ralpha2/MAPK pathway in rat intervertebral disc degeneration. Front Immunol. 2021;12:666361.
11. Zhao F, Guo Z, Hou F, Fan W, Wu B, Qian Z. Magnoflorone alleviates “M1” polarized macrophage-induced intervertebral disc degeneration through repressing the HMGB1/Myc88/NE-kappaB pathway and NLRP3 in inflammation. Front Pharmacol. 2021;12:701087.
12. Miyagi M, Uchida K, Takano S, et al. 2018. Macrophage-derived inflammatory cytokines regulate growth factors and pain-related molecules in mice with intervertebral disc injury. J Orthop Res. Online ahead of print.
13. Silva AJ, Ferreira JR, Cunha C, et al. Macrophages down-regulate gene expression of intervertebral disc degenerative markers under a pro-inflammatory microenvironment. Front Immunol. 2019;10:1508.
14. Lee S, Millecamps M, Foster DZ, Stone LS. Long-term histological analysis of innervation and macrophage infiltration in a mouse model of intervertebral disc injury-induced low back pain. J Orthop Res. 2019;38:1238-1247.
15. Miyagi M, Uchida K, Takano S, et al. 2021. Role of CD14-positive cells in inflammatory cytokine and pain-related molecule expression in human degenerated intervertebral discs. J Orthop Res 39:1755-1762.
16. Nakawaki M, Uchida K, Miyagi M, et al. Changes in nerve growth factor expression and macrophage phenotype following intervertebral disc injury in mice. J Orthop Res. 2019;37:1798-1804.
17. Nakazawa KR, Walter BA, Lauzier DM, et al. Accumulation and localization of macrophage phenotypes with human intervertebral disc degeneration. Spine J. 2018;18:343-356.
18. Wynn TA, Chawla A, Pollard JW. Macrophage biology in development, homeostasis and disease. Nature. 2013;496:445-455.
19. Zhang H, Cai D, Bai X. Macrophages regulate the progression of osteoarthritis. Osteoarthritis Cartilage. 2020;28:555-561.
20. Chanput W, Mes JJ, Wichers HJ. THP-1 cell line: an in vitro cell model for immune modulation approach. Int Immunopharmacol. 2014;23:37-45.
21. Ma Y, Mouton AJ, Lindsey ML. Cardiac macrophage biology in the steady-state heart, the aging heart, and following myocardial infarction. Transl Res. 2018;191:15-28.
22. Wang L, Zhang Y, Zhang N, Xia J, Zhan Q, Wang C. Potential role of M2 macrophage polarization in ventilator-induced lung fibrosis. Int Immunopharmacol. 2019;75:105795.
23. Zhang J, Muri J, Fitzgerald G, et al. Endothelial lactate controls muscle regeneration from ischemia by inducing M2-like macrophage polarization. Cell Metab. 2020;31:1136-1153.
24. Gensel JC, Zhang B. Macrophage activation and its role in repair and pathology after spinal cord injury. Brain Res. 2015;1619:1-11.
25. Bogatcheva NV, Coleman ME. Conditioned medium of mesenchymal stromal cells: a new class of therapeutics. Biochemistry (Mosc). 2019;84:1375-1389.
26. Monchaux M, Forforde S, Spreng D, Karol A, Forforde F, Wueertz Lankamp CLA. The contradictory effect of macrophage-related cytokine expression in lumbar disc herniations: a systematic review. Eur Spine J. 2019;28:1649-1659.
31. Tardito S, Martinelli G, Soldano S, et al. Macrophage M1/M2 polarization and rheumatoid arthritis: a systematic review. Autoimmun Rev. 2019;18:102397.

32. Vizcaino Revés N, Mogel HM, Stoffel M, Summerfield A, Forterre F. Polarization of macrophages in epidermal inflammation induced by canine intervertebral disc herniation. Front Vet Sci. 2020;7:32.

33. Kim JH, Studer RK, Vo NV, Sowa GA, Kang JD. p38 MAPK inhibition selectively mitigates inflammatory mediators and VEGF production in AF cells co-cultured with activated macrophage-like THP-1 cells. Osteoarthritis Cartilage. 2009;17:1662-1669.

34. Park HC, Quan H, Zhu T, Kim Y, Kim B, Yang HC. The effects of M1 and M2 macrophages on odontogenic differentiation of human dental pulp cells. J Endod. 2017;43:596-601.

35. Lee S, Millecamps M, Foster DZ, Stone LS. Long-term histological analysis of innervation and macrophage infiltration in a mouse model of intervertebral disc injury-induced low back pain. J Orthop Res. 2019;38:1238-1247.

36. Yang H, Liu B, Liu Y, et al. Secreted factors from intervertebral disc cells and infiltrating macrophages promote degenerated intervertebral disc catabolism. Spine (Phila Pa). 2018;1976(44):E520-E529.

37. Ni L, Zheng Y, Gong T, et al. Proinflammatory macrophages promote degenerative phenotypes in rat nucleus pulposus cells partly through ERK and JNK signaling. J Cell Physiol. 2019;234:5362-5371.

38. Long J, Wang X, Du X, et al. JAG2/Notch2 inhibits intervertebral disc degeneration by modulating cell proliferation, apoptosis, and extracellular matrix. Arthritis Res Ther. 2019;21:213.

39. Liu JAOX, Li SM. MiR-484 suppressed proliferation migration, invasion and induced apoptosis of gastric cancer via targeting CCL-18. Int J Exp Pathol. 2020;101:203-214.

40. Wang R, Xu B, Xu H. TGF-β1 promoted chondrocyte proliferation by regulating Sp1 through MSC-exosomes derived miR-135b. Cell Cycle. 2018;17:2756-2765.

41. Ding F, Shao ZW, Xiong LM. Cell death in intervertebral disc degeneration. Apoptosis. 2013;18:777-785.

42. Wang F, Cai F, Shi R, Wang XH, Wu XT. Aging and age related stresses: a senescence mechanism of intervertebral disc degeneration. Osteoarthritis Cartilage. 2016;24:398-408.

43. Zhao Y, Wang H, Zou L, et al. SIRT1 attenuates apoptosis of nucleus pulposus cells by targeting interactions between LC3B and Fas under high-magnitude compression. Oxid Med Cell Longev. 2021;2021:2420969.

44. Dai M, Sui B, Xue Y, Liu X, Sun J. Cartilage repair in degenerative osteoarthritis mediated by squid type II collagen via immunomodulating activation of M2 macrophages, inhibiting apoptosis and hypertrophy of chondrocytes. Biomaterials. 2018;180:91-103.

45. Dimri GP, Lee X, Basile G, et al. A biomarker that identifies senescent human cells in culture and in aging skin in vivo. Proc Natl Acad Sci USA. 1995;92:9363-9367.

46. Zhao Y, Jia Z, Huang S, et al. Age-related changes in nucleus pulposus mesenchymal stem cells: an in vitro study in rats. Stem Cells Int. 2017;2017:6761572.

47. Feng C, Liu H, Yang M, Zhang Y, Huang B, Zhou Y. Disc cell senescence in intervertebral disc degeneration: causes and molecular pathways. Cell Cycle. 2016;15:1674-1684.

48. Li S, Gao S, Jiang Q, et al. Clevudine attenuates bleomycin-induced early pulmonary fibrosis via regulating M2 macrophage polarization. Int Immunopharmacol. 2021;101:108271.

49. Du J, Pfannkuche JJ, Lang G, et al. Proinflammatory intervertebral disc cell and organ culture models induced by tumor necrosis factor alpha. JOR Spine. 2020;3:1104.

50. Pfannkuche JJ, Guo W, Cui S, et al. Intervertebral disc organ culture for the investigation of disc pathology and regeneration - benefits, limitations, and future directions of bioreactors. Connect Tissue Res. 2020;60:304-321.

51. Lang G, Liu Y, Gerisy J, et al. An intervertebral disc whole organ culture system to investigate proinflammatory and degenerative disc disease condition. J Tissue Eng Regen Med. 2018;12: e2051-e2061.

52. Alini M, Eisenstein SM, Ito K, et al. Are animal models useful for studying human disc disorders/degeneration? Eur Spine J. 2008;17:2-19.

53. Saka D, Nishimura K, Tanaka M, et al. Migration of bone marrow-derived cells for endogenous repair in a new tail-looping disc degeneration model in the mouse: a pilot study. Spine J. 2015;15:1356-1365.

54. Tian Z, Ma X, Yasen M, et al. Intervertebral disc degeneration in a percutaneous mouse tail injury model. Am J Phys Med Rehabil. 2018;97:170-177.

55. Ichi D, Taniguchi Y, Soma K, et al. A mouse intervertebral disc degeneration model by surgically induced instability. Spine (Phila Pa). 2018;1976(43):E557-E564.

56. Yang F, Leung VY, Luk KD, Chan D, Cheung KM. Injury-induced sequential transformation of notochordal nucleus pulposus to chondrogenic and fibrocartilaginous phenotype in the mouse. J Pathol. 2009;218:113-121.

57. Ji ML, Jiang H, Zhang XJ, et al. Preclinical development of a microRNA-based therapy for intervertebral disc degeneration. Nat Commun. 2018;9:5051.

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