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M2-like macrophages derived from THP-1 cells promote myofibroblast differentiation of synovial fibroblasts in association with the TGF-β1/SMAD2/3 signaling pathway

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Fibrosis occurs during progression of osteoarthritis (OA), and myofibroblasts are considered a key effector to drive the fibrotic response. Macrophages also play critical roles in OA progression. However, whether macrophage polarization is involved in OA-related fibrosis has not been adequately defined. Here, we investigated the effect of M2-like macrophages compared to M1-like macrophages on the myofibroblast differentiation of human synovial fibroblasts (HSFs). M1- and M2-like macrophages differentiated from the human monocytic THP-1 cells were co-cultured with HSFs for 72 h. Alpha-smooth muscle actin (α-SMA) positive cells and gene expression of pro-fibrotic and anti-fibrotic factors were quantified. The concentration of transforming growth factor-beta1 (TGF-β1) in the culture supernatant was also analyzed, and its effect on the regulation of the TGF-β/SMAD signaling pathway was further investigated. We found that, cocultured with M2-like macrophages increased the number of α-SMA positive cells and expression of pro-fibrotic genes and decreased the expression of anti-fibrotic genes in HSFs. TGF-β1 was highly secreted by M2-like macrophages and accelerated the phosphorylation of SMAD2/3 in HSF cells. Our results indicate the pro-fibrotic effects of M2-like macrophages in myofibroblast differentiation of HSFs, in association with the TGF-β1/SMAD2/3 signaling pathway activation. Thus, M2-like macrophages may play a role in OA fibrogenesis and its progression.

Keywords Osteoarthritis, Fibrosis, Macrophages, Human synovial fibroblast, Myofibroblast, TGF-β/SMAD signaling

Osteoarthritis (OA) is the most common chronic joint disease worldwide. Knee OA is the most prevalent, leading to disability in the aging population due to pain and impaired joint function. Rather than a "wear-and-tear" disease, OA is now considered a multifactorial disease that involves local and systemic factors and has various pathogenetic mechanisms. In addition to cartilage degeneration and osteophyte formation, accumulating evidence has revealed that fibrosis, involving synovial fibrosis and fibrocartilage formation, contributes significantly to OA pathogenesis and progression[1–4]. While chronic inflammation is predominant in the early stages of OA, fibrosis is frequently observed in the late stages[1,2,5]. In synovial fibrosis and fibrocartilage formation, myofibroblast is considered the effector cell that drives pathological fibrogenesis through excessive extracellular matrix (ECM) production and deposition[1–3]. However, how and which specific stimuli are responsible for myofibroblast differentiation is not fully understood.

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Macrophages have long been considered to play a critical role in the innate immune system of joints, along with synovial fibroblasts, the two dominant effector cells in the synovial lining. Polarized phenotypes of macrophages correlate with the progression of OA[6]. Two different macrophage polarization statuses have been identified when confronted with different stimuli. In the initiation phase of inflammation, classically activated M1 macrophages stimulated by lipopolysaccharide (LPS) and interferon-gamma (IFN-γ) are recruited and secrete large amounts of pro-inflammatory cytokines and chemokines, such as tumor necrosis factor-alpha (TNF-α), interleukin (IL)−1, and IL-6. M1 macrophages also promote the production of excess matrix metalloproteinases (MMPs), which enhances ECM degradation. Subsequently, cartilage degradation and bone erosion arise over time in response to proteases and inflammation. In contrast, alternatively activated M2 macrophages display anti-inflammatory properties and are implicated in the tissue repair and remodeling phase. M2 macrophages induced by IL-4 and IL-13 downregulate the inflammatory process by releasing anti-inflammatory mediators, such as IL-10, and secreting growth factors, such as transforming growth factor-beta 1 (TGF-β1)[7]. TGF-β1 is considered a key molecule in the activation of tissue generation and fibrotic progression. TGF-β1 targeting fibroblasts exerts their functions predominantly by activating canonical signaling, also known as the suppressor of mothers against decapentaplegic (SMAD) signaling pathway. The binding of the ligand to the receptor complex constitutively phosphorylates and induces the intracellular signaling molecules SMAD2 and SMAD3, which combine with common SMAD4, and then translocate into the nucleus where these molecules mediate TGF-β1-regulated gene expression[8]. Affected by these signals, fibroblasts can be activated and differentiated into myofibroblasts, characterized by the expression of alpha-smooth muscle actin (α-SMA) and the synthesis of ECM, such as type 1 collagen.

Through different stages of OA, the polarization of macrophages might account for various pathological processes, such as chronic inflammation, persistent fibroblast activation due to the hyperactivation of the TGF-β1 pathway, and unabated myofibroblast proliferation, coupled with excessive ECM deposition. The production of untracked fibrous tissue, which mainly contains type I collagen, can disrupt the typical healing cascade and ultimately lead to pathological fibrosis of joint tissue[9,10]. Nevertheless, it remains to be fully elucidated whether and how macrophages and mediators, particularly the TGF-β1 signaling pathway, regulate myofibroblast differentiation in the fibrosis stage of OA.

To better understand fibrosis progression in OA, we developed an in vitro fibroblast/macrophage co-culture system to mimic in vivo environments of two different stages, inflammation and fibrosis, of OA progression and reflect the cooperative interaction of the two cell types with a particular emphasis on their roles in fibrogenic activities. In this study, M1- and M2-like macrophages differentiated from human THP-1 cell lines were co-cultured with HSFs derived from patients with OA undergoing knee arthroplasty. The effect of M1- and M2-like macrophages on myofibroblast differentiation of HSFs was investigated, and the molecular mechanism based on the TGF-β/SMAD signaling pathway was further studied.

Material and method

This study was approved by the Research Ethics Committee of the University of Toyama (approval number: R2018059). Written informed consent was obtained from all the patients prior to specimen collection. All methods were performed in accordance with the relevant guidelines and regulations.

Cell culture and treatment

Human monocytic THP-1 cells were maintained in culture with Roswell Park Memorial Institute medium (RPMI 1640; Thermo Fisher Scientific, Waltham, MA, USA) containing 10% heat-inactivated fetal bovine serum (FBS; Thermo Fisher Scientific, Waltham, MA, USA) and supplemented with 1% antibiotics (100 U/mL penicillin, 100 mg/mL streptomycin). THP-1 monocytes were differentiated into uncommitted M0 macrophages by a 48-h incubation with 100 nM phorbol 12-myristate 13-acetate (PMA, #P8139; Sigma-Aldrich, St. Louis, MO, USA) followed by a 24-h incubation in PMA-free medium (Fig. 1A). Macrophages were then polarized into M1 macrophages by incubation with 20 ng/mL IFN-γ (#285-IF; R&D Systems, Minneapolis, MN, USA) and 10 pg/mL LPS (#8630; Sigma-Aldrich, St. Louis, MO, USA) for 24 h (Fig. 1A). Macrophage M2 polarization was obtained by incubation with 20 ng/mL IL-4 (#204-IL; R&D Systems, Minneapolis, MN, USA) and 20 ng/mL IL-13 (#213-ILB; R&D Systems, Minneapolis, MN, USA) in the presence of 5 nM PMA for 24 h after treatment with 100 nM PMA for 24 h, followed by incubation without PMA for an additional 48 h (Fig. 1A).

HSFs were harvested and isolated from synovium obtained from patients who underwent total knee arthroplasty at Toyama University Hospital. The synovium was cut into small fragments and subjected to enzymatic digestion with collagenase. The cells were then filtered through a 70 μm pore diameter nylon mesh (Corning Gilbert, Glendale, AZ, USA) and centrifuged at 1600 rpm. After the supernatant was discarded, the resulting pellet was washed twice with phosphate-buffered saline (PBS; Sigma-Aldrich, St. Louis, MO, USA). Cells were cultured in a 10 cm culture dish with Dulbecco's Modified Eagle Medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) at 37 °C in an atmosphere of 5% CO_2. The medium was supplemented with 10% FBS and 1% antibiotic mixture (100 U/mL penicillin and 100 mg/mL streptomycin); the medium is hereafter referred to as 'complete DMEM'.

Confirmation of differentiation of M1 and M2 macrophages

To characterize macrophage phenotypes prior to co-culture experiments, cell morphology, RT-qPCR, and flow cytometry analysis were performed. THP-1 cells were cultured in 12-well plates (1.0×10^5 cells/well) and polarized into M0, M1, and M2 macrophages. Phase contrast images of cells were captured using an Eclipse TE300 inverted microscope (Nikon, Tokyo, Japan).

For the confirmation of differentiation by real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR), total RNA was extracted using a PureLink RNA Mini Kit (Thermo Fisher Scientific,

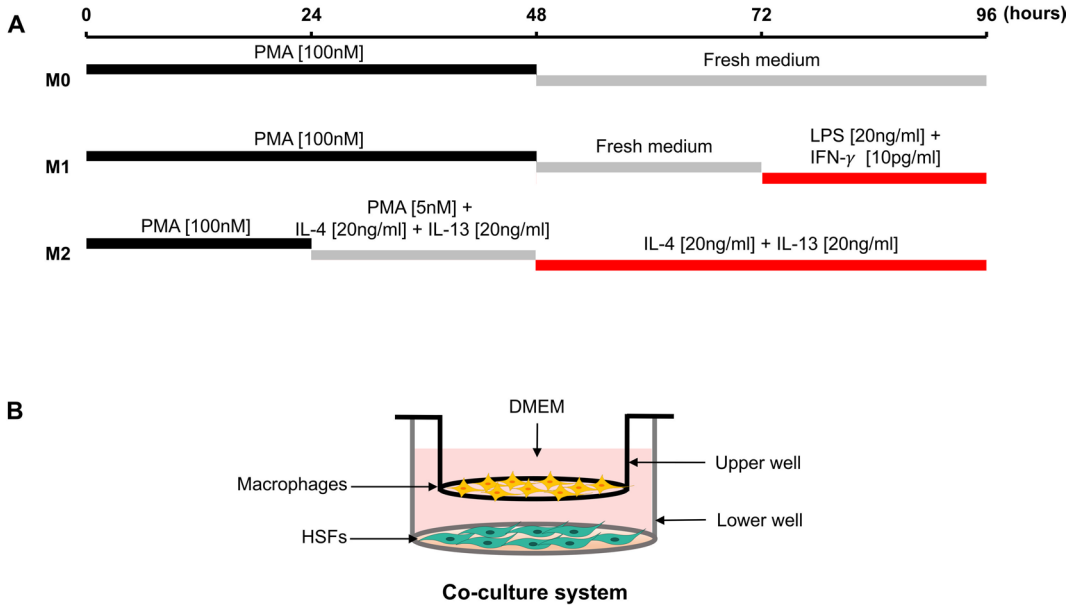

Fig. 1. Schematic illustration of macrophage differentiation and co-culture system. **(A)** Workflow for macrophage differentiation and activation towards M1 and M2-like macrophages from THP-1. **(B)** THP-1-derived macrophages and HSFs were co-cultured together using indirect contact in Transwell plates. HSFs were placed in the wells and macrophages were placed in the inserts.

Gene	Direction	Primer sequence (5'- 3')
CD80	Forward	ATCACCATCCAAGTGTCCATACCTC
	Reverse	AGAAACATTGTGACCACAGGACAG
CD206	Forward	TCGGGTTTATGGAGCAGGTG
	Reverse	TGAACGGGAATGCACAGGT
α-SMA	Forward	GAGAAGAGTTACGAGTTGCCTGATG
	Reverse	GGCAGCGGAAACGTTCA
COL1A1	Forward	CGAAGACATCCCACCAATCAC
	Reverse	TCATCGCACAACACCTTGC
CTGF	Forward	TCCACCCGGGTTACCAATG
	Reverse	CAGGCGGCTCTGCTTCTCTA
TIMP-1	Forward	CGCTGACATCCGGTTCGT
	Reverse	GTGGAAGTATCCGCAGACACTCT
MMP-1	Forward	TCGCTGGGAGCAAACACA
	Reverse	TTGGCAAATCTGGCGTGAA
MMP-2	Forward	TACAGGATCATTGGCTACACACC
	Reverse	GGTCACATCGCTCCAGACT
MMP-9	Forward	TGTACCGCTATGGTTACACTCG
	Reverse	GGCAGGGACAGTTGCTTCT
TGF-β1	Forward	ACCCACAACGAAATCTATGAC
	Reverse	GCTCCACTTTTAACTTGAGCC
GAPDH	Forward	ACCCACTCCTCCACCTTTGA
	Reverse	CTGTTGCTGTAGCCAAATTCGT

Table 1. Human primer sequences used in RT-qPCR.

Waltham, MA, USA). cDNA synthesis was performed using a High-Capacity RNA-to-cDNA Kit (Thermo Fisher Scientific, Waltham, MA, USA) with a GeneAmp PCR System 9700 thermal cycler (Thermo Fisher Scientific, Waltham, MA, USA). RT-qPCR analyses were performed using the iTaq Universal SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) with the CFX Connect (Bio-Rad, Hercules, CA, USA). Primers for *CD80* and *CD206* were used (Invitrogen, Waltham, MA, USA). Table 1 lists the primer sequences used for RT-qPCR. All experiments were performed in accordance with the manufacturer's instructions. The expression of target genes

was normalized to that of the reference gene, glyceraldehyde phosphate dehydrogenase (*GAPDH*), and evaluated using the $2^{-\Delta\Delta Ct}$ method.

To confirm differentiation by flow cytometry, polarized cells were analyzed using a FACS Canto^{II} (BD, Franklin Lakes, NJ, USA). Approximately 1.0×10^5 cells were resuspended in 0.5 mL PBS with 0.5% bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO, USA), followed by incubation with a 1:100 dilution of FcR blocking reagent (#130-059-901; Miltenyi Biotec, Bergisch Gladbach, Germany). Fluorescently labeled antibodies, a-CD14-fluorescein isothiocyanate (FITC) (M5E2, #555397; BD Bioscience, Heidelberg, Germany, 1:50), a-CD80-allophycocyanin (APC) (2D10, #305219; BioLegend, San Diego, CA, USA, 1:100), and a-CD206-phycoerythrin (PE) (19.2, #555954; BD Bioscience, Heidelberg, Germany, 1:50), were added to the samples at 4 °C in the dark according to the manufacturer's protocol. Isotype-specific controls, PE mouse IgG Isotype Control (MOPC-21, #551436; 1:200), APC mouse IgG1 K Isotype Control (MOPC-21, #550854; 1:200), and FITC mouse IgG Isotype Control (MOPC-21, #551954; 1:200), all from BD Bioscience (Heidelberg, Germany), were used. Dead cells were excluded by gating with 7-aminoactinomycin D (7AAD, Immunostep, Salamanca, Spain, 1:100). Live cells were selected for further analysis. The results were analyzed using FACS Diva software version 1.2 (BD, Franklin Lakes, NJ, USA).

Co-culture system

HSFs were seeded into the lower chamber of the 12-well culture plate (Corning Gilbert, Glendale, AZ, USA). THP-1 monocytes were differentiated in 12 Transwell inserts, with a membrane pore size of 0.1 μm (#3450; Corning Gilbert, Glendale, AZ, USA) (Fig. 1B). Both types of cells were co-cultured separately in complete DMEM at the same concentration of 1.0×10^5 cells/well. As negative and positive controls, 1.0×10^5 HSFs were mono-cultured with either basal medium alone or basal medium containing 10 ng/mL TGF-β1 (#240-B; R&D Systems, Minneapolis, MN, USA). After 72 h of culture, cells were harvested to examine the effects of macrophages on HSFs using flow cytometry and RT-qPCR analysis. The supernatant was stored at −80 °C for analysis of cytokines.

Flow cytometry quantitative analysis of α-SMA-positive cells

For the quantitative analysis of α-SMA-positive cells, HSFs mono- or co-cultured with M1 and M2-like macrophages for 72 h were collected, intracellular staining was performed, and cells were analyzed by flow cytometry using a FACS Canto II apparatus. Briefly, cells were fixed with 4% paraformaldehyde (#P6148; Sigma-Aldrich, St. Louis, MO, USA) followed by incubation with 0.1% Saponin (#S2149; Sigma-Aldrich, St. Louis, MO, USA) to permeabilize cell membranes. Cells were then incubated with FcR blocking reagent. Human α-SMA PE-conjugated antibody (1A4, #IC1420P; R&D Systems, Minneapolis, MN, USA, 1:50) was then added to the samples at 4 °C in the dark according to the manufacturer's protocol. Isotype-specific controls and PE mouse IgG isotype controls were used accordingly. Dead cells were excluded by gating with 7AAD. Live cells were selected for further analysis. The percentage of positive cells was determined using FACS Diva software.

Gene expression analysis of pro- and anti-fibrotic factors by RT-qPCR

To analyze the gene expression of pro- and anti-fibrotic factors, total RNA was extracted, and cDNA synthesis and RT-qPCR analyses were performed as described above. Primers for α-*SMA*, *COL1A1*, *TGF-β1*, connective tissue growth factor (*CTGF*), tissue inhibitor of metalloproteinases (*TIMP-1*), *MMP-1*, *MMP-2*, and *MMP-9* were purchased from Invitrogen (Carlsbad, CA, USA). Table 1 lists the primer sequences used for RT-qPCR. All experiments were performed in accordance with the manufacturer's instructions. The expression of the target genes was normalized to that of the *GAPDH* reference gene and evaluated using the $2^{-\Delta\Delta Ct}$ method.

Quantitative analysis of cytokine secretion profile

Released TNF-α, IL-10, and TGF-β1 cytokine concentrations in cell culture supernatants were analyzed using human TNF-α (#DTA00D), human IL-10 (#D1000B), and human TGF-β1 (#DB100B) enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's protocol.

Protein expression analysis of TGF-β signaling by Western blot

Cell lysates were prepared using RIPA Lysis and Extraction Buffer (#89900; Thermo Fisher Scientific, Waltham, MA, USA). Total protein concentration was determined using a BCA assay (Thermo Fisher Scientific, Waltham, MA, USA). Thirty milligrams of total protein were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and proteins were transferred to a polyvinylidene fluoride membrane. The membrane was blocked in 5% skim milk and then incubated with primary antibodies (1:1000 dilution) for the following target proteins: SMAD2/3 (polyclonal, #5678; Cell Signaling Technology, Danvers, MA, USA) and phosphorylate SMAD2/3 (D27F4, pSMAD2/3, #8828; Cell Signaling Technology, Danvers, MA, USA). β-Actin (13E5, #4970; Cell Signaling Technology, Danvers, MA, USA, 1:2000) served as the loading control. Anti-rabbit IgG conjugated to horseradish peroxidase (polyclonal, #7074; Cell Signaling Technology, Danvers, MA, USA; 1:2000) was used as the secondary antibody. Band detection was performed using the Amersham ECL Prime detection reagent (#RPN2232; Sigma-Aldrich, St. Louis, MO, USA) and a ChemiDoc MP touch chemiluminescence imaging system (Bio-Rad, Hercules, CA, USA). The relative protein expression of β-actin was quantified by densitometric analysis using ImageJ software version 1.52 (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM). All experiments were performed in triplicate. Statistical analysis was performed using an unpaired Student's t-test or one-way analysis of variance (ANOVA)

followed by Tukey's post hoc test using GraphPad Prism version 9 (GraphPad Software, Inc., San Diego, CA, USA). Statistical p-value < 0.05 was considered statistically significant.

Results

Establishment and characterization of THP-1-derived macrophages

The THP-1 cell line has been extensively used as a model for in vitro macrophage polarization[11–13]. We utilized and optimized these protocols to investigate macrophage differentiation and polarization into the M1 and M2 subtypes. Briefly, THP-1 cells were differentiated into macrophages using PMA and activated classically (M1) by LPS plus IFN-γ or alternatively (M2) by IL-4 plus IL-13.

THP-1-derived macrophages stimulated with cytokines to induce M1 or M2 polarization displayed markedly different cell morphologies. As shown in Fig. 2A, THP-1 cells displayed a small and oval shape with a partly clustered and non-adherent pattern, while macrophage-like THP-1 cells (M0, M1, M2) were larger and adherent, with round and flat shapes in M0 and dendritic elongated shapes in M1 and M2.

To determine the induction of macrophages from THP-1, M0, M1-, and M2-like macrophages were evaluated by flow cytometry using the macrophage surface marker CD14. The M0, M1, and M2 subtypes shared similar and significantly higher levels of CD14 positive populations as compared with THP-1 cells (Fig. 2B; $p < 0.0001$).

To further characterize and distinguish the polarization of M1 and M2-like macrophages, the expression of specific surface markers of M1 (CD80) and M2 (CD206) macrophages were measured in M0, M1, and M2 macrophages by flow cytometry and RT-qPCR. Flow cytometry analysis demonstrated that M1-like macrophages had the highest expression of CD80 (91.5%), while the expression of this marker was much lower in M0 macrophages (2.4%) and M2-like macrophages (0.4%) (Fig. 2C; $p < 0.0001$). In contrast, the expression of CD206 was the highest in M2-like macrophages (51.8%), followed by M0 macrophages (3.4%) and M1-like macrophages (0.4%) (Fig. 2C; $p < 0.0001$). Moreover, a high proportion of M1-like cells (85.3%) was double-

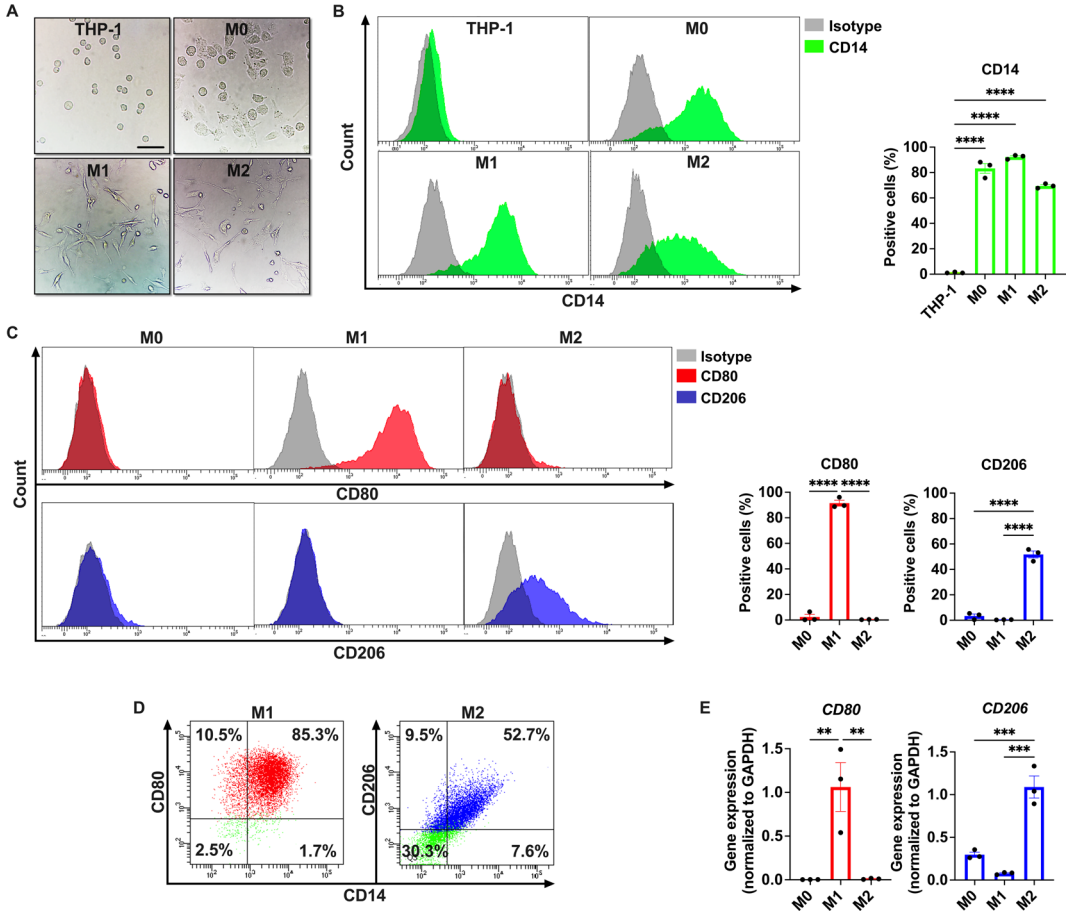

Fig. 2. Characterization of THP-1-derived M1- and M2-like macrophages. **(A)** Representative images of different morphologic appearances of THP-1 cells and M0, M1-, and M2-like macrophages. Magnification, ×100. Scale bar = 100 µm. **(B, C, and D)** Flow cytometry was used to analyze the percentages of positive cells using specific markers of **(B)** macrophages (CD14) and **(C)** M1 (CD80) and M2 (CD206) macrophages. The left panels are representative flow cytometry histograms. The right panels present the percentages of positive cells. **(D)** Representative flow cytometry plots of M1-like (CD14+, CD80+) and M2-like (CD14+, CD206+) macrophages. **(E)** Gene expression levels of M1 and M2 macrophage markers measured by RT-qPCR. Data are presented as the mean ± SEM from three independent experiments. $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

positive for CD14 and CD80, and a high proportion of M2-like cells (52.7%) was double-positive for CD14 and CD206 (Fig. 2D). Consistent with the flow cytometry results, RT-qPCR analysis revealed that CD80 was exclusively expressed in M1-like macrophages compared to M0 and M2-like cells ($p < 0.01$), whereas CD206 was significantly expressed in M2-like macrophages compared to M0 and M1-like cells (Fig. 2E; $p < 0.001$).

Collectively, these results confirm that THP-1 cells successfully differentiated into M0 macrophages after PMA treatment and further induced polarization into M1-like or M2-like macrophages after LPS plus IFN-γ or IL-4 plus IL-13 stimulation. Thus, these cells are suitable for further explorations of the influence of macrophage phenotypes on HSFs.

M2-like macrophages induce myofibroblast differentiation of HSFs

To distinguish between M1 and M2 phenotypes and assess their ability to regulate fibrogenesis during OA development, HSFs were co-cultured with M1 and M2 phenotypes for 72 h. Then, α-SMA expression was examined to evaluate fibroblast-to-myofibroblast differentiation. Mono-cultured HSFs in the absence and presence of 10 ng/mL TGF-β1 served as the negative and positive controls, respectively.

The expression of the *α-SMA* myofibroblast marker significantly increased when HSFs were cultured in the presence of TGF-β1 or co-cultured with M2-like macrophages compared to untreated HSFs (control) and those co-cultured with M1-like macrophages (Fig. 3A; both $p < 0.0001$). In contrast, co-culture of HSFs with M1-like

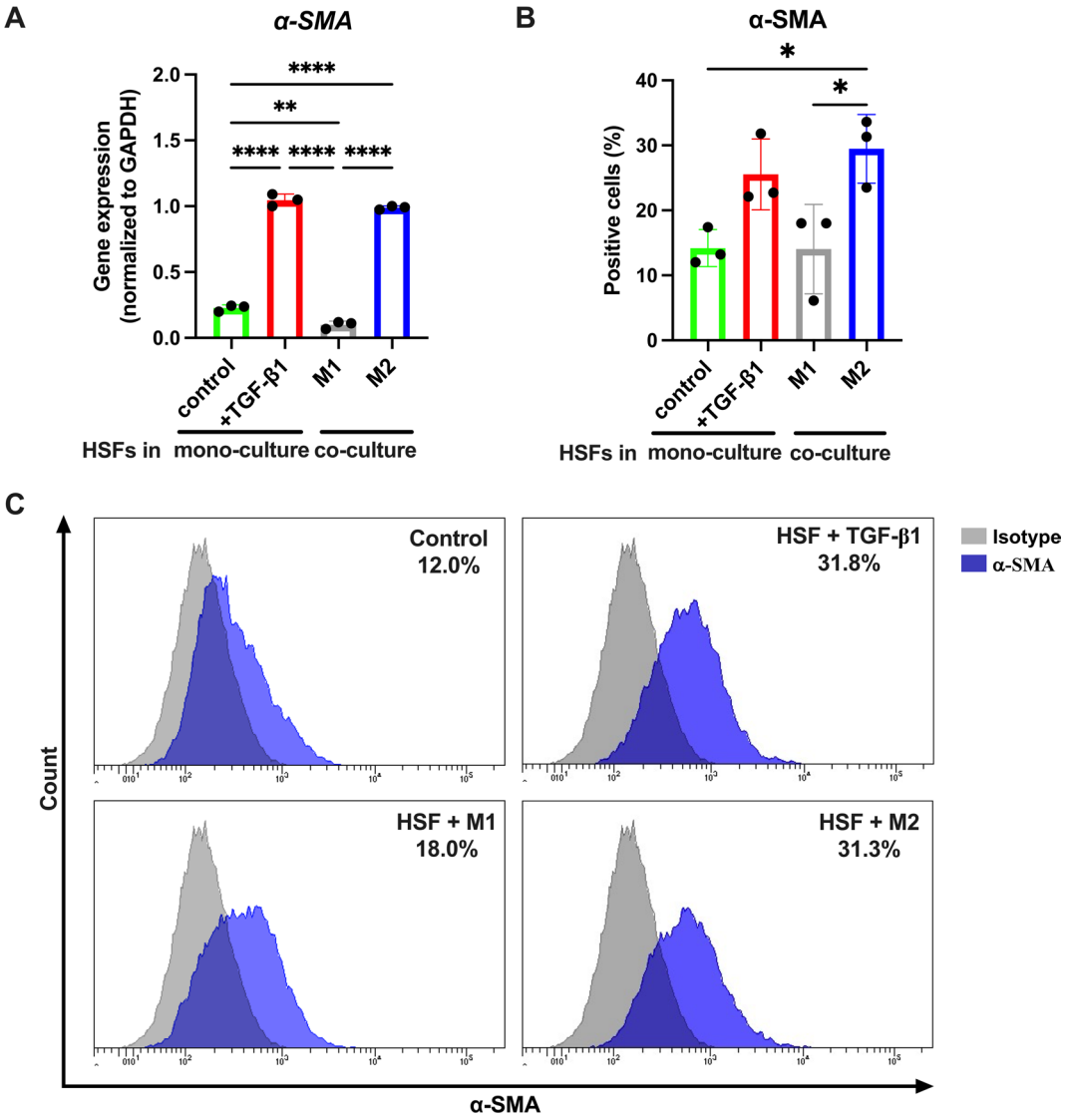

Fig. 3. M2-like macrophages induce myofibroblast differentiation of HSFs. **(A)** Gene expression analysis of *α-SMA* using RT-qPCR. Data are expressed as individual data points and mean values. **(B)** Percentages of α-SMA positive cells by flow cytometry after co-culture for 72 h. Data are averages of three independent experiments and presented as the mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{****}p < 0.0001$. **(C)** Representative flow cytometry histograms.

macrophages suppressed the expression of *α-SMA* to a significantly lower level than that of untreated HSFs (Fig. 3A; $p < 0.01$).

To confirm fibroblast differentiation, along with RT-qPCR analysis of *α-SMA* gene expression, we quantified α-SMA-positive cells using flow cytometry analysis (Fig. 3B and C). The number of α-SMA-positive cells and gene expression levels strongly correlated. α-SMA-positive cells were elevated in HSFs treated with TGF-β1 and co-cultured with M2-like macrophages. Each was significantly higher than the control and co-cultured with M1-like macrophages (Fig. 3B; $p < 0.05$).

Effect of M1- and M2-like macrophages on the expression of anti- and pro-fibrotic factors of HSFs

In addition to α-SMA expression, fibrosis-related genes produced by myofibroblasts (activated fibroblasts) and anti-fibrotic genes were investigated (Fig. 4). We first analyzed matrix protein production potential by analyzing the *COL1A1* gene. The expression of *COL1A1* followed the same trend as *α-SMA* expression, which was upregulated in HSFs treated with TGF-β1 or co-cultured with M2-like macrophages. We also analyzed other pro-fibrotic factors, such as *CTGF* and *TIMP-1*. *CTGF* expression in fibroblasts co-cultured with M2-like macrophages was significantly higher than that in untreated HSFs ($p < 0.05$) and fibroblasts co-cultured with M1-like macrophages ($p < 0.01$). TIMP-1 expression in fibroblasts co-cultured with M2 macrophages was significantly higher than that in untreated ($p < 0.05$) and TGF-β1-treated HSFs ($p < 0.01$). In contrast, HSFs co-cultured with M1-like macrophages did not show enhanced expression, and the expression levels of the matrix proteins *COL1A1*, *CTGF*, and *TIMP-1* were in the same range as those in untreated HSFs (Fig. 4A).

MMPs are proteolytic enzymes that play an important role in ECM remodeling, thus responsible for fibrosis resolution. Therefore, we investigated the effect of macrophage phenotypes on the production of anti-fibrotic MMPs (*MMP-1*, *MMP-2*, *and MMP-9*). We observed that in co-culture with M1-like macrophages, *MMP-1* expression in HSFs was significantly upregulated compared to monoculture ($p < 0.01$; Fig. 4B). In contrast, *MMP-2* and *MMP-9* expression levels were not significantly different between the M1 co-culture and monoculture groups. These findings suggest that M1-like macrophages selectively induce MMP-1 expression in synovial fibroblasts.

TGF-β1 derived from M2-like macrophages triggers myofibroblast differentiation of HSFs

To explore the mechanism underlying the induction of fibroblasts-to-myofibroblasts differentiation by M2-like macrophages, we investigated the gene expression of *TGF-β1* in HSFs and both macrophage phenotypes after

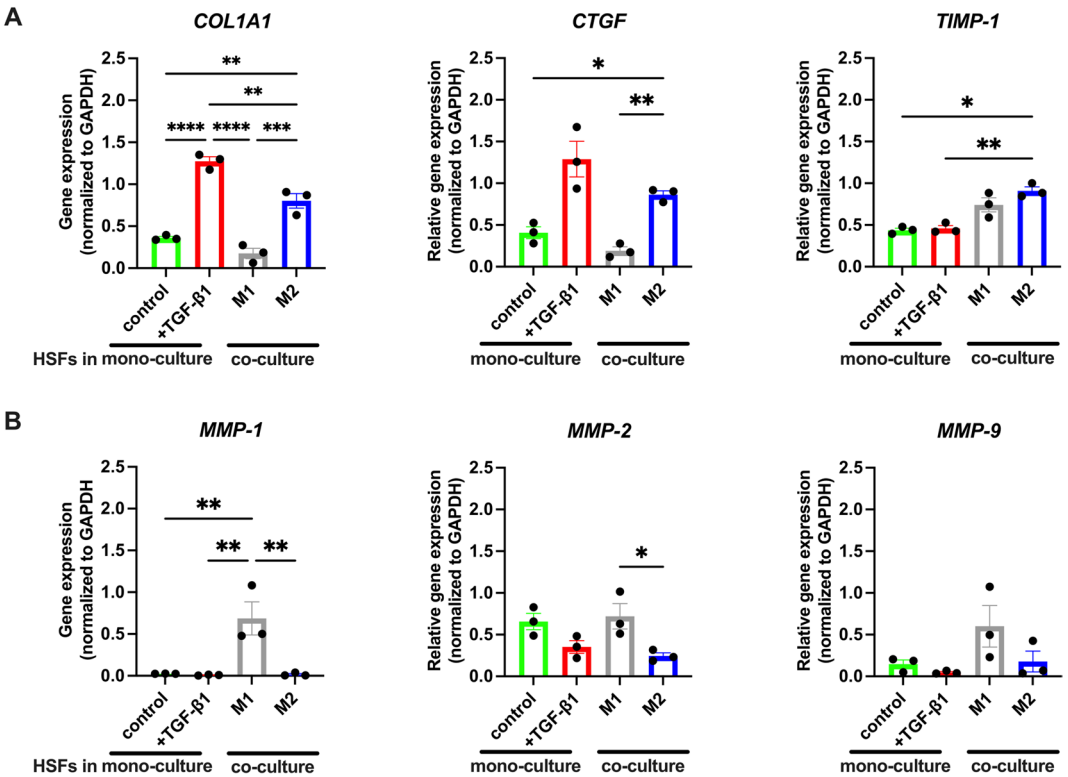

Fig. 4. Effects of M1- and M2-like macrophages on expression of pro- and anti-fibrotic factors by HSFs. The data are the fold change in gene expression of pro-fibrotic markers (*COL1A1*, *CTGF*, *TIMP-1*) and anti-fibrotic markers (*MMP-1*, *MMP-2*, *MMP-9*) in HSFs after co-culture for 72 h. Values are normalized relative to *GAPDH*. Data are expressed as individual data points and mean values from three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{****}p < 0.0001$.

co-culture. ELISA was performed to detect TGF-β1 and other cytokines released into the co-culture supernatant (Fig. 5).

The expression of *TGF-β1* in HSFs co-cultured with M2-like macrophages did not differ from that in HSFs cultured alone or with M1-like macrophages, as confirmed by RT-qPCR (Fig. 5A). However, treatment with TGF-β1 led to an increase in *TGF-β1* mRNA expression in HSFs, suggesting a potential autocrine feedback mechanism in which TGF-β1 enhances its own expression. In contrast, a significant difference was found between M2- and M1-like macrophages after co-culture with HSFs (Fig. 5B; $p < 0.01$). This variation implies the sustained release of TGF-β1 by M2-like macrophages, but not by HSFs.

Among the three cytokines examined, the TNF-α pro-inflammatory cytokine was the most predominantly secreted after co-culture of HSFs and M1-like macrophages (38.9 ± 6.8 pg/mL). In contrast, co-cultured HSFs and M2-like macrophages produced a significantly higher amount of the anti-inflammatory cytokine IL-10 (53.4 ± 11.4 pg/mL) than mono-culture and co-cultured HSFs and M1-like macrophages. Co-cultures of HSFs and M2-like macrophages also secreted a high amount of TGF-β1 (4816.3 ± 498.4 pg/mL) (Fig. 5C).

Together with the accelerated expression of the *TGF-β1* gene, the findings confirmed that M2-like macrophages secrete TGF-β1 into the culture medium and mediate fibrotic signals to HSFs.

HSFs differentiation into myofibroblasts triggered by TGF-β1 derived from M2-like macrophages is associated with the SMAD2/3 signaling pathway

To determine the mechanism of the myofibroblast differentiation abilities of HSF cells induced by M2-like macrophages, phosphorylation of SMAD proteins 2/3, which are downstream events in the canonical TGF-β signaling pathway, were examined by immunoblotting. As shown in Fig. 6A and B, the levels of pSMAD2/3 were significantly upregulated following M2 co-culture and TGF-β1 treatment. Thus, HSF cells seem to be affected by M2-like macrophages, with activation of the canonical TGF-β signaling pathway, as they are stimulated by TGF-β1.

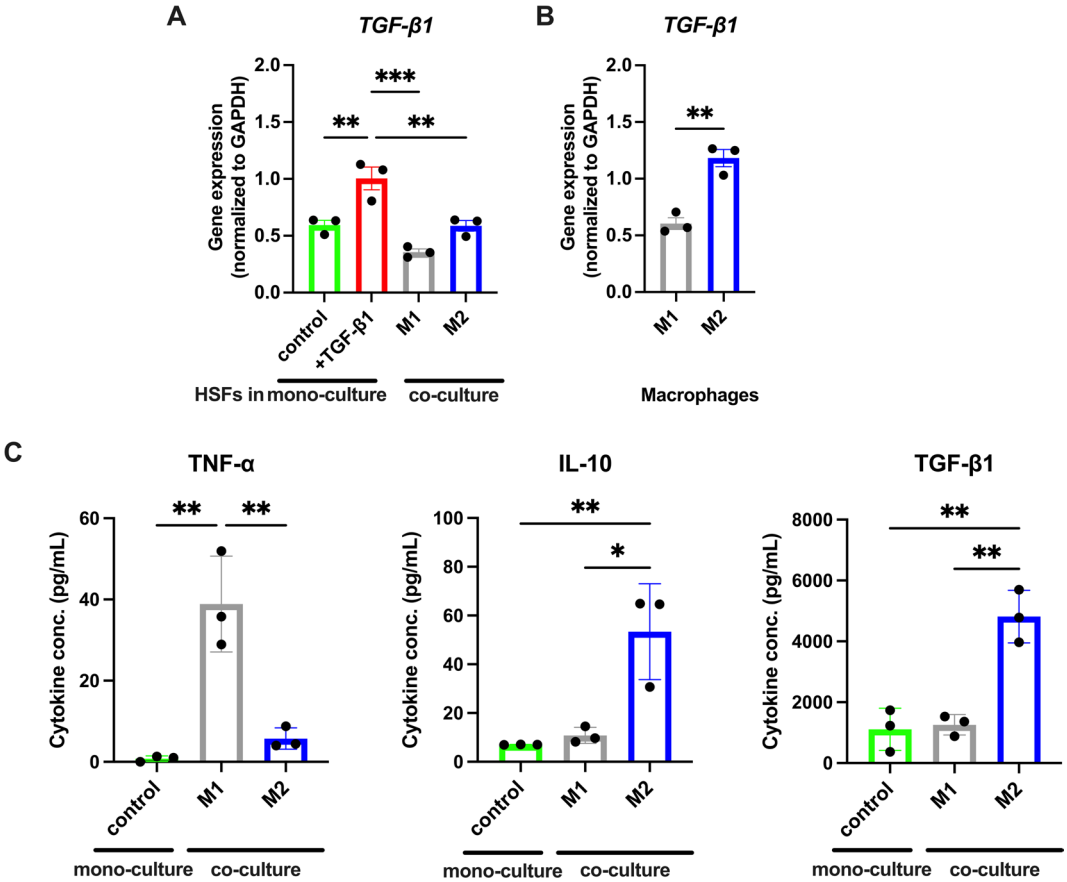

Fig. 5. TGF-β1 derived from M2-like macrophages triggers myofibroblast differentiation of HSFs. Fold change in gene expression levels of *TGF-β1* in **(A)** HSFs and **(B)** macrophages quantified using RT-qPCR. Values are normalized relative to *GAPDH*. **(C)** Secreted concentrations of TNF-α, IL-10, and TGF-β1 cytokines in cell culture supernatants after 72 h of co-culture determined by ELISA. Data are expressed as individual data points and mean values from three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

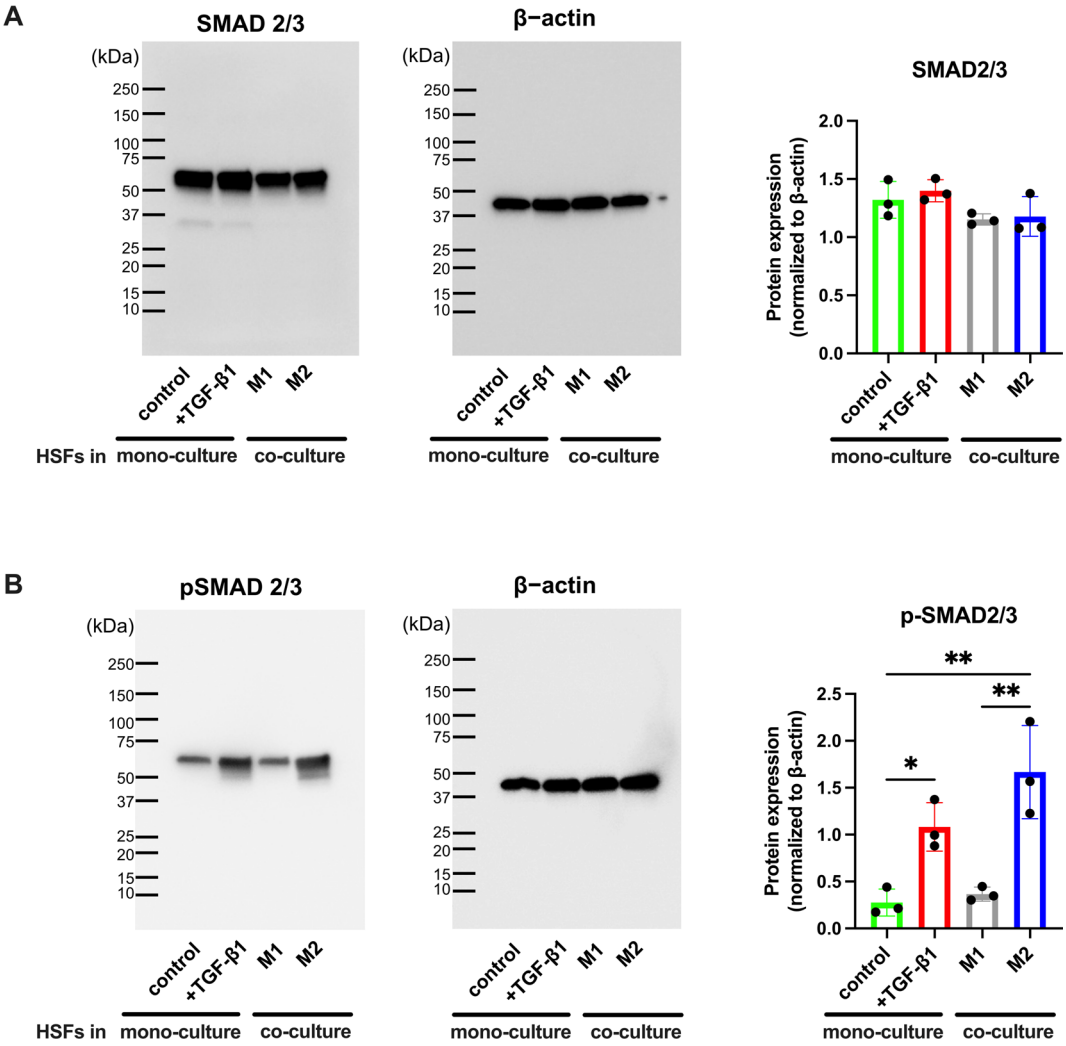

Fig. 6. TGF-β1 derived from M2-like macrophages triggers HSFs differentiation into myofibroblast is associated with the SMAD2/3 signaling pathway. Expression of the TGF-β pathway in HSF cells was assessed by western blot analysis. **(A and B)** Representative western blot images and results of statistical analysis of protein levels **(A)** and phosphorylation levels **(B)** of SMAD2/3 following the indicated treatment. Data are presented as the mean ± SEM from three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$.

Discussion

Although the pathogenesis of OA is complex and poorly understood, the use of in vitro models to study this disease continues to provide valuable insights. Given the importance of macrophages and fibroblasts in inflammatory regulation, tissue healing, and fibrotic reactions, extensive in vitro co-culture models of macrophages and fibroblasts have been established to study inflammation and fibrosis[14–16]. However, in OA pathogenesis, most studies have only focused on pro-inflammatory reactions, while fibrosis-related studies are largely missing[17]. Using co-culture models of HSFs with polarized M1- or M2-like macrophages, we investigated for the first time the critical role of macrophage polarization in the myofibroblast differentiation that may drive fibrosis in OA and the specific pathways involved in this process. The results demonstrate that co-culture with M2-like macrophages is associated with increased myofibroblast differentiation of HSF cells by secreting TGF-β1, leading to the upregulation of α-SMA and pro-fibrotic factors, in association with the activation of the SMAD2/3 pathway, when compared with HSFs co-cultured with M1-like macrophages. A hypothetical schematic summary of the results in different OA progression stages is presented in Fig. 7. The figure highlights the interplay between M1 and M2 macrophages in controlling the myofibroblast differentiation in the fibrotic process of OA. These findings provide novel insights into and potential therapeutic targets for the treatment of OA-related fibrosis.

Macrophages are phenotypically highly plastic. Their polarization state depends on the local microenvironment, as they respond to a wide range of stimuli under both physiological and pathological conditions. M1 (classically activated) macrophages are typically associated with inflammation, whereas M2 (alternatively activated) macrophages are involved in tissue remodeling and fibrosis[10,18,19]. Given the complicated heterogeneity in the polarization and function of macrophages, it is reasonable to suppose that different subsets of macrophages play

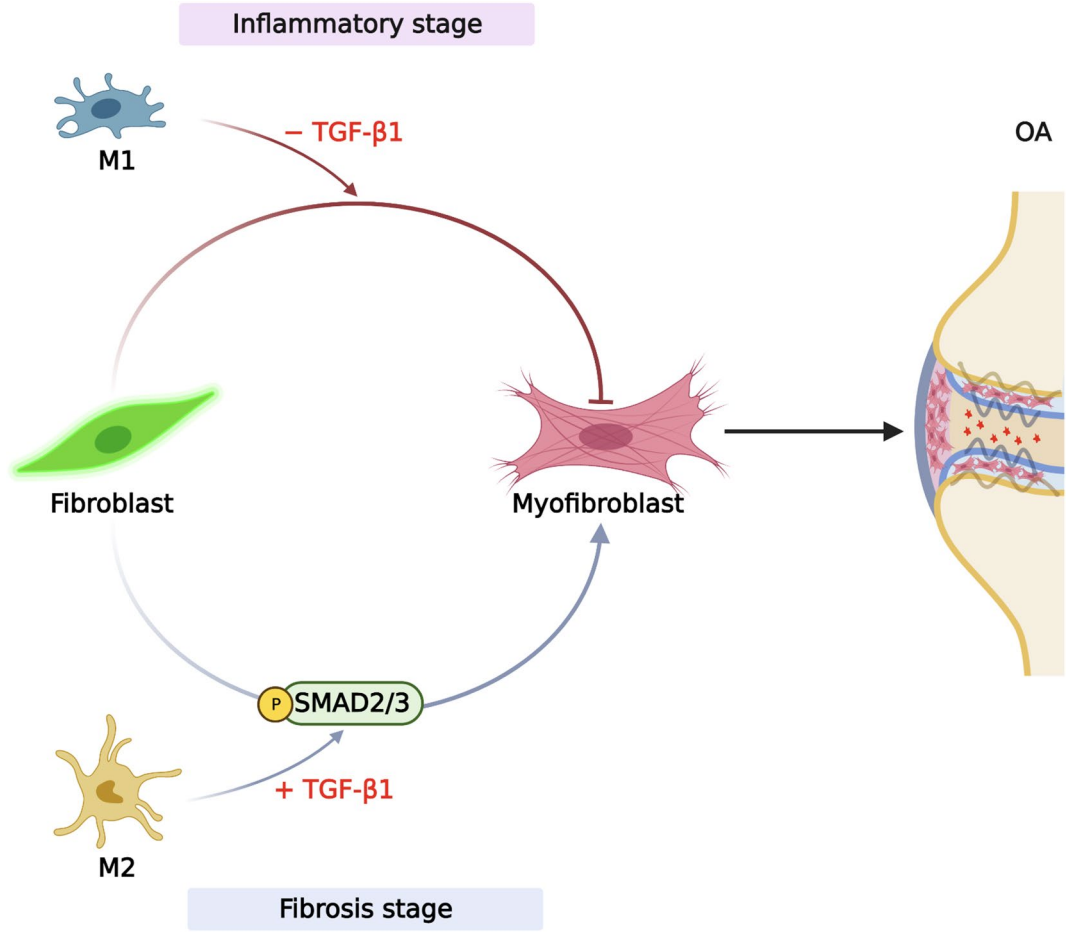

Fig. 7. Schematic illustration of a hypothetical model depicting the proposed functions of M1 and M2 macrophages in regulating myofibroblast differentiation of HSFs during the inflammation and fibrosis stages of OA progression.

different roles in OA fibrogenesis. Nevertheless, macrophage phenotypes in vivo likely exhibit a more diverse pattern, and mixed phenotypes of macrophages may exist during the transition between different stages of OA pathogenesis[20]. Furthermore, there is growing recognition of M2 macrophage subtypes, and that each subtype of M2 macrophages has a different activation state and its own products[21,22]. Thus, the role of M2 macrophages in OA pathogenesis in vivo appears to be complex. To overcome the complexity, the present study utilized THP-1-based macrophage models that have been extensively established in various biomedical studies, particularly co-culture studies[23,24]. Stimulation with LPS/IFN-γ and IL-4/IL-13 was used to induce the polarization of M1 and M2 macrophages, respectively. The initial results showed that the expression level of the CD14 pan-macrophage marker[25,26] was increased in THP-1-derived macrophages upon differentiation compared to undifferentiated cells. Furthermore, M2-like macrophages derived from the THP-1 cell line upregulated the M2 subtype surface marker CD 206, whereas M1-like macrophages upregulated the M1 subtype marker CD80. Our results are in accordance with the previously identified markers of phenotypic alterations of macrophages in the synovium and peripheral blood of patients with OA[27–29]. The findings indicate the successful transition from THP-1 cells to M1- and M2-like macrophage subtypes.

Subsequently, an in vitro co-culture system consisting of proliferated macrophages and HSFs was established. We observed that rather than M1-like macrophages, M2-like macrophages can induce myofibroblast differentiation of HSFs, as evidenced by the upregulated expression of *α-SMA* and *COL1A1*. α-SMA, a widely recognized marker of myofibroblasts[30]along with COL1A1, a well-known marker for fibrosis, has been detected in various fibrosis-related diseases, including pulmonary fibrosis, glomerulosclerosis, otosclerosis, and OA[31–33]. α-SMA and COL1A1 are key markers of myofibroblast in synovial fibrosis and fibrocartilage formation[4]. It has also been demonstrated that remodeled tissue in OA cartilage defects shows fibrocartilaginous characteristics evident by the increased levels of α-SMA and COL1A1[5,34,35]. In the present study, other pro-fibrotic factors, such as *CTGF* and *TIMP-1*, were increased in co-culture with M2-like macrophages. CTGF, also known as CCN2, is a potent enhancer of ECM adhesion and production[36,37]. *CTGF* expression is reportedly upregulated under pathological conditions, including fibrotic disorders[38]. In OA, CTGF is upregulated adjacent to damaged areas of the cartilage surface and is present in osteophytes of late-stage OA[39–41]. In contrast, TIMP-1 is an inhibitor of MMPs and is induced in fibrotic diseases, such as pulmonary, liver, and kidney fibrosis[42–44]. Notably, it has been

reported that TIMP-1 is also elevated in the synovium of both human end-stage OA patients and experimental OA mice[45]. The role of M2 macrophages in fibrosis has been reported in various tissues and organs[46,47]. Our study is consistent with previous studies and provides strong evidence that M2 macrophages promote the myofibroblast differentiation of HSFs, emphasizing their role in the fibrosis stage of OA progression. Interestingly, a study by Gao et al. also employed a 3D multi-cellular co-culture model to explore synovial changes in OA[48]. While their model incorporated macrophages, fibroblasts, and endothelial cells, its primary focus was on angiogenesis and immune regulation. Moreover, gene expression was analyzed at later time points (days 7–28), whereas our study focused on an earlier window (72 h), during which TGF-β1/SMAD2/3 signaling is known to be most active[49–51]. Re-analysis of their transcriptomic dataset showed no significant upregulation of canonical fibrotic genes such as *ACTA2 (α-SMA)*, *CTGF* in M2-treated fibroblasts, although a modest increase in *COL1A1* expression was observed. This suggests that their model may not have captured early fibrotic early fibrotic activation or that transient profibrotic signals had already diminished. In addition to timing, other factors including differences in macrophage polarization protocol, fibroblast origin, or the presence of multiple interacting cell types may contribute to the observed discrepancy. Taken together, our findings may offer a complementary perspective by highlighting early-stage activation of fibrotic pathways and myofibroblast differentiation induced by M2-like macrophages under defined in vitro conditions.

Next, we investigated the mechanism underlying myofibroblast differentiation of fibroblasts following M2-like macrophage treatment. Evidence supports that TGF-β regulates numerous intracellular signaling cascades to transmit its pro-fibrotic effects. Thus, to our knowledge, TGF-β plays a central role in all fibrosis organs researched so far, for example, in fibrotic lesions of the liver, lung, kidney, skin, and heart[52,53]. In patients with OA, TGF-β is present as three isoforms (TGF-β1–3), which are elevated and positively correlated with pain, loss of function, and radiographic staging[54]. Scharstuhl et al.. described the essential role of TGF-β in synovial thickening caused by fibrosis in experimental OA[55]. In addition, high concentrations of TGF-β exist in OA synovial fluids and are produced by synoviocytes[56]. Our results demonstrate that M2-like macrophages and their co-culture supernatants exhibit notably higher levels of TGF-β1. Simultaneously, co-cultured M2-like macrophages achieved similar effects to those induced by direct TGF-β1 protein treatment in myofibroblast differentiation performance, including increased expression of *α-SMA* and other fibrosis-related genes. These results implicate TGF-β1 as the key player in the crosstalk between M2 macrophages and HSF cells.

In various fibrotic settings, the inhibition of TGF-β signaling attenuates fibrosis[53,57,58]. TGF-β should be blocked at the beginning of the fibrotic cascade to prevent OA fibrosis. However, TGF-β regulates many crucial cellular processes, including chondrogenesis. High levels of TGF-β exist in healthy cartilage, whereas low levels of TGF-β are found in OA cartilage[59]. Adequate levels of TGF-β have protective effects on cartilage in animal models of arthritis, while an excessive amount of this growth factor has adverse effects[60]. Consequently, blocking TGF-β may have serious side effects on the cartilage and thus cannot be considered as the ultimate cure for fibrosis in OA. Therefore, it is important to identify the downstream targets of TGF-β that drive myofibroblast differentiation in OA-related fibrosis to minimize unwanted side effects.

The central mediators of intracellular TGF-β signaling are the SMAD proteins. However, the role of this signaling in OA fibrosis has not been fully investigated. Deroyer et al. recently reported that the SMAD2/3 pathway can drive pro-fibrotic response in OA conditions by inducing myofibroblast marker α-SMA expression in OA fibrocartilage[3]. In the current study, to demonstrate the TGF-β pathway activity in HSFs, the protein levels of pSMAD2 and pSMAD3 were measured. Co-culture with M2-like macrophages or TGF-β1 protein stimulation significantly increased SMAD2/3 phosphorylation. Although many effects of TGF-β signaling occur *via* the SMAD pathway, accumulating evidence indicates that TGF-β ligands also activate non-canonical pathways, such as the phosphoinositide 3-kinase/protein kinase B and extracellular signal-activated kinase pathways[4]which complement SMAD actions and have their own biological functions. A detailed investigation of the role of TGF-β non-canonical pathways in OA fibrosis requires further study. Nevertheless, the present study provides evidence that M2-like macrophages increase myofibroblast differentiation of HSF cells associated with the TGF-β1/SMAD2/3 pathway.

Our results also suggest that the TGF-β pathway may represents a potential molecular target for the therapeutic modulation of fibrosis in OA. Xue et al. reported that knockdown of SMAD4 in rat synovial cells and chondrocytes[61] decreased cell proliferation and expression of fibrosis-related markers, including vimentin, α-SMA, COL1A1, and TIMP1 induced by TGF-β1. Therefore, whether inhibition of the TGF-β1-SMAD2/3 pathway in an OA joint has anti-fibrotic effects may be worth investigating in future studies.

In contrast, M1-like macrophages appeared to exert partial inhibitory effects on myofibroblast differentiation and collagen synthesis in HSFs. This phenomenon could be explained by the pro-inflammatory stage of M1-like macrophages, particularly by the secretion of TNF-α in this co-culture system. Our results corroborate those of previous studies, which demonstrated that TNF-α can suppress TGF-β1-induced myofibroblast phenotypic genes, such as *α-SMA*, at the mRNA level as well as in the SMAD signaling pathway[62]. Furthermore, our M1-like macrophages influenced the expression of *MMP1* in HSFs. In general, MMPs are capable of ECM degradation during tissue repair[63,64]. However, excess MMPs damage tissue architecture. In OA, a series of MMPs, including MMP-1 and MMP-13, play critical roles in cartilage destruction through degradation of aggrecan and collagens[65,66]. In addition, other MMPs, such as MMP-2 (gelatinase A) and MMP-9 (gelatinase B), are overproduced in arthritis. These enzymes are believed to degrade cartilage as well as non-collagen matrix components of joints[67]. Thus, although M1-like macrophages may modulate fibrosis-related activity, enhancing their polarization is unlikely to be beneficial in OA because of the induction of tissue-degrading enzymes and the pro-inflammatory state after M1 stimulation.

Consequently, neither promoting M1 macrophage polarization nor blocking M2 macrophages to produce TGFβ1 will be the treatment for fibrosis in OA. So, how can we solve this problem? Ultimately, creating an environment that can comprehensively control the specific roles of M1 and M2 macrophages to prevent excessive

cartilage degradation and pathological fibrosis according to the stages of OA progression would be the key to developing novel treatments for OA.

This study has several important limitations that should be acknowledged. First, all experiments were conducted in vitro using a co-culture system of THP-1-derived macrophages and synovial fibroblasts. While this model enables mechanistic investigation under controlled conditions, it does not fully replicate the complexity of the osteoarthritic joint microenvironment. There is no direct evidence that the observed responses in this model accurately reflect OA pathogenesis in vivo. Second, relevant clinical data associated with the samples were not included, which limits the contextual interpretation and translational value of the findings. Third, although we observed upregulation and nuclear translocation of SMAD2/3 signaling in fibroblasts exposed to M2-like macrophages, we did not perform pharmacological inhibition or gene-silencing experiments to confirm whether this pathway is essential for myofibroblast differentiation. Future investigations incorporating targeted inhibition strategies will be critical to clarify the causal role of this pathway. In addition, α-SMA expression was assessed by RT-qPCR and flow cytometry, which do not provide spatial information on protein localization. The application of immunofluorescence imaging techniques in future investigations may help better characterize fibroblast activation and facilitate the assessment of anti-fibrotic strategies in translational models of OA.

Conclusion

We established an easy and reproducible co-culture model of differentiated THP-1 monocytes polarized into M1- and M2-like macrophages with HSFs to mimic in vivo environments in different stages of OA progression. Using this model, we show that M2-like macrophages display pro-fibrotic effects in HSFs, in association with activation of the TGF-β1/SMAD2/3 signaling pathway. Our study reveals the interplay between macrophages and HSFs on myofibroblast differentiation in pathological fibrosis of OA. Although the study provides some seeds for potential therapeutic targets to inhibit the initiation and progression of OA, further investigation is needed to develop a novel intervention procedure.

Data availability

The data presented in this study are available on request from the corresponding author.

Received: 22 March 2025; Accepted: 7 July 2025
Published online: 15 July 2025

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Acknowledgements
The authors thank Mrs.Mamiko Tsuji of the University of Toyama for her excellent technical assistance. This study was supported by JSPS KAKENHI [grant number JP19K09618].

Author contributions
Study conception and design (M.N., N.T.C.T, and Y.Y.). Data collection, assembly, and analysis (N.T.C.T., M.I., K.K., and Z.H.). Data interpretation (N.T.C.T., M.N., S.S., Y.Y., and H.M). Drafting and revision of manuscript (N.T.C.T., M.N., S.S., H.M., and Y.K.). Project administration (M.N., Y.K.). Funding acquisition (M.N). All authors have reviewed and approved the final version to be published.

Declarations

Competing interests
The authors declare no competing interests.

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