Received: 26 June 2023 | Revised: 17 August 2023 | Accepted: 23 September 2023

DOI: 10.1002/jsp2.1290

RESEARCH ARTICLE

JOR Spine OPEN ACCESS

M1 macrophage-derived oncostatin M induces osteogenic differentiation of ligamentum flavum cells through the JAK2/STAT3 pathway

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Funding information
National Natural Science Foundation of China, Grant/Award Number: 82002326

Abstract

Background: M1 macrophages (Mφs) are involved in osteogenic differentiation of ligamentum flavum (LF) cells and play an important role in heterotopic ossification. However, the mechanism by which M1 Mφs influence osteogenic differentiation of LF cells has not been studied.

Methods: The effect of conditioned medium including secretions of M1 Mφs (CM-M1) on LF cells was analyzed by GeneChip profiling and ingenuity pathway analysis (IPA). THP-1 cells were polarized into M1 Mφs and CM-M1 was used to induce LF cells. In addition, LF cells were induced by CM-M1 in the presence of cyclooxygenase 2 (COX-2) inhibitors or oncostatin M (OSM)-neutralizing antibodies. Based on the presence of OSM, knockout of OSMR or GP130 receptors, or addition of the Janus kinase 2 (JAK2) inhibitor AZD1480 or signal transducer and activator of transcription 3 (STAT3) inhibitor Stattic were examined for effects on osteogenic differentiation of LF cells. OSM secretion was quantified by ELISA, while qPCR and western blot were used to evaluate expression of osteogenic genes and receptor and signaling pathway-related proteins, respectively.

Results: GeneChip and IPA results indicate that the OSM signaling pathway and its downstream signaling molecules JAK2 and STAT3 are significantly activated. ELISA results indicate that OSM is highly expressed in cells treated with CM-M1 and lowly expressed in cells treated with CM-M1 and a COX-2 inhibitor. Besides, CM-M1 induces osteogenic differentiation of LF cells, which is weakened when COX-2 inhibitors or OSM-neutralizing antibody are added to it. Recombinant OSM could induce osteogenic differentiation of LF cells and upregulate expression of OSMR, GP130, phosphorylated (P)-JAK2, and P-STAT3. Upon knockdown of OSMR or GP130, or the addition of AZD1480 or Stattic, P-JAK2 and P-STAT3 expression were decreased and osteogenic differentiation was reduced.

Conclusion: M1 Mφ-derived OSM induces osteogenic differentiation of LF cells and the JAK2/STAT3 signaling pathway plays an important role.

KEYWORDS

JAK2/STAT3 signaling, macrophages, oncostatin M, ossification of the ligamentum flavum

1 | INTRODUCTION

Ossification of the ligamentum flavum (OLF) is an ossification disease of the spinal ligaments, commonly found in East Asia.[1,2] Thoracic ossification of the ligamentum flavum (TOLF) mainly involves the lower thoracic spine, leading to thoracic spinal stenosis and thoracic spinal cord compression,[3–6] which seriously affect patient quality of life. At present, surgery is the main treatment for TOLF.[7] However, there are many surgical complications, among which the incidence of cerebrospinal fluid leakage and nerve damage is high.[8] Because of the unclear pathogenesis of TOLF, there is no effective method to prevent or delay its progression at an early stage, making the diagnosis and treatment of related diseases difficult in the clinic.

TOLF is a heterotopic ossification of the spinal ligaments. The pathological process of TOLF involves fibrous tissue in the ligamentum flavum (LF) of the thoracic spine changing into osseous tissue, as characterized by typical endochondral ossification.[9] Previous studies found that stress stimuli,[10,11] degeneration,[12] genetics,[13,14] and endocrine metabolism[15,16] may be pathogenic factors of TOLF. However, the mechanism of occurrence and development of TOLF currently remain unclear.

The inflammatory microenvironment is an important factor affecting cell osteogenic differentiation. Monocytes, macrophages (Mφs), and their secreted inflammatory factors play an important role in bone formation.[17] In addition, Mφs are key regulators of inflammation that can participate in regulation of cell osteogenic differentiation and play an important role in heterotopic ossification. For example, Mφ-derived neurotrophin-3 can promote heterotopic ossification in rats after Achilles tendon injury.[18] Parishin-A can inhibit the occurrence of tendon heterotopic ossification by intervening in the secretion of inflammatory factors by Mφs.[19] Our previous study found that M1 Mφs can promote LF cell osteogenic differentiation,[20] although the specific mechanism was unclear.

Therefore, the aim of this study was to investigate the mechanism by which M1 Mφs promote osteogenic differentiation of LF cells. We found that M1 Mφs can secrete oncostatin M (OSM) cytokine and promote osteogenic differentiation of LF cells through the Janus kinase 2 (JAK2)/signal transducer and activator of transcription 3 (STAT3) signaling pathway

2 | MATERIALS AND METHODS

2.1 | Patient specimens

Samples used in this experiment were from the biological sample bank of the Spinal Surgery Institute of Peking University Third Hospital (Peking, China). All experimental procedures on human subjects were approved by the relevant medical research Ethics Committee (PUTH-REC-SOP-06-3.0-A27, 2014003). All methods were carried out in accordance with relevant regulations and guidelines. Individuals who signed informed consent and were enrolled in the study were patients with symptomatic thoracic stenosis diagnosed with TOLF that required surgical treatment under clinical guidelines. LF samples were taken from four patients with TOLF by holistic resection of the lamina and LF. LF samples were taken from seven patients with TOLF by holistic resection of the lamina and LF. The clinical information of these patients is shown in Table 1. Three patients were used for gene chip testing, patient numbers 1–3; four patients were used for subsequent experimental validation, patient numbers 4–7.

TABLE 1 Clinical information of ossification of the ligamentum flavum patients.

No	Sex	Age (years)	Level
1	M	49	T11/12
2	F	52	T9/11
3	M	46	T8/11
4	F	48	T10/11
5	M	47	T12/L1
6	M	50	T8–T11
7	F	55	T10/T11

2.2 | Cell culture, osteogenic differentiation, and M1 Mφ polarization

Ligamentum flavum samples were washed with phosphate-buffered saline (PBS; Gibco, Grand Island, NY) and the surrounding tissue was resected under an anatomical microscope to avoid osteoblast contamination as much as possible. Obtained ligaments were chopped into small pieces (approximately 0.5 mm^3) and digested with 0.25% trypsin, followed by 250 U/mL type I collagenase for 80 min. Specimens were washed with serum-containing medium and placed into a 100-mm dish containing Dulbecco's Modified Eagle Medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (Gibco), 100 U/mL penicillin, and 100 mg/mL penicillin/streptomycin (1% P/S) in humidified air at 37°C with 5% CO_2. Primary cells were digested with 0.25% trypsin and passaged. Second and third generations were used for experiments.

To induce osteogenic differentiation, cells were cultured in osteogenic medium consisting of DMEM supplemented with 50 μM ascorbic acid (Sigma-Aldrich, St. Louis, MO), 10 mM glycerophosphate (Sigma-Aldrich), and 10 nM dexamethasone (Sigma-Aldrich).

M1 Mφs were polarized from THP-1 cells using specific methods detailed in our previous report.[20]

2.3 | GeneChip profiling and ingenuity pathway analysis

Total RNA was extracted from LF cells using the RNeasy Mini Kit (#74134, QIAGEN) according to the manufacturer's instructions and tested by NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA) and Bioanalyzer 2100 (Agilent Technologies) with the following quality control standards: $1.7 < A260/A280 < 2.2$, RIN ≥7.0, and $28S/18S > 0.7$. Amplified RNA was prepared using a GeneChip 3′ IVT

PLUS Kit (Affymetrix). Sample hybridization and GeneChip wash/stain were performed with a GeneChip Hybridization Oven 645 and GeneChip Fluidics Station 450, respectively, using a GeneChip Hybridization Wash and Stain Kit (all from Affymetrix). GeneChips were scanned through a GeneChip Scanner 3000 (Affymetrix). Criteria for differentially expressed genes (DEGs) are fold-change >2 and *P*-value less than 0.05. DEGs are shown in a volcano plot and heat map. Data were analyzed using ingenuity pathway analysis (IPA) an online integrated analysis software (www.ingenuity.com) that includes Diseases and Functions Analysis, Canonical Pathway Analysis, and Upstream Regulator Analysis. P values indicate the significance of enriched elements, while Z scores represent the degree of activation or inhibition of enriched elements.

2.4 | Cell treatment

Conditioned medium (CM) secreted by M1 Mφs was used to evaluate the effect of M1 Mφs on osteogenic differentiation of LF cells. M1 Mφs were washed with PBS and cultured in serum-free RPMI 1640 medium for 24 h. Next, the culture supernatant was passed through a 0.22-μm sterile filter (Micropore, Billerica, MA). Supernatants from M1 Mφs were mixed with the same volume of osteogenic medium. CM generated from the supernatants of M1 Mφs is designated as CM-M1. A mixture of osteogenic medium and filtered RPMI 1640 medium was designed as a control, designated as CM-Control.

To analyze the effect of COX-2 inhibitors on OSM secretion by M1 Mφs and osteogenic differentiation of LF cells, celecoxib (HY-14398; MedChemExpress, Monmouth Junction, NJ), meloxicam (HY-B0261; MedChemExpress), or dimethyl sulfoxide (DMSO, Gibco) were added to polarized M1 Mφs, respectively, and after 24 h of incubation, CM was obtained by filtration. Subsequently, CM from each group was used to stimulate LF cells.

To detect whether OSM secreted by M1 Mφs is involved in the osteogenic differentiation of LF cells, CM-M1 was added to osteogenic differentiation medium in the presence of a neutralizing mouse anti-human OSM monoclonal antibody (601 653; Biolegend, San Diego, CA) or nonimmune isotype-matched mouse IgG2a (400 263, Biolegend).

To further analyze the effect of OSM on the JAK2/STAT3 signaling pathway and osteogenic differentiation of LF cells, a concentration of 25 ng/mL recombinant OSM (555202, Biolegend) was added to the osteogenic differentiation medium. Osteogenic differentiation medium without recombinant OSM was used as a blank control.

To determine whether OSM receptors affect osteogenic differentiation of LF cells, human OSMR shRNA (shOSMR, sc-40068-V), human GP130 shRNA (shGP130, sc-29333-V), and negative control shRNA (shNC, sc-108080) lentiviral particles (all from Santa Cruz Biotechnology, Dallas, TX) were transfected into LF cells with 5 μg/mL of Polybrene® (sc-134220, Santa Cruz Biotechnology) according to the manufacturer's protocol. Knockdown efficiency was detected by qPCR after 48 h and western blot (WB) after 72 h. Transfected LF cells were induced by adding 25 ng/mL recombinant OSM protein into osteogenic differentiation medium.

To investigate the effect of the JAK2/STAT3 signaling pathway on osteogenic differentiation of LF cells, the JAK2 inhibitor AZD1480 (10 μM, Santa Cruz Biotechnology), STAT3 inhibitor Stattic (5 μM, Santa Cruz Biotechnology), or blank control (DMSO) was added to the osteogenic differentiation medium with 25 ng/mL recombinant OSM protein to induce LF cells.

2.5 | Enzyme-linked immunosorbent assay

OSM levels in LF cells treated with CM-M1 and CM-M1 plus COX-2 inhibitors were determined by ELISA. Cell supernatants were harvested via centrifugation (800 × g, 15 min, 4°C). OSM cytokine levels in supernatants were measured using a sandwich ELISA kit (Human OSM ELISA Kit; SEKH-0275; Solarbio, Beijing, China) according to the manufacturer's protocols.

2.6 | Quantitative real-time polymerase chain reaction analysis

Expression levels of osteogenic marker genes and cytokine receptor genes in LF cells were assessed by quantitative real-time polymerase chain reaction (qRT-PCR). Total RNA was isolated using Trizol (Invitrogen, Carlsbad, CA). Reverse transcription and qPCR were performed using a BeyoFast™ SYBR Green One-Step qRT-PCR Kit (D7268; Beyotime, Shanghai, China) according to the manufacturer's instructions on a BioRad IQ5 system (BioRad, Hercules, CA). Values were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using the $2^{-\Delta\Delta Ct}$ method. Primer sequences are provided in Table 2.

2.7 | Western blot analysis

Cell lysates were obtained using radioimmunoprecipitation assay lysis buffer (Beyotime) containing 10 mM phenylmethylsulphonylfluoride as a protease inhibitor (Beyotime). WB analysis was carried out as previously described.[21] The following primary rabbit anti-human monoclonal antibodies were purchased from Abcam (Cambridge, UK): OSMR (1:1000, ab282577), GP130 (1:1000, ab283685), JAK2 (1:2500, ab108596), p-JAK2 (1:2500, ab32101), STAT3 (1:2000, ab68153), p-STAT3 (1:2000, ab76315), and β-catenin (1:5000, ab32572). Immunoreactive bands were semi-quantified using ImageJ software and normalized to the corresponding β-actin bands.

2.8 | Statistical analysis

Statistical analysis was conducted using SPSS version 13.0 software (SPSS, Chicago, IL). The Shapiro–Wilk normality test was used to assess the normality of the data distribution. Comparisons between two groups were performed with the student *t*-test. For multiple group comparisons, one-way analysis of variance (ANOVA) and least-significant difference (LSD) tests were carried out. Data obtained from

TABLE 2 Primer sequences for quantitative real-time polymerase chain reaction.

Gene	Primer (5′–3′) Fw	Rv
ALPL	CCAAGGACGCTGGGAAATCT	TATGCATGAGCTGGTAGGCG
RUNX2	GCGCATTCCTCATCCCAGTA	GGCTCAGGTAGGAGGGGTAA
SP7	AAACCCAAGGCAGTGGGAAA	TGCCCCCATATCCACCACTA
BGLAP	ATGAGAGCCCTCACACTCCT	CTTGGACACAAAGGCTGCAC
SPP1	CATACAAGGCCATCCCCGTT	GGGTTTCAGCACTCTGGTCA
GAPDH	TCAAGGCTGAGAACGGGAAG	TGGACTCCACGACGTACTCA

experiments are expressed as mean ± standard deviation. A *P*-value <0.05 is considered statistically significant.

3 | RESULTS

3.1 | GeneChip profiling and ingenuity pathway analysis identify mechanisms of CM-M1 in osteogenic differentiation of LF cells

To further understand the underlying molecular mechanisms by which CM-M1 mediates osteogenic differentiation of LF cells, we performed GeneChip profiling of LF cells incubated with CM-M1 or CM-Control for 7 days. In addition, IPA was performed to compare these two groups. After incubation with CM-M1, 619 genes were upregulated and 303 genes were downregulated, as shown in the volcano plot and heat map in Figure 1A, B.

Diseases and Functions Analysis evaluates the enrichment of DEGs associated with specific diseases and functions. As shown in Figure 1C–E, CM-M1 promotes the differentiation of osteoblasts and bone cells, leading to increased mineralization and bone quantity. Moreover, CM-M1 could decrease expression of genes associated with cell apoptosis, death, and necrosis; and increase expression of genes associates with cell survival and viability.

Canonical pathway analysis evaluates the enrichment of DEGs involved in classical signaling pathways (Figure 2A). Incubation with CM-M1 resulted in the OSM signaling pathway being the most significantly enriched pathway, with a *Z* value greater than 2. Moreover, the two transmembrane receptors of OSM, OSMR and GP130 (also called CD130 and IL6ST), and many downstream molecules were upregulated following incubation with CM-M1 (Figure 2B). Upstream Regulator Analysis evaluates all upstream regulators of DEGs. Upstream regulators can be any molecule that affects gene expression, which covers all molecular types, including transcription factors, cytokines, microRNAs, receptors, kinases, chemical molecules, and drugs. The prediction results of upstream regulator analysis show that the cytokine OSM, kinase JAK2, transcription factor STAT3, and JAK/STAT signaling pathway inhibitor AG490 all rank in the top-10 DEGs for their respective categories (Figure 2C–F), suggesting that CM-M1 promotes ossification of LF cells through OSM-mediated activation of the JAK2/STAT3 signaling pathway. In addition, upstream regulator analysis predicted that many growth factors may be involved in the process by which CM1 promotes osteogenic differentiation of LF cells (Figure 2G).

3.2 | M1 Mφs promote osteogenic differentiation of LF cells by secreting OSM

To investigate OSM secretion by M1 Mφs and the effect of CM-M1 on osteogenic differentiation of LF cells, we measured OSM levels in CM-M1 by ELISA. After 7-days osteogenic induction, WB and qPCR were used to measure the expression of receptors and osteogenic genes in LF cells induced by CM. As shown in Figure 3A, compared with the control group, the CM-M1 group had significantly increased OSM expression. Furthermore, compared with the control group cells, CM-M1-induced LF cells exhibited increased expression of OSMR, GP130, and osteogenic genes (Figure 3B–D).

We also investigated the effects of COX-2 inhibition on OSM secretion by M1 Mφs and osteogenic differentiation of LF cells. ELISA was used to measure OSM levels in the supernatant after the addition of celecoxib, meloxicam, or DMSO to CM-M1. After 7 days of osteogenic induction, WB and qPCR were used to measure the expression of receptors and osteogenic genes in LF cells induced by CM. As shown in Figure 3E, OSM concentrations were lower in celecoxib and meloxicam groups compared with those in the DMSO group. Moreover, expression of OSMR, GP130, and osteogenic genes alkaline phosphatase (*ALPL*), *RUNX2*, Sp7 transcription factor (*SP7*), bone gamma carboxyglutamate protein (*BGLAP*), and secreted phosphoprotein 1 (*SPP1*) was lower in celecoxib and meloxicam groups compared with that in the DMSO group (Figure 3F–H).

We further investigated the effect of OSM secretion by M1 Mφs on osteogenic differentiation of LF cells by stimulating them with CM-M1 for 7 days in the presence of a neutralizing anti-human OSM monoclonal antibody or non-immune isotype-matched mouse IgG2a. Analyses of OSMR, GP130 receptor, and osteogenic gene expression show reduced osteogenic differentiation of LF cells in response to induction by CM-M1 after addition of the OSM antibody (Figure 3I–K).

In summary, M1 Mφs can secrete OSM cytokine capable of promoting LF cell osteogenic differentiation. The semi-quantitative analysis of WB in Figure 3 are shown in the Supplementary Figure 1A–C.

3.3 | OSM promotes osteogenic differentiation of LF cells through the JAK2/STAT3 signaling pathway

Our analysis focused on the effect of OSM on the JAK2/STAT3 signaling pathway during osteogenic differentiation of LF cells. After 7 days of osteogenic differentiation, WB and qPCR were used to

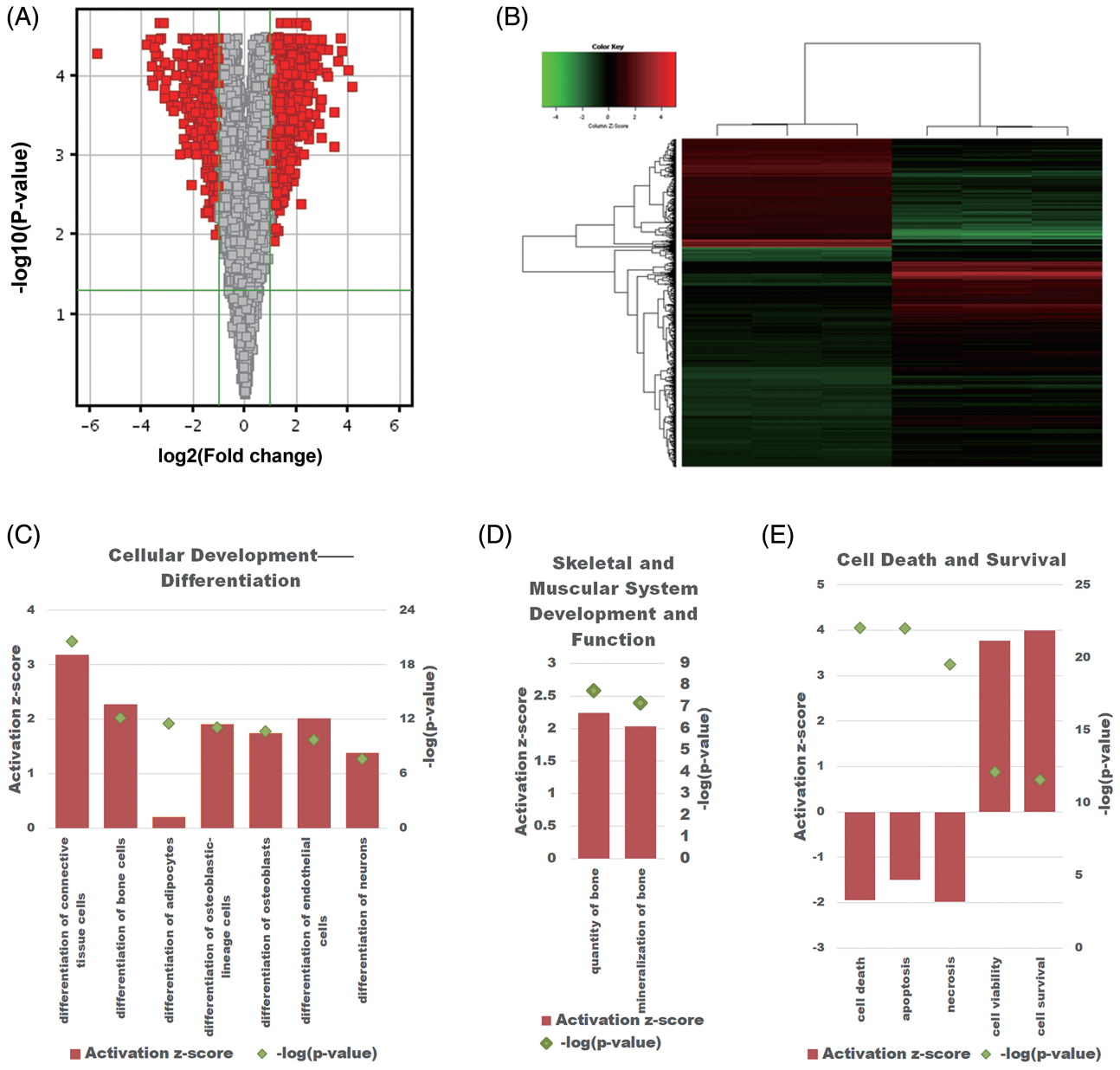

FIGURE 1 Conditioned medium containing secretions of M1 macrophages (CM-M1) can promote osteogenic differentiation of ligament flavum (LF) cells. (A, B) LF cells were induced by CM-M1. Differentially upregulated and downregulated genes are shown in volcano and heat maps. (C–E) Assessment of CM-M1 shows enrichment of factors involved in "Cellular Development-Differentiation", "Skeletal and Muscular System Development", and "Development by Upstream Regulator Function, Cell Death, and Survival", indicating notable functions.

measure the expression of membrane protein receptors OSMR and GP130, JAK2 and STAT3 proteins, and osteogenic genes. Compared with the control group, the OSM group had significantly increased expression of OSMR, GP130, P-JAK2, and P-STAT3 proteins, and osteogenic genes (Figure 4A–C).

Next, we investigated the impact of OSM receptors on the JAK2/STAT3 signaling pathway and osteogenic differentiation of LF cells. We used qPCR and WB to measure knockdown efficiency of the OSM receptor. After 7 days of osteogenic differentiation, we measured the expression of JAK2 and STAT3 proteins using WB, and osteoblast genes using qPCR. As shown in Figure 4D–G, following knockdown of OSMR and GP130 in LF cells, WB and qPCR results show that OSMR and GP130 were lowly expressed compared with those in the control group. Moreover, knockdown of *OSMR* and *GP130* genes in LF cells reduced expression of P-JAK2, P-STAT3, and osteogenic genes compared with the findings in the control group according to WB and qPCR analyses (Figure 4H, I).

To further investigate the impact of the JAK2/STAT3 signaling pathway on LF cell osteogenic differentiation, we employed JAK2/STAT3 signaling pathway inhibitors. Following 7 days of osteogenic differentiation in the presence of these inhibitors, expression of JAK2, STAT3, and osteogenic genes were detected by WB and qPCR,

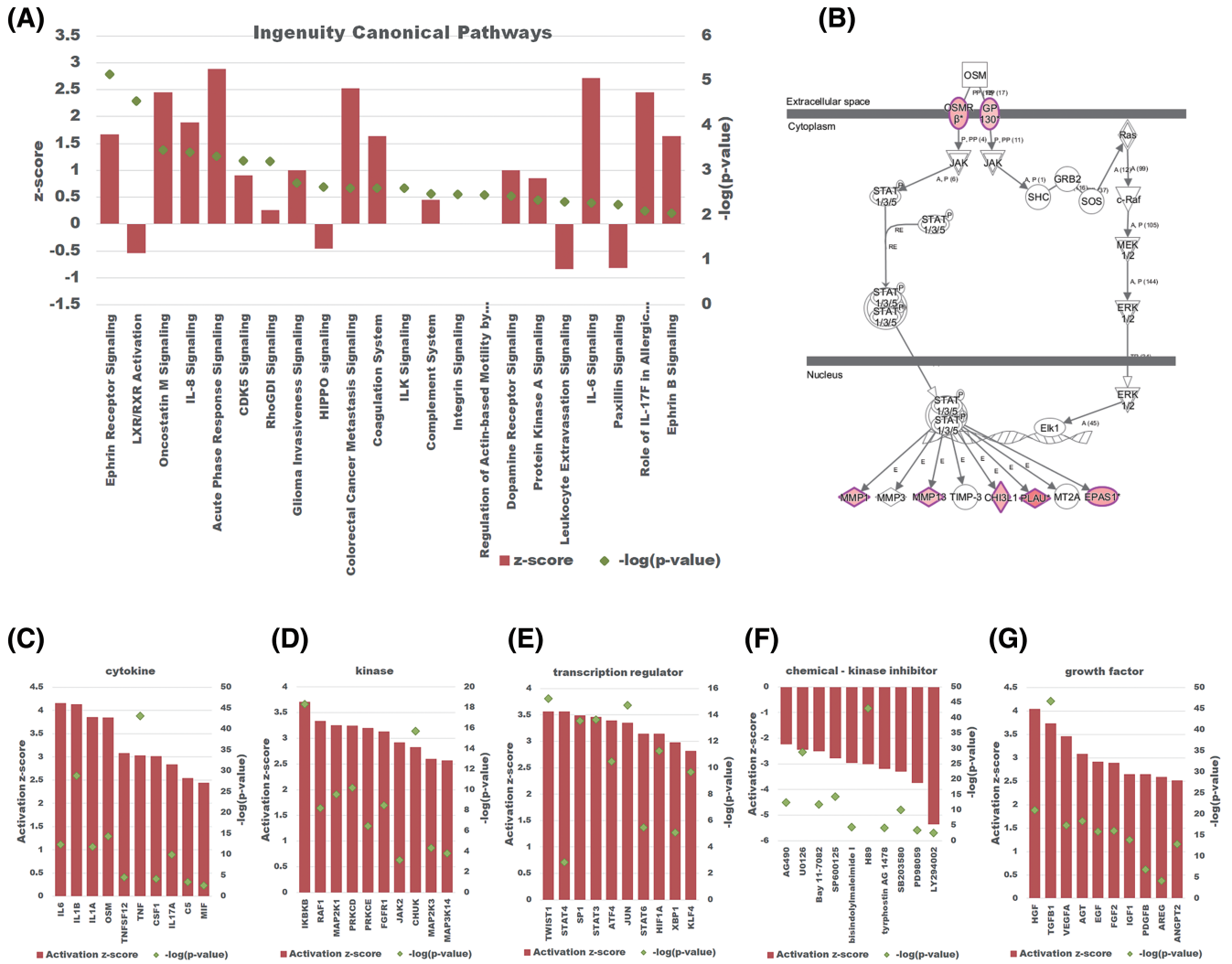

FIGURE 2 Oncostatin M (OSM) can promote the ossification of ligament flavum (LF) cells by activating the JAK2/STAT3 signaling pathway. (A) Upstream regulator analysis was used to evaluate the expression degree of each signaling pathway in osteogenic differentiation of LF cells. (B) Upregulated signal molecules downstream of OSM were analyzed by upstream regulator analysis. (C–F) Upstream regulator analysis was used to analyze the significance of the top 10 factors in each category of cytokines, kinases, transcription factors, and signaling pathway inhibitors. (G) Upstream regulator analysis was used to investigate the significance of growth factors other than OSM during osteogenic differentiation of LF cells.

respectively. Compared with the DMSO control group, both AZD1480 and Stattic groups had lower expression of P-JAK2, P-STAT, and osteogenic genes (Figure 4J, K).

In summary, OSM can promote LF cell osteogenic differentiation via the JAK2/STAT3 signaling pathway. The semi-quantitative analysis of WB in Figure 4 are shown in the Supplementary Figure 1D–H.

4 | DISCUSSION

Thoracic ligament ossification, a degenerative disease characterized by spinal heterotopic ossification, is caused by the alternation of ligament tissue damage and repair under chronic inflammation and long-term stress, which leads to an osteogenic differentiation process.[22,23] Mφs play an important role in heterotopic ossification and act as a bridge between inflammation and heterotopic ossification.[24–26] M1 Mφs can promote osteogenic differentiation of LF cells, although the specific mechanisms remain unclear. This study employed GeneChip and IPA to analyze the effect of CM-M1 on LF cells, which was then verified using in vitro experiments. The results show that M1 Mφs can secrete OSM cytokines that promote osteogenic differentiation of LF cells through the JAK2/STAT3 signaling pathway.

The results of GeneChip and IPA analysis show that the OSM signaling pathway was significantly activated in LF cells following stimulation with the supernatant of M1 Mφs. Moreover, two membrane protein receptors of the OSM signaling pathway (GP130 and OSMR) and several downstream inflammation-related genes were significantly upregulated. Upstream enrichment analysis of differentially expressed genes shows that JAK2 and STAT3 were significantly activated. Altogether, these results suggest that OSM signaling is involved in osteogenic differentiation of LF cells, and JAK2/STAT3 may be an important pathway downstream of OSM. In addition, expression of

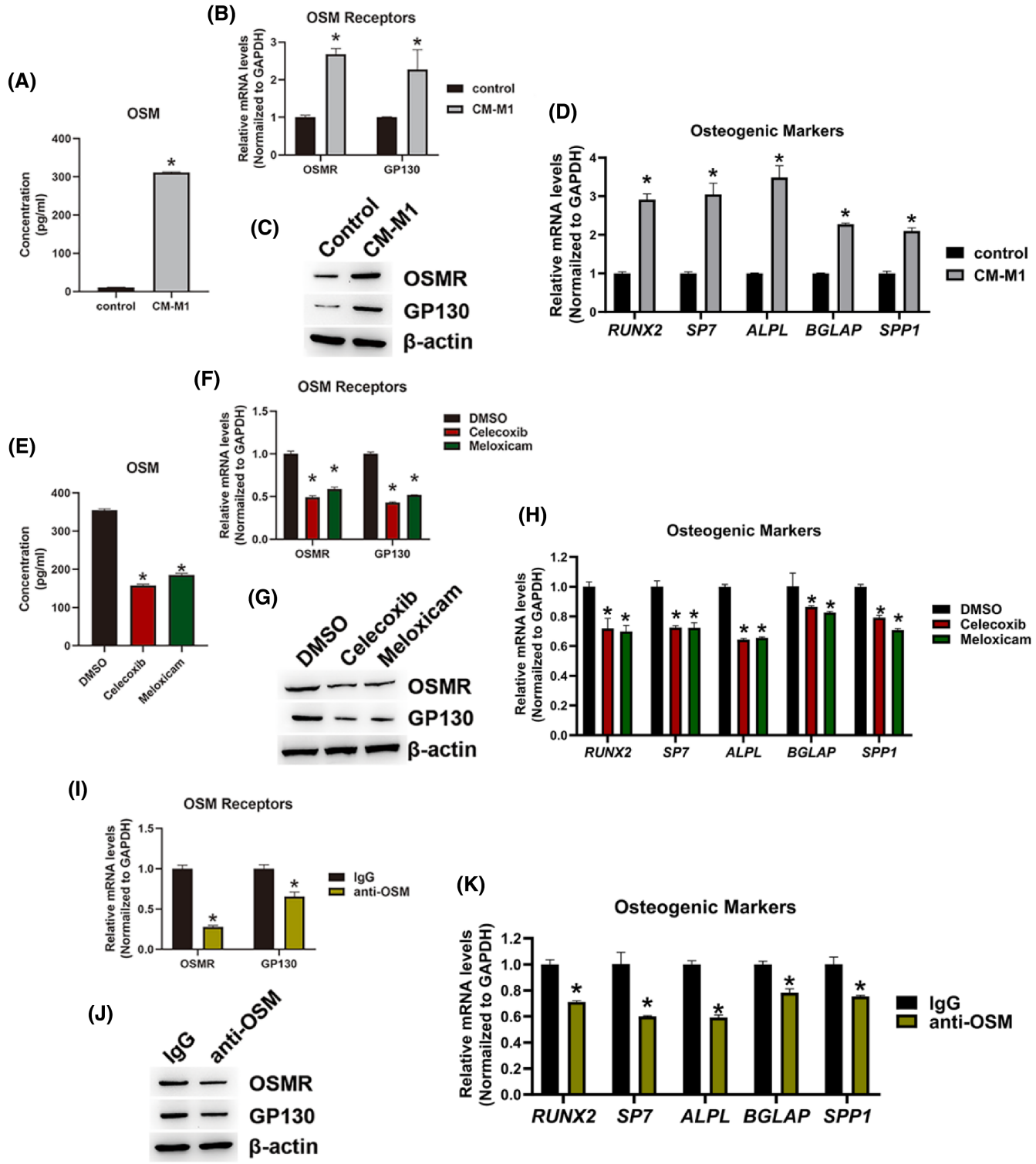

FIGURE 3 M1 macrophages promote osteogenic differentiation of ligament flavum (LF) cells by secreting oncostatin M (OSM). (A) ELISA detection of OSM in conditioned medium containing secretions of M1 macrophages (CM-M1) and the supernatant of control group cells ($n = 3$), two-tailed Student's *t*-test. (B–D) Expression of receptors and osteogenic genes in CM-M1-induced LF cells was detected by western blot (WB) and qPCR ($n = 3$), two-tailed Student's *t*-test. (E) ELISA was used to detect OSM contents in CM-M1 after dimethyl sulfoxide (DMSO) and cyclooxygenase 2 (COX-2) inhibitor were added ($n = 3$), one-way ANOVA analysis. (F–H) qPCR and WB were used to detect expression of LF cell receptor and osteogenic genes induced following the addition of DMSO or COX-2 inhibitors into CM-M1 ($n = 3$), one-way ANOVA analysis. (I–K) In the presence of OSM neutralizing antibody, LF cells were induced by CM-M1 for 7 days. Subsequently, expression of corresponding receptors and osteogenic differentiation genes in LF cells was evaluated by WB and qPCR ($n = 3$), two-tailed Student's *t*-test. All data were presented as means ± standard deviation. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

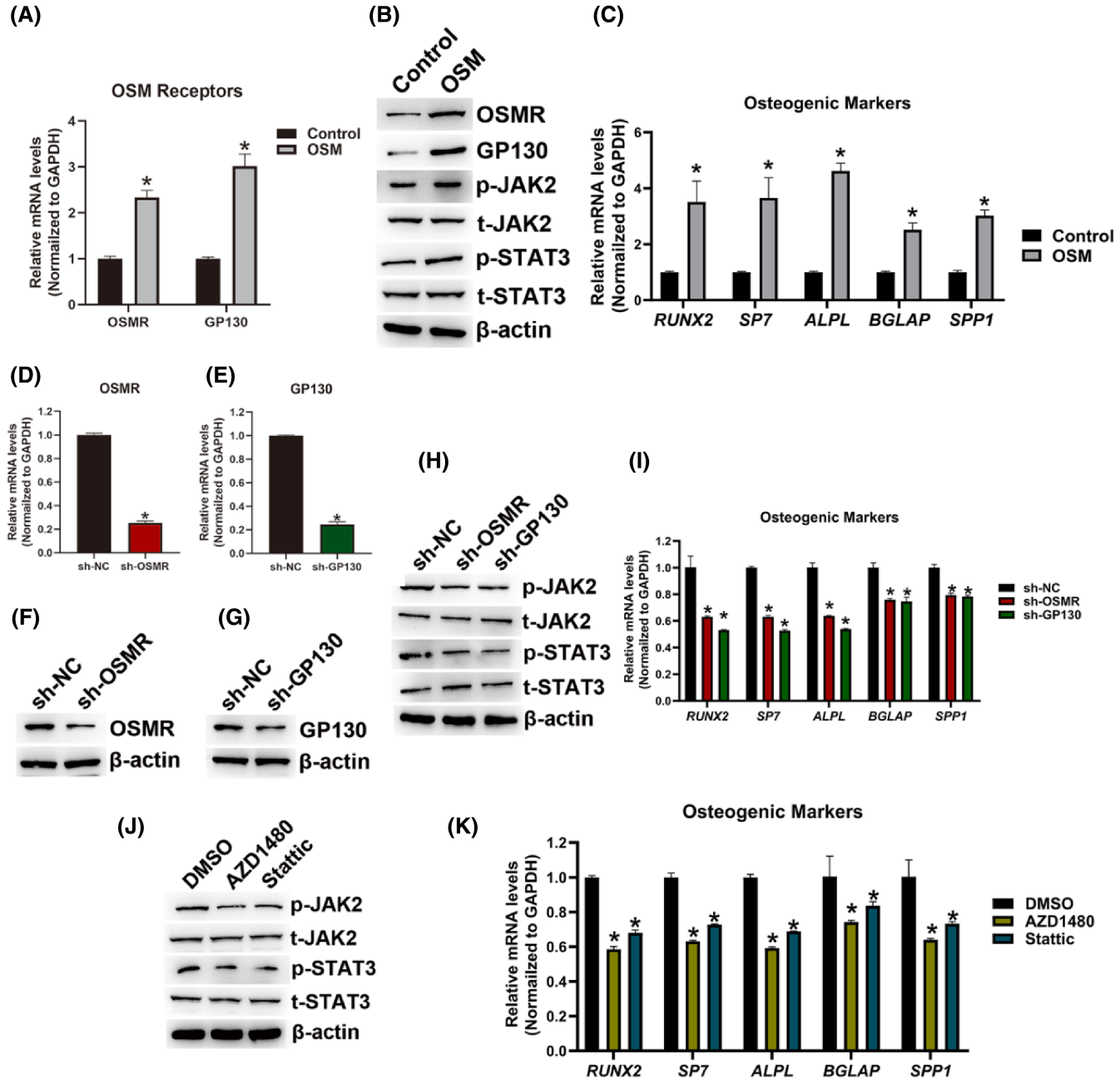

FIGURE 4 Oncostatin M (OSM) promotes osteogenic differentiation of ligamentum flavum (LF) cells through the JAK2/STAT3 signaling pathway. (A–C) Expression of OSMR, GP130, P-JAK2, P-STAT, and osteogenic genes was detected by western blot (WB) and qPCR after 7 days of OSM cytokine induction ($n = 3$), two-tailed Student's *t*-test. (D–G) Expression of OSMR and GP130 was evaluated after knockdown in LF cells ($n = 3$), two-tailed Student's *t*-test. (H, I) LF cells with knockdown of OSMR and GP130, and stimulation with OSM. WB and qPCR were performed to evaluate expression of P-JAK2, P-STAT3, and osteogenic differentiation genes ($n = 3$), one-way ANOVA analysis. (J, K) In the presence of AZD1480 and Stattic, expression of P-JAK2, P-STAT3, and osteogenic genes was detected by WB and qPCR after 7 days of OSM induction in LF cells ($n = 3$), one-way ANOVA analysis. All data were presented as means ± standard deviation. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

both GP130 and OSMR was upregulated in LF cells stimulated with CM of M1 Mφs; however, expression of the *OSM* gene itself was not upregulated, indicating that OSM receptors in LF cells may be activated by exogenous OSM. OSM is mainly produced and secreted by activated Mφs and can promote the occurrence of heterotopic ossification.[27] Therefore, we hypothesized that M1 Mφs activate the JAK2/STAT3 signaling pathway in LF cells by secreting OSM, thereby promoting osteogenic differentiation of LF cells during the occurrence and development of TOLF.

M1 Mφs play a key role in the occurrence and development of heterotopic ossification. Qu and colleagues[20] found that M1 Mφ-derived IL-6 can promote osteogenic differentiation of LF cells.

Xia[28] reported that M1 Mφs can promote osteogenic differentiation of aortic valve interstitial cells through mitophagy, providing a new target for the treatment of aortic valve calcification. Our findings indicate that supernatants of M1 Mφs induce osteogenic differentiation of LF cells and promote the progression of heterotopic ossification, in line with previous findings.[20,28]

OSM can induce osteogenic differentiation of cells. Guihard et al.[29] reported that OSM promotes osteogenic differentiation of MSCs, whereas an anti-OSM antibody attenuated their osteogenic differentiation. M1 Mφs can secrete OSM through the COX-2/PGE2 signaling pathway. COX-2 inhibitors, such as celecoxib and meloxicam, can inhibit the generation and secretion of OSM by M1 Mφs, thereby inhibiting their effect on osteogenic differentiation of MSCs.[30] The regulation of osteogenic differentiation of LF cells by M1 Mφs depends partly on the binding of OSM cytokine secreted by Mφs to their receptors on target cells. OSM cytokine secreted by M1 Mφs match the receptors on LF cells, suggesting their possible involvement in osteogenic differentiation of LF cells. COX-2 inhibitor and antibody neutralization experiments further reveal that OSM cytokine secreted by M1 Mφs promotes osteogenic differentiation of LF cells, consistent with the results of previous studies.[29,30] The osteoblast phenotype has two stages. In the early stage of osteogenic differentiation, ALP expression appears during proliferation and maturation of the cell matrix. In the late stage of osteogenic differentiation, bone markers OCN and OPN are mainly detected during bone mineralization.[31,32] Transcription factors Runx2 and osterix are upregulated during the early stage of osteogenic differentiation, whereby they regulate production of ALP, OCN, and OPN. For the induction of osteogenic differentiation of LF cells, the first 7 days represent the early stage[33] and ALP production is greater than that of OCN and OPN.

The JAK/STAT signaling pathway is involved in a variety of biological processes, including hematopoiesis, tissue repair, inflammation, differentiation, and apoptosis.[34] In addition, the JAK/STAT transduction pathway has been widely studied in osteogenic differentiation of cells.[35,36] A previous study showed that Mφ-derived OSM can bind to OSMR and promote osteogenic differentiation of cells in muscle tissue, thereby enhancing heterotopic ossification; moreover, silencing the *OSMR* gene can significantly reduce the incidence of heterotopic ossification.[37] Another study reported that the combination of Mφ-derived OSM and GP130/OSMR receptor complex during osteogenic differentiation of MSCs can activate JAK1/2 and mediate STAT3 phosphorylation to promote osteogenic differentiation, and the addition of JAK1/2 specific inhibitors can weaken osteogenic differentiation and inhibit the development of NHO in mice.[35] In addition, Zhang et al.[38] pointed out that blocking the JAK signaling pathway can effectively attenuate leptin-induced osteogenic differentiation of MSCs. Our results indicate that knockdown of OSMR or GP130 weakens osteogenic differentiation and decreases expression of P-JAK2 and P-STAT3 during OSM-induced osteogenic differentiation of LF cells. Moreover, addition of a JAK2 inhibitor or STAT3 inhibitor weakened osteogenic differentiation, and expression levels of P-JAK2 and P-STAT3 were decreased to different degrees. These experimental findings are consistent with the results of several previous studies.[34,36,37]

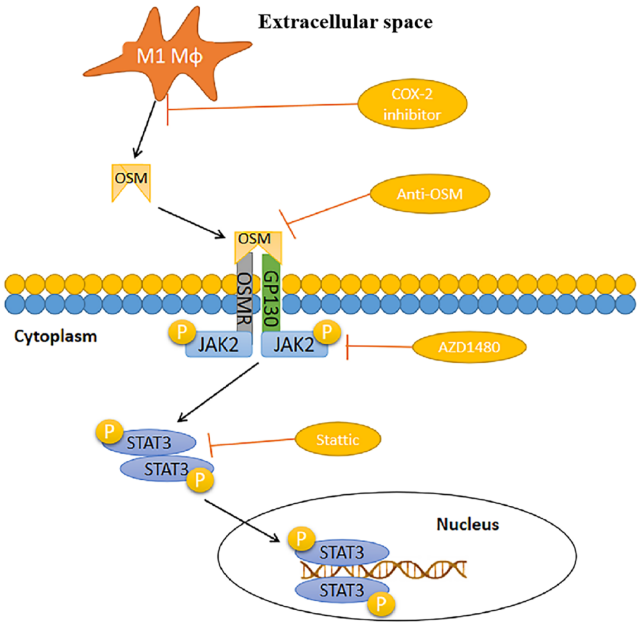

FIGURE 5 M1 macorphages (Mφs) can secrete oncostatin M (OSM) and induce osteogenic differentiation of ligamentum flavum (LF) cells via the JAK2/STAT3 signaling pathway. The cytokine OSM binds to the receptor OSMR/GP130, stimulating activation and phosphorylation of JAK2. Phosphorylated JAK2 subsequently stimulates phosphorylation of downstream STAT3, causing STAT3 to form a dimer that migrates to the nucleus and binds to DNA to regulate gene transcription. Cyclooxygenase-2 inhibitors reduce OSM secretion by M1 Mφs. OSM neutralizing antibody can inhibit the binding of OSM to its receptor. Knockdown of OSMR/GP130 can obstruct signal transmission. AZD1480 inhibits both expression and phosphorylation of JAK2. Stattic can block STAT3 activation and phosphorylation.

However, there are some limitations to this study. First, although osteogenic differentiation was reduced when JAK2 or STAT3 inhibitors were administered during OSM-induced osteogenic differentiation of LF cells, osteogenic markers were still expressed. These results indicate that other signaling pathways are involved and further research is needed. In addition, osteogenic differentiation of LF cells occurs in a three-dimensional environment in vivo, where multiple factors influence their differentiation. Because this study was conducted in vitro, further in vivo experiments are required to validate the findings. Finally, we only investigated the early stages of OSM on the osteogenic differentiation of LF cells, and further studies may be needed as to its effect on the later stages.

5 | CONCLUSIONS

This study found that M1 Mφs can secrete OSM cytokines, which promote osteogenic differentiation of LF cells mainly through the JAK2/STAT3 signaling pathway (Figure 5). This finding promotes

understanding of the effect and mechanism of Mφ-mediated inflammatory responses on OLF, which can further improve knowledge of related diseases and provide new ideas for treatments.

AUTHOR CONTRIBUTIONS

Jun Yang: Designed the study, performed the laboratory work, statistics and data analysis, and drafted the manuscript. Xiaochen Qu: Designed the study, secured funding, analyzed and interpreted data, and approved the final submitted manuscript. Guanghui Chen and Tianqi Fan: Specimen and clinical data acquisition, performed some of the laboratory work and statistics. All authors have read and approved the final submitted manuscript.

ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China (Grant Number: 82002326).We thank Liwen Bianji (Edanz) (www.liwenbianji.cn/) for basic language editing of a draft of this manuscript.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Yang, J., Chen, G., Fan, T., & Qu, X. (2024). M1 macrophage-derived oncostatin M induces osteogenic differentiation of ligamentum flavum cells through the JAK2/STAT3 pathway. *JOR Spine*, *7*(1), e1290. https://doi.org/10.1002/jsp2.1290