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# Mesenchymal stem cells derived from synovial fluid effectively alleviate immune imbalance in collagen-induced arthritis rats by regulating anti-inflammatory factor levels

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**Abstract**: **Background**: Rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by synovial inflammation and joint destruction. Mesenchymal stem cells (MSCs) derived from synovial fluid have shown immunomodulatory potential, but their therapeutic effects in RA models remain incompletely understood. **Objectives**: This study aimed to isolate and characterize synovial fluid-derived MSCs (SF-MSCs) from RA patients and evaluate their therapeutic efficacy in collagen-induced arthritis (CIA) rats. **Methods**: SF-MSCs were isolated from RA patient synovial fluid and characterized by flow cytometry. CIA was induced in Wistar rats. CIA rats were treated with either methotrexate (MTX, 0.2 mg/kg) or SF-MSCs ($1\times10^6$ cells/rat) via tail vein injection on days 21, 24, 27 and 30. Paw swelling, arthritis index scores, X-ray imaging, and histopathology were used to assess arthritis severity. *In vitro*, SF-MSCs were co-cultured with rat peripheral blood mononuclear cells (PBMCs) at a 1:10 ratio and cytokine levels were measured by enzyme-linked immunosorbent assay (ELISA) and quantitative real-time polymerase chain reaction (qPCR). **Results**: SF-MSCs expressed high levels of cluster of differentiation 44 (CD44, 98.2%), CD105 (96.5%) and CD166 (95.8%), but low levels of CD15, CD34 and CD45 (<2%). SF-MSC treatment significantly reduced paw swelling, arthritis index scores, joint space narrowing and bone erosion compared to CIA controls, with effects comparable to MTX. In co-culture experiments, SF-MSCs significantly suppressed interleukin-6 (IL-6), interleukin-17 (IL-17), interleukin-21 (IL-21) and interleukin-23 (IL-23) production while enhancing transforming growth factor-beta (TGF-β) expression in rat PBMCs. **Conclusion**: SF-MSCs derived from RA patients exhibit potent immunomodulatory effects, alleviating joint inflammation and destruction in CIA rats. These findings support SF-MSC-based therapy as a promising cell-based approach for RA treatment.

**Keywords**: Clinical data; Inflammatory factors; Immune imbalance; Rheumatoid arthritis; Synovial fluid

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## INTRODUCTION

Synovial fluid-derived mesenchymal stem cells (SF-MSCs) have strong self-renewal ability and the potential for multidirectional differentiation. Their differentiation direction is not restricted by the germ layer and their multi-directional differentiation potential can be maintained during long-term culture. Another important feature of MSCs is their immunomodulatory ability to inhibit immunological cell proliferation (Yu *et al*., 2023; Lin *et al*., 2025). Studies have shown that inflammatory conditions can induce MSCs to act as immunomodulators by inhibiting T cell proliferation and functional activity. Recent studies have demonstrated that MSCs can inhibit the production of inflammatory factors by inflammatory cells and promote the differentiation and functional induction of regulatory T cells (Tregs) by secreting regulatory molecules (Yang *et al*., 2023; Kao *et al*., 2026; Hou *et al*., 2025).

Rheumatoid arthritis (RA) is an autoimmune disease characterized by erosive arthritis, morning stiffness, swelling, pain and joint dysfunction. The disease is difficult to cure; only standard diagnosis and treatment can achieve a good prognosis and prevent joint deformity and loss of function (Ramos-Petersen *et al*., 2024; Yang *et al*., 2026). RA attacks the joints of the hands, wrists and knees due to an imbalance of the human immune system. The disease is most common between 30 and 50 years of age and approximately 75% of patients are women (Zhang *et al*., 2025; Venetsanopoulou *et al*., 2023). In RA, pro-inflammatory cytokines play a major role in the pathology. Clinical trials, animal models and cell culture studies have indicated that T cell balance in joint tissue is involved in the development and symptoms

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of RA. T helper 17 (Th17) cells and their effector cytokines are key participants in inflammation, autoimmunity and allergy and are closely related to the activity of Tregs. The imbalance between Th17 cells and Tregs is crucial for RA morbidity and disease progression. Thus, T helper 1 (Th1) and Th17 subsets mediate the occurrence of abnormal inflammatory responses. Therefore, the reduced number or functional defect of Tregs plays an important role in the immune imbalance associated with RA. Recent studies have shown that MSCs from various sources have good therapeutic effects in collagen-induced arthritis (CIA) models (Tang *et al*., 2025). In addition, the administration of human adipose tissue-derived MSCs before or during the early stage of induced arthritis can effectively play a preventive and therapeutic role. Therefore, we aimed to investigate the therapeutic effect and mechanism of synovial fluid-derived MSCs in RA treatment. In this study, we isolated SF-MSCs from the synovial fluid of RA patients, characterized their immunophenotype and evaluated their therapeutic effects and immunomodulatory functions using a CIA rat model and *in-vitro* co-culture experiments.

## MATERIALS AND METHODS

### *SF-MSC separation*

Synovial fluid-derived mesenchymal stem cells (SF-MSCs) were obtained from synovial fluid specimens provided by Nanjing First Hospital, Nanjing Medical University. The synovial fluid was diluted with an equal volume of phosphate-buffered saline (PBS), carefully layered over an equal volume of Ficoll-Paque Plus (GE Healthcare, USA) separation solution and centrifuged at 400 × g for 30 minutes. The mononuclear cell layer was collected, washed twice with PBS and resuspended in low-glucose Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS, Gibco, USA) and 1% penicillin/streptomycin. The cells were seeded into T25 culture flasks and incubated at 37ºC in a 5% carbon dioxide ($CO_2$) humidified incubator. The first medium change was performed after 48 hours to remove non-adherent cells, followed by medium changes every 3 days. When cells reached 80-90% confluence, they were detached using 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA) and passaged. Passage 3-5 cells were used for subsequent experiments (Jones *et al*., 2004).

### *Identification of mesenchymal stem cells*

Trypsin (Botto Biotechnology Co., Ltd., China) was used to detach the third-generation MSCs after washing with PBS. Fluorescently labeled antibodies were used to bind specifically to antigens on the cell surface and the fluorescent signal was detected. Isotype controls (same species source, same subtype, same dose and same immunoglobulin isoform as the experimental antibody) were used to eliminate non-specific background staining. Cell samples were divided into 1.5 mL Eppendorf (EP) tubes, centrifuged (1000 rpm, 4°C, 3 min) and the supernatant was discarded. Cells were resuspended in PBS containing 1% FBS to obtain a single-cell suspension at $1.0 \times 10^6$ cells/mL. Aliquots of 100 µL of the cell suspension were dispensed into labeled tubes and analyzed by fluorescence-activated cell sorting (FACS) for expression of cluster of differentiation 105 (CD105), CD90 and CD73 (Xian Ding Biotechnology Co., Ltd., China), CD31 and the hematopoietic marker CD45 (Leeham Biotechnology Co., Ltd., China).

Osteogenic differentiation: SF-MSCs were seeded at a density of $3 \times 10^4$ cells/well in 24-well plates. After cell attachment, the medium was replaced with osteogenic differentiation induction medium (α-MEM) containing 10% FBS, 10 mM β-glycerophosphate, 50 µg/mL ascorbate and 0.1 µM dexamethasone). The medium was refreshed every 3 days for 21 days. After induction, the cells were fixed with 4% paraformaldehyde and calcium nodule formation was evaluated using Alizarin Red S staining.

Chondrogenic differentiation: A total of $2 \times 10^5$ SF-MSCs were centrifuged into a pellet at the bottom of 15 mL centrifuge tubes. The supernatant was discarded and chondrogenic differentiation induction medium (α-MEM containing 1% insulin-transferrin-selenium (ITS), 50 ng/mL transforming growth factor-beta 3 (TGF-β3), 50 µg/mL ascorbate-2-phosphate, 100 nM dexamethasone, 1 mM sodium pyruvate and 40 µg/mL proline) was added. The medium was refreshed every 3 days for 21 days. After induction, the cell pellets were fixed with 4% paraformaldehyde, embedded in paraffin, sectioned and proteoglycan expression in the cartilage matrix was evaluated using Alcian Blue staining.

Adipogenic differentiation: SF-MSCs were seeded at a density of $2 \times 10^4$ cells/well in 24-well plates. After reaching 100% confluence, the medium was replaced with adipogenic differentiation induction medium (α-MEM containing 10% FBS, 10 µg/mL insulin, 1 µM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) and 100 µM indomethacin). The medium was refreshed every 3 days for 14 days. After induction, the cells were fixed with 4% paraformaldehyde and lipid droplet formation was evaluated using Oil Red O staining. Uninduced control groups (cells cultured in regular α-MEM complete medium) were included for all differentiation experiments and the medium was refreshed every 3 days. Staining results were observed and photographed under an inverted microscope (Pittenger *et al*., 1999).

***Flow cytometer***

Third-generation SF-MSCs ($5 \times 10^6$ cells) were collected and washed twice with PBS containing 1% FBS. The gating strategy was as follows: viable cell populations were first gated using forward scatter (FSC) and side scatter (SSC) dot plots to exclude cell debris and dead cells. The cells were stained with phycoerythrin (PE)-conjugated mouse anti-human CD44, CD105, CD166, CD15, CD34 and CD45 antibodies, as well as fluorescein isothiocyanate (FITC)-conjugated corresponding antibodies. FITC- or PE-conjugated mouse immunoglobulin G (IgG) isotype control antibodies were used as negative controls to eliminate non-specific binding background. After incubation for 30 minutes at room temperature in the dark, the cells were washed twice with PBS and analyzed by flow cytometry (FACS Calibur, BD Biosciences). A total of 10,000 events were acquired per sample. Data were analyzed using WINMDI software and positive expression rates were expressed as percentages (%). Each sample was tested in triplicate (Dominici *et al*., 2006).

***Laboratory animals and treatment***

Thirty healthy female Wistar rats (6-8 weeks old, weighing 140-150 g) were purchased from Huacuo Biotechnology Company. The rats were randomly divided into a normal group (n=6) and a CIA model group (n=24). CIA induction and treatment were performed as follows:

CIA model induction: On day 0, the rats were injected intradermally at the tail base with 200 μL of an emulsion containing equal volumes of bovine type II collagen (2 mg/mL) and complete Freund's adjuvant (CFA) containing Mycobacterium tuberculosis H37Ra (5 mg/mL). On day 21, a booster injection of the same dose was administered at the same site. CIA model induction was considered successful when the rats exhibited paw swelling, an increase in ankle diameter of more than 2 mm from baseline, and an increase in hind paw volume of more than 0.80 mL (Brand *et al*., 2007).

SF-MSC preparation and quality control: SF-MSCs were cultured to passage 3 (P3). At 80-90% confluence, the cells were detached with 0.25% trypsin-EDTA, washed twice with PBS and resuspended in PBS containing 1% rat serum albumin to a final concentration of $1 \times 10^7$ cells/mL. Cell viability was assessed using the trypan blue exclusion method: 20 μL of the cell suspension was mixed with 20 μL of 0.4% trypan blue solution, incubated for 2-3 minutes at room temperature and unstained (live) and stained blue (dead) cells were counted using a hemocytometer under an inverted microscope. Cell viability (%) was calculated as (number of live cells / total number of cells) × 100%. The overall mean cell viability was 96.8% ± 0.6% (range: 95.8%-98.0%), with all batches exceeding the 95% acceptance criteria for *in-vivo* injection. All cell suspensions were injected within 2 hours of preparation.

Animal groups and treatment: The normal group received no treatment. The CIA model group underwent CIA induction without treatment. The CIA + MTX group (positive control) received intraperitoneal injections of methotrexate (MTX, 0.2 mg/kg in 100 μL saline) on days 21, 24, 27 and 30. The CIA + SF-MSC group received tail vein injections of SF-MSC suspension ($1 \times 10^6$ cells/100 μL/rat) on days 21, 24, 27 and 30. The rats were euthanized at week 6 for specimen collection. Before euthanasia, the rats were deeply anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg). After confirming the absence of a pain reflex, the rats were euthanized by cervical dislocation. All animal experiments were approved by the Animal Ethics Committee of Nanjing First Hospital, Nanjing Medical University (Approval No.: 2022072605) and were performed in strict accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals and the Animal Research: Reporting of in-vivo Experiments (ARRIVE) guidelines (Percie du Sert *et al*., 2020). All surgeries were performed under anesthesia and every effort was made to minimize animal suffering and the number of animals used.

***Arthritis assessment and immunohistochemical***

The degree of arthritis and swelling in the rats was evaluated using an arthritis index score (Freeston *et al*., 2024). X-ray radiography was performed on the rats using X-ray equipment. At week 6, three experienced independent imaging physicians evaluated the radiological changes in the joints. The rats were then euthanized and the spleen and joints were removed for follow-up studies. The spleen of each rat was fixed in 4% paraformaldehyde (Hengdailao Biotechnology Co., Ltd., China), embedded in paraffin wax and synovial inflammation was detected by hematoxylin and eosin (H&E) staining (Hengdailao Biotechnology Co., Ltd., China) (Jianzhen *et al*., 2023).

***Separation and culture of peripheral blood mononuclear cells (PBMCs)***

Rat-derived blood samples were centrifuged at 20°C for 40 minutes. The monocyte layer was transferred and washed with PBS. Centrifugation at 200 × g for 15 minutes was performed to remove platelets. Serum samples were kept at -70°C until cytokine measurements were performed. Peripheral blood mononuclear cells (PBMCs) from rat blood were maintained in DMEM containing 10% FBS (Annorum Biotech, China) and 1% penicillin/streptomycin, and cultured in a constant-temperature, humidity-controlled incubato (Böyum A, 1968) r. The PBMCs used in the co-culture experiments were derived from CIA model rats and were used to evaluate the effects of SF-MSCs on rat immune cells.

***PBMC and MSC co-culture***

SF-MSCs derived from RA patients and rat-derived

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PBMCs were cultured alone or together according to the following groups: (1) PBMC alone, (2) MSC alone, (3) fibroblast alone, (4) PBMC alone with phytohemagglutinin (PHA) stimulation, (5) PBMC co-cultured with fibroblasts with PHA, (6) PBMC co-cultured with MSCs and (7) PBMC co-cultured with MSCs with PHA.

Fibroblasts were used as a non-MSC adherent-cell negative control to exclude non-specific activation or inhibition of PBMCs by the adherent-cell population itself in the co-culture system. A direct-contact co-culture system was used for the co-culture experiments. SF-MSCs and fibroblasts ($1 \times 10^5$/mL) were seeded into 12-well plates and PBMCs were added at a density of $1 \times 10^6$/mL. After 12 hours, all co-culture groups were established at a 1:10 ratio (SF-MSCs:PBMCs or fibroblasts:PBMCs). All cells were cultured for 24 hours in a 37°C, 5% $CO_2$ saturated humidity incubator, after which the PBMCs and their supernatants were collected.

***Enzyme-linked immunosorbent assay (ELISA) of cytokine secretion***

Supernatants from the co-culture experiments (section 2.7) were collected and the concentrations of IL-6, IL-17, IL-21, IL-23 and TGF-β were measured using ELISA kits. All ELISA kits were purchased from R&D Systems (USA). The catalog numbers were as follows: M6000B (IL-6), M1700 (IL-17), DY421 (IL-21), M2300 (IL-23) and DY1679 (TGF-β). The detection sensitivities were 2.0 pg/mL (IL-6), 5.0 pg/mL (IL-17), 3.0 pg/mL (IL-21), 4.0 pg/mL (IL-23) and 8.0 pg/mL (TGF-β). All kits were validated for mouse/rat samples. All operations were performed strictly according to the manufacturer's instructions. Absorbance (optical density, OD) was measured at 450 nm using a microplate reader (Multiskan FC, Thermo Fisher Scientific) and cytokine concentrations were calculated using standard curves. Each sample was tested in triplicate (Portilho *et al*., 2022).

***Quantitative real-time polymerase chain reaction (qPCR)***

Cells were collected and washed three times with pre-chilled PBS and an appropriate amount of lysis solution was added. After a 20-minute ice bath, the lysate was transferred to 1.5 mL EP tubes for subsequent steps or stored at -80°C.

An appropriate amount of tissue was fully shredded, placed into 2 mL thick-walled EP tubes and beads and 1 mL of TRIzol reagent were added. After centrifugation with chloroform at low temperature, the upper aqueous phase was collected to harvest RNA precipitate. RNA concentration and purity were then detected. Reverse transcription (RT) reaction solution was prepared on ice after collecting 1 μg of total RNA. A total of 1 μg of RNA was reverse-transcribed in IDTE buffer (pH 8.0, Integrated DNA Technologies, USA), diluted to 10 ng/μL, and qPCR was performed according to the manufacturer's instructions. The reaction conditions were as follows: 90°C for 60 seconds, pre-denaturation at 95°C for 90 seconds, followed by 40 cycles of denaturation at 95°C for 60 seconds, annealing at 95°C for 45 seconds and extension at 60°C for 45 seconds. The cycle threshold (Ct) value for each gene was determined after the reaction, corresponding to the initial cycle at which the gene entered the linear amplification phase. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression was used as an internal control and the expression of each sample and related genes was evaluated using the $2^{-}\Delta\Delta Ct$ method (Livak and Schmittgen, 2001).The primer sequences used for qPCR are listed in Table 1.

***Statistical analysis***

All statistical analyses were performed using SPSS 21.0 software (IBM Corp., USA). Data are presented as mean ± standard deviation (SD). Comparisons between the two groups were performed using independent-samples t-tests. For multiple group comparisons, a one-way analysis of variance (ANOVA) followed by Tukey's post hoc test was used. A p-value $< 0.05$ was considered statistically significant. All experiments were repeated at least three times independently.

## RESULTS

***Synovial fluid-derived mesenchymal stem cell (SF-MSC) identification***

Flow cytometry results showed that SF-MSCs expressed high levels of CD44 (98.2% ± 1.5%), CD105 (96.5% ± 2.1%) and CD166 (95.8% ± 1.8%), while the positive expression rates of the hematopoietic lineage markers CD15, CD34 and CD45 were all below 2% (1.2% ± 0.3%, 0.8% ± 0.2% and 1.5% ± 0.4%, respectively) (Fig. 1, Table 2). The staining background of the isotype control antibodies was below 1% for all markers. These results confirmed the mesenchymal stem cell characteristics of the isolated cells.

As shown in figure 1C, SF-MSCs demonstrated trilineage differentiation potential. After 21 days of osteogenic induction, Alizarin Red S staining revealed orange-red calcium nodule deposits. After 14 days of adipogenic induction, Oil Red O staining showed red lipid droplet accumulation. After 21 days of chondrogenic induction, Alcian Blue staining demonstrated blue proteoglycan deposition.

**Table 1**: Sequences of genes used for quantitative real-time polymerase chain reaction (qPCR).

| Gene | Upstream sequence (5'-3') | Downstream sequence (5'-3') |
|---|---|---|
| IL-6 | CAGGTTGTTTTCTGCCAGTG | GACCGACACTCACCTCTTCA |
| IL-17 | GGACTGTGATGGTCAACCTG | CTCCCAGATCACAGAGGGAT |
| IL-21 | AGGTCAAGATCGCCACAT | TTTGCTGACTTTAGTTGGGC |
| IL-23 | CAAGTGGAAGTGGGCAGAG | CAGCAACAGCAGCATTACAG |
| TGF-β | AACGAACTGGCTGTCTGC | CCTCTGCTCATTCCGCTTAG |
| GAPDH | TCGGAGTCAACGGATTTGGTC | GCCATGGGTGGAATCATATTGG |

Note: Primer sequences used for quantitative real-time polymerase chain reaction (qPCR). GAPDH was used as the internal reference gene.

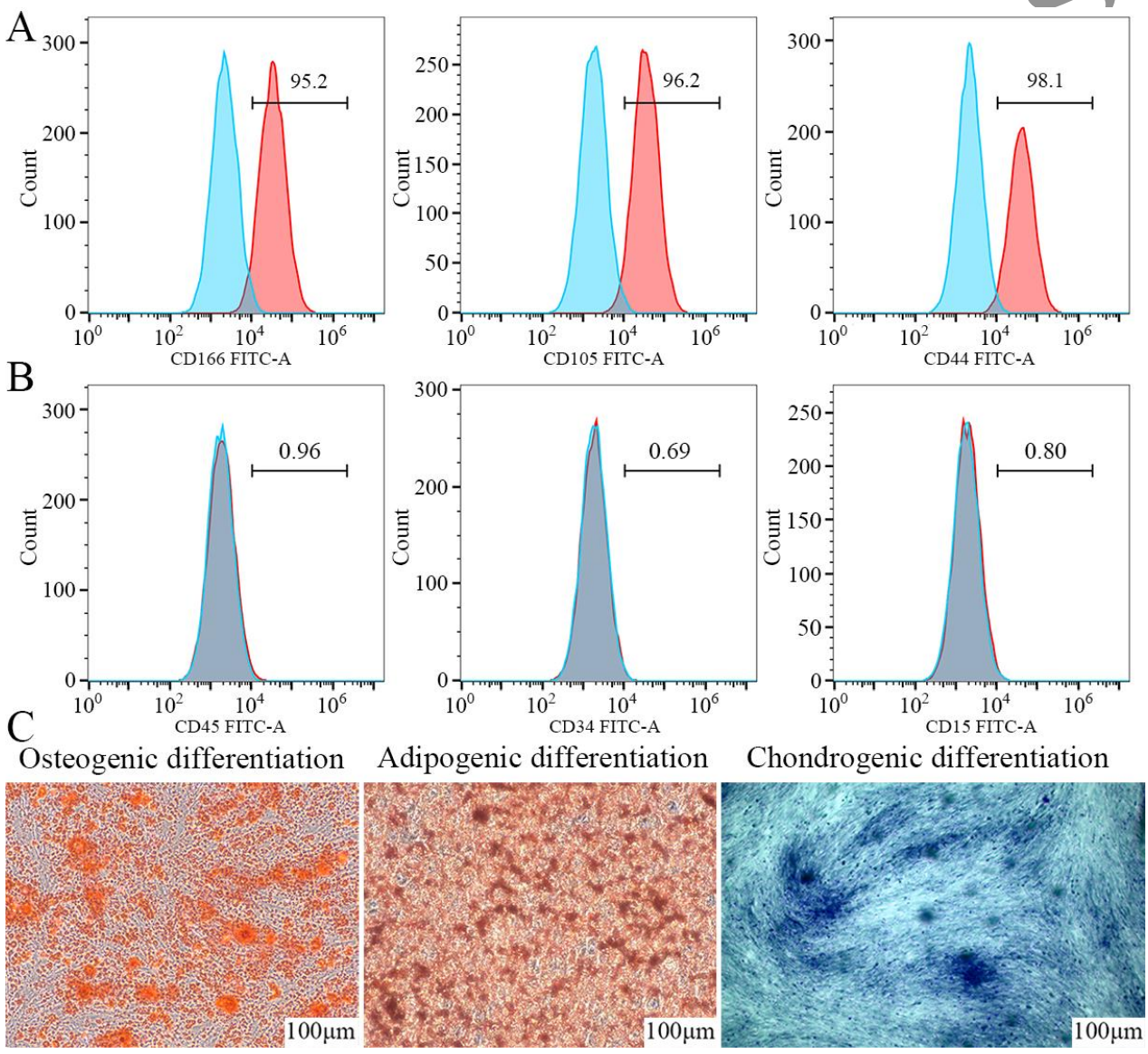



**Fig. 1**: Identification of synovial fluid-derived mesenchymal stem cells (SF-MSCs).
Flow cytometry analysis of surface marker expression and multilineage differentiation potential of SF-MSCs. (A) Positive expression of mesenchymal stem cell-associated markers CD166, CD105 and CD44 (positive rate > 95%). (B) Negative expression of hematopoietic lineage markers CD45, CD34 and CD15 (positive rate < 2%). (C) Multilineage differentiation potential of SF-MSCs (original magnification ×100): osteogenic differentiation (Alizarin Red S staining, orange-red calcium nodules after 21 days), adipogenic differentiation (Oil Red O staining, red lipid droplets after 14 days), and chondrogenic differentiation (Alcian Blue staining, blue proteoglycans after 21 days). Scale bar = 100 μm. Data are presented as mean ± SD from three independent experiments. Blue peaks represent isotype controls; red peaks represent specific antibodies.

**Table 2**: Detection of surface markers expression of mesenchymal stem cells.

| Surface markers | Positive expression rate (%) | Isomorphic control background (%) |
|---|---|---|
| Mesenchymal stem cell positive markers | | |
| CD44 | 98.2±1.5 | <1.0 |
| CD105 | 96.5±2.1 | <1.0 |
| CD166 | 95.8±1.8 | <1.0 |
| Hematopoietic lineage-negative markers | | |
| CD15 | 1.2±0.3 | <1.0 |
| CD34 | 0.8±0.2 | <1.0 |
| CD45 | 1.5±0.4 | <1.0 |

Note: SF-MSCs at passage 3 were stained with PE- or FITC-conjugated antibodies against the indicated surface markers and analyzed by flow cytometry. A total of 10,000 events were acquired per sample. Data are presented as mean ± standard deviation (SD) from three independent experiments. Isotype-matched IgG antibodies were used as negative controls, and the background staining was below 1.0% for all markers. Markers with a positive expression rate > 95% were considered positive, and those with a positive expression rate < 2% were considered negative, according to the minimal criteria for defining multipotent mesenchymal stromal cells proposed by the International Society for Cellular Therapy (Dominici et al., 2006). MSC, mesenchymal stem cell; CD, cluster of differentiation; PE, phycoerythrin; FITC, fluorescein isothiocyanate; SD, standard deviation.

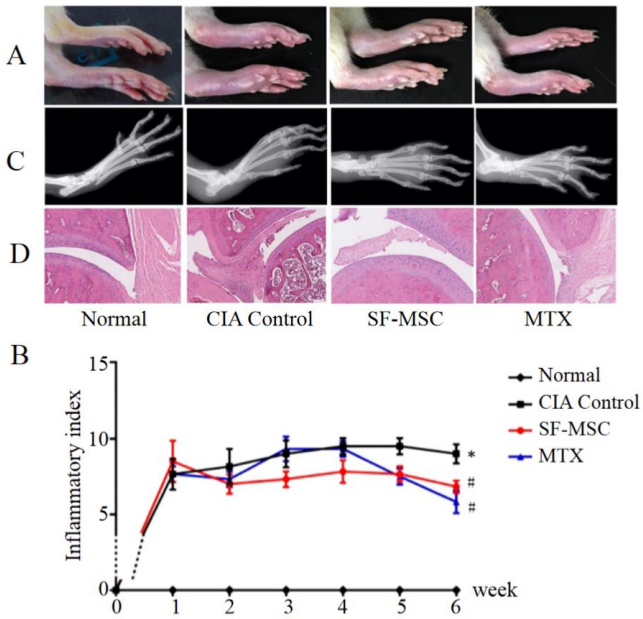



**Fig. 2**: SF-MSCs alleviated inflammatory response and prevented joint injury in collagen-induced arthritis (CIA) rats. Rats were divided into four groups: Normal (n = 6), CIA model (n = 6), CIA + MTX (0.2 mg/kg, n = 6), and CIA + SF-MSCs ($1 \times 10^6$ cells/rat, n = 6). (A) Representative images of paw swelling in normal and CIA rats. (B) Arthritis index scores at different time points after immunization (days 0, 21, 24, 27 and 30). (C) X-ray images of the posterior paw joint; arrows indicate joint space narrowing and bone erosion. (D) Hematoxylin and eosin (H&E)-stained ankle joint sections (original magnification × 40); arrows indicate synovial hyperplasia and inflammatory cell infiltration. Data are expressed as mean ± standard deviation (SD). *P < 0.05, **P < 0.01 compared with the CIA model group; #P < 0.05 compared with the normal group (one-way ANOVA followed by Tukey's post hoc test).

***Synovial fluid-derived mesenchymal stem cells (SF-MSCs) alleviated rheumatoid arthritis (RA) symptoms in collagen-induced arthritis (CIA) rats after transplantation***

At week 6, no swelling of the hind foot joints was observed in the normal group. CIA rats showed significant swelling and higher arthritis index scores, which were alleviated by SF-MSC intervention (Figs. 2A-C). Furthermore, the degree of swelling and arthritis index scores in the SF-MSC and MTX groups were similar and both lower than those in the CIA group. X-ray imaging showed clear joint space, no osteoporosis and no bone damage in the normal group. The CIA group showed blurred joint space, narrowing of joint space, osteoporosis, and bone erosion. In contrast, the SF-MSC and MTX groups showed only mild joint-space stenosis, with no osteoporosis or bone erosion, suggesting effective inhibition of radiographic progression in CIA rats (Fig. 2D).

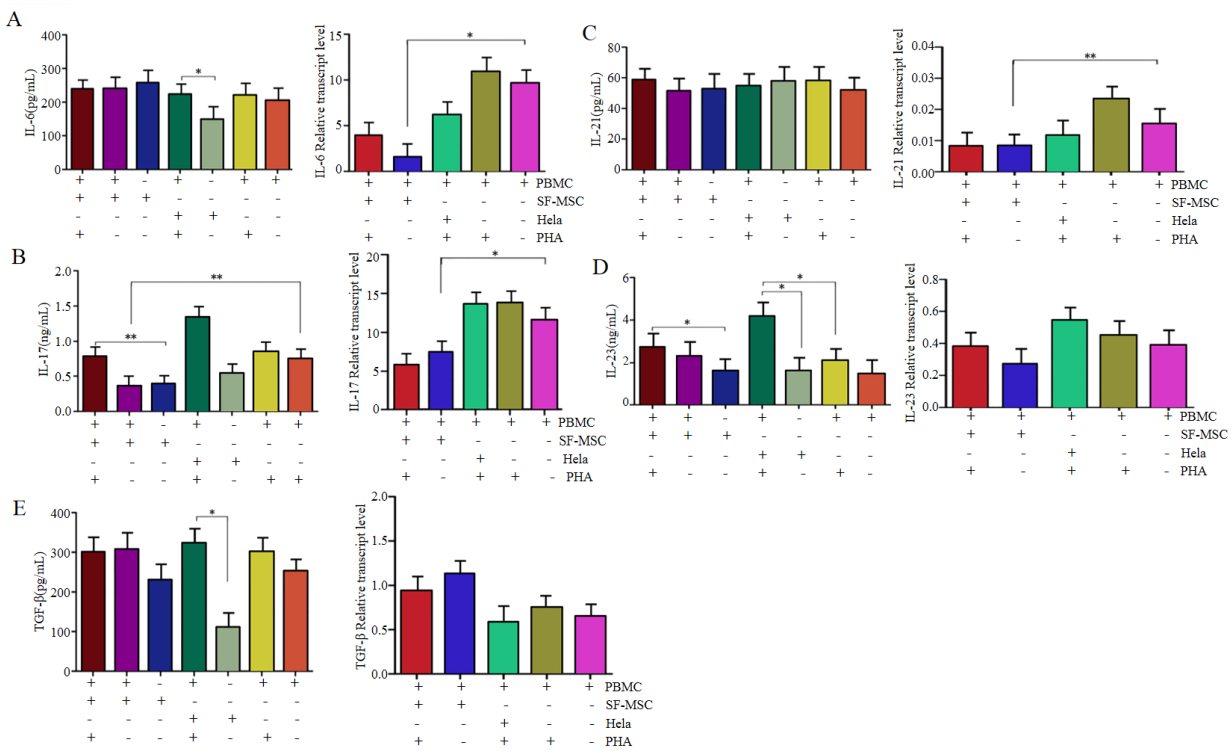



**Fig. 3**: Inhibition of pro-inflammatory cytokine expression in rat peripheral blood mononuclear cells (PBMCs) co-cultured with synovial fluid-derived mesenchymal stem cells (SF-MSCs).
SF-MSCs derived from rheumatoid arthritis (RA) patients were co-cultured with PBMCs derived from collagen-induced arthritis (CIA) rats at a 1:10 ratio (SF-MSCs:PBMCs) for 24 hours. The experimental groups were: (1) PBMC alone; (2) SF-MSC alone; (3) fibroblast alone; (4) PBMC + PHA; (5) PBMC + fibroblast + PHA; (6) PBMC + SF-MSC; and (7) PBMC + SF-MSC + PHA. The concentrations of (A) interleukin-6 (IL-6), (B) interleukin-17 (IL-17), (C) interleukin-21 (IL-21), (D) interleukin-23 (IL-23) and (E) transforming growth factor-beta (TGF-β) were measured by ELISA (left panels) and mRNA levels were measured by qPCR (right panels). Data are expressed as mean ± standard deviation (SD). *P < 0.05, **P < 0.01 compared with the PBMC + PHA group (n = 3 independent experiments; one-way ANOVA followed by Tukey's post hoc test).

***Synovial fluid-derived mesenchymal stem cells (SF-MSCs) exerted an immunomodulatory role by regulating peripheral blood mononuclear cell (PBMC) activation***

There was no significant difference in IL-6 expression in the supernatant of SF-MSC and PBMC co-cultures compared to PBMCs cultured alone (Fig. 3A). However, co-culture with SF-MSCs effectively inhibited IL-6 transcription levels compared to PBMCs cultured alone (Fig. 3A).

Compared with PHA-non-stimulated or PHA-stimulated PBMCs, co-culture with MSCs significantly inhibited IL-17 secretion and transcription levels (Fig. 3B). Compared with PBMCs alone, IL-23 production was significantly increased after PHA stimulation, while co-culture with fibroblasts had no significant effect on IL-23 production by PBMCs (Fig. 3C). Co-culture with SF-MSCs significantly inhibited IL-23 secretion in PBMCs, whereas IL-23 transcript levels were not significantly affected (Fig. 3D). In addition, TGF-β levels and transcription levels in PBMC supernatants were significantly increased after co-culture with MSCs compared to PHA-stimulated or non-stimulated PBMCs (Fig. 3E).

## DISCUSSION

Rheumatoid arthritis (RA) is an autoimmune inflammatory condition that not only affects the musculoskeletal system over the long term but also impacts patients' lives in many aspects. The global prevalence of the disease is approximately 1.5% and it ranges from 0.9% to 1.5% in the United States. MSCs have strong self-renewal ability and multidirectional differentiation potential. Their differentiation direction is not restricted by the germ layer and their multi-directional differentiation potential can be maintained during long-term culture (Xu *et al*., 2024; Peng *et al*., 2023; Jiang *et al*., 2023). Bone marrow-derived MSCs (BMSCs) are available from a wide range of sources, are low in immunogenicity, and are relatively easy to obtain, making them widely used in the treatment of diabetic complications, cirrhosis, and bone and joint diseases.

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In this study, we successfully isolated SF-MSCs from the synovial fluid of RA patients. Their immunophenotype was consistent with the typical characteristics of MSCs (positive for CD44, CD105 and CD166; negative for CD15, CD34 and CD45). SF-MSCs showed therapeutic effects comparable to MTX in the CIA rat model. *In-vitro* co-culture experiments further confirmed that SF-MSCs significantly inhibited the production of the pro-inflammatory cytokines IL-6 and IL-21 and promoted the expression of the anti-inflammatory cytokine TGF-β in rat PBMCs.

The synovium contains not only MSCs but also cells that contribute to synovial inflammation, which are associated with the pathogenesis of arthritis. Synovitis releases activated inflammatory cytokines that may contribute to the development of arthritis and ultimately lead to joint destruction. Therefore, synovectomy has been used as a disease-modifying procedure for osteoarthritis (OA) and RA patients. However, recent literature suggests that inflammatory cytokines produced by synovitis impair MSC cartilage formation, and that SF-MSCs may be an effective treatment for RA (Sui *et al*., 2025). In this study, we treated CIA rats with SF-MSCs and observed a significant relief of RA symptoms. Co-culture of SF-MSCs with rat-derived PBMCs resulted in inhibition of IL-6, IL-21 and IL-17 production and increased TGF-β expression compared to PBMCs cultured alone.

The immunomodulatory function of MSCs is mainly mediated through direct cell-cell interactions and the release of various immunomodulatory factors that act on immune cells. MSCs inhibit the proliferation, activation, differentiation and maturation of pro-inflammatory T lymphocytes, B lymphocytes, antigen-presenting cells and natural killer (NK) cells. They also promote the inhibition of inflammatory responses by Tregs and regulatory B cells (Bregs), as well as the differentiation of monocytes into anti-inflammatory, tissue-repairing M2 macrophages (or the conversion of pro-inflammatory M1 macrophages into M2 macrophages) (Cao *et al*., 2025; Liao *et al*., 2025). MSCs have been shown to induce immune tolerance and play a therapeutic role in RA. Moreover, it has been reported that when human MSCs are co-cultured with immune cells, they promote an anti-inflammatory phenotype (Li *et al*., 2024; Bayer *et al*., 2025). In this study, we used fibroblasts as another adherent cell control to compare with SF-MSCs. Compared to SF-MSCs, fibroblast cell lines exhibited stronger proliferative and inflammatory activity *in-vitro* and induced the production of Th17-related cytokines, indicating that the overall changes observed resulted from the specific effects of SF-MSCs rather than the co-culture system itself. The concentrations of IL-21 and IL-22 were significantly increased in RA patients. IL-21 produced by human CD4+ T cells auto-regulates its own secretion, facilitates Th17 proliferation, and is closely associated with autoantibody levels (McElroy, 2025). Culturing MSCs with allogeneic activated lymphocytes or pro-inflammatory cytokines affects MSC function and human leukocyte antigen (HLA) molecule expression, resulting in increased immunomodulatory capacity of SF-MSCs (Cequier *et al*., 2022; Dewhurst-Trigg *et al*., 2025). Moreover, the literature suggests that co-culture of SF-MSCs with PBMCs can enhance their ability to inhibit lymphocyte proliferation, while co-culture with pro-inflammatory cytokines further enhances their immunomodulatory capacity. Our data showed that the inflammatory conditions in RA patients induced an anti-inflammatory effect in SF-MSCs on patient PBMCs. In addition, TGF-β gene expression levels were significantly higher in the MSC treatment groups than in the untreated groups.

This study has several limitations. An MSC vehicle control group was not included; therefore, the potential impact of the injection procedure itself on joint inflammation could not be completely excluded. A healthy donor MSC control group was also not established. In addition, SF-MSCs and PBMCs were co-cultured using a direct-contact method. Future studies should use Transwell assays to verify further the mechanisms involved. Several important questions also warrant further investigation. The molecular mechanisms underlying SF-MSC-mediated immunomodulation, including the potential roles of the nuclear factor kappa-B (NF-κB) and mitogen-activated protein kinase (MAPK) signaling pathways, deserve further study. Preclinical studies comparing different administration routes (intra-articular vs. intravenous), optimal cell dosages and treatment timing are needed to establish standardized protocols. Long-term safety and efficacy should be assessed in larger animal models before clinical translation. Autologous SF-MSC transplantation using patient-derived cells is a promising strategy worthy of clinical evaluation. Finally, combination therapies involving SF-MSCs and conventional anti-rheumatic drugs (e.g., MTX, biologics) may produce synergistic effects and improve treatment outcomes.

## CONCLUSION

In summary, we found that SF-MSCs can play an immunomodulatory role by inhibiting pro-inflammatory cytokine levels and promoting anti-inflammatory factor expression. SF-MSC therapy demonstrated therapeutic effects in CIA rats comparable to conventional therapy with MTX. These findings suggest that SF-MSCs represent a potential cell-based therapeutic approach for RA.

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***Authors' contributions***

Bo Dong: Conceptualization (lead), Methodology (equal) and Investigation (lead). Meiqi Dong: Writing – original draft (lead) and Funding acquisition (lead); Kui Ma: Validation (lead), Formal analysis (lead); Haoyu Xu: Investigation (supporting), Data curation (lead) and Project administration (lead); Xiaofei Li: Methodology (supporting), Investigation (supporting) and Visualization (lead); Jianchao Gui: Conceptualization (supporting), Supervision (lead) and Writing – review & editing (lead). All authors participated in data interpretation and manuscript revision and agreed to the submission and publication of the final version.

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***Data availability statement***

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

***Ethical approval***

Human sample collection: The collection and use of human synovial fluid samples were approved by the Ethics Committee of Nanjing First Hospital, Nanjing Medical University (Approval No.: 2022072605). Written informed consent was obtained from all participants.

Animal experimentation: All animal experiments were approved by the Animal Ethics Committee of Nanjing First Hospital, Nanjing Medical University (Approval No.: 2022072605) and were performed in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals and adhered to the ARRIVE (Animal Research: Reporting of In-vivo Experiments) guidelines. This study was performed in adherence with the ARRIVE guidelines. See supplementary file for the ARRIVE checklist.

***Conflict of interest***

The authors declare no conflicts of interest.

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