

Synovial fluid niche promoted differentiation of dental follicle mesenchymal stem cells toward chondrogenesis in rheumatoid arthritis

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ABSTRACT

Objectives: In this study, we aimed to investigate the differentiation potential of dental follicle mesenchymal stem cells (MSCs) in the synovial fluid (SF) niche of early-onset or end-stage rheumatoid arthritis (RA).

Patients and methods: Between May 2020 and January 2021, six patients (1 male, 5 females; mean age: 57.5±11.2 years; range, 49 to 65 years) who were diagnosed with RA with the indication of SF aspiration were included in the study. The third passage dental follicle stem cells (DFSCs) were cocultured with fresh SF samples of end-stage or early-onset RA patients in micromass culture system for 21 days. SF samples were analyzed for secreted cytokines. Chondrogenic markers (CD49e, CD49f) were analyzed in DFSCs, gene expression analysis was performed for the expressions of Col I, Col II, Aggrecan and Sox-9, and histochemical analysis was performed by staining three-dimensional pellets with anti-collagen II antibody. The neutralization assay was performed with anti-interleukin (IL)-6, anti-interferon-gamma (IFN- γ), and anti-IL-1beta(β).

Results: The high levels of IL-1 β and IL-6 were observed in end-stage RA patients' SF samples compared to the early-onset patients (p<0.05). The CD49e and CD49f expressions in DFSCs were significantly higher in the SF samples of end-stage RA patients (p<0.05). Also, the Col II, Sox-9 and Aggrecan messenger ribonucleic acid (mRNA) expressions increased in the DFSCs, when cultured with end-stage RA patients' SF samples (p<0.01). Collagen-II expression in histochemical analysis of micromass pellets was higher in the DFSCs cultured with end-stage RA patients' SF samples. The neutralization of IL-6 significantly decreased the CD49e and CD49f expressions (p<0.05).

Conclusion: The high levels of IL-6 in SF niche of end-stage RA patients were found to differentiate DFSCs toward chondrogenesis. Based on these findings, DFSCs can be used as a new cell-based treatment in RA patients for the cartilage damage.

Keywords: Dental follicle mesenchymal stem cells, rheumatoid arthritis, synovial fluid.

Rheumatoid arthritis (RA) is a chronic autoimmune inflammatory disorder caused by inflammation of the synovial membrane. The impairment of peripheral tolerance followed by abnormal infiltration and activation of various

immune cells into the synovial membrane is also known to play a critical role in the development and progression of RA. The responses of lymphocytes secreting inflammatory cytokines have been shown to be directly related to RA

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immunopathogenesis. Current therapeutic applications include corticosteroids, disease-modifying anti-rheumatic drugs, methotrexate or tumor necrosis factor-alpha (TNF- α) inhibitors.¹ However, some of RA patients do not effectively respond to the conventional therapies, and new treatment options are still required.²

Mesenchymal stem cells (MSCs) are multipotent stromal cells that have an immunosuppressive effect on immune cells. They can be isolated from many tissues such as bone marrow, adipose tissue, umbilical cord blood, umbilical cord, placenta, dental tissues. Owing to their ability to suppress pathogenic immune responses, MSCs have been shown to be promising therapeutic cell candidates for the treatment of rheumatologic diseases.³ However, given the complexity of the RA and the mechanisms of the immunosuppressive and regenerative effects of MSCs, the uncovering of new MSC resources that may have a higher tissue regeneration capacity may contribute to the expansion of cellular therapy options.⁴

The inflammatory niche has an immunomodulatory effect on MSCs with a significant effect on their differentiation capacity. Several studies have shown that human bone marrow mesenchymal stem cells (BMSC) and adipose tissue mesenchymal stem cells (ATMSC) increase osteogenic and adipogenic differentiation capacity, when cultured with proinflammatory cytokines.⁵ The differentiation potential of MSCs may differ with pro- and anti-inflammatory cytokines. In a study investigating the effect of TNF- α , interleukin-6 (IL-6) proinflammatory cytokines, and IL-4, an anti-inflammatory cytokine, on the proliferation and osteogenic differentiation of human ATMSC, IL-6 was shown to strongly increase bone nodule formation by increasing the alkaline phosphatase activity, whereas TNF- α decreased the collagen type I (Col I) gene expression.⁶ Additionally, the differentiation capability may vary depending on the MSC source.⁷ In a study, MSCs isolated from the bone marrow, lung, liver, and spleen exhibited different potentials for differentiation into osteoblasts and adipocytes, despite having a similar immunophenotype.⁸ In this study, ATMSC had also a lower osteogenic and chondrogenic potential than BMSC.

The BMSC and ATMSC are the most commonly used and studied cell types in cell-based therapies. However, there are many challenges with the clinical application of BMSCs, given the fact that the cell numbers isolated and their potential for multiple differentiation are affected by age.⁹ Therefore, there is a need for new cellular resources that can be an alternative in cellular treatments. In the present study, we aimed to investigate the differentiation capacity of dental follicle stem cells (DFSCs) in the synovial fluid (SF) niche of both early-onset and end-stage RA patients by evaluating the gene expressions, chondrogenic markers, and histochemical properties and to contribute to the development of new cell-based therapies to be performed in distinct stages of RA.

PATIENTS AND METHODS

Study design and study population

This research study was conducted at Muğla Sıtkı Koçman University, Faculty of Health Sciences, Department of Nursing between May 2020 and January 2021. Six patients (1 male, 5 females; mean age: 57.5 \pm 11.2 years; range, 49 to 65 years) who fulfilled the 2010 American College of Rheumatology/European League Against Rheumatism (ACR/EULAR) Rheumatoid Arthritis Classification Criteria¹⁰ were included in the study. Prior to study, all patients were informed about the nature of the study and a written informed consent was obtained. The study protocol was approved by the Muğla Sıtkı Koçman University Clinical Researches Ethics Committee (No: 02/IV, 30/01/2020). The study was conducted in accordance with the principles of the Declaration of Helsinki.

All RA patients were evaluated according to their radiological findings, serology, joint involvement, disease duration, and clinical signs such as deformity or swelling in joints, and synovitis. The patients with synovitis were included in the study, while the end-stage RA group consisted of patients with high deformity as evidenced by the radiological findings and swelling in joints with a disease duration of longer than three years. The study flow chart is shown in Figure 1.

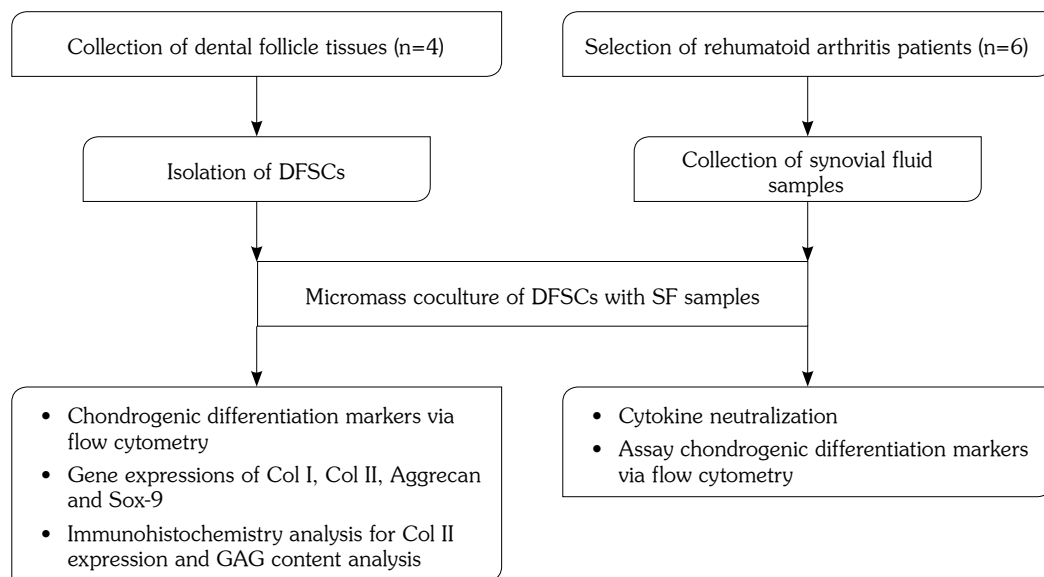


Figure 1. Study flow chart.

DFSCs: Dental follicle stem cells; SF: Synovial fluid; GAG: Glycosaminoglycan.

Collection and analysis of synovial fluid cytokine levels

A 6 to 10 mL of SF was aseptically collected from each patient by a single rheumatologist. The cytokine levels were analyzed as described in the cytokine bead array kit (BD™ CBA; BD Biosciences, CA, USA). In brief, SF samples were immediately centrifuged at 3,000 rpm for 5 min to eliminate cell contents and cellular debris. Supernatant was collected and stored at -80°C until analysis. The cytokine levels were measured by using cytokine bead array kit (BD™ CBA; BD Biosciences, CA, USA) via flow cytometry. A total of 50 μL of each collected SF was analyzed for IL-1 β , IL-6, TNF- α , interferon-gamma (IFN- γ), and IL-10 by using the Th1/Th2/Th17 CBA kit (BD Biosciences, CA, USA). Analysis was performed using the BD Accuri™ C6 Plus software (BD Biosciences, CA, USA). The results of ILs, TNF- α , and IFN- γ were given as pg/mL.

Characterization and differentiation potential of dental follicle stem cells

Dental follicle tissues were obtained from four healthy individuals aged between 19 and 23 years who applied to the Muğla Sıtkı Koçman University Dental Hospital for wisdom tooth extraction. Dental follicle tissues were mechanically and enzymatically extracted

as described previously.¹¹ In brief, dental follicle tissue was dissected into 1 to 2-mm pieces with a scalpel. The collagenase type II solution was prepared (3 mg/mL) in phosphate buffered saline (PBS) for the enzymatical digestion of tissue pieces. Dental follicle tissue was, then, enzymatically fragmented in 3% collagenase type II solution at 37°C for 45 min. After incubation period, enzymatic activation was stopped with 5 mL of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), and centrifuged at 1,500 rpm for 5 min. The pellet was resuspended in 5 mL DMEM supplemented with 10% FBS and 1% penicillin/streptomycin (100 U/mL), and cultured in T25 flask until reaching 80 to 90% confluency. The DFSCs in the third passage were analyzed for the positive cell surface markers of CD73, CD90, and CD105 by staining with anti-CD73 (APC), anti-CD90 (PerCp), and anti-CD105 (FITC), respectively. Negative markers of HLA-DR, CD3, and CD28 were analyzed by staining cells with anti-HLA-DR (APC), anti-CD3 (PerCp), and anti-CD28 (PE), respectively. All antibodies were supplied from the BD Biosciences, CA, USA. Analysis was performed via flow cytometry. The results were given in mean fluorescence intensity % (MFI%) for flow cytometry.

The third passage cells were subjected to osteogenic, chondrogenic, and adipogenic differentiation to evaluate the lineage differentiation criteria for MSCs, as previously described.¹² The cells were seeded in a six-well plates, each containing 2×10^4 cells, for 48 h at 37°C in a 5% carbon dioxide (CO₂) incubator. Subsequently, confluency cells were separately stimulated with osteogenesis, chondrogenesis, and adipogenesis using the StemPro™ Differentiation Kits (Thermo Fisher Scientific Inc., MA, USA). All cultures were incubated for 21 days for osteogenesis and chondrogenesis, and 14 days for adipogenesis, by changing the media three times a week. At the end of the culture period, the cells were fixed in 10% formaldehyde and stained with Alizarin Red S, Alcian Blue, and Oil Red O for the evaluation of osteogenic, chondrogenic, and adipogenic differentiation, respectively.

Micromass culture of dental follicle stem cells with synovial fluid

The third passage cells were trypsinized and placed into the 96 U-bottom well-plates each containing 5×10^5 cells in a 20- μ L volume of DMEM supplemented with 10% FBS, and 1% penicillin/streptomycin (100 U/mL), as described previously.¹³ The cells were, then, cultured for the initial 2 h at 37°C in a 5% CO₂ and 95% humidified chamber to obtain micromass cultures. After 2 h, 100 μ L of DMEM, chondrogenesis differentiation medium (StemPro™; Thermo Fisher Scientific Inc., MA, USA) or with SF samples (1:1 v/v) or only SF samples were added to the micromass culture wells. The DMEM was used as the negative control, while the chondrogenesis differentiation medium was used as the positive control. The cells were incubated at 37°C with 5% CO₂ for 21 days by changing the media twice a week.

Flow cytometry analysis of chondrogenic markers

The cultured cells were analyzed for chondrogenic markers of CD49e and CD49f in CD90+ cell population. The analysis protocol was performed as described previously.¹⁴ The spheroids were washed with PBS (Sigma-Aldrich GmbH, Merck, Darmstadt, Germany) and enzymatically digested with collagenase type II (Thermo Fisher Scientific Inc., MA, USA) in PBS solution (3 mg/mL) for 30 min at 37°C.

After incubation period, the enzymatical activity was ended with DMEM containing 10% FBS and centrifugated at 1,500 rpm for 5 min. The Cell pellet was finally resuspended in 100 μ L of PBS and stained with anti-CD49e (PE), anti-CD49f (APC), and anti-CD90 (PerCp) at 4°C for 30 min to evaluate the chondrogenic differentiation of CD90+ DFSCs. Cell surface markers were analyzed via flow cytometry. The gating strategy was performed as follows: CD90+ cells were analyzed for CD49e or CD49f expressing cells. The results were given in mean fluorescence intensity % (MFI%).

Messenger ribonucleic acid (mRNA) expression analysis by reverse transcriptase-polymerase chain reaction (RT-PCR)

The gene expression analysis was performed to analyze expression of genes related to chondrogenesis including Col I, collagen type II (Col II), Aggrecan and Sox-9. The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene. The sequences for genes were as follows: Col I Forward 5'-3' (CTCCGGCTCCTGCTCCTCTTA), Col I Reverse 5'-3' (GCACAGCACTCGCCCTCCC), Col II Forward 5'-3' (ATAAGGATGTGTGGAAGCCG), Col II Reverse 5'-3' (TTTCTGTCCCTTTGGTCCTG), Aggrecan Forward 5'-3' (TTGAGCAGTTCACCTTC), Aggrecan Reverse 5'-3' (CTCTTCTACGGGGACAGCAG), Sox-9 Forward 5'-3' (CCCAACGCCATCTTCAAGG), Sox-9 Reverse 5'-3' (CTGCTCAGCTCGCCGATGT), GAPDH Forward 5'-3' (GGGAGCCAAAAGGGTCATCATCTC), GAPDH Reverse 5'-3' (GAGGGCCATCCACAGTCTTC), as described previously.¹⁵ Total RNA was isolated from three-dimensional culture pellets using the RNA Isolation Kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) in accordance with the manufacturer's instructions. Both quantity and quality of isolated RNA samples were evaluated with the A260/280 ratio using the NanoDrop™ (Implen GmbH, München, Germany). The complementary deoxyribonucleic acid (cDNA) was, then, synthesized with 1 μ g of RNA templates using the First Strand cDNA Synthesis Kit (Biolabs Ltd., Ipswich, UK). Next, each cDNA template was mixed with the Go Taq™ qPCR Master Mix

(Promega Corp., WI, USA), and PCR was performed using the Roche LightCycler® 480 Real-Time PCR System (Roche Diagnostics GmbH, Mannheim, Germany). The mRNA expressions results were analyzed by using the $2^{-\Delta\Delta CT}$ methods.¹⁶ The analysis was performed by comparing gene expression levels in the samples with the negative control media. The results were recorded as fold changes in the samples.

Immunohistochemical staining of Col II

The Col II formation in micromass pellets were analyzed by immunohistochemical staining protocol as previously described.¹⁷ In brief, micromass pellets were fixed in 4% paraformaldehyde (Merck, USA) for 30 min, embedded in paraffin, and sectioned at 5 μ m. After deparaffinization, the slides were incubated with hydrogen peroxide (H₂O₂) (10%) (Sigma-Aldrich Corp., MO, USA) for 10 min to eliminate endogenous peroxidase activity and blocked with Super protein Block Solution (ScyTek Laboratories Inc., UT, USA) for 10 min at room temperature. Subsequently, the sections were incubated with human anti-Col II antibody (BT Lab, China; dilution 1/100) overnight at 4°C. On the following day, the slides were incubated for UltraTek Anti-Polyvalent and UltraTek HRP (ScyTek Laboratories Inc., UT, USA) for 20 min, respectively. The 3,3'-diaminobenzidin (DAB) was used to visualize the final product. Then, nuclei were counterstained with the Mayer's hematoxylin. All slides were photographed with a digital camera mounted on the Nikon Eclipse, 80i microscope (Nikon Instruments Inc., Tokyo, Japan). The intensity of anti-Col II expressions was given in percentile.

Determination of glycosaminoglycan (GAG) content

The density of GAGs was confirmed with the histological staining using the Safranin-O dye, which produced a red color in the constructs after 21 days of culture in the chondrogenic medium (positive control) and SF. The staining protocol was carried out as described previously.¹⁸ The slides were stained with 0.1% Safranin-O (Sigma-Aldrich Corp., MO, USA) dye solution for 10 min and washed twice with distilled water. Photos were taken using a digital camera mounted on the Nikon

Eclipse, 80i microscope (Nikon Instruments Inc., Tokyo, Japan).

Neutralization assays

To evaluate the effect of high-level cytokines (IL-6 and IL-1 β) on the chondrogenic differentiation potential of DFSCs, we performed a neutralization assay for these cytokines, as described previously.¹⁹ In addition, the neutralization of IFN- γ was done to examine the possible effect of IFN- γ on the chondrogenic differentiation potential of DFSCs. At the beginning of the culture period, a 5-mM anti-IL-6 neutralizing antibody (eBioscience Inc., CA, USA) or 5-mM anti-IL-1 β antibody (Invivogen, Toulouse, France) or 5 μ g/mL of anti-IFN- γ (PeproTech APAC, Rehovot, Israel) was added to the SF cocultures of both early- and end-stage patients and cultured with DFSCs in the micromass culture system and cultured for 21 days at 37°C in the 5% CO₂ incubator. After the incubation period, CD49e and CD49f expressions in DFSCs were analyzed via flow cytometry.

Statistical analysis

Statistical analysis was performed using the GraphPad Prism version 9.0 software (GraphPad Software Inc., CA, USA). Data were presented in mean \pm standard deviation (SD) or number and frequency. The Student t-test was performed to analyze the difference between the two samples. One-way analysis of variance (ANOVA) was used to determine the statistical significance between groups. A *p* value of <0.05 was considered statistically significant.

RESULTS

Demographic and baseline characteristics of the patients are shown in Table 1. Accordingly, five patients had rheumatoid factor (RF) positivity and four patients had anti-citrullinated protein antibody (ACPA) positivity. For anti-nuclear antibody (ANA), five patients were positive and the ANA status was unknown in one patient. None of the patients had comorbidities. The secreted cytokine profiles were shown in Table 2.

Table 1. Demographic data of RA patients

Patient ID	Sex	Treatment (years or months)	RF	ACPA	ANA	Comorbidity	Erosive changes in cartilage or bone (radiological findings)
P1	Female	Hydroxychloroquine+metilprednisolon (3 years)	Positive	Negative	Positive	No	No
P2	Female	Methotraxate+ metilprednisolon (12 years)	Negative	Positive	Positive	No	Bone and cartilage deformity in large joints (knees and ankles)
P3	Female	Methotrexate+metilprednisolon (15 years)	Positive	Positive	Positive	No	Bone and cartilage deformity in large joints (knees)
P4	Female	Methotrexate+hidroxyclorokine (2 years)	Positive	Negative	Positive	No	No
P5	Male	Methotrexate+metilprednisolon (8 years)	Positive	Positive	NA	No	Bone and cartilage deformity in large joints (knees)
P6	Female	Methotrexate+metilprednisolon+hydroxychloroquine (3 years)	Positive	Positive	Positive	No	No

RA: Rheumatoid arthritis; RF: Rheumatoid factor; ACPA: Anti-citrullinated protein antibody; ANA: Anti-nuclear antibody.

Cell surface markers and differentiation potential of dental follicle stem cells

The DFSCs in the third passage were analyzed for positive and negative markers as described by the International Society for Cellular Therapy (ISCT). They showed fibroblast-like colonies in passages 0-3, and expressed the positive markers of CD73, CD90, and CD105 over 95%, and negative markers lower than 5% in the third passage. The osteogenic, chondrogenic, and

adipogenic differentiation capacity of DFSCs were analyzed for the minimal criteria to define multipotent mesenchymal stromal cells. Alizarin Red staining was performed to evaluate the calcium deposits after 21 days of the osteogenic differentiation culture period. Extracellular calcium deposits and osteogenic colonies were observed in the monolayer cultures. Alcian Blue staining was performed to observe proteoglycans in chondrogenic differentiated cultures. Extracellular proteoglycans and chondrocytes were observed

Table 2. Secreted cytokine levels in the sf samples

Patient (Groups)	Number of patients	ANA	Secreted cytokines in SF pg/mL (mean±SD)
Early onset	3	2 patients positive	IL1 β (35.7±9.2) IL-6 (699.2±284.7) TNF- α (937.8±289.3) IFN- γ (91.3±25.7) IL-10 (13.8±6.9)
End stage	3	3 patients positive	IL1 β (63.8±12.5) IL-6 (1876.3±973.1) TNF- α (476.7±96.8) IFN- γ (97.2±91.9) IL-10 (7.3±1.8)

ANA: Anti-nuclear antibody; SF: Synovial fluid; SD: Standard deviation; All RA patients were divided into two groups according to joint swelling, synovitis, and bone erosion profiles. RA patients with bone erosion were included in the end-stage group.

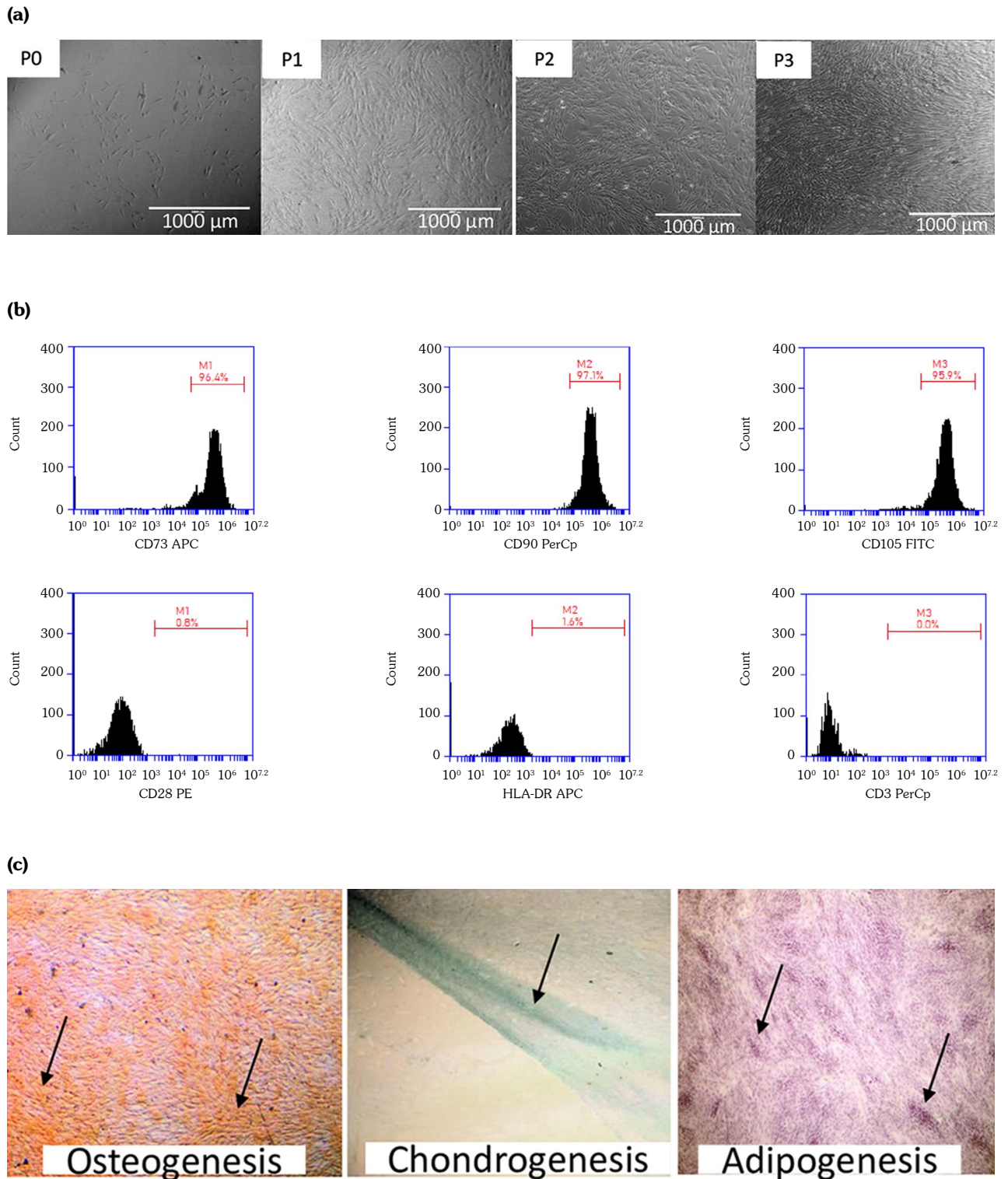


Figure 2. Inverted microscope images of DFSCs. **(a)** Fibroblast-like colonies formed in the passage 0 (P0), P1, P2 and P3. **(b)** Cell surface positive markers (CD73, CD90, CD105) expressed over 95% and lack the negative markers (HLA-DR, CD28, CD3). **(c)** The differentiation potential of DFSCs were evaluated with osteogenic, chondrogenic and adipogenic stimulation medium. Calcium deposits, cartilage formation and adipocytes were seen in osteogenic, chondrogenic and adipogenic stimulated cultures, respectively.

DFSCs: Dental follicle stem cells.

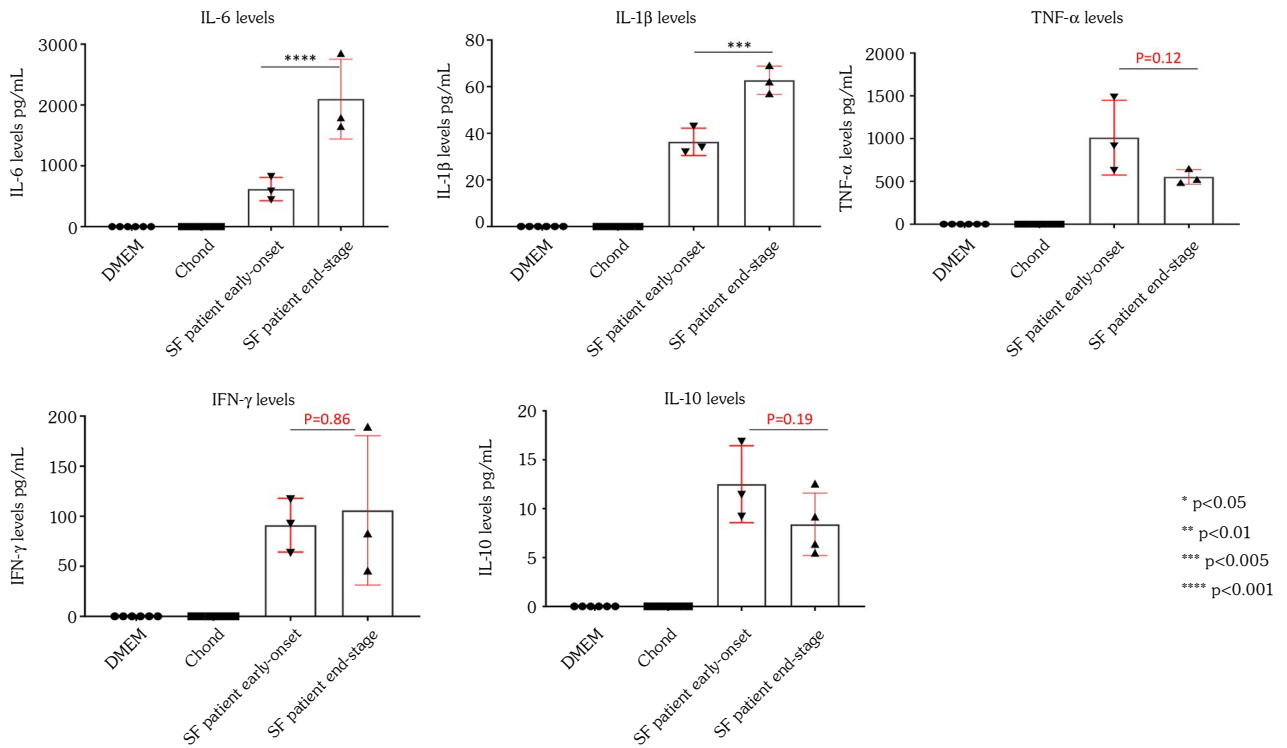


Figure 3. Cytokine levels in synovial fluid samples. IL-6 and IL-1 β levels were significantly higher in end-stage patients compared to early-onset subjects ($p < 0.001$ and $p < 0.005$, respectively), while no significant change was observed in IFN- γ or TNF- α levels ($p > 0.05$). IL-10 levels were significantly lower in end-stage RA patients, compared to early-onset patients ($p < 0.05$).

DMEM: Dulbecco's Modified Eagle Medium; IL: Interleukin; IFN: Interferon-gamma; TNF- α : Tumor necrosis factor-alpha; RA: Rheumatoid arthritis; SF: Synovial fluid.

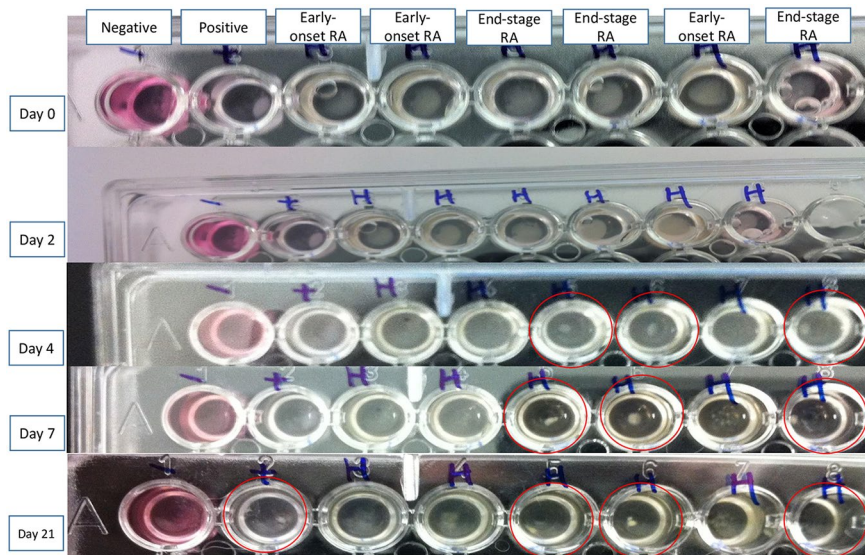


Figure 4. Micromass cultures of DFSCs in negative control medium (DMEM), positive control medium (Chondrogenic stimulated medium) and synovial fluid samples of end-stage and early-onset RA patients. DFSCs were cultured with end-stage RA synovial fluid samples formed chondrogenesis spheroids on Day 4, while the spheroids formed between Days 14 and 21 in early-onset RA synovial fluid cultures. The spheroids formed in positive control medium between Days 14 and 21, and there were no spheroid formation in negative control medium.

DFSCs: Dental follicle stem cells; DMEM: Dulbecco's Modified Eagle Medium; RA: Rheumatoid arthritis.

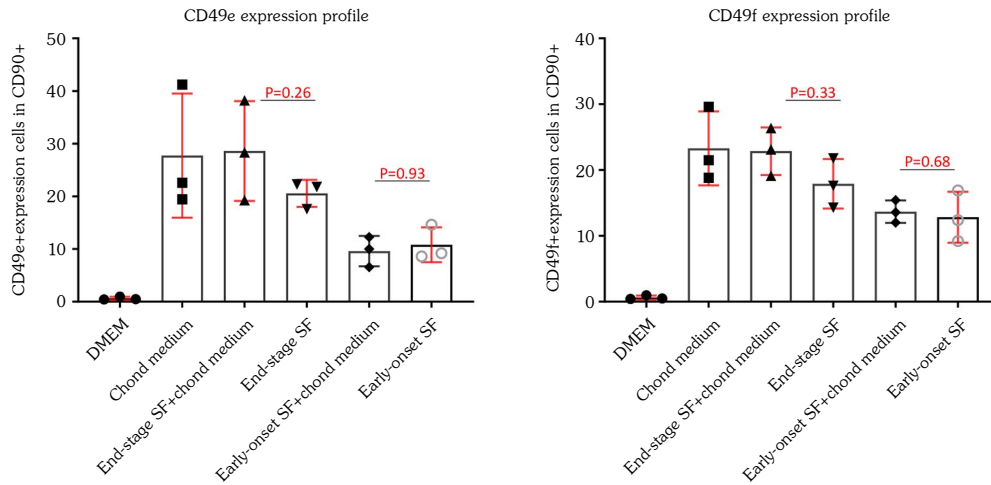


Figure 5. The evaluation of CD49e and CD49f expressions in CD90+ cell population with the culture of DFSCs in chondrogenesis medium and synovial fluid samples in 1:1 v/v or only synovial fluid samples. There were no significant differences in CD49e or CD49f expressions between 1:1 v/v cocultured DFSCs and only synovial fluid samples ($p > 0.05$).

DMEM: Dulbecco's Modified Eagle Medium; DFSCs: Dental follicle stem cells; SF: Synovial fluid.

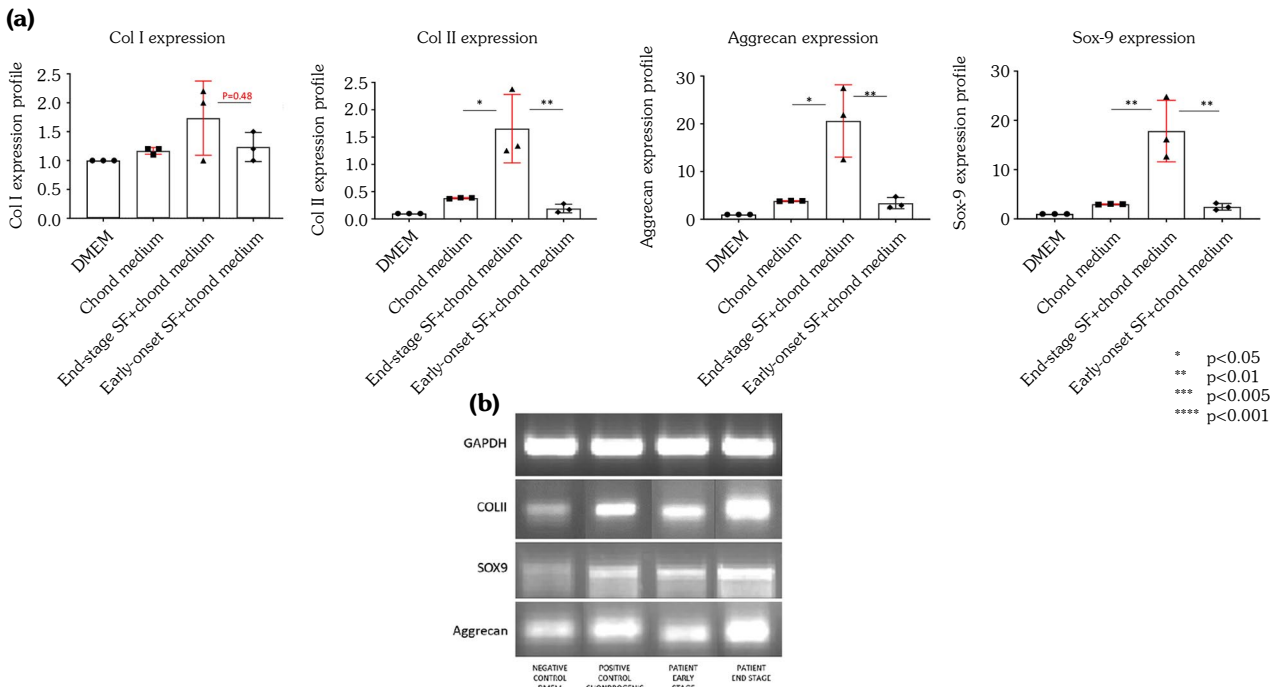


Figure 6. The mRNA expression profiles of Col I, Col II, Aggrecan and Sox-9 genes in DFSCs. **(a)** The statistical data of gene expressions. Col II, Aggrecan and Sox-9 gene expressions significantly increased in DFSCs in end-stage RA patients' synovial fluid samples, compared to those in early-onset subjects ($p < 0.01$, $p < 0.01$, and $p < 0.01$, respectively). Additionally, Col II, Aggrecan and Sox-9 mRNA expressions were significantly higher in DFSCs cultured with synovial fluid samples of end-stage RA patients, compared to chondrogenic culture medium ($p < 0.05$, $p < 0.05$, and $p < 0.01$, respectively). **(b)** Gel electrophoresis images of mRNA expressions.

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; DMEM: Dulbecco's Modified Eagle Medium; mRNA: Messenger ribonucleic acid; DFSCs: Dental follicle stem cells; RA: Rheumatoid arthritis.

after 21 days of the culture period. Also, cartilage formation was observed in the monolayer cultures. Oil Red O staining was performed to evaluate the oil drops. Oil drops and adipocytes were observed after 14 days of the adipogenic differentiation culture period of DFSCs (Figure 2).

Secreted cytokines in synovial fluid samples

To evaluate the effect of soluble mediators on differentiation of DFSCs, we first analyzed the levels of proinflammatory cytokines (IL-1 β , IL-6, TNF- α , IFN- γ) and IL-10 as an anti-inflammatory mediator. Cytokine expression profiles changed with the stage of RA patients. The mean IL-1 β levels were significantly higher in end-stage RA patients' SF samples (63.8 \pm 12.5 pg/mL) compared to early-onset patients

(35.7 \pm 9.2 pg/mL) (p<0.05). The mean IL-6 levels were notably higher in end-stage RA patients (1876.3 \pm 973.1 pg/mL), compared to early-onset patients (699.2 \pm 284.7 pg/mL) (p<0.01). The mean TNF- α levels were lower in end-stage RA patients (476.7 \pm 96.8 pg/mL), compared to early-onset patients (937.8 \pm 289.3 pg/mL), but no significant difference was observed (p>0.05). The mean IFN- γ levels were comparable in early- and end-stage patients (91.3 \pm 25.7 and 97.2 \pm 91.9 pg/mL, respectively) (p>0.05). The mean IL-10 levels were slightly lower in end-stage RA patients (7.3 \pm 1.8 pg/mL) compared to early-onset patients (13.8 \pm 6.9 pg/mL), indicating no significant difference (p>0.05) (Table 1 and Figure 3).

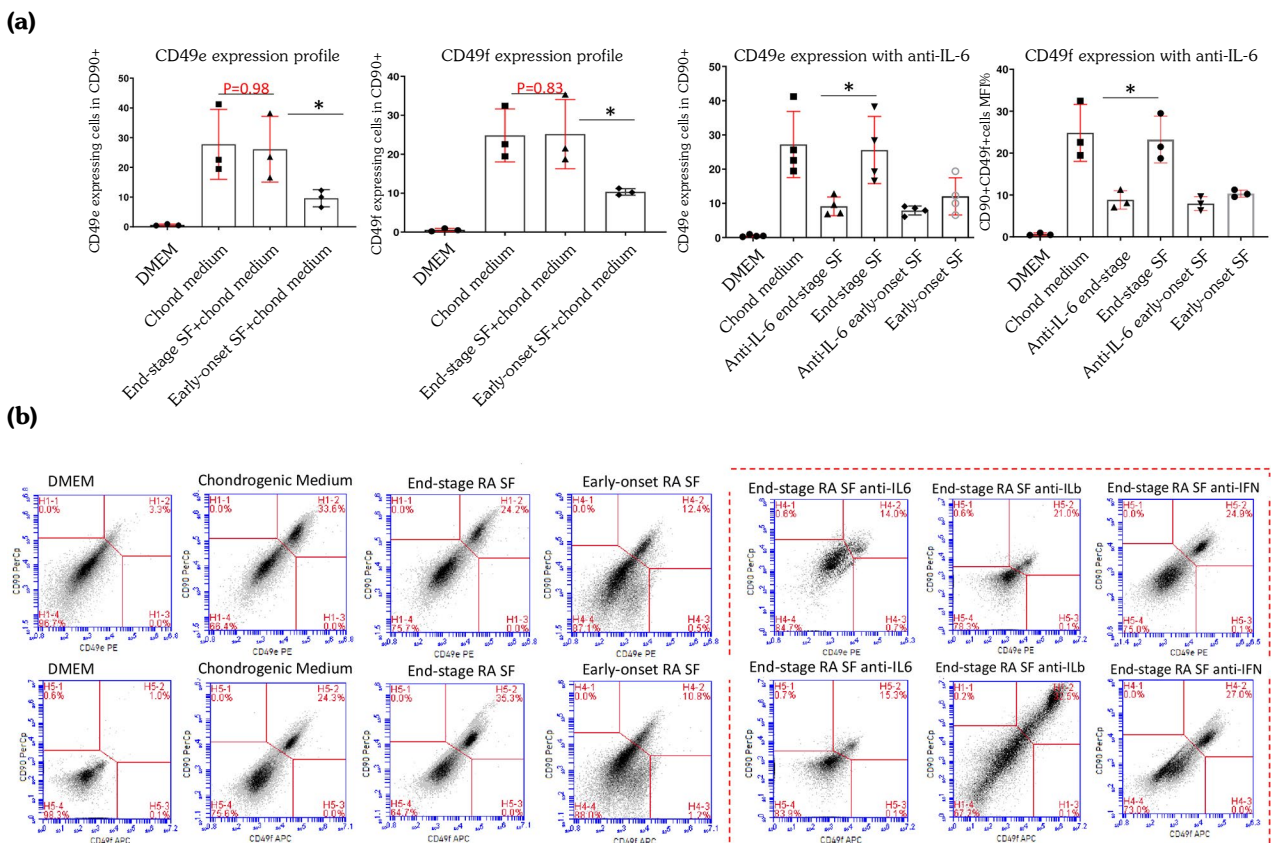


Figure 7. The expression profiles of chondrogenic markers of CD49e and CD49f in the CD90+ cell population. **(a)** The statistical data of CD49e and CD49f expressions. The expression of CD49e and CD49f significantly increased both in chondrogenic stimulated DFSCs and end-stage RA patients' synovial fluid samples, compared to negative control medium and early-onset RA patients (p<0.05). The expression of CD49e and CD49f in CD90+ cell population were comparable in end-stage RA synovial fluid samples and chondrogenic differentiation medium, and no significant change was observed (p>0.05). **(b)** The flow cytometry analysis of CD49e or CD49f positive cells in CD90+ population. DFSCs: Dental follicle stem cells; RA: Rheumatoid arthritis.

Chondrogenic differentiation capacity of dental follicle stem cells in synovial fluid niche of early-onset and end-stage rheumatoid arthritis patients

To assess whether DFSCs were candidates for use in RA cartilage damage, we evaluated the chondrogenic differentiation ability of DFSCs by comparing the cytokine profile of early-onset and end-stage RA patients' SF samples. The cultures with DMEM were used as the negative control and chondrogenic-stimulated

cultures as the positive control. The expression of genes related to chondrogenesis including Col I, Col II, Aggrecan and Sox-9 were analyzed by RT-PCR, and GAPDH was analyzed as the control. Interestingly, DFSCs cultured with SF started to differentiate on Day 4, whereas DFSCs cultured with chondrogenesis stimulation medium started to differentiate between Days 14 and 17 in concurrent cultures (Figure 4).

In addition, we analyzed DFSCs cocultures with SF alone or 1/1 (v/v) chondrogenesis differentiation

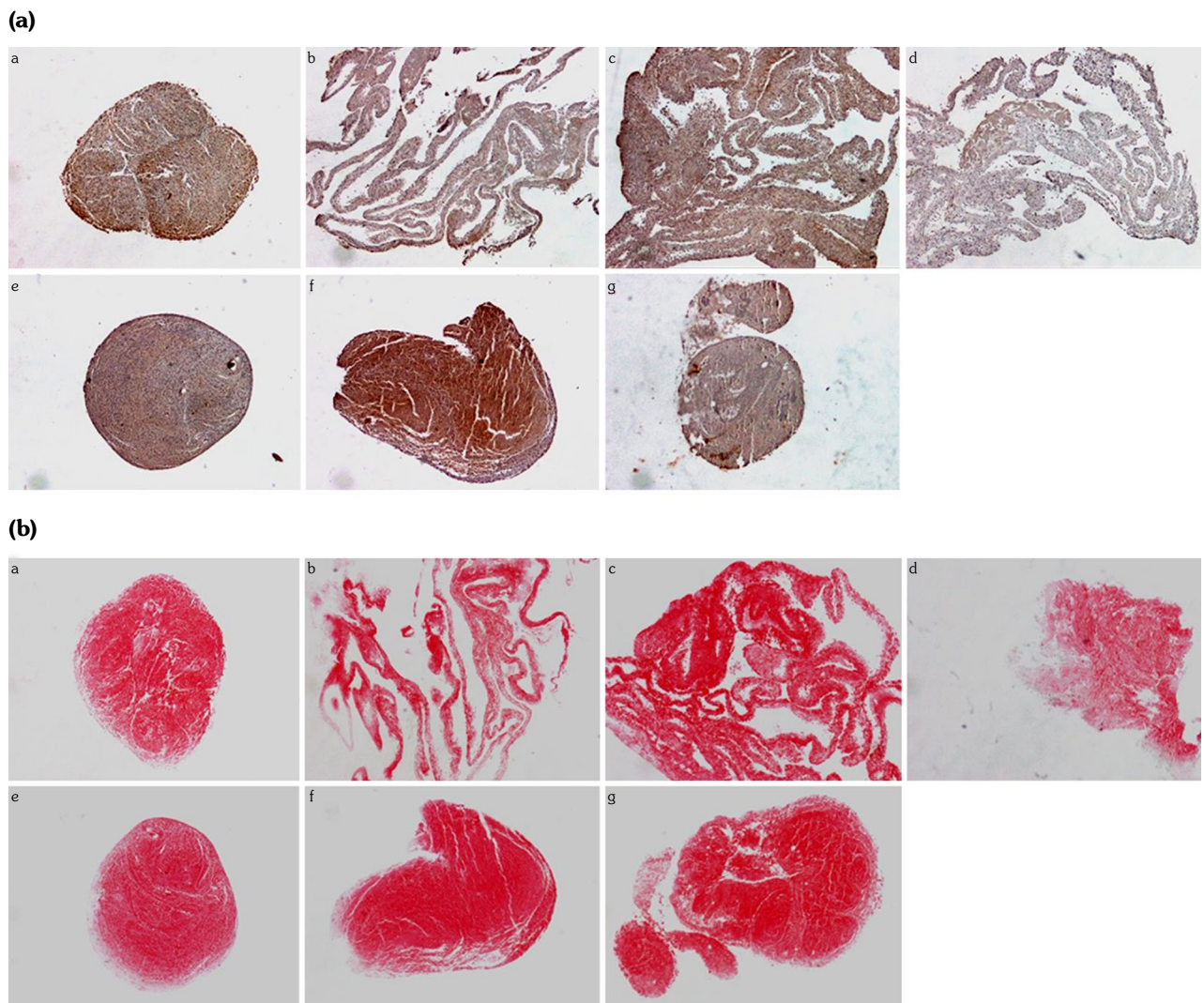


Figure 8. (a) Collagen type II and (b) Safranin-O staining of micromass pellet sections. Collagen type II expression was analyzed by staining the pellet with anti-collagen II antibody. a) DFSCs in the presence of chondrogenic medium after 21 days of culture period. b,c,d) Early-onset RA synovial fluid cultured micromass pellets. e,f,g) End-stage RA synovial fluid cultured micromass pellets, after 21 days of culture period for chondrogenic differentiation. Safranin-O staining intensity and collagen type II expression were higher in DFSCs micromass cultures with end-stage RA synovial fluid samples.

DFSCs: Dental follicle stem cells; RA: Rheumatoid arthritis.

medium/SF to evaluate whether the stimulation with chondrogenesis supplements changed the cell surface marker expression on DFSCs. The mean CD49e and CD49f expression in CD90+ DFSCs cultured with both chondrogenesis differentiation medium and SF samples of end-stage RA patients were $25.8\pm 6.9\%$ and $22.5\pm 4.8\%$ respectively, and only with SF samples were 19.4 ± 2.9 and 18.7 ± 3.1 respectively, indicating no significant difference ($p>0.05$). Similarly, the mean CD49e and CD49f expression in CD90+ DFSCs cultured with both chondrogenesis differentiation medium and SF samples of early-onset RA patients were $12.6\pm 2.7\%$ and $9.4\pm 3.3\%$, respectively and only with SF samples were $14.1\pm 2.7\%$ and $12.2\pm 2.4\%$, indicating no significant difference ($p>0.05$) (Figure 5).

Subsequently, we cultured DFSCs in SF samples in chondrogenic differentiation medium in 1:1 v/v ratio and compared the CD49e and CD49f expression profiles, gene expressions of Col I, Col II, Aggrecan and Sox-9, and histological staining for Col II expression, after the micromass culture period. Gene expression profile of DFSCs varied in early-onset and end-stage patients. The mean Col II mRNA expressions were higher in cocultures with end-stage RA patients' SFs (15.7 ± 8.0 fold increase), compared to cultures with SF samples of early-onset subjects (2.2 ± 0.8 fold increase) ($p<0.01$). Also, Aggrecan and Sox-9 mRNA expressions were significantly higher in end-stage RA patients' SFs (18.1 ± 4.3 and 16.5 ± 6.2 fold increase, respectively), compared to cultures with SF samples of early-onset subjects (3.0 ± 0.6 and 2.6 ± 0.4 fold increase, respectively) ($p<0.01$ for both). Interestingly, mRNA expression levels of Col II, Aggrecan and Sox-9 in DFSCs cultured with end-stage RA patients' SF samples were significantly higher compared to those in chondrogenic culture medium ($p<0.05$, $p<0.05$, and $p<0.01$, respectively) (Figure 6).

The expression of CD49e and CD49f, which are chondrogenic differentiation markers on MSCs, were analyzed via flow cytometry. The mean expression of CD49e and CD49f on CD90+ DFSCs was significantly higher in chondrogenesis differentiation medium (20.4 ± 5.7 and $28.2\pm 6.5\%$, respectively), compared to the negative control medium (DMEM) (0.8 ± 0.3 and $1.1\pm 0.4\%$, respectively) ($p<0.0001$). The expression of CD49e and

CD49f remarkably increased in CD90+ cells in the cocultures with SF samples of end-stage RA patients ($19.4\pm 2.9\%$ for CD49e and $18.7\pm 3.1\%$ for CD49f, respectively), compared to early-onset patients ($12.6\pm 2.7\%$ for CD49e and $10.4\pm 3.3\%$ for CD49f, respectively; $p<0.05$) (Figure 7).

To evaluate the effect of the cytokine profile of RA patients in SF medium on the chondrogenic differentiation potential of DFSCs, we neutralized the high-level cytokines in end-stage RA patients and analyzed for cell surface expressions of CD49e and CD49f. The neutralization of IL-6 resulted with the significant decrease in the expression of CD49e and CD49f in CD90+ DFSCs cultured with SF samples of end-stage RA patients (7.1 ± 1.5 and $9.8\pm 1.7\%$, respectively), compared to unneutralized cultures of end-stage RA patients (19.4 ± 2.9 and $18.7\pm 3.1\%$, respectively). The neutralization of IL-6 tended to decrease the expression of CD49e and CD49f in CD90+ DFSCs cultured with SF samples of early-onset RA patients (8.3 ± 1.4 , and $9.7\pm 2.1\%$, respectively), compared to unneutralized cultures of early-onset RA patients (12.6 ± 2.7 and $10.4\pm 3.3\%$, respectively) (CD49e: end-stage $p<0.005$, and early-onset $p>0.05$. CD49f: end-stage $p<0.01$, and early-onset $p>0.05$). The neutralization of IL-1 β tended to decrease the expression of CD49e and CD49f in CD90+ DFSCs cultured with SF samples of end-stage RA patients, although no significant difference was observed compared to the unneutralized cultures ($p>0.05$). Also, CD49e or CD49f expressions were similar in DFSCs in cocultures of early-onset RA patients' SF samples with the neutralization of IL-1 β compared to the unneutralized cultures, and no significant change was observed ($p>0.05$) (Supp. Figure 1). The neutralization of IFN- γ did not change CD49e or CD49f expressions in end-stage (22.3 ± 2.6 and $24.2\pm 3.8\%$, respectively) RA SF cultured CD90+ cell population, compared to unneutralized cultures ($p>0.05$). Figure 7.

To determine whether mRNA levels of high expressed Col II gene expressions correlated with Col II formation in DFSCs cultured with end-stage RA patients' SF samples, histological analysis was performed by staining paraffin-embedded sections with anti-Col II antibody and the GAG was analyzed with Safranin O staining. High-level Col II immunexpressions

were detected in the middle and border regions of the positive control ($75\pm 9\%$) and end-stage RA patients' SF cultures $63.3\pm 31.6\%$, while early-onset RA patients' SF samples showed a low level of Col II immunoeexpressions ($17.3\pm 8.1\%$) ($p < 0.005$). The intensity of GAG staining appeared to be higher in the positive control medium and cultures with end-stage RA SF samples, while the staining intensity of DFSCs was lower in early-onset patients (Figure 8).

DISCUSSION

Rheumatoid arthritis is a chronic systemic autoimmune disease that results in pain, loss of mobility, and erosion as a result of inflammation of the SF in the joints. Abnormal Th17 responses to synovium and Th17/Treg imbalance play an important role in the pathogenesis of RA.²⁰ The stages of RA can be defined as follows: Stage 1, No destructive changes; Stage 2, Presence of periarticular osteoporosis, subchondral bone destruction without joint deformity; Stage 3, Cartilage and bone destruction with joint deformity and periarticular osteoporosis; Stage 4, Bony or fibrous ankyloses and cartilage and bone destructions.²¹ Abnormal inflammatory responses in RA ultimately lead to the destruction of the cartilage, subchondral bone and soft tissues of the affected joint. At the same time, the rate of degeneration increases with the insufficient regeneration rate of synoviocytes in cartilage repair.²² Currently, corticosteroids, antirheumatic drugs or biological agents that modulate immune responses are frequently used in the treatment of RA. Long-term use of these drugs can cause side effects in a significant number of RA patients. In addition, some RA patients are resistant to these treatments.²³ In recent years, MSC-based therapies have largely been proposed as a new and promising stem cell therapeutic approach to treat RA.²⁴ Several studies have investigated the effects of bone marrow, adipose tissue or umbilical cord MSCs on cartilage damage and inflammatory responses in RA; however, the potential of differentiation in cartilage tissue regeneration in RA or SF niche of DFSCs has not been evaluated. The DFSCs can be isolated easily, have a high doubling time in culture and have an immunomodulatory effect, as well as a regenerative effect more than other

sources.^{9,16} In the present study, we evaluated the chondrogenic differentiation potential of DFSCs in the SF niche of RA patients to highlight the effects of distinct environmental conditions on differentiation potential of DFSCs and to suggest as a new cellular source for the chondrogenic regeneration in different stages of RA for personalized therapies. The results showed that cytokine levels of SF samples altered the chondrogenesis process in DFSCs. The DFSCs rapidly and fully participated in chondrogenesis in the high levels of IL-6 and IL-1 β , while they performed less chondrogenesis at low levels of these cytokines. As reported in previous studies whether cytokines can be used as a biomarker in RA, given the complexity and heterogeneous nature of RA, no single cytokine is responsible for the pathogenesis. Therefore, multiple biomarkers may be a more viable approach to the future of personalized medicine in RA.²⁵

Furthermore, the cytokine levels in the peripheral blood samples may differ from local sites, such as synovial tissue in RA; however, it is known that a large number of cytokines are detected in both synovial tissue and fluid, where they indicate an inflammation, cartilage or bone destruction associated with RA.²⁶ Also, the duration of the disease can change the cytokine profiles. The proinflammatory cytokines, i.e., TNF- α , IL-1 β , and IL-6 begin to be secreted locally in the initial stage of the disease and reach an increasing level toward the late stage.²⁷ Meanwhile, cytokines such as IL-1 β and TNF- α are critical for the development of synovitis.²⁸

The cytokines present in the microenvironment can influence the differentiation potential of MSCs. In previous studies, IL-1 β inhibited chondrogenic differentiation of SF MSCs²⁹ and MSCs isolated from intramedullary reaming.³⁰ In another study, IL-1 β pre-treatment of synovium derived MSCs showed increased chondrogenic differentiation potential.³¹ The difference between previous studies and the results obtained in the current study may have differed depending on the type of MSCs used and environmental conditions.

In the current study, we observed the correlation between high levels of IL-1 β and IL-6 cytokine levels in SF samples and the

chondrogenic differentiation potentials of DFSCs along with the differences in the results obtained in previous studies. Then, we conducted cytokine neutralization experiments to determine the responsible cytokine in the SF niche for chondrogenic differentiation. We analyzed the cell surface chondrogenic marker expressions on DFSCs with the neutralization of IL-6, IL-1 β or IFN- γ by using anti-IL-6, anti-IL-1 β and anti-IFN- γ neutralizing antibodies, respectively. The neutralization of IFN- γ was performed, since the effect of IFN- γ on the chondrogenic differentiation potential of DFSCs is unknown.

The TNF- α levels in SF samples did not significantly differ in early-onset and end-stage RA patients' SF samples, but was lower in end-stage patients compared to early-onset patients. In a study, equine BMSC reduced the levels of Sox-9, TGF- β 1, Aggrecan and Col II, while SF MSCs only reduced the expression of Aggrecan when cultured in chondrogenic medium with the stimulation of TNF- α .³² In another study, TNF- α did not change the chondrogenic gene expression significantly during chondrogenic differentiation of MSCs isolated from human femoral head spongiosa, but had slightly inductive effects on COL2A1, Sox-9, and Aggrecan gene expression.³³ In addition, pre-treatment of synovial MSCs with TNF- α did not affect the differentiation of MSCs into chondrogenesis.³⁴ In the present study, we observed low levels of TNF- α in the SF samples of end-stage RA patients without a significant difference in early-onset patients. However, the slightly higher values in the TNF- α level in the SF of early-onset RA patients may have prevented the chondrogenic differentiation of DFSCs. In addition, IFN- γ levels were closer in end-stage and early-onset RA SF samples. The expression levels of immunoregulatory genes such as IDO, PGE2 or TGF- β of ATMSC, BMSC, or Wharton's jelly-derived MSCs stimulated with proinflammatory cytokine cocktail (IL-1 β , TNF- α , IFN- α , and IFN- γ); however, there are no data for IFN- γ on chondrogenic differentiation of MSCs.³⁵ We, therefore, analyzed CD49e and CD49f expressions in end-stage RA SF cultured DFSCs with anti-IFN- γ neutralizing antibody, and there was no significant difference observed in the chondrogenic markers compared to the unneutralized cultures. Although IFN- γ levels

were closer in end-stage and early-onset RA SF samples, this finding indicates that IFN- γ has no effect on the chondrogenic differentiation of DFSCs in the SF microenvironment.

Interleukin-10 is a cytokine that have a suppressive effect on the inflammatory responses. The impaired regulation of IL-10 is associated with an increased risk for the development of many autoimmune diseases, as well as pathologies that develop in inflammatory responses.³⁶ In the previous study, Jagielski et al.³³ concluded that IL-10 had a slightly stimulatory effect on the expression of Col II, Aggrecan in BMSC. In the present study, significantly low levels of IL-10 were observed in end-stage patients' SF samples compared to early-onset patients. Unlike the previous study, we observed an increased chondrogenic differentiation potential in end-stage RA patients' SF niche, in whom the IL-10 levels were lower.

In the present study, steroid drugs did not prevent bone or cartilage damage in RA. Therefore, we attempted to develop new cellular therapies in cartilage damage occurring in RA disease. Our results showed that DFSCs could differentiate into cartilage at a high rate in the inflammatory SF environment of RA patients and could exert a regenerative effect on cartilage damage that occurs in RA disease.

Synovial fluid samples of patients using TNF or IL-6 inhibitors were not evaluated in this study. Therefore, data on MSCs differentiation in patient groups using these drugs are not presented.

In conclusion, our study results suggest that high levels of IL-6 in SF niche in end-stage RA patients affect the chondrogenic differentiation potential of DFSCs. Also, there may be other microenvironmental factors such as exosomes, paracrine factors or other inflammatory molecules that play a role in the differentiation of DFSCs toward chondrogenesis. Therefore, further studies are warranted to evaluate the molecular mechanisms that may affect the differentiation capacity of DFSCs. Finally, the present study demonstrates that DFSCs can be used for the cellular treatment of RA, particularly in end-stage patients who do not benefit from anti-inflammatory or steroid drugs. Further *in vivo* studies are needed to develop regenerative treatment options with DFSCs, which is easy to obtain with a high regenerative and immunomodulatory effects.

Declaration of conflicting interests

The authors declared no conflicts of interest with respect to the authorship and/or publication of this article.

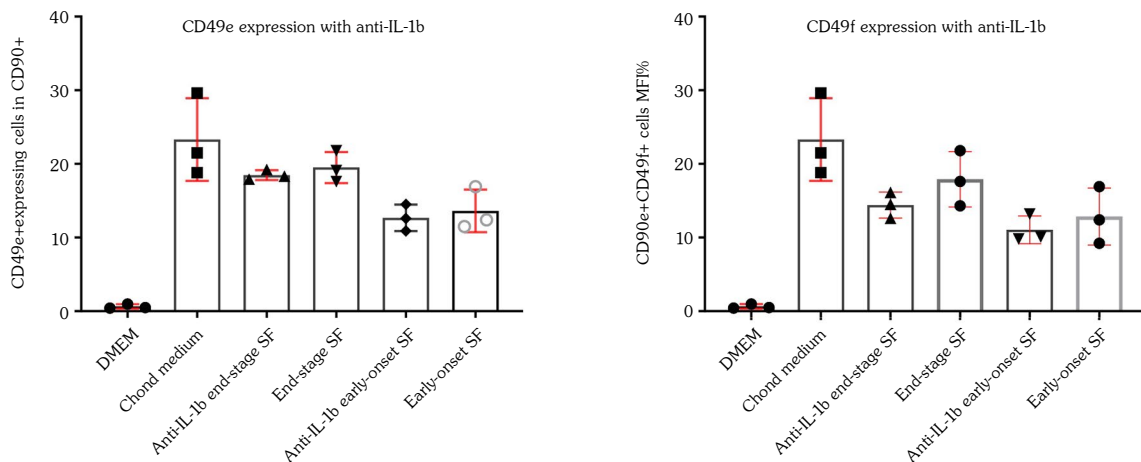
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Supplement Figure 1. Statistical data of the neutralization of IL-1 β in the cultures. The neutralization of IL-1 β did not change the cell surface expression of CD49e or CD49f in CD90+ cells.

DMEM: Dulbecco's Modified Eagle Medium; SF: Synovial fluid.