International Immunopharmacology 74 (2019) 105687

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Immunomodulatory effect of human umbilical cord mesenchymal stem cells on T lymphocytes in rheumatoid arthritis

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ARTICLE INFO

Keywords:
Mesenchymal stem cells
Extracellular vesicles
Rheumatoid arthritis
Collagen-induced arthritis
Immunoregulation
T lymphocytes

ABSTRACT

Rheumatoid arthritis (RA) is an autoimmune disease which is lack of effective therapies. Abnormal activation, proliferation, and differentiation of T lymphocytes are closely related to RA. Mesenchymal stem cells (MSCs) can be used for RA treatment due to their immunoregulatory effects. However, the specific molecular mechanisms have not been fully elucidated and the therapeutic effect has been inconsistent. This study investigated the immunomodulatory effect of human umbilical cord MSCs (hUCMSCs) on T lymphocytes in collagen-induced arthritis (CIA) rats and RA patients to clarify the possible mechanism of hUCMSCs in RA treatment. The effects of hUCMSCs on arthritis index, radiological and synovial pathological changes, T lymphocyte proliferation and apoptosis, RORγt and Foxp3 expression, Th17 and Treg cell ratios, and IL-17 and TGF-β levels were assessed in CIA rats. Further, we verified the effect of hUCMSCs in RA patients, and compared the effect of hUCMSCs with that of hUCMSC derived extracellular vesicles (EVs). The results showed that hUCMSCs inhibited the proliferation and promoted apoptosis in T lymphocytes, downregulated RORγt mRNA and protein expression, decreased Th17 cell ratio, upregulated Foxp3 mRNA and protein expression, and increased Treg cell ratio in the spleen. Furthermore, they downregulated RORγt and Foxp3 expression in the joints, and inhibited IL-17 and promoted TGF-β expression in the serum, thereby improving arthritis, delaying radiological progression, and inhibiting synovial hyperplasia in CIA rats. *In vitro* the effects of hUCMSCs and EVs were consistent with those *in vivo*. Therefore, hUCMSCs may be expected to serve as a new therapy for RA.

1. Introduction

Rheumatoid arthritis (RA) is an autoimmune disease characterized by chronic synovial inflammation and progressive destruction of cartilage and bone. It has a high rate of teratogenicity and disability. At present, commonly used therapeutic drugs include glucocorticoids, disease-modified antirheumatic drugs (DMARDs), and biological agents. In particular, the use of biological agents, to some extent, reduces the disability rate of RA patients, but 30–40% of patients still cannot achieve long-term remission and the treatment is expensive and prone to complications such as infection [1]. Further, these drugs only target the early, active inflammation and cannot repair broken joints in the late stage. Therefore, it is urgent to find a safe and economical treatment that not only control inflammation, but also inhibit and repair damaged joints.

The pathogenesis of RA has not yet been fully elucidated. It has been found that abnormal activation, proliferation, and differentiation of T lymphocytes are an important mechanism of inflammation, immune activation, and bone destruction in RA. A large number of $CD4^+$ T cells infiltrate synovial tissue, and under the action of antigens and cytokines, they are activated and differentiated into various T lymphocyte subsets which have immune effects. Hyperactivity of Th17 cells and an insufficient number or function of Treg cells play an important role in the immune disorder and joint destruction of RA [2]. Differentiation of $CD4^+$ T cells into each Th cell subset depends on the expression of specific transcription factors. Retinoic acid receptor-related orphan nuclear receptor-γt (RORγt) and forkhead or winged helix transcription 3 (Foxp3) are positive transcriptional regulators of differentiation between Th17 cells and Treg cells, respectively. When the body environment is stable, expression of Foxp3 is upregulated, which induces Treg cell differentiation and releases anti-inflammatory factors such as transforming growth factor-β (TGF-β). In the inflammatory environment, RORγt expression increases, which promotes Th17 cell differentiation, production of a large number of pro-inflammatory factors such as interleukin-17 (IL-17), and an aggravated inflammatory response [3,4].

Mesenchymal stem cells (MSCs) are multipotent adult stem cells widely distributed in the bone marrow, umbilical cord, fat, and other

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https://doi.org/10.1016/j.intimp.2019.105687
Received 21 February 2019; Received in revised form 23 May 2019; Accepted 5 June 2019
Available online 08 July 2019

tissues and have high proliferation, multi-differentiation, and immunoregulatory abilities. They can inhibit the proliferation of immune cells and the secretion of inflammatory factors [5]. Compared with MSCs from other sources, human umbilical cord MSCs (hUCMSCs) have many advantages, such as a wide source, easy access to materials, strong proliferation ability, low immunogenicity, and great differentiation potential. They are most likely to become pluripotent stem cells with clinical application prospects.

Some studies have reported that MSCs can improve arthritis symptoms in RA animal models *via* their immunoregulatory effects [5,6]. However, the specific molecular mechanism has not been fully elucidated, and some studies suggest that MSCs have no effect on arthritis [7,8]. The inconsistent results and unclear therapeutic mechanism limit the wide application of MSCs in RA treatment. Therefore, this study firstly re-evaluated the efficacy of hUCMSCs in collagen-induced arthritis (CIA) rats (RA animal model), and from the perspectives of the peripheral blood, immune organs, and inflamed tissues, aimed to clarify the immunoregulatory role of hUCMSCs. Secondly, recent studies have found that all cell types can secrete extracellular vesicles (EVs), which reproduce the effect of parental cells and avoid the limits of cell therapy [9]. Thus, this study verified the immunomodulatory effect of hUCMSCs in RA patients as well as compared the effect of hUCMSCs with that of hUCMSC derived EVs, to explore the possible mechanism of hUCMSCs in RA treatment.

2. Materials and methods

2.1. Reagents, materials and animals

hUCMSCs complete culture medium was purchased from Abmole Bioscience Inc. (Houston, TX, USA). Anti-human monoclonal antibodies CD29, CD44, CD73, CD166, CD90, CD105, CD34, and CD45, APC-labeled anti-rat IL-17 monoclonal antibody, FITC-labeled anti-rat CD4 monoclonal antibody, PE-labeled anti-rat CD25 monoclonal antibody, and APC-labeled anti-rat Foxp3 monoclonal antibody were purchased from BD Biosciences (San Diego, CA, USA). hUCMSC osteogenesis induction medium, adipogenic differentiation medium A and B solution were purchased from Cyagen Biosciences (Santa Clara, CA, USA). Alizarin red, oil red O, bovine type II collagen (CII), complete Freund's adjuvant (CFA), lymphocyte separation solution and bicinchonic acid (BCA) protein concentration determination kit were purchased from Sigma (St. Louis, MO, USA). DMEM-F12 was purchased from Hyclone (Logan, Utah, USA). Anti-RORγt monoclonal antibody, rabbit anti-mouse monoclonal antibody and sheep anti-rabbit IgG polymer antibody were purchased from Abcam (Cambridge, UK). The primers of RORrt, Foxp3, and GAPDH were synthesized by TaKaRa (Shiga, Japan). Wistar rats were supplied by an experimental animal center of the Military Medical Science Academy of the PLA (Beijing, China).

2.2. Isolation, culture, and identification of hUCMSCs [10]

2.2.1. Isolation and culture of hUCMSCs

This study was approved by the ethical committee of Shanxi Dayi Hospital.

Under aseptic conditions, the umbilical cord of normal full-term newborns was provided by the Department of Obstetrics and Gynecology in Shanxi Dayi Hospital. The tissue was washed with PBS and dissected into cubes of about 0.5 cm^3. Following this, hUCMSCs were added to complete culture medium and cultured in an incubator at 37 °C and 5% CO_2 incubator for 22–27 days. Under an inverted microscope, stem cells were fusiform. When the cells reached 80% fusion, they were digested with trypsin, centrifuged, and then passaged by 1: 3.

2.2.2. Identification of hUCMSCs surface antigen phenotype

The P3 generation of hUCMSCs was made into cell suspension (1×10^6 cells/mL), and l00 μL of each cell suspension was taken. The anti-human monoclonal antibodies CD29, CD44, CD73, CD166, CD90, CD105, CD34, and CD45 of mice labeled with PE or FITC were added to the cell suspension. Further, blank control tubes were prepared and detected by flow cytometry.

2.2.3. Osteogenic-induced hUCMSCs differentiation

The P3 generation of hUCMSCs was inoculated into 24-well plates at a density of $5 \times 10^3/cm^2$, and 1 mL of hUCMSC osteogenesis induction medium was added into each well. After incubating for 21 days in an incubator at 5% CO_2 and 37 °C, the hUCMSCs were fixed with 0.1% alizarin red. Finally, the cells were washed fully and observed under an optical microscope.

2.2.4. Lipid-induced hUCMSCs differentiation

The P3 generation of hUCMSCs was inoculated into 24-well plates at a density of $5 \times 10^3/cm^2$, and 1 mL of hUCMSC adipogenic differentiation medium A solution was added into each well. After incubating for 3 days in an incubator at 5% CO_2 and 37 °C, the medium was replaced with hUCMSC adipogenic induction medium B liquid for 1 day. The process was repeated for 4–5 rounds. When lipid droplets were observed, the cells were fixed with 4% paraformaldehyde for 10 min, stained with 0.5% oil red O for 20 min, and washed with double steaming water. Finally, the cells were observed under an optical microscope.

2.2.5. Preparation and characterization of EVs [11]

When the P3 generation of hUCMSCs reached 80% fusion, they were transferred to the DMEM-F12 culture medium (serum free). After 24 h, the conditioned medium was collected. The EVs were separated by gradient ultra-high speed centrifugation at 300 ×*g* for 10 min, 2000 ×*g* for 10 min, 10,000 ×*g* for 30 min, 100,000 ×*g* for 70 min and suspended in PBS. The EVs were visualized by transmission electron microscopy. The protein concentration of EVs was detected by the BCA protein concentration assay kit.

2.3. Effect of hUCMSCs on T lymphocytes in CIA rats

2.3.1. Experimental animals

Twenty-four healthy female Wistar rats (aged 6–8 weeks, weighing 140–150 g) were fed in a temperature-controlled (20 °C) room with a relative humidity of 55% and 12 h light/dark cycle per day in the animal care facility of Shanxi Medical University. All experimental procedures were performed according to the Institutional Animal Care and Use Committee guidelines of Shanxi Medical University.

2.3.2. Preparation of CIA rat model [12]

The rats were randomly allocated into the normal group (n = 6) and CIA model group (n = 18). The CIA model was induced in accordance with previous literature [10]. CII was dissolved in 0.1 M glacial acetic acid at a concentration of 2 mg/mL by stirring overnight at 4 °C. The solution was emulsified in equal volumes with CFA. This emulsified solution was made at a concentration of 1 mg/mL bovine type II collagen. On the first day, the rats were anesthetized by intraperitoneal injection with 5% chloral hydrate. Subsequently, they were separately injected intracutaneously with 0.1 mL emulsion at three points on the back, one point on the tail, and one point on the posterior palm. On day 14, rats received a second intraperitoneal injection with a 0.2 mL emulsion as a booster. CIA animal models were successfully established.

2.3.3. Grouping and intervention

Eighteen CIA rats were randomly assigned to three groups: CIA model group (n = 6), hUCMSCs treatment group (n = 6), and methotrexate treatment group (n = 6). The hUCMSCs group received intravenous injection of 2×10^6 hUCMSCs once after the immunization enhancement. The MTX group was administered four times intraperitoneally with 0.9 mg/kg/w MTX. The normal group and CIA

group received an equal volume of normal saline as MTX by intraperitoneal injection once a week for four times.

2.3.4. Arthritis assessment

Paw edema was measured by the mean volumes in both hind paws [13]. The rats were immersed from the shaved paw to a marked line above the ankles (internal malleolus of the lower tibia) in a water volumetric meter. The volumes of water rising were recorded. Data are presented as the mean paw volume per group. Each rat was measured in both hind paws once a week for 6 weeks.

Joint inflammation was scored by the arthritis index [13]. The arthritis lesion was graded on a scale of 0–4: 0, no evidence of hyperemia and/or inflammation; 1, hyperemia with little or no paw swelling; 2, swelling confined predominantly to the ankle region, with modest hyperemia; 3, increased paw swelling and hyperemia of the ankle and metatarsal regions; 4, maximal paw swelling and hyperemia involving the ankle, metatarsal, and tarsal regions. The scores for all 4 paws were summed as the arthritis index for each rat, for a maximum possible score of 16.

The radiographs in rats were performed by X-ray equipment (Philips Corporation, NY, USA) [14]. The radiologic change was assessed blindly by two independent experienced imaging physicians on the 6th week.

2.3.5. Preparation of specimens

The rats in each group received an intraperitoneal injection of 10% chloral hydrate for anesthesia at a dose of 4.5 mL/kg on the 6th week. The peripheral blood was collected by puncturing the venae angularis. After centrifugation, the supernatant was obtained and stored at −70 °C to detect the serum cytokines by flow cytometry. Subsequently, the rats were sacrificed by dislocation, and the spleen and joints were removed for the subsequent studies. The spleen of each rat was divided into three parts: the first part was fixed in 4% paraformaldehyde and then embedded in paraffin for immunohistochemical analysis, the second part was prepared to detect the T lymphocyte cycle by flow cytometry, and the third part was stored in a −200 °C liquid nitrogen container for reverse transcription-polymerase chain reaction (RT-PCR) analysis. The joints of each rat were divided into two parts: the first part was used to detect synovial inflammation by hematoxylin and eosin (HE) staining, and the second part was embedded in paraffin for immunohistochemical analysis.

2.4. Detection of T lymphocytes and inflammatory factors in rats

2.4.1. Detection of proliferation and apoptosis of T lymphocytes and proportion of Th17 and Treg cells in the spleen by flow cytometry

2.4.1.1. Splenocyte suspension. The spleen was fully dissected, and the filtrate was collected by a nylon filter. The splenocyte solution was added to the surface of the lymphocyte separation solution, centrifuged at 2500 rpm for 20 min, and the interface was collected. The splenocyte concentration was adjusted to 1×10^6/mL.

Proliferation and apoptosis of T lymphocytes: 100 μL splenocyte suspension was added to 1 mL 75% ethanol and fixed at 4 °C for > 24 h. After washing with PBS and centrifugation, the splenocytes were added to 3 μg/mL propidium iodide (PI) solution and incubated at 15–25 °C for 5 min. Flow cytometry was used to detect the proliferation index, *i.e.*, the ratio of G2 + S phase to G1 + G2 + S. In addition, 100 μL splenocyte suspension was mixed with 5 μL FITC-AnnexinV at room temperature for 10 min, and then 10 μL PI was added. The splenocyte suspension was incubated at room temperature for 10 min, and 400 μL PBS was added into the reaction tube. Flow cytometry was used to analyze the apoptotic rate.

2.4.2. Ratio of Th17 and Treg cells

Th17 cells were detected by adding FITC-labeled anti-rat CD4 monoclonal antibody (10 μL) and APC-labeled anti-rat IL-17 monoclonal antibody (2.5 μL). The following labels were sequentially added to Treg cells: FITC-labeled anti-rat CD4 monoclonal antibody, 10 μL; PE-labeled anti-rat CD25 monoclonal antibody, 10 μL; and APC-labeled anti-rat Foxp3 monoclonal antibody, 2.5 μL. The positive rate of 1×10^6 lymphocytes was detected by flow cytometry, and the ratio of Th17 and Treg cells was calculated.

2.4.3. Detection of RORγt and Foxp3 expression in the spleen by immunohistochemistry

The paraffin-embedded spleen tissue was cut into 5 μm sections, which were dried for > 4 h at 30 °C in the oven, dewaxed by gradient, incubated in 3% H_20_2 for 10 min at room temperature, washed with PBS, and repaired with antigen. Anti-RORγt monoclonal antibody was diluted 1: 100 and added to one part of the tissue, which was then dipped in rabbit anti-mouse monoclonal antibody. Anti-Foxp3 monoclonal antibody was diluted 1: 200 and added to the second part of the tissue, which was dipped in sheep anti-rabbit IgG polymer antibody. The sections were developed by DAB staining and observed with an optical microscope. When brown staining appeared in the cytoplasm, the reaction was stopped by tap water. Then, the tissue was re-dyed with hematoxylin, dehydrated, clarified, and finally sealed with gum. Image-Pro Plus 5.1 Graphic Analysis System (Media Cybernetics, Rockville, MD, USA) was used to capture histochemical images through optical microscopy (Olympus BX41 microscope). In the image analysis system, images were captured according to the standard resolution, and the OD value was calculated as Log (240/Gy value).

2.4.4. Detection of RORγt and Foxp3 mRNA in the spleen by RT-PCR

The spleen tissue was added to 1 mL RNAiso Plus solution, ground into a homogenate, and centrifuged at 12000 rpm for 5 min. Subsequently, the supernatant was extracted with a phenol-chloroform mixture, and the spleen RNA was precipitated with isopropanol. RNA quality and concentration were analyzed by absorbance analysis. The RNA quality was OD 260/OD 280: 1.8–2.0, and the RNA concentration was 1000–2000 μg/mL. Glyceraldehyde-3-phosphate dehydrogenase (GADPH) was used as an internal reference for the mRNA expression level and DNA synthesis in each sample experiment. The sequences were as follows: RORrt: F5′-GAGGCCATTCAGTACGTGGT-3′, R5′-GCTGTGCC AGAATGATCAGA-3′ 73 bp; Foxp3: F 5′-GGCAAACGGAGTCTGCAAG-3′, R 5′-TGCTCCAGAGACTGCACCAC-3′ 150 bp; and GAPDH: F5′-TGCACC ACCAACTGCTTAGC-3′, R5′-GGCATGGACTGTGGTCATGAG-3′ 87 bp. According to the TaKaRa PrimeScript RT reagent kit with gDNA and SYBR Premix Ex Taq II (TliRNaseH+) kit, reverse transcription of RNA and PCR amplification of cDNA were performed. Finally, the StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) was used for analysis. Each sample was analyzed in triplicate.

2.4.5. Detection of RORγt and Foxp3 expression in the joints by immunohistochemistry

The experimental steps were the same as those in 2.4.2.

2.4.6. Detection of cytokines in serum by flow cytometry

The serum levels of IL-17 and TGF-β were detected using a flow cytometer. Data were analyzed by Cell-Quest software and the Windows Multiple Document Interface flow cytometry application.

2.5. Detection of T lymphocytes and cytokines of peripheral blood in RA patients by flow cytometry

Eight RA patients (6 females and 2 males) were selected from the Shanxi Dayi Hospital. The study was approved by the ethical committee of Shanxi Dayi Hospital, and informed consent was obtained from the patients. The diagnostic method was based on the 1987 American College of Rheumatology (ACR) diagnostic criteria for RA. RA patients with other chronic diseases, such as infectious diseases, viral hepatitis, diabetes, hypertension, or coronary heart disease were excluded. The

peripheral blood mononuclear cells (PBMCs) were separated by gradient centrifugation over Ficoll. The hUCMSCs (2×10^6 cells per well) and PBMCs were co-cultured at a ratio of 1: 1 (2×10^6 cells per well). The EVs and PBMCs were co-cultured at a ratio of 30 μg: 2×10^6, 60 μg: 2×10^6, and 90 μg: 2×10^6, respectively. All culture sets were incubated in 6-well plates in a saturated humidity incubator at 37 °C with 5% CO_2 for 72 h. The PBMC suspensions were harvested. The cells were collected by centrifugation and the supernatant was kept at −70 °C for further analysis. The ratio of Th17 and Treg cells and the expression of cytokines were detected by flow cytometry. The experimental steps were the same as those in 2.4.1 and 2.4.5.

2.6. Statistical analysis

The statistical software SPSS 22.0 was used to analyze the data. Data that conformed to the normal distribution were expressed by mean ± standard deviation. One-way analysis of variance (LSD method: satisfying the homogeneity of variance, Dunnett T3 method: not satisfying the homogeneity of variance) was used to compare these data. Data that did not satisfy the normal distribution were compared by median (quartile spacing). The Kruskal-Wallis rank sum test was used for the overall comparison, and the Wilcoxon rank sum test was used for two samples in the intergroup comparison. $P < 0.05$ was considered significant.

3. Results

3.1. Isolation, culture, and identification of hUCMSCs

hUCMSCs adhered to the wall and grew uniformly in a long spindle shape. Fibroblast-like changes were observed under an inverted microscope (Fig. 1A). Flow cytometry was used to detect the surface phenotypes of hUCMSCs in the P3 generation. The results showed that CD29, CD73, CD166, CD90, CD105, and CD44 were positive, while CD34 and CD45 were negative. hUCMSCs showed osteogenic and adipogenic differentiation potential (Fig. 1B, C). Further, under a transmission electron microscope, the EVs could be visualized as round or oval cystic vesicles with a diameter of 40–300 nm, and their protein concentration was 2.05 μg/μL.

3.2. Therapeutic effect of hUCMSCs in CIA rats

3.2.1. Efficacy evaluation of hUCMSCs after transplantation

There were no adverse reactions in all groups. At the 6th week, there was no swelling in the hind foot joint of the normal group, but swelling was obvious in the CIA group. The swelling was alleviated after intervention with hUCMSCs and MTX (Fig. 2A). The swelling degree in the hUCMSCs and MTX groups was lower than that in the CIA group. There was no significant difference between the hUCMSCs group and MTX group. It was suggested that hUCMSCs can effectively improve the swelling degree of hind feet in CIA rats, and the effect was similar to that of MTX (Fig. 2B).

3.2.2. Comparison of arthritis index in rats

At the 6th week, the arthritis index of the CIA group was higher than that of the normal group. The arthritis index of the hUCMSCs group and MTX group was lower than that of the CIA group, and there was no significant difference between the hUCMSCs and MTX groups. These results suggested that hUCMSCs can effectively improve the arthritis index of CIA rats, and the effect was similar to that of MTX (Fig. 2C).

3.2.3. X-ray manifestations of foot joints in rats

The radiographs mirrored the progression from soft tissue swelling, joint space change (between the tibia and calcaneus, the tibia and talus, and the talus and calcaneus), articular bone erosion, and prominent new periosteal bone formation to bony ankylosis in the carpal, tarsal, metacarpal, metatarsal, and interphalangeal regions.

At the 6th week, X-ray showed clear articular space, no osteoporosis and no bone destruction in the normal group. In the CIA group, there was a blurry and narrow joint space, osteoporosis and bone erosion. The degree of joint space stenosis was lighter with no osteoporosis and bone erosion in the hUCMSCs and MTX groups. It showed that hUCMSCs suppressed the radiologic progression in CIA rats (Fig. 2D).

3.2.4. Pathology of ankle joint in rats

At the 6th week, the structure of the joint cavity in the normal group was clear with no synovial hyperplasia and inflammatory cell infiltration. In contrast, synovial hyperplasia was obvious in the CIA group, and a large number of inflammatory cells infiltrated. The synovial hyperplasia and inflammatory cell infiltration were significantly reduced in the hUCMSCs and MTX groups (Fig. 2E).

D

3.3. Immunomodulatory effect of hUCMSCs on T lymphocytes in rats

3.3.1. Effects of hUCMSCs on proliferation and apoptosis of T lymphocytes and proportion of Th17 and Treg cells in the spleen of rats

Compared with the normal group, the proliferation index of T lymphocytes in the CIA group was higher. However, it decreased in the CIA group after intervention with hUCMSCs or MTX, and the effect of the MTX group was greater than that of the hUCMSCs group (Fig. 3A, C). Compared with the normal group, the apoptotic index of T lymphocytes in the CIA group decreased but increased after intervention with hUCMSCs or MTX. There was no significant difference between the hUCMSCs and MTX groups (Fig. 3B, D). These results suggested that hUCMSCs played an immunoregulatory role by regulating the proliferation and apoptosis of T lymphocytes.

Compared with the normal group, the percentage of Th17 cells in the CIA group increased. Compared with the CIA group, the percentage of Th17 cells in rats treated with hUCMSCs or MTX decreased, and the effect of the MTX group was greater than that of the hUCMSCs group (Fig. 3E, G). Compared with the normal group, the proportion of Treg cells in the CIA group decreased, but there was no statistical difference. Compared with the CIA group, the proportion of Treg cells in rats treated with hUCMSCs or MTX increased. The proportion of Treg cells

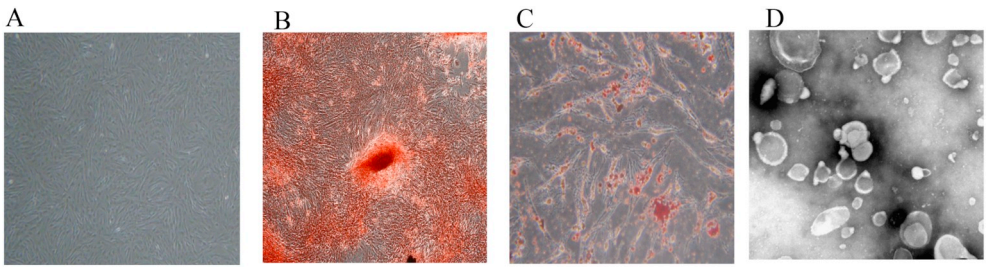

Fig. 1. Characteristics of hUCMSCs and EVs. (A) Cell culture of passage 3. Original magnification × 100. (B, C) Osteogenic differentiation of hUCMSCs indicated by alizarin red staining. Oil red O staining of hUCMSCs after induction of adipogenic differentiation. Original magnification × 100. (D) Under a transmission electron microscope, the EVs displayed a lipid bilayer, vesicular structure, and a diameter between 40 and 300 nm. Original magnification × 80,000. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

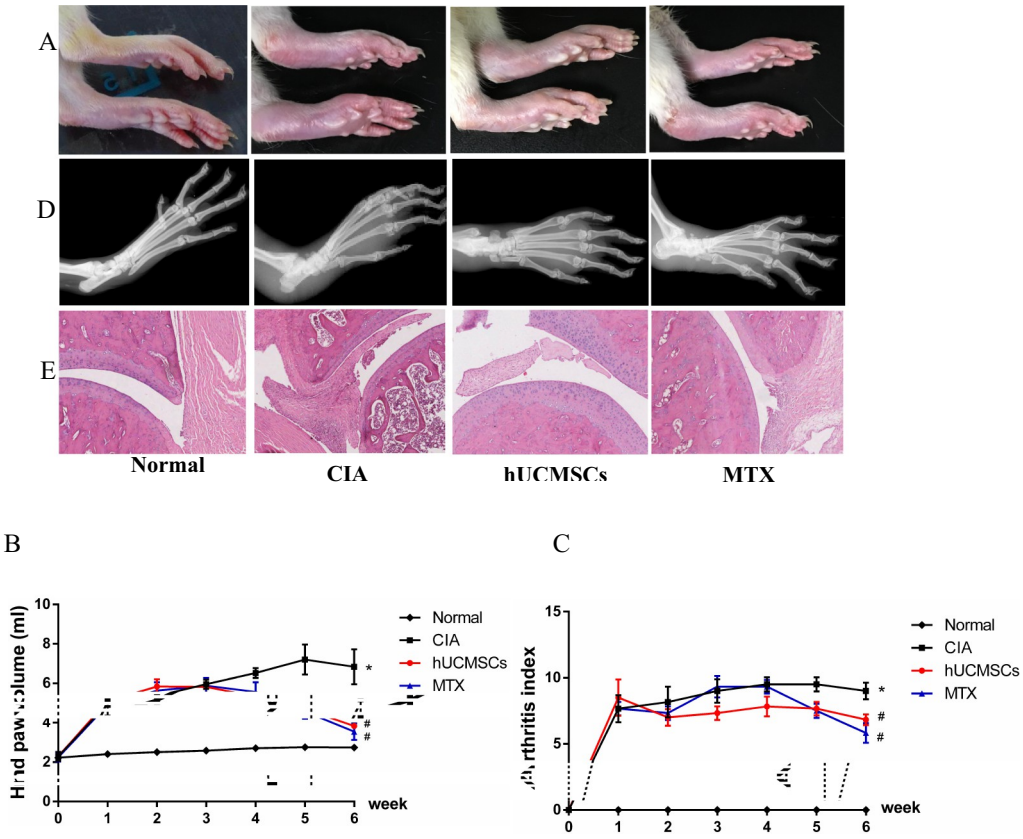

Fig. 2. hUCMSCs alleviated inflammatory responses and prevented joint damage in CIA rats. Female Wistar rats were immunized with type II collagen emulsified with Freund's complete adjuvant. The animal experiments were performed in four independent groups. n = 6 in each group. *P < 0.05 *vs* normal group. # P < 0.05 *vs* CIA group. Values are given as mean ± SD. On day 15 after immunization, 2×10^6 hUCMSCs were injected into the rats *via* the tail vein only once. MTX was intraperitoneally injected at 0.9 mg/kg/w for four times. The normal rats and CIA rats received an equal volume of normal saline by intraperitoneal injection for four times. (A) The swelling of the hind paw in each group. (B) Hind paw volumes were determined at various time points. (C) Arthritis index scores were valued at different time points. (D) X-ray manifestations of the hind paw joints in each group. (E) Pathology of ankle joint using HE staining (original magnification × 40).

in the hUCMSCs group was similar to that in the MTX group (Fig. 3F, H). Compared with the normal group, Treg/Th17 in the CIA group decreased. It increased after intervention with hUCMSCs or MTX. There was no significant difference between the hUCMSCs and MTX groups (Fig. 3I). These results suggest that hUCMSCs played an immunoregulatory role by balancing Treg/Th17.

3.3.2. Effects of hUCMSCs on the expression of RORγt and Foxp3 in the spleen of rats

The RORγt and Foxp3 were both localized in the cytoplasm of cells in the spleen of rats. The expression of RORγt was negative or weakly positive in the normal group, hUCMSCs group, and MTX group (Fig. 4A), while Foxp3 was strongly positive (Fig. 4B). However, the expression of RORγt was strongly positive in the CIA group (Fig. 4A), and Foxp3 was negative or weakly positive (Fig. 4B).

Compared with the normal group, the expression of RORγt in the CIA group increased, while the expression of Foxp3 decreased. Compared with the CIA group, both the hUCMSCs group and MTX group showed downregulated expression of RORγt and upregulated expression of Foxp3, and the effect on RORγt expression in the hUCMSCs group was greater than that in the MTX group (Fig. 4C). There was no significant difference in upregulation of Foxp3 between the two groups (Fig. 4D). These results suggest that hUCMSCs regulated T cell differentiation into Th17 and Treg cells by regulating the expression of transcription factors RORγt and Foxp3.

3.3.3. Effects of hUCMSCs on the expression of RORγt and Foxp3 mRNA in the spleen of rats

Compared with the normal group, the expression of RORγt mRNA in the CIA group increased, and Foxp3 mRNA expression decreased. Compared with the CIA group, both the hUCMSCs group and MTX group showed downregulated RORγt mRNA and upregulated Foxp3 mRNA expression. In the hUCMSCs group, the downregulation of RORγt mRNA expression was greater than in the MTX group, and the upregulation of Foxp3 mRNA expression was similar to that in the MTX group (Fig. 4E, F). These results suggest that hUCMSCs regulated T cell differentiation into Th17 and Treg cells at the transcriptional level.

3.3.4. Effect of hUCMSCs on the expression of RORγt and Foxp3 protein in the joints of rats

The RORγt and Foxp3 were both localized in the cytoplasm of cells in the joints of rats. The expression of RORγt and Foxp3 was negative or weakly positive in the normal group, hUCMSCs group, and MTX group, but was strongly positive in the CIA group (Fig. 4G, H).

Compared with the normal group, the expression of RORγt and Foxp3 in the CIA group increased. Compared with the CIA group, both the hUCMSCs group and MTX group showed downregulated expression of RORγt and Foxp3, and the effect in the MTX group was greater than that in the hUCMSCs group (Fig. 4I, J). These results also suggest that hUCMSCs regulated T cell differentiation into Th17 and Treg cells at the transcriptional level.

3.3.5. Effect of hUCMSCs on the levels of Th17- and Treg-related inflammatory factors in the serum of rats

Compared with the normal group, the IL-17 concentration in the CIA group B increased. Compared with the CIA group, the IL-17 concentration in the hUCMSCs and MTX groups decreased (Fig. 5A). The concentration of TGF-β in the CIA group was lower than that in the normal group, but there was no statistical difference. Compared with the CIA group, the concentration of TGF-β in the CIA group increased after intervention with hUCMSCs and MTX (Fig. 5B). There was no significant difference on the levels of IL-17 and TGF-β between the hUCMSCs and MTX groups. These results suggest that hUCMSCs played an immunoregulatory role through the inflammatory factor pathway.

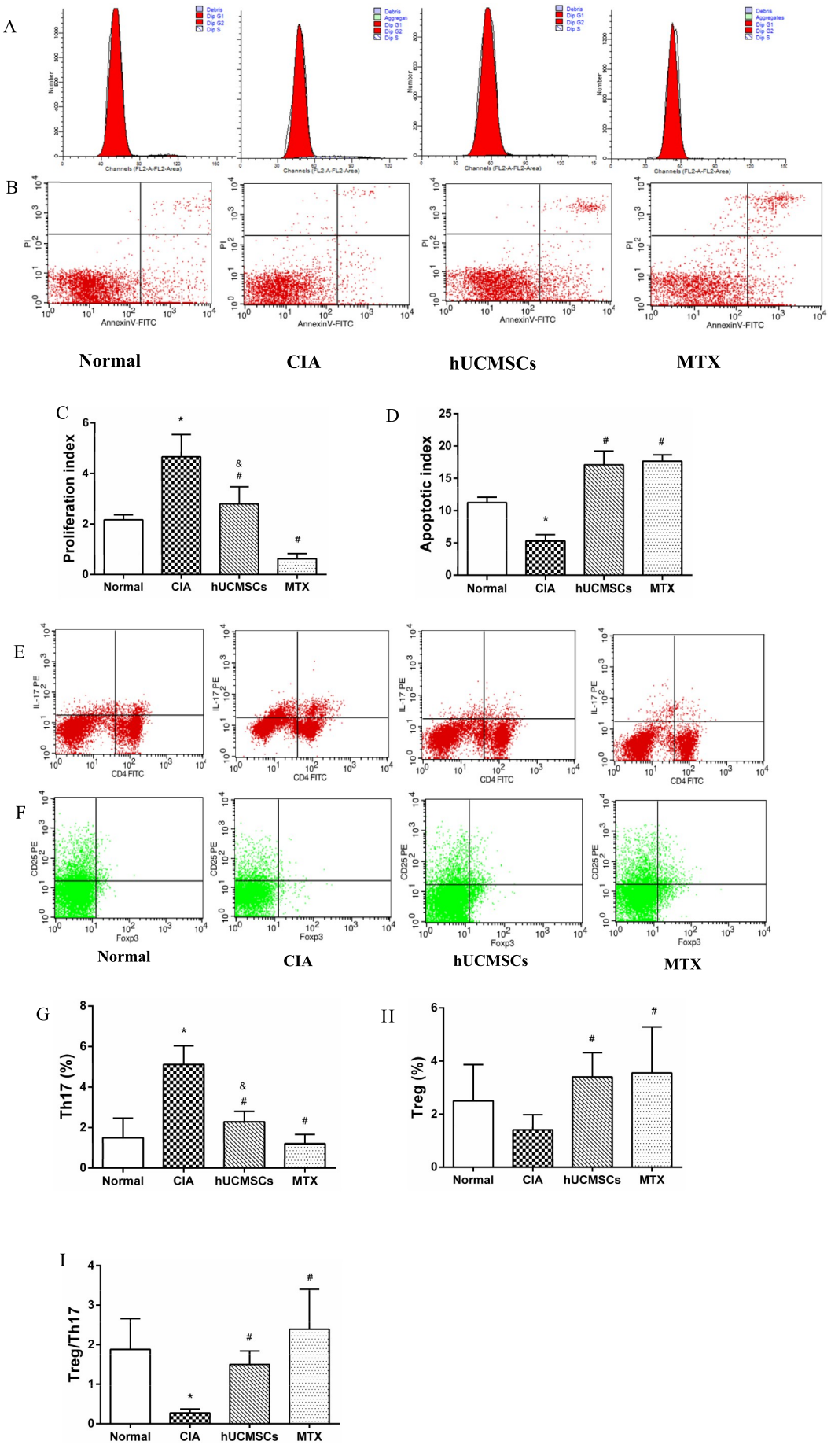

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Fig. 3. hUCMSCs transfer therapy influenced the proliferation and apoptosis of T lymphocytes and the ratio of Th17 and Treg cells in the spleen of rats. Values are given as mean ± SD. *P < 0.05 *vs* normal group. #P < 0.05 *vs* CIA group. &P < 0.05 *vs* MTX group. (A, C) Flow cytometry data demonstrating the proliferation of T lymphocytes in the spleen. (B, D) Flow cytometry data showing apoptosis of T lymphocytes in the spleen. (E, G) Flow cytometry data showing the proportion of Th17 cells in splenic mononuclear cells. (F, H) The ratio of Treg cells in splenic mononuclear cells. (I) The Treg/Th17 in splenic mononuclear cells.

3.4. Immunomodulatory effect of hUCMSCs and EVs on T lymphocytes and

3.4.1. Th17- and Treg-related inflammatory factors in RA patients

hUCMSCs and EVs downregulated the Th17 cell ratio (Fig. 6A, C), inhibited the production of IL-17 (Fig. 6F), upregulated the proportion of Treg cells and Treg/Th17 ratio (Fig. 6B, D, E), and promoted the expression of TGF-β (Fig. 6G). No significant difference between the hUCMSCs and EVs (90 μg) groups in decreasing the proportion of Th17 cells and the level of IL-17A was observed (Fig. 6C, F). The effect of the hUCMSCs group was greater than that of the EVs group (90 μg) in increasing the proportion of Treg cells and Treg/Th17 ratio and the level of TGF-β (Fig. 6B, D, E, G). The effect of the EVs (90 μg) group was superior than that of the EVs groups (30 μg, 60 μg). These results suggest that hUCMSCs played an immunoregulatory role by balancing Treg/Th17 and inflammatory factors in RA patients.

4. Discussion

It was previously believed that RA was driven by the Thl/Th2 imbalance. However, it was found that interferon-γ (IFN-γ) secreted mainly by Th1 cells in the synovial fluid of RA patients was rare; therefore, the Thl/Th2 imbalance could not explain all the mechanisms of RA. It has been found that Th17/Treg imbalance is closely related to the severity of RA [2,3]. Th17 cells mainly secrete IL-17, which can regulate Thl-mediated inflammatory response, promote the expression of tumor necrosis factor-α (TNF-α), IL-6, and IL-1, and eventually lead to persistent inflammation of the synovium. Meanwhile, IL-17 is also an anti-apoptotic factor that leads to the blockage of T and B cell apoptosis and induces a vicious cycle of inflammation [15]. Moreover, Th17 cells promote the release of CXC-chemokine 8 (CXCL8), C—C chemokine ligand 2 and 3 (CCL2 and CCL3), and matrix metalloproteinase (MMP) as well as the activation of osteoclasts and angiogenesis, eventually leading to the destruction of cartilage and bone. Treg cells mainly secrete TGF-β, which can inhibit the abnormal activation of T cells and secretion of inflammatory factors and maintain immune tolerance. Some studies have found that the proportion of Th17 cells and the levels of IL-17, IL-23, IL-6, and TNF-α in peripheral blood of RA patients are significantly increased [16,17] and blocking IL-17 can alleviate joint inflammation and bone destruction in CIA rats [3]. Nonetheless, results regarding whether the proportion of Treg cells and the expression of TGF-β are decreased in RA patients have been inconsistent [2,16]. This study showed that the proportion of Treg cells in spleen and the expression of TGF-β in serum had no significant differences between CIA rats and normal controls. However, the proliferation index of T lymphocytes increased, the apoptotic index decreased, the proportion of Th17 cells increased, the ratio of Treg/Th17 decreased, and the level of IL-17 increased in CIA rats, which is consistent with the literature [2]. Therefore, finding new agents that can regulate T lymphocytes may lead to a promising treatment for RA.

MSCs can immune-regulate T lymphocytes, providing a new hope for RA treatment. Some studies have evaluated the efficacy of MSCs in RA animal models, but the results are inconsistent [18–25], and the specific molecular mechanism is unclear. In this study, we re-evaluated the efficacy of hUCMSCs (2×10^6) in CIA rats after intravenous injection. It was found that hUCMSCs reduced swelling degree of hind foot and arthritis index, reduced synovial hyperplasia and inflammatory cell infiltration, and delayed radiological progression. The hUCMSCs therapy is safe without adverse reactions. Y. Sun [18] has reported that hUCMSCs and human synovial fibroblasts (FLS) were injected intraperitoneally into CIA mice; hUCMSCs decreased arthritis scores, and ameliorated synovial inflammation and joint damage compared to FLS. In another study, human gingiva-derived MSCs (G-MSCs) and human dermal fibroblasts were injected intravenously into CIA mice; G-MSCs reduced the severity of arthritis and the histopathology scores compared to dermal fibroblasts [23]. Furthermore, some other studies have shown that xenogenic or allogeneic MSCs provide similar benefits in experimental arthritis [20,22,25]. Because the positive effects of MSCs have been shown to be MHC-independent, combined with literature reports, it is speculated that the effects of hUCMSCs in this study are therapeutic effects rather than a xenograft response. The therapeutic effects may be achieved by regulating T lymphocytes. hUCMSCs inhibited the proliferation of splenic T cells in CIA rats, promoted T cells apoptosis, decreased the proportion of Th17 cells, increased Treg/Th17, and inhibited the expression of IL-17 in serum. Although the proportion of Treg cells in spleen and TGF-β expression in serum were not significantly different from those in the normal group, hUCMSCs increased the proportion of Treg cells and Treg/Th17 and promoted TGF-β expression. The study showed that hUCMSCs play an immunoregulatory role in T lymphocyte proliferation, apoptosis, differentiation, and inflammatory factor pathway. The inconsistency of the current study may be attributed to the source of MSCs, the culture conditions of MSCs, the timing of treatment, the number of injected cells, and the injection method. If these problems can be resolved in future, it will be greatly conducive to the clinical transformation of MSCs in the treatment of RA.

hUCMSCs improved the imbalance of Treg/Th17, but there are few studies on the specific mechanism. RORγt and Foxp3, as positive transcriptional regulators of Th17 and Treg cell differentiation, play a central role in the differentiation of naive $CD4^+$ T cells into Th17 and Treg cells [3]. Studies have shown that the expression of RORγt and Foxp3 are imbalanced in RA patients and animal models [3,26,27]. This study showed that the expression of RORγt protein and mRNA in spleen of CIA rats was higher than that in the normal rats, while the expression of Foxp3 protein and mRNA was decreased significantly. hUCMSCs downregulated the expression of RORγt protein and mRNA and upregulated the expression of Foxp3 protein and mRNA. Interestingly, this study showed that the expression of RORγt and Foxp3 in joints of CIA rats was increased. It has been confirmed that increased numbers of Th17 and Treg cells are present in the inflamed joints [28]. RORγt and Foxp3 are markers of Th17 and Treg cells, and hUCMSCs downregulated the expression of RORγt and Foxp3; thus it is speculated that hUCMSCs could downregulate the proportion of Th17 and Treg cells in inflamed joints; however, this needs to be confirmed in the future. In view of the inconsistent results between the spleen and joint, it is possible that the inflammatory environment and the activation of T cells promote Foxp3 expression, leading to the increased proportion of Treg cells. However, the increased expression of TNF-α, IL-6, and IL-1 in the inflammatory joints inhibited the activity of Treg cells and induced the production of Th17 cells, leading to the aggravation of joint inflammation. This study demonstrated from the immune organ and inflammatory joint of CIA rats that hUCMSCs could regulate T lymphocyte differentiation from the transcriptional level and balance the proportion of Treg/Th17 to play an immunomodulatory role.

As a type of cell therapy, certain practical difficulties are associated with MSCs therapy, such as the risk of capillary blockage, tumorigenesis, and genetic instability *in vivo* microenvironments, which limit clinical application. Recent studies have suggested that EVs derived from MSCs may resolve current problems with cell-based MSC therapies. EVs contain a large variety of proteins, mRNAs, and miRNAs and simulate the immune regulation and tissue repair of MSCs by interacting with other cells through transfer of information [9]. However,

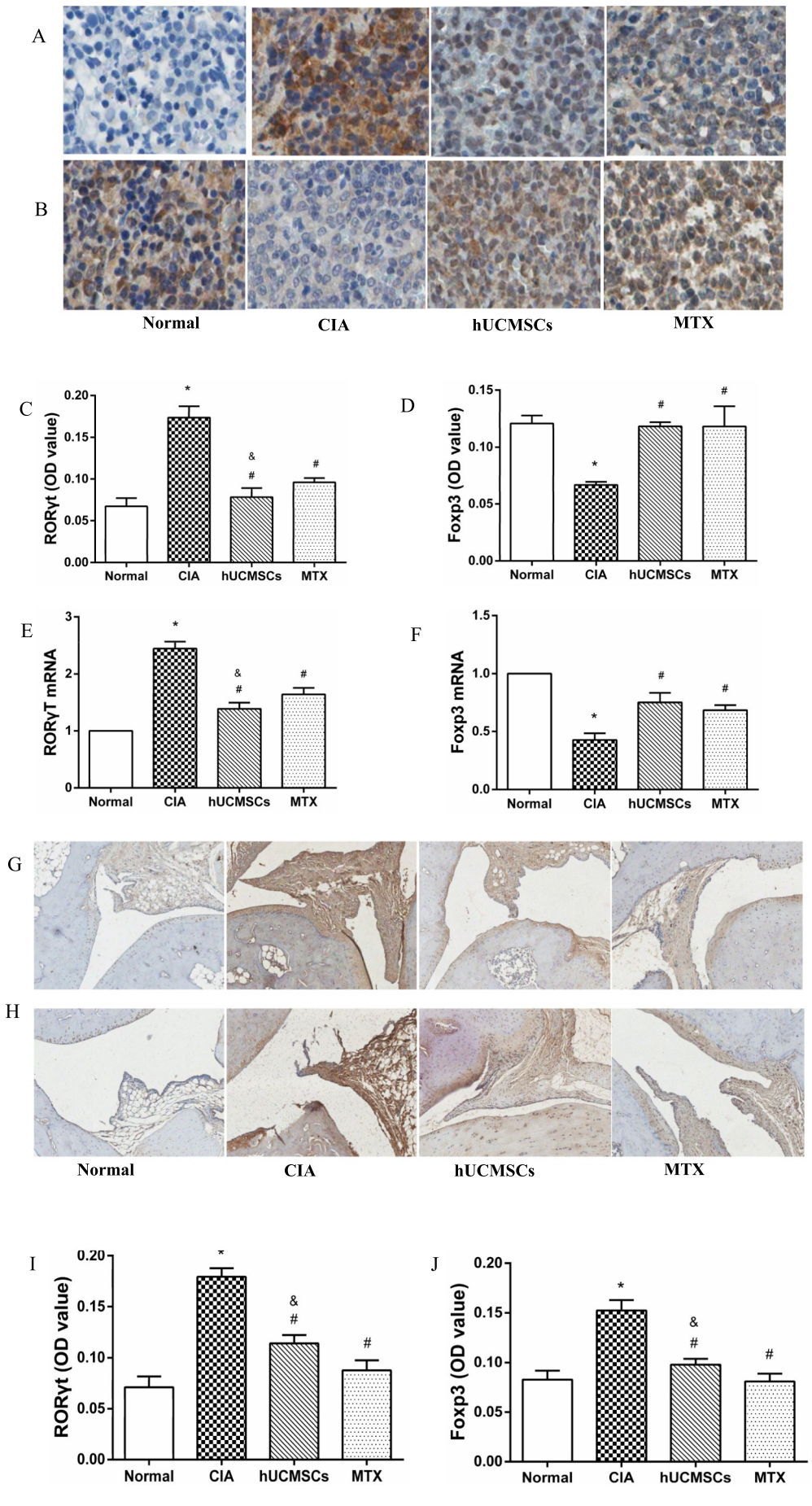

Fig. 4. hUCMSCs transfer therapy affected the expression of Th17- and Treg-related transcription factors in the spleen and joints of rats. Values are given as mean ± SD. *P < 0.05 *vs* normal group. #P < 0.05 *vs* CIA group. &P < 0.05 *vs* MTX group. (A, B) Immunohistochemical results showing staining for RORγt (A) and Foxp3 (B) in the spleen (original magnification, ×200). (C, D) The OD value of RORγt and Foxp3 in the spleen. (E, F) RT-PCR revealing RORγt and Foxp3 mRNA expression in the spleen. (G, H) Immunohistochemical results showing staining for RORγt (G) and Foxp3 (H) in the joints (original magnification, ×40). (I, J) The OD value of RORγt and Foxp3 in the joints.

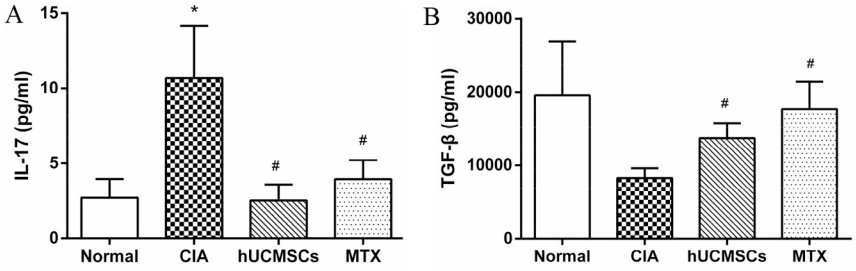

Fig. 5. hUCMSCs transfer therapy influenced Th17- and Treg-related inflammatory factors. Values are given as mean ± SD. *P < 0.05 *vs* normal group. #P < 0.05 *vs* CIA group. (A) Flow cytometry data showing IL-17 expression in the serum. (B) Flow cytometry data showing TGF-β expression in the serum.

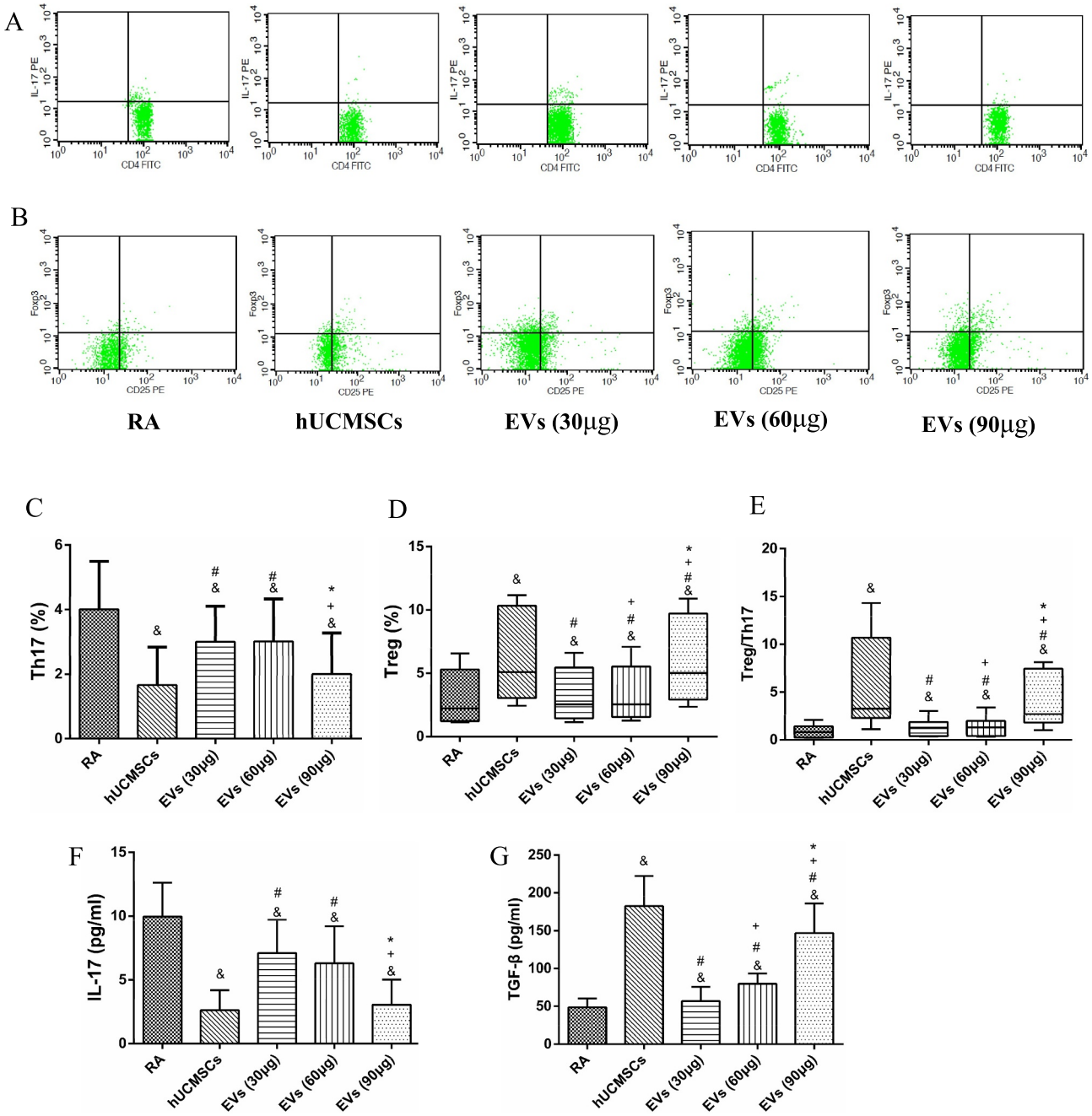

Fig. 6. Immunomodulatory effect of hUCMSCs and EVs on T lymphocytes in RA patients. Values are given as mean ± SD. &P < 0.05 *vs* RA group. #P < 0.05 *vs* hUCMSCs group. +P < 0.05 *vs* EVs (30 μg) group. *P < 0.05 *vs* EVs (60 μg) group. (A, C) Flow cytometry data showing the proportion of Th17 cells in PBMCs. (B, D) The ratio of Treg cells in PBMCs. (E) The Treg/Th17 ratio in PBMCs. (F, G) The expression of IL-17 and TGF-β in the supernatant.

only one study has assessed the immunomodulatory effect of EVs in CIA mice, and no study has evaluated their role in RA patients. It has been observed that EVs inhibit T lymphocyte proliferation, decrease the percentage of $CD4^+$ and $CD8^+$ T cell subsets, and increase Treg cell populations to improve arthritis symptoms in CIA mice [29]. Therefore, this study verified the immunomodulatory effect of hUCMSCs in RA patients and compared the immunomodulatory effect of hUCMSCs with that of the EVs. The result showed that both hUCMSCs and EVs regulated Treg/Th17 imbalance and inflammatory factor secretion in RA patients. Besides, the EVs concentration gradient was set by referring to the literature [29,30], it was found that the effects of EVs were dose-dependent. With respect to decreasing the proportion of Th17 cells and the level of IL-17A, the hUCMSCs group was similar to the EVs (90 μg) group; in case of increasing the proportion of Treg cells, Treg/Th17 ratio, and the level of TGF-β, the hUCMSCs group was superior to the EVs (90 μg) group. However, it is necessary to explore the optimal concentration of EVs, and need a large number of animals and clinical trials to further study its mechanism, efficiency and safety. This study demonstrated that hUCMSCs and hUCMSCs as seed source secreted EVs played an immunomodulatory effect in RA patients and may become a new treatment strategy for RA.

In addition, this study compared the therapeutic and immunomodulatory effects of hUCMSCs with those of MTX, a classical drug for RA, in CIA rats. The results showed that the therapeutic effect of hUCMSCs is similar to that of MTX; hUCMSCs ability to downregulate RORγt protein and gene expression in spleen is superior to that of MTX; In addition, similar to MTX, hUCMSC promoted T cell apoptosis, upregulated Treg cells ratio and Treg/Th17, upregulated Foxp3 protein and gene expression in spleen, inhibited the expression of IL-17, and promoted TGF-β in serum; Further, hUCMSC ability to inhibit T cell proliferation, downregulate the proportion of Th17 cells in spleen, and downregulate RORγt and Foxp3 in joints is slightly weaker than that of MTX. Moreover, hUCMSCs downregulated RORγt expression better than MTX did, but MTX downregulated the proportion of Th17 cells in spleen better than hUCMSCs did, which may be due to other transcription factors, such as Runt-related transcription factor 1 (RUNX-1) and aryl hydrocarbon receptor (Ahr), which promote differentiation of Th17 cells [3]. hUCMSCs ability to downregulate the proportion of Th17 cells in spleen was slightly more weaker than that of MTX, but its ability to inhibit IL-17 secretion by Th17 cells was similar to that of MTX; this needs to be further evaluated in animal samples and confirmed in the same tissue samples. hUCMSCs were similar to or weaker than MTX in some aspects of their immunomodulatory effects, which may be related to the number of injected cells and times of administration; this requires further studies to explore appropriate dosages and administration times. In addition, hUCMSCs have a beneficial effect on damaged joint repair due to their multi-differentiation ability, and thus may have a broader application prospect than MTX in RA treatment.

In conclusion, this study, from the perspectives of peripheral blood, immune organs, and inflamed joints, confirmed that hUCMSCs regulated T cell proliferation, apoptosis, and differentiation at the transcriptional level and T lymphocyte associated inflammatory factors *in vivo* and vitro to play an immunoregulatory role, ultimately providing a theoretical foundation for the clinical transformation and application of hUCMSCs in the treatment of RA.

Abbreviations

RA	Rheumatoid arthritis
hUCMSCs	human umbilical cord mesenchymal stem cells
CIA	collagen-induced arthritis
IL	interleukin
TGF-β	transforming growth factor-β
Th17	T helper cell 17
Treg	regulator T cell
RORγt	retinoic acid receptor-related orphan nuclear receptor-γT
Foxp3	forkhead or winged helix transcription 3
DMARDs	disease modified antirheumatic drugs
MTX	methotrexate
FITC	fluorescein isothiocyanate
DAB	diaminobenzidine
OD	optical density
GADPH	glyceraldehyde-3-phosphate dehydrogenase
IFN-γ	interferon-γ
MMP	matrix metalloproteinase
RUNX	runt-related transcription factor
Ahr	aryl hydrocarbon receptor

Ethics approval and consent to participate

The study was approved by the ethical committee of Shanxi Dayi Hospital. All experimental procedures were performed according to the Institutional Animal Care and Use Committee guidelines of Shanxi Medical University.

Consent for publication

Not applicable.

Availability of data and materials

The dataset supporting the conclusions of this manuscript is included within the manuscript.

Funding

This work was supported by the National Natural Science Foundation of China Grants (81202356, 81771768), the applied basic research project of Shanxi Science and Technology Department (201803D31136, 201801D221381).

Authors' contributions

LYZ directed the research. DM designed the research, performed the experiments, analyzed and interpreted data and drafted the manuscript. KX participated in the design and revised the manuscript. YL, MT and CW performed the experiments. GLZ, JFG and JL participated in the coordination of experimental work. All authors have read and approved the manuscript.

Declaration of Competing Interest

The authors declare that they have no competing interests.

Acknowledgements

The authors thank teachers in translational medicine research center of Shanxi Medical University for excellent technical assistance.

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