The expression of Th9 and Th22 cells in rats with cerebral palsy after hUC-MSC transplantation

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Abstract

Background: This study aimed to investigate the expression of Th9 and Th22 cells in rats with cerebral palsy (CP) after human umbilical cord-derived mesenchymal stem cell (hUC-MSC) transplantation.

Methods: First, hUC-MSCs were isolated from fresh umbilical cords and identified. Rats were divided into the normal group, CP group, and hUC-MSC transplantation group. The Morris water maze and balance beam tests were performed to evaluate the neurobehavioral ability of the rats. The levels of TNF-α, IL-6, IL-9, and IL-22 in rat brain tissues were detected by ELISA. Th9 and Th22 proportions in brain tissues were detected by flow cytometric analysis. The mRNA levels of IL-9, IL-22, PU.1, and AHR in brain tissues were determined by qRT-PCR.

Results: hUC-MSC transplantation enhanced the neurobehavioral ability of CP rats. Furthermore, Th9 and Th22 proportions were decreased in brain tissues from CP rats after hUC-MSC transplantation. The levels of proinflammatory cytokines (TNF-α and IL-6), Th9-related IL-9 and PU.1, and Th22-related IL-22 and AHR were markedly higher in brain tissues from CP rats than in brain tissues from control rats, but their levels were significantly decreased after hUC-MSC transplantation.

Conclusion: Our data indicate that Th9 and Th22 proportions are decreased in CP rats after hUC-MSC transplantation.

Keywords: Cerebral palsy; hUC-MSC transplantation; Th9; Th22

1. INTRODUCTION

Cerebral palsy (CP) is considered as a permanent disorder of movement and posture, and it causes activity limitation that results from nonprogressive damage to the developing brain.[1] CP dyskinesia is often accompanied by disturbances in sensation, perception, cognition, communication, and behavior and by secondary musculoskeletal problems.[2] The overall prevalence of CP, 2 to 3 cases per 1000 live births, has remained stable over the past 40 years.[3] Despite changes in antenatal and perinatal care, the childhood incidence of CP has risen in recent years.[4,5] There is no cure and few disease modifying interventions for CP and symptomatic treatment is the primary means of therapy. CP is one of the most serious diseases that cause childhood disability, and it presents a heavy spiritual and material burden to families and society. Therefore, there is an urgent need to identify predictive biomarkers to develop better therapies for CP treatment.

An increasing number of studies have indicated that intrauterine infection and hypoxic-ischemic brain injury, both of which contribute to CP through the inflammatory response mechanism, are the main pathogenic factors of CP.[6] Numerous cytokines are activated and interact with each other during the inflammatory process to participate in regulatory pathological and physiological processes, which are common pathways that underlie brain injury resulting from various pathogenic factors. Inflammatory reactions are always accompanied by immune function disorders, including an imbalance in helper T (Th) cell subgroups and the abnormal secretion of Th cell-related cytokines. Interleukin (IL)-9-producing CD4+ T helper (Th9) cells and IL-22-producing CD4+ T helper (Th22) cells are members of the most recently identified subsets of T helper cells. It is now clear that Th9 and Th22 cells play critical roles in immune-mediated diseases by secreting their respective signature cytokines, IL-9, and IL-22.[7,8] Th9 cells express the transcription factor PU.1, and Th22 cells express the transcription factor AHR.[9] Recently, emerging evidence has shown associations between these cells and some diseases, including type 1 diabetes,[10] lupus nephritis,[11] psoriasis,[12,13] and ulcerative colitis.[14] However, the expression and function of Th9 and Th22 cells in CP remain unclear.

Human umbilical cord-derived mesenchymal stem cells (hUC-MSCs) are a group of multipotent stem cells with significant self-renewing capacities and multi-lineage differentiation properties.[15,16] Since 2009, the therapeutic potential of the allogeneic transplantation of UC-MSCs has been under investigation.[17] Huajiang Dong et al revealed that prior treatment with UC-MSCs leads to better patient outcomes, suggesting that UC-MSC transplantation is a promising therapy for CP.[18]

In the present study, we aimed to explore the effects of Th9 and Th22 cells and the expression of related cytokines in rats with CP after hUC-MSC transplantation.

2. METHODS

2.1. Animals and CP model

Female Sprague-Dawley rats (10 to 15 g, 7 days old) were obtained from the Experimental Animal Centre of The First

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Affiliated Hospital of Anhui Medical University. The rats were caged individually in a temperature-controlled room (22°C ± 2°C) with 50% to 60% humidity and a 12-h/12-h light-dark cycle. Food and water were freely available during all stages of the experiment.

The induction of CP in rats was performed using unilateral carotid artery occlusion.[19] The rats were placed in the supine position after inhalational general anesthesia, a midline incision was made in the neck, the left common carotid artery was ligated, and the skin incision was sutured. The animals were allowed to recover from anesthesia before they were returned to their home cages. They were then exposed to hypoxic conditions (8% O_2 and 92% N_2) at 37°C for 150 minutes. Soda lime was used in the bottom of the cage to absorb moisture and CO_2. Then, the pups were returned to their cages and fed by female rats. The animal use protocol was reviewed and approved by the Institutional Animal Care and Use Committee of The First Affiliated Hospital of Anhui Medical University.

2.2. Culture and identification of hUC-MSCs

All of the healthy umbilical cord-donating puerperae were recruited from the Department of Obstetrics, The First Affiliated Hospital of Anhui Medical University, and all had negative scores on the hepatitis B-related 5-item examination and were negative for HCV, HIV, syphilis, and other related infectious diseases. The donors and their families were all informed of the purpose and content of the study, signed informed consent, and voluntarily donated umbilical cords. The procedure was approved by the ethical committee of The First Affiliated Hospital of Anhui Medical University.

The umbilical cord samples were rinsed with PBS containing 100 U penicillin/streptomycin, and the umbilical artery and vein were removed. The samples were then cut into approximately 1-mm^3 pieces and inoculated in Dulbecco's modified Eagle media/F12 (DMEM/F12; Gibco Invitrogen, Carlsbad, CA, USA) supplemented with 20% FBS (Gibco), 100 U/mL penicillin, and 100 U/mL streptomycin. The tissue suspension culture system was left in a 37°C incubator with 5% CO_2 for 7 days. The culture medium was replaced every 3 days. The hUC-MSCs were passaged when they reached 80% confluence by using 0.25% trypsin-EDTA solution. Flow cytometry was used to analyze the cell surface antigen markers of the hUC-MSCs as previously described.[20]

2.3. hUC-MSC transplantation

Seven days after CP model establishment, stereotactic agents were injected into the left sensorimotor cortex of the rats. The rats were fixed on a stereotaxic apparatus, and the transplantation site was located in the left sensorimotor cortex (coordinates, AP: −0.3 mm, ML: −2 mm, DV: −1.5 mm [corresponding to 0.3 mm posterior to bregma, 2 mm lateral to bregma, and a depth of 1.5 mm, respectively]).[21] Then, 10 μL of hUC-MSCs (2×10^6 cells/rat) was injected with a vertical microneedle. The injection process took more than 5 minutes, and the needle was fixed in place for 5 minutes after the injection. The scalp was sutured, and the rats were returned to their cages after rewarming.

2.4. Morris water maze

Four weeks after hUC-MSC transplantation, the cognitive performance of the rats was assessed by the Morris water maze (MWM) test as previously described.[22] The apparatus consisted of a circular pool (height: 40 cm, diameter: 120 cm, four quadrants) filled with water (24°C-26°C), and an escape platform (height: 28 cm, diameter: 10 cm) was submerged approximately 2 cm below the water surface. The rats were trained for five consecutive days with four trials per day. Each rat was allowed to swim for up to 60 seconds until it reached the escape platform, and the intertrial interval was 60 seconds, during which the rat remained on the escape platform. If the rat did not find the platform within the allowed time, it was guided to the platform by the observer. In each trial, the latency to escape onto the hidden platform was recorded by the observer. The movement of each rat was recorded with a computer program. The lighting conditions, environmental arrangement, and noise level of the laboratory were maintained throughout the entire test.

2.5. Balance beam test

The motor functions of these rats were assessed by beam walking and footprint analyses after the completion of the MWM test. The rats were trained to traverse a cylindrical beam with a length of 200 cm and a diameter of 2.5 cm. A black platform (7.0 cm × 4.0 cm) was positioned at one end of the beam at the starting point, and a black plastic box (15 cm × 15 cm × 8.0 cm) was placed at the other end as an incentive to traverse the beam. The time taken to traverse the beam was recorded using a stopwatch and was defined as the time from when the rat extended a forepaw from the first platform onto the beam until the rat had crossed the beam to the second platform and extended a forepaw into the black box. At each time point, the analyses were repeated three times.

2.6. Tissue preparation

The rats were euthanized after the balance beam test. The rats were anaesthetized with pentobarbital (Sigma) (50 mg/kg). The brain tissues were immediately frozen in liquid nitrogen. Frozen tissues were homogenized in PBS containing protease inhibitors (Mini-Complete; Roche). The supernatants were collected after centrifugation for further analysis.

2.7. Immunofluorescence staining

Transplanted hUC-MSCs were identified by immunofluorescence. One and 4 weeks after hUC-MSC transplantation, rat brain sections were incubated with a primary antibody against human nuclei (MAB1281; 1:125; Chemicon, Temecula, CA, USA) overnight at 4°C, followed by incubation with PE-labelled secondary antibody IgG (1:100; Santa Cruz Biotechnology, Dallas, TX, USA). The sections were counterstained with DAPI and then sealed and photographed under a fluorescence microscope (Nikon Corporation, Tokyo, Japan).

2.8. Brain leukocyte isolation and flow cytometry analysis

Mononuclear cells were isolated from rat brains as previously described.[23] Briefly, brain tissues were dissected and minced finely in RPMI 1640 and digested in 0.0625% trypsin (in Ca^{2+}/Mg^{2+}-free HBSS) for 20 minutes at room temperature. Single-cell preparations of the brains were suspended in 30% Percoll and banded on a 70% Percoll cushion at 900 × g at 15°C. Brain leukocytes obtained from the 30% to 70% Percoll interface were collected.

Following the preparation of single-cell suspensions, Th9 and Th22 cell proportions were evaluated. In brief, the supernatants were incubated with FITC-conjugated anti-human CD4 (eBioscience, San Diego, CA, USA) at room temperature for 20 minutes. After surface staining, intracellular staining for IL-22 (IgG1, Clone 142928, R&D, USA) and IL-9 (IgG1, Clone MH9A3, BD Pharmingen, USA) was performed with ready-to-use buffers according to the manufacturer's suggestions (BioLegend, USA). The expression of cell surface and intracellular markers was assessed using flow cytometry (LSRII, Becton–Dickinson, USA) after gating on live cells determined by scatter characteristics. Isotype controls were used to confirm appropriate compensation and antibody specificity. The data were analyzed using FlowJo software (Tree Star Inc. San Carlos, CA, USA).

2.9. ELISA

The levels of IL-6, TNF-α, IL-9, and IL-22 were measured using commercial ELISA kits (Abcam, Cambridge, UK) according to the manufacturer's instructions.

2.10. QRT-PCR

Total RNA was extracted from tissue supernatant with TRIzol (Invitrogen, Carlsbad, CA, USA). Reverse transcription was carried out using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). cDNA was subjected to real-time PCR through the use of Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. The results were calculated with the $2^{-\Delta\Delta Ct}$ method. The primers used in this study are as follows: IL-9, forward: 5′-CCAGGACCGCTATGACAGG-3′, reverse: 5′-GGACCCCAGAGTGTACTCCA-3′; IL-22, forward: 5′-GCTAAGGAGG CTAG CTTG-3′, reverse: 5′- CAGCA AA TCCAGTTCTCC-3′; AHR, forward: 5′-CTTCCAA GCGG CA TAGAGAC-3′, reverse: 5′-AGTT ATCC TGGCCT CCGTTT-3′; PU.1, forward: 5′-CCAGCT CAGATGA GGAGGAG-3′, reverse: 5′-CAGGTC CAACAGG AACTGGT-3′.

2.11. Statistical analysis

Statistical analysis of the data was carried out using SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA). All data are presented as the mean ± SD. Student's *t* test was used to analyze differences between two groups. One-way analysis of variance was used for multisample analysis. Differences at $p < 0.05$ were considered significant.

3. RESULTS

3.1. Morphologic and immunophenotypic characteristics of hUC-MSCs

hUC-MSCs isolated from fresh umbilical cords were cultured and identified. hUC-MSCs were observed migrating out from mesenchymal Wharton's jelly fragments approximately 1 week after isolation. After approximately 10 days of culture, the cells reached confluency, and the cells presented homogeneous spindle-shape morphology after 15 days of culture. After 2 to 3 passages, the cells displayed fibroblast-like morphology (Fig. 1A). Flow cytometry was performed to analyze the surface antigens of the hUC-MSCs, and the results showed that the cells in this study did not express the hematopoietic cell markers CD34 and

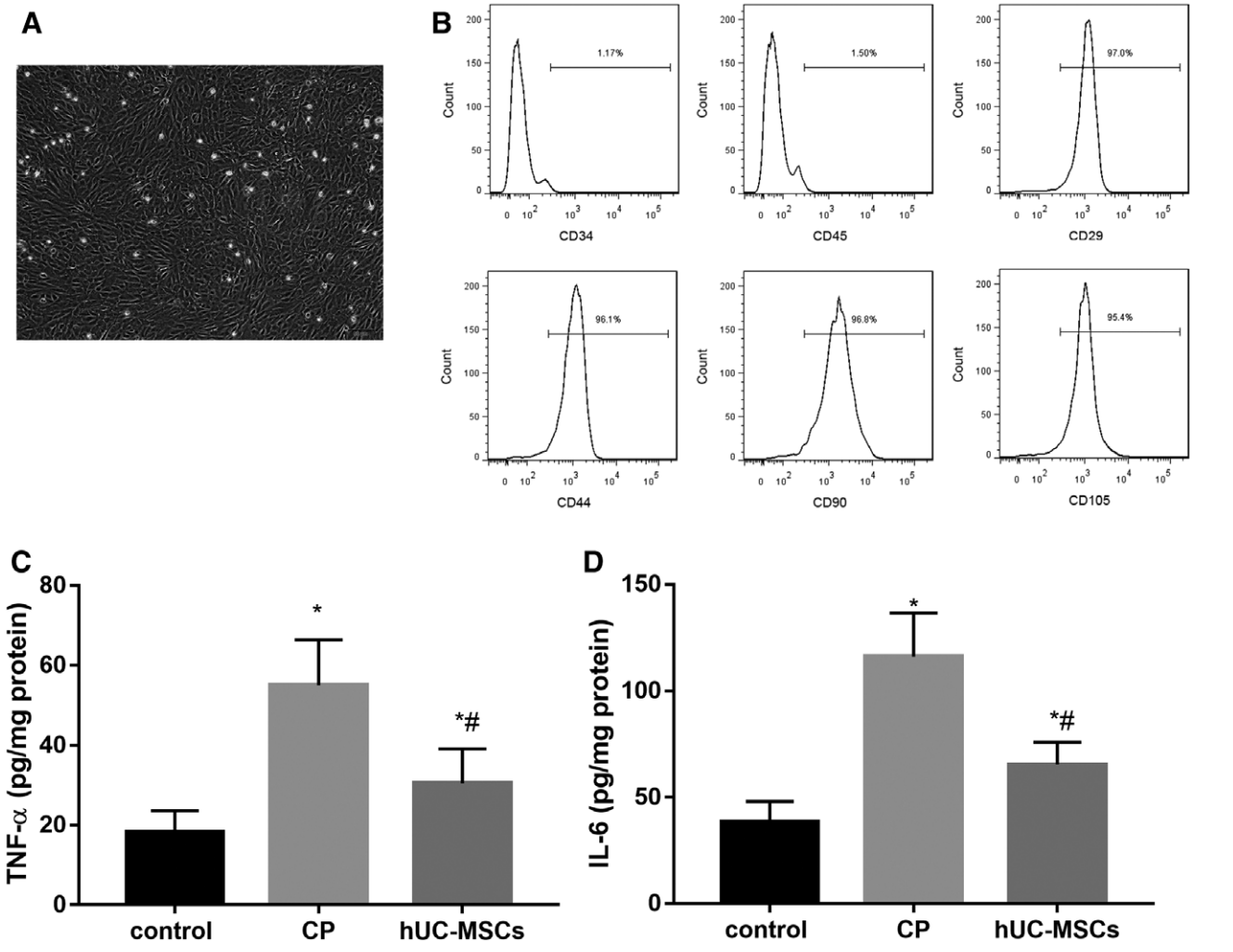

Fig. 1 The levels of inflammatory factors were decreased in CP rats after hUC-MSC transplantation. A, Morphologic characteristics of hUC-MSCs. B, Flow cytometric analysis of hUC-MSC surface biomarkers. C, TNF-α levels in brain tissues were detected by ELISA. D, IL-6 levels in brain tissues were detected by ELISA, n = 12; *$p < 0.05$ vs control; #$p < 0.05$ vs CP. CP, cerebral palsy; hUC-MSC, human umbilical cord-derived mesenchymal stem cell.

Table 1

Time spent to escape in the Morris water maze for rats at 4 weeks after hUC-MSC transplantation (s)

Group	Day 1	Day 2	Day 3	Day 4	Day 5
Control	23.12 ± 4.16	11.45 ± 2.89	8.35 ± 1.98	6.28 ± 1.69	5.57 ± 1.52
CP	38.55 ± 7.97*	19.85 ± 3.34*	14.86 ± 3.21*	11.79 ± 2.25*	8.89 ± 2.78*
hUC-MSCs	29.45 ± 4.98*,**	15.78 ± 2.76*,**	11.12 ± 2.25*,**	8.86 ± 1.87*,**	6.19 ± 2.51**

*$p < 0.05$ vs control; **$p < 0.05$ vs CP.
CP = cerebral palsy; hUC-MSC = human umbilical cord-derived mesenchymal stem cell.

Table 2

Balance beam results at 4 weeks after UC-MSC transplantation

Group	Time to pass (s)	Footslip number
Control	3.15 ± 0.38	0.26 ± 0.25
CP	9.45 ± 2.41*	1.42 ± 0.27*
hUC-MSCs	5.46 ± 1.84*,**	0.74 ± 0.26*,**

* $p < 0.05$ vs control; **$p < 0.05$ vs CP.
CP = cerebral palsy; hUC-MSC = human umbilical cord-derived mesenchymal stem cell.

CD45 but highly expressed the stromal cell antigens CD29, CD44, CD90, and CD105, which is consistent with hUC-MSC characteristics (Fig. 1B). As shown in Supplementary Fig. 1, one week after the transplantation of hUC-MSCs, MAB1281-labelled nuclei (red) were observed in the brain tissues, indicating that transplanted hUC-MSCs colonized the brain tissue and survived. Four weeks after hUC-MSC transplantation, the number of MAB1281-labelled nuclei was reduced, indicating that hUC-MSCs may gradually differentiate over time.

3.2. hUC-MSC transplantation enhanced the neurobehavioral ability of rats with CP

To investigate the effect of hUC-MSC transplantation in CP, the MWM and balance beam tests were performed to evaluate the neurobehavioral ability of the rats. SD rats were randomly divided into the normal group, CP model group, and hUC-MSC transplantation group. Four weeks after hUC-MSC transplantation, the rats in each group explored the margin of the pool from the starting point without direction, and then they swam diagonally through the pool to look for the escape platform and reached it. In subsequent experiments, the

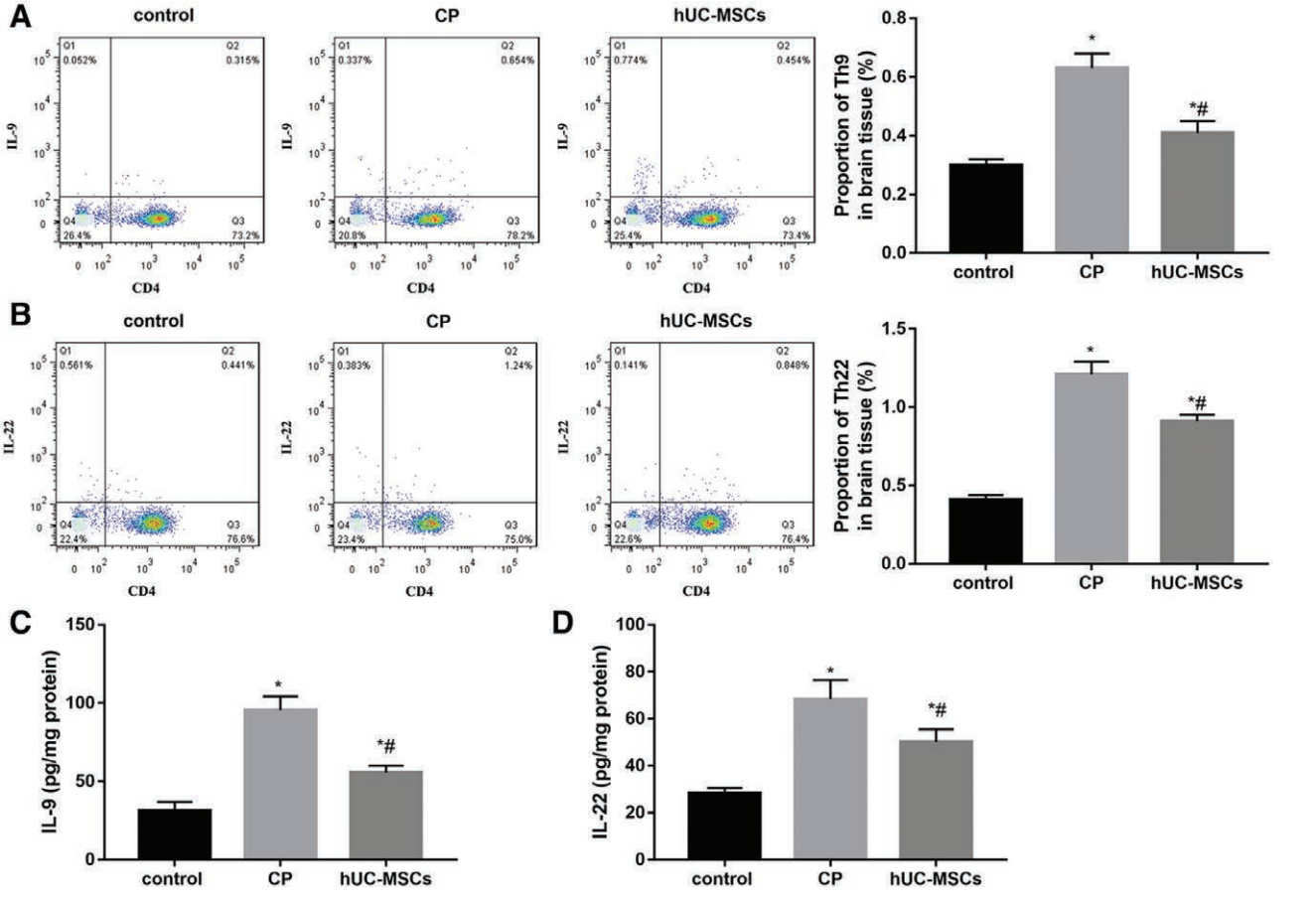

Fig. 2 Decreased Th9 and Th22 proportions in CP rats after hUC-MSC transplantation. A, Th9 proportions in brain tissues were detected by flow cytometric analysis. B, Th22 proportions in brain tissues detected by flow cytometric analysis. C, IL-9 levels in brain tissues were detected by ELISA. D, IL-22 levels in brain tissues were detected by ELISA, n = 12; *$p < 0.05$ vs control; #$p < 0.05$ vs CP. CP, cerebral palsy; hUC-MSC, human umbilical cord-derived mesenchymal stem cell.

number of random swim without direction around the wall was increased in the CP group. The rats in the normal group and transplant group looked for the platform by swimming backwards and in a narrowing circle and quickly reached the platform. The escape latency of each group became shorter as the number of trials increased. The data showed that both the navigational ability and spatial memory of the control and transplant groups were significantly better than those of the CP group ($p < 0.05$) (Table 1). In the balance beam test, the rats were placed on the beam to assess their balance and coordination by measuring the time required to traverse the beam. The CP rats exhibited hesitation and uncertainty in traversing the beam and showed searching behavior. The joints of the bilateral hind limbs lacked flexibility, particularly on the right side, and the interlimb coordination was poor. The time required for the CP rats to traverse the beam was significantly longer than that required for the control and transplant rats, and the number of hindpaw slips was significantly greater than that exhibited by the control and transplant rats ($p < 0.05$) (Table 2). In contrast, for the control and transplant rats, the movement of the hindpaw joints was smooth, and coordination was normal. Additionally, rat brain tissues were collected from each group, and ELISA results indicated that the levels of TNF-α and IL-6 in the brain tissues in the CP group were significantly higher than those in the brain tissues in the control group, but the TNF-α and IL-6 levels in the brain tissues were decreased by hUC-MSC transplantation (Fig. 1C, D). These observations indicated that hUC-MSC transplantation enhanced the neurobehavioral ability of the rats with CP.

3.3. Decreased Th9 and Th22 proportions in CP rats after hUC-MSC transplantation

Quantitative flow cytometric analysis of the Th9 subset in rat brain tissues revealed that the percentage of CD4+IL-9+ cells was higher in the CP group than in the control group but lower than in the hUC-MSC transplantation group compared with the CP group (Fig. 2A). As shown in Fig. 2B, the proportions of Th22 (CD4+ IL-22+) cells were markedly higher in brain tissues from CP model rats than in brain tissues from the control group, and this effect was rescued by hUC-MSC transplantation. Regarding IL-9 and IL-22 levels in the brain tissues, ELISA results showed a similar pattern for the Th9 subset and the Th22 subset (Fig. 2C, D). These data indicated that hUC-MSC transplantation resulted in a reduction in Th9 and Th22 proportions in CP rats.

3.4. Downregulation of IL-9, IL-22, AHR, and PU.1 mRNA levels in CP rats after hUC-MSC transplantation

To further identify the effect of hUC-MSC transplantation on the differentiation of Th9 and Th22 cells, we performed qRT-PCR to determine the mRNA levels of the Th9-related cytokine IL-9, the Th22-related cytokine IL-22, the Th9-specific transcription factor PU.1, and the Th22-specific transcription factor AHR in rat brain tissues. As shown in Fig. 3, the mRNA levels of IL-9, IL-22, AHR, and PU.1 were markedly higher in the CP group than in the control group, but their mRNA levels were significantly decreased in CP rats after hUC-MSC transplantation. Thus, these data further indicated that hUC-MSC transplantation suppressed Th9 and Th22 differentiation.

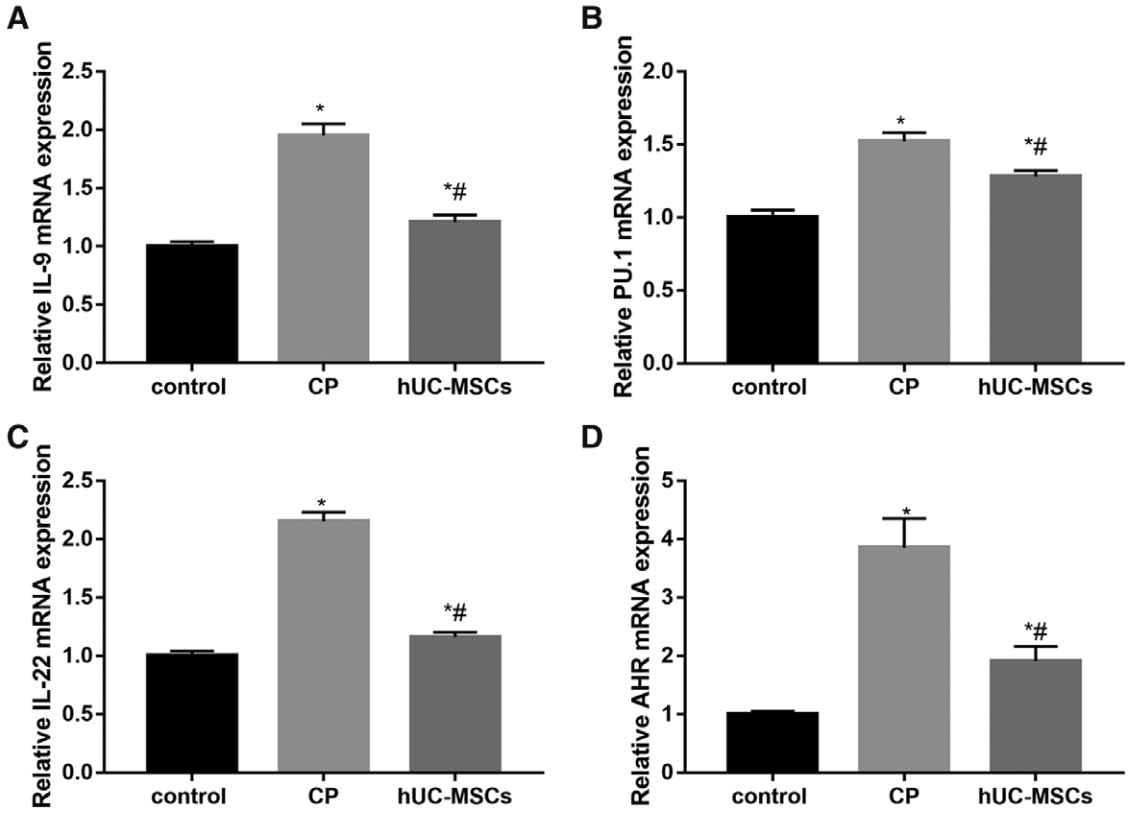

Fig. 3 Downregulation of IL-9, IL-22, AHR, and PU.1 levels in CP rats after hUC-MSC transplantation. qRT-PCR was performed on rat brain tissues from the control, CP, and hUC-MSC transplantation groups. A, IL-9 expression in brain tissues. B, IL-22 expression in brain tissues. C, AHR expression in brain tissues. D, PU.1 expression in brain tissues, n = 12; *$p < 0.05$ vs control; #$p < 0.05$ vs CP. CP, cerebral palsy; hUC-MSC, human umbilical cord-derived mesenchymal stem cell.

4. DISCUSSION

In this study, hUC-MSC transplantation was performed to treat CP rats. The results of the MWM and balance beam tests indicated that hUC-MSC transplantation enhanced the neurobehavioral ability of rats with CP. Further results showed that hUC-MSC transplantation resulted in a reduction in Th9 and Th22 proportions in CP rats.

hUC-MSCs are a class of cells that are easily separated and have a high proliferation rate and a stable genome. They are widely studied for their ability to facilitate the repair of various tissue lesions in vivo and their multipotent differentiation capacity in vitro.[24,25] Recently, increasing attention has been paid to stem cell transplantation and the subsequent directed differentiation of transplanted cells for gene therapy and for improving transplantation efficacy.[26] Our in vivo experiments suggested that hUC-MSC transplantation improves the navigational ability, spatial memory, balance and coordination of CP rats, suggesting that hUC-MSC transplantation is a promising therapy for CP; this finding is consistent with the conclusions of others.[18]

The Th22 and Th9 subsets are recently discovered effector populations that may contribute to autoimmune and inflammatory diseases. Th22 cells are the main producers of IL-22, which has been shown to regulate the pathogenesis of autoimmune diseases.[27] The profound pathogenic Th22 response is observed in chronic inflammatory disorders such as rheumatoid arthritis,[28] inflammatory bowel disease,[29] psoriasis, atopic dermatitis,[30] or multiple sclerosis.[31] Another new T-helper lymphocyte effector subset, Th9, has been identified in patients with some autoimmune as well as inflammatory diseases.[32,33] Th9 cells produce IL-9, which has pleiotropic functions.[34] Our study demonstrated that the activities of Th9 and Th22 were upregulated in rats with CP, and hUC-MSC transplantation further resulted in a reduction in Th9 and Th22 proportions in CP, which may have an impact on the chronic inflammation that contributes to the development of CP.

The mechanism by which transplanted hUC-MSCs reduce the proportions of Th9 and Th22 cells in CP remains unclear. Studies have shown that the transplantation of bone marrow-derived rat MSCs reduces the level of TGF-β in a rat model of contusive spinal cord injury.[35] The activation of the TGF-β/Smad signaling pathway may promote the differentiation of Th9 cells and IL-9 expression.[36] In addition, bone marrow-derived MSCs ameliorate diabetic nephropathy via inhibiting the Notch signaling pathway.[37] It has been reported that the inhibition of Notch signaling decreases the number of Th22 cells and IL-22 production and that this is accompanied by a reduction of the Th22-specific transcriptional factor AHR.[38] Thus, we speculate that the mechanism by which transplanted hUC-MSCs decrease the number of Th9 and Th22 cells may be related to the regulation of cytokines (eg, TGF-β) by hUC-MSCs or signaling pathways (eg, Notch signaling) that modulate Th9 and Th22 differentiation; this speculation requires further investigation.

In conclusion, our data indicate that the proportions of both Th22 cells and Th9 cells are elevated in brain tissues from rats with CP and are decreased after hUC-MSC transplantation. These findings suggest that Th22 cells and Th9 cells may be implicated in the pathogenesis of CP, and Th22 cells and Th9 cells may be reasonable cellular targets for therapeutic intervention.

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APPENDIX A. SUPPLEMENTARY DATA

Supplementary data related to this article can be found at http://doi.org/10.1016/j.jcma.2018.01.013.

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