

Original Article

Therapeutic effect of transplanted umbilical cord mesenchymal stem cells in a cynomolgus monkey model of multiple sclerosis

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Abstract: Multiple sclerosis (MS) is a demyelinating disease affecting 2.5 million young people worldwide because of its immune-mediated pathological mechanisms. Recent studies have shown that stem cell transplantation is a new potential therapy for MS. There has been renewed interest in cell therapy to improve quality of life for MS patients. In this study, the experimental autoimmune encephalomyelitis (EAE) model, which is the most commonly model to mimic MS, was successfully established in cynomolgus monkeys. To evaluate the therapeutic effect of human umbilical cord mesenchymal stem cells (UCMSCs) on MS, we intravenously transplanted UCMSCs into cynomolgus monkeys with EAE. Our results showed that UCMSC transplantation significantly ameliorated the clinical symptoms of MS. Magnetic resonance imaging and clinical signs indicated that demyelination was obviously decreased after UCMSCs therapy. Moreover, the present study showed that the mechanisms, involved in the effects of UCMSCs on MS, included their immunomodulatory functions to regulate cytokine secretion and affect functional differentiation of the T cell lineage.

Keywords: Umbilical cord mesenchymal stem cells, multiple sclerosis, therapeutic effect, cynomolgus monkey

Introduction

Multiple sclerosis (MS) causes the most disability in young adults, which is characterized as a chronic demyelinating disease of the central nervous system (CNS). This type of disease is considered to have an autoimmune etiology, which leads to differential clinical symptoms [1]. Typical pathological signs in the CNS include immune cell infiltration, perivascular inflammation, oligodendrocyte death, and axonal damage [2]. According to the variable progression of MS, four major clinical types are classified including relapsing remitting MS (RRMS), progressive MS (PMS), secondary progressive MS (SPMS), and progressive MS(PPMS) [3]. Over the past few years, MS symptoms have been treated by antibodies or glucocorticoid drugs, which are usually used to relieve acute exacerbations of MS in clinical practice [4].

Numerous studies have shown that mesenchymal stem cells (MSCs) can be used in therapeutic strategies as cell therapy for autoimmune disorders and degenerative diseases [5]. Our previous study showed that umbilical cord mesenchymal stem cells (UCMSCs) prevent the progression of MS patients and improve quality of life [6]. Because of their immunomodulatory and paracrine functions, more clinical trials have used MSC therapies for immune diseases [7, 8], indicating that stem cell transplantation is becoming an accepted treatment for autoimmune diseases including MS. Thus far, stem cells from bone marrow have served as the most frequent management for autoimmune disease. However, the use of stem cells from bone marrow is limited because of the immune status of donors and the quality and quantity of the isolated stem cells [7, 9, 10]. Therefore,

UCMSCs have become a superior choice for MS treatment because of their abilities of multipotent differentiation, immune regulation, and tissue repair [11].

Most recent clinical trials have been carried out in a severe phase of MS. Thus, it is difficult to investigate the primary role of UCMSCs in the initial stage. Therefore, in the present study, we used a non-human primate model of experimental autoimmune encephalomyelitis (EAE), which mimics the early clinical stage, to evaluate the effects of UCMSCs.

EAE has been the most commonly used animal model to explore the mechanisms of immune responses against self-antigens within the CNS and to test new therapies for the treatment of autoimmune diseases [12]. Non-human primate (NHP) models, whose clinicopathological features closely resemble those of MS patients, are more suitable for clinical evaluations than rodent models [13]. By comparing rapidly spontaneous remission during the progression of EAE in rodent models, NHP models can be a reliable choice to evaluate the efficacy MS treatments. The cynomolgus monkey is a neotropical primate species that shares significant immunological similarity with humans [14]. Moreover, it is highly susceptible to EAE, which is inactive in lower species, and offers a valid pre-clinical model to evaluate the efficacy of new therapies for MS. Evaluation of the lesions in the brain and spinal cord can be monitored by magnetic resonance imaging (MRI), which resembles the diagnosis of MS patients, making it useful for preclinical testing of MS therapies [15].

Our results showed that all cynomolgus monkeys immunized with MOG34-56 developed EAE that was confirmed by severe clinical symptoms such as paraplegia and demyelination of the CNS. We investigated the potential therapeutic effects of UCMSCs by examining cytokine secretion and remyelination functions. Moreover, time course differences were analyzed in the T cell lineage.

Material and methods

Isolation and culture of umbilical cord mesenchymal stem cells

This study was approved by the ethics committee of Yan'an Affiliated Hospital of Kunming Medical University. Umbilical cords were obtain-

ed after donors and their families provided informed consent. The umbilical cords were washed with sterile saline at 4°C, drained of blood, and stripped of vessels. Then, the remaining tissue was diced into cubes of about 1 mm³. The minced tissue was transferred to tissue culture dishes (Corning, Lowell, MA, USA) and incubated in 5 ml α -minimum essential medium (α -MEM, Hyclone, AU) supplemented with 20% fetal bovine serum (Hyclone), antibiotics (1% penicillin/streptomycin), and 5 ng/ml basic fibroblast growth factor (Life Technologies, CA, USA). Finally, the tissue was incubated in a humidified incubator containing 5% CO₂ at 37°C for 1-2 weeks. The culture medium was replaced every 3 days. When cells reached 80% confluence and numerous colonies were observed, 0.0625% trypsin was used to detach the cells, followed by subculture. Cells from third passages were employed in experiments.

Peripheral blood mononuclear cell and UCMSC cocultures

Peripheral blood mononuclear cells (PBMCs) were isolated using a Ficoll solution (STEM-CELL, Columbia, Canada), in accordance with the manufacturer's instructions, and cultured in 6-well plates with 10 ng/ml concanavalin A. UCMSCs were cocultured with PBMCs at a ratio of 1:10 (UCMSCs:PBMCs) in transwell plates (Corning Life Science, Costar, USA). α -MEM only was used as a negative control. Supernatants from both chambers were collected and analyzed for cytokines.

Flow cytometry

The surface markers of UCMSCs were stained and characterized by flow cytometry. Firstly, cells were prepared as a 1×10^6 /mL single cell suspension, washed with PBS twice, and resuspended in staining buffer (BD, San Diego, CA). The cells were stained with specific antibodies at the recommended concentration at room temperature for 30 min in the dark. Subsequently, the cells were washed once with 1 ml PBS and centrifuged at $2000 \times g$ for 3 min. Then, 100 μ l PBS was added to the cells, and they were analyzed by an FC500 flow cytometer (Beckman Coulter). The antibodies were PE-conjugated anti-CD34, PerCP-conjugated anti-CD45, FITC-conjugated anti-CD123, PE-conjugated HLA-DR, PE-conjugated anti-CD44, FITC-conjugated anti-CD49, PerCP-conjugated anti-CD29, FITC-conjugated anti-CD90, PerCP-conjugated anti-CD271, PE-conjugated anti-CD73,

Table 1. Information of the individual cynomolgus monkeys used in this study

Monkey	Animal ID	Sex ^a	Age ^b	Weight ^c	Original country
C1	140288	F	3.1	3.88	China
C2	140212	F	3.2	3.76	China
C3	140250	F	3.2	3.90	China
C4	130322	F	4.1	4.57	China
C5	130909	F	3.5	4.03	China
C6	120122	F	5.2	4.45	China
C7	130113	F	4.3	3.9	China
C8	140306	F	3.1	4.02	China
C9	120920	F	4.4	4.50	China

^aF, female. ^bAge in years at the beginning of the study.^cWeight in kilograms at the beginning of the study.

PE conjugated anti-CD166, and FITC-conjugated anti-CD105.

The same method was used to analyze surface markers of PBMCs. The following antibodies were used: CD3-FITC, CD4-PE, CD8-PerCp-Cy5.5, CD16-PerCp-Cy5.5, CD56-PE, and CD25-PerCp. Isotype-matched IgG served as the control. All antibodies were purchased from BD Biosciences (San Diego, CA). Cells were fixed and permeabilized using a Fix/Perm Kit (BD Bioscience), in accordance with the manufacturer's instructions, and stained for intracellular markers using anti-Foxp3-PE, IL17-FITC, and IFN- γ -PE antibodies. Isotype antibodies were used in accordance with the manufacturer's instructions.

EAE induction and UCMSC transplantation

All procedures involving animals were performed in accordance with the institutional guidelines for animal care of the Kunming Institute of Biology. Specific information of the female cynomolgus monkeys in our study is listed in **Table 1**. The MOG34-56 peptide (GMEVGYRPPFSRVVHLYRNGRD) synthesized by Qiangyao Biotechnology (Suzhou, China) was used to induce the EAE model. Nine cynomolgus monkeys were numbered from C1 to C9. The monkeys underwent EAE model establishment ($n = 6$). C7-C9 were used as the control group ($n = 3$). C1-C6 underwent subcutaneous injection with an emulsifier containing 200 μ g MOG34-56 in 300 μ l PBS, which was completely emulsified in 300 μ l incomplete found adjuvant (Sigma-Aldrich, Shanghai, China). C7-C9 were injected with the same dosage of emulsifier without MOG34-56. All cynomolgus monkeys

were monitored daily and evaluated for disease progression, according to the following scoring criteria (0 = no clinical signs; 0.5 = apathy, loss of appetite, and an altered walking pattern without ataxia; 1 = lethargy, anorexia, tail paralysis, and tremor; 2 = ataxia and optic disease; 2.5 = para- or mono-paresis, sensory loss, and brain stem syndrome; 3 = para- or hemi-plegia; 4 = quadriplegia; 5 = spontaneous death attributable to EAE [3, 16, 17]. Subsequent booster immunizations were conducted at 7-day intervals with the same dosage. The immunizations were administered four times until the cynomolgus monkeys had obvious clinical signs of EAE (score ≥ 2). The monkeys were scored twice a day. Their status was remotely monitored during experimental period by trained observers through web cameras installed in cages. At days 74 and 84, UCMSC suspensions were prepared in PBS and intravenously injected into each monkey according to their weight (1×10^6 cells/kg/ml). The cell suspension was carefully syringed with gentle agitation to avoid bubbles and foam.

Magnetic resonance imaging

MRI scans were operated by a 3.0T SIEMENS Magnetom version equipped with an 8-channel head phased array coil and maximum single-axis gradient field strength of ≥ 45 mT/m. Cynomolgus monkeys were anaesthetized with ketamine (30 mg/kg i.m., Sigma) and arranged for medical ventilation. A lower limb vein was prepared for injection of gadopentetate meglumine. Each monkey was fixed in a child holder and placed in a child cradle that interpolated to the nuclear MR spectrometer. During the inspection, the animals were anesthetized with isoflurane (1.5%-2.0%) in N_2O/O_2 (70/30). Body temperature was maintained at 37°C by an air conditioner.

Conditions for scans of the brain were T1W: FL2D sequence, TR/TE: 300 ms/2.52 ms, FOV read: 200 mm, layer thickness: 3 mm, and scanning times: 1 min and 58 s; T2W: TSE order column, TR/TE: 3000 ms/92 ms, layer thickness: 3 mm, and scanning times: 2 min and 44 s; FLAIR: TR/TE: 9000 ms/81 ms, layer thickness: 3 mm, and scanning times: 4 min and 32 s; DWI: TR/TE 4500 ms/94 ms, layer thickness: 3 mm, and scanning time: 59 s.

Histology

Cynomolgus monkeys were sacrificed by an overdose of ketamine and perfused transcardi-

ally with saline, followed by a fixative containing 4% of 0.1 M sodium phosphate-buffered paraformaldehyde. Histology included staining with hematoxylin and eosin (H&E), Luxol Fast blue (LFB), and Bielschowsky stain, and GFAP immunohistochemistry. The cryosections were washed with PBS and incubated with a primary rabbit anti-GFAP (Abcam, Honkong, China) overnight at 4°C. For visualization, the sections were incubated with a goat anti-rabbit antibody (Abcam, Hong Kong, China). Nuclei were counterstained with 4'-6-diamidino-2-phenylindole (Abcam). Images were acquired by digital sight DS-Ri1 (Nikon).

Evaluation of cytokines in culture supernatants and plasma

Plasma was collected at four time points. "BFT" was before treatment on day 72 when the MRI results showed that the EAE model was established successfully. "AFT" was after treatment on day 130, and "WT" was without treatment. The levels of cytokines and chemokines in co-culture supernatants from PBMCs of EAE cynomolgus monkeys treated with UCMSCs were quantified by the MILLIPLEX MAP Non-Human Primate Cytokine Magnetic Bead 96 well plate assay and PCYTMG-40 K-PX23 Cytokine-Chemokine Array kit (Millipore, Billerica, MA), following the manufacturer's instructions. Cytokines in plasma from monkeys were detected by the same panel, including G-CSF, GM-CSF, IFN γ , IL-1 β , IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12/23 (p40), IL-13, IL-15, IL-17A, MCP-1, MIP-1 β , MIP-1 α , sD40L, TGF- α , TNF- α , VEGF, and IL-18. The multiplex plate was read using the Bio-Plex 200 Suspension Array Luminex System (Bio-Rad, Hercules, CA, USA). Firstly, 96-well plates were washed with 50 μ L assay buffer. All washes were discarded with blotting on paper towels. Next, the samples were added as duplicates to the plate with 25 μ L pre-mixed antibody-immobilized beads. Then, the plates were incubated on a shaker at room temperature for 1 h. After incubation, the liquid was removed, and the plate was washed with 200 μ L wash buffer twice. Then, 25 μ L anti-human kappa and lambda-phycoerythrin was added to each well, followed by incubation at room temperature for 1 h. Finally, the liquid was removed, and 150 μ L sheath fluid was added to each well. The beads were briefly resuspended on a shaker. Data were obtained and analyzed by a

MAGPIX instrument (Millipore) and Milliplex Analyst software, respectively (version 5.1).

RT-qPCR analysis

Total RNA from purified PBMCs and CNS lesions was extracted by an RNeasy mini kit (Qiagen S.A., Courtaboeuf, France). RNA (500 ng) was reverse transcribed using GoScript reverse transcription mix (Promega, Madison, USA). Quantitative PCR was performed employing EVA Green Supermix (Bio rad, CA, USA). The expression level of each gene was normalized to β -actin expression. Specific primers for IFN- γ , IL-17A, GM-CSF, IL-4, IL-10, T-bet, ROR γ t, and Foxp3 were designed using Primer5 software. For quantification, values were expressed as the relative mRNA level of specific gene expression obtained using the 2- Δ Ct method.

Statistical analysis

Experimental data are presented as the mean $\pm$ SEM of three independent experiments. Statistical analysis of multiple groups was performed by Kruskal-Wallis one-way analysis of variance. Experimental data with two groups were analyzed for statistical significance by the unpaired t-test, unpaired, two-tailed, Student's t-tests, and two-way ANOVA. Statistical significance is indicated as *P < 0.05, **P < 0.01, and ***P < 0.001. Analyses were performed using GraphPad Prism6 software.

Results

The EAE model in the cynomolgus monkey is clinically homogeneous

The EAE model in cynomolgus monkey was successfully established by MOG34-56 containing the dominant encephalitogenic T cell epitope on days 0 and at 28 days (**Figure 1A**). The model developed the monophasic disease after four immunizations. The clinical score was up to 4 in MOG34-5-treated monkeys. MRI scans showed that monkeys treated with MOG34-56 developed obvious demyelination foci in their brain and spinal cord. The typical markers of the hyperintense lesion were observed in T2-weighted images of the periventricular area. In four continuous sections, demyelination in the cortical white matter, corpus callosum, and subpial white matter were the most severe lesions (**Figure 1C**). In terms of pathology, de-

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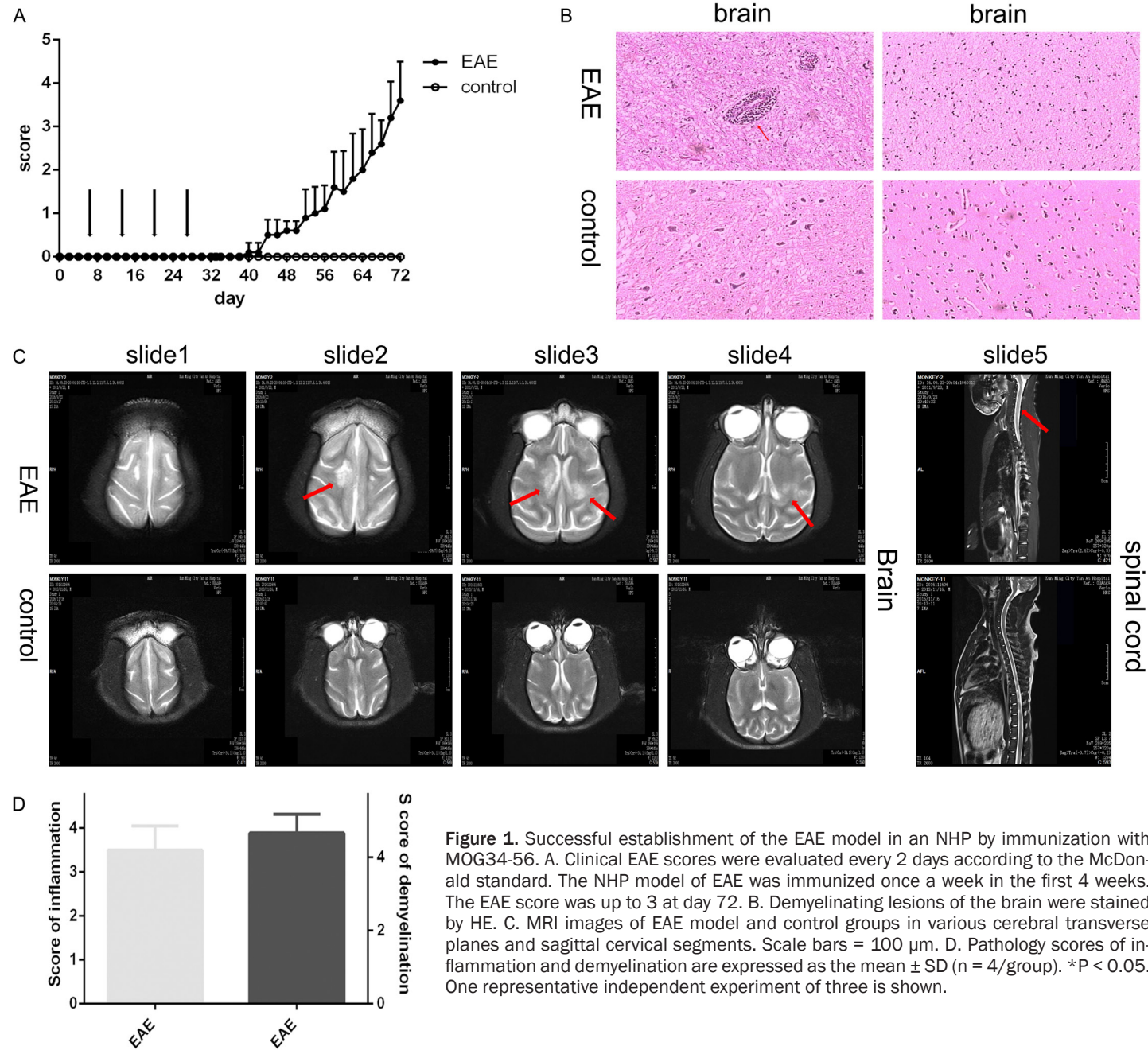


Figure 1. Successful establishment of the EAE model in an NHP by immunization with MOG34-56. **A.** Clinical EAE scores were evaluated every 2 days according to the McDonald standard. The NHP model of EAE was immunized once a week in the first 4 weeks. The EAE score was up to 3 at day 72. **B.** Demyelinating lesions of the brain were stained by HE. **C.** MRI images of EAE model and control groups in various cerebral transverse planes and sagittal cervical segments. Scale bars = 100 μ m. **D.** Pathology scores of inflammation and demyelination are expressed as the mean $\pm$ SD ($n = 4$ /group). * $P < 0.05$. One representative independent experiment of three is shown.

myelination plaques infiltrated by inflammatory cells including many myelin-laden neutrophils and macrophages according to H&E staining (**Figure 1B**). The characteristic pathological lesion contained profound perivascular infiltrates consisting of macrophages and neutrophils (**Figure 1B**). The lesions in the CNS showed perivascular inflammation and variable demyelination. In view of the above results, the model was successfully established and could be used to evaluate UCMSC therapy.

UCMSCs suppress CNS inflammation and demyelination

Among C1-C6 monkeys with EAE, three of them were treated with UCMSCs on days 74 and 84. The ability of UCMSCs to differentiate into osteoblasts, adipocytes, and nerve cells is shown in **Figure 2A**. Cells were positive for CD29, CD44, CD90, CD73, CD105, and CD166, but negative for CD34, CD45, CD123, and HLA-DR (**Figure 2B**). The other three monkeys were used for the concurrent control to exclude spontaneous retrograde EAE. The data in **Figure 3A** showed that UCMSCs prevented the clinical signs and sustained their effects during the therapy. Compared with the concurrent control, early improvement of the clinical score was observed after the two UCMSC treatments. Histological analysis of brain tissue sections from the control showed a healthy myelin sheath and no inflammatory lesions (**Figure 3B**). The EAE group showed typical demyelination and inflammation loci (**Figure 3B**), whereas UCMSC administration attenuated the CNS demyelination and inflammation remarkably. Transmission electron microscopy showed the ultrastructure of demyelinating lesions of the three groups (**Figure 3C**). The results supported those of histology. MRI scans indicated that the area of demyelination lesions shrank after the two UCMSC treatments. As shown in **Figure 3D**, the MRI results showed that the area of demyelination was decreased in the white matter of the brain and spinal cord after UCMSC therapy, indicating improvement of disease progression. These data demonstrated that UCMSCs reduced inflammation and demyelination, and delayed EAE progression effectively [18].

UCMSCs enhance the expression of immunoregulatory cytokines and decrease proinflammatory cytokines in the EAE model

Previous studies have shown that UCMSCs exert their effects through cytokines [18, 19].

Therefore, we evaluated the cytokine profile in plasma from monkeys with EAE (**Figure 4B**). In the initial stage of EAE progression, the results of the cytokine array showed that IL-17 was significantly increased, whereas SCD40L and IL-5 had declined compared with the control. In the later stages of disease progression, most cytokine levels showed a higher tendency, such as IFN- γ , IL-6, IL-10, IL-12/23 (p40), IL-13, IL-17A, MIP-1 β , MIP-1 α , TGF- α , VEGF, and IL-18, compared with the control. After UCMSC treatment, the level of IL-5 was significantly decreased in the treatment group compared with the initial stage. There was also a higher level of IL-8 compared with EAE models without UCMSC treatment. The other cytokines did not show significant changes during disease progression. To define the effect of UCMSCs on cytokine levels in the CNS of the EAE model, we extracted total RNA from the lesion site of the brain. The results were partially consistent with cytokine production in plasma. UCMSC treatment significantly enhanced the expression of IL-4 and IL-10, and attenuated the expression of IFN- γ , GM-CSF, and IL-17A (**Figure 5A**).

A previous study has shown that soluble factors are directly involved in T helper cell induction and the inflammatory environment [20]. Therefore, we quantified their production in supernatants of cocultures of UCMSCs with PBMCs (**Figure 4C**). The levels of 23 cytokines showed a decreased tendency in PBMCs from EAE models after coculture with UCMSCs compared with non-cocultured PBMCs of EAE models. In particular, proinflammatory cytokines, such as IL-15, IL-17A, IL-1 β , IL-4, and IL-5, marker of immune activation IL-2, and macrophage inflammatory protein-1 β (MIP-1 β) were decreased significantly. We also examined the proliferation ability of PBMCs after coculture with UCMSCs at various time points (**Figure 4A**). The results indicated that UCMSCs significantly enhanced the proliferative ability of PBMCs.

UCMSC treatment enhances Treg populations and NK cells, and suppresses astrocyte activation

MSCs affect the generation of immune cells in autoimmune diseases such as MS and SLE [21, 22]. To further examine the effects of UCMSCs on the proliferation, survival, and differentiation of the T cell lineage *in vivo*, we analyzed CD4⁺ T cells, CD8⁺ T cells, Tregs, Th1 cells, and Th17 cells by flow cytometry. The ratio of

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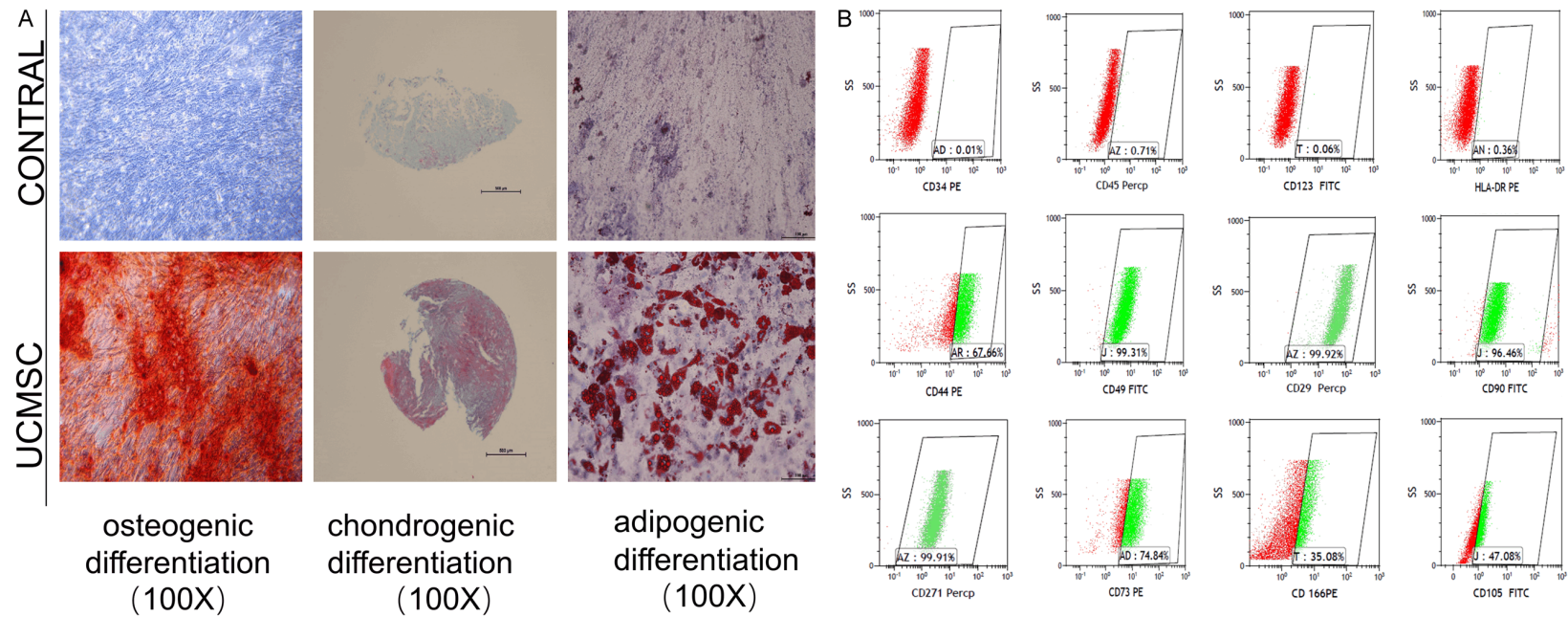


Figure 2. Multipotent differentiation ability of UCMSCs in vitro. A. Primary cultured UCMSCs differentiated into osteogenic, chondrogenic and adipogenic cells represented by staining with alizarin red, alcian blue, and oil red O. B. Immunophenotype of UCMSCs examined by flow cytometry, which were positive for CD49, CD90, CD29, CD271, CD73, CD105, CD166, and CD44, and negative for CD34, CD45, CD123, and HLA-DR.

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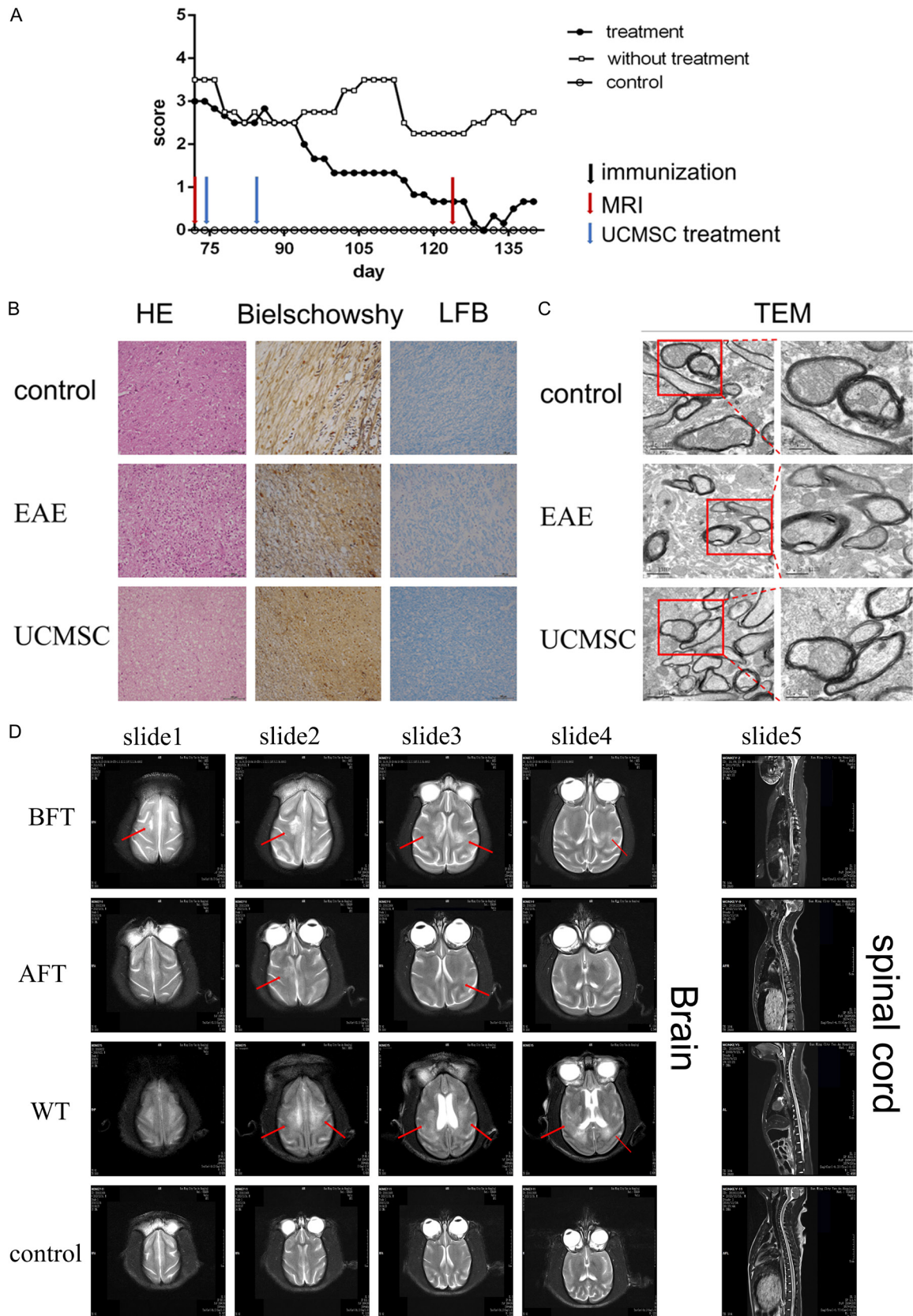


Figure 3. UCMSC therapy ameliorates clinical signs of EAE and reduces inflammation and demyelination in the CNS. A. Clinical scores were evaluated every 2 days from day 72, and UCMSC treatment was applied on days 74 and 84.

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B. The cynomolgus monkeys were sacrificed at day 140 p.i. and stained with HE for inflammation, LFB for demyelination, and Bielschowsky stain for the integrity of axons. C. TEM results showing the degree of demyelination. D. MRI scan on day 72 (BFT: before treatment) indicating the disease peak. MRI scan on day 124 (AFT: after treatment) showed improvement of disease progression. WT (without treatment) was regarded as the concurrent control of AFT.

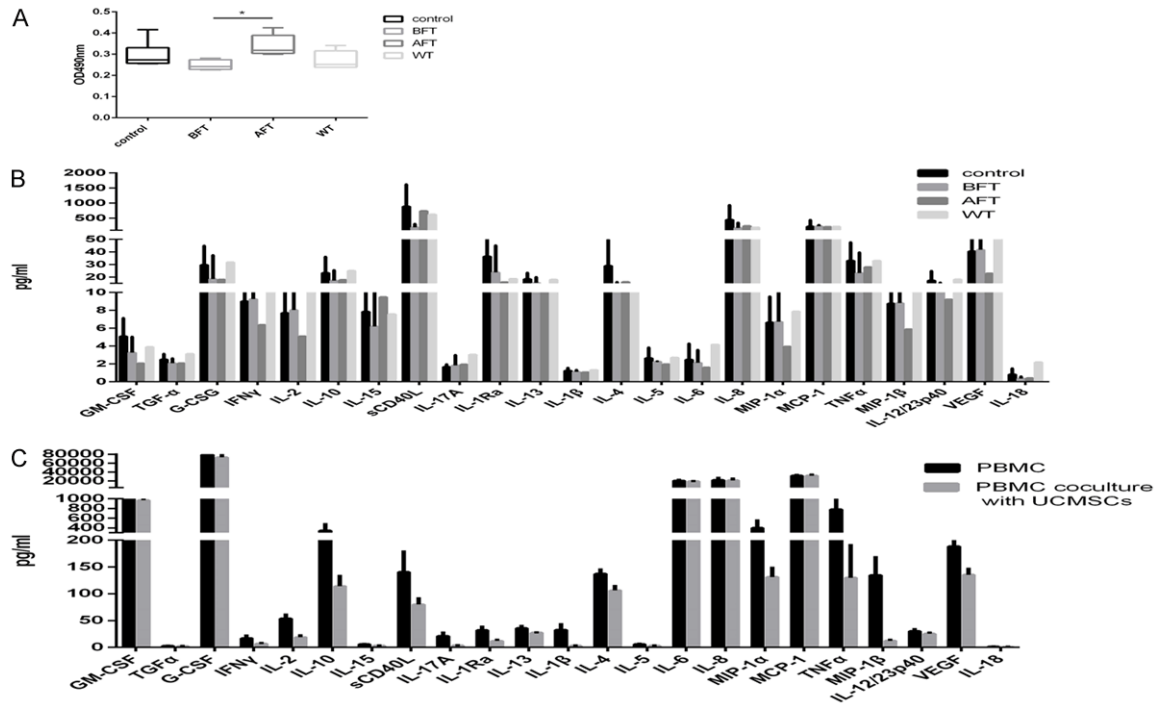


Figure 4. UCMSC therapy increases the expression of immunoregulatory cytokines and decreases proinflammatory cytokines in EAE development. A. Coculture of UCMSCs and PBMCs showed that UCMSCs promoted the proliferation of PBMCs after treatment. B. Cytokine secretion in plasma collected at four time points. C. Cytokine secretion in culture supernatants from cocultures with or without UCMSCs in a transwell system. Each treatment condition was analyzed in triplicate, and each experiment was repeated at least three times.

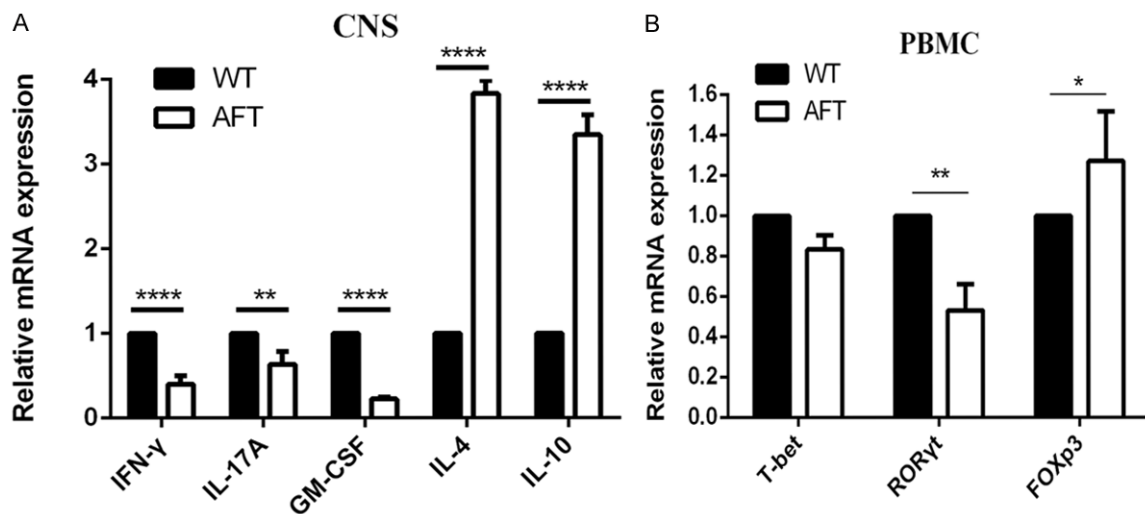


Figure 5. UCMSCs increase immunoregulatory cytokine expression in the CNS and PBMCs. A. Lesions in the CNS were scanned by MRI and mRNA expression of IFN- γ , IL-17A, GM-CSF, IL-4, and IL-10 was quantified by RT-PCR. B. mRNA levels of transcription factors, such as T-bet, ROR γ t, and Foxp3 genes, in PBMCs were analyzed by real-time PCR. Data represent the mean $\pm$ SEM ($n = 3$ /group). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$. One representative experiment of three is shown.

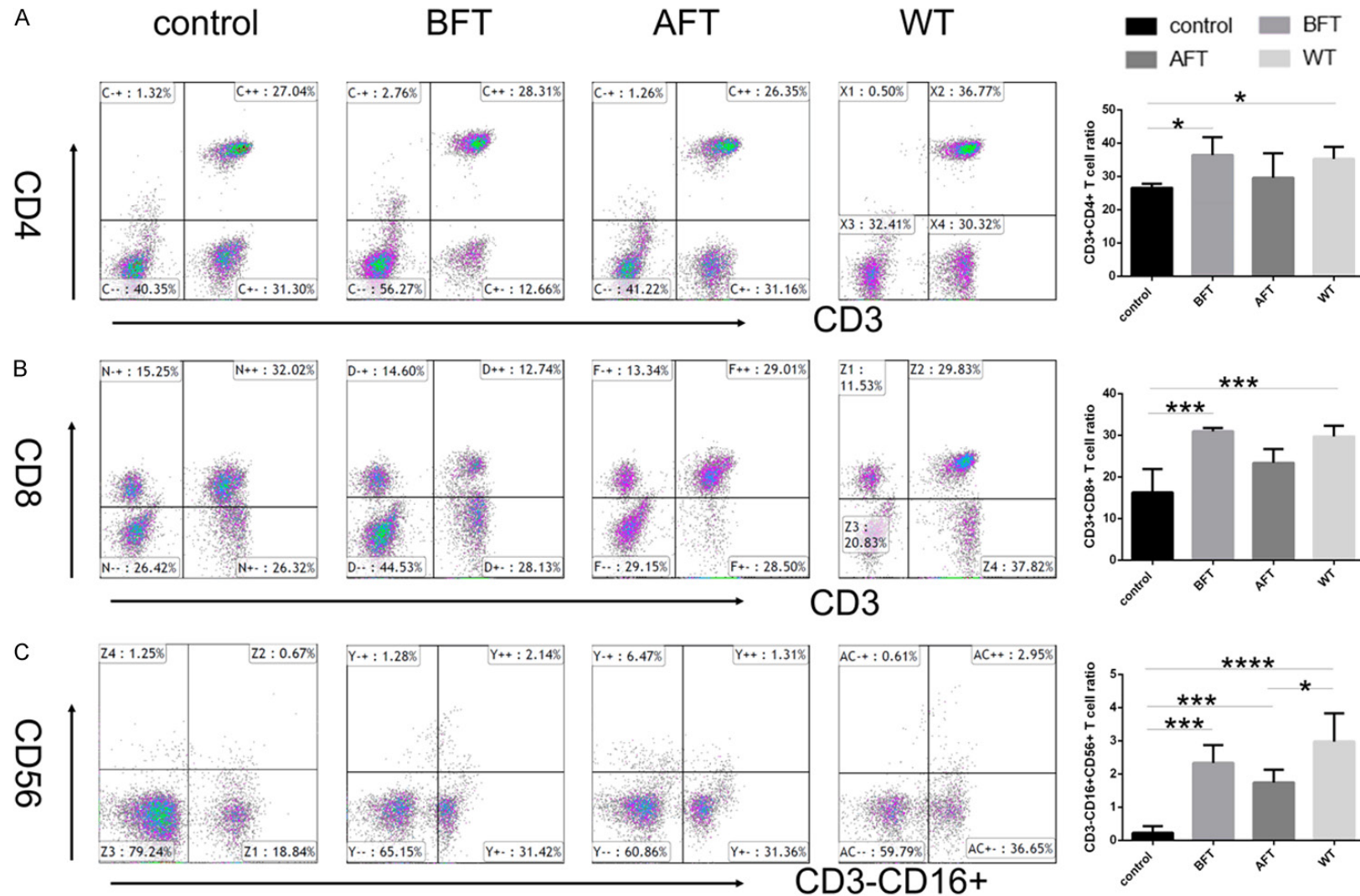


Figure 6. UCMSC influence on CD4⁺ T, CD8⁺ T, and CD3⁺CD16⁺CD56⁺ T cells during disease progression. A-C. Flow cytometric analyses. Percentages of CD4⁺ and CD8⁺ cells among gated CD3⁺ T cells and NK cells among gated CD3⁺ cells were determined and statistically analyzed. The unpaired t-test was used for statistical analysis. Statistical significance is denoted as *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001.

CD3⁺CD4⁺ cells was conspicuously increased to 30.96% when the EAE model in the cynomolgus monkey scored 3, indicating that the model mimicked the chronic phase of MS (**Figure 6A**). It is noteworthy that CD3⁺CD4⁺ cells were decreased to 23.40% after UCMSC therapy. In addition, CD8⁺ cytotoxic T cells showed a similar tendency in variation as T helper cells (**Figure 6B**). In the normal status of the cynomolgus monkey, NK cells were 1.468% of PBMCs, which increased to 4.487% when the EAE model was established and during disease progression (**Figure 6C**). However, UCMSC treatment decreased NK cells (**Figure 6C**).

Tregs only had a small augmentation at the onset of EAE compared with the control and increased continuously to 5.977% in the later period of EAE because of the immunogen reactivity (**Figure 7A**). Th1 cells were increased remarkably as well as Th17 cells. In addition, Th17 cells maintained a high level, while Th1 cells decreased slightly in the WT group. Th17 and Th1 cells declined to 1.326% and 2.113%, respectively after UCMSC treatment (**Figure 7B, 7C**). Analysis of T cells showed decreases of Th17 and Th1 cells, and an increase of CD4⁺CD25⁺Foxp3⁺ Tregs compared with the peak state of EAE. Compared with the control group, the percentage of T lineage cells was modified and the various examined T cell subpopulations had recovered to their normal status after MSC treatment. In parallel, the expression of key transcription factors, such as Foxp3 in Tregs, was increased significantly, while ROR γ t in Th17 cells and T-box in Th1 cells were decreased (**Figure 5B**).

Recent studies have shown major involvement of astrocytes in MS [23]. To assess whether astrocytes were activated in the EAE model of cynomolgus monkeys, we stained serial sections of the brain for GFAP. Activation of astrocytes in the EAE group was notably elevated compared with the control group. However, the green fluorescence intensity indicated that the activation of astrocytes was decreased significantly after UCMSC treatment (**Figure 8A**). The immunohistochemical staining of GFAP also indicated that UCMSCs reduced the activation of astrocytes (**Figure 8B**).

Discussion

The cynomolgus monkey shares significant genetic and immunological similarities with hu-

mans. It is an excellent model that mimics chronic MS in terms of clinical and neuropathological aspects [24, 25]. Because rodent animal models share properties with humans to some extent, studies have also used them to mimic the disease status in humans. However, there is still distinction concerning pathophysiological progression between them [26]. Our study showed that the EAE model in the cynomolgus monkey was characterized by acute clinical progression and perivascular inflammation, demyelination, and astrogliosis by pathological findings. A previous study has demonstrated that this model can be applied to evaluate a prospective therapy for MS [27]. Therefore, a nonhuman primate model of EAE is the most suitable model to assess the effects of UCMSCs on MS. The size of the demyelinated area shown by MRI was not reduced significantly after applying UCMSCs, although the clinical symptoms were obviously relieved. These results were consistent with our previous study of a MS patient who underwent complete regression without extensive relief of demyelination in the brain and spinal cord [6, 28]. Activation of astrocytes also has an important role in models of EAE [29]. GFAP⁺ astrocytes were increased obviously in the EAE group. UCMSC therapy significantly reduced astrogliosis. Astrocytes can contact with the BBB and respond to peripheral leukocytes [30]. Evidence has shown that T cells can alter the function of astrocytes through cytokines *in vitro*. These findings explain why infiltrated inflammatory cells and astrocytes decreased after UCMSC treatment, which may be because T cell-secreted cytokines modulated the activation of astrocytes in our study.

Recently developed techniques can obtain UCMSCs in large quantities [31]. The regulatory roles of MSCs in immune responses have been validated in preclinical and clinical studies [32]. Their application to immune diseases such as multiple sclerosis is promising [33]. Our results indicated that UCMSCs suppressed inflammatory T cell responses *in vivo* and the proliferation of PBMCs from EAE models *in vitro*. This may be associated with an increase in Th17 cytokines such as IL-17A *in vivo*. It is interesting to note that UCMSCs did not affect the secretion of IL-17A *in vitro*. After the treatment, the decrease in secretion of IL-5 was consistent with the decline of NK cells. Preclinical studies have provided clinical validation of this therapy

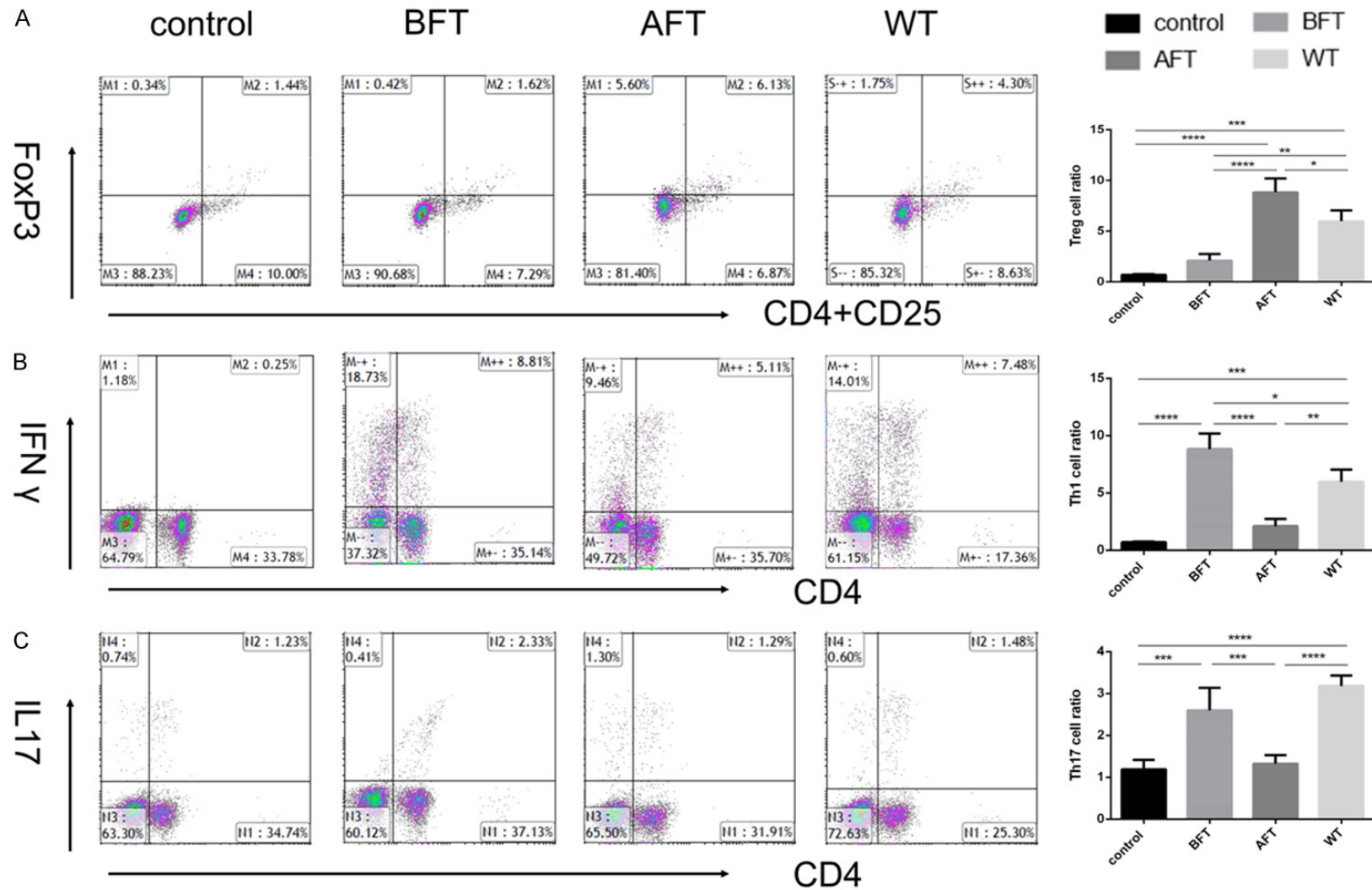


Figure 7. Th1 and Th17 cells are reduced by UCMSC therapy, while Tregs are increased. (A) Subsets of Tregs among gated CD4⁺ cells were analyzed by intracellular staining of Foxp3, IFN- γ (B), and IL-17 (C) at various time points. The percentages of positive cells are expressed as the mean $\pm$ SEM (n = 3). *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001. One representative experiment of three is shown.

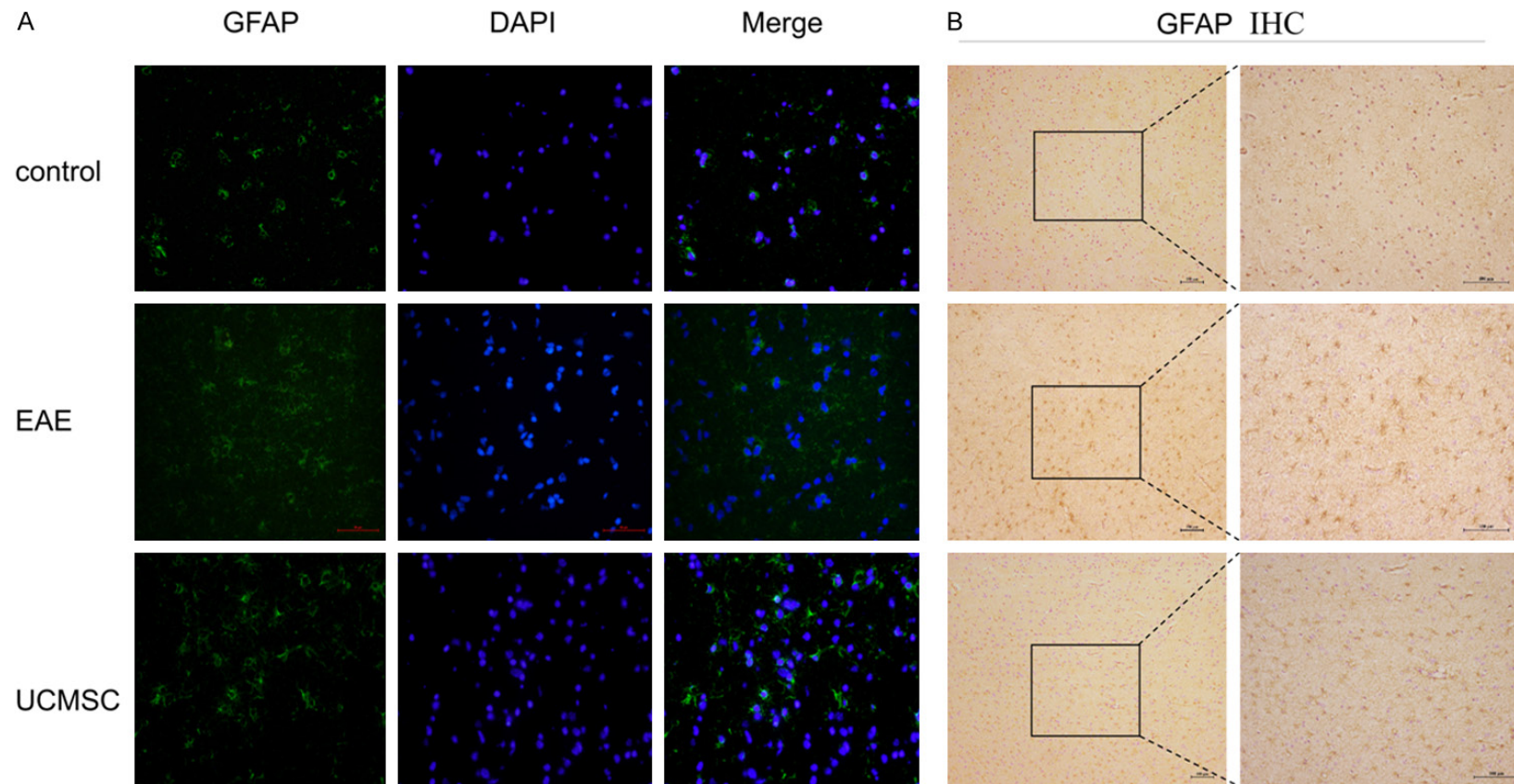


Figure 8. UCMSCs enhance the numbers of GFAP⁺ cells in the CNS. A. Representative images showing GFAP⁺ astrocytes. Nuclei were counterstained with DAPI. B. IHC staining of GFAP showing the same changes compared with immunostaining.

[6, 7, 19]. Our data showed reductions in Th1 cytokines, including IFN- γ , IL-2, and GM-CSF, after treatment, which were essential for the therapeutic effects of UCMSCs. Moreover, Th2 cytokines IL-4 and IL-10 were increased obviously, which was associated with the reduction in disease severity. In addition, the significant decreases in IL-5 and IL-13 demonstrated that the ability to phagocytize antigens in B cells might be inhibited by UCMSC therapy [34]. IL-8 increased after the final UCMSC administration, and EAE progression was constrained, which may influence the stability of the blood brain-barrier through endothelial cells of the choroid plexus in the brain [35-37].

Currently, it has been well elucidated that MSCs enhance functional Tregs, and that Tregs play a key role in controlling immune responses and protect against the development of EAE [38]. In our study, Tregs were increased among PBMCs of the EAE model that received UCMSCs. Elevated numbers of Tregs have also been observed among PBMCs from monkeys that received human amnion mesenchymal cells [39]. Studies of mesenchymal stem cells have obtained similar results, namely that Tregs increase, and Th1 and Th17 are downregulated in PBMCs of arthritis patients [40, 41]. UCMSCs induce Tregs from CD4⁺ T cells via cell contact and non-redundant contributions of PGE2 and TGF- β 1 [38]. These studies confirmed that Treg generation is associated with soluble factor-dependent priming by MSCs, which is essential for an immunosuppressive effect [42].

Collectively, our observations provide a basis to understand the immunomodulatory effects of UCMSC therapy for EAE, which were partly mediated through decreasing the numbers of T cells, inhibiting the production of proinflammatory mediators under strict regulatory control of Tregs, decreased proportions of Th1 and Th17 cells, and inhibited activation of astrocytes. Certainly, more studies are needed to further explain the mechanism of UCMSCs and promote their clinical application.

Conclusion

UCMSCs are emerging as a promising alternative stem cell type for the treatment of acute and chronic inflammatory neurological disorders, which are easily obtained in the large quantities required for therapeutic transplanta-

tion [43]. This study revealed that UCMSCs had immunomodulatory functions and protected the NHP model from developing signs of EAE. UCMSCs appeared to elicit therapeutic effects through promoting Tregs, maintaining peripheral T cells and NK cells, and inhibiting astrocyte activation, resulting in suppression of the production of proinflammatory factors in peripheral secondary lymphoid organs and inhibition of inflammation in the CNS. These features make UCMSCs an attractive cellular therapy for neurodegenerative diseases. In addition, we will further investigate the therapeutic mechanism of UCMSCs in MS and elucidate the relationship between the cytokine levels and treatment in a future study.

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Disclosure of conflict of interest

None.

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