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Original Article

Human adipose tissue-derived mesenchymal stem cells ameliorate experimental autoimmune myasthenia gravis by modifying the balance of Th1/Th2/Th17/Treg cell subsets

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Abstract: Myasthenia gravis (MG) is a T cell-mediated autoimmune disease characterized by acetylcholine receptor antibodies (AChR-Ab) selectively affecting AChR of the postsynaptic membrane. Mesenchymal stem cells (MSCs) have immunosuppressive qualities including suppressing effector T cell responses and have beneficial effects in various immune disorders. The aim of this study was to evaluate the therapeutic effects of human adipose tissue-derived mesenchymal stem cells (hASCs) in the model of MG. In this study, we demonstrated that intravenous injection of hASCs could ameliorate the pathological features of experimental autoimmune myasthenia gravis (EAMG). This effect was specifically dependent on the correction of imbalance among the Th1, Th2, Th17, and Treg cell subsets, which was involved in the development of EAMG. Our results suggested that hASCs emerge as potent regulators of immune/inflammatory responses with the capacity to suppress effector T cells in vivo and as attractive candidates for cell-based treatments for MG.

Keywords: Human adipose-derived mesenchymal stem cells, experimental autoimmune myasthenia gravis, immunotherapy

Introduction

Myasthenia gravis (MG) is a severe autoimmune disease characterized by loss of acetylcholine receptor (AChR) on the postsynaptic membrane of neuromuscular junction and resulted in impaired neuromuscular transmission and muscle weakness, which is antibody-mediated, T cell-dependent and complement involved [1]. Experimental autoimmune myasthenia gravis (EAMG), a reliable model of human MG, can be induced in Lewis rats by immunization with a synthetic peptide corresponding to region 97-116 of the rat AChRα subunit (R97-116 peptide) and is suitable for investigating the underlying pathogenesis and the novel therapeutic strategies of human MG [2].

Mesenchymal stem cells (MSC), multipotent stromal cells, exist in many tissues and have emerged as key regulators of immune/inflammatory responses [3]. Recent studies have shown that stem cell therapy protocols have therapeutic effects in many immune-mediated disorders, including systemic lupus erythematosus [4], inflammatory bowel disease [5], rheumatoid arthritis [6], and autoimmune encephalomyelitis [7]. Thus, bone marrow-derived MSCs (BMSCs) have recently been described as potent immunomodulators that have abilities to inhibit dendritic cell maturation, T cell activation and B cell function in vitro, and efficiently ameliorate experimental autoimmunity [8]. However, the critical issue that harvesting BMSCs is extremely painful for patients and large quantities of infused cells are required for treatment has become a bottleneck of its extensive application [9, 10]. Human adipose tissue-derived mesenchymal stem cells (hASCs) obtained from subcutaneous adipose tissue

are attractive alternative BMSC sources for cell treatment due to the abundant source and the more immunomodulatory properties [11]. Here, we characterized in vivo the immunoregulatory properties of hASCs in model of MG and provided a potential therapeutic strategy to MG.

Materials and methods

Animals

Female Lewis rats (six to eight weeks of age) were purchased from Vital River Laboratory Animal Co. Ltd. (Beijing, China) and maintained at the local animal house under specific-pathogen-free conditions. The protocols for in vivo study with rat were approved by the Ethics Committee of Wenzhou Medical University (Wenzhou, China), and the methods for in vivo study were carried out in accordance with the approved guidelines.

Antigens

R97-116 peptide (DGDFAIVKFTKVLLDYTGHI), a synthetic peptide corresponding to region α97-116 of the rat AChRα subunit, was synthesized by AC Scientific, Inc. (Xian, China) as described previously [2].

Elicitation of EAMG

Female Lewis rats were randomly divided into three groups with 8 rats in each. The EAMG and hASCs-administered groups were induced by subcutaneous injection at the base of tail with 200 μl inoculum containing 50 μg R97-116 peptide in 100 μl phosphate buffered saline (PBS) and 100 μl CFA (Sigma-Aldrich, USA) supplemented with 1 mg Mycobacterium tuberculosis (Difco, Detroit, MI, USA) on day 0, and then the rats were boosted on day 30 with the same peptide in IFA (Sigma-Aldrich, USA). The CFA control group was injected with the same emulsion except PBS was used instead of the peptide.

Clinical evaluation

After the first immunization, each animal was monitored on alternate days for weight changes until sacrificed. Disease severity was scored by measuring muscular weakness in a double-blind fashion. Clinical scoring was based on the presence of tremor, hunched posture, muscle weakness, and fatigability after exercise (through repetitive paw grips on the cage grid for 30 s). Disease severity was graded as follows [2, 12]: 0: normal muscle strength; 1: mildly decreased activity, weak grip, fatigable; 2: weakness, hunched posture at rest, decreased body weight, tremor; 3: severe generalized weakness, marked decrease in body weight, moribund; 4: dead. Rats with intermediate signs were assigned grades of 1.5, 2.5 or 3.5, respectively. Results were expressed as the mean score for each group at each time point.

Preparation of hASCs

The procedures for isolation, collection, and culture of hASCs were previously described [13]. Human adipose tissue samples aspirated from healthy donors were washed twice with cold PBS and digested with 0.2% collagenase type I (Worthington Biochem, Lakewood, Australia) for 30 min at 37°C with shaking at 80 rpm. Cell suspensions were obtained by passing the samples through a nylon mesh with a pore size of 100 mm. After precipitated by centrifugation of the filtrate at 200 g for 10 min, the stromal vascular fraction (SVF) was washed and plated in culture dishes at a density of 1×10^4 cells/cm^2 in DMEM supplemented with 10% fetal bovine serum (FBS; Gibco-BRL, USA). The hASCs were used between passages 2 and 3 for experiments. Ethical approval was approved by the ethics committee of the First Affiliated Hospital of Wenzhou Medical University. Written informed consents were obtained from all participating volunteers and all experiments were carried out in accordance to the approved ethical guidelines.

Transplantation of hASCs

On the day of the second immunization, animals in the hASC-administered group were injected with hASCs (5×10^6/rat in 0.5 mL PBS) via the tail vein. Rats from the CFA and EAMG groups were injected with the equal volume of PBS. All rats were weighed and monitored for clinical scores every other day until sacrificed.

Flow cytometric immunophenotyping

Different combinations of antibodies were used to characterize cells derived from the three groups. Detection of intracellular cytokines and cell markers was carried out by flow cytometry (FACS) analysis as described previously [14]. Isolated mononuclear cells from spleen were

Table 1. Primers used in real time RT-PCR

Gene name	Primer	Sequence
GADPH	Forward	5'-AATGCATCCTGCACCACCAAC-3'
	Reverse	5'-TGATGGCATGGACTGTGGTCAT-3'
T-bet	Forward	5'-AGCCGTTTCTACCCTGACCT-3'
	Reverse	5'-CTGCTCGGAACTCTGTTTCA-3'
RORγt	Forward	5'-TCTGGAAGCTGTGGGATAGA-3'
	Reverse	5'-GAGGAGCCTGTGGAGAAATAC-3'
Gata3	Forward	5'-CGGCCAGGCAAGATGAGAAAG-3'
	Reverse	5'-GGCACATAGGGCGGATAGGT-3'
Foxp3	Forward	5'-TCACCTATGCCACCCTCATC-3'
	Reverse	5'-CACTCTCCACTCGCACAAAG-3'

incubated with Brefeldin A (1:1000 dilution, eBioscience Inc., San Diego, CA, USA) for 4-6 h together with PMA (50 ng/ml) and ionomycin (500 ng/ml). Then T cells were firstly incubated with FITC-anti-rat-CD4 (eBioscience) for 30 min at 4°C, meanwhile, Tregs were identified by positive staining with APC-anti-rat-CD25 (eBioscience). After fixation and permeabilization, intracytoplasmic staining was carried out using one of the following fluorescently labeled Abs: eFluor660-anti-rat-IFN-γ (eBioscience), PE-anti-rat-IL-4 (BD Biosciences), PE-anti-rat-Foxp3 (eBioscience) and PE-anti-rat-IL-17 (eBioscience). Isotype-matched, FITC-, PE- and APC-conjugated mAbs of irrelevant specificity were used as negative controls. Samples were analyzed on a BD FACS Calibur flow cytometer (BD Biosciences, San Jose, CA) and data were analyzed with Flow Jo software (Ashland, OR, USA).

Quantitative real-time PCR

Total RNA was extracted from lymphocytes harvested from rats in the CFA, EAMG, and hASCs-administered groups 56 days after the first immunization using TRIzol as recommended by Life Technologies. RNA (1 μg) was reverse transcribed to cDNA using an RT-PCR kit from TIANGEN. Gene expression was evaluated by qRT-PCR,

using Fast SYBR Green Master Mix (TIANGEN BIOTECH CO., LTD., Beijing, China) on a StepOnePlus Real-Time PCR system (Applied Biosystems). Gene expression was normalized using GADPH. For comparisons, CFA groups were assigned an arbitrary value of 100. The primers used in RT-PCR are summarized in **Table 1**.

Histological staining

Snap frozen gastrocnemius muscle tissues from the CFA, MGFA, and hASCs-administered rats were sectioned (10 μm) longitudinal to the long axis of the muscle. After air-dried and blocked with 5% BSA at room temperature for 1 hr, sections were incubated for overnight at 4°C with Alexa Fluor 555-labeled-α-bungarotoxin (BTX, Invitrogen, Carlsbad, CA) and Anti-Synaptophysin antibody (Abcam, Cambridge, MA, USA). The sections were washed, followed by a second incubation with FITC-labeled anti-rabbit IgG for another 2 hr at room temperature. Finally, the sections were washed, viewed under a Nikon Ti Microscope (Nikon, Japan), and photographed at the same exposure and magnification.

Statistical analysis

The statistical software Statistical Program for Social Sciences (version 20.0, SPSS Inc, Chicago, IL, USA) was used for all analyses. Differences between groups were analyzed by a two-tailed Student's t-test for paired and unpaired data, respectively. All statistical tests were two sided and *p*-values less than 0.05 were considered statistically significant.

Results

hASCs ameliorates EAMG clinical presentation

To investigate the effects of hASCs on EAMG, 5 × 10^6 hASCs were injected into EAMG rat via tail vein. Compared with untreated model rats, hASCs-administered groups had lower average clinical scores and less weight loss (**Figure 1A** and **1B**).

As loss of AChR is the typical pathological change during EAMG development, we performed a histological examination of gastrocnemius muscle tissues from 3 groups rats. Muscle sections were incubated with Alexa-555-α-bungarotoxin and anti-synaptophysin antibodies, which bind to AChR and presynaptic nerve terminals at the NMJ respectively. Rats from the EAMG group showed significant AChR loss and endplate damage compared with those from the CFA group. Findings in the EAMG group were reversed following hASCs treatment (**Figure 2**).

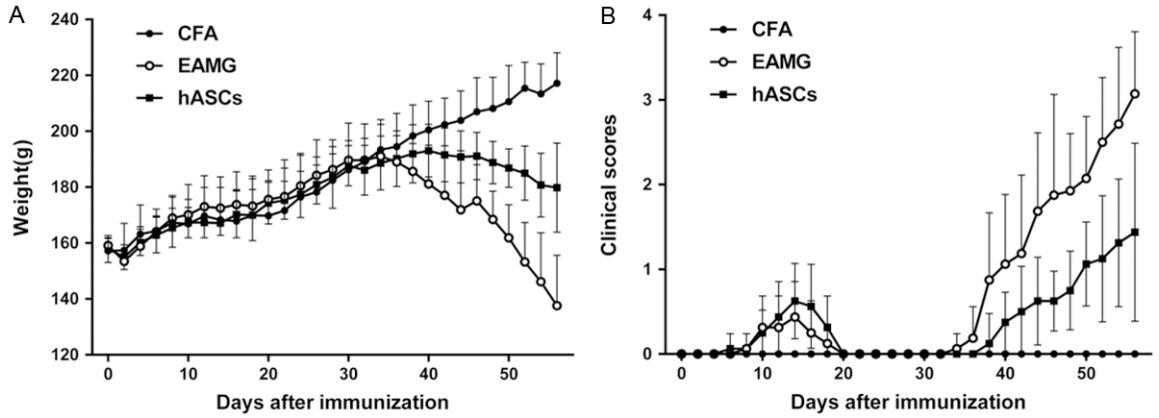

Figure 1. Human adipose-derived mesenchymal stem cells ameliorate experimental autoimmune myasthenia gravis (EAMG) symptoms. Body weight (A) and Clinical manifestations (B) were measured in normal rats (●), EAMG rats (○), and EAMG rats treated with BMSCs (■). The arrows indicate day of the booster injection with RAChR97-116 peptide and of simultaneous administration of hASCs. Note the difference in the timing and severity of EAMG symptoms when rats were treated with BMSCs. Data were obtained from 4 different experiments ($P_{clinical\ score} < 0.001$, $P_{body\ weight} < 0.001$).

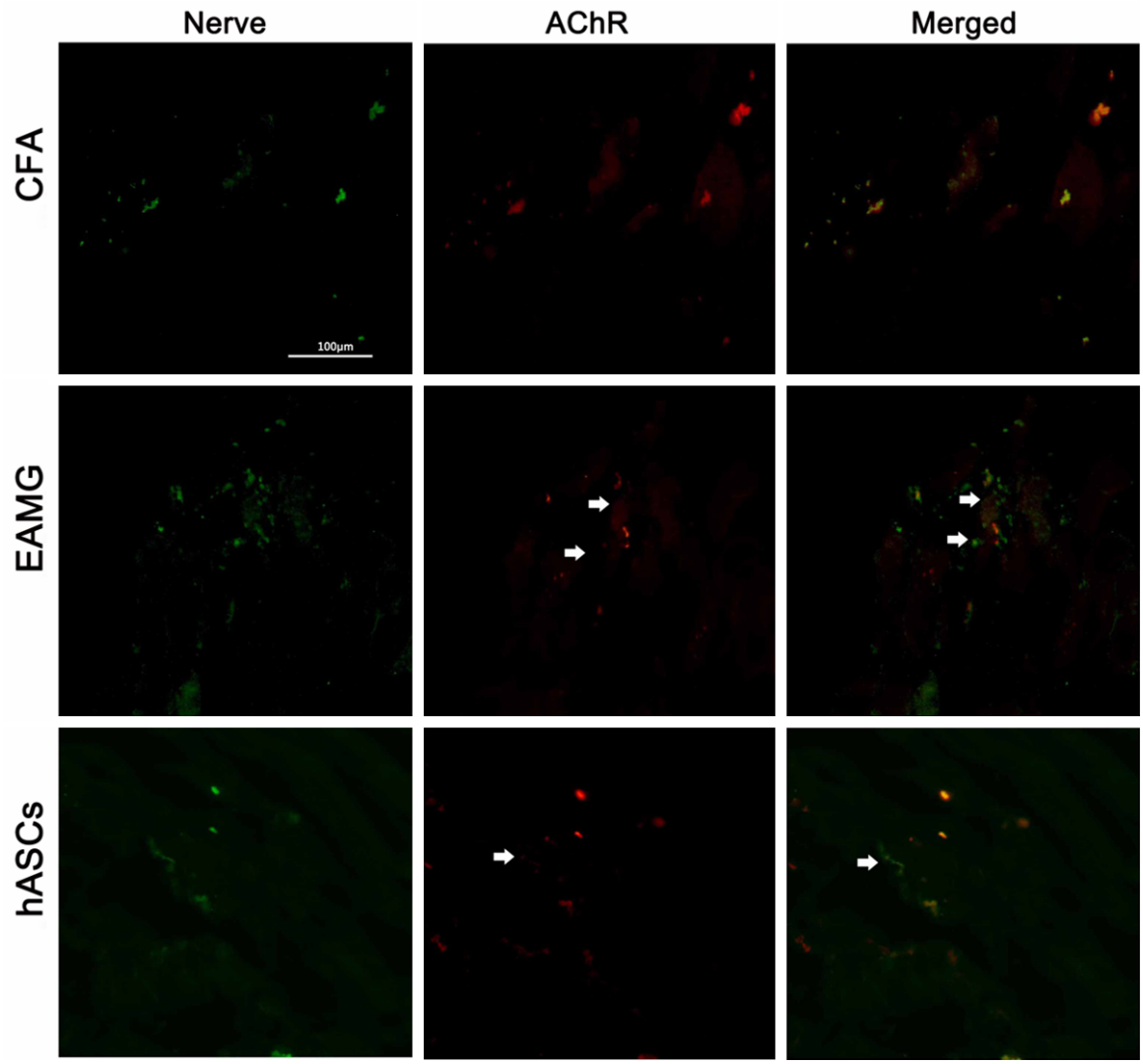

Figure 2. AChR and presynaptic nerve terminals at NMJs of gastrocnemius muscles of diseased EAMG rats and after hASCs treatment. Postsynaptic AChRs were stained with Alexa-555-α-bungarotoxin (red, middle panels) and presynaptic nerve terminals with anti-synaptophysin and FITC-labeled secondary antibody (green, left panels). Scale bars: 10 μm.

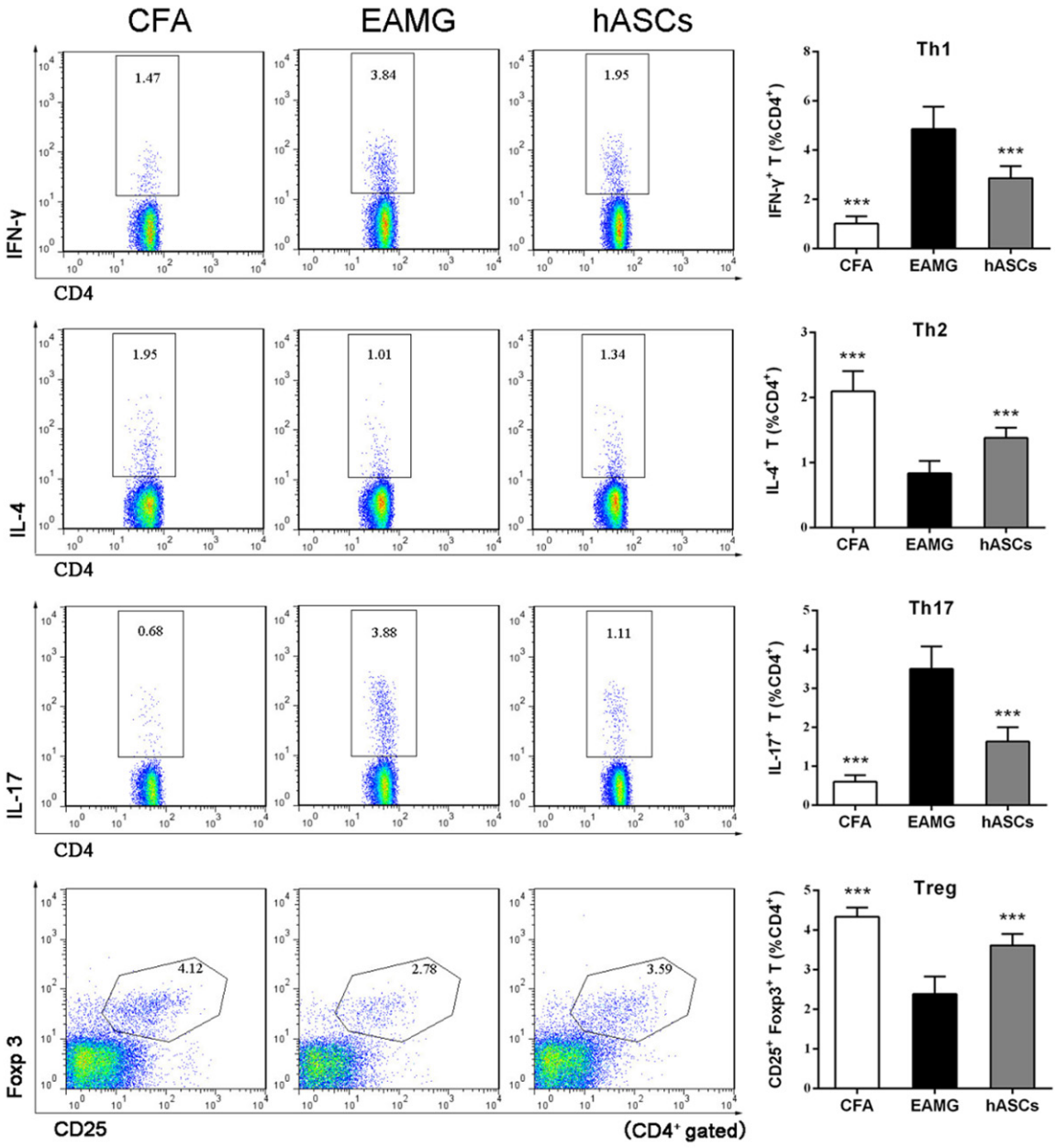

Figure 3. Human adipose-derived mesenchymal stem cells altered the T helper (Th) subset distribution in vivo. FACS analysis of expression of IFN-γ, IL-4, IL-17, and Foxp3 on $CD4^+$ T cells derived from: normal rats (left), EAMG group (middle), and EAMG rats treated with hASCs (right). Data are expressed as the mean ± SD of eight rats/group, *P < 0.05, **P < 0.01, ***P < 0.001.

hASCs altered the Th subset distribution in EAMG in vivo

Several studies have demonstrated that the imbalances between pro-inflammatory Th1 and Th17 populations and anti-inflammatory Th2 and Treg populations contribute to the pathogenesis in EAMG. Numerous evidences have also showed the role of hASCs in regulating these defined Th cells recently. Therefore, we

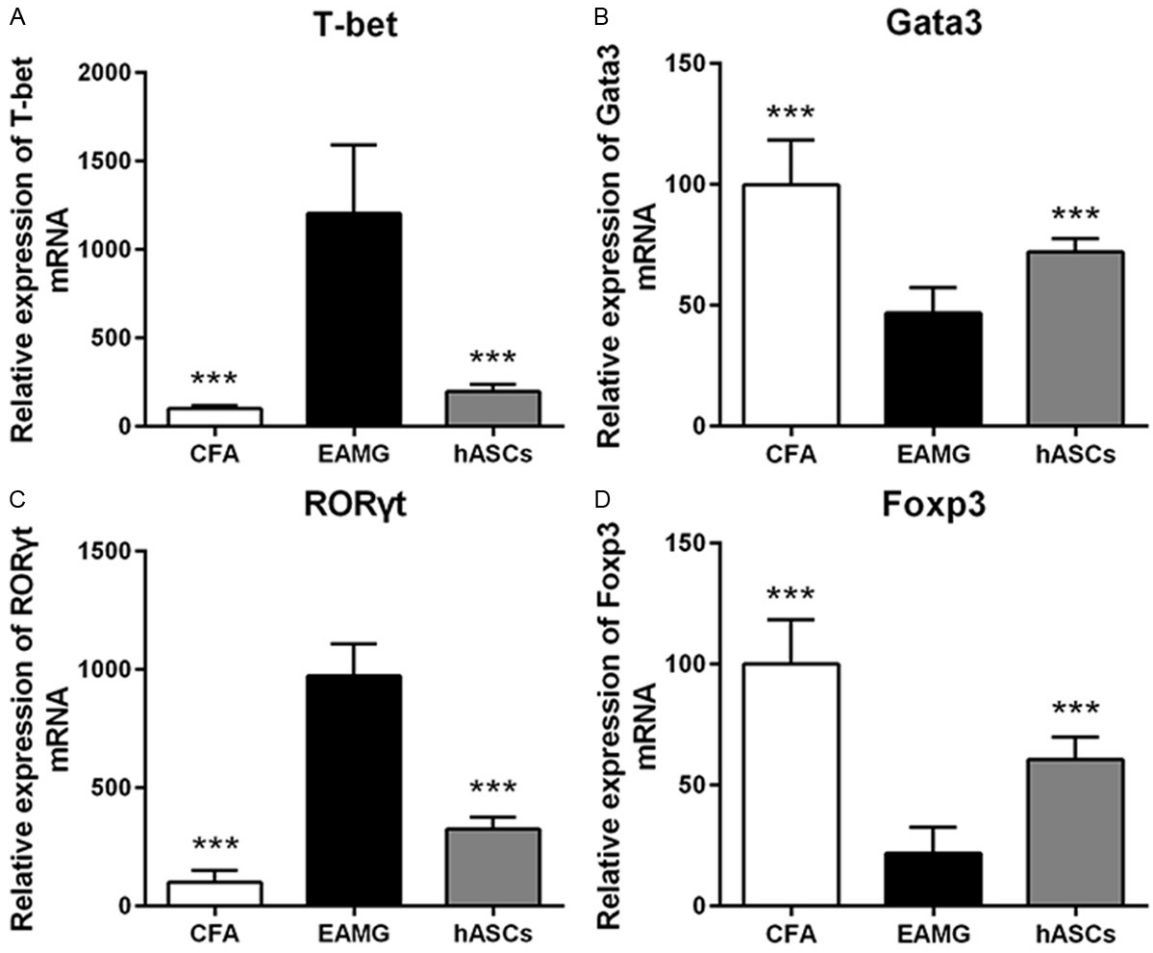

Figure 4. mRNA expression levels of Th1, Th17, Th2 and Treg related genes in CFA, EAMG and hASC-administered groups. T-bet, RORγt, Gata3 and Foxp3 mRNA expressions detected by quantitative real-time PCR. Data are expressed as the mean ± SD of eight rats/group, *P < 0.05, **P < 0.01, ***P < 0.001.

examined the effects of hASCs on AChR-specific Th cells in the respective treatment groups.

As compared to the proportion of Th1 ($CD4^+$-IFN-γ^+), Th17 ($CD4^+$-IL-$17A^+$), Th2 ($CD4^+$-IL-4^+) and Treg ($CD4^+$-$CD25^+$-$Foxp3^+$) of lymphocytes from CFA rats by FACS, EAMG rats had higher Th1 and Th17 expressing cells, and lower Th2 and Treg expressing cells (**Figure 3**). Administration of hASCs to EAMG rats reversed this phenomenon and led to a decline in Th1 and Th17 expressing cells, and an increase in Th2 and Treg expressing cells (**Figure 3**). In addition, we evaluated the changes in expression of Th1, Th17, Th2, Treg related transcriptional factors in lymphocytes from the CFA, MGFA, and hASCs-administered rats. As shown in **Figure 4**, the mRNA expression of T-bet (**Figure 4A**) and RORγt (**Figure 4C**) decreased significantly in hASCs treated EAMG compared with those in EAMG without treatment, while the mRNA expression of Gata3 (**Figure 4B**) and Foxp3 (**Figure 4D**) increased markedly.

Discussion

Due to no effective specific treatment for MG clinically, we tested the therapeutic effects of hASCs, an innovative therapeutic tool for MG, against EAMG in rats as the experimental model of autoimmune disease. To our knowledge, our study was the first report to demonstrate that the administration of hASCs could significantly ameliorated EAMG severity. The beneficial effects of hASCs treatment were involved in modulating the imbalance of the Th subset profiles in rats presenting with EAMG.

Accumulating data have implicated that immune dysregulation is involved in the development of various disease, including MG [15]. MG, as well as its animal model, EAMG, is a typical

antibody-mediated, T cell-dependent and complement involved autoimmune disorder, in which $CD4^+$ T cells play a critical role in the pathogenic process [1]. As exhibit some of the most varied effector options, $CD4^+$ T cells is well known to differentiate into a series of cell subsets, including Th1, Th2, Th17, and Treg cells [16]. The Th1 cytokine IFN-γ has a major role in the pathogenesis during the initiation and development of an organ-specific autoimmune disease and administration of IFN-γ had more severe EAMG [17], while mice with gene IFN-γ knockout are resistant to EAMG [18]. In addition, gene knockout of T-bet, a specific transcription factor controlling Th1 cell differentiation and IFN-γ production, leads to be less susceptible to EAMG [19]. Th17 cells, a pro-inflammatory T cell subset, with the production of the cytokine IL-17, contribute to EAMG development [20]. In contrast, Th2 cells have been shown to protect against EAMG [21] and its cytokine IL-4 can prevent the development of EAMG through the differentiation of AChR-specific regulatory $CD4^+$ T cells [22]. The Treg cells, a counter to the pro-inflammatory Th17 effects, were implicated to be involved in the tolerance of EAMG [23] and administration of ex-vivo generated Treg cells can inhibit the progression of EAMG [24]. Furthermore, alteration in the balance of Th1, Th2, Th17, and Treg is associated with the development of EAMG [4].

Since numerous evidences have identified the role of immunomodulatory capabilities, hASCs are believed to be therapeutic candidates to treat autoimmune diseases [6, 13, 25]. In the present study, our results demonstrated that hASCs also have therapeutic effects on the EAMG with the capacity to suppress AChR-specific Th1 and Th17 cells by inducing the generation of AChR-specific Th2 and Treg cells.

In conclusion, the present study demonstrated the capacity of hASCs to regulate EAMG pathogenesis by suppressing immune dysregulation as a result of partially reversing the imbalance of Th1, Th2, Th17, and Treg. These data suggested that hASCs might be attractive candidates for the use in ameliorating the severity of EAMG and would open up new perspectives for a cell-based treatment of MG.

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Disclosure of conflict of interest

None.

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