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Original Article

Mesenchymal stromal cells attenuate multiple sclerosis *via* IDO-dependent increasing the suppressive proportion of CD5+ IL-10+ B cells

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Abstract: Multiple sclerosis (MS), one of the autoimmune and inflammatory diseases, is a major cause of neurological disability worldwide. The existing clinical treatments are not curable, and better treatments are urgently needed. Mesenchymal stromal cells (MSCs) have shown promise for treating MS, but the favorable effects and mechanism of MSC therapy on MS are still not fully understood. In this study, we analyzed the phenotypic feature of peripheral blood mononuclear cells (PBMCs) in MS patients and found that the patients exhibited an increase in the frequency of B cells, but a markedly decrease in frequency of CD5+ and IL-10+ B cells compared to healthy controls. Infusion of MSCs exhibited a significant therapeutic effect on the experimental autoimmune encephalomyelitis (EAE) mice, infiltration of mononuclear cells and demyelination of the spinal cords were both reduced in CNS of the mice, the frequency of CD5+ IL-10+ B cells in the mice was significantly increased. Additionally, when PBMCs or B cells from MS patients were co-cultured with MSCs, the frequency of CD5+ IL-10+ B cells also increased, the proliferative and immunosuppressive capacity of CD5+ B cells were significantly enhanced while the apoptosis ratio of this cellular subset significantly decreased. Moreover, those effects could be eliminated while the indoleamine 2,3-dioxygenase (IDO) inhibitor, D/L-1MT, was added to the co-cultured cells. In summary, this study suggests that MSCs can control EAE via IDO pathway to promote the proportion and function of CD5+ IL-10+ B cells, providing a promise to treat patients with MS in the clinical setting.

Keywords: Multiple sclerosis, mesenchymal stromal cells, experimental autoimmune encephalomyelitis, indoleamine 2,3-dioxygenase, regulatory B cell

Introduction

Multiple sclerosis (MS) is an autoimmune disease that is characterized by central nervous system (CNS) inflammation, demyelination, axonal loss, and degeneration [1]. Approximately 2.5 million people suffered from MS worldwide. Current MS therapies can only relieve the inflammatory damage, but cannot recover the damage. There are more than 80% patients finally develop the progressive disability [2, 3]. Thus, it is imperative to develop new drugs or therapeutic approaches that are able to overcome or even cure the disease.

The etiology of MS is still not clear. It was originally thought to be a T cell-mediated disease [4-6], but accumulating data has also demonstrated the involvement of B cells [7, 8], the most convincing evidence comes from the clinical efficacy outcomes of selective B cell depletion therapies, such as rituximab, a depleting CD20 antibody in clinical trials in MS [9, 10]. B cells contribute to MS pathology by producing antibodies and cytokines, and by functioning as antigen-presenting cells. In addition to these potentially pathogenic roles, recent experimental evidence indicates that IL-10 producing B cells, a specific subset of B cells with negative

regulatory function (Bregs), suppress the progression of immune-mediated diseases. In experimental autoimmune encephalomyelitis (EAE) mice, the animal model for MS [11, 12], studies demonstrate that numbers of endogenous or adoptively transferred Bregs directly interfere with the outcome of EAE pathogenesis [13, 14]. While Bregs were showed to produce low levels of IL-10 in patients with MS [15, 16], the depletion and repopulation of B cells in rituximab-treated patients, seem to reset the production of IL-10 in B cells [15]. These studies indicated that Bregs play an important role in controlling the MS, development of targeting and promoting Bregs therapies could be beneficial for the treatment of patients with MS.

Recently, there has been increasing recognition of the potential of mesenchymal stromal cells (MSC)-based therapies for the treatment of MS [17-20]. Clinical trials of MSC transplantation in MS patient appears feasible, safe, well tolerated and effective [21, 22]. These exciting results implied that MSCs might be a potential therapeutic agent for MS. MSCs are multipotent progenitor cells that can be isolated from various adult tissues [23-27], in addition to their self-renewal capacity and multipotency [28], MSCs also have potent immunomodulatory effects on both innate and adaptive immune cells [29-33]. Studies in EAE have provided abundant evidence that MSCs decreased inflammatory infiltrates and demyelination [20, 34, 35]. Some studies suggested that MSCs could ameliorate EAE by preventing Th1 and Th17 cell population [36], or by enhancing the function of Tregs *in vitro* [37]. However, the immunomodulatory and neuroprotective effects of MSC therapy for MS on B cells has been less illustrated.

In this study, we demonstrated that a subset of CD5+ IL-10+ B cells was indeed decreased in PBMCs of patients with MS. Additionally, we observed that infusion of MSCs attenuated EAE through upregulation of CD5+ IL-10+ Breg cells. Moreover, the MSCs prompted upregulation of Breg cells *via* IDO pathway.

Materials and methods

Processing of peripheral blood cells

This study was approved by the Research Ethics Committee of the Third Affiliated Hospital at the Sun Yat-sen University and written informed consent was obtained from each participant according to the Declaration of Helsinki. Heparinized peripheral blood was obtained from MS patients and the healthy subjects. Ten patients (three men and seven women) along with age and sex matched controls enrolled in this study. Peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation using Ficoll-Paque PLUS media (GE Healthcare, USA) and stored in aliquots.

Cell culture

Human umbilical cord-derived MSCs (hUC-MSCs) and normal skin-derived fibroblast (NFs) were isolated and cultured as previously described [38, 39]. Briefly, fresh human umbilical cords were obtained after birth, with the written consent of parents, and collected in phosphate buffered saline (PBS; Sigma, USA) containing 100 UI/ml penicillin and streptomycin (Gibco-BRL, USA) at 4°C. The cords were washed twice and cut into pieces and floated in Dulbecco's modified Eagle's medium with low glucose (DMEM-LG) containing 10% FBS (Gbico), 5% HS, penicillin and streptomycin at 37°C in a humidified atmosphere with 5% CO_2. The medium was changed every 2 days, and non-adherent cells were removed by washing after 7 days. When well-developed colonies of fibroblast-like cells appeared after 10 days, the cultures were trypsinized and transferred (without dilution) into a new flask for further expansion. NFs were obtained from foreskin, the tissues were minced and digested in Roswell Park Memorial Institute 1640 (RPMI 1640; Invitrogen, USA) supplemented with 10% FBS, 1 mg/ml collagenase type I (Sigma) and 100 U/ml hyaluronidase (Sigma) at 37°C for 8 hours, washed twice with PBS (Sigma) and centrifuged at 450 g for 8 minutes each time. Cells were finally resuspended in RPMI 1640 supplemented with 10% FBS, 100 IU/ml penicillin, 100 mg/ml streptomycin, and then cultured at 37°C in a humidified 5% CO_2 environment.

EAE induction and MSC treatment

All animal studies were approved by the Institutional Animal Care and Use Committee of the Third Hospital at the Sun Yat-Sen University (Approve Number: 160520). Female mice (C57BL/6, 18-20 g, 8-10 weeks) were randomly divided into three groups: control group, EAE model group and hUC-MSC treatment group (*n*

= 6 per group). To induce EAE in mice, complete Freund's adjuvants (CFA) was prepared by mixing Mycobacterium tuberculosis (Difco, USA) (2 mg/mL) with Freund's adjuvants (Sigma). An equal amount of MOG35-55 peptide (GL Biochem, China) (2 mg/mL in ddH_2O) and CFA solution were mixed to have a final concentration of 1 mg/mL before injected into each mouse. 100 μL antigen/CFA emulsion was delivered to two different sites of each hind flank, immediately after that, 400 ng pertussis toxin (Enzo life sciences) was intraperitoneally injected. Another pertussis toxin was given to the mice two days later.

For the treatment of EAE, 2×10^6 hUC-MSCs in 200 μL PBS or PBS alone were intravenously injected into mice *via* the tail vein on 12th and 22nd days after immunization of the EAE model. Disease score was monitored every day for up to 30 days as follows: 0, no sign of disease; 1, loss of tone in the tail; 2, partial hind limb paralysis; 3, complete hind limb paralysis; 4, front limb paralysis; and 5, dead or moribund [40, 41].

Histology

Thirty days after cell injection, mice were sacrificed and perfused transcardially with 0.1 M PBS containing 4% paraformaldehyde (PFA, Sigma), spinal cords were dissected out and post-fixed overnight and then mounted in paraffin. The spinal cord sections were incubated in 0.1% luxol fast blue (LFB; Sigma) for 4 hours at 60°C, the excess stain will be differentiated with 0.01% Lithium carbonate (Sigma). The quantification of demyelination was performed on at least five spinal cord cross-sections per animal.

Co-culture experiment

PBMCs or B cells were isolated from untreated-MS patients or healthy person. Once MSCs or NFs reached 50% confluence, PBMCs or B cells were seeded onto MSCs or NFs monolayers at a density of 1×10^6 PBMCs or 1×10^5 B cells per 4 mL per well in 6-well plates, and MSC culture medium was replaced with RPMI 1640 supplemented with 5% FBS. To block the activity of indoleamine 2,3-dioxygenase (IDO), 0.5 mM D/L-1MT (Enzo life sciences, USA) was added after co-culture for 5 days. At day 9, B cells were separated from MSCs by spinning the cells in suspension and then washing them.

Flow cytometric analysis

The flow cytometric analysis of cell phenotypes and cell sorting were performed according to routine laboratory methods. Flow cytometric analyses or sorting were performed on LSR-Fortessa (BD, USA) or MoFlo Astrios EQs (Beckman, USA) flow cytometer. The antibodies used for flow cytometry were APC, FITC, PE-CF594 or PE-Cy7 conjugated anti-CD3, CD4, CD5, CD28, CTLA-4, PD1, CD19, B7-1, CD107a, TRAIL, CD8, CD56, NKG2D and IL-10, which were purchased from BD (USA), while anti-CD96, LAG-3, TIM3 and CD40 were purchased from BioLegend (USA), anti-TIGIT was brought from eBioscience (USA). The fresh isolated or cultured cells were collected, washed twice, and resuspended in 100 mL of PBS containing 0.1% BSA, and were stained and labeled with either specific antibodies or the appropriate isotype controls.

Sorted CD19+ B cells from healthy donors and MS patients were resuspended in RPMI-1640 medium. After 5 hours of stimulation with BFA (10 μg/mL; Sigma), PMA (50 ng/mL; Sigma), and ionomycin (1 μg/mL; Sigma), the cells were fixed, permeabilized, and stained for cell surface CD3, CD19, and CD5 and cytoplasmic IL-10 according to the manufacturer's instructions. The stained cells were then analyzed by flow cytometry.

Cell proliferation and cytokine production assays

To evaluate the suppressive functions of CD5+ B cells, 5×10^5 CD3+ T cells were labeled in 1 mL of PBS containing 2.5 μM carboxyfluorescein succinimidyl ester (CFSE; Invitrogen, USA) for 5 min at room temperature and seeded in a 96-well plate in 100 μL of RPMI 1640 medium containing 10% FBS. CD5+ B cells were purified by flow cytometry and added to CD3+ T cells at ratios of 1:1 (10^5 cells). Next, the CD3+ T cells were activated by the addition of 2 μg/mL of anti-CD3 and 5 μg/mL of anti-CD28 mAb beads per well for 3 days. Subsequently, T cell proliferation were measured by flow cytometry.

For cytokine production assays, 2×10^5 CD4+ T cells were co-cultured with CD5+ B cells and

Table 1. Demographic and baseline clinical characteristics of enrolled patients

Characteristic	MS patients (n = 10)	Health controls (n = 10)
Gender (Male: Female)	3:7	3:7
Age (Mean ± SEM)	34.10 ± 13.96	34.10 ± 13.96
EDSS Score (Mean ± SEM)	2.60 ± 1.05	0

then activated as same as the above mentioned. Expression of IL-17A, Foxp3 and IFN-γ were determined by qRT-PCR.

Cell apoptosis assay

B cell apoptosis was quantified using a PI/Annexin V apoptosis detection kit according to the manufacturer's instructions (Invitrogen). The binding of Annexin V-FITC and PI to the cells was measured by flow cytometry.

RT-PCR analyses

Using a commercial kit (Roche, Switzerland), total RNA was extracted from PBMCs in MS patients both before and after the MSC treatment. Reverse transcription was performed using the Quantitect Reverse Transcription kit (Qiagen, USA), according to the manufacturer's protocol, followed by qPCR using the DyNAmo ColorFlash SYBR Green qPCR kit (Thermo Fisher Scientific, USA) on a LightCycler 480 Detection System (Roche). The samples were transferred to the thermal cycler, and DNA was amplified using the following thermocycling conditions: 40 cycles of denaturation at 95°C for 10 seconds, annealing at 60°C for 10 seconds, and extension at 72°C for 30 seconds. GAPDH served as an internal control. The primers used for IL-17: forward 5'-CGAGATCTCCGAGATGCC-3' and reverse 5'-AGTTCACATGCGCCTTGA-3', IFN-γ: forward 5'-CGAGATCTCCGAGATGCC-3' and reverse 5'-AGTTCACATGCGCCTTGA-3', Foxp3: forward 5'-CGAGATCTCCGAGATGCC-3' and reverse 5'-AGTTCACATGCGCCTTGA-3'.

Statistical analysis

The results are expressed as the Means ± SEM. The statistical significance of differences between groups was determined by Student's t-test. SPSS statistical software (version 13.0) was used for all statistical analyses. All data were analyzed using two-tailed tests unless otherwise specified, and $P < 0.05$ was considered statistically significant.

Results

The frequency of CD5+ and IL-10+ B cells decreases in PBMCs of MS patients

To study the relationship between immunology dysfunction and patients with MS, we collected blood samples from 10 MS patients, including 9 who were clinically diagnosed with relapsing-remitting MS (RRMS) and 1 with secondary-progressive MS (SPMS). All patients were untreated before sample collection, the clinical features of the patients were listed in **Table 1**. In these samples, we did not detect any striking changes in CD4+ or CD8+ T cell subsets of the MS patients compared to healthy controls (**Figure 1A-C**). We also examined the T cell markers related to T cell activation and tolerance, such as Tim3, PD-1, CD107a, TIGIT, TRAIT, B7-1, LAG3, and CD28 (**Figure 2A** and **2B**), and found no significant changes as well. Nonetheless, the significant changes in natural killer (NK) cells (**Figure 1D** and **1E**) and B cells (**Figure 1F** and **1G**) were observed. Compared to the healthy donors, although the MS patients exhibited an increase in B cell and a decrease in NK frequency, detection of NKG2D and CTLA4 in NK showed non-significant changes (**Figure 2C** and **2D**). Interestingly, the frequency of CD5+ and IL-10+ B cells within the B cell population markedly decreased (**Figure 2E-H**). Importantly, the frequency of CD5+ and IL-10+ B cells in MS patients with an EDSS score of 2 or less was significantly greater than these in MS patients with an EDSS score greater than 2 (**Figure 2I** and **2J**). These results showed that MS progression is accompanied by an increase in B cells but a decrease in CD5+ and IL-10+ B cells in PBMCs of MS patients.

MSCs ameliorate symptoms and increase the proportion of CD5+ IL-10+ B cells in EAE mice

Given that the decrease of CD5+ IL-10+ B cells is possibly associated with the development and progression of MS and MSC therapy has an effect on controlling the animal model of MS, we next examined the therapeutic effects and alteration of CD5+ IL-10+ B cells in EAE mice that underwent MSC treatment. Interestingly, administration of MSCs significantly sup-

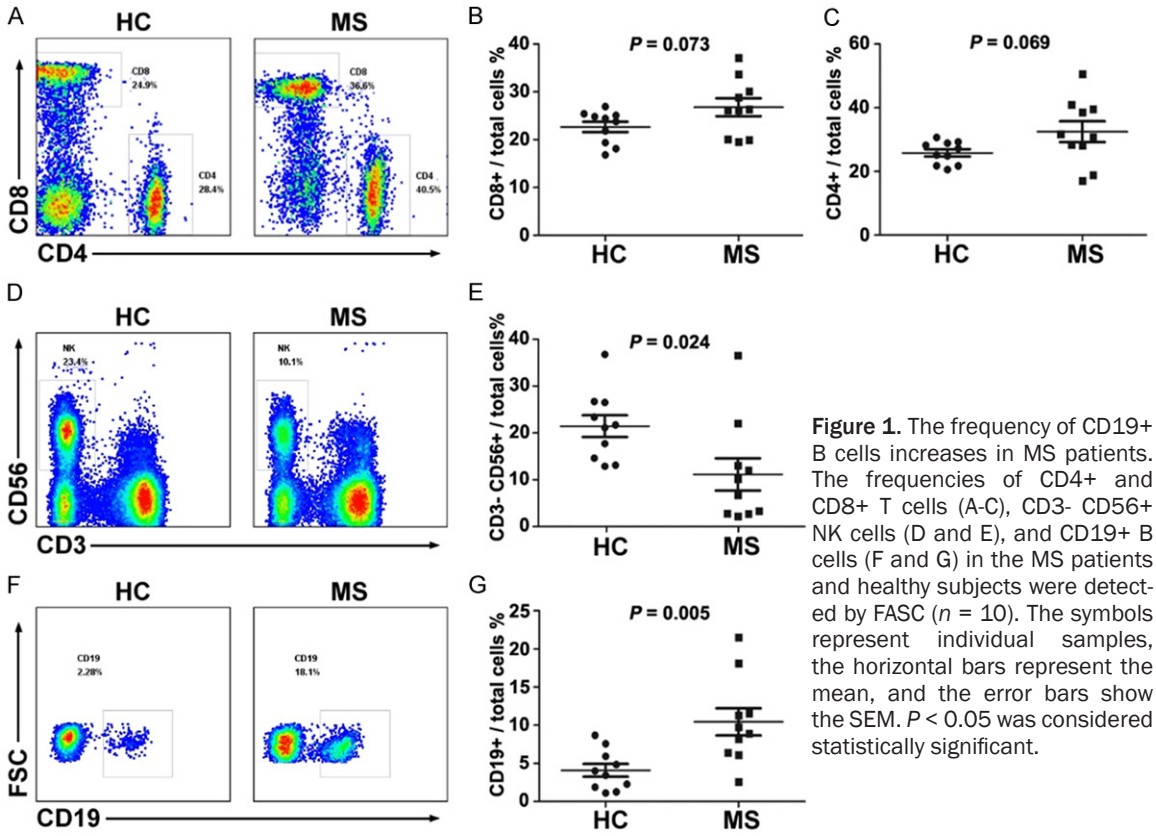


Figure 1. The frequency of CD19+ B cells increases in MS patients. The frequencies of CD4+ and CD8+ T cells (A-C), CD3- CD56+ NK cells (D and E), and CD19+ B cells (F and G) in the MS patients and healthy subjects were detected by FASC (n = 10). The symbols represent individual samples, the horizontal bars represent the mean, and the error bars show the SEM. $P < 0.05$ was considered statistically significant.

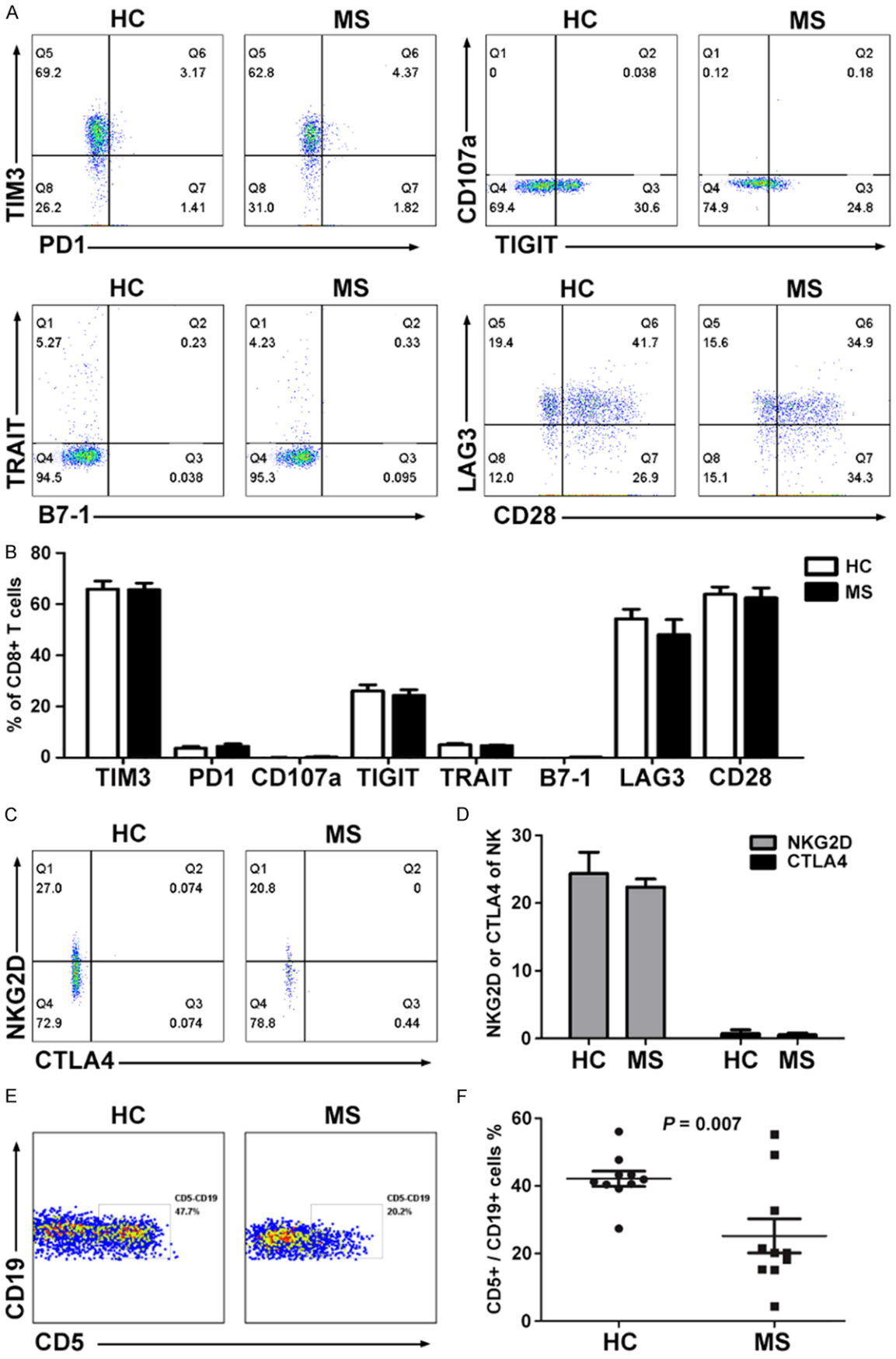
pressed the EAE clinical course compared to PBS (**Figure 3A**). Histological examination showed there was less infiltration of mononuclear cells and decreased demyelination in the spinal cords of MSC-treated mice as compared with those treated with PBS (EAE *vs.* EAE+MSCs, number of infiltrating mononuclear cells: 23.63 *vs.* 43.63, $P < 0.001$; demyelination score: 0.23 *vs.* 0.06, $P < 0.001$) (**Figure 3B-E**). Moreover, we found that MSC treatment significantly increased the frequency of CD5+ IL-10+ B cells in peripheral blood of EAE mice (**Figure 3F** and **3G**).

MSCs increase the frequency of CD5+ and IL-10+ B cells in vitro

To determine the effects of MSCs on CD5+ B cells, PBMCs from four MS patients were isolated and cultured either alone or with MSCs or with NFs. After 5 days of co-culture with MSCs or culture alone, the frequency of CD5+ B cells among CD19+ B cells from the MS patients increased from 38.1 ± 1.65 to 50.2 ± 2.01 (P = 0.0035, **Figure 4A** and **4B**), while the frequency of IL-10+ B cells among CD19+ B cells from the MS patients increased from 1.97 ± 0.24 to 4.02 ± 0.58 (P = 0.017) (**Figure 4D** and **4E**). Moreover, after 5 days of co-culture with MSCs, the frequency of CD5+ or IL-10+ B cells in CD19+ B cells from the MS patients were both markedly increased (38.6 ± 1.79 to 53.75 ± 1.89; 1.48 ± 0.28 to 3.68 ± 0.46, $P < 0.01$) (**Figure 4A**, **4C**, **4D** and **4F**). However, the effects of NFs on CD5+ or IL-10+ B cells were not observed. To further prove the effects of MSCs on CD5+ or IL-10+ B cells, PBMCs from the healthy person were isolated and cultured either alone or with MSCs. After 5 days of co-culture with MSCs or culture alone, the frequency of CD5+ B cells among CD19+ B cells from the healthy person increased from 38.2 ± 3.54 to 49.2 ± 1.64 (P = 0.0212, Figure S1A and S1B), while the frequency of IL-10+ B cells among CD19+ B cells from the healthy person increased from 2.13 ± 0.11 to 5.02 ± 0.14 (P = 0.0005, Figure S1C and S1D).

MSCs promote the proliferation, survival and function of CD5+ B cells

We next assessed the effect of MSCs on CD5+ B cell proliferation. CD5+ B cells from the MS patients exhibited a much greater proliferative


A
HC
MS
TIM3
PD1
Q5 69.2
Q6 3.17
Q8 26.2
Q7 1.41
Q5 62.8
Q6 4.37
Q8 31.0
Q7 1.82
CD107a
TIGIT
Q1 0
Q2 0.038
Q4 69.4
Q3 30.6
Q1 0.12
Q2 0.18
Q4 74.9
Q3 24.8
TRAIT
B7-1
Q1 5.27
Q2 0.23
Q4 94.5
Q3 0.038
Q1 4.23
Q2 0.33
Q4 95.3
Q3 0.095
LAG3
CD28
Q5 19.4
Q6 41.7
Q8 12.0
Q7 26.9
Q5 15.6
Q6 34.9
Q8 15.1
Q7 34.3
B
% of CD8+ T cells
HC
MS
TIM3
PD1
CD107a
TIGIT
TRAIT
B7-1
LAG3
CD28
C
NKG2D
CTLA4
Q1 27.0
Q2 0.074
Q4 72.9
Q3 0.074
Q1 20.8
Q2 0
Q4 78.8
Q3 0.44
D
NKG2D or CTLA4 of NK
NKG2D
CTLA4
E
CD19
CD5
CD5-CD19 47.7%
CD5-CD19 20.2%
F
CD5+ / CD19+ cells %
P = 0.007

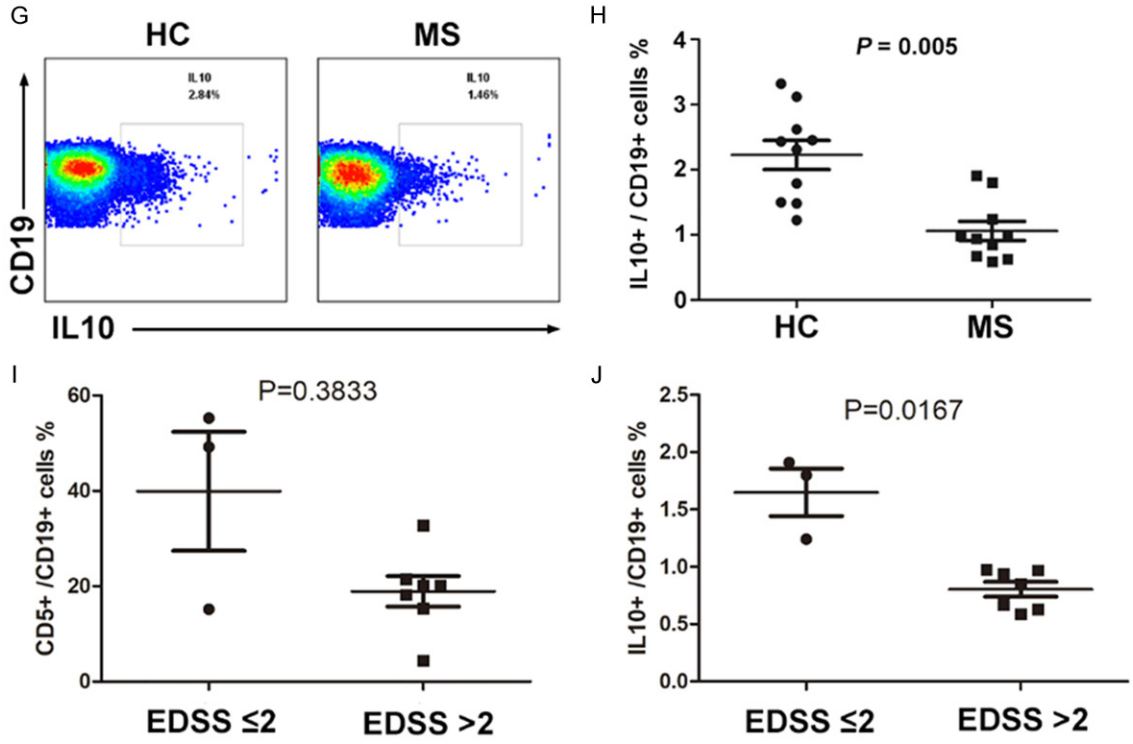


Figure 2. The frequency of CD5+ IL-10+ B cells decreases in MS patients. The expression of TIM3, PD1, CD107a, TIGIT, TRAIT, B7-1, LAG3 and CD28 in T cells (A and B), the NKG2D and CTLA4 expression in CD3- CD56+ NK cells (C and D), and the CD5 and IL-10 expression in CD19+ B cells (E-H) in the PBMCs of MS patients and healthy subjects were detected and analyzed by FASC (n = 10). The correlation between EDSS score and CD5+ or IL-10+ B cell ratio (I and J) in the PBMCs of MS patients. The symbols represent individual samples, the horizontal bars represent the mean, and the error bars show the SEM. $P < 0.05$ was considered statistically significant.

capacity in the presence of MSCs than in the absence of MSCs (5 ± 1.12 *vs*. 0.2 ± 0.08, $P < 0.001$); CD5- B cells also presented the same tendency, but the efficiency was much lower than that of the CD5+ B cells co-cultured with MSCs (1 ± 0.64 *vs*. 0.01 ± 0.008, $P = 0.022$) (**Figure 5A** and **5B**). Moreover, we analyzed the effects of MSCs on the survival of CD5+ B cells and found that the survival rate of CD5+ B cells from the MS patients after 96 hours was much lower in culture alone than in the presence of MSCs; MSCs also increased the survival rate of CD5- B cells, but the efficiency was relatively low (**Figure 5C**). To further prove the effects of MSCs on CD5+ B cell proliferation and survival, PBMCs from the healthy person were isolated and cultured either alone or with MSCs. CD5+ B cells from the healthy person exhibited a much greater proliferative capacity in the presence of MSCs than in the absence of MSCs (4.2 ± 0.25 *vs*. 1.8 ± 0.23, $P = 0.01$) (Figure S1E and S1F). The survival rate of CD5+ B cells from the healthy person after 96 hours was much lower in culture alone than in the presence of MSCs (Figure S1G and S1H).

In addition, we analyzed the effect of MSCs on the function of CD5+ B cells. As shown in **Figure 5D-F**, CD5+ B cells derived from the MS patients displayed a significant immunosuppressive capacity on T cells by inhibiting their proliferation, as well as the production of IL-17 (0.5 ± 0.12, $P < 0.001$) and IFN-γ (0.4 ± 0.15, $P < 0.001$). In contrast, after co-culturing with MSCs, CD5+ B cells showed a much more robust function of inhibiting T cell proliferation and IL-17 production (0.16 ± 0.04, $P = 0.0017$), and of producing a significantly higher level of Foxp3 (2.33 ± 0.58, $P = 0.009$). However, we did not observe the effect of NFs on the function of CD5+ B cells (**Figure 5E**).

MSCs act via IDO pathway to modulate the proliferation and function of CD5+ B cells

Our previous study found that MSCs can suppress human T cell-mediated diseases by

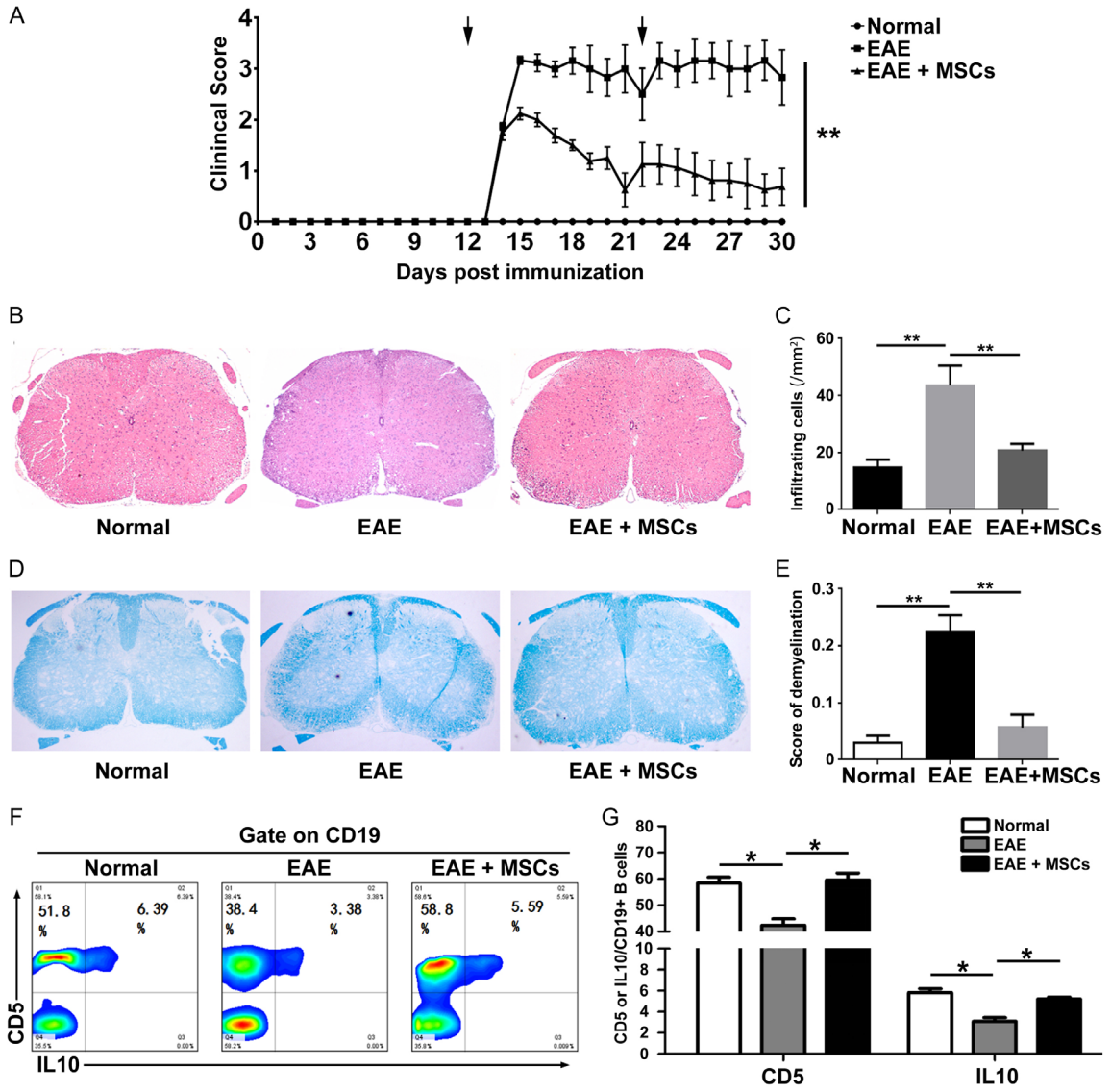


Figure 3. MSCs improve MS clinical symptoms and increase the proportion of CD5+ IL-10+ B cells *in vivo*. After EAE in mice was induced, 2×10^6 hUC-MSCs were injected into mice on days 12 and 22 (arrows). Administration of MSCs with a therapeutic protocol significantly improved the neurological function score on EAE mice compared with PBS (n = 6, each group, A). Histological examination showed there was less infiltration of mononuclear cells (B and C) and decreased demyelination in the spinal cords of MSC-treated mice as compared with those treated with PBS (D and E). MSCs significantly increased the frequency of CD5+ IL-10+ B cells in EAE model (F and G). *P < 0.05, **P < 0.01.

secreting IDO [27]. Thus, to gain further insight into the mechanism of the MSC-mediated proliferation, survival and function of CD5+ B cells, we tested IDO mediators using specific inhibitors for IDO, D/L-1MT. We found that D/L-1MT could significantly reverse the MSC-mediated effects on CD5+ B cell proliferation (2.29 ± 0.15 *vs*. 5.06 ± 0.58, P = 0.0037) (**Figure 6A** and **6B**), survival (28.03 ± 1.97 *vs*. 48.3 ± 2.05, P < 0.001) (**Figure 6C** and **6D**). The suppressive function of CD5+ B cells on proliferation of CD3+ T cells (40.85 ± 2.49 *vs*. 23.83 ± 1.99, P = 0.0017) (**Figure 6E** and **6F**), and also on production of IL-17 (0.47 ± 0.08 *vs*. 0.16 ± 0.04, P = 0.0044) and Foxp3 (1.08 ± 0.03 *vs*. 2.08 ± 0.38, P = 0.011) in CD4+ T cells (**Fiugre 6G**) were both reduced.

Discussion

The pathophysiology of MS involves several components, the role of CD4+ T cells has long

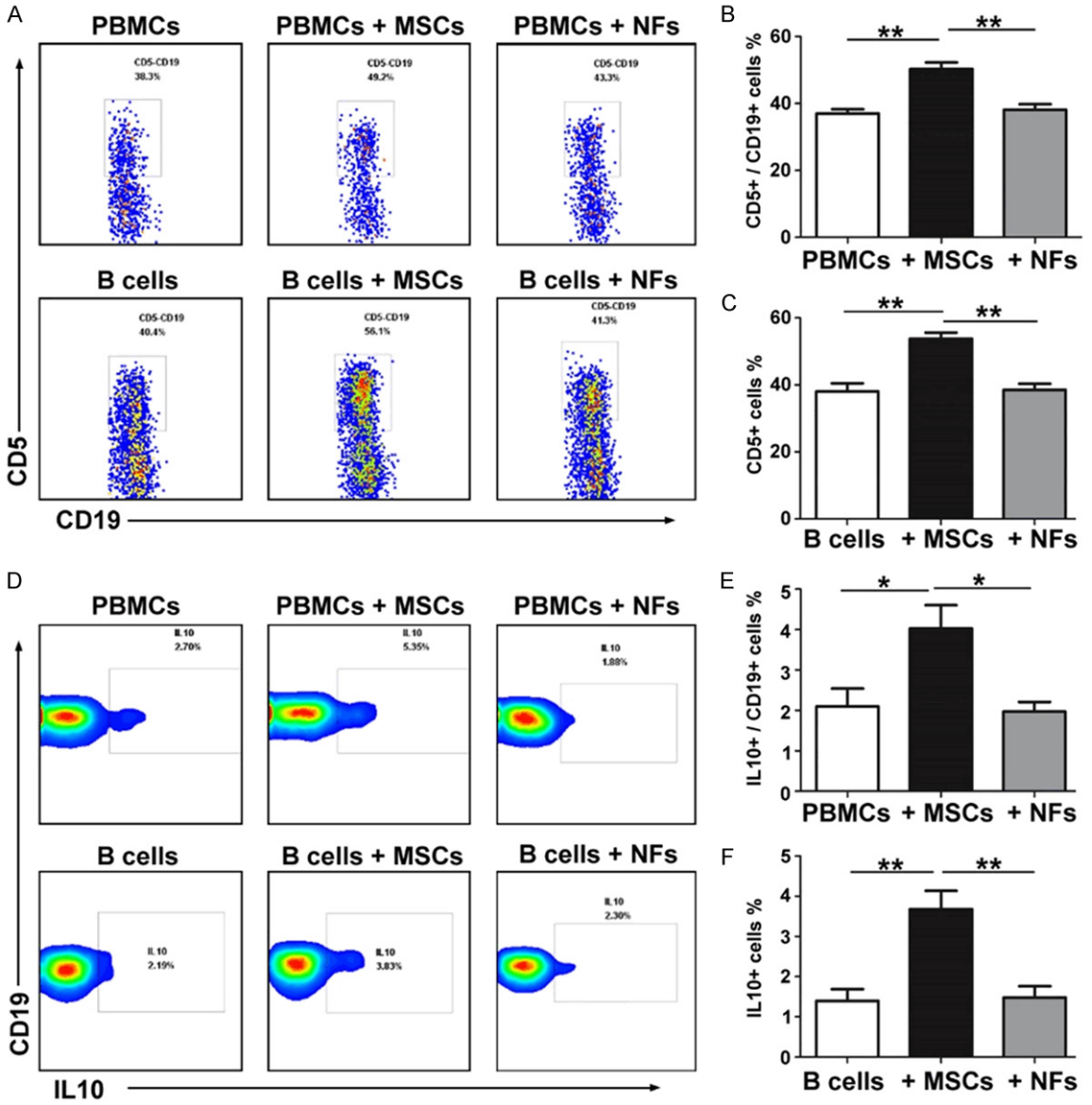


Figure 4. MSCs increase the frequency of CD5+/IL-10+ B cells *in vitro*. The CD5 expression of CD19+ B cells (A-C) in PBMCs or B cells alone and PBMCs or B cells co-culture with MSCs or NFs were detected by FASC. The IL-10 expression of CD19+ B cells (D-F) in PBMCs or B cells alone and PBMCs or B cells co-culture with MSCs or NFs were detected by FASC. The symbols represent individual samples, the horizontal bars represent the mean, and the error bars show the SEM. Significant differences are indicated as follows: $*P < 0.05$, $**P < 0.01$.

been considered to be central to MS pathophysiology [42-45]; increasing evidence also suggests additional immune cellularity, including B cells and NK cells, dendritic cells, and macrophages, play roles in this complex disorder [46]. However, the role of these immune cell subsets in the process of MS pathology has been both understudied and controversial. It is likely that different stages and different treatments have interfered with the explanation of these results.

In this study, we analyzed the phenotype distribution changes of PBMCs in 10 newly diagnosed patients with MS. Compared with the healthy subjects, we observed an increasing trend but not statistically significant in the T cell subsets in PBMCs of the MS patients (**Figure 1A-C**). MS had been viewed as a CD4+ T cell-mediated autoimmune disease, but studies found that the frequency of CD8+ T cells was greater than that of CD4+ T cells in inflamed plaques, CSF and blood of some MS patients

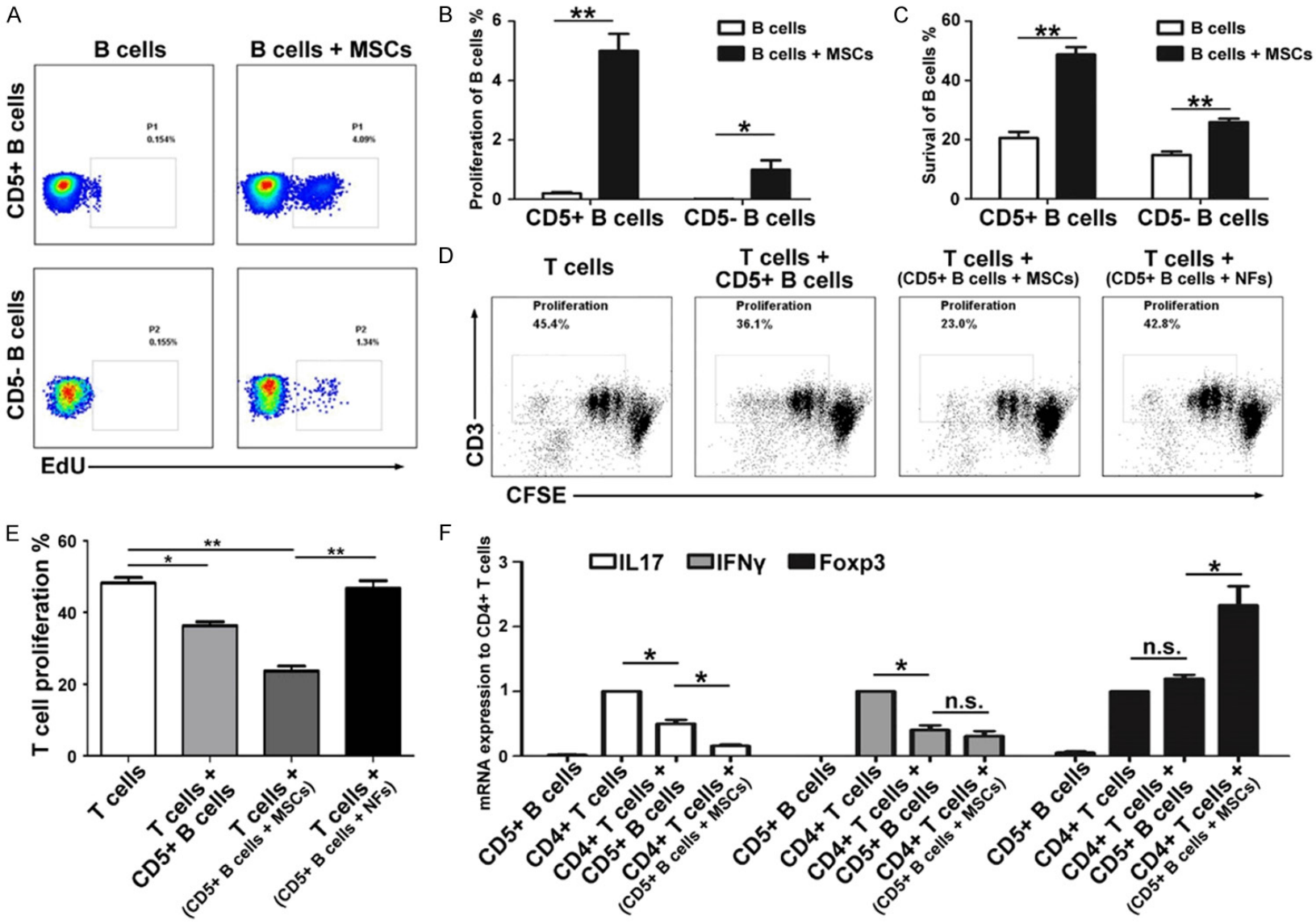


Figure 5. MSCs influence the proliferation, survival and function of CD5+ B cells. In the presence of MSCs, the proliferation (A and B) and survival rate (C) of CD5+ B cells were much higher than those of CD5- B cells in the MS patients and also much higher than those in the absence of MSCs. MSC-activated CD5+ B cells from MS patients markedly inhibit the proliferation of CD3+ T cells compared with the NF-activated or inactivated CD5+ B cells (D and E). MSC-activated CD5+ B cells

from patients markedly inhibit the IL-17 production by T cells, and markedly promoted the Foxp3 expression of T cells compared with the NF-activated or inactivated CD5+ B cells (F). The symbols represent individual samples, the horizontal bars represent the mean, and the error bars show the SEM. Significant differences are indicated as follows: $*P < 0.05$, $**P < 0.01$.

[5]. While there also were the reports of reduced frequency and function of CD4+ CD25+ Foxp3+ Tregs in MS patients [47, 48], our results demonstrated there is no significant difference on T cell subpopulations on newly diagnosed patients with MS. Thus, the immunologic mechanism of MS is complex and multifactorial. Future studies on different stages, dynamic change with increased sample sizes are warranted.

In MS patients, significant change in B cells was observed in their PBMCs (**Figure 1A-C**), this finding in accordance with previously reported results [49]. We also found a decrease in the frequency of NK cells (**Figure 1D** and **1E**), but the functional activity of NK cells seemed no significantly different between MS patients and healthy control (**Figure 2C** and **2D**). Previous studies have revealed that activity of NK cells dramatically changed in different stages of MS [50].

The MS patients exhibited an increase in B cell frequency in PBMCs (**Figure 1F** and **1G**), which indicates that MS patient's humoral immune system was activated, causing the corresponding immune damage, leading to the development of MS disease. But the frequency of CD5+ B cells within the B cell population of MS markedly decreased, B cells from MS patients were also shown to have a significantly diminished capacity to express IL-10 (**Figure 2E-H**), this alternation was recently observed by Piancone *et al* [51]. Importantly, the frequency of CD5+ and IL-10+ B cells in MS patients with an EDSS score of 2 or less is significantly higher than these in MS patients with an EDSS score greater than 2 (**Figure 2I** and **2J**), which indicates that MS progression is negatively correlated with the ratio of regulatory B cells. In EAE model, studies have found that specifically IL-10 producing B cells (Bregs) predominantly control the disease initiation [13, 14], these B cell subsets are necessary for EAE recovery [52], lack of these cells causes chronic inflammation [16]. Although other molecular markers including FSC, Foxp3, CD1d, $CD24^{hi}CD38^{hi}$, CD27+, Granzyme, PD1, and IL35 have been suggested to identify Breg cells, CD5+ IL-10+ are mostly applied molecular markers and they may somehow overlay with these molecules. In addition, Tregs are considered as an important regulator for immune response [53-55], nonetheless, Bregs were also able to alleviate the inflammation by a direct effect or an indirect effect that promotes Tregs differentiation and maintenance, and then reduces the differentiation of Th1 and Th17 cells [56]. These reports and our results indicate that the frequency and function of IL-10 producing B cells were both impaired in MS and EAE.

MSCs have been demonstrated in preclinical and clinical therapies for a variety of inflammatory and autoimmune diseases [57, 58]. In a recent clinical trial report, in comparison with placebo treatment, the frequency of CD19+ IL-10+ B cells was found an increase in the blood when MS patients were treated with MSCs [59], even if the percentage of this B cell subset respect to the total B population in MSC-treated patients was decreased. However, the mechanism underlying this observation remains to be elucidated. To verify the effect of MSCs on B cells, we established an MSC-treated EAE model. Administration of MSCs with a therapeutic protocol significantly decreased the infiltration of mononuclear cells and demyelination in the spinal cords compared with those treated with PBS (**Figure 3A-D**), and MSCs significantly increased the frequency of CD5+ IL-10+ B cells in EAE model (**Figure 3E** and **3F**). These results indicate that MSCs may improve the symptoms of MS patients by increasing Bregs.

We next analyzed the impact of MSCs on the phenotype and function of B cells from MS patients *in vitro*. Our results showed that co-culturing with MSCs increased the frequency of CD5+ IL-10+ B cells (**Figure 4**), enhanced the immunosuppressive capacity of CD5+ B cells (**Figure 5D-F**). In order to clarify how MSCs modulate the proportion of CD5+ B cells, we analyzed the effects of MSCs on CD5+ B cell proliferation and apoptosis. We found that MSCs not only enhanced proliferative capacity

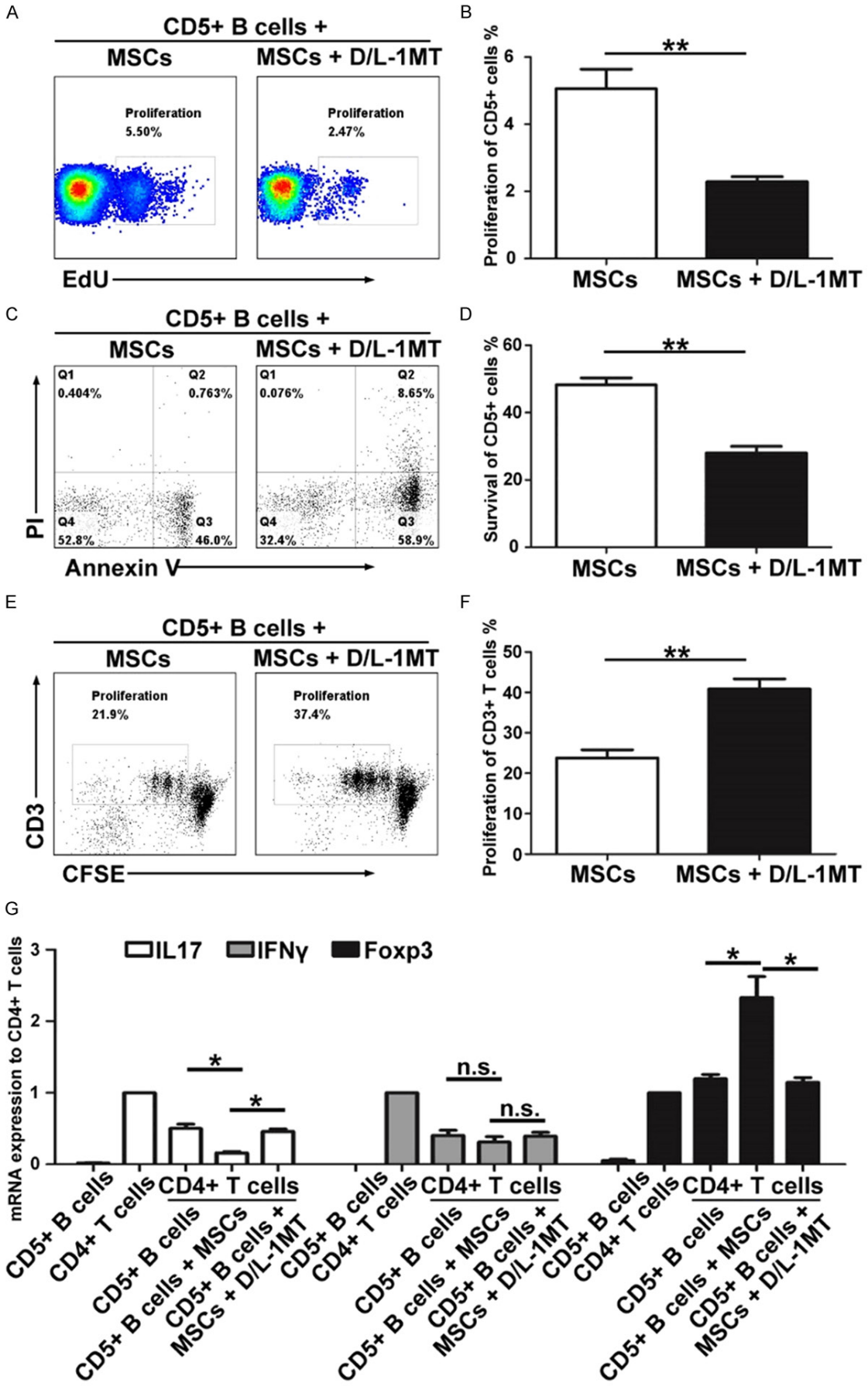

A
CD5+ B cells +
MSCs
MSCs + D/L-1MT
Proliferation
5.50%
Proliferation
2.47%
EdU
B
Proliferation of CD5+ cells %
**
MSCs
MSCs + D/L-1MT
C
CD5+ B cells +
MSCs
MSCs + D/L-1MT
Q1
0.404%
Q2
0.763%
Q4
52.8%
Q3
46.0%
Q1
0.076%
Q2
8.65%
Q4
32.4%
Q3
58.9%
PI
Annexin V
D
Survival of CD5+ cells %
**
MSCs
MSCs + D/L-1MT
E
CD5+ B cells +
MSCs
MSCs + D/L-1MT
Proliferation
21.9%
Proliferation
37.4%
CD3
CFSE
F
Proliferation of CD3+ T cells %
**
MSCs
MSCs + D/L-1MT
G
IL17
IFNγ
Foxp3
mRNA expression to CD4+ T cells
*
*
n.s.
n.s.
*
*
CD5+ B cells
CD4+ T cells
CD5+ B cells + MSCs
CD5+ B cells + MSCs + D/L-1MT
CD4+ T cells

Figure 6. MSCs influence the proliferation, survival and function of CD5+ B cells *via* the IDO pathway *in vitro*. D/L-1MT partially reversed the MSC-mediated effects on CD5+ B cell proliferation (A and B), survival (C and D), and function (E-G). The symbols represent individual samples, the horizontal bars represent the mean, and the error bars show the SEM. Significant differences are indicated as follows: $*P < 0.05$, $**P < 0.01$.

but also inhibited apoptosis in CD5+ B cells. These results indicate that MSCs are able to increase the proportion of Bregs by promoting proliferation but inhibiting apoptosis of CD5+ B cells of MS patients.

Several studies had demonstrated that MSCs were capable of producing a wide range of immunomodulatory factors, such as IDO, prostaglandin E2 (PGE-2), nitric oxide (NO) and TGF-β when they stimulated by an adequate pro-inflammatory [60]. In chronic graft-versus-host disease (cGVHD), MSC infusion was found to increase the number of IL-10 producing B cells in an IDO pathway dependent manner [61]. So we investigated potential mechanisms by which MSCs might regulate CD5+ B cells. Our results showed that D/L-1MT, the specific IDO inhibitor, could partially reverse the MSC-mediated effects on proliferation and survival of CD5+ B cells of MS patients, and the immunosuppressive function of CD5+ B cells which enhanced by MSCs was also blocked by D/L-1MT. These results suggest that MSCs may promote proliferation, survival, and function of CD5+ B cells *via* IDO pathway.

In summary, this study suggests that MSC treatment likely improves symptoms in EAE model and MS patients by increasing CD5+ IL-10+ B cells. In MS patients, we found that CD5+ IL-10+ B cells markedly decreased, although the sample size still needs to be enlarged to confirm this observation. For the first time, we found that MSCs could increase the frequency and function of CD5+ B cells by promoting proliferation and survival of CD5+ B cells from MS patient *via* IDO pathway. Our findings provide new clues for illustrating the therapeutic mechanisms of MSC-based treatments for patients with MS.

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Disclosure of conflict of interest

None.

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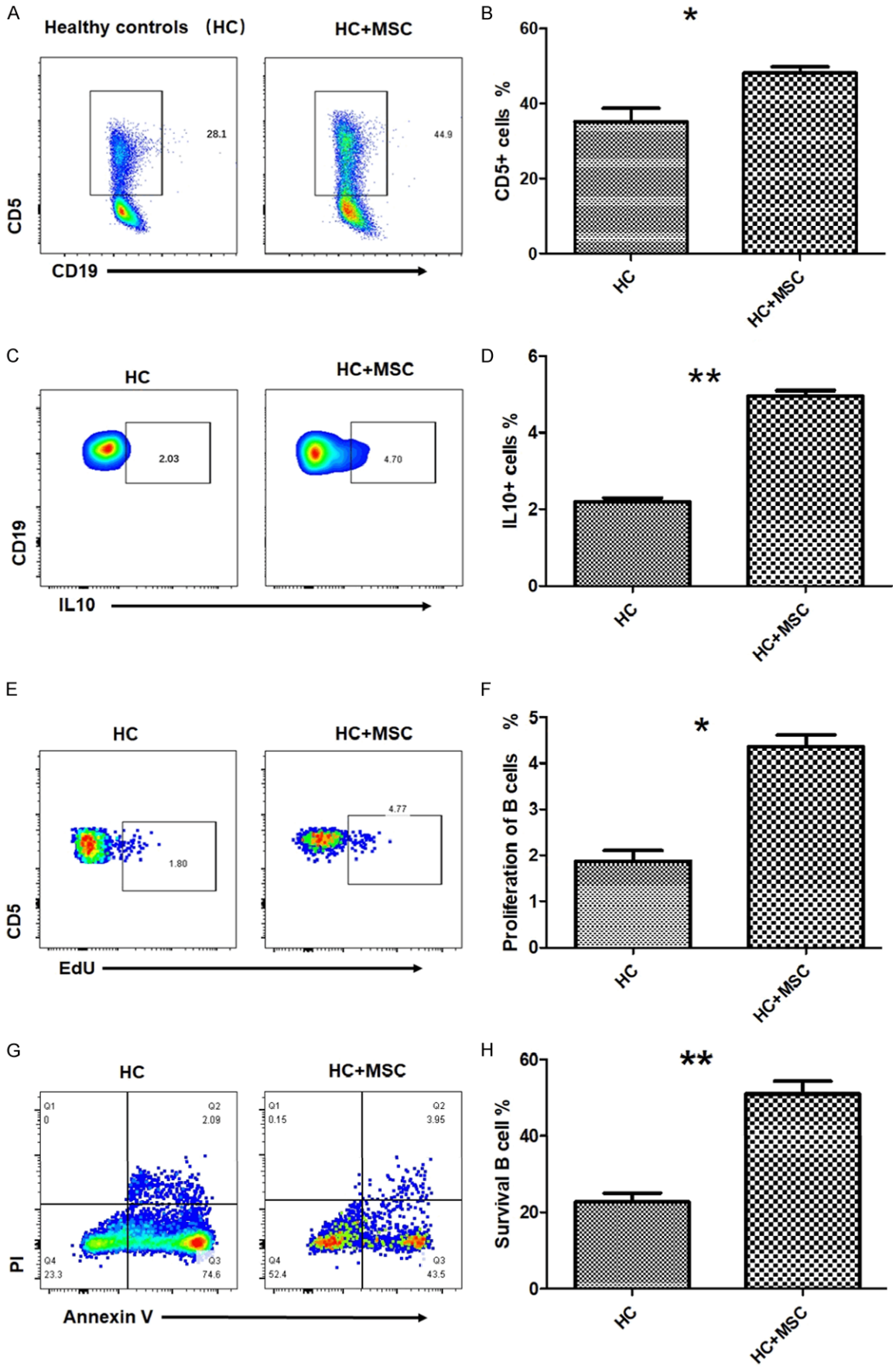
A
Healthy controls (HC)
HC+MSC
28.1
44.9
CD5
CD19
B
*
CD5+ cells %
60
40
20
0
HC
HC+MSC
C
HC
HC+MSC
2.03
4.70
CD19
IL10
D
**
IL10+ cells %
6
4
2
0
HC
HC+MSC
E
HC
HC+MSC
1.80
4.77
CD5
EdU
F
*
Proliferation of B cells %
5
4
3
2
1
0
HC
HC+MSC
G
HC
HC+MSC
Q1
0
Q2
2.09
Q4
23.3
Q3
74.6
Q1
0.15
Q2
3.95
Q4
52.4
Q3
43.5
PI
Annexin V
H
**
Survival B cell %
60
40
20
0
HC
HC+MSC

Figure S1. The effect of MSCs on CD5+ B cell differentiation, proliferation and survival *in vitro*. The CD5 expression of CD19+ B cells (A and B) in PBMCs alone and PBMCs co-culture with MSCs were detected by FASC. The IL-10 expression of CD19+ B cells (C and D) in PBMCs alone and PBMCs co-culture with MSCs were detected by FASC. In the presence of MSCs, the proliferation (E and F) and survival rate (G and H) of CD5+ B cells were much higher than those in the absence of MSCs. The symbols represent individual samples, the horizontal bars represent the mean, and the error bars show the SEM. Significant differences are indicated as follows: $*P < 0.05$, $**P < 0.01$.