Interferon-γ-Treated Mesenchymal Stem Cells Modulate the T Cell-Related Chemokines and Chemokine Receptors in an Animal Model of Experimental Autoimmune Encephalomyelitis

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ABSTRACT

Background Mesenchymal stem cells (MSCs) modulate immune responses, and their immunomodulatory potential can be enhanced using inflammatory cytokines. Here, the modulatory effects of IFN-γ-licensed MSCs on expression of T cell-related chemokines and chemokine receptors were evaluated using an experimental autoimmune encephalomyelitis (EAE) model.

Material and Methods EAE was induced in 3 groups of C57bl/6 mice and then treated with PBS, MSCs and IFN-γ-treated MSCs. The EAE manifestations were registered daily and finally, the brain and spinal cords were isolated for histopathological and gene expression studies.

Results The clinical scores were lowered in MSCs and IFN-γ-licensed MSCs groups, however, mice treated with IFN-γ-licensed MSCs exhibited lower clinical scores than MSCs-treated mice. Leukocyte infiltration into the brain was reduced after treatment with MSCs or IFN-γ-licensed MSCs compared to untreated group ($P < 0.05$ and $P < 0.01$, respectively). In comparison with untreated EAE mice, treatment with MSCs reduced CCL20 expression ($P < 0.001$) and decreased CXCR3 and CCR6 expression ($P < 0.02$ and $P < 0.04$, respectively). In comparison with untreated EAE mice, treatment with IFN-γ-licensed MSCs reduced CXCL10, CCL17 and CCL20 expression ($P < 0.05$, $P < 0.05$, and $P < 0.001$, respectively) as well as decreased CXCR3 and CCR6 expression ($P < 0.002$ and $P < 0.02$, respectively), whilst promoting expression of CCL22 and its receptor CCR4 ($P < 0.0001$ and $P < 0.02$, respectively). In comparison with MSC-treated group, mice treated with IFN-γ-licensed MSCs exhibited lower CXCL10 and CCR6 expression ($P < 0.002$ and $P < 0.01$, respectively), whereas greater expression of CCL22 and CCR4 ($P < 0.0001$ and $P < 0.01$, respectively).

Conclusion Priming the MSC with IFN-γ can be an efficient approach to enhance the immunomodulatory potential of MSCs.

Introduction

Multiple sclerosis (MS) is an autoimmune disease that is caused by inflammatory responses of the immune system against the central nervous system (CNS) [1, 2]. Various types of leukocytes, such as macrophages, dendritic cells (DCs), $CD4^+$ T, and $CD8^+$ T and B cells are infiltrated into the CNS during the early phases of MS [1, 2]. The pathogenic immune responses against myelin-related components play a crucial role in the development of MS [1–4]. T cell responses, in particular, play a key role in the pathogenesis of MS and experimental autoimmune encephalomyelitis (EAE), as an applicable animal model for MS [3, 4]. Th1 and Th17, as pathogenic $CD4^+$ T cells, mediate the demyelination of neurons in the CNS. In contrast, Th2 and regulatory T (Treg) cells regulate over-activated immune responses by their cytokines [2, 4, 5]. Accumulation of lymphocytes across the blood–brain barrier (BBB) is a critical step for the entry of these cells into the CNS [6]. In this scenario, chemokines, which are chemotactic cytokines, attract immune cells into the inflammatory site by binding to their specific receptors on the cellular surface [4, 7]. The C-X-C motif chemokine receptor 3 (CXCR3) on the surface of Th1 cells interacts with C-X-C motif chemokine ligand 9 (CXCL9), CXCL10, and CXCL11 producing after tissue inflammation [8]. Chemokine ligand 20 (CCL20) leads to the attraction of Th17 cells by binding to C-C motif chemokine receptor 6 (CCR6) expressed by these cells [9, 10]. On the other hand, the transit and retention of Th2 and Treg cells into the inflammatory site depends on the pairing of CCR4 on these cells with CCL17 and CCL22 chemokines [11, 12].

Mesenchymal stem cells (MSCs) are capable to exert immunomodulatory impacts on innate and adaptive immune systems by releasing various kinds of soluble molecules and direct cellular contacts [13–17]. Many pre-clinical and clinical studies of autoimmune diseases showed that treatment with MSCs can attenuate the function and expansion of pro-inflammatory Th1- and Th17 cells, whilst promoting the Th2 and Treg cell-mediated anti-inflammatory responses [18–20]. Nowadays, the clinical use of MSCs is one of the attractive approaches for the treatment of autoimmune diseases such as MS, due to their self-renewal ability and their capacity for differentiation into neural cells [21–23].

Some advanced clinical trials disclosed inappropriate results and limited efficacy of MSCs. One of the main reasons for this paradox is the different concentrations of inflammatory cytokines in the microenvironment, which influence the plasticity of MSC function [24]. Recent data clarified that the maximum immunomodulatory function of MSC is obtained in the presence of inflammatory cytokines, such as interferon-gamma (IFN-γ) [24]. Hence, priming of MSCs with inflammatory cytokines is now an alternative pathway to improve the therapeutic potential and immunomodulatory functions of these cells before administration [17]. It has been shown that priming the MSCs with IFN-γ remarkably enhances the production of immunoregulatory mediators including TGF-β and IDO (indoleamine 2,3-dioxygenase) that inhibit T-cell proliferation, whilst inducing the generation of Treg cells [25–29]. However, further research is needed to fully comprehend the immunomodulatory capabilities of IFN-γ-licensed MSCs.

EAE, a MS-like and T cell-dependent disorder, is extensively used as a proper and reliable animal model to study the pathological processes of MS [30, 31]. Moreover, EAE is used as a valid and reliable model to test novel treatment approaches for MS [30, 31]. Therefore, in this study, we treated EAE mice with IFN-γ-licensed MSCs to assess the effect of these cells on the expression of chemokines and chemokine receptors which have an undeniable role in the migration of different T cells into the inflamed CNS.

Materials and Methods

Materials

Female C57BL/6 mice (8–10 weeks) were purchased from Royan Institute (Isfahan, Iran). Type I collagenase was purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's Modified Eagle Medium/F12 (DMEM/F12) was supplied by Biowest (France). Fetal bovine serum (FBS) and penicillin-streptomycin were bought from Gibco (Waltham, Massachusetts, USA). Recombinant mouse IFN-γ was purchased from Biolegend (San Diego, California, USA). Adipogenic and osteogenic mediums were obtained from Sigma-Aldrich (St. Louis, Missouri, USA). Monoclonal antibodies against mouse CD73, CD105, CD90, CD34 and CD45 were purchased from eBiosciences (San Diego, California, USA). mRNA extraction and cDNA synthesis kits were obtained from Parstous (Mashhad, IRAN). SYBR Green Master Mix was bought from Takara Bio (Otsu, Japan).

Isolation, culturing and treatment of adipose tissue-derived mesenchymal stem cells (AD-MSC) with IFN-γ

The enzymatic method was used to obtain adipose tissue-derived MSCs (AD-MSCs). Firstly, epididymal fat was dissected from 8-weeks male C57BL/6 mice under sterile conditions. Then, fat tissues were placed in a petri dish containing cell culture medium (DMEM/F12) with 1 % penicillin/streptomycin and cut into small pieces. In the next stage, type I collagenase (0.075 %) was added to the samples and incubated at 37°C and 5 % CO2 for 30 minutes for tissue digestion [32]. During the incubation time, the sample tubes were shaken vigorously every 10 minutes. Then, DMEM was added to neutralize the enzyme and cell suspension was centrifuged at 1000 rpm for 5 min. After spinning, the supernatant was discarded and the AD-MSC pellet was resuspended in DMEM supplemented with heat-inactivated FBS, penicillin (100 U/ml) and streptomycin (100 U/ml) and cultured in a T25 flask. Forty-eight hours after culture, the medium was changed to remove all non-adherent cells. Afterward, the culture medium was changed every 2 days and Cells were detached when they reached 80 % confluency using Trypsin/EDTA solution. The harvested cells were transferred into the new flasks and expanded for further characterization or treated with 10 ng/ml recombinant mouse IFN-γ (for 48 h) as a stimulant of immunomodulatory functions of MSCs [33]. The dose of IFN-γ and the number of the infused MSCs were determined according to the previous experiments [33–37]. Wang et al. [38] reported that the long-time incubation with high IFN-γ doses suppressed MSCs-induced immunomodulatory activities. Moreover, Takeshita et al. [36, 39] also reported that IFN-γ impacted the MSC-induced modulatory effects in a dose-dependent process.

Flow-cytometry analysis of AD-MSCs

Five monoclonal antibodies were used to check the positive and negative cell surface markers which are necessary for the identification of the MSCs phenotype. Briefly, harvested cells from passage 3–5 were labelled with isotype control antibodies or monoclonal antibodies against mouse CD73, CD105, CD90 (as positive markers), CD34, and CD45 (as negative markers). Then, sample tubes were kept at 4°C for 1 h, treated with 1 % paraformaldehyde and cell markers were detected by flow cytometry (BD FACSVerse, BD Bioscience, USA). The interpretation of data was carried out using FlowJo 10 software.

Differentiation assays for AD-MSCs

The ability of AD-MSCs to differentiate into adipocytes and osteocytes was also assessed according to standard protocols as follows. MSCs were reseeded in 6 well plates (1×10^3 cells/cm2) and after proper confluency (more than 90 %), the culture medium was aspirated and differentiation mediums (Ready-to-use) were added. MSCs were cultured in the adipogenic or osteogenic medium for 3 weeks and the mediums were refreshed every 3 days. Afterward, Oil Red O and Alizarin red staining were applied to detect the intracellular lipid droplets of differentiated adipocytes and calcium deposits, respectively [40].

Animal immunization and clinical scoring

Female C57BL/6 mice (6–8 weeks old) were purchased from the Royan Institute, Isfahan, Iran. Animal care conditions in terms of circadian rhythm and access to water and food were in line with Kerman University of Medical Sciences rules. The Ethics Committee of the mentioned university verified the research protocol with the ethical approval code: IR.KMU.REC.1396.1542. The EAE induction was similar to the formerly published study [41]. Briefly, an emulsion of myelin oligodendrocyte peptide (MOG_{35-55}) and complete Freund's adjuvant was injected subcutaneously into the flank and between the shoulders of the mice. 250 ng pertussis toxin was also given twice intraperitoneally at the time of EAE induction and 48 h later. The animals' clinical disability scores were assigned as follows: 0 = normal, 0.5 = flaccidity and partial tail paralysis, 1 = entire tail paralysis, 1.5 = weakness in one hind limb, 2 = weakness in both hind limbs, 2.5 = partial hind limb paralysis, 3 = complete hind limb paralysis, 3.5 = partial forelimb paralysis, 4 = full forelimb paralysis, and 5 = death [42]. In addition to clinical scores, body weight was also recorded daily until day 30 post-EAE induction.

Cell therapy protocol

To assess the *in vivo* effects of IFN-γ-licensed MSCs on EAE disease, the mice were randomly divided into four groups (five mice in each group). The treatment protocol was started when the first clinical signs appeared in animals (on day 9). Two healthy and EAE control groups were injected with phosphate-buffered saline (PBS) intraperitoneally. The other two groups received 1×10^6 AD-MSCs or IFN-γ-licensed MSCs respectively. Primarily 10 mice were sacrificed for MSC isolation. Several thousands of MSCs were isolated from each mouse. The isolated MSCs were expanded *in vitro* and then 1×10^6 of the licensed- or non-licensed MSCs in 150 µl of PBS were administrated intraperitoneally on day 9 after MOG injection. Thus, all MSC-treated mice received the same cell therapy method.

Histopathological evaluation

At the end of the study, mice were euthanized with ketamine-xylazine. Brain tissues were removed and preserved in formalin. After dehydration, the tissues were embedded in a block of paraffin wax and prepared for sectioning and staining. Finally, all tissues were stained with hematoxylin as a basic dye and eosin as an acidic dye (H & E) and tissue infiltration of leukocytes was observed by an optical microscope. A 5-point scale was considered for the severity of leukocyte infiltration into the brain. Scale 0 was assigned to samples with no inflammation, scale 1 for cellular infiltrates around blood vessels, and scale 2 to 5 for mild, moderate, and serious cellular infiltrates in the parenchyma, respectively.

Assessment of the mRNA expression of chemokines and chemokine receptors

To assess the effects of IFN-γ-licensed MSCs on the expression of chemokines and chemokine receptors, mRNA was extracted from spinal cords based on the kit procedure. The cDNA was synthesized from 2 µg of total RNA via a cDNA synthesis kit. mRNA expression of chemokines, chemokine receptors and GAPDH (as a housekeeping gene) was evaluated with SYBR Green assay. All experiments were performed with the ABI StepOnePlus instrument. Eight pairs of primers were designed using Primer3 software (▶ **Table 1**). The relative expression of the evaluated genes was estimated to the GAPDH gene and computed using the $2^{-\Delta\Delta ct}$ formula.

Statistical analysis

The data of all experiments were analyzed by the SPSS 21 and GraphPad Prism V6.2 software. The differences in body weight data were assessed by one-way and two-way analysis of variance (ANOVA) tests. One-way ANOVA followed by the student's t-test was used to compare clinical scores, histological scores and real-time PCR data. All data were reported as mean ± standard error of

▶ **Table 1** Used primers to estimate the mRNA expression of chemokine and chemokine receptors by real-time PCR.

Genes	Primers	PCR product sizes (pb)
CXCR3	Forward: 5′-TTGCCCTCCCAGATTTCATC-3′	57
	Reverse: 5′-TGGCATTGAGGCGCTGAT-3′	
CXCL10	Forward:5′-GATGACGGGCCAGTGAGAAT-3′	57
	Reverse:5′-GCTCGCAGGGATGATTTCA-3′	
CCR4	Forward:5′- CACAGACACCACCCAGGAT-3′	151
	Reverse:5′- TCCAAACAGACCCAACAAGA-3′	
CCL17	Forward:5′- TCCAGGGCAAGCTCATCTGT-3′	59
	Reverse:5′-TCTGATGGCCTTCTTCACATGT-3′	
CCL22	Forward:5′- CTACATCCGTCACCCTCTGC -3′	142
	Reverse:5′- CTTCTTCACCCAGACCTGCC -3′	
CCR6	Forward:5′- CCTCACATTCTTAGGACTGGAGC -3′	151
	Reverse: 5′- GGCAATCAGAGCTCTCGGA -3′	
CCL20	Forward:5′- TTCACAAGACAGATGGCCGA -3′	143
	Reverse:5′- ATCTTCTTGACTCTTAGGCTGAGG -3′	
GAPDH	Forward:5′- CATGGCCTTCCGTGTTCCTA -3′	55
	Reverse:5′- GCGGCACGTCAGATCCA -3′	

the mean (SEM) and P-values ≤ 0.05 were considered statistically significant.

Results

Differentiation of AD-MSCs into osteocytes and adipocytes, and flow cytometry analysis

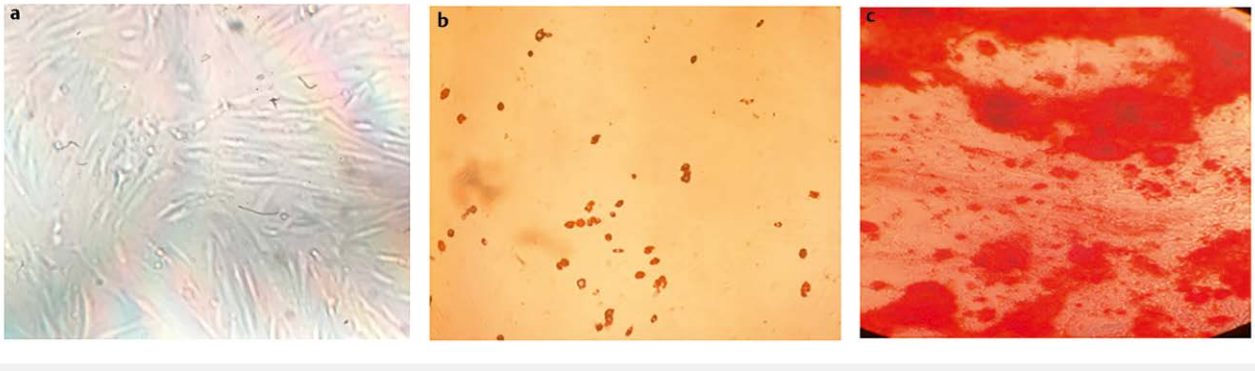
AD-MSCs were easily obtained from mice following isolation and enzymatic digestion of fat tissues. The spindle-shaped appearance of AD-MSCs was observed after the attachment of these cells to the inner surface of the flask (▶ **Fig. 1a**). After two passages, AD-MSCs differentiated into adipocytes under an adipogenic differentiation medium. The accumulated lipid droplets which filled or rounded differentiated cells were identified by Oil Red O staining (▶ **Fig. 1b**). The osteogenic potential of undifferentiated AD-MSC was also detected by alizarin red staining of calcified deposits after 3 weeks of incubation in osteogenic differentiation media (▶ **Fig. 1c**). As seen in the ▶ **Fig. 2**, the result of flow cytometry showed that these cells were positive for CD73 (99.6 %), CD90 (84.6 %) and CD105 (99.0 %) markers, and negative for CD34 (0.548 %), CD45 (0.670 %) markers. Similar to our studies, in other studies up to three positive surface markers and up to two negative surface markers were used to characterize MSCs [35, 43, 44].

Effect of IFN-γ-licensed MSCs on the clinical manifestation of EAE mice

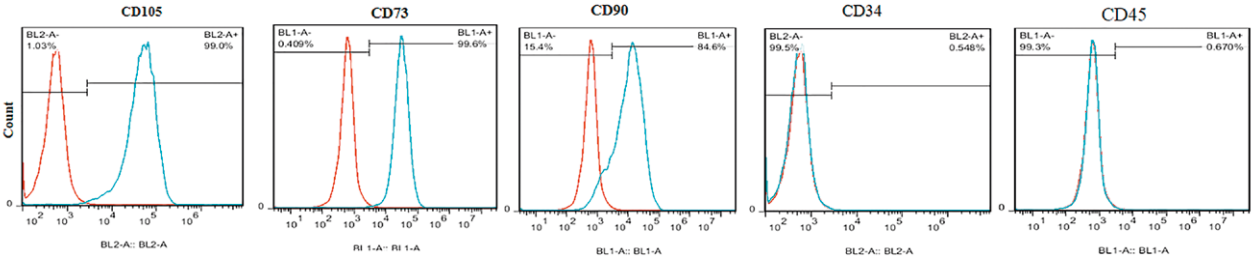
In this study, the therapeutic efficacy of AD-MSCs and IFN-γ-licensed MSCs were evaluated on EAE severity. Tail paresis as the first clinical sign of the disease was observed 8–12 days after MOG immunization. Overall, the severity of the disease was significantly different between all animal groups ($P < 0.0001$). The mean clinical scores were lowered in both AD-MSCs and IFN-γ-licensed MSCs groups than in untreated EAE control mice ($P < 0.001$ and $P < 0.0001$, respectively). Daily clinical scores in AD-MSCs and IFN-γ-licensed MSCs groups were considerably lower than the scores in the untreated EAE group ($P < 0.01$ and $P < 0.001$ at days 10–15, respectively and $P < 0.0001$ at days 16–30 for both groups). The clinical scores in the IFN-γ-licensed MSCs group were remarkably lower compared with AD-MSCs-treated mice at days 27–30 post MOG immunization ($P < 0.05$) (▶ **Fig. 3a**).

Effect of IFN-γ-licensed MSCs on body weight of EAE mice

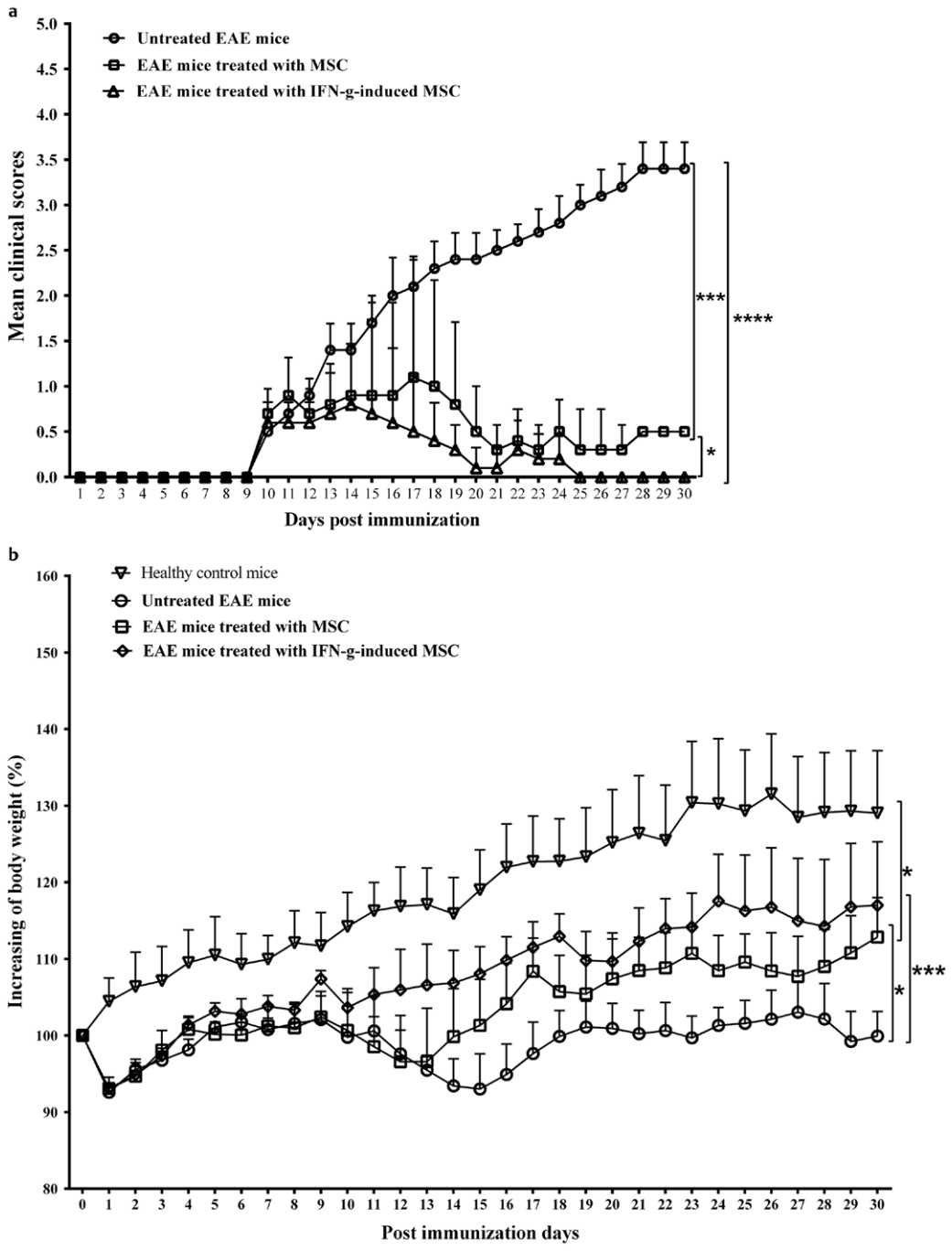
In this study, the body weight of mice was monitored daily for 30 days following immunization. As illustrated in ▶ **Fig. 3b**, in all groups, the weight of mice had an upward trend 2–9 days after MOG immunization. With the onset of disease symptoms, weight differences began to manifest between groups. Data analysis showed significant weight differences between all groups



▶ **Fig. 1** Isolation and characterization of AD-MSCs. **a**: Adipose tissue-derived mesenchymal stem cells after 3rd passage. **b**: After 21 days, adipogenic differentiation was approved via Oil Red O staining of AD-MSCs, intracellular lipid accumulation become stained bright red. **c**: After 21 days, osteogenic differentiation was observed using Alizarin Red S staining of AD-MSCs, calcium deposition became stained bright orange-red.

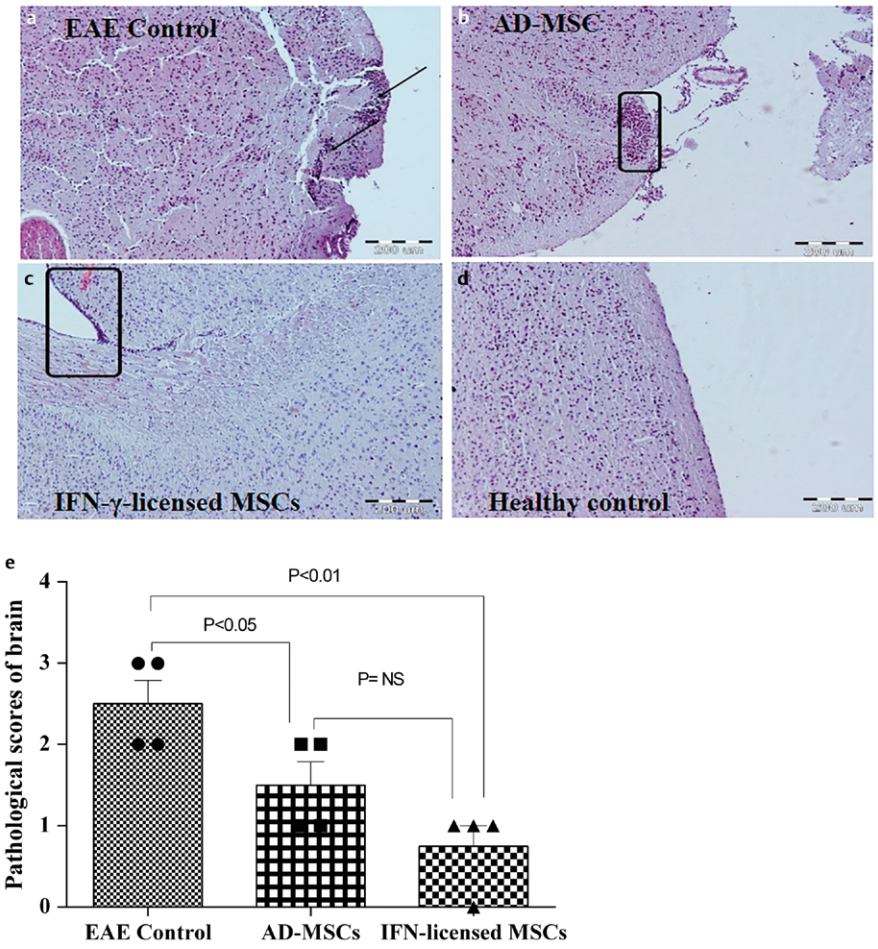


▶ **Fig. 2** Flow cytometry analysis of AD-MSCs. The expression pattern of CD105, CD73, CD90, CD34 and CD45 surface markers by AD-MSC.



▶ **Fig. 3** Clinical manifestations of EAE disease. **a**: Comparison of clinical scores.; Clinical manifestations of EAE disease. **b**: Comparison of body weight (**b**) between IFN-γ-licensed MSCs, AD-MSCs, EAE control and healthy groups. Results are presented as the Mean ± SEM. Results are presented as the mean ± SEM. One-way ANOVA followed by the student's t test was used to compare clinical scores. The difference of bodyweight data was assessed by two-way ANOVA tests.

($P < 0.005$). As expected, the healthy mice gained more weight during the study period than those in AD-MSCs, IFN-γ-licensed MSCs and untreated EAE groups ($P < 0.001$, $P < 0.05$ and $P < 0.0001$ respectively). The body weight was also higher in AD-MSCs ($P < 0.05$) and IFN-γ-licensed MSCs group ($P < 0.0001$) compared to the untreated EAE group. Overall, the body weight was higher in the IFN-γ-licensed MSCs group than in AD-MSCs-treated mice ($P < 0.001$).



▶ **Fig. 4** Histopathological patterns and leukocyte infiltration into the brain. EAE was induced on day 0 using MOG. Treatment with MSCs was performed on day 9 after MOG injection, when the first clinical EAE signs were appeared. On day 30 post EAE induction, the mice were sacrificed and the brain inflammation was assessed using hematoxylin and eosin staining. **a**: Untreated mice. **b**: AD-MSCs-treated mice. **c**: IFN-γ-licensed MSCs-treated mice. **d**: Healthy control mice.; Histopathological patterns and leukocyte infiltration into the brain. EAE was induced on day 0 using MOG. Treatment with MSCs was performed on day 9 after MOG injection, when the first clinical EAE signs were appeared. On day 30 post EAE induction, the mice were sacrificed and the brain inflammation was assessed using hematoxylin and eosin staining. **e**: The comparison of histopathological scores between groups. Results are presented as the mean ± SEM. One-way ANOVA followed by the student's t test was used to compare histological scores.

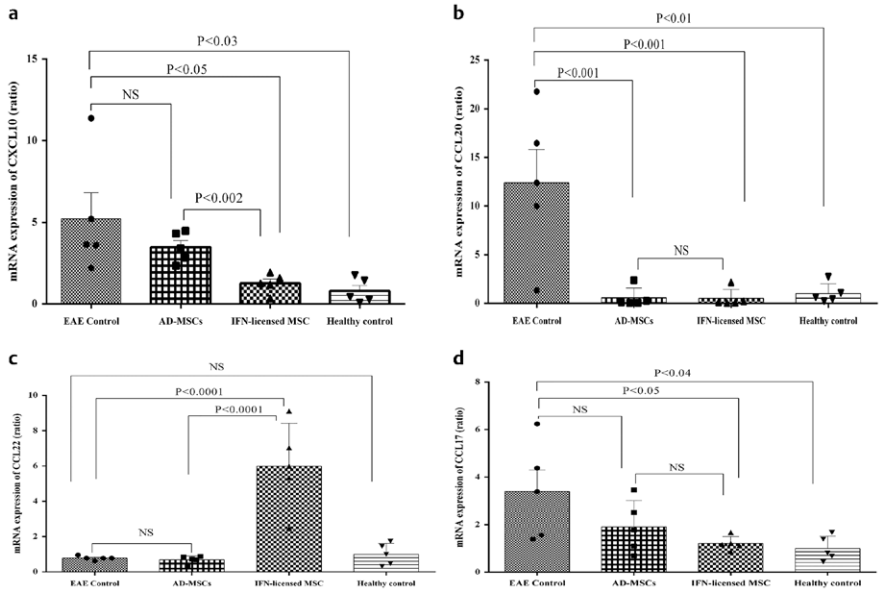
Effect of IFN-γ-licensed MSCs on leukocyte infiltration into the brain

Histopathological changes and leukocyte infiltration into the brain were evaluated by H&E staining after animal euthanasia and removal of brain tissues (▶ **Fig. 4**). Our data showed decreased infiltration of leukocytes into the brain after treatment with AD-MSCs or IFN-γ-licensed MSCs compared to the non-treated EAE group ($P < 0.05$ and $P < 0.01$, respectively). Fewer leukocyte infiltration was detected in tissue sections from EAE mice treated with IFN-γ-licensed MSCs compared to the AD-MSCs group, although the difference was not statistically significant (▶ **Fig. 4e**).

Effect of IFN-γ-licensed MSCs on the expression of chemokines

The effect of AD-MSCs or IFN-γ-licensed MSCs treatment on the mRNA expression of CXCL10, CCL20, CCL22, and CCL17 was evaluated in spinal cord tissues by real-time PCR. The mRNA expression amounts of CXCL10, CCL20 and CCL17 in spinal cord tissues collected from untreated EAE mice were substantially greater compared with those in the healthy control group ($P < 0.03$, $P < 0.01$ and $P < 0.04$, respectively).

As depicted in ▶ **Fig. 5a**, treatment of EAE mice with IFN-γ-licensed MSCs significantly decreased the expression of CXCL10 in comparison with the untreated control group ($P < 0.05$). CXCL10 expression was also lower in AD-MSCs rather than EAE group, although it was not significant. The expression of CXCL10 was remarkably lower in the IFN-γ-licensed MSCs group than in AD-MSCs-



▶ **Fig. 5** Expression of chemokine genes in the spinal cord from EAE control, AD-MSCs, IFN-γ-licensed MSCs, and healthy groups. EAE was induced on day 0 using MOG. Treatment with MSCs was performed on day 9 after MOG injection, when the first clinical EAE signs were appeared. On day 30 post EAE induction, the mice were sacrificed and the expression of chemokine genes was assessed using real time-PCR. One-way ANOVA followed by the student test was used to compare the results. **a**: The comparison of CXCL10 mRNA levels.; Expression of chemokine genes in the spinal cord from EAE control, AD-MSCs, IFN-γ-licensed MSCs, and healthy groups. EAE was induced on day 0 using MOG. Treatment with MSCs was performed on day 9 after MOG injection, when the first clinical EAE signs were appeared. On day 30 post EAE induction, the mice were sacrificed and the expression of chemokine genes was assessed using real time-PCR. One-way ANOVA followed by the student test was used to compare the results. **b**: The comparison of CCL20 mRNA levels.; Expression of chemokine genes in the spinal cord from EAE control, AD-MSCs, IFN-γ-licensed MSCs, and healthy groups. EAE was induced on day 0 using MOG. Treatment with MSCs was performed on day 9 after MOG injection, when the first clinical EAE signs were appeared. On day 30 post EAE induction, the mice were sacrificed and the expression of chemokine genes was assessed using real time-PCR. One-way ANOVA followed by the student test was used to compare the results. **c**: The comparison of CCL22 mRNA levels.; Expression of chemokine genes in the spinal cord from EAE control, AD-MSCs, IFN-γ-licensed MSCs, and healthy groups. EAE was induced on day 0 using MOG. Treatment with MSCs was performed on day 9 after MOG injection, when the first clinical EAE signs were appeared. On day 30 post EAE induction, the mice were sacrificed and the expression of chemokine genes was assessed using real time-PCR. One-way ANOVA followed by the student test was used to compare the results. **d**: The comparison of CCL17 mRNA levels. Results are presented as the mean ± SEM. One-way ANOVA followed by the student's t test was used to compare the results.

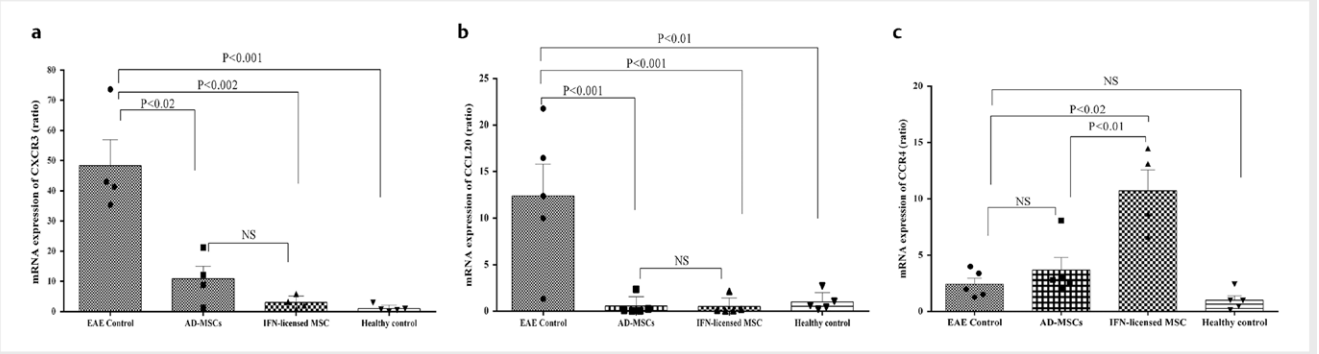
treated mice (P < 0.002). Furthermore, a significant decrease in the expression of CCL20 in mice receiving AD-MSCs and IFN-γ-licensed MSCs was observed when compared with the EAE group (P < 0.001). However, CCL20 is similarly expressed in AD-MSCs- and IFN-γ-licensed MSCs groups (▶ **Fig. 5b**).

The difference in the expression level of CCL22 was not statistically remarkable between AD-MSCs and the untreated EAE group. The obtained results also depicted higher expression of CCL22 in the IFN-γ-licensed MSCs group rather than the EAE group (P < 0.0001). The expression of CCL22 was remarkably higher in the IFN-γ-licensed MSCs group than in AD-MSCs-treated mice (P < 0.0001) (▶ **Fig. 5c**). Moreover, treatment of mice with AD-MSCs decreased the expression of CCL17, however, significantly lower expression of CCL17 was detected in the IFN-γ-licensed MSCs group than in the untreated EAE group (P < 0.05) (▶ **Fig. 5d**).

Effect of IFN-γ-licensed MSCs on the expression of chemokine receptors

The mRNA expression amounts of CXCR3 and CCR6 in spinal cord tissues collected from untreated EAE mice were remarkably greater compared with those in the healthy control group (P < 0.001, and P < 0.03, respectively). As depicted in ▶ **Fig. 6a**, treatment with AD-MSCs or IFN-γ-licensed MSCs significantly decreased the expression of CXCR3 in comparison with the untreated EAE group (P < 0.02 and P < 0.002, respectively). However, there was no significant difference between AD-MSCs- and IFN-γ-licensed MSCs groups regarding CXCR3 expression (▶ **Fig. 6a**). Treatment with AD-MSCs or IFN-γ-licensed MSCs also significantly reduced the CCR6 expression compared with untreated EAE mice (P < 0.04 and P < 0.02, respectively). The expression of CCR6 was remarkably lower in the IFN-γ-licensed MSCs group than in AD-MSCs-treated mice (P < 0.01) (▶ **Fig. 6b**).

There was no significant difference between untreated EAE mice and healthy group regarding CCR4 expression (▶ **Fig. 6c**). Moreover, no remarkable difference was found between untreated EAE mice and those treated with AD-MSCs regarding CCR4 expression. However, treatment of EAE mice with IFN-γ-licensed MSCs significantly promoted the CCR4 expression compared with untreated EAE mice (P < 0.02). The expression of CCR4 was remarka-



▶ **Fig. 6** Gene expression of chemokine receptor in the spinal cord from EAE control, AD-MSCs, IFN-γ-licensed MSCs, and healthy groups. EAE was induced on day 0 using MOG. Treatment with MSCs was performed on day 9 after MOG injection, when the first clinical EAE signs were appeared. On day 30 post EAE induction, the mice were sacrificed and the expression of chemokine receptor genes was assessed using real time-PCR. **a**: The comparison of CXCR3 mRNA levels.; Gene expression of chemokine receptor in the spinal cord from EAE control, AD-MSCs, IFN-γ-licensed MSCs, and healthy groups. EAE was induced on day 0 using MOG. Treatment with MSCs was performed on day 9 after MOG injection, when the first clinical EAE signs were appeared. On day 30 post EAE induction, the mice were sacrificed and the expression of chemokine receptor genes was assessed using real time-PCR. **b**: The comparison of CCR6 mRNA levels.; Gene expression of chemokine receptor in the spinal cord from EAE control, AD-MSCs, IFN-γ-licensed MSCs, and healthy groups. EAE was induced on day 0 using MOG. Treatment with MSCs was performed on day 9 after MOG injection, when the first clinical EAE signs were appeared. On day 30 post EAE induction, the mice were sacrificed and the expression of chemokine receptor genes was assessed using real time-PCR. **c**: The comparison of CCR4. Results are presented as the mean ± SEM. One-way ANOVA followed by the student's t test was used to compare the results.

bly greater in the IFN-γ-licensed MSCs group than in AD-MSCs-treated mice ($P < 0.01$) (▶ **Fig. 6c**).

Discussion

Nowadays, MSC therapy has attracted a lot of attention for the treatment of chronic neurodegenerative diseases, such as MS due to its immunomodulatory and neuroprotective properties [15, 45, 46]. Here, we evaluated the modulatory effects of the AD-MSCs on the expression of some chemokines/chemokine receptors in an EAE model. MSCs can be obtained from various sources, such as bone marrow, adipose tissue, umbilical cord-derived Wharton's jelly, umbilical cord blood and dental pulp [47]. Adipose tissue is abundant, and AD-MSCs can be collected in high numbers from rich adipose tissue using a minimally invasive manner [48, 49]. AD-derived MSCs possess several biological advantages, including great proliferative capability, easy *in vitro* expansion, high production of cytokines and immunomodulatory factors, high *in vitro* viability, and their potential to differentiate into several cell lineages [48–50]. The use of autologous AD-MSCs can provide promising therapeutic potential for the treatment of immune and degenerative disorders [48, 49].

MSCs require inflammatory cytokines to show their immunosuppressive functions. Previous investigations revealed that priming or licensing of MSC with IFN-γ enhances the immunosuppressive capacity of these cells [25, 28]. Hence, in the current study, we hypothesized that priming of MSCs with IFN-γ before the administration could efficiently regulate the expression of chemokines and chemokine receptors, which influence the immigration of leukocytes into the CNS and change the severity of inflammation. We observed that treatment with both MSC and IFN-γ-licensed MSC could significantly reduce the severity of clinical scores and leukocyte infiltration into the brain, while enhancing the body weight of mice after EAE induction. The clinical manifestation of EAE was better in the IFN-γ-licensed MSC group than the AD-MSC group. The body weight was considerably higher and the clinical score reached zero on the last five days of the study in mice treated with IFN-γ-licensed MSC. In accordance with our data, the preventive impacts of the IFN-γ-exposed MSCs on body weight loss and EAE-related clinical scores were indicated [34, 51]. It has also been reported that priming of MSCs with IFN-γ significantly improves the efficacy of MSC to reduce graft-versus-host disease (GVHD) mortality in mice [27]. Another study also pointed out that treatment with IFN-γ-licensed MSCs lowered clinical signs of an experimental model of colitis and enhance the survival rate and body weight of animals better than non-licensed MSC treatment [52].

In accordance with the expression levels of chemokine and their receptor, our histopathological data also demonstrated a significant reduction in the infiltration of leukocytes into the brain, especially in EAE mice treated with IFN-γ-licensed MSC. In agreement with our data, it was also revealed that the treatment with IFN-γ-primed MSCs reduced leukocyte infiltration into the CNS [51]. In this regard, the results from a study on a GVHD model showed that intravenously injection of IFN-γ-licensed MSC reduced leukocyte infiltration into the small intestine and skin of animals and enhanced the survival rate of animals [53].

The migration of circulating leukocytes into the CNS is directed by interactions between chemokines and their receptors on leukocytes, which is an essential step in disease onset [54]. Additionally, $CD4^+$ Th cells have been identified as the most important cells in the pathogenesis of MS and EAE [2, 5]. While specific cytokines of pathogenic Th1 and Th17 cells aid demyelination, anti-inflammatory cytokines of Th2 and Treg cells maintain self-tolerance and alleviate clinical manifestation [2, 4, 5]. Hence, we decided to evaluate

the effect of IFN-γ-licensed MSCs on the expression of chemokine and chemokine receptors, which are relevant to the attraction of different Th cells into the CNS.

Our results revealed that treatment with both MSC and IFN-γ-licensed MSC could decrease the expression of CXCR3, a Th1-related chemokine receptor, in spinal cords from EAE mice compared with the healthy group. However, only treatment with IFN-γ-licensed MSC significantly reduced the CXCL10 as a ligand for CXCR3. Therefore, it can be concluded that MSCs primed with IFN-γ than un-primed MSCs had a superior effect to decrease the entrance of Th1 cells into the CNS. We also observed that expression of the Th17-related chemokine and chemokine receptor (CCL20 and CXCR6 respectively) reduced following administration of MSC and IFN-γ-licensed MSC as compared to the control group, but IFN-γ-primed MSC was more efficient to reduce CCR6 expression. In addition, we assessed the expression of CCR4 as a chemokine receptor on Th2 and T-reg cells as well as its ligands, CCL17 and CCL22. In comparison with AD-MSCs, IFN-γ-licensed MSCs have more capability to promote the expression of CCR4 attracting Th2 and Treg cells which can result in attention of EAE-related symptoms. Although CCL22 (a Th2 and Treg- attracting chemokine) was similarly expressed in untreated EAE mice and the AD-MSC group, higher expression of this chemokine was detected in the IFN-γ-licensed MSC group, which may increase the infiltration of Treg and Th2 cells into the CNS and mitigate the CNS inflammation. However, our results indicated that IFN-γ-licensed MSCs exerted suppressive effects on the CCL17 expression.

The suppressive effects of the IFN-γ-induced MSCs on the expression of Th17- (IL-17 and RORγt) and Th1- (IFN-γ and T-bet) related markers in the brain of EAE mice were also indicated [34]. IFN-γ-induced MSCs significantly induced the expression of Treg cell-related markers (FOXP3 and IL-10) in the brain of EAE mice. Moreover, the MOG-stimulated lymph node cells and the MOG-stimulated splenocytes from EAE mice treated with IFN-γ-induced MSCs produced lower levels of IFN-γ and IL-17, whereas secrete higher levels of IL-4 and IL-10 compared with control cells from non-induced MSC-treated mice [34]. Thus, IFN-γ-induced MSCs can improve the Th17/Treg and Th1/Th2 imbalances in EAE mice [34]. The results from an *in vitro* experiment also indicated that IFN-γ promotes the capacity MSCs to induce Treg cell differentiation [55]. The enhancing effects of the treatment with IFN-γ-induced MSCs on the expression of Treg cell-related markers were also observed in a mouse model of allergic asthma [35]. The greater expression of CCR4 and its ligand, CCL22, can lead to the larger accumulation of the Treg and Th2 cells in the CNS. It has been indicated that treatment with IFN-γ-induced MSCs increases the number of Treg cells in the cervical and spleen of EAE mice [51].

In a preventive treatment protocol, the MSC administration before the disease onset (at day 3 and day 8 after MOG immunization), and at the disease peak (at day 15) remarkably attenuated the EAE symptoms, reduced the infiltration of inflammatory cells (T cells and macrophages) as well as decreased demyelination in CNS [56]. However, upon EAE stabilization (at day 24), MSC administration was ineffective [56], indicating that the time of treatment during the EAE course influences the effectiveness of the MSCs. Transfection of recombinant IL-23R mRNA into MSCs also promoted their immunosuppressive activities [57]. Treatment of EAE mice with IL-23R-transfected MSCs ameliorated the demyelination as well as the infiltration of inflammatory cells into the CNS [57], indicating that MSC responsiveness to other inflammatory cytokines, such as IL-23 can potentiate their immunomodulatory activities.

As mentioned, there are some studies indicating that priming of MSCs with inflammatory cytokines can improve their immunomodulatory functions. However, Dang et al. reported that MSC autophagy occurred during a preventive treatment using an EAE model [58]. Inflammatory cytokines, such as IFN-γ and TNF-α can synergistically induce autophagy in MSCs [58]. Despite these observations, the occurrence of autophagy in MSCs neither affected their capacity to modulate Th1, Th17, and Treg cell differentiation nor impacted their capability to regulate the expression of CXCR3 and CCR6 in the CNS [58]. However, autophagy inhibition promoted MSC-mediated immunosuppressive effects in T cell-induced EAE [58]. The impacts of the potent inflammatory microenvironment on MSC autophagy and MSC-mediated immunomodulation need to be clarified in future studies.

The present study may have several limitations: For example, in order to validate the licensing of MSCs, it was necessary to assess the expression of immunomodulatory markers such as IDO in the control and IFN-γ-exposed MSCs. However, it was previously indicated that the IFN-γ-exposed MSCs displayed greater expression of IDO compared to control MSCs [53, 59, 60]. There are some MSC-based therapies in humans worldwide, however, an FDA-approved MSC-based treatment does not exist, yet [61]. Before extrapolation of the animal studies to humans, the standardization of MSC-based therapy regarding some issues, such as the cellular number, injection time, lifelong of the injected cells, as well as their effect on other organs should be completely clarified.

Conclusion

Overall, the findings of our study depicted that treatment of EAE mice with IFN-γ-licensed MSC more effectively improved the clinical manifestations of the EAE, modulated the expression of some chemokines and their receptors, and reduced the leukocyte infiltration into the brain. The priming of MSC with IFN-γ can robust the MSC-mediated regulatory impacts on the expression of chemokines and their receptors. However, more studies are warranted to understand the precise mechanisms by which IFN-γ-licensed MSCs regulate immuno-inflammatory responses.

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Conflicts of Interest

The authors declare that they have no conflict of interest.

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