**ORIGINAL ARTICLE**

# Bone marrow mesenchymal stem cells to ameliorate experimental autoimmune encephalomyelitis via modifying expression patterns of miRNAs

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## Abstract

**Introduction** Clinical and experimental studies highlighted the significant therapeutic role of Mesenchymal stem cells (MSCs) in neurodegenerative diseases. MSCs possess potent immunomodulatory properties by releasing exosomes, which generate a suitable microenvironment. microRNAs (miRNAs), as one of several effective bioactive molecules of exosomes, influence cellular communication and activities in recipient cells. Recent studies revealed that miRNAs could control the progression of multiple sclerosis (MS) via differentiation and function of T helper cells (Th).

**Methods** Here, we investigated the therapeutic effects of syngeneic-derived BM-MSC in experimental autoimmune encephalomyelitis (EAE) mouse model of MS by evaluating expression profile of miRNAs, pro- and anti-inflammatory in serum and brain tissues. Three-time scheme groups (6th day, 6th & 12th days, and 12th day, of post-EAE induction) were applied to determine the therapeutic effects of intraperitoneally received $1*10^6$ of BM-MSCs.

**Results** The expression levels of mature isoforms of miR-193, miR-146a, miR-155, miR-21, and miR-326 showed that BM-MSCs treatment attenuated the EAE clinical score and reduced clinical inflammation as well as demyelination. The improved neurological functional outcome associated with enhanced expression of miR-193 and miR-146a, but decreased expression levels of miR-155, miR-21, and miR-326 were followed by suppressing effects on Th1/Th17 immune responses (reduced levels of IFN-$_\gamma$ and IL-17 cytokine expression) and induction of Treg cells, immunoregulatory responses (increase of IL-10, TGF-β, and IL-4) in treatment groups.

**Conclusion** Our findings suggest that BM-MSCs administration might change expression patterns of miRNAs and downstream interactions followed by immune system modulation. However, there is a need to carry out future human clinical trials and complementary experiments.

**Keywords** MicroRNA · Neuroinflammation · Multiple sclerosis · Experimental autoimmune encephalomyelitis · T cell differentiation

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## Introduction

Multiple sclerosis (MS) is a neurodegenerative and demyelinating autoimmune disease mediated by autoreactive T cells that affects the central nervous system (CNS). Experimental Autoimmune Encephalomyelitis (EAE) is a commonly known MS model induced by immunization with myelin antigens [1, 2]. Clinical and Cellular/macular Immunopathologia events in CNS in MS and EAE are similar. Pro-inflammatory cytokines are produced by Th1 and Th17 that have been raised in MS patients during an exacerbation of the disease [3, 4]. On the contrary, Th2/Treg cytokines with

anti-inflammatory capacity, including interleukin-4 (IL-4), interleukin-5 (IL-5), transforming growth factor (TGF-β), and interleukin-10 (IL-10), are predominant during disease remission and the recovery from the disease [5, 6].

Findings associated with the opinion that MSCs inhibit the immune response further need careful assessment of appropriate cell sources and additional scientific data. However, considering the exact mechanisms of stem cells' therapeutic and immunomodulation effects seems necessary [7].

Stem cell therapy has appeared as an innovative treatment for many diseases. MSCs, the capability to self-renew and differentiate into the variability of mesenchymal cell lineages, have emerged as a promising therapeutic intervention strategy [8]. Currently, suppressive and immunomodulatory effects of MSCs are demonstrated in in vitro and various clinical trials, although the smae mechanism by which MSCs modulate immune function remains largely unknown [8]. BM-MSCs have been proposed to treat MS and EAE, although the exact mechanisms underlying these cells' immunomodulatory functions are still largely unknown. Numerous previous studies have shown that the inhibitory effect of BM-MSCs is not exclusively reliant on cell-to-cell interaction, why so BM-MSCs possibly have other means of communication, and paracrine effects via soluble factors are responsible for the communication of secretory soluble factors [9]. Accumulating evidence shows that exosomes are one of the possible factors for intercellular communication in extracellular space, formed by MSCs, and exert their therapeutic properties in some animal disease models [10, 11]. In this regard, several dealings between exosomes and recipient cells indicate that exosomes perform a significant role in cell-to-cell interactions. Exosomes are small membrane vesicles created by a wide range of cells via inverse budding of the multivesicular bodies. Several mechanical/physical interactions have been described between exosomes and recipient cells. Exosomes contain RNA molecules, including mRNA and miRNA from the source cell, and proteins [12].

MiRNAs are short, 20–22 nucleotide non-coding RNA molecules acting as transcription and post-transcriptional regulators of several target gene expressions. The role of miRNA has been identified in various biological processes, including differentiation, cell proliferation, development, and apoptosis in different cell types. Because of their small size and constant structure in body liquids, miRNAs are an emerging group of promising biomarkers in many autoimmune diseases. Several studies have also found links between miRNAs and the onset and development of MS neurological disease [13].

Researchers discovered that some miRNAs are linked to disease action and duration, as well as different MS patterns, and have been related to the pathogenesis of MS by practically influencing the differentiation of CD4 + T cells to various T cell subtypes [14].

Some miRNAs may be reliable biomarkers and therapeutic targets for MS disease diagnosis, prognosis, and treatment monitoring. MiR-223, miR-146a, miR-155, miR-let7, miR-193, and miR-326 are among the miRNAs critical in the immunopathogenesis of MS and EAE by regulating CD4 + T cell differentiation [15–17].

miR-223 is an inflammatory miRNA, and its upregulation is associated with inflammation. In contrast to the healthy controls, it has been identified in the blood of MS patients [18]. Intriguingly, the Supplementary investigation revealed that miR-146a suppressed autocrine IL-6 and IL-21 release in T cells, preventing them from undergoing Th17 development. The autocrine IL-6 and IL-21-induced Th17 development pathways in autoreactive CD4 T cells are inhibited by miR-146a, a critical molecular brake [19]. miR-193a is increased in CD4+, CD8 + T cells, and B cells in the peripheral blood of MS patients in remission and has been associated with Treg cell induction [20, 21]. Studies have demonstrated that the miR-326 producer promoted Th17 development by targeting Ets-1, an undesired regulator of Th17 differentiation. Human autoimmune illnesses such as MS, systemic lupus erythematosus (SLE), and psoriasis have been linked to increased expression of miR-21, which promotes Th17 differentiation [22]. Through Th1 and Th17 induction, miR-155 plays a role in the activation of both T cells and macrophages, as well as the permeability of the BBB, resulting in immune-mediated destruction of the myelin sheath and neurodegeneration [23]. The role of let-7 miRNAs in the activity of diverse immune cells and the immune system has been postulated as a suppressive mechanism employed by regulatory T cells, which inhibits Treg cell formation and function while promoting Th1 and Th17 cell proliferation [24]. Understanding the processes by which exosomes play a role in immune modulation might aid the development of therapeutic programs for MSCs delivery. Here, we examined the therapeutic benefits of BM-MSCs in EAE by addressing possible impacts on miRNAs as well as pro- and anti-inflammatory cytokine production due to evidence of a critical role of exosomes in immune regulation.

## Materials and methods

The overall study methodology is depicted graphically (Fig. 1).
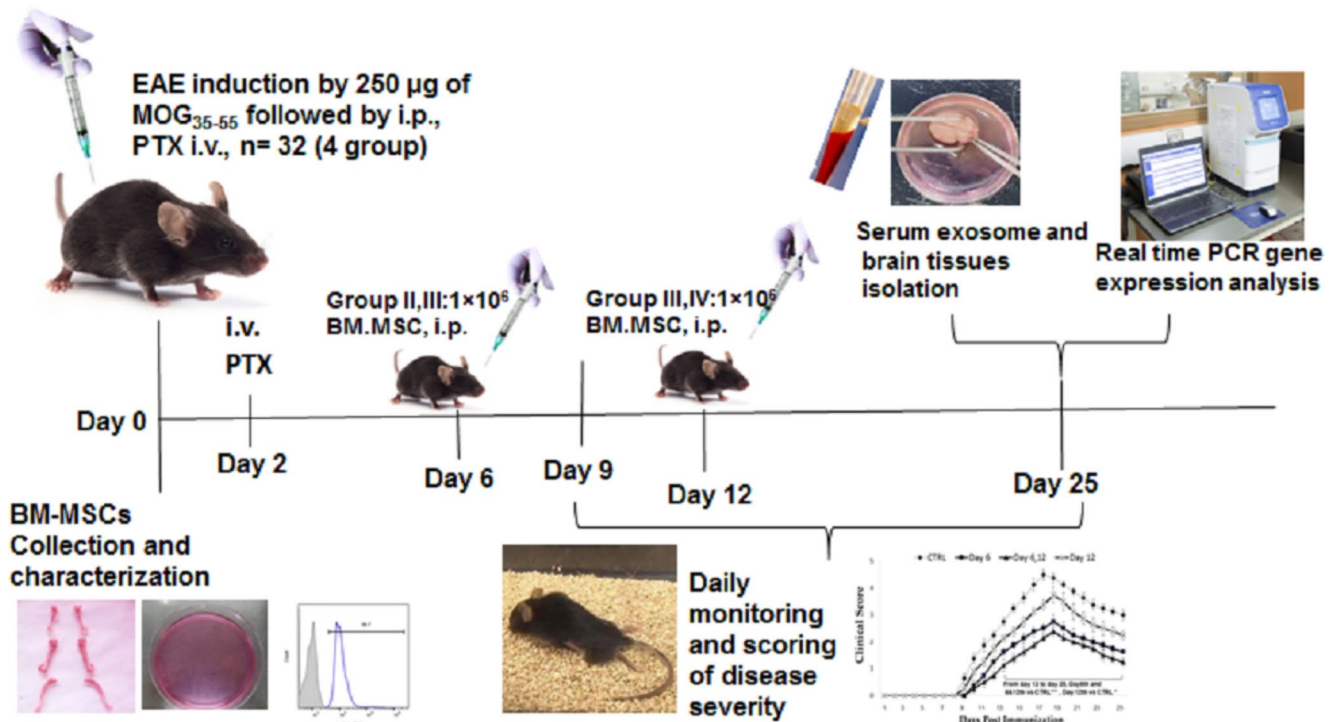
**Fig. 1** Graphical abstract of our study. The many phases of our investigation are depicted in this image, which also includes the separation of blood cells, induction and assessment of the MS animal model, grouping and treatment of the animals, and clinical, histological, and molecular assessments

## Animals

All mice were housed in a controlled environment with a temperature of 23 [25] °C, relative humidity of 50 [25] %, a 12 h light/dark cycle, and free access to water and pellet meals. All mouse handling methods were carried out in accordance with Semnan University of Medical Sciences ethical norms. All mice (n = 32) were assigned to one of four groups: 1-Control EAE group; 2-Treatment Day 6 group; 3- Treatment Days 6 and 12 group; 4- Treatment Day 12 group, consisting of 8 mice in each group.

## Isolation and expansion of BM-MSCs

Femora and tibiae were harvested from euthanized 6–8 weeks-old C57BL/6 mice. Following that, the whole bone marrow cells were collected by flushing each bone with 10 ml of complete culture medium (CCM) containing Dulbecco's modified eagles medium (DMEM; Gibco, low glucose), 20% fetal bovine serum (FBS, Gibco), 1% penicillin-streptomycin, and filtered through a 70 μm nylon cell strainer into T75 flasks. After 24 h of incubation at 37 °C with 5% $CO_2$, nonadherent cells were eliminated by changing the medium. The cell density reached 70–90% after 6 days, which was further diluted to avoid contact inhibition. The flasks were washed with PBS to remove all non-adhering cells; then,

the attached cells were treated with 0.25% Trypsin in PBS and cultured for 14 days. The CCM was replaced every three days or twice weekly to provide a suitable density of 70–90%. The homogeneous population of MSCs appeared and then was phenotyped by FACSCalibur Cytometer (BD Biosciences).

## BM-MSCs characterization by flow cytometry

Characterization of mouse bone marrow MSCs was performed using flow cytometry. The cell surface markers have been identified to characterize isolated cells. CD45, CD34 (blood cell markers), CD44, and CD105 (BM-MSC-specific markers) were detected in isolated mouse BM-MSCs. For this purpose, the cells at passage three were collected, and $1 \times 10^5$ cells in suspension were stained for 1 h at 4 °C with the fluorescence-conjugated antibodies, including PE Rat Anti-Mouse CD34 (#551387), PE Rat IgG2a Isotype Ctrl (#553930), FITC Rat Anti-Mouse CD44 (#561859), FITC Rat Anti-Mouse CD45 (#553079), FITC Rat IgG2a Isotype Ctrl (#556923, all from BD Pharmingen). In addition, the PerCP/Cy5.5 Rat IgG2a Isotype Ctrl (#400531), PerCP/Cy5.5 Rat Anti-Mouse CD105 (#120415) were obtained from Biolegend, CA, USA. The cells were then washed and collected using a centrifuge at 4 °C before resuspending in cold PBS and kept on ice until flow cytometry analysis. At

**Table 1** Scoring protocol for experimental autoimmune encephalomyelitis

| Score | Description |
| --- | --- |
| 0 | No detectable signs |
| 1 | Partial loss tail tonicity |
| 2 | Complete loss of tail tonicity |
| 3 | Flabby tail and abnormal manner of walking |
| 4 | Hind leg paralysis |
| 5 | Hind leg palsy with hind body partial immobility |
| 6 | Hind and foreleg paralysis |
| 7 | Death |

least 10000 events from each sample were collected by BD Biosciences FACSCalibur system and processed with Cell FlowJo version 10.5.3 software.

## Induction of EAE animal model and experimental groups

EAE was induced by immunizing each C57BL/6 mouse with 250 µg of MOG [35–52] synthetic peptide (MEVG-WYRSPFSRVVHLYRNGK) (BioBasic, Canada) that had been suspended in 100 µL PBS (pH 7.4) and emulsified in 100 µL Complete Freund's adjuvant (Sigma, St. Louis, MO) containing 4 mg/mL *M. tuberculosis* H37Ra (Difco Laboratories, Detroit, MI, USA). Volumes of 100 µL of this emulsion were subcutaneously injected into each flank of mice. The mice were injected intravenously with 250 ng pertussis toxin (Sigma-Aldrich, St. Louis, MO, USA) on the day of immunization and 48 h later. Eight included EAE mice were randomly selected for each group. Mice that have been placed in four specific groups include; three treatment groups that received BM.MSCs at Days 6, 6&12, and 12 of post-immunizations, respectively. In treatment groups, mice received $1 \times 10^6$ of MSCs intraperitoneally, and the EAE control group received 0.1 ml PBS as a vehicle [26, 27].

On day 25, mice were euthanized by ketamine/ xylazine. To anesthetize a mouse weighing 20 g, 2 mg of ketamine and 0.2 mg of xylosin are needed, and these amounts are present in 20 µl of ketamine solution and 10 µl of xylosin solution. Depending on the number of mice, we calculate the values and combine 2 solutions.The blood was allowed to clot at $4^0$ C overnight after which the samples were centrifuged, and the sera collected and stored at $-80^0$ C.

## Clinical observations and evaluation of EAE

All EAE-induced and not-induced control mice were housed in the same conditions from the day of inoculation. The clinical scores of EAE and the weight of the mice were assessed daily until day 25 following inoculation. The clinical scores

**Table 2** Scoring protocol for experimental autoimmune encephalomyelitis

| Score | Inflammation | Demyelination |
| --- | --- | --- |
| 0 | No inflammation | No demyelination |
| 1 | Small number of inflammatory cells | Rare Foci; Few sections with demyelination |
| 2 | Presence of perivascular infiltrates | Few areas of demineralization |
| 3 | Extending intensity of perivascular cuffing | Significant numbers of sections with demyelination |

were calculated using the usual scoring technique on a scale of 0–7, as indicated in Table 1; "0" means no discernible symptom, whereas "7" indicates death [28, 29]. Table 1 shows the Scoring protocol for experimental autoimmune encephalomyelitis,

## Histopathological assessment

EAE-induced mice were sedated with ketamine and xylazine and decapitated for the histopathological investigation. Tissues from the Brain was taken, fixed in 4% paraformaldehyde overnight at room temperature, and embedded in paraffin. After dissection, the 5 mm cerebrum was embedded in paraffin wax and sectioned to 5 µm (standard microtome HM355S; Microm, Walldorf, Germany) for staining with Hematoxylin and Eosin (H&E) for inflammation and Luxol fast blue (LFB) for demyelination. All slides were coded and read while blindfolded [30]. The area of LFB-stained sections of photographed images (Axioplan 2, Zeiss, Cologne, Germany) was measured by Fiji/ImageJ 1.46j software (NIH, Bethesda, US) for quantitative analysis of demyelination, and the area of demyelination was calculated as a percentage of the white matter area within a given section (Table 2).

## Isolation and purification of exosomes from serum

The whole serum exosome was isolated by the Total Exosome Isolation kit (Invitrogen Can: 4478360) in accordance with the manufacturer's instructions. In the first stage, collected and frozen serum samples from all study groups thaw them in a 25 °C water bath until it is completely liquid and placed on ice until needed; in the next step, samples were centrifuged at 2000 g for 30 min to remove cells and debris. The supernatant is transferred to a fresh tube without disturbing the pellet and kept on ice until the isolation is complete. A fresh microtube was filled with 100 µL of cleared serum and 20 µL of total exosome isolation. Combination of serum and reagents by pipetting up and down until a homogenous solution is achieved by thoroughly mixing the ingredients. After 30 min of incubation at 2 to 8 °C, the sample was centrifuged at 10000 g for 10 min at room temperature. The

supernatant was aspirated and discarded. Exosomes were collected in the pellet at the bottom of the tube. The pellet was then resuspended in a 25–50 µL of 1× PBS in the last step. The exosomes were used for total RNA purification and isolation once the pellet had been resuspended.

## RNA extraction and cDNA synthesis

Total RNA was obtained from the brain and serum exosomes using the miRNeasy Mini Kit Extraction Protocol and the QIAzol Lysis Reagent RNA isolation kit (Cat No./ ID: 79306). The miRNeasy Mini Kit extracted total RNA, including microRNAs, from mouse brain tissue samples, and the exosomes were separated (Qiagen). A Nanodrop was used to measure the concentration of RNA. First-strand cDNA was synthesized from 1 µg of total RNA using the miScript II RT Kit (Qiagen) for miRNA analysis and the PrimeScript RT Reagent (Takara Bio, Japan) kit for gene expression analysis, according to the manufacturer's instructions. To produce mRNA cDNAs from total RNA, Universal Stem-loop reverse (USLP primer 5′-CTCTCCTCTGGTGTGCAGGGTCCGAGGTATT CGCACCAGAG-3′) transcription and Oligo dT primers were utilized. The PrimeScriptTM RT reagent Kit (Takara Bio Inc., Otsu-Shiga, Japan) was used to synthesize cDNA following the manufacturer's instructions.

## Real-time PCR analysis

miRNAs (miR-146, miR-193, miR-223, miR-let7, miR-326, miR-155, and miR-21) and their predicted effects on IL-17, TGF-β, IL-12, IL-10, and IFN-γ expression levels were measured by real-time reverse transcription–PCR using SYBR Green dye on the ABI system. The expression levels of miRNAs and cytokine genes were normalized respectively to Snor202 and beta 2 microglobulin (β2m) as internal control genes using the $2^{-\Delta\Delta CT}$ method. All the reactions were conducted in duplicate, and the results presented as fold change compared to the EAE control group (Table 3. Real-time PCR primer sequences).

The PCR protocol consisted of 40 cycles of denaturation at 95 °C for 15 s, followed by 30 s at 60 °C to allow for extension and amplification of the target sequence, and products were identified using SYBR Green I dye (SYBR® Premix Ex TagTM II; TaKaRa, Otsu, Japan; StepOnePlus; Applied Biosystems, Foster City, CA).

## Statistical analysis

Comparison of the treatment groups vs. control mice on the development of clinical signs was conducted via two-way repeated measures ANOVA. One-way analysis of variance (ANOVA) followed by Tukey, multiple comparison tests, was performed for analysis between groups. SPSS 21 was used to analyze the data. Data were presented as mean $\pm$ SEM. The confidence level of the Type I error was defined as 95%. Statistical significance was defined as $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***).

## Results

### Bone-marrow mesenchymal stem cells (BM-MSCs) showed specific mesenchymal cell markers

After five to six in vitro passages, a homogenous BM-MSC population was established from C57BL/6 mice. The flow cytometry phenotyping confirmed the expression of CD44 and CD105 as specific markers for BM-MSC, with expression percentages of each CD44 and CD105 being 99.7% and 64.6%, respectively. Whereas CD34 and CD45 markers associated with Hematopoietic stem cells (HSCs) were expressed by only 1.48% and 3.61%, respectively, indicating the background or low levels of HSCs (Fig. 2).

**Table 3** Real-time PCR primer sequences

| Genes | Forwards | Reveres |
|---|---|---|
| **miR146a** | 5′-GCAATGAGAACTGAATTCCAT-3′ | 5′-GAGGAGACCACGCTTATG-3′ |
| **miR-193a** | 5′-CAACTGGCCTACAAAGTCC-3′ | 5′-GAGGAGACCACGCTTATG-3′ |
| **miR-223** | 5′-GCTGTCAGTTTGTCAAATACC-3′ | 5′-GAGGAGACCACGCTTATG-3′ |
| **miR-155** | 5′-TAATTGTGATAGGGGTTTTGG-3′ | 5′-GAGGAGACCACGCTTATG-3′ |
| **miR-Let7** | 5′-CCGCTGTGGAGATAACTG-3′ | 5′-GAGGAGACCACGCTTATG-3′ |
| **miR-21** | 5′-TGATGTTGACTGTTGAATCT-3′ | 5′-GAGGAGACCACGCTTATG-3′ |
| **MiR-326** | 5′-TTTGTGAAGGCGGGTTAT-3′ | 5′-GAGGAGACCACGCTTATG-3′ |
| **snor202** | 5′- AGATTAACAAAAATTCGTCAC-3′ | 5′-GAGGAGACCACGCTTATG-3′ |
| **IL-17** | 5′- GCTTCCCAGATCACAGAG-3′ | 5′- ACTACCTCAACCgTTCCA-3′ |
| **TGF-β** | 5′- CGCAACAACGCCATCTAT-3′ | 5′- TGCTTCCCGAATGTCTGA-3′ |
| **IL-12** | 5′- CCTGAGCA-GGATGGAGAATTACA-3′ | 5′-TCCAGAACATGCCGCAGAG-3′ |
| **IL-10** | 5′- GGTTGCCAAGCCTTATC-GGA-3′ | 5′-ACCTGCTCCACTGCCTTGCT-3′ |
| **IFN-γ** | 5′- GTCATTGAAAGCCTAGAAAGTC-3′ | 5′- TGCCAGTTCCTCCAGATA-3′ |
| **B2m** | 5′- TATCCAGAAAATCAAA-3′ | 5′-CTGAGCAGTATGTTC-3′ |

**Fig. 2** Bone marrow mesenchymal stem cells (BM-MSCs) displayed specific mesenchymal stem cell markers. BM-MSCs were isolated and phenotypically evaluated for the expression of stem cell particular markers. The cells are positive for BM-MSCs markers CD44 and CD105; the gray histograms show the isotype controls
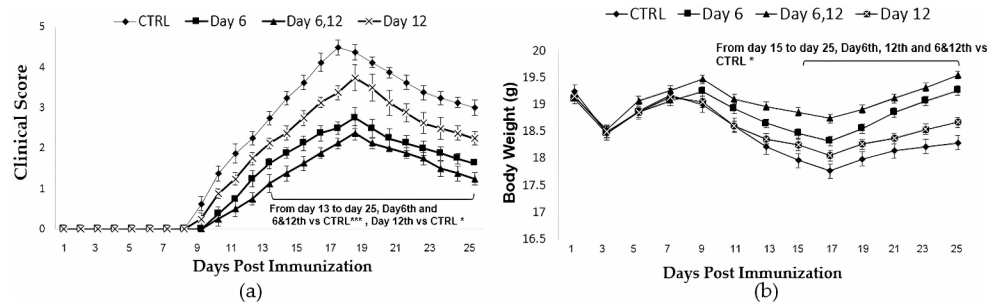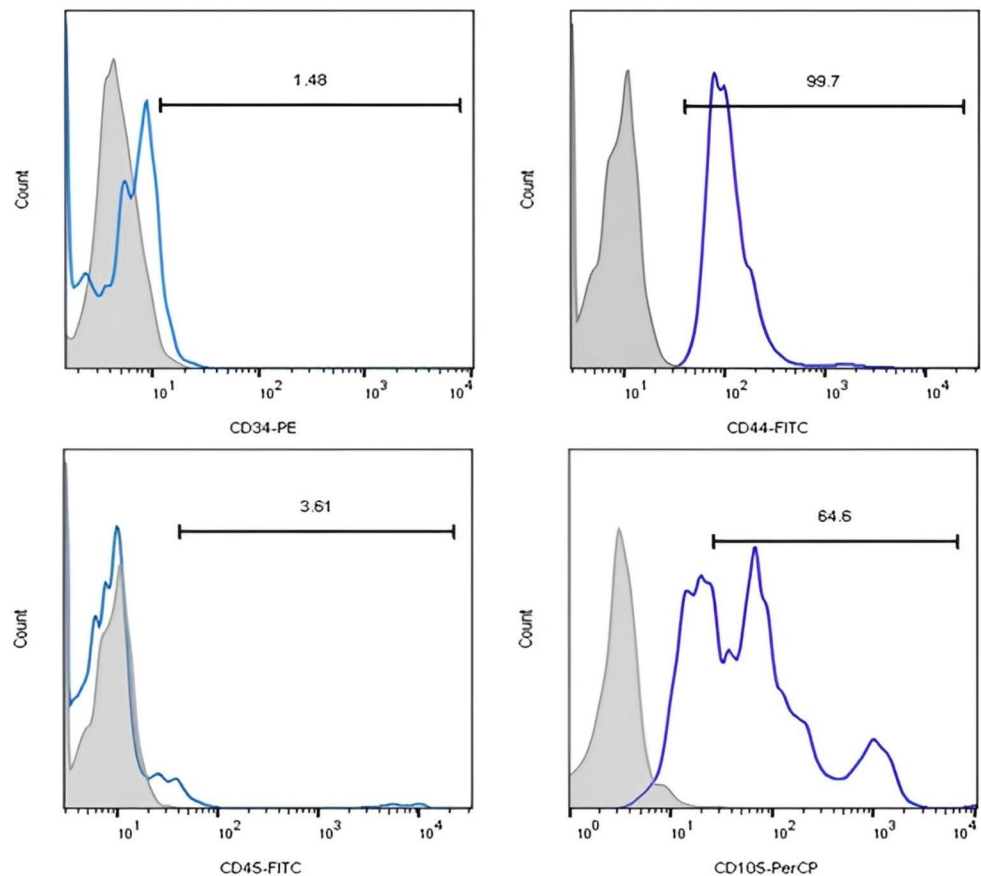


**Fig. 3** BM-MSCs inhibited the development of EAE. Female C57BL/6 mice were treated with BM-MSCs after EAE induction. Mice were monitored for signs of EAE, and the results for all mice were presented as **(a)** mean clinical score and **(b)** body weight. Results were expressed as mean ± SEM. *p < 0.05, ** p < 0.01, ***p < 0.001, compared with EAE control group. Mice were divided into five groups: (1) Control (CTRL); (2) Day 6th; (3) Day 12th; (4) Days 6th &12th

## Administration of BM-MSCs improved clinical manifestations

EAE untreated mice developed the first clinical signs of EAE at 9-day post-immunization and reached a maximum score of $4.5 \pm 0.19$ at 17-day post-immunization. While, clinical signs in all groups of group I control EAE, group II 6th day, group III 6th &12th days, and group IV 12th day, appeared at 11-day post-immunization and showed the maximum score of $2.5 \pm 0.16$ (p < 0.001 vs. CTRL), $3.38 \pm 0.18$ (p < 0.05 vs. CTRL), and $2.13 \pm 0.13$ (p < 0.001 vs. CTRL),

respectively at day 18 post-immunization (Fig. 3a). Administration of MSCs on day 6th was better than treatment on day 12, and the best treatment happens with twice injection of MSCs on days 6th &12th. Moreover, similar to clinical signs, treatment with MSCs improved animals' weight. The treatment groups with MSCs significantly prevented weight loss in EAE mice. The mean body weight of group II 6th day, group III 6th &12th days and group IV 12th day fason day 18 (maximal score) were $18.4 \pm 0.25$ and $18.7 \pm 0.2$ respectively (p < 0.05) compared to the CTRL group I with $17.7 \pm 0.15$ (Fig. 3b).

## BM-MSCs resulted in lower levels of immune cell infiltration and demyelination of CNS

H&E and LFB staining was employed to assess immune cell infiltration into brain tissue and the severity of demyelination and remyelination, respectively, as EAE progressed.

A semi-quantitative technique was used to determine the rate of leukocyte infiltration in various groups' brain tissue. In comparison to the treatment group with BM-MSCs, we identified large infiltrating cells and regions of leukocyte aggregation in the perivascular spaces into brain sections using H&E staining in EAE control group (Fig. 4a). Compared to EAE control groups with robust demyelination lesions, all therapy groups received BM.MSCs showed significantly reduced brain demyelination during illness progression (Fig. 4b). Furthermore, these results indicate a beneficial effect of BM.MSCs injections reduce inflammatory cell infiltration and demyelination compared to the EAE control group with severe demyelination and inflammatory cell infiltration. Consistent with clinical scores in the treatment groups, especially in group II 6 th day as well as group III 6th &12th days, had moderate inflammation and cell infiltration and low levels of demyelination in stained brain sections (Fig. 4c and d).

## Low levels of miRNAs (mir-21, mir-326, and mir-155) downregulates Th17- and Th1-related responses

The levels of miRNAs isolated from serum exosomes in the treated and the EAE control groups were evaluated using the Real-time PCR method. In the BM-MSCs treatment groups on group II 6 th day and group III 6th &12th days, the expression of mir-21, mir-326, and mir-155, which are involved in the induction of Th17 and Th1 cells, was reduced when compared to the EAE control groups (Fig. 5a-c). Administration of BM-MSCs on group IV 12th day resulted in no significant change in the amount of these miRNAs compared to the EAE control group. However, in the case of mir-223, it was observed that only the group IV 12th day treatment group was associated with an increase compared to the EAE control group. The study of mir-146, which is involved in the suppression of Th17 responses, showed that it was associated with a significant increase only in group I and group II, compared to other groups (Fig. 5d and e).

## Upregulation of mir-193 miRNAs promoted Treg-related responses

In this section, the evaluation of the pattern of miRNAs isolated from serum exosomes in the studied groups shows that the administration of BM-MSCs cells in the treatment groups led to increases in mir-193 expression. mir-193 involved in the induction of Treg cells [31]. This increase was significant on group II 6th day and group III 6th &12th days compared to the EAE control group. In the case of mir-193, it was expressed at significantly higher levels in the treatment group III 6th &12th days. In this part, the effect of treatment on group III 6th &12th days was much greater when compared to group II 6th day alone. Evaluation of mir-let7 expression, which is involved in suppressing Treg responses, showed that all treated groups were associated with a significant decrease compared to the EAE control group (Fig. 6a and b).

## Modified expression patterns of miRNAs targets transcript in EAE CNS tissues

Real-time PCR examined mRNA expression levels of T cell-associated cytokines to assess the impact of BM-MSCs administration in infiltrating activated T cells and define T helper responses in the CNS. In this regard, researchers looked at the expression of IL-17, IFN-γ, and IL-12 genes as pro-inflammatory responses and TGF-β and IL-10 genes as anti-inflammatory responses. Furthermore, when BM-MSCs were administered to all treated groups, followed by significantly reduced expression levels of Th1 and Th17 cell cytokines, especially on group II 6th day and group III 6th &12th days, compared to the EAE control group. Furthermore, these cytokine changes after the administration of BM. MSCs somehow related to the increase and decrease of miRNAs levels may be correlated with suppressing Th1 and Th17 differentiation and immune responses in treatment groups. In contrast to the EAE control group, there was increased production of Treg cell cytokines related to the miRNAs that trigger Treg cells and immunoregulatory responses. These findings corroborated the miRNAs data obtained with serum exosomes (Fig. 7a-e).

## Discussion

Cell therapy is an excellent candidate for therapeutic use, potentially revolutionizing the present pharmaceutical approaches. Between stem cell sources, MSC are stromal progenitor cells consequent from different tissues that denote a hopeful therapeutic instrument for autoimmune inflammatory disorders, such as MS, because of their immunomodulatory influence and neuroprotective ability [32]. However, the great variability in cell quality derived from diverse donors and tissues, inconsistent procedures, varying dosages and transfusion study designs, and the destiny of systemically injected MSC, target, and non-target organs with unpredictable outcomes can limit their therapeutic benefit [33].
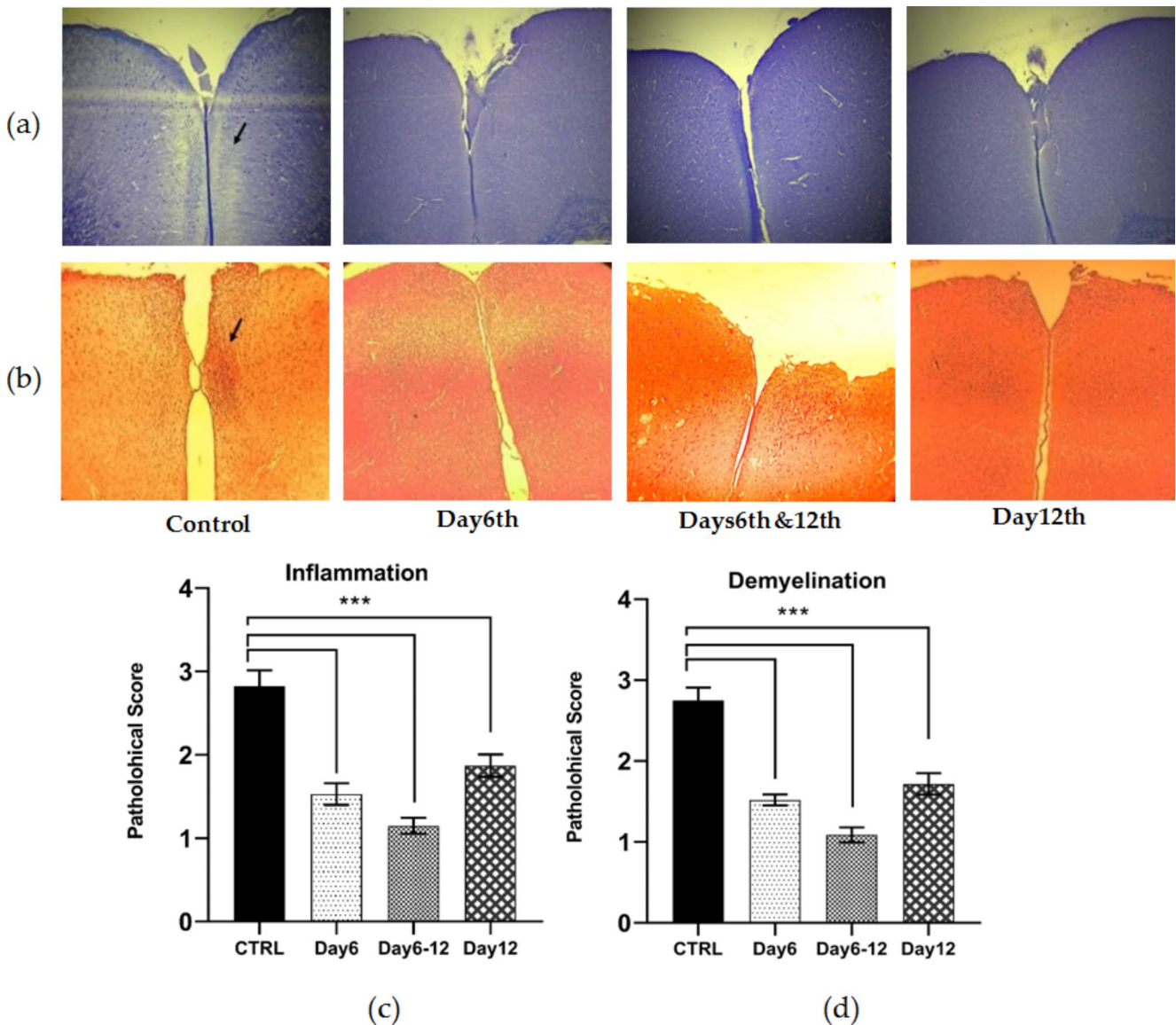
**Fig. 4** Comparative histopathology of the Brain demonstrated that treatment with BM-MSCs suppresses CNS inflammation and demyelination. Histopathological evaluation of brain tissues from treatment groups and control was performed. Brain tissues from each group were collected on day 25 post-immunization, fixed in paraformaldehyde, and embedded in paraffin. Five µm sections from different regions of the Brain from each of the groups were stained with **(a)** representative images of hematoxylin and eosin (H&E) staining, indicating the existence of robust inflammatory lesions (arrows) of pathology in EAE controls groups, in contrast to relatively milder and the (day "12th") and small infiltrate lesions (arrows) (day "6" and "6th 12th") ; **(b)** LFB staining indicates existence of high demyelination region (arrows) in contrast to very low and mild demyelination in the (day "6th", "6th&12th" and day "12th; the sections evaluated.by Light microscope (10X, Scale bar). **(c)** CNS inflammatory foci and infiltrating inflammatory cells were quantified, and CNS demyelination was quantified **(d)**. Pathological scores, including inflammation and demyelination, were analyzed and shown with a bar graph as mean scores of pathological inflammation or demyelination $\pm$ SEM. *$p < 0.05$, ** $p < 0.01$, ***$p < 0.001$ compared with EAE control group

MSCs generate immunological tolerance by boosting the recipients' endogenous immune regulatory system, which suppresses autoimmune reactions in MS models, according to previous research [34]. Furthermore, pre-clinical data collected in EAE models show that stem cell-based treatments help prevent and/or repair CNS damage by a twofold mechanism: regulating the immune system when used in the pre-clinical phase and endorsing remyelination when used in the clinical phase [35, 36].

MSCs, in particular, produce many potential therapeutic agents, such as cytokines, chemokines, and miRNAs, that can be delivered by extracellular vesicles (or exosomes). Exosomes produced by MSCs may retain the homing properties of their parent cells, which tend to home to wounded tissues. Indeed, various research has indicated
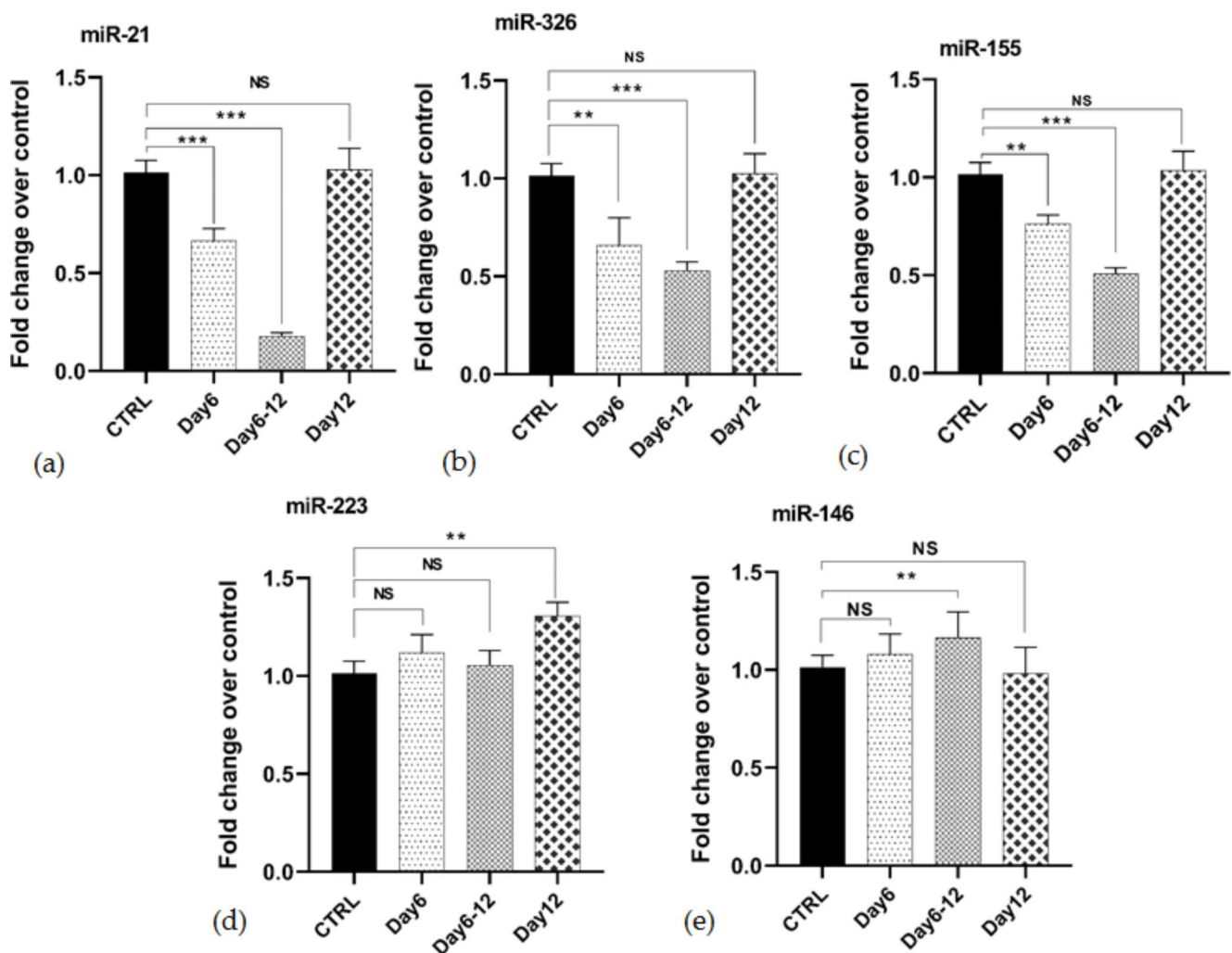
**Fig. 5** Exosomal miRNA expression pattern in serum. Expression of miRNAs was measured in serum exosomes in all groups by Real-time PCR. Expression of miRNAs **(a)** miR-21; **(b)** miR-326; **(c)** miR-155; **(d)** miR223; **(e)** miR-146 involved in regulation of Th17 and Th1 immune responses **(a-e)**. The assay was performed in triplicate, and fold change expression of genes was determined when compared to the EAE control group. Results are normalized versus snor202 as a reference gene and expressed as mean ± SEM. $p < 0.05$

that MSC-derived exosomes exhibit therapeutic effects in various syndrome models, indicating that MSC-derived exosomes may be a capable alternative to cell treatment for autoimmune illnesses. Growing evidence suggests that MSC-derived exosomes play an important role in cell-to-cell communication, horizontal transfer of proteins, miRNAs, and regulatory miRNAs. This has led to research into the immunological regulatory effects exerted by exosome release [37].

From several repeated experiments, using a wide variety of MSCs and their derived exosomes may play a role in the regulation of maturation, proliferation, and functional activation of lymphocytes, through the exact mechanisms. The previous significant studies investigated lymphocyte mRNA expression profile after incubation with MSC-derived Exosomes. MirRNAs carried by exosomes are one of the factors that perform this immunomodulation function by targeting intermediate molecular targets (transcription factors) and play a mediating role in the fate of effector T cells (Th17, Th1, Th2, and Treg) [5, 38–40].

Furthermore, miRNAs may be able to play a significant role in the regulation and alteration of immune responses in the central nervous system in neurodegenerative diseases, including; MS, Parkinson's disease (Pd), Amyotrophic lateral sclerosis (ALS) and Alzheimer's disease (AD) [41–43]. In this regard, growing evidence suggests that miRNA expression profiles may aid in recognizing the various forms of clinical progression of MS. Several approaches have been established to regulate the level of miRNAs in tissues or cells, which grasp the opportunity for disease management by pointing to dysregulated miRNAs. miRNAs are thought to have a role in vivo by targeting numerous functionally related proteins or a single protein target. miRNA expression regulation or inhibition of communication with

**Fig. 6** Exosomal miRNAs expression levels in serum are involved in immune suppressor immune responses through Treg cells. Expression of miRNAs: **(a)** miR-193; and **(b)** let-7, was measured in serum exosome in all groups by Real-time PCR. The assay was performed in triplicate, and the fold change expression of genes was determined when compared to the EAE control group. Results were normalized versus snor202 as a reference gene and expressed as mean ± SEM. $p < 0.05$



(a)

(b)
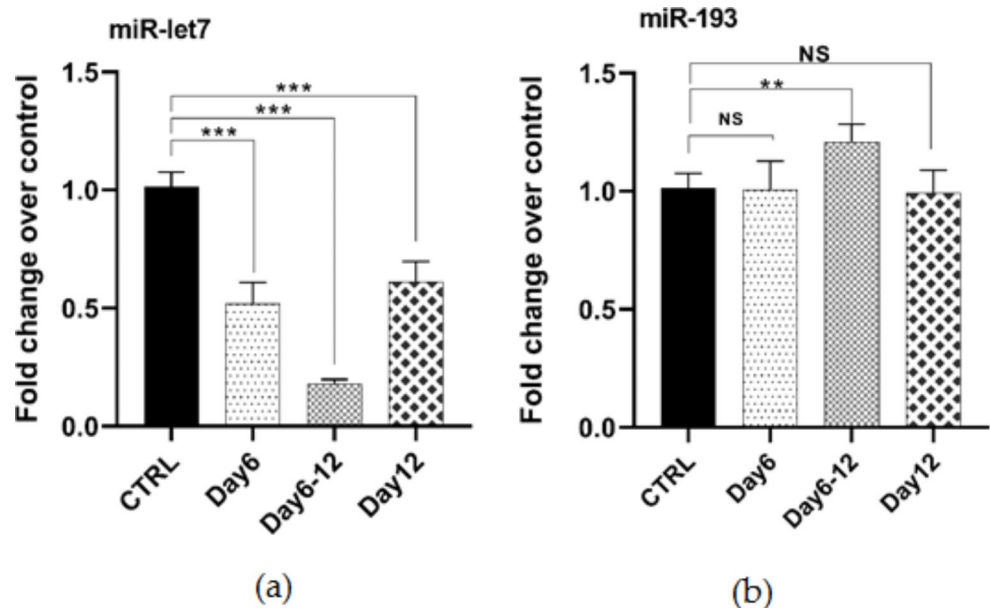
downstream actors using miRNA binding site blockers may represent a possible therapeutic option in autoimmune demyelination [44].

miRNAs are a wide class of endogenous non-coding RNAs that provide a critical layer of post-transcriptional gene expression control. During hematopoiesis and lymphoid cell development, miRNA expression is tightly controlled, and disruption of the overall miRNA network or specific miRNAs may result in dysregulated immunological responses [45].

MSCs have been demonstrated in several studies to have both inhibitory and stimulatory effects on T cell proliferation, differentiation, and antibody production. For these reasons, in this current study, we aimed to investigate specific immunological responses that BM-MSC may conduct through regulating immunological responses via miRNA evolution in serum-derived exosomes, specifically their effect on the production of certain pro and anti-inflammatory cytokines [46,47].

Because the balance and number of cells Th1 (with IFN) and Th17 (with IL-17), and Treg cells (with TGF and IL-10) are important mediators in EAE pathogenesis, the ability of miRNAs to influence the differentiation of these T helper subtypes, as well as their effects on pro- and anti-inflammatory mediators, was investigated in this study.

Modulating miRNA expression with certain medications may result in fewer Th17 cells or possibly suppression of the activities of pathogenic Th17 cells, making this a possible anti-inflammatory therapy for MS [48]. According to the current study and the results of other groups, the exosomal miRNAs have a role in inhibiting pathological immune responses in EAE. The administration of BM-MSCs in the treatment group in our study resulted in the downregulation of miR-21, miR-223, miR-146, and miR-155 in mouse serum exosomes which may be correlated with Th1- and Th17-associated cytokine reduction (IFN-γ, IL-17 A, and IL-12). Contrarily, the expression levels of TGF-β and IL-10 were higher in all treatment groups.

Autoreactive CD4 T cells stimulate TCR signaling, which activates NF-κB when CD4 cells encounter autoantigens. NF-κB stimulates STAT3 by inducing the release of autocrine IL-6 and IL-21 cytokines. STAT3 promotes the production of RORγt, the "master regulator" of Th17 cell development, which, in turn, promotes the expression of Th17 effector cytokines, such as IL-17 A and extra autocrine IL-21. As a result, autocrine IL-6 and IL-21 encourage autoreactive CD4 T cells to differentiate into pathogenic Th17 cells. miR-146a is a negative feedback regulator of NF-κB signaling that is activated by NF-κB and then suppresses NF-κB activity via the NF-κB signaling transducers TRAF6 and interleukin-1 receptor-associated kinase 1 (IRAK1) [19]. MiR-146a limits autocrine IL-6 and IL-21 signals in autoreactive CD4 T cells and inhibits their development into pathogenic Th17 cells by downregulating NF-κB activity.

CNS damage was caused by the differentiation of oligodendrocyte progenitor cells (OPCs) into remyelinating OLs. Remyelination is typically impeded in the CNS with neurodegenerative illnesses, such as MS. Toll-like receptor 2 (TLR2) and IRAK1 signaling, which are adversely regulated by miR-146a, are inhibitors of OPC differentiation. The decrease of the TLR2/IRAK1 signaling pathway by miR-146a was associated with increased OPC differentiation and remyelination. A study of the autoimmune symptoms in miR-146a–deficient mice found that these animals lacked natural regulatory T cells (Tregs), which failed to modulate Th1 pro-inflammatory responses and were likely
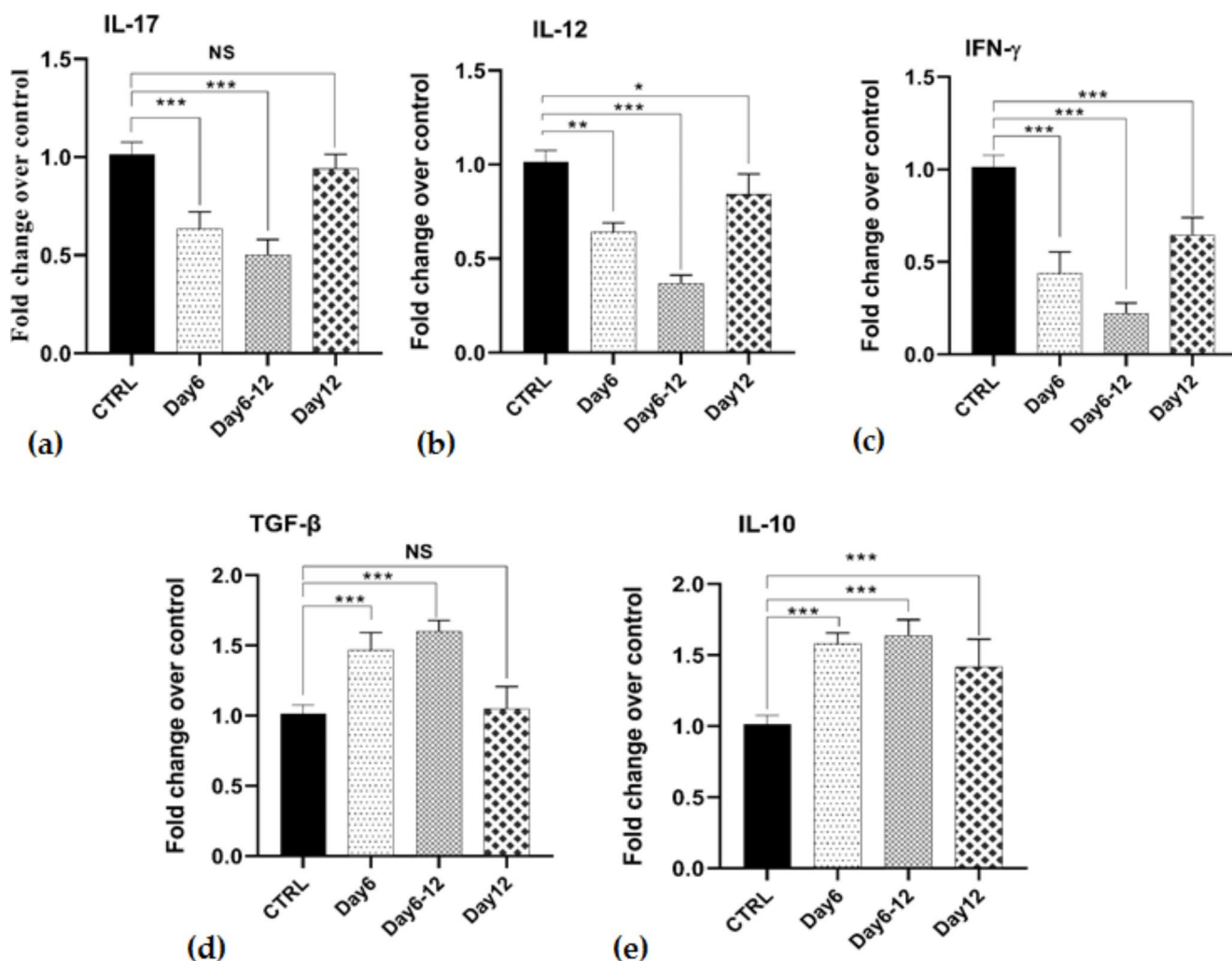
**Fig. 7** Treatment with BM-MSCs suppressed Pro-inflammatory-associated gene expression. On day 25 post-immunization, levels of cytokine mRNA in the Brain were assessed by Real-time PCR. The assay was performed in triplicate, and the fold change expression of genes was determined when compared to the EAE control group. Pro-inflammatory cytokines IL-17, IL-12, IFN-γ (**a**, **b**, and **c**); Anti-inflammatory cytokines TGF-β and IL-10 (d and e). Results are normalized versus B2m as a reference gene and expressed as fold change compared with the EAE control group. *p < 0.05, ** p < 0.01, ***p < 0.001 compared with EAE control group

caused by a dysregulated IFN-signaling pathway [49]. These results highpoint that miR-146a is a strong inhibitor of T cell-mediated autoimmunity. According to the results of our study, injection of two doses in group III 6th&12th days of stem cells resulted in a significant increase in the amount of mir-146, which may be correlated with a decrease of Th17-related inflammatory cytokines known as IL-17.

Expression of miR-155 is required for optimal Th1 function, and overexpression of this miRNA promotes Th1 differentiation, M1 macrophage polarization, and inflammation [50, 51]. The results of our studies also showed a decrease in the expression of this miRNA in both groups (group II 6th day and group III 6th &12th days) in comparison with the EAE control group. However, this reduction was greater in the group receiving twice the BM-MSC dose.

Systemic lupus erythematosus (SLE), multiple sclerosis (MS), and psoriasis, among other diseases, are all linked to miR-21 expression in T cells. SMAD-7, a negative modulator of TGF signaling, is targeted and depleted by miR-21. Furthermore, deficiencies in SMAD-2/3 activation and IL-2 inhibition were associated with reduced Th17 development by the miR-21–deficient T cell population. In vivo, miR-21 knockdown significantly decreased EAE illness and Th17 cell responses, and miR-21 silencing significantly improved EAE clinical symptoms [50]. Our findings show that the administration of BM-MSCs may contribute to modifying expression levels of miR-21, which in this way, may reduce the differentiation of Th17 cells and improve EAE clinical scores in treatment groups.

More and more evidence suggests that miR-223 is implicated in the pathophysiology of MS and is dysregulated

in EAE mice models, according to the current study. Treg cells, plasma, blood cells, PBMCs, and brain white matter tissue from MS patients and EAE animals have been shown to be elevated in miR-223. Through decreased Th1 and Th17 infiltration into spinal cords, a global miR-223 deletion (miR223$^{-/-}$) in mice delayed the start of EAE, reduced spinal cord damage, and reduced neurological symptoms [18].

The significance of miR-223 in controlling immune cells' function, development, and interaction was investigated. The quantity of miR-223 expression in the group II 6th day and group III 6th &12th days treatment groups remained unchanged. There was no significant change in miR223 expression overall, except for the "12-day" group, which increased.

miR-326 silencing resulted in fewer Th17 cells and mild EAE, whereas overexpression resulted in a higher number of Th17 cells and severe EAE. As a result, the expression of miR-326, a Th17 cell-associated miRNA, was strongly linked to the severity of illness in MS patients and mice with EAE. By targeting Ets-1, a negative regulator of Th17 development, miR-326 promoted Th17 differentiation [52]. Our data confirmed role of miR-326 in Th17 differentiation and the pathogenesis of EAE. The groups that received stem cells in our study demonstrated a reduction of symptoms and improvement in the mice.

miRlet7i might function as a negative regulator of Treg differentiation, and this regulation is likely to occur by reducing the expression of positive controllers of Treg cell differentiation. Let-7i was significantly down-regulated in the peripheral blood of all MS patient subtypes, including primary progressive, secondary progressive, and relapsing-remitting illness [52]. reduced levels of the anti-inflammatory cytokines IL-10 and TGF-β accompanied miRlet7i expression in all treatment groups of our study. The amount of miRNA, a positive Treg cell controller, was accompanied by increased expression when receiving BM-MSCs. Increased amounts of TGF-β and IL-10, following increasing and decreasing levels of miR-193 and miR-Let7 expression, suggest that these can promote the differentiation of Treg cells, which can, in turn, decline inflammation and alleviate clinical signs of EAE.

Overall, our findings support the idea that miRNAs play a role in the pathogenesis of EAE and MS, possibly by shifting the balance of T cell development toward pathogenic Th1 and Th17 cells. The findings of this study contribute to our knowledge of MSC-derived exosome immune-modulatory pathways and may help progress the therapeutic use of these exosomes in inflammatory illnesses. As a result of their immunomodulatory capabilities and neurodegenerative potential, stem cells are a viable method for treating

CNS autoimmune illnesses, allowing the opportunity to address multiple clinical elements of diseases like MS.

In conclusion, MicroRNAs (miRs) transferred by exosomes can function as non-invasive potential biomarkers for the diagnosis and prognosis in various types of autoimmune disorders such as MS. Therefore, Serum levels of exo-miRs were determined by quantitative PCR in samples obtained from patients with MS may function as a novel biomarker for predicting diagnosis and progression of this patients. However, extensive independent studies are required before applying the present study's results to clinical practice. We now provide compelling evidence that correlates with clinical signs that BM-MSCs limit induction and ameliorate chronic EAE, when it occurs after disease stabilization, causing the reduction of CNS inflammation, demyelination, and induction of local neurodegeneration. Our findings suggest that administration of BM-MSCs in treatment groups is associated with upregulation of miR-193, miR-146a, and downregulation of miR-155, miR-21, and miR-326. Overall, these changes in the number of miRNAs are implicated in the fall of Th1 and Th17 immune responses, and the augmentation of regulatory responses might be mediated by T cell differentiation into Treg cells, which reduces neuroinflammatory responses. Overall, our findings and observations suggest that MSC-derived miRNAs may play a role in the progression of immune-related disorders and shed light on the etiology of MS. The results of this study may contribute to the development of better and safer treatment methods by elucidating the processes through which exosomes influence immune modulation.

Furthermore, our findings suggest that BM-MSCs and miRNA compounds could be assessed as therapeutic targets for treating MS in clinical settings. Understanding the role of miRNAs as immune biomarkers helps with MS surveillance and diagnosis. Once this strategy is implemented and the effect of MSCs on improving the disease process is determined, we will see their usage in the treatment of MS.

## Declarations

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