BM-MSCs ameliorate experimental autoimmune encephalomyelitis via modifying the expression of miR-193, miR-146a, miR-155, miR-21 and miR-326

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Abstract

Background: Experimental Autoimmune Encephalomyelitis (EAE) is a demyelinating neurological illness having immunological, histological, and clinical parallels to MS (EAE). Cell-to-cell communication and exosomes are two mechanisms through which MSCs exert their effects. The purpose of this study was to see how effective BM-MSCs were at treating EAE patients.

Materials and Procedures: Myelin Oligodendrocyte Glycoprotein (MOG35-55) was used to induce EAE in C57BL/6 mice (n=32), and then BM-MSCs 1×10^6 cells were administered. Every day, clinical and weight examinations were performed. Histology will be used to assess inflammation and demyelination in mouse CNS parts. Using Real-time PCR, we investigated the expression of pro and anti-inflammatory genes, as well as miRNAs intricate in the differentiation and function of Th cells in the control and progression of EAE.

Results: In the EAE, BM.MSCs significantly reduced clinical symptoms, inflammation, and demyelination of the brain. Our findings suggest that improved expression of miR-193 miR-146a and decreased expression of miR-155 and miR-21 miR-326 was followed by an increase in cytokine expression levels of IL-10, TGF- β , and IL-4; however, IFN- γ and IL-27 levels were reduced in treatment groups. Treatment groups were also associated with suppressing effects on Th1 and Th17 immune responses, induction of Treg cells, and immunoregulatory responses.

Conclusions: These findings provide compelling evidence that MSC-derived exosomes modulate immune suppression and highlight the significance of BM.MSCs and miRNAs in affecting T cell differentiation and reducing CNS inflammation, demyelination, and local neurodegeneration.

Introduction

Multiple sclerosis (MS) is a chronic inflammatory disorder described by inflammatory cell infiltration in the CNS, demyelination, and axonal injury. Experimental Autoimmune Encephalomyelitis (EAE) is a commonly known model for MS that is induced by immunization with myelin antigens (Steinman, 2001). Clinical and Cellular/macular Immunopathologia events that occur in CNS in MS and EAE are similar. Pro-inflammatory cytokines produced by Th1 and Th17 that have been raised in MS patients during an exacerbation of the disease (12, 13). In contradiction, Th2/Treg cytokines with anti-inflammatory capacity including IL-4, IL-5, TGF- β , and IL-10, are predominant during disease remission and the recovery from the disease (14).

Findings associate with the opinion that MSCs inhibit the immune response further careful assessment of appropriate cell sources, additional scientific data, and a better mechanistic considerate of immunosuppression of MSCs is essential (Fletcher et al., 2010).

Stem cell therapy has appeared as an innovative treatment for many diseases. MSCs, in addition to their capability to self-renew and differentiate into a variability of mesenchymal cell lineages, have emerged as

a promising therapeutic intervention strategy (Ghannam et al., 2010). Currently, suppressive and immunomodulatory effects of MSCs demonstrated in vitro and a variety of clinical trials, although the exact mechanism by which MSCs modulate immune function remains largely unknown (Ghannam et al., 2010). BM.MSCs have been proposed to treat MS and EAE, despite the fact that the exact mechanisms underlying these cells' immunomodulatory functions are still largely unknown. Numerous studies have shown that the inhibitory effect of BM.MSCs is not solely reliant on cell-to-cell interaction. This suggests that paracrine effects of BM.MSCs, possibly via soluble factors, are responsible for communications (Wang et al., 2018). Accumulating evidence shows that exosomes that present in extracellular space, formed by MSCs exert their therapeutic properties in some disease models (Cheng et al., 2017). In this regard, several dealings between exosomes and recipient cells indicate that exosomes performance 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, in addition to proteins (Mathivanan et al., 2010).

MiRNAs are short, 20-22 non-coding nucleotide RNA molecules that act as transcription and post-transcriptional regulators of several target genes' expression. The role of miRNA has been identified in a variety of biological processes, including differentiation, cell proliferation, development, and apoptosis in a variety of 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 among miRNAs and the onset and development of MS neurological disease (Holley and Topkara, 2011).

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 (Yang et al., 2018).

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 that have been critical role in the immunopathogenesis of MS and EAE by regulating CD4⁺ T cell differentiation (Gandhi et al., 2013; Moore et al., 2013; Mortazavi-Jahromi et al., 2020).

miR-223 is an inflammatory miRNA, and its upregulation is associated with inflammation. In contrast to healthy controls, has been identified in the blood of MS patients (Gharibi et al., 2018). 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 (Liu et al., 2018). MiR-193a has been shown to be increased in CD4⁺, CD8⁺ T cells, and B cells in the peripheral blood of MS patients in remission, and has been associated to Treg cell induction (Fenoglio et al., 2012). Studies have demonstrated that the miR-326 producer promoted Th17 development by

targeting Ets-1, a regulator of Th17 differentiation that is undesired. 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. (Zhu et al., 2013). 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 (Maciak et al., 2021). 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 (Treg) cells, which inhibits Treg cell formation and function while promoting Th1 and Th17 cell proliferation (Angelou et al., 2020). Understanding the processes by which exosomes play a role in immune modulation might aid the development of therapeutic programs for MSCs delivery. We examined the therapeutic benefits of BM-MSCs in EAE by addressing possible impacts on miRNAs and pro and anti-inflammatory cytokine production due to evidence of a key role of exosomes in immune-regulation.

Materials And Methods

Animals

All mice were housed in a controlled environment with a temperature of 23 [±2] °C, a relative humidity of 50 [±5] %, a 12h 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 of the mice were assigned to one of four groups: 1-Control; 2-Treatment Day 6th; 3-Treatment Day 12th; 4-Treatment Day 6th & 12th, consisting of 8 mice in each group.

Isolation and Expansion of BM-MSCs:

6–8 weeks old mice C57BL/6 were sacrificed and Femurs and Tibias were harvested. Following that, 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 m nylon cell strainer into T75 flasks. After 24h of incubation at 37°C with 5% CO₂, nonadherent cells were eliminated by changing the medium. The cell density reached 70-90 % after 6 days, which should be passed due to contact inhibition. The flasks were washed with PBS to remove all non-adhering cells; then the attached cells were trypsinized with 0.25% Trypsin and cultured for 14 days with the twice-weekly exchange of CCM with 10% FBS to provide a suitable density of 70–90 % and was passaged again until a homogeneous polyclonal 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. Cell surface markers have been studied to identify isolated cells. The presence of CD45, CD34 (blood cells markers) CD44, CD105, and (BM.MSC specific markers) were investigated in isolated mouse BM.MSCs in this study. For

this purpose, cells at passage 3 were collected and 1×10^5 cell suspensions 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 of these antibodies were purchased from BD Pharmingen and PerCP/Cy5.5 Rat IgG2a Isotype Ctrl (400531), PerCP/Cy5.5 Rat Anti-Mouse CD105 (120415) obtained from Biolegend, CA, USA. The cells were then washed at 1500 RPM for 10 minutes at 4°C before being resuspended in cold PBS and kept on ice until flow cytometry analysis. Flow cytometry was used to collect a minimum of 10000 events from each sample. Data were collected using a BD Biosciences FACSCalibur system and processed with Cell FlowJo version 10.5.3 software.

Induction of EAE and treatment protocol with BM.MSCs:

EAE was induced by immunizing each C57BL/6 mice with 250 µg MOG35-55 synthetic peptide (MEVGWYRSPFSRVVHLYRNGK) (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) contain 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 also 48 hours later. Eight included EAE mice were randomly selected for each group. Mice were separated into 3 treatment groups that received MSCs at Day 6th, Day 12th, and both Day 6th & 12th -days post-immunization, respectively, and a control group. In treatment groups, mice received 1×10^6 MSCs intraperitoneally and the control group received 1 ml PBS as a vehicle (Shahla et al., 2021).

Clinical observations and evaluation of EAE:

All EAE induced and normal 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 21 days following inoculation. The clinical scores were calculated using the usual scoring technique on a scale of 0–7, as indicated in table 1: 0 indicates no discernible symptom, whereas 7 indicates death (Haghmorad et al., 2016). Table 3 shows the incidence, beginning day of disease, highest score (on the peak day), mean score (on the last day), and Cumulative Disease Index for mice (total disease score over experiment duration).

Histopathological assessment:

EAE-induced mice were sedated with ketamine and xylazine and decapitated for the histopathological investigation. Tissues from the brain and spinal cord were taken, post-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 (Berard et al., 2010). 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:

Serum samples from all groups were collected and chilled until totally liquid, then centrifuged at 2000×g for 30 min to remove cells and debris. The supernatant is then 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, thoroughly mix the ingredients. After 30 minutes of incubation at 2°C to 8°C, the sample was centrifuged at 10,000 × g for 10 minutes at room temperature. Aspirated and discarded supernatant Exosomes are present in the pellet at the bottom of the tube. The pellet is resuspended in a convenient amount of 1X PBS or a comparable buffer in the last step. The exosomes are suitable for total RNA purification and isolation once the pellet has been resuspended.

Extraction of RNA and cDNA synthesis:

Total RNAs were 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). Using the miRNeasy Mini Kit, total RNA including microRNAs was extracted from mouse brain tissue samples and separated exosomes (Qiagen). A Nanodrop was used to measure the concentration of RNA. First-strand cDNA was synthesized from 1 µg 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 manufacturers' instructions. For the production of mRNA cDNAs from total RNA, Universal Stem-loop reverse (USLP probe) transcription and Oligo dT primers were utilized. The PrimeScript™ RT reagent Kit (Takara Bio Inc., Otsu-Shiga, Japan) was used to synthesize cDNA in accordance with 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 microglobulins (β2m) as internal control genes using the 2-ΔΔCT method. All the reactions were conducted in duplicate and the results presented as fold change compared to the control group. (Table 3. Real-time PCR primer sequences).

The PCR protocol consisted of 40 cycles of denaturation at 95°C for 15 seconds in a total volume of 10 l, followed by 30 seconds 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 Tag™ II; TaKaRa, Otsu, Japan) (StepOnePlus; Applied Biosystems, Foster City, CA).

Statistical analysis:

To compare the overall difference in mouse scores as a result of the ANOVA For a significant study between the groups, the repeated measurement test was followed by the Tukey Post hoc test. For two-group comparisons, Mann–Whitney nonparametric unpaired t-tests were used. All data were presented as Mean SEM. In all experiments, the confidence interval was 95%. $P < 0.05$ and $P \geq 0.01$ were indicated with *. $P < 0.01$ and $P \geq 0.001$ were shown with ** and $P < 0.001$ were reported with ***. A p-value < 0.05 was recognized as statistical significant. SPSS 23 software (Chicago, IL), Graph Pad Prism 8.1, and Excel were utilized in this investigation to interpret and analyze the data acquired.

Ethical Statement

The ethics committee of Semnan University of Medical Sciences in Semnan, Iran, accepted this study. (IRCT: IR.SEMUMS.REC.1398.46, ID: 1587).

Results

MSC characterization:

After five to six in vitro passages, a homogenous BM.MSCs population was produced from C57BL/6 mice. Their flow cytometry phenotypic study confirmed their expression of CD44, CD105 as specific markers for BM.MSC, with expression percentages of each of the CD44, CD105 being were 99.2%, 50 %, respectively. Whereas CD34 and CD45 markers that are associated with HSCs are expressed by these cells was only .77% and 2.66% of the cells were, respectively indicating the presence of HSCs. (Figure 1).

Administration of BM-MSCs improved clinical manifestations:

EAE untreated mice developed the first clinical signs of EAE at 9.4 $\pm$ 0.5-day post-immunization and reached a maximum score of 4.5 ± 0.19 at 17-day post-immunization. While, clinical signs in treatment groups of Day 6th, Day12th, and Days 6th &12th, appeared at 18-day post-immunization, showed the maximum score of $2.5 \pm 0.16^{***}$, $3.38 \pm 0.18^*$, and $2.13 \pm 0.13^{***}$, respectively (**Figure 2a**). There were significant differences between treatment groups and control group (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Moreover, similar to clinical signs, treatment with MSCs improved animals' weight. Administration on day 6th was better than treatment on day 12, and the best treatment happens with twice injection of MSCs on days 6th &12th. Comparison of the mean of the treatment groups on different days compared to the control group found that the groups were significantly different from day 13 (Figure 2b).

BM-MSCs decreased immune cell infiltration and demyelination of CNS:

H&E and LFB staining were employed to assess immune cell infiltration into brain tissue, as well as 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 mononuclear cell infiltration into brain

sections using H&E staining (Figure 3a). Furthermore, as compared to normal control groups, all therapy groups showed a significant reduction in brain demyelination during illness progression, showing that the control group experienced greater demyelination (Figure 3b). The control group had severe demyelination and inflammation cell infiltration, whereas the days 6 and 6&12 groups had mild or moderate inflammation and cell infiltration on the brain as well as demyelination. (Figure 3c).

Decreased miRNAs molecules involved in Th17 and Th1 related responses:

The results of the evaluation of miRNAs patterns isolated from serum exosomes in the treated groups and the control group, which were evaluated by the Real-time PCR method. In the BM-MSCs treatment groups on day 6 as well as on days 6th &12th, the expression of mir-21, mir-326, and mir-155, which are involved in the induction of Th17 and Th1 cells, decreased compared to the control group, while administration of BM.MSCs on day 12th resulted in no significant change in the amount of these miRNAs compared to the control group. However, in the case of mir-223, it was observed that only the day 12 treatment group was associated with an increase compared to the 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 days 6th and 6th &12th in comparison to other groups (Figure 4a-e).

Increased miRNAs molecules involved in 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 MSCs BM cells in the treatment groups led to increases in mir-193 expression that involved in the induction of Treg cells. This increase was significant on day 6th as well as on days 6th &12th compared to the control group. In the case of mir-193, the results showed that in the treatment group, 6th &12th days were significantly associated with increased expression. In this part, the effect of treatment on days 6th and 12th was much greater compared to day 6 alone. Examination of mir-let7 value, which is involved in suppressing Treg responses, showed that all treated groups were associated with a significant decrease in comparison to the control group (Figure 5a and b).

BM-MSCs induced anti-inflammatory gene expression in the CNS.

mRNA expression levels of T cell-associated cytokines were examined by Real-time PCR to assess the impact of BM.MSCs administration in the infiltration of 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, as well as TGF- β and IL-10 genes as anti-inflammatory responses. Furthermore, when BM.MSCs were administered to all treated groups, the expression of Th1 and Th17 cell cytokines was significantly reduced, especially on days 6 and 6th &12th, compared to the control group, and this was correlated with miRNAs levels, which suppress Th1 and Th17 differentiation and immune responses. In contrast to the control group, there was increased production of Treg cell cytokines connected with miRNAs that have to trigger Treg cells and immunoregulatory responses. These findings corroborated the miRNAs data obtained with serum exosomes (Figure 6a-e).

Discussion

Cell therapy is an excellent candidate for therapeutic use that can potentially revolutionize 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 (Rawat et al., 2019). However, the great variability in cell quality derived from diverse donors and tissues, inconsistent procedures, varying dosages and transfusion study designs, the destiny of systemically injected MSC, target and non-target organs with unpredictable outcomes can limit their therapeutic benefit (Yousefi et al., 2016).

MSCs generate immunological tolerance by boosting the recipients' endogenous immune regulatory system, which suppresses autoimmune reactions in MS models, according to previous research. Furthermore, pre-clinical data collected in EAE models show that stem cell-based treatments help to the prevention and/or repair of 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. (Bassi et al., 2012).

MSCs, in particular, produce a large number of therapeutic agents in the form of extracellular vesicles, including as cytokines, chemokines, and miRNAs (exosomes). Exosomes produced by MSCs may retain the homing properties of their parent cells, which have a strong tendency to home to wounded tissues. Indeed, various research have indicated that MSC-derived exosomes exhibit therapeutic effects in a variety of 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 (Toh et al., 2018). Furthermore, miRNAs have been shown to play a significant role in the regulation and alteration of immune responses in the central nervous system. 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 downstream actors using miRNA binding site blockers may represent a possible therapeutic option in autoimmune demyelination. (Chen et al., 2018).

MSCs have been demonstrated in several studies to have both inhibitory and stimulatory effects on T cell proliferation, differentiation, and antibody production. As a result, we studied specific regulating immunological responses in BM. MSCs via miRNA evolution in serum-derived exosomes, specifically their effect on the production of certain pro and anti-inflammatory cytokines (Ha, 2011; Thamilarasan et al., 2012).

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 (Mathieu and Ruohola-Baker, 2013).

Because the balance and number of cells Th1 (with IFN- γ) and Th17 (with IL-17) and Treg cells (with TGF- β 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 (Raphael et al., 2015). According to the current study and the investigated results, which showed the potential role of exosomes miRNAs in inhibition of pathological immune responses in EAE, BM.MSCs administration in the treatment group in our study resulted in downregulation of miR-21, miR-223, miR-146, and miR-155 in mouse serum exosomes that are associated with Th1 and Th17 related cytokines (IFN- γ , IL-17A, and IL-12) reduction in comparison with TGF- β and IL-10 expression levels were related with higher expression in all treatment groups.

Autoreactive CD4 T cells stimulate TCR signaling, which activates NF- κ B, when they recognize autoantigens. NF- κ B stimulates STAT3 via inducing the release of autocrine IL-6 and IL-21 cytokines. STAT3 promotes the production of ROR γ t, the "master regulator" of Th17 cell development, which in turn promotes the expression of Th17 effector cytokines such IL-17A 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- κ B signaling that is activated by NF- κ B and then suppresses NF- κ B activity via the NF- κ B signaling transducers TRAF6 and interleukin-1 receptor-associated kinase 1 (IRAK1) (Liu et al., 2018). 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- κ B 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 like MS. Toll-like receptor 2 (TLR2) and IRAK1 signaling, both of 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 to 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 (n Tregs), which failed to modulate Th1 proinflammatory responses and were likely caused by a dysregulated IFN-signaling pathway (Zhang et al., 2019). These results highlight that miR-146a is a strong inhibitor of T cell-mediated autoimmunity. According to the results of our study, injection of two doses in reserves 6th and 12th days of stem cells resulted in a significant increase in the amount of miR-146, which is followed by a decrease of th17 related inflammatory cytokines known as IL-17.

miR-155 stimulates Th17 differentiation and promotes the production of 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. The results of our studies also showed a decrease in the expression of this miRNA in both groups (days 6th and 6th & 12th) in comparison with the control group, however, this reduction was greater in the group that received twice the dose of BM.MSC.

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 to decreases in Th17 development in miR-21-deficient T cells. In vivo miR-21 knockdown significantly decreased EAE illness and Th17 cell responses, and miR-21 silencing significantly improved EAE clinical symptoms (Wang et al., 2019). Our findings show that BM. MSCs injection lowers miR-21 expression, which leads to a deficit in Th17 differentiation and improves 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 (Gharibi et al., 2018).

The significance of miR-223 in controlling the function, development, and interaction of key immune cells was examined. The quantity of miR-223 expression in the 6th and 6th & 12th days' treatment groups remained unchanged. There was no significant change in miR223 expression overall, with the exception of the 12th-day group, which showed an increase.

MiR-326 silencing resulted in a low number of 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. (Du et al., 2009). Our data confirmed a serious role for miR-326 in Th17 differentiation and the pathogenesis of EAE, because the groups that received stem cells in our study had a decrease in the expression of this BM. MSCs, which was somehow accompanied by a reduction in symptoms and improvement in these mice.

miRlet7i function might as a negative regulator of Treg differentiation, and this regulation is likely to occur, through reducing the expression of positive controllers of Treg cell differentiation. Let-7i was found to be significantly down-regulated in the peripheral blood of all MS patient subtypes, including main progressive, secondary progressive, and relapsing-remitting illness, in recent studies.(Amici et al., 2017). It is interesting to note that miRlet7i expression in all treatment groups of our study was accompanied by a decrease. According to with present results of the anti-inflammatory cytokine expression of IL-10 and TGF- β . While the amount of miRNA, which is a positive controller of T reg cells, was accompanied by an

increase in the amount of expression when receiving BM. MSCs. Increased amounts of TGF.β and IL-10 following respectively increasing and decreasing amount of miR-193 and miR-Let7 expression suggest that these can promote the differentiation of Treg cells, which can decline inflammation and ameliorate 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 the treatment of CNS autoimmune illnesses, giving the opportunity to address multiple clinical elements of diseases like MS.

Conclusion

We now provide compelling evidence that correlates with clinical signs that BM.MSCs limit induction and ameliorate chronic EAE when prescribing after disease stabilization, causing the decrease of CNS inflammation, demyelination, and induction of local neurodegeneration. Our findings suggest that administration of BM.MSCs in treatment groups are associated with upregulation of miR-193 miR-146a and decrease of miR-155 and miR-21 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 findings 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 might be assessed as possible therapeutic targets for the treatment of MS in clinical settings. Understanding the role of miRNAs as immune biomarkers might also help with MS surveillance and diagnosis. It is hoped that once this strategy is implemented and the effect of MSCs on improving the disease process is determined, we will soon see their usage in the treatment of MS.

Declarations

Acknowledgments

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Conflict of interest

The authors have no conflict of interest.

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Tables

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

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 demyelination
3	Extending intensity of perivascular cuffing	Significant numbers of sections with demyelination

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'- AGATTTAACAAAAATTCGTCAC-3'	5'-GAGGAGACCACGCTTATG-3'
USLP	5'- CTCTCCTCTGGTGTGCAGGGTCCGAGGTATTCGCACCAGAG-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'

Figures

Fig.1

Figure 1

Phenotype identification of Bone marrow mesenchymal stem cells (MSCs) of through surface markers. Flow cytometry analysis results showed that these isolated cells were positive for BM.MSCs markers CD44 and CD105. Cells were are somewhat negative for HSCs cell markers CD34 and CD45.

Figure 2

BM-MSCs improved 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 (B) body weight. Results were expressed as mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared with control group. Mice were divided into five groups: 1. Control group (CTRL), 2. day 6th group, 3. Day12th group, 4. Days 6th & 12th.

Fig.3

Figure 3

Comparative histopathology of brain demonstrated 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, collected on day 25 post immunization, fixed in paraformaldehyde and embedded in paraffin. Five μ m sections from different regions of the Brain from each of the groups were stained with (A) Luxol fast blue to assess demyelination (B) H&E to enumerate infiltrating leukocytes and with. (C) CNS inflammatory foci and infiltrating inflammatory cells were quantified. Pathological scores including inflammation and demyelination were analyzed and shown with bar graph as mean scores of pathological inflammation or demyelination $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$,

***p <0.001 compared with control group. Mice were divided into five groups: 1. Control group (CTRL), 2. day 6th group, 3. Day12th group, 4. Days 6th &12th.

Figure 4

Exosomal miRNAs expression levels in serum involved in Th17 and Th1 immune responses. Expression of miRNAs a;miR-223, b;miR-146a, c;miR-21 d;miR155, e;miR-326 was measured in serum exosome in all groups by Real-time PCR. Results are normalized versus snor202 as reference gene and expressed as mean ± SEM. p < 0.05.

Fig.5

Figure 5

Exosomal miRNAs expression levels in serum involved in immune suppresser immune responses through Treg cells. Expression of miRNAs a;let-7 and b;miR-193 was measured in serum exosome in all groups by Real-time PCR. Results are normalized versus snor202 as reference gene and expressed as mean $\pm$ SEM. $p < 0.05$.

Fig.6

Figure 6

Treatment with BM.MSCs suppressed Pro-inflammatory related gene expression. On day 25 post immunization, brains mRNA levels of cytokines were assessed by Real-time PCR. Assay was run in triplicate and fold change expression of genes was determined compared 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 were expressed as fold change compared with control group. *p <0.05, ** p < 0.01, ***p <0.001 compared with control group. Mice were divided into five groups: 1. Control group (CTRL), 2. day 6th group, 3. Day12th group,4. Days 6th &12th.