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ORIGINAL ARTICLE

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Umbilical cord mesenchymal stem cells as well as their released exosomes suppress proliferation of activated PBMCs in multiple sclerosis

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Abstract

Multiple sclerosis (MS) is a central nervous system (CNS) degenerative disorder which is caused by a targeted autoimmune-mediated attack on myelin proteins. Previously, mesenchymal stem cells were considered as a novel and successful treatment of MS. One of the underlying mechanisms behind their immunomodulatory function is the release of extracellular vesicles, particularly exosomes. In this study, we aimed to evaluate the suppressive efficacy of MSCs and their exosomes on the proliferation of peripheral mononuclear blood cells (PBMC) in relapsing-remitting MS (RRMS) patients and healthy subjects. To do, mesenchymal stem cells were derived from human umbilical cord tissues and used for exosome isolation through ultracentrifugation. Suppressive function of MSCs and MSC-derived exosomes was examined in a coculture with CFSE-labelled PBMCs in vitro. PBMC proliferation of the patients and healthy individuals was measured using flow cytometry. We first demonstrated that proliferation of PBMCs decreased in the presence of MSCs and suppression was more efficient by MSC-derived exosomes, with a minimum alloreaction rate. However, suppression capacity of MSCs and their exosomes significantly decreased during extensive sub-culturing. The present study showed that MSC-derived exosomes as an effective cell-free therapy could prevent proliferation of PBMCs. However, further evaluations are need to move towards a functional approach that can be translated to the clinic.

1 | INTRODUCTION

Multiple sclerosis (MS) is an autoimmune disorder of the central nervous system (CNS) in which inappropriate activation of self-reactive T cells contributes to neurodegeneration.[1,2] There are more than 2.3 million affected individuals worldwide, and the exact physiological or environmental cause of the disease is still unknown.[3] Approximately 85% of the patients have shown relapsing-remitting MS clinical pattern.[4] Numerous efforts have been made to treat MS patients. Among them, Mesenchymal stem cells (MSCs) showed great promises to cure the disease.

The multipotent mesenchymal stem cells are important regulators of immune system.[5] One of the underlying

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mechanisms behind their immunomodulatory function is exosomes releasing.[6] The allogenic use of MSC-derived exosomes was considered safe and effective for autoimmune therapy due to their low or no expression of major histocompatibility complex (MHC) and costimulatory molecules as well as their ability to inhibit activity of various immune cell populations.[7,8] In addition, MSC-derived exosomes were therapeutically used in animal models of MS and successfully suppressed the brain atrophy, T cell proliferation and immune cell infiltration into the CNS. Their intravenous (iv) administration was also shown to re-induce the peripheral self-tolerance through upregulating of transforming growth factor beta (TGF-β), interleukin (IL)-4 and IL-10 in splenocytes.[9-12] However, in vitro and in vivo effects of MSC-derived exosomes have not been evaluated on MS individuals yet.

For the first time, the present study investigated the suppressive potential of MSCs and their exosomes on proliferation of peripheral blood mononuclear cells (PBMCs) isolated from RRMS patients compared to healthy subjects, with reference to the passage number of MSCs and allogenic capacity of the exosomes.

2 | MATERIAL AND METHODS

2.1 | Participants

Fifteen subjects with a definite MS (relapsing patients: 9 Female; mean age: 35.92 ± 2.1 years; EDSS: 3.01 ± 0.87, remitting patients: 4 Female, 2 Male; mean age: 35.40 ± 1.85 years; EDSS: 2.18 ± 0.54) were enrolled to the present study. All patients were referred to the Iranian Center of Neurological Research in Sina General Hospital, Tehran University of Medical Sciences (TUMS). The clinical interview and neurological examinations were carried out by a neurologist according to the McDonald's criteria (McDonald *et al*, 2011).[13] The key exclusion criteria for relapse patients were the use of disease-modifying treatments or any other immunomodulatory drugs in the last 3 months. However, all remitting patients were treated with interferon-β (IFN-β). Nine ethnically, age- and sex-matched healthy controls (9 Females; age: 36.53 ± 2.9 years), who had no history of autoimmune or inflammatory diseases themselves and in their families, were also recruited into the present study. All participants signed a written informed consent, and the study was approved by TUMS ethics committee (IR.TUMS.VCR.REC.1398.331).

2.2 | Isolation and expansion of umbilical cord tissue MSCs (UC-MSCs)

Having parents' written consents, twelve human umbilical cords were collected from females with healthy pregnancies and immediately transferred to the laboratory in phosphate buffer saline (PBS) supplemented with antibiotics, 300 U/mL penicillin and 300 μg/mL streptomycin (Biosera) at 2-8°C. The umbilical cords were washed with sterile PBS and cut longitudinally to remove blood vessels. It was excised into small pieces and all segments were then explanted on tissue culture-treated dishes containing DMEM-f12 supplemented with 10% foetal bovine serum (FBS; both from Gibco) and incubated at 37°C with 5% CO_2. Plates were left undistributed for 48 hours, and the culture media was changed every 2 days. The explants were removed after two weeks and the cells were allowed to expand until they reach to about 70% confluency. Morphology of isolated MSCs was regularly evaluated by an inverted microscope. Early passages of MSCs (passages 2 or 3) were used in all the next experiments unless otherwise stated.

2.3 | Multi-differentiation capacity of the MSCs in vitro

1×10^5 MSC cells were cultured in a 24-well plate containing either osteogenic or adipogenic differentiation medium (Cyagen), and the media were changed every 72 hours. After three weeks, the cells were fixed with formaldehyde, and stained with 2% alizarin red or 2% oil red in order to detect calcium deposition or lipid formation, respectively. A control sample was also cultivated in complete DMEM-f12 medium.

2.4 | Flow cytometric characterization of MSCs

At the end of passage 3, the adherent cells were harvested from cell culture flasks and stained for surface positive and negative markers including HLA-DR-PerCP, CD34-FITC, CD45-APC, CD73-FITC, CD90-PE-Cy5 and CD105-PE (all from Invitrogen), according to the manufacturer's guideline. Afterwards, the samples were examined by BD FACS Calibur™ flow cytometer and analysed using FlowJo software.

2.5 | Exosome isolation

To isolate exosomes, MSCs were cultivated in complete DMEM-f12 supplemented with 10% exosome-depleted FBS (SBI). After 72 hours, culture supernatant was collected and centrifuged at 300 *g* for 5 minutes to eliminate cellular debris. Subsequently, supernatants were passed through a 0.22 μm filter and also centrifuged at 17 000 *g* for 30 minutes to remove microvesicles. The supernatants were then carefully transferred to polycarbonate ultracentrifuge tubes and

centrifuged twice at 120 000 *g* for 90 minutes with an Optima L-90 K ultracentrifuge (Beckman Coulter, USA). Upon centrifugation, the supernatant was discarded, the exosome pellet was suspended in PBS and stored at −80°C for further analyses. The whole centrifugation process was taken at 4°C.

2.6 | Size characterization and quantification of exosomes

Size of the exosomes was evaluated through dynamic light scattering (DLS) using a ZetaSizer 3000-HA (Malvern Instruments, UK). To do, 100 μL of each exosome sample was first diluted 1:10 in PBS to a total volume of 1 mL. The whole volume was quickly transferred to a cuvette, and three independent measurements were performed for each sample.

Exosomes were also quantified by a CD63 ELISA kit (SBI System Bioscience) and a standard curve was generated to determine the number of exosomes, according to the manufacturer recommendations.

The pellet provided by ultracentrifugation was also solubilized and fixed with 2.5% glutaraldehyde-PBS solution. The solution was then washed twice with PBS and dehydrated by different concentrations of ethanol. Finally, the sample was left to dry and sent to investigate morphology of exosomes using a scanning electron microscope (SEM, Nova NanoSEM 450).

2.7 | Isolation of PBMCs and proliferation assay

Peripheral blood mononuclear cells (PBMCs) were isolated from blood samples using ficoll density gradient centrifugation (Lymphodex, Innotrain, Germany). To evaluate cell proliferation, 1×10^6 PBMCs were labelled with Cell Trace CFSE (BioLegend), as stated in the manufacturer's instruction. The PBMCs were then cultured in the presence of either MSCs (10:1 ratio) or 0.5×10^9 of the exosomes obtained from the equivalent number of MSCs. In addition, the same number of PBMCs cultured alone (in the absence of MSC or exosome) as untreated group and positive control of the proliferation assay. The PBMCs were also polyclonally activated by 1 μg/mL of anti-CD3 and 5 μg/mL of anti-CD28 monoclonal antibodies (Mabtech).

To assess the proliferation stimulating activity of MSCs and their exosomes, CFSE-labelled PBMCs were co-cultured with MSCs or exosome isolated from passage 3, 6 and 9. These PBMCs were isolated from healthy participants and had no further stimulation with anti-CD3 and anti-CD28.

After 72 hours, all PBMCs were harvested and analysed by BD FACS Calibur™ flow cytometer and FlowJo software. Finally, suppression index (S) was calculated based on the following Equation[14]:

$$S = \left(\frac{a - b}{a}\right) \times 100.$$

where a is the percentage of proliferation in the absence of suppressor (MSC and MSC-derived exosome) and b is the percentage of proliferation in the presence of each suppressor alone. CFSE dilution of PBMCs (proliferation percentage) on day 3 was calculated compared to their CFSE level on day 0.

2.8 | Statistics

SPSS software (IBM Corp.) was used for all statistical analyses, and data are expressed as means ± standard deviations (SD). Groups were compared using the repeated measures ANOVA, one-way ANOVA and Tukey's post hoc tests. *P* values <.05 were considered as statistically significant.

3 | RESULTS

3.1 | Isolation and characterization of MSCs and exosomes

Microscopic examination showed that the MSCs grew as adherent cells with spindle-shaped morphology. Immunophenotyping analysis also demonstrated that umbilical cord-derived MSCs (UC-MSCs) positively express CD73, CD90 and CD105 while they were negative for HLA-DR, CD34 and CD45 markers (Figure S1A). These cells illustrated multipotential characteristics when stained for calcium mineralization and lipid formation after 21 days of culture (Figure S1B).

Exosome size distribution assay indicated the exosomal average size of about 127 nm (Figure 1A). Moreover, the presence of CD63 was confirmed by ELISA to estimate the number of exosomes in each sample. In summary, after optimizing the procedure, 3 x 10^5 of UC-MSCs were cultured for 72 hours and the supernatant was collected for exosome isolation in each round. The total exosomes were aliquoted into 3 vials, and according to our ELISA data (Figure S2), the OD of each vial was about 0.29 which means there were approximately 0.5 x 10^9 exosomes in each vial. Eventually, only one of these vials (containing exosomes isolated from 1 x 10^5 of MSCs) was added to each exosome-PBMC group. Then, one of aliquots was used to investigate morphology of the exosomes isolated from UC-MSCs under a scanning electron microscope (Figure 1B).

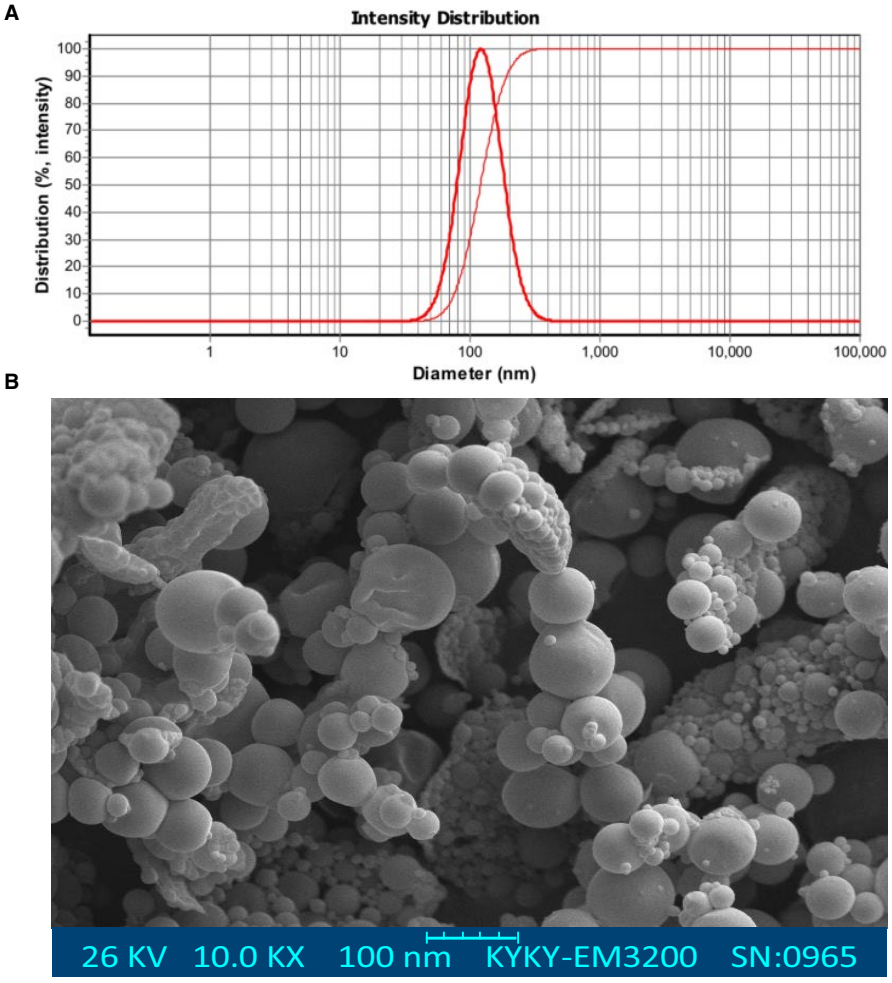

FIGURE 1 Characterization of UC-MSCs derived exosomes. A, Size distribution analysis by dynamic light scattering (DLS). MSC-derived exosomes were first dispersed in phosphate buffer saline, and then, the samples were measured at 30 μg/mL concentration (the figure shows representative line plot of one sample). B, Morphology of UC-MSC-derived exosomes under a scanning electron microscope (SEM, 20 000x magnification). UC-MSC, Umbilical cord tissue MSCs

3.2 | MSC-derived exosomes suppressed PBMC proliferation in vitro

It is well established that MSCs inhibit proliferation of autoreactive lymphocytes contributing to the MS pathogenesis. Here, we investigate if allogenic exosomes similarly display suppressive impact on PBMC proliferation. In this regard, CFSE-labelled PBMCs co-cultured with either MSCs or exosomes and analysed by flow cytometry. The gating strategy for proliferating cells is shown in Figure 2A. As shown in Figure 2C,D, relapse patients had higher proliferation capacity compared to healthy subjects and patients in remission phase. In addition, higher suppression index (S) was observed for exosome-treated PBMCs in patients and healthy controls ($S_{exo\text{-}relapse} = 77.69 \pm 4.27$; $S_{exo\text{-}remission} = 42.19 \pm 7.14$; $S_{exo\text{-}HC} = 57.21 \pm 6.32$) than MSC-treated PBMCs ($S_{MSC\text{-}relapse} = 47.53 \pm 8.13$; $S_{MSC\text{-}remission} = 25.60 \pm 7.80$; $S_{MSC\text{-}HC} = 42.55 \pm 7.50$; Figure 2E). Overall, this result supports the hypothesis that MSC-derived exosomes can similar to MSCs, alleviate proliferation of PBMCs in RRMS patients as well as healthy individuals.

3.3 | Both UC-MSCs and their exosomes have minor allostimulation effect on PBMC proliferation

Neither MSC nor exosome treatments cause a major proliferation stimulation on non-activated PBMCs isolated from RRMS patients and healthy subjects although exosomes demonstrated lower stimulation (Figure 3 A,B).

3.4 | Suppression capacity of MSCs as well as their exosomes are being decreased during extensive passages of the cells

We have assessed suppression characteristic of both MSCs and their isolated exosomes in different passages (3-9) and noticed that suppressive capacity of MSCs and their released exosomes significantly decreased after passage three (data are calculated only for fresh PBMCs isolated from healthy subjects; Figure 4A-C). Increased number of proliferating cells in higher passages, suggesting early passages of MSCs and their exosomes for therapeutic applications.

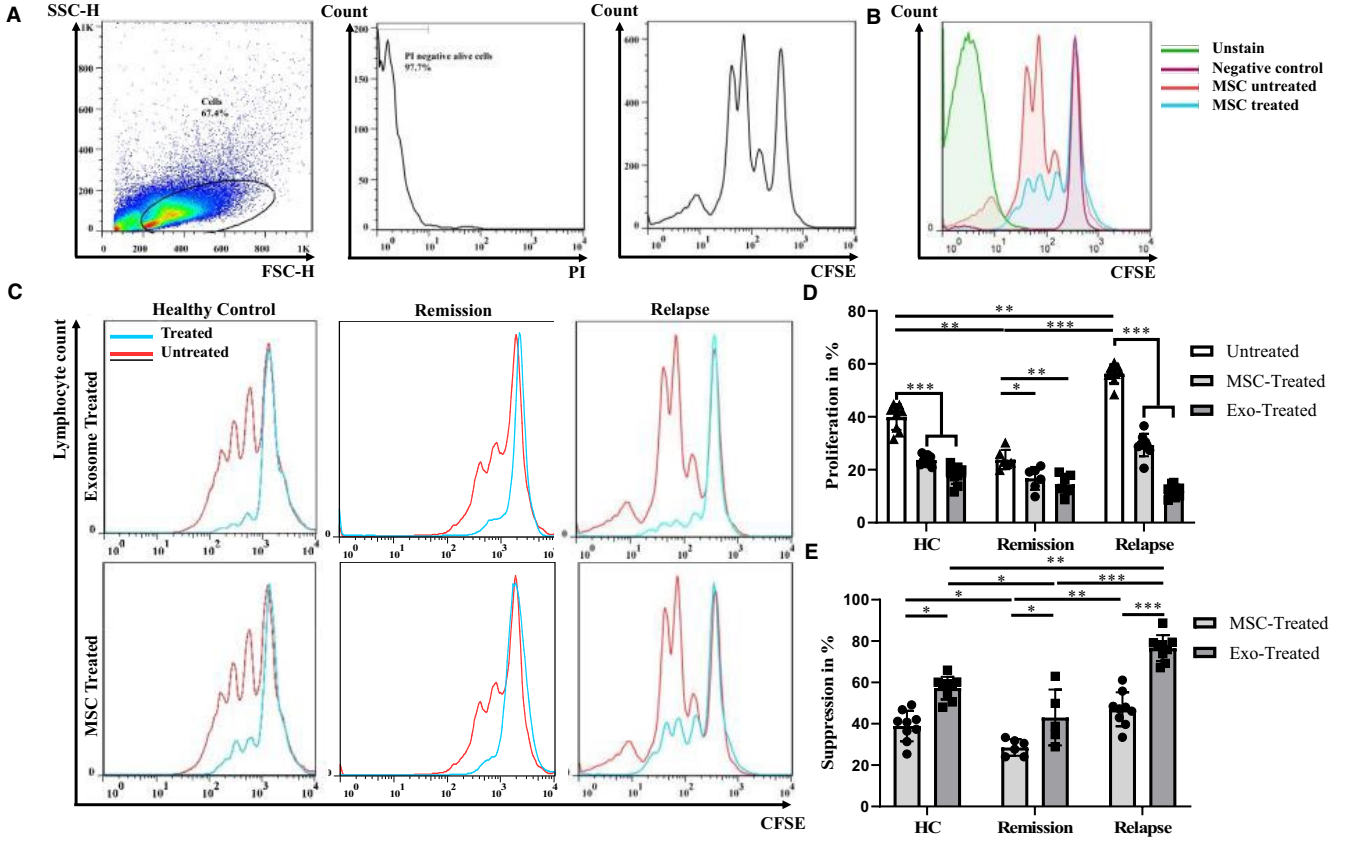

FIGURE 2 UC-MSC-derived exosomes exert immunosuppressive impact on in vitro proliferation of PBMCs. Human PBMCs were labelled with cell trace CFSE dye and polyclonally activated for 3 days in absence or presence of MSCs or exosomes. A, The gating strategy: the viable lymphocytes were selected according to the FSC/SSC and PI penetration. Then, the cells were gated for CFSE dilution to assess proliferation and finally (B) an overlay of unstain, negative control (untreated PBMCs which were not polyclonally activated), MSC-untreated (positive control, untreated PBMCs which were polyclonally activated), and also MSC-treated PBMCs are shown with green, violet, red, and blue lines, respectively. C, Representative FACS plots of CFSE dilution in untreated and MSC- or exosome- treated PBMCs in MS patients and a healthy individual (red lines: untreated PBMCs; blue blue: MSC or exosome-treated PBMCs). D, Quantification of cells proliferation: untreated PBMCs isolated from relapse patients demonstrate higher proliferation capacity, compared to remission and healthy PBMCs. Additionally, MSC or exosome treatment highly decreased proliferation of relapse PBMCs compared to remission PBMCs and healthy PBMCs). E, Suppression index of MSC- or exosome-treated PBMCs in healthy subjects and RRMS patients. Repeated measures ANOVA, one-way ANOVA and Tukey's post hoc were conducted to compare MSC-treated PBMCs and exosome-treated PBMCs in healthy controls and RRMS patients. MSC, Umbilical cord tissue MSCs; PBMC, Peripheral blood mononuclear cells; HC, Healthy control. *P* values <.05, .01 and .001 are summarized with *, ** and ***, respectively. The same gating strategy was used for Figures 3 and 4 as well

4 | DISCUSSION

Since first report of therapeutic effects for MSC-derived exosomes in an animal model of myocardial infarction,[6] these nanocarriers have extensively been on investigations for type one diabetes,[15-21] rheumatoid arthritis,[22] systemic lupus erythematosus[23] and uveitis.[24,25] We previously showed circulating MSCs increased in clinically active patients.[26] In addition, Treg-derived exosomes from MS patients and healthy individuals suppressed the proliferation and induced apoptosis in conventional T cells. However, the effect of MS-derived exosomes was significantly less than healthy controls.[14]

To the best of our knowledge, the current study for the first time reports comparative immunomodulatory effects of MSCs and MSC-derived exosomes in MS patients and healthy individuals in vitro. We have successfully demonstrated that the exosomes are capable to suppress lymphocytes proliferation. Moreover, we have shown that exosomes derived from the late passages of MSCs did not efficiently suppress PBMC proliferation, suggesting that anti-inflammatory phenotype of MSCs was attenuated during extensive sub-culturing. The first key finding of this study was inhibition of PBMCs proliferation caused by MSCs. Besides, MSC-derived exosomes significantly had higher suppression index than MSCs which is in line with a previous study on animal model of rheumatoid arthritis.[22]

High throughput data provided by RNA sequencing and mass spectrometry indicated that human MSC-derived exosomes contain high concentrations of anti-inflammatory factors like indoleamine 2 3-dioxygenase (IDO) transcripts and

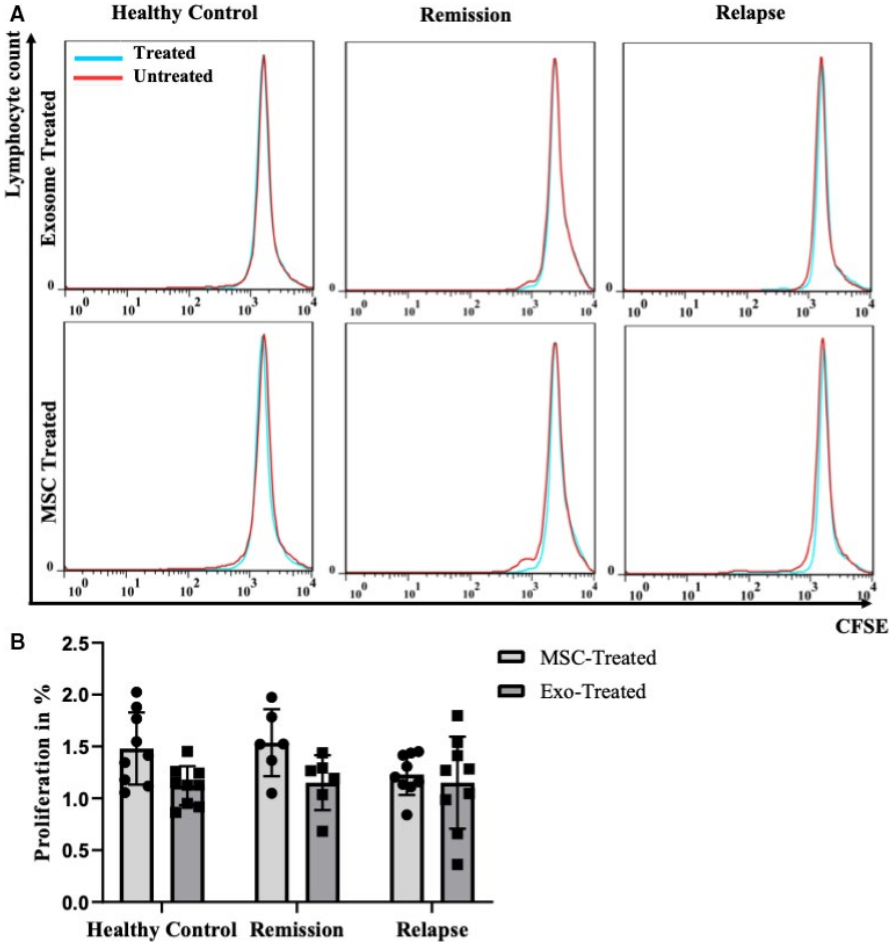

FIGURE 3 UC-MSCs and their exosomes do not display significant stimulation effect on PBMC proliferation. A, Representative flow cytometry plots of not activated PBMCs treated with MSC and exosome (B) and its quantification in a CFSE assay. Repeated measures ANOVA, one-way ANOVA and Tukey's post hoc were conducted to compare exosome-treated PBMCs and MSC-treated PBMCs between healthy controls and RRMS patients. MSC, Umbilical cord tissue MSCs; PBMC, Peripheral blood mononuclear cells. *P* values <.05, .01 and .001 are summarized with *, ** and ***, respectively

proteins including galectin-1, macrophage inhibitory cytokine 1, latent-transforming growth factor β-binding protein and heat shock protein 70.[27] Murine MSC-derived vesicles were also shown to carry galecin-1, PD-L1 and TGF-β.[10] The reason for quick and essential effect of MSC-derived exosomes could be due to the fact that these carriers contain final products provided by their maternal cells, while MSCs require to sense a degree of environmental inflammation and respond by releasing of cytokines and exosomes to decrease the inflammatory process.

In a literature review of previous investigations, we found that most studies have reported similar effects of MSC-derived vesicles on peripheral blood lymphocytes isolated from healthy donors.[27,28] Numerous studies have examined total extracellular vesicles (microvesicles and exosomes) derived from human MSCs in allogenic settings and reported efficient suppression on the proliferation of B and NK cells, although no or low functional impacts were observed on T cell proliferation.[29-31] Besides, some of animal studies exhibited that human MSC-derived exosomes failed to inhibit ConA-activated splenocytes and T cells in mice and rats, respectively. These investigations indicated that MSC-derived exosomes activated MYD88-dependent signalling through Toll-like receptor (TLR) 4 ligands which lead to M2 macrophage phenotype concomitant with increasing $CD4^+CD25^+FoxP3^+$ regulatory T cell polarization.[24,32] The conflicting outcomes regarding proliferation suppression might be on account of the various sources of MSCs, culture conditions, isolation protocols, inefficiency of the xenogeneic exosomes in the recipient organism, and the use of freeze and thawed exosomes.

Our results also showed that MSC-derived exosomes, similar to MSCs, suppress activated PBMCs in vitro, without subsequent stimulation of PBMC proliferation (alloreaction) reported for other cell therapies. In consensus with our data, Meisel *et al* used human bone marrow-derived MSCs to inhibit T cell responses in allogenic setting. They found that immunosuppressive properties of MSCs were mediated by IDO activity[33] which was similarly found in MSC-derived exosomes.[27]

Another important point for consideration is the fact that in vitro expansion of MSCs significantly changed suppressive capacity of MSCs and their exosomes. A study by Sareen *et al* have also reported that increase in passage number of MSCs (from passage 3 to 7) does not lead to any change in the immunological behaviour of allogenic MSCs. They showed no significant difference in amount of pro-inflammatory and anti-inflammatory cytokines secreted by MSC-treated leucocytes. Moreover, these indications were

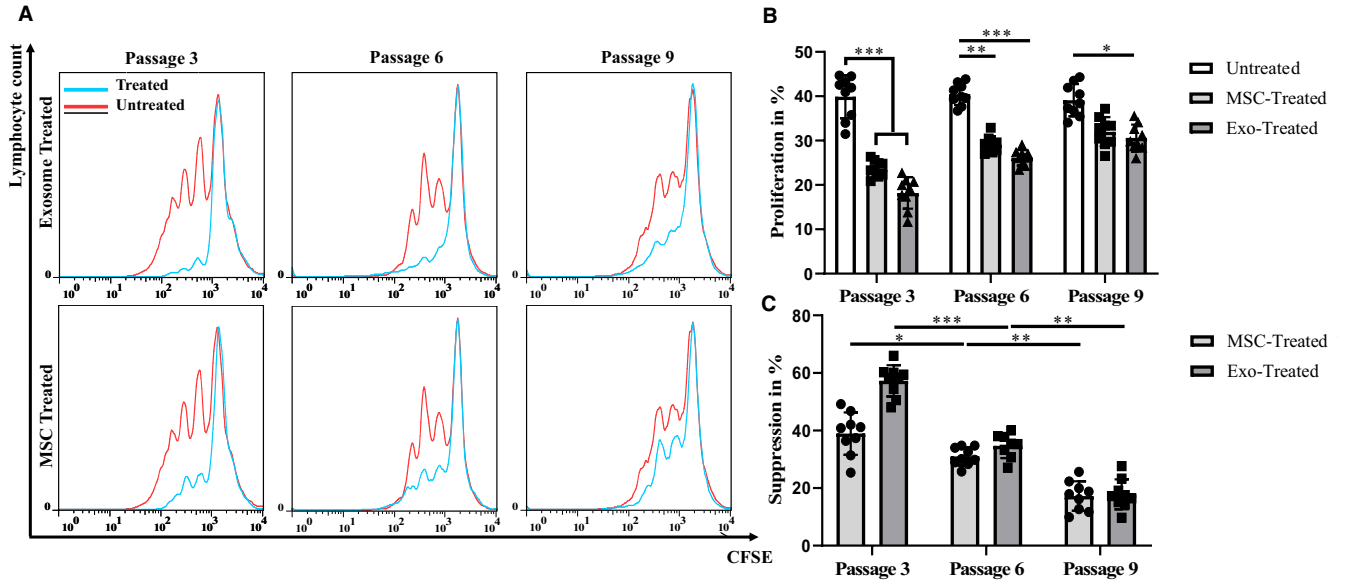

FIGURE 4 Suppression capacity of MSCs and their released exosomes is being decreased during extensive sub-culturing. CFSE dilution of healthy PBMCs was evaluated in presence of MSCs and exosomes isolated from passage 3, 6 and 9. A, Representative flow cytometry plots of activated PBMCs treated with MSC and exosome isolated from different passages (B) as well as quantification of proliferation cells. C, Suppression index of proliferating PBMCs in presence of MSCs and exosomes in different passages. Repeated measures ANOVA, one-way ANOVA and Tukey's post hoc were used to compare suppression of MSC- or exosome-treated PBMCs with reference to the passage number of MSCs and their exosomes. MSC, Umbilical cord tissue MSCs; PBMC, Peripheral blood mononuclear cells. *P* values <.05, .01 and .001 are summarized with *, ** and ***, respectively

confirmed in leucocyte proliferation[34] which is in contrast to our findings provided by suppression assay. On the other hand, numerous studies have demonstrated that senescent MSCs exhibit stronger immunosuppressive activities, with reference to a decreased proliferation of PBMC and cytokines produced by MSCs.[34-36] This confliction could be at least partly due to various culture conditions, sources of MSCs, and especially variations exist in MSCs isolated from different donors.

In the present study, we have depicted that human UC-MSC-derived exosomes mimic therapeutic benefits of their parental cells and can effectively suppress proliferation of the PBMC in both RRMS patients and healthy controls. These findings could introduce MSC-derived exosomes as an alternative of therapeutic MSCs, providing a novel approach for treating MS. However, considerable issues remain to be resolved and further investigations need to be done to validate this approach in a large-scale application, hopefully moving towards an effective module that can be translated to the clinic.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTIONS

Hussein Baharlooi, Maryam Azimi and Maryam Izad planned this research. The experiments and analyses performed by Hussein Baharlooi, Maryam Azimi, Mohammad Javad Tavassolifar and Batool Moradi. Hussein Baharlooi and Zeynab Nouraei contributed to collect the human samples (umbilical cords and blood samples). Naser Moghadasi and Mohammad Ali Sahraian diagnosed and introduced the patients.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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