Immunology Letters 212 (2019) 6–13

Contents lists available at ScienceDirect

Immunology Letters

journal homepage: www.elsevier.com/locate/immlet

Neuralized mesenchymal stem cells (NMSC) exhibit phenotypical, and biological evidence of neuronal transdifferentiation and suppress EAE more effectively than unmodified MSC

Moriel Ben-Zwi, Panayiota Petrou, Michelle Halimi, Dimitrios Karussis[1], Ibrahim Kassis[*,1]

Unit of Neuroimmunology and Multiple Sclerosis Center, Neurology department and The Agnes-Ginges Center for Neurogenetics, Hadassah University Hospital, Jerusalem, Ein-Kerem, Israel

ARTICLE INFO

Keywords:
Neuralized Mesenchymal Stem Cells
Mesenchymal Stem Cells
Transdifferentiation
Neurotrophic Growth Factors
Neuroprotection

ABSTRACT

In the last decade several studies employing stem cells-based therapies have been investigated as an optional treatment for multiple sclerosis. Several preclinical and few clinical studies tested the efficacy of mesenchymal stem cells as a potent candidate for such therapies. Here we suggest the option of "neuralization" of classical mesenchymal stem cells as a cellular structure that resembles neural stem cells as well as there differentiation by a unique procedure towards terminally differentiated neural cells suggesting that this cell population may be appropriate for clinical application in the CNS. We investigated whether neuralized MSC (NMSC) could promote repair and recovery after injection into mice with EAE. Injection of NMSC and differentiated NMSC starting at the onset of the chronic phase of disease improved neurological function compared to controls as well as compared to naïve MSC. Injection of NMSC and mainly differentiated correlated with a reduction in the inflammation as well as in the axonal loss/damage and reduced area of demyelination. These observations suggest that NMSC and differentiated NMSC may suggest a more potent cell-based therapy that naïve MSC in the treatment arsenal of multiple sclerosis.

1. Introduction

Mesenchymal stem cells (MSC) [1–4] are non-hematopoietic stromal cells, residing mainly in the bone marrow compartment (but also in the adipose tissue and other tissues) and their classical role is to support the process of hematopoiesis and hematopoietic stem cells (HSC) engraftment and to give rise to cells of the mesodermal lineage, such as osteoblasts, adipocytes and chondrocytes [5]. Their surface molecule profile includes, the expression of CD29, CD73, CD90, CD105 and CD166 and the absence of the CD34, CD45 and CD14 markers. Various studies have depicted additional properties of MSC, such as the ability to transdifferentiate into cells of the endodermal and ectodermal lineage 4,6,7] (including a possible- and debatable-neural transdifferentiation [2,8]) and a strong immunomodulating potential [9,10]. These immunomodulatory effects are mediated by both humoral mechanisms (production of soluble factors) and cell-to-cell contact dependent mechanisms 10]. The soluble factors, TGF-β1[11], IFN- γ [12], indolamine 2,3-dioxygenase (IDO) [13] and prostaglandin E2 [14] have been suggested to be involved in this process.

MSC were found to suppress EAE either administered intravenously [15] or intrathecally [16]. When adoptively transferred, encephalitogenic T cells activated against proteolipid protein (PLP) in the presence of MSC, induced a milder disease (EAE) as compared to that induced by untreated PLP-specific T cells [17]. The immunomodulatory properties of MSC along withn the trans-differentiation potential could be relevant for induction of remyelination and recovery of damaged myelin. Indeed, in rats, intravenous or spinal injection of freshly isolated MSC resulted in remyelination of the spinal cord following an induced focal demyelinating lesion [18].

In the previous EAE studies from our group and others, syngeneic, allogenic or xenogenic unmodified MSC were mostly used [15–17,19–22].

Although some pilot clinical trials [23,24] have indicated a degree of beneficial effects of unmodified MSC in clinical application in diseases like MS and ALS, there is still a wide controversy on the issue of the possible mechanisms of action of MSCs in neuroimmune and degenerative diseases. It is still not clear whether beneficial effects in such diseases are solely mediated by immunomodulatory mechanisms or also

* Corresponding author.
E-mail address: ibrahimk@ekmd.huji.ac.il (I. Kassis).
[1] Equal contribution as PIs.

https://doi.org/10.1016/j.imlet.2019.05.009
Received 2 May 2019; Received in revised form 15 May 2019; Accepted 20 May 2019
Available online 30 May 2019

by neurotrophic and neuroprotective effects. One of the major criticism about the use of MSC in neurological diseases concerns the mesodermal origin of these cells that differs substantially from cells of the neuronal lineage [3]. On the other hand, there is a broader consensus about the possible use of neuronal stem cells for neurological diseases. However, neuronal stem cells, although seemingly ideal for application in neurological diseases [1,25,26], are associated with significant safety and technical issues [27,28].

Several groups have tried to find an alternative solution, by modifying the, easily obtained and produced MSC (that have an obvious advantage for clinical applications over other types of stem cells and an accepted safety profile), in order to "neuralize" them and turn their profile closer to cells of the neuronal lineage or even to transform them into neuronal stem cells. Sadiq and colleagues [29,30] suggested a protocol (using neural progenitor maintenance medium (NPMM) containing epidermal growth factor (EGF) and basic fibroblast growth factor). MSC-NPs derived from all MSCs showed a pattern of gene expression that correlated with neural commitment and increased homogeneity, paralleled by a reduced expression of mesodermal markers and reduced capacity for adipogenic or osteogenic differentiation. The immunoregulatory function of MSC-NPs was similar to that of MSCs in terms of their ability to suppress T-cell proliferation and to promote expansion of FoxP3-positive T regulatory cells in vitro. MSC-NPs promoted oligodendroglial differentiation from brain-derived neural stem cells. The same group [29] showed that multiple injections of MSC-NPs starting at the onset of the chronic phase of EAE disease improved neurological function compared to control animals, whereas a single injection had no effect on disease scores. A pilot trial in 20 patients 30] showed that intrathecal MSC-NP treatment was safe and well tolerated with indications of possible beneficial effects.

Two other groups have shown earlier a technique (using methods for neuronal stem cells culture) for efficient generation of neural stem cell-like cells from adult human bone marrow stromal cells [31,32]. These cells expressed high levels of early neuroectodermal markers, such as the proneural genes NeuroD1, Neurog2, MSl1 as well as otx1 and nestin, but lost the characteristics of mesodermal stromal cells. In the presence of selected growth factors, hmNSCs could be differentiated into the three main neural phenotypes: astroglia, oligodendroglia and neurons. In general, various protocols have been suggested for such "differentiation" of the MSC [33,34].

Offen and colleagues tried another technique to enhance the production of neuronal growth factors by MSCs. These MSC-NTF showed efficacy in the models of EAE and ALS and in pilot clinical trials in patients with ALS [35,36,23].

In this study, we present a novel way of an in vitro, two stages transdifferentiation of MSCs into neuronal spheres(Neuralized MSC, NMSC) and subsequently using a unique technique of CSF-mediated differentiation into effective neuronal precursors and functional cells of the neuronal lineage. We present the phenotypical, and functional properties of these Neuralized MSC (NMSC) and differentiated NMSC and their biological in vivo activity in the model of chronic EAE.

2. Methods

2.1. Isolation and purification of mouse and human MSC

2.1.1. Mouse bone-marrow derived mesenchymal stem cells

Femurs and tibias were removed from naïve or 6–9 week old EAE-induced C57BL/6 mice (disease score 2.5–3.5) and bone marrow (BM) cells were collected by flushing the bones with Dulbecco's modified eagles medium (DMEM; low glucose formulation); supplemented with 10% fetal bovine serum (FBS); 1% vitamins; 1% glutamine; and 1% non-essential amino acids (NEAA; all from Biological Industries; Beit-Haemek; Israel). The cells were centrifuged; resuspended in fresh culture medium and counted. Subsequently; the isolated BM cells were seeded in 75 mm^2 flasks and grown in an incubator at 37 °C and 5% CO_2 for 48 h. The plates were washed with PBS to remove all non-adhering cells; and the attached cells cultured for 14 days with twice weekly exchange of medium; and then harvested and reseeded at a ratio of 1:3.

2.1.2. Human Bone-Marrow derived mesenchymal stem cells

100 ml of whole BM from were aspirated into heparin containing bone marrow collection bags (Macopharma, USA) and delivered to the GMP-facility for further processing. BM aspirates were transferred from the heparin containing bone marrow aspiration bags into sterile 50 ml conical tubes (Corning, USA) using two spike tubing sets (Macopharma, USA) and diluted 1:1 (v:v) in Hank's Balanced Salt Solution (HBSS, Sigma-Aldrich), and MNC were separated from total bone marrow cells by Ficoll density gradient (1.073 g/ml) centrifugation (GE Healthcare, USA). Diluted BM were transferred in barrier-containing 50 ml tubes (LEUCOSEP™, Greiner-bio one, Germany) prefilled with 15 ml of Ficoll and centrifuged for 10 min, 1000xg, 24oC. The MNC layer was removed using sterile pasture pipette (Greiner-bio one, Germany) and transferred in 50 ml sterile tubes and diluted with 30 ml CTS™ DPBS. Cells were centrifuged twice 10 min, 1000 rpm, 24oC. Cell were re-seeded in "complete culture media" containing DMEM-LG, 5% FBS, 1% L-Glutamine and 1% non-essential amino acids (biological Industries, Israel). MNC were counted using hematocytometer and cell viability was evaluated using trypan-blue dye staining (Sigma-Aldrich, Israel). MNC were washed and re-suspended with complete media media and seeded on T-175 culture vessels. The seeding density was 100,000 cells/cm2 and incubated in a 37 °C/5% CO2 humidified incubator for 48 h. The cell culture was examined under the microscope. At this stage, non-adherent, mononuclear cell floated in the culture supernatant and plastic-adherent MSC attached to the flask surface. The culture supernatant containing the non-adherent mononuclear cells was removed, and the adherent cells gently washed with 100 ml DPBS. The step from MNC seeding to hMSC harvesting is designated as Passage 0 (P0). The P0 cells were incubated in a 37 °C/5% CO2 humidified incubator and growth medium was replaced twice a week, with fresh complete medium as until the culture is 80–90% confluent.

2.2. Generation and propagation of neutralized MSC (NMSC) from hMSC

The generation of NMSC is a part of a patented method (united stated patent, US201361890481, European Patent EP3058063B1). Briefly, For the generation of NMSC, hMSC were cultured in DMEM-F12 serum free media (Biological Industries, Israel) containing 2% B-27 supplement (Gibco, USA), basic fibroblast growth factor (bFGF, Peprotech, Israel), epidermal growth factor (EGF, Peprotech, Israel). The cells were cultured for 14 days with medium exchange twice a week. Floating neurosphere-like structures (NMSC) were visible after 48 h. The suspension was then washed gently by centrifugation and cells pellet was re-suspended in DMEM-F12 serum free media supplemented with 20 ng/ml bFGF, 25 ng/ml EGF, 5 μg/ml heparin, 1% non-essential amino acids and 1% MEM-alpha vitamins and seeded in ultra Low-Adherence™ flasks for propagation (Corning, Mexico).

2.3. Characterization of MSC and NMSC by flow cytometry

Isolated cells were characterized by flow cytometry analysis (FACS) of surface antigen expression. The International Society for Cellular Therapy (ISCT) recommends that MSC should be characterized by a set of minimum criteria including the presence/absence of the below specific surface markers. Accordingly, MSC were tested for the markers CD105, CD73 and CD90 on the cell surface (> 95% positive) and the lack of expression (< 2% positive) of CD34, CD45, CD79alpha, CD19, and HLA-DR. For the identification of neuralized MSC (NMSC) cells were stained for Nestin and PSNcam antibodies. The stained cells were read and analyzed with CYTOMICS FC500 (Beckman Coulter, Germany).

2.4. CSF-induced differentiation of NMSC

The generation of NMSC is a part of a patented method (united stated patent, US201361890481, European Patent EP3058063B1). To terminally differentiate NMSC into neural cell lineages, intact NMSC from passage 2 were used (namely, after the NMSC propagated at least once in the renew medium, i.e. the medium was at least once replenished). The NMSC from passage 2 were cultured in DMEM-F12/GlutaMax™ serum free medium (Invitrogen, USA) supplemented with 1% non-essential vitamins (biological industries, Israel) and 0.2% Allogenic CSF seeded in regular attachment tissue vessels (NUNC, USA). Medium was changed twice a week and cells were culture for 17–21 days for differentiation progression.

2.5. Immunostaining of NMSC and naive MSC

The medium was aspirated and the NMSC were washed gently with 0.03% Tween 20 (Sigma-Aldrich, Rehovot, Israel) diluted in DPBS and then fixed with fresh paraformaldehyde, 4%, for 20 min at room temperature. To stain the intracellular components, the cells were permeabilized with Triton X-100 (Sigma-Aldrich, Rehovot, Israel), 0.1%, for 10 min. For blocking nonspecific binding, the cells were rinsed with 2.5% bovine serum albumin in DPBS for 45 min at room temperature on a slowly rotating plate. Then, the cells were washed 3 times with 0.03% Tween 20 diluted in PBS, and incubated for 120 min with the following primary antibodies for mouse: anti human CD34, anti human CD45, anti human CD90, anti human CD105 anti human Nestin, anti human PS-NCAM, anti human MAP2, anti human GFAP, anti human MAP2 and anti human MBP (all from Abcam, UK) diluted to the required concentrations with DPBS buffer containing 1% bovine serum albumin After washing, the cells were incubated with goat anti mouse fluorescein isothiocyanate-conjugated and goat anti rabbit tetramethylrhodamine isothiocyanate-conjugated secondary antibodies diluted in bovine serum albumin buffer, 1%, on a slowly rotating plate for 45 min in the dark at room temperature. The cells were mounted on slides with DAPI-mounting solution (Vectashield, Calif., USA) and examined under fluorescence and light microscopy. The number of immunoreactive cells was determined under a fluorescence microscope (Nikon, Japan) in relation to the nuclei stained with DAPI.

2.6. In vitro proliferation of mouse lymphocytes

Draining lymph nodes were excised from C57BL/6 mice on day 10 post MOG-immunization for EAE induction and cultured as single-cell suspensions. Lymph node cell (LNC) proliferation was assayed in vitro by ^{3}H-thymidine incorporation. All cultures were carried out in triplicate in 96-well, flat-bottom, microtiter plates. The assay was carried out by seeding 4 × 105 cells/well in 0.2 mL of RPMI medium (Sigma, Rehovot, Israel) supplemented with 2.5% FCS, 1 mM l-glutamine, and antibiotics. Basal ^{3}H-thymidine incorporation was determined in response to phytohaemagglutinin (PHA, 1 μg/mL). To examine the effect of unmodified or neuralized MSC on LNC proliferation, different numbers of MSC were added and co-cultured with the LNCs. The cultures were incubated for 48 h in a humidified atmosphere of 5% carbon dioxide at 37 °C and then pulsed for 16 h with ^{3}H-thymidine (1μCi/well). Cells were harvested on fiberglass filters using a multiharvester and the radioactivity was counted

2.7. Induction of chronic experimental autoimmune encephalomyelitis in C57BL/6 mice (cEAE)

Chronic EAE was induced in 6- to 7-week-old female C57BL/6 mice by immunization with an emulsion containing 300 μg of purified myelin oligodendrocyte glycoprotein (MOG) 35–55 peptide in phosphate-buffered saline (PBS) and an equal volume of complete Freund adjuvant containing 5 mg of H37Ra (Difco Laboratories, Detroit, Michigan). A 0.2-mL volume of the inoculum was injected subcutaneously on the day of induction (day 0) and on day 7. In addition, 300 ng of Bordetella pertussis toxin in 0.2 mL of PBS was injected intraperitoneally on days 0 and 2. Animals with EAE were scored daily for neurological symptoms according to the EAE clinical severity scale, as follows: 0, asymptomatic; 1, partial loss of tail tonicity; 2, tail paralysis; 3, hind limb weakness; 4, hind limb paralysis; 5, four-limb paralysis; 6, death.

2.8. Histopathology

The inflammatory process and axonal pathology of chronic EAE were quantified using axial frozen sections at predetermined levels. Sections were stained with the modified Bielschowsky technique for simultaneous evaluation of axonal pathology and inflammation. Brain sections were evaluated under (20×) magnification of optical fields. An examiner blinded to treatment and clinical severity counted the total number of perivascular mononuclear infiltrates in hematoxylin–eosin sections and estimated the axonal loss (modified Bielschowsky stain). To grade inflammation, the number of perivascular infiltrations were counted. For axonal loss we used the following scale: 0, normal axonal density; 1, focused mild to moderate axonal loss; 2, scattered mild to moderate axonal loss; 3, focused severe axonal loss; and 4, scattered severe axonal loss.

3. Results

3.1. Characterization of generated neuralized MSC (NMSC) derived from Mesenchymal stem cells

As described above (Methods), NMSC were generated by culturing naïve MSC (passage 1–2), derived from bone marrow, with growth factors (EGF and FGF-b) and cultured for 48 h. After 48 h, floating spheroid like structures were formed (Fig. 2). Their median diameter was ~70-100uM. The yield of NMSC formation was 65–80% from each batch of naïve MSC. While naïve MSC expressed classical mesenchymal stem cells markers ($CD90^{+}$/$CD105^{+}$) NMSC were found to express $Nestin^{+}$/$PSNCAM^{+}$ while loosing there mesenchymal markers $CD90^{-}$/$CD105^{-}$ (Fig. 1). Moreover, the generated NMSC lost their ability to differentiate to mesodermal lineage cells (i.e. adipocytes, chondrocytes and osteocytes) (data not shown). The propagation of the generated NMSC were dependent on the existence of both the growth factors EGF and FGF-b and withdrawal of one of the them, resulted in gradual death of the NMSC (data not shown).

3.2. CSF-induced neural differentiation of neuralized MSC

To induce neural differentiation of NMSC, Allogenic CSF was added to a fresh medium, which contained growth factors, and the cells were cultured for additional 7–10 days while changing the medium twice a week. A morphologic change was observed in the spheroid structures (Fig. 4). The differentiated NMSC were tested for expression of neuronal markers and found to express MAP2, beta III tubulin, MBP and GFAP. FACS analysis shown in Fig. 3 demonstrates the neural-differentiation potential of these NMSC after exposure to CSF containing differentiation medium. Differentiated NMSC were labeled by the beta III tubulin, MBP, MAP2 or GFAP markers. 37.61% of the cells were positive for the MAP2 labeling, pointing to their potential to differentiate into neurons. Similar results were obtained with the beta III tubulin labeling. 37.25% of the cells were positive for the Beta III tubulin, which again indicates a differentiation into neurons. MBP labeling showed 64.46% positivity, indicating a potential direction towards oligodendrocytes. The potential to differentiate into astrocytes was evaluated by GFAP labeling, which showed 71.17% positivity.

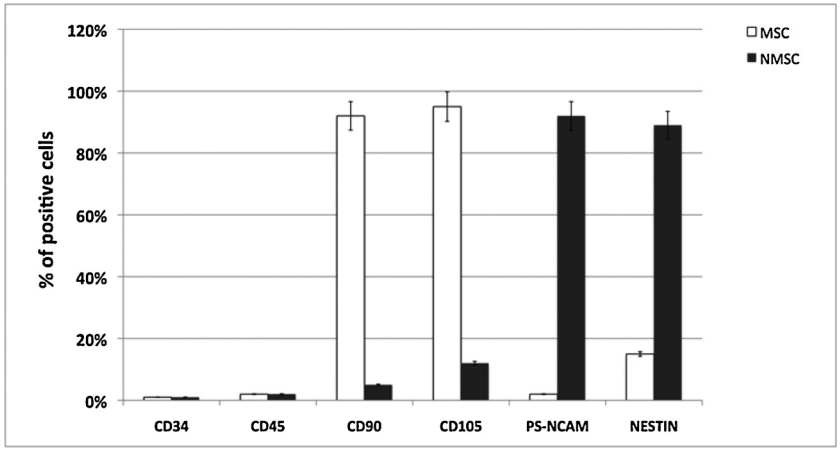

Fig. 1. Cell markers expression of naïve MSC and Neuralized MSC.
Characterization of NMSC. NMSC generated from hMSC were positively stained for the marker Nestin and PS-NCAM) Representative FACS analysis of hMSC and NMSC showing that hMSC stained positively for the mesodermal markers CD90 and CD105 and negative for the hematopoietic markers CD34 and CD45. NMSC were stained positively for Nestin and PSNCAM while showing low to negative staining for the mesenchymal and hematopoietic markers CD34, CD45,CD90 and CD105.

3.3. Generated NMSC suppress lymphocytes proliferation

In order to evaluate the immunomodulatory potential of the generated NMSC, a mixed lymphocyte reaction assay was used (MLR). As described in methods, different doses of NMSC were co-cultured with lymphocytes and the proliferation of the lymphocytes was measure in a ^{3}H-Thymidine radioactivity classical assay. NMSC suppressed in a dose-deepened manner the PHA-induced proliferation of lymphocytes (Fig. 5).

3.4. Neurotrophic factors secretion profile

In order to evaluate the neurotrophic potential of "neuralized" MSC (NMSC) at the different stages of our production process, we examined the secretion of neurotrophic factors by MSC, NMSC and differentiated NMSC. The cells were cultured with DMEM without any addidatives for 24 h and the medium was collected for ELISA testing. The secreted neurotrophic factors were examined in the medium, which was collected from the naïve MSC, the NMSC and the differentiated NMSC. We tested three factors: nerve growth factor (NGF), cilliary neurotrophic factor (CNTF) and brain derived neurotrophic factor (BDNF). The differentiated spheres showed a significant increase in the production of all these factors, as compared to naïve MSCs. BDNF showed the largest increase, (1008%), whereas the CNTF and the NGF showed a more modest but significant increase (approximately 400%), as shown in Fig. 6. The CNTF concentration in the soup collected from MSCs was 169.7 pg/ml, 276.4 pg/ml in the supernatant of the spheres and 816.5 pg/ml in the differentiated NMSC (increase of 163% and 481% vs the MSCs, accordingly). NGF concentrations showed a similar pattern of increase (1.57 pg/ml in the MSCs, 4.89 pg/ml in the spheres and 5.76 pg/ml in the diff. spheres) (100%, 310% and 365% accordingly). The most robust increase was found when BDNF secretion was almost undetectable in the soup collected from MSCs, but was high (500 pg/ml) in the NMSC supernatant and much higher (1008.5 pg/ml) in the differentiated NMSC (increase of 500% and 1008% vs the MSCs, accordingly).

3.5. In-vivo experiments: clinical improvement, immunomodulation and axonal protection following differentiated NMSC transplantation in EAE mice

The clinical course of chronic EAE was ameliorated in animals treated with MSCs following intraventricular administration (Fig. 7A). As presented in Table (Fig. 7B) mice treated with MSC had a mortality rate of 0% and a mean maximal clinical score of 1.42 ± 1.21 compared with 40% mortality and a mean maximal clinical score of 2.51 ± 1.93 in the saline-injected control group. In animals treated with NMSC, the mortality rate was again 0% vs. 40% in the controls; mean maximal clinical scores were 1.18 ± 0.87 vs. 2.51 ± 1.93, respectively. The lowest mean maximal clinical score (0.76 ± 0.53) was observed in animals treated with differentiated spheres. All deaths were related to EAE disease severity (Table 1).

Histopathologic evaluation of brains of MSC-treated mice at day 30 after chronic-EAE induction revealed a reduction in the total number of infiltrates in all treated animal groups (Fig. 8). The size and cellularity of the lymphocytic infiltrates were significantly reduced in all treated groups, with a greater degree of suppression in the differentiated spheres treated mice (60.1 ± 21.3 in the control group, 32.2 ± 7.25 in the MSCs group, 23.4 ± 7.89 in the NMSC group and 14.9 ± 4.73 in the differentiated NMSC group).

A neuroprotective effect was also observed following treatment with MSCs and was more profound in the group which was treated with the differentiated NMSC produced from MSCs (Fig. 9). In animals with chronic EAE that were treated with saline, the axons were significantly damaged (axonal loss degree of 3.5); whereas in mice treated with MSCs, NMSC or differentiated NMSC, the great majority of the axons

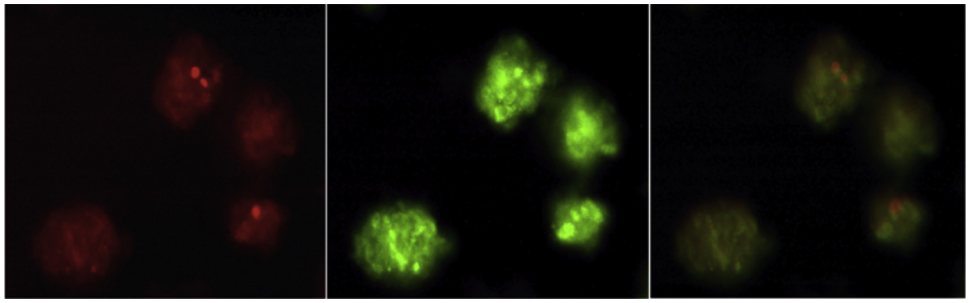

Fig. 2. Characterization of NMSC.
NMSC generated from MSC were positively stained for the markers Nestin (A) and PSNCAM (B). Merged micrograph (C) of the two markers is also shown.

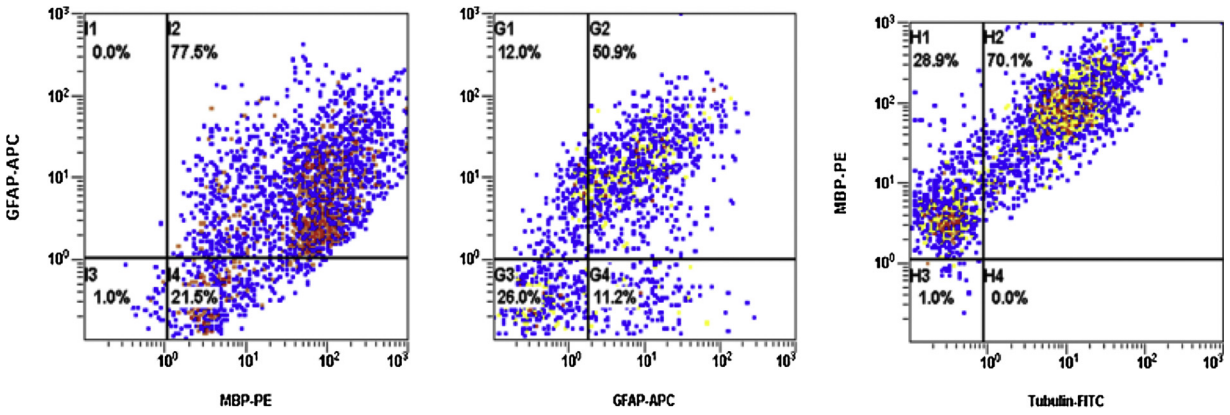

Fig. 3. Differentiated NMSC express neuronal differentiation markers.
Diff NMSC were labeled with markers of neuronal lineage and neuronal differentiation- β tubulin type III, a neuronal marker; glial fibrillary acidic protein (GFPA), an astrocytic marker and myelin basic protein (MBP), an oligodendrocyte marker. The labeled cells were examined by FACS. Some of the cells were shown positive for one of the markers, while others shown a double positive labeling, indicating their differentiation potential.

were found intact following treatment (2, 1.5 and 1). This neuroprotective effect is shown as axonal loss grades.

4. Discussion

In our study we describe a novel way (united stated patent, US201361890481, European Patent EP3058063B1) for production of neuronal-like stem cells ("neuralized" MSC, NMSC) and their further neuronal differentiation using CSF, from bone marrow mesenchymal stromal cells (MSC). NMCS showed a powerful potential to differentiate to astrocytes, oligodendrocytes or neurons using exposure to CSF, including the acquisition of genetic properties of terminally differentiated cells of the neuronal lineage. The NMCS and differentiated NMSC secret significantly higher quantities (than regular unmodified MSC) of neurotrophic factors, exhibiting powerful (in vivo and in vitro) neurotrophic effects paralleled by immunomodulatory properties. In vivo injection of NMSC ameliorated chronic EAE in a more effective way than regular MSC.

Some studies have raised the possibility that a fraction of the MSC derived from bone marrow can be directed to form spheres, [31,32,37–39] which are morphologically and phenotypically similar to neurospheres generated from neural stem cells. Kabos et al described the isolation and successful propagation of neural progenitor cells from adult rat bone marrow 37]. Unfractionated bone marrow cultured in vitro with epidermal growth factor and basic fibroblast growth factor gave rise to cellular spheres that differentiated into neurons and glia. The cellular spheres expressed nestin, a neural stem cell marker, as well as CD90, a marker of mesenchymal stem cells. Hermann et al. described the efficient conversion of human adult bone marrow stromal cells into a neural stem cell-like population [31]. These cells grown in neurosphere-like structures, expressed high levels of early neuroectodermal markers, such as the proneural genes NeuroD1, Neurog2, MSl1 as well as otx1 and nestin, but lose the characteristics of mesodermal stromal cells. In the presence of selected growth factors, hmNSCs could be differentiated into the three main neural phenotypes: astroglia, oligodendroglia and neurons. Fu et al reported that NSCs can be generated from human BM-derived mesenchymal stem cells 32]. When cultured in NSC culture conditions, 8% of MSCs were able to generate neurospheres. These MSC-derived neurospheres expressed characteristic NSC antigens, such as nestin and musashi-1, and were capable of self-renewal and multilineage differentiation into neurons, astrocytes, and oligodendrocytes [32] Furthermore, when these MSC-derived neurospheres were cocultured with primary astrocytes, they differentiated into neurons that possess both dendritic and axonal processes, form synapses, and were able to fire tetrodotoxin-sensitive action potentials [32]. Sadiq and colleagues described similar results by their technique, which generated MSC-NPs from MS patients and healthy donors [38]. They showed that these cells after culturing with neural differentiation medium, express higher levels of neuronal genes (Nestin, GFAP, NF-M and CXCR4). The generated cells exhibited immunoregulatory properties in evidenced by their ability to suppress T-cell proliferation and trophic effects supporting the ability of rat neural stem cells to differentiate into cells from the neural lineage. However, in all the above studies, the efficacy and yield of the produced neurosphere or "neuralized" cells was not compared (in vitro and in vivo) with unmodified MSCs. Moreover, there was no convincing evidence of true neural differentiation of these "neurospheres" and their resemblance to "real" neural-like stem cells. These studies also did not study the neurotrophic abilities of the neuropsherical-like cells that were produced. The viability of these cells in terms of long-term culturing was also not-well established. [38,30,29]. All these issues are important for possible clinical applications of neuralized MSC in human diseases.

The use of NMSCs and mainly the differentiated NMSC, as produced by our protocol, provides several advantages over conventional mesenchymal cells used in previous studies. NMSC can be produced by MSC that are obtained from adult bone marrow and easily expanded and effectively induced to transdifferentiate towards the neuronal lineages using a biological induction method without any recombinant

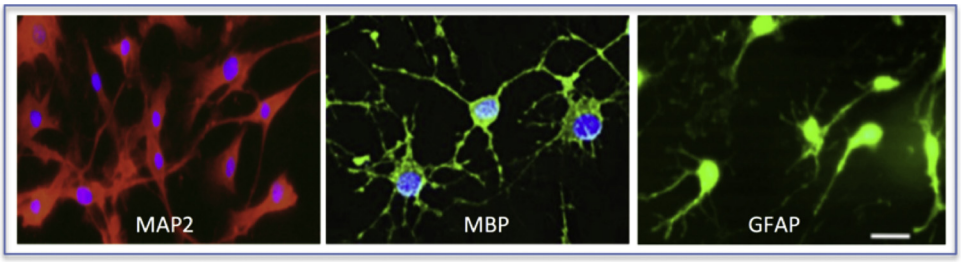

Fig. 4. Immunostaining of differentiated NMSC.
NMSC were cultured with neural differentiation medium for 14 days while changing medium twice. Obvious morphological changes were observed within the culture resembling neural and glial-like cells (A–C). A positive staining of the differentiated cells was observed for the neuronal marker MAP2 (A), the oligodendrocytic marker MBP (B) and for the astrocytic marker GFAP (C).

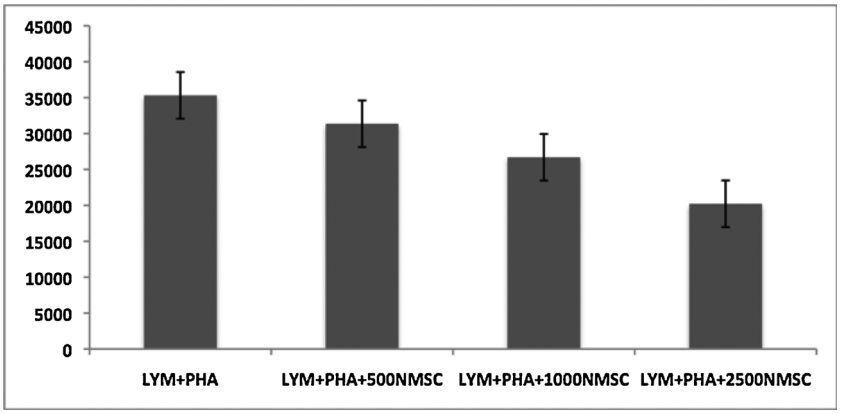

Fig. 5. Immunomodulatory effects of NMSC.
A significant dose-dependent suppression of the proliferation of lymphocytes obtained from peripheral blood of healthy donor by NMSC was observed using a ^{3}H-incorporation assay (* $p < 0.05$, **$p < 0.001$).

or chemical interference for neural differentiation induction. These NMSC biologically resembled "real" neurospheres in several aspects from the neuropheres markers they express (i.e Nestin and PSNcam) as well as the dependence of these generated sphere to the presence of the growth factors EGF and FGF for long-term viability and expansion (not shown) as well as to the diameter of the spheres (± 100-250 μm

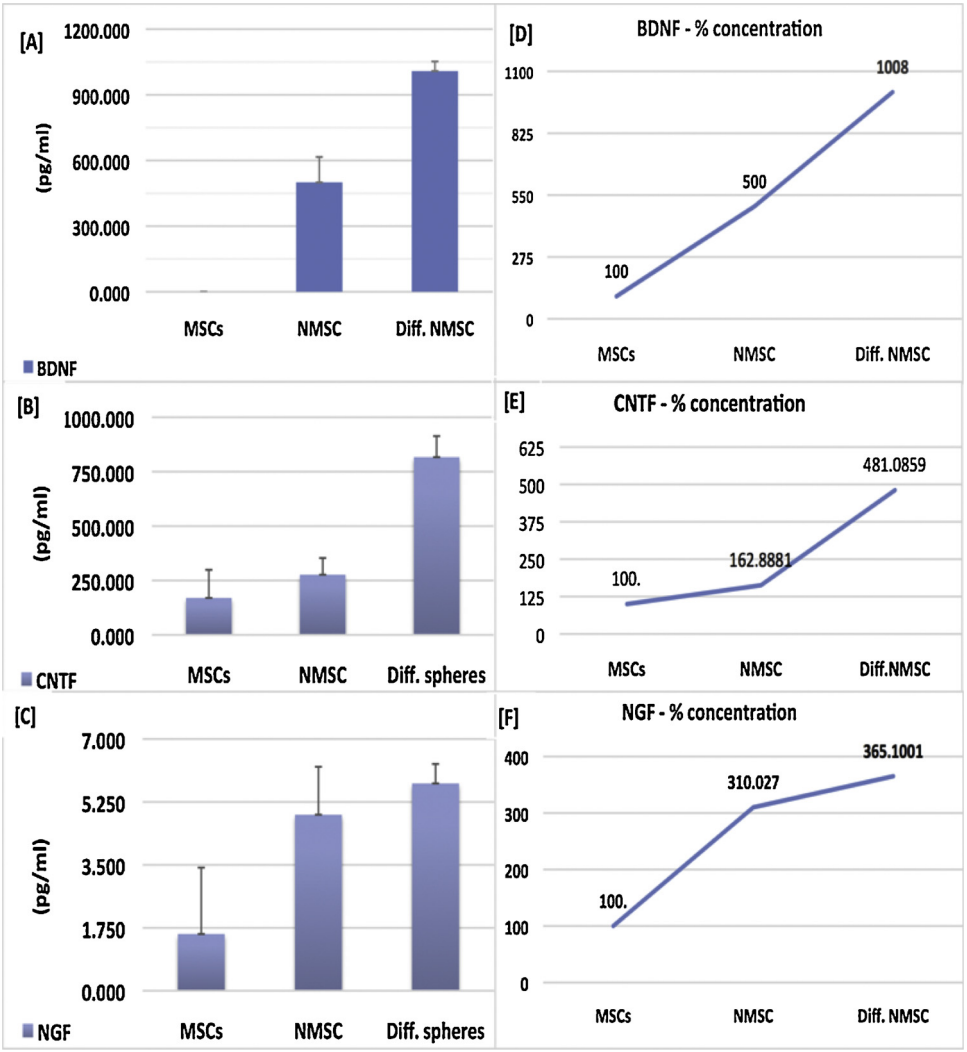

Fig. 6. Neurotrophic factors production of MSC, NMSC and Differentiated NMSC.
Neurotrophic factors production of MSC, NMSC and diff. NMSC as measured by their supernatants. The cells were cultured with DMEM without any additives for 24 h and the medium was collected for ELISA. 3 factors were measured- BDNF, CNTF and NGF. The results are shown in pg/ml and as a percentage of the factors secretion when compared with the naïve MSC, which represents the 100%.

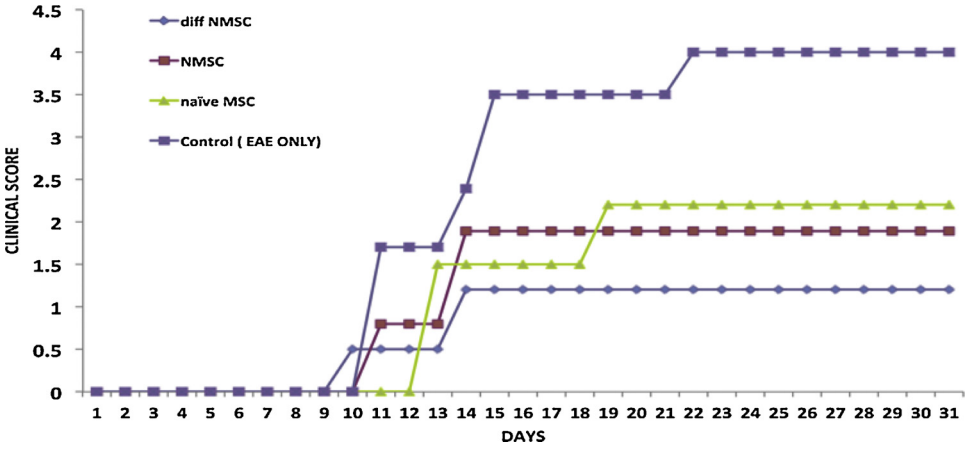

Fig. 7. Clinical effects of NMSC on chronic EAE vs unmodified MSC.
Clinical course of chronic experimental allergic encephalitis (EAE) in mice with no treatment (controls) or treated with MSC, NMSC or diff. NMSC 8 days after EAE induction. Treatments were administered intraventricularly. The EAE clinical severity scale ranges from 0 to 6: 0 = asymptomatic; 1 = partial loss of tail tonicity; 2 = tail paralysis; 3 = hind limb weakness; 4 = hind limb paralysis; 5 = 4-limb paralysis and 6 = death.

Table 1
Clinical parameters of EAE treated mice.

Group	N	Cumulative Score	Mean Clinical Score	Mortality
EAE	10	78	2.51 ± 1.93	40%
EAE + MSC	8	44.1	1.42 ± 1.21	0%
EAE + NMSC	8	36.6	1.18 ± 0.87	0%
EAE + Diff NMSC	8	23.6	0.76 ± 0.53	0%

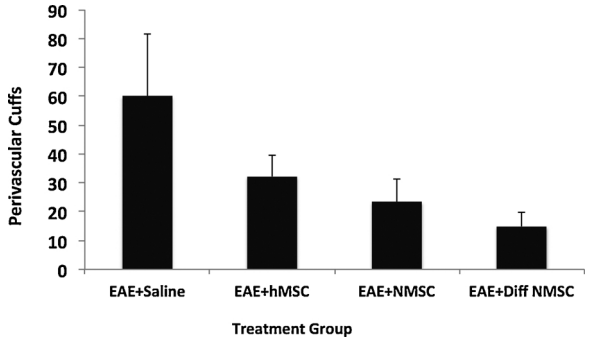

Fig. 8. Inflammation in EAE after treatment.
Lymphatic infiltration in the central nervous system in mice with EAE treated with either saline (controls), NAÏVE MSC, neuralized mesenchymal stem cells (NMSCs) or differentiated NMSCs. A significant reduction in the mean number of infiltrates is seen in the NMSC-treated animals and a further reduction is observed in the Diff. NMSCs' ones. (** $p < 0.01$), (* $p < 0.05$).

depending on the culture stage). These NMSC were shown to have immunomodulatory properties like neural stem cells derived neuropheres and the suppression of the proliferation was as well dose-dependent. NMSC produced this way, can be injected autologously without the need for immunosuppression to prevent rejection. And, most importantly, no genetic manipulation is involved in the process of NMSC production and therefore they seem to be similar to unmodified MSC in terms of the (low) risks for genetic abnormalities and carcinogenesis. Moreover, NMSC carrying a neuronal lineage profile are less prone to differentiation into mesodermal tissues (since loosing there mesodermal properties of cell markers and mesodermal differentiation abilities) and overriding the risky setting of injection of a wrong kind cell type (MSC) in a non-suitable environment (CNS).

The differentiated NMSC have the expression of surface markers specific for neurons, astrocytes and oligodendrocytes and strongly indicate a true neuronal-lineage transdifferentiation of the MSC by our protocol. These differentiated NMSC neural cells was shown to produce very high levels of neurotrophic factors compared to naïve MSC and naïve NMSC which make them to act as strong neuroprotective agents in neurodegenerative diseases resulted in axonal damage and eventually neural-death.

In the mouse model of chronic EAE, NMSC and differentiated NMSC injected intraventricularly suppressed the clinicopathologic manifestations of chronic EAE and induced a neuroprotection; their effects on all of the disease parameters were significantly more pronounced compared to unmodified MSC. In addition to the in vitro suppression of the proliferation of lymphocytes and the significant reduction in the number of infiltrating cells in the brains of NMSC-treated animals with chronic EAE, confirmed the immunomodulatory effects of NMSC. Based on our previous studies, in which we demonstrated the advantages of intraventricular MSC-transplantation, we followed a similar protocol of administration in the current study. The neuroprotective effect of the

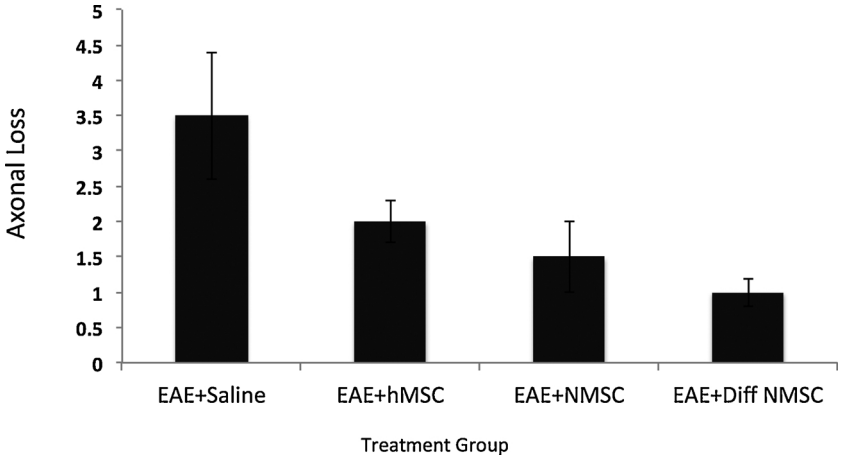

Fig. 9. Axonal loss in EAE after treatment.
EAE mice treated with saline vs. mice treated with naïve MSC, NMSC and Diff MSC was evaluated using modified Bielchwosky stain. It was found that in all treated groups a clear degree of axonal preservation was found. The group treated with diff NMSC was presented a very convincing degree of axonal preservation (** $p < 0.01$) compared to naïve MSC and NMSC (**$p < 0.05$).

NMSC can be either attributed to the injected cells that express neuronal markers and can further differentiate in situ, and/or to the activation of local resident CNS stem cells and progenitors through neuronal growth factors locally produced by the transplanted NMSC. The same mechanisms (ie production of new cells of the neuronal lineage in situ or activation of the intrinsic neuronal stem cells) seem also to be involved in the neuroregenerating and immunomodulatory effects, observed in EAE.

In summary, the above mentioned practical advantages of NSMC and differentiated NMSCs, along with their increased clinicopathological efficacy over “regular”, unmodified MSC, in the model of chronic EAE, may provide the scientific basis for the use of NMSC and differentiated NMSCs as a future treatment in diseases such as multiple sclerosis.

References

[1] S. Pluchino, G. Martino, The therapeutic plasticity of neural stem/precursor cells in multiple sclerosis, J. Neurol. Sci. 265 (2008) 105–110.
[2] J. Sanchez-Ramos, S. Song, F. Cardozo-Pelaez, et al., Adult bone marrow stromal cells differentiate into neural cells in vitro, Exp. Neurol. 164 (2000) 247–256.
[3] E.Y. Snyder, The risk of putting something where it does not belong: mesenchymal stem cells produce masses in the brain, Exp. Neurol. 230 (2011) 75–77.
[4] R. Talens-Visconti, A. Bonora, R. Jover, et al., Hepatogenic differentiation of human mesenchymal stem cells from adipose tissue in comparison with bone marrow mesenchymal stem cells, World J. Gastroenterol. 12 (2006) 5834–5845.
[5] M.F. Pittenger, A.M. Mackay, S.C. Beck, et al., Multilineage potential of adult human mesenchymal stem cells, Science 284 (1999) 143–147.
[6] P. Bossolasco, L. Cova, C. Calzarossa, et al., Neuro-glial differentiation of human bone marrow stem cells in vitro, Exp. Neurol. 193 (2005) 312–325.
[7] J. Oswald, S. Boxberger, B. Jorgensen, et al., Mesenchymal stem cells can be differentiated into endothelial cells in vitro, Stem Cells 22 (2004) 377–384.
[8] D. Woodbury, E.J. Schwarz, D.J. Prockop, I.B. Black, Adult rat and human bone marrow stromal cells differentiate into neurons, J. Neurosci. Res. 61 (2000) 364–370.
[9] I. Kassis, A. Vaknin-Dembinsky, D. Karussis, Bone marrow mesenchymal stem cells: agents of immunomodulation and neuroprotection, Curr. Stem Cell Res. Ther. 6 (2011) 63–68.
[10] A. Uccelli, L. Moretta, V. Pistoia, Immunoregulatory function of mesenchymal stem cells, Eur. J. Immunol. 36 (2006) 2566–2573.
[11] M. Di Nicola, C. Carlo-Stella, M. Magni, et al., Human bone marrow stromal cells suppress T-lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli, Blood 99 (2002) 3838–3843.
[12] M. Krampera, L. Cosmi, R. Angeli, et al., Role for interferon-gamma in the immunomodulatory activity of human bone marrow mesenchymal stem cells, Stem Cells 24 (2006) 386–398.
[13] R. Meisel, A. Zibert, M. Laryea, U. Gobel, W. Daubener, D. Dilloo, Human bone marrow stromal cells inhibit allogeneic T-cell responses by indoleamine 2,3-dioxygenase-mediated tryptophan degradation, Blood 103 (2004) 4619–4621.
[14] P.A. Sotiropoulou, S.A. Perez, A.D. Gritzapis, C.N. Baxevanis, M. Papamichail, Interactions between human mesenchymal stem cells and natural killer cells, Stem Cells 24 (2006) 74–85.
[15] E. Zappia, S. Casazza, E. Pedemonte, et al., Mesenchymal stem cells ameliorate experimental autoimmune encephalomyelitis inducing T-cell anergy, Blood 106 (2005) 1755–1761.
[16] I. Kassis, N. Grigoriadis, B. Gowda-Kurkalli, et al., Neuroprotection and immunomodulation with mesenchymal stem cells in chronic experimental autoimmune encephalomyelitis, Arch. Neurol. 65 (2008) 753–761.
[17] E. Gerdoni, B. Gallo, S. Casazza, et al., Mesenchymal stem cells effectively modulate pathogenic immune response in experimental autoimmune encephalomyelitis, Ann. Neurol. 61 (2007) 219–227.
[18] M. Inoue, O. Honmou, S. Oka, K. Houkin, K. Hashi, J.D. Kocsis, Comparative analysis of remyelinating potential of focal and intravenous administration of autologous bone marrow cells into the rat demyelinated spinal cord, Glia 44 (2003) 111–118.
[19] L. Bai, D.P. Lennon, V. Eaton, et al., Human bone marrow-derived mesenchymal stem cells induce Th2-polarized immune response and promote endogenous repair in animal models of multiple sclerosis, Glia 57 (2009) 1192–1203.
[20] G. Constantin, S. Marconi, B. Rossi, et al., Adipose-derived mesenchymal stem cells ameliorate chronic experimental autoimmune encephalomyelitis, Stem Cells 27 (2009) 2624–2635.
[21] J. Zhang, Y. Li, J. Chen, et al., Human bone marrow stromal cell treatment improves neurological functional recovery in EAE mice, Exp. Neurol. 195 (2005) 16–26.
[22] J. Zhang, Y. Li, M. Lu, et al., Bone marrow stromal cells reduce axonal loss in experimental autoimmune encephalomyelitis mice, J. Neurosci. Res. 84 (2006) 587–595.
[23] P. Petrou, Y. Gothelf, Z. Argov, et al., Safety and clinical effects of mesenchymal stem cells secreting neurotrophic factor transplantation in patients with amyotrophic lateral sclerosis: results of phase 1/2 and 2a clinical trials, JAMA Neurol. 73 (2016) 337–344.
[24] D. Karussis, C. Karageorgiou, A. Vaknin-Dembinsky, et al., Safety and immunological effects of mesenchymal stem cell transplantation in patients with multiple sclerosis and amyotrophic lateral sclerosis, Arch. Neurol. 67 (2010) 1187–1194.
[25] D. Karussis, P. Petrou, I. Kassis, Clinical experience with stem cells and other cell therapies in neurological diseases, J. Neurol. Sci. 324 (2013) 1–9.
[26] N.J. Scolding, M. Pasquini, S.C. Reingold, et al., Cell-based therapeutic strategies for multiple sclerosis, Brain 140 (2017) 2776–2796.
[27] D.J. Mathews, J. Sugarman, H. Bok, et al., Cell-based interventions for neurologic conditions: ethical challenges for early human trials, Neurology 71 (2008) 288–293.
[28] L. Anderson, R.M. Burnstein, X. He, et al., Gene expression changes in long term expanded human neural progenitor cells passaged by chopping lead to loss of neurogenic potential in vivo, Exp. Neurol. 204 (2007) 512–524.
[29] V.K. Harris, Q.J. Yan, T. Vyshkina, S. Sahabi, X. Liu, S.A. Sadiq, Clinical and pathological effects of intrathecal injection of mesenchymal stem cell-derived neural progenitors in an experimental model of multiple sclerosis, J. Neurol. Sci. 313 (2012) 167–177.
[30] V.K. Harris, J. Stark, T. Vyshkina, et al., Phase I trial of intrathecal mesenchymal stem cell-derived neural progenitors in progressive multiple sclerosis, EBioMedicine 29 (2018) 23–30.
[31] A. Hermann, R. Gastl, S. Liebau, et al., Efficient generation of neural stem cell-like cells from adult human bone marrow stromal cells, J. Cell. Sci. 117 (2004) 4411–4422.
[32] L. Fu, L. Zhu, Y. Huang, T.D. Lee, S.J. Forman, C.C. Shih, Derivation of neural stem cells from mesenchymal stemcells: evidence for a bipotential stem cell population, Stem Cells Dev. 17 (2008) 1109–1121.
[33] A. Hermann, S. Liebau, R. Gastl, et al., Comparative analysis of neuroectodermal differentiation capacity of human bone marrow stromal cells using various conversion protocols, J. Neurosci. Res. 83 (2006) 1502–1514.
[34] M. Krampera, S. Marconi, A. Pasini, et al., Induction of neural-like differentiation in human mesenchymal stem cells derived from bone marrow, fat, spleen and thymus, Bone 40 (2007) 382–390.
[35] Y. Barhum, S. Gai-Castro, M. Bahat-Stromza, R. Barzilay, E. Melamed, D. Offen, Intracerebroventricular transplantation of human mesenchymal stem cells induced to secrete neurotrophic factors attenuates clinical symptoms in a mouse model of multiple sclerosis, J. Mol. Neurosci. 41 (2010) 129–137.
[36] M. Dadon-Nachum, K. Ben-Yaacov, T. Ben-Zur, et al., Transplanted modified muscle progenitor cells expressing a mixture of neurotrophic factors delay disease onset and enhance survival in the SOD1 mouse model of ALS, J. Mol. Neurosci. 55 (2015) 788–797.
[37] P. Kabos, M. Ehtesham, A. Kabosova, K.L. Black, J.S. Yu, Generation of neural progenitor cells from whole adult bone marrow, Exp. Neurol. 178 (2002) 288–293.
[38] V.K. Harris, R. Faroqui, T. Vyshkina, S.A. Sadiq, Characterization of autologous mesenchymal stem cell-derived neural progenitors as a feasible source of stem cells for central nervous system applications in multiple sclerosis, Stem Cells Transl. Med. 1 (2012) 536–547.
[39] Q. Yang, J. Mu, Q. Li, et al., A simple and efficient method for deriving neurospheres from bone marrow stromal cells, Biochem. Biophys. Res. Commun. 372 (2008) 520–524.