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Therapeutic Administration of Mesenchymal Stem Cells Abrogates the Relapse Phase in Chronic Relapsing-Remitting EAE

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Abstract

Multiple Sclerosis (MS) is a neuroinflammatory and immune-mediated chronic disease of the Central Nervous System which progressively damages the axonal myelin sheath, leading to axonal transmission impairment and to the development of neurological symptoms. Most MS cases are characterized by a relapsing-remitting course, and current therapies rely only on the use of immunomodulating drugs which are, however, unable to reverse disease progression. Among the newly proposed alternative therapies, Mesenchymal Stem Cells (MSCs) are considered suitable for MS treatment due to their capacity to modulate the immune response and to modify the pattern of the released cytokines. So far, encouraging results have been obtained with the administration of MSCs before disease onset, mainly in animal models of acute Experimental Autoimmune Encephalomyelitis (EAE) in which MSCs were able to reduce inflammation, thus ameliorating also the disease's clinical symptoms.

On the contrary, only a very small number of studies have investigated the effect of MSCs on relapsing-remitting models of the disease.

Here, we investigated the therapeutic potential of MSC administration, both before and after the disease's onset, in an animal model of MS represented by Dark Agouti rats affected by chronic Relapsing-Remitting EAE. Our results demonstrated that in chronic Relapsing-Remitting EAE the administration of MSCs after the clinical disease's appearance is able to completely abrogate the relapsing phase and to strongly reduce spinal cord demyelination. These encouraging results have demonstrated that MSCs can provide a protective and reparative strategy for MS treatment.

Introduction

Multiple Sclerosis (MS) is a crippling chronic disease of the Central Nervous System (CNS) caused by an autoimmune reaction which progressively damages the axonal myelin sheath, both directly and through the creation of an inflammatory environment, thus leaving the axons exposed to possible damage [1]. This condition causes impairment of axonal transmission and leads to the development of neurological symptoms. Autoimmunity and inflammation is the sparks which kindle the fire and trigger the demyelination that progressively affects nervous transmission up to complete fiber degeneration. Moreover, immune cells also give rise to a pro-inflammatory milieu which further worsens nerve fiber structure and functionality and, for these reasons, both inflammation and demyelination are the hallmarks of MS [1].

As regards the clinical course, different types of MS have been identified, among which the most frequent is the Relapsing-Remitting form (RR-MS), and current therapies rely on the use of immune-modulating drugs which are, however, unable to effectively arrest the disease's progression [2]. For these reasons, the hunt for a valid therapeutic option is still ongoing, and attention is turning to the widely used animal model of MS, the Experimental Autoimmune Encephalomyelitis (EAE). Although it is undeniable that EAE and MS share several features, it must be kept in mind that EAE does not completely overlap with the human disease due to the complexity of the latter [3]. Moreover, EAE disease may be very heterogeneous, depending on the induction protocol and above all on the animal model used [4]. Typically, the immunization in different mouse strains as well as in

Lewis rats produces an acute monophasic disease that spontaneously resolves after several days, but which can be very useful for investigating the inflammatory phase of MS. This kind of monophasic model, however, does not really resemble MS, a chronic disease and, therefore, other animal models have been developed. The immunization in Dark Agouti (DA) rats or in SJL/J mice leads to the development of a RR disease, with the appearance of demyelinated lesions [4]. Due to these differences, it is mandatory to select an appropriate model in order to achieve reliable and convincing results.

Among the various therapeutic options suggested for MS treatment, encouraging results have been obtained in preclinical studies with the administration of Mesenchymal Stem Cells (MSCs), and a phase II clinical trial is currently under way (MESEMS, NCT01854957).

MSCs are adult stem cells characterized by many useful and distinguishing properties, in particular, the ability to modulate the

immune system by inducing T-cell anergy; for this reason it has been suggested that they may be able to counteract the autoimmune attack observed in MS [5]. Many studies have confirmed the efficacy of MSCs by using animal models of acute EAE that are characterized by the prevalence of the inflammatory component. However, recent findings have demonstrated that MSCs can also promote neuronal survival and restore axonal myelination [6]; they may, therefore, be useful also for relieving the chronic form of EAE.

Despite the large number of studies on acute EAE, only a few studies have evaluated the therapeutic effect of MSCs on RR models of the disease which is most similar to human MS [5,7-9], and even fewer studies have looked at the effect of administering them after the clinical manifestation of symptoms.

Focusing on the RR chronic form of EAE, the main aim of the present study was to investigate the effect of the administration of MSCs after the clinical manifestation of symptoms in order to shed light on their potential use after the first clinical appearance of the disease.

Materials and Methods

Relapsing-Remitting EAE (RR-EAE) Experimental model

Male Dark Agouti rats (175-200 g, Janvier SAS, Le Genest Saint Isle,France) were randomly divided into 4 groups of 10 animals (control rats, EAE rats, EAE rats +MSCs injected at T7 -preventive treatment-, and EAE rats +MSCs injected at T14 -therapeutic treatment). EAE was induced in each animal by the subcutaneous injection into both hind limb footpads of 100 mg of syngenic spinal cord homogenate dissolved in incomplete Freund's adjuvant. The peak of the first EAE episode was at day 14 after EAE induction.

MSCs were administered at doses of 10^6 cells/250 µl of saline solution into the tail vein 7 days post EAE induction (T7) to test the preventive protocol, or 14 days post EAE induction (T14) to test the therapeutic protocol.

EAE animals and healthy untreated animals were sacrificed on day 45 post EAE induction (pi) and histological analysis of spinal cords was performed at this time point to evaluate the degree of demyelinated lesions. The experimental plan was approved by the Ethics Committee of the University of Milano-Bicocca (n° 0014051/12) and animal care and treatment were conducted in conformity with the institutional guidelines, in compliance with national (DLn. 116/1992, Circ.n. 8/1994) and international laws and policies (EEC Council Directive 86/609, OJL 358, Dec 1987; NIH Guide for the Care and Use of Laboratory Animals, US NRC, 1996).

Clinical Score and body weight evaluation

Starting on day 7 pi and up to day 45 pi, the animals' body weight was registered and rats were blindly evaluated daily for neurological signs which were recorded on a scale from 0 to 5 using the following grading system: 0: healthy animals with no defective tail tonicity; 1:limp tail; 2: mild to moderate paraparesis of the hind limbs; 3:paraplegia of the hind limbs; 4: quadriplegia; 5: moribund or dead [10].

MSC cultures

MSCs were obtained from the bone marrow of male Sprague-Dawley rats by flushing the femur and tibia diaphisis with 2 ml/bone of alpha MEM with 2 mM glutamine and antibiotic. MSCs were cultured in alpha-MEM medium plus L-Glutamine, antibiotics and 20% of foetal bovine serum [11].

MSCs were administered at doses of 10^6 cells/250 µl of saline solution into the tail vein 7 days after EAE induction (T7), or 14 days post EAE induction (T14).

Histological examination

The spinal cord lumbar enlargement of each rat was fixed in 4% paraformaldehyde and embedded in paraffin. Three adjacent 5 µm thick sections were cut every 50 µm and placed on separate glass slides for a total of three series of 9 sections for each animal.

The first series of sections were stained with Luxol Fast Blue and hematoxylin-eosin to evidence demyelination and inflammation respectively; the second series of adjacent sections were processed for MBP detection through immunohistochemistry (IHC) to label myelin sheets, while the third series were immunostained for CD68 to label cells with macrophage activity infiltrating the spinal cord.

For the IHC, paraffin sections were deparaffinized with xylene, rehydrated and heated in a steamer (20 min in 1 mM EDTA pH 8.0) to retrieve antigens. Endogenous peroxidase activity was quenched by incubation in 3% H_2O_2 for 5 min at RT. The slides were washed in PBS and incubated with 5% bovine serum albumin (5% BSA in PBS) or in 5% NGS for 1 hour at RT. The sections were incubated with primary antibody overnight at 4°C (anti-MBP 1:50 in 1% BSA, Santa Cruz Biotechnology, Santa Cruz, CA; anti CD68 (ED1) 1:300 in 1% normal goat serum (NGS), Abcam, Cambridge, UK). Then the slides were washed and incubated with secondary anti-goat (1:250 in 1% BSA; Millipore, Billerica, MA) or anti-mouse (1:200 in 1% NGS, Millipore) antibody for 1 hour at RT. The antigen-antibody complex was visualized by incubating the sections with 3,3'-diaminobenzidine hydrochloride (Sigma, St. Louis, MO) dissolved in PBS with 10 µl of 3% H_2O_2. Negative controls were incubated only with the secondary antibody.

The percentage of demyelination was expressed as the ratio between the demyelinated plaque area and the total white matter area, both being measured on each section series for each animal by using Image J software (NIH, Bethesda, MD). The percentage of spinal cord sections with demyelinated areas out of the total sections analyzed was also evaluated.

The number of macrophagic cells infiltrating the tissue was expressed as the area of immunopositive cells measured on each section for each animal using ImageJ software (DAB plugin; NIH, Bethesda, MD). These measures were expressed as mean ± SD.

Statistical analysis

The statistical analysis was performed using the unpaired t test or Chi-square test with the GraphPad Prism (GraphPad Software, San Diego, CA) statistical package. A *P*-value of less than 0.05 was considered significant.

Results

Clinical score evaluation

The clinical score examination of DA rats induced with syngenic spinal cord homogenate was prolonged up to 45 days post induction. EAE-DA rats showed the clinical manifestation of disease symptoms starting from 12 days post induction, and a first peak of disease on day 14 (Figure 1A). The clinical course of RR-EAE overlapped with the model already described in the literature [12-14] in which all the animals developed the disease, with 80% of the animals showing a severe form of EAE (score 3) and 20% a mild EAE form (score 1-2). A
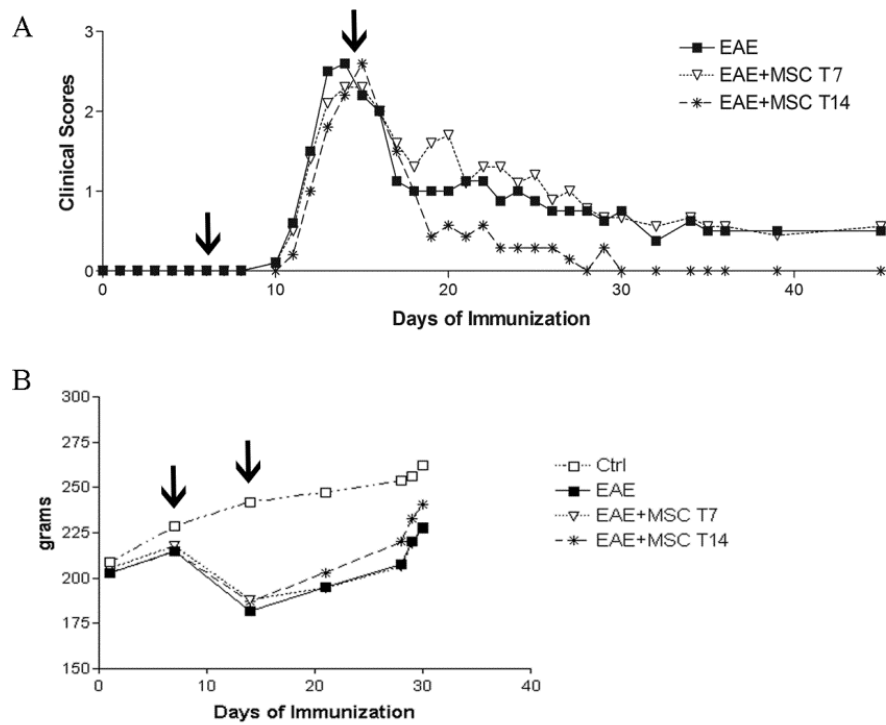
Figure 1: MSC effect on Clinical Score of RR-EAE rats. (A) Clinical score evaluation of chronic EAE rats. After immunization, animals were monitored for clinical symptoms of EAE as described in Materials and Methods. Arrow indicates the day of MSC administration. *P<0.05, EAE+MSC T14 vs. EAE. (B) Body weight daily evaluation in chronic EAE rats. Arrow indicates the day of MSC administration.

second relapse of disease occurred around day 20 post induction in 50% of the animals, followed by a third relapse at day 27-28 post induction, occurring in 30% of the animals. Only 10% of the animals developed a fourth relapse, the last one being at day 34 post induction (Figure 1A).

The group treated with MSC administration at day 7 post induction (before disease onset) was considered to be undergoing the "preventive treatment". In this case, the first disease peak developed by the animals was milder, with 30% of the animals scoring 3 (or more), and the majority (60%) having only a mild form of EAE (score 1-2). A small percentage of the animals (10%) did not develop the disease. However, subsequently the animals showed a clinical course very similar to untreated EAE rats, thus suggesting that the preventive treatment with MSCs was not able to modify the RR clinical course of EAE animals. In fact, the treated EAE rats underwent a second relapse (60%), followed by a third episode for 40% of the animals and a fourth for 20% of the rats. The relapses occurred at approximately the same time points observed for untreated EAE rats (Figure 1A).

The group treated with MSC administration at day 14 post induction (after the first disease peak) was identified as the "therapeutic treatment" group. The clinical score evaluation of EAE rats treated with MSCs at day 14 post induction demonstrated that the MSC injection determined a different clinical trend since, after the first disease peak, the treated EAE rats showed a complete recovery and overall no further relapses were observed, a statistically significant difference with respect to untreated EAE rats and with respect to the "preventive" MSC treatment group (Figure 1A). Similarly, the body weight trend suggested that EAE animals injected with MSCs at day 14 pi were healthier than EAE rats and EAE rats treated with the preventive schedule (Figure 1B).

### Inflammatory infiltrate analysis

The histopathological analysis of the inflammatory component infiltrating the lumbar tract of spinal cord parenchyma, which is known to be the most severely involved in EAE-induced alterations, was performed by using anti-ED1 immunocytochemistry, a marker of active microglia and macrophagic cells. As shown in Figure 2A, there was an important macrophagic infiltration in the spinal cord of the EAE rats which is, on the contrary, absent in the spinal cord of the untreated control rats. As shown in Figure 2A, in both the EAE groups treated with MSCs (T7 and T14) there was a marked reduction in active microglia and macrophagic cells infiltrating the parenchyma, with a limited presence of brown spots often restricted to the peripheral edge. However, a further quantitative analysis of the inflammatory infiltrate evidenced a better outcome of the therapeutic treatment (T14) which induced a statistically significant reduction of about 75% (P < 0.05) of the inflammatory infiltration observed in EAE rats, whilst the reduction observed with the preventive treatment (T7) was of about 40% (Figure 2B).

Demyelination analysis

Luxol Fast Blue and eosin staining evidenced the presence of unstained areas in the spinal cord white matter of EAE rats corresponding to demyelinated plaques, as well as the presence of an important parenchymal vacuolization (Figure 3A). On the contrary, the white matter of control untreated rats had uniform Luxol Fast Blue staining and an intact parenchyma. The spinal cords of EAE rats treated with the preventive treatment (MSC injection at day 7 pi) were very similar to those of untreated EAE rats and, therefore, this schedule was not able to reduce either the extension of demyelinated plaques or the
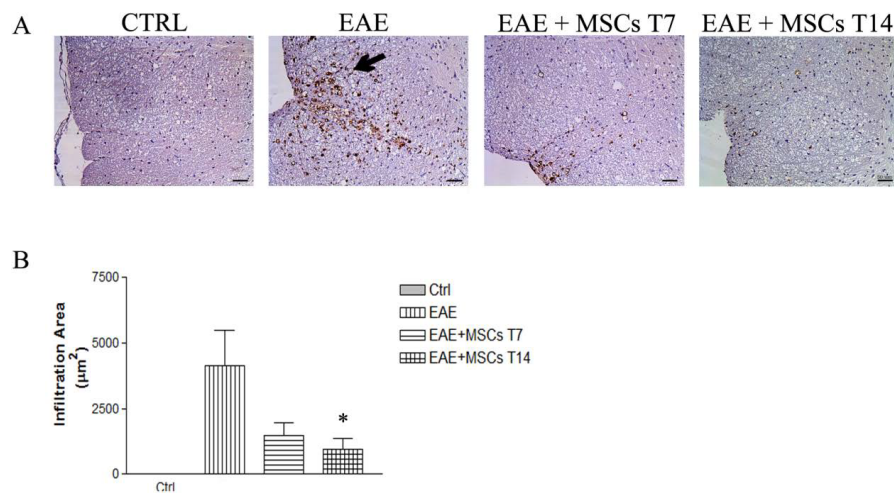
Figure 2: Histopathological examination of microglia infiltration in RR-EAE rats.A) Representative sections were obtained from lumbar spinal cords of untreated CTRL rats, EAE rats, and EAE rats treated with MSCs injected after 7 (T7) or 14 days after the immunization (T14). Sections were immunostained with anti- ED1, a marker of active microglia, and hematoxilin. Scale Bar 50 μm. Arrow indicated the microglial infiltration. B) The sections were then analyzed to quantify the inflammatory infiltrate ImageJ software. These measures were expressed as mean± SD. *$P< 0.05$, EAE+MSC T14 vs. EAE.
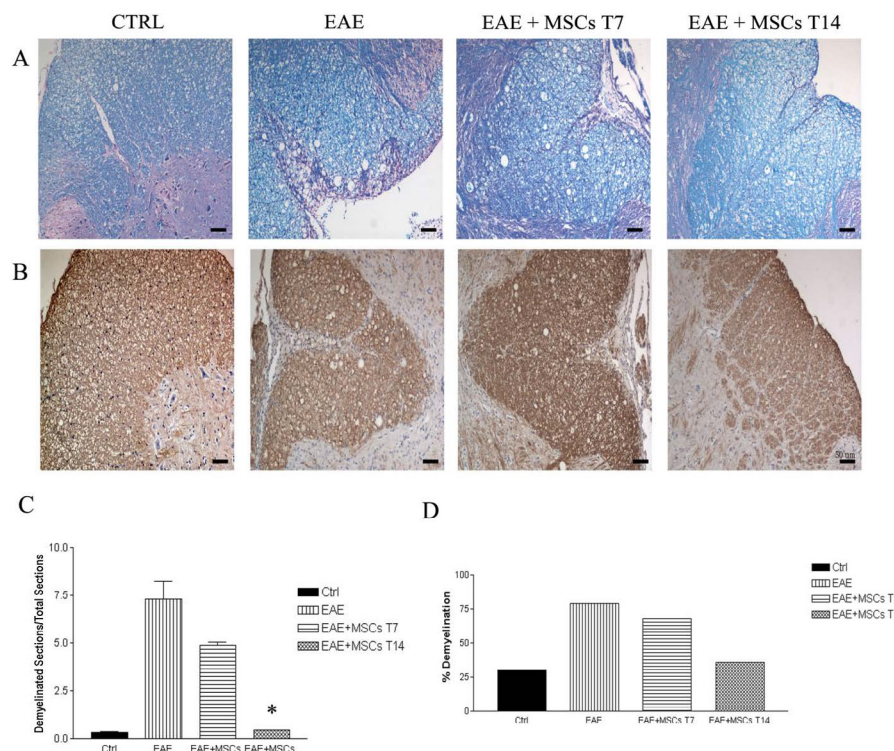
Figure 3:Histopathological examination of demyelination in RR-EAE rats.Representative sections were obtained from lumbar spinal cords of untreated CTRL rats, EAE rats, and EAE rats treated with MSCs injected after 7 (T7) or 14 days after the immunization (T14). Sections were stained with Luxol Fast Blue and eosin (A), or immunostained with anti- MBP and hematoxilin (B). Scale Bar 50μm. Arrow indicated demyelinated area, which resulted unstained by Luxol Fast Blue.C) The number of sections presenting demyelination on the full amount of sections was then analyzed, as well as the percentage of demyelinated lesions for each samples (D). * $P< 0.05$, EAE+MSC T14 vs. EAE.

vacuolization observed in the EAE group (Figure 3A). On the contrary, the spinal cords of EAE rats treated with the therapeutic schedule with MSCs (MSC injection at day 14 pi) no longer showed any vacuoles and had uniform Luxol Fast Blue staining, thus demonstrating that this treatment was able to significantly reduce the size of the demyelinated

areas (Figure 3A) and supporting the clinical score evaluations. Comparable results were obtained also at the IHC analysis by using anti-MBP antibodies (Figure 3B). The percentage of spinal cord sections with demyelinated areas out of the total sections analyzed was also evaluated. No significant differences were observed between EAE

and EAE+ MSCs T7 groups while the treatment with MSCs at day 14 pi determined a statistically significant marked reduction not only in the demyelinated area, but also in the percentage of demyelinated sections with respect to the other EAE groups, leading to values comparable to those observed in unaffected control rats (Figure 3C and 3D).

Discussion

In this study the positive role of MSCs for the treatment of RR-EAE has been demonstrated by using the rat as an experimental model, and by the use of treatment schedules which can be considered as "therapeutic", being initiated after the clinical manifestation of the disease, rather than "preventive", in view of its more realistic clinic application. These results have been achieved by using allogenic MSCs. Several authors have suggested the possible therapeutic role of MSCs for the treatment of MS because of their particular features [15-17]: first of all their immunomodulatory action [5] that is able to impede the damage produced by both inflammation and autoimmunity and, therefore, also to limit the appearance of neurological symptoms. The experimental *in vivo* models, however, only partly reflect the complex features of human MS where there is a close connection between inflammation, demyelination and axonal damage. For this reason, several animal models of EAE exist, and the induced disease has features which depend on the species, the strain, and the protocols used for the immunization [4]. The decision to exploit the MSCs' potential to treat EAE using the rat as an experimental model was based on reports in the literature which indicated that this model is more advantageous than the mouse model for studying RR EAE for several reasons [4]. More precisely, DA rats have the advantage of developing a RR disease very similar to MS with a simple immunization protocol [3]. Furthermore, almost all the immunized animals develop the disease with severe symptoms, even without the need for co-adiuvant reagents, i.e. pertuxis toxin and Micobacterium, that are pivotal for the development of the disease in mouse but which represent a further variable for the data interpretation [4,18].

In spite of a large number of studies on acute EAE, only a few studies have investigated the role MSCs play in the chronic form of EAE characterized by particular phases, a feature of the most common variant of MS. Our results evidenced the effectiveness of this treatment also for the chronic RR form of EAE. Promising evidence of the positive effect of MSCs for RR EAE have already been reported using mice models [7,8]. These papers reported a clinical score amelioration using SJC/J mice, but with the persistence of relapsing phases after MSC administration which was performed before the disease peak that is usually observed 14 days after the EAE induction. Our model, based on DA rats immunized to develop the chronic form of the disease and MSC administration after the first EAE peak, was able to completely abrogate the relapsing disease phase and to significantly reduce the spinal cord demyelination.

The main difference with respect to the previous reports, besides the experimental model, is represented by the schedule protocol. In our model, MSCs were administered after the disease reached its peak in order to verify the effectiveness of a "therapeutic" protocol rather than a "preventive" one, a condition closer to the clinical course of MS.

Interestingly, the greatest effect was achieved when MSCs were administered after the peak of EAE, while limited benefits were present in the event of an early administration (before the onset of neurological symptoms). This result is seemingly a contradiction, but it can be ascribed to the effect of the cytokine milieu which changes during the different temporal phases of EAE [13,19,20]. In particular,

it has been reported by several authors that some cytokines are able to drive Natural Killer (NK) cells to eliminate MSCs [19,21] or, at least, to create a microenvironment that may hamper MSC functionality. The expression of some of these cytokines is higher in the early phases of EAE [13,20] and, therefore, it is plausible that MSCs administered after the EAE peak may have a greater effect.

The reduction in demyelinated lesions reported in this paper is a controversial result: Semon et al. [22] confirmed the amelioration of the clinical score after MSC administration in a chronic mouse model of EAE but, differently from what we observed, they did not observe any effect on demyelination, and ascribed the protective effect of MSCs only to their immunomodulatory action. Other authors, however, have observed a demyelination reduction in EAE mice models treated with MSCs [23], but a recent paper by Nessler et al. [24] underlined the importance of inflammation for the achievement of this protective effect which was absent in non-inflammatory demyelination models. However, it is not clear if this protective effect is limited only to the reduction in inflammation, or rather that the damage to the Brain Blood Barrier (due to the inflammatory reaction) allows MSCs to reach the nervous system, thus achieving their protective effect.

Regarding the possible mechanisms involved in the reported positive effect, MSCs' immunomodulatory properties must certainly play an important role by stopping the immune attack against myelin; however, direct involvement of the MSCs in the repair of the myelin sheath cannot be ruled out. In fact, a recent paper has demonstrated that undifferentiated MSCs promote *in vitro* the survival and axonal myelination in sensory dorsal root ganglia neurons, thus strengthening the latter hypothesis [25]. It is more likely that there are several different mechanisms which cooperate in MSCs' neuroprotection ranging from immunomodulation and neuronal support to neurotrophic factor release and, for all these mechanisms, MSCs may represent a valid means of counteracting all EAE phases, the earlier as well as the later ones.

In addition, our results demonstrated that MSCs effectively abrogate EAE relapses also when administered after the first clinical manifestation of EAE symptoms, thus strengthening the possible clinical use of these cells for a protective and reparative strategy for the nervous system.

Acknowledgements

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