Molecular Medicine

Intravenous Mesenchymal Stem Cells Improve Survival and Motor Function in Experimental Amyotrophic Lateral Sclerosis

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Despite some advances in the understanding of amyotrophic lateral sclerosis (ALS) pathogenesis, significant achievements in treating this disease are still lacking. Mesenchymal stromal (stem) cells (MSCs) have been shown to be effective in several models of neurological disease. To determine the effects of the intravenous injection of MSCs in an ALS mouse model during the symptomatic stage of disease, MSCs (1×10^6) were intravenously injected in mice expressing human superoxide dismutase 1 (SOD1) carrying the G93A mutation (SOD1/G93A) presenting with experimental ALS. Survival, motor abilities, histology, oxidative stress markers and (^{3}H)D-aspartate release in the spinal cord were investigated. MSC injection in SOD1/G93A mice improved survival and motor functions compared with saline-injected controls. Injected MSCs scantly home to the central nervous system and poorly engraft. We observed a reduced accumulation of ubiquitin agglomerates and of activated astrocytes and microglia in the spinal cord of MSC-treated SOD1/G93A mice, with no changes in the number of choline acetyltransferase– and glutamate transporter type 1–positive cells. MSC administration turned around the upregulation of metallothionein mRNA expression and of the activity of the antioxidant enzyme glutathione *S*-transferase, both associated with disease progression. Last, we observed that MSCs reverted both spontaneous and stimulus-evoked neuronal release of (^{3}H)D-aspartate, a marker of endogenous glutamate, which is upregulated in SOD1/G93A mice. These findings suggest that intravenous administration of MSCs significantly improves the clinical outcome and pathological scores of mutant SOD1/G93A mice, thus providing the rationale for their exploitation for the treatment of ALS.

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INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a late-onset progressive neurodegenerative disease affecting motor neurons (MNs) and leading irreversibly to death (1). To date, approved treatments only modestly prolong survival in ALS patients (2). Development of more effective therapies is hampered by the poor understanding of the mechanisms of neuronal death. Most ALS cases are sporadic, while approximately 10% are inherited, sometimes because of mutations in the Cu/Zn superoxide dismutase (*SOD1*) gene (3) that induce MN killing by an unknown gain of toxicity. Selective vulnerability of MNs likely arises from a combination of mechanisms such as protein misfolding, mitochondrial dysfunction, oxidative damage, defective axonal transport, glutamate-mediated excitotoxicity, insufficient growth factor signaling and inflammation (4). Injury of MNs is enhanced by damage incurred by non-neuronal neighboring cells accelerating disease progression (5). Because sporadic and familial ALS affect the same neurons, studying SOD1 transgenic mice may provide a better understanding of pathogenic mechanisms and testing of therapies for ALS (6).

Mesenchymal stromal (stem) cells (MSCs) are a heterogeneous cell population recently demonstrated to display a remarkable therapeutic plasticity resulting in their ability of dampening inflammation, inhibiting pathogenic immune responses and releasing neuroprotective factors (7). However, MSCs have been mistakenly considered capable of repairing almost every tissue because of their

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intrinsic capacity of differentiating into other cell lineages (8). On the basis of these features, MSCs have been injected intravenously in mice with experimental autoimmune encephalomyelitis (EAE), a model of multiple sclerosis resulting in clinical amelioration, halting of autoimmunity, decrease of demyelination and preservation of axons (9,10). Importantly, intravenous injection of bone marrow–derived MSCs in patients with acute graft versus host disease led to a dramatic clinical improvement (11).

Early reports suggested that MSCs, administered during the presymptomatic phase of disease, improve motor performances of transgenic SOD1 mice when intravenously injected (12) or transplanted into the spinal cord (13) or intrathecally (14). In this study, we demonstrate that intravenous injection of MSCs after disease onset in mutant SOD1 mice results in a significant delay of symptoms and death and associates with an improvement of several histological and biochemical parameters.

MATERIALS AND METHODS

Animals

B6SJL-TgN SOD1/G93A1Gur mice expressing high copy number of mutant human SOD1 with a $Gly^{93}Ala$ substitution (SOD1/G93A) and B6SJL-TgN (SOD1)2Gur mice expressing wild-type human SOD1 (SOD1) (15) were originally obtained from Jackson Laboratories (Bar Harbor, ME, USA) and bred at the animal facility of the Department of Experimental Medicine in Genoa, where they were kept until experiments were carried out. Mice were intravenously injected into the tail with 10^6 MSCs or saline at d 90. Three groups of animals were used: saline-treated transgenic nonmutated SOD1, saline-treated and MSC-treated transgenic mutated SOD1/G93A mice. A total of 92 animals were used. Sexes were balanced in each experimental group to avoid bias due to sex-related intrinsic differences in disease severity. Animals were housed at a constant temperature (22 ± 1°C) and relative humidity (50%) under a regular dark–light schedule (lights on from 7:00 AM to 7:00 PM). Food (type 4RF21 standard diet obtained from Mucedola Settimo Milanese, Milan, Italy) and water were freely available. All experiments were carried out in accordance with the European Community Council Directive of 24 November 1986 (86/609/EEC). All efforts were made to minimize animal suffering and to use only the number of animals necessary to produce reliable results.

Isolation, Expansion, Transfection and Injection of MSCs

Bone marrow–derived MSCs were prepared as previously described (9). Briefly, MSCs were isolated from 6- to 8-wk-old female C57Bl/6J mice (Harlan Laboratories, Udine, Italy), maintained in culture in Mesencult basal medium supplemented with Mesenchymal Stem Cell Stimulatory Supplement (StemCell Technologies, Vancouver, BC, Canada) at 37°C in humidified 5% CO_2 incubator and expanded *in vitro* by plastic adherence at the appropriate plating concentration. Mature MSCs were defined by their ability to differentiate into bone, cartilage and fat and by the expression of $CD9^+$, $Sca\text{-}1^+$, $CD44^+$ and $CD73^+$ and the lack of hematopoietic markers $CD45^-$, $CD34^-$ and $CD11b^-$ as previously reported (9). To track cells *in vivo*, MSCs were transfected with a Luciferase gene reporter vector pL-Luc-IH, as reported elsewhere (16). Cells between culture passages 8–15 were suspended in 200 μL of phosphate-buffered saline (PBS) (Sigma-Aldrich, St. Louis, MO, USA) at a concentration of 1×10^6 and intravenously injected in SOD1/G93A mutant mice after the onset of motor symptoms (d 90). PBS-injected SOD1/G93A mice were used as controls.

Survival and Motor Performance

Survival time was identified as the time at which MSC- or saline-injected SOD1/G93A mice were unable to roll over within 20 s after being pushed on their side. Survival data originated from those animals scored in clinical tests only. Animal used in clinical tests were not used for other experiments, for which *ad hoc* MSC- or saline-injected mice were used. The effects of MSCs on the progression of the disease symptoms were investigated by Rotarod task, motor deficit scores and weighing, all of which are commonly used to evaluate SOD1/G93A animals (17). Beginning at d 60, MSC- or saline-injected animals were assessed every 5 d, until d 95, and then three times a week, until death. The clinical tests were performed in randomized order by an observer blinded to the treatment.

For the Rotarod test, the time for which an animal could remain on the rotating cylinder was measured using an accelerating Rotarod apparatus (Rota-Rod 7650; Ugo Basile, Varese, Italy). In this procedure, the rod rotation gradually increases in speed from 4 to 40 rpm over the course of 5 min. The time that the mice stayed on the rod until falling off or passively stayed on the rod was recorded. The first 2 wks of tests were considered training.

For motor deficits, MSC- or saline-injected mice were rated for disease progression by scoring the extension reflex of hind limbs and gait posture. Mice were evaluated for signs of motor deficit using a five-point score scale (5, no sign of motor dysfunction; 0, complete impairment). See Supplementary Table S1 for the complete score evaluation scale. Body weight was measured immediately before behavioral tests.

Histology

Animals were sacrificed at d 125 ± 2, and samples were collected for histological analysis, except for the analysis of MSC engraftment, where animals were also euthanized 24 and 48 h or 35 d after-MSC transplantation. SOD1/G93A mice treated with MSCs or PBS (control group) were transcardially perfused with 4% paraformaldehyde, and brain and spinal cord were removed, post-fixed with the fixative for 4 h, washed in PBS and embedded in paraffin (Bio Optica, Milan, Italy). All sections were cut at

5 μm on a microtome and stained for histological and immunofluorescence examination. Microglia and astrocytes were stained using, respectively, an isolectin IB4 conjugate (marker for microglia; Invitrogen, Milan, Italy) and a polyclonal rabbit anti-cow glial fibrillary acid protein (GFAP) (DAKO, Milan, Italy), both diluted 1:100, as primary antibody. Glutamate transporter 1 (GLT1), MNs and ubiquitin debris were stained using the following: guinea pig anti-GLT1 polyclonal antibody (Chemicon, Millipore, Vimodrone, Milan, Italy) diluted 1:100, a rabbit anti-choline acetyltransferase (ChAT) polyclonal antibody (as marker for MNs) (Chemicon, Millipore) diluted 1:500 and mouse anti-ubiquitin monoclonal antibody (Chemicon, Millipore) diluted 1:250. We also used a rabbit polyclonal anti-inducible nitric oxide synthase (iNOS) (Abcam, Cambridge, UK) diluted 1:100, a rabbit polyclonal anti-fractalkine receptor CX3CR1 (Abcam) diluted 1:100, a rabbit anti-insulin growth factor (IGF1) (Abcam) diluted 1:200 and an anti-human/mouse nuclear receptor–related 1 protein (Nurr1) (R&D, Milan, Italy) diluted 1:200. A rabbit anti-luciferase (Sigma-Aldrich) diluted 1:100 was used to identify the presence of MSCs inside the central nervous system (CNS). To quantify MSCs, a systematic random series of every tenth coronal section (20 μm) throughout the entire spinal cord and brain was obtained (a mean of 25 sections per animal). The numerical density of cells was then estimated as positive elements/mm^2 acquired at 20× magnification using a Zeiss Axio Imager M1 light microscope equipped with an AxioCam MRc5 digital camera and a 40×/0.75 Ec Plan objective lens set with the acquisition software Axio Vision 4.7 (Zeiss, Milan, Italy).

Quantification of Gene Expression

Mice were sacrificed at d 125 ± 2, and spinal cords were rapidly dissected, quickly frozen in liquid nitrogen and stored at –80°C until use. Total RNA was isolated by the acid phenol-chloroform procedure using the Trizol reagent (Sigma-Aldrich) according to the manufacturer instructions, converted to cDNA and amplified with the LightCycler 480 Sybr Green I Master (Roche, Milan, Italy) and 1 μmol/L of each primer pair (TIB MolBiol, Genoa, Italy) using a Light Cycler 480 (Roche). Hypoxanthine-phosphoribosyl-transferase (*HPRT*) was used as the housekeeping gene to normalize the expression data. The relative expression of target genes in comparison to the housekeeping reference gene was performed following the ΔC_t method by using the LightCycler 480 Software release 1.5.0 SP3 (Roche). Gene expression is represented as relative mRNA amount (fold induction) compared with the control sample. Analysis of the expression levels of metallothionein (MT) genes was carried out as described elsewhere using a Chromo 4™ System real-time polymerase chain reaction (PCR) apparatus (Bio-Rad, Milan, Italy) (17). Real-time (RT)-PCRs were performed in a final volume of 20 μL containing 10 ng cDNA, 10 μL iTaq SYBR Green Supermix with ROX (Bio-Rad) and 0.25 μmol/L of each primer pair (TIB MolBiol). Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was used as the housekeeping gene. The expression of MT-1, MT-2 and MT-3 in spinal cord was assessed in SOD1/G93A mice during disease progression and after MSC treatment. The list of all primer pairs is available in the supplementary material (Supplementary Table S2).

Determination of Glutathione *S*-Transferase Activity

Animals were sacrificed at d 125 ± 2, and glutathione *S*-transferase (GST) activity in spinal cord was measured as previously reported (18). Aliquots of pooled spinal cord samples from each experimental group were homogenized in 10 volumes (w/v) of ice-cold PBS (50 mmol/L, pH 7.8) and centrifuged at 700*g* for 5 min at 4°C. The supernatant was further centrifuged at 10,000*g* for 20 min at 4°C, and the resulting supernatant was used for determination of enzyme-specific activity. GST activity was evaluated using 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate. Aliquots diluted in 50 mmol/L PBS (pH 7.8) were incubated with 100 mmol/L GSH and 100 mmol/L CDNB. The formation of *S*-(2,4-dinitrophenyl)-glutathione conjugate was evaluated by monitoring the increase in absorbance at 340 nm at 25°C. GST-specific activity was expressed as micromoles per minute per milligram of sample protein. Spectrophotometric analyses were carried out at 25°C using a Varian Cary 50 spectrophotometer (Varian, Turin, Italy).

Preparation of Synaptosomes and Release Experiments

Animals were sacrificed at d 125 ± 2, and spinal cord synaptosomes were prepared and purified on Percoll® gradients as previously described (19). The tissue was homogenized in 14 volumes of 0.32 mol/L sucrose and buffered at pH 7.4 with Tris-HCl. The homogenate was centrifuged (5 min, 1,000*g* at 4°C) to remove nuclei and debris, and the supernatant was gently stratified on a discontinuous Percoll gradient (2, 6, 10 and 20% v/v in Tris-buffered sucrose). After centrifugation at 33,500*g* for 5 min, the layer between 10% and 20% Percoll (synaptosomal fraction) was collected. Synaptosomes were resuspended in physiological medium and labeled with 0.02 μmol/L [^{3}H]D-aspartate. Aliquots were distributed on microporous filters placed at the bottom of a set of parallel superfusion chambers maintained at 37°C. Superfusion was then started with physiological medium at a rate of 0.5 mL/min and continued for 48 min. After 36 min of superfusion to equilibrate the system, samples were collected as follows: two 3-min samples (t = 36–39 and 45–48 min; basal efflux) before and after one 6-min sample (t = 39–45 min; stimulus-evoked release). Stimulation with a 90-s pulse of 15–25 mmol/L KCl was applied at t = 39 min. Collected samples and superfused synaptosomes were counted for radioactivity. Tritium released in each sample was calculated as fractional rate × 100. The stimulus-evoked neurotransmitter overflow was estimated by subtracting the transmitter content of the two 3-min

samples representing the basal efflux from the content of the 6-min sample collected during and after the stimulating pulse.

Statistics

Data are expressed as mean ± standard error of the mean (SEM), and p values <0.05 were considered significant. The Kaplan-Meier plot was used to evaluate survival time, and the log-rank test was applied to compare the cumulative curves. The Mann-Whitney test was used for the analysis of two populations of means. Comparison of more than two mean populations was performed using the analysis of variance (ANOVA) followed by Bonferroni test (INSTAT software; GraphPad Software, San Diego, CA, USA).

All supplementary materials are available online at www.molmed.org.

RESULTS

MSCs Improve Survival and Motor Functions in SOD1/G93A Mice

Intravenous administration of 10^6 MSCs after the disease onset significantly prolonged the lifespan of SOD1/G93A mice compared with saline-treated mice (survival average: 116.1 ± 3.4 d for saline-treated mice versus 133.4 ± 3.8 d for MSC-injected mice; $p < 0.005$). The Kaplan-Meier survival curve is reported in Figure 1A. MSC injection significantly slowed disease progression, as depicted by a delayed decline of motor performances in all tests (Figures 1B–D). In detail, saline-injected SOD1 mice (healthy nonmutated control mice) maximally performed in the Rotarod task along the observation period (not shown). As expected, the performance of saline-treated SOD1/G93A mice declined starting around d 80 and rapidly worsened (Figure 1B). A similar decline was observed in the extension reflex of hind limbs (Figure 1C) and in the gait (Figure 1D) tests. MSC-treated SOD1/G93A mice performed significantly better than saline-treated SOD1/G93A mice in all tests

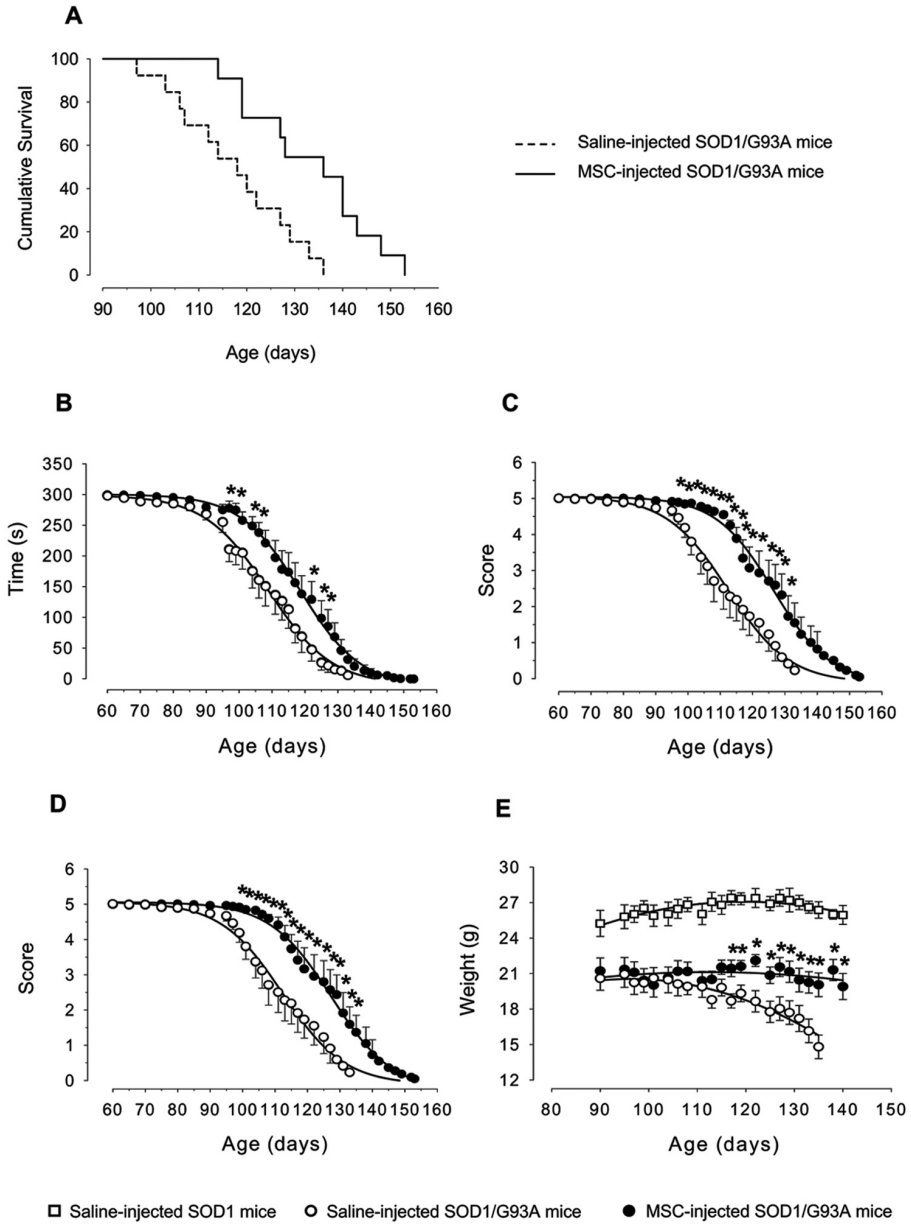

Figure 1. Effect of MSC administration on survival and disease progression of SOD1/G93A mice. Animals were intravenously injected with 10^6 MSCs or saline at d 90 and monitored for motor abilities and body weight. Survival time was determined when animals were unable to roll over within 20 s after being pushed on their side. Eleven MSC-treated and 13 saline-treated SOD1/G93A mice were used. (A) Survival. Survival time was examined by Kaplan-Meyer analysis. The difference between Kaplan-Meyer curves was significant at $p < 0.01$ (log-rank test). (B) Rotarod. Animals were tested starting on d 60 every 5 d until d 9 and were then examined 3 d/wk. Falling off time was recorded. (C) Extension reflex. Measures were obtained as reported in (A). Animals were rated according to a 5–0 score scale (see Supplementary Table S1). (D) Gait impairment. Measures were obtained as reported in (A). Each animal was rated according to a 5-0 score scale (see Supplementary Table S1). (E) Body weight. Body weight was measured immediately before behavioral testing. Values were compared with those from saline-injected SOD1 mice (n = 12). Means ± SEM are reported. *$p < 0.05$ at least (Mann-Whitney).

(Figures 1B–D). MSCs halted the decline in body weights of SOD1/G93A mice, which were already significantly lower than weights in SOD1 nonmutated mice (Figure 1E). Symptoms onset and survival values of saline-injected mice did not significantly differ from those routinely observed for the SOD1/G93A colony bred in our laboratory (not shown). These results demonstrate that MSC intravenous administration has a significant impact on survival and motor performances in mice with experimental ALS.

MSCs Migrate in the CNS After Intravenous Administration

Luciferase-positive cells were detected 24 and 48 h after injection throughout the entire spinal cord of animals injected with luciferase-gene reporter vector-transfected MSCs (83.3 ± 36.5 cells/mm^2 at 24 h after injection; 57.8 ± 52.2 cells/mm^2 at 48 h after injection. The number of labeled MSCs significantly decreased at d 20 after injection (7.03 ± 1.31 cells/mm^2) and almost vanished at d 35 after injection (1.66 ± 1.65 cells/mm^2) (Figures 2A–E). No significant difference among different cord sections was observed at all time points. A limited number of MSCs was identified in hypothalamus, especially in the subpial layer and around the ventral commissure at 24 h (9.34 ± 3.14 cells/mm^2), 48 h (4.70 ± 1.57 cells/mm^2), 20 d (2.61 ± 1.03 cells/mm^2) and 35 d (2.08 ± 1.62 cells/mm^2) after injection (Figures 2F–J). These findings prove that only a small number of MSCs can migrate into the CNS of SOD1/G93A mice after intravenous administration and suggest that their clinical effect is not likely to rely on long-term engraftment.

MSC Administration Improves Histological Pathology Traits

The number of cells immunoreactive for ubiquitin, a marker of abnormal protein degradation in neurons (20), was significantly reduced in the spinal cord of MSC-treated compared with saline-treated SOD1/G93A mice at the end stage of disease (d 125 of age) (Figure 3A

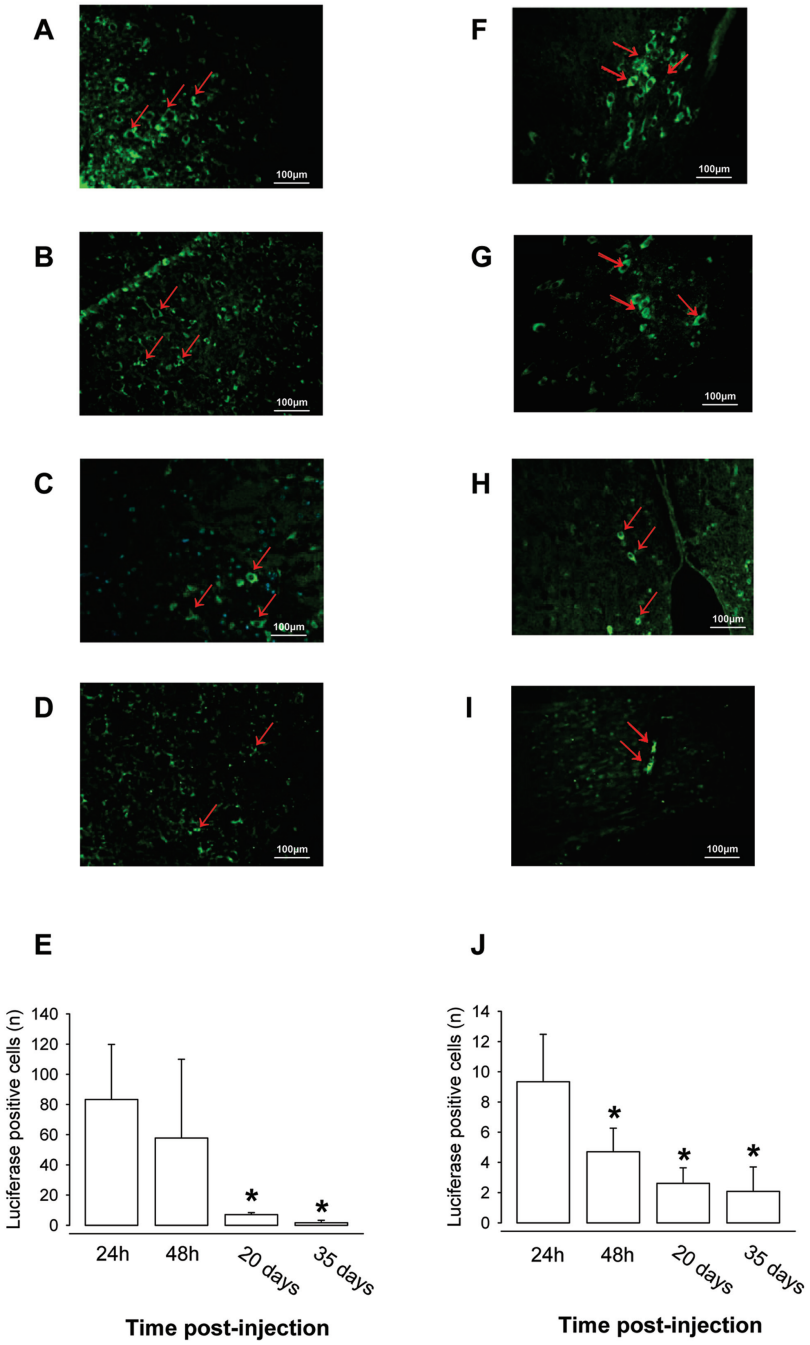

Figure 2. Tracking of MSCs *in vivo* after transfection with a luciferase gene reporter vector pL-Luc-IH. Luciferase-positive cells were detected at 20× magnification in the spinal cord (A–E) and in the hypothalamus (F–J). Mice were injected with LUC-positive MSCs and sacrificed 24 and 48 h or 20 and 35 d after injection. Histology was performed on 5-µm sections with a mouse anti-luciferase antibody. DAPI (4′,6-diamidino-2-phenylindole) was used for cell nucleus detection. Upper panels show representative images of tissues collected 24 h (A, E), 48 h (B, F), 20 d (C, G) or 35 d (D, H) after injection. Quantification (E, J) was performed on a systematic random series of six sections per animal, every tenth coronal section (20 µm), throughout the entire spinal cord and brain; data (means ± SEM) represent the number of positive cells/mm^2. Eight MSC-treated mice were used. *$p < 0.05$ at least (ANOVA plus Bonferroni). Scale bar, 100 µm.

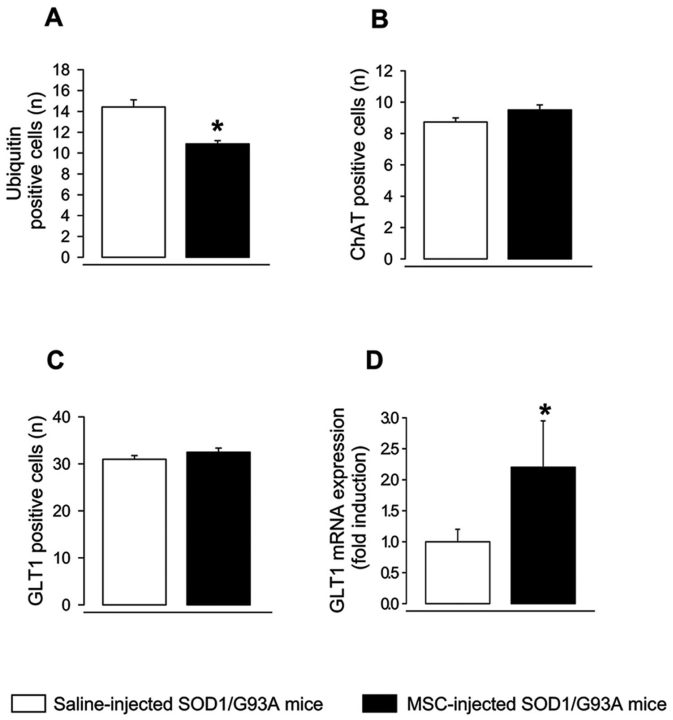

Figure 3. Effect of MSC administration on the number of ubiquitin-immunoreactive (A), ChAT-immunoreactive (B) and GLT1-immunoreactive (C) cells and on mRNA expression of GLT1 (D) in the spinal cord of SOD1/G93A mice. SOD1/G93A mice were treated with 10^6 MSCs or saline at d 90 and sacrificed at d 125. The following antibodies were used for histology: mouse anti-ubiquitin, 1:250; rabbit anti-ChAT, 1:500; guinea pig anti-GLT1, 1:100. Seven MSC-treated and seven saline-injected SOD1/G93A mice were used. Quantification was performed on six sections per animal, from three segments apart. mRNA was detected by RT-PCR using GAPDH mRNA as a reference. Data (means ± SEM) represent the number of positive cells/mm^2 (A–C) or fold induction with respect to controls (D). *$p < 0.01$ (Mann-Whitney).

and Supplementary Figure S1A; 14.4 ± 0.68 cells/mm^2 for saline-treated versus 10.9 ± 0.31 cells/mm^2 for MSC-injected animals; $p < 0.001$). Conversely, we could not observe significant modification of the number of cells positive for the MN-specific enzyme ChAT in MSC-treated mice compared with saline-treated SOD1/G93A mice (Figure 3B and Supplementary Figure S1B; 8.73 ± 0.25 cells/mm^2 for controls versus 9.50 ± 0.30 cells/mm^2 for treated animals) and for GLT1, the expression of which is selectively reduced in ALS (21) (Figure 3C and Supplementary Figure S1C; 31.3 ± 0.75 cells/mm^2 for controls versus 32.5 ± 0.87 for MSC-treated animals). However, GLT1 mRNA expression was almost doubled in SOD1/G93A mice in the end stage of disease after MSC treatment (Figure 3D). Next, we evaluated the effect of MSCs on reactive astrocytes and microglia. The number of GFAP-positive cells was significantly reduced in spinal cord (Figure 4A and Supplementary Figure S1D), brain cortex (Figure 4B) and hippocampus (Figure 4C) of MSC-treated SOD1/G93A mice compared with saline-treated controls (spinal cord: 18.97 ± 0.49 cells/mm^2 versus 15.93 ± 0.51 cells/mm^2; $p < 0.001$; cortex: 8.30 ± 0.67 cells/mm^2 versus 5.09 ± 0.60 cells/mm^2; $p < 0.01$; hippocampus: 24.48 ± 1.32 cells/mm^2 versus 7.54 ± 1.23 cells/mm^2; $p < 0.001$). Similarly, the number of microglia cells, identified as IB4-positive cells, was significantly decreased in the spinal cord after administration of MSCs (17.5 ± 1.55 cells/mm^2 versus 11.8 ± 1.52 cells/mm^2; $p < 0.01$) (Figure 4D and Supplementary Figure S1E), whereas no significant difference was observed in cortex (6.98 ± 4.00 cells/mm^2 versus 8.24 ± 3.74 cells/mm^2) and hippocampus (6.70 ± 1.93 cells/mm^2 versus 5.40 ± 2.47 cells/mm^2) (Figures 4E, F).

To evaluate whether this effect associates with a change in the expression of molecules involved in some functional activities of microglia (22), immunostaining of spinal cord sections for iNOS, IGF1, CX3CR1 and Nurr1 was performed. No significant changes were observed between saline- or MSC-treated SOD1/G93A mice for any of the markers under analysis (Supplementary Figures S2A–D). Similarly, no significant differences of related mRNA expression levels could be detected in spinal cord of both groups of mice (Supplementary Figures S3A–D). In contrast, we observed a significant downregulation of the RNA expression for interleukin (IL)-1β and tumor necrosis factor (TNF)-α in the mice treated with MSCs compared with controls (Supplementary Figures S3E and S3F).

These results demonstrate that MSC administration reduces ubiquitin inclusions as well as astrocyte and microglia proliferation and related inflammation in MNs of SOD1/G93A mice.

MSCs Reduce the Oxidative Stress Condition Occurring During Disease Progression in SOD1/G93A Mice

To address the impact of MSC administration on the oxidative stress occurring in SOD1/G93A mice, we measured the constitutive and progression-associated expression of MTs in the spinal cord. The constitutive expression of MT mRNA in control mice was higher for MT-3, intermediate for MT-1 and lower for MT-2 (Figure 5A). The mRNA expression of all MT isoforms increased in the spinal cord of SOD1/G93A mice during disease progression, and the increase was more pronounced for MT-3, the neuron-specific isoform (Figure 5B). MSC administration resulted in a striking downregulation of MT-2 and MT-3

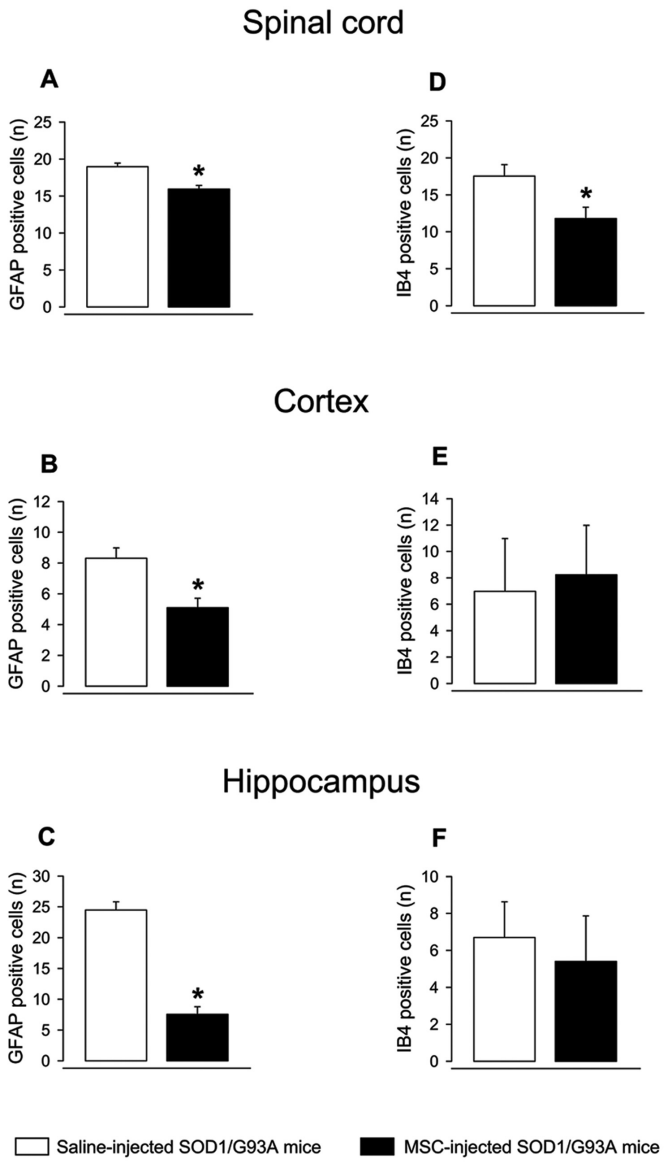

Figure 4. Effects of MSC administration on IB4-positive microglia cells and GFAP-positive astrocytes. SOD1/G93A mice were treated with 10^6 MSCs or saline at d 90 and sacrificed at d 125. Spinal cord (A, D), brain cortex (B, E) and hippocampus (C, F) 5-μm sections were stained for histological examination. Rabbit anti-cow GFAP, diluted 1:100 (A–C), or isolectin IB4 conjugated antibody, diluted 1:100 (D–F), was used. Seven MSC-treated and seven saline-injected mice were used for the experiments. Quantification was performed on six sections per animal, from three segments apart. Data (means ± SEM) represent the number of positive cells/mm^2. *$p < 0.01$ at least (Mann-Whitney).

isoforms at d 125 in treated compared with saline-treated SOD1/G93A mice; on the contrary, MT-1 expression was not modified (Figure 5C). Next, we evaluated the activity of the antioxidant enzyme GST in the spinal cord of SOD1 and saline- or MSC-treated SOD1/G93A mice at d 125. GST-specific activity was significantly increased in SOD1/G93A mice, when compared with SOD1 mice, and MSC treatment reverted such an increase by about 65% (Figure 5D). These results suggest that MSCs have a deep impact on the oxidative stress occurring in experimental ALS, as demonstrated by the ability to dampen the increase of antioxidant molecules.

MSCs Normalize the Excessive (^{3}H)D-Aspartate Release in the Spinal Cord of SOD1/G93A Mice

We previously observed that both the spontaneous and the stimulus-evoked release of [^{3}H]D-aspartate from prelabeled spinal cord synaptosomes were significantly elevated in SOD1/G93A mice at the late stage of disease, compared with SOD1 animals (23). Similarly, in the present experiments, the spontaneous and the 15 mmol/L (Figure 6A) or 25 mmol/L (Figure 6B) KCl-evoked release of [^{3}H]D-aspartate was significantly increased in saline-injected SOD1/G93A with respect to saline-treated SOD1 control mice. The increase of both basal and the 15 or 25 mmol/L KCl-stimulated release of [^{3}H]D-aspartate was reduced to control values after MSC administration. Thus, MSC injection results in the significant inhibition of the excessive release of glutamate in experimental ALS.

DISCUSSION

In this study, we demonstrated that allogeneic MSCs, isolated from C57B6 mice, prolong survival and improve motor performance in SOD1/G93A mice. Previous reports demonstrated that MSCs help motor functions and modestly increase survival of SOD1/G93A mice only when administered before disease onset (12–14). The first major achievement of this study was the observation that MSCs were able to significantly delay animal death when injected after the occurrence of clinical symptoms, a condition in which most treatments fail.

Survival improvement and behavioral tests in MSC-treated SOD1/G93A mice were associated with a significant decrease of the number of cells immunoreactive for ubiquitin, a protein adduct that targets proteins for degradation via the proteasome (24). The expression of mutant SOD1 protein leads to proteasome inhibition, which can contribute to MN

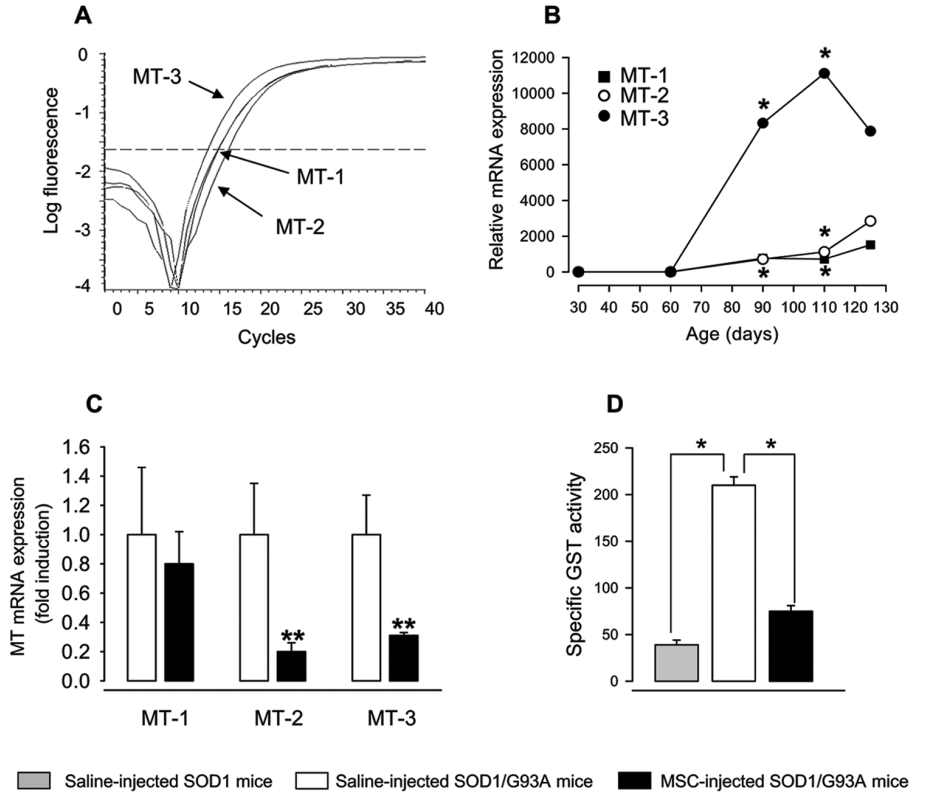

Figure 5. Effect of MSC administration on MT mRNA expression and GST specific activity in spinal cord of SOD1/G93A mice. Animals were intravenously injected with 10^6 MSCs or saline at d 90. (A) Basal expression of MT-1, MT-2 and MT-3 mRNA in spinal cord from control SOD1 mice. MTs were analyzed by RT-PCR using GAPDH mRNA as a reference. Amplification curves of MT-1, MT-2 and MT-3 are shown with their crossing point on the *x* axis. The difference between crossing points (ΔCt) are indicated. (B) Time course of the relative expression of MT-1, MT-2 and MT-3 mRNA. MTs were analyzed at d 30, 60 (presymptomatic mice), 90, 110 and 125 (symptomatic mice) by RT-PCR using GAPDH mRNA as a reference. Eight MSC-treated and seven saline-injected SOD1/G93A mice were used. Data are means ± SEM of two mRNA extractions from three experiments. (C) Expression of MT-1, MT-2 or MT-3 mRNA in MSC-injected SOD1/G93A mice at d 125. Data are reported as fold induction with respect to saline-injected SOD1/G93A mice and are means ± SEM of two mRNA extractions from three experiments. (D) Specific activity of GST in the spinal cord of saline-injected SOD1 and saline- or MSC-injected SOD1/G93A mice. GST activity was expressed as μmol/min/mg protein. Data are means ± SEM of four experiments in duplicate. *$p < 0.05$ at least (ANOVA plus Bonferroni); **$p < 0.001$ (Mann-Whitney).

suffering and death (25). Therefore, our data may indicate that MSCs may reduce the proteasome inhibition associated with ALS progression. This effect was observed regardless of any evidence of preservation of MNs positive for ChAT, an enzyme for which inhibition by misfolded SOD1 species leads to an impaired axonal transport (26), indicating no changes in the number of MNs after MSCs. Similarly, MSC administration did not affect the number of GLT1-expressing cells in spinal cord of SOD1/G93A mice. GLT1 is a glutamate transporter preferentially expressed on astrocytes and provides trophic support to MNs through the rapid recovery of synaptic glutamate (27). GLT1 expression and function is decreased in SOD1 mutant mice, and this reduction was proposed as a major cause of excessive glutamate and excitotoxicity in ALS (21). A recent study reported the ability of human adipose-derived stem cells to upregulate the expression of GLT1 in SOD1/G93A-bearing astrocytes, which resulted in enhanced glutamate uptake function (28). The histological data presented here do not allow defining whether tissue GLT1 levels are reestablished after MSC administration. However, quantitative RT-PCR results showed a positive effect of the MSCs on GLT1 mRNA expression in the spinal cord of SOD1/G93A mice, suggesting that this augment may be the result of an enhanced production by the single cell rather than of an increased number of GLT1-expressing cells. Regardless of a direct effect of MSCs on GLT1 expression, the observation that both basal and stimulus-evoked release of glutamate, which are increased in SOD1/G93A mice (23), were inhibited by MSC administration indicates that the treatment ameliorates the excitotoxic scenario during the development of pathology. These findings also suggest that an excess of glutamate, brought about by neurotransmitter release, plays a role in disease progression and further supports the therapeutic effect of MSCs.

A second major result of this study was the demonstration that MSCs are effective, even when injected intravenously. So far, the only study reporting on the effect of MSCs in symptomatic hSOD1(G93A) rats was on the basis of intrathecal cell delivery, leading to chimerism of endogenous astrocytes in the lumbar spinal cord (29). However, MSC intravenous administration has been shown to be effective in EAE, a model for multiple sclerosis, where inflammation is likely to play a role in the recruitment of cells to the CNS (10), and in experimental stroke (30) and other disease models (31), where the acute onset of tissue damage may lead to the release of chemokines (32) and other signal molecules possibly involved in the recruitment of MSC-bearing Toll-like receptors on their surface (33). Because of the wide consensus on the best cell dose for the treatment of experimental neurological diseases, the lack of reliable scales for

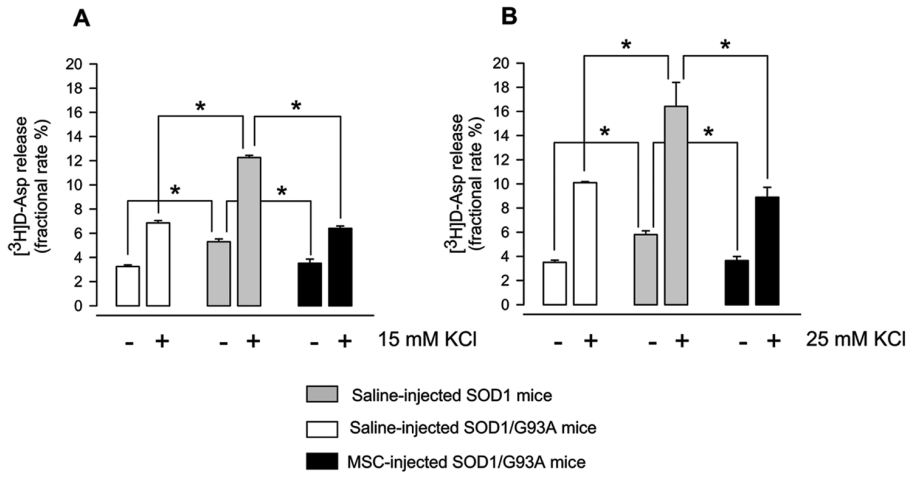

Figure 6. Effects of MSCs on the basal and depolarization-evoked release of (^{3}H)D-aspartate in synaptosomes purified from the spinal cord of control saline-injected SOD1 and saline- or MSC-injected SOD1/G93A mice. Animals were intravenously injected with 10^6 MSCs or saline at d 90 and sacrificed at d 125. Synaptosomes were labeled with the radioactive tracer and exposed in superfusion to 15 mmol/L (A) or 25 mmol/L (B) KCl. Results are expressed as fractional rate × 100. Six MSC-treated and six saline-injected SOD1/G93A mice were used. Saline-injected SOD1 mice were used as controls. Means ± SEM of six experiments run in triplicate are presented. *$p < 0.05$ at least (ANOVA plus Bonferroni).

translating mouse cell dose to humans and robust data suggesting that one injection suffices to achieve significant inhibition of inflammation and fostering of neuroprotection in EAE (31), we injected 10^6 MSCs only once after disease onset, a situation that may well represent future clinical scenarios for ALS patients. We showed that a small number of intravenously injected MSCs can be detected in the CNS of SOD1/G93A mice early after infusion, but they rapidly disappear over time. Although these results may appear surprising, they are in line with the most recent studies showing that MSCs act at a distance in many experimental diseases without or with limited engraftment (10,34–37). There is now overwhelming evidence that after intravenous administration, almost all MSCs are entrapped in the lungs, and the few passing through this filter are rapidly rejected, if of allogeneic or xenogeneic origin, and rapidly cleared from the CNS (38), where, in contrast to neural precursor cells, they are likely not to find an appropriate microenvironment to engraft and generate neurogenic ectopic niches (39). Thus, although MSCs may contribute to tissue repair by cell differentiation, mainly in circumstances where differentiation is oriented toward their physiological cellular fate (40), functional improvement is observed in most animal models by transient appearances of the cells, suggesting that paracrine mechanisms based on molecules secreted by MSCs (36,41–43) are likely to play a major role in the therapeutic plasticity of these cells (38,44).

In this study, we also assessed the dynamics of expression of MTs in the spinal cord of SOD1/G93A mice. MTs play a neuroprotective function through their intracellular role of radical and toxic metabolite scavengers and their extracellular release from astrocytes promoting axonal growth (45). There is substantial evidence supporting the hypothesis that oxidative stress plays a role in ALS pathogenesis, being one of the mechanisms by which MN death occurs (46). Therefore, changes in MT expression are consistent with the hypothesis that MTs may serve an important protective function in experimental ALS (47). Expression of all three MTs increased significantly during the course of experimental ALS, thus demonstrating a close relationship between disease progression and levels of MTs. The upregulation of MT mRNA paralleled with the increased activity of the antioxidant enzyme GST during disease progression. Here we show that MSC administration reverted the upregulation of MTs and the increased activity of GST in SOD1/G93A mice, thus suggesting that MSCs exert, similarly to that described in EAE (18), a remarkable antioxidative and neuroprotective effect.

Lastly, we observed that MSC systemic administration results in a decreased number of IB4-positive microglia and GFAP-positive astrocytes as well as a decreased expression of TNFα and IL1β in the spinal cord of MSC-treated SOD1/G93A mice compared with saline-treated animals. Because of the role of activated microglia in the oxidative stress–mediated and inflammatory insult leading to neurodegeneration in SOD1/G93A mice (5) and to the role of molecules released by astrocytes in the degeneration of spinal MNs (48), we postulate that MSCs may influence noncell autonomous processes involved in ALS pathogenesis.

CONCLUSION

The results presented here confirm that intravenous injection of MSCs, an adult stem cell type currently under clinical scrutiny for several neurological diseases (31), is an appealing treatment for human ALS. The results strongly support the clinical translation of this treatment. Recent reports have demonstrated the safety of MSC transplantation, when injected locally in the spinal cord, intraventricularly or intravenously in ALS subjects (49,50). Because of the lack of effective treatment for ALS and the known safety profile of MSCs (51), these data encourage careful clinical evaluation in the framework of rigorous phase II clinical trials.

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DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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