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A Combination of Intrathecal and Intramuscular Application of Human Mesenchymal Stem Cells Partly Reduces the Activation of Necroptosis in the Spinal Cord of SOD1^{G93A} Rats

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Key Words. Amyotrophic lateral sclerosis • Mesenchymal stem cells • Necroptosis • Apoptosis • Autophagy

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Received October 10, 2018; accepted for publication February 3, 2019; first published February 25, 2019.

http://dx.doi.org/10.1002/sctm.18-0223

ABSTRACT

An increasing number of studies have demonstrated the beneficial effects of human mesenchymal stem cells (hMSC) in the treatment of amyotrophic lateral sclerosis (ALS). We compared the effect of repeated intrathecal applications of hMSC or their conditioned medium (CondM) using lumbar puncture or injection into the muscle (*quadriceps femoris*), or a combination of both applications in symptomatic SOD1^{G93A} rats. We further assessed the effect of the treatment on three major cell death pathways (necroptosis, apoptosis, and autophagy) in the spinal cord tissue. All the animals were behaviorally tested (grip strength test, Basso Beattie Bresnahan (BBB) test, and rotarod), and the tissue was analyzed immunohistochemically, by qPCR and Western blot. All symptomatic SOD1 rats treated with hMSC had a significantly increased lifespan, improved motor activity and reduced number of Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) positive cells. Moreover, a combined hMSC delivery increased motor neuron survival, maintained neuromuscular junctions in *quadriceps femoris* and substantially reduced the levels of proteins involved in necroptosis (Rip1, mixed lineage kinase-like protein, cl-casp8), apoptosis (cl-casp 9) and autophagy (beclin 1). Furthermore, astrogliosis and elevated levels of Connexin 43 were decreased after combined hMSC treatment. The repeated application of CondM, or intramuscular injections alone, improved motor activity; however, this improvement was not supported by changes at the molecular level. Our results provide new evidence that a combination of repeated intrathecal and intramuscular hMSC applications protects motor neurons and neuromuscular junctions, not only through a reduction of apoptosis and autophagy but also through the necroptosis pathway, which is significantly involved in cell death in rodent SOD1^{G93A} model of ALS. STEM CELLS TRANSLATIONAL MEDICINE *2019;8:535–547*

SIGNIFICANCE STATEMENT

The results provide new evidence that a combination of repeated intrathecal and intramuscular application of human mesenchymal stem cells into symptomatic SOD1^{G93A} rats, prolongs life span and protects motor neurons and neuromuscular junctions, through a reduction of apoptosis, autophagy, and necroptosis pathways, which are involved in cell death in rodent model of ALS.

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is an adult neurodegenerative disease, which is defined by the loss of progressive motor neurons (MNs) in the spinal cord, cerebral cortex, and brain stem. Almost 90%–95% of ALS cases are sporadic (sALS) and the remaining 5%–10% are associated with a large number of gene mutations and variants in more than 30 human chromosomal regions [1, 2]. The first identified familial ALS (fALS) mutation is linked to the mutant Zn/Cu superoxide dismutase (SOD1), which accounts for 20% of fALS cases [3]. The transgenic animal ALS models over-express a human mutated SOD1^{G93A} gene (SOD1) which leads to the development of clinical and pathological features similar to human disease, including motor neuron pathology, loss of motor functions, muscle atrophy, progressive paralysis, and reduced lifespan. The proposed pathological mechanisms of disease include protein misfolding and aggregation,

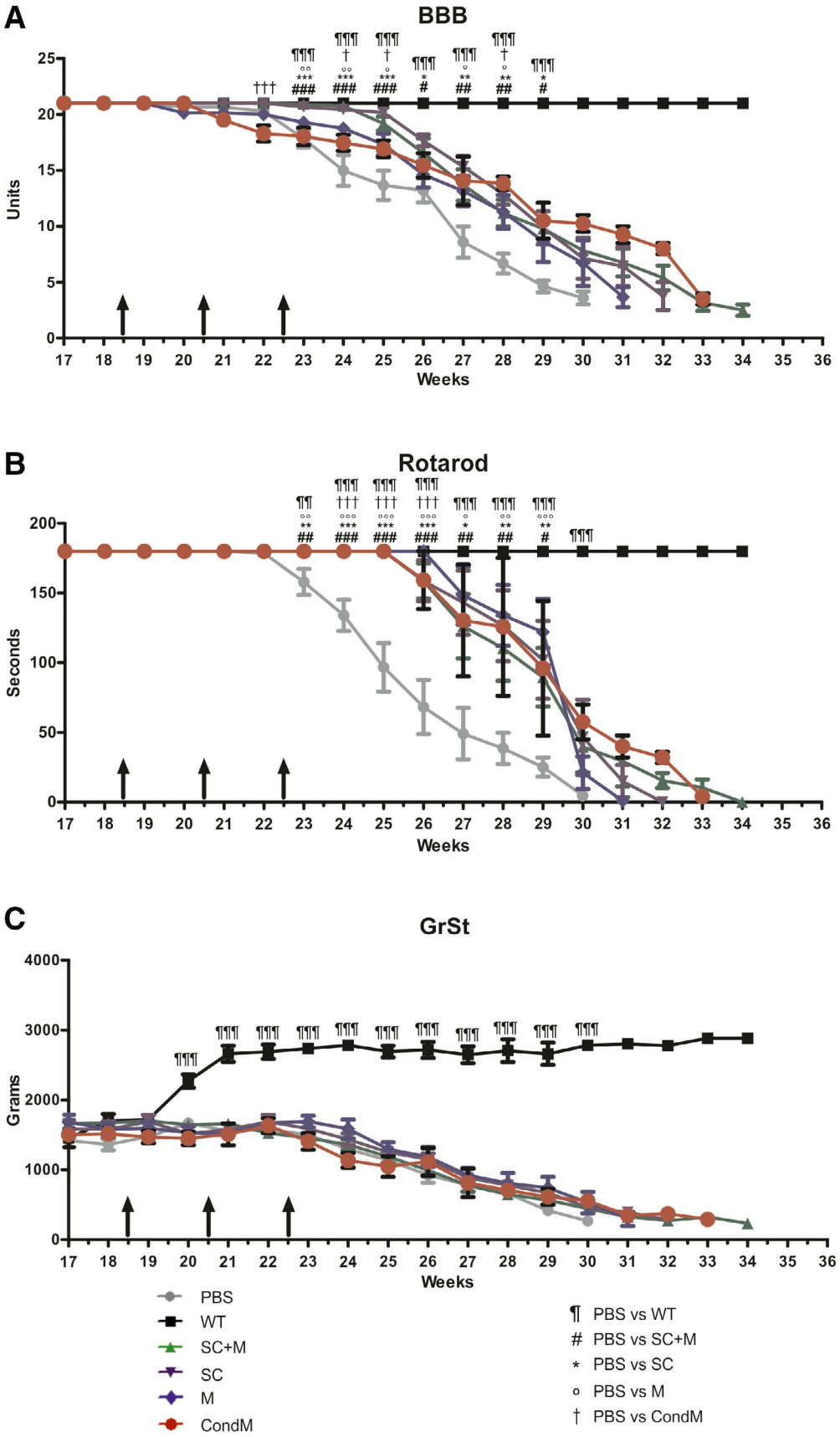

Figure 1. *(Legend appears on next page.)*

oxidative stress, glutamate excitotoxicity, mitochondrial dysfunction, glial cell activation, defective axonal transport, calcium homeostasis impairment, increased apoptosis and inflammatory cytokines, and neuromuscular junction (NMJ) denervation [4]. Despite scientific development, a successful treatment for ALS patients has not been found. However, a promising therapeutic option for ALS treatment is stem cell therapy. Human mesenchymal stem cells (hMSC) could be an ideal option due to their immunomodulatory and anti-inflammatory properties and the excretion of trophic factors and exosomes [5–7]. They are known to secrete a brain-delivered neurotrophic factor (BDNF), neural growth factor (NGF), vascular endothelial growth factor (VEGF), insulin-like growth factor 1 (IGF-1), a glia cell-line derived neurotrophic factor (GDNF) [8–11], and other substances, which are crucial for neuronal survival. Therefore, their application could lead to the generation of a protective environment for degenerating neurons, tissue repair and disease attenuation. The most recent clinical investigations, in patients with ALS who underwent hMSC transplantation, have shown procedural safety and clinical proof of principle with modest neurological benefits. The most frequently used route of delivery in patients is via lumbar puncture, either in single dose [12–14] or repeatedly [15, 16].

An intramuscular injection has also been applied in ALS patients in combination with an intrathecal application and, 6 months after treatment, a 25% improvement in the slope of progression was observed in 13 patients [17]. However, the mechanism of action remains to be elucidated. Instead of cells, an alternative approach is to use their exosomes or the conditioned media. Treating symptomatic SOD1 mice with a conditioned medium (CondM) from adipose stromal cells (ASC-CondM), significantly increased their postonset survival time and lifespan. Moreover, the SOD1 mice given ASC-CondM treatment showed, at an early symptomatic stage, high motor neuron counts and less activation of microglia and astrocytes [18]. Similarly, exosomes from ASC alleviated the aggregation of SOD1, in neuronal cells isolated from the SOD1 mice [19].

Many previous studies of fALS using mouse SOD1 models have provided evidence that the mechanism of motor neuron death during the manifestation of ALS is through apoptosis [20–22]. However, in recent years, several new types of cell death have been implicated with ALS, such as inflammasome (NLRP3)-mediated pyroptosis and necroptosis [23, 24]. The necroptosis pathway is dependent on three key proteins: receptor-interacting protein kinase 1/3 (Ripk1, Ripk3) and mixed lineage kinase-like protein (MLKL). Several studies were performed to unravel the role of apoptosis or necroptosis, however, the results remain controversial. Re et al. first demonstrated that necroptosis is a major driver in motor neuron cell death in both sALS and fALS. In the coculture of sALS astrocytes and human embryonic stem cell-derived MNs, it was found that zVAD-fmk, a pan-caspase inhibitor and inhibitor of caspase-3 activation, had no effect on the MNs survival. These results are inconsistent with a previous study showing that reduced caspase activity rescues MNs in SOD1 mouse models [25]. Ito et al. provided the first evidence regarding necroptosis in vivo in optineurin and SOD1 mice models, and described the alternative mechanism of the role of necroptosis in ALS [26]. Moreover, activated RIP1 and MLKL were identified in the spinal cord of post-mortem ALS patients. Overall, this evidence suggests that necroptosis plays an important role in the development of ALS [24, 26, 27]. We have previously reported the positive effect of a single application of hMSC into cisterna magna of SOD1 transgenic rats [28]. In this study, we aimed to investigate the effect of repeated intrathecal applications of hMSC in a SOD1 transgenic rat model of ALS. As a delivery route, we chose lumbar puncture and/or intramuscular injection, that is, applications which have already been used in clinical trials. We compared the obtained results with repeated applications of hMSC CondM, to determine if the neuroprotective effect can only be achieved via a CondM. Finally, we studied the effect of all the applied therapies on apoptosis, necroptosis, and autophagy.

Materials and Methods

An extended Materials and Methods section is available in Supporting Information (Supporting Information Methods).

Results

The Repeated Intrathecal hMSC Application Delays the Decline in Motor Activity and Extends the Overall Survival of SOD1 Rats

The progression of the disease in SOD1 rats was evaluated by monitoring motor activity using several behavioral tests, such as BBB, rotarod, and grip strength test (GrSt; Fig. 1). All the behavioral tests were performed every week, starting 2 weeks before the first application of hMSC or CondM. The first dose of hMSC was injected into approximately 18-week-old SOD1 rats at the onset of the disease via lumbar puncture only into the spinal canal (SC), or was combined with the application into *quadriceps femoris* (SC + M) or only injected into *quadriceps femoris* (M). The animals received second and third doses of hMSC, 14 days and 28 days later. The CondM was only applied into the SC according to the same protocol as hMSC. We found that the repeated intrathecal application of hMSC alone or in combination with an intramuscular injection, significantly improved motor activity. The significant difference in the BBB score during weeks 23–29 was seen between the animals with a combined treatment of SC + M and the animals with cells only injected to SC, compared with the phosphate-buffered saline (PBS)-treated SOD1 rats. Interestingly, the loss of motor function was also slowed down at weeks 23–25 and

Figure 1. The repeated intrathecal and intramuscular application of human mesenchymal stem cells (hMSC) or conditioned medium, delay the decline of the motor function of $SOD1^{G93A}$ rats. The motor ability of rats was assessed using BBB **(A)**, rotarod **(B)**, and GrSt **(C)**. The animals that were repeatedly treated with the application of hMSC into the spinal canal (SC) and *quadriceps femoris* (SC + M) but also the animals with applications only into the spinal cord or only into *quadriceps femoris* (M) showed a significantly higher improvement in the BBB test (A) and rotarod (B) in comparison with the control (phosphate-buffered saline [PBS]). GrSt did not reveal any differences between the treated SOD1 groups (C). The black arrows indicate the treatment application. Data are presented as mean and error bars indicate SEM. Differences in the groups were analyzed by the one-way analysis of variance test: statistical significance at *, $p < .05$; **, $p < .01$; ***, $p < .001$ (compared with PBS). The detailed statistical analysis of behavioral changes between the groups is presented in Supporting Information Table S2.

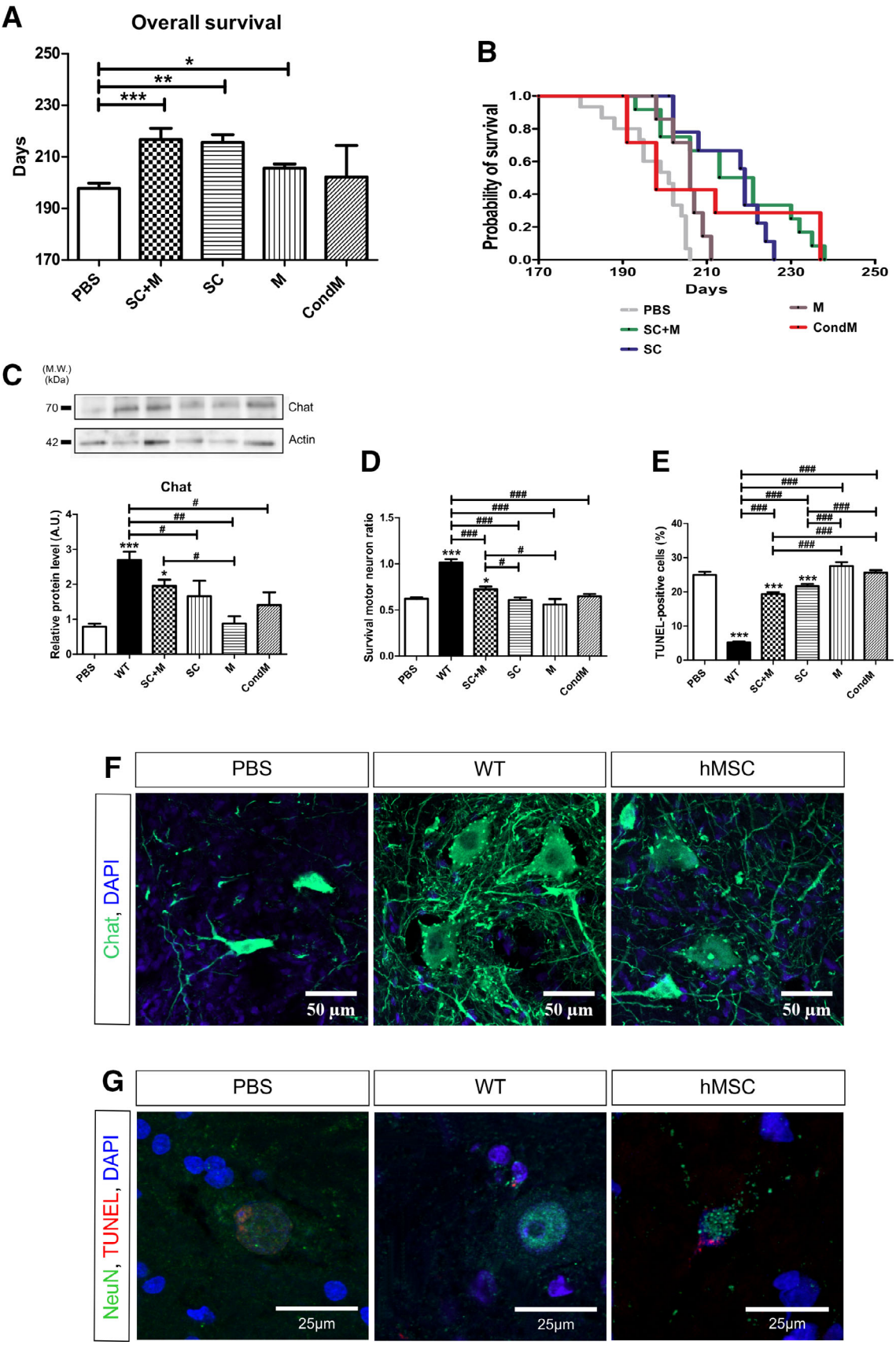

Figure 2. *(Legend appears on next page.)*

27–28 after repeated hMSC injections into the muscle, or a repeated application of CondM into the spinal cord canal (weeks 24, 25, and 28; Fig. 1A). Rotarod was used to measure balance, motor coordination, strength, and physical condition. All the treated animals scored better from weeks 23 to 29 than the PBS-treated group, except for the CondM group, which only scored significantly better until week 26 (Fig. 1B). Interestingly, the overall physical condition assessed by rotarod was similar to the wild type (WT) rats up to week 25 in the SC, CondM and SC + M groups, whereas the M group showed no decline for a further week (week 26). In contrast, the PBS-treated animals already showed a decline in rotarod performance from week 23 onward. The trend of body weight change in the SOD1 rats and grip strength test (Fig. 1C) showed no significant differences between the groups treated with PBS, hMSC, or CondM.

All the animal groups treated with hMSC survived significantly longer than the PBS-injected rats after repeated injections. The lifespan of the SOD1 rats was prolonged by hMSC applied to SC + M (group mean 217 days $\pm$ 4 days, $p \leq .001$), but SC (216 days $\pm$ 8 days, $p \leq .01$) or M applications also increased the animal survival time (206 days $\pm$ 6 days, $p \leq .05$) when compared with PBS (198 days $\pm$ 2 days) and CondM-treated animals (202 days $\pm$ 11 days; Fig. 2A, 2B). These results demonstrate that the combined application of hMSC most effectively improves motor activity and prolongs survival in SOD1 rats.

The Repeated Combined Application of hMSC to the Spinal Cord and Muscle Protects MNs

To establish whether hMSC could prevent motor neuron loss, we quantified the levels of choline acetyl transferase (Chat), which is a confirmed specific marker of MNs by Western blot and stained lumbar spinal cord sections with an antibody against Chat. The level of Chat as well as the number of Chat-positive neurons in the PBS-treated control rats, reflects a marked loss of MNs in the ventral horn of the lumbar spinal cord compared with the WT rats (Fig. 2C, 2D; $p \leq .001$). However, the motor neuron loss was reduced by hMSC treatment to SC + M ($p \leq .05$). hMSC treatment only to SC or only to M did not sufficiently protect MNs. Similarly, no difference in the level of protein and number of Chat-positive cells was detected between the PBS-control and CondM-treated groups. To detect motor neuron death in spinal cord ventral horns, we used TUNEL staining (Fig. 2E). Consistent with the anti-Chat staining results and Western blot, the WT rats had a significantly lower number of TUNEL-positive cells in the ventral horns compared with the PBS injected SOD1 rats ($p \leq .001$). We observed a decrease of TUNEL-positive cells in ventral horns after the application of hMSC to SC ($p \leq .001$) or SC + M in comparison to the PBS-injected animals ($p \leq .001$). No differences in the number of TUNEL-positive cells were detected between the CondM, M, and PBS-treated groups. Immunostaining confirmed the colocalization of TUNEL staining and NeuN (Fig. 2G).

The Survival of hMSC in the Spinal Cord and *Quadriceps Femoris*

We further investigated whether the transplanted hMSC were able to survive in the spinal cord of the SOD1 and WT rats. To identify the presence of viable transplanted hMSC, longitudinal spinal cord sections were stained with a human-specific marker for nuclei (HuNu; Supporting Information Fig. S1). We detected HuNu-positive cells 1 week and 2 weeks, but not at 4 weeks after hMSC applications in the SOD1 positive rats, whereas in the WT control rats few HuNu-positive cells were still detectable 4 weeks after the hMSC applications (Supporting Information Fig. S1A). The transplanted hMSC were predominantly found between the folds of arachnoidea in the cauda equina. One week after the hMSC applications only few cells were detected in *quadriceps femoris* of the SOD1 rats, while in the WT rats green, hMSC clusters were located in *quadriceps femoris* between the muscle fibers (Supporting Information Fig. S1B). At later time points, no grafted cells were found in muscles of both WT and SOD1 animals.

hMSC Grafts Induced the Downregulation of Necroptosis Related Genes in the Spinal Cord of SOD1 Rats

We studied the expression profile of several genes involved in the different pathways of cell death in the spinal cord of SOD1 rats at a late symptomatic phase of the disease (Supporting Information Fig. S2). A particularly, strong trend of downregulation of the RipK1, MLKL, and Casp-8 transcripts involved in necroptosis was observed after the repeated administration of hMSC into SC + M ($p < .05$) and their levels were close to the levels of WT animals (Supporting Information Fig. S2A, S2C, S2D). SC or M treatment did not show such a considerable expression reduction of these necroptosis related-genes. None of the treatments influenced the expression of RipK3 (Supporting Information Fig. S2B). Even in the WT rats, the transcripts were not significantly downregulated (Supporting Information Fig. S2A–S2D). With reference to the apoptotic genes, we detected a reduction in the RNA level of casp-3, casp-9, and Bak in the SC + M treated spinal cord, but this reduction was not statistically significant (Supporting Information Fig. S2E–S2G). On the contrary, the Bcl-2 gene showed an increase in all the treated groups, except for SC + M, compared with the PBS-treated controls (Supporting Information Fig. S2H).

Figure 2. The repeated combined application of human mesenchymal stem cells (hMSC) prolongs survival and protects the motor neurons of SOD1 transgenic rats. **(A):** The lifespan of SOD1 rats was significantly prolonged by hMSC applied to the spinal cord (SC) and *quadriceps femoris* (SC + M) but also after applications to the spinal canal or to *quadriceps femoris* (M), when compared with the control group (SOD1 + phosphate-buffered saline [PBS]) or conditioned medium-treated animals. **(B):** The Kaplan–Meier plot describes the cumulative probability of SOD1 transgenic rat survival after different treatments. **(C):** Representative image of immunoblots for chat and group and the Western blot analysis showed a significant decrease in the expression of chat in the spinal cord of SOD1 rats, which was reduced after treatment with hMSC to SC + M. Actin was used as a loading control **(D)**. The motor neuron loss was reduced by hMSC treatment to SC + M. **(E):** The number of TUNEL-positive cells was decreased in the spinal cord of SOD1 rats after applications of hMSC to SC + M or only to SC. **(F):** Representative images show an increased number of chat-positive neurons (green) in the spinal cord of SOD1-positive rats after applications of hMSC to SC + M. **(G):** Representative images show colocalization of TUNEL and NeuN in SOD1 rats. Cell nuclei were detected with DAPI staining (blue). Data are presented as mean and error bars indicate SEM. Differences in the groups were analyzed by one-way analysis of variance: statistical significance at *, $p < .05$; **, $p < .01$; ***, $p < .001$ (compared with PBS). The detailed statistical analysis of changes between the groups is presented in Supporting Information Tables S3, S4.

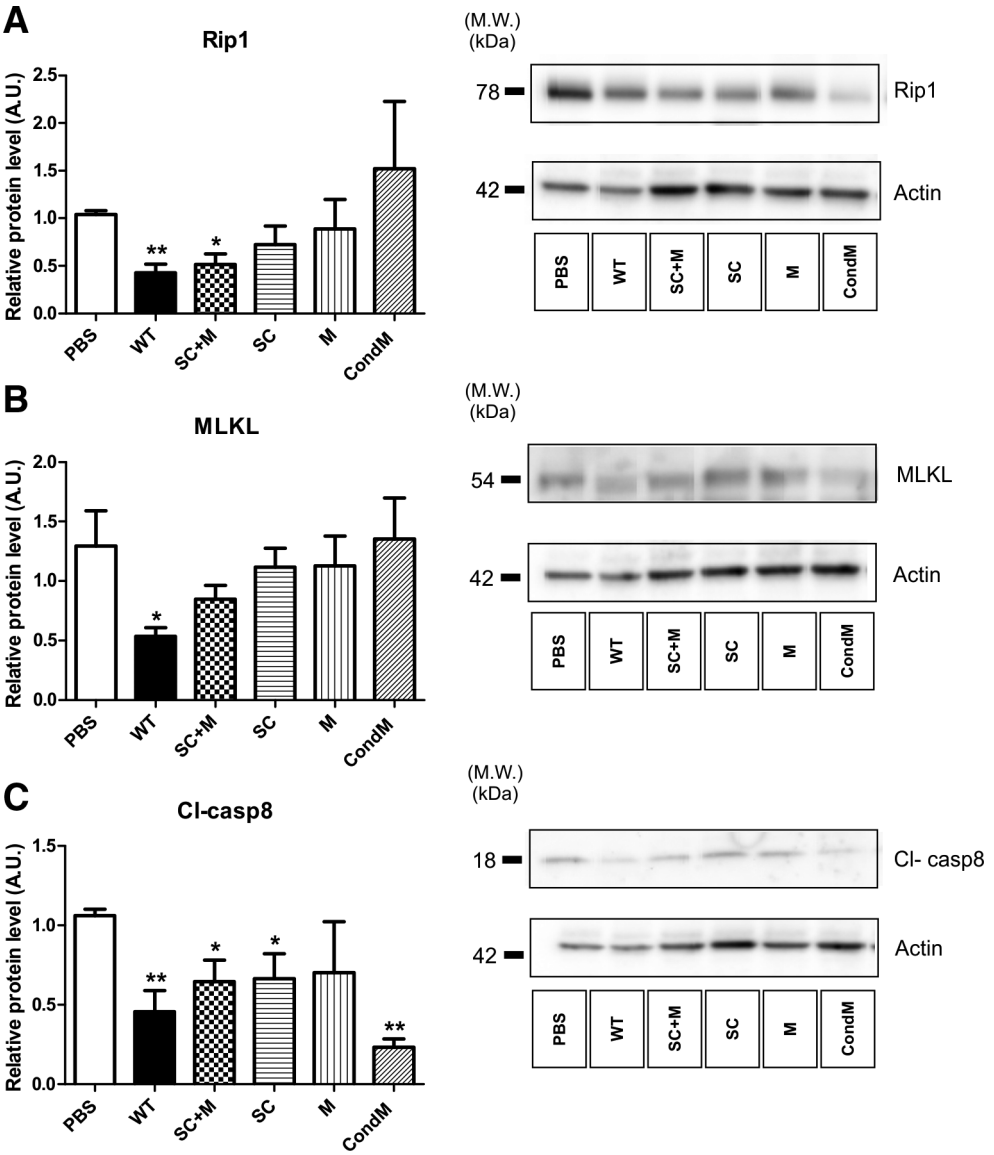

Figure 3. Representative immunoblots and quantitative analysis of proteins involved in the necroptosis pathway. The Western blot analysis showed a significant decrease in the expression of Rip1 **(A)** and cleaved casp-8 **(C)** in the spinal cord of SOD1 rats after treatment with human mesenchymal stem cells (hMSC) to SC + M, whereas the expression of MLKL **(B)** only showed the trend in reduction, which was not significant. The application of hMSC to SC or conditioned medium decreased the levels of cleaved casp-8. The application to M did not show any effect. Actin was used as a loading control. Differences in the groups were analyzed by one-way analysis of variance: statistical significance below *, $p = .05$; **, $p < .01$; ***, $p < .001$ (compared with phosphate-buffered saline control). The detailed statistical analysis of Western blot analysis; the groups are presented in Supporting Information Table S4.

In addition, we analyzed NF-κB and TNF-α expression, because these genes are one of the most important factors in the inflammatory pathway. NF-κB and TNF-α expression was reduced in the SC + M group and this downregulation was similar to the WT animals (Supporting Information Fig. S2I).

The Repeated Combined Application of hMSC Decreases the Level of Necroptosis, Apoptosis, and Autophagy-Related Proteins in SOD1 Rats

In order to further investigate the effect of transplanted hMSC on a programmed cell death, we assessed the expression of relevant necroptosis-related proteins. RIP, MLKL, and cleaved caspase 8 (cl-casp 8) were examined by Western blot (Fig. 3A–3C). Consistently with other publication (Ito et al., 2017), we observed a significant difference between the SOD1-PBS injected and WT animals in the protein levels of RIP1 ($p \leq .01$), MLKL ($p \leq .05$), and cl-casp-8 ($p \leq .01$). Although the amount of MLKL protein showed a nonsignificant reduction after hMSC treatment to SC + M, the amount of RIP1 and cl-casp 8 proteins were significantly decreased in the hMSC-treated rats to SC + M, when compared with the PBS treated animals ($p \leq .05$; $p \leq .05$). The application of CondM or hMSC to SC only led to the reduction of cl-casp 8 ($p \leq .01$; $p \leq .05$). Concurrently, we tested the expression profile of several proteins involved in the apoptosis pathways such as casp-3, casp-9, Bak, and Bcl-2 (Fig. 4A–4G). The WT animals had a lower amount of casp-3 ($p \leq .05$) and cleaved casp-9 ($p \leq .01$) than their SOD1 PBS-treated littermates. We observed a trend in reduction in the amount of casp-9 protein and a significant reduction of its active form, cleaved casp-9 ($p \leq .001$) after repeated

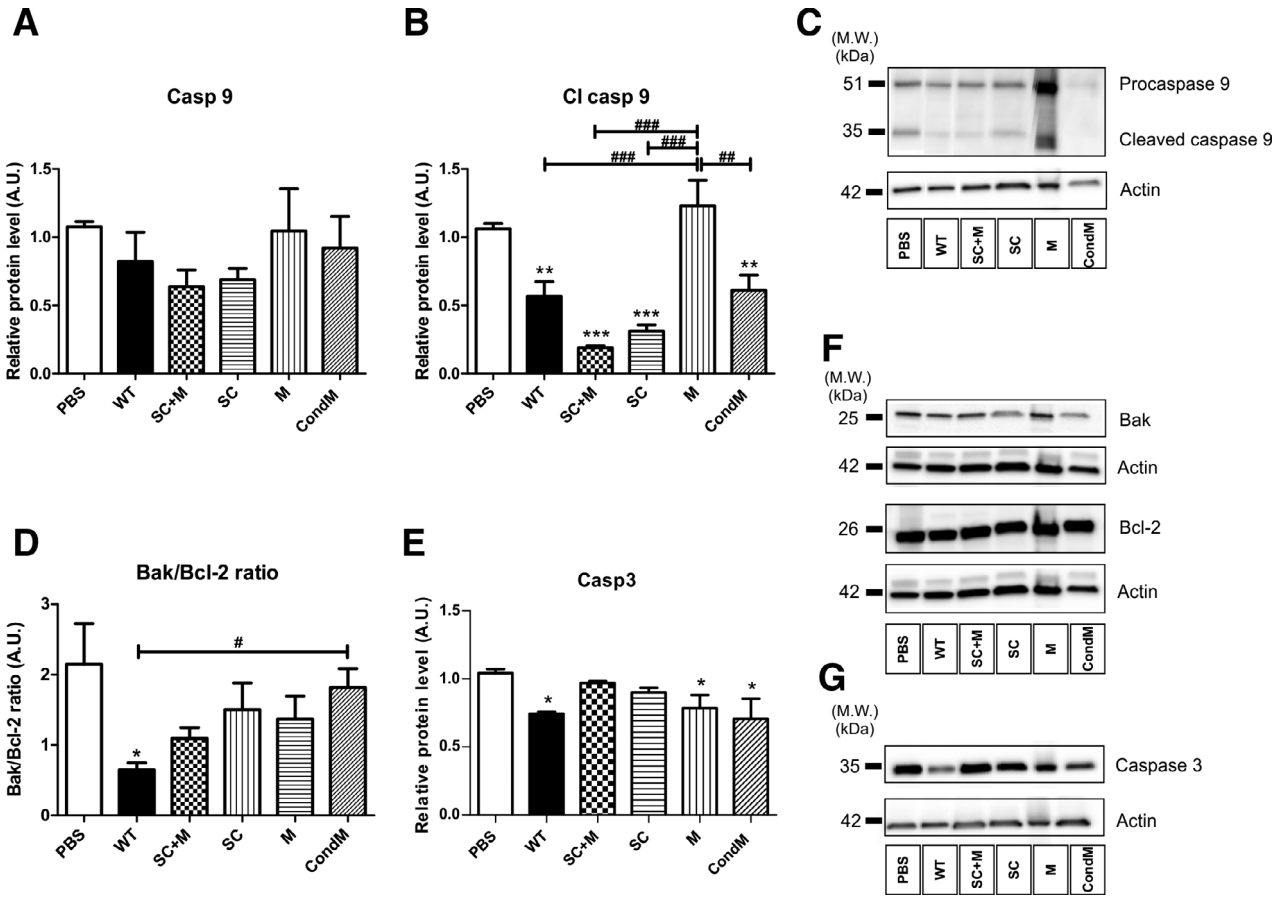

Figure 4. Representative immunoblots and quantitative analysis of proteins involved in the apoptosis pathway. The quantitative densitometry of cleaved casp-9 showed significantly lower levels of these markers in the spinal cord of SOD1 rats after applications of human mesenchymal stem cells (hMSC) to spinal canal (SC) and SC + M **(B)**. The applications of conditioned medium decreased the level of cleaved casp-9 and casp-3, which was also decreased after hMSC injections into the muscle **(E)**. Bak/Bcl-2 protein ratio **(D)** was >1 in SOD1 rats, but was slightly reduced in SC + M group. Casp-9 **(A)** did not differ among the groups. Representative images of immunoblots for each protein and group are presented in images **(C, F, G)**. Actin was used as a loading control. Data are presented as the mean ± SEM. Differences in the groups were analyzed by one-way analysis of variance: statistical significance at $^{\#}$, $p < .05$; $^{\#\#}$, $p < .01$; *, $p = .05$; **, $p < .01$; ***, $p < .001$ (compared with the phosphate-buffered saline control). The detailed statistical analysis of Western blot analysis changes between the groups, are presented in Supporting Information Table S4.

applications of hMSC to SC and SC + M when compared with the PBS-injected SOD1 control. The M group treated with hMSC had a significant reduction of casp-3 protein level ($p \leq .05$), whereas, the levels of casp-9 or cleaved casp-9 were upregulated in comparison with the PBS control. However, due to the high variance among the late stage samples, we did not observe any significance. The SOD1 rats treated with CondM had lower levels of cl-casp9 ($p \leq .01$) and casp-3 ($p \leq .05$). The ratio of Bak/Bcl-2 protein was >1 in all SOD1 animals, except for SC + M group, at which point it was nearly 1. Bak/Bcl-2 protein ratio <1 was only in WT animals.

Using Western blot analysis, we next tested the levels of proteins related to autophagy, Beclin-1, LC3B I and II, and p62 (Fig. 5A–5C) in spinal cords, and found that the amount of protein Beclin-1 was lower in the WT animals than in the PBS treated SOD1 rats. Significantly decreased levels were only measured after the administration of hMSC to SC + M ($p \leq .05$; Fig. 5A). Contrary to the WT animals, the ratio of LC3BII/I protein levels was elevated in all SOD1 animals, with the lowest level in SC group, whereas the highest value of LC3BII/I ratio was after the M injection (Fig. 5B). There were no significant changes between the groups in the expression of p62 protein. The lowest level of p62 protein was detected in WT animals, whereas no differences were found between SOD1 animals, except for a small trend in the increase in CondM treated animals. Immunohistochemistry confirmed the presence of p62 in SOD1 animals, which was more dispersed in hMSC treated animals than in SOD1 control (Fig. 5G).

The Repeated Combined Application of hMSC Ameliorates NMJs in SOD1 Rats

We next studied NMJs in the *quadriceps femoris* to determine whether hMSC provided any additional protection to muscle innervation. The level of NMJ denervation in the *quadriceps femoris* was estimated using Western blot (Fig. 6A–6F). The WT animals had significantly higher levels of synaptophysin (Syn) and nicotinic acetylcholine receptor α-7 (NAR) than any other animals ($p \leq .001$). The protein level of Syn was significantly higher ($p \leq .01$) and the trend in spared NAR was detected in the animals with SC + M treatment when compared with the PBS-treated control. In contrast, M treatment alone did not show a positive effect on the protein levels of SYN and NAR. These results suggest that the intrathecal applications of

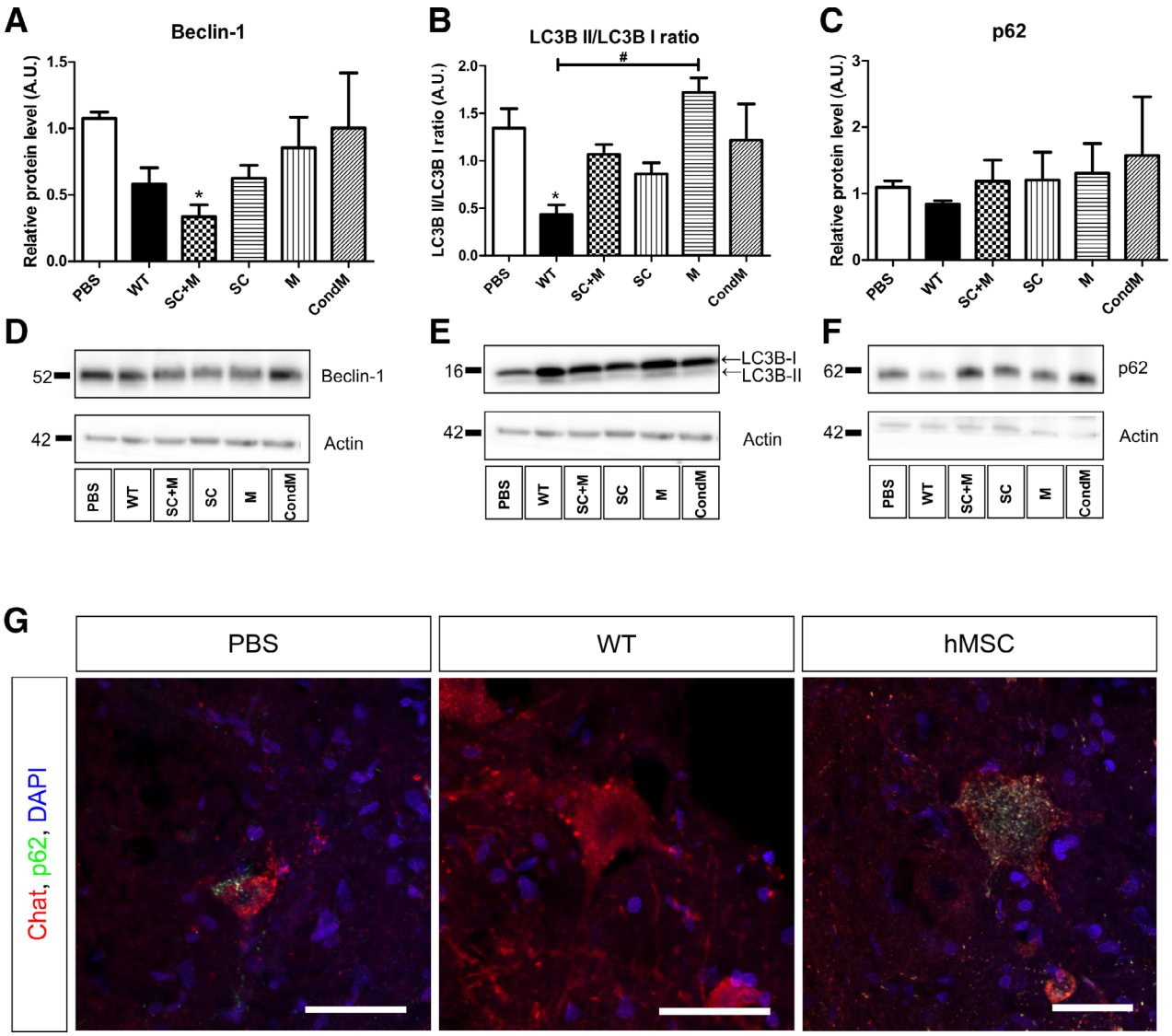

Figure 5. Representative immunoblots and quantitative analysis of protein in the autophagy pathway. The Western blot analysis showed a significant decrease of Beclin-1 expression **(A)** in the spinal cord of SOD1 rats after treatment with human mesenchymal stem cells (hMSC) to SC + M and a trend in reduction in spinal canal (SC) animals. LC3BII/LC3BI ratio was higher in all SOD1 rats when compared with WT animals. A significant difference was found between WT and M groups. The lowest values in the treated animals were in SC group, followed by SC + M group **(B)**. No significant differences between the groups were detected in expression of p62 protein **(C)**. Representative immunoblots for all proteins are shown in **(D)–(F)**. Actin was used as a loading control. Representative immunohistochemical images show colocalization of p62 and chat in SOD1 rats **(G)**. Cell nuclei were detected with DAPI staining (blue). Scale bar: 50 μm. Differences in the groups were analyzed by one-way analysis of variance: statistical significance at *, $p = .05$; **, $p < .01$; ***, $p < .001$ (compared with the phosphate-buffered saline control). The detailed statistical analysis of Western blot analysis between the groups is presented in Supporting Information Table S4.

hMSC are important for the protection of NMJs in *quadriceps femoris.* NMJ were also visualized by staining with α-Bungarotoxin, which binds to α unit of the NAR of NMJ and with an antibody against Syn. A very weak signal of Syn was detected in the SOD1 controls treated with PBS. The application of hMSC into SC + M partially rescued the NMJ innervation, as is visible in Figure 6G.

CondM from hMSC Contained Several Bioactive Molecules

Prior to application, CondM was analyzed for secreted proteins with Luminex xMAP Technology using ProcartaPlexImmunoAssays (Supporting Information Fig. S3). Six proteins (BDNF, bNGF, ICAM-1, SDF-1α, HGF, and VEGF-A), which are often present in CondM and can influence the progression of the disease, were chosen for the analysis. BDNF (96.57 pg/ml ± 11.61 pg/ml) and bNGF (118.10 pg/ml ± 17.16 pg/ml), were present in CondM in low concentrations, higher concentrations were detected for SDF-1α: (1138.10 pg/ml ± 133.04 pg/ml), sICAM-1 (1,078 pg/ml ± 131.53 pg/ml), HGF (2737.23 pg/ml ± 328.59 pg/ml), VEGF-A (4546.03 pg/ml ± 732.89 pg/ml). The complete culture medium, which included 10% fetal bovine serum, was used as a control. From the analyzed proteins, only very low levels of BDNF (4.41 pg/ml ± 0.79 pg/ml), bNGF (20 pg/ml ± 14.32 pg/ml), SDF-1α: (222 pg/ml ± 32 pg/ml), and sICAM-1 (125 pg/ml ± 10 pg/ml), were detected in the control unconditioned medium.

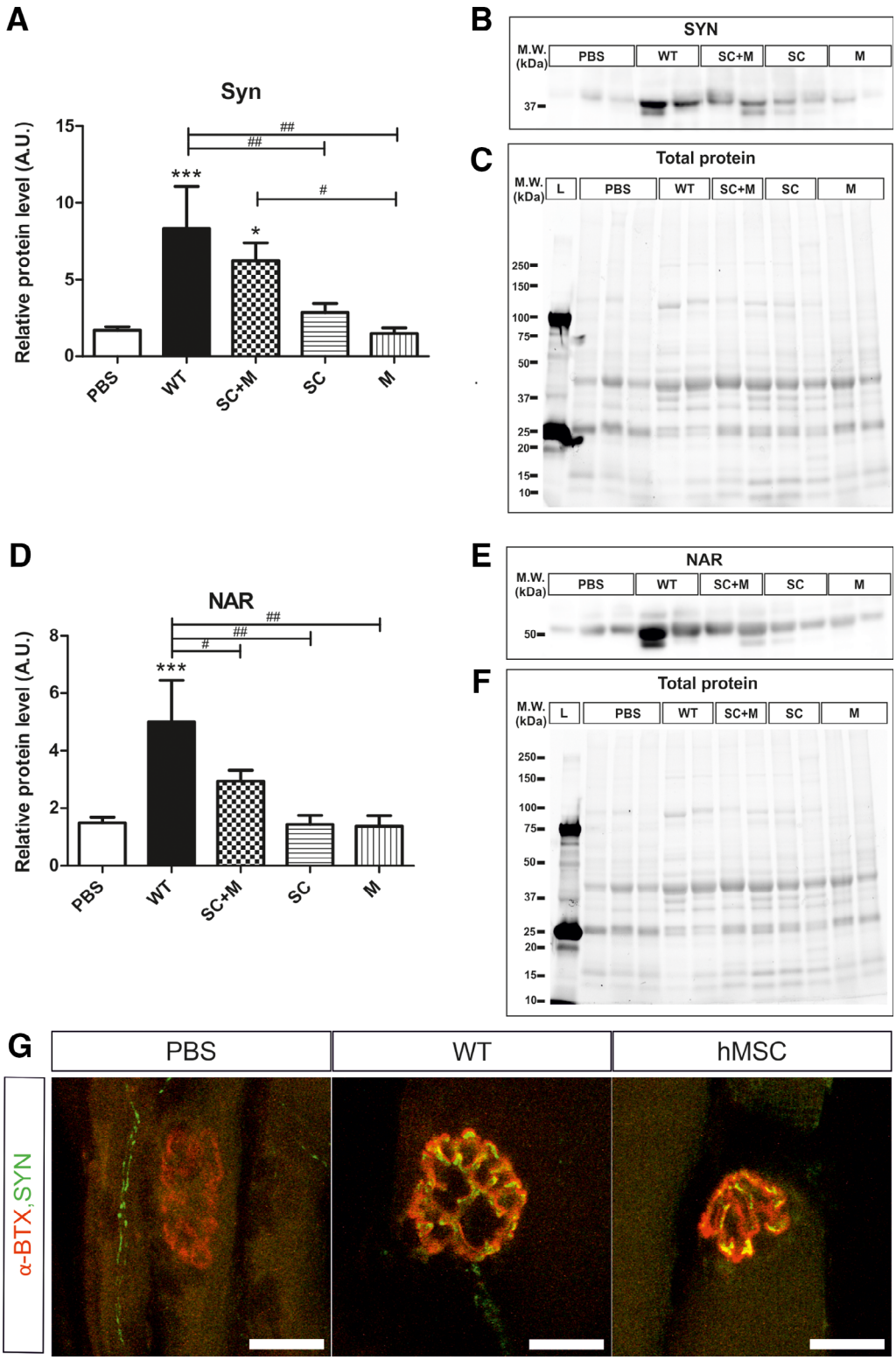

Figure 6. Neuromuscular junction (NMJ) denervation. NMJ in *quadriceps femoris* was evaluated by Western blot using an antibody against synaptophysin (Syn; **A**) and an antibody against nicotinic acetylcholine receptor α-7 (NAR; **D**). WT animals had higher levels of Syn and NAR proteins than any treatment or phosphate-buffered saline (PBS) control. A decrease in the reduction of Syn and NAR was observed only in SC + M group. The representative images of Western blot from SYN **(B)** and NAR **(E)**. The representative images of total protein for quantification of SYN **(C)** and NAR **(F)** protein. **(G):** Representative immunohistochemical images show NMJ denervation in SOD1 rats and a partial rescue of NMJ after applications of hMSC to SC + M (NAR visualized by staining with α-BTX, red; and Syn green). Scale bar: 20 μm. Differences in the groups were analyzed by one-way analysis of variance: statistical significance at *, $p = .05$; **, $p < .01$; ***, $p < .001$ (compared with the PBS control) and $^{\#}$, $p = .05$; $^{\#\#}$, $p < .01$. The detailed statistical analysis of Western blot analysis between the groups is presented in Supporting Information Table S4.

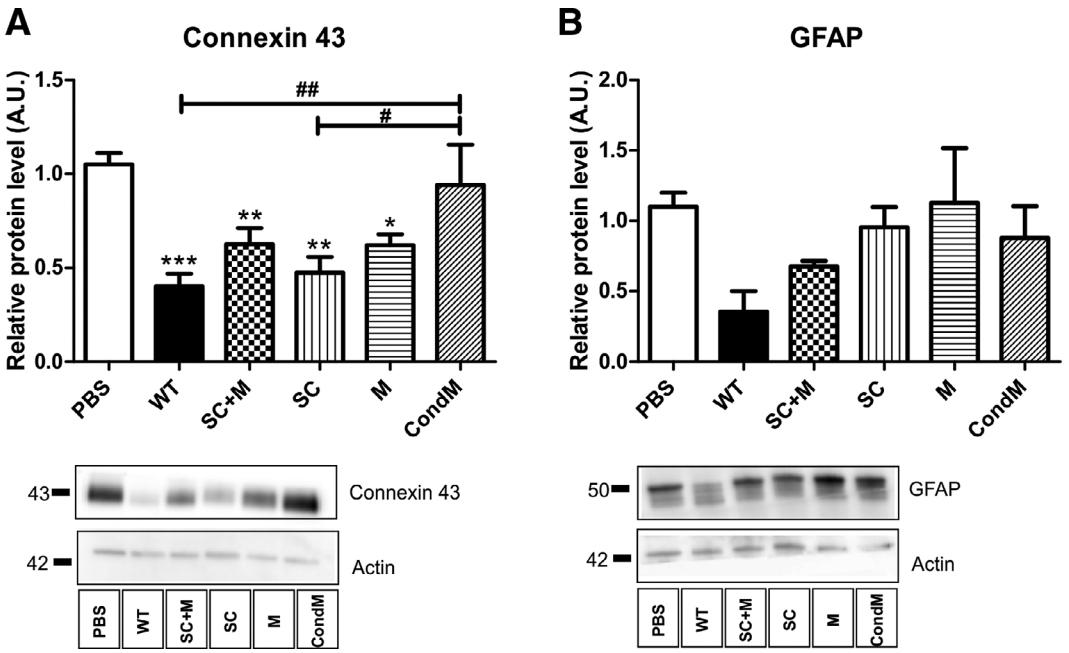

Figure 7. Representative immunoblots and quantitative analysis of astrocytic phenotype. The Western blot analysis showed an increase in the expression of Connexin 43 **(A)** and GFAP **(B)** in the spinal cord of SOD1 rats when compared with WT animals. Treatment with human mesenchymal stem cells (hMSC) to SC + M, spinal canal (SC) and M reduced the levels of Connexin 43 (A), whereas no difference was observed in conditioned medium (CondM) treated animals. The application of hMSC to SC + M decreased the levels of GFAP (B), whereas the application to SC, M, and CondM did not show any effect. Actin was used as a loading control. Differences in the groups were analyzed by one-way analysis of variance: statistical significance at $^{*,\#}$, $p = .05$; $^{**,\#\#}$, $p < .01$, ***, $p < .001$ (compared with the phosphate-buffered saline control). The detailed statistical analysis of Western blot analysis; the groups are presented in Supporting Information Table S4.

The Repeated Combined Application of hMSC Reduced the Expression of GFAP and Connexin 43

Provided that activated glial cells may directly affect the survival and/or activation of necroptosis of diseased neurons in ALS, we analyzed the phenotype of astrocytes in all experimental groups. Western blots revealed a significant upregulation of Connexin 43 in SOD1 animals, when compared with WT, which was strongly suppressed in all cell-treated animals (SC, SC + M, M, group; Fig. 7A). Rats treated with CondM had similar levels as SOD1 controls. Similarly, GFAP was upregulated in SOD1-PBS treated animals, when compared with WT, and this upregulation was reduced in SC + M group. These changes were, however, nonsignificant (Fig. 7B).

Discussion

We have studied the effect of hMSC isolated from bone marrow on disease progression in different settings in a SOD1^{G93A} rat model of ALS. We compared three injections of cells or their CondM applied at intervals of 14 days into SC, or cells injected into M, or a combination of both applications—SC + M. We used lumbar puncture as a route of delivery. This is the most common way of how ALS patients are treated with different types of stem cells [14–16, 29]. In our previous study, we reported a prolonged lifespan and an improved neurological outcome in the SOD1 rats treated with hMSC in one single injection into cisterna magna at the onset of the disease. The animals survived on average 13.5 days longer than the PBS treated rats and no grafted cells were found 2 weeks after the application [28]. The lifespan extension observed in our study was 18 and 19 days respectively for SC + M and SC groups. This finding supports the hypothesis that the cell mediated effect is transient and repeated application can prolong it. Our study confirmed that in the SOD1 animals the grafted cells only survived 2 weeks after application, whereas in the WT animals, the cells survived for at least 4 weeks. An even shorter survival was seen in muscles, where only a few grafted cells were detectable just 1 week after grafting, despite the fact that animals were immunosuppressed. The low survival rate was also confirmed by other studies [11, 30].

Although the majority of studies report on intraspinal or intrathecal injections, some studies showed interest in intramuscular injections, since the retraction of dying MN hampers the NMJ [31]. In our study, the combined application into the SC, together with intramuscular injection, showed the best effect in several parameters. In contrast, hMSC only injected into the *quadriceps femoris*, did not outperform any other cell-treated group, except for the rotarod test, where the decline in performance was observed 1 week later than in the other cell-treated groups (SC or SC + M). A higher lifespan of the SOD1 rats treated with hMSC into SC, SC + M or M was accompanied with less apoptosis detected in ventral horns and a higher number of MN. However, this neuroprotective effect was only observed when the cells were applied into the SC and was reinforced by simultaneous muscle injections. Muscle injections alone did not lead to a significant neuroprotective effect.

Since implanted cells do not survive for long, it is believed that the benefits of MSC therapy could be due to the vast array of bioactive factors they produce, which play an important role in the regulation of key biologic processes. Indeed, it was shown that MSC secretome is a potent modulator of neuronal and glial survival and differentiation, in both in vitro and in vivo environments [32]. Therefore, secretome derivatives,

such as conditioned media or exosomes, may present considerable advantages over cells for manufacturing, storage, handling, product shelf life, and their potential as a ready-to-go biologic product. We prepared CondM from the same batch of cells used for transplantation and applied it in three injections via lumbar puncture into the SC in the same experimental design, as hMSC. CondM treatment delayed the deterioration of motor functions, however, only a trend in prolonged lifespan was detected. A proteomic analysis confirmed the presence of growth factors BDNF, bNGF, VEGF A, and HGF. However, to obtain a similar effect as after cells transplantation, further optimization would be necessary, such as enrichment, culturing cells in hypoxic conditions, cell stimulation and/or the isolation of extracellular vesicles.

The pro-inflammatory factor NF-κB mediates the inflammation reaction via TNF-α and other inflammatory cytokines [33]. Its activation determines the pro-inflammatory phenotype of microglial cells in ALS [34], whereas its inhibition can slow down disease progression [35]. The levels of the mRNA NF-κB and TNF-α gene were reduced in the SC + M treated SOD1 rats. Our results are in agreement with previously published studies, that show MSCs can reduce the expression of COX-2 and NOX-2 and prevent microglia activation [11, 36, 37].

As ALS is characterized by progressive MN cell death, it is evident that research has focused on the different mechanisms of programmed cell death. The results are, however, often contradictory. The intrinsic apoptosis pathway is regulated by the Bcl-2 protein family [38]. After cytotoxic stimuli the antiapoptotic Blc-2, which inhibits dimerization of Bax and Bak protein, is silenced and the interaction between Bax/Bak dimer enables permeabilization of the mitochondrial membrane and the release of cytochrome c (cyt c) into cytoplasm. Cyt c activates casp-9 and through a cascade of caspases the apoptosis is executed by active casp-3. The extrinsic apoptosis pathway is dependent on activated casp-8, which then activates the caspase cascade [39]. Several studies proposed the involvement of apoptosis in ALS MN death. The presence of caspase and Bcl-2 aggregates in the SOD1 animals and rescued MN by different caspase inhibitors, suggest that apoptosis might be an underlying mechanism of MN cell death at least in the SOD1 models of ALS [20, 25, 40]. The over-expression of Blc-2 delays the onset of the disease in the SOD mice, but does not change the time from onset to death [41]. In contrast, the deletion of Bax and Bak protein prevented axonal degeneration and extended survival in the ALS mice [42]. The inhibitor of caspase 9 slowed the disease progression without delaying its onset [43]. In our study, we only observed the trend in mRNA downregulation of Casp3, Casp9, and Bak. However, on the protein level, a significant reduction of cl-casp-9 was detected in all the animals treated into the SC (SC + M, SC, and CondM group), confirming the involvement of casp-9 in ALS and its attenuation by a combined cell application. Bak/Bcl-2 ratio >1 indicate the induction of apoptosis in SOD1 animals. Only animals treated with a combined cell application had Bak/Bcl-2 ratio close to 1, supporting the hypothesis concerning the suppression effect of combined cell treatment on apoptosis induction.

Autophagy is an important cellular homeostatic pathway responsible for the clearance of misfolded or aggregated proteins. The inhibition of autophagy in MN of the SOD1 mice leads to the acceleration of the disease onset [44], and in contrast, autophagy inhibition in symptomatic animals resulted in the suppression of glial activation and prolonged survival [45]. Its role in ALS might therefore be positive at the earlier stage, however, at later stages the effect could be negative. The downregulation of beclin-1 corresponds to the reduced autophagy, whereas the increased levels of LC3BII correlated with the increased autophagosomes formation [46]. We observed reduced levels of beclin-1 in the SC + M group, when compared with the PBS-treated SOD1 rats, as well as a low level of LC3BII/LC3BI ratio, suggesting that the application of hMSC reduced autophagy in symptomatic rats. On the contrary, relatively high levels of beclin-1 and LC3BII/LC3BI ratio in the M and CondM treated rats may correspond with active autophagy, which might be detrimental for the animals. Immunostaining for p62 revealed protein accumulation in Chat positive cells of SOD1-PBS treated animals and we also found p62+ dots in hMSC treated animals. Protein levels of p62 determined by WB were not significantly increased in SOD1 animals which implicates that autophagy is not defective, even in the untreated SOD1 animals.

Recently necroptosis has been implicated in ALS as a primary mechanism driving MN cell death. In contrast to apoptosis or autophagy, necroptosis is characterized by swollen organelles, a disrupted plasma membrane and a lack of nuclear fragmentation [47]. The activation of RIP1 and RIP3 kinase, leads to necrosome formation and the subsequent phosphorylation of the pro-necroptotic MLKL protein. Necroptosis is initiated by phosphorylated MLKL which translocates into a plasma membrane. However, caspase-8 is able to disrupt the necrosome by cleaving RIP1 and RIP3, thereby effectively terminating necroptosis [48]. Thus, the low levels of casp-8 combined with sufficient levels of RIP3 and MLKL enables necroptosis execution. The analysis of the RNA levels of necroptosis genes (RIPK1, MLKL, casp-8) only revealed a trend in the downregulation in the SC + M treated SOD1 rats when compared with the PBS treated SOD1 animals, and these levels were close to the WT animals. In contrast, on the protein level, in the SC + M treated SOD1 rats, significantly lower levels of not only cl-casp8, but also RIP1 were detected, implying the suppression of necroptosis driven cell death. The SOD1 rats treated with CondM showed significantly lower levels of cleaved casp-8 than the PBS treated SOD1 controls and higher levels of RIP1 and MLKL when compared with the SC + M treatments (although these changes were not significant). We speculate that necroptosis can be driven by the HGF present in injected CondM (approximately 3,000 pg/ml) applied to the SOD1 rats. It was shown that HGF can reduce the levels of active caspase 8 and promote necroptosis in H9c2 cells under hypoxic conditions [49].

Degenerating MN in the ALS SOD1 model retract their axons from the NMJ and this axonal degeneration is followed by the loss of the motor neuron cell bodies in the spinal cord. Since neuromuscular degeneration precedes the onset of clinical symptoms and MNs death [50], some studies have focused on NMJ protection and reduction of MN degeneration by retrograde neurotrophism through axonal projections. In a mice model of ALS injections of mouse bone marrow MSC into *quadriceps femoris* resulted in a longer lifespan, improved motor function and slower MN degeneration, most likely due to the increased bioavailability of the neurotrophic factors, GDNF and neurotrophin 4 (NT4) in the skeletal muscle [51]. In contrast to our study, the grafted cells survived for 5 weeks and were not xenografts. Our WB analysis of

quadriceps femoris muscle, together with immunostaining for NMJ, revealed an increased amount of syn and partially rescued NMJ in the animals treated with hMSC into SC + M. Surprisingly hMSC only injected into the muscle did not show any effect on NMJ. One of the reasons might be the only 1 week of hMSC survival in *quadriceps femoris* of the SOD1 rats or the fact that the cells were grafted into symptomatic animals and the neuromuscular degeneration process had already started. If the process of neurodegeneration is taken as two subsequent events, then it is rational that the best results were obtained by the combined application of SC + M.

It is possible that the hMSC treatment may significantly affect not only MNs, but also astrocytes, which play a key role in maintaining the brain environment, participating in metabolic support, ionic balance, blood–brain barrier maintenance, and immune modulation [52]. Under disease or injury conditions, astrocytes change their morphology and properties and become "reactive astrocytes" [53]. Reactive astrocytes can be recognized by their enhanced expression of glial fibrillary acidic protein (GFAP) and their role switches from beneficial to detrimental [54]. We observed an increased expression of GFAP in SOD1 animals, confirming their reactive phenotype. hMSC combined application (SC + M) partly reduced astrogliosis in SOD1 animals. Astrocytes are interconnected through a gap of junction proteins—Connexins. Connexin 43 conducts crucial homeostatic functions in the CNS. However, in ALS, Connexin expression and functions are altered. A progressive increase in Cx43 expression in the SOD1 (G93A) mouse model of ALS during the disease course was reported. An elevated level of Connexin 43 induced toxicity to MN, partly through elevated intracellular calcium [55]. The repeated application of hMSC significantly reduced the levels of Connexin 43, suggesting that the neuroprotective effect of cell therapy can also be mediated via nonneuronal cells. To fully unravel the mechanisms of the cell therapy effect, further investigations of astrocytes and microglia and their role in ALS would be necessary.

Conclusion

Our study provides the new evidence that the combination of repeated intrathecal and intramuscular application with hMSC protected MNs and NMJ not only through reduction of apoptosis and autophagy, but also by the suppression of a necroptosis cell death pathway. This leads to the prolonged lifespan and improved motor activity of the SOD1 rats. The repeated application of naïve CondM without any further manipulation or repeated intramuscular injection of hMSC into *quadriceps femoris*, resulted in a limited improvement in motor activity and survival which was not supported by changes on a cellular and molecular level.

Acknowledgments

This work was supported by the grant GACR 15-06958S from Czech Science Foundation and by the Center of Reconstruction Neuroscience—NEURORECON (CZ.02.1.01/0.0/0.0/15_003/0000419). Some results were obtained due to "Advanced Bioimaging of Living Tissues" project, reg. no. CZ.2.16/3.1.00/21527, which was financed from the budget of the European Regional Development Fund and public budgets of the Czech Republic through the Operational Programme Prague—Competitiveness.

Author Contributions

M.R.: collection and/or assembly of data, data analysis and interpretation, manuscript writing; I. Vargová: collection and/or assembly of data; S.F.: conception and design; I. Vacková: data analysis and interpretation manuscript writing; K.T.: collection and/or assembly of data, manuscript writing; H.K.S: data analysis and interpretation, manuscript writing; P.V.: data analysis and interpretation, final approval of manuscript; E.S.: conception and design, final approval of manuscript; S.K.: conception and design, financial support; P.J.: conception and design, data analysis and interpretation, financial support, manuscript writing, final approval of manuscript.

Disclosure of Potential Conflicts of Interest

The authors indicated no potential conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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