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Amelioration of Amyotrophic Lateral Sclerosis in SOD1^{G93A} Mice by M_2 Microglia from Transplanted Marrow

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Abstract. *Background/Aim: The cause of fatal neuromuscular amyotrophic lateral sclerosis (ALS) is not known. Materials and Methods: Ninety-day-old superoxide-dismutase-1^{G93A} (SOD1^{G93A}) mice demonstrating level 1 paralysis, received 9.0 Gy total body irradiation (TBI) from a cesium source at 340 cGy per minute, and intravenous transplantation with 1×10^6 C57BL/6 green fluorescent protein (GFP)+ donor bone marrow cells. Results: Paralysis-free survival was prolonged in TBI and bone marrow-transplanted SOD1^{G93A} mice from 100 to over 250 days (p=0.0018). Other mice transplanted with SOD1^{G93A} marrow or marrow treated with the free-radical scavenger MMS350 showed no therapeutic effect. GFP+ macrophage-2 (M_2) microglial cells of bone marrow origin, were seen at sites of degenerating anterior horn motor neurons. SOD1^{G93A} mice had a disruption in the blood–brain barrier permeability which was reversed by marrow transplant from C57BL/6 mice. SOD1^{G93A} marrow showed unexpected robust hematopoiesis in vitro, and radioresistance. Conclusion: After TBI, M_2 microglial cells from transplanted donor marrow extended the paralysis-free interval in SOD1^{G93A} mice.*

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Amyotrophic lateral sclerosis (ALS) is a fatal neuromuscular disease caused by progressive neurodegeneration of the motor neurons in the brain and spinal cord (1, 2). While the cause of ALS is unknown, 10-15% of patients have a familial history of disease (3-7), while 85-90% develop the disease sporadically. There are nearly 40 genetic mutations associated with ALS, the second most common of which is a mutation in the superoxide dismutase-1 (*SOD1*) gene (3-30). The SOD1^{G93A} mouse model phenotypically recapitulates ALS in human patients and exhibits progressive muscle wasting, paralysis, and death (18-25). Existing therapeutic interventions only modestly extend the lifespan of patients with ALS. Recently, approved antioxidant therapies are believed to slow disease progression. Experimental therapeutic strategies, including administration of antioxidant drugs, biological response modifiers, cytokine inhibitors, cytokine receptor antagonists, and introduction into the spinal cord of neurotrophic growth factors or mesenchymal stem cells engineered to produce neuroleukines (31-46), have met with incomplete success (2, 32). Marrow transplant strategies, which were designed to target specific defects (47-56), have been either ineffective or of limited success in ALS (51, 52).

The histopathology of ALS-affected spinal cord demonstrates gliosis, including the accumulation of both inflammatory macrophage-1 (M1) and neurotrophic factor-producing (M_2) microglia at the site of neurodegeneration (8). However, the pathophysiology of homing of these cells to the site of motor neurons, and their mechanism of action is not known (8-13, 15, 19-22). Furthermore, the origin of microglial cells in the thoracolumbar spinal cord, where ALS typically begins the process of ascending muscle functional loss is not known. A summary of recent research suggests that microglial cell accumulation at the site of degenerating

neurons represents multiple phases in the degenerative process, one inflammatory potentially initiating the disease process (28), and a second response to degenerating neurons with a production of repair-related cytokines (9, 10). There remains controversy over the interaction of glial cells with motor neurons in the development of paralysis (57, 58).

In the present studies, we tested the hypothesis whether cells of donor bone marrow origin might provide a therapeutic effect if delivered to $SOD1^{G93A}$ mice after total body irradiation (TBI).

Materials and Methods

Mice and animal care. $SOD1^{G93A}$ and control SOD1 transgenic mice containing 4 copies of normal human SOD1 gene were obtained from the Jackson Laboratories, Bar Harbor, Maine. $SOD1^{G93A}$-green fluorescent protein (GFP^+) mice were bred according to published methods used to breed SMAD family member 3 (*Smad3*)$^{-/-}$ Fanconi anemia group D2 (*Fancd2*)$^{-/-}$ mice (59). Mice were derived from breeding pairs of $SOD1^{G93A}$ and control mice. Female mice were housed at five mice per cage and male mice four per cage according to institutional regulations. Mice were fed standard Purina laboratory chow. All work was performed under protocol 18022000, approved by the University of Pittsburgh Institutional Care and Use Committee. Veterinary care was provided by the Division of Laboratory Animal Resources at the University of Pittsburgh.

Paralysis was scored as described by Hatzipetros *et al*. (26). Mice were euthanized when reaching paralysis with neurological score (NS) of 3. At NS 3, the mice still have complete use of their front legs but show paralysis in the rear leg. Mice with NS 1 have no paralysis but exhibit some trembling in the rear legs and a collapsing of the rear legs toward the lateral mid line when picked up. NS 2 paralysis reflects the beginning of paralysis in the rear legs with a complete collapse of the rear legs to the lateral midline when picked up; mice begin to show an altered gait but are still able to move easily around in the cage.

Mice were given no irradiation, 9 Gy, or 7.0 Gy TBI using a Shephard Mark I ^{137}Cs γ-ray source (J. L. Shepherd, San Fernando, CA, USA), according to published methods (59). Other mice received 9 Gy cranial spinal irradiation. Subgroups were given 10^6 cells from B6 GFP+ or wild-type B6 mouse marrow intravenously after fractionated TBI, as described elsewhere (60).

TBI and craniospinal irradiation. Female and male mice received 9.0 Gy TBI from a cesium source at 340 cGy/min using a Shephard Mark 1 irradiator (J.L. Shepherd, San Fernando, Ca, USA). Bone marrow was isolated from C57BL/6 GFP^+ mice (Jackson Laboratories, Bar Harbor, ME, USA). The irradiated $SOD1^{G93A}$ mice were injected intravenously with 1×10^6 C57BL/6 GFP^+ donor bone marrow cells. Chimerism was determined from the presence of GFP^+ cells in peripheral blood according to (60). The mice were followed for the development of NS 3 paralysis at which time they were sacrificed and the spinal cord was isolated and fixed in 2% paraformaldehyde (PFA). Some of the spinal cords were analyzed by single photon, confocal, ribbon-scanning microscopy for quantitation of bone marrow origin cells. Other spinal cords were fixed in 2% PFA, sectioned, and stained for M_1 and M_2 microglial cells.

Control C57BL/6NTac mice were transplanted at the same age with $SOD1^{G93A}$ ALS bone marrow under the same conditions and followed for development of paralysis. To determine if spinal cord irradiation alone without bone marrow transplantation altered the development of paralysis, $SOD1^{G93A}$ mice were irradiated with 9.0 Gy to the spinal cord and brain while shielding the remainder of the body. All mice were followed for the development of paralysis. In a separate group of $SOD1^{G93A}$ mice, marrow transplant using B6 GFP+ or $SOD1^{G93A}$ GFP^+ 1×10^6 bone marrow cells was performed at 24 h after TBI.

To determine if TBI alone delay paralysis, $SOD1^{G93A}$ mice were irradiated to 7.0 Gy TBI without bone marrow transplantation. Mice were followed for development of paralysis.

Treatment with MMS350. The water-soluble radiation mitigator MMS350 was produced in the laboratory of Dr. Peter Wpif at the University of Pittsburgh, Pittsburgh, PA, USA and was administered at 400 mg/ml to mice in drinking water as described (61, 62) over days 60 until death from paralysis.

Continuous bone marrow culture. Long-term bone marrow cultures (LTBMCs) were established from the femur and tibia marrow of $SOD1^{G93A}$ or control transgenic mice, as described previously (63). The contents of a femur and tibia (N=6/genotype) were flushed into McCoy's 5A medium (Gibco, Gaithersburg, MD, USA) supplemented with 25% horse serum (Cambrex, Rockland, ME, USA) and 10^{-5} M hydrocortisone sodium hemisuccinate. Cultures were incubated at 33˚C in 7% CO_2. After 4 weeks, horse serum was replaced with 25% fetal bovine serum (FBS) (Gibco, Gaithersburg, MD, USA) (63). The cultures were observed weekly for hematopoietic cell production and cobblestone island formation. The number of cobblestone islands of 50 cells or more were scored weekly in each flask (63-64). A two-sided two-sample *t*-test was used to compare the number of cobblestone islands between culture groups each week. *p*-Values less than 0.05 were regarded as significant.

The cultures were observed weekly for hematopoietic cell production and adherent cell layer confluence. Non-adherent cell production data were expressed as the mean±standard error standard error of the mean (SEM) of weekly non-adherent cell number and cumulative non-adherent cell production. Confluence data were expressed as the mean±SEM of the percentage adherent cell layer confluence.

Establishment of clonal bone marrow stromal cell lines. Adherent cell layers from one 4-week-old LTBMC from $SOD1^{G93A}$ (63-64) and control mice were trypsinized and expanded by passage into Dulbecco's Modified Eagle's Medium (DMEM) with10% FBS to establish bone marrow stromal cell lines according to published methods (63). Cells were passaged for 10 weeks to establish cell lines. Culture were incubated at 37˚C in 5% CO_2.

Hematopoietic colony-forming cell lines and fresh marrow colony assays. Each week after explant and establishment of LTBMCs, 1×10^5 nonadherent cells were plated in triplicate in methylcellulose-containing medium, as previously published (63). Cells were incubated at 37˚C in 5% CO_2. At day 7 after plating, colonies of ≥50 cells were counted. The plates scored at day 7 were returned to the incubator for scoring at day 14. Data for days 7 and 14 are presented as the mean±SEM of weekly colony-forming cells and cumulative colony-forming cells (63).

Table I. *Source of antibodies and target cell identifiers*.

Primary antibody target	Known functions of target
Glial fibrillary acidic protein (Novus Biologics, NB300-141)	Intermediate filament expressed by numerous cell types in the central nervous system including astrocytes and ependymal cells.
Allograft inflammatory factor 1 (Abcam, ab5076)	Participates in membrane ruffling and phagocytosis in activated microglia.
Neurofilament heavy polypeptide (Abcam, ab8135)	Provides structural support to axons and regulate axon diameter.
Neurotracer (ThermoFisher, N21482)	A fluorescent tracer that adheres to neurons
Oligo2 (Novus Biologics, NBP1-28667)	Oligodendrocyte transcription factor found in motor neurons and oligodendrocytes
Platelet endothelial cell adhesion molecule (Abcam, ab134168)	Involved in leukocyte transmigration, angiogenesis and integrin activation.

Establishment of bone marrow stromal cell lines. Adherent cell layers from one LTBMC per treatment group were trypsinized and expanded by passage into DMEM supplemented with 10% FBS to establish bone marrow stromal cell lines according to published methods (63). Established criteria were used for authentication of cell lines (63). Genotyping of all bone marrow stromal cell lines established they were indeed from the $SOD1^{G93A}$ or control genotype. The established lines maintained stromal cell features including capacity for osteoblast differentiation, and support of hematopoietic cells in co-culture. We documented cell surface phenotype, biology, and absence of capacity to differentiate into hematopoietic cells. These cell lines were characterized as bone marrow stromal cells or mesenchymal stem cells (63). Cell lines were incubated at 37˚C in 5% CO_2 and passaged for 10 weeks to establish cell lines.

Establishment of single-cell-derived clonal marrow stromal cell lines. Single-cell cloning experiments were performed using fresh marrow, an adherent cell layer from a LTBMC at week 13 after explant, and bone marrow stromal cell lines that were established as described (63). Flow cytometry was used to sort single cells into the wells of a 96-well plate. Cells were grown in DMEM supplemented with 20% FBS, 1% antibiotic-anti-mitotic solution, and 1% L-glutamine. Plates were observed weekly for growth. Clonal lines were established from wells that showed growth after single-cell plating.

Histochemistry and ribbon scanning, confocal single-photon microscopy. The detailed methods for the microscopy techniques of the spinal cord have been published in the web-based textbook (65).

Microbead assay for blood–brain barrier permeability. Blood–brain barrier permeability was analyzed by intravenous perfusion with 0.2 μm Fluorospheres (F8810; Invitrogen) on day 125 in control $SOD1^{G93A}$ mice, $SOD1^{G93A}$ mice transplanted on day 90 with GFP+ C57BL6 bone marrow and C57BL/6 mice.

Immunofluorescence assay for detection of astrocytes, neurons, glial cells, and immunocytes. Spinal cord was removed from control C57BL/6 mice, and $SOD1^{G93A}$ mice with phase1, phase 2 and phase 3 paralysis. The spinal cords were fixed in 2% paraformaldehyde (PFA; Thermo Fisher Scientific, Waltham, MA, USA), sectioned, and stained with antibodies to astrocytes, neurons, glial cells and immunocytes. The antibodies used, their vendor/supplier and cellular target function are shown in Table I. Secondary antibodies used include goat anti-rabbit and donkey anti-goat (Thermo Fisher Scientific, Waltham, MA, USA). The antibodies specific for astrocytes, motor neurons, M_1 and M_2 microglial cells, endothelial cells, and hematopoietic cells have been described elsewhere (65). Cells were incubated with primary antibody mixture overnight at 4˚C then washed with PBS three times for 5 min each. Secondary antibody solution was prepared at manufacturer-suggested dilutions in PBS and added to cells for 1 hour at room temperature. After washing with PBS three times for 5 min each, 0.5 μg/ml 4',6-diamine-2'-phenylindole dihydrochloride (DAPI; Millipore Sigma, St. Louis, MO, USA) in PBS was added for 10-20 min at room temperature to label nuclei. Cells were mounted and allowed to dry for 30 min. Pictures were taken using fluorescence microscopes at the University of Pittsburgh Center for Biologic Imaging. For each sample, three fields were subjected to analysis. Data are presented as the mean±standard error of the percentage of cells staining positively for the target protein.

Statistical analysis. For *in vivo* experiments, the mean and standard error of the mean for each group was determined and graphed using GraphPad Prism (GraphPad Software, LaJolla, CA, USA) to compare experimental groups. Student's unpaired *t*-test was used to compare data of each group at different time points to those for the control group, as well as to compare all days for each experimental group (including the control) to day 0 (non-irradiated baseline) of the control group.

For LTBMCs, GraphPad Prism (GraphPad Software) was used for statistical analysis and plotting graphs. Graphs were plotted using the mean±SEM. The data were analyzed using two-tailed Student's *t*-test. In this exploratory experiment, *p*-values were not corrected for multiple comparisons. Differences were considered significant at $p<0.05$ (27). At each week of LTBMC for each of the endpoints (*i.e.* weekly non-adherent cells per flask, percent confluence, day-7 colonies, and day-14 colonies), data were summarized with mean±standard deviation. Comparisons were made using the one-way analysis of variation (ANOVA) F-test at each time point, followed by Tukey's multiple comparisons.

The description of calculation of D_0, which is the irradiation dose required to reduce survival to 37% on the linear portion of the survival curve, and ñ, which represents the shoulder on the survival curve as calculated by the back extrapolation of the linear portion of the survival curve to the y axis, for radiation survival curves has been published in detail elsewhere (63).

Results

Establishment of bone marrow chimeric GFP+ $SOD1^{G93A}$ mice. After marrow transplant, as described in the Materials

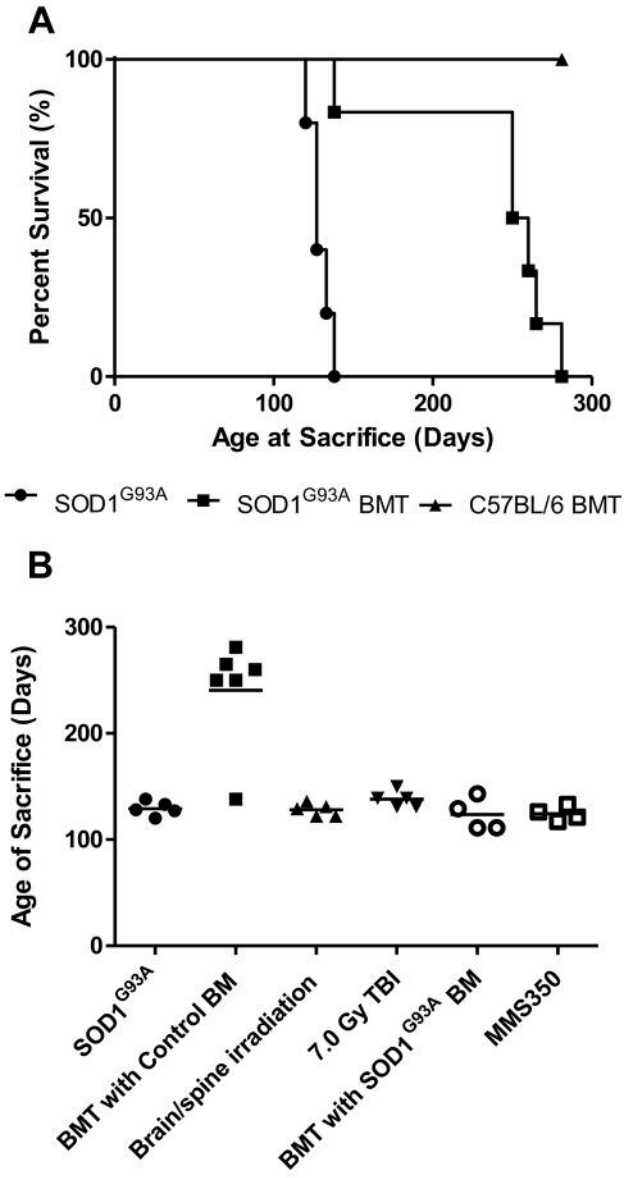

Figure 1. *Effect of total body irradiation (TBI) and bone marrow transplantation (BMT) on paralysis-free interval. Superoxide dismutase-1 (SOD1)^{G93A} mice at 90 days after birth received total body irradiation to 9 Gy. A: Mice were injected intravenously with 1×10^6 freshly removed tibia and femur bone marrow from C57BL/6-green fluorescent protein (GFP)+ mice and followed for development of stage 3 paralysis at which time the mice were sacrificed. Non-treated SOD1^{G93A} mice had an average survival of 129±3 days (n=5). In contrast, TBI-mice which received bone marrow transplant had a significant increase in mean survival to 233±23 days (p=0.0045) (n=6). Control wild-type C57BL/6 mice (n=10) were irradiated to 10 Gy and transplanted with bone marrow from SOD1^{G93A} mice and followed for development of paralysis. No paralysis, as suggested in several recent publications (21-24) was detected up to 280 days following transplantation (p=0.0018). B: Controls included mice which received 9.0 Gy brain and spine irradiation (n=5), sub-lethal TBI of 7.0 Gy alone (n=5), radiation mitigator MMS350 in drinking water (n=4), or transplant of SOD1^{G93A} marrow (n=4). The horizontal line for each group represents the average age at the time of sacrifice.*

and Methods, blood samples were checked for chimerism at day 60 and 90. Mice with over 80% donor origin GFP+ cells were considered to be successfully transplanted.

Quantitation of TBI and bone marrow transplantation delayed hind limb paralysis. TBI and marrow transplant significantly delayed paralysis and extended survival of SOD1^{G93A} mice (Figure 1A). TBI plus normal marrow transplant but not sub-TBI, reduced TBI dose, transplant of SOD1^{G93A} donor marrow nor administration of MMS350 prolonged the paralysis-free interval (p<0.0039) (Figure 1B).

The stages of paralysis are shown histologically in Figure 2. Bone marrow-derived M_2 microglia were found in the spinal cords of SOD1^{G93A} mice transplanted with GFP+ donor marrow at 120 days of age when other groups are developing stage 3 paralysis (Figure 3). GFP+ cells were seen throughout the spinal cord (Figure 4). The blood–brain barrier was permeable to microbeads in non-irradiated and non-transplanted SOD1^{G93A} mice (Figure 5) confirming findings of a prior publication (47). In contrast, TBI-treated, then GFP+ marrow-transplanted mice showed correction of the blood–brain barrier defect (Figure 5). Control wild-type mice transplanted with SOD1^{G93A} bone marrow demonstrated no paralysis (Figure 1A). There was no transfer of cells of SOD1^{G93A} GFP$^+$ donor bone marrow origin to the spinal cord of wild-type recipients. The blood–brain barrier was no longer disrupted in TBI-treated normal marrow-transplanted SOD1^{G93A} mice (Figure 5).

The results show that TBI and bone marrow transplantation significantly extended the survival of SOD1^{G93A} mice, and was a robust neuroprotective and translatable strategy to prevent motor neuron degeneration starting at the time of NS 1 paralysis in SOD1^{G93A} mice (Figure 1).

Analysis of subsets of cells of bone marrow origin in spinal cord of TBI-treated and bone marrow-transplanted SOD1^{G93A} mice. We identified microglia from GFP$^+$ marrow origin in the spinal cords of SOD1^{G93A} TBI/marrow-transplanted mice (Figures 3 and 4). We tested whether there were GFP$^+$ M_2 microglial cells in the anterior horns of the spinal cords at days 120, 200, and 280 in GFP$^+$ marrow-transplanted SOD1^{G93A} mice (Figure 3). The spinal cord showed significant infiltration with GFP+ cells of marrow origin at multiple levels (Figures 3 and 4). We did not see cells of marrow origin in the spinal cords of TBI-treated wild-type C57BL/6 mice that were transplanted with GFP+ SOD1^{G93A} marrow.

Demonstration of reversal of blood/brain barrier permeability in SOD1^{G93A} mice. SOD1^{G93A} mice demonstrated the blood–brain barrier was permeable to 2-μm diameter beads (Figure 5), confirming previous findings (47). In contrast, control C57BL/6 mice and C57BL/6 mice that

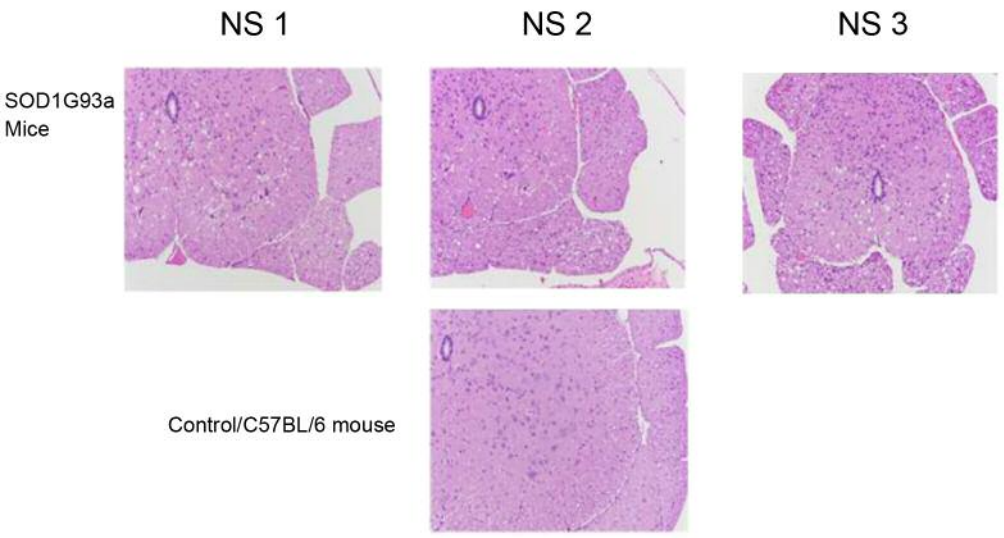

Figure 2. *Histopathological stages of spinal cord motor neuron loss in superoxide dismutase-1 (SOD1)^{G93A} mice (H&E ×20). With the advancement of each stage, there was increased degeneration and loss of motor neurons, astrogliosis in the areas of motor neuron loss, and vacuolation within the gray matter (n=3 at each stage). Lesions were more pronounced in the ventral horns (bar=100 μm).*

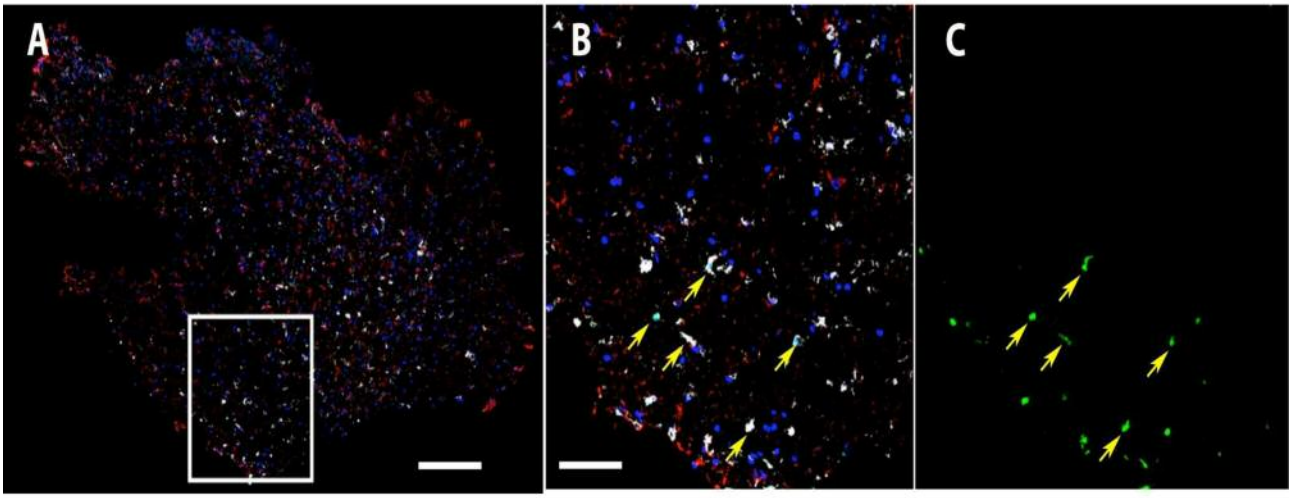

Figure 3. *Histochemical detection of M_2 microglial cells of bone marrow origin in the spinal cord of superoxide dismutase-1 (SOD1)^{G93A} mice. A: Cross section of spinal cord at recipient age 120 days which is 30 days post irradiation and transplantation of green fluorescent protein (GFP)+ bone marrow cells (n=3). Labelling is glial fibrillary acidic protein (GFAP) (red), nuclei (blue), ionized calcium-binding adapter molecule 1 (white), and stem cells (green). There is almost complete co-localization between the green cells of marrow origin and the white M_2 microglia (×100). B and C: Boxed inset region from A showing M_2 microglia (B) overlaying exactly with the GFP-labeled cells (C), highlighted with yellow arrows. Bar=500 μm (×500).*

had been transplanted with SOD1^{G93A} marrow showed no such detectable blood–brain barrier permeability (Figure 5).

Failure of isolated craniospinal irradiation and of sub-lethal TBI with no marrow transplant to prolong the paralysis-free interval in SOD1^{G93A} mice. As shown in Figure 1B, SOD1^{G93A} mice treated with 9.25 Gy to the brain and spinal cord only while shielding the rest of the body, and those receiving 7.0 Gy TBI without marrow transplant showed no therapeutic effect. Furthermore, SOD1^{G93A} mice treated with radiation mitigator MMS350 in drinking water starting at day 60 showed no therapeutic effect (Figure 1B).

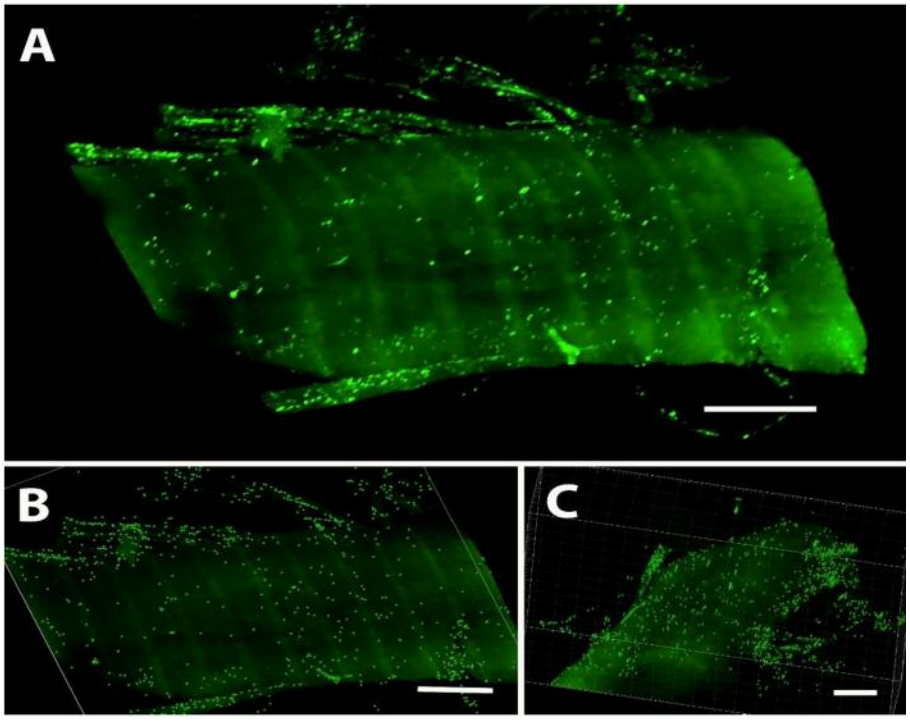

Figure 4. *Rendered three-dimensional image of superoxide dismutase-1 (SOD1)^{G93A} murine spinal cord following irradiation and transplantation of green fluorescent protein (GFP)+ bone marrow cells. Images were collected following CUBIC clearing and imaging using a Caliber XT ribbon scanning confocal microscope (25× 1.15 NA water objective, WD 2.4 mm) (n=3). A: En face reconstruction of the raw image. GFP+ cells can be seen throughout the tissue, but are more concentrated in the lumbar region in the lateral nerve branches. B: The GFP+ cells in the same image set in which the positive structures are highlighted as green spheres. C: The 3D nature of the reconstruction is clearly visible. Bar=1 cm. (Link to movie of Figure 4).*

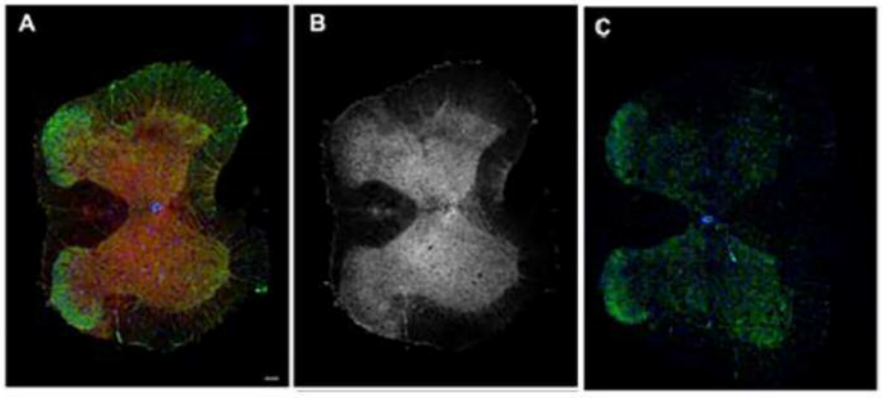

Figure 5. *The blood–brain barrier is permeable in superoxide dismutase-1 (SOD1)^{G93A} mice. A: Section though spinal cord of an ALS mouse (age 100 days) perfused with 0.2 μm Fluospheres (F8810, Invitrogen) shown in red (n=3). The actin cytoskeleton is green and blue defines the nuclei (bar=250 μm). Beads are present throughout the soma of the spinal cord (Panel B). However, in TBI/transplanted mouse at 30 days post transplantation (Panel C) no beads are present in the spinal cord.*

Hematopoiesis in LTBMCs from SOD1^{G93A} mice. We tested the effect of the SOD1^{G93A} genotype on limiting the duration of hematopoiesis in LTBMCs as a marker of defective antioxidant capacity according to prior publications (59, 61-64). Unexpectedly, SOD1^{G93A} mouse marrow had greater longevity than did that from SOD1 transgenic control mice. Stability of the adherent layer was equivalent in SOD1^{G93A} and SOD1 transgenic control mice (Figure 6A).

Table II. *Radioresistance of superoxide dismutase-1 (SOD1)G93A isolated bone marrow and bone marrow stromal cells.*

Mouse Strain	$SOD1^{G93A}$ bone marrow cells		$SOD1^{G93A}$ bone marrow stromal cells	
	Do (Gy)	n	Do (Gy)	n
SOD1 Transgenic	7.583±0.819	1.554±0.05989	8.564±1.564	1.319±0.0799
$SOD1^{G93A}$	4.791±0.5162	1.593±0.01132	3.803±0.3985	1.653±0.07226
p-Value	0.0488		0.0210	0.0228

Stem cell islands, cobblestones, were significantly greater in number (Figure 6B). There was a significantly greater production of total hematopoietic cells (Figure 6C), and greater duration of production of total cell forming day 7 (Figure 6D) and day 14 (Figure 6E) colony-forming progenitors (Supplemental Tables I-V may be found at https://pitt.box.com/s/ajzj6ov2cw9ttrbnspnfz3p7d7so7mob).

Radiation resistance of clonal bone marrow stromal cell lines from $SOD1^{G93A}$ mice. In prior studies, greater longevity of hematopoiesis in LTBMCs correlated with radioresistance of hematopoietic progenitors, suggesting a greater capacity of cells to tolerate oxidative stress (63). The data revealed that both $SOD1^{G93A}$ mouse isolated bone marrow CFU-GEM (Figure 7A, Table II) and marrow culture-derived stromal cell lines (Figure 7B, Table II) were radioresistant.

Number of motor neurons in thoracic and lumbar spinal cord of $SOD1^{G93A}$ mice is reduced compared to controls. The increased number of hematopoietic progenitor cells in LTBMCs and radioresistance of both fresh marrow colony-forming units-granulocyte, erythrocyte, monocyte, megakaryocyte and stromal cell lines from $SOD1^{G93A}$ mice suggested that the defective *SOD1* gene may not have reduced stem cell numbers in the marrow, but may have depleted the critical motor neurons in the spinal cord.

We counted motor neurons in spinal cords from mice of the $SOD1^{G93A}$ genotype in NS 0, 1, 2, 3. While the number of motor neurons was reduced at NS 1, 2, and 3 compared to stage 0, there was no clear direct stage-specific loss of motor neurons with progression of paralysis (Figure 8).

Discussion

In the present study with $SOD1^{G93A}$ ALS mice, we demonstrated that TBI and bone marrow transplantation during the symptomatic stage of the disease (day 90) exerted a profound therapeutic effect by delaying paralysis, and extending survival two-fold from 120 to over 250 days. This treatment regimen was significantly more effective than previous mesenchymal stem cell or bone marrow transplantation studies in $SOD1^{G93A}$ mice, and in other models of ALS (51-52). The present treatment paradigm more accurately recapitulates a therapeutic approach in patients with ALS since they often exhibit symptoms at the time of diagnosis. In other studies, from our laboratory using marrow transplant at day 60 before symptoms, we observed that the prolongation of survival was even better (data not shown). We found that $SOD1^{G93A}$ mice, but not transgenic mice with four copies of normal human *SOD1* displayed permeability of the blood–brain barrier, which was reversible by TBI and marrow transplant, and we identified bone marrow-derived M_2 microglia at the site of spinal motor neurons.

The present data suggest that the observed therapeutic effect of TBI and marrow transplant is mediated by M_2 microglia of marrow origin. In the present experiments, TBI and marrow transplant producing chimeric $SOD1^{G93A}$ mice resulted in M_2 microglial cells of bone marrow origin accumulating at the site of anterior horn cell degeneration. Mice receiving 9.0 Gy craniospinal irradiation or sub-lethal 7.0 Gy TBI irradiation alone did not show a therapeutic effect. Continuous antioxidant treatment with MMS350 (61,62) in drinking water also showed no beneficial effect.

Our data are the first to be reported of successful TBI and bone marrow transplantation in symptomatic $SOD1^{G93A}$ mice. Treatment did delay paralysis and more than doubled the lifespan. We determined that sub-lethal TBI, cranial spinal, or of the spinal cord only was not therapeutic. A marrow cell population facilitated accumulation of therapeutic M_2 microglial cells of marrow origin in the spinal cord at the site of motor neuron, but only after TBI. These studies are the first to demonstrate that ionizing irradiation in TBI is therapeutic for ALS.

Our novel $SOD1^{G93A}$ GFP+ mice and our unique single-photon, confocal ribbon-scanning microscopy allowed us to count all green motor neurons in the entire spinal cord. We have constructed a jig which allows non-anesthetized immobilized mice to be irradiated in specific areas of the spinal cord with precise radiation doses. In this way, less than 1% of the prescribed dose is delivered outside the irradiated field using our Stereotactic Radiosurgery Unit at our TrueBeam/Varian linear accelerator. The combination of

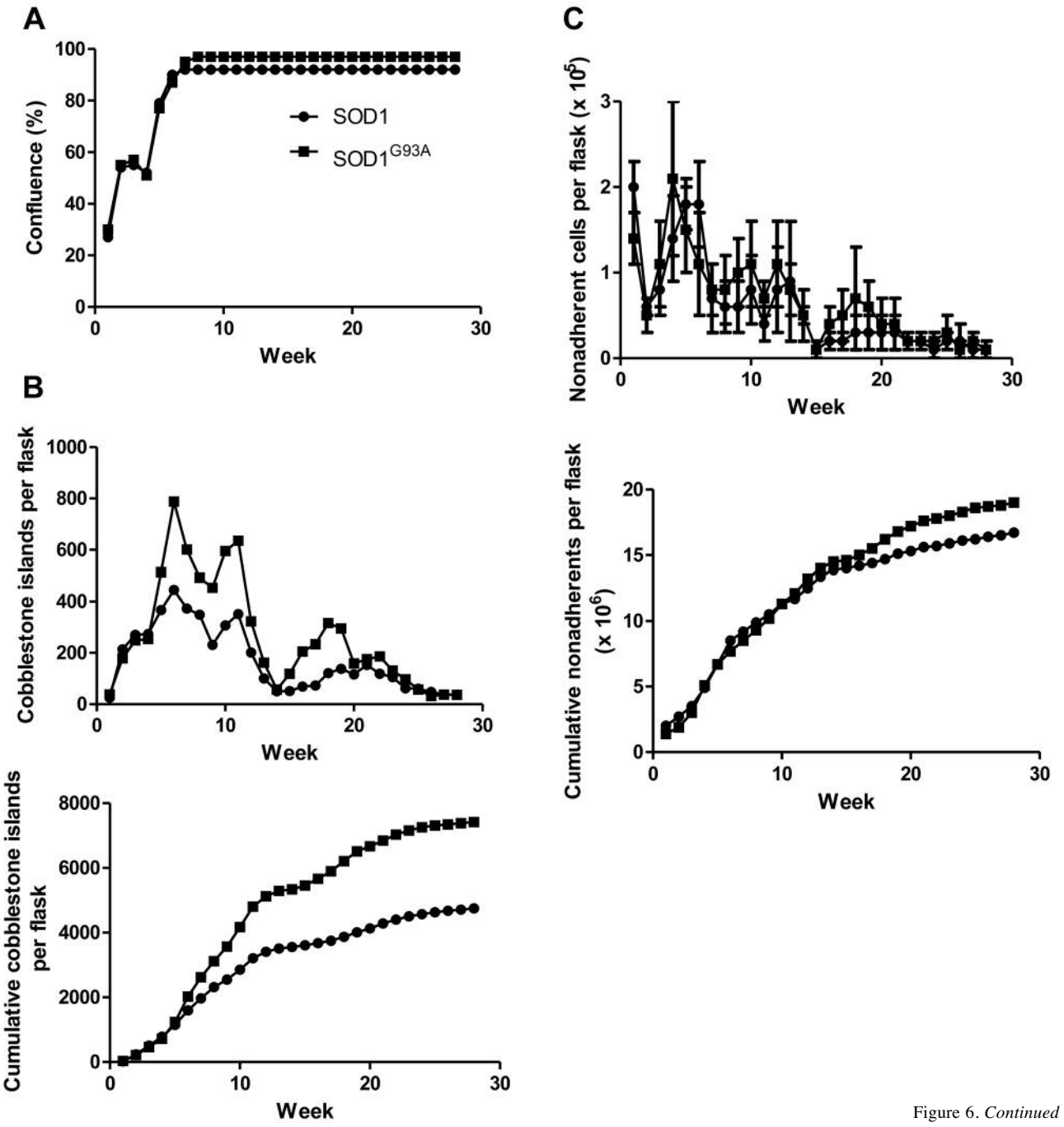

Figure 6. *Continued*

these novel techniques allowed us to quantitate the effect of each of the treatment modalities on preserving the number of intact and non-degenerating anterior horn motor neuron cells. Only TBI plus bone marrow transplantation facilitated the therapeutic effect in these $SOD1^{G93A}$ mice.

TBI for preparation of recipients for marrow transplant has been used for over 60 years in the treatment of not only cancer, but also autoimmune diseases and genetic disorders, including some that affect the central nervous system (49, 50). Use of bone marrow transplant in ALS has not been successful since results showed either no effect (51), or minimal effect (52). Irradiation effects on the spinal cord and the radiobiology of neurons have not been considered as a possible therapeutic modality for diseases of motor neurons in ALS (53). The role

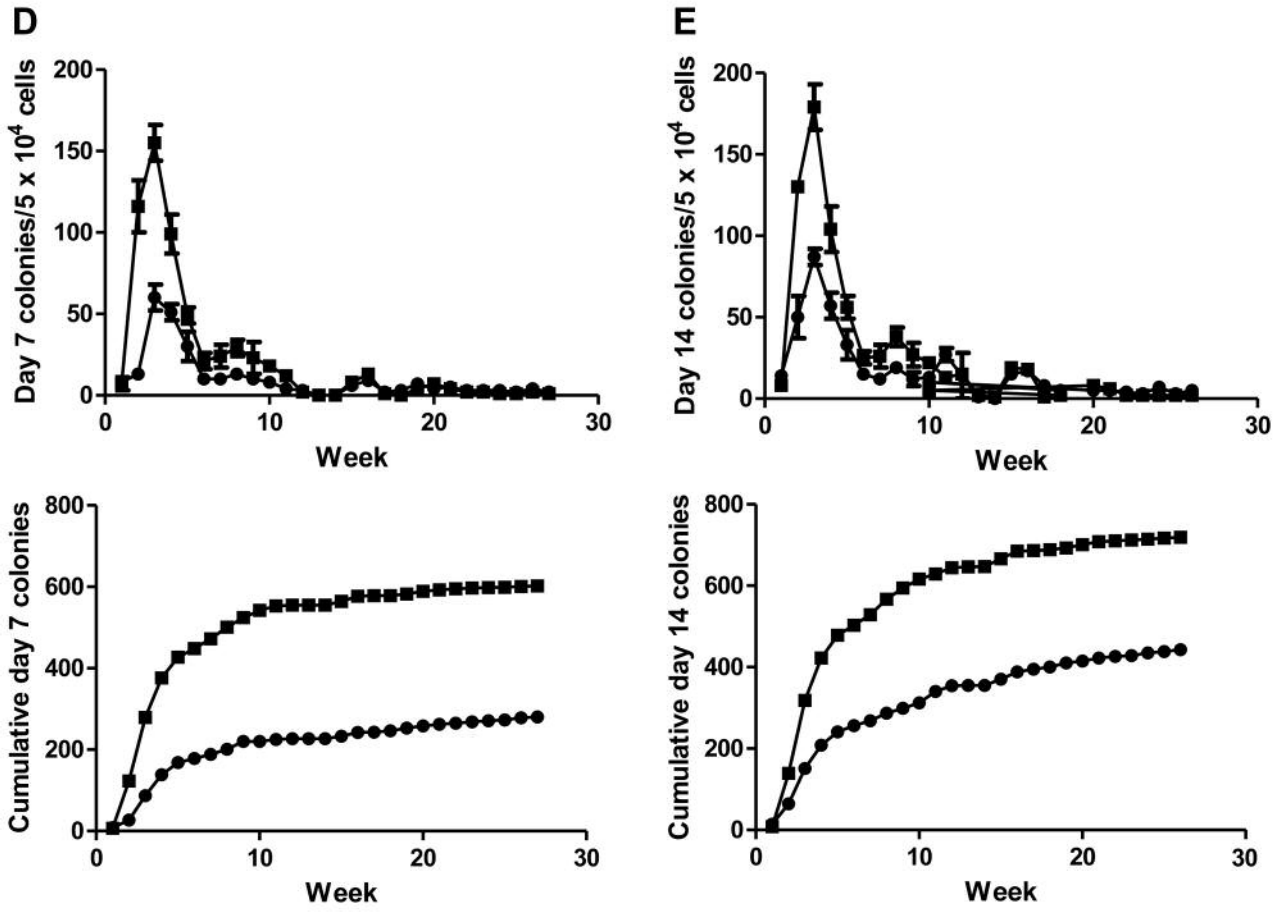

Figure 6. *Improved hematopoiesis in long-term bone marrow cultures from superoxide dismutase-1 (SOD1)^{G93A} mice compared to control SOD1 transgenic mice containing four copies of the normal human transgene (SOD1 transgene) (n=4). Absolute and cumulative data for surface area confluence (A), cobblestone islands per flask per week (B), non-adherent cells produced per flask per week (C), and colony-forming cells generated per flask per week at day 7 (D) and day 14 (E). All statistical analysis for the data is shown in Supplementary Tables I to V (https://pitt.box.com/s/ajzj6ov2cw9ttrbnspnfz3p7d7so7mob).*

of cells of marrow origin, both pro-inflammatory and anti-inflammatory, in the clinical evolution of ALS has not been mechanistically coupled with the role of irradiation relative to abnormalities in the blood–brain barrier in ALS, nor have the effects of transplanted marrow cells into patients with ALS been studied relative to prolongation of the paralysis-free interval (36, 41, 47, 54-58, 66-69).

Recent data with SOD1^{G93A} mice suggest that the inflammasome and significantly, interleukin 1 family cytokines, may mediate motor neuron cell death *via* induction of radical oxygen species pathways (70-78). How M_2 microglial from marrow might neutralize these interactions at the level of the microglial cell to motor neuron interface is unknown. Because 10% of ALS cases are familial, these SOD1^{G93A} mice may represent the most appropriate genetic model for future ALS studies.

The mechanism of cell death in ALS motor neurons is not known. Apoptosis, necroptosis, ferroptosis, parthanatos, and pyroptosis may be occurring. The role of M1 microglial cells in initiating motor neuron destruction and M_2 cells in ameliorating destruction is unknown. Use of TBI and bone marrow transplantation might be possibly in other neurodegenerative diseases including: multiple sclerosis, frontotemporal dementia, and the multiple etiologies of sporadic ALS such as that following trauma.

SOD1^{G93A} mice receiving bone marrow transplant at day 60 before onset of signs of paralysis fared much better, but translation of these data to the clinic is not practical since diagnosis of ALS is made after the onset of symptoms and weakness. Our mouse data do suggest that the course of degeneration might be further interrupted by earlier bone marrow transplantation. The mechanisms for blood–brain

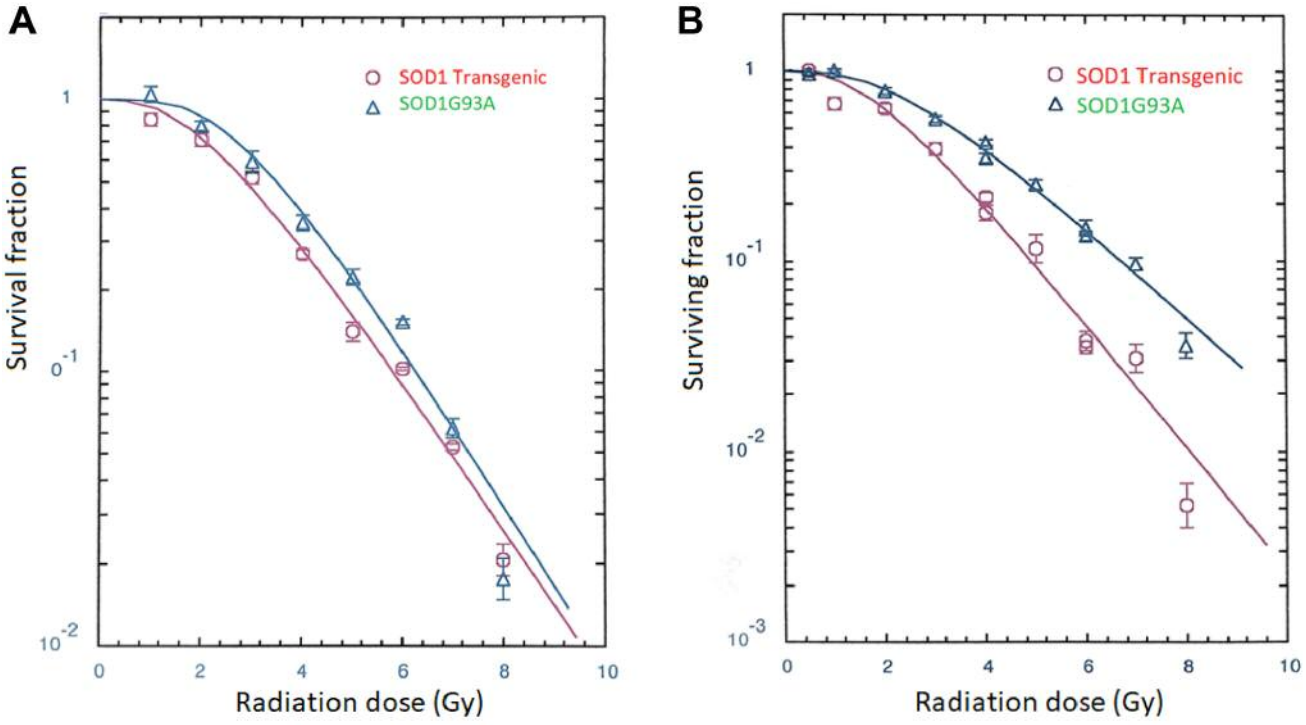

Figure 7. *Radiation resistance of fresh marrow hematopoietic progenitor cells from superoxide dismutase-1 (SOD1)^{G93A} mice (n=3) (A) and bone marrow stromal cell lines from SOD1^{G93A} and SOD1 transgenic (B6) mice (B). Radiation survival curves were carried out as described in the Materials and Method. The data are a composite of data from 3-6 experiments.*

barrier permeability in ALS, and its restoration by marrow transplant is at present not known.

The unexpected prolongation of LTBMC hematopoiesis and the radioresistance of cell lines and fresh marrow from SOD1^{G93A} mice appears to be unrelated to motor neuron defects in these same mice. SOD1^{G93A} mice with four copies of the mutant gene compared to copies of the normal human gene showed no increase in duration of hematopoiesis in LTBMCs, and cell lines were not radioresistant. The defective SOD1 may have caused changes in the bone marrow resulting in the up-regulation of SOD2, glutathione, or glutathione peroxidase-4 that may explain the differences in bone marrow biology in SOD1^{G93A} mice.

TBI combined with bone marrow transplantation is a potentially valuable therapeutic option for treating ALS. The subset of donor bone marrow cells (hematopoietic stem cells, committed granulocyte/macrophage progenitors, or mesenchymal stem cells) required to provide the therapeutic effect must also be determined (59, 60, 63, 64, 79-82).

Lrcc33$^{-/-}$ mice lack central nervous system vascular abnormalities. These mice have a different transformed growth factor-beta, develop ascending paraparesis and death attributable to microglia activation. Recently it was

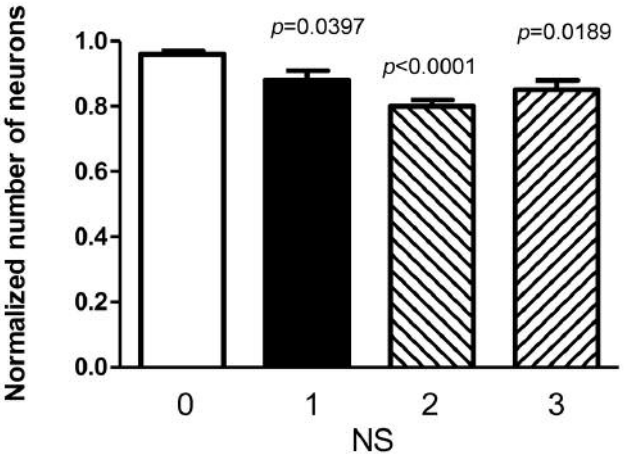

Figure 8. *Stages of spinal cord paralysis in superoxide dismutase-1 (SOD1)^{G93A} mice. Marrow-transplanted and control SOD1^{G93A} mice were sacrificed when developing amyotrophic lateral sclerosis with neurological score (NS) 0, 1, 2 or 3 paralysis (n=3). The spine was removed from euthanized mice, and the spinal cord was flushed from the vertebral column, fixed for 2 h in 2% paraformaldehyde, and then stored in 30% sucrose for 24 h. The number of neurons in sections at each stage of paralysis was normalized by dividing the number of neurons by the number of nuclei.*

demonstrated that transplantation of donor bone marrow from a wild-type mouse ameliorated these abnormalities due to the migration of wild type bone marrow origin microglia from the bone marrow (75). These data are a further indication in another model system that microglia arising from transplanted marrow can ameliorate a spinal cord degenerative disorder. Microglia appears to be a central component of the pathophysiology of ALS, as well as other neurodegenerative disorders (76), and bone marrow transplants may provide a cellular mechanism to correct such defects.

Supplemental Data

All supplemental data may be found at https://pitt.box.com/s/ajzj6ov2cw9ttrbnspnfz3p7d7so7mob

Conflicts of Interest

The Authors have no conflicts of interest in regard to this study.

Authors' Contributions

Michael Epperly, Ph.D., supervised all experiments. Joel Greenberger, M.D., planned and reviewed data for every experiment. Stephanie Thermozier scored radiation survival assay, and slides for immunostaining. Lora Rigatti, VMD, reviewed histopathology of spinal cords. Xichen Zhang, MD, carried out analysis of genotyping. Donna Shields carried out long-term bone marrow cultures. Wen Hou carried aided in staining of slides. Renee Fisher carried out all animal breeding and genotyping, and, also, observed animals daily and documented stages of paralysis. Simon Watkins, Ph.D., prepared fluorescent images. Andrew Henderson carried out the analysis of the numbers of neurons in spinal cords. Darcy Franicola carried out the assays for antioxidants and radiation survival curves of cell lines and fresh marrow. Christopher Donnelly, Ph.D., carried out review of all data, planned experiments in collaboration with radiation oncologists, and served as the neurosciences contributor for analysis and interpretation of all data. Hong Wang, Ph.D., completed all the biostatistics for the article. Hulya Bayir, M.D., analyzed histopathology and stages of paralysis. Peter Wipf, Ph.D., prepared drugs tested in Figure 9.

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