

Correction of Humoral Derangements from Mutant Superoxide Dismutase 1 Spinal Cord

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Objective: We sought to define molecular and cellular participants that mediate motor neuron injury in amyotrophic lateral sclerosis using a coculture system.

Methods: We cocultured embryonic stem cell–derived motor neurons with organotypic slice cultures from wild-type or SOD1G93A (MT) mice. We examined axon lengths and cell survival of embryonic stem cell–derived motor neurons. We defined and quantified the humoral factors that differed between wild-type and MT organotypic cultures, and then corrected these differences in cell culture.

Results: MT spinal cord organotypic slices were selectively toxic to motor neurons as defined by axonal lengths and cell survival. MT spinal cord organotypic slices secreted higher levels of nitric oxide, interleukin (IL)-1 β , IL-6, and IL-12p70 and lower levels of vascular endothelial growth factor. The toxicity of MT spinal cord organotypic cultures was reduced and axonal lengths were restored to near normal by coculturing in the presence of reactive oxygen species scavenger, vascular endothelial growth factor, and neutralizing antibodies to IL-1 β , IL-6, and IL-12.

Interpretation: MT spinal cord organotypic cultures overexpress certain factors and underexpress others, creating a nonpermissive environment for cocultured motor neurons. Correction of these abnormalities as a group, but not individually, restores axonal length to near normal. Such a “cocktail” approach to the treatment of amyotrophic lateral sclerosis should be investigated further.

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Amyotrophic lateral sclerosis (ALS) is a late-onset neurodegenerative disease characterized by the progressive loss of upper and lower motor neurons. Approximately 2 to 3% of all ALS cases are associated with mutations in the gene encoding Cu/Zn superoxide dismutase 1 (SOD1),¹ whereas others are associated with other genetic mutations² and most cases are sporadic. Although the molecular pathways that cause the death of motor neurons in familial ALS is still unknown, it is likely that accumulation of mutant SOD1 triggers creates a new gain-of-function toxicity to motor neurons. This toxicity could be entirely cell autonomous within motor neurons, noncell autonomous due to alterations in the environment around motor neurons, or a combination of the two. It is increasingly clear, however, that motor neuron death in ALS is not completely cell autonomous and that expression of mutant SOD1 within nonneuronal cells may result in dysfunction of motor neurons,

contributing to disease pathogenesis.³ *LoxSOD1^{G37R}/CD11b-Cre⁺* mice expressing a lower level of mutant SOD1 in microglia exhibit slower decline in function and extend survival.⁴

Proinflammatory cytokines are implicated in ALS pathogenesis, and if they are secreted by cells adjacent to motor neurons, this may explain, in part, the non-cell-autonomous death of motor neurons.⁵ The loss of motor neurons in transgenic ALS, as in mouse models⁶ and human postmortem tissue,⁷ is associated with a robust glial and microglial activation, both of which precede the onset of motor neuron death and motor axon degeneration.⁸ In addition, inducible nitric oxide (NO) synthase is upregulated in activated microglia in the spinal cord of transgenic ALS mice.⁹ Activated microglia are the primary source of both superoxide and NO in the central nervous system (CNS) in inflammatory degenerative disorders, and they lead to for-

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mation of the highly reactive and toxic derivative oxidant peroxynitrite.¹⁰ The compound *N*-acetyl-L-cysteine (NAC) inhibits reactive oxygen species (ROS), increasing the viability of cultured primary spinal motor neurons¹¹ and oligodendrocytes.¹² Another potential toxic factor, IL-1 β , is also increased in transgenic mouse models of ALS, and inhibition of IL-1 β activation slows disease progression in this model.¹³ Interleukin (IL)-6 level in the cerebrospinal fluid is statistically increased in ALS patients.¹⁴

Conversely, reduced expression of vascular endothelial growth factor (VEGF), which supports the survival of neurons, causes ALS-like motor neuron degeneration and increases the risk for ALS in humans.¹⁵ Transfer of the VEGF gene to muscle in transgenic SOD1 mice delays onset and prolongs survival.¹⁶

In total, these data suggest that, in ALS models, the initiation of proinflammatory cascades and the down-regulation of certain trophic factors create a nonpermissive state for motor neuron survival and function. This altered environment may, therefore, account for the non-cell-autonomous nature of motor neuron death in ALS. However, it is also clear that modulation of these factors individually does not substantially correct the environment and that the disease continues to progress. We sought, therefore, to define molecular characteristics of this toxic environment and to test whether correction of those derangements could rescue motor neurons. We created a cell culture system using embryonic stem (ES) cell-derived motor neurons and cocultured tissue slices from wild-type (WT) or transgenic SOD1G93A (MT) mice. We found that cocultured MT spinal cord slices (but not hippocampal slices) were toxic to cocultured motor neurons, resulting in substantially shortened neurite lengths. We further defined five molecular abnormalities that were distinct between WT and MT spinal cord slices and between MT spinal cord and hippocampal slices. Correction of these abnormalities resulted in increased neurite length to 80% of normal, suggesting that this combinatorial approach to ALS may represent a viable therapeutic strategy.

Materials and Methods

Materials used for cell cultures were purchased from Invitrogen (Carlsbad, CA). Unless stated otherwise, all other chemicals were obtained from Sigma (St. Louis, MO).

Cell Culture

ES cell-derived motor neurons were cultured following a previously described procedure.¹⁷

Hippocampus and Spinal Cord Organotypic Culture

The organotypic culture used in this study was performed through a modified procedure of that described previously.¹⁸ Animal care was conducted in accordance with protocols

and guidelines approved by the Johns Hopkins University Animal Care and Use Committee. Transgenic mice overexpressing human SOD1G93A (MT) were maintained by crossing B6SJL-Tg(SOD1G93A)1Gur/J male with matched B6SJL/J female mice obtained from Jackson Labs (Bar Harbor, ME). Seventy-day-old MT mice were used for organotypic culture. The SOD1 genotype was determined by polymerase chain reaction from tail samples. Mice were killed by an overdose of pentobarbital (100mg/kg Nembutal intraperitoneally; Abbott Laboratories, Abbott Park, IL).

BV2 Cell Line

BV2 cells are an immortalized mouse microglial cell line that exhibits the morphological and functional characteristics of microglia. BV2 cells were maintained in Dulbecco's modified Eagle medium with 10% fetal bovine serum, 50U/ml penicillin, and 50 μ g/ml streptomycin at 37°C in a 95% air/5% CO₂-humidified atmosphere.

Human SOD1 and SOD1G93A were subcloned into a cytomegalovirus-based expression vector, pNIT.¹⁹ The sequences of all constructs were confirmed by nucleotide sequencing. To generate stable cell lines that express SOD1 and SOD1G93A, we generated retroviruses that encoded each and infected BV2 cells, followed by selection with 400 μ g/ml G418 (BD Sciences, San Diego, CA). These studies resulted in the generation of the BV2-SOD1 and BV2-SOD1G93A lines. BV2 cells were plated into 96-well plates at a density of 5×10^3 cells per well, and *Escherichia coli* lipopolysaccharide (LPS; 011:A1) was added to the BV2 cell lines at 100ng/ml for 24 hours. Doxycycline was used at 500ng/ml to repress transgene expression. To coculture the BV2 cells with ES cell-derived motor neurons, we plated BV2 cells into each semipermeable membrane insert at a density of 2×10^5 cells; then the insert was transferred into wells of ES cell-derived motor neurons.

Immunohistochemistry

Spinal cord and hippocampal organotypic cultures were examined by immunohistochemistry for colocalized green fluorescent protein (GFP) to identify transplanted ES cell-derived motor neurons (GFP expression driven by the motor neuron-specific HB9 promoter) and neuronal nuclei (NeuN) staining to identify neurons. Organotypic sections were washed with 0.1M phosphate-buffered saline for 5 minutes, fixed with 4% (wt/vol) paraformaldehyde for 30 minutes, and permeabilized with 0.5% Triton X-100 (Sigma, St. Louis, MO) in phosphate-buffered saline. The slides were blocked with 1% goat serum for 30 minutes and incubated with a mixture of rabbit polyclonal anti-GFP antibody (1:100 dilution; Chemicon, Temecula, CA) and mouse monoclonal anti-NeuN antibody (1:100 dilution; Chemicon) at 4°C for 24 hours. After being washed with phosphate-buffered saline three times, organotypic sections were incubated with a mixture of fluorescent dye-conjugated goat anti-mouse IgG (1:1,000 dilution; Molecular Probes, Eugene, OR) and goat anti-rabbit IgG (1:1,000 dilution; Molecular Probes) for 3 hours at room temperature. To test the specificity of immunostaining, we treated control organotypic sections in an identical manner without the primary or secondary antibodies.

Cytokine Array

Mouse cytokine antibody assays were performed with RayBio Mouse Cytokine Antibody Array kit (RayBiotech, Norcross, GA), following the manufacturer's instructions. To extract proteins from organotypic cultures, we homogenized tissues using tissue lysis buffer with protease inhibitor (Roche, Nutley, NJ). The array membranes were incubated with 30 µg tissue lysates from spinal cord and hippocampal organotypic slices. Signal was analyzed and quantitated with a Fuji chemiluminescent detection system (Fujifilm Life Science, Stamford, CT).

IL-1β, IL-6, and IL-12p70 concentrations were measured with enzyme-linked immunosorbent assay kits (Quantikine; R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. Standards and samples were added to the wells, which were then incubated for 2 hours at room temperature. The concentration was measured with a kinetic microplate reader (Molecular Devices, Sunnyvale, CA) at 450nm. The concentrations of IL-1β, IL-6, and IL-12p70 in the samples were calculated based on a standard curve plotted on a log-log scale.

Measurement of Nitrite (NO₂⁻)

Nitrite concentration from culture media was measured with a total NO assay kit following the manufacturer's instruction (R&D Systems). Measurement of the concentration of each well was determined with a kinetic microplate reader at 550nm.

Microscopy

Immunohistochemical studies were conducted and evaluated using either two-color confocal imaging with a Zeiss LSM510 microscope (Carl Zeiss Inc., Germany), in which images were acquired in both red and green emission channels by an argon-krypton laser with a single-channel, line-switching mode, or with a CCD Nikon camera (Nikon, Japan).

Measurement of Neurite Length

Neurite outgrowth quantification was performed in a blinded fashion using National Institutes of Health image analysis software, *Image J*. Cells that did not produce axons were given a set value near zero. Cells with axons that were not in full view were not included. We counted the total number of cells and measured neurite lengths per cell within each test group.

Statistical Analyses

Data are expressed as means ± standard error of measurements. Differences in the mean values among the groups were analyzed, and significance was analyzed by Student's *t* test. Significance was defined as *p* < 0.05.

Results

Direct Coculture of Embryonic Stem Cell-Derived Motor Neurons with Hippocampal and Spinal Cord Organotypic Slices

We developed a coculture system using ES cell-derived motor neurons and organotypic cultures from WT or

transgenic MT mice. We differentiated murine ES cells that encode GFP driven by the motor neuron-specific HB9 promoter into motor neurons using retinoic acid and a chemical agonist of sonic hedgehog (HhAg1.3).¹⁷ Separately, we generated hippocampal or spinal cord organotypic slice cultures from 70-day-old WT or MT mice. Organotypic cultures derived from adult animals, unlike those from early postnatal animals, do not support the survival of motor neurons within the organotypic slice. However, we reasoned that these slice cultures may differentially support the survival of ES cell-derived motor neurons in coculture and, therefore, transplanted approximately 30,000 ES cell-derived motor neurons onto the organotypic slices using a Hamilton syringe. The ES cell-derived motor neurons were transplanted as partially disaggregated embryoid bodies, meaning that motor neurons could be identified individually, as well as within the three-dimensional structure of the embryoid body. Seven days after transplantation, organotypic cultures were probed with an anti-GFP antibody to detect survival of transplanted motor neurons immunohistochemically. We found that transplanted motor neurons were detected in both WT (Fig 1A; top left) and MT hippocampal organotypic cultures (bottom left) and in WT spinal cord organotypic slices (top right). However, when they were transplanted into MT spinal cord slices (see Fig 1A; bottom right), we consistently saw no surviving motor neurons, either singly or within transplanted embryoid bodies. Identified embryoid bodies (see arrowhead in Fig 1A, bottom right) invariably had no GFP immunoreactivity, suggesting that the motor neurons within the transplant had died. Dual-color confocal microscopy confirmed that doubly labeled NeuN⁺/GFP⁺ neurons could be identified within the WT hippocampal (see Fig 1B) and spinal cord (see Figs 1C, D) organotypic slices, but not within the MT spinal cord (see Fig 1E). This observation suggested to us that the MT spinal cord organotypic slices uniquely secreted toxic factors and/or failed to secrete trophic factors, therefore resulting in reduced motor neuron survival.

Neurite Outgrowth of Embryonic Stem Cell-Derived Motor Neurons in Coculture with Organotypic Slice Cultures

To further define this differential effect on transplanted motor neurons, we modified the coculture system so that the ES cell-derived motor neurons were cultured on the bottom of a culture dish and the organotypic slices were cultured on a semipermeable membrane insert. This variation allowed us both to assess neurite length of the motor neurons more quantitatively and to determine the importance of soluble factors secreted by the organotypic slices that might modify the motor neuron survival. As before, we found that after 7 days in coculture with MT spinal cord organotypic cultures, ES cell-derived motor neurons died, whereas motor

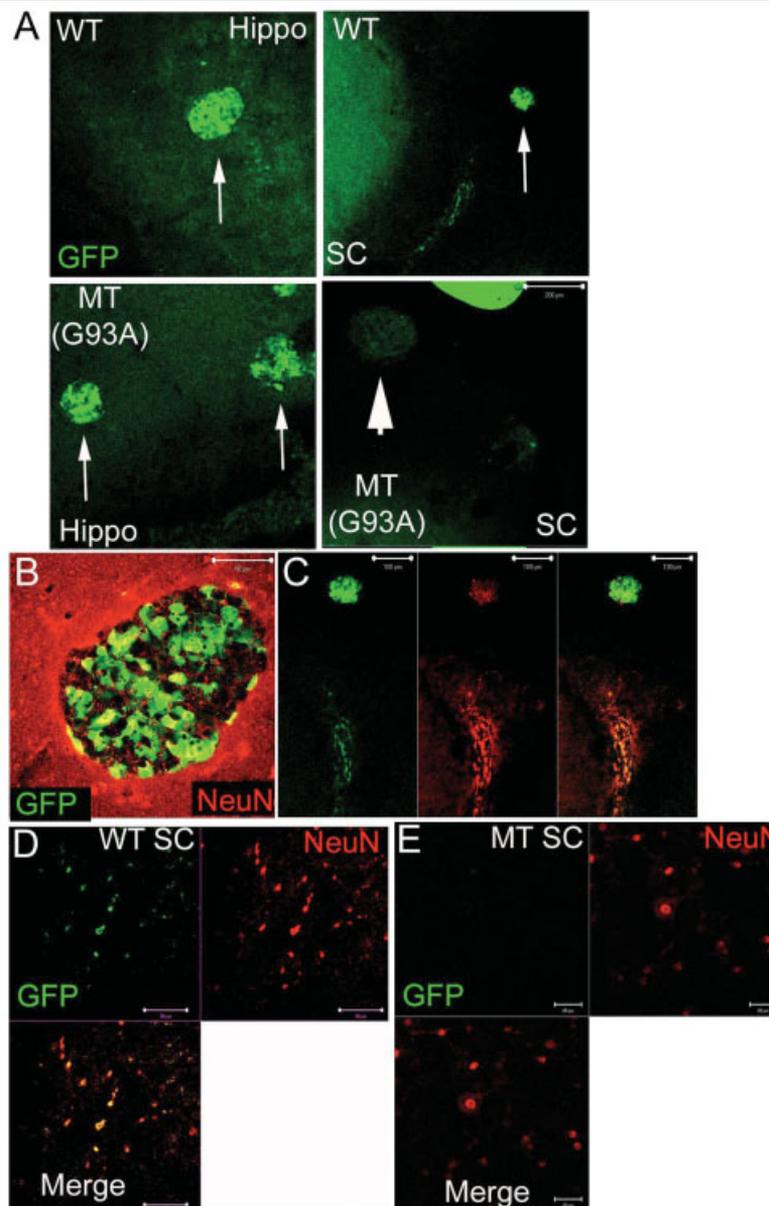


Fig 1. Survival of embryonic stem (ES) cell-derived motor neurons in organotypic cultures after transplantation. (A) Approximately 30,000 ES cell-derived motor neurons were transplanted into wild-type (WT) and transgenic SOD1G93A (MT) hippocampus (Hippo) and spinal cord (SC). Seven days after transplantation, organotypic cultures were probed with a green fluorescent protein (GFP) antibody to detect the survival of transplant-derived motor neurons by immunohistochemical analysis. Arrows denote transplant-derived GFP⁺ motor neurons, whereas the arrowhead (bottom right) depicts an embryoid body, which is largely devoid of GFP⁺ cells, transplanted into MT spinal cord organotypic cultures. (B, C) Higher power confocal microscopy analysis of GFP⁺/neuronal nuclei (NeuN)⁺ neurons in WT hippocampal (B) and spinal cord (C) organotypic culture. (D, E) Confocal microscopy of WT spinal cord (D) and MT spinal cord organotypic slices (E) after transplantation of dissociated ES cell-derived motor neurons. In (D), both host and transplant-derived neurons could be identified, whereas in (E), only host NeuN⁺ neurons could be identified.

neurons in coculture with WT or MT hippocampal slices or WT spinal cord organotypic slices remained alive with long axonal processes (data not shown). More importantly, we found that at shorter times of coculture (3–5 days), ES cell-derived motor neurons exhibited significant neurite shortening (Fig 2A) and irregularities (see Fig 2B), suggesting that neurite

length might be an appropriate and quantifiable read-out of coculture toxicity. Indeed, when we blindly quantified neurite length, we found that neurite lengths were approximately 60% reduced in coculture with MT spinal cord organotypic slices, compared with coculture with WT spinal cord slices ($p < 0.05$; see Fig 2C). Interestingly, neurite length in coculture

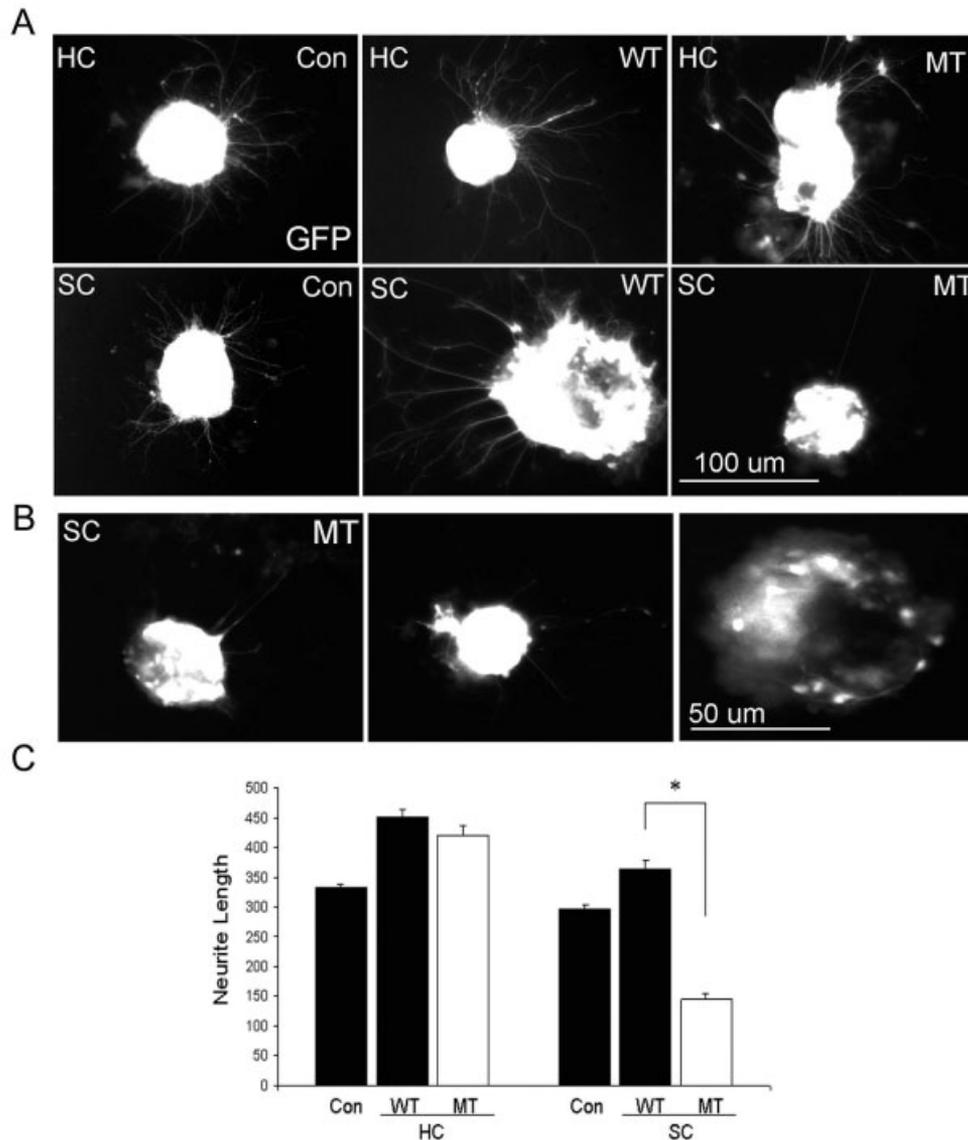


Fig 2. SOD1G93A (MT) spinal cord organotypic cultures secrete factors that reduce neurite lengths of cocultured motor neurons. Organotypic culture sections were grown on a semipermeable membrane, and cocultured motor neurons were grown adherent to the bottom of the culture dish. (A) Neurite length was examined in embryonic stem (ES) cell-derived motor neurons that had no cocultured organotypic sections (Con) or were cocultured with wild-type (WT) or MT hippocampus (HC) or spinal cord (SC). (B) Higher power microscopy showed reduction in the number of axons and abnormalities in motor neuron neurite morphology in the presence of cocultured MT spinal cord organotypic cultures. (C) Neurite lengths were assessed blindly using Image J and were quantified. Each experiment was done in quadruplicate wells per experiment and was conducted independently at least three times. Asterisk denotes that significance of the neurite reduction when cocultured with MT spinal cord organotypic cultures was $p < 0.05$.

with WT spinal cord organotypic sections was significantly longer than control cultures in which no organotypic tissue was present (Con), suggesting that spinal cord tissue from normal animals had a beneficial effect.

Nitrate Concentration from Coculture Media in the Embryonic Stem Cell-Derived Motor Neurons with SOD1G93A Spinal Cord Organotypic Culture

We sought to define the critical molecular differences between WT and MT spinal cord slices. Because ROS

have been implicated in ALS,⁹ we assessed total nitrate concentrations in organotypic coculture media. Total nitrate concentration was significantly higher in the MT spinal cord coculture media than in WT spinal cord coculture media and in WT and MT hippocampal coculture media (Fig 3A). Furthermore, addition of 5mM NAC, a ROS scavenger that has been shown to reduce the toxicity of NO and peroxynitrate,²⁰ conferred protection of neurite length when added to the culture media of ES cell-derived motor neurons and MT spinal cord sections ($p < 0.05$; see Figs 3B, C).

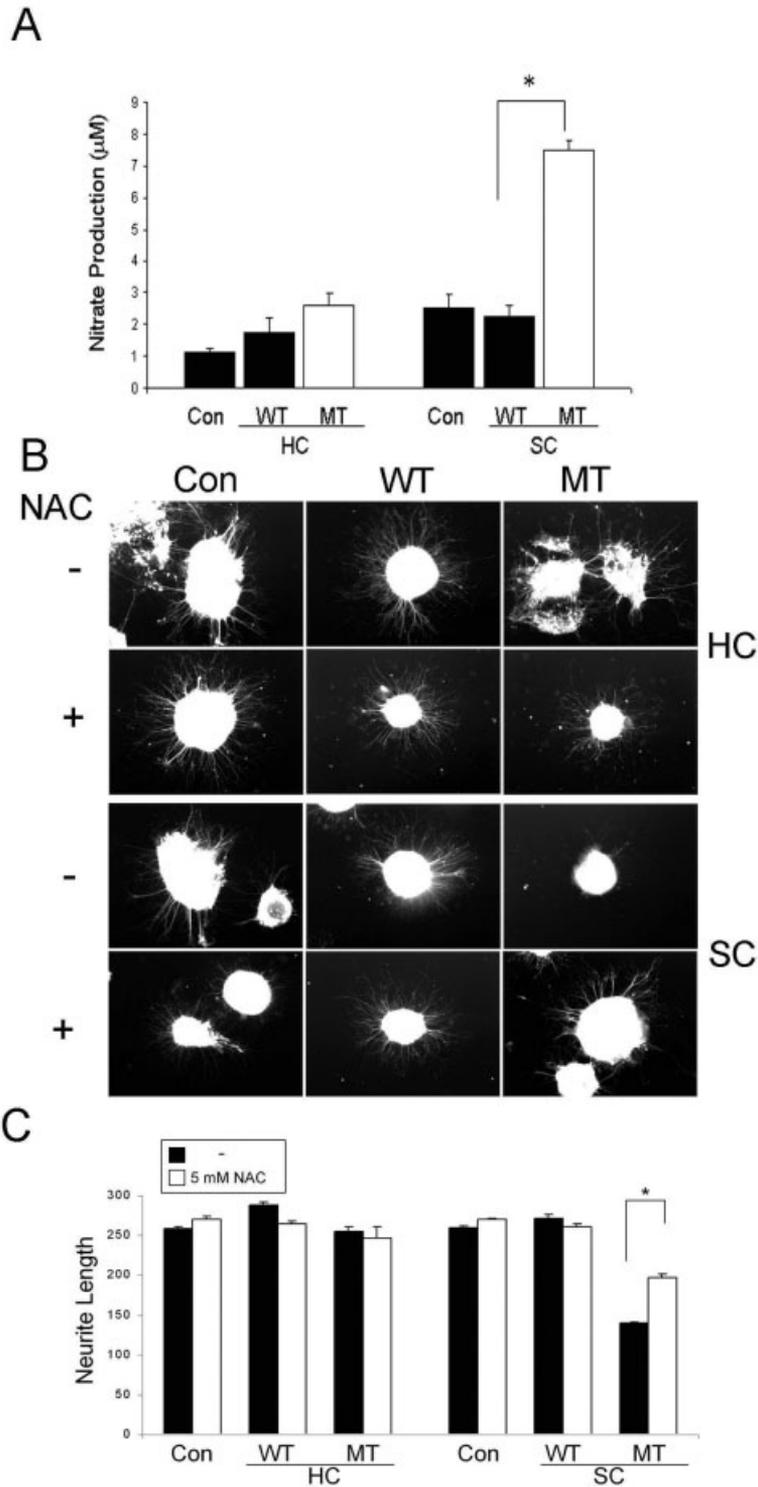


Fig 3. SOD1G93A (MT) spinal cord (SC) organotypic cultures secrete increased amounts of reactive oxygen species that contribute to neurite length shortening. (A) After 5 days of coculture, nitrate concentration in the culture media was measured using a total nitric oxide/nitrite/nitrate assay kit, plotted, and normalized to 100mg/tissue. MT spinal cord organotypic slices secreted more reactive oxygen species than WT spinal cord and wild-type (WT) or MT hippocampal (HC) cultures ($p < 0.05$). (B) N-acetyl cysteine (NAC) was added to the coculture medium at 5mM, and neurite length was assessed. (C) Blinded quantification of neurite length was conducted as above. The presence of NAC partially restored (approximately 35%) neurite length of motor neurons in coculture with MT spinal cord organotypic sections ($p < 0.05$).

Measurement of Inflammatory Factors in Wild-type and SOD1G93A Spinal Cord Organotypic Culture Sections

It was clear, however, that the difference in total nitrates could only partly explain the toxicity of MT spinal cord sections; therefore, we conducted a protein-based screen of organotypic tissue lysates to define other potential differences. We performed a cytokine protein array, comparing WT and MT spinal cord organotypic cultures, and found several consistent differences in each of three independent replicates. Most notably, IL-1 β , IL-6, and IL-12p70 were significantly elevated in MT spinal cord, whereas VEGF was reduced (Figs 4A, B). Quantification of each of the cytokines studied is shown in Figure 4B. Quantitative enzyme-linked immunosorbent assays were then performed to determine the concentration of each of the proinflammatory cytokines elevated in the coculture of ES cell-derived motor neurons and MT spinal cord organotypic slice cultures (see Figs 4C–E).

Combinatorial Correction of Toxicity in SOD1G93A Spinal Cord Organotypic Cultures

We reasoned that alterations in the levels of each of these four factors may participate in the shortened neurites seen on coculture with MT spinal cord organotypic slices. To test this hypothesis, we neutralized the three proinflammatory cytokines individually or in combination by adding neutralizing antibodies to the cell culture supernatant. In addition, we “corrected” the VEGF level by adding it to the culture medium and added NAC, as above, to reduce ROS. We confirmed immunodepletion of each proinflammatory cytokine by repeat enzyme-linked immunosorbent assay (data not shown) and found that elimination of each cytokine individually did not increase neurite length of motor neurons in coculture with MT spinal cord organotypic cultures (Figs 5A, B). Similarly, adding VEGF to the culture medium had no effect on neurite length. Triple immunodepletion of the proinflammatory cytokines had a modest, but significant, effect on neurite length, whereas addition of NAC alone, as above, had a modest effect. Notably, immunodepletion of the three proinflammatory cytokines and addition of NAC and VEGF had the most significant effect, restoring neurite length to approximately 80% of control levels (see Fig 5B). These studies strongly suggest to us that a combinatorial approach to the treatment of inflammation in ALS might protect motor neurons.

Highly Activated Microglia in SOD1G93A Spinal Cord Organotypic Slices

Because it appeared likely that these humoral derangements were mediated, at least in part, by differential microglial activation, we performed immunohistochemistry using a microglial antibody (Iba1) and hip-

pocampal and spinal cord organotypic cultures taken from 70-day-old WT and MT animal (see Supplemental Fig). We found that there was significantly more microglial activation within the MT spinal cord organotypic cultures compared with either WT spinal cord or WT or MT hippocampal organotypic slices, defined both by the number and morphology of Iba1⁺ cells. There was no difference in the microglial activation between WT and MT hippocampal cultures, suggesting that in this context the expression of mutant SOD1 was insufficient to fully activate microglia. Interestingly, when hippocampal cultures were treated with LPS to activate the microglial cells, these cultures were then toxic to cocultured motor neurons, suggesting that though these microglia were not activated *de novo*, they had the capacity to become activated and to become toxic to neurons.

hSOD1G93A-Expressing Microglial Cells Recreate Soluble Toxicity of Cocultured Motor Neurons

To determine whether microglial cells contribute to the humoral toxicity seen in this model, we used a BV2 microglial cell line and altered these cells such that they could inducibly express either hSOD1 or hSOD1G93A. We overexpressed BV2 cells with inducible expression plasmids, selected stable transfectants, and tested for inducible expression of the SOD1 (Fig 6A). We identified cell lines that had high levels of inducible and absent basal expression of SOD1 or SOD1G93A. Among several independent lines, we found no consistently different expression levels between SOD1 and SOD1G93A and further observed that levels of SOD1 and SOD1G93A expression were less than those found in SOD1G93A transgenic mice normalized by weight (data not shown). We generated BV2 cells overexpressing red fluorescent protein as a control (Con) to examine transfection efficacy. We then tested the secretion of IL-1 β , IL-6, IL-12p70, and NO from BV2-SOD1 (WT) and BV2-SOD1G93A (G93A) cells under both resting and activated conditions. To activate the BV2 cells, we incubated them with LPS at 100ng/ml for 24 hours before harvesting the supernatants. We found that when SOD1 expression was repressed (+doxycycline), cytokine expression by resting BV2 cells was low, and that LPS modestly induced the expression of IL-1 β , IL-6, IL-12p70, and NO in all cell lines to a similar degree (see Fig 6B). Similarly, when SOD1 expression was induced, resting expression of these cytokines was not increased and did not differ among control (no expression), BV2-SOD1, and BV2-SOD1G93A cell lines. Interestingly, however, when SOD1 and SOD1G93A expression was induced and the BV2 cells were activated with LPS, the expression of IL-1 β , IL-6, IL-12p70, and NO was dramatically and significantly increased in only BV2-

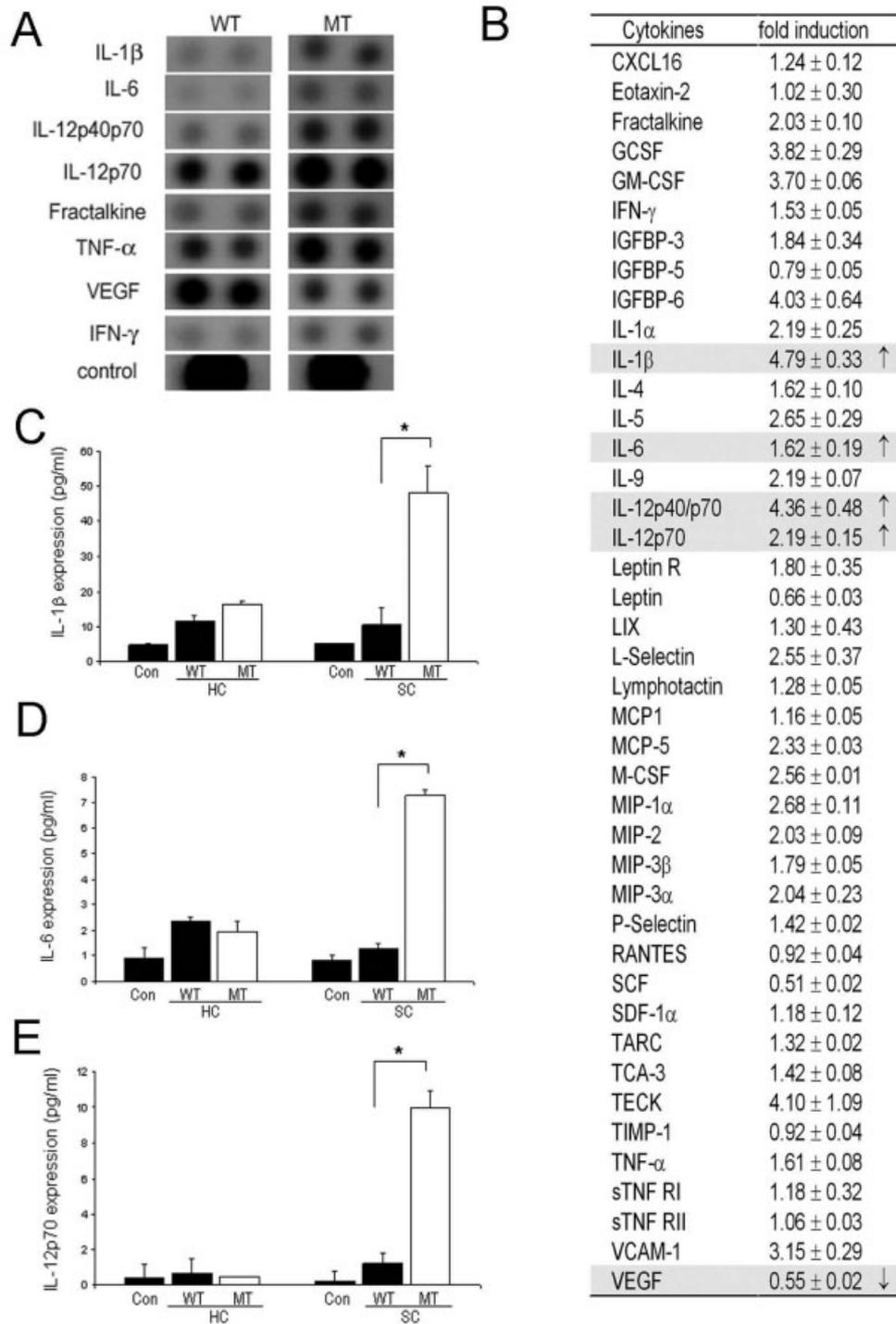


Fig 4. Measurement of inflammatory factors in wild-type (WT) and SOD1G93A (MT) spinal cord (SC) organotypic culture sections. (A) Cytokine protein array was used to profile 62 inflammatory proteins of WT and MT spinal cord organotypic cell lysates. The assay was performed three independent times, and representative changes in signal intensity are depicted. The expression levels were detected using a chemiluminescence image system. (B) Quantification of fold induction was calculated by averaging the fold induction of each individual experiment (WT vs MT). (C) Quantification of interleukin (IL)-1β, IL-6, and IL-12p70 levels was measured using enzyme-linked immunosorbent assay kits. HC = hippocampus; IFN-γ = interferon-γ; SC = spinal cord; TNF-α = tumor necrosis factor-α; VEGF = vascular endothelial growth factor.

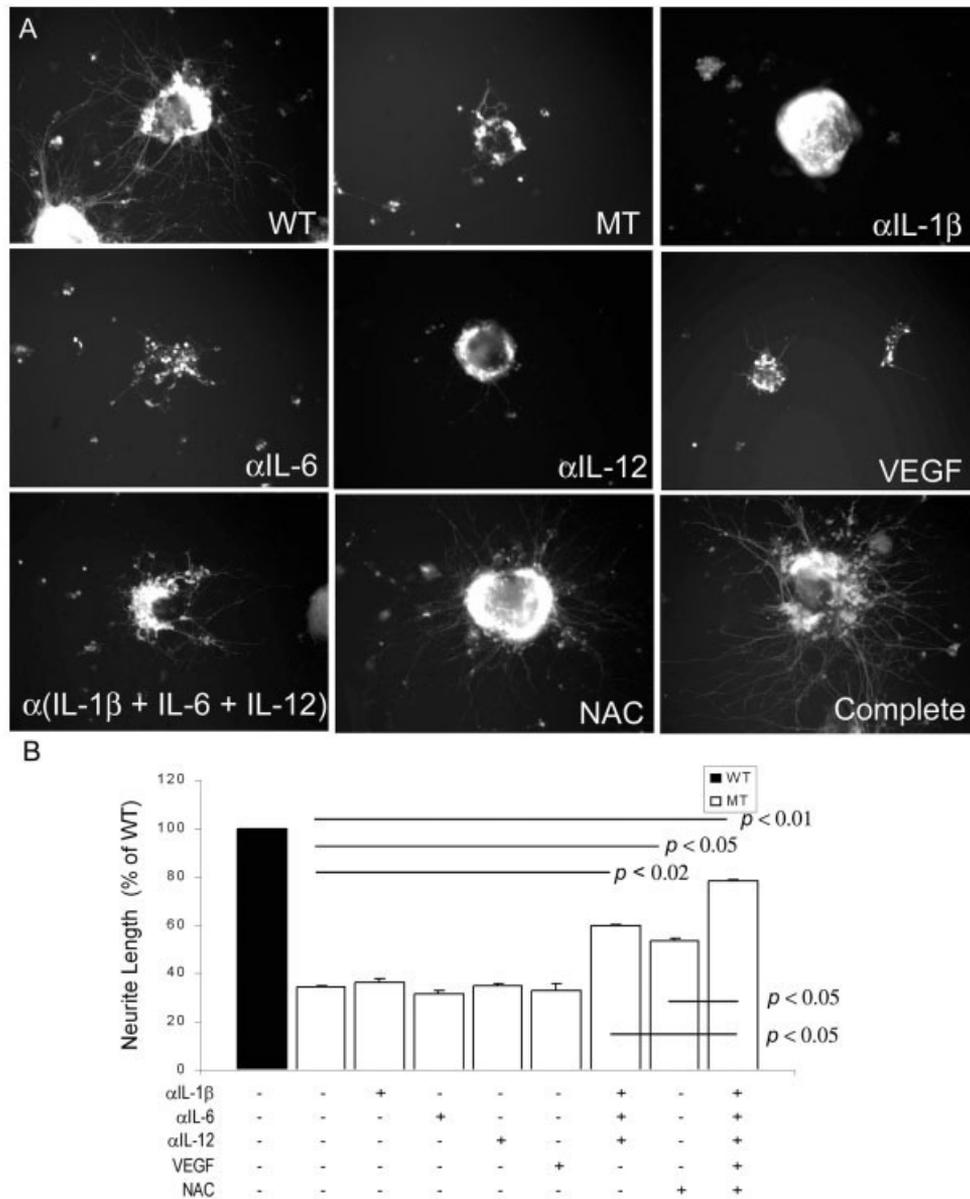


Fig 5. Combinatorial correction of humoral derangements in SOD1G93A (MT) spinal cord organotypic culture. (A) Embryonic stem (ES) cell-derived motor neurons were cocultured with MT spinal cord organotypic cultures supplemented with 5mM N-acetyl cysteine (NAC), interleukin (IL)-1 β (5 μ g/ml), IL-6 (1 μ g/ml), IL-12p70 (10 μ g/ml), or vascular endothelial growth factor (VEGF; 500ng/ml) singly or in combinations. Complete refers to all five treatments together. (B) Neurite lengths were blindly quantified as before. Each experiment was conducted in quadruplicate, and at least three independent experiments were performed for each condition. Significance of the protection is demonstrated by pairwise combination and the appropriate p value next to the horizontal line. MT = (white bars); WT = wild type (black bar); MT = SOD1G93A (white bar).

SOD1G93A. These data suggest that SOD1G93A expression within microglial-like cells induces a selective “irritability” in these cells: When they are activated, they secrete increased amounts of proinflammatory cytokines relative to control cells.

To determine whether this increased expression of proinflammatory cytokines is toxic to cocultured motor neurons, we cultured ES cell-derived motor neurons on the bottom of a culture dish and fibroblasts, BV2

(Con), BV2-SOD1 (WT), or BV2-SOD1G93A (G93A) cells were cultured on a semipermeable membrane insert and determined neurite length. All of the BV2 cells, under resting conditions, resulted in significantly increased neurite lengths of cocultured motor neurons (see Fig 6C). Similarly, all of the BV2 cells, under activated conditions (LPS), resulted in shortened neurites of cocultured motor neurons. Importantly, however, the activated BV2-SOD1G93A cells resulted

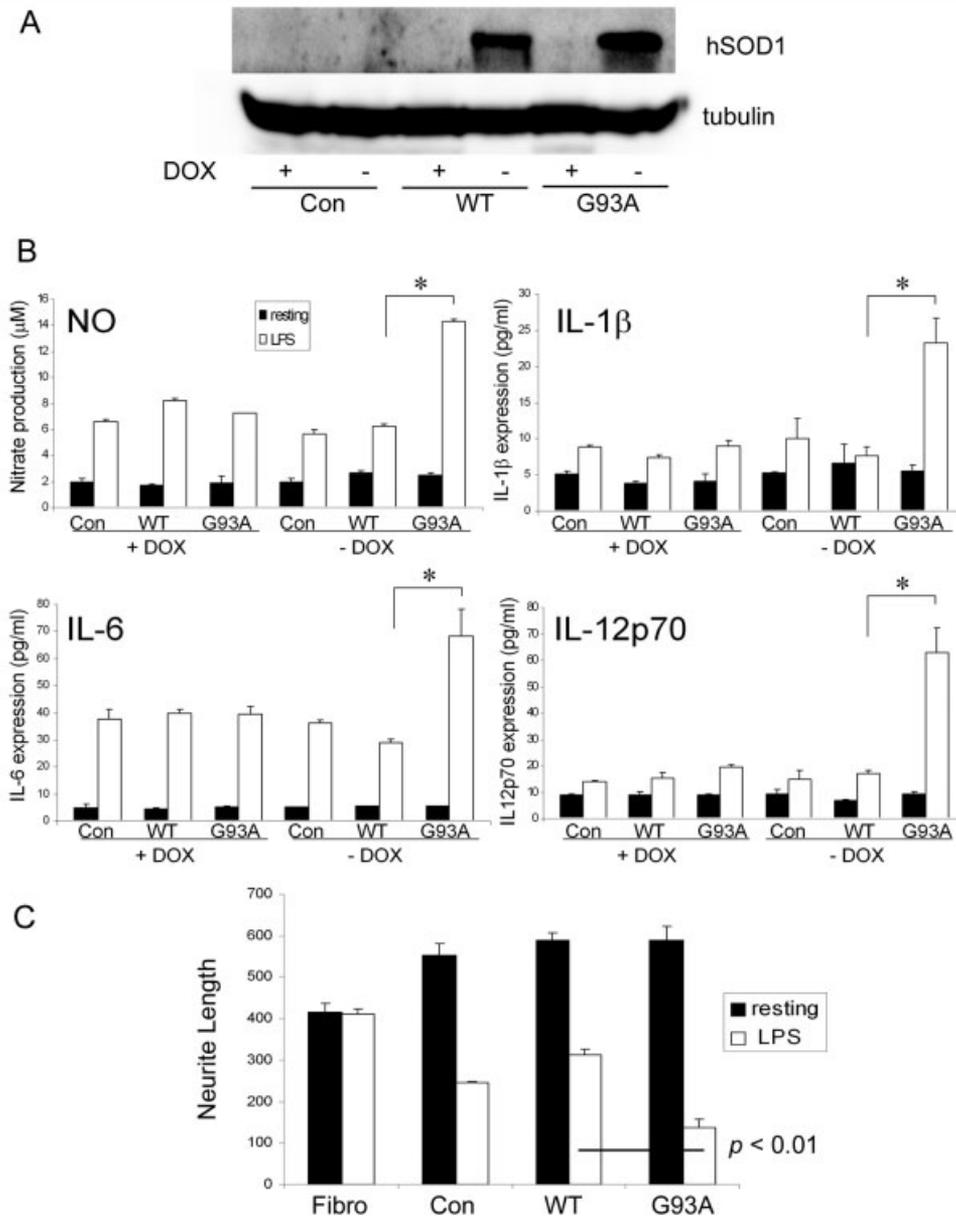


Fig 6. SOD1G93A-expressing microglial cells recreate soluble toxicity of cocultured motor neurons. We generated stable BV2 microglial cell lines that inducibly express either hSOD1 or hSOD1G93A on removal of doxycycline (DOX) from the culture media. (A) Immunoblot analysis shows tight control of hSOD1 expression within two stable sublines, one each for BV2-SOD1 (wild-type [WT]) and BV2-SOD1G93A (G93A). Two additional sublines for each were also analyzed and showed similar expression patterns (data not shown). Tubulin immunoreactivity is shown as a loading control for each of the lysates. (B) Levels of interleukin (IL)-1 β , IL-6, IL-12p70, and nitric oxide (NO) were quantified as before in each of two BV2-SOD1 and BV2-SOD1G93A sublines. BV2, BV2-SOD1, and BV2-SOD1G93A were cultured until they were 70% confluent and supernatants were harvested for analysis in 24 hours. BV2 sublines were cultured in a resting state (normal culture conditions; black bars) or in an activated state by the addition of lipopolysaccharide (LPS; white bars) at 100ng/ml for 24 hours before harvesting the supernatant. To control the expression of hSOD1, we added DOX to the culture media at 500ng/ml (* $p < 0.01$). (C) Neurite lengths were assessed and quantified as before. Fibro = fibroblast; Con = BV2-DsRed; WT = BV2-SOD1; G93A = BV2-SOD1G93A.

in the shortest neurite lengths of cocultured motor neurons ($p < 0.01$, compared with WT). These data suggest that BV2 microglial cells can recreate the toxicity of mutant spinal cord organotypic cultures, and that expression of SOD1G93A within microglial cells is required for this toxicity.

Discussion

Recent studies have supported the conclusion that motor neuron death in animal models of ALS is non-cell autonomous. Most notably, chimeric animals have demonstrated that normal motor neurons in the context of neighboring mutant SOD1-expressing cells are

injured, and that mutant SOD1-expressing motor neurons in the context of normal surrounding cells are capable of long-term survival.³ By extension, it is believed that nonneuronal cells that express mutant SOD1 have altered function, resulting in reduced function and survival of motor neurons. It is possible that nonneuronal cells may secrete toxic mediators or fail to secrete trophic factors, or both, resulting in motor neuron dysfunction. Indeed, although some groups found no differences in IL-6, tumor necrosis factor (TNF)- α , or IL-12 levels in patients with ALS,²¹ others found elevated levels of IL-6 and IL-1 β in the spinal cord and cerebrospinal fluid of patients with ALS,¹⁴ suggesting that an inflammatory cascade is important in both sporadic and familial ALS.

In this study, we sought to define humoral factors that distinguish WT from transgenic SOD1G93A (MT) spinal cord sections. We developed a cell-culture system using ES cell-derived motor neurons and organotypic slices from the spinal cord and hippocampus of adult WT and MT mice. We found that direct transplantation of ES cell-derived motor neurons onto MT spinal cord sections resulted in rapid death of the transplanted motor neurons, whereas transplantation onto WT spinal cord or WT or MT hippocampal organotypic cultures resulted in prolonged survival of the transplanted cells. This observation suggested to us that this culture system replicated some of the tissue-specific toxicity found in ALS, because neither the WT spinal cord nor the hippocampal cultures were toxic to motor neurons. We then modified this coculture system to better investigate the toxic features of MT spinal cord organotypic sections and found that the toxicity was mediated through soluble factors, resulting in axonal degeneration, followed by cellular death of cocultured motor neurons. We identified five soluble factors that were different between WT and MT spinal cord sections: increased total nitrate (a marker of increased ROS including NO), increased IL-1 β , increased IL-6, increased IL-12p70, and decreased VEGF. We found that correction of these abnormalities singly had modest to no effect on the axonal degeneration of cocultured motor neurons. By contrast, correction of all of these abnormalities restored motor axon length to approximately 80% of normal. We further identified that activation of microglia in MT spinal cord organotypic cultures was greater than those in the hippocampal organotypic slices, suggesting that regional-specific activation of microglia could mediate some or all of these findings (see Supplemental Fig). Interestingly, microglia in MT hippocampal organotypic cultures were not activated but could become activated with LPS, thereby becoming toxic. This means that it is not an inherent difference in the microglia from MT spinal cord and hippocampal organotypic sections, but rather a "state-of-activation" phenomenon.

This possibility is strengthened by the finding that inducible expression of SOD1G93A, but not SOD1,

within BV2 microglial cells recreates the soluble derangements identified in organotypic cultures and suggests a cell-autonomous function for abnormal microglial activation in response to BV2-SOD1G93A expression. The environment is critical to the microglial activation because BV2-SOD1G93A cells need a second stimulus, such as LPS, to become fully activated. Therefore, we conclude that expression of SOD1G93A within microglial cells induces an alteration in the inflammatory state within the spinal cord, including increased IL-1 β , IL-6, IL-12p70, and NO, which reduces the axon integrity and survival of motor neurons. Furthermore, we show reduced VEGF expression from MT spinal cord organotypic cultures, and that replacement of VEGF in the context of neutralizing the other four factors results in substantial correction of the neurite lengths. Therefore, the nonmotor neuron-autonomous injury of motor neurons involves these five factors, and therapeutic strategies that target all or many of these factors should be investigated.

Our study suggests: (1) "resting" microglial cells support motor neuron axons, and (2) "activated" microglial cells, expressing SOD1G93A, injure motor neuron axons. These data support and extend the findings from other studies that showed increased irritability of microglial cells in SOD1 animals, resulting in increased secretion of ROS and proinflammatory cytokines. These studies, however, did not distinguish between direct microglial activation due to SOD1G93A expression and the secondary effects of a degenerating environment that result in microglial activation. Our study, by contrast, addresses this issue and strongly supports the hypothesis that SOD1G93A expression within microglial cells induces a proinflammatory cascade that is toxic to neurons. One way this might occur is that SOD1G93A may directly promote the generation of ROS and reactive nitrogen species (RNS) by acting as a peroxidase or superoxide reductase or by producing O²⁻ to form peroxynitrite.¹⁰ The ROS or reactive nitrogen species could then presumably diffuse to nearby motor neurons and/or astrocytes, altering macromolecules, such as polyunsaturated fatty acids in membrane lipids, essential proteins, and DNA.²² In our study, we used NAC as an antioxidant and found that it reduced the toxicity of cocultured MT spinal cord sections. NAC increases intracellular glutathione, which provides a substrate for the detoxification of various peroxides, protecting cells from ROS, and reduces the degeneration of lower motor neurons and loss of choline acetyltransferase-positive neurons in the cervical spinal cord of *wobbler* mice.²³ NAC is particularly effective in enhancing cell survival and reducing free radical damage in neural cells¹¹ and, in humans, is modestly effective in enhancing function in ALS patients with limb-onset disease.²⁴

IL-1 β stimulates expression of a variety of growth factors in the brain, all of which generally have been believed to have a neuroprotective role. In contrast, in the

CNS, IL-1 β is produced initially by microglia and subsequently by astrocytes and invading macrophages under inflammatory conditions.²⁵ IL-1 β promotes inflammation by inducing glial production of additional cytokines, such as IL-6, TNF- α , cyclooxygenase-2, and inducible NO synthase.²⁵ In addition, IL-1 β can regulate synaptophysin expression in cortical neurons, increase Tau phosphorylation, and may be an important mediator of β -amyloid-induced neurotoxicity.²⁶ Treatment with IL-1 receptor antagonist or anti-IL-1 β antibody reduces neuronal death followed by ischemic brain damage.²⁷ The effect of IL-1 β may be activation of the prodeath mitogen-activated protein kinase, Jun-N-terminal kinase (JNK).²⁸ Jun-N-terminal kinase activation results in the accumulation of phosphorylated neurofilaments,²⁹ a well-described pathological feature of ALS and one that likely alters axonal transport. Axonal transport is increasingly thought to be central to the pathogenesis of ALS. In addition, mutations in axonal transport proteins, such as dynein, cause ALS-like pathology in mice and humans.² Therefore, it appears logical to propose that overactivation of this pathway from SOD1G93A-expressing microglial cells is critical to the pathogenesis of at least ALS.

Similarly, IL-6 is a glycoprotein cytokine, which, at low levels, may be a trophic factor that, under some circumstances, supports neuronal and glial differentiation and survival.³⁰ Introduction of members of the IL-6 superfamily, including IL-6 itself, in some systems has been shown to improve oligodendrocyte survival, perhaps by providing trophic support for oligodendrocytes without inducing an immune response.³⁰ However, recent investigation has shown the destructive potential of elevated levels of IL-6 in the CNS.³¹ IL-6 levels in the adult CNS are low or undetectable under baseline conditions, but they increase significantly in response to injury, inflammation, and CNS disease. TNF- α , IL-1 β , and neurotransmitters are the most important stimulators of IL-6 production from astrocytes and microglia within the CNS.³² Indirect evidence suggests that elevated IL-6 increases neural injury in Alzheimer's disease, Parkinson's disease, human immunodeficiency virus encephalopathy, multiple sclerosis, depression, and cognitive impairment.³² Indeed, IL-6 transgenic mice within astrocytes show ataxia, seizures, and hind-limb paralysis and have extensive neurodegeneration.³³ Neutralization of IL-6 attenuates traumatic spinal cord injury in rats and is associated with reduced inducible NO synthase activity.³⁴

IL-12p70 is a structurally unique heterodimer composed of two distinct disulfide-linked subunits, p35 and p40. IL-12p70 is significantly increased after activation of human CNS-derived microglia with LPS and interferon- γ , but it could not be detected in microglial cultures maintained under basal conditions.³⁵ IL-12p70 dose-dependently induces the production of TNF- α and

the expression of TNF- α messenger RNA in BV2 microglial cells.³⁶

Perhaps the most important finding of our study is that a combinatorial approach that neutralizes IL-1 β , IL-6, IL-12p70, and ROS and provides VEGF is required to normalize motor axonal lengths. These data suggest that the biochemical pathways initiated by each of these inflammatory factors is somewhat redundant and may be "compensated" for by other proinflammatory factors. It is for this reason that single inhibition of each factor has little to no effect, and that only in the setting of multiple, simultaneous inhibitions of proinflammatory factors is axonal length restored.

Finally, it is not only the increased expression of toxic factors, but also the reduced expression of trophic factors, such as VEGF, that underlies the difference between MT and WT spinal cords. VEGF and its receptors, on neurons and astrocytes, play a neuroprotective role against ischemia and spinal cord injury.^{15,37} VEGF induces neural progenitor proliferation, activation of neural maturation in the adult rat brain, and inhibition of apoptosis through the phosphatidylinositol 3-kinase/Akt/nuclear factor- κ B signaling pathway.³⁸ The induction of VEGF in mutant SOD1 transgenic animal is impaired from an early stage, and the low expression of VEGF has been detected in spinal cord of these mice.³⁹

That at least some of these injurious cytokines (notably IL-1 β and IL-6) have been found in the cerebrospinal fluid of ALS patients suggests that our findings are highly relevant to human disease for several reasons. First, cerebrospinal fluid is typically sampled early in the course of ALS, as clinicians attempt to rule out other causes of weakness. Therefore, abnormalities defined in this relatively early stage are more likely to be pathogenically relevant than those found altered in late-stage disease. Second, these cerebrospinal fluid findings confirm that although our studies are confined to an animal model of genetic ALS, at least some of these findings clearly apply to humans, particularly those with the more common sporadic form of ALS.

In summary, the interplay between motor neurons and glial cells is important in the clinical progression of both familial and sporadic motor neuron diseases. Release of ROS and cytokines and the reduced expression of trophic factors, such as VEGF from mutant SOD1 transgenic spinal cord, could contribute to the death of motor neurons. Taken together, a single endogenous factor is not responsible for ALS, and the future therapy of ALS may require combinatorial therapy.

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