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Extracellular Mutant SOD1 Induces Microglial-Mediated Motoneuron Injury

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Abstract

Through undefined mechanisms, dominant mutations in (Cu/Zn) superoxide dismutase-1 (*mSOD1*) cause the non-cell-autonomous death of motoneurons in inherited amyotrophic lateral sclerosis (ALS). Microgliosis at sites of motoneuron injury is a neuropathological hallmark of ALS. Extracellular mSOD1 causes motoneuron injury and triggers microgliosis in spinal cord cultures, but it is unclear whether the injury results from extracellular mSOD1 directly interacting with motoneurons or is mediated through mSOD1-activated microglia. To dissociate these potential mSOD1-mediated neurotoxic mechanisms, the effects of extracellular human $mSOD1^{G93A}$ or $mSOD1^{G85R}$ were assayed using primary cultures of motoneurons and microglia. The data demonstrate that exogenous $mSOD1^{G93A}$ did not cause detectable direct killing of motoneurons. In contrast, $mSOD1^{G93A}$ or $mSOD1^{G85R}$ did induce the morphological and functional activation of microglia, increasing their release of pro-inflammatory cytokines and free radicals. Furthermore, only when microglia were co-cultured with motoneurons did extracellular $mSOD1^{G93A}$ injure motoneurons. The microglial activation mediated by $mSOD1^{G93A}$ was attenuated using toll-like receptors (TLR) 2, TLR4 and CD14 blocking antibodies, or when microglia lacked CD14 expression. These data suggest that extracellular $mSOD1^{G93A}$ is not directly toxic to motoneurons but requires microglial activation for toxicity, utilizing CD14 and TLR pathways. This link between mSOD1 and innate immunity may offer novel therapeutic targets in ALS.

Keywords

mutant SOD1; CD14; Toll-like receptors; microglia; motoneurons

Introduction

Amyotrophic lateral sclerosis (ALS) is a devastating and chronic neurodegenerative disease, characterized by selective loss of lower and upper motoneurons (Lomen-Hoerth, 2008). Although the cause of sporadic ALS (sALS) is not known, approximately 10% of ALS cases

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are familial (fALS) with 20-25% of these cases resulting from various mutations in the *SOD1* (Gurney et al., 1994). Several human *mSOD1* genes have been over-expressed in mice and cause a disease that replicates many of the clinical and pathological features seen in ALS patients (Gurney et al., 1994; Wong et al., 1995). In addition, a recent study demonstrated an altered SOD1 species within the spinal cords of sALS patients that must have originated from the misfolded wild-type SOD1 ($SOD1^{WT}$), possibly linking a shared pathophysiologic pathway between sALS and fALS (Gruzman et al., 2007).

The toxicity of mSOD1 in ALS is not due to decreased enzymatic activity because mice lacking SOD1 expression did not develop a motoneuron disease (Reaume et al., 1996). Furthermore, motoneuron disease develops with different *SOD1* mutations regardless of their enzymatic activities (Cleveland and Rothstein, 2001; Wang et al., 2002). In addition, motoneuron specific expression of mSOD1 either does not initiate disease (Pramatarova et al., 2001; Lino et al., 2002) or, when expressed homozygously, causes a late onset and slowly progressing disease (Jaarsma et al., 2008). Thus, the aggressive development of disease in mSOD1 transgenic mice may be non-cell-autonomous (Lobsiger and Cleveland, 2007; Yamanaka et al., 2008a).

Current evidence suggests that glial over-expression of mSOD1 may contribute to disease progression in this model of inherited ALS. Several studies support the importance of both microglia and astroglia in mediating motoneuron injury (Zhao et al., 2004; Beers et al., 2006; Boillee et al., 2006; Nagai et al., 2007; Yamanaka et al., 2008b). Over-expression of mSOD1 in microglia enhances toxicity and accelerates disease progression compared with WT microglia (Beers et al., 2006; Xiao et al., 2007). Insights into the role of mSOD1 and microglia-mediated motoneuron injury are derived from the demonstration that mSOD1 can directly augment microglial NADPH oxidase-dependent superoxide production; mSOD1 stimulated microglial NADPH oxidase activity leading to increased production of toxic superoxide (Harraz et al., 2008). A key question not addressed by these studies is whether extracellular mSOD1 also interacts with and activates microglia which subsequently injures motoneurons.

A previous study suggests that the neurosecretory proteins, chromogranin A and B, interact with and mediate the secretion of mSOD1 protein from neurons and astrocytes (Urushitani et al., 2006). Unlike $SOD1^{WT}$ protein, extracellular mSOD1 protein triggered microgliosis and neuronal death in whole spinal cord mixed cultures. Furthermore, recent evidence suggests that oxidation of $SOD1^{WT}$ results in misfolded protein that may acquire the binding and toxic properties of mSOD1, suggesting a possible shared pathway between sporadic and inherited ALS cases (Ezzi et al., 2007; Gruzman et al., 2007; Kabashi et al., 2007). These studies, taken together with our data that $mSOD1^{G93A}$ expressing microglia are more neurotoxic than WT microglia (Beers et al., 2006; Xiao et al., 2007), indicate that mSOD1 is not only harmful within the cell, but also gains toxic functions when outside the cell. However, no study has investigated whether extracellular mSOD1 has direct effects on microglia and/or motoneurons, and by what mechanisms.

Materials and Methods

Materials

Culture media, sera and antibiotics were purchased from Gibco BRL (Rockville, MD), and all other reagents were from Sigma (St. Louis, MO) unless otherwise noted.

Recombinant protein purification

Recombinant human $mSOD1^{G93A}$, $mSOD1^{G85R}$ and $SOD1^{WT}$ proteins were purified from E. coli, metallated with copper and zinc (Urushitani et al., 2004). Briefly, E. coli were

transformed with the expression plasmid, pGEX6p-1 carrying human mSOD1^{G93A} or SOD1^{WT} gene. GST-fused hSOD1protein was induced by 1mM IPTG (Isopropyl β-D-1-thiogalactopyranoside) and absorbed with glutathione sepharose beads. After washing three times in PBS, the beads were incubated with a protease (Precision, Amersham-Pharmacia) to release hSOD1 from the GST tag. The hSOD1 proteins were then dialyzed against a buffer containing 50mM Tris-HCl pH7.5 and 100mM NaCl. Metallation was performed by incubation in two equivalent parts of zinc chloride for 24 hrs, followed by further incubation with two-equimolar copper chloride for 24 hrs. Metallated hSOD1 was dialyzed against the same buffer. The purity of the recombinant protein was verified by Western blotting and the activity of metallated recombinant SOD1 was confirmed using a SOD1 activity assay kit (Dojindo, Kumamoto, Japan), in which dismutase activity against the superoxide anion, generated from the reaction of xanthine with xanthine oxidase, was quantified (data not shown). Purified hSOD1 proteins were stored at −80°C.

Mice

Animal protocols were approved by the Methodist Research Institute's Institutional Animal Care and Research Advisory Committee in compliance with National Institutes of Health guidelines. mSOD1^{G93A} mice [C57B6.Cg-Tg(SOD1*G93A)1Gur/J] and CD14$^{-/-}$ mice were originally purchased from Jackson Laboratories (Bar Harbor, ME). All transgenic animals were bred and maintained in our animal facility. CD4$^{-/-}$ mice were initially bred with mSOD1^{G93A} mice for at least 8 generations. The mSOD1 and CD14 mice were identified by PCR (Eppendorf Mastercycler). mSOD1 copy number was determined by quantitative PCR.

Cell cultures

Primary motoneuron cultures were prepared from spinal cords of embryonic day 13-14 C57BL/6 or CD14$^{-/-}$ mice by HistoDenz gradient centrifugation (Arce et al., 1999; Zhao et al., 2006). Briefly, the ventral cords digested for 15 min with 0.025% trypsin at 37°C. After treatment with DNase, the cell suspension was centrifuged through a 6.8% HistoDenz cushion at 800g for 15 min, the sharp band on top of the HistoDenz cushion was collected and centrifuged through a 4% BSA cushion at 450g for 10 min. The cells were resuspended in the neurobasal medium supplemented with glutamate (25μM), glutamine (200mM), B27 (50×, 2ml), penicillin (100IU/ml), streptomycin (100μg/ml), and horse serum (2%). Cells were plated at a density of 15,000 cells/well in 24-well-plates containing coverslips coated with poly-L-ornithine (2μg/ml) and laminin (3μg/ml).

Microglia cultures were prepared from 7-8-day-old C57BL/6 or CD14$^{-/-}$ mice as previously described (Zhao et al., 2004). Greater than 95% of the floating cells were microglia as determined by OX42 immunocytochemical staining. Co-cultures of microglia and motoneurons were carried out by plating microglia (20,000/well) on motoneurons after motoneurons were incubated for 1 day. mSOD1^{G93A}, mSOD1^{G85R} or SOD1^{WT} was added to culture medium 4 hours after microglia plating. The addition of blocking antibodies or inhibitors was made 2 hrs prior to mSOD1^{G93A} or SOD1^{WT}.

Immunocytochemistry

For motoneuron staining, cultures were fixed for 25 min with 4% paraformaldehyde at room temperature. After washing, cultures were stained by SMI-32 antibody (1:1000, Covance) and M.O.M kit (Vector Laboratories) following the manufacturer's instructions. SMI-32, non-phosphorylated neurofilament H, is a commonly used specific marker for motoneuron staining (Carriedo, et al., 1996). Motoneuron survival was assessed by direct counting of large SMI-32 positive cells (cell bodies >25 μm) displaying intact neurites longer than three cell diameters. Microglial cultures were stained by OX42 antibody (1:100, Chemicon) and

biotinylated goat anti-rat IgG (1:200, Serotec). Images of microglia were obtained using a Zeiss Imager-Z1m microscope equipped with a Zeiss AxioCam MRc5 color camera and Zeiss digital image analysis system (Karl Zeiss, Oberkochen, Germany).

ELISA

ELISA Duoset kit (R & D Systems) was used to determine the concentration of TNF-α, IL-1β, and free IGF-1 protein in cell culture supernatant according to manufacture's instructions.

Superoxide assay

The release of superoxide was determined by WST-1 assay (Tan and Berridge, 2000). Briefly, microglia cultures grown in 96-well plates were treated with $mSOD1^{G93A}$ and $SOD1^{WT}$ with or without CD14-Ab for 4 days. After washing, WST-1 (250μM) and catalase (10U/ml) in HBSS were added to each well. The reaction was started by the addition of 800nM phorbol myristate acetate (PMA) (final volume of 200μl/well). The OD values at 450nm were measured at 0 min (served as background) and 5 hrs in a microplate reader. The increase of absorbance was used to determine $O_2\cdot^-$ generation.

Immunoprecipitation and western blot

$mSOD1^{G93A}$ or $SOD1^{WT}$ (12μg) was incubated with recombinant murine soluble CD14 (rCD14, Cell Sciences, 5.4 μg) at 4°C overnight. Anti-CD14 rabbit antiserum (Cell sciences, 2ul) was added and incubated for 30 min. Immunoprecipitation was performed by incubation with protein G-sepharose. After centrifugation, pellets were run through 15% gel (Bio-Rad Laboratories) and electroblotted onto PVDF membranes (Millipore). After blocking, blots were incubated with anti-SOD1 antibody (1:1000; Calbiochem) and peroxidase-conjugated anti-sheep IgG (1:2000, Vector Laboratories). The ECL plus detection system (Amersham Pharmacia Biotech) was used for protein detection. Negative control conditions include incubation of $mSOD1^{G93A}$ only or rCD14 only with anti-CD14 Ab. iNOS and β-actin were detected by anti- iNOS antibody (1:400; Chemicon) and anti- β-actin antibody (1:10,000; Sigma), respectively.

Quantitative RT-PCR

Cells were lysed in TRIzol Reagent (Invitrogen) and total RNA was prepared according to the manufacturer's instructions. RNA was further purified by RNeasy kit (Qiagen). Real-time RT-PCR was performed using one-step RT-PCR kit with SYBR Green (Bio-Rad Laboratories or Qiagen) and the iQ5 Multicolor Real-time PCR detection System (Bio-Rad Laboratories) according to the manufacturer's recommendations. The RT-PCR conditions were as follows: NOX2 primers: 5′-TGA ATG CCA GAG TCG GGA TTT-3′ and 5′-CCC CCT TCA GGG TTC TTG ATT T-3′, Tm=60°C; iNOS primers: 5′-CAG CAC AGG AAA TGT TTC AGC-3′ and 5′-TAG CCA GCG TAC CGG ATG A-3′, Tm=55°C; β-actin primers: 5′-TTG CTG ACA GGA TGC AGA AG-3′ and 5′-CAG TGA GGC CAG GAT AGA GC-3′, Tm=69.1°C or 58.8°C. Primer efficiency was assessed by analyzing a series dilution of RNA. The relative expression levels of each mRNA were calculated using the ΔΔCt method normalizing to β-actin and relative to the control samples. The presence of one product of the correct size was verified by both 2% agarose gel electrophoresis and melt curve analyses containing a single melt curve peak.

Statistics

Comparisons were performed by a one-way analysis of variance (ANOVA) when WT microglia were studied. Differences among WT and $CD14^{-/-}$ groups were analyzed using a two-way ANOVA. Onset and survival time differences determined using Kaplan–Meier

survival statistics (log-rank-sum test; Number Cruncher Statistical Systems, Kaysville, UT). Data are expressed as mean±SE and *p* values less than 0.05 were considered statistically significant.

Results

Extracellular mSOD1^{G93A} is not directly toxic to isolated motoneurons

Extracellular mSOD1^{G93A} has been documented to cause neuronal death in spinal cord mixed cell cultures (Urushitani et al., 2006). In the present study, 5 μg/ml metallated mSOD1^{G93A} was incubated with primary motoneuron cultures (>90% purity) to examine the direct effect of extracellularly applied mSOD1^{G93A} on motoneuron survival. After 7 days, motoneuron survival (94.7±3.2%) was not compromised compared with untreated-motoneuron (100%, representing 248±88 counted cells/coverslip; p=0.18) or SOD1^{WT}-treated cultures (104±9.0%; p=0.37; Fig. 1). Thus, after 7 days *in vitro*, mSOD1^{G93A} has no direct toxic effects on motoneurons.

Extracellular mSOD1 morphologically and functionally activates microglia

Initial experiments revealed that mSOD1^{G93A} increased microglial production of TNF-α in both a dose- and time-dependent manner (Fig. 2A,B). To determine the direct effects of mSOD1^{G93A} on microglia, 1 μg/ml mSOD1^{G93A} was added to primary microglial cultures and incubated for 48hrs. Incubation of microglia with mSOD1^{G93A} protein induced morphological changes of microglia with cell shape changing from a ramified configuration (Fig. 2C) into enlarged amoeboid cell bodies with vacuoles (Fig. 2E), suggestive of an activated microglial morphology. In contrast, most SOD1^{WT}-treated microglia maintained a ramified morphology; only a small percentage of SOD1^{WT}-treated microglia had an amoeboid shape (Fig. 2D). As an index of "functional" microglial activation, the levels of TNF-α and IL-1β were measured in the culture supernatants (Fig. 2G,H). TNF-α and IL-1β protein production increased 39-fold and 5.4-fold, respectively, in mSOD1^{G93A}-treated microglia compared with SOD1^{WT}-treated microglia (p=8E-5 and p=0.006, respectively). No changes in TNF-α and IL-1β production were observed between untreated and SOD1^{WT}-treated microglia. NOX2, a subunit of NADPH oxidase predominantly expressed in phagocytic cells, is the major source of microglial superoxide. Following treatment with mSOD1^{G93A}, microglial NOX2 mRNA was upregulated compared with SOD1^{WT}-treated microglia (p=0.0003; Fig. 2I). Superoxide released from microglia was also evaluated after treatment with mSOD1^{G93A} protein. While no difference was observed after 2 days, microglia incubated with mSOD1^{G93A} for 4 days produce more superoxide than SOD1^{WT}-treated microglia (4.8-fold increase, p=0.001; Fig. 2K); untreated and SOD1^{WT}-treated microglia released similar levels of superoxide. Since inducible nitric oxide synthase (iNOS) is upregulated after microglial activation (Zhao et al., 2004; Xiao et al., 2007), iNOS mRNA expression in microglia was examined using quantitative RT-PCR after a 2-day-treatment with mSOD1^{G93A} (Fig. 2J). Although it was undetectable in untreated microglia, iNOS mRNA increased 140-fold in mSOD1^{G93A}-treated microglia compared with SOD1^{WT}-treated microglia (p=0.001). Furthermore, after incubation for 2 days, mSOD1^{G93A} reduced microglial insulin-like growth factor-1 (IGF-1) production compared with SOD1^{WT} (p=0.0003; Fig. 2L). To determine whether the microglial response to mSOD1^{G93A} protein was a unique response to the specific mutation, microglia were treated with a different mutant SOD1 protein, mSOD1^{G85R}. The extracellular mSOD1^{G85R} morphologically activated microglia (Fig. 2F) and increased NOX2 and iNOS mRNA expression in microglia to a similar extent as mSOD1^{G93A} (Fig. 2I,J). The two mSOD1 proteins, which differ with respect to SOD1 enzymatic activity (Borchelt et al., 1994), had similar activating effects on microglia.

Since mSOD1^{G93A} has been reported to induce microglial proliferation (Urushitani et al., 2006), microglial numbers were counted to evaluate the influence of cell number on the increased markers of "functional" microglial activation. Microglial numbers in the mSOD1^{G93A}-treated cultures (1541±261 counted cells/coverslip) were only 2-fold greater than the numbers of microglia in untreated (720±65 counted cells/coverslip) or SOD1^{WT}-treated cultures (792±91 counted cells/coverslip). Therefore, the fact that the 4.8-140 fold increase in markers for microglia activation following treatment with mSOD1^{G93A} was greater than the two-fold microglia proliferative response induced by mSOD1^{G93A} suggests that mSOD1^{G93A} directly activates microglia and enhances the release of these toxic molecules from each microglial cell.

mSOD1^{G93A}-activated microglia are neurotoxic

To ascertain whether mSOD1^{G93A}-activated microglia are injurious to motoneurons, primary motoneurons were co-cultured with microglia in the presence of 1 μg/ml mSOD1^{G93A} or SOD1^{WT}, the identical concentration used to activate microglia. Incubating primary motoneurons and microglia with mSOD1^{G93A} for 2 days did not induce motoneuron injury when compared with untreated or SOD1^{WT}-treated co-cultures. However, prolonging the incubation time to 7 days and increasing the dose of mSOD1^{G93A} to 5 μg/ml, a dose shown above not to be directly toxic to motoneurons, resulted in motoneuron loss in mSOD1^{G93A}-treated co-cultures compared with SOD1^{WT}-treated co-cultures (p=0.007); motoneuron survival in SOD1^{WT}-treated co-cultures was similar to untreated co-cultures (p=0.61; Fig. 3A). Motoneuron morphology in the co-cultures was observed by immunostaining with anti-SMI-32 (Carriedo, et al., 1996). Surviving motoneurons were smaller and had fewer neurites in the mSOD1^{G93A}-treated microglia/motoneuron co-cultures. Motoneurons in SOD1^{WT}-treated co-cultures were uninjured and maintained large somas with extensive neuritic arborizations (Fig. 3B); motoneuron morphology was similar in appearance to the motoneurons in untreated co-cultures or when motoneurons were cultured in the absence of microglia. These data, in combination with the data presented in Fig. 1, suggest that the toxic effects of mSOD1^{G93A} are mediated predominantly through microglia; in the absence of microglia, 5 μg/ml mSOD1^{G93A} was not detectably toxic to motoneurons after 7 days.

Nitric oxide and superoxide mediates mSOD1^{G93A}-induced microglia toxicity

To explore why microglia treated with 1 μg/ml mSOD1^{G93A} for 2 days could not induce detectable motoneuron injury, the levels of superoxide and iNOS were assayed after different treatment periods. At 2 days, 1 μg/ml mSOD1^{G93A} did not increase the release of superoxide from microglia (p=0.2), while after 4 days, mSOD1^{G93A} treated microglia produced higher levels of superoxide than either untreated or SOD1^{WT}-treated co-cultures (p=0.001 and p=0.002, respectively; Fig. 3C). In addition, although iNOS protein was not detectable by western analyses from microglia after 2 days of incubation with 1 μg/ml SOD1^{WT} or mSOD1^{G93A}, at 7 days, 5 μg/ml mSOD1^{G93A}-treated microglia expressed more iNOS protein than SOD1^{WT}-treated microglia (p=0.03; Fig. 3D). As additional evidence for the important roles that superoxide and nitric oxide play in mSOD1^{G93A}-mediated neurotoxicity, apocynin, a NADPH oxidase inhibitor, and L-NIL, an iNOS inhibitor, were added to the microglial/motoneuron co-cultures. The combined application of apocynin and L-NIL to the mSOD1^{G93A}-treated microglial/motoneuron co-cultures restored motoneuron survival compared with mSOD1^{G93A}-treated co-cultures in the absence of apocynin and L-NIL (p=0.03; Fig. 3E).

Extracellular mSOD1^{G93A} activates microglia through CD14 and Toll-like receptors

Fassbender et al. (2004) previously demonstrated that β-amyloid interacts with CD14, a lipopolysaccharide (LPS) receptor known to be involved with cellular activation. Using the

immunoprecipitation technique used by Fassbender et al. (2004), mSOD1^{G93A} was co-incubated with recombinant soluble murine CD14 (rCD14) protein. The complexes were subsequently immunoprecipitated with anti-CD14 antibody, and western analyses were performed to detect SOD1. The results showed a clear SOD1 band, demonstrating binding of mSOD1^{G93A} to CD14 (Fig. 4A). Although anti-CD14 did immunoprecipitate SOD1^{WT}, the signal intensity was less compared with CD14 and mSOD1^{G93A}, suggestive of a stronger interaction between CD14 and mSOD1^{G93A} than CD14 and SOD1^{WT} proteins, possibly due to the misfolded confirmation of mSOD1. This weak interaction between CD14 and SOD1^{WT} may help explain the modest, though non-significant, increase in IL-1β and superoxide release from SOD1^{WT} stimulated microglia. The omission of rCD14 protein prevented the precipitation of mSOD1^{G93A}.

To determine whether CD14 mediates microglial activation induced by mSOD1^{G93A}, an antibody to CD14 was added to microglial cultures 2hrs prior to the addition of SOD1^{WT} or mSOD1^{G93A}. After incubating for 2 days, the CD14 blocking antibody attenuated TNF-α production by 86% and IL-1β release by 77% from mSOD1^{G93A}-treated microglia (p=0.0001 and p=0.0044, respectively; Fig. 4B,C). Increases in NOX2 mRNA levels after 2 days and superoxide release after 4 days were also inhibited by the CD14 antibody in mSOD1^{G93A}-treated microglial cultures (p=0.0009 and p=0.04, respectively; Fig. 4D); the change in microglial NOX2 mRNA expression closely mirrored the change in microglial superoxide production following treatment with either mSOD1^{G93A} or the combination of mSOD1^{G93A} and CD14 antibodies. In addition, increased iNOS expression by extracellular mSOD1^{G93A} to microglia cultures was suppressed 88% with CD14 blocking antibodies (p<0.01; Fig. 4E). CD14 blocking antibodies also inhibited the decrease in IGF-1 levels in mSOD1^{G93A}-treated microglia (p=0.016; Fig. 4F).

To further substantiate the role of CD14 and its involvement in mSOD1^{G93A}-mediated microglial activation, microglia were isolated and cultured from CD14$^{-/-}$ mice. Microglia lacking CD14 produced 96% less TNF-α following extracellular treatment with mSOD1^{G93A} than similarly cultured WT (CD14$^{+/+}$) microglia (p=0.0006; Fig. 4F). Thus, *in vitro*, the activating effects of mSOD1^{G93A} on microglia are possibly transduced through a CD14 dependent pathway.

Because CD14 mediates signal transduction in conjunction with TLR2 and TLR4 (Leung et al., 2005), the inhibitory effects of extracellular mSOD1^{G93A}-induced microglia activation were assayed using blocking antibodies specific for TLR2 and/or TLR4. TNF-α production in mSOD1^{G93A}-treated microglia was reduced following the addition of both TLR2 and TLR4 blocking antibodies (p=0.008; Fig. 5). Motoneurons express CD14, TLR2 and TLR4 mRNAs (Tang et al., 2007); therefore, the lack of direct toxic effects of mSOD1^{G93A} on motoneurons was not due to absence of these receptors.

CD14 is involved in mSOD1^{G93A}-induced neurotoxicity *in vitro*

To determine whether the neurotoxicity of mSOD1^{G93A}-activated microglia is also dependent on CD14, both microglia and motoneurons were isolated from CD14$^{-/-}$ mice. When CD14$^{-/-}$ motoneurons were co-cultured with CD14$^{-/-}$ microglia, motoneuron survival was partially increased compared with WT microglial/motoneuron co-cultures after incubation with 5 μg/ml mSOD1^{G93A} for 7 days (p<0.02; Fig. 6). These data indicate that CD14 is involved in the extracellular mSOD1^{G93A}-induced microglial neurotoxicity, although CD14 may not be the only pathway that mediates these neurotoxic effects.

Disease course is unaltered in mSOD1^{G93A}/CD14$^{-/-}$ mice

To examine whether the lack of CD14 expression alters the *in vivo* course of disease, mSOD1^{G93A} mice were bred with CD14$^{-/-}$ mice to generate both mSOD1^{G93A}/CD14$^{+/-}$ and mSOD1^{G93A}/CD14$^{-/-}$ mice. However, there were no significant differences in either symptom onset or lifespan between mSOD1^{G93A}/CD14$^{+/-}$ and mSOD1^{G93A}/CD14$^{-/-}$ mice using previously defined criteria (Beers et al., 2008; Beers et al., 2006; Fig. 7A,B). Furthermore, the disease progression profiles were similar between mSOD1^{G93A}/CD14$^{+/-}$ and mSOD1^{G93A}/CD14$^{-/-}$ mice (Fig. 7C).

Discussion

Previous studies have reported that mSOD1^{G93A} or oxidized SOD1^{WT} injure motoneurons and trigger microgliosis in spinal cord cultures (Urushitani et al., 2006; Ezzi et al., 2007). In those studies, the direct neurotoxic properties of mSOD1^{G93A} were not differentiated from the possible indirect toxic effects mediated through microglia, a component of the innate immune system. The present study demonstrates that exogenous mSOD1^{G93A} has minimal direct toxic effects on motoneurons and only when microglia were incubated with mSOD1^{G93A} was mSOD1^{G93A} toxic to motoneurons. Furthermore, mSOD1^{G93A} was demonstrated to bind CD14 and initiate microglia activation in concert with TLR2 and TLR4. Thus, in addition to the toxic intracellular effects of mSOD1 (Beers et al., 2006; Xiao et al., 2007), these data suggest an additional mechanism whereby mSOD1 gains an extracellular toxic function.

To test the hypothesis that microglia are involved in mSOD1^{G93A}-mediated neurotoxicity, motoneurons were co-cultured with microglia. Using the same conditions that were used in the motoneuron only cultures, co-cultures treated with the same dose of mSOD1^{G93A} over an identical time period, the presence of microglia caused motoneuron death. The ratio between microglia and motoneurons in these co-cultures was 4:3, a substantially lower number of microglia to motoneurons than that is found in lumbar spinal cords of mSOD1 mice. This reduces the possibility that excess microglia may lead to spurious neurotoxicity. Additionally, because both components of these co-cultures were greater than 95% pure, it is unlikely that other cells contributed to the toxicity. Therefore, the present study establishes that the neurotoxic properties of exogenous mSOD1^{G93A} were indirect and mediated through microglia; since the SOD1^{WT} protein was purified identically to mSOD1, the observed effects were due to mutations in the SOD1 protein and not due to the protein preparation.

Several lines of evidence document the importance of microglia in mSOD1-induced motoneuron death. Microglia isolated from mice over-expressing mSOD1^{G93A} induced more motoneuron injury than WT microglia (Xiao et al., 2007). *In vivo* studies have shown that replacing all mSOD1 expressing microglia with WT microglia or reducing expression of mSOD1 in microglia/macrophages, slowed motoneuron loss and prolonged disease duration and survival (Beers et al., 2006; Boillee et al., 2006). In addition, a recent study demonstrated the presence of microgliosis in mice homozygously expressing high levels of mSOD1^{G93A} specifically in neurons (Jaarsma et al., 2008). These data indicate that other cells such as microglia may actively contribute to the disease of ALS.

Treatment for 2 days with 1 μg/ml of mSOD1^{G93A} morphologically and functionally activated microglia. However, motoneuron death was only observed after 7 days of treatment with 5 μg/ml mSOD1^{G93A}. A comparison of superoxide and iNOS protein between microglia from 2-day-treatment and 4- or 7-day-treatment with mSOD1^{G93A} demonstrated that the levels of free radicals increased only after 4 days. The ability of apocynin and L-NIL to enhance motoneuron survival after 7 days also supports roles of

superoxide and nitric oxide in the mSOD1^{G93A} neurotoxicity. The fact that the loss of NOX2 expression or NADPH oxidase inhibition by apocynin slowed disease progression and prolonged survival of mSOD1^{G93A} mice (Marden et al., 2007; Harraz et al., 2008) emphasizes the importance of oxidative stress in ALS. Thus, the neurotoxic properties of mSOD1^{G93A} are temporal and dependent upon sufficient production of free radicals.

These data are in accord with our previous report documenting that motoneuron injury resulted from the reaction of superoxide and nitric oxide, which form the highly unstable and toxic compound peroxynitrite. Peroxynitrite initiates motoneuron injury by increasing the susceptibility of the motoneuron AMPA/kainate receptor to the toxic effects of glutamate; CNQX treatment prevented motoneuron injury (Zhao et al., 2004). Although other studies have reported that microglia-induced cortical neural injury is signaled through an NMDA receptor (Takeuchi et al., 2005), the NMDA receptor antagonist APV did not protect motoneurons from microglia-mediated injury (Zhao et al., 2004). Therefore, enhancing the vulnerability of motoneurons to glutamate by altering the AMPA/kainate receptor may be an underlying mechanism of extracellular mSOD1-induced neurotoxicity.

Because of the technical complexity, it is very difficult to determine the precise levels of extracellular mSOD1 in spinal cords of mSOD1 transgenic mice - lysed neurons within the spinal cord would contribute to the extracellular pool of mSOD1^{G93A}. However, the estimated mSOD1 concentration in the CNS of mSOD1^{G93A} mice is 4000 μg/ml (Gurney et al., 1994). Furthermore, extracellularly released mSOD1, mediated by chromogranin (Urushitani et al., 2006) or lysed motoneurons, may have a much higher effective local concentration. Thus, compared with the CNS concentration of 4000 μg/ml, the dose of mSOD1 used in the present study (1 or 5 μg/ml) is far below that observed in the CNS of mSOD1 mouse.

Since CD14 is a pattern recognition receptor and mutations in, or oxidation of, SOD1 leads to misfolded protein (Furukawa and O'Halloran 2005; Furukawa et al., 2006; Kabashi et al., 2007), mSOD1^{G93A} may be recognized by CD14 and in turn activate microglia. This study demonstrated that mSOD1^{G93A} binds to CD14 and that CD14 blocking antibody attenuated the production of several pro-inflammatory cytokines, free radicals and increase IGF-1 release from microglia treated with mSOD1^{G93A}. CD14$^{-/-}$ microglia treated with mSOD1^{G93A} produced less TNF-α and when co-cultured with CD14$^{-/-}$ motoneurons, motoneuron survival was enhanced compared with WT microglia/motoneuron co-cultures. When mSOD1^{G93A} treated CD14$^{-/-}$ microglia were co-cultured with WT motoneurons (data not shown), the increased motoneuron survival was modest, most likely due to the release of soluble CD14 from the motoneurons. Therefore, these data support the important role of CD14 in the activation and toxicity of mSOD1^{G93A}-treated microglia.

Two important CD14 co-receptors are TLR2 and TLR4. Using antibodies that block TLR2/TLR4 signal induction inhibited microglial activation caused by extracellular mSOD1^{G93A}, suggesting that TLRs are involved in the signal cascade of microglial activation. However, individually each inhibitory antibody resulted in only a slight inhibitory effect. Only with the combination of TLR2 and TLR4 inhibitory antibodies was the microglial suppression significant; therefore, the toxic signals induced by extracellular mSOD1^{G93A} are possibly transduced through both TLR2 and TLR4 pathways. Other lines of evidence indeed suggest that CD14/TLRs may contribute to the inflammatory responses initiated by microglia (Olson and Miller, 2004; Jack et al., 2005). Expression of CD14 is increased in spinal cords of both ALS patients and mSOD1 mice (Henkel et al., 2004; Henkel et al., 2006). Upregulation of CD14 and TLR2 in phagocytes are common in transgenic models of Alzheimer's disease, Parkinson's disease, and ALS (Letiembre et al., 2007). Aβ fibrils bind to CD14 and activate microglia (Fassbender et al., 2004), and anti-CD14 strategies reduced the Aβ-stimulated

microglia neurotoxicity (Bate et al., 2004). Additionally, chronic stimulation of CD14/TLR pathway by LPS was found to exacerbate disease in ALS mice (Nguyen et al., 2004).

An important component of the CD14/TLR signaling cascade is MyD88, which is utilized by all TLR except TLR3 and part of TLR4 (Watters et al., 2007). In agreement with our present data, Kang and Rivest (2007) demonstrated that mSOD1 injected into the central nervous system of mice activated microglia and increased TLR2 expression via a MyD88 dependent pathway. While the lack of MyD88 expression in mSOD1^{G37R} mice did not influence the disease course, MyD88-deficient bone marrow cells accelerated disease onset and reduced survival in mSOD1^{G37R} mice, suggesting that peripheral cells expressing MyD88 are beneficial; the absence of an altered disease course in MyD88 deficient mSOD1^{G37R} mice may be that any attributable protective effects of peripheral MyD88 cells are counteracted by the toxic effects of CNS MyD88 cells. In our study, the lack of CD14 expression in mSOD1 mice also did not influence disease onset or survival. Since CD14 and MyD88 share similar downstream pathways, a similar hypotheses could be applicable to CD14, namely that the peripheral and CNS CD14 cells may have opposite functions. Therefore, to further examine the effects of CD14 or the TLR in mSOD1 mice, these receptors may need to be specifically depleted in the CNS. Secondly, these *in vivo* results suggest that in the absence of CD14, other receptors may substitute for CD14 and mediate mSOD1-induced microglial neurotoxicity. Echchannaoui et al. (2005) reported that although knocking-out CD14 did not impair systemic host defenses, CD14 deficiency caused excessive meningeal inflammation and early death of CD14$^{-/-}$ mice after *S. pneumoniae* infection. In our *in vitro* systems, the lack of CD14 expression only partially reversed motoneuron injury and TLR2/TLR4 were also involved in mSOD1^{G93A}-induced microglial activation. Unlike CD14, TLR can bind to ligands and directly transduce cellular signals. In addition, Aβ interacts with microglia to induce neurotoxicity through numerous microglial receptors including scavenger receptors (El Khoury et al., 2003, 1996), CD40 (Townsend et al., 2005), and complement receptors (Eikelenboom and Veerhuis, 1996). Collectively, these data support the notion of putative compensatory mechanisms due to the developmental absence of CD14 and that other microglial receptors also mediate extracellular mSOD1 neurotoxicity.

Microgliosis is also a prominent neuropathological feature observed in the spinal cords of sALS patients (McGeer and McGeer, 2002). Because altered SOD1 species are found within the spinal cords of sALS patients and since oxidized SOD1^{WT} can acquire toxic properties similar to mSOD1 triggering microgliosis (Gruzman et al. 2007; Ezzi et al., 2007), possibly linking a shared pathophysiologic pathway between sALS and fALS, the present data may be relevant to sALS. Thus, this study demonstrates a link between extracellular mSOD1, microglial activation, and neurodegeneration and adds to the cumulative evidence supporting the non-cell-autonomous nature of motoneuron toxicity in ALS.

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Abbreviations list

ALS	amyotrophic lateral sclerosis
fALS	familiar ALS
sALS	sporadic ALS
IGF-1	Insulin-like Growth Factor-1
LPS	lipopolysaccharide
SOD1	Cu^{2+}/Zn^{2+} superoxide dismutase 1
mSOD1	mutant SOD1

mSOD1^{G93A}	G93A mutant form of SOD1
SOD1^{WT}	wild-type SOD1
WT	wild-type
TLR	Toll-like receptor

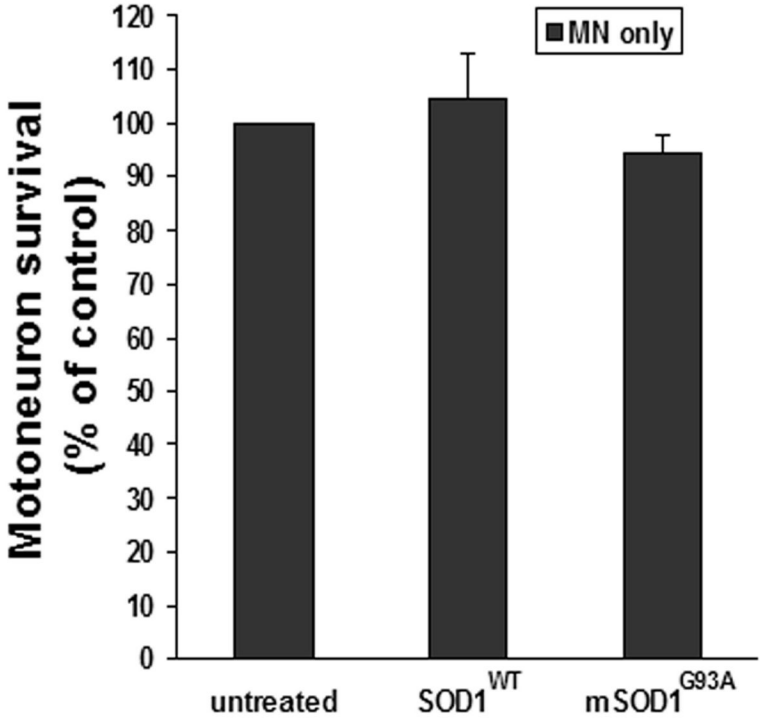

Fig. 1.

Extracellular mSOD1^{G93A} was not directly toxic to motoneuron cultures. Primary motoneuron cultures were treated with 5 μg/ml metallated (Cu/Zn) SOD1^{WT} or mSOD1^{G93A} for 7 days. Motoneuron survival was not decreased in mSOD1^{G93A}-treated motoneuron cultures compared with untreated or SOD1^{WT}-treated cultures. Motoneuron survival was expressed as a percentage of untreated cultures (100% represents 248±88 counted cells/coverslip). Data shown as mean±SE of three independent experiments with duplicate or triplicate wells. MN = motoneurons; SOD1^{WT} = wild-type SOD1; mSOD1^{G93A} = mutant SOD1^{G93A}.

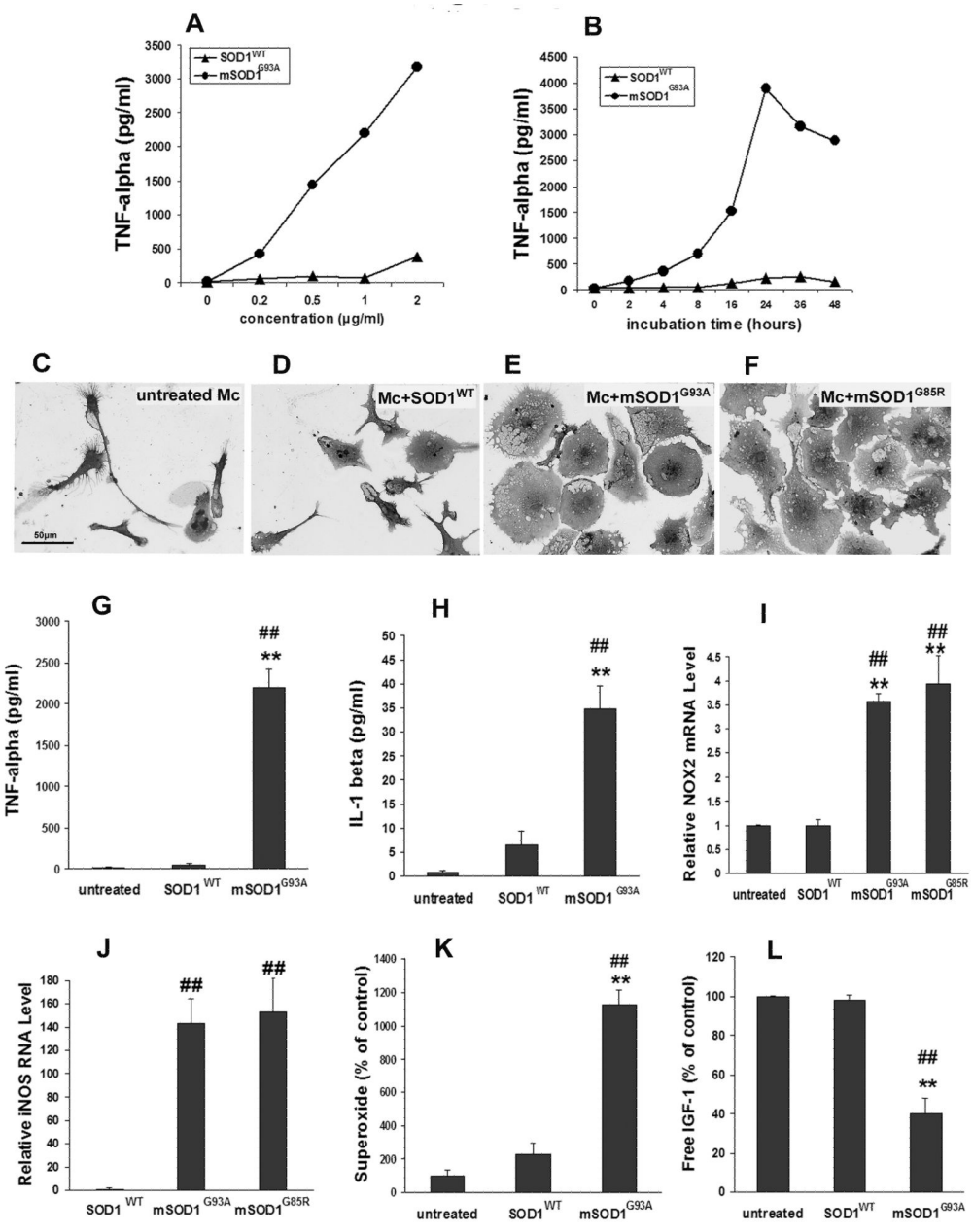

Fig. 2.

Effects of mSOD1^{G93A} and mSOD1^{G85R} on microglia cultures. **A-B:** Microglia were incubated with different concentrations (0-2 μg/ml) of SOD1^{WT} or mSOD1^{G93A} for 2 days **(A)** or with 1 μg/ml SOD1^{WT} or mSOD1^{G93A} for 0-48 hours **(B)**. TNF-α levels were measured as the index of microglial activation. **C-E:** Morphology of microglia without treatment **(C)**, treated with 1μg/ml SOD1^{WT} **(D),** 1 μg/ml mSOD1^{G93A} **(E)** or 1 μg/ml mSOD1^{G85R} **(F)** for 2 days. **G-L:** After a 2-day-treatment with 1 μg/ml SOD1^{WT}, mSOD1^{G93A}, or mSOD1^{G85R}, the production of TNF-α **(G)**, IL-1β **(H)**, NOX2 mRNA **(I)**, iNOS mRNA expression **(J)** increased in mSOD1^{G93A}- or mSOD1^{G85R}-treated microglial cultures compared with untreated or SOD1^{WT}-treated microglia cultures. **K:** mSOD1^{G93A}-treated microglia produced more superoxide than untreated or SOD1^{WT}-treated microglia at 4 days. **L:** Free IGF-1 was decreased in mSOD1^{G93A}-treated microglial cultures compared

with untreated or $SOD1^{WT}$-treated cultures. There was no difference between untreated and $SOD1^{WT}$-treated microglial cultures. Data shown as mean±SE of at least three independent experiments with duplicate or triplicate wells. Mc = microglia; $SOD1^{WT}$ = wild-type SOD1; $mSOD1^{G93A}$ = mutant $SOD1^{G93A}$, $mSOD1^{G85R}$ = mutant $SOD1^{G85R}$. $^{**}p<0.01$ vs untreated microglia; $^{\#\#}p<0.01$ vs $SOD1^{WT}$-treated microglia. Scale bar = 50 μm.

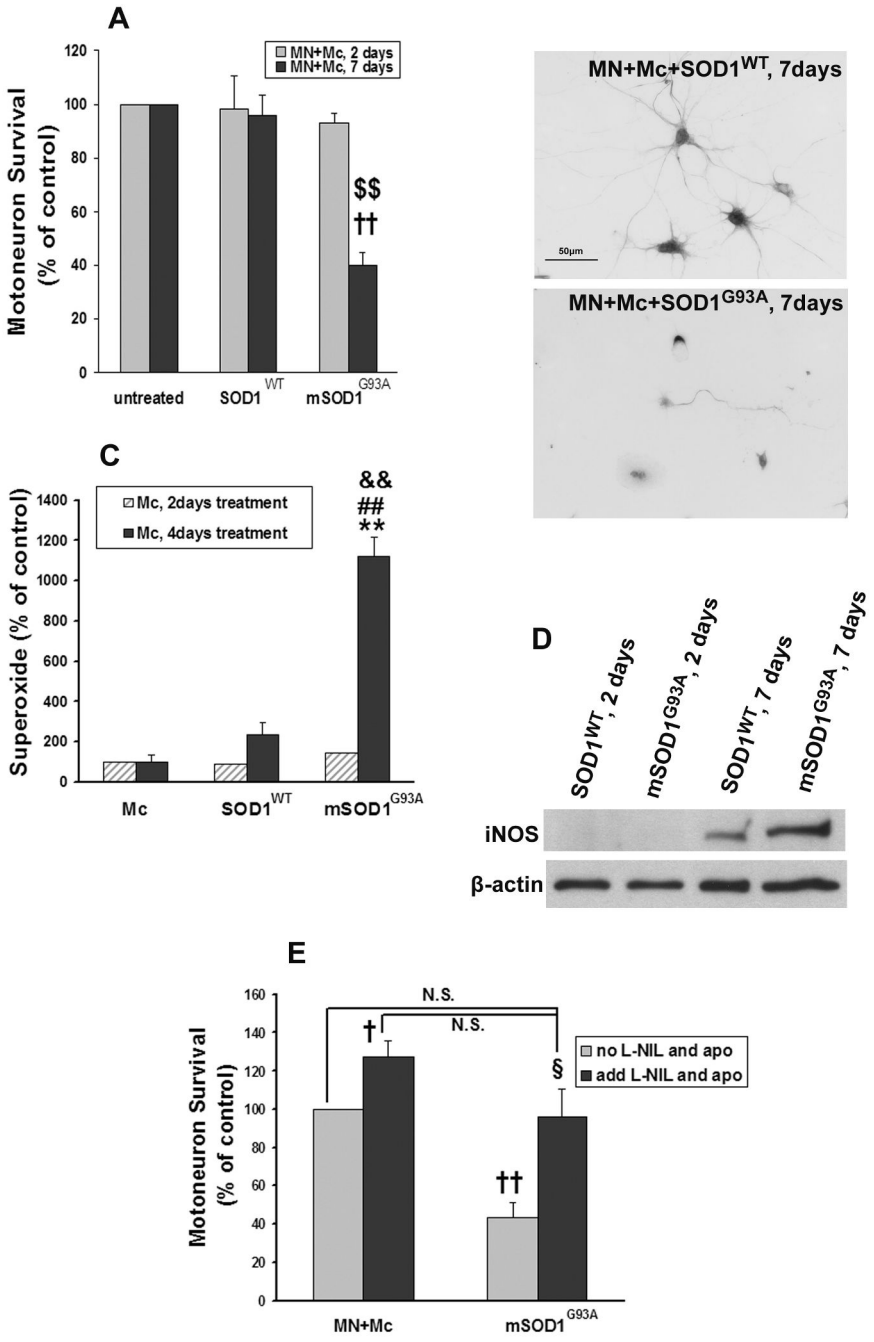

Fig. 3.

mSOD1^{G93A} induces motoneuron injury in the presence of microglia. **A:** mSOD1^{G93A} reduced motoneuron survival in microglial/motoneuron co-cultures after a 7-day-treatment with 5 μg/ml mSOD1^{G93A}. MN survival was expressed as a percentage of untreated co-cultures. **B:** Motoneuron morphology after a 7-day-treatment with 5 μg/ml SOD1^{WT} or mSOD1^{G93A}, stained by SMI-32. **C:** Microglia treated with mSOD1^{G93A} for 2 days did not produce more superoxide than untreated or SOD1^{WT}-treated microglia. After treatment for 4 days, superoxide levels in mSOD1^{G93A}-treated microglial cultures increased compared with untreated or SOD1^{WT}-treated microglia. **D:** iNOS was not detectible from microglia at 2 days with either SOD1^{WT} or mSOD1^{G93A}. At 7 days, mSOD1^{G93A}-treated microglia

express more iNOS than $SOD1^{WT}$-treated microglia. **E:** The addition of apocynin and L-NIL rescued motoneuron death induced by $mSOD1^{G93A}$-treated microglia. Motoneuron survival was expressed as a percentage of untreated co-cultures. MN = motoneurons; Mc = microglia; $SOD1^{WT}$ = wild-type SOD1; $mSOD1^{G93A}$ = mutant $SOD1^{G93A}$; Ab = antibody; apo = apocynin. $^{\dagger}p<0.05$, $^{\dagger\dagger}p<0.01$ vs untreated microglial/motoneuron co-cultures; $^{\$\$}p<0.01$ vs $SOD1^{WT}$-treated microglial/motoneuron co-cultures; $^{**}p<0.01$ vs untreated microglia; $^{\#\#}p<0.01$ vs $SOD1^{WT}$-treated microglia; $^{\&\&}p<0.01$ vs 2-day-treatment in each group; $^{\S}p<0.05$ vs $SOD1^{G93A}$-treated, “no apo and L-NIL” group; N.S. = no significant difference compared with “untreated groups with or without ‘apo and L-NIL’ ”. Data shown as mean±SE of three independent experiments with duplicate or triplicate wells.

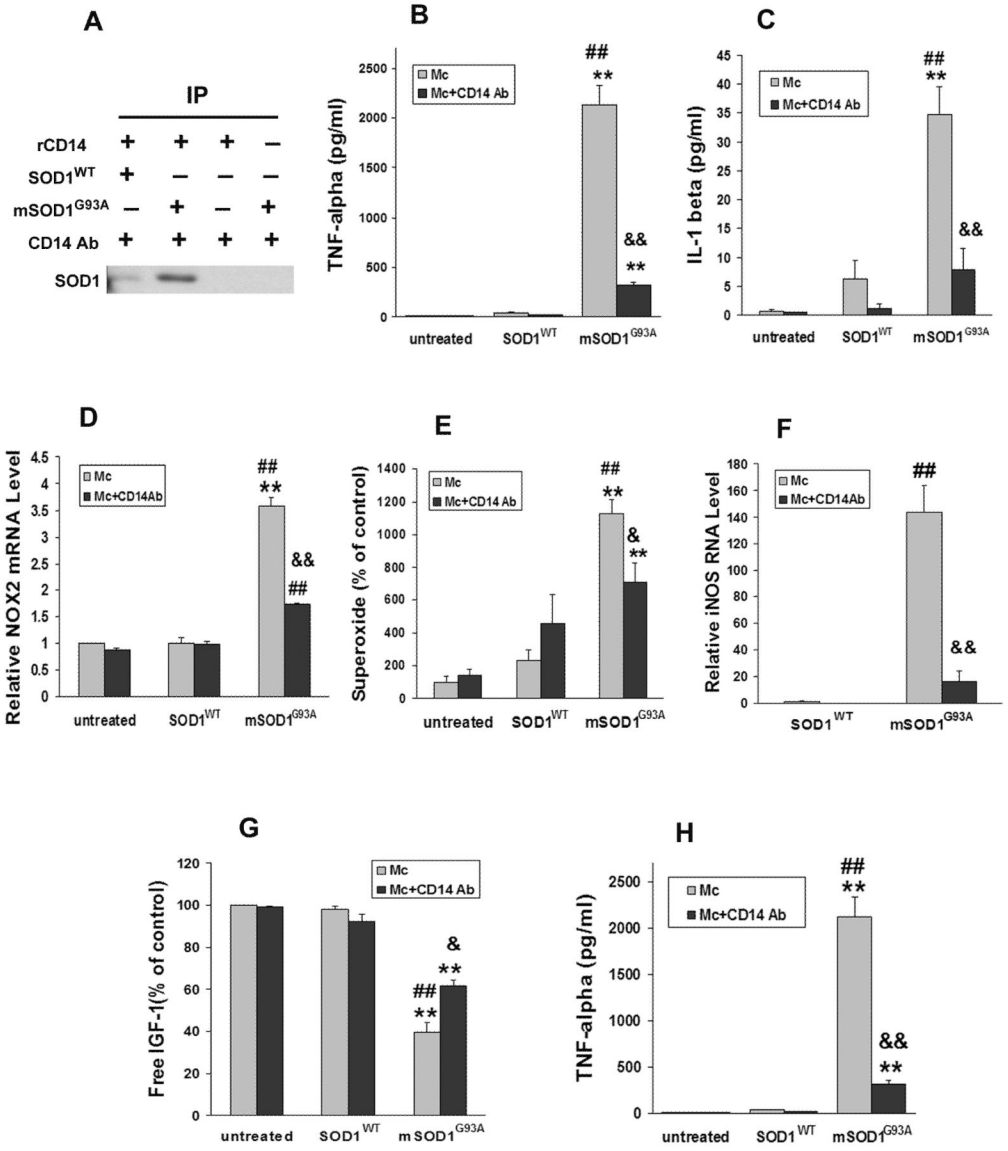

Fig. 4.

mSOD1^{G93A} activates microglia through CD14. **A:** Immunoprecipitation with anti-CD14 antibody and subsequent western blot with anti-SOD1 antibody showed binding of mSOD1^{G93A} to rCD14 was greater than SOD1^{WT}. Negative control included incubation of mSOD1^{G93A} only or rCD14 only with anti-CD14 antibody. **B-G:** The blocking CD14 antibody (1 μg/ml) attenuated mSOD1^{G93A}-induced microglial activation by suppressing the release of TNF-α **(B)**, IL-1β **(C)**, NOX2 mRNA **(D)**, superoxide **(E)**, iNOS expression **(F)**, and increasing the production of free IGF-1 **(G). H:** WT or CD14$^{-/-}$ microglia were treated with 1 μg/ml SOD1^{WT} or mSOD1^{G93A} for 2 days. mSOD1^{G93A} induced CD14$^{-/-}$ microglia to produce less TNF-α than WT microglia. Data shown as mean±SE of at least three independent experiments with duplicate or triplicate wells. Mc = microglia; SOD1^{WT} = wild-type SOD1; mSOD1^{G93A} = mutant SOD1^{G93A}; Ab = antibody. *p<0.05; **p<0.01 vs untreated microglia; $^{\#\#}p$<0.01 vs SOD1^{WT}-treated microglia; $^{\&}p$<0.05; $^{\&\&}p$<0.01 vs mSOD1^{G93A}-treated microglia.

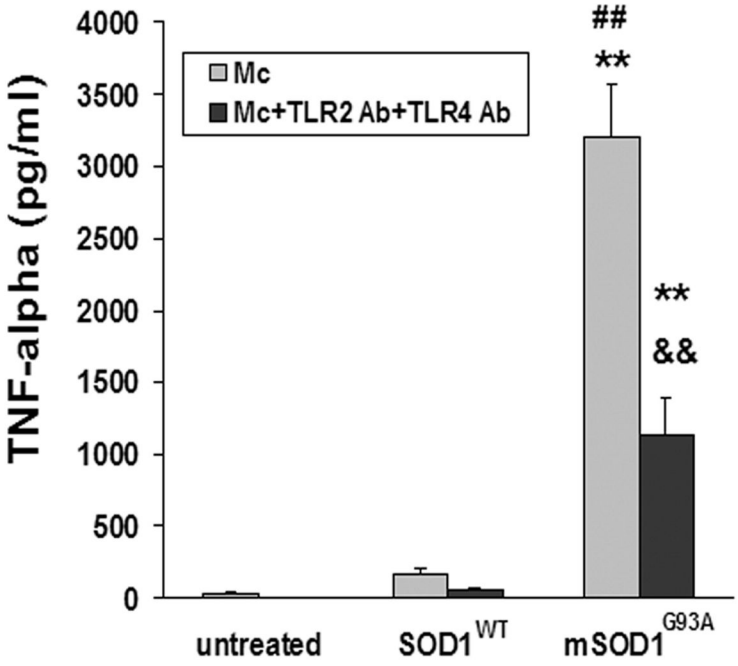

Fig. 5.

TLR2 and TLR4 participate in mSOD1^{G93A}-induced microglial activation. Combination of TLR2 and TLR4 blocking antibodies (1 μg/ml and 10 μg/ml, respectively) suppressed microglial production of TNF-α induced by mSOD1^{G93A}. Data shown as mean±SE of at least three independent experiments with duplicate or triplicate wells. Mc = microglia; SOD1^{WT} = wild-type SOD1; mSOD1^{G93A} = mutant SOD1^{G93A}; Ab = antibody. **p<0.01 vs untreated microglia; $^{\#\#}p$<0.01 vs SOD1^{WT}-treated microglia; $^{\&\&}p$<0.01 vs mSOD1^{G93A}-treated microglia.

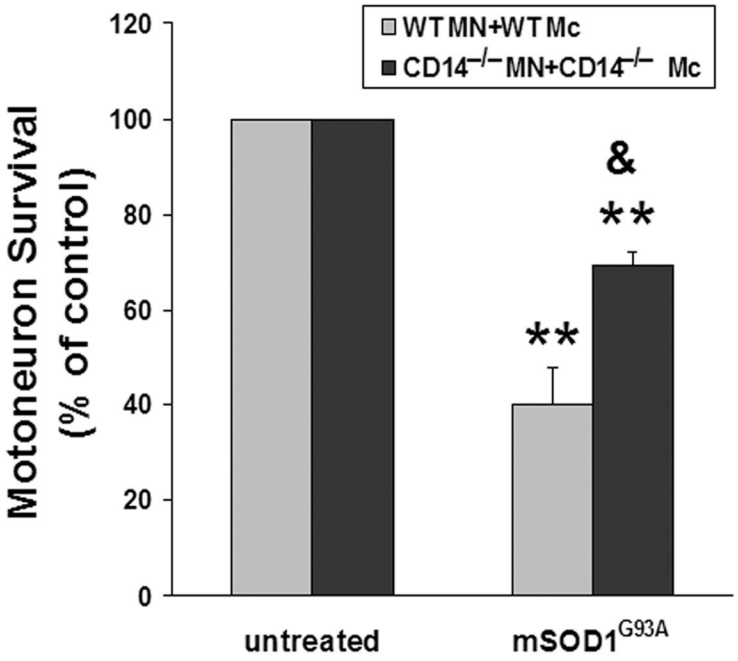

Fig. 6.

CD14 is involved in mSOD1^{G93A}-induced neurotoxicity *in vitro*. WT microglial/motoneuron co-cultures or CD14$^{-/-}$ microglial/CD14$^{-/-}$ motoneuron co-cultures were incubated with 5 μg/ml mSOD1^{G93A} for 7 days. Motoneuron survival was enhanced in mSOD1^{G93A}-treated CD14$^{-/-}$ co-cultures than mSOD1^{G93A}-treated WT co-cultures. Motoneuron survival was expressed as a percentage of untreated co-cultures in each genotype group. Data shown as mean±SE of three independent experiments with duplicate or triplicate wells. MN = motoneurons; Mc = microglia; SOD1^{WT} = wild-type SOD1; mSOD1^{G93A} = mutant SOD1^{G93A}. $^{**}p<0.01$ vs untreated co-cultures; $^{\&}p<0.05$ vs mSOD1^{G93A}-treated WT co-cultures.

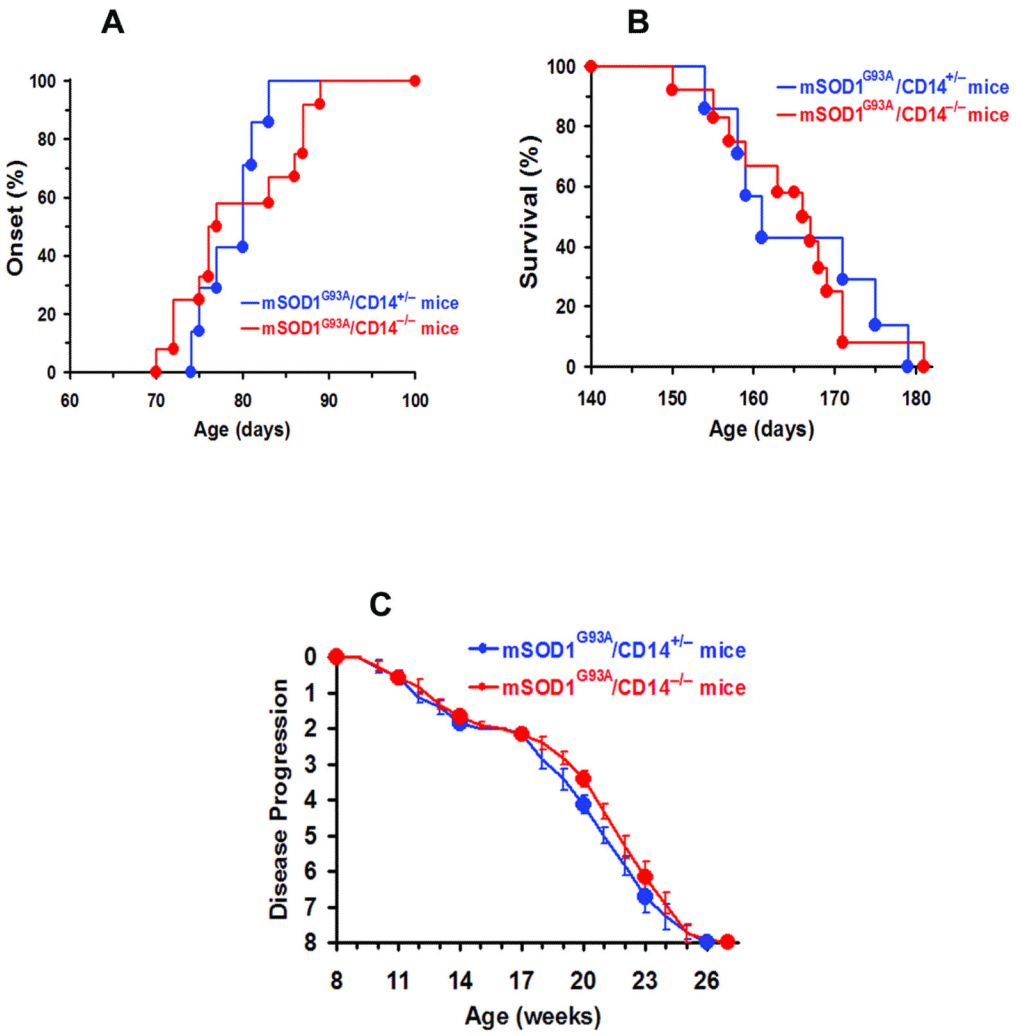

Fig. 7.

Disease course is unchanged in mSOD1^{G93A}/CD14$^{-/-}$ mice. The disease courses were compared between mSOD1^{G93A}/CD14$^{-/-}$ mice (n=12) and mSOD1^{G93A}/CD14$^{+/-}$ mice (n=7). The onset **(A)**, survival **(B)**, and disease progression **(C)** of these two groups were not different.