Supporting Information

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SI Materials and Methods

SOD1^{G93A} Mice. All transgenic animals were bred and maintained in our animal facility (1, 2). All mice were housed in microisolator cages within a modified pathogen-free barrier facility and had access to food and water *ad libitum.* All animal protocols were approved by the Methodist Research Institute's Institutional Animal Care and Use Committee in compliance with National Institutes of Health guidelines. Genomic tail DNA was isolated using a standard protocol.

Transgenic mice overexpressing the fALS associated G93A SOD1 mutation (G1H) (mSOD1^{G93A}) were originally purchased from Jackson Laboratories (Bar Harbor, ME); strain designations were as follows: B6SJL-Tg(SOD1*G93A)1Gur/J and B6.Cg-Tg(SOD1*G93A)1Gur/J]. mSOD1^{G93A} mice on a B6/SJL genetic background had a mean survival time of 132 days, whereas mSOD1^{G93A} mice on a C57BL/6 genetic background survived for 160 days. Transgenic mSOD1^{G93A} animals were identified and copy number verified by quantitative PCR using the antisense and sense SOD1 oligonucleotides 5′-AATTTGTGTCTACTCAGTCAA-3′ and 5′-TCACTTTGATTGTTAGTCGCG-3′, respectively. The PCR conditions for genomic tail DNA in a 50-μl reaction using an Eppendorf Mastercycler gradient thermocycler (Westbury, NY) were as follows: 2 min at 94°C; then 35 cycles of 20 sec at 94°C, 20 sec at 60°C, and 30 sec at 72°C; then 2 min at 72°C and held at 4°C until the samples were run on a 1% agarose gel in 1X TAE buffer. All transgenic mice were housed in microisolator cages within a modified pathogen-free barrier facility and had access to food and water *ad libitum.* PU.1$^{-/-}$ mice were bred with B6SJL-Tg(SOD1*G93A)1Gur/J, and RAG2$^{-/-}$ and CD4$^{-/-}$ mice were bred with B6.Cg-Tg(SOD1*G93A)1Gur/J.

PU.1 Mice. To prevent the possibility of graft-versus-host disease and/or alterations of onset and survival times resulting from differing genetic backgrounds, PU.1 heterozygous (PU.1$^{+/-}$) mice were first backcrossed with B6/SJL mice for 10 generations. PU.1$^{+/-}$ mice were then mated with mSOD1^{G93A} mice to produce PU.1$^{+/-}$ and mSOD1^{G93A}/PU.1$^{+/-}$ mice (2, 3). Finally, breeding mSOD1^{G93A}/PU.1$^{+/-}$ with PU.1$^{+/-}$ generated PU.1$^{-/-}$ and mSOD1^{G93A}/PU.1$^{-/-}$ mice. Within 12 h of birth, PU.1 litters were injected with antibiotic (Baytril, Bayer HealthCare). Giemsa staining was used to identify the PU.1$^{-/-}$ pups from a drop of tail blood smeared onto a glass slide and dried for 10 min at room temperature (RT). The Giemsa stain (Sigma) covered the blood smear and incubated for 5 min at RT. Phosphate-buffered saline (PBS) was added to the slide just to cover and incubated for 10 min at RT. The slides are briefly washed and dehydrated in 70% ethanol. The slides were then air dried and cover slipped using glycerol and were immediately examined. The presence or absence of the PU.1 gene was then confirmed by PCR (Eppendorph Mastercycler) using 250 ng tail DNA and using Eppendorf TaqDNA polymerase according to the manufacturer's instructions. The following primers were used: A) forward PUKO, 5′-GCCCCGGATGTGCTTCCCTTATCAAACC-3′; B) reverse 920, 5′-TGCCTCGGC-CCTGGGAATGTC-3′; C) reverse 0.Neo.1, 5′-CGCACGGGTGTTGGGTCGTTTGTTGG-3′. The A and B primers were used to identify mice with the WT PU.1 gene, and the A and C primers were used to identify mice with the PU.1 deletion. The PCR protocol was as follows: 92°C for 2 min; [denatured at 92°C for 1 min, annealed at 65°C for 2 min, and extended at 72°C for 3 min] for 45 cycles; then 72°C for 7 min, and finally held at 4°C. The PCR products were resolved by 1.0% agarose/ethidium bromide gel electrophoresis and photographed under UV illumination. PU.1$^{-/-}$ mice received daily antibiotic injections for the first 3 weeks after birth. PU.1$^{-/-}$, PU.1$^{+/-}$, and WT mice were weighed and observed for signs of motoneuron disease daily. Transgenic mSOD1^{G93A} mice animals were identified by quantitative PCR to ensure mSOD1^{G93A} transgene copy numbers remain stable.

RAG2 Mice. RAG2$^{-/-}$ mice were initially bred with B6.Cg-Tg(SOD1*G93A)1Gur/J for at least eight generations. The presence or absence of the RAG2 gene was determined by PCR (Eppendorf Mastercycler) using 250 ng of tail DNA and using Eppendorf TaqDNA polymerase according to the manufacturer's instruction. The following primers were used: Rag A) 5′-GGGAGGACACTCACTTGC-CAG-3′ and Rag B) 5′-AGTCAGGAGTCTCCATCTCAC-3′ and Neo C) 5′-CGGCGG-GAGAACCTGCGTGCAA-3′. Homozygotic mice will have one 350 bp band. Heterozygotic mice will have 350 and 263 bp bands. Wild-type mice will have one 263 bp band. The PCR protocol was: 95°C for 15 min (denatured at 94°C for 45 sec, annealed at 55°C for 1 min, and extended at 72°C for 1 min) for 35 cycles, then 72°C for 7 min, and finally held at 4°C. The PCR products were resolved by 1.0% agarose/ethidium bromide gel electrophoresis and photographed under UV illumination. Transgenic mSOD1^{G93A} mice animals were identified by quantitative PCR to ensure mSOD1^{G93A} transgene copy numbers remain stable. After the initial cross, mSOD1^{G93A}/RAG2$^{+/-}$ mice were bred with RAG2$^{-/-}$ mice that enabled a direct comparison of mSOD1^{G93A}/RAG2$^{-/-}$ mice with their mSOD1^{G93A}/RAG2$^{+/-}$ littermates.

CD4 Mice. CD4$^{-/-}$ mice were initially bred with B6.Cg-Tg(SOD1*G93A)1Gur/J for at least 8 generations. The presence or absence of the CD4 gene was also determined by PCR (Eppendorf Mastercycler) using 250 ng of tail DNA and using Eppendorf TaqDNA polymerase according to the manufacturer's instruction. The following primers were used: CD4 A) 5′-CCTCTTGGTTAATGGGGG-AT-3′ and CD4 B) 5′-TTTTTCTGGTCCAGGGTCAC-3′ and CD4 C) 5′-GTGTTGGG-TCGTTTGTTCG-3′. Knockout mice will have one band 225 bp; heterozygotic mice will have bands 380 and 225 bp; and wild-type mice will have one band 380 bp. The PCR protocol was as follows: 94°C for 3 min; [denatured at 94°C for 30 sec, annealed at 57°C for 1 min, and extended at 72°C for 1 min] for 35 cycles; then 72°C for 2 min, and finally held at 4°C. The PCR products are resolved by 1.0% agarose/ethidium bromide gel electrophoresis and photographed under UV illumination. Transgenic mSOD1^{G93A} mice animals were identified by quantitative PCR to ensure mSOD1^{G93A} transgene copy numbers remain stable. After the initial cross, mSOD1^{G93A}/CD4$^{+/-}$ mice were bred with CD4$^{-/-}$ mice to enable a direct comparison of mSOD1^{G93A}/CD4$^{-/-}$ mice with their mSOD1^{G93A}/CD4$^{+/-}$ littermates.

Bone Marrow Transplantation. The donor bone marrow was obtained from 9- to 12-week-old WT, mSOD1^{G93A}, or CCR2$^{-/-}$ mice and was transplanted into the mSOD1^{G93A}/PU.1$^{-/-}$ pups within 24 h of birth, as previously described (1). mSOD1^{G93A}/RAG2$^{-/-}$ mice were sublethally γ-irradiated (400 rads) and transplanted with WT, mSOD1^{G93A}, or RAG2$^{-/-}$ donor-derived bone marrow. Briefly, the donor mice were lethally anesthetized and their femurs removed under sterile conditions. The bone

marrow was flushed out of the femurs with Dulbecco's modified Eagle's medium (DMEM) plus 10% fetal calf serum. The hematopoietic cells were successively passed through 18-, 21-, 23-, and 25-gauge needles. The cells were then pelleted at 250 *g* for 10 min, washed with 5 ml DMEM without FCS, and resuspended at 2×10^8 cells/ml DMEM without FCS. Using a 27-gauge needle, a 50-μl aliquot (1×10^7 cells per mouse) was injected i.p. into mSOD1^{G93A}/PU.1$^{-/-}$ mice, or a 200-μl aliquot (3×10^7 cells per mouse) was injected i.v. via the tail vein for mSOD1^{G93A}/RAG2$^{-/-}$ mice.

Quantitative RT-PCR. RNA was isolated from homogenized flash-frozen murine lumbar spinal cords using TRIzol (Gibco) and purified using RNeasy (Qiagen) according to the manufacturer's recommendations. The concentrations were determined spectrophotometrically (NanoDrop 1000). Quantitative RT-PCR was performed on 10 ng mRNA using an iQ5 Multicolor Real-Time PCR Detection System (BioRad), all normalized with β-actin. The iScript One-step RT-PCR kit with SYBR Green (BioRad) or QuantiTect SYBR Green RT-PCR kit (Qiagen) was used to perform the quantitative RT-PCR according to the manufacturer's instructions. The primers sets were tested over a temperature gradient for amplification efficiency and specificity, and verified over 3 orders of magnitude for linearity. The relative expression levels of each mRNA were calculated using the $\Delta\Delta$Ct method normalizing to β-actin or GAPDH and relative to the control samples. The presence of one product of the correct size was verified by 1.5% agarose gel electrophoresis and by melting curve analyses containing a single melt curve peak. All samples were run in duplicate and were tested in a minimum of three separate experiments.

Immunohistochemistry and Antibodies. Lethally anesthetized mice were first perfused with ice-cold PBS and then ice-cold 3% paraformaldehyde. Spinal cords were removed, postfixed overnight in 3% paraformaldehyde, and placed in 30% sucrose. Sections 30 μm thick were cut from fixed spinal cord tissue, washed three times in PBS, and blocked for endogenous peroxidase activity (0.3% H_2O_2 in distilled water for 30 min). The sections were pretreated with 5% normal goat serum (Vector Laboratories, Burlingame, CA) for 1 h at RT to block nonspecific IgG binding. The CD3, CD4, CD8, CD11b, CD19, CD40, CD68, and MCH class II were rat anti-mouse, CD11c was hamster anti-mouse Ab (Serotec Inc., Raleigh, NC; 1:500, 1:500, 1:500, 1:1000, 1:500, 1:2000, 1:2000, 1:500, and 1:500 dilutions, respectively), and GFAP was rabbit anti-bovine (DAKO; 1:50,000) The primary Ab were diluted in PBS containing 5% normal goat serum and incubated with the sections overnight at 4°C. As a negative control, the primary Ab were omitted during the reaction. After rinsing in PBS, the sections were incubated with biotinylated goat anti-rat IgG (1:200 dilution in PBS containing 5% normal goat serum, Serotec), biotinylated goat anti-hamster IgG (1:200 dilution in PBS containing 5% normal goat serum, Serotec), or biotinylated goat anti-rabbit IgG (1:200 dilution in PBS containing 5% normal goat serum, Vector) for 2 h at RT. After washing in PBS, the sections were further incubated with biotin-avidin complex conjugated to HRP (Vector Elite kit, Vector Laboratories, Burlingame CA) for 1 h at RT. After washing in PBS, the peroxidase was visualized with the Immunopure Metal enhanced DAB substrate kit (Pierce, Rockford, IL) for 15 min. The reaction was stopped by washing in PBS. The sections were then mounted onto glass slides, dried overnight, dehydrated in graded series of ethanol, cleared in xylene, and coverslipped with Permount (Fisher Scientific, Fair Lawn NJ). The immunostained sections were examined using a Zeiss Imager-Z1m microscope equipped with a Zeiss AxioCam MRc5 color camera and Zeiss digital image analysis system.

Primary Microglia Cultures. Primary microglial cultures were prepared from 8- to 9-day-old mice and treated with LPS, as previously described (4). Briefly, after removal of the meninges, the cortexes were minced and digested with 0.25% trypsin and 0.01% DNase. After mechanical dissociation, the cells were resuspended in SATO's medium supplemented with 10% FBS and seeded in 150 cm^2 flasks at a density of 2×10^7 cells per flask. Twenty-four hours later, the medium was changed. After the cells were incubated at 37°C with 5% CO_2 for 1 week, the medium was changed. The flasks were then shaken at 100 rpm for 15 h. More than 95% of the floating cells were microglia, as determined by OX42 (Chemicon, Temecula, CA) immunocytochemical staining. Microglia monocultures were plated at a density of 20,000 cells per well. Microglia were activated with LPS. Microglial activation was determined by measuring the levels of TNF-α and IL-1β in the culture media using a sandwich enzyme-linked immunosorbent assay (ELISA; R&D Systems). A 5-μl quantity of medium from a microglia culture was incubated with 50 μl Assay Diluent RD1−41 in the TNF-α assay plate for 2 h at RT. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for rat TNF-α was added to the wells. Following the addition of the peroxidase substrate solution, the enzyme-reactive color product was detected by a microplate reader set to 450 nm with a wavelength correction set to 540 nm. The same procedure was used for IL-1β.

Statistical Analyses. Data were analyzed using two-tailed Student's t test using Excel (Microsoft) software. Data are expressed as mean ± SE.; $P < 0.05$ was considered statistically significant. Differences in onset and survival times were computed using Kaplan-Meier survival statistics (log-rank-sum test; Number Cruncher Statistical Systems). Disease progression and the *in vitro* studies were analyzed using one-way ANOVA with repeated measures (SigmaStat). Differences between groups were analyzed using a two-way ANOVA (SigmaStat).

1. Beers DR (2006) *et al* Wild-type microglia extend survival in PU 1 knockout mice with familial amyotrophic lateral sclerosis *Proc Natl Acad Sci USA* 103:16021–16026.
2. Beers DR (2001) *et al* Parvalbumin overexpression alters immune-mediated increases in intracellular calcium, and delays disease onset in a transgenic model of familial amyotrophic lateral sclerosis *J Neurochem* 79:499–509.
3. McKercher SR (1996) *et al* Targeted disruption of the PU 1 gene results in multiple hematopoietic abnormalities *EMBO J* 15:5647–5658.
4. Zhao W (2004) *et al* Activated microglia initiate motor neuron injury by a nitric oxide and glutamate-mediated mechanism *J Neuropathol Exp Neurol* 63:964–977.

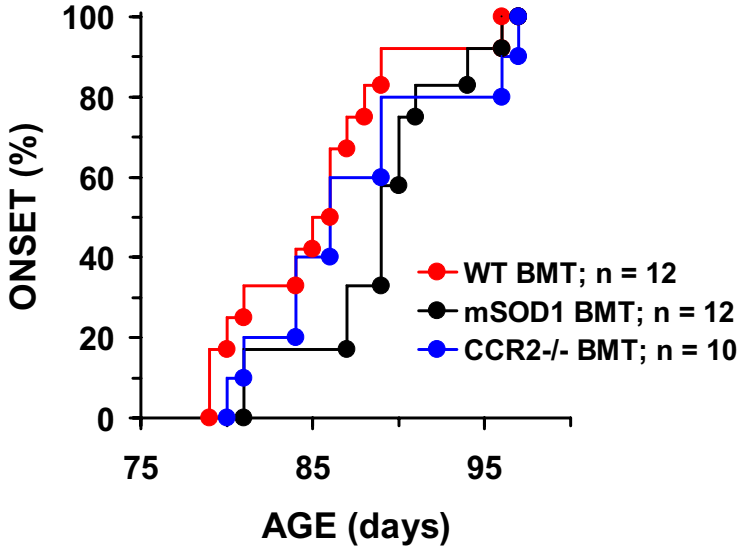

Fig. S1. mSOD1^{G93A}/PU.1$^{-/-}$ mice transplanted with CCR2$^{-/-}$ donor-derived cells have onset times (88 ± 2 days, n = 10) similar to mSOD1^{G93A}/PU.1$^{-/-}$ mice following WT bone marrow transplantation (85 ± 1 days, n = 12) and similar to mSOD1^{G93A}/PU.1$^{-/-}$ mice with mSOD1^{G93A} bone marrow transplants (89 ± 1 days, n = 12). The curve for mSOD1^{G93A}/PU.1$^{+/-}$ mice was eliminated for clarity but was similar to mSOD1^{G93A}/PU.1$^{-/-}$ mice with mSOD1^{G93A} bone marrow transplants (P = 0.49).

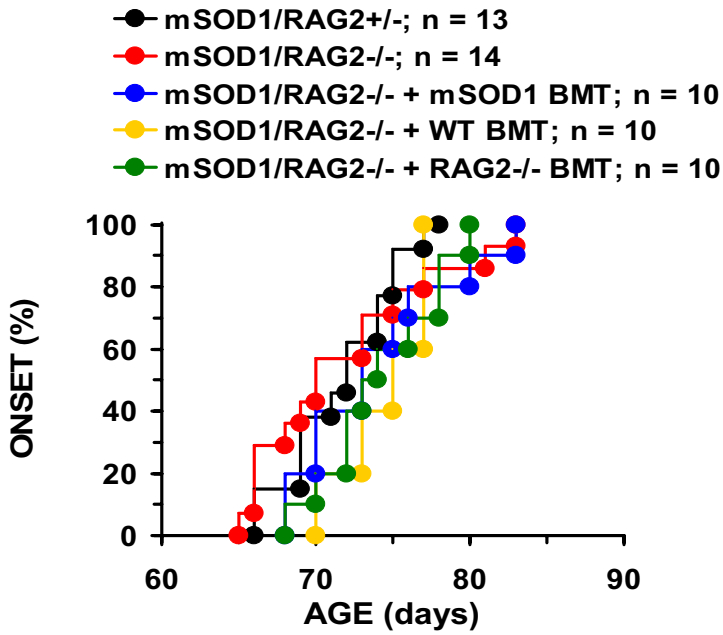

Fig. S2. Onset was not different in mSOD1^{G93A}/RAG2$^{-/-}$ mice lacking functional T and B cells (72 ± 2 days, $n = 14$) compared with mSOD1^{G93A}/RAG2$^{+/-}$ mice (71 ± 1 days, $n = 13$) or with mice receiving bone marrow transplants (mSOD1^{G93A}: 74 ± 2 days, $n = 10$; WT: 74 ± 1 days, $n = 10$; RAG2$^{-/-}$: 74 ± 1 days, $n = 10$).

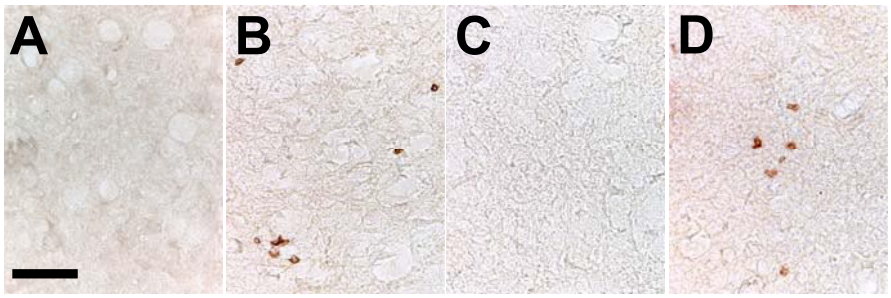

Fig. S3. CD3+ T-cells were documented at end-stage disease in mSOD1^{G93A}/RAG2$^{+/-}$ mice were absent in mSOD1^{G93A}/RAG2$^{-/-}$ mice (*A*). CD8+ T cells are observed in lumbar spinal cord sections of end-stage disease mSOD1^{G93A}/RAG2$^{+/-}$ mice (*B*), absent in sections from mSOD1^{G93A}/RAG2$^{-/-}$ mice (*C*), but were observed in mSOD1^{G93A}/RAG2$^{-/-}$ mice receiving BMT (*D*).

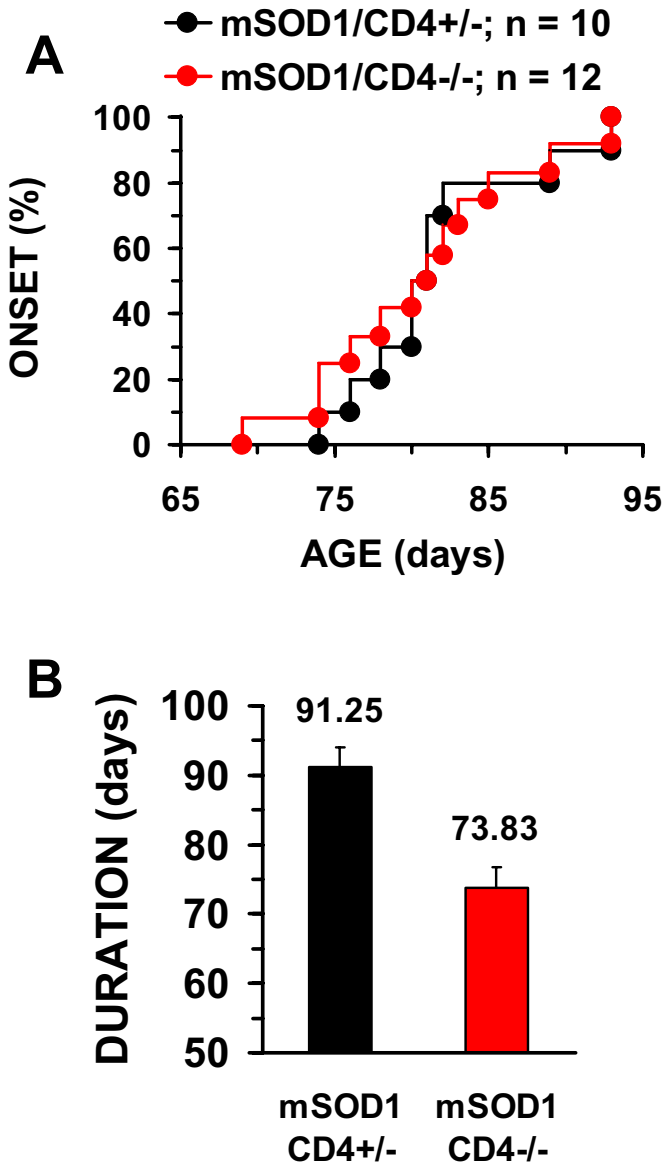

Fig. S4. (*A*) Onset was not different in mSOD1^{G93A}/CD4$^{-/-}$ mice lacking cells surface expression of CD4 (80 ± 2 days, $n = 12$) compared with mSOD1^{G93A}/CD4$^{+/-}$ mice (80 ± 2 days, $n = 10$). (*B*) Disease duration is attenuated in mSOD1^{G93A}/CD4$^{-/-}$ mice.

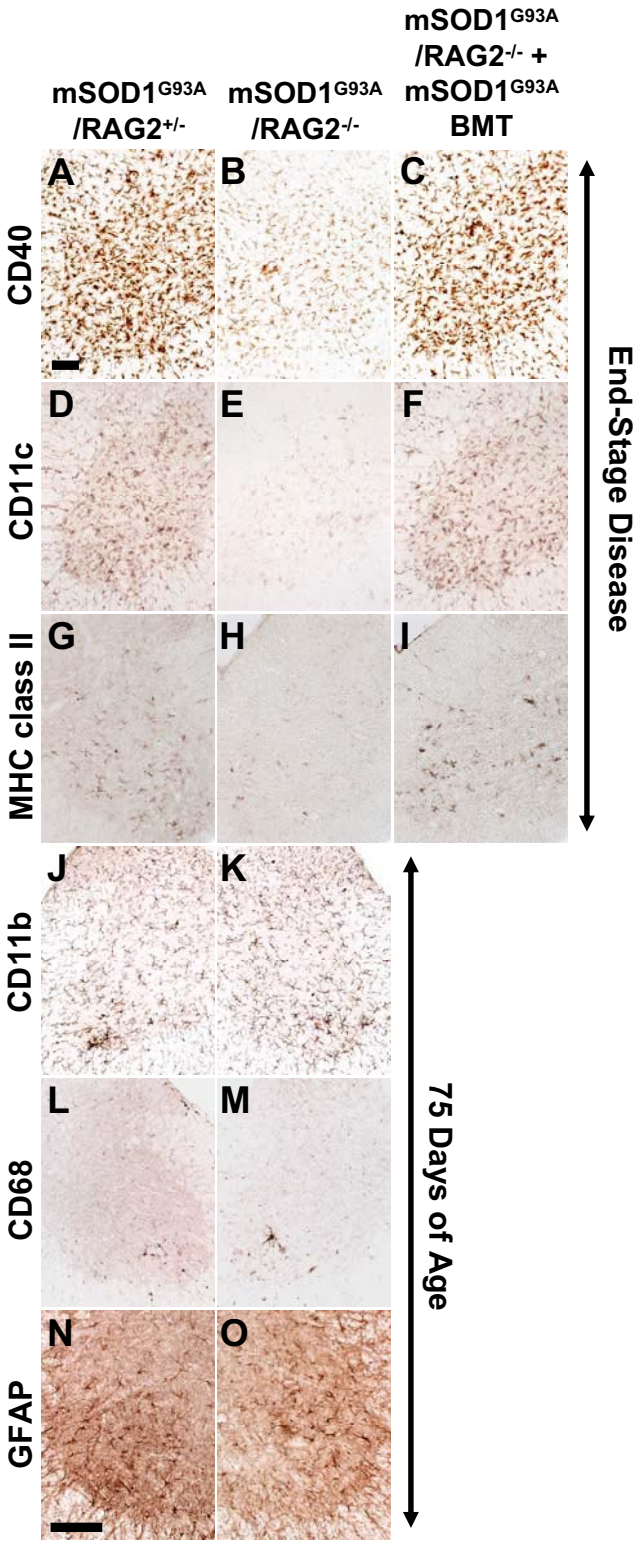

Fig. S5. Immunohistochemical evaluations of lumbar spinal cord sections. Activated microglia morphology (CD40 signal, a marker of dendritic cells) observed at end-stage disease in mSOD1^{G93A}/RAG2$^{+/-}$ mice (*A*) was decreased in mSOD1^{G93A}/RAG2$^{-/-}$ mice (*B*) and restored following mSOD1^{G93A} bone marrow transplantation (*C*) Similar results were observed at end-stage with antibodies to CD11c (*D–F*) and MHC class II (*G–I*). At 75 days of age, CD11b staining patterns were not different between mSOD1^{G93A}/RAG2$^{+/-}$ (*J*) and mSOD1^{G93A}/RAG2$^{-/-}$ mice (*K*). This was also observed with CD68 antibodies (*L* and *M*). At 75 days of age, compared with mSOD1^{G93A}/RAG2$^{+/-}$ mice (*N*), GFAP signal was less in mSOD1^{G93A}/RAG2$^{-/-}$ mice (*O*). (Scale bars: *A–M*, = 100 μm; *N* and *O*, 100 μm.)

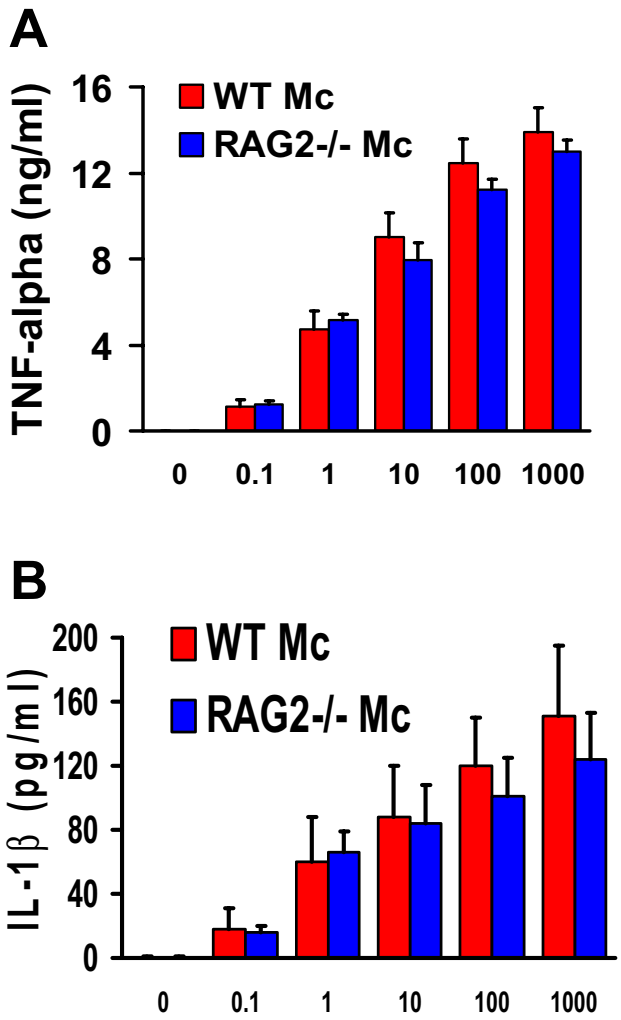

Fig. S6. Characterization of microglia *in vitro*. TNF-α (*A*) or IL-1β (*B*) released in cultures of primary microglia with and without LPS treatment. No differences were observed between RAG2$^{-/-}$ and WT microglia at any dose of LPS.

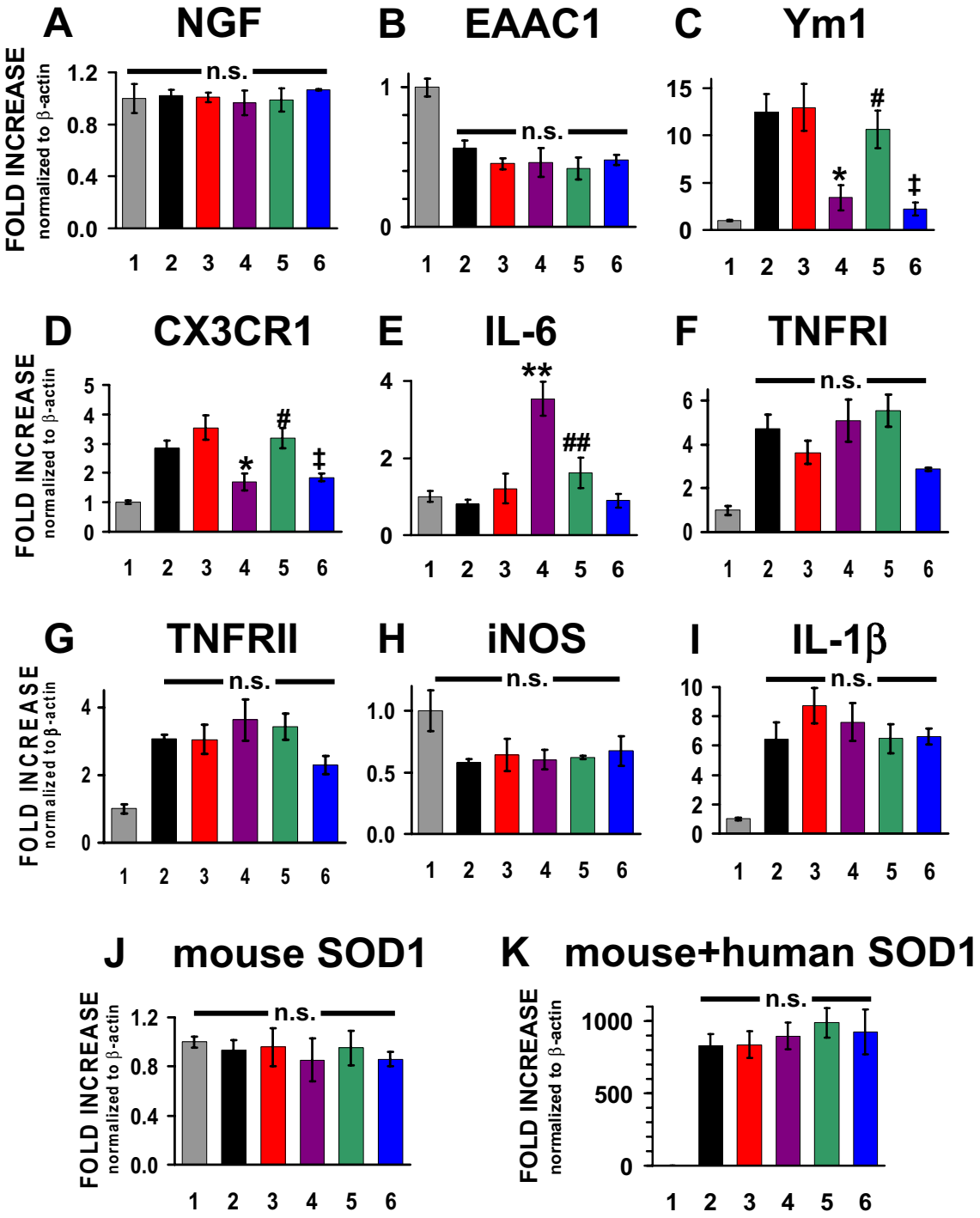

Fig. S7. Quantitative RT-PCR. Note the different ordinate for each graph. (*A*) There was no difference in message levels for NGF. (*B*) EAAC1/EAAT3 was reduced in all mSOD1^{G93A} genotypes compared with WT mice. (*C*) Ym1 mirrored the IL-4 results across all genotypes of mSOD1^{G93A} mice studied. (*D*) CX3CR1 (fractalkine receptor) was reduced in mSOD1^{G93A}/RAG2$^{-/-}$ mice compared with mSOD1^{G93A}/RAG2$^{+/-}$ mice, but was restored following BMT. mSOD1^{G93A}/CD4$^{-/-}$ mice also had a reduced mRNA level for CX3CR1. (*E*) IL-6 was increased in mSOD1^{G93A}/RAG2$^{-/-}$ mice. (*F* and *G*) TNF-α receptors I and II were elevated compared with WT mice, but were not different between all genotypes of mSOD1^{G93A} mice. (*H*) iNOS was not different between WT mice and any mSOD1^{G93A} genotypes. (*I*) IL-1β was elevated compared with WT mice, but were not different between all genotypes of mSOD1^{G93A} mice. (*J*) Mouse SOD1 was not different in any genotype. (*K*) Mouse+human SOD1 was elevated in all genotypes of mSOD1^{G93A} mice compared with WT mice. 1, WT mice; 2, mSOD1^{G93A} mice; 3, mSOD1^{G93A}/RAG2$^{+/-}$ mice; 4, mSOD1^{G93A}/RAG2$^{-/-}$ mice; 5, mSOD1^{G93A}/RAG2$^{-/-}$ mice + BMT; and 6, mSOD1^{G93A}/CD4$^{-/-}$ mice. $n = 3$ for all groups of mice. n.s., not significant. *, Decreased compared with mSOD1^{G93A} or mSOD1^{G93A}/RAG2$^{+/-}$ mice, $P < 0.05$; #, not different from mSOD1^{G93A}/RAG2$^{+/-}$ mice; ‡, not different from mSOD1^{G93A}/RAG2$^{-/-}$ mice; **, increased compared with mSOD1^{G93A} or mSOD1^{G93A}/RAG2$^{+/-}$ mice, $P < 0.05$; ##, decreased compared with mSOD1^{G93A}/RAG2$^{-/-}$ mice, $P < 0.05$.