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Transformation from a neuroprotective to a neurotoxic microglial phenotype in a mouse model of ALS

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Abstract

Neuroinflammation is a prominent pathological feature in the spinal cords of patients with amyotrophic lateral sclerosis (ALS), as well as in transgenic mouse models of inherited ALS, and is characterized by activated microglia. Earlier studies showed that activated microglia play important roles in both motoneuron protection and injury. More recent studies investigating the pathoprotection of disease in ALS mice have demonstrated that the *in vivo* activation states of microglia, including their anti- *versus* pro-inflammatory responses, are best characterized as a continuum between two extreme activation states which are represented as a neuro-protective M2 (alternatively-activated) phenotypic state or an injurious/toxic M1 (classically-activated) state; a more complete understanding and determination the temporal transformation of microglia activation states in the ALS disease pathoprotection is therefore warranted. In the current study, we demonstrated a phenotypic and functional transformation of adult ALS mice microglia that overexpress mutant superoxide dismutase (mSOD1). mSOD1 microglia isolated from ALS mice at disease onset expressed higher levels of Ym1, CD163 and BDNF (markers of M2) mRNA and lower levels of Nox2 (a marker of M1) mRNA compared with mSOD1 microglia isolated from ALS mice at end-stage disease. More importantly, when co-cultured with motoneurons, these mSOD1 M2 microglia were neuroprotective and enhanced motoneuron survival than similarly co-cultured mSOD1 M1 microglia; end-stage mSOD1 M1 microglia were toxic to motoneurons. Our study documents that adult microglia isolated from ALS mice at disease onset have an M2 phenotype and protect motoneurons whereas microglia isolated from end-stage disease ALS mice have adopted an M1 phenotype and are neurotoxic supporting the dual phenotypes of microglia and their transformation during disease pathoprotection in these mice. Thus, harnessing the neuroprotective potential of microglia may provide novel avenues for ALS therapies.

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Keywords

Amyotrophic lateral sclerosis; Microglia; Motor neurons; Neuroprotection; Neurotoxicity

Introduction

Amyotrophic lateral sclerosis (ALS) is a progressive and devastating neurodegenerative disease characterized by selective loss of upper and lower motoneurons in the cortex, brainstem, and spinal cord of the patient's central nervous system (CNS). Neuroinflammation is a prominent pathological feature in these CNS areas and characterized by the morphological activation of microglia (Appel et al., 2010). A similar set of neuroinflammatory responses, including microglia activation, occurs in mice overexpressing mutant Cu²⁺/Zn²⁺ superoxide dismutase (mSOD1), a model of inherited ALS. However, until recently, there has been controversy as to whether the proliferation and activation of microglia in ALS promoted motoneuron survival or exacerbated the neurodegenerative process (Moisse and Strong, 2006). This controversy has begun to be resolved by the demonstration that wild-type (WT) microglia or microglia expressing less mSOD1 promoted neuroprotection and extended survival of ALS mice (Beers et al., 2006; Boillée et al., 2006). Furthermore, our *in vitro* studies utilizing primary neonatal microglia and embryonic motoneuron co-cultures provided evidence that WT microglia were less neurotoxic than mSOD1 microglia due to their enhanced release of neurotrophic factors, and attenuated release of free radicals and proinflammatory cytokines (Beers et al., 2006; Weydt et al., 2004; Xiao et al., 2007).

Microglia, as a component of the innate immune system, are of hematopoietic origin and colonize the CNS during early development (Beers et al., 2006; McKercher et al., 1996; Ransohoff and Perry, 2009). These immunocompetent cells display functional plasticity during activation involving changes in cell number, morphology, surface receptor expression, and production of growth factors and cytokines. Cumulative studies using diverse animal models of CNS injury have demonstrated that microglia have very distinct and different phenotypic states, and in line with other tissue macrophage populations, may exert either neurotoxic or neuroprotective responses depending on the physiologic conditions they encounter. Although an over simplification, microglial activation states can be divided into classically activated microglia (M1) and alternatively activated microglia (M2); the *in vivo* functions of microglia are probably better represented as a continuum between these two extreme activation states. M1 microglia are cytotoxic due to their secretion of reactive oxygen species (ROS) and proinflammatory cytokines, and increased level of NOX2 expression. In contrast, M2 microglia block proinflammatory responses, and produce high levels of anti-inflammatory cytokines and neurotrophic factors (Buechler et al., 2000; Gordon and Martinez, 2010; Tiemessen and Kuhn, 2007). Thus, the M1/M2 distinction should not necessarily invoke a concept of a cellular differentiation state, but as a spectrum of pro- versus anti-inflammatory responses. Therefore, a more complete understanding of how these distinct microglial phenotypes temporally influence the ALS disease pathoprogession process is warranted (Appel et al., 2009; Henkel et al., 2009).

Because the phenotypic and functional states of adult microglia during the pathoprogession of ALS have not been fully characterized, and because an earlier study using chimeric mice demonstrated that healthy WT motoneurons develop features of ALS pathology when surrounded by mSOD1-expressing glia, which reinforced the hypothesis that alterations of glial properties by the overexpression of mSOD1 contributes to the motoneuron injury (Clement et al., 2003), we characterized the adult mSOD1 microglial phenotypic states that exist over the course of disease in ALS mice when studied *ex vivo* using WT motoneurons with and without WT astroglia; we isolated and assessed the mSOD1 microglia for their cytokine and neurotrophic factor mRNA levels, and whether they were protective or toxic to WT motoneurons. The adult mSOD1 microglia were obtained from ALS mice that were either at the clinical onset of disease, during the initial slowly progressing phase, or near end-stage disease, during the rapidly progressing phase (Beers et al., 2008, 2011b). The results of this study indicate that early in the disease process, mSOD1 microglia expressed more M2 related mRNAs compared with near end-stage mSOD1 microglia; near end-stage microglia expressed more M1 marker. More importantly, when co-cultured with WT motoneurons, mSOD1 M2 microglia were neuroprotective and enhanced WT motoneuron survival than similarly co-cultured mSOD1 M1 microglia; end-stage mSOD1 M1 microglia were toxic to WT moto-neurons. Although the addition of WT neonatal astroglia enhanced WT motoneuron survival when co-cultured with M2 microglia, they had only modest effects when included in cultures of end-stage disease M1 microglia and WT motoneurons. These data demonstrate the dual phenotypic and functional characteristics of mSOD1 expressing microglia over the course of disease, and again suggest that enhancing the M2 phenotypic state of microglia may have beneficial therapeutic effects.

Materials and methods

Experimental animals

mSOD1(G93A) mice on a C57Bl/6 genetic background were bred and maintained in The Methodist Hospital Research Institute's animal facility. All animals were housed in microisolator cages with access to food and water *ad libitum*, and all animals are specific pathogen free (SPF); sentinel mice are tested quarterly. All experimental procedures involving animals were approved by The Methodist Hospital Research Institute's Institutional Animal Care and Use Committee in compliance with National Institutes of Health guidelines. Transgenic mSOD1(G93A) mice were identified and copy number verified by quantitative RT-PCR (qRT-PCR) using the antisense and sense SOD1 oligonucleotides 5'-AAT TTG TGT CTA CTC AGT CAA-3' and 5'-TCA CTT TGA TTG TTA GTC GCG-3', respectively. The PCR conditions for genomic tail DNA in a 50 μ L reaction using an Eppendorf Mastercycler gradient thermocycler (Westbury, NY) were as follows: 2 min at 94 $^{\circ}$ C; then 35 cycles of 20 s at 94 $^{\circ}$ C, 20 s at 60 $^{\circ}$ C, and 30 s at 72 $^{\circ}$ C; then 2 min at 72 $^{\circ}$ C and held at 4 $^{\circ}$ C until the samples were run on a 1% agarose gel in 1 $\times$ TAE buffer. Both mSOD1 and WT mice were weighed and observed daily for signs of motoneuron disease; disease symptoms and course were assessed using the BASH scoring system (Beers et al., 2006, 2008). Specific cytokines/chemokines/neurotrophic factor mRNA levels were assessed at disease onset (11 weeks), during the initial slowly progressing phase, and at near end-stage disease; age-matched WT mice served as controls.

Cell isolation and culture procedures

Primary adult microglia were prepared from lumbar spinal cords of mSOD1 mice, as well as WT mice, by modification of a non-enzymatic procedure (Aloisi et al., 2000). Briefly, mice were lethally anesthetized and perfused intracardially with 40–50 mL of ice cold GKN buffer [8 g/L NaCl, 0.4 g/L KCl, 3.56 g/L Na₂HPO₄ 12H₂O, 0.78 g/L NaH₂PO₄ 2H₂O, and 2 g/L D-glucose, pH 7.4]. Spinal cord tissues were mechanically dissociated by passing through a 70 µm cell strainer using a 10 mL syringe plunger, and then strained through a 40 µm filter. Single cell suspensions were prepared by centrifugation over a 35%/70% discontinuous Percoll gradient (GE Healthcare); cells were isolated from the interface, and total cell counts determined. Flow cytometry was used to assess cell purification. Cells were pre-blocked with anti-CD16/CD32 (Fc Block, Serotec), and stained on ice for 30 min with anti-CD11b-PerCP-Cy5.5 (BD Biosciences); data collection was performed on a LSRII flow cytometer (BD Biosciences) and analyzed using FACSDiva (BD Biosciences) and FlowJo (Tree Star) softwares. The flow cytometry analyses determined the microglial cell population to be 95.2%±1.8% pure. Microglia were washed and resuspended in RPMI medium supplemented with 10% fetal bovine serum, 25 mM HEPES, 1 mM sodium pyruvate, 1× nonessential amino acids, 55 µM 2-mercaptoethanol, 100 units/mL penicillin and 100 µg/mL streptomycin.

Primary WT motoneuron cultures were prepared from spinal cords of embryonic days 13–14 (E13–E14) mice by Histodenz gradient (Sigma-Aldrich) centrifugation as previously described (Arce et al., 1999; Zhao et al., 2006). Briefly, ventral spinal cords were minced and digested for 15 min with 0.05% trypsin at 37 °C. After treatment with DNase, the cell suspension was layered onto and centrifuged through 6.8% Histodenz cushion. After centrifugation, a sharp band was collected and centrifuged through a 4% BSA cushion. The cells were resuspended in the Neurobasal culture medium (Gibco Invitrogen) supplemented with 25 µM glutamate, 0.5 mM glutamine, B27 (1×, Gibco Invitrogen), penicillin–streptomycin (1×), 25 µM 2-mercaptoethanol, and 2% horse serum. Cells were plated at a density of 15,000 cells/well in 24-well-plates previously coated with poly-L-ornithine (2 µg/mL) and laminin (4 µg/mL), and containing coverslips. The WT moto-neuron cultures were supplemented with 1 ng/mL BDNF, 1 ng/mL GDNF, and 10 ng/mL CNTF to promote neurite outgrowth. The purity of the primary motoneuron population used in this study was greater than 90% (Zhao et al., 2006, 2010).

Primary WT astroglial cultures were prepared from 7 to 9-day-old WT C57BL/6 mice according to Le et al. (2001). Briefly, after removing the meninges, cortices 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% fetal bovine serum and seeded in 75 cm² flasks at a density of 1×10⁷ cells per flask. After 1 week incubation, the flasks were shaken for 48 h at 200 rpm at 37 °C. The majority (>95%) of attached cells were astroglia as determined by glial fibrillary acidic protein (GFAP) immunoreactivity (Chemicon). Contamination with microglia was less than 3% as determined by Dil-Ac-LDL (Biomedical Technologies, Inc., Stoughton, MA).

WT motoneuron/microglia cultures with/without WT astroglia were carried out as follows: for WT motoneuron/mSOD1 microglia co-cultures without WT astroglia, microglia (2000

cells/well) were plated on top of WT motoneurons after the motoneurons were incubated for 1 day. After co-culturing for one day, motoneurons were fixed and examined immunocytochemically. For WT motoneuron/microglia/WT astroglia cultures, WT motoneurons were layered on top of WT astroglia (a density 10,000 cells/well) 1 day prior to adding microglia. After one day co-culturing, motoneurons were fixed and again examined immunocyto-chemically. After isolation, we determined by trypan blue exclusion and counting on a hemocytometer that we isolated approximately 50,000 microglia, 600,000 astrocytes, and 15,000 motoneurons per mouse.

Total mRNA isolation and qRT-PCR procedures

Total mRNA was isolated and purified from microglia immediately following cell isolation using Trizol (Gibco) and purified using RNeasy (Qiagen) according to the manufacturers' recommendations. The mRNA concentrations were determined spectrophotometrically (Beckman DU-64). qRT-PCR was performed on 1 ng of mRNA as previously described (Beers et al., 2008; Henkel et al., 2009). qRT-PCR primer sequences used were: Ym1: forward; 5'-TCA CTT ACA CAC ATG AGC AAG AC-3' and reverse; 5'-CGG TTC TGA GGA GTA GAG ACC A-3'. CD163: forward; 5'-TCA CTT CTC AGT GCC TCT GC-3' and reverse; 5'-AAC CAC GGA CAC TTC ATT CA-3'. BDNF: forward; 5'-CCA TAA GGA CGC GGA CTT GTA-3' and reverse; 5'-TTT GCG GCA TCC AGG TAA TTT-3'. Nox2: forward; 5'-TGA ATG CCA GAG TCG GGA TTT-3' and reverse; 5'-CCC CCT TCA GGG TTC TTG ATT T-3'. Primer design was based on the literature or Primer Bank (<http://pga.gh.harvard.edu/primerbank>). Primer efficiency was assessed by analyzing a dilution series of mRNA. The relative expression level of each mRNA was calculated using the Ct method normalizing to β -actin and relative to the control samples. The presence of one product of the correct size was verified by gel electrophoresis and melting curve analyses. All qRT-PCR reactions were performed using iScript SYBR Green RT-PCR Kit (Bio-Rad) and run on an iCycler iQ5 RT-PCR machine (Bio-Rad).

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, nonphosphorylated neurofilament H, is a commonly used specific marker for motoneuron staining (Carriedo et al., 1996). Motoneuron survival was assessed by directly counting large SMI-32 positive cells (cell bodies >25 μ m) displaying intact neurites longer than three cell diameters. Motoneuron counts were performed under bright-field microscopy in an area of 6 mm² along the diameter of a coverslip (15 fields/coverslip). To reduce the variation among motoneuron cultures in different preparation, percentages were normalized to the number of motoneuron in control cultures in the same spinal cord preparation. At the same time, the average numbers of neurites on each motoneuron were compared between groups. The average length of neurites on each motoneuron was measured by software (Image J) and compared between groups.

Statistical analyses

Data were analyzed using two-tailed Student's t-test using Excel software. Data are expressed as mean±S.E.; $p < 0.05$ was considered statistically significant. Differences between groups were analyzed using a two-way ANOVA (SigmaStat, Richmond, CA).

Results

End-stage adult mSOD1 microglia expressed diminished M2 mRNA levels

We previously demonstrated *in vitro* that in primary microglia/ motoneuron co-cultures WT microglia were less neurotoxic than mSOD1 microglia due to their enhanced release of neurotrophic factors, and attenuated release of free radicals and proinflammatory cytokines (Beers et al., 2006; Xiao et al., 2007). However, these previous studies were conducted using neonatal microglia and were activated with lipopolysaccharide (LPS). Furthermore, we recently established *in vivo* that specific M2 markers such as Ym1 and CD206 were increased in the lumbar spinal cords of ALS mice at disease onset and during the slowly progressing phase of disease compared with age-matched WT mice; the levels of these markers subsequently decreased during the rapidly progressing phase of disease (Beers et al., 2011a). To determine whether these M2 markers were increased specifically on microglia without prior LPS activation, we isolated adult microglia from the lumbar spinal cords of ALS mice at the initial slowly progressing disease phase (11 weeks of age) and at near end-stage disease, and immediately extracted their RNA and assessed the Ym1 and CD163 expression levels. End-stage mSOD1 microglia expressed decreased levels of both Ym1 and CD163 compared with 11 week old mSOD1 microglia (Ym1, $p = 0.016$; CD163, $p = 0.020$) and with age-matched WT microglia (Ym1, $p = 0.048$; CD163, $p = 0.007$) (Figs. 1A and B). Message levels of these M2 markers were similar between 11 week old mSOD1 microglia and their age-matched WT counterparts.

Since we recently demonstrated that brain derived neurotrophic factor (BDNF) was increased in lumbar spinal cords of ALS mice during the slowly progressing phase of disease and subsequently declined during the rapidly progressing phase, and since BDNF has been shown to protect motoneurons from injury, we determined whether the isolated microglia also differentially expressed BDNF (Beers et al., 2008; Serpe et al., 2005). Message levels for BDNF were decreased in end-stage mSOD1 microglia compared with 11 week old mSOD1 microglia ($p = 0.011$) and compared with age-matched WT microglia ($p = 0.006$) (Fig. 1C). The mRNA levels of this neurotrophic factor were not different in 11 week old mSOD1 microglia compared with 11 week old WT microglia.

End-stage adult mSOD1 microglia expressed an enhanced level of NOX2 mRNA, a prototypic M1 marker

We previously demonstrated that NOX2 mRNA levels increased during the pathoprogession of disease in the spinal cords of ALS mice; NOX2 is the subunit of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase found in macrophages producing superoxide anions ($O_2^{\bullet-}$) and a prototypic marker of M1 macrophages/microglia (Beers et al., 2011b). End-stage mSOD1 microglia expressed 5-fold more NOX2 mRNA than 11 week old mSOD1 microglia ($p = 0.028$) and 25-fold more than age-matched WT

microglia ($p=0.045$) (Fig. 1D). Thus, 11 week old mSOD1 microglia express M2 phenotypic markers whereas end-stage mSOD1 express more of an M1 marker. However, even 11 week old mSOD1 microglia expressed a 5-fold increase in NOX2 mRNA compared with 11 week old WT microglia ($p=0.009$) suggesting an inflammatory response caused by the possible onset of disease.

End-stage adult mSOD1 microglia are toxic to WT motoneurons

We have demonstrated that in microglia/motoneuron co-cultures mSOD1 microglia induced more motoneuron death and decreased neurite numbers and length than comparable co-cultures of WT microglia and motoneurons (Xiao et al., 2007). However, as previously mentioned, these studies were conducted with neonatal mSOD1 microglia that were activated with LPS. To determine whether adult mSOD1 microglia can induce motoneuron injury without prior LPS activation, we co-cultured the different aged mSOD1 microglia with WT motoneurons. When co-cultured with primary WT motoneuron cultures, untreated end-stage mSOD1 microglia were toxic to the motoneurons in terms of surviving motoneuron numbers and in terms of neurite numbers compared untreated 11 week old mSOD1 microglia (motoneuron numbers, $p=0.049$; neurite numbers $p=0.023$) and compared with untreated WT microglia (motoneuron numbers, $p=0.001$; neurite numbers $p=0.045$) (Figs. 1E and F). Interestingly, although 11 week old mSOD1 microglia expressed a 5-fold increase in NOX2 mRNA levels compared with age-match WT microglia, there was not a difference between surviving WT motoneurons and neurite numbers when co-cultured with either 11 week old mSOD1 microglia or WT microglia. Furthermore, immunocytochemical evaluations of WT motoneuron morphology showed that when co-cultured with untreated end-stage mSOD1 microglia, WT motoneurons were less healthy compared with motoneurons co-cultured with untreated 11 week old mSOD1 microglia or WT microglia; co-cultures of untreated end-stage mSOD1 microglia/WT motoneurons had smaller somas, and less and shorter neurites (Fig. 1G). Thus, the M1 phenotypic state of untreated end-stage mSOD1 microglia are toxic to WT motoneurons whereas untreated 11 week old M2 microglia are similar to untreated WT microglia.

Addition of astroglia further enhances WT motoneuron survival in microglia/motoneuron cultures

The previously described co-cultures of microglia and motoneurons were supplemented with BDNF, GDNF, and CNTF to promote neurite outgrowth. However, these growth factors may also modulate microglial phenotypes. Therefore, to avoid this potential artificial modulation, we cultured the WT motoneurons on a bed of astroglia and then added the different microglia. mSOD1 spinal cord microglia isolated at disease onset were protective to motoneurons in the presence of astroglia; motoneuron survival was enhanced in 11 week old mSOD1 microglia/WT motoneuron cultures compared with WT astroglia/WT microglia/WT motoneuron cultures ($p=0.024$) and when compared with WT astroglia/end-stage mSOD1 microglia/WT motoneuron cultures ($p=0.013$) (Fig. 1H). Furthermore, when 11 week old mSOD1 microglia/WT motoneuron cultures contained WT astroglia, the neurites were longer compared with cultures of WT astroglia, WT microglia, and WT motoneurons ($p=0.041$) (Fig 1I). Although WT motoneuron survival was decreased in cultures containing WT astroglia and end-stage mSOD1 microglia, there was not a

difference in neurite length between these cultures compared with cultures of WT astroglia/11 week old mSOD1 microglia/WT motoneuron.

Discussion

Previous studies have shown that motoneuron death in ALS is non-cell-autonomous. An earlier report using chimeric mice demonstrated that healthy motoneurons develop features of ALS pathology when surrounded by mSOD1-expressing glia, reinforcing the hypothesis that alterations of glial properties by the overexpression of mSOD1 contribute to the motoneuron injury (Clement et al., 2003). Our previous reports, and that of others, have established that different microglial responses can mediate motoneuron protection or injury (Beers et al., 2006; Boillée et al., 2006; Xiao et al., 2007). These studies demonstrated that *in vitro* WT microglia are relatively less toxic to motoneurons than mSOD1 microglia, and that *in vivo* WT microglia, or microglia expressing less mSOD1, protect motoneurons and prolong survival of ALS mice. In addition, cumulative studies using other diverse animal models of CNS inflammation have also demonstrated that microglia have very distinct and different phenotypic states, and in line with other tissue macrophage populations, are best characterized as a continuum between the two extreme activation states of M2 neuroprotective microglia and M1 neurotoxic microglia; the activation states of microglia fluctuates along this continuum contingent upon the physiologic conditions they encounter during the pathoprogession of each disease (Carson et al., 2007; Henkel et al., 2009; Murray and Wynn, 2011; Ransohoff and Perry, 2009). Thus, the M2/M1 distinction is not meant to represent a specific differentiation state of microglia, but as a spectrum of potential neuro-protective *versus* neurotoxic responses and that these responses may fall anywhere within this continuum.

In this study, we used Ym1 and CD163 to reflect an M2 response and NOX2 as a marker of an M1 response. Ym1 is a secretory protein synthesized by activated macrophages, and several studies have reported that Ym1 expression is strongly induced in M2 alternatively-activated macrophages as compared with M1 classically-activated macrophages (Henkel et al., 2009). CD163 is a monocyte/macrophage-restricted membrane protein which belongs to the scavenger receptor cysteine-rich (SRCR) domain family (Buechler et al., 2000). CD163 is also associated with an anti-inflammatory M2 macrophage phenotype (Buechler et al., 2000). NOX2 is a subunit of NADPH oxidase and locates on macrophage/microglia inner cell surface, and it is a well-known and widely used M1 marker (Zhao et al., 2010). Our results demonstrated changes in all of these three markers in terms of mRNA levels. Ym1 and CD163 mRNA levels were attenuated in near end-stage mSOD1 mice while NOX2 was elevated, suggesting a M1 microglial phenotypic state. We recently showed similar results in whole lumbar spinal cord extracts from ALS mice over the entire spectrum of disease pathoprogession compared with appropriate age-matched WT mice (Beers et al., 2011a). This study suggests that the changes observed in whole lumbar spinal cords were possibly due to the transformation of microglia over the course of disease.

In the current study, we provide evidence that during the pathoprogession of disease in ALS mice there is a phenotypic and functional transformation of adult mSOD1 microglia. During the early slowly progressing phase of disease in these mice, the adult mSOD1 microglia

display a neuroprotective M2 phenotype whereas during the rapidly progressing phase of disease, the adult mSOD1 microglia adopt an M1 neurotoxic phenotype. More importantly, the adult mSOD1 microglia isolated during this rapidly progressing phase are toxic to WT motoneurons, while the adult mSOD1 microglia obtained during the slowly progressing phase of disease behave and function more like WT microglia and are relatively non-toxic to WT motoneurons. Furthermore, in the presence of WT astroglia and slow phase mSOD1 microglia, motoneuron survival is enhanced. Thus, the transformation of mSOD1 microglia from a neuroprotective M2 to a cytotoxic state induces motoneuron injury.

We also observed that at an early stage of disease, the neuroprotection from M2 mSOD1 microglia was enhanced by astroglia. In WT motoneuron/mSOD1 microglial co-cultures, when WT motoneurons were co-cultured with near end-stage microglia, the numbers of live motoneurons were decreased compared with WT or 11 week old microglia; there was not an increase of WT motoneuron survival in 11 week old mSOD1 microglia group. However, in WT motoneuron/ mSOD1 microglia/WT astroglial cultures, there was an increase of motoneuron survival using 11 week old mSOD1 microglia. These results are particularly interesting in light of our data demonstrating that there was not a difference between 11 week old mSOD1 and WT microglia in terms of MN survival or neurite extension when co-cultured without astroglia. Thus, while the 11 week old mSOD1 microglia do not directly enhance motoneuron survival, the presence of mSOD1 microglia with WT astroglia, possibly through a direct communication between the two cell types, leads to increased motoneuron survival. These results also suggest that a microglia/astrocyte interaction was involved in M2 microglia protection; at an early stage of disease, astrocytes enhance M2 phenotype and/or function in microglia, leading to motoneuron protection. On the other hand, previous studies in ALS or in other neurodegenerative diseases have shown that astrocytes are neuroprotective, but the underlying mechanisms were not clear. Therefore, this study suggests that the neuroprotection of astrocytes may enhance motoneuron survival through microglia.

We have previously demonstrated that neonatal WT microglia are relatively more neuroprotective than neonatal mSOD1 microglia. However, that previous report used LPS to activate both sets of microglia. In this report, we demonstrated that adult microglia were more sensitive to the surrounding environment and thus may more accurately reflect their *in vivo* phenotypic states and functional attributes during the course of motoneuron degeneration. Furthermore, adult mSOD1 microglia isolated early in disease exhibit an alternatively-activated M2 phenotype which are potentially neuro-protective whereas mSOD1 microglia harvested from near end-stage disease present a more classically-activated M1 phenotype which are neurotoxic. These results suggest that there is a transformation of mSOD1 microglia from a neuroprotective M2 phenotype to a cytotoxic M1 phenotype during the pathoprogession of disease and thus enhances the burden of disease in ALS mice. Therefore, harnessing the protective phenotypic attributes of microglia provides potential novel therapeutic avenues for ALS patients.

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Fig. 1.

End-stage mSOD1 and M1 phenotype are toxic to motoneurons. (A) End-stage mSOD1 microglia express decreased levels of Ym1 mRNA than 11 week old mSOD1 or WT microglia. (B) CD163 mRNA expression is decreased in end-stage mSOD1 microglia compared with 11 week old mSOD1 or WT microglia. (C) End-stage mSOD1 microglia express less BDNF mRNA than 11 week old mSOD1 or WT microglia. (D) End-stage mSOD1 microglia express 5-fold more NOX2 mRNA than 11 week old mSOD1 microglia. Both ages of mSOD1 microglia express more NOX2 than their age-matched WT counterparts. (E) The number of surviving WT motoneurons in co-cultures with end-stage mSOD1 microglia is decreased compared with either motoneurons co-cultured with WT

microglia or 11 week old mSOD1 microglia. (F) When co-cultured with end-stage mSOD1 microglia, the average neurite number on the WT motoneurons was decreased compared with WT motoneuron co-cultured with WT microglia or 11 week old mSOD1 microglia. (G) Immunochemical evaluation with SMI-32 antibody showed motoneuron morphological alterations. When co-cultured with end-stage mSOD1 microglia, motoneurons were less healthy compared with motoneurons co-cultured with WT or 11 week mSOD1 microglia; there were fewer motoneurons and the remaining motoneurons had smaller somas and shorter neurites. (H) The numbers of surviving WT motoneurons in cultures with WT astroglia and 11 week old mSOD1 microglia were increased compared with similar cultures containing WT astroglia and WT microglia. (I) When cultured with 11 week old mSOD1 microglia and WT astroglia, WT motoneurons had longer neurites compared with cultures with WT microglia. ⁺*p*<0.05, end-stage WT MC *vs.* end-stage mSOD1 MC; [#]*p*<0.05, 11 weeks mSOD1 MC *vs.* end-stage mSOD1 MC; ⁺⁺*p*<0.01, end-stage WT MC *vs.* end-stage mSOD1 MC; ^{††}*p*<0.01, 11 weeks WT MC *vs.* 11 weeks mSOD1 MC; ^{*}*p*<0.05, MN co-cultured with WT MC *vs.* MN co-cultured with end-stage mSOD1 MC; ^{**}*p*<0.01, MN co-cultured with WT MC *vs.* MN co-cultured with end-stage mSOD1 MC; [&]*p*<0.05, MN co-cultured with 11 weeks mSOD1 MC *vs.* MN co-cultured with end-stage mSOD1 MC; [‡]*p*<0.05, MN/AC co-cultured with WT MC *vs.* MN/AC co-cultured with 11 weeks mSOD1 MC; [¶]*p*<0.05, MN/AC co-culture with 11 weeks mSOD1 MC *vs.* MN/AC co-culture with end-stage mSOD1 MC. Scale bar on panel G=50 μm. MN = motoneurons, MC = microglia, and AC = astroglia.