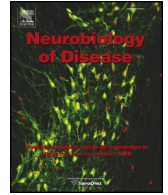




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# Spinal but not cortical microglia acquire an atypical phenotype with high VEGF, galectin-3 and osteopontin, and blunted inflammatory responses in ALS rats

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## ABSTRACT

Activation of microglia, CNS resident immune cells, is a pathological hallmark of amyotrophic lateral sclerosis (ALS), a neurodegenerative disorder affecting motor neurons. Despite evidence that microglia contribute to disease progression, the exact role of these cells in ALS pathology remains unknown. We immunomagnetically isolated microglia from different CNS regions of SOD1<sup>G93A</sup> rats at three different points in disease progression: presymptomatic, symptom onset and end-stage. We observed no differences in microglial number or phenotype in presymptomatic rats compared to wild-type controls. Although after disease onset there was no macrophage infiltration, there were significant increases in microglial numbers in the spinal cord, but not cortex. At disease end-stage, microglia were characterized by high expression of galectin-3, osteopontin and VEGF, and concomitant downregulated expression of TNF $\alpha$ , IL-6, BDNF and arginase-1. Flow cytometry revealed the presence of at least two phenotypically distinct microglial populations in the spinal cord. Immunohistochemistry showed that galectin-3/osteopontin positive microglia were restricted to the ventral horns of the spinal cord, regions with severe motor neuron degeneration. End-stage SOD1<sup>G93A</sup> microglia from the cortex, a less affected region, displayed similar gene expression profiles to microglia from wild-type rats, and displayed normal responses to systemic inflammation induced by LPS. On the other hand, end-stage SOD1<sup>G93A</sup> spinal microglia had blunted responses to systemic LPS suggesting that in addition to their phenotypic changes, they may also be functionally impaired. Thus, after disease onset, microglia acquired unique characteristics that do not conform to typical M1 (inflammatory) or M2 (anti-inflammatory) phenotypes. This transformation was observed only in the most affected CNS regions, suggesting that overexpression of mutated hSOD1 is not sufficient to trigger these changes in microglia. These novel observations suggest that microglial regional and phenotypic heterogeneity may be an important consideration when designing new therapeutic strategies targeting microglia and neuroinflammation in ALS.

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## Introduction

ALS is a neurodegenerative CNS disorder that selectively affects motor neurons, resulting in a progressive loss of motor function, paralysis and death. Although disease etiology is unknown, genetic mutations are associated with familial forms of ALS, including mutations in Cu/Zn superoxide dismutase-1 (SOD1; (Rosen et al., 1993)). Overexpression of mutant human SOD1 in animal models results in motor neuron disease. Some studies suggest that this pathology is driven by a non-cell autonomous toxicity mediated by astrocytes, neurons or microglia (Boillee et al., 2006a; Dibaj et al., 2011; Ilieva et al., 2009).

A hallmark of ALS pathology is microglial activation and proliferation (Beers et al., 2006, 2011; Fendrick et al., 2007; Philips and Robberecht

2011; Sanagi et al., 2010). Microglia are innate immune cells permanently residing in the CNS responsible for maintaining CNS homeostasis; however, their exact role in the ALS pathology or the mechanisms whereby microglia are activated have not yet been identified. Several lines of evidence indicate that while microglia do not significantly contribute to disease onset, they accelerate disease progression (Beers et al., 2006; Boillee et al., 2006b; Ilieva et al., 2009; Lee et al., 2012). Although microglial morphological activation is often interpreted as neuroinflammation, mostly based on *in vitro* studies or *in vivo* studies examining inflammatory markers in tissue homogenates (Beers et al., 2011; Hensley et al., 2003; Xie et al., 2004), there is little direct evidence that microglia are polarized toward the pro-inflammatory M1 phenotype in this disease *in vivo*. One recent study reported increased microglial expression of NOX2, but not other M1 markers, in SOD1 mutant mice (Liao et al., 2012). The failure of clinical trials targeting neuroinflammation in ALS (Benatar 2007; Cudkowicz et al., 2006; Gordon et al., 2007; Werdlein et al., 1990) suggests that a better understanding of microglial activities in ALS is needed.

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The severity and progression of ALS pathology is heterogeneous among motor neuron pools. In a rat SOD1<sup>G93A</sup> model, neurodegeneration is initially most prominent in the lumbar spinal cord, and gradually progresses to other spinal regions. By disease end-stage, more than 80–90% of motor neurons in the lumbar, 50–80% in the cervical and 40% in the thoracic spinal cord regions of SOD1<sup>G93A</sup> rats are lost, whereas brainstem hypoglossal motor neurons are unaffected at this time (Nichols et al., 2012). Thus, we hypothesized that microglial phenotype would be heterogeneous throughout the CNS, and that microglial activation would correlate with disease severity in specific regions. We further hypothesized that microglia would have a cytotoxic M1 phenotype in the most affected regions (lumbar and cervical spinal cord), but an anti-inflammatory/M2 phenotype in less affected regions (brain stem and cortex). If correct, these observations would suggest that therapies generally targeting all microglia, without regard to their specific phenotype, might be ineffective; it is even possible that detrimental microglial activities would be triggered in some CNS regions.

To address our hypotheses, we analyzed microglia isolated from the cortex, brain stem, and cervical, thoracic and lumbar spinal cord regions prior to, at symptom onset, and at disease end-stage (defined as 20% decrease in body mass) in SOD1<sup>G93A</sup> rats. Spinal microglial number was increased with morphologically activated microglia being present predominantly in ventral regions associated with motor neurons. Microglia were not polarized to typical M1 or M2 phenotypes at any disease stage, in any CNS region evaluated. Rather, they appeared to undergo a disease-specific transformation, characterized by high expression of galectin-3, osteopontin and VEGF, and a downregulation of TNF $\alpha$ , IL-6 and arginase-1 after symptom onset. This phenotype was restricted to the spinal cord as cortical microglia were not affected.

## Materials and methods

### Animals

Transgenic male Sprague Dawley rats bearing human SOD1<sup>G93A</sup> mutation were bred with wild-type females. For this study, SOD1<sup>G93A</sup> female offspring were used. Animals were weighed twice a week until they developed clinical signs and daily thereafter. Clinical signs included hind limb weakness, paresis and paralysis, loss of weight, and a decrease or loss of grooming abilities. In most animals (96%), the first clinical signs were observed in hind limbs, usually between 150 and 200 days of age. Very few animals developed front limb symptoms first. Symptom onset was defined as 5–10% loss of peak body weight, and in most cases, some weakness/paresis of hind limbs was also observed at this time. The end-stage of disease was defined as a loss of 20% body weight from peak body weight, and paralysis of at least one limb. Pre-symptomatic animals were harvested 3–8 weeks before expected symptom onset as approximated from clinical onset in sires. Wild-type littermates served as control groups. Animals were perfused with ice cold PBS followed by tissue dissection that included cortex, brain stem, cervical segments C1–C7, thoracic segments T2–T10 and lumbar segments L1–L5. Some SOD1<sup>G93A</sup> rats at disease end-stage and wild-type littermates were challenged with i.p. PBS or lipopolysaccharide (LPS) at a dose of 5 mg/kg body weight. Tissues were harvested 20 h later as described above. Animals were maintained in an AAALAC-accredited animal facility according to protocols approved by the University of Wisconsin Institutional Animal Care and Use Committee. All animals were housed under standard conditions, with a 12 hour light/dark cycle and *ad libitum* food and water.

### Isolation of microglial cells

Rats were perfused with ice-cold PBS followed by tissue dissection. Microglial cells were isolated as we have described in detail previously (Nikodemova and Watters 2011; Nikodemova and Watters 2012). Briefly, tissues were weighed and enzymatically digested using

Neurodissociation kit containing papain (Miltenyi Biotec, Germany), followed by centrifugation in 0.9 M sucrose to remove myelin. Cells were subsequently labeled with PE conjugated OX-42 (BD Biosciences, San Jose, CA) antibodies, incubated with anti-PE magnetic beads and separated on columns in a magnetic field. Cells in the CD11b<sup>+</sup> fraction were counted with a hemocytometer and the yield was normalized to tissue weight.

### RNA isolation and qRT-PCR

Isolated microglial cells or tissue homogenates were resuspended in Tri-reagent (Sigma) followed by total RNA isolation. Reverse transcription and quantitative PCR were performed as described in detail previously using SYBR Green (Applied Biosystems, Foster City, CA, USA) (Nikodemova and Watters 2012). Primer sequences are listed in Table 1. Relative gene expression normalized to 18S was determined by the  $\Delta\Delta C_t$  method. Data are expressed as the fold change relative to gene expression in the brainstem of wild-type rats. This allowed comparisons between wild-type and SOD1<sup>G93A</sup> rats at different stages of disease, as well as comparisons among CNS regions in the same animal.

### Flow cytometry

Animals were perfused with PBS followed by tissue dissection and enzymatic dissociation as described above. Cells were permeabilized with Cytofix/Cytoperm (BD Biosciences) for 25 min on ice followed by staining with OX42-APC (Biolegend, San Diego, CA, 1:100), CD45-FITC (BD Biosciences, 1:100) and galectin-3 (Novus, Littleton, CO, 1:100) antibodies for 25 min at 4 °C. After washing, cells were stained with anti-rabbit secondary antibodies (Invitrogen, Carlsbad, CA, 1:1000) for 20 min at 4 °C to detect galectin-3. Cells were fixed in 1.6% PFA and analyzed on a FACSCalibur. Data were analyzed using FlowJo software.

### Immunohistochemistry

Animals were perfused with ice-cold PBS followed by 4% PFA. The whole spinal cord was dissected and post-fixed with 4% PFA overnight. Tissues were cryoprotected by sequential incubation in 20 and 30% sucrose in PBS at 4 °C. 40  $\mu$ m sections were cut on a microtome. Free floating sections were stained with anti-CD11b-FITC (Biolegend), anti-ChAT (Millipore), anti-NeuN-Alexa488 (Millipore, Billerica, MA), anti-galectin-3 and anti-osteopontin antibodies (both from Novus) in staining buffer (PBS supplemented with 0.1% Triton-X100 and 0.5% BSA). Donkey secondary anti-rabbit-Alexa594 and anti-goat-Alexa647 antibodies (both from Invitrogen) were used to visualize galectin-3 and ChAT, respectively. Donkey secondary anti-mouse-Alexa647 antibody (Invitrogen) was used to visualize osteopontin. Images were taken on a Nikon Eclipse microscope and analyzed with ImageJ software (NIH).

**Table 1**  
Primer sequences for qRT-PCR analyses.

Gene name	Forward sequence (5')	Reverse sequence (5')	
MCP-1	ACGCTTCTGGGCTGTGTTC	TGG GGCATTAACTGCATCTGGCT	t1.1
Galectin-3	GCTTCACTGTGCTATGATTTGTG	CTGACAGTCCCTACGATATGC	t1.2
TNF- $\alpha$	TGCCATCTCATACAGGAGA	CCGAGTCCGTGATGTCTA	t1.3
Osteopontin	ATAGCTTGGCTTACGGACTGAG	GCATCGGGATACTGTTTCATCAGA	t1.4
Arginase-1	CCACGGTCTGTGGAAAAGCCAAT	TTGCCATCTGTGTTCTCCACCCA	t1.5
IL-6	GTGGCTAAGGACCAAGACCA	GGTTTCCGAGTAGACCTCA	t1.6
IL-10	AAGTGATGCCCGAGCAGAGAA	AAATCGATGACAGCGTCGCAGC	t1.7
BDNF	CCCATCAATCTACGGTAATC	TGCGGAGGGTCTCTATGAA	t1.8
VEGF	TTGAGACCTGTGGGACATCT	CACACAGGACGGCTTGAAGA	t1.9
COX-2	TGTTCCAACCATGTCAAAA	CGTAGAATCCAGTCCGGGTA	t1.10
iNOS	AGGGAGTGTGTTCCAGGTG	TCTGCAGGATGCTTGAACG	t1.11
18s	CGGGTGCTTCTAGCTGAGTGCCG	CTCGGGCTGCTTTGAACAC	t1.12

## Statistical analyses

Data are expressed as the mean  $\pm$  SE with 6–9 animals per group for each time point. In the LPS experiment, there were 4–6 animals/group. Statistical analyses were performed using SigmaStat software (Systat, San Jose, CA, USA). Multi-group comparisons were analyzed by one-way ANOVA followed by the Holm–Sidak test. A  $p$ -value  $< 0.05$  was considered statistically significant.

## Results

### Microglial distribution and gene expression are heterogeneous in wild-type rats

Although microglia are found throughout the CNS, they are not distributed homogeneously in all regions of the normal CNS. In wild-type rats, the density (number of cells/mg tissue) of CD11b<sup>+</sup> cells was highest in the cortex and gradually decreased along the spinal cord (Fig. 1A). Microglial density in the lumbar spinal cord was more than 50% lower than in cortex. In addition, there are significant regional differences in microglial gene expression, suggesting heterogeneous microglial phenotypes in distinct CNS regions. Basal expression of galectin-3 (Fig. 1B), MCP-1 (Fig. 1C) and TNF $\alpha$  (Fig. 1D) was significantly elevated in microglia from all spinal regions compared to brainstem or cortex. On the other hand, there were no regional differences in IL-6 expression (not shown). Thus, in the healthy CNS, spinal microglia

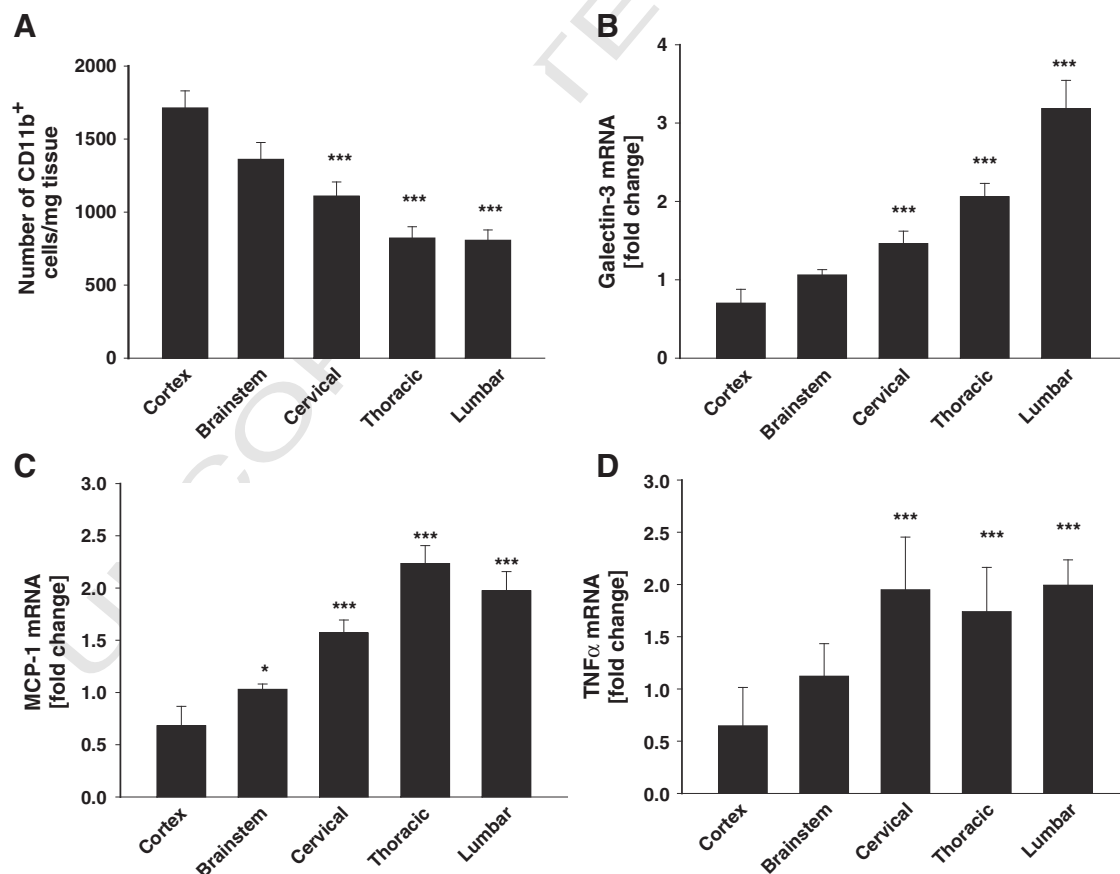
are fewer in number and express higher basal levels of at least some pro-inflammatory molecules.

### Spinal microglial number increases with disease progression in SOD1<sup>G93A</sup> rats

We did not observe differences in microglial number in pre-symptomatic rats versus age-matched wild-types in any CNS region. However, at symptom onset, there was a 2.5-fold increase in microglial number in the lumbar spinal cord, the most affected spinal region at this time (Fig. 2A). By disease end-stage, microglial number was increased 3-fold in the brainstem and cervical spinal cord, 2-fold in the thoracic spinal cord, and up to 6-fold in the lumbar spinal cord. Increased CD11b<sup>+</sup> cell number likely results from proliferation of resident microglia since there were no changes in CD11b/CD45 characteristics that would indicate peripheral macrophage infiltration (Fig. 2B).

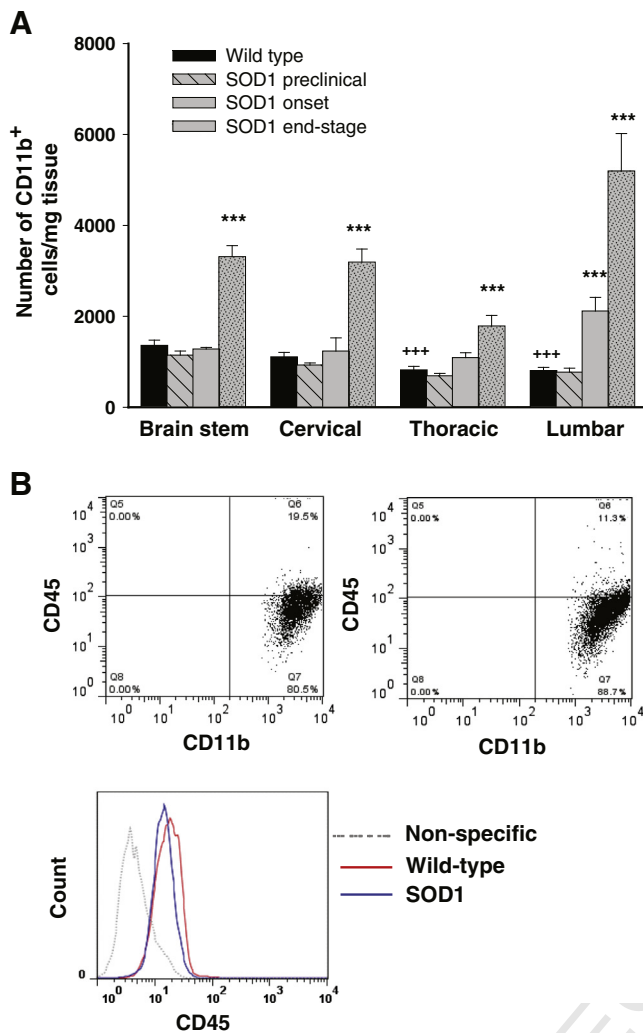
### Microglia acquire a motor neuron disease-specific phenotype

Whereas the activation and proliferation of microglia in ALS pathology are well-established, specific microglial activities are less well characterized. It is often presumed that morphologically activated microglia possess a pro-inflammatory M1 phenotype, and that microglia are pro-inflammatory at disease end-stage. Although we did not observe any changes in microglial gene expression in pre-symptomatic SOD1<sup>G93A</sup> rats, surprisingly, at disease onset and later, we found that microglia strongly suppressed expression of TNF $\alpha$  and IL-6, key pro-inflammatory



**Fig 1.** Microglial density and phenotype differ by region in the healthy CNS. Microglia were immunomagnetically isolated from cortex, brain stem and cervical, thoracic and lumbar spinal cord of wild-type rats and analyzed for gene expression by qRT-PCR. A) Microglial density (number of CD11b<sup>+</sup> cells/mg tissue) is significantly lower in all regions of the spinal cord compared to cortex or brain stem. B) Microglial galectin-3 expression is significantly higher in the spinal cord than in brainstem or cortex. Galectin-3 expression gradually increases along the spinal cord with lumbar microglia having 2 times higher mRNA levels than cervical microglia. Spinal microglia also express higher basal levels of the pro-inflammatory genes MCP-1 (C) and TNF $\alpha$  (D). Gene expression data are displayed relative to expression in brainstem microglia. \* $p < 0.05$  versus cortex; \*\*\* $p < 0.001$  versus cortex.





**Fig 2.** Microglial number increases with ALS disease progression. Microglia were isolated from wild-type rats and SOD1<sup>G93A</sup> rats at pre-clinical, onset and end-stage of disease. A) There were no changes in microglial number in the pre-symptomatic stage between wild-type and SOD1<sup>G93A</sup> rats. By disease end-stage, microglial number was significantly increased in all regions. B) Flow cytometry analysis revealed that most CD11b<sup>+</sup> cells had low levels of CD45 consistent with microglial characteristics. Quadrant 7 (Q7) shows CD11b<sup>+</sup>/CD45<sup>low</sup> cells and Q6 shows CD11b<sup>+</sup>/CD45<sup>high</sup> cells. The mean fluorescent intensity histogram indicates no differences between CD45 expression in wild-type and SOD1<sup>G93A</sup> microglia. <sup>+++</sup>*p* < 0.001 versus brainstem in wild-type rat; <sup>\*\*\*</sup>*p* < 0.001 versus wild-type of the same region.

cytokines. TNF $\alpha$  mRNA was significantly decreased in microglia from all spinal cord regions beginning at disease onset, an effect that further decreased with disease progression in both spinal cord and brainstem (Fig. 3A). At disease end-stage, IL-6 mRNA was significantly down-regulated by 40–60% in brainstem and spinal cord microglia (Fig. 3B). However, not all pro-inflammatory genes were down-regulated as MCP-1(CCL2) (Fig. 3C), COX2 and iNOS (Fig. 8, for cervical region) were unchanged. Interestingly, osteopontin, a marker usually associated with inflammatory Th1 responses was significantly increased by 8–10 fold at disease end-stage in all regions (Fig. 3D). Since there are no reports concerning the role of osteopontin in ALS pathology, the significance of this striking upregulation is uncertain. Overall, these data suggest that microglia do not possess a typical M1 phenotype, nor do they display clearly pro-inflammatory activities at any time during the course of disease. However, tissue production of some inflammatory mediators may be increased because of the increase in microglial cell number. For example, although microglial MCP-1 expression is not changed with disease progression, MCP1 is significantly increased 5-fold in cervical tissue homogenates (Fig. 3E). TNF $\alpha$  mRNA levels were

2 times higher in cervical homogenates from end-stage SOD1<sup>G93A</sup> rats than in age matched wild-type controls; however, this did not reach statistical significance (*p* < 0.057; Fig. 3F).

Because microglia are not polarized toward a typical M1 phenotype, we explored the possibility that they acquire an alternative, anti-inflammatory M2 phenotype. We analyzed microglia for arginase-I, IL-10 and brain derived neurotrophic factor (BDNF) expression, molecules associated with the M2 phenotype. We also evaluated vascular endothelial growth factor (VEGF) since this potent pro-survival growth factor is elevated in the spinal cords of ALS patients, although the cellular source of this factor was not identified (Gupta et al., 2011). Microglial arginase-1 levels were significantly decreased at disease end-stage in all spinal regions and in the brainstem (Fig. 4A). The anti-inflammatory cytokine IL-10 was significantly downregulated in the lumbar spinal cord at disease onset (Fig. 4B); at disease end-stage, IL-10 appeared to decrease in other spinal regions, but this apparent difference was significant only in the brainstem and lumbar spinal cord. BDNF and VEGF expressions were regulated in opposite ways. BDNF was downregulated by disease end-stage, but this effect was significant only in the lumbar spinal cord (Fig. 4C). VEGF mRNA was significantly increased in all regions of the spinal cord, beginning at symptom onset. By disease end-stage, VEGF was also elevated in the brainstem (Fig. 4D).

#### Galectin-3 is greatly upregulated in spinal microglia during disease

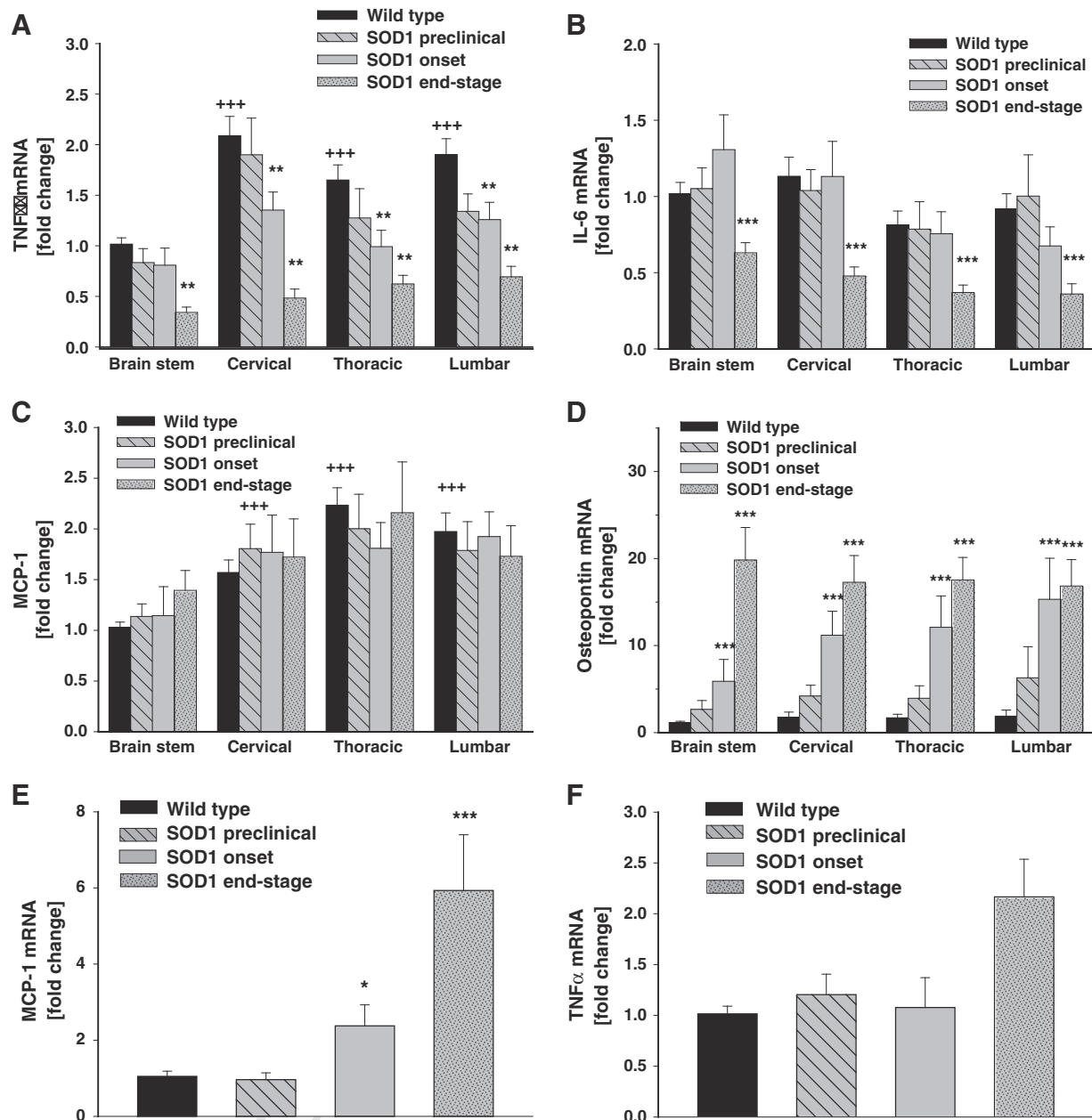
Recently, galectin-3 was identified as a potential biomarker for ALS diagnosis. In a murine ALS model, galectin-3 was primarily upregulated in microglial cells (Lerman et al., 2012). In the rat ALS model, we show that galectin-3 mRNA is highly upregulated (4 to 7-fold) in microglia at disease onset in brainstem and spinal cord, and it remained elevated until disease end-stage (Fig. 5A). In wild-type rats, galectin-3 protein is expressed only in a small proportion (9–10%) of brainstem and spinal cord microglia (Fig. 5B); after symptom onset, the frequency of galectin-3<sup>+</sup> microglia increased 4-fold in brainstem and 5 to 7-fold in spinal cord. However, there was still a significant proportion of CD11b<sup>+</sup> cells that did not express this protein. Thus, even in disease-affected CNS regions, there are several distinct CD11b<sup>+</sup> cell populations. In the wild-type CNS, galectin-3 is mostly expressed by cells other than microglia, including neurons (Fig. 6), although the function of galectin-3 in neurons remains unclear. At symptom onset, we found that galectin-3 was highly upregulated predominantly in CD11b<sup>+</sup> cells that were clustered in the ventral horn, where motor neurons are localized. Microglia in this region exhibited an activated morphology, whereas microglia in the white matter appeared to be less affected (Fig. 6). Interestingly, osteopontin was also predominantly expressed in neurons in the wild-type CNS, but it was greatly upregulated in galectin-3<sup>+</sup> microglia during disease.

#### Cortical microglia appear unaffected by disease

To determine if the microglial phenotype is driven by SOD1<sup>G93A</sup> overexpression, we evaluated microglial phenotype in the cortex, a region less affected by disease in this rat model. There was no change in microglial number (Fig. 7A), galectin-3 (Fig. 7B), TNF $\alpha$  (Fig. 7C), or osteopontin (Fig. 7D) expression during the course of disease. Since cortical microglia in SOD1<sup>G93A</sup> rats did not differ from wild-type microglia based on any parameter assessed here, it seems that SOD1<sup>G93A</sup> overexpression alone is not sufficient to promote the altered microglial phenotype.

#### Spinal microglia are functionally impaired at disease end-stage

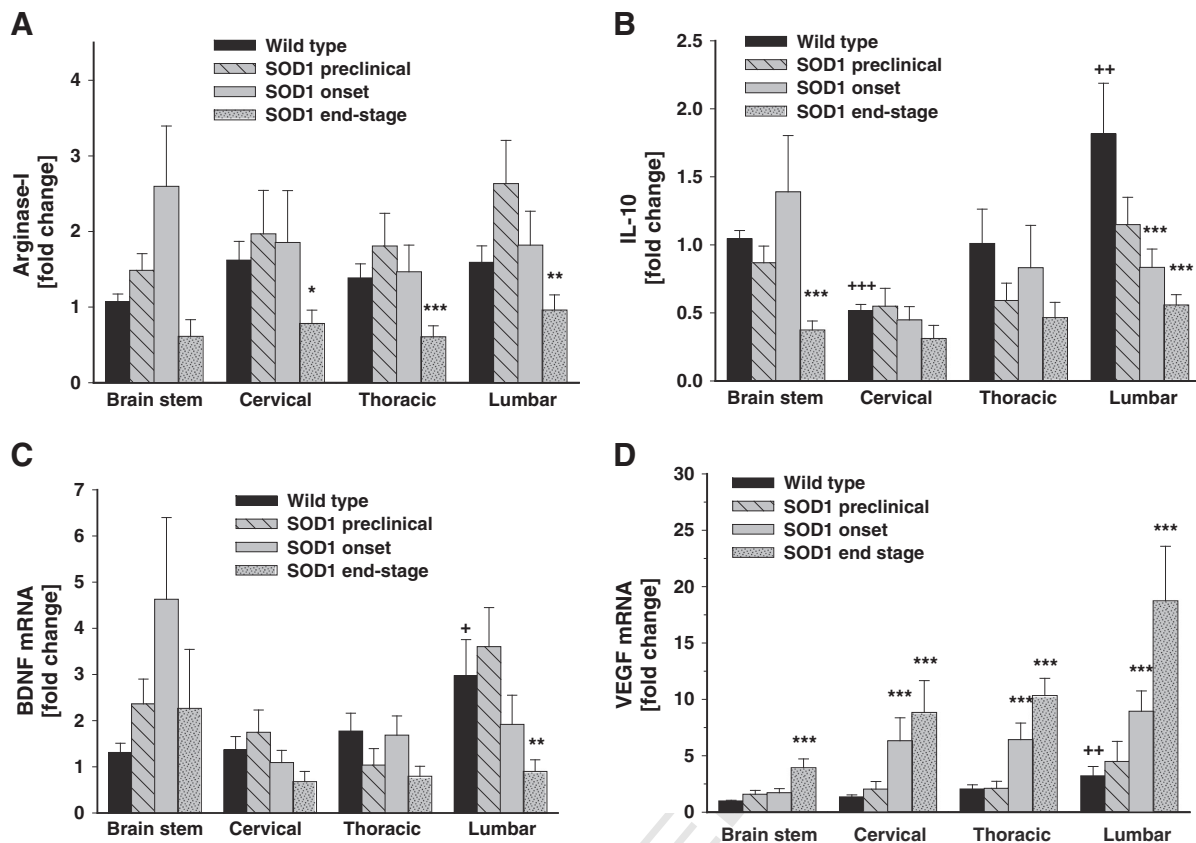
Since we observed significant differences in the microglial phenotype between cortex and spinal cord, we sought to determine if microglia in these regions are also functionally distinct. We exposed wild-type and end-stage SOD1<sup>G93A</sup> rats to LPS (i.p. 5 mg/kg) for 20 h, and



**Fig 3.** Pro-inflammatory gene expression in spinal microglia is differentially affected by disease. TNFα (A), IL-6 (B), MCP-1 (C), and osteopontin (D) mRNA levels were analyzed by qRT-PCR in microglia isolated from the brainstem and spinal cord. Spinal microglia expressed higher levels of TNFα and MCP-1, but not IL-6 or osteopontin compared to brain stem microglia. TNFα and IL-6 expression was significantly decreased by disease end-stage in all spinal regions, but here were no changes in MCP-1 mRNA levels with disease progression. In contrast, osteopontin expression was highly upregulated in microglia from all CNS regions, from symptom onset until end-stage. Data are displayed relative to expression in brainstem microglia from wild-type animals. (A–D): \*\*\*p < 0.001 versus brainstem in wild-type rat; \*\*p < 0.01 versus wild-type of the same region; \*\*\*p < 0.001 versus wild-type of the same region. Inflammation gene expression was also measured in cervical tissue homogenates. MCP-1 mRNA levels (E) were significantly increased after symptom onset and were further elevated by disease end-stage. TNFα mRNA levels (F) were elevated at end-stage, but not significantly. Data are graphed relative to expression in wild-type rats. (E–F): \*\*p < 0.01 versus wild-type; \*\*\*p < 0.001 versus wild-type.

analyzed microglial responses. In wild-type rats, microglia in all spinal regions displayed stronger pro-inflammatory responses to LPS than microglia from cortex. LPS-induced COX-2 and iNOS mRNA levels were increased approximately 3 to 5-fold in microglia from the cortex (Fig. 8A and B) and 6 to 12-fold (Fig. 8D and E) in the spinal cord of wild-type rats. Galectin-3 upregulation in response to LPS was similar in microglia from cortex and spinal cord (Fig. 8C and F). These findings correlate with the increased basal expression of pro-inflammatory genes in spinal microglia (Fig. 1), which may prime them toward enhanced inflammatory responses. Cortical microglial responses to systemic inflammation induced by LPS did not significantly differ between wild-type and SOD1<sup>G93A</sup> rats. However, in the spinal cord, LPS-induced upregulation

of iNOS and COX-2 was significantly lower in SOD1<sup>G93A</sup> versus wild-type spinal microglia (only cervical data are shown; similar responses were observed in microglia from the thoracic and lumbar spinal cord). Moreover, whereas galectin-3 expression increased in response to LPS in spinal microglia from wild-type rats, it was non-significantly downregulated in SOD1<sup>G93A</sup> microglia. Twenty hours post-LPS injection, no changes in TNFα or IL-6 were observed in any region in either wild-type or SOD1<sup>G93A</sup> rats. Together, these data show that, whereas microglial responsiveness in the cortex of SOD1<sup>G93A</sup> rats is unaffected at disease end-stage, spinal microglia are far less responsive to systemic inflammation, supporting the notion that they do not acquire the prototypical M1 phenotype at disease end-stage.



**Fig 4.** Microglial expression of anti-inflammatory genes and growth factors is differentially affected by disease. Arginase-I (A), IL-10 (B), BDNF (C) and VEGF (D) mRNA levels were analyzed by qRT-PCR in microglia isolated from the brainstem and spinal cord. In wild-type rats, microglia expressed higher mRNA levels of IL-10, BDNF and VEGF in the lumbar spinal cord compared to brainstem. At symptom onset, we observed significant upregulation of VEGF in the spinal cord, and by end-stage, elevated levels of VEGF were also found in the brainstem. At end-stage, there was also a significant down-regulation of arginase-1, a M2 phenotypic marker, in all spinal cord regions. Data are displayed relative to expression in brainstem microglia from wild-type animals.  $^+p < 0.05$  and  $^{++}p < 0.001$  versus brainstem in wild-type rats;  $^*p < 0.05$  versus wild-type of the same region;  $^{***}p < 0.001$  versus wild-type of the same region.

## Discussion

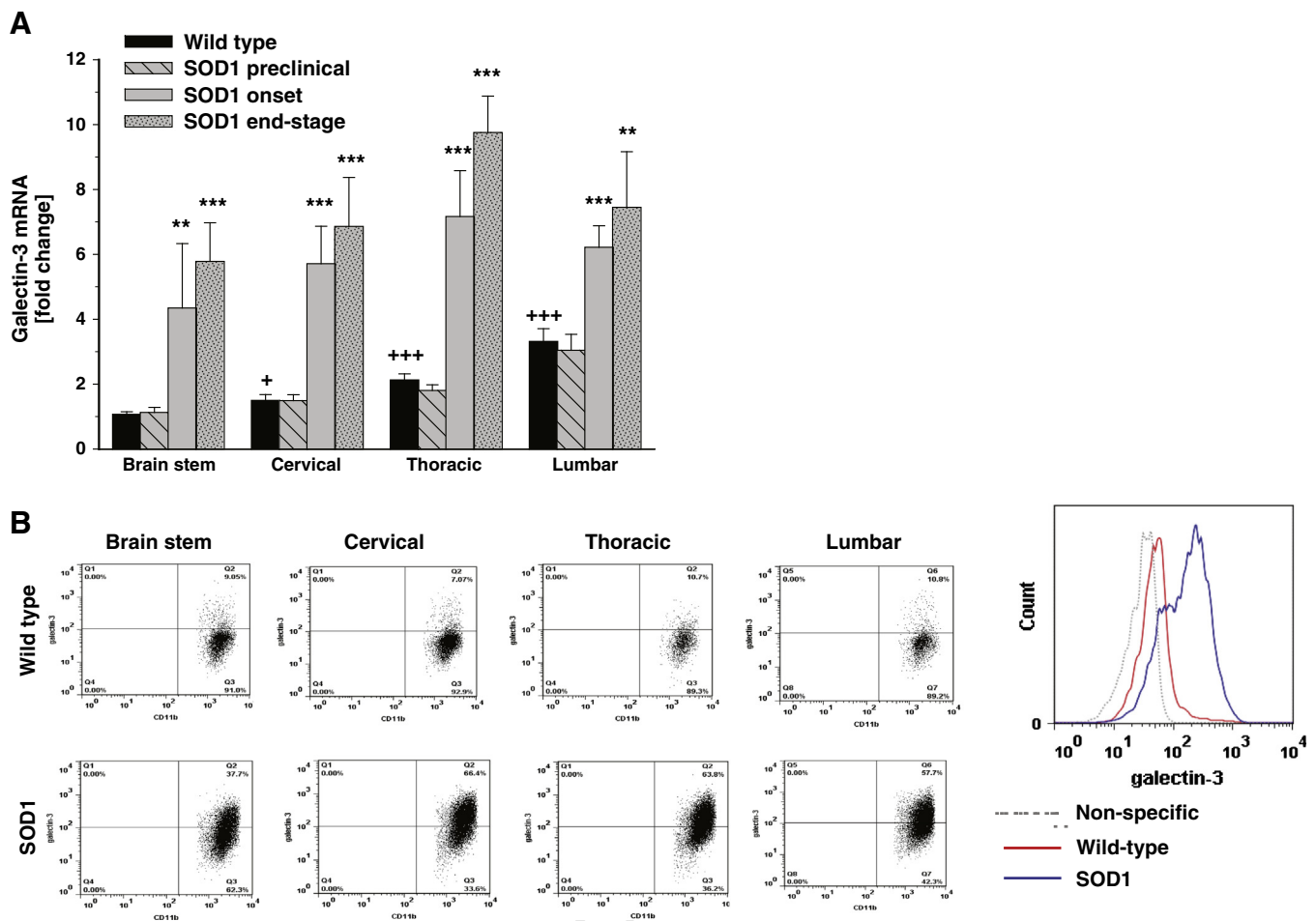
Although microglial activation is a hallmark pathology of all neurodegenerative disorders, it is still debated whether this activation is merely a response to distressed or dying neurons, or if microglia play an active role in disease etiology or progression. Another controversial question is whether microglial activities in CNS disorders are beneficial or detrimental.

In ALS, strong evidence suggests that overexpression of mutant SOD1 in microglia alone is not sufficient to cause motor neuron disease. For example, transplantation of SOD1<sup>G93A</sup> microglia into the spinal cord of wild-type mice does not induce motor neuron injury or ALS-like symptoms (Beers et al., 2006). On the other hand, microglia with mutant SOD1 accelerate disease progression in murine models of ALS (Beers et al., 2006; Boillee et al., 2006b; Lee et al., 2012). Based primarily on *in vitro* studies, it has been proposed that SOD1<sup>G93A</sup> microglia create a neurotoxic microenvironment due to increased inflammatory cytokine and reactive oxygen species production, with decreased phagocytic capacity (Hensley et al., 2003; Sargsyan et al., 2011, 2005; Weydt et al., 2004). However, it has not been shown that these microglial responses occur *in vivo*. In ALS mice, microglia exhibit increased NADPH oxidase (NOX2) expression in the lumbar spinal cord at disease end-stage (Liao et al., 2012), consistent with a compensatory response to the biochemical effects of increased SOD1 levels characteristic of SOD1 transgenic rodents (Nichols et al., 2012). On the other hand, we found elevated microglial levels of galectin-3, a carbohydrate-binding protein, which was shown to be necessary for the IL-4-induced alternative M2 (anti-inflammatory) phenotype in macrophage (MacKinnon et al.,

2008). These diverse findings leave us with several unanswered questions, including what microglial activities contribute to ALS pathology, how the microglia are themselves affected by the disease, and whether they have beneficial activities at some stages in disease progression. To increase our understanding of microglial activities in ALS, in this study we performed a detailed analysis of microglial phenotype, and their ability to respond to systemic inflammation in different CNS regions at different stages of disease.

Here, we report significant regional heterogeneity in microglial phenotype and number, even in the healthy CNS of wild-type rats. In specific, spinal microglia may be more reactive and macrophage-like than those in the cortex. Galectin-3 levels are increased in spinal microglia, and galectin-3 is an immunomodulator that increases during differentiation of monocytes into macrophages (Chen et al., 2005; Lund et al., 2009). Galectin-3 is also upregulated in activated microglia after ischemic brain lesions (Walther et al., 2000). Spinal microglia from wild-type rats have elevated basal galectin-3 levels and increased expression of pro-inflammatory genes, suggesting that they may be in a different activation state from cortical microglia. Thus, microglial responses to ALS progression may be region-specific, at least in part, due to differences in their intrinsic characteristics among CNS regions. Indeed, systemic inflammation induced by LPS elicited stronger pro-inflammatory responses in wild-type spinal versus cortical microglia, supporting the idea that microglia are more reactive in the spinal cord. How regional microglial heterogeneity affects the vulnerability of different CNS regions to neurodegeneration, and how this heterogeneity may contribute to the pathology of traumatic, ischemic and neurodegenerative CNS disorders remains to be answered.





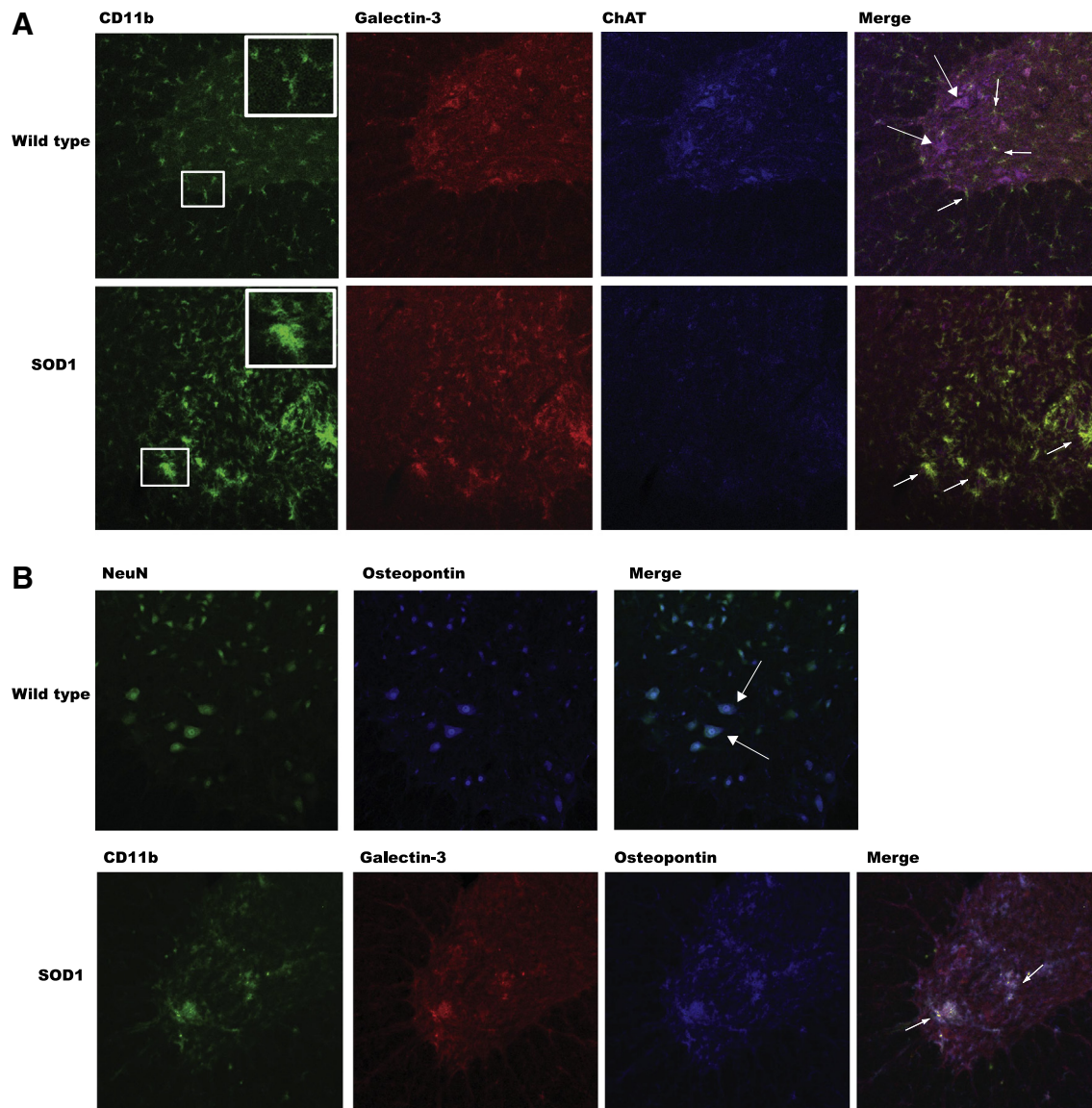
**Fig 5.** Rat SOD1<sup>G93A</sup> microglia upregulate galectin-3. Galectin-3 levels were assessed in microglia by qRT-PCR (A) and flow cytometry (B). In wild-type rats, galectin-3 mRNA levels were significantly higher in spinal microglia than in brainstem microglia (A). Only a small population (7–11%) was galectin-3<sup>+</sup> when analyzed by flow cytometry (B). However, galectin-3 mRNA and protein levels were significantly upregulated after symptom onset. Flow cytometry analysis also revealed that after disease onset, there are at least two major microglial populations in these regions; one that is galectin-3 positive and one that is negative, suggesting that they may also be functionally distinct. Gene expression data are graphically displayed relative to expression in brainstem microglia from wild-type animals. \**p* < 0.05 and \*\*\**p* < 0.001 versus brainstem in wild-type rats; \*\**p* < 0.01 versus wild-type of the same region; \*\*\**p* < 0.001 versus wild-type of the same region.

Prior to symptom onset in SOD1<sup>G93A</sup> rats, we did not observe differences in microglial number or gene expression in the brainstem, or in any region of the spinal cord compared to wild-type littermates. This finding correlates with other reports that microglia are not necessary for disease development (Boillee et al., 2006b), and further suggests that microglial changes observed at later time points are driven by local, ongoing pathology. However, in another study using the SOD1<sup>G93A</sup> rat model, increased CD11b immunoreactivity was reported prior to symptom onset (Graber et al., 2010). This apparent discrepancy with our results may be due to differences between animal colonies maintained in different environments. In their study, rats developed paralysis at 130 days of age, whereas symptom onset usually occurred between 150 and 200 days of age in our rat colony. Another important difference is that they used male rats, whereas we used females in our experiments. A recent study demonstrated that genetic background and sex are significant modifiers of disease in rodent SOD1 models (Pan et al., 2012; Suzuki et al., 2007). It is possible that environmental factors causing epigenetic regulation of microglial gene expression could account for colony-related differences in the same rodent strain. On the other hand, time relative to actual symptom onset may be an important modifier that distinguishes these studies. Because we harvested tissues 3–8 weeks before putative symptom onset, it is possible that changes in microglia would have been observed if harvested closer to actual symptom onset. This possibility is supported by changes in microglial phenotype and number in the brainstem at disease end-

stage, despite the absence of detectable neuronal death or dysfunction in this region (Nichols et al., 2012). Microglial activation may therefore be indicative of ongoing processes prior to the onset of clinical symptoms.

At symptom onset, increased microglial numbers were found in the lumbar spinal cord, the most affected region with the greatest motor neuron death. Analysis of CD11b and CD45 levels suggests that increased CD11b<sup>+</sup> cell numbers is likely due to proliferation of resident microglia, without significant macrophage infiltration. This interpretation is consistent with previous studies showing no contribution of peripheral macrophages to microglial proliferation and activation (Ajami et al., 2007; Solomon et al., 2006).

The most prominent microglial characteristic in the spinal cord was strong upregulation of galectin-3, VEGF and osteopontin. At present, we do not know the significance of these proteins in ALS pathology, and we can only infer possible roles based on sparse literature accounts. The role of galectin-3 in macrophage/microglial physiology is the most studied. Galectin-3 was recently identified as a possible biomarker for ALS diagnosis, as it is significantly increased in spinal tissues and cerebrospinal fluid of ALS patients (Zhou et al., 2010). Galectin-3 modulates the function of monocytes in an autocrine and/or paracrine manner (Chen et al., 2005), and it is important for phagocytosis (Sano et al., 2003). In macrophages, galectin-3 is also necessary for acquiring the anti-inflammatory/tissue reparative M2 phenotype in response to IL-4 (MacKinnon et al., 2008). In ischemic brain injury, the absence of



**Fig 6.** Galectin-3 and osteopontin co-localize in microglia at disease end-stage. (A) In the spinal cord of wild-type rats, galectin-3 is present predominantly in ChAT-positive neurons, including large motor neurons visible in the ventral horn (large arrowheads). At disease end-stage, there are no ChAT-positive motor neurons evident in the ventral horn. (B) Neurons (Neu-N<sup>+</sup> cells; large arrowheads), not microglia (small arrowheads) are a major source of osteopontin in healthy animals. Most microglia in wild-type rats display a ramified morphology, and are galectin-3 (A) and osteopontin (B) negative. At disease end-stage there are clusters of morphologically activated microglia (small arrowheads) that express both galectin-3 and osteopontin (A and B).

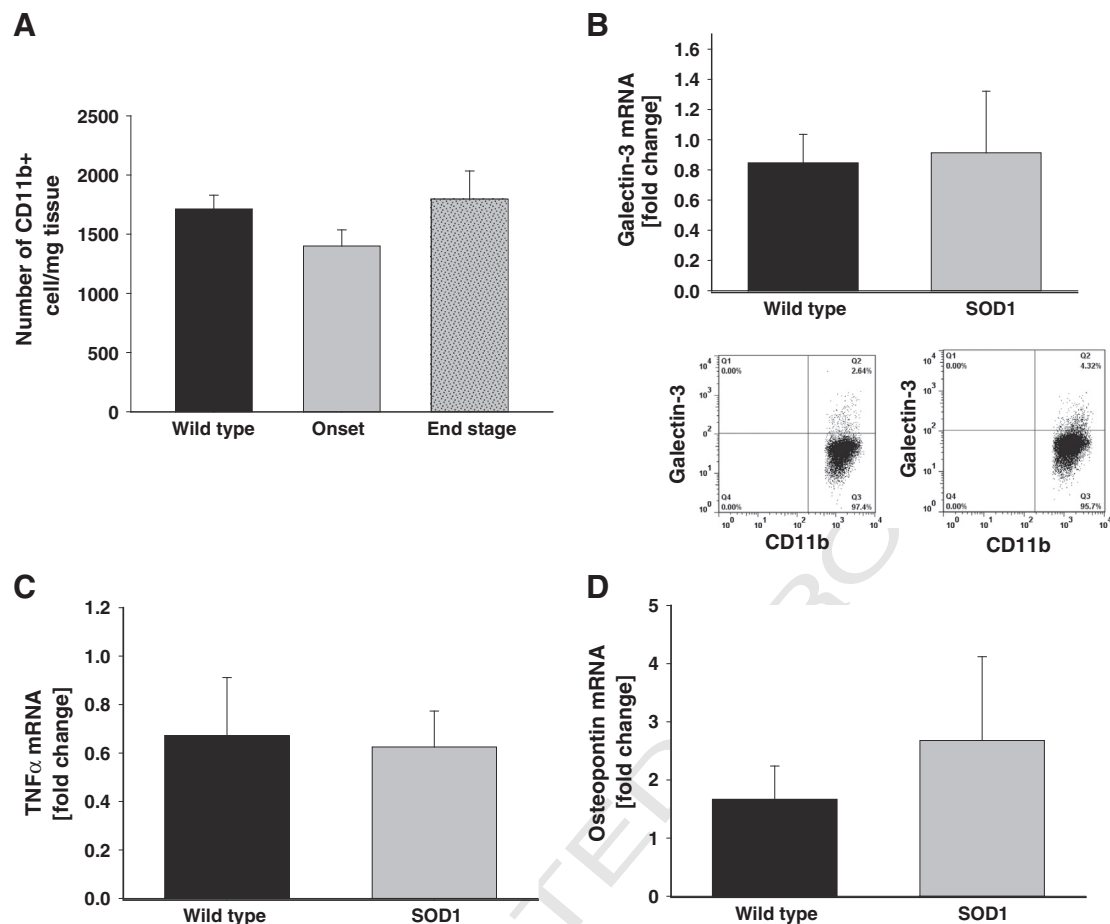
galectin-3 resulted in significant reductions in microglial proliferation in galectin-3<sup>-/-</sup> mice, an effect that significantly increased ischemic lesion size (Lalancette-Hebert et al., 2012). Therefore it is possible that upregulated galectin-3 in ALS contributes to increased microglial proliferation and/or phagocytosis. SOD1<sup>G93A</sup>/galectin-3<sup>-/-</sup> mice show more rapid disease progression with more severe symptoms (Lerman et al., 2012). However, since galectin-3 is widely expressed in the CNS by many cell types (including motor neurons), it is difficult to infer what cell type mediates this dramatic effect on disease progression.

VEGF administration slows disease progression and prolongs survival in ALS models (Azzouz et al., 2004; Zheng et al., 2004, 2007). In addition, increased VEGF levels are found in the serum and cerebrospinal fluids of North Indian ALS patients who have significantly higher survival rates (~10 years) compared to patients in the USA or Europe (3–5 years post diagnosis) (Gupta et al., 2011). However, the cellular source of increased VEGF production has not been identified. Besides its role in angiogenesis, VEGF is a potent neurotrophic factor that can protect motor neurons from oxidative stress *in vitro* (Li et al., 2003).

Here, we show that microglia significantly upregulate VEGF early in disease, and that this increase is maintained until end-stage. These observations suggest that at least some microglial activities may be neuroprotective, although more studies are needed.

Osteopontin is a matricellular protein with diverse functions. In immune cells it may act as a Th1 cytokine associated with inflammatory and autoimmune disease (Lund et al., 2009). However, the role of osteopontin in ALS pathology is completely unknown. A recent study showing significant upregulation of the osteopontin receptor, CD44, in microglia and astrocytes in SOD1<sup>G93A</sup> mice suggests that osteopontin plays an as yet undetermined role in ALS (Matsumoto et al., 2012). Osteopontin is highly expressed in the healthy CNS, mostly by neurons (Maetzler et al., 2010), consistent with our observations. In SOD1<sup>G93A</sup> rats, strong upregulation of osteopontin was found in galectin-3<sup>+</sup> microglia, predominantly in the spinal ventral horns. Increased osteopontin levels are also found in the CNS during Parkinson and Alzheimer disease, stroke and spinal cord injury; however, it seems that the effects of osteopontin can be opposite, depending on the specific pathology. For





**Fig 7.** Microglial number and inflammatory gene expression in the cortex of SOD1<sup>G93A</sup> rats are unaffected at disease end-stage. There were no differences in microglial number in the cortex between wild-type and SOD1 animals (A). At disease end-stage, no differences were observed in the mRNA levels of galectin-3 (B), TNFα (C) or osteopontin (D), genes that were significantly affected by disease in spinal microglia.

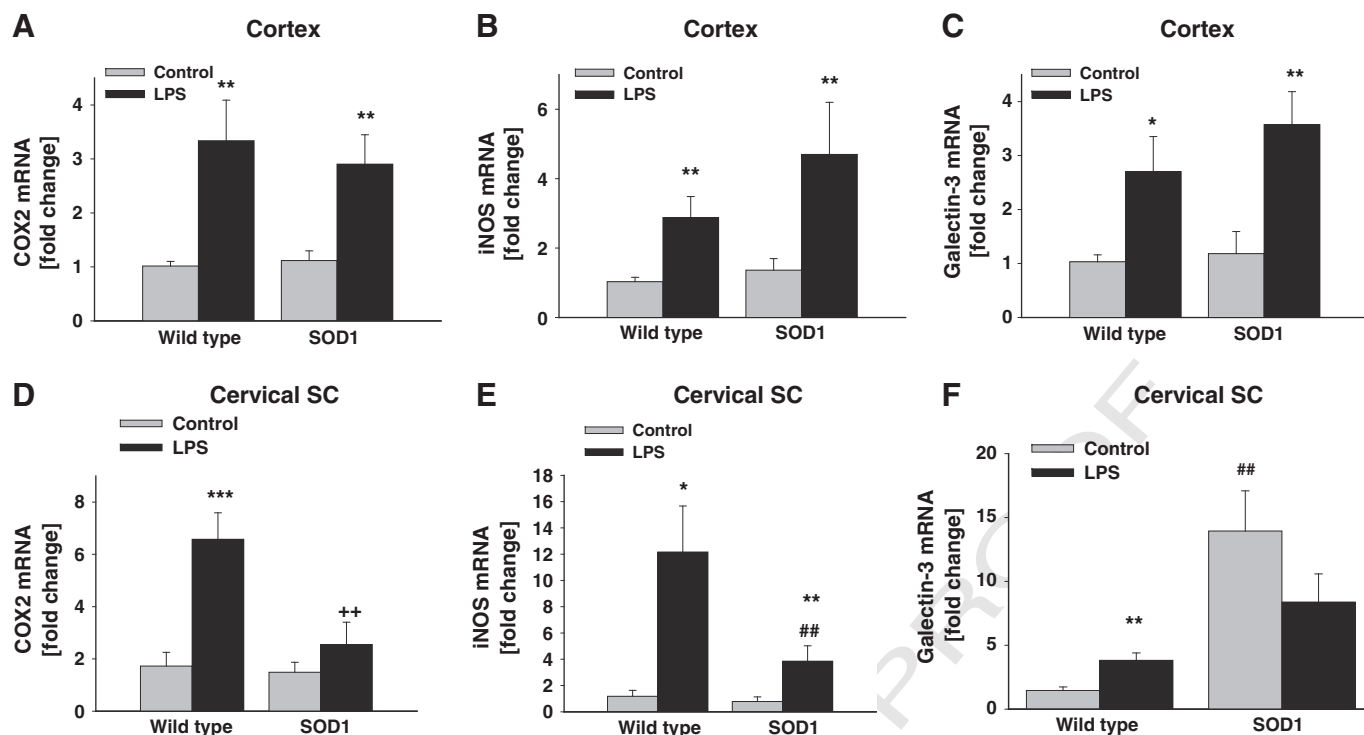
example, osteopontin is neuroprotective in spinal injury, and detrimental in Parkinson disease (Hashimoto et al., 2007; Maetzler et al., 2007).

At end-stage, decreased expression of TNFα and IL-6 (M1 markers) was observed, as well as decreased expression of the M2 markers arginase-1, IL-10 and BDNF. Thus, microglia in this ALS model have neither the typical M1 nor M2 phenotypes. We suggest that in ALS, microglia acquire an atypical phenotype not previously described in any neurodegenerative disorder. Although the expression of some pro-inflammatory genes in microglia was unchanged (iNOS, COX2, and MCP-1), tissue homogenates increase expression of these same genes, suggesting that there is tissue (*versus* microglial) inflammation. Our findings are consistent with other studies that found a 50–100% increase in TNFα in spinal tissues (Hensley et al., 2003; Xie et al., 2004). We suggest that spinal inflammation is a result of increased microglial cell numbers instead of an increase in expression of pro-inflammatory genes by individual microglia. It is likely that other CNS cell types also contribute to neuroinflammation in ALS. Collectively, our observations may help to explain the lack of clinical efficacy of anti-inflammatory drugs that target inflammatory gene regulation.

A recent study evaluating upper motor neuron loss in the rat SOD1 model found that although the number of corticospinal motor neurons in cortical layer 5 was not changed, their cell bodies were smaller compared to wild-type littermates, suggesting some degree of neurodegeneration in the motor cortex (Thomsen et al., 2012). We did not observe any changes in cortical microglia from SOD1<sup>G93A</sup> rats with regard to number, phenotype or responsiveness to inflammatory stimulation. Further, total SOD1 immunoreactivity is comparable in the cortex and spinal cord in SOD1<sup>G93A</sup> rats although in late presymptomatic to

late end-stage of disease, there are higher levels of monomeric (destabilized) forms that associate with the mitochondria in the spinal cord (Ahtoniemi et al., 2008). Thus, overexpression of mutated SOD1 alone is not sufficient to activate microglia. Other factors or signals, most likely originating from severely degenerating motor neurons, may be needed to transform microglia. However, spinal microglia may be naturally more sensitive to neuronal distress signals since they have higher basal expression of several pro-inflammatory genes *versus* cortical microglia. This concept is further supported by our findings that spinal microglia are more responsive to immune challenge in wild-type rats. Therefore, the initial responses of spinal microglia to ALS pathology may be more robust *versus* other CNS regions. On the other hand, we show here that spinal microglial responsiveness to LPS at disease end-stage is blunted. We speculate that with disease progression, microglia become impaired, and lose important properties necessary to sustain neuronal survival and/or properly respond to the needs associated with degenerating motor neurons. This idea is supported by recent findings showing diminished neuroprotective functions of microglial cells in response to hypoglossal nerve axotomy in adult SOD1<sup>G93A</sup> mice, but not in young, presymptomatic animals (Kawamura et al., 2012), or that SOD1<sup>G93A</sup> microglial cultures have reduced responsiveness to stimuli (Sargsyan et al., 2011).

In conclusion, microglia in the rat SOD1<sup>G93A</sup> ALS model do not acquire a typical M1 or M2 phenotype (as summarized in Table 2). Rather, they undergo a disease-specific transformation, predominantly near affected motor neurons. The existence of several phenotypically and functionally distinct microglial pools throughout the CNS (including heterogeneity in the affected regions) suggests that therapies targeting



**Fig 8.** SOD1<sup>G93A</sup> spinal microglia have blunted inflammatory responses to systemic LPS challenge. Wild-type or SOD1<sup>G93A</sup> rats at disease end-stage were challenged with LPS (5 mg/kg i.p.). After 20 h, cortical and spinal microglia were isolated, and mRNA levels of the inflammatory genes COX-2 (A and D), iNOS (B and E), and galectin-3 (C and F) were analyzed by qRT-PCR. Only data from cortical and cervical microglia are shown, although we observed similar responses in microglia from thoracic and lumbar spinal cord regions as well. In wild-type rats, LPS injection induced a stronger upregulation of COX2, iNOS and galectin-3 in spinal microglia than in cortical. In the cortex, there were no differences in microglial responsiveness to LPS between wild-type and SOD1<sup>G93A</sup> microglia (A–C). However, responses to LPS in cervical microglia were blunted in SOD1<sup>G93A</sup> microglia compared to wild-type (D–F). Data are displayed relative to expression in cortical microglia from wild-type animals. \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001 LPS versus control; +p < 0.01 LPS wild-type versus LPS SOD1; ##p < 0.01 control wild-type versus control SOD1.

all microglia might be ineffective at best, and may actually trigger inappropriate microglial activities at worst, in at least some CNS regions. Thus, anti-inflammatory drugs may have beneficial effects if delivered before symptom onset (*i.e.* before microglial transformation), but detrimental if administered after symptom onset (Keller et al., 2011; Kriz et al., 2002; Zhu et al., 2002), when phenotypic heterogeneity of microglial cells has been established. Since transplantation of wild-type microglia prolongs survival in the SOD1<sup>G93A</sup> model, better therapeutic strategies may involve identifying and targeting factor(s) responsible for microglial transformation, to prevent microglial disease-specific changes that lead to their impaired function.

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## References

- Ahtoniemi, T., Jaronen, M., Keksa-Goldsteine, V., Goldsteins, G., Kiostinaho, J., 2008. Mutant SOD1 from spinal cord of G93A rats is destabilized and binds to inner mitochondrial membrane. *Neurobiol. Dis.* 32, 479–485.
- Ajami, B., Bennett, J.L., Krieger, C., Tetzlaff, W., Rossi, F.M., 2007. Local self-renewal can sustain CNS microglia maintenance and function throughout adult life. *Nat. Neurosci.* 10 (12), 1538–1543.
- Azzouz, M., Ralph, G.S., Storkebaum, E., Walmsley, L.E., Mitrophanous, K.A., Kingsman, S.M., Carmeliet, P., Mazarakis, N.D., 2004. VEGF delivery with retrogradely transported lentivector prolongs survival in a mouse ALS model. *Nature* 429 (6990), 413–417.
- Beers, D.R., Henkel, J.S., Xiao, Q., Zhao, W., Wang, J., Yen, A.A., Siklos, L., McKercher, S.R., Appel, S.H., 2006. Wild-type microglia extend survival in PU.1 knockout mice with familial amyotrophic lateral sclerosis. *Proc. Natl. Acad. Sci. U. S. A.* 103 (43), 16021–16026.
- Beers, D.R., Zhao, W., Liao, B., Kano, O., Wang, J., Huang, A., Appel, S.H., Henkel, J.S., 2011. Neuroinflammation modulates distinct regional and temporal clinical responses in ALS mice. *Brain Behav. Immun.* 25 (5), 1025–1035.
- Benatar, M., 2007. Lost in translation: treatment trials in the SOD1 mouse and in human ALS. *Neurobiol. Dis.* 26 (1), 1–13.
- Boillee, S., Vande Velde, C., Cleveland, D.W., 2006a. ALS: a disease of motor neurons and their nonneuronal neighbors. *Neuron* 52 (1), 39–59.
- Boillee, S., Yamanaka, K., Lobsiger, C.S., Copeland, N.G., Jenkins, N.A., Kassiotis, G., Kollias, G., Cleveland, D.W., 2006b. Onset and progression in inherited ALS determined by motor neurons and microglia. *Science* 312 (5778), 1389–1392.

**Table 2**  
Summary of microglial gene expression changes in lumbar spinal cord in SOD1<sup>G93A</sup> rats compared to wild-type littermates. (↓ gene downregulated; ↑ gene upregulated).

	Preclinical	Onset	End-stage
<b>Typical M1 markers</b>			
TNFα	No change	↓	↓↓
IL-6	No change	No change	↓
MCP-1	No change	No change	No change
<b>Typical M2 markers</b>			
Arginase-1	No change	No change	↓
IL-10	No change	↓	↓↓
BDNF	No change	No change	↓
<b>Other markers</b>			
Osteopontin	No change	↑	↑
Galectin-3	No change	↑	↑
VEGF	No change	↑	↑↑

- Chen, H.Y., Liu, F.T., Yang, R.Y., 2005. Roles of galectin-3 in immune responses. *Archivum immunologiae et therapiae experimentalis* 53 (6), 497–504.
- Cudkowicz, M.E., Shefner, J.M., Schoenfeld, D.A., Zhang, H., Andreasson, K.I., Rothstein, J.D., Drachman, D.B., 2006. Trial of celecoxib in amyotrophic lateral sclerosis. *Ann. Neurol.* 60 (1), 22–31.
- Dibaj, P., Steffens, H., Zschuntzsch, J., Kirchhoff, F., Schomburg, E.D., Neusch, C., 2011. *In vivo* imaging reveals rapid morphological reactions of astrocytes towards focal lesions in an ALS mouse model. *Neurosci. Lett.* 497 (2), 148–151.
- Fendrick, S.E., Xue, Q.S., Streit, W.J., 2007. Formation of multinucleated giant cells and microglial degeneration in rats expressing a mutant Cu/Zn superoxide dismutase gene. *J. Neuroinflammation* 4, 9.
- Gordon, P.H., Moore, D.H., Miller, R.G., Florence, J.M., Verheijde, J.L., Doorish, C., Hilton, J.F., Spitalny, G.M., MacArthur, R.B., Mitsumoto, H., Neville, H.E., Boylan, K., Mozaffar, T., Belsh, J.M., Ravits, J., Bedlack, R.S., Graves, M.C., McCluskey, L.F., Barohn, R.J., Tandan, R., 2007. Efficacy of minocycline in patients with amyotrophic lateral sclerosis: a phase III randomised trial. *Lancet Neurol.* 6 (12), 1045–1053.
- Graber, D.J., Hickey, W.F., Harris, B.T., 2010. Progressive changes in microglia and macrophages in spinal cord and peripheral nerve in the transgenic rat model of amyotrophic lateral sclerosis. *J. Neuroinflammation* 7, 8.
- Gupta, P.K., Prabhakar, S., Sharma, S., Anand, A., 2011. Vascular endothelial growth factor-A (VEGF-A) and chemokine ligand-2 (CCL2) in amyotrophic lateral sclerosis (ALS) patients. *J. Neuroinflammation* 8, 47.
- Hashimoto, M., Sun, D., Rittling, S.R., Denhardt, D.T., Young, W., 2007. Osteopontin-deficient mice exhibit less inflammation, greater tissue damage, and impaired locomotor recovery from spinal cord injury compared with wild-type controls. *J. Neurosci.* 27 (13), 3603–3611.
- Hensley, K., Fedynyshyn, J., Ferrell, S., Floyd, R.A., Gordon, B., Grammas, P., Hamdheydari, L., Mhatre, M., Mou, S., Pye, Q.N., Stewart, C., West, M., West, S., Williamson, K.S., 2003. Message and protein-level elevation of tumor necrosis factor alpha (TNF alpha) and TNF alpha-modulating cytokines in spinal cords of the G93A-SOD1 mouse model for amyotrophic lateral sclerosis. *Neurobiol. Dis.* 14 (1), 74–80.
- Ilieva, H., Polymenidou, M., Cleveland, D.W., 2009. Non-cell autonomous toxicity in neurodegenerative disorders: ALS and beyond. *The Journal of cell biology* 187 (6), 761–772.
- Kawamura, M.F., Yamasaki, R., Kawamura, N., Tateishi, T., Nagara, Y., Matsushita, T., Ohya, Y., Kira, J., 2012. Impaired recruitment of neuroprotective microglia and T cells during acute neuronal injury coincides with increased neuronal vulnerability in an amyotrophic lateral sclerosis model. *Exp. Neurol.* 234 (2), 437–445.
- Keller, A.F., Gravel, M., Kriz, J., 2011. Treatment with minocycline after disease onset alters astrocyte reactivity and increases microgliosis in SOD1 mutant mice. *Exp. Neurol.* 228 (1), 69–79.
- Kriz, J., Nguyen, M.D., Julien, J.P., 2002. Minocycline slows disease progression in a mouse model of amyotrophic lateral sclerosis. *Neurobiol. Dis.* 10 (3), 268–278.
- Lalancette-Hebert, M., Swarup, V., Beaulieu, J.M., Bohacek, I., Abdelhamid, E., Weng, Y.C., Sato, S., Kriz, J., 2012. Galectin-3 is required for resident microglia activation and proliferation in response to ischemic injury. *J. Neurosci.* 32 (30), 10383–10395.
- Lee, J.C., Seong, J., Kim, S.H., Lee, S.J., Cho, Y.J., An, J., Nam, D.H., Joo, K.M., Cha, C.I., 2012. Replacement of microglial cells using clodronate liposome and bone marrow transplantation in the central nervous system of SOD1(G93A) transgenic mice as an *in vivo* model of amyotrophic lateral sclerosis. *Biochem. Biophys. Res. Commun.* 418 (2), 359–365.
- Lerman, B.J., Hoffman, E.P., Sutherland, M.L., Bouri, K., Hsu, D.K., Liu, F.T., Rothstein, J.D., Knoblach, S.M., 2012. Deletion of galectin-3 exacerbates microglial activation and accelerates disease progression and demise in a SOD1(G93A) mouse model of amyotrophic lateral sclerosis. *Brain and behavior* 2 (5), 563–575.
- Li, B., Xu, W., Luo, C., Gozal, D., Liu, R., 2003. VEGF-induced activation of the PI3-K/Akt pathway reduces mutant SOD1-mediated motor neuron cell death. *Brain Res. Mol. Brain Res.* 111 (1–2), 155–164.
- Liao, B., Zhao, W., Beers, D.R., Henkel, J.S., Appel, S.H., 2012. Transformation from a neuroprotective to a neurotoxic microglial phenotype in a mouse model of ALS. *Exp. Neurol.* 237 (1), 147–152.
- Lund, S.A., Giachelli, C.M., Scatena, M., 2009. The role of osteopontin in inflammatory processes. *Journal of cell communication and signaling* 3 (3–4), 311–322.
- MacKinnon, A.C., Farnworth, S.L., Hodgkinson, P.S., Henderson, N.C., Atkinson, K.M., Leffler, H., Nilsson, U.J., Haslett, C., Forbes, S.J., Sethi, T., 2008. Regulation of alternative macrophage activation by galectin-3. *J. Immunol.* 180 (4), 2650–2658.
- Maetzler, W., Berg, D., Funke, C., Sandmann, F., Stunitz, H., Maetzler, C., Nitsch, C., 2010. Progressive secondary neurodegeneration and microcalcification co-occur in osteopontin-deficient mice. *Am. J. Pathol.* 177 (2), 829–839.
- Maetzler, W., Berg, D., Schalamberidge, N., Melms, A., Schott, K., Mueller, J.C., Liaw, L., Gasser, T., Nitsch, C., 2007. Osteopontin is elevated in Parkinson's disease and its absence leads to reduced neurodegeneration in the MPTP model. *Neurobiol. Dis.* 25 (3), 473–482.
- Matsumoto, T., Imagama, S., Hirano, K., Ohgomi, T., Natori, T., Kobayashi, K., Muramoto, A., Ishiguro, N., Kadomatsu, K., 2012. CD44 expression in astrocytes and microglia is associated with ALS progression in a mouse model. *Neurosci. Lett.* 520 (1), 115–120.
- Nichols, N.L., Gowing, G., Satriotomo, I., Nashold, L.J., Dale, E.A., Suzuki, M., Avalos, P., Mulcrone, P.L., McHugh, J., Svendsen, C.N., Mitchell, G.S., 2012. Intermittent hypoxia and stem cell implants preserve breathing capacity in a rodent model of ALS. *Am. J. Respir. Crit. Care Med.* Q3
- Nikodemova, M., Watters, J.J., 2011. Outbred ICR/CD1 mice display more severe neuroinflammation mediated by microglial TLR4/CD14 activation than inbred C57Bl/6 mice. *Neuroscience* 190, 67–74.
- Nikodemova, M., Watters, J.J., 2012. Efficient isolation of live microglia with preserved phenotypes from adult mouse brain. *J. Neuroinflammation* 9, 147.
- Pan, L., Yoshii, Y., Otomo, A., Ogawa, H., Iwasaki, Y., Shang, H.F., Hadano, S., 2012. Different human copper-zinc superoxide dismutase mutants, SOD1G93A and SOD1H46R, exert distinct harmful effects on gross phenotype in mice. *PLoS One* 7 (3), e33409.
- Philips, T., Robberecht, W., 2011. Neuroinflammation in amyotrophic lateral sclerosis: role of glial activation in motor neuron disease. *Lancet Neurol.* 10 (3), 253–263.
- Rosen, D.R., Siddique, T., Patterson, D., Figlewicz, D.A., Sapp, P., Hentati, A., Donaldson, D., Goto, J., O'Regan, J.P., Deng, H.X., et al., 1993. Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis. *Nature* 362 (6415), 59–62.
- Sanagi, T., Yuasa, S., Nakamura, Y., Suzuki, E., Aoki, M., Warita, H., Itoyama, Y., Uchino, S., Kohsaka, S., Ohsawa, K., 2010. Appearance of phagocytic microglia adjacent to motoneurons in spinal cord tissue from a presymptomatic transgenic rat model of amyotrophic lateral sclerosis. *J. Neurosci. Res.* 88 (12), 2736–2746.
- Sano, H., Hsu, D.K., Appgar, J.R., Yu, L., Sharma, B.B., Kuwabara, I., Izui, S., Liu, F.T., 2003. Critical role of galectin-3 in phagocytosis by macrophages. *J. Clin. Invest.* 112 (3), 389–397.
- Sargsyan, S.A., Blackburn, D.J., Barber, S.C., Grosskreutz, J., De Vos, K.J., Monk, P.N., Shaw, P.J., 2011. A comparison of *in vitro* properties of resting SOD1 transgenic microglia reveals evidence of reduced neuroprotective function. *BMC neuroscience* 12, 91.
- Sargsyan, S.A., Monk, P.N., Shaw, P.J., 2005. Microglia as potential contributors to motor neuron injury in amyotrophic lateral sclerosis. *Glia* 51 (4), 241–253.
- Solomon, J.N., Lewis, C.A., Ajami, B., Corbel, S.Y., Rossi, F.M., Krieger, C., 2006. Origin and distribution of bone marrow-derived cells in the central nervous system in a mouse model of amyotrophic lateral sclerosis. *Glia* 53 (7), 744–753.
- Suzuki, M., Tork, C., Shelley, B., McHugh, J., Wallace, K., Klein, S.M., Lindstrom, M.J., Svendsen, C.N., 2007. Sexual dimorphism in disease onset and progression of a rat model of ALS. *Amyotroph. Lateral Scler.* 8 (1), 20–25.
- Thomsen, G.M., Gowing, G., Avalos, P., Latter, J., Staggenborg, K., Zedella, V., Lin, A., Svendsen, C., 2012. A study of corticospinal motor neurons in rat model of amyotrophic lateral sclerosis. (Program No.346.08), Neuroscience Meeting Planner. Society for Neuroscience, New Orleans, LA (Online).
- Walther, M., Kuklinski, S., Pesheva, P., Guntinas-Lichius, O., Angelov, D.N., Neiss, W.F., Asou, H., Probstmeier, R., 2000. Galectin-3 is upregulated in microglial cells in response to ischemic brain lesions, but not to facial nerve axotomy. *J. Neurosci. Res.* 61 (4), 430–435.
- Werdlein, L., Boysen, G., Jensen, T.S., Mogensen, P., 1990. Immunosuppressive treatment of patients with amyotrophic lateral sclerosis. *Acta neurologica Scandinavica* 82 (2), 132–134.
- Weydt, P., Yuen, E.C., Ransom, B.R., Moller, T., 2004. Increased cytotoxic potential of microglia from ALS-transgenic mice. *Glia* 48 (2), 179–182.
- Xie, Y., Weydt, P., Howland, D.S., Kliot, M., Moller, T., 2004. Inflammatory mediators and growth factors in the spinal cord of G93A SOD1 rats. *Neuroreport* 15 (16), 2513–2516.
- Zheng, C., Nennesmo, I., Fadeel, B., Henter, J.I., 2004. Vascular endothelial growth factor prolongs survival in a transgenic mouse model of ALS. *Ann. Neurol.* 56 (4), 564–567.
- Zheng, C., Skold, M.K., Li, J., Nennesmo, I., Fadeel, B., Henter, J.I., 2007. VEGF reduces astrogliosis and preserves neuromuscular junctions in ALS transgenic mice. *Biochem. Biophys. Res. Commun.* 363 (4), 989–993.
- Zhou, J.Y., Afjeji-Sadat, L., Asress, S., Duong, D.M., Cudkowicz, M., Glass, J.D., Peng, J., 2010. Galectin-3 is a candidate biomarker for amyotrophic lateral sclerosis: discovery by a proteomics approach. *Journal of proteome research* 9 (10), 5133–5141.
- Zhu, S., Stavrovskaya, I.G., Drozda, M., Kim, B.Y., Ona, V., Li, M., Sarang, S., Liu, A.S., Hartley, D.M., Wu, du, C., Gullans, S., Ferrante, R.J., Przedborski, S., Kristal, B.S., Friedlander, R.M., 2002. Minocycline inhibits cytochrome c release and delays progression of amyotrophic lateral sclerosis in mice. *Nature* 417 (6884), 74–78.