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# Arginase-1 expressing microglia in close proximity to motor neurons were increased early in disease progression in canine degenerative myelopathy, a model of amyotrophic lateral sclerosis

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ABSTRACT

Toxicity within *superoxide dismutase-1 (SOD1)*-associated familial amyotrophic lateral sclerosis (ALS) is non-cell autonomous with direct contribution from microglia. Microglia exhibit variable expression of neuroprotective and neurotoxic molecules throughout disease progression. The mechanisms regulating microglial phenotype within ALS are not well understood. This work presents a first study to examine the specific microglial phenotypic response in close association to motor neurons in a naturally occurring disease model of ALS, canine degenerative myelopathy (DM). Microglia closely associated with motor neurons were increased in all stages of DM progression, although only DM Late reached statistical significance. Furthermore, the number of arginase-1 expressing microglia per motor neuron were significantly increased in early stages of DM, whereas the number of inducible nitric oxide synthase (iNOS)-expressing microglia per motor neuron was indistinguishable from aged controls at all stages of disease. Fractalkine, a chemotactic molecule for microglia, was expressed in motor neurons, and the fractalkine receptor was specifically localized to microglia. However, we found no correlation between microglial response and lumbar spinal cord fractalkine levels. Taken together, these data suggest that arginase-1-expressing microglia are recruited to the motor neuron early in DM disease through a fractalkine-independent mechanism.

## 1. Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal adult-onset neurodegenerative disease with an incidence of 1.2–4 out of 100,000 people (Boillee et al., 2006; Kiernan et al., 2011). Mutations in *superoxide dismutase-1* (*SOD1*), the gene encoding cytosolic Cu/Zn superoxide dismutase, account for 20% of all inherited cases (Rosen et al., 1993; Valdmanis et al., 2009; Valentine et al., 2005). Although the mechanisms of SOD1-mediated toxicity in ALS remain unclear, disease progression is non-cell autonomous (Clement et al., 2003).

Microglia have been correlated with disease progression in both transgenic rodent ALS models and ALS patients (Beers et al., 2006; Boillee et al., 2006; Boillée et al., 2006; Brettschneider et al., 2012; Clement et al., 2003; Turner and Leigh, 2000). Selective silencing of mutant SOD1 in microglial cells delayed disease progression and extended survival of *SOD1* mutant mice (Beers et al., 2006; Boillée et al., 2006). Furthermore, spinal cord $SOD1^{G93A}$ microglia, isolated from mice at *disease onset,* promoted neuronal survival when both cell types were co-cultured, while *end-stage* $SOD1^{G93A}$ microglia decreased neuronal survival (Liao et al., 2012). These data suggest that microglia have a central role in disease progression, which may be disease stage-specific. Attempts to elucidate the phenotypic signature of *SOD1*-mutant microglia in transgenic rodent models have revealed a complex profile throughout disease (Chiu et al., 2013; Lewis et al., 2014; Liao et al., 2012; Nikodemova et al., 2014). While early studies suggested that the microglia phenotype in ALS is a continuum between neuroprotective and neurotoxic microglia (Beers et al., 2011; Liao et al., 2012), a recent in vivo study demonstrated increased protein expression of both inducible nitric oxide synthase (iNOS) and arginase-1 throughout disease progression in mouse $SOD1^{G93A}$ spinal cord

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microglia (Lewis et al., 2014). While this is the first in vivo study to examine the specific microglia phenotypic signature, it failed to demonstrate the relationship of the microglial phenotype to degenerating motor neurons.

Fractalkine ($CX_3CL1$) is a transmembrane chemokine expressed by neurons in the CNS, which can be cleaved to release an extracellular soluble fragment (Bazan et al., 1997; Garton et al., 2001; Maeda et al., 2012). In addition to inducing microglial chemotaxis and proliferation (Maciejewski-Lenoir et al., 1999), membrane-bound and soluble fractalkine have been shown to attenuate microglial neurotoxicity in neurodegenerative disease (Bhaskar et al., 2010; Pabon et al., 2011). Within ALS, $SOD1^{G93A}$/CX3CR1$^{-/-}$ mice exhibit accelerated disease progression and exacerbated neuronal death (Cardona et al., 2006). Importantly, functional variants of the human *CX3CR1* gene are associated with a shorter survival time in ALS patients and have been identified as the most potent ALS survival genetic factor reported to date (Lopez-Lopez et al., 2014). These results suggest that signaling to microglia via fractalkine may confer neuroprotection. Therefore, varying fractalkine levels throughout ALS disease progression may contribute to the disease associated microglial response.

Therapeutic efficacy in the available rodent models has failed to translate into human patients. Canine degenerative myelopathy (DM), a naturally occurring neurodegenerative disorder, shares similarities to ALS. The clinical spectrum of DM is homogeneous within and across dog breeds (Table 1). Clinical signs represent degeneration of the central and peripheral nervous systems (Awano et al., 2009; Braund and Vandevelde, 1978; Griffiths and Duncan, 1975; Jr., 1973; March et al., 2009; Morgan et al., 2013; Morgan et al., 2014; Ogawa et al., 2014; Shelton et al., 2012), similar to upper motor neuron-onset ALS (specifically $SOD1^{D90A}$ fALS) (Andersen et al., 1995; Parton et al., 2002). Like some forms of familial ALS, canine degenerative myelopathy (DM) results from mutations in *SOD1* (Awano et al., 2009; Wininger et al., 2011). Most DM-affected dogs are homozygous for the *SOD1:c.118A* mutation, which predicts an E40K substitution in SOD1 (Awano et al., 2009). Similar to most ALS mutations, $SOD1^{E40K}$ maintains normal enzymatic activity (Crisp et al., 2013). However, like ALS, the mechanism that $SOD1^{E40K}$ confers toxicity remains unclear. Recently, increased microglia have been documented within the spinal cord of DM-affected dogs (Ogawa et al., 2014), which may suggest that DM is also a non-cell autonomous disease. However, the specific examination of microglial responses throughout DM progression has yet to be explored.

In this study, we examined the microglial response in relation to motor neuron pathology within the lumbar spinal cord of DM-affected dogs. We tested the following hypotheses: 1) $SOD1^{E40K}$ microglia are progressively increased in close proximity to degenerating motor neurons of the lumbar intumescence, 2) microglia will transition from a predominantly neuroprotective to a neurotoxic phenotype in late stages of DM 3) increasing neuronal pathology correlates with decreased fractalkine levels and 4) decreased fractalkine correlates with a neurotoxic microglial phenotype.

**Table 1**
Clinical disease progression of degenerative myelopathy has four defined stages. Dogs were stratified according to the severity of neurologic deficits elicited upon neurologic examination.

| Stage | Neurological signs |
|---|---|
| 1 | Asymmetric, general proprioceptive ataxia and spastic paraparesis<br>Intact spinal reflexes |
| 2 | Non-ambulatory paraparesis to paraplegia<br>Reduced to absent pelvic limb spinal reflexes<br>Pelvic limb muscle atrophy<br>± urinary/fecal incontinence |
| 3 | Flaccid paraplegia, thoracic limb paresis<br>Absent spinal reflexes<br>Severe pelvic limb muscle atrophy<br>Urinary/fecal incontinence |
| 4 | Flaccid tetraplegia<br>Absent spinal reflexes<br>Severe generalized muscle atrophy<br>Urinary/fecal incontinence<br>Dysphagia, dysphonia, respiratory difficulty |

## 2. Materials and methods

### 2.1. Case selection and sample collection

Companion dogs were donated for inclusion in DM-related studies between July 2012 and March 2016. All pet owners signed an informed consent form (approved by the University of Missouri Animal Care and Use Committee, protocol #8339). In addition, tissues were obtained from collaborating primary care and neurology specialty veterinarians as mail-in samples.

Samples from DM-affected dogs were included if the following criteria were met: 1) clinical history and neurologic examination consistent with DM 2) homozygosity for the DM associated *SOD1* mutation (Awano et al., 2009), 3) histopathologic diagnosis of DM by a board-certified veterinary pathologist (GJC) confirming axonal degeneration and astroglial proliferation most severe in the dorsal portion of the lateral funiculus and dorsal funiculi of the caudal thoracic spinal cord, (Jr., 1973; March et al., 2009) and 4) SOD1-immunoreactive aggregates within ventral horn motor neurons (Morgan et al., 2013; Morgan et al., 2014). DM-affected dogs were also stratified according to disease stage (Table 1) (Coates and Wininger, 2010); however, DM Stage 3 and 4 were grouped into a single group 'DM Late'. The aged control cohort included dogs that were ≥8 years of age with a normal neurologic examination, wild-type *SOD1* genotype, and normal histopathology of the caudal thoracic spinal cord.

Dogs were humanely euthanized, and tissue samples were collected. The fourth lumbar spinal segment was isolated. The dura mater was removed, and the segment was hemisected. The left half of the spinal cord was designated for immunofluorescence studies. The tissue was immersion fixed in 10% neutral buffered formalin, cryoprotected with 30% sucrose solution, and stored at −80 °C until sectioning. 30-μm transverse serial sections were made through the segment with a Leica CM1900 cryostat at −20 °C. Three consecutive sections were pooled in each well and stored in a cryopreservative solution at −20 °C prior to immunostaining. The right half of the spinal cord was designated for immunoblot studies. The tissue was snap frozen in liquid nitrogen, and stored at −80 °C until analysis.

### 2.2. Immunofluorescence

Three sections/dog were randomly selected to represent cranial, middle and caudal L4 sections for immunostaining. Tissue was washed three times for 5 min with Tris-buffered saline with 0.5% Triton X-100 (TBST) (Sigma) at room temperature, and blocked for 1 h at room temperature (RT) on a shaker (10% normal goat serum, 1% bovine serum albumin in TBST). For neuronal pathology analysis, the following primary antibodies in fresh blocking buffer were used: chicken polyclonal antibody anti-neurofilament light, CPCA-NFL (Encor Biotechnology, Gainesville, FL, USA; 1:1000); mouse monoclonal antibody immunoreactive against phosphorylated neurofilament heavy (pNF-H) and medium (pNF-M), SMI 31 (Biolegend, San Diego, CA; 1:1000). For microglia identification, rabbit polyclonal antibody anti-ionized calcium-binding adaptor molecule (Iba-1) (Wako Pure Chemical Industries, Ltd., Chuo-Ku, Osaka, Japan; 1:1000) was used. To provide microglia phenotype information, co-application of mouse monoclonal antibody anti-arginase-1 (eBioscience, San Diego, CA, USA; 1:400) or mouse monoclonal anti-inducible nitric oxide synthase (iNOS) (EMD Millipore Corporation, Temecula, CA, USA; 1:400) was used. For fractalkine identification, goat polyclonal antibody anti-fractalkine (Santa Cruz Biotechnology, Inc.; 1:200) was used. To co-localize fractalkine to motor neurons, motor neurons were identified