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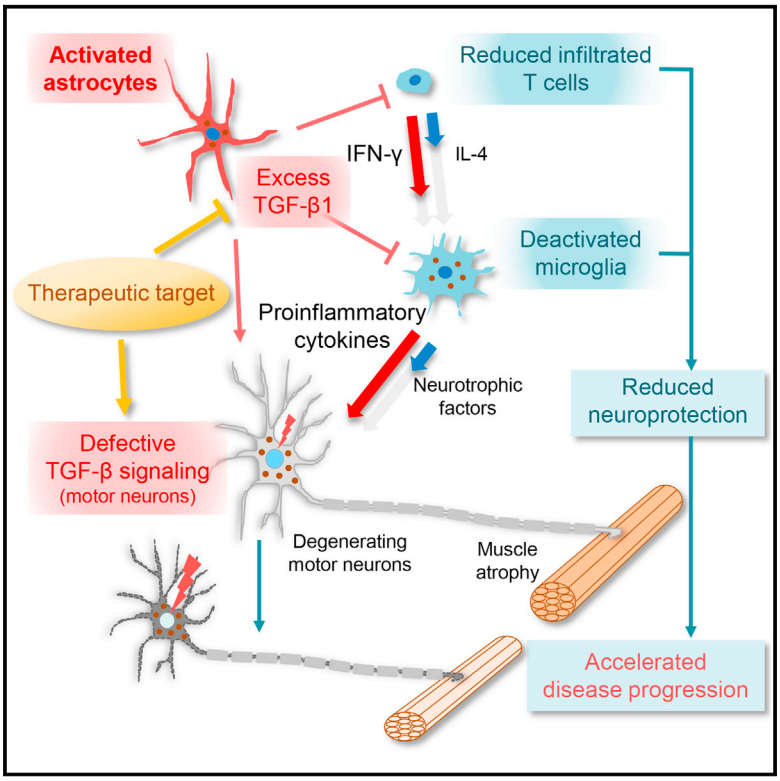
Astrocyte-Derived TGF-β1 Accelerates Disease Progression in ALS Mice by Interfering with the Neuroprotective Functions of Microglia and T Cells

Graphical Abstract



Highlights

- Astrocyte-derived TGF-β1 accelerates disease progression in ALS mice
- TGF-β1 inhibits neuroprotective inflammatory response by microglia and T cells
- TGF-β signaling inhibitor slows disease progression and extends survival of ALS mice
- Cell-type-specific dysregulation of TGF-β signaling is a therapeutic target for ALS

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In Brief

Endo et al. show that TGF-β1, upregulated in astrocytes of ALS patients and mice, negatively regulates neuroprotective inflammatory responses. Astrocyte-derived TGF-β1 accelerates disease progression in ALS mice, which is ameliorated by a TGF-β signaling inhibitor. These findings indicate that targeting glial TGF-β signaling may represent a therapeutic approach for ALS.

Endo et al., 2015, Cell Reports *11*, 592–604
April 28, 2015
http://dx.doi.org/10.1016/j.celrep.2015.03.053

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Astrocyte-Derived TGF-β1 Accelerates Disease Progression in ALS Mice by Interfering with the Neuroprotective Functions of Microglia and T Cells

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http://dx.doi.org/10.1016/j.celrep.2015.03.053

SUMMARY

Neuroinflammation, which includes both neuroprotective and neurotoxic reactions by activated glial cells and infiltrated immune cells, is involved in the pathomechanism of amyotrophic lateral sclerosis (ALS). However, the cytokines that regulate the neuroprotective inflammatory response in ALS are not clear. Here, we identify transforming growth factor-β1 (TGF-β1), which is upregulated in astrocytes of murine and human ALS, as a negative regulator of neuroprotective inflammatory response. We demonstrate that astrocyte-specific overproduction of TGF-β1 in SOD1^{G93A} mice accelerates disease progression in a non-cell-autonomous manner, with reduced IGF-I production in deactivated microglia and fewer T cells with an IFN-γ-dominant milieu. Moreover, expression levels of endogenous TGF-β1 in SOD1^{G93A} mice negatively correlate with lifespan. Furthermore, pharmacological administration of a TGF-β signaling inhibitor after disease onset extends survival time of SOD1^{G93A} mice. These findings indicate that astrocytic TGF-β1 determines disease progression and is critical to the pathomechanism of ALS.

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is an adult-onset, fatal neurodegenerative disease characterized by the selective loss of motor neurons. While most cases of ALS are of sporadic etiology, 10% of cases are familial ALS among which dominant mutations in the gene for Cu/Zn superoxide dismutase (SOD1) are the frequent causes. Mice overexpressing the *SOD1* gene with ALS-linked mutations recapitulate both the clinical and pathological characteristics of ALS.

Neuroinflammation, characterized by activated astrocytes, microglia, infiltrated T cells, and the subsequent overproduction of proinflammatory cytokines and other neurotoxic or protective molecules, is a pathological hallmark not only of mutant SOD1 mice but also of ALS patients (Engelhardt et al., 1993). Along with other researchers, we have demonstrated previously that the selective reduction of mutant *SOD1* expression in microglia (Beers et al., 2006; Boillée et al., 2006; Wang et al., 2009) or astrocytes (Wang et al., 2011; Yamanaka et al., 2008) significantly extends the survival time of ALS mice. Moreover, elimination of functional T cells from mutant SOD1 mice shortens the survival time (Beers et al., 2008; Chiu et al., 2008). Thus, non-neuronal cells, such as astrocytes, microglia, and T cells, are able to modify the course of disease through a non-cell-autonomous mechanism within the CNS (Ilieva et al., 2009; Lasiene and Yamanaka, 2011). On the other hand, the targeting of single proinflammatory cytokines has been tested as a treatment for ALS mice; however, the effectiveness of this strategy remains inconclusive. For example, some studies showed that deletion of TNF-α or IL-1β has marginal effects on the survival time of mutant SOD1 mice, while one study showed extended survival of IL-1β-deficient SOD1^{G93A} mice (Gowing et al., 2006; Meissner et al., 2010; Nguyen et al., 2001). In contrast, overexpression of neurotrophic factors, such as IGF-I and GDNF, extends the survival time of mutant SOD1 mice (Dodge et al., 2008; Henderson et al., 1994; Kaspar et al., 2003; Wang et al., 2002). Targeting a single proinflammatory cytokine may not be sufficient to modify disease course, but instead inducing a neuroprotective environment composed of glial cells and T cells may be more effective. However, it has not been elucidated what regulates the neuroprotective glia/immune response.

Transforming growth factor βs (TGF-βs) are pleiotropic cytokines that have key roles in immune homeostasis (Li et al., 2006), neurotrophic response (Katsuno et al., 2011), and

microglial development (Butovsky et al., 2014). In mouse models of Alzheimer's disease (AD) in which the amyloid precursor gene is overexpressed, increasing astrocytic TGF-β1 levels or blockade of TGF-β signaling in peripheral macrophages is shown to reduce senile plaque formation in the brain parenchyma (Wyss-Coray et al., 1995, 2001; Town et al., 2008). In ALS patients, TGF-β1 protein levels are elevated in the serum, plasma, and cerebrospinal fluid (CSF) (Houi et al., 2002; Ilzecka et al., 2002), and recent studies indicate that TGF-β signaling is implicated in ALS (Iida et al., 2011; Katsuno et al., 2011; Phatnani et al., 2013). However, the exact roles of TGF-β1 in the pathomechanism of ALS have not been elucidated.

In this study, we found TGF-β1 upregulation in the spinal cord astrocytes of sporadic ALS patients and ALS mice. By using SOD1^{G93A} mice with overexpression of TGF-β1 in astrocytes and SOD1^{G37R} mice with astrocyte-specific deletion of mutant gene, we identified astrocytic TGF-β1 negatively regulates neuroprotective inflammatory responses through microglia and T cells and accelerates disease progression of ALS mice. Moreover, pharmacological administration of TGF-β signaling inhibitor after disease onset extends the survival of ALS mice. On the other hand, a defect in canonical TGF-β signaling in motor neurons was unaffected by exogenous TGF-β1. These findings indicate that cell-type-specific dysregulation of TGF-β signaling is critical to the pathomechanism underlying ALS.

RESULTS

TGF-β1 Is Upregulated in the Spinal Cord Astrocytes of SOD1^{G93A} Mice and Sporadic ALS Patients

To explore the role of TGF-β1 in ALS pathogenesis, we examined expression levels of TGF-β1 mRNA in the spinal cord of mutant SOD1 mice. We found that, among its different isoforms, TGF-β1 mRNA level was highly upregulated during disease progression of SOD1^{G93A} mice (Figure 1A) as well as the mice expressing other mutant *SOD1* genes, SOD1^{G85R} and SOD1^{G37R} (Figure 1B). Immunoblotting also confirmed that TGF-β1 protein level was upregulated during disease progression of SOD1^{G93A} mice (Figure 2B). To further examine the cell types that produce TGF-β1 under an acute inflammatory condition, we examined expression levels of TGF-β1 in SOD1^{G93A}-bearing primary astrocytes and microglia induced by lipopolysaccharide (LPS) stimulation, since these glial cells were implicated to produce TGF-β1 under various neurological diseases (Flanders et al., 1998). We found a relatively elevated level of TGF-β1 mRNA in SOD1^{G93A}-primary astrocytes, but reduced TGF-β1 expression in SOD1^{G93A}-primary microglia upon LPS stimulation (Figure 1C). Immunofluorescence analysis revealed that TGF-β1 protein expressions in astrocytes were elevated at end stage compared with onset and that they were markedly observed compared with ones in microglia of end-stage SOD1^{G93A} mice (Figure 1D). More importantly, astrocytic TGF-β1 expression also was elevated in the spinal ventral horn and lateral column of sporadic ALS patients compared with control (Figure 1E). These results indicate that expression levels of endogenous TGF-β1 are upregulated in astrocytes during disease progression of ALS mice and sporadic ALS patients.

Astrocyte-Specific Overproduction of TGF-β1 Accelerates Disease Progression in SOD1^{G93A} Mice

We next crossed SOD1^{G93A} mice with mice modestly expressing the bioactive form of porcine TGF-β1 under the control of a glial fibrillary acidic protein (GFAP) promoter (GFAP-TGF-β1 mice) (Wyss-Coray et al., 1995) and monitored body weight, rotarod performance, and survival time. Astrocytic TGF-β1 expression in GFAP-TGF-β1 mice was confirmed by immunoblots, and total TGF-β1 protein level was elevated in end-stage SOD1^{G93A}/TGF-β1 mice compared with SOD1^{G93A} mice (Figures 2A and 2B). Although the time of disease onset remained unchanged in SOD1^{G93A}/TGF-β1 double-transgenic mice (Figure 2C), the mean survival time was unexpectedly shortened by about 10 days (SOD1^{G93A}/TGF-β1: 151.7 ± 10.4 days; SOD1^{G93A}: 161.9 ± 9.5 days) (Figure 2D). SOD1^{G93A}/TGF-β1 mice showed accelerated disease progression in the late stage (Figure 2E) of ALS, with faster weight loss (Figure 2F) and a decreased performance on the rotarod task (Figure 2G). On the other hand, heterozygous GFAP-TGF-β1 mice showed neither motor deficit nor weight loss with a normal lifespan (Figures 2D, 2F, and 2G). Additionally, analysis of motor axons in cross-sections of L5 ventral root revealed a trend toward more progressive axonal degeneration in SOD1^{G93A}/TGF-β1 mice compared to SOD1^{G93A} mice, while L5 motor axons in GFAP-TGF-β1 were not affected (Figures 2H and 2I). Together, these results suggest that astrocyte-specific overproduction of TGF-β1 plays a detrimental role in the disease progression of SOD1^{G93A} mice.

Dysregulated TGF-β Signaling in Spinal Motor Neurons of SOD1^{G93A} Mice

To determine whether TGF-β signaling is altered during disease progression of SOD1^{G93A} and SOD1^{G93A}/TGF-β1 mice, we examined Smad2, which is phosphorylated following stimulation of TGF-β signaling and transported to the nucleus (Shi and Massagué, 2003). High levels of phosphorylated Smad2 (pSmad2) were observed in motor neuron nuclei of non-transgenic mice; however, pSmad2 levels in SOD1^{G93A} mice were significantly decreased from pre-symptomatic stage and rarely observed in end stage (Figures S1A and S1B). Moreover, the loss of pSmad2 in motor neuron nuclei was not recovered by overexpression of TGF-β1 in SOD1^{G93A}/TGF-β1 mice (Figures S1A and S1B). Considering that the expressions of TGF-β receptor type 2 (TGFβR2) and type 1 (TGFβR1) and the level of cytoplasmic pSmad2 in the lumbar motor neurons of SOD1^{G93A} mice were preserved (Figures S1A and S1C), nuclear transport of pSmad2 is likely to be defective in spinal motor neurons of SOD1^{G93A} mice. In contrast, glial nuclear pSmad2 expressions were preserved in end-stage SOD1^{G93A} and SOD1^{G93A}/TGF-β1 mice (Figures S2A and S2B).

Astrocyte-Specific Overproduction of TGF-β1 Reduces the Levels of T Cell-Related Cytokines and Neurotrophic Factors in SOD1^{G93A} Mice

To identify the molecules linked to the rapid disease progression in SOD1^{G93A}/TGF-β1 mice, we performed qRT-PCR

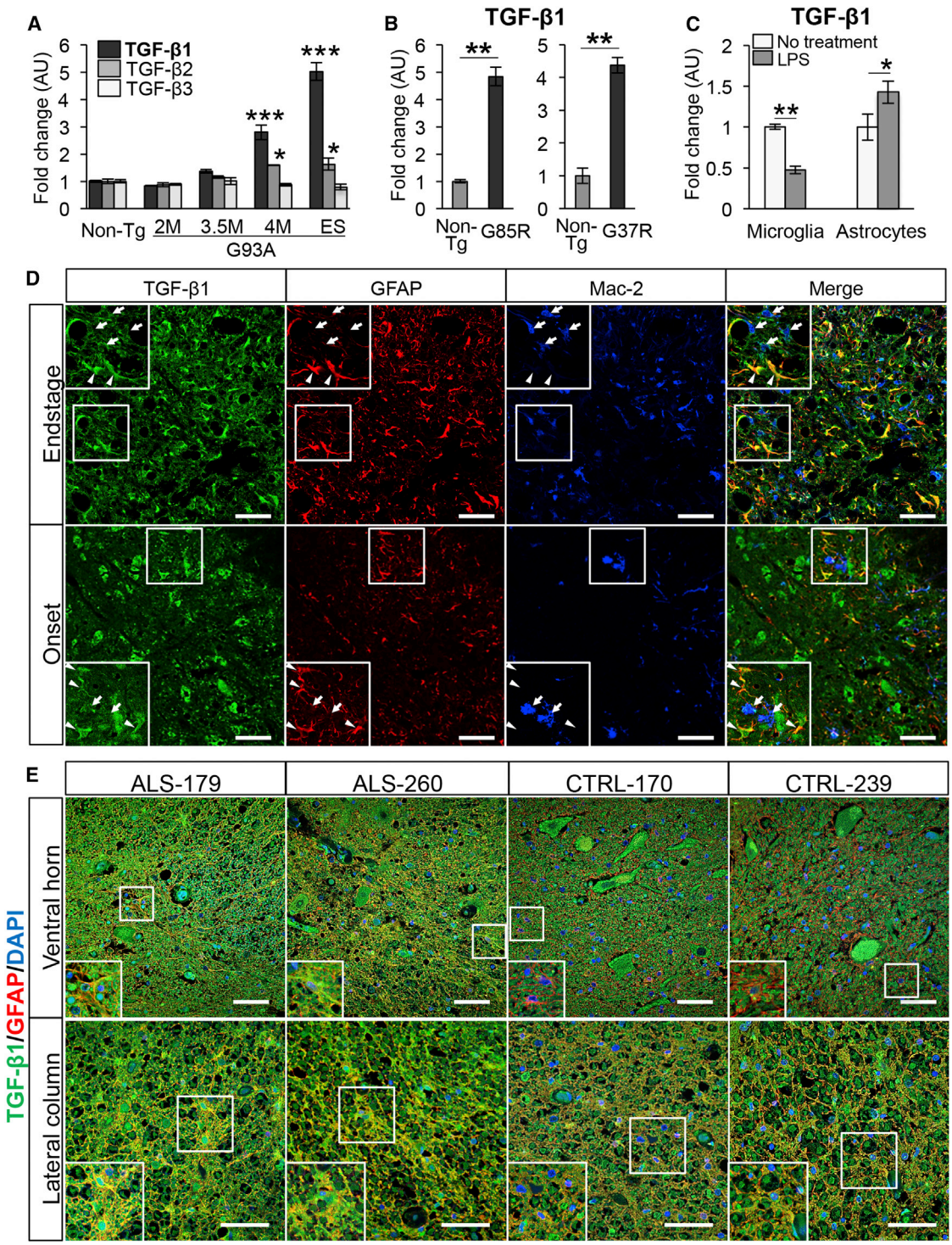

Figure 1. TGF-β1 Is Upregulated in Astrocytes in the Spinal Cords of Mutant SOD1 Mice and Sporadic ALS Patients

(A) Mean mRNA levels of TGF-β isoforms in the lumbar spinal cord of SOD1^{G93A} (2M, 2-month-old; 3.5M, 3.5-month-old; 4M, 4-month-old; and ES, end-stage) mice relative to the ones in 6-month-old non-transgenic (Non-Tg) mice are shown.

(B) Mean TGF-β1 mRNA levels in the lumbar spinal cord of end-stage SOD1^{G85R} (G85R) and SOD1^{G37R} (G37R) mice relative to the ones in age-matched Non-Tg mice are shown.

(C) Mean mRNA levels of TGF-β1 in SOD1^{G93A}-primary microglia and astrocytes with or without LPS treatment (1 μg/ml) are shown. (A–C) *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Error bars denote SEM (n = 3 in A and C and n = 3–5 in B).

(legend continued on next page)

analyses of the lumbar spinal cord of end-stage SOD1^{G93A} and SOD1^{G93A}/TGF-β1 mice. We observed significantly decreased mRNA levels of T cell-related cytokines, IFN-γ, and IL-4, in end-stage SOD1^{G93A}/TGF-β1 mice compared with SOD1^{G93A} mice (Figures 3A and 3B). Further, mRNA levels of the T helper 2 (Th2) cell-specific transcription factor GATA-3 tended to be reduced in SOD1^{G93A}/TGF-β1 mice, while ones of the Th1 transcription factor T-bet were unaffected (Table S2). The mRNA levels of the neurotrophic factors IGF-I and GDNF were decreased significantly (Figures 3C and 3D) in SOD1^{G93A}/TGF-β1 mice. We also observed lower mRNA levels of microglia activation marker CD68 in SOD1^{G93A}/TGF-β1 mice (Figure 3E), while the levels of Iba-1, classically activated (M1) microglia markers (iNOS, CD86, TNF-α, and IL-1β), and alternatively activated (M2) microglia markers (Arginase1 and CD206) were unaffected with TGF-β1 overproduction (Figures 3F and 3G; Table S2). Overexpression of TGF-β1 itself reduced expression levels of IFN-γ, IL-4, and GDNF in GFAP-TGF-β1 mice compared to non-transgenic mice (Figures 3A, 3B, and 3D), while the levels of others were unaffected. Intriguingly, expression levels of astrocytic marker GFAP measured by qRT-PCR (Figure S3A) and immunohistochemistry (Figure S3B) were unchanged during the disease progression between SOD1^{G93A} and SOD1^{G93A}/TGF-β1. With regard to astrocytic functions, mRNA levels of S100β, glutamate transporter (GLT-1), and neurocan were not significantly affected (Table S2). The levels of other glia- or immune-related molecules determined by qRT-PCR analyses are summarized in Table S2. In summary, T cell-related cytokines and neurotrophic factors were significantly affected by astrocyte-specific overproduction of TGF-β1 in SOD1^{G93A} mice, while the molecules linked to astrocytic function were relatively unaffected. Therefore, our next focus was on the roles of TGF-β1 in microglia and lymphocytes of SOD1^{G93A}/TGF-β1 mice.

Astrocyte-Specific Overproduction of TGF-β1 Inhibits Microglial Activation and Reduces the Neuroprotective Properties of Microglia/Macrophages of SOD1^{G93A} Mice

We first investigated the effect of astrocyte-secreted TGF-β1 on microglial activation in the lumbar spinal cord by immunohistochemistry. We observed that the expression of Mac-2, a marker for microglial activation, was markedly decreased in the lumbar ventral horn of SOD1^{G93A}/TGF-β1 compared with SOD1^{G93A} (Figure 4A, left) mice. Additionally, the number of Mac-2-positive cells in the ventral horn was significantly decreased in SOD1^{G93A}/TGF-β1 mice (Figure 4B), although there were no differences in expression of the microglial marker Iba-1 (Figure 4A, right) and the number of Iba-1-positive cells (Figure 4C) between the two genotypes. These data were in accordance with the lower CD68 mRNA levels observed in SOD1^{G93A}/TGF-β1 spinal cords (Figure 3E).

Moreover, to test whether TGF-β1 directly reduces microglial activation, morphology and Mac-2 mRNA levels were evaluated in SOD1^{G93A}-primary microglia cultured in astrocyte-conditioned media (ACM) from SOD1^{G93A} or SOD1^{G93A}/TGF-β1 mice. We found that the sizes of SOD1^{G93A}-primary microglia cultured with ACM from SOD1^{G93A}/TGF-β1 mice were smaller than those cultured with ACM from SOD1^{G93A} mice (Figure 4D). Mac-2 mRNA levels were noticeably reduced in SOD1^{G93A}-primary microglia with ACM from SOD1^{G93A}/TGF-β1 mice (Figure 4E). These findings indicate that TGF-β1 directly inhibits microglial activation. Furthermore, flow cytometric analysis of spinal microglia from end-stage SOD1^{G93A} and SOD1^{G93A}/TGF-β1 mice revealed no differences in cell number, cellular size, granularity (Figures S4A and S4B), surface expression of CD86 and CD206, and intracellular expression of TNF-α (Figure S4C) between the two genotypes, indicating no significant changes in M1 (CD86 and TNF-α) or M2 markers (CD206) in SOD1^{G93A} microglia by the in vivo expression of TGF-β1.

Having observed a decrease in the mRNA levels of CD68 and IGF-I in the lumbar spinal cord of SOD1^{G93A}/TGF-β1 mice (Figures 3C and 3E), we next evaluated their expression levels using immunofluorescence. We found that the expression levels of IGF-I, CD68, CD11c, a dendritic cell marker, and major histocompatibility complex (MHC) class II were significantly decreased in microglia of the ventral horn of SOD1^{G93A}/TGF-β1 mice compared with SOD1^{G93A} mice (Figure 4F). Moreover, expression of IGF-I in macrophages of the lumbar ventral root, which was markedly elevated in SOD1^{G93A} mice, was substantially decreased in SOD1^{G93A}/TGF-β1 mice (Figure 4G). Expression levels of both CD11c and CD68 in macrophages of the ventral root were also decreased in SOD1^{G93A}/TGF-β1 mice compared with SOD1^{G93A} mice (Figure 4G). In the intact ventral root of non-transgenic mice, IGF-I- and CD68-positive macrophages were both not detectable (Figure 4G). These findings indicate that astrocyte-specific overproduction of TGF-β1 inhibits activation and neuroprotective properties of microglia/macrophages in the lumbar spinal cord.

Astrocyte-Specific Overproduction of TGF-β1 Induces an IFN-γ-Dominant Milieu with Reduced Number of Infiltrated T Cells in SOD1^{G93A} Mice

Our observation of decreased mRNA levels for IFN-γ and IL-4 in the lumbar spinal cord of SOD1^{G93A}/TGF-β1 mice (Figures 3A and 3B) led us to evaluate infiltrated CD45^{hi} mononuclear cells in the spinal cord by flow cytometry. We found that total numbers of CD4+, CD8+, and CD4−CD8− double-negative

(D) Representative images show the ventral horn of the lumbar spinal cord of onset and end-stage SOD1^{G93A} mice stained for TGF-β1 (green), GFAP (red), and Mac-2 (blue), along with the merged image. Magnified images of the outlined areas (white square) also are shown. TGF-β1 expression was elevated in astrocytes (arrowheads) compared with microglia (arrows). Scale bars, 50 μm.

(E) Representative images show the ventral horn and the lateral column of the cervical spinal cord in sporadic ALS and control patients stained for TGF-β1 (green), GFAP (red), and DAPI (blue). Note that the signals double-positive for GFAP and TGF-β1 were more prominent in ALS spinal cords than controls. Magnified images of the outlined areas (white square) also are shown. Scale bars, 50 μm.

See also Table S1.

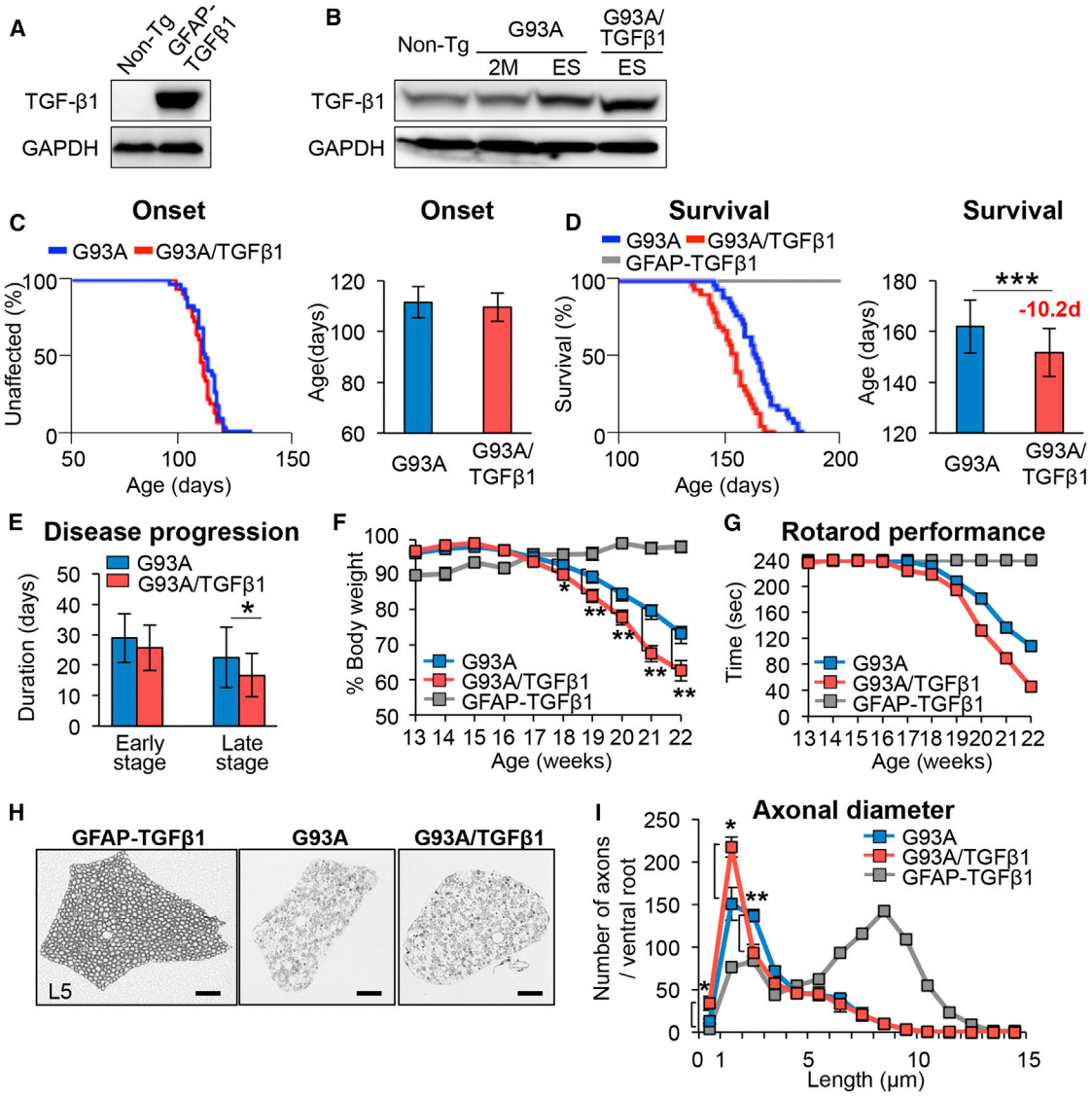

Figure 2. Astrocyte-Specific Overproduction of TGF-β1 Accelerates Disease Progression in SOD1^{G93A} Mice

(A and B) Immunoblot analysis for TGF-β1 in (A) primary astrocytes isolated from Non-Tg and GFAP-TGF-β1 mice and in (B) the lumbar spinal cords of Non-Tg, 2-month-old (2M), and end-stage (ES) SOD1^{G93A} (G93A) and SOD1^{G93A}/TGF-β1 (G93A/TGF-β1) mice. GAPDH was used as the internal loading control.

(C and D) Kaplan-Meier curves for (C) onset and plotted mean onset and (D) survival time and plotted mean survival time for G93A (n = 36), G93A/TGF-β1 (n = 33), and GFAP-TGF-β1 (n = 5) mice are shown.

(E) Plotted durations of early (from onset to 10% weight loss) and late (from 10% weight loss to end stage) stages for G93A and G93A/TGF-β1 mice are shown.

(F) Weekly mean body weight was plotted for GFAP-TGF-β1 (n = 5), G93A (n = 36), and G93A/TGF-β1 (n = 33) mice. (C–F) *p < 0.05, **p < 0.01, ***p < 0.001. Mean ± SD were plotted.

(G) Rotarod performances were evaluated weekly for GFAP-TGF-β1 (n = 5), G93A (n = 19), and G93A/TGF-β1 (n = 13) mice. Mean holding times on the rotating rod at indicated ages were plotted.

(H) Images show toluidine blue-stained axial sections of the L5 ventral root of GFAP-TGF-β1 mice and end-stage G93A and G93A/TGF-β1 mice. Scale bars, 50 μm.

(I) The distribution of motor axonal diameters in cross-sections of L5 ventral root of GFAP-TGF-β1 (n = 2), G93A (n = 6), and G93A/TGF-β1 (n = 6) mice were measured and plotted. *p < 0.05, **p < 0.01. Error bars denote SEM.

See also Figures S1 and S2.

(DN) T cells were decreased in SOD1^{G93A}/TGF-β1 mice compared to SOD1^{G93A} mice, while no significant difference was observed in the numbers of CD45^{hi}CD3− cells between the two genotypes (Figures 5A, 5B, and 5D). We also evaluated intracellular expression of IFN-γ and IL-4 in lymphocytes, and found that the numbers of IFN-γ/IL-4 producing total, CD4+,

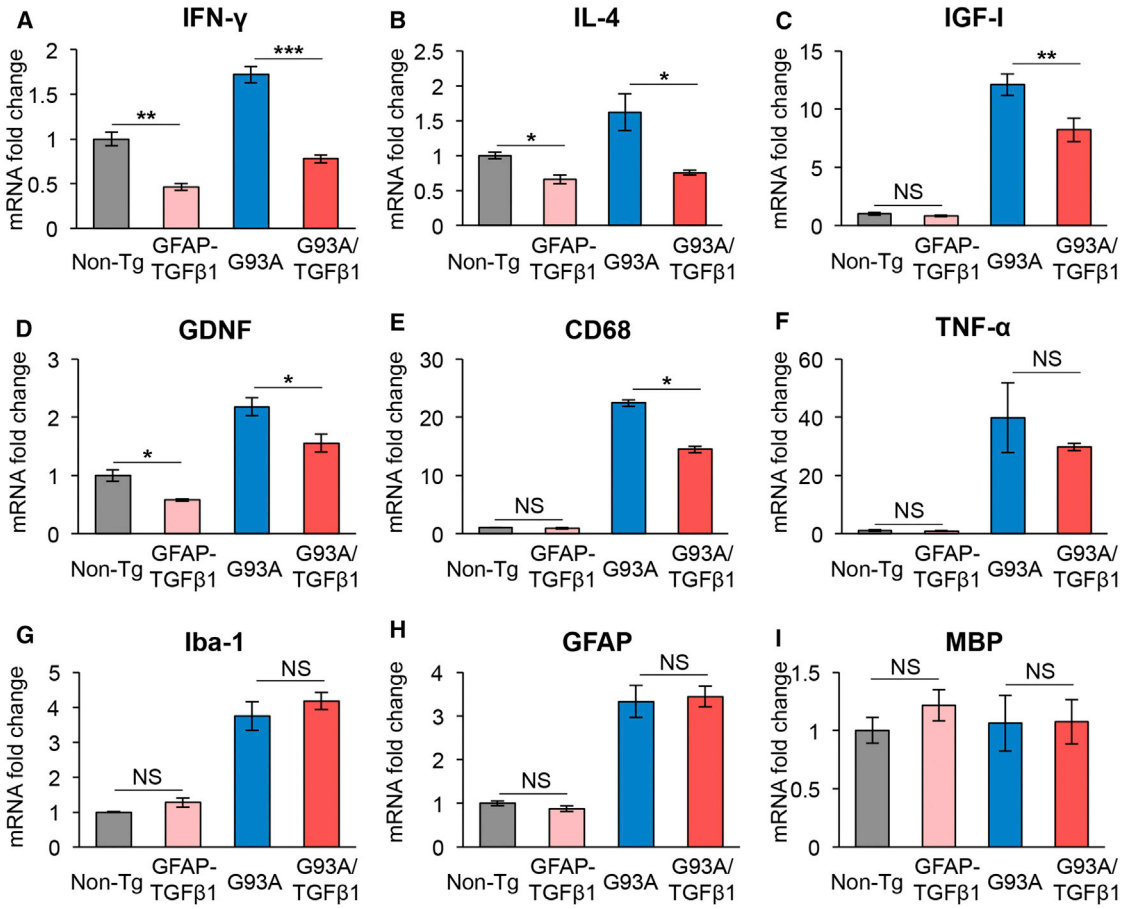

Figure 3. Quantitative RT-PCR Analyses of Glia/Immune System-Related Molecules in the Lumbar Spinal Cord of SOD1^{G93A} and SOD1^{G93A}/TGF-β1 Mice

(A–I) Mean mRNA levels of indicated glia/immune system-related molecules in the lumbar spinal cord of end-stage SOD1^{G93A} (G93A) and SOD1^{G93A}/TGF-β1 (G93A/TGF-β1) mice along with GFAP-TGF-β1 mice relative to the ones from Non-Tg littermates were plotted. Each result was normalized to β-actin. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Error bars denote SEM (n = 3–5).

See also Table S2 and Figure S3.

and DN T cells were significantly decreased in SOD1^{G93A}/TGF-β1 mice (Figures 5C, 5E, and 5F). Moreover, the ratio of IFN-γ/IL-4 in total and CD4+ T cells was significantly increased in SOD1^{G93A}/TGF-β1 mice (Figure 5G), indicating that overproduction of TGF-β1 induces an IFN-γ-dominant environment rather than IL-4. On the other hand, there were no significant differences in the numbers of T cells in the peripheral blood among all genotypes (Figure 5H). These findings indicate that astrocyte-specific overproduction of TGF-β1 reduces the number of infiltrated T cells and induces an IFN-γ-dominant milieu in the spinal cord of SOD1^{G93A} mice.

Astrocyte-Derived TGF-β1 Reduces Neuroprotective Microglial IGF-I Expression and Dendritic Cell-like Activation via a Dominant IFN-γ-Producing T Cell Environment

To uncover the mechanism through which astrocyte-derived TGF-β1 results in a decreased IGF-I expression in microglia and decreased number of infiltrated T cells with higher ratios of IFN-γ/IL-4-producing T cells in SOD1^{G93A}/TGF-β1 mice, we performed primary glial and peripheral blood mononuclear cell (PBMC) co-culture assays using primary glial cells obtained from non-transgenic, SOD1^{G93A}, and SOD1^{G93A}/TGF-β1 newborn mice and PBMCs from non-transgenic and end-stage SOD1^{G93A} mice (Figure 6A). In this assay, we examined intracellular expression of IGF-I and surface expressions of CD11c in microglia as well as intracellular expressions of both IFN-γ and IL-4 in T cells using flow cytometric analysis. We found that microglial IGF-I expression was significantly decreased in the SOD1^{G93A}/TGF-β1-primary glial cells when cultured with SOD1^{G93A}-PBMC (Figures 6B and 6C), while IGF-I levels were unaffected without PBMC (Figure 6C). Additionally, TGF-β1 itself marginally affected expression levels of IGF-I in microglia (Figures 6C and S5A). The numbers of T cells as well as IFN-γ/IL-4-producing T cells were significantly reduced when co-cultured with

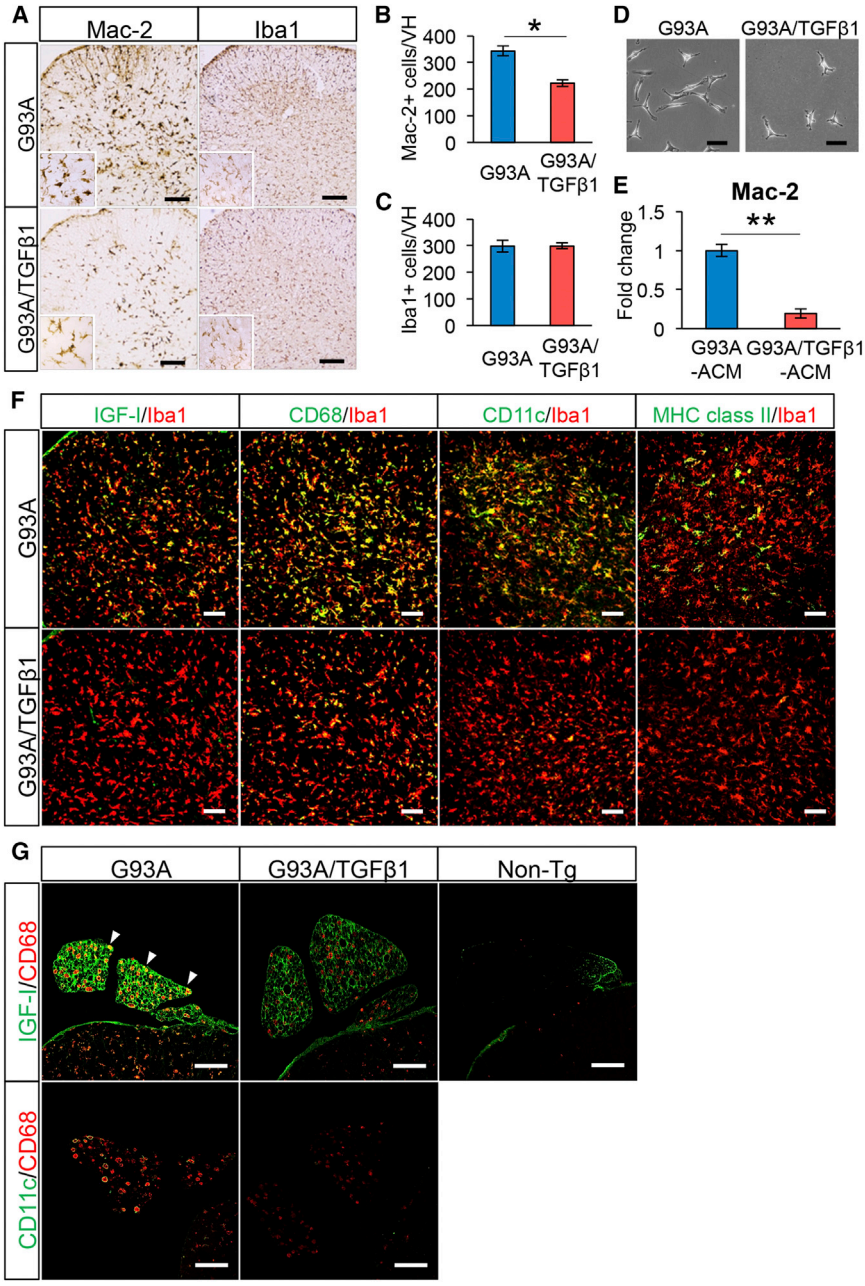

Figure 4. Decreased IGF-I and CD11c Expressions of Deactivated Microglia/Macrophages of SOD1^{G93A}/TGF-β1 Mice

(A) Images show the lumbar spinal cord of end-stage SOD1^{G93A} (G93A) and SOD1^{G93A}/TGF-β1 (G93A/TGF-β1) mice stained for Mac-2 (left) and Iba-1 (right). Scale bars, 250 μm.

(B and C) Numbers of (B) Mac-2- and (C) Iba-1-positive microglia within the ventral horn area from the lumbar spinal cord of end-stage G93A (blue) and G93A/TGF-β1 (red) mice are shown. *$p < 0.05$. Mean ± SEM were plotted (n = 3).

(D) Images show SOD1^{G93A} primary microglia cultured with ACM derived from G93A or G93A/TGF-β1 mice. Scale bars, 50 μm.

(E) Mac-2 mRNA levels of SOD1^{G93A} primary microglia cultured in the indicated conditions are shown. **$p < 0.01$. Mean ± SEM were plotted (n = 4).

(F) Images show the ventral horn of the lumbar spinal cord of G93A (top) and G93A/TGF-β1 (bottom) mice stained for IGF-I, CD68, CD11c, and MHC class II (green) together with Iba-1 (red). Scale bars, 100 μm.

(G) Images show the lumbar ventral root of end-stage G93A (left), G93A/TGF-β1 (middle), and Non-Tg (right) mice stained for IGF-I or CD11c (green) and CD68 (red). CD68-positive cells in the ventral root show IGF-I expression (arrowheads). Scale bars, 100 μm.

See also Figure S4.

SOD1^{G93A}/TGF-β1-primary glial cells (Figures 6E–6G). The ratio of IFN-γ/IL-4-producing T cells was significantly increased in the presence of SOD1^{G93A}/TGF-β1-primary glial cells (Figure 6H). Furthermore, microglial IGF-I expression was negatively correlated with the ratio of IFN-γ/IL-4-producing cells (Figure 6I), and expression of IGF-I in BV-2 microglia was altered by the IFN-γ/IL-4 ratio (Figure S5B). Lastly, microglial CD11c expression behaved like IGF-I (Figures 6B, 6D, and 6J). These results suggest that astrocyte-derived TGF-β1 reduces the neuroprotective properties of microglia, such as IGF-I expression and dendritic cell-like phenotype, alongside an IFN-γ-dominant milieu of T cells.

Higher Level of TGF-β1 Is a Negative Prognostic Factor by Determining the IFN-γ/IL-4 Ratio in SOD1^{G93A} Mice

We examined the mean mRNA levels determined by qRT-PCR in the lumbar spinal cord to evaluate the correlation among survival time, IFN-γ/IL-4 ratio, and endogenous TGF-β1 levels in SOD1^{G93A} mice. We found that the survival time of SOD1^{G93A} mice negatively correlated not only with TGF-β1 mRNA level (Figure 7A), but also with the ratio of IFN-γ/IL-4 mRNA (Figure 7B). Moreover, TGF-β1 levels significantly correlated with the ratio of IFN-γ/IL-4 (Figure 7C). In summary, these findings suggest that TGF-β1 is a negative prognostic factor in ALS mice likely through its determination of IFN-γ/IL-4 ratio.

Astrocytic TGF-β1 Is Downregulated in Slowly Progressive SOD1^{G37R} Mice with Astrocyte-Specific Deletion of Mutant *SOD1*

We previously demonstrated that the selective reduction of mutant *SOD1* in astrocytes significantly slowed the disease progression of loxSOD1^{G37R} mice using Cre-loxP system (Figure 7D; Yamanaka et al., 2008). However, astrocyte-derived molecules that are linked to neurotoxicity have not been fully elucidated.

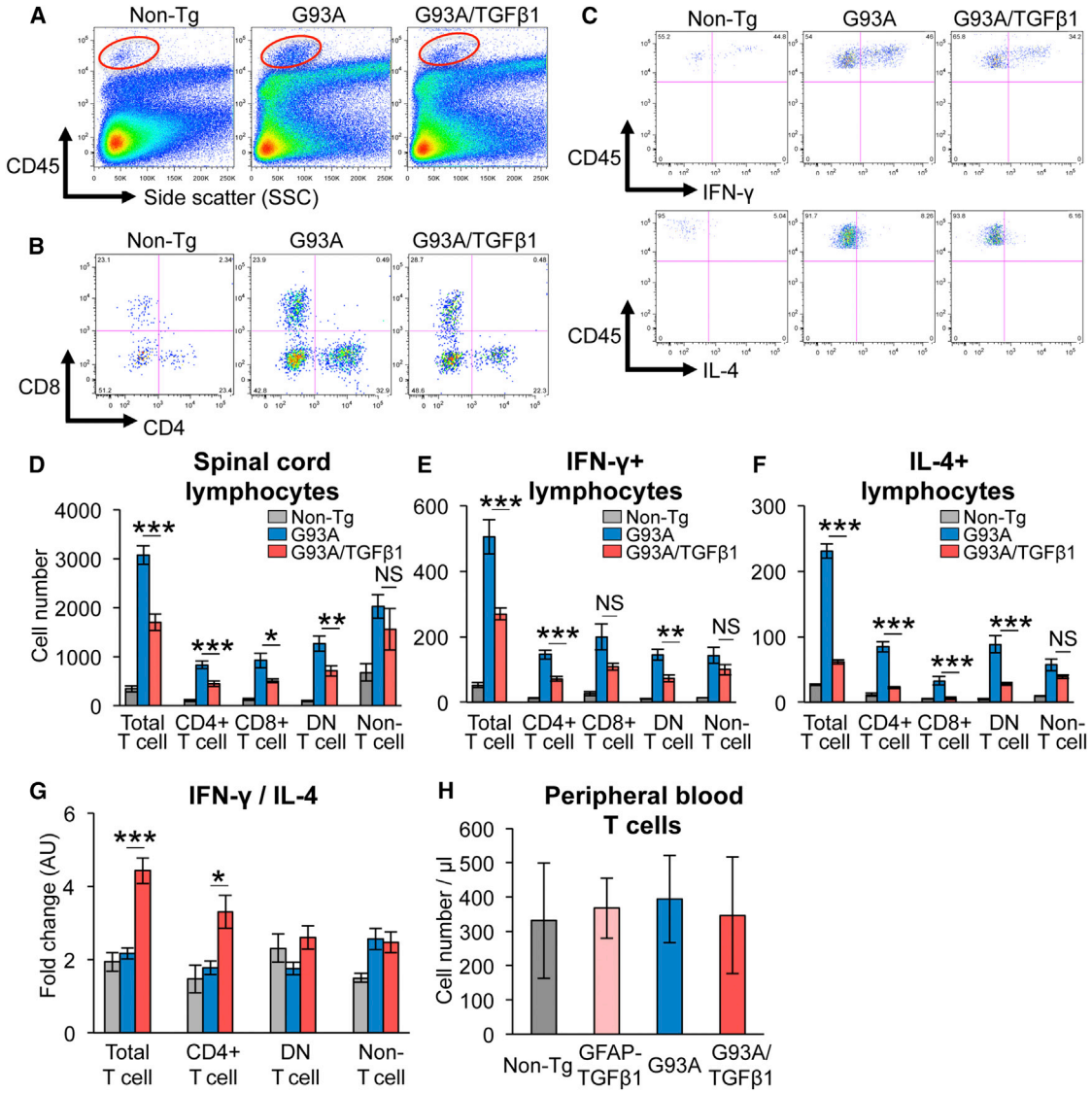

Figure 5. Decreased Number of Infiltrated T Cells and Increased Ratio of IFN-γ/IL-4-Producing T Cells in SOD1^{G93A}/TGF-β1 Mice

Flow cytometric analysis of spinal cord lymphocytes from non-transgenic (Non-Tg), end-stage SOD1^{G93A} (G93A), and SOD1^{G93A}/TGF-β1 (G93A/TGF-β1) mice.

(A–C) Flow cytometric analyses of (A) CD45^{hi} cells (cells in red-circled areas), (B) CD4/CD8 expression for T cells, and (C) cytokine staining of IL-4 or IFN-γ in T cells are shown.

(D) Numbers of each subset of lymphocytes in the spinal cord from each mouse line are shown.

(E and F) Numbers of each subset of (E) IFN-γ- or (F) IL-4-producing lymphocytes in the spinal cord from each mouse line are shown.

(G) Mean ratios of IFN-γ/IL-4-producing cells are shown.

(H) Cell numbers per microliter of CD3+ T cells in the peripheral blood of Non-Tg, GFAP-TGF-β1, end-stage G93A, and G93A/TGF-β1 mice are shown. (D–H) *p < 0.05, **p < 0.01, ***p < 0.001. NS, not significant. Mean ± SEM were plotted (n = 6–7 in D–G and n = 8 in H).

We found that TGF-β1 mRNA level was significantly reduced in loxSOD1^{G37R}/GFAP-Cre^{+} mice compared with loxSOD1^{G37R}/GFAP-Cre^{-} mice (Figure 7E). Furthermore, immunofluorescence analysis showed that TGF-β1 as well as GFAP expressions were reduced in the spinal cord astrocytes of loxSOD1^{G37R}/GFAP-Cre^{+} mice compared with loxSOD1^{G37R}/GFAP-Cre^{-} mice (Figures 7F and 7G). In summary, astrocytic TGF-β1 is one of the key factors induced by mutant SOD1 that is linked to accelerated disease progression in ALS mice.

Pharmacological Inhibition of TGF-β Signaling Slows Disease Progression and Extends Survival of SOD1^{G93A} Mice

To explore the therapeutic potential of modifying glial TGF-β signaling and to validate an adverse role of excess TGF-β in neuroinflammation, we administered TGF-β signaling inhibitor SB-431542 intraperitoneally to symptomatic SOD1^{G93A} mice. SB-431542 slowed disease progression and extended survival time of SOD1^{G93A} mice (Figure 7H). We also examined the

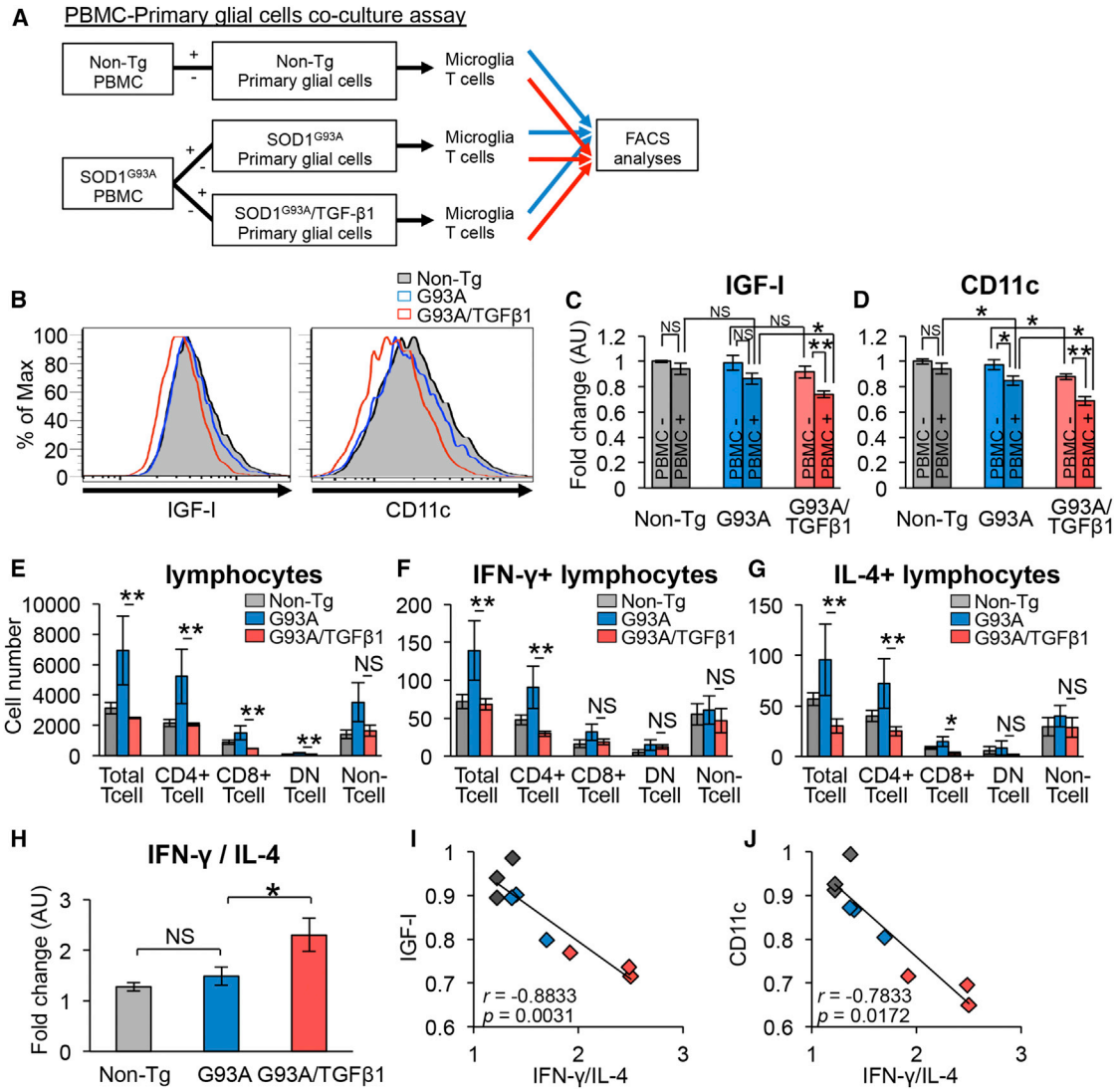

Figure 6. Correlation between Decreased Microglial IGF-I/CD11c Expressions and Decreased Number of T Cells with Increased IFN-γ/IL-4 Ratio in the Presence of TGF-β1 Overproduction in Astrocytes

Flow cytometric analyses of primary microglia from non-transgenic (Non-Tg), SOD1^{G93A} (G93A), and SOD1^{G93A}/TGF-β1 (G93A/TGF-β1) mice and T cells in PBMC from Non-Tg and end-stage G93A mice in the primary glial and PBMC co-culture assay.

(A) Experimental design for the primary glial and PBMC co-culture assay and flow cytometry is shown.

(B) Flow cytometric analysis shows IGF-I and CD11c for primary microglia cultured with PBMC (black, Non-Tg; blue, G93A; red, G93A/TGF-β1).

(C and D) Plotted relative expression levels of (C) IGF-I and (D) CD11c in primary microglia. Each datum was normalized to that of Non-Tg primary microglia cultured without PBMC.

(E) Plotted numbers of each subset of T cells and non-T cells in PBMC from Non-Tg, G93A mice cultured with primary glial cells from each mouse line are shown.

(F and G) Plotted numbers of each subset of (F) IFN-γ- and (G) IL-4-producing lymphocyte in PBMC from each mouse line are shown.

(H) Plotted mean ratios of IFN-γ/IL-4-producing T cells in PBMC from each mouse line are shown. (C–H) *$p < 0.05$, **$p < 0.01$. NS, not significant. Error bars denote SEM (n = 3).

(I and J) Plotted Spearman correlations between expression level of (I) IGF-I and (J) CD11c in primary microglia and the ratio of IFN-γ/IL-4-producing T cells in PBMC (n = 9, $r = -0.8833$, and $p = 0.0031$ in I and n = 9, $r = -0.7833$, and $p = 0.0172$ in J) are shown.

See also Figure S5.

effect of SB-431542 on the very late symptomatic SOD1^{G93A} mice (140 days old), revealing a trend toward extension of their survival times (Figure S6). These results indicate that inhibiting TGF-β signaling after disease onset ameliorates disease in ALS mice.

DISCUSSION

In this study, we have demonstrated that astrocyte-specific overproduction of TGF-β1 in mutant SOD1 mice results in accelerated disease progression in a non-cell-autonomous manner,

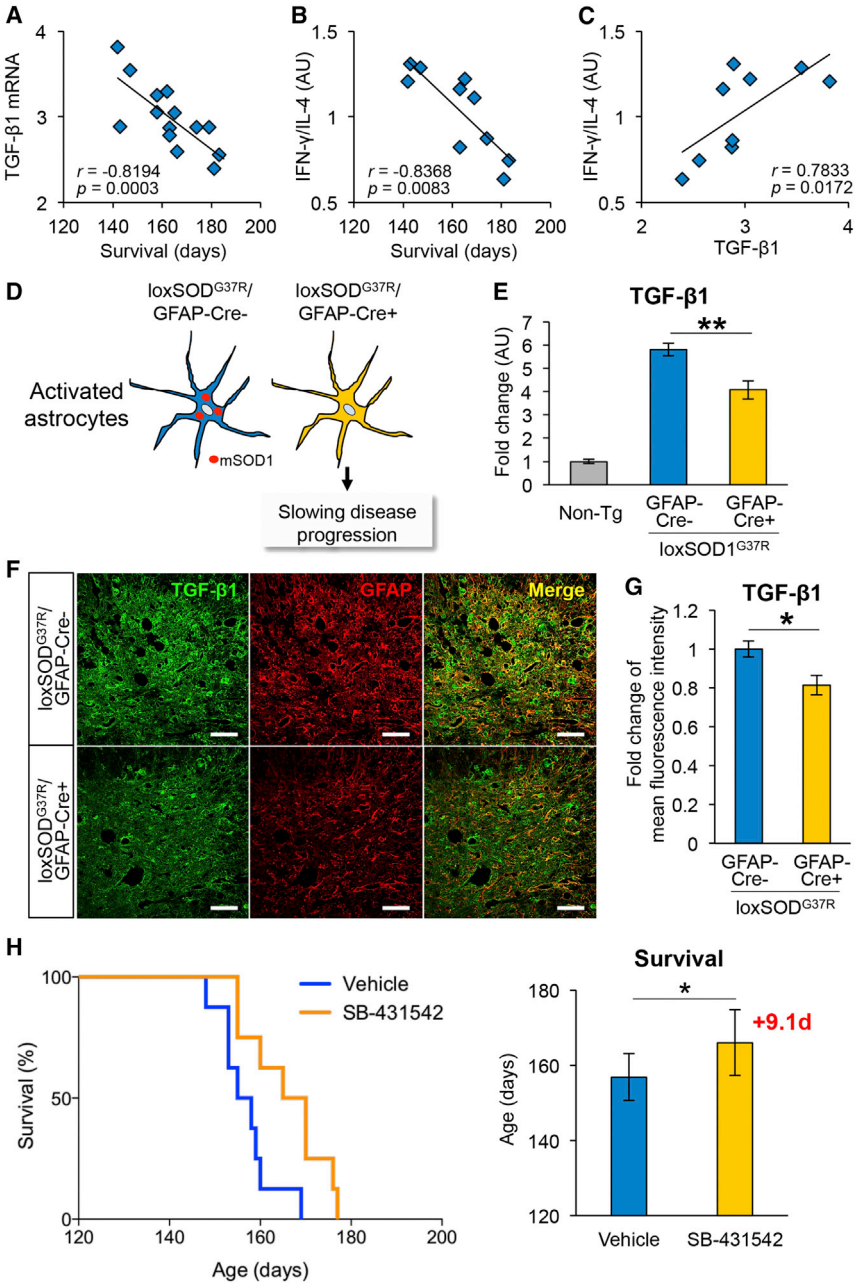

Figure 7. Astrocytic TGF-β1 Is a Determinant for Disease Progression and Survival for ALS Mice, and TGF-β Signaling Inhibitor Extends the Survival Time of ALS Mice

(A–C) Correlations between survival time, IFN-γ/IL-4 ratio, and TGF-β1 level in SOD1^{G93A} mice. (A) Plotted Spearman correlations (A) between TGF-β1 mRNA and the survival time of SOD1^{G93A} mice (n = 14, $r = -0.8194$, and p = 0.0003), (B) between ratio of IFN-γ/IL-4 mRNA and the survival time (n = 10, $r = -0.8368$, and p = 0.0083), and (C) between TGF-β1 mRNA and ratio of IFN-γ/IL-4 mRNA (n = 10, $r = 0.7833$, and p = 0.0172) are shown.

(D–G) Astrocytic TGF-β1 is downregulated in loxSOD1^{G37R} mice with astrocyte-specific deletion of mutant SOD1. (D) A schematic drawing shows astrocyte-specific deletion of mutant *SOD1* in loxSOD1^{G37R} mice using Cre-loxP system, which leads to slowing the disease progression.

(E) TGF-β1 mRNA levels in the lumbar spinal cord of end-stage loxSOD1^{G37R}/GFAP-Cre^{-} and loxSOD1^{G37R}/GFAP-Cre^{+} mice relative to age-matched Non-Tg mice are shown. **p < 0.01. Mean ± SEM were plotted (n = 6).

(F) Representative images show the ventral horn of the lumbar spinal cord of end-stage loxSOD1^{G37R}/GFAP-Cre^{-} and loxSOD1^{G37R}/GFAP-Cre^{+} mice stained for TGF-β1 (green) and GFAP (red), along with the merged image. Scale bars, 50 μm.

(G) Mean fluorescent intensities of TGF-β1 in the gray matter of lumbar ventral horn of loxSOD1^{G37R}/GFAP-Cre^{+} mice relative to loxSOD1^{G37R}/GFAP-Cre^{-} mice. *p < 0.05. Error bars denote SEM (n = 3).

(H) Peripheral administration of TGF-β inhibitor extended the survival time of SOD1^{G93A} mice. SOD1^{G93A} mice were intraperitoneally injected with SB-431542 (10 mg/kg, n = 8) or vehicle (DMSO/PBS, n = 8) five times per week from 16 weeks old (after disease onset). Kaplan-Meier analysis for the survival time and plotted mean survival showed that SB-431542 extended the survival of SOD1^{G93A} mice (SB-431542: 166.0 ± 8.7 days, vehicle: 156.9 ± 6.3 days, log-rank test: p = 0.0168). *p < 0.05. Error bars denote SD (n = 8, four males and four females in each group).

See also Figure S6.

with reduced neurotrophic factor production in deactivated microglia/macrophages and an IFN-γ-dominant environment of infiltrated T cells. Moreover, the lower level of TGF-β1 was achieved by astrocyte-specific deletion of mutant SOD1 from ALS mice, which slows disease progression. Furthermore, pharmacological inhibition of TGF-β signaling in symptomatic ALS mice extended survival time. These results provide compelling evidence that astrocytic TGF-β1 inhibits the neuroprotective inflammatory responses coordinated by microglia/macrophages and T cells.

Our study demonstrated elevated TGF-β1 levels in astrocytes of ALS patients and mice. Although TGF-β signaling has been implicated in the pathogenesis of ALS (Iida et al., 2011; Phatnani et al., 2013), the detailed mechanisms are yet to be elucidated (Katsuno et al., 2011). We demonstrated that decreased expression of pSmad2 in motor neuron nuclei occurred at the pre-symptomatic stage and was exacerbated during disease progression in ALS mice. Moreover, exogenous expression of TGF-β1 in SOD1^{G93A} mice did not improve pSmad2 level within motor neuron nuclei, the motor function, or disease course of mice. In spinobulbar muscular atrophy (SBMA), an inherited motor neuron disease caused by mutant androgen receptor, we previously reported a defect of TGF-β signaling in motor neurons (Katsuno et al., 2010). Intriguingly, while downregulation of TGFβR2 was observed in SBMA motor neurons, a defect in nuclear transport of pSmad2 was observed

in SOD1-ALS motor neurons rather than dysregulation of TGF-β receptors. In addition, dysregulated pSmad2/3 expression has been observed in motor neuron nuclei of patients with sporadic ALS (Nakamura et al., 2008). Our results along with these reports indicate that the dysfunction of TGF-β signaling, especially defects downstream of the TGF-β receptor in motor neurons, is involved in neurodegeneration in both familial and sporadic ALS.

Previous studies have shown that elimination of functional T cells in SOD1^{G93A} mice shortens their survival time (Beers et al., 2008; Chiu et al., 2008). However, what regulates the neuroprotective immune response in ALS mice remains unclear. In this study, we found that TGF-β1, known to inhibit T cell proliferation and differentiation (Li et al., 2006), regulates the number and IFN-γ/IL-4 balance in T cells both in vivo and in vitro and that microglia-related molecules were misregulated partly through altered IFN-γ/IL-4 balance. This finding suggests that TGF-β1 is likely to be one of the regulators responsible for controlling neuroprotective immune responses.

On the other hand, TGF-β1 has been reported to deactivate microglia (Lodge and Sriram, 1996; Suzumura et al., 1993) and to regulate antigen-presentation function of microglia in vitro (Abutbul et al., 2012; Suzumura et al., 1993). The influence of TGF-β1 on microglia, however, is unclear in the context of neurodegeneration (Flanders et al., 1998). Our results show that astrocyte-specific overproduction of TGF-β1 deactivates microglia/macrophages with reduced expression of Mac-2, CD68, CD11c, MHC class II, and IGF-I both in vivo and in vitro. Of note, T cell activation requires expression of MHC class II in antigen-presenting cells, including microglia and macrophages. Furthermore, IGF-I+ CD11c+ microglia have been reported to exert beneficial effects over neurodegeneration (Butovsky et al., 2006; Chiu et al., 2008). Thus, the current study suggests that astrocytic TGF-β1 inhibits the neuroprotective properties of microglia not only indirectly by regulating the number and balance of IFN-γ/IL-4 in T cells, but also directly through the deactivation of microglial functions, including antigen presentation. Previous studies have established that infiltration and activation of a large number of macrophages occurs in the peripheral nerves of SOD1^{G93A} mice (Chiu et al., 2009; Kano et al., 2012). In the current study, reduced expression of IGF-I, CD11c, and CD68 in macrophages was observed in the lumbar ventral root of SOD1^{G93A}/TGF-β1 mice, indicating that the TGF-β1-induced deactivation of macrophages in the ventral root also contributes to accelerated disease progression.

In addition, TGF-β1 is critical to the development of microglia (Butovsky et al., 2014). Although nuclear pSmad2 was preserved in both microglia and astrocytes of SOD1^{G93A} mice, expressions of microglia-related molecules such as CD68 were significantly reduced compared with those related to astrocytes in SOD1^{G93A}/TGF-β1 mice. These results implicate that TGF-β1 exhibits more robust effects on microglia than on astrocytes, likely because expressions of TGF-β receptors in microglia are highly dependent on TGF-β1 (Butovsky et al., 2014).

IGF-I has been found to exhibit neuroprotective properties in motor neurons. For example, IGF-I enhances axonal outgrowth of motor neurons, and microglia-derived IGF-I is required for the survival of motor and cortical neurons (Ozdinler and Macklis, 2006; Ueno et al., 2013). Moreover, IGF-I administration prolongs the survival time of SOD1^{G93A} mice (Dodge et al., 2008; Kaspar et al., 2003). Thus, in the current study, the marked reduction of IGF-I in the lumbar spinal cord of SOD1^{G93A}/TGF-β1 mice might have contributed to an accelerated disease progression. Although TGF-β1 itself slightly reduces expression levels of IGF-I, we observed that IFN-γ, and not TGF-β1, showed a strong antagonizing effect on the expression of IGF-I in microglia induced by IL-4 in vitro. Furthermore, IGF-I expression in microglia was regulated by the IFN-γ/IL-4 balance in vitro. We found that the level of GDNF, a potent survival factor for motor neurons that prolongs the survival of SOD1^{G93A} mice (Henderson et al., 1994; Kaspar et al., 2003; Wang et al., 2002), also was reduced in SOD1^{G93A}/TGF-β1 mice, suggesting that the neurodegenerative mechanism, similar to IGF-I reduction, might involve a low level of GDNF in SOD1^{G93A}/TGF-β1 mice. Together, decreased levels of these neurotrophic factors through enhanced expression level of TGF-β1 seem to have important roles in the accelerated disease progression in SOD1^{G93A}/TGF-β1 mice.

We demonstrated that expression levels of endogenous TGF-β1 mRNA at the end stage negatively correlates with the survival time of SOD1^{G93A} mice and positively correlates with the IFN-γ/IL-4 ratio. These findings suggest a functional relationship between astrocytes producing TGF-β1 and T cells producing IFN-γ/IL-4 in the disease progression, not only in SOD1^{G93A}/TGF-β1 mice but also in SOD1^{G93A} mice. Moreover, the negative correlation between TGF-β1 level and survival time of ALS mice is consistent with our observation that astrocyte-specific deletion of mutant *SOD1* extended survival time with a lower level of astrocytic TGF-β1. Our results indicate that astrocytic TGF-β1 is a determinant of disease progression in ALS mice, and TGF-β1 shall be evaluated as a candidate biomarker to predict disease progression of ALS.

Finally, pharmacological administration of TGF-β signaling inhibitor SB-431542 after disease onset extended the survival time of SOD1^{G93A} mice. Though peripherally administered, SB-431542 was presumably effective in the diseased spinal cord, since the blood-spinal cord barrier was damaged in the symptomatic mutant SOD1 mice (Zhong et al., 2008). In addition, an adverse effect on motor neurons by inhibiting TGF-β signaling is likely to be minimal, since TGF-β signaling in motor neurons is already defective at the late symptomatic stage. The effect of a TGF-β1 inhibitor on extension of the survival time might be more robust if TGF-β signaling in motor neurons would be simultaneously protected. Nevertheless, our data validated an adverse role of excess glial TGF-β1 in neuroinflammation and uncovered the therapeutic potential of modifying glial TGF-β signaling in ALS.

In conclusion, our study provides evidence that astrocytic TGF-β1 plays a key role in the neuroprotective inflammatory response in ALS mice by regulating microglial activation, T cell number, and IFN-γ/IL-4 balance. Our findings suggest that targeting TGF-β signaling in a cell-type-specific manner, such as restoration of TGF-β signaling in motor neurons and suppressing excess TGF-β1 in astrocytes, may represent a therapeutic approach for the treatment of motor neuron diseases.

EXPERIMENTAL PROCEDURES

Detailed information is available in the Supplemental Experimental Procedures.

Postmortem Human Tissues

Specimens of spinal cords from two patients with sporadic ALS and two other neurological disease patients as controls were obtained by autopsy with informed consent (Table S1). The diagnosis of ALS was confirmed by El Escorial diagnostic criteria as defined by the World Federation of Neurology. The collection of tissues and their use in this study were approved by the ethics committee of Nagoya University. For immunofluorescence analysis, sections were prepared from formalin-fixed and paraffin-embedded tissues, deparaffinized, and boiled for 30 min in 50 mM citrate buffer (pH 6.0).

Mice

Transgenic mice expressing familial ALS-linked SOD1 mutations, SOD1^{G93A} (B6.Cg-Tg (SOD1*G93A)1Gur/J, Jackson Laboratory), LoxSOD1^{G37R} (Boillée et al., 2006), and SOD1^{G85R} (Bruijn et al., 1997), were described previously. GFAP-TGF-β1 mice (a low-expressing line [line T64] on a C57BL/6 genetic background) (Wyss-Coray et al., 1995) and GFAP-Cre mice (Bajenaru et al., 2002) were described previously. To generate mice heterozygous for both SOD1^{G93A} and GFAP-TGF-β1 transgenes, SOD1^{G93A} males were bred with GFAP-TGF-β1 females. To generate loxSOD1^{G37R} mice with astrocyte-specific deletion of mutant SOD1, loxSOD1^{G37R} males were bred with GFAP-Cre females. The animal study was approved by the Animal Care and Use Committee of Nagoya University and RIKEN.

Analysis of Disease Progression, Survival, and Motor Function of Mice

SOD1^{G93A} mice always were compared with their SOD1^{G93A}/TGF-β1 littermates. Disease onset was determined as the time when mice reached maximum body weight, and the end stage was determined by the inability of an animal to right itself within 20 s when placed on its side. Definitions for early and late disease were described previously (Boillée et al., 2006). The motor function of mice was tested by an accelerated rotarod task with a rotarod device (MK-610A, Muromachi Kikai).

See the Supplemental Experimental Procedures for details.

Primary Glial Cell and PBMC Co-culture Assay

To isolate PBMCs, blood was collected from the central tail artery of mice. PBMCs were isolated using Lymphosepar II (IBL) by centrifugation at 400 × *g* for 30 min. Then, 5 × 10^5 PBMCs were cultured in 10% fetal bovine serum (FBS) RPMI-1640 medium with primary glial cells in 12-well plates for 4 days. For flow cytometric analyses, PBMCs were obtained from the culture media of this assay, and primary microglia were obtained from adhesive glial cells after treatment with trypsin-EDTA (Gibco).

Flow Cytometry Analyses of Spinal Cord Immune Cells, PBMCs, and Primary Microglia

Mice were transcardially perfused with PBS. Spinal cords were dissected, minced into 1-mm^3 pieces in collagenase working solution (1 mg/ml Collagenase IV [Worthington] and 0.4 mg/ml DNase I [Roche]), and incubated at 37°C for 15 min. For isolation of immune cells and microglia, cells were re-suspended in 37% Percoll (GE Healthcare) and centrifuged at 780 × *g* for 20 min. After centrifugation, myelin debris was removed and cell pellets were collected. Cells were then washed twice with PBS and suspended in 10% FBS RPMI-1640 medium or DMEM. Intracellular staining was performed as previously described (Komine et al., 2003) with modifications. Flow cytometry analyses were performed on FACS Aria and FACS Verse flow cytometer (Becton Dickinson). The data were analyzed by using FlowJo Software (Tree Star). Details are described in the Supplemental Experimental Procedures.

Administration of TGF-β Signaling Inhibitor In Vivo

SOD1^{G93A} mice were intraperitoneally injected with TGF-β signaling inhibitor SB-431542 (Sigma, 5 or 10 mg/kg) or vehicle (DMSO/PBS) five times per week either at 16 weeks old (early symptomatic phase) or 20 weeks old (late symptomatic phase).

Statistical Analysis

Statistical analyses of survival time and disease duration were performed with a log-rank test and unpaired t test, respectively. Statistical analysis of the fold change of relative mRNA levels determined by qRT-PCR was performed with a one-way ANOVA followed by Tukey's test or paired or unpaired t test. All analyses were carried out using GraphPad Prism.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.03.053.

AUTHOR CONTRIBUTIONS

F.E., O.K., and K.Y. designed the experiments and analyzed the data. F.E. performed the research with support from O.K., N.F-T., S.J., and S.W. M.K., G.S., M.D., and T.W.-C. contributed critical analytic tools and input. F.E. and K.Y. wrote the paper.

ACKNOWLEDGMENTS

The authors thank the Support Unit for Animal Resource Development and Biomaterial Analysis in RIKEN BSI Research Resources Center for supporting animal experiments and fluorescence-activated cell sorting (FACS) analysis, and BSI-Olympus Collaboration Center for supporting morphological analyses. This work was supported by Grants-in-Aid for Scientific Research on Innovative Areas (23111006) and Scientific Research (B) (26293208) from the Ministry of Education, Culture, Sports, Science and Technology of Japan; Grant-in-Aid for Research on rare and intractable diseases, the Research Committee on Establishment of Novel Treatments for Amyotrophic Lateral Sclerosis, from the Ministry of Health, Labour and Welfare of Japan; Japan Intractable Disease Research Foundation; Daiko Foundation; and "Inochi-no-Iro" ALS research grant (K.Y.).

Received: October 6, 2013
Revised: February 20, 2015
Accepted: March 24, 2015
Published: April 16, 2015

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