Neuroinflammatory mechanisms in amyotrophic lateral sclerosis pathogenesis

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Purpose of review
Neuroinflammation is increasingly recognized as an important mediator of disease progression in patients with amyotrophic lateral sclerosis (ALS), and is characterized by reactive central nervous system (CNS) microglia and astroglia as well as infiltrating peripheral monocytes and lymphocytes. Anti-inflammatory and neuroprotective factors sustain the early phase of the disease whereas inflammation becomes proinflammatory and neurotoxic as disease progression accelerates. Initially, motor neurons sustain injuries through multiple mechanisms resulting from harmful mutations causing disruptions of critical intracellular pathways. Injured motor neurons release distress signal(s), which induce inflammatory processes produced by surrounding glial cells in the CNS as well as peripheral innate and adaptive immune cells. This review will focus on mechanisms of neuroinflammation and their essential contributions in ALS pathogenesis.

Recent findings
Regulatory T lymphocytes (Tregs) are a subpopulation of immunosuppressive T lymphocytes that become reduced and dysfunctional as the disease progresses in ALS patients. Their degree of dysfunction correlates with the extent and rapidity of the disease. Treg numbers are boosted in transgenic mutant SOD1 (mSOD1) mice through the passive transfer of Tregs or through treatment with an interleukin-2/ interleukin-2 monoclonal antibody complex and rapamycin. Treating the transgenic mice with either of these modalities delays disease progression and prolongs survival. In addition, Treg function is restored when dysfunctional Tregs are isolated from ALS patients and expanded *ex vivo* in the presence of interleukin-2 and rapamycin. Based on these findings, a first-in-human phase 1 trial has been completed in which expanded autologous Tregs were infused back into ALS patients as a potential treatment. The infusions were safe and shown to 'hit target' by enhancing both Treg numbers and suppressive functions.

Summary
A delicate balance between anti-inflammatory and proinflammatory factors modulates the rates of disease progression and survival times in ALS. Tipping the balance toward the anti-inflammatory mediators shows promise in slowing the progression of this devastating disease.

Keywords
amyotrophic lateral sclerosis, microglia, neuroinflammation, regulatory T lymphocytes

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a fatal motor neuron disease characterized by relentless degeneration of upper and lower motor neurons. Following symptom onset, patients survive an average of 4 to 6 years, and there is minimal effective therapy. Although the cause(s) and pathogeneses of ALS are incompletely defined, increasing evidence indicates that peripheral and central inflammatory responses involving central nervous system (CNS) microglia and astroglia as well as infiltrating peripheral monocytes and lymphocytes contribute to motor neuron injury and rates of disease progression. Studies in the ALS transgenic mutant SOD1 (mSOD1) mice show that neuroprotective anti-inflammatory mediators are present during the early slow phase of the disease, and conversion to the fast phase of the disease is marked by an increase in cytotoxic proinflammatory mediators [1,2]. The immune/inflammatory constituents in early stages are characterized by anti-inflammatory microglia

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KEY POINTS

- Proinflammatory monocytes and CNS microglia are increased in ALS.
- Neuroprotective Tregs are decreased and dysfunctional in ALS.
- Following *ex vivo* expansion, the dysfunctional Tregs regain their suppressive function.
- In ALS transgenic mouse models, infusion of Tregs and upregulation of endogenous Tregs slow disease progression and prolong survival.
- A phase 1 trial of autologous infusions of expanded Tregs documented safety and possible clinical benefit.

and suppressive regulatory T lymphocytes (Tregs) whereas proinflammatory microglia and Th1 cells predominate during later stages.

EVIDENCE FOR DUAL NEUROPROTECTIVE/NEUROTOXIC ROLES OF PERIPHERAL AND CENTRAL INNATE IMMUNE CELLS

Anti-inflammatory microglia isolated from early stage ALS mouse spinal cords protect motor neurons in culture, while microglia isolated from late stage ALS mouse spinal cords exhibit a proinflammatory phenotype and are toxic to motor neurons. Transgenic mice produced by crossing mSOD1 mice with PU.1 knockout were either transplanted with mSOD1 or wild-type bone marrows, which allowed the populating CNS microglia to adopt either a proinflammatory mSOD1 phenotype or a wild-type anti-inflammatory phenotype. The transgenic mice that were transplanted with the mSOD1 bone marrow exhibited increased loss of motor neurons and decreased lifespans compared to the mice transplanted with the wild-type bone marrow, thereby substantiating the neuroprotective role of anti-inflammatory microglia and the neurotoxic role of proinflammatory microglia [3].

Increasing evidence suggests that systemic inflammation involving the innate immune system is present in ALS [4–6,7▪]. Peripheral blood innate immune monocytes exhibit a proinflammatory genotype in ALS patients, with greater expression in patients whose disease is progressing rapidly [8▪▪]. Whether and how these proinflammatory myeloid cells contribute to disease progression has not been definitively determined. Peripheral myeloid cells could migrate to the spleen, lymph nodes, or even to the CNS. Migration to the spinal cord parenchyma has been questioned under the assumption that the blood-brain-barrier (BBB) is intact. However, in ALS models and ALS patients the BBB is compromised [9], and alterations of the BBB would permit the entry of proinflammatory cells that could contribute to neurodegeneration and the pathogenesis of disease.

Denervation at the neuromuscular junction (NMJ) is an early event in the pathogenesis of ALS. Because the NMJ is outside the BBB, monocytes/macrophages can readily access the peripheral motor axon and the NMJ, and infiltration of degenerating peripheral nerve fibres has been described in the mSOD1 transgenic mouse [10]. However, the inflammatory response was not observed until after denervation had occurred, and the presence of such infiltrating peripheral macrophages may be the consequence rather than the cause of denervation.

The role of MHCI expression in amyotrophic lateral sclerosis models

The contribution of inflammation in the peripheral nervous system (PNS) contrasts with the contribution of inflammation in the CNS. In the early stages of disease in the mSOD1 transgenic mouse, disease progression is slow and CNS microglia and astrocytes provide anti-inflammatory responses that protect the injured motor neuron. During the later more rapidly progressive stages of disease in the CNS of ALS transgenic models and ALS patients, microglia, astrocytes and infiltrating immune cells release toxic proinflammatory mediators that exacerbate motor neuron injury and cell death. However, in the PNS the inflammatory response plays a role that appears to be toxic but is in fact enhancing the removal of injured motor axons to permit axonal regrowth. In later stages this 'protective' response is no longer present. In mSOD1 mice levels of the major histocompatibility complex class I (MHCI) are increased in peripheral motor axons and NMJs [11]. The MHCI is a key molecule of the immune system for antigen presentation to $CD8^+$ cytotoxic T cells. Following peripheral lesions of the axon, MHCI is rapidly translocated to motor axons and terminals, and the presence of MHCI has a positive effect on axonal regeneration. The greater is the neuronal MHCI expression, the more efficient is the axonal regrowth. The increased MHCI in the peripheral axon may activate cytotoxic T cells to create a growth-permissive milieu, which promotes the pruning of damaged motor axons and collateral sprouting.

Thus, increased MHCI expression is neuroprotective in peripheral nerves and depends on immune cells to remove motor axon debris. However, in the CNS as disease advances the increased

microgliosis and astrocytosis reduces MHCI in the lumbar spinal motor neurons of mSOD1 mice and ALS patients, and promotes neurotoxicity [12].

ADAPTIVE T-LYMPHOCYTES MODULATE THE BALANCE BETWEEN NEUROPROTECTION AND NEUROTOXICITY

A critical role for T lymphocytes was discovered by crossing transgenic mSOD1 mice with RAG2-/- mice that lacked mature T and B cells, or with CD4-/- mice that lacked $CD4^+$ T cells. In both lines of doubly transgenic mice, disease progression accelerated and was accompanied by increased messenger RNA (mRNA) expression levels of proinflammatory cytokines. The fact that transgenic mice lacking functional T cells died earlier suggested that T cells had been relatively protective. The key question was which subpopulation of $CD4^+$ T lymphocytes was protective. Subsequent studies of the T cell populations demonstrated that the numbers of Tregs as well as expression levels of their specific transcriptional factor, FOXP3, were increased during the early slowly progressive stages of disease in the transgenic mice, but were dramatically decreased during later more rapidly progressive stages. In addition, proinflammatory Th1 lymphocytes were markedly elevated during later rapidly progressing stages of disease. Evidence for the neuroprotective role of Tregs was provided by transplanting Tregs into mSOD1/RAG2-/- transgenic mice. Following the passive transfer of Tregs, disease duration and survival were substantially prolonged. These studies collectively support the neuroprotective contribution of Tregs during early stages of disease and the cytotoxic contribution of Th1 cells during later stages of disease.

Although the exact mechanisms through which Tregs exert their beneficial effects *in vivo* are not known, experiments have shown that Tregs suppress both the proliferation of responder T-lymphocytes (Tresp) and the activation of microglia isolated from the mSOD1 mice [13]. The proliferation of Tresp was suppressed by Tregs through a contact mediated mechanism and to a lesser extent the secretion of interleukin-4, interleukin-10 and TGF-β, whereas microglial activation was suppressed through interleukin-4 secretion. Further support for this potential mechanism was derived from human studies in which healthy control Tregs, through the secretion of interleukin-4, -10 and -13, caused monocytes to respond much less robustly to LPS [14].

A subsequent study in which transgenic mSOD1 mice were treated with an interleukin-2/interleukin-2 monoclonal antibody complex and rapamycin also resulted in increased Treg numbers with slowing of disease progression and prolongation of lifespan. The treatment also caused a reduction in microgliosis and an upregulation in mRNA expression levels of anti-inflammatory factors, providing further proof that Tregs modulate disease progression through a neuroprotective mechanism by producing an anti-inflammatory milieu [15▪▪].

REGULATORY T LYMPHOCYTES IN AMYOTROPHIC LATERAL SCLEROSIS PATIENTS

Tregs represent a subpopulation of T lymphocytes consisting of $CD4^+CD25^{high}FOXP3^+$ immunoreactivity. Tregs normally suppress proinflammatory responses and their dysfunction contributes to the development of many autoimmune disorders [16–19]. Tregs constitutively express the transcription factor FOXP3, which is a key regulatory gene for the development and function of natural Tregs in humans and mice [20,21], and the most specific marker for Tregs [17,22,23].

The importance of Tregs has also been documented in ALS patients. The numbers of Tregs and their FOXP3 mRNA expression levels in the blood were reduced in ALS patients who were progressing rapidly. In a cohort of 102 patients followed for 3.5 years, reduced FOXP3 mRNA expression levels during the early stages of disease were associated with decreased survival 3.5 years later [24]. In addition, Tregs derived from the blood of ALS patients were dysfunctional in that they failed to suppress the proliferation of Tresp *in vitro*, most markedly in ALS patients who were progressing rapidly [25▪▪]. *In vivo* the relative lack of functionally suppressive Tregs promoted an inflammatory state most likely mediated by both innate and adaptive immune cells. Even in ALS patients progressing slowly, the Treg suppressive function was also decreased; for a given FOXP3 expression level, the suppressive function was less than the suppressive function of age and sex-matched controls [25▪▪]. These data collectively showed that the lower the FOXP3 expression and more impaired the Treg suppressive function, the greater the neuroinflammation, the more rapid the disease progression, and the higher the burden of disease. Interestingly, Tregs isolated from fast-progressing ALS patients regained their suppressive capabilities on the proliferation of Tresp when expanded *ex vivo* in the presence of interleukin-2 and rapamycin [25▪▪,26].

The aforementioned findings that passive transfer of Tregs prolonged disease duration and survival of transgenic ALS mice, that decreased FOXP3

expression and Treg suppressive function correlated with more rapid disease progression, and that Treg function could be 'normalized' by *ex vivo* expansion all supported the potential for an adoptive therapy with expanded and highly purified autologous donor Tregs to slow disease progression in ALS patients. Hence, the first-in-man phase 1 study of autologous transplantation of expanded Tregs into ALS patients was completed and not only demonstrated safety, but also resulted in increased Treg numbers and enhanced Treg suppressive function, the latter of which correlated with disease progression rates and supported the utility of Treg suppressive function as a meaningful indicator of clinical status [27▪▪].

CONCLUSION

Tregs that express high levels of FOXP3 help suppress inflammation and protect motor neurons in ALS. However, as the disease progresses the Tregs lose their suppressive function, and this loss promotes a proinflammatory environment of activated microglia in the CNS and activated myeloid cells in the periphery. The exact factors that contribute to the loss of FOXP3 mRNA expression and impaired Treg function remain elusive, but are likely to result from the interaction of Tregs with proinflammatory myeloid cells themselves or soluble factors released from such cells that build up in the blood as the disease progresses. Eventually, during disease progression, endogenous suppressive Tregs cannot overcome the progressive proinflammatory milieu and a vicious cycle of Treg dysfunction and enhanced neurotoxic inflammation ensues ultimately resulting in motor neuron injury and accelerated disease progression. The dysfunctional Tregs in ALS lack the ability to suppress proinflammatory monocytes/macrophages in addition to the impaired ability to suppress proinflammatory T lymphocytes such as Th1 and Th17 cells. Thus, impaired suppression of both innate and adaptive immune systems may contribute to the acceleration of disease progression.

Expanded 'normalized' autologous Tregs have shown increased capabilities to suppress the proliferation of Tresp. Although Tregs suppress proinflammatory monocytes/macrophages in ALS transgenic mouse models, the specific suppressive mechanisms in ALS patients have not been delineated. Nevertheless, the major effects of Treg infusions likely occur in the periphery as well as in the CNS. Tregs may suppress proinflammatory monocytes/macrophages in the periphery and thus reduce proinflammatory signals that could potentially cross the BBB and exacerbate microglial activation and the ensuing motor neuron injury. Tregs can also readily enter the CNS and have the potential to suppress proinflammatory microglia. To date, there has been no direct demonstration in ALS patients that proinflammatory microglia can be suppressed by Tregs in the CNS. The recent advances in PET imaging of activated microglia in ALS patients offer a potential opportunity to determine whether Tregs can suppress neuroinflammation in living patients [28▪▪].

Balances or ratios of anti-inflammatory and proinflammatory mediators are critical in determining disease progression rates. Slowing of progression may be accomplished by tipping the balance toward anti-inflammatory mediators. Infusions of suppressive Tregs or other anti-inflammatory mediators such as monocytes/macrophages may be beneficial if their numbers and/or strengths outweigh those of the endogenous proinflammatory mediators. However, introducing anti-inflammatory cellular mediators into a proinflammatory environment could also be detrimental by hastening disease progression if the numbers and/or strengths of the anti-inflammatory mediators are insufficient to overcome the proinflammatory environment. None of the innate or adaptive immune cellular participants are end-stage differentiated. As a result, anti-inflammatory innate or adaptive cellular treatments could readily adopt a proinflammatory state when introduced into a proinflammatory environment if they are overwhelmed and unable to tip the balance. Therefore, cellular therapies aimed to treat this devastating disease show exciting potential, but their numbers and strengths may need to be escalated as the disease progresses to overcome a progressively proinflammatory environment.

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Conflicts of interest

E.P.S. receives publishing royalties from McGraw-Hill and is a speaker for Alexion Pharmaceuticals. S.H.A serves as a scientific consultant to Mitsubishi Tanabe Pharma, Neuraltus, and Brainstorm; and has served on the speaker's bureau of Avanir. J.R.T. has no conflicts of interest.

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- ■ of special interest
- ■■ of outstanding interest

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This study documents that PET scanning with PBR28 provides an important neuroimaging marker of microglial activation in brain regions compromised in ALS patients, and can be used as a biomarker in clinical studies of therapies such as Tregs.