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Immune-mediated Mechanisms in the Pathoprogression of Amyotrophic Lateral Sclerosis

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Abstract

Amyotrophic lateral sclerosis (ALS) is a devastating neurodegenerative disease with selective loss of upper and lower motor neurons. At sites of motor neuron injury, neuroinflammation is a prominent pathological finding and is characterized by microglial activation, astrogliosis, and infiltration of monocytes and T-cells. Both innate and adaptive immune responses actively influence disease progression in animal models and in ALS patients, and promote neuroprotection or neurotoxicity at different stages of disease. The early immune reaction to signals from injured motor neurons is to rescue and repair damaged tissue. As disease accelerates, a shift occurs from beneficial immune responses (involving M2 microglia and regulatory T-cells) to deleterious immune responses (involving M1 microglia and Th1 cells). In this review, we underscore the importance of immune-mediated mechanisms in the pathogenesis of ALS and discuss the alterations and distinct phenotypes of immune cells at the different stages of disease. The better we understand the dynamic changes that occur within the immune system over the course of disease, the better we will be able to develop effective therapeutic regimens in ALS.

Keywords

neuroinflammation; ALS; neuroprotection; neurotoxicity

Amyotrophic lateral sclerosis (ALS), known as Lou Gehrig's disease, is the most common adult motor neuron disease. Patients present with progressive muscle atrophy, paralysis, spasticity, and hyperreflexia. Failure of the respiratory muscles is generally the fatal event, occurring within 4–6 years. Pathologically, ALS is characterized by the selective loss of motor neurons in the motor cortex, brainstem and spinal cord. At the present time, there are no treatments that will arrest, or even substantially delay, the inexorable progression of ALS. More than 90% of ALS cases are sporadic with unknown cause. About 10% of ALS patients have a family history of a genetically dominant disorder. Familial cases have been linked to mutations in a number of genes, including Cu^{2+}/Zn^{2+} superoxide dismutase

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This chapter is dedicated to the memory of the late Dr. Jenny S. Henkel and to the legacy of brilliant writings and people she touched. She is greatly missed.

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(SOD1), TAR DNA binding protein (TDP-43) and chromosome 9 open reading frame 72 (C9ORF72) repeat expansions (Ince et al., 2011).

Mutant SOD1 (mSOD1) is the best characterized form of familial ALS, accounting for approximately 15–20% of familial ALS patients. Although SOD1 is the enzyme that converts superoxide to oxygen and hydrogen peroxide, the toxicity in ALS is not related to enzymatic activity; the absence of SOD1 does not initiate ALS disease in an animal model of this disorder. Both enzymatically active and inactive mutant forms cause motor neuron degeneration in animal models. Therefore, mSOD1 toxicity is not due to a loss of function, but rather to a toxic gain of function. Even in patients with sporadic ALS, alterations in wild-type SOD1 protein have been detected and implicated in the pathogenesis of disease. Oxidation results in aberrant conformation and misfolding of wild-type SOD1 protein that acquires the binding and toxic properties of mSOD1, such as damaging mitochondrial function and inhibiting axonal transport (Guareschi et al., 2012; Bosco et al., 2010; Ezzi et al., 2007). Thus, a SOD1-dependent toxic pathway is possibly shared between inherited and sporadic ALS cases.

Mutant TDP-43 is another protein that can initiate disease in ALS patients. TDP-43 is a ubiquitously expressed DNA- and RNA-binding protein that has multiple functions in transcriptional regulation, alternative splicing and microRNA processing. Mutant TDP-43 pathology in ALS patients is characterized by abnormal TDP-43 positive inclusions in the cytoplasm with diminished or absent nuclear TDP-43 staining. Even in the absence of mutation, abnormal accumulation of TDP-43 has been observed in most cases of sporadic ALS (Baloh, 2011; Da Cruz and Cleveland, 2011). Therefore, TDP-43 proteinopathy may be a common feature in pathogenesis of ALS.

In 2011, two independent groups reported a GGGGCC hexanucleotide repeat in a noncoding region of C9ORF72 (DeJesus-Hernandez et al., 2011; Renton et al., 2011). While C9ORF72 encodes a protein with unknown function, C9ORF72 repeat expansions are currently the major genetic mutation associated with ALS, accounting for about 30–40% of familial ALS cases and 6–10% of sporadic ALS cases (Van Blitterswijk et al., 2012). Of note, both TDP-43 pathology and C9ORF72 mutation are also identified in frontotemporal dementia (FTD), indicating ALS and FTD may share an underlying pathogenesis.

Several of the lethal mutations in the SOD1 gene found in ALS patients (human G93A, G85R, and G37R, and mouse G86R) have been over-expressed in transgenic mice, and these mice develop a disease that replicates many of the clinical and pathological hallmarks found in ALS patients. Recently, both wild-type and mutant TDP-43 have been over-expressed in numerous animal models, resulting in motor neuron pathology. These animal models are invaluable for investigating the pathogenesis of ALS.

The cause of motor neuron degeneration in ALS is still unclear. Numerous mechanisms have been proposed, including ER stress, mitochondrial dysfunction, oxidative stress, misfolded protein aggregation and impaired degradation, calcium overload, aberrant RNA/DNA regulation, and neuroinflammation. While the central nervous system (CNS) is conventionally thought to be immunologically privileged, considerable evidence supports

the presence of immune and inflammatory abnormalities in ALS. Neuroinflammation, characterized by activated microglia, astrogliosis, and infiltrating immune cells, is a prominent pathological finding in the spinal cords of both ALS patients and the transgenic mouse models of disease. The key question is whether such neuroinflammation is merely the consequence of motor neuron injury, or actively contributes to motor neuron neurodegeneration. In this review, we provide evidence that the dynamic immunological changes that occur during the course of disease do in fact actively contribute to the pathogenesis and progression of ALS disease.

Motor Neurons Do Not Die Alone: The Role of Immunity in ALS

The clinical symptoms of ALS are the result of motor neuron degeneration. However, specific expression of mSOD1 solely in motor neurons, does not initiate disease in mouse models (Pramatarova et al., 2001; Lino et al., 2002). Even when mSOD1 was expressed homozygously in neurons, it only caused minor motor neuron injury and a very late onset disease (Jaarsma et al., 2008). Thus, motor neuron degeneration and cell death requires the participation of non-neuronal cells, indicating that motor neurons do not die alone. This concept of non-cell autonomy provides a major rationale for implicating the immune system in motor neuron degeneration. A chimeric mouse experiment also supported the importance of nonneuronal cells in motor neuron death; motor neurons lacking mSOD developed features of ALS pathology when surrounded by mSOD1-expressing glia whereas motor neurons expressing mSOD1 but surrounded by wild-type glia appeared healthy (Clement et al., 2003). In mSOD1 transgenic mice, transplanting wild-type microglia prolonged disease duration compared with mice transplanted with mSOD1 microglia (Beers et al 2006). Reduction of mSOD1 expression in either microglia or astrocytes by a Cre/Lox approach delayed the progression of the disease and extended the lifespan of ALS mice (Yamanaka et al., 2008). Thus, despite the intracellular injury, motor neurons do not die alone; glia are required to mediate the progressive process of neurodegeneration.

Innate Immunity in ALS

The Role of Microglia

In the CNS, microglial activation is a major component of the neuroinflammation that follows CNS injury. Numerous studies have confirmed the presence of microglial activation at the site of motor neuron damage in both ALS patients and mSOD1 transgenic mice. PET scan of proteins expressed by activated microglia provide direct evidence that widespread microglial activation was present in the brain of living ALS patients (Turner et al. 2004; Corcia et al., 2012). Furthermore, increased microglial activation in the motor cortex has been shown to correlate with the severity of upper motor neuron signs in ALS patients (Turner et al. 2004). Studies using animal models of ALS also demonstrated that activated microglia were observed before disease onset and increased with disease progression through end-stage (Henkel et al., 2006; Alexianu et al., 2001). Overall, the intensity of microglial activation parallels the motor neuron degeneration and supports an active role for microglia.

Microglia are the primary immune cells in the brain and spinal cord. They act as the first line of immune defense in the CNS, surveying the surrounding environment through their processes. Microglia are sensitive to pathological changes in the CNS and respond to "danger signals" released from damaged tissue. In the context of ALS, misfolded proteins (such as mutant or oxidized SOD1) and ATP from injured motor neurons are very likely to function as such signals. It has been shown that mSOD1 can be released from motor neurons and astrocytes through chromogranin-mediated secretion (Urushitani et al., 2006). Evidence of oxidized wild-type SOD1 binding to chromogranin suggests a possible similar secretory mechanism for oxidized SOD1 as with mSOD1 (Ezzi et al., 2007). Data from our lab and others demonstrate that mutant or oxidized SOD1 effectively activate microglia through CD14/TLR2/TLR4 and scavenger receptor dependent pathways (Roberts et al., 2013; Zhao et al., 2010; Ezzi et al., 2007). In addition, ATP can be released from dying and dysfunctional cells as another neuron-to-microglia signal. ATP interacts with purinergic P2 receptors and activates microglia. Moreover, the presence of microgliosis has been shown in mice homozygously expressing high levels of mSOD1 specifically in neurons (Jaarsma et al., 2008), supporting the notion that microglial activation is induced by signals that arise from injured neurons. Once activated, microglia display very distinct and different phenotypes, which can be beneficial or injurious to motor neurons, depending on the stage and rate of progression of disease.

Microglial activation can be divided into classically activated microglia (M1) and alternatively activated microglia (M2). M1 microglia are cytotoxic due to their secretion of reactive oxygen species (ROS) and release of pro-inflammatory cytokines (such as IL-1β and TNF-α). The detrimental role of M1 microglia has been investigated in many *in vitro* and *in vivo* studies. Stimulation with LPS, a potent M1 stimulator, results in increased production and release of ROS from microglia, which are cytotoxic and mediate motor neuron death (Zhao et al., 2004). After LPS treatment, mSOD1-expressing microglia are more activated than wild-type microglia and produce more superoxide, nitric oxide and TNF-α (Xiao et al., 2007; Weydt et al., 2004). Of importance, these mSOD1 microglia are more injurious to primary cultured motor neurons (Xiao et al., 2007). Using mSOD1 transgenic mice, our lab has shown that replacing mSOD1 microglia with wild-type microglia by bone marrow transplantation significantly reduced motor neuron loss and prolonged disease duration and survival (Beers et al., 2006). In accordance with our results, reducing expression of mSOD1 in microglia/macrophages using a Cre-Lox approach also showed a significant delay in disease progression and an increase in survival (Wang et al., 2009; Boillée et al., 2006). A recent study demonstrated that extracellular mSOD1 could be endocytosed into microglia, activating caspase-1 and up-regulating IL-1β. Lack of IL-1β or treatment of IL-1β receptor antagonist extended the lifespan of mSOD1 mice and attenuated the inflammatory pathology (Meissner et al., 2010). Therefore, as disease progresses, mSOD1 microglia acquire a M1 phenotype and play a deleterious role promoting motor neuron degeneration and possibly increasing the rate of ALS disease progression.

In contrast, M2 microglia produce high levels of anti-inflammatory cytokines and neurotrophins (Gordon et al., 2010; Tiemessen and Kuhn, 2007; Buechler et al., 2000). In microglia and motor neuron co-cultures, IL-4 enhanced motor neuron survival by inducing

an M2 protective phenotype, suppressing M1 microglial activation, reducing the release of the ROS, and enhancing IGF-1 secretion (Zhao et al., 2006). Adult microglia isolated from spinal cords of mSOD1 mice at symptom onset posses higher levels of anti-inflammatory IL-1R antagonists and IGF-1 than wild-type microglia (Chiu et al., 2008). IL-1R antagonists blocked IL- 1 binding to its receptor and the downstream pro-inflammatory signaling. The protective effect of IGF-1 on motor neurons has been investigated in animal models of ALS. Delivery of IGF-1 by viral vectors or intrathecal infusion increased motor neuron survival, improved motor function and extended lifespans of mSOD1 mice (Dodge et al. 2008; Lepore et al. 2007; Kaspar et al. 2003; Nagano et al. 2005a). Unfortunately, the effect of intrathecal delivery of IGF-1 to a small number of ALS patients was not sufficiently robust to mandate a larger trial (Nagano et al. 2005b). Strategies with better IGF-1 delivery or combination with other neurotrophic therapies need to be tested. In addition, progranulin has neurotrophic and anti-inflammatory activity (Martens et al., 2012). Enhanced expression of progranulin in microglia was reported in mSOD1 mice as disease progressed (Philips et al., 2010). Although M2 microglia have been studied to a lesser extent than M1 microglia in ALS models, current evidence indicates that M2 microglia may protect motor neurons through increased production of neurotrophic and anti-inflammatory factors (Fig. 1).

Our recent studies in mSOD1 mice provided direct evidence of the plasticity of activated microglia in ALS. During the early slowly progressing phase of disease, the M2 markers, CD206 and Ym1, were up-regulated in the spinal cords of mSOD1 mice, indicating that microglia display an M2 phenotype at an early stage of disease. As disease transformed from a slowly progressing to a rapidly progressing phase, mSOD1 microglia in the spinal cords of mSOD1 mice adopted an M1 phenotype with increased levels of NOX2 and IL-1β (Beers et al., 2011b). Further study using isolated adult microglia from spinal cords of mSOD1 mice confirms this conclusion, namely M2 microglia are prevalent in the early slow phase while M1 microglia predominate during the late rapid phase. More importantly, when co-cultured with motor neurons, early stage M2 microglia enhanced motor neuron survival. In contrast, late stage M1 microglia were toxic to motor neurons (Liao et al., 2012). These data demonstrate the dual phenotypic and functional characteristics of microglia over the course of disease. During the early stage of motor neuron injury in ALS models, the surveying microglia exhibit an M2 phenotype and react to the signals (probably CD200 and fractalkine) with release of cytokines and trophic factors to promote repair and regeneration (Appel et al., 2010). However, as disease progresses, the injured motor neurons release “danger signals,” possibly misfolded oxidized proteins, that induce microglia to release ROS and pro-inflammatory cytokines and display an M1 phenotype. These pro-inflammatory cytokines are able to activate microglia to even higher extent through a self-propagating cycle of injury leading to excessive M1 neurotoxicity (Fig 2). Therefore, microglia can serve either neuroprotective or neurotoxic functions, depending on different states of activation elicited by local regulatory signaling at different stages of disease. Therapeutic approaches that broadly suppress both M2 and M1 microglia may reduce their protective properties and might not be an effective way to counter the neurodegenerative process. This dual role of microglia provides a potential explanation for why inhibition of microglial proliferation in mSOD1 mice did not change disease course. Therefore, potentially beneficial treatment

strategies need to be crafted to maintain microglia in an M2 state and to delay the M2 to M1 transformation.

The Role of Monocytes/Macrophages

Blood-spinal cord barrier disruption and altered pericytes are present in ALS patients (Winkler et al., 2013). Infiltration of peripheral immune cells has been documented in ALS. Several lines of evidence demonstrate the presence of infiltrating monocytes/macrophages in spinal cords of ALS mouse models. Studies of bone marrow transplantation in irradiated mSOD1 mice indicated that peripheral myeloid cells may be recruited into spinal cord and contribute to microgliosis (Philips and Robberecht, 2011). A recent study thoroughly investigated the changes of monocytes and their roles in mSOD1 mice (Butovsky et al., 2012). Splenic Ly6C^{high} monocytes exhibited a pro-inflammatory M1 phenotype two months prior to disease onset and during disease progression. These monocytes also expressed high levels of CCR2, the chemokine receptor for CCL2 (MCP-1, a monocyte chemoattractant). Meanwhile, CD39^{+} resident microglia in spinal cord had prominent expression of CCL2 and other chemotaxis-related molecules. Accordingly, we also demonstrated that CCL2 mRNA and immunoreactivity were upregulated in the neurons and glial cells of ALS mice early in disease (Henkel et al., 2006). The chemoattraction especially through CCL2-CCR2 appeared to be responsible for recruiting inflammatory Ly6C^{high} monocytes into the CNS. With disease progression, infiltrated Ly6C^{high} monocytes were reported to proliferate and become the major monocyte population in the spinal cord while CD39^{+} resident microglia were reported to undergo apoptosis. Importantly, treatment with an anti-Ly6C antibody exerted a beneficial effect on mSOD1 mice by delaying disease onset and prolonging survival. Anti-Ly6C antibody treatment attenuated neuronal loss by both down-regulating inflammatory splenic Ly6C^{high} monocytes and decreasing infiltration of these monocytes into the spinal cord (Butovsky et al., 2012). This study suggests that peripheral proinflammatory monocytes that enter the CNS rather than resident activated parenchymal microglia are the critical cells aggravating motor neuron injury. However, there are alternative explanations such as the possibility that peripheral monocytes entered the CNS because of prior irradiation, and that parenchymal microglia were still present but had lost expression of CD39. These possibilities mandate the need for additional studies to determine the relative role of peripheral monocytes and CNS microglia in mediating motor neuron cell death.

During neurodegeneration in ALS, dysfunction occurs not only in the cell bodies of motor neurons in CNS, but also in motor axons in the periphery. In mSOD1 mice, denervated endplates and motor axon degeneration are among the earliest pathological changes (Fischer et al., 2004). These alterations of neuromuscular junction and axons are accompanied by a robust response of peripheral macrophages detectable along the length of degenerating nerve fibers in ventral roots, sciatic nerves, and muscles at presympamatic stages, and increased during the course of disease (Chiu et al, 2009). In our own study, nerve degeneration occurred prior to macrophage infiltration and activation, indicating that inflammation in the PNS is in response to the neurodegenerative process, but did not initiate denervation (Kano et al., 2012). CCL2 was up-regulated and IgG, IgM, and complement deposition were also detected in sciatic nerves of mSOD1 mice concomitant with macrophage accumulation,

suggesting that they may regulate macrophage recruitment and activation in the altered nerve fibers (Chiu et al, 2009). The primary role of macrophages is thought to be the phagocytic removal of debris following axonal degeneration (Chiu et al, 2009). In addition, HLA-DR has been shown to be up-regulated on peripheral monocytes/macrophages in ALS patients (Holmoy, 2008). Thus, after digesting axonal debris, macrophages are capable of presenting antigens, but whether they do so in ALS models and ALS patients has not been definitively documented.

Although infiltrating monocytes/macrophages in CNS may contribute to motor neuron loss as previously described, there is evidence suggesting that peripheral macrophages may also exert neuroprotection under certain conditions.Kang et al. (2007) showed that while knocking-out MyD88 in mSOD1 mice did not influence the disease course, administration of MyD88-deficient bone marrow cells accelerated disease onset and reduced survival. MyD88 is the downstream signaling molecule of Toll-like receptors, which play a key role in the immune responses of macrophage. These results indicate that peripheral cells expressing MyD88 may have beneficial effects, while MyD88 in CNS cells may be involved in toxic signal transduction. In the PNS, recruited macrophages have been shown to participate in axon regeneration and functional recovery after sciatic nerve injury (Barrette et al., 2008). Similar beneficial effects of macrophages may apply in ALS since re-innervation of motor nerve branches was observed as disease developed in mSOD1 mice (Schaefer et al., 2005).

The Role of astrocytes

Astrocytes comprise the largest glial cellular component in the CNS. Reactive astrogliosis has been implicated in neurotoxicity and ALS disease progression, and has been thoroughly reviewed and will not be discussed here. Instead, we will focus on their role in neuroinflammation. Although astrocytes are not immune cells per se, they actively contribute to the immune response and motor neuron degeneration in ALS. Analyses of astrocytes in postmortem tissue from both familial and sporadic ALS patients revealed a set of 22 upregulated genes encoding chemokines, proinflammatory cytokines, as well as components of the complement cascade (Haidet-Phillips et al., 2011). Human mSOD1 astrocytes were shown to induce motor neuron toxicity that was correlated with an increased astroglial inflammatory response; mSOD1 astrocytes up-regulated NOX2 to produce superoxide, and the NOX2 inhibitor, apocynin, prevented motor neuron loss caused by mSOD1 astrocytes (Marchetto et al., 2008). Another group transplanted astroglial precursor cells into spinal cord of mSOD1 mice to establish healthy astroglial pools, which resulted in extended survival, attenuated motor neuron loss, and improved motor function (Lepore et al., 2008). Most importantly, reduced microgliosis was observed in these transplanted mice, suggesting that astrocytes may modulate the immune response elicited by microglia. Thus, astrocytes participate in immunological and inflammatory events, which ultimately mediate motor neuron toxicity in ALS.

The default role of astrocytes is to maintain and nourish neurons in CNS (Fig. 1). Release of neurotrophic factors (NTFs) and uptake of excess glutamate from the synaptic clefts are two major beneficial functions of astrocytes at early stages of disease. Astrocytes also release the

glutathione precursor (CysGly), which is utilized by motor neurons to synthesize the antioxidant glutathione to provide significant protection against oxidative stress (Dringen et al., 1999). Astrocytes can also help suppress cytotoxic microglial activation by releasing TGF-β (Liu et al., 2011). However, IL-1β, free radicals from M1 microglia, and ATP can promote astrocytic activation. Upon activation, astrocytes produce insufficient neurotrophic factors and down-regulate GLT1/EAAT2 transporter, which impairs glutamate clearance (Fig. 2). Activated astrocytes also release presently unidentified factors which are toxic to motor neurons.

The Role of dendritic cells

Immature and activated/mature blood-derived dendritic cells (DC) are present in ventral horn and corticospinal tracts of ALS patients as well as spinal cord of late symptomatic mSOD1 mice (Henkel et al., 2006; 2004). DC are highly involved in ongoing immune/inflammatory reactions; immature DC can take up and process antigen. As DC mature, they lose their phagocytic properties and their ability to capture antigen, but express MHC class II as well as costimulatory molecules and become potent antigen-presenting cells. The evidence that rapidly progressing ALS patients expressed significantly more dendritic transcripts than the slower progressing patients and the increase of DC markers in late symptomatic mSOD1 mice indicates that DC may be involved in accelerating disease (Henkel et al., 2006; 2004). In addition, degenerating and electrically silent motor neurons express MHC class I and induce MHC class II on surrounding glial cells (Neumann, 2001). Moreover, microglia from mSOD1 mice significantly up-regulate the dendritic markers, CD86, CD54 as well as MHC class II, suggesting mSOD1 microglia acquire dendritic features (Chiu et al., 2008). All of these studies indicate that the fundamental conditions for efficient antigen presentation may be present in degenerating areas of ALS CNS tissues. However, the exact functions of DC and DC-like microglia in ALS, especially their interactions with T-cells, and the ALS-specific antigens, have not yet been identified.

Adaptive Immunity in ALS

The Role of T Lymphocytes

Although investigations linking ALS and inflammation have mostly concentrated on microglia and innate immunity, T lymphocytes of the adaptive immune response are also present in areas of neurodegeneration. At early stage of disease, only $CD4^+$ T-cells are observed in lumbar spinal cords of mSOD1 mice; Even at end stage, $CD4^+$ T-cells still predominate, accounting for approximately 60% of the total T-cell population while remaining T-cells are CD8 positive (Beers et al., 2008; Chiu et al., 2008; Henkel et al 2006). The fact that $CD4^+$ T-cells first enter at an early stage of disease and increase as disease progresses, suggests that they are active participants throughout the course of disease in ALS mice.

To confirm a role for T-cells in mSOD1 transgenic mice, we bred mSOD1 mice with Rag2 knockout mice lacking functional T-cells. In these double transgenic mice, disease course accelerated and the mice died significantly earlier, indicating that T-cells are neuroprotective (Beers et al., 2008). We observed similar results when we crossed mSOD1 mice with CD4

knockout mice, indicating that $CD4^+$ T-cells contribute to the prolongation of disease duration (Beers et al., 2008). Our results were confirmed by breeding the mSOD1 mice onto a T-cell receptor β chain deficient background; the specific ablation of T-cells led to accelerated disease progression and significantly shorter lifespans (Chiu et al., 2008). The participation of $CD4^+$ T*cells has been further supported by the evidence that the passive transfer of *ex vivo* activated $CD4^+$ T-cells improves neurological function and lifespan of mSOD1 mice (Banerjee et al. 2008). Therefore, adaptive immune responses mediated by $CD4^+$ T-cells serve an important neuroprotective function in the ALS mouse.

The neuroprotective immunity associated with $CD4^+$ T-cells is mediated by interactions with microglia and astrocytes. When T-cells are depleted in mSOD1 mice, the more aggressive disease course was accompanied by increased proinflammatory cytokines and NOX2, and decreased levels of trophic factors and glutamate transporter; bone marrow transplantation reconstituted mice with functional T-cells, restored neuroprotective factors, and reduced toxic proinflammatory responses (Beers et al., 2008). Chiu et al (2008) also reported that deficiency of T-cells in mSOD1 mice resulted in decreased IGF-1 expression in microglia. These results suggest that $CD4^+$ T-cells provide neuroprotection by modulating the trophic/cytotoxic balance of glia.

Our recent studies systematically evaluated the dynamic changes of the $CD4^+$ subsets, Th1, Th2, Th17, and regulatory T-cells (Tregs) during progression of disease in mSOD1 mice. In lumbar spinal cords of mSOD1 mice at early slowly progressing stages, Tregs were increased accompanied by increased levels of IL-4, IL-10, and M2 microglia, and then decreased when disease rapidly accelerated. During the rapid stage of disease there was an increased expression of mRNA for toxic Th1 cells and decreased expression of mRNA for Th2 cells; microglia were predominantly M1 (Fig. 3). IL-17 expression was not detected at any time in the course of disease, suggesting that Th17 may not be involved in the pathogenesis of ALS (Beers et al., 2011b). At early stages of disease in mSOD1 mice, we observed increased expression of IL-4 and IL-10, suggesting that these cytokines were able to skew microglia towards an M2 phenotype. Although Th2 cells are one of the major sources of IL-4, Th2 are not increased in the lumbar region of ALS mice spinal cords. Tregs cells are the likely source of IL-4; our own studies as well as those from other laboratories have documented that Tregs are capable of releasing IL-4 and have the potential to maintain the protective M2 phenotype (Beers et al., 2011b; Tiemessen et al., 2007). In addition, passive transfer of early phase mSOD1 Tregs prolonged the slow phase of mSOD1 mice, augmented M2 markers, and suppressed M1 markers and their pro-inflammatory cytokines (Beers et al., 2011b). We further demonstrated that Tregs, through their secretion of IL-4, can directly suppress the toxic properties of microglia (Zhao et al., 2012); we have previously shown that suppressing the toxic attributes of microglia leads to prolonged survival in this model of ALS (Beers et al., 2008). Moreover, M2 cells also have the ability to induce $CD4^+$ Tregs with a strong suppressive function (Savage *et al*., 2008). Our data also demonstrated that mSOD1 microglia induced more IL-4 expressing Tregs isolated from mSOD1 mice (Zhao et al., 2012). Thus, Tregs during the early stages of disease are immunocompetent and actively contribute to neuroprotection through their interactions with microglia. As disease progresses, a transformation occurs from a supportive Tregs/M2

response to an injurious Th1/M1 response. It is known that Th1 cells produce IFN-γ, which promotes M1 microglial activation. Conversely, M1 cells can promote proliferation and function of Th1 cells (Gao and Tsirka, 2011). This vicious cycle is believed to be a significant driving force for acceleration of disease course. Therefore, the dialogue between T-cells and microglia modulates their phenotypic profiles and subsequently drives disease progression (Fig. 4).

When disease entered the rapidly progressing stage, transplantation of Treg cells could not reverse the acceleration of disease (Beers et al., 2011b). What causes the transformation and dysfunction of Tregs remains unknown, but cytokines released from activated microglia, astrocytes, and T-cells are likely candidates. Both Th1 cells and M1 microglia release large amounts of TNF-α, and TNF-α has been recently demonstrated to induce the dysfunction of Tregs by inhibiting phosphorylation of FoxP3, a functional marker of Tregs (Nie et al., 2013). IL- 1β was required to drive the conversion of Tregs to Th17-producting cells (Li et al., 2010). Moreover, IL-6 has been reported to inhibit the generation of $FoxP3^+$ Tregs (Bettelli et al., 2006). In our studies of the mSOD1 mouse model, the transition from protection to toxicity coincided with increased expression of IL-6 (Beers et al., 2011b); IL-6 can be produced by activated microglia, astrocyte, and Th1 cells. Nevertheless it is unlikely that any single cytokine such as IL-6 is solely responsible for the transformation from neuroprotective Treg/Th2/M2 cells to cytotoxic Th1/M1cells, and it appears much more likely that multiple pro-inflammatory cytokines mediate the transition (Fig. 4).

Although Th2 cells did not increase in lumbar spinal cord, more Th2 cells exist in the cervical spinal cord where disease starts later and progresses more slowly than in lumbar cord (Beers et al., 2011a). Th2 lymphocytes infiltrate the cervical region of ALS mice with enhanced IL-4; Tregs were also increased and sustained at elevated levels for a longer period in cervical than lumbar cord, suggesting that the higher levels of IL-4 in cervical region are attributable to both Tregs and Th2. The protective M2 response is maintained in the cervical spinal cords of these mice even when disease progressed rapidly. Thus, distinctly different regional and temporal neuroinflammatory responses are present in lumbar cord compared with the cervical cord, and may account for the earlier loss of function in hindlimbs than forelimbs.

The documented changes in T-cells in mSOD1 mice were also present in the ALS patient population. Diminished levels of naïve (CD45RA) T-cells and increased levels of memory (CD45RO) cells within the $CD4^+$ T-cell subset were reported in peripheral blood of ALS patients (Banerjee et al.2008). In our own study, definite T-cell alterations were present in the peripheral blood and spinal cord tissues of ALS patients (Henkel et al., 2013). The numbers of $CD4^+CD25^{high}$ Tregs, leukocyte FoxP3 (functional Treg marker), and CD25 mRNA levels were reduced in ALS patients with rapidly progressing disease. Similarly, leukocyte Gata3, TGF-β and IL-4 mRNA levels were also reduced in rapidly progressing patients. The levels of these Tregs and Th2 markers inversely correlated with rate of disease progression. Similar results on peripheral Tregs were reported in a recent study (Rentzos et al., 2012). Furthermore, in postmortem spinal cord tissues of ALS patients, Tbx21 levels (transcription factor of Th1), IFN-γ, and NOX2 levels were upregulated in rapidly progressing patients and FoxP3 expression was decreased (Henkel et al., 2012). These data

suggest that decreased Tregs/Th2 and enhanced Th1/M1 cells contribute to rapid disease progression. Most significantly, low leukocyte FoxP3 mRNA levels early in disease predicted a rapid progression and reduced survival. For the first time we may be able to predict rapid progression of disease in ALS patients, and such predictive ability may be of value in stratification for enrollment in clinical trials.

Another population of T-cells that participates in the systemic immune pathology of ALS is Natural Killer T (NKT) cells, which share properties of both T-cells and NK cells. NKT cells recognize lipids and glycolipids presented by CD1d molecules. Upon activation, these cells modulate different immune responses by rapidly releasing cytokines, such as IL-2, IFN-γ, TNF- α, IL-13, and IL-4. It has been shown that NKT cells were increased in peripheral blood of ALS patients (Rentzos et al., 2012). NKT cell levels and activation state were also increased in the spinal cords, spleens, and livers of mSOD1 mice. After treatment with PBS57, a ligand analog that induced hypo-responsiveness of NKT cells, mSOD1 mice had a delayed onset and extended lifespan as well as a reduction of motor neuron loss and astrogliosis (Finkelstein et al., 2011). The evidence of early recruitment of T-cells to the spinal cord after down-regulation of NKT cell activity by PBS57 suggests that NKT cells may contribute to the suppression of protective T-cell responses in ALS. Clearly, further studies are required to determine the role of NKT cells in the pathogenesis of ALS.

The Role of B-cells

B-cells are also significant components of immune system. In addition to antibody secretion, B-cells also function in antigen-presentation, immune suppression, and cytokine production. Auto-antibodies to neural antigens have been identified in CSF and serum of ALS patients (Niebroj-Dobosz et al., 2006). However, it is still unclear if these auto-antibodies have a pathological role in motor neuron degeneration or represent a secondary immunological consequence of neuronal death. A recent study demonstrated that the lack of mature B-cells did not change disease development in mSOD1 mice, arguing against an essential role for B-cells in ALS (Naor et al., 2009). Nevertheless, more detailed investigations are needed to determine whether B-cells contribute to the pathogenesis and progression of ALS.

Conclusion

Neuroinflammatory responses can be beneficial or harmful to motor neuron survival. These distinct effects are elicited by the different activation states of microglia/macrophages and astrocytes, and are modulated by infiltrating T-cells. The release of signals from motor neurons represents the earliest stages of ALS with microglia responding as an M2 phenotype, releasing neuroprotective factors to repair motor neurons and protect against further injury. As disease burden increases, motor neurons release “danger signals” that transform microglia from protective M2 to cytotoxic M1 phenotypes. M1 microglia release pro-inflammatory cytokines that cause astrocytic dysfunction, and enhance motor neuron degeneration. The activated glias also recruit peripheral monocytes/macrophages as well as T-cells to the injured cord. At the early stages, increased Tregs and Th2 cells maintain M2 neuroprotection and inhibit Th1/M1 neurotoxicity, and stabilize the progression of disease. However, with accelerating injury and a rapidly changing cytokine milieu, the balance

changes towards reduced Tregs/Th2/M2 and enhanced Th1/M1 cells, leading to exacerbation of disease. Therapeutic regimens that can down-regulate the harmful responses of innate and adaptive immune cells (M1 and Th1), and up-regulate the beneficial responses (M2 and Treg) may slow the progression of ALS and provide meaningful hope for our patients in the future.

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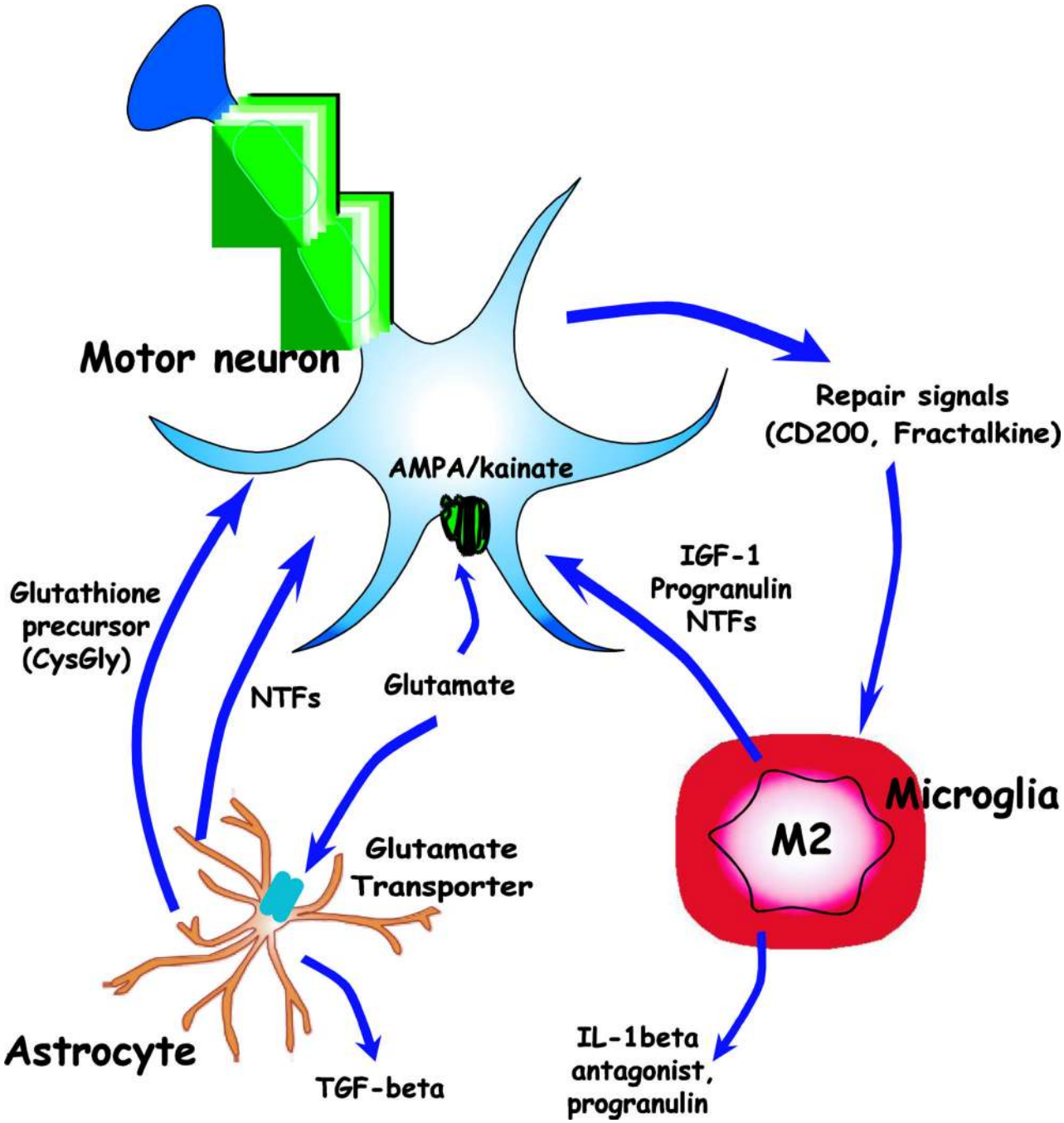

Figure 1.

The neuroprotective roles of microglia and astrocytes at early stages of disease. Abnormalities in motor neurons may initiate ALS disease and release repair signals (probably CD200 and fractalkine), which promote an alternatively activated (M2) microglial phenotype. M2 microglia are able to secrete high levels of neurotrophins, such as IGF-1, progranulin, and other neurotrphic factors (NTFs), thereby exerting a neuroprotective function. M2 microglia also release anti-inflammatory cytokines, including IL-1R antagonists and progranulin, to block proinflammatory responses. Astrocytes also participate

in the neuroprotective process with secretion of NTFs and uptake of excess glutamate from synaptic clefts. In addition, astrocytes enhance the antioxidant capacity of neurons by releasing the glutathione precursor (CysGly) which is taken up by motor neurons for the synthesis of glutathione. Thus, at early stages, both M2 microglia and astrocytes are involved in sustaining motor neuron health.

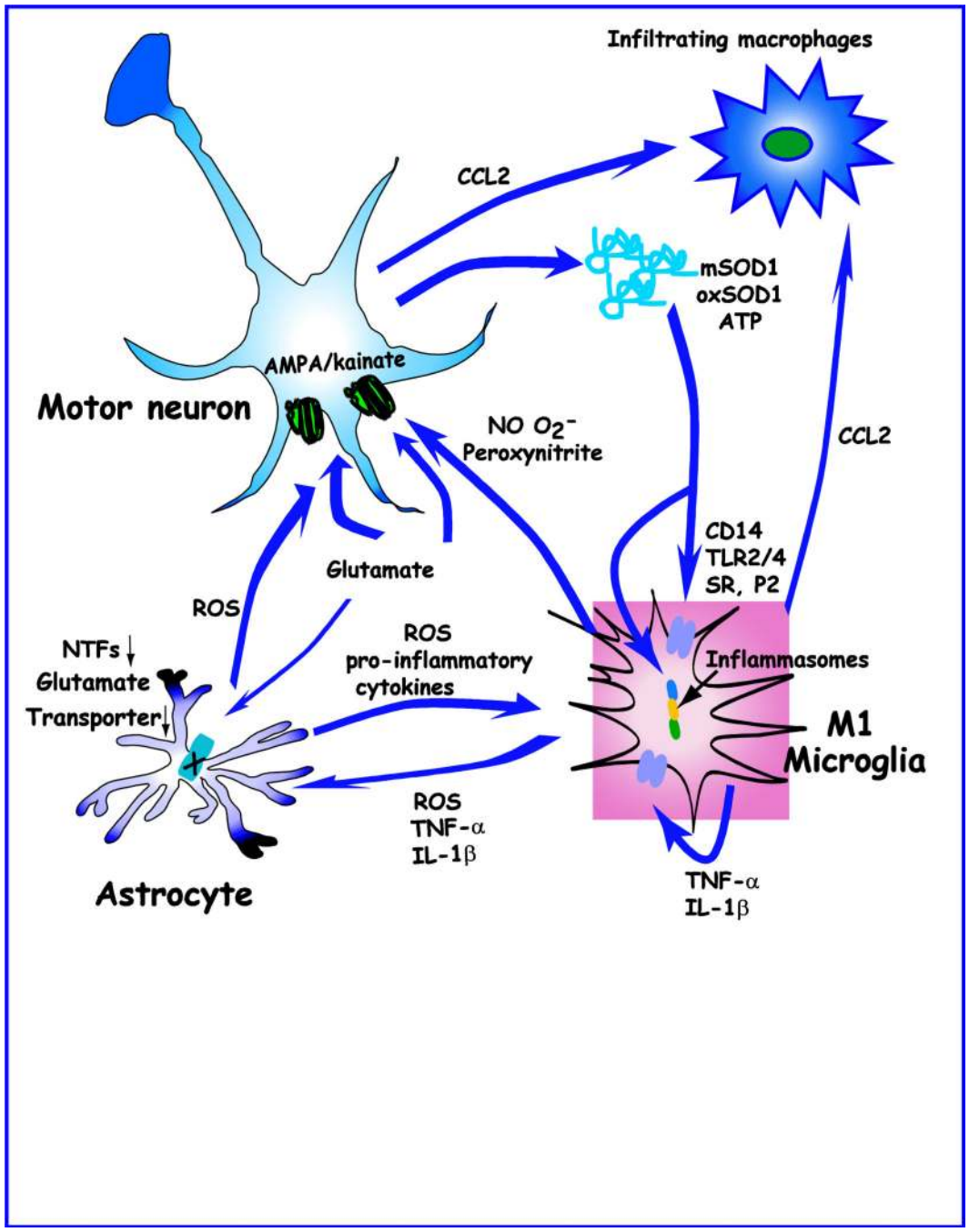

Figure 2.

The neurotoxic roles of microglia and astrocytes at rapidly progressing stages of disease. As disease progresses, "danger signals" released from motor neurons [possibly mSOD1 or oxidized SOD1 (oxSOD1) and ATP) induce M1 activation of microglia through CD14/ TLRs, scavenger receptors (SR), and purinergic P2 receptors. These misfolded proteins may also be endocytosed into microglia to activate inflammasomes. The result of this signaling is a transformation of microglia from an M2 to an M1 phenotype, which produces proinflammatory cytokines (TNF-α and IL-1β, etc), and promotes neurotoxicity by releasing

free radicals, including nitric oxide (NO), and superoxide ($O_2^{\cdot -}$). NO and $O_2^{\cdot -}$ form the more potent toxin peroxynitrite, which sensitizes AMPA/kainate receptors and increases motor neuron vulnerability to glutamate toxicity. M1 microglia also promote astrocyte activation through ROS and pro-inflammatory cytokines. Activated astrocytes acquire deleterious inflammatory phenotypes with release of ROS, pro-inflammatory cytokines, which, in turn, induce further microglial activation. Thus, ROS production, insufficient NTFs, and impaired glutamate clearance due to down-regulation of GLT1/EAAT2 transporter are the major neurotoxic components of activated astrocytes. Additional unidentified neurotoxic factors released from astrocytes have also been implicated. In addition, M1 microglia, activated astrocytes, and motor neurons produce CCL2, which attracts peripheral M1 monocytes/macrophages into the CNS, and further exacerbates motor neuron degeneration.

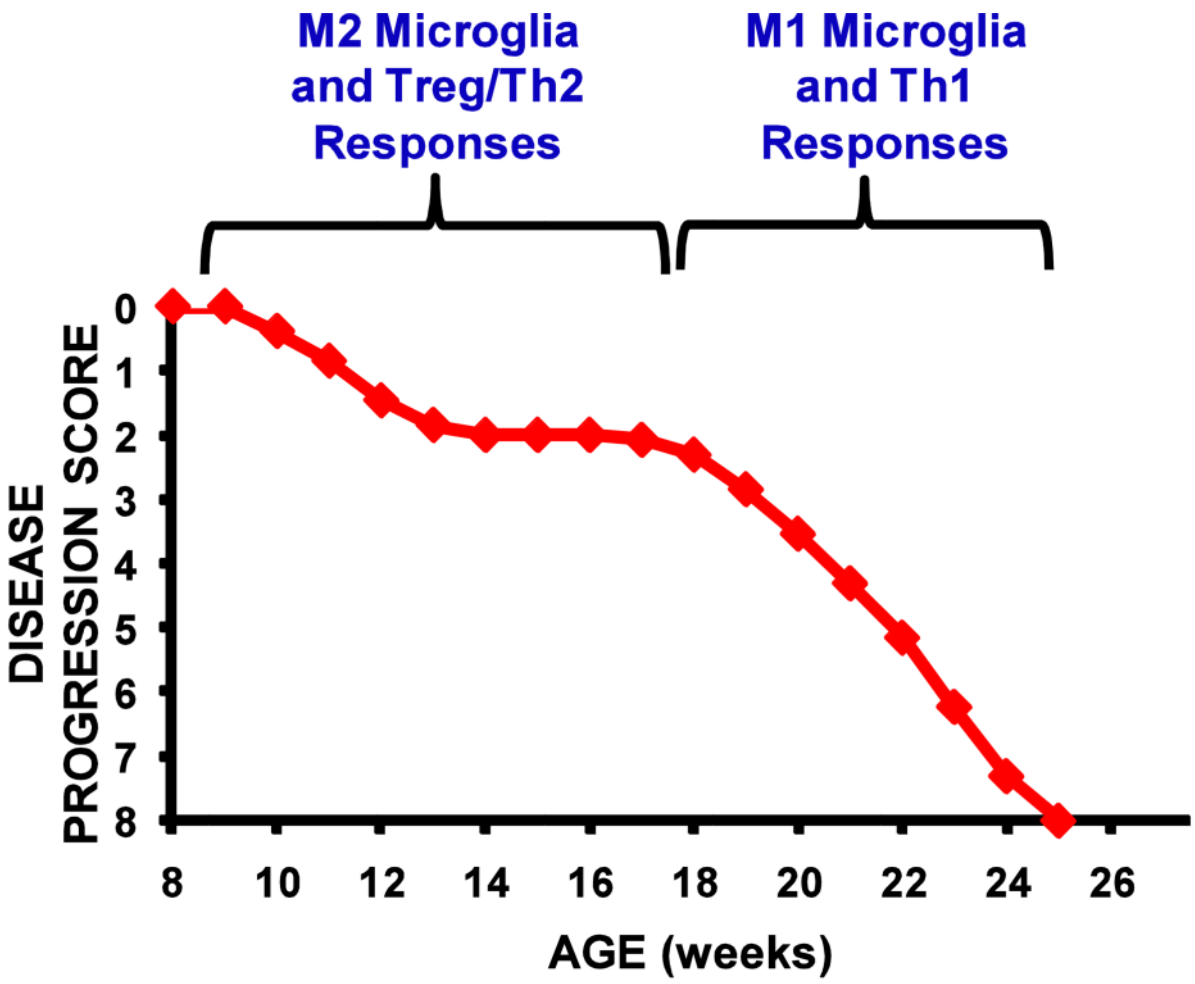

Figure 3.

In ALS mice, the disease progression curve can be divided into two stages. The early stable phase is associated with beneficial responses of M2 microglia and T regulatory/Th2 cells, while the later rapidly progressing stage is associated with injurious M1 microglia and Th1 responses.

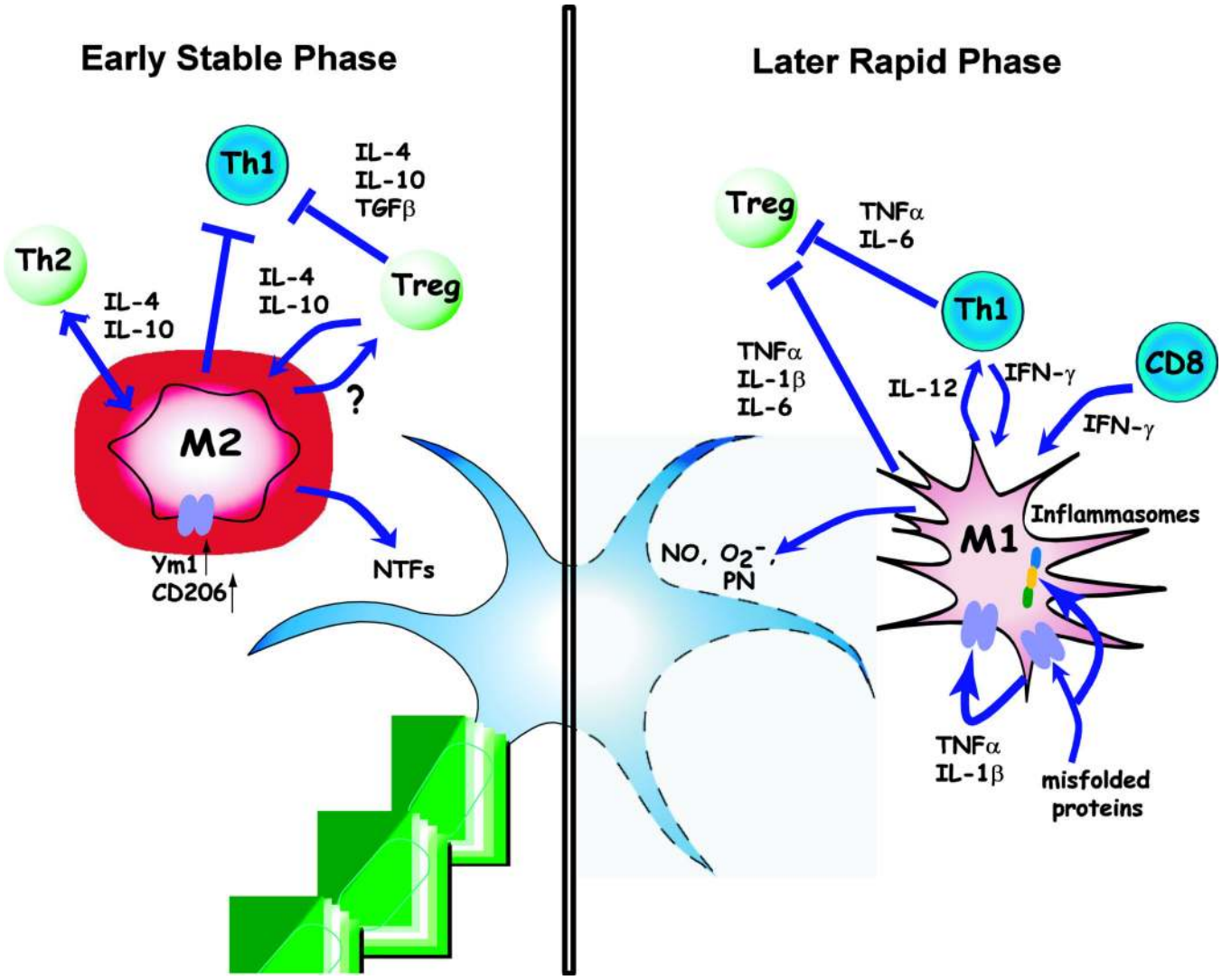

Figure 4.

Microglia and T-cell dialogues at different stages of disease. Tregs and Th2 cells predominate at early stable stages of disease. They release anti-inflammatory cytokines, such as IL-4, IL-10, and TGF-β. IL-4 and IL-10 promote an M2 phenotype, characterized by enhanced neurotrophic factors (NTFs) and up-regulation of Ym1 and CD206. M2 microglia in turn promote Treg and Th2 differentiation. In addition, Tregs and M2 microglia also suppress proliferation and cytotoxic function of Th1 cells. Overall, the immune responses at early stages favor neuroprotection through NTFs released from M2 microglia. As disease progression accelerates, the released misfolded proteins and self-propagation give rise to more M1 microglia. The interaction between Th1 and M1 further enhances pro-inflammatory responses, including the release of TNF-α, IL-6, and IL-1β, and downregulated Treg suppressive functions; the beneficial effects of M2 and Tregs/Th2 are lost and the detrimental effects of the Th1 and M1 cells become predominant, enhancing neurodegeneration.