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Abstract.

Because microglial cells, the resident macrophages of the CNS, react to any lesion of the nervous system, they have for long been regarded as potential players in the pathogenesis of several neurodegenerative disorders including Amyotrophic Lateral Sclerosis, the most common motor neuron disease in the adult. In recent years, this microglial reaction to motor neuron injury, in particular, and the innate immune response, in general, has been implicated in the progression of the disease, in mouse models of ALS. The mechanisms by which microglial cells influence motor neuron death in ALS are still largely unknown. Microglial activation increases over the course of the disease and is associated with an alteration in the production of toxic factors and also neurotrophic factors. Adding to the microglial/ macrophage response to motor neuron degeneration, the adaptive immune system can likewise influence the disease process. Exploring these motor neuron-immune interactions could lead to a better understanding in the physiopathology of ALS to find new pathways to slow down motor neuron degeneration.

Keywords: neuroinflammation; microglia; T lymphocytes; macrophages; cytokines; neurodegeneration; immune system.

Introduction

Amyotrophic Lateral Sclerosis (ALS) is a fatal neurodegenerative disease of the adult characterized by degeneration of motor neurons in the spinal cord, brain stem and motor cortex. Progressive muscle atrophy and weakness are the main clinical features of the disease leading to paralysis and death of the patient in mean 3-5 years after the onset of disease, usually around the age of 45-60. Death of the patients is generally due to respiratory failure linked to denervation of the respiratory muscles and diaphragm. Although the cause of the disease is currently unknown, several hypotheses including oxidative stress, protein aggregation, mitochondrial dysfunction, glutamate excitotoxicity and neuro-immune reactions are believed to participate in the motor neuron degenerative process (Boillee et al. 2006a). The majority of ALS cases are sporadic, and 10% are inherited (familial), but sporadic and familial ALS produces similar pathological hallmarks, including an immune response in the affected tissues. This reaction includes activation of astrocytes, microglia (the macrophage of the CNS) as well as lymphocyte infiltration and, therefore, implicates both the innate and adaptive immune system. Despite the important efforts to find new therapies to ALS, including targeting the immune system, very limited options are available today. This highlights the need to better understand the processes implicated, especially in the progressive phase of the disease to slow down the course of motor neuron degeneration. We review here the different faces of the immune response in ALS including what has been learnt so far from the rodent models and verified or not in human ALS patients concerning these neuroimmune interactions, the immune factors potentially implicated in motor neuron degeneration and the involvement of microglia/macrophages and the adaptive immune system on the disease progression.

SOD1 mutations and rodent models of ALS

Mutations in the Cu/Zn superoxide dismutase (or SOD1) gene are still the ones most frequently found in familial ALS (20% of inherited ALS, Rosen et al. 1993). As of today, more than 130 mutations have already been described in the human SOD1 gene involving all the regions of the 154 amino acid long protein. Beside for a few exceptions, mutations in the SOD1 gene are of dominant character and lead to motor neuron death through a gain of toxic function of the protein. Indeed, a large fraction of SOD1 mutants retain the dismutase activity of the enzyme (which functions as a radical oxygen species-superoxide scavenger) and patients carrying either a dismutase active or inactive form of the enzyme develop ALS with similar phenotypes. In addition, studies in mice showed that deleting the mouse endogenous SOD1 gene did not lead to ALS phenotypes (Reaume et al. 1996) while the transgenic expression of different forms of mutant human (or mouse) SOD1 in mice and rats recapitulated several features of the disease (Gurney et al. 1994; Ripps et al. 1995; Wong et al. 1995; Bruijn et al. 1997; Howland et al. 2002; Wang et al. 2002) that were not observed when expressing the human wild-type SOD1 at similar levels (Gurney et al. 1994; Wong et al. 1995). These mouse and rat lines expressing different forms of SOD1 represent, therefore, valuable tools and are currently the broadly used model to study ALS. However, the recent discoveries of new mutated genes involved in ALS like *TAR DNA-binding protein* (TDP-43)

and *fused in sarcoma/translated in liposarcoma* (FUS/TLS) (Gitcho et al. 2008; Kabashi et al. 2008; Rutherford et al. 2008; Sreedharan et al. 2008; Chow et al. 2009; Kwiatkowski et al. 2009; Vance et al. 2009), bring new comparative opportunities for designing novel animal models for studying the neurodegenerative processes occurring in ALS (Wils et al. 2010; Zhou et al. 2010; Wegorzewska et al. 2009).

ALS, a non-cell autonomous disease

Although death of motor neurons is responsible for ALS, important discoveries of the last years led to the concept that motor neuron degeneration is non-cell autonomous and cells other than motor neurons, for instance glial cells, actively participate in the neurodegenerative process (Fig 1) (Boillee et al. 2006a; Ilieva et al. 2009). First transgenic mice generated to express mutant SOD1 only in motor neurons did not show evidence of neurodegeneration (Pramatarova et al. 2001; Lino et al. 2002) and a second generation of the same type of mice had to be bred homozygously to increase the level of mutant SOD1 expressed in motor neurons to show a late onset motor neuron disease (Jaarsma et al. 2008) suggesting that mutant SOD1 expressed in other cells besides motor neurons could participate to the progression of the typical ALS phenotype in mice. However, selective expression of mutant SOD1 in astrocytes alone, although leading to astrogliosis (Gong et al. 2000), or in microglial cells alone (Beers et al. 2006) was not sufficient to induce motor neuron death suggesting the need for mutant SOD1 expressed by motor neurons and a cooperation between multiple cell types to induce motor neuron degeneration. The participation of cells in the neighborhood of motor neurons to neurodegeneration in ALS mouse models was shown using chimeric mice whose motor neurons expressing mutant SOD1 survived longer when surrounded by wild-type cells (Clement et al. 2003; Yamanaka et al. 2008a). However, the type of cells participating to the disease could not be identified in the chimeric mice and a Cre/Lox approach revealed that decreasing mutant dismutase active SOD1 ($SOD1^{G37R}$) levels in motor neurons (using Islet-Cre, Boillee et al. 2006b or VAChT-Cre, Yamanaka et al. 2008b mice) or decreasing dismutase inactive SOD1 in motor neurons and interneurons ($SOD1^{G85R}$, using Lhx3-Cre mice, Wang et al. 2009b) led to a delayed onset of the disease. Conversely, downregulating the amount of $SOD1^{G37R}$ or $SOD1^{G85R}$ in microglial cells and macrophages (CD11b-Cre, Boillee et al. 2006b; Wang et al. 2009b) or $SOD1^{G37R}$ in astrocytes (Yamanaka et al. 2008b) actively participated in slowing down the progression of the disease. Looking at other cell types that surround motor neurons, downregulating the expression of the dismutase active mutant $SOD1^{G37R}$ in muscle cells or endothelial cells did not have an impact on the disease while in Schwann cells, the peripheral myelinating cells and the ones most in contact with motor neuron axons, it surprisingly accelerated the disease (Miller et al. 2006; Lobsiger et al. 2009; Zhong et al. 2009). Therefore, mutant SOD1 in Schwann cells does not seem to have a detrimental effect that could influence motor neuron survival; however, the dismutase activity of SOD1 in this particular cell type seems to be neuroprotective during the course of the neurodegeneration in ALS mice (Lobsiger et al. 2009; Turner et al. 2009). These studies highlight the importance of targeting therapeutic strategies using downregulation of SOD1 at the right cell type, avoiding Schwann cells.

A complementary experiment by the group of Stanley Appel used an alternative technique of bone marrow transplant to replace mutant SOD1 (dismutase active, $SOD1^{G93A}$) expressing microglia with wild-type microglia. To be able to replace the entire myeloid lineage, including microglial cells, they used the PU.1 knockout mouse (McKercher et al. 1996) which, from birth, lacks myeloid cells, therefore macrophages and microglia as well as mature lymphoid B cells and reduced number of lymphoid T cells. Crossing $PU.1^{-/-}$ mice with $SOD1^{G93A}$ mice allowed them to reconstitute the myeloid lineage in ALS mice with either control or $SOD1^{G93A}$ cells and to compare the progression of the disease in ALS mice with microglia/macrophages (and possibly lymphocytes) expressing or not mutant SOD1. The outcome was that microglia/macrophages expressing mutant SOD1 accelerated the progression of the disease (Beers et al. 2006).

Altogether, these studies show that glial cells in the environment of motor neurons have an impact on their survival and on the disease progression (Fig 1). Knowing that the majority of ALS cases are sporadic and therefore diagnosed after showing symptoms of the disease, being able to act on the progression of the disease would provide pathways for potential therapeutic tools. Microglial cells, astrocytes and neuroinflammatory processes appear as valuable candidates to target ALS disease progression (Fig 1).

Implication of astrocytes in ALS disease process

Astrocytes, the main macroglia of the CNS that are known for their major functions as synaptic glutamate uptaker and glucose/metabolites provider for neurons, have long been suspected to be implicated in ALS (Barbeito et al. 2004; Di Giorgio et al. 2007; Nagai et al. 2007; Lepore et al. 2008; Van Den Bosch and Robberecht 2008; Vargas et al. 2008; Yamanaka et al. 2008b). Astrocytes were first shown to be activated and to display a decreased expression of the glutamate transporter EAAT2 in patients and animal models of ALS (Rothstein et al. 1995; Howland et al. 2002; Pardo et al. 2006) favoring glutamate-induced excitotoxicity (Van Den Bosch et al. 2006). Further evidence for a role of astrocytes in ALS came from work on primary astrocyte cell cultures carrying mutant SOD1 and showing toxicity towards motor neurons (Vargas et al. 2006; Di Giorgio et al. 2007; Nagai et al. 2007). In addition, *in vivo* evidence for an active role of astrocytes in ALS pathology came from the CreLox experiment described above where decreasing the expression of mutant SOD1 specifically in astrocytes increases survival of $LoxSOD1^{G37R}$ mice (Yamanaka et al. 2008b). Reducing the mutant SOD1 expressing astrocytes as well as increasing the number of wild-type astrocytes focally in the cervical spinal cord of $SOD1^{G93A}$ rats by grafting wild-type lineage-restricted astrocyte precursors (that efficiently differentiated into astrocytes) could prolong lifespan (Lepore et al. 2008). These studies not only showed the implication of astrocytes in the disease process, but also that they could be potentially used to increase motor neuron survival. In this line, boosting astrocytic antioxidant defenses by overexpressing the transcription factor Nrf2 (which is downregulated in the spinal cord of ALS patients) extended the lifespan of $SOD1^{G93A}$ mice (Sarlette et al. 2008; Vargas et al. 2008). Although astrocytes could be taken as a part of the neuroinflammatory process, the scope of this review will remain on the innate and adaptive immune response in the nervous

system during motor neuron degeneration in ALS models and will, therefore, focus on microglia and lymphocytes.

Microglial cell activation as a neuroinflammatory sign in ALS

Microglial cells are the intrinsic immune effector cells of the CNS. Under physiological conditions, they were previously considered as "resting" but after observing them with live imaging *in vitro* and *in vivo*, they had to be renamed "surveying" since they are actively screening their microenvironment most likely watching for a potential intrusion and are therefore in constant communication with surrounding cells (Kreutzberg 1996; Davalos et al. 2005; Nimmerjahn et al. 2005; Hanisch and Kettenmann 2007). Upon activation, microglial response will depend on the nature, amount and length of the stimulus and generally includes cell proliferation, phagocytosis, release of neurotransmitters, pro-inflammatory cytokines and reactive oxygen species as well as anti-inflammatory cytokines and neurotrophic factors (Fig 1) (Streit 2002; Hanisch and Kettenmann 2007). Microglial cells have, therefore, the capacities to affect neuronal survival and were shown to actively promote neuron death during development (Marin-Teva et al. 2004). Microglial cell activation occurs after any injury of the CNS including neurodegenerative disorders (McGeer et al. 1993; McGeer and McGeer 2002). In ALS patients, microglial activation has been largely documented in the early nineties from postmortem affected tissues and involves, besides proliferation and morphological changes, an up-regulation of cell surface molecules, such as complement receptor 3, major histocompatibility complex (MHC) antigens I and II, integrins and reactivity to immunoglobulins (Lampson et al. 1990; Troost et al. 1990; Kawamata et al. 1992; McGeer and McGeer 1995). The finding of increased expression of microglia cell markers in ALS tissues, however, represent a "snap-shot" at the end-stage pathology and does not give information about evolution of microglial activation during the course of the disease. Evidence from microglia activation during disease progression came from animal models where it is already apparent at early stages of the disease and increases with disease progression up to end-stage in several ALS mouse and rat lines (Hall et al. 1998; Alexianu et al. 2001; Elliott 2001; Olsen et al. 2001; Fendrick et al. 2007; Lobsiger et al. 2007; Gowing et al. 2008). More recently, PET imaging coupled to [11C](R)-PK11195, a ligand for the peripheral benzodiazepine binding site, which is expressed by activated microglia, detected a widespread microglial activation in motor (motor cortex and pons) as well as extra-motor (dorsolateral prefrontal cortex and thalamus) cerebral regions and showed evidence of increased microglial activation with the severity of the disease in ALS patients (Turner et al. 2004). Besides being able to show microglial reaction in living patients, this tool could also provide ways to define if potential therapeutic drugs act on inflammation.

In addition to activated microglia, large dendritic cells were also observed in affected areas of the CNS of ALS patients in particular in proximity to motor neuron cell bodies in the spinal cord (Fig 1) (Lampson et al. 1990; Henkel et al. 2004). Dendritic cells are antigen-presenting cells that control both innate and adaptive immunity and may infiltrate the CNS through blood vessels, choroid plexus and/or meninges. Increased dendritic cell marker transcripts (DEC205, CD1a, CD11c, CD123, CD83 and CD40) were detected both in ALS patients and in late symptomatic or end-stage mice (Henkel et al. 2004; Gowing et al. 2006;

Henkel et al. 2006). Although dendritic markers can also be expressed by activated microglial cells, dendritic cells with their stellate shape were already visible before symptoms in ALS mouse spinal cords (Henkel et al. 2004; Gowing et al. 2006; Henkel et al. 2006). However, the origin of these dendritic cells in ALS mice and patients, as well as their protective or otherwise injurious function has not yet been established. Interestingly, in ALS patients, the level of dendritic cell transcripts was higher in fast-progressing cases than in slow progressing ones supporting the involvement of the adaptive immune system in the disease process (Henkel et al. 2004). Implication of the innate immune system was also shown in ALS mice by stimulating the innate immune reactivity in SOD^{G37R} mice through chronic i.p. injections of lipopolysaccharides (LPS), which led to a speed up of the disease (Nguyen et al. 2004). However, the mechanisms by which immune cells including microglia could favor neurodegeneration in ALS are still largely unknown and the participation of potentially infiltrating monocytes/macrophages still under debate.

Activated microglial cells in ALS mice are of endogenous origin

Microglial cells originate from phagocyte progenitors generated in hematopoietic tissues including bone marrow and that enter the CNS during development, through different potential routes that are the blood vessels, the ventricles or the meninges (Perry et al. 1985; Cuadros and Navascues 1998). Several experimental designs have been used to analyze microglial turnover during physiological conditions or after lesions of the CNS. To summarize, under normal conditions in adulthood, microglial turnover seems to come from both endogenous dividing microglial cells and infiltrating monocytes but is very low and therefore monocytes entering the CNS are rare (Ling et al. 1980; Lawson et al. 1992). CNS lesions that highly compromise the blood-brain barrier (BBB) like ischemia, stab wounds or the injection of a toxin, favor blood monocytes infiltration through the damaged vessel wall (Andersson et al. 1991; Marty et al. 1991; Leong and Ling 1992). The current issue is whether similar infiltrations occur during neurodegenerative diseases such as ALS especially since the blood spinal cord barrier has been shown to be disrupted in different mutant SOD1 mice and this even before motor neuron degeneration (Fig 1) (Garbuzova-Davis et al. 2008; Zhong et al. 2008, 2009). So far, there are still no available antibodies capable of discriminating macrophages coming from outside and microglial cells because they share the same monocyte markers. To follow potential infiltration of peripheral monocytes into the CNS, the broadly used technique is irradiation of mice to deplete them from their immune cells and replenishment of the cells by bone marrow transplantation of marked (usually green fluorescent protein (GFP) positive) cells (Flugel et al. 2001; Priller et al. 2001; Simard and Rivest 2004; Ajami et al. 2007). Several authors have used this technique in $SOD1^{G93A}$ mice trying to understand if infiltration of monocytes was happening in the spinal cord during the course of the disease and found some GFP-positive cells, therefore cells originating from outside of the CNS, but the overall proportion of these GFP-positive microglial cells over the totality of microglial cells was very low indicating that their contribution to the local pool of microglia was limited and even if it increased over the course of the disease, the proportion of GFP versus non-GFP microglia showed that microglial expansion was rather coming from proliferation than peripheral recruitment (Solomon et al. 2006; Chiu et al. 2009). The higher

proportion of GFP-positive microglial cells in sick ALS mice as compared to controls showed that motor neuron degeneration (either directly or through increased inflammation) induced more attraction of cells from the outside. However, most of the GFP-positive cells in the spinal cord seemed to be associated with blood vessels, and therefore represented perivascular microglia (the cells located between glia limitans and endothelial cells) rather than parenchymal microglia (Lewis et al. 2009).

Recent studies, however, have revealed that this experimental paradigm of irradiation and bone marrow transplantation could lead to certain artifactual effects and actually modify what is happening in mice during the course of the disease. Indeed, first, bone marrow transplant could change the type of monocyte precursors in the blood stream and therefore the type of cells entering the CNS could be different (Mildner et al. 2007). In addition, irradiation seems to be the process provoking entrance of cells in the CNS, most probably by altering the BBB tightness (Ajami et al. 2007; Mildner et al. 2007). Mice whose brain was protected from the irradiation showed that microglial turnover from the periphery did not happen while it was the case in non-protected CNS (Mildner et al. 2007). Furthermore, a very elegant experiment used parabiosis (joining the circulatory systems of two animals leading to peripheral blood exchange to obtain 50% chimerism) between an $SOD1^{G93A}$ mouse and a control mouse expressing GFP in bone marrow cells and showed no evidence of microglia coming from cells recruited from the bloodstream in parabiotic chimeras (Ajami et al. 2007).

Bone marrow transplant to replace mutant SOD1 expressing myeloid cells by wild-type cells

Whether bone marrow derived cells enter or not the CNS is a question that remains important for ALS especially with regard to the potential benefit to motor neuron survival of replacing mutant SOD1 expressing bone marrow by wild-type bone marrow. Grafting wild-type bone marrow cells in irradiated presymptomatic $SOD1^{G93A}$ mice led to conflicting outcomes of either increased survival or no benefit (Corti et al. 2004; Solomon et al. 2006). To explain the discrepancy obtained by the different groups, technical aspects could be taken into consideration including strength of irradiation, number of cells grafted, route of transplantation (i.p, i.v or intra-bone) and the rate of engraftment. Indeed, increasing the percentage of wild-type bone marrow cells in grafted $SOD1^{G93A}$ mice could increase the survival of ALS mice (Ohnishi et al. 2009). In this study, wild-type (GFP+) bone marrow was transplanted directly in the bone marrow cavity (intra-bone marrow-bone marrow transplant) in $SOD1^{G93A}$ mice at onset (defined as leg tremor in 2 consecutive days) and showed an increase in survival. It is, therefore, interesting to see that transplantation even at onset could have a positive effect on the disease (Ohnishi et al. 2009). Therefore, bone marrow transplant after irradiation could still prove to be an interesting concept to test potential therapeutic tools. Cell replacement therapies or trophic cell therapies have also been tested in mice using hematopoietic cells derived from human umbilical cord blood showing an increased survival of $SOD1^{G93A}$ mice (Ende et al. 2000; Garbuzova-Davis et al. 2003).

The effect of wild-type myeloid cells on motor neuron survival could also be linked to a peripheral effect because bone marrow grafting experiments replaced also the peripheral macrophages. In addition, lower expression of mutant SOD1 in peripheral macrophages was

measured in CD11bCre/LoxSOD1^{G37R} mice as compared to LoxSOD1^{G37R} mice and CD11bCre/LoxSOD1^{G37R} mice survived longer (Boillee et al. 2006b). Macrophages at the periphery include the ones in the nerves, and activated macrophages are present in the peripheral nerves upon axonal degeneration in neurodegenerative conditions (Griffin et al. 1993) including in the sciatic nerves of ALS mice or rats (Graber et al. 2010; Chiu et al. 2009).

Allogeneic hematopoietic stem cell transplantation was used in a phase I trial in six ALS patients as a cell replacement therapy with the aim of slowing motor neuron degeneration and ALS disease. Two patients with 100% engraftment showed donor cells in the vicinity of affected motor neurons, but not in unaffected brain areas. Nevertheless, this trial did not show any benefit for the patients and the authors propose to transplant transduced bone marrow stem cells to deliver neurotrophic factors for therapeutic value (Appel et al. 2008).

Anti-inflammatory drugs in ALS clinical trials

Inflammatory cytokines could potentiate motor neuron degeneration and some of these factors are known to be produced by microglia/macrophages or even activated astrocytes. In addition to proinflammatory factors produced around motor neurons, as the blood spinal cord barrier has been shown to be disrupted in ALS mice (Garbuzova-Davis et al. 2008; Zhong et al. 2008; Zhong et al. 2009) proinflammatory factors from outside of the CNS could also enter the spinal cord. To lower the inflammatory reaction and potentially increase motor neuron survival, anti-inflammatory drugs were tested in ALS models and some of them brought to clinical trials. The most famous one is probably minocycline, a second generation tetracycline which had been previously shown to bear anti-inflammatory properties (Yrjanheikki et al. 1999) and that had prompted a lot of hope after several groups showed delayed onset of motor neuron degeneration and increased survival of different mutant SOD1 mouse models (Kriz et al. 2002; Van Den Bosch et al. 2002; Zhu et al. 2002). Although minocycline could have a direct anti-apoptotic effect on motor neurons (Zhu et al. 2002), it induced a concomitant decrease in microglial activation (Kriz et al. 2002). However, the positive results from the mice were not visible in a phase III randomized clinical trial that showed no benefit and maybe some adverse effects, although further studies would need to address the dose of minocycline to be used and the time frame of the administration (Gordon et al. 2007; Carri 2008; Leigh et al. 2008). This outcome has hampered the relevance of using SOD1 mice for testing drugs for potential clinical trials (Schnabel 2008). However and very importantly, patients are always treated after the onset of disease, and animals are often given the drug before motor neuron degeneration has started. When considering this major difference and the divergences in experimental protocols, some guidelines have been proposed for testing potential treatments in ALS mice (Ludolph et al. 2010; Scott et al. 2008).

Other anti-inflammatory drugs focused on the cyclooxygenase (COX) 2 enzyme that can participate in the activation of inflammatory pathways in the CNS through the production of prostaglandin E2 (PGE2) (Consilvio et al. 2004). Increased COX-2 expression was measured in ALS patient spinal cords where the COX-2 immunoreactive cells corresponded to activated microglial cells/macrophages and PGE2 levels were increased in the

cerebrospinal fluid and their receptors induced in the spinal cords of ALS patients (Almer et al. 2001; Yasojima et al. 2001; Maihofner et al. 2003; Yiangou et al. 2006; Liang et al. 2008). COX-2 and PGE2 levels were also found to be increased in $SOD1^{G93A}$ mice (Almer et al. 2001) and PGE2 receptors deletions or COX-2 inhibitors delayed the onset and prolonged survival of ALS mice together with decreasing both astroglia and microglial reactivity (Drachman et al. 2002; Pompl et al. 2003; Liang et al. 2008). Celecoxib, the selective COX-2 inhibitor used in mouse studies, however, proved no benefit to ALS patients although PGE2 levels were not downregulated, indicating that the dose of celecoxib used could have been too low (Cudkowicz et al. 2006).

Peroxisome proliferator-activated receptor-γ (PPARγ) agonists are ligand-dependent transcription factors that are known to downregulate pro-inflammatory cytokines and inducible nitric oxide synthase (iNOS) in both macrophages and microglial cells and can also protect against glutamate excitotoxicity (Colville-Nash et al. 1998; Ricote et al. 1998; Zhao et al. 2006b). The PPARγ agonist, pioglitazone, is a FDA-approved drug to treat type II diabetes, because it enhances insulin sensitivity. This drug previously showed improved symptoms in experimental autoimmune encephalomyelitis mice used to model multiple sclerosis (MS) and in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated mouse model for Parkinson's disease (Breidert et al. 2002; Dehmer et al. 2004). In $SOD1^{G93A}$ mice, pioglitazone was given orally, starting before onset and showed increased motor performance, protection from motor neuron loss and extension of survival accompanied by a reduced gliosis and immunoreactivity to inflammatory markers (iNOS, NFkB, 3-nitrotyrosine) in the spinal cord of treated mice (Kiaei et al. 2005; Schutz et al. 2005). Because this drug is well tolerated in patients chronically treated for diabetes and crosses the BBB, it makes it an attractive candidate for neurodegenerative diseases and is currently given to ALS patients in an ongoing phase II clinical trial (http://www.clinicaltrials.gov/).

Blocking the inflammatory response could, therefore, be a strategy to slow down motor neuron degeneration; however, inflammation and microglial activation cannot be assimilated only as a negative reaction. Indeed, elimination of a significant proportion of the proliferating pool of spinal cord microglial cells in $SOD1^{G93A}$ mice (crossing them with CD11b-thymidin Kinase mice) had no effect on motor neuron survival (Gowing et al. 2008). However, as previously described, boosting the inflammatory reaction by chronically injecting LPS exacerbated the disease (Nguyen et al. 2004). Defining the factors secreted by activated microglial cells and that are toxic or trophic for motor neurons is therefore the strategy that several groups have employed to define the types of anti-inflammatory drugs that could be used in the future.

Microglia-derived pro- or anti-inflammatory factors

The expression of several pro-inflammatory factors has been described in the spinal cord of ALS mice or patients over the course of the disease in ALS (Fig 1) and, interestingly, expression profiling of gene products modified between $SOD1^{G93A}$ and control mouse spinal cords show genes implicated in inflammatory processes as majorly changed in ALS mice (Yoshihara et al. 2002). Several strategies have been used to study genes involved in

neuroinflammation, including comparing isolated microglial cells (expressing or not mutant SOD1) in culture before or after adding activating factors such as LPS, but also crossing ALS mice with mice deleted for a gene implicated in neuroinflammation.

Tumor necrosis factor-alpha

Tumor necrosis factor-alpha (TNF-α) which can be released by microglial cells (Fig. 1) is a potent pro-inflammatory cytokine with direct effects on neurons, astrocytes and microglia and that can elicit toxic though sometimes also trophic responses, according to the receptor. TNF-α levels were shown to be increased in the blood and plasma of ALS patients (Poloni et al. 2000; Cereda et al. 2008). In $SOD1^{G93A}$ mice elevated TNF-α mRNA and protein levels are found in the spinal cord at pre-symptomatic stages which increases with the progression of the disease, suggesting that it could be related to the neurodegenerative process (Elliott 2001; Hensley et al. 2003). In addition, TNF-α release by microglial cell cultures was shown to be increased when adult (but not neonate) microglial cells isolated from $SOD1^{G93A}$ adult mouse brain were stimulated with LPS (Weydt et al. 2004). To analyze the role of TNF-α on motor neuron degeneration in ALS mice, $SOD1^{G93A}$ and $SOD1^{G37R}$ mice were crossed with TNF-α deleted mice that unexpectedly failed to decrease disease severity or even motor neuron death (Gowing et al. 2006). Neither axonal degeneration nor astrocytic or microglial activation were affected which suggests that TNF-α is not a key factor in motor neuron degeneration caused by SOD1 mutations. However, looking for potential compensatory processes, increased expression of transcripts for IL-1β (a pro-inflammatory factor) and TLR-2 (a receptor implicated in the innate immune response induction) (Fig. 1) were found in the spinal cord of $SOD1^{G93A}$ (but not in $SOD1^{G37R}$) mice lacking TNF-α, which could account for the absence of effect of TNF-α deletion, at least in $SOD1^{G93A}$ mice. The drugs thalidomide and lenalidomide that are known to inhibit TNF-α synthesis (but also modulate the expression of other cytokines) were neuroprotective and significantly increased lifespan in $SOD1^{G93A}$ mice, with a slightly better performance for lenalidomide, however, the administration (oral) started way before the onset of symptoms (Kiaei et al. 2006). In a second study, where mice were treated with lenalidomide starting at symptom onset it led to a slight improvement of motor performance and a slight increase in lifespan, but far less significant than when treated long before symptom onset (Neymotin et al. 2009). Thalidomide has recently been tested in a phase II clinical trial in ALS patients and showed no improvement of the symptoms, an increased mortality and several side effects experienced by the patients (Stommel et al. 2009).

Reactive Oxygen Species (ROS) and neuroinflammation

Oxidative stress has long been suspected to contribute to ALS pathology (Barber et al. 2006). Indeed, because SOD1 is a ROS scavenger, a loss of the dismutase activity function of the protein was first hypothesized to be part of the pathogenic process; however, it is a gain of toxic function of the different SOD1 mutants that leads to motor neuron degeneration (as described previously). Even though, downregulating the levels of dismutase active mutant

SOD1 in certain cell types, like Schwann cells, in ALS mice, still contributes to disease acceleration showing that an increase in oxidative stress most likely contributes to exacerbating motor neuron pathology (Lobsiger et al. 2009). Different markers of oxidative stress are induced during the course of ALS disease in human and mouse models. A marker of lipid peroxidation, 4-hydroxynonenal (HNE), is increased in serum, CSF and spinal cord (motor neurons and glia) of sporadic ALS patients (Smith et al. 1998; Shibata et al. 2001; Simpson et al. 2004) and expression of iNOS, mainly localized in astrocytes and microglia, is upregulated during the progression of motor neuron loss, starting at early symptomatic stages in mutant SOD1 mice (Almer et al. 1999). These observations are consistent with the fact that primary SOD1^{G93A} microglia, in culture, produce more nitric oxide (NO) than wild-type microglia (Fig. 1), and are toxic to motor neurons which can be partially reverted with the addition of an iNOS inhibitor (Beers et al. 2006; Xiao et al. 2007). NO has been shown to be necessary for Fas-triggered death of mutant SOD1 motor neurons in culture (Raoul et al. 2002). Exogenous NO leads to increased expression of the Fas ligand itself in primary motor neurons and of the *collapsin response mediator protein 4a* (CRMP4a) that can trigger motor neuron death (Duplan et al. 2010; Raoul et al. 2006). Crossing neuronal NOS (nNOS) knockout mice with the slow progressing line of transgenic mice SOD1$^{G93A\text{-}low}$ (low copy number, *B6SJL-Tg(SOD1-G93A)^{dl}1Gur/J*), did not affect motor neuron degeneration; however, these mice still produced β and γ isoforms of nNOS and of course iNOS (Facchinetti et al. 1999). Deletion of iNOS, this time, increased lifespan of the fast progressing and commonly used SOD1^{G93A} but had no effect on the SOD1$^{G93A\text{-}low}$ mice (Son et al. 2001; Martin et al. 2007). Pharmacological inhibition of iNOS could also delay disease onset and extend lifespan of SOD1^{G93A} mice (Chen et al. 2009). NO produced through iNOS seems, therefore, to have an impact on motor neuron survival in a fast-progressing ALS mouse line.

Microglial cells can also produce superoxide by the NADPH oxidase, a multiprotein complex including a transmembrane catalytic subunit, Nox, that transports electrons across biological membranes to reduce oxygen to superoxide (Bedard and Krause 2007). NADPH oxidase also modulates intracellular signaling in microglial cells since Nox2 (or phagocytic Nox), that is highly expressed by microglia, together with Nox1 are required to optimize microglial production of NO and Nox1-derived superoxide contributes to IL1β secretion by microglia (Cheret et al. 2008). During the course of ALS disease in mice, NADPH oxidase is upregulated in the spinal cord, increasing ROS production, that could lead to neuronal degeneration through protein oxidative damage (Fig. 1) (Wu et al. 2006; Marden et al. 2007). Crossing SOD1^{G93A} mice with mice either deleted for Nox2 or Nox1 significantly increased survival (with a greater efficiency for Nox2, Wu et al. 2006; Marden et al. 2007). In addition, apocynin, an NADPH oxidase blocker given in the drinking water to ALS mice remarkably prolonged their survival by more than 100 days (with the highest dose tested and with the treatment started at 2 weeks of age and therefore way before disease onset) what is to date the highest effect obtained with a drug treatment in this model (Harraz et al. 2008). However, given after onset, the outcome was less striking and this effect could well be linked to mutant SOD1 mediated ALS only (and maybe even a subpopulation of SOD1 mutants). Indeed, analyzing the mechanisms by which mutant SOD1 led to an increase in superoxide

production by Nox2, in transfected cell lines, dismutase active mutants of SOD1 but not dismutase inactive or wild-type forms hyperactivated Nox2, through binding to Rac1 (a regulatory subunit of Nox2) enhancing the production of superoxide (Harraz et al. 2008). Increased superoxide production has also been reported for microglial cells in culture expressing $SOD1^{G93A}$ compared with control microglia (Fig. 1) (Xiao et al. 2007); however, downregulation of overall microglial reaction by apocynin or Nox2 deletion was either not analyzed or reached divergent outcome (Wu et al. 2006; Marden et al. 2007; Harraz et al. 2008). Nox2 upregulation has also been reported in spinal cords of sporadic ALS cases (Wu et al. 2006), but considering the direct action of mutant SOD1 on Nox2 activation, it would be important to know if patients with SOD1 mutations generate similar or greater increase in Nox2 and if the NADPH oxidase pathway could be a target for potential therapeutics also in sporadic ALS (since activated microglial cells, even without expressing mutant SOD1 and can still produce superoxide through NADPH oxidase activation).

Anti-inflammatory and trophic factors produced by microglia.

When compared with the well studied pro-inflammatory factors, not much is known about the trophic factors potentially released by microglial cells and especially downregulated in ALS. IL-4 known to be released by T cells can also be produced by microglia (Fig. 1) and was shown to be protective to microglial-induced motor neuron injury through the reduction in microglial NO and superoxide production mediated by LPS (Zhao et al. 2006a). Microglial cells can also produce the trophic factor for motor neurons, insulin-like growth factor-1 (IGF-1) and primary $SOD1^{G93A}$ microglial cell cultures release less IGF-1 when compared with non-transgenic microglia (Fig. 1) and further maintains this difference upon treatment with LPS (Xiao et al. 2007). However, when microglial cells were directly isolated from adult mutant SOD1 mice, IGF-1 levels were found to be increased at pre-symptomatic stages and further increase later in disease when compared with non-transgenic or transgenic mice expressing wild-type SOD1 (Chiu et al. 2008). IGF-1 also showed anti-inflammatory properties, reducing the release of TNF-α and NO by BV2 microglial cell lines expressing mutant SOD1 and neuroprotective capacities against mutant SOD1 induced toxicity of astrocytes towards motor neurons in culture (Dodge et al. 2008).

Importantly, several groups have shown the motor neuron protective effect of IGF-1 in animal models of ALS, however, the site of effectiveness of the treatment is debated. Kaspar et al. (2003) showed that IGF-1 delivered by retrograde adeno-associated virus (AAV) prolonged survival and delayed disease progression of $SOD1^{G93A}$ mice when treated both before and at disease onset. The effect of IGF-1 was due to its presence in motor neurons themselves given that delivery of muscle-targeted IGF-1 viral vectors (which could not be retrogradely transported) only increased survival very modestly (Kaspar et al. 2003). However, muscle-specific expression of IGF-1 (by a transgenic mouse approach) in $SOD1^{G93A}$ mice reduced spinal cord inflammation and increased their survival (Dobrowolny et al. 2005), but this was not confirmed by a similar approach where IGF-1 was expressed exclusively in the skeletal muscle or in the CNS (Messi et al. 2007). Beneficial effects of IGF-1 were also obtained by intraparenchymal delivery of AAV in the lumbar spinal cord of presymptomatic $SOD1^{G93A}$ mice, leading to increased motor performance with delayed

disease onset but a modest increased survival selectively in males (Lepore et al. 2007). IGF-1 expression was found in neuronal cell bodies (including ventral horn motor neurons) but not in astrocytes, therefore in accordance with an absence of effect on the disease duration, which as previously discussed, is mostly dependent on glial cells. Injection of AAV-IGF-1 vectors this time, into the deep cerebellar nuclei of $SOD1^{G93A}$ mice at disease onset, to deliver IGF-1 in the brain stem and spinal cord through axonal transport led to increased motor neuron survival, reduction in microglial and astroglial activation, improved motor function and a significant extension of life span (Dodge et al. 2008). A different method for continuous delivery used infusion of IGF-1 into the intrathecal space (CSF) of the lumbar spinal cord in presymptomatic $SOD1^{G93A\text{-}low}$ mice and showed a positive outcome with improved performance of motor functions, delayed onset and extended survival (Nagano et al. 2005a).

A similar technique was used in a small number of ALS patients where intrathecal delivery of IGF-1 showed a modest, but significant benefit (Nagano et al. 2005b). Other clinical trials with recombinant human IGF-I used subcutaneous delivery that resulted in modest effects in one trial (Lai et al. 1997), or did not show significant differences in two other studies (Borasio et al. 1998; Sorenson et al. 2008).

IGF-1 levels have been shown to be altered in the blood and CSF of ALS patients, with circulating concentrations either decreased (Torres-Aleman et al. 1998), increased (Hosback et al. 2007; Pellecchia et al. 2009) but also unchanged (Braunstein and Reviczky 1987; Bilic et al. 2006; Corbo et al. 2009) as compared to controls. In addition, alterations in the levels of IGF-1 binding proteins, which regulate IGF-1 bioavailability have been reported (Torres-Aleman et al. 1998; Hosback et al. 2007). These alterations that could influence the effect of IGF-1 treatments point out the need to evaluate the best administration route for assuring a correct availability of IGF-1 to the CNS and whether it needs to be delivered to motor neurons and/or other non-neuronal cell types.

Increased expression of microglial mitogenic and chemoattractant factors

Colony stimulating factors (CSF) are implicated in myeloid cell production with macrophage-CSF (M-CSF or CSF-1) being the archetypal mitogenic factor for macrophages/microglial cells. Although secreted by microglial cells, a main source of CSF-1 is coming from astrocytes. Granulocyte macrophage CSF (GM-CSF) is also a mitogenic factor for macrophages while granulocyte-CSF (G-CSF) mostly affects neutrophil production. Lack of M-CSF in osteopetrotic (op/op) mice or lack of its receptor (fms) leads to severe deficiency in monocytes and tissue macrophages including microglia (Wiktor-Jedrzejczak et al. 1982; Wegiel et al. 1998; Dai et al. 2002). In op/op mice, macrophage production seems to increase with age but post-lesion proliferation of microglia remains impaired (Begg et al. 1993; Raivich et al. 1994; Kalla et al. 2001). M-CSF mRNA levels were found to be increased from onset of symptoms onward as disease progressed in the CNS of $SOD1^{G93A\text{-}low}$ mice, correlating with the increase in microglial reactivity (Elliott 2001) while levels of GM-CSF were measured in $SOD1^{G93A}$ mice and showed no alteration (Hensley et al. 2003). Intra-peritoneal administration of M-CSF to $SOD1^{G37R}$ mice induced an ameboid morphology and increased proliferation of microglia, but not sufficiently to

translate to a higher number of microglial cells in the spinal cord, or peripheral macrophages in the sciatic nerve. However, M-CSF treatment exacerbated the disease of ALS mice (Gowing et al. 2009). CSFs also have neuroprotective capabilities apparently due to direct effects on the neurons expressing their receptors (Schneider et al. 2005; Schabitz et al. 2008). This is the case for G-CSF, which improved motor neuron survival, delayed onset of symptoms and increased lifespan (though modestly) when administered subcutaneously after onset of denervation or when overexpressed transgenically in $SOD1^{G93A}$ mice (Pitzer et al. 2008). However, in a pilot clinical study G-CSF, which was well tolerated, showed no benefit in ALS patients (Nefussy et al. 2009).

Increased inflammatory processes also include chemoattraction of macrophages/microglial cells to the lesion site. Although as previously described, macrophages from the periphery do not seem to contribute to the increase in microglial cells number in the spinal cord, increased chemokine levels could still reflect extended microglial reactivity and attraction of local microglia to specific regions of the CNS. Monocyte chemoattractant protein-1 (MCP-1), a chemotactic factor for macrophages/microglia, was found to be increased in the CSF and serum of ALS patients while immunohistochemical studies localized its expression principally in glial cells in human ALS spinal cords (Wilms et al. 2003; Henkel et al. 2004; Simpson et al. 2004; Baron et al. 2005; Nagata et al. 2007; Kuhle et al. 2009). Interestingly, a trend to higher MCP-1 levels in the CSF was found in ALS patients with shorter lifespan which, if confirmed in a bigger cohort, could be of prognostic potential for ALS (Kuhle et al. 2009). MCP-1 was also found to be elevated in animal models of ALS (Nguyen et al. 2001; Henkel et al. 2006). Surprisingly, MCP-1 mRNA levels were already elevated at the age of 15 days in $SOD1^{G93A}$ mouse spinal cords and therefore before microglial activation revealing a very early intrinsic modification of MCP-1 synthesis in $SOD1^{G93A}$ mice and consistent with the increased production of MCP-1 by activated $SOD1^{G93A}$ cultured microglia from neonates when compared to control cells (Henkel et al. 2006; Sargsyan et al. 2009). MCP-1 immunoreactivity was found in neuronal and glial cells and overtly increased at end-stage of disease in ALS mice (Henkel et al. 2006). Although MCP-1 has been the most studied chemokine in ALS, other chemokines such as IL-8 in CSF, eotaxin in serum and RANTES in CSF and serum have also been found at higher concentrations in ALS cases (Rentzos et al. 2007; Kuhle et al. 2009). Accordingly, protein levels of RANTES/CCL5 but also GRO/KC (the mouse analog of IL-8) were also increased in the $SOD1^{G93A}$ mouse spinal cords (Hensley et al. 2003).

Motor neuron signals to microglial cells

Microglial cells through release of trophic or toxic factors could have an impact on motor neuron survival and disease progression. Initiation of the inflammatory process could in theory come from several sources. (1) Factors coming from the periphery through disrupted blood spinal cord barrier (Garbuzova-Davis et al. 2008; Zhong et al. 2008), however, the origin of this rupture is not yet clear and could likely be linked to the motor neuron degenerative processes. (2) Intrinsic pathways in microglial cells expressing mutations leading to ALS could be the second hypothesis since as described previously microglial cells expressing mutant SOD1 can produce more toxic and less trophic factors in

culture (Beers et al. 2006; Xiao et al. 2007) and downregulating mutant SOD1 in microglia/macrophages has a beneficial effect on disease progression in mice (Beers et al. 2006; Boillee et al. 2006b; Wang et al. 2009b). However, replacing wild-type microglia/macrophages by mutant expressing cells is not enough to induce death of motor neurons not expressing mutant SOD1 (Beers et al. 2006). (3) A likely pathway would, therefore, be signals coming from motor neurons when degenerating (and even when not yet showing signs of death that could explain early microglial activation in ALS mouse spinal cord) and an exacerbation of the inflammatory process by factors coming from microglia (described above) or even astrocytes and most likely also due to the intrinsic expression of ALS linked mutated proteins (Fig. 1). Factors coming from neurons and directly activating microglial cells are still not well defined in the context of ALS.

Communication between neurons and microglia is an important feature for normal function of the CNS. For example, fraktalkine and its receptor CX3CR1, as well as CD200-CD200R and SIRPα-CD47 actively maintain microglial cells in a resting state (Hoek et al. 2000; Cardona et al. 2006; Bessis et al. 2007). Microglia can sense a wide range of stimuli including the ones released from damaged cells in pathological conditions (Fig. 1), which is first believed to be protective to reconstitute the damaged area. However, its chronic persistence is most likely becoming deleterious and participates in the degenerative process of many neurodegenerative diseases including ALS (Wyss-Coray and Mucke 2002; Hanisch and Kettenmann 2007).

Among the factors released by damaged neurons, ATP can act on microglia through cell surface purinergic P2 receptors either the ionotropic (P2X) or the metabotropic (P2Y) subtypes (Fig. 1), which can activate inflammatory responses (Inoue 2006). Recently, upregulation of P2X4, P2X7 and P2Y6 receptors as well as down-regulation of ATP-hydrolyzing activities have been found in mutant SOD1 microglia. This activation translated first, into increased expression of TNF-α and COX-2 by mutant SOD1 microglia activated by ATP and second, into toxicity towards neuronal cell lines expressing human $SOD1^{G93A}$ and exposed to conditioned media of 2'-3'-O-(benzoyl-benzoyl) ATP-activated microglia (D'Ambrosi et al. 2009). P2X receptors have also been related to ALS pathology in humans, since a greater density of P2X7-immunoreactive microglial cells/macrophages was found in affected regions of ALS spinal cords (Yiangou et al. 2006).

One obvious candidate to participate in motor neuron-microglia interactions in ALS mouse models is mutant SOD1 itself, but this evidence only became clear when chromogranin A (CgA) was found as an interacting partner to $SOD1^{G93A}$ in a yeast two hybrid approach (Urushitani et al. 2006). Indeed, CgA which was expressed by motor neurons (but not microglial cells) seemed to act as a chaperone-like factor promoting secretion of mutant SOD1 in a CgA-SOD1 complex (Fig. 1). CgA and CgB co-localized with mutant SOD1, but not wild-type SOD1, in ALS mice in vivo. Of interest, a recent genetic screen showed implication of a mutation in the CgB gene as a risk factor and a modifier of onset in ALS patients (Gros-Louis et al. 2009). In mixed mouse spinal cord cell culture experiments adding mutant SOD1 was toxic for motor neurons in contrast to wild-type SOD1 and this toxicity was dependent on the presence of microglial cells (Urushitani et al. 2006). These observations, therefore, proposed a scheme where mutant SOD1 secreted by motor neurons would activate microglial cells that would in turn secrete neurotoxic factors and

affect motor neurons. In fact, mutant SOD1 added to microglial cell cultures changed their morphology towards a round activated shape and turned microglia to a pro-inflammatory type with increased production of TNF-α, IL-1β and superoxides (Zhao et al. 2010). Mutant SOD1 seemed to activate microglial cells through binding to CD14. CD14 is a LPS receptor that mediates signal transduction in concert with TLR2 and TLR4. These data are also consistent with studies on BV2 cells expressing mutant SOD1 and producing high amounts of TNF-α especially when the TLR2 pathway is activated (Liu et al. 2009). However, deletion of CD14 ($CD14^{-/-}$ mice) in $SOD1^{G93A}$ mice had no effect on disease progression and survival (Zhao et al. 2010).

Because mutant SOD1 release by motor neurons was implicated in the disease mechanism, vaccination approaches have been tested in different ALS lines. Using a bacterially purified recombinant mutant $SOD1^{G93A}$ protein as the immunogen, in the slowly progressing $SOD1^{G37R}$ line, the vaccination extended their lifespan by 4 weeks. This strategy did not impact the survival of the fast-progressing $SOD1^{G93A}$ mice (most probably due to too high expression levels of the transgenic protein). However, direct intraventricular infusion of antibodies raised against the $SOD1^{G93A}$ protein achieved to prolong lifespan of the fast-progressing $SOD1^{G93A}$ mice (Urushitani et al. 2007). Therefore, immunization could be considered for the treatment of ALS patients, but this strategy would only be pertinent for familial ALS linked to SOD1 mutations unless one could show that human SOD1 in sporadic ALS patients behaves like mutant SOD1. Indeed, studies in mice have shown that adding wild-type human SOD1 in mice expressing mutant human SOD1 could speed up the disease (Deng et al. 2006; Wang et al. 2009a) most likely through a stabilization of the mutant protein by the wild-type one, although wild-type SOD1 could also acquire a toxic deleterious conformation through oxidation (Rakhit et al. 2002; Ezzi et al. 2007; Kabashi et al. 2007). In any case, since microglial cells are activated in familial and sporadic ALS cases, finding the motor neuron pathways implicated in microglial activation could help in slowing motor neuron degeneration.

The role of the adaptive immune system in ALS mice

Besides microglia and astrocytes other cells participate in the inflammatory process occurring in ALS (Fig. 1). Evidence of T lymphocytes infiltration was described in ALS tissues (Lampson et al. 1990; Troost et al. 1990; Kawamata et al. 1992) and in mutant SOD1 mice (Alexianu et al. 2001; Beers et al. 2008; Chiu et al. 2008). In ALS patients, lymphocytic infiltration of both T-helper (CD4+) and cytotoxic (CD8+) classes (but predominantly CD8+ T cells) was described in the corticospinal tracts and anterior horns of the spinal cord (Troost et al. 1989; Lampson et al. 1990; Troost et al. 1990; Kawamata et al. 1992), leading to a proposed autoimmune reaction or viral infection as a cause of ALS disease (McGeer and McGeer 2002). However, these hypotheses were weakened by the lack of further evidence. The absence of specific antibodies or T cells directly attacking motor neurons or the failure of therapeutic immunization in mouse models and ALS patients (detailed below) are not in favor of an autoimmune process (Appel et al. 1993); moreover, the absence of viral DNA or RNA in tissues of ALS patients do not support a viral hypothesis (Swanson et al. 1995;

Rosener et al. 1996; Andrews et al. 1997, 2000; Walker et al. 2001). But still, the presence of T cells in the affected tissues of ALS patients and ALS mice raised the question of the possible role of these infiltrated cells and different mouse mating strategies were used to show the involvement of T cells in ALS mice. First, to block recruitment of peripheral cells in the CNS, PU.1$^{-/-}$ / SOD1^{G93A} mice were grafted with bone marrow cells lacking the receptor for MCP-1 (CCR2) which recruits CCR2 expressing cells meaning, activated T cells and monocytes. Because CCR2$^{-/-}$ bone marrow transplanted ALS mice had shortened lifespan as compared to the mice that received wild-type bone marrow, cells coming from the periphery into the CNS had an influence on the disease. However, this positive effect could have also come from CCR2$^{-/-}$ microglial cells in the CNS (since all the microglial cells in the PU.1$^{-/-}$ / SOD1^{G93A} got replaced by CCR2$^{-/-}$ cells). Therefore, SOD1^{G93A} mice were crossed with RAG2$^{-/-}$ mice that lack functional T and B cells which led to a shorter lifespan. When these mice received SOD1^{G93A} or WT bone marrow, they survived longer with a slower disease progression showing that lymphocytes had a beneficial effect on the disease (Beers et al. 2008). Of note, mutant SOD1 expressed in lymphocytes did not seem to affect the disease. Crossing SOD1^{G93A} mice with TCR$\beta^{-/-}$ mice that lack T cells, which led to decreased survival of ALS mice, showed the trophic capabilities of T cells in ALS mice (Chiu et al. 2008). To determine the role of subpopulations of T cells, SOD1^{G93A} mice were bred with CD4$^{-/-}$ mice. These mice showed the same disease acceleration than SOD1^{G93A} / RAG2$^{-/-}$ mice meaning that CD4+ T cells were responsible for the neuroprotection measured, probably by enhancing the expression of neurotrophic factors for motor neurons, such as IGF-1 and also by increasing the expression of glutamate transporters (GLT-1 and GLAST) as measured in the spinal cord of these mice (Beers et al. 2008). Furthermore, T cells also modulated the reactivity of microglial cells since SOD1^{G93A} / CD4$^{-/-}$ mice showed attenuated staining with microglial activation markers. This observation also means that acceleration of disease did not correlate with the level of microglial activation or that microglial activated morphology did not predict their toxic function (Beers et al. 2008).

Concerning the kinetics of infiltration of T cells that could be influenced by alterations in the BBB permeability described in ALS mice (Zhong et al. 2008; Zhong et al. 2009), CD4+ T cells were present at onset of disease and accumulated with disease progression while CD8+ T cells were only present at the end-stage of disease in SOD1^{G93A} mice consistent with observations in postmortem tissues of ALS patients, where the majority of the lymphocytes were CD8+ (Troost et al. 1990). No B cells were observed in the spinal cord of these ALS mice (Chiu et al. 2008) or in human ALS spinal cords (Troost et al. 1990; Engelhardt et al. 1993). The lack of contribution of B cells in SOD1^{G93A} mice was shown by mating them with Ig-$\mu^{-/-}$ mice (where B cell development is blocked at pro-B stage) resulting in no difference in survival. In addition, no evidence of altered phenotype of B cells expressing mutant SOD1 was found (Naor et al. 2009).

Analyzing the T cell immune response and lymphocyte proliferation in SOD1^{G93A} mice showed that ALS mice had impaired T cell function and increased number of apoptotic T cells (Banerjee et al. 2008). In addition, spleen size and weight as well as spleen cells counts were reduced at the end-stage of the disease in SOD1^{G93A} mice (Banerjee et al. 2008). This T cell malfunction was hypothesized to occur in parallel to the motor neuron dysfunction. Indeed, a significant reduction in thymic progenitor-cell content and abnormal

thymic histology was observed in ALS animals (Seksenyan et al. 2009). Importantly, ALS patients also showed reduction in blood levels of T cell receptor rearrangement excision circles (sjTRECs) and a restricted T cell repertoire accompanied by an increase in pro-apoptotic markers (Seksenyan et al. 2009).

A reconstitution strategy (adoptive transfer with polyclonal wild-type T regulator (Treg CD4+ CD25+) or T effector (Teff CD4+ CD25-)) cells beginning at the presymptomatic stage in ALS mice, added another evidence to the T cell impairment since it delayed motor neuron loss and extended survival with Treg rather delaying symptom onset and Teff increasing latency between disease onset and entry into late stage, suggesting that these two subsets act on independent pathways (Banerjee et al. 2008). This impairment in T cell response could explain the fact that copolymer-1 (cop-1), a glatiramer acetate derivate (GA), treatment was not efficient in ALS mice (or modestly in females only (Banerjee et al. 2008). Cop-1 is an FDA-approved treatment for MS that has also shown some benefit in mouse models of Alzheimer's and Parkinson's disease (Benner et al. 2004; Frenkel et al. 2005; Butovsky et al. 2006; Laurie et al. 2007). Although the action of cop-1 is not clearly established, it seems to act as an immunomodulatory agent by enhancing the CD4+ Th2-like response, resulting in anti-inflammatory effects. A previous study using different doses of TV-5010 (another GA derivate) on three different mutant SOD1 mouse lines had shown the same outcome of no delay in disease onset or survival of ALS mice (Haenggeli et al. 2007); conversely, another study reported that GA immunization in low copy ($SOD1^{G93A\text{-}low}$), but not high copy, $SOD1^{G93A}$ mice delayed disease progression (Angelov et al. 2003). A phase II clinical trial for GA was conducted on ALS patients and had no impact on disease progression, but since it was well tolerated GA could still be used as a supplement treatment for future trials targeting the immune system (Meininger et al. 2009).

Conclusion

Neuroinflammation is part of ALS physiopathology and immune cells participate in the disease progression. Although microglial cells are activated prior to the appearance of the symptoms, motor neuron dysfunction seems to be the primary affection in ALS. Factors coming from sick motor neurons would be at the origin of microglial activation leading to increased neurotoxicity and diminished neuroprotective action of the glial cells. This glial cell reactivity becoming self-sustained would amplify the neuroinflammatory process, hitting back at the suffering motor neurons and increasing their vulnerability to the primary source of damage (Fig. 1). Degenerating motor neurons or reactive glial factors would also influence the blood spinal cord barrier tightness allowing leakage of potential neurotoxic factors from the periphery and increasing the attraction of lymphoid cells from the periphery. CD4+ T lymphocytes entering the spinal cord can release trophic factors that could act directly on motor neurons or modify microglial reactivity to lead to a more trophic environment. However, mutant proteins that lead to ALS (in first instance mutant SOD1) act in different cell types and modify their response to the injury. The different studies highlighted in this review converge to the conclusion that the immune system participates to motor neuron degeneration in ALS. To this point, the main question is, therefore, why anti-infammatory drugs have not yet proven their benefice to ALS patients? Several explanations could be

emphasized including (1) a potential interaction with Riluzol in the clinical trials, (2) results in animal pre-clinical trials that are often based on the beneficial effect of presymptomatic administration of the drug. Indeed, all of the potential therapeutic compounds used in mice starting after the onset has shown less benefit than when used at presymptomatic stages. (3) A prescreening of the factors on mutant SOD1 linked ALS models that might not reflect the multifactorial causes of ALS and which explains the current keen interest on building new ALS models expressing new mutated genes responsible for ALS. (4) The adaptive immune response in ALS is defective which could cause imbalance of the immune response to the neurodegeneration and could influence the response to therapies targeting the immune system. (5) The immune reaction is finely tuned, microglial cells can exert both neurotoxic or neurotrophic functions on motor neurons, depending on their activation state and specific microenvironment therefore anti-inflammatory drugs could have beneficial effects at specific stages of the disease and for a defined period of time highlighting the need to better understand the mechanisms of cell interactions, to clarify the different actors of neuroinflammation and the time course of expression/regulation of the inflammatory pathways potentially implicated in motor neuron death.

Targeting the symptomatic phase of the disease being the only way to slow down motor neuron degeneration in a disease mainly of sporadic etiology like ALS, neuroinflammation is still one of the most promising candidate pathway for therapeutic intervention.

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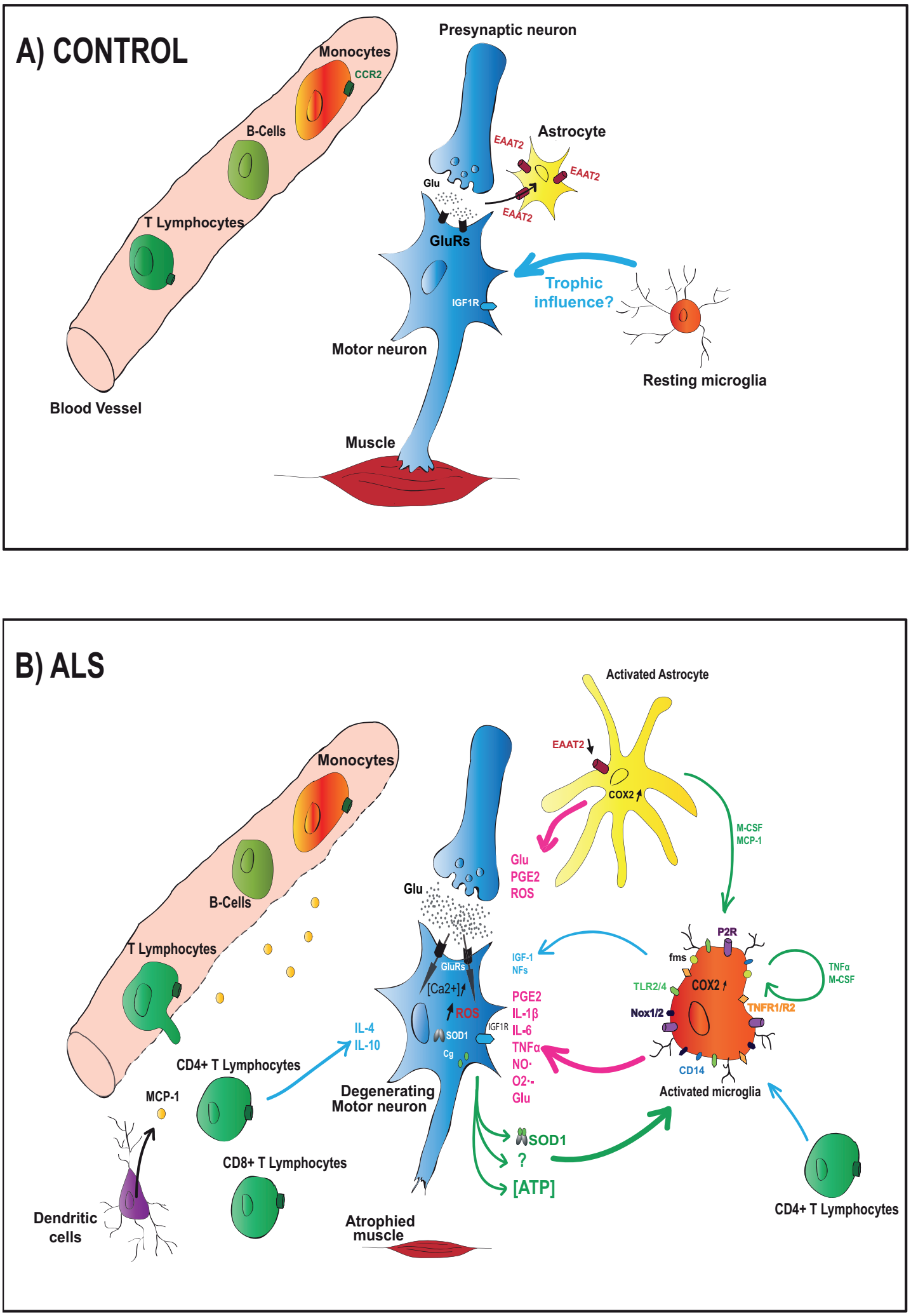
Figure Legend

Fig. 1 Immune cells of the CNS including microglia and T lymphocytes affect motor neuron survival. **a** Control situation: the presynaptic neuron releases glutamate (Glu), which binds to glutamate receptors (GluRs) on the postsynaptic motor neuron resulting in excitation through calcium influx. Extracellular glutamate is quickly cleared from the synaptic cleft by astrocytes through EAAT2 transporters. In a non-inflamed environment, microglial cells remain in a resting or "surveying" state, most likely releasing factors with neurotrophic influence (*blue arrows*) **b** ALS situation: decreased expression of astrocytic glutamate transporters EAAT2 could lead to prolonged glutamate excitation of motor neurons and participate in their degeneration (excitotoxic hypothesis). Activated microglial cells and astrocytes produce toxic factors (*pink arrows*). Among the factors released by astrocytes, macrophage- colony stimulating Factor (M-CSF) and monocyte chemoattractant protein-1 (MCP-1) are capable of activating microglial cells, increasing their proliferation (M-CSF) or migration (MCP-1) (*green arrows*). Microglial cells are also prone to self-activation by releasing Tumor Necrosis Factor-α (TNFα) for which they express the receptors 1 and 2 (TNFR1/2) and M-CSF acting on the receptor fms. Activated microglial cells will produce more reactive oxygen species (ROS) like nitric oxide (NO) through the inducible NO synthase and superoxide (O_2^-) through activation of NADPH oxidases (Nox1/2), but also proinflammatory cytokines like interleukines 1β and 6 (IL-1β, IL-6) and prostaglandins (PGE2) through activation of the cyclooxygenase 2 (COX2). Extracellular ATP, likely coming from damaged motor neurons, binds to microglial purinergic P2 receptors, therefore, contributing to microglial activation. Motor neurons can also participate to glial cell activation by releasing mutant SOD1 co-secreted with chromogranine (Cg) that can bind to CD14 acting in concert with the Toll-like receptors (TLR2/4). The adaptive immune system is also part of the degenerating motor neuron response. T lymphocytes (CD4+ and CD8+) coming from the periphery enter the spinal cord during the inflammatory process in ALS. CD4+ lymphocytes seem to have a neuroprotective effect by directly releasing anti-inflammatory factors like interleukines 4 and 10 (IL-4, IL-10) or by acting on microglial cells to increase their neurotrophic function (production of insulin-like growth factor-1 (IGF-1)). The role of infiltrating CD8+ T cells remains unclear and B cells are not present in ALS spinal cords. Dendritic (antigen-presenting) cells secrete MCP-1, which probably participates in the infiltration of peripheric immune cells. All together, the inflammatory environment and increased oxidative stress take part in the degeneration of the motor neurons that leads to muscle atrophy in ALS.



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