Chapter 4

The Neuroinflammation in the Physiopathology of Amyotrophic Lateral Sclerosis

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1. Introduction

Neuroinflammation is an inflammatory response that takes place within the central nervous system (CNS) during a neurodegenerative process or following a neuronal injury. The main effectors of neuroinflammation, which are astrocytes, microglia and immune cells can confer in a context- and time-dependent manner both neuroprotective and neurotoxic effects. It has now become evident that neuroinflammation is a prominent pathological hallmark of several neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease and Amyotrophic Lateral Sclerosis (ALS)(reviewed in [1, 2]). Indeed, reactive astrocytes and microglia as well as infiltrating T lymphocytes have been identified in ALS experimental models and patients. In the present chapter, we will describe the neuroinflammatory phenotype that characterizes ALS and discuss how the aberrant astrocytes, microglia and immune cells may actively participate in the neurodegenerative process. Further, we will examine the therapeutic potential of targeting neuroinflammation in both pre-clinical disease models and ALS patients.

2. The contribution of astrocytes in the neuroinflammatory response

2.1. Activation profile of astrocytes in human and animal models of ALS

Under normal and healthy conditions, astrocytes, which are the most abundant cell type within the CNS, are typically found in a resting state. Activation of astrocytes follows an acute or chronic injury, where the cells adopt a different morphology, become proliferative, express the intermediate filament glial fibrillary acidic protein (GFAP) release pro-inflammatory cytokines and growth factors as well as produce nitric oxide (NO)(reviewed in [3]). The phenomenon of astrocytosis has been well characterized in both ALS patients and animal models. Analysis of human ALS brains reveals the presence of reactive astrocytes within the subcortical white matter in a widespread fashion [4]. Importantly, the same brain regions from patients with non-ALS neurological disorders display a distinct histopathology, suggesting that the ALS astrocytosis is not simply an indirect result of the ongoing neurodegenerative process [4]. Similarly, the cortical gray matter tissue and the primary motor area from both sporadic and familial ALS patients are characterized by the omnipresence of reactive astrocytes [5, 6]. Studies performed on spinal cords from ALS patients show the occurrence of astrocytosis in both the ventral and dorsal horn region of the spinal cord [7, 8]. In addition to the above-mentioned post-mortem observations, in vivo brain imaging of ALS patients using deuterium-substituted [^{11}C](L)-deprenyl positron emission tomography has allowed the visualization of astrocytosis in live patients [9]. Hence, a thorough analysis of the CNS of ALS patients has uncovered and highlighted astrocytosis as a *bona fide* feature of ALS pathology, whether sporadic or inherited. While human ALS tissue represents most accurately the hallmarks that typify the disease, the caveat is that it limits our knowledge of the cellular events that occur prior to disease onset.

The generation of both mouse and rat models of ALS has helped elucidate more precisely the contributory role of astrocytosis during the neurodegenerative process. Analysis of different *superoxide dismutase 1* (*SOD1*) mutant mouse models identifies astrocytic alterations such as reactive morphological changes, proliferation as well as the presence of SOD1- and ubiquitin-positive inclusions, as occurring prior or close-to axonal degeneration and neuronal loss [10-13]. Furthermore, the process of astrocytosis significantly intensifies as the disease progresses [10, 11]. Three-dimensional reconstruction of *SOD1*^{G93A} spinal cord sections shows that astrocytic processes actually target and envelop pathological vacuoles within the degenerating neurons [11]. Similarly to the murine models, the transgenic *SOD1*^{G93A} rats also display signs of astrocytosis prior to significant motoneuron loss. As the disease progresses, there is an increase of astrocytic hypertrophy and proliferation as well as an accumulation of ubiquitin and tau-positive aggregates [14, 15]. Thus, while the human data provided the first insights into astrocytosis as a pathological hallmark of ALS, the observation in pre-clinical models of astrocytic inflammation prior to neurodegeneration strengthened the proposed contributory role of astrocytes in ALS pathogenesis.

2.2. A role for astrocytes in ALS pathogenesis

Once the astrocytic histopathology was thoroughly characterized in both human and animal ALS models, a comprehensive assessment of its functional influence on motoneuron loss thus ensued. One of the first indications of astrocyte-dependent neurodegeneration in ALS comes from the generation of chimeric mice, composed of both normal cells and SOD1 mutant-expressing cells [16]. This study demonstrates that mutant SOD1-positive motoneurons surrounded by wildtype non-neuronal cells have a better survival rate than those enclosed by mutant SOD1-positive non-neuronal cells [16]. A complementary approach consisting in deleting the human mutant SOD1 specifically within astrocytes of the *SOD1*^{G37R} mice suggests

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that mutant astrocytes contribute to progression, but not onset of the disease [17]. However, knocking down the mutant SOD1 in astrocytes of the *SOD1*^{G85R} mouse model results in increased survival by delaying disease onset as well as the early stage of the disease [18]. Despite minor differences between the targeted disease stages in both models, the key finding is that mutant SOD1-expressing astrocytes regulate the disease progression of murine ALS.

Another approach used to address the astrocytic-induced motoneuron loss in ALS is the *in vitro* co-culture of both cell types. Indeed, when cultured alone, primary *SOD1*^{G93A} astrocytes express high levels of pro-inflammatory effectors such as tumor necrosis factor alpha (TNFα), interferon gamma (IFNγ), interleukins (IL)-1 beta (IL-1β) and -18 (IL-18), 5-lipoxygenase (5-LOX), leukotriene B4, cyclooxygenase (COX-2) and prostaglandin E_2 (PGE_2), thus displaying an inflammatory phenotype with potential neurotoxic effects [19]. Consequently, primary wildtype and mutant motoneurons or motoneurons derived from murine or human embryonic stem cells show decreased survival when cultured in the presence of astrocytes expressing different mutated forms of SOD1 [20-24]. While the above-mentioned *in vivo* and *in vitro* studies suggest a contributory role for astrocytes in ALS pathogenesis, the targeted ablation of GFAP-expressing proliferating astrocytes in *SOD1*^{G93A} mice has no effect on the onset or the progression of the neurodegenerative process [25]. Recently, a subtype of astrocytes from spinal cord cultures of *SOD1*^{G93A} rats that displayed an aberrant phenotype has been isolated (termed Aba cells). Aba cells, that highly express S100β and connexin-43, but weakly express GFAP, are distinguished by their increased proliferative abilities and the absence of replicative senescence. Specifically, they are localized in proximity of motoneurons *in vivo*, increase drastically upon disease onset and demonstrate a greater neurotoxicity compared to non-Aba astrocytes isolated from *SOD1*^{G93A} rats [26]. Combined, these studies suggest that different subpopulations of astrocytes with different functional features and different cellular origin coexist during the pathological processes.

An additional important feature of the astrocytic contribution in ALS relates to the observation that the expression of SOD1^{G85R} solely in astrocytes does not give rise to motoneuron loss despite the fact that astrocytosis occurs prominently [27]. Likewise, the specific expression of SOD1^{G37R} in spinal cord motoneurons or the accumulation of SOD1^{G93A} in postnatal motoneurons does not impact motor function, neurodegeneration or disease onset and progression [28, 29]. Together, these observations therefore point to the critical communication that takes place between astrocytes and motoneurons, which might in turn lead to the initiation of neuronal death pathways.

2.3. Misregulation of neuronal transmission by astrocytes

The glutamate hypothesis proposes that a glutamate imbalance, leading to a calcium (Ca^{2+})-mediated excitotoxic insult, represents a major mechanism of motoneuron injury [30]. Astrocytes actively participate in modulating neuronal excitability and neurotransmission by controlling the extracellular levels of ions and neurotransmitters. The astroglial glutamate transporter excitatory amino-acid transporter 2 (EAAT2) in humans or glutamate transporter 1 (GLT-1) in rodents is the primary means of maintaining low extracellular glutamate levels. EAAT2/GLT-1 rapidly removes glutamate from the extracellular milieu and thereby prevents excitotoxic injury to neurons that occurs by overstimulation of the post-synaptic N-methyl-D-aspartic acid (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainate ionotropic glutamate receptors [31, 32]. Decreased expression of EAAT2/GLT-1, which leads to elevated levels of extracellular glutamate, has been found in a vast majority of sporadic and familial ALS patients as well as ALS mice and rats [10, 33-35], suggesting the participation of astrocytes in glutamate-induced excitotoxicity.

In addition to the relationship between glutamate excitotoxicity and glutamate transporter loss, other glutamatergic pathways have been implicated in motoneuron degeneration. Functional AMPA receptors consist of various combinations of four subunits (designated glutamate receptor (GluR)1-4) and are involved in fast excitatory synaptic transmission in the CNS [36]. The GluR2 subunit is functionally dominant and renders AMPA receptors impermeable to Ca^{2+}, preventing Ca^{2+} influx-induced toxicity. Thus, high levels of GluR2 in neuronal tissues might confer neuroprotection against glutamate-induced excitotoxicity. Within normal human spinal motoneurons, there is a low relative abundance of the GluR2 subunit mRNA compared to other GluR subunits and to other neuronal tissues, which may make them unduly susceptible to Ca^{2+}-mediated toxic events following glutamate receptor activation [37]. However, work from another group does not observe any significant quantitative changes in GluR2 mRNA within spinal cord motoneurons, suggesting that a selective decrease of the GluR2 subunit might not be the only mechanism mediating the AMPA receptor-dependent neurotoxicity in ALS [38]. Indeed, it has been demonstrated that RNA editing of GluR2 mRNA at the glutamine/arginine (Q/R) site is decreased in autopsy-obtained spinal motoneurons from patients with sporadic ALS [39], a molecular event that confers Ca^{2+} permeability to the GluR2 receptor [40]. Therefore, reductions in both GluR2 expression and GluR2 Q/R site editing may contribute to increased Ca^{2+} influx and neurotoxicity through AMPA receptors in ALS.

The molecular basis for lower GluR2 abundance in motoneurons compared to other CNS neurons has been investigated using two different rat strains that show differential vulnerability to AMPA-mediated excitotoxicity [41]. It has thus been demonstrated that astrocytes derived from the ventral spinal cord, but not those derived from the dorsal spinal cord, cerebellum, or the cortex, have the ability to regulate GluR2 expression in motoneurons. Interestingly, expression of mutant SOD1 abolishes their GluR2-regulating capacity. Although, the astrocytic factor responsible for GluR2 regulation in motoneurons remains to be identified, the regulation of motoneuron electrical activity through neuronal GluR2 expression and the uptake of glutamate by the glial transporter EAAT2/GLT-1 are major mechanisms by which astrocytes may mediate excitotoxic neurodegeneration in ALS.

2.4. Additional mechanisms of astrocytic neurotoxicity

While the astrocytic influence on neuronal excitability is seldom disputed, various reports suggest that they may also participate in the neurodegenerative process via the release of neurotoxic factors. Typically, the activation and/or reaction of astrocytes that characterize neuroinflammation occurs following a CNS injury, including chronic neurodegenerative diseases (reviewed in [42]). In experiments where the spinal cords of neonatal rats were injected with cerebrospinal fluid (CSF) from ALS patients, there is an increased GFAP immunoreac-

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tivity within the grey and white matter [43], suggesting that the astrocytosis in ALS might in fact be a responsive phenomenon. Conversely, many research groups have identified specific factors that are abnormally regulated in ALS astrocytes that could potentially trigger the motoneuron loss that typifies the disease.

2.4.1. The interferon response

Type I, II and III IFNs are an important family of immunomodulatory cytokines (reviewed in [44]). Elevated levels of IFNγ, a potent pro-inflammatory mediator, are found in the CSF of ALS patients, in the serum as the disease progresses and in spinal cord of sporadic ALS patients [45-47]. Further, the analysis of spinal cord sections from ALS patients shows that IFNγ is detected in ventral horn neurons, glial cells and plausibly immune cells [47]. In addition, the IFNγ-inducible protein, IP-30 and the interferon-stimulated gene 15 (ISG15) are significantly upregulated in human ALS spinal cord [48, 49]. In spinal cord extracts and serum of ALS mice, elevated levels of IFNγ mRNA and protein are also documented [24, 50, 51]. The expression of IFNγ is found within motoneurons and astrocytes of $SOD1^{G93A}$ and $SOD1^{G85R}$ spinal cords at both disease onset and symptomatic stages [24]. Similarly, a gene expression array analysis of pre-symptomatic $SOD1^{G93A}$ spinal cord reveals an induction of several genes regulated by type I IFNα, IFNβ and type II IFNγ, with specifically an increased expression of ISG15 in spinal cord astrocytes. Further, the phosphorylation of signal transducer and activator of transcription (STAT) 1 and 2, downstream effectors of IFNs [52], and STAT4, an inducer of IFNγ, is also elevated in $SOD1^{G93A}$ spinal cords [51]. Functionally, the genetic deletion of *Ifnα/β receptor 1* in $SOD1^{G93A}$ mice significantly prolongs life expectancy [49]. Importantly, astrocytic IFNγ triggers a motoneuron-selective death pathway via the activation of lymphotoxin beta receptor (LT-βR) by LIGHT. LIGHT is also upregulated in sporadic ALS spinal cords and the genetic ablation of *Light* in $SOD1^{G93A}$ mice delays disease progression [24]. Combined, these observations in rodent and human models of the disease suggest that the neuroinflammatory role of IFNs may contribute to the neurodegenerative process in ALS.

2.4.2. The contribution of nerve growth factor

The low affinity p75 neurotrophin receptor ($p75^{NTR}$) has a well-described role in mediating neuronal death signaling (reviewed in [53]). In symptomatic $SOD1^{G93A}$ mice and in ALS patients, $p75^{NTR}$ is overexpressed within spinal motoneurons [54]. Correspondingly, the immunoreactivity of nerve growth factor (NGF), a $p75^{NTR}$ ligand [55], is increased in spinal cord astrocytes of symptomatic $SOD1^{G93A}$ mice and in primary $SOD1^{G93A}$ astrocyte cultures [56, 57]. Further, the excessive expression of fibroblast growth factor 1 (FGF-1) by $SOD1^{G93A}$ motoneurons stimulates the nuclear accumulation of FGF receptor 1 (FGFR1) in astrocytes, consequently triggering astrocytic NGF production [58]. Importantly, primary $SOD1^{G93A}$ motoneuron cultures are hypersensitive to the NGF-$p75^{NTR}$ apoptotic signaling [59]. Thus, the astrocyte-dependent activation of the neurotoxic NGF-$p75^{NTR}$ pathway might participate to the neurodegeneration that typifies ALS.

2.4.3. Cyclooxygenase-2

COX-2 is a pro-inflammatory enzyme that converts arachidonic acid into prostanoids such as PGE_2, a potent inflammatory mediator (reviewed in [60]). In the anterior horn region of the spinal cord of *SOD1*^{G93A} mice, at both the early and end stage of the disease, COX-2 immunoreactivity is elevated in astrocytes [61]. Similarly, spinal cord astrocytes from sporadic ALS patients also display increased COX-2 expression [61, 62]. The expression of COX-2 can be modulated by the binding of CD40, a member of the TNF family (reviewed in [63]), with its ligand CD40L [64]. Interestingly, spinal cord astrocytes of symptomatic *SOD1*^{G39A} mice show an upregulation of CD40, concomitant with COX-2 astrocytic expression. Moreover, the activation of COX-2 in astrocytes upon CD40 stimulation leads to motoneuron death *in vitro* [65], suggesting that an astrocytic CD40-COX-2 pathway could also participate in ALS pathogenesis. The contribution of the CD40/CD40L pathway has recently been proposed in ALS mice, though its role in astrocytic neurotoxicity role has not been established [66]. Finally, another facet of the COX-2 pathway relates to the ability of PGE_2 to promote glutamate release from astrocytes, emphasizing further the complex multimodality of neuroinflammatory signals [67].

2.4.4. The Wnt/β-catenin signaling pathway

The canonical Wnt/β-catenin transduction pathway, which comprises multiple Wnt genes, regulates many biological functions (reviewed in [68]), including neuronal survival, as demonstrated by its involvement in other neurodegenerative disease such as Alzheimer's disease and Parkinson's disease [69, 70]. In the ventral region of symptomatic *SOD1*^{G93A} spinal cords, there is an increase in the number of Wnt3a- and β-catenin-positive astrocytes [71]. An upregulation of Wnt2 and Wnt7 within astrocytes of symptomatic *SOD1*^{G93A} spinal cords is also reported [72]. Among its biological functions, the Wnt/β-catenin pathway mediates the activity of cyclin D1 [73], a nuclear transcription factor important for cell cycle regulation (reviewed in [74]). The upregulation of cyclin D1 in *SOD1*^{G93A} astrocytes suggests that the increased activation of the Wnt/β-catenin/cyclin D1 may plausibly direct astrocytosis [71]. Interestingly, a study performed in colorectal cancer cell lines uncovers the possible regulation of COX-2 by the Wnt/β-catenin pathway [75]. Thus, an astrocytic increased activation of Wnt and β-catenin may not only impact cyclin D1 expression but potentially that of COX-2, for which a possible role in ALS neurodegeneration has been described above.

2.4.5. Monoamine oxidase-B

Monoamine oxidase-B (MAO-B) is an outer mitochondrial membrane-bound enzyme that catalyzes the oxidative deamination of biogenic amines, thus producing reactive oxygen species (ROS). MAO-B is primarily found in the CNS where it localizes mainly in astrocytes and radial glial [76]. The spinal cord lumbar region from symptomatic ALS patients displays more MAO-B, due to the general astrocyte proliferation and to a cell-intrinsic increased expression [77]. Using ^{3}H-L deprenyl *in vitro* autoradiography, a more in-depth follow-up study in the *post-mortem* ALS CNS reveals an increased expression in the corticospinal tract, the ventral white matter and in the vicinity of motoneurons. Further, reactive astrocytes

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displayed a higher content of MAO-B compared to microglial cells [8, 78]. Finally, an epidemiological analysis has uncovered that the MAO-B allelic phenotype influences the age of ALS onset [79]. Excessive astrocytic MAO-B expression, which results in elevations of extracellular ROS levels, may have damaging effects on neighboring motoneurons. Additional mechanisms could also involve mitochondrial dysfunction by the selective inhibition of respiratory complex I, which further leads to increased production of superoxide as well as microglial activation [80].

2.4.6. Mitochondrial dysfunctions

While there is a vast amount of research on the mitochondrial dysfunction in ALS motoneurons (reviewed in [81]), not much is known about the impact of toxic genetic mutations on the mitochondria of astrocytes. There is evidence however, that ALS astrocytes do in fact display pathological mitochondrial dysfunction that subsequently leads to oxidative damage, sustaining their reactive status. Indeed, primary astrocytes isolated from the cerebral cortex of neonatal rats and overexpressing $SOD1^{G93A}$ display a decreased mitochondrial respiration rate, an increased superoxide formation and a decreased membrane potential [82]. In co-culture experiments, modulating the mitochondrial defects of $SOD1^{G93A}$-expressing astrocytes via small chemical compounds improves astrocytic-dependent motoneuron survival. Conversely, induction of mitochondrial damage to wildtype astrocytes increases motoneuron death [82]. Thus, organelle dysfunction within ALS astrocytes may be an important contributor to the neurodegenerative process. In addition, a positive amplification system could take place during the degenerative process, since the inflammatory mediator NO, mainly produced by the inducible form of nitric oxide synthase (iNOS) in reactive astrocytes, can in turn induce mitochondrial dysfunction in astrocytes [83].

2.4.7. Activation of microglial cells

A fundamental role for astrocytes in the neuroinflammation process is the recruitment of microglia [84], the resident macrophages of the CNS (reviewed in [85]). In $SOD1^{G37R}$ mice where the mutant *SOD1* gene is specifically deleted in astrocytes, the delay in the progression of the later stages of disease is accompanied with an inhibition of microglial activation and microglia-dependent detrimental NO production [17]. Thus, astrocytosis in ALS may promote neuroinflammation events through microglial recruitment, which in turn may participate directly or indirectly to the motoneuron loss in ALS. Several pro-inflammatory contributors including TNFα, IFNγ, IL-1β and NO, which are aberrantly produced by mutant astrocytes can indeed enhance the activation of microglia. The specific role of microglia in the neuroinflammatory aspects of ALS will therefore be discussed below.

Figure 1 illustrates the potential non-cell-autonomous mechanisms implicating reactive astrocytes in the selective death of motoneurons in ALS.

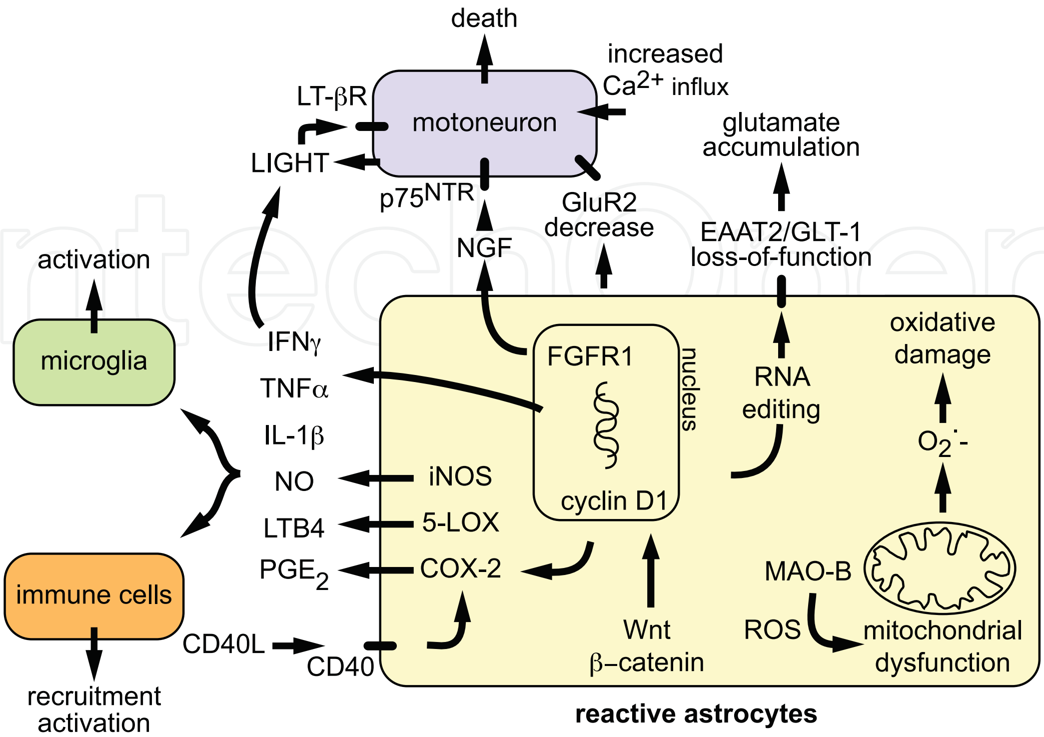

Figure 1. Proposed mechanisms for astrocytic-mediated neuroinflammation and toxicity towards motoneurons. Reactive astrocytes contribute to the degenerative process by influencing the activity of microglial and immune cells as well as by releasing soluble factors that are toxic to motoneurons (as described in section 2).

3. A role for microglia in neuroinflammation

3.1. Activation profile in human and animal models of ALS

Microglia are often termed the immune cells of the CNS as they constantly monitor the neuronal environment in a resting state and become activated upon acute or chronic neuronal damage, eliciting a strong pro-inflammatory response (reviewed in [86]). In ALS patients, reactive microglia are observed in the motor cortex, the motor nuclei of the brainstem, the ventral horn of the spinal cord, along the entire corticospinal tract and within the CSF [87-89]. Given the relationship between astrocytes and microglia [17, 84] and the importance of astrocytosis in ALS, it has been hypothesized that microgliosis may also participate in ALS pathogenesis.

To better understand at which developmental point of the disease reactive microglia appear, microgliosis has been characterized in rodent ALS models at various stages of the disease. Microgliosis occurs in pre-symptomatic and symptomatic *SOD1*^{G93A} spinal cords as well as within various CNS compartments [90-93]. Similarly, *SOD1*^{G37R} mice display microgliosis at both onset and early-stage of the disease [94]. An in-depth characterization of microgliosis in

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SOD1^{G93A} mice via *in vivo* imaging by two-photon laser-scanning microscopy shows that microglia are highly reactive in pre-symptomatic stages while they lose their ability to respond to injury and to monitor the environment as the disease progresses [95]. Indeed, comparison of microglia populations during disease progression reveals that microglia isolated from either neonatal or early onset *SOD1*^{G93A} mice display an alternatively activated M2 phenotype and enhance motoneuron survival while microglia isolated from either adult or endstage *SOD1*^{G93A} mice have a classically activated M1 phenotype and induce motoneuron death [96, 97]. In the pre-symptomatic and symptomatic *SOD1*^{G93A} rat model, microglia aggregates are detected in both the spinal cord and brainstem [98, 99]. Interestingly, the microglia in endstage *SOD1*^{G93A} rats display a degenerative and apoptotic phenotype [98]. Further, in the lumbar spinal cord of pre-symptomatic *SOD1*^{H46R} rats, the microglia express the proliferating marker Ki67 and the phagocytic markers ED1 and major histocompatibility complex (MHC) class II [100, 101]. The thorough investigation of microglial events in rodents therefore suggests that microgliosis not only typifies ALS but that the function of microglia changes during disease progression, thus exerting differential effects on the degenerating motoneurons.

3.2. A role for microglia in ALS pathogenesis

Experimental endeavors have been undertaken to better understand the precise contribution of microglia in the neurodegenerative process. A key finding in support of the proposed direct contribution of microglia to ALS pathogenesis is in ALS mice where the mutant SOD1 (G37R or G85R) is specifically deleted from macrophages and microglial lineages [94, 102]. This results in a delay in the progression but not onset of the disease and a significant extension in lifespan. The importance of microgliosis in ALS pathology was also ascertained in *SOD1*^{G93A} mice bred with *PU.1*$^{-/-}$ mice that lack CNS microglia at birth [103, 104]. While the bone marrow transplantation of *SOD1*^{G93A} microglia into *PU.1*$^{-/-}$ mice did not induce neurodegeneration, the bone marrow transplantation of wildtype microglia into *SOD1*^{G93A};*PU.1*$^{-/-}$ mice improved survival compared to the bone marrow transplantation of *SOD1*^{G93A} microglia [103]. Further, administration of extracellular murine SOD1^{G93A} to primary cultures of microglia activates these cells and renders them neurotoxic [105]. However, phenotypical analysis of microglia in different regions of *SOD1*^{G93A} spinal cord suggests that both neuroprotective and neurotoxic population of microglial cells may coexist during the disease [106]. In fact, the depletion of proliferative microglia does not prevent motoneuron degeneration [107]. Together, these studies suggest that microglia participate, through a complex balance between neuroprotective and neurotoxic signals, in the course of the disease.

3.3. Proposed mechanisms of microglial-derived neurotoxicity

While the injection of motoneuron-directed or ALS patient-derived immunoglobulin G into the spinal cord of mice initiates the recruitment of reactive microglia [108], a study looking at cerebral cortex of ALS patients shows that the phagocytosis of degenerating neurons is mediated by perivascular macrophages and not microglia [109]. This finding already suggested that reactive microglia might play a more complex function in ALS than simply eliminating dying motoneurons. Indeed, various misregulated pathways within ALS microglia have been identified that may influence motoneuron survival.

3.3.1. Endoplasmic reticulum stress

When a cell starts to excessively accumulate misfolded or unfolded proteins, the over-activated endoplasmic reticulum (ER) stress induces apoptosis (reviewed in [110]). Importantly, ER stress is an established characteristic of ALS pathogenesis (reviewed in [111]). In spinal cord microglia of both sporadic ALS patients and symptomatic $SOD1^{G93A}$ mice, there is an increased expression of C/EBP homologous protein (CHOP) [112], a member of the apoptotic ER stress pathway (reviewed in [113]). It remains unclear however if the aberrant levels of CHOP reflect an upstream defect in protein folding or if they directly participate in microglial neurotoxicity. It is noteworthy that the exposure of microglial cells to IFNγ induces iNOS expression, and the subsequent increased NO production can cause an ER stress response involving CHOP [114]. Interestingly, the analysis of selectively vulnerable motoneurons from low-expression $SOD1^{G93A}$, high-expression $SOD1^{G93A}$ and $SOD1^{G85R}$ mice shows the initiation of a specific ER stress response accompanied by microglial activation [115]. Thus, the interaction between ALS motoneurons and microglia may be important in the modulation of the neurodegenerative process.

3.3.2. CD14-toll-like receptor signaling

Once the ligand-dependent CD14 lipopolysaccharide (LPS) receptor located at the microglial surface [116] is activated, it initiates a pro-inflammatory signaling cascade dependent on Toll-like receptors (TLRs), specifically TLR2 and TLR4 [117, 118]. Interestingly, the neurotoxic activation of microglia by extracellular $SOD1^{G93A}$ is mediated by the CD14-TLR pathway [105, 119]. Indeed, immortalized microglia cells expressing mutant SOD1 display an increased TLR2 stimulation and subsequent release of pro-inflammatory cytokines, including TNFα and IL-1β. Importantly, an analysis of spinal cord microglia from sporadic ALS patients shows an enhanced TLR2 immunoreactivity [120]. Recently, it has been shown that the endocytosis of extracellular mutant SOD1 by microglia is required for the activation of caspase-1, which is required for the maturation of IL-1β [121]. This can be paralleled with the finding that the microgliosis caused by fibrillar amyloid beta (Aβ), the main component of the aggregates that are a pathological signature of Alzheimer's disease, also requires CD14, TLR2 and TLR4 [122]. All together, these studies suggest that microglia may participate in motoneuron loss following the specific activation of the CD14-TLR pathway by secreted SOD1 mutant, therefore propagating pro-inflammatory stimuli.

3.3.3. Purinergic signaling

The release of extracellular nucleoside di- and tri-phosphates by degenerating neurons can elicit the activation of microglia through the ionotropic P2X and metabotropic P2Y purinergic receptors. A general alarm signal for microglia is ATP, which can subsequently elicit a pro-inflammatory response, chemotaxis and phagocytosis (reviewed in [123, 124]). Embryonic immortalized microglia and neonatal primary microglial cultures isolated from mutant *SOD1* mice display an upregulation of $P2X_4$, $P2X_7$ and $P2Y_6$ receptors [125]. Notably, the immunoreactivity of P2X is increased within spinal cord microglia of ALS patients [126]. Activation of $P2X_7$ in *SOD1^{G93A}* microglial cells produces significantly higher levels of TNFα, which has a neurotoxic effect on motoneuron cultures [127], and of COX-2, compared to non-mutant microglia [125]. In addition, a reduced ATP hydrolysis activity, possibly implicating the ecto-NTPDase CD39, is observed in mutant SOD1 microglia, suggesting that a potentiation of a purinergic-mediated inflammation can participate to the neuroinflammatory state of microglial cells. Since ATP induces an astrocytic neurotoxic phenotype through $P2X_7$ [128], it is thus feasible to hypothesize that increased extracellular ATP in ALS, whether exacerbated by motoneurons and/or microglia contributes to the pathogenic microgliosis.

3.4. The potential influence of microglia on neuronal excitability

To our knowledge, there is presently no direct assessment of the influence of microglia on motoneuron electrophysiology. However, studies on peripheral nerve injury or spinal cord injury show that microglia activation has prominent effects on neuronal inhibitory control. Importantly, loss of inhibitory control is a contributing mechanism to the motoneuron hyperexcitability that typifies ALS pathogenesis in humans [129].

Loss of neuronal inhibitory control occurs by several means including decrease in gamma-aminobutyric acid (GABA)ergic interneurons [130] combined with changes in the expression of the $GABA_A$ receptor mRNA subunit [131]. $GABA_A$ and glycine receptors are chloride (Cl^-) channels and the expression of cation-chloride co-transporter contributes to inhibitory effects of these Cl^- currents [132]. Indeed, the entry of Cl^- following the opening of $GABA_A$ and glycine receptor-gated Cl^- channels inhibits neuron excitability by hyperpolarizing membrane potential. Under physiological condition, low $[Cl^-]_i$ is maintained by the potassium (K^+)-chloride co-transporter KCC2 that extrudes Cl^- from mature neurons [133]. Stimulation of spinal microglia following peripheral nerve injury induces a decrease in KCC2 expression among dorsal horn nociceptive neurons [134]. KCC2 decrease is induced by the brain-derived neurotrophic factor (BDNF) and this is consistent with the previous observation that BDNF can be produced by non-neuronal cells involved in immune responses, including T and B lymphocytes, monocytes and microglia [135, 136]. BDNF produces a depolarizing shift in the anion reversal potential of dorsal horn lamina I neurons due to an increase in $[Cl^-]_i$. This shift prompts an inversion of inhibitory GABA currents that contributes to neuropathic pain following nerve injury [135]. Decrease in KCC2 expression is thus responsible for the excitatory effects of GABA on neurons. Microglia activation and BDNF secretion are mediated through ATP activation of microglial P2X receptors. As described earlier, P2X receptors might be involved in ALS pathology since a higher density of $P2X_7$-immunoreactive microglial cells/macrophages are found in affected regions of spinal cords from ALS patients [126]. Levels of BDNF have been found to be increased in microglial cells isolated from ALS mice at the onset of disease and KCC2 is decreased in vulnerable motoneurons in *SOD1^{G93A}* mice [96, 137]. Additionally, BDNF might play a role in the microglia's influence on motoneuron electric activity as suggested by work on spasticity. Spasticity is characterized by a velocity-dependent increase in muscle tone resulting from hyperexcitable stretch reflexes, spasms and hypersensitivity to normally innocuous sensory stimulations. Spasticity develops following spinal cord injury and is also regarded as an ALS clinical symptom [138]. The main mechanisms hypothesized to be responsible for spasticity are increased motoneuron excitability and increased synaptic inputs in response to muscle stretch due to reduced inhibitory mechanisms. Recently, it has been demonstrated that, following spinal cord injury, increased levels of BDNF mediated spasticity, due to post-transcriptional down regulation of KCC2 [139]. Together, these studies suggest that reactive microglia in ALS may exert an aberrant effect on the electrical activity of motoneurons and highlight the importance of furthering our understanding of this functional interaction.

Lastly, a hypothetical scenario relates to the defect in astrocytic glutamate transporter and the neurotoxic accumulation of the excitatory amino acid that we have mentioned above. It has been demonstrated that TNFα promotes the release of glutamate by activated microglia through the cystine/glutamate exchanger (Xc)[140]. Though the implication of the Xc system in ALS has not yet been investigated, it is intriguing that the Aβ peptide induces a neurotoxic phenotype in microglia through the Xc-mediated release of glutamate Therefore, system Xc represents a potential mechanism of microglia-mediated excitotoxicity that warrants further study [141].

The potential non-cell-autonomous mechanisms involving microglial cells in the selective degeneration of motoneurons in ALS are illustrated in Figure 2.

4. Involvement of neuroimmunity in motoneuron degeneration

4.1. Pathological phenotype of the immune system in ALS

In addition to astrocytes and microglia, immune cells may also play synergistic and critical roles in ALS neuroinflammation and disease progression. Presence of a systemic immune activation is suggested by abnormalities observed in the blood and CSF of ALS patients such as increased numbers of circulating lymphocytes (CD4+ helper T cells, CD8+ cytotoxic T lymphocytes (CTL) and natural killer (NK) cells), increased expression of MHC class II molecules on monocytes as well as higher levels of inflammatory chemokines and cytokines (regulated on activation normal T cell expressed and secreted (RANTES), monocyte chemotactic protein (MCP-1), IL-12, IL-15, IL-17 and IL-23)[142-146]. Further, *post-mortem* studies of brain and spinal cord from ALS patients show that the activation and proliferation of microglia is associated with an infiltration of activated macrophages, mast cells and T lymphocytes which are found in close proximity to degenerating tissues [147-149]. An in-depth autopsy of six ALS patients reveals an enrichment of T-cell receptor Vβ2 positive T cells in the spinal cord and CSF, suggesting an antigen-driven T cell selection [150]. Finally, ALS patients with a more rapidly progressing pathology show decreased numbers of regulatory T lymphocytes (Tregs), suggesting that the numbers of Tregs are inversely correlated with disease progression [144, 151]. Tregs secrete anti-inflammatory cytokines such as IL-4, IL-10 and transforming growth factor beta (TGF-β) as well as the neurotrophic growth factors glial-derived neurotrophic factor (GDNF) and BDNF. Tregs are also able to dampen a Th1 pro-inflammatory response and

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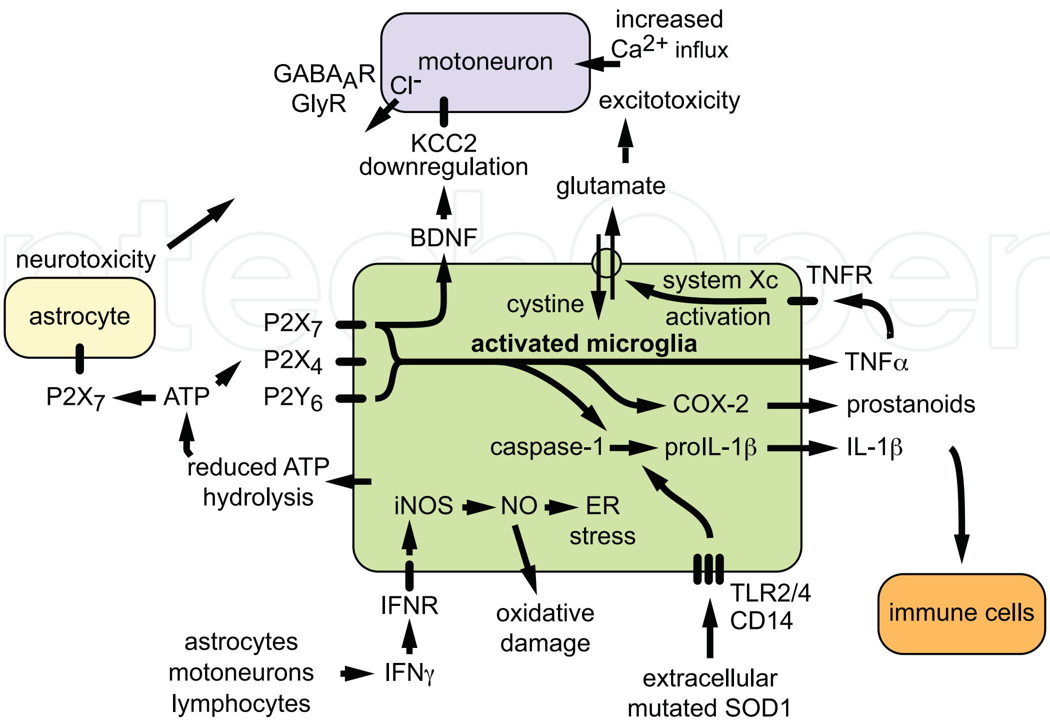

Figure 2. Proposed mechanisms by which microglial activation and inflammation contribute to the neurodegenerative process in ALS. Microglia can influence astrocytes and immune cells as well as directly impact motoneuron viability via several mechanisms.

attenuate toxic microglial responses. Contribution of the innate immune system is also suggested by the presence of immunoglobulins and complement deposition as well as a significant increase of NK cells in the blood of ALS patients [87, 144, 152]. While these investigations of ALS samples and tissues do not assess the contributory role of the immune system to the disease pathogenesis, they do highlight its active presence.

In support of what is observed in humans, ALS rodent models also display a particular immunological phenotype. Indeed, *SOD1^{G93A}* mice demonstrate that the inflammatory cell subtypes are phenotypicaly and functionally different depending upon the disease stage [96]. During the initial stages, infiltrating CD4+ T cells are almost mainly Th2 (IL-4+) while as the disease progresses there is a skew toward Th1 (IFNγ+) cells and CD8+ T cells (both IL-17A positive and negative)[106, 153]. Alteration in inflammatory cell subtypes is associated with, and maybe driven by, differences in Tregs. Interestingly, early symptomatic *SOD1^{G93A}* mice have increased numbers of Tregs and a decreased proliferation of effectors T lymphocytes (Teffs), whereas decreased numbers of Tregs and increased proliferation of Teffs is found in end-stage animals [151, 154]. The innate immune system is also affected in ALS rodents, displayed by the substantial increase of NK and NKT in the spinal cord of *SOD1^{G93A}* mice [155, 156].

Whether neuroinflammation is a cause or a consequence of motoneuron death is still debated. It is interesting to note that inflammation is not limited to the CNS but systemic with increased levels of plasma LPS associated with increased numbers of activated circulating monocytes and T lymphocytes that correlate with disease evolution [142, 157]. A thymic dysfunction is also observed in parallel to the neurodegenerative process in mutant SOD1 mice and ALS patient [158]. In the CNS of ALS patients, TAR DNA-binding protein 43 (TDP-43) increased and interacts with nuclear factor kappa B (NF-κB) in glial and neuronal cells. LPS-activation of NF-κB in microglial cells expressing the TDP-43 mutant is associated with the production of pro-inflammatory cytokines, including TNFα, IL-1β, IL-6 and IFNγ [159]. The central role of inflammation and NF-κB in ALS was recently confirmed by the description in familial ALS of mutations in the gene encoding optineurin, a negative regulator of TNF-induced NF-κB activation [160].

Altogether, the information from pre-clinical models and ALS patients suggest that systemic immune activation (innate and adaptive) might play a key role in ALS pathogenesis and may represent an interesting target for the development of novel treatments. However, a better understanding of the specific roles played by the different subtypes of immune cells is of utmost necessity. Indeed, accumulative evidence suggests that inflammatory cells mediate both protective and deleterious effects on motoneuron survival and that these functions vary during disease progression.

4.2. The protective function of the immune response in ALS

Protective immunity, a homeostatic phenomenon important in the repair of damaged tissues, results from both the clearance of debris and the effects of cytokines and growth factors delivered by inflammatory T-cells to the site of injury [161, 162]. The neuroprotective ability of immune cells is also evident in ALS. Indeed, when *SOD1*^{G93A} mice are bred with mice lacking functional T cells or CD4+ T cells, microglia skew towards an M1 inflammatory phenotype and disease progression accelerates, suggesting that CD4+ T cells provide neuroprotection by suppressing the cytotoxic activation of microglia. Accordingly, reconstitution of T cells following bone marrow transplantation of *SOD1*^{G93A} mice lacking functional T and B cells prolonged their survival and suppressed the activation of M1 microglia [163]. Further analysis shows that the increased numbers of CD4+/CD25+/Foxp3+ Tregs during early symptomatic stages secrete IL-4, thus promoting the M2 protective microglia while inhibiting the neurotoxic Th1 response and IFNγ secretion. As described above, these neuroprotective Tregs are decreased as the disease progression accelerates. Co-culture experiments show that Tregs suppress the expression of cytotoxic factors Nox2 and iNOS from *SOD1*^{G93A} microglia through IL-4. Tregs also inhibit the proliferation of *SOD1*^{G93A} Teffs via the combined secretion of IL-4, IL-10 and TGF-β[154]. The neuroprotective properties of Tregs are also reinforced by their ability to secrete GDNF and BDNF, thus attenuating toxic microglial responses [164]. Importantly, the passive transfer of endogenous Tregs into *SOD1*^{G93A} mice lengthens disease duration and prolongs survival, suggesting that Tregs is likely the neuroprotective subpopulation among CD4+ T lymphocytes. Therefore, a subtype of immune cells appear to have a beneficial

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role in ALS and targeting the Tregs/M2 signaling pathway may be an attractive therapeutic strategy for this neurodegenerative disease.

4.3. The neurotoxic function of the immune response in ALS

T lymphocytes could mediate motoneuron damage either directly through cell-cell contact, secretion of cytokines or indirectly through activation of microglia and macrophages [165]. As mentioned above, the effect of the immune system varies during disease progression from a protective role at early stages to a neurotoxic activity when disease accelerates [151]. Neuroprotective activity has been associated with a Tregs/M2 response and expression of trophic and anti-inflammatory factors such as BDNF, GDNF and IL-4 whereas neurotoxic effects are associated with an M1/Th1/CTL pro-inflammatory immune response [106]. Accordingly, mutated SOD1 Teffs proliferate to a greater extend and produce more IFNγ (Th1-driven) during the rapidly progressing phase than Teffs isolated during slowly progressing phase [154]. Different death pathways can be induced by Th1/CTL lymphocytes and promote motoneuron loss in ALS. For instance, activation of Fas (CD95) has been demonstrated to trigger a motoneuron-restricted death pathway. Motoneurons expressing ALS-linked SOD1 mutants showed an increased susceptibility to Fas-mediated death through activation of an amplification loop [166-168]. Accordingly, mutant *SOD1* mice with homozygous FasL mutation present a reduced loss of motoneurons and a prolonged life expectancy [169]. Likewise, the RNA interference-mediated silencing of Fas following intrathecal delivery of Fas-specific small interfering RNA improves motor function and survival in ALS mice [170]. While it remains unclear if T lymphocytes contribute to Fas-induced motoneuron degeneration, these studies suggest the possibility of their direct participation in the degenerative process.

T lymphocytes could also amplify the neuroinflammation in ALS via glial cells. Upon activation, microglia cells increase membrane expression of MHC class II molecules, becoming efficient antigen presenting cells able to actively drive T cell activation and differentiation. In turn, cytokines secreted by T cells modulate microglia phenotype and function. For instance, TNFα and IFNγ, two major pro-inflammatory cytokines produced by Th1 lymphocytes induce and activate M1 microglial cells and cause neurotoxicity toward motoneurons. Experimental studies in ALS mice demonstrated that inflammatory cell subtypes were phenotypicaly and functionally different depending upon the disease stage [96]. At initial stages, microglia exhibits anti-inflammatory M2 phenotype (Ym1+, CD163+) and infiltrating T cells are almost exclusively CD4+ while end-stage disease is associated with a skew of microglia toward a pro-inflammatory M1 phenotype (Nox2+) and T lymphocytes are mainly Th1 cells [106].

The neurotoxic effect of NK cells is suggested by the neuroprotective effect of the immunomodulation of NK cells, which increases lifespan of ALS mice and is accompanied by a reduced astrocytosis. While the pathological modalities of NK cells in ALS remain elusive, several hypothetical mechanisms can be raised. Indeed, activated NK (and to a lesser extent CD8+ T cells) inhibit neurite outgrowth of cerebellar neurons in a cell contact-dependent manner *in vitro* [171]. In sensory neurons, IL-2-activated NK cells have a killing activity that requires cellular contact and perforin [172]. Further, the production of IFNγ by activated NK cells might directly trigger motoneuron death through the LIGHT/LT-βR pathway or potentiate a cytotoxic Th1/CTL response via the combined action of other NK-related cytokines such as IL-17 or IL-22 [173]. Of note, NK cells also produce IL-4 upon activation, which as described earlier, mediates a neuroprotective effect. Therefore, NK cells represent an appealing branch of the immunopathology that could be considered as a therapeutic target for ALS.

In addition to the adaptive immune system, several studies suggest that humoral immunity and immunoglobulins could also contribute to the disease. Autoantibodies to voltage-gated Ca^{2+} or K^{+} channels have been described in ALS patients, which induce specific motoneuron alterations both *in vitro* and *in vivo* after passive transfer in mice [174-178]. Accordingly, C5a and other complement activation products released after activation of the classical complement pathway by antibodies are elevated in the CSF and spinal cord of ALS mice and patients and specific inhibition of C5a receptor ameliorates disease in *SOD1*^{G93A} mice [179, 180]. Additionally, abnormal levels of anti-Fas antibodies, able to induce neuronal apoptosis *in vitro*, have been detected in the serum of patients with ALS [181, 182]. Thus, both the innate and adaptive immune system appear to have deleterious consequences on the survival and maintenance of motoneurons in ALS.

Figure 3 illustrates the potential mechanisms implicating different populations of immune cells in ALS pathogenesis.

5. Pre-clinical therapies targeting neuroinflammation

5.1. Pharmacological targeting of the neuroinflammatory response

In light of the salient evidence supporting the contribution of neuroinflammation in ALS, several drug- or cell-based therapeutic approaches have been evaluated in ALS mice for their ability to modulate the pathologic process. Those that have shown a positive effect on astrocytosis and microgliosis are described below and have been categorized based on their desired functional target.

In order to mitigate the detrimental effects of the overactive $p75^{NTR}$ pathway in ALS, an antagonist that mimics the short NGF β loop region that binds the $p75^{NTR}$ has been utilized [183]. Unfortunately, the intraperitoneal (i.p.) delivery of the $p75^{NTR}$ antagonist from asymptomatic stage up until the endpoint of the disease does not improve the phenotype or survival of *SOD1*^{G93A} mice [183]. However, antisense peptide nucleic acid-based silencing of $p75^{NTR}$ following early systemic i.p. administration delays by about 10% both onset and progression of the disease [184]. Although, alternative route of administration or development of more efficient molecules should be assessed, $p75^{NTR}$ represents a therapeutic target that needs to be further explored.

COX-2 appears as an appealing therapeutic target for ALS as it promotes both pro-inflammatory events and astrocytic glutamate release [60, 67]. Celecoxib, a COX-2 inhibitor, fed to *SOD1*^{G93A} mice from asymptomatic to end-stage results in a delayed onset and an increased lifespan of approximately 25%. Celecoxib treatment prevents loss of spinal motoneurons and

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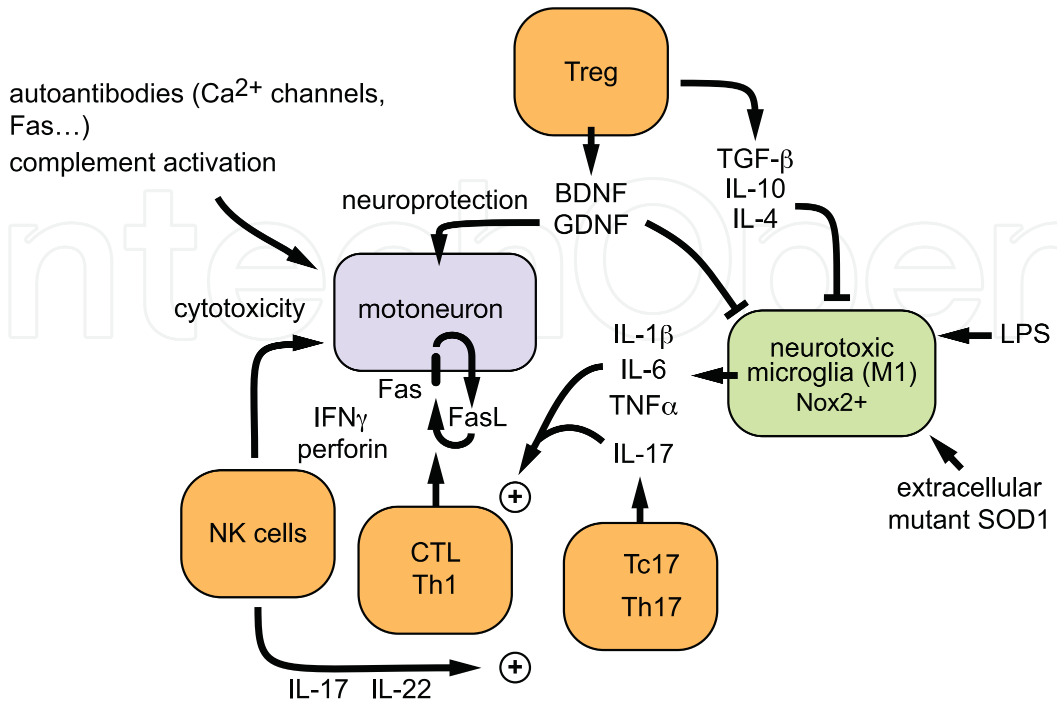

Figure 3. Potential Mechanisms by which peripheral and central immunity might contribute to the neurodegenerative process in ALS. Both neuroprotective and neurotoxic functions can be proposed for the involvement of lymphocytes in ALS pathogenesis (as described in section 3).

reduces astrocytosis [185]. Importantly, celecoxib-treated *SOD1*^{G93A} spinal cords display reduced levels of PGE2, a potent pro-inflammatory mediator as well as a signal for glutamate release from astrocytes [67].

Lenalidomide, an immunomodulatory drug with pleiotropic properties derived from thalidomide, has been evaluated in mutant SOD1 mice due to its inhibitory effect on TNFα production by monocytes [186]. A lenalidomide-diet given to *SOD1*^{G93A} mice retards disease onset, ameliorates motoneuron survival and extends survival by 18%. A significant decrease in IL-1α and TNFα as well as an increase in IL-1 receptor antagonist (IL-1RA) and TGF-β1 is observed in the spinal cord of Lenalidomide-treated mice [187]. In a similar study, the lifespan of ALS mice treated with lenalidomide at onset of symptoms is increased by 12%. Concomitantly, there is an improved survival of motoneurons, decreased levels of the pro-inflammatory cytokines TNFα and Fas associated Factor as well as an increased expression of the anti-inflammatory cytokines TGF-β3 and IL-1RA [188].

Epigallocathecin gallate (EGCG) is a green tea polyphenol that can prevent microglial neurotoxicity through the modulation of TNFα mRNA transcription and release as well as iNOS production [189]. The daily oral administration of EGCG to *SOD1*^{G93A} mice daily from asymptomatic to endstage delays disease onset and lifespan by approximately 10 and 14%, respectively. EGCG also moderately mitigates motoneuron loss and reduces microglia activation [190].

Pioglitazone is a drug that was initially developed to treat type II diabetes patients that also exerts ant-inflammatory and neuroprotective activities (reviewed in [191]). For these reasons, it has been hypothesized that it may improve ALS pathology. Indeed, pioglitazone-fed *SOD1*^{G93A} mice have a delayed onset of 10% and a prolonged lifespan of about 8% [192]. Pioglitazone significantly reduces microgliosis and astrocytosis in *SOD1*^{G93A} mice as well as alters the expression profile of spinal cord lysates from pro-inflammatory to anti-inflammatory [192, 193]. Further analysis of spinal cords reveals that pioglitazone may act through the inhibition of the p38 kinase, NF-κB and STAT3 pathways [167, 193, 194].

Olesoxime has previously been selected as a neuroprotective agent via a motoneuron survival-based screen [195]. Interestingly, *SOD1*^{G93A} mice fed an olesoxime diet from asymptomatic stage to end-stage survive 10% longer than non-treated mice and also demonstrate a reduction in both astrocytosis and microgliosis [195, 196].

Dicatechol nordihydroguaiaretic acid (NDGA) is a selective inhibitor of 5-LOX that presents TNFα antagonizing activity in microglial cells. *SOD1*^{G93A} mice on an NDGA-diet from pre-symptomatic stage to end-point have a 32% increase in median lifespan as well as a reduced motoneuron loss and astrocytosis [197].

Minocycline is a member of the tetracycline molecules that can enter the CNS and mediates inflammation and microgliosis (reviewed in [198]). Asymptomatic *SOD1*^{G93A} mice that received daily minocycline by i.p. injection have a delayed disease onset, a 16% increase in lifespan as well as a preservation of spinal motoneurons [199]. Similarly, minocycline-fed late pre-symptomatic *SOD1*^{G37R} mice display a 6% longer survival, an increased number of spinal cord motoneurons and a reduced microgliosis [200]. However, a minocycline diet in symptomatic *SOD1*^{G93A} has no effect on survival while amplifying both astrocytosis and microgliosis [201]. These results strikingly illustrate the time-dependent dynamics of the neuroinflammation response, highlighting not only the requirement to target the most pertinent therapeutic molecular and cellular effectors but to also do so at the proper stage of the disease.

5.2. Advances and possible applications of protein therapy

In addition to chemical compounds, the therapeutic delivery of proteins has also been assessed as a potential modulator of neuroinflammation in ALS. Indeed, the granulocyte-colony stimulating factor (G-CSF), a hematopoietic growth factor, has been delivered to *SOD1*^{G93A} mice by osmotic pump starting at asymptomatic stage for a continuance of 8 weeks [202]. G-CSF-recipient ALS mice display a delay in disease onset as well as an increased motoneuron survival. The time to clinical endstage is increased by 10% in *SOD1*^{G93A} mice receiving G-CSF. Importantly, while G-CSF was initially used for its neuroprotective effects and its ability to readily cross the blood-brain barrier [203, 204], further characterization of treated *SOD1*^{G93A} mice shows a reduced spinal cord astrocytosis and microgliosis as well as an increased availability of migratory healing monocytes, suggesting that G-CSF may be beneficial in ALS via its modulation of neuroinflammation [205].

Another potential protein therapy is the administration of the activated protein C (APC), a plasma protease with anti-coagulant, neuroprotective and anti-inflammatory functions (reviewed in [206]). A daily i.p. injection of APC to symptomatic *SOD1^{G93A}* mice until death slows disease progression, leading to a 25% increase in lifespan [207]. Further, APC appears to exert its beneficial effects via a downregulation of mutant SOD1 expression in both motoneurons and microglia, thus resulting in delayed neuroinflammatory events [207].

Anakinra (Kineret), a recombinant form of human IL-1RA, that inhibits the pro-inflammatory activity of both IL-1α and IL-1β, is approved by the U.S food and drug administration for rheumatoid arthritis [208]. When administrated by i.p. daily to asymptomatic stage to *SOD1^{G93A}* mice, it ameliorates motor function and prolongs lifespan by approximately 4% [121].

The CD40 costimulatory pathway, which plays an important role in B and T cell activation [209], has been proposed to contribute to ALS pathogenesis. The weekly delivery of a blocking anti-CD40L antibody by i.p. injection starting at an asymptomatic stage delays onset and prolongs survival by approximately 7%. Consistently, anti-CD40L delivery reduces significantly the percentage of peripheral CD8+ T cells as well as GFAP+ astrocytes and Mac2+ microglia in the spinal cord [66], suggesting that the CD40 pathway, an integral component of neuroimmunity, is a potential therapeutic target in ALS.

5.3. Cell therapy perspectives

While drugs and protein therapy target the misregulated pathways within astrocytes and microglia, the aim of cell therapy is to replace these aberrantly functional cells by healthy ones or use implanted cells as a therapeutic platform to deliver neurotrophic support, thus hopefully alleviating neuroinflammation in ALS.

5.3.1. Glial precursor cells

Glial cell therapy has indeed been evaluated by isolating human neural progenitor cells (hNPCs) and genetically modifying them to express GDNF [210, 211]. Prior to direct injection in the spinal cord of *SOD1^{G93A}* rats, hNPCs were pre-differentiated into astrocytes [210]. Despite the fact that the hNPC injection did not increase the lifespan of *SOD1^{G93A}* rats, they do localize within both the grey and white matter of the spinal cord and survive until the death of the animal. Further investigation of this method reveals that hNPCs preserve dying motoneuron cell bodies in *SOD1^{G93A}* rats without improving their innervations at the neuromuscular junction [212]. In *SOD1^{G93A}* mice, GDNF-expressing hNPCs also migrate to the spinal cord where a subset of them differentiates into astrocytes, again without improving survival or neurodegeneration [213]. However, the lack of beneficial outcome is most likely due to the regional specificity of GDNF's biological activity, as intramuscular but not intraspinal delivery of GDNF exerts its neuroprotective effect [214, 215]. Another astrocyte precursor with potential benefits is the glial-restricted progenitors (GRPs), isolated from the spinal cord of embryonic rats [216]. The transplantation of GRPs in the ventral horn of *SOD1^{G93A}* rats shows that these cells differentiate into astrocytes and can survive and migrate along the spinal cord [217]. Importantly, GRP-recipient ALS rats survive longer as well as show a slower cervical neurodegeneration and a reduced spinal cord microgliosis. In *SOD1*^{G93A} mice however, while the GRPs efficiently differentiate into astrocytes, survive, and locate to both grey and white matter of the spinal cord, they do not influence lifespan and do not prevent motoneuron loss [218].

Human umbilical cord blood cells (hUCBCs) also have the potential to differentiate into glial cells (reviewed in [219]). Pre-symptomatic *SOD1*^{G93A} mice received either native hUCBCs or cells engineered to overexpress vascular endothelial growth factor (VEGF) and/or FGF [220]. Two weeks following the orbital injection, analysis of the spinal cords reveals the presence of the transplanted hUCBCs with the non-modified cells preferentially differentiating into microglia while the cells expressing the growth factors became astrocytes [220]. Importantly, the administration of hUCBCs to pre-symptomatic and symptomatic *SOD1*^{G93A} mice via intravenous injections delays disease progression, increases lifespan by approximately 8%, prevents motoneuron loss and reduces both astrocytosis and microgliosis [221].

5.3.2. Mesenchymal stem cells

Mesenchymal stem cells (MSCs) are multipotent stem cells that can differentiate in a broad variety of cells and instigate a reparative environment. MSCs have been therapeutically assessed in light of their immunosuppressive capacities, limiting inflammatory responses in their surroundings [222]. MSCs isolated from rat muscle injected into the CSF of *SOD1*^{G93A} rats subsequently localize to the spinal cord and adopt astrocytic characteristics [223]. The injected ALS rats display motor deficits at the same age than vehicle-injected animals. However, the MSC-injected *SOD1*^{G93A} rats show an increased survival of approximately 11%, an increased number of motoneurons as well as a reduced neuroinflammation [223]. Similarly, the intravenous injection of MSCs after the onset of disease in *SOD1*^{G93A} mice increases lifespan by 13% and reduces both astrocytosis and microgliosis [224]. Alternatively, a combination of intraspinal and intravenous transplantation of MSCs has been evaluated in *SOD1*^{G93A} rats at disease onset and leads to a 6% increased in survival [225]. Similarly, the intracisternal delivery at asymptomatic stage of human MSCs derived from ALS patients also prolongs survival ALS mice by about 6% [226]. Further, intramuscular administration of MSCs genetically engineered to produce GDNF in asymptomatic *SOD1*^{G93A} rats does not influence the time of disease onset but prolongs survival of implanted ALS rats. However, the beneficial effect of MSCs transplantation was not correlated with decreased astrocytosis or microgliosis [227]. Although this pre-clinical evidence proposes MSC-based therapy as a potential means to intervene in the course of disease, the neuroprotective mechanisms involved remain elusive, especially regarding the immunosuppressive abilities of MSCs.

6. Clinical aspects

As discussed in the present book and chapter, the molecular pathways and cellular effectors responsible for ALS are numerous and their precise contributions to disease pathogenesis are still debated. While this has made translational therapy a challenge, it has shifted the devel-

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opment of neuronal-specific therapies toward those targeting more general phenomena characterizing ALS, including neuroinflammation.

The pattern of neurodegeneration in ALS has been described as overall linear, albeit with some variations [228, 229]. One of the biggest discrepancies between individuals is the evolution of the disease, which ranges from death in less than 6 months to a limited handicap after more than 10 years following the initial diagnosis. Either rapid or slow, the topography of neurodegenerative events is rather reproducible, usually spreading from one limb to the opposite one and then to another level. Thus, from the moment a patient presents himself at the clinic with symptoms, there is a progressive extension and diffusion of the pathological process. This spreading of neurodegeneration over time may result from the infiltration and migration of non-neuronal cells or by the exchange of molecules from one cell to another. This hypothesis, based on pre-clinical and clinical observations, highlights the importance of developing therapies that modulate immunity and/or neuroinflammation.

As described in section 2.3, the excitotoxic theory suggests that glutamate accumulates within the intercellular space and induce a pathological synaptic excitotoxic transmission, leading to motoneuron death [230]. This hypothesis motivated a series of clinical trials with riluzole, a potent glutamate antagonist, culminating in the demonstration that riluzole is efficient in slowing down disease progression [231]. To date, only riluzole is marketed as a *bona fide* treatment for ALS. While riluzole is thought to reduce glutamate release in neurons via the inhibition of voltage-gated sodium channels [232], riluzole may also have some important anti-inflammatory functions. Indeed, riluzole significantly decreases IL-1β, TNFα and iNOS levels as well as increases IL-10 levels in LPS-activated microglial cells [233]. Another neuroprotective mechanism mediated by riluzole may include the production of BDNF and GDNF by astrocytes, as demonstrated in cultures [234]. In experimental autoimmune encephalomyelitis (EAE), a commonly used murine model of multiple sclerosis, riluzole administration significantly ameliorates motor functions. Importantly, the decrease in the clinical severity of riluzole-treated EAE mice is associated with a diminished inflammatory response and a marked reduction in lymphocytes infiltrating the spinal cord [235]. Together, these results suggest a more complex mode of action for riluzole where a modulation of inflammation should be acknowledged as one of its therapeutic activity.

Other immunomodulatory agents have also been tested in ALS clinical trials, but their therapeutic benefits have not been as promising as those demonstrated by riluzole [236]. Indeed, immunosuppressants such as cyclosporine or cyclophosphamide as well as the more aggressive total lymphoid irradiation were not successful. The intravenous immunoglobulin G (IVIg) treatment has been proposed to suppress inflammatory responses by inducing an IFNγ-refractory state in macrophages [237]. Interestingly, an open-label pilot study of IVIg administration in ALS patients led to a transient clinical improvement in subjects with bulbar-ALS but not in patients with lower signs, suggesting that immunomodulation may have therapeutic potential [238]. Nevertheless, the combined administration of cyclophosphamide and IVIg in another cohort of 7 patients with upper and lower signs did not lead to clinical improvement [239]. These studies highlight the importance of a better identification of targets as well as a more efficient and specific design of therapies by specifically taking into account the clinical heterogeneity of the disease.

Among the drugs mentioned that have been evaluated in pre-clinical models, celecoxib and pioglitazone were both assessed in a randomized, double-blind, placebo-controlled trial, but gave disappointing results as there were no effects on motor function and survival rate [240, 241]. When minocycline was tested in a randomized placebo-controlled phase III trial, it not only did not show any benefits, it in fact displayed serious harmful effect in patients [242]. Thalidomide, an analogue of lenalidomide, which showed therapeutic potential in SOD1 mutant mice, was used in a single arm, open label phase II study. Unfortunately, similar to minocycline, thalidomide led to undesirable side effects, without any positive effects [243]. A pilot trial (double-blind, placebo-controlled, randomized) where G-CSF was administered to ALS patients for over 25 days does however show encouraging results on the prevention of degeneration of several white matter tracts [244]. This study supports a larger scale trial in which the immunomodulatory aspect of G-CSF should be further explored.

The translational therapy of neuroinflammatory and immunomodulatory effectors has thus shown both exciting and disappointing outcomes. There are many factors that could help explain the discrepancy between pre-clinical and clinical evaluations of potential therapies. Firstly, most drugs are typically assessed in the mutant SOD1 animal models. This poses an important caveat, as not only there exists an obvious difference between humans and animals, but SOD1 models also represent hereditary ALS, which account for only 4% of ALS cases. Thus, a drug may show a positive influence on an inherited disease model without having any effect on sporadic cases. There is therefore the risk of wrongly eliminating or pushing forward an ALS treatment due to the lack of diverse familial and sporadic pre-clinical models. Secondly, the exact timing of a specific treatment could also impact its efficiency. Indeed, as described in the present chapters, neuroinflammation consists of dynamic mechanisms, combining over time and space, different cell types with opposing neuroprotective and neurotoxic functions. Thus, depending on the desired target, the therapeutic window of various drugs may differ one from another. Thirdly, while establishing a dosage regimen (concentration of drug and treatment length) is amenable in pre-clinical models, determining the exact dose and duration of a therapy in human patients is somewhat more complex. Further, it remains unclear if the treatment of ALS patients should take place daily for several months or periodically in pulses. It thus becomes imperative to develop new analytical methods to adequately extract from pre-clinical studies the equivalent doses for humans as well as the optimal treatment protocol. Finally, in light of the high heterogeneity of ALS forms, it is possible that not all patients will respond equally to a particular therapy. Therefore, all of these parameters, including additional ones not mentioned herein, have to be thoroughly considered and analyzed to ensure that we do not wrongly disregard or promote a drug.

When dealing with neuroinflammation in ALS, the therapeutic intervention is of a different kind than the previous major clinical trials. It is not compulsorily a matter of influencing the disease process, that is the motoneuron death itself, but a matter of stopping a potential amplification and/or diffusion phenomenon. In animal models, this strategy has given

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interesting results but there still remains a lot of work before a successful therapy targeting neuroinflammation is translated into humans.

7. Future directions

In the present chapter, we have described the cellular and molecular events characterizing the neuroinflammation in ALS. We have also highlighted the beneficial potential of various therapeutic approaches specifically targeting these neuroinflammatory effectors. While the reports discussed herein support a role for astrocytes, microglia and immune cells in ALS, it remains unclear how they influence disease onset, progression or both. Hence, a thorough investigation of the neuroinflammatory pathways that impact neurodegeneration will ultimately enhance our understanding of how and when to therapeutically modulate this pathological process. Further, it is important to remember that the astrocytosis and microgliosis that typify ALS stem from the chronicity of this neurodegenerative disorder and thus, there is an active communication with the neurotoxic environment that is composed of neurons, glial cells and immune cells. Therefore, it is with caution that we should proceed with defining a causal or consequential role for neuroinflammation in ALS, but instead, our focus should be on identifying its exact pathological contribution.

List of abbreviations

5-LOX, 5-lipoxygenase; Aβ, amyloid beta; ALS, amyotrophic lateral sclerosis; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; APC, activated protein C; BDNF, brain-derived neurotrophic factor; CHOP, C/EBP homologous protein; CNS, central nervous system; COX-2, cyclooxygenase; CSF, cerebrospinal fluid; CTL, cytotoxic T lymphocytes; EAE, experimental autoimmune encephalomyelitis; EGCG, Epigallocathecin Gallate; excitatory amino-acid transporter 2; FDA, food and drug administration; FGF, fibroblast growth factor; G-CSF, granulocyte-colony stimulating factor; GABA, gamma-aminobutyric acid; GDNF, glial-derived neurotrophic factor; GFAP, glial fibrillary acidic protein; GLT-1, glutamate transporter 1; GRP, glial-restricted progenitor; glutamate receptor, GluR; IL-1RA, IL-1 receptor antagonist, IFN, interferon; IL, interleukin; i.p., intraperitoneal; ISG15, interferon-stimulated gene 15; IVIg, Intravenous immunoglobulin G; KCC, potassium (K^+)-chloride co-transporter; LPS, lipopolysaccharide; LT-βR, lymphotoxin beta receptor; MAO-B, monoamine oxidase-B; MHC, major histocompatibility complex; MSC, mesenchymal stem cell; NDGA, dicatechol nordihydroguaiaretic acid; NF-κB, nuclear factor kappa B; NK, natural killer; NMDA, N-methyl-D-aspartic acid; NPC, neural progenitor cell; NO, nitric oxide; NTF, neurotrophic factor; $p75^{NTR}$, p75 neurotrophin receptor; PGE_2 prostaglandin E_2; ROS, reactive oxygen species; SOD1, superoxide dismutase 1; STAT, signal transducer and activator of transcription; TDP-43, TAR DNA-binding protein 43; Teff, effectors T lymphocyte; TGF-β, transforming growth factor beta; TLR, Toll-like receptor; TNF, tumor necrosis factor; Treg, regulatory T lymphocyte; UCBC, umbilical cord blood cell; VEGF, vascular endothelial growth factor.

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