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Modulation of the inflammatory response in amyotrophic lateral sclerosis

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SUMMARY

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Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease that causes progressive paralysis and death to patients due to the degeneration of motor neurons in the spinal cord and the brain. At present, therapy is mainly symptomatic and fails to halt disease progression.

A common feature of ALS, and other neurological disorders, is the occurrence of an inflammatory reaction consisting of activated glial cells (microglia and astrocytes) within the central nervous system, and leukocytes, mainly macrophages, in the peripheral nerves. The inflammatory response is a physiological process with very precise control and plays an essential role in the removal of cell debris and the activation of repair processes in infected or injured tissues. However, immune cells also secrete cytotoxic mediators that exert damage in healthy neighboring cells and even lead to cell death. This dual sword edge of immune cells likely depends on regulatory mediators that are present in the milieu. However, in ALS, as well as, in other neurological conditions inflammatory response is believed to trigger greater hazardous than protective actions.

Based on these evidences, in the present thesis we aimed at assessing whether modulation of key aspects of inflammation could ameliorate the clinical course of ALS disease. In particular, we have focused our interest in three main targets: (i) the proinflammatory colony stimulating factor 1 receptor; (ii) the anti-inflammatory cytokine, interleukin-37; (iii) the immunoresolvent agent, Maresin-1. We provide novel data demonstrating that these approaches confer neuroprotection against the clinical course of ALS disease.

INTRODUCTION

INTRODUCTION

Motor neuron disease

Motor neuron diseases (MND) are a complex neurodegenerative condition clustered by a common hallmark: selective degeneration of lower and/or upper motoneurons (MNs) (Figure 1). Despite the ethology and the clinical spectra may vary, it manifests as muscle weakness, breathing, swallowing and speech problems along with spasticity of hind or forelimbs. The most common form of MND is amyotrophic lateral sclerosis (ALS; commonly referred to Lou Gehrig's disease in the United States), a fatal neurodegenerative disease of adult onset (Wijesekera and Leigh, 2009). The worldwide incidence for ALS (/100,000 population) is 2-3 cases, with the highest incidence around 55 years, and more commonly affecting men than women (3:2)(Al-Chalabi and Hardiman, 2013). In Europe, the median incidence rate (/100,000 population) is 2.08 corresponding to an estimated 15,355 individuals, whereas the median prevalence is 5.40 or 39,863 prevalent cases (Chiò et al., 2013).

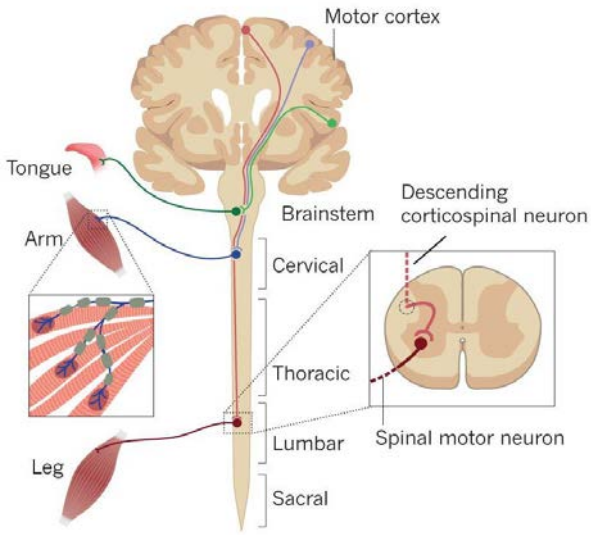

Figure 1. Specific areas affected by motoneuron degeneration. Extracted from (Taylor et al., 2016).

ALS was firstly defined by Jean-Martin Charcot in 1869 (Charcot and Joffroy, 1869). He described several clinical cases that showed progressive muscular atrophy of the limbs, tongue and lips. Anatomically, he found a specific and limited degeneration of the gray matter along with degeneration of the lateral columns of the spinal cord. He named the disease amyotrophic (greek word *'amyotrophia*,' meaning loss of muscle nourishment and sustenance) lateral (the side of the spinal cord affected, the lateral white matter) sclerosis (greek word *'sklerosis'* derived from *'skleroun'* meaning harden and

refers hardened or scarred tissue in affected region of the spinal cord replacing of healthy tissue) (Goetz, 2000). Nowadays, it is well known that ALS is characterized by a loss of MNs in the motor cortex, the brainstem and the spinal cord (Bruijn et al., 2004). MNs degeneration results in skeletal muscle weakness, spasticity, hyperreflexia and eventual paralysis leading to the death of patients by respiratory failure within 2 to 5 years of developing symptoms (Al-Chalabi and Hardiman, 2013). Every ALS patient, however, has an individual prognosis and disease progression because of its demographics, genetics, clinical factors and phenotypic variability (Chio et al., 2009). Most of ALS patients present a "Charcot ALS", with a spinal-onset of the disease. The initial clinical examination of these patients shows fasciculations and muscle weakness of the lower or upper limbs, until it lately spreads to supraspinal levels affecting speech and respiratory areas. Rarely (~20%), ALS patients show bulbar onset, which have a worse prognosis since it directly affects swallowing and breathing pools of motor neurons. It does manifest with dysphagia, dysarthria and tongue fasciculations. Other ALS phenotypes may include: progressive muscular atrophy, primary lateral sclerosis, pseudopolyneuritic ALS, hemiplegic ALS, flail arm/leg syndrome (Figure 2) (Swinnen and Robberecht, 2014).

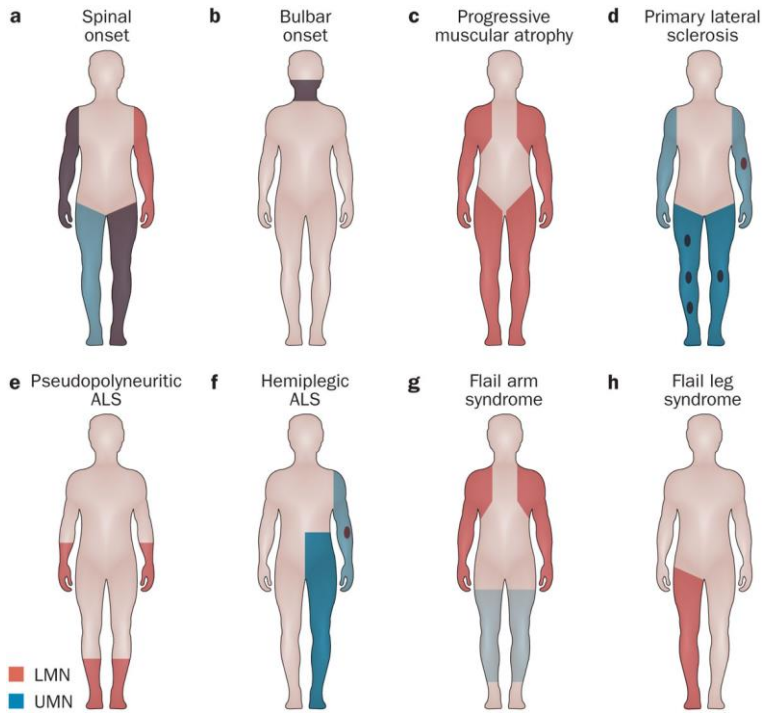

Figure 2. Schematic picture showing the involvement of lower motor neurons (LMN, red) or upper motor neurons (UMN, blue). Darker shading denotes more severe involvement. Extracted from (Swinnen and Robberecht, 2014).

Regardless of the disease onset or phenotype, there is currently no cure for ALS. The main treatment focuses on palliative care which includes: maintenance of physical function, pain-based therapies and providence of psychosocial support (Elman et al., 2007). At the end-stage, the use of assisted ventilation and nutrition is the only method to overcome the loss of primary functions (Wijesekera and Leigh, 2009). Despite the exhaustive research on pharmacotherapies, clinical trials have largely failed, leading to lose all hope for most ALS patients. Until 2017, there was only one drug approved by the food and drug administration (FDA): Riluzole©, an anti-glutamatergic drug. At the late eighties, several researchers hold that glutamate, the main excitatory neurotransmitter in the CNS, might become toxic when accumulates at synaptic level. In this line, various studies showed that metabolism of glutamate is highly altered in ALS patients (Plaitakis and Caroscio, 1987; Plaitakis, 1991). Furthermore, early in the nineties, two Riluzole clinical trials showed promising results since it extended survival of ALS patients by three to six months (Bensimon et al., 1994; Lacomblez et al., 1996). Nevertheless, over the last years, the efficacy of Riluzole has been questioned, and other anti-glutamatergic drugs such as gabapentin, have failed in clinical trials (Ludolph and Jesse, 2009). At the middle of 2017, 22 years later after the approval of Riluzole, the FDA approved Radicava© (Edaravone), a new drug to treat ALS patients. Edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one, MCI-186) is an anti-oxidant molecule that mediates neuroprotection by preventing oxidative damage (Shichinohe et al., 2004; Uno et al., 2005). In 2008, Japanese researchers showed that treatment with Edaravone initiated at symptomatic stage slowed motor decline by reducing mSOD1 aggregation in $SOD1^{G93A}$ mice (Ito et al., 2008). Later randomized clinical trials in Japan revealed that ALS patients receiving Edaravone treatment had less functional decline after 6 months of follow-up (Tanaka et al., 2016). Due to the promising beneficial effects observed in ALS Japanese patients, the FDA was rapidly engaged with the drug to treat ALS patients in the US.

Genetics

The exact causes of ALS are currently unknown. However, as occurs in other neurodegenerative disorders, it is associated to genetic and non-genetic factors. Approximately 90-95% of ALS cases are presented as sporadic forms of the disease (sALS), whereas the remaining 5-10% cases are considered familiar (fALS) (Figure 3) (Renton et al., 2014). In the last decades, several mutations inherited in an autosomal dominant fashion have been described as potential contributors to the development of familial ALS. In 1993, Rosen and colleagues described the first ALS susceptibility gene: mutations encoded in Cu/Zn superoxide dismutase (*SOD1*) gene (Rosen et al., 1993). This finding represented the first evidence that alterations in gene sequences could be related with the apparition of ALS. Nonetheless, later studies revealed that the prevalence of *SOD1* mutations was lower than expected. Population-based studies revealed that *SOD1* alterations were present in 1% of ALS patients with sporadic forms, and in approximately the 12% of the familial ones (Chio et al., 2009). It was not until 20 years later when mutations in the TAR DNA Binding Protein (*TARDBP*), were described as the second ALS-related gene (Sreedharan et al., 2008). In the last recent years, the development of high-sensitive techniques has allowed the identification of various genes involved in both fALS and sALS forms. These include mutations in Fused in Sarcoma (*FUS*), optineurin (*OPTN*), tank-binding kinase1 (*TBK1*), NIMA related kinase 1 (*NIK1*), Ubiquilin-2 (*UBQLN2*) or the hexanucleotide repeat expansion (GGGGCC) in Chromosome 9 open reading frame 72 (*C9orf72*) gene, among others. The latest, is found in more than 40% of fALS and 7% of sALS forms in people of European ancestry (summarized in Table 1) but it is extremely rare in patients from other origin (Renton et al., 2014; Cirulli et al., 2015).

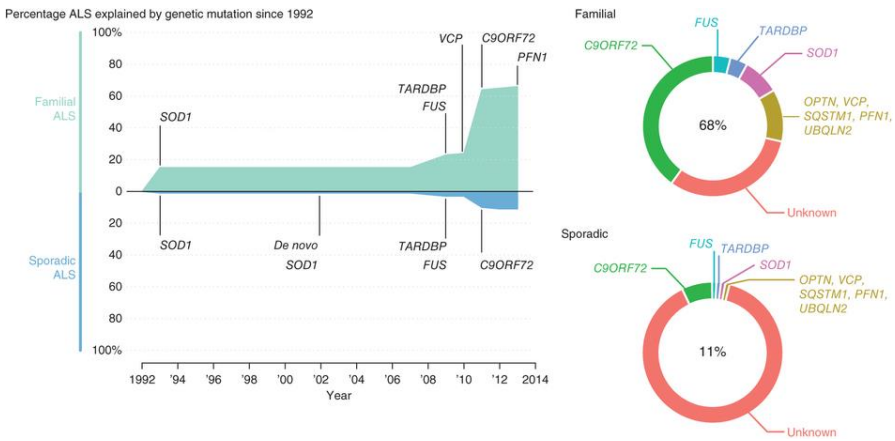

Figure 3. **Genetic contribution to ALS.** Proportion of the contribution of each gene in ALS cases of European ancestry. Extracted from (Renton et al., 2014).

Table 1. Genes linked to ALS

Locus	Gene	Chromosome	Percentage of prevalence	
			Familial ALS	Sporadic ALS
ALS 1	*SOD1*	21q22.11	4	1
ALS 2	*ALS2*	2q33.2	1	<1
ALS 3	*ALS3*	18q21	40	7
ALS 4	*SETX*	9q34.13	1	1
ALS 5	*SPG11*	15q14	<1	<1
ALS 6	*FUS*	16p11.2	4	1
ALS 7	*ALS7*	20p13	<1	<1
ALS 8	*VAPB*	20q13.33	12	1-2
ALS 9	*ANG*	14q11.1	<1	<1
ALS 10	*TARDBP*	1p36.22	5	<1
ALS 11	*FIG4*	6q21	?	?
ALS 12	*OPTN*	10p13	4	<1
ALS 13	*ATXN2*	12q23-q24.1	?	?
ALS 14	*VCP*	9p13	1-2	<1
ALS 15	*UBQLN2*	Xp11.21	<1	<1
ALS 16	*SIGMAR1*	9p13	?	?
ALS 17	*CHMP2B*	3p12.1	?	?
ALS 18	*PFN1*	17p13.3	<1	<1
ALS 19	*ERBB4*	2q33.3-q34	?	?
ALS 20	*HNRNPA1*	12q13.1	<1	<1
ALS 21	*MATR3*	5q31.2	<1	<1
ALS-FTD2	*CHCHD10*	22q11.23	<1	<1
ALS-FTD1	*C9orf72*	9p21.2	25	10
ALS	*DCTN1*	2p13	1	<1
ALS	*SQSTM1*	5q35	<1	?
ALS	*TUBA4A*	2q36.1	<1	<1
ALS	*TBK1*	12q14.1	?	?
ALS	*C21orf2*	21q22.3	?	?
ALS	*KIF5A*	12q13.3	?	?
ALS	NEK1	4q33	3	?

Data extracted from http://alsod.iop.kcl.ac.uk/index.aspx and (Takahashi et al., 2013; Taylor et al., 2016; Nicolas et al., 2018)

ALS experimental models

The discovery of the genetic mutations described in fALS patients led to the development of transgenic experimental animals to study the disease. The first ALS animal developed was the transgenic mouse carrying high copy numbers of the mutant human *SOD1* gene with a glycine-to-alanine conversion at the 93rd codon (*SOD1*^{G93A}) (Ripps et al., 1995). This mouse has been highly useful for mimicking clinical and histopathological features of both familial and sporadic human ALS, and is currently the most widely used experimental model of ALS (Ripps et al., 1995). *SOD1*^{G93A} mice develop an adult-onset form of ALS, with a rapid and progressive muscle denervation that precedes MN death. Phenotypically, animals manifest mostly hind limb weakness and gait deficits at rotarod test around 3-4 months of age that ultimately progress to muscle paralysis and early death at 5-6 months. At the histological and molecular level, the main features include: upper and lower MN degeneration, early astroglial and microglial reactivity, aggregation of misfolded SOD1, glutamate-induced excitotoxicity or axonal damage among others (Turner and Talbot, 2008).

Nevertheless, other rodent ALS models have been developed to shed light into the different sALS and fALS pathophysiology and phenotypes. In the recent years, came the appearance of new *SOD1*-gene based models with other codon conversions such as G37R, G85R or G86R rodent models. These mice showed different phenotypes and disease progression but similar pattern of motoneuron death (Philips and Rothstein, 2015). Moreover, other classical mendelian fALS genes are used to create a broad range of ALS murine models, including FUS or TDP43 mutations (Van Den Bosch, 2011; Philips and Rothstein, 2015). Despite the diversity and availability of ALS experimental models, *SOD1*^{G93A} rodent models are still the most used ALS model for molecular and preclinical studies.

Pathophysiology

The pathogenic process involved in MN degeneration in ALS is currently unknown, but similarly to other neurological disorders, it seems to involve several molecular processes and cell types (Ferraiuolo et al., 2011). Using ALS experimental models, several investigations have identified diverse abnormalities of cellular function in ALS MNs somas, axons and terminal end-plates. These alterations include glutamate-mediated excitotoxicity, oxidative stress, protein misfolding, impairment of axonal transport and neuroinflammation, among others (Pasinelli and Brown, 2006; Mancuso and Navarro, 2015; Taylor et al., 2016) (Figure 4).

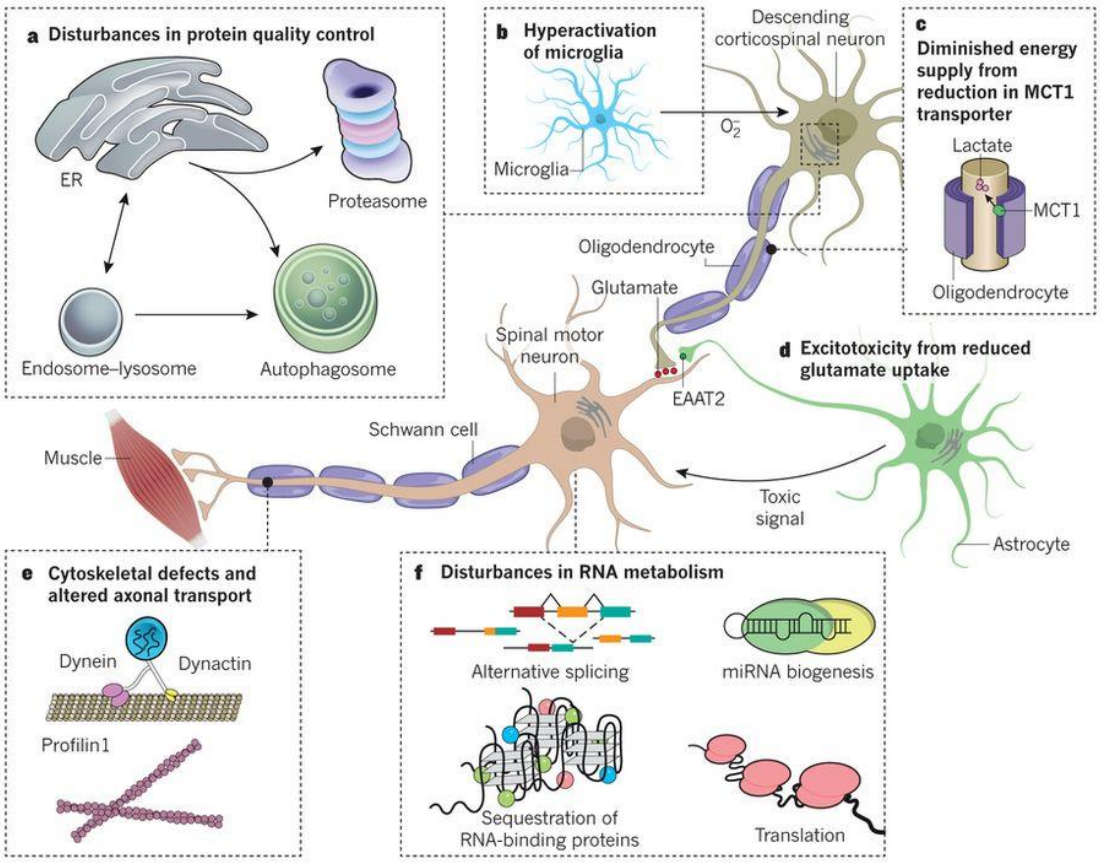

Figure 4. Pathophysiological processes involved in MN degeneration in ALS. Extracted from (Taylor et al., 2016).

Glutamate excitotoxicity

One of the first molecular alterations discovered in ALS was the glutamate-mediated excitotoxicity. This phenomenon is caused by the dysregulation, accumulation and overactivation of glutamate receptors due to a glutamate overload. Glutamate is the major excitatory neurotransmitter in the CNS. This amino acid exerts its effects through the activation of two types of receptors: ionotropics (AMPA, NMDA and kainate) and metabotropics (GluR1-5). In normal conditions, glutamate levels increase during synaptic transmission and then are rapidly cleared from the synaptic cleft by specialized transporters named excitatory aminoacid transporter 1 to 5 (EAAT1-5). Under some pathological conditions, such as ALS, these clearance systems may fail leading to a massive accumulation of glutamate in the intersynaptic milieu. This "excess" of glutamate overstimulates ionotropic receptors located at MNs, leading to massive calcium influx causing neuron degeneration (Foran and Trotti, 2009). Several alterations have been described in glutamate uptake, but downregulation of the EAAT2 (also not as glutamate transporter 1, GLT-1) in astrocytes seems to play a key role in glutamate excitotoxicity (Rothstein et al., 1995). Other alterations found in ALS patients or $SOD1^{G93A}$ mouse models include: increased release of glutamate by descending projections, dysregulation of AMPA subunit GluR2 and increasing amounts of glutamate in the cerebrospinal fluid (CSF) (Reviewed in (Ferraiuolo et al., 2011).

Oxidative stress

Mitochondrial dysfunction deregulates energy metabolism and produces reactive oxygen spices (ROS) (Kirkinezos and Moraes, 2001). ROS are chemicals composed by oxygen radicals such as superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH·)(Nathan and Cunningham-Bussel, 2013). At cellular level, ROS act as signaling molecules. However, under certain circumstances, impairment between anabolism and catabolism of ROS may occur, leading to their accumulation (Schieber and Chandel, 2014). ROS accumulation leads to oxidative damage and cell death by causing damage in several biochemical compounds and modification in the homeostasis of the cell (Cross et al., 1987). Oxidative stress heightened interest when mutations in *SOD1*, an antioxidant protein, were associated to fALS. Initial studies hypothesized that *SOD1* mutations may lead to the loss of dismutase activity, enhancing the production of free radicals and likely contributing to MN death. This hypothesis was supported by studies demonstrating oxidative damage in lipids (Simpson et al., 2004), proteins (Shaw et al., 1995) and DNA (Fitzmaurice et al., 1996; Bogdanov et al., 2000) in ALS patients. However, later studies

pointed out that SOD1 toxicity came from its aggregation rather than from its loss of function (Reaume et al., 1996; Subramaniam et al., 2002).

Protein misfolding and aggregation

Protein misfolding and aggregation is one of the hallmarks of neurodegenerative diseases, including ALS. However, whether they exert a toxic role, they are just products of neurodegeneration or they are being accumulated to avoid toxicity is yet to be defined. Post-mortem analysis has revealed that both familial and sporadic ALS cases present protein-ubiquitinated inclusions within the CNS, suggesting a pathogenic role of these proteins in the disease. These aggregates include protein products from mutated ALS familial genes such as *SOD1*, *TDP-43*, *FUS*, *OPTN*, *UBQLN2* or the *C9orf72* (Blokhuis et al., 2013).

Cytoplasmic inclusions of the mutant form of SOD1 were the first described in MNs of ALS patients and SOD1 murine models (Shibata et al., 1994; Bruijn et al., 1997). Mutations in *SOD1* can lead to the misfolding and then the formation of monomers, oligomers and lately insoluble aggregates of mSOD1 (Blokhuis et al., 2013). Interestingly, Sato and colleagues reported a correlation between protein aggregation and lifespan (Sato et al., 2005). However, the exact detrimental mechanisms underlying by mSOD1 is unknown, it is likely due to combination of protein sequestration, such as heat-shock proteins (HSP), and oxyradical production (Pasinelli and Brown, 2006).

It was not until 2006 when TDP-43 inclusions were discovered, the most abundant oligomeric protein in ALS patients (Neumann et al., 2006). TDP-43 aggregates are found within the CNS of the vast majority of sALS and fALS, except for those patients presenting mutations in the *SOD1* gene (Mackenzie et al., 2007). In non-pathological conditions, TDP-43 is mostly found in the nucleus. However, in neurodegenerative diseases, TDP-43 is highly phosphorylated and located at the cytoplasm of neuronal and non-neuronal cells (Hasegawa et al., 2008; Arai et al., 2009). To unmask its role, studies using transgenic animals have revealed that overexpression of WT or mTDP-43 produces motor impairments and shortens the survival of the animals (reviewed in (Wegorzewska and Baloh, 2010). Several other toxic inclusions have being identified in the last decades including FUS, OPTN, UBQN or C9orf72 that are reviewed in (Blokhuis et al., 2013).

Nonneuronal cells

Despite ALS is a disease that primary affects MNs, several studies have pointed out that neighboring glial cells play a harmful role in the disease. The first evidence came with the development of animals harboring *mSOD1* only in MNs. Unexpectedly, the specific

expression of m*SOD1* in MNs did not evocate MNs degeneration nor motor disease (Pramatarova et al., 2001; Lino et al., 2002). In this line, (Clement et al., 2003) an elegant work from Cleveland's laboratory demonstrated, by using chimeric mice which expressed mSOD1 either in MNs or glial cells, that *mSOD1* overexpression in MNs is insufficient to trigger MNs degeneration. Indeed, this study revealed that MNs-mSOD1 contributed to disease onset whilst glial-mSOD1 had a role in disease progression (Clement et al., 2003).

To unravel the role of microglia in ALS, the surveillance cells from the CNS, studies applying transgenesis technics were used. The selective removal of *mSOD1* from microglial cells effectively delayed the progression and extended the lifespan in $SOD1^{G93A}$ mice. Interestingly, they also showed that this slowed progression was not achieved when *mSOD1* was excised just from MNs (Boillée et al., 2006a). The same year, another work described that transplantation of WT microglia into *PU.1-*$SOD1^{G93A}$ knockout mice, which are animals lacking the ability to develop microglial cells, enabled to extend $SOD1^{G93A}$ lifespan (Beers et al., 2006). Since then, several authors have searched for the specific mechanisms by which microglial cells might exert their deleterious role.

Astrocytes are the largest population of cells in the CNS. Among its many functions, the following stand out: trophic support to CNS cells, maintenance of homeostasis and repairing role during traumatic injuries. In CNS injury and disease, astrocytes activate and become reactive, undergoing both, neurotoxic and neurorepairing roles. In ALS, the restricted expression of mSOD1 in astrocytes is not enough to lead to motor degeneration in mice (Gong et al., 2000), but they actively contribute to ALS pathogenesis. Indeed *in vitro* studies have consistently shown that astrocytes expressing mSOD1 are selectively toxic to MNs (Di Giorgio et al., 2007; Nagai et al., 2007; Marchetto et al., 2008). These findings were also supported by other studies using astrocytes derived from sALS and fALS patients (Haidet-Phillips et al., 2011). To further elucidate the exact mechanisms responsible for MNs degeneration,(Yamanaka et al., 2008) studies excising $SOD1^{G37R}$ exclusively in astrocytes have been performed. In line with previous results removing mSOD1 from microglial cells (Boillée et al., 2006a), the decreased expression of ALS-linked *SOD1* gene in astrocytes did not modified the disease onset but ameliorated the progression in mice (Yamanaka et al., 2008). Indeed, recent studies revealed that astrocytes from fALS and sALS patients release toxic factors that mediated MN death through necroptosis (Re et al., 2014). Interestingly, Re and colleagues described a complete rescue of MNs death when a caspase-independent death pathway was blockaded (Re et al., 2014). Moreover, they showed that pharmacological inhibition of either receptor-interacting protein 1 (RIP1) or mixed lineage kinase domain-like protein (MLKL),

which are effectors of necroptosis signaling, prevented the astrocyte-induced cell death of MNs (Re et al., 2014).

Oligodendroglia, the myelinating cells of the CNS, seems to be involved in ALS. They are distributed along the CNS surrounding the axons with myelin sheets and providing trophic support to neurons (Nave, 2010). Recent studies have pointed a deleterious role of oligodendrocytes in neurodegenerative diseases with axonopathy, including ALS (Lee et al., 2012). To uncover its role, in 2013 two independent works studied oligodendrocyte evolution during ALS progression in *SOD1*^{G93A} mice (Kang et al., 2013) and (Philips et al., 2013). They both showed that prior to disease onset, there was remarkably death of oligodendroglia located at the gray matter. Interestingly, they also found a marked proliferation of NG2+ cells, oligodendrocytes precursors, surrounding dying MNs. Despite the new formation of precursor cells, these cells were unable to mature resulting in demyelination. To finally unveil their pathological role, they removed *mSOD1* from oligodendrocytes and revealed that *SOD1*^{G93A} mice showed delayed disease onset and extended survival (Kang et al., 2013).

Not only CNS cells are affected in ALS, but cells from the peripheral nervous system (PNS) also contribute to the disease. Schwann cells are responsible for myelinating peripheral axons but also, cleaning the debris and guiding axons regeneration after injuries to PNS. Since ALS begins with a distal axonopathy that lately reaches MNs soma, it is expected that Schwann cells participate in ALS pathophysiology (Ilieva et al., 2009). To determine the contribution of Schwann cells, Cre-LoxP systems were used to specifically ablate *mSOD1* from P0 cells in *SOD1*^{G37R} mice (Lobsiger et al., 2009). *mSOD1* removal from around 70% of Schwann cells revealed unexpected results: it did not just fail to delay the disease, but accelerated its progression. Nonetheless, the authors related the protective role that Schwann cells may play in ALS with the dismutase activity of SOD1 enzyme. One of the products from dismutase reaction is the production of hydroxide peroxide which might modify gene transcription. As they showed, the lack of *mSOD1* from Schwann cells dramatically reduced the production of the insulin growth factor (IGF-1), a neuroprotective factor, without modifying other neuroprotective agents. They supposed that the lack of IGF-1 is promoting MNs degeneration. A parallel study overexpressing *wt*SOD1 in myelinating Schwann cells however, did not find any neuroprotection in normal nor ALS transgenic mice (Turner et al., 2010).

Inflammation in ALS

Since in 2003, when Clement and colleagues firstly described that ALS is not a cell-autonomous disease (Clement et al., 2003), several investigations appeared to shed light on the role of neighboring cells. Among the different processes that non-neuronal cells participate and that could contribute to ALS disease, inflammation has won prominence.

Overview of the inflammatory response

The inflammatory response is a physiological response of the body against injury and infections aimed to restore the affected tissue or organ to its homeostatic state. To carry out this action, it is needed the activation of a well-orchestrated events that involved innate and adaptive immunity (Serhan et al., 2010).

The innate immune system is an unspecific mechanism that appears within minutes or hours after damage or antigen exposure has appeared. Given these characteristics, the innate response is commonly considered the first-line of defense against pathogens by its rapid and nonspecific activation. Between its many functions, it highlights the activation of the adaptive immune system and the processing and removal of hazardous substances. To perform this response, the activation of several subsets of cells are needed: (i) polymorphonuclear leukocytes (PMN) or granulocytes including neutrophils, eosinophils, basophils and mast cells; (ii) monocytic cells such as macrophages, monocytes and most dendritic cells (DC); (iii) innate lymphocytes like natural killer (NK) cells, NK T cell (NKT); (iv) microglial cells in the CNS. To develop its response, each of this subset presents patter recognition receptors (PRR) in their cell surfaces that specifically recognize pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) that react against products from bacteria and cell injury (Parkin, J. and Cohen, 2001; Kumar et al., 2011). Despite innate response is an excellent first-line response against invaders, sometimes are required more specialized cells as adaptive immunity.

The adaptive immunity response is a specific mechanism orchestrated by lymphocytes (T and B cells) produced just after the innate response fails to heal the system *per se*. It therefore requires the direct presentation of a specific antigen by dendritic cells to execute its function. Due to the requirement of the specific activation by innate cells, the adaptive response takes several days and weeks to develop. Even though it needs more time to be able, its activation leads to the creation of memory mechanisms that will produce an efficient and rapid response against a second exposure of the same

antigen. The most important adaptive immunity responses are: the humoral or antibody response, mediated by the release of antigen-specific antibodies by B cells; and the cell-mediated response, which requires from a tight contact between the T cell and the virus or infected cell (Alberts et al., 1997; Bonilla and Oettgen, 2010).

In mammals, B cells or B lymphocytes develop in the bone marrow from a pluripotent hematopoietic stem cell. After antigen-presentation, naïve B cells activate, proliferate and differentiate to effector cells, which migrate to blood torrent and secrete antibody with unique antigen-binding site that will recognize the antigen from the pathogen (Alberts et al., 1997; Bonilla and Oettgen, 2010).

T cells also develop in the bone morrow, but they are activated in the thymus when antigen-presenting cells (APC) process and expose antigens to the cell surface through specialized molecules called major histocompatibility complex (MHC) proteins. Once the MHC proteins display the processed-antigen to the T cell, it binds the T-cell receptor (TCR) using co-stimulator molecules. Upon activation, T lymphocyte may produce different actions regarding on the T cell subtype. While T helper or CD4 cells release cytokines to evocate responses of other cells, CD8 or cytotoxic lymphocytes directly kill the cell harboring pathogens or tumor cells (Alberts et al., 1997).

Beside from its diverse cell subsets, the inflammatory response may be categorized depending on its duration: acute and chronic inflammation. When there is a trauma, pathogen infection or neoplasia, the innate immune response initiates several responses to prevent infection or tumor growing by triggering the inflammatory response. Upon its activation, several cytokines and chemokines are secreted to the blood to produce their effects throughout the body (Turner et al., 2014). Of the signals produced, stand out the release of interleukin-6 (IL-6), interleukin-1-beta (IL-1ß) and tumor-necrosis factor alpha (TNF-α) by innate immune cells. These signaling molecules will induce the recruitment of several other inflammatory subsets such as monocytes and neutrophils to the site of disturbance creating the acute-phase response. Once in the lesion area, the innate immune cells will create a cytotoxic milieu to destroy the source of inflammation while acting as APCs to facilitate the recruitment of adaptive immune cells. Finally, to produce the clearance of these immune cells from the lesion site, the recruitment of neutrophils is impeded and the recruitment of resolution-phase monocytes is promoted, in a phase known as resolution phase. These specialized monocytes will clear the resulting cellular debris to promote a healing area for tissue repair and functional restoration (Serhan and Savill, 2005). Unfortunately, in certain tissues and circumstances the resolution phase is blocked leading to persistent accumulation of the immune cells in the injured area and, therefore, chronifying inflammation (Schwab et al., 2015).

Since the inflammatory response may be triggered by cell death or degeneration, inflammation is therefore, a pathological hallmark of neurodegenerative diseases, including ALS. Several lines of evidence have provided data that immune cells are modified in ALS patients compared to healthy controls with other neurologic disease. In this line, some studies have recently showed that pro-inflammatory and anti-inflammatory mediators such as IL-6, IL-10, interferon gamma (IFN-γ), IL-8 or prostaglandin E_2 (PGE_2) are deregulated in CSF from ALS patients (Almer et al., 2002; Kuhle et al., 2009; Mitchell et al., 2009). Indeed, it has been showed that the release of IL-8 by microglial cells, correlates with a severe disease phenotype (Mitchell et al., 2009). In this line, it has been showed an important infiltration of T cells along with activated microglia, astrocytes, and deposits of IgG, presumably produced by plasma cells in spinal cord of ALS patients and SOD1 mice, predominately at final stages in the ALS animal model (Engelhardt and Appel, 1990; Kawamata et al., 1992; Chiu et al., 2008). Moreover, post-mortem studies have revealed the presence of activated-microglia and adaptive immune cells surrounding dying MNs in the areas of ongoing neurodegeneration such as the spinal cord, the motor cortex or the brainstem (Kawamata et al., 1992; Henkel et al., 2004b) and in transgenic rodent models as well (Hall et al., 1998; Nikodemova et al., 2014). The activation of immune cells in ALS patients represents therefore, a major component of its pathophysiology. Nevertheless, their contribution to the disease or whether these cells play an active role or they are just products of the neuronal degeneration is yet to be defined.

Microglial role in ALS

Microglial cells are the resident-myeloid cells of the CNS. Despite carrying out the same functions, they do not come from the same cellular progenitor. Contrary to macrophages, which derive from bone marrow cells, microglial cells are originated in the yolk sac at early stages of the development and migrate to the CNS (Ginhoux et al., 2010). Located throughout the CNS parenchyma, this immune cell subset executes the immune regulation, neuronal remodeling and extracellular signaling. In homeostatic or "resting" conditions, microglial cells exhibit ramified-shape with diverse cytoplasmic processes that surveil their surroundings. In response to injury, microglia become abruptly activated and switch its morphology to the classical amoeboid-shape contributing to the neuroinflammatory response (Ransohoff and Perry, 2009). Upon its activation, microglia proliferates and releases ROS, growth factors and pro-inflammatory cytokines/chemokines such as chemokine ligand 2 (CCL2), CCL3, granulocyte-macrophage colony stimulating factor (GM-CSF), macrophage colony stimulating factor (M-CSF), IL-1ß

or TNF-α (Pineau and Lacroix, 2007; Ransohoff and Perry, 2009) which contribute to the disruption of the blood-brain barrier and enhance the recruitment of peripheral leukocytes. This pro-inflammatory state of microglia is known as a "classical activated" or "cytotoxic" microglial cells. In general, classically activated microglial cells are found in pathologic environments and facilitate the activation of adaptive immunity. Despite its main function is to cope with the current pathologic agent, its continuous activation might lead to increase the damage. Nevertheless, microglia may be activated in a "neuroprotective" or "anti-inflammatory" way as well. In this context, alternatively-activated microglia carries out protection against diseases and tissue healing, including in the CNS (Saijo and Glass, 2011; Francos-Quijorna et al., 2016).

In neurodegenerative diseases, however, there is a dichotomy between whether microglial cells contribute to neurodegeneration or neuroprotection. Despite early studies using chimeric mice demonstrated that microglial cells played an essential purpose in ALS progression (Boillée et al., 2006b), other studies using other approaches have shown a neutral or even a neuroprotective role for these cells. Hence, the current role of microglial cells in ALS is poorly understood.

Approaches in which microglial proliferation has been targeted, either by the transgenic expression of thymidine kinase (TK) and later "suicide" of proliferating CD11b+ cells (Gowing et al., 2008), or by the administration of cytosine arabinoside (Ara-C), a known non-specific blocker of mitosis which it is clinically used as cytostatic (Audet et al., 2012), suggested a neutral or beneficial role of microglia in ALS. Nevertheless, the methods used in these studies did not consider some technical limitations of the approaches applied. First, the use of CD11b-TK mice leads to a massive and rapid death of microglia in a context of a CNS with on-going neurodegeneration (Gowing et al., 2008), hence not offering the optimal physiological environment. This sudden death may imply several cellular changes and release of neurotoxic factors that might modify the current process. On the other hand, when using a CD11b promoter not only microglial cells are ablated, but also myeloid cells, including macrophages or neutrophils. Second, the use of Ara-C causes a shift in the activation phenotype of microglia towards a detrimental pro-inflammatory profile (Audet et al., 2012; Gomez-Nicola et al., 2013). Supporting this hypothesis, *SOD1*^{G93A} transgenic mice whose microglial cells were no longer able to respond against fractalkine (CX3CL1), a neuroprotective chemokine, showed behavioral impairments and a shortened lifespan suggesting a neuroprotective role for these cells (Cardona et al., 2006). Highlight, that Ara-C not only reduces proliferation of microglia, but also of other glial cells that proliferate in the spinal cord such as astrocytes and oligodendrocytes.

By contrast, there are several studies in which microglial cells showed a neurotoxic role. The first studies showing microglia toxicity in ALS were approaches studying the role of a second-generation tetracycline with anti-inflammatory properties, minocycline. *In vitro* studies revealed that CSF from ALS subjects induced neuronal death and microglia activation in primary spinal cord cultures, and that minocycline attenuated neuronal death and microglial activation (Tikka et al., 2002). Later *in vivo* studies revealed similar results, since enriched-diet with minocycline slowed disease progression in a $SOD1^{G37R}$ mouse model and reduced the activation of microglia (Kriz et al., 2002). However, another study revealed that minocycline reduced caspase signaling, inducible nitric oxide synthetase (iNOS) and p38, but it also inhibited the release of cytochrome C from the mitochondria, suggesting that the beneficial effects of this drug could be due its ability to attenuate inflammation and apoptosis (Zhu et al., 2002). Other complementary studies indicated that the blockage of a pro-inflammatory enzyme such as cyclooxygenase-2 (COX-2), the enzyme responsible for the production of various pro-inflammatory eicosanoids such as prostaglandins and thromboxanes, ameliorated ALS motor impairments (Pompl et al., 2003). Furthermore, *PU.1* knock-out mice, animals whose ability of developing bone marrow cells (BMCs) was no longer functional, were crossed with $SOD1^{G93A}$. The resulting $SOD1^{G93A}/PU\text{-}1^{-/-}$ mice lacked both bone marrow-derived cells and microglial cells and thus, they required a graft of WT microglia and BMCs after birth. This approach led the authors to selectively study the contribution of WT microglia in a spinal cord infested with glial and MNs with *mSOD1* gene (Beers et al., 2006). Even though WT-BMCs transplant also replaced mutant circulating leukocytes, they concluded that WT microglia extended survival in *PU.1* knock-out mice pointing out a deleterious role for microglial cells in ALS (Beers et al., 2006).

Although several studies have indicated that microglia contribute to ALS pathology, just a few reports have unraveled the molecular mechanism by which they mediate toxicity. In 2009, an alternative approach studied the impact of increasing the proliferative activity of microglia. To this aim, they administered recombinant colony-stimulating factor 1 (CSF1). Both interleukin 34 (IL-34) and CSF1 mediate the activation of the colony-stimulating factor 1 receptor (CSF1R), a receptor located at the cell surface of mononuclear phagocytes. Once activated, it triggers the proliferation of both microglia and macrophages (Wei et al., 2010). Having increased microglial proliferation rates in $SOD1^{G93A}$ mice, this study suggested a detrimental role for microglia in the pathophysiology of ALS (Gowing et al., 2009). As it happened in a previous study from the same group (Gowing et al., 2008), however, this approach also modified the contribution from CSF1-responsive of peripheral cells such as circulating monocytes. More recently, it has been discovered that

one mechanism by which microglia specifically triggers motor neuron death is through the classical nuclear factor kappa B pathway (NF-κB) (Frakes et al., 2014). Upon any damage, there is a release of inflammatory signals that trigger the canonical NF-κB signaling. Once activated, it enhances the production of pro-inflammatory cytokines, adhesion molecules or enzymes that increase the inflammatory response in a positive feedback loop. Since many of these downstream effectors are upregulated in ALS patients, (Frakes et al., 2014) selectively inhibited NF-κB signaling in microglial cells. In this case, specific blockage of upstream NF-κB molecules using shRNA slowed disease progression, as well as, increased mice survival by rescuing MNs from death. Altogether, through the last years, several approaches have determined that microgliosis is a pathological hallmark found in both ALS patients and experimental models and it actively contributes for disease progression.

Peripheral leukocytes in ALS

Not only do CNS resident cells contribute to ALS pathophysiology, but peripheral leukocytes also participate in the disease. Several lines of evidence showed an important infiltration of T cells, monocytes, dendritic cells and deposits of IgG along with an increased production of chemoattractant cytokines as of the monocyte chemoattractant protein 1 (MCP-1/CCL2) (Engelhardt and Appel, 1990; Kawamata et al., 1992; Henkel et al., 2004a; Chiu et al., 2008). However, these immune cells may exert different roles in ALS depending on their subtype and time point.

In 2008, a report showed there is progressive recruitment of peripheral lymphocytes to affected areas in the CNS (Chiu et al., 2008). The characterization of the lymphocytes profiles revealed that most of the immune cells were T lymphocytes, including NK cells, CD4+ and CD8+ T cells, whereas only a small subset were B lymphocytes. To further understand the role of T cells in ALS pathology, authors bred $SOD1^{G93A}$ mice with T cell receptor ß chain (TCR-ß), which led to the specific ablation of T lymphocytes. T cell depletion resulted in accelerated ALS pathology and a decreased inflammatory response in microglial cells suggesting that the helpful effect of T cells in ALS might be related to modulation of glial cells. Indeed, a parallel study using immunodeficient-$SOD1^{G93A}$ mice showed CD4 T-helper cells were the only responsible of this neuroprotective effect by regulating neurotrophic/cytotoxic balance of glial cells (Beers et al., 2008). Novel studies revealed that another subset of T lymphocytes, the $CD4^{+}$, $CD25^{hi}$, $FoxP3^{+}$ known as regulatory T lymphocytes (Treg) strongly regulates the disease progression (Appel et al., 2011; Henkel et al., 2013). Treg are cellular mediators highly characterized for suppressing both innate and adaptive immune system and inducing immunological tolerance. In $SOD1^{G93A}$ mice, Treg are specially upregulated at early stages

of the disease, when they produce microglia polarization towards to an anti-inflammatory phenotype and produce neuroprotection by augmenting IL-4 production (Beers et al., 2011). However, these cells fail to sustain this neuroprotection along the disease since their levels dramatically reduce coinciding with the disease onset. In fact, it has been shown that Tregs mediate disease progression and survival in both ALS patients and experimental models. Besides, it was found strong correlation between a reduction of FoxP3 protein expression and a rapid disease progression compared to slowly progressing ALS patients (Henkel et al., 2013). A potential mechanism for this is that Tregs of ALS patients with rapid progression are dysfunctional because of the loss of FoxP3 due to the increased methylation of its locus (Beers et al., 2017).

Aside from adaptive immune response, innate immunity, which involves among others, the activation and recruitment of peripheral macrophages, is also involved in ALS pathology. Several studies have revealed an increase in the levels of circulating chemokines and cytokines in blood and CSF samples from ALS patients suggesting an activation of the peripheral immunity. IL-6, a well-known pro-inflammatory cytokine is also elevated in ALS (Moreau et al., 2005). However, the most up-regulated chemokine is the MCP-1/CCL-2, suggesting a clear role of monocytes/macrophages in ALS (Henkel et al., 2004a; Kuhle et al., 2009).

CCL2 through the activation of its receptor, the C-C chemokine receptor type 2 (CCR2), activates and recruits monocytes to the sites of inflammation. Peripheral monocytes originate from myeloid precursors in the bone marrow and are usually classified based on the expression of several surface markers. The most prevalent subset of circulating monocytes is known as “classical” or inflammatory monocyte, which expresses CD14+, CD16- markers in humans and its analogous in mice are the Ly6C^{high} monocytes. The second subset corresponds to the “non-classical” or resident monocytes which are CD14+, CD16+ in humans and express low levels of Ly6C in mice (Geissmann et al., 2003). In 2012, (Butovsky et al., 2012) showed that in SOD1^{G93A} mice inflammatory monocytes were activated and chemoattracted to the spinal cord, but not brain, prior to onset. Interestingly, they showed that Ly6C^{high} monocytes were recruited to the CNS because of the increased production of CCL2 by microglial cells. Besides, the invasion of peripheral monocytes highly correlated with the loss of spinal MNs which was attenuated using anti-Ly6C antibodies. Transcriptomic analysis of these inflammatory monocytes in both ALS patients and murine models, revealed a unique microRNA signature where miR-155 was highly up-regulated. Later studies showed that either genetic manipulation or administration of anti-miR-155 treatment prolonged the survival of SOD1^{G93A} mice by restoring the deregulated microglia and monocytes (Butovsky et al., 2015). Novel results

have revealed that peripheral monocytes are altered in ALS patients in both ALS diagnosed and ALS asymptomatic carriers, suggesting that these cells are affected before the disease manifestation and not consequently. This report showed that even though the number of total monocytes was unaltered, there was a shift in the percentage of populations: there was an increase of "classical" monocytes and a decreased of the resident ones compared to healthy subjects. Moreover, these inflammatory monocytes were functionally aberrant and invaded the CNS of ALS patients (Zondler et al., 2016). As Butovsky and colleagues pointed out, there was a strong correlation between the recruitment of the inflammatory monocytes and MNs loss in ALS murine models (Butovsky et al., 2012). Interestingly, when monocytes phenotype was switched towards "non-classical" or resident monocyte after the Fc-receptor activation using immunoglobulins type G (IgG) in $SOD1^{G93A}$ mice, there was greater preservation of spinal MNs and amelioration of the pathophysiology of the disease, raising the hypothesis of that monocyte could be modulated to exert neuroprotection (Zondler et al., 2016). However, these novel reports are in disagreement with previous parabiosis studies that demonstrated that circulating monocytes do not enter into the CNS of SOD1 mice (Ajami et al., 2007). Moreover, transcriptional analysis of CD11b+ cell sorted from the spinal cord of SOD1 mice did not reveal the presence monocytic markers at any stage of the disease (Chiu et al., 2013).

Although there is controversy over whether monocytes invade the CNS, it is widely accepted that monocytes do invade the PNS. It has been reported that there is a progressive recruitment of monocytes to the nerve bundles of $SOD1^{G93A}$ mice over disease progression (Chiu et al., 2009; Graber et al., 2010; Dibaj et al., 2011). Indeed, monocytes begin to invade the PNS coinciding with the "dying-back" process of the axons, before the onset of the disease, suggesting their possible involvement in early stages of the disease. Nevertheless, it still remains unknown whether macrophage influx into peripheral nerves contribute to ALS pathology directly or is secondary to axonal degeneration.

Overall, the ongoing degeneration along with the disruption of diverse cellular processes is highly accompanied by the proliferation of central and peripheral immune cells, release of pro-inflammatory cytokines and chemokines and the recruitment of leukocytes within the degenerating areas. As it occurs in other neurodegenerative disease, these immune reactions play an important role in the progression of ALS by boosting the degenerative events. Therefore, the involvement of immune system in neurodegeneration suggest that therapies aimed at modulating the inflammatory response could open a new avenue to slow disease progression, and thus, enhance the life quality of ALS individuals.

Interleukin 37

IL-37 is one of the eleven members of the IL-1 family. This cytokine family has a pivotal role in regulating inflammatory responses and encompasses seven ligands with agonist pro-inflammatory activity (IL-1α and IL-1β, IL-18, IL-33, IL-36a, IL-36b, IL-36g), three receptor antagonists (IL-1Ra, IL-36Ra, IL-38), and IL-37, which is the only member with anti-inflammatory effects. IL-37, initially known as IL-1 family member 7 (IL-1F7), was identified at the beginning of the 21st century, by sequencing of the IL-1 locus by three independent groups (Busfield et al., 2000; Kumar et al., 2000; Pan et al., 2001).

IL-37 structure

Members of the IL-1 family, excepting IL-18 and IL-33, are clustered within the human chromosome 2 map to 2q13. The computational analysis of the chromosome 2 led to the discovery of new family members with homology with the IL-1 family, which included IL-37 among others. Descending from the same ancestor, IL-37 gene and protein was found to share signature motifs and amino acid identity with other IL-1 members such as IL-1β, IL-18 and interleukin 1 receptor antagonist (IL-1Ra). In mice, the IL-1 family is also clustered at the chromosome 2, the IL-37 gene however, is absent, since no mouse ortholog of the human form of IL-37 has been found to date (Taylor et al., 2002). This may suggest that IL-37 might be found elsewhere in the mouse genome or it has been lost after some evolutionary events as it has already happened in other mammals (Newman et al., 2005). Within the 450kb containing the human IL-1 family, IL-37 locus has a size of 3.617kb and is composed by 6 exons which may rise to five different isoforms as a result of alternative splicing (Figure 5)(Boraschi et al., 2011).

- **IL-37a**: is composed by exons 3 to 6. This isoform is completely different from the other ones since exon 3 encodes for an amino terminus, which is absent in the other variants. The amino acids encoded in exons 4 to 6 leads to a formation of the highly conserved 12-strand β-trefoil structure also present in IL-1α, IL-1β and IL-1Ra, which suggest that IL-37a could be a functional cytokine. cDNA for IL-37a has been found in several tissues including: thymus, lymph nodes, placenta, testis, bone marrow, uterus, skin, colon, stimulated B cells, keratinocytes, NK cells and monocytes. Indeed, IL-37a is the only one found in the brain.

- **IL-37b**: is the largest isoform since it is encoded by all the IL-37 exons excepting the exon 3, which is exclusive of isoform a. As IL-37a, IL-37b contains exons 4 to 6 that form the IL-1-like domain which makes the cytokine biologically functional. Besides, IL-37b contains exons 1 and 2 that codify for a N-terminal pro-domain with cleavage sites to be processed for cytokine maturation. It is mainly found in the same tissues as IL-37a as well as in the kidney.
- **IL-37c**: is equal to IL-37b, but lacking exon 4 which produces an inactive form of the cytokine since it does not encompass the IL-1 conserved domain. Its expression is restricted to the heart.
- **IL-37d**: contains exons 1 and 4 to 6 so it may represent another functional isoform. It is only found in bone marrow and testis.
- **IL-37e**: as IL-37c, it does not have exon 4 and thus, it is not biologically active. It is found in bone marrow and testis.

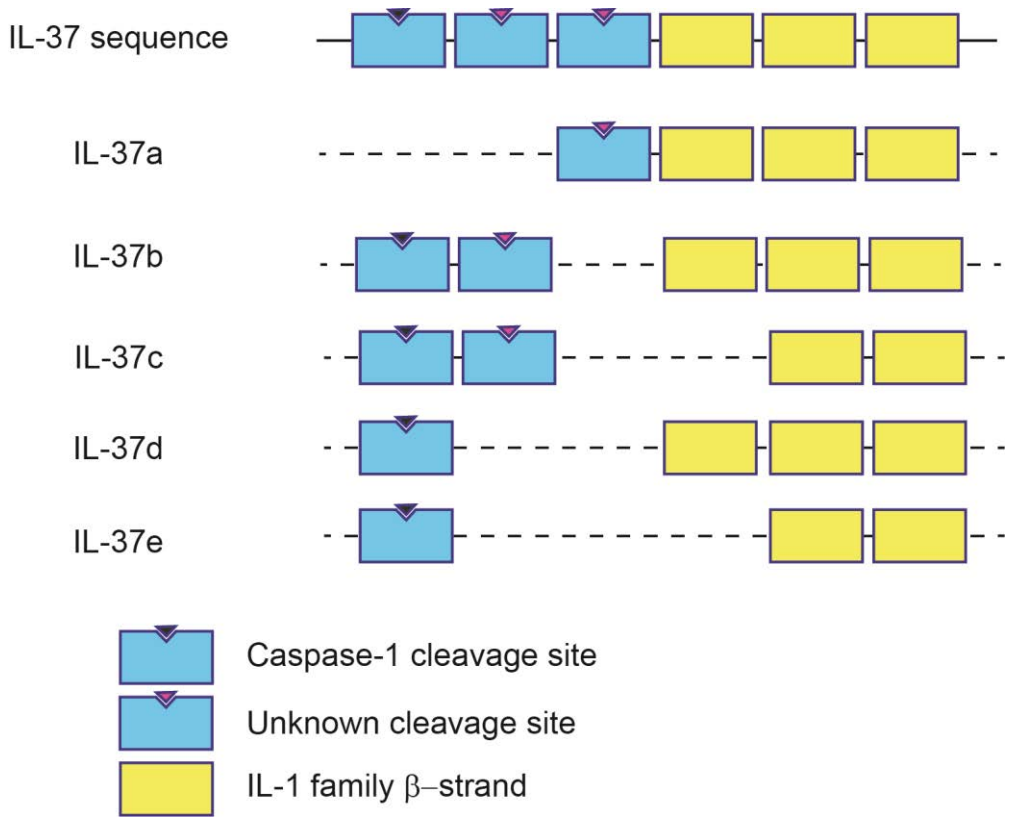

Figure 5. Gene sequence of IL-37 isoforms.

Regardless of isoform, IL-37 gene encodes for an immature protein, which might be active or not depending on the presence of the 12β-strand, and needs to be processed for the maturation of the cytokine. As other IL-1 family members, IL-37 possesses 3 cleavage sites located in different exons and will require the action of several enzymes to process the pro-peptide domains. The first cleavage domain is found in exon 1 between amino acid residues D20 and E21, which it is recognized by caspase-1 (Kumar et al., 2000). This caspase-1 dependent cleavage site is therefore found in IL-37 isoforms b, c, d and e as well as in IL-1β or IL-18. However, later experiments where caspase-1 was inhibited revealed that pro-IL-37 can be processed by other proteases as its intracellular processing was only partially blockaded (Sharma et al., 2008). As Pan and colleagues pointed out, pro-IL-37 has a second cleavage site within exon 4, between amino acids F45 and V46 (present in isoforms b and c) (Pan et al., 2001). Finally, since IL-37a does not encode for exons 1 and 2, it is likely that it presents an alternative cleavage site at exon 3. Using *in silico* techniques it has been predicted a possible elastase-mediated cleavage site between L21 and R22 within the N-terminal sequence (Boraschi et al., 2011). Besides these cleavage sites, IL-37 mRNA also presents functional instability elements within exon 5 that regulate the mRNA turnover. In non-inflammatory conditions, limited amounts of IL-37 mRNA or protein are constitutively expressed in human blood monocytes. However, upon lipopolysaccharide (LPS) stimulation, there is a rapid up-regulation of both mRNA and protein levels. The lack of steady-state mRNA for IL-37 under no inflammatory conditions, is due to a rapid degradation of its mRNA (Bufler et al., 2004). This transcript instability is because of an A-rich homology box found flanking coding regions of diverse stability determinants which under no inflammatory conditions will limit the half-life of IL-37 mRNA (Tierney and Medcalf, 2001). This regulatory mechanism is preserved in all IL-37 isoforms, since they all contain exon 5, but also in other IL-1-like cytokines such as IL-1β and IL-18. Hence, these mRNA turnover elements are an important mechanism to regulate the production of IL-1 family members only in inflammatory circumstances.

IL-37 function

Upon IL-37 discovery, several researchers aimed to unravel the cytokine function and its cellular signaling, being Charles Dinarello a pioneering in this field. To further study the biological function of this novel cytokine, murine leukocytes cell lines were transfected with hIL-37b plasmids. Firstly, macrophage-like RAW cells, were studied after a TLR-dependent stimuli was applied. These experiments demonstrated that after LPS stimulation, RAW cells broadly express several pro-inflammatory cytokines including IL-6, TNF-a, IL-1α, the macrophage inflammatory protein α (MIP-1α) or MIP-2 (Sharma et al.,

2008). Interestingly, when RAW cells expressed hIL-37b there was a marked reduction of all the pro-inflammatory cytokines, excepting MIP-1α upon stimulation with LPS (Sharma et al., 2008). These findings were confirmed when Nold and colleagues showed a similar decreased response to after an inflammatory stimuli in monocytic and epithelial human cell lines (Nold et al., 2010). Therefore, this data showed that hIL-37b is regulating somehow the inflammatory response when expressed in murine and human leukocytes lines. However, to further validate these results, it was necessary to prove this broad anti-inflammatory action in animal models. The main challenge when assessing the *in vivo* function of IL-37 was the lack of a murine ortholog for the human IL-37 in the mouse genome. To overcome this situation, researchers developed the human IL-37 transgenic mouse (hIL-37tg) by transfecting plasmids overexpressing the human IL-37 isoform b. As was it found previously *in vitro*, despite expressing hIL-37 under a cytomegalovirus (CMV) promoter, its steady-stage mRNA expression is low and it can only be up-regulated under an inflammatory stimuli due to the presence of the instability region (Bufler et al., 2004; Nold et al., 2010). Nowadays, it is widely accepted that IL-37 exerts an immunomodulatory role under inflammatory situations, but how it does carry out its function remains poorly understood. Most cytokines implement their actions as secreted mediators, but a small amount may be translocated to the nucleus and modify the transcription. Similar to some IL-1 family members including IL-1a or IL-33, IL-37 presents a dual function and can act both extracellularly and intracellularly (Busfield et al., 2000; Pan et al., 2001; Taylor et al., 2002).

Previously, (Sharma et al., 2008) demonstrated that when IL-37 is processed by caspase-1, it can be translocated to the nucleus and markedly reduce the levels of pro-inflammatory cytokines after LPS stimulation. Since caspase-1 processing is required to specifically translocate to the nucleus, human IL-37 was mutated at the caspase-1 cleavage site (IL-37D20A). Thus, to elucidate the nuclear role of IL-37, human peripheral blood mononuclear cells (PMBCs) and several lines of mouse monocytes were transfected with this genetically engineered IL-37 form. When stimulated with LPS, IL-37D20A translocation to the nucleus is highly impaired compared to WT IL-37-transfected cells. In terms of cytokines release, IL-37D20A was unable to decrease the levels of IL-6 upon LPS stimulation confirming that IL-6 inhibition is depending on its nuclear translocation (Bulau et al., 2014). Indeed, when IL-37 is cleaved, it interacts with phospho-SMAD3 and translocates to the nucleus to inhibit the expression of pro-inflammatory genes (Nold et al., 2010). However, it is still unclear to what degree the anti-inflammatory actions of IL-37 are mediated via intracellular function.

IL-37 can be also released extracellularly as a secreted molecule. Due to the highly amino acid resemblance with IL-18, when IL-37 is released, it interacts with the IL-18 receptor alpha (IL-18Rα). Under physiological conditions, IL-18 binds to IL-18Rα and recruits IL-18R subunit β (IL-18Rβ) to induce NF-κB signaling and release IFN-γ in T and NK cells (Bufler et al., 2002). However, unlike IL-18, when IL-37 binds to IL-18Rα, it fails to recruit the β chain (Bufler et al., 2002). The no formation of the IL-18Rα–IL-18Rβ heterodimeric complex could suggest that IL-37 acts as an IL-18 antagonist. Nevertheless, recent data dismisses this hypothesis since IL-37-IL-18R interactions does not inhibit neither IL-18 activity nor the IFN-γ transcription. Supporting this data, (Kumar et al., 2002) found that both pro-IL-37 and mature IL-37 presented much lower affinity to IL-18R compared to IL-18. Nonetheless, IL-37- IL-18Rα interaction is not completely useless, since IL-18Rα RNA silencing showed enhanced inflammatory responses suggesting that IL-18Rα may use another co-receptor to induce an anti-inflammatory signaling. To further determine which co-receptor was binding to IL-18Rα–IL-37 complex, researchers focused on other proteins that may interact with IL-18. Firstly, they focused their research on the IL-18 binding protein (IL-18BP), the natural antagonist of IL-18 (Novick et al., 1999). Despite not being part of the IL-18R signaling, IL-18BP using an Ig-like domain binds to IL-18 and neutralizes its interaction with IL-18Rα, and thus, inhibits its downstream effects. IL-37 structure shares with IL-18 two key amino acids, Glu-42 and Lys-98, which will allow it to interact with the IL-18BP Ig-like domain. As a result, IL-37-IL18BP complex will recruit IL-18Rβ preventing its dimerization with IL-18Rα and thereby depriving IL-18 signal transduction. However, recent studies revealed that IL-37 and IL-18BP interaction is very weak and it only enhances antagonistic effects when binding to IL-37 mature form (Bufler et al., 2002; Azam et al., 2003; Boraschi et al., 2011). To determine other possible candidates, researchers then focused in receptors and co-receptors with features similar to IL-18BP. Interestingly, IL-1 family receptors (IL-1R) present several Ig-like and intracellular Toll-IL-1R (TIR) domains, which are also present in TLRs (Gay and Keith, 1991). Notwithstanding, among all the IL-1R family members, the only one which produces anti-inflammatory effects is the IL-1R8. IL-1R8, formerly known as TIR8 or "SIGIRR", is an orphan receptor of the IL-1 family that mitigates inflammation by blocking TLRs and IL-1R mediated pathways. Unlike other IL-1R, IL-1R8-TIR domain does not activate the canonical signaling leading to NF-κB nuclear translocation and activation of JNK and p38 MAPK. Instead, IL-1R8-TIR domain activates IRAK and TRAF6 and thereby blocks the signaling of several pro-inflammatory cytokines such as IL-1, IL-33 or IL-18 among others (Garlanda et al., 2009). Indeed, animals lacking IL-1R8 present

exacerbated inflammatory responses, confirming its anti-inflammatory actions (Garlanda et al., 2009, 2013; Nold-Petry et al., 2015). Besides, IL-1R8 extracellular domain presents only one Ig-like domain which is the same as the IL-18BP domain. Therefore, due to the IL-1R8 unique structure and its broad anti-inflammatory responses, IL-1R8 was proposed as a co-receptor of the IL-37- IL-18Rα complex. However, to definitively confirm this hypothesis, both *in vivo* and *in vitro* experiments were performed. At first, *in vitro* silencing of IL-18Rα and IL-1R8 impaired the anti-inflammatory response after IL-37 administration when stimulated with LPS (Nold-Petry et al., 2015). *In vivo*, (Nold-Petry et al., 2015) revealed IL-37 activity was highly attenuated in IL-1R8-knock out mice upon LPS treatment. Later immunoprecipitation assays and immunostainings revealed a clear interaction between, IL-18Ra and IL-1R8, forming a tripartite complex along with IL-37 (Figure 6).

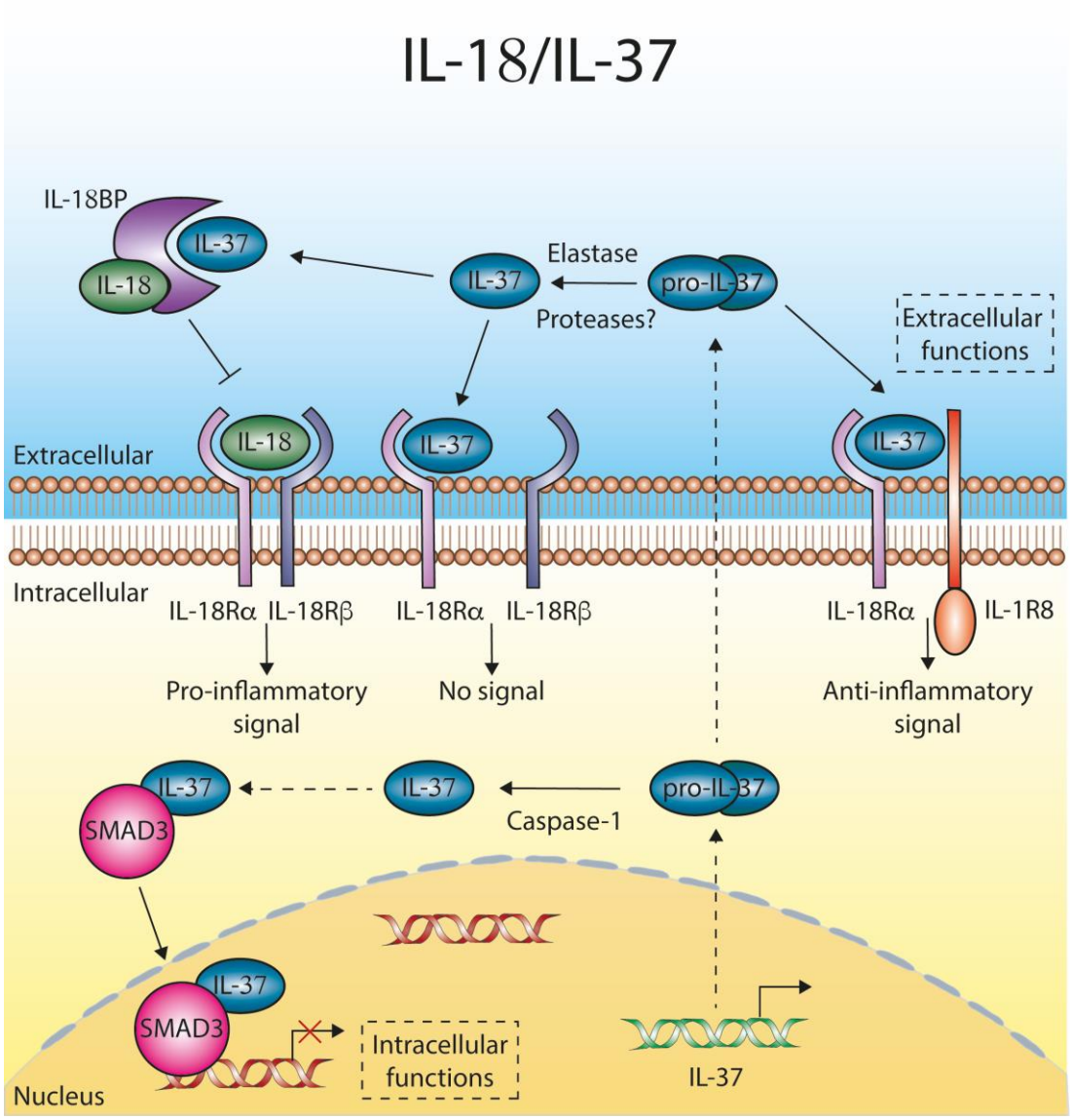

Figure 6. Summary of intracellular and extracellular IL-37 functions.

IL-37 in inflammatory diseases

Since IL-37 was discovered to be one of the most effective inhibitors of both innate and adaptive immune response, its role in several autoimmune and inflammatory-mediated diseases was studied.

In 2011, (McNamee et al., 2011) studied the anti-inflammatory effects of IL-37 in a mouse model of colitis, a bowel inflammatory disease (BID). BID results from the impairment of the bowel immune response and produces injuries in the lumen of several gastrointestinal structures. Hence, to study the protection mediated by IL-37 in a BID model, hIL-37tg animals were administrated with dextran sulfate sodium (DSS) to induce colitis. Interestingly, hIL-37tg mice conferred protection against the injuries caused by DSS administration compared to their WT counterparts. At immune level, hIL-37tg showed a marked reduction of pro-inflammatory cytokines as well as a decreased recruitment of leukocytes to the injuries sites. Surprisingly, IL-37 did not only reduce IL-1β and TNF-α, but also increased the production of IL-10. However, later administration of IL-10 blockers revealed that these anti-inflammatory effects were not IL-10 related.

hIL-37tg mice have been also studied in other diseases including ischemia, hepatic or metabolic diseases. For instance, hIL-37tg were shown to protect myocardium after ischemic injury by reducing MCP-1 and modulating the NF-kB pathway (Yousif et al., 2011). On the other hand, after a hepatic ischemia, hIL-37tg animals showed protection because of the reduction of several pro-inflammatory mediators as TNF-a, MIP-2 or KC (Sakai et al., 2012). Recent studies have also revealed that IL-37 is protective against obesity-induced inflammation and insulin resistance (Ballak et al., 2014). Overall, several studies confirmed the protection of IL-37 against systemic inflammation; however, little is known about its role in the CNS. In fact, there is only one study that tackles this question. Our laboratory provided the first evidence that IL-37 ameliorates the inflammatory response after spinal cord injury (SCI) (Coll-Miró et al., 2016). Traumatic injury to the spinal cord produces functional disabilities, in part, due to the inflammatory response that occurs as a consequence of the injury. To determine the role of IL-37, SCI was induced in hIL-37tg mice to assess their functional and inflammatory response. Not surprisingly, IL-37 was able to highly reduce several pro-inflammatory cytokines released after 24h post-injury (Coll-Miró et al., 2016). Indeed, reduction of these immune mediators, led to a decreased infiltration of peripheral macrophages to the lesion site and a reduced microglial activation. In line with the reduction of inflammatory response elicited by IL-37, hIL-37tg mice showed enhanced motor skills. Despite the multiple efforts to unravel the role of IL-37, there is no current knowledge about its role in neurodegenerative diseases.

Resolution of chronic inflammation

Resolution of inflammation is a crucial step to return tissue homeostasis after injury or infection. This event includes the clearance of immune cells from the challenged region, as well as, the activation of different mechanisms that lead to tissue healing. However, under some circumstances, as it occurs in CNS injuries and neurodegenerative disease, resolution of inflammation is impaired leading to chronic inflammation (Serhan and Savill, 2005; Schwartz and Baruch, 2014; Schwab et al., 2015).

Several theories have emerged to explain the lack of immune cell clearance from the CNS. Under physiological conditions and in most organs, leukocytes return to systemic circulation through the lymphatic drainage. Nevertheless, until 2015, there was no evidence of lymphatic vessels inside the CNS parenchyma (Louveau et al., 2015). Therefore, initial hypothesis postulated that immune cells get stuck within the parenchyma and behind the blood-brain barrier (BBB) (Lassmann, 2008). In this line, several authors showed that immune cells efflux to the cervical lymphatic nodes is very limited (Weller et al., 2008; Ransohoff and Engelhardt, 2012). However, there is now emerging evidence that suggest that the failure of the CNS to resolve the inflammatory response after injury and disease is due its inability to produce adequate levels of specialized pro-resolving lipid mediators (SPMs), the molecules that turn off inflammation these host tissues.

Specialized pro-resolving lipid mediators

SPMs are a family of bioactive lipids identified from inflammatory exudates over the last two decades that actively promote inflammation resolution. These chemical mediators are derived from omega-3 poly-unsaturated acids (PUFAs) such as eicosapentaenoic acid (EPA; C20:5n 3), docosahexaenoic acid (DHA C22:6n 3) and from the omega-6 PUFA, arachidonic acid (AA C20:4n 6). Through highly controlled spatiotemporal transcellular biosynthetic pathways, several SPMs are synthesized. From AA are derived the lipoxins (LX), whereas EPA biosynthesis produces the E series of resolvins (RvE). DHA leads to a broader range of SPMs since it is the precursor for the synthesis of D series of resolvins (RvD), protectins (PD) and maresins (MaR) (Serhan, 2007; Norling and Serhan, 2010). The identification of these bioactive lipids has opened a new field in immunology.

SPMs activate specific mechanisms that trigger the resolution of inflammation, which include: (i) down-regulation of pro-inflammatory cytokines; (ii) abrogation of

intracellular pathways that lead to inflammation; (iii) clearance of inflammatory cell detritus (such as apoptotic neutrophils) by macrophages and (iv) normalization of immune cells counts to basal levels also referred to as catabasis (Buckley et al., 2014; Serhan, 2014; Serhan et al., 2014). Failure to produce adequate amounts of SPMs or failure to bind to their receptors could lead to the persistence of inflammation leading to chronic inflammation. Several reports have shown that there is a defective synthesis of SPMs in different conditions characterized by excessive or chronic inflammation, and thus, the exogenous administration of different SPMs has resulted in potent therapeutic actions, including in cardiovascular diseases, chronic inflammatory conditions, inflammatory pain, infections and cancer (Rose and Connolly, 1999; Bazan, 2007; von Schacky, 2007; Spite et al., 2014; Martini et al., 2016; Sulciner et al., 2018). Several reports have shown that there is defective synthesis of SPMs central nervous in Alzheimer's disease and in multiple sclerosis (Pruss et al., 2013; Wang et al., 2015b). We have also reported defective production of SPMs in the spinal cord after contusion injury in mice, and that the administration of maresin 1 leads to beneficial effects (Francos-Quijorna et al., 2017). However, whether SPMs could exert therapeutic actions in ALS has not been studied yet.

Lipoxins

Lipoxins were firstly discovered at the early 80s (Serhan et al., 1984). Derived from the oxygenation of the arachidonic acid, to date, only two lipoxins have been identified: lipoxin A4 (LXA_4; 5S,6R,15S-trihydroxy-eicosa-7E,9E,11Z,13E-tetraenoic acid) and lipoxin B4 (LXB_4; 5S,14R,15S-trihydroxy-eicosa-6E,8Z,10E,12Etetraenoic acid). LXA_4 is an anti-inflammatory and immunomodulatory lipid mediator whose main function is to promote granulocytes clearance. Its resolution functions are mediated by the activation of LXA_4 receptor, ALX/FPR2 (ALX; also known as formyl peptide receptor (FPR), a G-protein coupled receptor (GPCR). Human ALX/FPR2 is highly expressed in myeloid cells but it is also found in lymphocytes, dendritic cells and resident cells (Serhan, 2007). Besides neutrophils efferocytosis, the activation of ALX/FPR2 through LXA_4 stimulates macrophage phagocytosis of pathogens and neutrophils (Chiang et al., 2006). Regarding LXB_4 signaling, it is thought to stimulate monocytes chemotaxis (Romano et al., 1996). However, it is unknown through which receptor produces its immunomodulatory actions.

Resolvins

After an inflammatory response, phospholipase enzymes (e.g., $cPLA_2$) produce PUFAs, including EPA and DHA. The resulting omega-3 fatty acids suffer several conversions to derive in a series of resolvins, protectins and maresins. First conversions of

EPA through aspirin-acetylated COX-2 or cytochrome P450 monooxygenase produce a compound termed 18-HEPE. Later sequential lipoxygenation of 18-HEPE produces the E series of resolvins (RvE) which encompasses resolvin E 1, (RvE1; 5*S*,12*R*,18*R*-trihydroxyeicosapentaenoic acid) (Arita et al., 2005a), resolvin E 2 (RvE2; 5S,18R-dihydroxy-6E,8Z,11Z,14Z,16E-eicosapentaenoic acid) (Tjonahen et al., 2006) and resolvin E 3 (RvE3;17R,18S-dihydroxy-5Z,8Z,11Z,13E,15E-eicosapentaenoic acid) (Isobe et al., 2012). Among their functions, E series of resolvins produce a potent inhibitory signal that stops the infiltration of blood-borne immune cells in a mouse model of aspiration pneumonia, being as potent as high doses of dexamethasone (Arita et al., 2005a; Tjonahen et al., 2006; Isobe et al., 2012). In the case of RvE1, its anti-inflammatory role is mediated by the activation of two receptors ChemR2344 and BLT1 (Arita et al., 2005b, 2007). Nevertheless, RvE2 and RvE3 receptors have not been discovered yet.

On the contrary, the metabolism of DHA produces the formation of D series of resolvins (RvD1-6). DHA is firstly metabolized by 15-lipoxygenase (15-LOX) producing an intermediate, 17S-H(p)DHA, which after several lipoxygenations will produce the following resolvins: resolvin D1 (RvD1 7S,8R,17S-trihydroxy-4Z,9E,11E,13Z,15E,19Z-), resolvin D2 (RvD2 7S,16R,17S-trihydroxy-4Z,8E,10Z,12E,14E,19Z-), resolvin D3 (RvD3 4S,11R,17S-trihydroxy-5Z,7E,9E,13Z,15E,19Z-), resolvin D4 (RvD4 4S,5,17S-trihydroxy-6E,8E,10E,13E,15Z,19Z-), resolvin D5 (RvD5 7S,17S-dihydroxy-4Z,8E,10Z,13Z,15E,19Z-) and resolvin D6 (RvD6 4S,17S-dihydroxy-5E,7Z,10Z,13Z,15E,19Z-) (Serhan et al., 2002)

RvD actions are mainly mediated by the activation of G protein-coupled receptors. For instance, RvD1 has been shown to activate ALX/FPR2, which also binds to LXA4, and is located at the cell surface of several immune cells such as microglia, endothelial or epithelial cells (Krishnamoorthy et al., 2010). Besides, RvD1 is also capable of interacting with GPR32, an orphan receptor found on polymorphonuclear cells as macrophages and vascular endothelial cells. Through the activation of both receptors, RvD1 modulates the acute inflammatory response by boosting macrophage efferocytosis and blocking PMN infiltration (Sun et al., 2007; Recchiuti et al., 2011; Serhan and Petasis, 2011). Moreover, it has also been shown that RvD1 is able to reduce pain (Xu et al., 2010). The molecular mechanisms involved in this immunomodulatory functions are related with the suppression of the NF-kB pathway by the peroxisome proliferator activated receptor gamma (PPARγ) (Arita et al., 2005b; Liao et al., 2012). On the other hand, RvD2 activates the GPR18 receptor, mainly expressed in human leukocytes (Krishnamoorthy et al., 2010; Chiang et al., 2015) and RvD3 binds to the human GPR32 (Dalli et al., 2013a). Regarding RvD2 activity, it can exert a wide range of functions which include the modification of cytokine balance, the shift on the eicosanoid profile, the modulation of the interaction

between leukocytes and endothelial cells and the reduction of pain (Park et al., 2011). Altogether indicates that at least RvD1 and 2 highly contribute to enhance leukocytes clearance on the resolution phase and control pain by acting on G protein-coupled receptors. However, the mechanisms by which other members of the D series of resolvins produce their actions are not fully understood.

Protectins

Aside from resolvins or maresins, DHA oxygenation can produce protectin D1 (PD1; 10(S),17(S)-dihydroxy-4Z,7Z,11E,13Z,15E,19Z-DHA) or neuroprotectin (NPD1) when produced in the nervous system (Serhan et al., 2006). As in the metabolism of resolvin D series, the catabolism of the ω-3 polyunsaturated fatty acid, DHA, firstly produces the intermediate 17S-H(p)DHA. This intermediate will produce PD1/NPD1 after sequential lipoxygenations as a response to different inflammatory signals produced in diverse tissues (Schwab et al., 2007). Otherwise, in the last years, a new biosynthesis pathway has been shown to produce an alternative form of PD1, termed AT-PD1, which is produced after the oxygenation of the 17R-epimeric form (Petasis et al., 2012). Both PD1/NPD1 have demonstrated a potent regulatory activity upon the polymorphonuclear leukocyte infiltration in a mouse model of peritonitis as well as facilitate the clearance of cellular debris by macrophages (Serhan et al., 2006). Beside its resolutory role, PD1 have also been described as an anti-apoptotic and neuroprotective agent. Recent studies performed in experimental models of Alzheimer's disease have revealed that PD1 may block pro-apoptotic signals and therefore preserve cells' degeneration (Zhao et al., 2011).

Maresins

The last product derived from the DHA catabolism is the maresin family, which encodes Maresin 1 (macrophage mediators in resolving inflammation) (MaR1; 7R,14S-dihydroxy-4Z,8E,10E,12Z,16Z,19Z-DHA) and Maresin 2 (MaR2; 13R,14S-dihydroxy-4Z,7Z,9E,11Z,16Z,19Z-DHA). Maresin formation comes from the enzymatically conversion of DHA by 12/15-lypooxigenase (12/15-LOX) to 14-HpDHA which will be next transformed to 13(S),14(S)-epoxy-maresin. Finally, this intermediate might be hydrolyzed to the Maresin 1 family: 7(R)-MaR1 or its epimer 7(S)-MaR1 (Serhan et al., 2009), which is an inactive form, or to the Maresin 2 family (Deng et al., 2014). MaR1, produced by human macrophages, is highly involved in the resolution of inflammation (Serhan et al., 2009). Appearing at later stages of the inflammatory response, MaR1 is released by resolution phase macrophages which have potent actions on resolution of inflammation, tissue regeneration and pain control (Serhan et al., 2012). Among its functions, it highlights the

inhibition of neutrophils infiltration, the enhancement of efferocytosis and the switch of the macrophage phenotype to an anti-inflammatory one (Serhan et al., 2009; Dalli et al., 2013b). Regarding its antinociceptive functions, MaR1 is able to reduce neuropathic pain by blocking the activity of the transient receptor potential cation channel subfamily V member 1 (TRPV1) and thus preventing the capsaicin-induced inward currents and ectopic neuronal excitation (Serhan et al., 2012). Similar to MaR1, MaR2 also induces anti-inflammatory and pro-resolving actions but in a less prominent fashion (Dalli et al., 2013b).

Conjugates in tissue regeneration (CTR)

In the last years it has been possible to discover a novel series of bioactive peptide-lipid conjugated mediators that not only orchestrate resolution of inflammation but also regulate tissue regeneration (Dalli et al., 2014). These molecules are produced by macrophages and appear later in time than classical SPMs, which facilitates tissue repair and regeneration. The first one discovered was the maresin conjugate in tissue regeneration (MCTR) (Dalli et al., 2016b). These macrophage-derived molecules are derived from the same biosynthetic pathway than maresins but, when 13(S),14(S)-epoxy-maresin is catalyzed, it is enzymatically converted to MCTR1 (13-glutathionyl, 14-hydroxy-docosahexaenoic acid), MCTR2 (13-cysteinylglycinyl, 14-hydroxy-docosahexaenoic acid) and MCRT3 (13-cysteinyl, 14-hydroxy-docosahexaenoic acid)(Dalli et al., 2016a). To produce these lipid effectors, the epoxide intermediate is converted to MCTR1 by the enzymes glutathione S-transferase Mu (GSTM4) and the leukotriene C4 synthase (LTC4S) enzymes. Once MCTR1 is produced, it is catalyzed by γ-glutamyl transferase (GGT) to MCTR2, which in turn, can be cleaved by dipeptidases to produce MCTR3 (Dalli et al., 2016b).

MCTRs are not the only sulfido-conjugated mediators produced during the resolution phase. Other molecules that also promote wound repair and tissue regeneration were recently discovered. Because they share biosynthetic pathways with their substrated precursors protectins and resolvins, they were coined resolving conjugates in tissue regeneration (RCTR) and protectin conjugates in tissue regeneration (PCTR) (Dalli et al., 2015). Similar to MCTRs, they are derived from the enzymatic conversion of their epoxide intermediate and carry potent tissue regeneration and pro-resolving actions in planaria and in human's cells *in vitro*. So far, it has been characterized as a new member of the family of protectin: PCTR1 (16-glutathionyl, 17-hydroxy-4Z,7Z,10,12,14,19Z-docosahexaenoic acid), PCTR2 (16-cysteinylglycinyl, 17-hydroxy-4Z,7Z,10,12,14,19Zdocosahexaenoic acid) and PCTR3 (16-cysteinyl, 17-hydroxy-

4Z,7Z,10,12,14,19Z-docosahexaenoic acid) (Dalli et al., 2015; Ramon et al., 2016) . The new series of RCTR include: RCTR1 (8R-glutathionyl-7S,17S-dihydroxy-4Z,9E,11E,13Z,15E,19Z-docosahexaenoic acid), RCTR2 (8R-cysteinylglycinyl-7S,17S-dihydroxy-4Z,9E,11E,13Z,15E,19Z-docosahexaenoic acid) and RCTR3 (8R-cysteinyl-7S,17S-dihydroxy-4Z,9E,11E,13Z,15E,19Z-docosahexaenoic acid) (Dalli et al., 2015; de la Rosa et al., 2018).

OBJECTIVES

OBJECTIVES

The general objective of the present thesis is to investigate different approaches focused on target inflammation that could lead to therapeutic actions in ALS. Our hypothesis is that inflammation exerts deleterious effects in ALS and thus its modulation leads to an amelioration of the pathophysiology of the disease. The present thesis has been further divided in three chapters according to the following specific aims:

Chapter I. CSF1R signaling mediates microglial proliferation and infiltration of monocytes to peripheral nervous system

- To characterize the expression of CSF1R and its ligands in ALS disease
- To assess the role of CSF1R in microglia proliferation in SOD1^{G93A} mice
- To study the role of CSF1R in monocyte influx to the peripheral nervous system
- To evaluate the contribution of CSF1R to the motor function in SOD1^{G93A} animals

Chapter II. Interleukin-37: a novel protective cytokine in amyotrophic lateral sclerosis

- To characterize the expression of IL-37 and its co-receptors in the spinal cord of amyotrophic lateral sclerosis patients and mice.
- To study the effects of transgenic expression IL-37 in disease progression in SOD1^{G93A} mice
- To evaluate whether IL-37 exerts anti-inflammatory actions in SOD1^{G93A} mice
- To unravel the importance of the extracellular functions of IL-37 in ALS disease
- To investigate genomic variants in IL-37 confers protection in ALS individuals

Chapter III. Maresin 1 improves motoneuron functions and increases lifespan of SOD1^{G93A} mice

- To evaluate whether Maresin 1 ameliorates the clinical course of ALS when its delivery is initiated at late stages of the disease
- To study the effects of Maresin 1 on ALS disease progression when treatments is initiated at pre-symptomatic stages of the disease.

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METHODOLOGY

METHODOLOGY

All the experimental procedures were approved by the Universitat Autònoma de Barcelona Animal Experimentation Ethical Committee (CEEAH 1188R3-DMAH 3131) and followed the European Communities Council Directive 2010/63/EU, and the methods for each procedure were carried out in accordance with the approved guidelines.

Mouse experimental models

All mice used in these studies were housed in the Universitat Autònoma de Barcelona animal facilities, in standard cages and feed *ad limitum* with a light-dark cycle of 12h.

Nowadays, there are several experimental mouse models that mimic ALS; however, the most widely used is the transgenic SOD1^{G93A} (B6-Tg[SOD1-G93A]1Gur) mouse model. Based on those patients with mutations in the *SOD1* gene, this experimental model recapitulates the most relevant histopathological features of the human disease. SOD1^{G93A} mouse presents a prominent loss of both cortical and spinal MNs that starts with a lumbar onset and spreads to upper levels as disease progresses (Mancuso et al., 2011b). Phenotypically, animals can be categorized into two main stages: pre-symptomatic and symptomatic. The pre-symptomatic stage ranges from the date of birth until the week 13th. During this period, animals do not present motor nor gait abnormalities despite having an ongoing degeneration. On the other hand, symptomatic stage ranges from the end of the pre-symptomatic stages until the death of the animal around the week 24th. In the symptomatic phase and due to MNs loss, animals present progressive muscle atrophy and paralysis that leads to their death (Turner and Talbot, 2008).

To study the contribution of the anti-inflammatory cytokine IL-37, we used the transgenic animals overexpressing the human form of IL-37b (hIL-37tg) provided by Prof. Charles Dinarello. To elucidate the importance of the extracellular function of IL-37, we used IL-1R8 null mice, provided by Prof. Cecilia Garlanda. None of these transgenic animals presented phenotypical alterations. All the transgenic animals were further crossed with SOD1^{G93A} mice to study their contribution in a mouse model of ALS, creating the strains SOD1^{G93A}-WT, SOD1^{G93A}-hIL-37tg, SOD1^{G93A}-IL-1R8 KO and SOD1^{G93A}-hIL-37tg-IL-1R8 KO mice.

Drug administration

Depending on the drug administered and their pharmacodynamics, different ways of administration were required:

- Intraperitoneal injection (IP): Maresin 1R (Cayman Chemical Company, Ann Arbor, MI) was administered via IP three times per week (1μg/mouse/day). When needed, IP injections of EdU (Invitrogen) were daily administered for 5 days prior to the experiment.
- Oral gavage: GW2580 was daily administered by oral gavage providing a daily dose of 75mg/kg.

Motor conduction studies

Electrophysiological studies were used to assess both central and peripheral motor conduction in ALS experimental rodent models. ALS is characterized by a progressive degeneration of upper and lower MNs which can be evaluated using extracellular electrophysiological tests. Therefore, conduction studies to study motor evoked potentials (MEPs) and compound muscle action potentials (CMAPs) are fundamental to assess disease progression.

In ALS, the major electrophysiological feature is the reduction of limbs' CMAPs due to the loss of spinal MNs. To electrophysiologically assess this degeneration, it is necessary to obtain fore or hindlimbs CMAPs. To evoke them, peripheral nerves are percutaneously stimulated through a pair of needle electrodes and the subsequent action potentials are recorded in the affected muscles using recording needle electrodes. Since $SOD1^{G93A}$ mice develop ALS with a lumbar onset that rapidly spreads to upper levels, to monitor the disease progression is necessary to record CMAPs in the hindlimb muscles. Therefore, sciatic nerve was percutaneously stimulated through a pair of needle electrodes placed at the sciatic notch, by means of single pulses of 0.02 ms duration (Grass S88). In this line, CMAPs were recorded from gastrocnemius *medialis* (GM), tibialis *anterior* (TA) and plantar *interossei* (PI) muscles. CMAPs signals were band pass filtered (3 Hz to 3kHz), amplified x100 for GM and TA muscles (P511AC amplifiers, Grass), and digitized with a PowerLab recording system (PowerLab16SP, ADInstruments) at 20 kHz. To assess CMAPs, the amplitude increment from baseline to the maximal negative peak and the latency from stimulus to the onset of the first negative deflection, to the maximal negative peak and to the end of the wave were measured.

For evaluation of the motor central pathways, MEPs were evoked by supramaximal electrical stimulation with rectangular pulses of 0.1 ms duration, delivered through needle electrodes inserted subcutaneously; the cathode was placed over the skull overlaying the sensorimotor cortex, and the anode at the nose. The evoked potentials were recorded from the GM in response to the transcranial electrical stimulation of the motor cortex. Since the degeneration of the motor descending pathways produces MEPs with polyphasic shapes, it is fundamental not to only analyze the maximum amplitude but, to further analyze the area under the curve. In this sense, the root mean square (RMS) was calculated from the input signal.

The recording needles were placed using a microscope and guided by anatomical landmarks, to ensure reproducibility of needle location on all animals. During the tests, the mouse skin temperature was maintained between 34 and 36 °C using a thermostatically controlled heating pad. All the evaluators were blinded to the experimental groups.

Behavioral assessment

Due to the progressive neuron degeneration, $SOD1^{G93A}$ mice develop sever motor impairments that can be evaluated using behavioral tests (Miana-Mena et al., 2005). One of the most widely used is the rotarod test. Rotarod test is useful in detecting abnormalities in balance, coordination and strength at early stages of the disease, which makes it a reliable tool to determine the onset of the disease (Miana-Mena et al., 2005; Mancuso et al., 2011a). Rotarod consists in a horizontal rotating cylinder placed above a cage. To perform the test, rodents are placed on the rotating rot and the length of time that the animal stays on the rotarod without falling to the ground is measured. An arbitrary maximum of 180 seconds at 14 rpm was recorded. Rotarod test was performed weekly from 8 to 16 or 20 weeks of age. To ensure reproducible results, animals were trained 3 times per week during the first week (8 weeks of age) to obtain baseline levels (180 seconds at 14 rpm). Besides behavioral tests, locomotion can be further assessed using an automated treadmill. The maximum speed test consists in the assessment of the maximum velocity that the animals are able to reach at the end-stage of the disease in a forced locomotion situation. The speed-controlled treadmill allows the researcher to set a constant belt speed ranging from 0 to 40 cm/s. Thus, rodents are placed on the treadmill and the velocity of the belt is gradually increased until the animal is no longer able to run. The maximum speed that the animal has achieved is then recorded.

Histology

At several time points during the disease progress, SOD1^{G93A} animals and WT littermates were transcardially perfused with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB). Several tissues including, sciatic nerves, spinal cord, muscles, ventral and dorsal roots or brain were harvested, post-fixed in 4% PFA for 2 hours and cryoprotected with 30% sucrose in 0.1 M PB at 4 °C for a minimum of 48h. Samples were then fast-frozen at -60ºC in a cryoembedding compound (Tissue-Tek® OCT, Sakura) and serially cut on a cryostat (Leica). Processed samples were further stained using different approaches.

Cresyl Violet

Frozen-tissue sections were placed in a hot plate during 30 minutes. Serially cut slides were rehydrated in deionized water for 1 min and stained for 2 h with an acidified solution of 3.1 mM cresyl violet at room temperature (RT). Following incubation, sections were then washed and dehydrated in 70%, 96% and 100% ethanol series for 1 min each and mounted with DPX (Fluka).

Immunostaining

Prior to the staining, slides were placed over a hotplate at 37ªC for 30 minutes. Then, samples were rehydrated in PBS for 5 minutes and further blocked with a blocking solution of 5% NDS in PBS-T 0,1% at RT. Once blocked, sections were incubated overnight at 4ºC with primary antibodies (Table 2). Sections were washed in PBS-T and further incubated with a specific secondary antibody bound to an alexa-594 or -488 flourocrom (Dilution 1:500, Invitrogen) for 1 hour at RT. After several washings in PBS-T, PBS and PB, slides were coverslipped with fluoromount mounting media containing DAPI (1μg/ml, Sigma).

Table 2. Immunostaining primary antibodies

Epitope	Dilution	Manufacturer
Iba1	1:1000	Wako
GFAP	1:1000	Dako
CSF1R	1:100	Santa Cruz
F4/80	1:150	Serotec
MHC-II	1:500	Ebioscience
PU.1	1:500	Cell Signalling

For histopathological analyses, slices were visualized with Olympus BX51 microscope and captured with Olympus DP50 digital camera using the Cell^A Image acquisition software. To quantify the staining, the NIH ImageJ (NIH, Bethesda, MD) was used:

- *Motoneuron sparing*: at 16 weeks of age, L4-L5 lumbar segments of $SOD1^{G93A}$ mice were harvested and stained with a cresyl violet staining to visualize lumbar MNs. MNs sparing was manually quantified counting only the those neurons that were within the laminae IX, well stained, larger than 20 µm, shaped in a polygonal fashion and with a preeminent nucleoli.
- *Immunoreactivity*: Either GFAP or Iba1 immunoreactivity was measured by quantifying the integrated density of the immunostaining at the ventral horns of L4-L5 segments using ImageJ software. The integrated density represents the area above the threshold for the mean density minus the background.
- *Cell counting*: cell counting (e.g. macrophages, PU.1, MHC-II…etc) was achieved by manually counting positive cells in the tissue. To avoid any bias through the size of the sample, all values were relativized to its own area and thus, the results were expressed as number of cells/area.

Real-time PCR

Spinal cords, sciatic nerves and hindlimb muscles were collected at 8, 12, 16 and 20 weeks of age from $SOD1^{G93A}$ transgenic mice and age-matched WT littermates after a transcardical perfusion with sterile saline. To RNA isolation, tissue was rapidly homogenized using a tissue rupture with QIAzol lysis reagent (Qiagen) and RNA was further extracted following the manufacturers' instructions of the RNeasy Lipid Tissue kit (Qiagen). From the isolated RNA, up to 1 μg of RNA was primed with random primers (Promega) and reverse transcribed using the Omniscript RT kit (Qiagen). For quantitative PCRs (qPCRs), reactions were prepared using either Brilliant II Ultra-Fast SYBR Green qPCR Master Mix or Taqman's master mix kit depending on the primer. The resulting analysis was achieved using a MyiQ Single-Color Real-Time PCR detection System (BioRad). Gene data was analyzed using the 2-ΔΔCt method with Excel, using GAPDH as housekeeping.

Table 3. SYBR-Green Gene specific primers

Gene	Nomenclature	Forward 5'-3'	Reverse 5'-3'
CSF1R	NM_001037859.2	GCAGTACCACCATCCACTTGTA	GTGAGACACTGTCCTTCAGTGC
IL-34	NM_001135100.1	CTTTGGGAAACGAGAATTTGGAGA	GCAATCCTGTAGTTGATGGGGAAG
CSF1	NM_007778.4	AGTATTGCCAAGGAGGTGTCAG	ATCTGGCATGAAGTCTCCATTT
PU.1	NM_011355.1	CAGAAGGGCAACCGCAAGAA	GCCGCTGAACTGGTAGGTGA
Cyclin D1	NM_007631.2	GGCTCCTCTCATGGCGCTGC	GTGGCATGCACAACAGGCCG
Cyclin D2	NM_009829	TCGATGGGCTGCGTTGCGTT	GGGAGCCTGCGTCAAAGGGG
RUNX1	NM_001111021.2	CAGGCAGGACGAATCACACT	CTCGTG CTGGCATCTCTCAT
IRF8	NM_008320.4	CGGGGCTGATCTGGGAAAAT	CACAGCGTAACCTCGTCTTC
IL-37	NM_014439.3	CTTAGAAGACCCGGCTGGAAG	TGTGATCCTGGTCATGAATGCT
IL-18R	NM_030688.2	TTTGCTGTGGAGACGTTACCC	GCCAGGCACCACATCTCTTT
IL-1R8	NM_023059	TGCTTTGGAAGCCTGGCTCCGT	GGTTTCCTGCAGTGGAGTTGGT

For human samples, anterior horn of the lumbar spinal cord from ALS patients was collected and kept at -80 ºC. For RNA isolation, RNA was extracted following the instructions of the manufacturer (RNeasy Mini Kit, Qiagen® GmbH, Hilden, Germany). RNA purity and concentration was determined using the NanoDrop™ Spectrophotometer

(Thermo Fisher Scientific). cDNA preparation was obtained using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA) following supplier's protocol. For gene expression, TaqMan qPCR were performed in duplicated for each gene in a thermocycler (BioRad) at settings appropriate (50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 sec and 60°C for 1 min). For each 5µL TaqMan reaction, 2.25µL cDNA was mixed with 0.25µL 20x TaqMan Gene Expression Assays and 5µL of 2x TaqMan Universal PCR Master Mix (Applied Biosystems). Hypoxanthine-guanine phosphoribosyltransferase (HPRT1) was used as housekeeping.

Fluorescent activated cell sorting (FACS) analysis

Immune cells from SOD1^{G93A} transgenic mice and age-matched WT littermates were analyzed by FACS at several time points during the disease. In brief, blood samples or nervous tissues were rapidly harvested after a perfusion of the animal with sterile saline. Collected samples were cut and manually dissociated and passed through a cell strainer of 70 µm (BD Falcon). The newly cell suspension was centrifuged at 300 g for 10 minutes at 4ºC twice. Pellet cells were suspended in 1 ml of DMEM with 5% of fetal bovine serum (FBS) and serially distributed according to its staining. Moreover, isotype-matched and negative control samples were used to avoid autofluorescence and nonspecific binding signals.

To label different immune cells types, the below listed primary antibodies were incubated 1:100 1h at RT (Table 4). Once labelled, cell suspensions were washed and fixed in 1% PFA.

Table 4. FACS primary extracellular antibodies

Epitope	Attached fluorophore	Dilution	Manufacturer
CD45	PerCP	1:100	BD Biosciences
CD11b	PE, PE-Cy7	1:100	BD Biosciences
F4/80	APC	1:100	BD Biosciences
EdU	FITC	1:100	Invitrogen
Arginase 1	Alexa 488	1:100	Santa Cruz
iNOS	Alexa 648	1:100	Abcam
CD86	APC	1:100	BD Biosciences

For intracellular staining, extracellular antibodies were fixed with 1% PFA and cells were further permeabilized with the Permeabilization Wash Buffer (Biolegend) for 30 minutes. Once permebialized, cells were incubated with intracellular antibodies (Table 5) for 1h at 4ºC. If the antibody was unconjugated, fluorophore-conjugated host-specific antibodies were incubated for 30 minutes at RT (dilutions 1:500, Invitrogen). Once labelled, stained cells were fixed with 1% PFA and analyzed on a FACSCanto flow cytometer (BD Biosciences).

Table 5. FACS primary intracellular antibodies

Epitope	Attached fluorophore	Dilution	Manufacturer
Arginase 1	Alexa 488	1:100	Santa Cruz
iNOS	Alexa 648	1:100	Abcam

All data was analyzed using FlowJo® software version 10. Immune cell populations were distinguished according to its relative expression of several antigens as listed below (Table 6).

Table 6. Immune cell types according to the expression of different markers

Immune cell type	Antibodies
Macrophages	$CD45^{High}$, $CD11b^{+}$, $F4/80^{+}$
Granulocytes	$CD45^{+}$, $CD11b^{+}$, $F4/80^{-}$
Microglia cells	$CD45^{Low}$, $CD11b^{+}$, $F4/80^{+}$
Neutrophils	$CD45^{High}$, $CD11b^{+}$, $F4/80^{-}$, $Ly6G^{High}$
Anti-inflammatory microglia/macrophages	CD45+, $CD11b^{+}$, $F4/80^{+}$, $Arg1^{+}$, $CD206^{+}$
Pro-inflammatory microglia/macrophages	CD45+, $CD11b^{+}$, $F4/80^{+}$, $iNOS^{+}$, $CD16/32^{+}$

Cytokine protein levels

Briefly, $SOD1^{G93A}$ transgenic mice and age-matched WT littermates were transcradically perfused with sterile saline and spinal cords and sciatic nerves were collected at several time points. Protein was extracted using a tissue rupture in protein extraction buffer (25 mM HEPES, IGEPAL 1%, 0.1 $MgCl_2$, 0.1 M EDTA pH=8, 0.1 M EGTA pH= 8, 0.1 M PMSF, 10 μl/ml of Protease Inhibitor cocktail (Sigma) and 1 pill of PhosphoSTOP). Protein was quantified and further concentrated at 4mg/ml using MicroCon centrifugation filters (Milipore).

Protein levels of several cytokines were then evaluated using an own-designed mouse Cytokine/Chemokine magnetic bead panel (Millipore) on a MAGPIX® system (Millipore). Data analysis was performed with the MILLIPLEX® Analyst 5.1 software (Millipore).

Post-mortem spinal cord tissue samples

Post-mortem fresh-frozen lumbar spinal cords were collected and provided by the Institute of Neuropathology HUB-ICO-IDIBELL Biobank following the guidelines of Spanish legislation and the local ethics committee. In brief, transversal sections of the spinal cord from human ALS patients and controls were post-mortem removed and kept at -80°C or fixed by immersion in 4% buffered formalin. Lumbar ventral spinal cords were processed on a dry-ice frozen plate under a binocular microscope at a magnification x4. The study included 14 sALS (6 men and 8 women; no *C9orf72, SOD1, TARDBP* and *FUS* mutations occurred in any case) and 14 control cases (6 men and 8 women). No cases with frontotemporal dementia were included. All ALS patients with associated pathologies including Alzheimer's disease (excepting neurofibrillary tangle pathology stages I-II of Braak and Braak), Parkinson's disease, tauopathies, vascular diseases, neoplastic diseases affecting the nervous system, metabolic syndrome, hypoxia and prolonged axonal states such as those occurring in intensive care units were excluded. Besides, cases with infectious, inflammatory and autoimmune diseases, either systemic or limited to the nervous system were excluded. Age-matched control cases had not suffered from neurologic or psychiatric diseases, and did not have abnormalities in the neuropathologic examination, excepting sporadic neurofibrillary tangle pathology stages I-II of Braak and Braak. All this data is summarized in table 7.

Table 7. Controls and ALS patients data

Group	Autopsy nº	Age	Gender	PM delay	RIN
Controls	A06/047	59	M	12 h 05 min	6,40
	A07/067	47	M	04 h 55 min	5,60
	A07/082	64	F	11 h 20 min	6,20
	A07/084	46	M	15 h 00 min	5,90
	A07/114	56	M	07 h 10 min	6,10
	A07/162	71	F	08 h 30 min	5,90
	A08/042	64	F	05 h 00 min	7,00
	A08/070	79	F	06 h 25 min	6,70
	A09/120	75	M	07 h 30 min	4,90
	A09/137	55	M	09 h 45 min	5,30
	A14/003	51	F	04 h 00 min	6,30
ALS	A07/010	56	M	10 h 50 min	7,10
	A07/013	70	M	03 h 00 min	7,30
	A07/071	77	M	04 h 30 min	7,40
	A07/072	56	F	03 h 45 min	8,20
	A07/121	59	M	03 h 15 min	7,50
	A07/139	63	F	13 h 50 min	6,80
	A09/012	59	F	14 h 15 min	6,40
	A11/054	64	M	16 h 30 min	6,30
	A12/020	57	F	04 h 00 min	6,20
	A12/040	75	F	04 h 05 min	6,80
	A12/083	79	F	02 h 10 min	7,00
	A13/003	57	F	10 h 00 min	6,50
	A15/029	46	M	07 h 00 min	7,00
	A15/033	69	F	17 h 00 min	6,40

Single nucleotide polymorphism (SNPs)

SNPs are small variations in single nucleotides in the DNA that are found at least in 1% of the population. To analyze the presence of the SNPs in the IL-37 gene (rs3811046), commercial predesigned TaqMan SNP Genotyping Assay (C__27487174_10, Thermo Fisher, MA, USA). The SPN rs3811046 (G/T) causes substitution of valine to glycine at 31th location the IL-37b protein amino acid sequence, and not the other way around.

DNA samples extracted from the skin of sporadic and familiar ALS patients and genotyping of the rs3811046 polymorphism within IL-37 was performed by means of a real-time polymerase chain reaction, Thermal cycling and endpoint PCR analysis was performed on an ABI PRISM 7900HT.

Cell sorting

Briefly, CNS from adult C57/Bl6 (8-10 weeks of age) were collected and enzymatically digested using a enzymatic cocktail containing collagenase B 0.2% (Roche Diagnostics GmbH) and trypsine-EDTA 0.2% at 37 ºC for 30 minutes. Digested solutions were further passed through a cell strainer of 40 μm mesh (BD Falcon) to obtain a cell suspension that was rapidly centrifuged twice at 300 g for 10 minutes at 4 ºC. To sort microglial cells, disaggregated cells were firstly separated by magnetic sorting using a CD11b antibody (MiltenyBiotec). Isolated CD11b-positive cells were further conjugated with anti-CD45 PerCP-Cy5.5-conjugated and anti-CD11b-PE-Cy7-conjugated antibodies for purification on cell sorter (FACSARIATM III, BD Bioscience). Only populations that presented >90% purity were used for gene expression.

CHAPTER I

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CSF1R blockade slows the progression of amyotrophic lateral sclerosis by reducing microgliosis and invasion of macrophages into peripheral nerves

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Inflammation is a common neuropathological feature in several neurological disorders, including amyotrophic lateral sclerosis (ALS). We have studied the contribution of CSF1R signalling to inflammation in ALS, as a pathway previously reported to control the expansion and activation of microglial cells. We found that microglial cell proliferation in the spinal cord of SOD1^{G93A} transgenic mice correlates with the expression of CSF1R and its ligand CSF1. Administration of GW2580, a selective CSF1R inhibitor, reduced microglial cell proliferation in SOD1^{G93A} mice, indicating the importance of CSF1-CSF1R signalling in microgliosis in ALS. Moreover, GW2580 treatment slowed disease progression, attenuated motoneuron cell death and extended survival of SOD1^{G93A} mice. Electrophysiological assessment revealed that GW2580 treatment protected skeletal muscle from denervation prior to its effects on microglial cells. We found that macrophages invaded the peripheral nerve of ALS mice before CSF1R-induced microgliosis occurred. Interestingly, treatment with GW2580 attenuated the influx of macrophages into the nerve, which was partly caused by the monocytopenia induced by CSF1R inhibition. Overall, our findings provide evidence that CSF1R signalling regulates inflammation in the central and peripheral nervous system in ALS, supporting therapeutic targeting of CSF1R in this disease.

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease caused by the loss of motoneurons (MNs) in the motor cortex, brainstem and spinal cord. It manifests with skeletal muscle weakness, spasticity and eventual paralysis, leading to the death of patients by respiratory failure 3 to 5 years after diagnosis[1]. ALS occurs sporadically in 90% of cases whereas the remaining 10% arises from inherited forms of the disease.

In the last decades, the generation of different ALS murine models has allowed the identification of several mechanisms leading to MNs death. The exact pathogenic process that triggers MN degeneration in ALS is currently unknown, but it is likely to be multifactorial, as for other neurodegenerative diseases[2].

One of the hallmarks of chronic neurodegenerative diseases, including ALS, is the contribution of non-neuronal cells to the progression of the pathology, especially those regulating the neuroimmune component[3]. Several studies indicate that astrocytes are harmful in ALS[4,5], whereas the role of microglial cells is unclear[6–9]. However, a recent report supports a detrimental role of microglia to the pathology of ALS, inducing MN death via NFκB activation[7]. Besides glial cells, peripheral leukocytes also contribute to ALS[10–12]. Evidence suggests that

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lymphocytes subsets contribute to slowing disease progression[13], whereas the function of macrophages, which invade the peripheral nerves during disease progression[14,15], is yet to be defined.

Colony-stimulating factor 1 receptor (CSF1R) is the cell surface receptor for IL-34 and CSF1. CSF1R has important roles in haematopoiesis, regulation of proliferation, cell survival and maturation of microglia and monocytes, as well as in controlling the overall immune response[16]. Recent evidence from a mouse model of prion disease supports that CSF1R controls microgliosis and contributes to neurodegeneration[17]. A previous study showed that systemic administration of CSF1 accelerates disease progression in the $SOD1^{G37R}$ mouse, suggesting that overactivation of CSF1R exerts detrimental actions in ALS, probably, by increasing the mitogenic activity of microglia[18,19]. It is important to establish whether pathological activation of CSF1R in ALS contributes to disease progression, and if so, which are the physiological mechanisms underlying its harmful effects, since CSF1 is increased in the spinal cord of ALS patients[19].

In the present study, we pharmacologically inhibited the activation of CSF1R to dissect the role of this receptor in ALS. We provide evidence that blocking CSF1R ameliorates the clinical course of ALS disease by reducing both the invasion of macrophages into peripheral nerves at pre-symptomatic stages of the disease, and by impeding microglia proliferation at late stages of the pathology.

Materials and Methods

Animals. All the experimental procedures were approved by the Universitat Autònoma de Barcelona Animal Experimentation Ethical Committee and followed the European Communities Council Directive 2010/63/EU, and the methods for each procedure were carried out in accordance with the approved guidelines. Experiments were performed in female transgenic mice carrying the G93A human SOD1 mutation (B6SJL-Tg[SOD1-G93A]1Gur) obtained from the Jackson Laboratory (Bar Harbor, ME, USA) and provided from the colony maintained at the University of Zaragoza. Hemizygous transgenic mice were identified by PCR amplification of DNA extracted from tail samples and then were maintained in local facilities. Mice were housed with food and water ad libitum at room temperature of 22 ± 2 °C under a 12:12-h light–dark cycle. It was considered that animals reached the endpoint of the disease when the righting reflex was lost for longer than 30 s.

At 8 weeks of age (prior to the beginning of the treatment), animals were electrophysiologically tested to obtain baseline levels. Animals were then distributed among the different experimental groups according to their progenitors, weight and electrophysiology baseline values in balanced groups, either GW2580-treated or untreated control $SOD1^{G93A}$ mice. Inhibition of CSF1R was achieved by administration of GW2580 as described previously[17]. Briefly, GW2580 (LC Laboratories) was dissolved in sterile 0.9% saline buffer with 0.1% tween80, and administered by oral gavage providing a daily dose of 75 mg/kg from 8 weeks of age until the end of the experiment. When required (assessment of microglia proliferation), mice received intraperitoneal injections of EdU (Invitrogen; 0.1 mg/kg in 0.2 ml of sterile PBS) daily for the 5 days before the end of the experiment.

Functional tests. Motor coordination, balance and strength of the animals were assessed using the Rotarod test[20]. All mice were trained three times a week on the rod rotating at constant speed of 14 rpm (rotating cylinder 3.4 cm diameter) for a maximum of 180 seconds to reach the baseline level of performance. Animals were then tested weekly from 8 until 16 weeks of age at the same speed, and the time for which each animal could remain on the rotating rod was measured. An arbitrary maximum time of remaining on the rotating rod of 180 s was considered ($n = 10$ per group).

The highest locomotion speed of the mice was tested on a controlled treadmill at the end of the follow up (16 weeks of age). Mice were placed over the treadmill and their capacity to run with increasing treadmill velocities, 5, 10, 15, 20, 25 and 30 cm/s, was recorded[21] ($n = 10$ per group).

Motor nerve conduction tests were performed every 2 weeks from 8 weeks to 16 weeks of age ($n = 18$ $SOD1^{G93A}$ control, $n = 19$ $SODG^{G93A}$ treated with GW2580). The sciatic nerve was percutaneously stimulated through a pair of needle electrodes placed at the sciatic notch, by means of single pulses of 0.02 ms duration (Grass S88). The compound muscle action potential (CMAP, M-wave) was recorded from the tibialis anterior (TA) and the plantar interossei (PL) muscles with microneedle electrodes[21]. All potentials were amplified and displayed on a digital oscilloscope (Tektronix 450S) at settings appropriate to measure the amplitude from baseline to the maximal negative peak and the latency from stimulus to the onset of the first negative deflection, to the maximal negative peak and to the end of the wave. The recording needles were placed using a microscope and guided by anatomical landmarks, to ensure reproducibility of needle location on all animals. During the tests, the mouse skin temperature was maintained between 34 and 36 °C using a thermostatically controlled heating pad. All the evaluators were blinded to the experimental groups.

Histology. At 16 weeks of age, female $SOD1^{G93A}$ GW2580-treated ($n = 9$), $SOD1^{G93A}$ untreated ($n = 7$) and age-matched WT littermates ($n = 7$) were transcardially perfused with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB). Lumbar segments of the spinal cords were harvested, post-fixed in 4% PFA for 2 hours and cryoprotected with 30% sucrose in 0.1 M PB at 4 °C. Samples were cut in transverse serial sections (40 μm thick) with a cryostat (Leica) between L2 and L6 segments. For each segment, series of 10 sections were sequentially collected on free-floating and kept in Olmos solution at −20 °C. To analyse MNs preservation, sections were rehydrated for 1 min and stained for 2 h with an acidified solution of 3.1 mM cresyl violet. Sections were then washed in distilled H20 for 1 min, dehydrated and mounted with DPX (Fluka). MNs were identified by their localization in the ventral horn of the stained spinal cord sections and quantified following size and morphological criteria: only MNs with diameters larger than 20 μm and with polygonal shape and prominent nucleoli were quantified. MNs present in the lateral site of both ventral horns were quantified in four serial sections of the L4 segment[21]. Spinal cord tissue sections were also immunostained with rabbit anti-Iba1 (1:500, Wako), goat anti-Iba1 (1:250, Abcam), rat anti-MCH II (1/500 Ebioscience) or rabbit anti-PU.1 (1/500 Cell Signalling)

and rabbit anti-CSF1R (1:100, Santa Cruz) primary antibodies for visualization of microglia, and detected with appropriate secondary antibodies conjugated with Alexa 488, 594 (Life Technologies) for immunofluorescence, or biotin for light microscopy (1:200, Vector Labs). For visualization of proliferative microglia, EdU was visualized using the Click-iT reaction coupled to an Alexa Fluor 568 azide following the instructions of the manufacturer (Invitrogen). Nuclei were visualized by DAPI staining and the sections were mounted with Mowiol/DABCO (Sigma-Aldrich) mixture. Quantification of Iba1 and MHCII cells was done as we reported previously[17,22].

Tibial nerves from female WT, treated and untreated SOD1^{G93A} mice were harvested at 12 and 16 weeks of age (n = 4 per group and time point). Nerve samples were cut in longitudinal serial sections of 15 μm thickness with a cryostat. Macrophages were detected by immunostaining using rat anti-mouse F4/80 antibody (1:150, Serotec) as described above. Similarly, a segment of L5 spinal nerve containing the dorsal root ganglia, as well as, the dorsal and ventral roots was removed from female untreated SOD1^{G93A} mice (n = 4), cut in longitudinal sections of 15 μm thickness with a cryostat, and stained against F4/80 for assessing the presence of macrophages in the dorsal and ventral roots. Macrophages were manually quantified in a 7.5×10^4 μm^2 area from 4 tissue sections of tibial nerve, sciatic nerve and dorsal and ventral roots.

Tissue sections were viewed with an Olympus BX51 microscope and images were captured using an attached Olympus DP50 digital camera, using the Cell^A Image acquisition software. Alternatively, sections were visualized in a Leica CTR 5000 microscope, coupled to a Leica DFC300FX microscope camera or in a Zeiss LSM 700 confocal microscope. Data was represented as number of positive cells/mm^2. All quantifications were performed blinded to the experimental groups with the help of the ImageJ image analysis software (NIH).

Fluorescent activated cell sorting (FACS) analysis. At 10, 12 and 16 weeks of age, cells from lumbar spinal cord of SOD1^{G93A} GW2580-treated (n = 4 per time point), SOD1^{G93A} untreated mice (n = 4 per time point) and age-matched WT littermates (n = 4; 16 weeks) were analysed by FACS analysis. Mice were terminally anesthetized with an overdose of sodium pentobarbital and transcardially perfused with 0.9% saline solution. Spinal cord lumbar segments were harvested, mechanically triturated and then passed through a cell strainer of 40 μm mesh (BD2 Falcon) with DMEM media with 10% of Fetal Bovine Serum (FBS). The cell suspension was centrifuged twice at 300 g for 10 min at 4 °C, to remove debris.

Samples were split in several tubes and immunostained. Primary antibody labelling was performed for 1 hour at 4 °C, using DMEM + 10% FBS as buffer. Cells were labelled with the following antibodies: PE or APC Cy7-conjugated anti-CD11b (1:100, eBioscience), PerCP or PerCP-Cy5 conjugated anti-CD45 (1:100, Biolegend), APC conjugated anti-CD86 (1:100, BD Biosciencies), APC conjugated anti-F4/80 (1:100, eBioscience), FITC conjugated anti-iNOS (1:100, BD Biosciences), FITC conjugated anti-CD206 (1:100, Biolegend), PE conjugated anti-CD16/CD32 (1:100, Biolegend) and goat anti-Arginase 1 (1:100, Santa Cruz) followed by secondary incubation with PE conjugated anti-goat (1:150, Abcam). For intracellular analysis of EdU, cells were fixed, washed, permeabilized and incubated with Alexa Fluor 488 conjugated anti-EdU following the Click-iT reaction according to the manufacturer's instructions (1:100, Life Technologies). Moreover, unstained cells and isotype-matched control samples were used to control for autofluorescence and/or non-specific binding of antibodies. Microglial cells were identified as CD45^{low}, CD11b$^+$ and further differentiated based on CD86, iNOS, CD16/32, Arg1 and CD206 expression relative to their activation state.

Sciatic nerve samples from untreated SOD1^{G93A} (n = 3) were also harvested at 16 weeks and processed as described above. Nerve macrophages (CD45$^+$, CD11b$^+$, F4/80$^+$ cells) were further differentiated based on iNOS, CD16/32, Arg1 and CD206 expression.

In addition, blood samples from 12 weeks old SOD1^{G93A} mice untreated or treated with GW2580 (n = 4 per group) were collected from the peroneal vein, erythrocytes were then lysed in RBC lysis buffer (eBioscience), and leukocytes were stained with PECy7-conjugated anti-CD11b (1:100, eBioscience), PerCP or PerCP-Cy5 conjugated anti-CD45 (1:100, Biolegend), APC conjugated anti-F4/80 (1:100, eBioscience) using the same method described above. Monocytes were identified as CD45$^+$, CD11b$^+$ and F4/80$^+$. Cells were analysed in a blinded fashion with respect to the experimental groups using FlowJo® software on a FACSCanto flow cytometer (BD Biosciences).

Isolation of microglia from CNS tissue. Briefly, spinal cord and brain from adult C57/Bl6 mice (8–10 weeks old) were removed and enzymatically digested with a collagenase B 0.2% (Roche Diagnostics GmbH) and trypsine-EDTA 0.2% at 37 °C for 30 min, and then passed through a cell strainer of 40 μm mesh (BD falcon). Cell suspension was centrifuged twice at 300 g for 10 minutes at 4 °C, and microglial cells were first isolated by magnetic sorting using a CD11b antibody (MiltenyiBiotec) and then stained with PerCP-Cy5.5-conjugated CD45 and PE-Cy7-conjugated CD11b antibodies for further purification on cell sorter (FACSARIATM III, BD Bioscience). Microglia cells were assessed on a flow cytometer (FACSCalibur; BD Biosciences), and only populations presenting >90% purity were used for gene expression analysis.

Analysis of gene expression by qPCR. At 12 and 16 weeks of age, SOD1^{G93A} GW2580-treated (n = 4 per time point), SOD1^{G93A} untreated mice (n = 4 per time point), and 16 weeks of age WT littermates (n = 3) were processed to obtain samples from the lumbar spinal cord. RNA from spinal cords and *in vivo* sorted microglia was extracted using the RNAqueous®-Micro Kit (Life Technologies), quantified using Nanodrop (Thermo Scientific), to be retro-transcribed using the iScript cDNA Synthesis Kit (Bio-Rad), following manufacturer's instructions, after checking its integrity by electrophoresis in a 2% agarose gel. cDNA libraries were analysed by qPCR using the iTaq Universal SYBR Green supermix (Bio-Rad) and the following custom designed gene-specific primers (Sigma-Aldrich)[17].

Quality of the primers and qPCR reaction were evaluated by electrophoresis in a 1.5% agarose gel, checking the PCR product size. Data was analysed using the 2-ΔΔCt method with Primer Opticon 3 software, using GAPDH as housekeeping gene.

Multiplex Assay. At 12 and 16 weeks of age, lumbar spinal cord of SOD1^{G93A} GW2580-treated (n = 4 per time point), SOD1^{G93A} untreated mice (n = 4 per time point), and WT littermates (n = 3; age 16 weeks) were homogenized and after centrifugation at 13000G for five minutes, supernatants were used for cytokine analysis. Protein levels of 11 cytokines (CSF1, TNF-α IL-1α, IL-1β, IL-4, IL-6, IL-10, IL-13, IP-10, KC, MCP-1 and MIP1α.) were assessed using a MILLIPLEX® MAPmagnetic bead-based multi-analyte panel Multiplex bead kit (Merck-Millipore). Standard curves were generated using the specifics standards supplied by the manufacturer. Samples were analysed on a MAGPIX® system (Millipore) using the MILLIPLEX® Analyst 5.1 software (Millipore).

Statistical analysis. Data are shown as mean ± SEM and analysed using the GraphPad Prism 6 software package (GraphPad Software). Electrophysiological and locomotion test results were analysed using two-way repeated measurements ANOVA with Tukey post-hoc test for multiple comparisons. T-Student was used for histological data comparing the 2 experimental groups. Two-way ANOVA was used for the gene expression data, FACS analysis and multiplex data, followed by a Tukey post-hoc test for multiple comparisons. Survival data was analysed using the Mantel-Cox test. Differences were considered significant at $p < 0.05$.

Results

Characterization of the components of the CSF1R pathway in the spinal cord of SOD1^{G93A} mice. Microgliosis is a major hallmark of the pathology in diverse neurodegenerative diseases, including ALS. Previous studies have highlighted the importance of CSF1R in microglial proliferation[17,18]. We therefore characterized the expression of the components of the CSF1R pathway in SOD1^{G93A} mice. We found that spinal cords and sorted microglial cells from C57Bl/6 mice have constitutive mRNA expression of CSF1R. CSF1R transcripts were enriched 39-fold in microglia as compared to total spinal cord homogenates suggesting that microglial cells are the main source, if not the unique, of CSF1R in the CNS (Fig. 1a). Interestingly, we found that mRNA levels of CSF1R increased in the spinal cord of SOD1^{G93A} mice at late stages of the disease (Fig. 1b). At this time point, histological images reveal that CSF1R expression was markedly increased in the ventral horn of the spinal cord (Fig. 1c). Confocal microscopy revealed that reactive microglia was source of the increased expression of CSF1R in this ALS mouse model (Fig. 1d–f). Analysing the expression of the CSF1R ligands, we observed that the mRNA levels of IL-34 remained unaltered in the lumbar spinal cord of SOD1^{G93A} mice, whereas the transcripts for CSF1 increased ~2 fold but only at late stages of the disease (16 weeks) (Fig. 1g,h). This data correlated with the measurement of the protein levels of CSF1 at the lumbar spinal cord of SOD1^{G93A} mice, with upregulated levels at 16 weeks when compared to WT littermates (Fig. 1i). Overall, these findings show that the activation of CSF1R signalling via CSF1 occurs in the spinal cord of SOD1^{G93A} mice at late stages of the disease.

Inhibition of CSF1R reduces microglial proliferation in SOD1^{G93A} mice. We have recently reported the importance of CSF1R pathway in inducing microgliosis in a mouse model of prion disease[17]. To determine whether CSF1-CSF1R signalling mediates microgliosis in ALS, we pharmacologically blocked CSF1R activation with GW2580, a selective CSF1R inhibitor[23]. FACS analysis of lumbar spinal cords of SOD1^{G93A} mice revealed that treatment with GW2580 reduced the number of microglial cells (CD45^{low}, CD11b^{+}) by ~30% at late, but not at earlier stages of the disease (Fig. 2a–c). Reduction of microgliosis by GW2580 treatment was further confirmed by immunohistochemical analysis of lumbar spinal cord of 16 weeks old SOD1^{G93A} mice (Fig. 2d–g). Attenuation of microgliosis by GW2580 was due to inhibition of microglia proliferation, since the number of EdU+ microglial cells was decreased by ~30% after treatment, as also revealed by FACS and histological analysis (Fig. 2h,j). Further evidence for the dependence of microgliosis on CSF1R signalling was observed: GW2580 treatment reduced the mRNA levels of three transcription factors that play a key role of microglia proliferation and lineage commitment, PU.1, IRF8, and RUNX1, and down-regulated the expression of the CSF1R downstream regulator of the cell cycle, cyclin D2, but not D1 (Fig. 3a–e). Histological studies revealed that PU.1+ microglial cells were reduced in the spinal cord of ALS mice after GW2580 treatment (Fig. 3f–i), corroborating our PCR data. Overall, these results demonstrate the importance of CSF1R activation in triggering microglial proliferation at late stages of the disease in the SOD1^{G93A} mouse.

Effects of CSF1R inhibition on the neuroinflammatory response in SOD1^{G93A} mice. We next assessed whether the inhibition of CSF1R activity modulated the inflammatory milieu in ALS. We first examined the effects of CSF1R on microglial cell activation and polarization. The expression of surface molecules related to antigen presentation is a hallmark of activated microglia[24]. Thus, we used CD86 and MHCII as markers for activated microglia[25]. FACS analysis revealed that GW2580 administration led to a significant reduction in the number of CD86+ activated microglial cells (Fig. 4a–c) and a reduction in MHCII+ microglia by the CSF1R inhibitor at the histological level (Fig. 4d–g), providing clear evidence that GW2580 attenuates activated microglia counts. However, reduction in CD86+ and MHCII+ cells is likely due to ability of the drug to hamper microglia proliferation since the proportion of these markers expressed in microglial cells is quite similar. FACS analysis revealed that CSF1R does not indiscriminately modulate microglia polarization in ALS, since the expression of CD16/32, iNOS, Arg1 and CD206 markers in microglial cells remained unaltered after GW2580 treatment (Fig. 4h). Similarly, we found that CSF1R inhibition had a minimal effect on cytokine production, since only the protein levels of one (IP10/CXCL10) out of the 11 cytokines evaluated were significantly reduced in the spinal cord homogenates of SOD1^{G93A} mice after GW2580 treatment (Fig. 4i).

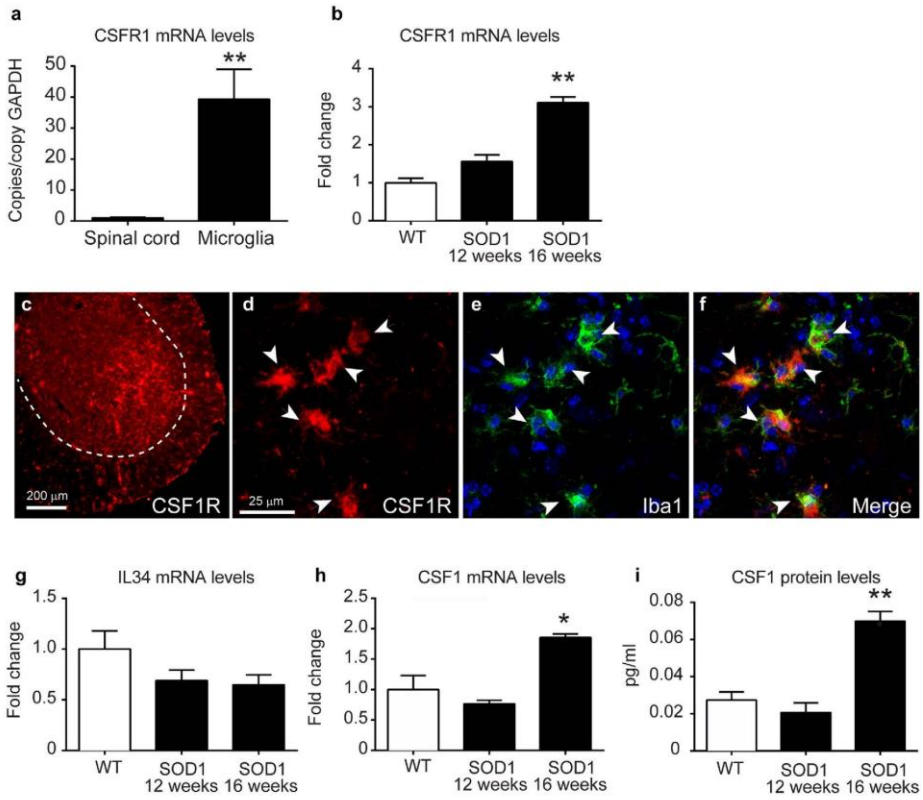

Figure 1. Characterization of the CSF1R pathway in the spinal cord of SOD1^{G93A} mice. (**a**) Analysis of the mRNA expression of CSF1R in the spinal cord and microglial cells sorted from adult CNS of C57/Bl6 mice (n = 4 per group). Note that CSF1R expression is highly enriched in sorted microglia as compared to spinal cord homogenates. (**b**) Expression of CSF1R in the ventral spinal cord of WT and SOD1^{G93A} mice at symptomatic (12 weeks) and end-stage (16 weeks) of the disease (n = 3 in WT, n = 4 in SOD1^{G93A} per time point). (**c**–**f**) Representative immunofluorescence image of CSF1R of the lumbar spinal cord of SOD1^{G93A} mice at 16 weeks of age (**c**) showing that CSF1R immunoreactivity is markedly increased in the ventral horn. Confocal high magnification images of the ventral horn showing the expression of CSF1R (**d**) and Iba1 (**d**). Note in the merged image (**f**) that microglial cell are the source of CSF1R in the SOD1^{G93A} mice at end stage of the disease. Analysis of mRNA levels of IL-34 (**g**) and CSF1 (**h**) in the ventral lumbar spinal cord of WT and SOD1^{G93A} at symptomatic (12 weeks) and end-stage (16 weeks) of the disease (n = 3 in WT, n = 4 in SOD1^{G93A} per time point). (**i**) Quantification of the protein levels of CSF1 in spinal cord homogenates of WT and SOD1^{G93A} mice at 12 and 16 weeks. (n = 3 in WT, n = 4 in SOD1^{G93A} per time point). Note that mRNA and protein levels of CSF1 are up-regulated in the spinal cord of SOD1^{G93A} mice at the end stage of the disease, but not earlier. *$p < 0.05$ and **$p < 0.01$ vs. WT. Error bars indicate SEM.

Selective inhibition of CSF1R slows the disease progression in SOD1^{G93A} mice. We next assessed the contribution of CSF1R to the functional outcome in the SOD1^{G93A} mouse model of ALS. GW2580 administration led to a significant preservation of the amplitude of the TA and PL CMAPs starting at 10 and 12 weeks, respectively, which was sustained until the end of the follow up (16 weeks of age) (Fig. 5a,b). We also assessed the effects of GW2580 treatment on motor skills. Rotarod testing revealed that GW2580 did not delay the onset of the motor impairment but slowed its progression (Fig. 5c). Moreover, mice treated with GW2580 were able to run at significant higher speeds on a treadmill at 16 weeks of age (Fig. 5d). In line with the functional data, treatment with GW2580 led to significant extension in the survival of SOD1^{G93A} mice, increasing the maximal lifespan by 12% (Fig. 5e). Histopathological analysis of the lumbar spinal cord from SOD1^{G93A} mice at 16 weeks of age revealed that GW2580 increased significantly the number of surviving MN compared to control SOD1^{G93A} mice (Fig. 5f–i). These data provide clear evidence of the detrimental contribution of CSF1R activation in the CNS of ALS mice.

CSF1R inhibition decreases the number of macrophages into the tibial nerve of SOD1^{G93A} mice. GW2580 treatment ameliorated disease progression in SOD1^{G93A} mice but the protective effects on muscle innervation were observed before the increase of CSF1 protein levels in the spinal cord, and consequently,

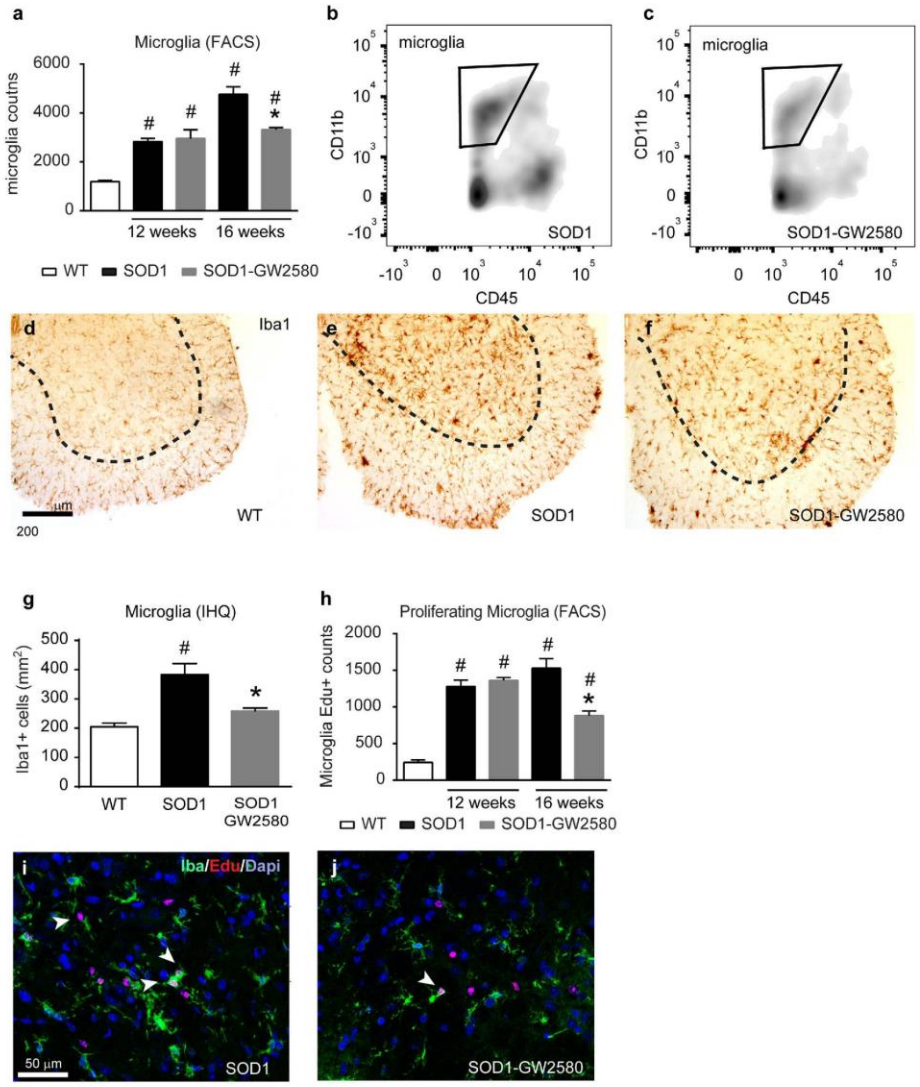

Figure 2. Assessment of microglial cells in lumbar spinal cord of SOD1^{G93A} mice. (**a**–**c**) FACS analysis from lumbar spinal cord of WT, SOD1^{G93A} untreated and treated with GW2580 showing microglial cell counts (CD45low, CD11b+) at 12 and 16 weeks. (n = 4 animals per group and time point). (**b**,**c**) Representative density FACS plots showing the reduction of microglia in 16 weeks old SOD1^{G93A} mice after GW2580 treatment. (**d**–**f**) Representative micrographs of L4-L5 ventral horns stained for Iba1 from (**d**) WT, (**e**) SOD1 control or (**f**) GW2580-treated SOD1 mice. (**g**) Graph showing immunohistochemical quantification of Iba1+ cells in the ventral horns of spinal cord tissue sections of 16 weeks of age SOD1^{G93A} mice untreated or treated with GW2580 (n = 4 per group). Note, that immunohistochemical analysis highly correlates with counts obtained by FACS analysis. (**h**) Quantification of microglial proliferation with EdU assay by FACS analysis. Note that selective blockade of CSF1R reduces proliferation of microglial cells in SOD1^{G93A} mice at 16 weeks of age (n = 4 animals per group and time point). (**i**,**j**) Representative images of microglial proliferation by double immunofluorescence for EdU (red), Iba1 (green) and DAPI (blue) in the ventral horn of L4-L5 spinal cord tissue sections of (**i**) SOD1^{G93A} control and (**j**) treated with GW2580 at 16 weeks of age. Note that CSF1R blockade significantly reduces microglia proliferation but only at 16 weeks of age. Scale bars: (**d**–**f**) 200 µm; (**i**,**j**) 50 µm. $^{*}p < 0.05$ compared to SOD1^{G93A} untreated, $^{\#}p < 0.05$ compared to WT. Error bars indicate SEM.

prior to its effects on microgliosis. These findings suggest that activation of peripheral CSF1R might be responsible for the early muscle denervation. We therefore assessed whether this effects could be due to recruitment of macrophages into the sciatic nerve of SOD1^{G93A} mice. In agreement with a previous work[14], we found that the

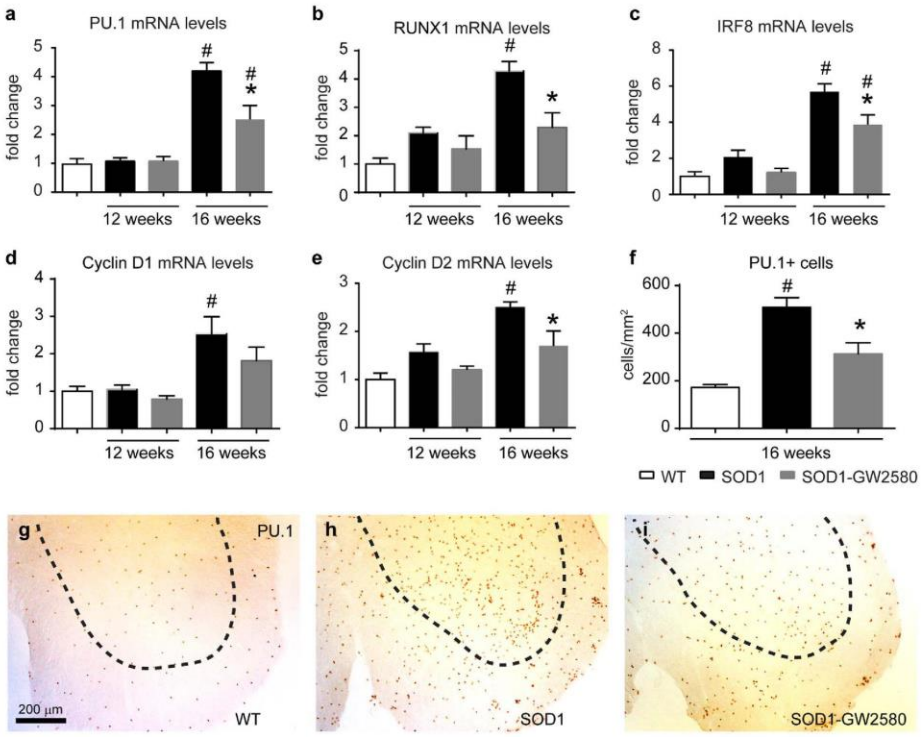

Figure 3. Effects of GW2580 treatment on CSF1R signalling in the spinal cord of SOD1^{G93A} mice. (**a**–**e**) Assessment of the mRNA levels of CSF1R transcription factors (**a**) PU.1, (**b**) RUNX1 and (**c**) IRF8 and cell cycle regulators (**d**) cyclin D2 and (**e**) D1 in the ventral lumbar spinal cord of SOD1^{G93A} mice. (**f**) Graph showing the reduction of PU.1+ cells in the ventral horn of spinal cords after GW2580 treatment in 16 weeks old SOD1^{G93A} mice. (**g**–**i**) Representative immunohistochemical images showing PU.1+ cells in the ventral horns of L4-L5 segments of WT (**g**), SOD1^{G93A} control (**h**) and GW2580-treated SOD1^{G93A} mice (**i**). G-I Scale bars 200μm. *$p < 0.05$ compared to SOD1^{G93A}, #$p < 0.05$ compared to WT. (n = 3 WT; n = 4 for SOD1^{G93A} control and SOD1^{G93A} treated with GW2580 per time point). Error bars indicate SEM.

presence of macrophages was markedly increased in the L5 spinal nerve of ALS mice at end-stage of the disease relative to WT controls. Macrophages were also abundant in the ventral roots, and to lower extent, in the dorsal roots (Fig. 6a–f). These macrophages showed predominant expression of CD16/32 and iNOS, whereas only a small subset of them displayed Arg1 and CD206 (Fig. 6g–i). Similar to that observed in microglia, GW2580 treatment did not change the expression of these markers in peripheral nerve macrophages (Fig. 6i). We then examined whether GW2580 treatment attenuated macrophage infiltration into the peripheral nerves of ALS mice prior to its anti-mitotic effects in microglia (12 weeks), as well as at end-stage of the pathology (16 weeks). We found, in comparison to control nerves, that macrophages were already abundant in the tibial nerve of SOD1^{G93A} mice at the clinical onset of the disease (12 weeks) that tended to increase in number over the course of the pathology (Fig. 7a), which is in agreement with a previous report[14]. Interestingly, GW2580 reduced macrophage counts in the tibial nerve of SOD1^{G93A} mice at 12 and 16 weeks (Fig. 7a–c), suggesting an important role of CSF1R for macrophage accumulation into peripheral nerves of ALS mice during early denervation stages. Since most macrophages in degenerating PNS derive from bloodstream in diverse conditions[26,27] including in ALS[14] it seems likely that reduction of PNS macrophages counts after GW2580 treatment is due to reduced invasion of monocytes. CSF1R has a key role in triggering hematopoietic stem cells to differentiate into monocytes, we thus assessed whether the reduced infiltration of macrophages into the nerve induced by GW2580 treatment of SOD1 mice was due to monocytopenia. FACS analysis of blood samples collected from 12 weeks old SOD1^{G93A} mice, the time point at which the reduction in nerve macrophages was already evident after GW2580 administration, revealed that the CSF1R inhibitor reduced the counts of circulating blood monocytes by about 2.5 fold (Fig. 7d–f). Overall, these findings highlight the importance of macrophage infiltration into the PNS in the pathophysiology of ALS.

Discussion

In the present study we assessed the role of CSF1R in a mouse model of ALS. We determined that the expansion of microglial cells that occurs during the pathological course of ALS pathology in the SOD1^{G93A} model is largely due to cell proliferation. We also observed that selective blockade of CSF1R inhibited microglial cell proliferation,

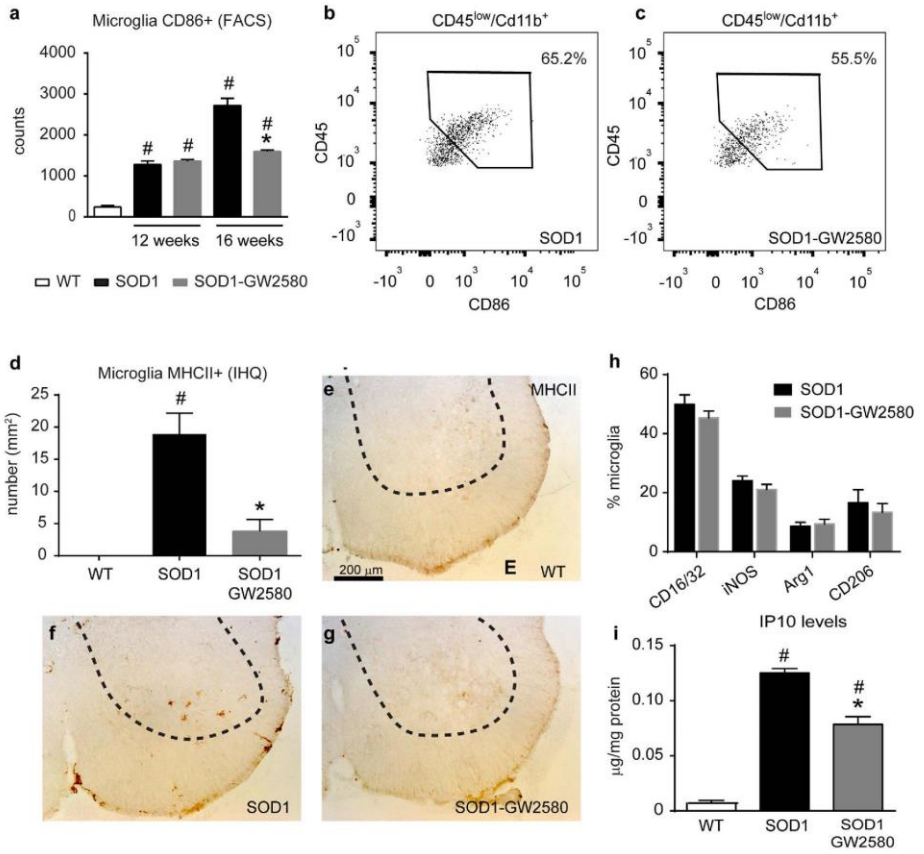

Figure 4. Effects of CSF1R blockade microglial cell activation and polarization in lumbar spinal cord of SOD1^{G93A} mice. (**a**) FACS analysis data showing CD86+ microglia in the lumbar spinal cord. Note that inhibition of CSF1R results in reduction of CD86+ microglial cells in SOD1^{G93A} mice at 16 weeks, but not at 12 weeks. (**b**,**c**) Representative dot plots showing CD86 expression in the gated microglia population (CD45^{low}, CD11b^{+}) of 16 weeks old SOD1^{G93A} control mice (**b**) and treated with GW2580 (**c**). Note the reduced counts of CD86+ microglia after the inhibition of CSF1R. (**d**–**g**) Quantification of MHCII immunostaining in the ventral horns of lumbar spinal cord tissue sections of 16 weeks old WT (**d**) SOD1^{G93A} control (**e**) and GW2580-treated SOD1^{G93A} (**g**) mice. (**h**) FACS analysis of different microglia activation markers from microglial cells from lumbar spinal cord at 16 weeks of age SOD1^{G93A} mice control or treated with GW2580. (n = 3 WT; n = 4 in SOD1^{G93A} mice untreated and treated with GW2580, per time point for each of the experiments). (**i**) Assessment of IP10 protein levels in the lumbar spinal cord of WT, SOD1^{G93A} control and treated with GW2580. *$p < 0.05$ compared to SOD1^{G93A}, $^{\#}p < 0.05$ compared to WT. Error bars indicate SEM.

and consequently, microgliosis. Treatment with GW2580 exerted beneficial effects in ALS pathology, slowing disease progression and extending mice survival. The protective actions of GW2580 are partially explained by its effect on microglial CSF1R, since the beneficial effects of GW2580 on motor conduction tests were achieved prior to its actions on microglia proliferation. Interestingly, we found that GW2580 treatment reduced the number of macrophages recruited to the peripheral nerves of SOD1^{G93A} mice before microgliosis occurred, which was likely due to the monocytopenia induced by CSF1R inhibition. Overall, these results demonstrate the importance of central and peripheral activation of CSF1R signalling in ALS pathophysiology, and support the strategies targeting CSF1R activation as possible therapeutic approaches.

Inflammation is a hallmark of chronic neurodegenerative diseases, including ALS. Two immune cell subsets predominate in two different compartments of the nervous system in ALS disease: microglia in the CNS and macrophages in the PNS. Microglia contribute to neurodegeneration in numerous neurological conditions[16,17,28,29], however, their role in ALS is currently under debate due to conflicting results. Macrophages play divergent roles in several PNS and CNS disorders[26,30], however, whether they exert detrimental, helpful or even neutral effects in ALS has not been elucidated yet.

In agreement with previous studies[31,32], we show that the prominent microgliosis that occurs in ALS is due to increased proliferative activity of resident microglial cells. Although the exact mechanisms that trigger the mitotic

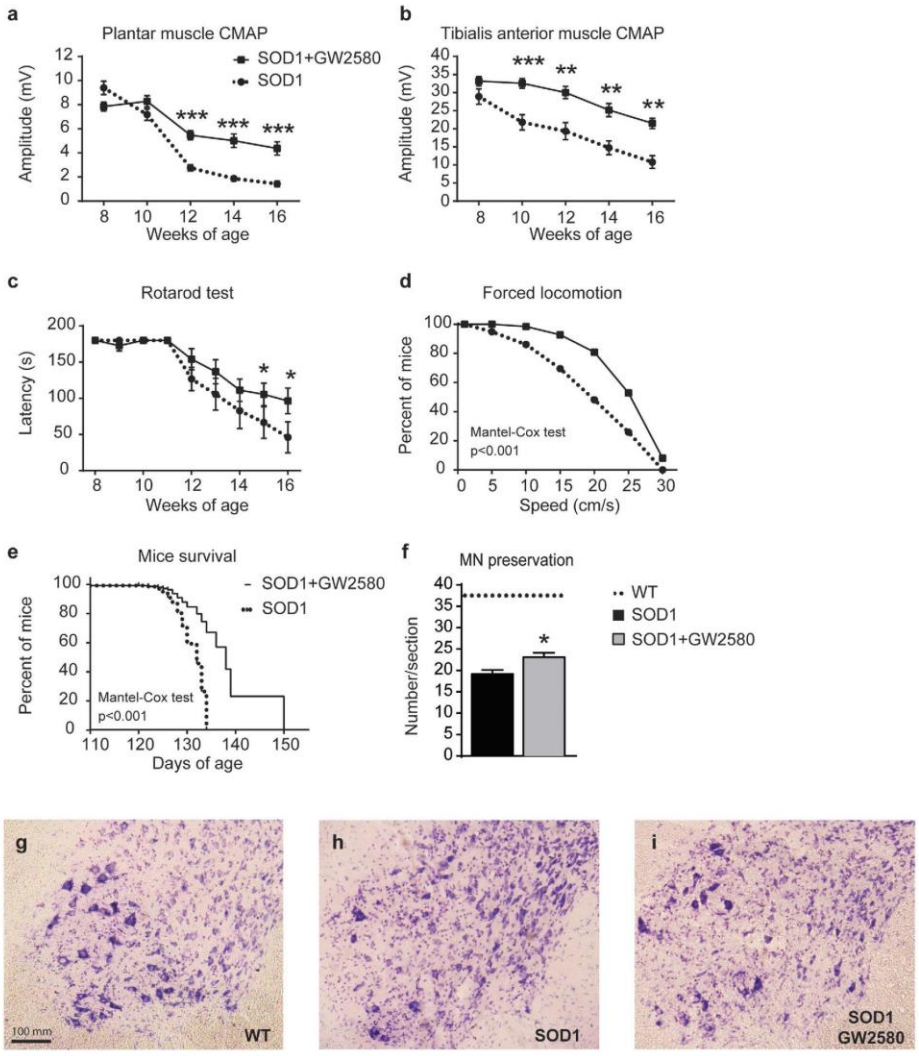

Figure 5. Administration of GW2580 preserves lower motoneuron function and extends SOD1^{G93A} mice survival. (**a**,**b**) Electrophysiological test showing preservation of the compound muscle action potential (CMAP) in both (**a**) plantar interossei and (**b**) tibialis anterior (TA) muscles (n = 18 control SOD1^{G93A} vs. 19 GW2580-treated SOD1^{G93A}). (**c**) Treatment with GW2580 leads to significant preservation in functional outcomes assessed by rotarod (n = 10 per group). (**d**) Locomotor performance, evaluated on a treadmill, also reveals that GW2580-treated 16 weeks old mice are able to run at higher velocities than age-matched untreated SOD1^{G93A} mice (n = 10 per group). (**e**) GW2580 treatment significantly extends life span of female SOD1^{G93A} mice with a maximum of 150 days. (n = 10 per group). (**f**–**i**) Representative micrographs of lumbar spinal cord taken from WT (**g**) SOD1^{G93A} control (**h**) and SOD1^{G93A} treated GW2580 (**i**) showing motoneurons stained with cresyl violet; arrows indicate surviving motoneurons. (**f**) Graph showing quantification of motoneurons in the lumbar spinal cord. Lines indicate the average number of motoneurons in WT mice. (**g**–**i**) Scale bars 200 μm. *p < 0.05, **p < 0.01, ***p < 0.001 compared to SOD1^{G93A} untreated mice. Error bars indicate SEM.

programme in microglia are not fully understood, recent works suggest that CSF1 signalling via CSF1R mediates the proliferation of microglia in various CNS disorders[17,33,34]. In the present experiments, we show that CSF1, but not IL-34, is up-regulated in the spinal cord of SOD1^{G93A} mice and that the treatment with GW2580, a selective CSFR1 inhibitor, attenuates microglial cell expansion and slows the progression of ALS disease. The reduction of microglial cells by GW2580 is due to inhibition of cell proliferation based on our analysis on EdU incorporation and gene expression analysis, highlighting the importance of CSF1R activation in microglia proliferation in ALS. Interestingly, GW2580 treatment led to significant sparing of lumbar MNs, suggesting that microgliosis induced by CSF1R signalling contributes to neurodegeneration in this ALS mouse model.

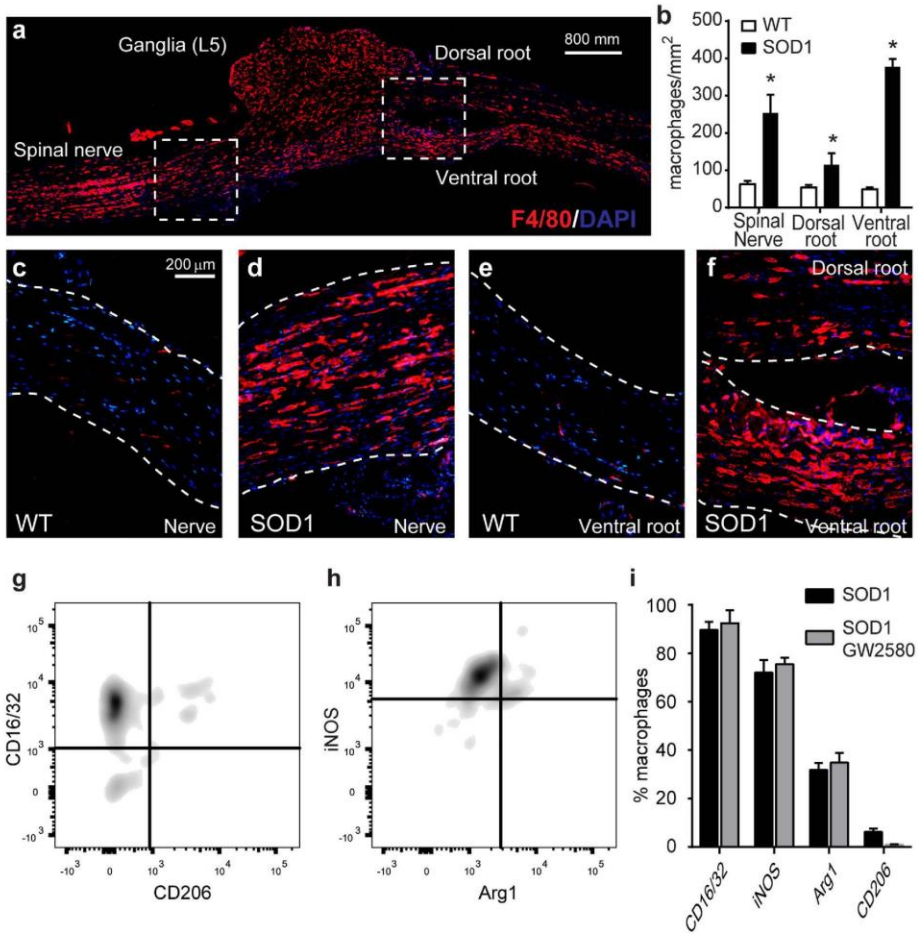

Figure 6. Macrophages infiltrate in the peripheral nerve of SOD1^{G93A} mice. (**a**) Representative image from L5 spinal nerve longitudinal section displaying the presence of macrophages in the nerve, as well as, in ventral and dorsal roots of SOD1^{G93A} mice at 16 weeks of age The area outlined in the boxes are shown in higher magnification in panels (**d**–**f**). (**b**) Quantification of macrophages in the L5 spinal nerve, dorsal and ventral roots of SOD1^{G93A} mice at 16 weeks of age (n = 4). (**c**–**f**) Representative images of L5 spinal nerve and ventral roots of WT and SOD1^{G93A} mice at 16 weeks of age. Note the macrophages are scarce in tissue sections from WT but abundant in those from ALS mice. (**g**–**i**) Evaluation by flow cytometry of CD16/32, iNOS, Arg1, and CD206 in macrophages present in the sciatic nerve of SOD1^{G93A} mice at 16 weeks of age untreated or treated with GW2580 (n = 3). *$p < 0.05$ vs WT.

The inhibitory effects of GW2580 on microgliosis were only observed at late stages of the disease in SOD1^{G93A} mice, which is due to the lack of CSF1 induction in early phases of the pathology. However, in agreement with others[14,18] we found that microgliosis was evident at early stages of the disease in this ALS mouse model. This data therefore suggest that there are various mediators involved in microglia proliferation during the course of ALS pathology, CSF1R signalling is a key player at late but not at early stages of the disease. Indeed a recent study reveals that IL-1R signalling has a key function in microglial cell proliferation following microglia ablation[35], which may explain the attenuated microgliosis observed in ALS mice treated with IL-1ra or lacking IL-1β expression[36]. The fact that CSF1R mediates microglia proliferation only at late stages of ALS disease is important in the present study, since the beneficial effects of GW2580 on motor function were already evident at the electrophysiological level from week 10–12, when CSF1 was not yet induced in the CNS. This observation suggests that although microgliosis triggered by CSF1R contributes to ALS disease, the early protective actions of GW2580 on muscle denervation are not mediated by microglial CSF1R but by peripheral CSF1R. Supporting these findings, a previous study reveals that systemic administration of CSF1 in another mouse model of ALS (SOD1^{G37R} mouse), which leads to over-activation of peripheral CSF1R, accelerated disease progression but not MN death, highlighting the importance of peripheral CSF1R in ALS pathology[18].

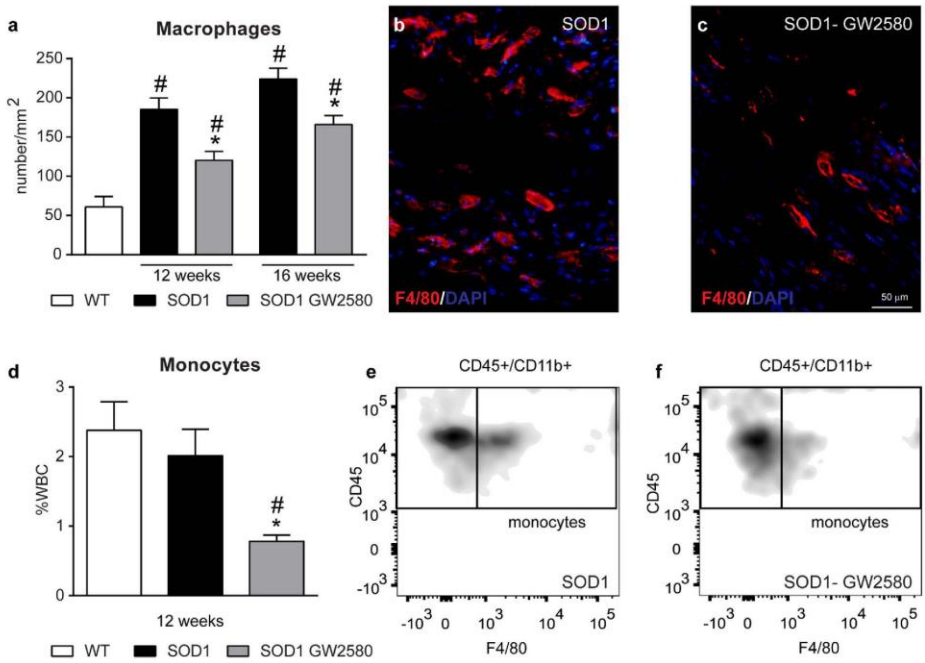

Figure 7. CSF1R attenuates infiltration of monocytes into tibial nerves of SOD1^{G93A} mice. (**a**) Quantification of F4/80+ cells in tibial nerve tissue sections of SOD1^{G93A} mice control or treated with GW2580 at the age of 12 and 16 weeks. (**b**,**c**) Representative images from tibial nerve longitudinal sections showing F4/80+ cells (red) inside nerve bundles in (**b**) SOD1^{G93A} control and (**c**) SOD1^{G93A} treated with GW2580. (**d**) Assessment by flow cytometry of circulating monocytes in both, GW2580-treated and control, SOD1^{G93A} mice. (**e**,**f**) Representative density plots showing blood monocytes (CD45+, CD11b high, F4/80+) in (**e**) control SOD1^{G93A} and (**f**) SOD1^{G93A} treated with GW2580. Note that CSF1R inhibition by GW2580 leads to monocytopenia. n = 4 per group, experiment and time. Scale bar 50 µm. *$p < 0.05$ vs. SOD1^{G93A} control mice; #$p < 0.05$ compared to WT. Error bars indicate SEM. Scale bar 50 µm.

Besides microglial cells, circulating monocytes show constitutive expression of CSF1R[37]. Although monocytes do not migrate into the spinal cord in ALS mice[31,38], there is robust presence of macrophages in the peripheral nerves of ALS mice prior to the clinical onset of the disease[14,15]. Indeed, PNS macrophages and microglia show similar dynamics of expansion in ALS[14]. In contrast to microglia, macrophages found within the nerves of ALS mice are derived from circulation (~75%), as shown by experiments where GFP bone marrow cells were transplanted into irradiated ALS mice[14]. Indeed, the expression of CCL2, one of the main chemoattractants for monocytes, in the nerve of ALS mice correlates with the accumulation of macrophages[14]. This is likely due to early distal axonal degeneration that occurs in the nerves of ALS mice and patients before the death of MN[39,40], which may trigger macrophage migration into the nerve. Although macrophages are crucial for proper axonal regeneration after PNS damage, they can also mediate detrimental effects in different PNS conditions[26]. Indeed, SOD1^{G93A} macrophages exert toxic effects on primary neuronal cell cultures[41], and chimeric mice have shown that myeloid cells express mutant SOD1 and that they are harmful to neurons[42,43]. These reports therefore suggest that macrophages expressing mutant SOD1 may not mediate repair in ALS. Indeed, here we show that macrophages that infiltrate into the sciatic nerve of ALS mice display a pro-inflammatory phenotype, associated with tissue damage[26]. Herein we found that GW2580 reduced the numbers of macrophages into the nerves of SOD1^{G93A} mice. Since PNS macrophages are mostly recruited from circulation in ALS[14], this data suggests that the entrance of monocytes into the PNS is attenuated when CSF1R signalling is blocked. In line with our results, systemic administration of CSF1 increased the numbers of macrophages in the sciatic nerves of ALS mice[18]. Similarly, a recent report indicates the CSF1R has a key role for the infiltration of macrophage in the sciatic nerve of a Charcot Marie Tooth mouse model, where they produce adverse effects[44]. The reduced migration of monocytes into the tissues after treatment with GW2580 is likely due to the importance of CSF1R in promoting the differentiation of bone marrow cells into monocytes[45] since we observed that the CSF1R blockade led to monocytopenia in the ALS mice. However, we cannot rule out the possibility that the disruption of CSF1R signalling could also interfere with monocyte chemotaxis and/or with the proliferation of resident macrophages. Interestingly, the reduction in the number of PNS macrophages observed in the SOD1^{G93A} mice treated with GW2580 prior to the clinical onset of the disease correlated with the greater preservation of the electrophysiological responses. These results indicate that, besides the harmful effects of microglia in ALS, macrophages have also significant impact on disease progression by acting in the PNS. This is a new inroad into the functions of macrophages in the pathophysiology

of ALS since the therapeutic effects of multiple anti-inflammatory approaches in ALS have been exclusively attributed to their ability to interfere with microgliosis, but not with PNS macrophages.

Despite GW2580 attenuated microgliosis and macrophage counts in the peripheral nerves of $SOD1^{G93A}$ mice, its administration only increased the maximal lifespan by 12%. This modest effect on mouse survival can be explained, in part, by the lower inhibitory activity of GW2580 on CSF1R as compared to novel compounds such as PLX3397. However, whereas GW2580 shows high selectivity for CSF1R[23], PLX3397 has also potent inhibitory effects on c-Kit, FTL3 and PDGFR, and thus, it does not allows one to precisely dissect out the role of CSF1R. It is likely that the generation of more potent and selective CSF1R inhibitors will result in greater beneficial effects[46].

In summary MN pathology in ALS disease, which begins with distal axonal pathology and proceeds in a dying back pattern, is likely to be the main trigger of inflammation in the PNS and in the CNS, which in turn contributes to the course of the pathology by accelerating muscle denervation and MN degeneration in a positive feedback loop. Our work reveals that CSF1R signalling has a crucial role in modulating the immune response in the CNS and PNS in $SOD1^{G93A}$ mouse model of ALS. Although further studies are needed to elucidate the exact molecular mechanisms underlying the combined deleterious actions of macrophages and microglia in ALS, our data suggest that CSF1R signalling could be a novel therapeutic target in ALS.

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Author Contributions

A.M.-M. and R.M. performed most of the experiments. I.F.-Q. performed FACS analysis, and A.O.-A. performed Q-PCR and histological analysis. R.O. provided the SOD1^{G93A} mice. V.H.P., X.N., D.G.-N. and R.L.-V. designed the study, supervised the project and wrote the manuscript. All the authors reviewed the manuscript.

Additional Information

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CHAPTER II

Interleukin-37: a novel protective cytokine in amyotrophic lateral sclerosis

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ABSTRACT

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disorder that affects upper and lower motoneurons (MNs). Several reports have shown that glial cells and leukocytes accelerate the disease progression in ALS animal models, suggesting neuroinflammation as a valuable therapeutic target. IL-37 is an IL-1 family member that exerts broad anti-inflammatory effects over innate and acquired immunity. We have previously demonstrated that IL-37 mediates anti-inflammatory actions after spinal cord injury, resulting in amelioration of functional deficits. However, whether IL-37 exerts similar beneficial effects in neurodegenerative conditions is not known yet. In the present study, we investigated the effects of IL-37 in amyotrophic lateral sclerosis (ALS). We found that IL-37 receptors (IL-1R8-IL-18Rα) were expressed in the spinal cord of post-mortem ALS patients, however, IL-37 was not induced by the disease. To assess the potential actions of IL-37 in ALS, we transgenically expressed the human form of IL-37 in the $SOD1^{G93A}$ mouse. These experiments revealed that IL-37 ameliorated the pathophysiological features of ALS disease and extended survival. IL-37 also modulated cytokine protein levels, attenuated microgliosis and astrogliosis and increased MN survival. We also reveal that the beneficial effects of IL-37 in ALS were mediated by acting as a secreted cytokine, since its protective actions were vanished in the lack of IL-1R8, the co-receptor required for IL-37 to carry out its extracellular functions. We finally found the presence of a genetic variant for IL-37 (rs3811046 SNPs) delays the onset of the disease in 13 years in familiar ALS patients. Overall, these findings demonstrate for the first time the beneficial effects of IL-37 in ALS and open a new avenue for the treatment of this neurodegenerative disease.

Keywords: interleukin 37, cytokines, inflammation, amyotrophic lateral sclerosis, IL-1R8

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease characterized by selective death of both upper and lower motoneurons (MNs). This motor degeneration results in loss of strength, atrophy and gradual muscle paralysis that leads to patients death in 2 to 5 years after its clinical diagnosis (Hardiman et al., 2017). The majority of ALS individuals present sporadic forms of the disease (sALS), whereas only 5 to 10% of cases correspond to familial forms (fALS) (Al-Chalabi and Hardiman, 2013). From the latest, several genetic variants have been identified and their study has led to the development of diverse fALS mouse models that mimic the clinical signs of the disease. Indeed, most of the current knowledge about the molecular processes involved in MNs degeneration comes from the $SOD1^{G93A}$ mouse model (Ripps et al., 1995). The exact mechanism by which *SOD1* mutations trigger MN degeneration in ALS remains to be defined, but it likely involves a complex interaction between neurons and non-neuronal cells (Boillée et al., 2006; Ferraiuolo et al., 2011).

Neuroinflammation is a common pathological feature found in degenerating areas of both ALS patients and experimental animal models (Engelhardt and Appel, 1990; Kawamata et al., 1992; Chiu et al., 2008, 2009). Several studies have reported that immune cells markedly contribute to MNs death (Clement et al., 2003; Beers et al., 2006; Re et al., 2014; Martínez-Muriana et al., 2016), suggesting that therapies aimed at targeting inflammation may be a valuable approach to treat this neurodegenerative disease.

Interleukin (IL-37) is one of the eleven members of the IL-1 cytokine family and exerts broad anti-inflammatory effects over innate and acquired immunity (Nold et al., 2010). The anti-inflammatory actions of IL-37 have been demonstrated in various experimental models of immune-related diseases, including endotoxin shock, colitis or lupus (Nold et al., 2010; McNamee et al., 2011; Ye et al., 2015). We recently provided the first evidence that IL-37 mediates potent anti-inflammatory effects in the injured central nervous system (CNS), ameliorating functional deficits after spinal cord injury in mice (Coll-Miró et al., 2016). However, whether IL-37 exerts similar beneficial effects in neurodegenerative conditions is not known yet.

Similar to IL-1α and IL-33, IL-37 translocates to the nucleus where it is thought to regulate the expression of different inflammatory factors (Nold et al., 2010). IL 37 is also released to the extracellular milieu and mediates anti-inflammatory actions by acting as a ligand for the IL-18Rα-IL-1R8 complex. However, it is currently unknown whether the

beneficial actions of IL-37 in the CNS are due to its intracellular function, extracellular functions, or both.

In the present chapter, we investigated the effects of IL-37 in ALS. Our data reveals IL-37 ameliorated the course of ALS disease and extended mice survival. We also show that the beneficial actions of IL-37 in ALS were correlated with its anti-inflammatory actions in the central and peripheral nervous system. We also demonstrate that the therapeutic actions of IL-37 require the activation of its extracellular components. Interestingly, we found that IL-37 is not up-regulated in the spinal cord of ALS patients, suggesting that this protective mechanism is not efficiently turned on in ALS individuals. Finally, we identified a single nucleotide polymorphism in the IL-37 gene that delays the disease onset in 13 years in fALS patients. Overall, we provide solid clinical and experimental evidence demonstrating the beneficial actions of IL-37 in ALS pathophysiology.

MATERIALS AND METHODS

Experimental animal models

All the experimental procedures were approved by the Universitat Autònoma de Barcelona Animal Experimentation Ethical Committee and followed the European Commission on Animal Care 2010/63/EU. Experiments were performed in both female and male transgenic SOD1^{G93A} mice (B6-Tg[SOD1-G93A]1Gur) obtained from the Jackson Laboratory (Bar Harbor, ME, USA). hIL-37tg animals were kindly provided by Professor Charles Dinarello from the University of Colorado Denver (Colorado, United States of America). IL-1R8 KO mice were gently provided by Professor Cecilia Garlanda from Humanitas Clinical and Research Center (Milan, Italy).

To study the role of IL-37 in ALS, hIL37tg were crossed with SOD1^{G93A} mice to generate double transgenic mice (SOD1^{G93A} -hIL-37tg). Only those mice that were homozygous for IL-37 gene were used (SOD1^{G93A}-hIL-37tg$^{+/+}$). To study the contribution of the extracellular function of IL-37 in ALS, SOD1^{G93A}-hIL-37tg were crossed with IL-1R8 knockout mice to generate a triple transgenic mouse (SOD1^{G93A}-hIL37tg-IL-1R8). These ALS mice, express the human form of IL-37 but cannot act extracellularly since they lack IL-1R8, the co-receptor needed to carry out its extracellular actions. All the transgenic animals were maintained in local animal facilities and were genotyped by PCR amplification of DNA extracted from tail samples. Mice were housed with food and water *ad libitum* at room temperature of 22 ± 2ºC under a 12:12-h light–dark cycle. Following the international guidelines, the endpoint criterion was considered when the animals were no longer able to perform the righting reflex for the following 30 seconds.

Functional tests

To test different parameters of the motor function of SOD1^{G93A} animals, we used the Rotarod and forced locomotion test (Miana-Mena et al., 2005). With rotarod test, we assessed the balance, strength and motor coordination of SOD1^{G93A} animals. Prior to the beginning of the weekly analysis, animals were trained 3 times the first week to obtain baseline levels of performance (an arbitrary maximum of 180 seconds at a constant speed of 14 rpm). Animals were then weekly tested from 8 to 16 weeks of age at the same speed. To quantify the motor performance of the animals, we measured the time for which each animal could remain on the rotating rod. The onset of the disease was calculated when the animals showed the first motor impairments in the rotarod test.

The forced locomotion test consists of an assessment of the highest locomotion speed that $SOD1^{G93A}$ are able to reach at the end of the follow up (16 weeks of age). For this purpose, mice were placed over the controlled-speed treadmill and their capacity to run at increasing treadmill velocities, 5, 10, 15, 20, 25, 30, 35 and 40 cm/s, was recorded (Mancuso et al., 2011).

Motor conduction tests

We performed motor nerve conduction tests from 8 to 16 weeks of age to assess MN function. Briefly, needle electrodes were placed within the sciatic notch and by means of single pulses of 0.02 ms duration (Grass S88) we percutaneously stimulated sciatic nerve. To assess the lower MN function, the compound muscle action potential (CMAP) was recorded in the hindlimb tested muscles (tibialis *anterior* (TA) and gastrocnemius *medialis* (GM)) with microneedle electrodes (Mancuso et al., 2011). To assess upper MN function, we perform motor evoked potentials (MEP). Briefly, we placed needle electrodes subcutaneously over the sensorimotor cortex and transcranially stimulated the descending motor pathways by single rectangular pulses of 0.1 ms duration. MEPs were recorded in the gastrocnemius muscle.

The obtained potentials were amplified and displayed on a digital oscilloscope (Tektronix 450S) at settings appropriate. We measured the CMAP and MEP amplitude from baseline to the maximal negative peak. Moreover, using the Labchart 8 software (ADInstruments), we also analyzed the root mean square from the MEPs. To ensure reproducibility, the recording and stimuli needle electrodes were placed under microscope, guided by anatomical landmarks. During the tests, the mouse skin temperature was maintained between 34 and 36 °C using a thermostatically controlled heating pad. All the researchers were blinded to the experimental groups.

Histology

At 16 weeks of age mice were sacrificed with an overdose of sodium pentobarbital and transcardically perfused with 4% paraformaldehyde (PFA) in 0.1M phosphate buffer (PB). Briefly, lumbar segments of spinal cord from ALS mice were collected and further post-fixed in 4% PFA for 1 hour and cryoprotected in 30% sucrose in 0.1M PB at 4ºC. Spinal cords were then serially cut in transverse sections (20 µm thick) with a cryostat (Leica) between L1 and L6 segments. To quantify the number of surviving MNs, fixed-frozen spinal cord slices were rehydratated and stained for 2 h with an acidified solution of 3.1 mM cresyl violet. Sections were then washed in deionized water for 1 min, dehydrated and mounted with DPX (Fluka).

Under the microscope, lumbar MNs were identified by their size, morphology and localization within the L4-L5 segments of the spinal cord. To ensure reliability, only those MNs that were located within the IX laminae, were larger than 20 μm and presented a polygonal shape and prominent nucleoli were quantified (Mancuso et al., 2011).

To assess the immunoreactivity in the spinal cord, transversal tissue sections were immunostained with goat anti-Iba1 (1:500, Abcam) and rabbit anti-GFAP (1:1000, Dako) for visualization of microglia and astrocytes, respectively. Primary antibodies were detected with appropriate secondary antibodies conjugated with Alexa 488 or 594 (1:200, Life Technologies).

Tissue sections were viewed with an Olympus BX51 microscope and images were captured using the Cell^A Image acquisition software through an attached Olympus DP50 digital camera. All quantifications were performed with the help of the ImageJ image analysis software (NIH).

Analysis of gene expression by qPCR

At 8, 12, 16 and 20 weeks of age, SOD1^{G93A}-WT, SOD1^{G93A}-hIL37tg and age-matched wild-type (WT) littermate mice were transcardially perfused with 60 mL of sterile saline and lumbar segments of spinal cords and sciatic nerves samples were harvested. Isolation of RNA from the tissue was achieved using the RNeasy Lipid Tissue Kit (Qiagen, Germany) and quantified with Nanodrop (Thermo Scientific). Then 1 μg of RNA was retro-transcribed using the omniscript cDNA Synthesis Kit (Qiagen). cDNA libraries were analyzed using the 5x SYBR green III (Bio-Rad) and the custom-designed primers: human *IL-37* transcript variant 1 (NM_014439.3, forward (FW) 5 '- CTTAGAAGACCCGGCTGGAAG - 3' ; reverse (RV), 5' - TGTGATCCTGGTCATGAATGCT - 3'), mouse single immunoglobulin and toll-interleukin 1 receptor (TIR) domain (*IL-1r8* or *Sigirr*) (NM_001355055.1, FW 5′- TGCTTTGGAAGCCTGGCTCCGT -3′; RV 5′- GGTTTCCTGCAGTGGAGTTGGT -3’), mouse interleukin 18 receptor 1 (*IL18r1*), transcript variant 1 (NM_008365.2, FW 5’- TTTGCTGTGGAGACGTTACCC -3’; RV 5’- GCCAGGCACCACATCTCTTT – 3’), mouse glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) (NM_001289726.1, FW 5' - TCAACAGCAACTCCCACTCTTCCA - 3', RV 5' - ACCCTGTTGCTGTAGCCGTATTCA - 3').

To assess gene expression from human samples, post-mortem fresh-frozen lumbar spinal cord from sALS patients were harvested at the Institute of Neuropathology HUB-ICO-IDIBELL Biobank following the guidelines of Spanish legislation on this matter and the approval of the local ethics committee. Age-matched cases who had not suffered from neurologic or psychiatric diseases, did not carry *C9orf72*, *SOD1*, *TARDBP* and FUS mutations, and did not have abnormalities in the neuropathologic examination, excepting

sporadic neurofibrillary tangle pathology stages I-II of Braak and Braak were used as controls. RNA was isolated using RNeasy Mini Kit (Qiagen) and retro-transcribed using cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA) following manufacturers' kit instructions. For qPCRs, we used TaqMan-designed primers (Thermo Fisher, MA, USA) for gene expression of human *IL-37*, human *IL-1R8* and hypoxanthine-guanine phosphoribosyltransferase. The mean value of the house-keeping gene, GAPDH and HPRT1 for mouse and human samples, respectively, was used as internal control for normalization of spinal cord samples. The double-delta cycle threshold (ΔΔCT) method was used to analyze the data.

Cytokine protein expression

At 12 weeks of age, $SOD1^{G93A}$-WT and $SOD1^{G93A}$-hIL-37tg and age-matched wild-type (WT) littermate mice were perfused with 60 mL of sterile saline. Sciatic nerves and lumbar spinal cords were rapidly harvested, snap-frozen and kept at -80 ºC. The two sciatic nerve for each mouse were pooled for protein tissue extraction. For tissue processing, spinal cords and nerves were homogenized using an electric tissue homogenizer followed by a pulse with an ultrasonic homogenizer for 4 seconds and at 40 kHz in HEPES buffer. Protein concentration was lately determined using the DC Protein Assay (Bio-Rad). Protein homogenates were concentrated to 4 μg/μL in spinal cord samples and to 1.5 μg/μL in sciatic nerve samples. The protein levels of G-CSF, IL-10, IL-3, IL-1β, M-CSF , IP-10, IL-4, IL-6, IFN-γ, IL-12p70, GM-CSF, RANTES, TNF-α, MCP-1, IL-17, MIP-2, IL-1α were then determined and analyzed using a custom-designed Milliplex Cytokine/Chemokine Magnetic Bead Panel on a MAGPIX system (EMD Millipore) in accordance with the manufacturer's protocol. Standard curves were generated using the specifics standards supplied by the manufacturer.

***Single nucleotide polymorphism (SNPs*)**

SNPs are small variations in single nucleotides in the DNA that are found at least in 1% of the population. To analyze the presence of the SNPs of IL-37 (rs3811046), commercial predesigned TaqMan SNP Genotyping Assay (C__27487174_10, Thermo Fisher, MA, USA). The SPN rs3811046 (G/T) causes substitution of valine to glycine at 31th location the IL-37b protein amino acid sequence, and not the other way around.

DNA samples extracted from the skin of sporadic and familiar ALS patients were obtained from the Institute of Neuropathology HUB-ICO-IDIBELL Biobank and genotyping of the rs3811046 polymorphism within IL-37 was performed by means of a real-time

polymerase chain reaction, Thermal cycling and endpoint PCR analysis was performed on an ABI PRISM 7900HT.

Statistical analysis

Data are shown as mean ± SEM and were analyzed using the GraphPad Prism 6 software package (GraphPad Software). Electrophysiological and locomotion test were analyzed using two-way repeated measurements ANOVA with Bonferroni post-hoc test. T-test of One-way ANOVA was used for histological, qPCR and SNP data comparing the 2 or more experimental groups, respectively. Two-way ANOVA was used for the gene expression data, qPCR and multiplex data, followed by a Tukey post-hoc test for multiple comparisons. Survival data was analyzed using the Mantel-Cox test. Differences were considered significant at $p < 0.05$.

RESULTS

IL-37 expression in the spinal cord of ALS patients

Since IL-37 is a potent anti-inflammatory cytokine that exert beneficial effects in several diseases (Nold et al., 2010; McNamee et al., 2011; Ye et al., 2015), we firstly characterized the expression of this cytokine and its co-receptor, IL1-R8, in the spinal cord of sALS patients and age-matched control. qPCR analysis revealed that IL-37 was detectable at very low levels (Ct>35) in 6 out 11 control individuals and in 11 out of the 13 ALS patients. However, in those samples that IL-37 transcripts were detected, IL-37 was not up-regulated in sALS individuals (Fig 1. A). When analyzing IL-37 expression according to the ALS onset form, we found that patients diagnosed with bulbar ALS tended to have lower IL-37 levels, although differences were not statistically significant. The expression of IL-1R8 was expressed at much higher levels than IL-37 in all the patients. However, this was not up-regulated in sALS patients, regardless the ALS onset form (Fig 1. B).

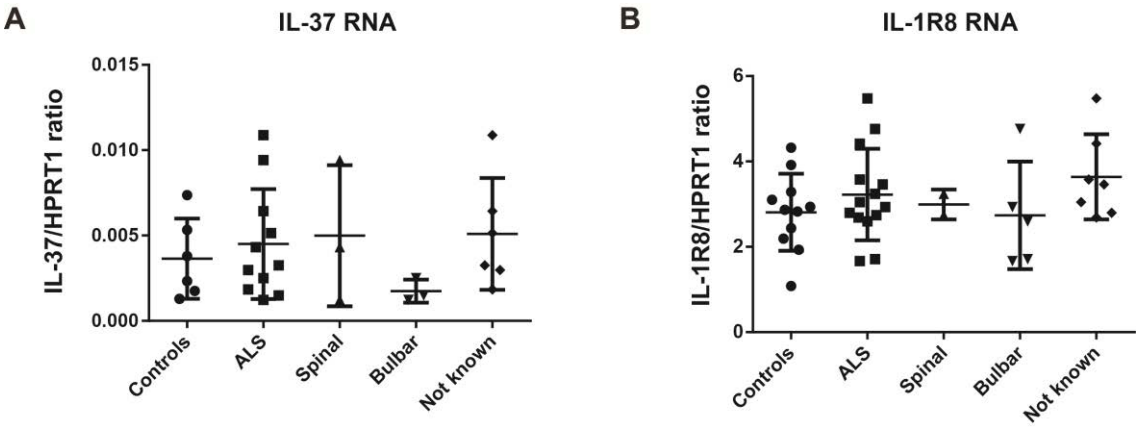

Figure 1. IL-37 and IL-1R8 expression in the spinal cord of SALS patients. A, B. Plots showing qPCR analysis of (**A**) IL-37 (n= 6 controls vs 11 ALS) and (**B**) IL-1R8 (n= 11 controls vs 14 ALS) mRNA levels relative to HPRT1 in ALS patients and age-matched controls. Error bars indicate SEM.

IL-37 is up-regulated in the nervous system of SOD1^{G93A} mice with transgenic expression of IL-37

Since the expression of IL-37 is not induced in the spinal cord of ALS, we next assessed whether increasing IL-37 levels in the nervous system could exert beneficial effects in ALS disease. One of the problematics when working with IL-37 in animal models is the fact that rodents do not have IL-37. To solve this limitation we crossed mice that have transgenic expression of human IL-37 (hIL-37tg) with SOD1^{G93A} mice. As previously

reported, IL-37 was found at very low levels in hIL-37tg animals (Coll-Miró et al., 2016). However, IL-37 transcripts were rapidly increased in the spinal cord of SOD1^{G93A}-IL-37tg mice as soon as 8 weeks of age (pre-symptomatic stage). However, IL-37 levels were especially abundant in the spinal cord from 12 to 20 weeks of age (Fig 2. A-C).

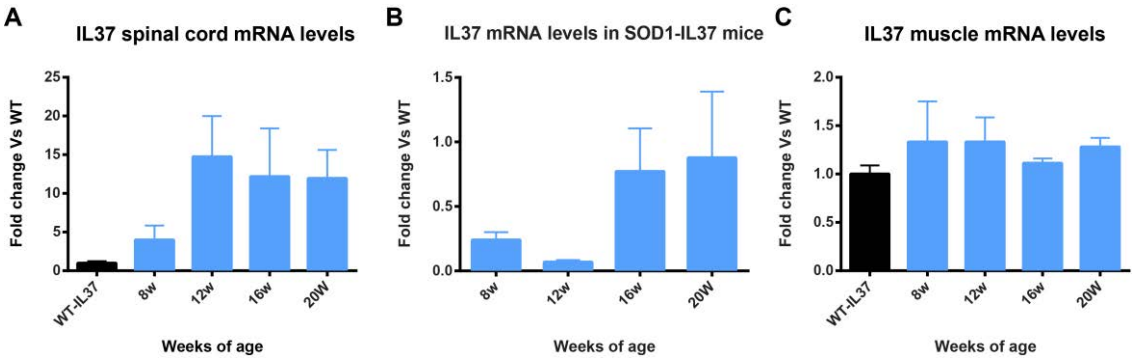

Figure 2. Characterization of IL-37 expression in SOD1^{G93A}-hIL-37tg mice. A-C. Graphs showing IL-37 mRNA levels in the spinal cord, (**B**) sciatic nerve and (**C**) gastrocnemius muscle of SOD1^{G93A}-hIL-37tg mice at 8, 12, 16 and 20 weeks of age (n=4 animals per group). Error bars indicate SEM.

IL-37 improves functional outcomes and increases lifespan of SOD1^{G93A} mice

ALS patients show motor impairments due to the loss of MNs that can be monitored using electrophysiological and behavioral approaches. To assess the effects of transgenic expression of IL-37 in ALS, we electrophysiologically analyzed the preservation of the lower MNs by recording compound muscle action potentials (CMAPs). We found that IL-37 significantly preserved the CMAP amplitude of the tibialis *anterior* (TA) and gastrocnemius (GM) muscles in ALS mice from pre-symptomatic stages of the disease until the end of the follow-up (Fig 3. A-C). We also assessed, by using motor evoked potentials (MEPs) whether IL-37 also prevented the loss of upper MN function at the end of the follow up (16 weeks). Similarly, we found that MEPs were markedly increased in SOD1^{G93A} animals expressing IL-37, indicating that this cytokine also conferred protection against the loss of upper MN function (Fig 3. D-F).

We next assessed the contribution of transgenic expression of IL-37 in functional outcomes. In accordance with the electrophysiological results, we found that IL-37 significantly improved motor function of SOD1^{G93A} mice in the rotarod and forced locomotion tests (Fig 3. H, I). In addition, rotarod test revealed that IL-37 delayed the onset of disease by more than 3 weeks in SOD1^{G93A} animals (Fig 3. G). Importantly, IL-37 increased the lifespan of SOD1^{G93A} (Fig 3. J-L), further supporting its beneficial actions in ALS.

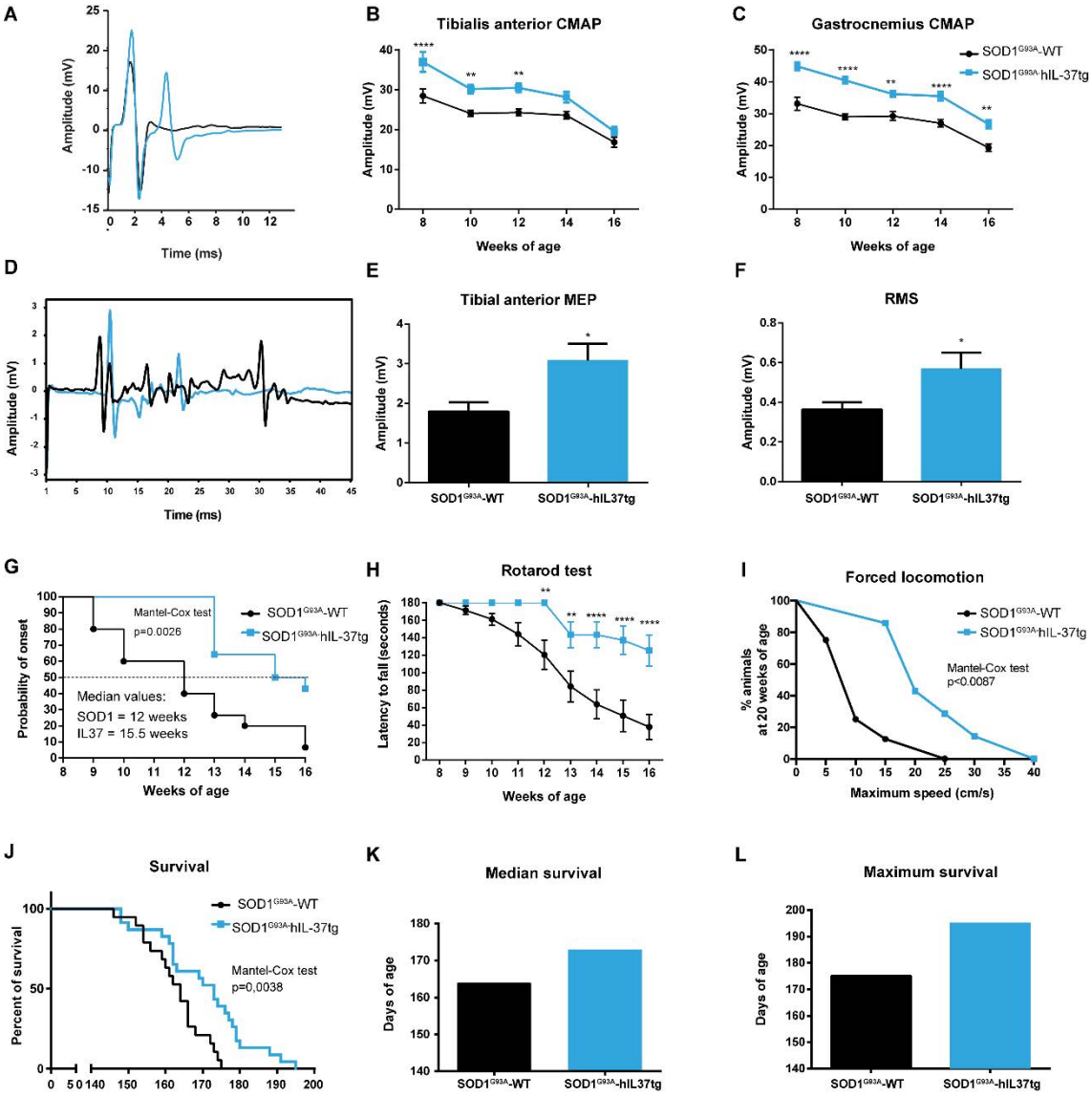

Figure 3. IL-37 expression slows disease progression in SOD1^{G93A} mice. A. Illustration of gastrocnemius muscle compound muscle action potential (CMAP) at 16 weeks of age. **B,C.** Electrophysiological test showing the preservation of the CMAP in the (**B**) TA and (**C**) GM (n = 20 SOD1^{G93A}-WT vs. 18 SOD1^{G93A}-hIL-37tg). **D.** Representative motor evoked potentials (MEPs) from SOD1^{G93A}-WT and SOD1^{G93A}-hIL-37tg mice. **E.** Preservation of MEPs' amplitude at 16 weeks of age. **F.** Quantification of the root mean square (RMS) (n = 13 per group). **G.** Probability of onset of SOD1^{G93A} mice (n = 15 SOD1^{G93A}WT vs. 14 S SOD1^{G93A}-hIL-L37tg). **H,I.** Preservation of functional outcomes assessed by (**H**) rotarod and (**I**) treadmill (n = 14 per group in the rotarod and n = 8 SOD1^{G93A}-WT vs. 7 SOD1^{G93A}-hIL-37tg in treadmill). **J-L.** Effects of IL-37 transgenic expression in lifespan (n = 19 SOD1^{G93A}-WT vs. 20 SOD1^{G93A}-hIL-37tg). *p <0.05, **p<0.01, ***p<0.001 compared to SOD1^{G93A}-WT mice. Error bars indicate SEM.

IL-37 preserves spinal motoneurons and reduces the inflammatory milieu in SOD1^{G93A} mice

Degeneration of MNs is the main feature found in both mice and ALS patients. Thus, we next assessed whether IL-37 was able to prevent the death of spinal MNs at the end-stage of the disease (16 weeks of age). Histological analysis of L4-L5 MNs pools revealed that IL-37 significantly preserved spinal MNs at 16 weeks of age (Fig 4. A-D). Indeed, SOD1^{G93A}-IL-37 mice had ~40% more MNs than SOD1^{G93A}-WT mice, indicating that the biological actions of this cytokine promoted neuroprotection

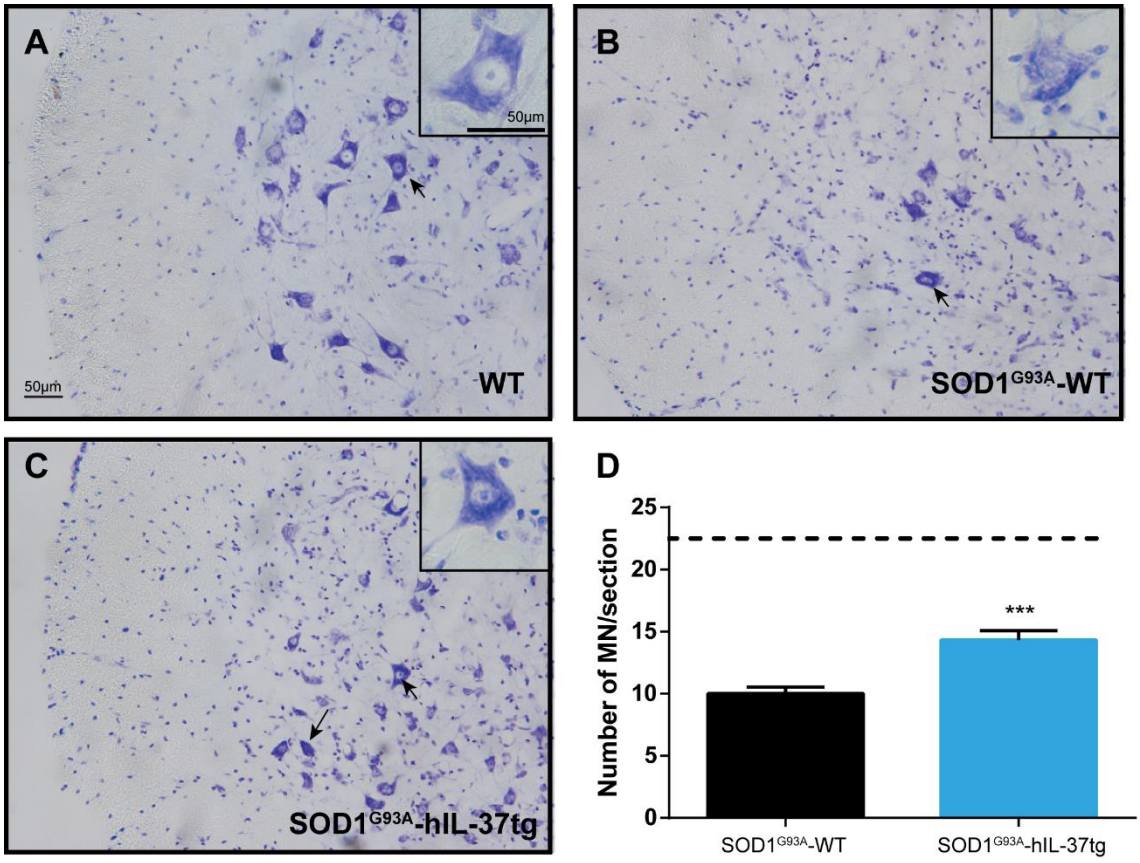

Figure 4. IL-37 attenuates MNs loss in SOD1^{G93A} mice. A-D. (**A-C**) Representative micrographs of lumbar spinal cord showing motoneurons stained with cresyl violet and (**D**) its quantification. Inset boxes show a magnification of single motoneurons; arrows indicate surviving motoneurons (n = 6 per group). Dotted lines indicate the average number of motoneurons in WT mice. ***p<0.001 compared to SOD1^{G93A}-WT mice. Error bars indicate SEM.

We next sought to determine whether the beneficial actions of IL-37 were associated to its ability to attenuate the inflammatory response. Since astrocyte and microglia are the main cell type involved in the inflammatory response that take place in the spinal cord of ALS mice and humans, we evaluated the immunoreactivity of GFAP and Iba1 in the ventral horns of the spinal cord of SOD1^{G93A} mice. We found that IL-37 drastically reduced the reactivity of both, astrocytes and microglial cells in the areas with ongoing neuronal degeneration at 16 weeks of age (Fig 5. A-C, E-G). Because cytokines/chemokines are one of the main factors that trigger glial activation in neurodegenerative disorders, we next studied the contribution of IL-37 to the production of these immunological mediators. Multiplex analysis of several cytokines revealed that IL-37 did not modify the protein levels of the pro-inflammatory cytokines evaluated in the ventral spinal cord at 12 weeks of age, but it did significantly increase (~5 fold) the expression of the anti-inflammatory cytokine, IL-10 (Fig 5. D). However, IL-37 reduced the protein levels of the pro-inflammatory cytokines IL-17 and M-CSF and the anti-inflammatory cytokine, IL-4 by 2 fold in the sciatic nerve of this ALS mouse model (Fig 5. H).

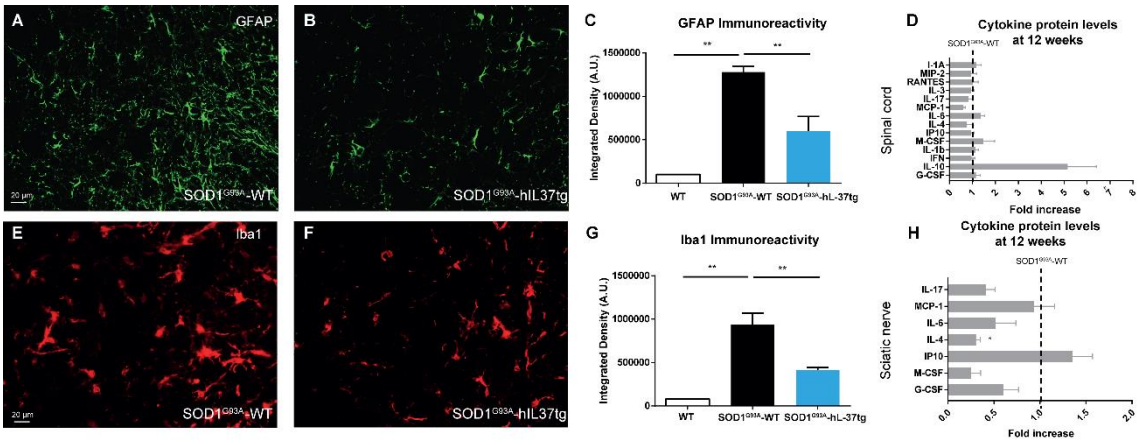

Figure 5. IL-37 expression reduced inflammatory milieu in SOD1^{G93A} mice. A,B. Representative images of lumbar spinal cord showing astroglial cells (GFAP, green) in (**A**) SOD1^{G93A}-WT and (**B**) SOD1^{G93A}-hIL-37tg. **C**. Graph showing quantification of GFAP immunoreactivity (n= 6 per group). (**E,F**) Lumbar spinal cords showing Iba1 positive cells in (**E**) SOD1^{G93A}-WT and (**F**) SOD1^{G93A}-hIL-37tg mice and (**G**) the quantification of Iba1 staining (n = 6 per group). (**G-H**) Assessment of cytokine protein levels in the (**D**) spinal cord and (**H**) sciatic nerve of SOD1^{G93A}-WT and SOD1^{G93A}-hIL-37tg (n = 3 SOD1^{G93A}-WT vs. 4 SOD1^{G93A}-hIL-37tg in the spinal cord; n = 4 per group in the sciatic nerve). *p<0.05, **p<0.01 compared to SOD1^{G93A}-WT mice. Error bars indicate SEM.

Since Luminex analysis revealed that IL-37 expression reduced the expression of M-CSF in the sciatic nerve, we therefore assessed the accumulation of macrophages within the nerve of SOD1^{G93A} mice at 16 weeks. Despite transgenic expression of IL-37 resulted in decreased protein levels of one of the main cytokines that trigger macrophages influx into the nerve in ALS (Martínez-Muriana et al., 2016), the accumulation of this myeloid cell subset in the peripheral nerves of ALS mice was not attenuated by IL-37, at least at end stages of the disease (Fig 6. A-D).

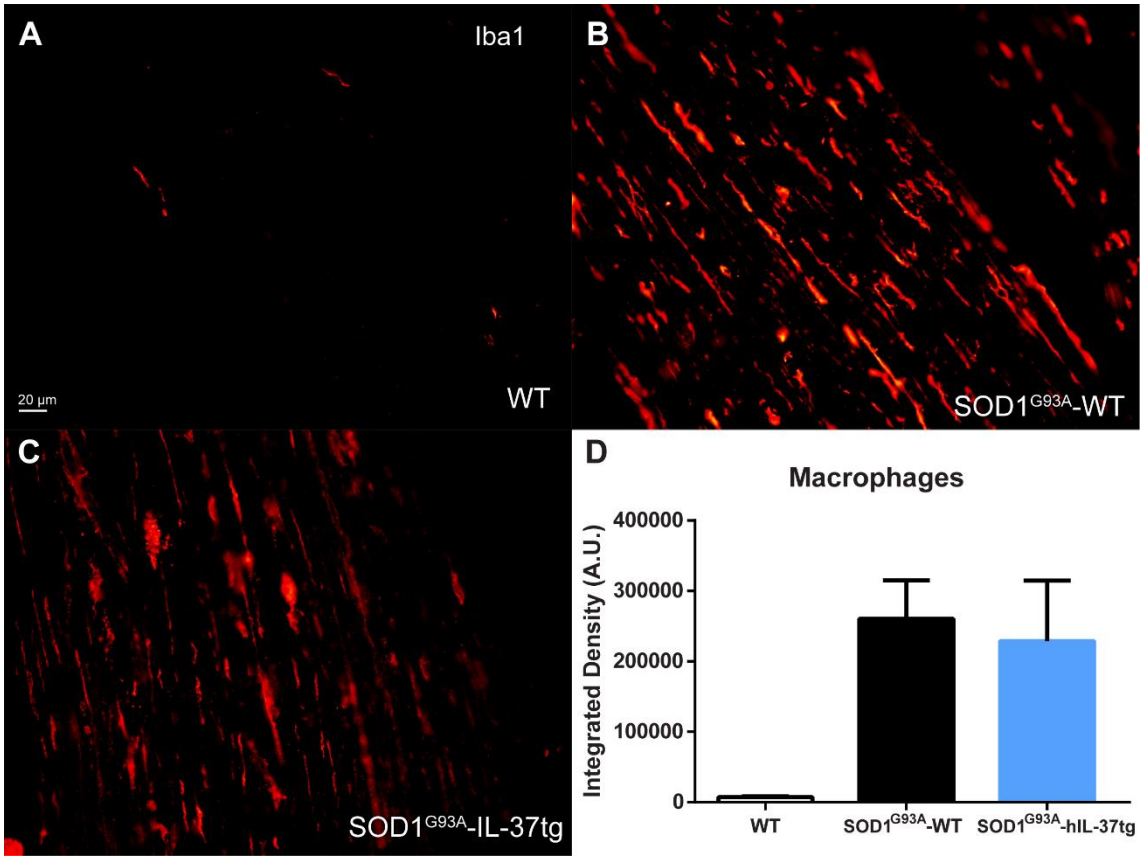

Figure 6. IL-37 expression did not reduce the amount of macrophages in the PNS of SOD1^{G93A} mice. A-C. Representative images of sciatic nerve sections showing the infiltration of macrophages (Iba1, red) into the nerve bundles of (**A**) WT, (**B**) SOD1^{G93A}-WT mice and (**C**) SOD1^{G93A}-hIL-37tg mice. **D.** Graph showing the quantification of Iba1 immunoreactivity in the sciatic nerve of ALS mice at 16 weeks of age (n = 3 per group). Error bars indicate SEM.

Beneficial effects of IL-37 in SOD1^{G93A} mice are mediated via IL-1R8

IL-37 can mediate its biological effects by acting as a ligand for the extracellular receptor complex IL-18Rα-IL-1R8 (Nold-Petry et al., 2015), as well as, by translocating to the nucleus upon binding SMAD3 (Nold et al., 2010). We then aimed at dissecting out to what degree the extracellular function of IL-37 is important to mediate its beneficial actions in ALS. For this purpose, we firstly characterized the expression of components of the IL-37 receptor complex in SOD1^{G93A} mice. We found that IL-18Rα and IL-1R8 were expressed in the spinal cord, sciatic nerve and GM muscle of C57Bl/6 mice and their expression did not increased in SOD1^{G93A} mice, although their levels tended to be higher in the GM muscle of ALS mice at the age of 20 weeks. (Fig 7. A-C).

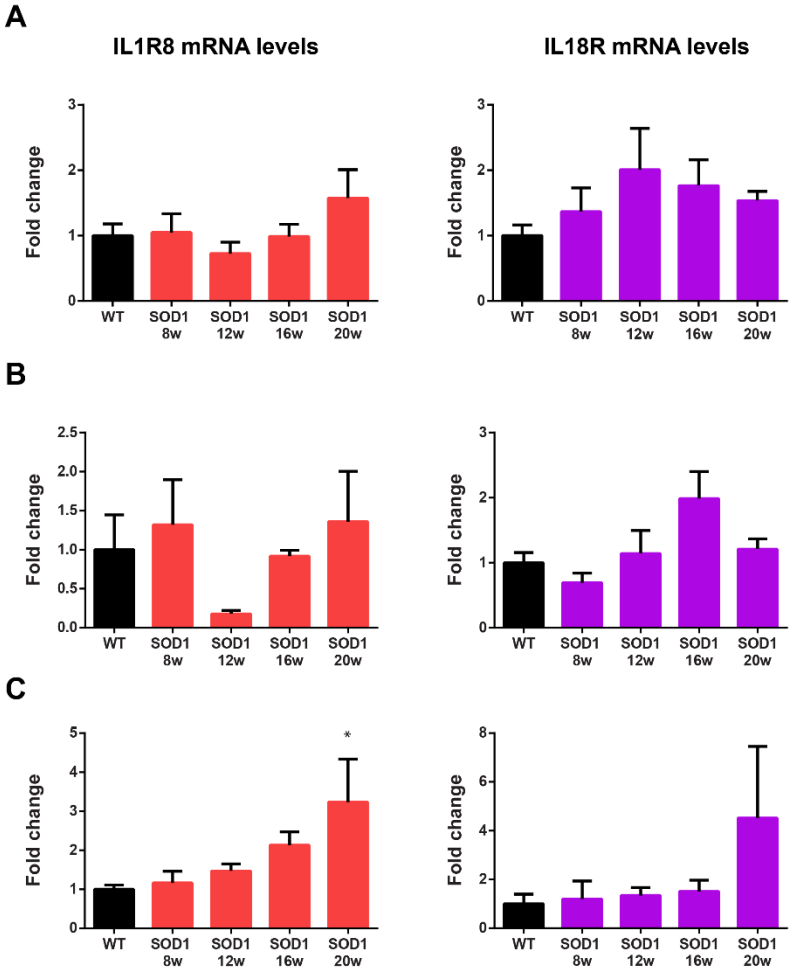

Figure 7. IL-37 extracellular receptors, IL-1R8 and IL-18Rα are expressed in SOD1^{G93A} mice. mRNA expression levels of IL37 receptor complex, IL1R8, and IL18Rα in the (**A**) spinal cord, (**B**) sciatic nerve and (**C**) gastrocnemius muscle of SOD1^{G93A} mice (n = 4 per group). *p<0.05 vs WT animals. Error bars indicate SEM.

To elucidate the importance of the extracellular function of IL-37 in SOD1^{G93A} mice, we crossed SOD1^{G93A}-hIL-37tg mice with IL-1R8 null mice. The resulting ALS mouse, despite expressing IL-37, it cannot act extracellularly since it lacks one of the main components of the IL-37 receptor complex.

Electrophysiological tests revealed that the beneficial effects of IL-37 on lower, but not upper, MN function were completely lost when removing IL-1R8 (Fig 9. A-D). Similarly, the protective actions of IL-37 on disease onset and progression, as well as, on mice survival gene were also fully abrogated in the lack of IL-1R8 (Fig 9. E-H).

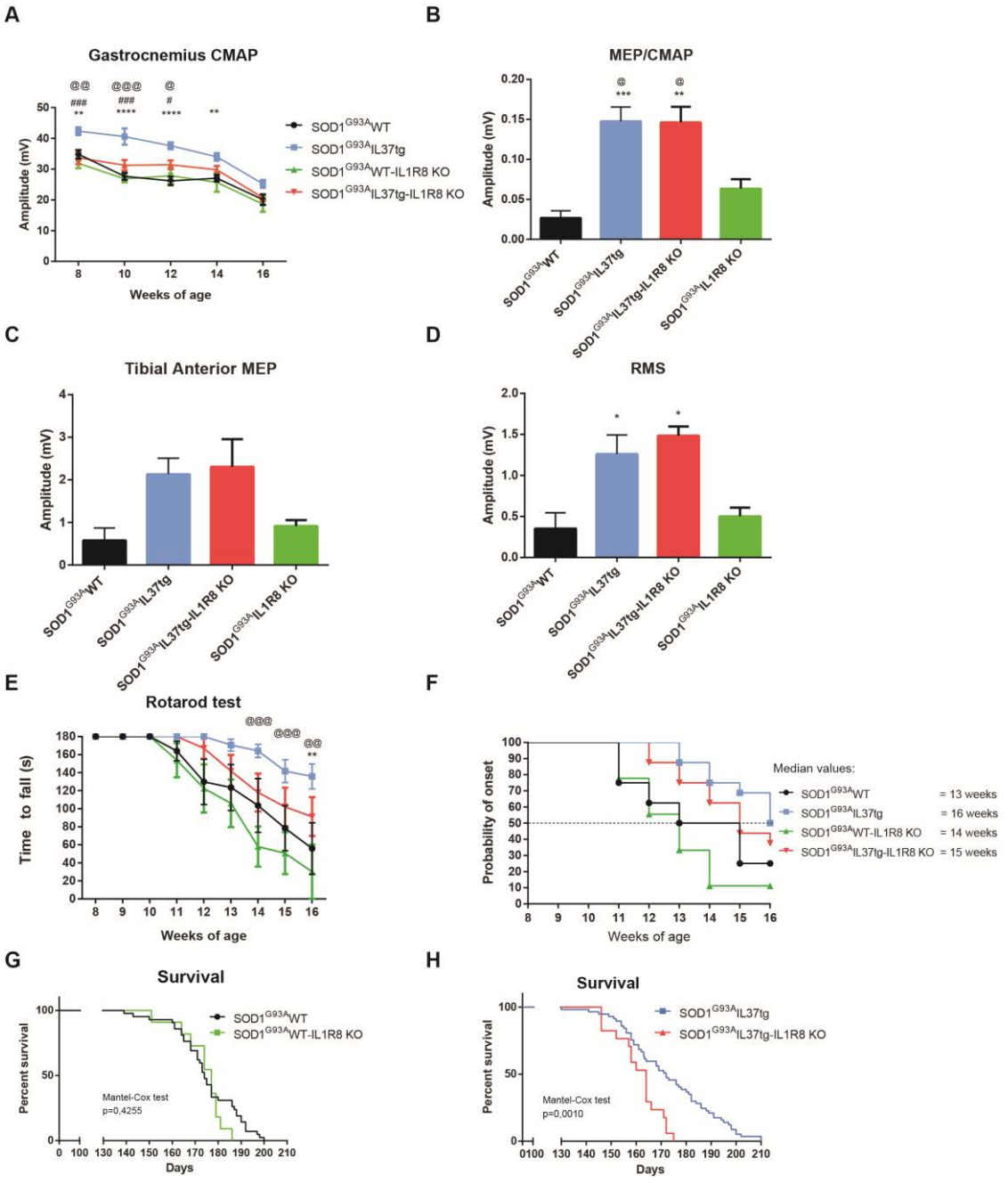

Figure 8. IL-37 exerts its beneficial effects through IL1R8. **A.** Electrophysiological test showing CMAPs' preservation in the gastrocnemius muscle (n=24 SOD1^{G93A}-WT, n=23 SOD1^{G93A}-hIL-37tg, n=25 SOD1^{G93A}WT-hIL-37tg-IL-1R8 KO, n=6 SOD1^{G93A}WT –IL-1R8 KO). **B-D.** Motor evoked potentials (MEPs) showing that SOD1^{G93A}IL37tg-IL1R8 null mice preserved upper MN function compared to SOD1^{G93A}-WT mice. **E**. Neuroprotection achieved by IL-37 expression is partially lost when removing IL1R8 in the (**E**) Rotarod test (n=8 SOD1^{G93A}-WT, n=16 SOD1^{G93A}-hIL-37tg, n=15 SOD1^{G93A}WT-hIL-37tg-IL-1R8 KO, n=9 SOD1^{G93A}WT –IL-1R8 KO). **F.** Blockade of IL-37 extracellular pathway delays the onset of the disease by 2 weeks. **G,F.** IL-37 increases SOD1^{G93A} mice survival through IL1R8 (n=42 SOD1^{G93A}-WT, n=57 SOD1^{G93A}-hIL-37tg, n=17 SOD1^{G93A}WT-hIL-37tg-IL-1R8 KO, n=11 SOD1^{G93A}WT –IL-1R8 KO). *p<0.05, **p<0.001, ***p<0.001, ****p<0.0001 compared to SOD1^{G93A}-WT mice. #p<0.05, ##p<0.01, ###p<0.001 compared to SOD1^{G93A}-hIL-37tg-IL-1R8 KO mice. @p<0.05, @@p<0.01, @@@p<0.001 compared to SOD1^{G93A}-IL-1R8 KO mice. Error bars indicate SEM.

At histological level, we also found the protective effects of IL-37 on neuronal survival (Fig 9. A-F), as well as, on glial cell immunoreactivity were completely vanished in the lack of IL-1R8 (Fig 10. A-L). Overall, this data demonstrates that the helpful actions of IL-37 in ALS disease are dependent on its extracellular function.

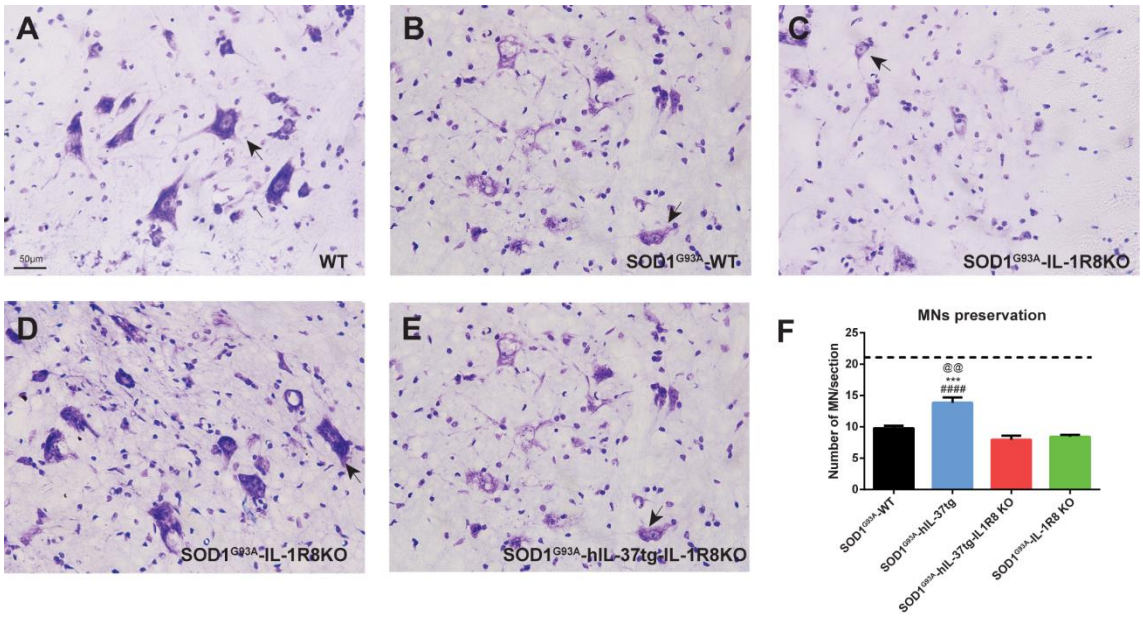

Figure 9. IL-37 protects spinal MNs by activating IL1R8. A-E. Representative spinal cord micrographs showing MNs preservation in (**A**) WT, (**B**) SOD1^{G93A}-WT, (**C**) SOD1^{G93A}WT-IL-1R8 KO, (**D**) SOD1^{G93A}-hIL-37tg and (**E**) SOD1^{G93A}-hIL-37tg-IL-1R8 KO mice. **F.** Quantification of neuronal sparing at 16 weeks of age. Dotted lines indicates the average of WT MNs (n=11 SOD1^{G93A}-WT, n=12 SOD1^{G93A} -hIL-37tg, n=7 SOD1^{G93A}WT-hIL-37tg-IL-1R8 KO, n=3 SOD1^{G93A}WT –IL-1R8 KO). ***p<0.001 compared to SOD1^{G93A}-WT mice, ####p<0.0001 compared to SOD1^{G93A}I-hIL37tg-IL-1R8 KO mice, @@p<0.01 compared to SOD1^{G93A}-IL-1R8 KO mice. Error bars indicate SEM.

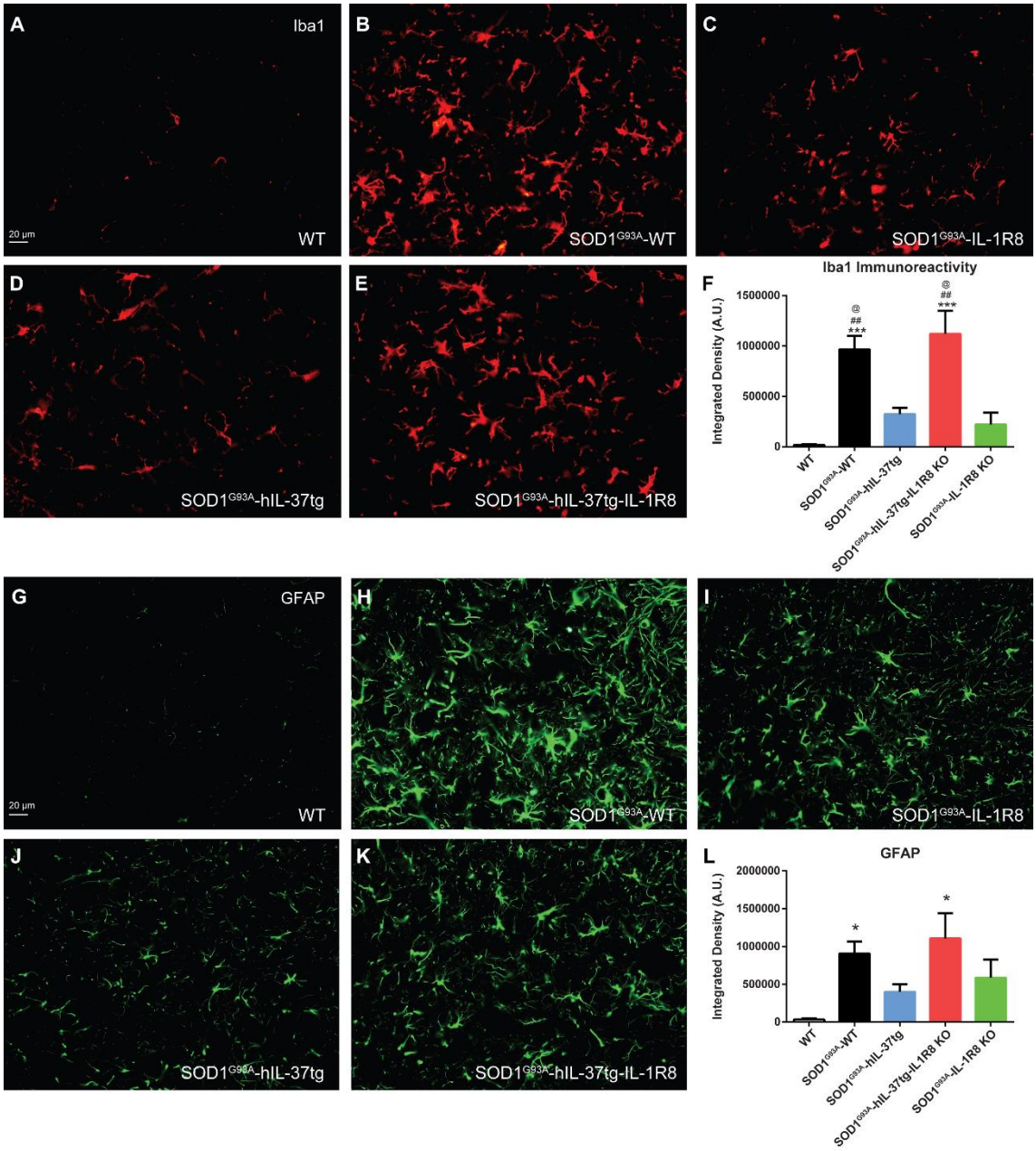

Figure 10. IL-37 reduces gliosis via IL-1R8. A-E. Representative images of Iba 1 staining in the lumbar cords of (**A**) WT, (**B**) SOD1^{G93A}-WT, (**C**) SOD1^{G93A}-IL-1R8 KO, (**D**) SOD1^{G93A}-hIL-37tg and (**E**) SOD1^{G93A}-hIL-37tg-IL-1R8 KO mice. **F.** Quantification of Iba1 immunoreactivity at 16 weeks of age (n=4 WT, n=4 SOD1^{G93A}-WT, n=7 SOD1^{G93A} -hIL-37tg, n=4 SOD1^{G93A}WT-hIL-37tg-IL-1R8 KO, n=3 SOD1^{G93A}WT –IL-1R8 KO). **G-K.** GFAP immunostaining showing astrogliosis in (**G**) WT, (**H**) SOD1^{G93A}-WT, (**I**) SOD1^{G93A}-IL-1R8 KO, (**J**) SOD1^{G93A}-hIL-37tg and (**K**) SOD1^{G93A}-hIL-37tg-IL-1R8 KO mice. **L.** Astroglial reactivity quantification in lumbar spinal cords at 16 weeks of age (n=4 WT, n=7 SOD1^{G93A}-WT, n=7 SOD1^{G93A} -hIL-37tg, n=5 SOD1^{G93A}WT-hIL-37tg-IL-1R8 KO, n=3 SOD1^{G93A}WT –IL-1R8 KO). *p<0.05, ***p<0.001 compared to SOD1^{G93A}WT mice. ##p<0.01 compared to SOD1^{G93A}IL37tg-IL1R8 KO mice, @p<0.05 compared to SOD1^{G93A}-IL-1R8 KO mice. Error bars indicate SEM.

A genomic variant in IL-37 delays disease onset in ALS patients

Having demonstrated that IL-37 exerts neuroprotective actions in SOD1^{G93A} mice, we next studied whether there is genetic association between IL-37 and ALS disease. Previous reports revealed that a single nucleotide polymorphism (SNP) in the IL-37 gene (rs3811046; T/G transition) was a protective factor in Grave's Disease (GD) (Yan et al., 2015). To unravel the role of this SNP in ALS, we examined the association between this SNP and clinical data of 158 ALS patients. We found that this SNP did not modify the onset, progression or survival of ALS patients. Nevertheless, when analyzing this SNP in those patients diagnosed with fALS (e.g. *SOD1*, *TARDBP*…), we found that the presence of the G allele was associated with a delay disease onset in 13 years (Fig 11. A).

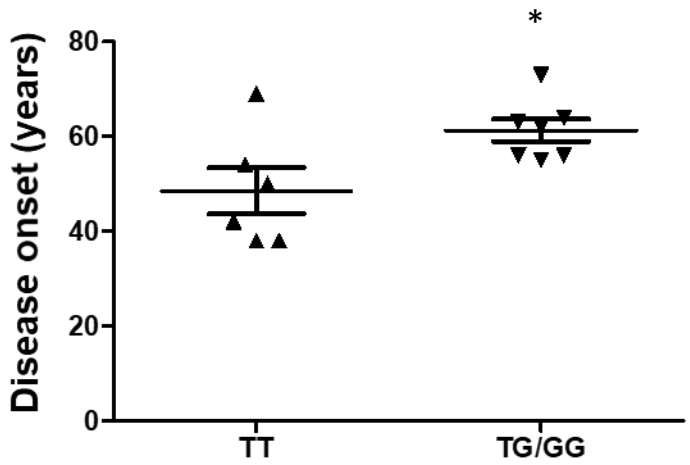

Figure 11. rs1138046 is associated with a delay onset of the disease of fALS patients. A. Relation between age ALS diagnosis and the presence of the G allele in the rs1138046 SNP (n=6 sALS and n=7 fALS). *p<0.05 compared to sporadic ALS. Error bars indicate SEM.

DISCUSSION

The aim of the present chapter was to study the role of the novel anti-inflammatory cytokine, IL-37, in a mouse model of ALS. Here, we reported for the first time that IL-37 attenuates the inflammatory response, ameliorates the clinical signs of the disease and increases lifespan of the $SOD1^{G93A}$ mouse. We also described that the protective actions of IL-37 are triggered via its extracellular receptor complex, IL-18Rα-IL-1R8, since the gene deletion of IL-1R8 results in complete loss of the protective actions mediated by this cytokine. Interestingly, we found that that IL-37 is not up-regulated in the spinal cord of sALS patients, despite its receptors are expressed. Consistent with these results, we also found that a SNP of IL-37 delays disease onset in 13 years in fALS but not in sALS. Overall, these results demonstrate a protective role for IL-37 in ALS.

Currently, it is widely accepted that inflammation highly contributes to ALS pathophysiology (Philips and Robberecht, 2011). The first insights of a deleterious role came from a landmark study using chimeric mice (Clement et al., 2003). In this work, Clement and colleagues elegantly demonstrated that MNs degeneration is triggered by neighboring non-neuronal cells expressing mutant SOD1. From then on, many other groups have extended the knowledge of the role of neuroinflammation in ALS. In this sense, it was recently discovered that microglia triggers MNs death via activation of the nuclear factor kappa B pathway (NF-κB) (Frakes et al., 2014). Due to the direct role of microglial cells in ALS pathophysiology and the deleterious contribution of the inflammatory response in both CNS and PNS, the development of immunomodulatory agents are likely to have therapeutic actions.

IL-37 is an anti-inflammatory cytokine with potent suppressive actions over the immune system (Nold et al., 2010). Despite not being present within the mouse genome, the transgenic expression of the human form of IL-37 notably protects mice from several immunological challenges (Nold et al., 2010; McNamee et al., 2011; Ye et al., 2015). In the hIL-37tg mouse, IL-37 expression is artificially regulated by a cytomegalovirus promoter which should increase IL-37 mRNA levels broadly (Nold et al., 2010). There is absent/low expression of IL-37 in the hIL-37tg mice in physiological conditions, as occurs in human tissues. This is due to a conserved ten nucleotide (adenosine) homology box in exon 5 of IL-37, which flanks a coding region of instability elements that are also found in IL-18, IL-1β, and in a variety of other mRNA (Bufler et al., 2004) such as plasminogen activator receptor or vascular endothelial growth factor. These instability elements overlap with the binding sites for proteins associated with mRNA stability and limits the half-life of IL-37

mRNA. However, after inflammatory challenges, mRNA levels increase due to the activation of mechanisms or sequences within the 3'-UTR (untranslated region) for mRNA stability. In agreement with these reports and our recent publication (Coll-Miró et al., 2016), we observed very low transcriptional levels of IL-37 in the spinal cord hIL-37tg mouse in physiological conditions. Nevertheless, IL-37 expression was markedly up-regulated in the nervous system of SOD1^{G93A} mice, especially, at symptomatic stages of the disease. Indeed, IL-37 levels were detected at early stages of the disease and raised as disease progressed, which may be due to several foci of inflammation that are likely stabilize IL-37 mRNA. First, at the periphery, and prior to MNs degeneration, there is a disconnection of the terminal end-plates with the skeletal muscles which is followed by a retrograde degeneration of the axons in a process known as "dying-back" (Fischer et al., 2004). This event occurs before the onset of the disease and coincides with the invasion of monocytes to the PNS (Chiu et al., 2009; Martínez-Muriana et al., 2016). At central level, transcriptomic analyses have previously revealed up-regulation of several pro-inflammatory cytokines and chemokines at early stages and late of the disease (Butovsky et al., 2012). This central and peripheral immune reaction may account for the early expression of IL-37 in the nervous system of SOD1^{G93A} in pre-symptomatic stages of the disease mice, as well as, its sustained levels during the disease course.

Here we showed that transgenic expression of IL-37 also exerted beneficial effects in functional and histological outcomes in SOD1^{G93A} mice. In accordance with our findings, recent studies revealed that overexpression of the anti-inflammatory cytokine, IL-10, significantly delays the disease and prolongs SOD1^{G93A} mice survival (Ayers et al., 2015; Gravel et al., 2016). Moreover, the blockade of IL-10R dramatically precipitates disease onset and increased the microglial response at early stages of the disease (Gravel et al., 2016). Reinforcing this data, it was found that higher IL-10 protein levels are related with an slower disease progression (Su et al., 2013). A recent report also showed that overexpression of the anti-inflammatory cytokine, IL-4, delayed the progression of the disease but failed to increase the survival of SOD1^{G93A} mice (Rossi et al., 2018). Since the neuroprotective effects of these anti-inflammatory cytokines were due to its modulatory actions over microglial cells, we therefore assessed whether IL-37 regulated glial reactivity in the lumbar spinal cord of SOD1^{G93A} mice. Interestingly, we observed that IL-37 expression attenuated microgliosis but also astrogliosis at the end-stage of the disease. Interestingly, we found that IL-37 did not attenuate the protein levels of pro-inflammatory cytokines within the spinal cord but increased in ~5-fold the expression of the anti-inflammatory cytokine, IL-10, as compared to SOD1^{G93A}WT mice. The induction of IL-10

by IL-37 is in accordance with previous works showing up-regulation of this cytokine by IL-37 in other animal models (McNamee et al., 2011; Luo et al., 2014; Moretti et al., 2014). However, the beneficial effects of IL-37 in ALS mice are unlikely due to IL-10 activity, since previous studies have revealed that the anti-inflammatory effects of IL-37 are maintained in the lack of IL-10 or after blocking IL-10R receptor, indicating that the effects of IL-37 are IL-10-independent (McNamee et al., 2011). Highlight that IL-37 also modulated cytokine expression in the PNS. Indeed, we observed that transgenic expression of IL-37 reduced the protein levels of IL-4, IL-17 and M-CSF in the sciatic nerves of $SOD1^{G93A}$ mice. This is interesting since results from chapter 1 revealed that M-CSF has a key role in triggering the recruitment of monocytes into the nerve bundles at the pre-symptomatic stage of the disease (Martínez-Muriana et al., 2016). This data may indicate that IL-37 is modulating inflammation in the CNS but also in PNS of ALS mice. However, we found that transgenic expression of IL-37 in $SOD1^{G93A}$ mice did not reduce macrophages accumulation in the sciatic nerve at 16 weeks of age. Since the reduction in M-CSF levels triggered by IL-37 was observed four weeks earlier (12 weeks of age), we do not discard that IL-37 could attenuate macrophage counts in the nerve at earlier stages of the disease. Due to time constrain, these studies have not been done in the present thesis, but they will be done in the laboratory. On the other hand, the reduction of IL-17 and IL-4 in the sciatic nerve by IL-37 is in accordance with previous reports (Nold-Petry et al., 2015; Ye et al., 2015). Interestingly, IL-17 is one of the pro-inflammatory cytokines that are more elevated in both serum and CSF of ALS patients (Fiala et al., 2010; Rentzos et al., 2010). Previous studies have shown that IL-17 increases the production of the pro-inflammatory Th17 lymphocytes. These IL-17-positive T cells play a critical role in autoimmunity, being one of the main immune effectors in the several diseases (Tzartos et al., 2008). However, whether they exert a deleterious role in ALS has not been investigated yet.

The anti-inflammatory actions of IL-37 may be triggered by two pathways: (i) extracellular activation of the complex IL-18Rα-IL-1R8 (Nold-Petry et al., 2015), or (ii) translocation to the nucleus by binding SMAD3 (Nold et al., 2010). In order to dissect out the relevance of the extracellular function of IL-37 in ALS, we then transgenically knocked-out IL-1R8 to remove the IL-37 extracellular pathway. We found that the lack of IL-1R8 in $SOD1^{G93A}$ mice did not have any effect in the progression of the disease, which can be explain by the absence of IL-37 in rodents. Interestingly, we observed that the beneficial effects of transgenic expression of IL-37 in ALS were vanished in the lack of IL-1R8, suggesting that the beneficial actions of this cytokine in this neurodegenerative disease are mediated by acting extracellularly. The only feature that was not affected by the lack of

IL-1R8 in SOD1^{G93A}-hIL-37tg mice was the preservation of MEPs. This data suggest that the intracellular function of IL-37 could be involved in upper MN survival. However, the fact that IL-37 exerts beneficial actions by acting extracellularly has important clinical relevance since it may suggest that the administration of recombinant IL-37 protein may open a new avenue for the treatment of ALS patients, especially, because IL-1R8 is expressed in the spinal cord of healthy and sALS individual. This is further reinforced by our data demonstrating that the induction of this protective mechanism is aberrant in sALS patients.

Although IL-37 expression is not up-regulated in the spinal cord of sALS, we found that a genetic variant of IL-37 (SNP rs3811046) is associated with delayed ALS onset in patients diagnosed with familial but not sporadic forms of the disease. It is currently unknown whether this SNP leads to greater IL-37 activity, expression or both. Since fALS forms only represent ~5-10% of ALS individual, none of the human spinal cord samples analyzed corresponded to fALS patients. However, we do not discard that fALS individual carrying this genetic variant could have increased IL-37 levels. This genetic variant has been described to be also a protective factor for a thyroid autoimmune disease known as Grave's disease (Yan et al., 2015). There are other genetic variants of IL-37 that are found in less frequency in the human population, and thus, we do not discard that they could be also associated with ALS disease. We are currently conducting a study to analyze all the genetic variants of the IL-37 and IL-1R8 genes in a cohort of 250 ALS patients. These data may provide relevant information about the involvement of IL-37 and ALS disease.

In summary, in the present chapter we provide clear evidence that IL-37 exerts beneficial actions in SOD1^{G93A} mice by exerting potent anti-inflammatory actions in the CNS and PNS by signaling via IL-18Rα-IL-1R8. Although further studies are needed to elucidate the involvement of IL-37 in ALS patients, our results suggest potential clinical application of this anti-inflammatory cytokine in ALS disease.

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CHAPTER III

Maresin 1 improves motoneuron functions and increases lifespan of SOD1^{G93A} mice

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ABSTRACT

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease that causes progressive paralysis and death to patients due to the degeneration of motoneurons in the spinal cord, brainstem and cortex. Although the mechanisms that cause the development of the disease are not fully known, recent studies indicate that inflammation contribute to ALS pathogenesis. Despite there are currently several potent anti-inflammatory drugs in clinical use, they lack therapeutic efficacy in ALS and in other diseases with persistent inflammation. There is therefore a need to develop novel interventions to attenuate inflammation in ALS. Recent studies have uncovered a family of lipids that actively promote resolution and tissue repair. Administration of these lipids have proven efficacy in inflammatory challenges in which current anti-inflammatory drugs fail to mitigate the bystander side effects of immune cells. We have recently reported that the administration of one of these lipids reduces inflammation in the injured spinal cord and leads to neurological recovery. Here, we aim to test the effectiveness of this resolution agonist in an animal model of ALS. We provide novel data that supports the efficacy of this approach. In particular, we show that exogenous administration at late stages of ALS disease slows progression of the disease and extends lifespan. We also reveal that administration of ALS before the clinical signs of disease markedly delays ALS onset. Moreover, we also show that MaR1 has greater efficacy than Riluzole in the SOD1^{G93A} mice. Overall, the results from this chapter suggest that the use of immunoresolvent agents could lead to the development of novel intervention to treat ALS.

Keywords: Resolution, inflammation, Maresin-1, specialized pro-resolving mediators, amyotrophic lateral sclerosis,

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease characterized by progressive degeneration of motoneurons in the spinal cord and brain (Philips and Robberecht, 2011; Robberecht and Philips, 2013). This leads to muscle atrophy, paralysis, spasticity and finally death of patients within 3–5 years after clinical diagnosis in most of the cases. At present, therapy is mainly symptomatic and fails to halt disease progression (Philips and Robberecht, 2011; Robberecht and Philips, 2013).

Despite ALS is a motoneuron disease, it is currently well accepted that non-neuronal cells strongly contribute to the development and progression of disease (Appel et al., 2010). A common feature observed in postmortem samples of familial and sporadic patients of ALS, as well as, in ALS animal models, is the occurrence of an inflammatory response in the central nervous system (CNS), mostly consisting in activated glial cells (microglia and astrocytes) (Mantovani et al., 2009; Appel et al., 2010; Philips and Robberecht, 2011; Robberecht and Philips, 2013), and in the peripheral nerves (PNS), preferably by circulating macrophages (Chiu et al., 2009; Martínez-Muriana et al., 2016). Although inflammation is a fundamental physiological process to clear pathogens and cell debris and to promote tissue healing, excessive or persistent inflammation as it occurs in most neurological conditions, including in ALS, leads to detrimental actions and contribute to the course of the pathology (Mantovani et al., 2009; Appel et al., 2010; Philips and Robberecht, 2011; Robberecht and Philips, 2013). There are currently very potent anti-inflammatory compounds in clinical use. Although these drugs relieve gross signs and symptoms of acute inflammation, they show limited efficiency in conditions with excessive inflammation and mediate several side effects, including severe immune suppression with opportunistic infections. In this line, clinical trials assessing the efficacy of various anti-inflammatory compounds in ALS patients have led to disappointing results. Therefore, there is a need to develop novel and effective interventions to minimize inflammation in conditions with persistent inflammation, such as ALS, and ideally, without resulting in immunosuppression.

The temporal events in self-limited acute inflammatory responses are known to resolve with the loss of inflammatory cells from the tissue and the return of function. Focus on the fundamental mechanisms in the resolution response has led to the identification of a new family of bioactive lipids known as 'specialized pro-resolving mediators' (SPMs). These are biosynthesized from essential polyunsaturated fatty acid (PUFA; Ω-3 and Ω-6) precursors by the action of cyclooxygenase-2 (COX-2) and 12/15-

lipoxygenase (12/15-LOX) enzymes to function as potent local-resolution agonists. SPMs include: lipoxins generated from arachidonic acid (AA), E-series resolvins from eicosapentaenoic acid (EPA), and D-series resolvins, neuroprotectins, and maresins from docosahexaenoic acid (DHA) (Serhan et al., 2015). SPMs actively turn off the inflammatory response by acting on distinct G protein coupled receptors expressed on immune cells that activates dual anti-inflammatory and pro-resolution programs (Serhan, 2014; Serhan et al., 2015) Interestingly, several reports reveal that conditions with sustained inflammation, such as atherosclerosis or arthritis, are associated to aberrant synthesis of SPMs, and the exogenous administration of SPMs reverses the clinical signs of the disease (Serhan et al., 2008).

Little is known about the role of SPMs in the CNS pathologies. Recent reports indicate that there is a deficit in the synthesis of SPMs in patients with active multiple sclerosis and Alzheimer's disease (Lukiw et al., 2005; Prüss et al., 2013) We have recently revealed that there is an aberrant synthesis of SPM in the spinal cord after injury and that administration of the SPM coined Maresin-1 (MaR1), results in significant benefit on functional and histological outcomes (Francos-Quijorna et al., 2017). However, no study has addressed yet whether stimulating the biological mechanisms that trigger the resolution of inflammation could result in a novel therapeutic intervention to slow the progression of the disease. In the present chapter we aim to evaluate for the first time the therapeutic actions of SPMs in ALS. Our preliminary data provides solid evidence indicating that that at least one of the SPM coined MaR1 has significant impact on disease progression.

MATERIAL AND METHODS

Experimental animal models

The experiments were performed in both female and male transgenic SOD1^{G93A} mice (B6-Tg[SOD1-G93A]1Gur) obtained from Jackson Laboratories (Bar Harbor, ME, USA) and maintained at our animal facilities. Animals were further genotyped and by PCR reaction using DNA extracted from the tail. Following the European guidelines, animals were housed at room temperature of 22 ± 2ºC under a 12:12-h light–dark cycle and food and water was provided *ad libitum*. The endpoint criterion was considered when the animals were no longer able to perform the righting reflex for the following 30 seconds. All the experimental procedures were approved by the Universitat Autònoma de Barcelona Animal Experimentation Ethical Committee following the European Commission on Animal Care 2010/63/EU.

Drug administration

At 8 weeks of age, animals were electrophysiologically tested and distributed according their baseline values and gender. 1 μg of MaR-1 (7R,14S-dihydroxy-4Z,8E,10E,12Z,16Z,19Z-docosahexaenoic acid; Cayman Chemical) in 100 μl saline containing 10% alcohol was administered intraperitoneally (IP) 3 days per week, starting at 8 or at 16 weeks of age. The solution was freshly prepared prior to the injection. Vehicle animals received the same solution without MaR1. Riluzole (Rilutek™ 50-mg tablets) was dissolved in drinking water at a concentration of 100 μg/ml and mice drunk water *ad libitum*. To ensure that all animals were exposed to the same conditions, riluzole-treated animals also received IP injections of the vehicle solution 3 days per week.

Motor conduction tests

To electrophysiologically evaluate the motor conduction of lower MNs of SOD1^{G93A} mice, we assessed the maximum amplitude of the compound muscle action potential (CMAP) in the hindlimb affected muscles: Gastrocnemius medialis (GM) and tibialis anterior (TA). Briefly, sciatic nerve of SOD1^{G93A} mice was percutaneously stimulated using a pair of needle electrodes placed at the sciatic notch by means of single pulses of 0.02 ms duration (Grass S88). The evoked M-wave was further amplified, recorded and displayed in a digital oscilloscope (Tektronix 450S) at settings appropriate for further analysis using recording electrodes placed at the studied muscles.

To ensure reproducibility, all the procedure was performed under microscope and the temperature was kept at 34-37 ºC using an electronic heating pad. All the researchers were blinded to the experimental groups.

Locomotor tests

To study different gait and motor parameters, $SOD1^{G93A}$ mice were weekly evaluated using the rotatod test (Miana-Mena et al., 2005). Rotarod test is a useful tool to assess the strength, coordination and balance as well as to determine the offspring of the disease. To achieve baseline results, all animals were trained three times prior to the beginning of the follow-up. Before starting the test, all animals must be able to stay on the rotating rod for 3 minutes at 14 rpm. Once animals learned the task, $SOD1^{G93A}$ animals were weekly tested from 8 to 16 weeks of age and the latency to fall from the rod was measured. To determine the onset of the disease, it was considered the week of age at which the animal was unable to run for 3 minutes on the rotarod. Regarding the forced locomotion test, it consists in an automated belt were the animals are placed and the maximum velocity that they are able to run at 16 weeks of age is measured. An arbitrary increasing velocity of 5, 10, 15, 20, 25, 30, 35 and 40 cm/s, was recorded (Martinez-Muriana et al., 2016).

Statistical analysis

Data are shown as mean ± SEM and were analyzed using the GraphPad Prism 6 software package (GraphPad Software). Electrophysiological and locomotion test were analyzed using two-way repeated measurements ANOVA with Bonferroni post-hoc test. Survival data was analyzed using the Mantel-Cox test. Differences were considered significant at $p < 0.05$.

RESULTS

Effects of the delayed treatment of MaR1 in ALS disease

To evaluate the potential beneficial actions of MaR1 in ALS, we first evaluated the effects of this SPM when treatment was initiated late stages of the disease (16 weeks), when experimental treatments fail to show any therapeutic benefit. At this time point, most SOD1^{G93A} mice show gross locomotor deficits and cannot perform on the rotarod test. We therefore performed electrophysiological analysis to evaluate the progression of the disease. We found that MaR1 slowed the decline of the compound muscle action potentials (CMAP) amplitude recorded in the gastrocnemius, suggesting that MaR1 conferred protection against the loss of neuro-muscular integrity (Fig. 1A). The beneficial effects of MaR1 in CMAPs were already evident at two weeks after the initiation of the treatment and were maintained for the next four weeks of the follow up (20 weeks of age). In line with the electrophysiological data, we also found that exogenous delivery of MaR1 led to significant extension in the survival of SOD1^{G93A} mice, increasing the median and maximal lifespan in 12 and 17 days, respectively, despite treatment was initiated at late stages of the disease (Fig 1B).

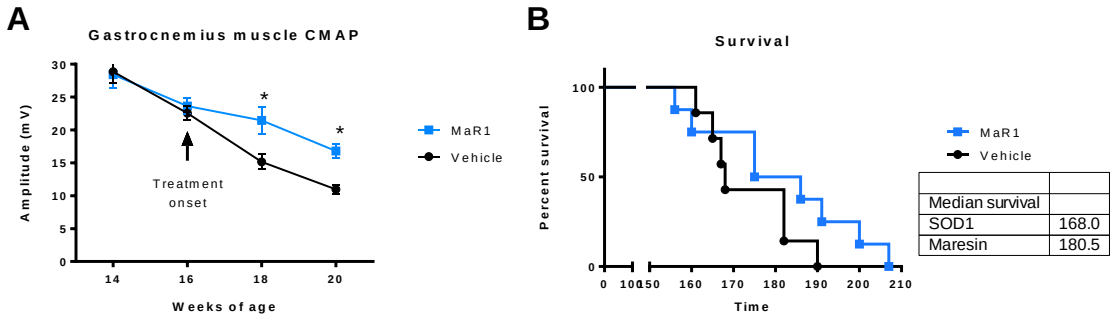

Figure 1. Administration of MaR1 at late stages slows disease progression. (**A**) Electrophysiological test showing preservation of the compound muscle action potential in the gastrocnemius muscle. (**B**) MaR1 treatment extends life span of female SOD1^{G93A} mice. (n=8 SOD1^{G93A} vehicle, n=9 SOD1^{G93A} Maresin). Error bars indicate SEM.

Functional characterization of MaR1 treatment at the pre-symptomatic phase of ALS disease

We next aimed at investigating whether administration of MaR1 starting at pre-symptomatic phases of the disease could exert greater therapeutic actions. For this purpose, MaR1 treatment was initiated in SOD1^{G93A} mice at the age of 8 weeks. At this point, ALS mice do not display any gross functional impairment in the rotarod test. However, at the electrophysiological level, CMAP amplitude in hindlimb muscles is reduced ~40-50%, indicating the integrity of the neuro-muscular functional is already strongly compromised.

Although these experiments are still ongoing, our current data provide clear evidence that the MaR1 markedly delays the onset of the gross motor impairments assessed in the rotarod test (Fig. 2A). At the electrophysiological level, we also found that MaR1 conferred protection against MN functional loss as revealed the amplitude of the CMAPs recorded in the gastrocnemius muscle (Fig. 2B). Highlight, that the therapeutic actions of MaR1 on ALS disease progression were more effective than those observed after Riluzole administration, the only approved treatment option for ALS patients in Europe.

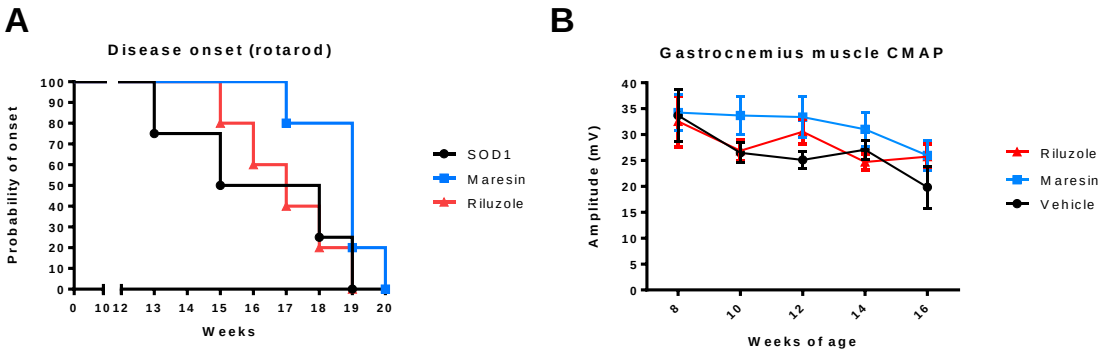

Figure 2. Administration of MaR1 at pre-symptomatic stages delays disease onset. (**A**) Treatment with MaR1 leads to significant preservation in functional outcomes assessed by rotarod (**B**) Electrophysiological test showing preservation of the compound muscle action potential in the gastrocnemius muscle (n=4 SOD1^{G93A} vehicle, n=6 SOD1^{G93A} Maresin, n=5 SOD1^{G93A} Riluzole). Error bars indicate SEM.

DISCUSSION

In the present chapter, we evaluate whether exogenous administration of SPMs may result in therapeutic actions in ALS. We found that MaR1 is effective in ameliorating ALS disease progression when treatment was initiated at late stages of the disease. Similarly, we found that delivery of MaR1 at pre-symptomatic phases of ALS strongly delayed the onset of the disease. Interestingly, the beneficial effects of MaR1 in ALS were more potent that those achieved after administration of Riluzole. This data reveals for the first time that administration of SPMs, in particular MaR1, could lead to the development of a new therapeutic approach to treat ALS disease.

Polyunsaturated fatty acids are key regulators of the inflammatory response, since they control several processes involved in the onset and resolution of this physiological process (David et al., 2012; Serhan et al., 2014, 2015). Among them, n-3 PUFA (omega 3-fatty acids) has been specially brought to the attention of the scientific community due to its therapeutic effects in several inflammatory diseases. In particular, the n-3 PUFAs, DHA and EPA, which are enriched in oils derived from fish and algae, are used extensively as dietary supplements, and found to exert beneficial actions in a number of conditions in which the inflammation contributes to the course of different pathologies, including after CNS trauma (King, 2006; Huang et al., 2007; Lopez-Vales et al., 2010).

More recently, EPA and DHA lipid-derived mediators known collectively as SPM, have been identified as key players in the resolution of inflammation and regulators of homeostasis (Schwab et al., 2007; Buckley et al., 2014; Serhan, 2014; Serhan et al., 2015). The importance of SPM in regulating inflammation is evident in many inflammatory disorders such as atherosclerosis, asthma, ulcerative colitis, among others, in which there is absence, or insufficient or delayed production of SPM (Serhan, 2014; Serhan et al., 2015). Importantly, the exogenous administration of SPM reduces inflammation and prevents the detrimental effects exerted by the immune cells, relating the failure in the production of SPM in the pathogenesis of different inflammatory diseases (Serhan, 2014; Serhan et al., 2015).

Among the different family members of SPM, maresins have been the less characterized. This family of SPM derived from macrophages consists of two members, MaR1 (Serhan et al., 2009) and the more recently identified MaR2 (Deng et al., 2014). MaR1 exerts potent actions in regulating inflammation resolution, but also in preventing nociception after inflammatory- and chemotherapy-induced neuropathic pain, and stimulating tissue regeneration in *planaria* (Serhan et al., 2012; Serhan, 2014).

A recent study from our laboratory revealed that there is also an aberrant production of SPMs in the spinal cord parenchyma after injury, and that administration of MaR1 enhanced different biological stages of the resolution of inflammation (Francos-Quijorna et al., 2017). These includes, down-regulation of cytokines, silencing of inflammatory pathways, reduction of neutrophil and macrophages counts, shift in macrophage phenotype, and stimulation of the phagocytic activity of macrophages. Importantly, all the biological effects induced by MaR1 treatment led to significant improvement in locomotor function and protection against secondary tissue damage (Francos-Quijorna et al., 2017). These results support the concept that the inappropriate biosynthesis of SPM in the injured CNS hampers resolution of inflammation and contributes to tissue damage.

It is currently unknown whether the production of SPM is impaired in ALS. However, DHA levels are significantly reduced in ALS patients (Yip et al., 2013), suggesting that the synthesis of resolution agonist of inflammation might be altered. We are currently performing lipidomic analysis from cerebrospinal fluid (CSF) samples of ALS individuals. These experiments will provide important information about the levels of SPM and various pro-inflammatory lipid mediators, such as prostaglandins, in ALS patients. Due to time constrains, these results have not been included in the present thesis. Based on our recent data in spinal cord injury, as well as, on previous reports demonstrating aberrant SPM production in the CFS of patients diagnosed with Alzheimer's disease or multiple sclerosis, we hypothesize that the synthesis of resolving agonists of inflammation in ALS patients will be defective.

SPMs are synthesized by cycloogynase-2 (COX-2) and lipoxygenase (LOX) enzymes, which are also responsible for the production of the pro-inflammatory prostaglandins and leukotrienes. Therefore, COX-2 and LOX generate lipid mediators that contribute to both, onset and resolution of inflammation. This is important because the administration of selective COX-2 or LOX inhibitors initially reduces the magnitude of inflammation during the early phase of inflammation, but they also prolong the inflammatory reaction due to the blockade of SPM production. This is of crucial importance since most of the broad anti-inflammatory therapies interfere with the expression or activity of COX-2 and LOX enzymes, and thus, they may adversely result in exacerbated inflammation when treatment has to be sustained on time. Indeed, administration of minocycline, which down-regulates COX-2 expression, is effective in delaying the onset of ALS pathology when administered in mice before the clinical symptom of disease (Zhu et al., 2002) but increases microglial and astroglial activation

and accelerates disease progression when given after clinical onset (Keller et al., 2011). This may explain, in part, the negative results found when minocycline or colecoxib, a selective COX-2 inhibitor, was administered in ALS patients (Cudkowicz et al., 2006; Gordon et al., 2007).

Here, we report that administration of MaR1 mediate therapeutic actions when given before the symptomatic phase of the disease but also when treatment is initiated at late stages. Although our results are still quite preliminary, they suggest that MaR1 may have clinical translation, especially, because it led to greater beneficial actions than Riluzole, the only prescribed drug for ALS individuals in Europe. Highlight that 1μg per mouse of MaR1 was enough to mediate these helpful actions, a dose 100-1000 lower than current anti-inflammatory drugs. This, together with the fact that MaR1 is produced by human host tissues, it suggests that this treatment might have minimal side effects, which is one of the main problems related to anti-inflammatory drugs. Future experiments in the laboratory will reveal key questions that may pave the way to collect funds from industry, private foundations, and public funds to carry out clinical trials in ALS individual. These include toxicological assays, elucidation of the mechanisms of action, pharmacokinetics analysis, and development of compounded formulation for administration in humans.

Altogether, our findings suggest for the first time that immunoresolvent therapies, in particular MaR1, could open a therapeutic avenue for the treatment of ALS.

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GENERAL DISCUSSION

GENERAL DISCUSSION

ALS represents one of the most challenging neurodegenerative diseases of the latest century. From the aggregation of SOD1 to the failure in the maintenance of neuromuscular synapsis, it encompasses the disruption of hundreds of molecular processes that makes the disorder a devastating neurodegenerative condition (Mancuso and Navarro, 2015). In the last decades, the inflammatory response emerged as a key factor that triggers MN degeneration (Philips and Robberecht, 2011). Under physiological conditions, the immune response plays an essential role leading to the clearance of cellular debris and the promotion of wound healing and homeostasis (Velnar et al., 2009). However, when the resolution of this process is hampered, the response becomes chronic and induces cell death and functional impairments. In ALS, this immune response becomes evident at early stages of the disease, with the first insights of peripheral degeneration (Chiu et al., 2009; Martínez-Muriana et al., 2016). This incipient immune reaction becomes more significant at the peripheral nerve and spinal cord at symptomatic and late stages of the disease, when the loss of MNs is prominent (Chiu et al., 2009; Martínez-Muriana et al., 2016). The increasing immune process results in an accelerated degeneration of the remaining neurons which leads to a rapid and overwhelming ending.

One of the main mechanisms that contribute to MN death is the activation of microglial cells, the resident immune cells of the CNS parenchyma. Among their functions, they play important roles in tissue homeostasis and surveillance against immune threats. However, under some pathological conditions, microglial cells exert detrimental roles and contribute to degeneration in multiple neurological diseases (Gomez-Nicola and Perry, 2015). Nevertheless, in ALS, whether microglial cells are neuroprotective, neutral or neurotoxic has generate some controversy. In 2003, Cleveland's lab first demonstrated that non-neuronal cells are necessary to trigger neuronal death (Clement et al., 2003). This groundbreaking research revealed that, when chimeric mice containing healthy MNs are surrounded with non-neuronal cells expressing mSOD1, they do develop aspects of ALS pathology. More important, they described that when toxic MNs are grafted with non-toxic glial cells, there is a delay in the progression of the disease and an extension of the lifespan. In the same line, later Cleveland's studies showed that microglial cells are key factors determining the onset and the early phase of disease progression (Boillée et al., 2006). From then on, later studies came out either corroborating or showing conflicting results. For instance, other groups revealed a neutral or even neuroprotective role of microglial cells. In this sense, Julien's Lab and colleagues showed that the ablation of

proliferating microglia does either not affect MN degeneration or has a protective role in SOD1^{G93A} mice (Gowing et al., 2008; Audet et al., 2012). In both studies however, the approaches applied to remove microglial cells also modified the contribution of peripheral leukocytes, glial cells or induced an unexpected inflammatory reaction and/or neurotoxicity. Due to these conflicting results, in the present thesis we firstly focused on the study of the contribution of proliferating microglial cells to ALS pathophysiology. To unravel its role, in chapter one, we evaluated the contribution of CSF1R. CSF1R is a cell surface receptor expressed in mononuclear phagocytes such as microglial cells and circulating monocytes. Through the interaction with its two main ligands, CSF1 and IL-34, it mediates the differentiation, survival and proliferation of these immune cells populations (Gomez-Nicola and Perry, 2015). Several reports have suggested that CSF1R activation triggers the proliferation of microglial cells in diverse neurodegenerative diseases (Conway et al., 2005; Kondo et al., 2007; Gomez-Nicola et al., 2013). However, whether this molecular pathway is involved in ALS pathology has not been addressed yet.

Here, we demonstrated that proliferative microglial cells exert a detrimental role in ALS through the activation of CSF1R. In agreement with previous studies (Hall et al., 1998; Nikodemova et al., 2014), we showed a marked microgliosis from symptomatic to late stages of the disease in SOD1^{G93A} mice. However, this inflammatory reaction was partially triggered by CSF1-CSF1R signaling. Here, we found that blockade of CSF1R reduced microglial proliferation only at late stages of disease, when CSF1 protein levels were up-regulated. However, CSF1-CSF1R pathway did not trigger microglia proliferation at early stages, since CSF1 was not up-regulated at this early phase and because CSF1R blockade did not have any effect on microgliosis. In ALS, microglial mitosis inducers at pre-symptomatic phases are currently unknown, but they might include the combination of mitotic factors and/or other cytokines. In this line, recent studies suggest that the activation of IL-1R signaling can induce microglia proliferation (Bruttger et al., 2015). This study, therefore, may explain why the inhibition of IL-1Rα or the down-regulation of IL-1β diminishes microgliosis in SOD1^{G93A} mice. In the present thesis, we found that CSF1R inhibition significantly slowed disease progression and extended survival of SOD1^{G93A} mice. Similar to our results, the lack of IL-1β significantly increased the lifespan of SOD1^{G93A} mice by reducing microglial proliferation (Meissner et al., 2010). Nonetheless, microglia CSF1R blockade could not fully explain why CMAPs were preserved at pre-symptomatic stages of the disease, since at these stages of the disease CSF1 was not up-regulated in the spinal cord, and thus, CSF1-derived microglial mitotic program could not be responsible for this early preservation. To identify which cells could have a role in the

pathology of ALS at early stages, we focused in the other immune population that constitutively expresses CSF1R, monocytes. In agreement with previous studies (Chiu et al., 2009; Graber et al., 2010; Dibaj et al., 2011), here we found that peripheral monocytes did infiltrate into nerve bundles. Moreover, after CSF1R inhibition, we found a reduction in macrophages counts in the nerve of SOD1^{G93A} mice. Interestingly, we demonstrated that the lower amount of macrophages was secondary to a blood monocytopenia that decreased the influx of monocytes to nerve bundles. Thus, CSF1R inhibition reduced circulating monocytes counts which resulted in reduced infiltration into the PNS. However, whether this invasion takes a direct role in ALS pathology has not been elucidated yet. Here, we found that myeloid infiltration into the PNS exacerbates ALS pathology and is responsible for the denervation found at pre-symptomatic stages. Similar to our results, a previous study showed that systemic administration of CSF1 in SOD1^{G37R} mice aggravated ALS pathology (Gowing et al., 2009). They also suggested that these detrimental actions were mediated by microglial proliferation and an increased denervation of hindlimb muscles. Based in our results, this increased denervation may be caused by a peripheral activation of CSF1R expressed in circulating monocytes, since this cytokine is unlikely to reach the CNS. Interestingly, they also found a reduction of spinal MNs and a shortened lifespan when administering CSF1. Therefore, our data from chapter 1 along with previous reports in the literature suggests that, aside from the reduction of the mitogenic program of monocytes and microglial cells, the selective blockade of both central and peripheral CSF1R signaling significantly preserves spinal MNs and extends lifespan of SOD1^{G93A} mice. This suggests for the first time the relevance of inflammation of peripheral nerves as key contributor of ALS disease.

Unfortunately, besides CSF1R activity, there are other crucial inflammatory pathways that exert deleterious roles in ALS pathophysiology (Philips and Robberecht, 2011). For instance, astrocytes are one of the key players in neuroinflammation. In ALS, it has been shown that astrocytes become toxic and can trigger MN death through the activation of necroptotic pathways (Re et al., 2014). Similar to microglial cells, the removal of mSOD1 from astrocytes slows diseases progression, suggesting a detrimental factor in late stages of the disease. Other neuroinflammatory effectors also include the activation of T-helper cells (Beers et al., 2008) or peripheral macrophages (Butovsky et al., 2012). Due to the complex scenario and the involvement of multiple cell types, therapies aimed at targeting more than one cell type at once may result beneficial in ALS. In this sense, the stimulation of wide range anti-inflammatory pathways may lead to the development of new therapies for patients with ALS.

In the last years, IL-37 was postulated as a modulator of both innate and adaptive immune response (Nold et al., 2010). This novel cytokine belongs to the IL-1 family and it has been widely studied due to its potent anti-inflammatory effects (Garlanda et al., 2013). In humans, IL-37 locus is located within the chromosome 2, surrounded up- and down-stream by other IL-1 family members genes. In mice, however, IL-37 rodent ortholog has not been identified yet. Therefore, the development of a transgenic mouse that expressed the human form of IL-37 was crucial to study its role. Once generated, Dinarello's lab revealed the potent anti-inflammatory effect of IL-37 upon LPS administration (Nold et al., 2010). Indeed, IL-37 expression reduced the pro-inflammatory cytokines IL-6, IL-1β, IL-α, MIP-1α or MIP-2, as well as, limited the pathological effects of the endotoxic shock. These immunomodulatory actions were further described in several models of immune-related diseases including hepatic failure, ischemia or diabetes (McNamee et al., 2011; Nold-Petry et al., 2015a; Ye et al., 2015). In line with these results, previous studies from our laboratory also showed that expression of IL-37 after spinal cord injury (SCI) limited functional deficits by reducing the secondary immune response caused by the traumatic injury (Coll-Miró et al., 2016). However, whether IL-37 may induce the same anti-inflammatory actions in ALS is yet to be defined.

In the present thesis, we studied the role of IL-37 in a mouse model of ALS and provide some clinical insights of its importance in ALS patients. To study IL-37 effects in a murine ALS experimental model, we crossed mice that expressed the human form of IL-37, the hIL-37tg mouse, with the $SOD1^{G93A}$ mouse, generating the double transgenic animal, $SOD1^{G93A}$-hIL-37tg mouse. First, we wanted to assess whether IL-37 was being expressed in $SOD1^{G93A}$-hIL-37tg mice. In hIL-37tg animals, IL-37 expression is artificially regulated by a CMV promoter that should increase its levels broadly (Nold et al., 2010). However, in physiological conditions, hIL-37tg animals show barely detectable transcripts of IL-37 in the spinal cord (Coll-Miró et al., 2016). This low/absent IL-37 mRNA levels are because IL-37 transcripts require an inflammatory challenge to be stabilized. Similar to other IL-1 family members, within exon 5, IL-37 sequence presents an instability region that blocks the binding between mRNA stability proteins and IL-37 mRNA. However, under inflammatory stimuli, the expression of mechanisms found in the 3'-UTR of the IL-37 locus, regulates its mRNA stability and concomitant protein levels (Bufler et al., 2004). Here we also show that IL-37 transcripts are very low in the post-mortem spinal cord of human samples not affected by neurological conditions. Our lab has previously revealed that IL-37 transcripts up-regulate after spinal cord injury due to the massive immune response generated after the trauma (Coll-Miró et al., 2016). However, whether IL-37

transcriptional levels increases/decreases in the areas with an ongoing degeneration was not known. Thus, by qPCR, we evaluated IL-37 mRNA levels in the CNS, PNS and hindlimb muscles of SOD1^{G93A}-hIL-37tg mice. Here, we found that IL-37 mRNA increased in the spinal cord and sciatic nerve of SOD1^{G93A}-hIL-37tg, as well as, in muscle samples at late stages of the disease. In SOD1^{G93A}-hIL-37tg, IL-37 levels showed a progressive up-regulation as disease progressed, showing maximum peaks at the end-stage. This gradual increase coincides with the ongoing MN degeneration and the infiltration/activation of immune cells. As discussed in the aforementioned chapter one, peripheral monocytes progressively invade PNS at pre-symptomatic stages of the disease (Chiu et al., 2009; Martínez-Muriana et al., 2016). This early peripheral influx is likely to be responsible of the IL-37 increase found in the sciatic nerve. In the same chapter, we also found a marked microgliosis from symptomatic to late stages of the disease. Here we found that, at central level, IL-37 transcripts were increased but at low levels at pre-symptomatic stages of the disease, when glial activation is barely found. However as disease progressed and MNs soma starts to degenerate, IL-37 mRNA stabilize and up-regulate correlating with this progressive gliosis.

Since IL-37 increased at pre-symptomatic stages of the disease at any affected area, we then wanted to determine whether its expression ameliorated ALS pathology. In the present thesis we therefore studied how IL-37 expression affected motor outcomes, progression and survival of SOD1^{G93A} mice. Here, we found that SOD1^{G93A}-hIL-37tg mice preserved upper and lower MN function, delayed the onset of the disease as well as increased mice survival. At histological level, IL-37 expression significantly preserved 30% more MNs than SOD1^{G93A} mice at late stage of the disease. These neuroprotective effects were in line with previous studies that found that overexpression of anti-inflammatory cytokines such as IL-10 or IL-4 slowed disease progression in SOD1^{G93A} mice (Gravel et al., 2016; Rossi et al., 2018). In these studies, these protective actions were triggered by a reduction in microgliosis in the spinal cord. Here, we found that SOD1^{G93A}-hIL-37tg animals showed a striking reduction of microglia, but also, astroglial reactivity. This finding is relevant due to the key role that both cell populations play over onset and disease progression in ALS. As previous studies from Cleveland's lab pointed out, microglial cells are determinants for both onset and progression whereas astrocytes are key effectors regulating disease progression (Boillée et al., 2006; Yamanaka et al., 2008). IL-37 expression delayed the onset of the disease by more than 3 weeks compared to SOD1^{G93A}WT animals. Besides, SOD1^{G93A}-hIL-37tg animals behaved better in locomotion tasks at late stages of the disease as well as had an increased survival, showing a slower

disease progression. The preservation of these motor outcomes could be therefore a consequence of glial modulation.

To gain further information of how IL-37 triggered these neuroprotective actions, we performed a Luminex analysis of the spinal cord and sciatic nerve of SOD1^{G93A}WT and SOD1^{G93A}-hIL-37tg mice at the onset of the disease. Interestingly, the study of several pro-inflammatory and anti-inflammatory cytokines revealed that IL-37 modulated different pathways depending on it was acting over the central or peripheral nervous system. At central level, IL-37 did not modify the expression of pro-inflammatory cytokines but increased 5-fold IL-10 levels. Previous studies showed that IL-10 overexpression slowed disease progression by specifically targeting microglial population (Gravel et al., 2016). Indeed, the administration of IL-10R antagonist had a deleterious effect in SOD1^{G93A} mice, suggesting a neuroprotective role of this cytokine. In line with our results, previous reports revealed an increase of IL-10 expression upon IL-37 expression. However, later experiments revealed that IL-37 protective effects were IL-10 independent (McNamee et al., 2011; Luo et al., 2014; Moretti et al., 2014). Here, despite these anterior studies demonstrated that IL-10 did not mediate IL-37 immunomodulatory actions, in this thesis we cannot rule out this possibility. To elucidate whether IL-10 is having a role in ALS pathology, further studies either crossing SOD1^{G93A}-hIL-37tg mice with IL-10R null mice or pharmacologically inhibiting of IL-10R are needed to fully unravel its role. Regarding Luminex analysis in the sciatic nerve, we found that IL-37 reduced the expression of IL-4, IL-17 and CSF1. The reduction of these cytokines was in accordance with reports that found a markedly decrease of these immune mediators upon IL-37 up-regulation (Nold-Petry et al., 2015b; Ye et al., 2015; Coll-Miró et al., 2016). Based on chapter one results, the reduction of CSF1 in the sciatic nerve could represent a decrease in the number of macrophages within the PNS of SOD1^{G93A} mice, since CSF1-CSF1R signaling plays an important role. In this sense, we therefore quantified the amount of these myeloid cells in the sciatic nerve at 16 weeks of age. Surprisingly, we found that IL-37 expression did not reduce the influx of monocytes to the nerve bundles at this time point. This result could suggest that CSF1 reduction after IL-37 expression, does not mediate the infiltration of monocytes. However, we cannot fully confirm this conclusion due to timing misleading. Luminex analysis was performed at 12 weeks of age, coinciding with the onset of the disease of SOD1^{G93A} mice. Per contra, macrophage quantification was performed at 16 weeks of age, the beginning of the end-stage. This timing difference may explain why we did not find a decrease in the number of macrophages within the sciatic nerve. To entirely elucidate whether IL-37 reduces the influx of monocytes by decreasing CSF1 expression in

the PNS, future studies are needed at 12 weeks of age, the same time-point as Luminex analysis.

Whether IL-37 immunomodulatory actions are mediated by the up or down-regulation of several cytokines is yet to be defined, in the present thesis we have demonstrated that IL-37 effects in ALS are mediated through the activation of its extracellular pathway, the IL-18Rα and IL-1R8 complex. Novel studies led by Dinarello's Lab revealed that IL-37 can have a dual action: (i) activation of an extracellular pathway through the interaction with the complex IL-18Rα and IL-1R8 or, (ii) translocation to the nucleus by binding to SMAD 3 (Sharma et al., 2008; Nold-Petry et al., 2015b). When binding to IL-18Rα-IL-1R8 complex, IL-37 mediates the majority of its anti-inflammatory actions. Nold-Petry and colleagues demonstrated that when IL-37 extracellular pathway is blockaded, IL-37 failed to reduce several pro-inflammatory cytokines such as IL-1α, IL-1β, IFNγ or MIP-2. Besides, the protection against endotoxic shock after LPS administration was lost *in vivo* (Nold-Petry et al., 2015b). However, IL-37 nuclear has also been demonstrated *in vitro*. The immature form IL-37 contains a cleavage site that can be processed by caspase 1. Upon processing, cleaved-IL-37 can bind to phospho-SMAD3 and translocate to the nucleus where regulates the transcription of diverse immune pathways. Among them, Sharma and colleagues showed that down-regulation of IL-6 after LPS stimulation was triggered by IL-37 nuclear actions (Sharma et al., 2008). Nevertheless, no one has ever demonstrated IL-37 nuclear function *in vivo*. To further elucidate whether IL-37 neuroprotective effects in ALS were due to its extracellular o intracellular pathways, in the present thesis we developed a triple transgenic animal, the SOD1^{G93A}-hIL-37tg-IL-1R8 KO mice. In chapter two, to block IL-37 extracellular pathway, we transgenically removed one of IL-37 extracellular effectors, the co-receptor IL-1R8. We found that preservation of motor outcomes mediated by IL-37 expression were lost when erasing IL-37 extracellular pathway. The only feature that was not affected by the lack of IL-1R8 in SOD1^{G93A}-hIL-37tg mice was the preservation of MEPs, and to a lesser extent, the clinical onset of disease. This finding could suggest that IL-37 nuclear pathway may be involved in upper MN sparing. However, further studies are needed to histologically determine whether there is neuronal survival in cortical regions. Nevertheless, this supraspinal preservation could explain why SOD1^{G93A}-hIL-37tg-IL-1R8 KO mice had a slight delay in disease onset. Upper MNs are responsible of voluntary motor control and balance maintenance. To determine the onset of the disease, we took advantage of rotarod test. This test evaluates the strength, coordination and balance of SOD1^{G93A} mice (Miana-Mena et al., 2005). Thus it assesses the functionality of both upper and lower MN function. In chapter two, we found

that SOD1^{G93A}-hIL-37tg-IL-1R8 KO mice slightly performed better than SOD1^{G93A} mice in the rotarod test which indicated a delay in the onset of the disease. If we compare the probability of onset of all experimental groups, we found 2 different behaviors: (i) SOD1^{G93A} and SOD1^{G93A}-IL-1R8 KO mice, which showed a probability of onset at around 13 weeks of age and (ii) SOD1^{G93A}-hIL-37tg mice and SOD1^{G93A}-hIL-37tg-IL-1R8 KO mice, which delayed the onset of the disease by 3 or 2 weeks of age, respectively. Despite further studies are needed to clarify these results, this suggest that IL-37 nuclear function may have a protective role in supraspinal regions, and consequently, in disease onset. However, this cortical protection is not enough to slower disease progression. Here, we demonstrated that SOD1^{G93A}-hIL-37tg-IL-1R8 KO animals did not increase their lifespan compared to either SOD1^{G93A} or SOD1^{G93A}-IL-1R8 mice. Besides, at histological level, we found that neuronal survival mediated by IL-37 expression was lost when IL-1R8 was removed. Similarly, the striking downregulation in glial reactivity found in SOD1^{G93A}-hIL-37tg mice, was completely lost in SOD1^{G93A}-hIL-37tg-IL-1R8 KO. As was previously discussed, glial reactivity has a key role determining onset and disease progression. Thereupon, this could explain why triple transgenic animals had the same disease progression as SOD1^{G93A} mice. Regarding SOD1^{G93A}-IL-1R8 null mice, we found that the lack of IL-1R8 in SOD1^{G93A} mice did not have any effect in the progression of the disease. Nevertheless, SOD1^{G93A}-IL-1R8 KO glial reactivity differed from SOD1^{G93A}. The lack of IL-1R8 did not modify astrocytes reactivity, but heavily decreased microglia activation. This was quite unexpected since previous reports showed that mice lacking IL-1R8 had an exacerbated immune response in some pathological conditions (Garlanda et al., 2009). IL-1R8, formerly known as TIR8 or "SIGIRR", is an orphan receptor of the IL-1 family that mitigates inflammation by blocking TLRs and IL-1R mediated pathways. Thus, the lack of IL-1R8 should aggravate immune response as was previously described in a mouse model of lupus (Lech et al., 2008). Giving these previous reports, we should have expected at least the same immunoreactivity as SOD1^{G93A} mice, but not less. However, we cannot fully confirm these results due to small amount of animals used to assess the immunostaining (n=3 in SOD1^{G93A}-IL-1R8 KO mice). Further studies are needed to elucidate whether this reduction in microgliosis was due to an immunostaining bias or to a real effect triggered by the lack of IL-1R8. Although future experiments are needed to elucidate the importance of IL-37 nuclear pathway in ALS, here we showed IL-37 extracellular pathway has potent beneficial actions in ALS pathology. This has important clinical relevance since it may suggest that the administration of recombinant IL-37 protein may open a new avenue for the treatment of ALS patients, especially, because our analysis in postmortem ALS spinal

cord revealed that IL-37 is not up-regulated in patients with sALS despite IL-1R8 is expressed in the spinal cord of healthy and sALS individual.

Finally, to gain further insights about the role of ALS in humans, we studied the contribution of a genetic IL-37 variant in the disease. Recently, it was described that the IL-37 SNP, rs3811046 (conversion of a T to G in the IL-37 gene sequence), is protective in Grave's disease and tuberculosis (Yan et al., 2015; Allam et al., 2016). Therefore, we wanted to assess whether the inheritance of this SNP can decrease or increase susceptibility in ALS patients. To unravel its role, we studied up to 158 patients and classified them according the presence of different SNPs and clinical scores. Unfortunately, we found that rs3811046 did not modify the onset or increased survival of ALS patients (data not shown). However, when we stratified the samples according to whether patients had fALS or sALS, we found that those fALS with the presence of rs3811046 had a delay of the onset of the disease by more than 13 years compared to those not carrying this SNP. Although these results are preliminary due to small number of samples from fALS patients, it may suggest that fALS patients carrying this SNP are partially protected from the disease.

Aside from anti-inflammatory inducers, there are other molecules that may have greater effectivity in attenuating the inflammatory response. Studies led by Dr. Charles Serhan revealed that inflammation is a well-orchestrated process where both initiation and resolution are time-limited and guided by immune mediators. When the organism is threatened by an injury or injection (trauma, ischemia, infectious organisms, cell death, etc.), the inflammatory response tries to restore the affected tissue or organ to its homeostatic state. To carry out its action, the immune system first triggers the induction of the innate and adaptive immunity to contain and heal the damage. Once the immunologic challenge has been solved, the infiltrating leukocytes leave the lesion site through the activation of pro-resolution programs (Serhan et al., 2010). This resolution phase is mediated by the production lipid mediators derived from omega-3 poly-unsaturated acids (PUFAs), such as eicosapentaenoic acid (EPA; C20:5n 3) and docosahexaenoic acid (DHA C22:6n 3), and from omega-6 fatty acid, such as arachidonic acid (AA C20:4n 6). Through the sequential lipoxygenation of AA, DHA and EPA by lipoxygenases (LOX) and cyclooxygenases (COX), these biolipid mediators are further derived in diverse SPMs superfamilies including lipoxins (LXA), resolvins (RvD and RvE), neuroprotectins (NP) and maresins (MaR1 and MaR2). By acting over G protein-coupled receptors, SPMs actively switch off the inflammatory response by activating both anti-inflammatory and pro-resolving programs (Schwab et al., 2007; Buckley et al., 2014). New

currents of knowledge have highlighted the importance of SPMs in regulating the inflammatory response in several disorders as asthma, colitis or atherosclerosis, in which there is a defective production of SPMs (Serhan, 2014; Serhan et al., 2014) In this line, the exogenous administration of SPMs mediates protection in inflammatory, cardiovascular or CNS injuries (Rose and Connolly, 1999; Bazan, 2007; von Schacky, 2007; Francos-Quijorna et al., 2017). Regarding neurological disorders, recent evidences have elucidated that SPMs production is deficient in CSF samples of multiple sclerosis (MS) and Alzheimer's disease (AD) (Prüss et al., 2013; Wang et al., 2015). However, little is known about their role in ALS. To shed light to this novel prospective, Ilieva and colleagues studied DHA production into the CNS of ALS patients. In this line, they showed that ALS patients had an impaired DHA production into the spinal cord but not the frontal cortex (Ilieva et al., 2007). Later on, the same authors described that the altered production of the omega-3 PUFA-derived lipid was due to a defective DHA synthesis because of enzyme malfunction (Cacabelos et al., 2016). The inefficient synthesis of DHA could further impair the anabolism of DHA-derived SPMs: resolvins, neuroprotectins and maresins, whose decreased levels in turn, may exacerbate the inflammatory response in ALS. It is crucial to highlight that the enzymes involved in the catabolism of SPMs (COX and LOX) are also involved in the production of pro-inflammatory eicosanoids including prostaglandins, leukotrienes and thromboxanes. This is important, since the majority of therapies aimed to decrease the inflammatory response directly target LOX or COX enzymes, and thus, they may also impair the production of these bioactive lipids. Nevertheless, whether SPMs superfamilies are involved in the clearance and resolution of inflammation in ALS is not known yet. To our knowledge, the only study that partially suggested a potential action of SPMs' role in ALS was performed by Liu and colleagues. Interestingly, they showed that the *in vitro* administration of RvD1 attenuates the release of pro-inflammatory cytokines after exposure to mSOD1 in PBMCs derived from ALS patients (Liu et al., 2012). However, whether the exogenous administration of SPMs mediates the same immunodulatory actions *in vivo*, and if so, it produces a beneficial effect in $SOD1^{G93A}$ mice has not been addressed yet. Therefore, in chapter three, we evaluated the therapeutic role of the DHA-derived biolipid, Maresin 1-R (MaR1). MaR1 exerts potent actions in regulating inflammation resolution, but also in preventing nociception after inflammatory- and chemotherapy-induced neuropathic pain and stimulating tissue regeneration in *Planaria* (Serhan et al., 2012; Dalli et al., 2016). Previous results from our lab have described that after spinal cord injury, there is an inappropriate production of SPMs which leads to an uncontrolled inflammatory response that produces functional detrimental outcomes. However, when MaR1 levels were increased, there was an enhanced resolution of the

inflammation that led to a significant protection against functional deficits and tissue damage (Francos-Quijorna et al., 2017). In the present thesis, we revealed that the delayed administration of MaR1 ameliorated ALS pathophysiology by slowering disease progression and increasing mice survival. Since MaR1 administration at late stages led to an extension of the lifespan of SOD1^{G93A} mice, we therefore wanted to test its therapeutic effects at pre-symptomatic stage. In order to translate it into a preclinical assay, we compared MaR1 results to Riluzole, the only drug approved by the FDA and EMA. In chapter three, we found that increasing levels of MaR1 at early stages of the disease significantly preserved upper and lower MN function and delayed the onset of the disease of SOD1^{G93A} mice. Interestingly, we found that Riluzole had a slight preservation of motor outcomes but to lesser extent to those achieved by MaR1. Currently, we do not have survival rates because we have not finished the follow up yet, so we cannot determine the effects of early treatment with MaR1 in the lifespan of SOD1^{G93A} mice. Nevertheless, how MaR1 triggers its beneficial effects in ALS is currently unknown. Due to time constrains, in the present thesis we have not elucidated the molecular mechanism underlying the beneficial effects behind MaR1 treatment, but further studies will be held in the future. However, based in previous results from our lab, MaR1 therapeutic outcomes might include the clearance of pro-inflammatory cytokines and leukocytes, and the switch of macrophages and microglial cells towards to an anti-inflammatory state (Francos-Quijorna et al., 2017).

Overall, the results presented in this thesis provide clear evidence of the deleterious role of peripheral and central immune response in SOD1^{G93A} mice. Here, we showed progressive infiltration of monocytes to nerve bundles from early to late stages of the disease. Besides, we found striking gliosis from symptomatic to the end-stage of the disease, being more prominent at late stages, when MNs loss is evident. Importantly, we demonstrated that microglial proliferation at late stages of the disease is triggered by CSF1-CSF1R signaling. Besides, we revealed a key role of CSF1R activation in monocytes that led to peripheral axonal degeneration at pre-symptomatic stages. The modulation of this inflammatory response either by blocking mitotic pathways such as CSF1 signaling, increasing the expression of anti-inflammatory cytokines as IL-37 or promoting inflammatory resolution by administering MaR1 ameliorates ALS pathophysiology and increases SOD1^{G93A} mice. Since inflammation detrimentally contributes to the course of ALS, administration of recombinant IL-37 or MaR1 may lead to new avenues for the treatment of ALS.

CONCLUSIONS

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Chapter I:

- CSF1R triggers microglial proliferation at late stages of the disease of SOD1^{G93A} mice
- CSF1R induces monocytes proliferation at early stages of the disease
- Selective blockade of CSF1R activity reduces influx of monocytes into the PNS and microglia proliferation in SOD1^{G93A} mice
- CSF1R inhibition slows ALS functional deficits and increases SOD1^{G93A} lifespan

Chapter II:

- IL-37 is not up-regulated in ALS patient but its co-receptor is expressed in the spinal cord of both ALS and healthy subjects
- IL-37 transcript levels are detected in the CNS, PNS and hindlimb muscles of SOD1^{G93A}-hIL-37tg mice
- Transgenic expression of IL-37 ameliorates ALS pathophysiology by modulating the levels of several cytokines at central and peripheral level
- IL-37 mediates neuroprotection by acting over the extracellular complex IL-18Rα-IL-1R8.
- rs3811046 SNP is associated with a delay disease onset in 13 years in fALS

Chapter III:

- MaR1 delayed treatment slows disease progression and increases SOD1^{G93A} lifespan
- MaR1 treatment at presymptomatic phases delays onset and disease progression of SOD1^{G93A}

ABBREVIATIONS

ABBREVIATIONS

12/15-LOX	12/15-lipoxygenase
AA	Arachidonic acid
AD	Alzheimer Disease
ALS	Amyotrophic Lateral Sclerosis
ALX/FPR2	Lipoxin A4 receptor
AMPA	A-Amino 3-hydroxy 5-methyl 4-isoxazolepropionic acid
APC	Antigen-presenting cells
AraC	Arabinosylcytosine
BBB	Blood-brain-barrier
BID	Bowel inflammatory disease
BMCs	Bone marrow cells
BMDM	Bone marrow derived macrophages
C9orf72	Chromosome 9 open reading frame 72
CCL2	Chemokine ligand 2
CCL3	Chemokine ligand 3
CCR2	Chemokine receptor type 2
CMAP	Compound muscle action potential
CMV	Cytomegalovirus
CNS	Central nervous system
COX-2	Cyclooxygenase-2
CSF	Cerebrospinal fluid
CSF1/M-CSF1	Colony-stimulating factor 1
CSF1R	Colony-stimulating factor receptor 1
CXCL3	Chemokine (C-X-C motif) ligand 3
DAMPs	Damage-associated molecular patterns

DC	Dendritic cells
DHA	Docosahexaenoic acid
DSS	Dextran sulfate sodium
EAAT2	Excitatory amino acid transporter 2
EPA	Eicosapentaenoic acid
FACS	Fluorescent activated cell sorting
fALS	Familiar Amyotrophic Lateral Sclerosis
FDA	Food and drug administration
FPR	Formyl peptide receptor
FUS	RNA-binding protein FUS/TLS
GGT	γ-glutamyl transferase
GluR	Glutamate Receptor
GM	Gastrocnemius medialis
GM-CSF	Granulocyte-macrophage colony stimulating factor
GPCR	G-protein coupled receptor
GSTM4	Glutathione S-transferase Mu
H2O2	Hydrogen peroxide
hIL-37tg	Human IL-37tg mice
HPRT1	Hypoxanthine-guanine phosphoribosyltransferase
HSP	Heat-shock proteins
IFN-γ	Interferon gamma
Ig	Immunoglobulins
IGF-1	Insulin growth factor
IL-18	IL-18 binding protein
IL-18Rα	IL-18 receptor alpha
IL-1F7	IL-1 family member 7
IL-1Ra	Interleukin 1 receptor antagonist

IL-1ß	Interleukin-1-beta
IL-34	Interleukin 34
IL-37	Interleukin 37
IL-6	Interleukin-6
iNOS	Inducible nitric oxide synthetase
IP	Intraperitoneal injection
LMN	Lower motor neuron
LPS	Lipopolysaccharide
LTC4S	Leukotriene C4 synthase enzymes
LX	Lipoxins
LXA4	Lipoxin A4
LXB4	Lipoxin B4
MaR1	Maresin 1
MaR2	Maresin 2
MCP-1/CCL2	Monocyte chemoattractant protein 1
M-CSF	Macrophage colony stimulating factor
MCTR	Maresin conjugate in tissue regeneration
MEP	Motor evoked potential
MHC	Major histocompatibility complex
MIP-1 α	Macrophage inflammatory protein α
MLKL	Mixed lineage kinase domain-like protein
MND	Motoneuron diseases
MNs	Motoneurons
NF-κB	Nuclear factor kappa B pathway
NIK1	NIMA related kinase 1
NK	Natural killer cells
NKT	NK T cell

NMDA	N-methyl-d-aspartic acid
NPD1	Neuroprotectin
O_2^-	Superoxide
OH^-	Hydroxide ion
OPTN	Optineurin
PAMPs	Pathogen-associated molecular patterns
PB	Phosphate buffer
PCTR	Protectin conjugate in tissue regeneration
PD	Protectins
PD1	Protectin D1
PFA	Paraformaldehyde
PGE2	Prostaglandin E2
PI	Plantar interossei
PMBCs	Peripheral blood mononuclear cells
PMN	Polymorphonuclear leukocytes
PNS	Peripheral nervous system
PPARγ	Peroxisome proliferator activated receptor gamma
PRR	Patter recognition receptors
PUFA	Omega-3 poly-unsaturated acids
RCTR	Resolvin conjugate in tissue regeneration
RIP1	Receptor-interacting protein 1
RMS	Root mean square
ROS	Reactive oxygen spices
RT	Room temperature
RvD	D series of resolvins
RvD1	Resolvin D1
RvE	E series of resolvins

sALS	Sporadic Amyotrophic Lateral Sclerosis
SCI	Spinal cord injury
SNPs	Single nucleotide polymorphism
SOD1	Superoxide dismutase gene
SPM	Specialized proresolving lipid mediators
TA	Tibialis anterior
TARDBP	TAR DNA Binding Protein
TBK1	Tank-binding kinase1
TCR	T-cell receptor
TCR-ß	T cell receptor ß chain
TGF-ß	Transforming growth factor beta
TIR	Toll-interleukin 1 receptor
TNF-a	Tumor-necrosis factor alpha
Treg	Regulatory T lymphocytes
TRPV1	Transient receptor potential cation channel subfamily V member 1
UBQLN2	Ubiquilin-2
UMN	Upper motor neuron
UTR	Untranslated region
WT	Wildtype

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