Therapeutic Strategies Under Development Targeting Inflammatory Mechanisms in Amyotrophic Lateral Sclerosis

Sebastiano Giuseppe Crisafulli [1] & Simona Brajkovic [1] & Maria Sara Cipolat Mis [1] & Valeria Parente[1] & Stefania Corti[1]

Abstract Amyotrophic lateral sclerosis (ALS) is a neurological disease characterized by the progressive loss of cortical, bulbar, and spinal motor neurons (MNs). The cardinal manifestation of ALS is a progressive paralysis which leads to death within a time span of 3 to 5 years after disease onset. Despite similar final output of neuronal death, the underlying pathogenic causes are various and no common cause of neuronal damage has been identified to date. Inflammation-mediated neuronal injury is increasingly recognized as a major factor that promotes disease progression and amplifies the MN death-inducing processes. The neuroimmune activation is not only a physiological reaction to cell-autonomous death but is an active component of nonautonomous cell death. Such injury-perpetuating phenomenon is now proved to be a common mechanism in many human disorders characterized by progressive neurodegeneration. Therefore, it represents an interesting therapeutic target. To date, no single cell population has been proved to play a major role. The existing evidence points to a complex cross talk between resident immune cells and nonresident cells, like monocytes and T lymphocytes, and to a dysregulation in cytokine profile and in phenotype commitment. After a summary of the most important mechanisms involved in the inflammatory reaction in ALS, this review will focus on novel therapeutic tools that rely on tackling inflammation to improve motor function and survival. Herein, completed, ongoing, or planned clinical trials, which aim to modify the rapidly fatal course of this disease, are discussed. Anti-inflammatory compounds that are currently undergoing preclinical study and novel suitable molecular targets are also mentioned.

Abbreviations

ALS	Amyotrophic lateral sclerosis
A-SMase	Acid sphingomyelinase ABC ATP-binding cassette
ALSFRS-R	ALS function rating scale revised
AMPA	α-Amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
AP1	Activator protein 1
APP	Amyloid precursor protein
Arg1	Arginase 1
ATP	Adenosine triphosphate
AUC	Area under curve
BDNF	Brain-derived neurotrophic factor
KIT	Receptor tyrosine-kinase
C(max)	Maximum serum concentration
C/EBP	CCAAT-enhancer-binding protein
C9ORF72	Chromosome 9 open reading frame 72
CAFS	Combined assessment of function and survival
CB2	Cannabinoid receptor 2
CCAAT	Cytidine-cytidine-adenosine-adenosine-thymidine
CD	Cluster of differentiation
Chi3l3	Chitinase-3-like-3
CNS	Central nervous system
COX	Cyclooxygenase
CRP	C-reactive protein

⁎ Stefania Corti
    stefania.corti@unimi.it

[1] Dino Ferrari Centre, Neuroscience Section, Department of Pathophysiology and Transplantation (DEPT), Neurology Unit, IRCCS Foundation Ca' Granda Ospedale Maggiore Policlinico, University of Milan, Via Francesco Sforza 35, 20122 Milan, Italy

CSF1	Colony-stimulating factor 1	MN	Motor neuron
CSF1R	Colony-stimulating factor 1 receptor	MnSOD	Manganese-dependent superoxide dismutase
CysGly	Cysteinylglycine	MRI	Magnetic resonance imaging
EAAT/ GLAST	Excitatory amino acid transporter/glutamate aspartate transporter	mRNA	Messenger RNA
		MS	Multiple sclerosis
EAAT/GLT1	Excitatory amino acid transporter/glutamate transporter 1	mSOD1	Mutant SOD1
		MyD88	Myeloid differentiation primary response gene 88
EDSS	Expanded Disability Status Scale	N-SMase	Neutral sphingomyelinase
EMA	European Medical Agency	NCAM	Neural cell adhesion molecule
ER	Endoplasmatic reticulum	Nec-1	Necrostatin-1
ERK	Extracellular signal-regulated kinases	NF-kB	Nuclear factor kappa-light chain-enhancer of activated B cells
FACS	Fluorescence-activated cell sorting		
FADD	Fas-associated protein with death domain	NFAT	Nuclear factor of activated T cells
fALS	Familial amyotrophic lateral sclerosis	NIV	Noninvasive ventilation
FAS	Fas cell surface death receptor	NMDA	N-methyl-D-aspartate
FasL	Fas ligand	NO	Nitric oxide
FDA	Food and Drug Administration	NOD	Nucleotide oligomerization domain
FEV1	Forced expiratory volume in the 1st second	NTG	Normal tension glaucoma
FGF	Fibroblast growth factor	OPTN	Optineurin
FIZZ1	Found in inflammatory zone 1	PDGFR	Platelet-derived growth factor receptor
FoxP3	Forkhead box P3	PET	Positron emission tomography
FTD	Frontotemporal dementia	PG	Prostaglandin
FUS	Fused in sarcoma	PI3K	Phosphatidylinositol 3-kinase
FVC	Forced vital capacity	PKA	Protein kinase A
GDNF	Glial cell-derived neurotrophic factor	PML	Progressive multifocal encephalitis
GIST	Gastrointestinal stromal tumor	PPAR	Peroxisome proliferator-activated receptor
HHD	Hand-held dynamometry	RAGE	Receptors for advanced glycation end products
HLA	Human leukocyte antigens		
HMGB1	High mobility group box 1	RIG	Retinoic acid-inducible gene
HSE	Herpes simplex encephalitis	RIP	Receptor-interacting protein
HSP	Heat shock protein	RIPK	Receptor-interacting serine/threonine kinase
IFN	Interferon	ROS	Reactive oxygen species
IGF	Insulin-like growth factor	RUNX	Runt-related transcription factor
IkB	Inhibitor of kB	S1P	Sphingosine-1-phosphate
IL	Interleukin	sALS	Sporadic amyotrophic lateral sclerosis
iNOS	Inducible nitric oxide synthase	SCID	Severe combined immunodeficiency
iPSC	Induced pluripotent stem cells	shRNA	Short hairpin RNA
IRAK	Interleukin-1 receptor-associated kinase	SOCS	Suppressor of cytokine signaling
IRF	Interferon regulatory factor	SOD1	Superoxide dismutase 1
JNK	c-Jun N-terminal kinase	STAT	Signal transducer and activator of transcription
KIR3DL2	Killer cell immunoglobulin-like receptor 3DL2		
LMN	Lower motor neuron	SVC	Slow vital capacity
LPS	Lipopolysaccharide	TACE	TNF-alpha converting enzyme
LTP	Long-term potentiation	TANK	TRAF family member-associated NF-kB activator
Mal	MyD88-adapter-like		
MAPK	Mitogen-activated protein kinase	TARDBP	Transactive response DNA-binding protein
MCP	Monocyte chemoattractant protein	TBK1	TANK binding kinase 1
mGluR	Metabotropic glutamate receptor	TDP-43	Transactive response DNA-binding protein 43 kDa
MHC	Major histocompatibility complex		
MIF	Migration inhibitory factor	TGF	Transforming growth factor
MIP	Maximum inspiratory pressure	Th	T helper
MLKL	Mixed lineage kinase domain-like protein	TIR	Toll/IL-1 receptor
MMT	Manual muscle testing		

TLR	Toll-like receptor
TNF	Tumor necrosis factor
TNFR	Tumor necrosis factor receptor
TRADD	TNFR1-associated death domain
TRAF2	TNF receptor-associated factor 2
TRAIL	TNF-related apoptosis-inducing ligand
Treg	Regulatory T cells
TSPO	Translocator protein 18 kDa
VEGF	Vascular endothelial growth factor
VZV	Varicella zoster virus
WT	Wild type
[11C]PBR28	[11C] peripheral benzodiazepine receptor 28

Introduction

Amyotrophic lateral sclerosis (ALS) is a degenerative neurologic disease with an adult-age onset. ALS is marked by the selective death of spinal, bulbar, and cortical motor neurons (MNs), ultimately causing progressive paralysis and precocious death usually occurring 3 to 5 years after the diagnosis [1].

In the majority of cases (~90%), no familial history of ALS is recognized, thus being classified as sporadic forms of ALS (sALS), while only 10% of ALS cases are familial (fALS). More than 10 different mutated genes have been associated with fALS [2] cases. The expansion in the hexanucleotide repeat $(GGGGCC)_n$ in the chromosome 9 open reading frame 72 (*C9ORF72*) gene (accounting for about 40% of fALS in Western countries) and mutations in the superoxide dismutase 1 (*SOD1*), fused in sarcoma

(*FUS*) and transactive response DNA binding protein 43 kDa (*TDP-43* or *TARDBP*) genes (identified in about 20, 1–5, and 1–5% of fALS patients, respectively) are recognized as the underlying cause of the majority of fALS cases.

Despite our current knowledge, the underlying pathogenic mechanism remains unclear, especially for sALS. Besides riluzole, which can extend the median survival of ALS patients only by 3 months, no other approved therapy exists for ALS. It is urgent to identify new potential therapeutic targets in order to develop treatments that can have a clinically meaningful impact.

The inflammatory reaction in the central nervous system (CNS) is emerging as a potential pathogenic and precipitating factor in a broad spectrum of degenerative diseases [3]. Neuroinflammation has been demonstrated in areas of neuronal loss in both animal and human tissues, even during the presymptomatic phase of the disease, but its role is far to be completely elucidated [4]. There is no definitive evidence whether inflammation is a major cause of cell death or if it represents a reaction to local damage signals derived from dying MNs, mutant proteins, or self-antigens. However, the emerging fact regarding neuroinflammation is its ambivalent action on ALS onset and course, being neuroprotective and repair driving during an early phase and becoming cytotoxic in an advanced phase (Fig. 1) [5]. Another interesting evidence of noncell-autonomous neuronal death is that neurons expressing mutant SOD1 (mSOD1) show no major pathological sign when supported by healthy macroglia and microglia, while MNs lacking SOD1 develop pathologic hallmarks of ALS when exposed to glial cells expressing mSOD1 [6].
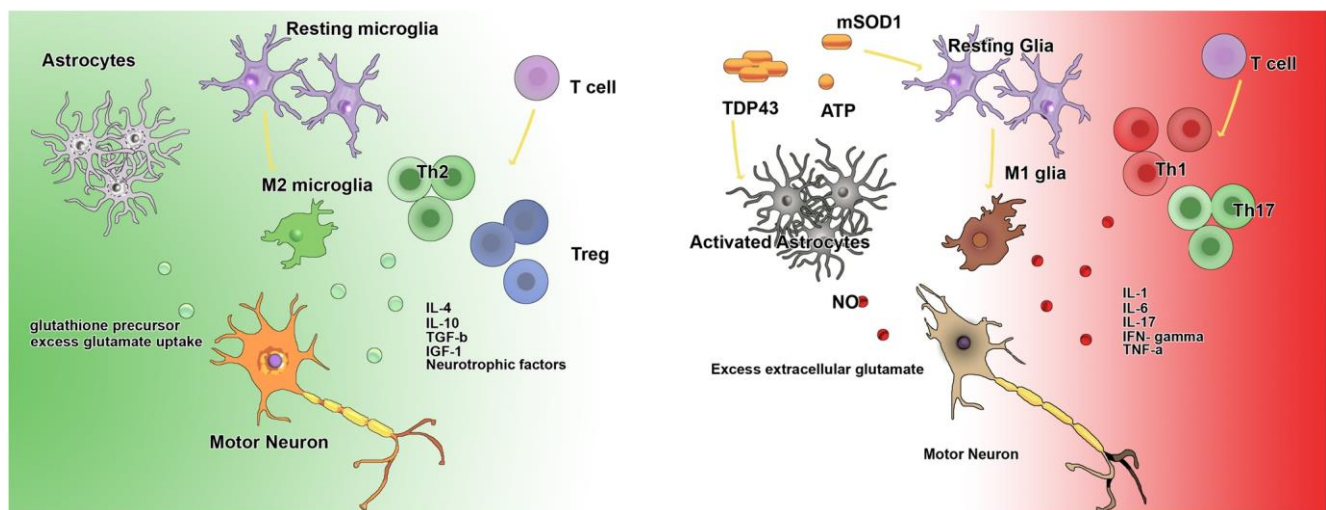

Fig. 1 The ambivalent role of inflammation on motor neuron survival. *Left*: Resting state astrocytes, M2 microglia, Th2, and Treg lymphocytes are predominantly present at an early stage of disease and support neuron function and viability by removing glutamate excess from the extracellular space and releasing anti-inflammatory and neurotrophic factors (IL-4, IL-10, TGF-β, IGF-1, BDNF, GDNF). *Right*: Danger signals and misfolded or mutated proteins (like mSOD1 and TDP-43) cause a switch of immune cells to a pro-inflammatory and neurotoxic state, which is predominant at a late stage of disease. Activated astrocytes, M1 microglia, and Th1 and Th17 lymphocytes secrete nitric oxide (NO) and pro-inflammatory factors (IL-1, IL-6, IFN-γ, TNF-α) that induce neurotoxicity and death of motor neurons. The impairment of glutamate uptake by activated astrocytes increases excitotoxicity and apoptosis of motor neurons

The major inflammatory pathways studied include microglia and astrocyte activation, recruitment of peripheral monocytes, the role of lymphocytes, and their ability to regulate the type and strength of immune response [7].

Given this evidence, several compounds targeting neuroinflammation such as celecoxib [8], ceftriaxone, thalidomide, and minocycline have been tested in preclinical stage and have been shown to exert positive effects on ALS transgenic mice [9–11] in the last years. However, none of them has been proved to be effective when transferred to human clinical trials. In a similar way, counteracting the negative effect of reactive oxygen species (ROS) exerted positive effects on ALS animal models but not on ALS patients [12]. Indeed, several well-known immunosuppressive drugs such as azathioprine, corticosteroids, cyclophosphamide, and calcineurin inhibitors like cyclosporine, which have a well-established efficacy on many immunological disorders and are routinely used, had not demonstrated any therapeutic efficacy in ALS [13, 14]. Also, some immunomodulatory drugs used for multiple sclerosis (MS), like glatiramer acetate, have been disappointing in a clinical setting [15].

The reasons of this failure are largely unknown. Such failure can be reasonably due to the fact that these drugs are generally administered when the disease has already progressed to an advanced phase. During this phase, inflammation could no longer represent an appropriate target or it is also likely that inflammatory pathways could contribute in a more complex way to ALS than to other neurological diseases with a clear inflammatory pathogenesis.

In this review, we propose a brief overview of the changes in innate and adaptive immunity and the evidence of their involvement in ALS. Then, we will discuss recent studies and ongoing or planned clinical trials that investigated the effects of anti-inflammatory and immune-modulating drugs on disease onset and progression. The different compounds are classified based on their main putative molecular/cellular targets.

Microglia and ALS

Several studies have demonstrated that glial cells, activated lymphocytes, and production of pro-inflammatory and neurotoxic agents have a role in the degeneration of MNs [16–18]. Increased activation of glial cells in ALS patients has been shown upon postmortem analysis [19] and with in vivo imaging techniques [20–22]. A good marker of enhanced glial activation is the translocator protein (TSPO), which is highly expressed in activated microglia [23]. Positron emission tomography (PET) imaging with the TSPO-binding radiotracer [11C] peripheral benzodiazepine receptor 28 ([11C]PBR28) has showed that the uptake of this compound was increased in the corticospinal tract and in the motor cortex of ALS patients compared to controls [24]. Also, this technique has

showed a strong link between the anatomical localization of active microglia and the clinical manifestation of the disease.

Microglial cells express different classes of receptors typically associated with the innate immunity. The most important and known of them are Toll-like receptors (TLRs), nucleotide oligomerization domain receptors (NOD), receptors for advanced glycation end products (RAGE), and retinoic acid-inducible gene (RIG) [25]. The activation of these receptors can be triggered by a variety of danger and damage signals, like the accumulation and aggregation of abnormal or misfolded proteins and the release of cellular components from damaged or dying cells. These factors are well-recognized pathogenic factors in different forms of ALS as previously described.

When activated, glial cells can acquire a M1 pro-inflammatory phenotype (classically activated microglia) or a M2 anti-inflammatory phenotype (alternatively activated microglia) [26]. M2-type glia has a positive effect on MN function and survival [27] and is mainly present at an early stage of disease, which is often a slow progression phase [28]. As the disease progresses, microglia strongly tend to switch to a M1 phenotype, enhancing the production of ROS and inflammation-inducing cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), and IL-6 and reducing the production of neurotrophic factors like insulin-like growth factor-1 (IGF-1) and IL-4 [29]. In fact, IGF-1 and, more recently, IGF2 [30] have been shown to prolong MN survival and ameliorate motor function in ALS mice [31]. Recently, RNAseq analysis has shown that TNF-α represents one of the most significant molecular alteration in the human ALS spinal cord [32].

TNF-α is first synthetized as a transmembrane protein and then cleaved by the TNF-α converting enzyme (TACE) to release its soluble form. Both the transmembrane and the soluble forms are active and able to bind to specific neuronal receptors, which are the effectors of the TNF-α-induced toxicity [33]. TNF-α has two different membrane receptors: tumor necrosis factor receptor 1 (TNFR1), which possesses a death domain in its cytoplasmatic portion, and TNFR2 [34]. The soluble form of TNF-α activates TNFR1, which in response recruits the adaptor protein TNFR1-associated death domain (TRADD). The latter in turn activates other adaptor proteins downstream, like receptor-interacting protein (RIP), TNF receptor-associated factor 2 (TRAF2), and Fas-associated protein with death domain (FADD), that ultimately lead to the activation of caspase 8 and caspase 10 and to apoptosis. Furthermore, TNFR1 is also responsible for the activation of other intracellular signal pathways related to cell survival and inflammatory response such as extracellular signal-regulated kinase (ERK), p38 mitogen-activated protein kinase (p38 MAPK), c-Jun N-terminal kinase (JNK), the acidic and neutral sphingomyelinase (A-SMase and N-SMase), and nuclear factor kappa-light chain-enhancer of activated B

cells (NF-kB) [35]. TNFR2 has been shown to induce apoptosis and activate NF-kB, ERK, p38 MAPK, and JNK through adaptor proteins TRAF2 and FADD [36]. The role of TNFR2 is still not completely understood, as other studies have showed that it could also have a neuroprotective effect [34]. Indeed, anti-TNF drugs are associated with neurological adverse events and negative effects on myelin [37]. The detrimental effect of TNF in neurodegeneration is amplified by its autocrine and paracrine action. The production of TNF-α is increased by TNF-α itself through binding to the TNFR1 [38] and to the group 2 metabotropic glutamate receptor (mGluR2) expressed by microglial cells. TNF signaling also increases the release of Fas ligand (FasL) [39] and glutamate from microglial cells [40]. Furthermore, glutamate toxicity is amplified by the impairment in the glutamate homeostasis system induced by TNF-α [41]. The downregulation of the excitatory amino acid transporter/glutamate aspartate transporter (EAAT1/GLAST) and excitatory amino acid transporter/glutamate transporter 1 (EAAT2/GLT1) on astrocytes, triggered by the activation of TNFR1 and secondarily of NF-kB [42], results in decreased glutamate uptake from the extracellular space [43, 44]. The reduced expression of these transporters has been demonstrated to occur both in rat models [45] and in ALS patients [46]. At the same time, it has been described that neurons exposed to TNF-α increase the expression of α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) [47] and N-methyl-D-aspartate (NMDA) receptors [48], which cause increased Ca^{+2} influx and excitotoxicity [49].

Microglial activation and polarization are highly dependent on the surrounding environment and signals from surrounding cells. Lipopolysaccharide (LPS) and interferon-γ (IFN-γ) promote a classical activation pathway (M1), whereas IL-4 and IL-13 induce the alternative activation pathway (M2) [50]. Notably, it has been speculated that IL-13 could also be able to modulate inflammation in the CNS by decreasing the survival of activated microglia [51]. The term M2 microglial also includes another microglial state called ʙacquired deactivation,ˆ which is induced by IL-10 and transforming growth factor-β (TGF-β) and the uptake of apoptotic bodies [52]. As well as alternatively activated ones, glial cells that undergo acquired deactivation appear to foster repair and neuron survival [53]. M2-related cytokines are not produced exclusively by Th2 lymphocytes but are also produced by microglia, astrocytes, and neurons themselves in the CNS and show a paracrine and autocrine activity [51, 54, 55].

The molecules Found in inflammatory zone 1 (FIZZ1), mannose receptor (CD206), chitinase-3-like-3 (Chi3l3), and arginase 1 (Arg1) are considered typical M2 markers. The most studied and interesting of them is Arg1. Along with inducible nitric oxide synthase (iNOS), Arg1 is a key enzyme in the metabolism of arginine in the nervous system, using this amino acid as its only substrate. iNOS uses arginine to produce nitric oxide (NO) and citrulline, while arginine metabolized by Arg1 gives urea and ornithine. Urea and ornithine are converted to hydroxyproline, proline, and polyamines [56]. These products not only contribute to cellular processes such as growth and differentiation [57], but proline and hydroxyproline are key components of the extracellular matrix and collagen [58, 59]. It has been described that Arg1 could reasonably have a neuroprotective effect that is not directly related to its products. Indeed, by consuming arginine, Arg1 could compete with iNOS for the use of available arginine, thus limiting the amount of this amino acid available for NO synthesis [60,61].

As microglial cells represent the primary immune cells in the CNS, microglial activation correlates with many stimuli and danger signals. mSOD1 is responsible for microglial activation through cluster of differentiation 14 (CD14), TLR2 and TLR4 [62], and adenosine triphosphate (ATP) released from damaged or dying cells.

The upregulation of TLRs in ALS patients [63], including TLR4 and its ligand high-mobility group box 1 (HMGB1), and the protective action of TLR4 antagonism have been studied in murine models of MN degeneration [64] and support the possible pathogenic role of TLR4. A preclinical study using mSOD1 transgenic mice has characterized the expression of both TLR4 and its ligand during determined stages of disease. The study has demonstrated that the deletion of TLR4 has a beneficial effect on disease progression. The levels of both HMGB1 and TLR4, along with the respective messenger RNA (mRNAs), were shown to be higher in mSOD1 mice than in wild-type (WT) controls. Also, they were shown to be especially upregulated in microglial and astrocytic cells. In this perspective, the authors have generated a transgenic mouse lacking TLR4 which exhibited a prolonged disease course and a significant improvement of motor performance, confirming that TLR4 is a notable player in MN degeneration [65]. Given these results, the inhibition of TLRs, particularly TLR4, could hold promise as a pharmacological target. Recently, the beneficial effect of TLR4 antagonists in cellular models has been studied. Two synthetic glycol-lipoic molecules have been tested in LPS and mSOD1 animal models. One of the two molecules inhibits LPS-induced TLR4 activation, while the other one directly interacts with TLR4 and prevents its activation. Protective effects against LPS and cytokine toxicity have been demonstrated on MNs in spinal cord cultures. Positive results have also been shown in cocultures of MNs and glial cells derived from mSOD1 mice, reducing glia-mediated MN death. We advise that more experimental data are needed in order to demonstrate the efficacy of such therapies in vivo.

Among the receptors crucial to innate immunity TLRs, TLR4 is expressed by the majority of both immune and non-immune cells, including CNS-resident immune cells [66, 67]. Activated TLR4 can enhance the secretion of many cytokines and so promote the neuroinflammatory mechanisms that have been demonstrated to be involved in ALS. The Toll/IL-1 receptor (TIR) region is the protein domain responsible for signal transduction and is present in all TLRs. Upon activation of TLRs, this region binds adaptor proteins such as myeloid differentiation primary response gene 88 (MyD88) and MyD88-adapter-like (Mal), which contain a TIR domain. Mal can form either homodimers or heterodimers with MyD88 and can activate NF-kB through the interaction with interleukin-1 receptor-associated kinase 2 (IRAK2). Instead, MyD88 seems to activate NF-kB via IRAK1 [68]. The products of the classical NF-kB activation are NO and TNF-α and can be measured as indicators of NF-kB activation. As proved by in vitro co-cultures of MNs with WT or mSOD1 microglia, the products downstream of NF-kB activation released by mSOD1 microglia are toxic to MNs. Interestingly, NF-kB activation in WT microglia decreased MN survival by 50% in co-cultures. This has been confirmed by the fact that the inhibition of NF-kB, either by overexpression of inhibitor of kB (IkB) or transgenically, can rescue MNs [69]. The importance of NF-kB activation in microglia is further supported by the fact that TDP-43 and FUS have been proved to be co-activators of NF-kB [70, 71]. Indeed, the inhibition of NF-kB in mice that express the mutant form of TDP-43 has resulted in an improvement of motor function and phenotype [70]. These studies seem to have recognized NF-kB as the common pathway activated by many pathogenic stimuli involved in ALS and as the crucial factor of microglia-mediated damage. Alongside the good therapeutic potential of NF-kB inhibitors, we think that this approach could require a good degree of specificity in targeting this nuclear factor just in microglia. In fact, the activity of NF-kB in neurons has been demonstrated to be important for many functions such as long-term potentiation (LTP) and, consequently, for memory, synaptic plasticity, and the ability to elaborate spatial information [72–74]. Many genes involved in neurite growth and migration [protein kinase A (PKA) [75], amyloid precursor protein (APP) [76], neural cell adhesion molecule (NCAM), β1-integrin [77]], calcium homeostasis (calbindin [78, 79]), and neurotrophic signals [brain-derived neurotrophic factor (BDNF) [80]] are target genes of NF-kB. NF-kB has also been demonstrated to enhance the expression of manganese-dependent SOD (MnSOD or SOD2) [81] both in neurons and nonneuronal cells and so could have a role in protecting neurons from apoptosis and from the damaging effects of ROS [82]. MnSOD is a mitochondrial protein and its function is to clear ROS, particularly superoxide, which is a toxic by-product of mitochondria [83]. The neuroprotective effect of SOD2 has been shown in animal models of Alzheimer's

disease [84] and in mice with traumatic brain damage [85]. Notably, the area of cortical damage has been shown to be larger in mice in which the *TNFR* gene is knocked-out as compared to WT [85]. In fact, abolishment of the signal downstream of the TNFR resulted in decreased activation of NF-kB and disrupted upregulation of MnSOD. The protective effect was also abolished in neurons in which NF-kB was inhibited by a kB decoy DNA [82].

Astrocytes

The major role of astrocyte is to nourish neurons and to sustain proper neuronal function. One of the ways by which astroglia exert its function is by regulating the extracellular glutamate concentration. In fact, glutamate clearance is impaired in ALS because of the lower levels of the molecule EAAT2, a glutamate transporter normally expressed by astrocytes, hence increasing extracellular glutamate [86]. This functional impairment is the rationale for the clinical use of riluzole, which is the only pharmacological therapy approved for ALS to date. Astrocytes also contribute to glutathione synthesis by secreting glutathione precursor cysteinylglycine (CysGly), thus enhancing neurons' defenses against oxidative injury [87]. This function has been shown to be compromised in astrocytes upon activation.

Astrocytes participate in the cellular response to damage and danger signals releasing inflammation-related molecules like NO, IL-6, and TNF-α and can induce the apoptosis of neurons through FasL [88] and TNF-α signaling [89] as described for microglia. TNF-α acts as previously described.

While activated microglial cells seem to be responsible for propagation and clinical progression of the disease after the onset, astrocytes could be directly involved in the disease onset. In fact, neural progenitor cells derived from induced pluripotent stem cells (iPSC) from ALS patients have been shown to differentiate into astrocytes once transplanted into the spinal cord of severe combined immunodeficiency (SCID) mice and induce a motor deficit [90]. Many other possible mechanisms through which astrocytes can trigger MN death have been proposed in the last years. Astrocytes have been shown to secrete factors that drastically reduce the expression of class I major histocompatibility complex (MHC I) on MNs [91]. The loss of MHC I expression has been demonstrated in MNs cultured with astrocytes expressing mSOD1, in MNs cultured with mSOD1 astrocyte conditioned medium, and in the mSOD1 mouse model. The same phenomenon has been described to occur in patients with fALS or sALS. In fact, spinal cord samples from these patients stained with antibodies against human leukocyte antigens (HLA) A, HLA-B, and HLA-C have showed a near complete loss of MHC I on MNs. The causative toxic factors have not been identified yet but are probably secreted only by astrocytes since mSOD1 microglia has not altered the expression of MHC I molecules on MNs in

co-cultures. Classical inflammatory mediators, such as TNF-α, IL-1, and IFN-γ produced by activated astrocytes, have proved to have no effect on MHC I expression. Instead, up to 76% of cultured MNs lost the expression of MHC I after being treated with thapsigargin. This compound triggers the stress of the endoplasmic reticulum (ER) by inhibiting the ER calcium-ATPase. We speculate that the loss of MHC class I molecules could be a key pathogenic factor in the selective vulnerability of MNs to astrocyte toxicity. Indeed, overexpression of MHC class I subclasses, especially H2k, in mSOD1 mice through a viral vector has been demonstrated to fully rescue MNs from astrocyte-related toxicity, both in co-cultures and in vivo, and to increase survival as well motor function. The same results have been obtained by overexpressing HLA-F in human MNs which were co-cultured with astrocytes derived from patients with fALS and sALS. MHC I molecules modulate astrocyte activation by binding to inhibitory receptors which have been shown to be expressed by astroglia in mSOD1 mice and in humans. Among many MHC I receptors, killer cell immunoglobulin-like receptor 3DL2 (*KIR3DL2*) has been found to be expressed only by astrocytes from ALS patients. The in vitro results have been confirmed by analysis of RNA expression in postmortem spinal cord specimens from individuals with ALS.

Furthermore, astrocytes have been shown to induce MN death by activating an alternative pathway of programmed cell death called necroptosis [92]. Necroptosis represents a form of programmed necrosis which is independent from the activation of caspases and involves the loss of the plasma membrane integrity. Two main effector proteins of necroptosis, receptor-interacting serine/threonine-protein kinase 1 (RIPK1) and mixed lineage kinase domain-like (MLKL), have been identified to date. In vitro inhibition of the necroptosis pathway, by the RIPK1 antagonist necrostatin-1 (Nec-1) or by direct silencing of RIPK1 via a short hairpin RNA (shRNA), has been reported to protect MNs from astrocyte-induced toxicity [93]. The inhibition of MLKL with necrosulfonamide has also been shown to rescue MNs from astroglia toxicity almost completely. Such results suggest that necroptosis could be required for MN degeneration induced by astroglial cells and thus could represent a new, interesting therapeutic target. However, we highlight that the toxic soluble factors released by astrocytes are yet to be identified with certainty. Although TNF-α, FasL, and TRAIL can trigger necroptosis and are also known to be released by astrocytes and microglia upon activation, some studies have reported that none of these factors was identified in mSOD1 or sALS astrocyte-conditioned media.

Since astrocytes are now recognized to contribute more than previously thought to MN loss, an interesting approach would be to replace ALS astrocytes with wild-type or modified cells that can mitigate the toxic environment, modulate neuroinflammation, and foster the MN repair process [16].

Several studies have been conducted grafting different types of cells in the mSOD1 murine model: human neural precursor overexpressing vascular endothelial growth factor (VEGF), BDNF, IGF-1, or glial cell-derived neurotrophic factor (GDNF) [94]; human umbilical cord blood cells overexpressing VEGF and fibroblast growth factor 2 (FGF2) [95]; rat glial restricted neural precursors [96]; and rat adult mesenchymal stem cells [97]. All cell types have been shown to differentiate into astrocytes, retain growth factor secretion, and reduce neuroinflammation. The effect on motor function and survival has been reported to be variable from no effect [94, 97] to increased survival and improved motor function [96] or has not been reported in other studies [95]. Currently, there is no consensus on which cell type could provide the best safety and efficacy profile, nor on the route of administration. Several clinical trials are now ongoing to evaluate this approach.

T Lymphocytes

Although T lymphocytes probably do not exert a direct action in the pathogenic cause of this disorder, they are involved in a complex cross talk with the CNS-resident immune cells and can drive the activation and polarization of the surrounding glia and astrocytes. CD4+ T lymphocytes are recalled to the brain and the spinal cord early during the disease and rise in number as ALS progresses. CD4+ lymphocytes remain the predominant T cell subpopulation.

As with microglia, T cells have been recognized to predominantly assume two phenotypes, one with anti-inflammatory and immune-modulating properties [(T helper (Th) 2 and regulatory T cells(Treg)) [98] and the other one with pro-inflammatory and toxicity-prone characteristics (Th1 and Th17). Th1 and Th17 secrete predominately IL-1, IL-6, and IFN-γ, while Th2 and Treg release TGF-β, IL-4, and IL-10. The neuroprotective action of T cells is supported by in vivo studies on mSOD1 transgenic mice lacking functional T cells, which showed a faster disease progression and a marked shortening in survival [99]. In many studies, mostly on mouse models, T cells have been proven to behave like glial cells. They have showed a preferred polarization toward Th2 and Treg early during the disease and a switch to Th1 and Th17 at a late, fast progression phase. This has been confirmed by the analysis of mRNA expression [28]. Given the similarity, not only in the type of behavior but also in the phase of the disease during which these phenotypic changes occur, it is likely that T lymphocytes and glial cells are interdependent and closely influence each other's properties. In fact, IL-4 produced by Treg cells can abolish the cytotoxic effect of microglia [100], and at the same time, M2 microglia can promote the acquisition of Th2 or Treg phenotype [101]. This phenomenon has been demonstrated to occur also in humans. In ALS patients, the blood count of Treg cells has been inversely associated with a faster disease progression. The same

correlation has been observed with the levels of the transcription factor forkhead box P3 (FoxP3) in leucocytes [102]. Interestingly, the factors that are specific of Th1 and Th17, such as IL-6, IL-1, and INF-γ, can inhibit the phosphorylation of FoxP3, thus inhibiting the immune suppression mediated by Treg cells [103] and facilitating their switch to Th17 lymphocytes [104].

Peripheral Immune System

The role of peripheral monocytes/macrophages remains controversial. There is no clear evidence that they can infiltrate the spinal cord and contribute to MN death [105] unless there is a disruption in the blood-brain barrier [106]. However, their damaging action on peripheral axons has been well recognized [107]. The increased activation of circulating monocytes has been described in ALS patients [108].

It is clear though that the characteristics of the many cellular components of the immune system are not the result of the influence of a single cell type or a unique cytokine or factor but the consequence of the complex milieu of signals and factors. These factors seem to be worsening over time and are now being recognized as major players in MN degeneration. What seems to be the most likely scenario is that an early slowly progressing phase, during which immune cells drive the repair processes and support MN function and viability, is followed by a late fast progressing phase. During this late stage, the cell-autonomous neuronal injury could also be responsible for a progressive switch in glia, astrocyte, and T cell phenotype through the release of apoptotic bodies, misfolded proteins, and ATP. This ultimately results in the amplification of injury and neurotoxic environment [5].

In this perspective, reducing the inflammation and modulating the reaction of immune cells appear as a promising strategy to consistently influence the progression and prognosis of ALS.

Experimental Drugs

Drugs with different mechanisms of action and molecular targets have been or are currently studied in clinical trials. Even if inflammation is a complex process and the altered behavior of a cell type can greatly influence that of other cells, many drugs can be classified based on their primary cellular target. Masitinib, ibudilast, and NP001 are reported to act predominantly on microglia by modulating microglial activation, survival, and production of pro-inflammatory mediators. Instead, T lymphocytes are the primary target of fingolimod. Fingolimod causes T cells to be retained to secondary lymphoid organs, consequently hindering their migration to the CNS and peripheral nerves.

Since cytokines are crucial mediators of inflammation, drugs targeting pro-inflammatory cytokines could represent a valuable tool and have been tested in clinical trials. Such drugs include anakinra, an anti-IL-1 receptor monoclonal antibody, and tocilizumab, a monoclonal antibody directed against the IL-6 receptor.

Other anti-inflammatory drugs with a wider spectrum of cellular targets have also been studied. Given the lack of a precise cellular target, they were classified as other anti-inflammatory drug in this work. These compounds include RNS60, pioglitazone, celecoxib, celastrol, AM-1241, and folic acid. The mechanism of action of the different drugs is described in the dedicated sections (Table 1, Fig. 2).

Drugs Targeting Microglia and Macrophages

Masitinib (AB1010)

A way to tackle inflammation is inhibiting the kinases involved in this process and in apoptosis. Masitinib, a molecule that can selectively inhibit the tyrosine-kinase mast/stem cell growth factor receptor (c-KIT) and a small number of other kinases [109], is a potential therapeutic drug and can be administered orally. Masitinib blocks microglia proliferation and activation and mast cell-mediated degranulation [110] and the release of cytotoxic factors that could worsen the injury to motor nerves. The inhibition of microglia is also mediated by the blockade of colony-stimulating factor 1 receptor (CSF1R) [111].

Compared to other molecules with inhibitory activity on tyrosine-kinases, masitinib is characterized by a relative selectivity for its target and requires half the concentration to exert its activity, thus suggesting that masitinib could have milder or less side effects. Indeed, genotoxicity or cardiotoxicity has not been reported [109]. Several clinical trials have been conducted on rheumatologic and neoplastic diseases such as gastrointestinal stromal tumors (GIST) [112, 113], in pancreatic ductal adenocarcinoma [114, 115], colorectal tumors (ClinicalTrials.gov Identifier: NCT02605044), and melanoma (ClinicalTrials.gov Identifier: NCT01280565). Furthermore, masitinib has been proved to reduce the resistance of cancerous cells to antineoplastic drugs by inhibiting transporter proteins of the ATP-binding cassette (ABC) type. This phenomenon is often responsible for the failure of chemotherapy [116]. Among neurologic diseases, masitinib has been studied in Alzheimer's dementia and MS, and some clinical trials are currently recruiting or completed (clinicaltrial.gov). Mast cells are likely to be crucial actors in the pathogenesis of MS and could be a target of masitinib, which has been proved to be well tolerated in a phase IIa clinical trial [117]. In Alzheimer's dementia, masitinib blocked the Fyn protein, a kinase involved in the signals

Table 1 Investigational anti-inflammatory drugs. The name of the drugs, their mechanism of action, and phase of clinical study are reported in the table

Product name	Company	Mechanism of action	Phase
Masitinib	AB Science	Tyrosine-kinase inhibitor	Phase III—active, not recruiting
NP001	Neuraltus Pharmaceuticals	Modulation of monocyte activation and downregulation of NF-kB in macrophages	Phase II—recruiting
Fingolimod	ALS Therapy Development Institute	Sphingosine-1-phosphate receptor modulator	Phase II—completed
RNS 60	Ravalesio Inc	Modulator of PI3K-Akt pathway	Phase I—recruiting Phase II start date: January 2018
Tocilizumab	Genentech, Inc.	mAb blocking IL-6 receptor	Phase II—recruiting
Anakinra	Swedish Orphan Biovitrum AB	IL-1 receptor antagonist	Phase II—completed
Ibudilast	MediciNova Inc.	Nonselective phosphodiesterase (PDE) inhibitor	Phase II—recruiting
GW2580	Calbiochem	CSF1R tyrosine-kinase inhibitor	Preclinical phase
Pioglitazone	Takeda Pharma GmbH	Peroxisome proliferator-activated receptor γ (PPARγ) agonist	Phase II—completed
Celecoxib	Pharmacia, Gaithersburg, MD	Cyclooxygenase-2 (COX-2) inhibitor	Phase II—terminated
Celastrol	Generic	Antioxidant triterpene—suppresses TNF-α, IL-1β, and iNOS and induces heat shock protein response	Preclinical phase
AM-1241	Tocris Bioscience	Selective cannabinoid receptor 2 (CB2) agonist	Preclinical phase
Folic acid	Generic	Methyl donor—reduces homocysteine levels	Preclinical phase

downstream of amyloid β plaques, and reduced the symptoms in a murine model [118]. Masitinib also decreased ischemic brain area and neurological deficits in rat models of postischemic stroke through the inhibition of the platelet-derived growth factor receptors (PDGFR) [119]. Preclinical experiments with masitinib have been performed. Oral therapy with masitinib initiated 7 days after the occurrence of paralysis in mSOD1 rats diminished gliosis and exerted a

neuroprotective effect on MNs [111]. This therapeutic regimen with masitinib increased postparalysis survival time by 40% (7 days).

A multicenter, phase II/III, double-blind clinical trial randomized 394 ALS patients to three experimental arms, two of them receiving 4.5 and 3 mg/kg of masitinib daily for 48 weeks, while the third group was the placebo control group (ClinicalTrials.gov Identifier: NCT02588677). All participants were taking riluzole 50 mg and they must have been on riluzole therapy for at least 30 days before screening. According to the results of the interim analysis, collected in April 2016 and based on data gathered from about 50% ($n = 191$) of the enrolled patients who completed the 48 weeks' therapy, masitinib seems to have met both its primary and secondary endpoint. The first was a change in the ALS function rating scale revised (ALSFRS-R) score, while the latter included respiratory function (forced vital capacity or FVC) and combined assessment of function and survival (CAFS). The dose used in this trial was below the maximum tolerated pharmacologic dose, which has not been clearly established yet, although pharmacokinetic data demonstrated a linear rising of the maximum serum concentration [C(max)] and of the area under curve (AUC) in a dose-dependent fashion [120]. Masitinib received orphan drug designation for ALS from the Food and Drug Administration (FDA) and the European Medical Agency (EMA). Apparently, the side effect profile was relatively satisfactory, while long-term side effects consisted in gastrointestinal, hematological, and dermatological manifestation and other systemic symptoms [112]. Recently, a case of autoimmune-like hepatitis has been reported in a patient with ALS who received masitinib for 6 months: transaminases and bilirubin kept increasing for 9 weeks after discontinuation of the drug. Other tyrosine-kinase inhibitors have been associated with drug-induced liver injury, but this is the first case of liver injury caused by masitinib to date [121]. In September 2016, AB Science applied for marketing authorization of masitinib in ALS at EMA.

On March 20, 2017, AB Science disclosed the results of the final analysis of the trial (Source: AB Science press release). The final analysis was based on data gathered from 394 patients and confirmed the positive results of the interim analysis regarding efficacy and safety. Indeed, the effects on change in ALSFRS-R score, progression-free survival, and patients' quality of life were statistically significant. Furthermore, the final analysis confirmed the safety profile of masitinib. The estimated completion date of the trial is June 2017.

AV411 or MN-166 (Ibudilast)

AV411 (or MN-166) is a relatively nonselective inhibitor of phosphodiesterase (PDE), available in an oral formulation and initially employed to treat asthma [122], along with first-line drugs such as steroids, β2-agonists, and leukotriene inhibitors.
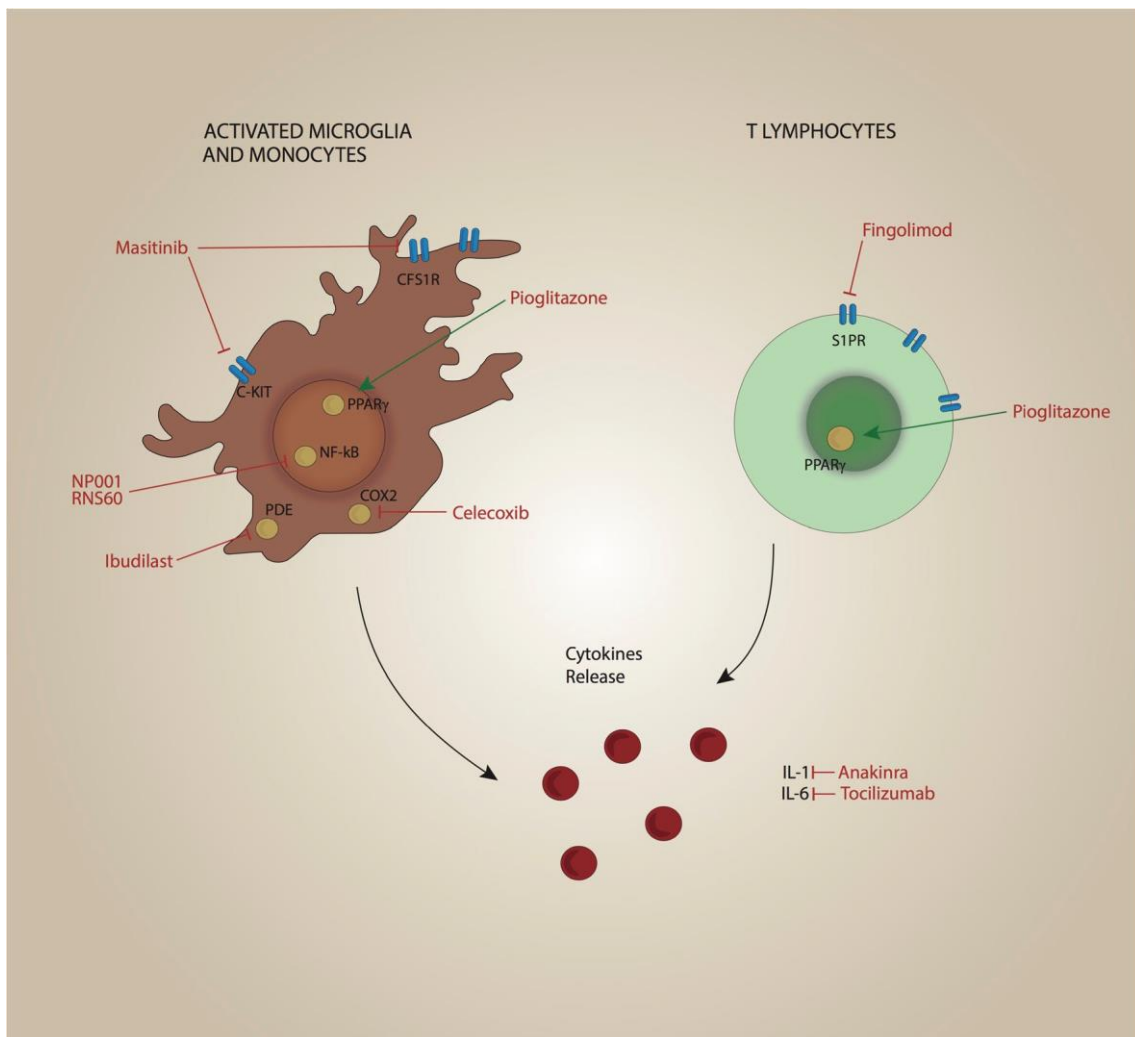
Fig. 2 Experimental drugs and their cellular and molecular target. Anti-inflammatory drugs studied in clinical trials can act predominantly on microglial cells and monocytes (masitinib, NP001, RNS60, ibudilast, celecoxib) or on T lymphocytes (fingolimod) or act on different cellular targets simultaneously (pioglitazone). By blocking different signaling pathways or enzymes, these anti-inflammatory drugs can also decrease the production and release of pro-inflammatory and cytotoxic cytokines. Other anti-inflammatory drugs act by blocking the action of pro-inflammatory cytokines (anakinra and tocilizumab), thus decreasing pro-inflammatory signals and inflammation-induced neurotoxicity

The role of ibudilast in reducing the release of leukotrienes, cytokines, and other molecules involved in bronchospasm [123] and inflammation is the basis for its indication as an anti-asthmatic medication [124, 125]. Furthermore, ibudilast seemed to be a useful treatment in poststroke dizziness, probably because of its vasodilator effect.

Further studies have shown that ibudilast could also attenuate inflammation in the CNS. MN-166 modulates survival and activation of immune cells [126] by preventing the production of pro-inflammatory agents from resident immune cells such as microglia [127, 128]. At the same time, it modulates how resident and peripheral immune cells interact with each other. It modulates the release of migration inhibitory factor (MIF), a strong pro-inflammatory molecule, which limits the activity of both peripheral and central macrophages. The results obtained studying animal models of autoimmune encephalomyelitis [129], cerebrovascular white matter lesions due to chronic microglial activation [130], and Krabbe's disease [131] have proved a globally favorable impact. This supports a possible beneficial role of ibudilast in neurological disorders [132] in which glial dysregulation has been demonstrated to contribute to pathogenesis and progression [133].

Initially, ibudilast has been tested in MS. A phase 2, multicenter, double-blind trial has been conducted on 297 patients affected by MS who showed a relapsing clinical pattern and with contrast-enhancing lesions at the magnetic resonance imaging (MRI). Patients have been randomized in three groups receiving respectively 30 or 60 mg of ibudilast or placebo on a daily regimen for a period of 12 months. The primary endpoint was the total number of new active brain lesions visible at the MRI obtained every 2 months over the study period, while the secondary endpoints considered the rate of relapse,

modification in the Expanded Disability Status Scale (EDSS) score, the volume of lesions seen as hyperintense in T2-weighted MRIs and hypointense in T1-weighted MRI sequences, and the percentage change in brain volume. This trial has proved that the drug was safe and well tolerated but it has not met the primary or the secondary endpoints, reasonably suggesting that ibudilast had no impact on the course of disease. On the other hand, a neuroprotective role of this molecule could have been detected in limiting brain atrophy and damage after acute inflammatory injury [134]. A multicenter, randomized controlled trial [ClinicalTrials.gov Identifier: NCT01982942] is currently ongoing to assess whether the drug is safe and tolerated and test its activity in 250 subjects diagnosed with either primary progressive or secondary progressive forms of MS, who are not undergoing long-term treatment with MS disease-modifying medications or who are treated with either glatiramer acetate or IFN-β. In the latter case, the drug is administered in combination. Randomization into two treatment groups is based on disease status and immune-modulating therapy status. The experimental drug (ibudilast 100 mg/day or placebo) is administered twice daily over 96 weeks. The pharmacological activity of the drug will be assessed at a time point of 96 weeks through the evaluation of the degree of atrophy of the entire brain using MRI and the evaluation of its overall safety and tolerability. The secondary endpoints are the examination of CNS structures via different MRI measures and the evaluation of disability, cognitive impairment, quality of life, and neuropathic pain. This trial could also highlight any possible interaction with eventually co-administered immune modulators.

Two trials on humans are now ongoing to evaluate the efficacy of ibudilast in ALS. The first is an open-label, single center phase II trial of MN-166 on ALS patients ($n = 15$) that is currently being carried out at Massachusetts General Hospital [ClinicalTrials.gov Identifier: NCT02714036]. The main purpose of this study is to evaluate the impact of ibudilast on inflammation in the CNS measured by imaging techniques. The study consists in a screening phase of 6 weeks followed by an open-label treatment phase of 36 weeks with 100 mg/day of ibudilast and a 4-week follow-up phase after the discontinuation of ibudilast. The primary endpoint includes the quantification of the drug uptake in the motor cortex and in the brain stem by PET imaging at 24 weeks and the effect of ibudilast on blood markers of inflammation, such as TNF-α, IFN-γ, IL-1, IL-6, and IL-10. The secondary outcome measures are safety, tolerability, the impact of the drug on motor function expressed as ALSFRS-R score, slow vital capacity (SVC), and hand-held dynamometry (HHD) as measures of the global respiratory function. At the same time, a phase II double-blind, placebo-controlled trial is ongoing and recruiting at Carolinas Healthcare System (ClinicalTrials.gov Identifier: NCT02238626). Its aim is to assess the general safety and tolerability profile and the clinical response to a

daily dosage of 60 mg of ibudilast, in combination with riluzole (100 mg/day), after a 6-month therapy. Sixty patients are randomly assigned to two experimental groups, receiving respectively MN-166 or placebo. Both groups will undergo a 3-month screening phase followed by a double-blind phase of 6 months for the MN-166 group, an open-label follow-up of 6 months only for the placebo control group, and a 2-week follow-up after the discontinuation of the drug. A periodic evaluation is performed at 3 and 6 months, while telephonic contacts will occur at 1, 2, 4, and 5 months to notice potential effects of concomitant medications or side effects. The primary endpoint is safety and tolerability of the drug when administered in association with riluzole, while the secondary outcome is the activity of MN-166 on disease progression, muscle strength, and respiratory function evaluated with the following scales or occurrence of clinical event: ALSFRS-R, SVC, maximum inspiratory pressure (MIP) and forced expiratory volume in the 1st second (FEV1), manual muscle testing (MMT), hand grip dynamometry, and the requirement of noninvasive ventilation (NIV). Even though these clinical trials on ALS patients are still at an early phase, the results of the use of ibudilast in MS allow us to hope in a potential effect in ALS, at least in combination with other molecules or the currently approved therapy.

NP001

Immunomodulatory therapy targeting monocytes/macrophages is the rationale of the use of NP001, produced by Neuraltus Pharmaceuticals, Inc. NP001 is a pH-adjusted formulation of sodium chlorite, available for IV administration, that is converted to taurine chloramine within monocytes and macrophages. It can regulate the function of monocytes both in vivo and in vitro by downregulating NF-kB expression, thus inhibiting the production of IL-1β. This inhibition causes a switch in monocytes from an inflammation-promoting phenotype to a basal, noninflammatory phenotype [135]. Safety, tolerability, and preliminary efficacy of NP001 have been assessed in a phase II, double-blind, placebo-controlled study (ClinicalTrials.gov Identifier: NCT01281631) [136]. The results were published in 2015. The study enrolled 136 patients who were randomly divided into three groups (1:1:1), receiving respectively 1 or 2 mg/kg or placebo in a total of 20 infusions over 6 cycles. The primary outcome of efficacy was expressed as ALSFRS-R score after 6 months of treatment, while secondary endpoints were tolerability and safety, change in pulmonary function, and other measures of patients' status such as survival and time to tracheotomy. At the beginning of each treatment cycle and after 6 months, plasma concentration of biomarkers of inflammation such as C-reactive protein (CRP) and monocyte chemoattractant protein 1 (MCP-1) were obtained. At the end of the study, other biomarkers of activation of macrophages to a pro-inflammatory

state such as IL-1, IL-18, IL-6, TNF-α, IFN-γ, LPS, and CRP were also quantified. The trial demonstrated that NP001 was generally safe and tolerated while the most common adverse effects were dizziness and infusion site pain, both of which had a higher incidence in the group receiving the higher dose regimen. Although 25% of patients that were assigned to the high dosage regimen did not show clinical signs of progression over 6 months, compared to 11% of patients who received the placebo, the trial failed to prove that NP001 could alter the progression of disease ($p = 0.55$). It is to note that the study was underpowered due to small sample size, as pointed out by the authors. It is also important to highlight that 70 to 80% of responders in the two groups receiving the NP001 formulation had elevated baseline markers of inflammation and that these markers, such as LPS and IL-18, decreased after treatment. This observation supports the fact that NP001 could be helpful and show a greater efficacy in slowing the progression of disease in a subset of patients who show relevant systemic neuroinflammation. In August 2016, Neuraltus Pharmaceuticals, Inc. initiated a phase II randomized, double-blind, placebo-controlled, multicenter study of NP001 in ALS patients (ClinicalTrials.gov Identifier: NCT02794857). Interestingly, inclusion criteria for this trial include a high concentration of CRP at screening with the aim to include only patients with elevated systemic inflammation. The primary endpoint of this study is the change in the ALSFRS-R score from baseline, while secondary outcomes are change in pulmonary function, time to tracheotomy, and change in the levels of blood markers of inflammation. The estimated primary completion date is September 2017.

Drugs Targeting Lymphocytes

FTY720 (Fingolimod)

FTY720 (fingolimod), the first approved oral drug for the treatment of relapsing-remitting MS [FDA, 2010, 2015], is a modulator of the sphingosine-1-phosphate (S1P) receptor [137]. The phosphorylated form of FTY720 causes the internalization and degradation of the S1P receptors, leading to the retention of lymphocytes to secondary lymphoid organs and the subsequent reduction of circulating naïve and central memory T cells [138]. Effector memory T cells are spared, thus preserving peripheral immune surveillance. The lipophilic characteristic of this compound makes it capable of crossing the blood-brain barrier and acting directly on CNS-resident cells where S1P receptors, except for S1P receptor 4, are highly expressed [139]. In vitro studies have demonstrated that FTY720 reduced the production of pro-inflammatory mediators in activated microglial cells in a dose-dependent manner by acting as a S1P receptor agonist. At the same time, it increased the

production of neurotrophic factors such as BDNF and GDNF [140]. Animal models of brain ischemia [141], epilepsy [142], and spinal cord damage [143] have showed a reduction of neuronal damage and microglial activation after treatment with FTY720, confirming the anti-inflammatory and neuroprotective properties of fingolimod. The primary effect of the drug on toxic neuronal death has been proved in cellular and animal models. Cultures of neurons without contact with the peripheral immune system and exposed to toxic concentrations of NMDA have showed a dose-dependent decrease in neuronal death after a prolonged treatment with fingolimod. The protective effect has been confirmed in vivo [144]. The toxicity due to the excess of excitatory stimuli is a crucial mechanism involved in ALS. Therefore, FTY720 seems to be a promising therapeutic drug because of its ability to act on at least two different processes involved in MN disease.

Recently, it has been described that treatment with fingolimod can improve the outcome of SOD1 murine model of ALS [145]; 0.1 or 1 mg/kg i.p. doses of fingolimod were administered to animals during the whole course of disease. The compound led to a significant improvement of clinical signs and survival rate of mSOD1 mice, even if only of 10–15 days. The positive effects have been linked to the modulation of inflammatory genes (CD11b, Foxp3, iNOS, IL-1β, IL-10, Arg1, and BDNF) in the CNS. These findings suggest that fingolimod could have a beneficial effect in ALS.

A phase IIA clinical trial, assessing the safety and tolerability of this molecule on ALS patients (ClinicalTrials.gov Identifier: NCT01786174), has been recently completed and the results should soon be published. The study enrolled 28 ALS patients, 18 of which received the treatment. The drug was administered for a short time span (28 days). No major safety issue was detected. ALSFRS-R score did not change significantly at 8 weeks (based on the data reported on clinicaltrial.gov), but the drug was administered only for 1 month.

Some adverse effects have been reported in MS patients both during clinical trials and postmarketing surveillance. Alterations in heart rate, especially bradycardia, were caused by the action of fingolimod on S1P receptors expressed on atrial myocytes and required the patient to be monitored for at least 6 h during the administration of the first dose [146]. A reduction of the number of lymphocytes up to 70% was another effect of the drug but was completely reversible after 6 weeks. Mild infections of the upper and lower airways were common but serious events were rare. A case of disseminated varicella zoster virus (VZV) infection and other severe herpes virus infections have been reported, thus making the serologic screening for VZV and the vaccination of antibody-negative patients necessary prior to treatment. Cases of cryptococcal meningitis and progressive multifocal encephalitis (PML) have been described. Finally, before and during the therapy,

an examination of fundus oculi could be required for early detection of macular edema, especially during the first 4 months [147].

Given these results and the fact that fingolimod has both direct and indirect neuroprotective effects, the next step would be a phase IIB/III clinical trial to test its efficacy and long-term safety.

Drugs Targeting Cytokines

Other inflammatory pathways have been proved to play a role in neuronal loss in ALS [3] and have been further investigated as pharmacological targets. Different compounds that can inhibit cytokines directly or indirectly have been investigated.

Anakinra

IL-1 has been shown to be particularly unregulated in response to the presence of mSOD1 [148]. The mutant protein induces the activation of a component of the cytosolic protein complex inflammosome [149] called caspase-1, a protein that is also necessary for IL-1β to be converted to its mature form through a proteolytic mechanism [148]. IL-1 stimulates macrophages and microglia in a dose-related fashion, leading to the acquisition of a pro-inflammatory phenotype and ultimately to inflammation-related neurotoxicity [17]. A study conducted by Meissner et al. [150] has demonstrated that the uptake of mSOD1 had a higher efficiency than that of the WT SOD1 and led to its cytoplasmic accumulation. mSOD1 can form oligomers that resemble those formed by amyloid protein, as described either in vitro and in animal models, and the misfolding degree was linked to IL-1β maturation and faster disease progression. In the same study, Meissner and colleagues have shown that the deficiency of IL-1β and caspase-1 and the treatment with the antagonist of the IL-1 receptor slowed disease progression in mice, providing further evidence that IL-1 could be a suitable therapeutic target.

A single-arm pilot study [151] (ClinicalTrials.gov Identifier: NCT01277315) has been conducted in Berlin at Charité University Hospital to assess safety and tolerability of the IL-1 receptor antagonist anakinra, a drug currently used to treat rheumatoid arthritis [152]. The results were published in 2015. Anakinra was administered in association with riluzole in ALS patients, and disease progression was measured using ALSFRS-R and forced expiratory vital capacity as secondary endpoint. Serum markers of inflammation such as IL-6 and TNF-α were also measured. Assuming that anakinra could reach a higher concentration in peripheral nerves, the study was designed to enroll only patients who showed predominantly or exclusively signs of degeneration of lower MNs (LMNs), even if there is evidence that anakinra can reach therapeutic concentration in the CNS [153]. Patients were enrolled at an early stage of disease (mean ALSFRS-R = 40.7 and mean delta ALSFRS-R = 0.35,

mean age = 57.9 years old) and had no sign of hypoventilation syndrome at baseline. The 52-week treatment with anakinra, 100 mg daily, was completed by 17 patients. Anakinra was generally safe, with the most common adverse effect being reaction at the site of injection, which occurred with increased frequency and intensity over the course of the study. Infections of the respiratory tract occurred in seven patients and resolved without the need of antimicrobial therapy. Fifteen patients were also screened for mutation in the SOD1 and C9ORF72 genes, in the attempt to identify a link between the genotype and the response to the treatment with anakinra. Only four patients were found to have the expansion of the hexanucleotide repeat (with 1700 repeat or more) in C9ORF72 and did not show any difference in the response to the treatment or any apparent sign of dementia compared to the rest of the group. The study did not prove any difference in disease progression, although the trial did not have sufficient power to show any statistically relevant difference and lacked a placebo control group. IL-6 and TNF-α levels were not significantly altered at the end of the treatment, but cytokine levels were measured only in plasma and not in the cerebrospinal fluid. It is otherwise relevant that 16 out of 17 patients developed anti-anakinra IgG after a mean of 4 weeks after treatment. These antibodies can halt the drug from acting on its target. The study proved the general safety and tolerability of anakinra and justifies further placebo-controlled studies on a broader cohort of patients to prove efficacy.

Tocilizumab

Another immunomodulatory drug, tocilizumab, is currently being studied for the treatment of ALS. Tocilizumab is a monoclonal antibody, currently approved to treat rheumatoid arthritis and juvenile idiopathic arthritis. It blocks the IL-6 receptor thus exerting a neuroprotective effect by decreasing cytokine production and the activation of the immune system. The in vitro efficacy of tocilizumab in reducing inflammation has been assessed by a pilot study [154]. Another study [155] has tested the effect of tocilizumab in sALS patients and has shown that the effects of Actrema[R] on inflammation were different in the two groups of patients. It reduced inflammation, as shown by downregulation of inflammatory genes as IL-1β, in patients who had strong baseline inflammation (eight key genes showed a more than 4-fold increased expression, $p < 0.05$), while it slightly increased inflammation in the second group which showed weak baseline inflammation. A phase 2 randomized, placebo-controlled trial is currently ongoing (ClinicalTrials.gov Identifier: NCT02469896), and its primary endpoint is to assess safety and tolerability of tocilizumab over a 16-week time frame, while evaluation of the expression of pro-inflammatory genes in monocytes from peripheral blood is the secondary endpoint. Patients are treated with 8 mg/kg tocilizumab infusion or placebo every 4 weeks for 3 months. The trial is currently recruiting participants and

the estimated completion date is August 2017. No update is available to date.

However, recent preclinical studies have revealed that the knockout of the IL-6 gene had no effect in modifying the outcome of mSOD1 mice [156]. The same has been observed with the pharmacological block of IL-6 through a murine analogous of tocilizumab, namely MR16-1 [157]. A significant increase in weight loss has been reported as a negative effect of MR16-1, despite a moderate anti-inflammatory effect.

These studies support the targeting of monocyte/macrophage activation as a strategy to slow the progression of disease but only in patients who show increased systemic inflammation and dysregulated activation of macrophages. This highlights that it could be needed to identify this subset of patients for this approach to be suitable. However, the possible negative effects observed in preclinical models represent a caveat for this approach.

Other Anti-inflammatory Drugs

RNS60: Charge-Stabilized Nanoparticles in Oxygen-Saline Solution

RNS60 has been proposed as a novel approach to attenuate NF-kB activation and the associated activation of glial cells.

RNS60 consists of a saline solution (0.9% NaCl) containing charge-stabilized nanostructures or nanobubbles, which have been shown to decrease inflammation and cell death. RNS60 is generated by subjecting normal saline solution to Taylor-Couette-Poiseuille flow under high oxygen pressure.

Nanobubbles were applied for the first time in the late 1990s by Tony Wood to increase plants' resistance to diseases. Thereafter, its therapeutic use for human diseases has been suggested.

RNS60 has been found to inhibit the production of NO and the expression of iNOS in activated microglia. It has been hypothesized that RNS60 could carry out its anti-inflammatory action by inhibiting NF-kB through the activation of type IA phosphatidylinositol 3-kinase (PI3K) and the phosphorylation of Akt protein. The latter would subsequently upregulate IκBα, which blocks NF-kB, ultimately preventing iNOS expression and the production of NO and neurotoxins [158]. iNOS upregulation has been related to MN loss and overactivation of microglia and astrocytes. Therefore, it has been proposed that the inhibition of iNOS could represent a fruitful direction to explore new therapeutic agents for neurodegenerative disorders [159]. It has been shown that RNS60 could enhance ATP synthesis by facilitating oxygen transport into the mitochondrial system in *Xenopus laevis* oocytes and on squid synapses [160].

Recently, positive effects of RNS60 have been reported on Alzheimer's disease, both in vitro and in the 5XFAD transgenic mouse model, by augmenting the expression of neuronal plasticity-associated proteins and AMPA-/NMDA-dependent hippocampal calcium inflow [161]. It also decreased neuron apoptosis, tau phosphorylation, and glial activation and the amount of amyloid tangles [162]. RNS60 has been reported to be a potential disease modulator in Parkinson's disease [163] and MS [164], improving neurotransmission [165] and neuroprotection on one hand while acting on the immune response on the other.

Preclinical studies on RNS60 toxicity have shown little or no side effects and three phase I clinical trials have provided satisfactory data on tolerability of inhaling and intravenous administration.

To date, an open-label phase I clinical trial is ongoing at Massachusetts General Hospital focusing on safety, tolerability, and efficacy in a group of ALS patients (ClinicalTrials.gov Identifier: NCT02525471). Inclusion criteria encompass riluzole naïve patients and patients who have not been on riluzole therapy for at least 30 days. The study combines the previously tested way of administration with a 7-day scheme for 24 weeks. Evaluation of the main endpoints will involve the measurement of blood biomarkers of inflammation, neuroimaging analysis (PET), and the assessment of the clinical outcome by testing of pulmonary function (SVC), strength (test of limb isometric strength), and functional status (ALSFRS-R).

Although the real biological impact of this compound is controversial, we are further concerned by the fact that RNS60 is basically saline solution. This raises doubt about the ethical basis of this trial and if the study should have been allowed to proceed to a phase II study.

Pioglitazone

Pioglitazone belongs to the class of thiazolidinediones and acts as a peroxisome proliferator-activated receptor γ (PPARγ) agonist [166]. Pioglitazone is currently used as an oral antidiabetic drug because it increases sensitivity to insulin and decrease glycemia. The PPARs are nuclear hormone receptors which present several isoforms. Isoforms share the major structural and functional properties but can show different ligand specificities and tissue distribution. For example, PPARα expression is predominant in the liver, skeletal muscle, kidney, heart, and vascular wall, while PPARγ is primarily found in adipose tissue, intestine, and macrophages. The activation of this class of receptors modulates the expression of several genes and influences the cellular response to different stimuli such as metabolic alterations or inflammation [167]. Interestingly, PPARs have been shown to regulate the inflammatory response by reducing the release of the proinflammatory cytokines TNF-α, IL-1β, and IL-6. PPARs have been reported to upregulate the expression of iNOS and metalloproteases in mononuclear and epithelial cells. Furthermore, PPARs inhibit various transcription factors and promote the expression of NF-κB, signal transducers and activators

of transcription (STATs), activator protein 1 (AP1), and nuclear factor of activated T cells (NFAT) [168]. Such effects have been demonstrated to be induced by PPARα thanks to the availability of PPARα knockout animal models [169]. Since the absence of PPARγ is not compatible with life, it is not possible to create a similar animal model. Consequently, the PPARα-induced anti-inflammatory effects have not been confirmed to be induced also by PPARγ. However, additional studies have been performed in vitro, using cerebellar granule cells and mononuclear cells [170, 171], and in vivo [172]. These studies have strongly suggested a pivotal role of PPARγ in attenuating inflammation-related neurotoxicity. Three independent groups have tested pioglitazone, the only molecule able to extensively penetrate the blood-brain barrier, in mSOD1 mice [173−175]. Since inflammation has been identified as an early event in disease pathogenesis, the treatment with oral pioglitazone was started before the onset of clinical signs. Treated animals have shown prolonged survival, decreased weight loss, and delayed decline in motor function compared to untreated mice. Neuropathological studies have demonstrated extended survival of MNs, which preserved their structural and functional properties, as well as a significant decrease in microglial activation. A reduced expression of two major pro-inflammatory enzymes cyclooxygenase-2 (COX-2) and iNOS and an increased expression of the anti-inflammatory genes SOCS-1 and SOCS-3 have been confirmed by Western blot analysis. The expression of SOD1 was not affected by the treatment. These promising results have encouraged the design of a phase II, multicenter, stratified, parallel-group, placebo-controlled trial to test the potential efficacy of pioglitazone in ALS patients who already received riluzole (ClinicalTrials.gov Identifier: NCT00690118) [176]. The primary endpoint of the trial was survival. The secondary endpoints included incidence of NIV and tracheotomy, decline of motor function measured as ALS-FRS-R score, modifications in SVC, and quality of life. Patients were matched for age, sex, type of disease onset, and functional and metabolic status. Patients were randomly assigned to two groups: one receiving placebo and the other one receiving pioglitazone. Unfortunately, on April 2010, the trial was terminated because of the increased number of deaths among the treated patients. However, further analyses have demonstrated that such deaths were not caused by pioglitazone. In fact, pioglitazone was globally well-tolerated. The failure of this clinical trial has been attributed to the fact that the drug should have been administered before the disease onset, as in preclinical studies.

COX-2 Inhibitors: Celecoxib

The COX-2 enzyme has been reported to play an important role in the pathogenesis of ALS. COX-2 catalyzes the synthesis of prostaglandins (PGs), especially PGE2, which are directly involved in glutamate-mediated neurotoxicity and inflammation. Specifically, PGE2 promotes the release of glutamate from astroglial cells via a calcium-dependent pathway,

feeding the mechanism of impaired glutamate turnover detected in ALS [177, 178]. Furthermore, COX-2 contributes to the inflammatory process by favoring the production of neuro-toxins, cytokines, and free radicals. COX-2 inhibitors, like celecoxib, have already been approved by the FDA for the treatment of arthritis and have been suggested to have a potential therapeutic effect in ALS. These observations have been supported by the fact that high levels of COX-2 and PGE2 have been detected in the cerebrospinal fluid and in the spinal cord of mSOD1 mice and of patients affected by sALS [179, 180]. The oral administration of celecoxib in mSOD1 mice has obtained promising results. The treatment with celecoxib extended the survival rate and prevented weight loss and motor function decline. Reduced expression of PGE2 in the spinal cord of treated animals has been demonstrated by neuropathological analysis [181]. A randomized, double-blind, placebo-controlled trial has been conducted to test the clinical effect of celecoxib in ALS [8]. The trial has been prematurely terminated because preliminary statistical analyses have showed no difference between the celecoxib and the placebo-treated groups. In fact, neither the primary nor the secondary endpoints have been met. Also, PGE2 levels in the cerebrospinal fluid have showed no changes between the two groups. The authors have claimed that PGE2 levels were not properly dosed because of unreliable biochemical tools.

Other Anti-inflammatory Compounds: Celastrol, AM-1241, and Folic Acid

Over the years, several well-known anti-inflammatory compounds have been tested for the treatment of ALS. Celastrol is a triterpine, a natural extracted molecule which is traditionally used in Southern China for its potent anti-inflammatory and neuroprotective effects. Celastrol has been shown to suppress the release of cytokines, decrease glial activation, and promote the synthesis of heat shock proteins (HSPs), especially HSP70 [182]. Preclinical studies have tested a 4-week treatment with celastrol in mSOD1 mice. Such studies have confirmed the ability of the drug to downregulate TNF-α expression and glial activity and to upregulate HSP70 expression and prevent the early loss of MNs in the spinal cord. Treated mice showed a delayed disease onset, slower motor function decline, and prolonged survival compared to controls [183].

Despite such promising results, no sufficient data on the safety of the drug and its capacity to penetrate the blood-brain barrier have been collected to date. Therefore, further studies are needed before celastrol could proceed to testing in humans [184].

The role of endocannabinoids in ALS pathogenesis is still controversial. They have been demonstrated to prevent the death of neuronal cells induced by excitotoxicity or oxidative damage and to have anti-inflammatory properties [185]. The selective activation of the cannabinoid receptor 2 (CB2) by

AM-1241 has demonstrated a positive effect against inflammatory hyperalgesia in animal models [186]. The first studies of AM-1241 in mSOD1 mice have demonstrated no psychotropic effect of the drug and a sex-dependent delay in the motor function impairment. Delayed motor function decline has been reported even if the treatment was started after the onset of the symptoms. No effect on weight loss and survival has been reported [187].

The upregulation of the CB2 receptors in glial cells and spinal cord specimens has been described in tissue samples from ALS patients [188]. Shoemaker's group has detected a significant increase of the CB2 receptor density in tissue samples from mSOD1 mice. The density of the CB2 receptor varied as the disease progressed, thus suggesting an early neuroprotective effect of such receptors [189].

Furthermore, a significant extension of life span has been obtained after the injection of AM-1241 in symptomatic mSOD1 mice. Despite these positive results, the exact role of endocannabinoids in ALS and the mechanism of action of compounds that bind to CB receptors remain to be elucidated. AM-1241 has been proposed to act as a ʙprotean agonist^ displaying a different mechanism of action, depending on the assay and/or tissue examined [190].

Folic acid has been evaluated for its ability to reduce the levels of homocysteine, which have been found to be elevated in astrocyte-derived ALS animal models [191]. It is still debated how homocysteine could be involved in the pathogenesis of ALS. However, its role in promoting inflammation and inducing cytokine release and excitotoxicity has been largely demonstrated [192]. Good preclinical data have been obtained from the treatment of mSOD1 mice with acid folic. Treated animals exhibited a delayed disease onset and an extended survival. Furthermore, a reduction of both MN degeneration and pro-inflammatory cytokine expression has been detected by histopathological and biochemical analysis [193]. Despite the good results obtained in the preclinical setting, we believe that the simple supplementation of folic acid would not have the same effects in humans. For this reason, we do not encourage the translation of this approach to clinical trial in humans.

Molecules in Preclinical Stages

Role of IL-10

Inflammation is a constant phenomenon in ALS and involves both systemic and CNS alterations in the immune response. In the CNS of ALS subjects, reactive astrocytes, activated microglia, levels of cytokines, and inflammatory mediators are typically increased.

Several in vitro and in vivo studies have shown that reactive microgliosis represents not only a hallmark of ALS but also correlates with the severity of MN degeneration. The clinical onset of the disease could depend on what type of cytokines is predominately expressed. Gravel et al. [194] have demonstrated that, in two mSOD1 murine models ($SOD1^{G93A}$ and $SOD1^{G37R}$ mice), microglial cells overexpress IL-10 during the presymptomatic phase of disease. The overexpression of IL-10 via viral vectors has been shown to slow the progression of disease and increase survival, while the opposite has been demonstrated by blocking this pathway with an anti-IL-10 receptor antibody.

Although it is a very fascinating and investigated aspect, the role of T cells in the pathogenesis and progression of ALS is not clear yet. There is evidence that microglial activation could have a toxic effect on neuronal progenitor by blocking their differentiation, while the activation of T cells could promote neurodifferentiation [195]. However, it is still unknown whether T lymphocytes are the primary inducers of microglia activation or vice versa. They could be just bystanders and be recalled to the site of inflammation by the death of neurons and chemokines released by resident cells [196].

Colony-Stimulating Factor 1 Receptor Inhibitor

CSF1R (or CD115) is a transmembrane receptor for IL-34 and colony-stimulating factor 1 (CSF1) and belongs to the type III growth factor receptor family with tyrosine-kinase activity. Signaling through this receptor leads to the activation of a downstream mitogenic pathway sustained by the activity of PU.1 and CCAAT/enhancer binding protein (C/EBPa) [197], a pathway that contributes to the maturation of microglia and monocytes and to hematopoiesis [198]. There is evidence that this pathway is among those involved in developing and maintaining the inflammatory response and the subsequent damage to neurons [199].

A recent study [200] on a mouse model of prion disease supported the role of CSF1R in microglial proliferation across different areas of the brain. This effect increased as the disease progressed and positively correlated with the progressively higher expression of CSF1 and CSF1R mRNAs. In the same study, GW2580, a CSF1R inhibitor, was shown to decrease the expression of CSF1R, PU.1, and C/ECBa and of the downstream effectors of this pathway cyclins D1 and D2. GW2580 also increased the acquisition of an M2 microglial phenotype. The block of microglial expansion resulted in slower disease progression, decreased number of damaged neurons, improved survival rate, and delayed onset of behavioral symptoms.

The same effect of the treatment with GW2580 has been shown in animal models of MS [201] and of Alzheimer's disease [202].

A preclinical study on mSOD1 mice has showed beneficial effects of GW2580 on disease progression and survival rate [203]. The inhibition of CSF1R reduced the number of microglial cells in the spinal cord by 30% and increased the

number of viable MNs at a late stage of disease compared to untreated mice. The results have been confirmed by fluorescence-activated cell sorting (FACS) and immunohistochemical analysis. Decreased levels of the transcription factors PU-1, IRF8, and RUNX1 have been detected. These transcription factors are involved in microglial maturation and proliferation. Furthermore, the cell cycle regulator cyclin D2 acts downstream of CSF1R. However, the treatment with GW2580 had no significant effect on the expression of the 11 cytokines investigated. The inhibition of this pathway has been proved to increase motor function and survival and delay the progression of the disease. No effect on disease onset has been observed. Instead, the impact on peripheral nerves was consistent, as suggested by the reduction of monocytes in the tibial nerve of treated mSOD1 mice, probably due to a 2.5-fold depletion of blood monocytes after treatment. The improvement in the peripheral nervous system could be beneficial to motor impairment by slowing early muscle denervation even before any measurable reduction in microglial proliferation. In fact, the upregulation of CSF1R has not been observed at an early stage of disease, suggesting that its role could be limited to a later stage. However, consistent microgliosis has been found to be an early mark of neurodegeneration and to be likely driven by the activation of other signaling pathways, some of which were previously discussed.

In contrast with the previously discussed studies on CSF1R, a study on a murine model of Alzheimer's disease has reported a favorable impact of the administration of human recombinant CSF1 and IL-34 and of the deletion of CSF1R in the forebrain on neuron survival and memory performance [204]. As reported by the authors, this could be due to the limited expression of CSF1R in a small group of cortical and hippocampal neurons under physiological conditions, while the receptor seems to be almost exclusively expressed by microglia in the CNS.

This evidence supports further studies on the role of the CSF1R signaling pathway in neurodegeneration, especially in ALS. The targeting of CSF1R could be evaluated as a therapeutic strategy for future trials on humans.

Novel Promising Molecular Targets

TANK Binding Kinase 1 (TBK1)

TBK1 is a serine/threonine kinase that plays an essential role in regulating the inflammatory response. When activated, this protein associates with TRAF3 and TANK and phosphorylates different IFN regulatory factors (IRFs). Phosphorylated IRFs can undergo homodimerization and translocate to the nucleus to activate the transcription of pro-inflammatory and antiviral genes such as IFN-α and IFN-β.

Recently, TBK1 has been implicated in the regulation of autophagy and inflammation. Mutations in the *TBK1* gene have been recognized to cause ALS, frontotemporal dementia (FTD) [205], normal tension glaucoma (NTG), or childhood herpes simplex encephalitis (HSE) [206]. In particular, mutations that cause the abnormal activation of TBK1 have been linked to NTG, while mutations that abolish its function lead to ALS/FTD or HSE. Genetic mutations that alter the signaling process between the TLR3 and IFN [207], like those in *TBK1*, have been shown to negatively affect IFN-γ levels and to be correlated with the onset of HSE in a group of children. Autophagy dysfunctions caused by loss of TBK1 [208] have also been described as possible underlying causes of ALS/FTD, but the possible perturbation of neuroinflammatory pathways should also be considered as potential therapeutic target in this case.

RIPK1

It has been recently described that optineurin (OPTN), the protein encoded by one of the ALS causative genes, inhibits the signaling downstream of RIPK1 by modulating its turnover [209]. RIPK1 is recruited to the TNFR1 receptor and consequently mediates TNF-α-mediated neurotoxicity. The lack of OPTN causes a gradual demyelination and progressive axonal damage by activating necroptosis signals, comprising RIPK1, RIPK3, and MLKL. RIPK1- and RIPK3-linked axonal damage has been detected in $Optn^{-/-}$ mice, in mSOD1 mice, and in tissue samples from ALS patients. Therefore, RIPK1 and RIPK3 are likely to exert a significant action on axonal degeneration. Ito et al. [209] have also demonstrated that the increased activation of RIPK1 in $Optn^{-/-}$ mice caused an increased activation of microglial cells toward an M1 phenotype in the spinal cord and increased the secretion of pro-inflammatory factors like TNF-α, IFN-γ, IL-1, IL-2, and IL-12. This suggests that the lack of OPTN and the impaired turnover of RIPK1 could induce neuronal injury either by causing axonal degeneration and necroptosis and increasing microglia-induced inflammation. To further confirm the involvement of this cell death pathway in vivo, the authors studied the effects of RIPK1 inhibition through the generation of $Optn^{-/-};Ripk1^{D138N/D138N}$ double mutant mice or the pharmacological inhibition of the RIPK1 kinase activity with Nec-1 in $Optn^{-/-}$ or mSOD1 mice. Either genetic disruption or pharmacological inhibition of RIPK1 activity rescued hind limb weakness and axonal pathology of $Optn^{-/-}$ mice, as well as axonal pathology and degeneration in mSOD1 mice. Furthermore, the myelination defects observed in mSOD1 mice were rescued by knockdown of *Ripk3*. Given these results, the inhibition of RIPK1 could represent a suitable strategy to rescue MNs from TNF-α-mediated death. Because RIPK1 has been shown to be specifically recruited by TNFR1 and modulate the negative effect of TNF-α, it has

been suggested that targeting RIPK1 could have a beneficial effect without involving TNFR2. Based on this evidence, the inhibition of the RIPK1 kinase activity could represent a promising strategy to slow or even stop the axonal injury and to improve MN function in ALS. Since an effective pharmacological inhibitor of RIPK1 is available, we encourage further in vivo studies with Nec-1 to confirm its efficacy and safety in ALS. These data would be helpful for a foreseeable application of Nec-1 in humans.

Conclusions

While no effective therapy for ALS is currently available, inflammation has come to light as an active injury mechanism and a new suitable target for a therapeutic effort. To date, many different molecules and antibodies have been tested in clinical trials, targeting astrocytes, microglial cells, monocytes, T cells, and cytokine pathways. Even though there is no major concern on the safety of all the studied drugs, almost all of them showed moderate or no efficacy in the phase II clinical trials that have been concluded, while others are still ongoing. Only masitinib has been proved to have a modest effect on motor performance. The final analysis of the phase II/III trial of masitinib in ALS patients has confirmed the positive results of the interim analysis and has proved that masitinib was effective in improving motor function, progression-free survival, and quality of life of patients. It is noteworthy that the studies that tested NP001, anakinra, and tocilizumab were not sufficiently powered to show a statistically relevant outcome. Furthermore, the trial that has been conducted with tocilizumab supported the fact that not all patients may present a strong inflammatory component. Therefore, this kind of approach could be consistently helpful for a relatively small group of patients. However, the identification of patients who have a strong baseline inflammation is still limited to subjects enrolled in a small number of clinical trials, thus making difficult to estimate the percentage of patients with a strong inflammatory component compared to all ALS cases. We suggest that recognizing this group of patients would be helpful to know what percentage of ALS subjects would be more likely to benefit from an anti-inflammatory treatment and would also allow to select a homogeneous group of patients for clinical trials. Moreover, this would permit to recognize if increased systemic inflammation is more frequent in subjects with fALS or sALS or if it could be associated with certain genetic mutations.

Immune modulation and promotion of an anti-inflammatory phenotype in microglia and T cells seems to be a relatively unexplored field to modify the course of this disease. Further studies could prove a positive impact of this strategy.

A considerable issue that emerged is the relatively frequent failure of clinical trials to replicate the results obtained in the animal model. Preclinical studies on ALS almost invariably employ the mSOD1 transgenic murine model (generally the $SOD1^{G93A}$ mouse model). Despite being a useful model for research, it is now clear that it is also a limited one. In fact, patients who harbor a recognized pathogenic mutation in the *SOD1* gene represent a very limited subgroup of all ALS patients. Moreover, the ALS phenotype showed by these mice is greatly dependent on four factors: gender, genetic background, *SOD1* mutation, and expression levels of the human *SOD1* transgene [210]. The standard SOD1^{G93A} mouse model carries 25 copies of the *mSOD1* transgene under the control of the human *SOD1* promoter [211], but it is known that loss of several transgene copies can occur spontaneously during meiosis [210]. This phenomenon can lead to a variable phenotype expression and cause a variable degree of severity and response to treatment [212]. The same phenomenon can occur in mice carrying a human *SOD1* transgene with a different mutation. Since the SOD1^{G93A} mouse model is the most used one, the effects of different *SOD1* mutations can be generally overlooked. The issue of the variable genetic background is more challenging because many genetic variations could modify the severity, the time of onset, and the progression of disease [213]. Further studies aimed to improve the available animal models of ALS or MN degeneration could help to improve the preclinical screening of novel therapeutic compounds.

Based on what is known about neuroinflammation and CNS-resident immune cells, we highlight that the NF-kB pathway seems to be particularly relevant for inflammation-induced neurotoxicity. The activation of NF-kB likely represents the converging point of several damage signals and could be a particularly fruitful target for therapeutics. However, this approach would likely require the precise target of NF-kB into microglial cells and astrocytes to have a significant impact.

Even if no major change in ALS progression has been achieved by targeting inflammation, some of the compounds undergoing preclinical investigation could have the potentiality to exert a meaningful impact on the disease and could be easily applied in clinical settings. Also, the discovery of novel molecular mechanisms warrants further investigation toward the development of an effective therapy for ALS. We postulate that a deeper knowledge of the molecules and cellular pathways involved in neuronal damage could lead to the selection and design of efficient therapies.

Based on the data presented herein, targeting inflammation in ALS would certainly have a weaker impact on the disease than a possible therapy directed against its pathogenic causes. However, we think that the development of efficient anti-inflammatory or immune-modulating therapies would at least lead to symptomatic improvement or to a relatively short

increase in survival, which would provide an acceptable clinical improvement for ALS patients. This possibility supports further efforts to improve this approach, since no pathogenic therapy is available to date.

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