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Abstract:

Purpose of review: Amyotrophic Lateral Sclerosis (ALS) is a degenerative motor neuron (MN) disease with a strong neuroinflammatory component. This review summarizes how the connection between neurodegeneration and the immune system is strengthened by new discoveries from ALS genetics and the analysis of subpopulations of immune cells in ALS.

Recent findings: Recent genes identified in ALS encode for proteins with direct immune roles, that when mutated lead to deregulation of immune functions, potentially influencing the disease. Although, neuroinflammation in the CNS of ALS has been well documented, new evidence suggests also direct malfunctions of immune cells in the CNS and at the periphery: While CD4+ T regulatory lymphocytes are protective in ALS, their number and function are altered over the disease course. CD8+ T cells are detrimental for MNs in the CNS, but show some protective roles at the periphery. Likewise, presence of mast cells in muscles of ALS models and patients, and impairments of monocyte functions reveal potential new players in ALS disease progression.

Summary: Although MN degeneration is considered the prime event in ALS, dysfunctions in immune processes can impact the disease, highlighting that targeting specific immune components is a strategy for developing biomarkers and ultimately new drugs.

Keywords: Amyotrophic Lateral Sclerosis (ALS), neuroinflammation, lymphocytes, monocytes, mast cells

Introduction

Amyotrophic Lateral Sclerosis (ALS) is the most common adult onset motor neuron (MN) disease, characterized by the degeneration of upper and lower MNs. ALS is a heterogeneous disease, multifactorial, with some known genetic causes, but for the majority of cases, multigenetic and probable environmental causes are likely present [1]. To date, mutations in more than 25 genes, mainly autosomal dominant, have been linked to ALS, the major genes being *C9orf72*, *SOD1*, *TARDBP* and *FUS*/*TLS* (http://alsod.iop.kcl.ac.uk) [2,3]. A hallmark of both familial and sporadic ALS, is the presence of reactive immune cells in postmortem tissues, with microglial cells, the macrophages of the CNS, found in the vicinity of degenerating MN cell bodies and axonal tracts [4]. Microglial activation (as part of the neuroinflammatory response) has also been correlated to MN damage in ALS patients [5,6]. To understand the role of these immune cells during the disease course, ALS mouse models have been extensively used, mostly mice expressing mutant Cu/Zn Super oxide Dismutase (SOD1), as they remained for the longest time the only model with clear recapitulation of progressive MN degeneration, and only in most recent time, alternative mouse models start to appear, although with variable efficiency and usability. Importantly, these studies with mutant SOD1 mice strongly suggested that ALS is a non-cell autonomous disease, where certain, but not all (non-neuronal) cell-types surrounding MNs contribute to disease toxicity, in particular and the focus of this review, microglial cells [7–9]. Interestingly, while downregulating mutant SOD1 in MNs delayed disease *onset*, in microglial cells, it influenced disease *progression* [7–9]. This specificity is of prime importance and led to three insights: ***(i)*** consistent with mutant SOD1 expressing microglial cells not being able, on their own, to kill control MNs in mice [9], ALS remains primarily a MN disease, and microglial cells act by *reacting* to neurodegeneration; ***(ii)*** but it also indicates that reactive microglial cells *can* contribute to MN degeneration; and thus, ***(iii)*** it suggests that targeting microglial cells to slow disease progression could be effective, even in the sporadic ALS population. Microglial cells are part of the myeloid lineage, but they have a different developmental origin than most other tissue macrophages and blood monocytes. Other immune cells coming from the lymphoid lineage take also part in the disease. While depletion of B cells did not impact ALS disease in mice, specific deletion of CD4+ or CD8+ T lymphocytes has emphasized their opposite impact, detailed thereafter [10–14**].

When ALS genes show immune functions

C9orf72 is the major ALS-linked gene in the western world, characterized by a hexanucleotide expansion in its intronic region. Following this discovery, several mouse lines deleted for the *C9orf72* ortholog were produced (C9orf72 KO mice), to understand the unknown function of *C9orf72* and assess a possible loss-of-function mechanism [15–21]. While only one study reported mild locomotor abnormalities [15], five described C9orf72 KO mice suffering from strong immune system defects, including splenomegaly, massive myeloid and lymphoid cell infiltration in the spleen and the lymph nodes, an elevated rate of autoantibodies, and elevated levels of several pro-inflammatory cytokines in the blood - phenotypes resembling an autoimmune syndrome [15–19]. These defects were reported in homozygous but not heterozygous mice. Importantly, ALS patients carry the mutant expansion in only one *C9orf72* allele, which leads to some decrease of C9orf72 mRNA and protein levels [20,22*]. In *C9orf72* ALS patients, the link between their MN disease and possible immune system dysfunctions is not obvious. Although, an increased number of autoimmune diseases in ALS/FTD patients has been reported, a specific link with *C9orf72* remains to be determined [23]. Microglial cells isolated from C9orf72 KO (but not from C9orf72$^{+/-}$) mice displayed a lysosomal transport defect, also noticed in microglial cells of motor cortex and spinal cord postmortem tissues of mutant *C9orf72* ALS patients [18]. Removing murine *C9orf72* from neuronal and glial cell precursors only (but not targeting immune cells, including microglial cells), did not lead to any pathological neuronal signs of ALS or neuroinflammatory reaction [24]. Therefore, the current hypothesis is a mechanism of gain-of-toxic function in neurons (and glial cells of neural origin), possibly potentiated by a downregulation of C9orf72 and a loss-of-function haplo-insufficiency effect in immune cells, which, however, remains to be further characterized in mutant C9orf72 ALS patients.

TBK1 (Tank-Binding Kinase-1) discovered in 2015 as a new ALS-linked gene, was previously known for its functions in autophagy but also immune system regulation (originally described as a mediator of NF-κB signalling pathway) [25–27]. Full deletion of *Tbk1* in mice on a C57Bl6 genetic background, is embryonic lethal (due to liver failure), but some *Tbk1* KO mice on a 129S5 genetic background get born and, interestingly, develop a phenotype very similar to the C9orf72 KO mice, including massive infiltrations of mononuclear cells in different organs, higher rate of circulating monocytes and greater amounts of pro-inflammatory cytokines in the blood [28]. Mutations in *TBK1*, discovered in familial ALS cases, result (in most cases) in a loss-of-function of the mutant protein, strongly

suggesting a mechanism of haploinsufficiency [25,26]. In mice, heterozygous deletion of *Tbk1*, did not lead to any obvious MN degeneration (at least, not until 6 months of age) [29*]. However, and surprisingly, in the neurodegenerative and inflammatory context of mutant $SOD1^{G93A}$ ALS mice, heterozygous deletion of *Tbk1* induced, *first,* an accelerated MN denervation, followed, *second*, by decreased microglial activation at late disease stages and, remarkably, an increased survival. This highlights a possible dual role of Tbk1 in MNs and microglial cells, that both can influence the ALS disease course separately and distinctly.

In this context, another gene previously reported for carrying rare (most likely loss-of-function) mutations in ALS, *OPTN* (encoding for Optineurin), implicated in autophagy and regulation of NF-κB, interacts and gets phosphorylated by TBK1 [30–32]. While *Optn* KO mice did not show a clear MN degeneration, they did display signs of distal axonopathy [33]. *Optn* KO mice also produced elevated amounts of pro-inflammatory cytokines, and transcriptional analysis of *Optn* KO microglial cells revealed a rather pro-inflammatory phenotype. Therefore, both mutations in *Optn* and *Tbk1* induce inflammatory dysregulations, potentially contributing to ALS.

Other genes expressed by immune cells have been suspected as causative or modifiers of ALS, including *CX3CR1*, encoding the receptor for the chemokine CX3CL1 (fractalkine), constitutively expressed by neurons including MNs. Microglia are the only CNS cells expressing CX3CR1, making CX3CL1/CX3CR1 a candidate pathway for a MN-microglia cross talk [34,35]. Membrane-bound fractalkine is considered a main actor to keep microglia in a homeostatic, non-activated state [36]. Deletion of *Cx3cr1* in $SOD1^{G93A}$ ALS mice revealed increased MN loss and a tendency to higher microglial activation. $SOD1^{G93A}/Cx3cr1^{-/-}$ males (but not females) displayed a faster decline in motor functions and a reduced survival compared to $SOD1^{G93A}/Cx3cr1^{+/-}$ males [36,37*]. In ALS patients, specific variants in the *CX3CR1* gene have been associated with a faster progression of the disease, although there was no association with ALS disease risk [38,39*].

TREM2, well-known for its mutations causing rare forms of Alzheimer's disease, encodes for Triggering Receptor Expressed Myeloid cell 2, expressed by myeloid cells and in particular microglial cells [40]. A specific variant in *TREM2* (p.R47H) was identified as a risk factor for ALS without, however, being correlated with the age of onset, the site of the first symptoms or disease progression [41]. *TREM2* encodes for an extracellular receptor involved in immune

functions, especially phagocytosis. The p.R47H variant was associated with maturation defects of the receptor causing impaired phagocytosis [42]. Increased expression of *Trem2* mRNA was measured in the spinal cord of symptomatic mutant SOD1^{G93A} mice, but also sporadic ALS patients [41]. Very recently, upregulation of *Trem2* mRNA was measured, at early disease stages in ALS mice, with a new technique of spatial transcriptomics, allowing full transcriptome analysis directly on tissue sections [43**]. Interestingly, this study revealed that Tyrobp, which forms a complex with Trem2 that triggers phagocytosis, was up-regulated on the mRNA level, even before *Trem2*. In addition, the Trem2-ApoE pathway (Trem2 is also known for inducing ApoE signalling) was previously shown to trigger a common molecular signature of microglial cells in several neurodegenerative conditions, including ALS [44**]. Deletion of *Trem2* in mutant SOD1-expressing microglial cells, induced downregulation of inflammatory genes and restored microglial homeostatic gene expression [44**]. The example of Trem2 is an interesting indicator of microglial implications in ALS, suggesting that such implication could be triggered both by internal defects, e.g. caused by genetic ALS-linked mutations (as *SOD1*), but also by (perturbed) external signalling, mediated, through the neurodegenerative/neuroinflammatory context.

Lymphocytes implicated in ALS neurodegeneration and a possible biomarker in ALS

Originally, CD4+ cells were the main T cell population thought to be implicated in ALS, with removal of CD4+ cells (CD4 KO mice) showing reduced survival of SOD1^{G93A} ALS mice [10,11]. Simplified, CD4+ cells can be subdivided in regulatory (Treg) and effector (Teff) T cells, with Tregs regulating the proliferation of Teff cells. In SOD1^{G93A} ALS mice, passive transfer of T cell populations enriched in Tregs compared to whole CD4+ T cells, led to a more pronounced increase in survival [45,46]. Treg expansion in SOD1^{G93A} mice (using peripheral injection of Interleukin 2 (IL-2)/IL2 antibody complex), decreased microglial activation and increased MN and mouse survival [47**]. In ALS patients, a higher proportion of Treg in the blood was correlated with a slower rate of disease progression. [46–48]. Functional assays with Tregs of ALS patients showed their reduced ability to suppress the Teff proliferation, compared to control Tregs [49**]. Therefore, both reduced numbers and impaired suppressive function could influence ALS progression. A phase II clinical study assessing the potential of IL-2 as a therapeutic agent is currently in progress in ALS (ClinicalTrials.gov NCT03039673). FoxP3, a marker of Treg was found downregulated in ALS patients' Tregs compared to controls. FoxP3 levels were also inversely correlated with the rate of disease progression, with FoxP3 expression being reduced in rapidly progressing

patients [48,49**]. Importantly, a prospective study pointed out low levels of FoxP3, not only reflecting a rapid disease progression at the time of blood collection, but also able to predict future rapid disease progression rate. Therefore, FoxP3 appears as a promising biomarker for ALS disease progression [48].

More recent studies have focused on the, so far in ALS understudied, CD8+ lymphocytes. While previous reports mentioned a very late infiltration of CD8+ T cells (compared to CD4+ cells) in the spinal cord of $SOD1^{G93A}$ ALS mice (arguing in favor of no possible impact of these cells), two recent studies measured the presence of infiltrating CD8+ T cells as early as disease onset [10,13**,14**]. To study CD8+ T cell involvement, ALS mice either lacking functional CD8+ T cells ($CD8a^{-/-}$ /$SOD1^{G93A}$) or defective for CD8+ T cell actions (ß2-microglobulin $(ß2M)^{-/-}$ /$SOD1^{G93A}$, displaying no MHC-I expression) were generated [13**,14**] or were injected with anti-CD8 antibodies [50**]. Although survival of $CD8a^{-/-}$ /$SOD1^{G93A}$ mice or of SOD1 mice injected with anti-CD8, was not modified, survival of $ß2M^{-/-}$ /$SOD1^{G93A}$ mice showed a different outcome in two independent studies with either, for the earlier study, no modified onset but a decreased survival [51], or in the more recent study, an earlier onset (measured by muscle weakness) followed by a prolonged survival [13**]. Interestingly, in $CD8a^{-/-}$ /$SOD1^{G93A}$ mice, MNs survived better in the lumbar spinal cord, while in $ß2M^{-/-}$ /$SOD1^{G93A}$ mice, it was not the case, however MNs were protected in the cervical spinal cord [13**,14**,51]. Since CD8+ T cell toxicity towards MNs was dependent on MHC-I recognition by CD8+ T cells *in vitro,* a compensatory mechanism could explain the difference obtained in $ß2M^{-/-}$ and $CD8a^{-/-}$ mice and the heterogeneity between cervical and lumbar MNs in $ß2M^{-/-}$ mice [13**,14**]. As MHC-I is expressed also by myeloid cells, an additional effect of deleting MHC-I could also come from deregulated microglia/macrophage reactivity. In addition, CD8+ T cells seem to have a dual role in ALS, with a protective effect at the periphery (accelerated muscle atrophy and axonal defects in $ß2M^{-/-}$ /$SOD1^{G93A}$ mice), and a toxic role on MN survival when infiltrating the CNS [13**,14**] (Fig1). Of note, for both CD4+ and CD8+ cells, it remains to be determined if their role in ALS disease is linked to mutant gene expression (e.g. mutant SOD1) or their neuroinflammatory responses.

Another subpopulation of lymphocytes (which are part, however, of the innate immunity, as they do not recognize specific antigens), the Natural killer (NK) cells with their cytotoxic activity, have recently also been studied in ALS. A longitudinal study showed an increased number of NK cells in the blood of ALS patients compared to controls, but also in ALS patients along the disease [52*]. Interestingly, infiltrating NK cells have been observed in the

spinal cord of ALS mice, however, decreasing the number of NK cells in $SOD1^{G93A}$ mice had no impact on disease [11,50**].

Mast cell involvement in ALS

Mast cells, granulated leucocytes involved in allergic reactions, have recently gained interest as the target cells of Masitinib (AB1010), a Tyrosine Kinase inhibitor, tested in clinical trials on ALS patients (ClinicalTrials.gov NCT02588677). Mast cells were observed in muscles (extensor digitorum longus) and sciatic nerves of symptomatic $SOD1^{G93A}$ ALS rats but not in the spinal cord. Attention has therefore been focused on their impact at the periphery [53**,54*]. Presence of mast cells (in variable amounts compared to controls), was also described in ALS patient quadriceps muscles with frequent degranulation, suggesting mast cell reaction and a potential role in ALS [54*]. In $SOD1^{G93A}$ rats, Masitinib treatment led to a decreased number of mast cells in muscles and sciatic nerves and delayed neuromuscular junction denervation, suggesting a deleterious role for mast cells towards axonal integrity in ALS [54*,55] (Fig. 1). However, Masitinib is also an inhibitor of Csf1-R, required for macrophage and microglia survival. Since selective inhibition of the Csf1-R (with GW2580) was also potent at slowing the disease course in $SOD1^{G93A}$ mice [56], the impact of Masitinib in ALS could be through mast cells and macrophage/microglial cells, and specific depletion or inhibition of mast cells would be needed to definitely conclude on mast cell contribution in ALS.

Deregulation of monocytes in ALS

For their easy accessibility in the blood, and since, as part of the myeloid lineage, they share common functions with microglial cells, monocytes have gained recent interest in ALS. While analysis of overall proportions of monocytes among all the leucocytes was not sensitive enough to show differences between ALS patients and healthy controls, a recent longitudinal study, performed over 3 years, highlighted an increased monocyte number (and also of the whole leucocyte content in the blood) during the course of ALS, although it was not correlated with the functional ALSFRS-R score [52*,57]. Expression of the specific marker, CX3CR1, in this longitudinal study, correlated, however, with the ALS Functional Rating Scale Revised (ALSFRS-R) score [52*]. Monocytes can be divided into two major populations, classical monocytes, considered to be inflammatory and recruited into the tissues upon inflammation, and non-classical or patrolling monocytes, in charge of tissue homeostasis. A higher proportion of classical to non-classical monocytes has been described

in ALS patients compared to healthy controls [58*]. One hypothesis would therefore be, that deregulated proportions of monocyte subtypes could lead to dysfunctions of immune responses, contributing to ALS disease. However, possible intrinsic dysfunctions in ALS monocytes have also been reported, and included alterations in phagocytosis, cytokine secretions upon external stimuli, and of their transcriptome [57,58*,59**] (Fig. 1). Upregulation of genes involved in inflammatory responses such as Interleukine-1ß, Interleukine-8 or *NLRP3*, and downregulation of genes involved in Tumor Necrosis Factor or Toll Like Receptor signaling (two important pathways of the innate inflammatory response), were revealed with RNAseq analyses in monocytes from ALS patients compared to controls, suggesting a possible dysfunction of myeloid cells in ALS [57,59**].

Conclusion

Although, MNs are the primary cells degenerating in ALS, rising evidence show that ALS has an inflammatory component, with some ALS related genes linked to different immune functions. Defects caused by mutations in those genes are likely to drive defects in their immune functions, potentially impacting ALS disease course. Modifying immune cells through bone marrow transplantation has been envisaged and targeting specific myeloid or lymphoid populations is the focus of ongoing clinical trials. However, better knowledge of the specific deregulations of the different subpopulations of immune cells (including CD4+ Tregs, CD8+ T cells, mast cells and monocytes) and this, over the course of the disease, between different ALS patient cohorts, are now necessary and the essential next step required for the development of immune-related therapies and biomarkers in ALS.

Key points:

- Recent genes discovered in ALS reinforce the link between the disease and alteration of the immune system.
- Blood lymphocytes and in particular regulatory T lymphocytes appear as promising predictors of the rate of disease progression.
- Presence of mast cells in muscles in ALS models and patients and deregulated monocytes in ALS patients highlight new immune cell players at the periphery to consider for future therapy.
- Failure of fundamental functions of monocytes calls on a possible systemic immune deregulation in ALS

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Conflicts of interest

The authors declare no conflict of interest

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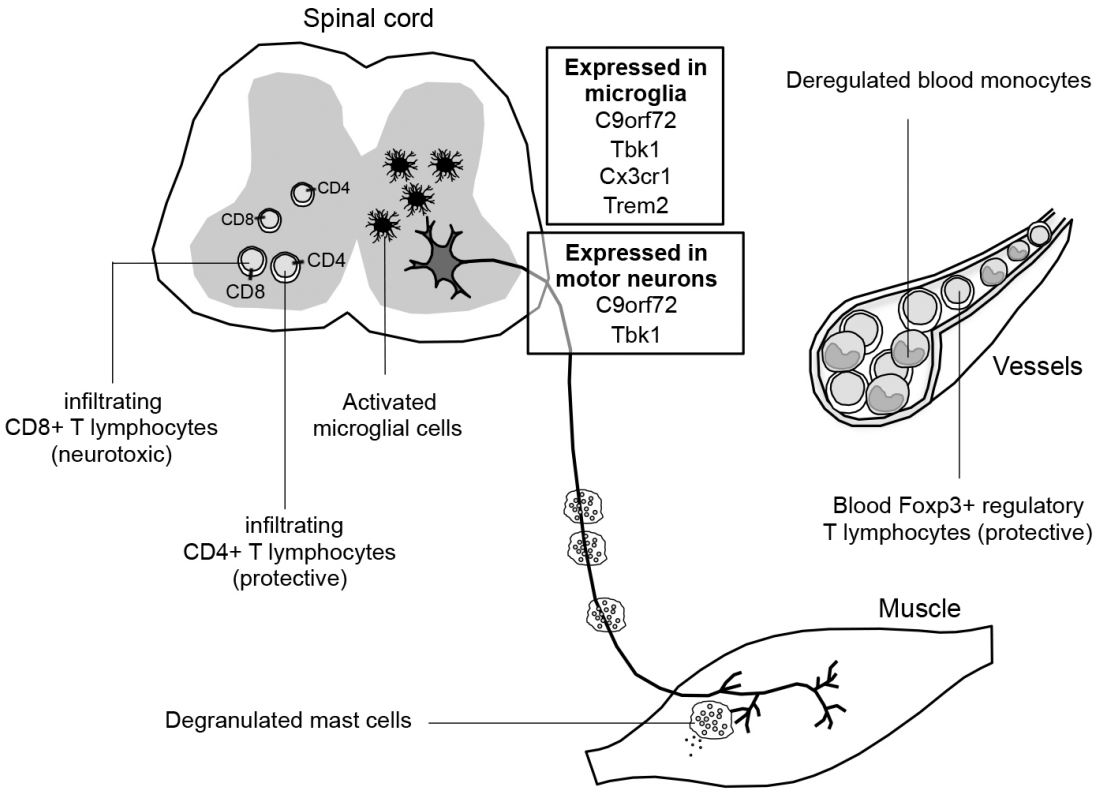

Figure 1. Evidence of involvement of neuroinflammatory processes in amyotrophic lateral sclerosis (ALS). New ALS-linked genes with important immune functions are shown in boxes. Different subpopulations of immune cells that have been linked to ALS: CD4+ (including Foxp3+ regulatory cells) and CD8+ T lymphocytes, mast cells, monocytes and microglial cells are depicted with their described functions in the different tissues.