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Advances on cellular clonotypic immunity in amyotrophic lateral sclerosis

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Abstract: Amyotrophic lateral sclerosis (ALS) is a fatal neuromuscular disease, characterized by progressive degeneration of upper and lower motor neurons in the cortex and spinal cord. Although the pathogenesis of ALS remains unclear, evidence on the role of the clonotypic immune system is growing. Adaptive immunity cells often appear changed in number or activation profile peripherally and centrally. However, their role in ALS appears conflicting. Data, from human and animal model studies, currently reported in literature show that each subset of lymphocytes and their mediators may mediate a protective or toxic mechanism in ALS, affecting both its progression and risk of death. In the present review article and attempt is made to shed light on the actual role of the cellular clonotypic immunity in ALS by integrating recent clinical studies and experimental observations.

Keywords: Amyotrophic lateral sclerosis (ALS); neurodegeneration; neuroinflammation; neuromuscular disease; autoimmunity; the clonotypic immune system

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1. Introduction

Amyotrophic lateral sclerosis (ALS) is a neurological disease characterized by the irreversible and progressive loss of motor neurons located both at the cortical level - the so-called *first motor neurons* - and in the gray matter of the spinal cord, and in the nuclei of the cranial nerves - the so-called *second motor neurons* (1). Sporadic ALS account for about 90% of cases of the disease, while in about 10% of cases ALS is familial being associated with genetic variants in numerous genes, including: the chromosome 9 open reading frame 72 (C9ORF72), Cu/Zn superoxide dismutase (SOD1), TAR DNA-binding protein 43 (TARDBP) and FUused in Sarcoma/Translocated in LipoSarcoma (FUS/TLS) genes, predominantly with autosomal dominant transmission (2).The pathogenesis of both forms, sporadic and familial, is complex, partially clear, and certainly not restricted to an unique factor, i.e. genetic factors, but strictly linked to several triggers and drivers. A strong involvement of immune system has been also documented. Precisely, it is well recognized the role of innate immunity, having a pivotal part in the homeostasis of central nervous system (CNS) in inducing neuroinflammation for restraining infections and eliminating pathogens, cell debris, and aggregated or misfold proteins, as well as in ASL, where neuroinflammation is continuous, harmful for CNS cells and constitutes the typical hallmark (3, 4). While, adaptive, or better clonotypic, immunity (5) is emerging in recent years in the studies on CNS health and disease, as a fundamental component with a double function, first mediating immune-surveillance and defense against neurotropic viruses (6, 7), as well as maintaining CNS homeostasis and integrity, and promoting neurogenesis and improving cognitive function. In CNS degenerative diseases, i.e., ALS, clonotypic immunity commonly shows dysregulation and abnormal immune responses (4). Accordingly, clonotypic immune cells often appear peripherally and centrally changed in number or activation profile. Data, from human and animal model studies, currently reported in literature, evidence that each subset of lymphocytes and their mediators can mediate a protective or toxic mechanism in ALS, affecting both its progression and risk of death. In

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the present review article and attempt is made to shed light on the actual role of the cellular clonotypic immunity in ALS by integrating recent clinical studies and experimental observations.

2. Recent evidence on clonotypic immunity in ASL

The interplay between clonotypic immune system and ALS stems from several clinical and experimental evidence. For example, ALS patients are more often affected by autoimmune diseases (8), and the presence or absence of cognitive impairment in ALS patients has been associated with a different peripheral immune profile, with lower total lymphocyte, CD4+, B cell counts and lower CD8+ lymphocytes in patients with cognitive decline than in those without objective cognitive impairment (9). Current evidence on the biological effects mediated by mutations in the genes abovementioned, additionally underlines a close relationship between the onset and progression of ALS and clonotypic responses of the immune system. Among them, mutations in the C9orf72 gene, which is the most frequent cause of inherited ALS, due to an expansion of the GGGGCC sequence within an intronic region (10), indirectly reveals some links of the disease to the clonotypic immune system. Interestingly, C9orf72 knockout (KO) mouse models, with mild motor deficits exhibit a dysregulated immune response, characterized by T-cell activation, overproduction of autoantibody and cytokines, and signs of massive leukocyte infiltration, such as lymphadenopathy and splenomegaly, developing a systemic lupus erythematosus-like disease (11). C9orf72 KO mice also show the absence of mitochondrial degradation of Stimulator of Interferon Genes (STING) signaling, leading to the maintenance of interferon production and the activation of adaptive immunity (12). In addition, ALS patients with C9orf72 mutation have higher levels of Interferon-α (INF-α) in cerebrospinal fluid (CSF), compared to ALS patients with other mutations (13).

The role of clonotypic immunity in the context of ALS seems to emerge and shows the involvement of different subsets with different functions depending on the stage of the disease. T cells have been shown to enhance survival of mononuclear cells (MNs) in SOD1 mutant mice through protective neuroinflammation, probably via Interleukin-4 (IL-4), and abundantly infiltrate the spinal cord and brain during ALS progression (14). Motor impairment has also been shown to

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be accompanied by a decline in the functions of the regulatory T cells that inhibits microglia activation in SOD1 mutant mice (15). Therefore, these data suggest that the neuroprotective functions of the immune system may prevail in an early stage of the disease. The progression of the disease is, however, accompanied by several changes in the immune system, such as: the acquisition of an inflammatory phenotype by microglia cells (16), thymic involution (17), increased levels of pro-inflammatory cytokines (18), leucocyte infiltration into the central nervous system (CNS) (14). In ALS, the infiltration of lymphocytes into the CNS has different consequences depending on the various cell types. Accordingly, CD4+ CD25+ regulatory T cells (Tregs) and CD4+ Th2 cells tend to mediate a neuroprotective effect, whereas the presence of CD4+ Th1, CD4+ Th17, cytotoxic CD8+, and Natural killer (NK) cells and effector T lymphocytes (Teffs) is associated with a more rapid course of ALS and an increased risk of death (19; 20).Few data are available on the role of B lymphocytes, plasma cells and antibodies in the pathogenesis of ALS. Cases of paraneoplastic ALS with specific anti-neuronal antibodies deserve particular attention, because these are extremely rare forms of the disease whose definitive diagnosis is difficult. However, the description of some of these forms in a recent review (21) suggests that there may be an interaction between cancer and neurodegeneration in ALS mediated by the immune system.

The evidence available in the literature for each of these cell types is described and discussed in this review.

3. T helper 17 cells

Changes in the clonotypic cellular composition of the immune system have been found in ALS patients. A peripheral increase in the number of Th17 T cells has been found to be positively correlated with symptom severity and disease progression (22). In addition, Interleukin-17 (IL-17) levels were assessed increased in both serum and CSF of ALS patients, likely indicative of Th17 activation (23). Accordingly, higher levels of IL-17 have been quantified in ALS patients compared with patients with primary progressive multiple sclerosis (PPMS) (24). Among the Th17-related

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cytokines, only the IL-17A has been shown to have a clear pathogenetic role in ALS models. Indeed, MNs of patients with ALS, derived from the differentiation of induced pluripotent stem cells, were found to express the receptor for IL-17A (IL-17AR) and to be vulnerable to its neurotoxic action in a dose-dependent manner, but not damaged by exposure to IL-17F. Furthermore, targeting IL-17A has shown to protect MNs from death (22). IL-17 secreting cells, CD8+ T cells and mast cells, have been observed to infiltrate the gray matter of the spinal cord in ALS patients. In these subjects, increased peripheral levels of IL-17A have been reported to parallel decreased serum levels of IL-10, which has anti-inflammatory effects. This could help explain the increased susceptibility to the effects of IL-17A (25).

4. CD8+ T cells

CD8 + cells have been shown to be activated both peripherally and intrathecally in ALS patients compared with healthy controls, dementia patients and PPMS (26). The percentage of CD8+ lymphocytes has been found to be negatively associated with ALS prognosis, and their increase has been correlated with the risk of death (27). Although it was initially reported that the infiltration of the spinal cord by CD8+ lymphocytes occurs later, more recent work has revealed their presence in a mouse model already in the early stage of the disease (28). CD8+ lymphocytes infiltrating the spinal cord of a $SOD1^{G93A}$ mouse model of ALS have been shown to interact with MNs through the major histocompatibility complex class I (MHC-I) and induce their killing by involving Fas and granzyme mechanisms (29). However, the role of CD8+ lymphocytes in ALS, mediated by their interaction with MHC-I, has proven to be rather complex and still unclear. Although CD8+ lymphocytes have been shown to be toxic for spinal cord MNs in ALS models, some protective effects have been observed at a more peripheral level. Specifically, using a $SOD1^{G93A}$ mice model, the absence in the sciatic nerve of the interaction between MHC-I on the surface of the motor axon and CD8+ lymphocytes appears to accelerate atrophy and denervation of hindlimb muscles, anticipating disease symptoms. However, it has been shown that the lack of

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interaction between CD8+ lymphocytes and microglia in the spinal cord protects cervical MNs from death and delays disease onset (30).

5. Natural killer cells

The presence of NK cells, which contribute to innate and adaptive immunity, was observed in the spinal cord and motor cortex of postmortem tissues from sporadic ALS (sALS) patients, whereas the presence of these cells was not found in tissues used as controls. In contrast, NK cells were shown to be reduced in the peripheral blood of sALS patients compared with controls (31; 32). Similarly, postmortem data in humans and SOD1G93A mice have reported that NK cells infiltrate the motor cortex and the spinal cord with a peak concentration during the early phase of the disease and a decrease in number during motor decline. In addition, they have been observed that such mice express high levels of activation markers on their cell surface. In $SOD1^{G93A}$ and $TDP43^{A315T}$ mice, early treatment with anti-NK cells antibodies has been shown to increase survival and delay disease onset (32). However, NK cell depletion has been shown to prolong survival in female but not male SOD1 mice, suggesting that NK cells are related to ALS in a sex-specific manner (33). In addition, it is also important to consider the subtype of NK cells rather than their total number. Indeed, although some authors noted no change in the total number of NK cells in the CSF of their study patients compared with controls, cell characterization in the slowly progressive ALS group showed an increase in the number of regulatory rather cytotoxic NK cells (22). In general, the immune aspects of NK cells in ALS are poorly explored and further investigations is needed, given the complexity of the topic, as shown by a possible paraneoplastic case of MN disease with rapidly progressive course associated with NK cells leukemia (34).

6. Regulatory T cells

It has been reported that of CD4+ CD25+Tregs levels correlate with the rate of disease progression in ALS patients and mice models. Using the Appel ALS score (AALS), 54 patients with ALS have been clinically evaluated and divided into two groups according to disease progression. The 28 patients with a slowly progressive clinical course (AALS points per month

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<1.5) had a percentage of T reg lymphocytes that did not vary from controls. In contrast, the 26 patients with rapidly progressive ALS (AALS points per month ≥1.5) showed a percentage of T-reg cells reduced by about one-third compared with controls and patients with a less aggressive clinical course (35). In another study from Sheean and colleagues, the levels of T-reg lymphocytes in 24 male and 9 female ALS patients were found to be inversely correlated with the rate of progression, although no clear difference was found between the T-reg cell levels of ALS patients and those of the control group (36).

In Cu^{2+}/Zn^{2+} superoxide dismutase (mSOD1) mutant ALS mice, an increase in the number of T-reg lymphocytes was found in the early stages of slowly progressive disease. This has been shown to improve the course of the disease by increasing the expression of IL-4 and enhancing the protective role of M2 microglia. In addition, transfer of CD4+ T lymphocytes from SOD1 mutated mice with increased number of T-reg to $SOD1^{-/-}$ mice has been shown to reduce disease progression compared with the transfer of wild-type CD4+ T lymphocytes (37).

Thus, the course of ALS appears to be characterized by a first phase in which the neuroprotective role of the immune system predominates, and a second phase characterized by the development of neurotoxicity by microglia and proinflammatory Teffs (19). Furthermore, it has been observed that T-reg cells support the neuroprotective phase of the disease by inhibiting microglia through the production of IL-4 and the Teffs through the production of IL-4, IL-10, and Transforming Growth Factor-β (15).

7. B-cells and immunoglobulins

Although some authors initially suggested that that B-cell and even anti-retroviral immune responses may be present in ALS (38), few data are available on the involvement of B cells in ALS, and in any case their role appears to be very limited. B cells isolated from SOD-1 mouse models of ALS before, during, and after the disease onset showed a phenotype and responsiveness like those from wild-type mice. In addition, SOD1 mice lacking mature B cells because they were blocked at the pro-B cell stage were shown to develop disease with clinical features identical to those of

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control SOD-1 mice (39). It has been shown that possible signs of B cell involvement in some mechanisms of the disease arise mostly from indirect signs. Indeed, autoantibodies against neurofilaments, actin and desmin have been found in the spinal cord of ALS patients, and these antibodies have been shown to positively correlate with disease the severity (40). In contrast, the presence of anti-SOD-1 antibodies has shown a positive association with survival in sALS patients (41). However, it should be noted that although T helper lymphocytes and cytotoxic T lymphocytes infiltrate the areas affected by degeneration, no infiltration by B lymphocytes has been found in the tissues of ALS patients (42), although the expression of IgG subclass in ALS has been found to be altered (43) and suggested some dysfunction of B lymphocytes.

Two animal models of autoimmunity developed in ALS were used to explore the role of IgG reactivity in ALS. One is experimental autoimmune motor neuron disease (EAMND), induced by inoculation of purified MNs and characterized by the loss of lower MNs. The second is experimental autoimmune gray matter disease (EAGMD), induced by inoculation of homogenate from the ventral horn of the spinal cord and leading to the death of upper and lower MNs. Both models mimic the ALS with respect to depletion of MNs and for neurophysiological findings (44). Passage of serum immunoglobulins isolated from both models and transferred to control mice appears to passively reproduce some alterations of the animal-derived models, such as increased calcium levels within MNs and increased release of acetylcholine from axons of spinal MNs; the latter suggests a possible role of the antibodies-mediated response in ALS in interaction with the neuromuscular junction (44; 45). In ALS, IgG accumulation in MNs has been described to induce altered calcium homeostasis, and inoculation of anti-MN antibodies induces similar alterations in mice (46). Similarly, Polgár and colleagues observed that serum transfer from ALS patients with C9orf72 mutation into ventral spinal cord mice causes increased calcium levels in MNs resulting in neurodegeneration (47); similar data were provided by the group of Obál and colleagues through long-term intraperitoneal injection of serum from ALS patients (48).

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8. Discussion and conclusions

Recent data seem to suggest that in ALS, the classic neurodegenerative disease with a usually rapid course, the documented contribution of inflammation may involve cells involved in clonotypic immunity in addition to glial activation and consequently to induction of innate immunity (49). At present, an initial pathogenetic role of such cells cannot be hypothesized, although it has been shown that in an animal model of SOD1-ALS T-lymphocytes localize to the CNS before the onset of symptoms (50). However, the balance between CD4+ and CD8+ and between T-reg and Teffs cells has been reported to influence the prognosis of the disease. Furthermore, there is not a clear association between the dysfunctions of the immune system, and particularly clonotypic immunity, and the different clinical forms of ALS. The higher incidence of autoimmune diseases, such as myasthenia gravis, polymyositis, dermatomyositis, and type I diabetes mellitus (8), in ALS patients suggests an increased reactivity of the immune system in these patients. We speculate that it is possible that progressive neurodegeneration causes the release of autoantigens and triggers the antibody-mediated response. In contrast, other authors have hypothesized that the formation of aggregates by SOD1 and TDP-43 triggered by CD4+ cells may elicit an immune response in the CNS towards certain antigens (51). However, the promising results reported on the potential role of the clonotypic immune system in negatively influencing the course of ALS has led to the development of substances that can act as immunomodulators, the effects of which are particularly interesting. Consistently, some authors have shown that Treg cells dysfunction is transient and that their expansion under different environmental conditions can lead to recovery of their functions. Subsequent autologous transplantation of these cells has also been shown to slow the progression of sALS in a phase I clinical trial (52). Such approaches therefore seem promising. The **Table 1**, below reported, shows the main immuno-modulatory agents and treatment options examined so far in ALS patients and animal models, which show interesting results and encourage further studies on large cohorts both to validate their effects and possible adverse reactions and to elucidate the actual role of clonotypic cells in ALS. This could enable the

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development of more appropriate treatments in preclinical and clinical stages of ALS to delay or halt its onset and progression. In addition, works based on omics technologies in the clonotypic subsets characterized and detected in ALS and its onset and progression stages could enable the development of personalized therapies. Overall, if confirmed, these research hypotheses will play an important role in terms of ALS treatment and prognosis, with significant health economics implications for national health care systems.

Table1 Main immune-modulating agents and treatment options evaluated in ALS patients and in animal models

Treatment	Dose and administration	Number of cases or types of animal model	Laboratory and/or clinical outcomes	References
Aldesleukin	Intravenous Low dose of Interleukin-2 for five days at week 1, 5 and 9	24 patients	Increase in Treg response; no significant change in ALSFRS-R score compared with placebo group	(53)
Dimethyl fumarate	Oral administration of 480 mg daily for 36 weeks	72 patients	No significant changes in ALSFRS-R score compared with placebo group	(54)
RNS60	Weekly intravenous infusion and daily nebulization	13 patients	No changes in biomarkers, (i.e., FOXP3 mRNA and IL-17 levels)	(55)
RNS60	300 µl/mouse intraperitoneally every other day	C57BL/6-SOD1^{G93A}	Increase in CD4+/Foxp3+ T regulatory cells and neuroprotection	(56)
Infusions of autologous Treg in ALS	Intravenous Tregs 10^6 cells/kg, initially 4 doses over 2 months and in later stages 4 doses over 4 months of disease (n. 8 total infusions)	Three patients with sALS	Increase Treg function and slowed disease progression as measured by Appel ALS scale for each patient	(52)
Fingolimod	Orally administration, dose of 0.5 mg/day for 4 weeks	18 patients with ALS	Riduction of circultaing lymphocytes in ALS patients. No effects on ALSFRS-R	(57)
Glatiramer acetate	Subcutaneous injection of 40 mg/day	366 patients with ALS enrolled in a phase II clinical trial	No effects on ALSFRS-R	(58)
Tocilizumab	Treatment of PMBCs overnight with 2 µg/ml apo-G37R + 10 µg/ml tocilizumab	4 patients with ALS	Reduction of secretion of cytokynes and chemiokynes from PMBCs of patients	(59)

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