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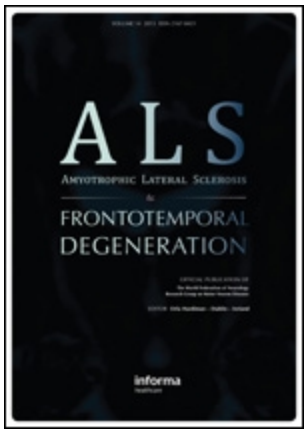

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The Involvement of Regulatory T Cells in Amyotrophic Lateral Sclerosis and Their Therapeutic Potential.

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Abstract

Neuroinflammation, meaning the establishment of a diffuse inflammatory condition in the CNS, is one of the main hallmarks of amyotrophic lateral sclerosis (ALS). Recently, a crucial role of regulatory T cells (Tregs) in this disease has been outlined. Tregs are a T cell subpopulation with immunomodulatory properties. In this review, we discuss the physiology of Tregs and their role in ALS disease onset and progression. Evidence has demonstrated that in ALS patients Tregs are dramatically and progressively reduced in number and are less effective in promoting immune suppression. In addition, Tregs levels correlate with the rate of disease progression and patient survival. For this reason, Tregs are now considered a promising therapeutic target for neuroprotection in ALS. In this review, the clinical impact of these cells will be discussed and an overview of the current clinical trials targeting Tregs is also provided.

Keywords: amyotrophic lateral sclerosis, neuroinflammation, neuroimmunology, regulatory T cells, clinical trials.

Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease characterised by the progressive loss of both upper and lower motor neurons which causes death usually within 3-5 years of diagnosis (1). Currently, there is no effective disease modifying treatment available (2). Riluzole, is the only available drug which has been approved by both the U.S. Food and Drug Administration (FDA) and the European Medicine Agency (EMA). However, riluzole only extends patients survival by 3 months on average (3, 4). Edaravone, a free-radical scavenger, was approved by the FDA for the treatment of ALS, although, longer-term effects of this drug are still to be evaluated (https://www.fda.gov/news-events/press-announcements/fda-approves-drug-treat-als, 5, 6).

ALS is considered a multifactorial disease as a series of mechanisms and pathways are implicated in the disease (7, 8). Among them, neuroinflammation is perceived as a dysregulation of the glial cells which surround neurons in the CNS and which can create an inflammatory milieu. Its main features are: activation of microglia and astrocytes, production of excessive quantities of pro-inflammatory cytokines and infiltration by T lymphocytes (9). Recently, a key role for regulatory T cells (Tregs) in the pathophysiology of ALS has been demonstrated.

Physiology of regulatory T cells

Tregs are fundamental modulators of the immune response: they maintain self-tolerance and homeostasis, preventing the onset of autoimmune diseases. These cells are generally classified into two subgroups: thymus-derived and peripheral inducible (10). The first subtype is produced in the thymus during the negative selection process; they then migrate into peripheral tissues to perform their functions. They are also known as

naturally occurring or $CD4^+CD25^+$ Tregs because of the surface molecules they express. The presence of $CD25^+$, also referred to as interleukin-2 receptor alpha (IL2RA), indicates the importance of IL-2 for Treg functions (10). Another key feature of these cells is the expression of Forkhead box protein 3 (FOXP3) (11). The second subtype of Tregs are peripheral in origin. These cells are derived by antigen-exposed $CD4^+$ T cells in peripheral tissues following the exposure to specific molecules. Induced $CD4^+$ Tregs can be further classified into $FOXP3^+$ or $FOXP3^-$ cells (12).

Clearly, FOXP3 has a crucial role for Tregs. It is a transcriptional regulator which is fundamental for their development and function. It can act both as a transcriptional activator or repressor because it interacts with several transcription factors and proteins involved in epigenetic regulation (13). In particular, it prevents the transcription of pro-inflammatory cytokines such as IL-2 and IFN-γ and it concomitantly activates immune suppressors including cytotoxic T lymphocyte antigen 4 (CTLA4) (13). Furthermore, other key markers for Tregs are glucocorticoid-induced tumour necrosis factor receptor (GITR) and inducible T cell co-stimulator (ICOS). GITR, also referred to as TNFRSF18, plays a role in Treg suppressive activities, in fact, antibodies against GITR can abrogate Treg immune modulatory functions, and it is also crucial for thymus Tregs differentiation process (14, 15, 16). ICOS is a costimulatory molecule which is known to exert various roles within the immune system, participating both to inflammatory and suppressive processes (17). However, ICOS appears to play a role in Treg functions. In fact, the blockage of ICOS interaction with its ligand (ICOSL) causes a decrease in the expression of CTLA4 and ICOS deficiency induces reduction in FOXP3 expression (18, 19).

Another key mediator of Treg activity is IL-2. This cytokine is known to be essential for the development and survival of these cells (20). Although IL-2 is known to exert

pleiotropic functions on the immune system, evidence from IL-2 or IL-2 receptor (IL-2R) knockout mouse models, demonstrates that these mice do not develop immune deficiency syndromes, but they do show features of autoimmune diseases (21). IL-2 administration promotes the activation of different pathways in effectors T cells (Teffs) and Tregs. In fact, the binding of this cytokine with its receptor in Teffs results in the activation of STAT5 and S6 kinases, which triggers PI3K-Akt and mTOR pathways. In contrast, in Tregs, IL-2 binding mediates direct activation of STAT5 and then FOXP3 becomes functional (22). Interestingly, it seems that after administration of low-dose IL-2 (ld-IL-2), there is a selective increase in phosphorylation, and thus activation, of STAT5 in Tregs, which corresponds to a decrease in the same modification in Teffs (23). Consistent with this, multiple studies demonstrate that low-dose IL-2-based treatment promotes the selective expansion of the Treg population in both mice and humans (24), (25), (26).

Tregs exert several immunosuppressive functions: they suppress Teffs, B cells, natural killer cells (NKs) and antigen-presenting cells by inhibiting their activation, proliferation and function (27). Moreover, they can alter the activation state of microglia/macrophages by promoting the M2 (or anti-inflammatory) phenotype (28). Several mechanisms have been proposed to mediate these functions (**Figure 1**):

- *Inhibitory cytokine release*: Tregs can secrete anti-inflammatory cytokines including IL-10, IL-35 and TGF-β which inhibit the function of Teffs (29).

- *Cytolysis induction*: Tregs are able to produce and release granzyme B and perforin which induce cytolysis and apoptosis in Teffs, B cells and NKs (30), (31), (32).

- *Metabolic disruption*: This proposed mechanism is based on the consumption of

local IL-2 by Tregs. This would cause the deprivation of this cytokine which is necessary for Teffs, leading to Teffs IL-2-deprivation-mediated apoptosis (33), (34).

- *CTLA4-mediated mechanism*: Tregs can physically interact with dendritic cells (DC) due to the binding of CTLA4 on the Treg surface and the co-stimulatory molecules CD80/CD86 expressed by DC. This leads to the production of indoleamine 2,3-dioxygenase (IDO) in DC, a potent immune regulator which suppress T cells and NKs (35), (36).

- *cAMP-mediated mechanism*: Tregs express high levels of cAMP and this molecule can be transferred to Teffs through gap junctions. This provokes the activation of inducible cAMP early repressor (ICER) which inhibits nuclear factor of activated T cells (NFAT), a transcription factor which is necessary for IL-2 production. Thus, this can lead to IL-2 deprivation-mediated apoptosis (37), (38).

Regulatory T cells in ALS

Evidence demonstrates a key role for Tregs in ALS disease onset and progression. In 2009, a dramatic decrease in the number of $CD4^+CD25^+$ Tregs in the peripheral blood of sporadic ALS patients was first reported (39). Since then, several laboratories have been studying these cell types and their possible role in ALS.

Changes in the Treg population over the disease course were demonstrated in mutant SOD1 (mSOD1) mouse models of ALS. In particular, in an early phase of the disease the number of $CD4^+CD25^+$ and $CD25^+FOXP3^+$ Tregs is increased while, as the disease progresses, they gradually decrease. More precisely, in 18-week old mice which reflect a later disease phase, a shift from the neuroprotective Treg/Th2 and M2 ("alternatively

activated" or anti-inflammatory) microglia population to a neurotoxic Th1/M1 ("classically activated" or inflammatory) phenotype is detected, and this enhances disease progression (40, 41). In fact, in the early phase of the disease Tregs appear to be functionally active and able to inhibit the activation of microglia through the secretion of IL-4. This cytokine is not entirely secreted by Tregs but also by Th2 and so, this latter cell type is also crucial for neuroprotection. Moreover, a mixture of IL-4, IL-10 and TGF-β is required to suppress Teffs. (41). On the contrary, as the disease progresses, an increased expression of NOX2, IL-1β and IL-12, which are markers of M1 microglia activation, and of IFN-γ, secreted by Th1, was reported. Moreover, an elevation in the levels of IL-6, which can completely inhibit Tregs, was detected (40). Interestingly, the passive transfer of healthy Tregs to mSOD1 mice seems to prolong the early phase and extend survival (40).

Given this background but also considering the limitation that findings in disease models may not translate to humans, it was questioned what was the role of Tregs in ALS patients. Tregs were found to be significantly decreased in their peripheral blood (42), perhaps as an attempt of the CNS to recruit Tregs to suppress neuroinflammation, which correlates with an increase of Tregs in the CNS in the early disease phase. However, as disease progresses, this compensatory attempt fails and Tregs are inhibited and decrease in number (42) (Figure 2). Moreover, the quantity of Tregs in the peripheral blood negatively correlates with the ALSFRS-R score, a questionnaire used to rate the stage and progression of the disease (43). Thus, a lower Treg count is associated with a poor ALSFRS-R score and a more aggressive disease course (40, 42). Moreover, the percentage of Treg in patient blood also negatively correlates with ALS progression expressed in terms of AALS (Appel ALS score) an ALS clinal rating scale

(44, 45, 46). This score is independent from ALSFS-R and thus this finding reinforces the hypothesis of a Treg disfunction occurring over time in ALS patients.

Interestingly, Tregs were found not only to be reduced in number but they also appear dysfunctional and less effective in promoting Teffs suppression and this dysfunction is more evident in rapidly progressing patients (44). In fact, the expression of key genes for the normal function of Tregs such as FOXP3, CD25, GATA3 and some anti-inflammatory cytokines including: TGF-β, IL-10 and IL-4, were found to be all downregulated in the peripheral blood of rapidly progressive ALS patients. Thus, their expression levels were found to be inversely correlated with the disease progression rate. Moreover, FOXP3 could be considered a prognostic factor since its levels can efficiently predict the speed of disease. (46). Furthermore, evidence of epigenetic alteration in TSDR, a CpG-rich regulatory region within the first intron of FOXP3 gene, was documented in ALS. This element is physiologically demethylated only in Tregs which stably express FOXP3 but it is fully methylated in $CD4^+$ T cells. TSDR is partially methylated in ALS patients and this modification occurred more significantly in rapidly progressive cases (44).

Recently, evidence demonstrated that Tregs isolated from ALS are not permanently impaired but their function can be restored by culturing them *in vitro* in the presence of IL-2 and rapamycin (47). This latter compound is known to suppress the activity of Th1 and Th17 because it suppresses the mechanistic target of rapamycin (mTOR) signalling pathway which is crucial for these cells. In contrast, rapamycin promotes Tregs differentiation and proliferation because they are independent from mTOR (48). After treatment with IL-2 and rapamycin, Tregs from ALS subjects and healthy controls have comparable suppressive functions. Evidence was provided of the generation of a large-

scale GMP-compliant method for Treg isolation and expansion in the presence of IL-2 and rapamycin (47).

More recently, specific Treg subtypes have been correlated with ALS progression rate (ALSFRS-R). Tregs can be classified into $CD45RO^+$, functionally active Tregs, and $CD45RA^+$ which are resting Tregs. In rapidly progressive patients, reduction in the number of $CD45RO^+$ Tregs was reported, and the amount of this cell type can be correlated with disease progression (49).

Furthermore, intraperitoneal injection of rapamycin and IL-2c (IL-2 combined with its monoclonal antibody which increases the magnitude and duration of IL-2 activity) in mSOD1 mice promoted $CD45RO^+$ Treg expansion and prolonged their survival. In addition, a reduction in astrogliosis and microgliosis by 40 and 50% respectively was demonstrated together with an increase in FOXP3 and M2 microglia in the spinal cord and the sciatic nerve, and increased levels of GATA3 in sciatic nerve of these treated mice. Thus, these latter findings establish that Tregs can not only promote neuroprotection in the CNS, but they can also exert an equivalent function in the periphery (49).

This body of evidence demonstrate a key role of Tregs and a dual trend during the disease course: these cells seem to be increased in an early disease phase, probably as an anti-inflammatory attempt made by the CNS, but as disease progresses, Tregs progressively and dramatically decrease leading to a worsening in neuroinflammation. Moreover, Treg levels can be considered as a prognostic factor of the disease progression and survival and enhancing Tregs can probably constitute a promising therapeutic strategy. However, the possible effect of this Tregs expansion is still to be investigated and, for this reasons, several clinical trials are currently trying to elucidate

their efficacy. In addition, research is being conducted alongside the clinical trials to elucidate the molecular and immunological effects of this drug on ALS patients.

Targeting regulatory T cells

Since the role of the immune system in ALS has been documented, different drugs, aiming to suppress neuroinflammation, have been screened for the treatment of this disease. For example, prednisone, celecoxib, minocycline and thalidomide have been evaluated in clinical trials for ALS. Unfortunately all of them failed to show disease progression modulation and some of these agents caused serious adverse reactions (50), (51), (52), (53). These compounds negatively regulate the overall function of the immune system, and thus, it is now believed that indiscriminate immunosuppression is not an appropriate therapeutic strategy for ALS. In contrast, drugs which exert immunomodulatory effects, aiming to restore the normal neuroimmune homeostasis, are considered promising. In this respect, Tregs constitute a new therapeutic target in ALS and different clinical trials, which aim to expand the immune modulatory Tregs, are reported in this section (**Table 1**).

Rapamycin

This drug has already been approved by both the FDA and EMA for the prevention of organ rejection after transplant. Rapamycin mediates mTOR inhibition which in turns promotes Treg differentiation and stimulation of autophagy. Rapamycin facilitates the formation of autophagosomes and thus it promotes the clearance of pathological protein aggregates which are a prominent feature in ALS (54). As previously mentioned, the mTOR pathway is essential for the differentiation of naïve CD4+ T cells into Th1 and Th17 subtypes but it is not necessary for Treg development and, for this reason, it is believed to induce the expansion of protective Tregs (48). However, conflicting

preclinical results have been reported. In fact, while different studies showed rapamycin to be neuroprotective by promoting the elimination of protein aggregates in several cellular and animal models; in an another one this drug appeared to be harmful for ALS mSOD1 mice causing increase in motor disfunctions, acceleration in MN degeneration and decrease survival (55, 56, 57, 58, 59). Nonetheless, a phase II clinical trial (RAP-ALS) is evaluating the effect of rapamycin as add-on therapy to riluzole on 63 ALS patients (54, https://clinicaltrials.gov/ct2/show/NCT03359538). Alongside clinical assessments, scientific and investigatory outcomes are also scheduled including: immune phenotyping, inflammatory cytokines measurements and blood/CSF biomarkers evaluation.

Autologous Treg transplant

Recently, a phase I trial consisting in the autologous transplantation of Tregs has been completed. Tregs from were isolated, expanded *in vitro* and then reinfused intravenously for a total of 8 injections. Concomitantly, subcutaneous administration of IL-2 ($2x10^5$ international units (IU)/m^2, 3 times weekly) was performed. Treatment was well tolerated although some infections were reported. At the end of the trial, increased suppressive properties of Tregs were observed, along with a slowing of disease progression (60), https://clinicaltrials.gov/ct2/show/NCT03241784). These promising results have boosted the interest in this type of treatment and a placebo-controlled phase IIa trial to investigate the effect of autologous Treg infusions in combination with low-dose-IL-2 in a larger number of patients is now underway (12, https://clinicaltrials.gov/ct2/show/NCT04055623).

RNS60

RNS60 is an experimental nanostructured drug which consists in different oxygen

nanobubbles. Preclinical studies indicate a significant effect of this compound in extending survival of mSOD1 mice, as well as protecting their spinal cord motor neurons and NMJs from degeneration. These results appear to be due to RNS60 action on several ALS pathological mechanisms: i) RNS60 activates the p-Akt pro-survival pathway in MN, astrocytes and Schwann cells; ii) it reduces mitochondrial alteration and oxidative stress; iii) it promotes M2/Th2 activation over M1/Th1; iv) it induces the overproduction of IL-4 and activates Tregs (61). In 2018, a pilot RNS60 phase I clinical trial was completed. Sixteen participants were involved who received RNS60 by infusions (375ml) once weekly and nebulized RNS60 (4ml/day) for the remaining 6 days of the week for a total of 18 weeks. Results showed that this drug is safe and well tolerated with no adverse events reported throughout the trial. Unfortunately, no significant change in IL-17 and FOXP3 expression or differences in neuroimaging markers were reported during the treatment period. This was probably due to the small patient size and the lack of a placebo group (62), https://clinicaltrials.gov/ct2/show/NCT02525471). Thus, to further investigate the therapeutic potential of RNS60, two different placebo-controlled phase II clinical trials are now active evaluating the effect of either intravenous and/or nebulized inhaled RNS60 in 142 (https://clinicaltrials.gov/ct2/show/NCT03456882) and 140 (https://clinicaltrials.gov/ct2/show/NCT02988297) ALS patients respectively.

Vitamin D

Vitamin D (VitD) is believed to have immune-modulatory properties. In particular, this vitamin appears to exert functions on Tregs: it promotes Treg differentiation over cytotoxic T cells, induces FOXP3 expression and IL-10 secretion (63). Recently, a role of VitD in ALS has been proposed. However, contradictory results have emerged. While some studies reported that VitD deficiency correlates with a decreased survival,

another reported the association between high levels of VitD and worse ALS prognosis (64), (65), (66). Despite these discrepancies, a clinical trial called "T cell phenotype in ALS, influence of vitamin D" or VITALS is currently in the recruiting phase. Seventy ALS patients and 27 healthy controls will be screened for VitD levels in their blood and patients with VitD deficiency will be supplemented with this molecule. (https://clinicaltrials.gov/ct2/show/study/NCT02756104). Immune phenotyping will be conducted throughout the trial to monitor changes within the T cell population.

Low-dose IL-2

Although IL-2 is known to exert pleiotropic functions on the immune system, it is a crucial mediator of Treg differentiation and survival (20). In particular, ld-IL-2 seems to specifically promote Treg expansion without having any significant effects on Teffs (23). This can be explained by the different IL-2 receptors these two cell types show on their surface membranes. In fact, while Tregs constitutively express high-affinity IL-2R (made of three subunits: IL-2RA, IL-2RB and IL-2RG), Teffs express it only after T cell receptor (TCR) activation. Without this stimulus, Teffs constitutively express only the intermediate-affinity receptor (IL-2RB and IL-2RG). This means that Teffs require much more IL-2 to become active compared to Tregs (23, 67). In particular, a study shows that the amount of IL-2 required for the activation of Teffs is about 5000 higher than the dose required for Tregs (67). For this reason, IL-2 at low doses is able to exert a specific expansion effect only on Tregs and it is now under investigation for ALS treatment.

A pilot phase II trial, IMODALS, using two different concentrations of ld-IL2 (1MIU and 2MIU) in combination with riluzole was undertaken. Although the recruiting phase of this trial is completed, the results of the study are still to be published (https://clinicaltrials.gov/ct2/show/NCT02059759).

However, MIROCALS, a ld-IL-2 phase II clinical trial using 2MIU IL-2 is currently in the recruiting phase. Its purpose is to evaluate the clinical efficiency and safety of 2MIU IL-2 as an add-on therapy to riluzole in 216 ALS patients, recruited at diagnosis (https://clinicaltrials.gov/ct2/show/NCT03039673). Together with clinical evaluations, additional research including deep immune-phenotyping, genomics, blood transcriptomics as well as CNS biomarker analysis will be conducted to investigate the effect of ld-IL-2 on this patients (http://www.mirocals.eu/en/).

Dimethyl fumarate (DMF)

This drug is currently approved by both the FDA and the EMA for the treatment of psoriasis and relapsing-remitting forms of multiple sclerosis (MS) with the commercial name of Tecfidera. Evidence shows that DMF exerts its beneficial effects by a dual mechanism. It promotes the antioxidant response through the activation of the nuclear 1 factor (erythroid-derived 2)-like 2 (NRF2) pathway and it has immune modulatory properties. In fact, it stimulates type II dendritic cells which secrete the anti-inflammatory IL-10, reduces in the synthesis of pro-inflammatory cytokines and promotes a shift in the T lymphocyte population (68, 69, 70). In particular, MS patients treated with DMF showed a significantly decrease Th1 cells proportion, while an increase in Th2 and Treg cells is documented, although, a different study reported the effect of Tregs to be of short term (71, 72). Moreover, DMF seems to increase the sensibility of Teffs to Tregs and in turns this promotes their suppression (73, 74). Given this background, DMF or Tecfidera is currently being investigated in a phase II clinical trial called TEALS. The aim of the study is to evaluate safety and efficacy of this treatment on 90 sporadic ALS patients (https://www.australianclinicaltrials.gov.au/anzctr/trial/ACTRN12618000534280) (75).

Conclusions

ALS is a fatal neurodegenerative disease and currently, there is no effective disease modifying therapy for this condition. A range of mechanisms contribute to the disease onset and progression. Amongst these, neuroinflammation has a key role and its potential efficacy as a therapeutic target is now of growing interest. However, drugs which indiscriminately suppress the immune system do not appear to be effective (50, 51, 52, 53). Therefore, immunomodulatory treatments, which can re-establish the normal immune phenotype, could represent a more appropriate target. In particular, several trials, with the aim of expanding Tregs, are currently ongoing and after their completion we will understand whether this type of treatment has an effect on disease progression and/or patient survival. However, from preliminary studies, regulatory T cells seem to represent a promising therapeutic opportunity to restore the physiological equilibrium among immune cell types.

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Declaration of interest

No conflicts of interest.

FIGURE AND TABLE LEGENDS

Figure 1: **Mechanisms of action of Tregs.** Summary of the different mechanisms of Treg-mediated immune suppression. **A:** Inhibitory cytokine release, **B**: Cytolysis induction, **C**: Metabolic disruption, **D**: CTLA4-mediated mechanism, **E**: cAMP-mediated mechanism.

Figure 2: **The early phase versus the later phase of disease: focusing on the role of Tregs.** The early phase is characterized by a recruitment of regulatory T cells in the CNS from the periphery (a). This is a neuroprotective attempt made by the CNS to reduce neuro-inflammation. At this stage, Tregs secrete anti-inflammatory compounds which promote the activation of M2 microglia known for their anti-inflammatory properties. In the later stage, as the disease progresses, the number of Tregs in the CNS decreases, while inflammatory mediators prevail (b). This includes Th1, which secretes cytokines that activate M1 microglia and promote inflammation and neurotoxicity.

Table 1: Targeting Tregs: ongoing clinical trials

These ongoing clinical trials aim to expand the protective regulatory T cells. Names and IDs are reported together with drug information, phase of the study, status and number of participants involved. For more information, group divisions and treatment regimens are also described.

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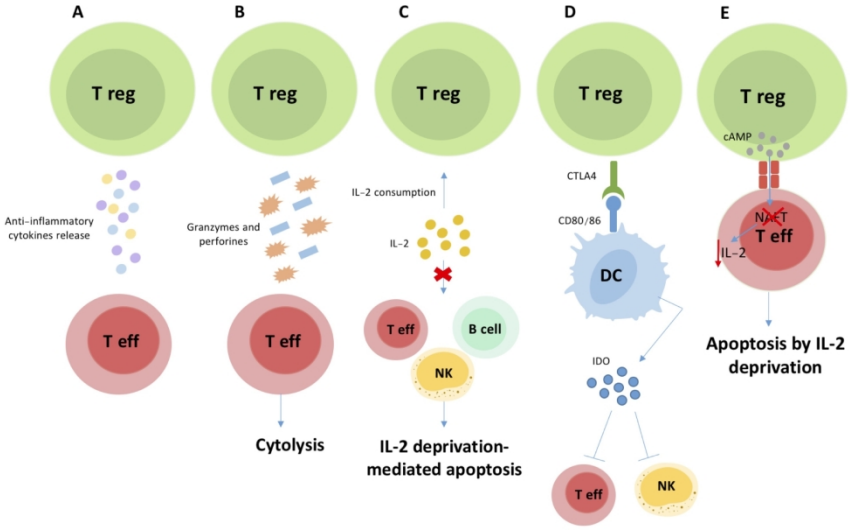

Figure 1: Mechanisms of action of Tregs. Summary of the different mechanisms of Treg-mediated immune suppression. A: Inhibitory cytokine release, B: Cytolysis induction, C: Metabolic disruption, D: CTLA4-mediated mechanism, E: cAMP-mediated mechanism.

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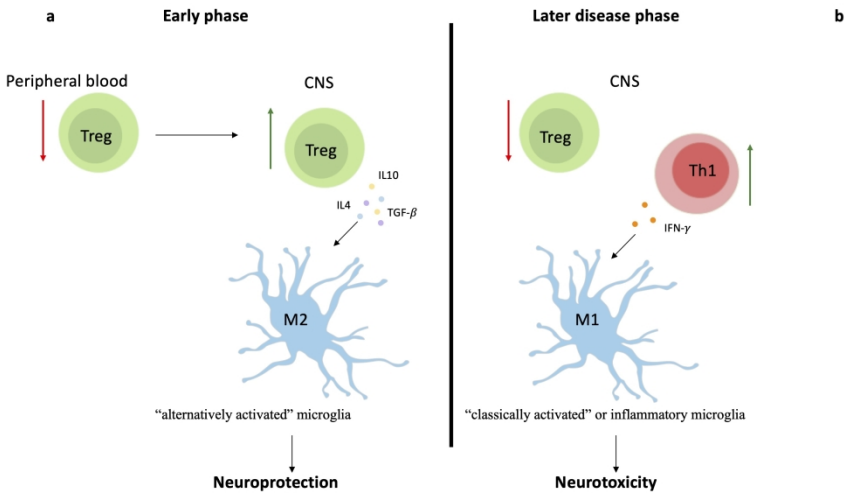

Figure 2: The early phase versus the later phase of disease: focusing on the role of Tregs. The early phase is characterized by a recruitment of regulatory T cells in the CNS from the periphery (a). This is a neuroprotective attempt made by the CNS to reduce neuro-inflammation. At this stage, Tregs secrete anti-inflammatory compounds which promote the activation of M2 microglia known for their anti-inflammatory properties. In the later stage, as the disease progresses, the number of Tregs in the CNS decreases, while inflammatory mediators prevail (b). This includes Th1, which secretes cytokines that activate M1 microglia and promote inflammation and neurotoxicity.

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Trial name	Drug	Clinical trial ID	Trial phase	Status	Participants	Group division and treatment types
RAP-ALS	Rapamycin	NCT03359538	Phase II	Active, not recruiting	63	21 patients: placebo 21 patients: 1mg/m^2/day rapamycin 21 patients: 2mg/m^2/day rapamycin 1 administration daily for 18 weeks
T-regulatory in ALS (Tregs in ALS)	Autologous Treg + IL2	NCT04055623	Phase IIa	Active, not recruiting	12	The study is divided in 2 parts: 1. First 6 months: -Treated patients: Treg will be taken, expanded in lab and reinjected each month + ld-IL2 subcutaneous injections 3 times per week -Placebo patients: monthly placebo infusions + 3 placebo subcutaneous injection per week. 2. Second 6 months: all participants will receive autologous expanded Tregs monthly + ld-IL2 3 times weekly.
The effect of RNS60 on ALS biomarker	RNS60 (intravenous and inhaled)	NCT03456882	Phase II	Active, not recruiting	142	71 patients: placebo 71 patients: RNS60 (normal saline plus oxygen in nanobubbles) 1 intravenous infusion per week and 1 nebulized inhalation daily for 24 weeks.
Nebulised RNS60 for the treatment of ALS	RNS60 (inhaled)	NCT02988297	Phase II	Not yet recruiting	140	70 patients: placebo 70 patients: RNS60 Daily inhalation for 24 weeks
VITALS	Vitamin D (VD)	NCT02756104	NA	Recruiting	97	27 healthy volunteers 70 ALS patients: VD-deficient patients will be supplemented for 6 months
MIROCALS	ld-IL-2	NCT03039673	Phase II	Active, not recruiting	216	108 patients: placebo 108 patients: 2MIU IL-2 1 injection daily for 5 days every 4 weeks for 18 months
TEALS	Dimethyl fumarate (Tecfidera)	ACTRN12618000534280	Phase II	Not yet recruiting	90	30 patients: placebo 60 patients: 240mg of Tecfidera (oral tablets) Two administrations daily for 36 weeks.