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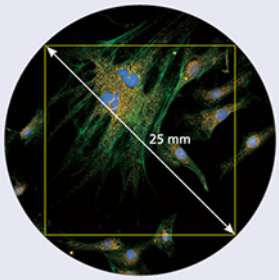

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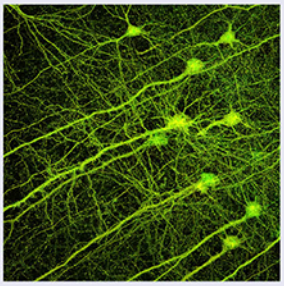

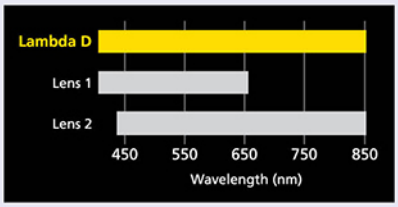

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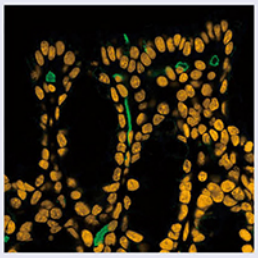

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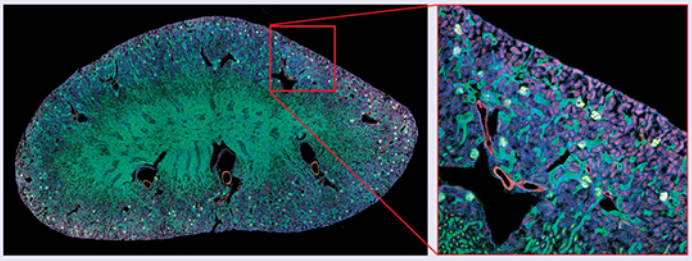

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REVIEW ARTICLE

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Regulatory T cells for amyotrophic lateral sclerosis/motor neuron disease: A clinical and preclinical systematic review

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Abstract

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disorder characterized by neuronal degeneration and inflammation in the nerves. The role of the immune system has been concentrated by researchers in the etiopathogenesis of the disease. Given the inhibitory roles of regulatory T cells (Tregs), it is expected that increasing or activating their populations in patients with ALS can have significant therapeutic effects. Here we searched databases, including CENTRAL, MEDLINE, CINAHL Plus, clinicaltrials.gov, and ICTRP for randomized clinical trials (RCTs) and non-RCTs until March 2019. For preclinical studies, we searched PubMed, Scopus, and Google Scholar up to June 2019. We also included preclinical studies, due to the lack of clinical information available, which used Tregs (or directly targeting them) for treating mice models of ALS. We identified 29 records (CENTRAL 7, MEDLINE 4, CINAHL Plus 8, and clinicaltrials.gov 10) and removed 10 duplicated publications. After screening, we identified one RCT which had been published as an abstract, three non-RCTs, and four ongoing studies. We also identified 551 records (PubMed 446, Google Scholar 68, and Scopus 37) for preclinical studies and performed a meta-analysis. Finally, we found three papers that matched our inclusion criteria for preclinical studies. Results indicated the effectiveness of the application of Tregs in the treatment of ALS. Our meta-analysis on preclinical studies revealed that Tregs significantly prolonged survival in mice models of ALS. Overall, our analysis testified that exertion of Tregs in the treatment of ALS is a promising approach, that notwithstanding, requires further evaluations.

KEYWORDS

amyotrophic lateral sclerosis, motor neuron disease, preclinical study, randomized controlled trial, regulatory T cells

1 | INTRODUCTION

Amyotrophic lateral sclerosis (ALS), also known as Lou Gehrig's and motor neuron disease (MND), is a fatal neurodegenerative disease. This disorder is very heterogeneous and many of its causes are still unknown. However, several factors involved in its pathogenesis have been identified, including accumulation and collapse of miss fold proteins, oxidative stress, defective axon

Abbreviations: AEs, adverse events; ALS, amyotrophic lateral sclerosis; ALSFRS-R, ALS Functional Rating Scale-Revised; ALSSQOL-R, amyotrophic lateral sclerosis-specific quality of life-Revised; CNS, central nervous system; CTLA4, cytotoxic T-lymphocyte associated protein 4; FOXP3, forkhead box P3; FVC, forced vital capacity; IFN-γ, interferon-γ; IL, interleukin; RCT, randomized controlled trial; ROS, reactive oxygen species; SAE, serious adverse event; sALS, sporadic amyotrophic lateral sclerosis; SD, standard deviation; SOD, superoxide dismutase.

transfer function, abnormal activation of astrocyte, neuron inflammation, cytoskeletal protein abnormalities, mitochondrial function impairment, and glutamate excitotoxicity (Moisse & Strong, 2006; Van Dyke et al., 2016). ALS is characterized by the destruction of the upper and lower neurons that begins in adults. The disease begins with the weakening, of the hands and feet in 70% of the patients and in 35% of the remaining patients with a bulbar weakening which causes defective swallowing and speaking. The degeneration of the neuronal motor leads to the loss of respiratory muscles and the average death within 3–5 years (Ajroud-Driss & Siddique, 2015). Almost 90% of ALS cases are sporadic (sALS) and do not have a family history of the condition. The familial type of the disease is responsible for approximately 10% of all cases, and so far about 70–80% of the responsible genes have been identified (containing approximately 20 different genes). Interestingly, mutations in some of these genes are also observed in patients without a family history (Talbot, Feneberg, Scaber, Thompson, & Turner, 2018).

Various studies clearly indicate that ALS is the result of interactions between the environment and genetic abnormalities (Beers & Appel, 2019; Zou et al., 2017). Advances in genetic studies have led to the identification of many of the genes responsible for this disease. Many proteins encoded by these mutated genes directly or indirectly affect the function of the immune system, which is important evidence of the role of immune system disorders in the pathogenesis of ALS. Extensive inflammation in the central nervous system (CNS) is observed in patients with mutations in *superoxide dismutase 1* (*SOD1*; the first identified the genetic cause of ALS), *TAR DNA binding protein* (*TARDBP*), and *chromosome 9 open reading frame 72* (*C9orf72*) genes. Several studies have also shown that mutations in genes, including *optineurin*, *sequestosome-1 protein*, *TANK-binding kinase 1*, *valosin-containing protein*, *tumor necrosis factor α-induced protein 3 interacting protein-1*, and *chemokine receptor 1* may also impair immune function and increase inflammation in patients or experimental models of ALS. In addition, there are reports indicating that autophagy, *nuclear factor-κB*, and the *nucleotide-binding domain-like receptor protein 3* may involve in the pathogenic process of ALS by altering the immune system function and inflammatory response (Beers & Appel, 2019).

The global outbreak of the disease is ~8 per 100,000 people (Moisse & Strong, 2006). Studies about the environmental factors that cause the ALS are still unconvincing, but a recent meta-analysis study shows that there are different patterns of genes responsible for the disease in different geographic regions (Zou et al., 2017). In this meta-analysis, which included 111 different studies, a significant difference was identified between European and Asian patients regarding the frequencies of mutations in major genes responsible for ALS. In European populations, the most common mutations were found in *C9orf72* (familial 33.7% and sporadic 5.1%), *SOD1* (familial 14.8% and sporadic 1.2%), *TARDBP* (familial 4.2% and sporadic 0.8%), and *FUS* (familial 2.8% and sporadic 0.3%) genes, while *SOD1* (familial 30.0% and sporadic 1.5%), *FUS* (familial 6.4% and sporadic 0.9%), *C9orf72* (familial 2.3% and sporadic 0.3%), and *TARDBP* (familial 1.5% and sporadic 0.2%) were the most common mutations in Asian populations.

1.1 | Regulatory T cells in ALS

Both innate and adaptive immunity play an important and interdependent role in the progression of ALS and the process of the inflammation. The most important clinical finding at the site of neuronal damage in ALS is neuronal inflammation, which is caused by the activation of microglia (resident macrophage within the CNS), astrocytes, and infiltration of monocytes and T cells (Zhao, Beers, & Appel, 2013), and some reports suggest that reducing the rate of the inflammatory response can delay the onset of the disease and increase survival (A McCombe & D Henderson, 2011; Moisse & Strong, 2006; Nguyen, D'Aigle, Gowing, Julien, & Rivest, 2004; Phani, Re, & Przedborski, 2012).

In a normal CNS, resting microglia and regulatory T cells (Tregs) provide immune surveillance in the neuronal environment (Lasiene & Yamanaka, 2011; Moisse & Strong, 2006). In the early stages ALS, the phenotype M2 of microglial cells have a major role and able to secrete high levels of neurotrophins such as insulin-like growth factor-1 and progranulin, which have a neuroprotective function. Moreover, M2 microglial cells release anti-inflammatory cytokines, such as transforming growth factor-β (TGF-β), interleukin-10 (IL-10), and interleukin-1 receptor antagonists, which inhibit the proinflammatory response. Tregs also release anti-inflammatory cytokines to maintain the phenotype M2 of microglial cells. In addition, astrocytes participate in this protective process by secreting neurotrophic factors and taking extracellular glutamate. Astrocytes also increase the antioxidant capacity of neurons by releasing glutathione precursors (CysGly), which are harvested by the neuronal engine. Thus, in the early stages of ALS, microglia, and astrocytes can maintain the survival of the neuronal engine. However, as the disease progresses, the risk signals, including mSOD1, oxidized SOD1, and ATP are released from the neurons. These factors induce the activation of M1 microglia via CD14/toll-like receptors, scavenger receptors, and purinergic P2 receptors. The result of the downstream signaling of these receptors in the production of proinflammatory cytokines, such as IL-1β and tumor necrosis factor-α (TNF-α) and enhanced neurotoxicity through the release of the free radicals. In addition, M1 microglia cells activate astrocytes via reactive oxygen species (ROS) and proinflammatory cytokines. Activated astrocytes contribute to the generation of the harmful inflammatory phenotype by releasing ROS and proinflammatory cytokines, which induces more activation of the microglia. In addition, activated astrocytes and neurons produce C–C motif chemokine ligand 2 that recruits peripheral monocytes/macrophages into CNS and exacerbates neuronal degeneration (Lasiene & Yamanaka, 2011; Moisse & Strong, 2006; Zhao et al., 2013). Overall, the primary immune response to damaged nerves helps repair tissues, which is due to the presence of Tregs and their effects on the differentiation of M2 microglia. As the disease progresses, immune responses from M2 microglia and Tregs shift to M1 microglia and T helper (Th) 1/Th17 cells and this causes

more inflammation and damage to the neurons (Zhao et al., 2013; Figure 1).

Tregs are a population of $CD4^+$ cells, which are known as $FOXP3^+CD25^{high}CD127^{-/low}CTLA\text{-}4^+GITR^+CD45RA^+$ ($CD45RA^-CD45RO^+$ in memory and effector Tregs; Azimi et al., 2016). These cells are divided into two subgroups, including forkhead box $P3^+$ ($FOXP3^+$) natural Tregs (nTregs) and $FOXP3^-$-induced Tregs (iTregs). nTregs originate from thymocytes located in the thymus (primary lymphoid organ) due to agonist selection, and iTregs (Tr1 and Th3) are derived from peripheral T cells in the presence of TGF-β, or IL-10, and retinoic acid in the secondary lymphoid organs or mucosa-associated lymphoid tissue (Mohr, Malhotra, Mayer, Gorochov, & Miyara, 2018). These cells are the main reservoirs of maintaining peripheral tolerance by secreting anti-inflammatory cytokines, such as IL-10, IL-35, IL-4, and TGF-β, expressing inhibitory receptors like cytotoxic T-lymphocyte associated protein 4 (CTLA-4) and up taking IL-2 through CD25. Studies on mouse models of ALS showed that the deletion of $TCD4^+$ cells accelerated the disease progression, and the presence of $TCD4^+$ cells reduced the level of proinflammatory cytokines. This study also found that activated $CD4^+CD25^+$ cells prolonged the life span of SOD1 mutant mice (Lasiene & Yamanaka, 2011). Other investigations show that $CD4^+CD25^{hi}FOXP3^+$ cells are increased in the early stages of disease progression and decreased during the rapid phase of disease progression, which is probably due to the reduction of FOXP3 expression in Tregs. In addition, a recent study in ALS mice shows that Tregs inhibit microglia cells through an IL-4 dependent mechanism. Furthermore, Tregs in the slow phase of ALS increase the level of anti-inflammatory cytokines and suppress T cells with IL-4, IL-10, and TGF-β (Zhao, Beers, Liao, Henkel, & Appel, 2012).

Studies in the patients with ALS also show a similar pattern compared with studies in the mouse models, and it has been observed that Tregs are significantly decreased with progression of the disease (Table 1) and gradually lose their regulatory functions (Bargh et al., 2018; Henkel et al., 2013; Menon et al., 2014; Sheean et al., 2018). Recently, in a cohort study performed on 33 patients and 38 healthy controls, it was shown that the progression rate of ALS in patients was inversely correlated with total Treg count, although naive Tregs ($CD45RA^+$) were not correlated with the rate of disease progression (Sheean et al., 2018). They also used transgenic $SOD1^{G93A}$ mice models to confirm their study results in humans. The study showed that if these mice were treated with IL-2 monoclonal antibody complexes (IL-2c), the amount of effector Treg population ($CD45RA^-CD45RO^+$) was significantly increased, and the inflammation of the neurons and the progression of the disease would be reduced. In addition, another study, which was accomplished in 2018 on 37 patients with ALS and 30 healthy controls, also confirmed the results of the previous study on reducing regulatory cells with disease progression. This study reported a significantly higher proportion of $CD4^+$ T cells, along with a significantly lower proportion of $CD4^+CD25^+$ Tregs, in the peripheral blood of patients with ALS compared with the control group (Bargh et al., 2018).

In addition to studies that show a decrease in the number of Tregs during the disease progression, there are also studies indicating that they are functionally defective (Alsuliman et al., 2016; Beers et al., 2017). These studies have shown that Tregs from patients with ALS failed to suppress T-cell proliferation and cytokine production (including interferon-γ [IFN-γ], TNF-α, and IL-2). In one of these studies, it was shown that circulating Tregs in patients with ALS had less suppressive activity than that of the control group (Beers et al., 2017). Despite the fact that this suppressive dysfunction was observed in both slowly and rapidly progressing patients with ALS, the severity of this dysfunction was higher in rapidly progressing patients with ALS than in slowly progressing patients with ALS. In addition, rapidly progressing patients had also less FOXP3 messenger RNA (mRNA) expression

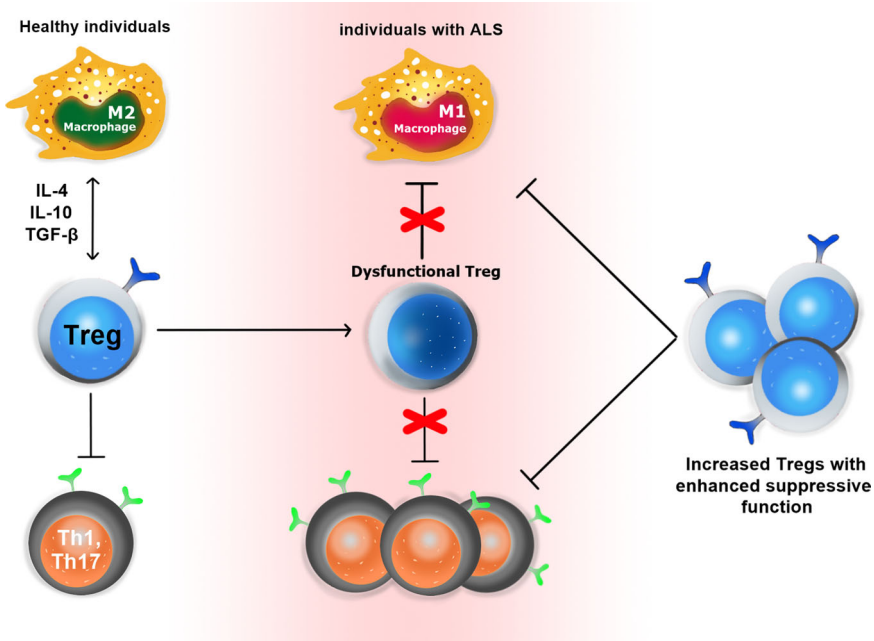

FIGURE 1 Hypothetical model of Tregs function in ALS. ALS, amyotrophic lateral sclerosis; IL, interleukin; TGF-β, transforming growth factor-β; Tregs, regulatory T cells

TABLE 1 Studies demonstrating a decrease in the number of Tregs with disease progression

Study	Year	Number of patients	Number of healthy control	ALSFRS-R score (Mean ± *SD*)	Disease duration at the time of studies (Mean ± *SD*)	Decrease in the number of Tregs compared with healthy control (*p* value)
Sheean et al.	2018	33	38	43	14 month	$p = .01$ ($CD45^+$ Tregs)
Bargh et al.	2018	37	30	34.6 ± 8.1	27.7 ± 16.3 month	$p = .02$ (Total Tregs)
Menon et al.	2014	38	40	NA	NA	$p = .036$ ($CD45^+$ Tregs)
Henkel et al.	2013	54	33	NA	NA	$p = .003$ (Total Tregs in rapidly progressing patients)

Abbreviations: ALS, amyotrophic lateral sclerosis; ALSFRS-R, ALS Functional Rating Scale-Revised; NA, not applicable; *SD*, standard deviation; Tregs, regulatory T cell.

than slowly progressing patients and healthy controls. This study also showed that the suppressive function of Tregs from patients with ALS could be restored. After in vitro expansion, Tregs from patients with ALS were able to regain their suppressive potency and reach the level of Treg cells from the control group. The results of this study showed that the future of the autologous transfer of Tregs could be promising in treating the ALS.

Consequently, according to the results of studies in both human and mouse models, it can be expected that Tregs will be an important therapeutic target for patients with ALS. Moreover, it is expected that by increasing their population in patients or targeting them with drugs, Tregs can have positive therapeutic outcomes in patients. For this reason, we decided to systematically summarize the results of studies that used Tregs (or directly targeting them) for the treatment of patients with ALS. The aim of this study is to provide acceptable preclinical or clinical evidence of the usefulness of Tregs (as disease-modifying agent) for the treatment of the patients with ALS and help to understand whether the data of experimental studies implying to the beneficial effects of Tregs in the treatment of ALS are reliable.

1.2 | Description of the intervention

An increased number of Tregs in the body can be due to different pharmacological drugs or even autologous transplantation of these cells. In this review, all of the drugs that directly increase the Tregs population or their activation in patients were investigated. These factors may include cytokines, transcription factors, biological drugs (antibodies), or immunomodulatory drugs.

2 | OBJECTIVES

We set out to review the evidence from randomized clinical trials (RCTs) and non-RCTs and in vivo preclinical studies for the efficacy of Tregs in the treatment of ALS disease.

3 | METHODS

3.1 | Inclusion criteria

3.1.1 | Types of studies

All controlled clinical trials that are double-blind RCTs and used Tregs (or directly targeting them) for treating patients with ALS were included in our review. Studies that are randomized controlled trials but they clearly do not indicate which method they use, were referred in our study. After the initial searches, because of the question of interest cannot be answered only by RCTs, we decided to also review non-RCTs and discussed the clinical results in two RCTs and non-RCTs. Our strategy for non-RCTs was to include the best available studies that can answer to our study question. In addition, due to the risk of bias, we would prefer to include studies that were double or triple-blind and used the masking methods in the study; but if sufficient results from blind studies were not available, also open-labeled studies were included in our review. In addition, we decided to include in vivo preclinical studies, due to the lack of clinical information available, which used Tregs (or directly targeting them) for treating mice models of ALS and discussed them in the preclinical result section.

3.1.2 | Types of participants

Eligible studies had to include participants of any age or sex with ALS having all of the following clinical criteria.

- Diagnosis of ALS according to the criteria for the disease diagnosis, known as the El Escorial World Federation of Neurology criteria (also known as Airlie House criteria; Brooks, 1994; Brooks, Miller, Swash, & Munsat, 2000). Additional tests may include electromyogram, magnetic resonance imaging, lumbar puncture, muscle biopsy, and blood and urine tests.
- Diagnosis of ALS with the onset of less than or equal to 5 years from the first onset of clinical symptoms. Unfortunately, most of the patients with ALS die from respiratory failure, usually within

3–5 years from when the symptoms first appear, and only 14% of patients with ALS survive 5 years or more (Mateen, Carone, & Sorenson, 2010). For this reason, people who live more than 5 years after first clinical symptoms are generally in advanced and severe conditions and many of the indicators and markers in their bodies are probably changed due to severe disorders. In addition, many studies in the field of ALS are aimed at finding a cure for increasing survival and preventing disease progression in newly diagnosed patients.

- Forced vital capacity (FVC) ≥65% of that predicted for age and height. FVC is an index of respiratory function that can be an important predictor of survival in patients with ALS. Studies have shown that patients with FVC < 70% had a faster disease progression with an average survival of 2.91 years, compared with 4.08 years for patients with FVC > 70% (Czaplinski, Yen, & Appel, 2006). For this reason, we preferred the studies which had patients with FVC ≥ 65%.
- Not taking riluzole (a common drug in the treatment of the patients with ALS) or on a stable dose if undergoing treatment with this agent.
- Without medical disorders, such as autoimmunity, primary or secondary immunodeficiency, malignancy, allergy, acute or chronic infections, and history of transplantation or use of immune-suppressive drugs.

For preclinical studies, our inclusion criteria were as follows:

- Studies on mice models of ALS (mSOD1^{G93A}) that exerted Tregs (or any drugs directly targeting Tregs) for treatment.
- Studies with starting treatment after the onset of symptoms.
- Studies with assessment of outcomes compared with a control group (untreated transgenic mice).

3.1.3 | Types of outcome measures

Primary clinical outcomes

1. Treatment-related change in the percentage of Tregs ($CD4^+CD25^+FOXP3^+$) assessed by multicolor flow cytometry or gene expression methods. It can also include $FOXP3^-$ Tregs (Th3 and Tr1) as well as markers identifying subtypes (like CD45RA and CD45RO).

Secondary clinical outcomes

1. Treatment-related change in levels of blood inflammatory biomarkers (including TNF-α, IFN-γ, IL-1, IL-6, etc.) or in levels of blood anti-inflammatory biomarkers (including IL-10, TGF-β, etc).
2. Treatment-related change in score of ALS Functional Rating Scale-Revised (ALSFRS-R).

Progress and severity of the ALS are determined by physicians using ALSFRS-R (Cedarbaum et al., 1999). This test consists of 12 questions that are scored from 0 to 4 per question, depending on the patient's condition (0 indicates the worst condition [no ability] and 4 indicates the best condition [normal ability]). ALSFRS-R measures speech, salivation, swallowing, handwriting, cutting food and handling utensils, dressing and hygiene, turning in bed and adjusting bedclothes, walking, climbing stairs, dyspnea, orthopnea, and respiratory insufficiency.

3. Change in quality of life.
   Treatment-related change in ALS Specific Quality of Life-Revised (ALSSQOL-R). The ALSSQOL-R is a specific test with 50 questions (46 of the questions are used in scoring) that is completed by the patients with ALS. Each question is rated by the patient using a 0–10 point (0 indicates the worst condition and 10 indicates the best condition; Simmons et al., 2010).
4. A number of participants with treatment-related serious adverse events (SAEs), which is defined as any unwanted medical occurrence that results in death (at any dose) or hospitalization.

For preclinical studies, outcomes include:

1. Treatment-related change in the percentage of Tregs (as described in primary clinical outcome).
2. Treatment-related change in the levels of blood inflammatory biomarkers.
3. Treatment-related change in survival.

3.2 | Electronic searches

For clinical trials, we searched the Cochrane Central Register of Controlled Trials (CENTRAL; 2019, Issue 3 of 10) in the Cochrane Library (12 March 2019), MEDLINE (January 1966–March 2019), Cumulative Index to Nursing and Allied Health Literature plus (CINAHL; January 1982–March 2019), www.clinicaltrials.gov and the World Health Organization International Clinical Trials Registry Platform (ICTRP; www.who.int/ictrp/en/), and as well as the reference lists of review publications. For the preclinical studies, we searched PubMed, Scopus, and Google Scholar until June 28, 2019. There was not any restriction on searching. We also tried to get more information by contacting the authors of relevant publications. The detailed search strategies for clinical trials are in the appendices: MEDLINE (Appendix 1), CINHAL plus (Appendix 2), CENTRAL (Appendix 3), clinicaltrials.gov (Appendix 4), and ICTRP (Appendix 5).

3.3 | Data collection and analysis

3.3.1 | Selection of studies and data extraction

Two of the review authors (M. R. N. and A. R. M.) independently reviewed all the records of the search and identified those that were fully published and matched with our inclusion criteria. If there was a

discrepancy in the results and the decision to include or exclude a study, we would solve it with discussion among the authors, and if needed, we would contact the author to obtain further information.

3.3.2 | Measures of treatment effect

Statistical analysis was performed on the preclinical trials using the Cochrane software Review Manager v5.3 (RevMan v5.3). For continuous variables, standardized mean difference and *p* value were calculated based on the random-effects model, with a corresponding 95% confidence interval (CI), according to mean, standard deviation (*SD*), and the number of participants reported in papers. $p < .05$ were considered to be statistically significant. We only performed meta-analyses for the outcomes that were similar enough to be meaningful, and the mean, *SD*, and the number of participants in the study had been clearly reported.

For clinical results that were not homogenous enough for meta-analyses, we used the Grading of Recommendations, Assessment, Development, and Evaluation model (via GRADpro/GTD online software) to access the quality of the evidence (Puhan et al., 2014). We assessed the risk of bias of the included studies as described in Chapter 8 (Assessing the risk of bias in included studies) of the *Cochrane Handbook for Systematic Reviews of Interventions* (Higgins, 2011). In addition, we evaluated the statistical heterogeneity between trials using both the *Q*-test of heterogeneity and the I^2 test of inconsistency. Any disagreements were resolved by discussion among the review authors.

4 | RESULTS

4.1 | Preclinical results

After the initial searches, we identified 551 records (PubMed 446, Google Scholar 68, and Scopus 37). After removing duplicates and unrelated papers, we found three papers that matched our inclusion criteria for in vivo preclinical studies (Beers et al., 2011; Sheean et al., 2018; Vallarola et al., 2018). All three articles were conducted on transgenic mSOD1^{G93A} mice (ALS experimental model) and fulfilled our inclusion criteria for preclinical studies.

Beers et al. study was conducted on mSOD1^{G93A} and RAG2 knockout mice, C57BL/6 strain, and used age-matched wild-type mice as controls. In this study, T cells were isolated from the spleen, lymph nodes, and blood. CD4$^+$ T cells and Tregs were separated using the magnetic-activated cell sorting. Tregs were transferred into mSOD1/RAG2$^{-/-}$ mice without ex vivo activation (at 70 days of age) via tail vein injections and repeated every 28 days. The passive transfer of Tregs significantly increased the mRNA expression levels of IL-4 ($p = .025$) and decreased the mRNA expression levels of IFN-γ ($p = .026$) in the spinal cords of 20-week-old mSOD1/RAG2$^{-/-}$ mice compared with untreated mSOD1 mice. Furthermore, passive transfer of Tregs into mSOD1/RAG2$^{-/-}$ mice prolonged survival by 33 days (46%) compared with untreated mSOD1/RAG2$^{-/-}$ mice ($p < .01$; Beers et al., 2011).

Sheean et al's. (2018) study was conducted on mSOD1^{G93A} mice (C57BL/6 strain) and used age-matched wild-type mice as controls. Mice were divided into three groups (IL-2/IL-2c [IL-2 monoclonal antibody complex] + rapamycin, rapamycin alone, and placebo [normal saline] alone). Mice in the first group were intraperitoneally injected with 100 μl of IL-2/IL-2c + rapamycin once a day for 3 days (at 60 days of age), followed by twice-weekly injections. Treatment with IL-2c significantly increased the percentage of Tregs ($p < .001$) and the mRNA expression level of Foxp3 ($p = .003$) in SOD1^{G93A} mice compared with placebo-treated SOD1^{G93A} mice. In addition, treatment with IL-2c prolonged survival by 15.7 days in SOD1^{G93A} mice compared with placebo-treated SOD1^{G93A} mice ($p = .003$; Sheean et al., 2018).

Vallarola et al's. (2018) study was conducted on mSOD1^{G93A} mice (C57BL/6 strain) and used age-matched wild-type mice as controls. Mice were intraperitoneally injected with 300 μl of RNS60 or normal saline (as placebo; started from the onset of the disease). RNS60 is an immune modulator drug that has been studied in an experimental model of neurodegenerative diseases like experimental autoimmune encephalomyelitis (the mouse model for MS), which can increase the number of Tregs in the body. Treatment with RNS60 significantly increased the mRNA expression levels of Foxp3 and IL-4 ($p < .05$ and $p = .003$, respectively) and decreased (not statistically significant) the mRNA expression levels of IL-1β ($p = .7$) in SOD1^{G93A} mice compared with placebo-treated SOD1^{G93A} mice. In addition, treatment with RNS60 prolonged survival by 10 days in SOD1^{G93A} mice compared with placebo-treated SOD1^{G93A} mice ($p = .254$, not statistically significant; Vallarola et al., 2018).

Given that all three articles reported the mean survival of mice in days, the mean mRNA expression levels of FOXP3 and *SD*, these studies can be considered homogeneous only in two of the outcomes (treatment-related change in the percentage of Tregs and survival), which allows for a comparison using meta-analytical methods. Forest plot (Figure 2) of the preclinical use of Tregs (or agents that directly increase Tregs) in the treatment of mice models demonstrates a

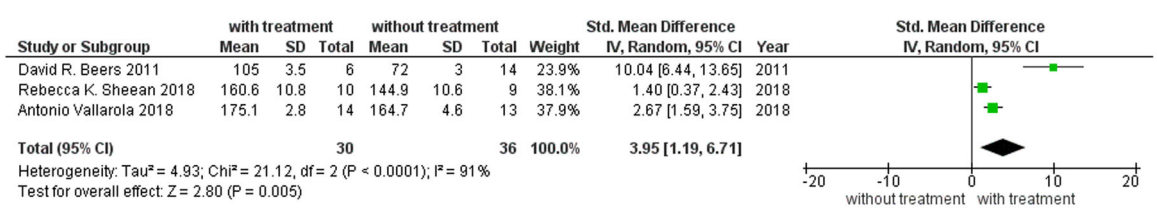

FIGURE 2 Forest plot for analyses of the effect of treatment-related change in survival. CI, confidence interval; *SD*, standard deviation

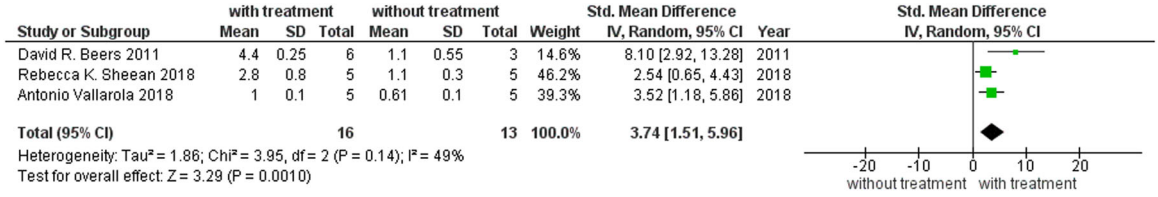

FIGURE 3 Forest plot for analyses of the effect of treatment-related change in mRNA expression of FOXP3. CI, confidence interval; FOXP3, forkhead box P3; mRNA, messenger RNA; *SD*, standard deviation

standardized mean difference of survival of 3.95 days (95% CI: 1.19–6.71), which favored using Tregs over placebo (statistically significant difference [$p = .005$]). In addition, Figure 3 represents a forest plot for treatment-related change in the mRNA expression levels of FOXP3 (data expressed as fold change), which indicates a standardized mean difference of 3.74 (95% CI: 1.51–5.96), demonstrating statistically significant difference ($p = .001$) in the mRNA expression of FOXP3 after treatment compared with untreated transgenic mice.

4.2 | Clinical results

See Figure 4 for a PRISMA flow chart, showing the study selection process for this review. We identified 29 records (CENTRAL 7, MEDLINE 4, CINAHL Plus 8, and clinicaltrials.gov 10) and removed 10 duplicate. After screening, we identified one RCT which is published as an abstract (not containing the outcomes; Bensimon et al., 2017), three non-RCTs (Paganoni et al., 2019; Thonhoff et al., 2018), one of them was a protocol paper, which is why it was counted in the ongoing studies (Mandrioli et al., 2018), and four ongoing studies. We excluded 12 studies which were not fulfilled our inclusion criteria.

4.2.1 | Randomized clinical trials

Only one study (Bensimon et al., 2017) was fulfilled our selection criteria for RCTs, which was published as an abstract (not containing the outcomes; Table 2). This study is a double-blind, placebo-controlled, randomized (12 patients/groups), a parallel-group study of IL-2 (1 million international units [MIU] and 2 MIU/days subcutaneous) for 5 days every 4 weeks for 3 cycles. Assessment times were: Before each cycle, 3 days after Cycle 1 and 3, 3 weeks after 3 cycles, and 3 months after the last dosing. As mentioned above, a low dose of IL-2 can induce Tregs expansion and survival. All 36 randomized participants were alive at the end of this study, with no drug-related SAEs reported. Drug-related nonserious adverse events were also similar to those reported in other conditions and were generally mild to moderate and transient. Compared with placebo, both IL-2 doses significantly expanded Tregs

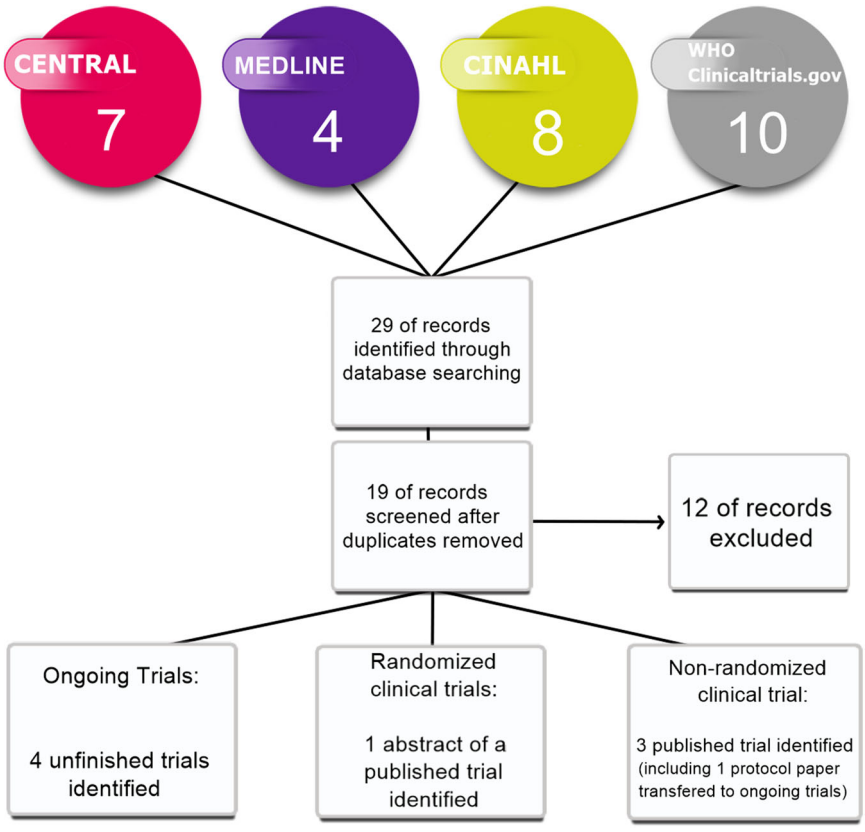

FIGURE 4 Study flow diagram (PRISMA flow chart) for clinical searches. CENTRAL, Cochrane Central Register of Controlled Trials; CINAHL, Cumulative Index to Nursing and Allied Health Literature; WHO, World Health Organization

TABLE 2 Characteristics of included randomized clinical trial

Study name	Bensimon 2017
Methods	Double-blind, placebo-controlled, randomized, and parallel-group study
Participants	36 Participants
Interventions	IL-2 or placebo
Outcomes	Assessment times were: Before each cycle, 3 days after cycle 1 and 3, 3 weeks after 3 cycles, and 3 months after the last dosing 1. Increase in Tregs proportion 2. No drug-related SAE 3. No changes in ALSFRS or FVC
Bias	Study findings presented in abstract form but currently unpublished. The abstract may provide selective reporting of the results. It is unclear who was or was not blinded. It is unclear what information was obtained by which laboratory methods
GRADE	Moderate quality (⊕⊕⊕O)

Abbreviations: ALS, amyotrophic lateral sclerosis; ALSFRS, ALS functional rating scale; FVC, forced vital capacity; GRADE, Grading of Recommendations, Assessment, Development, and Evaluation; IL-2, interleukin-2; SAE, serious adverse event; Tregs, regulatory T cells.

($p < .00001$). No significant changes were reported for the ALSFRS and FVC. The published abstract did not report the evaluation of proinflammatory or anti-inflammatory biomarkers.

4.2.2 | Nonrandomized clinical trials

As previously mentioned, we found three non-RCTs according to our inclusion criteria, one of which was excluded from the study because that was a protocol paper. Characteristics of included non-RCTs are shown in (Table 3).

Thonhoff et al's. (2018) study was a Phase I trial with no placebo controls and blinding methods. This study was conducted on only three patients with ALS (with no family history of ALS). For Patient 1, 2, and 3 the score of ALSFRS-R and the time from symptom onset to the first Treg infusion (month) were (44, 14), (36, 24), and (41, 38), respectively. Tregs were isolated and expanded ex vivo with leukapheresis approximately 1 month before the first injection. In total, patients were injected eight times intravenously (every 2 weeks at an early stage and every 4 weeks at a later stage) with expanded autologous Tregs (1×10^6 cells/kg) plus subcutaneously injection of low dose IL-2 (2×10^5 IU/m^2) and followed up for almost 2 years. Treatment with expanded autologous Tregs plus low dose IL-2, which was used to stabilize the infused expanded Tregs, increased Tregs proportion and suppressive function during the first and the second round of infusions. As well, increased Tregs suppressive function was associated with decelerated disease progression (correlation for Patient 1, 2, and 3 was [$p = .003$], [$p = .0026$], and [$p = .016$], respectively). This study reported no significant changes in FVC and a slower decrease in ALSFRS-R score during the first and the second round of infusions. Moreover, no infusion-related adverse events (AEs) were reported (Thonhoff et al., 2018).

Paganoni et al's. (2019) study was an investigator-initiated, open-label, and pilot clinical trial. In this study, 16 participants with ALS (13 participants completed 23 weeks of treatment) received 375 ml of RNS60 via intravenous infusion once a week (nebulization at home the remaining 6 days a week with 4 ml/day). The score of ALSFRS-R and the time (month) from symptom onset of participants were (34.1 ± 6.2) and (30.3 ± 17.8), respectively. As previously mentioned, RNS60 is an immune modulator agent with a potential for increasing the Tregs population. After the end of the treatment period, there were no statistically significant changes in IL-17 ($p = .3$) or FOXP3 mRNA expression ($p = .9$). As outcomes, the most common AEs included falls (75%), headaches (50%), nasopharyngitis (38%), and contusions (31%). No serious AEs related to RNS60 occurred and no participant withdrew from the trial due to treatment-related AEs. ALSFRS-R declined (not statistically significant) over the 23 weeks of observation and there was no statistically significant change in slow vital capacity (Paganoni et al., 2019).

4.2.3 | Ongoing clinical trials

We found four ongoing trials including Mandrioli et al's. (2018) study which was a Phase II clinical trial and published as a protocol paper (not containing the results). Characteristics of ongoing studies are shown in Table 4.

5 | DISCUSSION AND CONCLUSION

In this systematic review, we performed a meta-analysis to investigate the efficacy of Tregs (or agents directly targeting them) in improving survival in preclinical trials, while the number of clinical

TABLE 3 Characteristics of included nonrandomized clinical trials

Study	*N*	Type	Average score of ALSFRS-R	Average time from symptom onset (month)	Treatment-related change in Tregs proportion	Treatment-related change in ALSFRS-R	Treatment-related change in FVC	SAE	GRADE
Thonhoff et al. (2018)	3	Phase I trial	40.33	25.33	Yes	Slower reduction (Not significant)	No	No	Low quality (⊕⊕OO)
Paganoni et al. (2019)	16	Pilot trial	34.1	30.3	No	Reduction (Not significant)	No	No	Low quality (⊕⊕OO)

Abbreviations: ALS, amyotrophic lateral sclerosis; ALSFRS-R, ALS Functional Rating Scale-Revised; FVC, forced vital capacity; GRADE, Grading of Recommendations, Assessment, Development, and Evaluation; SAE, serious adverse event; Tregs, regulatory T cell.

TABLE 4 Characteristics of ongoing studies

Study ID	NCT02988297
Study name	MIROCALS: Modifying immune response and outcomes in ALS
Methods	A randomized, placebo-controlled, double-blind, and parallel-group trial
Participants	216 Participants
Interventions	Treatment arms • Riluzole • IL-2 • Placebo
Outcomes	• Time to death from date of randomization to date of death
Starting date	June 19, 2017
Contact information	Gilbert Bensimon, Christine Payan
Study ID	**NCT02988297**
Study name	Nebulized RNS60 for the treatment of ALS
Methods	A randomized, double-blind study
Participants	140 Participants
Interventions	Treatment arms • RNS60 • Placebo
Outcomes	• ALSFRS-R score • Deaths or tracheostomies • The proportion of Treg • SVC • ALSAQ-40 score • AEs
Starting date	July 2019
Contact information	No contacts or locations provided
Study ID	**NCT03359538**
Study name	Rapamycin treatment for ALS
Methods	Phase II randomized, double-blind, placebo-controlled, multicenter clinical trial
Participants	63 Participants
Interventions	Treatment arms • Rapamycin 2 mg/m^2 • Rapamycin 2 mg/m^2 • Placebo
Outcomes	• Tregs number • Number of SAEs and AEs in placebo and treatment arms • Rapamycin capacity to pass through the blood-brain barrier • Rapamycin efficacy in inhibiting the mTOR pathway • Changes in activation and homing capabilities of different T, B, natural killer (NK) cell subpopulations • Changes in CSF neurofilaments • Changes in blood biomarkers • Rapamycin-induced changes in inflammatory status • ALSFRS-R • Tracheostomy-free survival rate • Changes in FVC • Change in quality of life
Starting date	September 19, 2017
Contact information	Jessica Mandrioli
Study ID	**NCT03456882**
Study name	The effect of RNS60 on ALS biomarkers
Methods	Multicenter, randomized, double-blind, placebo-controlled, parallel group, and Phase II trial
Participants	142 Participants
Interventions	Treatment arms • RNS60 • Placebo
Outcomes	• Pharmacodynamics biomarkers, include Tregs, MCP-1, and IL-17 • ALSFRS-R scale • Survival • FVC • Incidence of adverse event (tolerability) related to RNS60 • Quality of life
Starting date	November 18, 2016
Contact information	Ettore Beghi, Elisabetta Pupillo

Abbreviations: AEs, adverse events; ALS, amyotrophic lateral sclerosis; ALSAQ, ALS assessment questionnaire; ALSFRS-R, ALS functional rating scale-revised; CSF, cerebrospinal fluid; FVC, forced vital capacity; IL-2, interleukin-2; IL-17, interleukin-17; MCP-1, monocyte chemoattractant protein-1; mTOR, mechanistic target of rapamycin; SAE, serious adverse event; SVC, slow vital capacity; Tregs, regulatory T cells.

trials (randomized and nonrandomized) is still not enough to assess their exact effectiveness. However, Thonhoff et al's. (2018) study showed that the treatment with expanded autologous Tregs plus low dose IL-2 can increase Tregs proportion. Furthermore, the suppressive function and increased Tregs function were associated with decelerated disease progression, which supports the potential of this approach for the treatment of patients with ALS (Thonhoff et al., 2018). Nevertheless, due to the low number of participants and the lack of the control group, there is a potential for publication bias in this study.

After statistical analysis in preclinical studies (Beers et al., 2011; Sheean et al., 2018; Vallarola et al., 2018), a significant increase was observed in the mean difference of the mRNA expression levels of

FOXP3 and survival of mice (about 3.95 days) due to the treatment. Some of these studies also showed an increase in anti-inflammatory cytokines and a reduction in inflammatory cytokines after treatment, which indicates the usefulness of Tregs in treating mice model of ALS. However, some researchers still have concerns about the generalization of the results of preclinical studies into effective human treatments (Mancuso & Navarro, 2015). This concern could be acceptable, because transgenic mSOD1^{G93A} mice are more likely to develop familial ALS than sALS. Because ALS is a heterogeneous disease with little-known etiopathogenetic implications, it is possible that familial and sporadic types of disease differ in some important mechanisms, which makes them different in the effectiveness of treatments.

In addition, it should be considered in future studies that Tregs have a heterogeneous phenotype so that only the examination of mRNA expression of genes (such as FOXP3) is not reliable, and more precise techniques, such as flow cytometry and more reliable markers should be exerted. Because there are also FOXP3^{-} Tregs that should be considered in the studies. Another point to be taken into account in future studies is the time it takes for the drugs to increase the population of Tregs. Because patients with ALS generally do not have proper clinical conditions, the time required for the efficacy of the drug should be taken into account.

In conclusion, ALS is a lethal heterogeneous disease, in which our knowledge of the causes of its occurrence has not yet been completed. In addition, the management and treatment of ALS have not been successful until today. Preclinical studies, as well as some of the results of clinical studies, indicate the effectiveness of Tregs application in the treatment of this disease. Tregs can be the future of ALS therapy. However, more RCTs have to be done to ensure their effectiveness in the treatment of ALS. Moreover, the results of the ongoing clinical trials, which have not yet been completed, can largely contribute to understanding whether the Tregs are beneficial for the treatment of ALS.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS

M. R. N. and S. R. conceived the study and wrote the manuscript. They performed the literature search and selected the relevant studies. F. S. and A. G. K. provided methodological support in the study design and classified retrieved articles according to the level of evidence. A. R. and L. A. H. critically revised the manuscript and provided the final approval.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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