RESEARCH ARTICLE OPEN ACCESS

Combined Regulatory T-Lymphocyte and IL-2 Treatment Is Safe, Tolerable, and Biologically Active for 1 Year in Persons With Amyotrophic Lateral Sclerosis

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Neurol Neuroimmunol Neuroinflamm 2022;9:e200019. doi:10.1212/NXI.0000000000200019

Abstract

Background and Objectives

In a phase 1 amyotrophic lateral sclerosis (ALS) study, autologous infusions of expanded regulatory T-lymphocytes (Tregs) combined with subcutaneous interleukin (IL)-2 were safe and well tolerated. Treg suppressive function increased and disease progression stabilized during the study. The present study was conducted to confirm the reliability of these results.

Methods

Participants with ALS underwent leukapheresis, and their Tregs were isolated and expanded in a current Good Manufacturing Practice facility. Seven participants were randomly assigned in a 1:1 ratio to receive Treg infusions (1×10^6 cells/kg) IV every 4 weeks and IL-2 (2×10^5 IU/m^2) injections 3 times/wk or matching placebo in a 24-week randomized controlled trial (RCT). Six participants proceeded into a 24-week dose-escalation open-label extension (OLE). Two additional participants entered directly into the OLE. The OLE included dose escalation of Treg infusions to 2×10^6 cells/kg and 3×10^6 cells/kg at 4-week intervals.

Results

The Treg/IL-2 treatments were safe and well tolerated, and Treg suppressive function was higher in the active group of the RCT. A meaningful evaluation of progression rates in the RCT between the placebo and active groups was not possible due to the limited number of enrolled participants aggravated by the COVID-19 pandemic. In the 24-week OLE, the Treg/IL-2 treatments were also safe and well tolerated in 8 participants who completed the escalating doses. Treg suppressive function and numbers were increased compared with baseline. Six of 8 participants changed by an average of −2.7 points per the ALS Functional Rating Scale–Revised, whereas the other 2 changed by an average of −10.5 points. Elevated levels of 2 markers of peripheral inflammation (IL-17C and IL-17F) and 2 markers of oxidative stress (oxidized low-density lipoprotein receptor 1 and oxidized LDL) were present in the 2 rapidly progressing participants but not in the slower progressing group.

Discussion

Treg/IL-2 treatments were safe and well tolerated in the RCT and OLE with higher Treg suppressive function. During the OLE, 6 of 8 participants showed slow to no progression. The 2 of 8 rapid progressors had elevated markers of oxidative stress and inflammation, which may help delineate responsiveness to therapy. Whether Treg/IL-2 treatments can slow disease progression requires a larger clinical study (ClinicalTrials.gov number, NCT04055623).

Classification of Evidence

This study provides Class IV evidence that Treg infusions and IL-2 injections are safe and effective for patients with ALS.

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Class of Evidence
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Go to Neurology.org/NN for full disclosures. Funding information is provided at the end of the article.

Glossary

AALS = Appel ALS Rating Scale; **AE** = adverse event; **ALS** = amyotrophic lateral sclerosis; **ALSFRS-R** = ALS Functional Rating Scale–Revised; **cGMP** = current Good Manufacturing Practice; **FDA** = Food and Drug Administration; **FVC** = forced vital capacity; **HMH** = Houston Methodist Hospital; **IL** = interleukin; **IP** = infusion product; **IRB** = Institutional Review Board; **MGH** = Massachusetts General Hospital; **MIP** = maximal inspiratory pressure; **OLE** = open-label extension; **OLR1** = oxidized low-density lipoprotein receptor 1; **ox-LDL** = oxidized low-density lipoprotein; **RCT** = randomized controlled trial; **SAE** = serious adverse event; **TEAE** = treatment-emergent adverse event; **UTHealth** = University of Texas Health Science Center at Houston.

Regulatory T-lymphocytes (Tregs) are a subpopulation of T lymphocytes that are defined by their $CD4^+CD25^+FOXP3^+$ markers and immunosuppressive ability.[1-4] In multiple diseases, Tregs lose their suppressive function and their neuroprotective capabilities.[5-8] In mutant SOD1 amyotrophic lateral sclerosis (ALS) mice, infusions of Tregs slowed disease progression.[9] In people with ALS, the expression of the Treg master transcription factor FOXP3 was reduced, and Treg suppressive function was impaired.[10,11] These reductions correlated with increased burden of disease and rate of disease progression.[10] When expanded ex vivo in the presence of interleukin (IL)-2 and rapamycin, Treg suppressive function was restored.[10,12] These data cumulatively suggest that infusions of expanded autologous Tregs together with injections of IL-2 may improve Treg suppressive functions in persons with ALS and slow their rates of disease progression.

A first-in-human phase 1 study was conducted in 3 persons with ALS to determine whether IV infusions of expanded autologous Tregs were safe and tolerable.[13] IL-2 was administered subcutaneously to enhance the function and longevity of the infused Tregs. The Treg infusions, in combination with IL-2, were safe and well tolerated, and Treg suppressive function and numbers increased following the infusions. In these 3 subjects, slowed progression rates also correlated with suppression of peripheral markers of oxidative stress and inflammation at early and later stages of disease.[14] Thus, enhancing Treg suppressive function could stabilize the subject's clinical status.[13]

The primary goals of the present trial were to determine whether Treg/IL-2 treatments increased Treg suppressive function and numbers and were safe and tolerable compared with placebo. Furthermore, this study aimed to refine processes for the isolation and ex vivo expansion of Tregs at a central current Good Manufacturing Practice (cGMP) facility, followed by cryopreservation and transportation of the final autologous Treg products to each site for repeated administrations in persons with ALS.

Methods

Standard Protocol Approvals, Registrations, and Patient Consents

Approvals from the Food and Drug Administration (FDA) and Institutional Review Boards (IRBs) at Houston Methodist Hospital (HMH) and MGH were obtained before study initiation. All participants provided written informed consent before screening. The study was registered on ClinicalTrials.gov (NCT04055623).

Trial Design and Oversight

A 24-week randomized, double-blind, placebo-controlled trial (RCT) followed by a 24-week ascending dose open-label extension (OLE) was conducted at 2 centers in the United States, HMH and Massachusetts General Hospital (MGH), from August 2019 to May 2021. Oversight of study progress and monitoring of unblinded safety data were provided by an independent data and safety monitoring board.

Trial design and data collection and analysis were performed through the collaborative efforts of HMH and MGH investigators. Statistical analyses were performed by the MGH Biostatistics Center. The Tregs and placebo were prepared at the University of Texas Health Science Center at Houston (UTHealth)—The Judith R. Hoffberger Cellular Therapeutics Laboratory, a FDA-registered, CAP- and FACT-accredited cGMP facility, transported from UTHealth to HMH on the days of infusions, and shipped frozen to MGH within 1 week before each infusion. All procedures were compliant with FDA regulations and guidelines. The IL-2 was provided by donation from the drug's manufacturer, Clinigen. Clinigen was not involved in the trial design, data analysis, or manuscript development. The IL-2/matching placebo syringes were prepared by investigational pharmacists at HMH and MGH for their institution's respective participants. All investigators and research personnel, other than personnel working in the cGMP facility and investigational pharmacies and the unblinded biostatistician, were blinded to the treatment for each participant during the RCT.

This study was powered to detect the change in Treg suppressive function in the RCT based on an observed increase in Treg suppressive function following Treg infusions and IL-2 injections in the phase 1 study.[13] All amendments to the protocol were approved by the FDA and IRBs at each institution before implementation.

Trial Participants

Adults with ALS meeting El Escorial criteria for possible, probable, laboratory-supported probable, or definite ALS were enrolled in the trial. Additional eligibility criteria included a decline in the ALS Functional Rating Scale–Revised (ALSFRS-R) total score of at least 2 points in the 90 days before screening or at least 4 points over the 180 days before screening, a forced vital capacity (FVC) ≥ 65% of predicted capacity at screening, and either no use of riluzole and willingness to refrain from initiating the agent for the duration of the trial or on a stable regimen of riluzole for at least 30 days at the time of screening.

If participants were on edaravone at the time of screening, they had to refrain from receiving edaravone on the same day as the infusion of the study drug. If the participants were not on edaravone, they would refrain from initiating the drug for the duration of the trial. The complete inclusion and exclusion criteria are found at ClinicalTrials.gov (NCT04055623).

Trial Interventions and Procedures

Eligible participants were randomized at a 1:1 ratio to receive Treg infusions/IL-2 injections or placebo infusions/placebo injections during the RCT. Randomization sequences were computer generated by the study statistician using permuted blocks, stratified by site. During the OLE, all participants received Treg infusions/IL-2 injections. All participants from both sites underwent leukapheresis at Houston Methodist Hospital within 6 weeks of their screening visit. Tregs were isolated from the leukapheresis products, expanded in IL-2 and rapamycin for 15–20 days, harvested, and cryopreserved in CryoStor CS10 10% DMSO-based medium (STEMCELL Technologies, Cambridge, MA) inside CellSeal cryogenic vials (Sexton Biotechnologies, Indianapolis, IN) containing 1×10^6 cells/kg for the respective participant. The Treg dose of 1×10^6 cells/kg/infusion and IL-2 dose of 2×10^5 IU/m^2/injection were administered during the RCT with Treg/placebo infusions every 4 weeks and the IL-2 subcutaneous injections 3 d/wk. During the OLE, the Treg dose was 1×10^6 cells/kg/infusion every 4 weeks for 2 infusions, then double and triple the starting dose for 2 infusions each.

On the days of Treg/placebo infusions, a cryovial(s) was thawed using a CellSeal Automated Thawing System (Sexton Biotechnologies, Indianapolis, IN), and the cells were diluted in 50 mL normal saline containing 5% human serum albumin. The final concentration of DMSO in the infusion product (IP) was 0.2%. The placebo IP was identical to the treatment IP, but without the cells. For infusions at MGH, a cryovial was shipped in liquid nitrogen vapor phase in a Cryoport shipment container, thawed and formulated in the Bone Marrow and Cellular Therapy Center at MGH, and infused within 6 hours of thawing. Quality assurance at both UTHealth and HMH ensured that all products met the criteria for release before infusion. Participants were closely monitored for any infusion-related adverse reactions.

Outcomes

The primary outcome measure was the change in Treg suppressive function from screening (baseline value) to week 24 during the RCT. The secondary outcome measures were the changes in Treg numbers from screening to week 24, safety, and tolerability. Exploratory outcome measures were changes in disease progression from baseline to week 24 as measured by the ALSFRS-R and Appel ALS Rating Scale (AALS)[15] and changes in respiratory measures of the FVC and maximal inspiratory pressure (MIP). Other exploratory outcome measures were to assess blood samples for levels of inflammation and oxidative stress and their responses to treatment.

Peripheral blood was drawn for assessments of Treg suppressive function and Treg numbers at screening, immediately before each infusion, 1 day after the first and sixth infusions of the RCT and OLE, 7 days after the first, third, and sixth infusions of RCT and OLE, the final visit of the RCT (week 24), the final visit of the OLE (week 50), and the final safety visit for the study (week 54). Treg suppressive function of the proliferation of autologous responder Tresp was assessed by [^{3}H]-thymidine incorporation. The assays were performed at the sites of the respective participants, but all plates were read at HMH to reduce intersite variability due to differences in machine calibration. Treg numbers were assessed by measuring the percentage of $CD4^+CD25^+FOXP3^+$ Tregs within the total $CD4^+$ population by flow cytometry at HMH. Serum samples were assayed for 48 inflammatory proteins (Olink Target 28 Cytokine, Olink Proteomics, Boston, MA). Oxidized LDL levels in the sera were assayed by ELISA per the manufacturer's instructions (Invitrogen kit L34357, Fisher Scientific).

Participants were asked about adverse events (AEs) at each visit. Safety was assessed by the occurrence of AEs, serious adverse events (SAEs), treatment-emergent adverse events (TEAEs), and clinically significant abnormalities in clinical and laboratory values. Tolerability was defined as the percentage of participants who completed all infusions during the 24-week RCT and each ascending dose during the OLE. The ALSFRS-R, AALS, and FVC and MIP measurements were performed at screening/baseline, immediately before each infusion, the final visit of the RCT (week 24), the final visit of the OLE (week 50), and the final safety visit for the study (week 54). All evaluators underwent training to ensure a consistent approach to each measurement scale. The ALSFRS-R and AALS were completed by trained clinical research personnel. The FVC and MIP were measured with a spirometer by a trained evaluator.

Statistical Analyses

Statistical analyses were performed using SAS (SAS Institute, NC) or R (R Foundation for Statistical Computing, Vienna, Austria). A single primary end point, 24-week change in Treg suppression function during the RCT was tested at 2-tailed $p < 0.05$. A single secondary end point of 24-week change in Treg numbers during the RCT was tested at 2-tailed $p < 0.05$ using a closed testing procedure to maintain an overall 2-tailed family-wise error rate less than 5%. Nominal comparison-wise p values were reported for all analyses. Treatment groups were compared for progression of continuous end points (Treg suppressive function and Treg numbers) during the RCT using a shared-baseline, linear mixed model. The model included fixed terms for discrete visit and treatment group × postbaseline visit interaction and random participant-level intercepts and slopes with unstructured covariance. The primary estimate was the treatment-dependent difference in change from the screening visit to the week 24 visit. The estimate and its 95% Wald confidence bounds were obtained by a linear contrast of adjusted means. For the OLE, data were analyzed in a mixed model with fixed terms for discrete visit and random participant-level intercepts and slopes with unstructured covariance. Data from the screening visit were

used as the start of follow-up for all participants, imputed at the time of the week 24 visit for participants who went through the RCT. Estimates of change from the screening visit and comparisons between dosage levels were obtained by linear contrasts of adjusted means at applicable visits.

Data Availability

The Study Protocol and Statistical Analysis Plan are available in the Supplement. The individual deidentified participant data including clinical evaluations, AE logs, and Treg suppressive function and numbers will be shared with any qualified investigator through requests made to the corresponding author.

Results

Trial Participants

The initial plan for the study included 12 participants, 6 from HMH and 6 from MGH, randomized 1:1 on active drug to placebo who would proceed from the RCT to the OLE. However, the COVID-19 pandemic limited the enrollment to 9 participants, 4 at HMH and 5 at MGH. Two participants' Tregs could not be expanded. One of these 2 received IL-2 briefly and was included in the safety analysis of AEs. Seven successfully began treatment in the RCT. Six of these participants successfully completed the RCT and OLE. One participant discontinued the study after receiving the second Treg or placebo infusion due to an SAE classified as an allergic reaction. At the study's conclusion, the participant was revealed as having been in the placebo group. Anaphylactic shock has been described from constituents of the placebo formulation, including albumin and DMSO. There were no similar SAEs in participants receiving Treg infusions either in the RCT or OLE. Two additional participants entered the OLE directly and successfully completed the OLE. Data were collected at each site and analyzed for 7 participants in the RCT and 8 participants in the OLE. The overall trial schematic is shown in Figure 1.

Baseline demographic and disease characteristics for the RCT are summarized in eTable 1, links.lww.com/NXI/A738. The 4 participants in the placebo group and 3 participants in the Treg/IL-2 group were well matched for all the parameters assessed other than the ALSFRS-R respiratory subscore, which was lower in the placebo group.

Safety and Tolerability During the RCT

A summary of the TEAEs during the RCT is organized by organ class and shown in eTable2, links.lww.com/NXI/A738. There were no major differences in the AE profiles between the placebo and treatment groups. Eight participants were included in the safety analysis. Adverse events related to the IL-2/placebo injection sites were experienced in both groups.

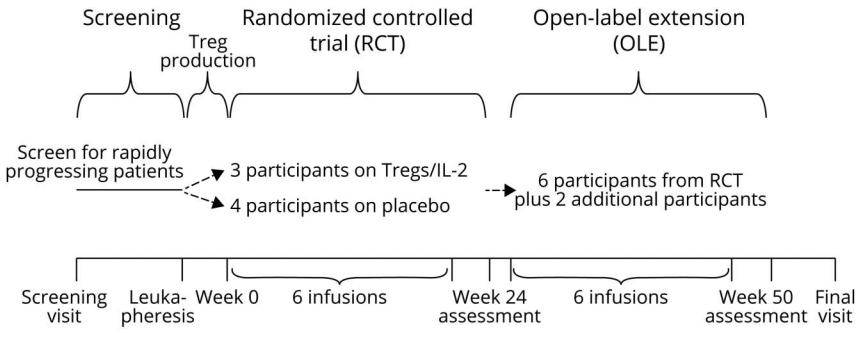
Treg Suppressive Function and Numbers During the RCT

The primary end point of the study, change in Treg suppressive function from screening to week 24 (4 weeks following the final RCT Treg/placebo infusion), decreased by 6.4% in the placebo arm (screening, 27.6% ± 5.8; week 24, 21.2% ± 13.3) and increased by 20.0% in the Treg/IL-2 arm (screening, 27.6% ± 5.8; week 24, 47.6% ± 13.8), equaling a difference of 26.4% between the groups (95% CI −10.6 to 63.4, $p = 0.16$; Figure 2A). The Treg numbers increased by 2.2% in the placebo arm (screening, 3.9% ± 0.6; week 24, 6.1% ± 0.8) and decreased by 1.3% in the Treg/IL-2 arm (screening, 3.9% ± 0.6; week 24, 2.6% ± 0.9), equaling a difference of −3.5% (95% CI −5.8 to −1.2, $p = 0.004$; Figure 2B).

Exploratory End Points During the RCT

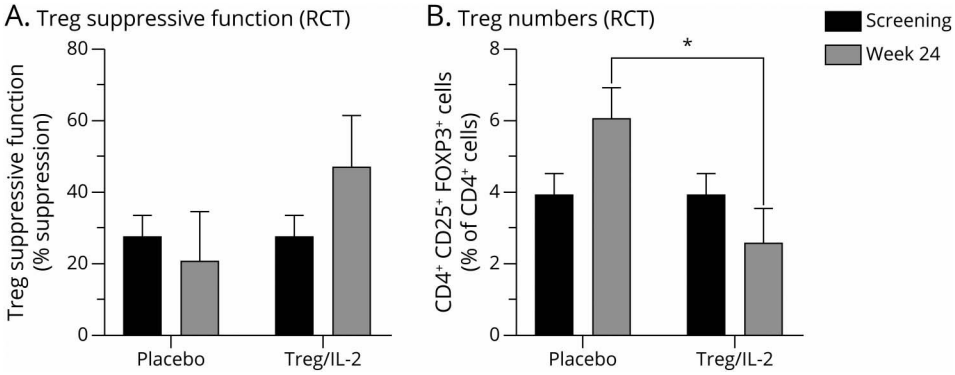
Due to limitations placed on pulmonary function evaluations caused by the COVID-19 pandemic, not all AALS, FVC, and MIP measurements were performed during the RCT. Disease progression during the RCT was assessed using the ALSFRS-R and is shown for each participant in the placebo and treatment groups in Figures 3A and B, respectively.

Figure 1 Study Design



Seven participants with rapidly progressing ALS were enrolled and underwent leukapheresis. The participants were randomized at a 1:1 ratio to active drug or placebo. Participants received IL-2 or placebo subcutaneous injections between 2 and 4 weeks before week 0. In the RCT, placebo or Treg infusions were administered every 4 weeks beginning week 0 for a total of 6 infusions. The final assessment of the RCT was performed on week 24. Six participants from the RCT entered the open-label extension (OLE). Two additional participants were directly enrolled into the OLE. All 8 participants received IL-2 beginning 2–4 weeks before the first Treg infusion. Treg infusions were administered every 4 weeks in the OLE beginning week 26 (week 0 for 2 participants who entered directly into the OLE) for a total of 6 infusions. The first 2 infusions were a 1× dose of Tregs, the next 2 infusions were a 2× dose, and the final 2 infusions were a 3× dose. The final assessment of the OLE was performed on week 50, and the final study visit occurred on week 54. IL = interleukin; RCT = randomized controlled trial; Treg = regulatory T-lymphocyte.

Figure 2 Treg Suppressive Function and Numbers in the Randomized Controlled Trial



(A) Treg suppressive function was assessed in each participant in the placebo and Treg/IL-2 treatment groups at screening and week 24 (4 weeks after the last infusion of the RCT). (B) Treg numbers were assessed in each participant in the placebo and Treg/IL-2 treatment groups at screening and week 24 (4 weeks after the last infusion of the RCT). Data were depicted as visit-specific estimates ±standard error and were compared for progression of continuous end points using a shared-baseline, linear mixed model. A $p < 0.05$ was considered significant. IL = interleukin; RCT = randomized controlled trial; Treg = regulatory T-lymphocyte.

Trial Participants in the OLE

Baseline demographic and disease characteristics for the OLE are summarized in eTable 3, links.lww.com/NXI/A738. Six participants proceeded from the RCT to the OLE. Two participants were directly enrolled into the OLE. The demographics differed only in that the 2 direct enrollees in the OLE were older.

Safety and Tolerability During the OLE

Treg/IL-2 treatments were safe and well tolerated in all participants, including the 3 participants who received Treg/IL-2 treatments in the RCT and OLE over a 1-year period. The TEAEs from the OLE are summarized by organ class for the 8 participants in eTable 4, links.lww.com/NXI/A738. All TEAEs were observed to be mild, and all participants tolerated the escalating doses up to 3× the initial Treg dose.

Treg Suppressive Function and Numbers During the OLE

During the OLE, the Treg suppressive function (Figure 4A; screening, 33.5% ± 6.2; week 34, 56.0% ± 7.4, difference 22.5%, 95% CI 8.0 to 37.0, $p = 0.003$; week 42, 56.3% ± 9.0, difference 22.8%, 95% CI 5.3 to 40.3, $p = 0.011$; week 50, 50.2% ± 11.1, difference 16.7%, 95% CI -4.6 to 38.1, $p = 0.123$) and Treg numbers (Figure 4B; screening, 3.6% ± 0.9; week 34, 5.3% ± 0.9, difference 1.7%, 95% CI 0.04 to 3.4, $p = 0.045$; week 42, 5.3% ± 1.0, difference 1.7%, 95% CI -0.03 to 3.5, $p = 0.054$; week 50, 4.4% ± 1.1, difference 0.8%, 95% CI -1.0 to 2.7, $p = 0.36$) were higher compared with baseline measurements obtained at screening. Evidence of a dose-response was not observed in either Treg suppressive function or numbers.

Disease Progression of Participants During the RCT and OLE

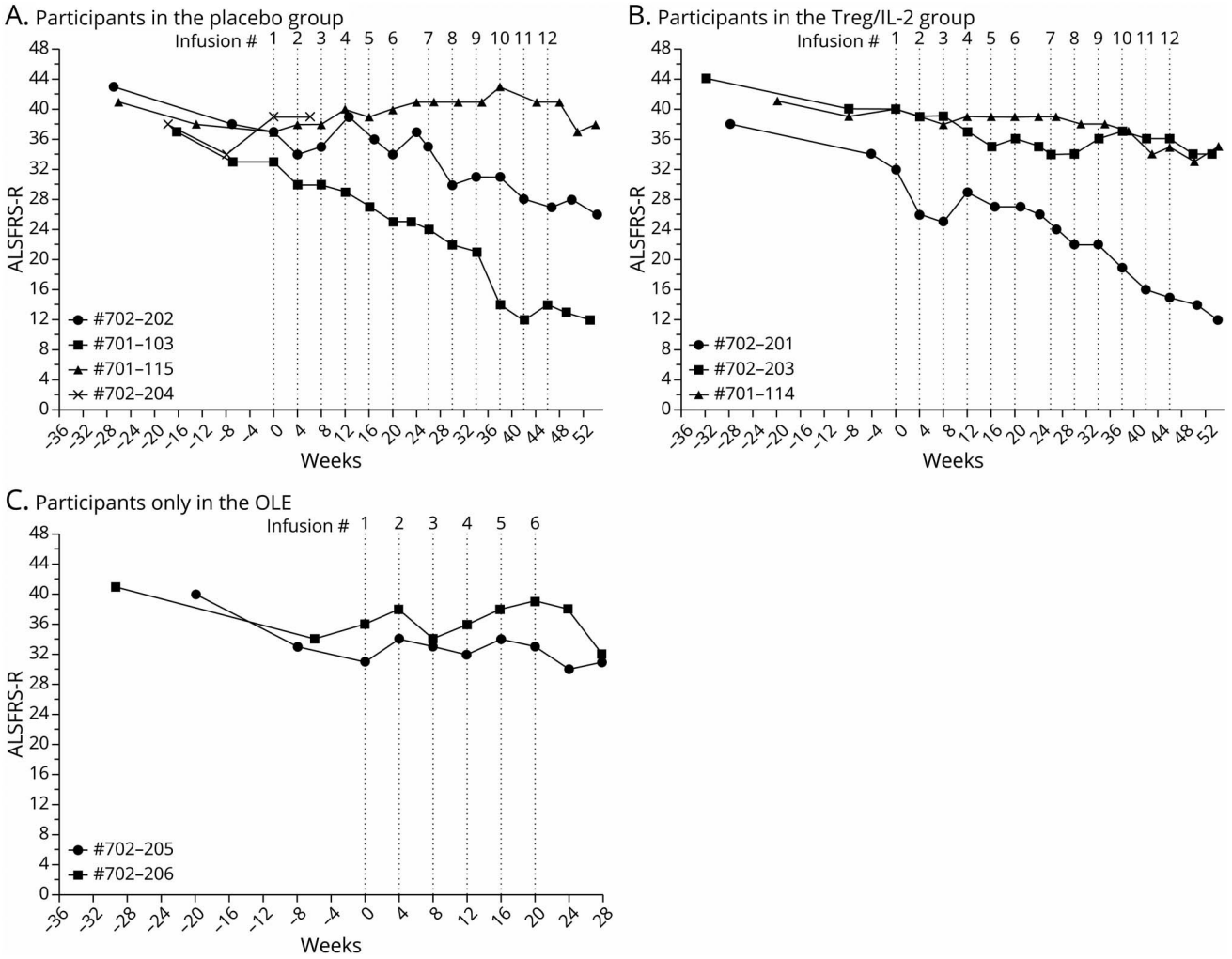
Disease progression curves per the ALSFRS-R for each of the 7 participants in the RCT are shown in Figure 3 (Figure 3A placebo arm; Figure 3B treatment arm). In the placebo group, participant #702-204 was removed after the second infusion. Of the 3 remaining participants in the placebo group (Figure 3A), 1 participant remained relatively stable (#701-115), 1 participant progressed at an intermediate rate (#702-202), and 1 participant progressed at a rapid rate (#701-103). During the RCT, participant #701-115 increased from an ALSFRS-R score of 38 to 41, participant #702-202 declined from 38 to 37, and participant #701-103 declined from 33 to 25. During the OLE, with escalating Treg doses, participant #701-115 declined from an ALSFRS-R score of 41 to 37, participant #702-202 declined from 35 to 28, and participant #701-103 declined from 24 to 13. Of note, participant #701-115 appeared to improve during the OLE but contracted COVID-19 at week 47; participant #701-115 declined 4 points between week 46 and week 50. Furthermore, although participant #701-115 did meet the criteria for clinically possible ALS per the El Escorial criteria, this participant was the only person in the study who appeared to improve over 50 weeks from the RCT to the OLE before contracting COVID-19. Of the 3 participants in the Treg/IL-2 group (Figure 3B), 2 participants remained relatively stable (#702-203 and #701-114), and 1 participant progressed at a rapid rate (#702-201). During the RCT, participant #702-203 declined from an ALSFRS-R score of 40 to 35, participant #701-114 was stable at 39, and participant #702-201 declined from 34 to 26. During the OLE, participant #702-203 had a stable ALSFRS-R of 34, participant #701-114 declined from 39 to 33, and participant #702-201 declined from 24 to 14.

Disease progression curves per the ALSFRS-R for the 2 participants who enrolled directly into the OLE are shown in Figure 3C. Both participants (#702-205 and #702-206) remained stable through 24 weeks (Figure 3C). Participant #702-205 declined from an ALSFRS-R score of 31 to 30, and participant #702-206 improved from 36 to 38.

Disease Progression in All Participants During the OLE

During the OLE, a post hoc analysis separated the 8 participants into 2 separate groups; 6 participants (#701-114, #701-115, #702-202, #702-203, #702-205, and #702-206) who showed an average ALSFRS-R change of −2.7 points (−0.45 points/month) and 2 participants (#701-103 and #702-201) who showed rapid progression with an average ALSFRS-R

Figure 3 Disease Progression During the RCT and OLE



(A) Four participants were randomized to the placebo group and received infusions every 4 weeks as depicted by the vertical dotted lines. Infusions #1–6 were placebo infusions in the RCT, and #7–12 were Treg infusions in the OLE. (B) Three participants were randomized to the active group (Treg/IL-2 treatment) and received infusions every 4 weeks. Infusions #1–6 were Treg infusions in the RCT, and #7–12 were Treg infusions in the OLE. Disease progression was documented in each participant by the ALSFRS-R over the duration of the 54-week study including the screening period. Placebo or IL-2 subcutaneous injections were administered beginning 2–4 weeks before the first infusion at week 0 for the RCT and 2 weeks before the 7th infusion at week 26 for the OLE. (C) Two participants were directly enrolled into the OLE and received Treg infusions every 4 weeks as depicted by the vertical dotted lines. Disease progression was documented in each participant by the ALSFRS-R over the duration of the 28-week study including the screening period. IL-2 subcutaneous injections were administered beginning 4 weeks before the first infusion at week 0. ALSFRS-R = ALS Functional Rating Scale–Revised; IL = interleukin; OLE = open-label extension; RCT = randomized controlled trial; Treg = regulatory T-lymphocyte.

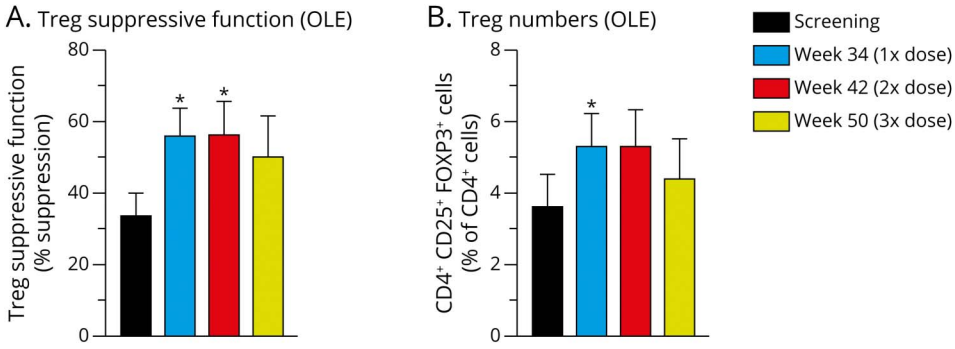
change of −10.5 points (−1.75 points/month) as represented in Figure 5.

Peripheral Levels of Inflammation and Oxidative Stress

To determine why participants #701-103 and #702-201 differed substantially from the other 6 participants, peripheral markers of inflammation and oxidative stress were assessed in the participants' sera through an unbiased approach using Olink proteomic profiles. Four of these potential markers were found to differentiate the 6 participants who showed intermediate to no progression from the 2 participants who progressed rapidly during the OLE (Figure 6). IL-17F (Figure 6A) and IL-17C (Figure 6B) were elevated in the 2 rapidly progressing participants at baseline compared with the 6 other participants. Furthermore, the 6 participants had IL-17F and IL-17C levels within or close to the range of healthy controls. The IL-17F and IL-17C levels did not fluctuate with Treg/IL-2 treatments in the 6 participants who did well during the OLE. However, in the 2 rapidly progressing participants, both IL-17F and IL-17C levels remained high during the study and further increased toward the end of the OLE. The levels of IL-17F and IL-17C for each participant throughout the RCT did not substantially differ from the baseline values obtained at screening (data not shown).

One marker of oxidative stress, oxidized low-density lipoprotein receptor 1 (OLR1), was elevated in the sera of the 2 rapidly progressing participants compared with the 6 participants and the controls (Figure 6C). OLR1 levels in the 6 participants were within the range of the controls and showed a trend of

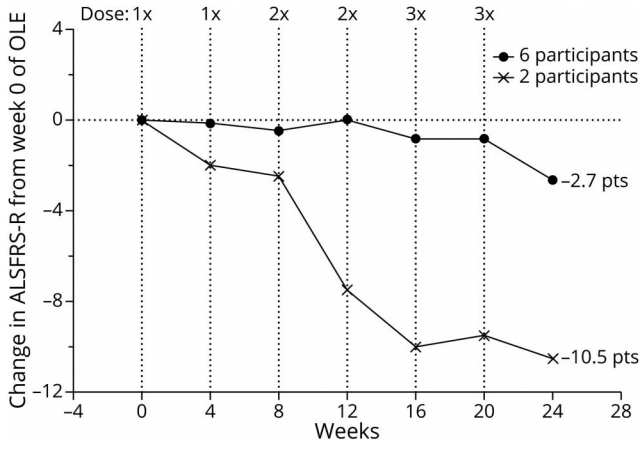
Figure 4 Treg Suppressive Function and Numbers in the Open-Label Extension (OLE)



(A) Treg suppressive function was assessed in each participant at screening, 4 weeks after the second infusion of a 1× dose of Tregs (week 34), 4 weeks after the second infusion of a 2× dose (week 42), and 4 weeks after the second infusion of a 3× dose (week 50). (B) Treg numbers were assessed in each participant at screening, 4 weeks after the second infusion of a 1× dose of Tregs (week 34), 4 weeks after the second infusion of a 2× dose (week 42), and 4 weeks after the second infusion of the 3× dose (week 50). Data were depicted as visit-specific estimates ±standard error and were compared for progression of continuous end points using a shared-baseline, linear mixed model. A $p < 0.05$ was considered significant. Treg = regulatory T-lymphocyte.

decreasing in response to treatment with Tregs/IL-2. In the 2 rapidly progressing patients, OLR1 decreased following the first 2 infusions, but increased to higher levels over the remainder of the OLE. Based on the OLR1 findings, further evaluation of oxidized low-density lipoprotein (ox-LDL) was performed by an ELISA (Figure 6D), and changes in ox-LDL levels relative to controls were calculated. ox-LDL levels were elevated in the 2 rapidly progressing participants. Of the 6 other participants, all were near or within the range of controls, except for participant #701-115 who had elevated levels similar to the 2 rapidly progressing participants. The levels of OLR1 in the 2 rapidly progressing participants, #701-103 (placebo arm) and #702-201 (treatment arm), substantially increased during the RCT (data not shown), but their levels of ox-LDL remained similar to the baseline levels (data not shown). The levels of OLR1 and ox-LDL did not appreciably differ from the baseline levels in the other 4 participants during the RCT (data not shown).

Figure 5 Disease Progression of Participants During the Open-Label Extension (OLE)



Disease progression was documented in each participant by the ALSFRS-R over the duration of the 24-week OLE. The timing of the Treg infusions was depicted by the vertical dotted lines with the corresponding Treg dosages. The baseline ALSFRS-R value for the OLE was taken at week 26 for the 6 participants who went through the RCT and week 0 for the 2 participants who entered directly into the OLE. The total change in the ALSFRS-R was calculated after 24 weeks. Six of the 8 participants showed intermediate to no progression of the ALSFRS-R (average of −2.7 points). Two of the 8 participants showed rapid progression (average of −10.5 points). ALSFRS-R = ALS Functional Rating Scale–Revised; Treg = regulatory T-lymphocyte.

Classification of Evidence

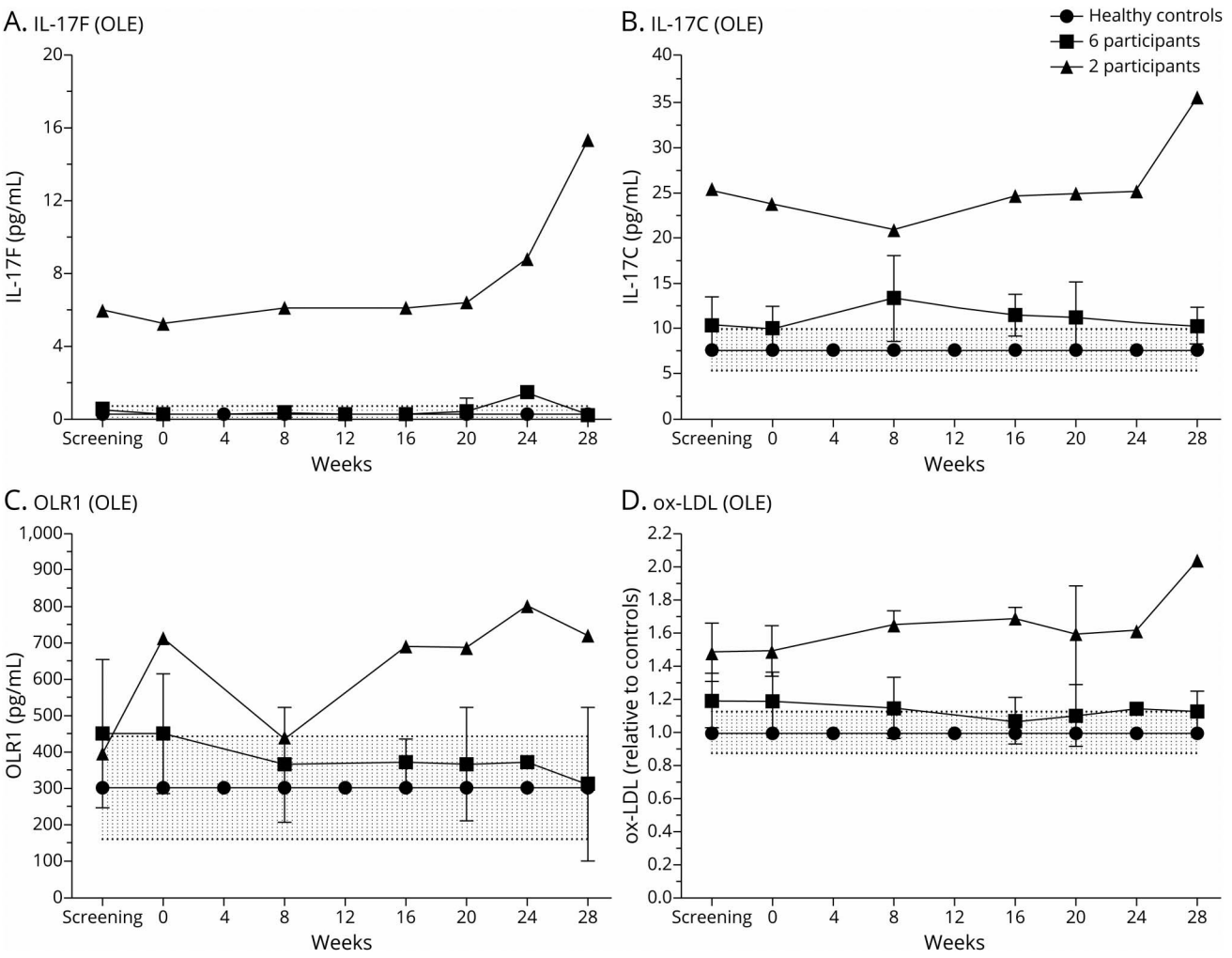
This study provides Class IV evidence that Treg infusions and IL-2 injections are safe and effective for patients with ALS.

Discussion

This study demonstrates that Treg/IL-2 treatments are safe, tolerable, and biologically active in persons with ALS over 1 year. Due to the unpredictable nature of the COVID-19 pandemic, only 7 participants were initiated in the RCT, 4 in the placebo group and 3 in the Treg/IL-2 treatment group, thus providing limited statistical power to detect changes in the primary and secondary end points, Treg suppression function, and numbers, respectively, in the RCT. Nevertheless, the primary end point of the RCT, change in Treg suppressive function from screening to week 24, was 26 percentage points higher in participants receiving Treg/IL-2 therapy compared with placebo. During the RCT, the Treg/IL-2 therapy was 100% tolerable and appeared safe, with only mild AEs; mild and transient inflammation at the sites of the IL-2 injections were common. The single anaphylactic reaction in the placebo group has been observed in other studies using excipients of albumin and/or DMSO, and the participant recovered fully. This reaction highlights a need for close monitoring of patients with infusions that include albumin and/or DMSO in IV formulations. The RCT was not powered to evaluate efficacy, and the COVID-19 pandemic negatively affected the number of participants, not allowing for a meaningful efficacy analysis.

In the 8 participants who went through the OLE, Treg/IL-2 treatments were safe and 100% tolerable even with escalating Treg doses. Treg suppressive function and numbers were higher compared with the participants' baseline values obtained

Figure 6 Peripheral Markers of Inflammation and Oxidative Stress During the Open-Label Extension (OLE)



Sera from all 8 participants were collected during the OLE and assayed through an Olink proteomic study for markers of inflammation and oxidative stress. Two markers of inflammation, (A) IL-17F and (B) IL-17C, were higher in the 2 participants who progressed rapidly during the OLE compared with the 6 participants who showed intermediate to no progression. One marker of oxidative stress, (C) oxidized low-density lipoprotein receptor 1 (OLR1), was elevated in the 2 rapidly progressing participants compared with the other 6 participants. An ELISA for another marker of oxidative stress, (D) oxidized low-density lipoprotein (ox-LDL), showed higher levels in the 2 rapidly progressing participants compared with the other 6 participants. Levels of IL-17F, IL-17C, and OLR1 in sera from 9 healthy controls were depicted as shaded areas of the mean ± SD. Levels of ox-LDL in sera from 26 healthy controls were depicted as a shaded area of the mean ± SD. The levels of ox-LDL in the participants were standardized to the control levels. These data were not statistically analyzed as there were only 2 participants in the rapidly progressive group. IL = interleukin.

at screening. In a post hoc analysis of the OLE, 6 of 8 participants showed intermediate to no progression as measured by the ALSFRS-R, whereas the other 2 participants showed rapid progression while receiving the Treg/IL-2 treatment. An unbiased assay of the OLE specimens with Olink documented that the 2 rapidly progressing participants had higher levels of inflammation (IL-17F and IL-17C) and oxidative stress (OLR1 and ox-LDL) compared with the other 6 participants, representing important findings that may aid in the stratification of future trials evaluating immunomodulatory therapies.

Tregs have the potential to suppress inflammation in the periphery, and in theory, Treg/IL-2 treatments should suppress the ever-worsening proinflammatory milieu that develops in people with ALS. In specimens collected throughout the study, post hoc analyses identified 4 markers of inflammation and oxidative stress that differentiated the 2 participants who progressed rapidly from the 6 participants who showed intermediate to no progression. These 2 participants, 1 randomized to the placebo arm and 1 to the active Treg/IL-2 arm, progressed rapidly throughout the RCT and OLE and did not respond to the active therapy, even with escalating doses in the OLE. They both showed elevated levels of proinflammatory IL-17F and IL-17C cytokines as well as oxidative stress markers, OLR1 and ox-LDL, throughout the RCT and the OLE. The high levels of inflammation and oxidative stress observed in these 2 participants could have limited the effectiveness of the Tregs resulting in shorter half-lives and negating their downstream suppressive effects. Larger and/or more frequent doses of Tregs may be needed to combat a more hazardous in vivo environment to alter its rapidly progressing clinical trajectory. The frequency and dose of the Treg

infusions in future trials might need to be tailored to the degree of each participant's inflammatory milieu.

IL-17F is released from Th17 cells as a proinflammatory cytokine and in turn can induce the production of many other proinflammatory cytokines including IL-6, IL-1β, TNFα, as well as chemokines, IL-8, GRO-α, and MCP-1, and prostaglandins from many cell types including macrophages.[16] IL-17C is produced primarily by epithelial cells rather than hematopoietic cells and induces the expression of proinflammatory cytokines and chemokines.[17] Tregs are not end stage differentiated, and IL-6, which is increased in ALS sera, has been documented to convert $FOXP3^+$ Tregs to Th17 cells.[18] Th17 cell numbers are increased in the peripheral blood of patients with ALS, and IL-17 levels are increased in the CSF and sera of patients with ALS.[19] For instance, in the sera of the rapidly progressing participant, #701-103, IL-6 was more than 4 times greater at baseline compared with controls with levels maintained at more than a 2.5-fold increase over controls throughout the study (data not shown), thus possibly converting the infused Tregs to Th17 cells. Increased Th17 cells aggravate the inflammatory cascade by increasing IL-17 levels, which induces further release of IL-6 from activated macrophages and promotes a self-propagating inflammatory milieu.

The question that needs further clarification and confirmation is whether proinflammatory cytokines may be so elevated as to create an inflammatory milieu unresponsive to Treg/IL-2 treatments. The results of this study suggested that levels of oxidative stress and inflammation were higher in a subpopulation of patients with rapidly progressing ALS (2 of 8 participants) who were unresponsive to Treg/IL-2 treatments. If this result is confirmed in larger, sufficiently powered studies, elevated IL-17F and IL-17C levels could be used as exclusion criterion or for participant stratification in future studies of immunomodulatory therapies in ALS. Furthermore, IL-6 levels may need to be evaluated in the stratification process. Regardless of whether this initiating inflammatory milieu is driven by genetic or environmental factors, suppression of activated myeloid proinflammatory cytokine production and release could mitigate the conversion of Tregs to Th17 cells and foster prolonged neuroprotection.

IVIg suppresses many of the aforementioned proinflammatory factors, but it is not effective in patients with rapidly progressing ALS.[20] Treatments with expanded Treg infusions and IL-2 injections are potentially superior due to their enhanced abilities to suppress proinflammatory myeloid cells as well as Th1 and Th17 signals.[5,6,10] In addition, in contrast to Tregs, IVIg does not prevent the range and extent of cytokine production and secretion from proinflammatory cells, nor does it inhibit the proinflammatory effects of reactive oxygen species and lipid peroxides.[14] Along the same lines, targeting a single cytokine such as TNFα or IL-6 with monoclonals may not be as effective as therapeutic approaches that simultaneously suppress multiple cytokines, reactive oxygen species, and lipid peroxides.

This study provides additional evidence that IV administered expanded autologous Tregs in combination with subcutaneous IL-2 injections are safe and well tolerated in persons with ALS. This study also provides the proof of concept that dysfunctional Tregs from patients with ALS can be manufactured into robust numbers of highly functional Tregs, cryopreserved, shipped, thawed, and then infused back into patients at regular intervals to enhance Treg suppressive function.[10-13] Furthermore, safety and tolerability of monthly infusions of expanded, cryopreserved, and thawed autologous Tregs can be sustained for, at least, 1 year. The RCT was limited by the small number of participants and the fact that the COVID-19 pandemic precluded reaching the planned number of enrollments as well as the completion of several clinical assessments. However, the fact that 6 of 8 participants in the OLE showed slow to no progression is a promising result. Whether the prolongation of the slow phase of the disease in study participants supports the hypothesis that Treg/IL-2 treatments exert a clinically meaningful benefit requires additional investigation. A larger clinical trial would allow for the proper evaluation of clinical efficacy and further characterization of the long-term safety of this therapy.

Acknowledgment

The authors are extremely grateful to all the participants in this study as well as their families and caregivers. They are also very appreciative of the funding from ALS Finding a Cure, ALS Association, and Muscular Dystrophy Association and their continuing support throughout the COVID-19 pandemic. They are especially thankful to Clinigen for their generous donation of the IL-2 that was used to treat the participants in this study. They thank the cGMP operators who performed the Treg manufacturing including David Vo and Aiping Gao from HMH and Dannelys Perez Bello and Cecilia Martin from UTHealth; Sufira Kiran and Deepa Bhattarai who provided the Quality Assurance for the Treg products at UTHealth as well as Russell Wilson and Christopher Lincoln who provided Quality Assurance at HMH; Naama Toledano Furman and Mitra Nair for performing QC flow testing at UTHealth; Shixiang Wen, Douglas Casey, Rangarajan Sreenivasan, and Jinghong Wang at HMH and Olivia L. Venezia at MGH for performing all assays on the clinical study samples including Treg suppressive function and flow cytometry; Dr. Christopher Leveque and Dr. Eric Salazar for performing the leukapheresis at the HMH Blood Donor Center; Dr. Jeremy Shefner for serving as the Medical Monitor; the members of the Data Safety and Monitoring Board including Dr. Lorne Zinman (Chair), Dr. Eric Macklin, Dr. Christina Nicole Fournier, and Dr. Matthew Frigault; and the NCRI coordination center including Matthew Sexton, Stephanie Berry, and Lindsay Pothier.

Study Funding

ALS Finding a Cure, ALS Association, and Muscular Dystrophy Association.

Disclosure

The Treg manufacturing process used in this study has been licensed from the Houston Methodist Hospital Research Institute to Coya Therapeutics, Inc. Drs. Thonhoff, Beers, Zhao, Thome, and Appel are listed as inventors of the Treg

manufacturing process in a patent application. Dr. Appel serves as chair of the Scientific Advisory Board for Coya Therapeutics, Inc. Coya Therapeutics, Inc., was not involved in the study concept and design, acquisition, analysis, and interpretation of data, or drafting of the manuscript. J. R. Thonhoff reports no other disclosures. J. D. Berry and E. A. Macklin report grants from ALS Finding a Cure, ALS Association, and Muscular Dystrophy Association. D. R. Beers, P. A. Mendoza, W. Zhao, A. D. Thome, F. Triolo, and J. J. Moon report no disclosures. S. Paganoni reports grants from ALS Finding a Cure and ALS Association. M. E. Cudkowicz reports personal fees from Biogen, Takeda, Cytokinetics, Immunity Pharma, Lilly, Anelixis, Aclipse, Orion, Biohaven, Sunovion, ALS Pharma, MT Pharma, Avanir, Wave, Avexis, Revalesio, and Disarm. S. H. Appel reports personal fees from Mitsubishi Tanabe Pharma, Neuraltus, and UCB Biopharma. Go to Neurology.org/NN for full disclosures.

Publication History

Received by *Neurology: Neuroimmunology & Neuroinflammation* January 26, 2022. Accepted in final form June 14, 2022. Submitted and externally peer reviewed. The handling editor was Marinos C. Dalakas, MD, FAAN.

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