**A Randomized, Placebo-Controlled Phase IIa Trial to Evaluate the Biological Activity, Safety, and Tolerability of Autologous Regulatory T Lymphocytes (Tregs) Expanded Ex-Vivo and Returned Intravenously in Combination with Low-Dose IL-2 in People with Amyotrophic Lateral Sclerosis (ALS)** Protocol Version: 9.0 (10Aug2020)

IND 16614

Funded by: ALS Association, ALS finding a Cure and Muscular Dystrophy Association


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## SIGNATURE PAGE

I have read the attached protocol entitled, A Randomized, Placebo-Controlled Phase IIa Trial to Evaluate the Biological Activity, Safety, and Tolerability of Autologous Regulatory T Lymphocytes (Tregs) Expanded Ex-Vivo and Returned Intravenously in Combination with Low-Dose IL-2 in with Amyotrophic Lateral Sclerosis (ALS), dated 10Aug2020 (Version 9.0) and agree to abide by all described protocol procedures. I agree to comply with the International Council on Harmonization Tripartite Guideline on Good Clinical Practice (FDA GCP), applicable FDA regulations and guidelines identified in 21 CFR Parts 11, 50, 56, and 312, local Institutional Review Board (IRB) guidelines and policies, and the Health Insurance Portability and Accountability Act (HIPAA).

Site Principal Investigator Printed Name: _____

Signed: _____

Date: _____

## STATEMENT OF COMPLIANCE

The trial will be carried out in accordance with Food and Drug Administration Good Clinical Practice (FDA GCP) and the following:

- United States (US) Code of Federal Regulations (CFR) applicable to clinical studies (45 CFR Part 46, 21 CFR Part 50, 21 CFR Part 56, 21 CFR and Part 312)

National Institutes of Health (NIH)-funded investigators and clinical trial site staff who are responsible for the conduct, management, or oversight of NIH-funded clinical trials have completed Human Subjects Protection Training.

The protocol, informed consent form(s), recruitment materials, and all participant materials will be submitted to the Institutional Review Board (IRB). Approval of both the protocol and the consent form must be obtained before any participant is enrolled. Any amendment to the protocol will require review and approval by the IRB before the changes are implemented to the study. In addition, all changes to the consent form will be IRB-approved; a determination will be made regarding whether a new consent needs to be obtained from participants who provided consent, using a previously approved consent form.

## 1 PROTOCOL SUMMARY

### 1.1 SYNOPSIS

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|---|---|
| Title: | A Randomized, Placebo-Controlled Phase IIa Trial to Evaluate the Biological Activity, Safety, and Tolerability of Autologous Regulatory T Lymphocytes (Tregs) Expanded Ex-Vivo and Returned Intravenously in Combination with Low-Dose IL-2 in People with Amyotrophic Lateral Sclerosis (ALS) |
| Study Description: | Increasing evidence demonstrates that dysregulation of the immune system hastens ALS disease progression. In particular, Treg numbers and function are reduced in patients with ALS and more marked reduction is associated with more rapid progression. We hypothesize that enhancing Treg numbers and function will slow disease progression. We have completed a single- center, open-label phase I study of autologous Tregs from people with ALS, expanded ex-vivo and returned to study participants in multiple doses every 2 to 4 weeks. This early study provided evidence that treatment with autologous Tregs in this manner may be efficacious in slowing ALS progression. |

As a stepping stone toward efficacy trials, we are now proposing this study, a multicenter (two-site) trial in which Tregs will be expanded ex-vivo at a central facility, cryopreserved and then administered at two sites. This is a two-period study with two cohorts. The first period consists of a screening, followed by leukapheresis, and then a 6-month, randomized, placebo-controlled clinical trial (RCT) evaluating the biological activity, safety and tolerability of an expanded population of autologous Tregs administered intravenously in combination with subcutaneous low-dose IL-2 versus matching "Treg placebo" (containing normal saline, 5 % human serum albumin and 0.2% DMSO per infusion) and matching "IL-2 placebo" (containing normal saline). The second period is a 6-month open-label extension (OLE) in which participants will receive autologous Tregs administered intravenously in combination with subcutaneous low-dose IL-2. Cohort 1 will consist of 6 participants who will go through period 1 and period 2. Cohort 2 will consist of 2 participants who will only go through period 2 after successfully completing the screening and leukapheresis.

Objectives:

Primary Objectives:

1. To evaluate the biological activity of IV infusion of an expanded population of autologous Tregs and subcutaneous low-dose IL-2 in people with ALS by evaluating Treg suppressive functions.

Secondary Objective:

1. To evaluate the biological activity of IV infusion of an expanded population of autologous Tregs and subcutaneous low-dose IL-2 in people with ALS by evaluating Treg numbers.
2. To evaluate the safety and tolerability of IV infusion of an expanded population of autologous Tregs and subcutaneous low- dose IL-2 in people with ALS.

Exploratory Objectives:

1. To characterize the effects of IV infusion of an expanded population of autologous Tregs and subcutaneous low-dose IL-2 on clinical outcome measures of ALS, including: Appel ALS Rating Scale (AALS) and ALS Functional Rating Scale-Revised (ALSFRS-R) scores, forced vital capacity, maximum inspiratory pressure, and fasciculations.
2. To bank blood and urine for future analysis of ALS biomarkers.
3. To assess the Combined Assessment of Function and Survival (CAFS) from baseline to 1 year after the last Treg infusion.

Endpoints:

Primary Endpoints:

1. Change in Treg suppressive function in the blood from baseline to week 24 during Period 1 and from start to finish of each dosage level during Period 2.

Secondary Endpoints:

1. Change in Treg numbers in the blood from baseline to week 24 during Period 1 and from start to finish of each dosage level during Period 2.
2. Safety: Defined as the occurrence of adverse events (AEs), serious adverse events (SAEs), treatment-emergent adverse events (TEAEs), and clinically significant abnormalities in clinical and laboratory values.
3. Tolerability: Defined as the percentage of participants who complete the 6-month RCT (RCT Tolerability) and who complete the 6-month OLE and each ascending dose on study treatment (OLE Tolerability).

Exploratory Endpoints:

1. Change in Appel ALS (AALS) score from baseline to week 24 during Period 1 and from start to finish of each dosage level during Period 2.
2. Change in ALS Functional Rating Scale-Revised (ALSFRS-R) from baseline to week 24 during Period 1 and from start to finish of each dosage level during Period 2.
3. Change in forced vital capacity (FVC) and maximum inspiratory pressure (MIP) from baseline to week 24 during Period 1 and from start to finish of each dosage

level during Period 2.

4. Tracheostomy-free survival between baseline and week 24 during Period 1 and from start to finish of each dosage level during Period 2.
5. Proportion of days free of fasciculations between baseline and week 24 during Period 1 and from start to finish of each dosage level during Period 2.
6. Combined Assessment of Function and Survival from baseline to 1-year after the last Treg infusion.

**Study Population**

This study will be conducted in patients with a diagnosis of ALS. Patients may be recruited from participating study centers, on social media, and through web and print advertisements.

**Inclusion Criteria**

Patients must meet all of the following criteria to be eligible for study participation:

1. ALS meeting El Escorial criteria for possible, probable, lab- supported probable, or definite ALS.
2. At least 18 years old.
3. Provided informed consent and authorized use of protected health information (PHI) in accordance with national and local patient privacy regulations.
4. Capable of complying with all study procedures, including the study drug delivery procedure, in the Investigator's opinion.
5. On a stable regimen of riluzole for at least 30 days at the time of screening. If not on riluzole at the time of study entry, willing to refrain from initiation of the agent for the duration of the trial.
6. Patients on edaravone willing to refrain from taking edaravone on the same day as they will receive the Tregs infusion for the duration of the trial. If not on edaravone at the time of study entry, willing to refrain from initiation of the agent for the duration of the trial.
7. Medical record documentation of a decline in ALSFRS-R total score of at least two points in the 90 days prior to screening or at least four points over the 180 days prior to screening.
8. Forced vital capacity (FVC) ≥65% of predicted capacity for age, height, and gender at screening.
9. Patient able and willing to undergo leukapheresis.

**Exclusion Criteria**

Patients meeting any of the following criteria are not eligible for study participation:

1. Presence of any of the following clinical conditions that would interfere with the safe conduct of the study, as determined by the Investigator:
    a. Unstable neurological, cardiovascular, cerebrovascular, pulmonary, renal, hepatic, endocrine, or hematologic disease; active malignancy or infectious disease; or other

medical illness.

b. Unstable psychiatric illness defined as psychosis (hallucinations or delusions), unstable major depression, or substance abuse within 180 days prior to screening.
c. Persistent asthma, prior history of acute systemic reactions involving IgE-dependent mechanisms, history of angioedema, or history of anaphylactic reactions to any medication.

2. Serum alanine aminotransferase (ALT) or aspartate aminotransferase (AST) > 3× the upper limit of normal (ULN) , or complete blood count (CBC) with white blood cells (WBC) <4 or >13, hematocrit (HCT) <30 or >54, or platelets <90 or >500 at screening.
3. Serum creatinine >1.8 mg/dL or creatinine clearance <40 mL/min at screening.
4. History of, or positive test result for human immunodeficiency virus (HIV), hepatitis C virus, or hepatitis B virus (i.e. positive for both hepatitis B surface antigen and hepatitis B core antibody) at screening.
5. Tracheostomy.
6. If female, breastfeeding, known to be pregnant, planning to become pregnant during the study, or unwilling to use effective contraception for the duration of the trial and for 90 days after treatment.
7. If male of reproductive capacity, unwilling to use effective contraception for the duration of the trial and for 90 days after treatment.
8. Enrollment in any other interventional study.
9. Treatment with another investigational drug, biological agent, or device within 30 days or 5 half-lives of screening, whichever is longer. Patient participation in an observational/non-interventional clinical study is allowed and to be discussed prior to study enrollment with the Medical Monitor.
10. Prior gene or cell therapy treatments for ALS.

| | |
|---|---|
| Phase: | Phase IIa |
| Description of Sites/Facilities Enrolling Participants: | A total of 2 sites through the Northeast ALS (NEALS) Consortium will participate. The consortium is made up of medical institutions that specialize in performing clinical trials for ALS.<br><br>1) Houston Methodist Hospital (Overall Project Medical Director: Stanley H. Appel, MD; Clinical Investigator: Jason Thonhoff, MD, PhD)<br>2) Massachusetts General Hospital (Clinical Investigators: James Berry, MD, MPH and Sabrina Paganoni, MD, PhD) |
| Description of study Intervention | Two Cohorts:<br><br>Cohort/Group 1: up to 6 subjects<br><br>In Period 1 (Randomized Controlled Trial (RCT)), participants will receive either active intervention (Treg |

infusions and IL-2) or matching placebos (randomized 1:1) for 6 months.

Cohort/Group 1:

In Period 2 (Open Label Extension (OLE)), all participants who complete 5 of the 6 cycles in the RCT will be eligible to receive an ascending-dose of expanded autologous Tregs for up to 6 months, or as long a duration as can be supported using the available expanded autologous Tregs for a given participant; total of up to 13 months participation.

Cohort/Group 2: up to 2 subjects

In Period 2 (Open Label Extension (OLE)), all participants will enter the OLE directly and receive an ascending-dose of expanded autologous Tregs for up to 6 months, or as long a duration as can be supported using the available expanded autologous Tregs for a given participant; total of up to 7 months participation. Due to the COVID-19 pandemic's impact on the study, all subjects enrolled after March 23, 2020, will be included in cohort/group 2.

**Period 1 – Randomized Controlled Trial (RCT) – Cohort 1 only:**

The first 6 months of the trial will be randomized, placebo-controlled and consist of monthly infusions. Participants will be randomized 1:1 to receive either IV infusions of expanded autologous Tregs and subcutaneous IL-2 injections, or matching placebo infusions and placebo subcutaneous injections.

- The MGH site PI, and Study Team, Medical Monitor, the Clinical Research Associations monitoring the study, and the third-party biostatistician will be blinded through the end of the trial (data lock).
- After the final subject has completed his/her last visit in Period 1 (Week 24 V9 visit) and once data monitoring has been completed and all queries resolved for the RCT portion, the OPMD and HMH site PI will be unblinded to all clinical assessment and biomarker data in order to begin optimizing the Treg manufacturing process and trial design for the next clinical trial. The COVID-19 pandemic limited subject travel, study funding, and visit procedures for this study, which affected the ability of the study to meet the primary and secondary outcome measures. It is imperative that the investigators review the data and begin making changes to the Treg manufacturing processes in order to prepare for a subsequent clinical trial without the unnecessary delay of waiting for all subjects in cohort 1 to complete Period 2 (OLE).
- The HMH and MGH investigational pharmacists and the UTHealth cGMP facility personnel will

be unblinded throughout the study for subject IP preparation and product management.

- Members of the DSMB supporting data safety oversight will be unblinded.
- During Period 1: Subjects randomized to active investigational treatment will be infused with Tregs intravenously once a month (Treg dose = $1x10^6$ cells/kg/infusion) and also receive concomitant subcutaneous administration of IL-2 at a dose of $2x10^5$ IU/$m^2$/injection three times weekly.
- Subjects randomized to the placebo group will receive a matching "Treg placebo" consisting of normal saline infusions containing 5% human serum albumin and 0.2% DMSO and subcutaneous injections of normal saline per the same schedule as the active treatment group. Infusions should ideally occur on a Thursday for MGH or Thursday/Friday for HMH throughout the trial.
- Subjects will start IL-2 or placebo injections at least 2 weeks and preferably as much as 4 weeks prior to the first Treg infusion. IL-2 or placebo injections will be discontinued 4 weeks after the $6^{th}$ Treg infusion (V24). Subcutaneous injections should ideally start on Mondays for dosing on Mondays, Wednesdays, and Fridays but may also be on Tuesday, Thursday, and Saturday if needed.
- Participants who complete the RCT and received at least 5 of the 6 scheduled Tregs infusions will be eligible to complete a second leukapheresis and Treg expansion, if needed to obtain more Treg product for infusion.

**Period 2 - Open Label Extension (OLE) – Cohorts 1 and 2:**

The OLE will consist of up to 6 monthly infusions during which all participants from Cohort 1 and 2 will receive IV infusions of ascending doses of expanded autologous Tregs and IL-2 subcutaneous injections three times per week. Day 0 for the two participants in Cohort 2 corresponds to the Week 26 V11 OLE1 visit in the Schedule of Activities.

- To the extent that a given participant's expanded autologous Tregs are available, all participants during the OLE phase will receive 2 monthly infusions at a dose of $1x10^6$ cells/kg/infusion, then 2 monthly infusions at a dose of $2x10^6$ cells/kg/infusion and finally 2 monthly infusions at a dose of $3x10^6$ cells/kg/infusion. Infusions should ideally occur on a Thursday or Friday throughout the trial.
- All participants in the OLE, Cohorts 1 and 2, will receive IL-2 at least 2 weeks and preferably as much as 4 weeks prior to the first Treg infusion of the OLE. IL-2 will be administered subcutaneously 3 times per week at a dose of $2x10^5$IU/$m^2$/injection and then will be discontinued at the final visit for the open label extension portion of the study (Schedule of Events Week 50 for Cohorts 1 and 2). Subcutaneous injections should ideally start on Mondays for generally dosing on Mondays, Wednesdays, and Fridays.
- All subjects will return for a final safety visit (Week 54).

## 1.2 SCHEDULE OF ACTIVITIES (SOA)

**Study Table for Period 1 – Randomized Controlled Trial (RCT) – Cohort 1 Only**

| | V1 Screening | V2 Leuka-pheresis | V3[13] RCT 1 w/in 8 weeks from screening & 6 weeks of Leukapheresis | | | V4[13] RCT 2 | V5[13] RCT 3 | | V6[13] RCT 4 | V7[13] RCT 5 | V8[13] RCT 6 | | | V9[13] Follow-Up Visit |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Week -8 to -3 | Week -6 to -3 | Infusion Week 0 Day 0 | Week 0 Day 1 +3 Days ^^ | Week 1 Day 0 ±3 Days | Infusion Week 4 ±7 Days | Infusion Week 8 ±7 Days | Week 9 ± 3 Days | Infusion Week 12 ±7 Days | Infusion Week 16 ±7 Days | Infusion Week 20 ±7 Days | Week 20 Day 1 +3 days | Week 21 Day 0 ±3 Days | Week 24 ±7 Days |
| Written Informed consent | X | | | | | | | | | | | | | |
| Eligibility Review | X | X | | | | | | | | | | | | |
| Medical history | X | X | | | | | | | | | | | | |
| Demographics | X | | | | | | | | | | | | | |
| Screening Labs[1] | X | | | | | | | | | | | | | |
| ALS Diagnosis History | X | | | | | | | | | | | | | |
| EI Escorial Criteria | X | | | | | | | | | | | | | |
| Vital Signs, Height and Weight[2] | X | | X | | | X | X | | X | X | X | | | X |
| Physical Exam | X | | | | | | | | | | | | | X |
| Neurological Exam[3] | X | | | | | | | | | | | | | X |
| 12-Lead ECG (Electrocardiogram) | X | | | | | | | | | | | | | X |
| C-SSRS[4] | X | | X | | | X | X | | X | X | X | | | X |
| ALSFRS-R and Appel ALS Scale | X | | X | | | X | X | | X | X | X | | | X |
| Forced Vital Capacity (FVC) and maximum inspiratory pressure (MIP) Measurements | X | | X | | | X | X | | X | X | X | | | X |
| Assess Survival | | X | X | X | X | X | X | X | X | X | X | X | X | X |
| Concomitant Medications | X | X | X | X | X | X | X | X | X | X | X | X | X | X |

| | V1 Screening | V2 Leuka-pheresis | V3[13] RCT 1 w/in 8 weeks from screening & 6 weeks of Leukapheresis | | | V4[13] RCT 2 | V5[13] RCT 3 | | V6[13] RCT 4 | V7[13] RCT 5 | V8[13] RCT 6 | | | V9[13] Follow-Up Visit |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Week -8 to -3 | Week -6 to -3 | Infusion Week 0 Day 0 | Week 0 Day 1 +3 Days ^^ | Week 1 Day 0 ±3 Days | Infusion Week 4 ±7 Days | Infusion Week 8 ±7 Days | Week 9 ± 3 Days | Infusion Week 12 ±7 Days | Infusion Week 16 ±7 Days | Infusion Week 20 ±7 Days | Week 20 Day 1 +3 days | Week 21 Day 0 ±3 Days | Week 24 ±7 Days |
| Adverse Events | X | X | X | X | X | X | X | X | X | X | X | X | X | X |
| Safety Labs[5] | X | | X | | | X | X | | X | X | X | | | X |
| Leukapheresis | | X | | | | | | | | | | | | |
| Blood & Urine biomarkers[6] | X | | X+ | X | X | X | X | X | X | X | X | X | X | X |
| Randomization[7] | | X ~ | | | | | | | | | | | | |
| Intravenous Treg or placebo infusion[8] | | | X | | | X | X | | X | X | X | | | |
| Teaching of IL-2 or placebo administration[9] (administer prior to infusion; at least 2 weeks to 4 weeks – preferably) | | X~ | | | | | | | | | | | | |
| First dose of IL-2 or placebo[10] (3x/wk dosing throughV9.IL-2 dosing for all subjects restarting at least 14 days prior to Week 26(V11) through Week 50 (V17) follow-up visit) | | X~ | | | | | | | | | | | | |
| Return of IL-2 or placebo supplies | | | X | | | X | X | | X | X | X | | | X |
| Shipping of IL-2 (Pharmacy*) | | X~ | X | | | X | X | | X | X | X | | | |
| Distribute fasciculations diary | X | | X | | | X | X | | X | X | X | | | |
| Review fasciculations diary | | | X | | | X | X | | X | X | X | | | X |
| IP accountability compliance and Diary Review & IL2-re- education | | | X | | | X | X | | X | X | X | | | X |
| Blindedness Questionnaire | | | | | | | | | | | | | | X |

**Study Table for Period 2 – Open Label Extension (OLE) ^ – Cohorts 1 and 2**

| | Optional V10[12] Leuka-pheresis #2 (if needed) **** | V11 OLE 1 | | | V12 OLE 2 | V13 OLE 3 | | V14 OLE 4 | V15 OLE 5 | V16 OLE 6 | | | V17 OLE 7 | V18 Final Safety Visit |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | □Not needed<br><br>Week 25 ±7 Days | Week 26 +14 Days ***** | Week 26 Day 1 +3 Days | Week 27 Day 0 ±3 Days | Week 30 ±7 Days | Week 34 ±7 Days | Week 35 Day 0 +3 Days | Week 38 ±7 Days | Week 42 ±7 Days | Week 46 ±7 Days | Week 46 Day 1 +3 Days | Week 47 ±3 Days | Week 50 ±7 Days | End of Participation/End of Study Week 54 ±7 Days |
| Written Informed consent | | | | | | | | | | | | | | |
| Vital Signs, Height and Weight[2] | | X | | | X | X | | X | X | X | | | X | X |
| Physical Exam | | | | | | | | | | | | | | X |
| Neurological Exam[3] | | | | | | | | | | | | | X | X |
| 12-Lead ECG | | | | | | | | | | | | | | X |
| C-SSRS[4] | | X | | | X | X | | X | X | X | | | X | X |
| ALSFRS-R and Appel ALS Scale | | X | | | X | X | | X | X | X | | | X | X |
| Forced Vital Capacity (FVC) and maximum inspiratory pressure (MIP) Measurements | | X | | | X | X | | X | X | X | | | X | X |
| Assess Survival | X | X | X | X | X | X | X | X | X | X | X | X | X | X |
| Concomitant Medications | X | X | X | X | X | X | X | X | X | X | X | X | X | X |
| Adverse Events | X | X | X | X | X | X | X | X | X | X | X | X | X | X |

| | Optional V10[12] Leuka-pheresis #2 (if needed) **** | V11 OLE 1 | | | V12 OLE 2 | V13 OLE 3 | | V14 OLE 4 | V15 OLE 5 | V16 OLE 6 | | | V17 OLE 7 | V18 Final Safety Visit |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | □Not needed<br><br>Week 25 ±7 Days | Week 26 +14 Days ***** | Week 26 Day 1 +3 Days | Week 27 Day 0 ±3 Days | Week 30 ±7 Days | Week 34 ±7 Days | Week 35 Day 0 +3 Days | Week 38 ±7 Days | Week 42 ±7 Days | Week 46 ±7 Days | Week 46 Day 1 +3 Days | Week 47 ±3 Days | Week 50 ±7 Days | End of Participation/End of Study Week 54 ±7 Days |
| Safety Labs[5] | X | X | | | X | X | | X | X | X | | | X | X |
| Leukapheresis | X | | | | | | | | | | | | | |
| Blood & Urine biomarkers[6] | X | X+ | X | X | X | X | X | X | X | X | X | X | X | X |
| Intravenous Treg infusion[8] | | X | | | X | X | | X | X | X | | | | |
| Teaching of IL-2 administration[9&11] | X | | | | | | | | | | | | | |
| First dose of IL-2[10] | X | | | | | | | | | | | | | |
| Shipping of IL-2 | X | X | | | X | X | | X | X | X | | | | |
| Return of IL-2 supplies | | X | | | X | X | | X | X | X | | | X | X |
| Review fasciculations diary | | X | | | X | X | | X | X | X | | | X | X |
| Drug accountability compliance | | X | | | X | X | | X | X | X | | | | |
| Exit Questionnaire[12] | | | | | | | | | | | | | | X |

1. Screening laboratory tests will be performed locally and include HIV, Hepatitis B, Hepatitis C, Complete Blood Count with Differential, Full Chemistry Panel (Sodium, Potassium, Chloride, Blood Urea Nitrogen, Creatinine, Glucose, Calcium, Aspartate Aminotransferase,

Alanine Aminotransferase, Alkaline Phosphatase, Total Bilirubin, Total Protein, Albumin, and Carbon dioxide/bicarbonate), Urine, International Normalized Ratio/Prothrombin Time/Partial Thromboplastin Time, and Pregnancy Test (urine) for Women of Childbearing Potential.

2. Vital signs include systolic and diastolic pressure in mmHg, respiratory rate/minute, heart rate/minute and temperature. Height is only recorded once at the Screening Visit. During Treg infusions, vital signs will be obtained pre-infusion and monitored and recorded once every hour from the infusion start time for up to 4 hours. There is a +/- 10-minute window for each hour vital signs are taken during the infusion.
3. The standard Neurological Exam will be used for all subjects.
4. C-SSRS Screening Version to be completed at Screening Visit only. C-SSRS since Last Visit version to be completed at all other visits.
5. Safety laboratory labs will be performed locally and include Complete Blood Count with Differential, Full Chemistry Panel (Sodium, Potassium, Chloride, Blood Urea Nitrogen, Creatinine, Glucose, Calcium, Aspartate Aminotransferase, Alanine Aminotransferase, Alkaline Phosphatase, Total Bilirubin, Total Protein, Albumin, and Carbon dioxide/bicarbonate), Urinalysis, and Pregnancy Test (urine) for Women of Childbearing Potential.
6. Biomarker blood and urine samples (non-fasting) will be obtained from each participant for biomarker analyses and labeled with a subject identifier code and date at/with:

    - Immediately prior to each infusion at Screening, the final RCT-week 24 visit, OLE-week 50, and the End of Study Visit -week 54;
    - Weeks 0, 20, 26, and 46- 1 day +3 days & 7 days ± 3 days following the infusion day/date;
    - Week 8 & Week 26 - 7 days ±3 days following the infusion day/date.
    - Specimen collection: (six tubes) 10 mL sodium heparin green tops, (one tube) 10 mL serum red top, (one tube) 4 mL sodium heparin green top, and a urine sample (5 mL to 10 mL).
        - (five) 10 mL sodium heparin green tops will be used at the site of collection to perform the Treg Suppression Assay per SOP. Serum will be stored at -80°C at the site of collection per SOP.
        - (one) 10 mL sodium heparin green top and the 4 mL sodium heparin green top will be shipped overnight at room temperature to HMH for flow cytometry and messenger RNA collection per SOP. Urine specimens will be frozen at -80°C at the site of collection per SOP. All biospecimens will be labeled with a subject identifier code and date.
    - Primary Biomarkers: Treg suppressive function;
    - Secondary biomarkers: Treg numbers by flow cytometry and; Exploratory biomarkers: Banking of urine, serum, plasma, and messenger RNA. These samples will be collected and stored at -80°C. Banked samples will be analyzed after the study has completed.
    - After all analyses have been completed, the remaining samples will be transported to the NEALS biorepository.

**+** Participants will also return to their enrollment sites 1 day (+3 days) and 7 days (± 3 days) after the first infusion of the RCT and OLE (Week 0, Week 20, Week 26 and week 46) to collect the same amount of blood and urine for post-infusion analyses of blood biomarkers.

7. Randomization will occur after the Leukapheresis procedure is completed within 7 days.

8. Safety monitoring & TReg or matching placebo infusion: For the RCT, 4 hours post-infusion monitoring is required for the first infusion but is otherwise optional at the discretion of the Investigator. For the OLE, 4 hours post-infusion monitoring is required following the first infusion. Post-infusion monitoring for at least 1 hour is then required after each escalating Treg dose in the OLE but is otherwise optional at the discretion of the Investigator. Following each infusion, the PI or study staff will provide a follow up phone call within 24 to 48 hours to query subjects for symptoms of a delayed anaphylaxis response.

9. Il-2 or Matching Placebo: The teaching of IL-2 subcutaneous administration will be provided to all participants following the manufacturer's directions on the days of leukapheresis. The dispensation of IL-2 or placebo and diary will take place at the enrollment site. For additional syringe dispensation, IL-2 and placebo for subjects will be shipped directly to the subjects from the HMH or MGH, respectively. Sites will provide the subjects with a sharps container and instructions on discarding used syringes and returning unused syringes with their IL-2/Placebo dosing diary to the next visit.

10. During RCT and OLE, IL-2 or placebo will begin after Leukapheresis and will start as early as 4 weeks or at least 2 weeks prior to the first Treg infusion. IL-2/placebo will be administered subcutaneously 3 times per week and then discontinued 4 weeks after the last Treg infusion of the RCT (Week 24) and at the final visit for the OLE phase (Week 50).

11. For MGH patients, teaching of IL-2 will occur at screening.

12. If performed as part of an early termination visit before the V9 Week 24 Follow-Up Visit, the Exit Questionnaire will include the Blindedness Questionnaire. If performed as part of an early termination visit after the V9 Week 24 Follow-Up Visit or as part of the V18 Week 54 Final Safety Visit, the Exit Questionnaire will not include the Blindedness Questionnaire.

13. RCT applies to Cohort 1 only

**~** These activities will only apply to HMH patients.

* IL-2 or matching placebo administration is ongoing throughout the study and not associated with a study-specific visit.

** Any study visit that cannot be done in person will be done by phone, collecting as much safety data as possible.

*** Early termination visits for randomized subjects who have received at least one dose of investigational product; where possible perform safety labs, physical and neurological exam, vital signs, ECG, AE, and SAE assessment, collect IL-2 or placebo syringes and materials and collection of fasciculation diary.

**** Second leukapheresis will be performed ONLY if Treg collection from initial leukapheresis is insufficient.

***** OLE 1 for Cohorts 1 & 2 must occur at least 14 days after participant has initiated IL-2 dosing for the OLE phase.

^ For subjects in Cohort 1, visit windows for the OLE are to be calculated from Week 24 of the RCT. For subjects in Cohort 2, visit windows for the OLE are to be calculated from the Week 26 infusion (i.e., the Week 26 V11 OLE1 will be Day 0).

^^ Visit windows for visits following infusions are relative to the date of the infusion

# 2 INTRODUCTION

## 2.1 STUDY RATIONALE

$CD4^+CD25^+FOXP3^+$ regulatory T lymphocytes (Tregs) are a subpopulation of T lymphocytes that are immunosuppressive and maintain tolerance to self-antigens, with their dysfunction playing a pivotal role in the development of autoimmune disorders[1-4]. In ALS mice, infusions of Tregs slow disease progression and prolong survival, and Tregs suppress the proliferation of responder T lymphocytes and the activation of microglia[5,6]. In ALS patients, the expression of the Treg master transcription factor FOXP3 is reduced in rapidly progressing patients[7], with subsequent impairment of Treg suppressive functions; FOXP3 expression and Treg suppressive functions correlate with the extent and rapidity of disease progression[8]. When expanded *ex vivo* in the presence of interleukin (IL)-2 and rapamycin, Treg suppressive function is restored[8,9]. These data suggest that infusions of expanded autologous Tregs will improve Treg suppressive function *in vivo* and slow rates of disease progression in ALS patients.

To test these hypotheses, a first-in-human phase 1 study was conducted to determine whether infusions of expanded autologous Tregs into ALS patients were safe and tolerable during early and later stages of disease[10]. IL-2 was administered concomitantly in the participants in an effort to stabilize and possibly enhance the suppressive functions of the infused Tregs. Infusions of Tregs were safe and well-tolerated in all participants. Treg numbers and suppressive function increased after each infusion. The infusions slowed progression rates during early and later stages of disease, and Treg suppressive function correlated with slowing of disease progression per the Appel ALS scale. In addition, respiratory measures of maximal inspiratory pressure also stabilized, particularly in two participants, during Treg infusions. The phase I results demonstrated the safety and potential benefit of expanded autologous Treg infusions, warranting further clinical trials in ALS patients. The correlation between Treg suppressive function and disease progression underscored the significance of using Treg suppressive function as an indicator of clinical status[10].

We propose to build on these promising results by conducting a multi-center (two sites), randomized, placebo-controlled phase IIa trial of expanded autologous Treg infusions followed by an ascending-dose open label extension. The primary goal of the trial is to measure the biological activity of Tregs in patients with ALS by measuring their impact on Treg suppressive function and number. Further, we will broaden our safety and tolerability data by administering ascending doses of Tregs with longer exposure times. Finally, we will refine our operational abilities. Tregs will be expanded ex-vivo at a central facility, cryopreserved, and then administered at two sites in preparation for a larger, multi-center efficacy trial.

## 2.2 BACKGROUND

**Amyotrophic Lateral Sclerosis**

Amyotrophic Lateral Sclerosis (ALS) is a progressive devastating motor neuron disease characterized by

the degeneration of upper and lower motor neurons, culminating in respiratory failure. Patients live an average of 4-6 years after symptom onset, and there is minimal effective therapy. Although the cause and pathogenesis of ALS are incompletely defined, increasing evidence indicates that immune/inflammatory reactions contribute to motor neuron injury in ALS. In ALS spinal cord tissue, we and others have demonstrated the presence of T cells and activated microglia[11]. In the mSOD1 transgenic mouse model of familial ALS (fALS), immune/inflammatory responses are present throughout the course of disease, raising the question as to whether such inflammation contributes to the pathogenesis of motor neuron injury[12]. In fact, we have documented in the ALS mouse model that inflammation plays a neuroprotective role in early stages of disease with slow progression, whereas in later stages of disease inflammation plays a cytotoxic role and disease progression is rapid. The immune/inflammatory constituents in early stages are characterized by anti-inflammatory microglia and suppressive regulatory T cells; whereas at later stages proinflammatory microglia and neurotoxic Th1 cells predominate.

**Anti/Pro-inflammatory microglia modulate the equilibrium of neuroprotection/cytotoxicity**

*In vitro* studies of anti-inflammatory microglia isolated from early stage ALS mouse spinal cords protected motor neurons in culture, while microglia isolated from late stage ALS mouse spinal cord had a pro-inflammatory phenotype and were toxic to motor neurons in culture. *In vivo* evidence utilized mSOD1 mice crossed with PU.1 knockout mice; such transgenic mice were then transplanted with mSOD1 or Wild-Type (WT) bone marrows and the CNS microglia were then either pro-inflammatory mSOD1 phenotypes or WT anti-inflammatory phenotypes. The mice transplanted with the mSOD1 microglia had greater motor neuron cell loss and died much earlier than mice transplanted with the WT microglia, thereby substantiating the neuroprotective role of anti-inflammatory microglia and the cytotoxic role of pro-inflammatory microglia[13].

**T-cells modulate the equilibrium of neuroprotection and cytotoxicity**

To define a potential role for T cells, we bred mSOD1 mice with RAG2-/-mice that lacked functional T cells or with CD4-/- mice that lacked $CD4^+$ T cells. In both lines of doubly transgenic mice, motor neuron disease was accelerated, accompanied by increased mRNA levels of proinflammatory cytokines, as well as enzyme producing superoxide anion, and decreased levels of trophic factors. The fact that transgenic mice lacking functional T cells died earlier suggested that T cells had been relatively protective. The key question was which subpopulation of $CD4^+$T lymphocytes was protective? Additional studies of T cell populations suggested that regulatory T lymphocytes were elevated in numbers and in their specific functional marker, FoxP3, during the early slowly progressive stages of disease in the SOD1 mice, and were dramatically decreased during later more rapidly progressive stages; the cytotoxic Th1 lymphocytes were markedly elevated during later rapidly progressing stages of disease.

The subpopulation of $CD4^+CD25^+$ regulatory T cells has been determined to play a crucial role in the control of autoimmune processes[3]. Tregs constitutively express the transcription factor FOXP3, which is a key regulatory gene for the development and function of natural Tregs in humans and mice[14,15], and thus far, intracellular expression of FOXP3 is the most specific marker for Tregs[1,16,17]. Definitive proof for the neuroprotective role of Tregs in ALS models was provided by transplanting Tregs into mSOD1/RAG2-/-

transgenic mice. Following transplantation of Tregs, disease duration and survival were significantly prolonged (by more than 80%). Thus, we concluded that regulatory $CD4^+$ T lymphocytes could enhance neuroprotection *in vivo* and prolong disease duration and survival. *In vitro* the Tregs not only suppressed the proliferation of Th1 cells, but also suppressed pro-inflammatory microglia. These studies collectively document the neuroprotective contribution of Tregs during early stages of disease and the cytotoxic contribution of Th1 cells during later stages of disease.

**Regulatory T Cells in ALS patients**

To determine whether Tregs were similarly protective in ALS patients, we used flow cytometry to assay numbers of Tregs and the expression of FOXP3 protein per cell in the blood of ALS patients compared to controls. We also isolated RNA from the blood of ALS patients and healthy controls and determined the expression of FOXP3. We found that Tregs and their expression of FOXP3 protein were reduced in ALS patients who were progressing rapidly; levels of FOXP3 and progression rates were inversely correlated. To confirm these results, we examined an additional cohort of 102 patients, followed for 3.5 years, and documented that FOXP3 levels, that were reduced in the early stages of disease, were associated with decreased survival 3.5 years later; 35% of ALS patients with low FOXP3 at early stages of disease were deceased, while only 13% of ALS patients with high FOXP3 were deceased 3.5 years later.

As noted above in the studies with the ALS mouse model, transplanting Tregs into mSOD1/RAG2-/- transgenic mice significantly enhanced disease duration and survival by more than 80%. These data confirm that regulatory $CD4^+$ T lymphocytes can enhance neuroprotection *in vivo* and prolong disease duration and survival. The fact that transplanting Tregs in the ALS mouse model enhanced disease duration and survival, together with the demonstration that a high level of Tregs in the blood of ALS patients was associated with longer survival supports the reasonable expectation that adoptive therapy with highly purified autologous donor Tregs could similarly increase disease duration and survival in ALS patients.

We have also documented that Tregs derived from the blood of ALS patients are dysfunctional and fail to suppress the proliferation of responder T lymphocytes *in vitro*. This suppressive dysfunction is most marked in ALS patients progressing rapidly[8]. Yet even in ALS patients progressing slowly suppressive function is decreased-i.e. for a given FOXP3 expression level, the suppressive function is less than the suppression function of age and sex-matched control blood specimens. The Treg suppressive function is negatively correlated with the burden of disease-i.e. the more significant the burden of disease measured by the Appel ALS score, the lower the suppressive function. Also, this dysfunction is a property of the ALS Treg population since suppression of ALS responder T cells by control Tregs is completely normal.

**Infusion after Expansion**

Infusions of expanded Tregs were first reported in 2009 principally for combating the effects of graft-versus-host disease (GVHD)[18]. Trzonkowski, et al performed this first-in-man study treating 2 patients with *ex vivo* expanded T regulatory cells. A larger and more compelling safety study with infusion of *ex vivo* T regulatory cells was carried out in 23 adults with GVHD[19]. No infusion toxicities were seen in this study and no deleterious effects were reported on risks of infection, relapse, or early mortality. Thus, there is

precedence for the safe infusion of *ex vivo* expanded T regulatory cells in man and this first in ALS use.

**Clinical Low-Dose IL-2 Infusions**

Several centers have used low-dose recombinant human IL-2 infusions for the purpose of providing anti-tumor immunity post allogeneic stem cell transplantation (alloSCT). One such group evaluated 29 patients with hematologic malignancies treated with low-dose IL-2 after T-cell-depleted alloSCT[20]. They found that daily infusion of IL-2 at doses of 2 to 4 x $10^5$ units/$m^2$/day can be tolerated for up to 3 months by most patients. The most common toxicities observed were fatigue, fever, nausea, and vomiting. Side effects were more frequently noted at the higher starting doses (doses greater than 2 x $10^5$/$m^2$/dose).

Reasons for early discontinuation were catheter-related sepsis (n = 3), bronchiolitis (n = 2), gastrointestinal toxicity (n = 2), disease relapse (n = 2), pancytopenia (n = 1), hypersensitivity (n = 1), and acute GVHD (n = 1). However, these are also side effects commonly seen after SCT. The single patient who developed acute GVHD had grade III disease, presenting with an erythematous rash and profuse watery diarrhea at 90 days post-BMT and did not develop until 7 weeks after beginning IL-2 and persisted long after discontinuation. Although several other patients developed rashes while receiving IL- 2 therapy, they were transient and usually resolved spontaneously without histologic evidence of GVHD when their skin was biopsied. NIH-FDA Clinical Trial Protocol Template V2.0 1 November 2018

The same group evaluated 13 BMT recipients (7 autologous and 6 T-cell depleted allogeneic) who received recombinant human IL-2 at a dose of 2 x $10^5$/$m^2$/day for up to 90 days by continuous infusion beginning a median of 85 days after BMT[21]. Toxicity was minimal in this cohort of patients and the therapy was delivered via an outpatient setting. No patient developed GVHD, hypotension, or pulmonary capillary leak syndrome. Early and late adverse events were noted. In two patients, fever developed and resolved within 3 days of discontinuing therapy, and in both patients, IL-2 was restarted and escalated back to the original starting dose. Late adverse events included peripheral edema, cough, transient localized rashes that resolved prior to IL-2 discontinuation, and thyroid abnormalities.

**Role of IL-2**

Interleukin-2 is critical for Treg development, expansion, activity, and survival. Treg constitutively express high levels of high affinity IL-2 (CD25) receptors. In recent clinical trials, administration of low- dose IL-2 has been shown to result in the selective expansion of Treg and clinical improvement in symptoms of auto and allo-immunity. Daily therapy with low-dose IL-2 (1x$10^6$ IU/$m^2$) for 8 weeks in patients with chronic GVHD led to a rapid increase in circulating Treg, without a significant increase in CD4 Tcon or CD8 T cells. This was associated with clinical improvement in approximately 50% of patients and no patients experienced GVHD progression[22]. Patients with severe chronic GVHD had elevated levels of the cytokines IL-7 and IL-15 and higher levels of phosphorylated Stat5 (pStat5) in Tcon than Treg. This imbalance of Stat5 signaling was rapidly reversed in patients receiving low-dose IL-2, resulting in increased thymic generation and proliferation of Treg and reduced susceptibility to apoptosis. These results demonstrate that daily administration of IL-2 at physiologic doses can restore Treg homeostasis *in vivo* and promote immune tolerance[23]. In this same study in patients with GVHD, low- dose IL-2 also had

profound effects on Treg homeostasis *in vivo*, which varied with the duration of IL-2 therapy. The initial effect was primarily on Treg proliferation, which peaked 1 week after starting IL-2. The high affinity receptor expressed by Treg rapidly binds exogenous IL-2 and increasing numbers of Tregs likely limit the levels of free ligand *in vivo*. Moreover, exogenous IL-2 has a short half-life *in vivo* that may limit the ability to maintain high systemic cytokine levels during continued treatment. IL-2- induced Treg proliferation was followed by a 6–7 fold increase in thymic-derived Treg, that peaked 4–6 weeks after starting IL-2 and waned by week 8[23].

Adoptive therapy with highly purified donor Tregs can also be combined with low dose IL-2 therapy. In this setting, low dose IL-2 may be used to selectively expand infused Tregs *in vivo* and this may reduce the need to expand large numbers of Tregs *in vitro* for adoptive therapy. In our own laboratory, we have determined that Tregs can be preferentially expanded directly from PBMCs isolated from serum in healthy donors using very low doses of IL-2 pulsed three times weekly in culture for 14 days. These Tregs demonstrate excellent suppressive function *in vitro*. From other published data and our own preclinical data using low dose IL-2 to expand Tregs *in vitro*, it is feasible that low-dose IL-2 injections may lead to the preferential *in vivo* expansion of Tregs, thereby reducing the pro-inflammatory response that may aggravate ALS.

**Our prior experiences with IL-2 in ALS patients**

In 2010-2011, we performed an IL-2 study (IRB Pro00002825) in ALS patients. The following strategy was implemented: To favor the repolarization of the immune response to that of a regulatory profile in patients, we depleted the lymphocyte count in patients with a single dose of cyclophosphamide 800mg/m$^2$ intravenously. At this dose, cyclophosphamide produces a transient depletion of T cells and allows for a more robust and homogeneous expression of Tregs after IL-2 administration. Cyclophosphamide is well tolerated, not myeloablative, and widely used for the treatment of autoimmune disorders, including multiple sclerosis.

While the combination of cyclophosphamide and low dose IL-2 appeared promising, analysis of the first 5 patients treated in our study failed to show significant expansion of the Treg compartment. In retrospect, this may have been predicted by our present data that ALS Tregs are dysfunctional. Nevertheless, no patient showed any adverse effects of low dose IL-2 subcutaneous injections.

Recent studies have also shown that rapamycin favors the *in vitro* and *in vivo* expansion of natural Treg (nTreg) cells, which are continuously derived from the thymus, and the conversion of induced Treg (iTreg) cells, which arise from naive T cells, at the expense of conventional T cells. This provided further rationale for its application to suppress autoimmune phenomena. Rapamycin was therefore added at the time IL-2 injections were begun, at an initial dose of 2 mg/day and the dose adjusted to maintain blood levels between 8-16 ng/ml. These levels of rapamycin were lower than those targeted when this drug is used as a single agent and thus we did not expect increased toxicities even in combination with the other drugs. Interim analysis did not show significant increase in Tregs with the addition of rapamycin. Moreover, one patient complained of worsening weakness while on the drug, which improved after its discontinuation. In view of that, we discontinued administration of rapamycin. On the other hand, 4 of 5 subjects initially treated

reported improvement in symptoms while on IL-2 and worsening symptoms after its discontinuation and requested continuation of IL-2: extension of their treatment period was sought and approved from the IRB. However, there was no statistically significant change in clinical status as measured by AALS or ALSFRS-R that could be attributed to low-dose IL-2. The study was considered a negative study and has not been published.

**Summary of phase I study**

We recently completed a first-in-human phase 1 study in three participants with ALS during which we isolated autologous Tregs from patients with ALS through leukapheresis, expanded the Tregs *ex vivo*, and then infused the expanded autologous Tregs back into participants intravenously at a dosing schedule of every 2 to 4 weeks. Participants received treatment during both early and more advanced stages of ALS. IL-2 was administered concomitantly in the participants to stabilize and possibly enhance the suppressive functions of the infused Tregs. In this pilot trial, we enrolled three participants with arm, bulbar, and leg-onset ALS, respectively.

Safety

In these participants, the treatment appeared safe and well-tolerated. No infusion-related adverse events were observed, there were no significant changes in safety labs or ECGs, and there were no serious adverse events related to the Treg infusions or low-dose IL-2 injections.

Treg percentage and suppressive function increased during infusions

In all participants, Treg percentage (Figure 1A-C) and suppressive function (Figure 1D-F) increased during the first round of infusions, declined between each round of infusions, and increased again during the second round.

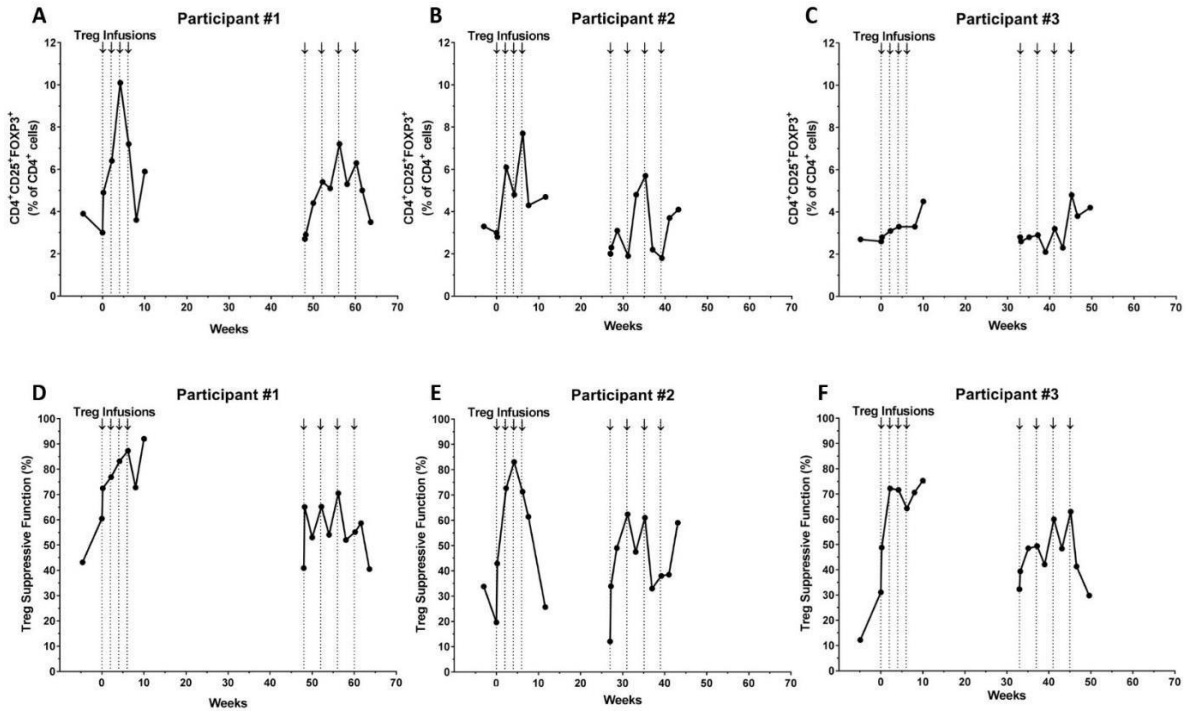


**Figure 1:** Treg percentage and suppressive function increased during each round of Treg infusions. Arrows and vertical dotted lines represent Treg infusions. The 1st Treg infusion was administered on week 0 and then every 2 weeks for a total of 4 infusions. The 5th Treg infusion was administered in each participant on weeks 48, 27 and 33, respectively, and then every 4 weeks for a total of 4 infusions. (A - Participant #1, B - Participant #2 and C - Participant #3) The percentage of $CD4^+CD25^+FOXP3^+$ Tregs within the total $CD4^+$ cell population is shown. Treg percentages are shown at baseline (weeks -4.6, -3.0 and -4.9 in each participant, respectively), the days of the 1st and 5th Treg infusions, the day after each Treg infusion, every 2 weeks during each round of infusions, and 1 month after each round. The data point collected the day after the 4th Treg infusion (week 6) in participant #3 was not d e te rm in e d due to a flow staining error. (D - Participant #1, E - Participant #2 and F - Participant #3) Treg suppressive function is shown on simultaneous days as the Treg percentage.

Enhanced Treg suppressive function correlated with slowing of functional decline

In all participants, the rate of decline of the ALSFRS-R and AALS slowed for 2 months during the first round of infusions, accelerated between each round of infusions, and slowed again over 4 months during the second round (Figure 2A-C). Spearman's correlation showed an inverse relationship between the change in Treg suppressive function and the change in AALS (Figure 2D-F; $\rho = -0.60$, $p = 0.003$ in participant #1; $\rho = -0.71$, $p = 0.0026$ in participant #2; and $\rho = -0.54$, $p = 0.016$ in participant #3). The larger the increase in Treg suppressive function, the smaller the decline in the AALS at the next clinical evaluation.

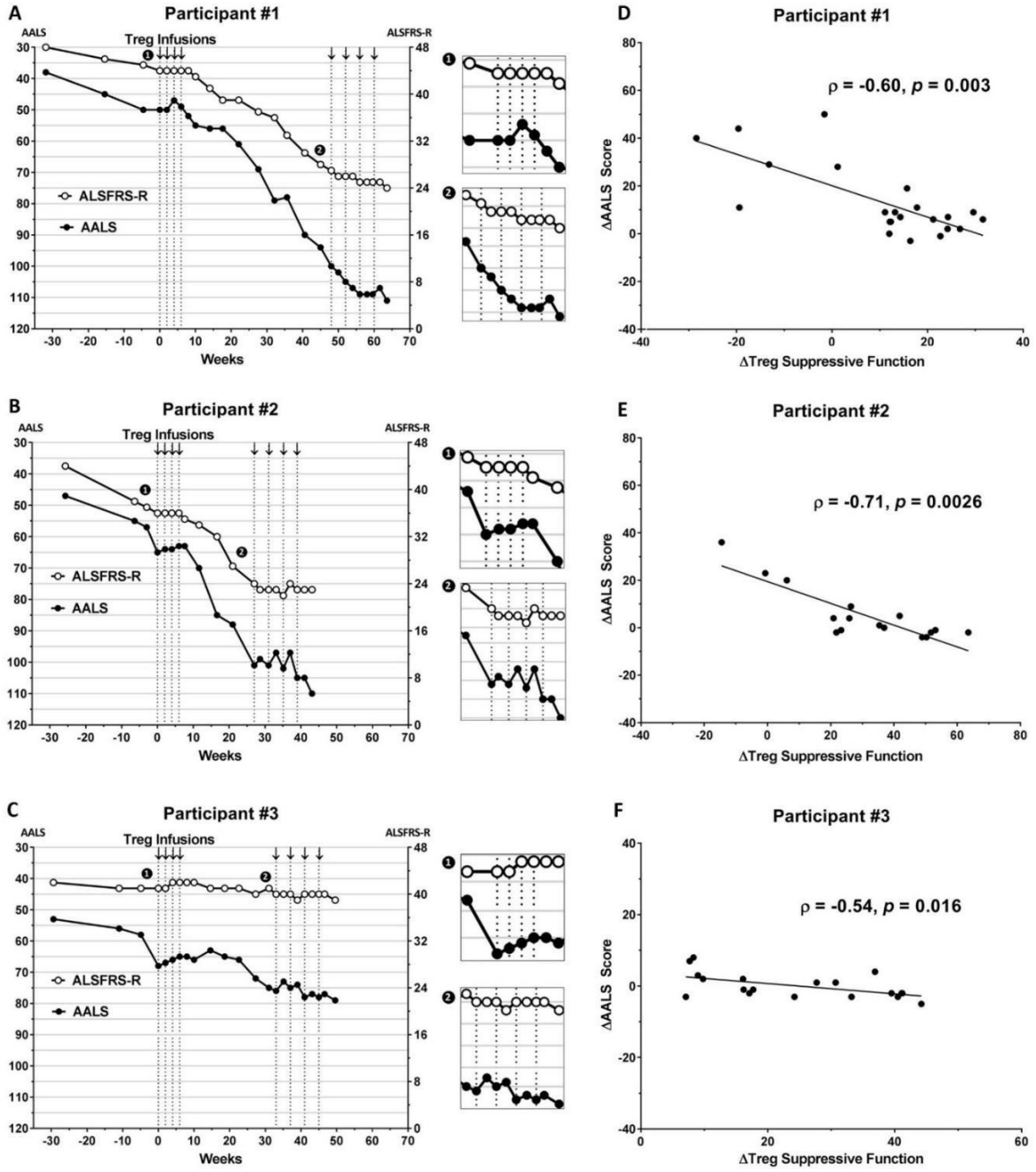


***Figure 2:*** Disease progression slowed during each round of Treg infusions and correlated with increased Treg suppressive function. Arrows and vertical dotted lines represent Treg infusions. (A - Participant #1, B – Participant #2 and C - Participant #3) Clinical progression is depicted by the ALSFRS-R (white points) and AALS (black points). Clinical progression lines during each round of Treg infusions are enlarged in side panels for the early (1) and later (2) stages of disease. (D - Participant #1, E - Participant #2 and F - Participant #3) Correlation between changes in the AALS and Treg suppressive function is shown (Participant #1; ρ = -0.60, p = 0.003, Participant #2; ρ = -0.71, p = 0.0026, and Participant #3; ρ = -0.54, p

= 0.016). Lines represent the best fit as determined by linear regression analysis. Data were analyzed by Spearman's correlation and p values less than 0.05 were considered significant.

**Summary:**

The first-in-man study of expanded autologous Treg infusions and low-dose IL-2 injections provided evidence that treatment with Tregs, given intravenously, is likely safe and well tolerated, and appears to exert a beneficial biological effect that may be efficacious in slowing ALS disease progression. As a steppingstone toward an efficacy trial, we are now proposing this phase IIa trial in which Tregs will be expanded *ex vivo* at a central facility, cryopreserved, and then administered at two sites, Houston Methodist Hospital (HMH) and Massachusetts General Hospital (MGH). This phase IIa study is designed to further explore the biological activity, safety and tolerability, of autologous Tregs in combination with low dose IL-2 in patients with ALS.

## 2.3 RISK/BENEFIT ASSESSMENT

### 2.3.1 KNOWN POTENTIAL RISKS

In our phase I study, no Treg infusion-related adverse events or clinically significant changes in safety labs or ECG findings were observed. Participant #1 reported increased muscle cramps in his legs, two falls, pharyngitis and a urinary tract infection. Participant #2 underwent placement of a percutaneous endoscopic gastrostomy tube due to progressive dysphagia. He also experienced an episode of aspiration pneumonia. His progressive dysphagia and episode of aspiration pneumonia were likely due to his bulbar ALS. Participant #2 dropped out of the study due to his progressive disease, was placed in hospice care, and he expired due to respiratory failure secondary to ALS. Participant #3 reported two suspected gastrointestinal infections and an upper respiratory infection. She also reported mild dyspnea on exertion during the study.

Common to all participants in the phase 1 study was the occurrence of infections. A potential increased risk of infections with Treg and IL-2 treatment is concerning and requires further study in a larger number of ALS patients. Also, of concern is the potential for acceleration of disease progression upon cessation of the Treg infusions. Dramatic clinical deterioration occurred between each round of infusions in the phase 1 study, especially in participants #1 and #2, who were progressing more rapidly prior to starting the trial. A larger trial with more subjects needs to be conducted over a longer time period to determine whether the acceleration of disease progression can be minimized with continuous monthly Treg infusions. Another potential long-term risk of Treg treatment is the occurrence of malignancy, although it has not been observed in early published pilot trials.

In another phase 1 trial assessing the safety of autologous Treg therapy in patients with type 1 diabetes, no infusion reactions or cell therapy-related serious adverse events were reported in 14 adult subjects after a mean follow up of 31 months[4]. There were also no opportunistic infections or malignancies reported. Out of a cumulative 144 adverse events, 36 were related to infections (all grade 1 or 2). There were 11 grade 3 or 4 adverse events, the majority related to metabolic abnormalities due to diabetes. Four serious adverse events were reported, which included three episodes of severe hypoglycemia and one episode of diabetic

ketoacidosis[4].

Additional risks in this study include toxicities from the cryoprotectant, dimethyl sulfoxide (DMSO). DMSO has been used extensively for cryopreservation of autologous stem cell grafts. Infusions of autografts containing DMSO are associated with well-known adverse effects that increase with the amount of DMSO infused. Mild adverse events include nausea, vomiting, headache, flushing, chest tightness, hypotension, bradycardia and abdominal cramps. Serious adverse events include hypertension, arrhythmias, cardiac arrest, multi-organ failure, encephalopathy and stroke[24]. Autologous stem cell infusions usually consist of high volumes more than 50 mL.

In this phase 2a study, Tregs will be cryopreserved in 10 % DMSO in 2 mL CellSeal cryogenic vials (manufactured by Cook Regentec). Immediately prior to each Treg infusion, the cryovial will be thawed in a regulated manner, Tregs will be removed from the cryovial and resuspended in an IV bag per the Preparation of TRegs for Infusion SOP. The matching Treg placebo will consist of 50 mL Normal Saline containing 5 % human serum albumin and 0.2% DMSO per infusion. Details of the IP and placebo preparations are described in the Preparation of TRegs for Infusion SOP.

Adverse reactions to human albumin are rare and include hypersensitivity/anaphylactic reactions, hypervolemia and/or hemodilution, and pulmonary edema. As the product is made from human plasma, there is also a potential risk that the product may contain infectious agents. Other reported adverse reactions include headache, dysgeusia, myocardial infarction, atrial fibrillation, tachycardia, hypotension, flushing, dyspnea, nausea, vomiting, urticaria, rash, pruritis, pyrexia and chills. Please refer to the Prescriber's Information handout for FLEXBUMIN for further details. In this phase 2a study, the final product for infusion will contain 2.5 grams of albumin (human) in a total of 50 mL normal saline solution (5 % albumin). The relative amount of albumin that will be administered is low, thus minimizing the risks of albumin-related adverse reactions.

Patients enrolled in this study will also be receiving subcutaneous injections of low-dose IL-2 for approximately 6 to 12 months depending on whether they fall in the placebo or treatment groups. In our phase 1 study, one participant experienced bruising at the injection sites, which resolved with more education regarding rotation of the sites of injection. The other two participants reported no adverse effects with IL-2 administration. Common toxicities observed with IL-2 include inflammation at the site of injection, fatigue, fever, nausea, vomiting, diarrhea, myalgias, cough, dyspnea, peripheral edema, weight gain and rash. More rare and serious adverse reactions include pancytopenia, encephalopathy, azotemia, jaundice, hypotension, pulmonary capillary leak syndrome, and thyroid abnormalities[20].

Potential adverse events due to the leukapheresis procedure include nausea, vomiting, fainting or dizziness, hematoma at the access site, seizures, blood loss, pain, infection, hypotension, tingling in the lips, hands, and face, and other vasovagal symptoms. Platelet counts may temporarily decrease; however, the body normally replaces platelets in one to two days after the procedure. Citrate anticoagulation may be used as an anticoagulant in order to prevent blood from clotting during the procedure. Possible side effects of citrate anticoagulation include muscle cramping, numbness, sensation of feeling cold, tingling in the hands, lips, and face, feeling of anxiety, and red blood cell damage.

Blood and urine will be collected for biobanking. Risks of blood collection include the possibility of fainting, pain, bruising, infection, and blood clot at the site of intravenous (IV) catheter insertion. Despite study investigators taking all appropriate precautions, there is still a small chance of compromise of identifying information. Standards for subject confidentiality, particularly with regard to genetic analysis, will be tightly followed according to the IRBs at all participating sites.

To minimize the risk of anxiety with procedures including the leukapheresis, blood draws and ECGs, participants will be reassured that these are standard hospital procedures. To safeguard confidentiality, personal health information and compliance with HIPAA during this multicenter trial, all research blood products and data will be de-identified and coded. Coded information will be kept in electronic form (password protected and encrypted) in the Houston Methodist Neurological Institute.

### 2.3.2 KNOWN POTENTIAL BENEFITS

In our prior phase 1 study in ALS patients, three participants underwent two rounds of autologous Treg infusions with each round consisting of 4 infusions[10]. The rates of decline according to the ALSFRS-R and Appel ALS scores (AALS) slowed for all participants during each round of Treg infusions[10].

Each participant also reported subjective benefits during each round of infusions. Participant #1 reported improved endurance and proximal leg strength during the first round of infusions. During the second round, he noted stabilization of his leg strength and dysarthria. Participant #2 reported improvements in chewing, swallowing and endurance during the first round of infusions. During the second round of infusions, he felt that his strength was stable, and endurance improved. Participant #3 reported improvements in grip strength, gait stability, abdominal core strength and endurance throughout the first round of infusions. During the second round, she also felt that her strength stabilized, and endurance improved.

Potential benefits may include stabilization or slowing of disease progression including respiratory decline as well as subjective improvements in symptoms as observed in patients during the phase 1 study.

### 2.3.3 ASSESSMENT OF POTENTIAL RISKS AND BENEFITS

ALS is a relentlessly progressive and fatal motor neuron disease for which there are only a few treatments that are minimally effective. Patients survive an average of 4 to 6 years following symptom onset. Although efficacy is not the focus of the trial, participation in this trial would allow for the potential benefit of slowing of disease progression as well as many subjective benefits noted by participants in the phase 1 study.

The phase 1 study provided evidence that autologous infusions of expanded Tregs are safe and well-tolerated at both early and later stages of disease. It also showed that Treg infusions engaged the target biomarkers by increasing both Treg numbers and suppressive function in the blood, and the increase in Treg suppressive function correlated with slowing of disease progression. The phase 1 study also provided evidence that slowing of progression and engagement of biomarkers could still be observed when the infusion frequency was decreased from every 2 weeks to every 4 weeks. This observation allowed us to design the phase 2 study with less frequent infusions, every 4 weeks over a 1-year period, thus reducing

the risk of infusion-related adverse events. Participants in the phase 2 study will be monitored throughout the Treg infusions for up to 4 hours post-infusion end time and discharged if vital signs are stable and no serious adverse events are reported. Vital signs will be obtained pre-infusion and monitored and recorded once every hour from the infusion start time required for the first infusion but is otherwise optional at the discretion of the Investigator.

In addition, throughout the phase 2 study, safety will be continuously reviewed by an independent Medical Monitor in consultation with the study investigators. AEs will be reported to the Medical Monitor from the study electronic data capture system, MGH Neurological Clinical Research Institute EDC system, at regular intervals throughout the study, and in real time when SAEs occur or when questions arise. Site Principal Investigators will also report SAEs to the site IRB and Coordinating Center IRB per Good Clinical Practice. An independent Data Safety and Monitoring Board (DSMB) will be convened for this study and will review unblinded data quarterly and in real-time as needed. The DSMB will have the authority to recommend study discontinuation or modification based on medical concerns, including AE or SAE profiles.

Risks of the study include infusion-related reactions and adverse reactions to both the IL-2 and Tregs themselves as discussed above. The potential for an increased risk of infections and long-term risk of malignancy due to the treatment being studied is also concerning. In the phase 1 study, dramatic deterioration was observed in each participant in between each round of Treg infusions and after completion of the second round. It was not clear whether the progression was related to the cessation of Treg infusions or would have occurred spontaneously. The potential risk of acceleration of demise due to the treatment also exists.

The purpose of this study is to determine whether the treatment is safe and tolerable over a longer time period and at higher doses in a larger number of ALS patients, and to determine whether the treatment engages biomarkers that we hypothesize would be beneficial in slowing the progression of the disease. In addition, we will begin collecting data on efficacy in a double-blinded, placebo-controlled manner that will help us to power a larger phase 2 or phase 3 trial to test the efficacy of treatment. The information gained from this study would be imperative to developing a meaningful therapy for this terminal disease. Exposures to the risks of the treatment are acceptable given that ALS is a terminal condition and there are currently no treatments that drastically change the fatal course of the disease.

# 3 OBJECTIVES AND ENDPOINTS

| OBJECTIVES | ENDPOINTS | JUSTIFICATION FOR ENDPOINTS |
|---|---|---|
| Primary | | |
| 1) To evaluate the biological activity of IV infusion of an expanded population of | 1) Treg suppressive function is increased in the blood after Treg infusions compared to | We hypothesize that increasing Treg suppressive function will slow the progression of ALS, |

| | | |
|---|---|---|
| autologous regulatory T-lymphocytes and low-dose IL-2 in people with ALS by evaluating Treg suppressive function. | placebo. | and we will determine whether this marker increases in response to the Treg infusions and IL-2 treatment. These values will be determined immediately prior to leukapheresis and each infusion during the RCT, and 1 day and 7 days after infusions on week 0, and 7 days after infusions on week 20. |
| Secondary | | |
| 1) To evaluate the biological activity of IV infusion of an expanded population of autologous regulatory T-lymphocytes and low-dose IL-2 in people with ALS by evaluating Treg number.<br>2) To evaluate the safety and tolerability of IV infusion of an expanded population of autologous regulatory T-lymphocytes and low-dose IL-2 in people with ALS.<br>• Safety will be assessed by the occurrence of adverse events (AEs), serious adverse events (SAEs), treatment-emergent adverse events (TEAEs), clinically significant abnormalities in clinical and laboratory values.<br>• RCT Tolerability will be defined as the percentage of participants who complete the 6-month RCT and each ascending dose on study treatment. | 1) Treg numbers are increased in the blood after Treg infusions compared to placebo.<br>2) Safety: Defined as the occurrence of adverse events (AEs), serious adverse events (SAEs), treatment-emergent adverse events(TEAEs), clinically significant abnormalities in clinical and laboratory values.<br>3) Tolerability: Defined as the percentage of participants who complete the trial on study treatment (RCT and OLE periods analyzed individually). | We hypothesize that increasing Treg numbers and suppressive function will slow the progression of ALS, and we will determine whether these markers increase in response to the Treg infusions and IL-2 treatment.<br><br>We will determine whether safety and tolerability are acceptable in order to proceed with the next phase. Safety and tolerability will be assessed throughout the study |

| | | |
|---|---|---|
| • OLE Tolerability will be defined as the percentage of participants who complete the 6-month OLE and each ascending dose on study treatment. | | |
| Exploratory | | |
| 1) To characterize the effects of IV infusion of an expanded population of autologous regulatory T-lymphocytes and low-dose IL-2 on clinical outcome measures of ALS including Appel ALS Scale (AALS) and ALS Functional Rating Scale-Revised (ALSFRS-R) scores, and forced vital capacity (FVC), maximum inspiratory pressure and fasciculations. To assess efficacy, the Combined Assessment of Function and Survival (CAFS) will be evaluated. | 1) Change in Appel ALS Scale (AALS) is slowed after Treg infusions compared to placebo.<br>2) Change in ALS Functional Rating Scale (ALSFRS-R) is slowed after Treg infusions compared to placebo.<br>3) Change in forced vital capacity (FVC) and maximum inspiratory pressure (MIP) are slowed after Treg infusions compared to placebo.<br>4) Tracheostomy-free survival is increased after Treg infusions compared to placebo<br>5) Frequency of fasciculations over the course of the study treatment is increased after Treg infusions compared to placebo.<br>6) To bank blood and urine for future analysis of ALS biomarkers.<br>7) To assess the Investigational Product, a Combined Assessment of Function and Survival (CAFS) will be assessed at baseline to 1 year post last Treg infusion. | Validated measures of ALS progression were chosen to begin collecting data on treatment efficacy.<br><br>These scores will be determined at screening and immediately prior to each Treg infusion. |

# 4 STUDY DESIGN

## 4.1 OVERALL DESIGN

Our overall hypothesis is that treatment with expanded autologous Tregs and low-dose IL-2 will enhance Treg numbers and suppressive function and slow disease progression in ALS. We completed a phase 1 trial, which provided evidence that the treatment is safe and tolerable at both early and later stages of disease. We propose to build on these promising results by conducting a **multi-center (two site), randomized, placebo-controlled phase IIa trial of expanded autologous regulatory T-cells infusions followed by an ascending-dose open label extension**. The primary goal of the trial is to measure the biological activity of Tregs infusions in people with ALS by measuring their impact on Treg suppressive functions. Further, we will broaden our safety and tolerability data by administering ascending doses of Tregs with longer exposure times. For Cohort 1: the randomized controlled trial (RCT) portion of the study will take place during the first 6 months and the open label extension (OLE) portion will occur during the last 6 months of the trial. Cohort 2: will enter the OLE directly after screening and leukapheresis. The planned duration of the trial for each participant is 66 weeks. Finally, we will refine our operational abilities. Tregs will be expanded ex-vivo at a central facility, cryopreserved, and then administered at two sites in preparation for a larger, multi-center efficacy trial.

Participants in **Cohort 1** will receive either active drug or placebo (randomized 1:1) for 6 months (Randomized Controlled Trial-RCT). Participants who complete the first 6 months will then be eligible to receive ascending-doses of expanded autologous Tregs for 6 months (Open Label Extension- OLE); for a total of up to 13 months study participation. **Cohort 2** will be eligible to enter the ascending-doses of expanded autologous Tregs for 6 months (Open Label Extension- OLE) for up to 7 months of study participation.

**Randomized Controlled Trial (RCT): - Cohort 1 only**

The first 6 months of the trial will be randomized, placebo-controlled and consist of 6 monthly infusions. Participants will be randomized 1:1, stratified by site using a permuted-block randomization schedule, to receive either infusions of expanded autologous Tregs and injections of IL-2 or matching placebo (50 mL Normal Saline containing 5 % human serum albumin and 0.2% DMSO) per infusions and injections (normal saline). Tregs will be infused intravenously every 4 weeks (Tregs dose = $1x10^6$ cells/kg/infusion). Randomization will occur after the leukapheresis procedure (+7 days).

Participants who are randomized to active treatment will also receive concomitant subcutaneous administration of low-dose IL-2. IL-2 will be administered subcutaneously 3 times weekly at a dose of $2x10^5$ IU/$m^2$/injection. Placebo subcutaneous injections of Normal Saline will be administered 3 times weekly to maintain blinding. The IL-2 or placebo injections will begin at least 2 weeks and preferably as much as 4 weeks prior to the first infusion. Blinding within this study will be maintained until the database is locked. Participants will not be notified regarding their randomization to the Treg product or matching Treg placebo.

Participants who complete the randomized, placebo-controlled (RCT) portion of the trial and received 5 out of the 6 scheduled Treg infusions will be eligible to complete a second leukapheresis and Treg expansion. Participants who missed ≥2 Treg infusions will be eligible to enter the OLE phase; however, they will not undergo a second leukapheresis nor expansion of cells, and they will only be eligible to receive their existing banked cells.

**Period 2 - Ascending Dose Open Label Extension (OLE): - Cohorts 1 and 2**

The OLE will consist of 6 monthly infusions during which all participants will receive ascending doses of expanded autologous Tregs.

- To the extent that a given participant's expanded autologous Tregs are available, all participants will receive 2 monthly infusions at a dose of $1x10^6$ cells/kg/infusion, then 2 monthly infusions at a dose of $2x10^6$ cells/kg/infusion and finally 2 monthly infusions at a dose of $3x10^6$ cells/kg/infusion.
- All participants in the OLE will receive IL-2 subcutaneously 3 times weekly at least 2 weeks and preferably as much as 4 weeks prior to the first infusion for this portion of the trial.

**Collection of blood and urine samples for ALS biomarkers**

Participants will provide blood and urine samples for exploratory biomarkers as detailed on the Schedule of Activities (SoA) following standard clinical protocols. These samples will be used for other future research both within and outside of motor neuron diseases. All samples will be labeled with a code. The code will not include any identifiable information. Deidentified blood and urine samples will be stored in the NEALS biorepository at the Massachusetts General Hospital (MGH) for future analysis.

## 4.2 SCIENTIFIC RATIONALE FOR STUDY DESIGN

The control group will receive "Treg placebo" infusions consisting of 50 mL normal saline, 5% human serum albumin and 0.2%DMSO per infusion, and placebo subcutaneous injections of normal saline. A placebo group was chosen in order to determine whether the treatment (Treg infusions and IL-2 injections) demonstrates safety and tolerability and shows biomarker engagement compared to the control. The ascending dose OLE portion of the study is included in order to determine whether higher doses maximize target biomarker engagement while maintaining acceptable safety and tolerability to the participants.

If the trial demonstrates safety and tolerability and shows biomarker engagement, we will select the dose that provides maximum target engagement while ensuring safety for the participants in preparation for a larger efficacy trial. If no biomarker engagement is detected, we will seek to understand this before moving clinical development forward. If safety or tolerability are not acceptable, development will not carry forward. Efficacy analyses will be treated as exploratory and will not provide rationale to proceed with further trials.

In summary, this phase IIa study will lay the groundwork for a definitive phase II/III trial in several ways:

1. We will obtain evidence of biological activity on important pharmacodynamic biomarkers.
2. We will obtain broader safety and tolerability data up to 12 months (Cohort 1), including safety data at higher doses for up to 6 months (Cohorts 1 and 2).
3. We will establish the infrastructure and processes for Treg production, cryopreservation, and distribution.

## 4.3 JUSTIFICATION FOR DOSE

The starting Treg dose ($1x10^6$ cells/kg/infusion) was empirically determined but was selected within the range of doses that has been shown to be safe and tolerable in patients with type 1 diabetes[4]. Ascending doses up to 3 times higher than the starting dose will also be evaluated in the open label extension portion of the study. These doses also remain well within the range of doses previously shown to be safe and tolerable in patients with type 1 diabetes[4]. It is imperative that these higher doses be assessed in this study in order to determine whether they will maximize target biomarker engagement while maintaining acceptable safety to the participants.

The maximum doses of Tregs that can be used in this study are limited by the Treg manufacturing process. Tregs will be isolated and expanded from the patients' leukocytes collected through leukapheresis procedure(s). We estimate that two leukapheresis procedures over a 12-month period will yield enough Tregs to complete the planned 12 monthly infusions in this study. Adequacy of Treg production and cryo-preservation will determine if a second leukapheresis procedure would be required within a 12-month period in order to follow the dosing schedule as outline for the RCT and the OLE. Higher doses would require additional leukapheresis procedures for each patient, which would pose additional procedure-related risks to the patients.

Tregs will be administered intravenously, which allows the cells to fully maximize their beneficial effects through their proposed mechanisms of action. The major effects of Tregs are within the peripheral circulation, and numbers of Tregs as well as suppression of responder T lymphocytes and monocytes/macrophages occurs systemically and can turn a proinflammatory milieu into an anti-inflammatory environment. Nevertheless, Tregs can readily enter the central nervous system (CNS) and potentially suppress proinflammatory microglia. However, we do not have direct data in ALS patients that activated pro-inflammatory microglia are being suppressed by Tregs in the CNS. In the ALS mouse model, Tregs can suppress proinflammatory macrophages in the periphery as well as microglia in the spinal cord[5]. In addition, Tregs may suppress proinflammatory monocytes/macrophages in the periphery and thus reduce proinflammatory signals that could potentially cross the brain blood barrier and exacerbate microglial activation. Overall, we hypothesize that circulating functional Tregs may slow disease progression by suppressing proinflammatory microglia, monocytes/macrophages and responder T lymphocytes.

Low-dose IL-2 will be administered subcutaneously. The major effects of IL-2 are for "stabilizing" peripheral Tregs, which can enter the CNS. Thus IL-2 need not enter the CNS to have a major effect.

Normal Saline only (50 mL) containing 5% human serum albumin and 0.2% DMSO, to better maintain the

blinding between the two groups, will be administered intravenously to the placebo group. The placebo group will also receive placebo subcutaneous injections containing Normal Saline only. In order to maximize Treg viability post-thawing, the cells must be cryopreserved at a high density in 10% DMSO. Tregs will be cryopreserved at a dose of $1x10^6$ cells/kg per 1 mL of cryopreservant containing 10% DMSO. The low volume of only 1 mL of cryopreservant minimizes risks of DMSO-related adverse reactions during the infusions. The cryopreserved Tregs (1 mL in 10 % DMSO) will be thawed and immediately resuspended in 49 mL of Normal Saline with 5 % albumin (human) to make a 50 mL solution. The addition of 5 % albumin improves the viability of Tregs after thawing compared to Normal Saline alone. The Normal Saline with 5 % albumin (human) 50 mL solution containing the thawed Tregs will be administered to the active treatment group.

IL-2 / Matching Placebo: Low-dose IL-2 will be administered subcutaneously. The major effects of IL-2 are for "stabilizing" peripheral TRegs, which can enter the CNS. Thus, IL-2 need not enter the CNS to have a major effect. The placebo group during the RCT portion of the study will receive placebo subcutaneous injections containing Normal saline only. During the OLE all subjects will receive low-dose subcutaneous Il-2 three times per week. Dosing for the Il-2 will be based on patient height and weight to determine body surface area using the Mosteller calculation at screening or adjusted weight, as needed throughout the study.

### 4.4 END OF STUDY DEFINITION

A participant is considered to have completed the study if he or she has completed all relevant phases of the study (Cohort 1: RCT and OLE; Cohort 2: OLE only) including the last visit shown in the Schedule of Activities (SoA), Section 1.3.

## 5 STUDY POPULATION

### 5.1 INCLUSION CRITERIA

In order to be eligible to participate in this study, an individual must meet all of the following criteria:

1. ALS meeting El Escorial criteria for possible, probable, lab-supported probable, or definite ALS.
2. At least 18 years old.
3. Provided informed consent and authorized use of protected health information (PHI) in accordance with national and local patient privacy regulations.
4. Capable of complying with all study procedures, including the study drug delivery procedure, in the Investigator's opinion.
5. On a stable regimen of riluzole for at least 30 days at the time of screening. If not on riluzole at the time of study entry, willing to refrain from initiation of the agent for the duration of the trial.
6. Patients on edaravone willing to refrain from taking edaravone on the same day as they will receive the Tregs infusion for the duration of the trial. If not on edaravone at the time of study entry, willing to refrain from initiation of the agent for the duration of the trial.

7. Medical record documentation of a decline in ALSFRS-R total score of at least two points in the 90 days prior to screening or at least four points over the 180 days prior to screening.
8. Forced vital capacity (FVC) ≥65% of predicted capacity for age, height, and gender at screening.
9. Patient able and willing to undergo leukapheresis.

## 5.2 EXCLUSION CRITERIA

Patients meeting any of the following criteria are not eligible for study participation:

1. Presence of any of the following clinical conditions that would interfere with the safe conduct of the study, as determined by the Investigator:
    a. Unstable neurological, cardiovascular, cerebrovascular, pulmonary, renal, hepatic, endocrine, or hematologic disease; active malignancy or infectious disease; or other medical illness.
    b. Unstable psychiatric illness defined as psychosis (hallucinations or delusions), unstable major depression, or substance abuse within 180 days prior to screening.
    c. Persistent asthma, prior history of acute systemic reactions involving IgE-dependent mechanisms, history of angioedema, or a history of anaphylactic reactions to any medication.
2. Serum alanine aminotransferase (ALT) or aspartate aminotransferase (AST) > 3× the upper limit of normal (ULN), or complete blood count (CBC) with white blood cells (WBC) <4 or >13, hematocrit (HCT) <30 or >54, or platelets <90 or >500 at screening.
3. Serum creatinine >1.8 mg/dL or creatinine clearance <40 mL/min at screening.
4. History of or positive test result for human immunodeficiency virus (HIV), hepatitis C virus, or hepatitis B virus (i.e., positive for both hepatitis B surface antigen and hepatitis B core antibody) at screening.
5. Tracheostomy.
6. If female, breastfeeding, known to be pregnant, planning to become pregnant during the study, or unwilling to use effective contraception for the duration of the trial and for 90 days after treatment.
7. If male of reproductive capacity, unwilling to use effective contraception for the duration of the trial and for 90 days after treatment.
8. Enrollment in any other interventional study.
9. Treatment with another investigational drug, biological agent, or device within 30 days or 5 half-lives of screening, whichever is longer. Patient participation in an observational/non- interventional clinical study is allowed and to be discussed prior to study enrollment with the Medical Monitor.
10. Prior gene or cell therapy treatments for ALS.

## 5.3 LIFESTLYE CONSIDERATIONS

During this study, participants are asked to:

- Abstain from strenuous exercise for a day before each clinical evaluation, leukapheresis procedure,

Treg infusion and blood collection for clinical laboratory tests. Participants may participate in light recreational activities (e.g., watching television, reading).

- Minimize interactions with household contacts who may be ill due to an infectious agent.

### 5.4 SCREEN FAILURES AND RANDOMIZATION FAILURES

Screen failures are defined as participants who consent to participate in the clinical trial but are not subsequently randomly assigned to the study intervention or entered into the study. A minimal set of screen failure information is required to ensure transparent reporting of screen failure participants, to meet the Consolidated Standards of Reporting Trials (CONSORT) publishing requirements and to respond to queries from regulatory authorities. Minimal information includes demography, screen failure details, eligibility criteria, and any serious adverse event (SAE).

Individuals who do not meet the criteria for participation in this trial (screen failure) because of an abnormal lab result (e.g. serum creatinine, ALT or AST) meeting the exclusion criteria may be rescreened. Re-screening will be discussed with the medical monitor. If participants are allowed to re-enter the study, re-screened participants should be assigned a new participant number which will link the prior and new study ID.

Randomization failures are defined as participants who consented to participate in the study, met trial eligibility criteria, underwent leukapheresis and were randomized but were not treated with Treg or matching placebo infusion and IL-2 or matching placebo subcutaneous injections. A participant may be randomized but not receive investigational products due to Treg cell growth failure, product concern by the principal investigator or Sponsor, important medical event, or other unforeseen events that make the trial impractical, unacceptable, or unsafe for the participant in the opinion of the participant or PI.

### 5.5 STRATEGIES FOR RECRUITMENT AND RETENTION

This study will be conducted in patients with a diagnosis of ALS who may be recruited from participating study centers (Houston Methodist Hospital or Massachusetts General Hospital), on social media, and/or through web and print advertisements. The potential participants may be identified and/or approached by clinical research teams or PIs at the participating study centers in order to enroll 8 patients into the study. The anticipated accrual rate is 1 to 2 patients per month in order to allow time for Treg manufacturing for each participant. Once patients are enrolled in the study, multiple methods may be utilized to enhance participant retention including email and phone calls for visit reminders. Patients will not be compensated or provided any incentives for study participation.

## 6 STUDY INTERVENTION

### 6.1 STUDY INTERVENTION(S) ADMINISTRATION

### 6.1.1 STUDY INTERVENTION DESCRIPTION

The study intervention will be expanded autologous regulatory T-lymphocytes (Tregs) in a 50 mL solution of Normal Saline with 5 % albumin (human) and 0.2 % DMSO (residual from the cryopreservation process). Tregs will be manufactured in a centralized facility operated by Houston Methodist Hospital personnel with collaboration from the Cell Therapy Core at The University of Texas Health Science Center at Houston (UTHealth). Following cryopreservation, Treg products will be transported to Houston Methodist Hospital and shipped to Massachusetts General Hospital, where the products will be thawed and resuspended in Normal Saline with 5 % albumin (human). A 50 mL solution of Normal Saline with 5 % albumin (human) containing the Tregs will be infused intravenously each month during the study. The study intervention will also include IL-2 administered subcutaneously 3 times weekly at least 2 weeks and preferably 4 weeks prior to the first Treg infusion. IL-2 is commercially available and is being used in accordance with approved labeling.

The control products will be monthly intravenous administration of the Normal Saline only (50 mL) containing 5 % human serum albumin and 0.2% DMSO per infusion, and three times weekly subcutaneous administration of Normal Saline.

Each cryovial of expanded autologous Tregs contains one infusion dose of Tregs. The density of the cropreserved Tregs in each cryovial equals (($1x10^6$ cells)*((patient weight in kg) + (0.3)*(patient weight in kg))/mL) in a total of 1.2 mL. The IP will be prepared in an IV bag per the Preparation of Tregs for Infusion SOP. The Tregs are cryopreserved in each cryovial at a dose 30% higher than the treatment dose of $1x10^6$ cells/kg to account for losses by cell death after thawing and decreased recovery from the cryovial, which occurs during the preparation process prior to infusion.

The final dose in each IV bag will be slightly variable due to these factors and may be minimally higher or lower than the required dose of $1x10^6$ cells/kg. If the total number of recovered Tregs is more than 10% above the required dose, then the extra cells will be removed and the volume replenished to 50 mL with Normal Saline with 5 % human serum albumin per SOP. If the total number of Tregs is more than 10% less than the required dose, then a second cryovial will be thawed and the cells will be used to reach the required dose in the same 50 mL volume per SOP. The total number of Tregs in each IV bag will be assessed and recorded on a Certificate of Analysis (CoA) of the final product prior to each infusion. Other release criteria parameters for the final product will include visual inspection, Gram stain, endotoxin, viability, mycoplasma, immunophenotyping, and residual beads. Tests for mycoplasma, residual beads and immunophenotyping will be performed on the product immediately prior to cryopreservation. Visual inspection, Gram stain, endotoxin and viability will be performed on the final product just prior to infusion. The final Treg product has an expiration time of 6 hours after thawing. The Release Criteria Table is provided below.

cGMP Quality Assurance personnel at both UTHealth and HMRI will review the results of the Release Criteria and will release the final product for infusion into the study participant if all criteria are met. If the final product does not meet all Release Criteria, then the product will be quarantined, and the Principal Investigator will be notified. In the event that the 14-day sterility or PCR mycoplasma results on the product

return positive after the final product has been infused into the patient, then the Laboratory Director, Quality Assurance, Principal Investigator, and the Investigational New Drug (IND) Office Sponsor representative will be notified, and the cryopreserved product will be quarantined. Quality Assurance will conduct an investigation to identify any corrective and/or preventative actions. The Principal Investigator at Houston Methodist will be responsible for notifying the appropriate regulatory agencies (FDA) and PI's from their respective institution will notify their local IRB of deviations and adverse events. All Certificates of Analysis will be made available to the Data and Safety Monitoring Board (DSMB). It will not be considered a protocol deviation if the dose is not exactly $1x10^6$ cells/kg in each infusion bag.

**Release Criteria**

| **Test** | **Specification** | **Results** |
|---|---|---|
| Visual Inspection | No Evidence of Contamination | |
| Gram Stain | No Organism Seen | |
| Endotoxin | < 5 EU/kg | |
| Viability | ≥ 70% | |
| Mycoplasma (Pre-Freeze) | Negative | |
| Immunophenotyping (Pre-Freeze) | < 20% $CD8^+$ | |
| | ≥ 70% $CD4+CD25^+$ | |
| Residual Beads (Pre-Freeze) | ≤ 100 beads / 3 x $10^6$ cells | |
| Treg Dose (CD4+CD25+ Cells) | 1 x $10^6$ cells/kg ± 10 % | |

### 6.1.2 DOSING AND ADMINISTRATION

Cohort 1: The first 6 months of the trial will be randomized, placebo-controlled (RCT) and will consist of 6 monthly infusions. During the RCT, participants will be randomized 1:1 to receive either the expanded autologous Tregs and IL-2 or matching placebos (note: the Treg placebo infusion will contain 50 mL Normal Saline containing 5 % human serum albumin and 0.2% DMSO per infusion). Tregs will be infused intravenously once a month (Tregs dose = $1x10^6$ cells/kg/infusion). The starting dose of Tregs is the same as the dose studied in the phase 1 trial[10]. This dose was found to be safe and tolerable, and shown to engage target biomarkers including Treg numbers and suppressive function. Tregs and matching placebo are to be administered within 6 hours of preparation.

Subjects will be observed at the study facility for four hours after their first infusion in Period 1 (RCT). Thereafter, in the RCT, subjects will be monitored following infusions only as long as the investigator deems necessary.

Subjects randomized to active treatment will also receive concomitant subcutaneous administration of low-dose IL-2 and provided training by qualified research personnel to self-administer subcutaneous IL-2 injections. IL-2 will be administered subcutaneously 3 times weekly at a dose of $2x10^5$ IU/$m^2$/injection. Placebo subcutaneous injections containing normal saline will be administered 3 times weekly to maintain blinding. The training sessions to self-administer IL-2 or matching placebo subcutaneous injections will include: verification that the pre-measured dose of IL-2 or matching placebo provided by the study is accurate, wash hands first, selecting an appropriate injection site, i.e. the right or left lower abdominal quadrant area alternating sites for each injection, cleaning the site with alcohol provided by the site prior to the injection, administer only the pre-measured dose of IL-2 or matching placebo provided by the study, and discarding the empty syringe in the sharps container provided by the study. Subjects will be trained to document doses administered, missed doses, contact their study contacts for any questions or concerns related to dosing of the investigational product (IL-2 or placebo), and monitor injection sites for local irritation. Should the local injection site become inflamed or hard, the subject should contact the study team for direction related to dosing. IL-2 or matching placebo syringes are to be administered within the expiration date provided by the pharmacy, generally less than nine days from pharmacy release. Subjects will be instructed to store the IL-2 or matching placebo in the refrigerator toward recommended temperatures, 2ºC to 8ºC (36ºF to 46ºF).

Subjects will receive either active Treg infusion and IL-2 drugs or matching placebo (randomized 1:1) for 6 months (period 1: Randomized Controlled Trial). All participants will be eligible to receive an ascending-dose of expanded autologous Tregs for up to 6 months (period 2: Open Label Extension), depending on availability of each participant's expanded autologous Tregs. Participants who complete the 6-month RCT and who received at least 5 of the 6 scheduled Treg infusions will be eligible to complete a second leukapheresis and Treg expansion.

Cohorts 1 & 2: All OLE participants will receive 2 monthly infusions at a dose of $1x10^6$ cells/kg/infusion, then 2 monthly infusions at a dose of $2x10^6$ cells/kg/infusion and finally 2 monthly infusions at a dose of $3x10^6$ cells/kg/infusion and IL-2 subcutaneously 3 times weekly for the duration of the OLE portion of trial.

Single doses up to $3x10^6$ cells/kg/infusion as well as the total number of Tregs from all 6 doses have previously been shown to be safe and tolerable in patients with type 1 diabetes who received up to $26x10^8$ cells in one IV infusion[4]. Higher doses of Tregs are being tested in the phase 2a trial to determine whether maximum engagement of target biomarkers (e.g., Treg numbers and suppressive function) can be obtained while maintaining an acceptable safety and tolerability profile.

Subjects will be observed at the study facility for 4 hours after their first infusion in Period 2 (OLE). The subjects will also be monitored for at least 1 hour following each escalating dose during the OLE. For the remaining infusions in the OLE, subjects will be monitored only as long as the investigator deems necessary if they receive a dosage of cells that they have previously tolerated.

## 6.2 PREPARATION/HANDLING/STORAGE/ACCOUNTABILITY

### 6.2.1 ACQUISITION AND ACCOUNTABILITY

Leukapheresis will be performed at Houston Methodist Hospital for all study participants. The leukapheresis product will be delivered to the Cellular Therapy Core at UTHealth, an experienced FDA inspected manufacturing site located across the street from Houston Methodist Hospital. Houston Methodist Neurological Institute Investigators and UTHealth will conduct the manufacturing of Tregs in a cGMP-compliant manner and cryopreserve the cellular product post-expansion. The cryopreserved product will be stored and made readily accessible at the prescribed doses for extended timelines.

The cryopreserved Treg products will be transported to Houston Methodist Hospital via a courier and shipped to Massachusetts General Hospital via the Cryoport system for shipping frozen biological materials. The system is comprised of a pre-packaged liquid nitrogen dry vapor dewar that contains no free liquid nitrogen. The system also includes a Web-based Cryoportal™ that provides live monitoring and reporting on the location and condition of the shipment, change of custody and complete visibility of shipping. Chain of custody of the IP to the investigators at Massachusetts General Hospital and Houston Methodist Hospital will occur following an established SOP. The placebo and final Treg products will be prepared at the site of administration according to an established SOP. The cryopreserved Tregs will be thawed and resuspended in Normal Saline with 5 % albumin (human) in a cGMP-compliant manner at each respective site. Houston Methodist Hospital and Massachusetts General Hospital pharmacies will prepare the IL-2 or placebo and provide it to the clinical research staff. The clinical research staff will ship IL-2 or placebo to their respective study participants, including participants that live out of state. If applicable, the PI and clinical research staff at each institution will be responsible for sending or delivering the products to the participant.

Documented instructions on IL-2 dosing, storage and management will be provided to subjects. IP accountability for the Tregs and IL-2 will be documented in subjects' records.

### 6.2.2 FORMULATION, APPEARANCE, PACKAGING, AND LABELING

The investigational product, the expanded autologous Tregs, will be cryopreserved in 1.2 mL of cryopreservant, CryoStor CS10, in a 2 mL CellSeal cryogenic vial (Cook Regentec). One mL of Tregs will be thawed and resuspended in 49 mL of Normal Saline with 5 % albumin (human). The final product that will be infused into the treatment group will be 50 mL of Normal Saline with 5 % albumin (human) containing Tregs at a dose of $1x10^6$ cells/kg. Placebo will consist of 50 mL Normal Saline containing 5 % human serum albumin and 0.2% DMSO per infusion. The bags containing products will be labeled with the MRN or a global unique identification number (GUID) for each participant. The bags will also be labeled with the recipient's name, date of birth, patient medical record number, global unique identification number, harvest date, cell type, "For Autologous Use only," per 21CFR 1271, and contain the statement, "Caution: New Drug-Limited by Federal Law to Investigational Use," per 21 CFR 312.6(a).

IL-2 will be labeled per each institution's internal policies.

### 6.2.3 PRODUCT STORAGE AND STABILITY

Expanded autologous Tregs will be cryopreserved in 2 mL cryogenic vials and stored in liquid nitrogen, vapor phase. Treg products will be thawed and resuspended in Normal Saline with 5 % albumin (human), prior to being administered intravenously per an established SOP. The Treg products will be infused immediately upon verification that they meet the release criteria, with a target administration time within 1 hour of thawing. The products do not need to be protected from light or humidity.

### 6.2.4 PREPARATION

Cryopreserved Treg products will be thawed and suspended in Normal Saline with 5 % albumin (human) and will undergo sterility and viability testing prior to IV administration according to the Thawing of Cryopreserved Tregs in Saline (5 % HSA) SOP. Each IV bag will contain a dose of $1x10^6$ cells/kg in 50 mL Normal Saline with 5% albumin (human). Thus, a participant receiving a dose of $3x10^6$ cells/kg will receive infusions from three separate IV bags with each bag labeled per section 6.2.2. When study participants receive doses of $2x10^6$ cells/kg or $3x10^6$ cells/kg, at least 2 cryovials will be thawed and their contents combined in order to achieve the required total dose for that particular infusion. Sterility and viability testing will be performed on the combined sample as all cryovials were originally prepared on the same day from the same lot following leukapheresis. Then, the IP will be divided into 2 or 3 IV bags, each containing 50 mL of Normal Saline with 5% albumin, corresponding to a total dose of $2x10^6$ cells/kg or $3x10^6$ cells/kg, respectively.

## 6.3 MEASURES TO MINIMIZE BIAS: RANDOMIZATION AND BLINDING

Cohort 1: Randomization will take place after informed consent is obtained and within seven days after the patient has completed leukapheresis. The MGH biostatistician will construct a randomization schedule stratified by site using permuted random blocks and a 1:1 allocation ratio. All participants will undergo leukapheresis prior to being randomized to the treatment or placebo group. Tregs will be manufactured for every participant, but the placebo containing normal saline only will be prepared for the placebo group. All products (Tregs and placebo) will be appropriately labeled with the participant's MRN or unique identifying number as indicated in Section 6.6.2. The Tregs will be transported or shipped to the appropriate study site for each participant just before each scheduled infusion during both the randomized, placebo-controlled portion of the trial and the open-label extension.

Cohort 2: study participation in the OLE will take place after informed consent is obtained, eligibility is verified by the Clinical Investigator, and post completed leukapheresis assessing cell product utility. Tregs product management will occur as above.

The Houston Methodist Hospital and Massachusetts General Hospital Investigational Drug Services pharmacies will be unblinded to prepare the IL-2 or placebo (normal saline) injections appropriately for each participant according to the participants' unique identifying numbers.

Safety will continuously be reviewed by an independent Medical Monitor who will be kept abreast of subject enrollment and trial activities via regular meetings. In consultation with the study investigators, the Medical Monitor has the authority to unblind any participant due to safety concerns (e.g., serious adverse events (SAEs)).

Trained research personnel at the Houston Methodist Neurological Institute and Massachusetts General Hospital will assay biomarkers of biological activity including Treg numbers and suppressive function within blood samples obtained from their respective participants at HMH or MGH per validated SOPs. The investigators will not evaluate any data relating to the primary end points until the end of the randomized, placebo-controlled portion of the trial as the results may cause unblinding to occur. However, investigators will closely monitor subjects, record, and report any AEs or SAEs, expected or unexpected, for any IP-related activities. Investigators will report any inadvertent unblinding to the NEALS Project Management group and action measures may need to be taken to minimize bias in the evaluations.

Cohort 1: Once the randomized, placebo-controlled portion of the trial has completed, the participants may enter the open label extension portion of the trial. Once all subjects in Cohort 1 have completed the RCT portion of the study, the Treg assay results and randomization schedule will be unblinded to the OPMD and Houston Methodist PI so that they can begin to optimize the Treg manufacturing process for subsequent clinical trials without delay. The plan for unblinding following completion of the RCT by all subjects in cohort 1 was discussed with and agreed upon by the DSMB and study biostatistician.

Cohort 2 subjects will directly enter the open label extension portion of the trial. If the participants in Cohort 1 were in the placebo group, then they will begin receiving infusions of Tregs that were manufactured following their initial leukapheresis. All compliant participants in Cohort 1 completing the RCT portion of the trial may undergo a second leukapheresis if needed in order to achieve the required doses for the OLE portion of the trial.

## 6.4 STUDY INTERVENTION COMPLIANCE

Treg or matching placebo infusions will take place in the clinical research infusion centers at Houston Methodist Hospital and Massachusetts General Hospital. All evaluations will take place in person or by phone. IL-2 or placebo injections will be provided to each participant for self-administration after the appropriate training has taken place. Each participant will be required to record a drug diary of the dates and times the injections were administered at home. Study intervention compliance will be determined by the participants' adherence to the schedule of activities, Treg infusion compliance, and their maintenance of the drug log for IL-2/placebo injections.

Participants should receive Treg infusions within the visit window according to the Schedule of Activities. If an infusion cannot be conducted within the visit window, attempts should be made to bring the subject back for dosing as soon as possible; however, the dosing must not occur within 2 weeks prior to the next scheduled dose. If the participant cannot return for dosing in this timeframe, the dose should be missed, and the next visit should be conducted per the Schedule of Activities.

Participants will receive either active drug or placebo (randomized 1:1) for 6 months (period 1: Randomized Controlled Trial). All participants will be eligible to receive an ascending-dose of expanded autologous Tregs for up to 6 months (period 2: Open Label Extension), depending on availability of each participant's expanded autologous Tregs. Participants who complete the 6-month RCT and who received at least 5 of the 6 scheduled Treg infusions will be eligible to complete a second leukapheresis and Treg expansion.

Due to COVID-19 concerns, every effort will be made to comply with the trial schedule of events. However due to concerns related to COVID-19 restrictions, protocol deviations will continue to be recorded in the subject case records and every effort will be made to monitor participant safety in person, if possible, or remotely via electronic / mobile communications.

## 6.5 CONCOMITANT THERAPY

For this protocol, a prescription medication is defined as a medication that can be prescribed only by a properly authorized/licensed clinician. Medications to be reported in the subject record and/or Case Report Form (CRF) are concomitant prescription medications, over-the-counter medications, and supplements.

### 6.5.1 RESCUE MEDICINE

The study sites will supply rescue medications that will be obtained locally. The following rescue medications may be used as needed in the event of an adverse reaction to the product infusion (Tylenol 325 mg po x2, Benadryl 25 mg po/IV x2, SoluMedrol 125 mg IV x2, Epinephrine 0.3 mg IM x1). Other medications will be used as clinically indicated.

**Screening for the need to premedicate subjects:**

Subjects will receive the dose of IP at each infusion per clinical trial protocol. Rescue medications may be used as needed in the event of an adverse reaction to the product infusion (Tylenol 325 mg po, Benadryl 25 mg po/IV, SoluMedrol 125 mg IV, Epinephrine 0.3 mg IM). Other medications will be used as clinically indicated.

**For subjects who exhibit any allergic response to prior infusions and the determination is made to proceed with the Treg or Matching Placebo infusion:**

Subjects will be assessed for risk factors (hypersensitivity to medications, prior unreported allergies, concomitant medications that may affect the immune response) and asked to report any sign or symptom of a response to the infusion (e.g., itching, throat or tongue swelling, hypotension, increased respiratory rate, tachycardia. dyspnea, dizziness, N/V, anxiety, numbness). The research team will be at bedside monitoring the subject's physical state, vital signs Q15min for the first hour, and communicating with the subject as is their standard practice during IP delivery. Pre-infusion premedications will be administered as needed per PI assessment: oral and/or IV diphenhydramine or corticosteroids. The use of rescue medications is allowable during the product infusion and for 4 hours following each product infusion while the

participant is being monitored in each site's clinical research infusion center. The date and time of rescue medication administration as well as the name and dosage regimen of the rescue medication must be recorded. Participants should be urgently transported to an appropriately equipped emergency department for adverse reactions that do not respond to the rescue medications.

# 7 STUDY INTERVENTION DISCONTINUATION AND PARTICIPANT DISCONTINUATION/WITHDRAWAL

## 7.1 DISCONTINUATION/WITHDRAWAL

Discontinuation from the study intervention is permitted in specific circumstances while the subject remains in the study to complete remaining study procedures as indicated by the study protocol. If a clinically significant finding is identified (including, but not limited to changes from baseline) after consent, the investigator or Medical Monitor will determine if any change in participant management is needed. Any new clinically relevant finding will be reported as an adverse event (AE).

Study interventions may be halted if the patient experiences a moderate-severe infection according to the investigator's and Medical Monitor's evaluations. Treg infusions and IL-2 injections will be discontinued until the infection has resolved. The participant will be asked to continue all regularly scheduled trial activities if possible until the infection resolves and the PI and Medical Monitor approve the resumption of the study intervention. Once medically cleared, the participants will resume study intervention according to their schedule of activities.

In the event of study discontinuation, final data will be collected in person, or if an in-person visit is impractical, as much data will be collected as possible by telephone, at least updating concomitant medications and adverse events. End of study events will include:

- Screen Failures (i.e. randomization did not occur) – Collect as much data as required to determine that the participant is ineligible.
- Cohort 1: Randomization Failures (i.e. randomization occurred but investigational product (cells/placebo) not delivered) – Conduct a Final Safety Visit. Early Discontinuation (i.e. randomized and received investigational product) – Conduct a Final Safety Visit. In all cases of discontinuation:
    - Relevant medical notes pertaining to a medical reason for discontinuing, if available
    - Communication with the participant pertaining to his/her study discontinuation
    - Investigator review of study discontinuation

## 7.2 PARTICIPANT DISCONTINUATION/WITHDRAWAL FROM THE STUDY

Participants are free to withdraw from participation in the study at any time. An investigator should withdraw a participant from the study only if continued participation places the participant at undue risk. Any risk that can be mitigated by discontinuing study intervention alone should be managed without

withdrawing the participant from the study.

If provided by the participant or known to the investigator, the reason for participant withdrawal from the study will be recorded on the Case Report Form (CRF) and MGH Neurological Clinical Research Institute EDC.

Subjects who sign the informed consent form and who do not receive the investigational product (cells/placebo) will be replaced.

### 7.3 LOST TO FOLLOW-UP

A participant will be considered lost to follow-up if he or she fails to return for 6 scheduled visits and is unable to be contacted by the study site staff.

The following actions must be taken if a participant fails to return to the clinic for a required study visit:

- The site will attempt to contact the participant and reschedule the missed visit within 7 days and counsel the participant on the importance of maintaining the assigned visit schedule and ascertain if the participant wishes to and/or should continue in the study.
- Before a participant is deemed lost to follow-up, the investigator or designee will make every effort to regain contact with the participant (where possible, 3 telephone calls and, if necessary, a certified letter to the participant's last known mailing address or local equivalent methods). These contact attempts should be documented in the participant's medical record or study file.
- Should the participant continue to be unreachable, he or she will be considered to have withdrawn from the study with a primary reason of lost to follow-up.

## 8 STUDY ASSESSMENTS AND PROCEDURES

### 8.1 EFFICACY ASSESSMENTS

Screening procedures and evaluations will be performed within 8 weeks of the first infusion. Screening procedures will be performed during an office visit to determine the subject's eligibility for the study. The patients' medical charts and results of diagnostic tests will be used for screening purposes, where applicable. All Health Insurance Portability and Accountability Act (HIPAA) rules, relevant federal and state laws, and local institutional requirements will be followed when reviewing the patients' medical charts. Inclusion and exclusion criteria will be assessed. Medical history and demographics will be obtained, and a physical examination will be performed. If there is any question regarding peripheral vascular access, consultation will be obtained from the Houston Methodist Blood Donor Center or a trained nurse to make sure that the patient will be able to undergo leukapheresis. Informed consent will be obtained from eligible patients electing to proceed with the study.

The study participants will undergo leukapheresis at Houston Methodist Hospital within 5 weeks of

screening and the participants will undergo their first infusion of either placebo or Tregs within 6 weeks of the leukapheresis and undergo infusions every 4 weeks for a total of 6 infusions during the randomized controlled portion of the study.

Participants will undergo infusions at their sites of enrollment, Houston Methodist Hospital or Massachusetts General Hospital. Participants will check-in at the designated site for the infusion. Upon arrival, subjects will be queried for any changes in medication or health status, vital signs will be collected, and any concerns related to the study will be reviewed and documented. The participants will have blood drawn and urine collected for safety and biomarker studies. ALS functional outcome assessments including the Appel ALS score and the ALSFRS-R will also be performed.

Patients may be premedicated with Benadryl (diphenhydramine) up to 1mg/kg IV (max 50mg), SoluMedrol (max 250mg IV) and Tylenol (acetaminophen) 10 mg/kg po (max 650mg) at the discretion of the Principal Investigator. The study therapy or matching placebo will be infused into the patients according to an established SOP over approximately 1-10 minutes via a peripheral IV line. Monitoring will be undertaken according to institutional standards for administration of blood products. Patients will be monitored for up to 4 hours post-infusion from the start time and discharged if vital signs are stable and no serious adverse events are reported. Vital signs will be obtained pre-infusion and monitored and recorded once every hour from the infusion start time required for the first infusion but is otherwise optional at the discretion of the Investigator.

**Prior to Treg or matching placebo infusions:**

1. Assess subject for concomitant medications use, ACE Inhibitors and NSAIDs:

   Subjects will be screened and queried for concomitant use of ACE inhibitors [e.g. benazepril (Lotensin, Lotensin Hct),captopril (Capoten), enalapril (Vasotec), fosinopril (Monopril), lisinopril (Prinivil, Zestril), moexipril (Univasc), perindopril (Aceon), quinapril (Accupril), or others ACE inhibitors not listed here]; and chronic use of non-steroidal anti-inflammatory drugs (NSAIDs), [e.g. aspirin, celecoxib (Celebrex), diclofenac Cambia, Cataflam, Voltaren-XR, Zipsor, Zorvolex), ibuprofen (Motrin, Advil), indomethacin (Indocin), and other NSAIDs not listed here] which suggests the need for closer monitoring to assess for and prevent the potential for an anaphylactic infusion reaction.

2. Assess subject for recent severe upper respiratory infection.

   A recent severe URI may require the subject to be rescheduled for their infusion to a later date due to the potential for infusion reaction related to a further impaired immune response. The Investigator will assess the need to reschedule the infusion. Site personnel will educate research participants to notify the site regarding a severe URI occurrence prior to an upcoming infusion visit.

**Subject monitoring for intravenous infusions of Treg or Matching Placebo:**

Infusions of drugs and cell products have the potential for serious adverse infusion reactions and subject safety must consider several potential infusion reaction responses. All subjects will be assessed for the need to premedicate with Tylenol, diphenhydramine and corticosteroids, based on medical history, current healthcare status, and previous infusion response. Subjects will be monitored and assessed for the potential of: **biphasic anaphylaxis** (i.e., two phase anaphylactic response whereby the first anaphylactic response to the Treg or Placebo infusion is treated and resolves, and later an anaphylactic response occurs which may be milder or more severe without being re-exposed to the initial immune trigger); **protracted anaphylaxis** (i.e., anaphylaxis lasting hours to days after the initial Treg or Matching Placebo infusion exposure, which does not completely resolve; and **delayed anaphylaxis** (i.e., an adverse infusion reaction that occurs 2-6 hours to days later following the Treg or Placebo infusion exposure) to prevent the potential for serious adverse infusion reactions. Sites will document the need to premedicate subjects based on Investigator assessment.

**Pre-, During-, and Post-infusions:**

Subjects will be monitored for infusion response, instructed to report any signs or symptoms of infusion reaction, e.g., itching, change in vital signs, change in breathing or airway compromise, inflammation, diaphoresis, nausea, &/or erythema, and continue to have periodic vital sign measurements collected. Sites will continue to monitor subjects throughout the infusion and actively query subjects for infusion responses. Prior to infusions, site personnel will instruct subjects to immediately report any signs and symptoms of infusion reaction throughout the infusion and for 1 week following the infusion.

Pre-Post infusion monitoring if noted Treg or Matching Placebo reaction: discontinue infusion immediately, continue monitoring vital signs Q 2-5 min, administer O2 at 15ml/min, recline subject in comfortable position, leaving legs elevated of possible, and monitor subject's airway to prevent aspiration if vomiting potential, and for diminished airway; be prepared to call a Code Blue/Code, the emergency medications including epinephrine at the bedside, and Crash Cart near infusion room.

**Post-infusion, if allergic response noted:**

Collect a WBC, lactic acid, and blood cultures to evaluate for an infection due to a potentially contaminated Treg or Matching Placebo infusion. The IP undergoes sterility testing including visual inspection, Gram stain, and endotoxin immediately prior to each infusion. Mycoplasma testing is performed immediately prior to cryopreservation. The IP is not released for the infusion by QA at UTHealth and Houston Methodist until these sterility tests are confirmed negative. Fourteen day anaerobic and aerobic cultures are also initiated from the IP immediately prior to each infusion. If cultures come back positive, the IRB will be immediately notified. The residual product in the infusion bag will not need to be tested for sterility as these tests are performed on the product prior to the infusion

**Following each infusion:**

The PI or study staff will provide a follow up phone call, or face-to-face review if in clinic, within 24 to 48 hours to query subjects for symptoms of a delayed anaphylaxis response.

**For subjects who experience anaphylaxis related to the Treg or Matching Placebo infusion:**

Subjects experiencing an adverse event such as anaphylaxis will be treated as clinically indicated to fully ensure their safety. Standard practice would likely include monitoring and overnight observation in the hospital for possible biphasic and protracted anaphylaxis responses.

In addition, eligible participants will self-administer placebo or commercially available IL-2 ($2 \times 10^5$ IU/$m^2$) injections subcutaneously 3 times weekly. The injections will begin after the leukapheresis, at least 2 weeks and preferably as much as 4 weeks prior to the first Treg infusion. Following the leukapheresis, participants will be taught to inject the medication subcutaneously and will do so at home with medication supplied by the Methodist Hospital Investigational Drug Services Pharmacy or Massachusetts General Hospital Pharmacy.

Cohort 1: The subcutaneous injections will be discontinued 4 weeks after at the final visit for the randomized controlled trial portion of the study (week 24). Prior to the open label extension portion of the study, participants may undergo a second leukapheresis (week 25). Cohorts 1 and 2: Participants will start or re-initiate IL-2 injections at least 2 weeks and preferably as much as 4 weeks prior to the first Treg infusion for the open label extension portion of the trial. Participants will undergo Treg infusions every 4 weeks for a total of 6 infusions. IL-2 injections will be discontinued at the final visit for the open label extension portion of the study (Week 50). The patients will return for a final safety visit during week 54.

The following procedures/evaluations will be performed at the appropriate times within the clinical trial (see Schedule of Activities section 1.3):

- **Informed Consent discussion, review, and execution**: A discussion of the study will occur with the potential subject by the Clinical Investigators and delegated study team members prior to any research related activity. The informed consent **contains two optional statements** which are not required for study participation. (1) Consent to full frontal and whole-body photographs and/or video to track ALS progression in which the images will be maintained, on a secure, encrypted and backed-up server. The photographs and/or video will not be shared or displayed outside the study without additional written permission by the subject. (2) Attestation by the subject for future donation of their tissue/organs (this attestation is not consent for future tissue donation or autopsy which will be obtained from next of kin per state regulations). Subjects will complete the applicable consent form documentation options during the consent form review and execution process.
- **Physical examination.** A physical and neurological examination will be performed by a physician and include examination of general appearance, skin, neck (including thyroid), eyes, ears, nose, throat, heart, lungs, abdomen, lymph nodes, extremities, and nervous system. All clinically significant changes following the start of the first infusion will be considered an adverse event and

recorded as appropriate unless deemed as a newly identified non-reported baseline condition which has not worsened. The subject's height (cm) and weight (kg) will be recorded at the screening visit.

- **Vital Signs**. Vitals signs will be checked by the clinical research coordinator, nurse, or physician. Blood pressure, heart rate, respiratory rate, and temperature should be measured in the sitting position after 5 minutes of rest. Alternatively, measurements can be taken in recumbent position.
- **The Appel ALS Rating Scale (AALS).** The Appel ALS Rating Scale (AALS) will be completed by **blinded** trained clinical research personnel and physicians and consists of five sub- scores: bulbar, respiratory, muscle strength, and lower extremity and upper extremity function. Each group is composed of individual tests. A group score of six is assigned if there is no dysfunction and group scores of 30-36 points are assigned for maximal dysfunction. The AALS total score ranges from 30 for healthy subjects to 164 for those with maximum impairment. The rate of change in the AALS is a significant predictor of survival for subjects with ALS. The AALS should be one of the first assessments conducted at screening, prior to the start of each infusion, at the end of the randomized controlled and open label extension portions of the study, and at the End-of-Study or Discontinuation Visit.
- **ALSFRS-R.** The ALS Functional Rating Scale-Revised (ALSFRS-R) will be performed by a designated, **blinded in-clinic evaluator** at each site for the assessment of disease progression. All evaluators will undergo training in order to ensure a consistent approach to measurement during the preparation for the study. ALSFRS-R evaluators will not be involved in the management of adverse events, laboratory assessments, and reports in order to avoid operational bias. To the extent possible, a participant's rater should be the same throughout the study. The ALSFRS-R should be one of the first assessments conducted at screening, prior to the start of each infusion, at the end of the randomized controlled and open label extension portions of the study, and at the End-of-Study or Discontinuation Visit.
- **FVC and MIP.** Forced vital capacity (FVC) and maximum inspiratory pressure (MIP) will be measured via spirometer by a **blinded evaluator**. At screening, prior to the start of each infusion, at the end of the randomized controlled and open label extension portions of the study, and at the End-of-Study or Discontinuation Visit, subjects will be asked to make three efforts to record FVC and MIP. If the best two of three readings differ by greater than 10%, the subject will be asked to make a fourth effort. If the best two of the four readings differ by greater than 10%, the subject will be asked to make a fifth (final) effort. The best reading of the set will be recorded. All readings should be printed and saved as source documents. The absolute values as well as the % predicted values are to be recorded.
- **Fasciculations.** Subjects will be provided with a Fasciculation diary to record if they had muscle twitching on any given day and the maximum severity of the fasciculations at home daily. At each subsequent visit, the site staff will review the diary and record whether the subject had fasciculations and the maximum intensity before providing the subjects with a new diary.
- **Screening and safety laboratory tests.** Screening labs will include HIV, Hep B, Hep C, CBC with differential, full chemistry panel, urine, INR/PT/PTT, and pregnancy test (urine) for women of childbearing potential. Safety labs will be drawn prior to each leukapheresis and infusion, and at the final RCT, OLE and End-of-Study visits, and will include a CBC with differential, full chemistry panel, and pregnancy test (urine) for women of childbearing potential.

- **Biomarkers.** Blood and urine samples will be obtained from each participant for biomarker analyses at screening, immediately prior to each infusion, during the final RCT (Week24) and OLE (Week 50) visits; and during the End of Study visit (Week 54). Biomarkers will be also collected on Week 0 and Week 26, 1 (+3 days) and 7 days (±3 days) post-infusion. On Week 20, Week 34 and Week 46, biomarkers will be collected 7 (±3 days) days post-infusion. Samples may be collected from subjects in a non-fasted state.
- **Blindedness Questionnaire.** The subject and Site Principal Investigators will answer questions regarding their perception on trial blindedness.
- **Exit Questionnaire.** The subject will answer questions regarding their overall experience with the trial. If performed as part of an early termination visit before the V9 Week 24 Follow-Up Visit, the Exit Questionnaire will include the Blindedness Questionnaire. If performed as part of an early termination visit after the V9 Week 24 Follow-Up Visit or as part of the V18 Week 54 Final Safety Visit, the Exit Questionnaire will not include the Blindedness Questionnaire. If included, the subject and Site Principal Investigators will answer the questions regarding blindedness.

At the conclusion of the study, remaining samples and ALS Biomarker primary and exploratory samples will be donated to the NEALS Biorepository and made available to other ALS researchers for further analyses. No results of any study specific procedures related to biomarkers will be provided to the participants.

## 8.2 SAFETY AND OTHER ASSESSMENTS

The following procedures and evaluations will be done as part of the study to monitor safety and support the understanding of the study intervention's safety:

- **Physical examination.** A physical and neurological examination will be performed by a physician and include examination of general appearance, skin, neck (including thyroid), eyes, ears, nose, throat, heart, lungs, abdomen, lymph nodes, extremities, and nervous system. All clinically significant changes following the start of the first infusion will be considered an adverse event and recorded as appropriate.
- **Vital signs.** Blood pressure, heart rate, respiratory rate, and temperature should be measured in the sitting position after 5 minutes of rest. Alternatively, measurements can be taken in recumbent position during infusions.
- **Electrocardiograms (ECGs**). 12-lead ECGs will be conducted using a machine with built-in diagnostic capabilities (Marquette MAC 5500 or similar). Investigators will determine and document clinical significance. If clinically significant changes are noted in ECG parameters, this should be documented in the ECG source document.
- **Infusion Site/Vein Assessment.** Prior to each infusion and after infusion just prior to discharge, the infusion site will be assessed clinically for signs (redness, temperature, and swelling) and symptoms (pain and tenderness).
- **Clinical Safety Laboratory Tests.** The clinical safety laboratory analyses will be performed at a qualified hospital laboratory. Screening labs will include HIV, Hep B, Hep C, CBC with

differential, comprehensive chemistry panel, urinalysis, and pregnancy test (urine) for women of childbearing potential. Safety labs drawn prior to all leukapheresis procedures and infusions will include a CBC with differential, full chemistry panel, and pregnancy test (urine) for women of childbearing. Any laboratory variables that are not within normal limits will be reviewed by the Investigator and marked as clinically significant or not clinically significant. Clinically significant laboratory abnormalities observed at Pre-Screening will not necessarily exclude the subject from participation in the study. However, such findings should be reviewed with the PI and Medical Monitor to determine eligibility for the trial. All clinically significant laboratory abnormalities newly observed or markedly changed after infusions will be reported as adverse events and must be followed until resolved or judged as stable. Subjects terminated from the trial due to clinically significant abnormal laboratory values will be referred (with appropriate recommendations) for follow-up with their primary care physician.

- **C-SSRS (Columbia-Suicide Severity Rating Scale).** The C-SSRS is designed to quantify the severity of suicide ideation and behavior. The C-SSRS will be administered by the Investigator or a trained designee. This will be performed at screening and prior to all infusions.
- **Assessment of adverse events.** All AEs/SAEs will be monitored until resolution. Subjects may be referred (with appropriate recommendations) for follow-up with their primary care physician until resolution is achieved.

## 8.3 ADVERSE EVENTS AND SERIOUS ADVERSE EVENTS

### 8.3.1 DEFINITION OF ADVERSE EVENTS (AE)

An adverse event (AE) is any unfavorable and unintended sign (including a clinically significant abnormal laboratory finding, for example), symptom, or disease temporally associated with a study, use of a drug product or device whether or not considered related to the drug product or device.

Adverse drug reactions (ADR) are all noxious and unintended responses to a medicinal product related to any dose. The phrase “responses to a medicinal product” means that a causal relationship between a medicinal product and an adverse event is at least a reasonable possibility, i.e., the relationship cannot be ruled out. Therefore, a subset of AEs can be classified as suspected ADRs, if there is a causal relationship to the medicinal product.

Examples of adverse events include: new conditions, worsening of pre-existing conditions, clinically significant abnormal physical examination signs (i.e. skin rash, peripheral edema, etc.), or clinically significant abnormal test results (i.e. lab values or vital signs), with the exception of outcome measure results, which are not being recorded as adverse events in this trial (they are being collected, but analyzed separately). Stable chronic conditions (i.e., diabetes, arthritis) that are present prior to the start of the study and do not worsen during the trial are NOT considered adverse events. Chronic conditions that occur more frequently (for intermittent conditions) or with greater severity, would be considered as worsened and therefore would be recorded as adverse events.

Adverse events are generally detected in two ways:

- Clinical → symptoms reported by the subject or signs detected on examination.
- Ancillary Tests → abnormalities of vital signs, laboratory tests, and other diagnostic procedures (other than the outcome measures, the results of which are not being captured as AEs).

For the purposes of this study, symptoms of progression/worsening of ALS, including 'normal' progression, will not be recorded as adverse events.

The following measures of disease progression will not be recorded as adverse events even if they worsen (they are being recorded and analyzed separately): vital capacity results, ALSFRS-R, and AALS results.

If discernible at the time of completing the AE log, a specific disease or syndrome rather than individual associated signs and symptoms should be identified by the Site Principal Investigator and recorded on the AE log. However, if an observed or reported sign, symptom, or clinically significant laboratory anomaly is not considered by the Site Principal Investigator to be a component of a specific disease or syndrome, then it should be recorded as a separate AE on the AE log. Clinically significant laboratory abnormalities, such as those that require intervention, are those that are identified as such by the Site Principal Investigator.

Subjects will be monitored for adverse events from the time they sign consent until completion of their participation in the study (defined as death, consent withdrawal, loss to follow up, early study termination for other reasons, or following completion of the entire study).

An unexpected adverse event is any adverse event, the specificity or severity of which is not consistent with the current Investigator's Brochure. An unexpected, suspected adverse drug reaction is any unexpected adverse event for which, in the opinion of the Site Principal Investigator or Sponsor (or their designee), there is a reasonable possibility that the investigational product caused the event.

### 8.3.2 DEFINITION OF SERIOUS ADVERSE EVENTS (SAE)

A serious adverse event (SAE) is defined as an adverse event that meets any of the following criteria:

1. Results in death.
2. Is life threatening: that is, poses an immediate risk of death as the event occurred.
    a. This serious criterion applies if the study subject, in the view of the Site Principal Investigator or Sponsor, is at immediate risk of death from the AE as it occurs. It does not apply if an AE hypothetically might have caused death if it were more severe.
3. Requires in-patient hospitalization or prolongation of existing hospitalization.
    a. Hospitalization for an elective procedure (including elective PEG tube/g-tube/feeding tube placement) or a routinely scheduled treatment is not an SAE by this criterion because an elective or scheduled "procedure" or a "treatment" is not an untoward medical occurrence.
4. Results in persistent or significant disability or incapacity.
    a. This serious criterion applies if the "disability" caused by the reported AE results in a substantial disruption of the subject's ability to carry out normal life functions.
5. Results in congenital anomaly or birth defect in the offspring of the subject (whether the subject is

male or female)

6. Necessitates medical or surgical intervention to preclude permanent impairment of a body function or permanent damage to a body structure.
7. Important medical events that may not result in death, are not life-threatening, or do not require hospitalization may also be considered SAEs when, based upon appropriate medical judgment, they may jeopardize the subject and may require medical or surgical intervention to prevent one of the outcomes listed in this definition. Examples of such medical events include blood dyscrasias or convulsions that do not result in in-patient hospitalization, or the development of drug dependency or drug abuse.

An in-patient hospital admission in the absence of a precipitating, treatment-emergent, clinical adverse event may meet criteria for "seriousness" but is not an adverse experience, and will therefore, not be considered an SAE. An example of this would include a social admission (subject admitted for other reasons than medical, e.g., lives far from the hospital, has no place to sleep).

A suspected unexpected serious adverse reaction (SUSAR) is an SAE for which, in the opinion of the Site Principal Investigator or Sponsor, there is a reasonable possibility that the investigational product caused the event. SUSARs should follow expedited SAE reporting requirements.

The Site Principal Investigator is responsible for classifying adverse events as serious or non-serious.

### 8.3.3 CLASSIFICATION OF AN ADVERSE EVENT

#### 8.3.3.1 SEVERITY OF EVENT

For adverse events (AEs) not included in the protocol defined grading system, the following guidelines will be used to describe severity. The clinical research team will collect sufficient information to enable the PI's determination of severity.

- **Mild** – Events require minimal or no treatment and do not interfere with the participant's daily activities.
- **Moderate** – Events result in a low level of inconvenience or concern with the therapeutic measures. Moderate events may cause some interference with functioning.
- **Severe** – Events interrupt a participant's usual daily activity and may require systemic drug therapy or other treatment. Severe events are usually potentially life-threatening or incapacitating. Of note, the term "severe" does not necessarily equate to "serious."

#### 8.3.3.2 RELATIONSHIP TO STUDY INTERVENTION

The Site Principal Investigator(s) will carefully monitor each subject throughout the study for possible AEs. All AEs will be documented on source document templates and eCRFs designed specifically for this purpose. All AEs will be collected and reported in the electronic data capture (EDC) system and compiled into reports for periodic reviewing by the Medical Monitor. The Medical Monitor shall promptly review all

information relevant to the safety of the investigational product, including all serious adverse events (SAEs). Special attention will be paid to those that result in permanent discontinuation of the investigational product being studied, whether serious or non-serious.

**Assessment of Adverse Events**

At each visit (including telephone interviews), the subject will be asked if they have had any problems or symptoms since their last visit in order to determine the occurrence of adverse events. If the subject reports an adverse event, the Investigator will probe further to determine:

1. Type of event
2. Date of onset and resolution (duration)
3. Severity (mild, moderate, severe)
4. Seriousness (does the event meet the above definition for an SAE)
5. Causality, relation to investigational product and disease
6. Action taken regarding investigational product
7. Outcome

**Relatedness of Adverse Event to Investigational Product**

The relationship of the AE to the investigational product should be specified by the Site Principal Investigator, using the following definitions:

| | |
|---|---|
| 1. Not Related: | Concomitant illness, accident or event with no reasonable association with treatment. |
| 2. Unlikely: | The reaction has little or no temporal sequence from administration of the investigational product, and/or a more likely alternative etiology exists. |
| 3. Possibly Related: | The reaction follows a reasonably temporal sequence from administration of the investigational product and follows a known response pattern to the suspected investigational product; the reaction could have been produced by the investigational product or could have been produced by the subject's clinical state or by other modes of therapy administered to the subject. (Suspected ADR) |

4. Probably Related: The reaction follows a reasonably temporal sequence from administration of investigational product; is confirmed by discontinuation of the investigational product or by re-challenge; and cannot be reasonably explained by the known characteristics of the subject's clinical state. (Suspected ADR)

5. Definitely Related: The reaction follows a reasonable temporal sequence from administration of investigational product; that follows a known or expected response pattern to the investigational product; and that is confirmed by improvement on stopping or reducing the dosage of the investigational product, and reappearance of the reaction on repeated exposure. (Suspected ADR)

#### 8.3.3.3 EXPECTEDNESS

The PIs will be responsible for determining whether an adverse event (AE) is expected or unexpected. An AE will be considered unexpected if the nature, severity, or frequency of the event is not consistent with the risk information previously described for the study intervention.

### 8.3.4 TIME PERIOD AND FREQUENCY FOR EVENT ASSESSMENT AND FOLLOW-UP

The occurrence of an adverse event (AE) or serious adverse event (SAE) may come to the attention of study personnel during study visits and interviews of a study participant presenting for medical care, or upon review by a study monitor.

All AEs including local and systemic reactions not meeting the criteria for SAEs will be captured on the appropriate case record or subject's data collection forms. Information to be collected includes event description, time of onset, clinician's assessment of severity, relationship to study product (assessed only by those with the training and authority to make a diagnosis), and time of resolution/stabilization of the event. All AEs occurring while on study must be documented appropriately regardless of relationship. All AEs will be followed to adequate resolution.

Any medical condition that is present at the time that the participant is screened will be considered as baseline and not reported as an AE.

Changes in the severity of an AE will be documented to allow an assessment of the duration of the event at each level of severity to be performed. AEs characterized as intermittent require documentation of onset and duration of each episode.

The PIs and clinical research team will record all reportable events with start dates occurring any time after

informed consent is obtained until the final follow-up study visit. At each study visit, the clinical research team and Investigator will inquire for the occurrence of AE/SAEs since the last visit and the PI will determine whether the event is an AE or SAE. Events will be followed for outcome information until resolution or stabilization.

### 8.3.5 ADVERSE EVENT REPORTING

Adverse events (AEs) will be reported to the Medical Monitor from the study electronic data capture system approximately every 90 days throughout the study or sooner if deemed by the PI and/or trends are identified. Site Principal Investigators will also report AEs to the site IRB, the lead Project Medical Director at Houston Methodist for reporting to the IND, and NEALS Project Management group per Good Clinical Practice, IND requirements, and institutional policies. An independent Data Safety and Monitoring Board (DSMB) will be convened for this study and will review unblinded data quarterly and in real-time as needed. In addition, disease-related events (DREs) common in the study population (e.g., expected), will not be reported per the standard process for reporting, but will be tracked, recorded and monitored per scales of clinical outcome measures for ALS (e.g., ALSFRS-R and Appel ALS scores).

### 8.3.6 SERIOUS ADVERSE EVENT REPORTING

The study clinician or their designee will immediately report to the sponsor any serious adverse event, whether or not considered study intervention related, including those listed in the protocol or investigator brochure and must include a Clinical Investigator assessment of whether there is a reasonable possibility that the study intervention caused the event. Study endpoints that are serious adverse events (e.g., all-cause mortality) must be reported in accordance with the protocol unless there is evidence suggesting a causal relationship between the study intervention and the event (e.g., death from anaphylaxis). In that case, the investigator must immediately report the event to their IRB as required per institutional policy, Project Medical Director at Houston Methodist for reporting to the FDA, and the NEALS Project Management group.

All serious adverse events (SAEs) will be followed until satisfactory resolution or until the Site Principal Investigator deems the event to be chronic or the participant is stable. Other supporting documentation of the event may be requested by the NEALS Project Management group and should be provided as soon as possible.

The study sponsor will be responsible for notifying the Food and Drug Administration (FDA) of any unexpected fatal or life-threatening suspected adverse reaction as soon as possible, but in no case later than 7 calendar days after the sponsor's initial receipt of the information. In addition, the sponsor must notify FDA and all participating investigators in an Investigational New Drug (IND) safety report of potential serious risks, from clinical trials or any other source, as soon as possible, but in no case later than 15 calendar days after the sponsor determines that the information qualifies for reporting.

### 8.3.7 REPORTING EVENTS TO PARTICIPANTS

Participants will be informed about AEs and SAEs encountered during the study as well as study-related results as an aggregate once the study has concluded and the data has been analyzed.

### 8.3.8 REPORTABLE EVENTS

Clinically significant laboratory test abnormalities arising during the study will be treated as AEs and will be reported to the medical monitor per section 8.3.5.

The following are considered reportable events and must be reported to the Medical Monitor and Coordination Center within 24 hours of the site being notified of the event.

- All events that meet the above criteria for Serious Adverse Events (SAEs)
- Dosage Changes (Dose Management)
    - Investigational Product Suspension, Reduction or Re-challenge
    - Investigational Product Discontinuation
- Key Study Events:
    - Subject Final Disposition
    - Feeding Tube Placement
    - Permanent Assisted Ventilation (PAV)*
    - Tracheostomy
    - Mortality
    - Pregnancy
    - Diaphragm Pacing System (DPS) device implantation
    - Emergency or Accidental Unblinding Events

* Permanent Assisted Ventilation (PAV) is defined as more than 22 hours daily of non-invasive mechanical ventilation for more than one week (7 days). The date of onset of PAV is the first day of the seven days.

### 8.3.9 REPORTING OF PREGNANCY

Pregnancy or planning to become pregnant is an exclusion criterion for this study. If a participant becomes pregnant during the study, the participant will immediately discontinue the study intervention, while continuing safety follow up. The pregnancy will be reported to the Medical Monitor who will relay critical information relating to the study to the participant's primary physician. The Medical Monitor will request permission to follow pregnant women to pregnancy outcome.

## 9 DATA AND SAFETY MONITORING AND STATISTICAL CONSIDERATIONS

### 9.1 DATA AND SAFETY MONITORING BOARD

An independent Data and Safety Monitoring Board (DSMB) will be assembled for the trial. The DSMB may hold open meetings that include participation by trial personnel to discuss the trial progress, however formal DSMB activities for monitoring the trial will occur during closed sessions. The DSMB receives the blinded and unblinded summary reports of the frequency of all clinical adverse events and safety laboratory tests for planned periodic meetings as specified in the Data and Safety Monitoring Board Charter (DSMB charter). In addition, the DSMB Chair may call ad hoc meetings. Meetings will be held via teleconference. A DSMB Charter will detail the processes of this group.

Summaries of serious adverse events and enrollment will be provided to the DSMB by the Study Biostatistician ahead of scheduled DSMB meetings. Any possibly, probably or definitely study drug related, unexpected, serious adverse events (i.e. serious unexpected adverse drug reactions, SUSARs) are considered reportable events and will be reported in real-time (within 1 business day of Coordination Center (CC) awareness) to the DSMB. All adverse events and abnormal laboratory values results will be listed and will be completely identified (using MedDRA adverse reaction codes) by subject and center. The DSMB can ask to receive the SAE reports more frequently. As necessary, the DSMB can review the frequencies of clinical and laboratory abnormalities. Recommendations for modification or termination of the trial based on safety data will be made by the DSMB to the PIs. The DSMB will review safety data throughout the trial and may stop the trial for safety if they determine that there is a significant difference in the rate of a particular adverse event that would indicate a risk that is greater than the possible benefit of the study drug. A notable increase in the frequency of any adverse event should be examined by the DSMB although it may not lead to a recommendation by the DSMB.

Prior to each DSMB meeting, the CC will provide an update to the DSMB on enrollment, data quality (missing data) and protocol adherence. The CC will be responsible for communication with the DSMB.

Complete information can be found in the DSMB Charter.

## 9.2 STATISTICAL HYPOTHESES

- Primary Efficacy Endpoint(s):

    1) Change in Treg suppressive function in the blood from baseline to week 24 during Period 1 and from start to finish of each dosage level during Period 2.

- Secondary Efficacy Endpoint(s):

1) Change in Treg numbers in the blood from baseline to week 24 during Period 1 and from start to finish of each dosage level during Period 2.
2) Safety: Defined as the occurrence of adverse events (AEs), serious adverse events (SAEs), treatment-emergent adverse events (TEAEs), and clinically significant abnormalities in clinical and laboratory values compared to the placebo group.
3) Tolerability: RCT Tolerability will be defined as the percentage of participants who complete

the 6-month RCT on study treatment compared to the placebo group. OLE Tolerability will be defined as the percentage of participants who complete the 6-month OLE on study treatment.

- Safety and Tolerability Endpoint(s):

    1) The safety and tolerability profiles of treatment with expanded autologous Treg infusions and subcutaneous IL-2 injections will be identical to those of the placebo group.

## 9.3 SAMPLE SIZE DETERMINATION

Power analyses are based on the pharmacodynamic biomarker Treg suppression, safety, and tolerability. We also present a statement of power for efficacy, although this trial is not designed to test efficacy.

Treg suppression assay

With 6 participants in the six-month RCT period, the study has 80 percent power to detect a treatment difference in change from baseline to week 24 Treg suppression assay at a two-sided 0.05 significance level if the Treg therapy causes a true increase in Treg suppression levels relative to placebo that is 3.1 times the standard deviation of the baseline to week 24 change in Treg suppression assay.

Safety

Using the calculation power = 1-(1-p)n, where p is probability of an event and n is the number of subjects in a treatment arm, with 12 participants exposed to the treatment (6 in the first arm of the study and all 12 in the open label extension), we will have more than an 80% chance of seeing at least one occurrence of any serious adverse event from the Treg treatment that occurs with a frequency of at least 13%.

Tolerability

RCT Tolerability will be defined as the percentage of participants who complete the 6-month RCT on study treatment.

OLE Tolerability will be defined as the percentage of participants who complete the 6-month OLE and each ascending dose on study treatment.

If more than 2 of the 6 participants cannot tolerate the Treg treatment, we will consider the treatment intolerable. Using that rule, there is less than a 5% probability that we would reject the treatment as intolerable if its true tolerability were at least 85%, and there is an 80% probability that we would declare the Treg treatment intolerable if the true tolerability were 41%.

Efficacy

Efficacy is not the focus of this trial. Evaluations of clinical efficacy endpoints will be exploratory.

## 9.4 POPULATIONS FOR ANALYSES

All participants who receive as least one infusion of Tregs or placebo will be included in the analysis datasets.

## 9.5 DATA SAFETY MONITORING BOARD AND STATISTICAL ANALYSES

### 9.5.1 GENERAL APPROACH

Safety and tolerability, and tracheostomy-free survival data will be presented as percentages. Treg suppressive function, and all other secondary endpoint data will be presented as means with standard deviations. At the conclusion of the study, the de-identified data will be delivered to the MGH Biostatistics Center for analyses. Details of the planned analyses will be specified in a separate statistical analysis plan (SAP).

### 9.5.2 ANALYSIS OF THE PRIMARY EFFICACY ENDPOINT(S)

Treg suppressive function on the proliferation of responder T-lymphocytes (Tresp) will be assessed by $^{3}$H-thymidine incorporation according to our previously described protocol[8]. Data will be presented as visit-specific means and standard deviations and analyzed in a shared-baseline linear mixed model. Associations with time will be modeled as both fixed and random effects using a partial linear spline with a knot at week 24. Fixed effects will include a time-dependent, dose-specific treatment term. Linear contrasts of least-square estimates will be used to compare active and placebo arms during the 6-month RCT period and to estimate dose-dependent effects from the combined data from both arms and both RCT and OLE periods.

### 9.5.3 ANALYSIS OF THE SECONDARY ENDPOINT(S)

The percentage of $CD4^{+}CD25^{high}FOXP3^{+}$ Tregs within the total $CD4^{+}$ population will be assessed by multicolor flow cytometry. Treg percentages will be summarized as visit-specific means and standard deviations and analyzed in the same model as specified for Treg suppression function.

### 9.5.4 SAFETY ANALYSES

<u>Safety</u>

Safety will be assessed by the occurrence of adverse events (AEs), serious adverse events (SAEs), treatment-emergent adverse events (TEAEs), clinically significant abnormalities in clinical and laboratory values. AEs will be coded to preferred terms from a consistent version of the MedDRA library and summarized as counts of events and proportions of participants experiencing a given type of event. Rates will be compared using an exact trend test based on the proportion of patients with 0,1,2… instances of each type of event.

Tolerability

RCT Tolerability will be defined as the percentage of participants who complete the 6-month RCT period of the trial and each ascending dose on study treatment and will be summarized as simple percentages with exact 95% confidence intervals and compared by Fisher's exact test.

OLE Tolerability will be defined as the percentage of participants who complete the 6-month OLE period of the trial and each ascending dose on study treatment and will be summarized as simple percentages with exact 95% confidence intervals.

The distribution of severity, frequency, relationship to the study intervention, and action taken with respect to study intervention of AEs will be tabulated by treatment group and period. The start date, stop date, severity, relationship, expectedness, action taken, and outcome of each AE will be listed.

### 9.5.5 BASELINE DESCRIPTIVE STATISTICS

Treatment groups will be compared on baseline characteristics including age, sex, weight, site of symptom onset, time from symptom onset to diagnosis, time from symptom onset to first Treg infusion, riluzole use, edaravone use, noninvasive ventilation use, forced vital capacity prior to first Treg infusion, and measures of disease burden (ALSFRS-R and Appel ALS scores) prior to first Treg infusion.

### 9.5.6 PLANNED INTERIM ANALYSES

Safety will be continuously reviewed by an independent Medical Monitor in consultation with the study investigators. Adverse events (AEs) will be reported to the Medical Monitor from the study electronic data capture system at regular intervals throughout the study, and in real time when SAEs occur or when questions arise. The Medical Monitor will have the authority to recommend study discontinuation or modification based on medical concerns, including AE or SAE profiles.

Following the last subject's completion of the RCT portion of the study, and once data monitoring has been completed and all queries resolved for the RCT portion, the biomarker data will be unblinded to the OPMD and Houston Methodist PI to allow immediate assessment of the biological effects of the IP. The OPMD and Houston Methodist PI need to review this data prior to completion of the phase 2a trial in order to begin optimizing the Treg manufacturing procedures as soon as possible for subsequent clinical trials. The plan to unblind following the last participant's completion of the RCT has been discussed with and agreed upon by the DSMB and study biostatistician.

During the OLE portion of the trial, all biomarker and clinical assessment data will be available to the investigators in real time. Real-time access to the results of the OLE would not unblind the investigators to the RCT results, since there is a substantial washout period between the final RCT infusion and the first OLE infusion. Real-time access to the OLE results will allow the investigators to make more rapid decisions about Treg dosing for future studies based on target engagement and clinical status. This will hasten critical decisions regarding the IP development, particularly given the trial's limitations in the setting

of the COVID-19 pandemic.

### 9.5.7 SUB-GROUP ANALYSES

Sub-group analyses are not planned due to the small study size.

### 9.5.8 TABULATION OF INDIVIDUAL PARTICIPANT DATA

Individual participant data will be listed by measure and time point.

### 9.5.9 EXPLORATORY ANALYSES

Exploratory outcomes will be summarized as visit-specific (or week-specific for fasciculations) means and standard deviations and analyzed in the same model as specified for Treg suppression function.

## 10 SUPPORTING DOCUMENTATION AND OPERATIONAL CONSIDERATIONS

### 10.1 REGULATORY, ETHICAL, AND STUDY OVERSIGHT CONSIDERATIONS

#### 10.1.1 INFORMED CONSENT PROCESS

##### 10.1.1.1 CONSENT/ASSENT AND OTHER INFORMATIONAL DOCUMENTS PROVIDED TO PARTICIPANTS

Consent forms describing in detail the study intervention, study procedures, and risks are given to the participant and written documentation of informed consent is required prior to starting intervention/administering study intervention. The informed consent form was submitted with the original protocol submission to the Agency and Site PI IRB's.

##### 10.1.1.2 CONSENT PROCEDURES AND DOCUMENTATION

Informed consent is a process that is initiated prior to the individual's agreeing to participate in the study and continues throughout the individual's study participation. Consent forms will be Institutional Review Board (IRB)-approved and the participant will be asked to read and review the document. The investigator and appropriately delegated clinical research personnel will explain the research study to the participant and answer any questions that may arise. A verbal explanation will be provided in terms suited to the participant's comprehension of the purposes, procedures, and potential risks of the study and of their rights as research participants. Participants will have the opportunity to carefully review the written consent form and ask questions prior to signing. The participants should have the opportunity to discuss the study with their family or surrogates or think about it prior to agreeing to participate. The participant will sign the informed consent document prior to any procedures being done specifically for the study.

Participants must be informed that participation is voluntary and that they may withdraw from the study at any time, without prejudice. A copy of the informed consent document will be given to the participants for

their records. The informed consent process will be conducted and documented in the source document (including the date), and the form signed, before the participant undergoes any study-specific procedures. The rights and welfare of the participants will be protected by emphasizing to them that the quality of their medical care will not be adversely affected if they decline to participate in this study.

### 10.1.2 STUDY DISCONTINUATION AND CLOSURE

This study may be temporarily suspended or prematurely terminated if there is sufficient reasonable cause. Written notification, documenting the reason for study suspension or termination, will be provided by the suspending or terminating party to study participants, investigator, funding agency, the sponsor of the Investigational New Drug (IND), and regulatory authorities. If the study is prematurely terminated or suspended, the site-specific Principal Investigator (PI) will promptly inform study participants, the Institutional Review Board (IRB), the BNI Medical Monitor, MGH, and the FDA to the IND, and will provide the reason(s) for the termination or suspension. Study participants will be contacted, as applicable, and be informed of changes to study visit schedule.

Circumstances that may warrant termination or suspension include, but are not limited to:

- Determination of unexpected, significant, or unacceptable risk to participants
- Demonstration of efficacy that would warrant stopping
- Insufficient compliance to protocol requirements
- Data that are not sufficiently complete and/or evaluable
- Determination that the primary endpoint has been met
- Determination of futility

Study may resume once concerns about safety, protocol compliance, and data quality are addressed, and satisfy the sponsor, IRB and/or Food and Drug Administration (FDA).

### 10.1.3 CONFIDENTIALITY AND PRIVACY

Participant confidentiality and privacy is strictly held in trust by the participating investigators, their staff, and the sponsor. This confidentiality is extended to cover testing of biological samples in addition to the clinical information relating to participants. Therefore, the study protocol, documentation, data, and all other information generated will be held in strict confidence. No information concerning the study or the data will be released to any unauthorized third party without prior written approval of the sponsor.

All research activities will be conducted in as private a setting as possible.

The Medical Monitor, other authorized representatives of the sponsor, representatives of the Institutional Review Board (IRB), or regulatory agencies may inspect all documents and records required to be maintained by the investigator, including but not limited to, medical records (office, clinic, or hospital) and pharmacy records for the participants in this study. The clinical study site will permit access to such records.

The study participant's contact information will be securely stored at each clinical site for internal use during the study. At the end of the study, all records will continue to be kept in a secure location for as long a period as dictated by the reviewing IRB, Institutional policies, or sponsor requirements.

Study participant research data, which is for purposes of statistical analysis and scientific reporting, will be transmitted to and stored at the NEALS Systems and Data Management Center. The data capture system is Part 11-compliant. This will not include the participant's contact or identifying information. Rather, individual participants and their research data will be identified by a unique study identification number (Global Unique Identifier GUID).

A patient Global Unique Identifier (GUID) will be used as the identifier for participants included in the study. The GUID is an 11-character string that is generated using encryption technology licensed by the NCRI from the National Institutes of Health (NIH) in 2013.

The GUID is generated on a secure website that utilizes 128-bit Secure Socket Layer (SSL). On the website, the GUID is generated using an irreversible encryption algorithm – it accepts twelve identifying data elements, (e.g. last name at birth, first name at birth, gender at birth, day, month and year of birth, city and country of birth, etc.), and produces a unique random-generated character string, or GUID. No identifying information is stored in the system; it is simply used to generate the GUID. If the same information is entered into the secure website in the future, the same GUID will be generated.

The study data entry and study management systems used by clinical sites and by the NEALS Systems and Data Management Center research staff will be secured and password protected. At the end of the study, all study databases will be de-identified and archived at the NEALS Systems and Data Management Center.

### 10.1.4 FUTURE USE OF STORED SPECIMENS AND DATA

Data collected for this study will be analyzed and stored at the NEALS Systems and Data Management Center. After the study is completed, the de-identified, archived data will be transmitted to the MGH Biostatistics Center for analyses.

With the participant's approval and as approved by local Institutional Review Boards (IRBs), de- identified biological samples will be stored at the NEALS Biorepository. These blood samples could be used to research the mechanisms of action of the study intervention and to improve treatment. The NEALS Biorepository will also be provided with a code-link that will allow linking the biological specimens with the phenotypic data from each participant, maintaining the blinding of the identity of the participant.

During the conduct of the study, an individual participant can choose to withdraw consent to have biological specimens stored for future research. However, withdrawal of consent regarding biosample storage may not be possible after the study is completed. When the study is completed, access to study data and/or samples will be provided through the NEALS Biorepository.

### 10.1.5 KEY ROLES AND STUDY GOVERNANCE

| **Overall Project Medical Director** | **Medical Monitor** |
|---|---|
| Stanley H. Appel, M.D. | Jeremy Shefner, M.D., Ph.D. |
| Houston Methodist Neurological Institute | Barrow Neurological Institute |
| 6560 Fannin St, Suite 802<br>Houston, TX, 77030 | 240 West Thomas Road, Suite 400<br>Phoenix, Arizona 85013 |
| 713-441-3765 | 855-777-5797 |
| sappel@houstonmethodist.org | Jeremy.shefner@dignityhealth.org |

### 10.1.6 SAFETY OVERSIGHT

Throughout the study, safety will be continuously reviewed by an independent Medical Monitor in consultation with the study investigators. Adverse events (AEs) will be reported to the Medical Monitor from the study electronic data capture system at regular intervals throughout the study, and in real time when SAEs occur or when questions arise. Site Principal Investigators will also report SAEs to the site IRBs and, if necessary, the Sponsor per Good Clinical Practice and the IND requirements. An independent Data Safety and Monitoring Board (DSMB) will be convened for this study and will review unblinded data quarterly and in real-time as needed. The DSMB will have the authority to recommend study discontinuation or modification based on medical concerns, including AE or SAE profiles.

Additional reporting may be required for unanticipated problems involving risks to subjects or others which includes events that are unexpected, related or possibly related, and suggest the research places subjects or others at greater risk of harm (including physical, psychological, economic, or social harm) that was previously known or recognized. Unanticipated problems will be promptly reported to the sponsor and NEALS Coordinating Center, and according to local IRB reporting requirements.

Safety oversight will be under the direction of a Data and Safety Monitoring Board (DSMB) composed of individuals with appropriate expertise. Members of the DSMB should be independent from the study conduct and free of conflict of interest, or measures should be in place to minimize perceived conflict of interest. The DMSB will operate under the rules of an approved charter that will be written and reviewed at the organizational meeting of the DSMB. At this time, each data element that the DSMB needs to assess will be clearly defined. The DSMB will provide its input to the NEALS Project Management group.

### 10.1.7 CLINICAL MONITORING

The study will be performed in accordance with US 21 Code of Federal Regulations Parts:

312, Investigational New Drug (IND); 50, Protection of Human Subjects; 54, Financial Disclosure by Clinical Investigators; and 56, Institutional Review Boards. In addition, the study will be performed in accordance with the guidelines of the Food and Drug Administration (FDA), the Health Insurance Portability and Accountability Act of 1996 (HIPAA), and all other applicable medical privacy laws and regulations.

Clinical site monitoring is conducted by the study monitors to ensure that the rights and well-being of trial participants are protected, that the reported trial data are accurate, complete, and verifiable, and that the conduct of the trial is in compliance with the currently approved protocol/amendment(s), with Food and Drug Administration Good Clinical Practice (FDA GCP), and with applicable regulatory requirement(s).

- Monitoring for this study will be performed by the NEALS Project Management group.
- On-site or remote and centralized monitoring throughout the study will occur with comprehensive data verification. Remote monitoring was put in place during the COVID-19 pandemic.
- PIs will be provided copies of monitoring reports within 30 days of visit and monitoring reports will be submitted to the site PI's IRB.
- Details of clinical site monitoring are documented in a Clinical Monitoring Plan (CMP). The CMP describes in detail who will conduct the monitoring, at what frequency monitoring will be done, at what level of detail monitoring will be performed, and the distribution of monitoring reports.
- Independent audits will be conducted by the NEALS consortium to ensure monitoring practices are performed consistently across all participating sites and that monitors are following the GCP.

### 10.1.8 QUALITY ASSURANCE AND QUALITY CONTROL

Each clinical site will perform internal quality management of study conduct, data and biological specimen collection, documentation and completion. An individualized quality management plan will be developed to describe a site's quality management.

Quality control (QC) procedures will be implemented beginning with the data entry system and data QC checks that will be run on the database will be generated. Any missing data or data anomalies will be communicated to the site(s) for clarification/resolution.

Following written Standard Operating Procedures (SOPs), the monitors will verify that the clinical trial is conducted, and data are generated, and biological specimens are collected, documented (recorded), and reported in compliance with the protocol, Food and Drug Administration Good Clinical Practice (FDA GCP), and applicable regulatory requirements.

The investigational site will provide direct access to all trial related sites, source data/documents, and

reports for the purpose of monitoring and auditing by the NEALS Project Management group, Houston Methodist, and inspection by local and regulatory authorities.

### 10.1.9 DATA HANDLING AND RECORD KEEPING

#### 10.1.9.1 DATA COLLECTION AND MANAGEMENT RESPONSIBILITIES

NEALS Core Services will be utilized to oversee study execution covering the following areas and provide reports to Houston Methodist, the sponsor of record for assessment, review, concurrence, and acceptance:

The Barrow Neurological Institute group will be responsible for monitoring of study activities, specifically to ensure quality control, high performance, protocol compliance, and good clinical practice. They will also monitor IRB approvals and regulatory materials from sites, perform adverse event monitoring, enrollment supervision, study analyses and quality assurance issues. Site Monitoring will include onsite review for source data verification, protocol compliance, and good clinical practice and for study close-out visits. The NEALS Systems and Data Management Center has a Part 11-compliant web-based electronic data capture system for the trial. Standard Operating Procedures are in place for all data management activities. The data management staff will design the electronic case report forms (eCRFs) that will capture all data collected as part of the protocol. The Data Management team will also be responsible for tracking and developing programs to check the integrity of the data, including range, error, logic checks, missing data reports, and locking the study database at study conclusion and delivering the data to the MGH Biostatistics Center.

Data collection is the responsibility of the PI and clinical trial staff at the site under the supervision of the site PI. The investigator is responsible for ensuring the accuracy, completeness, legibility, and timeliness of the data reported. All written source documents should be completed in a neat, legible manner to ensure accurate interpretation of data per GCP.

Hardcopies of the study visit worksheets will be provided for use as source documents and data collection worksheets for recording data for each participant enrolled in the study. Data recorded in the electronic case report form (eCRF) derived from data collection worksheets and other source documents should be consistent with the data recorded on the source documents.

Clinical data (including adverse events (AEs), concomitant medications, and expected adverse reactions data) and clinical laboratory data will be entered into a 21 CFR Part 11-compliant data capture system provided by the NEALS Systems and Data Management Center. The data system includes password protection and internal quality checks, such as automatic range checks, to identify data that appear inconsistent, incomplete, or inaccurate. Clinical data will be entered directly from the source documents.

#### 10.1.9.2 STUDY RECORDS RETENTION

Study documents should be retained for a minimum of 7 years after formal discontinuation of the clinical study. These documents should be retained for a longer period, however, if required by local regulations. No records will be destroyed without the written consent of the site PI and the NEALS Project Management group. It is the responsibility of the NEALS Project Management Group to inform the investigator when

these documents no longer need to be retained.

### 10.1.10 PROTOCOL DEVIATIONS (PD)

A protocol deviation is any noncompliance with the clinical trial protocol, or Food and Drug Administration Good Clinical Practice (FDA GCP) requirements. The noncompliance may be either on the part of the participant, the investigator, or the study site staff. As a result of a trend in protocol deviations, corrective actions are to be developed by the site and implemented promptly. No changes to the protocol should occur without prior sponsor and IRB review, unless deviations from the protocol occur in relation to the subject's welfare and safety. All significant deviations are to be reported to the sponsor and the NEALS Project Management group.

It is the responsibility of the Site Principal Investigator to use continuous vigilance to identify and report deviations within 30 working days of identification of the protocol deviation, or within 30 working days of the scheduled protocol-required activity. All deviations must be addressed in study source documents and reported to the NEALS Project Management group. Protocol deviations must be sent to the reviewing Institutional Review Board (IRB) per their policies. The Site Principal Investigator is responsible for knowing and adhering to the reviewing IRB requirements, and the clinical research team is responsible for supporting reporting of PDs.

### 10.1.11 PUBLICATION AND DATA SHARING POLICY

This study will be conducted in accordance with the following publication and data sharing policies and regulations:

National Institutes of Health (NIH) Public Access Policy, which ensures that the public has access to the published results of NIH funded research. It requires scientists to submit final peer-reviewed journal manuscripts that arise from NIH funds to the digital archive PubMed Central upon acceptance for publication.

This study will comply with the NIH Data Sharing Policy and Policy on the Dissemination of NIH- Funded Clinical Trial Information and the Clinical Trials Registration and Results Information Submission rule. As such, this trial will be registered at ClinicalTrials.gov, and results information from this trial will be submitted to ClinicalTrials.gov. In addition, every attempt will be made to publish results in peer-reviewed journals. Data from this study may be requested from other researchers 2 years after the completion of the primary endpoint by contacting the Site PI's and the NEALS Project Management group.

### 10.1.12 CONFLICT OF INTEREST POLICY

The independence of this study from any actual or perceived influence is critical. Therefore, any actual conflict of interest of persons who have a role in the design, conduct, analysis, publication, or any aspect of this trial will be disclosed and managed. Furthermore, persons who have a perceived conflict of interest

will be required to have such conflicts managed in a way that is appropriate to their participation in the design and conduct of this trial. The study sponsor in conjunction with the NEALS Project Management group have established policies and procedures for all study group members to disclose all conflicts of interest and will establish a mechanism for the management of all reported dualities of interest.

## 10.2 ADDITIONAL CONSIDERATIONS

**Institutional Review Board**

In accordance with the US Code of Federal Regulations (CFR), 21 CFR 56, the protocol, subject recruitment advertisements (if applicable), and ICF will be submitted to the IRB for review and subsequent written approval by the IRB must be received before proceeding. The Investigator will supply and submit relevant material to the IRB for the protocol's review and approval. Verification of the IRB unconditional approval of the protocol and the written ICF statement will be transmitted to the Investigator.

The IRB will be informed by the Investigator of subsequent protocol amendments and of serious and unexpected AEs. Approval for protocol amendments will be transmitted in writing to the Investigator. If requested, the Investigator will permit audits by the IRB and regulatory inspections by providing direct access to source data/documents.

The Investigator will provide the IRB with progress reports at appropriate intervals (not to exceed one year) and a Study Progress Report following the completion, termination, or discontinuation of the Investigator's participation in the study. A copy of the IRB review and approvals will be entered into the database or sent to the site PI. Any changes to the protocol and/or consent submitted to the local IRB with IRB review correspondence will also be entered into the database or sent to the site PI.

## 10.3 ABBREVIATIONS

| | |
|---|---|
| AE | Adverse Event |
| ANCOVA | Analysis of Covariance |
| AALS | Appel ALS Scale |
| ALS | Amyotrophic Lateral Sclerosis |
| alloSCT | Allogenic stem cell transplantation |
| ALSFRS-R | ALS Functional Rating Scale-Revised |
| ALT | Alanine aminotransferase |
| AST | Aspartate Aminotransferase |
| CFR | Code of Federal Regulations |
| cGMP | Current Good Manufacturing Practices |
| CLIA | Clinical Laboratory Improvement Amendments |
| CMP | Clinical Monitoring Plan |
| CoA | Certificate of Analysis |
| CONSORT | Consolidated Standards of Reporting Trials |
| CRF | Case Report Form |
| C-SSRS | Columbia-Suicide Severity Rating Scale |
| DCC | Data Coordinating Center |
| DHHS | Department of Health and Human Services |
| DRE | Disease-Related Event |
| DMSO | Dimethyl sulfoxide |
| DSMB | Data and Safety Monitoring Board |
| EC | Ethics Committee |
| ECG | Electrocardiogram |
| eCRF/EDC | Electronic Case Report Forms/Electronic Data Capture |
| fALS | Familial Amyotrophic Lateral Sclerosis |
| FDA | Food and Drug Administration |
| FDAAA | Food and Drug Administration Amendments Act of 2007 |
| FFR | Federal Financial Report |
| FVC | Forced Vital Capacity |
| GCP | Good Clinical Practice |
| HIPAA | Health Insurance Portability and Accountability Act |
| HMH | Houston Methodist Hospital |
| HMNI | Houston Methodist Neurological Institute |
| HMRI | Houston Methodist Research Institute |
| IB | Investigator's Brochure |
| IDE | Investigational Device Exemption |

| | |
|---|---|
| IL-2 | Interleukin-2 |
| IND | Investigational New Drug Application |
| IRB | Institutional Review Board |
| ISM | Independent Safety Monitor |
| ISO | International Organization for Standardization |
| ITT | Intention-To-Treat |
| LSMEANS | Least-squares Means |
| MedDRA | Medical Dictionary for Regulatory Activities |
| MGH | Massachusetts General Hospital |
| MIP | Maximum Inspiratory Pressure |
| MSDS | Material Safety Data Sheet |
| nTregs | Natural Tregs |
| NCT | National Clinical Trial |
| NEALS | Northeast Amyotrophic Lateral Sclerosis (Consortium) |
| NIH | National Institutes of Health |
| NIH IC | NIH Institute or Center |
| OLE | Open Label Extension |
| OHRP | Office for Human Research Protections |
| OPMD | Overall Project Medical Director |
| PD | Protocol Deviations |
| PI | Principal Investigator |
| PHI | Protected Health Information |
| po | Per Os (by mouth) |
| QA | Quality Assurance |
| QC | Quality Control |
| RCT | Randomized Controlled Trial |
| SAE | Serious Adverse Event |
| SAP | Statistical Analysis Plan |
| SMC | Safety Monitoring Committee |
| SOA | Schedule of Activities |
| SOC | System Organ Class |
| SOP | Standard Operating Procedure |
| TEAEs | Treatment-emergent adverse event |
| Tregs | Regulatory T-Lymphocytes |
| WT | Wild-Type |
| UP | Unanticipated Problem |
| US | United States |

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# STATISTICAL ANALYSIS PLAN (SAP)

| | |
|---|---|
| **Title** | A Randomized, Placebo-Controlled Phase IIa Trial to Evaluate the Biological Activity, Safety, and Tolerability of Autologous Regulatory T Lymphocytes (Tregs) Expanded Ex-Vivo and Returned Intravenously in Combination with Low-Dose IL-2 in People with Amyotrophic Lateral Sclerosis (ALS) |
| **Project Medical Director** | Stanley H. Appel, MD |
| **Protocol Version** | 9.0, 10 Aug 2020 |
| **SAP Version** | 1.0, 30 July 2021 |

SAP APPROVAL SIGNATURES

DocuSigned by Stanley Appel, MD
Stanley Appel, MD | I approve this document 07/30/2021 | 4:13:03 PM CDT
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07/30/2021

Stanley H. Appel, MD — Date
Overall Project Medical Director and Sponsor Representative

DocuSigned by Jason Thonhoff, MD, PhD
Jason Thonhoff, MD, PhD | I approve this document 07/30/2021 | 4:10:02 PM CDT
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07/30/2021

Jason R. Thonhoff, MD, PhD — Date
HMH Principal Investigator

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DocuSigned by Eric A. Macklin
Eric A. Macklin | I approve this document 07/30/2021 | 1:38:23 PM PDT
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07/30/2021

Eric A. Macklin, PhD — Date
Study Biostatistician

SAP REVISION HISTORY

| Version | Date | Description of Changes |
|---|---|---|
| 1.0 | 30 Jul 2021 | Initial version |

## Table of Contents

# 1. Governing Documents

This statistical analysis plan (SAP) specifies the outcome measures, analysis samples, and planned analyses for the T-Reg IIa trial. The SAP supplements the clinical protocol. Please refer to the clinical protocol for details on the rationale for the intervention, eligibility criteria, conduct of the trial, clinical assessments and the timing of their use in the trial, definitions and reporting of adverse events, data management conventions, and regulatory oversight and compliance procedures. In case of discrepancies between the SAP and the clinical protocol concerning matters of data analysis, the SAP is authoritative. On all other matters, the clinical protocol is authoritative.

# 2. Study Design

## 2.1 Overview

This is a phase IIa, two-period, multicenter trial to evaluate the biological activity, safety, and tolerability of autologous regulatory T lymphocytes (Tregs) expanded ex-vivo and returned intravenously in combination with low-dose IL-2 in people with amyotrophic lateral sclerosis (ALS). Participants in Cohort 1 enrolled in a six-month, two-arm, double-blind, placebo-controlled randomized controlled trial (RCT, Period 1) followed by a six-month open-label extension (OLE, Period 2) with exposure to three dosage levels. Participants in Cohort 2 enrolled directly into the OLE. The trial is registered at Clinicaltrials.gov as study NCT04055623.

## 2.2 Study Objectives

Primary Objective:

- To evaluate the biological activity of IV infusion of an expanded population of autologous regulatory T lymphocytes and low-dose IL-2 in people with ALS by evaluating Treg suppressive function.

Secondary Objectives:

- To evaluate the biological activity of IV infusion of an expanded population of autologous regulatory T-lymphocytes and low-dose IL-2 in people with ALS by evaluating Treg number.
- To evaluate the safety and tolerability of IV infusion of an expanded population of autologous regulatory T lymphocytes and low-dose IL-2 in people with ALS.

Exploratory Objectives:

- To characterize the effects of IV infusion of an expanded population of autologous regulatory T lymphocytes and low-dose IL-2 on clinical outcome measures of ALS, including Appel ALS Rating Scale (AALS) and ALS Functional Rating Scale Revised (ALSFRS-R) scores, forced vital capacity (FVC), maximum inspiratory pressure (MIP), and fasciculations. To assess efficacy, the Combined Assessment of Function and Survival (CAFS) will be evaluated.

## 2.3 Study Population

Individuals eligible for trial participation are men or women at least 18 years old who meet the El Escorial criteria of possible, laboratory-supported probable, probable, or definite for a diagnosis of sporadic or familial ALS, experienced a decline in ALSFRS-R total score of at least two points in the 90 days prior to screening or at least four points over the 180 days prior to screening, possess a forced vital capacity (FVC) at least 65% of that expected based on age, sex, and height, are treatment naïve or on a stable dose of riluzole, edaravone, or both, lack unstable clinical or psychiatric conditions, and were not recently exposed to other investigational treatments. Detailed inclusion and exclusion criteria are specified in the protocol.

Participants will be recruited from two centers that are part of the Northeast ALS (NEALS) Consortium: Houston Methodist Hospital (HMH), Houston, TX and Massachusetts General Hospital (MGH), Boston, MA.

## 2.4 Participant Flow

Cohort 1 participation included screening, leukapheresis, initiation of subcutaneous IL-2 at $2x10^5$ IU/$m^2$/injection or placebo three times weekly, randomization, initiation of monthly Treg intravenous infusions at $1x10^6$ cells/kg/infusion or placebo, follow-up visits every 4 weeks to 24 weeks with additional biospecimen sampling 1 and 7 days post-infusion at selected visits, followed by entry into OLE, initiation of subcutaneous IL-2 at $2x10^5$ IU/$m^2$/injection three times weekly, a dose escalation of monthly Treg infusions at $1x10^6$ cells/kg/infusion for two infusion, at $2x10^6$ cells/kg/infusion for two infusion, and at $3x10^6$ cells/kg/infusion for two infusion, and follow-up visits every 4 weeks to 54 weeks with additional biospecimen sampling 1 and 7 days post-infusion at selected visits.

Cohort 2 participation included screening, leukapheresis, initiation of subcutaneous IL-2 at $2x10^5$ IU/$m^2$/injection three times weekly, a dose escalation of monthly Treg infusions at $1x10^6$ cells/kg/infusion for two infusions, at $2x10^6$ cells/kg/infusion for two infusions, and at $3x10^6$ cells/kg/infusion for two infusions, and follow-up visits every 4 weeks to 28 weeks (corresponding to Week 54 for Cohort 1 participants) with additional biospecimen sampling 1 and 7 days post-infusion at selected visits.

Detailed descriptions of study procedures and timing are specified in the protocol.

## 2.5 Treatment Allocation

Prior to the baseline visit, eligible Cohort 1 participants were randomly allocated in a 1:1 ratio to active or placebo treatment. Randomizations followed a permuted-block schedule, stratified by site. The randomization schedule was prepared by computer program by an unblinded study statistician.

## 2.6 Treatment Administration

Details of treatment administration are described in the protocol.

## 2.7 Allocation Concealment

Allocation to treatment group of Cohort 1 participants is concealed to participants and investigators. The randomization schedule is known only to the unblinded study statistician who generated the schedule and personnel at the central cGMP laboratory responsible for processing

cells harvested by leukapheresis and generating the population of expanded autologous regulatory T lymphocytes and tracking completed activities. An independent Data and Safety Monitoring Board (DSMB) received unblinded data as described in the DSMB Charter. Concealment of the true treatment assignment of specific participants was achieved by using matching placebo syringes containing saline in place of syringes containing IL-2 and using matching infusion bags containing saline, 5% human serum albumin, and 0.2% DMSO in place of infusion bags containing Tregs.

[continued on next page]

## 2.8 Schedule of Assessments

### 2.8.1 Study Table for Period 1 – Randomized Controlled Trial (RCT) – Cohort 1 Only

| | V1 Screening | V2 Leuka-pheresis | V3[13] RCT 1 w/in 8 weeks from screening & 6 weeks of Leukapheresis | | | V4[13] RCT 2 | V5[13] RCT 3 | | V6[13] RCT 4 | V7[13] RCT 5 | V8[13] RCT 6 | | | V9[13] Follow-Up Visit |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Week -8 to -3 | Week -6 to -3 | Infusion Week 0 Day 0 | Week 0 Day 1 +3 Days ^^ | Week 1 Day 0 ±3 Days | Infusion Week 4 ±7 Days | Infusion Week 8 ±7 Days | Week 9 ± 3 Days | Infusion Week 12 ±7 Days | Infusion Week 16 ±7 Days | Infusion Week 20 ±7 Days | Week 20 Day 1 +3 days | Week 21 Day 0 ±3 Days | Week 24 ±7 Days |
| Written Informed consent | X | | | | | | | | | | | | | |
| Eligibility Review | X | X | | | | | | | | | | | | |
| Medical history | X | X | | | | | | | | | | | | |
| Demographics | X | | | | | | | | | | | | | |
| Screening Labs[1] | X | | | | | | | | | | | | | |
| ALS Diagnosis History | X | | | | | | | | | | | | | |
| EI Escorial Criteria | X | | | | | | | | | | | | | |
| Vital Signs, Height and Weight[2] | X | | X | | | X | X | | X | X | X | | | X |
| Physical Exam | X | | | | | | | | | | | | | X |
| Neurological Exam[3] | X | | | | | | | | | | | | | X |
| 12-Lead ECG (Electrocardiogram) | X | | | | | | | | | | | | | X |
| C-SSRS[4] | X | | X | | | X | X | | X | X | X | | | X |
| ALSFRS-R and Appel ALS Scale | X | | X | | | X | X | | X | X | X | | | X |
| FVC and MIP Measurements | X | | X | | | X | X | | X | X | X | | | X |
| Assess Survival | | X | X | X | X | X | X | X | X | X | X | X | X | X |
| Concomitant Medications | X | X | X | X | X | X | X | X | X | X | X | X | X | X |
| Adverse Events | X | X | X | X | X | X | X | X | X | X | X | X | X | X |

| | V1 Screening | V2 Leuka-pheresis | V3[13] RCT 1 w/in 8 weeks from screening & 6 weeks of Leukapheresis | | | V4[13] RCT 2 | V5[13] RCT 3 | | V6[13] RCT 4 | V7[13] RCT 5 | V8[13] RCT 6 | | | V9[13] Follow-Up Visit |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Week -8 to -3 | Week -6 to -3 | Infusion Week 0 Day 0 | Week 0 Day 1 +3 Days ^^ | Week 1 Day 0 ±3 Days | Infusion Week 4 ±7 Days | Infusion Week 8 ±7 Days | Week 9 ± 3 Days | Infusion Week 12 ±7 Days | Infusion Week 16 ±7 Days | Infusion Week 20 ±7 Days | Week 20 Day 1 +3 days | Week 21 Day 0 ±3 Days | Week 24 ±7 Days |
| Safety Labs[5] | X | | X | | | X | X | | X | X | X | | | X |
| Leukapheresis | | X | | | | | | | | | | | | |
| Blood & Urine biomarkers[6] | X | | X+ | X | X | X | X | X | X | X | X | X | X | X |
| Randomization[7] | | X ~ | | | | | | | | | | | | |
| Intravenous Treg or placebo infusion[8] | | | X | | | X | X | | X | X | X | | | |
| Teach IL-2/placebo administration[9] | | X~ | | | | | | | | | | | | |
| First dose of IL-2 or placebo[10] | | X~ | | | | | | | | | | | | |
| Return of IL-2 or placebo supplies | | | X | | | X | X | | X | X | X | | | X |
| Shipping of IL-2 (Pharmacy*) | | X~ | X | | | X | X | | X | X | X | | | |
| Distribute fasciculations diary | X | | X | | | X | X | | X | X | X | | | |
| Review fasciculations diary | | | X | | | X | X | | X | X | X | | | X |
| IP accountability compliance and Diary Review & IL2-re-education | | | X | | | X | X | | X | X | X | | | X |
| Blindedness Questionnaire | | | | | | | | | | | | | | X |

### 2.8.2 Study Table for Period 2 – Open Label Extension (OLE) ^ – Cohorts 1 and 2

| | Optional V10 Leuka-pheresis #2 (if needed) **** | V11 OLE 1 | | | V12 OLE 2 | V13 OLE 3 | | V14 OLE 4 | V15 OLE 5 | V16 OLE 6 | | | V17 OLE 7 | V18 Final Safety Visit |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | □Not needed<br>Week 25 ±7 Days | Week 26 +14 Days ***** | Week 26 Day 1 +3 Days | Week 27 Day 0 ±3 Days | Week 30 ±7 Days | Week 34 ±7 Days | Week 35 Day 0 +3 Days | Week 38 ±7 Days | Week 42 ±7 Days | Week 46 ±7 Days | Week 46 Day 1 +3 Days | Week 47 ±3 Days | Week 50 ±7 Days | End of Participation/End of Study Week 54 ±7 Days |
| Written Informed consent | | | | | | | | | | | | | | |
| Vital Signs, Height and Weight[2] | | X | | | X | X | | X | X | X | | | X | X |
| Physical Exam | | | | | | | | | | | | | | X |
| Neurological Exam[3] | | | | | | | | | | | | | X | X |
| 12-Lead ECG | | | | | | | | | | | | | | X |
| C-SSRS[4] | | X | | | X | X | | X | X | X | | | X | X |
| ALSFRS-R and Appel ALS Scale | | X | | | X | X | | X | X | X | | | X | X |
| FVC and MIP Measurements | | X | | | X | X | | X | X | X | | | X | X |
| Assess Survival | X | X | X | X | X | X | X | X | X | X | X | X | X | X |
| Concomitant Medications | X | X | X | X | X | X | X | X | X | X | X | X | X | X |
| Adverse Events | X | X | X | X | X | X | X | X | X | X | X | X | X | X |
| Safety Labs[5] | X | X | | | X | X | | X | X | X | | | X | X |

| | Optional V10 Leuka-pheresis #2 (if needed) **** | V11 OLE 1 | | | V12 OLE 2 | V13 OLE 3 | | V14 OLE 4 | V15 OLE 5 | V16 OLE 6 | | | V17 OLE 7 | V18 Final Safety Visit |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | □Not needed<br>Week 25 ±7 Days | Week 26 +14 Days ***** | Week 26 Day 1 +3 Days | Week 27 Day 0 ±3 Days | Week 30 ±7 Days | Week 34 ±7 Days | Week 35 Day 0 +3 Days | Week 38 ±7 Days | Week 42 ±7 Days | Week 46 ±7 Days | Week 46 Day 1 +3 Days | Week 47 ±3 Days | Week 50 ±7 Days | End of Participation/End of Study Week 54 ±7 Days |
| Leukapheresis | X | | | | | | | | | | | | | |
| Blood & Urine biomarkers[6] | X | X+ | X | X | X | X | X | X | X | X | X | X | X | X |
| Intravenous Treg infusion[8] | | X | | | X | X | | X | X | X | | | | |
| Teaching of IL-2 administration[9&11] | X | | | | | | | | | | | | | |
| First dose of IL-2[10] | X | | | | | | | | | | | | | |
| Shipping of IL-2 | X | X | | | X | X | | X | X | X | | | | |
| Return of IL-2 supplies | | X | | | X | X | | X | X | X | | | X | X |
| Review fasciculations diary | | X | | | X | X | | X | X | X | | | X | X |
| Drug accountability compliance | | X | | | X | X | | X | X | X | | | | |
| Exit Questionnaire[12] | | | | | | | | | | | | | | X |

1. Screening laboratory tests will be performed locally and include HIV, Hepatitis B, Hepatitis C, Complete Blood Count with Differential, Full Chemistry Panel (Sodium, Potassium, Chloride, Blood Urea Nitrogen, Creatinine, Glucose, Calcium, Aspartate Aminotransferase, Alanine Aminotransferase, Alkaline Phosphatase, Total Bilirubin, Total Protein, Albumin, and Carbon dioxide/bicarbonate), Urine, International Normalized Ratio/Prothrombin Time/Partial Thromboplastin Time, and Pregnancy Test (urine) for Women of Childbearing Potential.

2. Vital signs include systolic and diastolic pressure in mmHg, respiratory rate/minute, heart rate/minute and temperature. Height is only recorded once at the Screening Visit. During Treg infusions, vital signs will be obtained pre-infusion and monitored and recorded once every hour from the infusion start time for up to 4 hours. There is a +/- 10-minute window for each hour vital signs are taken during the infusion.
3. The standard Neurological Exam will be used for all subjects.
4. C-SSRS Screening Version to be completed at Screening Visit only. C-SSRS since Last Visit version to be completed at all other visits.
5. Safety laboratory labs will be performed locally and include Complete Blood Count with Differential, Full Chemistry Panel (Sodium, Potassium, Chloride, Blood Urea Nitrogen, Creatinine, Glucose, Calcium, Aspartate Aminotransferase, Alanine Aminotransferase, Alkaline Phosphatase, Total Bilirubin, Total Protein, Albumin, and Carbon dioxide/bicarbonate), Urinalysis, and Pregnancy Test (urine) for Women of Childbearing Potential.
6. Biomarker blood and urine samples (non-fasting) will be obtained from each participant for biomarker analyses and labeled with a subject identifier code and date at/with:
    - Immediately prior to each infusion at Screening, the final RCT-week 24 visit, OLE-week 50, and the End of Study Visit -week 54;
    - Weeks 0, 20, 26, and 46 - 1 day +3 days & 7 days ± 3 days following the infusion day/date;
    - Week 8 & Week 26 - 7 days ±3 days following the infusion day/date.
    - Specimen collection: (six tubes) 10 mL sodium heparin green tops, (one tube) 10 mL serum red top, (one tube) 4 mL sodium heparin green top, and a urine sample (5 mL to 10 mL).
        - Five (5) 10 mL sodium heparin green tops will be used at the site of collection to perform the Treg Suppression Assay per SOP. Serum will be stored at -80°C at the site of collection per SOP.
        - One (1) 10 mL sodium heparin green top and the 4 mL sodium heparin green top will be shipped overnight at room temperature to HMH for flow cytometry and messenger RNA collection per SOP. Urine specimens will be frozen at -80°C at the site of collection per SOP. All biospecimens will be labeled with a subject identifier code and date.
    - Primary Biomarkers: Treg suppressive function;
    - Secondary biomarkers: Treg numbers by flow cytometry; and
    - Exploratory biomarkers: Banking of urine, serum, plasma, and messenger RNA.
    - These samples will be collected and stored at -80°C. Banked samples will be analyzed after the study has completed.
    - After all analyses have been completed, the remaining samples will be transported to the NEALS biorepository.
    + Participants will also return to their enrollment sites 1 day (+3 days) and 7 days (± 3 days) after the first infusion of the RCT and OLE (Week 0, Week 20, Week 26 and week 46) to collect the same amount of blood and urine for post-infusion analyses of blood biomarkers.
7. Randomization will occur after the Leukapheresis procedure is completed within 7 days.
8. Safety monitoring & TReg or matching placebo infusion: For the RCT, 4 hours post-infusion monitoring is required for the first infusion but is otherwise optional at the discretion of the Investigator. For the OLE, 4 hours post-infusion monitoring is required following the first infusion. Post-infusion monitoring for at least 1 hour is then required after each escalating Treg dose in the OLE but is otherwise optional at the discretion of the Investigator. Following each infusion, the PI or study staff will provide a follow up phone call within 24 to 48 hours to query subjects for symptoms of a delayed anaphylaxis response.

9. Il-2 or Matching Placebo: The teaching of IL-2 subcutaneous administration will be provided to all participants following the manufacturer's directions on the days of leukapheresis. The dispensation of IL-2 or placebo and diary will take place at the enrollment site. For additional syringe dispensation, IL-2 and placebo for subjects will be shipped directly to the subjects from the HMH or MGH, respectively. Sites will provide the subjects with a sharps container and instructions on discarding used syringes and returning unused syringes with their IL-2/Placebo dosing diary to the next visit.
10. During RCT and OLE, IL-2 or placebo will begin after Leukapheresis and will start as early as 4 weeks or at least 2 weeks prior to the first Treg infusion. IL-2/placebo will be administered subcutaneously 3 times per week and then discontinued 4 weeks after the last Treg infusion of the RCT (Week 24) and at the final visit for the OLE phase (Week 50).
11. For MGH patients, teaching of IL-2 will occur at screening.
12. If performed as part of an early termination visit before the V9 Week 24 Follow-Up Visit, the Exit Questionnaire will include the Blindedness Questionnaire. If performed as part of an early termination visit after the V9 Week 24 Follow-Up Visit or as part of the V18 Week 54 Final Safety Visit, the Exit Questionnaire will not include the Blindedness Questionnaire.
13. RCT applies to Cohort 1 only

**~** These activities will only apply to HMH patients.

* IL-2 or matching placebo administration is ongoing throughout the study and not associated with a study-specific visit.

** Any study visit that cannot be done in person will be done by phone, collecting as much safety data as possible.

*** Early termination visits for randomized subjects who have received at least one dose of investigational product; where possible perform safety labs, physical and neurological exam, vital signs, ECG, AE, and SAE assessment, collect IL-2 or placebo syringes and materials and collection of fasciculation diary.

**** Second leukapheresis will be performed ONLY if Treg collection from initial leukapheresis is insufficient.

***** OLE 1 for Cohorts 1 & 2 must occur at least 14 days after participant has initiated IL-2 dosing for the OLE phase.

^ For subjects in Cohort 1, visit windows for the OLE are to be calculated from Week 24 of the RCT. For subjects in Cohort 2, visit windows for the OLE are to be calculated from the Week 26 infusion (i.e., the Week 26 V11 OLE1 will be Day 0). Visit windows for visits following infusions are relative to the date of the infusion.

# 3. General Considerations for Data Analysis

## 3.1 Statistical Software

Statistical analyses will be performed using SAS (SAS Institute, NC, USA) or R (R Foundation for Statistical Computing, Vienna, Austria).

## 3.2 Summary Statistics

Data will be summarized with respect to disposition, demographics, pre-treatment characteristics, efficacy endpoints, safety endpoints, and tolerability. Summary statistics for continuous variables will include the number of observations, the mean, median, standard deviation, inter-quartile range, and range. Summaries of categorical data will include counts, denominators, and percentages.

## 3.3 Precision

Results will generally be reported to 3 significant figures. Percentages will generally be reported to 0.1 percentage points. P-values will be reported to two digits when greater than or equal to 0.095, to three digits when greater than or equal to 0.00095 and less than 0.095, and as <0.001 for all smaller values.

## 3.4 Transformations

Data that are strictly positive, continuous, and strongly right skewed will typically be log-transformed prior to any inferential testing. Skewness greater than 3 will be used as a guide in determining which variables to transform. Original, untransformed values will be used for all summaries.

## 3.5 Multiplicity Adjustments

A single primary endpoint, 24-week change in Treg suppression function during Period 1, is specified and will be tested at two-tailed $p < 0.05$. A single secondary endpoint of biological activity, 24-week change in Treg cell number during Period 1, will be tested at two-tailed $p < 0.05$ using a closed testing procedure to maintain an overall two-tailed family-wise error rate less than 5%. Tests of clinical response are exploratory. Nominal comparison-wise p-values will be reported for all analyses.

## 3.6 Missing Data

If baseline values of a given measure are missing, the last observed pre-treatment value will be used. Missing baseline covariates will be imputed using the mean of the respective covariate. Off-schedule results, e.g., those collected as part of an Early Termination Visit, will be used in place of the closest missing scheduled visit (while preserving visit sequence) for analyses that depend on visit-specific data. Other results will not be carried forward.

The planned mixed model analyses, where applied, yield estimates that are unbiased conditional on the observed values under a missing at random assumption.

# 4. Study Endpoints

## 4.1 Endpoints of Biological Activity

- Change in Treg suppressive function in the blood from baseline to week 24 during Period 1 (overall primary endpoint) and from start to finish of Period 2 and each dosage level during Period 2, and
- Change in Treg numbers in the blood from baseline to week 24 during Period 1 (overall key secondary endpoint) and from start to finish of Period 2 and each dosage level during Period 2.

## 4.2 Exploratory Clinical Endpoints

- Change in Appel ALS Rating Scale (AALS) score from baseline to week 24 during Period 1 and from start to finish of Period 2,
- Change in ALS Functional Rating Scale Revised (ALSFRS-R) from baseline to week 24 during Period 1 and from start to finish of Period 2 and each dosage level during Period 2,
- Change in forced vital capacity (FVC) and maximum inspiratory pressure (MIP) from baseline to week 24 during Period 1 and from start to finish of Period 2 and each dosage level during Period 2,
- Tracheostomy-free survival between baseline and week 24 during Period 1 and from start to finish of Period 2 and each dosage level during Period 2,
- Proportion of days free of fasciculations between baseline and week 24 during Period 1 and from start to finish of Period 2 and each dosage level during Period 2, and
- Combined Assessment of Function and Survival from baseline to 1-year after each participant's first Treg infusion.

## 4.3 Safety and Tolerability Endpoints

- Safety: Defined as the occurrence of adverse events (AEs), serious adverse events (SAEs), treatment-emergent adverse events (TEAEs), and clinically significant abnormalities in clinical and laboratory values.
- Tolerability: Defined as the percentage of participants who complete the 6-month RCT (RCT Tolerability) and who complete the 6-month OLE and each ascending dose on study treatment (OLE Tolerability).

# 5. Measurement Definitions

## 5.1 AALS

The Appel ALS Rating Scale (AALS, Appel et al. 1987) is a 19-item clinician-completed instrument for assessing participant function in five groups: bulbar, respiratory, muscle strength, lower extremity function, and upper extremity function. Each group is scored based on one to six individual assessments (bulbar = 2, respiratory = 1, muscle strength = 4, lower extremity function = 6, upper extremity function = 6). Group scores are calculated as the sum of all items

within the group. A group score of six (6) points indicates no dysfunction, and group scores of 30 to 36 points (maximum group score: bulbar = 30, respiratory = 30, muscle strength = 36, lower extremity function = 35, upper extremity function = 33) indicate maximal dysfunction. The AALS total score is the sum of all items (range 30 for healthy subjects to 164 for those with maximum impairment). Any missing item causes the corresponding group score and the total score to be missing.

## 5.2 ALSFRS-R

The ALS Functional Rating Scale Revised (ALSFRS-R, Cedarbaum et al. 1999) is a 12-item instrument completed by clinician interview for assessing participant function in four domains: bulbar, fine motor, gross motor, and respiratory. Each item is scored from 0 to 4 with higher scores indicating greater function. The ALSFRS-R total score is the sum of all items (range 0 to 48). Domain scores are the sum of their respective items (range 0 to 12): bulbar function = Q1-Q3, fine motor function = Q4-Q6, gross motor function = Q7-Q9, and respiratory function = QR1-QR3 (Q10-Q12). Any missing item causes the domain score and the total score to be missing.

The average rate of disease progression prior to the Baseline Visit as measured by ALSFRS-R (delta-FRS) will be calculated as 48 minus the ALSFRS-R total score measured at the Baseline Visit, that difference divided by the number of months from onset of symptom weakness due to ALS to the date of the Baseline Visit (with months calculated as days x 12 / 365.25).

## 5.3 FVC

Forced vital capacity (FVC) is the maximum volume of air that can be forcefully exhaled after maximal inhalation. Trained technicians coach participants through 3 to 5 maneuvers using a spirometer. A minimum of 2 completed FVC maneuvers are required to calculate an estimate of maximum FVC for a given participant visit. The maximum volume expired is converted to percent of predicted normal using normal values calculated based on age, sex, age, and height (Knudson et al. 1983). Higher values indicate greater respiratory function.

## 5.4 MIP

Maximum inspiratory pressure (MIP) is the maximal pressure achieved by inspiration in a seated position. Trained technicians coach participants through 3 to 5 maneuvers using a spirometer. A minimum of 2 completed MIP maneuvers are required to calculate an estimate of maximum MIP for a given participant visit. MIP is reported in units of cm $H_2O$. Higher values indicate better pulmonary function.

## 5.5 Survival

Survival will be measured by time to the earlier of death or tracheostomy. Follow-up for analysis of survival during Period 1 among participants who do not die or receive a tracheostomy prior to the Week 24 Visit will be censored at the Week 24 Visit, if completed, the date of consent withdrawal, if withdrawn, or the last date at which vital status is known prior to the end of the Week 24 Visit window for participants lost to follow-up. Follow-up for analysis of survival during Period 2 among participants who do not die or receive a tracheostomy prior to the Week 54 Visit will be censored at the Week 54 Visit, if completed, the date of consent withdrawal, if

withdrawn, or the last date at which vital status is known prior to the end of the Week 54 Visit window for participants lost to follow-up.

# 6. Statistical Methodology

## 6.1 Analysis Sets

Two analysis sets will be used for summaries and analyses of biological activity, safety, and tolerability:

- RCT Set: Cohort 1 participants who were eligible, randomized during Period 1, and received at least one double-blind Treg infusion, and
- OLE Set: Cohort 1 and 2 participants who were eligible and received at least one open-label Treg infusion.

## 6.2 Baseline Characterization

Each analysis set will be summarized overall and by treatment group in the RCT set and by cohort and treatment group (for Cohort 1) in the OLE set. Baseline characteristics of both the RCT and OLE sets are those assessed at the Screening Visit. The following characteristics will be summarized: enrollment site; age, sex, race, ethnicity, weight, El Escorial diagnosis, years since ALS symptom onset, delay between symptom onset and ALS diagnosis, site of ALS symptom onset, use of noninvasive ventilation, use of riluzole, use of edaravone, Treg suppression function, Treg cell number, AALS total score, ALSFRS-R total score, delta-FRS, FVC, and MIP.

## 6.3 Biological Activity and Clinical Endpoints

### 6.3.1 Primary Analysis Model

Treatment groups will be compared for progression of continuous endpoints (Treg suppressive function, Treg cell number, AALS total score, ALSFRS-R total score, FVC, and MIP) during Period 1 using a shared-baseline, linear mixed model in the RCT set. The model will include fixed terms for discrete visit and treatment group × post-screening visit interaction and random participant-level intercepts and slopes with unstructured covariance. Omitting a treatment-group main effect enforces a shared-baseline assumption. Use of a shared baseline reflects the true state of the population sampled prior to randomization and has the advantage of adjusting for any chance differences at baseline and increasing efficiency in a manner similar to analysis of covariance (Liang and Zeger, 2000). To enforce the shared-baseline assumption, the interaction between treatment group and visit will be restricted to post-baseline visits by including a numeric indicator variable (0 pre-treatment, 1 post-treatment) in the interaction.

The following SAS code specifies the model:

```
proc mixed data=xxx method=reml; class regimen id
    trtrnd visit;
    model Value = visit post*trtrnd*visit / solution; random intercept month /
    subject=id type=un;
```

where `id`is a participant study identifier, `trtrnd`is the randomly assigned treatment group, `visit`is the visit identifier, `Value`is value of the efficacy endpoint being tested for a given

participant at a given visit, postis the indicator of visits occurring after baseline, dFRSis delta-FRS centered at the sample median, and monthis time in months from the Screening Visit (assuming 12 months in an average of 365.25 days per year). The primary estimate will be the treatment-dependent difference in change from the Screening Visit to the Week 24 Visit. The estimate and its 95% Wald confidence bounds will be obtained by a linear contrast of adjusted means. The following SAS code specifies the linear contrast for Treg suppressive function assessed at the Screening Visit and 12 follow-up visits assuming that the sort order for treatment group has the active group last and visits are sorted chronologically:

```
estimate "3|Act vs Plb|dWk 24"
   post*trtrnd*visit 0 0 0 0 0 0 0 0 0 0 0 0 -1  0 0 0 0 0 0 0 0 0 0 0 0 0 1 / cl;
```

A significant positive difference in 24-week change would support inference that the infusion of autologous T regulatory cells improved Treg suppressive function over 24 weeks.

### 6.3.2 OLE Analysis Model

Progression of continuous endpoints (Treg suppressive function, Treg cell number, AALS total score, ALSFRS-R total score, FVC, and MIP) during Period 2 will be estimated as a single group using a linear mixed model in the OLE set. The model will include fixed terms for discrete visit and random participant-level intercepts and slopes with unstructured covariance. Data from the Screening Visit will be used as the start of follow-up for both Cohort 1 and 2 participants, imputed at the time of the Week 24 Visit for Cohort 1 participants. Estimates of change from the Screening Visit and comparisons between dosage levels will be obtained by linear contrasts of adjusted means of applicable visits.

### 6.3.3 Survival

The proportion of participants who die or receive a tracheostomy will be summarized overall and by treatment group in the RCT set and by cohort and treatment group (for Cohort 1) in the OLE set. Kaplan-Meier product-limit estimates with complimentary log-log confidence bounds will be estimated in the RCT set at 24 weeks (end of Period 1), 34 weeks (end of OLE $1x10^6$ cells/kg/infusion dosage interval), 42 weeks (end of OLE $2x10^6$ cells/kg/infusion dosage interval), 50 weeks (end of OLE $3x10^6$ cells/kg/infusion dosage interval), and 54 weeks (end of Period 2). Time to death or tracheostomy will be compared between treatment group in the RCT set by log-rank test.

### 6.3.4 Fasciculations

The proportion of days per week with no fasciculations, fasciculations only after exertion, fasciculations at rest or after exertion, and fasciculations that substantially interfere with daily activities will be summarized as means and standard deviations by treatment group and week in the RCT and OLE sets.

### 6.3.5 CAFS

To account for potentially informative loss to follow-up due to death, treatment groups will be compared by the Combined Assessment of Function and Survival (CAFS, Berry et al. 2013) in the RCT set to the end of Period 1. CAFS is a joint-rank test (Finkelstein and Schoenfeld 1999) of a composite endpoint combining function as measured by change in ALSFRS-R total scores and time to death or tracheostomy. The test consists of calculating a rank-sum score for each individual relative to pair-wise comparisons with all other participants. Participants are ranked

according to time to death or tracheostomy when that is observed for both members of a pair or when one is censored after the observed event time for the other. Pairs that cannot be ranked by time to death or tracheostomy are ranked by absolute change from baseline in ALSFRS-R total score at the maximum follow-up time at which both participants have an observation. Inference of treatment-dependent difference in the hierarchical composite of death and disease progression is drawn by calculating a U-statistic from the rank-sum scores and testing as a Z-score.

### 6.3.6 Correlations between Biological Activity and Clinical Endpoints

Changes in measures of biological activity (Treg suppressive function and Treg cell number) and measures of clinical progression (AALS total score, ALSFRS-R total score, FVC, and MIP) will be compared by Spearman correlation. The correlation of change from the Screening Visit to the Week 24 Visit will be estimated among participants in the RCT set as a single group. The correlation of change from the Screening Visit to the Week 54 Visit will be estimated among all participants in the OLE set as a single group.

## 6.4 Safety and Tolerability Summaries

### 6.4.1 Treatment-emergent Adverse Events

The incidence of TEAEs will be summarized by the number of events of a given classification experienced by participants in each treatment group and by the number and proportion of participants experiencing such an event in each treatment group in the RCT and OLE sets. TEAEs will be summarized by MedDRA system organ class and preferred term for all TEAEs and all serious TEAEs.

Aggregate summaries of TEAE characteristics in the RCT and OLE samples will include: (a) seriousness, (b) severity, (c) relationship to IL-2/placebo injection, (d) action taken with respect to IL-2/placebo, (e) relationship to Treg infusion, (e) action taken with respect to Treg infusion, (f) use of a concomitant medication, and (g) outcome. For each level of a given TEAE characteristic, summaries will include the number of events of a given classification and the number and proportion of participants for which that level of a characteristic was the worst they experienced. Any unknown characteristic of a TEAE will not be classified as worst. The level for participants who experienced no TEAE will be coded as the zero-level of each characteristic.

### 6.4.2 Safety Labs

The absolute level and the absolute change from baseline for each safety laboratory assay will be summarized as means, standard deviations, medians, and ranges at each visit by treatment group in the RCT and OLE sets. The proportion of participants with abnormal and clinically significant safety lab levels will be summarized by treatment group by visit and at any post-baseline visit in all safety samples.

### 6.4.3 Vital Signs and Weight

The absolute level and the absolute change from baseline for vital signs and weight will be summarized as means, standard deviations, medians, and ranges at each visit by treatment group in the RCT and OLE sets.

## 6.5 Other Summaries

### 6.5.1 Participant Disposition

The number of Cohort 1 participants consented to the study, failed screening, determined eligible, completing leukapheresis, randomized, initiated on IL-2/placebo study drug, receiving an initial Treg/placebo infusion, completed scheduled Period 1 follow up, prematurely terminated study participation due to TEAE, withdrawal of consent, or administrative termination, and entered into the OLE will be summarized overall and by treatment group for the RCT set. The number of OLE participants initiated on IL-2, completing leukapheresis for Period 2 participation, receiving an initial Treg infusion, completed scheduled Period 2 follow up, and prematurely terminated study participation due to TEAE, withdrawal of consent, or administrative termination will be summarized overall and by cohort for the OLE set. Reasons for screen failure and for withdrawal from study will be presented.

### 6.5.2 Tolerance

Tolerability will be defined for Period 1 as the percentage of participants who complete the 6-month RCT on study treatment. RCT Tolerability will be compared between treatments in the RCT set. Tolerability will be defined for Period 2 as the percentage of participants who complete the 6-month OLE on study treatment. OLE Tolerability will be compared between cohorts in the OLE set.

### 6.5.3 Concomitant Medication Use

Concomitant medications taken during the study period will be coded using the World Health Organization (WHO) Drug Dictionary Enhanced and Default Anatomical, Therapeutic, and Chemical (ATC) class. The percentage of participants who initiate a concomitant medication after first Treg/placebo infusion will be summarized by WHO drug classified medication and each ATC class of medications overall and by treatment group in the RCT set and overall and by cohort in the OLE set.

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| Stephanie Berry<br>SMBERRY@PARTNERS.ORG<br>PM<br>Stephanie Berry<br>Security Level: Email, Account Authentication (Required)<br>**Electronic Record and Signature Disclosure:**<br>Accepted: 8/24/2020 4:21:51 AM<br>ID: 5608aae2-4c1f-4510-a07a-1648108fee4e | COPIED | Sent: 7/30/2021 1:36:08 PM<br>Viewed: 7/30/2021 1:43:06 PM |

| Witness Events | Signature | Timestamp |
|---|---|---|

| Notary Events | Signature | Timestamp |
|---|---|---|

| Envelope Summary Events | Status | Timestamps |
|---|---|---|

| Envelope Summary Events | Status | Timestamps |
| --- | --- | --- |
| Envelope Sent | Hashed/Encrypted | 7/30/2021 1:36:08 PM |
| Certified Delivered | Security Checked | 7/30/2021 2:12:42 PM |
| Signing Complete | Security Checked | 7/30/2021 2:13:30 PM |
| Completed | Security Checked | 8/2/2021 5:50:31 AM |

| Payment Events | Status | Timestamps |
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eTable 1: Baseline Clinical Characteristics of Participants in the Randomized Controlled Trial

| | | | Treatment Group | | |
|---|---|---|---|---|---|
| Variable | Level | Overall | Placebo | Treg/IL-2 | P-value |
| Participants | N | 7 (100.0%) | 4 (100.0%) | 3 (100.0%) | |
| Site | 701: MGH | 3 (42.9%) | 2 (50.0%) | 1 (33.3%) | 1.000 |
| | 702: HMH | 4 (57.1%) | 2 (50.0%) | 2 (66.7%) | |
| Sex | Female | 1 (14.3%) | 0 (0.0%) | 1 (33.3%) | 0.429 |
| | Male | 6 (85.7%) | 4 (100.0%) | 2 (66.7%) | |
| Race | White | 7 (100.0%) | 4 (100.0%) | 3 (100.0%) | |
| Ethnicity | Non-Hispanic or Latino | 7 (100.0%) | 4 (100.0%) | 3 (100.0%) | |
| El Escorial | 1 = Clinically Possible | 1 (14.3%) | 1 (25.0%) | 0 (0.0%) | 1.000 |
| | 3 = Clinically Probable | 5 (71.4%) | 3 (75.0%) | 2 (66.7%) | |
| | 4 = Clinically Definite | 1 (14.3%) | 0 (0.0%) | 1 (33.3%) | |
| Onset site | Bulbar | 1 (14.3%) | 1 (25.0%) | 0 (0.0%) | 1.000 |
| | Limb | 6 (85.7%) | 3 (75.0%) | 3 (100.0%) | |
| Riluzole use | No | 3 (42.9%) | 2 (50.0%) | 1 (33.3%) | 1.000 |
| | Yes | 4 (57.1%) | 2 (50.0%) | 2 (66.7%) | |
| Edaravone use | No | 6 (85.7%) | 3 (75.0%) | 3 (100.0%) | 1.000 |
| | Yes | 1 (14.3%) | 1 (25.0%) | 0 (0.0%) | |
| NIV use | No | 6 (85.7%) | 3 (75.0%) | 3 (100.0%) | 1.000 |
| | Yes | 1 (14.3%) | 1 (25.0%) | 0 (0.0%) | |
| Age at screening (yrs) | | 49.8±6.7 (39.9,61.9) | 52.5±6.8 (46.2,61.9) | 46.2±5.6 (39.9,50.3) | 0.251 |
| Weight (kg) | | 81.8±17.0 (54.5,101) | 89.3±14.8 (68.3,101) | 71.8±16.6 (54.5,87.5) | 0.2 |
| Months since symptom onset | | 32.7±14.8 (17.7,59.5) | 30.5±11.2 (17.7,44.4) | 35.6±21.0 (19.9,59.5) | 0.689 |
| Diagnostic delay (months) | | 16.9±12.4 (5.7,42.0) | 12.9±5.6 (8.0,19.7) | 22.2±18.3 (5.7,42.0) | 0.37 |
| Treg suppressive function (%) | | 28.0±15.9 (1.4,46.1) | 26.3±18.3 (1.4,45.1) | 30.2±15.8 (14.5,46.1) | 0.78 |
| Treg cell number (%) | | 3.90±1.84 (1.70,7.50) | 4.45±2.38 (1.70,7.50) | 3.17±0.55 (2.60,3.70) | 0.411 |
| Appel ALS Total score | | 63.7±14.9 (47.0,86.0) | 60.0±15.5 (47.0,82.0) | 68.7±15.5 (56.0,86.0) | 0.497 |
| Appel ALS Bulbar | | 6.86±227 (6.00,12.0) | 7.50±3.00 (6.00,12.0) | 6.00±0.00 (6.00,6.00) | 0.437 |
| Appel ALS Respiratory | | 9.43±3.21 (6.00,12.0) | 9.00±3.46 (6.00,12.0) | 10.0±3.46 (6.00,12.0) | 0.721 |
| Appel ALS Muscle Strength | | 18.4±6.73 (6.00,28.0) | 15.8±6.65 (6.00,21.0) | 22.0±6.00 (16.0,28.0) | 0.257 |
| Appel ALS Lower Extremity | | 15.3±9.01 (7.00,30.0) | 16.0±10.5 (7.00,30.0) | 14.3±8.74 (7.00,24.0) | 0.833 |
| Appel ALS Upper Extremity | | 13.7±4.61 (10.0,23.0) | 11.8±1.50 (10.0,13.0) | 16.3±6.51 (10.0,23.0) | 0.219 |
| ALSFRS-R Total score | | 36.6±2.8 (33.0,40.0) | 35.8±2.6 (33.0,38.0) | 37.7±3.2 (34.0,40.0) | 0.423 |
| ALSFRS-R Bulbar | | 10.4±2.88 (4.00,12.0) | 9.50±3.70 (4.00,12.0) | 11.7±0.58 (11.0,12.0) | 0.371 |
| ALSFRS-R Fine motor | | 7.71±2.63 (5.00,12.0) | 8.50±2.52 (6.00,12.0) | 6.67±2.89 (5.00,10.0) | 0.41 |

| | | | | | |
|---|---|---|---|---|---|
| ALSFRS-R Gross motor | | 7.57±3.10 (4.00,12.0) | 7.75±3.50 (4.00,12.0) | 7.33±3.21 (5.00,11.0) | 0.878 |
| ALSFRS-R Respiratory | | 10.9±121 (9.00,12.0) | 10.0±0.82 (9.00,11.0) | 12.0±0.00 (12.0,12.0) | 0.009 |
| FVC (max %-predicted) | | 91.2±18.2 (74.0,120) | 92.7±20.7 (74.0,120) | 89.3±18.4 (75.9,110) | 0.834 |
| MIP (max cm $H_20$) | | 69.0±35.0 (12.0,119) | 71.0±45.5 (12.0,119) | 66.3±23.6 (44.0,91.0) | 0.879 |

eTable 2: Summary of Treatment-Emergent Adverse Events by System Organ Class in the Randomized Controlled Trial

| System Organ Class / Preferred Term | Placebo N=4 (%) | Treg/IL-2 N=4 (%) | Overall N=8 (%) |
|---|---|---|---|
| Any TEAE | 4 (100) | 3 (75) | 7 (88) |
| Immune System Disorders | 1 (25) | 0 | 1 (13) |
| Anaphylactic Reaction | 1 (25) | 0 | 1 (13) |
| Eye Disorders | 1 (25) | 0 | 1 (13) |
| Eye Inflammation | 1 (25) | 0 | 1 (13) |
| Gastrointestinal Disorders | 2 (50) | 0 | 2 (25) |
| Gastroesophageal Reflux Disease | 1 (25) | 0 | 1 (13) |
| Salivary Hypersecretion | 1 (25) | 0 | 1 (13) |
| General Disorders and Administration Site Conditions | 2 (50) | 2 (50) | 4 (50) |
| Injection Site Bruising | 1 (25) | 0 | 1 (13) |
| Injection Site Discomfort | 0 | 1 (25) | 1 (13) |
| Injection Site Erythema | 0 | 2 (50) | 2 (25) |
| Injection Site Pain | 1 (25) | 0 | 1 (13) |
| Injection Site Pruritus | 1 (25) | 0 | 1 (13) |
| Oedema Peripheral | 1 (25) | 0 | 1 (13) |
| Swelling | 1 (25) | 0 | 1 (13) |
| Infections and Infestations | 0 | 2 (50) | 2 (25) |
| Upper Respiratory Tract Infection | 0 | 1 (25) | 1 (13) |
| Urinary Tract Infection | 0 | 1 (25) | 1 (13) |
| Injury, Poisoning and Procedural Complications | 2 (50) | 1 (25) | 3 (38) |
| Exposure to Toxic Agent | 0 | 1 (25) | 1 (13) |
| Fall | 1 (25) | 1 (25) | 2 (25) |
| Post-Procedural Hematoma | 0 | 1 (25) | 1 (13) |
| Skin Abrasion | 1 (25) | 0 | 1 (13) |

| | | | |
|---|---|---|---|
| Musculoskeletal and Connective Tissue Disorders | 1 (25) | 2 (50) | 3 (38) |
| Arthralgia | 1 (25) | 1 (25) | 2 (25) |
| Bursitis | 0 | 1 (25) | 1 (13) |
| Limb Discomfort | 0 | 1 (25) | 1 (13) |
| Myalgia | 0 | 2 (50) | 2 (25) |
| Nervous System Disorders | 3 (75) | 1 (25) | 4 (50) |
| Dizziness | 0 | 1 (25) | 1 (13) |
| Dysarthria | 1 (25) | 0 | 1 (13) |
| Dysgeusia | 1 (25) | 0 | 1 (13) |
| Syncope | 1 (25) | 0 | 1 (13) |
| Psychiatric Disorders | 0 | 1 (25) | 1 (13) |
| Anxiety | 0 | 1 (25) | 1 (13) |
| Renal and Urinary Disorders | 1 (25) | 0 | 1 (13) |
| Urine Odor Abnormal | 1 (25) | 0 | 1 (13) |
| Respiratory, Thoracic and Mediastinal Disorders | 1 (25) | 1 (25) | 2 (25) |
| Respiratory Alkalosis | 0 | 1 (25) | 1 (13) |
| Rhinorrhea | 1 (25) | 0 | 1 (13) |
| Skin and Subcutaneous Tissue Disorders | 2 (50) | 1 (25) | 3 (38) |
| Dermatitis Contact | 0 | 1 (25) | 1 (13) |
| Erythema | 1 (25) | 0 | 1 (13) |
| Pruritus | 1 (25) | 0 | 1 (13) |
| Rash Maculo-Papular | 1 (25) | 0 | 1 (13) |
| Vascular Disorders | 1 (25) | 0 | 1 (13) |
| Peripheral Coldness | 1 (25) | 0 | 1 (13) |

eTable 3: Baseline Clinical Characteristics of Participants in the Open-Label Extension

| | | | Cohort | | |
|---|---|---|---|---|---|
| Variable | Level | Overall | Participants from RCT | Participants only in OLE | P-value |
| Participants | N | 8 (100.0%) | 6 (100.0%) | 2 (100.0%) | |
| Site | 701: MGH | 3 (37.5%) | 3 (50.0%) | 0 (0.0%) | 0.464 |
| | 702: HMH | 5 (62.5%) | 3 (50.0%) | 2 (100.0%) | |
| Sex | Female | 1 (12.5%) | 1 (16.7%) | 0 (0.0%) | 1.000 |
| | Male | 7 (87.5%) | 5 (83.3%) | 2 (100.0%) | |
| Race | White | 8 (100.0%) | 6 (100.0%) | 2 (100.0%) | |
| Ethnicity | Non-Hispanic or Latino | 8 (100.0%) | 6 (100.0%) | 2 (100.0%) | |
| El Escorial | 1 = Clinically Possible | 1 (12.5%) | 1 (16.7%) | 0 (0.0%) | 1.000 |
| | 3 = Clinically Probable | 5 (62.5%) | 4 (66.7%) | 1 (50.0%) | |
| | 4 = Clinically Definite | 2 (25.0%) | 1 (16.7%) | 1 (50.0%) | |
| Onset site | Bulbar | 1 (12.5%) | 1 (16.7%) | 0 (0.0%) | 1.000 |
| | Limb | 7 (87.5%) | 5 (83.3%) | 2 (100.0%) | |
| Riluzole use | No | 2 (25.0%) | 2 (33.3%) | 0 (0.0%) | 1.000 |
| | Yes | 6 (75.0%) | 4 (66.7%) | 2 (100.0%) | |
| Edaravone use | No | 7 (87.5%) | 5 (83.3%) | 2 (100.0%) | 1.000 |
| | Yes | 1 (12.5%) | 1 (16.7%) | 0 (0.0%) | |
| NIV use | No | 6 (75.0%) | 5 (83.3%) | 1 (50.0%) | 0.464 |
| | Yes | 2 (25.0%) | 1 (16.7%) | 1 (50.0%) | |
| Age at screening (yrs) | | 52.8±10.2 (39.9,71.3) | 47.8±4.3 (39.9,52.2) | 68.0±4.8 (64.6,71.3) | 0.001 |
| Weight (kg) | | 85.9±16.3 (54.5,103) | 84.0±17.4 (54.5,101) | 91.7±16.0 (80.4,103) | 0.603 |
| Months since symptom onset | | 30.1±14.5 (17.7,59.5) | 32.6±16.2 (17.7,59.5) | 22.6±2.8 (20.6,24.6) | 0.442 |
| Diagnostic delay (months) | | 15.8±12.0 (4.0,42.0) | 18.4±12.8 (5.7,42.0) | 8.0±5.7 (4.0,12.1) | 0.331 |
| Treg suppressive function (%) | | 33.4±18.1 (1.4,56.5) | 28.1±17.5 (1.4,46.1) | 49.1±10.5 (41.6,56.5) | 0.171 |
| Treg cell number (%) | | 3.60±1.86 (1.70,7.50) | 3.82±2.00 (1.70,7.50) | 2.95±1.77 (1.70,4.20) | 0.609 |
| Appel ALS Total score | | 66.0±15.0 (47.0,86.0) | 64.5±16.1 (47.0,86.0) | 70.5±14.8 (60.0,81.0) | 0.661 |
| Appel ALS Bulbar | | 7.13±2.23 (6.00,12.0) | 7.00±2.45 (6.00,12.0) | 7.50±2.12 (6.00,9.00) | 0.807 |
| Appel ALS Respiratory | | 9.00±3.21 (6.00,12.0) | 10.0±3.10 (6.00,12.0) | 6.00±0.00 (6.00,6.00) | 0.134 |
| Appel ALS Muscle Strength | | 19.9±6.81 (6.00,28.0) | 18.5±7.37 (6.00,28.0) | 24.0±2.83 (22.0,26.0) | 0.362 |
| Appel ALS Lower Extremity | | 13.5±8.67 (7.00,30.0) | 14,8±9.79 (7.00,30.0) | 9.50±2.12 (8.00,11.0) | 0.494 |
| Appel ALS Upper Extremity | | 16.5±6.65 (10.0,29.0) | 14.2±4.88 (10.0,23.0) | 23.5±7.78 (18.0,29.0) | 0.081 |
| ALSFRS-R Total score | | 36.1±2.9 (33.0,40.0) | 37.0±2.8 (33.0,40.0) | 33.5±0.7 (33.0,34.0) | 0.150 |
| ALSFRS-R Bulbar | | 10.5±2.73 (4.00,12.0) | 10.3±3.14 (4.00,12.0) | 11.0±1.41 (10.0,12.0) | 0.790 |
| ALSFRS-R Fine motor | | 6.88±2.85 (4.00,12.0) | 7.67±2.88 (5.00,12.0) | 4.50±0.71 (4.00,5.00) | 0.192 |

| | | | | | |
|---|---|---|---|---|---|
| ALSFRS-R Gross motor | | 8.00±2.83 (4.00,12.0) | 7.83±3.31 (4.00.12.0) | 8.50±0.71 (8.00,9.00) | 0.797 |
| ALSFRS-R Respiratory | | 10.8±1.75 (7.00,12.0) | 11.2±0.98 (10.0,12.0) | 9.50±3.54 (7.00,12.0) | 0.275 |
| FVC (max %-predicted) | | 87.3±12.6 (74.0,110) | 86.4±14.2 (74.0,110) | 90.0±9.4 (83.3,96.6) | 0.759 |
| MIP (max cm $H_20$) | | 72.6±33.4 (12.0,119) | 70.0±38.3 (12.0,119) | 80.5±17.7 (68.0,93.0) | 0.731 |

eTable 4: Summary of Treatment-Emergent Adverse Events by System Organ Class in the Open-Label Extension

| System Organ Class / Preferred Term | Treg/IL-2<br>N=8 (%) |
|---|---|
| Any TEAE | 8 (100) |
| Eye Disorders | 1 (13) |
| Lacrimation Increased | 1 (13) |
| Gastrointestinal Disorders | 3 (38) |
| Abdominal Discomfort | 1 (13) |
| Constipation | 2 (25) |
| Nausea | 1 (13) |
| General Disorders and Administration Site Conditions | 4 (50) |
| Chills | 1 (13) |
| Fatigue | 2 (25) |
| Injection Site Bruising | 1 (13) |
| Injection Site Erythema | 2 (25) |
| Injection Site Pruritus | 1 (13) |
| Injection Site Reaction | 1 (13) |
| Injection Site Urticaria | 1 (13) |
| Infections and Infestations | 1 (13) |
| Coronavirus Infection | 1 (13) |
| Injury, Poisoning and Procedural Complications | 5 (63) |
| Arthropod Bite | 1 (13) |
| Contusion | 2 (25) |
| Eye Contusion | 1 (13) |
| Fall | 2 (25) |
| Skin Abrasion | 3 (38) |
| Skin Laceration | 1 (13) |
| Investigations | 1 (13) |
| Alanine Aminotransferase Increased | 1 (13) |

| | |
|---|---|
| Musculoskeletal and Connective Tissue Disorders | 5 (63) |
| Back Pain | 2 (25) |
| Muscle Atrophy | 1 (13) |
| Muscle Spasms | 2 (25) |
| Muscle Weakness | 1 (13) |
| Myalgia | 1 (13) |
| Nervous System Disorders | 5 (63) |
| Cervical Radiculopathy | 1 (13) |
| Dysgeusia | 2 (25) |
| Headache | 2 (25) |
| Hypoesthesia | 1 (13) |
| Paresthesia | 1 (13) |
| Respiratory, Thoracic and Mediastinal Disorders | 3 (38) |
| Hypercapnia | 1 (13) |
| Nasal Congestion | 2 (25) |
| Skin and Subcutaneous Tissue Disorders | 3 (38) |
| Dermatitis Contact | 1 (13) |
| Hyperhidrosis | 1 (13) |
| Seborrhea | 1 (13) |
| Skin Odor Abnormal | 1 (13) |
| Vascular Disorders | 1 (13) |
| Hypotension | 1 (13) |