

First-in-human study of high-dose gp96 in ALS: Safety and preliminary immunological effects

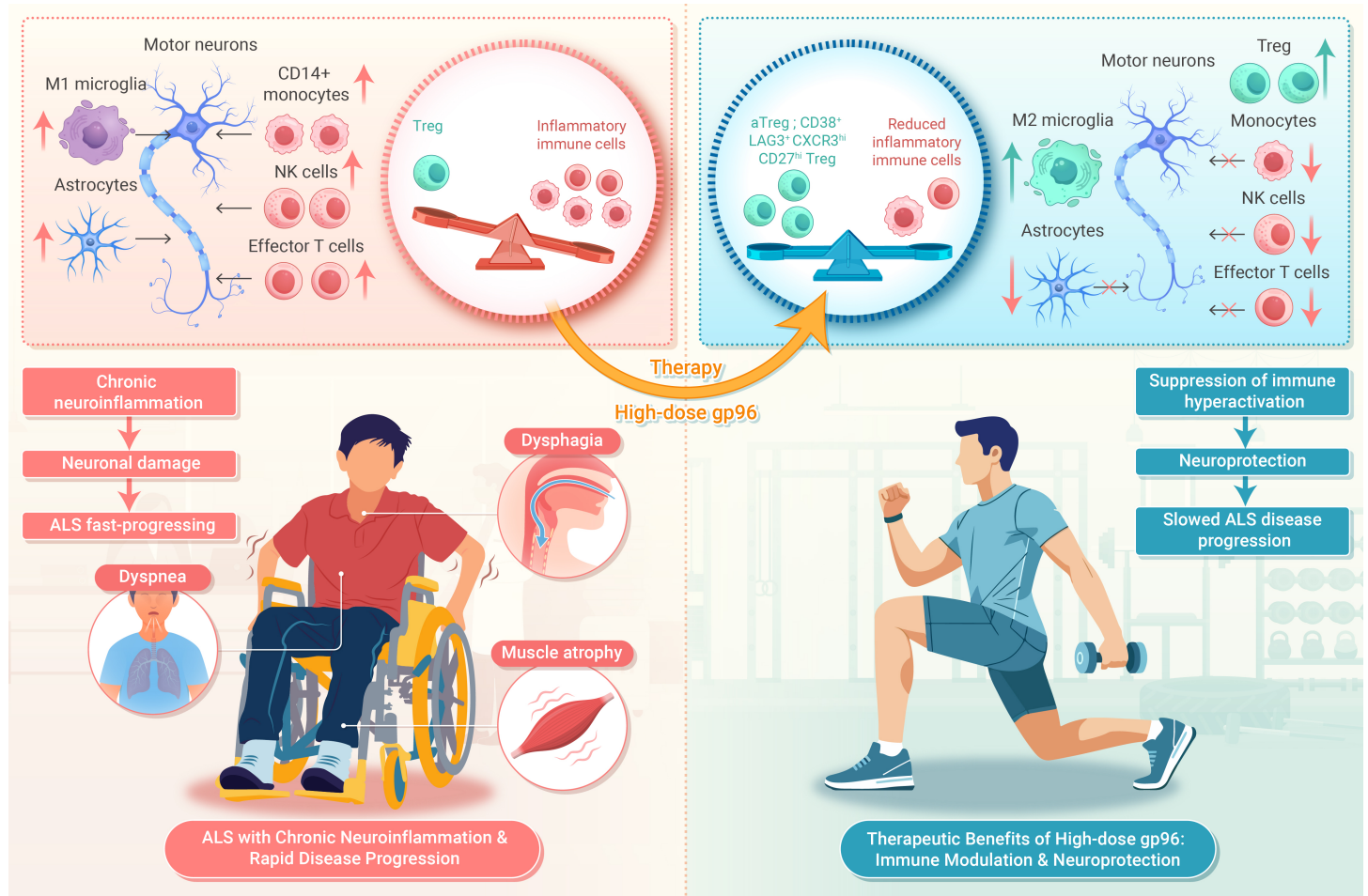
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GRAPHICAL ABSTRACT



PUBLIC SUMMARY

- High-dose gp96 suppresses inflammatory CD14 monocytes, natural killer (NK) cells, and T cells in ALS patients.
- gp96 boosts regulatory T cells (Treg) and activated Treg (aTreg), enhancing their suppressive function.
- 40% (4/10) of patients showed slower ALS Functional Rating Scale-Revised (ALSFRS-R) decline with gp96 therapy.
- gp96 efficacy links to Treg activation and reduced innate and effector T cell activity.

First-in-human study of high-dose gp96 in ALS: Safety and preliminary immunological effects

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Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease associated with immune dysregulation. Based on the robust therapeutic effect and immunoregulatory capability of gp96 administration in the SOD1G93A mouse model, we conducted a prospective one-arm, open-label, proof-of-concept clinical trial in Hainan, China. High-dose gp96 was well tolerated in all 10 enrolled patients with ALS, and pronouncedly reduced the levels of inflammatory immune cells—CD14⁺ monocytes, NK cells, and CD4⁺ and CD8⁺ effector T cells—to levels comparable to those seen in healthy individuals. Treatment with gp96 also enhanced the suppressive function of Tregs and promoted the expansion of both activated Tregs and the CD38⁺LAG3⁺CXCR3^{hi}CD27^{hi} Treg subset. All participants experienced a slower decline in ALSFRS-R scores during the first 16 weeks post-treatment, and this slower decline was sustained in 40% (4/10) of patients at the end of follow-up in week 72. Treatment duration and Treg activation were positively correlated with the efficacy of gp96 treatment in alleviating the disease progression rate. These preliminary results emphasize the importance of the early identification of fast-progressing ALS patients and the need for long-term treatment. Our findings support further evaluation of gp96 as an immunomodulatory agent against ALS in randomized controlled trials with standardized outcome measures.

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disorder characterized by the progressive loss of motor neurons in the brain and spinal cord, leading to muscle deterioration, paralysis, and, typically, death within 3–5 years. Studies have established a strong link between ALS and neuroinflammation or immune dysfunction.^{1–4} Early ALS is marked by neuroinflammation, with activated microglia and astrocytes releasing pro-inflammatory molecules that cause motor neuron death.^{5–7} Several studies have suggested that ALS may even involve autoimmune processes, where antigen-dependent T cells and other immune components mistakenly attack healthy motor neurons, contributing to a decline in motor function.^{8,9} Immune cells, including T cells and macrophages, infiltrate the spinal cord and brain, worsening neuroinflammation and neuronal damage.^{8–12} Interestingly, similar patterns of immune imbalance have been documented in several other neurodegenerative diseases.¹² Besides, some genetic mutations associated with ALS, such as those in the superoxide dismutase (*SOD1*), *TDP43*, and *C9orf72* genes, have been linked to immune system dysfunction and neuroinflammation.^{13–15} These mutations promote protein misfolding and aggregation, leading, in turn, to the activation of microglia and astrocytes, which typically exhibit dysregulation of the cGAS/STING pathway and the NLRP3 inflammasome.

Emerging evidence supports that regulatory T cells (Tregs) play a crucial role in maintaining immune homeostasis and preventing autoimmunity, as well as in neuroinflammation and ALS disease progression.^{16–20} Treg numbers and Foxp3 expression are reduced in patients with rapidly progressing ALS, and these decreases correlate with immune dysregulation and increased inflammation. Moreover, defects in Treg function are linked to disease severity. In contrast, a high frequency of activated Tregs (aTregs) and an elevated aTreg-to-resting Treg (rTreg) ratio have been validated as robust prognostic biomarkers for disease progression in patients with ALS. Studies involving ALS mouse models and clinical trials have shown that increasing Treg numbers and function can suppress abnormal immune activation, delay disease progression, and improve motor function.^{18–20} These findings highlight the potential of Tregs as both biomarkers of immune status and potential therapeutic targets in ALS.

Heat shock protein gp96 is a molecular chaperone essential for protein folding and quality control. In addition, it contributes to immune responses, including the suppression of autoimmunity, by activating Tregs.^{21–27} We and others have demonstrated that the administration of gp96 leads to Treg activation in a dose-dependent manner. In mice, treatment with high-dose gp96 significantly increases the frequency of Tregs and enhances their suppressive capacity, while largely abrogating T cell-mediated hepatic injury.^{24,25} High-dose gp96 demonstrated therapeutic benefits in mouse models of systemic lupus erythematosus and experimental autoimmune encephalomyelitis. These effects were achieved by binding to TLR1/2 on Tregs or CD91 on plasmacytoid dendritic cells (pDCs), thereby activating Tregs and promoting Foxp3 expression.^{26,27} However, Treg activation following gp96 administration has not yet been assessed in a clinical study of ALS or any other neuroinflammatory or autoimmune disease.

Several clinical trials have evaluated the safety and effectiveness of autologous tumor-derived gp96 as an active specific immunotherapy in cancer patients.²⁸ Building on this, we undertook a prospective, one-arm, open-label clinical trial to evaluate the safety of human placenta-derived gp96 in ALS. Our primary goal was to determine, within a small pilot study, whether high-dose gp96 therapy could activate Tregs and inhibit the hyperactivation of innate and cellular immunity in the context of ALS, while maintaining an acceptable safety profile. The integration of disease progression rate and immune target response or gp96 treatment duration in our trial design offered a novel approach to control for ALS disease heterogeneity. The results are expected to inform the development of innovative immunotherapeutic strategies for ALS.

MATERIALS AND METHODS

Preparation of gp96

Human placenta-derived gp96 were purified by ConA-Sepharose affinity

chromatography followed by anion exchange chromatography human placenta tissue, according to a previous report.²⁹

Participants in this study

This single-arm, single-center open-label trial of high-dose gp96 included ALS patients at BOAO EVERGRANDE INTERNATIONAL HOSPITAL from 2 August 2022 to 18 March 2024 in China. The study was designed for ALS adults of less than 70 years old with probable or laboratory-supported probable or definite ALS as defined by Gold Coast diagnostic criteria. The main inclusion criteria consisted of a disease duration of less than 3 years, all ALSFRS-R scores of more than 2 points (≥ 2), and respiratory function items of more than 3 points (≥ 3) before enrollment. ALSFRS-R decreased by 1–4 points (≥ 1 and ≤ 4) within 3 months of the screening period, and respiratory function before enrollment forced vital capacity (%FVC) $\geq 70\%$. All participants were required to provide signed informed consent before being included in the study. Patients with severe pulmonary insufficiency or hepatic function impairment, acute myocardial infarction, heart failure, other serious primary diseases of the nervous system, heart, lungs, hematopoietic system or endocrine system, and psychosis, pregnant or lactating women were excluded, as well as those who were suspected or had a history of alcohol or drug abuse.

Procedures

Upon inclusion by an investigating physician, baseline assessments were performed, which included blood biochemistry, routine examination, clotting function tests, erythrocyte sedimentation rate (ESR), thyroid function tests, immunologic detections, pulmonary function tests, routine urine tests, tumor markers detection, cerebrospinal fluid (CSF) tests, ALS Functional Rating Scale Revised (ALSFRS-R), molecular genetic tests, magnetic resonance imaging scan, electrocardiogram, and electromyography. Following enrollment (a week before the first administration on day 0), ten participants started a 15-week cycle of once-weekly subcutaneous injections of 200 μg of placental gp96 protein. The 15-week cycles were repeated twice continuously, for a total of two cycles of treatment per participant. After the second cycle, five participants chose to continue another two cycles of the open-label extension treatment. All ten participants were followed up for safety monitoring and therapeutic effect at the 18th month.

Placental gp96 protein was prepared at Foshan Heat Shock Therapeutics Laboratory, a good manufacturing practice-compliant facility. Gp96 protein was extracted from placenta of healthy pregnant women who were detected negative for common infectious diseases (hepatitis B virus, hepatitis C virus, human immunodeficiency virus, treponema pallidum, hepatitis A virus, cytomegalovirus, and Epstein-Barr virus) by ConA-Sepharose affinity and anion exchange chromatography as previously described.²⁹ Placental donation and collection were approved by the Committee (Ethics permit number ChiCTR2400089654), and written consents were obtained from all the pregnant donors. The purity of gp96 protein was above 95% as determined by SDS-PAGE and HPLC analysis. The concentration of endotoxin was lower than 1 EU/mg protein, measured by Dynamic Turbidimetry assay.

Primary endpoints

This clinical study obtained safety data on the use of high-dose gp96 in treating ALS. Adverse events (AEs), including any events that occurred during the clinical trial period, total treatment-emergent adverse events (TEAEs) during the administration period of placental gp96, serious TEAEs, TEAEs that resulted in death, TEAEs that led to the discontinuation of placental gp96, and TEAEs related to gp96 administration (side effects), were included in safety assessments.

Efficacy of high-dose gp96 injection in the treatment of ALS was obtained. ALSFRS-R scores were recorded every four weeks during treatment and follow-up periods.

Secondary endpoints

To explore immunoregulatory activity of subcutaneous injections of high-dose gp96 protein in the treatment of ALS, detection of Tregs, Th17 and Th1/Th2 cells and single-cell RNA sequencing were conducted. The immunogenicity of the gp96 injection was determined, and the level of anti-

body against the gp96 protein in the blood was measured.

Data collection

Epidemiological, clinical and laboratory data were reviewed from electronic medical records, and data were entered into a standardized collection form. The data were reviewed by a trained team of physicians. The final date of follow-up was Mar 18th, 2024. The researchers filled the collected patient data into the designated case report form (CRF).

Single-cell library sequencing

Ten PBMC samples were utilized for single-cell sequencing. Beads, each carrying a unique molecular identifier (UMI) and a cell barcode, were loaded near saturation into Gel Beads-in-emulsion (GEM), ensuring each cell was paired with a bead. Upon exposure to cell lysis buffer, polyadenylated RNA molecules hybridized to the beads. The beads were then collected into a single tube for reverse transcription, during which each cDNA molecule was tagged at the 3' end—corresponding to the 5' end of a messenger RNA transcript—with a UMI and a cell label denoting its origin. This process utilized 10x beads and included second-strand cDNA synthesis, adaptor ligation, and universal amplification. Sequencing libraries were prepared from randomly fragmented whole-transcriptome amplification products to enrich the 3' ends of transcripts associated with the cell barcode and UMI. All subsequent steps, including library construction, adhered to the Chromium Single Cell 3' v3.1 standard protocol. The libraries were quantified using a High Sensitivity DNA Chip on a Bioanalyzer 2100 and the Qubit High Sensitivity DNA Assay (Thermo Fisher Scientific). Sequencing was conducted on an Illumina Xplus platform using 2x150 chemistry, facilitated by Majorbio Co., Ltd (Shanghai, China).

Statistics

FACS data were collected and processed using FACS software (FlowJo). GraphPad Prism 6 software was used to analyze data using a two-tailed Student's t-test, Wilcoxon rank-sum test or two-way ANOVA.

Descriptive statistical analyses included the calculation of means, standard deviations, medians, and interquartile ranges for continuous variables and frequencies and proportions for categorical variables. To assess the change from baseline in the ALSFRS-R score, paired-samples t-tests were performed at each follow-up time point. To control for type I error due to multiple comparisons, all *P* values were corrected using the Bonferroni method. In addition, to quantify the effect size, we calculated Cohen's *d* for each time point as compared with baseline. According to Cohen's criteria, $|d| \geq 0.2$, ≥ 0.5 , and ≥ 0.8 represent small, medium, and large effect sizes, respectively.

A linear mixed-effects model was used to analyze the changes in ALSFRS-R score over time and the influence of related factors. We used the lme4 and lmerTest packages to build models that took into account Time and key clinical covariates. Baseline percentage of predicted FVC (BFVCP), baseline FRS-R score (BFRSR), exposure duration of treatment (EXDRU), age (AGE), disease duration (DUR), and ALS subtype or location classification (ALSL) were included. To account for interindividual differences, the model incorporated subject-specific random intercepts and random slopes, allowing for variation in baseline status and rate of disease progression within each subject. The statistical significance level was set at a two-sided $\alpha = 0.05$. All analysis results were presented as regression coefficients (β), standard errors, *t*-values, and corresponding *P*-values. To visually interpret the magnitude of the fixed effect, we also calculated and reported the 95% confidence intervals. To visually present the trend of ALSFRS-R scores over time, we plotted individual subject trajectories and superimposed the average trajectory curve of all subjects. All analyses were performed using R software (version 4.4.2, R Foundation for Statistical Computing, Vienna, Austria).

RESULTS

High-dose gp96 treatment activated Tregs, reduced central nervous system inflammation, and ameliorated disease in SOD1^{G93A} mice

In this study, gp96 was extracted from human placenta using ammonium sulfate precipitation, ConA-Sepharose affinity chromatography, and anion exchange chromatography.²⁹ The resulting preparation routinely yielded gp96

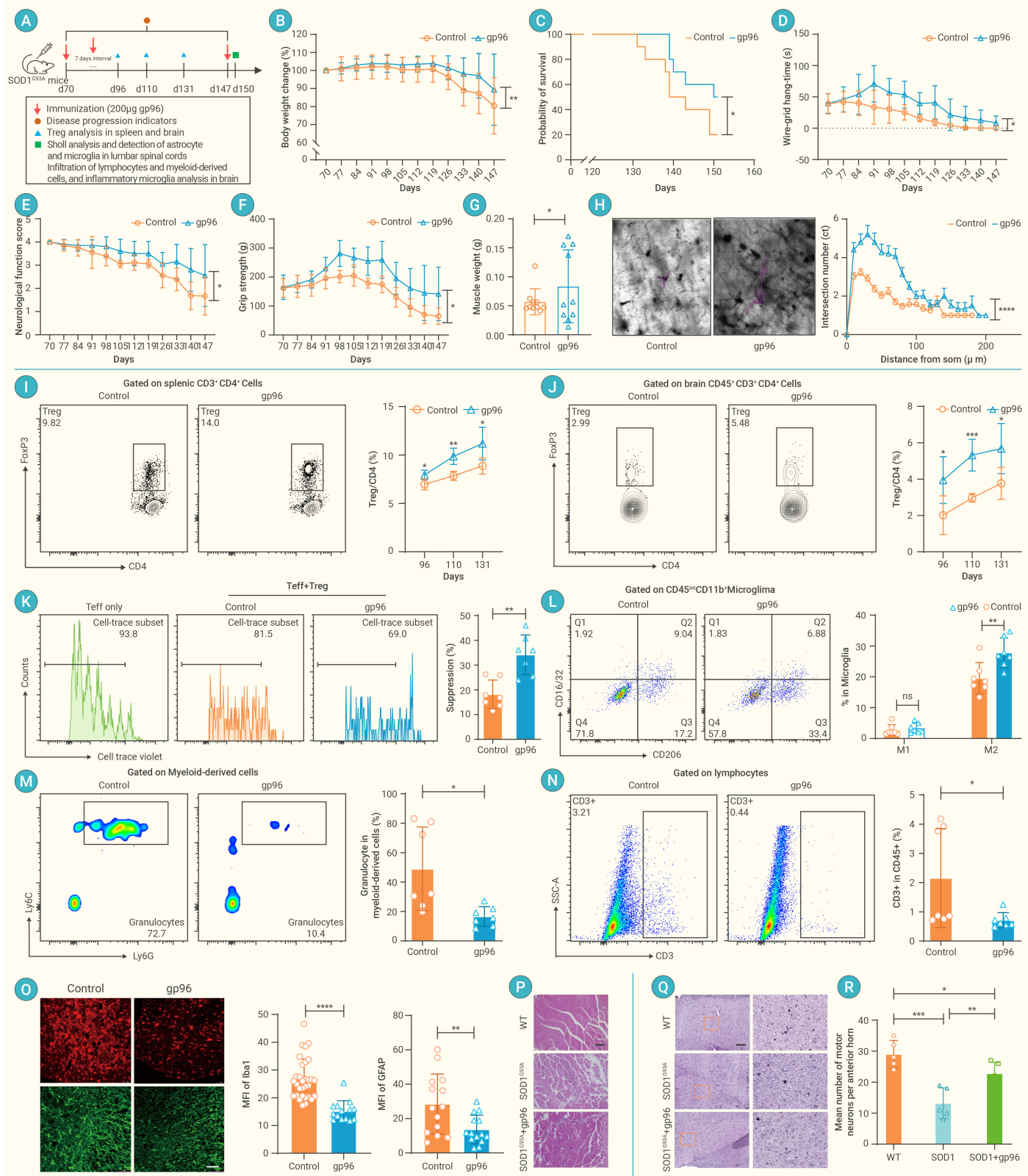


Figure 1. Effect of high-dose gp96 treatment on Treg activation, inflammatory astrocyte and microglia, and disease progression in SOD1^{G93A} mice Male SOD1^{G93A} mice were injected subcutaneously with 200 μg of gp96 or saline (control) weekly starting at the age of 70 days (n=10/group). (A) Experimental design. (B-F) Mice body weight (B) and survival time (C) were monitored weekly from day 70 to day 147. Wire grid hang analysis (D), neurological function detection (E), and grip strength tests (F) were performed. (G) Weight of the gastrocnemius muscle at day 150. (H) Neuronal cell complexity quantification by Sholl analysis at day 150. (I-J) FACS analysis of Treg cell populations in mice spleen (I) and brain (J) (n=5/group) at indicated days. (K) Treg suppression analysis at day 110. A total of 1 × 10⁵ cell trace violet-labeled CD4⁺CD25⁺T (Teff) cells were cultured with Tregs at a ratio of 1:1 for 3 days. Teff cell division cycle was measured using FACS (left). Suppression rate of proliferation was calculated (right). (L-N) FACS analyses of the microglia subsets (L), myeloid cell subsets (M) and CD3⁺T lymphocytes (N) in brain single cell suspensions (n=7/group). (O) Immunofluorescence analysis of Iba1-positive microglia cells (upper, red) and GFAP-positive astrocytes (lower, green) in the whole lumbar spinal cords of SOD1^{G93A} mice. Bar, 20 μm. Mean ± SD is shown. (P) The representative graph of gastrocnemius cross-sectional area by H&E staining. (Q and R) The representative image of motor neuron in spinal cord by Nissl staining (Q) and quantitative analysis (R). Bar, 100 μm. Mean ± SD is shown. Statistical significance was determined using Two-Way ANOVA or two-tailed unpaired t test. Ns, no significant, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

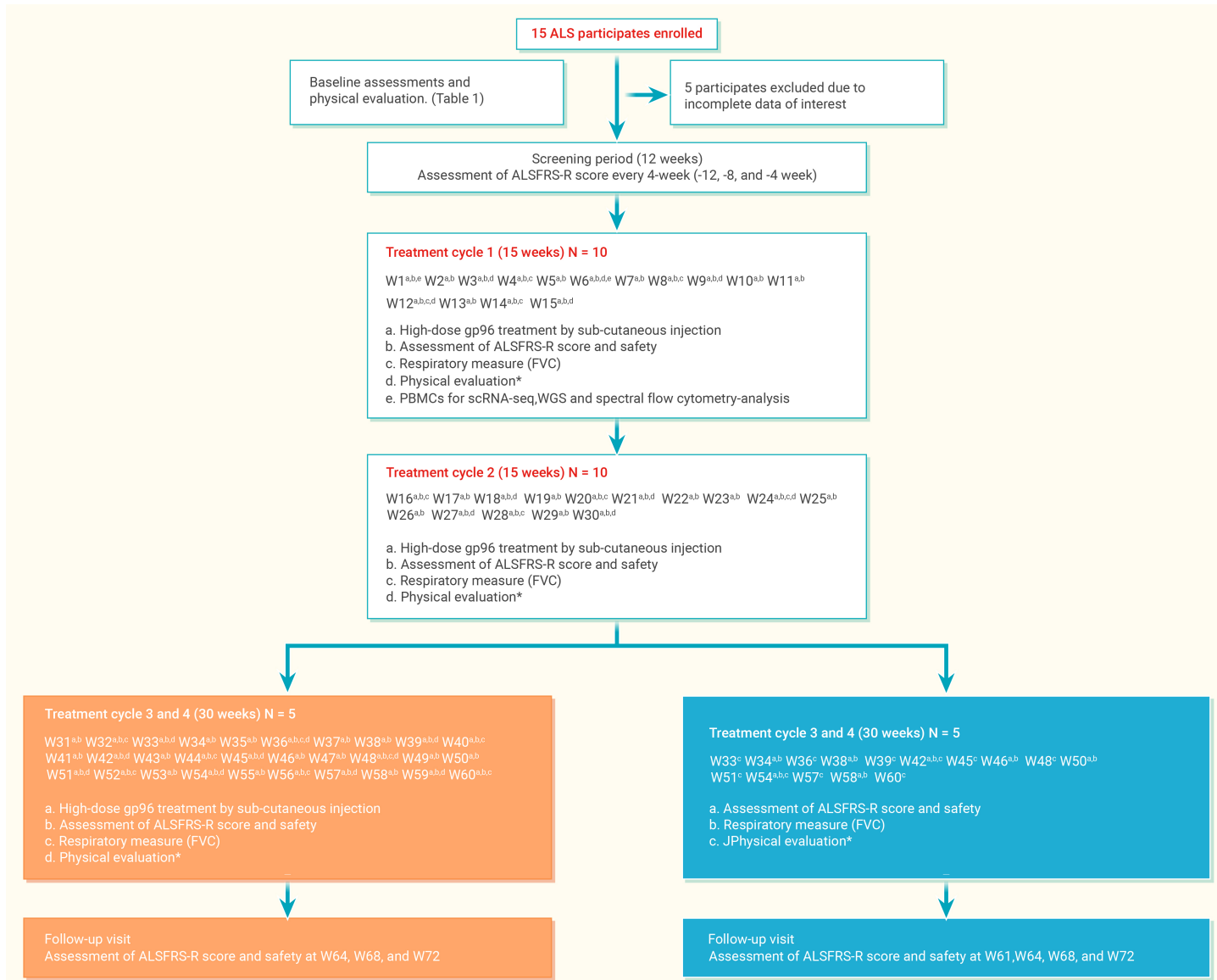


Figure 2. Trial profile ALS: Amyotrophic Lateral; N: Number of subjects; PBMCs: Peripheral Blood Mononuclear Cells; scRNA-seq: Single-cell RNA sequencing.

protein with a purity greater than 90%, as determined by SDS-PAGE and reverse-phase HPLC (Figure S1).

SOD1^{G93A} mice were used as a working model to assess the effectiveness of high-dose gp96 therapy for ALS.³⁰ Our previous study showed that gp96 administration led to a dose-dependent increase in the frequency of Foxp3⁺ Tregs, with significant Treg expansion observed under high-dose (200 or 400 µg/mouse) gp96 treatment.²⁶ Here, SOD1 mice were subcutaneously injected with 200 µg of gp96 weekly starting at the age of 70 days, which corresponds to disease onset (Figure 1A). Compared to controls, gp96-treated mice had significantly greater body weight (Figure 1B) and longer survival time (Figure 1C). Additionally, muscle strength and motor neuron function were assessed using the wire grid hang test (Figure 1D), neurological function scores (Figure 1E), and grip strength tests (Figure 1F). These tests all demonstrated that gp96 treatment effectively slowed the progression of ALS symptoms. Furthermore, the weight of the gastrocnemius muscle was greater in gp96-treated mice than in untreated controls at the end of the experiment (Figure 1G). Sholl analysis of imaged neurons revealed that dendrite length and the number of branch nodes on basal dendrites of neurons were increased in gp96-treated mice relative to control animals (Figure 1H).

Under gp96 treatment, the proportion of CD4⁺Foxp3⁺ Tregs was significantly increased in both the peripheral immune organ (spleen), and the

central nervous system (brain, (Figures 1I–J)). Flow cytometric analysis revealed that gp96 treatment primarily increased the levels of effector Treg subsets (by 43% in cluster 1 and 64% in cluster 2), characterized by elevated expression levels of ICOS, CD44, TIGIT, and/or KLRG1 (Figures S2A–C).³¹ Compared to control animals, Tregs from gp96-treated SOD1 mice exerted greater inhibitory effects on effector T cell (Teff) proliferation *ex vivo* (Figure 1K). Conversely, the infiltration of total CD45⁺ lymphocyte and myeloid-derived cells in the brain was significantly decreased following gp96 treatment (Figures S2D–E). The proportion of anti-inflammatory M2 microglia was increased in gp96-treated mice, accompanied by reductions in the proportions of granulocytes among myeloid-derived cells and CD3⁺ cells among CD45⁺ lymphocytes (Figures 1L–N). Gp96 treatment also led to a significant decline in the numbers of both Iba1-positive microglia and GFAP-positive astrocytes (Figure 1O) in the spinal cord, indicating that neuroinflammation had been suppressed. Furthermore, gp96 treatment contributed to the preservation of neuromuscular integrity and led to an increase in the number of Nissl bodies in spinal motor neurons (Figures 1P–R), indicating that gp96 administration led to the attenuation of neuronal damage. Mechanistically, *in vitro* experiments showed that gp96 treatment promoted Treg proliferation (Figure S2F) and suppressive activity (Figure S2G). Importantly, these effects were almost abolished when cells were co-incubated with a TLR2-neutralizing antibody, suggesting that gp96 activates Tregs primarily through the

Table 1. Demographic and clinical baseline characteristics of the study participants.

	Patients of the study cohort (n = 10)
Age (years)	
Mean (SD)	45.7 (13.45)
Median (range)	44.5 (31–70)
Sex (female)-no. (%)	2 (20%)
BMI	
Mean (SD)	23.15 (15.07)
Median (range)	24.65 (14.69–27.34)
Disease duration (months)	
Mean (SD)	23 (13.15)
Median (range)	15 (13–45)
Familial form-no. (%)	1 (10%)
Site of onset-no. (%)	
Limb	9 (90%)
Bulbar	1 (10%)
ALS stage-no. (%)	
I	0
II	2 (20%)
III	6 (60%)
IV	2 (20%)
V	0
Riluzole or edaravone use-no. (%)	
Riluzole	6 (60%)
Edaravone	4 (40%)
Both	4 (40%)
Other interventions-no. (%)	
Chinese traditional medicine	4 (40%)
Massage	2 (20%)
Acupuncture	1 (10%)
ALSFRS-R score at onset	
Mean (SD)	35.7 (9.59)
Median (range)	37.5 (9–42)
Changes in ALSFRS-R scores in the 3-month screening period	
Mean (SD)	–2.6 (1.04)
Median (range)	–3 (–1 to –4)
FVC in ml (range)	3125 (2190–4990)
ppFVC in % (range)	89.35 (50.7–106.9)
Supportive care	
IV	0
NIV	1 (10%)

BMI = body mass index; FVC = forced vital capacity; ppFVC = percentage of predicted FVC; ALSFRS-R = Amyotrophic Lateral Sclerosis Functional Rating Scale-Revised; IV = invasive ventilation; NIV = non-invasive ventilation.

TLR1/2 signaling pathway, consistent with prior mechanistic models.²⁶

Trial participants

From September 1 to 13, 2022, a total of 15 patients with ALS were assessed for inclusion in the study. Five were excluded due to incomplete ALSFRS-R score data, which was assessed every 4 weeks during the 12-week screening period (Figure 2). The remaining 10, all of whom had a 1- to 4-point decrease in ALSFRS-R scores, received two cycles of weekly high-dose gp96 injections, with five continuing into an open-label extension phase. Participant details are listed in Table 1. At trial entry, 60% of the participants were receiving riluzole and 40% were receiving edaravone.

Safety and tolerability during ALS patient treatment

We and other groups have observed, through titration of the gp96 dose (0, 0.5, 5, 10, 20, 50, 100, and 200 µg), that treatment with 10–20 µg (low doses) of gp96 elicited the strongest CTL response in mice. In contrast, when the immunization dose was increased to 50–100 µg (high doses), the CTL response was markedly decreased, accompanied by Treg induction and activation.^{24,26,27} In clinical trials, a comparable dose of 25 µg of tumor-derived gp96 preparations has generally been used to induce antitumor T cell immune responses.^{32,33} Accordingly, we selected a dose of 200 µg for the induction of Treg cells in this clinical trial.

No drug-related serious adverse events (SAEs) occurred during the entire high-dose gp96 administration period or follow-up. Only 1 (10%) non-serious adverse event (NSAE) was reported, which was a slight fever. No elevation of anti-gp96 antibodies was detected in the blood of any participant following gp96 treatment.

Treatment with high-dose gp96 suppressed innate immunity hyperactivation in participants with ALS

Single-cell RNA sequencing (scRNA-seq) was employed to assess changes in peripheral blood mononuclear cell (PBMC) composition, gene expression, and the immune repertoire before and following high-dose gp96 treatment. A total of 120,562 cells were captured from the 15 samples, including pre- and post-gp96 treatment samples from five randomly selected participants and five publicly available datasets from healthy individuals³⁴ with an age distribution (20 to 60 years) comparable to that of the ALS patients in this study; 119,454 cells remained after quality control. We identified 11 cell types based on marker gene expression (Figures 3A–B). Following gp96 treatment, the proportions of most cell types, particularly monocytes and NK cells, shifted towards the distribution observed in healthy controls, a change that correlates with the restoration of immune homeostasis (Figure 3C). Specifically, notable differences in the ratios of CD14⁺ monocytes and NK cells were observed between pre- and post-treatment samples, as suggested by a more refined Milo algorithm (Figures 3D–E). Correlation analysis revealed that genes that were differentially expressed between healthy controls and ALS participants were positively correlated with those showing differential expression between post- and pre-gp96 treatment in participants with ALS across most cell types (Figures 3F–G). Pathways related to innate immunity were upregulated in ALS participants relative to those in healthy controls (Figure S3A). GO pathway enrichment analysis of the differentially expressed genes (DEGs) revealed that pathways involved in cell activation and cytokine production were downregulated in innate immune cells of both healthy controls and ALS participants following gp96 treatment, with the most pronounced effects detected in CD14⁺ monocytes and NK cells (Figures 3H–I & S3B). Finally, flow cytometric analysis of PBMCs from ALS participants identified sharp decreases in the proportions of CD14⁺ (classical) monocytes, CD14⁺CD16⁺ (intermediate) monocytes, and NK cells in ALS participants following gp96 treatment (Figures 3J–K).

Treatment with high-dose gp96 suppressed monocyte hyperactivation in ALS participants

Despite distinct transcriptional signatures between participants with ALS and healthy controls, ALS participants who received gp96 treatment and healthy controls shared transcriptional signatures in all monocyte subsets (Figure S4A). Analysis of the enriched functional modules among the DEGs in the various cell types indicated that the shared downregulation module was

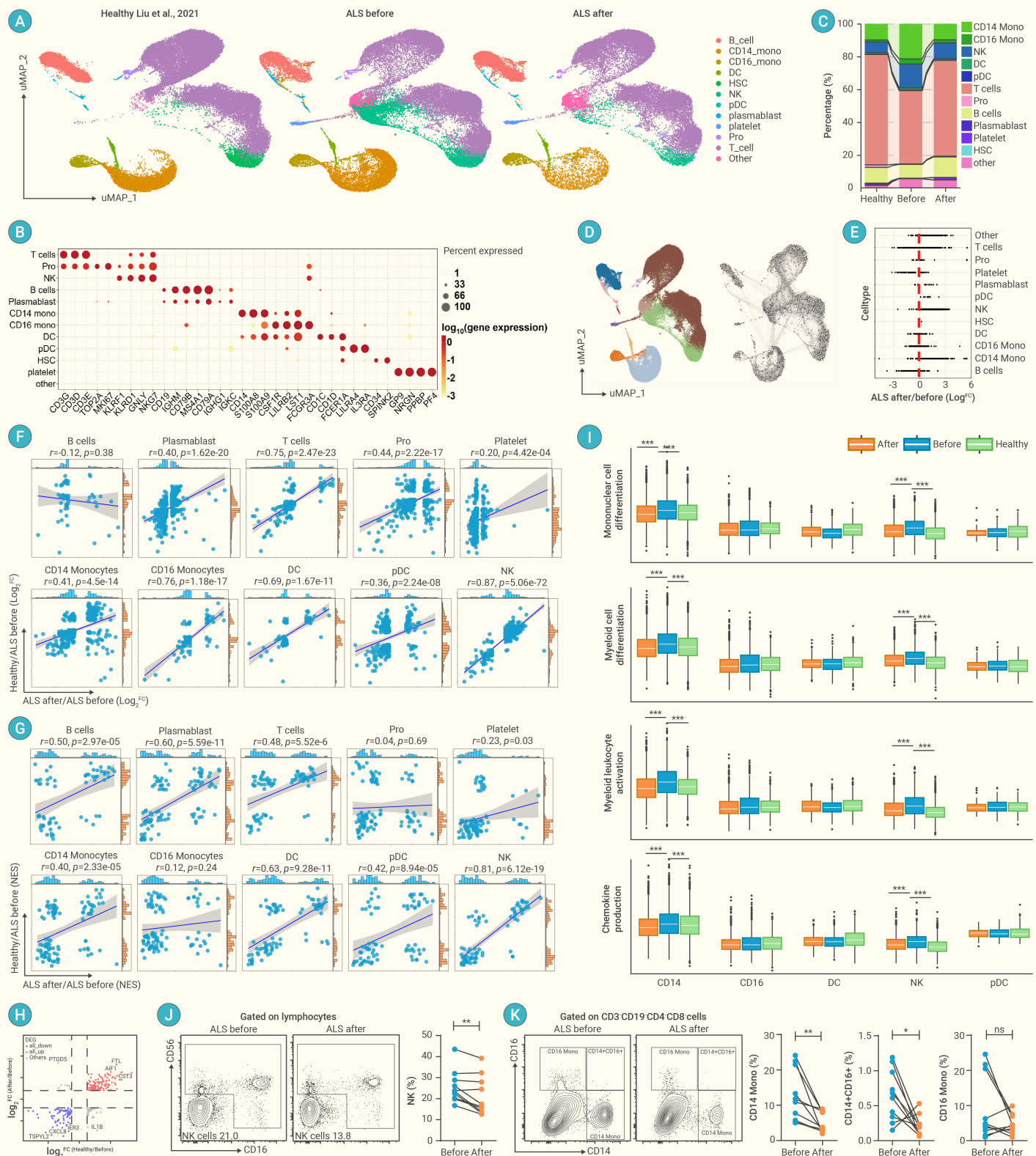


Figure 3. Characterization of innate immune cells in healthy controls and ALS participants before and following gp96 treatment (A) UMAP projection of participants with ALS before and after gp96 treatment and healthy controls. Each dot corresponds to a single cell, colored based on cell type. (B) Dot plots show the expression distribution of selected canonical cell markers in the 15 clusters. (C) Stacked bar plots showing proportions of each cell type derived from (A). (D) UMAP plot shows differences in cell composition between ALS participants before and after gp96 treatment, with samples identified using the Milo algorithm. (E) Fold change of cell composition in participants following gp96 treatment was determined based on the Milo algorithm. (F) Correlation analysis of Log₂ fold changes of DEGs between healthy controls versus ALS participants before gp96 treatment and ALS participants post-treatment versus pre-treatment among different cell types. (G) Correlation analysis of pathway scores. GSEA analysis for each cell type was performed, and NES values were used for the correlation analysis. (H) Scatter plot showing DEGs in monocytes, DC, NK, and pDC cells of ALS patients before and after gp96 treatment, and healthy controls. Each red dot represents a gene with a Benjamini–Hochberg adjusted *P* value ≤ 0.01 and average log₂ fold change ≥ 0.5 , comparing ALS patients after gp96 treatment to before treatment or healthy controls to ALS patients before treatment. (I) Box plots of expression levels for indicated GO biological process terms in monocytes, DC, NK, and pDC cells across clusters. Conditions are color-coded. Horizontal lines indicate medians, with whiskers extending to the furthest data point within 1.5 $\times$ interquartile range. (J–K) Flow cytometry analysis of NK cells (J) and mono cell subtypes (K) in PBMCs from ALS participants before and following gp96 treatment (*n*=10/group). All differences with *P* < 0.05 are indicated. ****P* < 0.001; using two-sided unpaired Dunn's (Bonferroni) test.

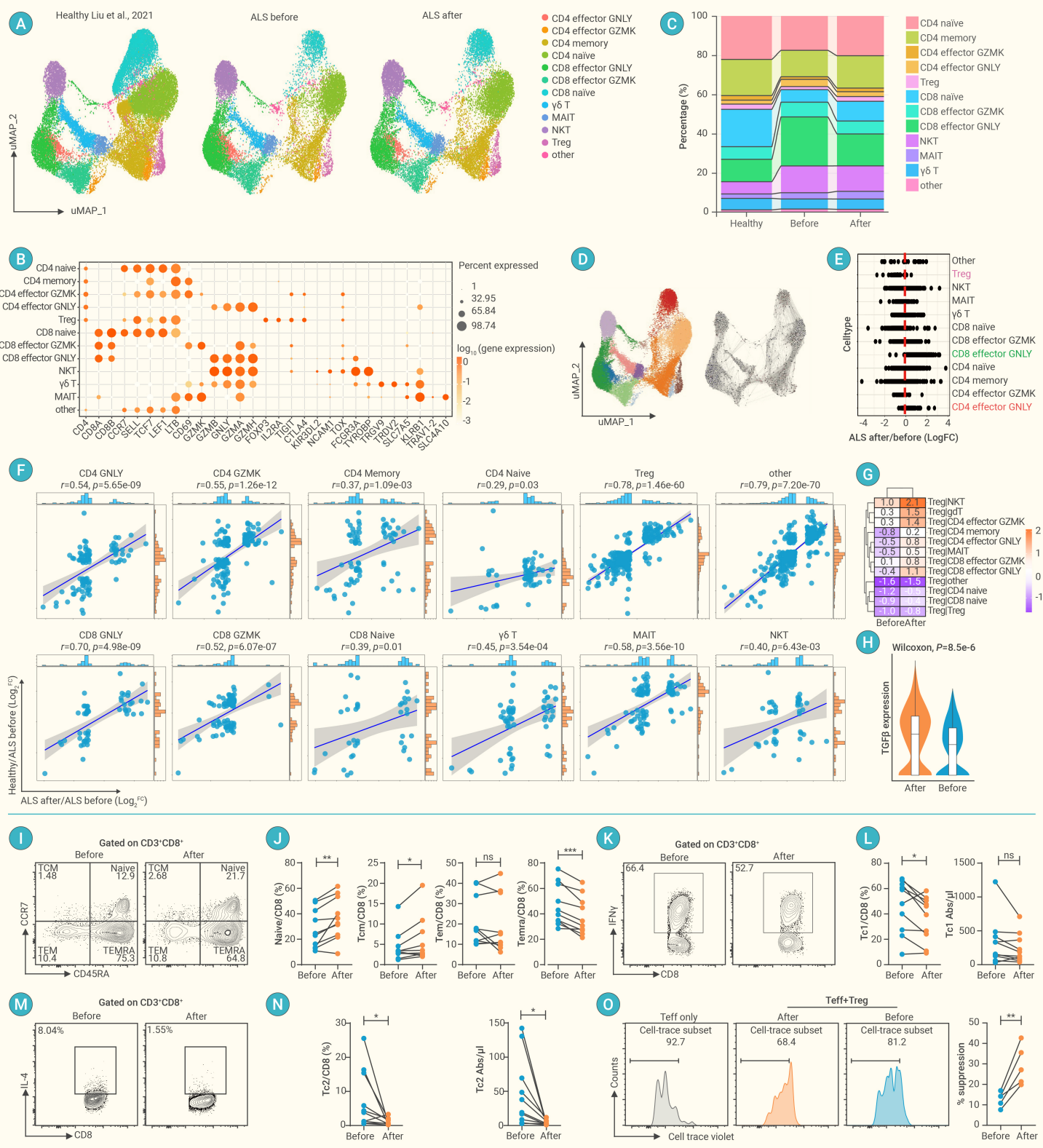


Figure 4. Immunological features of T cell subsets in ALS participants before and after treatment with high-dose gp96 (A) UMAP projection of ALS participants before and after gp96 treatment and healthy controls. Each dot corresponds to a single cell and is colored according to cell type. (B) Dot plots showing expression distribution of canonical cell markers in 12 T cell subsets. (C) stacked bar plots showing proportions of each cell type derived from (A). (D) UMAP plot shows differences between cell composition of ALS participants before and after gp96 treatment groups identified based on Milo algorithm. (E) Fold change of cell composition based on Milo algorithm. (F) Correlation analysis of Log₂ fold changes of DEGs between healthy controls versus ALS participants before gp96 treatment and ALS participants post-treatment versus pre-treatment among different cell types. (G) Heatmap displaying cell-cell contact analysis between Treg and non-Treg cells using CellPhoneDB. The color represents the intensity of the interaction between cell types. (H) Violin plot displaying the expression levels of the TGF- β gene in Treg cells before and following treatment in patients. (I-N) Representative flow cytometry plots and quantification of changes in the frequencies of naive and non-naive CD8⁺T cells (I, J), Tc-1 (CD8⁺IFN γ) (K, L) and Tc-2 (CD8⁺IL4⁺) (M, N) cells from ALS participants before and following gp96 treatment ($n=10$ /group). (O) Treg suppression analysis. A total of 1×10^4 cell trace violet-labeled CD4⁺CD25⁻T (Teff) cells were cultured with Tregs at a ratio of 1:1 for 3 days. Teff cell division cycle was measured using FACS (left). Suppression rate of proliferation was calculated (right). Wilcoxon rank-sum test or Two-tailed paired t test was used. Ns, no significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

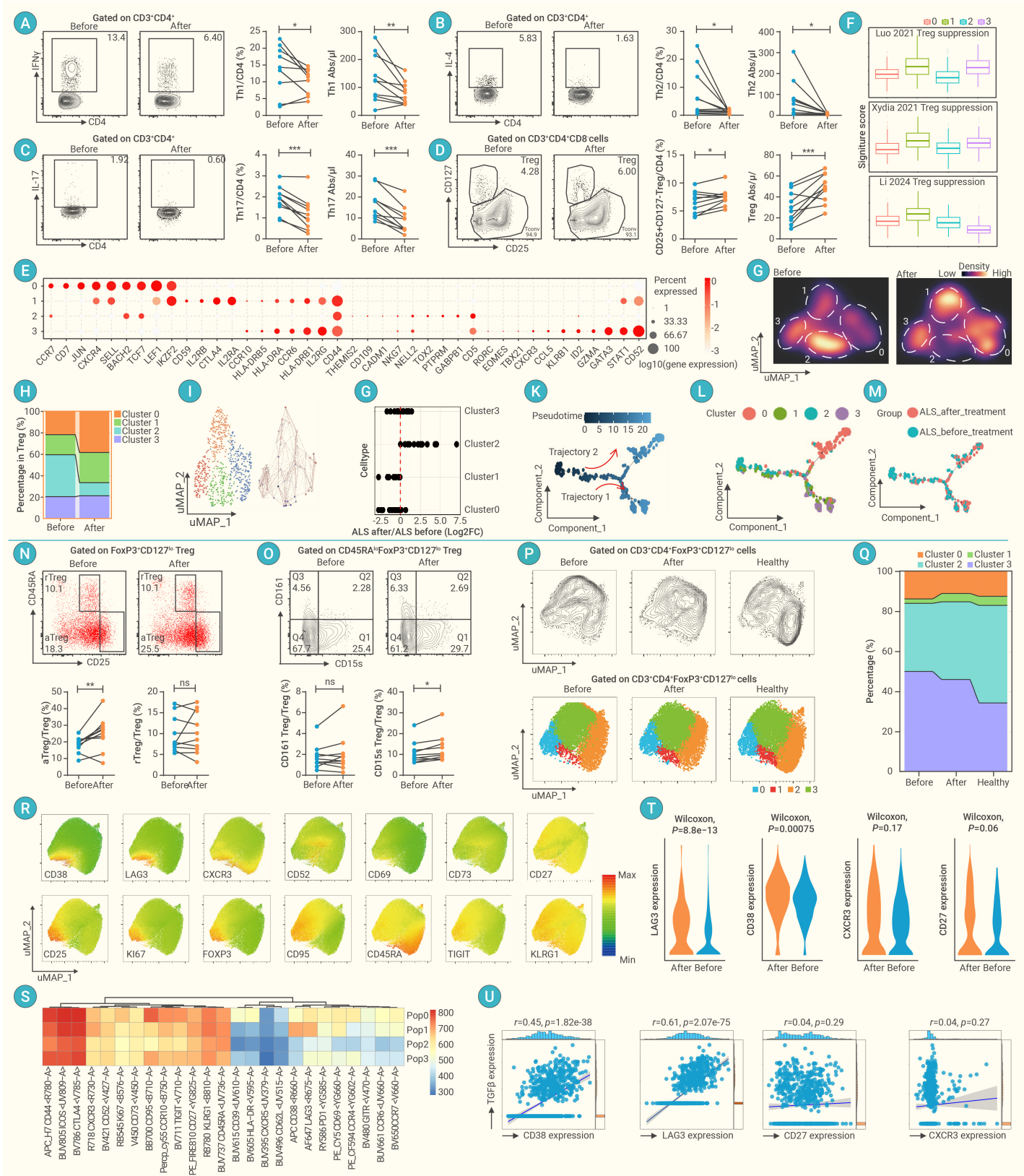


Figure 5. Immunological features of CD4⁺T cell and Treg (A-D) Flow cytometry analysis of CD4⁺T cell subtypes from ALS participants before and following gp96 treatment. Th-1 (CD4⁺IFN γ) cells (A). Th-2 (CD4⁺IL-4⁺) cells (B). Th-17 (CD4⁺IL-17⁺) cells (C). Treg (CD4⁺CD8⁺CD25⁺CD127⁺) cells (D). (E) Dot plots showing expression distribution of cell markers in Treg cell subsets. (F) Difference in Treg suppression signature scores across Treg cell subsets. (G) Galaxy plots displaying the distribution of Treg cell subsets between ALS participants before and following gp96 treatment. (H) Stacked bar plots showing proportions of Treg cell subsets. (I) UMAP plot shows differences in Treg cell subsets composition based on Milo algorithm. (J) Fold change of Treg cell subsets based on Milo algorithm. (K-M) Pseudotime-ordered analysis of Treg subclusters (K, L). Graph of each cluster from pre- and post-treatment are shown (M). (N-O) Representative flow cytometry plots and quantification of aTregs (N) and CD155⁺ and CD161 Tregs (O) from ALS participants before and following gp96 treatment. (P-S) Spectral flow cytometry analysis of Treg. UMAP graph displaying Treg cell subpopulations and clustering (P). Stacked barplots showing proportions of Treg cell subsets derived from P (Q). UMAP visualization shows the distribution of indicated markers in Treg cells before and following treatment in patients. (R) Heatmap showing expression distribution of indicated markers in Treg cell subsets (S). (T) Violin plot displaying the expression levels of the indicated genes in Treg cells before and following treatment in patients. (U) Correlation analysis of the expression levels of the indicated genes and TGF- β in Treg cells. $n=10/\text{group}$. Wilcoxon rank-sum test or Two-tailed paired t test was used. Ns, no significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

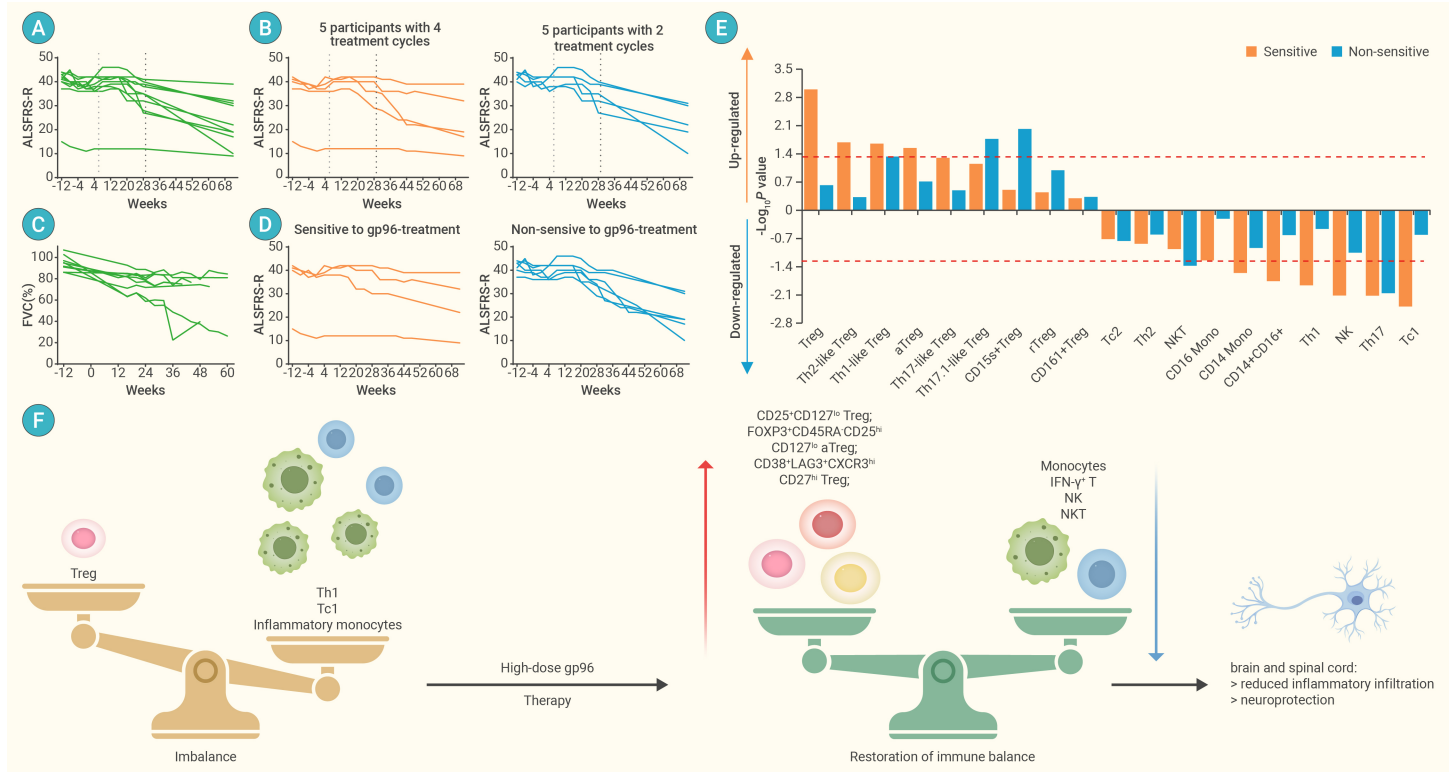


Figure 6. Therapeutic efficiency analysis of high-dose gp96 treatment for ALS (A) The ALSFRS-R total scores of the 10 included participants were monitored every 4 weeks during the screening period, treatment, and follow-up. (B) The ALSFRS-R scores of participants who completed four or two cycles of treatment. (C) Changes in FVC of the participants. (D) ALSFRS-R scores of the participants grouped into sensitive and non-sensitive to gp96 treatment, according to the comparison of disease progression rates in the screening period and treatment/follow-up period. (E) Bar plot of $\log_{10} P$ value of indicated cell types in gp96-treatment sensitive and non-sensitive subgroup. Data above or below dashed lines indicate a P value < 0.05 . (F) A working model. In patients with ALS, an imbalance between Tregs and inflammatory monocytes, along with effector T cells, results in immune disorders and hyperactivation, potentially contributing to the immunopathogenesis of neurological dysfunction. Treatment with high-dose gp96 therapy enhances the population of Tregs, including FOXP3⁺CD45RA⁺CD25^{hi}CD127^{lo} aTreg and CD38⁺LAG3⁺CXCR3^{hi}CD27^{hi} Treg, while also decreasing inflammatory monocytes, NK cells, and T cells. This correlates with restoration of immune homeostasis and safeguard neurons from immunopathogenesis in the brain and spinal cord.

also predominantly enriched in CD14⁺ monocytes, and was focused on cell differentiation and activation, particularly in the regulation of oxidative stress-induced neuron death (Figures S4B-C). GO pathway enrichment analysis of the DEGs revealed that pathways involved in mononuclear cell activation and differentiation were downregulated in CD14⁺ monocytes of ALS participants following gp96 treatment (Figures S4D-E). Additionally, downregulated DEGs associated with myeloid cell differentiation, activation, and cytokine production showed significant enrichment in CD14⁺ monocytes in ALS participants following gp96 treatment (Figure S4F). Sub-clustering analysis of monocytes, performed to uncover disease-specific transcriptional signatures, identified a subset of gp96 treatment-responsive monocytes^{35,36} that were present at lower frequencies in healthy controls than in ALS participants. This type of monocyte was decreased after gp96 treatment in ALS participants (Figures S4G-H). STRING-based protein interaction network analysis indicated that the upregulated genes in this type of monocytes were predominantly associated with monocyte activation (Figure S4I).³⁷ Meanwhile, GO enrichment analysis of the most strongly upregulated genes suggested that pathways related to monocyte activation were enriched in gp96 treatment-responsive monocytes (Figure S4J).

High-dose gp96 activated Tregs and suppressed the hyperactivated response of T cells

Next, we subclustered T cells from ALS participants, both before and after treatment with high-dose gp96, as well as from healthy controls, and obtained 12 subsets based on the expression and distribution of canonical T-cell markers (Figures 4A-B).³⁸ As shown in Figure 4C, the proportions of naïve CD4⁺ T cells, memory CD4⁺ T cells, and naïve CD8⁺ T cells—which were lower in ALS participants than in healthy controls—significantly increased after gp96 treatment. In contrast, the proportions of two effector CD8⁺ T cell subsets (granzyme K- and granzysin-expressing CD8⁺ T cells) and one effector CD4⁺ T cell subset (granzysin-expressing CD4⁺ T cells) were higher

in ALS participants than in healthy controls, but their proportions significantly decreased after gp96 treatment. Additionally, following gp96 treatment, the Treg percentage, which was lower in ALS participants than in healthy controls, increased to a level similar to that of healthy controls. Milo analysis revealed that there was a significant difference in the ratios of Tregs and effector GNLY cells when comparing participants before and after treatment (Figures 4D-E). Enrichment analysis of GO biological pathways revealed that inflammation response-related pathways, such as leukocyte-mediated cytotoxicity, leukocyte-mediated immunity, and immune response-regulating cell surface receptor signaling, were upregulated in ALS patients compared to healthy controls. However, these pathways were significantly downregulated in ALS participants following gp96 treatment (Figure S5A). Analysis of the DEGs indicated that genes that were differentially expressed between healthy controls and ALS participants were positively correlated with those showing differential expression between post-and pre-treatment in all T-cell subtypes (Figure 4F). Additionally, we found that the downregulated DEGs were involved in T cell activation and T cell receptor (TCR) signaling pathways in ALS participants following gp96 treatment (Figures S5B-C). Functional enrichment analysis revealed that CD8⁺ effector GNLY cells were the most affected by gp96 treatment, as evidenced by the downregulation of pathways related to TCR signaling, antigen receptor-mediated signaling, and immune response-activating signaling pathways following gp96-treatment (Figures S5D-E). Interactions between Tregs and all types of effector cells were increased following gp96-treatment, particularly through TGF- β 1 and TGF- β receptor (Figures 4G-H & S6), suggesting that treatment enhanced the ability of Tregs to suppress effector cell activity. Analysis of changes in different T-cell subpopulations revealed that the percentage of naïve CD8⁺ T cells and central memory T (TCM) cells significantly increased in ALS participants after gp96 treatment, whereas the percentage of effector memory cells re-expressing CD45RA (TEMRA) decreased (Figures 4I-J). Furthermore, there were notable decreases in the percentages of IFN γ CD8⁺ Tc1 and IL4⁺CD8⁺

Table 2. The effect of treatment duration on the therapeutic efficacy of high-dose gp96.

	Parameter	EXDUR = 30			EXDUR = 60		
		Estimate	95% CI	P-value	Estimate	95% CI	P-value
Time (-12,0)	(Intercept)	34.08	[7.76, 60.39]	0.1112	2.78	[-0.19, 5.76]	0.0864
	Time	-0.86	[-1.34, -0.38]	0.0188	-1.26	[-1.79, -0.73]	0.0003
	BFRSR	0.15	[-0.47, 0.77]	0.6782	0.93	[0.86, 1.00]	<0.0001
	BALSL						
	ALSL = 2	Reference					
	ALSL = 3	-0.07	[-3.57, 3.42]	0.9699	0.09	[-1.53, 1.71]	0.9151
	Marginal R^2	0.24					
	Conditional R^2	0.78					
Time (1,12)	(Intercept)	7.65	[-4.78, 20.07]	0.2752	4.28	[-3.32, 11.88]	0.4318
	Time	0.54	[-0.10, 1.18]	0.1683	0.80	[0.33, 1.27]	0.0261
	BFRSR	0.84	[0.54, 1.13]	0.0017	0.86	[0.68, 1.04]	0.0348
	BALSL						
	ALSL = 2	Reference					
	ALSL = 3	-1.2	[-2.85, 0.45]	0.2075	1.37	[-2.91, 5.65]	0.6214
	Marginal R^2	0.75					
	Conditional R^2	0.81					
Time (1,72)	(Intercept)	6.62	[-11.41, 24.65]	0.5446	-5.00	[-10.32, 0.32]	0.0991
	Time	-1.08	[-1.47, -0.69]	0.0056	-0.67	[-1.21, -0.13]	0.0730
	BFRSR	0.97	[0.54, 1.39]	0.0458	1.17	[1.05, 1.29]	<0.0001
	BALSL						
	ALSL = 2	Reference					
	ALSL = 3	-2.52	[-4.91, -0.14]	0.1732	1.86	[-1.07, 4.78]	0.2362
	Marginal R^2	0.77					
	Conditional R^2	0.89					

Estimate: model coefficient for each fixed effect; *95% CI*: 95% confidence interval for the estimate; *P-value*: significance level of the *t*-test, with values <0.05 typically indicating statistical significance; *Reference*: the reference category for the categorical variable; *Marginal R^2* : fixed effects only; *Conditional R^2* : fixed + random effects EXDUR: EXposure DURation.

Tc2 cells, as well as a reduction in the absolute number of Tc2 cells post-gp96 treatment (both $P < 0.05$) (Figures 4K–N). Consistent with the above findings, the suppressive ability of Tregs against Teffs in PBMCs significantly increased following gp96-treatment (Figure 4O).

As seen in Figures 5A–C, both the percentages and absolute numbers of CD4⁺IFN γ ⁺ effector Th1, CD4⁺IL-4⁺ Th2, and CD4⁺IL-17⁺ Th17 cells were significantly decreased following gp96 treatment, whereas the opposite trend was observed for CD25⁺CD127^{lo} Tregs (Figure 5D). Four clusters of Tregs were further defined in ALS participants (Figure 5E). Cluster 0 was characterized by a naïve phenotype (CCR7^{hi}TCF7^{hi}HLA-DR^{lo}). Cluster 1 showed activated/effector features, including low/absent CCR7 and TCF7 expression, along with high expression of CXCR4 and CTLA4. Cells in cluster 2 had a distinct transcriptome signature defined by a CD5⁺CD109⁺ profile, leading to their classification as non-classical Tregs. Cluster 3 comprised memory KLRB1⁺RORC⁺ Tregs expressing CCR4 and HLA-DR but not IKZF2.³⁹ Cells in clusters 0 and 1, both of which exhibited high IKZF2 expression, were considered classical Tregs. Each of these clusters had a unique gene expression and functional profile (Figure S7A). Cluster 1 showed the strongest suppres-

sive signature based on previous studies (Figure 5F).^{40–42} Cluster 2 cells had the highest number of DEGs and the greatest number of affected pathways post-treatment, indicating that this population was the most influenced by high-dose gp96 treatment (Figures S7B–C). Following gp96 treatment, the proportions of classical Tregs increased, while those of non-classical Tregs decreased (Figures 5G–J). Additionally, Tregs exhibited a different distribution post-treatment compared to pre-treatment, characterized by fewer non-classical Tregs and a greater number of classical Tregs (Figures 5K–M & S8A–D). This suggested that non-classical Tregs had been transformed into classical Tregs following gp96 treatment. As shown in Figure S8E, the expression of Treg lineage-associated genes (e.g., *IL2RA*, *IL2RB*, *FOXP3*, *IKZF2*) and Treg function-associated genes (e.g., *CCR4*, *TIGIT*, *CD40LG*, *IL1R*) increased along the pseudotime trajectory post-gp96 treatment. This also included genes involved in the regulation of Treg migration and homing, especially in the context of CNS autoimmune diseases (e.g., *ITGA4*), and genes encoding transcription factors with roles in the maintenance of Treg proliferation and stability (e.g., *JUN*, *FOXP1*, *ID3*).⁴³ In line with these findings, transcription factors such as FOS and JUN, which regulate immune

responses and maintain FOXP3 activity, showed enhanced activity in Tregs following gp96 treatment (Figure S8F).

Based on the expression levels of CD45RA and CD25, peripheral blood Tregs can be categorized into rTregs, aTregs, and non-Tregs.⁴⁴ As seen in Figure 5N, the Teff population significantly increased following gp96-treatment ($P < 0.05$), as detected by flow cytometry. There was also a mild increase in the number of CD155⁺ (Lewis X antigen) Tregs following gp96 treatment (Figure 5O, $P < 0.05$), suggesting that gp96 treatment led to the induction of Treg subpopulations with robust proliferative and immunosuppressive capabilities. Besides, Th1-like and Th17/Th1-like Treg (defined based on the expression of chemokine receptors) numbers were also increased following gp96-treatment (Figure S8G).⁴⁵ Finally, spectral flow cytometric analysis showed that a subpopulation of Tregs, characterized by high expression of LAG3, CD38, CD27, and CXCR3, increased nearly two-fold in ALS participants following gp96 treatment (Figures 5P–S). Consistent with these observations, we found that LAG3 and CD38 expression levels were increased in Tregs following gp96 treatment, and the expression of both LAG3 and CD38 showed a positive correlation with TGF- β expression at the transcriptomic level (Figures 5T–U). This suggested that these Tregs may serve as indicators of the response to gp96 treatment.

The therapeutic efficiency of high-dose gp96 was correlated with Treg activation

During the screening period, treatment, and follow-up, changes in total ALSFRS-R scores were recorded every 4 weeks for all participants receiving two (EXDUR = 30 weeks) or four (EXDUR = 60 weeks) treatment cycles. First, the ALSFRS-R scores at different follow-up time points were compared with those at baseline. At the beginning of the follow-up (baseline), the average ALSFRS-R score of the subjects ($n = 10$) was 36.00 ± 9.02 points (95% CI [29.55, 42.45]). Within the first 16 weeks of treatment, the functional status of the patients was relatively stable, with a slight increase in the score, which peaked at 37.90 ± 9.49 points (95% CI [31.11, 44.69]) on week 16. However, the differences were not statistically significant when compared to baseline (adjusted $P > 0.05$) (Figures 6A–B, Table S1). For four ALS participants (40%), the disease progression rate was lower during treatment and follow-up than during the screening period. At the end of follow-up at week 72, stable FVC was observed in seven patients, with a marked decline in this parameter occurring in only 2 patients (Figure 6C).

As listed in Table S2, ALS patients showed a trend toward disease progression during the pre-treatment period (–12 to 0 weeks), with the ALSFRS-R score decreasing by 1.06 points every 4 weeks (95% CI: –1.40, –0.72; $P < 0.0001$). In the early stage after gp96 administration (1 to 12 weeks), this trend was significantly reversed, with the score increasing by 0.67 points per month (95% CI: 0.30, 1.04; $P = 0.0062$), indicating that gp96 exerted a positive therapeutic effect. During the long-term treatment and the follow-up period (1 to 72 weeks), the score again slowly declined, decreasing 0.87 points per month (95% CI: –1.21, –0.53; $P = 0.0007$). Age (AGE) showed a weak positive effect at the baseline period ($\beta = 0.10$, 95% CI: 0.02, 0.18; $P = 0.0157$), no significant effect in the early treatment stage, and a strong positive correlation with gp96 treatment in the long-term follow-up stage ($\beta = 0.22$, 95% CI: 0.13, 0.31; $P < 0.0001$). Disease duration (DUR) showed significant negative effects in both the early and long-term follow-up stages (early: $\beta = -0.59$, 95% CI: –0.83, –0.35, $P = 0.0001$; long-term: $\beta = -0.83$, 95% CI: –1.11, –0.55, $P < 0.0001$), indicative of a possible negative correlation between disease duration and drug effect. The baseline score (BFRS-R) showed a strong positive correlation with treatment in all stages ($P < 0.0001$), suggesting that the baseline status is an important indicator for predicting the gp96 treatment response. Regarding baseline severity (BALSL), no significant difference was observed between the ALSL = 3 group and the ALSL = 2 reference group ($\beta = -0.89$, 95% CI: –8.12, 6.34; $P = 0.8109$). However, significant negative effects were noted in the ALSL = 3 category in both the early treatment stage ($\beta = -14.78$, 95% CI: –20.97, –8.60; $P = 0.0001$) and the long-term follow-up stage ($\beta = -16.77$, 95% CI: –24.31, –9.23; $P < 0.0001$), indicating that patients with higher baseline severity might have a poorer response to treatment. The ALSL = 4 category did not show statistically significant differences in all periods compared with the reference group.

As seen in Table 2, during the screening period, the regression coefficients

of the time variable in the EXDUR = 30 and EXDUR = 60 subgroups were –0.86 ($P = 0.0188$) and –1.26 ($P = 0.0003$), respectively, both reaching statistical significance ($P < 0.05$). This indicated that the ALSFRS-R score significantly decreased before gp96 treatment, consistent with ongoing disease deterioration at this stage. This result reflects the rate of natural disease progression in the absence of treatment and provides a baseline reference against which to evaluate subsequent intervention effects. During the long-term treatment and the follow-up period (weeks 1 to 72), the regression coefficient of the time variable was –1.08 ($P = 0.0056$) in the EXDUR = 30 subgroup, which was highly statistically significant, and –0.67 ($P = 0.0730$) in the EXDUR = 60 subgroup. This indicated that the rate of decline of the ALSFRS-R score in the EXDUR = 60 subgroup was slightly slower than that in the EXDUR = 30 subgroup, suggesting that a longer treatment duration may be associated with slower disease progression.

Mixed-effects modeling of the longitudinal changes in the ALSFRS-R score revealed a clinically significant phenomenon. During the baseline observation period (–12 to 0 weeks), patients in the EXDUR = 60 group showed a more significant functional decline ($\beta = -1.26$, $P = 0.0003$) compared to the EXDUR = 30 group ($\beta = -0.86$, $P = 0.0188$). However, after the initiation of treatment (1 to 12 weeks), the EXDUR = 60 group showed a significant slope reversal ($\beta = 0.80$, $P = 0.0261$), and, although the EXDUR = 30 group also showed a tendency toward improvement, this trend did not reach statistical significance ($\beta = 0.54$, $P = 0.1683$). This “fast progressors-superior response” phenomenon suggested that the intensity of the treatment effect may be positively correlated with the rate of natural disease progression. Collectively, these data indicated that patients with a faster disease progression at baseline may derive more significant clinical benefits from gp96 treatment, supporting a clinical strategy of early identification of fast-progressing patients and the timely administration of long-term treatment.

To analyze treatment efficacy regarding Treg activation and the suppression of immune cell hyperactivation by high-dose gp96 therapy, ALS participants were divided into two subgroups: those sensitive and those not sensitive to treatment, with disease progression rates during treatment and follow-up lower than those during the screening period (Figure 6D). As shown in Figures 6E & S9, significant increases in the proportions of Tregs, aTregs, Th17-like Tregs, and Th2-like Tregs, along with decreases in those of Th1 cells, Tc1 cells, NK cells, CD14 monocytes, and CD14⁺CD16⁺ monocytes were observed in the gp96 treatment-sensitive subgroup, but not in the non-sensitive subgroup.

DISCUSSION

In this study, we confirmed that in SOD1 mice, high-dose gp96 treatment activated Tregs, reduced neuroinflammation, and slowed ALS progression. Based on these preclinical results and the reported safety of gp96 in clinical trials, we conducted a single-arm, open-label trial to assess the safety and immune regulatory efficacy of gp96 in patients with ALS. The trial showed that high-dose gp96 was well-tolerated and that gp96 treatment produced clear clinical benefits in most ALS participants at the early treatment stage. Additionally, the overall therapeutic efficiency was positively associated with treatment duration and negatively correlated with disease duration and severity. As the extensive heterogeneity of ALS results in a highly variable clinical response to therapy, these results may help to define the responder population that will most benefit from gp96 treatment.

Innate and T cell immune hyperactivation are thought to contribute to the progression of ALS by promoting chronic inflammation and causing neuronal damage.^{46,47} In our study, ALS patients had higher levels of inflammatory monocytes, NK cells, and T cells relative to healthy controls; however, the numbers of these cell types decreased following gp96 therapy. This reduction is significant given that these cells contribute to ALS-related neuroinflammation.^{48–50} Meanwhile, gp96 treatment increased the frequencies of various Treg subsets, promoting a regulatory immune environment that countered immune hyperactivation and neuroinflammation in ALS (Figure 6F). The CD38⁺LAG3⁺CXCR3^{hi}CD27^{hi} Treg subtype was identified as a potential prognostic immune biomarker for gp96 treatment efficacy in ALS. After treatment, the interaction between Tregs and Tregs via TGF- β and its receptors was enhanced, and an increase in TGF- β expression in Tregs was also observed (Figures 4G & S6). In addition, the expression levels of LAG3 and

CD38 were increased in Tregs following gp96 treatment, and were positively correlated with those of TGF- β (Figures 5T-U). Notably, several studies have shown a reciprocal induction between TGF- β and CD38, LAG3, or CXCR3, which promotes the stability and suppressive function of Tregs.⁵¹⁻⁵⁴ Given that TGF- β is a key immunosuppressive cytokine secreted by Tregs, these results suggest that LAG3 and CD38 may serve as potential biomarkers for identifying gp96-responsive Tregs.

Interestingly, our results suggest that high-dose gp96 may promote the interaction between pDCs and Treg through the pDC-Nrp1-VEGFA-Treg axis, based on the analysis of PBMC samples from ALS participants before and after gp96 treatment (Figure S10A). This was consistent with previous work showing that exogenous high-dose gp96 promotes the development of Nrp1⁺ pDCs, which enforce Treg-mediated tolerance.²⁷ In addition, treatment with the myelin oligodendrocyte glycoprotein (MOG)-derived immunogenic epitope MOG₃₅₋₅₅ complexed with gp96 protein was found to induce antigen-specific Tregs (Figure S10B), a response with potential for attenuating disease progression.⁵¹ GO analysis indicated that MHC class II antigen processing and presentation pathways were upregulated in Tregs in ALS participants after gp96 treatment (Figure S10C), suggesting that gp96 promoted the induction of antigen-specific Tregs.

Notably, gp96 functions as an immune modulator capable of activating both Tregs and Teffs in a context-dependent manner. We propose two key explanations for the apparent selectivity of the immunosuppressive effects of high-dose gp96 in the ALS setting. First, gp96 activates suppressive Tregs and inflammatory Teffs via pathways distinct from those employed by IL-2, which selectively regulates the activation of Tregs or Teffs/NK cells through the high- or intermediate-affinity receptor IL-2R.⁵⁵ In cancer immunotherapy, the immunostimulatory activity of gp96 is largely dependent on its binding to tumor-specific antigenic peptides and their subsequent presentation to conventional DCs via CD91, thereby stimulating antigen-specific CTL responses.⁵⁶ In contrast, high-dose gp96 activates and expands functional Tregs through direct interaction with TLR1/2 on Tregs, leading to enhanced Foxp3 expression, as well as by upregulating the expression of neuropilin-1 on pDCs, thus enabling the long-term interactions between pDCs and Tregs.^{26,27} Importantly, the placental gp96 preparation used in this study was devoid of neuron- or inflammation-associated antigenic peptides. Second, the immunomodulatory effects of gp96 are dose-dependent, and the weekly administration of 200 μ g gp96 employed in this study greatly exceeded the threshold dose for Treg activation.²⁴⁻²⁶ This dose-dependent divergence highlights the importance of context and concentration in determining the immune effects of gp96. Nevertheless, no extensive activation of immune cells and inflammatory responses was observed in clinical trials using low doses (20–50 μ g) of tumor-derived gp96 as active specific immunotherapy, indicating that gp96 has a relatively low capacity for activation of innate and inflammatory immunity.²⁸ Together, these findings strongly imply that high-dose placental gp96, in the absence of neuron antigenic peptides, predominantly activates Tregs rather than CTLs in the context of ALS. Future studies are warranted to dissect the molecular and cellular pathways underlying these effects of gp96 across different disease settings and antigenic backgrounds.

Our study provides preliminary evidence that high-dose gp96 treatment correlates with rebalancing of the peripheral immune landscape in ALS and may slow disease progression in a subset of patients. While these preliminary findings support a potential immunomodulatory role for gp96, the observed associations should be interpreted with caution, as causal relationships have yet to be established in randomized controlled studies. Several limitations of our study should be acknowledged. While we observed that high-dose gp96 treatment induced notable immune changes and exhibited trends toward clinical benefit in ALS patients, the open-label, non-randomized design of the study, along with the small sample size ($n = 10$), inherently limit the strength of our conclusions. The slower rate of decline in ALSFRS-R scores after gp96 treatment, although encouraging, may reflect natural fluctuations in disease course or placebo effects. Besides, multiple factors, including environmental conditions or blood collection timing, can affect the expression profile of PBMCs during treatment, resulting in PBMC transcriptomic data variability. This, together with the small sample size, may preclude reliable conclusions. To mitigate these limitations, future studies should

adopt a randomized controlled trial design, involving larger sample sizes (e.g., $n > 50$), the inclusion of a placebo group or using crossover designs, and appropriate blinding to better assess the specific therapeutic and immunoregulatory efficacy of gp96. Further stratification of patients by disease subgroup or genetic background (e.g., hereditary vs. non-hereditary) or ALS-associated gene mutations (e.g., *SOD1*, *TDP-43*) will be needed to define ALS subtypes, thereby guiding gp96-based treatment prioritization and maximizing treatment responses. Additionally, longitudinal monitoring of immunological biomarkers, including functional Treg subtypes and gene expression dynamics, may help identify predictive indicators of treatment responsiveness and guide personalized therapeutic strategies. The lack of more objective clinical endpoints, such as survival analysis, comprehensive pulmonary function measures, quantitative muscle strength testing, and predictive biomarker detection, also limits the interpretation of gp96 efficacy, as ALSFRS-R scoring is susceptible to observer bias in small, open-label studies. These limitations will be addressed in future trials with longer follow-up periods for collecting survival data, and with objective and multi-dimensional endpoints, such as quantitative muscle strength testing or hand-held muscle strength tests, comprehensive pulmonary function testing with the rate of change of FVC with maximal inspiratory pressure as the parameter, imaging-related examination (e.g., MRI), and neuronal and axonal damage assessment via the detection of neurofilament light chain (NFL) and phosphorylated neurofilament heavy chain (pNfH) levels in cerebrospinal fluid and blood from ALS patients. Finally, considering the complexity of ALS pathogenic factors, exploring synergies between gp96-based immunotherapy and existing therapies with different mechanisms of action could offer a more comprehensive disease management strategy. For instance, gp96 therapy targeting chronic neuroinflammation could be explored in combination with the existing ALS drug riluzole, which exhibits anti-glutamatergic and neuroprotective properties.

CONCLUSION

In summary, our research indicates that high-dose gp96 possesses immunomodulatory effects that may benefit patients with ALS. The findings that gp96 treatment suppressed immune hyperactivation and that a link exists between treatment efficacy and treatment duration or Treg activation underscore the value of further exploring gp96-based immunotherapy for ALS.

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AUTHOR CONTRIBUTIONS

SM and XL conceived the project. ZZ, YL, BM, and HZ contributed to the sample collection, XL and FC performed the experiments. YJ, ZH and ZW analyzed the data. RL, BW, YX, CX and SX assisted with FACS staining and data analysis. ZY, YM, XL, SY and DM assisted with the Bioinformatics analysis. XL and SM completed the first draft of the article. SM, CL, DF, and XZ supervised and interpreted clinical data. XL had full access to all the data in the study and verified the underlying data. All authors contributed to and approved the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

ETHICAL STATEMENT AND PATIENT CONSENT

This clinical study was an investigator-initiated trial (IIT), approved by the Ethics Committee of Boao Evergrande International Hospital (approval number: Heng Jian Bo Yi 000015) and registered in the Chinese Clinical Trial Registry (ChiCTR2200062823). The placental tissue used for gp96 extraction was separately registered under ChiCTR2400089654 for retrospective material use documentation. Placental donation and collection were approved by the Committee (Ethics permit number

ChiCTR2400089654), and written consents were obtained from all the pregnant donors.

All participants in the clinical trial were fully informed about the study's objectives, procedures, potential risks, and benefits, and provided signed informed consent prior to inclusion. For participants unable to provide consent themselves, consent was obtained from their legally authorized representatives. All personal data were anonymized to ensure privacy protection.

DATA AND MATERIALS AVAILABILITY

All data reported in this study will be shared by the lead contact upon request. This paper does not report original code. Any additional information required to reanalyze the data reported in this work paper is available from the lead contact upon request. The single-cell sequencing data generated in this study have been deposited in the Genome Sequence Archive database under the accession code HRA007732 (<https://ngdc.cncb.ac.cn/gsa-human>). The raw single-cell sequencing data is protected and restrictively available due to data privacy laws. Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Songdong Meng (mengsd@im.ac.cn).

SUPPLEMENTAL INFORMATION

It can be found online at <https://doi.org/10.59717/j.xinn-med.2025.100157>

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