Tregs Attenuate Peripheral Oxidative Stress and Acute Phase Proteins in ALS

David R. Beers, PhD, Jason R. Thonhoff, MD, PhD, Alireza Faridar, MD, Aaron D. Thome, PhD, Weihua Zhao, MD, PhD, Shixiang Wen, BS, and Stanley H. Appel, MD

Oxidative stress (OS) induces inflammation, which in turn exacerbates OS and the expression of acute phase proteins (APPs). Regulatory T lymphocyte (Treg) therapy was assessed for suppression of OS and APP responses in longitudinal serum samples from subjects with amyotrophic lateral sclerosis (ALS) enrolled in a phase I clinical trial. The first round of Treg therapy suppressed levels of oxidized low-density lipoprotein (ox-LDL). During a 6-month washout period, ox-LDL levels increased. A second round of therapy again suppressed ox-LDL levels and then rose following the cessation of treatment. Serum levels of APPs, soluble CD14, lipopolysaccharide binding protein, and C-reactive protein, were stabilized during Treg administrations, but rose during the washout period and again after therapy was discontinued. Treg therapy potentially suppresses peripheral OS and the accompanying circulating pro-inflammatory induced APPs, both of which may serve as peripheral candidates for monitoring efficacies of immunomodulating therapies.

Materials and Methods

Patients

Subjects, study design, and subject selection, have been previously described. For the Olink and ELISA assays, the demographics for the subjects with ALS were (n = 30) (mean [SD]) 58.8 [1.57] years; 63.3% were men and 36.7% were women;...
86.7% were white, 6.7% were Hispanic, 3.3% were black, and 3.3% were Asian. Control individuals (n = 10) were similar in age (57.6 [2.15] years) with 60% men and 40% women; 90.0% were white, none were Hispanic, none were black, and 10% were Asian. For the Treg therapy study, three subjects with ALS were selected based on their differing sites of disease onset and rates of progression. Subjects underwent leukapheresis, and Tregs were subsequently isolated and expanded ex vivo. Tregs (1 x 10^6 cells/kg) were administered IV at early stages (four doses over 2 months) and later stages (four doses over 4 months) of disease. Concomitant interleukin-2 (2 x 10^5 IU/m²/injection) was administered subcutaneously three times weekly over the entire study period. Approval from the Food and Drug Administration and Institutional Review Board at Houston Methodist Hospital was obtained before study initiation. Written informed consent was obtained before enrollment. The study was registered on clinicaltrials.gov (NCT03241784).

Olink and ELISAs. Lectin-type oxidized LDL receptor 1 (LOX-1) was assayed by Olink (Boston MA). Human soluble CD14 (sCD14), C-reactive protein (CRP), and lipopolysaccharide binding protein (LBP) ELISA Kits from R&D Systems, and an oxidized LDL (ox-LDL) ELISA kit from Fisher Scientific, were used to determine their concentrations in sera of subjects with ALS and healthy controls (HC) according to manufacturer’s instructions.

Statistics
Comparisons were performed using an ANOVA for more than two groups or Student’s t-test for two groups. The ANOVA is presented with the degrees of freedom, F value, and p value. The Student’s t-test is presented with a p value. Correlations were done using Spearman Rank Order in SigmaStat software and presented with a t (rho) and p values.

Results
A blinded non-biased assay demonstrated that soluble LOX-1, a receptor for ox-LDL, was elevated in sera of subjects with ALS (n = 16) compared with sera from age-matched HC (n = 9, p < 0.001) (Fig 1A). When separated into rapidly (n = 8) and slowly progressing (n = 8) subjects, LOX-1 was elevated in sera from rapidly progressing subjects compared with slowly progressing subjects (p = 0.011) or HC (p < 0.001) [F(2, 22) = 15.69, p < 0.001]; LOX-1 was elevated in slowly progressing subjects compared with HC (p = 0.038) (Fig 1B). Since LOX-1 was elevated in these subjects, its ligand, ox-LDL, was also assayed in the serum of the subjects. ox-LDL levels were increased in subjects with ALS (n = 30) compared with HC (n = 10, p < 0.001) (Fig 1C). ox-LDL was elevated in sera from rapidly progressing subjects (n = 13) compared with slowly progressing subjects (n = 17, p < 0.001) or HC (p < 0.001) [F(2, 37) = 49.78, p < 0.001]; ox-LDL was not increased in sera from slowly progressing subjects compared with HC (p = 0.243) (Fig 1D). LOX-1 levels positively correlated with disease progression rates (r = 0.618, p = 0.011) (Fig 1E). ox-LDL levels also positively correlated with disease progression rates (r = 0.729, p < 0.001) (Fig 1F). LOX-1 levels positively correlated with ox-LDL levels in the 16 subjects that were assayed for both LOX-1 and ox-LDL (r = 0.829, p < 0.001) (Fig 1G).

ox-LDL levels were evaluated in longitudinal sera samples following infusion of autologous Tregs in subjects (n = 3) enrolled in a phase I ALS clinical trial. In the first subject, infusions of Tregs every 2 weeks for 8 weeks in combination with subcutaneous injections of IL-2 three times a week, suppressed ox-LDL levels (Fig 2A). With the first round of infusions, the subject’s Appel ALS Score (AALS) remained stable for 10 weeks. During the 6 months Treg “washout” period, when subjects were still receiving IL-2, ox-LDL levels rose toward the baseline level with a coinciding deterioration of the AALS. With a second round of infusions, administered every 4 weeks for 16 weeks, the therapy suppressed ox-LDL levels again and the subject’s clinical status stabilized. At the end of the study when Treg infusions ceased and only IL-2 was continued, ox-LDL level gradually increased. Similarly, in the second subject, ox-LDL levels were suppressed following the first round of Treg infusions, increased during the washout period, decreased again during the second round of Treg infusions, and rose following the cessation of Treg infusions (Figs 2B). The stabilization and deterioration of the subject’s clinical status mirrored the decline and rise of serum ox-LDL levels. In the third subject, who was progressing slowly, ox-LDL levels declined during the first round of therapy, rose during the washout, fell with the second round of infusions, and rose again after cessation of therapy. However, the subject’s clinical status remained stable throughout treatment (Fig 2C).

As was observed with ox-LDL, Treg infusions suppressed serum sCD14 levels with a concomitant increase in sCD14 when not on therapy in two of the three phase I study subjects (Fig 3). The third subject never had increased levels of sCD14, but the levels trended downward during the first round of infusions (Fig 3C). LBP levels were comparable to ox-LDL levels; LBP levels stabilized during the first round, increased during the washout period, decreased during the second round, and increased following Treg cessation (Figs 3). The changes in CRP levels were like those of ox-LDL and LBP levels in all subjects (Figs. 3). In the first subject, who was rapidly progressing, CRP levels did not decrease again until after the second infusion of the second round, which mirrored the time of disease stabilization.
FIGURE 1: LOX-1 and ox-LDL are elevated in subjects with ALS. A, B. LOX-1 is increased in the serum of all subjects with ALS, and in rapidly and slowly progressing subjects, when compared with HC. C, D. ox-LDL is increased in the serum of all subjects with ALS, and in rapidly progressing subjects, when compared with HC. ox-LDL is not increased in the serum of slowly progressing subjects with ALS compared with HC. The progression rate was determined using the Appel ALS (AALS) scoring system. In this scoring system, less than 1.5 AALS points per month is a slowly progressing subject. Equal to or greater than 1.5 points per month is a rapidly progressing subject. E. LOX-1 positively correlated with the rate of progression in subjects with ALS. F. ox-LDL positively correlated with the rate of progression in subjects with ALS. G. LOX-1 positively correlated with ox-LDL in the 16 subjects with ALS that were assayed for both LOX-1 and ox-LDL. *p < 0.05, **p < 0.001, and n.s. = not significant.
FIGURE 2: A, B. Subjects’ clinical statuses reflect the level of the subjects’ serum ox-LDL levels. These subjects were enrolled and completed a phase I clinical trial with Treg + IL-2 therapy. ox-LDL levels were suppressed following the first round of Treg infusions, increased during the washout period, were then suppressed during the second round of Treg infusions, and rose following the cessation of Treg + IL-2 treatment. The stabilization and deterioration of the subject’s clinical status mirrored the decline and rise of serum ox-LDL levels. C. Subject 3, a slowly progressing subject, had stable ox-LDL serum levels and reflects a stable clinical status. Arrows indicate Tregs + IL-2 infusion times. IL-2 was administered 3X/week throughout the study. The red-dotted lines demarcate Treg + IL-2 therapy or IL-2 only intervals. During the Treg “washout” period, the subjects received IL-2 injections. Red line = mean value of the ox-LDL level in HC. Black lines = ± one standard deviation of the ox-LDL levels in HC.

FIGURE 3: sCD14, LBP, and CRP in the serum of subjects enrolled in a phase 1 Treg + IL-2 clinical study. A, B. sCD14, LBP, and CRP fell and rose with Treg + IL-2 treatment. C. sCD14 was unchanged in a slowly progressing subject with ALS. LBP and CRP fell and rose with Treg + IL-2 treatment. Arrows indicate Tregs + IL-2 infusion times. IL-2 was administered 3X/week throughout the study. The red-dotted lines demarcate Treg + IL-2 therapy or IL-2 only intervals. During the Treg “washout” period, the subjects received IL-2 injections. Red line = mean value of each APP level in HC. Black lines = ± one standard deviation of each APP level in HC.
Discussion

In ALS, as disease progresses, there is a concomitantly escalating cascade of pro-inflammatory responses that exacerbate oxidative stress.2,6,14,15 This report demonstrates that soluble LOX-1 was increased in sera from subjects and was mirrored by increased ox-LDL levels; ox-LDL is a commonly assessed serum marker for OS. The increased ox-LDL was exclusively increased in rapidly progressing subjects with ALS; ox-LDL was also increased in rapidly progressing subjects with ALS that express the C9orf72 mutation (data not shown). The levels of ox-LDL fell and rose following infusions and cessation, respectively. IL-2 treatment alone did not appear to be beneficial at the given dose and frequency.9 The fall or rise of ox-LDL levels mirrored the stabilization or deterioration of the subject’s clinical status. These similar patterns were observed for the APPs; APP levels fell and rose following infusion and cessation, respectively, of Tregs. All three APPs were increased in rapidly progressing subjects with ALS that express the C9orf72 mutation (data not shown). Serum soluble LOX-1 and ox-LDL were increased in mild cognitively impaired and Alzheimer’s disease subjects (data not shown).16

LOX-1, the main receptor for ox-LDL, binds, internalizes, and degrades ox-LDL in macrophages and other cells. This is consistent with the finding that as serum ox-LDL increases so does serum LOX-1; at higher concentrations, ox-LDL upregulates LOX-1 expression.17 Although the ox-LDL levels in subject 3 are elevated relative to subjects 1 and 2, so are the mean and standard deviation. However, the levels of ox-LDL in subject 3 remained relatively stable and that is reflected in the clinical scores; the important finding is that the ox-LDL levels between subjects is relative to their respective controls.

LOX-1 expression is normally low, but ox-LDL and TNF-α increase LOX-1 expression. Binding of ox-LDL to LOX-1 activates NF-κB in macrophages, which in turn stimulates the downstream production of IL-1β and IL-18. TNF-α, IL-1β, and IL-18 are pro-inflammatory cytokines elevated in the blood of subjects with ALS.15 Macrophage affinity for unmodified LDL particles is low but is increased in the presence of oxidized LDL, thus exacerbating the peripheral pro-inflammatory milieu.12 It has been postulated that elevations in soluble LOX-1 may reflect increased expression of the membrane-bound form.18 Another possible explanation for the increased serum soluble LOX-1 is that membrane-bound LOX-1 is cleaved from the surface of activated pro-inflammatory monocytes/macrophages; it is known that activated macrophages/monocytes shed surface proteins. The initiating sources of increased OS are unclear, but 4-hydroxy-2, 3-nonenal, another marker of OS, was elevated in the sera and spinal fluid of subjects with ALS and positively correlated with disease burdens.19,20 Thus, soluble LOX-1 may indeed be a biological indicator of activated monocytes/macrophage that are involved with the ever-intensifying pro-inflammatory responses in subjects with ALS.

The current study also showed that serum APPs were increased in subjects with ALS and that these increased levels were attenuated by Treg therapy. Interestingly, the two rapidly progressing subjects exhibited elevated sCD14 levels that were suppressed by Treg infusions whereas the slowly progressing subject had normal levels of sCD14 although the levels still trended downward during the first round of infusions; slowly progressing subjects have little to no increased sCD14 in their sera.8 Treg infusions also suppressed LBP and CRP levels in the sera of these subjects.

Subjects with ALS have chronic and persistent low-grade systemic inflammation that is associated with a worse disease prognosis.21 This report confirms that there are ongoing OS and pro-inflammatory responses in subjects with ALS, and that expanded autologous Treg therapy suppresses these responses while potentially stabilizing the subject’s clinical status. ox-LDL levels correlated with disease progression rates; the higher the levels, the more rapid the progression. Thus, in addition to APPs, LOX-1 and ox-LDL are possible candidates to monitor the effectiveness of immunomodulatory therapies in subjects with ALS.8,9

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Author Contributions

DRB, JRT, and SHA contributed to the conception and design of the study. DRB and SW contributed to the acquisition and analysis of data. DRB, JRT, AF, ADT, WZ, and SHA contributed to drafting the text or preparing the figures.

Potential Conflict of Interest

DRB declares a conflict of interest as a consultant with Implicit Bioscience and Coya Therapeutics, Inc. ADT declares a conflict of interest as a consultant with Implicit Bioscience and Coya Therapeutics, Inc. SHA declares a conflict of interest as a consultant with Implicit Bioscience and scientific advisory board chair of Coya Therapeutics, Inc. The remaining authors have no conflict of interest.

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