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Review

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Oxidative stress-mediated inflammation promotes the pathogenesis of amyotrophic lateral sclerosis

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Abstract

Neuroinflammation in amyotrophic lateral sclerosis (ALS) is characterized by activation of monocytes/macrophages and T lymphocytes in the periphery and microglia and astrocytes within the central nervous system. This review emphasizes the role of oxidative stress in promoting systemic inflammation and the early stages of neurodegeneration. Motor axon terminals of ALS patients have significantly increased intraluminal calcium and dysfunctional mitochondria, increasing the formation of lipid peroxides and ferroptosis programmed cell death. Serum lipid peroxides and acute phase proteins are elevated, and regulatory T lymphocytes (Tregs) are dysfunctional, impairing immune-mediated neuroprotection. Macrophages are pro-inflammatory; the expression of genes involved in inflammation is increased in peripheral monocytes/macrophages of ALS patients. Suppressing these multiple components of inflammation is an important therapeutic goal and provides an opportunity to interrupt the self-propagating cytotoxic cycle. Two clinical trials with autologous infusions of ex vivo expanded Tregs have been safe and well tolerated, with promising clinical results associated with suppression of pro-inflammatory lipid peroxides.

Keywords: Amyotrophic lateral sclerosis, ferroptosis, oxidative stress, lipid peroxides, 4-hydroxynonenal, oxidized LDL, acute phase proteins, regulatory T lymphocytes

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a devastating disease characterized by relentless degeneration of upper and lower motor neurons. Following symptom onset, patients survive an average of 3 to 5 years. The widespread use of non-invasive ventilation, early attention to proper nutrition, and promotion of safe exercises and prevention of falls have made a difference in patients' quality and length of life, but the ability of therapy to significantly modify the pathogenesis of the disease is still limited. Although the cause(s) and pathogeneses of ALS are still to be completely defined, advances in gene sequencing have led to the discovery of mutant genes that cause ALS, many of which encode proteins that compromise immune function. In fact, linkage of these mutations to ALS provides the most cogent evidence that immune dysregulation contributes to the pathogenesis of ALS[1]. Even in 90% of ALS patients without a positive family history of the disease, innate and adaptive immune cells are pro-inflammatory and modulate motor neuron injury and disease progression.

In ALS, motor neuron cell injury and death are initiated by multiple cell-autonomous pathways leading to misfolded proteins, mitochondrial dysfunction with increased intramitochondrial calcium, oxidative stress, impaired autophagy and altered RNA metabolism[1]. In ALS transgenic mouse models, injured motor neurons interact with surrounding glia and peripheral and central immunomodulatory cells, which receive the message to protect and repair. The initial glial and immune reactivities are neuroprotective[2]. However, as the intraneuronal injury process continues, the message from the motor neuron changes. The new message promotes a pro-inflammatory cascade. Most investigations of neuroinflammation have focused on the central nervous system (CNS), where microglia and astrocytes are pro-inflammatory; neuroprotection is impaired, and release of pro-inflammatory cytokines promotes further injury to the motor neuron. However, it is becoming increasingly clear that peripheral inflammation may contribute significantly to motor neuron injury and cell death[3]. In the following sections, the factors that initiate and sustain both peripheral and CNS inflammation are reviewed, and our clinical efforts to slow the evolving pathogenesis of disease in ALS patients are described.

PATHOLOGICAL CHANGES IN MOTOR AXON TERMINALS

The motor neuron projects outside the blood-brain barrier to the muscle and the neuromuscular junction may be one of the early sites of pathology, initiating a "dying back" from the neuromuscular junction[4]. To determine the potential contribution of motor neurons in initiating widespread oxidative stress, we used electron microscopy to examine the ultrastructure of ALS patient motor nerve terminals in muscle biopsy specimens. Seven ALS patients, ten non-denervating disease control subjects, and five patients with denervating neuropathies were studied[5]. Following oxalate-pyroantimonate fixation to preserve in situ calcium distribution, we noted swollen calcium-containing mitochondria, increased density of synaptic vesicles, increased active-zone vesicle density, and increased intraluminal calcium precipitates within membranous organelles. These changes were not present in either denervating or non-neuropathic controls. There was minimal Schwann cell envelopment of the ALS motor terminals compared to the neuropathic controls, possibly due to impaired neuronal Schwann cell signaling [Figure 1].

At the time we published these findings, the concept of ferroptosis as a significant pathway of programmed cell death had not been recognized. In 2012, ferroptosis was described as a cause of cell death distinct from apoptosis, necrosis, and necroptosis[6]. Ferroptosis is now recognized as a form of iron-dependent regulated cell death driven by lipid peroxidation. Polyunsaturated fatty acids (PUFAs), such as arachidonic acid within phospholipid-containing membranes, undergo peroxidation, which yields neurotoxic moieties such as 4-hydroxynonenal (HNE). Free PUFAs are not themselves drivers of ferroptosis and are not intrinsically toxic. It is the accumulation of oxidized PUFA-containing lipids within cell membranes that drives lipid

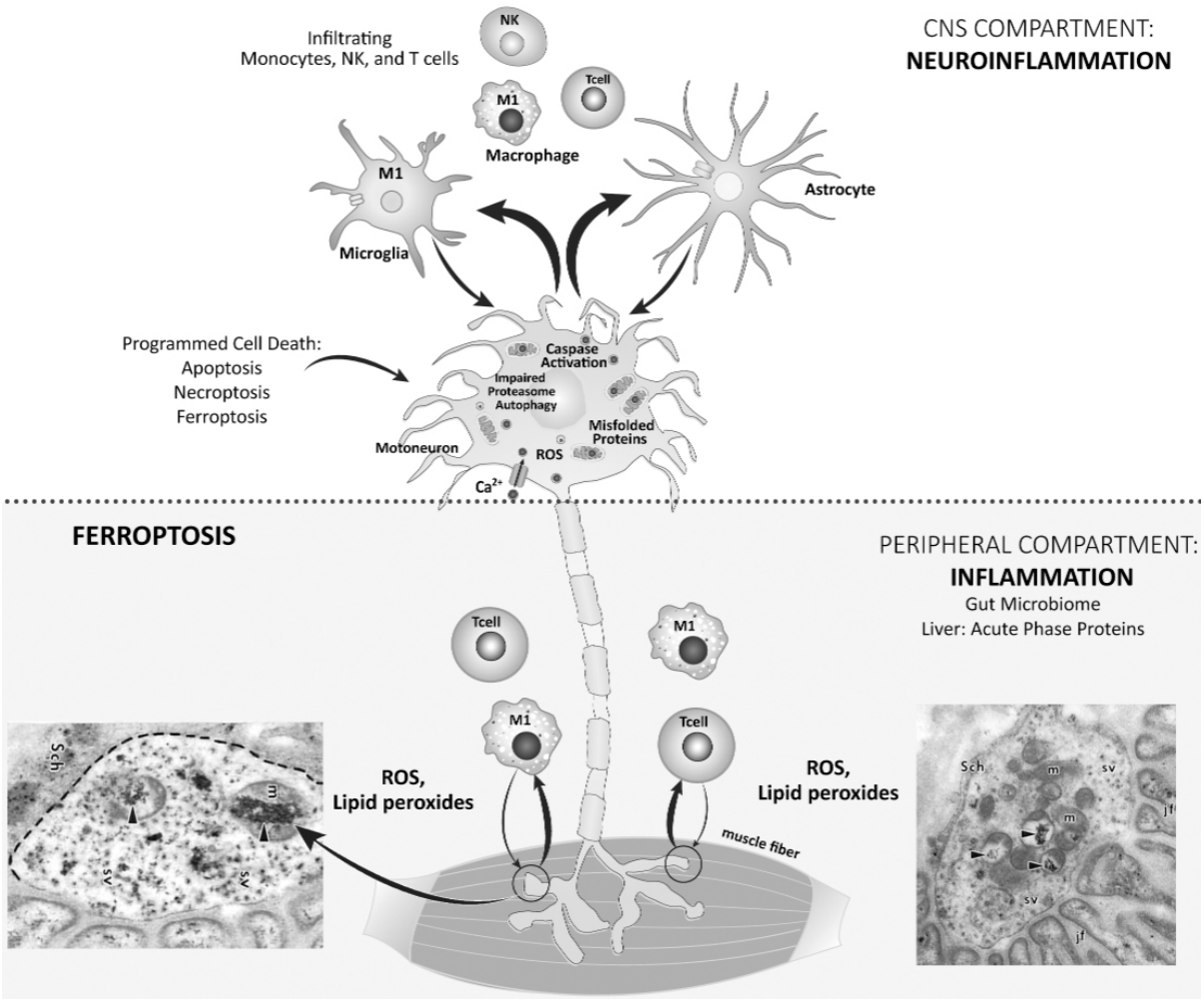

Figure 1. Systemic inflammation is initiated in motor axon terminals of the peripheral compartment In ALS patients. On the lower panel L- and R-sides we present ultrastructural illustrations of terminals from two different ALS patients[5]. Calcium precipitates are present in intraluminal membrane bound organelles and mitochondria. The dysfunctional mitochondria induce reactive oxygen species (ROS) and lipid peroxides, and promote ferroptosis, which activates macrophages and T lymphocytes. In turn, the activation by oxidized lipids induces macrophage production and secretion of IL-6, IL-1β, and TNF-α, followed by synthesis and release of acute phase proteins by the liver. The gut microbiome directly interacts with the immune system at this early stage. In the upper panel neuroinflammation in the CNS compartment is the consequence of "dying back" from the axon terminal as well as the spread of pro-inflammatory immune cells from the periphery to the CNS. The spread of pro-inflammatory immune cells from the CNS back to the periphery then promotes a self-propagating cytotoxic cascade. The result is activation of programmed cell death pathways involving apoptosis, necroptosis, as well as ferroptosis (Modified from Figure 1, Ref. 3[3]). ALS: Amyotrophic lateral sclerosis; CNS: central nervous system.

peroxide formation and ferroptosis.

The most basic characteristics of ferroptosis are iron overload and a lethal accumulation of intracellular lipid peroxides and reactive oxygen species (ROS)[7]. The peroxidation of membrane-bound PUFA-containing lipids is driven by both the labile iron pool facilitating the Fenton reaction, which propagates non-enzymatic lipid peroxidation[8], and by iron-dependent enzymes, such as arachidonate lipoxygenases, that initiate the formation of lipid hydroperoxides as substrates for the Fenton reaction[9]. The significantly elevated serum levels of HNE, as well as the increased levels of ferritin and decreased levels of transferrin as reported in a meta-analysis review, provide evidence for ferroptosis as a cell programmed cell death pathway in ALS[10]. Other sources of iron result from the compromised blood-brain barrier and the entry of

hemoglobin into the CNS parenchyma[11]. Microvascular lesions reported in SOD1G93A mice contain blood-derived hemoglobin that releases free iron, which can catalyze the formation of neurotoxic free radical species[12]. Ferroptosis is also immunogenic and can promote inflammation[13]. Ferroptosis promotes the release of inflammatory factors and damage-associated molecular patterns, enhancing the pro-inflammatory environment, inducing inflammation by releasing IL-33, and activating additional pathways[14]. Ferroptosis has also been reported to accelerate the metabolism of arachidonic acid, which stimulates the synthesis of bioactive inflammatory mediators such as prostaglandins and leukotrienes.

Mitochondrial changes in ALS are not limited to motor axon terminals. In ALS mouse models and ALS patients, motor neuron mitochondria are dysfunctional and intracellular calcium is dysregulated[15]. Mitochondria are reported as fragmented. Calcium homeostasis is impaired, and respiratory chain activity and ATP synthesis are decreased. Levels of glutathione (GSH) are significantly reduced in ALS. In one study, GSH levels were reduced to 22.25 ± 0.99 μg/mL, compared to controls 131.54 ± 12.05 μg/mL[16]. With significantly reduced levels of GSH, mitochondria can initiate or enhance cell susceptibility to ferroptosis by promoting lipid peroxidation or enhancing ROS production. Ultimately, lipid peroxides spread to the plasma membrane, where they trigger the rupture of the plasma membrane and cell death. The electron transport chain may also be involved secondary to the leakage of electrons that produce superoxide and H_2O_2, which can then react with Fe (II) to drive Fenton chemistry and non-enzymatically-mediated lipid peroxidation. Ultimately the significantly increased levels of lipid peroxides provide the most compelling evidence for ferroptosis-mediated programmed cell death in ALS.

MONOCYTES/MACROPHAGES

Innate immune myeloid cells are the first line of defense, mediating both anti-inflammatory and pro-inflammatory functions. Monocyte/macrophage myeloid cells are often designated as anti-inflammatory M2 or pro-inflammatory M1. However, myeloid cells do not exist either as M2 or M1 but are an overlapping continuum from anti-inflammatory to pro-inflammatory phenotypes. A similar state exists within the CNS where innate immune cells, namely microglia, can express a continuum of anti- or pro-inflammatory states. Monocytes can be differentiated from inducible progenitor stem cells and can then be differentiated into anti-inflammatory or pro-inflammatory macrophages[17]. The anti-inflammatory macrophages are neuroprotective in vitro and suppress pro-inflammatory cytokine signaling, while the pro-inflammatory macrophages are neurotoxic in vitro and enhance pro-inflammatory cytokine synthesis and secretion. Thus monocytes have the potential to be protective or toxic, with the phenotype dictated by environmental signaling.

In ALS mouse models, microglia are initially neuroprotective in the early stages of the disease and subsequently transit to a pro-inflammatory state[18]. The specific molecular determinants of this transition are far from clearly understood but appear to derive from communications between the motor neuron projections outside the blood-brain barrier at the neuromuscular junction and the peripheral immune cells. Within the CNS, the dialogue is between injured motor neurons and glia. There is also a continual dialogue between peripheral and CNS compartments. Neuroprotection and neurotoxicity are overlapping responses of myeloid populations to signals initiated by motor neurons which may vary with intensity of injury. Both peripheral and central compartments become readily involved as immunomodulatory signaling spreads from the periphery to CNS and from CNS back to the periphery.

In ALS patients, an understanding of the specific myeloid phenotypes at the earliest stages of the disease is presently limited. We can only determine the phenotypes after the disease process is underway, and then primarily in the peripheral compartment. In ALS, serum monocytes are activated and pro-inflammatory. To

define the pro- or anti-inflammatory signaling of peripheral circulating monocytes in an unbiased manner, we used high-throughput deep RNA sequencing (RNA-seq)[19]. Our deep RNA-seq and qRT-PCR data demonstrated that monocytes isolated from patients with ALS expressed a unique genetic profile associated with pro-inflammatory immune responses [Figure 2]. Interleukin-1β (IL-1β), interleukin-8 (IL-8), nicotinamide phosphoribosyltransferase (NAMPT), FosB proto-oncogene-AP-1 transcription factor (FOSB), and CD83 were prominent upregulated disease-related genes involved in ALS monocyte-mediated pro-inflammatory responses. IL-1β is a major inflammatory cytokine produced by inflammasome activation in monocytes/macrophages. Blockade of the IL-1β receptor decreases microglial activation, reduces motor neuron loss and prolongs survival in ALS mice[20]. NAMPT promotes interleukin-6 (IL-6) production. Silencing *NAMPT* gene expression in monocytes reduces IL-6 production, decreases T helper 17 cells and decreases infiltration of monocytes/macrophages[21]. CD83 is involved in regulating antigen presentation, and in monocytes of rapidly progressing ALS patients, CD83 promotes upregulation of antigen presentation. The expressions of several cytokines and chemokines, namely, IL-8, CXC motif chemokine ligand 1 (CXCL1), and CXCL2, are increased in $CD14^+$/ $CD16^-$ ALS monocytes and promote migration to sites of inflammation[22]. Peripheral monocytes/macrophages and lymphocytes infiltrate the CNS and can combine with central inflammatory responses to promote a self-propagating amplification of inflammation and injury.

Our unbiased investigation clearly documents the pro-inflammatory phenotype of ALS monocytes. Although several studies assert that pro-inflammatory cytokines are elevated in the serum of ALS patients, the reported results from ELISA assays are quite variable. However, a meta-analysis did report that the pro-inflammatory cytokines IL-6, IL-1β, and tumor necrosis factor-α (TNF-α) are increased in ALS blood[23]. The increased gene expression of inflammatory markers adds to the compelling evidence that ALS monocytes are skewed toward a pro-inflammatory phenotype and could influence rates of disease progression; monocytes of rapidly progressing patients expressed more inflammation-related differentially expressed genes than slowly progressing patients.

Our further studies documented that ALS monocytes are more activatable than monocytes from healthy controls[24]. We differentiated peripheral monocytes into pro-inflammatory and anti-inflammatory macrophages and determined their gene expressions. ALS pro-inflammatory-derived macrophages produced more pro-inflammatory cytokines than healthy control pro-inflammatory-derived monocytes. IL-6 mRNA and TNF-α mRNA expressions were significantly increased in ALS macrophages, as were IL-6 and TNF-α proteins, compared to healthy control activated macrophages. The increased IL-6 protein correlated with the burden of ALS disease, and TNF-α protein correlated with rates of disease progression. Collectively these data document the loss of macrophage-mediated neuroprotection and the predominant neurotoxic pro-inflammatory phenotype of ALS monocytes/macrophages.

The factors initiating the pro-inflammatory phenotype of peripheral monocytes/macrophages in ALS have not been clearly delineated. The presence of significantly increased levels of HNE lipid peroxides as well as oxidative stress could drive the activation of peripheral monocytes/macrophages, enhancing the synthesis and release of pro-inflammatory cytokines[5]. In turn, the activation of macrophages can enhance the production of free radicals and the synthesis of HNE [Figure 3]. The ferroptosis-mediated lipid peroxide synthesis in ALS motor axon terminals could be an initiating event, but this remains to be proven. The gut microbiome could also be an early event initiating the self-propagating cycle of HNE-pro-inflammatory cytokine reactivity[3]. Suppressing ferroptosis or the resulting pro-inflammatory myeloid cells certainly should represent an important component of any disease-modifying therapy for ALS.

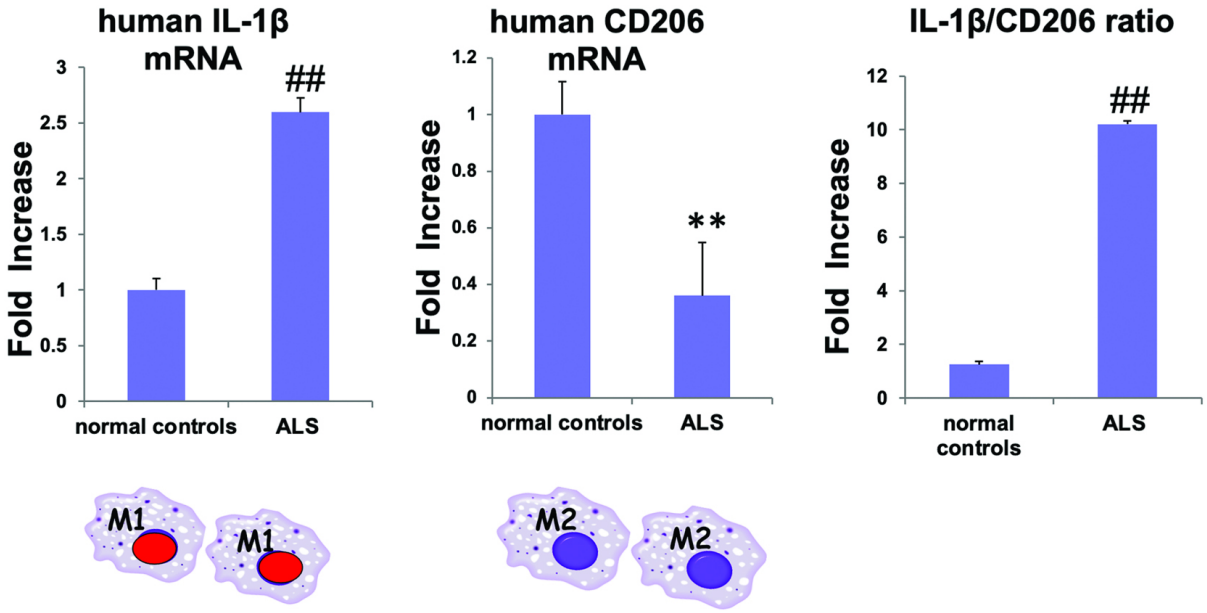

Figure 2. ALS peripheral blood monocytes are pro-inflammatory. Expression of pro-inflammatory cytokine IL-1β gene as a marker of M1 macrophages in ALS monocytes is compared to normal control monocytes in the left panel. The expression of CD206 gene as a cell-surface protein marker of M2 is compared with normal control monocytes in the middle panel. The ratio of IL-1β/CD206 gene expressions is presented in the right panel. Monocytes of ALS patients (n = 43) were verified by qPCR and normalized to β-actin and normal control monocytes (n = 22). Error bars indicate the standard error, $^{**}P < 0.01$ and $^{**}P < 0.05$. ALS: Amyotrophic lateral sclerosis.

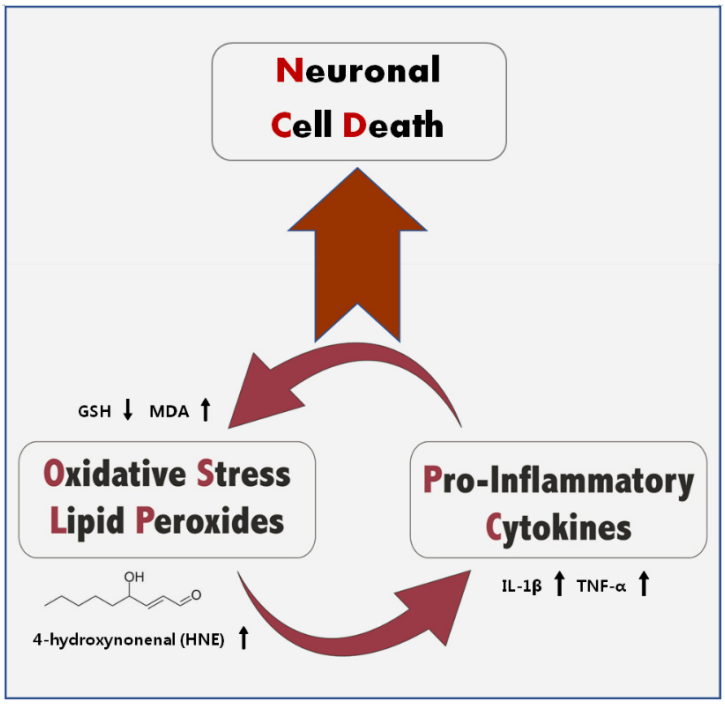

Figure 3. The significantly increased HNE lipid peroxides as well as oxidative stress could drive the activation of peripheral monocytes/macrophages enhancing the synthesis and release of pro-inflammatory cytokines. In turn, pro-inflammatory cytokines promote the production of free radicals and the synthesis of HNE. This vicious circle promotes the motor neuronal cell death in ALS including ferroptosis. IL-1β: Interleukine-1β; TNF-α: tumor necrosis factor α; GSH: glutathione; MDA: malondialdehyde; HNE: 4-hydroxynonenal; ALS: amyotrophic lateral sclerosis.

PERIPHERAL BIOMARKERS OF *INFLAMMATION*-ACUTE PHASE PROTEINS

Activation of circulating monocytes to a pro-inflammatory state induces the shedding of membrane-bound CD14 (mCD14), increasing serum levels of the acute phase protein (APP) soluble CD14 (sCD14) and enhancing the production of interleukins and TNF-α[25]. The pro-inflammatory state of macrophages was also associated with the production of the APP, C reactive protein (CRP)[26]. Elevated serum CRP levels were associated with faster disease progression in ALS patients[27]. Lipopolysaccharide binding protein (LBP) is another APP whose synthesis is increased in ALS and is enhanced as a component of the acute phase response to tissue trauma or inflammation[28]. The acute phase response accompanies chronic as well as acute inflammatory states. The APPs are synthesized and secreted from liver hepatocytes stimulated by inflammatory cytokines including IL-6, IL-1β, and TNF-α.

In ALS patients, serum levels of APPs, including sCD14, LBP, and CRP, are elevated and correlate positively with increased disease burden and faster disease progression[29]. Levels of APPs predicted survival times. In a 3-year follow-up, 72% of the patients with sCD14 levels above the Receiver Operating Characteristic cutoff values were deceased, whereas only 28% below the cutoff were deceased. Thus, the increased levels of APPs in ALS patients accurately reflect disease burden, progression rates, and survival times. The APPs were not elevated in the blood of patients with Alzheimer's Disease (AD), Parkinson's Disease (PD) or frontotemporal dementia (FTD). The increased levels of these liver-synthesized proteins confirm the concept of ALS as a widespread systemic disorder, which is distinctive from other neurodegenerative disorders such as AD, PD, or FTD. The fact that the acute phase response and the increased APPs produced by the liver are initiated by IL-6, IL-1β, and TNF-α, clearly suggests that activated pro-inflammatory macrophages, which release these cytokines, also play a key role in stimulating the increased synthesis of APPs.

BIOMARKERS OF OXIDATIVE STRESS- LIPID PEROXIDE HNE

Activation of macrophages in ALS not only enhances the synthesis and secretion of pro-inflammatory cytokines, but also increases markers of oxidative stress including superoxide anion and nitric oxide. These latter free radicals can non-enzymatically lead to lipid peroxides, specifically HNE[30]. A meta-analysis of markers of oxidative stress in ALS showed significantly increased blood levels of 8-hydroxyguanosine, malondialdehyde, and advanced oxidation protein product[31]. Levels of GSH were significantly reduced. Thus, both pro-inflammatory responses and markers of oxidative stress are increased; the pro-inflammatory responses exacerbate oxidative stress, and the oxidative stress exacerbates pro-inflammatory responses[32,33]. One of the major markers of oxidative stress in neurodegenerative diseases is HNE, which results from peroxidation of PUFAs, especially arachidonic acid. HNE promotes the formation of toxic protein adducts, which are increased in the spinal cord and ventral horn motor neurons in ALS patients[34].

HNE was significantly elevated in the cerebrospinal fluid (CSF) of sporadic ALS (sALS) patients; levels of HNE were 1.82 ± 0.15 ng/mL in 186 sALS patients compared with 0.51 ± 0.05 ng/mL in 236 patients with other neurological diagnoses[35]. Levels of HNE were considerably less increased in patients with familial ALS, PD, and AD or patients with immune or nonimmune neurological diseases. No differences were noted in sALS patients comparing those with limb *vs.* bulbar onset. 130 limb-onset sALS patients had mean CSF HNE values of 1.77 ± 0.17 ng/mL, and 56 patients with bulbar-onset sALS had levels of 1.79 ± 0.25 ng/mL. Nanomolar concentrations of free HNE or sALS.

HNE is neurotoxic in vitro. CSF samples containing equivalent HNE or HNE adducts were toxic to a motor neuron cell line in vitro. Incubation with the VSC4.1 cell line caused significant cell loss, which could be rescued by co-incubation with GSH[35]. HNE is also cytotoxic *in vivo*. Intrathecal administration of HNE to

rats increased CSF HNE and was toxic to spinal motor neurons. Total calcium was reduced in the surviving, structurally intact motor neurons, but only if GSH synthesis was concomitantly inhibited. Thus the *in vivo* toxic effects of HNE are dependent on a reduction of GSH. GSH is neuroprotective and reduced GSH level causes increased CSF HNE and enhanced motor neuron loss[36].

To determine whether oxidative stress was systemically increased in ALS, we analyzed serum as well as CSF levels of HNE using high-performance liquid chromatography and ELISA and compared them with levels in disease and normal control subjects[37]. HNE levels were significantly elevated in the sera and spinal fluid of sALS patients compared with control populations. sALS HNE serum and CSF levels were elevated above all control values [Figure 4]. CSF HNE levels were significantly increased compared with serum HNE levels in sALS patients ($P < 0.001$). As the burden of the disease increased from early to mid to late stages, HNE was increased and correlated with disease extent but not rates of progression[37]. HNE protein adducts have been reported to result from increased intraneuronal calcium and mitochondrial dysfunction, thereby supporting an important role for motor neurons in initiating the cytotoxic environment[38,39].

BIOMARKERS OF OXIDATIVE STRESS- OX LDL

Oxidative stress is an important trigger of lipid oxidation[40]; the presence of oxidized low-density lipoprotein (ox-LDL) in the serum reflects significantly increased inflammation. Lectin-like oxLDL receptor-1 (LOX-1) is the main receptor for oxLDL on macrophages, and internalizes and then degrades ox-LDL. Scavenger receptors on macrophages can also bind oxLDL. Binding of ox-LDL to LOX-1 on macrophages activates NF-κB, stimulating the production of the pro-inflammatory cytokines IL-1β and IL-18[32]. In ALS patients, serum levels of oxLDL are increased, primarily in patients with rapidly progressing disease[32] [Figure 5].

IMMUNOMODULATORY THERAPY FOR ALS

Given the evidence for neuroinflammation and oxidative stress systemically as well as within the CNS compartment, our own therapeutic efforts have been to suppress activated macrophages and microglia as well as T-effector lymphocytes that promote neuroinflammation. We have focused on regulatory T lymphocytes (Tregs), which are the $CD4^{+}CD25^{high}FOXP3^{+}$ subpopulation of T-lymphocytes that promote neuroprotection by suppressing pro-inflammatory responses. Tregs are dysfunctional in ALS; their ability to suppress the release of cytokines from pro-inflammatory myeloid macrophages and microglia is impaired, as is their ability to suppress the proliferation of T-effector lymphocytes[41]. The failure to effectively suppress central and peripheral inflammation promotes the pathogenesis of the disease, increasing the rate of disease progression and disease burden.

Our discovery of the central role of Tregs in ALS came as a result of crossing the *mSOD1* transgenic mouse model of ALS with a $Rag2^{-/-}$ transgenic mouse and a $CD4^{-/-}$ transgenic mouse. We had assumed that the offspring mSOD1/$Rag2^{-/-}$ or SOD1/$CD4^{-/-}$ double transgenic mice would live longer because we had deleted pro-inflammatory T-effector cells. However, the double transgenic mice died earlier than expected, suggesting the loss of a neuroprotective population. In these transgenic mice, pro-inflammatory macrophages and microglia were significantly increased, as were CNS pro-inflammatory cytokines. Adoptive transfer of mouse Treg cells into these doubly transgenic mice prolonged survival by 88%, and pro-inflammatory phenotypes were suppressed[2,42].

In patients with ALS, inflammation is associated with decreased numbers of circulating Tregs, and decreased expression of FoxP3, the key transcription factor in the development and function of Tregs. As a result, neuroprotective functions were diminished. The ability of circulating Tregs to suppress either

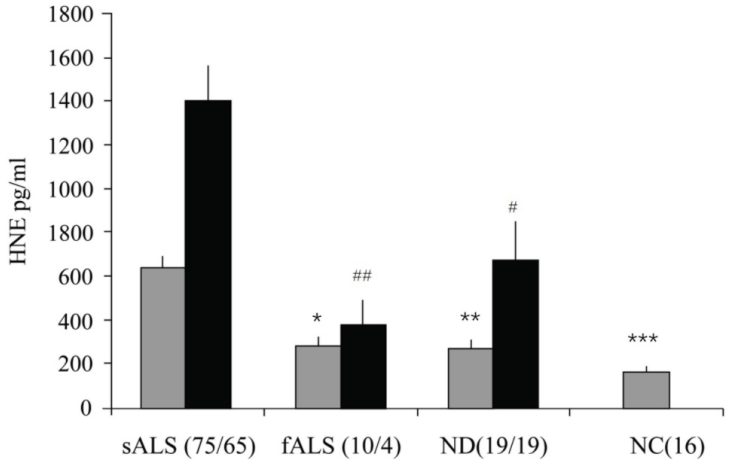

Figure 4. Serum and CSF levels of HNE in patients with sporadic ALS (sALS), familial ALS (fALS), non-ALS neurodegenerative diseases (ND) compared with normal controls (NC). Serum HNE levels in 75 patients with sALS (grey columns) were significantly increased compared to NCs ($P < 0.0001$, 10 fALS ($P < 0.05$), and 19 NDs ($P < 0.001$). CSF HNE levels (black columns) were significantly increased compared with serum HNE levels in sALS patients ($P < 0.001$). ***$P < 0.0001$, **$P < 0.001$, *$P < 0.05$, compared with sALS sera; ##$P < 0.001$, #$P < 0.05$, compared with sALS CSF (Modified from Figure 1, Ref. 37[37]). CSF: Cerebrospinal fluid; HNE: 4-hydroxynonenal.

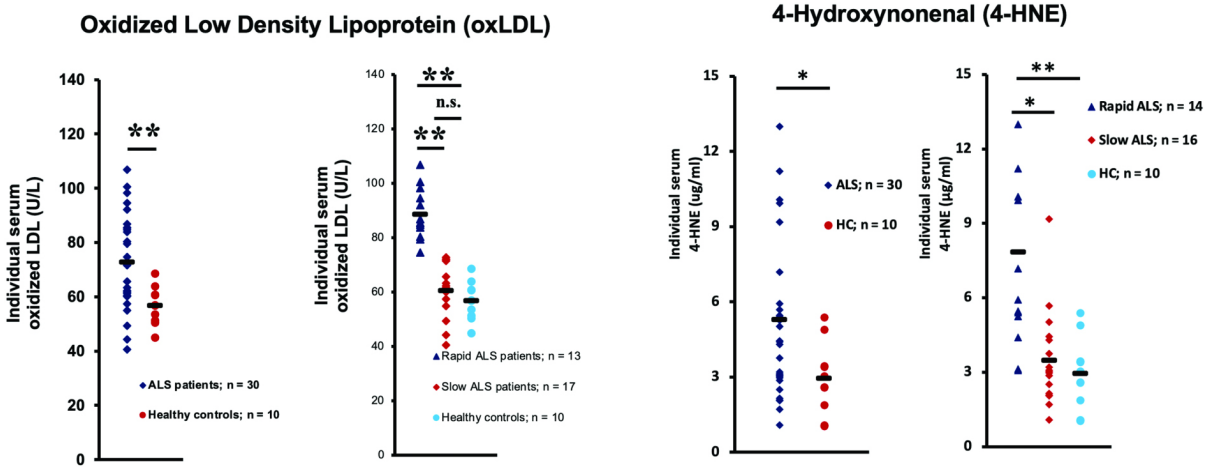

Figure 5. Serum oxLDL levels were increased in ALS compared to healthy controls. When fast progressing ALS patients were compared with slowly progressing patients, only fast progressors differed from controls. Slowly progressing patients did not differ from controls (**$P < 0.001$; n.s.= not significant). Serum HNE levels were also significantly different from controls (*$P < 0.05$), but only fast progressors differed from controls (**$P < 0.01$). Fast *vs.* slow progression was based upon changes of > 1.5 points *vs.* < 1.5 points on our AALS clinical scale[45] (Modified from Figure 1, Ref. 32[32]). ALS: Amyotrophic lateral sclerosis; HNE: 4-hydroxynonenal.

myeloid cells or T-effector cells was impaired. The more advanced the disease, the less the suppressive function. Reduced FoxP3 expression levels predict rapidly progressing disease and attenuated survival; increased FoxP3 expression levels were associated with longer survival. The impaired suppressive function also predicted shortened survival. If Tregs suppressive function was increased at baseline, three years later, 13% of ALS patients had expired, whereas decreased Treg suppressive function at baseline resulted in 35% of ALS patients having expired[43]. Thus, Treg suppressive functions are impaired in ALS, but withdrawal of Tregs from the blood of ALS patients and expansion ex vivo in the presence of IL-2 restored and enhanced their suppressive functions[44].

Autologous infusions of these expanded Tregs, together with subcutaneous IL-2 injections, formed the basis of two ALS clinical trials. In the first trial, three patients were selected to participate in an FDA-approved pilot study of autologous infusions of expanded Tregs[45]. Each of the patients was progressing at a different rate: the first patient with arm onset was progressing at an intermediate rate, the second patient with bulbar onset was progressing at a rapid rate, and the third patient was progressing at a slow rate. Regulatory T lymphocytes from all three patients had decreased ability to suppress T-effector proliferation in vitro. However, following ex vivo expansion, the in vitro suppressive function in all three patients was restored. The patient's own expanded Tregs were infused intravenously every 2 weeks for a total of four infusions, together with subcutaneous injections of IL-2 three times weekly. When the infusions stopped, even though IL-2 was continued, the patient's clinical status deteriorated. After a 4-6 months hiatus, four monthly autologous infusions again slowed clinical progression. Once again, when infusions were stopped, the clinical condition deteriorated.

In all three patients, infusions were safe and well-tolerated and slowed progression rates during early and later stages of the disease. Treg numbers and suppressive function increased after each infusion and correlated with slowing of disease progression. However, the limited duration of the clinical benefit was initially unexplained. Only in retrospect did it become apparent that the serum biomarkers of oxidative stress, HNE and oxidized LDL provided a potential explanation. These lipid peroxide biomarkers were increased prior to Treg infusions, fell with Treg infusions and slowing of disease progression, rose again as disease progression accelerated in the absence of infused Tregs, then fell again when Tregs were reinfused[32]. Thus, the fall or rise of HNE and ox-LDL levels were effectively responsive to Treg infusions and mirrored the stabilization or deterioration of the subject's clinical status.

A Phase 2A study of autologous infusions of expanded Tregs in combination with subcutaneous IL-2 injections was undertaken at Houston Methodist and Massachusetts General Hospitals[46]. The trial was planned for 12 ALS patients enrolled in a 24-week double-blind placebo-controlled trial (RT) followed by a 24-week open-label extension (OLE). In the RT portion, Treg/IL-2 treatments were safe and well-tolerated with increased Treg suppressive function in the active group. However, the COVID-19 pandemic reduced the number of ALS patients enrolled to six and precluded a meaningful statistical comparison of the efficacy of Treg infusions versus control infusions. However, the six patients plus two additional OLE-only ALS participants were able to complete the 24-week OLE; Treg/IL-2 treatments were safe and well-tolerated and Treg suppressive function and numbers were increased. Six patients showed minimal clinical progression in the OLE with the ALS Functional Rating Scale (ALSFRS) decreasing by only 2.7 points over 24 weeks, while two patients were unresponsive to Treg/IL-2 infusions with the ALSFRS decreasing by 10.5 points over 24 weeks [Figure 6]. These two rapidly progressing patients had elevated levels of two markers of peripheral inflammation (IL-17C and IL-17F) as well as a marker of oxidative stress, oxLDL. Normal levels of IL-17C and 17F, as well as oxLDL, were present in the six participants that responded to Treg/IL-2 infusions with slowed progression. The two ALS participants that were unresponsive to Treg/IL-2 infusions and progressed rapidly also had significantly increased levels of HNE, while the six responder participants had normal HNE levels [Figure 7]. Thus, in the open-label Phase 1 trial and the open-label extension of the Phase 2 trial, the biomarkers of oxidative stress were reduced, paralleling the responsiveness to therapy; unresponsiveness of Treg/IL-2 infusions to significantly elevated levels of HNE and oxLDL was associated with lack of therapeutic benefit. Levels of neurofilament light were unchanged throughout both studies and did not serve either as a prognostic or responsiveness biomarker. HNE and oxLDL not only contribute to the pathogenesis of the disease, but also serve as meaningful biomarkers of responsiveness to immunomodulatory therapy.

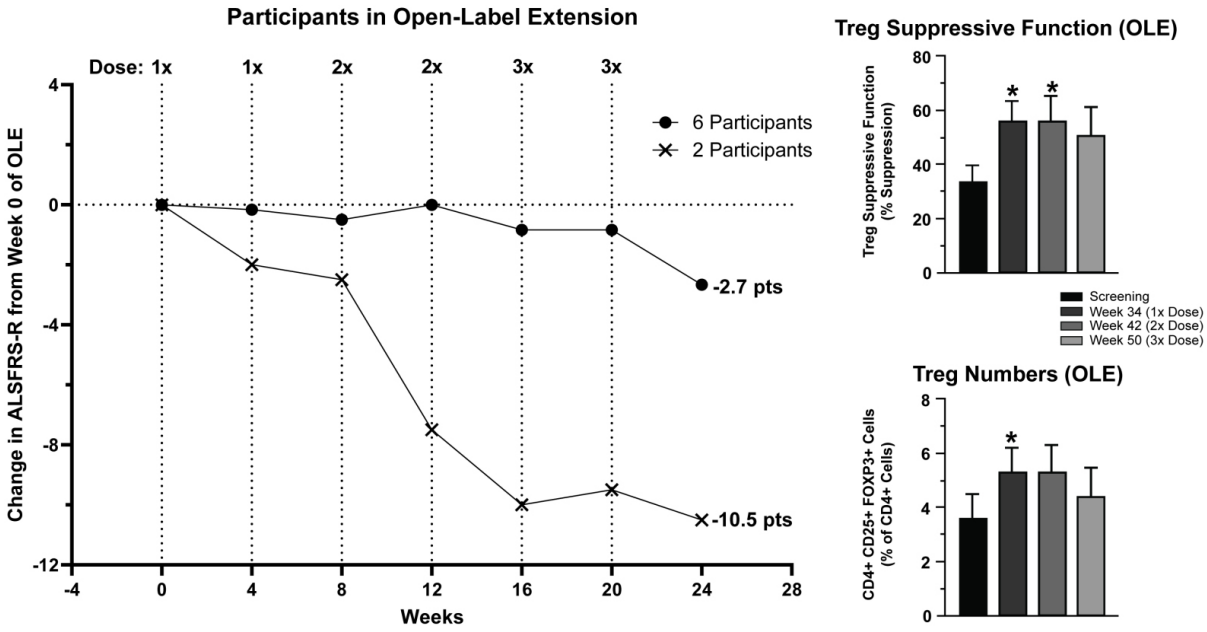

Figure 6. Treg numbers and suppressive function of Tresponder proliferation and disease progression in ALS patients in the Open Label Extension (OLE) of the Phase2A study[46]. The dose escalation was 1X, 2X, and 3X q2 months during the 24 weeks of the Open Label Period. Both Treg numbers and suppressive functions were increased (*$P < 0.05$) for at least the first 2 weeks. During the OLE the progression rate was slowed in the 6 ALS patients that appeared to have responded to the Treg/IL-2 infusions as monitored by the ALS Functional Rating Scale-Revised; whereas 2 ALS patients were unresponsive to the Treg/IL-2 infusions. ALS: Amyotrophic lateral sclerosis.

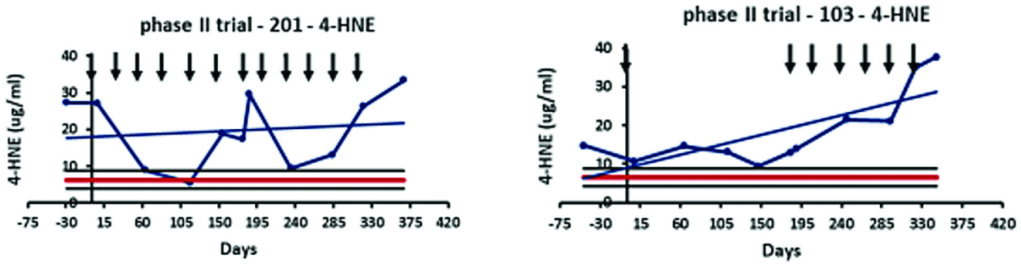

Figure 7. Serum HNE levels were evaluated throughout the Phase 2 A trial. HNE was significantly elevated in the 2 ALS patients who progressed rapidly despite escalating doses of Treg/IL-2 infusions during the Open Label Extension (OLE). The down arrows indicate dates of infusions Patient 201 (Left) had received 12 monthly Treg/IL-2 infusions while patient 103 (Right) received 6 monthly infusions only during the OLE. Both patients progressed rapidly and both had markedly increased HNE. The 6 ALS patients whose progression appeared to have slowed during the OLE had HNE levels within the normal range averaging less than 10 μg/mL. The red line indicates the mean of healthy controls and the green lines ± 1 standard deviation. ALS: Amyotrophic lateral sclerosis; HNE: 4-hydroxynonenal.

CONCLUSIONS

Systemic inflammation drives the pathogenesis of the disease in ALS. Alterations at the neuromuscular junction represent the early stages of neurodegeneration. Axon terminal mitochondria are swollen and disrupted, and intraluminal and intramitochondrial calcium are increased and promote ferroptosis. Lipid peroxides and oxidative stress are increased, monocyte/macrophages and cytokines are pro-inflammatory, and Tregs are dysfunctional. Oxidative stress promotes pro-inflammatory immune activation, and pro-inflammatory immune activation promotes oxidative stress. Peripherally activated macrophages and T lymphocytes spread from the periphery to the CNS and from the CNS back to the periphery, amplifying microglia/T lymphocyte-mediated neuroinflammation and self-propagating neurotoxicity. Lipid peroxides

not only drive the systemic inflammation, but also are biomarkers of the ongoing inflammatory cascade. Dysfunctional Tregs fail to provide neuroprotection. However, following ex vivo expansion, Treg suppressive functions are restored; autologous infusions of expanded Tregs in two small open-label studies suppressed pro-inflammatory lipid peroxides with promising clinical results. Only a large double-blind placebo-controlled clinical trial can determine whether Treg/IL-2 autologous infusions can slow disease progression in ALS patients. Our studies suggest that suppressing peripheral oxidative stress-mediated inflammation may provide disease-modifying therapy in ALS.

DECLARATIONS

Author's contribution

The author contributed solely to the article.

Availability of data and materials

Not applicable.

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Conflicts of interest

"No conflicts of interest influencing the representation or interpretation of reported research results". However, I do have the following disclosures-none of which are involved with this manuscript. Mitsubishi Pharma- Scientific Advisory Board; Eledon- Consultant; Implicit, Inc - Consultant; Coya Therapeutics- Scientific Advisory Board; Coya Therapeutics- Treg data licensed from Methodist Research Institute.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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