**Abstract**

ALA (α-lipoic acid) is a natural, endogenous antioxidant that acts as a PPAR-γ (peroxisome-proliferator-activated receptor-γ) agonist to counteract oxidative stress. Thus far, the antioxidative and immunomodulatory effects of ALA on EAE (experimental autoimmune encephalomyelitis) are not well understood. In this study, we found that ALA restricts the infiltration of inflammatory cells into the CNS (central nervous system) in MOG (myelin oligodendrocyte glycoprotein)-EAE mice, thus reducing the disease severity. In addition, we revealed that ALA significantly suppresses the number and percentage of encephalitogenic Th1 and Th17 cells and increases splenic Treg-cells (regulatory T-cells). Strikingly, we further demonstrated that ALA induces endogenous PPAR-γ centrally and peripherally but has no effect on HO-1 (haem oxygenase 1). Together, these data suggest that ALA can up-regulate endogenous systemic and central PPAR-γ and enhance systemic Treg-cells to inhibit the inflammatory response and ameliorate MOG-EAE. In conclusion, our data provide the first evidence that ALA can augment the production of PPAR-γ in vivo and modulate adaptive immunity both centrally and peripherally in EAE and may reveal further antioxidative and immunomodulatory mechanisms for the application of ALA in human MS (multiple sclerosis).

**Key words:** α-lipoic acid, experimental autoimmune encephalomyelitis, immunomodulation, multiple sclerosis, proliferator-activated receptor-γ, regulatory T-cell

INTRODUCTION

MS (multiple sclerosis) is a chronic disease of the CNS (central nervous system) that primarily affects young adults. The pathogenesis of MS is characterized by inflammation, demyelination and axonal injury [1]. EAE (experimental autoimmune encephalomyelitis) is the most widely recognized animal model of MS. Autoreactive pathogenic Th cells (e.g. Th1 and Th17 cells) have been shown to be involved in the immunopathogenesis of both MS and EAE [2–4]. Activated T-cells recruit invading macrophages, resident astrocytes and microglia, leading to the release of inflammatory mediators and toxic molecules, including nitrogen free radicals [5] and/or ROS (reactive oxygen species) [6]. These substances contribute to axonal damage, which is followed by complement activation or the antibody-mediated phagocytosis of axons [7,8]. Considering the confirmed pathogenic roles of ROS and nitrogen free radicals in MS and EAE, antioxidants may have therapeutic potential by preventing free radical-mediated tissue destruction and inhibiting certain early pro-inflammatory events, such as T-cell activation and trafficking into the CNS [9].

**Abbreviations:** ALA, α-lipoic acid; CAF, complete Freund’s adjuvant; CNS, central nervous system; EAE, experimental autoimmune encephalomyelitis; FBS, fetal bovine serum; HBSS, Hanks’ balanced salt solution; HO-1, haem oxygenase 1; IFN, interferon; IL, interleukin; mAb, monoclonal antibody; MFI, mean fluorescence intensity; MMP, matrix metalloproteinase; MOG, myelin oligodendrocyte glycoprotein; MS, multiple sclerosis; NK, natural killer; PE, phycoerythrin; PLP, proteolipid protein; PPAR-γ, peroxisome-proliferator-activated receptor γ; PTX, pertussis toxin; ROS, reactive oxygen species; Treg-cell, regulatory T-cell; TBS, Tris-buffered saline; TNF, tumour necrosis factor.

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ALA (α-lipoic acid) is a natural, endogenous antioxidant that is produced by both eukaryotic and prokaryotic cells and is found in foods such as broccoli, spinach and tomatoes [10]. ALA is a necessary cofactor for mitochondrial α-ketoacid dehydrogenases and thus plays an essential role in mitochondrial energy metabolism. Furthermore, ALA acts as a PPAR-γ (peroxisome-proliferator-activated receptor-γ) agonist to enhance PPAR-γ activity and counteract oxidative stress [11]. Klotz et al. [12] demonstrated that PPAR-γ is a key negative regulator of human and mouse Th1 differentiation and selectively inhibits Th17 differentiation in a T-cell-intrinsic fashion and suppresses CNS autoimmunity. Hontecillas and Bassaganya-Riera [13] subsequently confirmed that decreased levels of PPAR-γ resulted in impaired Treg-cells (regulatory T-cells) activity, leading to a failure to suppress IFN-γ (interferon-γ) production and the consequent prolongation of the inflammatory response.

However, the mechanisms of the immunomodulatory and antioxidative effects of ALA on EAE remain unclear. In the present study, we demonstrate that ALA ameliorates EAE through either the up-regulation of splenic Treg-cells and Th2 cells or the down-regulation of Th1 and Th17 cells in ALA-treated MOG (myelin oligodendrocyte glycoprotein)-EAE mice in situ. Strikingly, ALA enhances the endogenous production of the nuclear receptor PPAR-γ both peripherally and centrally in EAE, which implies that PPAR-γ may play an important immunomodulatory role in ALA-mediated protection against EAE in mice.

MATERIALS AND METHODS

Mice
C57BL/6 mice (6–8 weeks old) were purchased from the National Laboratory Animal Center, Taiwan. All animal protocols were approved by the IACUC (Institutional Animal Care and Use Committee) of Taiwan.

EAE induction and treatment protocol
A MOG-(35–55) peptide (M-E-V-G-W-Y-R-S-P-F-S-R-O-V-H-L-Y-R-N-G-K) that corresponded to the sequence of the mouse MOG in 100 μl of an emulsion of CFA (complete Freund’s adjuvant) and 20 mg of xylazine/kg of body mass (Rompun; Bayer) and were perfused transcardially through the left ventricle with 20 ml of ice-cold PBS. The spleens were harvested, placed in RPMI-1640 medium (Invitrogen Life Technologies) and minced, and the erythrocytes were depleted by treatment with Tris-buffered ammonium chloride. After centrifugation, the pellets, which represented the total splenic mononuclear cell population, were resuspended in RPMI-1640 medium. The non-adherent lymphocyte population was collected, washed and resuspended in PBS containing 1 % (v/v) FBS (fetal bovine serum) (all supplements were from Invitrogen Life Technologies).

The brains and spinal cords were dissociated by glass homogenization through a fine mesh screen using a syringe plunger and collected in 10 ml of HBSS (Hanks’ balanced salt solution) containing 0.05 % collagenase D (Boehringer Mannheim Biochemicals), 0.1 μg/ml of the trypsin inhibitor N-α-TLCK (tosyllysylchloromethane) (Sigma), 10 μg/ml DNase I (Sigma) and 10 mM Hepes buffer, pH 7.4. The resulting tissue slurry was mixed at room temperature for 60 min and allowed to settle at under gravity for 30 min to remove any undisgested debris. The supernatant was collected and centrifuged at 200 g for 5 min, and the pellet was resuspended in 10 ml of Ca2+/Mg2+-free HBSS per brain. Next, 5 ml of this suspension was layered carefully onto 10 ml of a modified density gradient medium (a mixture of 75 % RPMI-1640, 10 % FBS, 10 mM Hepes and 50 μg of gentamicin with 25 % Ficoll-Paque) in a 50-ml centrifuge tube. Following a 30-min centrifugation at 500 g, the overlying medium and the tissue interface were removed. The entire 10 ml of gradient medium was diluted 10-fold with HBSS and centrifuged at 300 g for 10 min [14].

SDS/PAGE and Western blotting
Proteins were extracted from the brains and spinal cords, separated via SDS/PAGE (12 % gels) and electroblotted on to nitrocellulose membranes. The membranes were incubated for 1 h in 20 ml of blocking buffer [TBS (Tris-buffered saline) and 5 % (w/v) non-fat dried skimmed milk], washed three times

Clinical EAE score
The clinical EAE score was assessed using the following scale: 0 = no symptoms, 0.5 = distal weak or spastic tail, 1 = completely limp tail, 1.5 = limp tail and hindlimb weakness (feet slip through cage grill), 2.0 = unilateral partial hindlimb paralysis, 2.5 = bilateral partial hindlimb paralysis, 3.0 = complete bilateral hindlimb paralysis, 3.5 = complete hindlimb and unilateral partial forelimb paralysis, 4.0 = moribund, and 5 = dead [14].

Isolation of lymphocytes from spleens, brains and spinal cords
The mice were anaesthetized with an intramuscular injection of 100 mg of ketamine/kg of body mass (Imalgene 1000; Merial Laboratoire de Toulouse) and 20 mg of xylazine/kg of body mass (Rompun; Bayer) and were perfused transcardially through the left ventricle with 20 ml of ice-cold PBS. The spleens were harvested, placed in RPMI-1640 medium (Invitrogen Life Technologies) and minced, and the erythrocytes were depleted by treatment with Tris-buffered ammonium chloride. After centrifugation, the pellets, which represented the total splenic mononuclear cell population, were resuspended in RPMI-1640 medium. The non-adherent lymphocyte population was collected, washed and resuspended in PBS containing 1 % (v/v) FBS (fetal bovine serum) (all supplements were from Invitrogen Life Technologies).

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in TBS with 0.1% Tween-20 and incubated overnight at 4°C with rabbit anti-HO-1 (haem oxygenase 1) antibody (Stressgen) and anti-PPAR-γ antibody (BioVision). The membranes were washed three times and incubated for 1 h with HRP (horseradish peroxidase)-conjugated goat anti-rabbit antibody (Novus) at room temperature. Following three additional washes, the membrane-bound antibody was detected with Western Lighting Chemiluminescent Reagent Plus (PerkinElmer Life Sciences) and visualized on X-ray film. The molecular masses of HO-1, PPAR-γ and β-actin are 32, 33 and 43 kDa respectively.

Flow cytometric analysis
Prepared cells (10⁶ cells in 0.1 ml of PBS) were incubated on ice and stained with the following marker-specific antibodies (0.5 μg of antibody/10⁶ cells): FITC-conjugated anti-HO-1 antibody (Stressgen), FITC-conjugated anti-PPAR-γ antibody (Santa Cruz) and APC (allophycocyanin)-conjugated anti-mouse CD4 antibody (eBioscience). Either lymphocytes isolated from the CNS or erythrocyte-depleted splenocytes were stained with anti-mouse CD4 and stimulated with 20 ng/ml PMA plus ionomycin for 4 h, with the final 2 h in the presence of monensin [15]. Erythrocyte-depleted splenocytes were used to perform the ex vivo study, MOG stimulation (25 μg MOG/ml overnight and last 4 h with monensin) and MOG-specific Th lineages were measured. After fixation in 4% (v/v) formaldehyde for 20 min, the cells were stained with an FITC-conjugated mAb (monoclonal antibody) to IFNγ, an FITC-conjugated mAb to FoxP3, a PE (phycoerythrin)-conjugated mAb to IL-17 (interleukin 17), a PE-conjugated mAb to IL-4, a PE-conjugated mAb to CD25 or isotype control mAbs, according to the manufacturer’s instructions (BD Biosciences), in the presence of 0.5% saponin for permeabilization (eBioscience). Flow cytometric analysis was performed with a fluorescence-activated cell sorter (FACS; FACSCalibur, Becton Dickinson), and the data were analysed with CellQuest software. The results were analysed using WinMDI software.

Antigen-specific proliferation
Splenocyte cell suspensions were isolated on day 21 from MOG-(35–55)-immunized mice that had been treated with ALA or left untreated. The pooled splenocytes from six mice from the same group were plated in triplicate in 96-well round-bottomed plates at 2×10⁴ cells/well in 200 μl of complete RPMI-1640 medium supplemented with 2 mM l-glutamine, 25 mM Hepes, 100 units/ml penicillin, 100 μg/ml streptomycin, 5.5×10⁻⁵ M 2-mercaptoethanol and 5% (v/v) FBS (all the supplements were from Invitrogen Life Technologies) that contained 0–10 μg/ml MOG-(35–55) (Enzo Life Sciences). On day 3, 1 Ci/well [³H]-thymidine (Amersham Pharmacia Biotech) was added, cells were cultured for an additional 6 h, and harvest proliferation was assessed by [³H]-thymidine incorporation detected with a TopCount scintillation counter (Packard, PerkinElmer). The supernatants were collected after a 72-h culture at 37°C in 5% (v/v) CO₂, and the cytokine concentrations were determined with ELISA using antibody pairs specific for IL-4, IL-17, IFNγ and TNF-α (tumour necrosis factor α) (BD Biosciences).
Figure 1  ALA ameliorates EAE and enhances PPAR-γ expression in situ
(A) The clinical scores of EAE in C57BL/6 mice treated intraperitoneally with either PBS (100 μl/mouse per injection) or ALA (100 unit/100 μl per mouse per injection diluted in sterile PBS, equivalent to 5000 units/kg of body mass per mouse per injection) on days 1, 3, 5 and 7 after subcutaneous (s.c.) immunization with MOG(35–55)/CFA on day 0 and intraperitoneal PTX on days 0 and 2. Each group contained 10 mice. Results are means ± S.E.M. (B) Western blots of PPAR-γ in brains and spinal cords from ALA-treated and control mice on day 14 after MOG injection. (C) The expression of HO-1 and PPAR-γ proteins in brains, and spinal cords isolated from the CNS of ALA-treated and PBS-treated (control) MOG-EAE mice obtained from the density of Western blotting. Results are means ± S.E.M. from three independent experiments. ∗P < 0.05. (D) H/E (haematoxylin/eosin) staining of spinal cord tissues from ALA-treated mice (right) or controls (left) on day 14 after MOG injection. The images are at ×200 magnification.

determined by flow cytometry. However, we found a significantly higher proportion of Th2 cells, a lower level of Th17 cells and a trend towards decreased Th1 cells in situ in the ALA-treated MOG-EAE mice compared with the controls (Figure 2), which suggests that ALA has the capacity to suppress Th17 and Th1 cells and to enhance Th2 cells in situ to protect mice from EAE.

ALA up-regulates HO-1 and PPAR-γ peripherally, but only PPAR-γ in situ
Interestingly, we disclosed a significantly elevated expression of endogenous PPAR-γ in both the brain and the spinal cord from the ALA-treated MOG-EAE mice compared with the controls (Figure 1C). Upon further examination of the antioxidant potential of ALA via the peripheral expression of HO-1 and PPAR-γ, we observed marked increases in the levels of splenic HO-1 and splenic PPAR-γ in the ALA-treated mice with EAE (Figures 3A and 3B). We used flow cytometry to address whether ALA affected the intensity of cellular HO-1 and PPAR-γ expression in splenocytes. We observed a significantly increased MFI (mean fluorescence intensity) for PPAR-γ and a slight trend towards an increased MFI for HO-1 using equal numbers of splenic lymphocytes from the ALA-treated MOG-EAE mice and the control mice (Figure 3C). Overall, we observed that ALA not only causes a central up-regulation of PPAR-γ in the CNS, but is also a potent inducer of PPAR-γ and HO-1 in the peripheral immune system, particularly in the augmentation of PPAR-γ expression.

ALA enhances splenic Treg-cells and suppresses MOG-specific Th1
Both the PPAR-γ activation caused by the administration of a PPAR-γ agonist and the up-regulation of endogenous HO-1 have been shown to enhance the expression of Th2 cytokines, such as IL-4, to ameliorate inflammation in CNS-demyelinating diseases such as MS [18,19]. Therefore we analysed subsets of splenic
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Figure 2 Distribution of Th lymphocyte subsets in situ
(A) Flow cytometric analysis of intracellular cytokines in CD4+ T-cells isolated from the CNS of mice treated with either ALA or PBS. The CD4+ T-lymphocytes isolated from the CNS of either ALA-treated mice or controls on day 21 (the peak disease stage) were stained intracellularly for IL-17, IFNγ, and IL-4 and assessed with flow cytometry. The data presented are representative of three experiments. (B) The percentages of the total IL-17-producing CD4+ T-cells (Th17), IFNγ-producing CD4+ T-cells (Th1) and IL-4-producing CD4+ T-cells (Th2) obtained from three experiments.

Th lineages using flow cytometry on day 21 after EAE induction. RBC (red blood cell)-depleted splenocytes were stained with anti-mouse CD4 and stimulated with 20 ng/ml PMA plus ionomycin for 4 h, with the final 2 h in the presence of monensin. Interestingly, we observed a significantly higher proportion of splenic Th2 cells (Figure 4A) and a markedly increased number of splenic Treg-cells (CD25+ FoxP3+ CD4+ cells) from the ALA-treated MOG-EAE mice (Figures 4B and 4C). However, no significant difference in either splenic Th1 or Th17 cells was observed in this non-specific stimulation assay. In addition, we conducted further ex vivo study where we isolated RBC-depleted splenocytes from mice of EAE on day 14 for antigen specific stimulation via MOG (25 μg of MOG/ml overnight and last 4 h with monensin) to measure these MOG-specific Th lineages. We gated CD4+ cells for analysis and revealed a significant elevated ratio of Th1 cells (IFNγ+/CD4+ cells) and mild elevation of Th17 (IL17+ /CD4+ cells) in the control group compared with the ALA treatment group (see Supplementary Figure S1 at http://www.clinsci.org/cs/125/cs1250329add.htm). Together, the up-regulation of splenic Treg-cells and down-regulation of inflammatory response suggests a role for the anti-inflammatory potential of ALA in EAE.

Splenocytes from ALA-treated mice inhibit T-cell proliferation and ameliorate EAE severity after adoptive transfer
To investigate the suppressive potential of ALA on T-cell-mediated effector functions in EAE, we further analyse the MOG-specific T-cell response and in vivo adoptive transfer examination. We isolated splenocytes from ALA-treated MOG-EAE mice and from control mice on day 14 post-EAE. The cultured splenocytes were treated with various concentrations of MOG as
specific antigen. A mild suppressive effect on T-cell proliferation was observed in the ALA group compared with the control (Figure 5A). We also investigated further pro- and anti-inflammatory cytokines in terms of the response of IL-17, IFN-γ, TNF-α and IL-4 to antigen-specific stimulation in ALA-treated and control groups, respectively (Figure 5B). Surprisingly, we found that all the cytokines were significantly suppressed in the ALA-treated group compared with the controls. To further elucidate the in vivo immunomodulation of T-cell function by ALA, we isolated splenocytes from the ALA-treated MOG-EAE mice or the controls on day 21 after MOG-(35–55)/CFA induction and passively transferred these cells into C57BL/6 recipients. The recipients of splenocytes from the ALA-treated MOG-EAE mice had a lower clinical score and a more rapid recovery from disease than the recipients of splenocytes from the controls (Figure 5C). This result indicates that splenocytes from ALA-treated mice preserve their immunomodulatory competency to decrease neuroinflammation in EAE, which implies that the immunomodulation of ALA may directly repress immunopathogenic T-cells in MS patients. Nevertheless, additional evidence is needed to confirm this phenomenon.

**DISCUSSION**

The immunopathogenesis of MS is an extremely complicated process and difficult to explore adequately through in vitro analysis. EAE has become the most popular animal model of MS for CNS inflammation and demyelination research into complex pathogenic mechanisms and for testing novel or developing therapeutic agents [20]. ALA has been shown to have a protective role against oxidative injury in various diseases, including...
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Figure 4 Splenic T-cell lineages and Treg-cells

(A) Splenic lymphocytes either from ALA-treated mice or from controls were stained for Th1, Th2 and Th17 for flow cytometric analysis to determine the proportion of CD4^+ T-cells. The data presented are representative of four experiments. An increased level of splenic Th2 cells was detected on day 21 in ALA-treated MOG-EAE mice compared with the controls. (B and C) These splenic lymphocytes were also stained for CD25^+ forkhead box P3^+ CD4^+ cells (Treg cells) and analysed with flow cytometry. An increased number of splenic Treg cells was detected on day 21 in the ALA-treated MOG-EAE mice compared with the controls. The Treg-cell data are representative of six experiments.
Figure 5  Splenocytes from ALA-treated mice inhibit T-cell proliferation and ameliorate EAE severity after adoptive transfer

(A) Splenocytes from either ALA-treated mice or from controls on day 14 were restimulated with various concentrations of MOG(35–55) for 72 h. Proliferation was measured by [3H]-thymidine incorporation and is displayed as counts/min (cpm).

(B) The supernatants of cultured splenocytes from mice on day 14 were collected to analyse IL-17, IFN-γ, TNF-α and IL-4 expression. The vertical bars signify the S.E.M. ∗P < 0.05. The results represent triplicate experiments. (C) Splenocytes were collected from either mice treated with ALA or controls, and each recipient received by adoptive transfer 2×10⁷ splenocytes obtained from the indicated mice on day 21 after MOG(35–55)/CFA immunization. Each group contained eight mice. Results are means ± S.E.M.
diabetes [21,22], hypertension [23,24], ischaemia-reperfusion [25], diabetic polyneuropathy [26–28] and AD (Alzheimer’s disease) [29]. However, the treatment of MS with ALA has been considered as a complementary adjunctive therapy only [30,31].

The protective mechanism of ALA in EAE has been partially elucidated by previous studies [16,17,32], but there are limited data on the immunologic effects on EAE of the inhibition of cytokine secretion and adhesion molecule expression. In the study by Marracci et al. [16], ALA inhibited the migration of T-cells into the CNS by acting as an MMP (matrix metalloproteinase) inhibitor in the PLP-induced EAE model. In the report by Morini et al. [17], ALA was used to treat MOG-induced EAE through a mechanism involving the inhibition of MMP and IFNγ and IL-4 production by MOG-reactive T-cells. The MOG-specific T-cells exhibited decreased production of IFNγ and IL-4, suggesting an immunosuppressive activity of both the Th1 and Th2 cytokines.

In a pilot clinical study, Yadav et al. [31] demonstrated that oral ALA may exert its immunological benefits by down-regulating adhesion molecules.

We did not test the role of adhesion molecules in the ALA-treated MOG-EAE model; however, our data showed a corresponding decrease in the infiltration of inflammatory leucocytes into the CNS and a trend towards an increased amount of Th2 cells in situ in ALA-treated MOG-EAE mice. Previously, Mestre et al. [33] demonstrated that a cannabinoid agonist interferes with the progression of a chronic model of MS by down-regulating adhesion molecules [ICAM-1 (intercellular adhesion molecule 1) and VCAM-1 (vascular cell adhesion molecule)] through the up-regulation of PPAR-γ. This may imply that PPAR-γ produced by ALA has the ability to inhibit the migration of peripheral proinflammatory Th17 and Th1 cells into the CNS. In our study, Th1 and Th17 subsets in CNS were significantly suppressed (Figure 2A). Accordingly, we hypothesize that ALA inhibits the infiltration of inflammatory cells through the decreased expression of adhesion molecules via enhancing the endogenous production of PPAR-γ. Nevertheless, further study is needed to identify the adhesion molecules associated with the PPAR-γ-mediated immunomodulation of ALA-treated MOG-EAE mice.

ALA is a PPAR-γ agonist in CV-1 monkey epithelial cells [11]. ALA can induce PPAR-γ expression and prevent a decrease in PPAR-γ protein levels in the aorta and heart in chronically glucose-treated rats, preventing an increase in systolic BP (blood pressure) and aortic superoxide production and attenuating the development of insulin resistance [23]. ALA also significantly induced PPAR-γ activation in platelets and increased intracellular amounts of PPAR-γ, thus enhancing the anti-platelet activity [34]. Several studies have reported that PPAR-γ agonists possess both anti-oxidative and anti-inflammatory properties in EAE [19,35,36]. PPAR-γ agonists suppressed the proinflammatory molecules produced by microglial cells and astrocytes [37]. PPAR-γ also inhibits the interaction between leucocytes and endothelial cells via the inhibition of adhesion molecule expression [38]. The increased expression of PPAR-γ and the subsequent inhibition of glial cell activation were hypothesized to have an anti-inflammatory effect in EAE [35]. Previously, an association between the PPAR-γ gene polymorphism P12A and the delayed onset of MS was reported [39]. Natarajan et al. [40] observed that PPAR-γ-deficient heterozygous mice exhibited an increased Th1 response compared with wild-type controls, implying that the induction of PPAR-γ during local inflammation may produce anti-inflammatory effects, such as changes in the local IL-4 concentration, and may down-regulate the Th1 inflammatory response, similar to our observations (Figure 2). Even Th1 and Th17 subsets in the CNS were significantly suppressed (Figure 2A); however, these phenomena are not consistent in splenic Th subsets under non-specific stimulation (Figure 4A). Nevertheless, we found further evidence of consistent expression of splenic Th subsets under antigen-specific stimulation and we also revealed Th1 and Th17 subsets were repressed correspondingly under MOG-(35–55) stimulation in the ALA-treated group (see Supplementary Figure S1). Accordingly, we hypothesize that PPAR-γ produced by ALA had the ability to inhibit the inflammation whether in the CNS or in the peripheral environment.

The antioxidant effects of ALA are mediated by the induction of HO-1 in aortic smooth muscle cells, which provides a protective effect in vascular disease [23,41]. Our previous study demonstrated that erythropoietin induces the production of endogenous HO-1 and represses the inflammatory response in EAE [42]. Similarly, PPAR-γ agonists inhibit activated microglia via the production of HO-1 [43]. However, in the present study, ALA induced elevated HO-1 levels in EAE mice only peripherally, not centrally. One possible explanation for this finding is that ALA decreased the infiltration of inflammatory cells into the CNS via the production of PPAR-γ both centrally and peripherally, but the role of HO-1 during ALA-treated MOG-EAE was overwhelmed by the dominant effects of PPAR-γ.

Encephalitogenic Th17 cells are the essential pathogenic cells of EAE [44]. Th1 cells can facilitate the invasion of Th17 cells into the CNS during EAE [45], and the CNS-derived Th2 cytokine IL-4 is crucial for the regulation of inflammation in EAE [46]. We explored the immunomodulatory mechanisms of this phenomenon. Our data confirmed that ALA counteracts both the Th17- and Th1-mediated inflammatory responses in situ during EAE. ALA increased IL-4 markedly in the spinal cord and decreased inflammation and the EAE clinical score, suggesting that this immunomodulation may be partly dependent on the increased level of IL-4 [47]. Ponomarev et al. [46] demonstrated that an increased expression of IL-4 in glial cells was associated with a reduced severity of EAE and that IL-4 production in the CNS is essential for controlling autoimmune inflammation by inducing an alternative regulation of microglial cells.

Kohm et al. [48] observed that Treg-cells effectively inhibit both the proliferation of MOG-specific Th1 cells and their production of cytokines and that the adoptive transfer of Treg-cells confers significant protection from EAE. The expansion of myelin-reactive Treg-cells can prevent disease relapse when it occurs after the onset of clinical EAE [49]. In our study, a high level of splenic Treg-cells may provide an immunosuppressive effect to protect ALA-treated MOG-EAE mice. Accordingly, the enhancement of PPAR-γ expression may contribute to the overexpression of peripheral Treg-cells and inhibit the increased inflammation in ALA-treated MOG-EAE mice, as observed in our study. Furthermore, we identified a significant suppression of MOG-specific T-cell proliferation and a lower clinical severity.
after the adoptive transfer of splenocytes from the ALA-treated group. However, the in vitro data on IL-4 in Figure 5(B) showed somewhat inconsistent results with Figure 2, but the in vivo transfer study supported decreased inflammation in the ALA treatment group. These effects may be attributed, at least in part, to the up-regulation of peripheral T<sub>reg</sub>-cells and down-regulation of the Th1 lineage in the ALA-treated MOG-EAE mice.

However, some limitations of this work remain. Dunn et al. [50] first revealed that PPAR-δ limits the expansion of encephalitogenic Th cells in EAE in a murine gene knockout model. Our study did not investigate the role of PPAR-δ in an ALA-treated MOG-EAE model. Secondly, Schillace et al. [51] found that ALA can induce an increase in cAMP, a known immunosuppressant, in human T-cells and NK (natural killer) cells, which may suppress the inflammation associated with EAE or MS. We did not study the role of NK cells in this ALA-treated MOG-EAE model. Nevertheless, the results presented here advance our understanding of the anti-inflammation effect of ALA in EAE, and further studies should be conducted to address the concerns raised.

In conclusion, ALA can augment the induction of endogenous PPAR-γ centrally and peripherally, repressing Th1 and Th17 responses in situ and enhancing systemic Th2 and T<sub>reg</sub>-cell responses to ameliorate MOG-EAE severity. We also provide further evidence of the immunomodulatory and antioxidant effects of ALA via up-regulating endogenous PPAR-γ to modulate the central and peripheral immune responses to contribute to neuroprotection in EAE.

CLINICAL PERSPECTIVES

- ALA had been used as a complementary and alternative medicine treatment for MS patients, but direct evidence of its effects is lacking.
- In our MOG-EAE model, ALA up-regulated the production of endogenous PPAR-γ in vivo and modulated adaptive immunity both centrally and peripherally in EAE.
- These findings suggest that PPAR-γ may play an important immunomodulatory role in ALA-mediated protection against EAE in mice and potentially in human MS.

AUTHOR CONTRIBUTION

Kai Chen Wang conceived and designed the research, analysed and interpreted the data, performed the statistical analysis and drafted the paper. Ching-Piao Tsai acquired the data and made critical revisions of the paper. Chao-Lin Lee conceived and designed the research, analysed and interpreted the data, handled funding and supervision, and made critical revisions to the paper. Shao-Yuan Chen and Mao-Hsiung Yen analysed, acquired and interpreted the data, and made critical revisions of the paper. Huey-Kang Sytwu acquired the data and made critical revisions of the paper. Shy-Jou Chen conceived and designed the research, interpreted the data, drafted and made critical revision of the paper. All authors agree with its contents and approved submission of the paper.

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SUPPLEMENTARY ONLINE DATA

α-Lipoic acid enhances endogenous peroxisome-proliferator-activated receptor-γ to ameliorate experimental autoimmune encephalomyelitis in mice

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Figure S1 Splenic T-cell lineages stimulated ex vivo with MOG
A significant elevated ratio of Th1 cells (IFNγ+ /CD4 cells) and a small elevation of Th17 cells (IL17+ /CD4 cells) in the control group compared with the ALA treatment group, supporting the specific anti-inflammatory response of ALA.

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