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RESEARCH

Proteomic Profiling and Therapeutic Targeting of Oxidative Stress in Autoimmune Encephalitis

Fan Zhang[1] · Cong Zhang[1] · Wei Sun[1] · Shuhua Xie[1] · Pengcheng Wu[1] · Guoyong Zeng[1] · Xianghong Liu[1]

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Abstract

Autoimmune encephalitis (AE) is an immune-mediated non-infectious disease, and novel and robust biomarkers are needed to improve the diagnosis and prognostic outcomes of AE. Oxidative stress is a ubiquitous cellular process causing damage to various biological molecules. The aim of our study was to understand the clinical implication and mechanism underlying oxidative stress in AE. Liquid chromatography-mass spectrometry analysis was conducted on the serum of eight patients with AE and seven healthy controls, and oxidative stress was characterized. Experimental autoimmune encephalitis (EAE) models were established in C57BL/6 and SJL mice for investigation of the therapeutic effect and mechanism of anti-oxidative stress *N*-acetylcysteine (NAC). We provided proteomic landscape in the serum of AE and identified antioxidant ALB, APOE, GPX3, and SOD3 as serum diagnostic markers of AE. The antioxidant markers were lowly expressed both in the serum of AE patients and central nervous system (CNS) of EAE mice. NAC administration improved clinical signs and motor function and alleviated nerve injury of EAE mice as well as lowered oxidative stress (decreased MDA content and ROS accumulation and elevated SOD activity and GSH content). ALB, APOE, GPX3, and SOD3 expressions were elevated by NAC in the CNS of EAE mice. Moreover, NAC reduced tissue-resident $CD4^+$ and $CD8^+$ T cells and GFAP-marked astrocytes and Iba-1-marked microglia in EAE mice, thus alleviating autoimmunity-mediated damage and neuroinflammation. Our findings facilitate the discovery of novel oxidative stress-related biomarkers for AE and reveal the promise of anti-oxidative stress for AE management.

Keywords Autoimmune encephalitis · Oxidative stress · Proteomics · *N*-acetylcysteine · T cells · Neuroinflammation

Introduction

Autoimmune encephalitis (AE) is an immune-mediated non-infectious disease that mainly affects the gray matter of the central nervous system (CNS). Anti-GABABR AE was first described in 2010 (Lamblin et al. 2024). First described in 2010, clinical features of anti-GABABR AE typically include limbic encephalitis, antiepileptic drug-resistant seizures, confusion, and anterograde amnesia, but can also manifest as rapidly progressive dementia without significant seizures (Coevorden-Hameete et al. 2019; Maureille et al. 2019). About 50 ~ 60% of patients with anti-GABABR AE have underlying malignant tumors, primarily small-cell lung cancer in older smoking males, often before the onset of neurological symptoms and ultimately resulting in the cancer diagnosis (Ronchi and Silva 2022; Jiang et al. 2022). However, AE is still a rare disease with potentially devastating life and functional prognostic outcomes. With the increasing understanding of AE, two unintended consequences have emerged: high frequency of misdiagnosis and improper use of diagnostic criteria for antibody-negative disease (Flanagan et al. 2023; Dalmau and Graus 2023). Hence, novel and robust biomarkers are required to improve the diagnosis and prognostic outcomes of AE.

Oxidative stress describes a ubiquitous cellular process that can cause damage of a variety of biological molecules and functions as a central part of innate immune-mediated neurodegeneration (Mendiola et al. 2020; Qiu et al. 2024), which has been implicated in the etiology of AE (Yong and Yong 2022; Fan et al. 2023; Dąbrowska-Bouta et al. xxxx). For example, a prior study reported that in autoimmunity

✉ Guoyong Zeng
hssgy@126.com

✉ Xianghong Liu
lxhong7176@126.com

[1] Department of Neurology, The Affiliated Ganzhou Hospital of Nanchang University, Ganzhou 341000, China

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individuals, increased mitochondrial oxidative stress and DNA damage response related to cell death occur in regulatory T cells (Tregs) (Alissafi et al. 2020). In experimental autoimmune encephalitis (EAE) mouse models, dysfunction of Tregs recapitulates the characteristics of autoimmune Tregs and has a notable mitochondrial oxidative stress signature. Clearance of mitochondrial oxidative stress in Tregs of EAE mice can reverse DNA damage response and prevent death of Tregs, and attenuate Th1/Th17 autoimmune responses. The activity of system xC^- can be increased by ROS and inflammatory stimuli, and the increase can result in the release of toxic amounts of glutamate, thus inducing excitotoxicity and neuronal degeneration. The lack of system xC^- on immune cells that invade the CNS weakens EAE (Merckx et al. 2017). Leukadherin1 exerts protection in an EAE rat model through potentially decreasing leukocyte infiltration and oxidative stress (Hemmati et al. 2020). Liraglutide enables to delay the onset of an EAE rat model and is related to improved protective ability against oxidative stress (DellaValle et al. 2016). Despite previous research, the clinical implications and mechanisms of oxidative stress in AE require further exploration.

Proteomics is a common unbiased method evaluating protein abundance in serum, with the potential to discover novel biomarkers facilitating early diagnosis and prognostic prediction, in turn impacting treatment options. Herein, this study provides an unbiased proteomic analysis of treatment-naïve AE patients compared with healthy controls aiming to improve the pathophysiological understanding of AE and promote the discovery of novel oxidative stress-related biomarkers for AE.

Materials and Methods

Study Subjects and Sample Collection

We enrolled eight patients with anti-GABABR AE who were treated at The Affiliated Ganzhou Hospital of Nanchang University between 2022 and 2023. Meanwhile, seven age- and sex-matched volunteer subjects without a history of neurological or systemic autoimmune disorders were included as healthy controls. The patients were diagnosed with anti-GABABR AE in accordance with expert consensus (Graus et al. 2016). Anti-GABABR antibodies were identified in cerebrospinal fluid and/or serum with the use of commercial assay. All subjects signed written informed consent before participating in our study. This study was performed in accordance with the Declaration of Helsinki, and the protocols gained approval by the Ethics Committee of The Affiliated Ganzhou Hospital of Nanchang University, the ethical number is TY-DKY2022-001–01. Whole blood was harvested from the included AE patients and control subjects, and serum was enriched and stored at − 80 °C prior to assay.

Liquid Chromatography-Mass Spectrometry (LC–MS) Analysis

The specimens were lysed by adding 1% sodium dodecyl sulfate (Amresco) supplemented with 1% protease inhibitors (Merck Millipore) in lysis buffer, boiled at 100 °C for 10 min, with subsequent sonication lysis. After centrifugation at 12,000 g for 10 min, the supernatants were harvested. The protein concentration was quantified utilizing a BCA kit (Beyotime). The samples were treated with pre-cooled acetone and precipitated at − 20 °C for 2 h. Following centrifugation at 4500 g for 5 min, the supernatants were removed, with the pellet being washed twice using pre-cooled acetone. Next, the samples were added with 200 mM TEAB. After sonication of the precipitate, the samples were digested with trypsin (1:50) overnight. A total of 5 mM dithiothreitol was added, and the samples were lowered at 56 °C for half an hour and incubated with 11 mM iodoacetamide at room temperature for 15 min in the dark.

The peptides were dissolved in mobile phase A (an aqueous solution supplemented with 0.1% formic acid and 2% acetonitrile), and separated with the use of an ultra-high performance liquid phase system, followed by high-resolution TOF analysis. The scanning ranges of secondary mass spectrometry were set as 100–1700. After a first-order mass spectrometer collection, ten times PASEF mode was conducted to harvest the second-order spectrum. Secondary mass spectrometry data were obtained utilizing Maxquant. The false positive rate of protein identification and PSM identification was set as 1%. The accuracy FDR of the three levels of the spectrum, peptide, and protein identification was set as 1%, and ≥ 1 unique peptide was included in the protein identification. Library search analysis of the mass spectrometry data provided signal intensity values for peptides in each sample, followed by calculation of relative quantitative values for proteins.

Differential Expression Analysis

Differentially expressed proteins in patients with AE in comparison to controls were selected with the use of a limma package (Ritchie et al. 2015). The screening criteria were fold change > 1.5 and $p < 0.05$. On the basis of protein-expressing profiling, dimensionality reduction was carried out utilizing principal component analysis (PCA).

Functional Enrichment Analysis

Enrichment analysis on GO terms and KEGG pathways was conducted with the use of clusterProfiler package (Ge et al. xxxx; Yu et al. 2012). Moreover, oxidative stress level was quantified with single sample gene set enrichment analysis (ssGSEA) approach on the basis of expression profiling

of oxidative stress-related biomarkers (ALB, APOE, B2M, CAT, GCLC, GPX3, GSTP1, KRT1, MBL2, PDLIM1, PRDX1, PRDX2, PRDX6, SOD3, and TXN) (Hänzelmann et al. 2013).

Diagnostic Model Establishment

The findCorrelation function in the caret package was employed for screening and eliminating highly correlated genes. Utilizing the rfe function, genes that contributed more to the classification of AE and control samples were identified. Next, with the use of the createDataPartition function, the samples were separated into the training set and test set. Furthermore, several classification algorithms: NaiveBayes, RandomForest, and linear kernel support vector machine (SVM), were chosen by the train function for model training. Eventually, the classification outcomes were inferred utilizing the predict function, and the receiver operating characteristic curve (ROC) analysis was performed utilizing the pROC package to calculate the area under the curve (AUC) value (Wang et al. 2022).

Transcriptional Regulation Analysis

On the basis of ChEA (Lachmann et al. 2010), ENCODE (Rosenbloom et al. 2010), hTFtarget (Zhang et al. 2020), TRANSFAC (Wingender et al. 1996), and TRRUST (Han et al. 2015) databases, transcription factors of target genes were comprehensively inferred. The interactions of transcription factors with target genes that co-existed in at least two databases were selected to map a transcriptional regulatory network.

Prediction of Small Molecule Compounds

Through the Comparative Toxicogenomics Database (CTD) (Davis et al. 2023), literature-based, manually curated relationships between small molecule compounds and target genes were obtained.

Immune Composition Estimation

The fractions of immune cells were quantified from expression profiling with the use of a deconvolution algorithm (CIBERSORT) (Newman et al. 2015).

Experimental Animals and Drug Administration

The animal study was conducted at The Affiliated Ganzhou Hospital of Nanchang University and approved by the Animal Ethics Committee of The Affiliated Ganzhou Hospital of Nanchang University. To construct a mouse model of EAE, as previously described (Song et al. 2022), female C57BL/6 mice of 8 ~ 10 weeks old were subcutaneously injected with an emulsion containing 200–250 μg MOG35-55 peptide, 0.05 mL complete Freund's adjuvant, 0.05 mL sterile saline, and 4 mg/mL Mycobacterium H37Ra via paracanthosis. Then, the mice were intraperitoneally injected with 250–200 ng pertussis toxin at the time of immunization and again 48 h later. Female SJL mice of 8 ~ 10 weeks old were also used to construct an EAE mouse model, as previously described (Rashid Khan et al. 2023). In total 200 μg proteolipid protein peptide (PLP-amino acids 139–151), 50 μg/site emulsified with complete Freund's adjuvant, was injected subcutaneously at four sites. Furthermore, 200 ng lyophilized pertussis toxin was injected intraperitoneally on the same day of immunization. On the third day of post-encephalitogenic injection, the mice were repeatedly injected with pertussis toxin. After 17 days of EAE model construction, the mice were given 2 mg/mL of *N*-acetylcysteine (NAC) orally daily for 7 days, as previously described (Lehmann et al. 1994). The control mice were given the same amount of normal saline.

Clinical scores of EAE severity were assessed daily as follows: 0 scores, no clinical symptoms; 0.5 scores, partial soft tail; 1 score, paralysis tail; 2 scores, not coordinated movement and hind limb paralysis; 2.5 scores, paralysis of one hind limb; 3 scores, paralysis of both hind limbs; 3.5 scores, hind limb paralysis and front limb weakness; 4 scores, front limb paralysis; and 5 scores, barely alive.

After 7 days of NAC treatment, the sports injury of the mice was detected by rotating rod method. Before the test, the mice were adapted to the test environment for 1 ~ 2 days, and the following training was carried out on the day before the experiment: 3*10 rpm uniform training for 5 min. Each mouse needed a rest time of more than 30 min between each training, during which if the mouse fell, it immediately put the rotating rod. Holding the mouse body, the front and back paws were gently put on the rotating rod, with the back facing the direction of the rod rotation. During the test, the rotation speed of the rod was gradually elevated, 5 rpm*1 min → 3 rpm/min → 40 rpm*5 min, and the test time was 5 min. The latency to fall time of the mice was recorded: the time from when the mice boarded the rotating rod, and the timing stopped after the mice fell or completed the time that met the conditions. The time of each mouse was repeatedly measured to determine the average. After 7 days of NAC treatment, the spinal cord tissues of the mice were harvested after euthanasia.

Western Blot

Overall, 10 mg tissue specimens were lysed utilizing 200 μL RIPA lysate (P0013B; Beyotime) for 2 h and centrifuged at

12,000 g for 10 min. The supernatants were harvested for protein quantification via a BCA kit (BL521A; Biosharp) and stored at − 80 ℃. The extracted protein was exposed to 5 × SDS loading buffer and bathed in boiling water for 5 min. The denatured proteins were electrophoretically separated utilizing 10% ~ 12% SDS-PAGE, with subsequent transference onto the PVDF membrane (IPVH00010; Millipore). After blocking at room temperature for 1 h, incubation with primary antibodies was conducted at 4 °C overnight, with subsequent HRP-bound goat anti-mouse or anti-rabbit secondary antibodies (1:5000; ZB2305 or ZB2301; ZSGB-BIO) at room temperature for 1 h. The used primary antibodies against ALB (1:1000; 16,475–1-AP), APOE (1:1000; 18,254–1-AP), GPX3 (1:1000; 3947–1-AP), SOD3 (1:1000; 14,316–1-AP), and GAPDH (1:5000; 60,004–1-Ig) were purchased from Proteintech. Immunoreactive protein bands were detected via ECL solution (ECL-0011; Beijing Dingguo Changsheng) and then photographed.

TUNEL Staining

Apoptosis was investigated with the use of a TUNEL staining kit (C1098, Beyotime) under the manufacturer's instructions. The slices were exposed to 100 μL of 20 mg/mL protease K reaction solution at room temperature 37 ℃ for 20 min, and sealed with 3% H_2O_2 for 20 min. After exposure to 50 μL of 20 U/μL TdT enzyme reaction solution at 37 ℃ for 1 h without light, they were treated with 50 μL of reaction stop solution at room temperature for 20 min. Each slice was dripped with 50 μL of 5 μg/mL Streptavidin-HRP working solution and reacted at room temperature for half one hour, with subsequent exposure to 50 μL DAB solution for 5 min. After being re-stained by 0.5% hematoxylin for 5 min, they were immersed in 1% hydrochloric methanol to differentiate for 5 s. Subsequently, the slices were dehydrated with 70%, 85%, and 95% alcohol, soaked twice with xylene for 10 min each time, dried and sealed. Positive and negative controls were included to validate the results. Images were investigated and photographed under a fluorescence microscope (Olympus). Six fields of view per slice were randomly selected.

Glutathione (GSH) and Malondialdehyde (MDA) Content and Superoxide Dismutase (SOD) Activity Assays

Under the ratio of weight (g)/volume (mL) = 1:9, nine times the volume of normal saline was added to the samples, followed by homogenization in an ice water bath. After centrifugation at 2500 g for 10 min, the supernatant was harvested. Under the manufacturer's instructions of GSH content assay kit (A006-2–1; Nanjing Jiancheng), MDA content assay kit (A003-1; Nanjing Jiancheng), and SOD activity assay kit (A001-1; Nanjing Jiancheng), we measured GSH and MDA content and SOD activity, respectively. Optical density (OD) values were estimated at 405 nm, 532 nm, and 550 nm using a microplate detector, respectively (ThermoFisher Scientific).

Reactive oxygen Species (ROS) Experiment

ROS assay kit (E004-1–1; Nanjing Jiancheng) was adopted for ROS detection in accordance with the manufacturer's instructions. Following the tissue samples were trimmed and digested, they were centrifuged at 500 g for 10 min, and the supernatants were discarded, with subsequent collection of the precipitates. The samples without any treatment were re-suspended with 0.01 M PBS as a negative control. In a positive control, DCFH-DA was utilized for precipitating the suspended cells, and ROS hydrogen donors were added to induce the cells. The cell precipitates were resuspended with diluted DCFH-DA, with a cell density of 1×10^6 to 2×10^7/mL. Following the 30-min incubation, cellular suspension was centrifuged at 1000 g for 5 min. The supernatants were removed for harvesting the precipitation and washed with PBS 2 times. Cell precipitates were harvested by centrifugation for immunofluorescence.

Immunofluorescence

After treatment with blocking buffer, the sections were incubated with primary antibodies of ALB (1:50; 16,475–1-AP; Proteintech), APOE (1:50; 18,254–1-AP; Proteintech), GPX3 (1:200; 3947–1-AP; Proteintech), SOD3 (1:50; ab80946; Abcam), GFAP (1:50; 16,825–1-AP; Proteintech), and Iba-1 (1:50; 66,827–1-Ig; Proteintech) at 4 ℃ overnight, with subsequent fluorochrome-conjugated secondary antibodies at room temperature for 1 h. The nuclei were counterstained with DAPI (C1002; Beyotime). Images were acquired under a fluorescence microscope (Olympus).

Flow Cytometry

The tissues were trimmed and digested, followed by centrifugation at 500 g for 10 min. After discarding the supernatants, the precipitates were harvested, with incubation with CD4 (FITC-65104; Proteintech) and CD8 (FITC-65069; Proteintech) at 37 ℃ for 30 min. $CD4^+$ and $CD8^+$ T cells were measured via a flow cytometer (FACSVerse; BD).

Enzyme-Linked Immunosorbent Assay

Under the manufacturer's instructions of mouse interferon (IFN)-γ kit (ml002277; mlbio), mouse tumor necrosis factor (TNF)-α kit (mlC50536; mlbio), and mouse interleukin (IL)−1β kit (mlC50300; mlbio), IFN-γ, TNF-α and IL-1β

levels were analyzed, respectively. OD values were measured at 450 nm using a microplate detector (ThermoFisher Scientific).

Statistical Analysis

All the analyses were achieved utilizing R v4.2.1 and GraphPad Prism v9.0.1. The Kolmogorov–Smirnov normality test was performed to confirm if the data followed a Gaussian distribution for comparison. If data were Gaussian, a parametric test was conducted (unpaired student's test, one-way ANOVA, or Pearson correlation test). If data were non-Gaussian, a nonparametric test was conducted (Wilcoxon rank test or Spearman correlation test). P-value < 0.05 was statistically significant.

Results

Proteomic Analysis of Patients with AE Than Healthy Controls

In total, serum samples of eight patients with AE and seven healthy controls were included for proteomic analysis. After normalization (Supplementary Fig. 1A, B), differential expression analysis was conducted. As a result, 47 highly expressed proteins and 61 lowly expressed proteins were determined in the serum of patients with AE versus that of controls (Fig. 1A–C; Supplementary Table 1). Immune-associated biological processes (e.g., immune responses) and oxidative stress-related pathways (e.g., carbon metabolism) were notably enriched by the proteins (Fig. 1D–F; Supplementary Fig. 2A–D). Dimensionality reduction was conducted with the use of PCA. Serum protein profiling of patients with AE was notably distinct from that of healthy controls (Fig. 1G). Moreover, we quantified oxidative stress scores in the serum of AE patients and controls. Oxidative stress score was higher in AE than controls (Fig. 1H), indicating the involvement of oxidative stress in AE.

Oxidative Stress-Related Models for Diagnosing Patients with AE

Given the implication of oxidative stress in AE, we established NaiveBayes, RandomForest, and SVM models for accurately diagnosing patients with AE on the basis of oxidative stress-related biomarkers (Fig. 2A–F). After intersecting the results from the three models, antioxidants ALB, APOE, GPX3, and SOD3 were finally identified (Fig. 2G). All of them enabled an accurate diagnosis of AE in serum samples (Fig. 2H), and they were significantly related to oxidative stress score (Fig. 2I). Hence, serum levels of ALB, APOE, GPX3, and SOD3 may be potential diagnostic biomarkers of AE.

Antioxidant ALB, APOE, GPX3, and SOD3 Are Lowly Expressed Both in the Serum of AE Patients and CNS of EAE C57BL/6 Mice

Antioxidant ALB, APOE, GPX3, and SOD3 were lowly expressed in the serum of AE patients versus that of controls (Supplementary Table 2). Moreover, their expression was also confirmed to be attenuated in the CNS of EAE mice than controls (Fig. 3A–E). Transcription factors of the antioxidant genes were predicted, indicating the potential transcriptional regulatory mechanisms (Fig. 3F; Supplementary Table 3). It was observed that there were notable interactions between ALB, APOE, GPX3, and SOD3 and immune cells (Fig. 3G–K). Potential small molecule compounds of ALB, APOE, GPX3, and SOD3 were inferred (Supplementary Table 4), indicating their potential as pharmacological targets.

NAC Improves Clinical Signs and Motor Function and Alleviates Nerve Injury of EAE C57BL/6 Mice Through Suppressing Oxidative Stress

NAC is a synthetic derivative of the endogenous amino acid L-cysteine and a precursor of GSH, which has been utilized as a mucolytic and antidote for acetaminophen poisoning (Raghu et al. 2021). To investigate whether inhibition of oxidative stress by NAC ameliorated AE, after 17 days of EAE mouse model establishment, the mice were given 2 mg/mL of NAC orally daily for 7 days. Clinical score of EAE severity was notably decreased and latency to fall was notably elevated by NAC administration in EAE mice (Fig. 4A and B), indicating the improvement of clinical signs and motor function by NAC administration. There were increased apoptotic levels in the CNS of EAE mouse models, which was ameliorated by NAC (Fig. 4C and D). In addition, oxidative stress was enhanced in the CNS of EAE mouse models on the basis of increased MDA content, reduced SOD activity and GSH content (Fig. 4E–G) as well as elevated ROS accumulation (Fig. 4H and I), which was ameliorated by NAC. Altogether, NAC may improve clinical signs and motor function and alleviate nerve injury in EAE mice by suppressing oxidative stress.

Anti-Oxidative Stress by NAC Elevates the Expression of Antioxidant ALB, APOE, GPX3, and SOD3 in the CNS of EAE C57BL/6 Mice

Similar to the western blot results, immunofluorescence proved the low expression of antioxidant ALB, APOE, GPX3, and SOD3 in the spinal cord of EAE mouse models in comparison to that of controls (Fig. 5A–H). Their

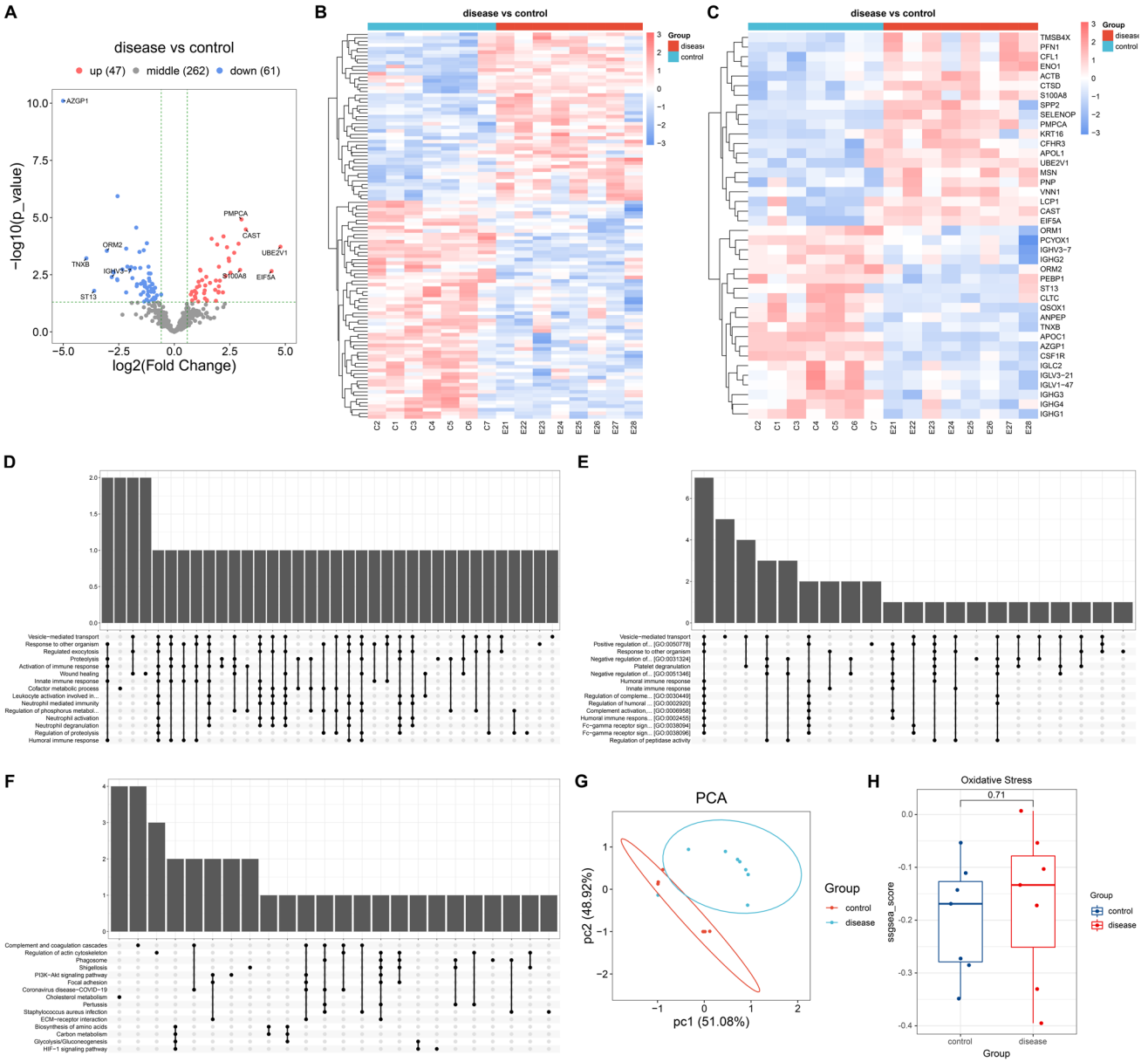

Fig. 1 Proteomic analysis of patients with AE than healthy controls. **A** and **B** Differentially expressed proteins in the serum of patients with AE in comparison to that of controls. **C** Top 20 highly/lowly expressed proteins in serum of patients with AE compared with that of controls. **D** and **E** Biological processes enriched by highly/lowly expressed proteins, respectively. **F** Enrichment of KEGG pathways by differentially expressed proteins. **G** Dimensional reduction of serum protein profiling from patients with AE and controls via PCA. **H** Quantification of oxidative stress score and comparison between AE patients and controls

expression was notably elevated by NAC administration in the spinal cord of EAE mouse models, indicating that NAC ameliorates AE progression possibly through increasing the antioxidant defense proteins.

Anti-Oxidative Stress by NAC Reduces Tissue-Resident CD4$^+$ and CD8$^+$ T Cells in EAE C57BL/6 Mice

AE describes a unique inflammatory disorder of the CNS, with the features of the production of a variety of autoimmune antibodies that target neuronal proteins. We found that the levels of immune cells in the serum of patients with AE were notably distinct from those of controls (Fig. 6A and B). Increasing evidence demonstrates that lymphocytes, T cells, exert a critical role in the onset and progression of AE (Chen et al. 2024). CD4$^+$ T cells affect AE progression through the secretion of associated cytokines, while CD8$^+$ T cells possess a cytotoxic effect and cause irreversible damage to neurons. The proportions of tissue-resident CD4$^+$ and CD8$^+$ T cells presented the

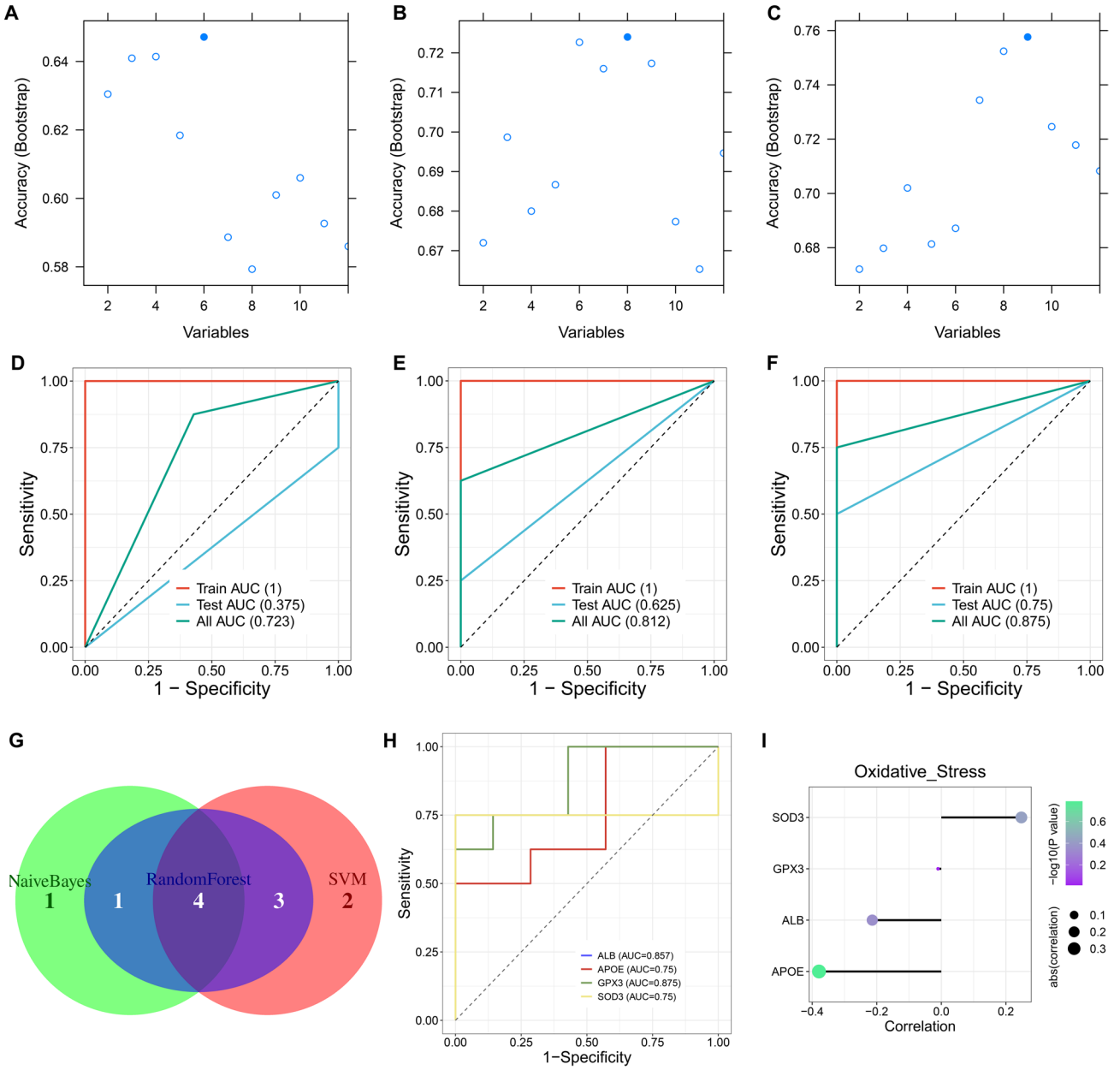

Fig. 2 Oxidative stress-related models for diagnosing patients with AE. **A**–**C** NaiveBayes, RandomForest, and SVM for selection of oxidative stress-related variables for differentiating patients with AE from controls. **D**–**F** ROCs of NaiveBayes, RandomForest, and SVM models in diagnosing patients with AE in training, test, and total sets. **G** Venn diagram of the key oxidative stress-related variables (ALB, APOE, GPX3, and SOD3) through intersecting the three models. **H** ROCs of ALB, APOE, GPX3, and SOD3 in diagnosing patients with AE. **I** Correlation analysis of ALB, APOE, GPX3, and SOD3 with oxidative stress score

elevation in the spinal cord of EAE mice than controls (Fig. 6C–F). NAC administration notably attenuated the proportions of tissue-resident $CD4^+$ and $CD8^+$ T cells in the spinal cord of EAE mice. It was also observed that there is a remarkable increase in proinflammatory cytokines (IFN-γ, TNF-α, and IL-1β) in the spinal cord from EAE mice (Fig. 6G–I). Following the administration of NAC, their levels in the spinal cord from EAE mice presented a reduction. The above data indicate that NAC may lower tissue-resident $CD4^+$ and $CD8^+$ T cells, thus alleviating progressive autoimmune-mediated damage.

Anti-Oxidative Stress by NAC Alleviates Neuroinflammation in EAE C57BL/6 Mice Through Decreasing Astrocytes and Microglia

Activation of astrocytes and microglia is an essential defense mechanism of the brain against damaged tissues and harmful

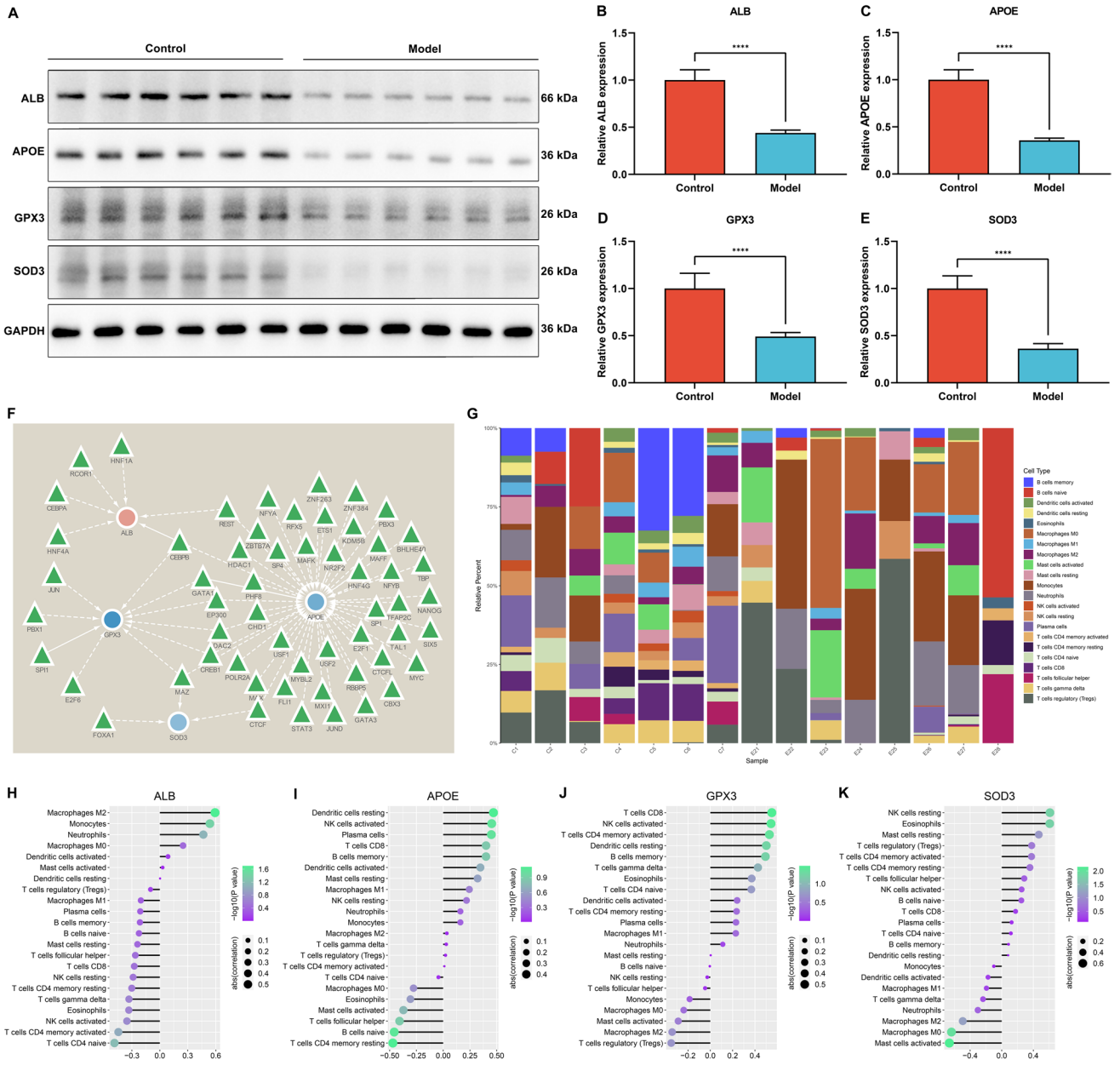

Fig. 3 Antioxidant ALB, APOE, GPX3, and SOD3 are lowly expressed both in the serum of AE patients and CNS of EAE C57BL/6 mice. **A**–**E** Western blot of ALB, APOE, GPX3, and SOD3 in spinal cord tissues of control and EAE mice. **F** Transcriptional regulatory network. Triangles represent predicted transcription factors, and the circle represents the four target genes (red, high expression in autoimmune encephalitis, and green, low expression in autoimmune encephalitis). Dashed lines represent the predicted transcriptional regulatory relationships in the two databases, and solid lines represent the predicted transcriptional regulatory relationships in the three databases. **G** Quantification of the fractions of immune cells in patients with AE and controls. **H**–**K** Correlations between ALB, APOE, GPX3 and SOD3, and immune cells; $^{****}p < 0.0001$

pathogens (Kwon and Koh 2020). Nevertheless, their activation elicits neuroinflammation, which can aggravate or trigger CNS damage (Hasel et al. 2023). The CNS of EAE mice exhibited a notable increase in GFAP-labeled astrocytes and Iba-1-labeled microglia (Fig. 7A–D), indicating the activation of astrocytes and microglia in the CNS of EAE mice. Following the administration of NAC, the levels of GFAP-labeled astrocytes and Iba-1-labeled microglia were lowered in the CNS of EAE mouse models. We indicate that NAC potentially alleviates the neuroinflammation of an EAE mouse model by decreasing the activation of astrocytes and microglia.

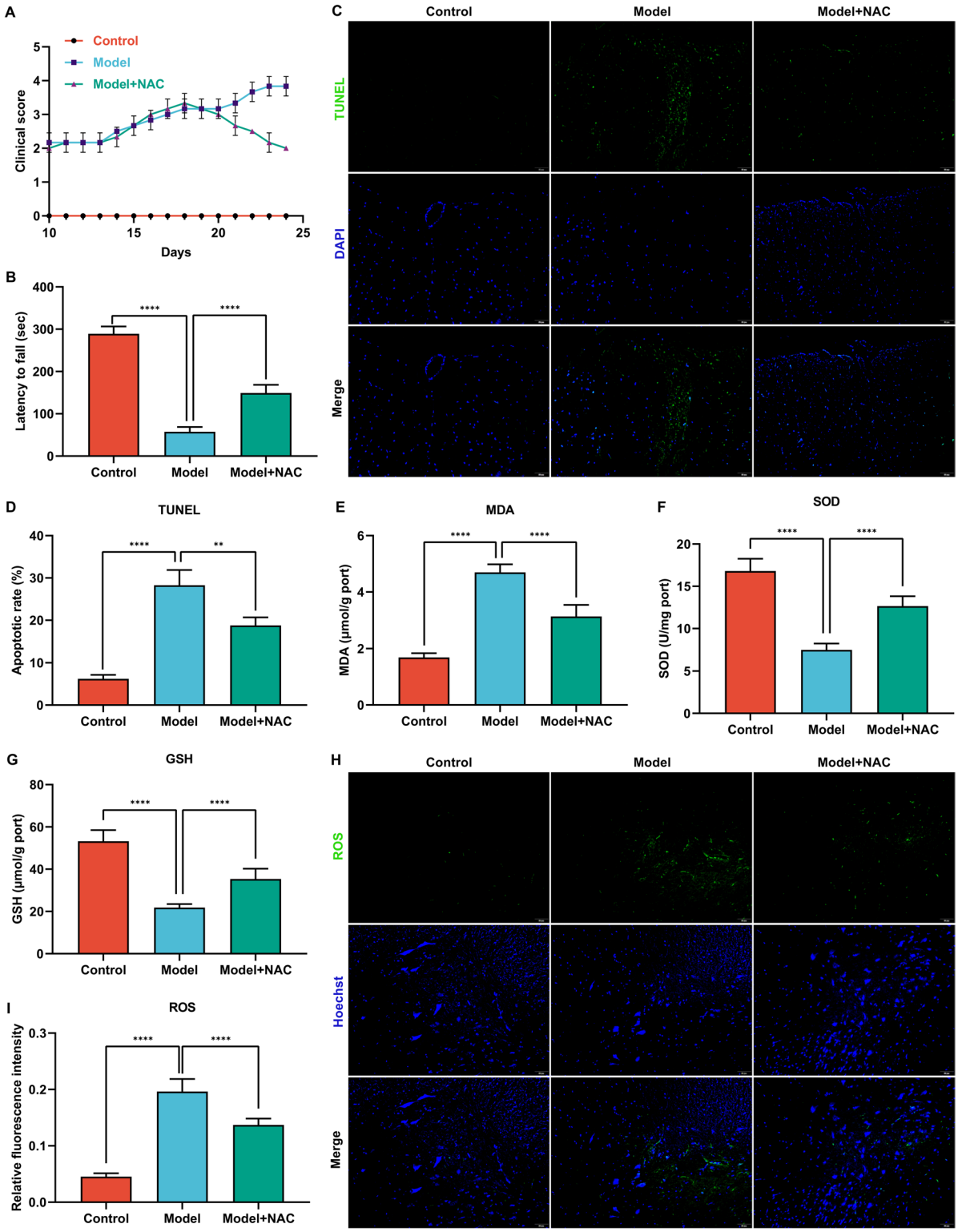

Fig. 4 NAC improves clinical signs and motor function and alleviates nerve injury of an EAE mouse model by suppressing oxidative stress. **A** and **B** Clinical score of EAE severity and latency to fall of control mice, EAE mice, and NAC-administrated EAE mice. **C** and **D** TUNEL staining of apoptosis in spinal cord tissues of the above mice. Scale bars, 50 μm. **E**–**G** Detection of **E** MDA content, **F** SOD activity, and **G** GSH content in spinal cord tissues of the above mice. **H** and **I** DCFH-DA probe for measuring ROS level in spinal cord tissues of the above mice. Scale bars, 50 μm; $^{**}p < 0.01$; $^{****}p < 0.0001$

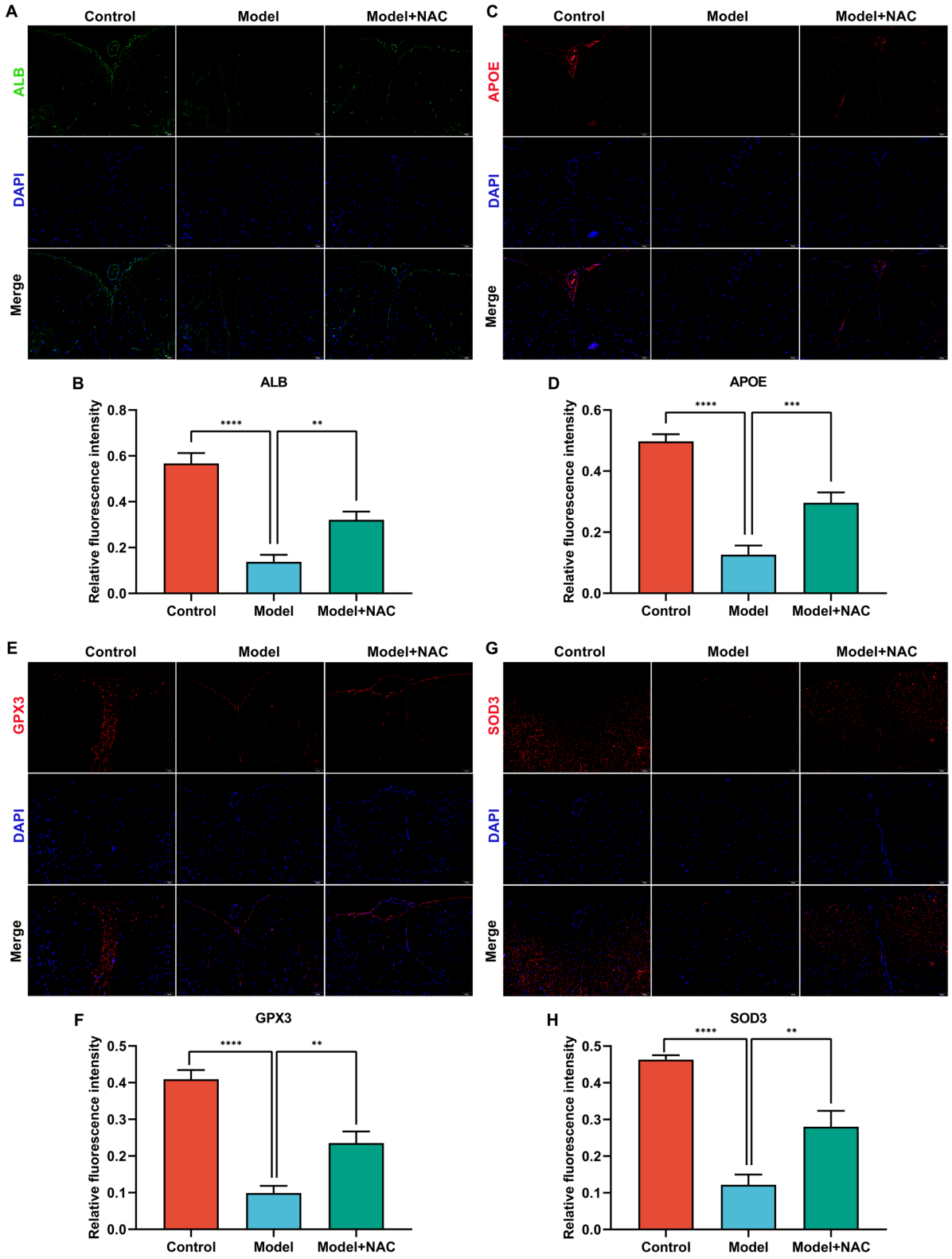

Fig. 5 Anti-oxidative stress by NAC elevates the expression of anti-oxidant ALB, APOE, GPX3, and SOD3 in the CNS of EAE C57BL/6 mice. **A**–**H** Immunofluorescence of **A** and **B** ALB, **C** and **D** APOE, **E** and **F** GPX3, and **G** and **H** SOD3 in spinal cord tissues of control mice, EAE mice, and NAC-administrated EAE mice. Scale bars, 50 μm; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$

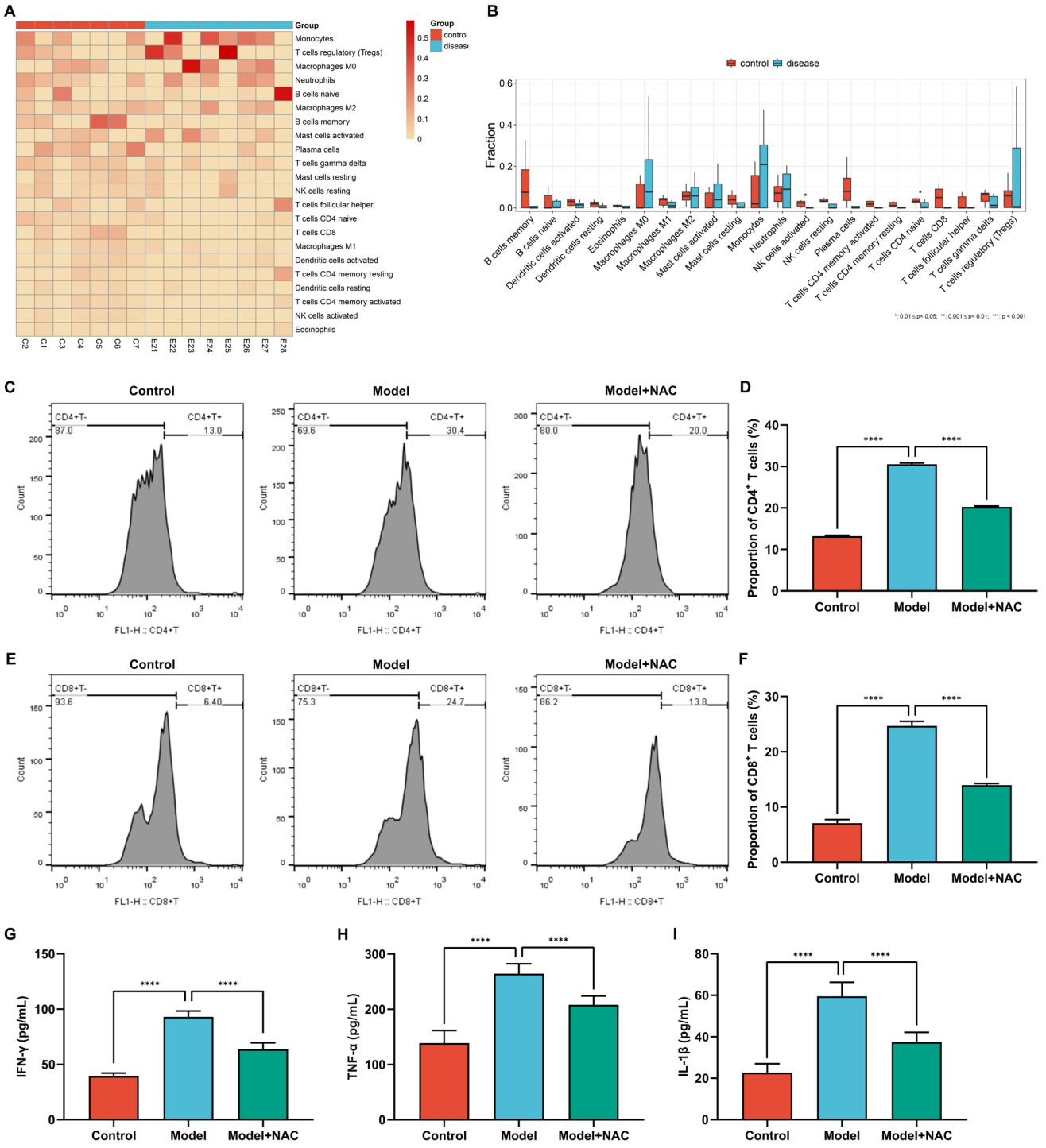

Fig. 6 Anti-oxidative stress by NAC reduces tissue-resident $CD4^+$ and $CD8^+$ T cells in EAE C57BL/6 mice. **A** and **B** Comparison of the fractions of immune cells in the serum of patients with AE in comparison to that of controls. **C**–**F** Flow cytometry of the proportions of tissue-resident **C** and **D** $CD4^+$ and (**E** and **F**) CD8.$^+$ T cells in spinal cord tissues of control mice, EAE mice, and NAC-treated EAE mice. **G**–**I** ELISA of the levels of **G** IFN-γ, **H** TNF-α, and **I** IL-1β in spinal cord tissues of the above mice. $^{****}p < 0.0001$

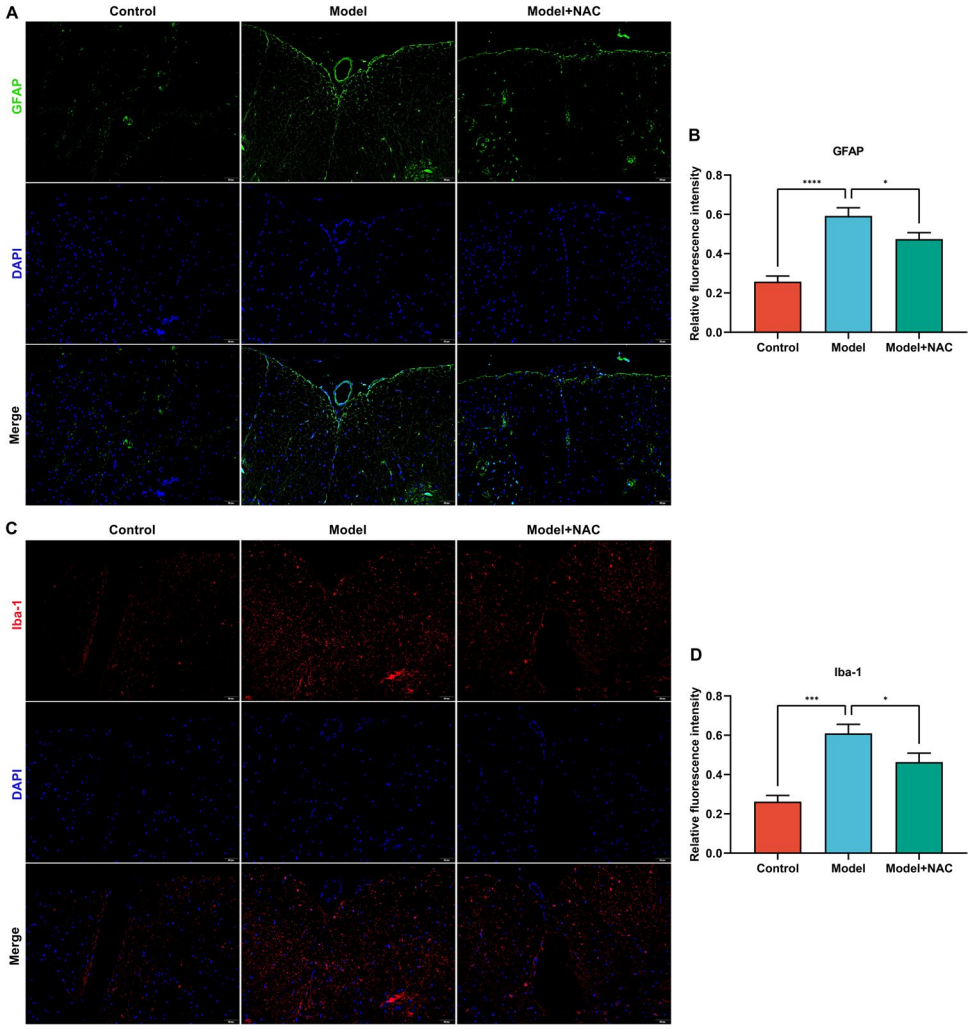

Fig. 7 Anti-oxidative stress by NAC alleviates neuroinflammation in EAE C57BL/6 mice through decreasing astrocytes and microglia in the CNS. **A**–**D** Immunofluorescence of **A** and **B** GFAP (a marker of astrocytes) and **C** and **D** Iba-1 (a marker of microglia) in spinal cord tissues of control mice, EAE mice, and NAC-administrated EAE mice. Scale bars, 50 μm; $^{*}p < 0.05$; $^{***}p < 0.001$; $^{****}p < 0.0001$

NAC Treatment Attenuates Oxidative Stress, Tissue-Resident CD4^{+} and CD8^{+} T Cells and Neuroinflammation in EAE SJL Mice

To further validate the effects and mechanisms of NAC in the treatment of EAE, SJL mice were also used to establish an EAE model and orally administrated with 2 mg/mL of NAC daily for 7 days after 17 days of EAE mouse model establishment. Consequently, NAC administration remarkably reduced the clinical score of EAE severity and increased latency to fall in EAE mice (Fig. 8A and B). This indicated that clinical signs and motor function were improved by NAC administration. Moreover, NAC administration attenuated MDA content and elevated SOD activity and GSH content in spinal cord tissues of EAE mice (Fig. 8C–E). The expression levels of antioxidant ALB, APOE, GPX3, and SOD3 in the spinal cord of EAE mice were also increased after NAC administration (Fig. 8F–J), indicating the reduction in oxidative stress by NAC. It was also observed that NAC administration reduced the proportions of tissue-resident CD4^{+} and CD8^{+} T cells (Fig. 8K–N) as well as reduced the levels of proinflammatory cytokines (IFN-γ, TNF-α, and IL-1β) (Fig. 8O–Q) in spinal cord tissues of EAE mice. These results further confirm that NAC treatment can attenuate oxidative stress and neuroinflammation in the treatment of EAE.

Discussion

Oxidative injury is a pathological feature associated with neurodegenerative diseases and oxidative stress is critical for the pathogenesis of AE (Yong and Yong 2022; Fan et al. 2023; Dąbrowska-Bouta et al. xxxx). Here, we provided serum proteomic landscape of AE patients and controls, and characterized oxidative stress characteristics from the proteomic perspective, reflecting the importance of oxidative stress in diagnosing and therapeutically targeting AE.

While our understanding of the pathophysiological characteristics of AE is increasing, novel diagnostic biomarkers

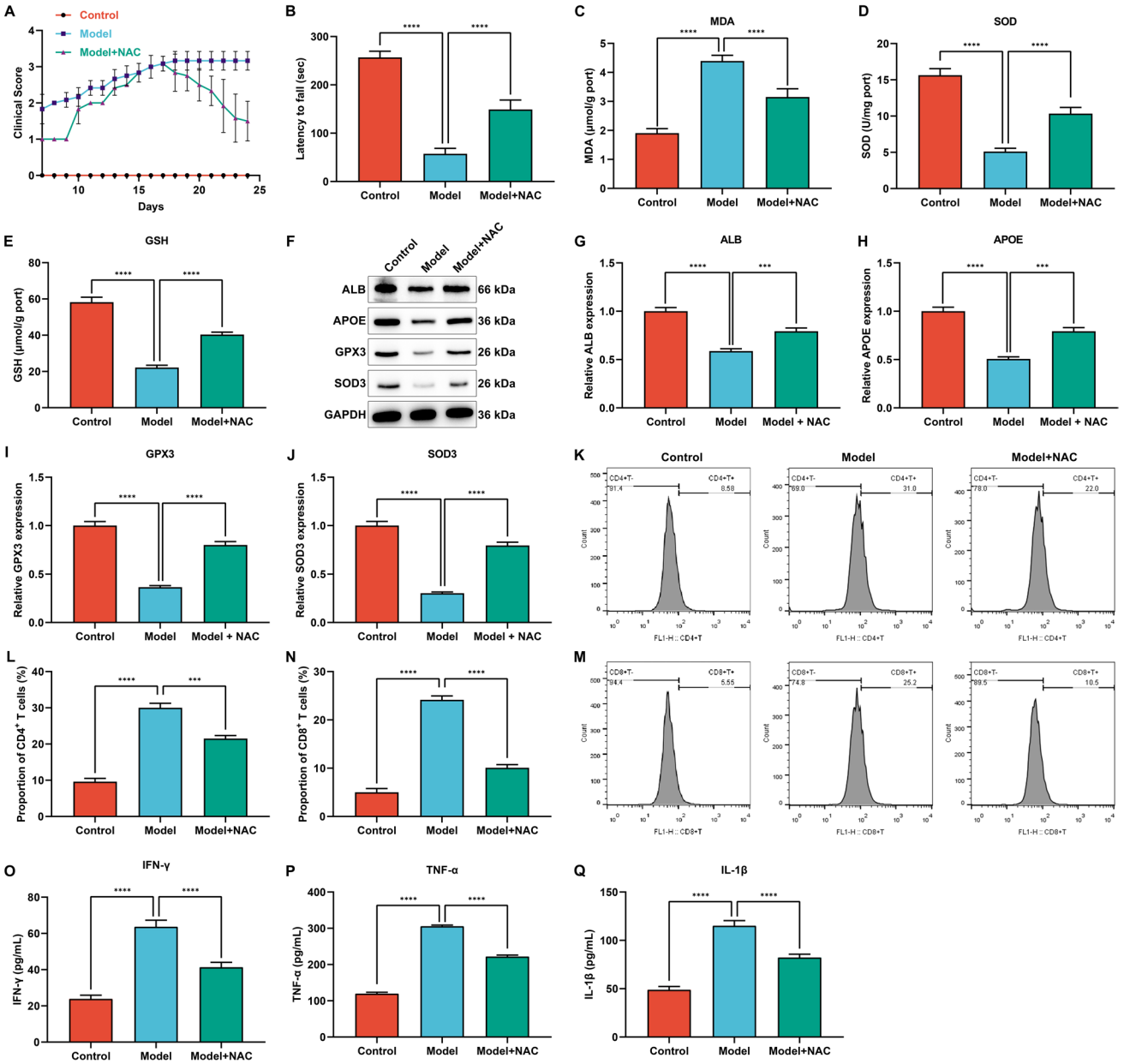

Fig. 8 NAC treatment attenuates oxidative stress, tissue-resident $CD4^+$ and $CD8^+$ T cells and neuroinflammation in EAE SJL mice. **A** and **B** Clinical score of EAE severity and latency to fall of control, EAE, and NAC-treated EAE mice. **C**–**E** Detection of **C** MDA content, **D** SOD activity, and **E** GSH content in spinal cord tissues of the above mice. **F**–**J** Western blot of **F** and **G** ALB, **F** and **H** APOE, **F** and **I** GPX3, and **F** and **J** SOD3 in spinal cord tissues of the above mice. **K**–**N** Flow cytometry of the proportions of tissue-resident **K** and **L** $CD4^+$ and **M** and **N** $CD8.^+$ T cells in spinal cord tissues of the above mice. **O**–**Q** ELISA of the levels of O IFN-γ, **P** TNF-α and **Q** IL-1β in spinal cord tissues of the above mice; $^{***}p < 0.001$; $^{****}p < 0.0001$

are required to support early diagnosis and optimize treatment. Patients with AE were found to possess a unique serum protein profile distinct from controls. Through Naive-Bayes, RandomForest, and SVM models, antioxidant ALB, APOE, GPX3, and SOD3 were determined for potentially diagnosing patients with AE in the clinic. All of them were notably related to immune cells, further revealing the central role of oxidative stress in innate-immune-mediated neurodegeneration (Mendiola et al. 2020). Antioxidative defense proteins ALB, APOE, GPX3, and SOD3 were lowly expressed in the serum of both patients with AE and CNS of EAE mice. Among them, the clinical value of ALB has been evidenced in several studies. When the blood–brain barrier is dysfunctional, serum albumin (ALB), a blood-borne protein, extravasates into the brain parenchyma (Preininger et al. 2023). The increase in the cerebrospinal fluid/serum

albumin quotient has been reported as an independent indicator for poor prognostic outcomes of patients with neuronal surface antibody-associated AE (Lai et al. 2022). A retrospective study showed that ALB is related to non-responses to short-term first-line treatments for AE patients (Liu et al. 2022). However, another study reported elevated albumin levels as an indicator of good immunotherapeutic responses in AE (Jang et al. 2018). Apolipoprotein E (APOE) traffics lipids in the CNS (Windham and Cohen 2024). The E4 variant of APOE is a main genetic risk factor for many neurodegenerative diseases (Krasemann et al. 2017). In EAE mouse models, deficient APOE results in the increased proportions of Th1 and Th17 cells in the circulating system and brain, and increases the expression of proinflammatory cytokines in the CNS (Wei et al. 2013). Furthermore, the deficiency of APOE facilitates blood–brain barrier disruption in EAE mice through modulating MMP-9 (Zheng et al. 2014). Low expression of glutathione peroxidase 3 (GPX3) in cerebrospinal fluid or serum has been evidenced to be correlated to neurodegenerative diseases, e.g., Alzheimer's disease (Panyard et al. 2024). Superoxide dismutase (SOD3) can catalyze the dismutation of the superoxide radical in the extracellular space, thus preventing oxidative damage of lipids/proteins and preserving the bioavailability of nitric oxide (Carmona-Rodríguez et al. 2020). Adeno-associated viral SOD3 gene therapy augments the levels of SOD3 in cerebrospinal fluid and protects the brain from the toxicity of chemotherapy (Jang et al. 2022). Combining prior studies, the antioxidative defense proteins ALB, APOE, GPX3, and SOD3 were potential diagnostic biomarkers of AE and therapeutic targets.

NAC is a synthetic derivative of the endogenous amino acid L-cysteine and a precursor of GSH, with widespread application as a mucolytic and antidote for acetaminophen poisoning (Raghu et al. 2021). In EAE mouse models using C57BL/6 and SJL mice, NAC administration was evidenced to improve clinical signs and motor function as well as alleviate nerve injury through attenuating oxidative stress, which was consistent with prior studies (Ljubisavljevic et al. 2011; Khalatbari Mohseni et al. 2023). Antioxidant ALB, APOE, GPX3, and SOD3 were elevated by NAC in the CNS of EAE C57BL/6 and SJL mice, indicating their potential as NAC targets. T cells exert an indispensable function in the onset and progression of AE (Chen et al. 2024): $CD4^+$ T cells affect AE progression through the secretion of associated cytokines, while $CD8^+$ T cells possess a cytotoxic function and contribute to irreversible damage to neurons. Tissue-resident $CD8^+$ T cells are determined as a critical driver of chronic CNS autoimmunity. An elevated proportion of brain-infiltrating $CD8^+$ T cells display a T^{RM}-like characteristic in patients with AE (Frieser et al. xxxx). The autoreactive tissue-resident $CD8^+$ T cells maintain local neuroinflammatory responses and progressive neuronal loss, which are independent of recirculating $CD8^+$ T cells. Persistent tissue-resident $CD8^+$ T cells in the CNS and their functional export critically depend upon $CD4^+$ T cells. NAC lowered tissue-resident $CD4^+$ and $CD8^+$ T cells in the CNS of EAE C57BL/6 and SJL mice, thus alleviating progressive autoimmune-mediated damage. Moreover, NAC alleviated neuroinflammation by decreasing the activation of astrocytes and microglia. Adjuvant therapy with NAC can be considered an effective strategy for the management of AE. Further clinical trials are urgently needed.

However, the sample size for proteomics analysis is relatively small and represents a limitation of our study. Larger studies may explore the diagnostic utility of proteomic biomarkers for more clinically useful timeframes and estimate improvement using clinically meaningful performance metrics. Although we identified antioxidant ALB, APOE, GPX3, and SOD3 as serum diagnostic biomarkers, our results require external validation in independent studies. Given that our study included limited samples, generalizability must be assessed in future work.

Conclusion

Collectively, this study provides a proteomic analysis of AE patients and healthy controls and proposes antioxidant ALB, APOE, GPX3, and SOD3 as serum diagnostic biomarkers. Anti-oxidative stress by NAC enabled the alleviation of the progression of EAE through ameliorating chronic CNS autoimmunity and neuroinflammation. Therefore, our proteomics and drug discovery methods may identify oxidative stress-related diagnostic markers and therapeutic targets for AE and contribute to the development of selective therapeutic strategies for AE.

Abbreviations AE: Autoimmune encephalitis; CNS: Central nervous system; Tregs: Regulatory T cells; EAE: Experimental autoimmune encephalitis; LC-MS: Liquid chromatography-mass spectrometry; PCA: Principal component analysis; ssGSEA: Single-sample gene set enrichment analysis; SVM: Support vector machine; ROC: Receiver operating characteristic curve; AUC: Area under the curve; CTD: Comparative Toxicogenomics Database; NAC: *N*-Acetylcysteine; GSH: Glutathione; MDA: Malondialdehyde; SOD: Superoxide dismutase; OD: Optical density; ROS: Reactive oxygen species; IFN: Interferon; TNF: Tumor necrosis factor; IL: Interleukin; ALB: Albumin; APOE: Apolipoprotein E; GPX3: Glutathione peroxidase 3; SOD3: Superoxide dismutase

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Data Availability No datasets were generated or analysed during the current study.

Declarations

Ethics Approval and Consent to Participate The protocol of the study was approved by the Ethics Committee of the Affiliated Ganzhou Hospital of Nanchang University (TY-DKY2022-001–01).

Consent for Publication All authors have agreed to publish this manuscript.

Competing Interests The authors declare no competing interests.

References

Lamblin F, Kerstens J, Muñiz-Castrillo S et al (2024) Comparative study of paraneoplastic and nonparaneoplastic autoimmune encephalitis with GABA(B)R antibodies. Neurol Neuroimmunol Neuroinflamm 11:e200229

van Coevorden-Hameete MH, de Bruijn M, de Graaff E et al (2019) The expanded clinical spectrum of anti-GABABR encephalitis and added value of KCTD16 autoantibodies. Brain 142:1631–1643

Maureille A, Fenouil T, Joubert B et al (2019) Isolated seizures are a common early feature of paraneoplastic anti-GABA(B) receptor encephalitis. J Neurol 266:195–206

Ronchi NR, Silva GD (2022) Comparison of the clinical syndromes of anti-GABAa versus anti-GABAb associated autoimmune encephalitis: a systematic review. J Neuroimmunol 363:577804

Jiang C, Zhu M, Wei D et al (2022) SCLC and anti-GABABR encephalitis: a retrospective analysis of 60 cases in China. Thorac Cancer 13:804–810

Flanagan EP, Geschwind MD, Lopez-Chiriboga AS et al (2023) Autoimmune encephalitis misdiagnosis in adults. JAMA Neurol 80:30–39

Dalmau J, Graus F (2023) Diagnostic criteria for autoimmune encephalitis: utility and pitfalls for antibody-negative disease. Lancet Neurol 22:529–540

Mendiola AS, Ryu JK, Bardehle S et al (2020) Transcriptional profiling and therapeutic targeting of oxidative stress in neuroinflammation. Nat Immunol 21:513–524

Qiu C, Tang C, Tang Y et al (2024) RGS5(+) lymphatic endothelial cells facilitate metastasis and acquired drug resistance of breast cancer through oxidative stress-sensing mechanism. Drug Resist Updat 77:101149

Yong HYF, Yong VW (2022) Mechanism-based criteria to improve therapeutic outcomes in progressive multiple sclerosis. Nat Rev Neurol 18:40–55

Fan H, Yang Y, Bai Q et al (2023) Neuroprotective effects of sinomenine on experimental autoimmune encephalomyelitis via anti-inflammatory and Nrf2-dependent anti-oxidative stress activity. Neuromolecular Med 25:545–562

Dąbrowska-Bouta B, Strużyńska L, Sidoryk-Węgrzynowicz M, Sulkowski G (2021) Memantine modulates oxidative stress in the rat brain following experimental autoimmune encephalomyelitis. Int J Mol Sci 22:11330

Alissafi T, Kalafati L, Lazari M et al (2020) Mitochondrial oxidative damage underlies regulatory T cell defects in autoimmunity. Cell Metab 32:591-604.e597

Merckx E, Albertini G, Paterka M et al (2017) Absence of system x(c)(-) on immune cells invading the central nervous system alleviates experimental autoimmune encephalitis. J Neuroinflammation 14:9

Hemmati S, Sadeghi MA, Yousefi-Manesh H et al (2020) Protective effects of leukadherin1 in a rat model of targeted experimental autoimmune encephalomyelitis (EAE): possible role of P47phox and MDA downregulation. J Inflamm Res 13:411–420

DellaValle B, Brix GS, Brock B et al (2016) Glucagon-like peptide-1 analog, liraglutide, delays onset of experimental autoimmune encephalitis in Lewis rats. Front Pharmacol 7:433

Graus F, Titulaer MJ, Balu R et al (2016) A clinical approach to diagnosis of autoimmune encephalitis. Lancet Neurol 15:391–404

Ritchie ME, Phipson B, Wu D et al (2015) limma powers differential expression analyses for RNA-sequencing and microarray studies. Nucleic Acids Res 43:e47

Ge C, Zhu X, Niu X et al (2021) A transcriptome profile in gallbladder cancer based on annotation analysis of microarray studies. Mol Med Rep 23:1

Yu G, Wang LG, Han Y, He QY (2012) clusterProfiler: an R package for comparing biological themes among gene clusters. OMICS 16:284–287

Hänzelmann S, Castelo R, Guinney J (2013) GSVA: gene set variation analysis for microarray and RNA-seq data. BMC Bioinformatics 14:7

Wang Z, Yao J, Dong T, Niu X (2022) Definition of a novel cuproptosis-relevant lncRNA signature for uncovering distinct survival, genomic alterations, and treatment implications in lung adenocarcinoma. J Immunol Res 2022:2756611

Lachmann A, Xu H, Krishnan J et al (2010) ChEA: transcription factor regulation inferred from integrating genome-wide ChIP-X experiments. Bioinformatics 26:2438–2444

Rosenbloom KR, Dreszer TR, Pheasant M et al (2010) ENCODE whole-genome data in the UCSC genome browser. Nucleic Acids Res 38:D620-625

Zhang Q, Liu W, Zhang HM et al (2020) hTFtarget: a comprehensive database for regulations of human transcription factors and their targets. Genomics Proteomics Bioinformatics 18:120–128

Wingender E, Dietze P, Karas H, Knüppel R (1996) TRANSFAC: a database on transcription factors and their DNA binding sites. Nucleic Acids Res 24:238–241

Han H, Shim H, Shin D et al (2015) TRRUST: a reference database of human transcriptional regulatory interactions. Sci Rep 5:11432

Davis AP, Wiegers TC, Johnson RJ et al (2023) Comparative toxicogenomics database (CTD): update 2023. Nucleic Acids Res 51:D1257-d1262

Newman AM, Liu CL, Green MR et al (2015) Robust enumeration of cell subsets from tissue expression profiles. Nat Methods 12:453–457

Song S, Guo R, Mehmood A et al (2022) Liraglutide attenuate central nervous inflammation and demyelination through AMPK

and pyroptosis-related NLRP3 pathway. CNS Neurosci Ther 28:422–434
Rashid Khan M, Fayaz Ahmad S, Nadeem A et al (2023) Cathepsin-B inhibitor CA-074 attenuates retinopathy and optic neuritis in experimental autoimmune encephalomyelitis induced in SJL/J mice. Saudi Pharm J 31:147–153
Lehmann D, Karussis D, Misrachi-Koll R et al (1994) Oral administration of the oxidant-scavenger N-acetyl-L-cysteine inhibits acute experimental autoimmune encephalomyelitis. J Neuroimmunol 50:35–42
Raghu G, Berk M, Campochiaro PA et al (2021) The multifaceted therapeutic role of N-acetylcysteine (NAC) in disorders characterized by oxidative stress. Curr Neuropharmacol 19:1202–1224
Chen J, Qin M, Xiang X et al (2024) Lymphocytes in autoimmune encephalitis: pathogenesis and therapeutic target. Neurobiol Dis 200:106632
Kwon HS, Koh SH (2020) Neuroinflammation in neurodegenerative disorders: the roles of microglia and astrocytes. Transl Neurodegener 9:42
Hasel P, Aisenberg WH, Bennett FC, Liddelow SA (2023) Molecular and metabolic heterogeneity of astrocytes and microglia. Cell Metab 35:555–570
Preininger MK, Zaytseva D, Lin JM, Kaufer D (2023) Blood-brain barrier dysfunction promotes astrocyte senescence through albumin-induced TGFβ signaling activation. Aging Cell 22:e13747
Lai QL, Cai MT, Zheng Y et al (2022) Evaluation of CSF albumin quotient in neuronal surface antibody-associated autoimmune encephalitis. Fluids Barriers CNS 19:93
Liu F, Zhang B, Huang T et al (2022) Influential factors, treatment and prognosis of autoimmune encephalitis patients with poor response to short-term first-line treatment. Front Neurol 13:861988
Jang Y, Lee ST, Kim TJ et al (2018) High albumin level is a predictor of favorable response to immunotherapy in autoimmune encephalitis. Sci Rep 8:1012
Windham IA, Cohen S (2024) The cell biology of APOE in the brain. Trends Cell Biol 34:338–348
Krasemann S, Madore C, Cialic R et al (2017) The TREM2-APOE pathway drives the transcriptional phenotype of dysfunctional microglia in neurodegenerative diseases. Immunity 47:566-581.e569
Wei J, Zheng M, Liang P et al (2013) Apolipoprotein E and its mimetic peptide suppress Th1 and Th17 responses in experimental autoimmune encephalomyelitis. Neurobiol Dis 56:59–65
Zheng M, Wei J, Tang Y et al (2014) ApoE-deficient promotes blood-brain barrier disruption in experimental autoimmune encephalomyelitis via alteration of MMP-9. J Mol Neurosci 54:282–290
Panyard DJ, Reus LM, Ali M et al (2024) Post-GWAS multiomic functional investigation of the TNIP1 locus in Alzheimer's disease highlights a potential role for GPX3. Alzheimers Dement 20:5044–5053
Carmona-Rodríguez L, Martínez-Rey D, Mira E, Mañes S (2020) SOD3 boosts T cell infiltration by normalizing the tumor endothelium and inducing laminin-α4. Oncoimmunology 9:1794163
Jang A, Petrova B, Cheong TC et al (2022) Choroid plexus-CSF-targeted antioxidant therapy protects the brain from toxicity of cancer chemotherapy. Neuron 110:3288-3301.e3288
Ljubisavljevic S, Stojanovic I, Pavlovic D et al (2011) Aminoguanidine and N-acetyl-cysteine supress oxidative and nitrosative stress in EAE rat brains. Redox Rep 16:166–172
Khalatbari Mohseni G, Hosseini SA, Majdinasab N, Cheraghian B (2023) Effects of N-acetylcysteine on oxidative stress biomarkers, depression, and anxiety symptoms in patients with multiple sclerosis. Neuropsychopharmacol Rep 43:382–390
Frieser D, Pignata A, Khajavi L et al (2022) Tissue-resident CD8(+) T cells drive compartmentalized and chronic autoimmune damage against CNS neurons. Sci Transl Med 14:eabl6157