Original Article

Integration of Single-cell RNA Sequencing and Mendelian Randomization Analysis for Identifying Potential Immune Therapeutic Targets in Amyotrophic Lateral Sclerosis

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Abstract

Objective Adaptive immune responses play a critical role in the pathogenesis of amyotrophic lateral sclerosis (ALS). In this study, we investigated the functional mechanisms of T cell subtypes and assessed the causal links between CD4+ cytotoxic T cell-related genes and ALS risk.

Methods Single-cell RNA sequencing (scRNA-seq) of peripheral blood mononuclear cells (PBMCs) from patients with ALS and healthy controls (HC) was used to identify differentially expressed genes (DEGs) in CD4+ cytotoxic T cells. Comprehensive analyses of CD4+ cytotoxic T cells, including pseudotemporal trajectory, intercellular communication, and metabolic pathway analysis, were performed. Mendelian randomization (MR) analysis evaluated the causal effects of DEGs on ALS risk, with validation using independent genome-wide association study (GWAS) data. Expression patterns of the causal genes were further verified using scRNA-seq, bulk-seq, and clinical samples.

Results CD4+ cytotoxic T cells were significantly expanded in patients with ALS. The upregulated genes *S100A6*, *SERPINB6*, *SMAD7*, and *TPST2* were positively correlated with ALS susceptibility, whereas *DIP2A* showed a protective association.

Conclusion *S100A6*, *SERPINB6*, *SMAD7*, *TPST2*, and *DIP2A* were identified as causal genes and potential therapeutic targets in ALS, implicating CD4+ cytotoxic T cells in the disease mechanisms. Further studies targeting these genes and neuroinflammatory pathways are warranted.

Key words: Amyotrophic lateral sclerosis; CD4+ cytotoxic T cells; Drug target; Mendelian randomization; Single-cell RNA sequencing

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INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disorder, marked by the loss of upper and lower motor neurons within the central nervous system (CNS)[1,2]. Clinically, ALS manifests as progressive motor system degeneration characterized by muscle weakness, atrophy, and ultimately paralysis. Most patients develop fatal respiratory failure within 2−5 years of diagnosis and experience significant deterioration in quality of life[3]. Although research on ALS has advanced considerably, treatment options remain limited due to an incomplete understanding of its

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underlying pathophysiological mechanisms[4]. This knowledge gap underscores the critical need for a better understanding of the molecular basis of ALS pathogenesis and progression.

Growing evidence suggests that T cells, key regulators of adaptive immunity, contribute to the pathogenesis of ALS[5-7]. Flow cytometry analyses utilizing well-defined surface markers have revealed significant alterations in peripheral T cell populations in both patients with ALS and mouse models[8-12]. Notably, these studies have consistently reported elevated T cell activation in patients with ALS, implicating immune dysregulation in disease progression[10,12]. Activated peripheral T cells may exacerbate neuroinflammation through multiple mechanisms, including the secretion of proinflammatory mediators into the CNS or direct crosstalk with glial cells, thereby amplifying neurodegenerative cascades[10,13]. Recent investigations have identified the enrichment of cytotoxic CD4+ T cells in the cerebrospinal fluid (CSF) of patients with ALS, further underscoring their potential involvement in disease pathogenesis[10,13]. Supporting this observation, subsequent research demonstrated that CD4+ cytotoxic T cell infiltration in the spinal cord of ALS mouse models is positively correlated with disease progression[5]. Mechanistically, this study proposed that CD4+ T cell activation within the CNS microenvironment may be mediated through MHC class II-dependent antigen presentation by microglia, with subsequent T cell receptor engagement triggering proinflammatory cascades that ultimately contribute to motor neuron degeneration[5]. Despite these advances, the pathogenic mechanisms of CD4+ cytotoxic T cells in ALS remain incompletely characterized, and the causal links between T cell-related genetic factors and ALS susceptibility are still elusive. Addressing these unanswered questions is essential to comprehensively understand the complex immunopathological network in ALS and develop targeted immunomodulatory therapies.

Recent advances in drug development have emphasized the genetic underpinnings of diseases, as evidenced by numerous studies demonstrating that disease-related genetic variants modulate gene expression and contribute to pathological mechanisms[14]. Although genome-wide association studies (GWAS) have successfully identified single nucleotide polymorphisms (SNPs) linked to ALS susceptibility and progression, this methodology alone remains insufficient for definitive causal gene identification or direct therapeutic target discovery[15,16]. To address this limitation, researchers have proposed combining GWAS data with expression quantitative trait loci (eQTL) analyses to enhance the detection of disease-relevant genes[17]. Mendelian randomization (MR) has emerged as a powerful genetic statistical approach that enables drug target discovery by simulating randomized controlled trial conditions[14].

In this study, we aimed to investigate T cell-related immune mechanisms as potential therapeutic targets for ALS. We performed a comprehensive analysis of publicly accessible single-cell RNA sequencing (scRNA-seq) data from peripheral blood, revealing a notable increase in CD4+ cytotoxic T cells in patients with ALS compared to healthy controls (HC). Differential gene expression analysis between CD4+ cytotoxic T cells and other T cell subtypes identified candidate targets, followed by MR analysis to explore potential drug targets in ALS. The MR results were further validated using multiple analytical approaches, including Steiger filtering, reverse MR, Bayesian colocalization, and phenotype scanning. External validation was achieved by analyzing an independent ALS GWAS dataset. Finally, the observed gene expression differences between patients with ALS and HC were additionally verified through both single-cell and bulk transcriptome sequencing data, with further corroboration from clinical samples.

MATERIALS AND METHODS

Data Source and Study Design

The flowchart of the study is shown in Figure 1.

Peripheral blood mononuclear cell (PBMCs) scRNA-seq data comprising samples from 30 patients with ALS and 10 HC (GSE244263) were obtained from the GEO database (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE244263)[18]. A summary of the data is shown in Supplementary Table S1. The cell types were annotated manually, and T cells were further extracted and annotated with reference to previous literature[19-21]. Finally, 16 T cell subsets were identified, with CD4+ cytotoxic T cells exhibiting a significantly higher abundance in patients with ALS. The differentially expressed genes (DEGs) between CD4+ cytotoxic T cells and other T cell subtypes were subsequently screened.

We then obtained comprehensive cis-eQTL data, including summary statistics and allele frequencies, from the eQTLGen Consortium (https://www.eqtlgen.

org/cis-eqtls.html), which encompassed 16,987 genes derived from 31,684 blood samples (Supplementary Table S2)[22]. The cis-eQTL instrument variables (IVs) for the DEGs were selected based on stringent criteria: SNPs significantly associated with DEGs ($P < 5 \times 10^{-8}$), independent linkage disequilibrium ($r^2 < 0.001$), and located within 10,000 kb of the target gene. This selection process yielded 364 SNPs that qualified for subsequent MR analysis.

Two independent ALS GWAS datasets (GWAS ID: ebi-a-GCST90027164 and ebi-a-GCST005647) were obtained from the IEU Open GWAS project (https://gwas.mrcieu.ac.uk/datasets) as outcome datasets (Supplementary Table S2). The primary discovery cohort (GWAS ID: ebi-a-GCST90027164) consisted of 27,250 patients with ALS and 110,881 HC[15], whereas the secondary validation cohort (GWAS ID: ebi-a-GCST005647), consisting of 20,806 patients with ALS and 59,804 HC, was used for external validation[23].

ScRNA-Seq Data Processing and Analysis

The workflows of scRNA-Seq data processing are detailed in Supplementary Figure S1. The cell types were annotated using marker genes obtained from published articles. Based on the annotation results, the ratios of all T cell subtypes between patients with ALS and HC were analyzed. We also conducted pseudotime trajectory and intercellular communication analyses using the Slingshot[24] and CellChat[25] R packages. Subsequently, the DEGs between the cytotoxic CD4+ T cells and the other T cell subtypes were screened using "FindMarkers" function in the "Seurat" R package, with adjusted $P < 0.05$ and $|\log FC| > 0.5$ as screening criteria. GO and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses of the DEGs were conducted using the KOBAS platform (https://bioinfo.org/kobas/genelist/)[26]. Cellular metabolic profiles across T cell subtypes were comparatively analyzed using the "scMetabolism" R package with "AUCell"

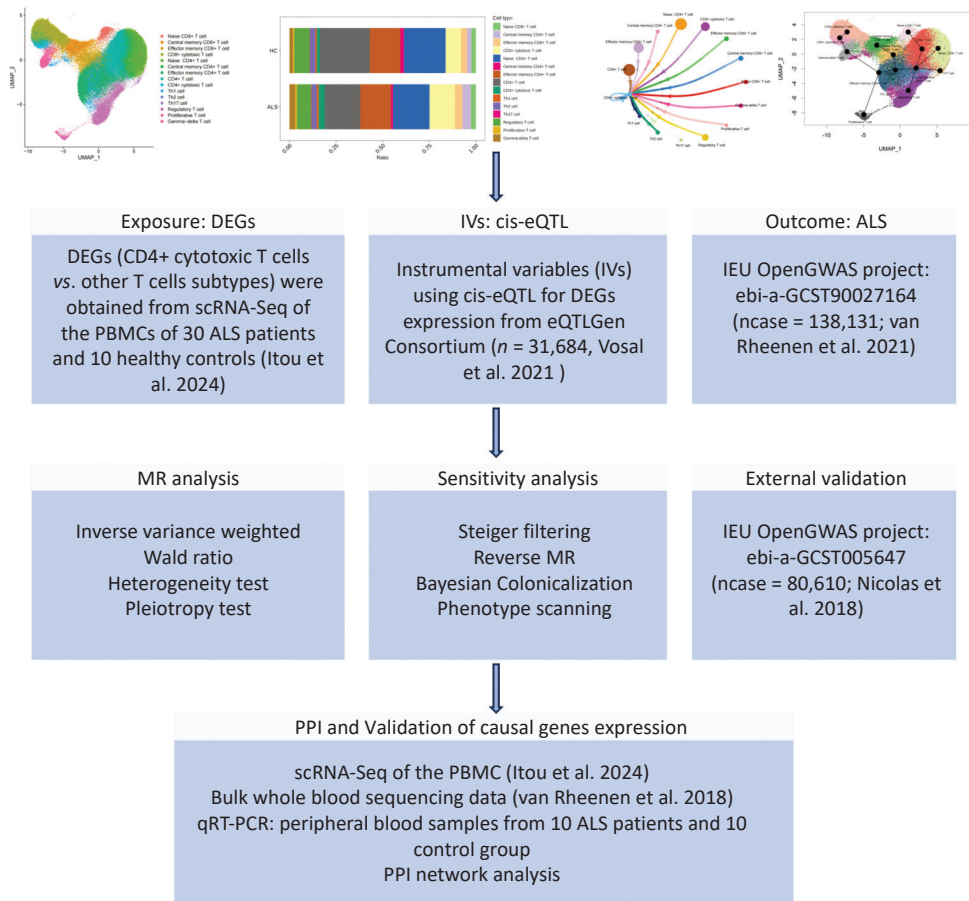


Figure 1. Study design outlining the identification of therapeutic targets causally associated with amyotrophic lateral sclerosis (ALS).

methodology, employing KEGG metabolic pathways as reference.

MR Analysis

MR analysis was performed using the "TwoSampleMR" package in R. Causal relationships between DEGs (exposure) and ALS risk (outcome) were assessed through inverse variance weighted (IVW) and Wald ratio methods. The IVW approach was applied to DEGs with multiple cis-eQTLs, whereas the Wald ratio method was used to analyze DEGs with only one cis-eQTL. For DEGs with more than three qualified SNPs, supplementary analyses, including MR-Egger, weighted median, and simple mode methods, were performed to assess the robustness of the causal estimates. DEGs demonstrating statistically significant associations ($P < 0.05$) were validated using the replication cohort as the outcome dataset. To evaluate the robustness of the findings, we implemented a heterogeneity test using Cochran's Q statistic ($P < 0.05$)[27] and assessed potential horizontal pleiotropy using MR-Egger intercept analysis ($P < 0.05$)[28]. No multiple testing correction was applied to maximize the detection of potential associations.

Sensitivity Analysis

To assess the directionality of the causal relationships between the identified DEGs and ALS, Steiger filtering was applied using eight genetic IVs for ALS (exposure, GWAS ID: ebi-a-GCST90027164) and the causal DEGs (outcome) identified in our primary analysis, with statistical significance set at $P < 0.05$[29]. Bayesian co-localization analysis was subsequently performed to evaluate the probability of shared causal variants between the identified DEGs and ALS using the "coloc" R package[30]. Additionally, the relationship between the causal DEGs and other phenotypes was investigated through comprehensive screening of GWAS data using the "phenoscanner"[31].

Differential Expression of Causal DEGs in Single-cell and Bulk Transcriptome Sequencing

We analyzed the differential expression patterns of five causal genes between patients with ALS and HC using both scRNA-Seq and bulk transcriptome sequencing data. The scRNA-Seq results were visualized as bubble plots generated with the "DotPlot" function in "Seurat" R package, clearly demonstrating expression differences between patients and HC. Bulk whole-blood transcriptome data from 508 HC and 233 patients with ALS (GSE112676; Supplementary Table S3) were downloaded from the GEO database[32]. Following standard preprocessing including background calibration and normalization procedures, we employed the "ggboxplot" function to generate comparative boxplots illustrating expression variations of the causal DEGs between patients with ALS and HC.

Protein–Protein Interaction (PPI) Network and Drug Prediction

The PPI network between proteins encoded by the identified DEGs was mapped using the GeneMANIA analysis platform (https://genemania.org/) to reveal potential mechanistic associations. To identify potential therapeutic drugs for ALS, we used the Drug Signatures Database (DSigDB) on the Enrichr platform (https://amp.pharm.mssm.edu/enrichr/). This approach enabled the systematic discovery of candidate drugs targeting the identified DEGs, with a particular focus on those capable of modulating cytotoxic CD4+ T cell pathology in ALS.

Acquisition of the Clinical Sample

The study protocol was approved by the Ethics Committee of the Chinese PLA General Hospital (Ethics Approval No. S2024-405-1), and written informed consent was obtained from all participants. During the six-month recruitment period from June to December 2024, we a cohort comprising ten patients with ALS meeting the revised EI Escorial diagnostic criteria and ten demographically matched HC were enrolled. Venous blood samples were collected using strict standardized protocols.

RNA Extraction and RT-qPCR

Total RNA isolation from whole blood samples was carried out employing TRIzol reagent (Invitrogen, USA), followed by reverse transcription into cDNA using the ReverTra Ace qPCR RT Master Mix (TOYOBO, FSQ-301). For quantitative gene expression analysis, we conducted RT-qPCR with SYBRGreen qPCR master mix (ES science, RN001) on a Real-Time PCR Detection System (Bio-Rad Laboratories). Gene-specific primers (Generay, China), with sequences provided in Supplementary Table S4, were utilized in conjunction with GAPDH as an internal control to normalize expression data. Quantitative measurements of target gene expression were calculated using the comparative threshold cycle ($2^{-\Delta\Delta CT}$) method.

Statistical Analysis

Statistical analyses were conducted using R

software (version 4.3.0) and GraphPad Prism (version 9.5). Data distribution was first evaluated for normality, with normally distributed variables analyzed using Student's *t*-test for two-group comparisons and non-normally distributed variables examined using the Mann-Whitney U test. A *P*-value < 0.05 was considered statistically significant.

RESULTS

Analysis of scRNA-seq Reveals Elevated CD4+ cytotoxic T cell Populations in ALS

Our analysis utilized publicly available scRNA-seq data of PBMCs from the GEO dataset, including 30 patients with ALS and 10 matched HC. Following standard quality control procedures, we retained 122,743 high-quality cells from patients with ALS and 23,001 cells from HC for subsequent analysis (Supplementary Table S1, Supplementary Figure S2A). After normalizing the scRNA-Seq data, we identified the top 3,000 highly variable genes using the "FindVariableFeatures" function (Supplementary Figure S2B). Subsequent principal component analysis (PCA) enabled dimensionality reduction while preserving biological variation for effective batch integration. We then performed uniform manifold approximation and projection (UMAP) for further dimensionality reduction and cluster identification (Supplementary Figure S3A–B). We further selected immune T cells from the human scRNA-seq data and identified 24 distinct T cell clusters (Supplementary Figure S3C) for visualization. Cell types were manually annotated based on the expression patterns of classical marker genes across clusters (Supplementary Figure S3D) and their positions in 2D space. After cell type annotation, we identified 15 T cell subsets: gamma-delta T cells, naïve CD8+ T cells, naïve CD4+ T cells, central memory CD8+ T cells, effector memory CD8+ T cells, CD8+ cytotoxic T cells, central memory CD4+ T cells, effector memory CD4+ T cells, Th1 cells, Th2 cells, Th17 cells, CD4+ T cells, CD4+ cytotoxic T cells, proliferating T cells, and regulatory T cells (Figure 2A). Figure 2B shows the varying proportions of T cell subtypes in patients with ALS relative to HC. CD4+ cytotoxic T cells were significantly more abundant in patients with ALS than in HC (Figure 2C), suggesting their potential involvement in ALS pathogenesis.

Pseudotime, Cell–cell Communication, and Metabolic Pathways Analyses

Pseudotemporal ordering of all T cell subtypes revealed that CD4+ cytotoxic T cells occupied the terminal position in the differentiation trajectory (Supplementary Figure S4A). To assess potential crosstalk among immune T cells in ALS, we quantified intercellular communication probabilities (Supplementary Figure S4B−D). Further investigation of ligand-receptor-mediated interactions identified several critical signaling axes, including CCL5-CCR4, CD40LG-(ITGAM+ITGB2), GZMA-F2R, and IL16-CD4, which mediate communication between CD4+ cytotoxic T cells and other immune subtypes (Supplementary Figure S4E−F). We conducted a metabolic analysis of the T cell subtypes to further characterize their functional profiles. The results indicated that the glycosaminoglycan biosynthesis-chondroitin sulfate/dermatan sulfate metabolism pathway was the predominantly enriched process in CD4+ cytotoxic T cells (Supplementary Figure S5).

DEGs Identification and Enrichment Analysis

We identified 158 DEGs following differential expression analysis between CD4+ cytotoxic T cells and other subtypes in ALS (Supplementary Table S5). GO and KEGG enrichment analyses illustrated that these DEGs were implicated in inflammatory responses, innate/adaptive immune responses, leukocyte migration, T cell-mediated cytotoxicity, cell-cell adhesion, adhesion molecules, natural killer cell-mediated cytotoxicity, and TNF signaling pathway, among others (Figure 3).

Identification of T Cell-related Causal Genes in ALS

Five causal genes of ALS were identified among these DEGs (Figure 4): *S100A6*, *SERPINB6*, *SMAD7*, *TPST2*, and *DIP2A*. Increased expression levels of *S100A6* (*OR* = 1.283; 95% *CI*, 1.001–1.645; *P* = 0.049), *SERPINB6* (*OR* = 1.072; 95% *CI*, 1.033–1.114; *P* = 0.0003), *SMAD7* (*OR* = 1.363; 95% *CI*, 1.057–1.756; *P* = 0.017), and *TPST2* (*OR* = 1.186; 95% *CI*, 1.038–1.355; *P* = 0.012) correlated significantly with higher ALS risk. Conversely, high *DIP2A* expression was associated with a lower risk of ALS (*OR* = 0.947; 95% *CI*, 0.906–0.991; *P* = 0.017). No heterogeneity or horizontal pleiotropy was observed for these causal genes (Supplementary Tables S6 and S7).

PPI Network and Drug Prediction

The PPI network constructed using GeneMANIA comprised 25 genes (including 5 causal genes and 20 interacting partners) with 345 connections (Supplementary Figure S6A). These interactions were predominantly physical, with additional associations through co-expression, prediction-based links, co-

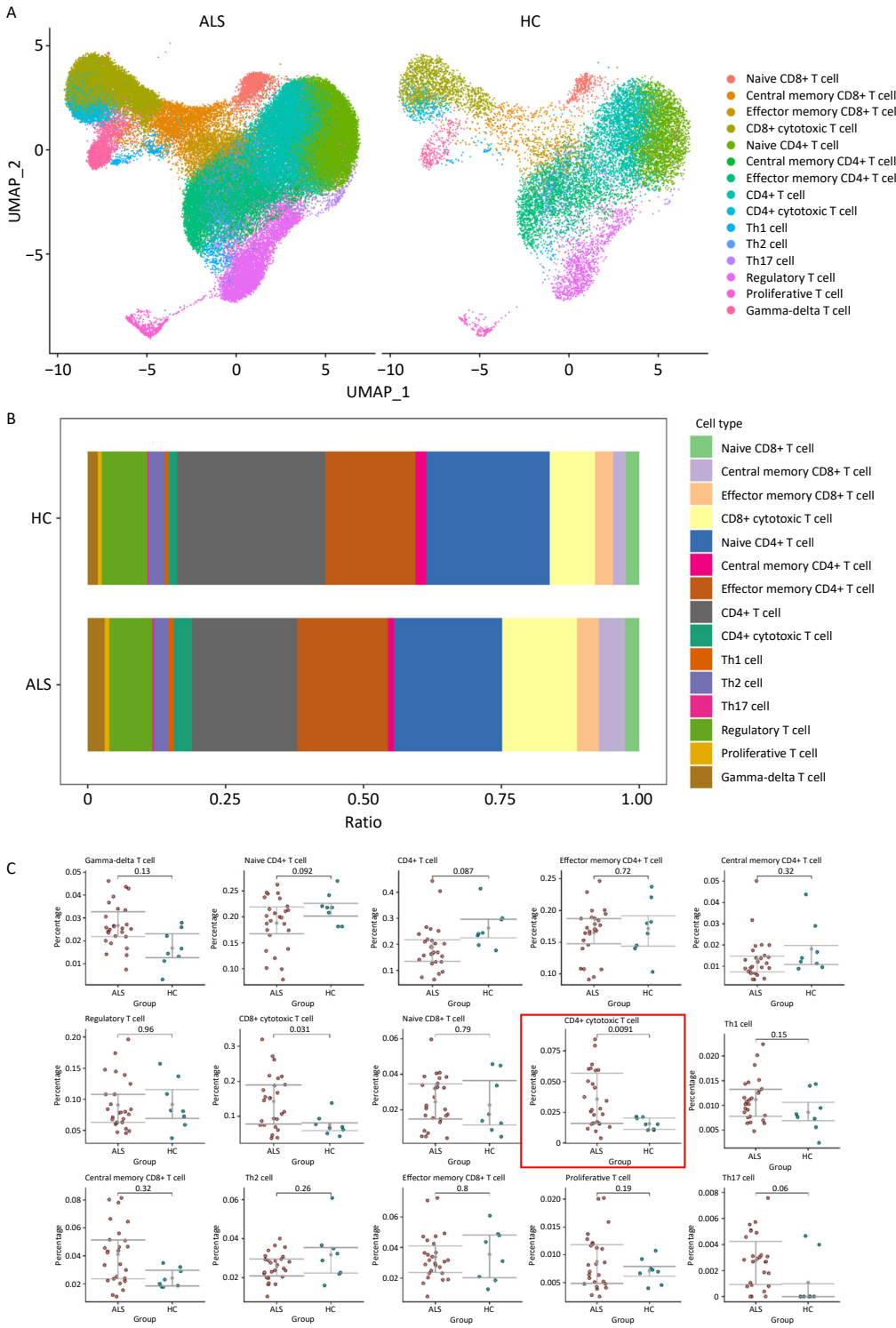


Figure 2. scRNA-seq analysis of T cell subtypes in patients with ALS and HC. (A) UMAP plot showing the distribution of different T cell subtypes in patients with ALS and HC. (B) Bar chart showing the ratio of 15 T-cell subtypes between patients with ALS and HC. (C) Statistical analysis of the proportion of T cells subtypes in patients with ALS and HC. ALS, amyotrophic lateral sclerosis; HC, healthy controls; UMAP, uniform manifold approximation and projection.

localization, genetic interactions, pathway involvement, and shared protein domains. Moreover, this search yielded a list of the top 10 promising drug candidates targeting five causal genes, including glibenclamide, ampyrone, tamoxifen, chlorzoxazone, quercetin, MIGLITOL, lobeline, methaneseleninic acid, ajmaline, and deltamethrin, offering new avenues for therapeutic interventions in ALS (Supplementary Figure S6B).

Sensitivity Analysis

Steiger filtering strengthened the direction of the causality between the five causal genes and ALS (Supplementary Table S8). Consistently, the reverse MR analysis showed no causal effect of ALS on these causal genes (Supplementary Figure S7A). Bayesian co-localization analysis also revealed minimal evidence for shared genetic variants between these genes and ALS (Supplementary Table S9, Supplementary Figure S7B–F). The results of phenotype scanning (Supplementary Table S10) showed that *SERPINB6* (rs318493) and *DIP2A* (rs2096507) were mainly associated with phenotypes related to blood cells, such as platelet,

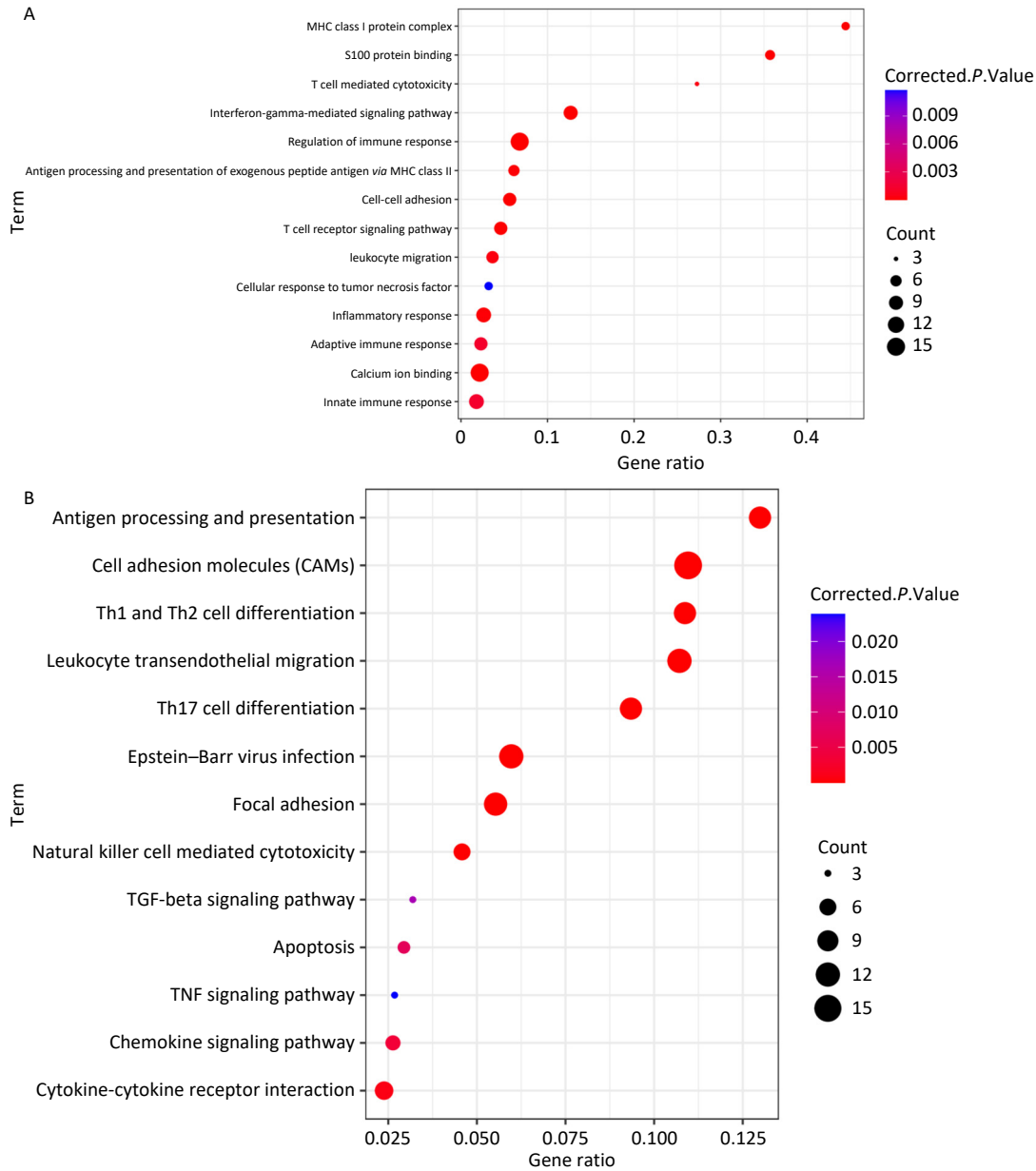
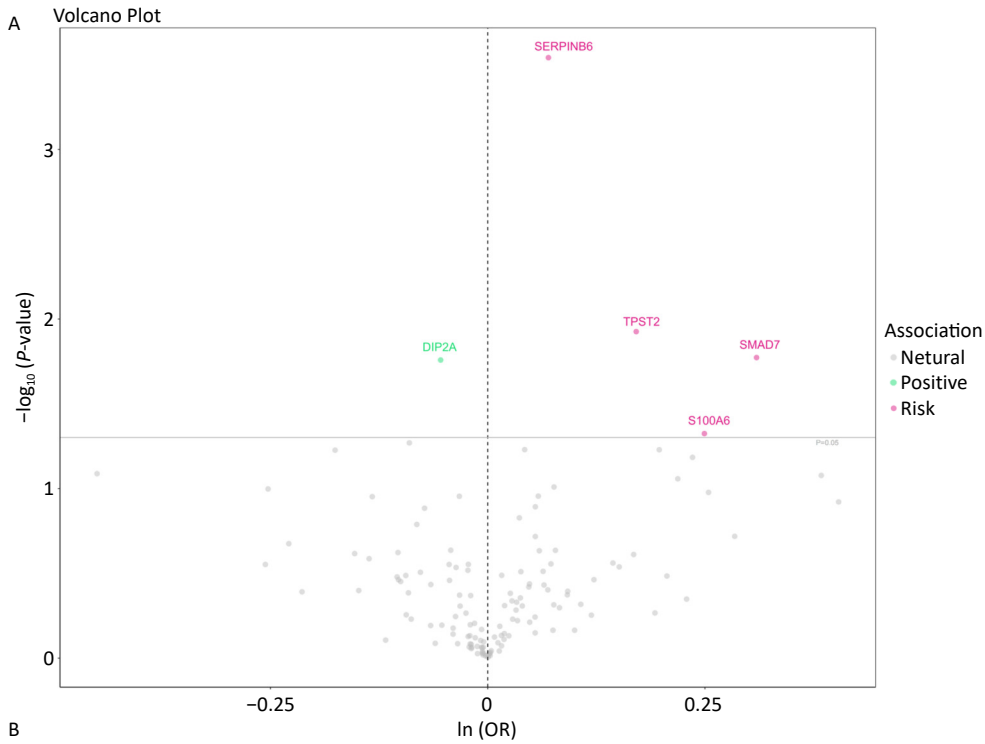


Figure 3. Functional enrichment analyses of the DEGs. (A) GO analysis. (B) KEGG analysis. DEGs, differentially expressed genes; KEGG, Kyoto Encyclopedia of Genes and Genomes; GO, gene ontology.

monocyte, lymphocyte, and neutrophil counts.

External Validation of MR Analysis and Differential Expression of Causal Genes

We tested five causal genes in the replication cohort (ebi-a-GCST005647, outcome) and detected a significant causal effect of *S100A6* (*OR* = 1.296; 95% *CI*, 1.086–1.583; *P* = 0.015), *SERPINB6* (*OR* = 1.056; 95% *CI*, 1.002–1.176; *P* = 0.033), and *DIP2A* (*OR* = 0.915; 95% *CI*, 0.845–0.989; *P* = 0.043; Figure 5A) in ALS. scRNA-seq analysis of peripheral blood CD4+ cytotoxic T cells demonstrated significant upregulation of *S100A6*, *SERPINB6*, *SMAD7*, and *TPST2*, coupled with reduced DIP2A expression in patients with ALS compared to HC (Figure 6B). We also investigated the expression changes of the five causal DEGs in the whole blood transcriptomic dataset. As illustrated in Figure 6C, transcriptomic



Exposure	No.of SNP	Method	*OR* (95% *CI*)	*P*
S100A6	**1**	**Wald ratio**	**1.283 (1.001–1.645)**	**0.049**
SERPINB6	4	MR Egger	1.057 (0.994–1.124)	0.220
SERPINB6	4	Weighted median	1.072 (1.031–1.114)	0.000
SERPINB6	**4**	**Inverse variance weighted**	**1.072 (1.033–1.114)**	**0.000**
SERPINB6	4	Simple mode	1.079 (1.022–1.138)	0.070
SERPINB6	4	Weighted mode	1.075 (1.033–1.119)	0.038
SMAD7	**2**	**Inverse variance weighted**	**1.363 (1.057–1.756)**	**0.017**
TPST2	**2**	**Inverse variance weighted**	**1.186 (1.038–1.355)**	**0.012**
DIP2A	4	MR Egger	0.934 (0.856–1.018)	0.262
DIP2A	4	Weighted median	0.934 (0.889–0.981)	0.007
DIP2A	**4**	**Inverse variance weighted**	**0.947 (0.906–0.991)**	**0.017**
DIP2A	4	Simple mode	0.937 (0.884–0.992)	0.113
DIP2A	4	Weighted mode	0.938 (0.888–0.990)	0.104

Figure 4. MR analysis identifying potential ALS causal genes using eQTL data based on DEGs. (A) Volcano plot showing the MR results for 158 DEGs and their association with ALS risk. (B) Forest plot illustrating the MR results for the five identified causal genes and ALS risk. ALS, amyotrophic lateral sclerosis; DEGs, differentially expressed genes; eQTL, expression quantitative trait loci; MR, mendelian randomization.

profiling identified the consistent upregulation of three genes (*S100A6*, *SERPINB6*, and *TPST2*) in the ALS cohort, suggesting their potential involvement in disease pathogenesis.

Experimental Validation of Key Gene Expression Levels

The qRT-PCR results revealed significant differential expression of the four genes between patients with ALS and HC. Specifically, the expression levels of *S100A6*, *SERPINB6*, and *TPST2* were markedly upregulated in the ALS group compared to HC, whereas *DIP2A* expression was significantly downregulated, which was consistent with the expression trends observed in the bioinformatics analysis ($P < 0.05$; Figure 6).

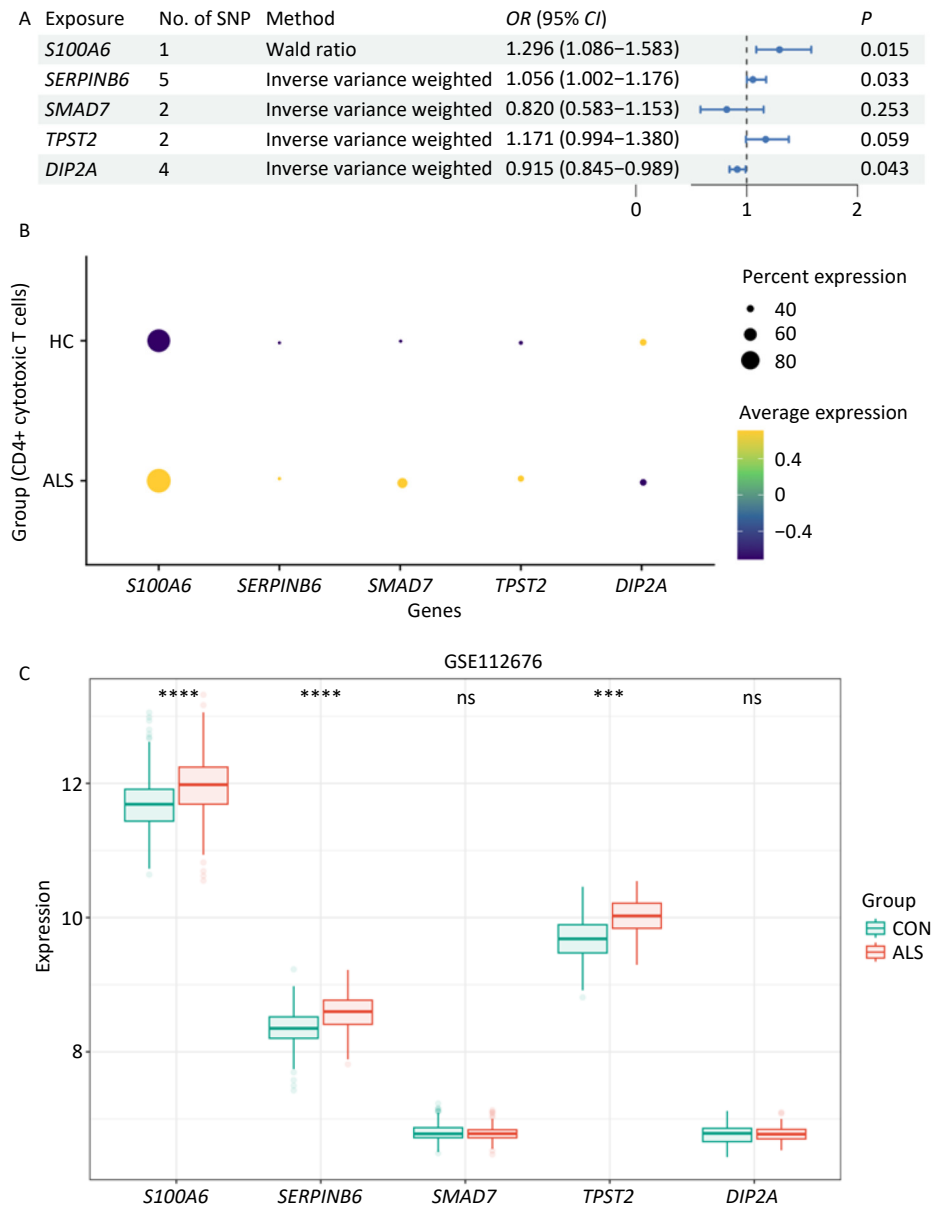


Figure 5. External validation of MR analysis for the five causal genes. (A) Forest plot illustrating MR results for the five identified causal genes and ALS risk using an external validation cohort (ebi-a-GCST005647). (B) Dot diagram showing the average expression levels of the five causal genes in patients with ALS and HC. (C) Box plot demonstrating the external validation of the five causal genes by comparing their expression levels between ALS and HC groups using whole-blood transcriptomic data. $^{***}P < 0.001$, $^{****}P < 0.0001$. ALS, amyotrophic lateral sclerosis; HC, healthy controls; MR, mendelian randomization.

DISCUSSION

Our study is the first to explore potential drug targets for ALS based on peripheral blood T cell-related immune differences between patients with ALS and HC using the MR approach. Our findings indicated that CD4+ cytotoxic T cell-related immune responses may be involved in the pathogenesis of ALS. We also identified five causal genes in CD4+ cytotoxic T cells, *S100A6*, *SERPINB6*, *SMAD7*, *TPST2*, and *DIP2A*, which could be further analyzed as potential drug targets for ALS. Overall, this study identified potential mechanisms linking genetic loci, gene expression, and CD4+ cytotoxic T cell-related immune mechanisms in ALS. MR analysis identified *S100A6*, *SMAD7*, *TPST2*, and *SERPINB6* as risk factors for ALS, and *DIP2A* as a protective factor.

Neuroinflammation may play a pivotal role in the pathogenesis of ALS *via* several interconnected mechanisms[33-35]. First, the infiltration of immune cells into the spinal cord triggers the release of inflammatory mediators, which not only disrupts neuronal function but also induces oxidative damage in neurons by promoting the generation of reactive oxygen species[36]. As the main lymphocyte subtype infiltrating the CNS in ALS, T cells have been shown to be associated with increased levels of CCL2 and microglial activation[37]. Activated microglia produce inflammatory cytokines and neurotoxic substances that exacerbate neuroinflammation and induce neuronal death, leading to the development of ALS[38]. These findings are also supported by the fact that regular use of non-steroidal anti-inflammatory drugs, such as aspirin and acetaminophen, significantly reduces the risk of ALS[39,40].

Previous studies have established the crucial involvement of diverse T cell subtypes (naïve, helper, regulatory) in ALS pathogenesis, traditionally characterized by marker-based flow cytometry analysis[9,12,41]. Recent advances in scRNA-seq have enabled unbiased, high-resolution profiling of disease-associated T-cell states, revealing novel functional subtypes beyond conventional classifications[42]. Our study, based on scRNA-seq data, uncovered a significant expansion of CD4+ cytotoxic T cells in patients with ALS, implicating these cytotoxic T cells in disease pathogenesis. CD4+ cytotoxic T cells, initially characterized in chronic viral infections[43], may represent a critical immunological link between persistent viral exposure and ALS pathogenesis. This hypothesis is supported by recent evidence demonstrating CD8+ T cell infiltration in the anterior horns of patients with ALS-like syndrome, which has been associated with Human T-cell lymphotropic virus type I infection[44]. Considering the well-documented association between viral infections and ALS[45], we propose that chronic antigenic stimulation could promote CD4+

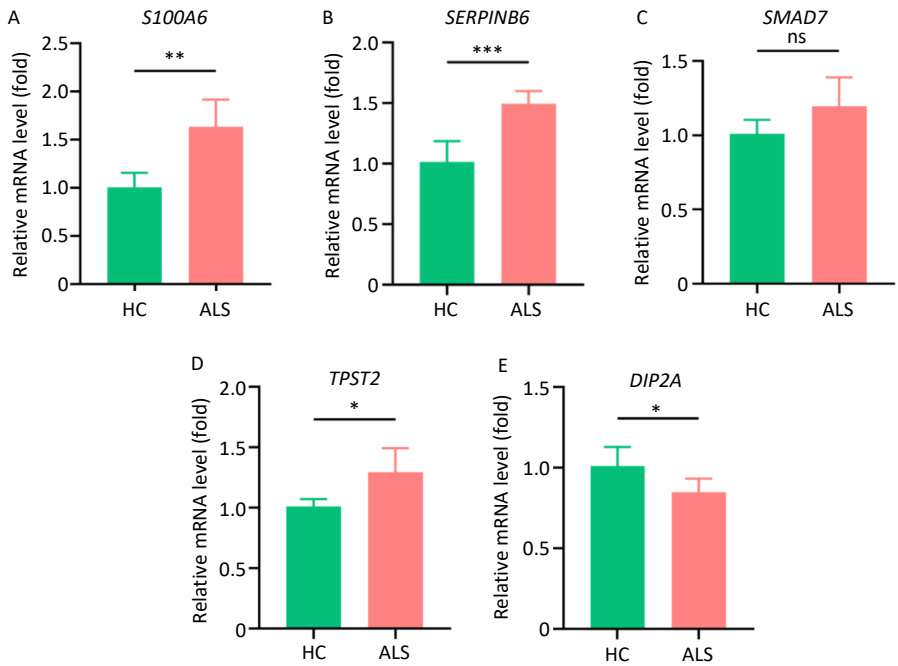


Figure 6. qRT-PCR verification of mRNA expression of *S100A6* (A), *SERPINB6* (B), *SMAD7* (C), *TPST2* (D), and *DIP2A* (E). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

cytotoxic T cell accumulation, potentially contributing to disease progression. In addition to viral triggers, CD4+ cytotoxic T cell expansion has been observed in multiple neurodegenerative disorders, including Alzheimer's disease (AD)[46], Parkinson's disease (PD)[17], and multiple sclerosis[47], suggesting a role in chronic neuroinflammation. In ALS, neuroinflammation represents a key pathological driver involving both resident glial cells (innate immunity) and infiltrating T cells (adaptive immunity)[48]. Our findings align with previous reports of altered CD4+ T-cell differentiation in patients with ALS, with a shift toward cytotoxic subtypes[5]. Expansion of CD4+ cytotoxic T cells in ALS likely reflects persistent immune dysregulation, highlighting their dual potential as diagnostic biomarkers and novel therapeutic targets.

Furthermore, we identified two ALS-specific signaling pathways in CD4+ cytotoxic T cells: the protease-activated receptor (PARs; GZMA-F2R) and macrophage migration inhibitory factor (MIF; CD74+CXCR4) pathways. The PARs pathway, which plays a pivotal role in synaptic modulation and neuroinflammation[49], is significantly upregulated under inflammatory conditions[50]. Activation of this pathway promotes the recruitment of protease-releasing cells and disrupts the blood-brain barrier (BBB), thereby facilitating the entry of peripheral coagulation proteases into the CNS[50]. Although dysregulation of PARs signaling has been implicated in other neurodegenerative disorders, such as AD and PD[51], its GZMA/F2R-mediated mechanism in ALS had not been previously investigated. Our study revealed that GZMA, secreted by gamma delta T cells and proliferative T cells, may interact with the coagulation factor II thrombin receptor (F2R) expressed on CD4+ cytotoxic T cells in ALS, suggesting a novel pathogenic mechanism.

CD4+ cytotoxic T cells exert cytotoxic effects by releasing perforin and granzyme B[52]. Although this subtype constitutes only a minor proportion of peripheral CD4+ T cells under physiological conditions, its expansion has been documented in neurodegenerative diseases and with advancing age[46,53]. Notably, expanded CD4+ cytotoxic T cells have been detected in the CSF of patients with ALS, implying dysregulated T cell immune responses in the CNS[7]. Supporting this observation, in vivo ALS models have indicated that CNS-infiltrating CD4+ cytotoxic T cells may derive from peripheral circulation[5]. Therefore, peripheral CD4+ cytotoxic T cells may be involved in ALS pathogenesis. To further improve our understanding of the effect of CD4+ cytotoxic T cells in ALS, we identified 158 DEGs in CD4+ cytotoxic T cells relative to other subtypes. These DEGs were significantly enriched in pathways associated with leukocyte transendothelial migration, chemokine signaling, innate/adaptive immune responses, inflammatory activation, and cell-cell adhesion. The upregulation of migration- and adhesion-related genes in CD4+ cytotoxic T cells may facilitate their traversal across the BBB in patients with ALS. This notion is further supported by studies in PD, where peripheral CD4+ cytotoxic T cells were shown to secrete interferon-gamma (IFN-γ), thereby activating endothelial cells, compromising BBB integrity, and recruiting additional immune cells to exacerbate neurodegeneration[54]. Although these findings underscore the potential role of CD4+ cytotoxic T cells in ALS progression, the exact mechanisms governing CNS infiltration and its pathogenic contributions remain unclear. Future studies should aim to delineate these processes and evaluate the therapeutic potential of targeting CD4+ cytotoxic T cells in ALS.

Furthermore, through MR analysis, we identified five genes–*S100A6*, *SERPINB6*, *SMAD7*, *TPST2*, and *DIP2A*–whose genetic regulation in CD4+ cytotoxic T cells may have a causal impact on ALS. As a member of the S100 calcium-binding protein family, S100A6 has been extensively characterized as a mediator of inflammatory responses. This protein exerts its pro-inflammatory effects by inducing key cytokines including IL-1β and TNF-α, primarily through activation of the p38/MAPK signaling pathway[55]. Recently, accumulating clinical evidence has established significant correlations between S100A6 expression levels and disease progression in various malignancies (colorectal, gastric, breast, and lung cancers), as well as chronic inflammatory conditions such as chronic obstructive pulmonary disease and Sjögren's Syndrome[56-59]. Of particular relevance to neurodegenerative diseases, S100A6 has emerged as a potential contributor to ALS pathogenesis[60]. Notably, postmortem studies and animal model investigations have consistently revealed elevated S100A6 expression, with pronounced accumulation in activated astrocytes within the spinal cord[61]. These observations strongly suggest that S100A6-mediated neuroinflammation may represent a critical component of ALS pathophysiology. In addition, recent in vitro findings have demonstrated that S100A6 can promote SOD1 protein aggregation, a well-established pathological hallmark of ALS, thereby potentially leading to the degeneration of

motor neurons[62].

SMAD7, a key regulator of TGF-β signaling, is closely associated with inflammatory diseases, such as inflammatory bowel diseases[63]. Recent studies have also suggested a potential role of SMAD7 in neuroinflammation. In a mouse model of postoperative cognitive dysfunction, elevated SMAD7 levels in the hippocampal CA1 region significantly suppressed TGF-β signaling by blocking SMAD2/3 phosphorylation, thereby exacerbating hippocampal neuroinflammation and neuronal apoptosis, which ultimately contributed to postoperative learning and memory impairments[64]. In addition, Kleiter et al. confirmed that SMAD7 is overexpressed in peripheral-blood CD4+ T cells of patients with multiple sclerosis[65]. Notably, SMAD7-transgenic mice exhibited an exacerbated pathology with enhanced CNS inflammation and Th1 activation, whereas SMAD7 silencing in T cells attenuated CNS inflammation and disease severity in mice, establishing SMAD7 as a key regulator of neuroinflammation in autoimmune demyelination[65]. Our findings demonstrated significantly elevated SMAD7 expression in CD4+ cytotoxic T cells from patients with ALS relative to HC, implicating SMAD7-mediated TGF-β signaling dysregulation in the pro-inflammatory mechanisms underlying ALS-associated neuroinflammation[66]. Although these findings position peripheral SMAD7 as a promising ALS biomarker, comprehensive investigations are needed to elucidate its temporal expression patterns in the CNS and potential correlations with disease progression.

Serine protease inhibitor B6 (SERPINB6), a member of the superfamily of proteases inhibitors[67], is implicated in the regulation of numerous physiological processes, including inflammation, autophagy, and apoptosis[68,69]. Previous studies have shown that *SERPINB6* is upregulated in patients with AD and is a susceptibility gene for AD[70,71]. Similarly, *SERPINB6* expression was significantly higher in the peripheral blood of patients with ALS compared to that in HC in our study, indicating that it may be a potential therapeutic target for the treatment of neurodegenerative diseases. Furthermore, previous studies have suggested that TPST2 is involved in cancer[72], with high TPST2 levels correlating with low survival rates in patients with invasive breast carcinoma and stomach adenocarcinoma[72].

Disco interacting protein 2 homolog A (DIP2A) was the only protective factor identified in this study, which is consistent with other reports[73]. As a member of the DIP2 protein family, DIP2A is widely expressed in various tissues, with particularly high levels in the brain. Its activity is modulated by Follistatin-like 1 (FSTL1), and once activated, DIP2A engages with key signaling pathways, including AMPK/mTOR and AKT-to, to regulate critical biological processes such as oxidative stress, transcriptional control, and apoptosis[74-76]. Notably, aberrant DIP2A activation has been linked to multiple brain disorders, including stroke, AD, and glioma[77]. In a stroke model, middle cerebral artery occlusion resulted in elevated levels of both FSTL1 and DIP2A, and recombinant FSTL1 reduced infarct size, improved neurological function, and suppressed apoptosis-effects reversed by FSTL1 or DIP2A inhibition. Specifically, FSTL1 activates DIP2A to enhance Akt phosphorylation, thereby attenuating neuronal apoptosis and neurological deficits, suggesting DIP2A mediates neuroprotection via the Akt pathway.

Our study has certain limitations. First, while these findings indicated a potential involvement of five casual genes in ALS, it's worth emphasizing that the precise mechanisms by which these molecules influence ALS pathology remain to be fully elucidated. Second, our results were restricted to peripheral blood-based cis-eQTL data and T-cell subtypes. However, there may be different T-cell subtypes and genetic regulatory mechanisms in the brain and spinal cord tissues. Thus, future investigations utilizing brain- and spinal cord-based data could yield a superior understanding of the pathogenesis of ALS. Third, although we investigated the association between these five candidate genes and ALS susceptibility, we did not examine their relationship with disease progression rates. Fourth, our findings were based on data from individuals of European descent, which may not apply to other ethnic groups.

The findings of this study may provide a scientific rationale for the development of therapeutic interventions targeting these five potential biomarkers. In subsequent studies, we intend to undertake both in vitro and in vivo studies to further elucidate the functional mechanisms and roles of these genes in ALS pathology. Then, the selected drug candidates will undergo rigorous preclinical evaluation to determine their safety profiles and therapeutic efficacy. By implementing these methodologies, we aspire to translate our scientific findings into tangible therapeutic strategies that can be clinically implemented to offer novel treatment modalities for ALS.

CONCLUSION

Our findings showed that CD4+ cytotoxic T cells are highly abundant in patients with ALS compared to HC, which is indicative of a significant role of this cell subset in ALS-related neuroinflammation. The core DEGs associated with CD4+ cytotoxic T cells, *S100A6*, *SERPINB6*, *SMAD7*, *TPST2*, and *DIP2A,* were causally related to ALS risk and might be potential drug targets for ALS. Enrichment analysis further revealed the important role of cytotoxic CD4+T cell-mediated neuroinflammation in ALS pathogenesis. These findings provide new insights into the pathogenesis of ALS, and further preclinical and clinical research based on these five potential drug targets and neuroinflammation may offer new treatment options.

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Competing Interests The authors declare no conflict of interest.

Ethics This study was conducted after ethical approval was obtained (S2024-405-1). The study protocol was approved by the Ethics Committee of Chinese PLA General Hospital and the Declaration of Helsinki were strictly adhered to during the conduct of this study. All participants signed an informed consent form.

Authors' Contributions Conceptualization, Methodology, Software, and Writing-original draft preparation: Xinyuan Pang. Data curation, Visualization, Investigation: Xinyuan Pang, Hongfen Wang and Jiongming Bai. Supervision, Writing, Reviewing, and Editing: Xusheng Huang. All authors have read and agreed to the published version of the manuscript.

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Data Sharing The data supporting the findings of this study are openly available in the GEO database at https://www.ncbi.nlm.nih.gov/geo/. The supplementary materials will be available in www.besjournal.com.

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