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The correlation of GluR3B antibody with T lymphocyte subsets and inflammatory factors and their role in the progression of epilepsy

Qingwei Lai[1,2*†], Nuan Wang[3,4†], Binbin Wang[5] and Yue Chen[2]

Abstract

Objective To investigate changes in proportions of peripheral blood lymphocyte subsets, the correlation between the lymphocyte subsets and cytokine levels in patients with GluR3B antibody-positive epilepsy, analyze the role of GluR3B antibodies and cytokines in the progression of epilepsy. In addition, the immunotherapeutic effect in patients with GluR3B antibody-positive epilepsy will be evaluated.

Methods Patients with epilepsy hospitalized in the Department of Neurology of the affiliated Hospital of Xuzhou Medical University from December 2016 to May 2023 were recruited. GluR3B antibody levels were measured by enzyme-linked immunosorbent assay (ELISA). Lymphocyte subset proportions were determined using flow cytometry, and serum concentrations of 12 cytokines were measured using cytometric beads array. Differences in T lymphocyte subsets and inflammatory factors were analysed between GluR3B antibody positive and negative patients. Structural equation modeling (SEM) was used to analyse the role of GluR3B antibodies and inflammatory factors in drug-resistant epilepsy (DRE). Finally, the therapeutic effect of immunotherapy on epilepsy patients with GluR3B antibodies was assessed.

Results In this study, sixty-four cases of DRE, sixty-six cases of drug-naïve epilepsy (DNE), and forty-one cases of drug-responsive epilepsy were recruited. (1) DRE patients with positive GluR3B antibody were characterized by a significant increase in the proportion of cluster of differentiation (CD)4^+ T lymphocytes, a decrease in $CD8^+$ T lymphocytes, and an increase of $CD4^+/CD8^+$ ratio. Similar alterations in T lymphocyte subsets were observed in GluR3B antibody-positive patients with DNE. GluR3B antibody levels correlated positively with $CD4^+$ T lymphocytes ($r=0.23$) and negatively with $CD8^+$ T lymphocytes (r=-0.18). (2) In patients with DRE, the serum concentrations of interleukin-1β (IL-1β), IL-8, and interferon-gamma (IFN-γ) were significantly higher in those with positive GluR3B antibody compared to those with negative GluR3B antibody. Serum IL-1β levels were also higher in GluR3B antibody-positive DNE patients compared to antibody-negative DNE patients. In drug-responsive epilepsy patients with GluR3B antibody-positive, both serum IL-1β and IFN-γ levels were higher than those with GluR3B antibody-negative. Moreover, the concentrations of serum GluR3B antibody were positively correlated with the levels of IL-1β, IL-8, and IFN-γ. (3) SEM

†Qingwei Lai and Nuan Wang contributed equally to this work.

*Correspondence:
Qingwei Lai
lqw8696@163.com

Full list of author information is available at the end of the article

analysis indicated that GluR3B antibody may be a direct risk factor for DRE (direct effect = 4.479, 95%CI 0.409–8.503), or may be involved in DRE progression through affecting IFN-γ and IL-8 levels (total indirect effect = 5.101, 95%CI 1.756–8.818). (4) Immunotherapy significantly decreased seizure frequency and serum GluR3B antibody levels, and the seizure frequency was positively correlated with the levels of GluR3B antibody levels in patients receiving immunotherapy.

Conclusions This study demonstrates that GluR3B antibody may influence the progression of epilepsy through altering the proportion of $CD4^+$ and $CD8^+$ lymphocyte subsets and increasing proinflammatory cytokines. The seizure suppression of immunotherapy is associated with the decrease of GluR3B antibody levels. Thus, the present study contributes to a better understanding of the immunoregulatory mechanisms of autoimmune-associated epilepsy and provides a potential target for DRE.

Keywords Epilepsy, GluR3B antibody, Lymphocyte subsets, Proinflammatory cytokines, Structural equation modeling, Immunotherapy

Introduction

Epilepsy is a common chronic paroxysmal neurological disorder that poses significant physical and mental health risks to patients. Currently, there are approximately 70 million epilepsy patients worldwide, with over 10 million patients in China, and an annual increase of about 400,000 new patients [1]. Approximately 30% of patients with epilepsy do not achieve effective control of disease and develop DRE subsequently [2].

An increasing number of studies suggest that the occurrence and development of epilepsy may be associated with abnormal autoimmune mechanism [3, 4]. The amygdala may also be involved in epileptogenesis [5]. It is estimated that approximately 5% of focal epilepsies of unknown etiology may be immune-mediated [6–8]. Autoimmune antibodies targeting neuronal cell receptors in the brain enhance immune reactions, leading to increased neuronal excitability and abnormal synaptic transmission, thereby triggering or exacerbating epileptic seizures [9–13]. GluR3B antibodies, as one of the early-discovered autoantibodies against neurons, bind to specific amino acid sequences of neuronal α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, causing calcium overload and mitochondrial dysfunction, generating excitotoxicity that damages neurons and glial cells, ultimately triggering epileptic seizures [14–17]. Additionally, GluR3B antibodies may interact with the complement system, triggering inflammatory responses, disrupting the blood-brain barrier, and promoting neuronal damage [18]. Previous research had found that GluR3B antibodies were present in patients with early-onset, severe, or DRE [19, 20], with approximately 24% of epilepsy patients having GluR3B antibodies [21]. Clinical studies have shown a significant correlation between GluR3B antibodies and seizure frequency [19], and animal experiments have demonstrated that specific GluR3B antibodies produced in GluR3B peptide-immunized mice can lower the seizure threshold, leading to motor disturbances and abnormal behaviors [22]. Furthermore, some epilepsy patients with GluR3B antibody positivity showed symptom improvement after receiving non-specific immunotherapy [23–25]. Our study also indicates that elevated GluR3B antibody levels can serve as a biomarker for predicting DRE [26]. Taken together, these research findings highlight the significant role of GluR3B antibodies in the process of epilepsy development.

Clinical studies have shown significant disturbances in T lymphocyte populations in patients with epilepsy [27–29]. Levite and colleagues demonstrated that AMPA-GluR3 is highly expressed in $CD4^+$ T lymphocytes and $CD8^+$ T lymphocytes of healthy volunteers, and that autoantibodies against GluR3B peptide can induce damage to normal human T lymphocytes in vitro, particularly with more significant damage observed in the T lymphocytes of patients with autoimmune diseases such as nodding syndrome [30]. In addition to producing GluR3B-specific antibodies, 3B peptide-specific T cells [17]. Whether GluR3B antibodies damage T lymphocytes in patients with epilepsy has not been reported. Previous studies have also found increased expression of $CD8^+$ T lymphocytes in patients with Rasmussen's encephalitis, marginal encephalitis, and glutamic acid decarboxylase-65 (GAD65) antibody positivity [31, 32]. Furthermore, intriguingly, there is an increased proportion of $CD4^+$ T lymphocytes in the cerebrospinal fluid (CSF) of temporal lobe epilepsy (TLE) patients and in the serum of patients with DRE [32, 33]. In the central nervous system (CNS), marginal encephalitis driven by $CD8^+$ T lymphocytes may contribute to the development of TLE [34]. Additionally, serum IL-6 concentrations were significantly elevated in autoimmune epilepsy patients related to GAD65 antibodies [35]. However, the characteristics of peripheral blood lymphocyte subpopulations and inflammatory factors in GluR3B antibody-positive epilepsy patients remain unclear.

There is no consensus on whether epilepsy patients positive for GluR3B antibodies should receive

immunotherapy. Previous studies have shown that some epilepsy patients with positive GluR3B antibody experienced symptom improvement after receiving nonspecific immunotherapies such as plasma exchange [25, 36, 37], intravenous immunoglobulin (IVIG) [24, 38], or immunoadsorption therapy [23]. However, other studies have found that 1 case of GluR3B antibody-positive seizures failed to improve following immunotherapy [39]. It has also been suggested that neuroautoantibodies act as a by-product of the immune response rather than a direct causative factor [40]. Therefore, whether immunotherapy is suitable for GluR3B antibody-positive epilepsy patients still requires further in-depth research and clinical validation. This study aims to analyze the changes in peripheral blood T lymphocyte subpopulations and cytokine expression in GluR3B antibody-positive epilepsy patients, evaluate the therapeutic effects of immunotherapy, and explore the potential mechanisms of GluR3B antibody involvement in epilepsy progression. Therefore, this research contributes to a better understanding of the immunoregulatory mechanisms of autoimmune-associated epilepsy and provides a potential target for DRE.

Materials and methods

Ethical approval of the study protocol

The study protocol was ratified (XYFY2016-KL017-02) by the Ethics Committee of the Affiliated Hospital of Xuzhou Medical University (Xuzhou, China). Each participant (or his/her guardian) provided written informed consent.

Study cohort

171 patients suffering from epilepsy hospitalized from December 2016 to May 2023 were recruited. These patients underwent extensive clinical examination, including magnetic resonance imaging (MRI), electroencephalography (EEG), and biochemical tests. Two experts in epilepsy management assessed the diagnosis and seizure type of epilepsy according to International League Against Epilepsy (ILAE) guidelines [41]. Also, 40 healthy volunteers matched for age and sex were recruited during the same period. Autoimmune encephalitis, demyelinating diseases, the acute phase of CNS infection, severe cognitive impairment, psychogenic nonepileptic seizures, systemic lupus erythematosus, or paraneoplastic syndrome were the exclusion criteria.

A comprehensive search for relevant data was conducted on PubMed and Web of Science databases using various search terms such as 'GluR3B antibody', 'epilepsy', 'lymphocyte subsets', 'proinflammatory cytokines', and 'immunotherapy' from database inception to July 2024.

Patients with epilepsy were defined as "drug naïve" if they had never been treated with antiseizure drugs at the time of enrolment. DRE was defined as seizures of any frequency in the previous 12 months after taking ≥ 2 optimal doses [42].

Evaluating whether epilepsy patients with high titers of GluR3B antibodies should receive immunotherapy has strict inclusion criteria. The inclusion criteria are as follows: (1) Seizures are the only or main symptom. (2) Seizures are frequent, with poor response to antiseizure medications and high serum GluR3B antibody titers (OD value > 2 cut-off value). Seizure frequency: at least 2 seizures per month in the preceding 3 months before consultation. (3) Complete CSF examination with normal white cell count. (4) No inflammatory lesions on cranial MRI, no enhancement. (5) Informed consent from the patient or guardian. The patients enrolled in the group were randomly divided into the immunotherapy + antiseizure drug group (immunotherapy group) and antiseizure drug group (control group), and the frequency of seizures was evaluated at 1, 3, and 6 months post-treatment. Serum GluR3B antibody levels before and after immunotherapy were detected by ELISA kits.

The antiseizure drug group is defined as receiving ≥ antiseizure drugs at optimal doses, with no change in dose or type of antiseizure drugs within 6 months of enrolment. The immunotherapy group is defined as patients receiving IVIG, steroids, or combined immunotherapy, follow-up for at least 6 months. The dose and type of antiseizure drugs remained unchanged. Response to immunotherapy is defined as a 50% reduction in seizure frequency compared to baseline.

Blood collection and isolation of Peripheral-Blood Mononuclear Cells (PBMCs)

Samples of peripheral venous blood were harvested from each participant. PBMCs were isolated as described previously [43]. For serum separation after coagulation, samples underwent centrifugation (1000 × *g*, 10 min, 4 °C) and kept at −80 °C.

Detection of GluR3B antibodies

GluR3B antibodies in serum were detected by ELISA as described previously by our research team [26]. The amino-acid sequence of the human GluR3B peptide segment 372–395 is NEYERFVPFSDQQISNDSSSSENR, and was synthesized by Shanghai Qiang Yao Biotechnology (Shanghai, China).

Flow Cytometry (FCM)

The acquisition, processing, and analyses of cells for FCM were undertaken as stated previously [44]. Lymphocyte subsets were identified by FCM using 2 mL of EDTA-anticoagulated blood. Cell clusters for differentiated antigens were assayed by FCM using CD3/CD16^{+}56/CD45/CD4/CD19/CD8 Reagent (batch number: 20200001; Beijing Tongsheng Biotechnology, Beijing, China). FCM was

carried out using a Navios™ 10 Color system (Beckman Coulter, Fullerton, CA, USA).

For the detection of T-regulatory cells (Tregs), 5 mL of EDTA-anticoagulated blood was added to culture medium (RPMI 1640, 100 µL). This action was followed by mixing, placement in an incubator in an atmosphere of 5% CO_2, and incubation for 5 h at 37 °C. Then, after addition of monoclonal antibodies (CD3, CD4, CD25), samples underwent incubation in the dark for 30 min at room temperature. Next, lysis buffer (2 mL) was added to red blood cells, followed by thorough mixing. Finally, cells underwent incubation in the dark for 10 min at room temperature, and FCM was employed for detection.

Detection of cytokines with a Cytometric Bead Array (CBA)

Circulating levels of cytokines were measured with a CBA in compliance with manufacturer protocols (Qingdao Riskell Biotechnology, Qingdao, China). EDTA-anticoagulated blood (5 mL) was centrifuged at 1000 g for 10 min. Sample and calibration tubes were filled with 25 µL each of buffer, Matrix B, sample, and calibrator. Capture microspheres and detection antibodies (25 µL each) were added and incubated for 2 h at room temperature in the dark (400 r/min). Subsequently, SA-PE (25 µL) was added, incubated for 0.5 h at room temperature, and 500 µL of 1x washing buffer was added. After mixing and centrifugation at 400 g for 5 min, samples were processed according to flow cytometer requirements before immediate machine detection. Data were analysed in duplicate on a plate reader (QuickPlex™ SQ120; Meso Scale Discovery, Rockville, MD, USA) [45]. The pooled coefficient of variation for the multiplex immunoassays was 8.25%.

Statistical analyses

Statistical analyses were carried out using SPSS 23.0 (IBM, Armonk, NY, USA) and R (version 3.4.2). The Mann-Whitney *U* test or independent samples *t*-test was used to analyze the differences in peripheral blood T lymphocyte subpopulations and inflammatory factors between antibody-positive and -negative groups, and Spearman rank correlation test was used to evaluate the association between GluR3B antibody levels and T lymphocyte subpopulations and inflammatory factors. The Kruskal-Wallis *H* test and Bonferroni method were used to screen the differential inflammatory factors between DRE and drug-responsive epilepsy, which were then included in SEM using Mplus 7.4 software to analyze the direct or indirect effects of GluR3B antibody levels and inflammatory factors in DRE. $P<0.05$ was considered significant.

Table 1 Demographics of drug-resistant epileptic patient according to the serum concentration of GluR3B antibodies. Notes: serum concentration of GluR3B antibodies: group A ≥ 0.335 OD; group B < 0.335 OD

	Drug-resistant patients (*n* = 64)		
	Group A (*n* = 36)	Group B (*n* = 28)	*P*
Age (years)	35.0 ± 15.8	29.2 ± 13.6	0.14
Gender (M,%)	21.0 (58.5)	17.0 (60.7)	0.85
Epilepsy duration, y [M (P_{25},P_{75})]	10.0 (2.0,18.5)	4.5 (0.78,108)	0.18
Age at first seizure, y [M (P_{25},P_{75})]	18.0 (13.0, 37.5)	19.5 (13.0, 32.8)	0.68
Abnormal EEG (n, %)	35.0 (97.2)	26.0 (92.9)	0.41
Abnormal MRI (n, %)	21.0 (58.3)	21.0 (75.0)	0.16
Number of AEDs	2.4 ± 0.5	2.3 ± 0.6	0.44

Table 2 Demographics of drug-naïve epileptic patient according to the serum concentration of GluR3B antibodies. Notes: serum concentration of GluR3B antibodies: group A ≥ 0.335 OD; group B < 0.335 OD

	Drug-naïve patients (*n* = 66)		
	Group A (*n* = 9)	Group B (*n* = 57)	*P*
Age (years)	41.1 ± 25.0	36.7 ± 21.0	0.43
Gender (M,%)	4.0 (44.4)	33.0 (57.9)	0.45
Epilepsy duration, y [M (P_{25},P_{75})]	0.01 (0.01, 0.7)	0.3 (0.03, 2.0)	0.079
Age at first seizure, y [M (P_{25},P_{75})]	51.0 (15.0, 61.0)	33.0 (15.0, 52.0)	0.51
Abnormal EEG (n, %)	8.0 (88.9)	43.0 (75.4)	0.37
Abnormal MRI (n, %)	4.0 (44.4)	18.0 (31.6)	0.45
Number of AEDs	-	-	-

Results

Clinical characteristics of individuals suffering from epilepsy

In this study, a total of 171 epilepsy patients were enrolled, including DRE ($n=64$), DNE ($n=66$), and drug-responsive epilepsy ($n=41$). Among them, there were 100 male and 71 female patients, with an average age of 34.5 ± 18.2 years. Serum levels of GluR3B antibody were considered to be positive if it exceeded the mean optical density (OD) values + 2 standard deviations values of the respective levels of GluR3B antibody in healthy controls. The calculated OD value for the cut-off of GluR3B antibody was found to be 0.335. Subsequently, patients were categorized into 2 groups based on their GluR3B antibody levels: Group A with GluR3B antibody levels OD ≥ 0.335, and Group B with GluR3B antibody levels OD < 0.335. The clinical characteristics of patients with DRE, DNE, and drug-responsive epilepsy were presented in Tables 1, 2 and 3.

Among patients with DRE, DNE, and drug-responsive epilepsy, there were no statistically significant differences between GluR3B antibody-positive and -negative

Table 3 Demographics of drug-responsive epileptic patient according to the serum concentration of GluR3B antibodies. Notes: serum concentration of GluR3B antibodies: group A ≥ 0.335 OD; group B < 0.335 OD

	Drug-responsive patients (*n* = 41)		
	Group A (*n* = 8)	Group B (*n* = 33)	*P*
Age (years)	34.5 ± 13.2	32.9 ± 17.8	0.44
Gender (M,%)	3.0 (37.5)	22.0 (66.7)	0.13
Epilepsy duration, y [M (P_{25},P_{75})]	7.5 (3.3,13.8)	10.0 (7.0,19.0)	0.27
Age at first seizure, y [M (P_{25},P_{75})]	20.0 (16.5, 27.5)	15.0 (11.5, 29.5)	0.19
Abnormal EEG (n, %)	7.0 (87.5)	28.0 (84.8)	0.85
Abnormal MRI (n, %)	2.0 (25.0)	17.0 (51.5)	0.18

patients in terms of patient age, gender, age at first seizure, disease duration, abnormal video EEG, and cranial MRI results.

Distribution of T lymphocyte subsets was altered in the peripheral blood of patients with GluR3B antibody positive epilepsy

Flow cytometry was used to assess the distribution of T lymphocyte subsets in peripheral blood of epilepsy patients with positive GluR3B antibodies. The results revealed that compared to patients with negative GluR3B antibodies, DRE patients with positive GluR3B antibodies showed an increase in $CD4^+$ T lymphocyte proportion (41.3 ± 4.9% vs. 37.2 ± 6.4%, P = 0.007), a significant decrease in $CD8^+$ T lymphocyte proportion (23.5 ± 6.1% vs. 27.5 ± 6.3%, P = 0.013), and an increase in $CD4^+$ T/$CD8^+$ T ratio (1.9 ± 0.7% vs. 1.5 ± 0.5%, P = 0.002) (Fig. 1A-D). In drug-naïve patients, those with positive GluR3B antibodies also exhibited similar changes in T lymphocyte subsets, with an increase in $CD4^+$ T lymphocyte proportion, an increased $CD4^+$/$CD8^+$ ratio (46.9 ± 5.6% vs. 39.6 ± 7.8%, P = 0.006; 2.5 ± 0.6 vs. 1.9 ± 1.0, P = 0.011), and a significant decrease in $CD8^+$ T lymphocyte proportion (19.9 ± 3.9% vs. 25.2 ± 9.8%, P = 0.043) (Fig. 1E-H). However, in drug-responsive epilepsy patients, there were no significant differences in T lymphocyte subsets between those with positive and negative GluR3B antibodies (Fig. 1I). Finally, correlation analysis of serum GluR3B antibody levels with the proportions of $CD4^+$ and $CD8^+$ T lymphocytes showed a positive correlation between serum GluR3B antibody levels and $CD4^+$ T lymphocyte proportion (r = 0.23, P = 0.0021), and a negative correlation with $CD8^+$ T lymphocyte proportion (r = -0.18, P = 0.018) (Fig. 1J).

Levels of proinflammatory cytokines were increased significantly in epilepsy patients with positive GluR3B antibody

The proportion of $CD4^+$ T lymphocytes in peripheral blood of epilepsy patients with positive GluR3B antibodies was increased. Subsequently, we analyzed whether $CD4^+$ T lymphocytes tended to differentiate towards pro-inflammatory helper T cells, and detected 12 cytokines associated with T lymphocyte polarization. Results showed that in DRE patients, those with positive GluR3B antibodies had significantly higher serum concentrations of IL-1β, IL-8, and IFN-γ (7.8 ± 4.5 vs. 5.5 ± 4.0, P < 0.0001; 13.3 ± 18.0 vs. 7.8 ± 10.5, P = 0.02; 10.1 ± 5.4 vs. 5.0 ± 3.7, P < 0.0001, Fig. 2A-D), while the concentrations of anti-inflammatory cytokines IL-10 and IL-4 did not differ significantly between the two groups (Fig. 2A). In DNE patients, those with positive GluR3B antibodies had significantly higher serum IL-1β concentrations compared to antibody-negative patients (10.5 ± 5.6 vs. 5.1 ± 5.3, P = 0.001, Fig. 2H-I). Furthermore, evaluation of cytokine levels of drug-responsive epilepsy patients revealed that patients with positive GluR3B antibodies had significantly higher serum IL-1β and IFN-γ concentrations compared to the antibody-negative group (8.7 ± 4.3 vs. 4.4 ± 3.5, P = 0.018; 6.4 ± 2.0 vs. 2.8 ± 1.7, P < 0.0001, Fig. 2E-G). Correlation analysis of serum GluR3B antibody levels with the concentrations of the cytokines revealed positive correlations between serum GluR3B antibody levels and IL-1β, IL-8, and IFN-γ (r = 0.40, P < 0.0001; r = 0.29, P = 0.0001; r = 0.50, P < 0.0001, Fig. 2J-L). These findings suggest that T lymphocytes from GluR3B antibody-positive epilepsy patients are more inclined to differentiate into Th17 and Th1 cells with pro-inflammatory factor secretion. Flow cytometry analysis further demonstrated that circulating cytokine levels associated with differentiation of $CD4^+$ T lymphocytes toward pro-inflammatory phenotypes were elevated in GluR3B antibody-positive epilepsy patients.

The proportion of DRE patients with positive GluR3B antibodies was significantly higher

Among DRE and drug-responsive epilepsy patients, the proportion of DRE patients with positive GluR3B antibodies was significantly higher than in antibody-negative patients (81.8% vs.45.9%, P < 0.0001, Fig. 3A). Meanwhile, the rate of positive GluR3B antibodies was significantly higher in DRE patients compared to drug-responsive epilepsy patients (56.3% vs. 19.5%, P < 0.0001, Fig. 3B). Furthermore, the level of GluR3B antibodies was also significantly higher in DRE patients compared to drug-responsive epilepsy patients (0.37 ± 0.15 OD vs. 0.22 ± 0.11 OD, P < 0.0001, Fig. 3C). These findings suggest that DRE patients have a higher rate of positive GluR3B antibodies as well as higher antibody levels.

Distribution of T lymphocyte subsets and inflammatory factors in peripheral blood of DRE patients

First, we compared the changes in peripheral blood T lymphocyte subsets and found no significant differences

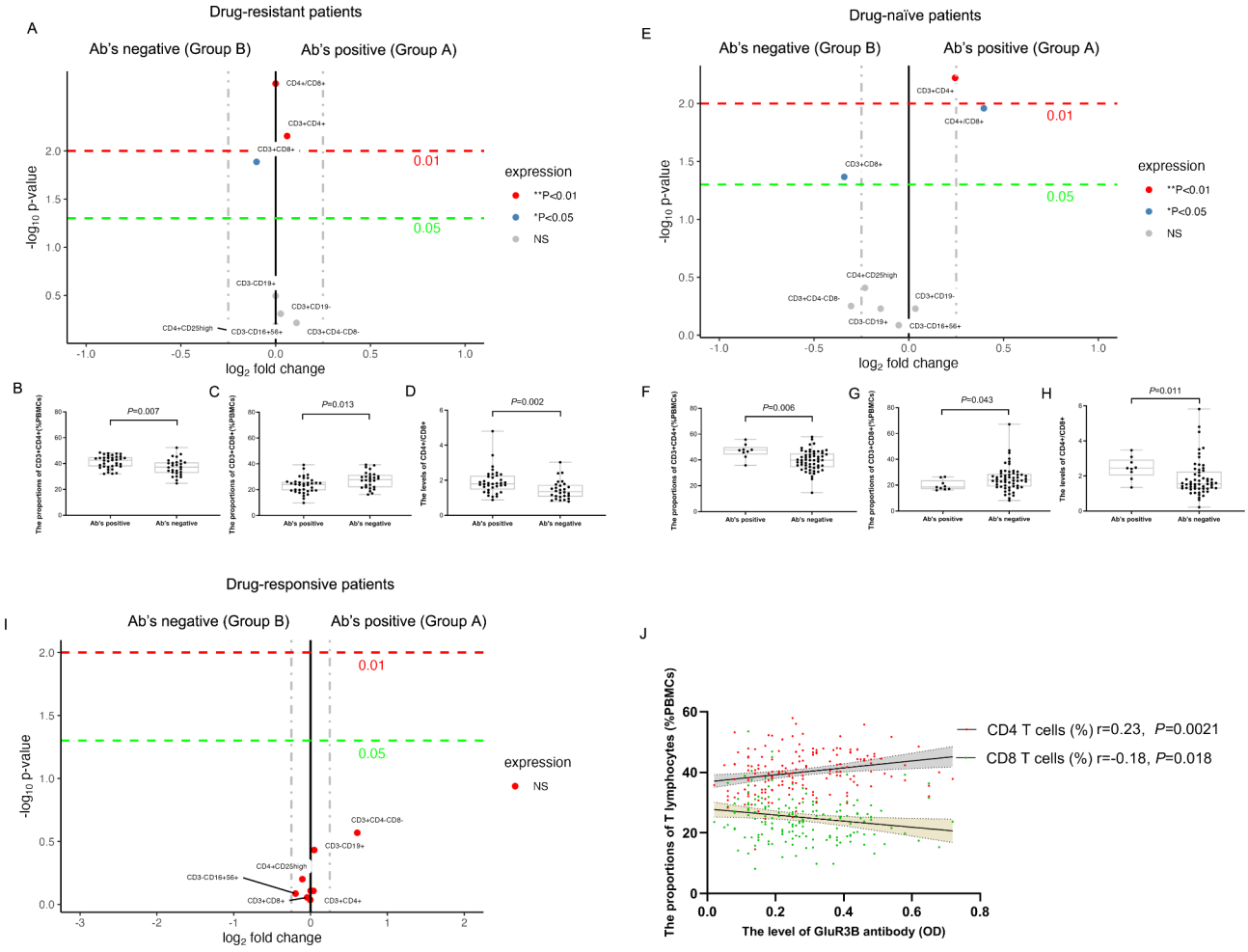

Fig. 1 Distinct immune signature of GluR3B antibody positive and antibody negative epilepsy patients. Representation of the mean fold change and corresponding *P* values in parameters between GluR3B antibody positive ($n=36$) and GluR3B antibody negative ($n=28$) peripheral blood in drug-resistant epilepsy (**A**). The proportions of $CD4^+$ and $CD8^+$ T lymphocytes, and the $CD4^+/CD8^+$ ratio are shown in antibody-positive and antibody-negative drug-resistant patients (**B-D**). Representation of the mean fold change and corresponding *P* values in parameters between GluR3B antibody positive ($n=9$) and GluR3B antibody negative ($n=57$) peripheral blood in drug-naïve epilepsy (**E**). The proportions of $CD4^+$ and $CD8^+$ T lymphocytes, and the $CD4^+/CD8^+$ ratio are shown in antibody positive and antibody negative drug-naïve patients (**F-H**). Representation of the mean fold change and corresponding *P* values in parameters between GluR3B antibody positive ($n=8$) and GluR3B antibody negative ($n=33$) peripheral blood in drug-responsive epilepsy (**I**). Correlation (Spearman) of the serum concentration of GluR3B antibody with proportions of $CD4^+$ and $CD8^+$ T lymphocytes (**J**). Statistical analysis was performed by Mann-Whitney *U* test

in T lymphocyte subsets between the groups ($P>0.05$, Table 4). Next, we examined the changes in inflammatory factors in peripheral blood of the different groups, and the results showed statistically significant differences in serum IL-8 and IFN-γ levels among the groups (both $P<0.05$, Table 5). Further pairwise comparisons with Bonferroni correction (α'=0.05/3=0.0167) revealed that DRE patients had significantly higher levels of IL-8 and IFN-γ than drug-responsive epilepsy patients, suggesting that IFN-γ and IL-8 may be involved in the progression of DRE. Therefore, we included IFN-γ and IL-8 in the subsequent SEM analysis.

Structural equation modeling analysis of the role of GluR3B antibody levels and inflammatory factors in DRE

There were significant correlations between GluR3B antibody and IFN-γ, IL-8 and DRE, and IFN-γ and IL-8 were also associated with DRE. To reveal the potential relationship between GluR3B antibody, IFN-γ, IL-8, and their role in DRE, we conducted a SEM analysis.

The results of the path coefficient table (Table 6) showed that GluR3B antibody level had a significant positive influence on DRE (B=4.479, $P=0.039$) and also had a significant positive influence on IL-8 level (B=18.426, $P=0.015$), but the positive influence of IL-8 level on DRE was not significant (B=0.078, $P=0.084$). GluR3B antibody level had a significant positive influence on IFN-γ

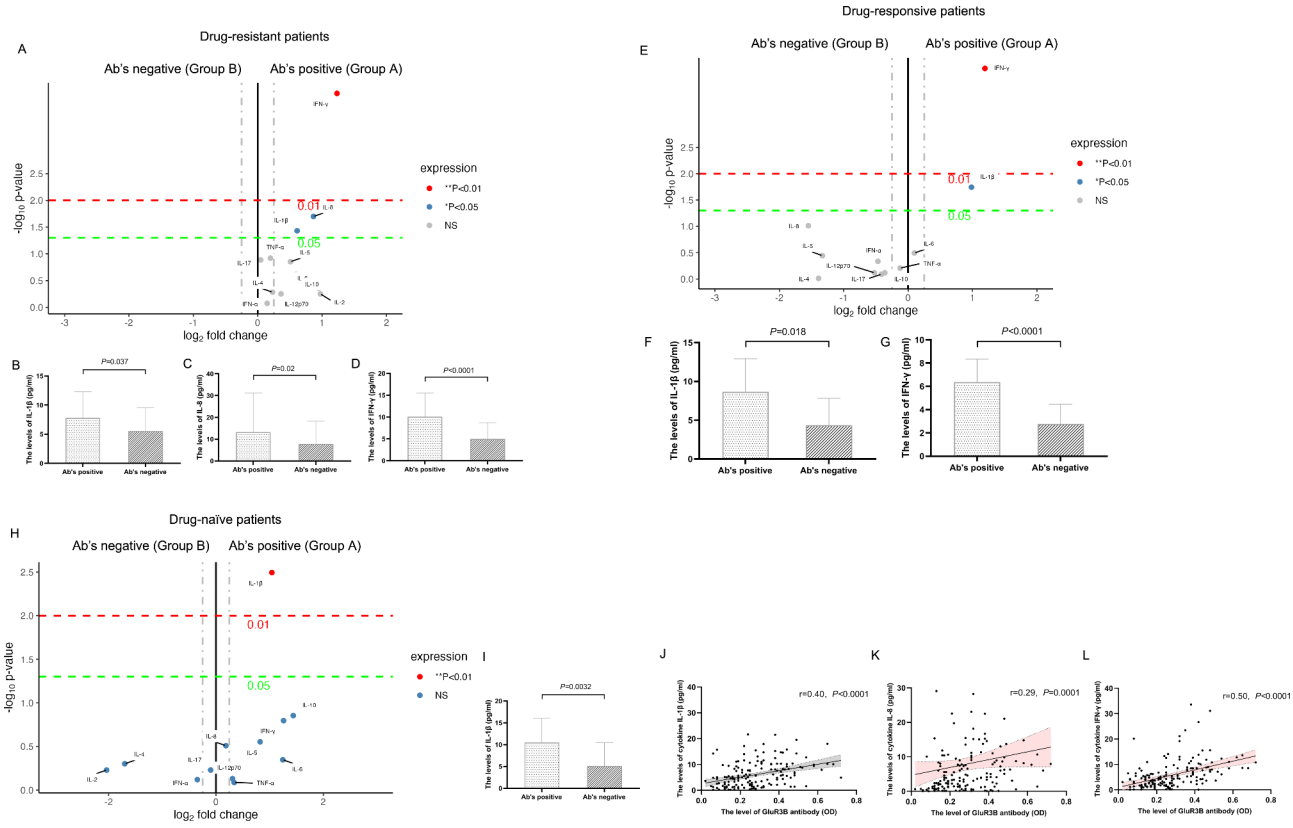

Fig. 2 Circulating levels of anti- and pro-inflammatory cytokines in GluR3B antibody-positive and antibody-negative epilepsy patients. Representation of the mean fold change and corresponding *P* values in parameters between GluR3B antibody positive ($n=36$) and GluR3B antibody negative ($n=28$) peripheral blood in drug-resistant epilepsy (**A**). The circulating levels of IL-1β, IL-8 and IFN-γ are shown in antibody-positive and antibody-negative drug-resistant patients (**B-D**). Representation of the mean fold change and corresponding *P* values in parameters between GluR3B antibody positive ($n=8$) and GluR3B antibody negative ($n=33$) peripheral blood in drug-responsive epilepsy (**E**). The circulating levels of IL-1β and IFN-γ are shown in antibody-positive and antibody-negative drug-responsive patients (**F-G**). Representation of the mean fold change and corresponding *P* values in parameters between GluR3B antibody positive ($n=9$) and GluR3B antibody negative ($n=57$) peripheral blood in drug-naïve epilepsy (**H**). The circulating levels of IL-1β are shown in antibody-positive and antibody-negative drug-naïve patients (**I**). Correlation (Spearman) of the serum concentration of GluR3B antibody with circulating levels of multiple cytokines (IL-1β, IL-8, and IFN-γ) (**J-L**). Statistical analysis was performed by Mann-Whitney *U* test

level (B=17.379, $P=0.015$), and IFN-γ level also had a significant positive influence on DRE (B=0.211, $P=0.024$) (Fig. 3D).

The mediation effect results (Table 7) showed that the total effect of GluR3B antibody level on DRE was significant (total effect=9.580, 95% CI 3.765–13.344). The direct effect was 4.479 (95% CI 0.409–8.503). The mediation effect of IL-8 level between GluR3B antibody level and DRE was 1.431 (95% CI 0.399–6.863), and the proportion of indirect effect accounted for by IL-8 was 14.92% (0.081/0.543). The mediation effect of IFN-γ was 3.670 (95% CI 0.618–7.193), indicating that the mediation effect was established, and the proportion of indirect effect accounted for by IFN-γ was 38.31% (0.208/0.543). The total indirect effect was 5.101 (95% CI 1.756–8.818), accounting for 53.22% (0.289/0.543) of the total effect. Therefore, GluR3B antibody level may directly influence the risk of DRE (direct effect=4.479, 95% CI 0.409–8.503), and may also indirectly influence the risk of DRE through IFN-γ level and IL-8 level (total indirect effect=5.101, 95% CI 1.756–8.818).

In summary, IFN-γ and IL-8 play important roles in GluR3B antibody-positive DRE. In the mechanism with IFN-γ and IL-8 as mediating variables, as the GluR3B antibody level increases, the levels of IFN-γ and IL-8 also increase, further increasing the risk of DRE in patients.

The efficacy of immunotherapy in patients with GluR3B antibody-positive epilepsy

This section of the study included a total of 38 patients, with 19 patients in the immunotherapy group and 19 patients in the control group. There were no statistically significant differences in baseline characteristics between the immunotherapy group and the control group (Table 8).

In this study, 6 patients received IVIG therapy, 7 patients received steroid therapy, and 6 patients received a combination of IVIG and steroids. The results showed

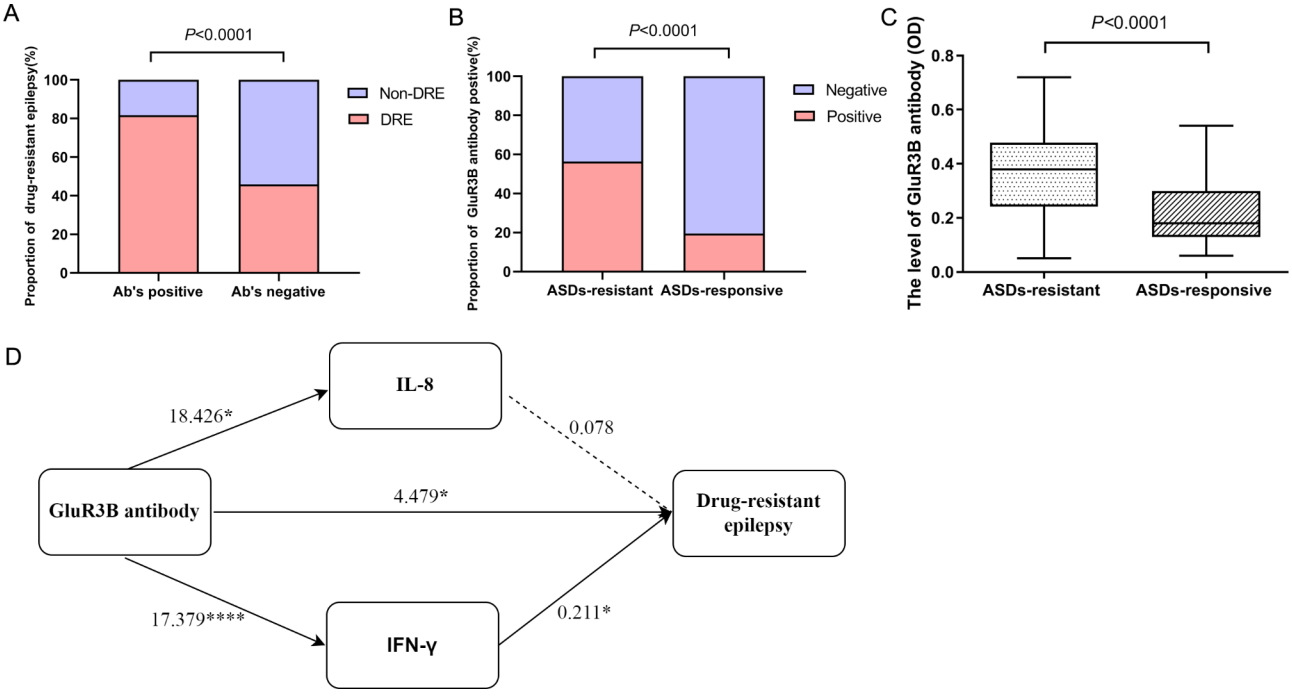

Fig. 3 Structural equation modelling analysis of the role of GluR3B antibody in DRE. (**A**) Proportion of DRE patients among GluR3B antibody-positive and negative patients. (**B**) Proportion of GluR3B antibody-positive patients among DRE and drug-responsive epilepsy patients. (**C**) Box plots representing GluR3B antibody levels in DRE and drug-responsive epilepsy patients. (**D**) Plot of path coefficients for structural equation modeling. *$P<0.05$, ****$P<0.0001$. DRE: drug-resistant epilepsy; ASD: represents antiseizure drugs

Table 4 Comparison of peripheral blood lymphocyte subpopulations in drug-resistant epilepsy, drug-responsive epilepsy, drug-naïve epilepsy, and healthy controls

	units	Drug-resistant patients ($n=64$)	Drug-responsive patients ($n=41$)	Drug-naïve patients ($n=66$)	Healthy control ($n=40$)	*P*
$CD3^+CD19^-$	%PBMCs	70.7 ± 6.6	72.2 ± 8.0	70.0 ± 8.6	69.4 ± 5.7	0.30
$CD3^-CD19^+$	%PBMCs	13.8 ± 4.5	12.0 ± 4.8	14.1 ± 6.4	13.1 ± 2.8	0.15
$CD3^-CD16^+56^+$	%PBMCs	14.1 ± 5.2	13.9 ± 7.3	13.7 ± 7.3	12.3 ± 3.7	0.35
$CD3^+CD4^+$	%PBMCs	39.5 ± 5.9	40.1 ± 7.3	40.6 ± 7.9	37.7 ± 3.7	0.11
$CD3^+CD8^+$	%PBMCs	25.2 ± 6.5	26.1 ± 7.8	24.5 ± 9.3	23.9 ± 4.5	0.37
$CD4^+/CD8^+$		1.7 ± 0.7	1.7 ± 0.6	1.9 ± 1.0	1.8 ± 0.5	0.61
$CD3^+CD4^-CD8^-$	%PBMCs	6.9 ± 2.9	6.9 ± 4.3	6.1 ± 3.8	6.0 ± 3.2	0.15
$CD4^+CD25^{high}$	%PBMCs	2.3 ± 1.4	2.7 ± 1.4	2.7 ± 1.3	2.5 ± 1.2	0.28

that the proportion of patients with effective control of seizures at 1, 3, and 6 months after immunotherapy was significantly higher in the immunotherapy group compared to the control group (42.1% vs. 10.5%, $P=0.027$; 63.2% vs. 21.1%, $P=0.009$; 63.2% vs. 15.8%, $P=0.003$, Fig. 4A). Moreover, further analysis of the effects of different immunotherapy programs on seizure frequency showed no statistically significant differences ($P>0.05$, Fig. 4B).

Serum GluR3B antibody levels decreased significantly after immunotherapy (0.44±0.33 OD vs. 0.98±0.19 OD, $P<0.0001$, Fig. 4C), while non-immunotherapy patients showed no significant decrease in GluR3B antibody levels (0.82±0.32 OD vs. 0.97±0.19 OD, $P=0.25$, Fig. 4C). Steroid therapy or combined therapy (IVIG combined with steroids) were more effective in reducing GluR3B antibody levels (Fig. 4D).

Analysis of the relationship between GluR3B antibody levels and seizure frequency revealed that 83.3% of patients with significantly lower serum GluR3B antibody levels had a significant reduction in seizure frequency. Patients with significantly lower GluR3B antibody levels were more likely to reduce seizure frequency ($P<0.0001$, Fig. 4E), indicating that the decrease in serum GluR3B antibody levels reduced seizure frequency.

Discussion

Previous studies have suggested that the imbalance of peripheral lymphocyte subpopulations and increased levels of inflammatory cytokines may be related to the

Table 5 Comparison of peripheral blood cytokine levels in drug-resistant epilepsy, drug-responsive epilepsy, drug-naïve epilepsy, and healthy controls

	units	Drug-resistant patients ($n=64$)	Drug-responsive patients ($n=41$)	Drug-naïve patients ($n=66$)	Healthy control ($n=40$)	*P*
IL-1β [M (P_{25},P_{75})]	pg/ml	6.4 (2.5,9.7)	4.7 (1.6,8.2)	4.3 (1.3,8.3)	2.8 (2.1,6.6)	0.069
IL-2 [M (P_{25},P_{75})]	pg/ml	2.1 (1.0, 36.4)	1.7 (1.0, 3.2)	1.6 (0.8, 11.2)	1.7 (1.3, 2.5)	0.28
IL-4 [M (P_{25},P_{75})]	pg/ml	1.4 (0.8,10.0)	1.2 (0.9,2.7)	1.3 (0.8,4.0)	1.0 (0.9,2.6)	0.44
IL-5 [M (P_{25},P_{75})]	pg/ml	2.6 (1.5, 4.3)	2.5 (1.2, 5.8)	1.9 (1.1, 4.3)	2.4 (1.2, 3.1)	0.12
IL-6 [M (P_{25},P_{75})]	pg/ml	2.6 (1.6, 4.6)	3.0 (2.1, 6.1)	2.4 (1.4, 4.9)	2.3 (1.6, 3.8)	0.31
IL-8 [M (P_{25},P_{75})]	pg/ml	7.4 (2.5, 12.6)	1.4 (0.4, 4.8)	3.1 (0.8, 8.1)	1.0 (0.6, 2.4)	**< 0.0001**
IL-10 [M (P_{25},P_{75})]	pg/ml	1.4 (0.8, 1.8)	1.3 (0.8, 1.7)	1.3 (0.8, 1.7)	0.9 (0.8, 1.5)	0.11
IL-12p70 [M (P_{25},P_{75})]	pg/ml	1.8 (1.0, 3.5)	1.6 (1.0, 2.6)	1.7 (0.8, 2.5)	1.4 (0.9, 2.0)	0.20
IL-17 [M (P_{25},P_{75})]	pg/ml	1.6 (1.1, 2.8)	1.6 (1.1, 3.1)	1.5 (0.9, 2.4)	1.3 (1.1, 1.9)	0.52
IFN-α [M (P_{25},P_{75})]	pg/ml	1.9 (1.0, 4.9)	1.6 (0.9, 4.5)	1.5 (0.8, 4.3)	1.2 (1.1, 1.5)	0.14
IFN-γ [M (P_{25},P_{75})]	pg/ml	7.1 (3.7, 10.8)	2.9 (1.6, 5.5)	3.1 (1.2, 5.6)	2.5 (1.3, 3.6)	**< 0.0001**
TNF-α [M (P_{25},P_{75})]	pg/ml	2.4 (1.5, 3.9)	2.3 (1.1, 3.9)	2.3 (1.2, 4.7)	2.1 (1.3, 2.5)	0.34

Table 6 Table of path coefficients for structural equation modeling

pathway	STD Estimate	Estimate	S.E.	C.*R*.	*P*
GluR3B–> DRE	0.254	4.479	2.171	2.063	0.039
GluR3B–> IL-8	0.227	18.426	7.596	2.426	0.015
IL-8 --> DRE	0.357	0.078	0.045	1.727	0.084
GluR3B–> IFN-γ	0.56	17.379	2.377	7.310	< 0.0001
IFN-γ --> DRE	0.372	0.211	0.094	2.258	0.024

Notes: DRE, Drug-resistant epilepsy; SE, standard error; CR, critical ratio; STD, Standardized

Table 8 Clinical characteristics of patients in the immunotherapy and control groups

	Epilepsy patients ($n=38$)		
	Control ($n=19$)	Immunotherapy ($n=19$)	*P*
Age (years)	38.89 ± 16.60	47.84 ± 19.66	0.129
Gender (M,%)	7.0 (36.8)	12.0 (63.2)	0.105
Epilepsy duration, y [M (P_{25},P_{75})]	3.0 (2.0,7.0)	2.0 (1.0,4.0)	0.230
Age at first seizure, y [M (P_{25},P_{75})]	31.0 (20.0, 47.0)	48.0 (30.0, 57.0)	0.129
Abnormal EEG (n, %)	15.0 (78.9)	17.0 (89.5)	0.374
Abnormal MRI (n, %)	12.0 (63.2)	15.0 (78.9)	0.283
Number of AEDs	2.16 ± 0.50	2.21 ± 0.42	0.433

Note: AEDs: antiseizure drugs

progression of epilepsy [32–35]. However, the characteristics of peripheral lymphocyte subpopulations and inflammatory factors in GluR3B antibody-positive epilepsy patients are not clear. Our study found that DRE patients with positive GluR3B antibody showed a significantly increased proportion of $CD4^+$ T lymphocytes, a significantly decreased proportion of $CD8^+$ T lymphocytes, and an elevated $CD4^+/CD8^+$ ratio. Patients with DNE also exhibited similar changes in T lymphocytes, and the serum concentration of GluR3B antibodies was positively correlated with the proportion of $CD4^+$ T lymphocytes, and negatively correlated with the proportion of $CD8^+$ T lymphocytes, suggesting that GluR3B antibodies may exacerbate the imbalance of T lymphocyte subpopulations. Interestingly, previous studies have found that patients with hippocampal sclerosis TLE and DRE have an increased proportion of $CD4^+$ T lymphocytes [32, 33, 46]. Patients with GAD65-positive limbic encephalitis showed an increased proportion of $CD8^+$ T lymphocytes [32], and patients with glutamate receptor antibody-positive epilepsy showed a significant increase in $CD4^+CD25^{high}$ Treg [47]. AMPA-GluR3 is highly expressed in $CD4^+$ and $CD8^+$ T lymphocytes, and autoimmune GluR3B antibodies can specifically kill T lymphocytes in patients with nodding syndrome [30],

Table 7 Table of mediating effects of structural equation modeling

Pathway (effect)	STD Estimate	Estimate	S.E.	C.*R*.	*P*	95%CI
Total indirect effect	0.289	5.101	1.832	2.785	0.005	1.756~8.818
GluR3B—> IL-8–> DRE	0.081	1.431	1.186	1.206	0.228	0.399~6.863
Proportion of indirect effect of IL-8	14.92% (0.081/0.543)					
GluR3B—> IFN-γ --> DRE	0.208	3.670	1.680	2.184	0.029	0.618~7.193
Proportion of indirect effect of IFN-γ	38.31% (0.208/0.543)					
Direct effect	0.254	4.479	2.171	2.063	0.039	0.409~8.503
Total effect	0.543	9.580	2.284	4.194	< 0.0001	3.765~13.344
Proportion of total indirect effect	53.22% (0.289/0.543)					

Notes: DRE, Drug-resistant epilepsy; SE, standard error; CR, critical ratio; STD, Standardized

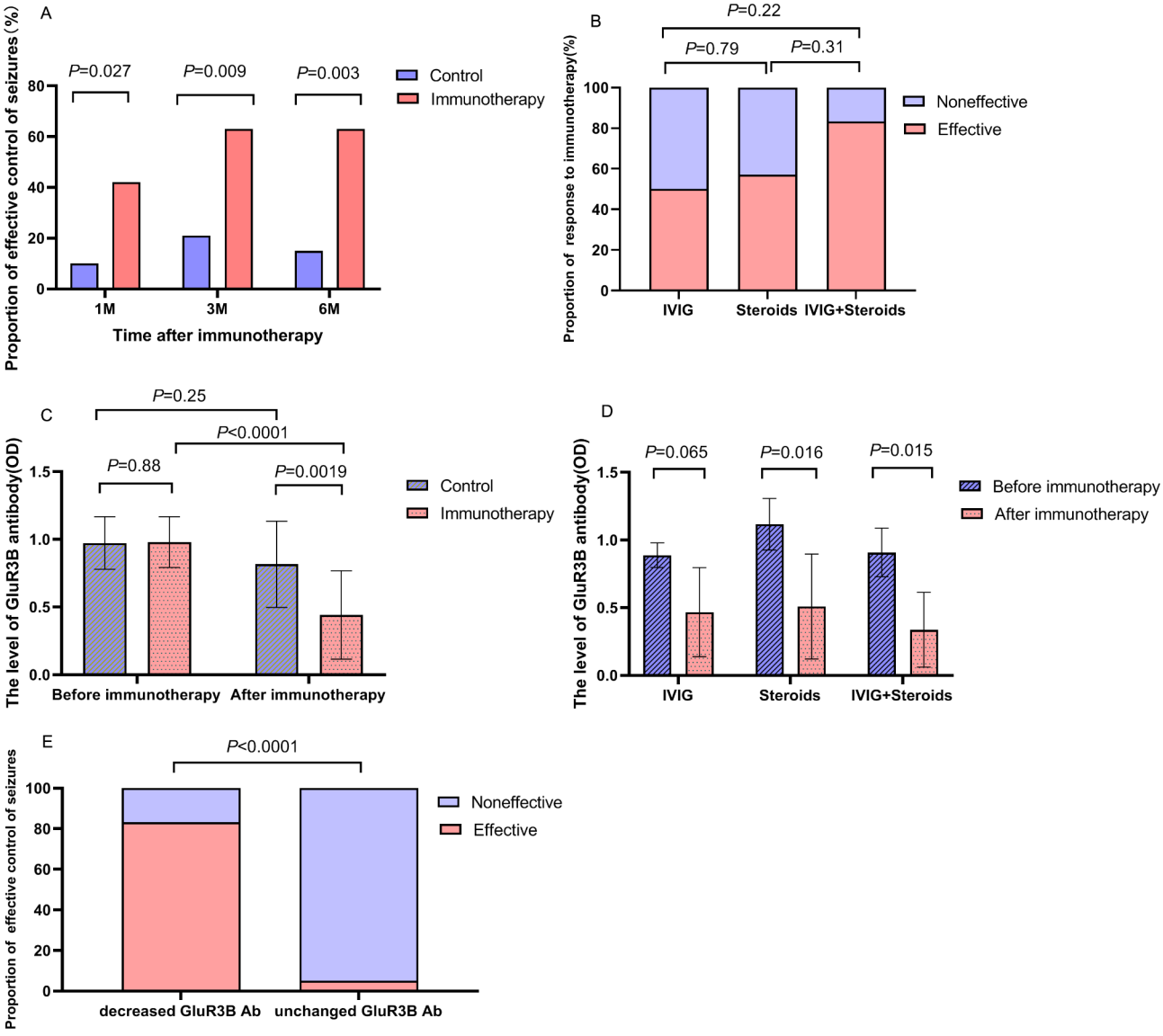

Fig. 4 Effect of immunotherapy on seizure frequency. (**A**) Changes in seizure frequency at 1, 3, and 6 months after immunotherapy. (**B**) Effects of IVIG therapy, steroid therapy, or combination therapy on seizure frequency. (**C**) Changes in GluR3B antibody levels in patients before and after immunotherapy. (**D**) Effects of IVIG therapy, steroid therapy, or combination therapy on GluR3B antibody levels. (**E**) Effect of reduced serum GluR3B antibody titer on seizure frequency. IVIG: intravenous immunoglobulin

suggesting that GluR3B antibodies may tend to damage $CD8^+$ T lymphocytes in epilepsy patients.

$CD4^+$ T cells can differentiate into various subsets such as helper T cells 1 (Th1), Th2, Th17, and Treg. Among these, Th1 and Th17 cells are pro-inflammatory cells closely associated with the development of various autoimmune diseases and are pathogenic under neuroinflammatory conditions [48]. In contrast, Treg and Th2 cells are believed to have neuroprotective and anti-inflammatory effects under neuroinflammatory conditions [49]. The differentiation of Th1 cells is promoted by IFN-γ, IL-18, IL-12, and type 1 interferon, while the differentiation of Th17 cells is promoted by IL-1β, IL-6, IL-23, and TGF-β. We further analyzed the levels of cytokines in the serum of GluR3B antibody-positive epilepsy patients to assess the possible polarization of $CD4^+$ T lymphocytes. The results indicated a significant increase in the levels of pro-inflammatory cytokines IL-1β associated with Th17 cell differentiation, and a significant increase in the levels of IFN-γ and IL-8 associated with Th1 cell differentiation in the serum of GluR3B antibody-positive epilepsy patients. There was no significant difference in the levels of anti-inflammatory cytokines IL-10 and IL-4 related to Treg and Th2 polarization between antibody-positive and antibody-negative epilepsy, suggesting the presence of an excessive inflammatory response mediated by Th1/Th17

cells in GluR3B antibody-positive epilepsy patients. Previous studies have shown similar elevation of pro-inflammatory cytokines in different subtypes of autoimmune epilepsy, including patients with positive anti-neuronal cell membrane antigen antibodies and GAD65 antibody-associated epilepsy [35, 50, 51]. Additionally, elevated levels of pro-inflammatory cytokines in the CSF and serum have been observed in patients with N-methyl-D-aspartate receptor (NMDAR) encephalitis [52, 53]. Our study supports that GluR3B antibody-positive epilepsy is associated with an increase in circulating proinflammatory cytokines IL-1β, IL-8, and IFN-γ associated with Th1 and Th17 differentiation, suggesting that Th1 and Th17 cell-mediated proinflammatory responses may play an important role in the pathogenesis of GluR3B antibody-mediated epilepsy.

The significant increase in pro-inflammatory cytokines IL-1β, IL-8, and IFN-γ in the peripheral blood of GluR3B antibody-positive epilepsy patients not only increases the CNS excitability, lowers the seizure threshold, and promotes the development of epilepsy [54], but also affects the production of autoantibodies [35, 55]. IL-1β, IL-8, and IFN-γ are important inflammatory mediators, chemokines, and immune regulatory factors, involved in regulating inflammation and immune responses. They promote the secretion of immunoglobulins by B cells directly or indirectly and play a role in the generation of antibodies [55]. Particularly in autoimmune diseases, excessive expression of pro-inflammatory cytokines may lead to the secretion of pathological autoantibodies, possibly including GluR3B antibodies. In the present study, serum levels of pro-inflammatory cytokines IFN-γ and IL-8 were significantly higher in patients with DRE than in patients with drug-responsive epilepsy. In addition, our current study also supported the involvement of the GluR3B antibody in the progression of DRE. Therefore, we speculate that GluR3B antibodies may interact with inflammatory factors, collectively contributing to the progression of epilepsy.

We further analyzed the role of GluR3B antibody levels with IFN-γ and IL-8 in epilepsy progression using SEM. The results showed that GluR3B antibody levels may directly impact the risk of DRE, and they may also indirectly affect the risk of DRE through the levels of IFN-γ and IL-8. This important finding emphasizes the significant role of GluR3B antibodies in the progression of epilepsy and suggests a crucial role of peripheral immune inflammatory activity in GluR3B antibody-mediated epilepsy progression. Some researchers have suggested that high levels of IL-6 and high titers of GAD65 may be dual factors in the occurrence of autoimmune epilepsy [35]. Therefore, we speculate that high titers of GluR3B antibodies and high levels of IFN-γ and IL–8 may be dual factors contributing to the occurrence of DRE.

In our study, the results indicated that immunotherapy significantly decreased seizure frequency and serum GluR3B antibody levels, and the seizure frequency was positively correlated with the levels of GluR3B antibody levels in patients receiving immunotherapy, further supporting the association of GluR3B antibodies with the development of epilepsy. Previous studies have also found that GluR3B antibody levels in epileptic patients decreased significantly after the use of immunotherapy, accompanied by an improvement in clinical symptoms [23–25]. This suggests that monitoring GluR3B antibody levels may be useful in predicting the effects of immunotherapy and may serve as a biomarker for assessing response to treatment.

Timing of immunotherapy plays a crucial role in its effectiveness for autoimmune-associated epilepsy. In our study, delayed immunotherapy (the time from onset to immunotherapy was >1 year) was ineffective in 71.4% of GluR3B antibody-positive epilepsy patients. Previous studies have also reported that early immunotherapy benefited of GAD65 antibody-positive patients by reducing seizures, while delayed treatment led to DRE [56]. The timing of immunotherapy, antibody titer, and structural lesions influence treatment outcomes. Our findings show a 63.2% seizure reduction in GluR3B antibody-positive patients post-immunotherapy, supporting its potential as an effective treatment for autoimmune-associated epilepsy, warranting further investigation.

In conclusion, this study demonstrates that GluR3B antibody may influence the progression of epilepsy through altering the proportion of $CD4^+$ and $CD8^+$ lymphocyte subsets and increasing proinflammatory cytokines. The seizure suppression of immunotherapy is associated with the decrease of GluR3B antibody levels. Thus, the present study contributes to a better understanding of the immunoregulatory mechanisms of autoimmune-associated epilepsy and provides a potential target for DRE.

However, this study also has certain limitations: (1) Due to the difficulty in acquiring CSF samples from epilepsy patients, there is a lack of characterization of the changes in CNS immune cells and cytokines in GluR3B antibody-positive epilepsy patients. (2) There is a lack of a control group consisting of non-epileptic individuals stratified based on GluR3B antibody-positive status (autoimmune/non-autoimmune), which prevents the accurate assessment of the specificity of T lymphocyte subset proportions and the "characteristics" of proinflammatory cytokines in GluR3B antibody-positive epilepsy. (3) Although the results suggest the involvement of GluR3B antibodies in the progression of DRE, detailed experimental data explaining its potential mechanisms were not provided.

Although our study shows the promise of the GluR3B antibody as a potential therapeutic target, further clinical trials are needed to validate its efficacy and safety as an alternative to existing therapies. Currently, immunotherapy has shown some efficacy in DRE, but there is a lack of data from large-scale, multicentre, randomised controlled trials to support its widespread use. Future studies should aim to standardise immunotherapy regimens and investigate their long-term effects and potential side effects in order to provide a reliable alternative treatment for patients with epilepsy.

Supplementary Information

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Supplementary Material 1

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Author contributions
QL designed the study. QL wrote the manuscript. BW and YC carried out the experiments. NW analyzed the data.

Data availability
The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate
The study received approval from the Ethics Committee of the Affiliated Hospital of Xuzhou Medical University (XYFY2016-KL017-02), and all participants or their legal guardians provided informed consent.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

Author details
[1]Jiangsu Key Laboratory of New Drug Research and Clinical Pharmacy, Xuzhou Medical University, Xuzhou, China
[2]Department of Neurology, Affiliated Hospital of Xuzhou Medical University, 99 West Huaihai Road, Xuzhou, Jiangsu 221002, China
[3]China University of Mining and Technology, Xuzhou, China
[4]Department of Neurology, First People's Hospital of Xuzhou, Xuzhou, China
[5]Department of Neurology, People's Hospital of Suining, Xuzhou, China

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