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Distinct T Cell Dysregulation Reflects Disease Severity and Progression in Infantile Epileptic Spasms Syndrome and Lennox-Gastaut Syndrome

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ABSTRACT

Developmental and epileptic encephalopathies (DEEs), including Infantile Epileptic Spasms Syndrome (IESS) and Lennox-Gastaut Syndrome (LGS), are severe pediatric conditions characterized by profound developmental delays and treatment-resistant epilepsy. Although steroid therapies provide some clinical benefits, the underlying immunological mechanisms remain poorly understood. In this study, we performed comprehensive immune profiling using multi-parametric flow cytometry on PBMCs from IESS (n=25) and LGS (n=9) patients, comparing them with age-matched healthy controls (n=54). Our findings identified distinct patterns of immune dysregulation: IESS patients exhibited reduced naïve $CD4^+$ T cells, an altered CD4/CD8 ratio, and diminished TNFα production in $CD4^+$ T cells. Conversely, LGS patients demonstrated an increase in central memory $CD4^+$ T cells, marked dysfunction of Tregs, and heightened activation of $CD8^+$ T cells. Notably, elevated activated $CD8^+$ T cells in IESS patients correlated significantly with clinical severity and demonstrated enhanced responsiveness to viral peptides, suggesting prior viral infections may exacerbate disease progression. Collectively, our findings demonstrate distinct immune signatures associated with disease severity and progression in DEE, suggesting their potential utility as biomarkers. Further studies are necessary to determine whether targeting these immune pathways could provide clinical benefits.

Keywords: Epilepsy; T-lymphocytes; Spasms, infantile; Lennox Gastaut syndrome; Epileptic syndromes

INTRODUCTION

Developmental and epileptic encephalopathies (DEEs) encompass a spectrum of severe pediatric epilepsies characterized by profound developmental delays that significantly

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Conflict of Interest

The authors declare that they have no conflict of interest.

Abbreviations

ACTH, adrenocorticotrophic hormone; CM, central memory; CMV, cytomegalovirus; DEE, developmental and epileptic encephalopathy; EEG, electroencephalogram; EM, effector memory; IESS, Infantile Epileptic Spasms Syndrome; LGS, Lennox-Gastaut Syndrome; MRI, magnetic resonance imaging; RT, reverse transcription; Tc, T cytotoxic; TEMRA, terminally differentiated effector memory; UMAP, Uniform Manifold Approximation and Projection.

Author Contributions

Conceptualization: Chang L, Kim SH, Cheon KA, Kwon HK; Data curation: Chang H, Sang HD, Kang YM, Ha S; Formal analysis: Chang L; Funding acquisition: Chang L, Kim SH, Kwon HK; Methodology: Chang L, Jeong YJ, Kwon KN, Lee SB, Kim SY; Project administration: Kim SH, Cheon KA, Kwon HK; Resources: Jeong YJ, Chang H, Sang HD, Kwon KN, Lee SB, Kim SY, Kang YM, Ha S; Supervision: Kim SH, Cheon KA, Kwon HK; Writing - original draft: Chang L, Kwon HK; Writing - review & editing: Kim SH, Kwon HK.

impact individuals and their families (1). Infantile Epileptic Spasms Syndrome (IESS) and Lennox-Gastaut Syndrome (LGS) are well established DEEs which begin in childhood. IESS, also known as West syndrome, typically emerges between 3 to 12 months of age and is characterized by spasms and potential developmental delays, intellectual disability, and other neurological complications (2,3). LGS, typically diagnosed between 3 and 5 years of age, is a severe childhood-onset epilepsy which often develops from earlier epileptic syndromes such as IESS (4,5). It is characterized by its complex seizure types and distinctive electroencephalogram (EEG) patterns, particularly slow spike-and-wave complexes (4,5). The transition from IESS to LGS in some patients represents significant, yet poorly understood, clinical progression within DEE (6,7).

Recent research has increasingly focused on the role of the immune system in the pathophysiology of epilepsies, including DEE (8,9). Elevated levels of pro-inflammatory cytokines in the serum of epilepsy patients suggest a link between immune dysregulation and epileptogenesis (10). Further, evidence of T cell infiltration into the brain parenchyma in patients with drug-resistant epilepsy indicates a localized immune response at epileptic foci (11). Additionally, the heightened risk of epilepsy associated with autoimmune diseases (12) underscores the significant role of immune processes in the pathology of these treatment-resistant variants.

In both IESS and LGS, immune-related therapies such as steroid treatments with prednisolone and adrenocorticotrophic hormone (ACTH) have demonstrated efficacy, supporting the hypothesis of an immunological component in their pathogenesis (13,14). The relationship between the peripheral immune system and DEE, particularly notable in IESS, has been a focal point of numerous studies, emphasizing the importance of immunological investigations in understanding these disorders (15-17). Despite these advances, the detailed phenotyping and functional characterization of the peripheral immune system in DEE patients remain incomplete.

Our study aims to explore the intricate connections between immune dysregulation and DEEs, particularly focusing on IESS and LGS. By comprehensively phenotyping and characterizing the functionality of peripheral T cells, we seek to elucidate the immune mechanisms potentially underlying the severity and progression of these diseases. Our findings indicate significant alterations in T cell dynamics, cytokine production, and T cell polyfunctionality, suggesting a complex immunopathology that may inform targeted therapeutic strategies. These findings offer insights into the immunological landscape of these disorders and may contribute to developing novel immunomodulatory approaches.

MATERIALS AND METHODS

Study population

Patients with IESS or LGS treated at Severance Children's Hospital from September 23, 2020, to September 22, 2023, were included based on recent International League Against Epilepsy diagnostic criteria (18). Patients were under 18 years old, with age-matched healthy controls also enrolled. Controls included neurologic clinic patients diagnosed with non-pathologic conditions like sleep myoclonus, or healthy siblings of children with autism spectrum disorder (19). Patients who had received steroid within 1 month prior to the study were excluded. Consent was obtained from the caregivers and patients unless they had severe intellectual disability.

Clinical data

The phenotype spectra of patients were retrospectively reviewed from patient medical records and included demographics, seizure onset, epilepsy syndrome, serial EEG and magnetic resonance imaging (MRI) findings. Seizure frequency was also collected. For patients who showed increased response, additional clinical data including microcephaly, hearing test, ophthalmologic test results, perinatal history, and perinatal body weight were obtained, additionally.

PBMCs isolation and cryopreservation

PBMCs were isolated from whole blood using Ficoll density gradient centrifugation. Briefly, blood was diluted 1:1 with PBS and transferred to conical tubes and layered on the Ficoll-Paque PLUS (Cytiva, Marlborough, MA, USA) solution. Following centrifugation at 1,100 g for 20 min at 23°C in a swinging bucket rotor with no brake, the layer containing mononuclear cells was taken and diluted to 20 ml with PBS and then centrifuged at 700 g for 10 min at 23°C in a swinging bucket rotor. PBMCs were resuspended at 2×10^6 cells per vial (1 ml) in freezing media containing 70% fetal bovine serum (Gibco, Waltham, MA, USA), 20% RPMI-1640 (Welgene, Gyeongsan, Korea) and 10% DMSO (Sigma-Aldrich, St. Louis, MO, USA), and stored at −80°C as previously described (20).

Immune profiling by multiparameter flow cytometry analysis

One vial of frozen PBMC stock was cryorecovered by rapid thaw at 37°C water bath followed by careful dilution to 10 ml in RPMI containing 10% fetal bovine serum (Gibco) prewarmed to 37°C, and centrifuged at 520 g for 5 min at 23°C in a swinging bucket rotor. A half of the recovered cells cultured in 96-well round bottom dish with T cell media and stimulated by eBioscience™ Cell Stimulation Cocktail plus protein transport inhibitors (Invitrogen, Carlsbad, CA, USA) for 4 h at 37°C in the incubator (5% CO_2). The other half of the cells were rested in the same media without stimulation for 3 h at 37°C in the incubator (5% CO_2).

For the evaluation of T cell population, unstimulated cells were surface-stained with following antibodies: CD3-BV421 (UCHT1, 1:200), CD4-BV605 (OKT4, 1:200), CD8-APC-Cy7 (SK1, 1:200), CD45RA-BV785 (HI100, 1:200), CCR7-APC (G043H7, 1:200), CXCR3-BV650 (G025H7, 1:200), CCR6-APC-R700 (11A9, 1:200), CCR4-PerCP-Cy5.5 (L291H4, 1:200), CXCR5-FITC (J252D4, 1:200), CD25-PE-Cy7 (BC96, 1:200). LIVE/DEAD™ Fixable Aqua (Thermo Fisher Scientific, Waltham, MA, USA) was added to staining solution to discriminate dead cells. After incubation at 4°C for 20 min, the cells were fixed and permeabilized with eBioscience™ Foxp3/Transcription Factor Staining Buffer (Invitrogen) and were stained with following antibodies at reverse transcription (RT) for 40 min: FOXP3-PE (236A/E7, 1:100).

To evaluate cytokine production from T cell upon stimulation, stimulated cells were washed with PBS and surface-stained with following antibodies: CD4-BV785 (OKT4, 1:200), CD8-BV650 (SK1, 1:200). LIVE/DEAD™ Fixable Aqua (Thermo Fisher Scientific) was added to staining solution to discriminate dead cells. After incubation at 4°C for 40 min, the cells were fixed and permeabilized with Bioscience™ Intracellular Fixation & Permeabilization Buffer (Invitrogen) and were stained with following antibodies at RT for 40 min: TNFα-APC-Cy7 (MAb11, 1:100), IFNγ-BV421 (B27, 1:100), IL-17a-PE-Cy7 (BL168, 1:100), IL-6-PerCP-Cy5.5 (MQ2-13A5, 1:100), IL-5-PE (TRFK5, 1:100), GZMB-FITC (QA16A02, 1:100), IL-2-APC-R700 (MQ1-17H12, 1:100).

After staining, cells were washed with PBS and data acquisition was performed by using BD FACSCelesta (BD Biosciences, San Jose, CA, USA) or Sony ID7000 (Sony, Tokyo, Japan). Flow cytometric data were analyzed in FlowJo v10.9.0 (BD Biosciences). The gating strategy for flow cytometric data is described in **Supplementary Figs. 1** and **2**. Uniform Manifold Approximation and Projection (UMAP) algorithm for dimensionality reduction was applied using the UMAP plugin (v3.1) available on FlowJo.

Viral peptide stimulation

Six control participants, 6 IESS patients with low frequency of seizures and low $CD8^+$ T cell activity, and 4 IESS patients with high frequency of seizures and high $CD8^+$ T cell activity were selected based on the clinical data and obtained flow cytometry data. PBMCs of were cryorecovered as mentioned above, resuspended in T cell media and divided into 6 wells into 96-well plate. Cells were stimulated by addition of following peptide mixture to each well: PepMix™ EBV (BARF1), PepMix™ Influenza A [MP1/California (H1N1)], PepMix™ HRSVB (MSG), PepMix™ HCMVA (pp65), PepMix™ SARS-CoV-2 (Spike Glycoprotein) (JPT Peptide Technologies, Berlin, Germany). Cells with no stimulation by adding DMSO were also prepared for the negative control. Cells were incubated in the presence of Golgiplug and Golgistop (BD Biosciences) for 16 h at 37°C in the incubator (5% CO_2). Cells were washed with PBS and surface-stained with following antibodies: CD4-BV785 (OKT4, 1:200), CD8-BV650 (SK1, 1:200). LIVE/DEAD™ Fixable Aqua (Thermo Fisher Scientific) was added to staining solution to discriminate dead cells. After incubation at 4°C for 40 min, the cells were fixed and permeabilized with Bioscience™ Intracellular Fixation & Permeabilization Buffer (Invitrogen) and were stained with following antibodies at RT for 40 min: TNFα-APC-Cy7 (MAb11, 1:100), IFNγ-BV421 (B27, 1:100), GZMB-FITC (QA16A02, 1:100). After staining, cells were washed with PBS and data acquisition was performed by using BD FACSCelesta (BD Biosciences) or Sony ID7000 (Sony). Flow cytometric data were analyzed in FlowJo v10.9.0 (BD Biosciences). Cells treated with DMSO only were included as negative controls, and T cell frequencies responding to each peptide mix were adjusted by subtracting background values from these controls.

Statistical analysis

Group comparisons were conducted using GraphPad Prism 10 (GraphPad Software, San Diego, CA, USA). Immunological variables between patients and controls were compared with an unpaired Student's *t*-test. For multiple group comparisons, either one-way ANOVA with Sidak's *post hoc* test or the Kruskal-Wallis test followed by Dunn's multiple comparisons test was applied. Pearson's correlation analysis was performed using the rcorr function in the Hmisc package (v5.0.1) within R Version 4.1.3 (The R Foundation, Vienna, Austria). A 2-sided p-value of < 0.05 was considered statistically significant. Ages are presented as median (min, max), and data as mean ± SEM.

Ethics approval and consent to participate

This study was approved by the Institutional Review Board and Ethics Committee of Severance Hospital, affiliated to Yonsei University College of Medicine (4-2020-0899). Informed consent was obtained from participants or their legal representatives.

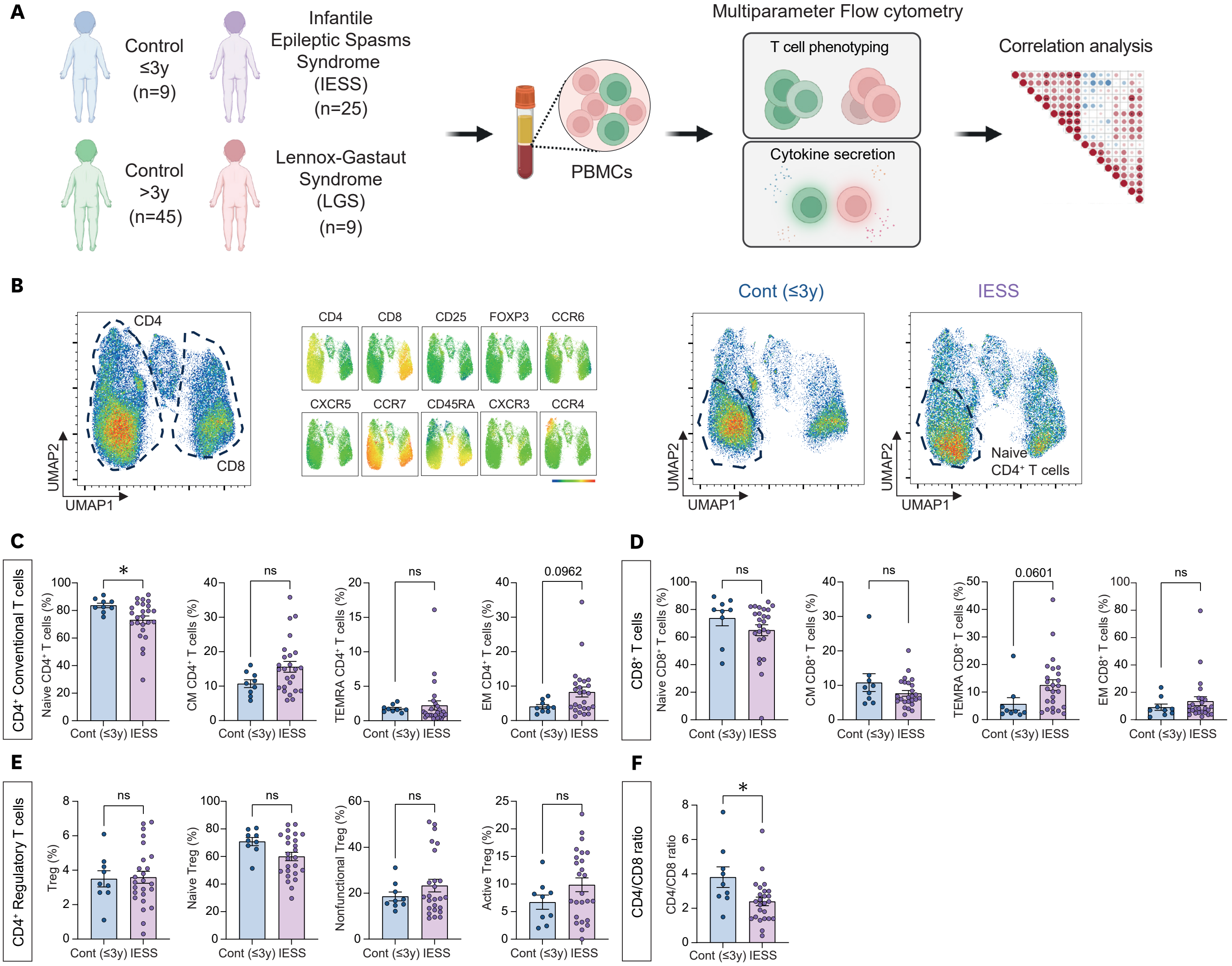

Figure 1. T cell dynamics in IESS patients. (A) A schematic representation of the experimental design is shown. T cell phenotyping was conducted using PBMCs isolated from the blood of individuals with IESS, patients with LGS, and age-matched control subjects. The correlation between immune phenotypes and clinical information was assessed. (B) UMAP visualization of T cell populations for each study group. (C-F) Comparison of CD4$^+$ conventional T cell populations (C), CD8$^+$ T cell populations (D), CD4$^+$ regulatory T cell populations (E), and CD4/CD8 ratios (F) in IESS patients compared to age-matched controls. Data are presented as mean ± SEM.
Cont, control; ns, not significant.
$^*p<0.05$; Student's *t*-test.

RESULTS

Patient population and demographics

A total of 25 patients with IESS (14 males, 11 females), 9 with LGS (5 males, 4 females), and 54 controls (33 males, 21 females) were included (**Fig. 1A**). We compared IESS and LGS patients with age-matched controls, considering the known age-related influence on T cell distribution and function (21,22). For comparisons, 25 IESS patients were age-matched with 9 controls, and 9 LGS patients with 45 controls. Median ages were similar between groups: IESS patients had a median age of 13 months (range: 2–33) vs. 9 months (range: 2–41) in controls (p=0.54); LGS patients had a median age of 74 months (range: 40–209) vs. 78 months (range: 52–161) in controls (p=0.53). Detailed data is presented in **Table 1**.

Table 1. Characteristics of groups

Characteristics	IESS[*] (n=25)	LGS[†] (n=9)
Age at evaluation (months)	13 (2–33)	74 (40–209)
Age of seizure onset (months)	4 (2–6)	31 (9–43)
Etiology		
Cancer	1 (4)	1 (11)
Genetic	6 (24)[‡]	3 (33)[§]
Hypoxic-ischemic encephalopathy	9 (36)[‖]	2 (22)
Inflammatory (meningoencephalitis)	3 (12)	1 (11)
Structural	1 (4)	
Vascular	2 (8)	
Unknown	5 (20)	2 (22)
Seizure/day	3 (0, 10)	8 (4, 20)
Development to LGS	5 (20)	

Values are presented as median (range or Q1, Q3) or number (%).

[*] IESS patients were compared with 9 age-matched controls.

[†] LGS patients were compared with 45 age-matched controls.

[‡] 9q24 deletion (n=1), STXBP1 (n=2), SCN2A (n=1), SCN8A (n=1), DEPDC5 (n=1).

[§] MECP2-related (n=2), STXBP1 (n=1).

[‖] With inflammatory (n=1), with vascular (n=1).

Profound alterations in T cell dynamics

For IESS patients, we embarked our analysis using an advanced machine learning approach, employing unbiased UMAP to the $CD45^+CD3^+$ population within PBMCs (**Fig. 1B**). This analysis revealed a markedly reduced population of naïve $CD4^+$ T cell populations in IESS patients (**Fig. 1B**), a finding that was also statistically significant in manual gating (83.67±1.65 for Cont ≤3 years, 73.21±2.86 for IESS, p=0.041; **Fig. 1C**). Alongside, IESS patients exhibited increased tendency in central memory (CM) (10.72±1.13, 15.63±1.57, p=0.081) and effector memory (EM) $CD4^+$ T cells (4.07±0.58, 8.29±1.45, p=0.096), as illustrated in **Fig. 1C**. While $CD8^+$ T cell populations did not significantly differ, a trend toward an increased proportion of $CCR7^-$ $CD45RA^+$ terminally differentiated effector memory (TEMRA) $CD8^+$ T cells was observed in IESS patients (5.70±2.28, 12.55±1.93, p=0.0601; **Fig. 1D**). The percentage of $CD4^+$ Tregs remained comparable between IESS and controls (3.50±0.47, 3.59±0.35, p=0.88; **Fig. 1E**). Furthermore, the CD4/CD8 ratio was markedly decreased in IESS patients (3.81±0.60, 2.40±0.25, p=0.015; **Fig. 1F**), suggesting potential immune activation driven by previous immune challenges, such as infections (23).

In LGS patients, UMAP analysis showed reduction of naïve T cell population with altered CM $CD8^+$ T cell profiles in the LGS cohort (**Fig. 2A**). LGS patients paralleled IESS patients in having decreased naïve $CD4^+$ T cells (68.79±1.23 for Cont >3 years, 58.73±3.71 for LGS, p=0.0028; **Fig. 2B**) but elevated CM $CD4^+$ T cells (14.08±0.46, 19.74±1.87, p<0.0001; **Fig. 2B**) and CM $CD8^+$ T cells frequencies (3.07±0.21, 4.36±0.63, p=0.022; **Fig. 2C**). Intriguingly, LGS patients exhibited a significant decrease in naïve Tregs (62.31±1.36, 33.73±5.77, p<0.0001) but an increase in non-functional Tregs (25.72±1.30, 41.92±3.15, p<0.0001), which are characterized by diminished immunosuppressive capabilities and heightened pro-inflammatory cytokine production (**Fig. 2D**) (24). This particular dysregulation of Tregs might suggest an LGS-specific immune alteration. The CD4/CD8 ratio in LGS patients remained unchanged compared to controls (1.48±0.10, 1.53±0.17, p=0.83; **Fig. 2E**), underscoring the unique immune dysregulation within each condition.

Collectively, our results demonstrate significant dysregulation in both the proportion and functionality of T cells in IESS and LGS patients. These differences not only delineate

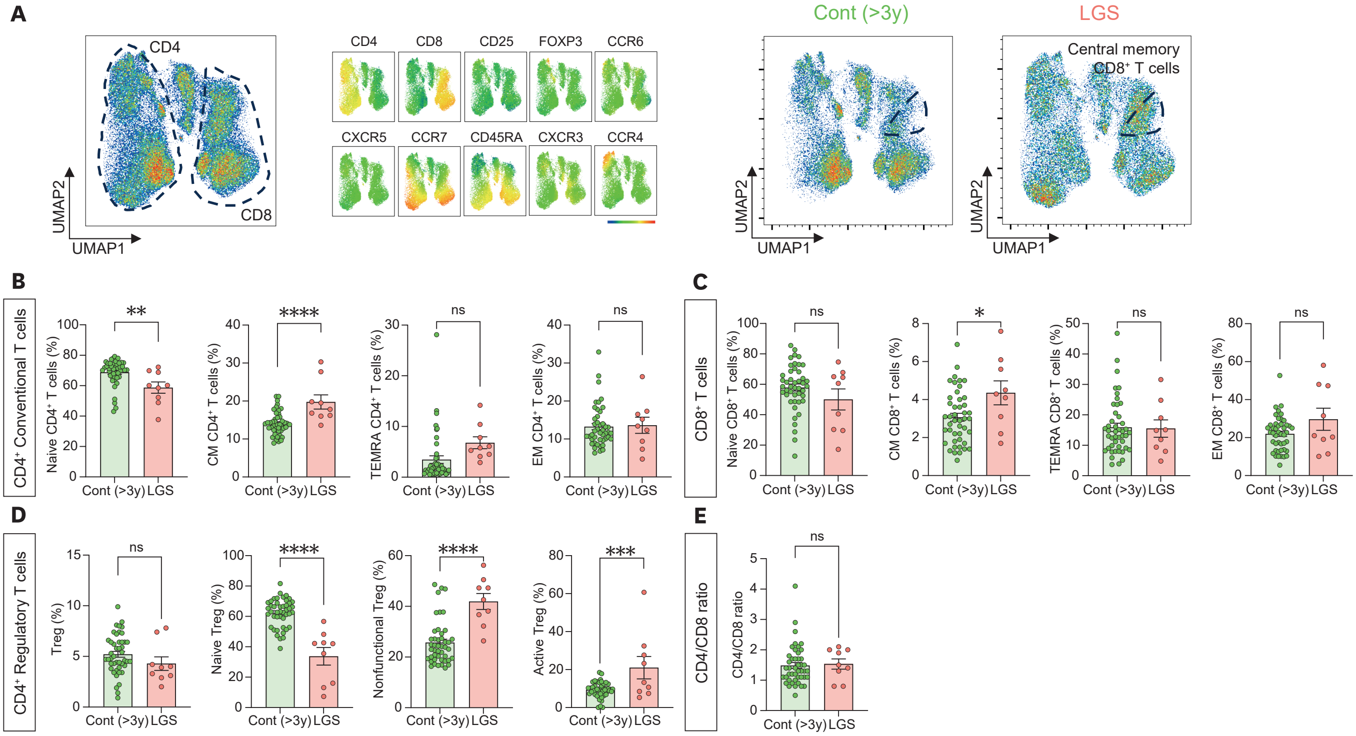

Figure 2. T cell dynamics in LGS patients. (A) UMAP visualization of T cell populations for each study group. (B-E) Comparison of CD4$^+$ conventional T cell populations (B), CD8$^+$ T cell populations (C), CD4$^+$ regulatory T cell populations (D), and CD4/CD8 ratios (E) in LGS patients compared to age-matched controls. Data are presented as mean ± SEM.
Cont, control; ns, not significant,
$^*p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, $^{****}p<0.0001$; Student's *t*-test.

the unique T cell landscapes of each syndrome but also underscore the complexity of their respective immune responses.

Altered cytokine signatures and polyfunctionality in T cells

We further focused on cytokine production to identify deviations in helper T cell functions. In IESS cohort, UMAP analysis demonstrated a substantial reduction in the production of TNFα by CD4$^+$ T cells (**Fig. 3A**), which was consistent with the results from manual gating (22.97±1.68, 13.08±1.03, p<0.0001; **Fig. 3B**). A modest decrease in IL-2 production was also observed (20.96±2.17, 16.68±1.32, p=0.104; **Fig. 3B**). In contrast, these CD4$^+$ T cells showed elevated levels of IFNγ (2.96±0.39, 5.17±1.20, p=0.28) and GZMB (0.92±0.13, 2.50±0.89, p=0.30; **Fig. 3B**) while not significant. Subsequently, we pivoted to the analysis of cytokine production in CD8$^+$ T cells, given their established pathogenic influence in epilepsy (25). Examination of these cells in IESS patients revealed no significant deviations in cytokine profiles when compared to controls (**Fig. 3C**). Although significantly reduced percentage of IL-6 (0.29±0.07, 0.15±0.03, p=0.043) and IL-17A (0.63±0.06, 0.33±0.05, p=0.0045) producing CD8$^+$ T cells was observed, these differences were not considered due to the inherently low frequency of these cells (**Supplementary Fig. 3**). The polyfunctionality assessment, pivotal for delineating T cell behavior in a spectrum of infectious and inflammatory scenarios (26,27), identified a trend towards a reduced capability of CD4$^+$ T cells to produce multiple cytokines. In the IESS group, specifically noting a decline in TNFα$^+$ and TNFα$^+$/IL-2$^+$ cells (**Fig. 3D**). Polyfunctional CD8$^+$ T cells did not vary significantly between IESS patients and controls (**Fig. 3D**).

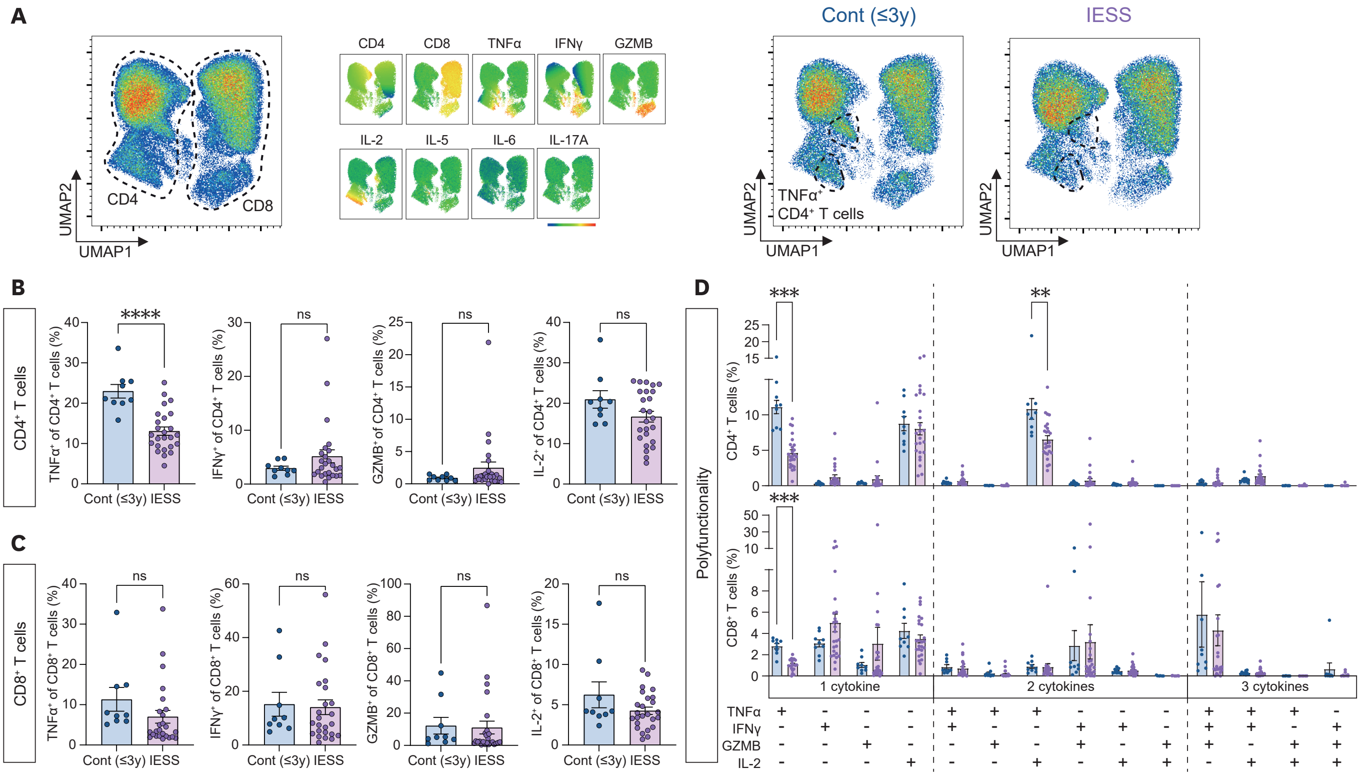

Figure 3. Cytokine signatures of T cells in IESS patients. (A) UMAP visualization of cytokine production from T cells for each study group. (B, C) Comparison of cytokine production from CD4$^+$ (B) and CD8$^+$ (C) T cells between IESS patients and age-matched controls. (D) Frequencies of CD4$^+$ and CD8$^+$ T cells expressing combinations of TNFα, IFNγ, GZMB, and IL-2 in IESS patients compared to age-matched controls. Data are presented as mean ± SEM.
Cont, control; ns, not significant.
$^{**}p<0.01$, $^{***}p<0.001$, $^{****}p<0.0001$; Student's *t*-test.

In LGS patients, UMAP analysis revealed a marked increase in cytokine-producing CD8$^+$ T cells, particularly those co-producing TNF, IFNγ and GZMB, underscoring a vital shift within T cell dynamics (**Fig. 4A**). A notable decrease in IL-2 production by CD4$^+$ T cells was also observed (23.63±1.09, 17.70±2.34, p=0.030), with a tendency for reduced TNFα$^+$ cells (19.70±1.08, 16.19±2.32, p=0.19; **Fig. 4B**). In contrast to CD4$^+$ T cell response, patients with LGS exhibited a pronounced elevation in the proportion of TNFα (14.69±1.34, 25.24±5.90, p=0.0099), IFNγ (24.29±1.62, 34.76±6.50, p=0.027), and GZMB-producing CD8$^+$ T cells (15.75±1.79, 38.61±8.53, p=0.0001) in comparison to the control group (**Fig. 4C**). This increase was also evident in polyfunctional CD8$^+$ T cell populations producing TNFα, IFNγ, and GZMB, indicating a broad upregulation of cytokine production in LGS (**Fig. 4D**).

Altogether, our analysis delineates a diminished CD4/CD8 T cell ratio and lower TNFα$^+$ CD4$^+$ T cells in IESS, pointing to potential functional anomalies. Conversely, LGS is characterized by a significant upregulation of TNFα$^+$, IFNγ$^+$, and GZMB$^+$ CD8$^+$ T cells, along with an increase in CD8$^+$ T cell polyfunctionality. These distinct immune signatures offer insights into the divergent immunopathology of IESS and LGS.

Association of disease severity with hyper-T$_H$1/T$_C$1 responses in IESS

We observed significant heterogeneity in T cell subtype proportions and functions among patients, but the link to disease pathogenesis remains unclear. For this, we correlated clinical data with immune phenotyping results. Seizure frequency (seizures per day) showed a negative correlation with naïve CD8$^+$ T cells and a positive correlation with EM CD8$^+$ T cells

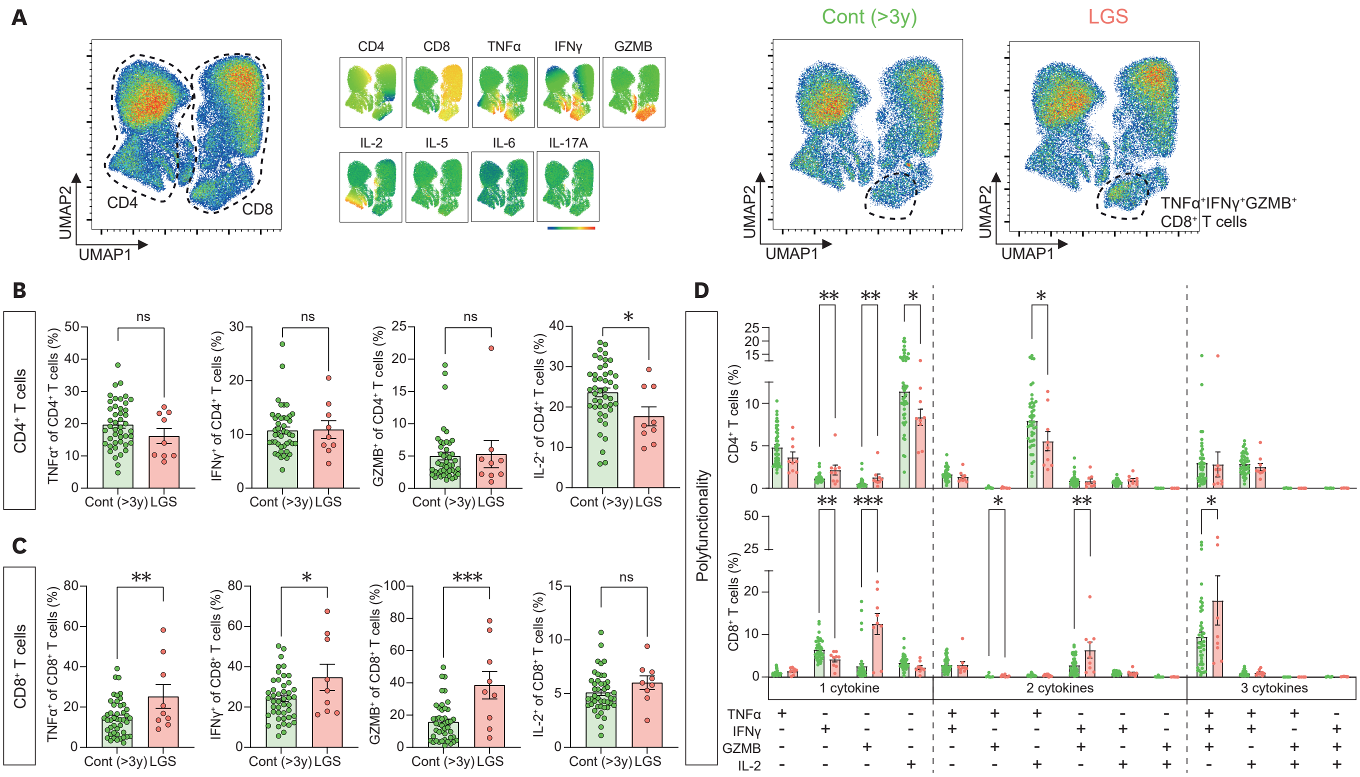

Figure 4. Cytokine signatures of T cells in LGS patients. (A) UMAP visualization of cytokine production from T cells for each study group. (B, C) Comparison of cytokine production from CD4$^+$ (B) and CD8$^+$ (C) T cells between LGS patients and age-matched controls. (D) Frequencies of CD4$^+$ and CD8$^+$ T cells expressing combinations of TNFα, IFNγ, GZMB, and IL-2 in LGS patients compared to age-matched controls. Data are presented as mean ± SEM.
Cont, control; ns, not significant.
$^*p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$; Student's *t*-test.

(**Fig. 5A**). Additionally, it was positively correlated with the frequency of cytokine-producing CD8$^+$ T cells, including TNFα, IFNγ, and GZMB (**Fig. 5A-C**). Stratifying patient samples based on seizure frequency per day, we observed a marked increase in the proportion of TNFα$^+$ CD8$^+$ T cells (3.45±0.79 for 0, 4.86±1.35 for 1–9, 5.74±2.28 for 10–39, 20.30±5.87 for ≥40; **Fig. 5D**) and TNFα$^+$IFNγ$^+$GZMB$^+$IL-2$^-$ CD8$^+$ T cells (1.33±0.57 for 0, 2.20±1.14 for 1–9, 2.75±1.60 for 10–39, 16.63±5.91 for ≥40; **Fig. 5E**) in patients experiencing 40 or more seizures per day compared to those with fewer seizures.

Furthermore, we compared IESS patients who later developed LGS to those who did not, to identify factors linked to progression to LGS. Interestingly, progression to LGS was associated with a higher proportion of EM and TEMRA CD4$^+$ T cells, as well as TEMRA CD8$^+$ T cells (**Fig. 5A**). These patients also exhibited increased frequencies of cytokine-producing T cells, including IFNγ$^+$ CD4$^+$ and CD8$^+$ T cells (**Fig. 5A**). Direct comparison between 2 groups revealed elevated IFNγ$^+$ CD4$^+$ T cells (3.49±0.62 for N, 11.90±4.67 for Y, p=0.0028) and IFNγ$^+$ CD8$^+$ T cells (10.63±2.43, 27.86±7.19, p=0.0083) in patients who progressed to LGS (**Fig. 5F**). In addition, the frequencies of TEMRA CD4$^+$ (1.35±0.28, 5.72±2.80, p=0.0055) and TEMRA CD8$^+$ T cells (9.97±1.63, 22.9±5.32, p=0.0047) were significantly higher in these patients (**Fig. 5F**), suggesting the potential association between activated immune status and the transition from IESS to LGS.

Our analysis also revealed notable correlations among immune parameters, especially within CD4$^+$ and CD8$^+$ T cell subsets defined by surface markers and cytokine production profiles

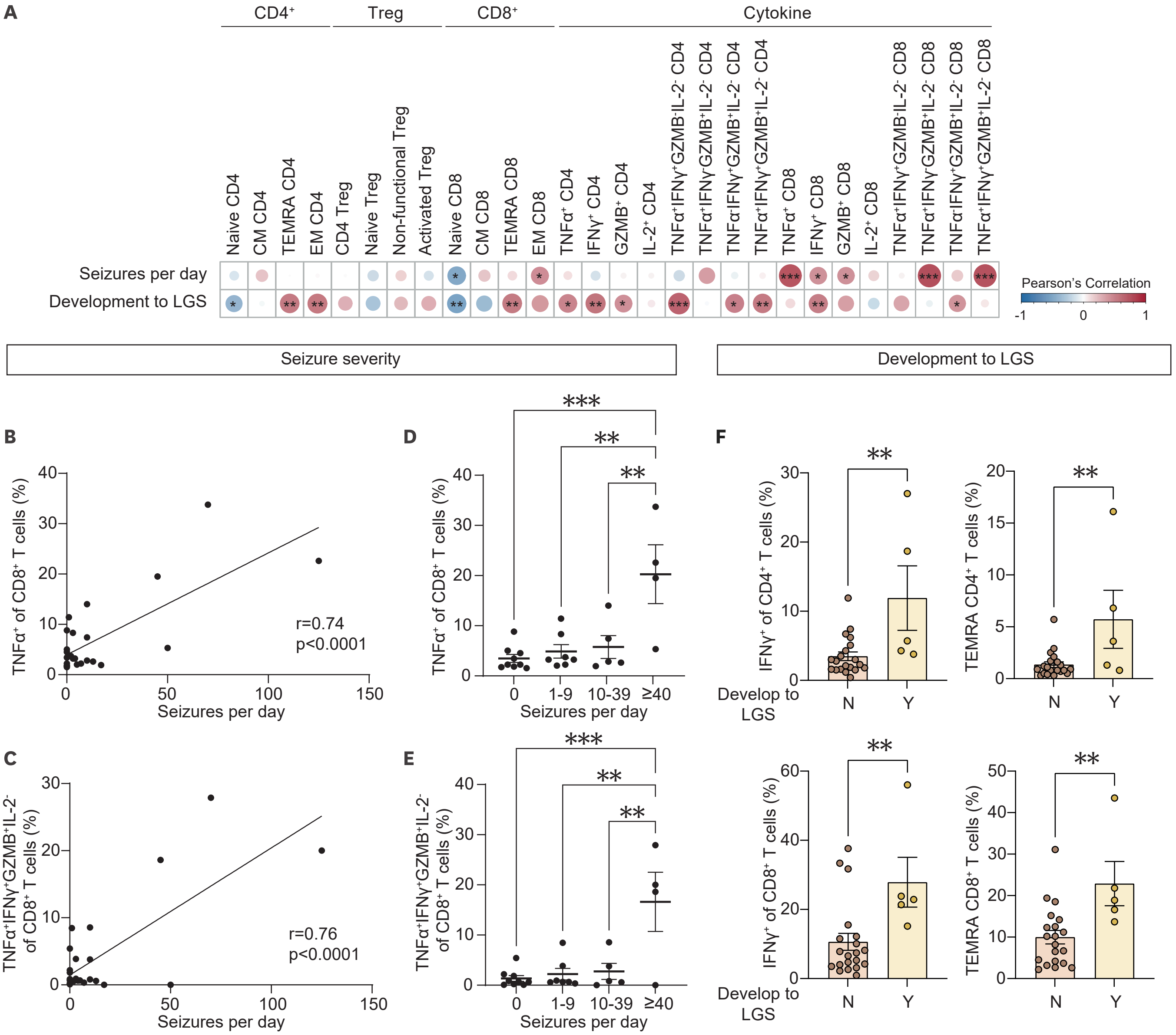

Figure 5. Correlation among immune populations and clinical data in IESS patients. (A) Correlation matrix for T cell populations and clinical data (column) in IESS patients. Color and circle size indicate the Pearson correlation coefficient value. (B, C) Scatter plot depicting the relationship between TNFα$^+$ CD8$^+$ T cells (B), TNFα$^+$IFNγ$^+$GZMB$^+$IL-2$^-$ CD8$^+$ T cells (C) and seizure frequency. (D, E) Proportions of TNFα$^+$ CD8$^+$ T cells (D) and TNFα$^+$IFNγ$^+$GZMB$^+$IL-2$^-$ CD8$^+$ T cells (E) among groups categorized by seizure frequency. (F) Comparisons between IESS patients who progressed to LGS and those who did not. Data are presented as mean ± SEM.
Y, IESS patients who progressed to LGS; N, IESS patients who did not progressed to LGS; ns, not significant.
$^{**}p<0.01$, $^{***}p<0.001$; Pearson's correlation analysis (A-C), one-way ANOVA followed by a Sidak's *post hoc* test (D, E) and Student's *t*-test (F).

in IESS patients. We found that the proportion of naïve CD4$^+$ and CD8$^+$ T cells negatively correlated with the frequency of T cells expressing TNFα, IFNγ, or GZMB, as well as those producing combinations of these cytokines (**Supplementary Fig. 4**). In contrast, EM and TEMRA CD4$^+$ T cell populations showed positive correlations with IFNγ$^+$ and GZMB$^+$ CD4$^+$ T cells—a trend also seen in CD8$^+$ T cells (**Supplementary Fig. 4**). A negative correlation was apparent between the CD4/CD8 ratio and markers indicative of T cell activation, including TEMRA/EM CD4$^+$ T cells, cytokine-producing CD4$^+$ T cells (TNFα$^+$, IFNγ$^+$, GZMB$^+$), EM CD8$^+$ T cells, and GZMB$^+$ CD8$^+$ T cells (**Supplementary Fig. 4**). This suggests that a lower CD4/CD8 ratio in IESS patients may signify an activated immune status. Similar correlations were observed in LGS, where TNFα$^+$, IFNγ$^+$, and GZMB$^+$ producing CD8$^+$ T cells correlated negatively with naïve, and positively with EM CD8$^+$ T cells (**Supplementary Fig. 5**).

In summary, these results indicate a strong link between IESS severity and an activated Th1/type 1 T cytotoxic (Tc1) immune response, highlighting the potential of certain immune markers as indicators of disease progression.

Infection history and Tc1 cell dynamics in IESS

Given the observed correlation between elevated Tc1 responses and disease severity in IESS patients, we speculated that Tc1 responses might contribute to the development of severe epilepsy in this group and therefore focused our analysis on these patients. To explore potential drivers of this immune activation, we investigated virus-specific CD8$^+$ T cell responses. Intriguingly, IESS patients with frequent seizures and high CD8$^+$ T cell reactivity (≥40 seizures/day, n=4) showed a significant increase in GZMB production when stimulated with cytomegalovirus (CMV) peptides, compared to patients with rare seizures and low T cell reactivity (0 seizure/day, n=6; 2.10±0.81, 0.015±0.015, p=0.041; **Fig. 6A**). A similar trend, although not statistically significant, was also observed when compared with age-matched controls (Cont ≤3 years, n=6; 2.10±0.81, 0.093±0.072, p=0.15; **Fig. 6A**). There was a minor increase in the response to SARS-CoV-2 in the control group compared to IESS groups (1.83±0.99, 0.0017±0.0017, 0.15±0.15; **Fig. 6A**). However, no differences were observed with other viral peptides such as human respiratory syncytial virus group B, influenza A, and Epstein-Barr virus (**Fig. 6A**). A closer examination of the clinical profiles of 4 patients who responded positively to CMV (**Fig. 6B**) revealed potential indicators of congenital CMV infection, including low birth weight below the 10th percentile (2 of 4), microcephaly (3 of 4), sensorineural hearing loss (1 of 4), and optic atrophy (1 of 4), as summarized in **Supplementary Table 1**. MRI findings in 2 patients revealed radiologic features consistent with congenital CMV infection, including bilateral polymicrogyria, ventriculomegaly, and diffuse white matter abnormalities with calcifications (**Fig. 6C**, **Supplementary Table 1**). One patient with microcephaly and low perinatal body weight, but no identified etiology, also raised the possibility of uninvestigated congenital CMV infection (**Supplementary Table 1**). The notable increase in immune responses to CMV peptides in severe IESS patients suggests that prior viral infections, including CMV, may play a role in the pathology of IESS.

DISCUSSION

This study discloses complex immunopathological landscape of DEEs, highlighting distinct T cell dysfunctions in IESS and LGS. Our study's insights into T cell polyfunctionality and cytokine production in IESS and LGS pave the way for potentially transformative immunomodulatory therapies that could significantly improve patient outcomes by targeting these identified pathways.

Patients with IESS and LGS had marked deviations in T cell dynamics and cytokine profiles compared to age-matched healthy controls (**Fig. 6D**). In IESS patients, specific immune dysregulation was observed, including a decreased CD4/CD8 ratio (**Fig. 1F**) and a reduced proportion of TNFα-producing CD4$^+$ T cells (**Fig. 3B**). To date, there have been few studies that have performed immune profiling in IESS patients, reporting an increase in plasma cells and CD8$^+$ T cells (8). Our results of detailed immune phenotyping are in line with previous findings, suggesting adaptive immune dysfunction in these patients. Notably, specific

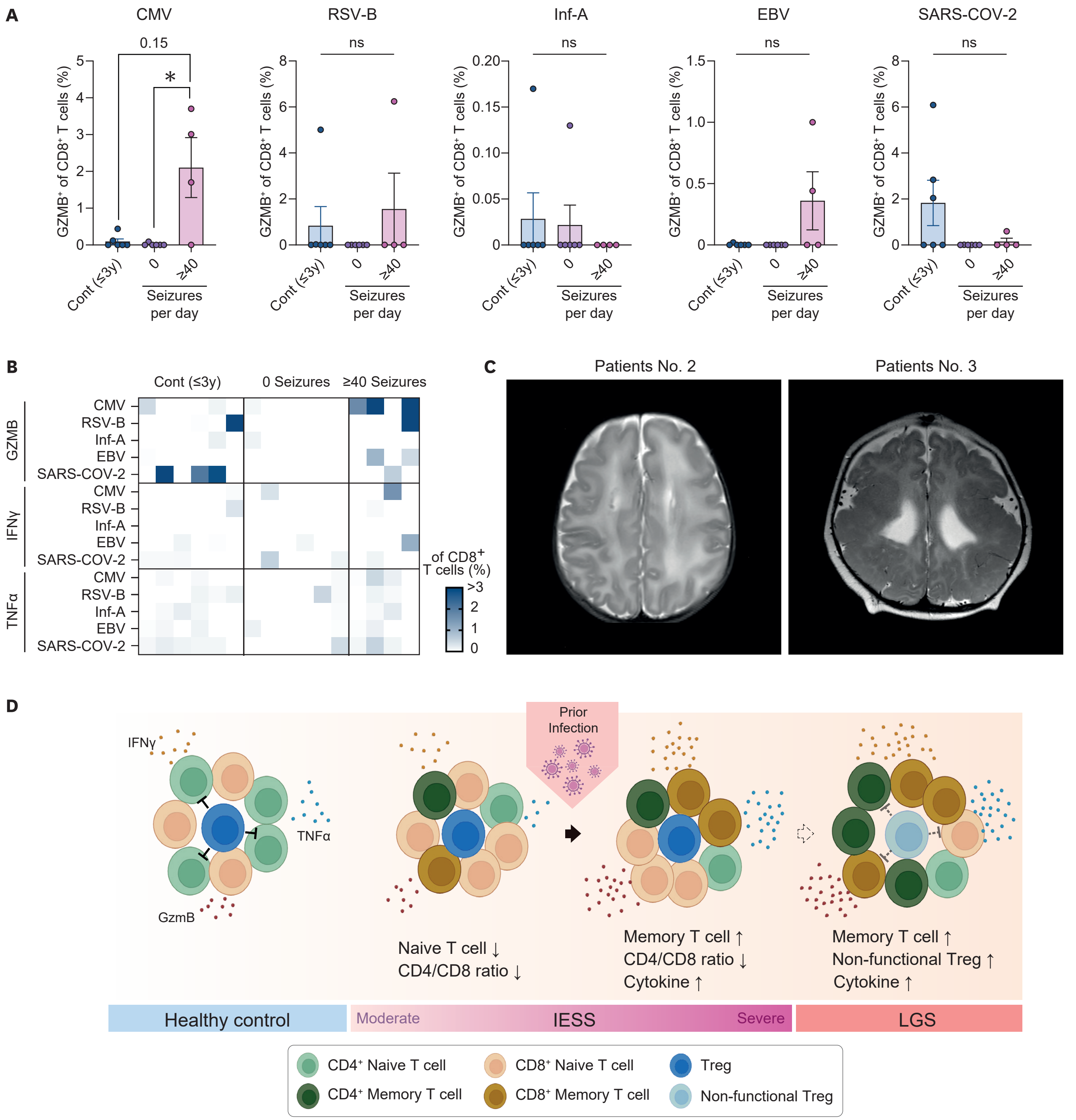

Figure 6. $CD8^+$ T cells response to viral specific peptides in IESS patients. (A) Comparison of $GZMB^+$ $CD8^+$ T cells following peptide stimulation. Data are presented as mean ± SEM. (B) Heatmap illustrating the proportions of cytokine-producing $CD8^+$ T cells in response to virus peptides. (C) Representative MRI images of IESS patients showing diffuse white matter abnormality (left) and bilateral symmetric polymicrogyria (right). (D) A schematic representation of T cell dysfunctions in IESS and LGS.
RSV-B, human respiratory syncytial virus group B; Inf-A, influenza A; EBV, Epstein-Barr virus; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; ns, not significant.
$^*p<0.05$; Kruskal-Wallis test followed by Dunn's multiple comparisons test.

immune anomalies in IESS correlated with disease progression and clinical severity in IESS patients. Significantly, immune variables including $TNF\alpha^+$, $IFN\gamma^+$, or $GZMB^+$ $CD4^+$ T cells, IFNγ$^+$ CD8$^+$ T cells, and TEMRA CD4$^+$ and CD8$^+$ T cells showed a positive association with progression from IESS to LGS (**Fig. 5F**, **Supplementary Fig. 4**), highlighting a potential mechanistic link between immune system behavior and disease evolution. Further, the correlation between disease severity and specific immune responses, particularly the Th1/Tc1 cell responses, is of considerable interest. The association of increased TNFα$^+$, IFNγ$^+$, and GZMB$^+$ T cell proportions with higher seizure frequencies and more severe disease courses in IESS patients (**Fig. 5A-E**, **Supplementary Fig. 4**) implies a link between an activated immune profile and clinical outcomes. This raises the possibility that modulating these specific immune pathways could mitigate disease severity. The identification of a distinctive increase in CD8$^+$ T cell activity and polyfunctionality in LGS also suggests that these cells may play a critical role in the persistence and exacerbation of this condition, offering a potential target for therapeutic intervention.

The observed increase in non-functional Tregs (**Fig. 2D**), specifically fraction III cells (28), within the LGS cohort is significant. Traditionally, Tregs are crucial for maintaining immune tolerance in both the peripheral system and the CNS (29,30). However, in LGS, there is an upsurge in Tregs that, despite expressing FOXP3, paradoxically produce pro-inflammatory cytokines and lack the typical suppressive function of conventional Tregs (28,31,32). This aligns with evidence showing that Tregs depletion negatively impacts temporal lobe epilepsy and correlates with the severity of drug-resistant epilepsy in pediatric patients (33,34). This suggests that Tregs functionality may inversely relate to disease severity. The broader scientific literature supports this, emphasizing Tregs' role in modulating CD8$^+$ T cell responses. A deficiency in Tregs regulatory capacity could intensify epileptogenic processes through heightened CD8$^+$ T cell responses (35-37). Investigating the mechanisms behind Tregs dysfunction and their contribution to epilepsy pathogenesis is crucial. Restoring Tregs function presents a promising therapeutic opportunity, potentially reducing inflammation and seizure severity in LGS patients.

Notably, elevated responses to viral antigens were observed in patients with IESS. The decreased CD4/CD8 ratio in IESS patients, as shown in **Fig. 1F**, and the significant presence of CMV-specific CD8$^+$ T cell responses in patients with high seizure rates (**Fig. 6**) suggest a potential link between IESS disease severity and viral infection. The causality or temporal relationship remains unrevealed. However, the fact that some patients had features suggestive of congenital CMV infection raises the possibility that a remote viral infection, such as undiagnosed asymptomatic congenital CMV infection, may have played a role. Previously, few literatures have shown that congenital CMV infection was related to IESS (38,39) but the evidence linking congenital CMV infection to an increase in CD8$^+$ T cell activity and the severity of IESS remains inconclusive. The accurate temporal relationship between CMV infection and IESS have to be further investigated in human and animal models.

Activated CD8$^+$ T cells in peripheral blood have been documented in various epilepsy syndromes, including temporal lobe epilepsy and other treatment-resistant epilepsies (40,41). Recent studies indicate that activated CD8$^+$ T cells could exacerbate epilepsy through multiple mechanisms, such as disruption of the blood-brain barrier, induction of neuroinflammation, and direct neuronal damage mediated by cytotoxic molecules, including GZMB and perforin, upon infiltration into the central nervous system (34,42,43). Notably, both human studies and animal models demonstrate that congenital viral infections, particularly congenital CMV infection, can induce infiltration of activated virus-specific CD8$^+$ T cells into brain parenchyma, potentially contributing to neuronal injury, chronic inflammation, and subsequent epileptogenesis (44,45). Moreover, recent findings suggest that even in cases with identifiable genetic etiologies of epilepsy, a heightened CD8$^+$ T cell response may serve as an additional pathogenic factor, amplifying disease severity rather than representing the primary causal mechanism. Consistent with this concept, our cohort included severe IESS patients exhibiting enhanced virus-specific CD8$^+$ T cell responses alongside genetic abnormalities (**Supplementary Table 1**). Thus, we interpret our findings as reflecting an immunological state of heightened activation, possibly triggered by previous viral encounters, which aggravates the clinical phenotype. Future longitudinal studies will be critical for determining whether this immunological pre-activation directly influences disease progression and whether immunomodulatory interventions targeting activated CD8$^+$ T cells could improve clinical outcomes in these epilepsy syndromes.

As our understanding of the immunological aspects of epileptogenesis grows (46), drugs targeting specific immune targets are already being utilized in select epilepsy patients. Tocilizumab, an anti-interleukin 6 receptor inhibitor, and Anakinra, an anti-interleukin 1 antibody, have demonstrated effectiveness in immune-related epileptic syndromes such as new-onset refractory status epilepticus (47) and febrile infection-related epilepsy syndrome (48). Additionally, Natalizumab, an anti–α4-integrin antibody that blocks leukocyte migration to the brain, is currently under clinical trial for drug-resistant epilepsies (49), with some successful cases reported in Rasmussen's encephalitis (50). In the case of IESS, use of immunotherapy such as prednisolone and ACTH, is common. Our findings of increased inflammatory responses in IESS patients support the use of immunosuppressants like steroids. However, it has a broad spectrum of efficacy and various adverse effects necessitating a more meticulous stratified treatment approach (51,52). In particular, mechanisms of steroid resistance in Tc1/Th1-type inflammatory diseases might limit their efficacy (53). This highlights the need to identify specific immune targets and patient immune profiles to develop immune-based therapies that can potentially overcome steroid resistance and improve treatment outcomes.

In conclusion, our study identifies distinct immune profiles in IESS and LGS that correlate with clinical parameters, highlighting potential immune dysregulation associated with disease severity and progression in DEEs. While these findings enhance our understanding of immune involvement in these disorders, further mechanistic studies and controlled clinical trials are required to clarify the exact roles of these immune alterations and to evaluate the feasibility and efficacy of immunomodulatory strategies.

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SUPPLEMENTARY MATERIALS

Supplementary Table 1

Clinical observations related with congenital CMV infection in IESS patients who showed CD8 CMV response

Supplementary Figure 1

Gating strategy for T cell population profiling. A representative example of a control sample is shown. Tregs are defined as CD25$^+$FOXP3$^+$ CD4$^+$ T cells. Subtypes of Tregs were defined by expression of FOXP3 and CD45RA. Characterization of CD4$^+$ and CD8$^+$ cells were defined by expression of CD45RA and CCR7. Th1/Tc1, Th17/Tc17, Th2/Tc2 and Tfh cells were defined by expression of CXCR3, CCR6, CCR4 and CXCR5, respectively, within the CD25$^-$FOXP3$^-$CD4$^+$ conventional T cells and CD8$^+$ T cells.

Supplementary Figure 2

Gating strategy for cytokine production from CD4$^+$ and CD8$^+$ T cells. A representative example of a control sample is shown. Upon PMA/ionomycin stimulation, the proportion of cytokine (TNFα, IFNγ, IL-2, GZMB, IL-5, IL-17A, IL-6) producing cells were analyzed within CD4$^+$ T cells and CD8$^+$ T cells.

Supplementary Figure 3

Additional T cell phenotypes and cytokine production in IESS and LGS patients. Data not displayed in **Figs. 1-4** are presented. Data are presented as mean ± SEM.

Supplementary Figure 4

Correlation among immune populations and clinical data in IESS patients. Correlation matrix displaying associations among immune cell populations and clinical data. Color and circle size indicate the Pearson correlation coefficient value.

Supplementary Figure 5

Correlation among immune populations and clinical data in LGS patients. Correlation matrix displaying associations among immune cell populations and clinical data. Color and circle size indicate the Pearson correlation coefficient value.

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