Proportion of peripheral regulatory T cells in patients with autoimmune encephalitis

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Purpose
Regulatory T cells (Tregs) play a crucial role in maintaining immune tolerance. Any deficiency or dysfunction of the Tregs can influence the pathogenesis of autoimmune disease. This study aimed to assess the role of Tregs among patients with autoimmune encephalitis (AE) with different autoantibody types and to evaluate their association with clinical features.

Methods
This was a cross-sectional observational study involving 29 patients with AE. Peripheral blood was sampled from each patient for flow cytometric analysis. Proportions of CD4⁺CD25⁺ and CD4⁺CD25⁺Foxp3⁺ Tregs were calculated and compared between the antibody types (synaptic, paraneoplastic, and undetermined). Associations between the proportion of Tregs and clinical features were also evaluated.

Results
Five patients had synaptic autoantibodies, five had paraneoplastic autoantibodies, and the others were of an undetermined type. The proportion of CD4⁺CD25⁺ Tregs tended to be higher in those with paraneoplastic antibodies than in those with synaptic antibodies (post-hoc p = 0.028) and undetermined antibody status (post-hoc p = 0.043). A significant negative correlation was found between the proportion of Tregs and the initial modified Rankin score (r = −0.391, p = 0.036). Those who received intravenous immunoglobulin had lower proportions of Tregs than those who did not.

Conclusion
The results of the present study suggest that Tregs may play different roles according to the type of AE and may be linked to disease severity.

Keywords: Regulatory T-lymphocytes, Autoimmune encephalitis, Antibodies, Intravenous immunoglobulins

Introduction
Autoimmune encephalitis (AE) causes focal or diffuse neural inflammation mediated by autoantibodies [1]. The diagnosis of AE is based on clinical symptoms and diagnostic tests, including autoantibodies [2]. Based on autoantibody findings, AE can

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largely be classified into three categories according to (1) the presence of antibodies against intracellular targets (paraneoplastic autoantibodies), (2) the presence of antibodies against membrane surface antigens (synaptic autoantibodies), and (3) the absence of any detectable antibodies (undetermined). The pathophysiology of AE, clinical features, and patients’ response to immunotherapy among between the autoantibody types [3].

Regulatory T cells (Tregs) are known to suppress inflammatory autoimmune responses [4]. The Tregs inhibit the proliferation and function of inflammatory cells [5]. Known Tregs include CD4+CD25+ cells and express transcription factor forkhead box P3 (Foxp3). Dysfunction or deficiency of the Tregs can lead to microglial activation, inflammation, and neural injury. Tregs are known to be involved in the pathogenesis of autoimmune diseases, including type 1 diabetes, rheumatoid arthritis, systemic lupus erythematosus [5,6]. Tregs also play a crucial role in autoimmune diseases involving the central nervous system (CNS), such as multiple sclerosis (MS) [7]. A meta-analysis study showed that the proportion of CD4+CD25+Foxp3+ Tregs was lower in MS patients than in control subjects [8]. The Tregs are also known to be associated with both development and exacerbation of MS [9].

AE also occurs when the CNS immune system becomes dysregulated. The proportion of Tregs, which suggests the maintenance of immune tolerance, may be involved in the pathogenesis of AE and may differ between the autoantibody types. Thus, in the present study, we compared proportions of Tregs between different antibody types and evaluated their association with clinical characteristics in patients with AE.

Methods

Patients and clinical characteristics

Thirty patients diagnosed with AE were enrolled between January 2017 and July 2018. The study inclusion criteria were based on established criteria for diagnosing AE [2] as follows: (1) subacute onset of working memory deficits, altered mental status, or psychiatric symptoms and (2) new focal CNS findings, unexplained seizures, pleocytosis in the cerebral spinal fluid (CSF), or abnormalities on magnetic resonance imaging, electroencephalography, and CSF evaluation, at the time of diagnosis as well as at the time of the last follow-up were obtained. Moreover, the results of initial diagnostic tests, including brain magnetic resonance imaging, electroencephalography, and CSF evaluation, were evaluated.

This study was approved by the Institutional Review Board of Seoul National University Hospital (No. 1603-047-747). Written informed consent to participate was obtained from the patients enrolled or their next of kin.

Serum regulatory T cell measurements

About 10 mL of venous blood was collected from each patient into heparin-anticoagulated vacuum tubes, and peripheral blood mononuclear cell (PBMC) preparation was conducted on the same day as sampling. The PBMCs were incubated with a cocktail containing anti-human CD4+ and CD25+ for 30 minutes at room temperature and then were stained for fluorescence-activated cell sorting. The percentages of CD4+CD25+ and CD4+CD25+Foxp3+ cells were calculated using the FACSCalibur software program (BD Biosciences, San Jose, CA, USA).

Statistical analysis

The Kruskal-Wallis test was used to compare Treg proportions between the three antibody groups. The Mann-Whitney U-test was used for post-hoc analysis to determine the significance of the differences between pairs of groups. Categorical variables were compared using Fisher exact test. Spearman correlation coefficient was used to examine correlations between Treg proportions and clinical variables, and the level of significance was set at p < 0.05. For the post-hoc analysis, Bonferroni correction was used to adjust for multiple comparisons, and the level of significance was set at p ≤ 0.05/3 (0.017). Statistical analyses were performed using the IBM SPSS version 22.0 (IBM Corp., Armonk, NY, USA).

Results

Clinical features and demographics

A total of 29 patients, including five with synaptic antibodies (i.e., four with anti-N-methyl-D-aspartate receptor and one with anti-LGI1 antibodies), five with paraneoplastic autoantibodies (i.e., two with anti-Yo, two with anti-glutamic acid decarboxylase [GAD], and one with anti-SOX1 antibodies), and the remaining of
whom were undetermined, were analyzed and one patient was excluded from the undetermined antibody group because of an inadequate number of PBMCs. The mean age of participants was 46.7 years, 10 (34.5%) were male, and the mean disease duration was 26 months. After a mean follow-up duration of 16 months, mean mRS values and CASE scores were significantly reduced (2.7 ± 1.0 to 2.1 ± 1.4 points \( p = 0.004 \) and 5.3 ± 3.8 to 3.7 ± 4.0 points \( p < 0.001 \), respectively). Demographics and mean mRS values and CASE scores were similar among the groups (Table 1).

**Proportion of Tregs (CD4^+CD25^+ and CD4^+CD25^+Foxp3^+)**
The mean PBMC count was \( 157.2 \times 10^5 \) cells/mL and was also similar between the groups. The mean proportions of CD4^+CD25^+ and CD4^+CD25^+Foxp3^+ Tregs were 8.8% ± 4.9% and 1.8% ± 1.9%. The proportion of CD4^+CD25^+ Tregs tended to be higher in those with paraneoplastic antibodies than in those with an undetermined antibody status (\( p_{\text{post-hoc}} = 0.043 \)) and synaptic antibodies (\( p_{\text{post-hoc}} = 0.028 \)) (Table 2).

**Correlation between the proportion of Tregs and clinical features**
A significant negative correlation was observed between the proportion of Tregs (CD4^+CD25^+Foxp3^+) and the initial mRS value (\( r = -0.391, p = 0.036 \)) (Figure 1). No significant correlation was found between the disease duration or CASE score and proportion of Tregs. Those who received IVIg had higher initial mRS values (3.0 ± 1.0 vs. 2.1 ± 0.7 points; \( p = 0.028 \)), lower proportions of CD4^+CD25^+Foxp3^+ Tregs (1.1% ± 0.8% vs. 3.2% ± 2.5%; \( p = 0.029 \)), and lower proportions of Foxp3^+/CD4^+ Tregs (15.7% ± 10.9% vs. 39.3% ± 27.7%; \( p = 0.031 \)) than those who did not (Table 3).

**Discussion**
The results of the present study suggest that the role of Tregs

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### Table 1 Clinical characteristics of patients with autoimmune encephalitis according to autoantibody type

| Characteristic          | Undetermined | Synaptic | Paraneoplastic | p-value |
|-------------------------|--------------|----------|----------------|---------|
| No. of patients         | 19           | 5        | 5              |         |
| Age (yr)                | 47.9 ± 17.8  | 36.2 ± 19.5 | 52.8 ± 16.1 | 0.325   |
| Male sex                | 7 (36.8)     | 1 (20.0)  | 2 (40.0)       | 0.749   |
| Disease duration (mo)   | 30.9 ± 47.5  | 9.5 ± 9.9 | 24.1 ± 13.8    | 0.287   |
| Follow-up duration (mo) | 18.4 ± 14.8  | 6.8 ± 8.6 | 17.0 ± 8.9     | 0.180   |
| mRS value               |              |          |                |         |
| Initial                 | 2.4 ± 1.0    | 3.2 ± 1.1 | 3.0 ± 0.7      | 0.164   |
| Follow-up               | 1.8 ± 1.2    | 2.4 ± 1.5 | 2.8 ± 1.9      | 0.499   |
| CASE score              |              |          |                |         |
| Initial                 | 4.8 ± 3.8    | 8.0 ± 4.6 | 4.4 ± 1.7      | 0.094   |
| Follow-up               | 3.4 ± 4.1    | 5.8 ± 4.8 | 2.5 ± 1.3      | 0.168   |
| Treatment received      |              |          |                |         |
| Immunotherapy           | 11 (57.9)    | 5 (100)  | 5 (100)        | 0.055   |
| IV steroid              | 3 (15.8)     | 4 (80.0)  | 2 (40.0)       | 0.020   |
| IVIg                    | 11 (57.9)    | 4 (80.0)  | 4 (80.0)       | 0.492   |
| Rituximab               | 4 (21.1)     | 2 (40.0)  | 3 (60.0)       | 0.220   |

Values are presented as mean ± standard deviation or number (%).

mRS, modified Rankin scale; CASE, Clinical Assessment Scale in Autoimmune Encephalitis; IV, intravenous; IVIg, IV immunoglobulin.

### Table 2 Flow cytometry results of patients with autoimmune encephalitis according to autoantibody type

| Variable                  | Undetermined (n = 19) | Synaptic (n = 5) | Paraneoplastic (n = 5) | p-value |
|---------------------------|-----------------------|------------------|------------------------|---------|
| WBC (/μL)                 | 7,215 ± 2,592         | 6,062 ± 1,400    | 6,468 ± 1,847          | 0.586   |
| ANC (/μL)                 | 4,647 ± 2,551         | 3,810 ± 1,596    | 4,019 ± 1,804          | 0.865   |
| PBMC (× 10^9)/mL          | 160.4 ± 126.9         | 124.6 ± 78.3     | 178.0 ± 143.4          | 0.838   |
| CD4^+CD25^+ (%)           | 8.2 ± 5.1             | 7.3 ± 3.5        | 12.8 ± 3.4             | 0.073   |
| CD4^+CD25^+Foxp3^+ (%)    | 1.7 ± 1.8             | 1.5 ± 1.7        | 2.6 ± 2.3              | 0.319   |
| Foxp3/CD4^+ (%)           | 26.7 ± 24.5           | 17.1 ± 11.0      | 19.7 ± 15.2            | 0.769   |

Values are presented as mean ± standard deviation.

WBC, white blood cell count; ANC, absolute neutrophil count; PBMC, peripheral blood mononuclear cell count; Foxp3, forkhead box P3.
may vary with autoantibody type in AE. Those with paraneo-
plastic antibodies tended to present higher Treg proportions
than those with synaptic antibodies. The proportion of Tregs
was negatively correlated with initial disease severity and dif-
ered between those who were treated with IVIg and those who
were not, which may be further used as a biomarker for disease
activity.

Although the proportion of CD4⁺CD25⁺ Tregs tended to be dif-
ferent between AE patients with different antibody types, no dif-
ference was found in the proportion of CD4⁺CD25⁺Foxp3 Tregs.
Foxp3 plays a crucial role in the development and function of
Tregs and is considered one of the most reliable markers of
Tregs [12]. Although the expression of Foxp3 is evident in natu-
ral Tregs, it is known to be unstable in peripherally induced
Tregs [13], which may explain our result. Proportions of Treg
subpopulations in AE should be elucidated in further studies.

Variable proportions of Tregs among AE patients with different
antibody types may suggest different pathophysiologies. AE pa-
tients with paraneoplastic antibodies usually have no pathogen-
ic role, but their condition involves cytotoxic T cells that directly
cause neuronal impairment. In contrast, T-cell involvement in
those with synaptic antibodies is unclear. Synaptic antibodies
bind to neuronal cell surface receptors and alter synaptic signal-
ning processes [3]. One study reported that the number of CD4⁺ T
cells in AE patients with an undetermined antibody status was
greater than in those with confirmed antibodies, regardless of
the antibody type. However, the ratio of CD4⁺ and CD8⁺ T cells
were lower only in those with paraneoplastic autoantibodies
[14]. A lower CD4/8⁺ T-cell ratio has been linked to blood–brain
barrier dysfunction in AE patients with temporal lobe epilepsy
[15]. Our data also support that T-cell involvement may be more
prominent in those with paraneoplastic antibodies than in those
with synaptic antibodies or undetermined antibody status.

The proportion of Tregs was also negatively associated with
functional status in AE patients, with lower Treg proportions
correlating with higher mRS values. In line with our study, the
frequency of Tregs was found by Correale and Villa [16] to be
lower during disease exacerbation in patients with MS than
during remission or in healthy controls. Moreover, the propor-
tion of Tregs has also been reported to be associated with the
severity of graft-versus-host disease [17]. One recent study sug-
gested that the proportion of CD4⁺ T cells in peripheral blood
was associated with frontal lobe function in GAD65 and volt-
age-gated potassium channel antibody-related encephalitis
[18].

Treg proportions differed according to IVIg treatment but not
rituximab treatment. IVIg therapy can increase cytokine secre-
tions to enhance immune tolerance [19] and modulates the
function of dendritic cells to expand the number of Tregs [20].
In previous research, IVIg promoted the expansion of CD4⁺
CD25⁺Foxp3⁺ Tregs in patients with Guillain-Barré syndrome
[21]. Theoretically, rituximab can also lead to Treg expansion
following B-cell depletion and the suppression of autoreactive T
cells [22], which was not shown in our study. Recently, a Treg-
based immunotherapeutic approach has been suggested as an
experimental treatment for autoimmune or neurodegenerative
disease [23], and our study suggests that this concept may also
be applicable in AE patients.

The results of our study should also be interpreted cautiously in
light of their limitations. This study was a single-center investi-
gation considering a relatively small number of AE patients
without a comparison with healthy controls. Because this was a
cross-sectional study, the time and type of immunotherapy were
not controlled, which may have influenced our findings.
In conclusion, this study suggests different roles of Tregs exist according to autoantibody type in patients with AE. Moreover, it proposes novel therapeutic models for AE by expanding the numbers of immunoregulatory and anti-inflammatory Tregs. Future prospective longitudinal research is necessary to confirm the role of Tregs in AE and the response to immunotherapy.

**Conflicts of Interest**

Jangsup Moon, Soon-Tae Lee, Keun-Hwa Jung, Kyung-II Park, Sang Kun Lee, Kon Chu have been editorial board of *Encephalitis* since October 2020. They were not involved in the review process of this original article. No other potential conflict of interest relevant to this article was reported.

**Author Contributions**

Conceptualization: JI Byun, J Moon, K Chu; Data curation: JI Byun; Formal analysis: JI Byun, JY Bae; Resources, Funding acquisition: K Chu; Methodology, Investigation: JY Bae; Project administration: JI Byun, K Chu; Supervision: JI Moon, ST Lee, KH Jung, KI Park, M Kim, SK Lee, K Chu; Writing - original draft: JI Byun; Writing - review & editing: J Moon, ST Lee, KH Jung, KI Park, M Kim, SK Lee.

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