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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 suggestive of encephalitis. Those with inadequate amounts of serum samples were excluded. Antibodies were assessed using an indirect immunofluorescence test performed on serum or CSF (Euroimmune Ag., Lübeck, Germany), as described previously [10]. Immunotherapy, including intravenous (IV) steroids or IV immunoglobulin (IVIg), was administered based on the treating physician's preference and acceptance of the treatment by the patient's family. Patients were enrolled regardless of the treatment they received.

Clinical characteristics, including the modified Rankin scale (mRS) values and the Clinical Assessment Scale in Autoimmune Encephalitis (CASE) score [11], 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 $CD4^+CD25^+$ cells 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 $p \leq 0.05/3$ (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 × 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 (*post-hoc* p = 0.043) and synaptic antibodies (*post-hoc* p = 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

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.

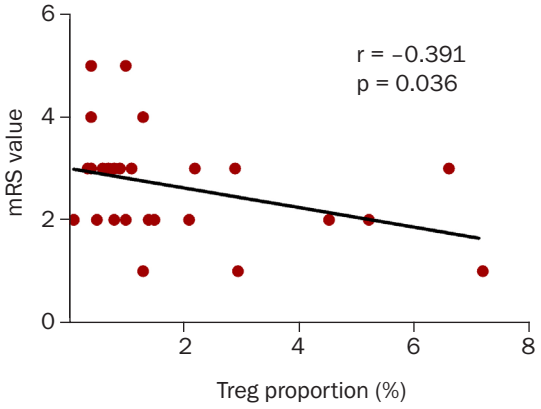
Table 2 Flow cytometry results of patients with autoimmune encephalitis according to autoantibody type

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 (× 10^5/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.

Figure 1 Correlation between the proportion of peripheral Treg (%) and the initial mRS in patients with autoimmune encephalitis



Treg, regulatory T-cell; mRS, modified Rankin scale.

Table 3 Proportions of peripheral regulatory T cells in patients who received IVIg and those who did not

Variable	No IVIg (n = 10)	Received IVIg (n = 19)	p-value
$CD4^+CD25^+$ (%)	10.4 ± 6.0	8.0 ± 4.1	0.271
$CD4^+CD25^+Foxp3^+$ (%)	3.2 ± 2.5	1.1 ± 0.8	0.029
$Foxp3/CD4^+$ (%)	39.3 ± 27.7	15.7 ± 10.9	0.031

Values are presented as mean ± standard deviation.
IVIg, intravenous immunoglobulin; Foxp3, forkhead box P3.

may vary with autoantibody type in AE. Those with paraneoplastic antibodies tended to present higher Treg proportions than those with synaptic antibodies. The proportion of Tregs was negatively correlated with initial disease severity and differed 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 different between AE patients with different antibody types, no difference 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 natural 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 patients with paraneoplastic antibodies usually have no pathogenic 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 signaling 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 proportion of Tregs has also been reported to be associated with the severity of graft-versus-host disease [17]. One recent study suggested that the proportion of $CD4^+$ T cells in peripheral blood was associated with frontal lobe function in GAD65 and voltage-gated potassium channel antibody-related encephalitis [18].

Treg proportions differed according to IVIg treatment but not rituximab treatment. IVIg therapy can increase cytokine secretions 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 investigation 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-Il 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: J 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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