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Numerical status of $CD4^+CD25^+FoxP3^+$ and $CD8^+CD28^-$ regulatory T cells in multiple sclerosis

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ABSTRACT

Objective(s): Regulatory T cells, including CD4+CD25+Fox3+ and CD8+CD28- cells play an important role in regulating the balance between immunity and tolerance. Since multiple sclerosis is an inflammatory autoimmune disease, regulatory T cells are considered to be involved in its pathogenesis. In this study, we investigated the circulatory numbers of the two mentioned types of regulatory T cells and also their association with different clinical characteristics in 84 multiple sclerosis patients.

Materials and Methods: 84 patients with multiple sclerosis and 75 normal individuals were studied. Demographic and clinical information of all participants were collected via questionnaire and clinical examination as well as MRI. The peripheral blood frequency of two different subgroups of regulatory T cells (CD4+ CD25+Foxp3+ and CD8+CD28- cells) were analyzed by flow cytometry using anti-human antibodies conjugated with CD4-FITC / CD25-PE/Foxp3-PE-Cy5, CD3-PE/CD8a-PE-Cy5/CD28-FITC.

Results: The frequency of CD4+CD25+Foxp3+ cells in multiple sclerosis patients was significantly less than that in healthy controls (P=0.006) and in mild forms less than that in sever forms (P=0.003). There was not any correlation between the frequency of regulatory T cells and different clinical variables.

Conclusion: Our results showed that the number of CD4+CD25+Foxp3+ cells decreases significantly in multiple sclerosis patients, which probably shows the regulatory role of these cells in multiple sclerosis.

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Introduction

Multiple sclerosis (MS), as the most common inflammatory demyelinating disease of the central nervous system (CNS) and also the most common cause of neurological disability at young age, is an inflammatory demyelinating disease characterized by lymphocyte infiltration and inflammation of the CNS white matter. Such demyelinating process typically shows 4 clinical courses including: relapsing remitting (RRMS) which is the most common type (80%), characterized by unpredictable relapses followed by periods of months and years of relative quiet with no sings, and usually beginning with a clinical isolated syndrome (CIS) not fulfilling the criteria of MS; secondary progressive (SPMS) occurring in around 65% of those with initial RRMS, who eventually have progressive neurologic decline between acute attacks; primary progressive (PPMS) occurring in 10-20% of all, with no remission after the initial symptoms; and progressive relapsing (PRMS) describing a steady neurologic decline with superimposed attacks (1). The most widely used scale evaluating the severity of MS is the EDSS (expanded disability status scale) defined by a set of rules (2) and providing a numerical quantification of the neurological examination. The etiology of MS is unclear; however, it is thought that both genetic and environmental factors play a role in its pathogenesis (3). MS may result from the failure of tolerance mechanisms including CD4+CD25+ T regulatory cells (Treg) (4), which prevent expansion of pathogenic T cells directed against myelin determinants, or other self-tissue antigens. A lot of studies have reported

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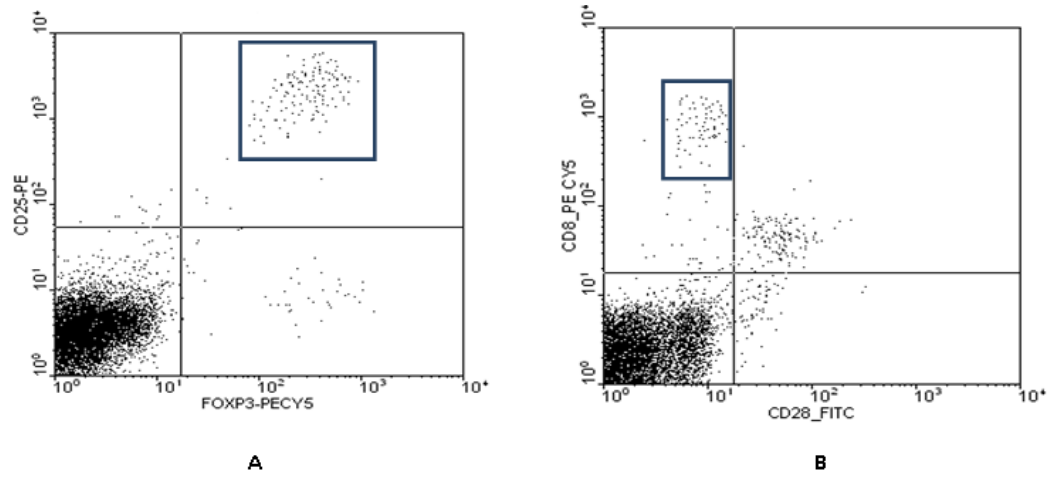

Figure 1. Flow cytometry results in a sample patient. A) Having gated on CD4+ cells, we counted CD4+CD25+Foxp3+ cells B) Having gated on CD3+ cells, we counted CD8+CD28- cells

numeric or functional deficiencies of Tregs in various human autoimmune diseases including inflammatory demyelinating disorders of the CNS (5). Although there is not yet a specific surface marker for Treg subsets, naturally occurring CD4+CD25+Treg (nTreg) cells can be characterized on the basis of their high expression of CD25 (in contrast to the intermediate expression in recently activated T cells) and the intracellular expression of the fork head transcription factor 3 (Foxp3) (6). Another Treg population, CD8+CD28- Treg (7) which lacks co-stimulatory molecule of CD28, is associated with some suppressor abilities and its failure has been recognized in numerous animal (8–10) as well as human autoimmunities (11–12).

The role of CD4+CD25+Foxp3+ and CD8+CD28- Treg cells (7, 13, 14) has been studied in MS. The present study has evaluated both of these Treg populations in all types of MS and has compared the results with healthy controls.

Materials and Methods

Study subjects

Peripheral blood samples were obtained from a total of 84 MS patients in remission phase, who were diagnosed according to clinical examination and MRI, and 75 sex and age-matched healthy controls. None of the patients and controls suffered from any other autoimmune or inflammatory states. The EDSS (Expanded Disability Status Scale) (15), the type of MS (CIS, RRMS, PPMS, SPMS, PRMS), duration as well as treatment of the illness, and the number of recurrences were determined for each patient before sampling. Blood samples of all subjects were taken after signing the informed consent form approved by the local ethical committee. The study protocol conformed to the ethical guidelines of the 1975 Helsinki Declaration.

Flow cytometric analysis

At least one million fresh peripheral blood mononuclear cells (PBMCs) were separated from 2 ml of anticoagulated blood by Ficoll-Hypaque (Lymphodex, Inno-Train, Germany) density gradient centrifugation. CD4+CD25+Foxp3+ Tregs were detected in one tube by staining with a cocktail of anti-human surface CD4-FITC / CD25-PE, and intracellular Foxp3-PE-Cy5 according to the manufacturer's instructions (eBioscience, USA). In this procedure, CD4+-FITC/CD25+-PE-stained cells were fixed, permeabilized and washed twice. Then the cells were stained by 10 µl anti-human FoxP3 antibody and were incubated at 4°C for 30 min. Inducible CD8+CD28- Tregs were detected in another tube by staining with anti-human CD3-PE, CD8a-PE-Cy5, and CD28-FITC. All lymphocytes in each sample were gated on the basis of light scattering properties and then at least 20,000 events were obtained to count each subtype of Tregs. Having gated CD4+CD25+cells, we counted CD4+CD25+Foxp3+cells on the basis of simultaneous expression of CD25 and Foxp3. To count CD8+CD28- Tregs, gated CD3-expressing

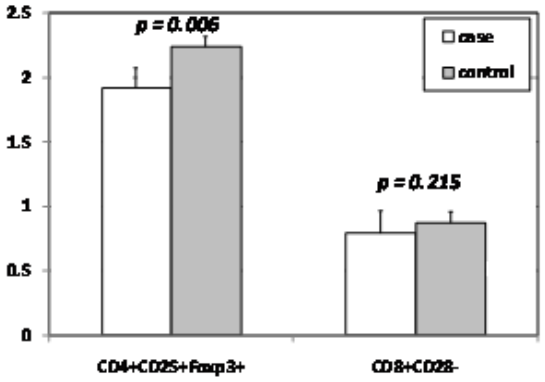

Figure 2. Numbers of CD4+CD25+Foxp3+ cells and CD8+CD28-cells in peripheral blood samples of 84 MS patients and 75 healthy controls

Table 1. Basic and clinical characteristic of MS patients and controls

		MS patients	Healthy controls	*P*- value
Number of subjects		84	75	-
Male/female		15/69	15/60	0.73
Age (years) mean±SD		34.2±8.8	31.4±10	0.4
Family history (%)	Positive	77 (91.7%)	-	-
	Negative	7 (7.3%)		
Disease duration (years) mean±SD		5.8±4.4	-	-
Treatment duration (years) mean±SD		3.1±3.31	-	-
Number of recurrences mean±SD		3.7±4	-	-
Number of patients in different types of MS (%)	CIS	5(6.1)	-	-
	RRMS	69(82.1)		
	PPMS	1(1.2)		
	SPMS	7(8.5)		
	PRMS	2(2.4)		
Number of patients using different kinds of drugs (%)	Cinovex	63(76.8)	-	-
	Rebief	10(12.2)		
	Betaferon	2(2.4)		
	Others	2(2.4)		
	No drug	5(6.1)		
EDSS (mean±SD)	CIS	0.5±1.1	-	-
	RRMS	2±1.44		
	PPMS	6		
	SPMS	5±1.42		
	PRMS	6.25±0.35		
Tregs±SD	CD4+CD25+Foxp3+	1.91±0.71	2.24±0.74	0.006
	CD8+CD28-	0.79±0.37	0.87±0.38	0.215

CIS: clinical isolated syndrome, RRMS: relapsing remitting multiple sclerosis, PPMS: primary progressive multiple sclerosis, SPMS: secondary progressive multiple sclerosis, PRMS: progressive relapsing multiple sclerosis, EDSS: expanded disability status scale, Tregs: regulatory T cells

lymphocytes were counted on the basis of expression of CD8 and no expression of CD28 (Figure 1). All antibodies and their isotype-matched controls were purchased from eBioscience, USA. The percentages of CD4+CD25+Foxp3+ and CD8+CD28- cells were analyzed by a 3-color flow cytometry using BD FACSCalibur Flow Cytometer and CELLQuest software version 3 (BD, USA).

Statistical analysis

Data was expressed as mean±SD. The statistical indices of Tregs were analyzed using independent *t* and chi-square tests. Correlations between variables were calculated by Pearson's correlation coefficient, and simultaneous effects of various factors on Tregs were analyzed by multiple linear regressions with backward method. Adjusted R Squared was determined as a criterion of goodness-of-fit test, and $P<0.2$ was considered for exclusion from the model. *P*-values < 0.05 were considered statistically significant. All analyses were performed using the SPSS 16 software.

Results

Patient characteristics and Treg frequencies

Basic and clinical characteristics of MS patients and controls enrolled in our study are shown in Table 1. The most affected ages were between 18 and 30. The least EDSS score was related to the most benign type of MS named CIS and then it raised in more sever types of MS. There were not any significant differences in the frequency of Tregs in terms of age and gender (Table 1). The frequency of CD4+CD25+Foxp3+Tregs in MS patients was significantly lower than that in healthy controls ($P=0.006$) (Table 1). The frequency of CD4+CD25+Foxp3+ Tregs was different in MS patients according to type of MS in such a way that the frequency of CD4+CD25+Foxp3+ Tregs in severe forms of MS (PPMS, SPMS, PRMS) was significantly higher than that in the mild forms (CIS, RRMS) ($P=0.03$) (Table 2).

Table 2. Values of Tregs according to different variates in MS patients

		Tregs			
		CD4+CD25+Foxp3+	*P*- value	CD8+CD28-	*P*- value
EDSS	Mild (0.5-4.5)	1.83±0.7	0.08	0.75±0.4	0.48
	Sever (5-9.5)	2.23±0.7		0.83±0.23	
Type	Mild (CIS, RRMS)	1.86±0.72		0.8±0.4	
	Sever (PPMS, SPMS, PRMS)	2.25±0.57	0.03	0.7±0.26	0.72
sex	Male	2.24±0.8	0.054	0.71±0.34	0.36
	Female	1.84±0.68		0.81±0.38	
Family history	Positive	1.85±0.78	0.764	0.84±0.2	0.323
	Negative	1.92±0.72		0.79±0.39	

CIS: clinical isolated syndrome, RRMS: relapsing remitting multiple sclerosis, PPMS: primary progressive multiple sclerosis, SPMS: secondary progressive multiple sclerosis, PRMS: progressive relapsing multiple sclerosis, EDSS: expanded disability status scale

Table 3. Correlation between the number of Treg subtypes with age and different clinical variates in MS patients

	Tregs			
	CD4+CD25+Foxp3+		CD8+CD28-	
	Correlation coefficient	*P*- value	Correlation coefficient	*P*- value
Age	-0.043	0.7	-0.07	0.5
EDSS	0.15	0.2	0.09	0.45
Number of recurrence	-0.03	0.8	0.16	0.13
Disease duration	0.008	0.94	0.2	0.08
Treatment duration	0.17	0.12	0.14	0.2

Correlation of Treg frequencies with different parameters

There was not any significant correlation between the frequency of Tregs with age and various clinical variables including EDSS scores, number of recurrences, duration of the disease, and duration of the treatment (Table 3). Also, no correlation was found between the frequencies of Tregs with age in healthy group (data not shown). Using linear multiple regression analysis, we found a significant goodness-of-fit for both subsets of Treg frequencies (CD4+CD25+FoxP3+Treg: Adjusted R^2=0.845, P<0.001; CD8+CD28-Treg: Adjusted R^2=0.759, P<0.001). CD4+CD25+FoxP3+ Treg frequencies remained not significant in the presence of factors of positive family history (P=0.519), number of recurrences (P=0.656), and EDSS score (P=0.939). Although the factors of age, sex, disease duration, and treatment duration stayed in the model, the frequency of such cells turned significant in the presence of age (P<0.001) and treatment duration (P=0.016). The model also revealed that CD8+CD28-Treg frequencies remain insignificant in the presence of factors of positive family history (0.315), EDSS score (0.908), disease duration (0.466), and treatment duration (0.409). Although the factors of age, sex, and number of recurrences stayed in the model, the frequency of such cells turned significant only in the presence of age (P<0.001) (Table 4).

Discussion

We evaluated the frequency of two subtypes of Tregs and showed that the frequency of CD4+CD25+Foxp3+ cells is significantly lower in MS patients than in healthy controls; it is also significantly lower in mild forms of MS than in sever forms.

Although inconsistent with some other studies (16–19) that showed no significant differences in the circulatory number of CD4+CD25+ cells in patients with MS compared to that in healthy controls, our study showed lower circulatory number of CD4+CD25+Foxp3+ cells in MS patients, which may be due to their disturbed thymic development (20) and/or their recruitment into the inflamed organ i.e. CNS. It may be considered that the reduction in the percentage of CD4+CD25+Foxp3+ cells could be one probable reason (or at least a predisposing factor) of MS. Restoring the frequency as well as function of regulatory cells in MS patients after taking INF-β, strengthens the likelihood of this idea (21). Evaluating the percentage of Tregs in different types of MS, we found that the number of CD4+CD25+Foxp3+ cells in mild forms of MS (CIS and RRMS) was significantly lower than in severe forms (SPMS, PPMS, and PRMS), yet we found a study inconsistent with ours (16). It is likely that in more sever types of MS, more de novo or induced production of CD4+CD25+Foxp3+Tregs, as a manifestation of their immunomodulatory function, constitutes a circulatory reservoir that replaces the CNS-redistributed ones. Such observation has also been made in rejection episodes of organ transplantation, in which more circulatory number of Tregs, as a demonstration of their generation, tries to compensate both the residing ones in lymphoid tissues (to block initiation of aggressive immune responses) and migrating ones to graft sites (to inhibit the aggressive cells that have escaped from the regulation) (22). However, despite several studies which have demonstrated that rather functional defects of Tregs are present in MS patients (7, 16–19, 23), we showed that numbers of such cells may also be important in determining the severity of the disease. In other words, according to their immunomodulatory roles, naturally increasing numbers of Tregs in sever forms, in line with therapeutic procedures which increase the number of Tregs such as INF-β (21) and inhibitors of Histone Deacetylase (22), may delay the progression of the process to more severe types.

Table 4. Linear multiple regression analysis evaluating the effect of different factors on the frequency of Tregs in MS patients

	Variables	Coefficients		t	Sig. P	Adjusted R Squared
		B	Std. error			
CD4+CD25+Foxp3+Tregs	Age	0.0489	0.005	9.1	<0.001	0.845
	Sex	0.458	0.252	1.8	0.07	
	Disease duration	-0.041	0.025	-1.63	0.10	
	Treatment duration	0.072	0.029	2.47	0.016	
CD8+CD28-Tregs	Age	0.018	0.002	8.64	<0.001	0.759
	Sex	-0.178	0.134	-1.32	0.18	
	Number of recurrences	0.021	0.012	1.79	0.07	

Although not statistically significant, the circulatory frequency of CD8+CD28-Tregs was lower in our MS patients than in healthy controls. However, some other studies found a significant decrease (7, 24–26). According to our knowledge, the exact pathophysiologic basis of such reduction is not clear, but it may simply be a demonstration of tolerance failure in MS patients.

We did not find any correlation between the frequencies of Tregs with different parameters. However, adjusting the effects of different factors, we showed that age affects the frequency of both subsets of Tregs and treatment duration affects the frequency of CD4+CD25+Foxp3+Tregs. The effect of age also has been shown in other studies (17, 20).

The limitation of our study was that we did not monitor the changes of Tregs longitudinally. This limitation allowed just a cross-sectional analysis of Treg profiles of only limited robustness. Secondly, functional assays which provide further information on the immunoregulatory status of the patients were not performed. It should also be mentioned that we studied a greater number of MS patients in comparison with others working on numerical status of Tregs in MS patients.

Conclusion

Our study shows that although the number of CD4+CD25+Foxp3+ cells is reduced in MS patients, there is no statistical correlation between the number of such cells and the type/severity of MS according to EDSS scale. According to our results it seems that Treg cells may play an important role in the pathogenesis of MS.

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The authors have no conflicts of interest to declare.

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