The Cd4(+) Cd25(+) Regulatory T Cell Profile and Foxp3 Expression and Clinic Associations of in Various Stage and Types of Multiple Sclerosis

Multipl Skleroz'un Değişik Klinik Tiplerinde ve Farkli Evrelerinde Cd4(+) Cd25(+) Regülatuar T Hücre Profili ve Foxp3 Ekspresyonu

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Abstract: In the pathogenesis of MS, impairment of peripheric immune tolerance plays an important critical role in the emergence of autoimmunity. The regulatory T cells play a crucial role in the healthy function of immune tolerance. FoxP3 is a transcription factor which assignment for enough regulatory T cell expression. We compared the total percentage CD4(+) and FoxP3 expression in T lymphocytes in 31 cases including 12 RRMS, 11 SRMS and eight attack which has a definite MS regarding Mc Donald's criteria 12 healthy subjects for regulatory T cell subtypes with analysis flow cytometry in haematology laboratory. Also, we compared 7 cases in a group of 8 RRMS with an attack for before and after 1000 mg/day IVMP. The results have assesment as statistical. The regulatory cell profiles CD4(+) CD25(+), CD4(+) foxP3(+), CD4(+) CD25(+) foxP3(+),CD4(+) CD25(+) foxP3(-), and CD4(+) CD25(-) foxP3(+)of all patients were compared with the healthy control group.No significant differences existed between the groups ($p>0.005$). The results of regulatory cell profiles and foxP3 expression of 7 patients with attacks before treatment and after IVMP treatment were not significant ($p>0.05$). However, high-dose intravenous methylprednisolone therapy (IVMP) treatment was observed to cause a slight numerical increase in regulatory cell subtypes. As a consequence, we thought that the regulatory T cells play an important role in the immunopathogenesis of MS as well as its numerical sufficiency.
Key Words: MS, immune tolerance, foxP3, regulatory cell.

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Özet: Multipl Skleroz patogenezinde otoimmunitenin ortaya çıkışında periferik immun toleransın bozulması önemli rol oynamaktadır. İmmun toleransın sağlıklı işleyişinde regülatuvar T hücreleri ise anahtar rol oynamaktadır. FoxP3 ise regülatuvar T hücreler yeterli ekpresyonu için transkripsiyon faktörü olarak görev almaktadır. Biz bu çalışmayla Mc Donald's kriterlerine göre klinik olarak kesin MS tanısı almış 12 RRMS (relapsing Remitting Multipl skleroz), 11 SRMS (Sekonder Relapsing Multipl skleroz), 8 atak RRMS hastası olmak üzere toplam 31 hasta ile 12 sağlıklı kontrol gurubunun regülatuvar T hücre alt tipleri hematoloji laboratuarında akım sitometrisi ile analiz ederek total CD4(+) T lenfosit içerisindeki yüzdeleri ve FoxP3 ekspresyonunu karşılaştırdık. Ataklı 8 RRMS hastasının ise 7 tanesi 1000 mg/ gün İVMP tedavisinden önce ve tedaviden sonra kendi içinde karşılaştırıldı. Sonuçlar istatiksel olarak değerlendirildi. Regülatuvar T hücre profili CD4(+) CD25(+), CD4(+) foxP3(+), CD4(+) CD25(+) foxP3(+),CD4(+) CD25(+) foxP3(-), ve CD4(+) CD25(-) foxP3(+) karşılaştırıldı, hastalar ve kontrol grubu arasında anlamlı fark bulunmadı ($p>0.005$). Atak öncesi ve atak sonrası IVMP tedavisi sonrası 7 hastanın regülatör hücre profilleri ve foxP3 ekspresyonu arasında anlamlı fark saptanmadı ($p>0.05$). Ancak, yüksek doz intravenöz metilprednizolon (IVMP) tedavisinin regülatör hücre alt tipleri içinde hafif bir sayısal artış neden olduğu gözlenmiştir. Regülatuvar T hücrelerin MS imunopatogenezinde sayısal yeterliliği yanında işlevsel özelliklerinin önemli rol oynadığı düşünüldü.
Anahtar Kelimeler: MS,immun toleransı, foxP3, regülatuvar hücre

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1. Introduction

Multiple sclerosis (MS) is an inflammatory, demyelinating disease that appears to result from genetic and environmental factors and is an autoimmune character (1). The process which causes a series of autoimmune events leading to pathology in the central nervous system (CNS) and the presence of the clinical picture, as a result, is thought to commence in the peripheral immune system. Findings of numerous studies on the pathogenesis of autoimmune diseases in recent years have demonstrated the first stage as a break in peripheral immune self-tolerance at the onset of the disorder (2).

The immune anomaly in MS has been established by the expression of chemokines that leads to activation of T cells and their products against myelin in cerebrospinal fluid (CSF).

Among the lymphocytes observed in the CNS in MS patients are CD4(+) cells, CD8(+) cells, and B lymphocytes. The pro-inflammatory cytokines, such as interleukin (IL)-1, IL-2, and tumor necrosis factor (TNF)-α, cause a deterioration in the blood-brain barrier by activating immune cells, increasing the expression of adhesion molecules, and affecting the chemokines and vascular adhesions in leucocytes, and thus facilitating passage of activated immune cells into the CNS and inflammation. Monocytes and such mediating agents released from macrophages, complement, and B lymphocyte cell-based antibodies collaborate, and the resulting inflammation causes axonal damage and demyelination (3,4).

It is known that formation of all the immunopathologic events is initiated by T cells that become autoreactive through a break in peripheral self-tolerance. Regulatory cells play an important role in preventing the activation, and thus controlling this first stage of autoimmune events. Many of the subtypes of these cells, of which the major type is formed by CD4(+), have been discovered5. Regulatory CD4(+) cells are of the following two main subtypes: CD4(+) CD25(+)regulatory T cells (Tr) and type 1 regulatory T cells (Tr1). Although both subtypes are essential to perpetuating peripheral tolerance, CD4(+) CD25(+) Tr also act in contact-dependent interactions and can prevent cytokine release via an increase in CD4(+) cells (5).

CD4(+) CD25(+) cells prevent autoimmunity via forkhead box protein-3 (foxP3) transcription factor. Some studies have reported a decrease in foxP3 expression in patients that follows an early course with attacks (6). Our study aimed at demonstrating the relationship in MS patients between clinical condition, the number of immune cells, and transcription factor foxP3, with the ultimate goal of identifying potential treatment methods for these patients.

2. Materıal And Methods

The study commenced following approval by the local research ethics committee of Eskisehir Osmangazi University Medical School. Of 31 MS patients who were being followed in the Neurology Department of Eskisehir Osmangazi University Medical School or referred with complaints of attacks, and 12 healthy individuals who served as a control group, were included in the study. Of the MS patients, 12 were grouped as relapsing-remitting MS (RRMS) without attacks, 11 as secondary progresif MS (SPMS) without attacks, and eight were grouped as RRMS patients with attacks (RRMS-A). All the patients were selected among the patients diagnosed with MS based on Mc Donald's criteria (7). All the patient groups were compared with the healthy control group. One patient from the attack group was excluded from the study due to early discharge.

The regulatory T cell profiles and foxP3 expression of the remaining 7 patients were compared before the daily 1000 mg/day intravenous methylprednisolone (IVMP) treatment, at the first day of treatment and at the end of treatment.

Inclusion criteria

Patients who were diagnosed with clinically definite MS or laboratory-supported definite MS based on Mc Donald's criteria7. Patients with an Expanded Disability Status Scale (EDSS) score between 0 and 6.5. Symptoms occured before the 1-month period following the IVMP therapy was not accepted as a new attack.

Exclusion criteria:

Patients who were not diagnosed with clinically definite MS or laboratory-supported definite MS based on7. Patients with an inflammatory focus. Patients who were pregnant or were nursing. Patients with a hematologic malignant disease. Patients with a severe liver disease. Patients with medical conditions (uncontrolled blood glucose, advanced osteoporosis, and medication allergies) that might prove to be a contraindication for corticosteroid treatment.

Study protocol:

Two ml of venous blood sample was obtained in a haematology tube from patients without attacks and from patients before and after IVMP treatment. Around the same days, the EDSS scores of patients were estimated according to clinical examination and findings. The collected blood samples were analysed by flow cytometry. Anti-human CD4 PerCP monoclonal antibody marker, CD25 FITC marker, anti-human foxP3 PE marker, and a foxP3 Staining Buffer set from eBioscience were used.

Statistical analysis:

SPSS for Windows 15.0 and Sigmastat 3.1 were utilised for analyses. The data shown are the mean ± SD. The normality hypotheses of data were tested by the Shapira-Wilk test. For the data demonstrating a normal distribution, a paired sample t-test and one-way ANOVA were used. For data not showing a normal distribution, the Wilcoxon t-test and Kruskal-Wallis were used. P value of < 0.05 was considered significant.

3. Results

The age range of the 12 healthy control group members (8 females and four males) was 22-41 years(mean, 34.26±6.85 years). The age range of the 12 RRMS patients (all women) was 21-42 years (mean, 31.75±2.22 years); the EDSS scores ranged between 0 and 2 (mean, 0.59±0.17) and the disease period ranged between 6 and nine years (mean, 2.45±0.73 years). The age range of the 11 SPMS patients (4 males and seven females) was 23-53 years (mean, 37.6±2.7), the EDSS scores ranged between 2 and 6.5 (mean, 3.5±0.35), and the disease period ranged between 2 and 14 years (mean, 6.8±1.13 years).

The age range of the 8 RRMS-A patients (one male and seven females) was 23-46 years (mean, 36.2±2.8), the EDSS scores pre-treatment ranged between 2 and 3.5 (mean, 2.9±0.22), the EDSS scores post-treatment ranged between 0.5 and 2 (mean, (1.14±0.8), and the disease period ranged between 6 months and 10 years (mean, 5.2±1.3) (Table 1).

The regulatory cell profiles CD4(+) CD25(+), CD4(+) foxP3(+), CD4(+) CD25(+) foxP3(+), CD4(+) CD25(+) foxP3(-), and CD4(+) CD25(-) foxP3(+) of all patients were compared with the healthy control group. No significant differences existed between the groups ($p>0.05$) (Table 2).

The results of regulatory cell profiles and foxP3 expression of 7 patients with attacks before treatment and after IVMP treatment were not significant ($p>0.05$). However, IVMP treatment was observed to cause a slight numerical increase in regulatory cell subtypes (Table 3).

Table 1.
Demographic and clinical data

	Patient group	***Control group***
Age *(mean ± SD;years)*	*35.18 ± 2.4*	*34.26 ± 6.85*
RRMS	*31.75 ± 2.2*	
SPMS	*37.60 ± 2.7*	
RRMS	*36.20 ± 2.8*	
Sex		
Female	*26*	*8*
Male	*5*	*4*
Disease duration *(mean ± SD; years)*		
RRMS	*2 . 4 5 ± 0.73*	
SPMS	*6 , 8 0 ± 1.13*	
RRMS-A	*5 . 2 0 ± 1.3*	
Subgroup members (n)		
RRMS	*12*	*12*
SPMS	*11*	
RRMS-A	*8*	
EDSS level *(mean ± SD; years)*		
RRMS	*0.59 ± 0.17*	
SPMS	*3.50± 0.35*	
RRMS-A(pre-attack)	*2 . 9 0 ± 0 . 2 2*	
RRMS-A(post-attack)	*1 . 1 4 ± 0 . 8*	

Table 2.

CD4(+) CD25(+), CD4(+) foxP3(+), CD4(+) CD25(+) foxP3(+), CD4(+) CD25(+) foxP3(-), and CD4(+) CD25(-) foxP3(+) profiles in individual subgroups in MS patients

Regulatory cell profiles	***Control***	***RRMS***	***SPMS***	***RRMS-A***	***P value ****
CD4(+)CD25(+)	*6.61 ± 1.7*	*7.65 ± 2.4*	*7.13 ± 3.5*	*5.50 ± 2.5*	*0.344**
CD4(+)FoxP3(+)	*3.67 ± 1.6*	*4.51 ± 1.5*	*4.89 ± 2.3*	*3.08 ± 2.7*	*0.107**
CD4(+)CD25(+) FoxP3(+)	*1.82 ± 0.9*	*1.86 ± 0.9*	*1.74 ± 0.8*	*1.27 ± 1.3*	*0.590**
CD4(+)CD25(+) FoxP3(-)	*5.85 ± 1.9*	*5.59 ± 1.9*	*6.25 ± 3.8*	*4.90 ± 2.5*	*0.739**
CD4(+)CD25(-) FoxP3(+)	*1.79 ± 0.8*	*1.95 ± 0.6*	*2.69 ± 1.2*	*1.64 ± 1.4*	*0.117**

**P>0.05*

Table 3.
The distribution of pre- and post-treatment Tr into groups in patients with attacks

	Pre-treatment	***Post-treatment***	***P value***
CD4(+)CD25(+)	*5.38 ± 1.04*	*8.27 ± 1.24*	*0.670*
CD4(+)FoxP3(+)	*2.78 ± 1.05*	*3.98 ± 1.02*	*0.725*
CD4(+)CD25(+) FoxP3(+)	*1.21 ± 0.53*	*1.51 ± 0.47*	*0.550*
CD4(+)CD25(+)FoxP3(-)	*5.17 ± 0.98*	*7.15 ± 0.83*	*0.236*
CD4(+)CD25(-)FoxP3(+)	*1.63 ± 0.59*	*2.23 ± 0.79*	*0.322*

**P>0.05*

4. Discussion and Conclusions

CD4+ CD25+ regulatory T cells are a subpopulation of suppressor T cells that play an important role in downmodulating the activation and effector function of potential auto-aggressive T cells. The transcriptional repressor FOXP3 plays a key role in the development and function of naturally occurring CD4+ CD25+ Tregs. Lossof-function mutations in the FOXP3 gene lead to the development of a severe lymphoproliferative disease and autoimmune manifestations in mice (scurfy model) and in patients with the immuno-dysregulatory, polyendocrinopathy, enteritis X-linked (IPEX) syndrome (8).

Although in studies carried out thus far a statistical difference in Tr cells in the peripheral blood and CSF of MS patients has not been shown to exist, there is significant evidence suggesting a defect in the suppression of autoreactive T lymphocytes and thus in the prevention of autoimmunity (8).

A study by Viglietta et al. (9) established that in a comparison of regulatory CD4(+) CD25(+) T cell populations between 15 MS patients with ages ranging from 15-57 years and 21 healthy control group members with no autoimmune disease, a numerically significant difference was not observed in cells; the suppressible characteristics of Tr cells of these patients had a low capacity relative to the healthy control group when the T cells were compared both autologous and effector T cells. Viglietta et al.9 drew the conclusion that regulatory cells with a possible defective function play a role in the pathogenesis of MS. The researchers found there was a difference in the suppressive ability of Th1 cytokine production in Tr cells of these patients. Within this scope, while Tr cells are able to suppress INF-γ production in the healthy control group, such a characteristic was not observed in the Tr cells in MS patients. As the other defective functional regulatory cell indication in MS patients, the expression of IL-2 was significantly lower when compared with the control group.

Also, the fact that these cells are deficient in recognising specific antigens and in a genetic modulation of their activation was demonstrated as the basis for the functional deficiency in Tr cells. As a result, the loss of inhibition capabilities of these cells causes the appearance of an immune regulation disorder induced by these cells (10). In recent years, Seddon et al. (11) observed in an experimental study that autoimmunity of lab rats with dysfunctional Tr cells were deteriorated in 4 weeks by injecting functional CD4(+) CD25(+) regulatory cells. Another accentuated issue was the induction of functional regulatory cells through bone marrow transplant, and thus prevention of MS attacks. A study on this issue was conducted by Herman et al. (12) based on an EAE model, and it was found that the bone marrow transplant performed on autoimmune rats decreased relapses observed in the clinical picture of the disease. With analysis of peripheral blood samples from rats, they demonstrated that functional CD4(+) CD25(+)foxP3(+) expression was increased. CD4(+) CD25(+) Tr cells show functional heterogeneity.

The reason for functional heterogeneity is related to the function of expressing such different molecules as HLA-DR and intracellular CTLA4. These molecules are not expressed at the same level in all regulatory cells, and while expression increases in some cells, they would never be expressed in others. While this result causes the appearance of the disease in patients with autoimmune deficiency, even though regulatory cells exist in same amounts in all the patients and healthy individuals, it may offer an explanation for the lack of autoimmunity. The insufficient expression of TGF-γ and IL-10 in Tr cells would suffice and negatively affect the suppression capabilities, and thus regulatory functions of the cells (13,14,15).

Another opinion expressed on the role of these cells in MS pathogenesis is that these cells decrease in number at the onset of the disease and their function deteriorates (16,17). In their study that supports this opinion,

Jensen et al. (18) established the percentages of autoreactive T cell count of 44 patients with a clinically isolated syndrome assessed to have a MS onset attack increased relative to MR imaging and clinical findings, however, the CD4(+) CD25(+) Tr cell count was low in both the blood and in the BOS. Interestingly, in the study they found the Tr cell percentage in BOS demonstrated a negative correlation with oligoclonal bands, myelin basic protein (MBP) concentrations, and plaques and lesions showing demyelinating lesions.

In our study, unlike this study, no significant difference was found between CD4 + CD25 + regulator T-cell function and FOXP3 expression between SPMS and RRMS. The difference in the study effort may be related to the low number of patients between groups.

A high dose of IVMP treatment was proven to ensure a clinically significant improvement in RRMS attack patients, and it is being utilised in numerous clinics today for treating attacks. It was established the treatment enabled beneficial effects of corticosteroids, inhibition of the release of autoreactive T cells, antigen presenting cells (APC), and inflammatory cytokines, and thus improved blood-brain barrier permeability and decreased the number of MR lesions showing contrast involvement (19,20). In a treatment by Wang et al. (21) applied on 16 RRMS attack patients by performing 1g/day IVMP for 3-5 days following 60 mg orally (discontinued in 2 weeks), a significant decrease (p=0.028) was established in EDSS scores in the entire group compared with pre-treatment.

In our study, a statistically significant decrease was found in EDSS scores of patients. In regulatory cell percentages of our patients, we established an increase in regulatory cell subtypes with no statistical significance.

A similar study to the current study was conducted by Navarro et al. (22) and 1g/day IVMP was administered to 20 relapse MS patients; they were compared to a group of 18 healthy individuals. The results they obtained included an increase in CD8(+) CD25(+), CD4(+) CD25(+) and regulatory cell profile in contrast to a significant decrease in autoreactive CD8(+) T lymphocytes in the treatment group.

In our study, the ratio of male to female ratio was 26/5 in the study group, 8/4 in the control group, and there was a difference between the gender distribution in the patient and control group and the small number of the control group. In addition, the lack of subgroup analysis of patients with RRMS and SPMS according to the immunomodulatory and immunosuppressive agents they receive and the absence of treatment treatments is another limitation of our study.In light of all these studies, the conclusion we have drawn is a deterioration of quality and/or functionality of these cells for some reason, even though they were normal or close to normal numerically, and that this plays a key role in the pathogenesis of MS. Even though a percentage difference did not exist between the healthy control group, the patient group, and the attack group, normal foxP3 expression existed. In a few studies, the result concerning the decrease in the regulatory cell count of MS patients is probably true for patients at the onset stage when immune regulation began to deteriorate. However, MS patients in our study were composed of follow-up patients that passed the onset stage. This brings to mind these cells were rendered insufficient in terms of function even though the Tr cell profile did not pose much of a difference numerically compared to the healthy control group.

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