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Increased frequency of $CD4^+CD25^{high}$ $CD127^{low/-}$ regulatory T cells in patients with multiple sclerosis

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ABSTRACT

Background: Multiple sclerosis (MS), one of the most common diseases of the central nervous system (CNS), is characterized by demyelination and chronic inflammation of the CNS. Failure of immune tolerance and induced autoimmune processes are involved in MS immunopathogenesis. Regulatory T (Treg) cells play an important role in maintaining peripheral tolerance and immune homeostasis.

Objective: The aim of this study was to evaluate the frequency of $CD4^+CD25^{high}CD127^{low/-}$ Treg cells in MS patients.

Methods: The study population was composed of 25 healthy controls (HCs), 35 patients with relapsing remitting multiple sclerosis (RRMS) and 25 patients with progressive multiple sclerosis (PMS). Frequency of $CD4^+CD25^{high}CD127^{low/-}$ Treg cells in RRMS and PMS patients was compared with HC by flow cytometry.

Results: Treg cells frequency in PMS patients was significantly higher compared to RRMS patients ($P < 0.001$) and HCs ($P < 0.001$). It was lower in RRMS patients than HCs ($P = 0.005$). A Significant direct correlation between Treg cells frequency and expanded disability status scale (EDSS) in PMS patients ($P = 0.001$, $r = 0.6$) was observed. Reverse correlation between Treg cells frequency and EDSS in RRMS patients was found ($P = 0.01$, $r = -0.4$).

Conclusion: More detailed clarification of the role of Treg cells in MS patients could provide a basis for development of Treg cells-mediated therapeutic strategies.

1. Introduction

Multiple sclerosis (MS) is one of the most common autoimmune debilitating neurologic diseases that is characterized by demyelination and chronic inflammation of the CNS (Trapp and Nave, 2008). There are two main categories of MS: relapsing remitting multiple sclerosis (RRMS) and progressive multiple sclerosis (PMS). RRMS, the most common type, is typified by irregular relapses followed by month-and-year-periods of relative quiet without symptoms. Clinically isolated syndrome (CIS), which does not fulfill the MS criteria, is frequently its starting point. PMS includes three types: secondary progressive multiple sclerosis (SPMS), primary progressive multiple sclerosis (PPMS) and progressive relapsing multiple sclerosis (PRMS) (Hawkes and Giovannoni, 2010; Confavreux and Vukusic, 2006). The etiology of MS is not clearly but genetic, environmental, immunological factors and some viruses, e.g. EBV, CMV, HBV, HSV, human herpetic viruses 6 and 7, measles viruses, coronaviruses, may have a role in the occurrence of MS (Sospedra and Martin, 2005). Myelin sheath surrounding nerve fibers will be damaged in MS (Jiang and Chess, 2006; Dendrou et al., 2015). The major immune cells involved in the immunopathogenesis of MS are T cells, especially $TCD8^+$, Th1 and Th17. Amounts of Th1 and Th17 cells and their associated cytokines, e.g. IL-1, IL-6, IL-17, Interferon gamma (IFN-γ), and tumor necrosis factor (TNF-α), have been shown to be increased in MS patients (Raphael et al., 2015; Li et al., 2014). In recent years, it became clear that Treg cells also participate in immunopathogenesis of MS (Buc, 2013). Treg cells are essential for

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maintaining peripheral tolerance against self antigens through a variety of soluble mediators including IL-10, IL-35, TGF-β, and cell surface molecules such as CD25, and CTLA-4 (Tai et al., 2012; Collison et al., 2010). There are multiple Treg subsets and a variety of suppressive mechanisms . Treg cells decrease activity and expansion of conventional T cells by suppressing their biological activities such as inhibition of proliferation and blocking the production of proinflammatory cytokines (Liu, 2006; Lan et al., 2005). Quantitative or functional defect of Treg cells is associated with many autoimmune diseases, including MS, rheumatoid arteritis (RA), and Type 1 diabetes (T1D) (Sakaguchi et al., 2006; Long and Buckner, 2011). The role of Treg cells in MS is controversial. While several studies reported a decrease in Treg cells frequency in MS patients (Huan et al., 2005; Haas et al., 2007; Venken et al., 2008a; Jamshidian et al., 2013; Kouchaki et al., 2014), a number of studies reported the same frequency compared to healthy subjects (Venken et al., 2006; Haas et al., 2005; Feger et al., 2007; Viglietta et al., 2004; Frisullo et al., 2009). Moreover, other studies indicated a functional defect in Treg cells (Venken et al., 2006; Haas et al., 2005; Feger et al., 2007; Frisullo et al., 2009; Sellebjerg et al., 2012; Chen et al., 2012; Venken et al., 2008b). Further studies are needed to understand the potential of Treg-based therapies in MS patients. In this study, we determined the frequency of $CD4^{+}CD25^{high}CD127^{low/-}$ Treg cells in RRMS and PMS patients compared toHC in Ahvaz, the center of Khuzestan province, which is host for living of Fars and Arab ethnic populations.

2. Methods

2.1. Ethics statement

Blood samples were taken from patients and healthy controls after signing an informed consent. This study was approved by the Ethics Committee of Ahvaz Jundishapur University of Medical Sciences (IR.AJUMS.REC.1395.191).

2.2. Study population

Peripheral blood samples were obtained from 60 patients with MS and 25 HCs, who were the same age and sex matched at Golestan Hospital in Ahvaz, Iran. Diagnosis was based on clinical trials, magnetic resonance imaging (MRI), and according to the McDonald criteria 2010 (Polman et al., 2011) and were diagnosed as having either RRMS or PMS. None of the patients and HCs suffered from any other autoimmune disease or inflammatory status. MS patients were either untreated or under treatment with Cinovex, Rebif (IFN-β1a), Betaseron (IFN-β1b), Methotrexate, Glatiramer acetate (GA) at the time of blood sampling. Blood samples of MS patients were obtained during a clinically stable phase (remission) at least 1 month after the last active relapse. EDSS score and disease duration were asked at time of blood sampling. According to standard scale of EDSS, the patients' scale was determined by neurologist.

Table 1
Demographic and clinical characteristics of MS patients and HCs.

characteristic	RRMS	PMS	HC
Number	35	25	25
Male/ Female	13/22	8/17	10/15
Ethnicity (Fars/ Arab)	18/17	8/17	12/13
Age (Year, Mean ± SD)	32.14 ± 7.26	34.6 ± 8.2	32.76 ± 7.56
Disease Duration (Year, Mean ± SD)	3 ± 2.68	6.68 ± 4.51	-
EDSS (Mean ± SD)	1.61 ± 0.97	5.29 ± 4.74	-
Drug			-
Cinovex	12	-	
Rebif (IFN-β1a)	9	10	
Betaseron (IFN-β1b)	6	1	
Methotrexate	-	3	
Glatiramer acetate (GA)	2	3	
No treatment	6	8	

RRMS: Relapsing remitting multiple sclerosis, PMS: Progressive multiple sclerosis, HC: Healthy control, EDSS: Expanded disability status scale, GA: Glatiramer acetate.

2.3. Preparation, surface staining of cells, and analysis by flow cytometry

Peripheral blood mononuclear cells (PBMCs) were isolated from 2.5 ml of heparinized blood samples by Ficoll-hypaque density gradient centrifugation (density, 1.077 ± 0.002) (Sigma, Germany) of patients and HCs. Antibodies were purchased from eBioscience company (eBioscience, USA). The cells were stained according to the manufacturer's instructions. PBMCs were incubated for 30 min at 4 °C with 10 µl monoclonal antibodies. The cells were washed twice with phosphate-buffered saline (PBS) and resuspended in 500 µl PBS and stored in the dark at 4 °C prior to do flow cytometry analysis. The monoclonal antibodies used were as follows: FITC-conjugated mouse anti-human CD4 (Clone RPA-T4), PE-conjugated mouse anti-human CD25 (clone BC96), and APC-conjugated mouse anti-human CD127 (clone eBioRDR5). Mouse IgG1 K Isotype control FITC (clone P3.6.2.8.1), Mouse IgG1 K Isotype control PE (clone P3.6.2.8.1) and Mouse IgG1 K Isotype Control APC (clone P3.6.2.8.1) were used as isotype controls. The cells were analyzed by using flow cytometry (Becton Dickinson, San Diego, CA, USA). At least 100,000 events were recorded for each sample, and the data were analyzed by FlowJo™ software (Tree Star, Ashland, USA).

2.4. Statistical analysis

Statistical analysis was done by SPSS version 17 (SPSS Inc., Chicago, IL, USA). Comparisons of Treg cells frequency between different groups of patients and HCs were performed by using the one-way analysis of variance (ANOVA) and Tukey Post Hoc test. Correlations between parameters were calculated by Pearson's correlation coefficient test and independent *t*-test. Data are expressed as mean ± standard error of the mean. *P*-value < 0.05 was considered as significant.

3. Results

3.1. Patients' characteristics

Demographic and clinical characteristics of MS patients and HCs are shown in Table 1. Some patients due to expense of medicine and economical condition could not supply medicine, considered as no treatment.

3.2. Identification of Treg cells population

Because a single marker cannot describe Treg cells, this population was defined by flow cytometry as $CD4^+CD25^{high}CD127^{low/-}$ cells (Fig. 1).

3.3. Frequency of Treg, $TCD4^+CD25^+$, and $TCD4^+CD25^-$ cells in RRMS patients

Lower frequency of Treg cells in RRMS patients than the subject in their age and sex matched HCs was observed (1.25 ± 0.23 *vs* 1.57 ± 0.34, $P = 0.005$). There was no difference of $CD4^+$ $CD25^+$ and $CD4^+$ $CD25^-$ T cells frequency between RRMS patients and HCs, (2.5 ± 0.9 *vs* 2.18 ± 0.66, $P = 0.14$ and 38 ± 6.5 *vs* 39 ± 10.49, $P = 0.81$, respectively, as shown in Fig. 2.

3.4. Frequency of Treg, $TCD4^+CD25^+$, and $TCD4^+CD25^-$ cells in PMS patients

Treg cells frequency in PMS patients was significantly higher than RRMS patients and HCs (2.12 ± 0.53 *vs* 1.25 ± 0.23 and 1.57 ± 0.34, respectively, $P < 0.001$ in all cases).

Also T $CD4^+CD25^-$ cells frequency in PMS patients was significantly higher than HCs (44.7 ± 5.2 *vs* 39 ± 10.49, $P = 0.03$). The frequency of T $CD4^+CD25^+$ cells in PMS patients versus HCs was not different (2.69 ± 0.82 *vs* 2.18 ± 0.66, $P = 0.07$), (Fig. 2).

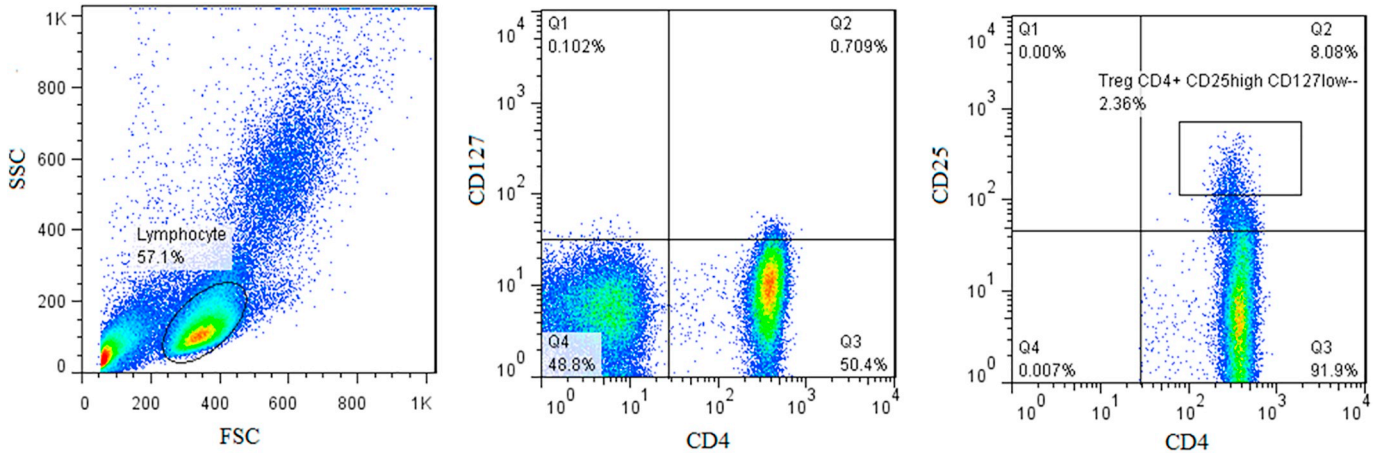

Fig. 1. Flow cytometry analysis. Identification of Treg cells in peripheral blood. PBMCs stained for CD4, CD25 and CD127 and analyzed by flow cytometry. Treg cells characterized by CD4, high CD25 and low/− CD127 expression. Treg: regulatory T cell, PBMC: peripheral blood mononuclear cell

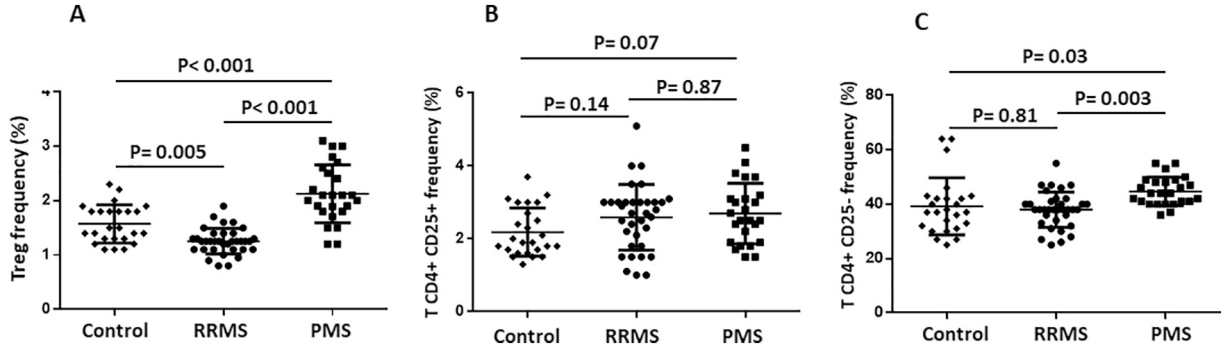

Fig. 2. Frequency of A) Treg cells B) T $CD4^+$ $CD25^+$ cells C) T $CD4^+$ $CD25^-$ cells in peripheral blood samples of RRMS and PMS patients. Treg: regulatory T cell, HC: healthy control, RRMS: relapsing remitting multiple sclerosis, PMS: progressive sclerosis.

3.5. Correlation of Treg cells frequency with different parameters

There was not any correlation between Treg cells frequency with age, sex and ethnicity in RRMS and PMS patients (data not shown). Using Pearson's correlation coefficient test, significant inverse correlation between Treg cells frequency and EDSS in RRMS patients was

observed (P = 0.01, r = - 0.4), but there was not any correlation between Treg cells frequency and disease duration (P = 0.55, r = 0.41). Direct correlation between Treg cells frequency and EDSS, and disease duration in PMS patients have been shown, ($P = 0.001$, $r = 0.6$) and ($P = 0.03$, $r = 0.41$), respectively, as shown in Table 2.

Table 2
Correlation of Treg cells frequency with EDSS, Age and Disease duration in RRMS and PMS patients.

Characteristic		RRMS			PMS	
		Treg: 1.25 ± 0.23			Treg: 2.12 ± 0.53	
		p-value	r		p-value	r
EDSS	1.61 ± 0.97	0.01	-0.4	5.29 ± 1.47	0.001	0.6
Disease duration	3 ± 2.68	0.55	0.41	6.68 ± 4.51	0.03	0.41

RRMS: Relapsing remitting multiple sclerosis, PMS: Progressive multiple sclerosis, Treg: Regulatory T cell, EDSS: Expanded disability status scale.

4. Discussion

Previous studies have controversial results on the quality and quantity of Treg cells in MS patients. More often, characterization of Treg cells has been based on the quantification of $CD4^+$ $CD25^+$ FoxP3 $^+$ cells. The obtained frequency by this way will be more than the actual value; because active T cells without regulatory activity up-regulate the FoxP3 expression (Allan et al., 2007; Tran et al., 2007). To rule out these phenomena, we used the combination of CD4, high expression of the CD25, and low levels of IL-7 α-receptor (CD127) expression for identification of Treg cells in peripheral blood (Seddiki et al., 2006; Liu et al., 2006).

In this study, we have shown that $CD4^+$ $CD25^{high}$ $CD127^{low/-}$ Treg cells frequency in RRMS patients was significantly lower than PMS patients and HCs. In accordance with our results, others demonstrated that Treg cells frequency was reduced in RRMS patients compared to the HCs (Huan et al., 2005; Haas et al., 2007; Venken et al., 2008a; Kouchaki et al., 2014; Jamshidian et al., 2013) and the reduced number of Treg cells have been considered as a possible reason for the onset of MS or as a predisposing factor (Venken et al., 2008b; Namdar et al., 2010). Consistent with this observation, may due to the clonal or functional exhausting and more migration of the most potent suppressor cells in RRMS patients to inflammatory sites, the function and frequency of Treg cells is lower in RRMS patients rather than PMS patients (Venken et al., 2006). As shown in the MOG35–55 EAE model, in the CNS during remission, there were Treg cells with a high suppressive capacity (Kouchaki et al., 2014). These reports are in accordance with observation of high levels expression of ICAM-1 (CD54) by Treg cells (Venken et al., 2006), and detection of $TCD4^+$ $CD25^{high}$ cells in the CSF of RRMS patients (Haas et al., 2005). According to some studies, Treg cells frequency was not significantly different between MS patients in comparison to HCs (Venken et al., 2006; Haas et al., 2005; Feger et al., 2007; Viglietta et al., 2004; Frisullo et al., 2009). Other studies have shown that even if the number of Treg cells in MS patients is normal, their function is not normal (Venken et al., 2006; Haas et al., 2005; Feger et al., 2007; Frisullo et al., 2009; Venken et al., 2008b; Sellebjerg et al., 2012; Chen et al., 2012). Lower Treg suppressive capabilities may cause increase the production of proinflammatory cytokines, for instance, IL-6, IL-17, and IFN-γ, and activation of B cells-producing autoantibody. Via contact-dependent and contact-independent suppression of effector T cells (Teff) known to produce IL-6, IL-17 and IFN-γ, Treg cells usually suppress the production of these pro-inflammatory cytokines (Wang et al., 2010; van Mierlo et al., 2008).

Some studies also reported a reduction in Treg cells frequency in MS patients, not necessarily in RRMS patients. Kouchaki et al. showed that reduction of Treg cells frequency in MS patients compared to HCs, refers to higher frequency of these cells in severe types (SPMS, PPMS, and RPMS) compared to milder types (RRMS and CIS) (Kouchaki et al., 2014).

The marked paradox results between different studies due to different sample size, patients' characteristics, different include and exclude criteria, different patient categories, comparators, markers of Treg identification and the method used.

So far there is no clear indication of Treg cells frequency in PMS patients. In this study we found a significant increase in the Treg cells frequency in PMS patients compared to RRMS patients and HCs.

Increase frequency of Treg cells in PMS patients may be a defensive approach to progressive trend of the disease; the immune system increases Treg cells to control self-reactive cells and inflammation with negative feedback.

Taking into account of the reports of Treg cells increase in the elderly (Kouchaki et al., 2014; Venken et al., 2006) also an early immune-senescence in MS disease similar to other autoimmunities (Haas et al., 2005; Feger et al., 2007), the enhancement of Treg cells may occur as a result of premature activation of mechanisms which normally cause immune-senescence in the elderly people.

Some studies demonstrated that Treg cells can not properly infiltrate in CNS during the disease course (de Oliveira et al., 2015; Müller et al., 2007; Fritzsching et al., 2011). Inefficient migration of Treg cells to neuroinflammatory sites lead to profound implications (Keil et al., 2016; Buckner, 2010). Inefficient migration can be the reason for the higher frequency of Tres cells in PMS patients compared to RRMS patients and HCs.

In present study we have seen a significant direct correlation between Treg cells frequency and both EDSS and disease duration in PMS patients. As Venken et al. expressed it is likely the restored function of Treg cells among PMS patients is related to their long-duration treatment (Venken et al., 2006) and increase of Treg cells frequency in PMS patients may also due to disease duration and long-duration treatment.

We have shown an inverse correlation between Treg cells frequency and EDSS in remission RRMS patients. Our result is in agreement with an inverse correlation between Treg cells frequency and EDSS in remission MS patients, has been noted in a study conducted by Bjerg et al. (2012), and another study that has been reported in relapse of MS patients by Jamshidian et al. (2013).

We found a direct correlation between frequency of Treg cells with disease duration in PMS patients but not about RRMS.

Since patients with SPMS have a relapsing remitting course at the disease onset and there was a strong correlation between the activity of Treg cells and disease duration , in the early phase of the disease the function of Treg cells is seemingly more affected (Li et al., 2014). As one reported the enhancement of the frequency of Treg cells has also been seen as a function of disease duration (Liu, 2006).

In this study, we have shown that there was no significant correlation between Treg cells frequency and age, sex and ethnicity in RRMS and PMS patients.

In this study, a quantitative comparison was done on Treg cells between RRMS and PMS patients with each other and with healthy controls via CD4, CD25, and CD127 markers for the first time.

Abbreviations list

MS	Multiple sclerosis
CNS	Central nervous system
RRMS	Relapsing remitting multiple sclerosis
PMS	Progressive multiple sclerosis
CIS	Clinically isolated syndrome
SPMS	Secondary progressive multiple sclerosis
PPMS	Primary progressive multiple sclerosis
PRMS	Progressive relapsing multiple sclerosis
IFN-γ	Interferon gamma
TNF-α	Tumor necrosis factor

RA	Rheumatoid arteritis
HC	Healthy control
MRI	Magnetic resonance imaging
GA	Glatiramer acetate
EDSS	Expanded disability status scale
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline

Declaration of competing interest

The authors declare no conflict of interests.

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