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Biased Treg/Th17 balance away from regulatory toward inflammatory phenotype in relapsed multiple sclerosis and its correlation with severity of symptoms

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ABSTRACT

The opposing immune functions of Treg and Th17 lymphocytes and the plasticity of Treg/Th17 differentiation, has led us to investigate the effects of their fluctuations and counterbalance in autoimmune condition of multiple sclerosis (MS). Evaluation of Treg and Th17 frequency in peripheral blood of a group of relapsed MS patients, showed a decrease in Treg/Th17 ratio compared to that of healthy controls. A reverse correlation between these subsets was observed in controls but not in patient groups. Both Treg frequency and Treg/Th17 ratio were negatively correlated with severity of symptoms. There was shown to be an enduring increase in Treg frequency associated with MS disease.

1. Introduction

Multiple sclerosis (MS) is known as a neurotropic autoimmune disease where a coordinated attack of innate and adaptive immune cells inflames the central nervous system (CNS), and interrupts signal transduction through demyelinating (destruction of myelin cover) of the nerve fibers. Neurological symptoms may result from distinct attacks (relapsing–remitting MS) or accumulate over time (progressive subtypes). The first clinical manifestation of relapsing–remitting MS (RR-MS) is the clinically isolated syndrome (CIS). Most of the CIS patients are at high risk for MS, but the definitive MS diagnosis depends on the collective criteria of magnetic resonance imaging (MRI), cerebral–spinal fluid (CSF) analysis and clinical data (Polman et al., 2011).

In addition to precise central mechanisms in the thymus which are responsible for removing the auto-reactive T cells, induction of regulatory T cells and suppression of effector T cells as the most important peripheral regulatory mechanisms, are crucial for establishing and maintaining self-tolerance in the immune system. Defective suppression of auto reactive pathogenic T cells due to alterations in one or more of these peripheral check points has been considered as a main cause of autoimmunity in MS (Venken et al., 2010). Regulatory T (Treg) cells, a specific suppressor subtype of $CD4^+$T cells, are differentiated in the thymus during overall T cell developmental pathway (natural Treg), or induced in the peripheral immune system organs under peripheral regulatory mechanisms (inducible Treg). Both natural and inducible Tregs are characterized by a high expression of IL-2 receptor α-chain (CD25) on their surface, and a transcription factor named Forkhead box P3 (FOXP3) in their nucleus which is considered as their most exclusive lineage marker (Sakaguchi et al., 2010).

Th17 cells are a more recently discovered effector subset of $CD4^+$ T cells, characterized by IL-17A cytokine production. In contrast to Tregs, Th17 cells play a potent pro-inflammatory role in the immune system, through stimulating a range of inflammatory mediators including IL-9, IL-17A, IL-17F, IL-21, IL-22, TNF and granulocyte–macrophage colony-stimulating factor (GM-CSF), also provoking chemokine expression and neutrophil recruitment. Besides Th17 effector function in defense against extracellular pathogens, these cells can promote many autoimmune inflammatory conditions, unless they are efficiently controlled by regulatory cells (Wilson et al., 2007).

Interestingly, Th17 and inducible Tregs, originate from common naïve $CD4^+$ T cell precursors in the peripheral immune system organs under the influence of a key cytokine, transforming growth factor β1 (TGFβ1) (Weaver et al., 2006), although the full attainment of pathogenic potential by effector Th17 cells is dependent on the other cytokines such as IL-23 (McGeachy et al., 2007). Moreover, under certain in vitro inflammatory conditions, differentiated regulatory T cells have a tendency to be reprogrammed and convert to the inflammatory Th17 subset (Koenen et al., 2008). Therefore, with setting the boundaries of mediation and regulation of inflammatory reactions,

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Table 1
Clinical and demographic characteristics of study groups.

	Normal controls	CIS	RR-MS
Age ± SD, year (range)	31.4 ± 9.1 (19–50)	30.6 ± 7.9 (17–42)	32.8 ± 7.7 (19–51)
Female/male (ratio)[a]	19/4 (4.67)	6/5 (1.20)	22/1 (22.00)
Disease duration ± SD, month (range)	NA	<1 (0–1)	56.3 ± 58.4 (6–252)
EDSS score ± SD (range)	NA	2.37 ± 0.77 (1.5–3.5)	2.97 ± 1.22 (1.6–6.0)
Immuno-modulatory drugs	No	No	IFNβ-a, IM[b], weekly
Total	23	11	23

[a] The women to men ratio in the patient groups overall is 28/6 (4.75) and is matched with controls.
[b] Intra muscular injection.

Treg/Th17 balance, distinct from the number and function of each subset alone, is an imperative checkpoint in immune homeostasis. The present study was conducted to evaluate Treg/Th17 ratio in peripheral blood of MS patients in context of both subset frequency measurements.

2. Methods

2.1. Human subjects

Peripheral blood samples were obtained from MS patients attending a MS outpatient's clinic and from age and sex matched healthy volunteers at Kashani University Hospital in Isfahan, Iran. According to the McDonald diagnostic criteria for MS (Polman et al., 2011), the involved patients were diagnosed as having either RR-MS or CIS, with a clinically documented attack of neurological symptoms (relapse) within the last 2–7 days, but still were free of corticosteroids or other immunosuppressive treatments in the present attack. Relapse was defined as the appearance of one or more neurological abnormalities in both CIS and RR-MS groups and worsening of pre-existing neurological symptoms in RR-MS group, in the absence of any underlying infectious disease. The abnormalities had to persist for more than 24 h after a period of at least 30 days of disease stability to be considered as a new relapse. All patients had white matter lesions on their brain and/or spinal cord identified via MRI. Clinical severity of disease was measured by the Kurtzke's Expanded Disability Status Scale (EDSS) scores (Kurtzke, 1994). All RR-MS patients had been previously treated with interferon-β-a during the last remission, however patients who had received a drug in the last 5 days were not included in this study. In the CIS group, no patient had a history of receiving immuno-modulatory treatments. Informed consents were obtained from all subjects. The study was approved by the institutional review boards of Isfahan University of Medical Sciences and the local ethics committee.

2.2. Preparation and stimulation of PBMCs

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood samples immediately following blood collection, by density gradient centrifugation on lymphocyte cell separation medium (Lymphodex; Inno-Train Diagnostik, Kronberg, Germany). For Th17 assays, 2×10^6 PBMCs were stimulated in 1 mL RPMI 1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% FCS, for 6 h at 37 °C and 5% CO_2 in the presence of 25 ng/mL phorbol myristate acetate (PMA) and 1 μg/mL ionomycin (both from Sigma-Aldrich, MO, USA). Cytokines were maintained inside the cells by adding 1.5 μg/mL monensin (Sigma-Aldrich, MO, USA) in the final 4 h of stimulation.

2.3. Surface and intracellular-staining of cells and analysis by flow cytometry

To identify Treg cells, 1×10^6 PBMCs were surface labeled within 2 h of isolation, with PerCP-Cy5.5 conjugated anti-human CD4 (clone RPA-T4, eBioscience, CA, USA) and fluorescein isothiocynate (FITC) conjugated anti-human CD25 (clone 2A3, BD Biosciences Pharminogen, CA, USA) antibodies. Surface labeling was followed by an overnight fixation with 4% w/v para-formaldehyde (PFA) in 4 °C and then washed with 0.1% w/v saponin. Permeabilization was performed by 0.1% w/v triton (t-octylphenoxypolyethoxyethanol) X-100 solution (Sigma-Aldrich, Steinheim, Germany) for 30 min at room temperature. All washing buffers used after permeabilization contained 0.1% w/v saponin. Intracellular staining was achieved by 60 minute incubation of permeabilized cells with a phycoerythrin (PE) labeled anti-FOXP3 antibody (clone 236A/E7, eBioscience, CA, USA).

For detecting Th17 cells, cells were stimulated for 6 h as above, then harvested, washed with PBS, and surface-labeled with PerCP-Cy5.5 conjugated anti-CD4 (clone RPA-T4, eBioscience, CA, USA). Intracellular cytokine staining was conducted after 2 hour fixation with 4% PFA and then, 1 hour permeabilization with 0.1% w/v saponin solution. All wash buffers used after permeabilization contained 0.1% w/v saponin. Intracellular labeling was performed as above with anti-IL17a — FITC (clone eBio64DEC17, eBioscience, CA, USA).

Isotype matched control antibodies (all from eBioscience) were used in parallel for PerCP-Cy5.5, PE and FITC mouse IgG1 K isotype antibodies to determine nonspecific binding of antibodies, also to help find out the levels of background emittance and setting gates and quadrants.

Flow cytometry was carried out using BD FACSCalibur instrument. $3–5 \times 10^5$ events were acquired from each sample tube, and lymphocytes were gated based on their side and forward light scatter characteristics. Cell Quest analysis program (BD Biosciences, NSW, Australia) was used to analyze acquired data. Complete blood cell count was determined with hematology analyzer Sysmex XE2100 (Sysmex, Kobe, Japan) and the absolute counts of lymphocytes were used for calculating absolute numbers of Treg and Th17 cells.

2.4. Statistical analysis

Data were analyzed using IBM SPSS Statistics version 20.0 (SPSS Inc., San Francisco, CA, USA). The statistical differences between control and MS patient groups were evaluated using the nonparametric two-tailed Mann–Whitney ranked sum test. Data were verbalized as mean ± standard error of the mean. Spearman's rank correlations were used to assess the relationships between variables. P value of <0.05 was considered significant.

3. Results

3.1. Study groups

Clinical and demographic characteristics of patients and controls are summarized in Table 1. CIS and RR-MS patient groups were significantly different in gender ratio, disease duration, EDSS score, and history of IFN-β therapy.

3.2. Reduced frequency of Tregs in MS relapses

Treg cells were delineated as $CD4^+CD25^+FOXP3^+$ cells. The $CD4^+$ population of lymphocytes was gated (Fig. 1-A), and frequency of $CD25^+FOXP3^+$ cells in this population was evaluated (Fig. 1-B). The mean percentage of these cells in healthy subjects was significantly higher than in all patients groups, also it was slightly but not significantly higher in RR-MS patients compared with CIS group, (Table 2) (Fig. 1-D).

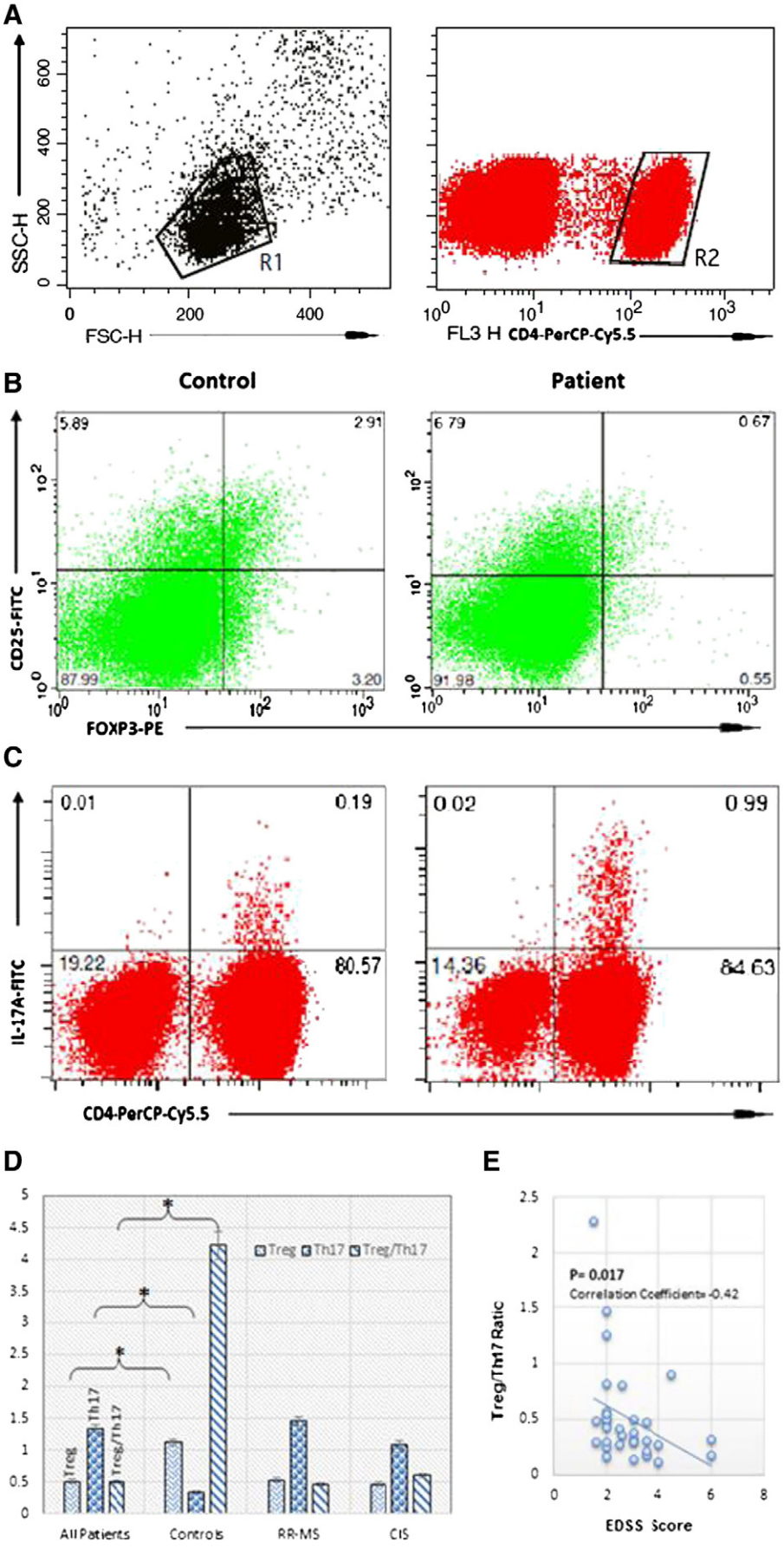
A
SSC-H
FSC-H
R1
R2
FL3 H CD4-PerCP-Cy5.5
B
Control
Patient
CD25-FITC
FOXP3-PE
5.89
2.91
87.99
3.20
6.79
0.67
91.98
0.55
C
IL-17A-FITC
CD4-PerCP-Cy5.5
0.01
0.19
19.22
80.57
0.02
0.99
14.36
84.63
D
Treg
Th17
Treg/Th17
All Patients
Controls
RR-MS
CIS
E
P= 0.017
Correlation Coefficient= -0.42
Treg/Th17 Ratio
EDSS Score

Table 2
Frequencies, ratios and absolute counts of studied subpopulations in controls and patient groups.

Subpopulations	Controls N = 23	All patients N = 34	RR-MS patients N = 23	CIS patients N = 11
Treg (%)	1.12 ± 0.68	0.51 ± 0.20***	0.53 ± 0.20***	0.47 ± 0.20**
Th17 (%)	0.34 ± 0.16	1.33 ± 0.67***	1.45 ± 0.71***	1.08 ± 0.52***
Treg/Th17 ratio	4.22 ± 3.32	0.51 ± 0.49***	0.46 ± 0.35***	0.60 ± 0.62***
$CD4^+CD25^+$cells (%)	3.04 ± 1.02	2.25 ± 0.84**	2.43 ± 0.81	1.87 ± 0.80**
$FOXP3^+$cells (%) in $CD4^+CD25^{High}$cells	80.83 ± 11.97	62.05 ± 8.05***	62.85 ± 8.47***	60.37 ± 7.17***
Lymphocyte count ($\times 10^3/mm^3$)	2.09 ± 0.51	1.74 ± 0.60*	1.65 ± 0.62*	1.94 ± 0.58
Treg count ($\times 10^3/mm^3$)	12.33 ± 10.25	4.93 ± 2.81***	4.77 ± 1.74***	5.24 ± 4.05**
Th17 count ($\times 10^3/mm^3$)	5.58 ± 3.16	16.42 ± 8.58***	16.57 ± 7.51***	16.11 ± 10.63***

* $P < 0.05$, in comparison with controls.
** $P < 0.01$, in comparison with controls.
*** $P < 0.001$, in comparison with controls.

Considering the fact that $CD4^+CD25^{intermediate}$Tcells also include activated Tcells that transiently express FOXP3 but do not have suppressive function, we captured the highest expression of CD25 through gating on top 0.5% of $CD4^+CD25^+$lymphocytes (Fig. 2). The mean percentage of $FOXP3^+$cells in $CD4^+CD25^{high}$ population in healthy subjects was significantly greater when compared to RR-MS, CIS, and total patients. No significant difference was observed between RR-MS and CIS patients.

A higher frequency of $CD4^+CD25^+$Tcells was seen in controls in comparison with CIS and total patients. The observed difference of $CD4^+CD25^+$Tcells between RR-MS and CIS patients was not significant ($P = 0.060$).

3.3. Increased frequency of Th17 cells in MS relapse

To identify the Th17 subset, the capacity of these cells to produce detectable amounts of IL-17A cytokine upon stimulation, was used in flow cytometry analysis. $CD4^+$IL-17^+Tcells in the lymphocyte gate were considered as Th17 cells and their frequency was compared between study groups (Fig. 1-C). Th17 cells showed greater frequency in the patient groups in comparison to healthy controls (Fig. 1-D). Also the frequency of these cells in RR-MS group was slightly elevated as compared to CIS patients, although this difference was not significant (Table 2).

3.4. Multiple fold decrease in Treg/Th17 ratio in relapsed MS

To investigate the relation between changes of two subpopulations, the Treg/Th17 ratio in each blood sample was calculated. The Treg/Th17 ratio was shown to be almost nine and seven folds greater in control subjects than in RR-MS patients and CIS patients, respectively (Table 2) (Fig. 1-D). Although both Treg and Th17 subsets frequency are a bit elevated in RR-MS in comparison with CIS patients (both not significantly as mentioned above), the Treg/Th17 ratio is slightly less in RR-MS, but this difference was not significant (Table 2).

3.5. Negative correlation of Th17 frequency with FOXP3 expression in $CD4^+CD25^{High}$Tcells in healthy subjects but not in MS patients

A strong inverse relationship was found between the frequency of Th17 cells and FOXP3 expression in $CD4^+CD25^{High}$Tcells in total study cohort ($P < 0.001$, $R = -0.519$). When the healthy and patient groups were separately analyzed, the inverse correlation disappeared in patients and remained significant in healthy subjects ($P = 0.008$, $R = -0.536$) (Fig. 3).

3.6. Treg frequency and Treg/Th17 balance, but not Th17 frequency is closely correlated with patients' EDSS scores

We also examined the effects of Treg and Th17 frequency both distinctly and in context of their balance, on the severity of present attack in MS patients. The EDSS score as a clinical scale of symptom severity was used for this purpose. There was a significant inverse correlation between EDSS and both Treg frequency ($P = 0.003$, $R = -0.501$) and Treg/Th17 ratio ($P = 0.017$, $R = -0.418$) in MS patients (Fig. 1-E). As expected, the relationship tended toward positive for Th17 cells, but the correlation was not significant ($P = 0.215$, $R = +0.226$). There wasn't any significant relationship between EDSS and other scalable variables like age and disease duration.

3.7. Tregs frequency and FOXP3 expression by $CD4^+CD25^{High}$Tcells are significantly increased with disease duration enhancement

A positive correlation was observed between disease duration and Treg frequency ($P = 0.034$, $R = +0.443$) and FOXP3 expression in $CD4^+CD25^{High}$Tcells ($P = 0.038$, $R = +0.435$) in RR-MS patients. All CIS patients were newly diagnosed, however when they were added to RR-MS for this analysis, the observed positive correlation between disease duration and Treg frequency, also FOXP3 expression in $CD4^+CD25^{High}$Tcells became more significant and less strong ($P = 0.024$, $R = +0.387$ and $P = 0.020$, $R = +0.398$, respectively). There wasn't any significant correlation between disease duration and Th17 frequency or Treg/Th17 ratio. Control and patient groups were tested for possible relationships between age and cell frequencies and no significant correlation was observed.

3.8. Absolute counts of lymphocytes and lymphocyte subtypes

From the statistical point of view, total changes in lymphocyte numbers may significantly affect the frequency of subtypes. To refute such error in the frequency results, the absolute count of lymphocyte subsets were also calculated in each blood sample as the product of subset frequency in lymphocyte gate and the absolute number of lymphocytes. The mean lymphocyte absolute numbers, gained by hem-analyzer complete blood cell counts, is shown in Table 2. The differences between controls and all patients ($P = 0.049$) and controls and RR-MS patients ($P = 0.02$) were significant and other groups didn't show a significant difference in the total count of

Fig. 1. Peripheral Treg and Th17 cells. (A) Gating strategy is demonstrated. The lymphocytes were gated in R1 region based on their side and forward light scattering properties (Right), then the $CD4^+$ population in the lymphocyte region was gated in R2 (Left). (B, C) Representative dot plots from one control and one patient are shown. Numbers represent the percentage of related population in each quadrant. (B) Tregs were identified as $CD25^+FOXP3^+$ cells in $CD4^+$ lymphocytes gate. (C) Th17 cells were identified as $CD4^+$ $IL17A^+$ cells in lymphocyte gate. (D) Treg and Th17 cells mean frequency and ratio in study groups. Only comparisons between all patients and controls are shown. *$P < 0.001$ (E) Treg/Th17 ratio is significantly correlated with symptoms severity indicated by EDSS score.

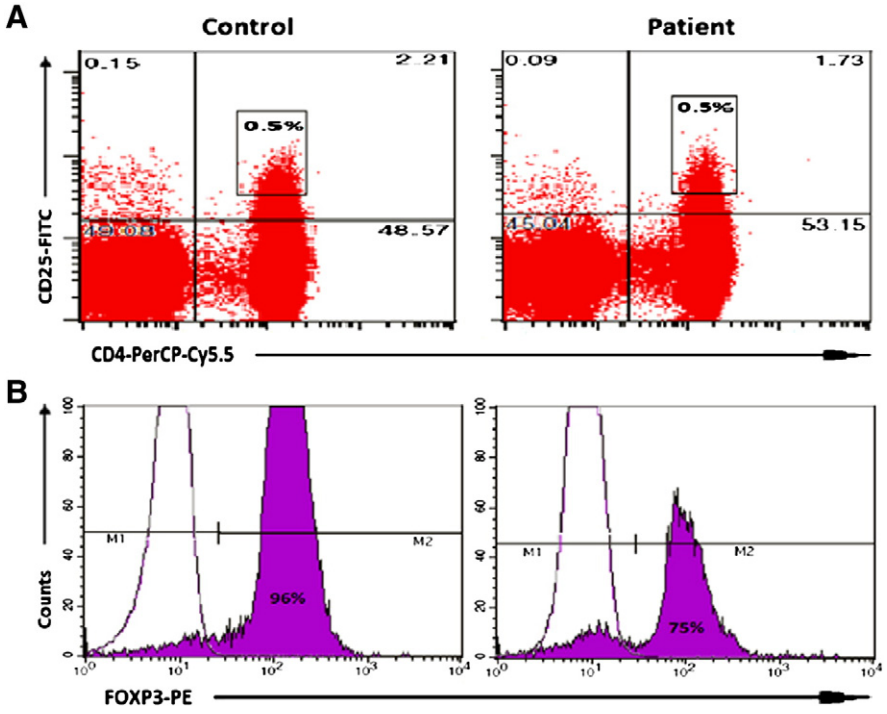

Fig. 2. Percentages of FOXP3 expression in $CD4^{+}CD25^{Highest}$Tcells in representative patient and control samples. (A)The top 0.5% of $CD25^{High}$ cells is gated on $CD4^{+}CD25^{+}$ lymphocytes. (B) The percentages of $FOXP^{3}$ expressing cells in these gates are shown. The overlaid histograms are the same samples stained with isotype matched control antibodies.

lymphocytes. In all patient groups, the mean count of Tregs was significantly less and the mean count of Th17 cells was significantly more than controls. There wasn't any significant difference in Treg and Th17 absolute counts between RR-MS and CIS patients.

4. Discussion

There have been controversial reports about the frequency of regulatory Tcells and pro-inflammatory Th17 cells in peripheral blood of MS patients, with the majority of the discrepancy in Treg data. While most previous studies have been conducted on clinically stable state of disease (Putheti et al., 2004; Venken et al., 2007; Cosentino et al., 2012; Praksova et al., 2012), more questions emerge around such fluctuations in acute attacks of MS. Of the few studies that have involved relapsed-MS, some reported a compensatory peripheral increase of Tregs approaching normal levels in comparison with remission state, while others showed no difference between both states of relapse and remission and health (Feger et al., 2007; Edwards et al., 2011). Studying documented relapses of MS, our Treg frequency data suggests a decreased number of Tregs, also lower expression of their functionality marker FOXP3, and significant association of both with severity of new attack.

We have also examined the $CD4^{+}CD25^{+}$ population, ignoring FOXP3 staining. While a higher frequency of these cells was seen in healthy subjects versus all patients and CIS patients groups, this difference was not significant in the case of RR-MS patients. Consistent with these results, other groups who studied RR-MS patients only in relapse, also reported no significant change in the percentages of $CD4^{+}CD25^{+}$cells (Putheti et al., 2004; Haas et al., 2005; Braitch et al., 2009). A key preference of our study to previous ones is intracellular staining of the transcription factor FOXP3 as the most exclusive marker for Tregs. It has been shown that the expression levels of FOXP3 is directly correlated with Treg suppressive activity (Fontenot et al., 2003; Venken et al., 2007), and a deficiency exists in suppressive function of $CD4^{+}CD25^{High}$Tregs despite their normal count, during both relapse and remission in MS patients (Haas et al., 2005; Venken et al., 2008). Also, the lower Treg frequency in remitted MS has been evident only with FOXP3 staining (Venken et al., 2007). In a more recent study, Bjerg et al. have shown a dichotomy in Treg frequency among remitting RR-MS patients (Bjerg et al., 2012). While they observed no change in $FOXP3^{+}$Tregs frequency in total patients against all controls, when patients were segregated in two groups with $FOXP3^{+}$Treg percentages higher and lower than the median in total controls, the two patient groups were significantly different from each other, also from their matched controls. The lower Treg frequency was related to the higher median of disease severity score indicated by EDSS. Although they have included just remitting patients, considering the elevated EDSS scores in MS relapses versus remission states, our results of lower Treg frequency in relapsed MS versus health are in agreement with this study. Also, the observation of a significant reverse correlation between Treg percentages and EDSS scores in our study, represents lower Treg frequency relates to more severity of symptoms in relapsed MS.

The observed Treg frequency enhancement as a function of disease duration has also been reported previously (Venken et al., 2008). Considering the reports of Treg augmentation in the elderly (Gregg et al., 2005; Rosenkranz et al., 2007) along with an early immune-senescence in MS disease like other autoimmunities (Hug et al., 2003; Thewissen et al., 2007), this Treg enhancement may occur as a consequence of premature activation of mechanisms which normally cause immune-senescence in the elderly. Naturally, T cell generation declines with the age due to thymus involution, as the first appearing aspect of immune-senescence. Considering the accelerated immune-senescence in MS autoimmunity, reduced repertoire of naïve T cells with increasing disease duration may cause memory Tregs enrichment by comparison. Entirely in line with this hypothesis, our study has shown a significant decrease in total lymphocyte count in RR-MS patients and a slight but non-significant decline in newly diagnosed CIS patients in comparison with healthy subjects. Our findings are in agreement with previous reports of reduced frequency of naïve Tregs in early stages and enhanced memory Tregs in later-stage MS (Venken et al., 2008). There was no significant correlation between cell frequencies and age in either control or patient groups. Additionally, there was no correlation in cell frequencies between IFN-β treated and untreated patient groups. Nevertheless, it is

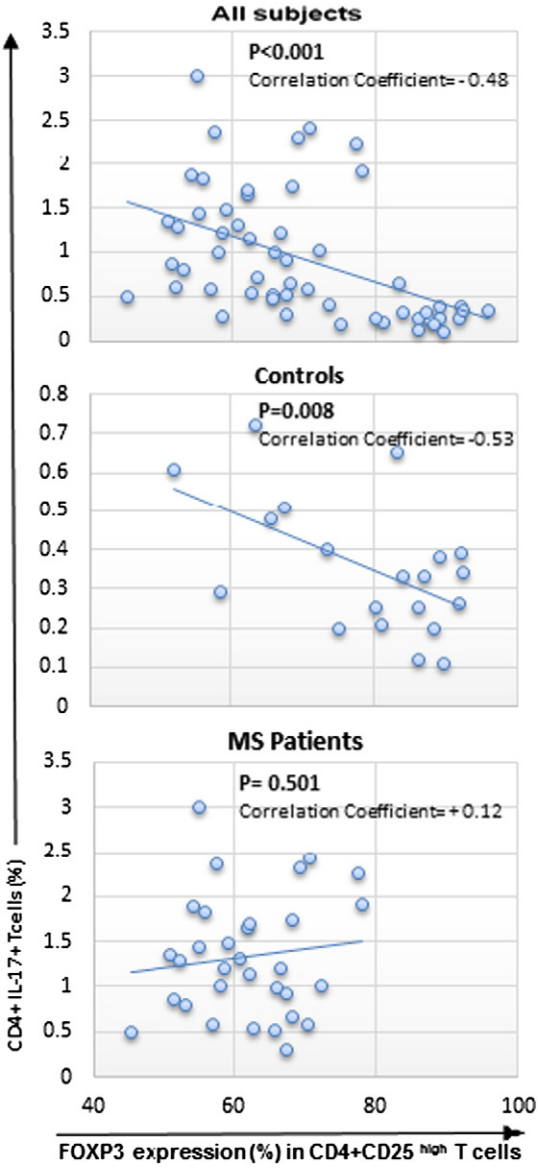

Fig. 3. FOXP3$^+$Tregs and Th17 cells frequency relation curves in study groups. The negative correlation in healthy bodies is lost in MS patients. All subjects (N = 57); healthy controls (N = 23); MS patients (N = 34).

still plausible that the observed Treg enhancement with disease duration may be affected by direct relation between disease duration and the length of time to be in IFN-β therapy as previously shown (Praksova et al., 2012).

Consistent with our results, an obvious increase in Th17 cells in PBMCs of relapsed MS patients has been reported by others (Durelli et al., 2009; Edwards et al., 2010; Peelen et al., 2011). However in another study, a highly significant increase of Th17cells in CSF, despite a non-significant increase in the peripheral blood, has been shown in relapsed-MS versus remission and other neurological diseases (Brucklacher-Waldert et al., 2009). The difference in PBMC evaluation results may be due to comparison between relapsed MS and remitted patients, also other neurological diseases, but not healthy subjects. Although we did not evaluate CSF samples as well, considering the disruption of blood–brain barrier (BBB) as a main and early phenomenon in MS pathogenesis (Morales et al., 2006), it is interpretable that the enhancement of Th17 cells in peripheral blood, could result in relative enhancement in the CSF. In addition, one of the main mechanisms through which Th17 cells promote CNS inflammation, is through a breakdown in the BBB (Kebir et al., 2007).

The hallmark cytokine of Th17 subset, IL-17A was used for identifying this subset. However, new evidences are emerging about the role of other cytokines in the pathogenicity of Th17 cells. Studies in experimental autoimmune encephalomyelitis (EAE), an animal model of MS, have revealed that Th17 pathogenicity is independent of IL-17A production. Indeed, the cytokine GM-CSF, is the only T cell-cytokine which is necessary for susceptibility to EAE induction. This cytokine is produced by Th1, Th2 and Th17 cells and is known as a critical element in both TH1 and TH17 pathogenicity. Pathogenic TH17 cells which are re-stimulated with IL-23, have been shown to produce higher amounts of GM-CSF than non-pathogenic Th17 cells (El-Behi et al., 2011).

In addition to extending previous knowledge of both subset discrepancies in MS relapses, our study clarified a reverse relationship between the percentages of FOXP3 expressing cells in CD4$^+$CD25^{High} population and Th17 cell frequency in circulation (Fig. 3). Interestingly, this correlation tends to be lost in patients, when they are analyzed separately from healthy subjects. It reveals that in healthy individuals, Th17 cells differentiation is effectively controlled by FOXP3$^+$Tregs, so higher frequency of one subset is associated with lower counts of the other subset. In contrast, in relapsed-MS the increase of Th17 cells was offset by the enhancement in the number of FOXP3$^+$Tregs, but the Treg portion was not sufficient to regulate the enhanced induction of Th17 phenotype under the pathologic MS. Comparable to our results, Peelen et al. have reported a significant direct correlation between another regulatory phenotype (CD39$^+$Tregs) and Th17 cells in remitted-MS which has been lost in relapsed disease (Peelen et al., 2011).

In accordance with a deficient regulatory function in MS patients indicated by many studies (Venken et al., 2007; Venken et al., 2008; Smolders et al., 2009), it is to be expected that higher frequency of FOXP3$^+$Tregs does not result in a lower frequency of Th17 cells in individual patients. It is also conceivable that effector Th17 cells have an unusual resistance to suppression by Tregs in MS patients. Such resistance has been reported in other immune-pathogenic conditions (Schneider et al., 2008; Chauhan et al., 2009), although to the best of our knowledge it has not yet been investigated in MS. The reversal of the Treg/Th17 ratio, from more than fourfold higher frequency of Tregs in healthy subjects to an approximately one-half prevalence in proportion to Th17 cells in relapsed MS, is the other remarkable aspect of our results. This shift in ratios is in full accord with the above mentioned probabilities of Treg deficiency or Th17 cell resistance in these patients. Also, considering the plasticity in T-helper subsets (Koenen et al., 2008; Weaver and Hatton, 2009; d'Hennezel and Piccirillo, 2012), it may be the case that the inflammatory conditions in MS promotes more conversion of differentiated Treg cells to Th17 phenotype. However, the rate of IL-17 production by FOXP3$^+$ Tcells in MS patients versus healthy subjects is unclear. It has been shown that epigenetic modifications induced by histone deacetylase enzymes has an extreme influence on the emergence of IL-17 producing FOXP3$^+$ Tcells (Koenen et al., 2008; Weaver and Hatton, 2009). Beside the certified role of these enzymes in MS disease (Gray and Dangond, 2006; Kazantsev and Thompson, 2008), this finding implies a connection between Treg-Th17 plasticity and MS pathogenesis.

The Treg/Th17 balance has been shown to be similarly disturbed in other autoimmune and inflammatory conditions, with decreasing Tregs versus increasing Th17 cells (Eastaff-Leung et al., 2010; Li et al., 2010; Ma et al., 2010; Liu et al., 2011; Shen et al., 2011). While a previous study in MS patients reported no significant difference in this ratio despite increased Th17 and decreased Treg frequency in these patients and a positive correlation between Treg and Th17 frequencies in the total study cohort (Edwards et al., 2011). The inconsistencies may be partly explained by dissimilarities in study design, MS types and courses, and case volumes, as the study involved progressive MS, RR-MS and CIS in remission, and a lower number of relapsed patients. Even so, the need for more investigation is undeniable.

In conclusion, our results suggest an important role for Treg/Th17 imbalance in MS relapses. There are still many issues which remain to be better clarified about this disease: 1) Treg efficiency in control of Th17 cells, 2) the contribution of Th17 resistance to Treg suppressive effect, and 3) Treg to Th17 conversion and relative rates of IL-17 production by FOXP3$^+$Tcells. In addition to these clues for basic research, our data confirms the changes in Treg/Th17 balance as an informative biomarker for evaluating or comparing the effectiveness of MS therapies, and highlights the Treg-Th17 interaction as a target for new therapeutic approaches.

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