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## **Characterization of CD4+ and CD8+ T Cell Subsets and Interferon Regulatory Factor 4 (IRF4) in MS Patients Treated with Fingolimod (FTY-720); A Follow-up Study**

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### **ABSTRACT**

Fingolimod is a novel immunomodulatory drug used in patients with relapsing multiple sclerosis (MS) which reversibly inhibits egress of lymphocytes from lymph nodes.

In this longitudinal study, the frequency of Interferon- gamma (IFN- $\gamma$ )+, IL4+, IL17+ and IL10+ CD4+ and CD8+ T cell subsets were measured in Fingolimod treated patients before and after 12 months' (12M) therapy using flow cytometry and compared to those of naive, Betaferon treated MS patients and healthy individuals. Additionally, the level of transcription factor IRF4 and IL-6, IL-23, TGF- $\beta$ 1 cytokines, required for differentiation of IL-17+ T cells, were assessed by RT-PCR and ELISA, respectively.

In Fingolimod treated MS patients, we observed a significant decrease in the percentage of IFN- $\gamma$ +/IL17+ CD4+ and CD8+ T cell subsets. In contrast, Fingolimod increased IL10+ CD4+ T cells. We also showed that IFN- $\gamma$ +IL17+ co-producing CD8+ T cells were reduced in patients under fingolimod therapy. Furthermore, Fingolimod could reduce the expression level of IRF4 in patients while IL6 was increased in the supernatant of cultured peripheral blood mononuclear cells.

Our data showed that Fingolimod treatment alters CD4+ and CD8+ T cell subsets and reduces expression of IRF-4, which affects the proportion of pathogenic memory T cells in peripheral blood.

**Keywords:** Fingolimod; IFN regulatory factor 4; Multiple sclerosis; T cells

### **INTRODUCTION**

Multiple sclerosis (MS) is an immune-mediated

disorder characterized by demyelination of the central nervous system (CNS) because of autoreactive lymphocyte activation in genetically susceptible

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individuals.<sup>1</sup> It has been now determined that in addition to Th1 cells, interleukin 17 producing CD4+ T (Th17) cells has a pathogenic role in the development of MS and its animal model, experimental Autoimmune encephalomyelitis (EAE).<sup>2-5</sup> Th1 and Th17 cells exist in MS lesions and can promote neurodegeneration and Blood-brain barrier (BBB) disruption.<sup>6,7</sup> A combination of several cytokines, including IL-6, TGF- $\beta$ , IL-23, IL-1b, and IL-21 stimulate Th17 cell differentiation.<sup>8</sup> Some of the Th17 cell clones are able to co-produce IL-17 and IFN- $\gamma$ . These non-classical Th17/Th1 subsets, which share features of both Th17 and Th1 cells, may have a particular effector role in the CNS inflammation.<sup>9-11</sup> In addition to Th1 and Th17 cells, CD8+ T cells are also suspected to involve in MS pathogenesis. Similar to Th cells, CD8+ T cells categorize into different subsets based on their cytokine profile.<sup>12-14</sup> Recently, IL-17 producing CD8+ T cells (Tc17) have been found among cells infiltrating active lesions, in peripheral blood of relapsing-remitting multiple sclerosis (RRMS) patients in the relapse phase and Cerebrospinal Fluid (CSF) of patients with early-stage MS.<sup>15-18</sup>

Interferon regulatory factor 4 (IRF-4), a member of IRF family of transcription factors, has been reported to be necessary for IL-17 induction. IRF-4 has the essential role in autoimmune disease models especially in EAE.<sup>19-22</sup> IRF4 deficient mice have a marked decrease in Th17 cells in the brain.<sup>20</sup> Moreover, IRF4 is also required for the differentiation of Tc17 cells during CNS autoimmunity.<sup>16,20,23</sup> These data show the importance of IRF4 in T-cell differentiation and autoimmune diseases.

Fingolimod (FTY720) is a sphingosine 1-phosphate (S1P<sub>1</sub>) receptor antagonist, which was the first oral drug for the treatment of RRMS.<sup>24,25</sup> Binding of fingolimod to S1P<sub>1</sub> receptors, causes their internalization and degradation, which retains T and B lymphocytes within secondary lymphoid organs (SLOs).<sup>26-28</sup> This drug selectively retains CC-chemokine receptor 7 (CCR7) positive naive and T central memory (TCM) cells including autoreactive Th17 cells within lymphoid nodes, but not CCR7 negative T effector memory (TEM) cells and affects CD8 T cells less.<sup>29-32</sup> Previous studies in Fingolimod-treated MS patients showed that the frequency and counts of T cell subsets changed in

circulation, including a decrease in naive and TCM cells and an increase of TEM and memory regulatory CD4+ T (Treg) cells.<sup>29,30,33,34</sup> fingolimod also reduces the percentage of memory B cells but increases naive and transitional B cells, plasma cells and regulatory B cell subsets.<sup>32</sup> Additionally, the frequency of CD56 bright natural killer cells was reduced after Fingolimod therapy.<sup>35</sup> However, there are a few studies investigating the effect of fingolimod on the frequency of CD8+ and CD4+ T cells subsets in MS patients, which are different in drug dosage and study duration. A study showed a decrease in the percentage of IL17+ CD4+ and IFN- $\gamma$ + CD8+ T cells in patients after 1-month fingolimod therapy.<sup>36</sup> In a recent study, the percentages of both CD4+ and CD8+ IFN- $\gamma$  producing T cells as well as IL17+CD4+ T cells increased transiently 2 weeks after initiation of fingolimod and then decreased gradually after 3 months.<sup>33</sup> A study reported that Fingolimod (1.25 mg /day) could reduce the frequency of IL17+ CD4+ T cells in MS patients at long-term therapy.<sup>30</sup> While other study showed an increase in circulating Th17 cells in half of patients after short-term therapy with less dosage of Fingolimod (0.5 mg/day).<sup>37</sup> These results indicate some controversy among studies which makes essential the further evaluation of Fingolimod treatment on cytokine-producing T cell subsets at a usual dose (0.5mg/day) in a long-term treatment.

Here we examined the effect of Fingolimod on frequency of CD4+ and CD8+ T cells subsets including Th1, Th2, Th17, Th10, Th1/17 and Tc1, Tc2, Tc17, Tc10, Tc1/17. We also evaluated IL-6, IL-23 and TGF- $\beta$ 1, necessary cytokines for induction of IL-17 producing CD4+ and CD8+ T cells, in patients under Fingolimod therapy in a 12 months follow-up study. We also provide new evidence of the effect of Fingolimod on the expression of IRF-4.

## MATERIALS AND METHODS

### Patients and Control Subjects

A total of 45 Relapsing remitting (RR) MS patients were involved in this study, including 10 treatment-naive MS patients, 15 MS patients on Interferon- $\beta$  (IFN- $\beta$ ) treatment and 20 MS patients on fingolimod treatment (0.5 mg/once a day). A

longitudinal follow-up study was performed on patients initiating fingolimod drug. IFN- $\beta$  treated MS patients had an average treatment of 5.8 years equal to average disease duration. Treatment-naive MS Patients were not under any immunosuppressive or immunomodulatory treatments at least 3 months before the sampling. All MS patients were diagnosed according to the revised McDonald criteria at the MS research center of Sina General Hospital, Tehran.<sup>38</sup> Disability was assessed by an experienced neurologist using expanded disability status scale (EDSS). Fifteen age-matched healthy volunteers who had no history of MS or other autoimmune diseases in their families were also participants in this research. All patients and controls were Iranian Caucasian origin.

### Cell Preparation and Culture

Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation over Lymphodex (innoTrain, Germany).  $10^6$  cells/mL were cultured in RPMI 1640 (Sigma-Aldrich, US) supplemented with penicillin (100 IU/mL), streptomycin (100  $\mu$ g/mL), 1% L-glutamine 100 mM and 10% fetal calf serum (Gibco, US). Cells were stimulated with anti-CD3 (1 mg/mL; Mabtech, Sweden) and anti-CD28 (0.1mg/mL; Mabtech, Sweden) monoclonal antibodies (mAbs). The cells were incubated in 5% CO<sub>2</sub>, at 37°C. After 66h, the supernatants (SNs) were collected and stored in -70°C until the cytokine measurements and the cells were additionally incubated in the presence of phorbo-12-myristate-13-acetate (PMA 50 ng/ml, Sigma-Aldrich, US), ionomycin (500 ng/ml, Sigma-Aldrich, US) and Golgi Plug (1  $\mu$ l/ $10^6$  cells, BD Biosciences, US) for 6h. After incubation time cells were harvested for flow cytometric analysis.<sup>18</sup>

### Multicolor Flow Cytometry Assay

To analyze the frequency of CD4+ and CD8+ T cell subsets, the harvested cells were washed once with PBS and after staining evaluated by flow cytometry. In brief, the  $10^6$  cells/100ul staining buffer (PBS+2% FCS) were first stained for surface antigens with anti-CD4-APC-eflour780 and CD8-PerCP-cyanin5.5 (BioLegend, US) monoclonal antibodies for 30 minutes in the dark at 4°C and then the cells were fixed and permeabilized with Cytotfix/Cytoperm (BIO-RAD, BUF09B, US) according to the manufacturer's instructions. Intracellular cytokine staining was performed using anti-IFN- $\gamma$ -PE-CY-7, IL-

17-FITC, IL-4-APC (BD Biosciences, US) and IL-10-PE (BioLegend, US) monoclonal antibodies. The stained cells were analyzed with a FACS Aria II flow cytometer (BD Biosciences, US) using FACSDiva software.

### Cytokine Measurement

For analysis of Th17 and Tc17 polarization cytokines, IL-6, IL-23 (eBioscience, CA) and TGF- $\beta$ 1 (Mabtech, Sweden), were measured in the supernatant of stimulated cells by commercial ELISA kits following the manufacturer's recommendations. The detection limits of the studied cytokines were 2 pg/mL for IL-6, 10 pg/mL for IL-23, and 10.4 pg/mL for TGF- $\beta$ 1.

### Quantitative Real-time PCR (qPCR)

Total RNA was isolated from PBMCs using RNX plus (RN7713C) according to the manufacturer's instructions. To eliminate any residual genomic DNA from the sample, extracted RNAs were treated with RNase-free DNase I (Invitrogen Life Technologies). Reverse transcription into cDNA was performed with cDNA synthesis kit (fermentase, lithuania). Real-time PCR analysis for quantification of expression of IRF-4 was performed with EvaGreen PCR Master Mix plus (ROX) (Solis BioDyne, Tartu, Estonia) on a Light Cycler 480II (Applied Biosystems, US) according to the manufacturer's recommendations. Relative expression level of IRF-4 was normalized by GAPDH as a housekeeping gene and calculated by the  $2^{-\Delta\Delta C_t}$  method.<sup>39</sup> The data were presented as fold change relative to control samples.

The sequences of the primers in the 5' to 3' direction were as follows: IRF4 forward: 5'-AG-CGC-ATT-TCA-GTA-AAT-GTA-AAC-ACA-T-3' and IRF4 reverse: 5'-TCT-TGT-GTT-CTG-TAG-ACT-GCC-ATC-A-3', GAPDH forward: 5'-TCT-TTT-GCG-TCG-CCA-GCC-GA-3' and GAPDH reverse: 5'-AGT-TAA-AAG-CAG-CCC-TGG-TGA-CCA-3'.

### Statistical Analysis

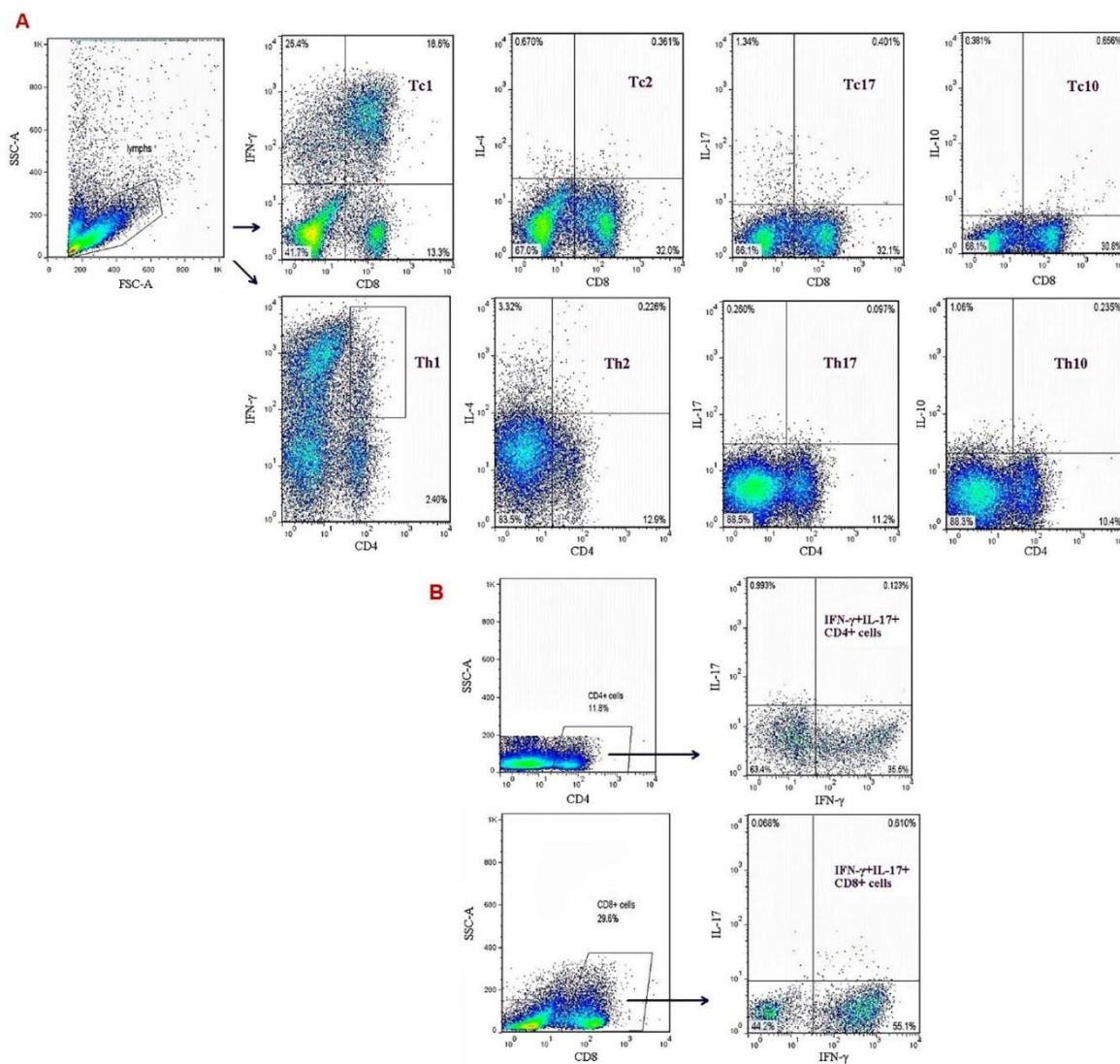
Statistical analysis was performed using SPSSv23 (SPSS Inc; Chicago, IL, USA). Paired T-test or non-parametric Wilcoxon signed-rank test was used to compare results of experiments before and 12 months after fingolimod therapy according to the normality of the data as assessed by Kolmogorov-Smirnov/Shapiro-Wilk test. The comparisons between multiple groups were performed using one-way ANOVA with Welch's

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correction and Tukey's multiple comparison post-hoc test. Pearson correlation was used for correlation assessment unless the data were not normally distributed, in which case Spearman correlation analysis was performed. A p-value less than 0.05 was considered significant. The figures were drawn using GraphPad Prism, Version 6.00 software.

### Ethical Considerations

This work supported by Tehran university of medical sciences [94.01.30,27866]. The study was conformed to the Helsinki declaration and approved by the Ethics Committee of the Tehran University of Medical Sciences. Written informed consent was obtained from all patients and healthy controls.



**Figure 1. Representative gating strategy for different CD4+ and CD8+ T cell subsets. In this sample gating, cells were first gated for lymphocytes (SSC-A vs. FSC-A). The lymphocyte gate was further analyzed for IFN- $\gamma$ , IL-4, IL-17, IL-10 and IFN- $\gamma$ /IL-17 CD4 and CD8 positive cells fractions.**

**Table 1. Clinical Characteristics of Study Population**

Group	Gender			EDSS ( range)
	age <sup>a</sup> (range)	F/M	Disease duration(years)	
Total ( n=60)	31 (19-52)	42/18	5.6 (1-20)	1.9 (0.5-4)
Treatment naïve ( n=10)	30 (25-52)	8/2	2.5 (1-6)	1.6 (1-2.5)
Betaferon ( n=15)	33 (23-49)	11/4	5.8 (1-19)	1.7 (1-3)
<sup>b</sup> Fingolimod ( n=20)	29 (19-43)	15/5	7.2 (2-20)	2.06 (0.5-3.5) <sup>b</sup>
<sup>c</sup> Fingolimod (n=16)	30 (20-44)	11/5	5.9 (3-21)	2.2 (0.5-4) <sup>c</sup>
Healthy control ( n=15)	30 ( 23-43)	8/7		

a. Mean age in years.

b. Before Fingolimod initiation

c. After Fingolimod initiation

Data are expressed as mean (range).

Abbreviations: F = female; M = male; EDSS= expanded disability status scale

## RESULTS

A total of 60 study population were recruited in this study. A longitudinal follow-up study was performed on 20 patients under Fingolimod therapy.

The patients were in remission and discontinued the other immunomodulatory drugs for wash-out of the previous therapies, almost 3 weeks before fingolimod initiation. They did not have any relapse during therapy. (Descriptive and clinical characteristics of Fingolimod-treated MS patients and controls are presented in Table 1. Four Fingolimod-treated patients did not finish the study due to side effects caused by the treatment and pregnancy. Different groups of controls were recruited in this study to compare the percentage of cell subsets between controls and patients before and after Fingolimod therapy to confirm the validity that shows the previous treatment was not probably effective.

For flow cytometry analysis, lymphocytes were first gated on a forward vs. side scatter dot plot. Then, CD4<sup>+</sup> and CD8<sup>+</sup> subsets were determined on gated cells. Post-acquisition analysis was done using the Flow Jo software package v.7.6.1 (Tree Star). At least 50,000 events were acquired within the cultured cells. A representative example of gating strategy is illustrated in Figure 1.

### Fingolimod Therapy Alters CD4<sup>+</sup> and CD8<sup>+</sup> T Cell Frequencies in Peripheral Blood with A Decreased CD4/CD8 T Cell Ratio

The mean percentage of CD4<sup>+</sup> T lymphocytes was reduced in Fingolimod-treated MS patients after 12 months therapy (9.77%) compared to baseline (35.23%), treatment naïve (36.32%), Betaferon treated

(32.23%) MS patients and healthy individuals (32.45%) ( $p < 0.0001$ ). The frequency of CD8<sup>+</sup> T lymphocytes also decreased in Fingolimod-treated MS patients after 12M therapy (21.75%) compared to baseline (28.31%) ( $p < 0.05$ ), treatment-naïve (26.04%), Betaferon treated (24.72%) MS patients and healthy individuals (23.75%) (Table 2).

We also compared the CD4/CD8 T cells ratio between studied groups. We found a reduction of the CD4/CD8 T cells ratio in patients under Fingolimod therapy (0.522) in comparison with baseline (1.366,  $p < 0.0001$ ), treatment naïve (1.41,  $p < 0.01$ ), Betaferon treated (1.40,  $p < 0.0001$ ) MS patients and healthy individuals (1.39,  $p < 0.0001$ ) (Table 2).

### Fingolimod Reduces IL-17 and IFN- $\gamma$ and Increases IL-10 Producing CD4<sup>+</sup> T Cells in Peripheral Blood

To characterize the influence of Fingolimod treatment on cytokine-producing CD4<sup>+</sup> T cell subsets, we determined the percentages of IFN- $\gamma$ , IL-4, IL-17, IL-10 cytokine producing CD4<sup>+</sup> T helper cells (Th1, Th2, Th17 and Th10 respectively) and IFN- $\gamma$ /IL17 co-producing cells as a non-classical T cell subset in PBMCs of Fingolimod-treated MS patients and controls.

The frequency of IFN- $\gamma$  and IL-17 producing CD4<sup>+</sup> T cells from Fingolimod-treated MS patients after 12M decreased compared to baseline in total lymphocytes (0.236% vs. 0.698 and 5.63% vs. 25.53 respectively,  $p < 0.0001$ ) (Figure 2A-B). In addition, we observed an increase in frequency of IL-10 secreting CD4<sup>+</sup> cells after Fingolimod-therapy (0.312% vs. 0.193,  $p = 0.043$ ), while there was no difference between the mean percentage of IL-4 and IFN- $\gamma$ /IL17 co-producing CD4<sup>+</sup>

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T cells before Fingolimod initiation and after therapy ( $p>0.05$ ) (Figure 2, C-D-E).

Fingolimod treated patients after therapy had lower IFN- $\gamma$ + CD4+ T cells while, pre-treated Fingolimod and treatment naïve MS patients had a higher percentage compared to healthy controls ( $p<0.0001$ ,  $p=0.02$ ,  $p=0.003$  respectively) (Figure 3 A). In addition, IL-4+ CD4+ T cells decreased in Fingolimod-treated compared to betaferon treated MS patients ( $p=0.0018$ ) (Figure 3 B). The percentage of IL17+ CD4+ T cells also were lower in Fingolimod-treated than Betaferon treated and treatment-naïve MS patients ( $p=0.001$ ,  $p<0.0001$ ; respectively) (Figure 3C). Furthermore, the percentage of IFN- $\gamma$ /IL17 co-producing CD4+ T cells in Fingolimod-treated patients before therapy and treatment naïve MS patients were higher than healthy controls ( $p=0.02$ ,  $p=0.01$ ; respectively) (Figure 3E).

### Fingolimod Treatment Decreases the Frequency of IFN- $\gamma$ and IL-17 as Well as IFN- $\gamma$ /IL17 Co-producing CD8+ T Cells

To characterize the Effects of Fingolimod treatment on cytokine-producing CD8+ T cell subsets, we determined the percentages of IFN- $\gamma$ , IL-4, IL-17, IL-10 cytokine producing CD8+ T cells (Tc1, Tc2, Tc17 and Tc10 respectively) and IFN- $\gamma$ /IL17 co-producing cells as a non-classical T cell subset in PBMCs from Fingolimod-treated MS patients and controls.

The percentage of IFN- $\gamma$  and IFN- $\gamma$ /IL-17 co-producing CD8+ T cells decreased after Fingolimod therapy compared to the baseline in total lymphocyte population (20.52% vs. 35.49%,  $p=0.001$  and 0.314% vs. 0.437%,  $p=0.03$ , respectively) (Figure. 4 A and E), whereas no significant alterations in IL-4 and IL-10 secreting CD8+ T cells were observed ( $p=0.46$  and  $p=0.08$ , respectively). In addition, the percentage of IL17+ CD8+ T cells was lower in Fingolimod treated patients compared to baseline (0.31% vs. 0.51%,  $p=0.009$ ) (Figure 4B).

The percentage of IFN- $\gamma$ + CD8+ T cells in Fingolimod-treated patients after therapy was lower than treatment naïve MS patients ( $p=0.001$ ) and higher than healthy controls ( $p=0.004$ ) (Figure 5A). Also, the percentages of IL-17+ CD8+ T cells were lower in Fingolimod-treated than treatment naïve MS patients ( $p=0.03$ ) (Figure 5 C). Furthermore, the percentages of IFN- $\gamma$ /IL-17 co-producing CD8+ T cells in Fingolimod-treated patients before and after therapy

and treatment naïve MS patients were higher than healthy controls ( $p<0.0001$ ,  $p=0.002$ ,  $p=0.0004$ ; respectively) (Figure 5E).

### Fingolimod Therapy Reduces the Expression of Transcription Factor IRF4

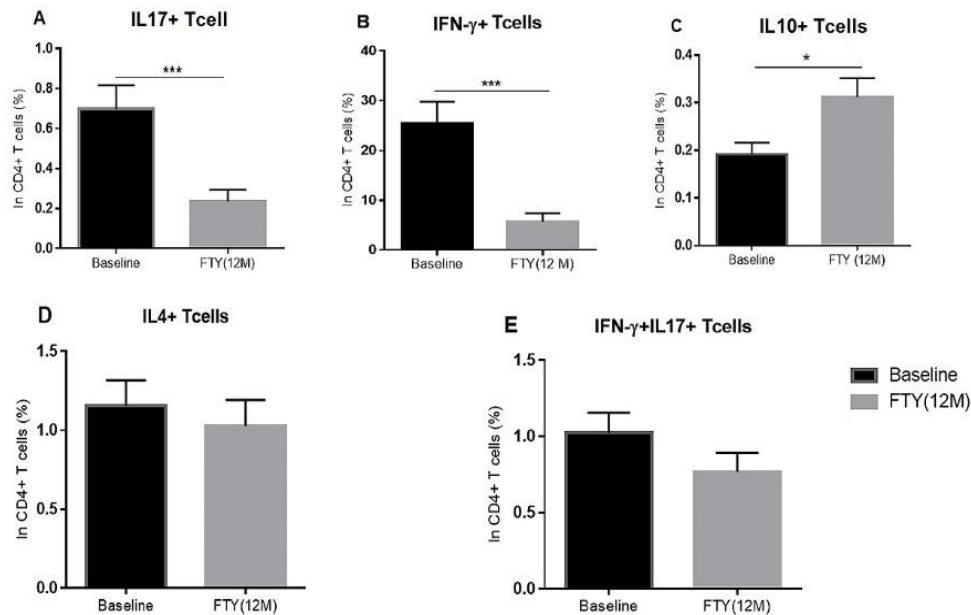
We evaluated the influence of Fingolimod on mRNA expression level of IRF-4, an essential transcription factor involved in Th cell differentiation, specifically Th17 cells. Our data revealed that the mRNA expression level of IRF-4 decreased in Fingolimod-treated patients compared to baseline (Before initiation) ( $p=0.02$ ) (Figure 6A). Healthy controls (HCs) had the lowest mRNA levels (Mean=0.92), and the highest mRNA level was found in treatment-naïve MS patients (Mean=25.76) (Figure 6B). Fingolimod-treated patients showed decreased expression levels compared to treatment naïve MS patients ( $p=0.04$ ). Furthermore, the expression level of IRF-4 in Fingolimod-treated patients before therapy and treatment naïve MS patients were higher than healthy controls ( $p<0.0001$ ,  $p=0.029$ , respectively) (Figure 6B). There was no significant correlation between the expression level of IRF-4 and frequency of Th17 or Tc17 cells in Fingolimod-treated patients and all different groups as well. Interestingly, we have seen an increase in expression of IRF-4 in two cases after Fingolimod treatment and sought their results of T cell subsets and cytokines. In both patients, the levels of IL-6 and IL-23 cytokines and EDSS score were high after Fingolimod treatment. In the patient with very high IRF-4 expression, the percentages of CD4+ T cells and IFN- $\gamma$ + CD8+ T cells was higher than the other cases in Fingolimod-treated group.

### Fingolimod Treatment Alters Th17 Cell Differentiating Cytokines

We analyzed the levels of several cytokines involved in 17+ T cell differentiation including IL-6, IL-23, and TGF- $\beta$ 1 in culture supernatants. Our results showed an elevated level of IL-6 in Fingolimod-treated MS patients after 12M therapy in comparison with baseline ( $p=0.03$ ), while the increase in TGF- $\beta$ 1 and IL-23 did not reach significance (Figure 7A). Betaferon treated patients had reduced concentrations of IL-23 cytokine in comparison to treatment naïve and Fingolimod-treated MS patients ( $p=0.04$  and  $p=0.036$ , respectively) (Figure 7B). Concentrations of TGF- $\beta$ 1 cytokine in Fingolimod-treated and betaferon treated

MS patients significantly increased compared to healthy individuals ( $p=0.046$  and  $p=0.04$ , respectively) (Figure 7B). We also investigated the associations between IRF-4 and IL-6, IL-23 and TGF- $\beta$ 1 cytokines.

However, we observed no association between the expression level of IRF-4 and those of cytokines in Fingolimod-treated patients and all different groups.



**Figure 2.** Fingolimod therapy alters the frequency of cytokine producing CD4+ T cell subsets. The percentages of IFN- $\gamma$ , IL17, IL4, IL-10 and IFN- $\gamma$ /IL17 cytokine producing CD4+ T cells in multiple sclerosis (MS) patients before starting Fingolimod (Baseline) and 12-months after therapy (FTY(12M)). Histogram figures represent mean  $\pm$ S.E.M. \* $p<0.05$ , \*\*\* $p<0.0001$ .

**Table 2.** Mean percentage of lymphocyte subsets in peripheral blood of MS patients and controls

Study Group	CD4+	CD8+	CD4+:CD8+ratio
FTY (12 M)	9.77 $\pm$ 3.8	21.75 $\pm$ 6.3	0.522 $\pm$ 0.34
FTY (0M)	35.23 $\pm$ 4.3	28.31 $\pm$ 7.6	1.366 $\pm$ 0.53
Betaferon treated	32.23 $\pm$ 2.7	24.72 $\pm$ 5.8	1.409 $\pm$ 0.52
treatment naive	36.32 $\pm$ 4.6	26.04 $\pm$ 5.3	1.414 $\pm$ 0.25
HC	32.45 $\pm$ 5.2	23.75 $\pm$ 3.5	1.397 $\pm$ 0.31
<i>p</i> -value	$p < 0.0001^*$	$p < 0.05^*$	$p < 0.0001^*$

FTY (0M): before initiation of Fingolimod treatment

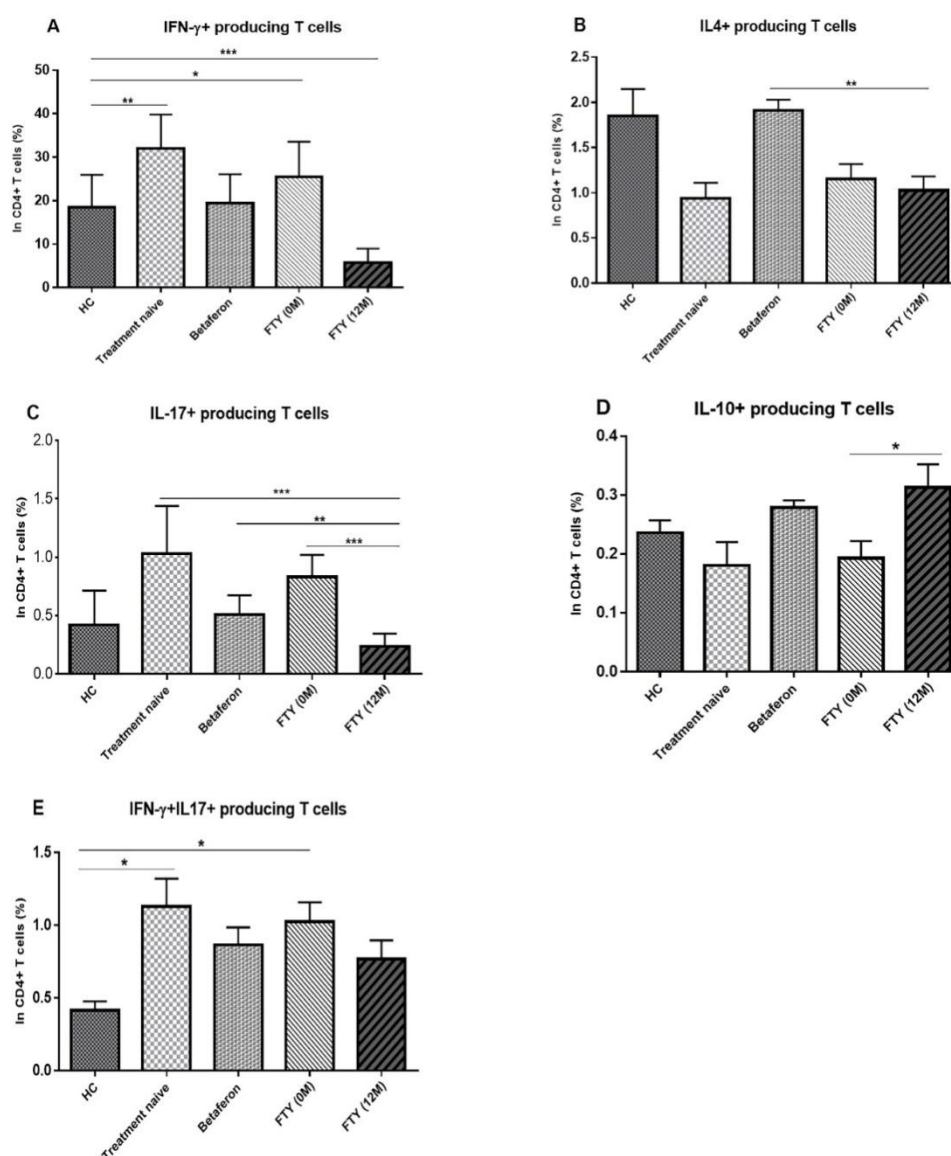
FTY (12 M): after 12 months Fingolimod treatment

HC: healthy controls

Data are expressed as mean $\pm$  SD.

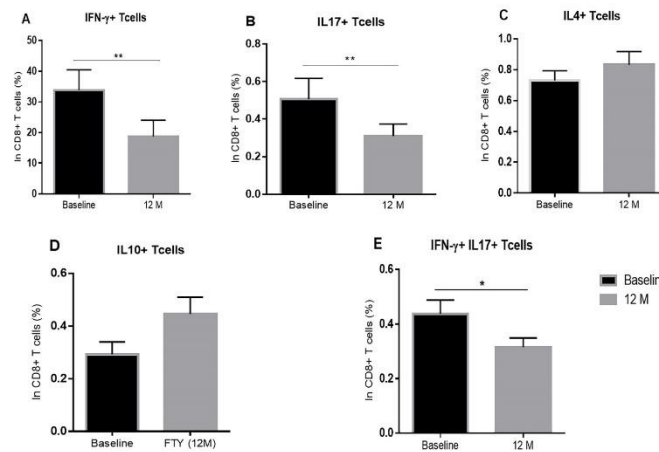
\* shows significant *p* values which calculated using One-Way ANOVA.

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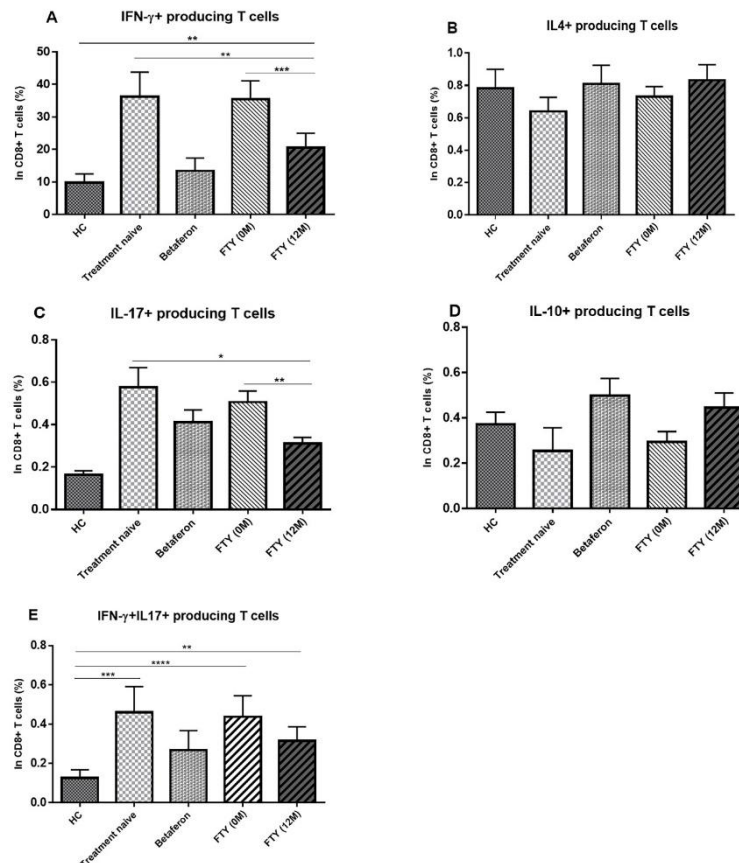


**Figure. 3** The frequency of cytokine producing CD4+ T cell subsets in multiple sclerosis (MS) patients and healthy controls (HC). The percentages of IFN- $\gamma$  (Th1), IL4 (Th2), IL17 (Th17), IL10 (Th10) producing and IFN- $\gamma$ /IL17 co-producing CD4+ T cells (Panels A-B-C-D-E) were analyzed in peripheral blood mononuclear cells (PBMCs) from healthy controls (HC; n=15), treatment naive MS patients (n=5), betaferon treated MS patients (n=15) and MS patients before starting Fingolimod (FTY; 0M, n=20) and after 12M Fingolimod therapy (FTY; 12M, n=16) by flow cytometry. Histogram figures represent mean $\pm$ S.E.M. \* $p$  < 0.05, \*\* $p$  < 0.01, \*\*\* $p$  < 0.0001.



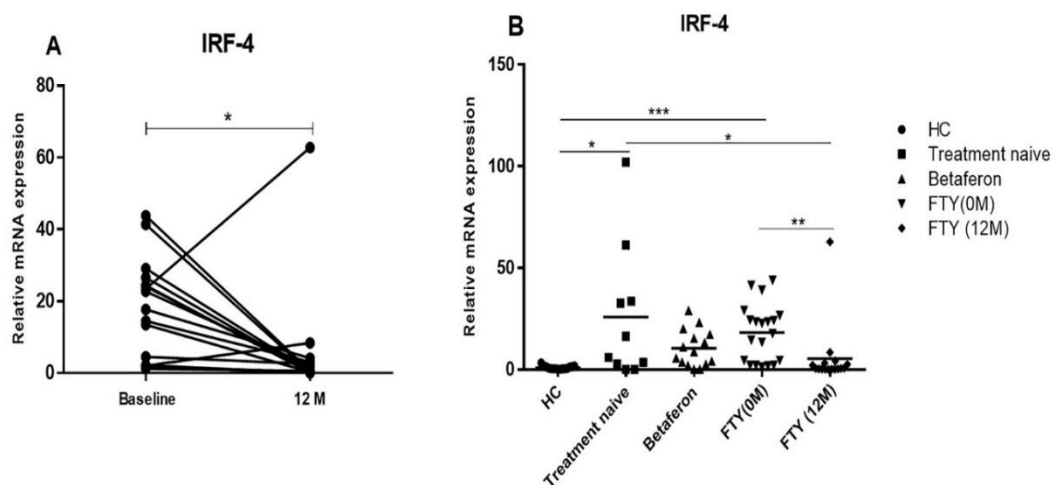


**Figure 4.** Fingolimod therapy alters the frequency of cytokine producing CD8+ T cell subsets. Percentages of IFN- $\gamma$ , IL17, IL4, IL10 and IFN- $\gamma$ /IL17 cytokine producing CD8+ T cells in MS patients before starting Fingolimod (Baseline) and 12 months after Fingolimod therapy (12M). Histogram figures represent mean  $\pm$  S.E.M. \* $p < 0.05$ , \*\* $p < 0.01$ .

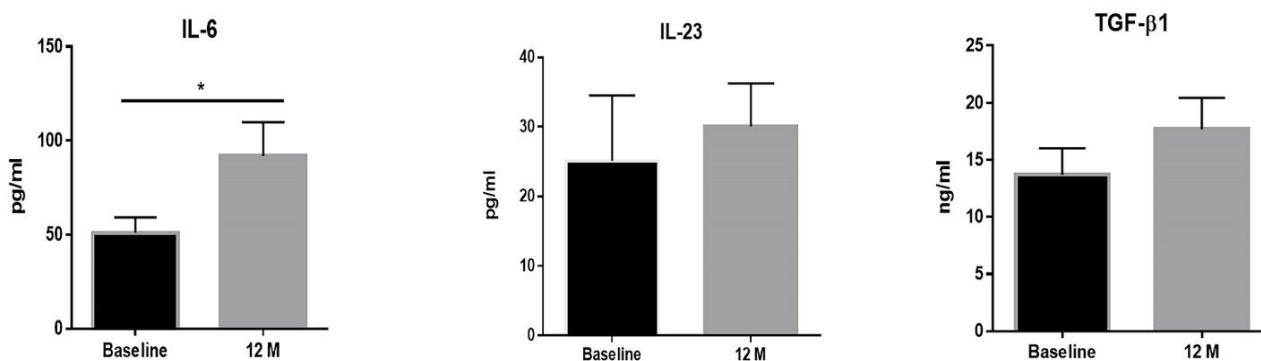


**Figure 5** The frequency of cytokine producing CD8+ T cell subsets in MS patients and HCs. Percentages of IFN- $\gamma$  (Tc1), IL4 (Tc2), IL17 (Tc17), IL10 (Tc10) producing and IFN- $\gamma$ /IL17 co-producing CD8+ T cells (Panels A\_B\_C\_D\_E) were analyzed in PBMCs of healthy controls (HC; n=15), treatment naive MS patients (n=5), betaferon treated MS patients (n=15) and MS patients before Fingolimod therapy (FTY 0M; n=20) and after 12M Fingolimod therapy (FTY 12M; n=16) by flow cytometry. Histogram figures represent mean  $\pm$  S.E.M. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ .

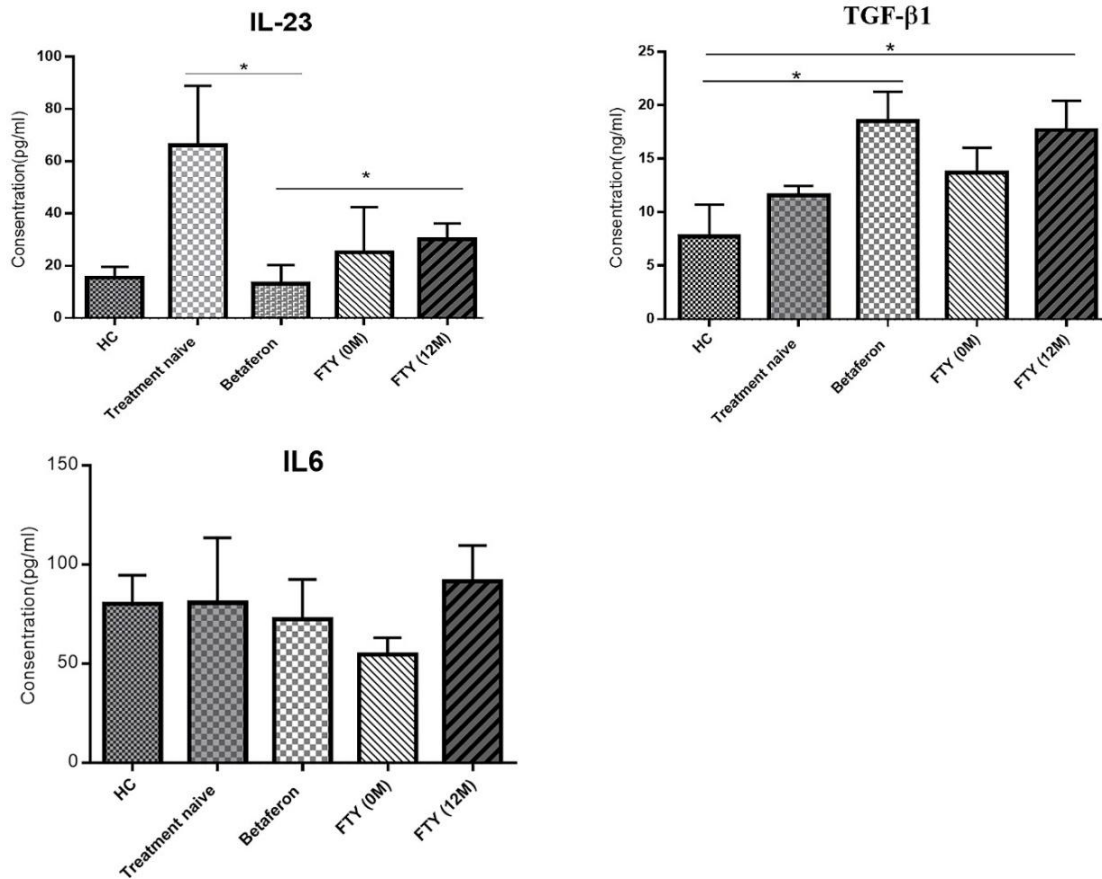
## T Cell Subset and IRF4 in Fingolimod Treated Patients



**Figure 6** (A) Fingolimod therapy reduces the expression level of transcription factor IRF-4. Fingolimod treated patients had the lower expression level after 12M therapy compared with baseline. (B) The expression level of IRF-4 in Fingolimod-treated patients and other groups. HCs had the lowest mRNA levels and mRNA levels increased in treatment-naïve and MS patients before Fingolimod therapy. Scatter dot plots show relative mRNA expression levels analyzed by Realtime-PCR. Horizontal lines represent the Mean value in all subgroups. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.0001$ .



**Figure 7** (A) 17+ T cells differentiating cytokines changed in fingolimod-treated multiple sclerosis (MS) patients after therapy. Fingolimod treated patients had an increase in IL-6, while the increase in TGF-β1 and IL-23 did not reach significance. Histogram figures represent mean ±S.E.M. (\* $p < 0.05$ ).



**Figure 7. (B) Production of 17+ T cell differentiating cytokines in Fingolimod-treated patients and other groups. Production of IL-6, IL-23 and TGF-β1 cytokines in anti-CD3/CD28 activated peripheral blood mononuclear cells (PBMCs) of Fingolimod-treated patients after 12 M therapy and other groups. Histogram figures represent mean±S.E.M. \**p*<0.05.**

## DISCUSSION

Multiple sclerosis is an inflammatory disorder of the central nervous system mediated by an autoimmune T cell response against myelin antigens. Besides Th1 and Th17 cells, recent data demonstrated that Tc17 cells are also implicated in MS immunopathogenesis.<sup>15-17</sup> Among immunomodulatory drugs for MS treatment, Fingolimod is the only oral drug which efficiently reduces MS relapses by inhibiting CD4+ and CD8+ T cells egress from lymph nodes resulting in a marked lymphopenia in the peripheral blood.<sup>40-42</sup>

In this 12M follow-up study, we determined the effect of Fingolimod on the frequency of different cytokine-producing CD4+ and CD8+ T cell subsets. Consistent with other studies,<sup>29,40,43</sup> we also found decreased percentages of both CD4+ and CD8+ T lymphocytes in the peripheral blood of MS patients with a predominant reduction in the CD4+ T cells.

Accordingly, CD4+/CD8+ T cell ratio decreased in peripheral blood as well.

Our data showed that the frequency of IL-17 and IFN-γ producing CD4+ T cells were reduced in Fingolimod-treated patients after 12M therapy compared to those of control groups. These results are in line with the study of Mehling et al.<sup>30</sup> They showed that FTY720 reduced the number of IL17+ CD4+ TCM cells, in peripheral blood of MS patients. However, a recent study by Song et al.,<sup>33</sup> showed that the percentages of both CD4+ and CD8+ IFN-γ producing T cells and Th17 cells increased transiently in the 2 weeks after initiation of therapy and decreased gradually to the pre-treatment levels which is compatible with Sato et al. 's studies.<sup>37</sup> They had explained that this increase might be due to a relative increase in TEM cells in pre-treatment stage, but after that, they are limited because of the absence of Th1 and Th17 replacement by naïve T cells which were trapped

in lymph nodes.

Interestingly, we found a higher frequency of IL-10 producing CD4+ T cells in Fingolimod-treated patients after 12M therapy in comparison with baseline. The reduction of Th1 and Th17 cells and higher frequency of Th10 cells could be the mechanism by which the drug alleviates the disease progression in MS.

In the present study, we assessed the frequency of different cytokine producing CD8+ T cell populations. Our data showed that frequency of IFN- $\gamma$ + and IL-17+, as well as IFN- $\gamma$ /IL-17 co-producing CD8+ T cells, decreased markedly in Fingolimod-treated patients after 12M, while the percentages of IL-4+ and IL-10+ CD8+ T cells did not differ between MS patients before and after 12M therapy. Consistent with our results, Serpero et al.<sup>36</sup> observed a significant decrease in IFN- $\gamma$  producing CD8+ T cells in Fingolimod-treated patients after 1M. However, in their experiments, the reduction of IL-17 producing T cells alone or in combination with IFN- $\gamma$  was not statistically significant. Song et al. reported that the percentage of IFN- $\gamma$  producing CD8+ T cells increased transiently at 2 weeks after initiation of Fingolimod and then decreased to the pre-treatment levels.<sup>33</sup> They also revealed that IFN- $\gamma$  and IL-4 producing CD8+ T cells increased in MS patients pre-treated with Fingolimod compared to healthy controls which is partly similar to our work.<sup>33</sup> Tc17 cells have been found among infiltrating cells in MS active lesions and peripheral blood of RRMS patients in relapse phase.<sup>15-18</sup> A decrease in frequency of Tc1 and Tc17 cells as well as IFN- $\gamma$ /IL17 cytokine co-producing CD8+ T cells, support the clinical benefits of Fingolimod treatment in MS disease.

In our study, the percentage of Tc1 cells decreased in Betaferon treated compared to treatment naive MS patients, while Tc17 cells increased in the blood of these patients compared to healthy controls. However, Peelen et al. showed lower Tc1 cell percentage, but no difference in Tc2, Tc10 or Tc17 cell frequencies in Betaferon treated compared to treatment naive MS patients.<sup>44</sup> The difference between the studies may be due to patient selection criteria.

The present study, for the first time, showed reduced transcript levels of transcription factor IRF-4 in Fingolimod-treated patients after therapy compared to baseline and treatment naive MS patients. In addition to the induction of ROR $\gamma$ t and activation of STAT3 in the differentiation of Th17 cells, it has now revealed that transcription factor IRF-4 also can acts as a

molecular activator of IL17 induction by cooperating with other factors.<sup>19</sup>

In previous results, patients treated with Fingolimod experienced profound lymphopenia in the peripheral blood, especially for memory T cells. Since IRF4 is preferentially expressed in memory T cells,<sup>45</sup> the present observation of a decreased expression of IRF4 in PBMC of Fingolimod-treated patients is probably a direct consequence of the decreased number of memory T cells in the circulation. Recent investigations in several autoimmune disease models indicated the essential role of IRF4 in the development of pathogenic Th17 cells. IRF4-deficient mice were protected from induction of EAE which is correlated with lack of Th17 differentiation.<sup>20</sup> Moreover, it was proposed that IRF4 has a role in effector CTL response and clearance of intracellular pathogens.<sup>45-47</sup> IRF4-deficient mice failed to produce CD8+ T cell-mediated immune responses during viral or bacterial infection.<sup>45</sup> These findings suggest that Fingolimod through decreased expression of transcription factor IRF-4 may prevent the development of pathogenic T cells.

According to our knowledge, few studies have investigated the production of pro-inflammatory cytokines in T cells from Fingolimod treated MS patients. In this respect, we evaluated the levels of differentiating cytokines related to IL17+ T cells. Our results showed that the level of IL6 significantly increased in Fingolimod treated patients after 12M therapy compared to baseline. A recent study by Blumenfeld et al.,<sup>32</sup> indicated that production of IL-6 and IL-10 from stimulated B cells significantly increased after 3 months of fingolimod therapy.

IL-6 is a pleiotropic cytokine that is present during inflammation and contributes to host defense.<sup>48</sup> We propose that an increase of this cytokine in Fingolimod treated patients may exert its effects in the host defense response by stimulating other cells in Fingolimod-treated patients. IL-6 is considered as a double-edged sword mediator because of both pro-inflammatory and potential neuroprotective functions. The elevated levels of IL-6 were seen in the serum of MS patients and were found to be significantly correlated with the number of relapses in MS patients.<sup>49,50</sup>

In this study, the levels of TGF- $\beta$ 1 was high in Fingolimod-treated, and betaferon treated MS patients compared to healthy individuals. However, the increase in the TGF- $\beta$ 1 and IL23 did not reach significance in Fingolimod treated patients compared to baseline.

TGF- $\beta$  is a regulatory cytokine which contributes to the differentiation of regulatory T cells (Tregs) and could ameliorate autoimmune disease.<sup>51</sup> On the other hand, it is well established that differentiation of IL17 producing T cells requires TGF- $\beta$  cytokine which represents the pro-inflammatory role of this cytokine.<sup>52</sup> A recent study showed an increase of TGF- $\beta$  during the remission phase of MS, pointing out this cytokine may be a good option in the treatment of MS.<sup>18</sup>

We have shown that Fingolimod-treated MS patients after 12-Months have reduced pro-inflammatory T cell subsets in the peripheral blood, including IL-17 and IFN- $\gamma$  producing CD4+ T cells and IFN- $\gamma$ + and IL-17+ as well as IFN- $\gamma$ /IL-17 co-producing CD8+ T cells. Fingolimod increased the frequency of IL-10 producing CD4+ T cells as well. This study for the first time has demonstrated the reduced level of transcription factor IRF-4 in MS patients after Fingolimod treatment.

These changes in T cell subsets, along with reduced expression of IRF-4, represents beneficial effects of this drug and may prevent the development of pathogenic T cells. However, these beneficial effects accompany some potential complications in MS patients concerning the immune response. While there is a decrease in relapses and progress of disease, on the other hand, patients have reduced CD4+ and CD8+ T lymphocyte in the peripheral Blood, which may affect the cellular immune response especially in controlling viral infections in long-term therapy. However, the remaining lymphocytes in the periphery have the potential to activate and secrete more IL-6 cytokine that helps immune defense in Fingolimod-treated MS patients.

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