Journal of Neuroimmunology 337 (2019) 577065

Contents lists available at ScienceDirect

Journal of Neuroimmunology

journal homepage: www.elsevier.com/locate/jneuroim

Impact of fingolimod on CD4+ T cell subset and cytokine profile of relapsing remitting multiple sclerosis patients

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ARTICLE INFO

Keywords:
Fingolimod
Sphingosine-1-phosphate receptor
Cytokine
Chemokine
T cell

ABSTRACT

Fingolimod inhibits the egress of lymphocytes from lymphatic tissues and also directly affects their functions by modulation of the sphingosine-1-phosphate receptor 1 (S1P1). Our aim was to evaluate the impact of fingolimod on diverse CD4+ T cell subsets, and cytokines.

Sixty-six relapsing remitting multiple sclerosis (RRMS) patients were treated with oral fingolimod (0.5 mg) for 6 months, and blood samples were collected at baseline, 3 months, and 6 months. Serum levels of seven cytokines and five chemokines were measured by multiplex immunoassay, and frequencies of peripheral blood mononuclear cell subsets were assessed by flow cytometry, and compared with those of 60 healthy controls.

CCL2 ($p = 0.039$), and CCL5 ($p = 0.001$) levels were significantly higher in fingolimod-treated patients than healthy controls, whereas end-of-study serum levels of IL-6, IL-8, IL-17A, IL-22, IL-23, TNF-α, CXCL10, and CXCL13 were comparable to the baseline levels. Six months of fingolimod treatment reduced CD3+ T cell (mean ± standard deviation, 72.9% ± 5.5 vs. 60.1% ± 11.1, $p < 0.001$), CD4+ T cell (62.2% ± 8.5 vs. 24.6% ± 12.9, $p < 0.001$), CD4+CD25hi regulatory T cell (Treg) (3.4% ± 1.3 vs. 2.0% ± 1.4, $p < 0.01$), and CD19+ B cell (13.2% ± 5.8 vs. 5.3% ± 2.7, $p < 0.001$) frequencies, while CD8+ T cells (31.8% ± 7.8 vs. 57.8% ± 13.2, $p < 0.001$) were increased, and NK and NKT cells remained unchanged. The proportions of intracytoplasmic IL-4, IL-10, IFN-γ, and TNF-α-producing T cells were increased, whereas IL-17-producing cells remained relatively constant as measured by flow cytometry.

Fingolimod appears to primarily diminish lymphocyte subsets involved in antigen presentation (CD19+ B and CD4+ T cells) rather than immune cells (CD8+ T, NK, and NKT cells) in charge of host defense against pathogens. In contrast, a relative increase is observed in pro- and anti-inflammatory cytokine-producing T helper subsets (IFN-γ, TNF-α, IL-4, and IL-10-producing CD4+ T cells), suggesting that effector T cells are suppressed to a lesser degree by S1P1 modulation.

1. Introduction

Multiple sclerosis (MS) is a demyelinating and neurodegenerative disease of autoimmune origin (Tahmasebinia and Pourgholaminejad, 2017; Sumida et al., 2018; Dargahi et al., 2017). A complex interplay of both innate (mainly macrophages and microglia) and adaptive (B and T cells) immune cell interactions, in which many cytokines, chemokines, transcription factors, and receptors are involved, occurs throughout the inflammatory process in MS (Dargahi et al., 2017; Reich et al., 2018).

CD4+ T helpers appear to be the key drivers of the neuroinflammation in MS. It is well established that Th1 (producing IL-2, IL-12, IFN-γ, and TNF-α) and Th 17 cells (IL-17A, IL-17F, IL-21, IL-22, IL23, IL-6, IL-9, and IFN-γ) are the CD4+ T cell subsets that activate the neuroinflammatory cascade via proinflammatory cytokines they release (Tahmasebinia and Pourgholaminejad, 2017; Dargahi et al., 2017). In recent years, CD8+ (T cytotoxic cells) lymphocyte subsets with distinct

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https://doi.org/10.1016/j.jneuroim.2019.577065
Received 25 July 2019; Received in revised form 7 September 2019; Accepted 10 September 2019

pro-inflammatory or anti-inflammatory cytokine profiles were identified and shown to play a role in the immunopathogenesis of MS, as well. CD8+ cells are abundant within demyelinating lesions and cerebrospinal fluid (CSF), much more than CD4+ cells, irrespective of the stage and activity of the disease (Dargahi et al., 2017; Dendrou et al., 2015). The other cellular component of adaptive immunity, CD19+ B cells also contribute to neuroinflammation in MS not only by secreting autoantibodies and pro-inflammatory cytokines but also by activating T cells due to their antigen presenting cell properties. (Dargahi et al., 2017; Li et al., 2017; Claes et al., 2014). Moreover, regulatory T cells (Treg) are important in maintaining tolerance against self-antigens and preventing autoimmunity. Although the frequency of Tregs in MS patients is usually comparable to that in healthy individuals, the cells are functionally impaired especially in the early stages of the disease (Dargahi et al., 2017; Luckheeram et al., 2012).

In parallel to the advances in elucidating the pathogenesis of MS, new immunomodulatory therapeutic options targeting different mechanisms were developed since mid-1990s with an intention to modify the course of disease (Dargahi et al., 2017). Fingolimod is one of these disease modifying agents approved for the treatment of relapsing remitting MS (RRMS) with demonstrated beneficial effects in reducing the flare ups, disability progression, and MRI outcome measures (Calabresi et al., 2014; Cohen et al., 2010; Kappos et al., 2010). It mainly exerts its effects via inhibiting autoreactive immunological response at lymph node level. It acts as a 'super agonist' of the sphingosine-1-phosphate receptor 1 (S1P1) on lymphocytes inducing internalization of the receptor by the cell. This renders these cells unresponsive to S1P1 signaling, thus depriving them of a signal necessary for egress from secondary lymphoid tissues (Rudnicka et al., 2015; Scott, 2011) This in turn results in marked reduction in the number of both B and T lymphocytes in the intravascular compartment and a decrease in migration of the activated effector lymphocytes to extravascular compartments, including the central nervous system.

In addition to inhibiting the egress of lymphocytes from lymphatic tissues, fingolimod alters the functions of specific lymphocyte subsets through S1P1 modulation (Dominguez-Villar et al., 2019). In this study, we investigated the impact of fingolimod on the composition of phenotypically and functionally distinct CD4+ T cell subsets and profile of associated cytokines and chemokines in the peripheral blood of RRMS patients. Although impact of fingolimod on lymphocyte functions is extensively studied, the entire panel of cytokines and immunity types that are affected by fingolimod treatment still need to be characterized. The distinguishing features of our study were the high number of fingolimod-treated participants, the broad panel of cytokines and chemokines investigated through serum level and/or intracellular measurements and the special emphasis given to Treg subsets.

2. Methods

2.1. Subjects

In this multicenter, prospective, interventional, open label study, 66 RRMS patients were treated with oral fingolimod 0.5 mg/day for six months and assessed every three months. Blood samples for cytokine measurements and flow cytometry were collected at baseline, three and six months after initiating treatment with fingolimod. A single blood sample was taken from each age and gender matched healthy volunteer (n = 60) who served as healthy control (HC) group.

Included patients were (i) previously non-treated/newly-diagnosed patients (having displayed ≥2 serious attacks within one year or ≥3 serious attacks within two years and ≥1 gadolinium-enhancing lesions in cranial MRI or significantly increased T2 lesions compared to the previous cranial MRI) or (ii) non-responders to first-line treatments (beta-interferon or glatiramer acetate) or (iii) patients who were unable to tolerate first-line treatments due to adverse effects associated with parenteral administration or (iv) not having a relapse in the last two months and not having been treated with immunosupressive or immunomodulating agents in the last three months. Criteria for being a non-responder were absence of a change or increase in number of attacks or having more severe attacks or displaying ≥1 contrast enhancing lesions in cranial MRI or displaying an increase in T2 lesions identified with successive MRIs despite treatment with an adequate dose of beta-interferon, glatiramer acetate or teriflunomide for at least one year. Patients with secondary progressive MS, coexisting autoimmune diseases, cardiovascular conditions, history of malignancy, pregnancy, history of previous fingolimod therapy, clinically active infections, and known contraindications for fingolimod treatment were excluded. All patients were screened for varicella-zoster virus IgG and antibody-negative patients were not included.

The study protocol was approved by the Clinical Research Ethics Committee of the Istanbul University Medical Faculty and the Turkish Medicines and Medical Devices Agency of the Ministry of Health. The study was conducted in accordance with the ethical principles of the Declaration of Helsinki. All subjects provided written informed consent prior to any study related procedure.

2.2. Serum cytokine measurements

All sera were stored and kept frozen at −80 °C until assayed. The serum levels of the cytokines and chemokines were measured using multiplex immunoassays (Invitrogen, Carlsbad, CA, USA). The levels of IL-4, IL-6, IL-17A, IL 22, IL-23, IFN-γ, TNF-α, IL-8, CCL2 (MCP-1), CCL5 (RANTES), CXCL10 (IP-10), and CXCL13 were quantified by reference to standard curves and results were expressed as pg/ml or ng/ml.

2.3. Flow cytometry for lymphocyte subgroups

Peripheral blood ratios of selected lymphocyte subgroups were measured by flow cytometry only in 28 RRMS patients and 20 HCs with sufficient available peripheral blood samples. Fresh venous blood stained for 30 min at 4 °C with anti-human monoclonal antibodies: anti-CD3-PE, anti-CD4-FITC, anti-CD8-APC, and anti-CD25-APC obtained from Becton Dickinson (BD) (Franklin Lakes, NJ, USA). At the same time, absolute counting tubes (BD) and four-color direct immunofluorescence agents (anti-CD3-FITC, anti-CD16/anti-CD56-PE, anti-CD45-PerCP, anti-CD19-APC, BD) was also used.

For intracytoplasmic cytokine measurements, peripheral blood mononuclear cells (PBMCs) were separated by Ficoll density gradient centrifugation. Cells were incubated 4 h with 0.25 μg/mL PMA (AdipoGen, San Diego, CA, USA), 1 μg/mL ionomycin (Santa Cruz, Dallas, TX, USA), and 10 μg/mL brefeldin (Ebioscience, Santa Clara, CA, USA) in complete medium (RPMI 1640 enriched with 10% fetal calf serum, 1% minimum essential medium vitamin, 1% L-glutamine, 1% Na-pyruvate, 1% nonessential amino acids, 1% penicillin-streptomycin; Gibco, Waltham, MA, USA) at 37 °C in humidified 5% CO_2 chamber. Afterwards, cells were surface stained with anti-CD4-FITC, anti-CD8-APC, and anti-CD25-APC (all from BD). Then, PBMCs were fixed/permeabilized (FoxP3 Staining Buffer Set, eBioscience) and stained with an anti-IFN-γ-PE, anti-IL-10-PE, anti-TNF-α-PE, anti-IL-17A-PerCP, and anti-IL-4-PerCP (eBioscience) in accordance with the manufacturer's instructions. All immune cell staining experiments were utilized in BD FACS Calibur and data were analyzed using the FlowJo software.

2.4. Statistical analysis

Cytokine/chemokine levels and peripheral blood proportions of immune system cells of HC and RRMS groups were compared with ANOVA and Tukey's post hoc test. Immunological parameters of treatment subgroups were compared with Student's t-test. Correlation studies were conducted with Pearson's correlation test. $p < 0.05$ was considered as statistically significant.

3. Results

3.1. Demographics and baseline characteristics

Serum samples of 66 RRMS patients [mean age: 35.7 ± 8.2 years, gender (Female/Male): 46/20] and age/gender matched 60 HCs [mean age: 34.7 ± 9.6 years, gender (Female/Male): 40/20] were analyzed. While mean age of onset of disease was 29.0 ± 8.1 years, mean duration of disease was 6.7 ± 4.9 years. There were 64 treated (97%) and 3 untreated (3%) RRMS patients in the study. During the previous 2 years, mean number of relapses requiring the use of corticosteroid treatment was 1.2 ± 0.9 and MS relapse number was 1.5 ± 0.9 in RRMS patients. Out of 66 RRMS patients, 38 had switched from beta-interferon, 23 had switched from glatiramer acetate and 5 had switched from teriflunomide to fingolimod treatment.

3.2. Cytokine and chemokine levels

The baseline serum levels of all cytokines and chemokines were statistically comparable among RRMS patients and HCs. Likewise, there were no significant differences among baseline and month six values of RRMS patients for any of the investigated mediators. Only the serum CCL2 (from 76 ± 79 to 93 ± 101 ng/ml; $p = 0.039$) and CCL5 (from 368 ± 273 to 487 ± 469 ng/ml; $p = 0.001$) levels showed a significant gradual increase under fingolimod treatment. After six months of fingolimod treatment, RRMS patients had significantly higher CCL2 and CCL5 levels than HCs. Levels of IL-6 and IL-23 showed trends towards declining after fingolimod treatment without attaining statistical significance (Table 1). IL-4 and IFN-γ levels of RRMS patients were below the detection level of the immunoassay and therefore could not be measured.

3.3. Peripheral blood immunophenotyping

First major PBMC subgroup proportions (CD3+, CD19+, NK, and NKT cells) were evaluated in the lymphocyte gate and then CD4+, CD8+ and CD4+CD25+ cell populations were analyzed in the CD3 gate. At baseline, the frequencies of peripheral blood CD3+ T cells, CD4+ T cells, CD8+ T cells, CD19+ B cells, CD4CD25hi Treg cells, CD16CD56hi natural killer (NK) cells, and NKT cells were similar in HC and RRMS groups. The frequencies of CD3+ T cells (72.9% ± 5.5, 56.8% ± 12.7, and 60.1% ± 11.7 at baseline, month 3, and month 6, respectively; $p < 0.0001$), CD4+ T cells (62.2% ± 8.5, 23.7% ± 12.7, and 24.6% ± 12.9 at baseline, month 3, and month 6, respectively; $p < 0.0001$), CD19+ B cells (13.2% ± 5.8, 5.9% ± 4.0, and 5.3% ± 2.7 at baseline, month 3, and month 6, respectively; $p < 0.0001$), and CD4+CD25hi Treg cells (3.4% ± 1.3, 2.0% ± 1.4, and 1.9% ± 1.4 at baseline, month 3, and month 6, respectively; $p = 0.0004$) sharply declined 3 months after starting fingolimod and remained significantly lower compared with baseline at month 6 as well. The reduction was most prominent in B cells. While RRMS patients exhibited significantly lower proportions of CD3+, CD4+, and CD19+ cells than HCs at months 3 and 6, CD4CD25hi Treg cell frequencies of RRMS patients were not significantly different than those of HCs at any time point. In contrast with CD3+, CD4+, CD19+, and CD4+CD25hi cells, relative frequencies of CD8+ T cells (31.8% ± 7.8, 58.6% ± 12.6, and 57.9% ± 13.2 at baseline, month 3, and month 6, respectively; $p < 0.0001$), NK cells, and NKT cells increased after initiation of fingolimod both at months 3 and 6. The absolute numbers of NK cells and NKT cells were not affected (Figs. 1 and 2).

Next, T helper subsets producing specific cytokines were measured in the CD4 gate. Before fingolimod treatment, the frequencies of CD4+ and CD4+CD25+ cell subsets producing different cytokines were comparable among RRMS patients and HCs. Fingolimod treatment significantly increased CD4+ T cell subsets producing anti-inflammatory cytokines IL-4 and IL-10. At month six, RRMS patients had significantly increased CD4+IL-10+ cell proportions as compared to baseline values (0.74% ± 0.75, 1.4% ± 1.6, and 2.4% ± 2.2 at baseline, month 3, and month 6, respectively; $p = 0.0019$) and higher CD4+IL-4+ cell frequencies than HCs (1.4% ± 1.2), baseline and month 3 values (0.55% ± 0.46, 1.5% ± 1.2, and 2.5% ± 1.8 at baseline, month 3, and month 6, respectively; $p < 0.0001$). As for CD4+ T cell subsets producing inflammatory cytokines, fingolimod treatment significantly increased frequencies of CD4+IFN-γ+ (3.6% ± 4.8, 7.0% ± 9.1, and 13.7% ± 12.4 at baseline, month 3, and month 6, respectively; $p = 0.0019$) and CD4+TNF-α+ (24.8% ± 20.8, 29.2% ± 23.7, and 48.6% ± 24.0 at baseline, month 3, and month 6, respectively; $p = 0.0010$) but not CD4+IL-17+ cells. At month 6, RRMS patients under fingolimod treatment had higher frequencies of IFN-γ and TNF-α-producing CD4+ cells than non-treated RRMS patients. There was also a significant difference among frequencies of CD4+TNF-α+ cells at 3 and 6 months of fingolimod treatment (Fig. 3).

Fingolimod treatment also enhanced frequencies of IL-4 and IL-10-producing CD4+CD25+ T cells. After six months of fingolimod treatment, RRMS patients showed significantly increased CD4+CD25+IL-4+ (0.58% ± 0.68, 1.2% ± 0.9, and 1.46% ± 1.3 at baseline, month 3, and month 6, respectively; $p = 0.0016$) and CD4+CD25+IL-10+ (1.7% ± 1.8, 2.8% ± 2.1, and 4.0% ± 3.7 at baseline, month 3, and

Table 1
Serum cytokine and chemokine levels of healthy controls (HC) and relapsing remitting multiple sclerosis (RRMS) patients at 0, 3, and 6 months (M) of fingolimod treatment.

					p values for Tukey's post-hoc test						
	HC	RRMS 0 M	RRMS 3 M	RRMS 6 M	ANOVA p-value	HC vs 0 M	HC vs 3 M	HC vs 6 M	0 M vs 3 M	0 M vs 6 M	3 M vs 6 M
IL-6 (pg/ml)	0.9 ± 2.3	1.7 ± 5.5	1.4 ± 5.7	0.8 ± 3.1	0.653	ns	ns	ns	ns	ns	ns
IL-17A (pg/ml)	1.2 ± 0.2	1.1 ± 0.2	1.1 ± 0.3	1.0 ± 0.2	0.123	ns	ns	ns	ns	ns	ns
IL-22 (pg/ml)	22 ± 4.3	23 ± 4.6	23 ± 5.1	23 ± 6.3	0.879	ns	ns	ns	ns	ns	ns
IL-23 (pg/ml)	1.3 ± 3.9	5.0 ± 2.0	2.8 ± 6.3	2.1 ± 5.0	0.064	ns	ns	ns	ns	ns	ns
TNF-α (pg/ml)	0.9 ± 0.6	1.2 ± 0.9	1.1 ± 0.7	1.1 ± 1.0	0.459	ns	ns	ns	ns	ns	ns
CCL2 (ng/ml)	53 ± 31	76 ± 79	74 ± 74	93 ± 101	0.039	ns	ns	*	ns	ns	ns
IL-8 (pg/ml)	3.2 ± 3.3	3.6 ± 3.4	4.0 ± 3.6	4.3 ± 3.9	0.455	ns	ns	ns	ns	ns	ns
CCL5 (ng/ml)	212 ± 159	368 ± 273	374 ± 272	487 ± 469	0.001	ns	ns	***	ns	ns	ns
CXCL10 (ng/ml)	16 ± 12	22 ± 22	20 ± 22	21 ± 22	0.385	ns	ns	ns	ns	ns	ns
CXCL13 (pg/ml)	111 ± 158	103 ± 88	117 ± 175	107 ± 159	0.967	ns	ns	ns	ns	ns	ns

Cytokine and chemokine levels are denoted as mean ± standard deviation.
ns: statistically not significant.
* $p < 0.05$.
*** $p < 0.001$.

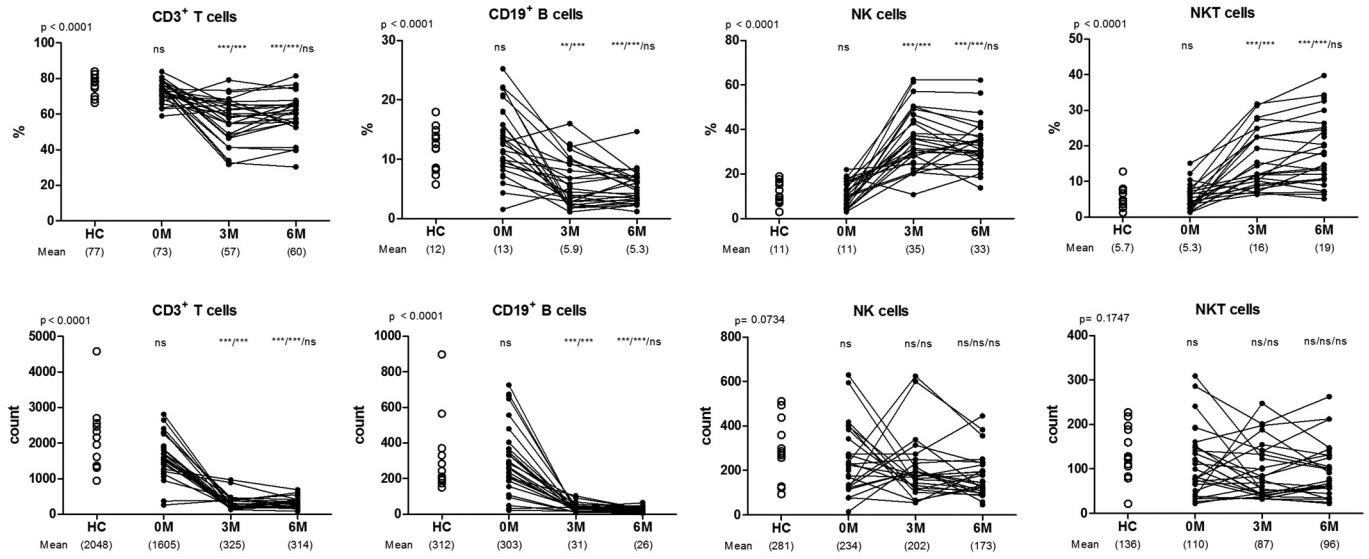

Fig. 1. Analyses of the percentage and counts of T cells, B cells, NK cells, and NKT cells (analyzed at the lymphocyte gate) in the peripheral blood of healthy controls (HC) and relapsing remitting multiple sclerosis (RRMS) patients obtained at baseline (0), 3, and 6 months (M) of fingolimod treatment. p-values for ANOVA are indicated on the upper left corner of the panel. Numbers in brackets indicate mean values for each group. Asterisks on the top of the panel indicate Tukey's post-hoc test p-values for comparisons with HC, 0 M, and 3 M, consecutively from left to right. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ns, not significant ($p \geq 0.05$).

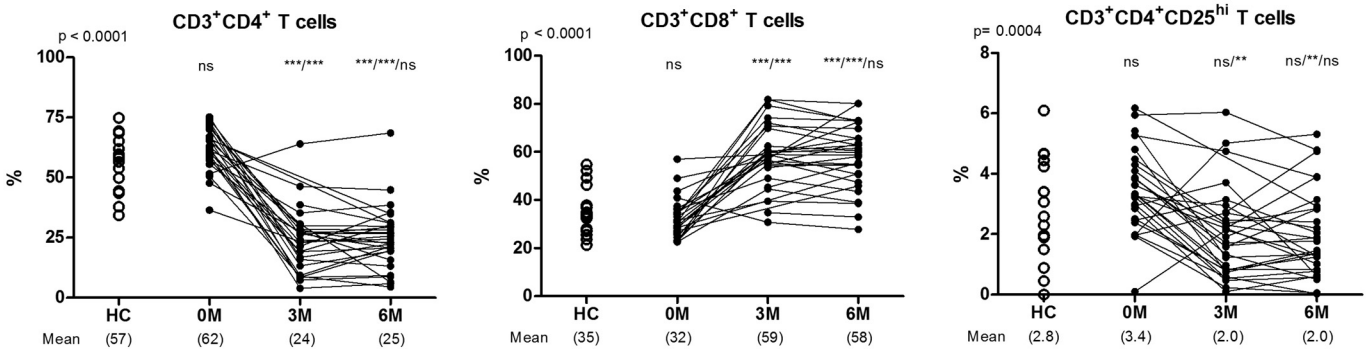

Fig. 2. Analyses of the percentage of CD4+ T cells, CD8+ T cells, and CD4+CD25hi regulatory T cells (analyzed at the CD3 gate) in the peripheral blood of healthy controls (HC) and relapsing remitting multiple sclerosis (RRMS) patients obtained at baseline (0), 3, and 6 months (M) of fingolimod treatment. p-values for ANOVA are indicated on the upper left corner of the panel. Numbers in brackets indicate mean values for each group. Asterisks on the top of the panel indicate Tukey's post-hoc test p-values for comparisons with HC, 0 M, and 3 M, consecutively from left to right. **, $p < 0.01$; ***, $p < 0.001$; ns, not significant ($p \geq 0.05$).

month 6, respectively; $p = 0.0163$) cell frequencies than their baseline values. Frequencies of these cell subsets were comparable to those of HCs at all time points (Fig. 4).

3.4. Association between clinical features and immunological results

To find out the impact of clinical features on immunological parameters in fingolimod-treated patients, we investigated the correlations between duration of MS, MS relapses in the last two years and MS relapses requiring steroid treatment in the last two years versus all investigated immunological parameters measured at month 6. Among cytokines only IL-6 levels showed significant correlation with the total number of MS relapses in the last two years ($R = 0.352$, $p = 0.008$). Moreover, proportions of CD4+IL-17+ cells were correlated with the total number of MS relapses ($R = 0.493$, $p = 0.014$) and number of MS relapses requiring steroid treatment in the last two years ($R = 0.476$, $p = 0.017$). Secondly, MS patients were divided as those that switched from beta-interferon to fingolimod treatment (n = 38) and those that switched from other medications to fingolimod treatment (n = 28) and all investigated immunological parameters were compared among these two groups using Student's *t*-test. No significant changes were found between two treatment subgroups.

4. Discussion

Levels of most of the examined immunological mediators did not show a continual and significant alteration under fingolimod treatment. Thus, serum cytokine evaluation suggests that, different from other immunomodulating agents such as interferon-beta (Mei et al., 2006), fingolimod does not tip the balance to any of the well-characterized T helper immunity types. However, fingolimod treatment gradually increased serum levels of chemokines CCL2 and CCL5, which are well associated with MS pathogenesis (van Veen et al., 2007; Semple et al., 2010). Particularly, CCL2 is highly expressed in MS lesions and according to experimental animal studies of MS models, CCL2 released by glial cells significantly contribute to the recruitment of inflammatory infiltrates (monocytes, T cells, and dendritic cells) into the central nervous system (Mahad and Ransohoff, 2003; O'Sullivan et al., 2018). As to the flow cytometry results, peripheral blood CD3+, CD4+, and CD19+ lymphocyte counts were significantly decreased by fingolimod treatment and the most notable reduction occurred in CD19+ cell percentages indicating that fingolimod is both a T and B-cell inhibitor. By contrast, CD8+ and CD16+CD56+ NK cell percentages were increased by fingolimod treatment. These results are generally in agreement with previous studies (Claes et al., 2014; Dominguez-Villar et al.,

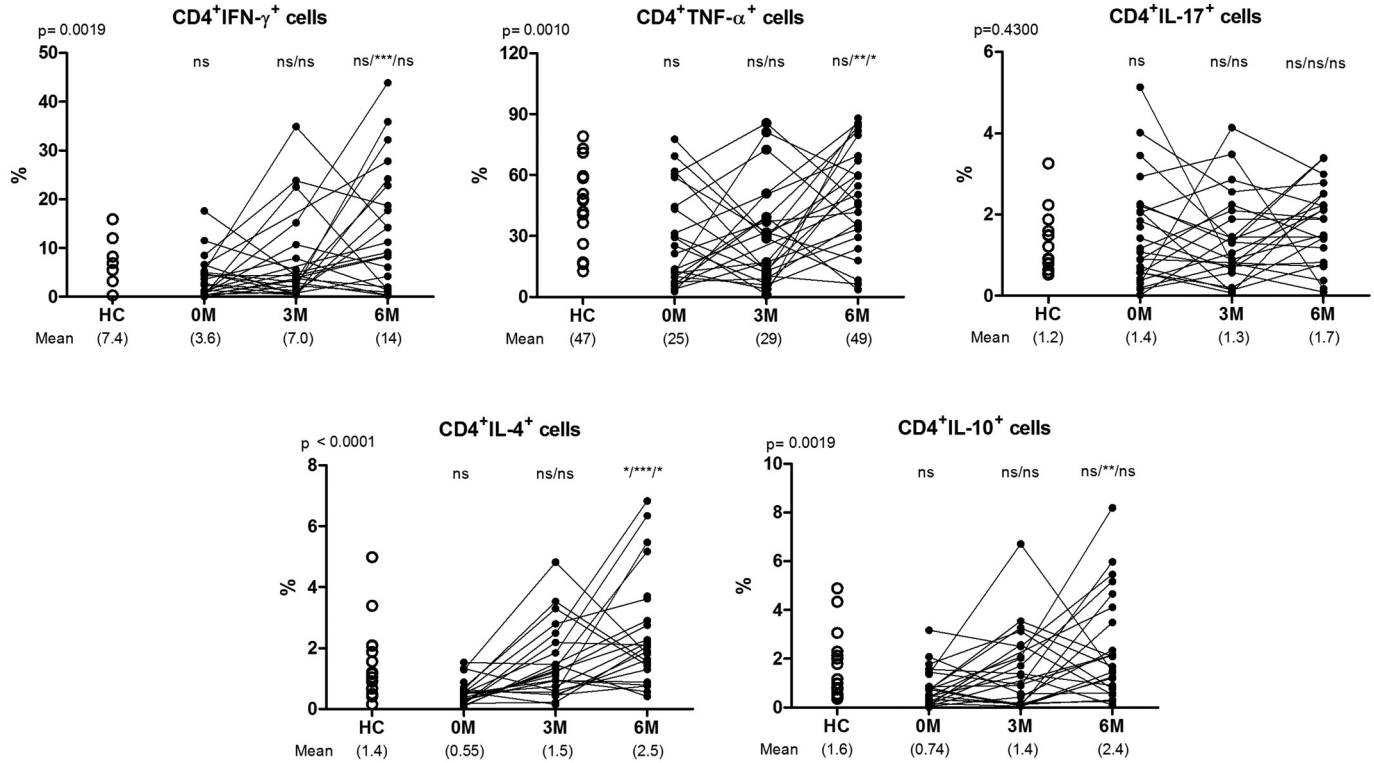

Fig. 3. Impact of fingolimod treatment on CD4+ T cell subset proportions in peripheral blood. Open circles indicate healthy controls (HC), whereas closed circles represent relapsing remitting multiple sclerosis (RRMS) patients at baseline (0), 3, and 6 months (M) of fingolimod treatment. p-values for ANOVA are indicated on the upper left corner of the panel. Numbers in brackets indicate mean values for each group. Asterisks on the top of the panel indicate Tukey's post-hoc test p-values for comparisons with HC, 0 M, and 3 M, consecutively from left to right. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ns, not significant ($p \geq 0.05$).

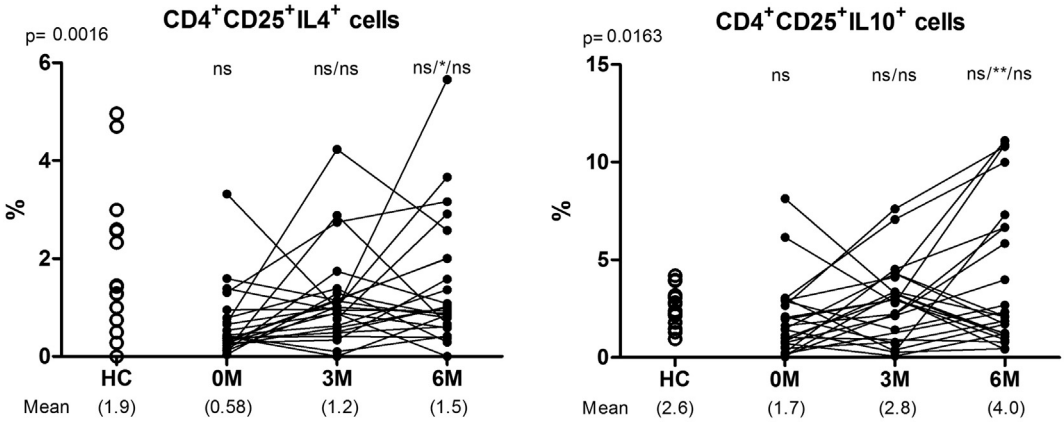

Fig. 4. Impact of fingolimod treatment on CD4+CD25+ T cell subset proportions in peripheral blood. Open circles indicate healthy controls (HC), whereas closed circles represent relapsing remitting multiple sclerosis (RRMS) patients at baseline (0), 3, and 6 months (M) of fingolimod treatment. p-values for ANOVA are indicated on the upper left corner of the panel. Numbers in brackets indicate mean values for each group. Asterisks on the top of the panel indicate Tukey's post-hoc test p-values for comparisons with HC, 0 M and 3 M, consecutively from left to right. *, $p < 0.05$; **, $p < 0.01$; ns, not significant ($p \geq 0.05$).

2019; Muls et al., 2014; Song et al., 2015; Nakhaei-Nejad et al., 2017; Grützke et al., 2015) and might suggest that fingolimod treatment does not notably affect the immune cell types that are associated with cytotoxic responses directed against pathogen microorganisms (e.g. CD8+ T cells, NK cells) and yet effectively suppress cell types involved in autoimmunity (e.g. CD4+ T cells and B cells). By this way, fingolimod treatment does not seem to affect host defense mechanisms and at the same time prevents effector immune cells from reaching the central nervous system.

In contrast with major lymphocyte subtypes, considerable discrepancies are observed among different studies when specific CD4+ T cell subsets are examined. Our results showed a gradual increase in IL-4 (Th2), IL-10 (Treg), IL-17 (Th17), IFN-γ, and TNF-α (Th1)-producing CD4+ cells indicating that fingolimod does not favor any particular Th-type immune response. A global increase in prevalence of major Th types also suggests that fingolimod has a lesser effect on the egress of cytokine-producing effector T cells and mostly prevents the migration of naïve T cells that have not yet polarized to a specific Th-type response, as reported previously (Dominguez-Villar et al., 2019; Song et al., 2015). Relative frequencies of TNF-α, IFN-γ (Sugimoto et al., 2016), IL-17 (Song et al., 2015; Sato et al., 2014), and IL-10 (Dominguez-Villar et al., 2019; Laribi et al., 2018) producing CD4+ T cells are enhanced by fingolimod treatment. Likewise, we found increased proportions of IL-4, IL-10, IFN-γ, and TNF-α-producing T cells

in fingolimod-treated patients, whereas proportions of IL-17-producing T cells were not significantly altered.

By contrast, some other studies have shown decreased frequencies of IL-17 or IFN-γ-producing CD4+ T cells in peripheral blood of fingolimod-treated MS patients (Dominguez-Villar et al., 2019; Laribi et al., 2018). However, these alterations have occurred 12 months after fingolimod treatment, whereas, in our study, a substantial alteration occurred in frequencies of these cells after six months of fingolimod treatment. The enhancing effect of fingolimod on IL-17 and IFN-γ-producing cells is probably transient, emerging in the first few months of treatment and gradually fading away thereafter (Song et al., 2015). Moreover, proportions of CD4+ cell subsets show a significant variation among fingolimod-treated MS patients (Sato et al., 2014). A determinant of this variation is disease exacerbation, which probably makes patients more prone to display increased CD4+ subset proportions (Sato et al., 2014). Secondly, MS patients who have switched from interferon-beta to fingolimod show trends towards displaying higher peripheral blood CD4+IL-17+ and CD4+IFN-γ + cell proportions as compared to treatment-naïve patients (Song et al., 2015). However, we did not find a significant difference between previously interferon-beta treated or non-treated patients by means of levels of cytokines and cell subsets ruling out this assertion. Nevertheless, several clinical variables may modulate CD4+ cell subset frequencies towards an increase or decrease and may cause the discrepancies among different studies. In any case, fingolimod does not appear to preferentially interfere with Th1 and Th17 responses and thus Th1 cells find an opportunity to relatively increase in the peripheral blood. However, since the number of CD4+ cells in the peripheral blood is drastically reduced in fingolimod-treated MS patients, this relative enhancement of inflammatory cytokine-producing T cells probably does not have an immunopathological implication.

An important finding of our study was the gradual decrease in CD4+CD25hi Tregs under fingolimod treatment and was in accordance with previous reports (Muls et al., 2014; Serpero et al., 2013). While some other fingolimod studies have reported increased Treg subset proportions (CD127 negative or CD39 positive Tregs) (Dominguez-Villar et al., 2019; Muls et al., 2014; Song et al., 2015), these and other Treg subsets were not included in our analysis.

Although elevation of IL-4 and IL-10 production is associated with favorable clinical course in both MS and its animal model (Casella et al., 2016; Valenzuela et al., 2007; Putheti et al., 2003; Krakauer et al., 2008; Zorzella-Pezavento et al., 2017), T cell populations producing these cytokines were relatively understudied in MS patients under fingolimod treatment. In our study, proportions of IL-4 and IL-10-producing CD4+ T cell and CD4+CD25+ Treg subsets were increased. Previously, IL-10-producing CD4+ T cells were increased at 12 months under fingolimod treatment (Laribi et al., 2018). Fingolimod-induced enhancement of IL-4 and IL-10-producing anti-inflammatory T cell subsets is a notable finding and this effect may one of the mechanisms by which fingolimod exerts its beneficial influence on MS course.

On the other hand, our study also showed an increase in serum levels of CCL2 and CCL5, and an enhancement in peripheral blood proportions of inflammatory cytokine-producing T cell subsets. These elevations might be a transient and putatively compensatory response to S1P1 modulation. Therefore this early response might possibly be followed by a decrease in the levels of these chemokines. This assertion needs to be tested by future studies evaluating chemokine levels with longer term follow-ups. Another potential explanation is that fingolimod may not be acting properly early in the treatment course of some patients thus permitting increased production of inflammatory factors. This may be one of the reasons of observing MS relapses in the earlier stages of fingolimod treatment in some patients.

A limitation of fingolimod treatment studies in general is absence of correlation of immunological variables with clinical progression, attack status and clinical outcome measures under treatment. In our study, we found significant positive correlations between final visit levels of IL-6 and CD4+IL-17+ cells versus the number of MS relapses. In other words, MS patients with higher serum IL-6 levels and higher proportions of CD4+IL-17+ cells in the peripheral blood showed trends towards experiencing more MS relapses. Thus, these two parameters may be used as biomarkers of resistance to fingolimod treatment.

Due to the paucity of treatment-naïve patients in our cohort, cytokine and lymphocyte profiles of patients with or without previous immunomodulating agents treatment could not be compared and need to be addressed in future studies. Secondly, CD4+ T cell subset proportions appear to remarkably fluctuate throughout the study period (Dominguez-Villar et al., 2019; Song et al., 2015). Nevertheless, in previous studies, CD4+ T cell subset measurements were done in widely differing time points ranging between 1 week and 12 months leading to contradictory results. Therefore, a longer assessment period duration and increased number of visits might yield a clearer profile of CD4+ T cell subset alterations.

In brief, fingolimod treatment preferentially suppresses antigen-presenting immune cells, while not interfering with cytotoxic immune cells and thus preserves inborn immunological functions while suppressing autoimmunity in MS. Fingolimod treatment does not explicitly affect specific Th-type responses and therefore plausibly does not act through the Th1/Th17-Th2 shift paradigm. Clinical implications of these immunological alterations need to be further studied by clinical follow-up studies.

Role of the funding source

This study was sponsored by Novartis Pharmaceuticals.

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