



Fingolimod modulates T cell phenotype and regulatory T cell plasticity *in vivo*



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ABSTRACT

Fingolimod is an approved therapeutic option for patients with relapsing-remitting multiple sclerosis that primarily functions by sequestering T cells in lymph nodes inhibiting their egress to the central nervous system. However, recent data suggests that Fingolimod may also directly affect the immune cell function. Here we examined the *in vivo* effects of Fingolimod in modulating the phenotype and function of T cell and Foxp3 regulatory T cell populations in patients with multiple sclerosis under Fingolimod treatment. Besides decreasing the cell numbers in peripheral blood and sera levels of pro-inflammatory cytokines, Fingolimod inhibited the expression of Th1 and Th17 cytokines on CD4⁺ T cells and increased the expression of exhaustion markers. Furthermore, treatment increased the frequency of regulatory T cells in blood and inhibited the Th1-like phenotype that is characteristic of patients with multiple sclerosis, augmenting the expression of markers associated with increased suppressive function. Overall, our data suggest that Fingolimod performs other important immunomodulatory functions besides altering T cell migratory capacities, with consequences for other autoimmune pathologies characterized by excessive Th1/Th17 responses and Th1-like regulatory T cell effector phenotypes.

1. Introduction

Multiple sclerosis is an autoimmune disease of the central nervous system (CNS) with infiltration of activated autoreactive T cells into the CNS that damage both myelin and axons [1]. This complex genetic disease is associated with as yet unknown environmental factors that appear to drive a predominantly T cell autoimmune response against the CNS [1,2]. The CD4⁺ T cell response in patients with multiple sclerosis is characterized by an increased frequency of memory Th1 and Th17 cells with a pathogenic phenotype [3–6]. Elevated frequencies of IL-17 and IFN γ production by T cells in the peripheral blood of multiple sclerosis patients have been associated with disease activity [7,8], with IFN γ being associated with relapses [9,10]. Besides this activated phenotype, relapsing-remitting multiple sclerosis patients show a functional loss of peripheral tolerance mechanisms characterized by the presence of dysfunctional regulatory cell populations [11–17]. Among these cell populations, it has been shown that Foxp3⁺ regulatory T cells (Tregs) are essential in maintaining immune homeostasis and inhibiting

autoimmune responses (reviewed in Refs. [18,19]). Human Tregs are characterized by the expression of the master transcription factor Foxp3 [20,21], high level of expression of the IL-2 receptor alpha chain (CD25) [22] and low expression of the IL-7 receptor alpha chain (CD127) [23]. Tregs have been shown to be deficient in function in several autoimmune diseases [16,24–31]. In multiple sclerosis, we have previously shown that Tregs from patients with relapsing-remitting disease display an increased frequency of IFN γ ⁺ Th1-like Tregs [12,32] and are defective in function *ex vivo* [12,16]. These Tregs are characterized by an activation of the PI3K/AKT/FoxO1/3 signaling pathway *in vivo*, which represents a major driver of Th1-like Treg generation in relapsing-remitting multiple sclerosis patients [17].

Fingolimod (Gilenya[™], FTY720) is an immunomodulating agent that was first described as an immunosuppressant in transplant settings [33,34], where it was shown to be efficacious in prolonging allograft survival in various experimental allotransplantation models. Soon after it was discovered that its major mechanism of action was to modulate sphingosine-1-phosphate (S1P) receptor signaling, which is a family of

Abbreviations: CNS, Central nervous system; IL, Interleukin; IFN, Interferon; S1P, Sphingosine-1-phosphate; Th, T helper; Treg, Regulatory T cell

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five receptors present on the surface of thymocytes, lymphocytes and neural cells [35]. Its active compound, Fingolimod, is the first orally approved therapeutic option for patients with relapsing-remitting multiple sclerosis [36] and has been shown to be an effective treatment in reducing disease activity reflected in significant reductions in the relapse rate and in the number of CNS lesions measured by Magnetic Resonance Imaging (MRI) [37,38]. Fingolimod is phosphorylated by a sphingosine kinase to the active compound, Fingolimod-phosphate [39]. Phosphorylated Fingolimod acts as a functional antagonist of all S1P receptors except for S1PR2 [35], inducing their internalization and thus blocking lymphocyte egress from secondary lymph organs to the peripheral blood circulation [40–43]. This results in sequestration of inflammatory cells in lymph nodes and reduction of peripheral lymphocyte counts, including potential encephalitogenic T cells, blocking their entrance to the CNS.

Besides its clear effect on lymphocyte egress from secondary lymphoid organs [40–42], there is an increasing number of reports suggesting that Fingolimod can modulate immune cell function besides their migratory properties. Thus, Fingolimod renders antigen-presenting cells less immunogenic and increases their anti-inflammatory potential [44,45], interferes with effector functions of human monocyte-derived dendritic cells *in vitro* [46], and alters B cell viability and cytokine release [47].

Here, we examined the *in vivo* effects of Fingolimod on T cells and regulatory T cells from patients with relapsing-remitting multiple sclerosis during 12 months of treatment with Fingolimod. Besides decreasing sera levels of pro-inflammatory cytokines including TNF α and the numbers of T cells in the periphery, Fingolimod directly affected the phenotype of T cells and Tregs. Effector CD4⁺ T cells showed a decreased expression of IL-17 and IFN γ , while increasing TGF β and IL-10 production, and upregulated the expression of markers associated with exhausted T cells including PD-1 and Tim-3. Furthermore, Fingolimod also exerted direct effects on Tregs. Thus, the Th1-like phenotype that characterizes Tregs from relapsing-remitting multiple sclerosis patients was abrogated by Fingolimod, with downregulation of T-bet and IFN γ expression and upregulation of Tim-3 receptor, which has been associated with increased suppressive function [48]. Overall, these data suggest a direct anti-inflammatory effect of Fingolimod on T cell populations and improvement of peripheral tolerance mechanisms in multiple sclerosis besides its role in modulating lymphocyte migration to the CNS.

2. Materials and methods

2.1. Experimental design

20 patients with relapsing-remitting multiple sclerosis were recruited from the Yale Multiple Sclerosis Center (CT) or Griffin Hospital (CT) following the decision made by their physician to start them on Fingolimod treatment. Subjects were recruited as outpatients after providing a full informed consent form in accordance to Yale University IRB approval. Patients demographics are summarized in Table 1. Inclusion criteria were 18–55 years old, diagnosed with relapsing-remitting multiple sclerosis, not on any immunosuppressant or disease-modifying drugs, and at least 3 months without any previous treatment, and able to and willing to donate blood at four different visits (0, 3, 6, 12 months). Exclusion criteria were: patients receiving class Ia or class III anti-arrhythmic drugs, beta blockers, calcium channel blockers, those with low heart rate, history of syncope, sick sinus syndrome, patients with acute or chronic infections, or another autoimmune disease, pregnant. According to FDA, oral Fingolimod was administered at 0.5 mg per day. Blood was collected at the time of first visit before starting Fingolimod treatment, and 3, 6 and 12 months thereafter.

Table 1

Baseline demographics. All multiple sclerosis patients had relapsing-remitting disease. EDSS: Expanded Disability Status Scale. BMI: Bone Mass Index. SD: standard deviation.

Age in years mean (\pm SD), range	44 (\pm 19.5), 24–64
Gender	
Male	2 (11.8%)
Female	15 (88.2%)
EDSS mean (\pm SD), range	1.8 (\pm 0.9), 0–3
BMI mean (\pm SD), range	24.6 (\pm 7.4), 19.3–35.4
Weight mean (\pm SD), range	144.8 (\pm 45), 112–213
Ethnicity	
Caucasian	15 (88.2%)
African american	2 (11.8%)

2.2. Cell culture reagents and antibodies

Cells were cultured in X-Vivo 15 media (Biowhittaker, Walkersville, MD).

2.3. PBMC isolation and cell sorting

Peripheral blood was obtained from patients with multiple sclerosis after informed consent was obtained in accordance with the Declaration of Helsinki. Peripheral blood mononuclear cells (PBMC) were isolated using CPT tubes (BD Biosciences). After 1 step centrifugation, plasma (upper phase) and PBMC (lower phase) were both collected and PBMC were washed and counted using a two-laser Guava cytometer; cell viability was determined using Viacount. Total PBMC were stained for fluorescence-activated cell sorting (FACS) with anti-CD3 V450, anti-CD4 PE-Cy7, anti-CD8 APC-H7, anti-CD127 APC, anti-CD25 PE. The Treg (CD4⁺CD25^{high}CD127^{neg}), and responder T cell (Tresp, CD4⁺CD25^{dim/low}CD127⁺) populations were sorted on a FACS Aria (BD Biosciences).

2.4. RNA isolation and gene expression analysis by TaqMan real-time PCR

RNA was isolated using QIAGEN RNeasy Micro Kit (QIAGEN, Valencia, CA), following manufacturer's guidelines and converted to cDNA by reverse transcription (RT) with random hexamers and Multiscribe RT (TQMN, Reverse Transcription Reagents; Applied Biosystems, Foster City, CA). For mRNA gene expression assays, probes were purchased from Applied Biosystems and the reactions were set up following manufacturer's guidelines and run on a StepOne Real-Time PCR System (Applied Biosystems). Values are represented as the difference in Ct values normalized to β 2-microglobulin for each sample as per the following formula: Relative RNA expression = $(2^{-\Delta\Delta Ct}) \times 1000$.

2.5. Cytometry by time of flight (CyTOF)

Due to the dramatic decrease in numbers of lymphocyte populations after Fingolimod treatment, we opted for the use of mass cytometry to immunophenotype the cells rather than the classic flow cytometry assays. This technique allowed interrogation at the single cell level of 40 markers (Table 2) while running simultaneously 20 different samples at the same time after assigning molecular barcodes to each sample.

PBMC were collected from relapsing-remitting multiple sclerosis patients at baseline, 3, 6 and 12 months after Fingolimod treatment, cryopreserved and stored in liquid nitrogen until all time point collections were finished. Samples were thawed using automation and robotics (Biomek FXP); so all the collected samples were processed consistently, the same day using the same reagents and the same instrument settings. PBMC concentration was counted and adjusted using automated solutions (Biomek FXP, Guava) to 10^6 cells/well in a 96 well plate containing 50 ng/ml PMA and 250 ng/ml ionomycin or diluent. After 4 h in the presence of GolgiStop™, the 96-well plate was

Table 2
List of mass cytometry markers and isotopes.

Marker	isotope	Marker	isotope	Marker	isotope	Marker	isotope
CD45	89Y	TNF α	152Sm	IFN γ	165Ho	DNA1	191Ir
CCR6	141Pr	Tim3	153Eu	CD223	166Er	DNA2	193Ir
CD 19	142Nd	CXCR5	154Sm	Granz. B	167Er	Viability	195Pt
CD 127	143Nd	CD27	155Gd	CD49d	168Er	CD11b	209Bi
CCR5	144Nd	CD14	156Gd	IL17	169Tm		
CD4	145Nd	CCR4	158Gd	CD3	170Er		
CD8	146Nd	CCR7	159Tb	CD226	171Yb	Mass barcoding	102Pd
CD20	147Sm	CD28	160Gd	CD274	172Yb	Mass barcoding	104Pd
CD 16	148Nd	CD 152	161Dy	TIGIT	173Yb	Mass barcoding	105Pd
CD25	149Sm	FoxP3	162Dy	HLA-DR	174Yb	Mass barcoding	106Pd
Ki67	150Nd	CXCR3	163Dy	PD1	175Lu	Mass barcoding	108Pd
CD278	151Eu	CD45RO	164Dy	IL10	176Yb	Mass barcoding	110Pd

washed and the cells stained with a viability dye (Cis¹⁹⁵Pt) for 1 min. After washing, the cells were stained for CyTOF surface markers (CD25, CD127, CD11b, CCR6, CCR5, CXCR5, CXCR3, CD45RO), fixed, permeabilized and barcoded. Barcoded samples were pooled and stained with the remaining CyTOF antibodies stained for surface and intracellular markers (Table 2). After washing, the cells were stained with DNA intercalator (¹⁹¹Ir and ¹⁹³Ir), washed 3 times and analyzed by mass cytometry on a CyTOF II instrument (Fluidigm). CyTOF data were analyzed using Cytobank software.

2.6. Intracellular staining and flow cytometry analysis

PBMC were collected from relapsing-remitting multiple sclerosis patients at baseline and after 3 months of Fingolimod treatment. PBMC were cryopreserved and stored in liquid nitrogen until all time point collections were finished. Samples were thawed using automation and robotics (Biomek FXp) and stimulated as for CyTOF staining. After 4 h in the presence of GolgiStop[™], the 96 well-plate were washed and the cells stained with a viability dye (LIVE/DEAD viability dye, Invitrogen, CA) for 20 min on ice. After washing, the cells were stained for surface markers (CD3, CD4, CD8, Tim-3), fixed, permeabilized and stained for intracellular markers (IL-10, TNF α , IFN γ , IL17, Granzyme B) for 20 min at room temperature. After washing, the samples were run on a LSRII Fortessa instrument (BD Biosciences). Flow cytometry data were analyzed using FlowJo software.

2.7. Cytokine measurements in sera by Luminex assays

Fresh plasma was collected from patients at all their scheduled visits and frozen down at -80°C . At the end of the study, all plasma aliquots were thawed and assayed as one batch for inflammatory cytokines, using the Th17 Luminex[™] kit and following manufacturer recommendations.

2.8. Statistical analysis

This is a paired before/after study, and data were analyzed by paired Student's *t*-test correcting for multiple comparisons when appropriate. *P* values below 0.05 were considered significant.

3. Results

3.1. Fingolimod treatment decreases the numbers of circulating T and B cells

It is widely believed that the major therapeutic effect of Fingolimod

occurs through sequestration of lymphocytes in lymph nodes with a subsequent reduction in peripheral blood cell counts. To confirm this previous observation in our cohort of patients, we measured total blood cell population counts at each visit on fresh blood (within one hour of blood draw) using flow cytometry and Truecount[™] tubes. Consistent with previous observations, there was a sharp decrease in the frequency of total lymphocytes, CD3⁺ T cells, CD4⁺ T cells, CD8⁺ T cells and B cells at 3, 6 and 12 months after starting treatment. Fingolimod[™] reduced both the relative frequency and the absolute numbers of circulating lymphocytes (Fig. 1A and Supplementary Fig. 1A). NK cells, monocytes and granulocytes were unaffected, with their relative frequency being increased (Fig. 1A and Supplementary Fig. 1A), but their total numbers remained unchanged (Fig. 1B). However, in two individuals the lymphocyte populations did not decrease as sharply after Fingolimod treatment. Interestingly, both patients reported having worse symptoms and their EDSS remained stable at 2 (data not shown). We also examined the frequency of Tregs (measured as percentage of CD25^{high}CD127[−]) within the CD4⁺ compartment at all time points after treatment initiation (Fig. 1C and Supplementary Fig. 1B). Interestingly, we found that Fingolimod treatment significantly increased the number of circulating Tregs in peripheral blood, and this increase was statistically significant at 3 (89%), 6 (142%) and 12 (187%) months after treatment initiation. Treg percentages within the CD4⁺ population were 3.32 ± 0.71 ; 6.28 ± 1.33 ; 8.05 ± 1.55 and 9.53 ± 1.80 at baseline; 3; 6 and 12 months, respectively.

3.2. Fingolimod treatment decreases TNF α and IL-27 concentrations in plasma

To assess whether Fingolimod affects the levels of inflammatory cytokines in the circulating blood, fresh plasma was collected from each patient at 0, 3, 6 and 12 months after treatment start, and cytokines were examined by Luminex-based immunoassay. Most of the cytokines examined were below detection levels in plasma at all time points except for the two Th1-related cytokines TNF α and IL-27, which were significantly decreased at all time points examined after Fingolimod treatment (Fig. 2). There was a 33% decrease in TNF α concentration (baseline: 5.35 ± 0.53 pg/ml; 3 months: 3.89 ± 0.46 pg/ml) and a 55% decrease in IL-27 (baseline: 231.34 ± 73.51 pg/ml; 6 months: 110.12 ± 32.48 pg/ml).

3.3. Effector T cells display an exhausted phenotype in vivo after fingolimod treatment

Several reports in the literature have suggested that Fingolimod might have a direct effect on immune cell phenotype and function besides its well-known role in affecting lymphocyte migration to the CNS. To test this hypothesis, we examined the phenotype of effector T cells and Tregs from patients with relapsing-remitting multiple sclerosis after Fingolimod treatment. Effector T cells (CD4⁺CD25^{+/low}CD127⁺) and Tregs (CD4⁺CD25^{high}CD127^{neg}) were FACS-sorted (Supplementary Fig. 2) and stimulated *ex vivo* with PMA and ionomycin as described [12]. RNA was isolated and the expression of pro- and anti-inflammatory cytokines, as well as exhaustion markers was examined by real-time PCR (Fig. 3). Effector T cells significantly downregulated the expression of *IL17* after 3 months of treatment (Fig. 3A) and this trend was maintained after a year (Supplementary Fig. 3). Although we did not observe a significant effect of Fingolimod on *IFNG* expression at 3 months after beginning of the treatment, there was a significant decrease after one year (Supplementary Fig. 3), suggesting that treatment was downregulating the expression of individual pro-inflammatory cytokines with different kinetics of inhibition. Concomitant with this decrease, the expression of the anti-inflammatory cytokine *TGFB1* was slightly upregulated after 3 months and this difference was significant after one year of treatment. In contrast, *IL10* expression was significantly downregulated at all time points examined as compared to

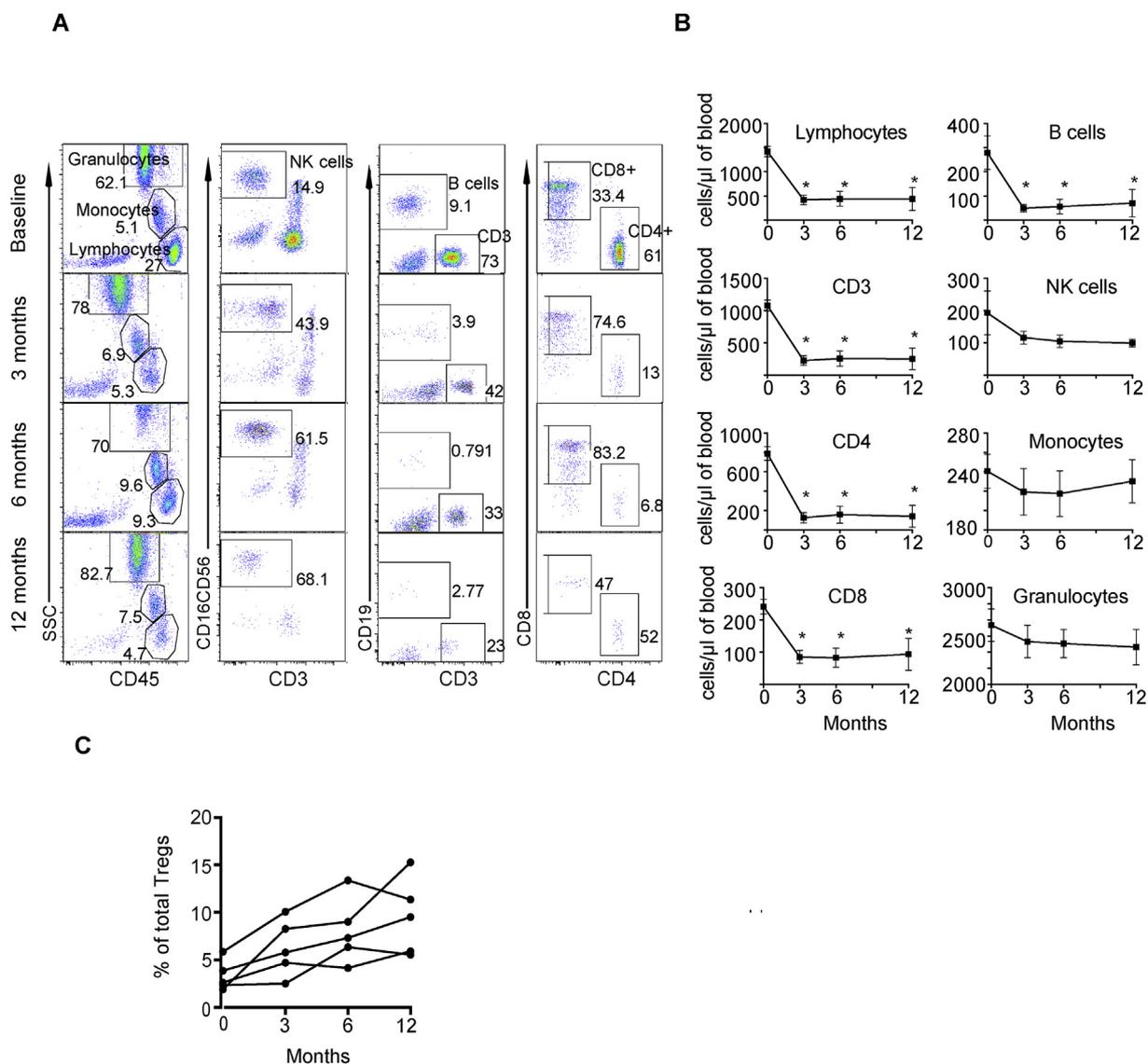


Fig. 1. Fingolimod treatment decreased both B and T cells and increased Tregs in the peripheral blood. Blood was collected from 17 MS patients at baseline and after 3, 6 and 12 months of Fingolimod treatment. Proportion and absolute numbers of PBMC populations were measured in freshly collected whole blood by flow cytometry using Trucount tubes. (A) Representative example of percentage of different populations of white blood cells in whole blood of one MS patient before and at different times after fingolimod treatment. (B) Absolute counts of PBMC populations in peripheral blood (n = 17). *p < 0.0001, paired t-test (compared to baseline, 0 months). (C) Proportion of Tregs in CD4⁺ population before and after Fingolimod treatment.

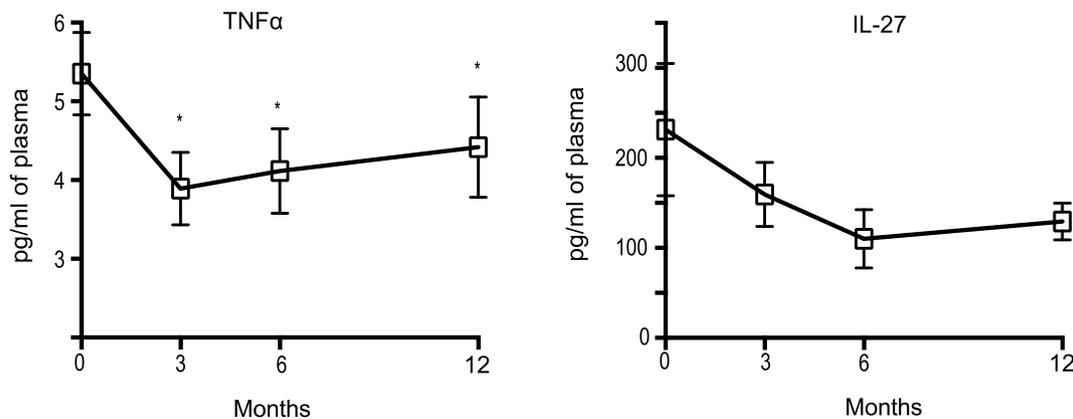


Fig. 2. Fingolimod treatment decreases the concentration of TNFα and IL-27 in the plasma of MS patients. Concentrations of inflammatory cytokines were measured in the plasma of MS patients at baseline (0), and after 3, 6 and 12 months of Fingolimod treatment using Luminex-based assay. Data are shown as mean ± SEM (n = 17). *p < 0.03, paired t-test (compared to baseline levels).

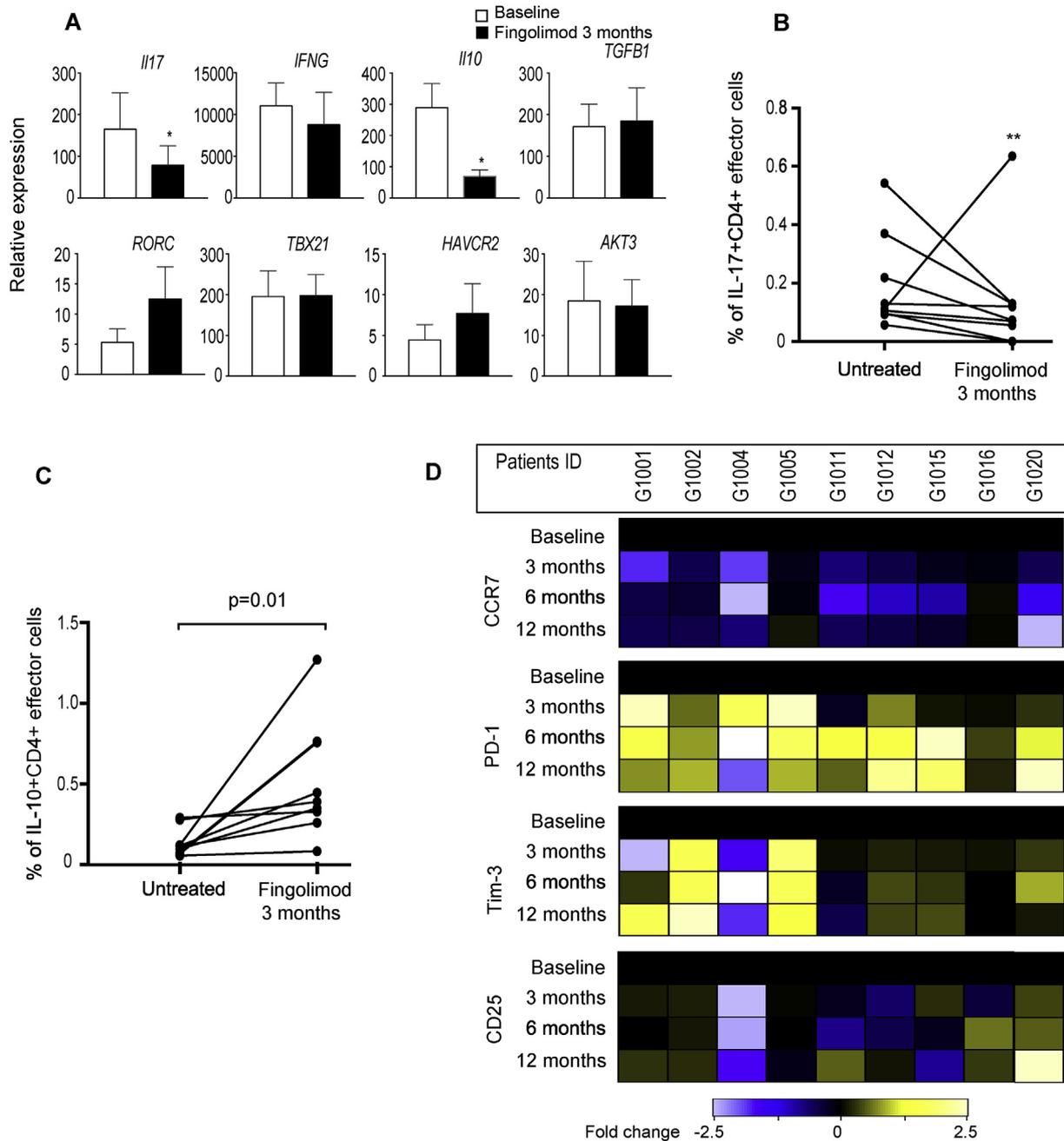


Fig. 3. Effect of Fingolimod on gene and protein expression in effector T cells. PBMC were isolated from MS patients at baseline and 3 months after Fingolimod treatment, and labeled with T cell regulatory and effector marker antibodies. (A) T effector cells were sorted (Supplementary Fig. 2) and stimulated with PMA and ionomycin, for 4 h. RNA expression was analyzed by real-time PCR. *p < 0.05, paired t-test (compared to baseline levels, n = 9). (B) IL-17 and (C) IL-10 production was measured by flow cytometry in CD4⁺ effector cells from patients after stimulation with PMA and ionomycin. (D) Protein expression of CCR7, PD-1, Tim-3 and CD25 was measured by mass cytometry on CD4⁺ effector cells of 9 patients, before and 3, 6, 12 months after Fingolimod treatment.

baseline (Fig. 3A). Transcription factors *RORC* and *TBX21*, master regulators of Th17 and Th1 cells, respectively, were also down-regulated, while the expression of the exhaustion marker Tim-3 (*HAVCR2*) was significantly upregulated as compared to baseline expression.

In order to confirm these data at a protein level, we stimulated effector T cells *ex vivo* with PMA and ionomycin and examined IL-10 and IL-17 expression by intracellular staining. Interestingly, while the decrease in IL-17 gene expression was confirmed at protein level (Fig. 3B), the frequency of IL-10-producing effector T cells was significantly increased after treatment as compared to baseline (Fig. 3C). We further explored the expression of the two major exhausted markers, Tim-3 and

PD-1, on effector T cells isolated *ex vivo* by CyTOF. Of note, the expression of both markers was significantly upregulated in most of the patients examined as early as 3 months after beginning of treatment and maintained during the 12 months of follow-up. Of note, the expression of CCR7, another signaling system that promotes retention of lymphocytes in secondary lymphoid organs [49,50] was decreased in all patients. No significant changes were observed in the expression of CD25 (Fig. 3D). The decrease in pro-inflammatory cytokine secretion, the increased in anti-inflammatory cytokine expression and the upregulation of exhaustion markers, suggests that Fingolimod affects the remaining circulating T cells in patients with relapsing-remitting multiple sclerosis by inducing an exhausted phenotype.

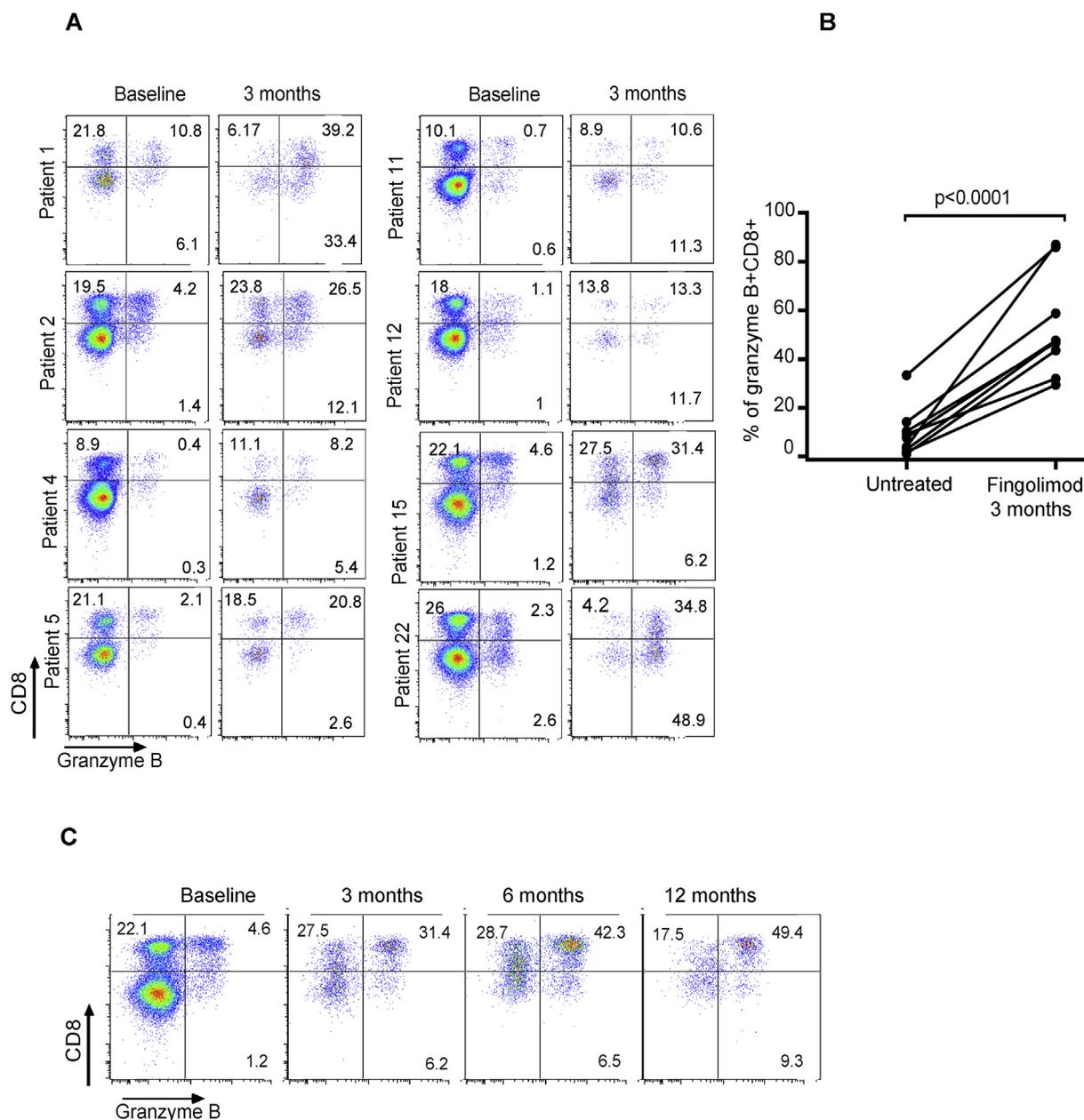


Fig. 4. Increased secretion of Granzyme B by CD8⁺ T cells after Fingolimod treatment. PBMC from patients were stained for CD8 and granzyme B at baseline and 3 months after Fingolimod treatment. (A) Granzyme B was measured intracellularly by flow cytometry after gating on live CD3⁺ cells. (B) Statistical analysis of the proportion of CD8⁺ cells producing granzyme B after gating on CD8⁺ cells. (C) Flow plots of granzyme B production in a representative patient before and at 3, 6 and 12 months after fingolimod treatment.

3.4. Increased secretion of granzyme B by CD8⁺ T cells after Fingolimod treatment

As another important player in multiple sclerosis pathogenesis, we were interested in examining the function of CD8⁺ T cells after treatment. Besides the significant decrease in percentage and total cell numbers observed after initiation of therapy (Fig. 1), we sought to determine whether CD8⁺ T cell functionality was altered by Fingolimod. For this, *ex vivo* isolated PBMC from patients at baseline, 3, 6 and 12 months after treatment were stained for Granzyme B, as a marker of cytotoxic capacity [51]. As previously reported [52], we observed a dramatic increase in the frequency of Granzyme B⁺ CD8⁺ T cells at all time points after treatment initiation, and this was statistically significant (Baseline: 12.5 ± 3; 3 months: 41.4 ± 6.9; 6 months: 46.9 ± 5.8; 12 months: 46.8 ± 5.7, Fig. 4).

3.5. Treg plasticity in relapsing-remitting multiple sclerosis patients is inhibited by Fingolimod treatment

We previously observed that Tregs isolated *ex vivo* from patients with relapsing-remitting multiple sclerosis were functionally defective [16] and displayed a Th1-like phenotype characterized by the upregulation of the transcription factor T-bet, the secretion of IFN γ and a defect in their suppressive capacity [12,17]. In order to examine whether Fingolimod inhibits this characteristic phenotype of Tregs from patients, sorted Tregs were stimulated with PMA and ionomycin *ex vivo* and the expression of pro- and anti-inflammatory cytokines, as well as their master transcription factors, were examined by real time PCR, at 3 and 12 months after the beginning of treatment (Fig. 5A). Interestingly, the expression of pro-inflammatory cytokines such as IL-17 and IFN γ was significantly downregulated in Tregs after 3 months of Fingolimod

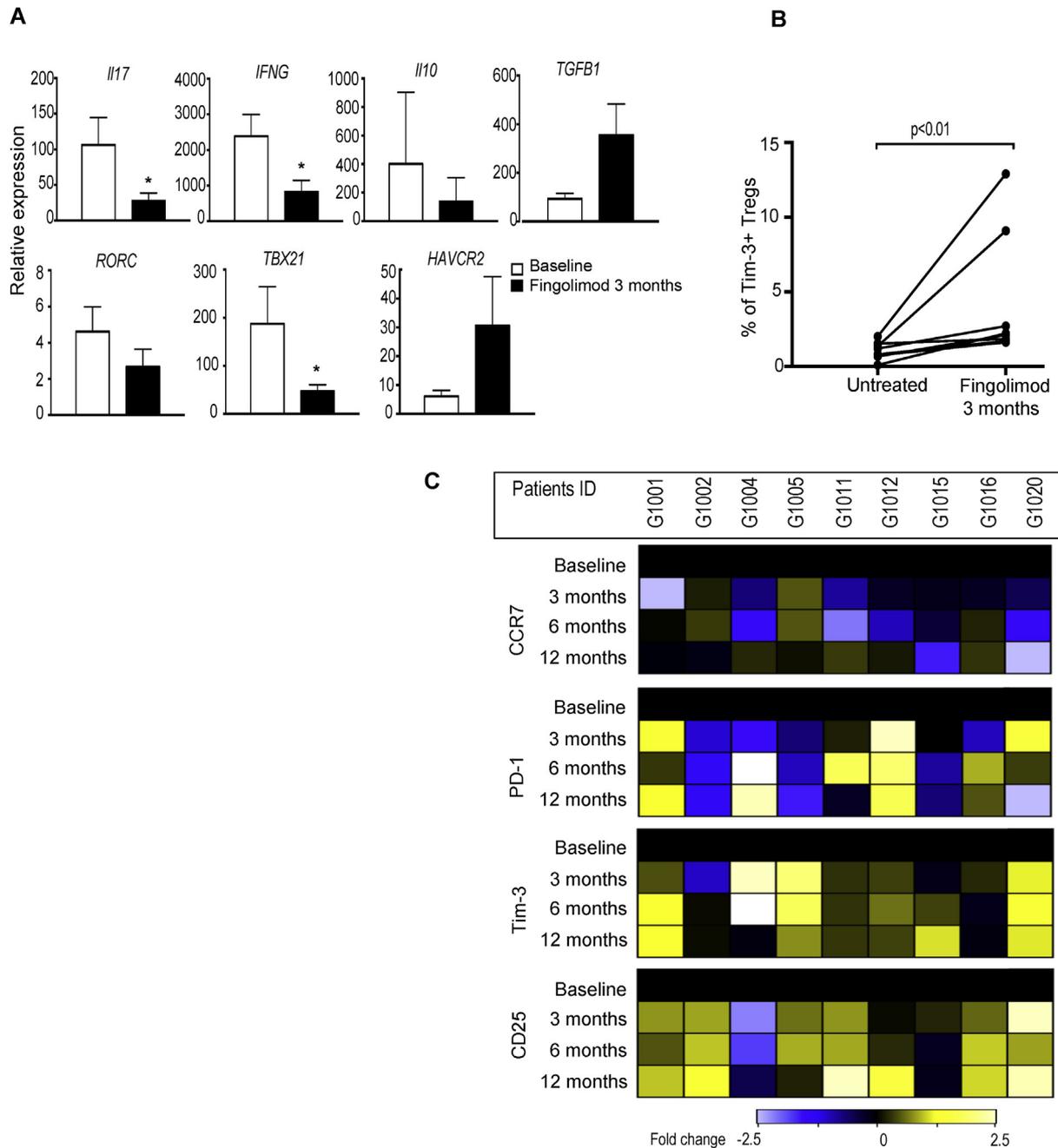


Fig. 5. Effect of Fingolimod on gene and protein expression in Tregs. PBMC were isolated from MS patients at baseline and 3 months after Fingolimod treatment, labeled with T cell regulatory and effector marker antibodies. **(A)** Tregs were sorted (Supplementary Fig. 2) and stimulated with PMA and ionomycin for 4 h. RNA expression was analyzed in batches by qRT-PCR. * $p < 0.05$, paired t tests (compared to baseline levels), $n = 9$. **(B)** Tim-3 expression was measured by flow cytometry in CD4 from patients before and 3 months after Fingolimod treatment. **(C)** Protein expression of CCR7, PD-1, Tim-3 and CD25 was measured by mass cytometry on Tregs of 9 patients, before and at 3, 6, 12 months after Fingolimod treatment.

treatment as compared to baseline levels, and this was accompanied by a concomitant downregulation of both *RORC* and *TBX21*. Furthermore, while the expression of *IL10* did not change at any time point, *TGFB1* was upregulated after Fingolimod treatment (Fig. 5A) and this increased expression was maintained at 12 months after beginning of treatment. Furthermore, *IL17* and *RORC* expression were upregulated after one year of treatment, as it occurs in patients with multiple sclerosis after IFN β treatment (Supplementary Fig. 4) [12]. As Tim-3 expression defines a population of Tregs with increased suppressive function [48,53], we examined the expression of Tim-3 as a marker of suppressive function. Tregs from relapsing-remitting multiple sclerosis patients displayed increased expression of *HAVCR2* at 3 months after

treatment as compared to baseline (Fig. 5A), and this increase was significant at 12 months (Supplementary Fig. 4). The increased expression of *HAVCR2* was confirmed at protein level by cell surface staining (Fig. 5B).

We further examined the expression of PD-1, CCR7, CD25 and Tim-3 on Tregs at times 0, 3, 6 and 12 months of treatment by CyTOF (Fig. 5C). Similarly to effector T cells, Tregs showed a decrease in CCR7 expression and increased levels of Tim-3, confirming our RNA and surface staining data. However, the expression of PD-1 at the different time points examined was variable between donors and did not reach statistical significance at any time point, in contrast with the clear increase in effector T cells (Fig. 3D). Lastly, CD25 expression was

augmented in patients after Fingolimod treatment as compared to baseline and this increase was maintained for the duration of the study (Fig. 5C).

The decrease in pro-inflammatory cytokine expression and the up-regulation of markers that correlate with increased suppressive function suggests that Fingolimod is correcting the aberrant Th1-like Treg phenotype observed in patients with relapsing-remitting multiple sclerosis.

4. Discussion

Here, we performed a longitudinal study with relapsing-remitting multiple sclerosis patients treated for 12 months with Fingolimod to examine the effects of the drug on T cell and Treg phenotype and function. We describe here specific roles of Fingolimod in T cell and Treg populations in patients with relapsing-remitting multiple sclerosis *in vivo*, and we demonstrate the function of Fingolimod at inducing an exhausted-like phenotype on circulating effector T cells and at inhibiting the Th1-like Treg phenotype that is characteristic of Tregs from patients with relapsing-remitting multiple sclerosis [12].

Fingolimod is an approved oral therapy for relapsing-remitting multiple sclerosis and is thought to act primarily by interacting with sphingosine receptors and sequestering lymphocytes in the lymph nodes, thus inhibiting egress to the CNS [35]. Nevertheless, growing evidence suggests additional effects beyond impact on lymphocyte migration, highlighting further promising targets in multiple sclerosis therapy. Thus, secondary effects on immune cells such as dendritic cells [44,45], NK cells [54] and B cells [47] among others, have been described. We thus examined the direct effects of Fingolimod on T cells populations *in vivo*, with a focus on cytokine secretion and expression of exhaustion markers. We first confirmed published data demonstrating the decrease in T cell numbers in the circulation of patients under Fingolimod treatment [38,55–59] and the lack of effects on monocyte and NK cell counts [60]. These differential results on the effect of Fingolimod on T cells as compared to NK cells and monocytes are in agreement with recent reports [46,54], and could be due to differential levels of expression of the five S1P receptors in these cell populations [61,62].

Our data suggest that Fingolimod affects the phenotype of T cells *in vivo* by inducing an exhausted-like phenotype on T cells, characterized by the inhibition of IL-17 and IFN γ expression, augmentation of IL-10 and TGF β , and increased expression of exhaustion markers such as PD-1 and Tim-3. These data are in agreement with recent reports that demonstrate that Fingolimod reduces the frequency of IL-17- and IFN γ -producing T cells [59,63]. It remains to be determined whether the effect observed in T cells is due to direct signaling of Fingolimod on them, or if, by the contrary, the change in T helper phenotypes is due to an indirect effect through, for example, antigen-presenting cell modulation [44,45]. However, Fingolimod was able to downregulate the expression of S1PR1 *in vitro* when added to Treg and T cell cultures (data not shown), which supports the hypothesis that there are direct effects of the drug on T cell populations. In this regard, we observed a discrepancy between cultures of stimulated whole mononuclear cells where we observed decreases in IL-10 secretion (data not shown) while *ex vivo* measurement of IL-10 from isolated CD4⁺ T cells revealed an increase. This may be due to interactions between antigen presenting cells in the culture system as compared to direct measurement in isolated CD4⁺ T cells. Moreover, there are well documented discrepancies between protein and RNA expression levels for specific cytokines related to kinetics of RNA transcription, which might explain the difference in *IL10* gene and protein expressions on CD4⁺ T cells. Finally, Fingolimod addition to activated T cell cultures *in vitro* did not affect per se the frequency of IL-17-secreting cells [63], suggesting that the inhibition of IL-17 secretion by T cells in patients with relapsing-remitting multiple sclerosis might be an indirect effect on another cell population.

We also observed an unexpected increase in Granzyme B expression

by CD8⁺ T cells after Fingolimod treatment. While Granzyme B and Perforin has been shown to be increased after treatment in CD56⁺ T cells [52], Fingolimod has been reported to decrease Granzyme B by CD8⁺ T cells in an *in vivo* model of multiple sclerosis [64]. However, we and others have described non-apoptotic functions of Granzyme B [65,66] and further investigations are warranted to examine the potential protective roles of increased Granzyme B levels after Fingolimod treatment in patients with multiple sclerosis.

With regards to Tregs, we and others have demonstrated that Tregs are functionally impaired in patients with relapsing-remitting multiple sclerosis [11–13,16] and display a Th1-like phenotype characterized by the expression of IFN γ , upregulation of *Tbx21* and decreased suppressive capabilities. Treatment of patients with Fingolimod significantly reduced the expression of *IFNG* and *TBX21* on Tregs as compared to baseline (Fig. 5). One limitation of our study is the lack of *ex vivo* functional assays to demonstrate this increase in function after *in vivo* treatment with Fingolimod, as the low number of cells recovered from peripheral blood made these experiments infeasible. However, with one patient we were able to perform a suppression assay with sorted Tregs after 3 months of treatment, and they were significantly better suppressors as compared to the baseline suppressive function (data not shown). To have an indirect indicator of suppressive capacity, we examined the expression of Tim-3 on Tregs, which has been associated with superior suppressive capacity [48,53], and we observed that Fingolimod increased significantly the expression of Tim-3 on Tregs from patients, suggesting that these cells also regained suppressive capacities. In agreement with this, Fingolimod has been shown to inhibit the PI3K/AKT signaling pathway [58,67], which is activated in Tregs from relapsing-remitting multiple sclerosis patients and contributes to the Th1-like phenotype and defect in suppression as compared to healthy individuals [17,32]. Thus, Fingolimod might be restoring the Treg phenotype and increasing their suppressive capacities by inhibition of this pathway. The specific molecular mechanisms underlying this restoration require further investigation.

In summary, our data demonstrates that Fingolimod not only affects migration of lymphocytes to the CNS and sequesters them in the lymph nodes, but it also modulates the phenotype and function of T cell and Tregs in patients with relapsing-remitting multiple sclerosis *in vivo*. This modulation affects two major axes: on one hand, there is a global attenuation of the pathogenic phenotype of T helper cells characterized by decreased expression of IL-17 and IFN γ and their respective master regulators RORC and T-bet, and on the other hand, an inhibition of the Th1-like phenotype that is characteristic of Tregs from relapsing-remitting multiple sclerosis patients. While it is currently unknown whether the effects of Fingolimod observed in peripheral immune cells are also taking place in secondary lymph nodes where most of T cells are after treatment, it is reasonable to think that Fingolimod also changes the phenotype of sequestered immune cells. These results open new avenues to the potential therapeutic use of Fingolimod in other diseases in which there is exacerbated Th1/Th17 responses and/or dysfunctional Th1-like Tregs, as it has been shown in pre-clinical models of arthritis [68,69], autoimmune diabetes [70–72], colitis [56,73], and thyroiditis [74].

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.jaut.2018.08.002>.

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