

Dimethyl fumarate treatment alters circulating T helper cell subsets in multiple sclerosis

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Catharina C. Gross,
PhD*
Andreas Schulte-
Mecklenbeck*
Svenja Klinsing
Anita Posevitz-Fejfar,
PhD
Heinz Wiendl, MD
Luisa Klotz, MD

Correspondence to
Dr. Klotz:
Luisa.klotz@ukmuenster.de

ABSTRACT

Objective: To evaluate the effect of dimethyl fumarate (DMF; Tecfidera, Biogen, Weston, MA) on CD4⁺ and CD8⁺ T cell subsets in patients with multiple sclerosis (MS).

Methods: Peripheral lymphocyte subsets, including CD4⁺ and CD8⁺ memory cells and T helper (T_H) cells T_H1, T_H2, T_H17, and peripheral regulatory T cell (pT_{reg}) subpopulations were analyzed before and 6 months after onset of DMF treatment.

Results: CD4⁺ and CD8⁺ memory T cells were preferentially decreased compared to naive CD4⁺ and CD8⁺ T cell populations. Within the CD4⁺ memory T cell population, frequencies of T_H1 cells were decreased, whereas those of T_H2 cells were increased and those of T_H17 cells remained unaltered. Accordingly, we observed decreased production of interferon γ , granulocyte-macrophage colony-stimulating factor, tumor necrosis factor α , and interleukin (IL)-22 by CD4⁺ T cells under DMF treatment, whereas the frequency of IL-4- and IL-17A-producing CD4⁺ T cells remained unchanged. With regard to regulatory T cells, proportions of pT_{reg} increased following DMF treatment.

Conclusion: Our data demonstrate that DMF treatment of patients with MS affects predominantly memory T cells accompanied by a shift in T_H cell populations, resulting in a shift toward anti-inflammatory responses. These findings indicate that monitoring of memory subsets might enhance vigilance of impaired antiviral immunity and that patients with T_H1-driven disease might preferentially benefit from DMF treatment.

Classification of Evidence: This study provides Class IV evidence that DMF might preferentially reduce CD4⁺ and CD8⁺ memory T cells in MS. *Neurol Neuroimmunol Neuroinflamm* 2016;3:e183; doi: 10.1212/NXI.000000000000183

GLOSSARY

DMF = dimethyl fumarate; **EDTA** = ethylenediaminetetraacetic acid; **IFN** = interferon; **IL** = interleukin; **MS** = multiple sclerosis; **Nrf2** = nuclear factor erythroid 2-related factor 2; **PBMC** = peripheral blood mononuclear cell; **PML** = progressive multifocal leukoencephalopathy; **pT_{reg}** = peripheral regulatory T cell; **RRMS** = relapsing-remitting multiple sclerosis; **SOP** = standard operating procedure; **T_H** = T helper cell; **TNF α** = tumor necrosis factor α .

Delayed-release dimethyl fumarate (DMF; Tecfidera, Biogen, Weston, MA) is a newly approved immune-modulatory drug for treatment of relapsing-remitting multiple sclerosis (RRMS) whose mechanism of action has not been fully resolved.^{1,2} Anti-inflammatory and neuroprotective effects of DMF have been documented, including a reduction in lymphocyte cytokine production, a reduction in lymphocyte counts presumably by an apoptosis-related mechanism, a downregulation of the migratory activity of immune cells at the blood-brain-barrier, and activation of the nuclear factor erythroid 2-related factor 2 (Nrf2) transcriptional pathway mediating antioxidative and potentially neuroprotective effects.³⁻⁷

Immunologic data from patients with RRMS treated with DMF are still sparse. In the clinical study program, a mean reduction in lymphocyte counts of about 50% after 1 year of treatment

Supplemental data
at Neurology.org/nn

*These authors contributed equally to this manuscript.

From the Department of Neurology, University Hospital Münster, Münster, Germany.

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has been described,^{1,2} which could be replicated by others.⁸ The reason a more pronounced reduction in lymphocytes occurs in about 6% of individuals⁹ remains unclear but should be highlighted because lymphopenia in the context of fumaric ester treatment has been associated with rare cases of progressive multifocal leukoencephalopathy (PML) in both patients with psoriasis and RRMS.^{9–13} Recently, it has been observed that CD8⁺ T cells are more affected by DMF treatment-induced lymphopenia than CD4⁺ T cells.⁸ However, a detailed analysis of lymphocyte subset changes under DMF treatment in patients with RRMS has not been provided. We therefore aimed to provide a detailed characterization of changes in lymphocyte subset composition as a consequence of DMF treatment in order to increase our knowledge of DMF-mediated immune alterations in the context of MS.

METHODS Patients. All patients were recruited at the Department of Neurology at the University Hospital Münster, Germany. Fifteen stable patients with RRMS (ages 24–54 years, mean age 40.7 years; 7 female, 8 male) were included and treated with a standard treatment regimen of DMF for 6 months. Forty-six percent of the patients were treatment naive, whereas 27% each had been previously treated with glatiramer acetate or interferon (IFN) α . All patients switching from glatiramer acetate or IFN- α underwent a washout period of at least 4 weeks.

Standard protocol approvals, registrations, and patient consents. This study was performed according to the Declaration of Helsinki and was approved by the local ethics committee (# 2010-236-F-5). All patients gave written informed consent.

Cells. Ethylenediaminetetraacetic acid (EDTA) blood was taken from each patient immediately before the first dose of DMF as well as after 6 months of therapy. Peripheral blood mononuclear cells (PBMCs) were isolated and stored in liquid nitrogen according to our standard operating procedure (SOP).¹⁴ Samples from baseline and after 6 months of therapy were thawed following our SOP.¹⁴

Cell culture. For cytokine stimulation assays, freshly thawed PBMCs were centrifuged at 300g for 5 minutes, resuspended in X-Vivo 15 \pm 10 μ L/mL Leukocyte Activation Cocktail (phorbol 12-myristate 13-acetate, ionomycin, and Brefeldin A; BD Biosciences, Franklin Lakes, NJ) at a concentration of 5 \times 10⁶ PBMC/mL, and incubated at 37°C/5% CO₂ for 6 hours. Finally, PBMCs were washed and stained for flow cytometry.

Flow cytometry. Freshly thawed or stimulated PBMCs were centrifuged at 300g for 5 minutes, resuspended in phosphate-buffered saline (Sigma-Aldrich, St. Louis, MO) supplemented with 2% heat-inactivated fetal bovine serum (GE Healthcare/PAA, Little Chalfont, UK) and 2 mM EDTA (Sigma-Aldrich) with fluorochrome-conjugated antibodies at the indicated working concentrations (see table e-1 at Neurology.org/nn) or isotype-matched controls, and incubated at 4°C for 30 minutes. Staining of chemokine receptors was performed at

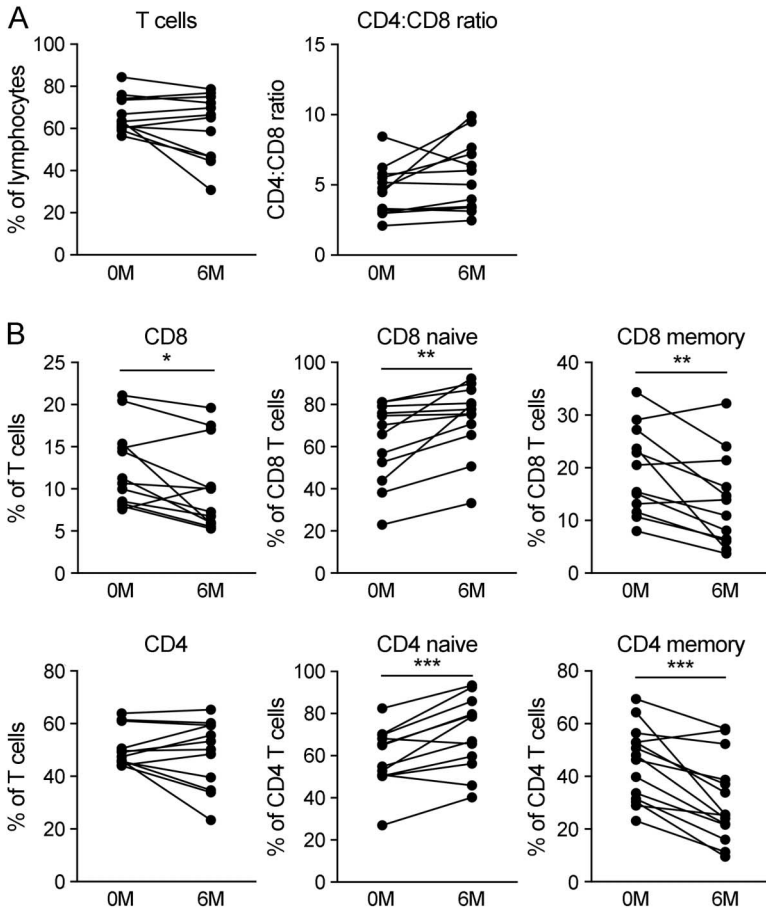
37°C for 30 minutes. Subsequently, cells were washed twice and either analyzed by flow cytometry (Navios; Beckman Coulter, Brea, CA) or stained for intracellular proteins with fixation/permeabilization solution (eBioscience, San Diego, CA) following the manufacturer's instructions. Resulting data were analyzed using Kaluza Flow Cytometry Analysis software version 1.2 (Beckman Coulter) and Prism software version 5.04 (GraphPad, La Jolla, CA).

Gating strategy. CD14⁻ lymphocytes were selected in a CD14 vs side scatter plot. Lymphocytes were then displayed in a CD3 vs CD56 plot and CD3⁺CD56⁻ T cells were selected. T cells were further divided into CD4⁺CD8⁻ and CD8⁺CD4⁻ subsets using a CD4 vs CD8 plot. Naive T cells were defined as CD45RO⁻CD27⁺, whereas memory T cells were defined as CD45RO⁺CD4⁺ or CD8⁺ T cells and distinguished by expression of CCR4/CD194, CCR6/CD196, and CXCR3/CD183 into CCR4⁻CCR6⁻CXCR3⁺ T helper (T_H)1 cells, CCR4⁺CCR6⁻CXCR3⁻ T_H2 cells, and CCR4⁺CCR6⁺CXCR3⁻ T_H17 cells according to the literature.¹⁵ Peripheral regulatory T cells (pT_{reg}) were defined as CD127^{low}CD25^{high}FoxP3⁺Helios⁻CD4⁺ T cells.^{16,17}

RESULTS Six months of DMF treatment did not alter the proportion of CD3⁺CD56⁻ T cells within the lymphocyte population (figure 1A, left), whereas it resulted in a significant decrease in CD8⁺ T cells within the T cell subpopulation (figure 1B, left, 19.39%, $p = 0.024$), in accordance with an earlier study.⁸ Further analysis revealed that memory T cells (CD45RO⁺) were predominantly affected within the CD4⁺ and the CD8⁺ T cell population, resulting in a highly significant reduction in CD8⁺ as well as CD4⁺ memory T cells (figure 1B; 29.90%, $p = 0.0079$ and 31.13%, $p = 0.0002$, respectively). Of note, DMF resulted in a decrease of both CD45RO⁺CD27⁺ central memory as well as CD45RO⁺CD27⁻ effector memory cells (data not shown). In contrast, percentages of naive (CD45RO⁻CD27⁺) CD4⁺ and CD8⁺ T cell subsets increased under DMF treatment (19.26%, $p = 0.001$ and 18.26%, $p = 0.0035$, respectively).

We further addressed the impact of DMF treatment on distinct CD4⁺ T cell subsets (figure 2A). Whereas proportions of T_H1 and T_H17 cells were significantly reduced at 6 months after onset of DMF treatment (36.77%, $p = 0.0045$ and 32.10%, $p = 0.0059$, respectively), the proportion of T_H2 cells was not altered (figure 2A, top). Because DMF treatment decreased CD4⁺ memory T cells in general (figure 1B, bottom), we also analyzed DMF-induced alterations within the cytokine-producing CD4⁺ memory subset (figure 2A, middle). We observed a significant decrease of T_H1 cells (17.02%, $p = 0.035$) accompanied by an increase of T_H2 cells (53.67%, $p = 0.0003$), whereas T_H17 cells remained unchanged. With regard to the regulatory subsets, the proportion of pT_{reg} cells was significantly increased (24.6%, $p = 0.0087$). As a

Figure 1 Effect of DMF therapy on T cell subsets



Peripheral blood mononuclear cells from 12 patients with relapsing-remitting multiple sclerosis (RRMS) at baseline (0M) and after 6 months of therapy (6M) with dimethyl fumarate (DMF; Tecfidera, Biogen, Weston, MA) were thawed and analyzed by flow cytometry for changes in the T cell compartment. (A) Left: CD3⁺CD56⁻ T cells as percentage of total lymphocytes; right: ratio of CD4⁺ to CD8⁺ T cells. (B) DMF-induced changes in CD8⁺ T cells (top left: CD8⁺CD4⁻ T cells as percentage of total T cells; top center: CD8⁺CD45RO⁻ naive T cells as percentage of total CD8⁺ T cells; top right: CD8⁺CD45RO⁺ memory T cells as percentage of total CD8⁺ T cells) and in CD4⁺ T cells (bottom left: CD4⁺CD8⁻ T cells as percentage of total T cells; bottom center: CD4⁺CD45RO⁻ naive T cells as percentage of total CD4⁺ T cells; bottom right: CD4⁺CD45RO⁺ memory T cells as percentage of total CD4⁺ T cells). Statistical analysis was done by paired Student t test, **p* < 0.05, ***p* < 0.01, ****p* < 0.001.

consequence, DMF treatment resulted in a significant reduction of the T_H1/pT_{reg} (52.14%, *p* = 0.0029) and T_H17/pT_{reg} (48.21%, *p* = 0.002) ratios (figure 2A, bottom).

Based on these findings, we investigated the functional capacity of CD4⁺ T cells to produce different cytokines upon ex vivo stimulation (figure 2B). We observed that in patients treated with DMF, the percentage of CD4⁺ T cells producing tumor necrosis factor α (TNFα) (reduction of 14.21%, *p* = 0.0231), IFN-γ (47.71%, *p* = 0.0014), interleukin (IL)-22 (24.41%, *p* = 0.0244), and granulocyte-macrophage colony-stimulating factor (GM-CSF) (48.05%, *p* = 0.0008) was significantly reduced when compared to baseline (figure 2, B and C). In

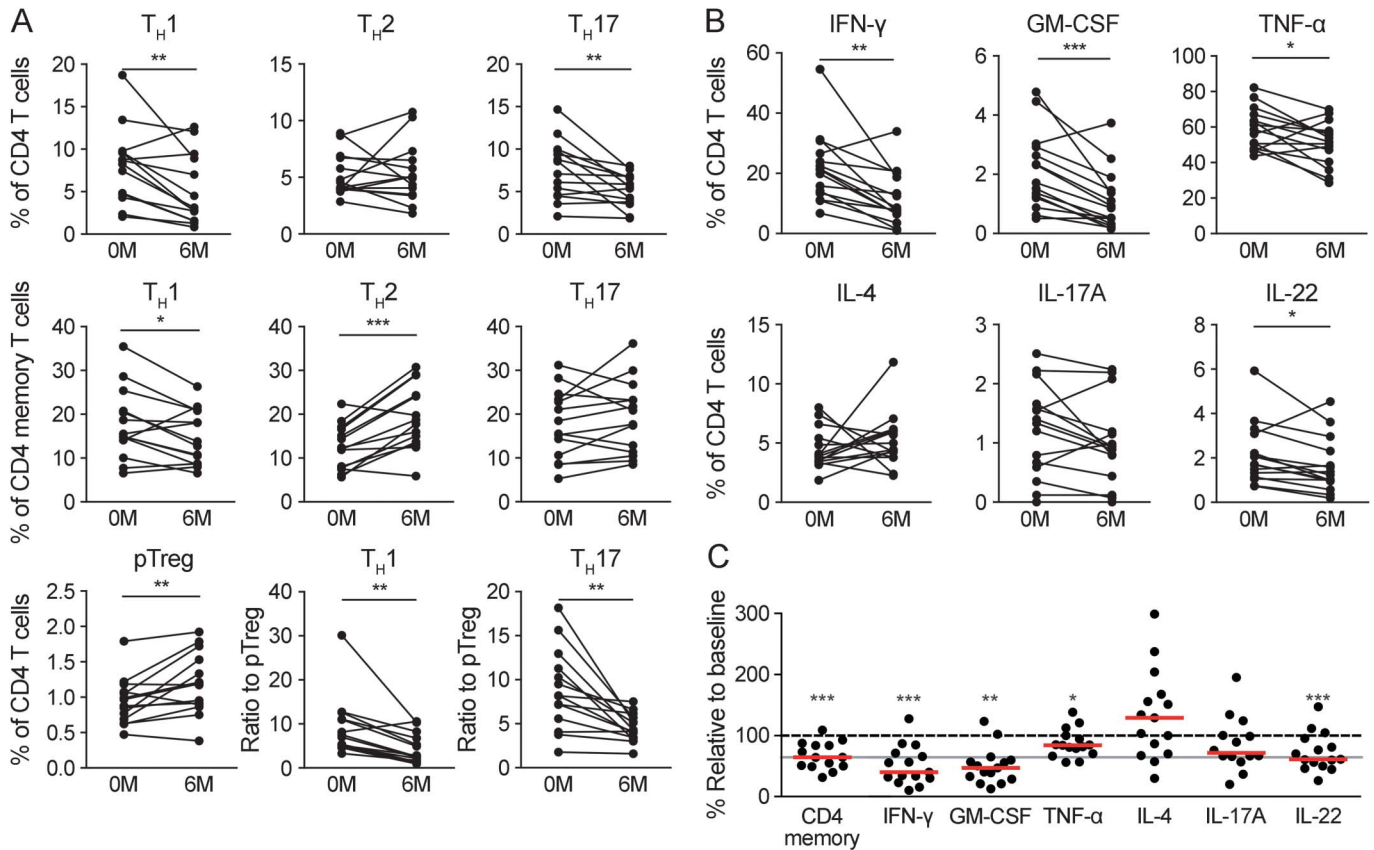
contrast, production of IL-17A and IL-4 was not affected.

DISCUSSION Inhibitory effects of fumaric esters, in particular DMF, on lymphocyte counts are well known and have already been described as frequent adverse events in phase 3 clinical trials in patients with RRMS.^{1,3} DMF-related lymphopenia has been a recent area of focus, as it is suspected to be associated with rare cases of PML in patients receiving fumaric esters for different indications.^{10–13} However, the underlying cause of such lymphopenia and the impact on different lymphocyte subpopulations have not been addressed in great detail. Recently, it was described that this effect preferentially affects CD8⁺ T cells rather than CD4⁺ T cells.⁸ We now extend these investigations by demonstrating that DMF treatment in patients with RRMS causes distinct and reciprocal alterations of different CD4⁺ T cell subsets, characterized by reduced T_H1 and T_H17 cells and increased pT_{reg} cell populations. The composition of CD4 memory T cells was skewed from T_H1 toward T_H2 cells by DMF treatment. These changes in subset composition were paralleled by a change in the CD4⁺ T cell cytokine secretion pattern, with decreased IFN-γ, GM-CSF, and TNFα production.

This study addresses the impact of in vivo DMF treatment on different effector vs regulatory T cell subsets in RRMS. We demonstrated that the impact of DMF treatment on antigen-experienced memory CD4⁺ and CD8⁺ T cells is more pronounced compared to total CD4⁺ and CD8⁺ T cells. Although the reason for this preferential effect still needs to be elucidated, our study revealed that additional analysis of these populations is important not only with regard to the mechanisms of DMF-mediated immune regulation but also with regard to immune vigilance aspects such as lymphopenia-associated PML,^{13,18,19} because memory T cell subsets are particularly relevant for maintenance of antiviral immunity.²⁰

In order to elucidate the potential impact of in vivo DMF treatment on different CD4⁺ T cell subpopulations in more detail, we used established strategies to determine T_H1, T_H2, T_H17,¹⁵ and pT_{reg}¹⁶ subpopulations via multicolor flow cytometry without further manipulation of cells by short-term culturing and stimulation of cells. Our analysis revealed a distinct and reciprocal regulation of different subsets. This interesting observation has 2 important implications. First, it indicates a rather selective effect of DMF treatment on distinct subsets rather than a broad suppressive activity on all T cells, as suggested by several authors.^{5,21} Second, it might reflect a normalization of disturbed effector vs regulatory T cell subpopulations in MS. Such a disturbance characterized by augmented proinflammatory T_H1 or T_H17

Figure 2 Effect of DMF therapy on the T helper cell repertoire and cytokine production



Peripheral blood mononuclear cells (PBMCs) from patients at baseline (OM) and after 6 months of therapy (6M) with dimethyl fumarate (DMF) were analyzed by flow cytometry for changes in the T cell compartment with focus on helper T (T_H) cells (A, $n = 14$) and their cytokine production (B, C, $n = 15$). (A) T_H1 (left), T_H2 (center), and T_H17 cells (right) as percentage of CD4⁺ T cells (top) and memory CD4⁺ T cells (middle) at baseline and after 6 months of treatment; bottom: peripheral regulatory T cells (pT_{reg}) as percentage of total CD4⁺ T cells (left) and T_H1 as well as T_H17 (in percent of CD4⁺ T cells) cells as ratio to pT_{reg} (center and right). (B) PBMCs were stimulated with Leukocyte Activation Cocktail for 6 hours and analyzed by flow cytometry for the intracellular amount of interferon (IFN)- γ (top left), granulocyte-macrophage colony-stimulating factor (GM-CSF) (top center), tumor necrosis factor α (TNF- α) (top right), interleukin (IL)-4 (bottom left), IL-17A (bottom center), and IL-22 (bottom right). The given results are the percentage of total CD4⁺ T cells. (C) CD4⁺ memory cells and data from (B) depicted as percentage relative to baseline after 6 months of treatment. Red bars indicate the median, and the gray line marks the median reduction of CD4⁺ memory T cells. Statistical analysis was done by paired Student *t* test for matched samples and Wilcoxon signed rank test for the comparison of the median cytokine production to baseline, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

responses, impaired regulatory T cell functions, and an association of disease remission with pronounced T_H2 responses has already been proposed by several studies in the context of MS.^{22–28}

With regard to the impact of DMF treatment on T cell functions in vivo, only 1 pilot study comprising 10 patients with RRMS, of whom only 6 completed the study, showed enhanced production of IL-10 by restimulated CD4⁺ T cells at different time points after onset of DMF treatment, whereas IFN- γ production was found to be unchanged.²⁹ The small sample size might explain the discrepancy compared to our data with regard to IFN- γ production. Other studies investigated the impact of in vitro DMF or mycophenolate mofetil exposure on cytokine production by human T cells. Some of these studies observed reduced proinflammatory cytokine production (i.e., IFN- γ or TNF α) with DMF exposure,^{21,30} whereas others did not observe such reductions.³¹ It is

interesting that at least a few studies also suggested an enhancement of anti-inflammatory cytokine production with DMF,^{29–32} which is in line with our finding of enhanced T_H2 and pT_{reg} frequencies in patients treated with DMF. Together, these data support the notion that DMF exerts differential effects on T cell cytokine production, although the different experimental settings in these in vitro studies did not reveal a conclusive picture of DMF-induced cytokine changes and did not take into account the impact of MS-specific immunologic characteristics. Hence, our study extends these findings by providing data on DMF-induced changes in T cell cytokine production in a defined and larger cohort of patients with MS. It has been shown that DMF acts on different intracellular pathways, such as interference with nuclear factor κB -mediated transcription of proinflammatory genes,³³ induction of Nrf2 antioxidant pathways,³⁴ and induction of alternatively activated anti-inflammatory

microglial cells through activation of the hydroxycarboxylic acid receptor 2.³⁵ Future studies will reveal whether these important transcriptional pathways might be differentially affected in different T_H cell subsets during DMF treatment in patients with RRMS.

We are aware that interpretation of this confined immunologic pilot study is limited by several aspects. First, we cannot distinguish between direct DMF-mediated effects on T cells (e.g., during differentiation) and indirect DMF effects via influence on antigen-presenting cells, which has already been suggested in 2 studies, albeit not involving immune cells from patients treated with DMF.^{36,37} Second, we were able to address effects on peripheral blood-derived immune cells but not on immune cells within the CNS or CSF from patients treated with DMF. Third, because our observational period was limited to 6 months after treatment onset, a correlation with the treatment response was not possible.

Our study demonstrates distinct effects of DMF treatment on different T cell subsets in patients with MS, with a pronounced reduction of memory subsets and a differential effect on T_H cell subsets with a shift toward anti-inflammatory subsets. Our findings suggest that closer monitoring of memory T cell populations might enhance vigilance toward immune-mediated side effects such as PML and that patients with more T_H1-driven disease might preferentially benefit from DMF treatment compared to those with T_H17-driven disease.

AUTHOR CONTRIBUTIONS

C.C.G., A.S.-M., and S.K. performed research and collected and analyzed data. C.C.G. and L.K. conceptualized the study and analyzed and interpreted the data. A.P.-F. and S.K. organized patient recruitment and logistics and provided clinical information. L.K. designed the project, was responsible for the concept, designed research, generated funding, organized patient recruitment, provided clinical information, and wrote the manuscript. H.W. helped to conceptualize the project, generated funding, and critically edited the manuscript. All authors wrote the manuscript.

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DISCLOSURE

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University of Münster. A. Schulte-Mecklenbeck and S. Klinsing report no disclosures. A. Posevitz-Fejfar received speaker honoraria and travel funding from Genzyme. H. Wiendl serves on the scientific advisory board for Bayer, Biogen, Sanofi-Genzyme, Merck Serono, Novartis, Roche, and Teva; is on the editorial board for *Journal of Clinical Practice*, *Journal of Neuroinflammation*, and *PLOS ONE*; has consulted for Biogen, Merck Serono, Novartis, Omnamed, Roche, Sanofi-Genzyme; and received research support from Bayer Healthcare, Bayer Vital, Biogen Idec, Merck Serono, Novartis, Sanofi-Genzyme, Sanofi US, Teva, German Ministry for Education and Research, Deutsche Forschungsgesellschaft, European Union, Else Kroner Fresenius Foundation, Fresenius Foundation, Hertie Foundation, NRW Ministry of Education and Research, Interdisciplinary Center for Clinical Studies Muenster, RE Children's Foundation, Sanofi Aventis, and NovoNordisk. L. Klotz is on the scientific advisory board for Genzyme; received travel funding and speaker honoraria from Novartis, Merck Serono, and CSL Behring; and received research support from Novartis, Biogen, and CRC. Go to Neurology.org/nn for full disclosure forms.

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