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Dimethyl Fumarate Selectively Reduces Memory T Cells and Shifts the Balance between Th1/Th17 and Th2 in Multiple Sclerosis Patients

Qi Wu,* Qin Wang,* Guangmei Mao,* Catherine A. Dowling,* Steven K. Lundy,†‡ and Yang Mao-Draayer*‡

Dimethyl fumarate (DMF; trade name Tecfidera) is an oral formulation of the fumaric acid ester that is Food and Drug Administration approved for treatment of relapsing-remitting multiple sclerosis. To better understand the therapeutic effects of Tecfidera and its rare side effect of progressive multifocal leukoencephalopathy, we conducted cross-sectional and longitudinal studies by immunophenotyping cells from peripheral blood (particularly T lymphocytes) derived from untreated and 4–6 and 18–26 mo Tecfidera-treated stable relapsing-remitting multiple sclerosis patients using multiparametric flow cytometry. The absolute numbers of CD4 and CD8 T cells were significantly decreased and the CD4/CD8 ratio was increased with DMF treatment. The proportions of both effector memory T cells and central memory T cells were reduced, whereas naive T cells increased in treated patients. T cell activation was reduced with DMF treatment, especially among effector memory T cells and effector memory RA T cells. Th subsets Th1 (CXCR3⁺), Th17 (CCR6⁺), and particularly those expressing both CXCR3 and CD161 were reduced most significantly, whereas the anti-inflammatory Th2 subset (CCR3⁺) was increased after DMF treatment. A corresponding increase in IL-4 and decrease in IFN-γ and IL-17—expressing CD4⁺ T cells were observed in DMF-treated patients. DMF in vitro treatment also led to increased T cell apoptosis and decreased activation, proliferation, reactive oxygen species, and CCR7 expression. Our results suggest that DMF acts on specific memory and effector T cell subsets by limiting their survival, proliferation, activation, and cytokine production. Monitoring these subsets could help to evaluate the efficacy and safety of DMF treatment. The Journal of Immunology, 2017, 198: 3069–3080.

Multiple sclerosis (MS) is an autoimmune disease of the CNS that leads to multifocal inflammatory lesions in the brain and spinal cord. MS pathogenesis is thought to involve inflammatory responses against oligodendrocytes mediated by activated myelin-specific T cells, resulting in demyelination of the axon. Investigations using the mouse model of MS, experimental autoimmune encephalomyelitis (EAE), have shown that activation of CD4⁺ Th cells through cognate interactions with myelin peptide–loaded APCs can lead to a breakdown in immune tolerance and the formation of memory T cells capable of transferring disease to naive recipients. Pathogenesis in EAE has been associated with either activation of Th1- or Th17-type immune responses and involves upregulation of the surface adhesion molecule VLA4, which enables Th cells to cross the blood–brain barrier (BBB) and infiltrate into the CNS. Additionally, activated CD8⁺ CTLs, B lymphocytes, and myeloid cells infiltrate the CNS and participate in inflammation and tissue damage. Both CD8⁺ and CD4⁺ T cells have been visualized in acute inflammatory lesions and to a lesser extent in chronic demyelinated lesions in humans (1).

Immunological memory is an important property of the adaptive immune system. Naive T (Tn) cells (CCR7⁺CD45RA⁺) and central memory T (Tcm) cells (CCR7⁻CD45RA⁻) exhibit high proliferative potential but lack immediate effector functions, whereas effector memory T (Tem) cells (CCR7⁻CD45RA⁻) and effector memory RA (Temra) cells (CCR7⁺CD45RA⁻) have less proliferative potential but produce cytokines and exert competent effector functions, respectively (2, 3). Tcm cells, marked by coexpression of the memory marker CD45RO and the chemokine receptor CCR7, arise in response to autoantigen exposure. Loss of CCR7 expression facilitates the egress of T cells out of lymphoid organs into the periphery, where they are designated as Tem cells. Enecephalitogenic Tem cells represent a pool of CNS Ag-primed T cells that are capable of extravasation across the endothelial wall of the BBB, migrating into the CNS where they can mediate inflammatory responses (4). In contrast, Tcm cells usually reside in the T cell areas of secondary lymphoid organs. They have little...
or no direct effector function, but can support development of other effector cells or readily proliferate and differentiate into Tem cells upon secondary antigenic challenge to generate a new wave of reactions. CD45RA<sup>+</sup> effector Temra cells represent the most differentiated type of memory cells; they have high susceptibility to apoptosis and express high levels of cytotoxic molecules such as perforin and Fas ligand. As CD4<sup>+</sup> Tn cells become activated by APCs and differentiate into memory Th cells, they encounter costimulatory signals that drive their differentiation into subsets, that is, Th1, Th17, Th2, and regulatory T (Treg) cells, with distinct patterns of gene transcription, cytokine production, chemokine receptor expression, and effector functions. A major therapeutic goal in treating MS is to re-establish tolerance toward CNS Ags, which in theory may be achieved through controlling the development, differentiation, and survival of Ag-specific memory Th1 and Th17 cells, or skewing their responses toward Th2 or Treg cell functions.

Dimethyl fumarate (DMF; also known as BG-12, trade name Tecfidera) is an oral formulation of the fumaric acid ester that showed remarkable efficacy and low adverse effects in two phase III clinical trials, leading to its approval by the Food and Drug Administration for treating relapsing-remitting MS (RRMS) (6, 7). DMF is also an active ingredient of Fumaderm, a drug that has been used to treat psoriasis in Germany for several decades (8–10). Clinical trials of DMF-treated RRMS patients showed significant reductions in the proportion of patients with relapse, disability progression, and numbers of new magnetic resonance imaging (MRI) lesions (6, 7, 11). We and others have shown that the clinical effects of DMF may involve its direct antioxidant effects on the nervous system and neuroprotection (12, 13). The effectiveness of DMF in psoriasis also suggests an immune regulatory role, which prompted us to study the effects of DMF treatment on human peripheral lymphocytes in DMF-treated RRMS patients. We recently reported that RRMS patients treated with DMF had reduced levels of naive and memory B cells, whereas there was an increase in B cells with phenotypes reported to produce IL-10 and to have regulatory capacity (14). Rare cases of progressive multifocal leukoencephalopathy (PML) have been reported to be associated with long-term DMF treatment of psoriasis and MS patients (15–18), which makes it important to understand the immunological changes induced by DMF treatment to find reliable biomarkers that can predict JC virus activation and prevent PML.

To assess the balance between memory and effector cells, we analyzed the phenotypes and functional response profile of T cells within various memory and helper subsets in RRMS patients treated with DMF. Collectively, our cross-sectional and prospective longitudinal studies indicate that DMF decreases frequencies and absolute numbers of peripheral T lymphocytes, particularly Tem and Tcm cells. Additionally, T cell activation was reduced with DMF treatment, especially among Tem and Temra cells. Th1 (CXCR3<sup>+</sup>), Th17 (CCR6<sup>+</sup>), and particularly those subsets expressing both CXCR3 and CD161 were reduced most significantly, whereas the anti-inflammatory Th2 subset (CCR<sup>+</sup><sub>3</sub>) was increased after DMF treatment. DMF in vitro treatment also led to increased T cell apoptosis and decreased activation, proliferation, reactive oxygen species (ROS), and CCR7 expression. These findings implicate T cell involvement in the action of DMF in treating RRMS. Our studies help to establish the link between the efficacy of DMF and its effects on memory T cells and Th subtypes. The relevance to the therapeutic effects of DMF and its possible relationship to rare incidences of PML are also discussed.

### Materials and Methods

#### RRMS patients and PBMC isolation

We enrolled all patients in this study from the Multiple Sclerosis Center at the University of Michigan Health System. All subjects had a clinical diagnosis of RRMS and were treated with Tecfidera. Informed consent was obtained from patients prior to participation in the study, which was approved by the University of Michigan Institutional Review Board. Table I summarizes the demographic characteristics and clinical data of patients in this study. Prior disease-modifying therapies (DMTs) for those patients who were treated within a 6-mo time point of starting Tecfidera are also listed in Table I. All patients in this study discontinued other DMTs while taking Tecfidera. Blood samples were collected in tubes containing sodium citrate (BD Biosciences) and processed immediately as they were collected. PBLs and monocytes (PBMCs) were isolated from peripheral blood by a density gradient centrifugation according to the manufacturer’s suggested protocol.

#### Flow cytometry

Isolated PBMCs were stained with panels of mAbs against human cell surface and intracellular markers of major cell lineages and lymphocyte subsets. Abs and reagents used in this study are summarized in Supplemental Table I, and the specific Abs used for each figure are outlined in the figure legends. 7-Aminoactinomycin D (7AAD) was used to exclude dead cells from the analysis. Only included in Supplemental Fig. 2, FOXP3 was stained intracellularly after cell surface staining of CD3, CD4, and CD25 using a FOXP3 staining buffer set (eBioscience, San Diego, CA) according to the manufacturer’s suggested protocol. For other intracellular staining, surface-stained cells were fixed and permeabilized using a BD Cytofix/Cytoperm kit (BD Biosciences). Stained PBMCs were analyzed on a BD FACSVerse II flow cytometer. Flow cytometry data were analyzed using FlowJo version 7.6.5 software (FlowJo, Ashland, OR). Cell lineage surface markers used in this study are summarized in the Results section.

#### Ex vivo T cell cytokine analysis

Frozen PBMCs from patients were thawed quickly in 37°C prewarmed complete RPMI with 5 U/ml Benzonase (EMD Millipore, Billerica, MA). After 3 min incubation at 37°C, cells were washed twice with complete RPMI. The cells were then co-cultured at 2.5 × 10<sup>5</sup> cells/ml with 80 ng/ml PMA, 1 μg/ml ionomycin, and 1 μg/ml brefeldin A for 6 h with 5% CO2 at 37°C. At the end of the culture, cells were collected and washed with PBS containing 2% FBS and surface stained with an Abs panel against human IL-17A and IL-17F (clones eBio64CAP17 and SHLR17; eBioscience) and FITC-conjugated anti-human IFN-γ (clone B27; Thermo Fisher/Invitrogen, Waltham, MA) in BD Cytofix/Cytoperm Perm/Wash buffer twice to remove dead cells from the analysis. Only included in Supplemental Fig. 2, FOXP3 staining of IL-4 was carried out using anti–IL-4-allophycocyanin (BioLegend). Cells were then fixed for 15 min using BD Cytofix fixation buffer at room temperature, washed twice with PBS containing 2% FBS, and stored overnight at 4°C. Before intracellular staining, cells were permeabilized using BD Cytofix/Cytoperm Perm/Wash for 15 min and stained with PE-conjugated Abs against human IL-17A and IL-17F (clones eBio64CAP17 and SHLR17; eBioscience) and FITC-conjugated anti-human IFN-γ (clone B27; Thermo Fisher/Invitrogen, Waltham, MA) in BD Cytofix/Cytoperm Perm/Wash buffer for 30 min with occasional vortex. Cells were then washed with BD Cytofix/Cytoperm Perm/Wash buffer, resuspended in unbound Abs before analysis using the BD FACSVerse II flow cytometer in staining buffer (PBS with 2% FBS). To assay the production of IL-4 by ex vivo CD4<sup>+</sup> T cells, thawed frozen PBMCs derived from patients before or after DMF treatment were rested in complete RPMI at 37°C with 5% CO2 for 18 h, followed by an additional 6 h with 1 μg/ml brefeldin A before being harvested and stained with anti–CD3-PE-Cy7 (BioLegend) and anti–CD4-Brilliant Violet 510 (BioLegend). Intracellular staining of IL-4 was carried out using anti–IL-4-allophycocyanin (BioLegend) with the BD Cytofix/Cytoperm method as described above.

#### In vitro DMF treatment assays

PBMCs of healthy donors were prepared from plasmapheresis filters by washing in reverse flow with 30 ml of PBS followed by standard Ficoll-Hypaque density centrifugation. After washing with PBS, PBMCs were cultured in complete RPMI (RPMI 1640 with 10% human AB plasma, 2 mM glutamine, and 100 U/ml penicillin and streptomycin) at 2.5 × 10<sup>6</sup> cells/ml for 48 h in a six-well plate. DMF (10, 100 μM) or DMSO (vehicle control) was added at the beginning of the culture.

For apoptosis analysis, unstimulated cells were first stained with anti–CD8-FITC/anti–CD3-PE-Cy7 (BioLegend) and FITC-conjugated anti-human IFN-γ (clone B27; Thermo Fisher/Invitrogen, Waltham, MA) in BD Cytofix/Cytoperm Perm/Wash buffer for 30 min with occasional vortex. Cells were then washed with BD Cytofix/Cytoperm Perm/Wash buffer, resuspended in BD Cytofix/Cytoperm Perm/Wash buffer, and stained intracellularly after cell surface staining of CD3, CD4, and CD25 using a BD Cytofix/Cytoperm Perm/Wash 3-color staining buffer set (eBioscience) and anti–CD8-FITC/anti–CD3-PE-Cy7/anti–CD4-Brilliant Violet 510 (BioLegend). Intracellular staining of IL-4 was carried out using anti–IL-4-allophycocyanin (BioLegend) with the BD Cytofix/Cytoperm method as described above.
For T cell activation, ROS, cell proliferation, and CCR7 expression analysis, T cells were activated by anti-CD3 and anti-CD28. Briefly, the six-well plates were precoated with 3 μg/ml anti-CD3 for 4 h at 37°C and washed once with PBS. PBMCs were then seeded at 2.5 × 10^6 cells/ml with 5 μg/ml soluble anti-CD28. After 48 h, cultured PBMCs were harvested and stained with 7AAD/anti-CD3-PE-Cy7/anti-CCR7-allophycocyanin/anti-CD45RA-Pacific Blue/anti-CD4-Brilliant Violet 510. For ROS assays in T cells, stained cells were then washed with complete RPMI and incubated with 5 μM of a chloromethyl derivative of 2',7'-dichlorodihydrofluorescein diacetate (CM-H2DCFDA; Thermofisher/Invitrogen) in complete RPMI at 37°C for 10 min, and then washed with staining buffer before being analyzed on a flow cytometer. For analyzing cell activation and proliferation, cultured cells were surface stained with anti-CD3-PE-Cy7/anti-CD45RO-allophycocyanin-Cy7/anti-CD69-Brilliant Violet 510. After washing, cells were fixed and stained intracellularly using anti-Ki67-EFlour 450 with a BD Cytofix/Cytoperm kit according to the manufacturer’s recommended protocol. CCR7 expression was measured using the value of geometric mean fluorescence of CCR7 on a gated T cell population calculated by FlowJo.

Statistical analysis

For cross-sectional study of DMF treatment groups, nontreated RRMS, 4–6 mo DMF-treated, and 18–26 mo DMF-treated groups were compared using nonparametric tests and a post hoc Dunn multiple comparison test to assess the differences between groups. For longitudinal samples, we implemented the Friedman test with a Dunn multiple comparison test to find the pairwise difference between paired data. For in vitro experiments, a Wilcoxon signed-rank test was used to assess the difference between control and DMF groups. All statistical analyses were performed using GraphPad Prism6 software (GraphPad Software, La Jolla, CA).

Results

DMF treatment reduced circulating lymphocyte count

All RRMS patients treated with Tecfidera in this study cohort have been clinically stable without further relapses, new MRI activities, or disease progression measured by the Expanded Disability Status Scale up to 26 mo during our study (Table I). Previously, these patients were either treatment naive (8 of 18 in the baseline cohort), intolerant to other DMTs (8 of 10 who had been on prior DMT), or had some breakthrough disease activities on other DMTs (5 of 10). More specifically, among the 18 subjects in the baseline cohort, 13 had new MRI lesions and 12 had new symptoms (mostly sensory) in the months to year prior to initiating Tecfidera. There were no statistical differences in the characteristics of the three study groups in the cross-sectional study (Table I). To determine the effect of DMF on peripheral blood counts, we first compared cross-sectionally RRMS patients treated with DMF for either the short term (4–6 mo, n = 20) or long term (18–26 mo, n = 18) with age- and sex-matched untreated RRMS patients (n = 18). Furthermore, prospective data from nine of the RRMS patients from whom a sample was collected at all time points were analyzed and presented in separate graphs. Complete blood counts were obtained at the University of Michigan hospital pathology laboratory and the results were retrieved from the patients’ records. As shown in Table II, the total WBC counts and PBMC counts were reduced significantly with DMF treatment at both short-term and at long-term time points. Among all WBC lineages, lymphocyte counts were significantly reduced at 4–6 mo and remained so up to 26 mo with DMF treatment. DMF treatment did not show significant effects on neutrophil, eosinophil, basophil, and monocyte populations.

DMF treatment reduced both circulating CD4+ T cell and CD8+ T cell counts

To further understand the effects of DMF treatment on subtypes of lymphocytes, PBMCs were isolated and stained with fluorochrome-conjugated Abs against cell surface markers for T cells, B cells, monocytes, and NK cells, followed by multicolor flow cytometric analysis. Representative staining profiles are shown in Fig. 1A, and the gating was consistent for all of the samples analyzed in the study. As shown in Fig. 1B, compared with the untreated group, CD4+ T lymphocyte counts were significantly reduced in the 4–6 mo treated group (p = 0.002) and in the >18 mo (i.e., 18–26 mo) treated group (p = 0.002). Absolute numbers of CD8+ lymphocytes were lower in the 4–6 mo treatment group (median, 0.22 × 10^6/ml) compared with the untreated group (median, 0.40 × 10^6/ml; p = 0.045), but these were even lower in the >18 mo treated group (median, 0.17 × 10^6/ml; p = 0.0002). With more extensive reductions of CD8+ T cells, the CD4/CD8 T cell ratio was significantly higher for the >18 mo group (median, 6.3) than that of the untreated group (median, 3.9; p = 0.010). Moreover, NK cell counts, identified as forward scatter (FSC)3CD14+ TCRαβCD56+, were progressively decreased and the differences were significant between the long-term >18 mo treatment group and the untreated group (p = 0.0007). B cell counts were significantly decreased after >18 mo of treatment, and the detailed B cell subtype changes were described in our recent paper (14). Total NK cell counts showed no significant differences among the three groups.

To further validate the changes to the lymphocyte profiles noted in the cross-sectional cohort with DMF treatment over time, we analyzed the longitudinal data from 9 out of these 18 RRMS patients who were able to be followed at all three time points before and after DMF treatment (Fig. 1C). The results for individual patients showed similar trends as were observed in the cross-sectional analysis. CD4+ T, CD8+ T, B, and NK cell counts were significantly decreased, especially with long-term treatment. More significant reductions were seen of CD8+ T cells compared with CD4+ T cells, resulting in a significant increase of the CD4/CD8 ratio after >18 mo of treatment with DMF.

DMF treatment reduced memory T cells, particularly Tem cells

To determine the effect of DMF on subsets of T cells with memory or nonmemory phenotypes, PBMCs from patients before and after DMF treatment were analyzed for their expression of CD45RA, CD45RO, and CCR7 on T cells. A representative flow cytometry staining profile for one patient before and after treatment is shown in Fig. 2A. Compared with the untreated group, the memory fractions (CD45RA-CD45RO+) of T cells were both significantly reduced from a median of 45.2% (CD3+CD4+) and 37.7% (CD3+CD8+) in the untreated group to 28.4% (p = 0.0007) and 19.2% (p = 0.0093), respectively, after 4–6 mo of DMF treatment. These subsets were further reduced to 24.7% (p = 0.0006) and 17.5% (p = 0.0004), respectively, after 18 mo of treatment (Fig. 2B). Using analysis of chemokine receptor CCR7 expression, the central memory fraction of CCR7+CD45RO−CD3+CD4+ T (CD4+ Tem) cells was found to be significantly lower in both the 4–6 mo group (p = 0.028) and >18 mo group (p = 0.009) than in the untreated patient group (Fig. 2C). In contrast, the CD4− Tem cell fraction was not altered significantly with DMF treatment. Compared to the untreated group, the fractions of CD45RO−CCR7− Tem of both CD4+ and CD4− T cells were decreased from a median of 15.5 and 30.0% in the untreated group to 6.8% (p = 0.0008) and 14.0% (p = 0.002), respectively, in the 4–6 mo group, and they were sustained at a low level in the long-term (>18 mo) treatment group (p < 0.0001 and p = 0.0007, respectively). Reciprocally, we found that the naive fractions (CCR7+CD45RA+Tn) of both CD4+ and CD4− T cells were relatively higher in both short-term and long-term treatment groups than in the untreated control group. Temra cell (CD45RO−CCR7+) fractions were less affected in both CD4+ and CD4− T cells by DMF treatment.
suggesting that DMF inhibits T cell activation. Furthermore, the CD4 persists after DMF treatment, whereas Tcm cell fractions of is correlated with [3H]thymidine incorporation (21). To study whether T cell activation could be affected by DMF treatment, we analyzed the frequency of CD69+ cells among T cells. The percentage of CD69+ Temra cells (Fig. 3). This indicated that DMF reduced functional activation of Temra cells.

**DMF treatment reduced Th1, Th17, and Th1/Th2 cells, but did not alter frequencies of Treg cells**

Th1 and Th17 subsets have been found to be pathogenic in MS, whereas Th2 cells and Treg cells may suppress autoimmune responses and have protective effects in MS (22, 23). Peripheral Th subsets from patients before and after treatment with DMF were analyzed using Abs against CXCR3, CCR6, CCR3, CD161, CD25, and FOXP3 that mark specific Th cell functional subsets from patients before and after treatment with DMF were analyzed using Abs against CXCR3, CCR6, CCR3, CD161, CD25, and FOXP3 that mark specific Th cell functional subsets (Table III). CCR3, CCR4, and CRTh2 have all been used as surface markers of Th2 cells, and we found in preliminary studies that most IL-4+ Th cells ex vivo were enriched in the CCR3+ (Table III). CCR3, CCR4, and CRTh2 have all been used as surface markers of Th2 cells, and we found in preliminary studies that most IL-4+ Th cells ex vivo were enriched in the CCR3+CD4+CCR7−CD45RA+ (CD4+ Temra) and CD4− Temra cells were not changed during the duration of the study (Fig. 2D).

**DMF inhibited T cell activation**

CD69 is a marker commonly used to evaluate T cell activation (19, 20). Its expression on T cells is induced by TCR engagement and is correlated with [3H]thymidine incorporation (21). To study whether T cell activation could be affected by DMF treatment, we analyzed the frequency of CD69+ cells among T cells. The percentage of CD69+ of total CD4+ and CD4− T cells was decreased significantly after >18 mo of treatment as compared with the untreated group (p = 0.004 and p = 0.03 respectively, Fig. 3), suggesting that DMF inhibits T cell activation. Furthermore, the percentage of CD69+ cells in Tem and Temra cells was decreased significantly after 18 mo of treatment with DMF, particularly for CD4+ T cells (Fig. 3). Although the proportion of reduction of Temra cells was not as significant compared with Tcm and Tem cells (Fig. 2), there was a significant reduction of the percentage of CD69+ Temra cells (Fig. 3). This indicated that DMF reduced functional activation of Temra cells.

<table>
<thead>
<tr>
<th>Table I. Demographic characteristics of RRMS patients</th>
</tr>
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<tbody>
<tr>
<td>Untreated (n = 18)</td>
</tr>
<tr>
<td>Age, y&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Gender&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Female</td>
</tr>
<tr>
<td>Male</td>
</tr>
<tr>
<td>Race&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>White</td>
</tr>
<tr>
<td>African-American</td>
</tr>
<tr>
<td>Disease duration, y&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Baseline EDSS score&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>Tx&lt;sup&gt;f&lt;/sup&gt;, naïve</td>
</tr>
<tr>
<td>Prior Tx&lt;sup&gt;g&lt;/sup&gt; (&gt;6 mo)</td>
</tr>
<tr>
<td>Prior Tx&lt;sup&gt;g&lt;/sup&gt; (3–6 mo)</td>
</tr>
<tr>
<td>Prior Tx&lt;sup&gt;g&lt;/sup&gt; (&lt;3 mo)</td>
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</tbody>
</table>

<sup>a</sup>Data are presented as mean ± SD; p value was from ANOVA test.
<sup>b</sup>Data are presented as number (%); p value was from χ² or Fisher exact test.
<sup>c</sup>Data are presented as median (Q1, Q3); p value was from Kruskal–Wallis ANOVA test.
<sup>d</sup>DMT prior to baseline draw.
<sup>e</sup>Treatment prior to baseline draw, not time point.
<sup>f</sup>Prior treatment for ankylosing spondylitis.

Table II. Effects of DMF on complete blood counts of RRMS

<table>
<thead>
<tr>
<th>Untreated Median (IQR)</th>
<th>4–6 mo DMF</th>
<th>18–26 mo DMF</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBCs</td>
<td>7.2 (3.27)</td>
<td>5.4 (1.1)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.022</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>4.4 (2.33)</td>
<td>3.7 (1.8)</td>
<td>NS</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>1.8 (1.3)</td>
<td>1.3 (0.6)b</td>
<td>0.002</td>
</tr>
<tr>
<td>Monocytes</td>
<td>0.60 (0.35)</td>
<td>0.6 (0.3)</td>
<td>NS</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>0.20 (0.18)</td>
<td>0.10 (0.1)</td>
<td>NS</td>
</tr>
<tr>
<td>PBMCs</td>
<td>2.6 (1.33)</td>
<td>1.7 (0.7)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.003</td>
</tr>
</tbody>
</table>

Numbers represent cell counts (×10<sup>3</sup>/μl blood). The absolute counts of PBMCs are deduced from the complete blood count value by subtracting absolute counts of neutrophils, eosinophils, and basophils from that of WBCs. PBMC values are the sum of lymphocytes, monocytes, and early granulocytes.

<sup>a</sup>p < 0.05, <sup>b</sup>p < 0.01 compared with RRMS by Kruskal–Wallis ANOVA with a Dunn multiple comparison test.

IQR, interquartile range.
Because CD161 was also found to be associated with the IL-17–producing T cell subset (27–29), its expression was also analyzed. With DMF treatment, we found that the fraction of CD161+ cells among CD4+ T cells was reduced significantly after 18 mo of treatment with DMF (data not shown). Significant reductions were observed in those CD4+ T cells that were triple positive for CXCR3, CCR6, and CD161 (cross-sectional, \( p = 0.0052 \); longitudinal, \( p = 0.0066 \)) (Fig. 4C) and in double-positive cells expressing CXCR3 and CD161, but not CCR6 (cross-sectional, \( p = 0.0004 \); longitudinal, \( p = 0.0029 \)) (Fig. 4D). Based on these findings, the functional capacity of CD4+ T cells to produce pathogenic cytokines IFN-\( \gamma \) and IL-17 upon ex vivo stimulation was evaluated (Fig. 4E, Supplemental Fig. 1A). The percentages of CD4+ T cells producing IFN-\( \gamma \) or IL-17 were gradually reduced and more significantly reduced for >18 mo of treatment when compared with baseline, whereas the frequency of ex vivo CD4+ T cells producing IL-4 without stimulation was increased after DMF treatment (Fig. 4E, Supplemental Fig. 1B). Interestingly, the frequencies of the two CD4+ T cell subsets (CD161+/CCR6+/CXCR3+ and CD161+/CCR6−/CXCR3+) that were preferentially reduced by DMF treatment were found closely correlated with the frequency of CD4+ T cells producing IFN-\( \gamma \), but not IL-17 (Fig. 4C, 4D).

Frequencies of CD25+FOXP3+CD4+ Treg cells were not changed significantly at both 4–6 mo and at >18 mo of DMF treatment (Supplemental Fig. 2A). Because the balance between Th1 and Th17 to Th2 is important for the pathogenesis of RRMS,
FIGURE 2. DMF treatment reduced memory T cells. PBMCs were isolated from blood of patients and stained with Abs specific to surface markers CD3, CD4, CD45RO, CD45RA, and CCR7 to analyze subsets of memory cells. (A) Representative profile depicting the frequencies of memory and nonmemory phenotypes among CD4+CD3+ T cells (top panels) and CD3+CD4+ T cells (lower panels) from an RRMS patient prior to DMF treatment and after 4–6 mo and >18 mo of continuous DMF treatment. (B) Frequencies of memory (CD45RA−CD45RO+) fractions as a percentage of CD3+CD4+ (upper panels) and CD3+CD4− (lower panels) T cells in cross-sectional (left panel) untreated (UNT, n = 18), 4–6 mo DMF-treated (4–6M, n = 20), and 18–26 mo (18M, n = 18) DMF-treated groups, as well as longitudinal cohort (n = 9, right panel). (C) Frequencies of naive Tn (CD45RO−CCR7+), Tcm (CD45RO+CCR7+), Tem (CD45RO+CCR7−), and CD45RA+ Temra (CD45RO+CCR7−) cells of CD3+CD4+ (upper panels) and CD3+CD4− (lower panels) T cells in three cross-sectional patient groups. Scatter plots show data for individual patients as dots and the median of each group as a line. The p values are from a Kruskal–Wallis ANOVA with a Dunn multiple comparison test and are shown above the data for each cell type. (D) Tn, Tcm, Tem, and Temra cells as a percentage of CD3+CD4+ (upper panels) or CD3+CD4− (lower panels) T cells in the longitudinal cohort at the three time points. The p values are from a Friedman test with a Dunn. (Figure legend continues)
the ratios of Th1 (CXCR3⁺) and Th17 (CCR6⁺ or CD161⁺) to Th2 (CCR3⁺) were calculated. All of these ratios were found significantly reduced with DMF treatment (Supplemental Fig. 2B). However, the ratio of these Th1/Th17 subsets to Treg cells was unchanged with DMF treatment (Supplemental Fig. 2C).

Effects of DMF on T cell apoptosis, activation, ROS production, and proliferation in vitro

To further investigate potential mechanisms underlying the treatment effects of DMF in MS patients, we cultured fresh unstimulated or anti-CD3/anti-CD28–stimulated PBMCs derived from healthy individuals and treated the cells with DMF. As shown in Fig. 5A, DMF treatment significantly increased apoptosis (percentage annexin V⁺/7AAD⁺) of both unstimulated CD4⁺ T cells (10 μM, p = 0.0156; 100 μM, p = 0.0156) and CD4⁻ T cells (10 μM, p = 0.0078; 100 μM, p = 0.0078).

To investigate whether DMF will have direct effects on T cell activation, we analyzed the percentage of enlarged blasts (CD3⁺, FSC-areahi) in the PBMC culture activated by anti-CD3 and anti-CD28. T cell blasts are activated T cells expressing CD69. Few of these activated T cells (CD69⁺) were positive for 7AAD staining, confirming that they were alive (Supplemental Fig. 3). A significant reduction of the percentage T cell blasts was observed in cultures treated with 100 μM DMF (median, 23.05%; p = 0.0156) compared with the vehicle control group (median, 30.15%) (Fig. 5B).

T cell activation triggers the production of superoxides that can alter the behavior of the cells in positive or negative ways. Because DMF was reported as an antioxidant, we measured the effect of DMF on ROS level in T cells by staining anti-CD3/anti-CD28–stimulated PBMCs with CM-H₂DCFDA, which can penetrate into live cells and get oxidized by ROS to produce fluorescence. As shown in Fig. 5C, compared with vehicle control, the mean fluorescence intensity of ROS of both CD4⁺ and CD4⁻ activated T cells was reduced at 10 μM DMF (CD4⁺, p = 0.0078; CD4⁻, p = 0.0391) and significantly at 100 μM concentrations (CD4⁺, p = 0.0078; CD4⁻, p = 0.0078). Reduction of ROS further confirmed that DMF played an antioxidant role in activated T cells.

The effect of DMF on proliferation of activated T cells was determined by Ki67 expression (Fig. 5D). The percentage of Ki67⁺ cells among activated CD4⁺ and CD4⁻ T cells was significantly reduced in the culture with 100 μM DMF treatment (CD4⁺ T cells, p = 0.0156; CD4⁻ T cells, p = 0.0078), but not with 10 μM DMF.

Because CCR7 is important for lymphocyte migration into secondary lymphoid organs, we analyzed the effects of DMF on CCR7 surface expression on T cells. Anti-CD3– and anti-CD28–stimulated PBMCs were treated with DMF for 48 h. As shown in Fig. 5E, 10 μM DMF exerts a suppressive effect on the expression of CCR7, particularly on CD4⁺ T cells (p = 0.0181 for 10 μM, p = 0.0181 for 100 μM). There was also a reduction of CCR7 expression for CD4⁻ T cells at 100 μM DMF treatment (p = 0.0199).

Discussion

Understanding the lymphocyte subpopulation changes associated with DMF treatment is important for monitoring treatment efficacy and potential long-term side effects. Such studies could also lead to discovery of surrogate markers for evaluating MS disease activity and progression. We thus conducted a cross-sectional study of RRMS patients on DMF treatment for up to 2 y, along with a smaller longitudinal study within the same cohort of patients. Utilizing multiparametric flow cytometry, we recorded comprehensive immunophenotyping results following short-term (4–6 mo) and long-term (18–26 mo) DMF treatment in RRMS patients. There were reductions of multiple peripheral immune cell subtypes, including CD4⁺ T cells, CD8⁺ T cells, B lymphocytes, and NKT cells. Compared with CD4⁺ T cells, we observed a more significant reduction of CD8⁺ T cells. Among all T cells, memory T cells (especially Tem cells) were the most affected by DMF treatment, both short term and long term. In addition to the reduction of both frequency and absolute numbers, the activation potential of Tem and Temra cells was also reduced. Furthermore, DMF treatment was also shown to increase the abundance of anti-inflammatory Th2 cells while decreasing proinflammatory Th1 and Th17 cells. Correspondingly, DMF also led to an increase in IL-4 and a decrease in IFN-γ and IL-17⁺ Th cells.
Our study followed RRMS patients on DMF treatment for > 2 y, contrasting with an earlier report that followed DMF treatment for only 6 mo (30). In our study of DMF-treated patients, ~15% of the RRMS patients (3 out of 20) had grade 2–3 lymphopenia (absolute lymphocyte count, <800 cells/µl) at 4–6 mo, whereas at 18–24 mo only 1 out of 18 patients had grade 2–3 lymphopenia; none of our patients had grade 4 lymphopenia (lymphocyte count, <200 cells/µl; Fig. 1, Table II). This differs from the patient cohort in a previous study, where 17 of 41 (41%) patients were lymphopenic (31). Our cohort did not show such a significant degree of lymphopenia overall. As initially reported by Spencer et al. (32), there was an increased CD4/CD8 ratio at 12 mo with DMF treatment. We found that there was a more pronounced reduction of CD8+ T cells than CD4+ T cells after 18 mo of DMF treatment as well, resulting in an elevation of the CD4/CD8 ratio in the 18–26 mo group but not in the 4–6 mo group. A recent cross-sectional study (31) demonstrated that only in patients with grade 2–3 lymphopenia were CD4+ and CD8+ T cells decreased and the CD4/CD8 ratio increased. In our group of patients without significant lymphopenia, a significant reduction of CD4+ T cells was observed as early as 4–6 mo, whereas a more significant reduction of CD8+ T cells and an increase of the CD4/CD8 ratio became more compelling with longer term (>18 mo) DMF treatment (Fig. 1). DMF showed more profound suppressive effects on CD4+ T cells compared with CD4+ T cells in our in vitro study (Fig. 5), which was consistent with the in vivo DMF treatment effect in RRMS patients (as most CD4+ T cells are CD8 cells). The findings in the cross-sectional study were consistently confirmed in individual patients that were followed longitudinally. Thus, a longer and more detailed follow-up of DMF-treated MS patients, as was shown in this study, could be beneficial in detecting differences in the effect of DMF on the immunological profile in individual patients, especially for those without marked lymphopenia.

Earlier studies have shown that RRMS patients had fewer peripheral naive T cells and more effector memory T cells than did healthy controls (33, 34), emphasizing the potential importance of Tem cells in the pathogenesis of RRMS. In our study, DMF suppressed both Tcm and Tem cells as early as 4–6 mo of treatment, supporting previous results that showed >6 mo treatment of DMF decreased the frequency of Tcm and Tem cells with relative expansion of Tn cells in DMF-treated patients with or without lymphopenia (31). Compared to previous publications, our study provided a more long-term follow-up of DMF treatment that in some cases was >2 y. Our data showed interesting kinetics of CD4+ memory T cells: the percentage of CD4+ Tcm cells decreased after 4–6 mo and then recovered significantly after 18 mo of treatment, whereas the percentage of CD4− Tem cells stayed low with long-term DMF treatment (>18 mo) (Fig. 2). Although the absolute number and proportional reduction of Temra cells were not as significant compared with Tcm and Tem cells, there was a significant reduction in the percentage of CD69+ Temra cells. This indicates that DMF reduced functional activation of Temra cells. Temra cells are a very interesting subset of memory T cells that has not been previously studied following DMF treatment. Temra cells represent the most differentiated type of memory cells and are highly susceptible to apoptosis. They also express high levels of cytotoxic molecules such as perforin and Fas ligand (2). Similar to Tem cells, Temra cells in particular are more competent to carry out effector functions than are Tcm cells; they exhibit potent ex vivo cytotoxicity and produce Th1 cytokines upon stimulation (2). Therefore, the reduction of CD69+ frequency among CD4+ Temra cells is consistent with reductions of Th1 cells observed for patients treated with DMF.

Considering the significant decrease of the absolute CD8 count, the recovery of CD4− Tcm cells (which contains mostly CD8+ T cells) may reflect lymphopenia-induced homeostatic proliferation (35). CD8+ Tcm cells were found to be at low frequencies but with high proliferative ability, playing an important role in self-renewal and long-term survival (36, 37). Thus, the recovery of CD8+ Tcm cells found in this study may suggest that DMF at current dosing does not completely shut off homeostatic proliferation, which is usually driven by a relative overabundance of IL-7 and self Ag–MHC complexes (35). CD4+ Tcm cells were not increased, possibly due to the fact that they were not depleted as much as CD8+ T cells by DMF treatment.

Table III. Cell surface lineage markers used

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FIGURE 4. DMF specifically reduced Th1 and Th17, particularly those expressing CXCR3 and CD161, but increased Th2 cells. PBMCs from patients were stained with Abs against CD3, CD4, CXCR3, CCR6, CCR3, and CD161 to evaluate frequencies of CXCR3+ (Th1), and CCR6+ (Th17) on CD4+ T cells (UNT, n = 15; 4–6M, n = 19; >18M, n = 18). The CCR3+ (Th2) of total CD4+ T cells were also analyzed (UNT, n = 11; 4–6M, n = 20; >18M, n = 18). These frequencies were plotted as dots at three time points with the median of each group as a line in three cross-sectional groups (A) or in the longitudinal cohort (B). DMF effects on the frequencies of CD4+ T cells expressing CD161 and CXCR3 with (C) or without (D) CCR6 in cross-sectional (left panels) and longitudinal (middle panels) studies, and corresponding correlation (right panels) with frequencies of CD4+ T cells producing IFN-γ but not IL-17. (E) IFN-γ (left panel) and IL-17 (middle panel) production by CD4+ T cells was analyzed in our longitudinal study following stimulation of PBMCs with PMA and ionomycin for 6 h in the presence of brefeldin A. Cells were then stained with fluorochrome-conjugated Abs against CD3 and CD4, followed by intracellular staining of Abs against IFN-γ, IL-17A, and IL-17F. IL-4 (right panel) production was measured from ex vivo PBMCs without further in vitro stimulation because it was clearly detectable (Supplemental Fig. 1B). PBMCs were incubated in complete RPMI for 24 h with brefeldin A added in the last 6 h of culture. The p values are from a Kruskal–Wallis ANOVA with a Dunn multiple comparison test and are shown above the data for each cell type group. *p < 0.05, **p < 0.01, ***p < 0.001. ns, not significant. 4–6M, patients after 4–6 mo of DMF treatment; >18M, patients after 18–26 mo of DMF treatment; UNT, untreated patients.
The reduction of CD4+ T cells expressing both CXCR3 and CD161 suggests that this cell population was also preferentially depleted with DMF treatment. Because this population is more proinflammatory in nature compared with other CD4+ T cells (42), its preferential depletion may play an important therapeutic role for the success of DMF as an RRMS disease-modifying medication. With the knowledge that IL-17-producing cells are also within the CD161+ fraction of CD4 T cells, and that CD161+ cells could also produce IFN-γ (44), the analysis of double marker CXCR3+CD161+ and triple marker CXCR3+CCR6+CD161+ expression of Th cells might further refine the Th1/Th17 subsets. The fact that DMF-treated MS patients have both a reduced level of Th1 and Th17 cytokines produced by CD4+ T cells and a reduced Th subset defined by CXCR3, CD161, and CCR6 provided us a unique opportunity to correlate these cell surface markers with the level of IFN-γ and IL-17 production by CD4+ T cells. We found that CXCR3+CD161+ with or without CCR6 correlate closely with the frequency of CD4+ T cells producing IFN-γ, but not IL-17, suggesting that these markers more closely define Th1 cells.

The phenotypical and functional reduction of Th1, Th17, as well as the Th1/Th17 population, accompanied by the boosting effects on Th2, might play pivotal anti-inflammatory roles contributing to the therapeutic effects of DMF in MS. Although earlier in vitro studies suggested that DMF mainly suppresses Th1 responses in psoriasis (45), a more recent study of DMF-treated psoriasis patients has shown that DMF modulates Th17 cells in addition to Th1/Th2 cells (46). DMF seems to exert similar multifaceted effects on Th cells in MS, as we show in this study. More research is needed to address the question of whether DMF is capable of reducing numbers of myelin-specific Th17 cells, especially those within the CNS.

CD69 is a marker commonly used to evaluate T cell activation (19, 20). We found that the percentages of CD69+CD4+ and CD69+CD4+ T cells were both reduced after 4–6 mo of DMF.
The inhibitory effects on T cell activation were found most on memory T cells and the effects sustained beyond 18 mo (Fig. 3), suggesting that there were fewer newly activated T cells in circulation with DMF treatment.

DMF was found to be able to modestly reduce CCR7 expression on T lymphocytes in vitro (Fig. 5E). The CCR7 ligands CCL19 and CCL20 have been detected in venules surrounded by inflammatory cells at the BBB (47). CCR7+ Tcm cells were found to be enriched in the cerebrospinal fluid of subjects with inflammatory neurologic diseases, including MS (48). Downregulation of CCR7 by DMF may thereby affect Tcm cell trafficking to the CNS and could be another mechanism that contributes to the therapeutic effect of DMF in MS. A recent study by Kihara et al. (49) has demonstrated that DMF inhibits integrin α4 expression on CD3+ T cells and B220+ B cells in EAE mice, as well as on activated human Jurkat T cells. The adhesion molecule VLA4, which is the αβ1 integrin, has been shown to be involved in the mechanism of T lymphocytes crossing the BBB. By downregulating VLA4 on T cells, DMF would be expected to limit lymphocyte infiltration through the BBB into CNS, therefore curtailing inflammation in the CNS.

We and others have shown that DMF treatment could be associated with varying degrees of lymphopenia (32, 50). The mechanism underlying lymphopenia is not entirely clear. Our study was consistent with a previous report showing that DMF can induce T cell apoptosis (51). By performing an in vitro assay using PBMCs from healthy controls treated with DMF, we showed that there was increased T cell apoptosis and decreased proliferation after DMF treatment. Furthermore, we showed that CD4+ T cells were more susceptible to these effects than were CD4+ T cells (Fig. 5). These results are consistent with our in vivo data, which demonstrate that over time DMF suppresses CD8+ T cell counts more than do CD4+ T cells. DMF has been shown to activate the Nrf2-antioxidant response element signaling pathway (12, 52). Consistent with this, we showed that ROS level in activated T cells declined with the increasing concentration of DMF. DMF suppressed T cell activation both in vivo and in vitro, which is consistent with an earlier study by Turley et al. (53), who determined that tert-butylhydroquinone, an Nrf2 activator, inhibited primary human CD4+ T cell activation. However, DMF was unable to intercept T cell activation in Tcm cells of CD8+ T cells, which seemed to have undergone homeostatic proliferation induced by lymphocyte depletion (Fig. 2) (54). A separate study demonstrated that DMF can induce apoptosis in hematopoietic cells by inhibition of NF-κB nuclear translocation and downregulation of Bcl-2, Bcl-xI, and XIAP (55). Our group recently showed that DMF prevented apoptosis in neural stem/progenitor cells by activating the Nrf2-antioxidant response element signaling pathway (13), suggesting that the effects of DMF on apoptosis are perhaps cell type specific and may involve different signaling pathways in different cell types. A recent mouse study comparing DMF effects on EAE induced in WT and Nrf2 knockout mice showed that DMF induced both adaptive and innate immune modulation independent of Nrf2 (56). Further studies are needed to demonstrate the exact signaling pathways and mechanism for this differential inhibitory effect of DMF on selected subsets of T cells.

Several cases of PML have been reported in psoriasis and MS patients who have been treated with DMF (57). PML is a potentially lethal viral disease caused by JC virus, a type of polyomavirus. JC virus infects a substantial fraction of the general population, initially as an asymptomatic kidney infection. However, a mutated form of JC virus can become pathogenic in immunocompromised individuals by invading the CNS and eliciting progressive damage to white matter in the brain (58). DMF could facilitate JC virus reactivation by reducing the memory and cytotoxic CD8+ T cells targeting the mutated JC virus, potentially reducing their ability to infiltrate into the CNS to fight against the virus. The suppressive effects of DMF on peripheral lymphocytes and particularly CD8+ Tem cells were seen as early as 4 mo, and sustained after 18–26 mo of treatment in our study. The depletion of CD8 Tem cells, as well as the diminished activation potential of Temra cells by DMF, may be particularly devastating, because it would weaken the normal immune surveillance against JC virus. Careful monitoring of peripheral T cells, particularly JC virus–specific CD8+ as well as CD4+ T cells, could be clinically important for making therapeutic decisions regarding continual and long-term DMF usage for treating RRMS. Further studies should also be conducted to investigate the correlation of these specific changes in the immunological profile with clinical efficacy after long-term treatment, and how it might play a role in relapse and potential side effects. Because we only tested circulating lymphocytes in this study, the effects of DMF on immune cells in the cerebrospinal fluid or within the CNS still remain to be addressed.

In conclusion, our results suggest that efforts should be made to monitor the immune modulating effects of DMF on the CD4+ and CD8+ memory T cells, Th1, Th17, Th1/Th17 cells, as well as NKT and B cell compartments. These could be an important link in understanding how depletion of these cell lineages might be associated with drug efficacy as well as the risk of PML. Future longitudinal studies with larger patient cohorts should allow for teasing out the predictors of drug response and rare incidence of PML.

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