Immunological effects of dimethyl fumarate treatment in blood and CSF of patients with primary progressive MS

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ABSTRACT

Dimethyl fumarate is an efficient therapy used widely in patients with relapsing-remitting multiple sclerosis (RRMS). However, lacking effect of treatment has recently been reported in patients with primary progressive MS (PPMS) (Højsgaard Chow et al., 2021). In order to further analyze the immunological treatment response we investigated the systemic and intrathecal immunological effects of dimethyl fumarate (DMF) treatment in 50 patients with PPMS who participated in a 48-week randomized controlled trial with dimethyl fumarate vs placebo. We found substantial systemic immunomodulatory effects of DMF treatment comparable with those observed in patients with RRMS. However, intrathecal effects were limited and restricted to CD4+ T cells presumably resulting in higher concentrations of intrathecal IL-7.

1. Introduction

Immunosuppressive and immunomodulatory treatment strategies have largely failed in patients with primary progressive multiple sclerosis (PPMS), especially in the later stages of the disease (Sorensen et al., 2020). We recently reported results from a randomized controlled trial investigating dimethyl fumarate (DMF) treatment in patients with PPMS (Højsgaard Chow et al., 2021). The study investigated effects on biomarkers of neuroaxonal damage, demyelination and inflammation in the cerebrospinal fluid (CSF) (neurofilament light chain [NFL], myelin basic protein [MBP], soluble B cell maturation antigen [sBCMA], soluble CD27 [sCD27], chitinase-3-like-1 protein [CHI3L1] and soluble CD14 [sCD14]), without observing evidence of treatment effects (Højsgaard Chow et al., 2021).

It is well established that DMF ameliorates clinical and radiological disease activity and disability worsening in patients with relapsing-remitting MS (RRMS) (Fox et al., 2012; Gold et al., 2012; Kappos et al., 2008). In addition, patients diagnosed with RRMS treated with DMF show reduced concentrations of CSF NFL and depletion of several immune cell subsets linked to disease activity (Gold et al., 2012; Huglind et al., 2018; Kappos et al., 2008; Sejbaek et al., 2019). Immunological effects of DMF treatment include depletion of B and T cells (especially Th1-like Th17 CD4+ T cells and CD8+ T cells) and an increase in monocyte frequencies in blood (Carlström et al., 2019; Fleischer et al., 2018; Mansilla et al., 2019; Wu et al., 2017). Although the precise pharmacodynamics of DMF have not been fully elucidated, interference with aerobic glycolytic metabolic pathways seems essential for the reduction in effector lymphocyte subsets that may contribute to the drug’s disease-modifying effects in RRMS (Kornberg et al., 2018). Additionally, DMF’s modulation of nuclear factor (erythroid derived 2)-like 1/2 (Nrf-2) and nuclear factor-κB (NF-κB) also leads to reduction of inflammatory activity, and DMF activation of the hydroxycarboxylic acid receptor 2 ameliorates disease development in the murine MS model experimental autoimmune encephalomyelitis (Chen et al., 2014; Gillard et al., 2015). In this study, we examined immunological effects of DMF on immune cell subsets in CSF and blood. Furthermore, we explored how CSF cytokine and chemokine concentrations respond to DMF treatment in patients with PPMS and analyzed associations between effects on cytokine and CSF cells.

2. Methods

2.1. Patients and samples

All enrolled patients participated in a randomized, controlled trial conducted at the Danish Multiple Sclerosis Center, Copenhagen University Hospital - Rigshospitalet, Denmark from December 2018 to November 2020 (Højsgaard Chow et al., 2021). The trial compared
treatment with DMF versus placebo for 48 weeks. Key inclusion criteria were: a diagnosis of PPMS; age between 18 and 65 years; and no immunomodulatory or immunosuppressive treatment within the last 6 months before inclusion, and no steroid treatment within 3 months before inclusion. At baseline and week 48 visits, we collected 10 to 12 ml CSF in a polypropylene tube on ice of which three milliliters was sent for routine analysis of cell count, IgG index, CSF-serum albumin quotient, and oligoclonal bands. The remaining CSF was separated into cells and supernatant by centrifugation within 30 min after collection, and stored at −80 °C. At week 48 visits, approximately 10 ml of blood was drawn in ethylenediaminetetraacetic acid (EDTA) vacutainer blood collection tubes (BD, San Jose, CA, USA) and peripheral blood mononuclear cells were isolated using density gradient centrifugation (Lymphoprep, Axis-Shield, Oslo, Norway). The study was approved by the Capital Region Ethics Committee, Denmark (protocol number H-16047666), and informed consent was obtained from all participants. The trial is registered at ClinicalTrials.gov (NCT02959658).

2.2. Flow cytometry analysis

Blood and CSF cells were collected as previously described and washed twice in phosphate-buffered saline (PBS)/2 mM EDTA (Holm Hansen et al., 2019a). Aferwards, we stained the cells with the following fluorochrome-conjugated antibodies (fluorochrome; clone) in PBS/2% fetal bovine serum/0.02% NaN3 (APC/Cy7; HIT3a), CD4 (PerCP/Cy5.5; RPA-T4), CD25 (PE; M-A251), and CD127 (APC; A019D5 or BV421; A019D5) in combination with either CXCXR3 (PC/Cy7; G025H7 or AlexaFlour488; G025H7) and CCR6 (BV421; G034E3 or BV605; G034E3) or CCR5 (AlexaFlour488; J252D4) and PD1 (BV605; EH12.2H7) for CD4+CD8 T helper cell (T4) or CD4+/CD8+ follicular T cell (T3) phenotyping, respectively. In addition, we used CD19 (PerCP/Cy5.5; HIB19) or CD20 (BV421; 2H7) for phenotyping of B cells, and CD56 (PC/Cy7; CMSB) for NK cells. In peripheral blood mononuclear cells we further characterized B cells by staining with CD27 (FITC; 323), CD38 (BV421; H1T2), IgD (BV605, IAD–2), and IgM (APC, MHM-88), and we used CD14 (FITC; M5E2) and CD16 (BV605; 3G8) to characterize monocytes. Isotype controls were applied when appropriate to correct for nonspecific antibody binding and spectral overlap. All antibodies were from BioLegend (San Jose, CA, USA) except CD56 (Invitrogen, USA). In blood, we also analyzed absolute counts of monocytes, T cells, B cells, and NK cells with TrueCount Beads (BD BioSciences, NJ, USA) according to the manufacturer’s instructions. To estimate absolute counts of cell subsets in the CSF, we multiplied the proportion of each cell subset with the corresponding leucocyte count reported by the routine laboratory. Due to technical inaccuracy when measuring low cell counts, we also reported as “<3” cells/µL. For these cell counts, we multiplied corresponding cell subset proportions by two to estimate absolute counts. All flow cytometry data were acquired on a FACS Canto II flow cytometer (BD Biosciences). All flow cytometry data analysis and graphs were performed with FlowJo software (Treestar, Inc.).

2.3. Electrochemiluminescence analysis

CSF was collected as described above, and aliquots were thawed on ice. Concentrations of the following cytokines and chemokines were analyzed with the same batch of a 22-plex kit (Meso Scale Diagnostics, Rockville, MD) according to manufacturer’s instructions: vascular endothelial growth factor-A (VEGF-A), tumor necrosis factor-α (TNF-α), lymphotixin-α (LT-α), interleukin (IL)-5, IL-7, IL-10, IL-12, IL-15, IL-16, IL-17, IL-21, IL-22, IL-23, IL-27, IL-31, CC chemokine ligand (CCL)-3, CCL-20, CCL-22, CCL-26, CXC chemokine ligand (CXCL)-8, CXCL-10 interferon-γ (IFN-γ). Lower and upper limits of quantification (LoQ and ULoQ, respectively) were determined by the manufacturer based on batch analysis of coefficients of variation (CV) and recovery (supplementary data). We excluded cytokines with more than 50% of the samples below or above the limit of quantification (LoQ). For all cytokines we also compared changes in detectability from baseline to week 48.

2.4. Statistics

Data are given with mean and standard deviation, median with interquartile range or frequencies as appropriate. We used Wilcoxon’s rank-sum test to detect differences between treatment groups at baseline. To analyze differences in cytokine and chemokine concentrations, we applied a general linear model with change in concentrations from baseline to W48 as dependent and treatment group and log-transformed baseline concentration as independent variables. To detect differences in detectability between the number of samples that changed from within or outside LoQ from baseline to W48, we applied chi-square test of independence. To analyze peripheral blood and CSF cell subsets for differences between patients treated with DMF and placebo after 48 weeks of treatment, we applied Wilcoxon’s ranked-sum test. We used false discovery rate correction to correct for multiple testing (Benjamini-Hochberg method) and considered a q-value below 0.05 statistically significant. Post-hoc, we analyzed whether CSF cytokine concentrations were associated with CSF cell counts with spearman’s signed rank test. All statistics were conducted with RStudio v1.2.5(R Core Team, 2017; Sjoberg et al., 2020; Wickham, 2016).

2.5. Data availability

Anonymized data can be made available by request from a qualified investigator and will require a data transfer agreement between the responsible parties.

3. Results

Baseline comparisons of demographics, disease, and CSF characteristics revealed that groups were comparable. However, the baseline CSF-serum albumin quotient was higher in patients treated with DMF (p = 0.021) (Table 1).

3.1. DMF therapy effects on blood lymphocyte subtypes

We used flow cytometry to characterize peripheral blood T cells, B cells, monocytes, and NK cells in 46 patients treated with DMF or placebo (Table 2). For flow cytometry gating strategy, see supplementary data (Figs. e1-e5).

Compared with patients receiving placebo, patients treated with DMF had lower absolute counts of CD4+ and CD8+ T cells including

| Table 1 | Baseline demographic, disease and routine CSF characteristics. |
|---------|---------------------------------------------------------------|
| Characteristic | PLB, N = 24 | DMF, N = 26 | p-value |
| Age | 54.9 (6.2) | 55.6 (5.6) | 0.6 |
| Sex, Female | 10 (42%) | 9 (35%) | 0.8 |
| Disease duration | 14.4 (9.9) | 14.2 (9.5) | 0.9 |
| EDSS | 4.0 (3.5, 6.0) | 4.25 (4.0, 6.0) | 0.5 |
| IgG-index | 0.76 (0.40) | 0.66 (0.20) | 0.8 |
| Albumin quotient | 6.78 (3.09) | 8.38 (3.44) | 0.021 |
| OCB | 21 (88%) | 25 (96%) | 0.3 |
| CSF cell count | | | |
| ≤4 | 20 (83%) | 18 (69%) | |
| >4 | 4 (17%) | 8 (31%) | |

Legend (Table 1): Abbreviations: PLB = placebo, DMF = dimethyl fumarate, EDSS = expanded disability status scale; IgG = immunoglobulin G; OCB = oligoclonal bands; CSF = cerebrospinal fluid. Data are presented as mean (SD), n (%), or mean (Q1, Q3).

a Statistics presented: mean (SD); n (%); median (Q1, Q3).

b Statistical tests performed: Wilcoxon rank-sum test; chi-square test of independence; Fisher’s exact test.
Table 2

Absolute counts and proportions of blood cell subsets in patients receiving PLB or DMF.

| Cell population | PLB | DMF |
|----------------|-----|-----|
| **n** | **Value a** | **n** | **Value a** | **Difference (95% CI) b** |
| Lymphocytes | 21 | 1989 (565) | 25 | 938 (632) | -960 [-1315 to -604]*** |
| CD4 | 21 | 859 (287) | 25 | 373 (246) | -485 [-646 to -324]*** |
| CD25 ‘CD127’ (non-reg) | 21 | 740 (231) | 25 | 325 (219) | -415 [-554 to -276]*** |
| CD25 ‘CD127’ (reg) | 21 | 74 (17) | 25 | 30 (127) | -44 [-62 to -26]*** |
| CD8 | 21 | 423 (221) | 25 | 128 (127) | -294 [-409 to -179]*** |
| CD25 ‘CD127’ (non-reg) | 21 | 254 (141) | 25 | 78 (101) | -176 [-250 to -101]*** |
| CD25 ‘CD127’ (reg) | 21 | 3.2 (0.3) | 25 | 1.5 (1.8) | -1.7 [-3.0 to -0.4]6 |
| NK cells | 21 | 154 (75) | 25 | 120 (115) | -34 [-91 to -23] |
| B cells | 21 | 170 (71) | 25 | 117 (129) | -54 [-115 to -7.5] |
| Monocytes | 21 | 355 (136) | 25 | 357 (140) | 1.5 [-82.5 to -82] |
| CD4 | 21 | 47 (8) | 25 | 44 (12) | -2.8 [-8.7 to 3.2] |
| CD4 ‘CD25 ‘CD127’ (non-reg) | 20 | 87 (6) | 25 | 86 (4) | -0.2 [-3.5 to 3.0] |
| CXCR3 ‘CCR6’ (Th1) | 19 | 23 (10) | 24 | 15 (9) | -7.7 [-14 to 0] |
| CXCR3 ‘CCR6’ (Th17) | 19 | 10 (4) | 24 | 8.1 (5.0) | -2.4 [-5.0 to 0.3] |
| CXCR3 ‘CCR6’ (Th17.1) | 19 | 14 (5.8) | 24 | 5.3 (5.7) | -8.3 [-12 to -4.7]*** |
| CXCR3 ‘CCR6’ | 19 | 53 (14) | 24 | 72 (15) | 18 [9.3 to 27.3]*** |
| CXCR5 ‘(folicular)’ | 20 | 12 (5.5) | 25 | 9.1 (4.4) | -3.2 [-6.2 to 0.3] |
| PD1 | 20 | 4.0 (2.2) | 25 | 3.1 (1.9) | -1.1 [-2.1 to 0.4] |
| CD4 ‘CD25 ‘CD127’ (reg) | 20 | 8.4 (2.2) | 25 | 8.5 (2.0) | 0.1 [-1.2 to 1.3] |
| CXCR5 ‘(folicular)’ | 20 | 28 (9.9) | 25 | 25 (8) | -8.0 [-8.2 to 0.0] |
| PD1 | 20 | 4.6 (2.3) | 25 | 4.9 (2.5) | 0.3 [-1.1 to 1.7] |
| CD8 | 21 | 25 (9) | 25 | 15 (6.6) | -10 [-15 to -5.4]*** |
| CD8 ‘CD25 ‘CD127’ (non-reg) | 55 | 55 (19) | 25 | 48 (20) | -7.6 [-13.9 to 3.9] |
| CXCR3 ‘CCR6’ (Th1) | 19 | 69 (10) | 24 | 67 (13) | 2.7 [-9.8 to 4.3] |
| CXCR3 ‘CCR6’ (Th17) | 19 | 28 (2.4) | 24 | 19 (5.1) | -9.3 [-4.6 to 0] |
| CXCR3 ‘CCR6’ (Th17.1) | 19 | 15 (10) | 24 | 8.5 (7.2) | -6.8 [-12 to -1.4] |
| CXCR3 ‘CCR6’ | 19 | 22 (6.6) | 24 | 20 (10) | 2.2 [1.5 to 3.9]*** |
| CXCR5 ‘(folicular)’ | 20 | 3.8 (2.0) | 25 | 1.2 (1.2) | -2.5 [-3.5 to -1.5]*** |
| PD1 | 20 | 1.9 (1.0) | 25 | 0.6 (0.6) | 0.1 [-1.8 to 0.8]*** |
| CD8 ‘CD25 ‘CD127’ (reg) | 25 | 0.7 (0.3) | 25 | 0.5 (0.7) | 0.0 [0.2 to 0.8]*** |
| NK cells | 21 | 75 (4.1) | 25 | 11 (5.6) | 3.2 [0.3 to 6.1] |
| CD56+ | 21 | 91 (6.5) | 25 | 87 (7) | -4 [-8.3 to 0.2] |
| CD56+ (B cells) | 21 | 8.8 (6.5) | 25 | 13 (7.1) | 4.3 [0.2 to 8.3] |
| Memory (CD38+) | 20 | 9.2 (5.6) | 25 | 5.2 (6.6) | -4.1 [-7.7 to -0.4] |
| Memory (CD38+) | 20 | 9.2 (5.6) | 25 | 5.2 (6.6) | -4.1 [-7.7 to -0.4] |

Table 2 (continued)

| Cell population | PLB | DMF |
|----------------|-----|-----|
| **n** | **Value a** | **n** | **Value a** | **Difference (95% CI) b** |
| Naïve | 20 | 60 (16) | 24 | 58 (20) | -2.0 [-9.0 to 0] |
| CD25 ‘CD38’ | 20 | 11 (4.3) | 24 | 10 (5.2) | -0.6 [-2.5 to 2.3] |
| Transitional | 20 | 5.9 (2.9) | 24 | 14 (8.2) | 7.7 [4.1 to 11]*** |
| Plasmablasts | 20 | 0.3 (0.3) | 24 | 0.3 (0.3) | 0.0 [-0.2 to 0.2] |
| IgM ‘IgD’ | 20 | 82 (8.5) | 24 | 85 (13) | 3.7 [-2.7 to 10] |
| IgM ‘IgD’ | 20 | 13 (4.3) | 24 | 8.9 (10) | -3.9 [-9.3 to 1.5] |
| IgM ‘IgD’ | 20 | 2.9 (2.2) | 24 | 4.3 (3.4) | 1.4 [-0.3 to 3.1] |
| Monocytes | 20 | 17 (5.7) | 25 | 32 (16) | -1.2 [8.2 to 22]*** |
| CD16+ | 20 | 17 (5) | 25 | 16 (7) | -1.2 [-4.3 to 1.9] |
| CD16+ | 20 | 85 (5.1) | 25 | 86 (6.1) | -1.0 [-1.8 to 4.3] |

Legend (Table 2): Abbreviations: PLB = placebo, DMF = dimethyl fumarate; non-reg = non-regulatory; reg = regulatory; Th1 = T helper; Tc = CD8+ cytotoxic T helper; IgM = immunoglobulin M; IgD = immunoglobulin D. Flow cytometry gating and additional cell subset definitions can be found in supplementary data (Figs. e1 to e5).

a Statistics presented: Mean (SD).

b Statistical tests performed: Wilcoxon rank-sum test with false discovery rate correction for multiple testing.

q < 0.05.

*** q < 0.01.

*** q < 0.001.

3.2. DMF reduces CD4+ T cells in CSF of patients treated with DMF

We investigated CSF frequencies and estimated absolute counts of CD4+ T cells, CD8+ T cells, NK cells, and B cells in patients treated with DMF or placebo (Table 4). For flow cytometry gating strategy, see supplementary data (Figs. e1-e4).

Absolute counts of T cells were available from 42 patients, B cell counts from 40 patients, and NK cell counts from 26 patients. There was a reduction in CD4+ T cell counts (q < 0.05) and a decrease in the percentage of CD4+ T cells in CSF of patients treated with DMF (q <
0.001). We found no changes in other cell subsets analyzed in the CSF, i.e. CD8+ T cells, B cells or NK cells.

### 3.3. Higher CSF concentrations of interleukin-7 in patients treated DMF

In the same cohort, we have previously shown that there was no difference in the CSF cell count, the IgG-index, the QAb or in the CSF concentrations of NFL, MBP, sBCMA, sCD27, CHI3L1 or sCD14 in patients treated with DMF or placebo (Høgskjold Chow et al., 2021). We applied electrochemiluminescence to analyze changes in concentrations of cytokines and chemokines in the CSF of patients treated with DMF or placebo for 48 weeks (Table 3). Six out of 22 analyzed cytokines had 50% of samples or more within the detection limits based on limits of quantification. We analyzed whether DMF induced changes in these six cytokines. After 48 weeks, the mean concentration of interleukin-7 (IL-7) was higher in DMF-treated patients compared with patients receiving placebo (mean difference [95% CI] 0.2 ng/L [0.1–0.4 ng/L], q < 0.001). The remaining cytokines and chemokines within the specified LoQ (CXCL8, CXCL10, IL-12p40, IL-15 and IL-27) showed no differences, although there was a trend towards down-regulation of IL-12p40 (p = 0.05, q = 0.151) and up-regulation of IL-15 (p = 0.076, q = 0.152) Neither did we observe differences in the number of samples changing from below the LLoQ to within LoQ or vice versa during the 48 week treatment period in any cytokines besides IL-7 (Table e1).

### 3.4. CSF concentrations of IL-7 and CSF CD4+ T cell counts are associated

We analyzed associations between CSF IL-7 concentrations and CSF CD4+ T cells counts (Fig. 1). This analysis revealed a moderate association between concentrations of IL-7 and CD4+ T cells in blood compared with patients treated with DMF, accompanied by increases in some regulatory T cell subsets expressing CXCR3 and CCR6 (Th1-like Th17 cells) and decreased concentrations of IL-7.

The effects of DMF treatment on peripheral lymphocytes in patients with RRMS have previously been investigated thoroughly (Ghdiri et al., 2017; Gross et al., 2016; Høglund et al., 2018; Holm Hansen et al., 2019a; Li et al., 2017; Mansilla et al., 2019; Mehta et al., 2019; Späth et al., 2020; Wu et al., 2017). We studied indications that many effects of DMF on peripheral blood cell subsets are caused by effects on both effector and regulatory T cell subsets. However, both CD4+ and CD8+ T cells expressing CXCR3 and CCR6 (Th1-like Th17 cells) were downregulated upon treatment with DMF. Reductions in this subset have been suggested to mediate beneficial effects of DMF treatment (Mansilla et al., 2019).

We observed an increase in concentrations of IL-7 in CSF in PPMS patients treated with DMF. IL-7 is a lymphopoietic cytokine which is

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### Table 3

| Cell population | PLB | DMF | Difference [95%CI] |
|-----------------|-----|-----|--------------------|
| **n** | **Value** | **n** | **Value** |
| Leucocytes | 24 | 2.9 (1.2) | 25 | 2.4 (0.9) | 0.5 | [−1.1–0.1] |
| CD4+ | 19 | 1.8 (0.9) | 23 | 1.0 (0.6) | 0.8 | [−1.3–0.3] |
| CD8+ | 12 | 1.4 (0.7) | 14 | 0.8 (0.6) | 0.0 | [−1.1–0.0] |
| CD4+ | 12 | 0.1 (0.1) | 14 | 0.1 (0.1) | 0.0 | [−0.1–0.1] |
| CD8+ | 19 | 0.7 (0.3) | 23 | 0.7 (0.3) | 0.0 | [−0.1–0.1] |
| NK cells | 12 | 0.0 (0.0) | 14 | 0.0 (0.0) | 0.0 | [−0.0–0.0] |
| B cells | 19 | 0.0 (0.0) | 21 | 0.1 (0.1) | 0.0 | [−0.0–0.1] |
| CD4+ | 19 | 62 (11) | 24 | 43 (14) | −19 [−27 to −11] | **<0.001** |
| CD4+ | 12 | 81 (7) | 14 | 71 (7) | −9.2 [−14 to −4.3] | **<0.001** |
| CXCR3+CCR6 (Th1) | 7 | 54 (4.5) | 10 | 46 (7) | 4 (3.2) | [−0.5–0.0] |
| CXCR3+CCR6 (Th17) | 7 | 7.0 (2.1) | 10 | 4.4 (3.2) | −2.6 (2.4) | [−3.3–2.6] |
| CXCR3+CCR6 (Th17.1) | 7 | 34 (4.2) | 10 | 45 (11) | 11 (2.4) | [1.4–19.2] |
| CXCR3+CCR6 | 7 | 5.6 (2.2) | 10 | 4.4 (4.0) | 4 (0.0) | [−3.9–2.6] |
| CD4+CCR5 (follicular) | 12 | 18 (6.0) | 14 | 19 (6.1) | 1.4 | [−3.6–6.3] |
| CD4+CCR5 (reg) | 12 | 14 (3.8) | 14 | 14 (5.1) | −0.2 (2.4) | [−1.6–5.6] |
| CD8+CCR5 (reg) | 12 | 6.4 (1.9) | 14 | 8.8 (4.2) | 2.4 (2.4) | [0.4–4.4] |
| CD8+CCR5 (follicular) | 12 | 22 (8.8) | 14 | 25 (10) | 2.8 (10) | [4.8–10.4] |
| CD8+CCR6 (reg) | 12 | 14 (5.7) | 14 | 12 (10) | −1.6 (10) | [8.1–4.9] |
| CD8+CCR6 (Th17) | 12 | 25 (6.8) | 24 | 31 (8.7) | 6.2 (14.1) | [3.0–9.4] |
| CD8+CCR6 (Th17.1) | 12 | 48 (14) | 14 | 40 (10) | −4 (2.8) | [−20.0–3.0] |
| CD8+CCR6 (Th17) | 7 | 84 (6.8) | 10 | 76 (7.6) | −8 (7.6) | [−13.2–1.1] |
| CD8+CCR6 (reg) | 7 | 0.7 (0.7) | 14 | 1.4 (1.6) | −0.7 (16) | [−0.5–2.0] |
| CD8+CCR6 (Th1) | 12 | 11 (7.7) | 15 | 15 (7.6) | 4.3 (15) | [−3.8–12.8] |
| CD8+CCR6 | 7 | 4.1 (2.6) | 14 | 4.4 (4.2) | 0.4 (2.4) | [−3.2–3.9] |
| CD8+CCR5 (reg) | 12 | 4.8 (3.2) | 14 | 3.6 (3.2) | 1.2 (3.2) | [−3.9–1.4] |
| CD8+CCR5 (Th1) | 12 | 2.4 (1.6) | 14 | 1.8 (1.6) | 0.6 (1.6) | [−1.9–0.7] |
| CD8+CCR5 (Th17) | 12 | 4.9 (3.7) | 14 | 5.2 (2.8) | 0.3 (2.8) | [−2.5–3.0] |
| CD8+CCR5 (reg) | 12 | 2.6 (1.3) | 14 | 2.1 (1.2) | 1.9 (1.2) | [−0.4–3.3] |
| CD8+CCR5 (follicular) | 12 | 46 (22) | 14 | 43 (20) | −3.3 (20) | [−21.1–14.4] |
| CD8+CCR5 (Th1) | 54 | 58 (22) | 14 | 58 (20) | 3.5 (20) | [−14.21–14.21] |
| CD8+CCR5 (reg) | 19 | 52 (13) | 22 | 3.0 (2.2) | 1.9 (2.2) | [−0.6–4.3] |

Legend (Table 3): Abbreviations: PLB = placebo; DMF = dimethyl fumarate; non-reg = non-regulatory; reg = regulatory; Th1 = helper; Th2 = CD8+ cytotoxic T helper; IgM = immunoglobulin M; IgD = immunoglobulin D. Leucocyte concentrations in the CSF were estimated using routine diagnostic cell count (see
methods). For flow cytometry gating and cell phenotype definitions see supplementary data (Figs. e1 – e5).

* Statistics presented: Mean (SD).

† Statistical tests performed: Wilcoxon rank-sum test with false discovery rate correction for multiple testing.

q < 0.05

†† q < 0.001.

Table 4

|                | PLB (N = 24) | DMF (N = 26) | Estimate (95% CI)* | p-value * | q-value * |
|----------------|-------------|-------------|--------------------|-----------|-----------|
| CXCL10         |             |             |                    |           |           |
| >ULoQ (W0; W48) | 6; 9        | 13; 10      |                    |           |           |
| W0; ng/l       | 329.5       | 299.2       |                   | >0.9      |           |
| change; ng/l   | −1.9        | −51.8       | −38.9              | 0.112     | 0.167     |
| <LLoQ (W0; W48)| 11; 11      | 13; 4       |                    |           |           |
| W0; ng/l       | 0.7 (0.6, 0.8) | 0.7 (0.6, 0.9) | 0.5               |           |           |
| change; ng/l   | 0.0 (0.1)   | 0.3 (0.1)   | 0.2 (0.1–0.4)      | 0.000     | 0.002     |
| CXCL8          |             |             |                    |           |           |
| W0; ng/l       | 43.3 (37.0, 47.9) | 48.5 (40.6, 56.0) | 0.2              |           |           |
| change; ng/l   | 1.2 (9.5)   | −2.1 (10.1) | −2.0 (−7.4–3.4)    | 0.461     | 0.554     |
| IL12p40        |             |             |                    |           |           |
| W0; ng/l       | 3.8 (3.0, 6.3) | 4.5 (3.4, 6.7) | 0.4               |           |           |
| change; ng/l   | −0.6 (3.1)  | −2.6 (4.5)  | −1.5 (−3.0–0.0)    | 0.050     | 0.151     |
| IL15           |             |             |                    |           |           |
| W0; ng/l       | 2.2 (2.0, 2.6) | 2.3 (2.0, 2.6) | 0.8               |           |           |
| change; ng/l   | −0.0 (0.2)  | 0.1 (0.4)   | 0.2 (−0.0–0.3)     | 0.076     | 0.152     |
| CXCL9          |             |             |                    |           |           |
| <ULoQ (W0; W48)| 1; 0        | 7; 2        |                    |           |           |
| W0; ng/l       | 92.9 (62.7, 115.3) | 88.7 (59.9, 117.8) | 0.5          |           |           |
| change; ng/l   | 18.9 (34.3) | 17.4 (33.6) | 2.45 (−20.8–25.7)  | 0.832     | 0.832     |

Legend (Table 4): Abbreviations: PLB = placebo, DMF = dimethyl fumarate, CCL = chemokine ligand; LLoQ = lower limit of quantification; ULoQ = upper limit of quantification; W0 = week 0 visit (baseline); W48 = week 48 visit; CXCL = CXC chemokine ligand; IL = interleukin; TNF = tumor necrosis factor; VEGF = vascular endothelial growth factor. W0 (baseline) are presented as median (Q1, Q3) while changes and estimated between group differences are presented as mean (SD) and mean (95% CI), respectively.

* Statistical tests performed: generalized linear models adjusted for baseline concentrations.

† False discovery rate correction for multiple testing (Benjamini-Hochberg).

Number of samples above ULoQ (week 0 [n], week 48 [n]).

Number of samples below LLoQ (week 0 [n], week 48 [n]).

crucial for the expansion of CD4+ T cells (Surd and Sprent, 2008). We therefore suggest that this increase is related to the CD4+ T cell depletion within the CSF, presumably due to reduced cytokine consumption (Gillard et al., 2016; Holm Hansen et al., 2020). This decrease may be a result of peripheral lymphocyte depletion and hence reduced migration to the CNS or a direct depletion within the CNS by the metabolite of DMF, monomethyl fumarate (MMF) which is capable of crossing the blood brain barrier (Mills et al., 2018). We also found associations between IL-7 concentration and CSF CD4+ T cell counts. This supports the evidence for an association between IL-7 concentrations and the extent of CD4+ T cell activity in the CNS. Although neither of the remaining cytokines were significantly suppressed upon DMF treatment, we did observe a trend towards reduction of IL-12p40 concentrations. Interestingly, IL-12p40 concentrations are decreased by DMF in studies of both dendritic cells and alloreactive T cells from human peripheral blood mononuclear cells (Lehmann et al., 2007; McGuire et al., 2016; Peng et al., 2012). Processes that may also be mediated by inhibition of NFκB and extracellular signal-regulated kinase (ERK) 1/2 transcription (McGuire et al., 2016). Furthermore, there was a trend towards upregulation of IL-15 concentrations. IL-15 has been associated with proliferation and activation of NK cells that were increased in the CSF of DMF-treated patients, although this finding was not significant (Wang and Zhao, 2021).

Meningeal inflammation and slowly expanding lesions compose key elements in the pathogenesis of progressive MS (Elliott et al., 2019; Magliozzi et al., 2018). These processes involve innate immune cell subsets, B cells, cytotoxic T cells, and the formation of ectopic lymphoid follicles (Lassmann, 2019). Unfortunately, we did not investigate monocyte subsets in the CSF directly. However, we previously reported concentrations of C3H1L1 and sCD14, that are associated with innate immune activation, both of which were not affected by treatment with DMF (Højgaard Chow et al., 2021). Furthermore, we found no changes in B cells, CD8+ T cells, or NK cells in CSF in the present study. Similar results have been found regarding CD8+ T cells in patients with RRMS (Holm Hansen et al., 2020). Together these findings indicate that CSF immune cell subsets associated with disease mechanisms in progressive MS do not seem to be affected by treatment with DMF.

It has previously been suggested that DMF treatment depletes CD4+ T cells expressing CNS homing markers (Holm Hansen et al., 2020). Our findings of decreased CD4+ T cell counts in CSF and unaffected CD8+ T cells and B cells support these findings, which are in agreement with other studies showing evidence of accumulation of CD4+ T cells in the CNS due to migration of central memory T cell from the periphery to the CNS (Rivisakk et al., 2004).

Our study has several limitations: the CSF cell subset analysis was conducted with considerably fewer samples than the blood analysis. Therefore, the analysis of CSF cell subsets may be subject to type 2 errors (Sedgwick, 2014). However, the lack of changes in the CSF concentrations in five out of six cytokines in addition to the information previously reported from the randomized controlled trial support our findings of limited effects on intrathecal inflammation. In our flow cytometry protocol, we did not include a CD8+ antibody but defined CD8+ T cells as CD3+CD4- T cells due to a limitation in the number of fluorochromes that could be used in the FACS Canto flow cytometer. This may result in the inclusion of a regulatory CD4+CD8- cell subset. This population accounts for approximately 1% of the CD3+ T cell population (Fischer et al., 2005). Also, we did not analyze B cell subpopulations or monocytes in the CSF, which could have provided valuable additional information to our findings.

We analyzed an extensive panel of cell subsets and cytokines/chemokines, and although we performed false discovery rate correction for multiple testing, the analysis is still susceptible to type 1 errors. However, the main findings of this study are the absence of specific immunological effects induced by DMF. These findings may be more prone to type 2 errors.

In summary, we conducted a detailed analysis of peripheral and intrathecal DMF-induced immunological effects in a cohort of 50 patients with PPMS. Our findings suggest that DMF treatment of patients with PPMS primarily suppresses adaptive T cells in blood, and that effects on CNS inflammation are restricted to depletion of CD4+ T cells. These findings advance our understanding of the effects of DMF and provide important information regarding the absence of DMF treatment effects in patients with PPMS.
Fig. 1. Relationship between IL-7 concentration and CD4\(^+\) T cell counts in CSF. Legend (Fig. 1): Abbreviations: IL = interleukin.

Declaration of Competing Interest

JT reports non-financial support from Biogen, non-financial support from Sanofi Genzyme, during the conduct of the study.

H. Højsgaard Chow reports non-financial support from Merck, non-financial support from Teva, non-financial support from Biogen, non-financial support from Roche, outside the submitted work;

RHF: Nothing to disclose.

MRvE: Reports no conflicts of interest.

FS has served on scientific advisory boards for, served as consultant for, received support for congress participation or received speaker honoraria from Alexion, Biogen, Merck, Novartis, Roche and Sanofi Genzyme. His laboratory has received research support from Biogen, Merck, Novartis, Roche and Sanofi Genzyme.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jneuroim.2021.577756.

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