Dimethyl fumarate as a first- vs second-line therapy in MS

Focus on B cells

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**Abstract**

**Objective**

To elucidate the immunomodulatory effects of dimethyl fumarate (DMF) on B cells in patients with relapsing MS receiving DMF as a "1st-line" vs "2nd-line" therapy.

**Methods**

B cells were isolated from 43 patients with MS at baseline and after 15-week DMF therapy. Phenotype and functional markers and cytokine profile were assessed by flow cytometry. Analysis included clinical and MRI parameters recorded during a 1-year follow-up.

**Results**

1st-line and 2nd-line patients presented several differences in their baseline immune profile, which corresponded with differences in their immunologic response to DMF treatment. DMF reduced the proportions of B cells and CD8 T cells whereas increased monocytes. DMF reduced memory B cells, including plasma cells in 2nd-line patients only, whereas strongly increased transitional B cells. Several IL10" B-cell subsets and TGFβ" B cells were increased. Proinflammatory LTα" and TNFα" B cells were reduced, while IL4" B cells elevated, whereas IFNγ" B cells showed opposite effects in 1st-line and 2nd-line patients. HLA and ICAM-1 expression was increased, but % CD86" B cells reduced. The expression of B-cell activating factor receptor and the proportion of activated CD69 B cells were increased.

**Conclusions**

DMF is associated with increased transitional and IL10" and TGFβ" regulatory B cells and a shift toward a more anti-inflammatory immune profile. Cell activation with reduced costimulatory capacity may induce immune hyporesponsiveness. Carryover effects of preceding therapies in 2nd-line patients and the stage of disease influence the immune profile of the patients and the immunomodulatory effects of DMF.
MS is an immune-mediated neurodegenerative disease of the CNS. Accumulating evidence has demonstrated the importance of B cells in MS pathology, including most convincingly the beneficial clinical outcomes of selective B-cell therapies. Dimethyl fumarate (DMF) is an oral MS drug, with a not yet fully elucidated mechanism of action. DMF appears to act through immunomodulation of various cells and through neuroprotection, inducing the nuclear factor (erythroid-derived 2)-related factor 2 (Nrf2) pathway, while downregulating the NF-κB pathway. DMF therapy was shown to reduce the numbers of CD4+, CD8+ T cells, and B cells in the periphery and to reduce the lymphocyte count by ~30%. Recent reports have found that DMF alters several B-cell subsets. DMF is approved as either a 1st-line or 2nd-line medication for patients with relapsing-remitting (RR) MS. 2nd-line drug patients are generally in a more advanced disease stage and may present yet undetermined, “carryover effects” from previous MS drugs. This may affect the immunologic profile of the patients and thereby the mode the disease-modifying therapy (DMT) affects the patients. We therefore, in this study, aimed at examining how DMF affects B cells in “1st-line” and “2nd-line” patients with MS (PwMS). The study further elucidates the mechanism of action of DMF and demonstrates how the individual patient disease and immune profile may affect the modulatory action of a medication.

Methods

Standard protocol approvals, registrations, and patients consents
The study obtained approval from the Institutional Ethical Committee on human experimentation (0034-13-CMC). Written informed consent was obtained from all patients participating in the study.

Study participants
Forty-three patients with RRMS (aged >18 years) fulfilling the revised McDonald criteria were recruited at the Carmel Medical Center, Israel. Blood were obtained before and 15 weeks after initiating DMF therapy. Patients were free of previous DMT or steroid treatment for at least 1 month, in remission, with an Expanded Disability Status Scale score (EDSS) of ≤6.

Isolation of B cells and culture
Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation (Novamed) and B cells isolated by negative selection (EasySep kit [Stemcell]) with a purity >90%. Cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum, penicillin-streptomycin-nystatin (100 U/mL), and l-glutamine (2 mM) (Biological Industries) in a 37°C humidified 5% CO2 incubator.

Flow cytometry
Cells were stained with monoclonal antibodies against CD14, CD3, CD8 (Biolegend), CD4, and CD19 (BD Bioscience) for immune cell subsets; against CD19 (BD Bioscience), CD27, IgD, CD24, CD138, and CD38 (Biolegend) for B-cell subsets; against IL10, CD1D (BD Bioscience), CD5 and CD25 (Biolegend) for regulatory markers; against CD80 (Biolegend) and CD86 (BD Bioscience) for costimulatory molecules; against human leukocyte antigen (HLA)-DR, CD40, and intercellular adhesion molecule-1 (ICAM-1) for antigen-presenting markers; and against B-cell activating factor receptor (BAFF-R) and CD69 (Biolegend) for activation markers. For cytokines, B cells stimulated with or without 10 μg/mL anti-immunoglobulin M (IgM) (SouthernBiotech) and 1 μg/mL anti-CD40 (BioLegend) were cultured for 40 hours (with 4 hours Golgistop) and stained against CD19, CD27, IL10, IL4, LTA, TGFβ, TNFα (BD Bioscience), and interferon γ (IFNγ) (Biolegend) using a Fix & Perm kit (Invitrogen). Unstained cells and appropriate isotype controls were used as negative control for staining, and BD CompBeads (BD Bioscience) were used for compensation. Analysis was performed on an LSRFortessa (BD bioscience), and results were analyzed using FlowJoX. Cytometer Setup & Tracking beads (BD Bioscience) were used at baseline and after 3.5 months to keep the cytometer performance consistent. Gating strategy is presented in figure e-1-I, links.lww.com/NXI/A78. The level of secreted IL10 after 24 hours culture was assessed using the Human Th1/Th2/Th17 Cytometric Bead Array (CBA) kit (BD bioscience) according to the manufacturer’s protocol.

Statistical analysis
Statistical analysis was performed using SPSSv22 or GraphPad Prism 5. Data before and after 3.5-month therapy were compared using the paired t-test or Wilcoxon signed-rank test, and 1st-line vs 2nd-line patients were compared using the unpaired t test or Mann-Whitney test, according to the normality of the data as assessed by the Kolmogorov-Smirnov test. Correlation tests were performed using GraphPad Prism 5. A p value < 0.05 was considered statistically significant.

Results
Forty-three patients were recruited for this study, 22 patients receiving DMF as 1st-line medication (without previous...
DMF as 2nd-line medication (with at least 1 previous DMT). The demographic and clinical data of the patients are summarized in table (full data are presented in e-1, links.lww.com/NXI/A79). There was no significant difference in age, sex, or the baseline EDSS score between the 1st-line and 2nd-line patients. Interferon-β was the previous DMT in most 2nd-line patients, whereas 4 patients had received fingolimod, and the median time since previous DMT was 1 month. Eleven patients switched from previous therapy to DMF because of disease activity, whereas 9 switched because of adverse events and 1 patient for oral treatment. Within 1-year follow-up, no significant change was observed in the EDSS score in all patients or in 2nd-line patients; however, the EDSS score was reduced in 1st-line patients after 6 months (by 29.3%; p = 0.003). While 18 and 7 relapses occurred in 1st-line and 2nd-line patients, respectively, during 1 year before DMF, only 6 patients experienced a relapse after DMF initiation, 3 from each group with two 2nd-line patients relapsing twice during the follow-up. Three relapses occurred within 3 months of drug initiation. The annual relapse rate (ARR) 1 year after DMF initiation was significantly reduced in all and in 1st-line patients, but not

### Table: Summary of demographic and clinical data

|                          | All patients | **1st-line** patients | **2nd-line** patients | p Value 1st vs 2nd line (baseline) |
|--------------------------|-------------|---------------------|----------------------|-----------------------------------|
| Age (y)                  | 38 ± 2 (35) | 34.9 ± 2.7 (34)     | 41.2 ± 2.9 (42)      | ns                                |
| Sex                      | 28 F/15 M   | 14 F/8 M            | 14 F/7 M             |                                   |
| Disease duration (y)     | 5.8 ± 1     | 2.1 ± 1             | 9.7 ± 1.5            | <0.0001                           |
| Time since previous DMT (mo) | —           | —                   | 12.3 (1)             | —                                 |
| EDSS 0 mo                | 2.26 ± 0.3  | 2.39 ± 0.4          | 2.12 ± 0.4           | ns                                |
| EDSS 3.50 mo             | 2.18 ± 0.3 ns | 2.12 ± 0.3 ns     | 2.24 ± 0.5 ns        | —                                 |
| EDSS 6 mo                | 2.17 ± 0.3 p = 0.0 | 1.69 ± 0.3 p = 0.003 | 2.76 ± 0.5 p = 0.07 | —                                 |
| EDSS 12 mo               | 2.22 ± 0.3 ns | 1.80 ± 0.4 p = 0.06 | 2.71 ± 0.5 ns        | —                                 |
| ARR 1 year before DMF therapy | 0.58 ± 0.09 (25) | 0.82 ± 0.11 (18) | 0.33 ± 0.13 (7)      | 0.035                             |
| ARR 1 year after DMF therapy initiation | 0.2 ± 0.08 (8) p = 0.0042 | 0.14 ± 0.07 (3) p = 0.0003 | 0.26 ± 0.14 (5) ns | ns (at 1 y)                      |
| MRI disease activity (1 y) | 7 patients | 3 patients (14%)    | 4 patients (24%)     | —                                 |
| ALC (×10⁹/L) baseline    | 1.94 ± 0.1  | 2.11 ± 0.13         | 1.75 ± 0.14          | ns                                |
| ALC (×10⁹/L) 3.5 mo      | 1.81 ± 0.1 ns | 1.96 ± 0.17 ns     | 1.67 ± 0.12 ns       | —                                 |
| ALC (×10⁹/L) 6 mo        | 1.51 ± 0.1 p = 0.012 | 0.53 ± 0.17 p = 0.088. | 1.49 ± 0.12 p = 0.057 | —                                 |
| ALC (×10⁹/L) 12 mo       | 1.55 ± 0.1 p = 0.0001 | 1.59 ± 0.14 p = 0.002 | 1.51 ± 0.15 p = 0.019 | —                                 |
| % Lymphocytes baseline   | 28.2 ± 1.3  | 28.90 ± 1.8         | 27.51 ± 1.9          | ns                                |
| % Lymphocytes 3.5 mo     | 27.7 ± 1.2 ns | 29.32 ± 1.9 ns     | 26.28 ± 1.6 ns       | —                                 |
| % Lymphocytes 6 mo       | 25.7 ± 1.4 ns | 26.40 ± 2.2 ns     | 24.97 ± 1.8 p = 0.009 | —                                 |
| % Lymphocytes 12 mo      | 25.9 ± 1.4 ns | 26.70 ± 2.2 ns     | 25.14 ± 1.7 p = 0.099 | —                                 |
| AMC (×10⁹/L) baseline    | 0.43 ± 0.02  | 0.40 ± 0.03         | 0.46 ± 0.04          | ns                                |
| AMC (×10⁹/L) 3.5 mo      | 0.48 ± 0.003 p = 0.052 | 0.49 ± 0.04 p = 0.014 | 0.46 ± 0.04 ns       | —                                 |
| AMC (×10⁹/L) 6 mo        | 0.44 ± 0.02 ns | 0.40 ± 0.02 ns     | 0.48 ± 0.05 ns       | —                                 |
| AMC (×10⁹/L) 12 mo       | 0.44 ± 0.02 ns | 0.41 ± 0.03 ns     | 0.48 ± 0.04 ns       | —                                 |
| % Monocytes baseline     | 6.41 ± 0.3  | 5.69 ± 0.4          | 7.18 ± 0.4           | 0.017                             |
| % Monocytes 3.5 mo       | 7.35 ± 0.3 p = 0.008 | 7.32 ± 0.4 p = 0.006 | 7.38 ± 0.4 ns       | —                                 |
| % Monocytes 6 mo         | 7.36 ± 0.5 p = 0.019 | 6.42 ± 0.3 p = 0.04 | 8.37 ± 0.5 ns       | —                                 |
| % Monocytes 12 mo        | 7.55 ± 0.4 p = 0.007 | 6.96 ± 0.4 p = 0.022 | 8.14 ± 0.5 ns       | —                                 |

Abbreviations: ALC = absolute lymphocyte count; AMC = absolute monocyte count; ARR = annual relapse rate; DMT = disease-modifying therapy; EDSS = Expanded Disability Status Scale; ns = not significant.
DMF therapy alters the proportions of immune cells

We assessed the proportions of B cells, CD4 and CD8 T cells, and monocytes within PBMCs to confirm previous reports on DMF effects,\textsuperscript{4-10} and to compare the effects in 1st-line vs 2nd-line patients, in 20 PwMS (10 1st line and 10 2nd line) before and after DMF therapy. Gating strategies are presented in figure e-1-I, links.lww.com/NXI/A78 and results in figure e-1-II, links.lww.com/NXI/A78. Table e-2, links.lww.com/NXI/A80 summarizes baseline differences between the 1st-line and 2nd-line patients. DMF caused a reduction in % CD8 T cells in all patients (15.2%, \(p = 0.003\)) and in 1st-line patients (18.6%, \(p = 0.0096\)), while not in 2nd-line patients, who at baseline had less % CD8 cells (28%, \(p = 0.045\), figure e-1-IIA, links.lww.com/NXI/A78, table e-2, links.lww.com/NXI/A80). Percent CD4 T cells was increased in 1st-line patients only (26%, \(p = 0.047\) (figure e-1-IIB, links.lww.com/NXI/A78), and there was an increase in the ratio of CD4/CD8 T cells in all patients (28.6%, \(p = 0.003\)) and in 1st-line patients (47.1%, \(p = 0.001\)), whereas 2nd-line patients had a trend higher ratio at baseline (\(p = 0.07\)) (figure e-1-IIC, links.lww.com/NXI/A78, table e-2, links.lww.com/NXI/A80). Percent monocytes was increased in 1st-line patients only (35%, \(p = 0.034\)) (figure e-1-IIIE, links.lww.com/NXI/A78). Percent B cells was reduced by 34% in 1st-line patients (\(p = 0.008\), however increased by 49% in 2nd-line patients (\(p = 0.027\)) (figure e-1-IID, links.lww.com/NXI/A78), although no difference was found on the baseline level between the groups (table e-2, links.lww.com/NXI/A80). There was no change in the absolute cell number of B cells or CD8 T cells, but an increase in the number of monocytes (39%, \(p = 0.0057\)) and CD4 T cells (34%, \(p = 0.017\)) (data not shown). Plotting all patients, we found a significant correlation between the change in % B cells after 15-week therapy and the change in the EDSS score after 12 months (\(p = 0.014, r^2 = 0.3\)) (figure e-1-IIE, links.lww.com/NXI/A78), suggesting that the reduction in % B cells is associated with a reduction in the EDSS score.

DMF therapy increases IL10\(^+\) B regulatory subsets

B regulatory cells (Bregs) are not yet clearly defined, and several subsets have been shown to have regulatory capacities (reviewed in\textsuperscript{1}). We assessed the effect of DMF therapy on IL10\(^+\) B cells and markers associated with Bregs, such as CD25, CD5, CD1D, CD80, and CD86. DMF increased % IL10\(^+\) B cells in all patients (176%, \(p = 0.021\) and in 2nd-line patients (1,143%, \(p = 0.0098\)), whereas the change was not significant in 1st-line patients, who had higher % IL10\(^+\) cells at baseline (\(p = 0.019\)) (figure 2A, table e-2, links.lww.com/NXI/A80). Several IL10\(^+\) B-cell subsets were also increased after DMF therapy, such as CD80\(^+\)IL10\(^+\) (114%, \(p = 0.049\) all patients; 498%, \(p = 0.022\) 2nd line) (figure 2B), CD1D\(^+\)IL10\(^+\) (891%, \(p = 0.039\), 2nd line) (figure 2C), and CD5\(^+\)IL10\(^+\) (1,104%, \(p = 0.008\), 2nd line) (figure 2D). Furthermore, IL10 expression was increased in B-cell subsets such as CD86\(^+\) cells (1.2 fold change [FC], \(p = 0.002\); 1.3 FC, \(p = 0.039\); 1.2 FC, \(p = 0.032\) (figure 2E) and CD5\(^+\) cells (1.14 FC, \(p = 0.005\); 1.2 FC, \(p = 0.039\); 1.1 FC, \(p = 0.039\)) (figure 2F) in all, 1st-line and 2nd-line patients, respectively, in CD80\(^+\) cells (1.16 FC, \(p = 0.007\); 1.3 FC, \(p = 0.008\) (Figure 2G), and in CD1D\(^+\) cells (1.13 FC, \(p = 0.005\); 1.21 FC, \(p = 0.027\)) (figure 2H) in all and 1st-line patients, respectively, and in CD25\(^+\) cells in 1st-line patients (1.3 FC, \(p = 0.038\)) (data not shown). The baseline level of IL10 in these subsets was higher in 2nd-line patients (figure 2, E-H, table e-2, links.lww.com/NXI/A80). The absolute number of IL10\(^+\) B cells was increased in all and 2nd-line patients (data not shown). Assessment of IL10 secretion in the media by CBA confirmed a 185% increase (\(p = 0.059\), trend) (figure 2I).

DMF therapy reduces proinflammatory and increases anti-inflammatory cytokines

We compared the cytokine profile of B cells before and after 3.5-month DMF therapy in 23 PwMS (12 1st-line and 11 2nd-line patients), culturing cells for 40 hours with or without stimulation. We confirmed in this additional cohort and setup that DMF increases % IL10\(^+\) B cells (data not shown). Furthermore, DMF increased % TGFβ\(^+\) B cells in all patients (26%, \(p = 0.06\), trend) (figure 3A) and the expression of...
TGFβ (median fluorescence intensity [MFI]) in 1st-line patients (25%, \( p = 0.028 \)) (figure 3B). We found a correlation between % IL10+ and % TGFβ+ B cells after DMF therapy (\( p = 0.0004, r^2 = 0.5 \)) (figure 3C). % IFNγ+ B cells were increased in 2nd-line patients (294%, \( p = 0.005 \)) whereas reduced in 1st-line patients (48%, \( p = 0.077 \), trend) (figure 3D). Percent LTα+ B cells was reduced in 1st-line patients (71%, \( p = 0.002 \)) (figure 3E); however, the expression of LTα was increased within LTα+ cells (FC = 1.5, \( p = 0.02 \), all patients; FC = 1.78, \( p = 0.012 \), 1st line) (figure 3F). Percent TNFα+ B cells was reduced in 1st-line patients (36%, \( p = 0.06 \), trend) (figure 3G), whereas % IL4+naive B cells was elevated in all patients (45%, \( p = 0.046 \)) (figure 3H).

DMF modulates markers of antigen presentation and activation

We assessed the effect of DMF on molecules involved in antigen presentation such as ICAM-1, HLA-DR, and CD40, CD80, and CD86 (figure 4A-D). We found no change in % HLA+, ICAM-1+, or CD40+ cells (data not shown), but the expression of HLA-DR and ICAM-1 was increased in all patients (1.2 FC, \( p = 0.04 \); 1.6 FC, \( p = 0.014 \) respectively) (figure 4, A–B). In contrast, % CD86+ B cells was decreased by 60% (\( p = 0.047 \)) in 1st-line patients (figure 4C). The baseline level of CD86+ B cells was significantly lower in 2nd-line patients (\( p = 0.026 \), figure 4C, table e-2, links.lww.com/NXI/A80). We found no effect on the expression of CD86 or CD80 (data not shown) or on % CD80+ cells (figure 4D). HLA-DR and CD86 are also markers of activation in B cells, along with CD69. There was a 2.9 times increase in % CD69+ B cells in all patients (\( p = 0.007 \)) and a 6.5 times increase in 1st-line patients (\( p = 0.0001 \)), whereas no effect was found in 2nd-line patients, who had a higher baseline level (\( p = 0.035 \), figure 4E, table e-2, links.lww.com/NXI/A80). The expression of CD69 (MFI) was increased by 123% in all patients (\( p = 0.001 \)) and by 129% in 1st-line patients (\( p = 0.06 \), trend) (figure 4F), whereas in 1st-line patients, the baseline level was higher (\( p = 0.001 \), table e-2, links.lww.com/NXI/A80). There was no difference on % receptor for BAFF-R+ B cells (data not shown), but the expression of BAFF-R was increased by 109% in 2nd-line patients (\( p = 0.018 \)) (figure 4G).

Discussion

DMF is an oral therapeutic approved for patients with RRMS, with more than 4 years in “real-world” usage, although its mechanism(s) of action still to be fully elucidated. This study aimed at further understanding the immunomodulatory effects of DMF therapy on B cells, specifically comparing the effects in patients receiving DMF as a 1st-line vs 2nd-line therapy. 1st-line
patients are usually in a relatively early phase of the disease, shortly after diagnosis, whereas 2nd-line patients are generally in a more advanced disease stage. Because MS is believed to follow a chronic disease course, where the inflammatory processes are gradually replaced by neurodegenerative processes, the immunologic profile of patients at different disease stages may differ. Furthermore, 2nd-line patients have previously been exposed to at least 1 other MS DMT and may present immunologic carryover effects, depending on the length of time since preceding immunotherapy and the mode of action of the specific DMT. We found several differences in baseline values of various subsets and markers between the 1st-line and the 2nd-line patients, which could underlie the differences in response to DMF therapy, as observed on some of these parameters. Thus, our results elucidate how the individual, baseline immunologic profile of the patient may affect the immunomodulatory effect of DMF. An important message of this observation is that pooling of the 1st-line and 2nd-line patients in a study on drug response may mask biological relevant changes and potential biomarkers of treatment response. This observation may also have consequences for drug development and for implementation of personalized therapy.
Clinically, 1st-line patients showed a reduction in the EDSS score after 6 months, whereas in 2nd-line patients, the EDSS score remained stable. This difference may represent more beneficial clinical response in the 1st-line group vs 2nd-line group. However, it may also represent better recovery after the first relapse compared with recovery after repeated relapses. Although there was no difference in the relapse rate between the 2 groups after DMF therapy, there was a significant reduction in the ARR in 1st-line patients, but not 2nd-line patients, who in most cases were under treatment before DMF, and only half of them switched to DMF because of insufficient response to the previous DMT. Of 4 patients who ceased fingolimod therapy before DMF, 2 patients relapsed within 6 months of DMF therapy, and again after 10 months, suggestive of a rebound effect of disease activation after fingolimod cessation.

There was also a lower percentage of 1st-line patients with MRI disease activity after 1-year treatment compared with the 2nd-line group; however, not all 2nd-line patients had available MRI. Together, these observations can suggest that the...
clinical effect of DMF may be more favorable in 1st-line patients; however, long-term clinical follow-up (>2 years) and larger patient cohorts are necessary to reach conclusions on this matter.

We confirmed that DMF therapy is associated with reduced proportion of CD8+ T cells, but not CD4+ T cells, as previously shown.8,10 The lack of effect on CD8 T cells in the 2nd-line patients may be due to the lower % CD8 T cells already at baseline. We showed that although B cells were reduced in 1st-line patients, they were increased in 2nd-line patients, which also included patients previously receiving fingolimod, a drug shown by us and others to strongly reduce circulating B cells.20,21 Without the previous fingolimod-treated patients, the increase in B cells was still a trend in 2nd-line patients, thus confirming that the effect on B cells may depend on the disease stage and previous therapies. Previous studies have reported similar results on immune cell numbers.8–10,14–16 The exact mechanism responsible for the reduction in lymphocytes during treatment is not yet known, although DMF has been shown in vitro to induce apoptosis in B cells15 and T cells.8,22 Of interest, a significant correlation was found between the reduction of B cells after 15 weeks and a reduction in the EDSS score after 12 months and thus with more beneficial clinical effect. Whether this may serve as a surrogate marker for response to therapy is to be verified in further studies.

We showed that DMF therapy reduced memory B cells, whereas increased naive cells, specifically transitional B cells, in line with previous reports.14–16 Of interest, a similar effect is found after fingolimod therapy,21 despite the different modes of actions of these 2 drugs, suggesting that they reset the B-cell composition toward a more naive and regulatory state. In line with this, the 2nd-line patients had higher % naive and transitional cells at baseline, likely because of carryover effects of previous therapies such as fingolimod and IFNβ.1 The observed reduction in plasma cells, found in 2nd-line patients only, has not previously been reported. We suggest that this could be a result of DMF stabilizing an effect already induced by previous therapy.

Because transitional B cells have been associated with regulatory capacities, including high production of IL10,23–25 their
increase suggests that DMF may enhance Bregs. Several B-cell subsets have been described as having regulatory capacities, including CD27 CD24hiCD38hi transitional cells, CD27 CD24hi memory cells, and CD27 CD25+ memory cells. This indicates that either various Breg subsets exist or that distinct B-cell subsets can adopt anti-inflammatory capacity in response to appropriate stimuli. Bregs can inhibit the expansion of inflammatory T cells, through particularly IL10, but also IL35 or TGFβ. We found that DMF increased IL10 expression and % IL10+ B cells within several subsets, such as CD11b+, CD80+, CD5+, CD86+, and CD25+ B cells, all markers associated with Breg subsets. Two previous reports did not find an increase in IL10+B cells; however, this may be masked when pooling 1st-line and 2nd-line patients in the analysis. The higher baseline level of IL10 expression in 2nd-line patients could be due to carryover effects from previous therapies because both fingolimod and IFNβ increase IL10 in B cells. We showed here for the first time that DMF increases TGFβ+ B cells and TGFβ expression. Because DMF also reduced proinflammatory LTA+ and TNFα+ B cells and increased IL-4+ cells, our results suggest a shift toward a more anti-inflammatory, regulatory profile of B cells. This is in line with a recent report of a reduced ratio of GM-CSF, TNFa, and IL6 to IL10 in B cells in DMF-treated patients. We found an increase in IFNg+ B cells in 2nd-line patients, which could result from the lower baseline level of this cytokine than in 1st-line patients, who in contrast showed a trend reduction in this cytokine. Thus, DMF therapy may reset the cytokine profile of the patients, which has been shown to be altered in B cells in MS.

One of the major roles of B cells is the presentation of antigen and stimulation of T cells. Efficient activation of T cells requires a first signal through the T-cell receptor and a second costimulatory signal through CD28-CD80/CD86 binding. In the absence of a second signal, T cells become unresponsive or anergic. Although DMF therapy was associated in this study with increased antigen-presenting molecules CD54 and HLA-DR, the reduction in % CD86+ B cells, and no change in CD86, CD80, or CD40 expression, may suggest that DMF could induce T-cell insensitivity. The increased proportion of peripheral activated CD69 B cells may contribute to retain a functional immune system, as demonstrated by the good safety profile of DMF.

We are aware of certain limitations of this study. The 3.5-month therapy duration may not be sufficient to capture all immunomodulatory effects of DMF on B cells; however, those detectable after 3.5 months are likely to be of biological relevance and potential biomarkers. A recent report showed that observations on B-cell subsets after 3-month therapy were persistent also after 12 months, thus confirming the relevance of our findings. Another limitation is the relative small cohorts of the subanalysis of 1st-line vs 2nd-line patients, especially with the variability among the latter group, due to differences in previous DMT and time since previous DMT cessation. Furthermore, time between the onset of symptoms to diagnosis may vary greatly in MS, thus limiting the timing of disease cause of a patient. However, the differences in baseline immune profile and in DMF immunomodulation between the 1st-line and 2nd-line patients demonstrated in this study call for further validation in larger cohorts and for careful consideration of such variance in studies of drug biomarkers.

To conclude, DMF therapy is associated with reduced memory and increased transitional B cells. The drug increases IL10 and TGFβ Bregs and promotes an anti-inflammatory cytokine profile. An increased proportion of activated B cells may allow retained functional immunity; however, with a reduction in CD86, these cells might potentially induce a restricted immune hypo-responsiveness. The 1st-line and 2nd-line patients present differences in their baseline immunologic profiles, supposedly because of carryover effects from previous DMTs and changes in disease mechanisms occurring along the disease course, and these differences may affect the individual immune response to DMF therapy.

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**Appendix 1. Author Contributions**

| Name                  | Location                                                                 | Role          | Contribution                                                                 |
|-----------------------|--------------------------------------------------------------------------|---------------|------------------------------------------------------------------------------|
| Elsebeth Staun-Ram, PhD | Rappaport Faculty of Medicine, Technion-Israel Institute of Technology, Haifa, Israel; Neuroimmunology Unit & Multiple Sclerosis Center, Department of Neurology, Carmel medical Center, Haifa, Israel | Author        | Designed and conceptualized the study, obtained, analyzed, and interpreted the data, and drafted the manuscript |
| Eiman Najjar          | Rappaport Faculty of Medicine, Technion-Israel Institute of Technology, Haifa, Israel | Author        | Obtained, analyzed, and interpreted the data                                  |
| Anat Volkowich        | Neuroimmunology Unit & Multiple Sclerosis Center, Department of Neurology, Carmel Medical Center, Haifa, Israel | Author        | Major role in acquisition of the data                                        |
| Ariel Miller, MD, PhD  | Rappaport Faculty of Medicine, Technion-Israel Institute of Technology, Haifa, Israel; Neuroimmunology Unit & Multiple sclerosis Center, Department of Neurology, Carmel Medical Center, Haifa, Israel | Author        | Designed and conceptualized the study, interpreted the data, and revised the manuscript for intellectual content |