We evaluated the effect of dimethyl fumarate (DMF) treatment on B-cell memory and cytokine production in 18 patients with relapsing remitting multiple sclerosis (RRMS) using peripheral blood mononuclear cells obtained prior to and at 6 months post-DMF initiation. We noted a decline in the absolute B-cell number with DMF treatment, with a preferential depletion of memory B cells and a concurrent increase in naïve B cells. We noted significant reductions in GM-CSF, TNF-α, and IL-6 producing B cells with DMF treatment. These effects on the B-cell compartment may underlie the beneficial effects of DMF in RRMS.

Our study utilized a prospectively collected longitudinal cohort to study the immunological effects of DMF treatment. We demonstrate the preferential depletion of memory B cells with DMF treatment and a reduction in B cells producing pro-inflammatory cytokines including granulocyte macrophage colony-stimulating factor (GM-CSF), tumor necrosis factor -α (TNF-α), and interleukin-6 (IL-6).

Methods

Participants, Standard protocol approvals, and Patient consents

We enrolled patients with RRMS, who were initiating DMF, from the Johns Hopkins MS Center. The protocol for this study was approved by the Johns Hopkins Institutional Review Board. All participants provided informed consent prior to enrollment. Participants underwent phlebotomy at baseline and at 6 months postinitiation of
DMF. Additionally, demographic and disease-related information was collected from all participants.

**Isolation of Peripheral blood mononuclear cells (PBMCs)**

Blood obtained at each visit was processed using a standard protocol and PBMCs were obtained by density gradient separation. These were cryopreserved until the end of the study.

**B-cell culture and flow cytometry**

PBMCs were thawed and stained for viability (Live/Dead Aqua, Life Technologies) followed by surface markers. For intracellular cytokine detection, PBMCs were thawed and cultured in AIMV media (Life Technologies) overnight. The next morning, cells were stimulated with 25 ng/mL PMA, 475 ng/mL ionomycin, and BD GolgiStop (BD Biosciences) for 5 h at 37°C. This protocol is similar to a previously described strategy to identify B-cell cytokine production. Following stimulation, cells were stained for viability (Viobility Green, Miltenyi), Fc blocked, stained for surface markers, fixed and permeabilized using IC fixation buffer (eBioscience), then stained for intracellular cytokines. Following staining, cells were analyzed on a MACSQuant flow cytometer (Miltenyi). Fluorescence minus one staining controls were used to determine gating. Data analysis was performed using FlowLogic (Inivai Technologies). Antibodies used included CD20, GM-CSF, CD19, CD27, TNFα, CD38, IgD, CD45, CD24, CD16/32 (Biolegend), IL-6, and IL-10 (BD Biosciences). The gating strategy for various B-cell populations is shown in Figure S1.

**Statistical analysis**

We compared the baseline and end-of-study values for the various immune cell populations using a Wilcoxon signed-rank test. We utilized a nonparametric test since some variables were not normally distributed (based on Shapiro-Wilk test). We considered a P-value of <0.05 as significant. We also performed sensitivity analyses excluding two participants who received intravenous steroids within 1 month prior to the end-of-study visit, and was excluded from our analyses.

**Results**

We enrolled 18 RRMS patients in the study. The demographic and disease characteristics are summarized in Table 1. The majority of the patients were either treatment naive or treated with an injectable disease-modifying therapy prior to DMF initiation. Two patients received intravenous steroids within a month prior to study initiation. One participant discontinued DMF, more than 1 month prior to the end-of-study visit, and was excluded from our analyses.

**DMF treatment reduced the absolute number of B cells in RRMS patients**

DMF treatment resulted in a significant drop in absolute lymphocyte counts (2036 cells/mm³ vs. 1255 cells/mm³, P = 0.002), and while the proportion of lymphocytes that were B cells was not altered (P = 0.93); the absolute B-cell counts decreased with DMF treatment (272 cells/mm³ vs. 151 cells/mm³, P = 0.005). This is in keeping with previous reports of reductions in total B-cell numbers with DMF treatment.6

**DMF treatment reduced the proportion of memory B cells and increased the proportion of naive B cells**

As demonstrated in Figure 1, DMF treatment led to a reduction in the proportion of CD27⁺ memory B cells (39.3%, P = 0.003) and a concomitant increase in the proportion of CD27⁻ IgD⁺ naïve B cells (12.2%, P = 0.001) in RRMS patients (Table 2). This suggests that within the B-cell compartment, DMF preferentially targets the memory population; and this was true for both CD27⁺ IgD⁻ class-switched and CD27⁺ IgD⁺ nonclass-switched memory B cells (Table 2).

We also examined the change in proportion of Transitional B cells (CD24high CD38high), B-1 cells (CD43⁺ CD27⁻), and plasmablasts (CD19⁺ CD24⁻ CD38high) with DMF treatment and noted no significant change in these populations (Table 2).

**DMF treatment reduced the proportion of B cells producing pro-inflammatory cytokines (GM-CSF, TNF-α, and IL-6)**

We then examined the production of proinflammatory cytokines by B cells using intracellular staining and flow

### Table 1. Demographic and disease characteristics of participants.

| Characteristic                  | RRMS             |
|--------------------------------|------------------|
| Number                         | 18               |
| Age, years (mean ± SD)         | 43.9 ± 10.8      |
| Female sex, n (%)               | 13 (72.2)        |
| EDSS, median (IQR)             | 2.0 (1.5)        |
| Disease duration, years (mean ± SD) | 9.8 ± 6.2 |
| Previous treatment             |                  |
| None                           | 6                |
| Glatiramer acetate             | 5                |
| Interferon beta                | 5                |
| Natalizumab                    | 2                |

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DMF treatment alters B-cell memory subsets and reduces proinflammatory cytokine-producing B cells. PBMCs isolated from RRMS patients (baseline and after 6 months of dimethyl fumarate treatment) were cryopreserved at the time of collection. PBMCs were thawed and then either stained for viability and surface markers, or cultured overnight then restimulated with PMA/Ionomycin/BD GolgiStop for 5 h before being stained for viability, Fc blocked, and stained for cytokine production. B cells were defined as singlet viable CD19+ lymphocytes, and this gated population of cells is shown in the representative flow cytometry plots (A, C, E, G, I). Line graphs with each line representing an individual participant depict the changes in B-cell subpopulations (B, D, F, H, J). RRMS patients treated with dimethyl fumarate had a significant decrease in memory B cells (CD27+) (A, B) and an increase in naïve B cells (CD27+ IgD+) (C, D). Dimethyl fumarate treatment also led to a significant decrease in TNF-α (E, F), GM-CSF (G, H), and IL-6 (I, J) producing B cells. Wilcoxon signed-rank tests were utilized to compare data from the two study time-points. *P < 0.05, **P < 0.01.
cytometry. We noted reductions in the proportion of B cells producing proinflammatory cytokines GM-CSF (35.3%, \( P = 0.004 \)), TNF-\( \alpha \) (32.1%, \( P = 0.007 \)), and IL-6 (23.9%, \( P = 0.044 \)) (Fig. 1, Table 2). Increased production of these cytokines by B cells has been previously noted in MS.\(^7,8\) We observed no change in the proportion of B cells producing the anti-inflammatory cytokine IL-10 (\( P = 0.58 \)) (Table 2). Previous studies have demonstrated that short stimulation protocols only identify a subset of B cells that produce IL-10,\(^9\) and hence we also quantified transitional B cell and B-1 cell populations which are thought to have regulatory properties related to IL-10 production.\(^10,11\) Neither of these populations changed significantly with DMF treatment (Table 2).

Exclusion of the two patients who received steroids within a month of the baseline visit from the analyses did not significantly change any of the presented results.

### Discussion

This study utilized a prospectively collected cohort of RRMS patients to demonstrate that DMF treatment leads to a preferential depletion of memory B cells and reduces the proportion of GM-CSF, IL-6, and TNF-\( \alpha \) producing B cells. These changes in pathogenic B cell subsets are potentially a part of the immunological mechanism of action of DMF.

We noted a reduction in the absolute number of B cells with DMF treatment, in keeping with previous studies.\(^6\) However, within the B-cell compartment, we noted a preferential depletion of memory B cells which is consistent with the results of a recent study.\(^5\) Memory B cells are thought to be pathogenic in MS and previous studies have noted the production of proinflammatory cytokines such as GM-CSF and TNF-\( \alpha \) predominantly from memory B cells, while anti-inflammatory cytokines such as IL-10 are derived mainly from the naïve B-cell population.\(^12\) Memory B cells are also more efficient antigen presenting cells and provide help to T cells involved in autoimmunity. Other disease-modifying therapies have also been noted to selectively target the memory B-cell population.\(^13\) Depletion of memory B cells by DMF would thus be expected to have beneficial effects on inflammatory disease activity in patients with MS.

The mechanism for this selective targeting is unknown, but may be related to the modification of reactive cysteine residues by DMF in various proteins involved in immune cell function.\(^14\) Several of these proteins identified in T cells, including NF-kB and interferon regulatory factor 4 (IRF-4), are also important in B-cell maturation and could explain the preferential reduction in the B-cell memory population.\(^15\)

We also demonstrate striking effects of DMF on B-cell cytokine production. We noted a reduction in GM-CSF-producing B cells with DMF treatment. This subset of B cells provides proinflammatory signals to myeloid cells and is enriched in RRMS patients.\(^7\) The efficacy of B-cell depletion appears to be related to the depletion of this particular B-cell subset. Additionally, other B-cell subsets that declined with DMF treatment included IL-6-producing and TNF-\( \alpha \)-producing B cells. Both these subsets of B cells have been implicated in MS disease pathogenesis.\(^4\) B cell IL-6 production can lead to increased differentiation of Th17 cells which are important players in MS pathogenesis.\(^3\) Similarly, TNF-\( \alpha \) production from B cells also promotes inflammatory T-cell responses.\(^4\) Thus, the reduced production of these proinflammatory cytokines from the B-cell compartment, could lead to a reduction in myeloid and T-cell inflammatory responses.

DMF treatment did not change the proportion of IL-10-producing B cells. Our protocol for assessing cytokine
production may not identify a subset of B cells capable of producing IL-10 that require more prolonged stimulation in vitro, however, we also quantified other B-cell subtypes (transitional B cells and B-1 cells) that are associated with regulatory function and noted no significant change in these subtypes. The lack of change in transitional B cells and B-1 cells could be due to the short duration of this study, since another study noted changes in these B-cell subsets primarily after 12 months of DMF therapy. The lack of a reduction in IL-10-producing B cells in combination with the decline in proinflammatory cytokine-producing B cells, could shift the balance between these cell populations resulting in an anti-inflammatory state. Our findings are in concordance with those from a recent study that noted an anti-inflammatory shift in B cells in RRMS patients with DMF treatment.

DMF treatment produced multiple changes in the B-cell compartment that target B-cell functions related to MS pathogenesis; these changes may underlie the beneficial effects of DMF on MS disease activity.

**Author contributions**

MDS performed the experiments and wrote the manuscript. KAM performed experiments and provided critical feedback on the manuscript, PAC conceived the study, obtained funding, provided supervision, designed the study and provided critical feedback on the manuscript. PB designed the study, collected samples, performed statistical analysis, and wrote the manuscript.

**Conflicts of Interest**

MDS reports no disclosures. KAM reports no disclosures. PAC reports grants from Biogen, during conduct of the study; grants from Teva, grants from Novartis, grants from MedImmune, personal fees from Vertex, and grants and personal fees from Biogen, outside the submitted work. PB reports no disclosures.

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**Supporting Information**

Additional Supporting Information may be found online in the supporting information tab for this article:

**Figure S1.** Flow cytometry gating strategy.