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PII: S2211-0348(18)30310-9
DOI: <https://doi.org/10.1016/j.msard.2018.08.032>
Reference: MSARD 956

To appear in: *Multiple Sclerosis and Related Disorders*

Received date: 26 April 2018
Revised date: 22 August 2018
Accepted date: 30 August 2018

Please cite this article as: Rune A. Høglund , Justyna Polak , Frode Vartdal , Trygve Holmøy , Andreas Lossius , B-cell composition in the blood and cerebrospinal fluid of multiple sclerosis patients treated with dimethyl fumarate, *Multiple Sclerosis and Related Disorders* (2018), doi: <https://doi.org/10.1016/j.msard.2018.08.032>

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Highlights

- DMF impacts the composition of B cells in the blood and CSF of MS patients
- Treatment with DMF is associated with reduced CSF cell counts
- Treatment with DMF leads to a relative reduction of plasmablasts in the CSF

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B-cell composition in the blood and cerebrospinal fluid of multiple sclerosis patients treated with dimethyl fumarate

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Keywords: multiple sclerosis, B cell, dimethyl fumarate, memory B cell, cerebrospinal fluid, plasmablast

Words (main text): 2249

Figures: 3

Tables: 1

Abstract

Background: B cells may contribute to the immunopathogenesis of multiple sclerosis (MS). Dimethyl fumarate (DMF) has recently been shown to reduce the frequency of memory B cells in blood, but it is not known whether the drug influences the cellular composition in the cerebrospinal fluid (CSF).

Methods: A cross-sectional study examining the cellular composition in blood and cerebrospinal fluid (CSF) from 10 patients treated with DMF and 18 patients receiving other disease modifying drugs or no treatment.

Results: Patients treated with DMF had reduced proportions of memory B cells in blood compared to other MS patients ($p = 0.0007$), and the reduction correlated with treatment duration ($r_s = -0.75$, $p = 0.021$). In the CSF, the absolute number of mononuclear cells were significantly lower in DMF-treated patients compared to the other patients ($p = 0.023$), and there was a disproportionate decrease of plasmablasts ($p=0.031$).

Conclusion: The results of this exploratory study support a B-cell mediated mechanism of action for DMF in both blood and CSF.

1 Introduction

B cells have an important role in multiple sclerosis (MS), as is evident from several successful clinical trials of B-cell depletion¹⁻³. Several potential mechanisms have been proposed, including antigen presentation, cytokine secretion, and antibody production⁴. In MS, the proportion of immunoglobulin class-switched CD27⁺ B cells is increased in the cerebrospinal fluid (CSF) compared to the peripheral blood⁵, and in particular the frequency of plasmablasts expressing CD38 and CD138 have been shown to be elevated^{6,7}. Studies have demonstrated that immunoglobulin class-switched B cells in the CSF are related to B cells in the brain parenchyma⁸, cervical lymph nodes⁹, and peripheral blood^{10,11}, indicating a dynamic exchange between these compartments.

Dimethyl fumarate (DMF) is an oral disease-modifying treatment (DMT) approved for treating relapsing-remitting MS (RRMS). The mechanism of action is not fully clarified¹², but includes a reduction of peripheral blood CD19⁺CD27⁺ memory B cells¹³⁻¹⁶. Upon intake, DMF is rapidly metabolized to monomethyl fumarate (MMF), which crosses the blood-brain barrier in animal models and in humans, however only at 15% of plasma concentration^{17,18}.

The penetration of MMF into the CSF, and the exchange of B cells between the periphery, CSF and the central nervous system (CNS), suggest the possibility that DMF may exert a direct or indirect effect on intrathecal B cells. To address this, we examined blood and CSF B-cell subsets from MS patients treated with DMF and patients not receiving immunomodulatory treatment. We found that while DMF treatment is associated with a decrease of CD19⁺CD27⁺ memory B cells in the periphery, it is associated with a significant reduction of CD19⁺CD27⁺CD38⁺ plasmablasts in CSF.

2 Materials and methods

2.1 Patients

MS patients ($n = 28$) were recruited at the Departments of Neurology at Akershus University Hospital and Oslo University Hospital; other data based on the same cohort have been previously published ¹⁹. All patients met the 2010 McDonalds criteria for MS ²⁰; one patient was classified as secondary progressive MS and the remaining as RRMS. All patients had intrathecal synthesis of immunoglobulin G (IgG) at the time of diagnosis, detected as oligoclonal IgG bands by isoelectric focusing. Lumbar puncture was performed either as a part or outside of diagnostic investigations, reflected by the variation in disease duration (Table 1). The study was approved by the Regional Ethical Committee South East (2009/23 S-04143a). All patients gave written informed consent before inclusion.

2.2 Cell subset analysis

For each individual included in this study, blood, serum and CSF samples were collected once, during the same consultation. Blood and CSF mononuclear cells were isolated as described previously, counted using a Neubauer improved hemocytometer, and immediately processed for flow cytometry, which was performed within two hours after sample collection ^{11, 19}. For B-cell subset identification we used fluorochrome-conjugated antibodies as described ¹⁹, including anti-human CD19, CD27, CD38, CD138, IgG, Ki-67, and HLA-DR, and additionally CD3 and CD14 for dump-channel purposes (BD Biosciences). Paired cell samples from CSF and blood were stained for surface antigens, and after fixation and permeabilization following the manufacturer's instructions (Fixation/Permeabilization Solution Kit, BD Biosciences), the cells were stained for Ki-67. The flow cytometry dataset was reanalyzed for the present study using FlowJo Version 10.2 (FlowJo, LLC). Of note, flow

cytometry data from one DMF-treated and one untreated patient were excluded due to technical reasons.

2.3 Quantification of IgG and albumin

For each patient, blood and CSF were collected simultaneously. After centrifugation and removal of cells for immediate flow cytometry analysis, the serum and CSF supernatants were frozen at -80°C . The supernatants were thawed and analyzed as a single batch within 36 months after the collection and freezing of the first patient sample. The collection and freezing procedures followed the published consensus protocol for the standardization of cerebrospinal fluid collection and biobanking ²¹. IgG and albumin in CSF were quantified by nephelometry (BN ProSpec Systems, Siemens). IgG and albumin in serum were determined by turbidimetry and colorimetry, respectively (Vitros 5.1 FS, Ortho Clinical Diagnostics).

2.4 Statistics

Statistical analyses were performed in JMP® pro 12.1 (StataCorp, LLC). Due to non-normality of data, non-parametric 2-sample Wilcoxon exact tests were used to compare groups unless otherwise specified. The significance level was set at 5%, and the tests were two-sided. No correction for multiple testing was performed. Results are presented as median [range]. Figures were made in FlowJo and JMP pro 12.1.

3 Results

3.1 Patients

Ten patients received treatment with DMF at inclusion, whereas 18 received other DMTs or no treatment (seven patients received glatiramer acetate, four received teriflunomide, and seven received no treatment). Clinical and demographic data at the time of CSF and blood

(including serum) collection are shown in Table 1. With the exception of treatment durations, which was shorter for the patients treated with DMF than other DMTs, there were no significant differences in clinical characteristics between DMF-treated patients and the other patients. All DMF-treated patients had normal total lymphocyte count before treatment start, with a median value of $1.7 \times 10^9/L$ [1.2–2.6], and two patients developed lymphopenia during DMF treatment ($<0.9 \times 10^9/L$).

3.2 DMF-treatment is associated with lower numbers of mononuclear cells in the CSF

DMF-treated patients had lower total CSF mononuclear cell counts compared to the other patients (560/mL [80–3000] vs 680/mL [264–6736], $p=0.023$). Similar results were obtained when comparing DMF-treated patients to those not receiving any treatment (Fig S1 A). The CSF mononuclear cell count in DMF-treated patients tended to correlate inversely with the treatment duration, but this was not statistically significant (Spearman r_s -0.6, $p=0.07$).

3.3 DMF reduces the frequency of memory B cells in blood and plasmablasts in the CSF

Previous studies have shown that DMF reduces the number of memory B cells in blood¹³⁻¹⁶, typically identified as CD19⁺CD27⁺ cells. To identify these cells in blood and CSF, a gating strategy based on available data was devised (Figure 1). Confirming previous studies, we found that DMF-treatment was associated with a relative reduction in CD19⁺CD27⁺CD38⁻ memory B cells in blood (12.8% [2.2–25.2] in DMF-treated vs 26.6% [6.9–46.6] in the other MS patients, $p=0.0007$, Figure 2A). Moreover, the reduction correlated strongly with treatment duration ($r_s = -0.75$, $p=0.021$, Figure 2B). There were no significant differences between the two groups in the proportions of CD19⁺CD27⁺CD38⁺ plasmablasts in blood (Figure 2A). In the CSF, in contrast, there was a selective depletion of CD19⁺CD27⁺CD38⁺ plasmablasts among DMF-treated patients (0% [0–13.6] vs 5.4% [0–69.8], $p=0.031$, Figure

2A), whereas the relative proportion of CD19⁺CD27⁺CD38⁻ memory B cells was similar in both groups (68.9% [0–100] in DMF-treated vs 66.7% [17.4–80] in other MS-patients, $p=0.534$, Figure 2A). There were no differences between the two groups in the proportion of total CD19⁺ B cells in blood or CSF (Figure 2A). Including only DMF-treated and untreated patients in the analyses yielded similar results (Fig. S1 B).

3.4 DMF reduces HLA-DR expression among memory B cells in blood

To further characterize the B-cell subsets, we examined antigen-presenting potential (HLA-DR), IgG expression, proliferation status (Ki-67), and the expression of the differentiation marker CD138. In patients treated with DMF, we found that blood CD19⁺CD27⁺CD38⁻ memory B cells express lower levels of the HLA class II molecule HLA-DR (median fluorescence intensity (MFI) 2025 [1702–2410] vs 2279 [1360–3649], $p=0.04$, Figure 3). The majority of blood plasmablasts expressed Ki-67 and IgG, and there were no significant differences between the patient groups (Figure 3). We found overall low levels of CD138 expression among blood plasmablasts.

In the CSF, the HLA-DR expression among CD19⁺CD27⁺CD38⁻ memory B cells was similar in both groups (MFI 1634 [1337–2391] vs 1570 [666–2414], $p=0.68$). Virtually all intrathecal CD19⁺CD27⁺CD38⁺ plasmablasts in both patient groups expressed Ki-67 and the majority expressed IgG (100% [83.3–100] vs 100% [50–100], Figure 3). In most patients in both groups we found that CSF plasmablasts expressed moderate to high levels of CD138 (MFI 1223 [-392–2998] vs MFI 5437 [-142–20911]). There were no significant differences for these variables between the patient groups (Figure 3).

3.5 DMF treatment did not influence intrathecal IgG production

Since DMF-treated patients exhibited preferential reductions of CD19⁺CD27⁺CD38⁺ antibody-secreting cells in CSF, we compared the intrathecal IgG production among DMF-treated patients ($n = 10$) with that among untreated patients ($n = 7$). There were no differences in IgG or albumin levels or indices of these, also indicating a similar blood-brain barrier integrity (Table 1).

4 Discussion

This cross-sectional study demonstrates that while DMF-treatment causes a time-dependent reduction in CD19⁺CD27⁺CD38⁻ memory B cells in blood, it leads to a depletion of CD19⁺CD27⁺CD38⁺ plasmablasts in the CSF. Our findings in blood are in agreement with recent publications¹³⁻¹⁶, and the present work demonstrates how the peripheral effects of DMF translate into the CSF. These data point to a possible B-cell mediated mechanism of action for DMF, in addition to the previously discussed pluripotent immunomodulatory effects¹².

The almost immediate effect of anti-CD20 treatment in MS is thought to be mediated by a reduction of B cells participating in antigen presentation and/or cytokine production^{22, 23}. Accordingly, the intrathecal IgG production does not seem to be affected by the treatment in the short run^{1, 24}. Memory B cells express HLA class II molecules and are potent antigen-presenting cells²⁵. Interestingly, we found that blood CD19⁺CD27⁺CD38⁻ memory B cells express lower levels of HLA-DR in DMF-treated than in untreated MS patients, suggesting that memory B cells in these patients are less efficient in presenting antigens. One could speculate that a reduced amount of memory B cells, with additionally reduced expression of HLA class II molecules, may result in less maturation and development of

CD19⁺CD27⁺CD38⁺ antibody-secreting cells in the CSF. This possibility is supported by the dynamic exchange of B cells between the CSF and periphery⁸⁻¹¹. However, a not mutually exclusive possibility is that the observed alterations in the composition of CSF B cells could be due to a local intrathecal effect of MMF.

Despite depletion of CD19⁺CD27⁺CD38⁺ plasmablasts in the CSF of DMF-treated patients, we did not observe a reduced intrathecal IgG production. This could be due to persistent secretion from end-differentiated plasma cells protected within tertiary lymphoid tissue in the meninges of MS patients²⁶. In line with this thought, we found that all antibody-secreting cells in the CSF express high levels of Ki-67, compatible with recently derived proliferating plasmablast. Interestingly, a similar persistence of intrathecal IgG synthesis has been observed in patients receiving anti-CD20 therapies²⁷. In accordance with the present findings, no difference in circulating IgG, IgA, or IgM in blood of DMF treated patients has been observed, indicating that end differentiated plasma cells could be protected from the effects of the drug also in the periphery in a short-term perspective¹⁴.

The strength of this study is the comparison of CSF B-cell subsets between two groups of MS patients similar in clinical and demographic characteristics. This was made possible by slight different treatment strategies between the two clinical departments contributing to the study. Samples from both hospitals were transported directly to the same external laboratory and processed identically, and the findings are not influenced by differences in inflammatory activity. Although we cannot exclude that differences in treatment durations could have influenced the results, treatment durations were long enough to allow full immunological effect in the majority of patients receiving DMF and other DMTs.

This study has several limitations. First, the number of patients is small. Given the risk of lumbar puncture headache, as well as rare but more serious risks such as infection, bleeding and herniation, we cannot perform lumbar puncture solely for the purpose of studying drug effects. While properly understanding the mechanism of action of DMF in MS patients is important, such studies must due to the above reasons be part of studies on disease mechanisms, which was the primary aim of this study¹⁹. This is therefore an exploratory study, the p-values were not corrected for multiple testing, and the results need to be reproduced in an independent study. Second, the control group is heterogeneous from a treatment standpoint, which potentially could confound our results. Nevertheless, as the sub-analysis with untreated controls ($n = 7$) demonstrated similar results, and lymphocyte depletion and reduced memory B-cell population in blood are analogue to previous reports¹³⁻¹⁶, we believe our findings have merit. While reduction in blood plasmablasts similar to what we observed in CSF have not been extensively described, a recent interim analysis of the PROCLAIM study demonstrated a decline in blood plasmablasts that had a delayed onset compared to memory B cell depletion²⁸. This delay, in addition to the relatively low proportions of circulating plasmablasts in blood compared to CSF of MS patients can potentially have hidden this phenomenon in studies with shorter time-frames. Lastly, as our data did not contain markers for IgD, we were unable to differentiate between class-switched and unswitched memory B cells. A recent publication limited to blood found that both populations are affected similarly, with a corresponding increase in CD27⁻IgD⁺ naïve B cells²⁹. New studies as to how these subpopulations are distributed in the CSF could also enlighten whether the effects of DMF are mainly peripheral or intrathecal.

5 Conclusion

This is the first study on the effects of DMF treatment on B cells in CSF, suggesting that while memory B cells are reduced in blood, plasmablasts are preferentially depleted in CSF. These findings, which need to be verified in an independent study, support the hypothesis that DMF mediates positive effects through B-cell modulation.

Abbreviations

CSF – cerebrospinal fluid

DMT – disease modifying therapy

IgG – immunoglobulin G

M/DMF – mono-/dimethyl fumarate

MFI – median fluorescence intensity

MS – multiple sclerosis

RRMS – relapsing remitting MS

TFM - teriflunomide

Funding

The study was supported by the South-Eastern Norway Regional Health Authority (grant 2016079) and the Norwegian Research Council (grant 250864/F20).

Declaration of conflicting interests

RH has received speaker honoraria from Merck and Roche, and unrestricted research grants from Biogen and Novartis. TH has received unrestricted research grants or speaker honoraria from Biogen, Merck, Novartis, Sanofi Genzyme and Teva. AL has received speaker honoraria

from Roche, and unrestricted research grants from Sanofi Genzyme. JP and FV declare no conflict of interest.

Author contributions

Study concept and design: AL, FV, RH, TH; data acquisition and/or analysis: AL, JP, RH; drafting the manuscript AL, RH. All authors revised the manuscript for intellectual content.

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Table 1 Demographic and clinical characteristics of patients.

Treatment	DMF	Other	Statistical test
Participants	10	18	-
Age (years)	41 (27–58)	47.5 (25–57)	p=0.75 ¹
Sex (M/F)	4/6	5/13	p=0.68 ²
Disease duration (months)	47.5 (10–153)	102 (2–283)	p=0.10 ¹
Treatment duration (months)	8 (2–13)	All: 35 (3–152)* GA: 79 (18–152) TFM: 12.5 (3–27)	p=0.0012 ¹
EDSS score	1.25 (1–3.5)	1.25 (0–5.5)	p=0.78 ¹
Number of relapses	1.5 (1–5)	3 (1–6)	p=0.11 ¹
Time since last relapse (months)	18.5 (2–118)	5 (1–164)	p=0.60 ¹
Albumin ratio**	4.4 (3.6–9.24)	4.6 (2.3–6.9)	p=0.67 ¹
IgG index**	0.7 (0.5–2.1)	1.2 (0.5–1.9)	p=0.74 ¹

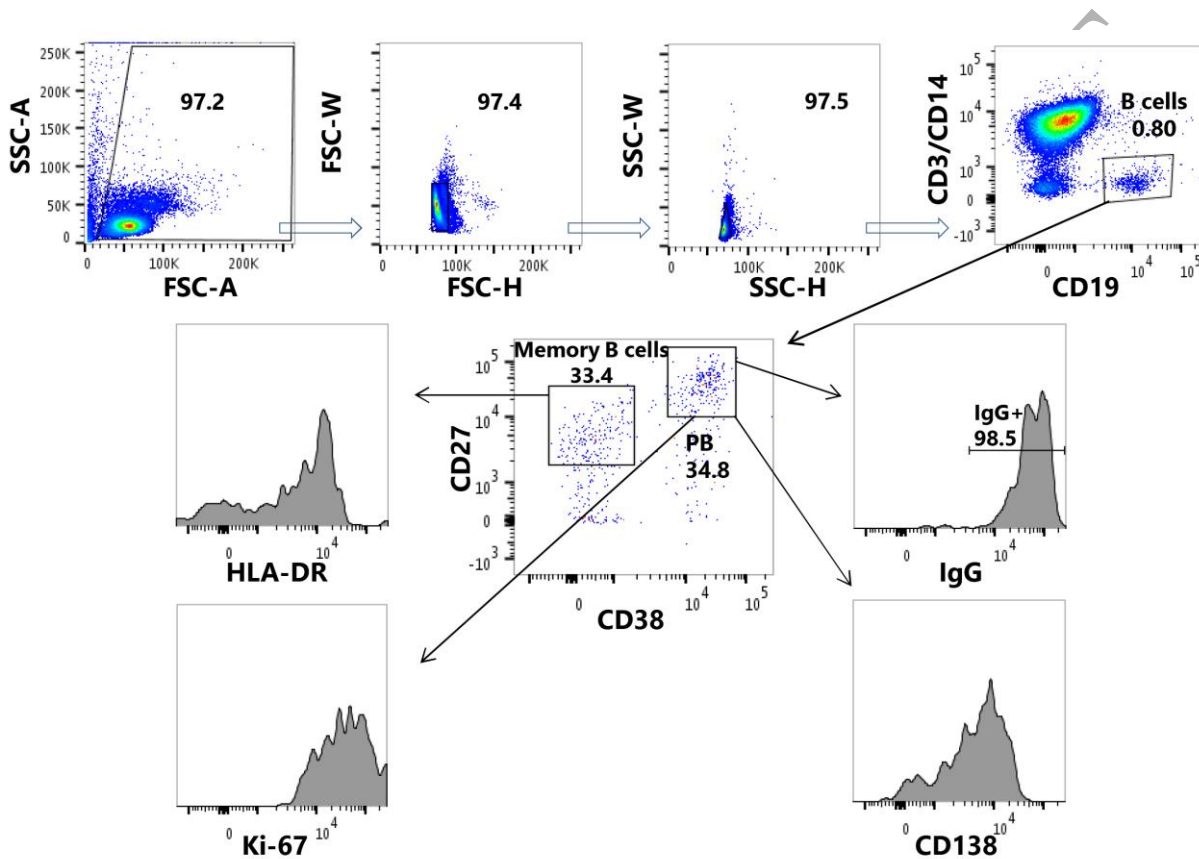
Numbers given as median (range). DMF: dimethyl fumarate. EDSS: expanded disability status scale. GA: glatiramer acetate. MRI: magnetic resonance imaging. TFM: teriflunomide.

¹Wilcoxon 2-sample exact test, ²Fishers exact test. *untreated patients excluded **compared between DMF treated and no-treated

Figure legends

Figure 1 - Gating strategy

We devised a gating strategy to identify single lymphocytes expressing CD19, CD27, CD38, CD138, HLA-DR, IgG and/or Ki-67. Cells expressing CD3 or CD14 were excluded in a dump channel.



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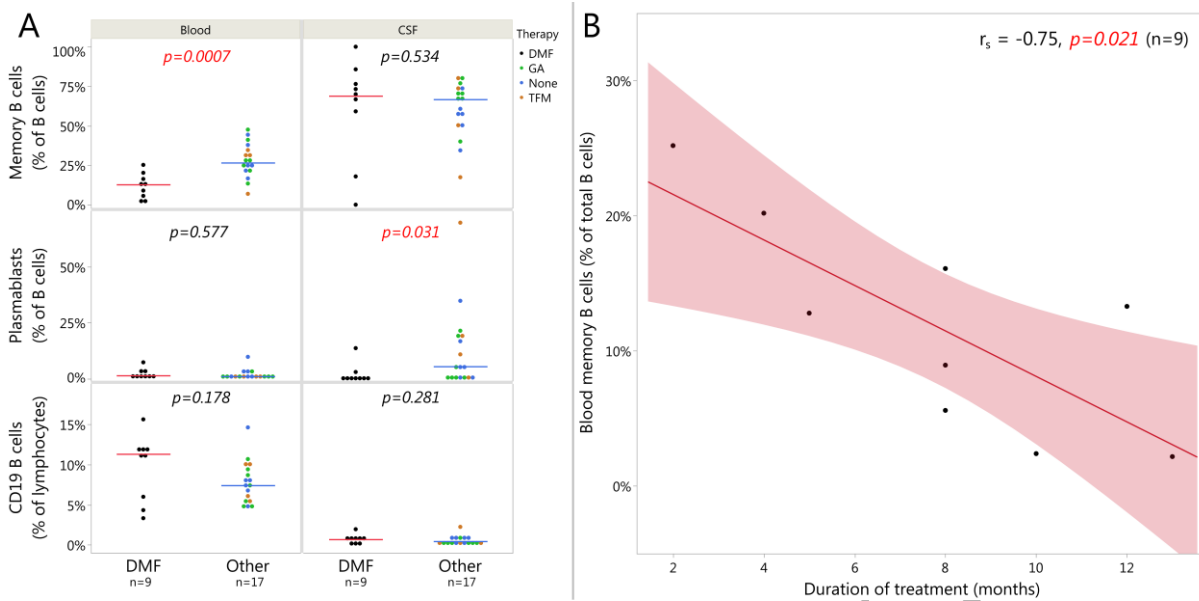


Figure 2 - DMF preferentially reduces the frequency of memory B cells in blood and plasmablasts in the CSF

(A) Proportion of $CD19^+CD27^+CD38^-$ memory B cells and $CD19^+CD27^+CD38^+$ plasmablasts of $CD19^+$ B cells, and proportion of $CD19^+$ B cells of lymphocytes in blood and CSF. Each symbol represents the cell frequencies in a given individual, and the bars depict the median.

(B) Proportion of memory B cells in DMF treated patients, in linear fit with treatment duration (boundaries depict 95% confidence interval). DMF, dimethyl fumarate; GA, glatiramer acetate; TFM, teriflunomide.

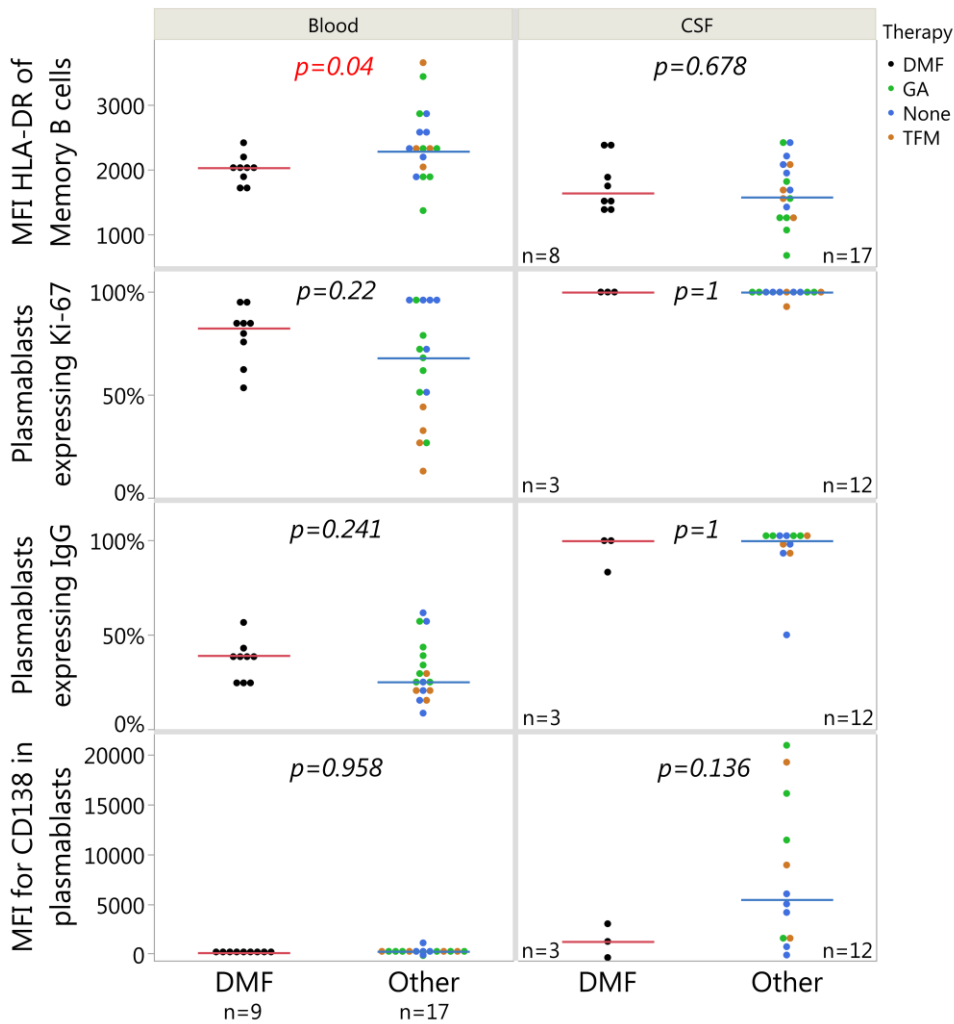


Figure 3 - DMF reduces HLA-DR expression among memory B cells in blood

MFI of HLA-DR for CD19⁺CD27⁺CD38⁻ memory B cells, proportion of CD19⁺CD27⁺CD38⁺ plasmablasts expressing Ki-67 and IgG, and MFI of CD138 for plasmablasts. Each symbol represents the cell frequencies or MFI in a given individual, and the bars depict the median.

DMF, dimethyl fumarate; GA, glatiramer acetate; TFM, teriflunomide.

Supplementary Figure – Sub analysis of DMF vs untreated patients for B cell subsets in blood and CSF

(A) Number of mononuclear cells per mL of cerebrospinal fluid from patients treated with DMF or no disease modifying therapy. (B) Proportion of $CD19^+CD27^+CD38^-$ memory B cells and $CD19^+CD27^+CD38^+$ plasmablasts of $CD19^+$ B cells, and proportion of $CD19^+$ B cells of lymphocytes in blood and CSF. Each symbol represents the cell count or -frequencies in a given individual and the bars depict the median. DMF, dimethyl fumarate.