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Original article

Dimethyl fumarate therapy reduces memory T cells and the CNS migration potential in patients with multiple sclerosis

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ABSTRACT

Background: Dimethyl fumarate (DMF) is a disease-modifying therapy for patients with relapsing-remitting multiple sclerosis (RRMS). T cells are major contributors to the pathogenesis of RRMS, where they regulate the pathogenic immune response and participate in CNS lesion development.
Objectives: In this study we evaluate the therapeutic effects of DMF on T cell subpopulations, their CNS migration potential and effector functions.
Methods: Blood and CSF from untreated and DMF-treated patients with RRMS and healthy donors were analyzed by flow cytometry.
Results: DMF reduced the prevalence of circulating proinflammatory CD4+ and CD8+ memory T cells, whereas regulatory T cells were unaffected. Furthermore, DMF reduced the frequency of CD4+ T cells expressing CNS-homing markers. In coherence, we found a reduced recruitment of CD4+ but not CD8+ T cells to CSF. We also found that monomethyl fumarate dampened T cell proliferation and reduced the frequency of TNF-α, IL-17 and IFN-γ producing T cells.
Conclusion: DMF influences the balance between proinflammatory and regulatory T cells, presumably favoring a less proinflammatory environment. DMF also reduces the CNS migratory potential of CD4+ T cells whereas CD8+ T cells are less affected. Altogether, our study suggests an anti-inflammatory effect of DMF mainly on the CD4+ T cell compartment.

1. Introduction

Multiple Sclerosis is a chronic demyelinating disease of the central nervous system (CNS) characterized by T cell-mediated neuroinflammation and tissue damage (Compston and Coles, 2008; Dendrou et al., 2015). Dimethyl fumarate (DMF) is one of the disease modifying treatments approved for treating patients with relapsing-remitting multiple sclerosis (RRMS) (Bar-Or et al., 2013; Fox et al., 2012; Gold et al., 2012). DMF is administered orally and metabolized to monomethyl fumarate (MMF) in the small intestine (Horssen and Vries, 2013). Various studies report protective effects of DMF in inflammatory diseases, possibly through an apoptosis-induced reduction of circulating lymphocytes and modulation of lymphocyte effector functions (Ghadiri et al., 2017; Breuer et al., 2017; Diebold et al., 2018; Lim et al., 2016; Longbrake et al., 2016; Mathias et al., 2017). Additionally, DMF has been suggested to have neuroprotective effects by agonistic properties on the G-protein-coupled receptor hydroxycarboxylic acid receptor 2 (HCA2) and hereby activating the nuclear factor erythroid 2-related factor 2 (Nrf2) pathway. Activation of the Nrf2 pathway induces downregulation of adhesion molecules and suppression oxidative damage; processes related to lymphocyte trafficking to the CNS and tissue damage in MS. DMFs agonistic effect on HCA2 have further been reported to inhibit the transcription factor nuclear factor kappa B (NfκB) that plays a major role in inducing inflammatory cytokine production facilitating T cell maturation (Breuer et al., 2017; Lim et al., 2016; Mathias et al., 2017; Berkovich and Weiner, Jul 2015; Linker et al., 2011; Tahvili et al., 2015; Gerdes et al., 2007). With this study we aimed to clarify the mode of action of DMF in patients with RRMS, with special regard to the treatment effect on peripheral T cell subpopulations, the CNS migratory potential of T cells, and T cell proliferation and cytokine production.

2. Materials and methods

2.1. Study participants and ethics

We included 18 untreated patients with RRMS (4 males and 14 females; mean 34 years [range 22–52]; mean EDSS 1.75 [range 0–3.5]) and 21 patients treated with delayed release DMF (Tecfidera) 240 mg BID for more than 12 months (5 males and 16 females; mean 39 years [range 25–50]; mean EDSS 1.5 [range 0–4]). There was no difference in sex ($p = = 0.99$) or EDSS ($p = = 0.50$) and only suggestive significance of age ($p = = 0.03$) between groups (Mann–Whitney test). Untreated patients were defined as at least one month since last steroid treatment,

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Table 1
Characteristics of the subjects included in the study.

Subject	Sex ($p = = 0.99$)	Age ($p = = 0.03$)	EDSS ($p = = 0.50$)	Previous treatment	Number of relapses within one year prior to acquisition ($p < 0.0001$)
Untreated patients					
1	F	31	1	None	1
2	F	44	3	None	2
3	F	38	3	None	2
4	M	42	2.5	None	1
5	F	26	0	None	0
6	F	47	1.5	None	1
7	F	29	2	None	1
8	F	24	0	None	1
9	M	42	1	None	1
10	M	28	1	None	1
11	F	31	2.5	None	3
12	F	36	0	None	3
13	F	23	3	None	1
14	F	44	0	Interferon beta 1a, Teriflunomide	0
15	M	29	2.5	None	2
16	F	41	3.5	None	1
17	F	37	1	None	0
18	F	22	3	None	2
Dimethyl fumarate treated patients					
1	F	50	1.5	Interferon beta-1a, Peginterferon beta-1a	0
2	F	27	1	None	0
3	F	33	0	Interferon beta-1a	0
4	M	47	2	Interferon beta 1-a, Interferon beta 1-b	0
5	M	47	0	Interferon beta 1-a, Glatiramer acetate	0
6	F	47	2.5	Interferon beta 1-a	0
7	F	34	1	Interferon beta 1-a	0
8	F	25	0	None	0
9	F	46	1.5	Interferon beta 1-a	0
10	F	38	0	Interferon beta 1-a, Teriflunomide, Glatiramer acetate	0
11	F	49	2	Interferon beta 1-a	0
12	F	39	2	None	0
13	M	32	1.5	Interferon beta 1-a	0
14	F	46	4	Interferon beta 1-a, Teriflunomide	0
15	F	47	2.5	Teriflunomide, Glatiramer acetate	0
16	F	33	2	Interferon beta 1-a	0
17	F	47	4	Interferon beta 1-a	1
18	M	31	0	Interferon beta 1-a	0
19	M	49	1	Teriflunomide	0
20	F	31	1.5	Interferon beta 1-a	0
21	F	32	1	None	0

EDSS: Expanded disability status scale.
Given are the subjects sex, age, EDSS at the date of blood drawing, previous treatments and number of relapses within a year prior to acquisition.

and more than three months since last immunomodulatory treatment. The majority of the untreated patients where newly diagnosed. None of the patients had ever received strong immunosuppressive drugs, e.g., mitoxantrone or cyclophosphamide, or cell-depleting monoclonal antibody therapy. Number of relapses within one year prior to acquisition and further demographic information is listed in Table 1. The same cohort of patients with RRMS was used in a previous study (Holm Hansen et al., 2018). Total lymphocyte count was performed on 11 of the untreated and 6 of the DMF-treated patients with RRMS. Cerebrospinal fluid (CSF) samples was collected from 7 of the untreated and 3 of the DMF-treated patients with RRMS. In addition, 12 healthy controls were included for *in vitro* studies. The healthy controls had no autoimmune, neurological, or other chronic illnesses. All participants gave informed, written consent to participation. The study was approved by the regional scientific ethics committee (protocol number H-16047666).

2.2. Blood and cerebrospinal fluid samples

Venous blood was drawn and peripheral blood mononuclear cells (PBMC) were isolated using density gradient centrifugation (Lymphoprep, Axis-shield, Oslo, Norway) and washed twice in cold PBS/2mM EDTA.10 ml of cerebrospinal fluid (CSF) was collected in polypropylene tubes on an ice bath and immediately centrifuged for 10 min at 400 g to separate cells from fluid. Cells were then instantly stained and analyzed by flow cytometry.

2.3. Flow cytometric analysis of freshly isolated cells

For flow cytometric analysis freshly isolated PBMC were incubated with FcR blocking reagent (Miltenyi Biotec, Bergisch Gladbach, Germany) to avoid nonspecific Ab binding and next stained with fluorochrome-conjugated antibodies targeting the surface molecules of interest. For T cell phenotyping the following Ab were used: CD3 (APC; UCHT1), CD8 (BV605; RPA-T8), CCR7 (PE; G043H7), CD45RA (FITC; HI100), CXCR3 (AF488; G025H7), CCR6 (PerCP/Cy5.5; G034E3), CD49d (BV605; 9F10), MCAM-1 (BV421; P1H12), CD25 (PE; M-A251), CD127 (BV421; A019D5), CCR2 (BV605; K036C2), CCR5 (AF647; HEK/1/85a) all from BioLegend (San Diego, CA, USA) and CD4 (APC/AF750; S3.5) from ThermoFisher Scientific (Waltham, MA, USA). Corresponding isotype controls were used to correct for nonspecific Ab binding and overlaps in spectra. Absolute counts of T cell subpopulations of 6 of the included DMF-treated and 11 of the untreated patients with RRMS were determined using TruCount beads (BD Biosciences, CA, USA) according to manufacturer. Data were acquired on a FACS Canto II flow cytometer (BD Biosciences, CA, USA) and data analysis

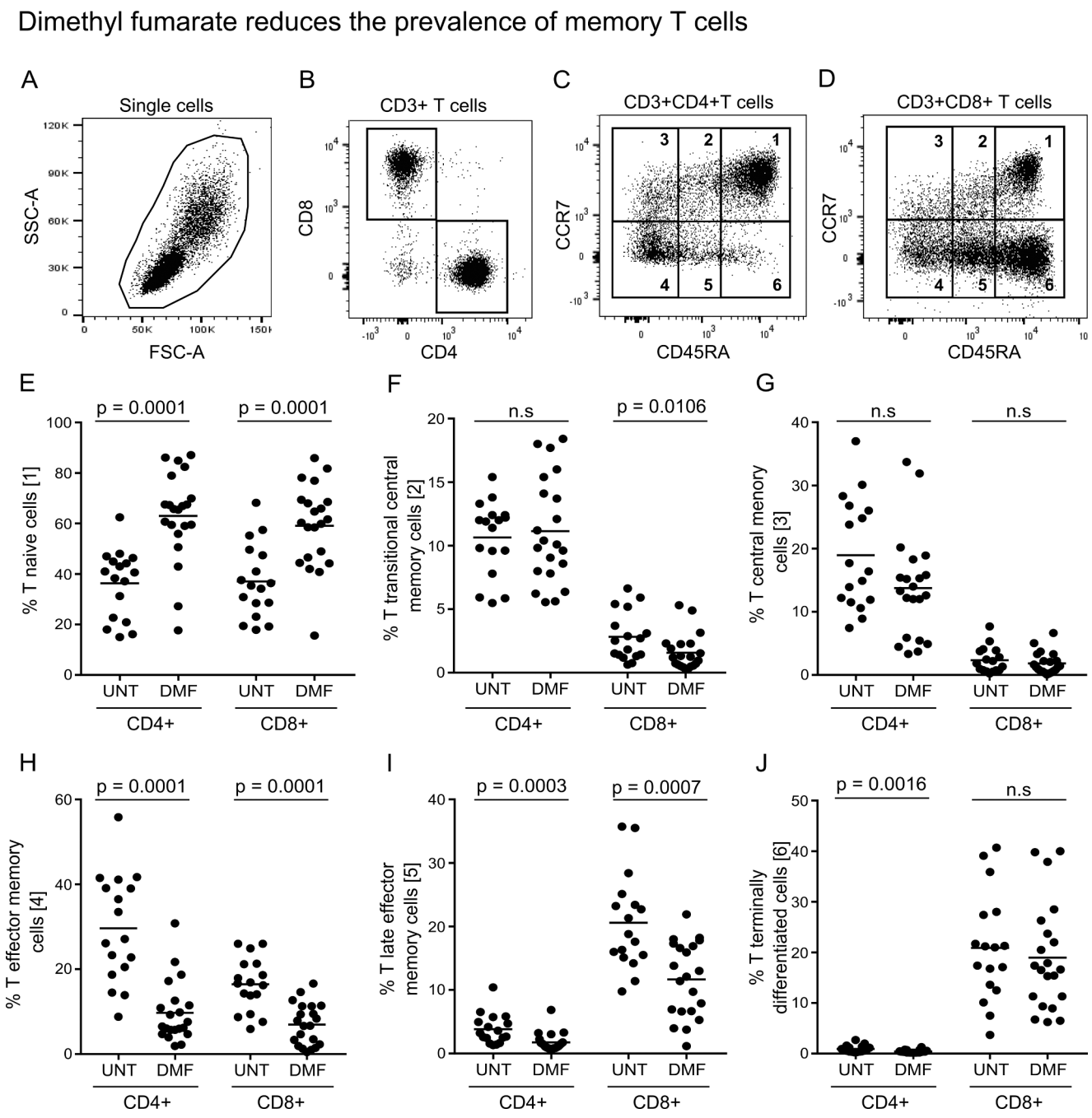

Fig. 1. Dimethyl fumarate reduces the prevalence of memory T cells.
(A–D) Gating strategy. Flow cytometry analysis of T cell subpopulations includes a lymphocyte gate in a FSC-A/SSC-A dot plot (A), a FSC-A/FSC-H dot plot for single cell gating, a CD3/SSC-A plot for gating of CD3+ T cells followed by a CD4/CD8 dot plot from where CD4+ and CD8+ T cells were defined (B). Flow cytometry dot plot example of the six T cell differentiation stages of CD4+ (C) and CD8+ (D) T cells in blood; the cell population numbers 1–6 refer to the individual T cell subpopulations also indicated in the scatterplots E-J. (E-J) Distribution of naïve ($CD45RA^{++}CCR7^{+}$) (E), transitional central memory ($CD45RA^{+}CCR7^{+}$) (F), central memory ($CD45RA^{-}CCR7^{+}$) (G), effector memory ($CD45RA^{-}CCR7^{-}$) (H), late effector memory ($CD45RA^{+}CCR7^{-}$) (I), and terminally differentiated ($CD45RA^{++}CCR7^{-}$) (J) T cells within the CD4+ and CD8+ compartment in blood from DMF-treated (DMF) and untreated (UNT) patients with RRMS. The mean value is shown for all groups analyzed.

performed using the software FlowJo (TreeStar, Ashland, OR, USA).

2.4. PBMC stimulation

Freshly isolated PBMC were cultured in growth media RPMI 1640/5% Human AB serum (Invitrogen, Carlsbad, CA, USA)/penicillin/streptomycin (50 units/ml) (Gibco, Waltham, MA, USA) in 24-well flat bottomed plates (1.5 mio./well). The cells were stimulated for 4 days with Dynabeads Human T-Activator CD3/CD28 (Gibco) at a bead to cell ratio of 1:8 and the culture supplemented with 0, 5, 10, 20 or 30 μg/ml monomethyl fumarate (MMF: Sigma-Aldrich, St. Louis, MO, USA).

2.5. T cell proliferation and phenotyping after in vitro stimulation

Freshly isolated PBMC were stained with carboxyfluorescein diacetate succinimidyl ester (CFSE: (Molecular Probes, Eugene, OR, USA) and stimulated for 4 days as described above. Cells were then surface stained with fluorochrome-conjugated antibodies specific for TCRαβ

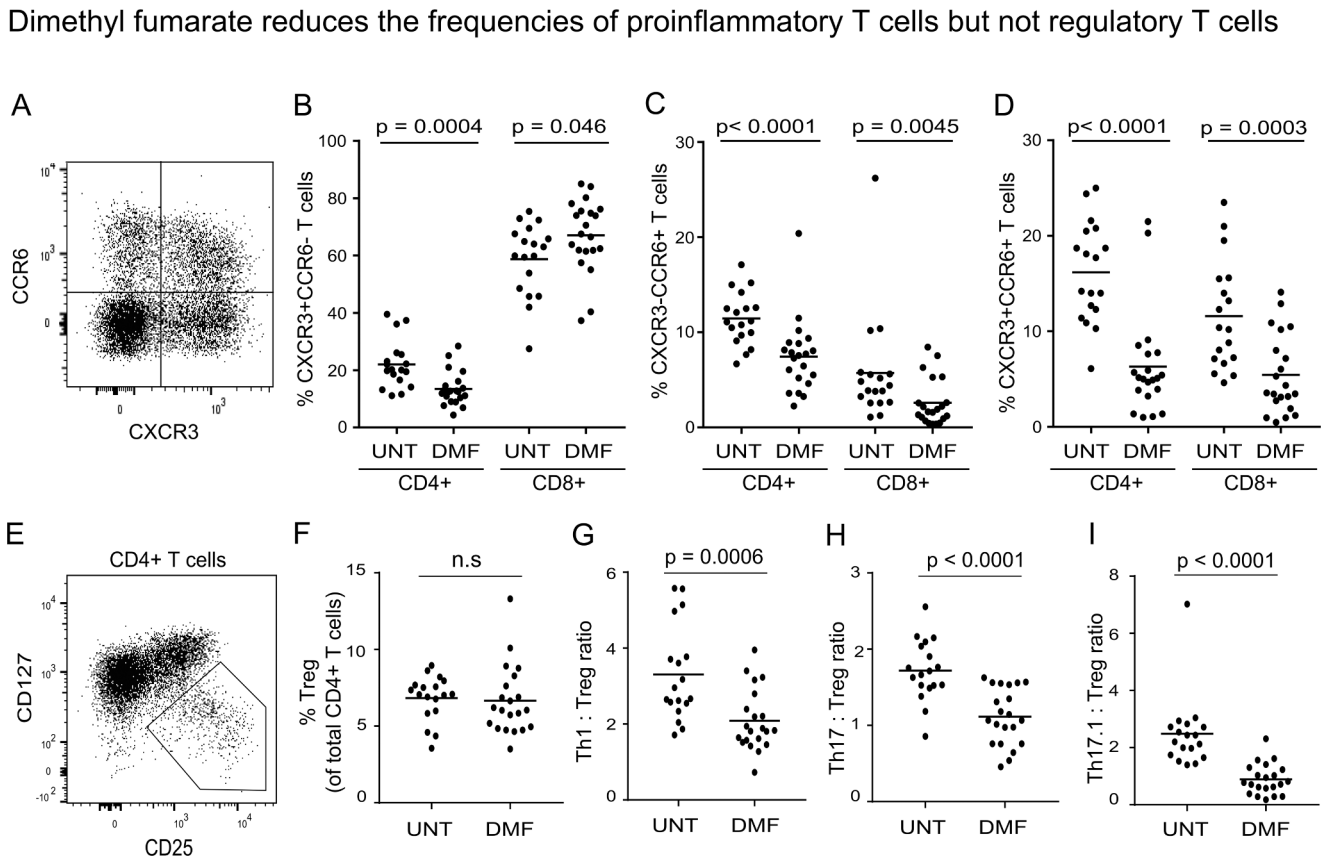

Fig. 2. Dimethyl fumarate reduces the frequencies of proinflammatory T cells but not regulatory T cells.
(A) Flow cytometry dot plot example of CXCR3 and CCR6 T cell populations in blood. (B–D) Distribution of CD4+ and CD8+ T cells in the CXCR3+CCR6- (B), CXCR3-CCR6+ (C) and CXCR3+CCR6+ (D) T cell populations in blood from DMF-treated (DMF) and untreated (UNT) patients with RRMS. (E) Flow cytometry dot plot example of CD4+CD25^{hi} T regulatory (T_{reg}) cells in the blood. (F) Frequency of T_{reg} cells in blood from DMF-treated (DMF) and untreated (UNT) patients with RRMS. (G–I) Scatterplots of Th1:T_{reg} (G), Th17:T_{reg} (H), and Th17.1:T_{reg} (I) ratios in blood from DMF-treated and UNT patients with RRMS. The mean value is shown for all groups analyzed.

(PE; IP26), CD49d (PerCP/Cy5.5; 9F10), CXCR3 (BV421; G025H7), CCR6 (APC; G034E3), MCAM-1 (BV421; P1H12) all from BioLegend and CD4 (APC/AF750; RM4-5) and live/dead stain from ThermoFisher Scientific. Corresponding isotype controls were used to correct for nonspecific Ab binding and overlaps in spectra. Proliferation were defined according to the strength of the CFSE signal. Data were acquired on a FACS Canto II flow cytometer and data analysis performed using FlowJo software.

2.6. Intracellular cytokine staining

To measure T cell cytokine production PBMC were stimulated for 4 days as described above. Thereafter the cells were re-stimulated with 10 ng/ml phorbol myristate acetate (PMA: Sigma-Aldrich) and 0.5 μg/ml ionomycin (Sigma-Aldrich) for 30 min at 37 °C, 5% CO2 after which 5 μg/ml brefeldin A (Sigma-Aldrich) was added to the cell culture and the cells incubated for an additional 4 h. Cells were then surface stained with fluorochrome-conjugated antibodies specific for TCRαβ (AF488; IP26) and CD4 (BV421; OKT4) from BioLegend and CD8 (APC/AF750; 3B5) and live/dead stain from ThermoFisher. Cells were fixed, and permeabilized (fixation buffer and permeabilization wash buffer from BioLegend) according to manufacturer. Finally, cells were intracellularly stained with fluorochrome-conjugated antibodies specific for IFN-γ (PE/Cy7; B27), TNF-α (PerCP/Cy5.5; MAb11) GM-CSF (APC; BVD2-21C11), IL-13 (PE; JES10-5A2), and IL-17 (PerCP/Cy5.5; BL168) all from BioLegend. Matching isotype controls were included. Data were acquired on a FACS Canto II flow cytometer and data analysis were performed using FlowJo software.

2.7. Statistics

Statistical analyses were performed using GraphPad Prism 7 software (GraphPad software Inc, La Jolla, CA, USA). Mann-Whitney unpaired tests were used for comparison of T cell subpopulations between DMF-treated and untreated patients. Unpaired *t*-test were used for comparison of CSF samples between DMF-treated and untreated patients. Friedman paired tests were used for *in vitro* analysis of T cell cytokines, adhesion molecules and chemokine receptors. $P < 0.005$ was considered as statistically significant and $p < 0.05$ was considered as suggestive of significance (Benjamin et al., 2018).

3. Results

3.1. Dimethyl fumarate reduces the prevalence of memory T cells

To investigate the effect of DMF on the peripheral pool of T cells, we compared CD4+ and CD8+ T cells from 18 untreated and 21 DMF treated patients with RRMS by flow cytometry. Using the differentiation markers CCR7 and CD45RA to discriminate T cell subpopulations, six different phenotypes were identified: naïve T cells ($CD45RA^{++}CCR7^{+}$), central memory T cells ($CCR7^{+}CD45RA^{-}$), effector memory T cells ($CCR7^{-}CD45RA^{-}$), terminally differentiated T cells ($CCR7^{-}CD45RA^{++}$), and two other distinct intermediate populations referred to as transitional central memory T cells ($CCR7^{+}CD45RA^{+}$) and late effector memory T cells ($CCR7^{-}CD45RA^{+}$) (Fig. 1(A)–(D)). These T cells subpopulations represent six different differentiation states as demonstrated by control staining of the

activation markers; CD69, HLA-DR, CD25, CXCR3 and PD-1 (Supp. Fig. 1(A)–(O)). We found that DMF treatment significantly increases the frequency of naïve T cells in both the $CD4^+$ and $CD8^+$ compartment (Fig. 1(E)). In contrast the frequency of effector memory and late effector memory T cells both in the $CD4^+$ and $CD8^+$ compartment were significantly decreased in DMF-treated patients (Fig. 1(H)–(I)). Frequencies of terminally differentiated T cells were significantly decreased by DMF-treatment in $CD4^+$ T cells, but not in $CD8^+$ T cells (Fig. 1(J)). Altogether, these data demonstrate an increased frequency of naïve T cells and a decreased frequency of memory T cells in the blood of DMF-treated patients. We next calculated the absolute lymphocyte counts in 11 of the untreated and 6 of the DMF-treated patients and found a reduced total T cell count ($p = = 0.001$) in DMF-treated patients. This reduction was not observed in the naïve T cell population; however, the absolute cell count of memory T cells was significantly reduced by DMF-treatment (Supp. Fig. 2(A)–(F)).

3.2. Dimethyl fumarate reduces the frequencies of proinflammatory T cells but not regulatory T cells

The cytokines IFN-γ and IL-17 are critically involved in MS development (Paroni et al., 2017). Both CD4+ T helper cells (Th cells) and CD8+ cytotoxic T cells (Tc cells) are producers of IFN-γ and IL-17 and can be identified according to the surface expression of CXCR3 (IFN-γ production) and CCR6 (IL-17 production) (Paroni et al., 2017). To explore the capacity of DMF to affect these proinflammatory T cell subsets in the periphery, we evaluated the frequencies of Th1 and Tc1 (CXCR3+CCR6-) cells, Th17 and Tc17 (CXCR3-CCR6+) cells, and Th17.1 and Tc17.1 (CXCR3+CCR6+) cells from 18 untreated and 21 DMF-treated patients with RRMS (Fig. 2(A)). This showed a reduction in the frequency of Th1, Th17, Th17.1, Tc17 and Tc17.1 cells (Fig. 2(B)–(D)); and was further supported by absolute T cell counts showing a significant reduction in Th1, Th17, Th17.1, Tc1, Tc17 and Tc17.1 cells (Supp. Fig. 3(A)–(F)).

We further investigated whether the effect of DMF was exclusively found within proinflammatory T cells or whether a similar effect could be observed for regulatory T cells (T_{reg}). For this, we analyzed the frequencies of T_{reg} cells defined as CD4+CD127-$CD25^{hi}$ T cells (Fig 2(E)) and found no significant difference between DMF-treated and untreated patients with RRMS (Fig 2(F)), suggesting that DMF mainly affects proinflammatory T cell subsets. Calculating the ratio between proinflammatory and regulatory CD4+ T cells showed a significant reduced Th1:T_{reg}, Th17:T_{reg}, and Th17.1:T_{reg} ratio in DMF-treated RRMS patients (Fig 2G-I); indicating an increased capacity to control the proinflammatory environment

3.3. Dimethyl fumarate reduces the CNS migration potential of blood T cells

To investigate the influence of DMF treatment on the CNS migratory potential of blood T cells, the surface expression of chemokine receptors and adhesion molecules assumed to navigate peripheral T cells to the CNS were analyzed on T cells from 18 untreated and 21 DMF-treated patients with RRMS. This showed that the frequency of peripheral T cells expressing CCR5, CCR6 and high levels of CD49d ($CD49d^{hi}$) was significantly decreased in both CD4+ and CD8+ T cells from DMF treated compared to untreated patients (Fig. 3(G), (K) and (L)). Likewise, the frequency of MCAM-1+, CXCR3+ and CCR2+ CD4+ T cells was significantly decreased in DMF treated patients compared to untreated (Fig. 3(H)–(J)).

In coherence with the observed reduced CNS migration potential of CD4+ T cells we found a 47% reduced frequency of CD4+ T cells in CSF from 3 DMF-treated patients, whereas DMF treatment had no effect on the prevalence of CD8+ T cells in CSF (Fig. 3(O)).

3.4. Monomethyl fumarate reduces the CNS migration potential in vitro

To verify the effect of DMF on the CNS migration potential of peripheral blood T cells, we analyzed the expression of CCR6, $CD49d^{hi}$, CXCR3 and MCAM-1 on T cells from healthy donors following *in vitro* stimulation with anti-CD3/anti-CD28 stimulatory beads and various concentrations of the DMF metabolite MMF. Here we found a significant decrease in the frequency of CCR6+, $CD49d^{hi}$, CXCR3+ and MCAM-1 + + T cells in both the CD4+ and CD8+ T cell compartment at high MMF concentrations (20–30 μg/ml) (Fig. 4(A)–(H)). The most extensive effect of MMF observed was a 70–75% reduction in CD4+ and CD8+ T cells expressing $CD49d^{hi}$ ($p < 0.0001$; Fig. 4(B) and (F)). In contrast, we only observed a decreased frequency of CCR6+, $CD49d^{hi}$, CXCR3+ and MCAM-1+ T cells in the CD4+ T cell compartment in response to low concentrations of MMF (5–10 μg/ml) (Fig. 4(A)–(D)). Altogether, these data confirmed our *ex vivo* observation and emphasizes the effect of DMF/MMF on the CNS migratory potential of CD4+ T cells.

3.5. Monomethyl fumarate reduces T cell proliferation and modifies the cytokine profile

We next investigated the impact of MMF on T cell proliferation. For this, PBMC from 12 healthy donors were stained with CFSE and cultured in growth media including anti-CD3/anti-CD28 stimulatory beads and various concentrations of MMF. This showed a proportional decrease in CD4+ and CD8+ T cell proliferation with increasing concentration of MMF (Fig. 5(A)–(C)).

To analyze the impact of MMF on T cell cytokine production, the cultured PBMC were re-stimulated with PMA/ionomycin and the frequency of T cells producing TNF-α, GM-CSF, IL-17, IFN-γ and IL-13 evaluated. At high concentrations of MMF (20–30 μg/ml) we found a reduced frequency of CD4+ and CD8+ T cells producing TNF-α and IL-17 (Fig. 5(I), (K), (N), (P)), and CD4+ T cells producing IFN-γ (Fig. 5(L)). The largest effect observed was a 74% reduced frequency of CD4+ T cells producing IL-17, a 25% reduced frequency in CD4+ T cells producing TNF-α, and a 21% reduced frequency in CD4+ T cells producing IFN-γ (Fig. 5(I), (K), (L)). At low concentrations of MMF (5–10 μg/ml) we observed a 47% decrease in IL-17 producing CD4+ T cells (Fig. 5(K)) and an increased frequency of CD4+ and CD8+ T cells producing IL-13 and GM-CSF (Fig. 5(J), (M), (O), (R)). Altogether, this analysis indicates a complex effect of MMF on the proinflammatory cytokine profile of CD4+ T cells.

4. Discussion

In this study we investigated the impact of DMF on T cells in patients with RRMS. In agreement with other studies (Ghadiri et al., 2017; Diebold et al., 2018; Longbrake et al., 2016; Wu et al., 2017) we found that the frequency of circulating memory T cells was reduced and conversely that the frequency of naïve T cells was increased in DMF-treated patients. Previous studies have reported a DMF-induced lymphopenia in DMF-treated patients with RRMS (Ghadiri et al., 2017; Diebold et al., 2018; Longbrake et al., 2016; Wu et al., 2017; Fleischer et al., 2018), and we therefore included absolute T cell counts in our analysis. This analysis showed no difference in the absolute count of naïve T cells, in contrast to memory T cells in which a significant decrease in the number of cells was observed despite a higher mean age of DMF-treated patients. This indicated that one of the beneficial treatment effects of DMF is a preferential loss of memory T cells.

We furthermore investigated the effect of DMF on specific effector T cell subsets assumed to play a major role in neuroinflammation and tissue damage in MS, including Th1 (CXCR3+CCR6-), Th17 (CXCR3-CCR6+), and Th17.1 (CXCR3+CCR6+) cells (Paroni et al., 2017; Stromnes et al., 2008). In line with previous observations (Diebold et al., 2018; Longbrake et al., 2016; Wu et al., 2017) we

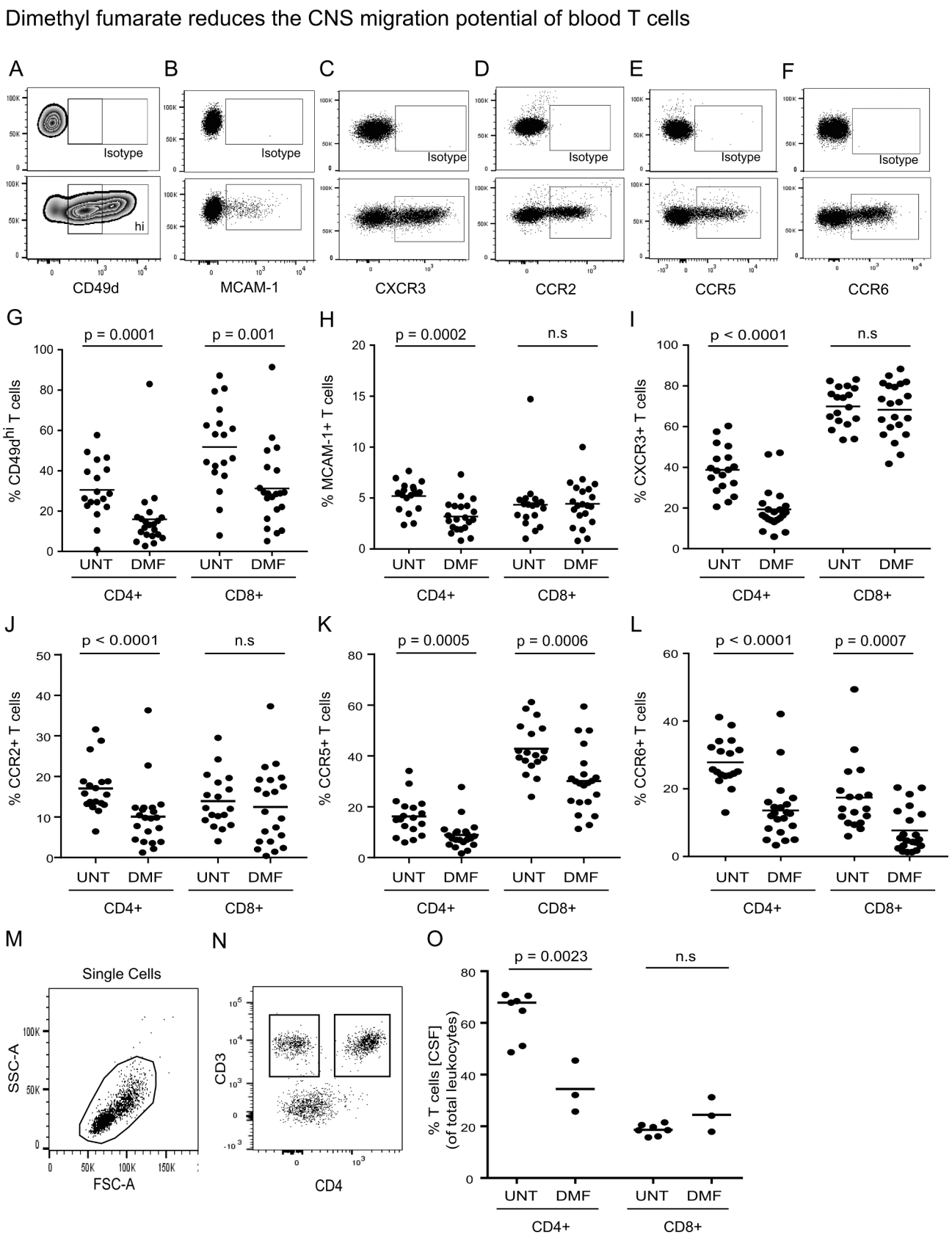

Fig. 3. Dimethyl fumarate reduces the CNS migration potential of blood T cells.
(A–F) Flow cytometry dot plot examples of gating of CD49d^{hi} (A), MCAM-1+ (B), CXCR3+ (**C**), CCR2+ (D), CCR5+ (E), CCR6+ (F) T cells; the upper panel showing the isotype control staining and the lower panel staining with the primary antibody. (G–L) Frequency of CD49d^{hi} (G), MCAM-1+ (H), CXCR3+ (I), CCR2+ (J), CCR5+ (K), CCR6+ (L) CD4+ and CD8+ T cells in blood from DMF-treated (DMF) and untreated (UNT) patients with RRMS. (M) Flow cytometry dot plot example of T cell gating in cerebrospinal fluid (CSF). (O) Frequency of CD4+ and CD8+ T cells in CSF from DMF-treated and UNT patients with RRMS. The mean value is shown for all groups analyzed.

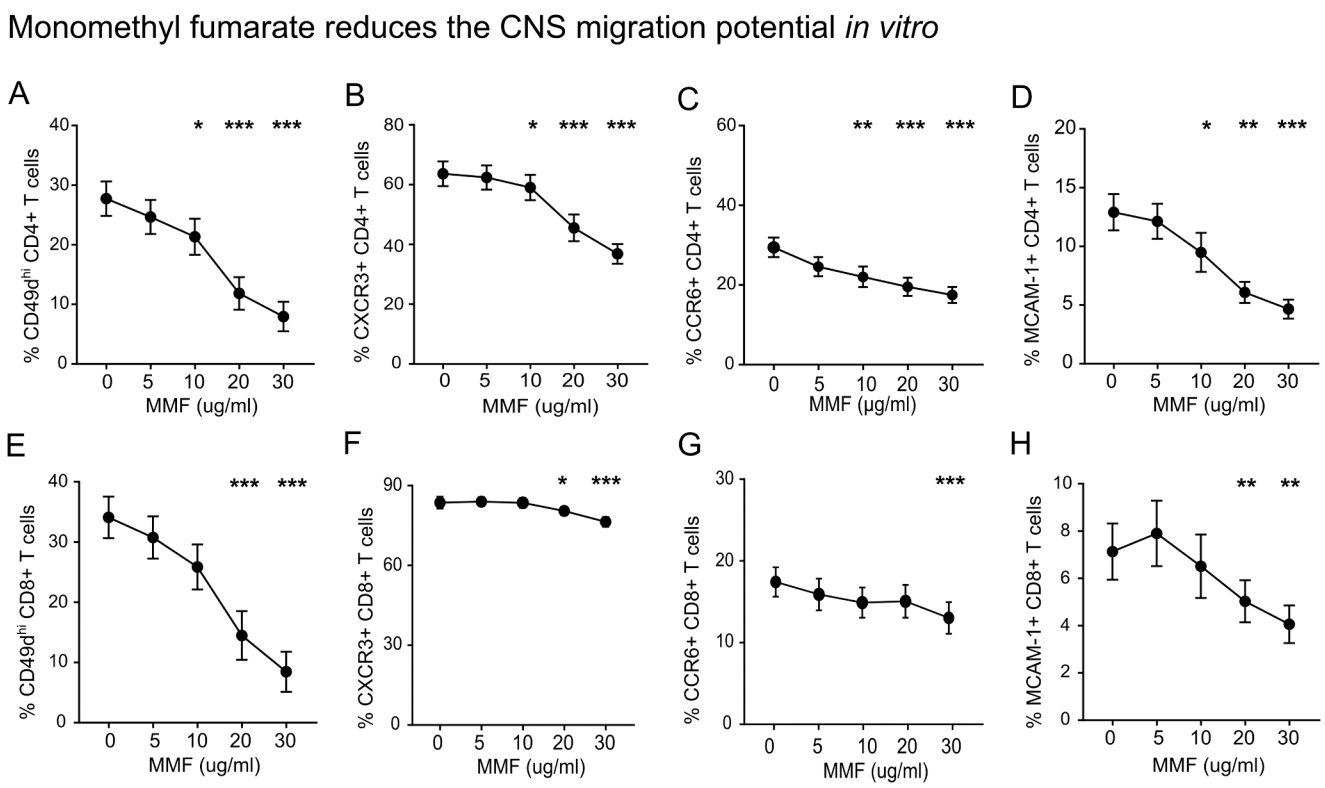

Fig. 4. Monomethyl fumarate reduces the CNS migration potential *in vitro*.
(A–H) Frequency of CD49d^{hi}, CXCR3+, CCR6+, MCAM-1+ CD4+ T cells (A-D) and CD8+ T cells (E–H) from healthy donors cultured with anti-CD28/CD3 stimulatory Dynabeads and various concentrations of MMF for 4 days. ***$p < 0.0001$; **$p < 0.005$; *$p < 0.05$.

showed a DMF-induced down-regulation of proinflammatory Th1 and Th17 cells and in particular Th17.1 cells. Th17.1 cells have recently been shown to be associated with MS disease activity likely due to their cytokine profile producing IFN-γ, IL-17 and GM-CSF and high expression level of VLA-4 which enhances the potential of CNS infiltration (van Langelaar et al., 2018). We further elucidated whether these inhibitory properties of DMF were exclusively directed towards the proinflammatory T cell subsets or whether their regulatory counterpart T_{reg} cells were similarly affected. This analysis showed no effect of DMF on the T_{reg} population in patients treated for more than 12 months, suggesting a skewing in the ratio between proinflammatory and regulatory T cells. In line with this we found that both the Th1:T_{reg}, Th17:T_{reg} and Th17.1:T_{reg} ratios in DMF-treated patients were decreased indicating an improved capacity to control proinflammatory T cells. This shift in the balance between T helper and T_{reg} cells was supported by recent studies by Wu et al. (2017) and Longbrake et al. (2016). Furthermore, Kornberg et al. (2018) described a DMF-induced favoring of differentiation of T_{reg} cells. In coherence, a study by Diebold et al. found an increased frequency of T_{reg} cells after three months of treatment (Diebold et al., 2018). This may indicate an even stronger effect of DMF on the T helper cell:T_{reg} ratio after a shorter period of treatment (Longbrake et al., 2016, 2018; Wu et al., 2017).

A central step in the pathogenesis of MS is migration of peripheral T cells to the CNS (Dendrou et al., 2015). Analyzing the CNS migration potential of blood T cells from DMF-treated patients with RRMS greatly support and expand previous findings (Breuer et al., 2017; Lim et al., 2016; Mathias et al., 2017) showing a lower frequency of CD4+ T cells expressing the chemokine receptors CXCR3, CCR6, CCR2, CCR5 and the adhesion molecule MCAM-1 and CD49d^{hi} all assumed to be mediators of CNS migration (Barrau et al., 2000; Sporici and Issekutz, 2010; Schneider-Hohendorf et al., 2014; Reboldi et al., 2009; Larochelle et al., 2012; Kara et al., 2015; Gu et al., 2016; Fife et al., 2000). Intriguingly, the expression of CXCR3, CCR2 and MCAM-1 was not affected by DMF treatment in CD8+ T cells. T cells co-expressing CCR2 and CCR5 has been associated with MS pathology and are further found enriched in the CSF of patients with MS (Mexhitaj et al., 2019; Sato et al., 2012). Interestingly, we found a significant decrease in frequencies of peripheral CD4+ and CD8+ T cells co-expressing CCR2 and CCR5 in DMF treated patients (data not shown) emphasizing the likelihood that DMF therapy inhibits the entry of T cells into CNS. Validating these *ex vivo* observations with *in vitro* experiments confirmed that CXCR3 and MCAM-1 expression was mainly affected by fumarates in CD4+ T cells. CXCR3 is a major receptor used to attract peripheral T cells to the CNS (Sporici and Issekutz, 2010) and MCAM-1 is important for adhesion of T cells to the endothelial cells of the blood-brain-barrier (Larochelle et al., 2015). Our data therefore suggest that DMF-treatment reduces the CNS migration potential of CD4+ T cells whereas the potential of CD8+ T cells is less compromised. This suggestion was strengthened by our observation of a significantly reduced frequency of CD4+ T cells in the CSF of DMF-treated patients whereas no change in the frequency of CD8+ T cells was observed.

One major effector mechanism of T cells is production of cytokines. Assessing the T cell cytokine profile *in vitro* revealed an MMF-induced reduction in the proinflammatory cytokines TNF-α, IL-17 and IFN-γ; an effect significantly pronounced in CD4+ T cells. In addition, we found an increase in IL-13 even at the lowest concentration of MMF applied. IL-13 is a cytokine associated with protection against demyelination and loss of the myelinating oligodendrocytes (Guglielmetti et al., 2016). Our data therefore indicate that MMF induces a skewing of the proinflammatory cytokine profile towards a more anti-inflammatory profile. This observation is in line with previous studies showing that DMF treatment reduces the frequency of CD4+ T cells producing IFN-γ, TNF-α and IL-17 (Wu et al., 2017; Longbrake et al., 2018; Galli et al., 2019). We did, however, also find a paradoxical increase in the production of GM-CSF, which is a proinflammatory cytokine and crucially involved in inflammatory demyelination (Codarri et al., 2011; Hartmann et al.,

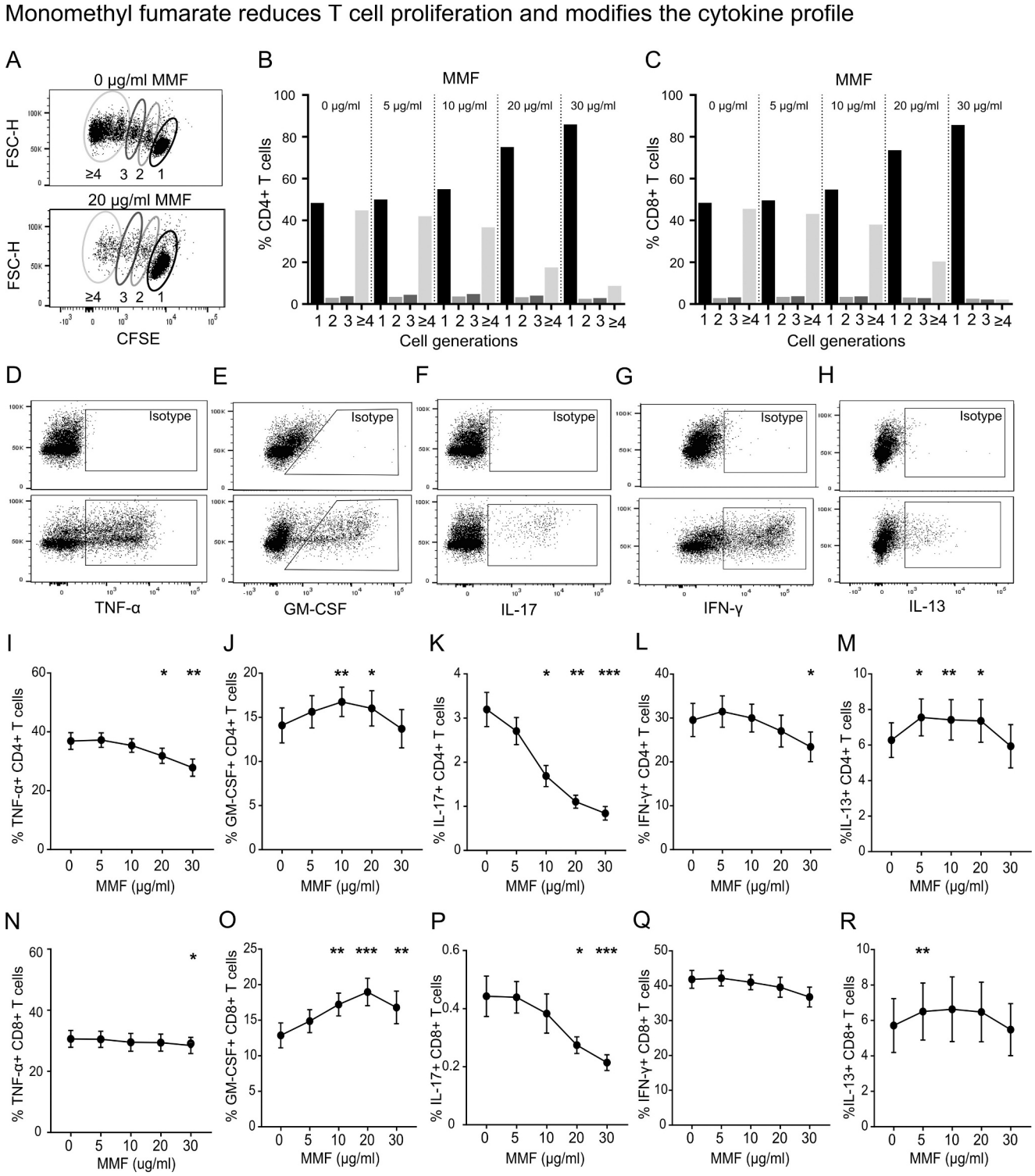

Fig. 5. Monomethyl fumarate reduces T cell proliferation and modifies the cytokine profile.
(A) Flow cytometry dot plot example of identification of proliferating CD8+ T cells from healthy donors stained with CFSE and cultured with anti-CD28/CD3 stimulatory Dynabeads and various concentrations of MMF for 4 days; the numbers 1–4 refer to the number of cell generations. Upper panel shows CD8+ T cells cultured with 0 µg/ml MMF and lower panel CD8+ T cells cultured with 20 µg/ml MMF. (B–C) Bar charts showing frequencies of the cell generation numbers of CD4+ (B) and CD8+ (C) T cells at each MMF concentration. (D–H) Flow cytometry dot plot examples of gating TNF-α+ (D), GM-CSF+ (E), IL-17+ (F), IFN-γ+ (G), and IL-13+ (H) T cells from healthy donors cultured with stimulatory beads and various concentrations of MMF for 4 days followed by a 4 h re-stimulation with PMA/ionomycin; the upper panel showing the isotype control staining and the lower panel staining with the primary antibody. (I–R) Frequencies of TNF-α+, GM-CSF+, IL-17+, IFN-γ+, and IL-13+ CD4+ T cells (I–M) and CD8+ T cells (N–R). ***$p < 0.0001$; **$p < 0.005$; *$p < 0.05$.

2014). If this effect is also observed *in vivo*, it might counteract the beneficial effects of DMF therapy.

A recent study reported anti-proliferative properties of DMF treatment (Diebold et al., 2018). In line with this we found a dose-dependent inhibition of T cell proliferation. This observation could possibly explain the decrease in memory T cells observed in this and

many other studies. It may also explain the DMF-induced lymphopenia reported by several studies since T cell proliferation is essential for T cell maintenance.

In conclusion, this study demonstrates that DMF treatment targets the differentiation of naïve T cells to effector T cells, modifies the cytokine profile of CD4+ T cells, and suppresses the CNS migration potential of CD4+ T cells. It also shows that CD8+ T cells are less affected by the treatment. CD8+ T cells play an important role in the pathogenesis of MS (Babbe et al., 2000), and our data therefore propose that the lack of CD8+ T cell control may explain residual disease activity in patients treated with DMF.

Declaration of Competing Interest

None.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.msard.2019.101451.

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