RESEARCH ARTICLE

The NRF2 pathway as potential biomarker for dimethyl fumarate treatment in multiple sclerosis

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Abstract

Objective: Immunological studies have demonstrated a plethora of beneficial effects of dimethyl fumarate (DMF) on various cell types. However, the cellular and molecular targets are incompletely understood and response markers are scarce. Here, we focus on the relation between nuclear factor (erythroid-derived 2)-like 2 (NRF2) pathway induction under DMF therapy and the composition of the blood immune cell compartment and clinical efficacy in relapsing-remitting multiple sclerosis (MS) patients. **Methods**: We explored effects of DMF on peripheral immune cell subsets by flow cytometric and transcriptional analysis of serial blood samples obtained from 43 MS patients during the first year of therapy. **Results**: Gene expression analysis proved activation of NRF2 signaling under DMF therapy that was paralleled by a temporal expansion of FoxP3$^+$ regulatory T cells, CD56^{bright} natural killer cells, plasmacytoid dendritic cells, and a decrease in CD8$^+$ T cells, B cells, and type 1 myeloid dendritic cells. In a subgroup of 28 patients with completely available clinical data, individuals with higher levels of the NRF2 target gene NAD(P)H quinone dehydrogenase 1 (NQO1) 4–6 weeks after DMF therapy initiation were more likely to achieve no evidence of disease activity status 1 year later. The degree of NQO1 induction further correlated with patient age. **Interpretation**: We demonstrate that positive effects of DMF on the clinical outcome are paralleled by induction of the antioxidant NRF2 transcriptional pathway and a shift toward regulatory immune cell subsets in the periphery. Our data identify a role of the NRF2 pathway as potential biomarker for DMF treatment in MS.

Introduction

Multiple sclerosis (MS) is the most frequent chronic neuroinflammatory disease of the central nervous system (CNS) that leads to multifocal demyelinated lesions in the brain and spinal cord.[1,2] Studies using the mouse model of MS, experimental autoimmune encephalomyelitis (EAE), suggest a correlation between disease development/progression and activation of T helper (Th) 1, Th17 cells, and CNS infiltrating CD8$^+$ T cells, B lymphocytes, natural killer (NK) cells, and myeloid cells which participate in inflammation and tissue damage.[3] A major therapeutic goal in treating MS is to re-establish tolerance toward the CNS, which may be achieved not only through directly inhibiting the development of autoreactive Th1 and Th17 cells but also by skewing

immune system responses toward Th2 or Treg functions via modulating antigen-presenting cells (APC).

Dimethyl fumarate (DMF) is an oral formulation of fumaric acid esters that has been approved for the treatment of relapsing-remitting MS based due to its highly significant effects on relapses, disability progression, and CNS lesions.[4–7]

We and others have shown that clinical effects of DMF involve immunomodulatory and direct antioxidant actions on the CNS as well as neuroprotection.[8,9] DMF reduces cytokine production,[10] downregulates the migratory activity of immune cells at the blood–brain barrier[11] and activates the nuclear factor (erythroid-derived 2)-like 2 (NRF2) transcriptional pathway.[8,12] The antioxidant transcription factor NRF2 was described as critical regulator responsible for maintenance of redox homeostasis and prevention of oxidative stress by modulating the expression of cytoprotective genes, such as NAD(P)H quinone dehydrogenase 1 (NQO1), Aldo-keto reductase family 1 member C1 (AKR1C1), and glutathione.[13,14] However, other studies demonstrated that DMF treatment also promoted immune modulation and provided clinical benefits in Nrf2 knockout mice suggesting an anti-inflammatory activity of DMF through alternative pathways, independent of Nrf2.[15] For example, DMF is a potent agonist of the hydroxycarboxylic acid receptor 2 (HCAR2) which is also essential for DMF efficacy in the animal model.[16,17]

Although immunological studies show that DMF has a plethora of beneficial effects on various cell types in the CNS and periphery, the cellular and molecular target structures are not fully understood. In this study, we focus on the relation of NRF2 pathway induction under DMF therapy to the composition of the blood immune cell compartment and clinical efficacy in MS patients.

Material and Methods

Patients and sample collection

The study was approved by the Ethics Committee of the Friedrich-Alexander University Erlangen-Nürnberg (no. 81_15B). Written informed consent was obtained from all participants in accordance with the Declaration of Helsinki. To allow inclusion in this study, patients were required to be >18 years and diagnosed with relapsing-remitting MS according to the revised McDonald criteria.[18] Patients were excluded in case of immunization <3 months or clinical evidence of ongoing bacterial infection. The majority of patients were treatment-naïve to disease-modifying drugs (60.5%) or switching from an ongoing interferon-beta or glatiramer acetate therapy; 44.2% percent of participants had been newly diagnosed within the year preceding initiation of Tecfidera® therapy. The

Table 1. Baseline demographic and disease characteristics of the study cohort.

Characteristic	Patient cohort (*n*=43)
Age, mean (SD)	36.3 (11.7)
Female, *n* (%)	31 (72.1)
Newly diagnosed within previous year, *n* (%)	19 (44.2%)
Patients with prior treatment, *n* (%)	17 (39.5%)
Interferon *β*, *n* (%)	11 (25.6%)
Glatiramer acetate, *n* (%)	6 (13.9%)
Patients with relapses in prior year, *n* (%)	30 (69.8%)
EDSS score, median (IQR)	1.5 (1.0)

SD, standard deviation; IQR, interquartile range; EDSS, Expanded Disability Status Scale.

median age was 34a (20–63a) with a female to male ratio of 2.58 (see also Table 1). At each visit, number of relapses, clinical course, DMF side effects, concomitant medication, and Expanded Disability Status Scale (EDSS) were recorded. Furthermore, MRI data were collected.

Peripheral blood was obtained by venipuncture using EDTA prefilled tubes for PBMC analysis or PAXgene blood RNA tubes (Qiagen, Venlo, Netherlands) before initiation of therapy (T0), after 4–6 weeks (T1), 3 months (T2), 6 months (T3), and 12 months (T4) of Tecfidera® treatment. In addition, age and sex-matched healthy donors (HD) were recruited for blood donation.

Isolation and phenotyping of PBMC

PBMC were isolated by density gradient centrifugation (Lymphoprep; Axis Shield, Oslo, Norway). 1×10^6 PBMC were incubated with fixable viability dye eFluor®780 (eBioscience), FcR block (Miltenyi), and the respective fluorochrome-conjugated antibodies: αCD3-eFluor450 (UCHT1, eBioscience), αCD4-eFluor450 (SK3, Biolegend), αCD8-APC (SK1, BD Biosciences), αCD19-PE/Cy7 (HIB19, Biolegend), αCD56-PE (HCD56, Biolegend), αFoxp3-PE (206D, Biolegend), αIFNγ-APC (B27, BD Biosciences), αIL-4-FITC (MP4-25D2, Biolegend), αIL-17A-PE (eBio64DEC17, eBioscience), and analyzed on a BD FACSCantoII using FACSDiva software. For intracellular cytokine staining, cells were prestimulated for 3h with ionomycin (1 *μ*mol/L, Sigma-Aldrich) and PMA (50 ng/mL, Sigma-Aldrich) in the presence of monensin (2 *μ*mol/L, eBioscience), fixed and made permeable with the Fixation/Permeabilization buffer set (eBioscience) according to the manufacturer's protocol.

Peripheral blood DC were analyzed for the expression of CD1c (BDCA-1), CD303 (BDCA-2), and CD141 (BDCA-3) with the Blood Dendritic Cell Enumeration Kit (Miltenyi) in a subset of patients (frozen samples) according to the manufacturer's instructions.

Real-time PCR

PCR reactions were performed with a qTower real-time PCR System (Analytik Jena, Jena, Germany). Relative quantification was performed by the $\Delta\Delta CT$ method, normalizing target gene expression on *GAPDH* as housekeeping gene. The following TaqMan® real-time PCR assays from Thermo Fisher Scientific were used: KEAP1 Hs00202227_m1, NFE2L2 Hs00975961_g1, NQO1 Hs02512143_s1, AKR1C1 Hs04230636_sH, HMOX1 Hs01110250_m1, HCAR2 Hs02341584_s1, GAPDH Hs02758991_g1.

Statistical analysis

Statistical testing was performed using GraphPad Prism (GraphPad Software Inc, San Diego, California). All data were analyzed by either unpaired t-test, one-way ANOVA followed by Dunnett's posttest, or linear regression analysis after checking for normal distribution (unless otherwise indicated). Statistical significance was tested between T0 and the different time points after DMF treatment. HD were not included in the statistical analysis as indicated by dotted lines. Data are presented as mean ± SEM; *$P < 0.05$, **$P < 0.01$, or ***$P < 0.001$ were considered to be statistically significant.

Results

DMF therapy induces the NRF2 transcriptional pathway in PBMC

Gene expression analyses from whole blood samples of DMF-treated patients showed a transient increase in KEAP1 (Kelch-like ECH-associated protein 1) and NRF2 (*NFE2L2*) mRNA as well as elevated levels of the NRF2 target genes *NQO1* and *AKR1C1* (Fig. 1A–D) but not *HO-1* (Fig. S1A) that was most pronounced 4–6 weeks after initiation of therapy. In contrast, the expression level of HCAR2, a niacin receptor involved in the regulation of inflammation and oxidative stress independently from NRF2 signaling, was not altered during the course of treatment (Fig. 1E).

DMF increases the frequencies of regulatory immune cell subsets in MS patients

Circulating FoxP3^{+} Tregs transiently increased following DMF treatment initiation with peak levels 3 months after therapy induction (T2) (Fig. 2A). FoxP3^{+} Treg frequencies returned to baseline after 1 year of therapy (T4) (Fig. 2A), while the proportion of regulatory CD56^{bright} NK cells was observed to gradually rise during the observation period (Fig. 2B). Similar observations were made

for plasmacytoid dendritic cells (pDC), whereas type 1 myeloid dendritic cells (mDC) but not type 2 mDC were significantly decreased during the first 3 months (Fig. 2C–E). The frequency of total CD4^{+} T cells started to increase after 6 months of therapy (Fig. 2F), whereas the percentages of Th1, Th2, and Th17 differentiated cells as well as CD56^{dim} NK cells remained stable in our longitudinal analysis (Fig. 2G–J). In line with other publications, DMF therapy was associated with reduced CD8^{+} T cell and B cell counts among total PBMC (Fig. 2K and L).[19–21]

In accordance with cell frequencies, cell counts of mDC1, CD8^{+} T cells, and B cells were significantly reduced during DMF therapy (Fig. S2). CD56^{bright} NK cells were slightly increased after 1 year of treatment, whereas pDC and Treg cell counts remained stable at least until 6 months (Fig. S2). Therefore, the observed increase in Treg, pDC, and CD56^{bright} NK cell frequencies may rather result from a relative reduction in other cell counts.

Immune effects and clinical outcome of DMF therapy correlate with NRF2 transcriptional pathway induction

DMF treatment significantly reduced the absolute lymphocyte count in 21 of 24 patients by $0.59 \pm 0.49 \times 10^3/\mu L$ (Table 2). Still, the mean absolute lymphocyte number remained within the normal range ($1.2 \pm 0.5 \times 10^3/\mu L$). During the first year of therapy, 22 of 28 (78.6%) patients showed no new MRI lesions, 19/28 (67.9%) patients had no relapses, and the EDSS remained stable in 16 out of 28 (57.1%) patients. In the end, 9 of 28 (32.1%) patients gained no evidence of disease activity (NEDA) status (Table 2).

Since the transcription factor NRF2 is mainly regulated via its binding to KEAP1, which is followed by proteasomal degradation,[22] and not by alterations on the mRNA level, we used the NRF2 target gene NQO1 to further analyze the NRF2 transcriptional pathway as potential early biomarker for DMF treatment effectiveness. Patients who achieved NEDA after 1 year of therapy showed significantly higher NQO1 expression levels after 4–6 weeks and a trend toward higher Treg frequencies during the first 3 months of the study (Fig. 3A and B). Furthermore, the observed increase in Treg frequencies at time point T2 significantly correlated with the degree of NQO1 induction (Fig. 3C).

Interestingly, also the age of the patient at the time of treatment initiation showed a trend to influence the chance to achieve NEDA (Fig. 3D). Moreover, patient age negatively correlated with the degree of NQO1 and Treg induction following DMF treatment (Fig. 3E and F). In
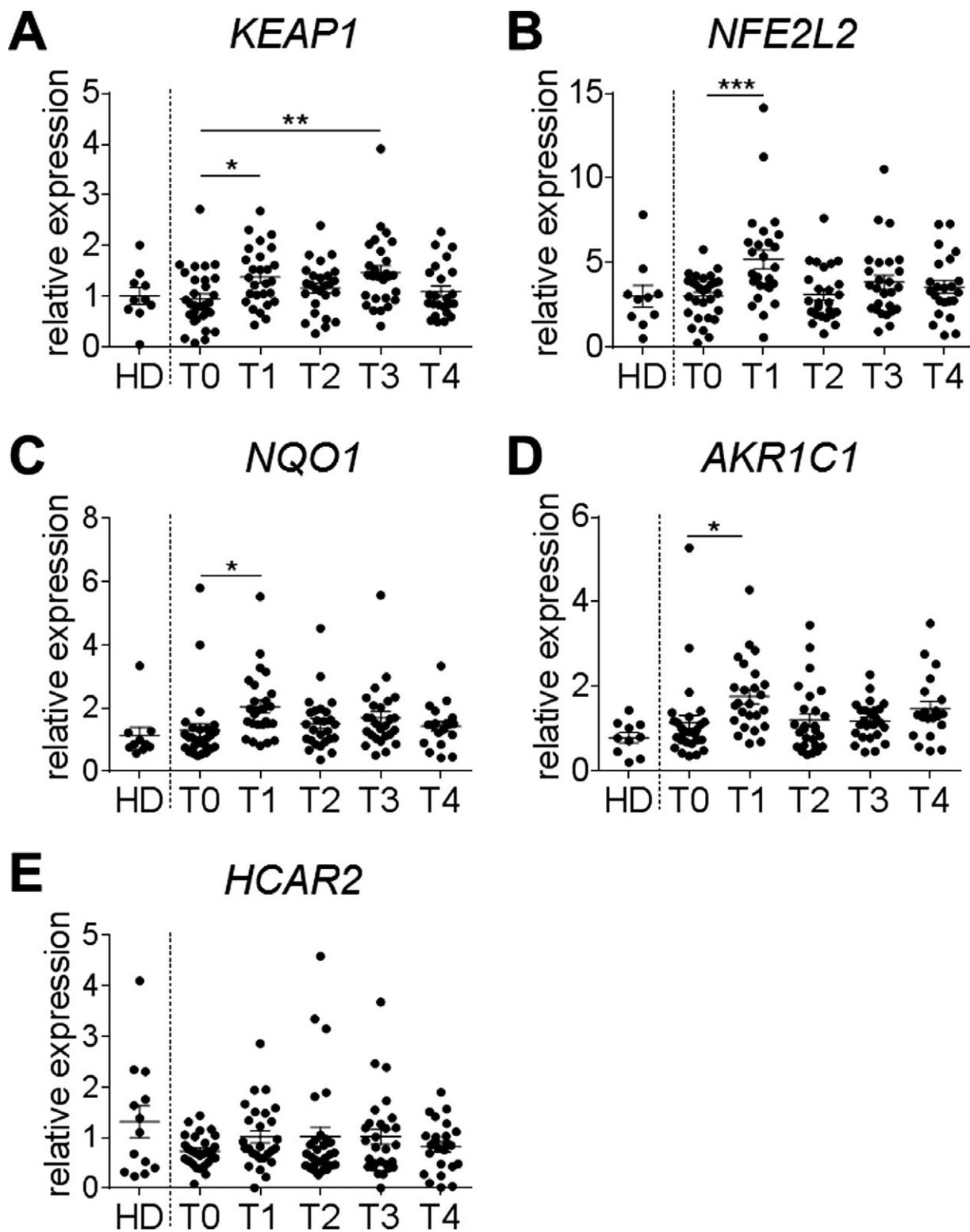
Figure 1. DMF induces the NRF2 transcriptional pathway but not HCAR2 expression in PBMC. mRNA expression levels of *KEAP1* (A), *NFE2L2* (NRF2), (B) and its target genes *NQO1* (C) and *AKR1C1* (D) as well as *HCAR2* (E) were analyzed by qRT-PCR (9–11 HD and 25–30 DMF-treated MS patients per time point, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). HD, healthy donor; baseline (T0), 4–6 weeks (T1), 3 months (T2), 6 months (T3), and 1 year (T4) of DMF intake.
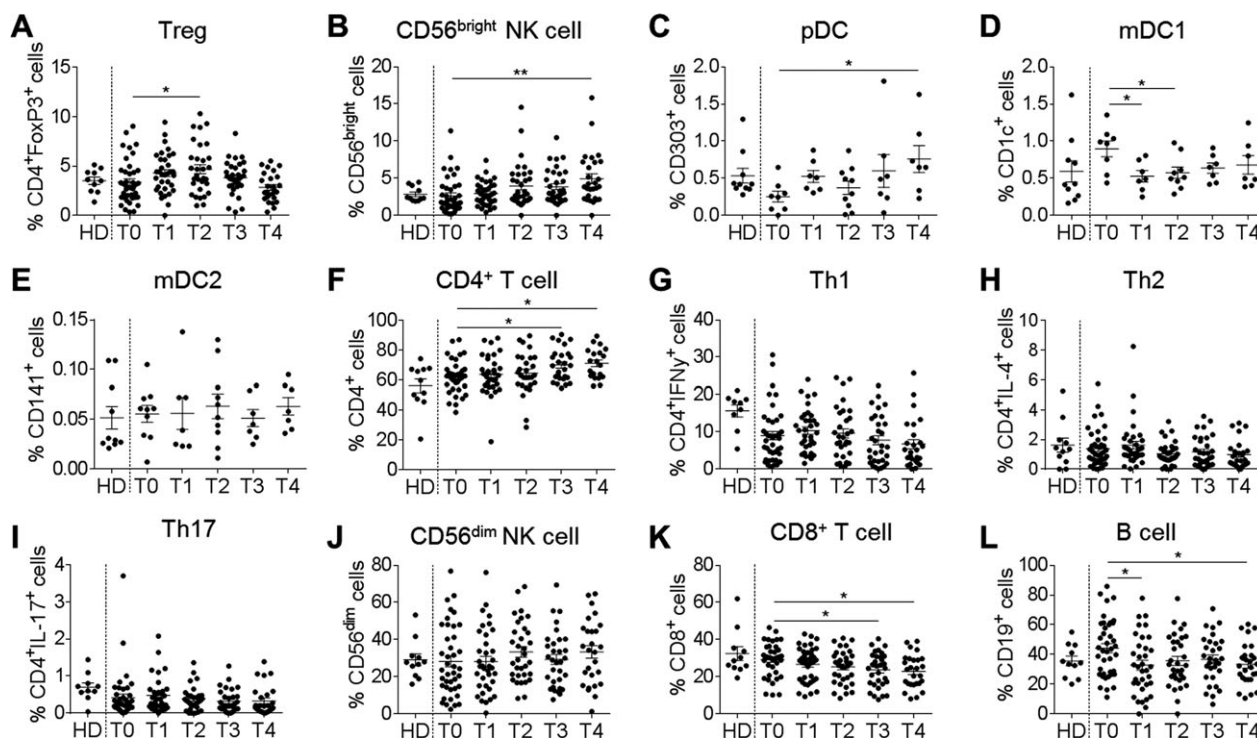
Figure 2. DMF therapy modulates the frequency of immune cell subsets in the blood of MS patients. The frequency of Treg (A), $CD56^{bright}$ NK cells (B), pDC (C), mDC1 (D), mDC2 (E), $CD4^+$ cells (F), Th1 (G), Th2 (H), Th17 (I), $CD56^{dim}$ NK cells (J), $CD8^+$ T cells (K), and B cells (L) in the peripheral blood of HD and DMF-treated MS patients as assessed by flow cytometry (9–11 HD and 10–30 DMF-treated MS patients per time point; $*P < 0.05$, $**P < 0.01$). HD, healthy donor; baseline (T0), 4–6 weeks (T1), 3 months (T2), 6 months (T3), and 1 year (T4) of DMF intake.

Table 2. Evaluation of clinical outcome after 1 year of DMF therapy.

Clinical outcome after 1 year	Patient cohort ($n = 28$)
Absolute lymphocyte count	
T0, mean (SD)	$1.9\ (0.7) \times 10^3/\mu L$
T4, mean (SD)	$1.2\ (0.5) \times 10^3/\mu L$
Patients with new lesions in MRI, n (%)	6 (21.4%)
EDSS score	
T0, median (IQR)	1.5 (1.0)
T4, median (IQR)	1.5 (1.5)
Patients with relapses, n (%)	9 (32.1%)
Patients with NEDA, n (%)	9 (32.1%)
Clinically stable (self-evaluation patient), n (%)	14 (50.0%)

SD, standard deviation; IQR, interquartile range; MRI, magnetic resonance imaging; NEDA, No evidence of disease activity; EDSS, Expanded Disability Status Scale; T0, baseline; T4, 1 year after treatment onset.
Only patients who participated until the end of the study (T4) were included (28/43).

contrast, NQO1 levels did not correlate with EDSS score (Fig. S3), lymphocyte counts, treatment discontinuation, or side effects (data not shown). Levels of AKR1C1 and HO-1 expression shortly after treatment onset did not correlate with NEDA status or Treg frequency, however, AKR1C1 induction showed a trend to be reduced with increasing patient age (Fig. S1 and S4).

Discussion

Here, we show that DMF activated the NRF2 transcriptional pathway inducing an anti-inflammatory shift in circulating immune cell subsets in MS patients. NQO1 induction 4–6 weeks after treatment initiation correlated with Treg frequencies in PBMC after 3 months as well as NEDA status 1 year later.

In our cohort, DMF enhanced the frequency of Tregs in peripheral blood during the first 3 months of treatment. Although there are no numerical deficits in Treg cells, they have repeatedly been reported to be dysfunctional in MS.[23,24] An increase in Treg numbers may therefore help to compensate for a reduced functionality and contribute to early clinical effects of DMF. Together with Treg cells, pDC are considered to be safeguards of peripheral T-cell tolerance and display an altered maturation and regulatory function in MS.[25,26] Our findings suggest that DMF favors a late expansion of pDC which may promote a tolerogenic microenvironment. Indeed, pDC
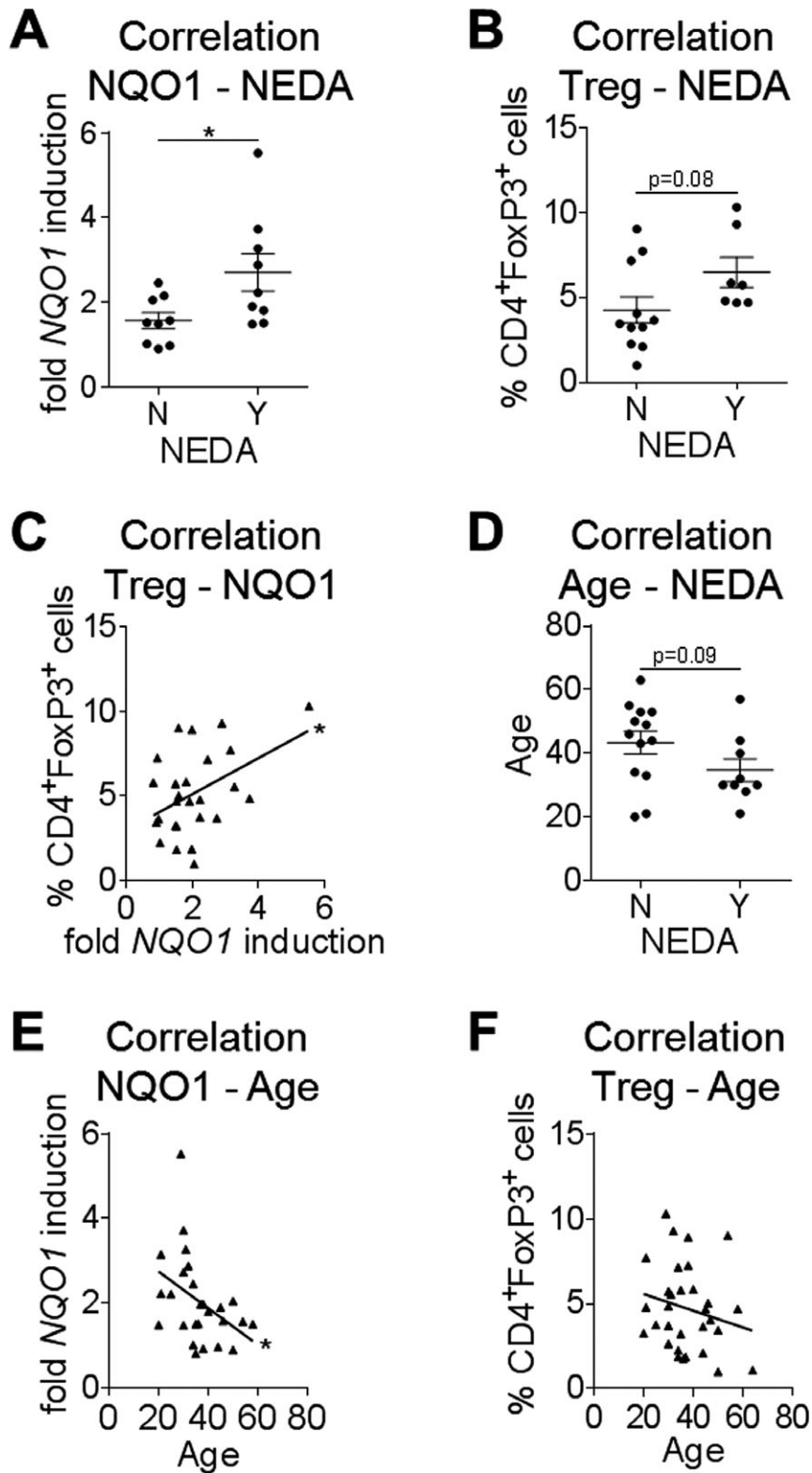
Figure 3. Age-dependent NQO1 induction correlates with Treg frequencies and NEDA status after 1 year of DMF therapy. Analysis of relations between NEDA status (T4), NQO1 induction (T1), Treg frequency (T2), and patient age (T0) (18–25 DMF-treated MS patients were included in the 1 year analysis; *$P < 0.05$). N = no, Y = yes; baseline (T0), 4–6 weeks (T1), 3 months (T2), 6 months (T3), and 1 year (T4) of DMF intake.

have been shown to suppress mDC-dependent induction/expansion of CNS Th1 and Th17 cells and rather promote Treg activation.[27] Furthermore, mDC from DMF-treated MS patients exhibit a rather immature phenotype with reduced expression of costimulatory molecules and impaired T-cell activation capacity.[28] Lower numbers of circulating mDC, CD8^{+} cytotoxic T cells and B lymphocytes but enhanced CD56^{bright} NK cell frequencies in the blood of DMF-treated MS patients further support the concept of an anti-inflammatory shift in the peripheral immune cell compartment probably mediated by NRF2 activation.[19] Close associations between a reduction in B lymphocytes, respectively, an increase in regulatory CD56^{bright} NK cells and a good response to treatment were previously described for other immunomodulatory drugs.[29,30] Therefore, our and previous data suggest that activation of the NRF2 transcriptional pathway and respective regulation of specific cell types is key to achieve a complete response to DMF in MS.

In good accordance with previously published data, DMF treatment activates the NRF2 pathway and induces NQO1 expression in the blood of our MS patient cohort.[31] However, in our study, the gene expression already peaked after 4–6 weeks and was not significantly different from baseline values after 3 months of DMF therapy. This discrepancy might be caused by differences in technical approaches, the patient cohort, compliance, or study design and underlines the overall complexity of defining the mechanism of action of this therapy. Additionally, we demonstrated a correlation between the induction of NQO1 expression upon DMF treatment initiation and enhanced Treg frequencies as well as clinical outcome. The fact that AKR1C1 and HO-1 induction did not show any correlation with Treg counts or NEDA status may indicate a diverging or cell type-specific role for different NRF2 target genes in MS pathogenesis.

Interestingly, the chance to achieve NEDA status after 1 year of therapy was lower in older MS patients. Reduced NQO1 induction following DMF treatment with increasing age may explain this observation, since we showed that activation of the NRF2 transcriptional pathway during the first weeks of therapy is crucial for later Treg induction and clinical outcome. Therefore, a comparison between peripheral NQO1 levels before treatment onset and after 1–2 months may represent an early biomarker for clinical efficacy and response to DMF therapy of the individual patient.

Due to its role in managing oxidative stress responses, NRF2 is involved in several other autoimmune diseases besides MS, including psoriasis, asthma, and diabetes, as well as in neurodegeneration and different forms of cancer (as reviewed in 32–34). Our data further add to a growing body of literature on how the NRF2 transcriptional pathway contributes to balance T-cell and APC polarization during human autoimmunity in the periphery.[8,35,36] Yet, some recently published data employing Nrf2 knockout mice suggest that the anti-inflammatory activity of DMF in treatment of MS patients may additionally occur through alternative pathways, independent of NRF2.[15]

Furthermore, we observed no effect of DMF intake on the expression of the niacin receptor HCAR2. Although HCAR2 signaling seems to be important for the efficacy of DMF treatment in neuroinflammation,[16] transcriptional regulation of HCAR2 may not be relevant for DMF therapy in MS. Additionally, Tang and colleagues already showed that the expression of HCAR2 is rather low in the peripheral immune system and that it may play a role in the central nervous system instead.[17]

In conclusion, we demonstrated that DMF effects in relapsing-remitting MS are paralleled by induction of the antioxidant NRF2 transcriptional pathway and a shift toward regulatory immune cell subsets in the periphery. Investigating the role of NRF2 and its target genes on efficacy as well as side effects of DMF therapy in larger cohorts may further advance our understanding of its mechanism of action and contribute to a safer treatment of MS but also other autoimmune and neurodegenerative diseases.

Acknowledgments

Conflict of Interest

None declared.

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Supporting Information

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

Figure S1. DMF therapy does not alter HO-1 expression in PBMC.

Figure S2. DMF therapy modulates the absolute number of immune cells in the blood of MS patients.

Figure S3. Induction of the NRF2 target genes does not correlate with disability progression (EDSS) after 1 year of DMF therapy.

Figure S4. AKR1C1 induction does not correlate with Treg frequencies and NEDA status after 1 year of DMF therapy.