BRAIN
ORIGINAL ARTICLE

Dimethyl fumarate treatment restrains the antioxidative capacity of T cells to control autoimmunity

Marie Liebmann,[1] Lisanne Korn,[1] Claudia Janoschka,[1] Stefanie Albrecht,[2] Sarah Lauks,[1] Alexander M. Herrmann,[3] Andreas Schulte-Mecklenbeck,[1] Nicholas Schwab,[1] Tilman Schneider-Hohendorf,[1] Maria Eveslage,[4] Brigitte Wildemann,[5] Felix Luessi,[6] Stephan Schmidt,[7] Martin Diebold,[8] Stefan Bittner,[6] Catharina C. Gross,[1] Stjepana Kovac,[1] Frauke Zipp,[6] Tobias Derfuss,[8] Tanja Kuhlmann,[2] Simone König,[9] Sven G. Meuth,[3] Heinz Wiendl[1] and Luisa Klotz[1]

See Mousa and Jones (doi:10.1093/brain/awab361) for a scientific commentary on this article.

Dimethyl fumarate, an approved treatment for relapsing-remitting multiple sclerosis, exerts pleiotropic effects on immune cells as well as CNS resident cells.

Here, we show that dimethyl fumarate exerts a profound alteration of the metabolic profile of human $CD4^+$ as well as $CD8^+$ T cells and restricts their antioxidative capacities by decreasing intracellular levels of the reactive oxygen species scavenger glutathione. This causes an increase in mitochondrial reactive oxygen species levels accompanied by an enhanced mitochondrial stress response, ultimately leading to impaired mitochondrial function. Enhanced mitochondrial reactive oxygen species levels not only result in enhanced T-cell apoptosis *in vitro* as well as in dimethyl fumarate-treated patients, but are key for the well-known immunomodulatory effects of dimethyl fumarate both *in vitro* and in an animal model of multiple sclerosis, i.e. experimental autoimmune encephalomyelitis. Indeed, dimethyl fumarate immune-modulatory effects on T cells were completely abrogated by pharmacological interference of mitochondrial reactive oxygen species production.

These data shed new light on dimethyl fumarate as *bona fide* immune-metabolic drug that targets the intracellular stress response in activated T cells, thereby restricting mitochondrial function and energetic capacity, providing novel insight into the role of oxidative stress in modulating cellular immune responses and T cell-mediated autoimmunity.

1 Department of Neurology with Institute of Translational Neurology, University Hospital of Münster, Münster 48149, Germany
2 Institute of Neuropathology, University Hospital Münster, Münster 48149, Germany
3 Department of Neurology, University Hospital Düsseldorf, Düsseldorf 40225, Germany
4 Institute of Biostatistics and Clinical Research, University of Münster, Münster 48149, Germany
5 Molecular Neuroimmunology Group, Department of Neurology, University of Heidelberg, Heidelberg 69120, Germany
6 Department of Neurology, Focus Program Translational Neuroscience (FTN) and Immunotherapy (FZI), Rhine Main Neuroscience Network (rmn2), University Medical Center of the Johannes Gutenberg University Mainz, Mainz 55131, Germany
7 Bonn Neurological Practice, Bonn 53111, Germany

Received March 08, 2021. Revised June 11, 2021. Accepted July 25, 2021

8 Laboratory of Clinical Neuroimmunology, Neurologic Clinic and Policlinic, Departments of Biomedicine and Clinical Research, University Hospital Basel, and University of Basel, Basel 4031, Switzerland
9 Core Unit Proteomics, Interdisciplinary Clinical Research Center, University of Münster, Münster 48149, Germany

Correspondence to: Luisa Klotz, MD
University Hospital Münster, Department of Neurology with Institute of Translational Neurology
Albert-Schweitzer-Campus 1, Building A1; 48149 Münster, Germany
E-mail: luisa.klotz@ukmuenster.de

Keywords: autoimmunity; multiple sclerosis; antioxidative T-cell capacities; dimethyl fumarate; T-cell metabolism

Abbreviations: DMF = dimethyl fumarate; EAE = experimental autoimmune encephalitis; MitoQ = mitoquinone mesylate; NAC = *N*-acetylcysteine; Nrf2 = nuclear factor erythroid 2-related factor 2; ROS = reactive oxygen species; RRMS = relapsing-remitting multiple sclerosis; T_M = memory T cell; T_N = naïve T cell

Introduction

Multiple sclerosis is a chronic inflammatory demyelinating disease of the CNS that primarily affects young adults.[1] According to current pathophysiological concepts, lymphocytes play a key role in disease initiation, as autoreactive T cells promote disruption of the blood–brain barrier and facilitate the development of perivascular inflammatory infiltration within the CNS, ultimately resulting in demyelination and axonal damage.[1]

Dimethyl fumarate (DMF) is an established immunomodulatory treatment for relapsing-remitting multiple sclerosis (RRMS), and its efficacy has been demonstrated in two pivotal clinical trials.[2,3] Animal studies and human cohorts have shown that DMF exerts a number of direct effects on lymphocytes, such as the inhibition of lymphocyte proliferation, proinflammatory cytokine production and impaired immune cell migration across brain endothelial cells.[4–7] However, its mode of action has not been fully elucidated yet, and there are currently some inconclusive findings in the literature. Some studies have shown that the effect of DMF on astrocytes is at least partly linked to activation of the transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) via interference with the inhibitory molecule kelch-like ECH-associated protein 1 (KEAP1).[8,9] However, another study demonstrated that the protective effect of DMF in experimental autoimmune encephalitis (EAE), a classical animal model of multiple sclerosis, as well as its effects on lymphocyte activation during EAE, did not depend on the presence of Nrf2.[10] Furthermore, several antioxidative effects of DMF have been characterized in brain resident cells including astrocytes and oligodendrocytes.[8,9,11] Notably, DMF has recently been associated with the induction of oxidative stress in myeloid cells, as well as in T_c17 cells, a small population of $CD8^+$ T cells.[12–16] The latter findings are of particular interest in light of the acknowledged role of intracellular antioxidative pathways, such as the glutathione pathway for shaping T-cell responses during inflammation in different mouse models.[17] However, it is still unclear whether DMF might generally affect the antioxidative capacities of human T cells.

Recently, it has been demonstrated that DMF succinates and inactivates the glycolytic enzyme GAPDH (glyceraldehyde 3-phosphate dehydrogenase) both *in vitro* and *in vivo*, thereby downregulating aerobic glycolysis in myeloid as well as lymphoid cells.[18] This not only sheds light on the pleiotropic effects of DMF on distinct molecular targets that contribute to its effects *in vivo*, but also underlines the relevance of metabolic processes in immune cells in the context of autoimmune diseases. Metabolic processes are essential for lymphocyte activation, expansion, acquisition of effector functions, and they are further important for the generation of memory T cells.[19] Accordingly, in recent years, metabolic disturbances have been observed in several autoimmune diseases, including rheumatoid arthritis, systemic lupus erythematosus and multiple sclerosis.[20–22] Although different autoimmune diseases exhibit distinct metabolic signatures, these findings underscore the relevance of immune-metabolic changes in the pathophysiology of autoimmunity and support the concept of targeted intervention of such metabolic disturbances as a novel treatment strategy.

DMF is a derivative of the Krebs cycle intermediate fumarate, and we wondered whether DMF exerts other metabolic effects in T cells in addition to its direct effect on GAPDH. Metabolomic screening of isolated T cells in the presence of DMF revealed distinct changes in many metabolites, including glutathione, an enzyme that protects cells from oxidative damage and maintains redox homeostasis. We observed a profound increase of intracellular and mitochondrial reactive oxygen species (ROS) leading to impaired mitochondrial function. This mitochondrial dysfunction in T cells was associated with a decrease in mitochondrial respiratory activity and subsequent lymphocyte apoptosis. Notably, due to their distinct metabolic profile, memory T cells were particularly susceptible to such oxidative stress and stress-related cell death mechanisms both *in vitro* and in patients with multiple sclerosis who were treated with DMF. Mechanistically, these effects were not mediated via Nrf2 but were directly linked to an enhanced intracellular stress response due to interference with the antioxidative capacities of glutathione and subsequent increase in mitochondrial ROS levels. Notably, rescue of intracellular antioxidative capacities not only prevented mitochondrial dysfunction and apoptosis, but completely abrogated the well-known anti-inflammatory effects of DMF *in vitro* and in an animal model of multiple sclerosis. These findings closely link the DMF-mediated immunomodulatory effects to disturbed oxidative stress responses resulting in mitochondrial dysfunction in lymphocytes.

Materials and methods

DIMAT-MS study design

DIMAT-MS was an exploratory, open-label, multicentre, phase four clinical trial (NCT02461069) that recruited patients with RRMS ($n = 42$) from four sites in Germany. All age and sex-matched healthy donors ($n = 15$) were recruited from one site in Germany. Study subjects were 21–54 years of age and met the McDonald 2010 criteria for RRMS. DMF treatment was initiated by daily

administration orally (Week 0: 120 mg once a day; Week 1: 120 mg twice daily; Week 2: 240 mg in the morning and 120 mg in the evening; Week 3: 240 mg twice daily; Weeks 4 and 24: 240 mg twice daily). Healthy subjects did not receive any treatment during the study. For data analysis, all patients were included that had received at least one dose of DMF ($n = 34$; Supplementary Table 1). A subgroup of the study cohort with sufficient material from patients and matched healthy donors was analysed for T-cell metabolism ($n = 14$; Supplementary Table 2) as well as apoptosis ($n = 16$; Supplementary Table 3). The responsible ethics committee of the Ärztekammer Westfalen-Lippe and the Westfälische Wilhelms-Universität Münster (2016–053-f-S) previously approved the study protocol, and all participants provided written informed consent prior to entry into the study according to the Declaration of Helsinki. Specific inclusion and exclusion criteria details are described in Supplementary Table 4.

Study subjects independent from the clinical trial

An independent small cohort of RRMS patients, who either did not develop lymphopenia during treatment ($n = 8$) or became lymphopenic ($n = 8$) during the course of DMF treatment (Supplementary Table 5), were analysed for T-cell subset composition, T-cell metabolism and apoptosis. These samples were collected at the Multiple Sclerosis Center of the University Hospital of Basel. Blood sampling of patients with clinically definite RRMS according to the McDonald 2010 criteria was approved by the Ethics Committee of Northwest and Central Switzerland (EKNZ 2014–48/12). Moreover, the effect of DMF treatment on the T-cell response *in vitro* was investigated using 30healthy donors (Supplementary Table 6). The local ethics committee (Ärztekammer Westfalen-Lippe, 2016–053-f-S) approved blood sampling of healthy donors for *in vitro* experiments. All subjects (patients and healthy controls) provided signed informed consent. Samples were pseudonymized and their origin was obscured by using coded vials before sample storage and subsequent analysis.

Cell isolation and T-cell phenotyping

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood using Ficoll-Paque (Merck) density-gradient centrifugation and analysed by flow cytometry. Flow cytometric measurement was performed on a Gallios Flow Cytometer or a CytoFLEX (both from Beckman Coulter), and the results were analysed with FlowJo (FlowJo LLC) or Kaluza (Beckman Coulter) software. For a detailed description of the surface markers that were used for the T-cell subsets see the Supplementary material.

Analysis of human immune cell responses *in vitro*

Human $CD4^+$ and $CD8^+$ T cells were isolated from the PBMCs of patients with RRMS and healthy donors by immunomagnetic separation using $CD4^+$ or $CD8^+$-labelled magnetic-activated cell sorting (MACS) microbeads (Miltenyi Biotec), respectively. For the purification of the T-cell subsets [memory (T_M) and naïve (T_N)], $CD4^+$ or $CD8^+$ T cells were enriched using a negative selection technique (Miltenyi Biotec) and subsequently T_M cells ($CD45RO^+$) and T_N cells ($CD45RO^-$) were isolated using $CD45RO^+$-labelled MACS microbeads. All T cells were cultured in X-VIVO™ Media 15 (Lonza). When indicated, 5 μg/ml DMF, 5 μg/ml monomethyl fumarate, 250 nM *N*-acetylcysteine (NAC), 250 μM glutathione (all from Merck) and 100 μM mitoquinone mesylate (MitoQ; MedChem Express) were used. Sulphoraphane (500 nM, Merck) was applied for pharmacological activation of Nrf2.[23] For pharmacological inhibition of Nrf2, 5 μg/ml ML385 (Merck) was used.[24] Buthionine sulphoximine (3 mM, Merck) was utilized as a specific inhibitor of glutamate cysteine ligase, the rate-limiting step in glutathione synthesis.[25] For a detailed description see the Supplementary material.

Metabolomics

Human $CD4^+$ and $CD8^+$ T cells were isolated from PBMCs of healthy donors and subsequently stimulated with α-CD3 and α-CD28 (both 4 μg/ml plate-bound) in the presence and the absence of 5 μg/ml DMF. After 72 h, cells were washed and stored at –80°C until shipping. T cells were frozen and thawed several times to lyse the cells; subsequently, the cells were centrifuged and supernatants were processed for liquid chromatography-mass spectrometry (LC-MS)-based untargeted metabolomics analysis at Creative Proteomics using ultra-high performance LC-quadrupole time-of-flight MS (UHPLC-QTOF-MS, WatersCorp). In addition, $CD4^+$ T-cell samples were measured at the IZKF Core Unit Proteomics using Pall Nanosep® 10K Omega units (3.5 kDa cut-off). Mass spectrometry was performed using SynaptG2Si/M-ClassnanoUPLC (WatersCorp) using PharmaFluidics C18μPAC columns (trapping and 50 cm analytical; PharmaFluidics) in HDMSe mode. For LC-tandem MS, samples were split in two aliquots that were analysed in either positive or negative ionization mode. For a detailed description of the statistical analysis, see the Supplementary material.

Metabolic assays of human T cells

Measurements of the oxygen consumption and extracellular acidification rates were performed with a Seahorse XFp or XFe96 Extracellular Flux Analyzer (Agilent Technologies), as described previously.[26] $CD4^+$ and $CD8^+$ T cells were isolated from fresh blood of healthy donors and stimulated with α-CD3 and α-CD28 (both 4 μg/ml plate-bound) for indicated time points. Activated T cells were cultured in XF Base Medium Minimal Dulbecco's Modified Eagle's Medium (Agilent Technologies) containing 10 mM glucose, 2 mM L-glutamine and 1 mM sodium pyruvate (all Merck). The oxygen consumption and extracellular acidification rates were simultaneously acquired under basal conditions and in response to 1 μM oligomycin, 1 μM carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP), 100 nM rotenone plus 1 μM antimycin A and 50 mM 2-desoxy-glucose (all Merck). When indicated, $CD4^+$ and $CD8^+$ T cells were isolated from frozen PBMCs of healthy donors and patients (Supplementary Tables 1–6). Before the oxygen consumption and extracellular acidification rates were simultaneously assessed using the XF96 Extracellular Flux Analyzer, T cells were either left unstimulated or were short-term stimulated with 50 ng/ml phorbol 12-myristate 13-acetate (PMA, Merck) and 500 ng/ml ionomycin (Cayman Chemical) for 2.5 h. Data analysis was performed with Wave Software (Agilent Technologies).

Transmission electron microscopy

In vitro cultured $CD8^+$ T memory cells were fixed in Karlsson-Schultz fixative, embedded in low gelling agarose (Merck) and processed according to Albrecht *et al.*[27] with the following modifications: cells were centrifuged at 300*g* between steps, cells were infiltrated in Epon (Serva) and ultrathin sections were cut at thickness of 90 nm. After uranyl acetate/lead citrate staining, the micrographs were acquired with a Philips electron microscope 208S equipped with the Morada transmission electron microscopy (TEM) camera.

Experimental autoimmune encephalomyelitis

Active EAE was performed with age and sex-matched C57BL/6 mice at the age of 10 to 12 weeks. EAE was induced as previously

described.[28] In parallel, mice received 100 mg/kg body weight of DMF (Merck) or 200 mg/kg body weight of NAC (Merck), both prepared in 0.5% carboxymethylcellulose, or vehicle only (dimethylsulphoxide in 0.5% carboxymethylcellulose) by daily oral gavage from 3 days before immunization and throughout the whole disease course. Moreover, active EAE was induced in wild-type mice treated with DMF as described and/or intraperitoneal (i.p.) injected with vehicle (PBS) or MitoQ (100 nmol/mouse) twice a week from 3 days before immunization and throughout the whole disease course. For a detailed description see the Supplementary material.

Analysis of murine immune cell responses *ex vivo*

On Day 10 of EAE, $CD4^+$ T cells from draining lymph nodes were isolated by immunomagnetic separation (Miltenyi Biotec) and subsequently stimulated with or without 50 ng/ml PMA (Merck) and 500 ng/ml ionomycin (Cayman Chemical) for 2.5 h prior to the measurement of mitochondrial respiration and glycolysis inside the Extracellular Flux Analyzer (Agilent Technologies). For a detailed description of further *ex vivo* analyses, see the Supplementary material.

Statistical analysis

All results are shown as the mean ± standard error of the mean (SEM) unless otherwise indicated. Sample size was determined based on previous studies of a similar nature. Inferential statistics are intended to be exploratory (hypotheses generating), not confirmatory. GraphPad Prism software (GraphPad Software), SAS® software version 9.4 for Windows (SAS Institute) and R version 3.2.5 were used to perform the statistical analyses. To compare the values obtained from two groups, a two-tailed Student's *t*-test was used for normally distributed data, whereas a two-sided Mann-Whitney test was conducted for non-normally distributed datasets. For multiple comparisons, a one-way ANOVA followed by either Dunnett's *post hoc* test or Tukey's *post hoc* test was performed, as indicated in figure legends. The experiments depicted in Figs 2I, 6A and B were statistically analysed by two-way ANOVA plus the indicated *post hoc* test. Since the DIMAT study was exploratory, no randomization was performed, but investigators were blinded throughout data acquisition and data analysis to the participant demographics. The change from baseline to Weeks 24 and 48 was analysed using a Wilcoxon signed-rank test. **P*-values of 0.001 to 0.05 reflected statistically significant differences; ***P*-values of 0.001 to <0.01 were considered highly significant; and ****P*-values <0.001 reflected extremely significant differences. Nevertheless, all inferential statistics are intended to be exploratory and must be interpreted accordingly.

Data availability

All raw data used for figure generation in this manuscript can be obtained by contacting the corresponding author. There is no restriction in the availability of materials described in the study.

Results

DMF broadly affects the metabolic profile of activated T cells

It has been shown that DMF treatment alters the aerobic glycolysis of myeloid and lymphoid cells.[5,18] Since T-cell activation depends not only on glycolysis but also on other metabolic pathways, including beta-oxidation, mitochondrial respiration and the pentose phosphate pathway, we evaluated the potential metabolic effects of DMF in T-cell receptor-activated $CD8^+$ and $CD4^+$ T cells from healthy donors using an untargeted metabolomics approach. As illustrated by principal component analysis (PCA) and corresponding heat maps (Fig. 1A, B and Supplementary Fig. 1A and B), DMF profoundly affected a variety of metabolic pathways in both T-cell subsets based on filtered mass spectrometric metabolite positive ion signals (ANOVA $P \leqslant 0.05$). Following PCA and hierarchal clustering, we performed a metabolite set enrichment analysis (MSEA) [29] to identify affected pathways. Notably, strong alterations upon DMF treatment were mainly associated with glutathione metabolism and purine and glutamate metabolism in $CD8^+$ T cells (Fig. 1C) and to a lesser extent in $CD4^+$ T cells (Supplementary Fig. 1C), suggesting that DMF is implicated in distinct intracellular metabolic pathways. We were particularly interested in glutathione in light of its function as the main source of cellular antioxidant power, and therefore determined glutathione levels in DMF-treated $CD4^+$ and $CD8^+$ T cells from healthy donors upon stimulation. We confirmed that DMF reduced intracellular glutathione levels as well as the ratio of reduced to oxidized glutathione in both $CD4^+$ and $CD8^+$ T cells (Fig. 1D). This decrease in glutathione was preferentially observed in T_M cell subsets as compared to T_N cell subsets (Fig. 1E). Together, these data identify DMF as an important regulator of a broad range of metabolic processes, which might limit the antioxidative capacity of activated T cells.

DMF alters the mitochondrial stress response in T cells leading to impaired mitochondrial function

We next investigated whether DMF affects cellular ROS levels as a consequence of impaired antioxidative capacity due to decreased glutathione levels (Fig. 2A). Indeed, DMF resulted in enhanced cellular (Fig. 2B) as well as mitochondrial (Fig. 2C) ROS levels 16 h after stimulation compared with those of untreated activated $CD4^+$ and $CD8^+$ T cells from healthy donors. Notably, this elevated ROS production was more pronounced in memory $CD4^+$ and $CD8^+$ T cells compared with their naïve counterparts (Fig. 2B and C). Elevated mitochondrial ROS levels in memory T cells were accompanied by a significant increase in the maximum mitochondrial membrane potential,[30] an indicator of mitochondrial stress (Fig. 2D and Supplementary Fig. 2A). In light of the close and reciprocal interaction between ROS and both mitochondrial stress and mitochondrial function, we next determined the impact of DMF on oxidative respiration in activated $CD4^+$ and $CD8^+$ T_N and T_M cells from healthy donors. *In vitro*, DMF induced a significant reduction in mitochondrial respiration in both T_N and T_M cells 72 h after stimulation; however, the extent of this inhibitory effect was much more pronounced in T_M cells due to their generally increased metabolic activity, which was consistent with another study[19] (Fig. 2E and Supplementary Fig. 2B). Notably, mitochondrial mass correlated with metabolic activity, as DMF-treated $CD4^+$ and $CD8^+$ T cells exhibited decreased MitoTracker™ Green signals after 48 h of stimulation (Fig. 2F). Furthermore, electron micrographs of $CD8^+$ T_M cells cultured for 48 h *in vitro* with DMF displayed mitochondrial alterations, showing blunted cristae and swollen mitochondria (Supplementary Fig. 2C). Given the known association between mitochondrial ROS production and the induction of apoptosis via cytochrome *c* elevation,[31,32] DMF treatment resulted in significantly increased cytochrome *c* levels in stimulated $CD4^+$ and $CD8^+$ T_M cells but not T_N cells (Fig. 2G and Supplementary Fig. 2D). Furthermore, DMF selectively induced apoptosis 72 h after stimulation in activated $CD4^+$ and $CD8^+$ T_M cells but not T_N cells via induction of caspase 9 and 3/7 (Fig. 2H). Time course experiments revealed that DMF-mediated mitochondrial dysfunction in T cells occurred 24 h after stimulation, whereas T-cell apoptosis was observed 48 h after stimulation, indicating that decreased mitochondrial function is not a direct consequence of enhanced T-cell

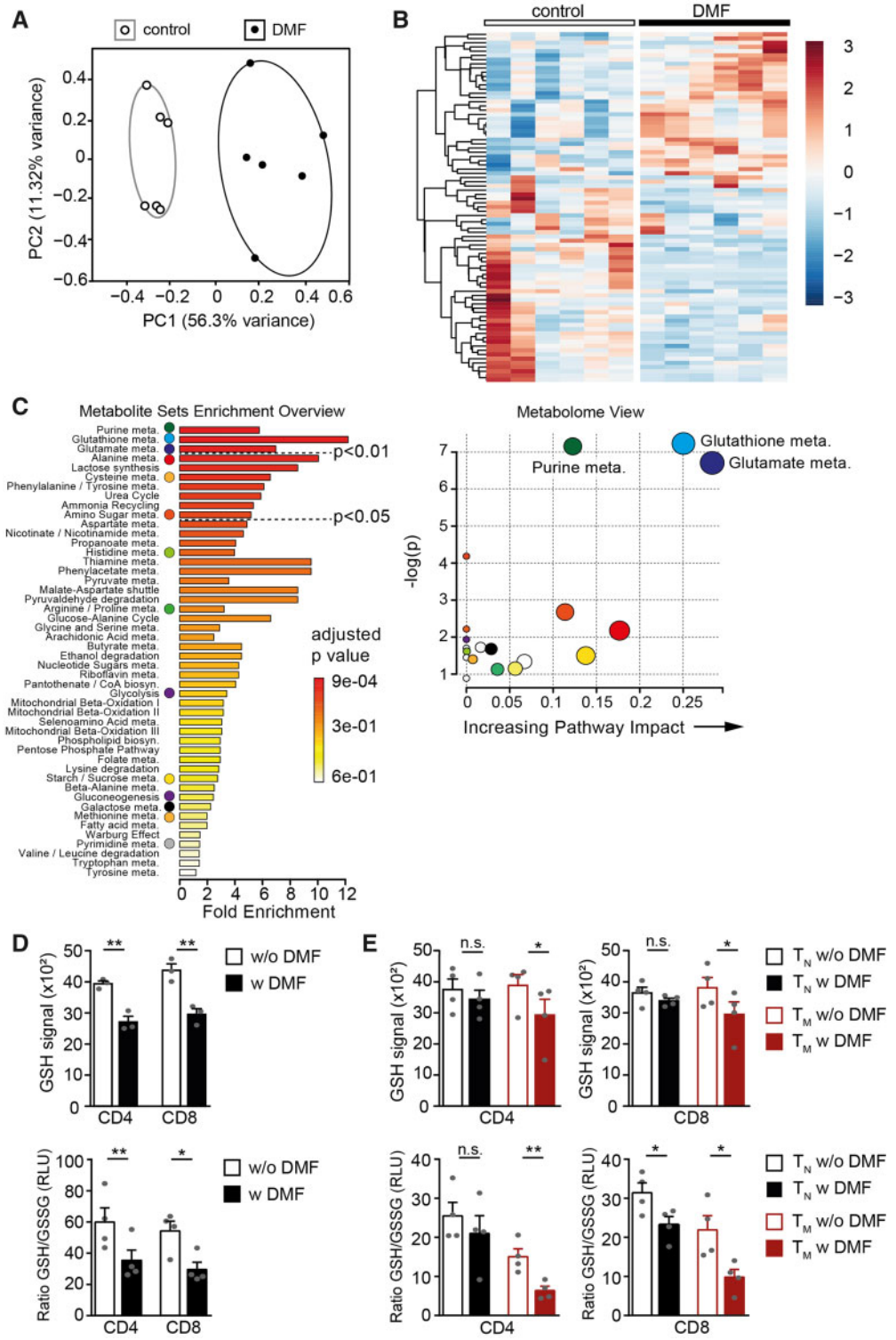

Figure 1 Influence of DMF on the metabolic profile of activated T cells. (**A–C**) Unbiased metabolome analysis was performed with α-CD3/α-CD28-activated human CD8⁺ T cells from healthy donors in the presence or absence of 5 μg/ml DMF for 72 h ($n = 6$ group). (**A**) PCA of mass spectrometric signals of activated T-cell metabolite positive ion profiles in the presence or absence of DMF. (**B**) Heat map depicting the normalized and transformed read counts of mass spectrometric signals that met the filter of ANOVA $P \leqslant 0.05$ of data from **A**. (**C**) Summary plot of MSEA of identified metabolites ranked according to the adjusted *P*-values (*left*). Metabolome view of increasing metabolic pathway impact according to *P*-values (from MSEA) (*right*). (**D**) Intracellular glutathione (GSH) levels of α-CD3/α-CD28-stimulated human CD4⁺ and CD8⁺ T cells from healthy donors in the presence or absence of DMF for 6 h were determined using monochlorobimane ($n = 3$) (*top*). Bar graph displays the ratio of reduced to oxidized glutathione (GSH/GSSG) contents of stimulated T cells in the presence or absence of DMF for 16 h ($n = 3$–4) (*bottom*). (**E**) Memory (T_M) and naïve (T_N) T-cell subsets were isolated from CD4⁺ and CD8⁺ T cells (healthy donors) and stimulated with α-CD3/α-CD28 in the presence or absence of DMF for 6 h (*top*) or 16 h (*bottom*) ($n = 4$). Graphs showing cellular GSH levels (*top*) or GSH/GSSG contents (*bottom*) of depicted subsets. All data are displayed as the mean ± SEM. Statistical analysis was conducted by Student's *t*-test (**D** and **E**). *$P < 0.05$ and **$P < 0.01$.

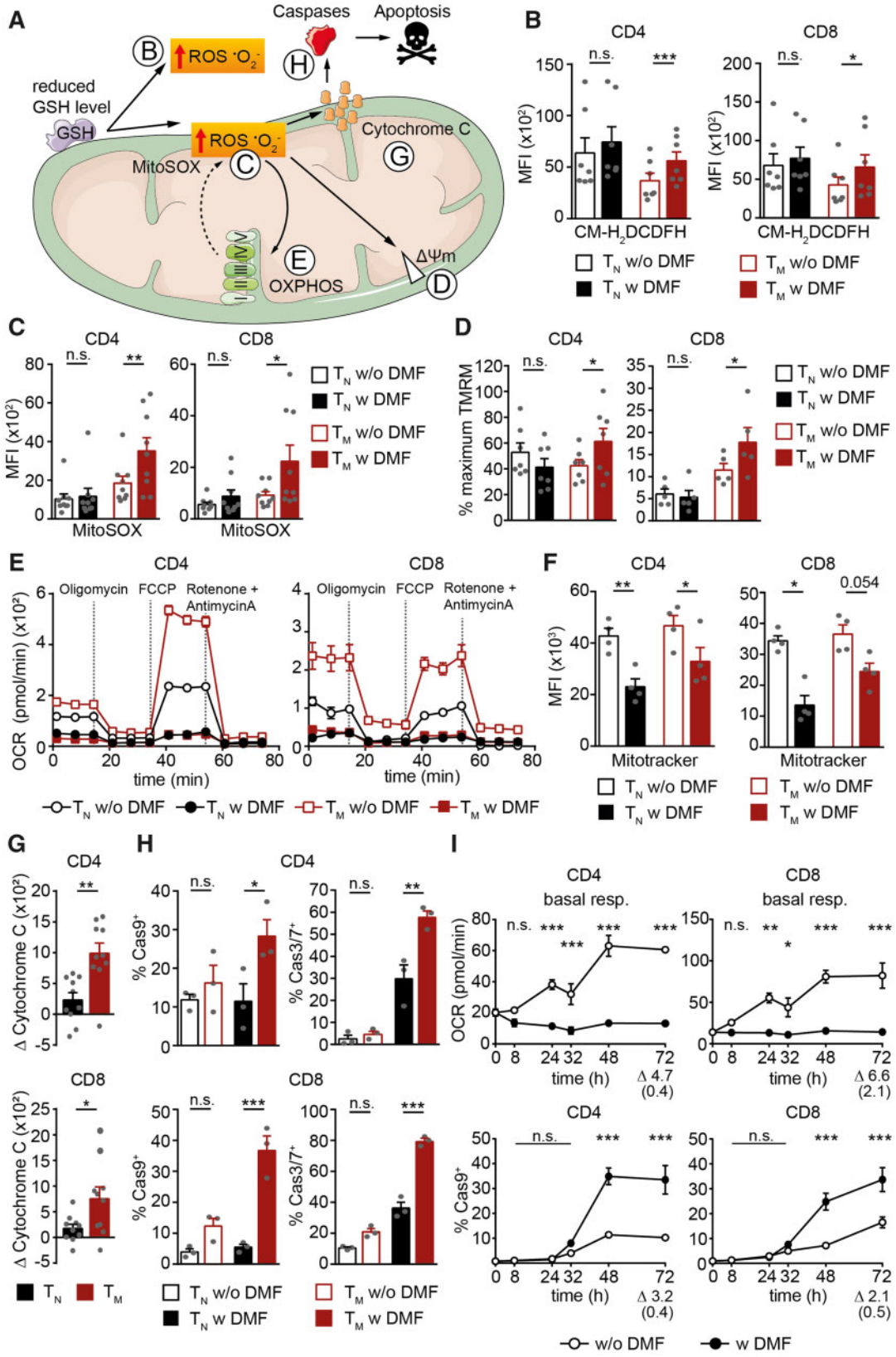

Figure 2 Differential influence of DMF on the cellular stress response, mitochondrial respiration and apoptosis in distinct T-cell subsets. (**A**) Graphical illustration of the impact of glutathione (GSH) depletion on cellular ROS levels, mitochondrial respiration and apoptosis in T cells. (**B–H**) Memory (T_M) and naïve (T_N) subsets were isolated from $CD4^+$ and $CD8^+$ T cells from healthy donors and stimulated with α-CD3/α-CD28 in the presence or absence of DMF. (**B**) Intracellular ROS levels in T cells following 16 h of activation were determined by flow cytometric analysis using CM-H_2DCFDA [chloromethyl-dichlorodihydrofluorescein diacetate, acetyl ester, mean fluorescence intensity (MFI), $n = 7$]. (**C**) Rate of mitochondrial ROS production in T cells following 16 h of activation was determined by flow cytometric analysis using MitoSOX™ Red ($n = 9$). (**D**) Flow cytometric

(continued)

death (Fig. 2I and Supplementary Fig. 2E). Taken together, DMF affects mitochondrial ROS levels, restricts mitochondrial energy generation and induces caspase-mediated T-cell apoptosis with a pronounced effect on T_M cells. Notably, similar effects were observed when using monomethyl fumarate, suggesting that this serves as the active metabolite of DMF as described previously (Supplementary Fig. 3).[10,18]

DMF treatment causes distinct alterations in T-cell metabolism and cellular apoptosis in patients with RRMS

Next, we investigated the effects of DMF treatment on the $CD4^+$ and $CD8^+$ T-cell subset composition ($n = 34$), T-cell cytokine production ($n = 34$), T-cell metabolism ($n = 14$) and apoptosis ($n = 16$) in patients with RRMS before and during DMF treatment over a period of 48 weeks within a multicentre clinical trial (NCT02461069; Fig. 3A and Supplementary Fig. 4A). In accordance with earlier studies,[4,5] DMF treatment resulted in a decrease in $CD4^+$ and $CD8^+$ T-cell numbers (Supplementary Fig. 4B and Supplementary Table 1), a marked reduction in central memory, effector memory subsets and terminally differentiated effector memory cells (TEMRAs, Supplementary Fig. 4B) as well as decreased numbers of interferon gamma (IFN-γ) and granulocyte-macrophage colony-stimulating factor (GM-CSF) producing $CD4^+$ and $CD8^+$ T cells in DMF-treated RRMS patients (Supplementary Fig. 4C). We next determined the effect of DMF treatment on the mitochondrial respiratory activity of $CD4^+$ and $CD8^+$ T cells at different time points before and during DMF treatment in a subgroup of the study cohort who provided sufficient material for this specific analysis. Notably, we observed that stimulated $CD4^+$ and $CD8^+$ T cells from DMF-treated patients exhibited a significant reduction in mitochondrial respiratory capacity compared with the pretreatment levels (Fig. 3B, Supplementary Fig. 4D and Supplementary Table 2). As described above, DMF treatment resulted in a decrease in glycolysis and the glycolytic capacity in stimulated $CD4^+$ and $CD8^+$ T cells compared with pretreatment levels (Supplementary Fig. 4E). The analysis of apoptosis in distinct lymphocyte subsets of DMF-treated patients revealed that $CD4^+$ and $CD8^+$ T_M cells displayed increased frequencies of caspase 9- and 3/7-positive cells compared with pretreatment levels (Fig. 3C, Supplementary Fig. 4F and Supplementary Table 3). In contrast, apoptosis of T_N cells was not affected by DMF treatment (Fig. 3C and Supplementary Fig. 4F). To address this finding in more depth, we compared the T-cell subset composition, T-cell metabolism and apoptosis in eight patients with RRMS who had no signs of lymphopenia under DMF treatment and eight patients who eventually developed lymphopenia under DMF treatment (i.e. lymphocyte count < 800) from a different, independent study cohort (Fig. 3D and Supplementary Table 5). The analysis of absolute T-cell numbers revealed that DMF treatment decreased $CD8^+$ T-cell numbers and reduced memory subsets in both lymphopenic and non-lymphopenic patients (Supplementary Fig. 5A). Similar findings for memory markers were observed for $CD4^+$ T cells in lymphopenic patients (Supplementary Fig. 5A). Importantly, $CD8^+$ T cells from lymphopenic patients exhibited significantly lower mitochondrial respiratory capacity and glycolysis compared to those of T cells from non-lymphopenic patients (Fig. 3E and Supplementary Fig. 5B). Notably, the mitochondrial respiratory activity of T cells from patients who later developed lymphopenia was already reduced at baseline (i.e. before DMF exposure) (Supplementary Fig. 5C). $CD4^+$ and $CD8^+$ T_M cells from lymphopenic DMF-treated patients exhibited a slight increase in apoptosis than T_M cells from patients before treatment (Fig. 3F and Supplementary Fig. 5D). In contrast, the apoptosis of T_N cells from DMF-treated lymphopenic patients was not affected (Fig. 3F and Supplementary Fig. 5D). These data indicate that DMF has a crucial impact on both mitochondrial respiratory activity and the apoptosis of memory T cells, which might explain their enhanced vulnerability in the context of DMF treatment in RRMS patients.

Glutathione restores DMF effects on mitochondrial function but abrogates its immune-modulatory impacts

In light of the literature, we evaluated whether DMF-related effects in T cells were mediated via Nrf2 activation.[8–10] Indeed DMF induced Nrf2 target genes in $CD8^+$ T cells *in vitro*, which was abolished upon co-incubation with the Nrf2 inhibitor ML385 (Supplementary Fig. 6A).[24] However, interference by the pharmacological inhibitor ML385 did not abrogate DMF-mediated effects on mitochondrial respiration or apoptosis induction (Fig. 4A, B and Supplementary Fig. 6B and C). Moreover, pharmacological Nrf2 activation using sulphoraphane did not alter mitochondrial respiration or apoptosis of $CD4^+$ and $CD8^+$ T cells (Supplementary Fig. 6D and E), together indicating that Nrf2 is not responsible for DMF-related effects on T cells.

Given the observed effects of DMF on cellular ROS levels, we next asked whether glutathione supplementation might prevent DMF-mediated effects on mitochondrial dysfunction. Glutathione supplementation in $CD4^+$ as well as $CD8^+$ T cells abrogated the DMF-mediated reduction in mitochondrial respiratory activity (Fig. 4C and Supplementary Fig. 7A), aerobic glycolysis (Supplementary Fig. 7B) and T-cell apoptosis (Fig. 4D and Supplementary Fig. 7C), thus illustrating that DMF-associated T-cell apoptosis is indeed caused by elevated intracellular ROS levels. Unexpectedly, glutathione supplementation also abrogated DMF-mediated effects on proliferation (Fig. 4E and Supplementary Fig. 7D) as well as proinflammatory cytokine production (IFN-γ and GM-CSF) (Fig. 4F and Supplementary Fig. 7E), suggesting that the well-known immune-modulatory effects of DMF are linked to altered intracellular stress responses. In a similar line of evidence, inhibition of the rate-limiting enzyme in glutathione production, i.e. glutamate cysteine ligase, by buthionine sulphoximine also impaired mitochondrial respiration and aerobic glycolysis (Fig. 4G and Supplementary Fig. 7F and G), increased T-cell apoptosis (Fig. 4H and Supplementary Fig. 7H) and reduced T-cell proliferation as well as cytokine production (Fig. 4I and J and

Figure 2 Continued
analysis of the mitochondrial membrane potential of T cells following 16 h of activation and stained with tetramethylrhodamine methyl ester perchlorate (TMRM) alone or in the presence of oligomycin or carbonyl cyanide-p-trifluoromethoxyphenylhydraz (FCCP). Graphs depict the proportion of the maximum membrane potential ($n = 5–7$). (**E**) Oxygen consumption rate (OCR) of activated T cells in the presence or absence of DMF after 72 h. Shown is one representative example. (**F**) Flow cytometry of MitoTracker™ Green staining of T cells assessed 48 h after stimulation ($n = 4$). Bar graphs display MFI values. (**G**) Analysis of intracellular cytochrome c levels of T_M and T_N cell subsets assessed 18 h after stimulation ($n = 10$). Bar graphs display MFI values data normalized to cells without DMF treatment. (**H**) Frequencies of apoptotic (caspase-9^+ and $3/7^+$) T cells 72 h after stimulation ($n = 3$). (**I**) $CD4^+$ and $CD8^+$ T cells from healthy donors were stimulated with α-CD3/α-CD28 in the presence or absence of DMF. The basal respiration of activated T cells and frequencies of apoptotic (caspase-9^+) stimulated T cells was assessed at the indicated time points. Delta values (Δ) indicate normalized data from cells without treatment ($n = 3$). All data are displayed as mean ± SEM. Statistical analysis was conducted by Student's *t*-test. $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$. Panel **A** was made using components from SMART – Servier Medical ARTA) (https://smart.servier.com/).

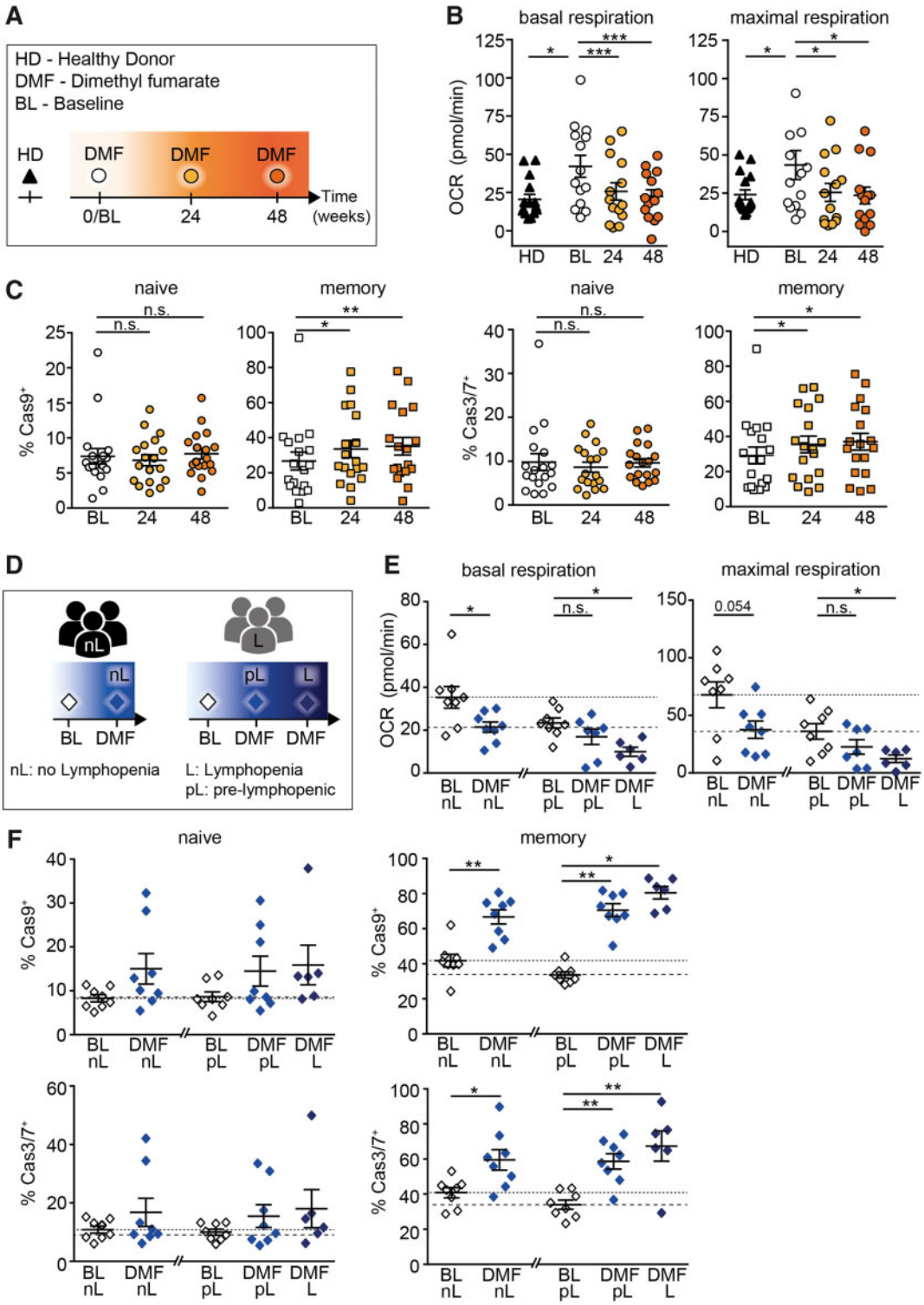

Figure 3 Impact of DMF treatment in patients with RRMS on mitochondrial respiration and apoptosis of T cells. (**A–C**) Study setup from the clinical DIMAT study. The data were assessed in patients with RRMS at baseline (BL; white) and after 24 weeks (24; yellow), as well as after 48 weeks (48; orange) of DMF treatment. Healthy donors (HD; black) represent the control group. *P*-values were calculated with the Wilcoxon signed-rank test or Student's *t*-test (HD versus BL). (**B**) Basal and maximal respiration of $CD8^+$ T cells isolated from frozen PBMC samples of healthy donors and a subgroup of patients from the DIMAT study cohort (Supplementary Table 2) were determined upon short-term stimulation with PMA and ionomycin for 2.5 h. (**C**) Frequencies of apoptotic (caspase-9^+ and $3/7^+$) $CD8^+$ memory T cells and $CD8^+$ naïve T cells of a subgroup of patients from the DIMAT study cohort (Supplementary Table 3). (**D–F**) Analysis of T cell subpopulations from an independent small cohort of DMF-treated RRMS patients, who either did not develop lymphopenia during treatment [non-lymphopenic (nL)] or became lymphopenic (L) during the course of DMF treatment (Supplementary Table 5) ($n = 8$ patients/group). *P*-values were determined with the Wilcoxon signed-rank test. (**E**) Basal and maximal respiration of $CD8^+$ T cells isolated from frozen PBMC samples of study subjects from the cohort depicted in **D** upon short-term stimulation with PMA/ionomycin for 2.5 h. (**F**) Frequencies of apoptotic $CD8^+$ memory and naïve T cells of the cohort depicted in **D**. All data are displayed as the mean ± SEM. $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

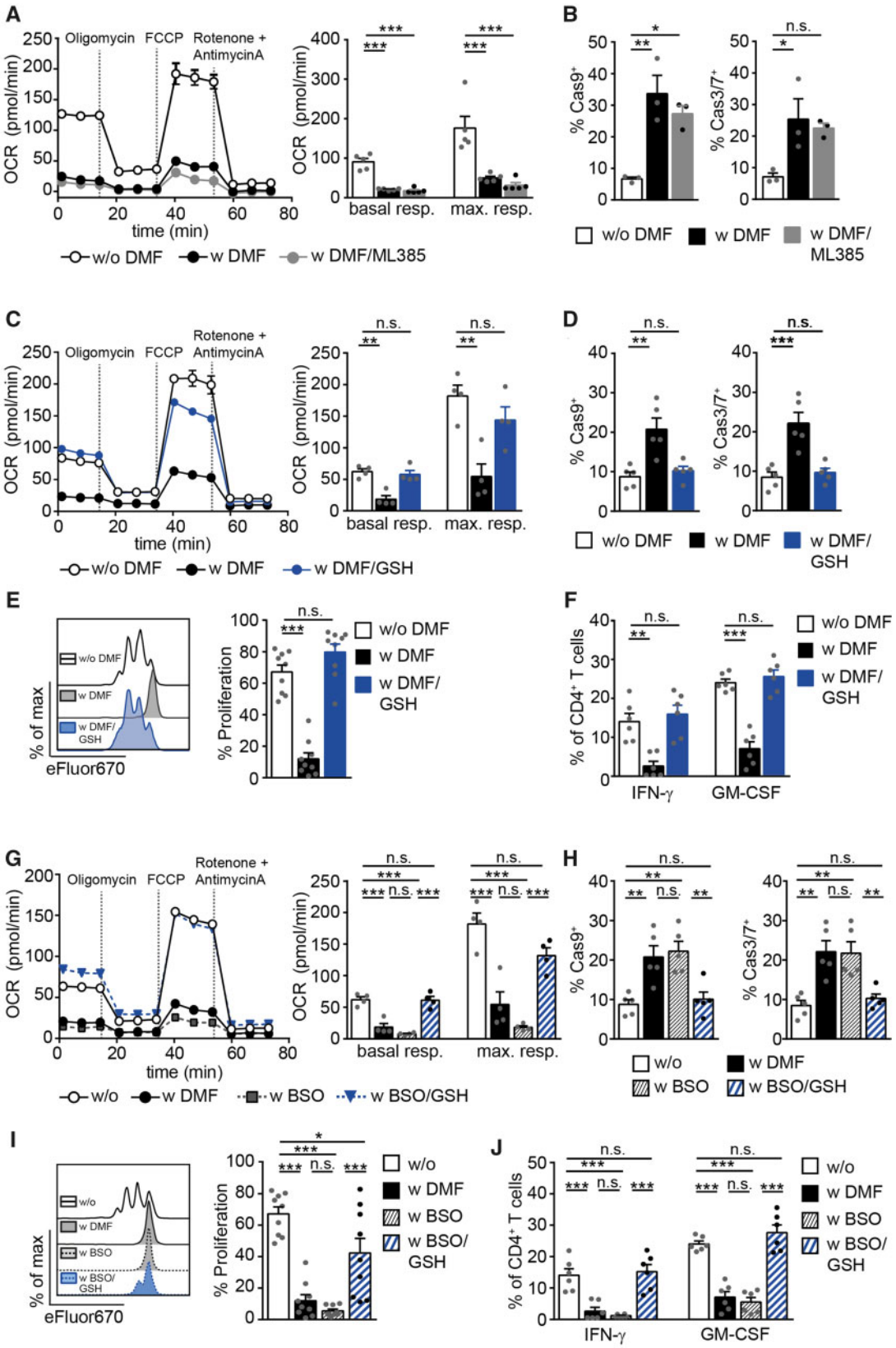

Figure 4 Glutathione supplementation, but not Nrf2 inhibition, restores DMF-mediated effects on T-cell metabolism, apoptosis induction, T-cell proliferation and cytokine production. (**A** and **B**) $CD4^+$ T cells of healthy donors were stimulated with α-CD3/α-CD28 in the presence or absence of DMF and the pharmacological Nrf2 inhibitor ML385 (5 μg/ml). (**A**) OCR of activated $CD4^+$ T cells in the presence or absence of DMF and ML385 after 72 h ($n = 5$). (**B**) Frequencies of apoptotic (caspase-9^+ and $3/7^+$) $CD4^+$ T cells were determined by flow cytometry ($n = 3$). (**C–F**) $CD4^+$ T cells were stimulated with α-CD3/α-CD28 in the presence or absence of DMF and glutathione (GSH, 250 μM). (**C**) OCR of activated $CD4^+$ T cells in the presence or absence of DMF and GSH after 72 h ($n = 4$). (**D**) Frequencies of apoptotic (caspase-9^+ and $3/7^+$) $CD4^+$ T cells were determined by flow cytometry ($n = 5$). (**E**) T-cell

(continued)

Supplementary Fig. 7I and J). This effect was at least partially reverted upon addition of glutathione, again pointing towards a key role of intracellular ROS levels for modulating T-cell responses.

Reduction of intracellular and mitochondrial ROS levels reverses DMF-mediated effects on T cells

Along this line, we evaluated the effects of two distinct ROS scavengers on DMF-mediated effects on $CD4^+$ and $CD8^+$ T-cell function. To this end, we used NAC, a well-known intracellular ROS scavenger, as well as MitoQ, a specific inhibitor of mitochondrial ROS production.[33,34] Application of NAC and MitoQ reduced intracellular as well as mitochondrial ROS levels (Fig. 5A and B and Supplementary Fig. 8A and B) restored mitochondrial respiration and mitochondrial mass (Fig. 5C–F and Supplementary Fig. 8C–F), and reduced DMF-induced T-cell apoptosis (Fig. 5G and H and Supplementary Fig. 8G and H). Importantly, reduction in intracellular as well as mitochondrial ROS levels also abrogated DMF-mediated anti-inflammatory effects on $CD4^+$ and $CD8^+$ T-cell proliferation and cytokine production (Fig. 5I–L and Supplementary Fig. 8I–L). These data indicate that known key immunological effects of DMF on T-cell function are closely linked to its alteration of cellular antioxidative capacities.

NAC abrogates DMF-mediated effects in experimental autoimmune encephalomyelitis

We finally examined whether restoration of cellular antioxidative capacities interfered with DMF-mediated immune-modulatory effects in a well-known mouse model of multiple sclerosis, i.e. EAE. Indeed, both NAC and MitoQ supplementation in parallel with DMF treatment in mice during active MOG-induced EAE completely abrogated the DMF-induced disease amelioration (Fig. 6A and B). In contrast, NAC or MitoQ supplementation alone did not affect the disease course of EAE (Fig. 6A and B). Furthermore, NAC supplementation abrogated the effects of DMF on lymphocyte counts in the peripheral blood, whereas NAC alone did not alter lymphocyte counts compared with control animals (Supplementary Fig. 9A). At the peak of disease (Day 14), $CD4^+$ T cells were isolated from the CNS and periphery, and *ex vivo* cytokine secretion was analysed by flow cytometry upon short-term restimulation. Again, NAC supplementation completely abrogated the effects of DMF on proinflammatory cytokines [IFN-γ, GM-CSF and interleukin (IL)-17A], while NAC supplementation alone did not affect cytokine production in $CD4^+$ T cells (Fig. 6C and Supplementary Fig. 9B). Similar findings on the production of proinflammatory cytokines were observed in peripheral $CD8^+$ T cells (Supplementary Fig. 9C). Additionally, NAC supplementation *in vivo* restored the decreased mitochondrial respiration of $CD4^+$ T cells from DMF-treated mice (Fig. 6D) and abrogated the DMF-mediated reduction in glutathione levels in $CD4^+$ T cells that were isolated from the spleens of EAE animals (Fig. 6E). Finally, NAC supplementation abrogated DMF-mediated effects on $CD4^+$ T-cell apoptosis *in vivo* (Fig. 6F). Taken together, these data support the concept that both DMF-mediated immunomodulatory effects as well as its impairment of antioxidative capacities of T cells were reversed by restoring intracellular antioxidative capacities.

Discussion

The relevance of immune-metabolic functions in shaping immune responses and their disturbance in the context of autoimmune diseases has been increasingly acknowledged. Accordingly, there is accumulating evidence about the metabolic effects of well-known immunomodulatory drugs such as teriflunomide, sirolimus and methotrexate.[18,22,35,36] Moreover, with respect to DMF, metabolic effects on immune cells have been described. In monocytes for example, DMF increases intracellular ROS levels impacting transcriptional changes during inflammation, while the targeted inactivation of the key glycolytic enzyme GAPDH via succination by DMF has been discovered in human T cells.[16,18] To achieve a more comprehensive overview of DMF-induced metabolic alterations in T cells in general, we employed a global metabolomics approach, which revealed profound metabolic alterations affecting a number of distinct metabolic pathways. We were particularly interested in the observed DMF-mediated decrease in glutathione levels, as glutathione is the main source of antioxidant power in cells, and it has previously been demonstrated that DMF can reduce intracellular glutathione by covalent bond formation in tumour cells.[14,15,37] Notably, a reduction in glutathione levels has already been associated with increased mitochondrial ROS levels, resulting in enhanced cytochrome c levels that ultimately induce cellular apoptosis via the induction of caspase 9 and 3/7.[31,32] Furthermore, elevated mitochondrial calcium and ROS levels in B cells have been linked to structural mitochondrial alterations, resulting in mitochondrial dysfunction and increased apoptosis.[30] Indeed, we show that DMF not only reduced glutathione levels but also enhanced mitochondrial ROS levels, led to structural alterations of mitochondria and elevated cytochrome c levels in T cells, which was accompanied by enhanced induction of apoptosis, thus supporting the concept that DMF-associated glutathione reduction facilitates the apoptosis of activated T cells.[38] It is known that DMF-mediated depletion of glutathione results in the upregulation of endogenous ROS in T_c17 cells, monocytes and several cancer cell lines; however, these studies did not investigate the consequences for cell survival.[13–16]

Our proposed cascade might explain the clinically well-known phenomenon of lymphopenia in DMF-treated patients with multiple sclerosis.[4,39,40] A detailed analysis of lymphocyte subsets in the peripheral blood of patients with multiple sclerosis revealed that T_M cells were particularly susceptible to DMF treatment-associated cell loss.[4,39] Consistent with our hypothesis, we showed that DMF had a preferential impact on mitochondrial respiratory activity and T_M cell apoptosis both *in vitro* and in DMF-treated patients with multiple sclerosis. From a metabolic point of view, this is not surprising, given the enhanced metabolic potential of T_M cells that underlies their inherent capacity to rapidly respond to T-cell receptor-mediated activation.[19,22] Furthermore, T_M cells exhibit an enhanced susceptibility to ROS-mediated cell death.[41]

Figure 4 Continued
proliferation after 72 h was determined by flow cytometry. Representative histograms and percentages of proliferated cells are shown ($n = 9$). (**F**) Intracellular cytokine production of IFN-γ and GM-CSF after 48 h was assessed by flow cytometry. Graph displays percentages of cytokine-producing $CD4^+$ T cells ($n = 6$). (**G–J**) $CD4^+$ T cells were stimulated with α-CD3/α-CD28 in the presence or absence of DMF, GSH and/or the specific glutamate cysteine ligase inhibitor buthionine sulfoximine (BSO, 3 mM). (**G**) OCR of activated $CD4^+$ T cells in the presence or DMF, GSH and/or BSO after 72 h ($n = 4$). Bar graph shows basal and maximal respiration. (**H**) Frequencies of apoptotic $CD4^+$ T cells after 72 h were determined by flow cytometry ($n = 5$). (**I**) T-cell proliferation after 72 h was assessed by flow cytometry. Representative histograms and percentages of proliferated $CD4^+$ T cells are shown ($n = 9$). (**J**) Intracellular cytokine production of IFN-γ and GM-CSF after 48 h was determined by flow cytometry. Graph displays percentages of cytokine-producing T cells ($n = 6$). All data are displayed as mean ± SEM. Statistical analysis was conducted by one-way ANOVA plus Tukey *post hoc* test (**G–J**) or plus Dunnett's *post hoc* test (**A–F**). $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

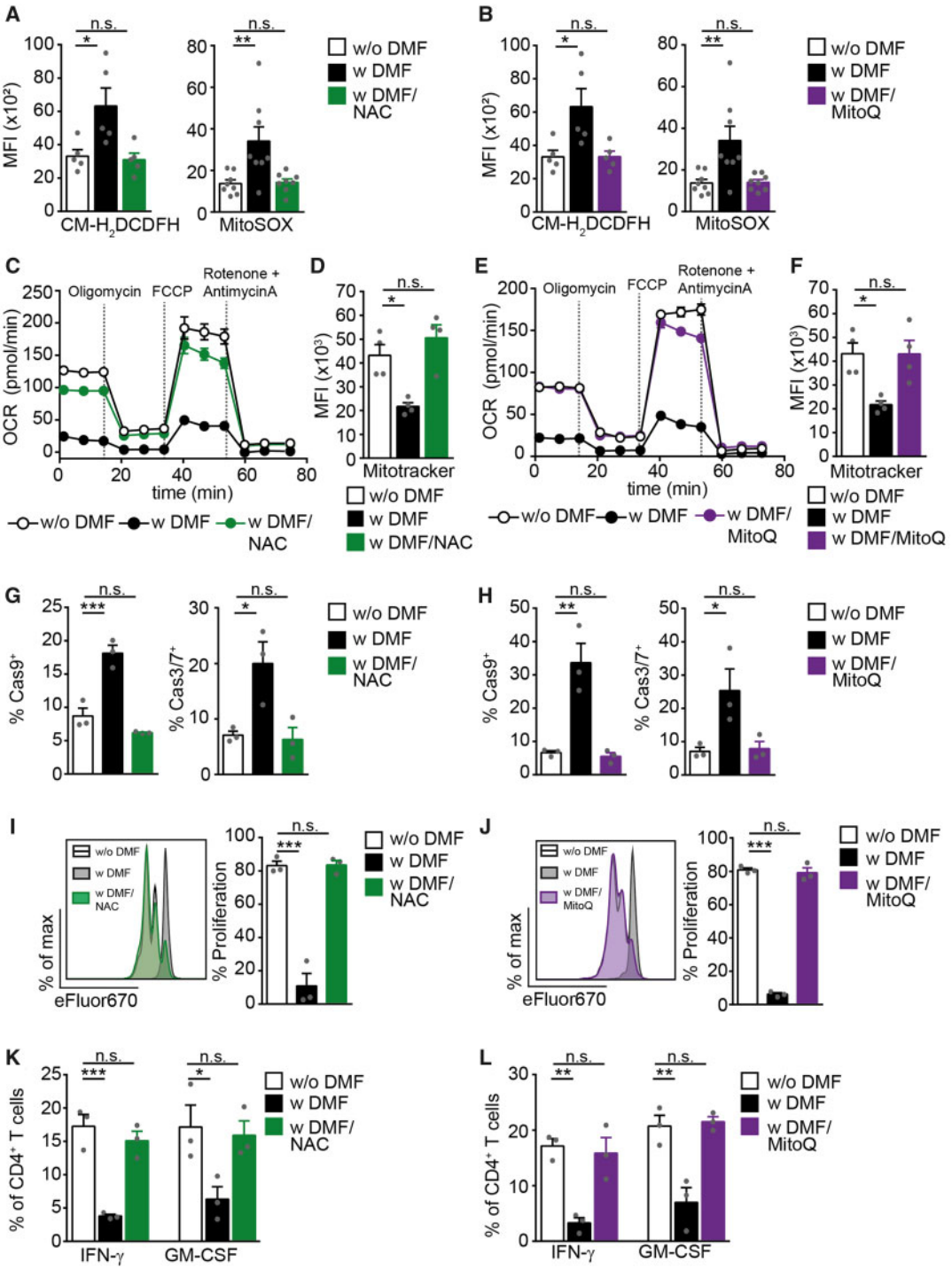

Figure 5 Reduction of intracellular as well as mitochondrial ROS levels restores the DMF-mediated effects on T-cell metabolism, apoptosis induction, T-cell proliferation and cytokine production. $CD4^+$ T cells of healthy donors were stimulated with α-CD3/α-CD28 in the presence or absence of DMF and 250 nM of the intracellular ROS scavenger NAC (**A**, **C**, **D**, **G**, **I** and **K**) or 100 nM of the mitochondrial ROS scavenger MitoQ (**B**, **E**, **F**, **H**, **J** and **L**). (**A** and **B**) Intracellular and mitochondrial ROS levels were determined by flow cytomteric analysis using CM-H_2DCFDA (chloromethyl-dichlorodihydrofluorescein diacetate, acetyl ester; *left*) and MitoSOX Red (*right*). Bar graphs display MFI of stimulated T cells in presence or absence of DMF and NAC (**A**) or MitoQ (**B**) for 16 h ($n = 5$–8). (**C**) OCR of activated T cells in the presence or absence of DMF and NAC after 72 h. Shown is one representative example. (**D**) Flow cytometric analysis of MitoTracker™ Green staining of stimulated T cells in the presence or absence of DMF and NAC after 48 h. Bar graph displays MFI values ($n = 4$). (**E**) OCR of activated T cells in the presence or absence of DMF and MitoQ after 72 h ($n = 4$). (**F**) Flow cytometric analysis of the MitoTracker™ Green signal of stimulated T cells in presence or absence of DMF and MitoQ for 48 h. Bar graph displays MFI values ($n = 4$). (**G** and **H**) Frequencies of apoptotic (caspase-9^+ and $3/7^+$) cells in presence or absence of DMF and NAC (**G**) or MitoQ (**H**) after 72 h ($n = 3$). (**I** and **J**) T-cell proliferation in the presence or absence of DMF and NAC (**I**) or MitoQ (**J**) was determined by flow cytometry after 72 h. Representative histograms and percentages of proliferated cells are shown ($n = 3$). (**K** and **L**) Intracellular cytokine production of IFN-γ and GM-CSF was determined by flow cytometry after 72 h in T cells stimulated in the presence or absence of DMF and NAC (**K**) or MitoQ (**L**). Graphs depict percentages of cytokine-producing T cells ($n = 3$). All data are displayed as the mean ± SEM. Statistical analysis was conducted by one-way ANOVA plus Dunnett's *post hoc* test. $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

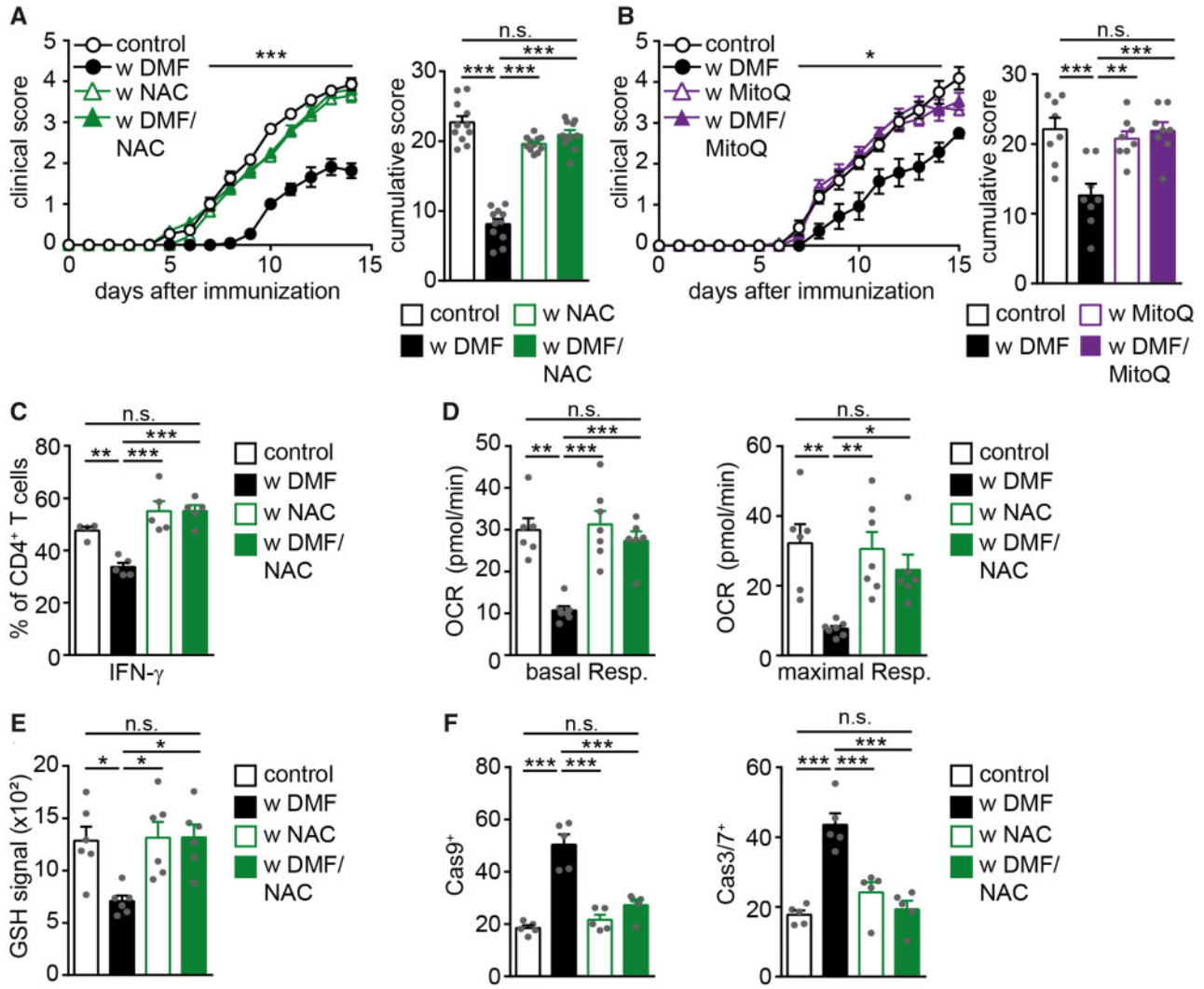

Figure 6 The ROS scavenger NAC abrogates the protective effects of DMF during EAE. (**A**) Active EAE was induced by MOG_{35-55} immunization in wild-type mice. In parallel, 100 mg/kg body weight DMF, 200 mg/kg body weight NAC or vehicle only were administered daily by oral gavage throughout the experiment. The mean clinical score is shown. The graph on the *right* depicts the mean cumulative clinical score (n = 10–11/group). (**B**) Active EAE was induced in wild-type mice treated with DMF as described in **A** and/or injected with vehicle or MitoQ (100 nmol/mouse, i.p.) twice a week. The mean clinical score and the mean cumulative clinical score are shown (n = 7–8/group). (**C–F**) *Ex vivo* analyses were performed from EAE mice treated with DMF and/or NAC. (**C**) At the peak of EAE (Day 14), the frequencies of CNS-isolated IFN-γ-producing $CD4^+$ T cells were determined after *ex vivo* short-term stimulation with a leucocyte activation cocktail for 3 h (n = 4–5/group). (**D**) On Day 10 after EAE induction, $CD4^+$ T cells were isolated from draining lymph nodes and basal and maximal respiration were determined after *ex vivo* stimulation with PMA and ionomycin for 2.5 h (n = 6–7/group). (**E**) Cellular glutathione (GSH) levels were determined in $CD4^+$ T cells isolated from spleens of EAE mice at Day 10 using monochlorobimane (n = 6/group). (**F**) On Day 14 post-injection, the frequencies of apoptotic (caspase-9^+ and $3/7^+$) T cells were determined in $CD4^+$ T cells from draining lymph nodes (n = 5/group). All data are displayed as the mean ± SEM. Statistical analysis was performed using a two-way ANOVA (**A** and **B**) and one-way ANOVA plus Tukey's *post hoc* test (**C–F**). $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

The preferential impact of DMF treatment on mitochondrial stress responses, mitochondrial respiratory function and ultimately caspase-dependent apoptosis of T_M cells might help to explain why DMF treatment in the context of treatment-associated lymphopenia is associated with an increased risk of developing progressive multifocal leukoencephalopathy.[42] The progression of this opportunistic infection has previously been linked to the number of $CD8^+$ T_M cells specific to the underlying virus.[43] It should be noted that in the case of DMF, lymphocyte blood counts in affected cases were not as low as those of other multiple sclerosis immunotherapies, suggesting additional contributing factors beyond a mere quantitative reduction.[44] Although we cannot prove this directly, we hypothesize that DMF-mediated T-cell apoptosis is only the final step in DMF-mediated disturbance of T-cell metabolism, oxidative balance and function, so that T_M cells in the presence of DMF *in vivo* might be functionally impaired but still alive. This idea is supported by data from our longitudinal analysis of samples from DMF-treated patients who ultimately developed lymphopenia; here, lymphopenia was indeed preceded by disturbed T-cell metabolism.

When investigating the underlying mechanisms of DMF-mediated disruption of mitochondrial respiration and enhanced oxidative stress, we evaluated whether DMF acted via KEAP1 and Nrf2, which is regarded as a key mechanism of DMF-mediated effects in the context of multiple sclerosis. However, interference with this pathway in T cells by using the pharmacological inhibitor ML385[24] did not abrogate DMF-mediated effects on mitochondrial respiration, proliferation, proinflammatory cytokine production or apoptosis induction (Supplementary Fig. 10). Furthermore, pharmacological Nrf2 activation using sulphoraphane did not

affect T-cell metabolism, cytokine production and survival, together indicating that Nrf2 is not responsible for DMF-mediated effects on T-cell functions (Supplementary Fig. 10). Notably, restoration of the antioxidative capacities of DMF-treated T cells either (i) via supplementation of glutathione as the most abundant intracellular antioxidant; (ii) via NAC as a precursor of glutathione; or (iii) via MitoQ, a mitochondrial ubiquinone derivative that acts as specific inhibitor of mitochondrial ROS production[33,34] not only restored DMF-mediated effects on mitochondrial function but also abrogated its immune-modulatory effects on T-cell activation and proliferation. Furthermore, the well-known DMF-mediated protective effects in the EAE mouse model were also abrogated in the presence of NAC and MitoQ supplementation. These data indicate that altered mitochondrial ROS production and resulting oxidative stress are directly involved in DMF-mediated modulation of key immune functions of T cells. In support of this notion, previous studies described that supplementation of glutathione or NAC restored DMF-mediated effects on the cellular stress response in cancer cells as well as in T_c17 cells *in vitro*.[13,15] In T_c17 cells, DMF altered their transcriptional profile at least partly via ROS, resulting in decreased IL-17 expression; however, this was limited to a small $CD8^+$ T-cell subset and not observed in T helper 17 cells, their CD4 counterparts.[13] Several studies have demonstrated that ROS display concentration-dependent effects on T cells leading to their activation at physiologically relevant levels via activation of oxidation-dependent transcription factors including NFAT, NF-κB and AP-1.[45,46] However, constantly elevated ROS levels, e.g. by genetic ablation of glutathione production, prevented metabolic reprogramming of T cells during inflammation and elicited activation-induced cell death via expression of apoptosis-related genes and altered mitochondrial function, indicating that ROS exert dose-dependent and opposing effects during T-cell activation.[17,38,47] Of interest, patients with systemic lupus erythematosus inherently display elevated intracellular ROS levels, resulting in mitochondrial dysfunction in T cells leading to enhanced apoptosis, which is a hallmark of this autoimmune disease.[48]

Our findings have several important implications: first, they shed novel light on the molecular mechanisms underlying DMF-mediated control of T-cell responses and CNS autoimmunity in the EAE model and multiple sclerosis. Second, they underscore the emerging relevance of metabolic processes in immune cells in the pathophysiology of autoimmune diseases such as rheumatoid arthritis, multiple sclerosis and systemic lupus erythematosus, and consequently, the still underestimated potential of immune-metabolic interventions as possible therapeutic strategies in the field of autoimmunity. Several studies have previously described metabolic disturbances in different autoimmune diseases,[20–22,49] and it has therefore been suggested that targeted modulation of metabolic pathways in immune cells represents a highly attractive therapeutic concept in immunology.[50,51] Our data, together with those from other studies, contribute to this concept by providing evidence that immunomodulatory drugs approved for the treatment of multiple sclerosis at least partly exert their effects via the modulation of metabolic pathways.[18,22,52]

Finally, our findings unveil the underestimated relevance of equilibrated antioxidative capacities in immune cells as a prerequisite for improved immune regulation in multiple sclerosis. In multiple sclerosis lesions within the CNS, signs of enhanced oxidative stress resulting in mitochondrial dysfunction have previously been described.[53–55] To date, disequilibrated antioxidative capacities have not been extensively studied in immune cells from patients with multiple sclerosis; however, one recent publication described that T cells from primary progressive patients with multiple sclerosis exhibited alterations in glycolysis and mitochondrial respiratory function,[56] while another publication described elevated nitric oxide production by peripheral blood leucocytes in patients with multiple sclerosis.[57]

From a more general point of view, our data suggest that an optimal immune-metabolic treatment for multiple sclerosis should maintain the balance between targeted modulation of imbalanced immune responses while maintaining antioxidative capacities of immune cells to prevent oxidative stress-associated cellular dysfunction and apoptosis. One option for individualized optimization of therapeutic responses in the context of DMF might be dose adaptation according to lymphocyte subset counts. To date, data on the association between lymphocyte counts and disease control have been inconclusive. While the clinical trials DEFINE and CONFIRM did not provide evidence of higher efficacy in lymphopenic patients,[2,3] two real life cohorts from the USA, Italy and Germany suggested such a correlation.[58–60] Based on our data, we propose that rather than total lymphocyte counts, the effects on memory T cells combined with alterations in mitochondrial respiration, ROS production and glutathione levels might help to define a threshold of desired lymphocyte reductions as a consequence of DMF-mediated effects on lymphocyte proliferation with only a limited increase in oxidative stress and consequently enhanced T-cell apoptosis. Currently, this remains speculative; however, we propose that the relevance of such biomarkers of DMF-associated oxidative stress should be prospectively evaluated.

We aimed to focus specifically on DMF-mediated effects on T lymphocytes. However, in light of the increasing acknowledgement of B cells as another important driver of multiple sclerosis pathophysiology, it seems reasonable that DMF might also exert relevant effects on B cells not only with regard to immune-modulation but also potentially with regard to a disturbed oxidative equilibrium.

Taken together, our data support the concept that DMF-mediated immunomodulatory effects on T cells and T cell-mediated autoimmune responses are linked to disturbed antioxidative functions, resulting in altered mitochondrial metabolism. The other side of that coin is enhanced mitochondrial apoptosis of memory T cells upon enhanced oxidative stress due to the DMF-mediated depletion of glutathione, which is a major antioxidant in mammalian cells. These data provide new insights into the mechanism of action of DMF and its major unwanted effect of lymphocyte depletion, which is associated with potential opportunistic progressive multifocal leukoencephalopathy infection in susceptible patients.

Acknowledgements

We thank Annika Engbers and Andrea Pabst (Department of Neurology with Institute of Translational Neurology, Münster) as well as Rebecca Ley and Luzia Buchholz (Institute of Neuropathology, Münster) for excellent technical assistance.

Funding

This study was funded by the Kompetenznetz Multiple Sklerose project 01GI1603A (H.W., L.K. and C.C.G.) and by the Deutsche Forschungsgemeinschaft (DFG) – Project-ID 213904703 – CRC TR128, Projects A08 (L.K.), A09 (H.W. and C.C.G.), B01 (N.S.), B06 (S.G.M.) and Z2 (H.W., L.K. and T.K.), and – Project-ID 194468054 – CRC 1009, Project A03 (L.K. and H.W.). The clinical trial (DIMAT) was supported by Biogen GmbH Germany.

Competing interests

M.L. received travel support from Biogen (2018). C.J. received travel support from Novartis. A.S.M. received travel expenses and research support from Novartis (2018–19). N.S. received research and research support from Novartis and Biogen. T.S.-H. received

research and travel support from Novartis and Biogen. B.W. received grants from the German Ministry of Education and Research, Deutsche Forschungsgemeinschaft, Dietmar Hopp Foundation and Klaus Tschira Foundation, grants and personal fees from Alexion, Merck Serono, Sanofi Genzyme, Novartis pharmaceuticals, and personal fees from Bayer Healthcare, Biogen and Teva; none related to this work. F.L. received consultancy fees from Roche and support with travel costs from Teva Pharma. S.S. received speaker's honoraria from Bayer, Biogen, Genzyme, Merck, Novartis, Roche, Sanofi, Teva and financial research support from Bayer and Merck. M.D. received speaker honoraria and research funding from Biogen Switzerland used exclusively for research purposes. S.B. received honoria and compensation for travel from Biogen Idec, Merck Serono, Novartis, Sanofi-Genzyme and Roche. C.C.G. received speaker honoraria from MyLan, Bayer Healthcare, and Sanofi-Genzyme and travel/accommodation/meeting expenses from Bayer Halthcare, Biogen, EUROIMMUN, Novartis and Sanofi-Genzyme. She also received research support from Biogen, Roche, and Novartis. S.Kov. received research funding support from Biogen and honoraria from Sanofi, Esai and Genzyme. F.Z. received research grants and/or consultation funds from the DFG, BMBF, PMSA, Novartis, Octapharma, Merck Serono, ONO Pharma, Biogen, Genzyme, Celgene and Roche. T.D. received research funding support from Novartis and Biogen. His institution received financial support from Novartis, Merck and GeNeuro for his activities as a steering committee or advisory board member or consultant from Biogen, Roche, Genzyme, MedDay, Celgene and Actelion for his activities as advisory board member or consultant from Mitsubishi Pharma for his activities on a steering committee, all outside the submitted work; his wife is an employee of Novartis and holds stock options in Novartis. T.K. received research funding from the German Research Foundation, Interdisciplinary Center for Clinical Studies (IZKF) Münster, National MS Society, European Leukodystrophy Association, Progressive MS Alliance, European Commission (H2020-MSCA-ITN-2018) and Novartis. She received compensation for serving on scientific advisory boards (Frequency Therapeutics, Inc.) and speaker honoraria from Novartis. S.G.M. received honoraria for lecturing and travel expenses for attending meetings from Almirall, Amicus Therapeutics Germany, Bayer Health Care, Biogen, Celgene, Diamed, Genzyme, MedDay Pharmaceuticals, Merck Serono, Novartis, Novo Nordisk, ONO Pharma, Roche, Sanofi-Aventis, Chugai Pharma, QuintilesIMS and Teva. His research is funded by the BMBF, Bundesinstitut für Risikobewertung (BfR), DFG, Else Kröner Fresenius Foundation, Gemeinsamer Bundesausschuss (G-BA), German Academic Exchange Service, Hertie Foundation, IZKF Muenster, German Foundation Neurology and Alexion, Almirall, Amicus Therapeutics Germany, Biogen, Diamed, Fresenius Medical Care, Genzyme, HERZ Burgdorf, Merck Serono, Novartis, ONO Pharma, Roche and Teva. H.W. received honoraria for acting as a member of Scientific Advisory Boards Biogen, Evgen, Genzyme, MedDay Pharmaceuticals, Merck Serono, Novartis, Roche Pharma AG and Sanofi-Aventis as well as speaker honoraria and travel support from Alexion, Biogen, Cognomed, F. Hoffmann-La Roche Ltd, Gemeinnützige Hertie-Stiftung, Merck Serono, Novartis, Roche Pharma AG, Genzyme, TEVA and WebMD Global. He acts as a paid consultant for Abbvie, Actelion, Biogen, IGES, Johnson & Johnson, Novartis, Roche, Sanofi-Aventis and the Swiss Multiple Sclerosis Society. His research is funded by the BMBF, DFG, Else Kröner Fresenius Foundation, Fresenius Foundation, Hertie Foundation, NRW Ministry of Education and Research, IZKF Muenster and RE Children's Foundation, Biogen, GlaxoSmithKline GmbH, Roche Pharma AG and Sanofi-Genzyme. L.Kl. received compensation for serving on scientific advisory boards for Alexion, Genzyme, Janssen, Merck Serono, Novartis and Roche. She received speaker honoraria and travel support from Bayer, Biogen, Genzyme, Grifols, Merck Serono, Novartis, Roche, Santhera and Teva. She receives research support from the German Research Foundation, the IZKF Münster, IMF Münster, Biogen, Novartis and Merck Serono.

Supplementary material

Supplementary material is available at *Brain* online.

References

1. McFarland HF, Martin R. Multiple sclerosis: A complicated picture of autoimmunity. *Nat Immunol*. 2007;8(9):913–919.
2. Fox RJ, Miller DH, Phillips JT, et al. Placebo-controlled phase 3 study of oral BG-12 or glatiramer in multiple sclerosis. *N Engl J Med*. 2012;367(12):1087–1097.
3. Gold R, Kappos L, Arnold DL, et al. Placebo-controlled phase 3 study of oral BG-12 for relapsing multiple sclerosis. *N Engl J Med*. 2012;367(12):1098–1107.
4. Gross CC, Schulte-Mecklenbeck A, Klinsing S, Posevitz-Fejfár A, Wiendl H, Klotz L. Dimethyl fumarate treatment alters circulating T helper cell subsets in multiple sclerosis. *Neurol Neuroimmunol Neuroinflamm*. 2016;3(1):e183.
5. Diebold M, Sievers C, Bantug G, et al. Dimethyl fumarate influences innate and adaptive immunity in multiple sclerosis. *J Autoimmun* 2018;86:39–50.
6. Mathias A, Perriot S, Canales M, et al. Impaired T-cell migration to the CNS under fingolimod and dimethyl fumarate. *Neurol Neuroimmunol Neuroinflamm*. 2017;4(6):e401.
7. Breuer J, Herich S, Schneider-Hohendorf T, et al. Dual action by fumaric acid esters synergistically reduces adhesion to human endothelium. *Mult Scler J*. 2018;24(14):1871–1882.
8. Linker RA, Lee DH, Ryan S, et al. Fumaric acid esters exert neuroprotective effects in neuroinflammation via activation of the Nrf2 antioxidant pathway. *Brain*. 2011;134(Pt 3):678–692.
9. Scannevin RH, Chollate S, Jung M.-y, et al. Fumarates Promote Cytoprotection of Central Nervous System Cells against Oxidative Stress via the Nuclear Factor (Erythroid-Derived 2)-Like 2 Pathway. *J Pharmacol Exp Ther*. 2012;341(1):274–284.
10. Schulze-Topphoff U, Varrin-Doyer M, Pekarek K, et al. Dimethyl fumarate treatment induces adaptive and innate immune modulation independent of Nrf2. *Proc Natl Acad Sci U S A*. 2016;113(17):4777–4782.
11. Huang H, Taraboletti A, Shriver LP. Dimethyl fumarate modulates antioxidant and lipid metabolism in oligodendrocytes. *Redox Biol*. 2015;5:169–175.
12. Lehmann JCU, Listopad JJ, Rentzsch CU, et al. Dimethylfumarate induces immunosuppression via glutathione depletion and subsequent induction of heme oxygenase 1. *J Invest Dermatol*. 2007;127(4):835–845.
13. Lückel C, Picard F, Raifer H, et al. IL-17+ CD8+ T cell suppression by dimethyl fumarate associates with clinical response in multiple sclerosis. *Nat Commun*. 2019;10(1):5722–5715.
14. Sullivan LB, Martinez-Garcia E, Nguyen H, et al. The Proto-oncometabolite fumarate binds glutathione to amplify ROS-dependent signaling. *Mol Cell*. 2013;51(2):236–248.
15. Zheng L, Cardaci S, Jerby L, et al. Fumarate induces redox-dependent senescence by modifying glutathione metabolism. *Nat Commun*. 2015;6:6001.
16. Carlström KE, Ewing E, Granqvist M, et al. Therapeutic efficacy of dimethyl fumarate in relapsing-remitting multiple sclerosis associates with ROS pathway in monocytes. *Nat Commun*. 2019;10(1):3081.

17. Mak TW, Grusdat M, Duncan GS, et al. Glutathione primes T cell metabolism for inflammation. *Immunity*. 2017;46(4):675–689.
18. Kornberg MD, Bhargava P, Kim PM, et al. Dimethyl fumarate targets GAPDH and aerobic glycolysis to modulate immunity. *Science*. 2018;360(6387):449–453.
19. van der Windt GJW, O'Sullivan D, Everts B, et al. CD8 memory T cells have a bioenergetic advantage that underlies their rapid recall ability. *Proc Natl Acad Sci U S A*. 2013;110(35): 14336–14341.
20. Yin Y, Choi S-C, Xu Z, et al. Normalization of CD4+ T cell metabolism reverses lupus. *Sci Transl Med*. 2015;7(274):274ra18.
21. Yang Z, Shen Y, Oishi H, et al. Restoring oxidant signaling suppresses proarthritogenic T cell effector functions in rheumatoid arthritis. *Sci Transl Med*. 2016;8(331):331ra38.
22. Klotz L, Eschborn M, Lindner M, et al. Teriflunomide treatment for multiple sclerosis modulates T cell mitochondrial respiration with affinity-dependent effects. *Sci Transl Med*. 2019; 11(490):eaao5563.
23. Geisel J, Brück J, Glocova I, et al. Sulforaphane protects from T cell–mediated autoimmune disease by inhibition of IL-23 and IL-12 in dendritic cells. *J Immunol*. 2014;192(8):3530–3539.
24. Singh A, Venkannagari S, Oh KH, et al. Small molecule inhibitor of NRF2 selectively intervenes therapeutic resistance in KEAP1-deficient NSCLC tumors. *ACS Chem Biol*. 2016;11(11): 3214–3225.
25. Griffith OW. Mechanism of action, metabolism, and toxicity of buthionine sulfoximine and its higher homologs, potent inhibitors of glutathione synthesis. *J Biol Chem*. 1982;257(22): 13704–13712.
26. Liebmann M, Hucke S, Koch K, et al. Nur77 serves as a molecular brake of the metabolic switch during T cell activation to restrict autoimmunity. *Proc Natl Acad Sci U S A*. 2018;115(34): E8017–E8026.
27. Albrecht S, Korr S, Nowack L, et al. The K 2P -channel TASK1 affects Oligodendroglial differentiation but not myelin restoration. *Glia*. 2019;67(5):870–883.
28. Hucke S, Floßdorf J, Grützke B, et al. Licensing of myeloid cells promotes central nervous system autoimmunity and is controlled by peroxisome proliferator-activated receptor g. *Brain*. 2012;135(Pt 5):1586–1605.
29. Xia J, Wishart DS. MSEA: A web-based tool to identify biologically meaningful patterns in quantitative metabolomic data. *Nucleic Acids Res*. 2010;38(Web Server issue):W71–77.
30. Akkaya M, Traba J, Roesler AS, et al. Second signals rescue B cells from activation-induced mitochondrial dysfunction and death. *Nat Immunol*. 2018;19(8):871–884.
31. Green DR, Galluzzi L, Kroemer G. Metabolic control of cell death. *Science*. 2014;345(6203):1250256.
32. Kagan VE, Tyurin VA, Jiang J, et al. Cytochrome C acts as A cardiolipin oxygenase required for release of proapoptotic factors. *Nat Chem Biol*. 2005;1(4):223–232.
33. Bavarsad Shahripour R, Harrigan MR, Alexandrov AV. N-acetylcysteine (NAC) in neurological disorders: Mechanisms of action and therapeutic opportunities. *Brain Behav*. 2014;4(2):108–122.
34. Smith RAJ, Murphy MP. Animal and human studies with the mitochondria-targeted antioxidant MitoQ. *Ann N Y Acad Sci*. 2010;1201:96–103.
35. Lai ZW, Kelly R, Winans T, et al. Sirolimus in patients with clinically active systemic lupus erythematosus resistant to, or intolerant of, conventional medications: A single-arm, open-label, phase 1/2 trial. *Lancet*. 2018;391(10126):1186–1196.
36. Bedoui Y, Guillot X, Sélambarom J, et al. Methotrexate an old drug with new tricks. *Int J Mol Sci*. 2019;20(20).
37. Held KD, Epp ER, Clark EP, Biaglow JE. Effect of dimethyl fumarate on the radiation sensitivity of mammalian cells in vitro. *Radiat Res*. 1988;115(3):495–502.
38. Hildeman D. A, Mitchell T, Teague TK, et al. Activation-induced T cell apoptosis. *Immunity*. 1999;10(6):735–744.
39. Ghadiri M, Rezk A, Li R, et al. Dimethyl fumarate-induced lymphopenia in MS due to differential T-cell subset apoptosis. *Neurol Neuroimmunol Neuroinflamm*. 2017;4(3):e340.
40. Spencer CM, Crabtree-Hartman EC, Lehmann-Horn K, Cree BAC, Zamvil SS. Reduction of CD8+ T lymphocytes in multiple sclerosis patients treated with dimethyl fumarate. *Neurol Neuroimmunol Neuroinflamm*. 2015;2(3):e76.
41. Mougiakakos D, Johansson CC, Kiessling R. Naturally occurring regulatory T cells show reduced sensitivity toward oxidative stress-induced cell death. *Blood*. 2009;113(15):3542–3545.
42. Klotz L, Havla J, Schwab N, et al. Risks and risk management in modern multiple sclerosis immunotherapeutic treatment. *Ther Adv Neurol Disord*. 2019;12:1756286419836571.
43. Du Pasquier RA, Kuroda MJ, Zheng Y, Jean-Jacques J, Letvin NL, Koralnik IJ. A prospective study demonstrates an association between JC virus-specific cytotoxic T lymphocytes and the early control of progressive multifocal leukoencephalopathy. *Brain*. 2004;127(Pt 9):1970–1978.
44. Gieselbach RJ, Muller-Hansma AH, Wijburg MT, et al. Progressive multifocal leukoencephalopathy in patients treated with fumaric acid esters: a review of 19 cases. *J Neurol*. 2017; 264(6):1155–1164.
45. Sena LA, Li S, Jairaman A, et al. Mitochondria are required for antigen-specific T cell activation through reactive oxygen species signaling. *Immunity*. 2013;38(2):225–236.
46. Kaminski MM, Sauer SW, Klemke C-D, et al. Mitochondrial reactive oxygen species control T cell activation by regulating IL-2 and IL-4 expression: mechanism of ciprofloxacin-mediated immunosuppression. *J Immunol*. 2010;184(9):4827–4841.
47. Kaminski M, Kiessling M, Süss D, Krammer PH, Gülow K. Novel role for mitochondria: protein kinase Ctheta-dependent oxidative signaling organelles in activation-induced T-cell death. *Mol Cell Biol*. 2007;27(10):3625–3639.
48. Gergely P, Grossman C, Niland B, et al. Mitochondrial hyperpolarization and ATP depletion in patients with systemic lupus erythematosus. *Arthritis Rheum*. 2002;46(1):175–190.
49. Bantug GR, Galluzzi L, Kroemer G, Hess C. The spectrum of T cell metabolism in health and disease. *Nat Rev Immunol*. 2018; 18(1):19–34.
50. Bettencourt IA, Powell JD. Targeting metabolism as a novel therapeutic approach to autoimmunity, inflammation, and transplantation. *J Immunol*. 2017;198(3):999–1005.
51. O'Sullivan D, Pearce EL. Targeting T cell metabolism for therapy. *Trends Immunol*. 2015;36(2):71–80.
52. De Riccardis L, Ferramosca A, Danieli A, et al. Metabolic response to glatiramer acetate therapy in multiple sclerosis patients. *BBA Clin*. 2016;6:131–137.
53. Mahad D, Ziabreva I, Lassmann H, Turnbull D. Mitochondrial defects in acute multiple sclerosis lesions. *Brain*. 2008;131(Pt 7): 1722–1735.
54. Witte M, Bo L, Rodenburg R, et al. Enhanced number and activity of mitochondria in multiple sclerosis lesions. *J Pathol*. 2009; 219(2):193–204.
55. Adiele RC, Adiele CA. Metabolic defects in multiple sclerosis. *Mitochondrion*. 2019;44:7–14.
56. De Biasi S, Simone AM, Bianchini E, et al. Mitochondrial functionality and metabolism in T cells from progressive multiple sclerosis patients. *Eur J Immunol*. 2019;49(12):2204–2221.

57. Koch M, Mostert J, Arutjunyan A, et al. Peripheral blood leukocyte NO production and oxidative stress in multiple sclerosis. *Mult Scler*. 2008;14(2):159–165.
58. Manni A, Iaffaldano A, Lucisano G, et al. Lymphocyte count and body mass index as biomarkers of early treatment response in a multiple sclerosis dimethyl fumarate-treated cohort. *Front Immunol*. 2019;10:1343.
59. Wright K, Winkler MD, Newton BD, Sormani MP, Okuda DT. Patient outcomes influenced by reduced lymphocyte counts after dimethyl fumarate initiation. *Neurol Neuroimmunol Neuroinflamm*. 2017;4(6):e397.
60. Fleischer V, Friedrich M, Rezk A, et al. Treatment response to dimethyl fumarate is characterized by disproportionate CD8+ T cell reduction in MS. *Mult Scler J*. 2018;24(5):632–641.