Dimethyl fumarate treatment induces adaptive and innate immune modulation independent of Nrf2

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Dimethyl fumarate (DMF) (BG-12, Tecfidera) is a fumaric acid ester (FAE) that was advanced as a multiple sclerosis (MS) therapy largely for potential neuroprotection as it was recognized that FAEs are capable of activating the antioxidative transcription factor nuclear factor (erythroid-derived 2)-like 2 (Nrf2) pathway. However, DMF treatment in randomized controlled MS trials was associated with marked reductions in relapse rate and development of active brain MRI lesions, measures considered to reflect CNS inflammation. Here, we investigated the antiinflammatory contribution of Nrf2 in DMF treatment of the MS model, experimental autoimmune encephalomyelitis (EAE). C57BL/6 wild-type (WT) and Nrf2-deficient ($Nrf2^{-/-}$) mice were immunized with myelin oligodendrocyte glycoprotein (MOG) peptide 35–55 (p35–55) for EAE induction and treated with oral DMF or vehicle daily. DMF protected WT and $Nrf2^{-/-}$ mice equally well from development of clinical and histologic EAE. The beneficial effect of DMF treatment in $Nrf2^{-/-}$ and WT mice was accompanied by reduced frequencies of IFN-γ and IL-17–producing $CD4^+$ cells and induction of antiinflammatory M2 (type II) monocytes. DMF also modulated B-cell MHC II expression and reduced the incidence of clinical disease in a B-cell–dependent model of spontaneous CNS autoimmunity. Our observations that oral DMF treatment promoted immune modulation and provided equal clinical benefit in acute EAE in $Nrf2^{-/-}$ and WT mice, suggest that the antiinflammatory activity of DMF in treatment of MS patients may occur through alternative pathways, independent of Nrf2.

multiple sclerosis | dimethyl fumarate | Nrf2 | EAE | M2 monocytes

Dimethyl fumarate (DMF) (BG-12, Tecfidera) is an oral fumaric acid ester (FAE) that is frequently prescribed for treatment of relapsing forms of multiple sclerosis (MS). DMF was developed for treatment of MS based upon knowledge that Fumaderm, a combination of DMF and monoethyl fumarate used in treatment of psoriasis, exhibits immunomodulatory and antioxidative properties (1, 2). FAEs can reduce oxidative stress by activating the transcription factor nuclear factor (erythroid-derived 2)-like 2 (Nrf2) (3, 4), which promotes gene expression of detoxification enzymes such as glutathione S-transferase A2 (GSTA2), hemoxygenase, and NADPH quinone oxidoreductase 1 (NQO1) (4–6). FAEs, which are electrophilic, can covalently link to essential thiol groups (nucleophiles) on macromolecules, including Kelch-like ECH-associated protein 1 (Keap1), the functional inhibitor of Nrf2 pathway activation (3, 7). The phase III MS trials that led to the approval of DMF treatment in MS demonstrated a marked reduction in relapse rate and development of active brain MRI lesions with DMF treatment (8, 9), measures considered to reflect CNS inflammation. Those findings indicated that DMF also exhibits antiinflammatory activity that may contribute to its benefit in treatment of relapsing–remitting MS, although the role of Nrf2 in this process has not been established. Recent in vitro studies have indicated that Nrf2 may not be involved with DMF effects on nuclear factor-κB (NF-κB) inflammatory responses (10).

FAEs can cause immune modulation. Fumaderm, an FAE combination of DMF and three different monoethylfumarate salts, is approved for treatment of psoriasis in Germany, and is known to suppress proinflammatory T-cell responses. In vitro, DMF treatment inhibited proliferation and production of Th1 cytokines by human blood lymphocytes (1). In vivo, DMF treatment of experimental autoimmune encephalomyelitis (EAE) was also associated with a Th2 bias, which may be a consequence of induction of antigen-presenting antiinflammatory type II dendritic cells (DCs) (11). Consistent with these observations, results indicate Nrf2 itself may regulate innate and adaptive T-cell immune responses in models of organ-specific inflammation (12, 13), including EAE (14, 15). Nevertheless, although DMF demonstrated prominent reduction of inflammatory measures of MS (8, 9), it is not clear that this benefit is mediated through activation of Nrf2. Our current study of DMF treatment of EAE in $Nrf2^{-/-}$ and WT mice highlights the importance of Nrf2-independent DMF-mediated immune modulation.

Results

DMF Reduces Development of Clinical and Histological Signs of EAE. Multiple proinflammatory T-cell subsets, including Th1, Th17,

Significance

Dimethyl fumarate (DMF) (BG-12, Tecfidera), a fumaric acid ester (FAE), is a commonly prescribed oral therapy for multiple sclerosis (MS), a CNS autoimmune inflammatory demyelinating disease that may result in sustained neurologic damage. It is thought that the benefit of DMF in MS therapy is mediated through activation of the antioxidative transcription factor nuclear factor (erythroid-derived 2)-like 2 (Nrf2) pathway. However, the role of Nrf2 in the antiinflammatory effects of DMF has not been fully elucidated. Here, we investigated the role of Nrf2 in DMF treatment of the MS model, experimental autoimmune encephalomyelitis (EAE), and demonstrated DMF can modulate T cells, B cells, and antigen-presenting cells, and reduce clinical and histologic EAE, independent of Nrf2.

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and GM-CSF–producing T cells, can contribute to the development of EAE (16). We therefore examined the influence of DMF treatment on activation of proinflammatory myelin-specific T cells in EAE. Oral DMF treatment ameliorated clinical EAE (Fig. 1*A*) and reduced accumulation of CNS inflammatory lesions (Fig. 1*B*). This benefit was associated with decreases in CNS infiltrating Th1, Th17, and GM-CSF–producing T cells (Fig. 1*C*). Of interest, we did not detect a significant difference in $CD4^+CD25^+Foxp3^+$ regulatory T cells in WT mice treated with DMF (Fig. S1). Our results underscore DMF's capability to inhibit multiple proinflammatory T-cell subsets that participate in development of EAE.

DMF Reduces EAE Susceptibility and Promotes Immune Modulation in the Absence of Nrf2. Although it has been suggested that the influence of DMF treatment in MS and EAE may occur, at least in part, through activation of the Nrf2 pathway, the extent to which Nrf2 contributes to the antiinflammatory properties of DMF is not clear. Thus, we examined DMF treatment in $Nrf2^{-/-}$ mice. As exposure to DMF's active metabolite, monomethyl fumarate (MMF), may differ in $Nrf2^{-/-}$ and WT mice, we first evaluated blood and tissue MMF levels after treatment with different doses of DMF (Fig. 2*A*). Similar MMF levels were detected when WT and $Nrf2^{-/-}$ mice were treated with 100 mg/kg DMF. Thus, 100 mg/kg DMF was chosen for the subsequent experiments.

When tested in EAE, oral DMF treatment of MOG p35–55-immunized $Nrf2^{-/-}$ mice reduced both clinical (Fig. 2*B*) and histological signs of disease (Fig. 2*C*). Strikingly, the magnitude of the DMF treatment effect observed in $Nrf2^{-/-}$ and WT mice was nearly identical. DMF treatment had no effect on total number of $CD4^+$ T cells in WT or $Nrf2^{-/-}$ mice. Although there was no change in the proportion of $CD44^+CD62L^{hi}$ central memory cells (T_{CM}) in DMF-treated mice, there was a reduction of in $CD44^+CD62L^{lo}$ effector memory cells (T_{EM}) in both WT and $Nrf2^{-/-}$ mice (Fig. 2*D*). DMF treatment was associated with similar reductions in Th1 and Th17 cells in WT and $Nrf2^{-/-}$ mice. These results clearly demonstrate that a significant contribution of the clinical and immunologic benefit of DMF treatment in CNS inflammatory disease can occur independently of Nrf2.

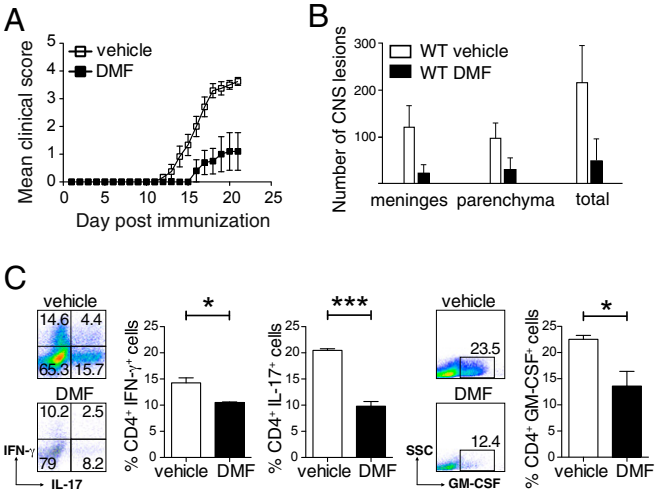

Fig. 1. DMF reduces development of clinical and histological disease. C57BL/6 WT mice were immunized with MOG p35–55 and treated with DMF (100 mg/kg) or vehicle (0.6% Methocel) by oral gavage. (*A*) Mean disease score (±SEM) of $n = 5$ mice per group is displayed. (*B*) Lesion quantification of Luxol fast blue–H&E staining of total number of meningeal and parenchymal inflammatory foci in C57BL/6 and $Nrf2^{-/-}$ mice treated with DMF ($n = 4$ mice per group). (*C*) After disease onset, CNS infiltrating cells were evaluated for secretion of IFN-γ, IL-17, and GM-CSF. Representative FACS staining is shown including quantification ($n = 3$ mice per group). Data are displayed as mean values (±SEM). $^*P \leq 0.05$, $^{***}P \leq 0.001$, Student's *t* test.

DMF Induces Antiinflammatory Type II Monocytes and DCs Independent of Nrf2. Previously, it was observed that in vitro treatment of human DCs by DMF promoted an antiinflammatory type II phenotype (11). As it was not clear whether the influence of DMF on antigen-presenting cells (APCs) was unique to DCs or reflected a general mechanism of action of DMF treatment on myeloid cell subsets, we evaluated how in vivo DMF treatment influenced separate myeloid APC populations. We examined classical $CD11c^{hi}$ dendritic cells (cDCs) in mice treated with DMF or vehicle for 9 days after immunization with MOG p35–55. There were no significant alterations in the frequencies of cDCs (Fig. 3*A*). Interestingly, upon in vivo DMF treatment, we found a significant decrease in frequency of $CD11b^+CD11c^-$ monocytes, whereas the population of $CD11b^+Ly6C^{hi}Ly6G^-$ cells, known as myeloid-derived suppressor cells (17), was increased (Fig. 3*A*). Expression of MHC II and costimulatory molecules on $CD11b^+CD11c^-$ monocytes tended to be lower in vehicle-treated $Nrf2^{-/-}$ than WT mice (Fig. 3*B*). In vivo DMF treatment caused a significant reduction of MHC II on $Nrf2^{-/-}$ $CD11b^+CD11c^-$ monocytes. In contrast, there was a significant reduction in CD80 and CD86 costimulatory molecules on $CD11b^+CD11c^-$ monocytes from DMF-treated WT, but not $Nrf2^{-/-}$ mice. $CD11b^+CD11c^-$ monocytes from DMF-treated WT mice exhibited an M2 (type II) phenotype, characterized by a reduction in production of proinflammatory-polarizing cytokines.

Next, we examined whether the type II APCs induced by DMF treatment influenced proinflammatory and antiinflammatory cytokine production by myelin-specific T-cell responses. First, spleen cells were depleted of $CD3^+$ T cells and used as APCs for activation of naïve ($CD4^+CD44^-CD62L^+$) MOG p35–55 TCR-transgenic (2D2) T cells under nonpolarizing conditions. As shown in Fig. 3*C*, splenic APCs from mice treated with DMF inhibited differentiation of Th1 cells and promoted Th2 polarization. Furthermore, purified $CD11b^+$ monocytes from DMF-treated mice were used as APCs under proinflammatory T-cell–polarizing conditions (Fig. 3*D*). Here, the $CD11b^+$ monocytes from DMF-treated mice inhibited differentiation of Th1 cells.

Whether DMF-mediated M2 polarization was Nrf2 dependent was also evaluated. As in DMF-treated WT mice, the reduction of $CD11b^+CD11c^-$ monocytes and increase of $CD11b^+Ly6C^{hi}$ monocytes occurred in a similar manner in $Nrf2^{-/-}$ mice (Fig. 3*A*). As for $CD11b^+CD11c^-$ monocytes from DMF-treated WT mice, there was a reduction of cell surface MHC II molecules on monocytes from DMF-treated mice, although expression of CD80 and CD86 was not altered. Further, the DMF-induced M2 cytokine polarization profile did not require Nrf2 (Fig. 3*B*). Thus, our results clearly demonstrate that Nrf2-independent mechanism(s) of action participate in M2 monocyte polarization.

DMF Modulates B-cell Function and Suppresses Development of Spontaneous CNS Autoimmunity. Although data suggest that continuous DMF treatment of MS may influence B cells (18, 19), little is known regarding its potential immunomodulatory effects on those lymphocytes in either MS or in EAE. It is recognized that B cells serve an important role as APCs in T-cell activation and the development of acute and chronic CNS inflammation (20). Thus, as an initial evaluation, we examined whether DMF treatment influenced the numbers of B cells or expression of molecules involved in B-cell APC function. WT and $Nrf2^{-/-}$ mice were treated orally with DMF or vehicle for 9 days. There was no alteration in the frequency of $B220^+$ B cells in either WT or $Nrf2^{-/-}$ mice. However, DMF treatment was associated with a similarly marked reduction of cell surface MHC II expression molecules in both mouse strains (Fig. 4*A*). Although there were no significant changes in expression of CD40 or CD80 costimulatory molecules in either WT or $Nrf2^{-/-}$ mice, there was a small, but significant, reduction of CD86

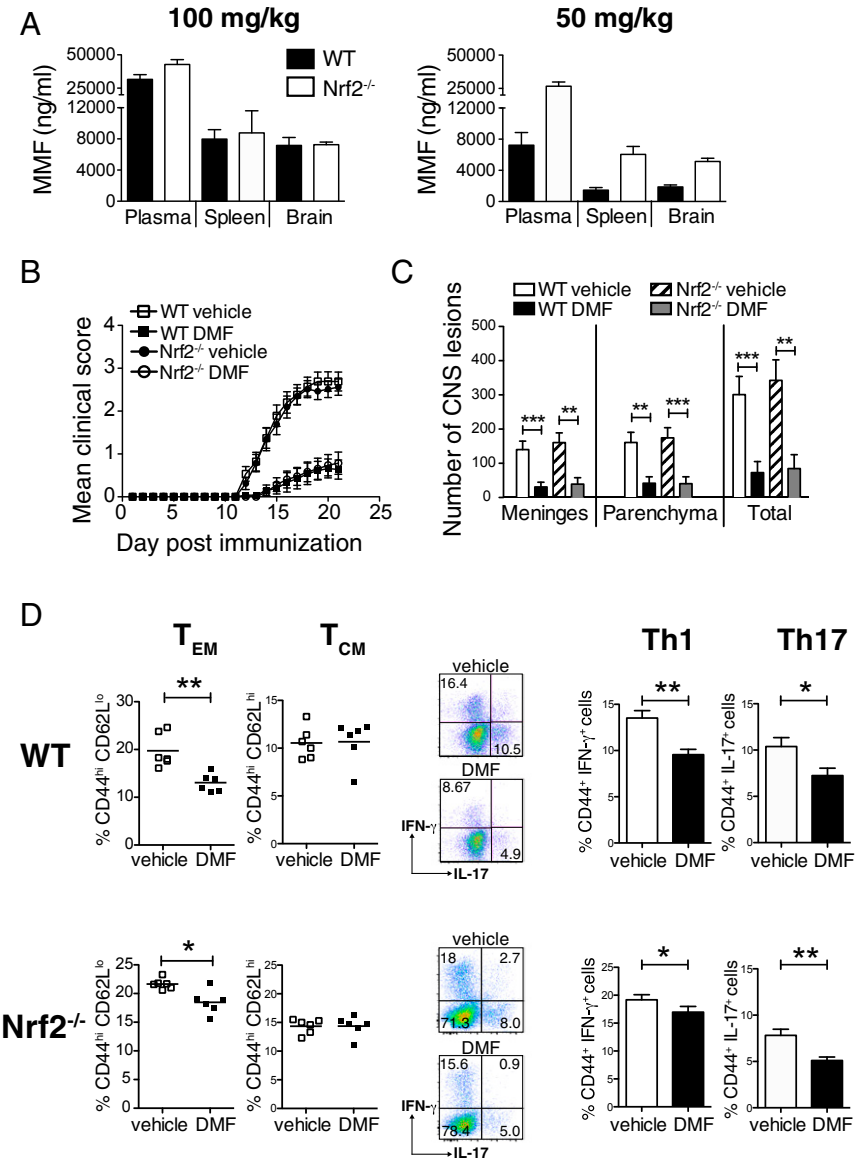

Fig. 2. DMF treatment reduces development of clinical and histological disease in both WT and Nrf2$^{-/-}$ mice. (*A*) MMF levels were quantitated in plasma, brain, and spleen samples ($n = 3$ mice per group) collected 30 min after oral administration of DMF (50, 100 mg/kg). (*B–D*) C57BL/6 WT and Nrf2$^{-/-}$ mice were immunized with MOG p35–55 and treated with 100 mg/kg DMF or vehicle (0.6% Methocel) daily by oral gavage. (*B*) Composite disease course of three independent experiments is shown ($n = 21$ mice per group). (*C*) Composite lesion quantification from two independent experiments measuring Luxol fast blue–H&E staining of total number of meningeal and parenchymal inflammatory foci in C57BL/6 and Nrf2$^{-/-}$ mice treated with DMF ($n = 14$ mice per group). (*D*) Nine days after immunization, CD4$^+$ spleen cells were evaluated for their activation state and cytokine production. FACS analysis of CD4$^+$ cells for expression of CD44, CD62L. Displayed are percentages of $n = 6$ mice per group of effector memory T cells (T_{EM}) and central memory T cells (T_{CM}). CD4$^+$CD44$^+$ spleen cells were further evaluated for secretion of IFN-γ, and IL-17. Representative FACS staining and quantification ($n = 6$ mice per group) is shown. Data are shown as mean values (±SEM). *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, Mann–Whitney *U* test.

costimulatory molecules in WT, but not Nrf2$^{-/-}$ mice. Therefore, although Nrf2 expression may participate in DMF-mediated modulation of certain costimulatory molecules, Nrf2 does not participate in DMF-induced reduction of B-cell MHC II expression.

Having observed that in vivo DMF treatment influences B-cell expression of molecules involved in antigen presentation, we evaluated in vivo DMF treatment in a model of spontaneous chronic opticospinal EAE (OSE) (20–22) that is dependent upon B-cell APC function (20). In comparison with treatment with vehicle, oral DMF delayed the onset and reduced the incidence and severity of spontaneous OSE (Fig. 4*B* and Table 1). Collectively, these results indicate that DMF can modulate B-cell–dependent participation in CNS autoimmunity.

Discussion

The purpose of this investigation was to evaluate the degree of Nrf2 dependency in DMF treatment of CNS inflammatory autoimmune disease. It is recognized that Nrf2, a transcription cofactor that activates detoxifying enzymes (5, 6), is inhibited by Keap1, which binds Nrf2 and facilitates its degradation (3, 7). FAEs, including DMF, are electrophiles and serve as "Michael acceptors" that covalently link to nucleophilic thiol groups on macromolecules, including cysteine residue 151 on Keap1 (3, 23). Upon FAE conjugation to Keap1, Nrf2 is released, permitting nuclear translocation and initiation of gene transcription (5, 6, 24). DMF therefore activates Nrf2 by "inhibiting its inhibitor" (25). It has also been observed that DMF induces Nrf2

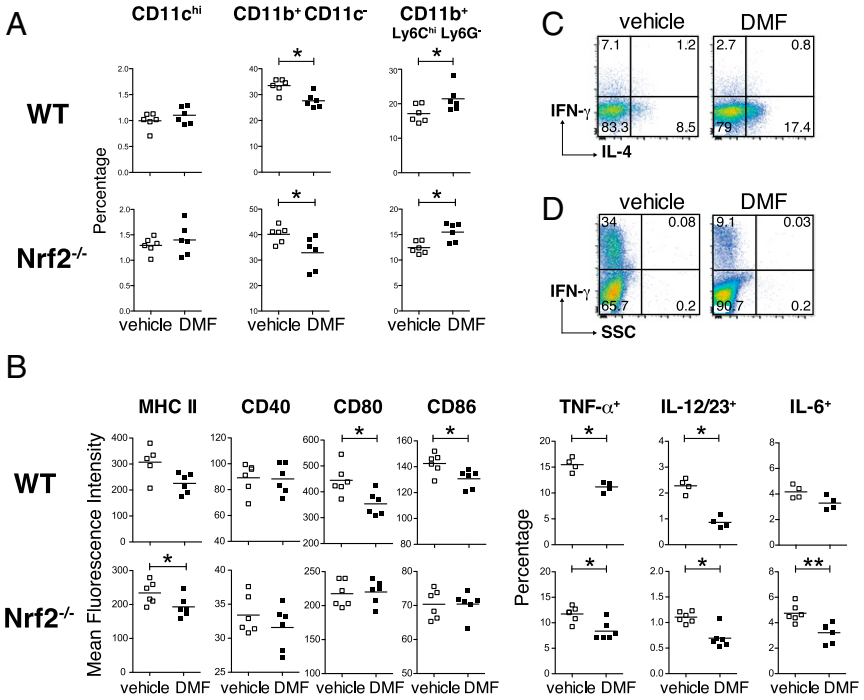

Fig. 3. DMF treatment induces type II (M2) monocytes in both WT and Nrf2$^{-/-}$ mice. FACS analysis of CD11b^{+}CD11c^{-} cells isolated from spleens of (*A*) WT and (*B*) Nrf2$^{-/-}$ mice treated with DMF or vehicle for 9 days after immunization with MOG p35–55. (*A* and *B*) Quantification of CD11c^{hi}, CD11b^{+}CD11c^{-}, and CD11b^{+}Ly6C^{hi}Ly6G^{-} cells is shown. CD11b^{+}CD11c^{-} cells were analyzed for intracellular production of TNF-α, IL12/23 (p40), and IL-6. (*C*) CD3-depleted spleen cells from DMF- or vehicle-treated mice were used as APCs and cocultured with naïve (CD4^{+}CD44^{-}CD62L^{+}) T cells from 2D2 mice under non-polarizing conditions in the presence of antigen (Ag) (MOG p35–55). (*D*) Purified CD11b^{+} cells isolated from in vivo DMF-treated or untreated mice were used as APCs in coculture with naive 2D2 T cells and Ag (MOG p35–55) under Th1-polarizing conditions. (*C* and *D*) Intracellular cytokine staining for IFN-γ and IL-4 after 4 days in culture is shown. *$P \leq 0.05$, **$P \leq 0.01$, Mann–Whitney *U* test.

expression in glial cells (3, 4, 26) and that Nrf2 is up-regulated in MS lesions (27). In addition to stimulating antioxidative and potentially neuroprotective pathways with DMF, earlier studies of Fumaderm treatment in psoriasis demonstrated that FAEs also reduce lymphocyte proliferation and secretion of proinflammatory cytokines (1). Furthermore, DMF treatment caused significant reduction in inflammatory measures of disease activity in MS clinical trials (8, 9), and data reported after its approval for MS indicate that sustained DMF treatment may restrict proliferation of certain lymphocyte subsets (18, 19). Here, we evaluated antiinflammatory and immunomodulatory activity of DMF treatment of acute EAE in WT and Nrf2$^{-/-}$ mice. Our results demonstrated that DMF protected WT and Nrf2$^{-/-}$ mice equally well from development of acute "inflammatory" EAE. The clinical benefit of DMF treatment in both Nrf2$^{-/-}$ and WT mice was associated with a reduction of Th1 and Th17 cells as well as the induction of antiinflammatory M2 monocytes. Our results clearly show that DMF exhibits potent antiinflammatory and immunomodulatory activity in CNS autoimmunity and that Nrf2 may not be required for many of those beneficial effects.

Although our study emphasizes the contribution of Nrf2-independent immune modulation of DMF therapy, previous studies have shown that Nrf2 itself has immunomodulatory and antiinflammatory activities. Nrf2 deficiency has been associated with hyperproliferation of hematopoietic stem cells (28), proinflammatory T-cell differentiation (15), and exacerbation of EAE (14). Because Nrf2$^{-/-}$ mice can develop more severe EAE, investigators who previously evaluated the role of Nrf2 in DMF-mediated treatment of chronic EAE, used an attenuated protocol in Nrf2$^{-/-}$ mice (3). In that study, DMF mediated beneficial effects on clinical course, axon preservation, and astrocyte activation in WT mice, and these effects were lost in mice deficient for Nrf2. Other in vitro studies have demonstrated the necessity of Nrf2 in mediating the cytoprotective effects of DMF and MMF in astrocyte toxic oxidative stress challenge models (4), and together these data suggest a role for Nrf2 in mediating some of the beneficial effects of DMF in chronic EAE, presumably a reflection of neuroprotection. Our results do not conflict with those studies, as we focused on the influence of DMF treatment in acute EAE, which is considered to represent the most inflammatory phase of this disease model. We observed that most, but not all, clinical and immunologic effects of DMF in this model occurred in a similar manner in WT and Nrf2$^{-/-}$ mice. Although DMF treatment promoted M2 monocyte polarization in both WT and Nrf2$^{-/-}$ mice, decreased expression of CD80 and CD86 costimulatory molecules was observed in WT, but not Nrf2$^{-/-}$ mice, indicating that those effects were Nrf2 dependent. Based upon the earlier study (3), we had also used an attenuated protocol of EAE induction in Nrf2$^{-/-}$ mice to match EAE severity in WT mice. However, when we induced EAE in precisely the same manner in both WT and Nrf2$^{-/-}$ mice, those mice developed similar EAE severity and, again, oral DMF produced equal benefit in WT and Nrf2$^{-/-}$ mice (Table S1). Thus, the pronounced benefit of DMF treatment in WT and Nrf2$^{-/-}$ mice was not dependent upon the protocol used for EAE induction.

B cells contribute to pathogenesis of MS and certain forms of EAE (20, 29). However, knowledge regarding the influence of DMF treatment on B cells has been limited. Thus, we initially examined how in vivo DMF treatment affected B-cell expression of MHC II and costimulatory molecules. A marked reduction of B-cell MHC II expression was observed in both DMF-treated WT and Nrf2$^{-/-}$ mice, along with a reduction of B-cell CD86 in WT, but not Nrf2$^{-/-}$ mice, suggesting that DMF may exert both Nrf2-dependent and -independent effects on B cells, respectively. We also observed that in vivo DMF treatment delayed

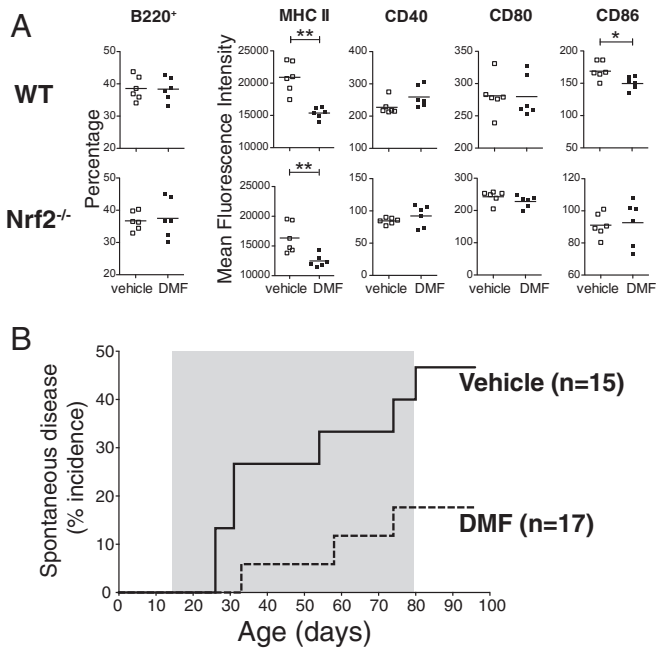

Fig. 4. DMF treatment reduces incidence of spontaneous EAE. (*A*) B cells from mice treated with DMF or vehicle were examined for cell surface expression of MHC II and costimulatory molecules by FACS. B220$^+$ cells were isolated from spleens of WT and Nrf2$^{-/-}$ mice (six per group) who had been treated orally with DMF or vehicle (0.6% Methocel) daily for 9 days after immunization with MOG p35–55. (*B*) IgH$^{MOG\text{-}ki}$ (Th) × TCR^{MOG} (2D2) mice were treated with oral DMF (dashed line) or vehicle (solid line) daily beginning 18 days after birth and monitored daily for the development of spontaneous EAE (DMF, n = 17 mice per group; vehicle, n = 15 mice per group). *$P \leq 0.05$, **$P \leq 0.01$, Mann–Whitney U test.

the onset and reduced the incidence and severity of B-cell–dependent spontaneous EAE in 2D2 × Th mice. Thus, our results suggest that DMF exerts important immunomodulatory effects on B cells. Of interest, when another group of investigators tested the MS therapy glatiramer acetate (GA) (Copolymer-1, Copaxone) in this model, they did not detect a therapeutic effect, and concluded that it is not a suitable model for evaluating clinical disease modification after therapeutic interventions (30). In that study, the investigators administered GA by i.p. injection, and not s.c. as it is administered in MS patients, as well as in many EAE studies demonstrating therapeutic benefit (31–33). As our results demonstrated that oral DMF treatment was clinically beneficial in B-cell–dependent spontaneous EAE, it may have been premature for those investigators to conclude that the 2D2 × Th model is not a useful model to evaluate the clinical benefit of therapeutics.

DMF probably has multiple therapeutic targets. In this regard, MMF is a potent agonist of the hydroxycarboxylic acid receptor 2 (HCAR2) (GPR109A) (34). It was also observed that HCAR2 deficiency prevented the beneficial effects of DMF treatment in acute EAE in mice, suggesting that HCAR2 may, indeed, be a principal target in DMF therapy of EAE (35). Our results in this report, highlighting the importance of the Nrf2-independent immunologic and clinical effects of DMF, are complementary with studies that identified HCAR2 as a potential target for DMF (34–36). However, the clinical and immunologic effects of DMF treatment of EAE were not completely inhibited by HCAR2 deficiency (35), indicating that HCAR2 is not the sole target of DMF therapy. One should recognize that the therapeutic response to DMF in MS is dose dependent (37), and it is possible that individual targets may vary in their sensitivity to different levels of MMF exposure. In this study, the plasma MMF levels obtained in DMF treatment of mice were severalfold higher than those in healthy volunteers treated with DMF doses used in MS (38). Of interest, when DMF was administered in vivo at a higher dose than was used in either our investigation of Nrf2-deficient mice or the study that evaluated HCAR2-deficient mice (35), it was observed that a majority of genes induced in spleen cells by DMF treatment were Nrf2 dependent (4). Thus, in vivo DMF treatment likely mediates its effects through activation of both Nrf2 and HCAR2, and possibly additional targets. Just as MMF covalently attaches to cysteine 151 of Keap1, it also conjugates to other Keap1 cysteine residues (3) and may therefore also modify other cysteine-containing proteins involved in immune regulation. Our results in this report should stimulate exploration for additional potential targets of DMF therapy.

Materials and Methods

Mice. C57BL/6 female mice, aged 5–8 weeks, were purchased from The Jackson Laboratory. Nrf2-deficient mice were provided by Jeff Chan (University of California, Irvine, CA). C57BL/6J MOG p35–55-specific T-cell receptor Tg (TCR^{MOG}, 2D2) mice (39) were provided by V. K. Kuchroo (Harvard, Boston, MA). C57BL/6J MOG-BCR knock-in (IgH$^{MOG\text{-}ki}$, also referred to as Th) mice (40) were provided by H. Wekerle (Max Planck Institute, Munich, Germany).

Peptide. Mouse MOG p35–55 (MEVGWYRSPFSRVVHLYRNGK) was synthesized by Auspep.

EAE Induction. Seven- to 10-wk-old female C57BL/6 mice were injected s.c. with 50 μg of MOG p35–55 in complete Freund's adjuvant (CFA) (DIFCO Laboratories) containing 400 μg of *Mycobacterium tuberculosis* H37Ra. Nrf2$^{-/-}$ mice were immunized with 25 μg of MOG p35–55 in CFA containing 133 μg of H37Ra or immunized with 50 μg of MOG p35–55 containing 400 μg of H37Ra. After immunization and 2 days later, mice received 200 ng of pertussis toxin i.p.

DMF Treatment. DMF (Sigma-Aldrich), used in an emulsion of 0.6% Methocel, was administered daily (100 mg/kg) by oral gavage. Treatment began on the day of EAE induction. Control mice received a similar volume of vehicle (0.6% Methocel) daily. In other experiments (in vitro APC–T-cell assays), mice were treated for 9 days with DMF or vehicle before isolation of specific cell subsets.

Determination of Blood and Tissue MMF Concentrations. Plasma, brain, and spleen samples were collected 30 min after oral administration of DMF. Sodium fluoride (10 mg/mL) was added to each sample. Samples were centrifuged within 30 minutes of collection at 4 °C for 15 minutes at 1,500 × *g*, transferred into prechilled tubes, immediately frozen on dry ice, and maintained frozen (less than or equal to −80 °C) until analysis. MMF levels were quantitated by HPLC coupled to tandem mass spectrometry.

Monocyte Isolation and Coculture with Naive T Cells. Splenic CD11b$^+$ cells were separated from DMF- or vehicle-treated mice using magnetic beads (Miltenyi). Monocyte preparations were evaluated for expression of CD11b, CD11c, B220,

Table 1. Oral DMF treatment inhibits development of spontaneous EAE

Treatment*	Incidence	Mean day of onset	Median day of onset	Mean disease score†	Mean maximal score‡
Vehicle	7/15	47 ± 8.57	32	1.37 ± 0.47	2.93 ± 0.58
DMF	3/17	56 ± 12.24	59	0.35 ± 0.21	2.00 ± 0.58

*IgH$^{MOG\text{-}ki}$ (Th) × TCR^{MOG} (2D2) mice were treated with oral DMF or vehicle daily beginning 18 days after birth and monitored daily.
†Mean clinical scores of DMF-treated mice were reduced in comparison with vehicle-treated mice. $P = 0.024$, two-sample t test.
‡Mean scores for mice with EAE only. Maximal and final scores were identical.

CD3 (BD Biosciences). Purity of monocytes used as APCs was routinely around 95%. In some experiments, spleen cells were depleted of $CD3^+$ cells by magnetic cell sorting and used as APCs. APCs were cocultured with $CD4^+CD62L^+CD44^+$ naive T cells, magnetically sorted from TCR^{MOG} mice (purity greater than 96%), and their respective antigen for 3 days at a ratio of 25:1. For Th1 cells, 10 ng/mL IL-12 (R&D Systems) was added.

Isolation of CNS Infiltrating Mononuclear Cells. Isolation of CNS infiltrating cells was performed as described previously (41). Mice were perfused using PBS, CNS tissue was cut into small pieces and incubated for 20 min in Hanks' buffered saline solution containing collagenase. Homogenate was resuspended in 30% (vol/vol) Percoll (Sigma-Aldrich) and underlain with 70% (vol/vol) Percoll and centrifuged for 25 min, and lymphocytes were harvested from the resulting interface and analyzed by flow cytometry on a FACS Canto (BD Biosciences).

Histopathology. Brains and spinal cords of mice were fixed in 10% (vol/vol) neutral-buffered formalin, sectioned, and stained with Luxol fast blue and hematoxylin and eosin (H&E). Meningeal and parenchymal inflammatory lesions and areas of demyelination were quantified as previously described (32, 42).

Flow Cytometry. Single-cell suspensions were incubated with anti-CD16/CD32 (1:100) to prevent nonspecific antibody binding, stained with anti-CD4, -CD62L, -CD44, -CD11c, -CD11b, -B220, -Gr1 (Ly6C/G), and -CD3 (all 1:100) (eBioscience). Intracellular cytokine production by $CD4^+$ T cells and APCs was analyzed by monitoring the expression of IFN-γ, IL-17, GM-CSF, IL-6, IL-12/23 (p40) TNF, and iNOS (1:100) (eBioscience). For intracellular cytokine staining, T cells were stimulated with phorbol 12-myristate 13-acetate (50 ng/mL) plus ionomycin (500 ng/mL) in the presence of monensin (GolgiStop; BD Biosciences). $CD11b^+$ cells were stimulated with LPS (1 μg/mL) for 12 h in the presence of monensin. Cells were analyzed using a FACSCanto flow cytometer.

Statistical Analysis. Data are shown as mean ± SEM or SD. Significance between groups was examined using Student's *t* test or the Mann–Whitney *U* test. A value of $P \leq 0.05$ was considered significant.

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Supporting Information

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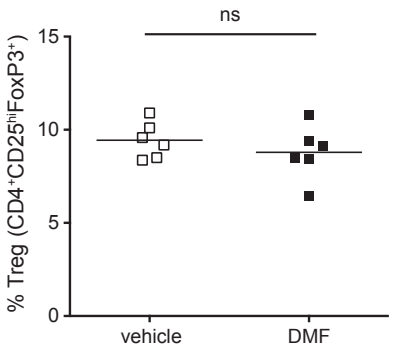

Fig. S1. DMF treatment does not alter regulatory T-cell frequency in WT mice. FACS analysis of $CD4^+CD25^+FoxP3^+$ regulatory T cells (Treg) isolated from spleens of WT mice treated with DMF (filled squares) or vehicle (open squares) for 9 days after immunization with MOG p35–55. Quantification of Treg cells is shown. Individual data points and mean values of $n = 6$ mice per group are displayed.

Table S1. Oral DMF treatment inhibits development of EAE in both WT and $Nrf2^{-/-}$ mice

Mice	Treatment	Incidence	Mean day of onset	Mean maximal severity*	Mean severity (day 14)*	Mean severity (day 20)*	Cumulative score (days 0–23)*
WT	Vehicle	4/5	12.5 ± 2.50	3.00 ± 0.89	2.20 ± 0.97	2.15 ± 0.89	149.25
	DMF	2/5	16.5 ± 3.50	1.20 ± 0.73	0.55 ± 0.55	0.80 ± 0.51	36.25
$Nrf2^{-/-}$	Vehicle	5/5	14.8 ± 1.46	3.15 ± 0.22	1.95 ± 0.81	2.45 ± 0.41	120.25
	DMF	2/5	16.5 ± 1.50	0.65 ± 0.53	0.00 ± 0.00	0.30 ± 0.30	10.25

EAE was induced in WT and $Nrf2^{-/-}$ mice by immunization with 50 μg of MOG p35–55 containing 400 μg of *M. tuberculosis* H37Ra. After immunization and 2 days later, mice received 200 ng of pertussis toxin, i.p.
*Includes all five mice per group.