

# Dimethyl fumarate suppresses granulocyte macrophage colony-stimulating factor–producing Th1 cells in CNS neuroinflammation

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## Abstract

### Objective

To study the immunomodulatory effect of dimethyl fumarate (DF) on granulocyte macrophage colony-stimulating factor (GM-CSF) production in CD4<sup>+</sup> T cells in experimental autoimmune encephalomyelitis (EAE) and human peripheral blood mononuclear cells (PBMCs).

### Methods

We collected splenocytes and CD4<sup>+</sup> T cells from C57BL/6 wild-type and interferon (IFN)- $\gamma$ -deficient mice. For human PBMCs, venous blood was collected from healthy donors, and PBMCs were collected using the Percoll gradient method. Cells were cultured with anti-CD3/28 in the presence/absence of DF for 3 to 5 days. Cells were stained and analyzed by flow cytometry. Cytokines were measured by ELISA in cell supernatants. For in vivo experiments, EAE was induced by myelin oligodendrocyte glycoprotein<sub>35–55</sub> and mice were treated with oral DF or vehicle daily.

### Results

DF acts directly on CD4<sup>+</sup> T cells and suppresses GM-CSF–producing Th1 not Th17 or single GM-CSF<sup>+</sup> T cells in EAE. In addition, GM-CSF suppression depends on the IFN- $\gamma$  pathway. We also show that DF specifically suppresses Th1 and GM-CSF–producing Th1 cells in PBMCs from healthy donors.

### Conclusions

We suggest that DF exclusively suppresses GM-CSF–producing Th1 cells in both animal and human CD4<sup>+</sup> T cells through an IFN- $\gamma$ -dependent pathway. These findings indicate that DF has a better therapeutic effect on patients with Th1-dominant immunophenotype. However, future longitudinal study to validate this finding in MS is needed.

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## Glossary

DF = dimethyl fumarate; EAE = experimental autoimmune encephalomyelitis; GM-CSF = granulocyte macrophage colony-stimulating factor; MNC = mononuclear cell; RRMS = relapsing-remitting MS; WT = wild type.

MS, the leading cause of disability in young adults, is an inflammatory demyelinating disease with axonal injury in the CNS.<sup>1</sup> The rapidly growing number of diagnosed cases and available immune-modifying therapies in recent years is motivating scientists and clinicians to further study the mechanism of action of currently approved medications to discover novel underlying pathways that can be targeted to develop new and more efficient treatments for MS.

Patients with relapsing-remitting MS (RRMS) patients are treated with oral dimethyl fumarate (DF) since 2013 in the United States,<sup>2</sup> and DF is added to the armamentarium of disease-modifying therapies for MS. In terms of an underlying mechanism of action, DF inhibits interleukin (IL)-12p35 and IL-23p19 transcription in dendritic cells. This leads to the generation of type-2 dendritic cells, which indirectly increases IL-4<sup>+</sup> Th2 cells in both experimental autoimmune encephalomyelitis (EAE) and human cells and ameliorates inflammatory responses.<sup>3,4</sup> In addition, EAE mice treated with DF show a significant reduction in the total number of interferon (IFN)- $\gamma$ -, IL-17-, and granulocyte macrophage colony-stimulating factor (GM-CSF)-producing CD4<sup>+</sup> T cells among CNS-infiltrating cells.<sup>5</sup> These findings add to the role of DF in decreasing the inflammatory profile of T cells indirectly by changing the phenotype of antigen presenting cells, but the direct effect of DF on pathogenic T-cell subtypes has not been adequately studied.

Among the various types of immune cells involved in the pathogenesis of EAE, T cells have been the main focus of research because of their pathogenic role in animal models of demyelination and the abundance of T cells in active demyelinating brain lesions in patients with MS.<sup>6,7</sup>

Th1 and Th17 cells are considered the main culprits in EAE pathogenicity,<sup>8,9</sup> and their signature cytokines, IFN- $\gamma$  and IL-17, play a role in disease pathogenesis.<sup>10,11</sup> Lack of IFN- $\gamma$  leads to more severe EAE, and the absence of IL-17 does not affect EAE development.<sup>12,13</sup> Given that neither Th1 (IFN- $\gamma$ ) nor Th17 (IL-17) signature cytokines are required for the development of EAE,<sup>14</sup> we and others have shown that GM-CSF is an essential cytokine for EAE induction. GM-CSF-producing CD4<sup>+</sup> T cells can effectively induce EAE by passive transfer, and lack of GM-CSF in Th1 or Th17 cells abrogates their encephalitogenicity. In addition, GM-CSF-deficient mice are resistant to EAE induction.<sup>15,16</sup>

Here, we studied the direct effect of DF on CD4<sup>+</sup> T cells and their cytokine profiles. We demonstrate that DF significantly decreases GM-CSF in CD4<sup>+</sup> T cells in vitro and in vivo. Further evaluation showed that the decrease in GM-CSF is more prominent in Th1 than that in Th17 or single GM-

CSF<sup>+</sup>CD4<sup>+</sup> T cells. In addition, the suppressive effect of DF on GM-CSF was abrogated by the lack of IFN- $\gamma$ . We also evaluated the effect of DF on human PBMCs and confirmed that DF significantly decreases GM-CSF in Th1 cells.

## Methods

### Mice

Female C57BL/6 mice, 7–9 weeks old, were obtained from Jackson Laboratory (Bar Harbor, ME). Mice were housed at animal facility at Thomas Jefferson University with water and food ad libitum. All experimental procedures were approved by the Institutional Animal Care and Use Committee.

### EAE induction and clinical evaluation

Mice were immunized subcutaneously with 200  $\mu$ g of myelin oligodendrocyte glycoprotein (MOG)<sub>35–55</sub> (GenScript, Piscataway, NJ) emulsified in complete Freund's adjuvant (DIFCO Laboratories) containing *Mycobacterium tuberculosis* H37Ra (5 mg/mL; DIFCO Laboratories, Detroit, MI). In addition, mice were intraperitoneally injected with 200 ng of pertussis toxin at 0 and 48 hours after immunization. Clinical EAE was assessed daily in a blind fashion using the clinical scoring system from 0: normal to 5: death as described previously.<sup>17</sup>

### DF treatment

DF (Sigma-Aldrich, St. Louis, MO) was used in an emulsion of 0.8% methylcellulose. For in vivo treatment, DF solution was administered by oral gavage twice daily (750  $\mu$ g for 25 g mice in 200  $\mu$ L volume), starting the day of EAE induction until termination (day 23 post immunization). The solution was prepared early everyday and stored at 4°C. Mice receiving vehicle were used as controls.

### Isolation of CNS-infiltrating mononuclear cells and splenocytes

To collect mononuclear cells (MNCs), mice were extensively perfused at day 23 post immunization with ice-cold phosphate buffered saline. For spleen cells, spleens were collected and disrupted in 70  $\mu$ m cell strainer before RBC lysis. To collect CNS-infiltrating cells, brains and spinal cords were removed, thinly minced in Liberase TL (Roche, Indianapolis, IN), and incubated at 37°C for 30 minutes. Then, the CNS was strained through a 70  $\mu$ m cell strainer, and MNCs were enriched by centrifugation on a 70/30 Percoll gradient for 30 minutes at 2,000 rpm.<sup>17</sup>

### Splenocyte and lymphocyte culture and ELISA experiments

Spleen cells were cultured in Iscove Modified Dulbecco medium (Gibco, Gaithersburg, MD) supplemented with 10% fetal bovine

serum (Gibco), 5% L-glutamine (Gibco), 5% penicillin/streptomycin (Gibco), and  $\beta$ -mercaptoethanol (Sigma). Spleen cells were stimulated with 1  $\mu\text{g}/\text{mL}$  anti-CD3/CD28 agonistic antibodies for 72 hours at 37°C, 5% CO<sub>2</sub>. For antigen-specific recall response, CNS-infiltrating cells from EAE mice, at a concentration of 1  $\times 10^6$  cells/mL, were stimulated with 10  $\mu\text{g}/\text{mL}$  MOG<sub>35–55</sub> at 37°C, 5% CO<sub>2</sub>. At the indicated time points, supernatants were collected and centrifuged to eliminate cellular debris. Cytokine levels in supernatants were measured by ELISA. We analyzed the following cytokines: GM-CSF (R&D Systems, Minneapolis, MN, DY415), human GM-CSF (R&D Systems DY215), IFN- $\gamma$  (R&D Systems DY485), human IFN- $\gamma$  (R&D Systems DY285), IL-17 (R&D Systems DY421), and human IL-17 (R&D Systems DY317).

### Purification of CD4<sup>+</sup> T cells

MNCs from spleens were subjected to positive selection according to the manufacturer's recommendation (CD4 microbeads; Miltenyi Biotec, Auburn, CA, 130-049-201).

### Proliferation assay

Splenocytes were cultured in 96-well plates at a concentration of 1  $\times 10^6$  cells/mL and stimulated with anti-CD3/28 (1  $\mu\text{g}/\text{mL}$  each) for 48 hours. Cells were pulsed with 0.5  $\mu\text{Ci}$  of <sup>3</sup>H-thymidine for the last 18 hours. Thymidine incorporation was measured using a scintillation counter.

### Human blood samples and cell culture

All subjects provided informed consent before their participation in the current study. All human studies were approved by the Thomas Jefferson University Institutional Review Board. Blood was obtained from 8 healthy donors at the Department of Neurology, Thomas Jefferson University. PBMCs were collected by Ficoll-Paque Plus density gradient centrifugation. PBMCs were washed and cultured at a density of 1  $\times 10^6$  cells/mL in X-VIVO 15 serum-free medium. Cells were stimulated with 1  $\mu\text{g}/\text{mL}$  of anti-CD3 (HIT3a; BD Biosciences, San Jose, CA) and 1  $\mu\text{g}/\text{mL}$  anti-CD28 (CD28.2; BD Biosciences) for 120 hours in the presence of 0.1  $\mu\text{g}/\text{mL}$  of DF (Sigma-Aldrich).

### Flow cytometry experiments

For analysis of surface markers, MNCs were surface stained in flow cytometry (FACS) buffer (phosphate buffered saline containing 3% fetal bovine serum and 0.02% NaN<sub>3</sub>) with fluorescence antibodies for 20 minutes at 4°C. For analysis of cytokine production, MNCs were activated with phorbol 12-myristate 13-acetate (50 ng/mL), ionomycin (500 ng/mL), and Golgi-Stop (1  $\mu\text{g}/\text{mL}$ ) for 4 hours. Then, cells were surface stained as mentioned previously, before being fixed and permeabilized with buffers (Fix/Perm; ThermoFisher, Frederick, MD). Finally, cells were incubated with cytokine-specific antibodies for 30 minutes at 4°C. All antibodies and reagents used in this section were purchased from BD Biosciences, except for phorbol 12-myristate 13-acetate and ionomycin (both from Sigma-Aldrich). Samples were acquired on an FACS Aria equipment (BD Biosciences). Analyses were performed with FlowJo software (Tree Star, Ashland, OR).

### Statistical analysis

Data are presented as mean  $\pm$  standard error of the mean. Statistical analysis was performed using one-way analysis of variance or the Kruskal-Wallis test (all analyses performed with GraphPad Prism software, San Diego, CA). A probability level (*p* value) of \*: *p* < 0.05, \*\*: *p* < 0.01, and \*\*\*: *p* < 0.001 was statistically significant for all tests. All error bars represent standard error of the mean. For human results, we used pre-treatment and posttreatment paired *t* test analysis.

### Data availability

Raw FACS and ELISA files are not included in this article. Any unpublished and anonymized data will be shared upon request from a qualified investigator.

## Results

### DF acts directly on CD4<sup>+</sup> T cells and decreases GM-CSF production

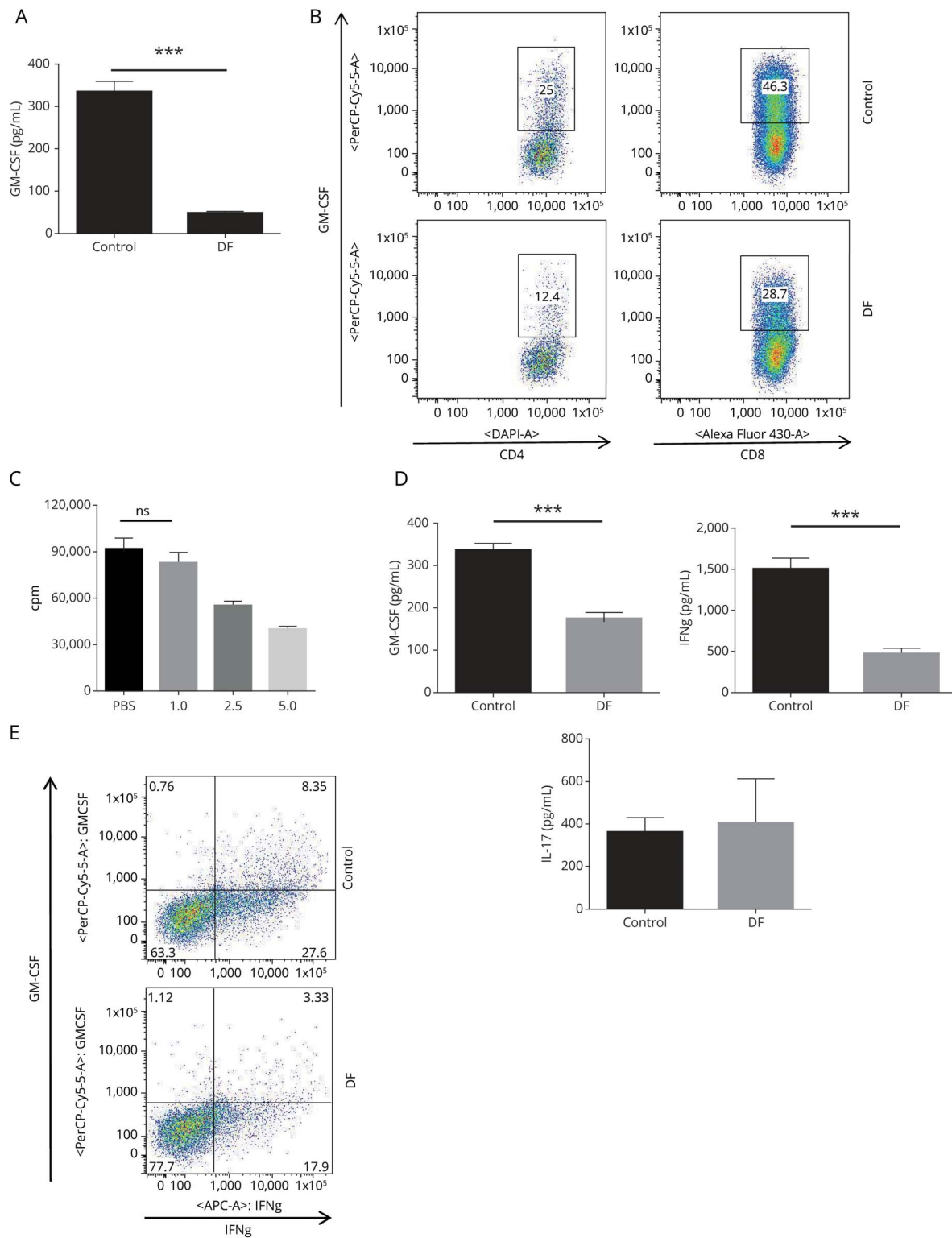
To evaluate the role of DF on GM-CSF production in T cells, we first collected splenocytes from B6 wild-type (WT) mice and cultured them with anti-CD3/CD28 in the presence and absence of DF (1  $\mu\text{g}/\text{mL}$ ) for 72 hours. We found that GM-CSF in culture supernatants was significantly lower in DF-treated cells compared with controls (figure 1A). FACS analysis of the same cells showed a significant reduction of GM-CSF in both CD4<sup>+</sup> and CD8<sup>+</sup> T cells (figure 1B). As previously shown in several studies, DF induces T-cell lymphopenia in patients with MS.<sup>18</sup> To confirm that the effect of DF on the T-cell cytokine profile is not dependent on its lymphopenic effect, we performed a proliferation assay on activated splenocytes with anti-CD3/CD28 in the presence or absence of varying doses of DF. Administration of DF at a dose of 1  $\mu\text{g}/\text{mL}$ , which we used in all our in vitro experiments, did not show a significant suppressive effect on splenocyte proliferation (figure 1C).

Following the above-mentioned experiment, we studied how DF directly affects purified CD4<sup>+</sup> T cells from mice. CD4<sup>+</sup> T cells were isolated and cultured with anti-CD3/CD28 Abs with or without DF for 72 hours. We measured IFN- $\gamma$ , GM-CSF, and IL-17 in the supernatant after the incubation period. We found that DF significantly decreased GM-CSF and IFN- $\gamma$ , but not IL-17, in purified CD4<sup>+</sup> T cells compared with controls (figure 1D). In addition, FACS analysis of the same cells demonstrated that DF decreased GM-CSF in IFN- $\gamma$ <sup>+</sup>CD4<sup>+</sup> T (Th1) cells (figure 1E).

### DF treatment significantly decreased GM-CSF<sup>+</sup> Th1 cells in CNS-infiltrating cells

To analyze the immunomodulatory effect of DF treatment in EAE, we first immunized all mice and then treated half of them with oral DF in an emulsion of 0.8% methylcellulose (DF group) and the other half with a similar volume of 0.8% methylcellulose vehicle (control group). Mice in the control group developed EAE, with an average severity of around 2.5 in scoring (score range from 1.25 to 4 with a median score of 2.625). By contrast, DF-treated mice showed very mild clinical

**Figure 1** DF decreases GM-CSF production and GM-CSF+Th1 cells in mouse splenocytes



Splenocytes from wild-type (WT) mice were stimulated with 1  $\mu\text{g/mL}$  of anti-CD3/28 antibody in the presence or absence of DF (1  $\mu\text{g/mL}$ ) for 72 hours. (A) GM-CSF levels in culture supernatants were determined by ELISA. Data are representative of 3 experiments (mean  $\pm$  SEM;  $n = 4$  replicates per group). (B) Cells were stimulated with ionomycin/PMA and GolgiPlug in the last 4 hours of culture. After intracellular staining for GM-CSF, flow cytometry analysis of gated CD4<sup>+</sup> and CD8<sup>+</sup> T cells showed that DF administration decreased GM-CSF production in both CD4<sup>+</sup> and CD8<sup>+</sup> T cells. (C) Proliferation assay on splenocytes treated with different DF doses. DF did not affect proliferation of splenocytes at a dosage of 1  $\mu\text{g/mL}$ . (D) CD4<sup>+</sup> T cells were purified from WT spleen cells and stimulated with anti-CD3/28 in the presence or absence of DF. IL-17, IFN- $\gamma$ , and GM-CSF were measured in cell supernatants. DF decreased IFN- $\gamma$  and GM-CSF, but not IL-17. (E) Flow cytometry analysis demonstrated that DF significantly reduced GM-CSF production in IFN- $\gamma$ -producing CD4<sup>+</sup> (Th1) cells. DF = dimethyl fumarate; GM-CSF = granulocyte macrophage colony-stimulating factor; IFN = interferon; IL = interleukin; PMA = phorbol 12-myristate 13-acetate; SEM = standard error of the mean. \*\*\* $p < 0.001$ .



signs with an average clinical score of  $\leq 1$  (score range from 0 to 1.5 with a median score of 0.75) (figure 2A).

We killed the mice at disease peak (day 23) and assessed the number of infiltrating MNCs in the CNS of both DF-treated and control groups. In addition to counting the total number of infiltrating cells, we evaluated the immunophenotype of those cells by flow cytometry. The total number of CD4<sup>+</sup> T cells and GM-CSF<sup>+</sup>CD4<sup>+</sup> T cells was dramatically decreased in the CNS of the DF-treated group (figure 2B). We also looked at IFN- $\gamma$  and IL-17 in CNS-infiltrating CD4<sup>+</sup> T cells and, interestingly, found that DF decreases GM-CSF<sup>+</sup>CD4<sup>+</sup> cells that coproduce IFN- $\gamma$ , but not IL-17 (figure 2C), given that DF has a greater suppressive effect *in vivo* on GM-CSF-producing Th1 cells than that on Th17 cells.

Splenocytes from treated and control mice were isolated, cultured, and stimulated with MOG<sub>33–35</sub> for 72 hours. GM-CSF, IFN- $\gamma$ , and IL-17 were measured in culture supernatants by ELISA. As shown in figure 2D, splenocytes from DF-treated mice produced a significantly decreased amount of GM-CSF and IFN- $\gamma$ , but not IL-17.

### DF requires an intact IFN- $\gamma$ pathway to suppress GM-CSF in CD4<sup>+</sup> T cells

Based on the observation that DF suppresses GM-CSF predominantly in Th1 cells, and to further evaluate the underlying mechanism of the suppressive effect of DF on GM-CSF, we investigated whether IFN- $\gamma$  plays a role in this phenomenon.

WT and IFN- $\gamma$ -deficient splenocytes were cultured and stimulated with anti-CD3/28 in the presence or absence of DF. Although DF suppressed GM-CSF-producing CD4<sup>+</sup> T cells and GM-CSF cytokines in the supernatant of WT splenocytes, the lack of IFN- $\gamma$  abrogated the effect of DF on GM-CSF in CD4<sup>+</sup> T cells. This finding implies that IFN- $\gamma$  plays a crucial role in the suppressive effect of DF on GM-CSF (figure 3, A and B).

### DF decreases Th1 and GM-CSF-producing Th1 cells in human PBMCs

To determine the best dosage of DF in human *in vitro* experiments, we performed a proliferation assay and treated cells with 10, 1, and 0.1  $\mu\text{g}/\text{mL}$  of DF as described previously. Based on our result, 0.1  $\mu\text{g}/\text{mL}$  DF had the least toxic effects on human PBMCs (data not shown). To evaluate the effect of DF on human PBMCs, we cultured and stimulated PBMCs from healthy donors with human anti-CD3/28 with or without DF (0.1  $\mu\text{g}/\text{mL}$ ) for 120 hours. Cells were analyzed with flow cytometry after 5 days, and multiple cytokines were measured by ELISA in cell supernatants. For statistical analysis, we used the paired *t* test to compare pretreatment and posttreatment results.

DF treatment significantly reduced IFN- $\gamma$ <sup>+</sup>CD4<sup>+</sup> (Th1) T cells, and ELISA analysis of PBMC culture supernatants demonstrated that DF significantly decreased IFN- $\gamma$  production (figure 3C). In terms of GM-CSF-producing CD4<sup>+</sup> T cells, DF significantly decreased GM-CSF<sup>+</sup> Th1 cells with no effect on total GM-CSF-producing CD4<sup>+</sup> T cells, implying that the suppressive effect of DF on GM-CSF is mostly on Th1 cells (figure 3D).

## Discussion

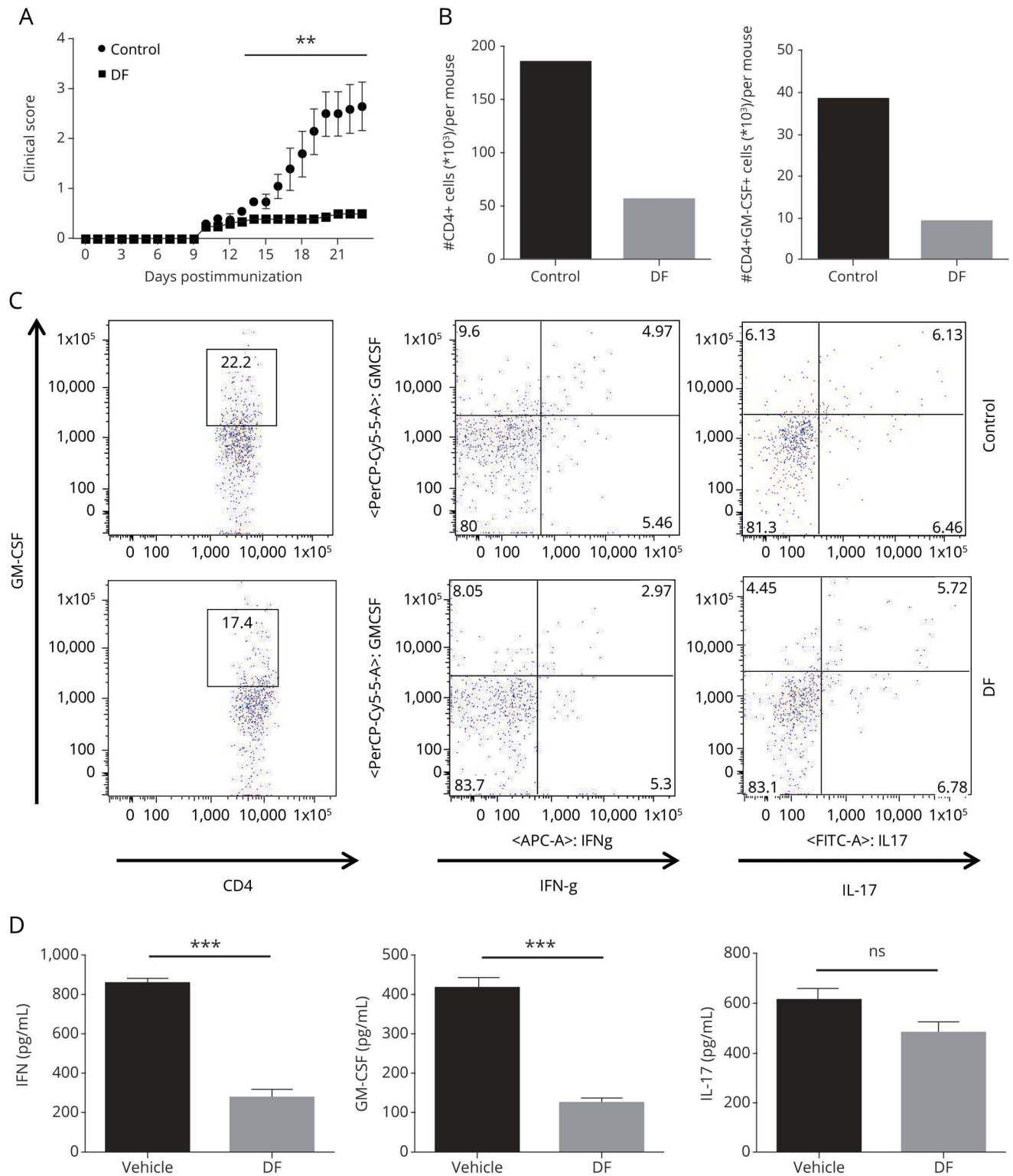
DF was approved by the Food and Drug Administration for the treatment of MS in 2013, and its efficacy has been well demonstrated. During 2 years of treatment, DF reduced clinical relapse and lesion frequency while improving health-related quality of life in adults with RRMS.<sup>19,20</sup> However, the underlying therapeutic mechanism of DF is still under investigation. DF has neuroprotective effects,<sup>4,21,22</sup> including reducing spinal cord inflammation and protecting myelin and neurons.<sup>4,23</sup> Also, researchers who have studied the Nrf2 transcriptional pathway and oxidative stress that DF modulates<sup>4,21,24</sup> found that both DF and its active metabolite, monomethyl fumarate, induce the reactive oxygen species scavenger glutathione in oligodendrocytes, astrocytes, and hippocampal cells.<sup>22,25,26</sup> Other pathways have also been reported, including the hydroxycarboxylic acid receptor 2 pathway and nuclear factor kappa-light-chain-enhancer of activated B cells.<sup>23,27,28</sup> DF has been shown to promote Th2 cytokine profiles,<sup>3,22,29</sup> and transferring DF-induced IL-17A<sup>low</sup>IFN- $\gamma$ <sup>low</sup>IL-4<sup>+</sup>CD4<sup>+</sup> T cells has demonstrated its therapeutic effect in EAE.<sup>30</sup>

In patients with RRMS after 6 months of DF treatment, the proportion of Th2 cells was increased among memory T cells but with a decrease in the proportion of Th1 and with no change in Th17 cell proportion among memory T cells.<sup>31</sup> Another study, however, showed a decrease in CD4<sup>+</sup> T cells producing IFN- $\gamma$ , IL-17, and GM-CSF, with no significant change in IL-10 and IL-4 after 12 months.<sup>32</sup> We also found that direct administration of DF decreased proinflammatory cytokine production in the supernatant of human PBMCs obtained from healthy subjects. When we took a closer look at CD4<sup>+</sup> T-cell subsets, DF suppressed only GM-CSF<sup>+</sup>Th1 cells, but not GM-CSF<sup>+</sup>Th17 or GM-CSF<sup>only</sup>CD4<sup>+</sup> T cells. One limitation of our study is that the human data are based on very small number of samples from healthy donors. Further studies are necessary to confirm the effect of DF on GM-CSF-producing cells from patients with MS.

Although IL-17 and IFN- $\gamma$  are implicated in MS, mice still develop EAE even in the absence of these cytokines.<sup>33,34</sup> Previous studies have shown that GM-CSF secreted by CNS-infiltrating T helper cells is essential for EAE, even more so than IL-17 or IFN- $\gamma$ . Lack of GM-CSF or GM-CSF receptor abrogates EAE development, and adoptively transferred GM-CSF-deficient Th1 or Th17 cells do not induce EAE.<sup>15</sup> We showed that DF caused a decrease in GM-CSF<sup>+</sup> Th1 cells in both the periphery and CNS-infiltrating cells during EAE, and given the fact that GM-CSF-expressing cells are the crucial determinant of EAE susceptibility and progression, one of the underlying therapeutic mechanisms of DF appears to be through suppression of GM-CSF<sup>+</sup> Th1 cells.

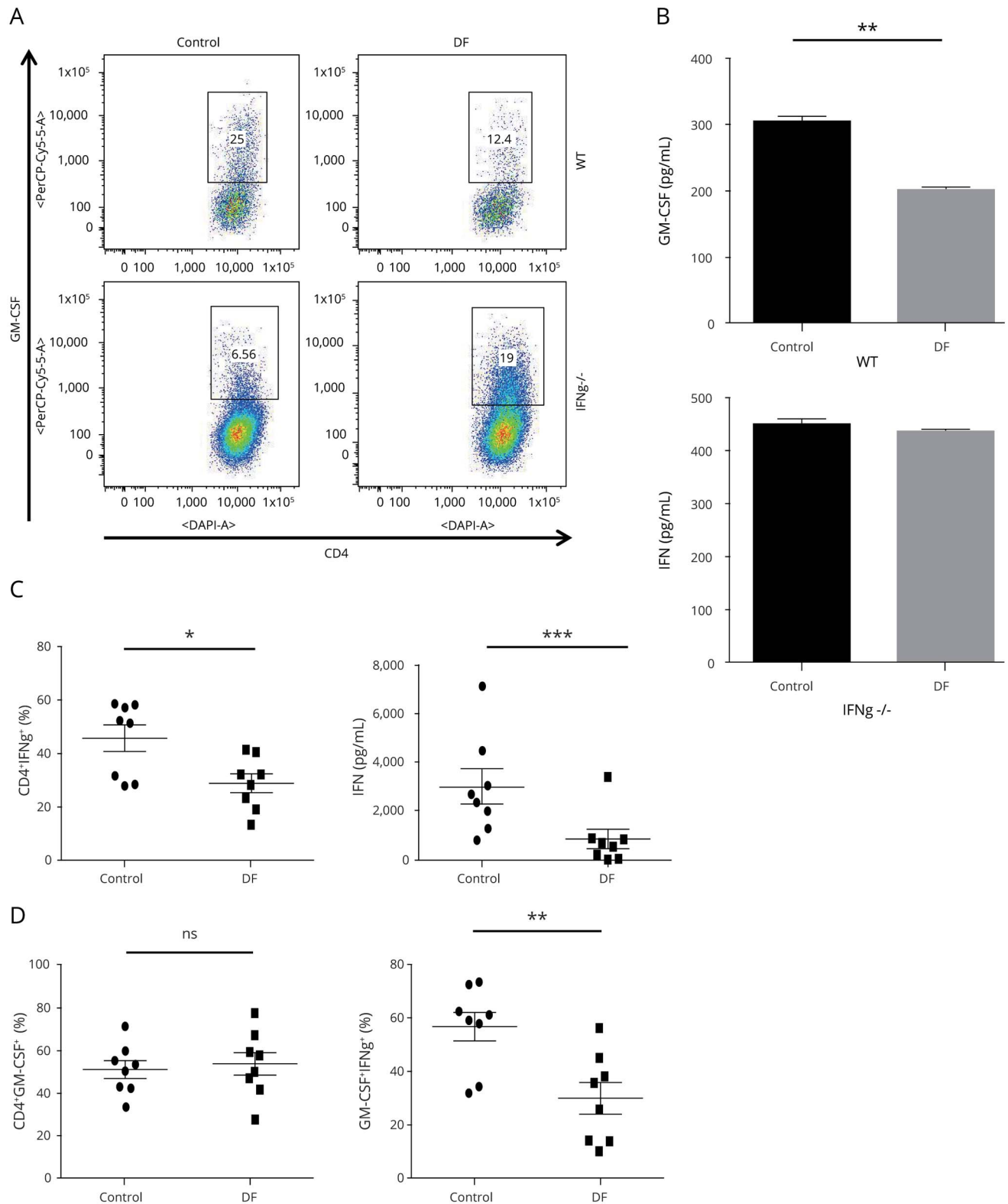
In a variety of T helper cell populations, different transcription factors, including bhlhe40, ROR $\gamma$ t, T-bet, GATA-3, and STAT5,<sup>35–38</sup> are active at multiple time points. It has been reported that IL-7-activated STAT5 promotes GM-CSF generation of CD4<sup>+</sup> T cells, which differ from ROR $\gamma$ t-, T-bet-, and GATA-3-promoted GM-CSF-producing CD4<sup>+</sup> T cells (Sheng

**Figure 2** DF ameliorates experimental autoimmune encephalomyelitis and suppresses GM-CSF-producing Th1 cells in the CNS



(A) Wild-type B6 mice were immunized with 200  $\mu\text{g}$  of MOG<sub>35-55</sub> and were treated with DF (treatment group) or placebo (control group) by oral administration starting on the day of immunization. Clinical signs were scored daily following a 0–5 scale. Data represent 1 of 3 experiments and the mean clinical scores  $\pm$  SEM ( $n = 5$  each group). (B) CNS-infiltrating cells from DF-treated and control mice were isolated on day 23 of disease. DF treatment significantly decreased total CD4<sup>+</sup> T cells and GM-CSF-producing CD4<sup>+</sup> T cells among CNS-infiltrating cells. (C) Cells were stained with GM-CSF, IFN- $\gamma$ , and IL-17A antibodies and analyzed by flow cytometry. We found that GM-CSF was decreased in Th1 cells, but not Th17 cells, after DF treatment. (D) Splenocytes from both treated and control groups were collected and cultured/stimulated with 25  $\mu\text{g}/\text{mL}$  MOG<sub>35-55</sub> for 72 hours. Concentrations of GM-CSF, IFN- $\gamma$ , and IL-17A in culture supernatants were measured by ELISA. There was significantly less GM-CSF and IFN- $\gamma$  in treated mice compared with controls.  $^{**}p < 0.01$ ; and  $^{***}p < 0.001$ . One of 2 experiments is shown. DF = dimethyl fumarate; GM-CSF = granulocyte macrophage colony-stimulating factor; IL = interleukin; MOG = myelin oligodendrocyte glycoprotein; SEM = standard error of the mean.

**Figure 3** The suppressive effect of DF on GM-CSF depends on IFN- $\gamma$  in murine cells



(A) Splenocytes were isolated from wild-type (WT) and IFN- $\gamma$ <sup>-/-</sup> mouse and stimulated with 1  $\mu$ g/mL of anti-CD3/28 antibodies in the presence or absence of DF (1  $\mu$ g/mL) for 72 hours. DF treatment significantly decreased GM-CSF in CD4<sup>+</sup> T cells from WT but not CD4<sup>+</sup> T cells from IFN- $\gamma$ <sup>-/-</sup> mice. (B) Measurement of GM-CSF in cell supernatants by ELISA confirmed the same finding. Data are mean  $\pm$  SEM and representative of 1 of 3 experiments. DF reduced GM-CSF production and GM-CSF<sup>+</sup>Th1 cells in human PBMCs. PBMCs from healthy donors (n = 8) were stimulated with 1  $\mu$ g/mL of anti-CD3/anti-CD28 antibody in the presence or absence of DF (0.1  $\mu$ g/mL) for 120 hours. (C) Cells were stimulated with PMA/ionomycin and GolgiPlug in the last 4 hours of culture. Flow cytometry analysis showed that DF treatment decreased IFN- $\gamma$ <sup>+</sup>CD4<sup>+</sup> (Th1) cells. Measurement of IFN- $\gamma$  in cell supernatants confirmed that DF reduced IFN- $\gamma$  in human PBMC culture. (D) DF treatment did not affect the total percentage of GM-CSF<sup>+</sup>CD4<sup>+</sup> T cells but significantly decreased GM-CSF-producing Th1 cells. A paired t test was used for the statistical analysis of human PBMC results before and after treatment. DF = dimethyl fumarate; GM-CSF = granulocyte macrophage colony-stimulating factor; PMA = phorbol 12-myristate 13-acetate; SEM = standard error of the mean. \* $p$  < 0.05; \*\* $p$  < 0.01, and \*\*\* $p$  < 0.001.

et al., 2014). Assay for transposase-accessible chromatin using sequencing analysis of GM-CSF–expressing cells showed an open IFN- $\gamma$  locus in both GM-CSF– and ex-GM-CSF–expressing cells, and single-cell RNA-sequencing showed that pathogenic Th17 cells gain Th1-like characteristics once they enter the mouse-inflamed CNS.<sup>34,39</sup> On the epigenetic basis, GM-CSF–producing T cells are also more related to IFN- $\gamma$ <sup>+</sup> T cells. Here, we show that DF affects only GM-CSF<sup>+</sup>IFN- $\gamma$ <sup>+</sup>CD4<sup>+</sup> T cells, and its GM-CSF suppressive effect relies on an intact IFN- $\gamma$  pathway. Determining whether DF affects one or several transcription factors in this pathogenic T-cell subset could provide insights into its precise therapeutic mechanisms in MS. Future research on DF should focus on the molecular pathway of GM-CSF and IFN- $\gamma$  generation after DF treatment.

In conclusion, we evaluated the role of DF on CD4<sup>+</sup> T cells and its effects on GM-CSF production. For mouse splenocyte experiments, GM-CSF–producing Th1 T cells were reduced, but other GM-CSF–producing T cells were not. For EAE model experiments, DF decreased the percentage of GM-CSF<sup>+</sup> Th1 cells in the CNS. In human ex vivo experiments, GM-CSF<sup>+</sup> Th1 cells were reduced in healthy donor PBMCs after DF treatment; however, a larger study in patients before and after DF treatment is needed to validate current findings in MS. We also demonstrated that the suppressive effect of GM-CSF is through the IFN- $\gamma$  pathway. The molecular basis of the effects of DF on GM-CSF–producing T cells is unknown and merits further study.

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## Disclosure

F. Safavi, R. Thome, Z. Li, G.-X. Zhang, and A. Rostami report no disclosures relevant to the manuscript. Go to [Neurology.org/NN](http://Neurology.org/NN) for full disclosures.

## Publication history

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## Appendix Authors

Name	Location	Contribution
<b>Farinaz Safavi, MD, PhD</b>	Thomas Jefferson University, Philadelphia	Performed the experiments; analyzed the data; and drafted the manuscript for intellectual content
<b>Rodolfo Thome, PhD</b>	Thomas Jefferson University, Philadelphia	Revised the manuscript for intellectual content
<b>Zichen Li, MSc</b>	Thomas Jefferson University, Philadelphia	Major role in the acquisition of data

## Appendix (continued)

Name	Location	Contribution
<b>Guang-Xian Zhang, MD, PhD</b>	Thomas Jefferson University, Philadelphia	Interpreted the data and revised the manuscript for intellectual content
<b>Abdolmohamad Rostami, MD, PhD</b>	Thomas Jefferson University, Philadelphia	Interpreted the data and revised the manuscript for intellectual content

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