Dimethyl fumarate inhibits integrin α4 expression in multiple sclerosis models
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Abstract
Dimethyl fumarate is an orally bioavailable compound for the treatment of multiple sclerosis and psoriasis. A mechanism involving nuclear factor erythroid 2-like 2 activation has been proposed to account for its efficacy in multiple sclerosis. Here, we report that dimethyl fumarate inhibits expression of integrin α4 on circulating lymphocytes in experimental autoimmune encephalomyelitis mice and also on activated human Jurkat T cells in a manner distinct from nuclear factor erythroid 2-like 2 activation. Our results offer an alternative mechanism for the efficacy of dimethyl fumarate in multiple sclerosis.

Introduction
Dimethyl fumarate (DMF, Tecfidera) is an FDA-approved oral multiple sclerosis (MS) therapy that is one of a growing number of oral disease modifying therapies now available for the treatment of relapsing forms of MS.1 DMF is proposed to function through a transcription factor, nuclear factor erythroid 2-like 2 (Nrf2), that can regulate antioxidant genes, thereby preventing neurodegeneration by inhibiting reactive oxygen and nitrogen species.1 DMF is also used clinically in the treatment of psoriasis, where it alters cell adhesion molecules.2 Although DMF is efficacious in MS and psoriasis, DMF-associated progressive multifocal leukoencephalopathy (PML) cases were reported in psoriasis patients and in an MS patient.3,4 PML was also reported in MS patients receiving natalizumab (Tysabri), a monoclonal antibody against integrin α4 (ITGA4/CD49d). Natalizumab blocks very late antigen-4 (VLA-4, α4β1 heterodimer), which is expressed on leukocytes including lymphocytes and monocytes, and prevents lymphocyte entry into the central nervous system (CNS).5,6 In view of natalizumab’s action and the reported alteration of adhesion molecules by DMF, we tested the hypothesis that DMF might affect ITGA4 expression on circulating lymphocytes during murine experimental autoimmune encephalomyelitis (EAE) and in human Jurkat T cells.

Material and Methods
EAE induction and treatment
EAE was induced in 8-week-old C57BL/6 female mice with MOG35–55 peptide and clinical scores were recorded...
DMF (Sigma, cat. no. 242926, St. Louis, MO, USA) was suspended in 0.5% methylcellulose or 0.8% hydroxypropyl methylcellulose solution and sonicated to produce a uniform suspension. For prevention studies, mice were treated with DMF (50 and 100 mg/kg, p.o., s.i.d. or b.i.d.) as compared to fingolimod (0.1 mg/kg, p.o., s.i.d.) from days 0 to 30 after immunization. For therapeutic studies, mice were treated with DMF (100 mg/kg, p.o., b.i.d.) or fingolimod (0.1 mg/kg, p.o., s.i.d.) from days 17 to 30 after immunization. Control mice were treated with vehicle solution.

### Analysis of blood cells

Complete blood counts (CBCs) were carried out on fresh blood samples collected in BD Microtainer tubes (BD Biosciences, San Jose, CA, USA) containing EDTA (Ethylene-diaminetetraacetic acid) and processed at the University of California, San Diego Hematology and Coagulation Core Laboratory. White blood cells (WBCs) were immunolabeled with FITC (fluorescein isothiocyanate)-anti-CD3, PE (phycoerythrin)-anti-B220, or PerCP (Peridinin Chlorophyll Protein Complex)-Cy5-anti-CD49d antibodies (eBioscience, San Diego, CA, USA) and analyzed using a BD LSR-II flow cytometer (BD Biosciences, San Jose, CA, USA), with appropriate controls.

### Jurkat T cells

Jurkat T cells were maintained in (Roswell Park Memorial Institute) RPMI-1640 supplemented with 10% (fetal bovine serum) FBS, 50 μmol/L 2-mercaptoethanol and penicillin/streptomycin. Jurkat T cells (2.5 × 10^5 cells) were added to 96-well round bottom plates with varied concentrations of DMF and tert-Butylhydroquinone (tBHQ), an activator of Nrf2, in the presence or absence of anti-CD3 and anti-CD28 antibodies (1 μg/mL; BioLegend, San Diego, CA, USA). The cells were immunostained with FITC-anti-CD49d antibody (BioLegend) 42 h after stimulation, and analyzed using a BD LSR-II flow cytometer.
Statistical analyses

Results were expressed as means ± SEM. As appropriate, data were analyzed statistically by Student’s t test, Kruskal–Wallis test with Dunn’s multiple comparisons test, and two-way analysis of variance (ANOVA) with Bonferroni’s multiple comparisons test.

Results

DMF shows prevention but not therapeutic activity in EAE

Preventative effects of DMF on EAE were assessed by b.i.d. dosing as compared to a positive control (fingolimod). DMF and fingolimod significantly affected the development and severity of EAE as shown by lower clinical scores, decreased incidence, and delayed onset (Fig. 1A and B). We also tested the preventative effects of daily (s.i.d.) DMF dosing on EAE (Fig. 1C), however, this paradigm showed inefficient (albeit significant) prevention of EAE (Fig. 1B and C).

To investigate therapeutic effects of DMF, DMF was delivered b.i.d near peak clinical scores. DMF treatment had no effect on therapeutic clinical scores even after 14 days of b.i.d. treatment (Fig. 1D). As expected, fingolimod (positive control) produced a reduction in clinical scores as compared to control groups.

DMF inhibits ITGA4 expression on EAE lymphocytes, without changing total WBC counts

CBC tests were carried out to examine if DMF shared fingolimod’s known lymphopenic effects on EAE (Fig. 2A). DMF treatment did not significantly affect blood cell

Figure 2. DMF reduces ITGA4 expression on T and B lymphocytes from EAE mice with insignificant effects on blood cell subsets. (A) Total numbers of leukocyte subsets from DMF (days 0–30) and fingolimod (days 17–30)-treated EAE mice. Each point represents a single animal and the data represent means ± SEM. P values were analyzed by Kruskal–Wallis test with Dunn’s multiple comparisons test. (B) The relative proportion of CD3+ T cells and B220+ B cells in the blood of DMF (b.i.d.) and fingolimod-treated EAE mice (days 0–30) as compared to controls. Each point represents a single animal and the data represent mean ± SEM. P values were analyzed by Kruskal–Wallis test with Dunn’s multiple comparisons test. (C and D) Flow cytometric analysis of ITGA4 expression on CD3+ T cells (C) and B220+ B cells (D) from the blood of control and DMF (b.i.d.)-treated EAE mice (days 0–30). Unstained cells serve as negative controls and are represented by the filled histogram in each plot. Percentages of ITGA4high population (gate indicated in the histograms) are plotted. Each point represents a single animal and the data represent mean ± SEM. P values were analyzed by Student’s t test. DMF, dimethyl fumarate; ITGA4, integrin α4; EAE, experimental autoimmune encephalomyelitis.
suppression of VLA-4 on activated lymphocytes by DMF

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In this study, we show that DMF inhibited ITGA4 expression on CD3⁺ T and B220⁺ B cells in EAE mice, implicating downregulation of α4β1 integrin (VLA-4) as well as a distinct integrin heterodimer, α4β7 integrin. Consistent with these results, natalizumab-treated mice and both CD4⁺ T-cell-specific and CD19⁺ B-cell-specific ITGA4 knockout mice exhibit milder EAE disease.10-12 Supporting relevance of DMF in reducing ITGA4 expression, which appears to be distinct from and counter-regulatory to Nrf2 activation.

Discussion

In this study, we show that DMF inhibited ITGA4 expression on CD3⁺ T and B220⁺ B cells in EAE mice, implicating downregulation of α4β1 integrin (VLA-4) as well as a distinct integrin heterodimer, α4β7 integrin. Consistent with these results, natalizumab-treated mice and both CD4⁺ T-cell-specific and CD19⁺ B-cell-specific ITGA4 knockout mice exhibit milder EAE disease.10-12 Supporting relevance of DMF in reducing ITGA4 expression, which appears to be distinct from and counter-regulatory to Nrf2 activation.

Our data that ITGA4⁴ʰ populations were selectively affected by DMF treatment may have clinical implications. The DEFINE14 and CONFIRM studies15 on relapsing MS observed that DMF was more effective in younger patients than older patients. VLA-4 levels on CD3⁺ T cells are significantly higher in younger adults (~20%),16 therefore younger patients may be more responsive to DMF exposure that selectively affects ITGA4⁴ʰ populations. It is also notable that, in the PRISMS trial,17 interferon-β-1a (IFN-β1a) treatment also reduced VLA-4 expression by about 25% and the responders to IFN-β1a also showed higher baseline levels of VLA-4 than nonresponders. These results support the reduction in VLA-4 expression.
on lymphocytes as a shared mechanism amongst several MS-modifying drugs including DMF. The observed differences in DMF efficacy between preventative and therapeutic paradigms are also consistent with this shared mechanism as an established anti-VLA-4 antibody treatment is similarly ineffective in a therapeutic paradigm and moreover, exacerbates EAE.\textsuperscript{18}

VLA-4 inhibition is associated with a serious adverse event, PML, as has been well documented in natalizumab use.\textsuperscript{5} From this perspective, VLA-4 inhibition by DMF could help to explain recent PML cases.\textsuperscript{3,4} Considering results of the PRISMS trial,\textsuperscript{17} a recent case of PML in an MS patient (with a common variable immunodeficiency syndrome) after IFN/β1a monotherapy supports the role of VLA-4 in PML independent of natalizumab or DMF treatment.\textsuperscript{19} However, the much lower incidence of PML in both the interferon and DMF cases, as compared to natalizumab, underscores the existence of additional variables contributing to PML. The blockade of VLA-4 on lymphocytes by natalizumab dampens immuno-surveillance that may cause PML,\textsuperscript{6} whereas selective actions of DMF on ITGA4\textsuperscript{high} lymphocytes could reduce the number of circulating ependymaligenic cells,\textsuperscript{20} which is also consistent with the preventative but not therapeutic effects of DMF in EAE. Thus, this putative mechanism offers a possible explanation for both DMF efficacy and rare cases of PML, underscoring a need for balance between efficacy and adverse events mediated by the same molecular target.

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Author Contributions
Y. K., A. G., R. R. R., and J. C. designed research and wrote the manuscript. Y. K. and A. G. performed experiments and analyzed data. J. C. supervised the study.

Conflict of Interest
Y. K., A. G., and R. R. R. declare no competing financial interests. J. C. received honoraria, consulting fees and/or grant support from Biogen-Idec and Novartis.

References

