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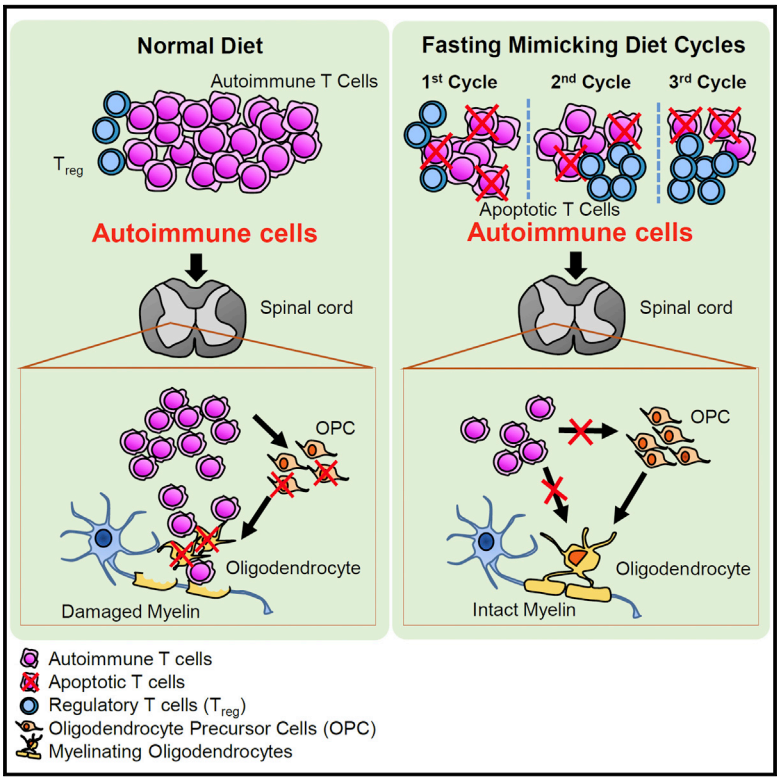
A Diet Mimicking Fasting Promotes Regeneration and Reduces Autoimmunity and Multiple Sclerosis Symptoms

Graphical Abstract



Highlights

- FMD reduces pro-inflammatory cytokines and increases corticosterone levels
- FMD suppresses autoimmunity by inducing lymphocyte apoptosis
- FMD promotes regeneration of oligodendrocyte in multiple MS models
- FMD is a safe, feasible, and potentially effective treatment for MS patients

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In Brief

Choi et al. show that cycles of a fasting mimicking diet (FMD) ameliorate disease severity by suppressing autoimmunity and stimulating remyelination via oligodendrocyte regeneration in multiple sclerosis (MS) mouse models. They also show that a similar FMD is a safe, feasible, and possibly a potentially effective treatment for patients with relapsing-remitting MS.

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A Diet Mimicking Fasting Promotes Regeneration and Reduces Autoimmunity and Multiple Sclerosis Symptoms

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SUMMARY

Dietary interventions have not been effective in the treatment of multiple sclerosis (MS). Here, we show that periodic 3-day cycles of a fasting mimicking diet (FMD) are effective in ameliorating demyelination and symptoms in a murine experimental autoimmune encephalomyelitis (EAE) model. The FMD reduced clinical severity in all mice and completely reversed symptoms in 20% of animals. These improvements were associated with increased corticosterone levels and regulatory T (T_{reg}) cell numbers and reduced levels of pro-inflammatory cytokines, T_H1 and T_H17 cells, and antigen-presenting cells (APCs). Moreover, the FMD promoted oligodendrocyte precursor cell regeneration and remyelination in axons in both EAE and cuprizone MS models, supporting its effects on both suppression of autoimmunity and remyelination. We also report preliminary data suggesting that an FMD or a chronic ketogenic diet are safe, feasible, and potentially effective in the treatment of relapsing-remitting multiple sclerosis (RRMS) patients (NCT01538355).

INTRODUCTION

Multiple sclerosis (MS) is an autoimmune disorder characterized by T cell-mediated demyelination and neurodegeneration in the CNS (Friese and Fugger, 2005; Pender and Greer, 2007; Sospedra and Martin, 2005). In experimental autoimmune encephalomyelitis (EAE), an animal model for MS, activated myelin-specific T_H1 and T_H17 cells cross the blood-brain barrier and migrate into the CNS, where they are activated by local antigen-presenting cells (APCs) and promote inflammation (Dhib-Jalbut, 2007; Fletcher et al., 2010; Goverman, 2009; Hemmer et al., 2002). This inflammatory process leads to oligodendrocyte death, demyelination, and axonal damage, which eventually cause neurologic damage (Lucchinetti et al., 1999; Raine and Wu, 1993). Although oligodendrocyte precursor cells (OPCs) can migrate to the sites of MS lesions, they often fail to differentiate into functional oligodendrocytes (Chang et al., 2002; Wolswijk, 1998). Several MS treatment drugs have been effective in reducing immune responses, but their impact on long-term disease progression, accrual of irreversible neurological disability, and immune system function remains largely unclear, underlining the need for novel therapeutic strategies (Wingerchuk and Carter, 2014). Therefore, effective treatments for MS may require not only the mitigation of autoimmunity but also the stimulation of oligodendrocyte regeneration and restoration of a functional myelin sheath. Periodic cycles of prolonged fasting (PF) or of a fasting mimicking diet (FMD) lasting 2 or more

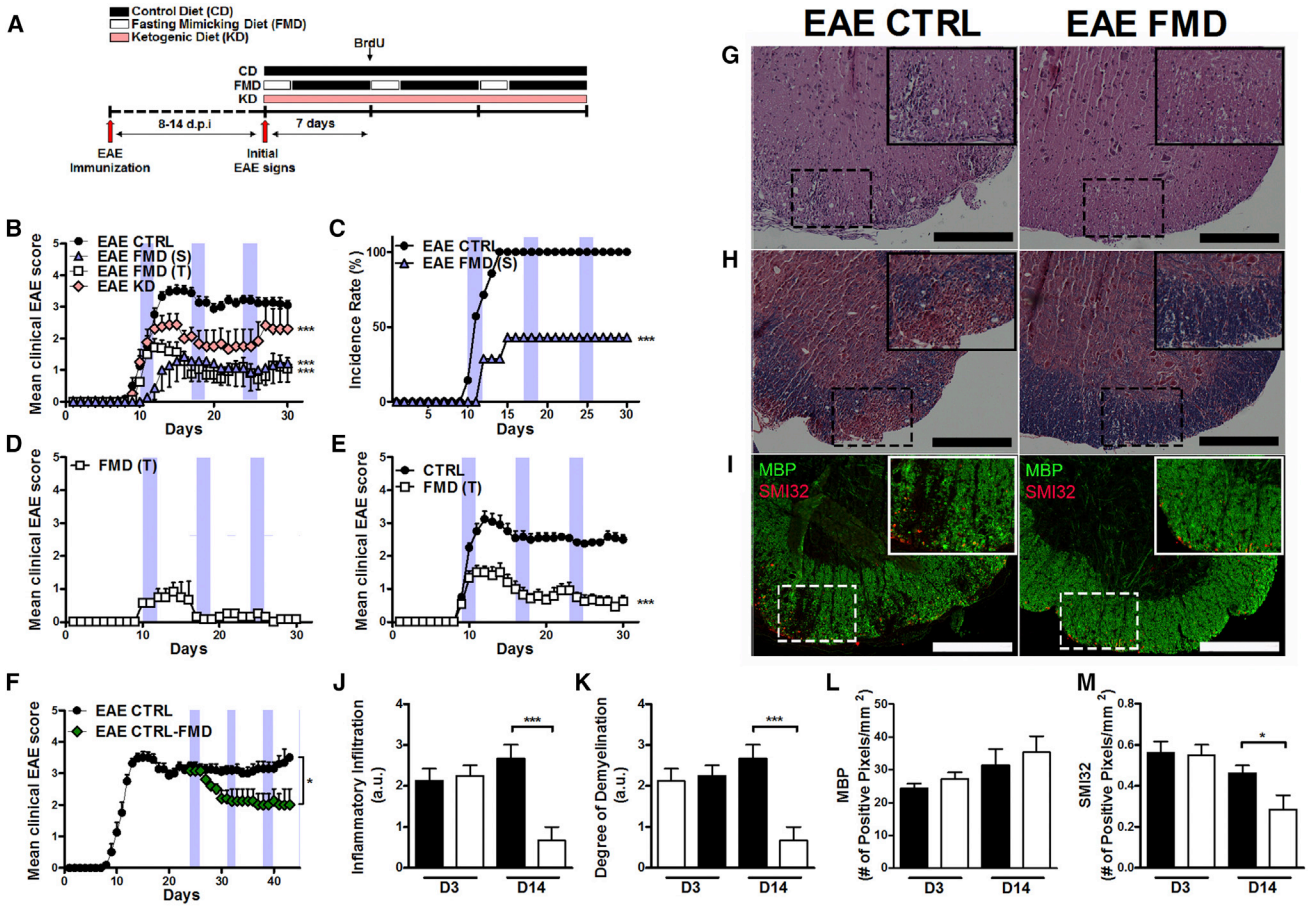


Figure 1. FMD Cycles Decrease Disease Severity of the $MOG_{35–55}$-Induced EAE Model

(A) Diagram displaying the time course of the immunization and the diet interventions.

(B) The EAE severity scores of the control diet (EAE CTRL; n = 23), ketogenic diet (EAE KD; n = 13), semi-therapeutic FMD cycles (EAE FMD (S); n = 7), or therapeutic FMD cycles (FMD(T); n = 23).

(C) Incidence rate of EAE CTRL and EAE FMD (S) (n = 7–23).

(D) EAE severity score in mice for which FMD(T) completely reversed EAE severity, with no observable disease (score = 0; 5 out of 23 mice).

(E) EAE severity score of the best-performing control mice (n = 12) and FMD(T) mice (n = 12).

(F) EAE severity score of the mice treated with FMD after chronic EAE development (EAE CTRL-FMD; n = 6).

(G–M) Spinal cord sections of EAE CTRL and EAE FMD (T) mice with quantification of H&E staining (G), solochrome cyanine staining (H), and MBP (myelin basic protein)/SMI32 (I) double staining of spinal cord sections isolated at day 14.

Data are presented as mean ± SEM; *p < 0.05, **p < 0.01, and ***p < 0.001, Student's t test, one-way or two-way ANOVA, and Bonferroni post test. Scale bar represents 200 μm.

days can increase protection of multiple systems against a variety of chemotherapy drugs in mice and possibly humans (Fontana et al., 2010; Guevera-Aguirre et al., 2011; Lee et al., 2010; Longo and Mattson, 2014). Moreover, PF or an FMD reverses the immunosuppression or immunosenescence of either chemotherapy or aging through hematopoietic stem cell-based regeneration (Brandhorst et al., 2015; Cheng et al., 2014). Chronic caloric restriction, a ketogenic diet (KD), and intermittent fasting have been shown to help prevent EAE by reducing inflammation and enhancing neuroprotection when administered prior to disease induction or signs (Esquifino et al., 2007; Kafami et al., 2010; Kim do et al., 2012; Piccio et al., 2008), but dietary interventions have not been reported to be effective as therapies for EAE or MS or to promote myelin regeneration.

Here, we report on the effects of low-calorie and low-protein FMD cycles as a treatment in MS mouse models, and we investigate the mechanisms involved. Furthermore, we report preliminary results on the safety and feasibility of a FMD and a KD in patients with relapsing-remitting multiple sclerosis (RRMS).

RESULTS

FMD Cycles Reduce Disease Severity in the $MOG_{35–55}$-Induced EAE Model

We examined the effects of 3 cycles of a very-low-calorie and low-protein FMD lasting 3 days every 7 days or a KD continued for 30 days in EAE-induced by active immunization with myelin oligodendrocyte glycoprotein 35–55 ($MOG_{35–55}$) (Figure 1A).

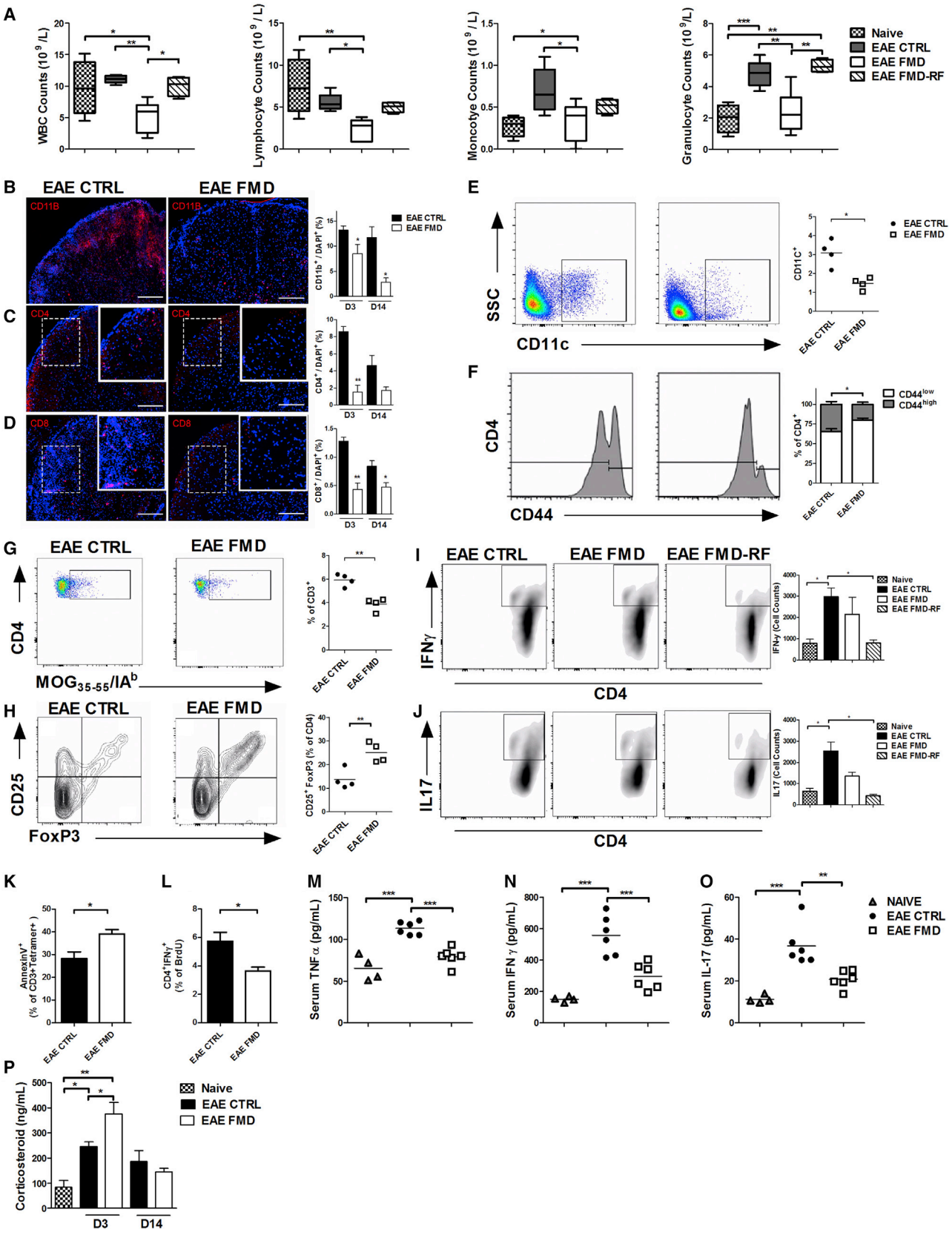
Groups of mice were treated semi-therapeutically (EAE FMD (S), where FMD treatment started after 10% of the immunized population showed signs of EAE) or therapeutically (EAE FMD (T), where FMD treatment started after all of the immunized population showed signs of EAE). FMD and KD treatment decreased disease severity compared to that in the control group (Figure 1B). However, the FMD reduced the mean severity score to ~1, whereas a KD reduced the severity score to ~2 at the later stages (Figure 1B). In the EAE FMD (S) group, FMD treatment not only delayed the onset of disease but also lowered the incidence rate (100% versus 45.6%; Figure 1C). In the EAE FMD (T) group, FMD cycles completely reversed the severity score to 0 in 21.7% of the cohort (no observable signs; Figure 1D) and reduced the severity score to <0.5 in >50% of mice (12 out of 23 mice; Figure 1E). To address whether the FMD cycles also have beneficial effects in chronic EAE models that have established disease, we initiated FMD treatment 2 weeks after the initial signs of EAE were observed (EAE CTRL-FMD). Prior to treatment, the control diet EAE group (EAE CTRL) and EAE CTRL-FMD cohorts had similar severity scores (3.19 ± 0.52 versus 3.30 ± 0.27; day 24). After three FMD cycles, we observed a significant reduction in severity score in the EAE CTRL-FMD cohort compared to the EAE CTRL cohort (3.3 ± 0.57 versus 2.1 ± 0.89; day 42; $p < 0.05$; Figure 1F). As infiltration of immune cells and demyelination are histopathological hallmarks of EAE and MS, spinal cord sections from control and FMD(T) mice were stained with H&E to visualize infiltrating immune cells (Figure 1G) or solochrome cyanine to visualize myelin (Figure 1H). To assess demyelination and axonal damage, immunohistochemistry was performed using antibodies against myelin basic protein (MBP) or dephosphorylated neurofilaments (SMI-32; Figure 1I). At day 3, levels of infiltrating immune cells and demyelination were similar in the EAE CTRL and EAE FMD groups (Figures 1J and S1H). At day 14, sections of EAE CTRL mice displayed severe immune cell infiltration corresponding to demyelinated lesions, reduced MBP expression, and increased SMI-32 expression (Figures 1J–1M). By contrast, sections of EAE FMD mice at day 14 displayed significantly reduced immune cell infiltration and demyelination (Figures 1J–1M). Although MBP staining showed no significant difference between EAE CTRL and EAE FMD mice at day 14 (Figure 1L), neurofilament dephosphorylation in EAE FMD mice was reduced compared to EAE CTRL mice (Figure 1M). Overall, these results suggest that FMD cycles reduce EAE disease severity in part by reducing inflammation and preventing demyelination and axonal damage.

FMD Cycles Reduce Infiltration of Immune Cells in the Spinal Cord

To investigate the capacity of FMD cycles to reduce potential autoimmune T cells, we measured circulating white blood cells (WBCs), lymphocytes, monocytes, and granulocytes in naive, EAE CTRL, EAE FMD, and EAE FMD:RF (measured 4 days after returning to a standard ad lib diet) mice after three cycles of the FMD regimen (Figure 2A). The FMD resulted in a temporary 40%–50% reduction in total WBCs, lymphocytes, monocytes, and granulocytes. Upon returning to the standard ad lib diet (EAE FMD:RF), all complete blood counts (CBCs) returned to either naive or lower levels than those observed in the EAE CTRL, with the exception of granulocytes, indicating that the FMD cycles cause both WBC death and regeneration (Figure 2A). Next, we measured the inflammatory markers associated with EAE pathophysiology. Day 3 and day 14 spinal cord sections of the EAE CTRL mice were extensively populated with $CD11b^+$ cells (Figure 2B). However, at day 14, the EAE FMD mice displayed a 75% reduction ($p < 0.05$) in spinal cord-associated $CD11b^+$ cells compared to mice on the control diet (11.7% versus 2.8%; Figure 2B). Since myelin-specific effector T cells migrate into the CNS and initiate demyelination, we investigated the accumulation of $CD4^+$ or $CD8^+$ T cells in the spinal cord. A large number of $CD4^+$ T cells were detected in the white matter of spinal cord sections from the control diet cohort (Figure 2C). In contrast, the FMD-treated cohort displayed a >4-fold reduction ($p < 0.01$) in $CD4^+$ T cells at day 3 (8.6% versus 1.5%; Figure 2C) compared to the control diet cohort, which remained lower even at day 14. The FMD group also had reduced $CD8^+$ T cells (day 3: 1.3% versus 0.4%; $p < 0.01$; Figure 2D) compared to the control diet group. To investigate whether the FMD affects APCs, we isolated splenocytes from EAE CTRL and EAE FMD mice at day 3, stained them for CD11c and F4/80, and characterized them by flow cytometry. We observed a significant decrease ($p < 0.05$) in $CD11c^+$ dendritic cells in the EAE FMD cohort compared to the EAE CTRL cohort (3.08% ± 0.70% versus 1.46% ± 0.31%), but we did not observe any changes in the number of $F4/80^+$ macrophage cells in the control or FMD-treated groups (Figures 2E and S2B). To determine the effects of the FMD treatment on T cell infiltration in the spinal cord, we measured T cell activation levels. The number of $CD4^+$ T cells and $CD8^+$ T cells in EAE CTRL and EAE FMD mice was similar (Figures S2C and S2D), but the ratio of splenic naive ($CD44^{low}$) to activated ($CD44^{high}$) $CD4^+$ T cells was increased ($p < 0.05$) in the FMD group compared to the control group (1.95 versus 3.67; Figure 2F). No difference in $CD8^+$ T cells was observed (Figure S2E). Moreover, the total number of effector ($CD44^{high}$ and $CD62L^{low}$) T cells was reduced in the FMD compared to the control group, but the ratio of effector ($CD44^{high}$ and $CD62L^{low}$) to memory T ($CD44^{high}$ and $CD62L^{high}$) cells did not change (Figures S2F–S2H). These results indicate that FMD cycles reduce the number of dendritic cells and increase the relative number of naive T cells, which may explain the reduced autoimmunity caused by the FMD.

FMD Cycles Induce Autoreactive Lymphocyte Apoptosis and Increase the Number of Naive Cells

To determine whether FMD cycles also reduce the number of MOG-specific antigen-reactive cells, we used a major histocompatibility complex (MHC) tetramer ($MOG_{35–55}/IA^b$) to identify antigen-reactive cells after an FMD cycle in vivo. The number of $CD4^+$ $MOG_{35–55}$ $/IA^{b+}$ cells was reduced in the EAE FMD cohort compared to the EAE CTRL cohort (5.75% ± 0.51% versus 3.83% ± 0.66% of lymphocytes; * $p < 0.05$; Figure 2G). To determine whether the reduced active T cell number is due to an increase in the number of regulatory T (T_{reg}) cells, we isolated lymphocytes from draining lymph nodes and spleens of EAE CTRL or EAE FMD mice and analyzed them for $CD4^+$ $CD25^+$ $FoxP3^+$ T_{reg} cells. The FMD cohort showed a 2-fold increase ($p < 0.01$) in the number of $CD25^+$ $FoxP3^+$-expressing



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T_{reg} cells (13.6% ± 4.2% versus 25.1% ± 4.2%; Figure 2H). Moreover, the FMD cohort showed a 27.8% reduction ($p < 0.05$) in the number of interferon γ (IFN-γ)- expressing T_H1 cells (2,974.4 ± 708.0 versus 2,148.1 ± 1,396.1; Figure 2I) and a 46.5% reduction ($p < 0.05$) in the number of interleukin-17 (IL-17)-expressing T_H17 cells (2,535.9 ± 722.0 versus 1,357.1 ± 256.2; Figure 2J), both of which are known to be central mediators of EAE. Interestingly, upon re-feeding of the control diet, the EAE FMD treatment group (EAE FMD:RF) showed a 72.9% reduction ($p < 0.05$) in the number of IFN-γ-expressing T_H1 cells (2,974.4 ± 708.0 versus 805.8 ± 251.5; Figure 2I) and a 82.9% reduction ($p < 0.05$) in the number of IL-17-expressing T_H17 cells (2,535.9 ± 722.0 versus 432.4 ± 117.4; Figure 2J), suggesting that the FMD can prevent autoimmunity in part by reducing the levels of pro-inflammatory T cells implicated in EAE.

In order to assess how FMD cycles may reduce the number of T cells, we measured apoptosis in MOG-specific T cells ($CD3^+$ $MOG_{35–55}$/IAb) in vivo. We observed a significant increase ($p < 0.05$) in apoptotic $CD3^+$ $MOG_{35–55}$/IAb levels in the EAE FMD cohort compared to the EAE CTRL cohort (28.3% ± 4.94% versus 39.1% ± 4.79%; Figure 2K), which was consistent with the major reduction in the number of WBCs and lymphocytes observed in the FMD group (Figure 2A). To investigate whether these apoptotic cells are replaced by newly generated cells, we treated the mice with bromodeoxyuridine (BrdU) during the re-feeding period (four injections within 48 hr, at 1 mg of BrdU per injection). Splenocytes were isolated 4 days after the re-feeding of the regular diet and stained for BrdU (Figure S2I). We observed no difference in levels of total $BrdU^+$ lymphocytes (8.11% ± 1.99% versus 12.02% ± 2.72%; Figure S2J), but we observed a significantly reduced proliferation of T_H1 ($BrdU^+$ $CD4^+IFN\gamma^+$) (5.74% ± 1.07% versus 3.65% ± 0.63%; *$p < 0.05$; Figure 2L) and no difference in proliferation of T_H17 ($BrdU^+CD4^+IL17^+$) (4.71% ± 1.53% versus 5.01% ± 1.66%; Figure S2K). Taken together, these data indicate that FMD cycles may promote apoptosis of autoreactive T cells, leading to an increase in the proportion of naive T cells and regulatory T cells. In addition, FMD cycles may interfere with proliferation and differentiation of T_H1 cells, but not T_H17 cells. To investigate whether the FMD's effects on CNS infiltrating immune cells are associated with suppression of T_H1- and T_H17-dependent cytokine production (IL-17, IFN-γ, and tumor necrosis factor α [TNF-α]), we analyzed serum from naive, EAE CTRL, and EAE FMD mice (Figures 2K–2M). We observed significant reductions in serum TNF-α (113.3 ± 7.9 versus 79.3 ± 10.5 pg/ml; $p < 0.001$; Figure 2M), IFN-γ (558.43 ± 124.5 versus 296.0 ± 83.4 pg/ml; $p < 0.001$; Figure 2N), and IL-17 (36.8 ± 9.67 versus 20.75 ± 4.2 pg/ml; $p < 0.01$; Figure 2O). To identify a potential mediator for the effects of FMD cycles on the suppression of autoimmune responses, we measured serum corticosterone levels. Corticosterone is a glucocorticoid hormone with broad anti-inflammatory and immunosuppressive effects affecting leukocyte distribution, trafficking, and death (Ashwell et al., 2000; Herold et al., 2006; Planey and Litwack, 2000; Vegiopoulos and Herzig, 2007). Serum corticosterone levels were elevated in association with the first signs of EAE (EAE day 1, before treatment) (data not shown). FMD treatment caused a further increase in corticosterone levels at day 3 compared to those of controls (245.9 ± 38.8 versus 375.0 ± 94.1 ng/ml; $p < 0.01$), which returned to EAE basal levels by day 14 in both groups (Figure 2P). These results indicate that FMD cycles reduce the number of T_H1 and T_H17 effector cells and the production of pro-inflammatory cytokines. These effects of the FMD may be regulated in part by the temporary elevation of corticosterone levels, dampening of T cell activation, and reduced APC and T cell infiltration in the spinal cord.

FMD Reverses EAE Symptoms by Reducing the Level and Reactivity of Established Autoimmune Cells

To determine how the FMD affects the initiation of EAE, splenocytes were isolated from EAE CTRL and EAE FMD mice, re-activated with $MOG_{35–55}$ peptide and IL-23 ex vivo, and transferred into naive recipient mice to induce EAE. The mice were then subjected to either a control diet or FMD cycles (Figure 3A). The supernatant from ex vivo splenocyte cultures derived from EAE FMD mice showed no difference in TNF-α levels (110.8 ± 14.9 pg/ml versus 97.1 ± 8.4 pg/ml; Figure 3B) but a major reduction ($p < 0.01$) in the levels of IFN-γ (342.0 ± 29.8 pg/ml versus 46.6 ± 16.6 pg/ml Figure 3C) and IL-17 (850.5 ± 442.0 pg/ml versus 257.4 ± 36.4 pg/ml; Figure 3D). Interestingly, upon in vitro reactivation, both EAE CTRL and EAE FMD had similar levels of T_H1 and T_H17 differentiated cells (Figures 3E and 3F). To determine whether the immune cells from EAE CTRL and EAE FMD mice

Figure 2. FMD Cycles Decrease the Number of Infiltrating T Cells in the Spinal Cord

(A) Total white blood cell (WBC), lymphocyte, monocyte, and granulocyte counts of naive, EAE-CTRL, EAE-FMD, and EAE-FMD:RF (after 3 days of re-feeding) mice after three cycles of the FMD and a matched time point for EAE-CTRL mice.

(B–D) Spinal cord sections (day 14) and quantification at days 3 and 14 after the first sign of EAE for $CD11b^+$ (B), $CD4^+$ (C), and $CD8^+$ (D) (at least six sections per mouse).

(E) $CD11c^+$ isolated from EAE CTRL or EAE FMD mice on day 3, and quantification of cells from the total isolated splenocyte.

(F) $CD4^+$ gated for $CD44^{low}$ or $CD44^{high}$ cells isolated from EAE CTRL or EAE FMD mice, and quantification of percent splenocytes in $CD4^+$ $CD44^{low}$ (inactive) or $CD4^+$ $CD44^{high}$ (active) cells.

(G) $CD3^+$ lymphocytes gated for CD4 and $MOG_{35–55}/IA^b$ from EAE CTRL or EAE FMD mice, and quantification of MOG-specific $CD4^+$ cells.

(H) $CD4^+$ $CD25^+$ $FoxP3^+$ isolated from EAE CTRL or EAE FMD mice, and quantification of $CD25^+$ $FoxP3^+$ in $CD4^+$ cells.

(I and J) Intracellular staining for either IFNγ (I) or IL17(J) after gated for $CD4^+$ of the naive, EAE CTRL, EAE FMD, EAE FMD:RF and quantification of cell counts.

(K) Quantification of Annexin V^+ apoptotic $CD3^+$ MOG_{35-55}/IA^b cells.

(L) Quantification of $CD4^+IFN\gamma^+$ of $BrdU^+$ lymphocytes.

(M–O) Serum TNF-α (M), IFN-γ (N), and IL-17 levels (O) (pg/ml) in naive, EAE CTRL, and EAE FMD mice on day 3 after the first sign of EAE.

(P) Serum corticosterone levels (ng/ml) before immunization, at the time of symptom occurrence, or 3 or 14 days after the initial symptom appeared in the control or FMD group.

n = 4–8 per group; mean ± SEM. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$, Student's t test, one-way ANOVA, and Bonferroni post test. Scale bar represents 200 μm.

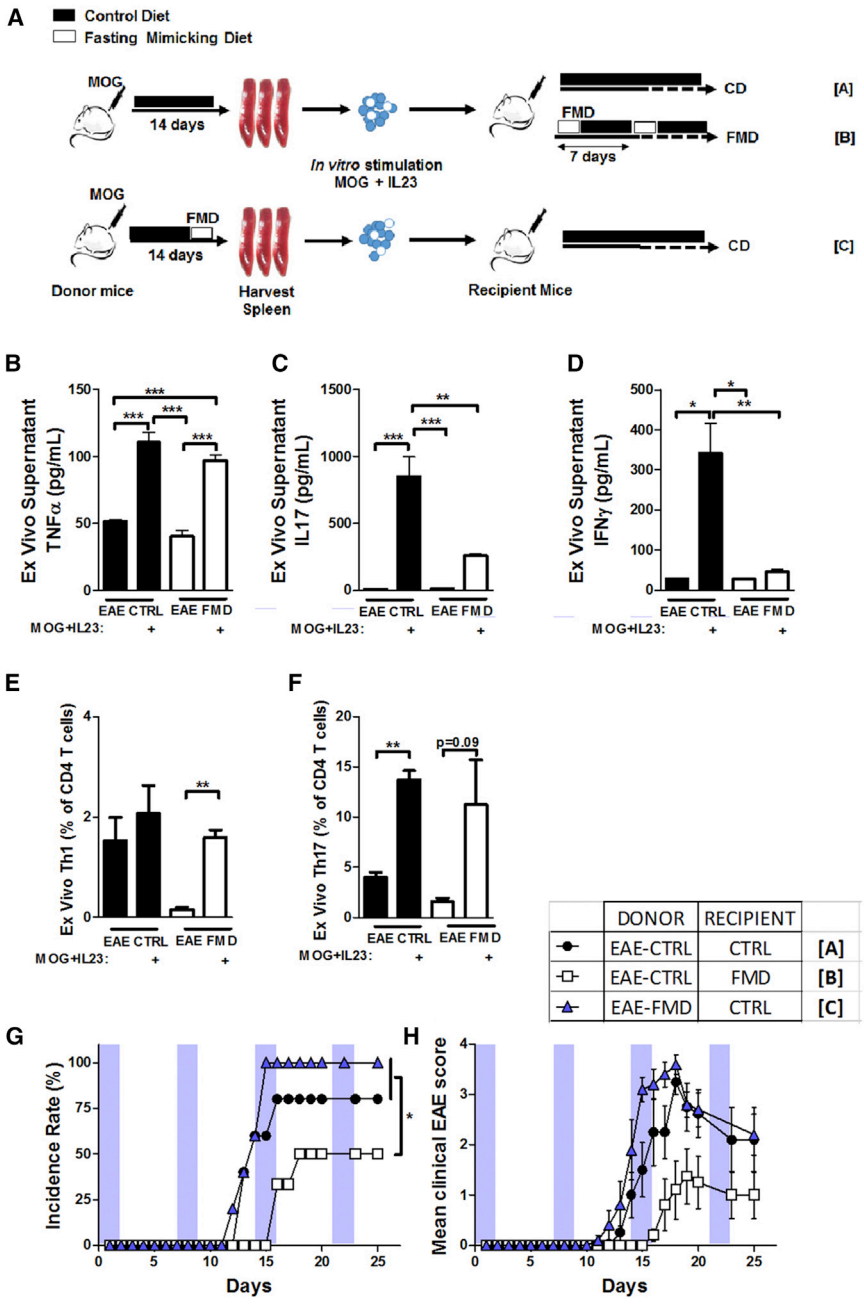

Figure 3. Antigen-Activated Splenocytes from EAE-CTRL and the EAE-FMD Mice Had Similar Encephalitogenic Effects

(A) Diagram for the adoptive transfer EAE model.

(B–D) Quantification of TNF-α (B), IFN-γ (C), and IL-17 (D) (pg/ml) in the supernatant from ex vivo cultures of splenocytes from naive, EAE CTRL, and EAE FMD mice either with or without $MOG_{35–55}$ and IL-23 re-activation.

(E and F) Quantification of T_H1 or T_H17 (represented by percentage of $CD4^+$) from lymphocyte culture of EAE CTRL and EAE FMD mice with or without $MOG_{35–55}$ and IL-23 re-activation.

(G) Incidence rate of adoptive transfer EAE groups.

(H) EAE severity score of adoptive transfer EAE groups.

n = 5–6 per group; mean ± SEM. *p < 0.05, **p < 0.01, and ***p < 0.001, Student's t test, one-way ANOVA, and Bonferroni post test.

have similar encephalitogenic effects, we transferred splenocytes from either donor group (EAE CTRL or EAE FMD) into naive recipients (A, EAE CTRL donor to control diet recipient; and C, EAE FMD donor to control diet recipient). This resulted in a similar disease incidence rate (Figure 3G) and an equally severe EAE disease severity by day 20 (2.38 ± 0.48 versus 2.70 ± 0.75; Figure 3H), indicating that the FMD did not affect the development and function of reactive immune cells in vivo or ex vivo. However, when FMD treatment was initiated after transfer of control donor splenocytes (B, EAE CTRL donor to naive mice with FMD treatment), recipient mice displayed a delayed disease onset (day 12 versus day 16 post-transfer; Figure 3G) and a major reduction in EAE severity scores compared to control mice (2.38 ± 0.48 versus 0.75 ± 0.87; Figure 3H). Taken together, these results suggest that T cell priming in response to myelin antigen occurred normally in the EAE CTRL and EAE FMD groups, but the FMD can reduce the level of the existing autoimmunity.

FMD Cycles Stimulate Remyelination by Promoting Oligodendrocyte Regeneration

To investigate whether the reduced demyelination in FMD mice may also be related to enhanced oligodendrocyte regeneration, we first carried out a quantitative image analysis of $NG2^+$ (an oligodendrocyte progenitor cell [OPC] marker) and GST-π^+ (a mature oligodendrocyte marker) in spinal cord sections from control or FMD mice (Figure 4A). We observed no difference in the number of $NG2^+$ OPCs in sections taken from EAE CTRL and EAE FMD mice (Figure S3A). However, at day 14, the number of GST-π^+ oligodendrocytes was reduced in the EAE CTRL group, but not in the EAE FMD group (886.7 ± 41.6 versus 1,273 ± 200.3; cells per spinal cord section area; p < 0.01; Figure 4B). To assess whether the normal levels of mature oligodendrocytes in the EAE FMD group were due to enhanced regeneration and/or differentiation, EAE CTRL or EAE FMD mice were injected with BrdU at the time of re-feeding (day 10). We observed a major increase (p < 0.01) in the percentage of cells that are double positive for $BrdU^+$ and GST-π^+ in the EAE FMD group compared to the EAE CTRL group (42.9% ± 11.2% versus 83.0% ± 13.2%; p < 0.01), suggesting that the FMD promotes oligodendrocyte differentiation from precursor

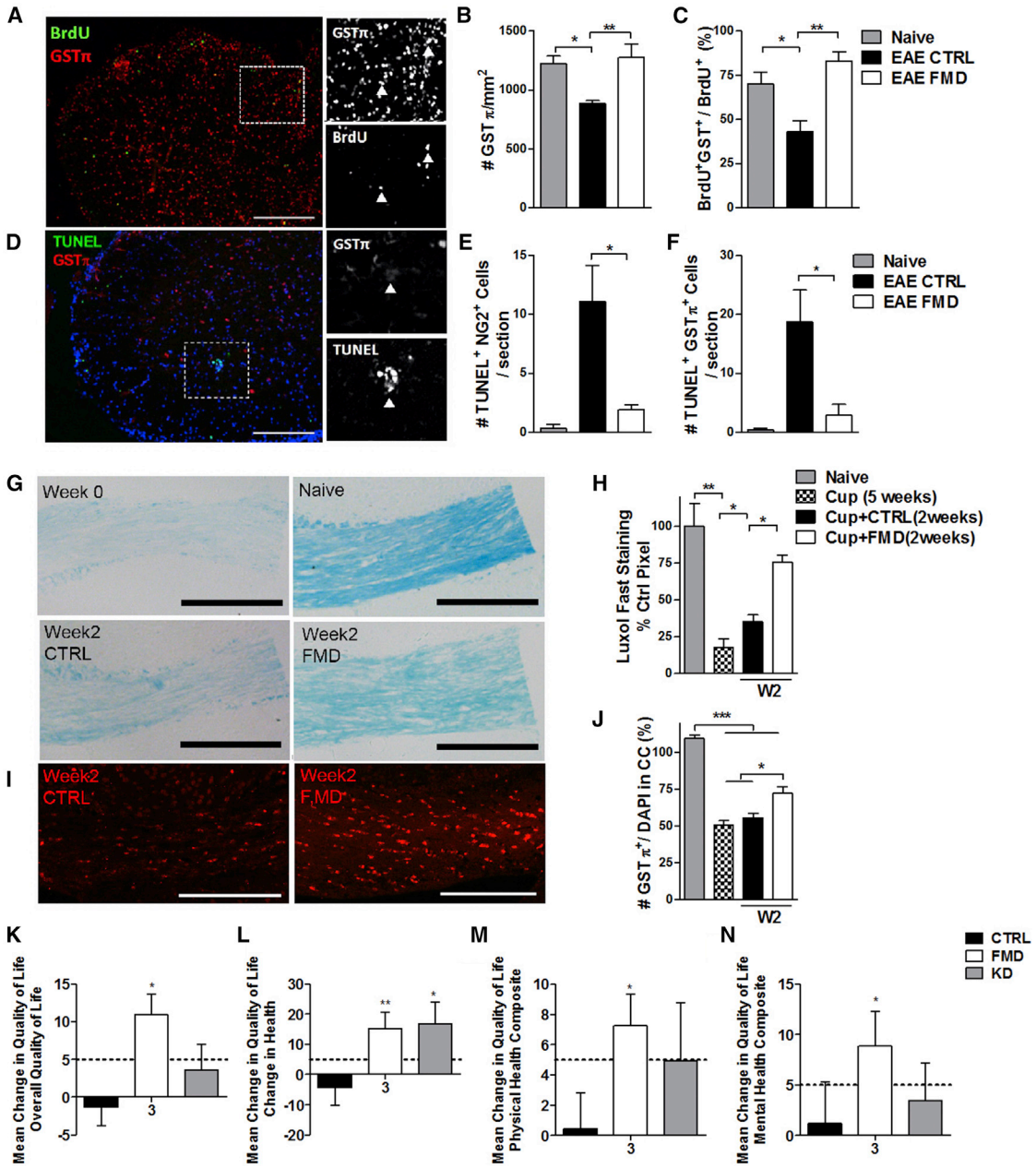

Figure 4. FMD, which Protects the Mouse Spinal Cord from Loss of Oligodendrocytes and Enhances Remyelination, Is Safe and Potentially Effective in the Treatment of MS Patients

(A–C) Spinal cord sections isolated at day 14 and quantification for GST-π (mature oligodendrocyte) and BrdU (A), TUNEL and NG2 (oligodendrocyte precursor cells) (B), and TUNEL and GST-π (C) in naive, EAE-CTRL, or EAE-FMD mice.

(F–H) Sections from the corpus callosum region and quantification of cuprizone treated brains, stained with Luxol Fast Blue of the naive control, end of 5 weeks of cuprizone diet (week 0), cuprizone (5 weeks) plus regular chow (2 weeks), and cuprizone (5 weeks) plus FMD cycle (2 weeks).

(I and J) Section from the corpus callosum region and its quantification of the cuprizone treated brains stained with GST-π^+ of cuprizone (5 weeks) plus regular chow (2 weeks), and cuprizone (5 weeks) plus FMD (2 weeks). Quantification is normalized to percent naive GST-π^+ level.

(K–N) Change in quality of life at 3 months in terms of overall quality of life (K), change in health (L), physical health composite (M), and mental health composite (N). The dotted line represents a threshold that is thought to be clinically important ($\geq$5 points). Data represent mean ± standard error of the difference (SED); *$p < 0.05$, Mann-Whitney *U* test. An increase of $\geq$5 points is considered clinically important.

At least 12 sections per mouse were used for quantification; n = 4; mean ± SEM, *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$, one-way ANOVA and Bonferroni post test.

cells (Figure 4C). To assess the effects of the FMD on either OPCs or mature oligodendrocytes, sections were stained with TUNEL, an apoptotic marker, and GST-π^+ or NG2$^+$ (Figure 4D). We observed a significant increase in the number of TUNEL$^+$ NG2$^+$ (11.2 ± 12.2 versus 1.9 ± 1.4 cells/section) and TUNEL$^+$ GST-π^+ (18.8 ± 15.2 versus 2.9 ± 5.3 cells/section) cells in the control group compared to the FMD group ($p < 0.05$; Figures 4E and 4F). Taken together, these results indicate that the FMD not only stimulates regeneration and differentiation of oligodendrocytes but also protects OPCs and mature oligodendrocytes from apoptosis.

To investigate whether the FMD-dependent stimulation of oligodendrocyte differentiation and remyelination can occur independent of the observed effects on T cell number and activity, we used the cuprizone-induced demyelinating mouse model (Ransohoff, 2012; Torkildsen et al., 2008). Addition of 0.2% (w/w) cuprizone to the regular mouse diet for 5–6 weeks results in demyelination in the corpus callosum followed by spontaneous remyelination upon re-feeding with regular chow. After 5 weeks of cuprizone treatment, mice were switched to either the control diet or FMD cycles for 5 weeks, and some were euthanized weekly to assess the degree of myelination by Luxol fast staining and GST-π^+ (Figures 4G and 4I). As expected, after 5 weeks of the cuprizone diet, a significant reduction in myelin staining was observed in the corpus callosum compared to the naive controls (Figures 4H and 4J). After two cycles, the FMD-treated group displayed increased myelin staining and an increased number of GST-π^+ oligodendrocytes compared to the control diet group (Figures 4H and 4J). However, at later time points, we did not observe differences in spontaneous re-myelination between the control diet and FMD cohorts, as it is well established that cuprizone-dependent myelin damage can be fully reversed after removal of the toxin (Figures S3C and S3D). These results indicate that the FMD promotes OPC-dependent regeneration and accelerates OPC differentiation into oligodendrocytes while enhancing remyelination independently of its modulation of the inflammatory response.

A Randomized Pilot Trial to Test the Effects of a FMD or KD in Relapsing-Remitting MS Patients: Evidence for Safety and Feasibility

A randomized, parallel-group, three-arm pilot trial (NCT01538355) was conducted to assess the safety and feasibility of FMD or KD treatment on health-related quality of life (HRQOL) in RRMS patients. 60 patients were randomly assigned to a control diet (CD; n = 20), KD for 6 months (n = 20), or a single cycle of a modified human FMD for 7 days (n = 20) followed by a Mediterranean diet for 6 months (Figure S4). Baseline characteristics were balanced among the three groups (Tables S1 and S2). The FMD and KD cohorts displayed clinically meaningful improvements in the HRQOL summary scales at 3 months, which included the overall quality of life (Figure 4K) change in health (Figure 4L), a physical health composite (Figure 4M), and a mental health composite (Figure 4N). Also, similar changes were observed in the total HRQOL scales at different time points (Figure S5). Adverse events (AEs) and serious adverse events (SAEs) were reported for 92% (8%) of CD cohort individuals, 78% (16%) of FMD cohort individuals, and 78% (11%) of KD cohort individuals (Table S5). The most common AE was airway infection, and the most frequent SAE was lower urinary tract infection. No indication of an increase in liver enzymes exceeding the normal range was observed in any of the three treatment groups. Also, the interventions were well tolerated, as evidenced by high compliance rates (CD, 60%; KD, 90%; and FMD, 100%). During the 6-month study period, we observed a total of eight relapses: four in the CD group, one in the KD group, and three in the FMD group. In addition to increased β-hydroxybutyrate levels in plasma, we observed a slight reduction in lymphocytes and WBC counts and detected a mild reduction in expanded disability status scale (EDSS) scores in the FMD and KD groups (measured at baseline, month 3, and month 6; Tables S1 and S6). Thus, there was an inverse association between EDSS and HRQOL scores (Table S7). In MS patients the FMD treatment lead to an over 20% drop in the total lymphocyte count (baseline versus day 8; Table S4) in 72% of the patients (13 of 18 FMD-treated patients). WBC counts returned to the baseline levels after these patients were switched to the Mediterranean diet (month 3). Based on the mouse studies, these results raise the possibility that the FMD alleviated symptoms in MS patients by reducing the number of autoimmune lymphocytes. Overall, our study indicates that the administration of FMD and KD is safe, feasible, and potentially effective, but further studies, including analyses such as magnetic resonance imaging (MRI), blinded clinical assessments, and immune assays, are required to determine efficacy.

DISCUSSION

An FMD administered every week was effective in ameliorating EAE symptoms in all mice and completely reversed disease progression in a portion of animals after the onset of EAE signs. By contrast, the KD had more modest effects and did not reverse EAE progression in mice. FMD cycles appear to be effective in the treatment of EAE in mice by (1) promoting oligodendrocyte precursor-dependent regeneration and (2) reducing the levels of microglia/monocytes and T cells contributing to autoimmunity and encephalomyelitis. Our results support an FMD-mediated anti-inflammatory effect possibly involving the upregulation of AMPK or the downregulation of mTORC1, which sense nutrient availability and dictate cell fate (Laplante and Sabatini, 2012). It was shown that mTORC1 couples immune signals and metabolic programming to establish T_{reg} cell function (Zeng et al., 2013). In fact, treatment with the mTORC1 inhibitor rapamycin or the AMPK activator metformin attenuates EAE symptoms by modulating effector T cells and T_{reg} cells and restricting the infiltration of mononuclear cells into the CNS (Esposito et al., 2010; Nath et al., 2009). Therefore, FMD treatment could interfere with T cell proliferation and differentiation and with recruitment of other immune cells, resulting in a decreased recruitment at lesion sites (Figure 5). Some of these effects of the FMD may be triggered by endogenous glucocorticoid production. Glucocorticoids are used to treat MS relapses, but they are generally administered in short bursts, since they can cause AEs such as osteoporosis and metabolic syndrome (Brusaferri and Candelise, 2000; Ce et al., 2006; Roth et al., 2010; Uttner et al., 2005). The FMD may avoid these adverse effects by promoting additional and coordinated endogenous responses. Importantly, FMD cycles also activated OPCs, resulting in myelin regeneration, as demonstrated

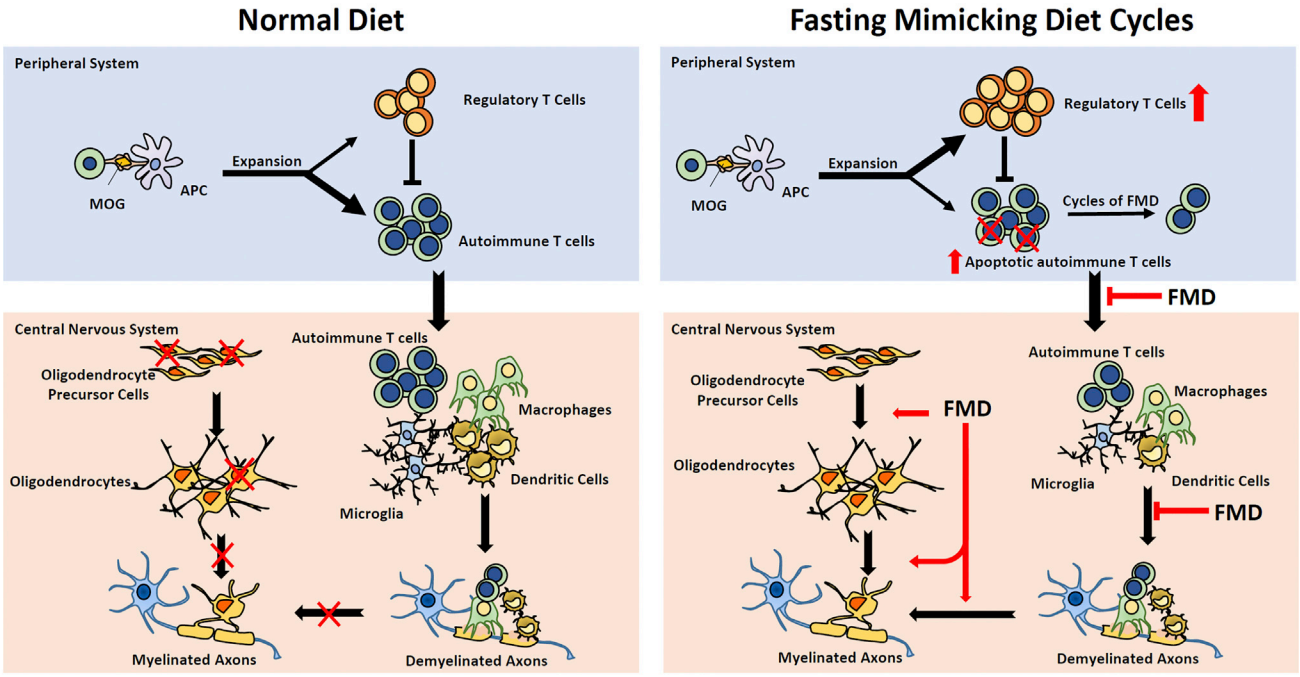

Figure 5. A Simplified Model of FMD-Mediated Effects on Immune Suppression, Oligodendrocyte Regeneration, and Differentiation in MS

FMD treatment promotes endogenous glucocorticoid production, increases T_{reg} cell numbers, blocks T cell activation, and promotes T cell death. In the lesion area, FMD treatment reduces autoimmune T cell and microglia infiltration and promotes oligodendrocyte-precursor-dependent regeneration and differentiation of myelinating oligodendrocytes, which engage with demyelinated axons to promote the formation of myelin sheaths.

by accelerated remyelination rate in the cuprizone model (Figure 5). Notably, because it is the alternation of FMD cycles and re-feeding and not the FMD alone that promotes the regeneration and replacement of autoimmune cells with naive cells, the use of chronic restriction or even a chronic KD may not be effective, or as effective, in the treatment of EAE and MS.

Finally, we report that the administration of the FMD and KD in MS patients was safe and well tolerated and resulted in high compliance. We observed positive effects of FMD cycles or KD treatment in RRMS based on changes in self-reported HRQOL and a mild improvement in EDSS (Table S6). However, the lack of a proper Mediterranean diet control makes it difficult to establish whether FMD cycles alone are sufficient to produce these effects. In addition, MRI analyses and adequately blinded clinical assessments (EDSS and multiple sclerosis functional composite [MSFC]), as well as immune function analyses would greatly enhance the strength of the clinical findings. Because, unlike for the mouse experiments, the FMD was only administered to patients only once, it will be important to test the effects of multiple FMD cycles on MS patients in larger, randomized, and controlled trials.

EXPERIMENTAL PROCEDURES

EAE Model

C57Bl/6 (10-week-old female) mice were purchased from The Jackson Laboratory and immunized subcutaneously with 200 μg $MOG_{35–55}$ (GenScript) mixed 1:1 with supplemented complete Freund's adjuvant followed by 200 ng pertussis toxin (PTX; List Biological Laboratories) intraperitoneally (i.p.) at days 0 and 2. For adoptive transfer, spleens from active immunized mice were isolated and red blood cells (RBCs) were lysed. Spleen cells were cultured in the presence of $MOG_{35–55}$ (20 μg/ml) with rmIL-23 (20 ng/ml) for 48 hr. Cells were collected and re-suspended in PBS, and 15 million cells were injected intravenously. See Supplemental Experimental Procedures for a detailed description of disease severity scoring. All experiments were performed in accordance with approved Institutional Animal Care and Use Committee (IACUC) protocols of the University of Southern California.

Mouse Fasting Mimicking Diet

Mice were fed ad lib with irradiated TD.7912 rodent chow (Harlan Teklad), containing 15.69 kJ/g digestible energy (animal-based protein 3.92 kJ/g, carbohydrate 9.1 kJ/g, and fat 2.67 kJ/g). The experimental FMD is based on a nutritional screen that identified ingredients that allow high nourishment during periods of low calorie consumption. The FMD diet consists of two different components, day 1 diet and day 2–3 diet, that were fed in this order, respectively. See Supplemental Experimental Procedures for a detailed explanation of the FMD. Mice consumed all the supplied food on each day of the FMD regimen and showed no signs of food aversion. After the end of FMD, we supplied TD.7912 chow ad lib for 4 days before starting another FMD cycle. Prior to supplying the FMD, animals were transferred into fresh cages to avoid feeding on residual chow and coprophagy.

Clinical Trial Design

This study was a three-armed, parallel-group, single-center, controlled, and randomized clinical pilot trial to assess the effects of dietary interventions on HRQOL in RRMS patients. The permuted-block randomization was generated online at http://randomization.com. An investigator blind

to the randomization plan determined the patients' randomization number before they underwent the randomization step. This study is registered at http://www/clicaltrials.gov as NCT01538355. The study was approved by the local ethics committee. All participants gave informed written consent according to the 1964 Declaration of Helsinki. See Supplemental Experimental Procedures for detailed descriptions of the clinical trial and diet compositions.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, five figures, and seven tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.05.009.

AUTHOR CONTRIBUTIONS

I.Y.C., L.P., M.W., and V.D.L. designed mouse experiments. I.Y.C., S.B., and P.C. performed the mouse experiment. I.Y.C., L.P., P.C., B.B., and A.G. performed and processed immunohistochemistry. I.Y.C., L.P., and B.B. performed qualitative and quantitative analysis. I.Y.C. and J.S. performed fluorescence-activated cell sorting (FACS) analysis. I.Y.C. processed the cytokine assay. A.M., F.P., and M.B. designed the human study; M.B. acquired human clinical data; A.M., F.P., and M.B., analyzed and interpreted data; and M.B. performed, interpreted, and presented the statistical analysis. A.M., F.P., M.B., A.H.C., T.E.M., M.W., and V.D.L. were involved in discussing the results and editorial support. I.Y.C., M.B., and V.D.L. wrote the paper. All authors discussed the results and commented on the manuscript.

CONFLICTS OF INTEREST

The University of Southern California has licensed intellectual property to L-Nutra that is under study in this research. As part of this license agreement, the University has the potential to receive royalty payments from L-Nutra. V.D.L. has equity interest in L-Nutra, a company that develops medical food.

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