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Diet mimicking fasting promotes regeneration and reduces autoimmunity and multiple sclerosis symptoms

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Summary

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Author Contributions: I.C., L.P., M.W., and V.D.L designed mouse experiments. I.C., S.B. and P.C performed the mouse experiment. I.C, L.P., P.C., B.B. and A.G performed and processed immunohistochemistry. I.C., L.P. and B.B. performed qualitative and quantitative analysis. I.C. and J.S. performed FACS analysis. I.C. processed cytokine assay. AM, FP, and MB designed the human study; MB acquired human clinical data; AM, FP and MB, analyzed and interpreted data; MB 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.C., M.B., and V.D.L. wrote the paper. I.C. and L.P. contributed equally to this work. M.B. and V.D.L. contributed equally to this work. All authors discussed the results and commented on the manuscript.

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 the animals. These improvements were associated with increased corticosterone levels and T_{reg} cell number, 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 response to both EAE and cuprizone MS models, supporting its effects on both suppression of autoimmunity and remyelination. We also report preliminary data suggesting that a 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 central nervous system (CNS) (Friese and Fugger, 2005; Pender and Greer, 2007; Rasmussen et al., 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 neurological 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 the function of the immune system 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 the restoration of a functional myelin sheath. Periodic cycles of prolonged fasting (PF) or of a fasting mimicking diet (FMD) lasting 2 or more days can increase protection of multiple systems against a variety of chemotherapy drugs in mice and possibly humans. Moreover, PF or FMD reverse the immunosuppression or immunosenescence of either chemotherapy or aging through hematopoietic stem cell-based regeneration (Brandhorst et al., 2015; Cheng et al., 2014; Fontana et al., 2010; Guevara-Aguirre et al., 2011; Lee et al., 2010; Longo and Mattson, 2014). Chronic caloric restriction, a ketogenic diet (KD), and intermittent fasting have been shown to prevent EAE by reducing inflammation and enhance 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 as a therapy 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 of MS mouse models, and 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 MS (RRMS).

Results

The FMD cycles reduce disease severity in the MOG_{35-55}-induced EAE model

We examined the effects of periodic cycles of a very low calorie and low protein fasting mimicking diet (FMD) lasting 3 days every 7 days (3 cycles) or a ketogenic diet (KD) continued throughout the 30 days on EAE model induced with active immunization with myelin oligodendrocyte glycoprotein 35-55 (MOG_{35-55}) (Fig. 1a). Groups of mice were treated both semi-therapeutically -EAE FMD (S); in which FMD treatment started after 10% of the immunized population showed EAE signs- or therapeutically -EAE FMD (T), in which FMD treatment started after all of the immunized population showed EAE signs. FMD and KD treatment decreased the disease severity compared to the control (Fig. 1b); however, the FMD reduced the mean severity score to approximately 1, whereas the KD group reduced the severity score to approximately 2 at the later stages (Fig. 1b). In the EAE FMD (S) group, FMD treatment not only delayed the onset of disease but also lowered the incidence rate (100% *vs.* 45.6%; Fig. 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; Fig. 1d), and reduced the severity score to below 0.5 in over 50% of the mice (12 out of 23 mice; Fig. 1e). To address whether the FMD cycles also have beneficial effects on the chronic EAE models that have established disease, we initiated FMD treatment two weeks after initial EAE signs (EAE CTRL-FMD). Prior to the treatment, both the EAE CTRL and EAE CTRL-FMD cohorts had similar severity scores (3.19 ± 0.52 *vs.* 3.30 ± 0.27; Day 24). After three FMD cycles, we observed a significant reduction of severity score in the EAE CTRL-FMD cohort compared to the EAE CTRL cohort (3.3 ± 0.57 *vs.* 2.1 ± 0.89; Day 42; $p < 0.05$; Fig. 1f). As infiltration of immune cells and demyelination are histopathological hallmarks of EAE and MS, spinal cord sections were stained with hematoxylin and eosin to visualize infiltrating immune cells (H&E; Fig. 1g) or solochrome cyanine to visualize myelin (Fig. 1 h). To assess demyelination and axonal damage, immunohistochemistry was performed using antibodies against myelin basic protein (MBP) or dephosphorylated neurofilaments (SMI-32; Fig. 1i). At D3, the level of infiltrating immune cells and demyelination were similar in the EAE CTRL and EAE FMD groups (Fig. 1j; Supplemental Fig. 1h). At D14, sections of EAE CTRL mice displayed severe immune cell infiltration corresponding with demyelinated lesions, reduced MBP expression and increased SMI-32 expression (Fig. 1j–m). By contrast, sections of EAE FMD mice at D14 displayed significantly reduced immune cell infiltration and demyelination (Fig. 1j–m). Although MBP staining showed no significant difference between EAE CTRL and EAE FMD at D14 (Fig. 1l), neurofilament dephosphorylation in the EAE FMD mice was reduced compared to the EAE CTRL group (Fig. 1m). Overall, these results suggest that FMD cycles reduce EAE disease severity in part by reducing inflammation, and preventing demyelination and axonal damage.

The 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 of the naive, EAE CTRL, EAE FMD and EAE FMD:RF (measured 4 days after returning to a standard *ad lib* diet) groups after 3 cycles of the FMD regimen (Fig. 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 CBC counts returned to either naive level or lower levels than those observed in the EAE CTRL with exception of granulocytes, indicating that the FMD cycles cause both white blood cell death and regeneration (Fig. 2a). Next, we measured the inflammatory markers associated with EAE pathophysiology. D3 and D14 spinal cord sections of the EAE CTRL were extensively populated with $CD11b^+$ cells (Fig. 2b). However, at D14, 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% *vs.* 2.8%; Fig. 2b). Since the 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 was detected in the white matter of the spinal cord from the control diet cohort (Fig. 2c). In contrast, the FMD treated cohort displayed an over 4-fold reduction ($p< 0.01$) in $CD4^+$ T cells at D3 (8.6% *vs.* 1.5%; Fig. 2c) compared to the control diet cohort, which remained lower even at D14. The FMD group also had reduced $CD8^+$ T cells (D3: 1.3% *vs.* 0.4%; $p<0.01$; Fig. 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 D3, 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% *vs.* 1.46±0.31%), but did not observe any changes in the number of $F4/80^+$ macrophage cells in the control or the FMD treated groups (Fig. 2e; Supplemental Fig. 2b). 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 the EAE CTRL and EAE FMD mice was similar (Supplemental Fig. 2c–d), 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 *vs.* 3.67; Fig. 2f). No difference in $CD8^+$ T cells was observed (Supplemental Fig. 2e). 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 (Supplemental Fig.2f–h). These results indicate that FMD cycles decrease dendritic cells and increases the relative number of naïve T cells which may explain the reduced autoimmunity caused by the FMD.

The FMD cycles induce autoreactive lymphocytes apoptosis and increase the number of naive cells

To determine whether FMD cycles also reduce the MOG-specific antigen reactive cells, we used a MHC Tetramer (MOG_{35-55} /IA^b) to identify antigen-reactive cells after a FMD cycle *in vivo*. $CD4^+$ MOG_{35-55} /$IA^{b\,+}$ cells were reduced in the EAE FMD cohort compared to the EAE CTRL cohort (5.75 ± 0.51 % *vs.* 3.83 ± 0.66 % of lymphocytes; * $p < 0.05$; Fig. 2g). To determine whether the reduced active T-cells numbers are due to an increase in the

number of regulatory T cells (T_{reg}), we isolated lymphocytes from draining lymph nodes and spleens of the EAE CTRL or EAE FMD mice and analyzed for $CD4^+$ $CD25^+$ $FoxP3^+$ T_{reg} cells. The FMD cohort resulted in a two-fold increase ($p<0.01$) in $CD25^+$ $FoxP3^+$ expressing T_{reg} cells (13.6±4.2 % *vs.* 25.1±4.2%; Fig. 2h). Moreover, the FMD cohort resulted in a 27.8% reduction ($p<0.05$) in IFN-γ expressing T_H1 cells (2974.4±708.0 *vs.* 2148.1±1396.1; Fig. 2i) and a 46.5% reduction ($p<0.05$) in IL-17 expressing T_H17 cells (2535.9±722.0 *vs.* 1357.1±256.2; Fig. 2j), both 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 IFN-γ expressing T_H1 cells (2974.4±708.0 *vs.* 805.8±251.5; Fig. 2i) and 82.9% reduction ($p< 0.05$) in IL-17 expressing T_H17 cells (2535.9±722.0 *vs.* 432.4±117.4; Fig. 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 the FMD cycles may reduce the number of T cells, we measured apoptosis in MOG-specific T cells ($CD3^+MOG_{35-55}/IA^b$) *in vivo*. We observed a significant increase ($p < 0.05$) in apoptotic $CD3^+$ MOG_{35-55}/IA^b level in the EAE FMD cohort compared to the EAE CTRL cohort (28.3 ± 4.94 % *vs.* 39.1 ± 4.79 %; Fig. 2k), which was consistent with the major reduction in the number of white blood cell and lymphocytes in the FMD group (Fig. 2a). To investigate whether these apoptotic cells are replaced by newly generated cells, we treated the mice with BrdU during the re-feeding period (4 injections within 48 hours, 1 mg of BrdU/injection). Splenocytes were isolated 4 days after the re-feeding of the regular diet, and stained for BrdU (Supplemental Fig. 2i). We observed no difference in levels of total $BrdU^+$ lymphocytes (8.11 ± 1.99% *vs. 12*.02 ± 2.72%; Supplemental Fig. 2j), but a significantly reduced proliferation of T_H1 ($BrdU^+$ $CD4^+IFN\gamma^+$) (5.74 ± 1.07% *vs.* 3.65 ± 0.63 %; * $p < 0.05$; Fig. 2l), and no difference in proliferation of T_H17 ($BrdU^+CD4^+IL17^+$) (4.71 ± 1.53% *vs.* 5.01 ± 1.66 %; Supplemental Fig. 2k). Taken together, these data indicate that FMD cycles may promote apoptosis of the autoreactive T cells, leading to an increase in the proportion of naïve 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 effects on CNS infiltrating immune cells are associated with suppression of T_H1- and T_H17-dependent cytokine production (IL-17, IFNγ, and TNF-α), we analyzed serum from the naive, EAE CTRL and EAE FMD mice (Fig. 2k–m). We observed a significant reductions in serum TNF-α (113.3±7.9 *vs.* 79.3±10.5 pg/mL; $p<0.001$; Fig. 2m), IFNγ (558.43±124.5 *vs.* 296.0±83.4 pg/mL; $p<0.001$; Fig. 2n), and IL-17 (36.8±9.67 *vs.* 20.75±4.2 pg/mL; $p<0.01$; Fig. 2o). To identify a potential mediator for the effects of FMD cycles on the suppression of autoimmune responses, we measured serum corticosterone. 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 EAE signs (EAE Day1; before the treatment). The FMD treatment caused a further increase in corticosterone levels at D3 compared to those in controls (245.9±38.8 *vs.* 375.0±94.1 ng/mL; $p < 0.01$), which returned to EAE basal levels by D14 in both groups (Fig. 2p). These results indicate that the FMD cycles reduce 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 APCs and T cell infiltration in the spinal cord.

FMD reverses EAE symptoms by reducing the levels/reactivity of established autoimmune cells

To determine how FMD affects the initiation of EAE, splenocytes were isolated from EAE CTRL and EAE FMD, re-activated with MOG_{35-55} peptide and IL-23 *ex-vivo* and transferred into naïve recipient mice to induce EAE. Then the mice were subjected to either the control diet or FMD cycles (Fig. 3a). The supernatant from *ex-vivo* splenocyte cultures derived from the EAE FMD showed no difference in TNFα level (110.8±14.9 pg/mL *vs.* 97.1±8.4 pg/mL; Fig. 3b) but showed a major reduction ($p < 0.01$) in IFNγ (342.0±29.8 pg/mL *vs.* 46.6±16.6 pg/mL Fig. 3c) and IL-17 (850.5±442.0 pg/mL *vs.* 257.4±36.4 pg/mL; Fig. 3d). Interestingly, upon *in vitro* reactivation, both EAE CTRL and EAE FMD had similar levels of T_H1 and T_H17 differentiated cells (Fig. 3e–f). To determine whether the immune cells from EAE CTRL and EAE FMD mice 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 (Fig. 3g), and an equally severe EAE disease severity by day 20 (2.38±0.48 *vs.* 2.70±0.75; Fig. 3h), indicating that the FMD did not affect the development and function of reactive immune cells *in vivo* or *ex vivo*. However, when the FMD treatment initiated after transfer of control donor splenocytes [B; EAE CTRL donor to naïve mice with FMD treatment], the recipient mice displayed a delayed disease onset (Day 12 *vs.* Day 16 post-transfer; Fig. 3g) and a major reduction in EAE severity scores compared to the control (2.38±0.48 *vs.* 0.75±0.87; Fig. 3h). Taken together, these results suggest that T-cell priming in response to myelin antigen occurred normally in EAE CTRL and EAE FMD groups.

The 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 $NG2^+$ (oligodendrocyte progenitor cells marker; OPC) and GST- π^+ (mature oligodendrocyte marker) in the spinal cord sections from the control or FMD mice (Fig. 4a). We observed no difference in the number of $NG2^+$ OPC in the sections from EAE CTRL and EAE FMD group (Supplemental Fig. 3a). However, at D14, the number of GST- π^+ oligodendrocytes was reduced in the EAE CTRL group but not in the EAE FMD group (886.7±41.6 *vs.* 1273±200.3; cells/ spinal cord section area; $p < 0.01$; Fig. 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 (Day10). We observed a major increase ($p < 0.01$) in the percentage of cells that are double positive for $BrdU^+$ and $GST\pi^+$ in the EAE FMD group compared to the EAE CTRL (42.9±11.2% *vs.* 83.0±13.2%; $p < 0.01$) suggesting that the FMD promotes oligodendrocyte differentiation from precursor cells (Fig. 4c). To assess the effects of the FMD on either OPC or mature oligodendrocytes, sections were stained with TUNEL, an apoptotic marker, and GST- π^+ or $NG2^+$ (Fig. 4d). We observed a significant increase in $TUNEL^+$ $NG2^+$ (11.2±12.2 *vs.* 1.9±1.4 cells/section) cells and $TUNEL^+$ GST-

π^+ (18.8±15.2 *vs.* 2.9±5.3 cells/section) in the control group compared to FMD group ($p < 0.05$; Fig. 4e–f). Taken together, these results indicate that the FMD not only stimulates regeneration and differentiation of oligodendrocytes but also protects OPC 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^+ (Fig. 4g, i). 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 (Fig. 4h, j). After 2 cycles, the FMD-treated group displayed increased myelin staining and in the number of GST- π^+ oligodendrocytes compared to the control diet group (Fig. 4h, j). However, at later time points, we did not observe differences between spontaneous re-myelination of the control diet cohort and FMD cohort, since it is well established that cuprizone-dependent myelin damage can be fully reversed after removal of the toxin (Supplemental Fig. 3 c, d). 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 3 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 relapsing-remitting MS patients. 60 patients were randomly assigned to: control diet (CD n=20), KD for 6 months (n=20) or a single cycle of modified human FMD for 7 days (n=20) followed by a Mediterranean diet for 6 months (Supplemental Fig. 4). Baseline characteristics were balanced between the 3 groups (Supplemental Tab. 1 and Supplemental Tab. 2). The FMD and KD cohorts displayed clinically meaningful improvements in the HRQOL summary scales at 3 months which included the overall quality of life (Fig. 4k) change in health (Fig. 4l), physical health composite (Fig. 4m), and mental health composite (Fig. 4n). Also, similar changes were observed in the total HRQOL scales at different time points (Supplemental Fig. 5). 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 (Supplemental Tab. 5). The most common AEs was airways infection (common adverse events) and the most frequent SAE was lower urinary tract infection (serious adverse events). No indication of increase in liver enzymes exceeding the normal range was observed in any of the three treatment groups. Also, the interventions were well tolerated, as evident from high compliance rates (CD: 60%; KD: 90%; FMD: 100%). During the 6-month study period we observed a total of 8 relapses: 4 in the CD, 1 in the KD, and 3 in the FMD group. In addition to increased β-hydroxybutyrate

levels in plasma, we observed a slight reduction in lymphocytes and white blood cell counts and we detected a mild reduction in EDSS scores in the FMD and KD groups (measured on Day 7 for FMD and Day 30 for KD; Supplemental Tab. 4 and Supplemental Tab. 6). Thus, there was an inverse association between EDSS and HRQOL scores (Supplemental. Tab. 7). Overall, our study indicates that the administration of FMD and KD is safe, feasible and potentially effective, but further studies including analyses such as MRI, blinded clinical assessments, an appropriate control diet, and immune assays are required to determine efficacy.

Discussion

A FMD administered every week was effective in ameliorating EAE symptoms in all mice and completely reversed disease progression 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 of T cells contributing to the autoimmunity and encephalomyelitis. Our results support a FMD-mediated anti-inflammatory effects possibly involving the up-regulation of AMPK or down-regulation of mTORC1, which sense nutrient availability and dictate cell fate (Laplante and Sabatini, 2012). It was shown that mTORC1 couples the immune signals and the metabolic programming to establish T_{reg}-cell function (Zeng et al., 2013). In fact, treatment with the mTORC1 inhibitor rapamycin or AMPK activator metformin attenuates EAE symptoms by modulating effector and regulatory T cells and restricting the infiltrating mononuclear cells into the CNS (Esposito et al., 2010; Nath et al., 2009; Zeng et al., 2013). Therefore, the FMD treatment could interfere with T-cell proliferation, differentiation, and with recruitment of other immune cells, resulting in a decreased recruitment at the lesion sites (Fig. 5). Some of these effects of the FMD may be triggered by an endogenous glucocorticoid production. Glucocorticoids are used to treat MS relapses, but are generally administered in short bursts since they can cause adverse effects including 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 oligodendrocyte precursor cells resulting in myelin regeneration, as demonstrated by accelerated remyelination rate in the cuprizone model (Fig. 5). Notably, because it is the alternation of FMD cycles and re-feeding and not the FMD alone that promotes both the regeneration and the replacement of autoimmune cells with naïve cells, the use of chronic restriction may not be effective or as effective in the treatment of EAE/MS.

Finally, we report that the administration of the FMD and KD in MS patients was safe, well tolerated and resulted in high compliance. We observed potentially positive effects of FMD cycles or KD treatment in RRMS based on changes in self-reported HRQOL, and a mild improvement in EDSS (Supplemental Tab. 6). However, the lack of a proper Mediterranean diet control makes it difficult to establish whether the FMD cycles alone are sufficient for these effects. In addition, MRI analyses and an adequately blinded clinical assessments (EDSS, MSFC) and of 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 once, it will be important to test the effects of multiple FMD cycles on MS patients in larger, randomized and controlled trials.

Materials and Methods

EAE model

C57Bl/6 (10-week-old female) mice were purchased from The Jackson Laboratory, immunized subcutaneously with 200 μg myelin oligodendrocyte glycoprotein peptide (MOG_{35-55}; GenScript) mixed 1:1 with supplemented complete Freund's Adjuvant followed by 200 ng of mouse pertussis toxin (PTX; List Biological Laboratories) i.p. at day 0 and day 2. For adoptive transfer, spleens from active immunized mice were isolated and RBC were lysed. Spleen cells were cultured in presence of MOG35-55 (20 μg/mL) with rmIL-23 (20 ng/mL) for 48 hours. Cells were collected and re-suspended in PBS and 15 million cells were injected intravenous. See supplemental material and methods for detailed description of disease severity scoring. All experiments were performed in accordance with approved Institutional Animal Care and Use Committee (IACUC) protocols of University of Southern California

Fasting Mimicking Diet (Mouse)

Mice were fed *ad lib* with irradiated TD.7912 rodent chow (Harlan Teklad), containing 15.69 kJ/g of digestible energy (animal-based protein 3.92 kJ/g, carbohydrate 9.1 kJ/g, fat 2.67 kJ/g). The experimental FMD is based on a nutritional screen that identified ingredients which allow high nourishment during periods of low calorie consumption. The FMD diet consists of two different components designated as day 1 diet and day 2–3 diet that were fed in this order respectively. See supplemental material and methods for 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 grouped, 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 the website randomization.com. An investigator blind to the randomization plan determined the patients' randomization number before they underwent the randomization step. This study is registered with ClicalTrials.gov. NCT01538355. See supplemental material and methods for detailed descriptions of the clinical trial and the diet compositions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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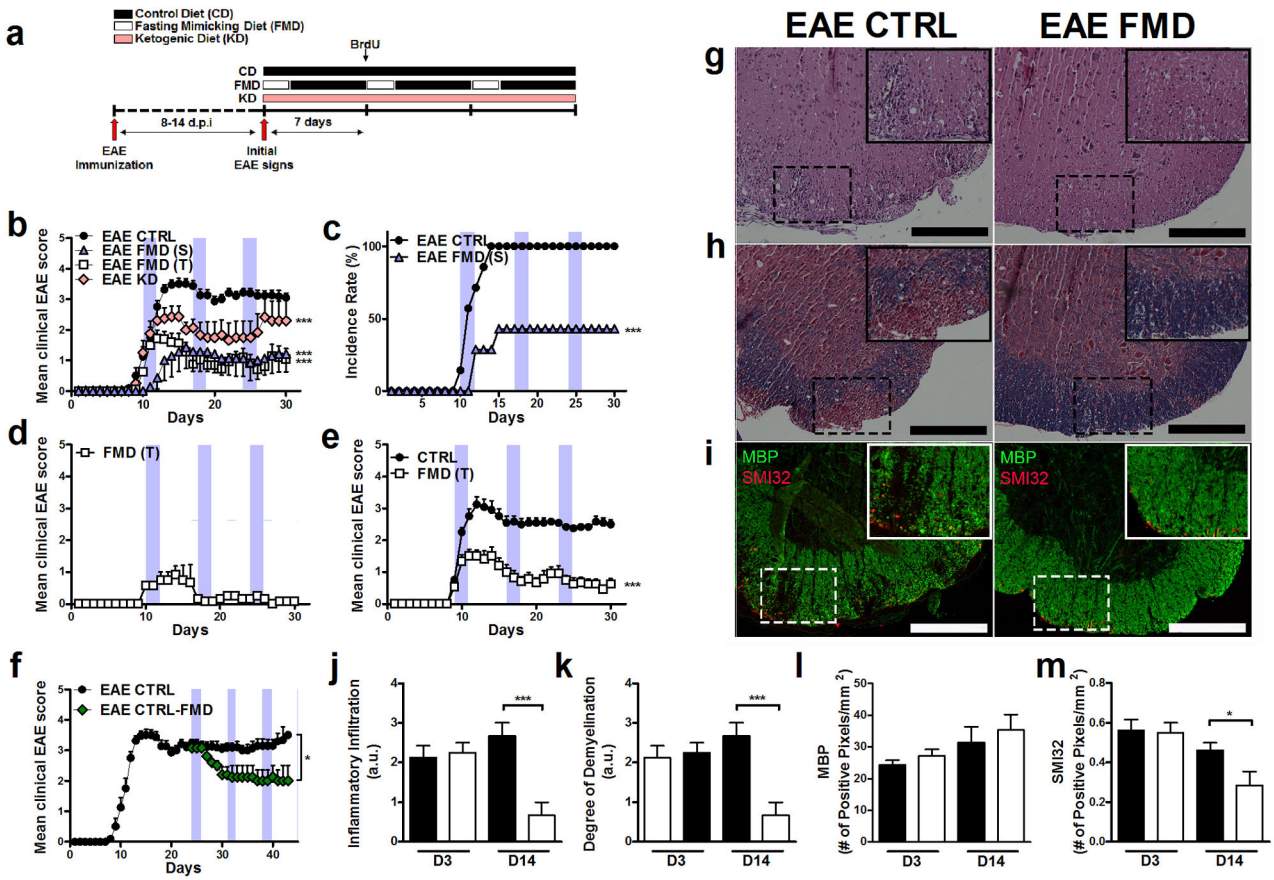

Figure 1. FMD cycles decrease disease severity of the MOG_{35-55}-induced EAE model

(mean ± S.E.M, * p < 0.05, ** p<0.01; *** p < 0.001; *t test*, 1-way or 2-way ANOVA & Bonferroni Post Test; Scale bar represents 200 μm).

a. Diagram displaying the time course of the immunization and the diet interventions.

b. The EAE severity scoresof 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 the EAE CTRL and EAE FMD (S) (n=7–23).

d. EAE severity score of the EAE FMD(T) mice that completely reversed the EAE severity and scored 0, no observable disease (n=5).

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

f. EAE severity score of the EAE CTRL mice that treated with FMD upon chronic EAE development(EAE CTRL-FMD) (n=6).

g–m. Spinal cord of the EAE CTRL and the EAE FMD (T) mice with quantification of (**g**) H&E staining, (**h**) solochrome cyanine staining, and (**i**) MBP (Myelin Basic Protein) and SMI32 staining of spinal cord sections isolated at day 14.

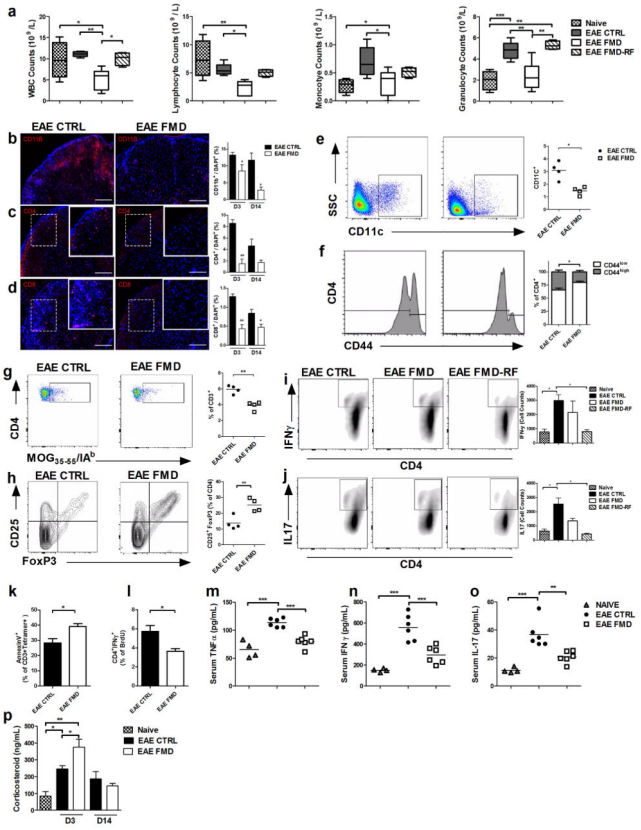

Figure 2. FMD cycles decrease the number of infiltrating T cells in the Spinal Cord

(n=4–8 / group; mean ± S.E.M, * $p < 0.05$, ** $p<0.01$; *** $p < 0.001$; *t test*, 1-way ANOVA & Bonferroni Post Test; Scale bar represents 200 μm).

a. Total white blood cells (WBC), lymphocytes, monocytes and granulocyte counts of the naïve, EAE-CTRL, EAE-FMD, and after 3 days of the re-feeding (EAE-FMD: RF) mice after 3 cycles of the FMD and matched time point for the EAE-CTRL.

b–d. Spinal cord sections (D14) and quantification at D3 and D14 post the first EAE sign for (**b**)$CD11b^+$, (**c**) $CD4^+$, and (**d**) $CD8^+$ (at least 6 sections / mouse).

e. $CD11c^+$ isolated from the EAE CTRL or EAE FMD mice on D3 and the quantification of cells from the total isolated splenocyte.

f. $CD4^+$ gated for $CD44^L$ or $CD44^H$ isolated from the EAE CTRL or EAE FMD and quantification of % splenocyte of $CD4^+$ $CD44^L$ (Inactive) or $CD4^+$ $CD44^H$ (Active) cells.

g. $CD3^+$ lymphocytes gated for CD4 and MOG_{35-55}/IA^b from EAE CTRL or EAE FMD and quantification of the MOG specific $CD4^+$ cells.

h. $CD4^+$ $CD25^+$ $FoxP3^+$ isolated from EAE CTRL or EAE FMD and the quantification of $CD25^+$ $FoxP3^+$ of $CD4^+$ cells.

i–j. Intracellular staining for either IFNγ (**i**) or IL17(**j**) after gated for $CD4^+$ of the naïve, EAE CTRL, EAE FMD, EAE FMD:RF and quantification of cell counts.

k. Quantification of Annex in V^+ apoptotic $CD3^+$ MOG_{35-55}/IA^b cells.

l. Quantification of $CD4^+IFN\gamma^+$ of $BrdU^+$ lymphocytes.

m–o. Serum (**m**)TNFα, (**n**)IFNγ, and (**o**)IL-17 level (pg/mL) of the naive, EAE CTRL and EAE FMD mice on D3 post first sign of EAE.

p. Serum corticosterone level (ng/mL) of before immunization, at the time of the symptom, 3 or 14 days after initial symptom of the control or FMD.

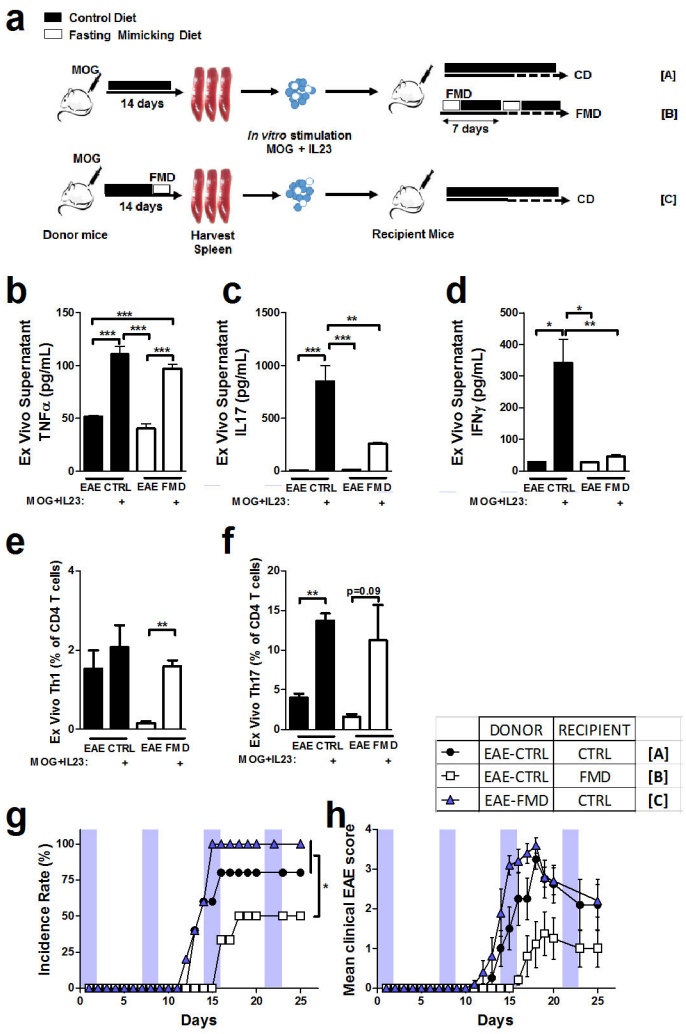

Figure 3. Antigen activated splenocytes from the EAE-CTRL and the EAE-FMD mice had similar encephalitogenic effects

(n=5–6 / group; mean ± S.E.M, * $p < 0.05$, ** $p<0.01$; *** $p < 0.001$; *t test*, 1-way ANOVA & Bonferroni Post Test).

a. Diagram for the adoptive transfer EAE model.

b–d. Quantification of the (**b**) TNFα, (**c**) IFNγ, and (**d**)IL-17 (pg/mL) of the supernatant from e*x-vivo* culture of splenocytes from naïve, EAE CTRL, and EAE FMD either with or without MOG_{35-55} and IL-23 re-activation

e–f. Quantification of T_H1 or T_H17 (represented by % of $CD4^+$) from lymphocytes culture of EAE CTRL and EAE FMD 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.

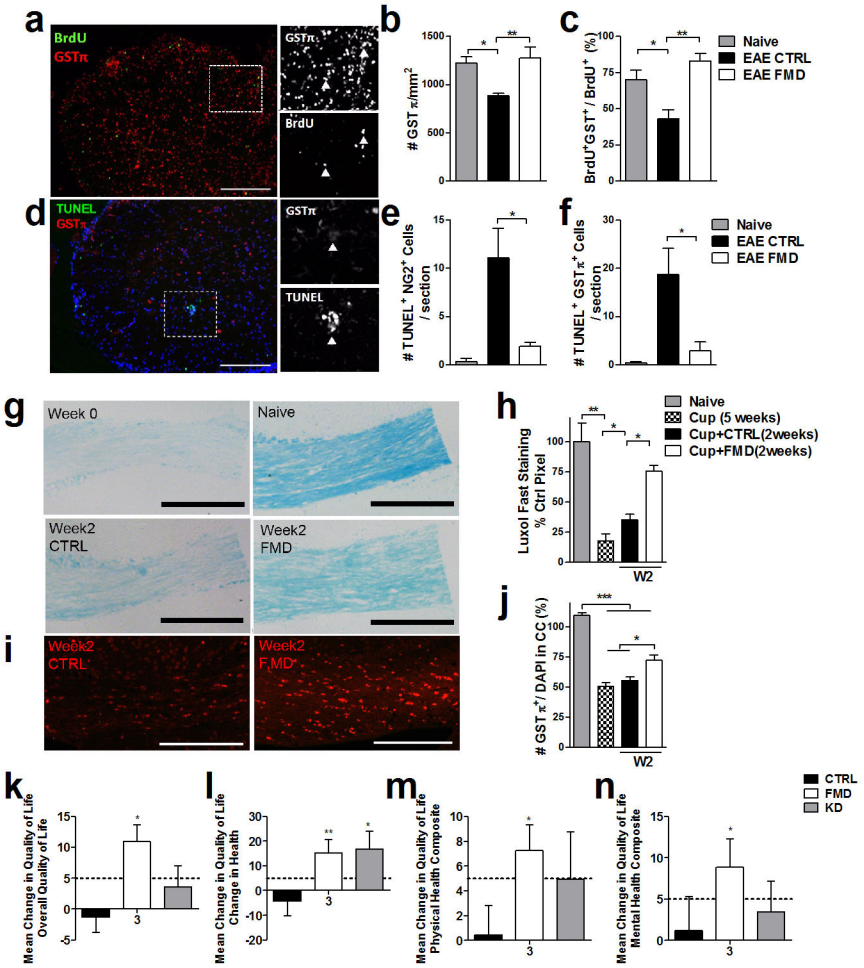

Figure 4. The FMD cycles protect the spinal cord from loss of oligodendrocyte precursor cells and oligodendrocytes and enhances remyelination in the cuprizone model

(At least 12 sections/ mouse were used for quantification; n=4; mean ± S.E.M, * $p < 0.05$, ** $p<0.01$; *** $p < 0.001$; 1-way ANOVA & Bonferroni Post Test).

a – c. Spinal cord sections isolated at Day 14 and quantification for (**a**) GST-π (mature oligodendrocyte) and BrdU, for (**b**) TUNEL and NG2 (oligodendrocyte precursor cells), and for (**c**) TUNEL and GST-π of the naive, EAE-CTRL or EAE-FMD.

f–h. Sections from the corpus callosum region and quantification of cuprizone treated brains, stained with Luxol Fast Blue of the naïve control, end of 5 weeks of cuprizone diet (week 0), cuprizone (5 weeks) + regular chow (2 weeks), cuprzione (5 weeks) + FMD cycle (2 weeks).

i–j. Section from the corpus callosum region and its quantification of the cuprizone treated brains stained with GST-π^+ of cuprizone (5 weeks) + regular chow (2 weeks), cuprzione (5 weeks) + FMD (2 weeks). Quantification is normalized to % of the naïve GST-π^+ level.

k–m. Change in the quality of life at 3 month of (**k**) overall quality of life, (**l**) change in health, (**m**) physical health composite, and (**n**) mental health composite. The dotted line represents a threshold that is thought to be clinically important (≥ 5 points) (mean ± SED; * $p<0.05$; Mann-Whitney-U test. Increase of ≥ 5 points are considered as clinically important).

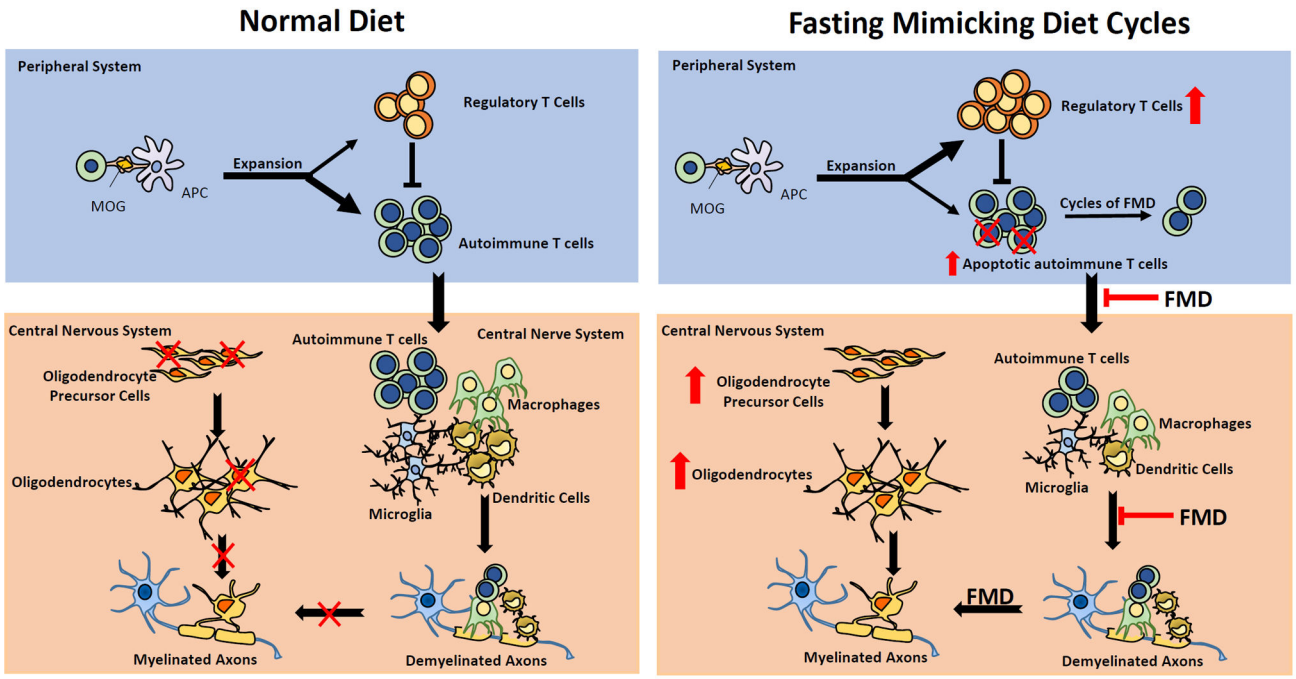

Figure 5. A simplified model of FMD-mediated effects on glucocorticoid, immune suppression & oligodendrocyte regeneration and differentiation in MS

The 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, promotes oligodendrocyte precursor dependent regeneration and the differentiation of myelinating oligodendrocyte which engage with demyelinated axons to promote the formation of myelin sheaths.