



Hydroxycarboxylic acid receptor 2 mediates dimethyl fumarate's protective effect in EAE

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Taken orally, the drug dimethyl fumarate (DMF) has been shown to improve functional outcomes for patients with MS; however, it is unclear how DMF mediates a protective effect. DMF and, more so, its active metabolite, monomethyl fumarate, are known agonists of the hydroxycarboxylic acid receptor 2 (HCA₂), a G protein-coupled membrane receptor. Here, we evaluated the contribution of HCA₂ in mediating the protective effect afforded by DMF in EAE, a mouse model of MS. DMF treatment reduced neurological deficit, immune cell infiltration, and demyelination of the spinal cords in wild-type mice, but not in *Hca2*^{-/-} mice, indicating that HCA₂ is required for the therapeutic effect of DMF. In particular, DMF decreased the number of infiltrating neutrophils in a HCA₂-dependent manner, likely by interfering with neutrophil adhesion to endothelial cells and chemotaxis. Together, our data indicate that HCA₂ mediates the therapeutic effects of DMF in EAE. Furthermore, identification of HCA₂ as a molecular target may help to optimize MS therapy.

Introduction

Recently, orally available drugs that modify the disease process of MS have come into routine use in clinical practice. Dimethyl fumarate (DMF) stands out as a compound that is more effective than standard parenteral treatment (1, 2). DMF has been used to treat psoriasis since the 1990s; during that time, the drug has had a well-documented safety record (3). Of its adverse effects, flushing and gastrointestinal side effects at the beginning of therapy are most common.

Although the clinical efficacy of DMF has been established and the drug has been approved by the FDA and European Medicines Agency, its mode of action is still incompletely understood. In EAE, an animal model of MS (4), DMF has shown immunomodulatory and neuroprotective effects and improved the clinical score (5–7). DMF is rapidly metabolized to monomethyl fumarate (MMF). DMF and MMF covalently modify glutathione and KEAP-1, the inhibitor of the antioxidant transcription factor nuclear factor erythroid-derived 2-related factor 2 (NRF2), and induce hemoxygenase-1 (*HMOX1*), NAD(P)H quinoline oxidoreductase-1 (*NQO1*), and other genes (6, 7). Whether the covalent modifications occur in vivo and eventually ameliorate MS pathology is still unclear.

Recently, the carboxylic acid MMF was shown to be a potent agonist of the hydroxycarboxylic acid receptor 2 (HCA₂; GPR109A) (8). This G_i-coupled membrane receptor mediates the lipid-lowering effects of nicotinic acid but is also expressed by keratinocytes and immune cells and especially by neutrophils (9). In this study, we aimed to elucidate the role of HCA₂ in the disease-modifying action of DMF in EAE. Our data suggest that HCA₂ is an important drug target for the treatment of MS.

Results and Discussion

To investigate the potential role of HCA₂ in the protective effect afforded by DMF, we used wild-type and *Hca2*^{-/-} mice that show no overt phenotype if unchallenged (10). Starting 3 days postimmunization (dpi), we administered vehicle or DMF (30 mg/kg, twice per day) by gavage. DMF treatment improved the motor score of wild-type mice but lost its effect in *Hca2*^{-/-} mice (Figure 1A). DMF also reduced the area under the curve of the motor deficit, the peak scores, and the day of disease onset in wild-type mice but not in *Hca2*^{-/-} mice (Figure 1, B–D). We reproduced the experiment twice with mice coming from 2 different animal facilities and with littermates in one case (Supplemental Figure 1, A and B; supplemental material available online with this article; doi:10.1172/JCI72151DS1). Although the efficacy of DMF varied among experimental series, it always depended on HCA₂.

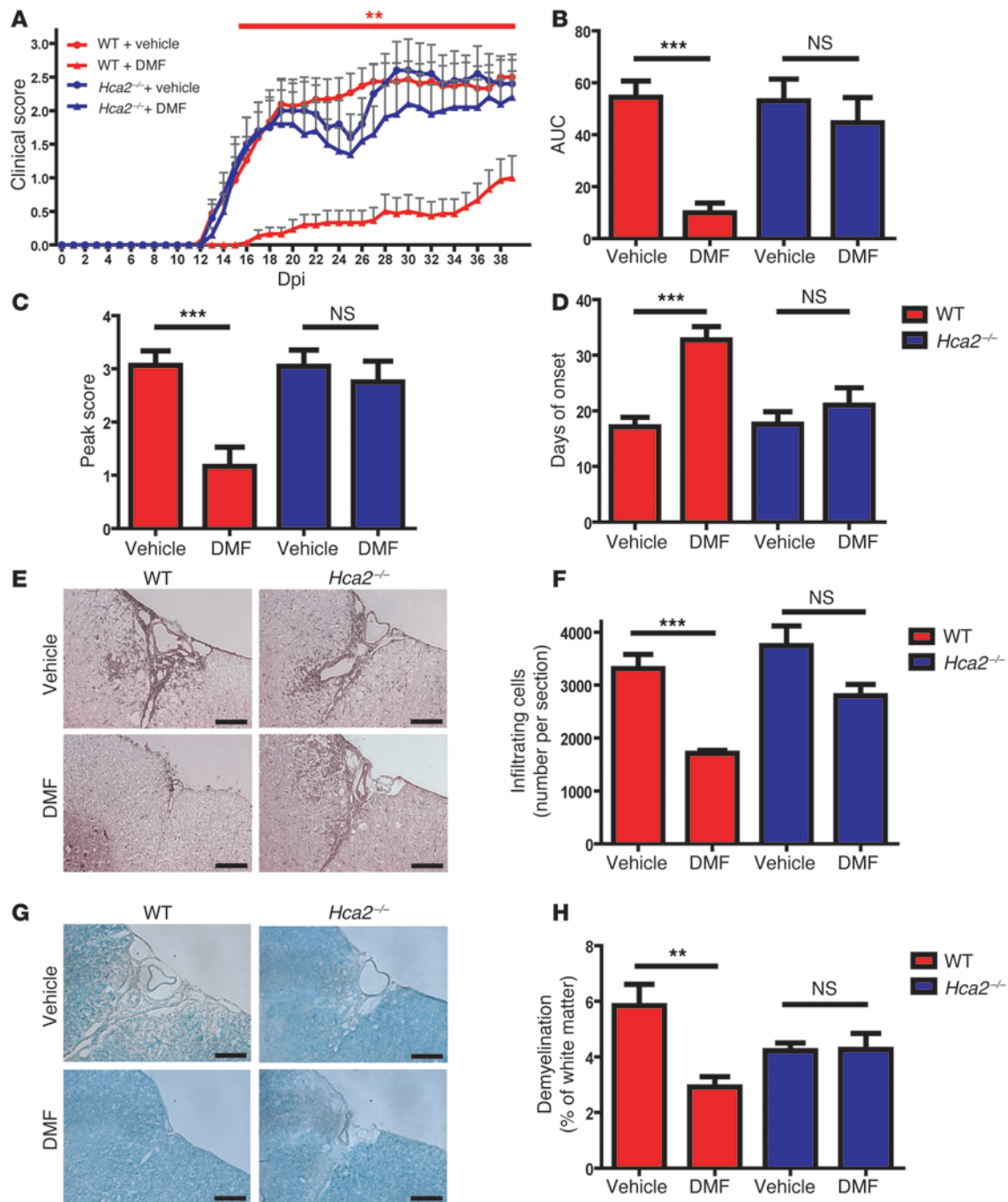
On dpi 28, we evaluated the histopathology of the spinal cord (for the clinical scores of these mice, see Supplemental Figure 1A). Treating wild-type mice with DMF lowered the number of inflammatory cells (Figure 1, E and F). In contrast, there was no significant difference in the number of inflammatory cells between vehicle- and DMF-treated *Hca2*^{-/-} mice (Figure 1, E and F). Furthermore, the area of demyelination was reduced by DMF treatment in wild-type mice but not *Hca2*^{-/-} mice (Figure 1, G and H). These data show that HCA₂ is required for the protective effect of DMF in EAE.

As HCA₂ is expressed in immune cells, such as macrophages and neutrophils (11), we characterized the action of DMF on immune cell infiltration into the spinal cord by using flow cytometry on dpi 17 (Figure 2). DMF treatment reduced the number of CD45⁺Ly-6G⁺ neutrophils in the spinal cords of wild-type mice but had no effect in *Hca2*^{-/-} mice (Figure 2D). In addition, a nonsignificant trend toward lower numbers of CD4⁺ T cells, CD11b⁺ macrophages, and CD11c⁺ dendritic cells was observed in wild-type mice but not in *Hca2*^{-/-} mice (Figure 2, A, C, and E).

Authorship note: Nina Wettschureck and Markus Schwaninger contributed equally to this work.

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**Figure 1**

DMF treatment attenuates the severity of EAE in wild-type mice but not in *Hca2*^{-/-} mice. (A) EAE clinical scores for wild-type and *Hca2*^{-/-} mice ($n = 10$ – 15 per group) that were treated orally with vehicle or DMF (30 mg/kg body weight, twice per day). The transient partial remission observed in this experiment in the *Hca2*^{-/-} groups was not reproduced in other experiments and therefore is not a general feature of *Hca2*^{-/-} mice (see Supplemental Figure 1, A and B). $^{**}P < 0.01$ for comparison between “WT + Vehicle” and “WT + DMF” by nonparametric Mann-Whitney test. (B–D) DMF reduced the area under the curve (AUC), the peak score, and the day of disease onset in wild-type mice but not in *Hca2*^{-/-} mice. $P = 0.0001$, Kruskal-Wallis for AUC; $P = 0.0003$, Kruskal-Wallis test for peak scores; $P = 0.0002$, Kruskal-Wallis test for day of onset. $^{***}P < 0.001$ (Dunn’s multiple comparison test). NS, nonsignificant. (E and F) Immune cell infiltration was assessed by H&E staining of spinal cord sections on dpi 28 ($n = 6$ – 7). $P = 0.0002$ (2-way ANOVA for drug treatment); $^{***}P < 0.001$ (Bonferroni post-hoc test). Scale bar: 100 μm . (G and H) White matter demyelination was determined by Luxol Fast Blue staining of spinal cord sections on dpi 28 ($n = 6$ – 7). $P < 0.0125$ (2-way ANOVA for drug treatment); $^{**}P < 0.01$ (Bonferroni post-hoc test). Scale bar: 100 μm . (E–H) Clinical scores of mice are given in Supplemental Figure 1A. Data represent mean \pm SEM.

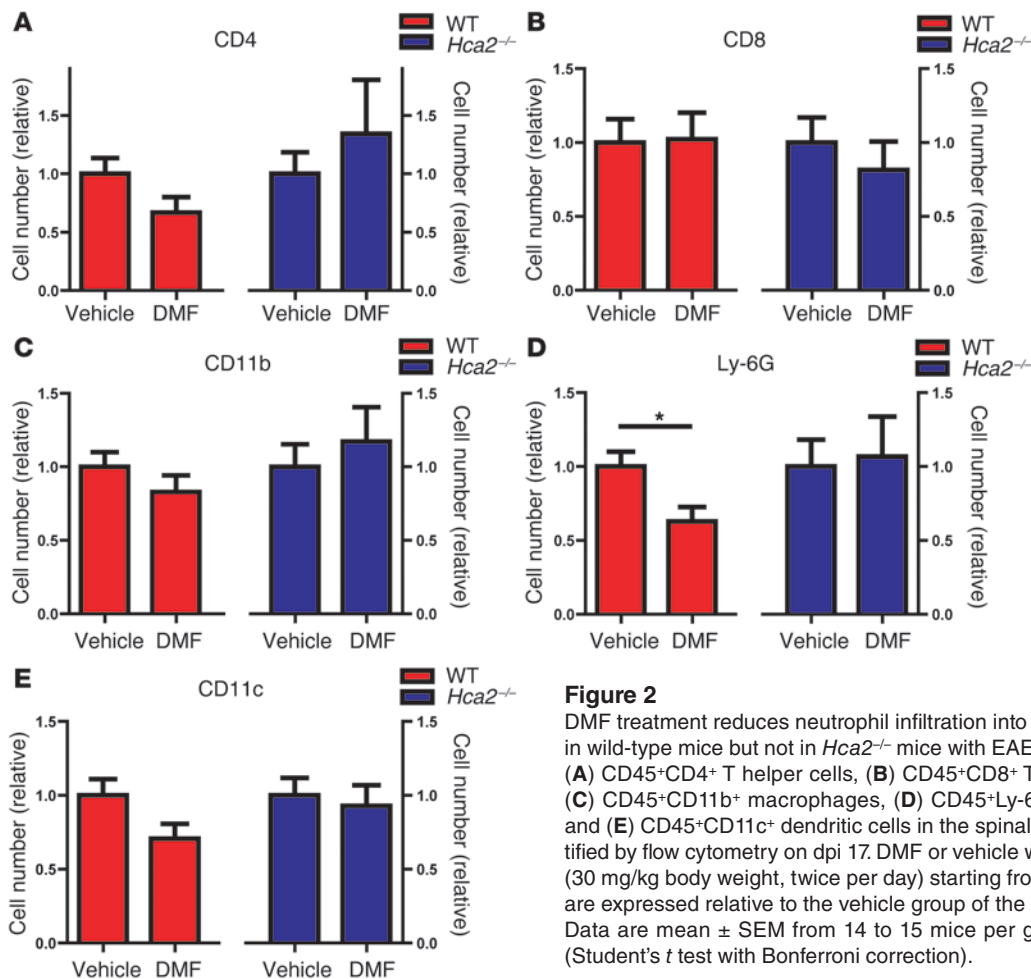
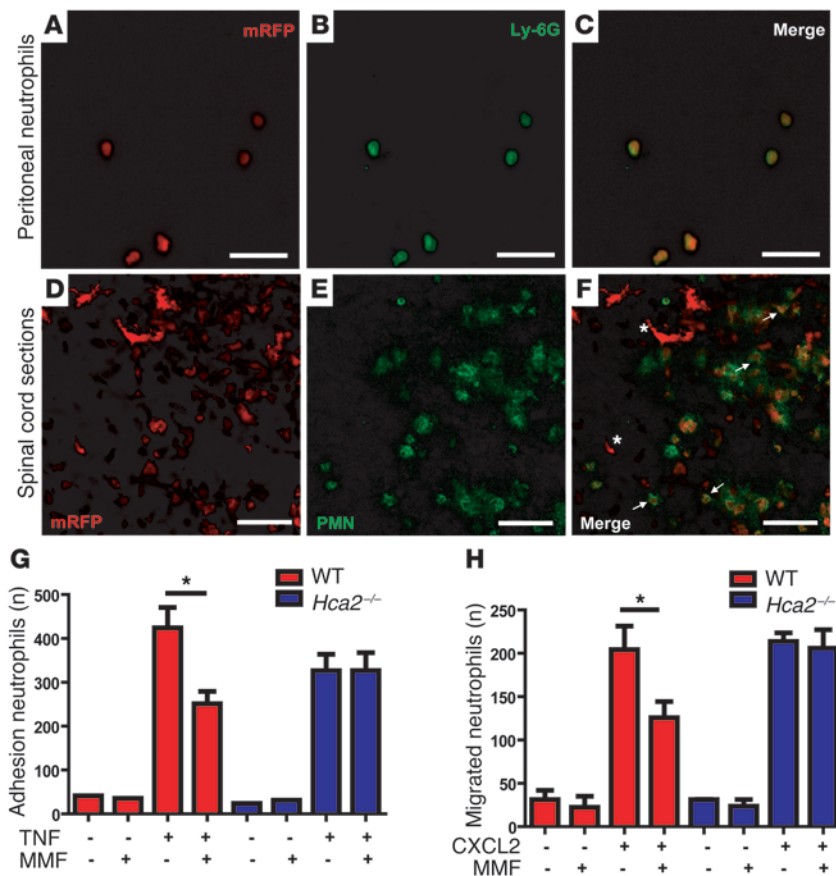


Figure 2
DMF treatment reduces neutrophil infiltration into the spinal cords in wild-type mice but not in *Hca2*^{-/-} mice with EAE. The number of (A) CD45⁺CD4⁺ T helper cells, (B) CD45⁺CD8⁺ T cytotoxic cells, (C) CD45⁺CD11b⁺ macrophages, (D) CD45⁺Ly-6G⁺ neutrophils, and (E) CD45⁺CD11c⁺ dendritic cells in the spinal cord was quantified by flow cytometry on dpi 17. DMF or vehicle were given orally (30 mg/kg body weight, twice per day) starting from dpi 3. Results are expressed relative to the vehicle group of the same genotype. Data are mean ± SEM from 14 to 15 mice per group. **P* < 0.05 (Student's *t* test with Bonferroni correction).

In EAE, neutrophils infiltrate the CNS and have been shown recently to play an important role in the disease, suggesting that reduced neutrophil infiltration may underlie the protective effect of DMF (12–15). To confirm that the *Hca2* locus is expressed in mouse neutrophils, we made use of the reporter mouse line *Hca2*^{mRFP}, in which expression of the monomeric red fluorescent protein (mRFP) is driven by the *Hca2* locus (16). Peritoneal neutrophils from this mouse line were mRFP-positive, indicating that they express HCA₂ (Figure 3, A–C). In addition, neutrophils that infiltrated spinal cords in EAE were mRFP-positive. mRFP-positive cells that were not stained by anti-polymorphonuclear leukocyte (anti-PMN) most likely represent macrophages or microglia (Figure 3, D–F). HCA₂ is coupled to G_i-type G proteins (10). As other G_i-coupled receptors are able to interfere with neutrophil adhesion to endothelial cells and with neutrophil migration (17), we hypothesized that DMF treatment may reduce adhesion and chemotaxis of neutrophils by activating HCA₂. Because DMF is immediately hydrolyzed to MMF in the body (18), we used MMF in vitro. When purified neutrophils were added to cultured brain endothelial bEnd.3 cells, pretreatment of endothelial cells with TNF increased the number of adherent neutrophils (Figure 3G). Interestingly, MMF reduced the number of wild-type neutrophils adhering to TNF-stimulated endothelial cells in a concentration-dependent manner (Supplemental Figure 2) but had no effect

on *Hca2*^{-/-} neutrophil adhesion (Figure 3G). These experiments demonstrated that MMF is able to inhibit neutrophil adhesion to brain endothelial cells by activating HCA₂. As the next step in neutrophil recruitment, we investigated chemotaxis. In a transmigration assay, MMF partially blocked the migration of wild-type neutrophils toward the chemokine CXCL2. However, in neutrophils from *Hca2*^{-/-} mice, MMF had no effect on the chemotactic activity of CXCL2. A recent report showed that, in human neutrophils, nicotinic acid induces cell death, and this effect was linked to the HCA₂ receptor (19). MMF also slightly induced cell death in wild-type neutrophils in vitro but had no effect in *Hca2*^{-/-} neutrophils (Supplemental Figure 3A). DMF treatment did not affect neutrophil counts in the blood of mice (Supplemental Figure 3B), suggesting that, if DMF treatment triggered apoptosis in circulating neutrophils, the effect was balanced by the mobilization or distribution of neutrophils. Thus, it is unclear whether the small HCA₂-dependent induction of cell death by MMF contributes to the beneficial effect in EAE.

DMF has been shown to activate the antioxidant transcription factor NRF2 and to induce its target genes, *NQO1* and *HMOX1* (6, 7). To investigate whether HCA₂ is required for this effect, we treated peritoneal macrophages and primary microglia that express HCA₂ (data not shown) with MMF in vitro. In accordance with previous reports, MMF induced *Nqo1* and *Hmox1* in wild-

**Figure 3**

MMF inhibits neutrophil adhesion and migration via HCA₂. (A–F) Neutrophils expressed HCA₂. Purified (A–C) peritoneal neutrophils and (D–F) spinal cord sections from *Hca2^{mRFP}* reporter mice subjected to EAE were stained with (B) anti-Ly-6G and (E) anti-PMN (Ly-6G/Ly-6C) antibodies specific for neutrophils (green). (A and D) mRFP signal (red) represents HCA₂ expression. Arrows indicate neutrophils stained by anti-PMN and expressing HCA₂. Asterisks indicate mRFP⁺ and anti-PMN⁻ cells most likely representing macrophages or microglia. Scale bar: 40 μm. (G) MMF (100 μM) reduced the adhesion of wild-type neutrophils but not of *Hca2^{-/-}* neutrophils to TNF-stimulated bEnd.3 cells. Values are mean ± SEM (*n* = 4) in 1 of 2 experiments with similar results. *P* < 0.05 (2-way ANOVA for drug treatment); **P* < 0.05 (Bonferroni post-hoc test). (H) MMF inhibited the CXCL2-induced migration in wild-type neutrophils but not in *Hca2^{-/-}* neutrophils. Values are mean ± SEM (*n* = 3–4) in 1 of 3 experiments with similar results. *P* = 0.05 (2-way ANOVA for drug treatment); **P* < 0.05 (Bonferroni post-hoc test).

type cells (Supplemental Figure 4). In *Hca2^{-/-}* macrophages, MMF did not stimulate *Nqo1* expression (Supplemental Figure 4B), but otherwise induction was not impaired in *Hca2^{-/-}* cells (Supplemental Figure 4, A, C, and D), demonstrating that MMF exerts HCA₂-independent effects in addition to the HCA₂-dependent reduction in neutrophil adhesion and chemotaxis.

In summary, we obtained evidence for the first time that the protective effect of DMF in EAE is mediated by HCA₂. This finding does not exclude additional actions of DMF in EAE and MS. DMF or its metabolite MMF may directly form conjugates with the NRF2 inhibitor KEAP-1 or glutathione to induce *NQO1* and *HMOX1* gene expression (6, 7). Notably, we found that the induction of *Nqo1* and *Hmox1* was largely HCA₂-independent, whereas the improvement of the clinical outcome and the neutrophil recruitment did depend on HCA₂. The conclusion from our study that HCA₂ mediates the protective effect of DMF in EAE and MS is supported by previous data. First, DMF and another chemically unrelated agonist of HCA₂, nicotinic acid, share various clinical effects, including the well-known cutaneous flushing response. In mice, there is direct evidence that HCA₂ mediates the DMF-induced cutaneous flushing (16), suggesting that HCA₂ is activated by DMF treatment of patients with MS. Second, it has been reported that nicotinic acid ameliorates EAE pathology (20). This effect of nicotinic acid, a prototypical agonist of HCA₂, is well explained by the pivotal role of HCA₂.

Concerning the potential cellular mode of action, it is interesting to note that HCA₂ expression is high in human neutrophils (11, 19). Although the role of neutrophils in MS is not fully understood, it

is clear that neutrophils show a primed phenotype in patients with MS (21). Moreover, neutrophils are involved in disease progression of EAE (12–15). G_i-coupled receptors are known to modulate chemotaxis of neutrophils (22). More specifically, HCA₂ activates the protein kinase ERK (23) that is able to transmit a “stop” signal for neutrophil migration (22). By interfering with neutrophil recruitment, DMF may exert secondary effects on other immune cells in EAE and MS (24). Importantly, the identification of HCA₂ as a drug target in EAE and MS opens up a rich therapeutic armamentarium. Several structurally diverse agonists of HCA₂ are already available, which may help to further optimize therapy (25, 26).

Methods

Animals. Female mice aged 8 to 12 weeks were used for in vivo experiments. As *Hca2^{-/-}* mice (10) were backcrossed with C57BL/6 mice for at least 8 generations, we used C57BL/6 mice as wild-type controls, if not indicated otherwise. *Hca2^{mRFP}* (*Gpr109a^{mRFP}*) mice have been described previously (16).

Induction of EAE. Active EAE was induced by subcutaneous immunization with MOG_{35–55} peptide (100 μg) emulsified in incomplete Freund’s adjuvant (BD Difco) containing *Mycobacterium tuberculosis* (400 μg, BD Difco). In addition, mice were treated with pertussis toxin (PTX; 200 ng, List Biological Laboratories) administered intraperitoneally on the day of immunization (day 0) and on dpi 2. In the experiments depicted in Figure 1, A–D, and Supplemental Figure 1B, we used the EAE Induction Hooke Kit (MOG_{35–55}/CFA Emulsion PTX, Hooke Labs) according to the manufacturer’s instructions. Animals were examined daily and scored as follows: 0, no disease; 0.5, tail weakness; 1, limp tail; 2, hind limb weakness; 2.5, partial hind limb paralysis; 3, hind limb paralysis; 3.5, hind limb paralyzed and



weakness in forelimbs; 4, forelimb paralyzed; and 5, moribund. Paralyzed animals were given easier access to food and water. Mice were treated with vehicle or DMF (30 mg/kg body weight, twice per day, Sigma-Aldrich) that was suspended in 0.8% Methocel (Sigma-Aldrich Fluka). Starting on dpi 3, vehicle (0.8% Methocel) or DMF was given by gavage until the end of the experiments. The therapeutic time window of DMF probably started only between dpi 5 and 7, because PTX treatment of mice on day 0 and dpi 2 reduced the inhibitory effect of MMF on neutrophil adhesion *in vitro* until dpi 5 (Supplemental Figure 5). This effect is explained by the fact that PTX irreversibly ADP-ribosylates G_i proteins and consequently interferes with the actions of the G_i-coupled HCA₂ (10). In all experiments, investigators were blinded to the genotype of mice or to both genotype and treatment.

Supplemental Methods. Additional procedures and details are described in the Supplemental Methods.

Statistics. EAE scores were evaluated by using the nonparametric Mann-Whitney or Kruskal-Wallis tests. Histology, neutrophil migration, adhesion assays, gene expression, and neutrophil viability were analyzed by 2-way ANOVA with Bonferroni post-hoc test, with the only exception of the concentration-response relationship (Supplemental Figure 2), which was analyzed with 1-way ANOVA followed by Bonferroni post-hoc test. As indicated in the figure legends, Student's *t* test with Bonferroni correction was used in flow cytometry experiments. *P* values of less than 0.05 were considered significant.

Study approval. All experiments were performed according to animal protection law in Germany and approved by the local animal welfare authorities (Regierungspräsidium Karlsruhe; Ministerium für Energiewende, Landwirtschaft, Umwelt und ländliche Räume, Kiel, Germany).

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