Supplemental Methods

Histology

On dpi 28, mice were anesthetized and perfused intracardially with Ringer's solution and 4% paraformaldehyde (PFA). The lumbar part of spinal cords were dissected and fixed in 4% PFA overnight at 4ºC, followed by incubation in 30% sucrose for 24 h. Cryosections (14-µm thick) were stained with hematoxylin & eosin (H&E) or Luxol fast blue (LFB). Quantification was performed as previously reported (1).

Immunohistochemistry

PFA fixed spinal cord cryosections or isolated neutrophils were incubated with anti-PMN (specific for Ly-6G and Ly-6C, Abcam) or Ly-6G antibody (BD Pharmingen) overnight at 4ºC, followed by Alexa-488 conjugated secondary antibody (Invitrogen) incubation at room temperature for 45 min. Sections were then mounted with Mowiol and analyzed with a confocal microscope (TCS SP5, Leica).

Flow cytometry analysis and counting of neutrophils

Mice were anesthetized and perfused with Hank's Balanced Salt Solution (HBSS) on dpi 17. After spinal cords were homogenized in HBSS, cells were isolated by Percoll gradient centrifugation and incubated with the following antibodies for 20 to 30 min in the dark: PE-labeled rat anti-CD45 (eBioscience) and APC-labeled rat anti-CD11b, FITC-labeled rat anti-Ly-6G, PerCP-labeled rat anti-CD4, FITC-labeled rat anti-CD8 (BD Pharmingen), APC labeled hamster anti-CD11c (AbD Serotec), or isotype controls, respectively. Cells were fixed with 4% PFA and measured on a FACSCanto II flow cytometer. Data was analyzed using FACSDiva software. We counted the number of neutrophils in blood taken from the tail of mice with the Hemavet 950 analyzer.

Cell culture

For the preparation of peritoneal neutrophils we used a previously reported method with minor changes (2). Briefly, casein (9%, 1 ml) was injected intraperitoneally two times with an interval of 12 h. Mouse neutrophils were separated from peritoneal exudate cells using continuous Percoll density gradient centrifugation. Three h after the second injection, peritoneal cells were collected by lavage with PBS containing 0.02% EDTA and washed once with PBS. The cell pellet was resuspended in PBS (3.4 ml per mouse) and mixed with 5.1 ml Percoll gradient solution (90% Percoll in PBS, Sigma). Neutrophils were obtained from the second opaque layer after ultracentrifuge at 60,650 g for 20 min at 4ºC. The purity of neutrophil preparation was higher than 95%, as confirmed by Ly-6G immunostaining and FACS analysis.

Primary microglia culture was performed as previously described (3). Peritoneal macrophages were elicited by intraperitoneal injection of 1 ml of 3% Brewer's thioglycollate medium (BBL)(4). After 4 days, peritoneal cells were harvested by lavage from peritoneal cavity with 10 ml of cold DMEM/F12 medium. CD11b staining indicated that the purity of macrophages was greater than 90%.

Neutrophils adhesion and transmigration assays

Neutrophil adhesion and transmigration assays were performed as previously described (5). For adhesion assays, mouse brain endothelial cells (bEnd.3) were cultured to confluence on coverslips in 24-well plates and pre-treated with mouse TNF (10 ng/ml; Peprotech) for 6 h. Freshly isolated neutrophils were incubated with calcein AM (5 µg/ml; eBioscience) in the presence of MMF (Sigma) for 30 min. Then calcein AM-labeled neutrophils ($5x10^5$ cells/ well) were allowed to interact with activated bEnd.3 cells for another 30 min. After removing the nonadherent cells, the coverslips were mounted with Mowiol and analyzed with a fluorescence microscope (DMI 6000B, Leica). Adherent neutrophils were counted in 3 random fields using a 10x objective in each coverslip. After investigating the concentration-response relationship of MMF in adhesion assays (Supplemental Figure 2), we chose a MMF concentration of 100 µM for further in vitro experiments.

For transmigration assays, neutrophils were preincubated with 100 µM MMF for 30 min. To induce chemotaxis, RPMI containing 100 ng/ml recombinant mouse CXCL2 (Peprotech), 100 µM MMF, and 0.01% BSA was added to 24-well plates. Neutrophils (10^6 cells in 0.2 ml RPMI containing 0.01% BSA) were added into the upper chambers of inserts (ThinCerts, pore size 3 µm, Greiner). After 1 h incubation, neutrophils in the upper side of ThinCert were removed and the membranes were stained with Diff-Quick solution (Siemens). Migrated neutrophils were counted in 3 random fields using a 40x objective. The results are shown as the mean number of neutrophils per field.

Induction of mRNA expression

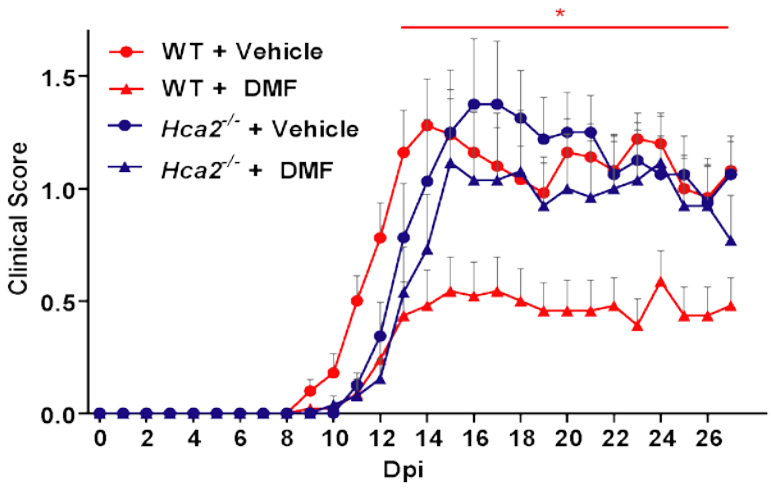
Microglia and macrophages were treated with 100 µM MMF or the solvent DMSO for 30 min, followed by LPS stimulation (20 ng/ml) for 24 h. Total RNA was extracted from primary microglia and macrophage cells using ABI Prism 6100 Nucleic Acid PrepStation (Applied Biosystems) according to the manufacturer's protocol. RNA was transcribed with Moloney murine leukemia virus reverse transcriptase and random hexamer primers. For real-time PCR the following primers were used: *Ppia* forward, 5'-AGG TCC TGG CAT CTT GTC CAT-3'; *Ppia* reverse, 5'- GAA CCG TTT GTG TTT GGT CCA-3' (PCR product, 51 bp); *Hmox1* forward, 5'-CAC AGA TGG CGT CAC TTC GTC-3'; Hmox1 reverse: 5'-GTG AGG ACC CAC TGG AGG AG-3' (PCR product, 130 bp); *Nqo1* forward: 5'-AGC CCA GAT ATT GTG GCC G-3'; *Nqo1* reverse: 5'-CCT TTC AGA ATG GCT GGC AC-3' (PCR product, 101 bp). Amplification and data collection was carried out by ABI Prism 7000 Sequence Detection System (Applied Biosystems) using Platinum SYBR Green qPCR Supermix (Invitrogen). The purity of the amplified products was confirmed by the dissociation curve. Relative expressions of target genes were normalized to *Ppia* using the ΔΔCt method.

Cell death assay

Isolated neutrophils were incubated with MMF (100 µM), the solvent DMSO, GM-CSF (50 U/ml, Peprotech), or TNF (10 ng/ml) for 24 h. Neutrophil viability was determined

by flow cytometry using the Annexin V-APC apoptosis detection kit (eBioscience). Cells that were either annexin V- or propidium iodide-positive were counted.

A



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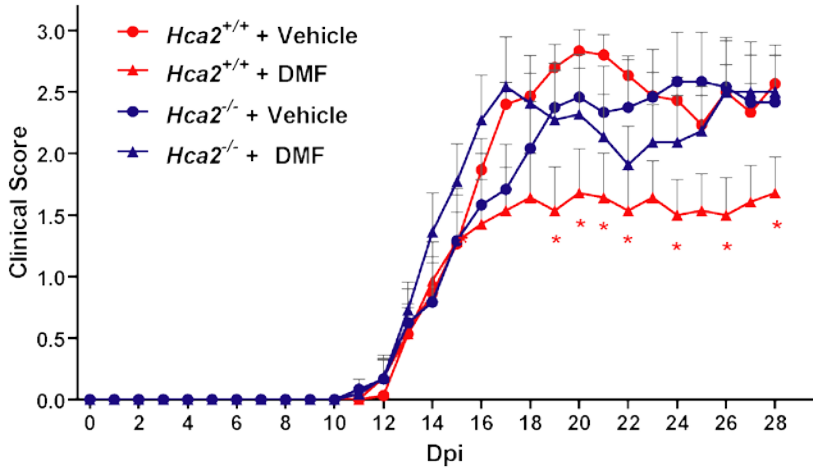
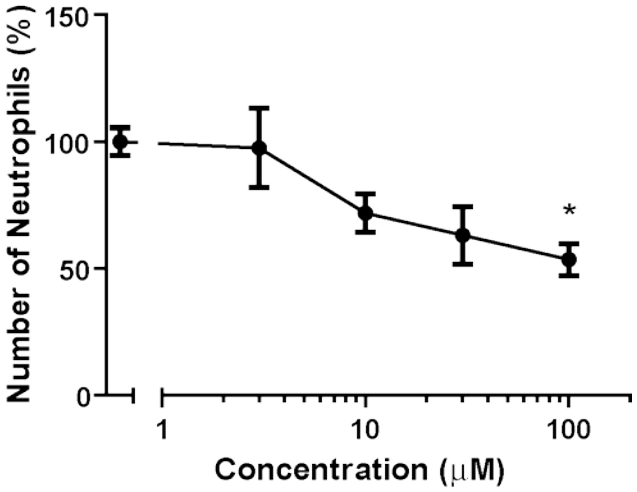


Supplemental Figure 1

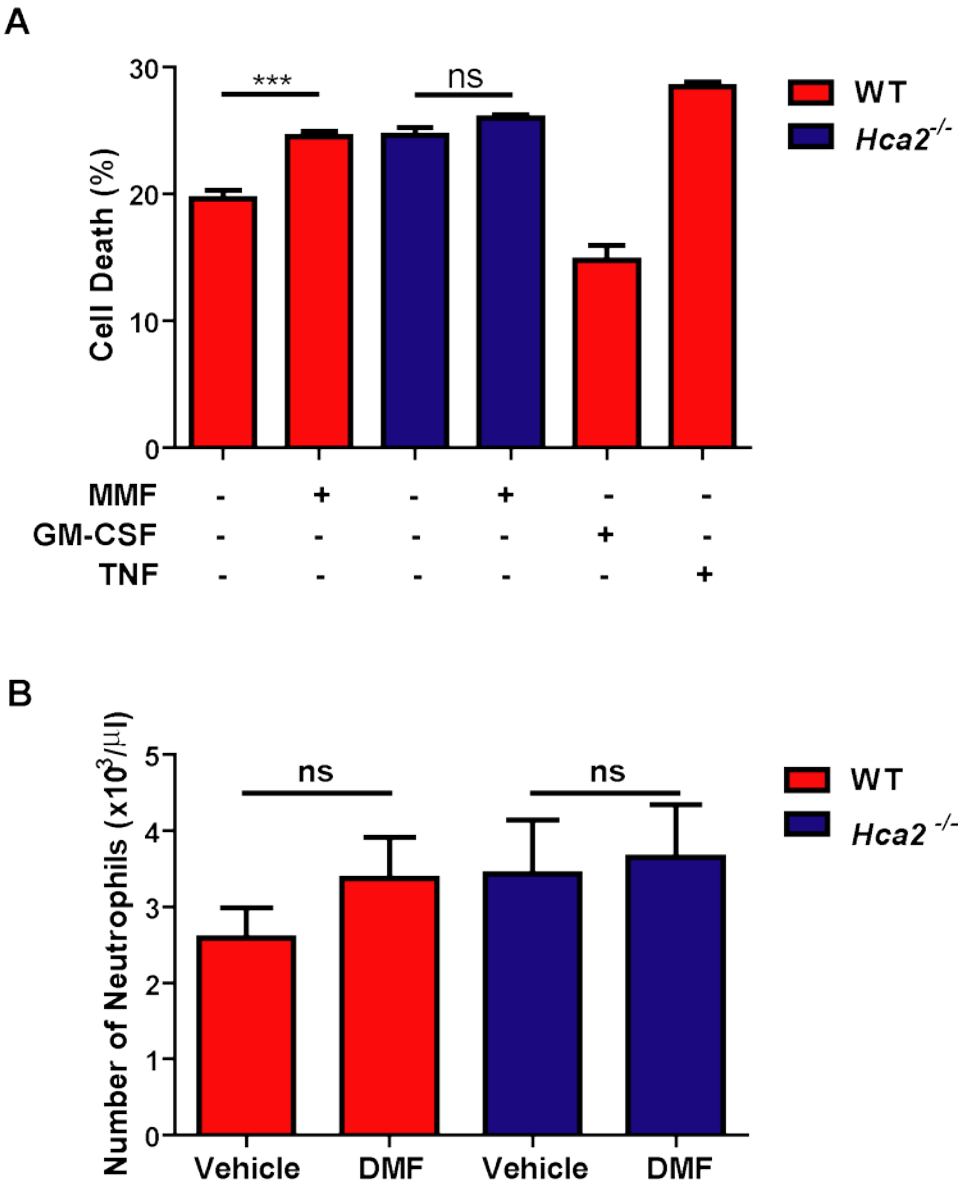
(**A**) Clinical scores after EAE induction for wild-type (WT) and *Hca2*$^{-/-}$ mice (n = 13-25 per group), that were treated orally with vehicle or DMF (30 mg/kg body weight, twice per day). Data represent means ± SEM. **P* < 0.05 for comparison between 'WT vehicle' and 'WT DMF' group by non-parametric Mann–Whitney test.

(**B**) Clinical scores after EAE induction for *Hca2*$^{+/+}$ and *Hca2*$^{-/-}$ littermates (n = 11-15 per group), that were treated orally with vehicle or DMF (30 mg/kg body weight, twice per day). Data represent means ± SEM. **P* < 0.05 for comparison between '*Hca2*$^{+/+}$ + vehicle' and '*Hca2*$^{+/+}$ + DMF' group by non-parametric Mann–Whitney test.



Supplemental Figure 2

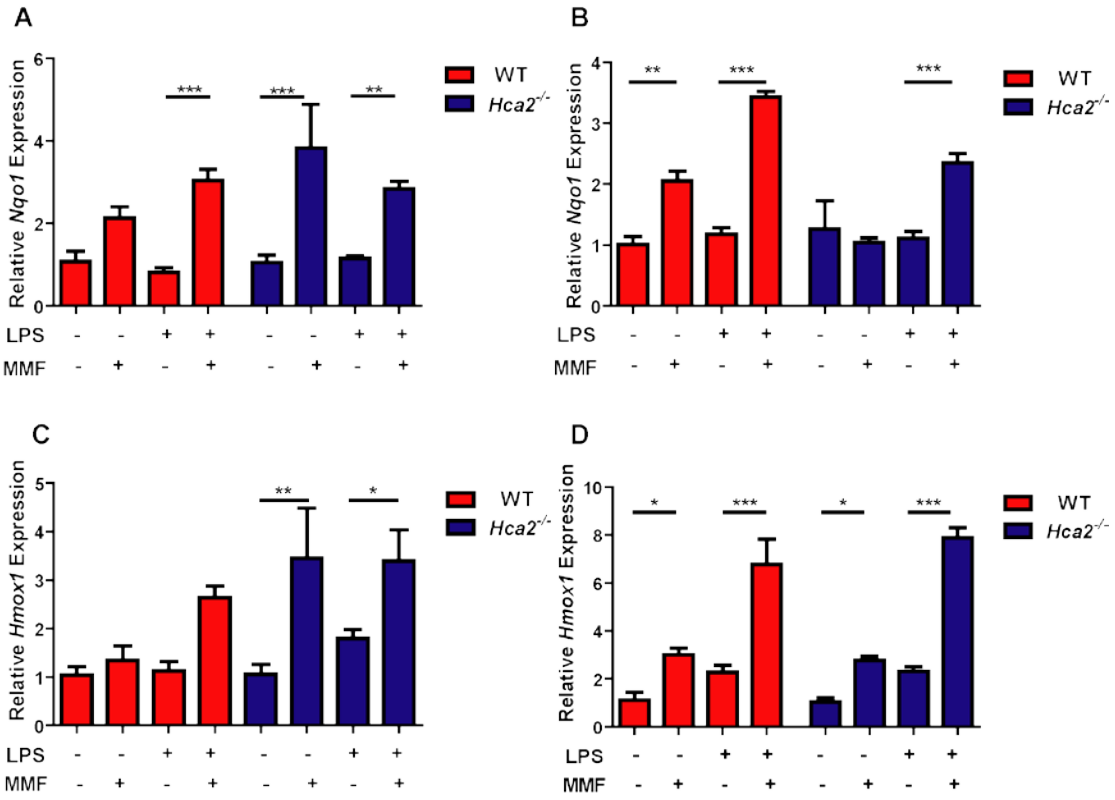
Effect of various concentrations of MMF on neutrophil adhesion to bEnd.3 endothelial cells that were pretreated with TNF. Values are mean ± SEM. P = 0.0078 (one-way ANOVA), *$P < 0.05$ in comparison to the vehicle-treated group (Bonferroni posthoc test).



Supplemental Figure 3

(**A**) MMF (100 µM) slightly increased cell death of wild-type (WT) but not *Hca2*$^{-/-}$ neutrophils. Peritoneal neutrophils were stimulated with the indicated compounds for 24 h before cell death was determined. GM-CSF (50 U/ml) and TNF (10 ng/ml) served as controls that reduce or increase cell death, respectively. Values are means ± SEM (n = 10 per group). $P < 0.0001$ (two-way ANOVA for treatment), ***$P < 0.001$ (Bonferroni posthoc test). ns, nonsignificant.

(**B**) DMF treatment (30 mg/kg twice a day) over 21 days did not affect the neutrophil count in peripheral blood of wild-type (WT) and *Hca2*$^{-/-}$ mice. Values are means ± SEM (n = 10-15 mice per group). ns, nonsignificant.



Supplemental Figure 4

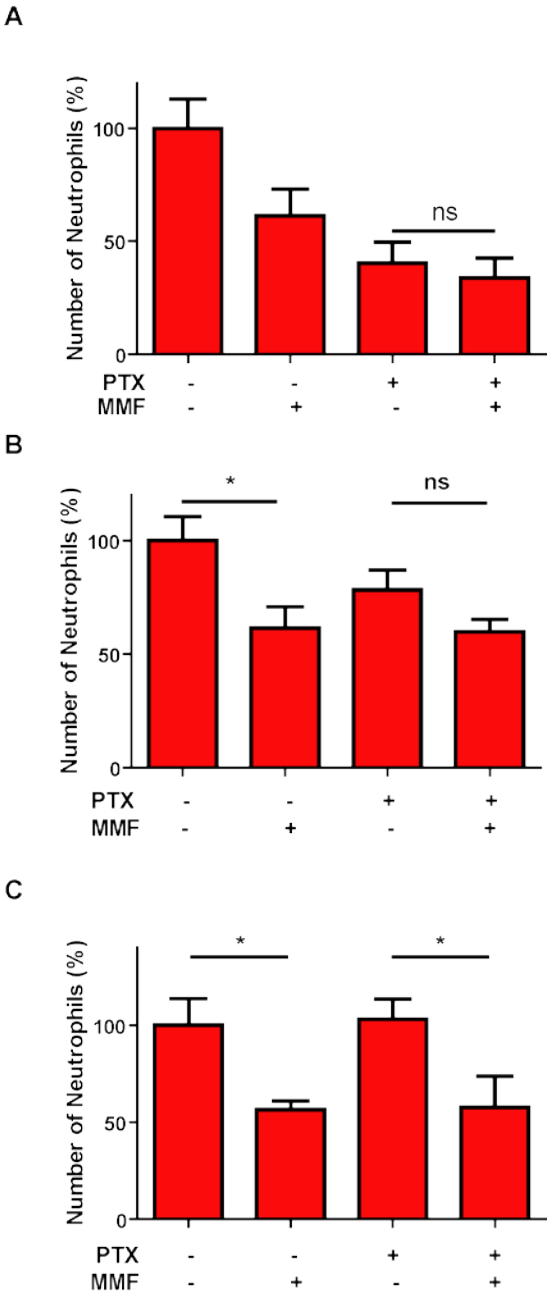
Effect of MMF (100 µM) on *Nqo1* and *Hmox1* expression is not mediated by HCA_2. *Nqo1* and *Hmox1* expression at the mRNA level were determined in microglia (**A** and **C**) and macrophages (**B** and **D**) by real-time RT-PCR, respectively.

(**A**) *Nqo1* in microglia. Values are means ± SEM (n = 4) in one out of two experiments with similar results. $P < 0.0001$ (two-way ANOVA for treatment), $^{**}P < 0.01$, $^{***}P < 0.001$ (Bonferroni posthoc test).

(**B**) *Nqo1* in peritoneal macrophages. Values are means ± SEM (n = 4) in one out of three experiments with similar results. $P < 0.0001$ (two-way ANOVA for treatment), $^{**}P < 0.01$, $^{***}P < 0.001$ (Bonferroni posthoc test).

(**C**) *Hmox1* in microglia. Values are means ± SEM (n = 4). $P < 0.01$ (two-way ANOVA for treatment), *P<0.05, **P<0.01 (Bonferroni posthoc test).

(**D**) *Hmox1* in macrophages. Values are means ± SEM (n = 4) in one out of two experiments with similar results. $P < 0.0001$ (two-way ANOVA for treatment), $^{*}P < 0.05$, $^{***}P < 0.001$ (Bonferroni posthoc test).



Supplemental Figure 5

Pertussis toxin (PTX) treatment transiently interferes with the effect by MMF on neutrophil adhesion. After injecting mice with PTX (200 ng) at day 0 and day postimmunization (dpi) 2 we prepared primary neutrophils on dpi 3 (**A**), dpi 5 (**B**), and dpi 7 (**C**), and tested the effect of MMF (100 µM) on neutrophil adhesion. Values are means ± SEM (n = 5-6), expressed as percent of the vehicle-treated control without PTX treatment in vivo. $P < 0.01$ (two-way ANOVA for treatment), $^*P < 0.05$ (Bonferroni potshoc test).

1. Berard, J.L., Wolak, K., Fournier, S., and David, S. 2010. Characterization of relapsing-remitting and chronic forms of experimental autoimmune encephalomyelitis in C57BL/6 mice. *Glia* 58:434-445.
2. Luo, Y., and Dorf, M.E. 2001. Isolation of mouse neutrophils. *Curr Protoc Immunol* Chapter 3:Unit 3 20.
3. Elzer, J.G., Muhammad, S., Wintermantel, T.M., Regnier-Vigouroux, A., Ludwig, J., Schutz, G., and Schwaninger, M. 2010. Neuronal estrogen receptor-alpha mediates neuroprotection by 17beta-estradiol. *J Cereb Blood Flow Metab* 30:935-942.
4. Zhang, X., Goncalves, R., and Mosser, D.M. 2008. The isolation and characterization of murine macrophages. *Curr Protoc Immunol* Chapter 14:Unit 14 11.
5. Murikinati, S., Jüttler, E., Keinert, T., Ridder, D.A., Muhammad, S., Waibler, Z., Ledent, C., Zimmer, A., Kalinke, U., and Schwaninger, M. 2010. Activation of cannabinoid 2 receptors protects against cerebral ischemia by inhibiting neutrophil recruitment. *FASEB J.* 24:788-798.