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RESEARCH ARTICLE

Effects of fumarates on circulating and CNS myeloid cells in multiple sclerosis

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Abstract

Objective: Dimethyl fumarate (DMF), a therapy for relapsing-remitting multiple sclerosis (RRMS), is implicated as acting on inflammatory and antioxidant responses within both systemic immune and/or central nervous system (CNS) compartments. Orally administered DMF is rapidly metabolized to monomethyl fumarate (MMF). Our aim was to analyze the impact of fumarates on antiinflammatory and antioxidant profiles of human myeloid cells found in the systemic compartment (monocytes) and in the inflamed CNS (blood-derived macrophages and brain-derived microglia). **Methods**: We analyzed cytokine and antioxidant expression in monocytes from untreated or DMF-treated RRMS patients and controls, and in monocyte-derived macrophages (MDMs) and microglia isolated from adult and fetal human brain tissue. **Results**: Monocytes from multiple sclerosis (MS) patients receiving DMF had reduced expression of the proinflammatory micro-RNA miR-155 and of antioxidant genes HMOX1 and OSGIN1 compared to untreated MS patients; similar changes were observed in patients receiving FTY720 and/or natalizumab. *In vitro* addition of DMF but not MMF to MDMs and microglia inhibited lipopolysaccharide-induced production of inflammatory cytokines and increased expression of the antioxidant gene HMOX1 in the absence of significant cytotoxicity. **Interpretation**: Our *in vivo*-based observations that effects of DMF therapy on systemic myeloid cell gene expression are also observed with FTY720 and natalizumab therapy suggests that the effect may be indirect, reflecting reduced overall disease activity. Our *in vitro* results demonstrate significant effects of DMF but not MMF on inflammation and antioxidant responses by MDMs and microglia, questioning the mechanisms whereby DMF therapy would modulate myeloid cell properties within the CNS.

Introduction

Multiple sclerosis (MS) often manifests clinically as a relapsing disorder that evolves into a progressive course. The activation and entry of peripheral immune cells into the central nervous system (CNS) is thought to initiate lesion formation. However, a compartmentalized immune response within the CNS is considered to sustain the initial inflammatory response and contribute to subsequent disease evolution into a progressive phase. Constituents of the innate immune system in both the peripheral and CNS compartments are implicated as contributors to all phases of the disease. Peripheral blood-derived monocytes have a proinflammatory bias in both relapsing and progressive-course patients.[1,2] Within the CNS, myeloid cells are comprised of both brain-resident microglia and infiltrating macrophages, and show an activated, proinflammatory phenotype.

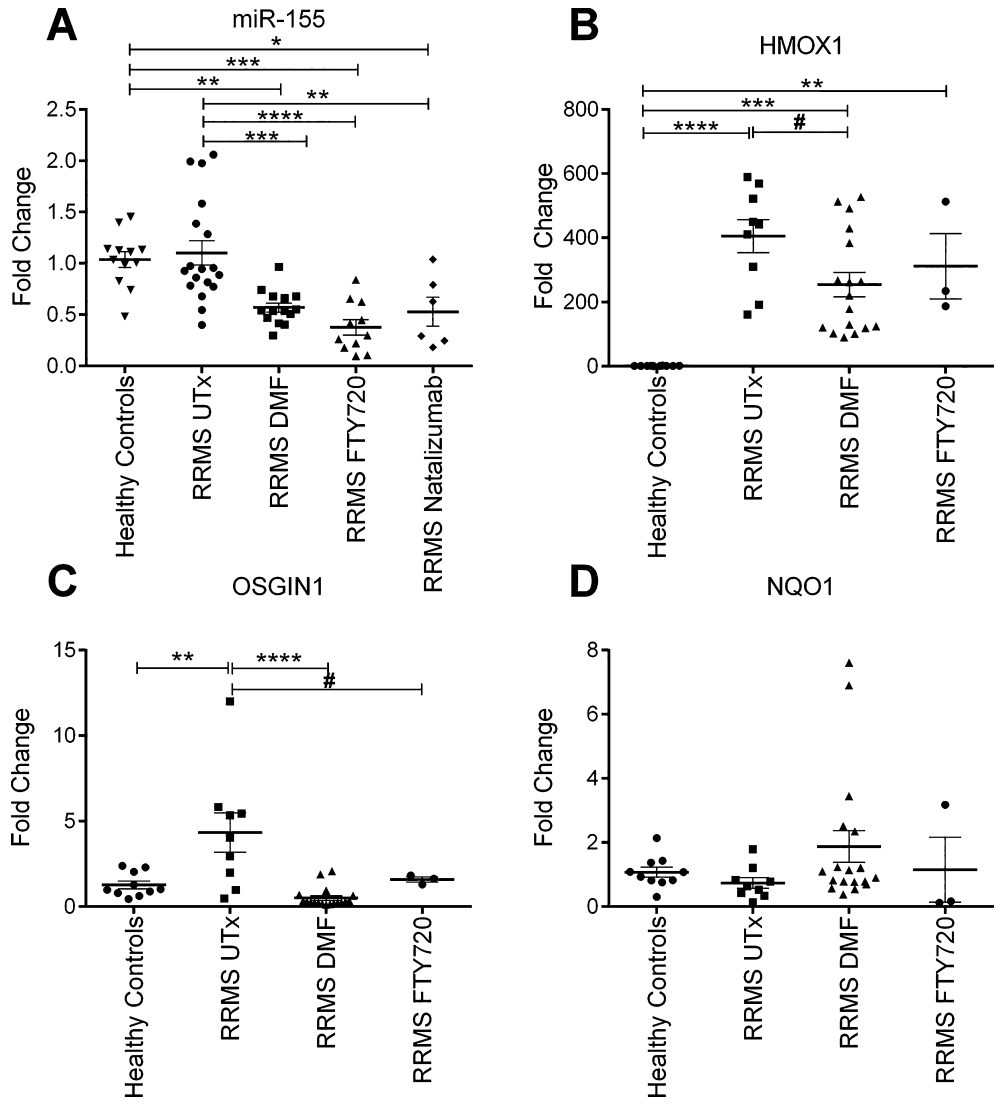

Figure 1. Antiinflammatory and antioxidative effects of DMF treatment in monocytes isolated from RRMS patients. (A) DMF-treated, FTY720-treated, and natalizumab-treated patients were observed to have reduced miR-155 levels compared to untreated RRMS patients and healthy controls. Data are expressed as fold change relative to healthy controls. (B–D) HMOX1 and OSGIN1 but not NQO1 expression was significantly higher in untreated MS patients relative to healthy controls. HMOX1 and OSGIN1 expression levels were reduced in DMF and FTY compared to untreated MS patients. Levels of HMOX1 remained increased in comparison to healthy controls, whereas OSGIN1 stabilized to healthy control levels. Similar levels of miR-155, NQO1, HMOX1, and OSGIN1 were observed in monocytes from patients treated with DMF and FTY720. For all panels, each data point indicates a single individual. One-way ANOVA compares means of all groups against each other with Dunnett's multiple comparisons test, $\alpha = 0.05$; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. #Comparison using ANOVA against Untreated MS patients with Holm–Sidak multiple comparisons test ($\alpha = 0.05$, $\#P < 0.05$). DMF, dimethyl fumarate; RRMS, relapsing-remitting multiple sclerosis; ANOVA, analysis of variance; MS, multiple sclerosis.

The mechanisms underlying the clinical efficacy of dimethyl fumarate (DMF) therapy have been linked to induction of antioxidant genes and inhibition of NFκB linked proinflammatory responses.[3–5] DMF therapy decreases the severity of experimental autoimmune encephalomyelitis (EAE); this effect is reduced in animals lacking Nuclear Factor (Erythroid-Derived 2)-Like 2 (*NFE2L2*, Nrf2),[5,6] a regulator of antioxidant response factors including HMOX-1, NQO1, and OSGIN 1.[3,7] Linker et al. and Scannevin et al. found that DMF and monomethyl fumarate (MMF) increased Nrf2 activity and reduced susceptibility to oxidative stress in astrocytes *in vitro*; the latter effect was linked to up-regulation of HMOX-1 and NQO1.[5,6] Hydroxycarboxylic acid receptor 2 (*GP109A*, HCAR2) is recognized as a high-affinity G-protein coupled receptor for MMF.[8–10]

In vitro studies have also implicated MMF and DMF as mediating the inflammation-related responses of DMF therapy. MMF is reported to induce tumor necrosis factor (TNF) and IL-10 in human monocytes *in vitro*.[11] However, Lehman et al. found that only DMF decreased inflammatory cytokine production in peripheral blood-derived mononuclear cells (PBMCs),[12] whereas DMF is also shown to decrease TNF and IL-6 mRNA in rat microglia *in vitro*.[13] The antioxidant and inflammation-related effects may be interrelated as the antioxidant gene HMOX1 can act as a regulator of inflammation.[14–16] However, inhibition of the inflammatory response may also occur independently of induction of Nrf2 through inhibition of NFκB activity.[4,17] Pharmacokinetic studies in healthy subjects indicate that conversion of DMF to MMF occurs prior to entering the circulation.[18,19] However, studies of psoriasis patients demonstrate that specific breakdown products of DMF can be detected in urine, indicating the potential for absorption of DMF in chronic inflammatory conditions.[20]

Our aim was to analyze the impact of DMF and MMF on the antiinflammatory and antioxidant profiles of human myeloid cells including CD14+ monocytes, monocyte-derived macrophages (MDMs), and brain-derived microglia. As a marker of myeloid cell inflammatory activity, we chose micro-RNA 155 (miR-155), an established inducer of proinflammatory cytokine secretion, previously shown to have increased expression in myeloid cells from MS patients.[21] We present data demonstrating *in vivo* effects of DMF therapy on these responses in monocytes of MS patients, but also observe similar effects with FTY720 and natalizumab therapy. *In vitro* studies demonstrate functional effects of fumarate therapy including on CNS compartmentalized myeloid responses (macrophages/microglia) mediated by DMF rather than MMF. Our combined *in vivo* and *in vitro* observations raise the issue of the exact mechanisms underlying this therapy.

Methods

Subjects and recruitment

Peripheral blood samples were collected into K_2EDTA-coated plastic tubes from a total cohort comprised of healthy subjects (n = 22, mean age 45 years, 15 female) and MS patients who were either untreated (n = 27, mean age 41 years, 18 female) or were receiving DMF (n = 32, mean age 43 years, 23 female) or FTY720 (n = 16, mean age 44 years, 10 female) or natalizumab (n = 6). All patients were on therapy for >4 months and none had clinical relapses within 3 months of study.

Quantitation of blood cell populations

Complete blood counts were obtained retrospectively for 24 DMF-treated patients from our patient information database. On average, pretreatment counts were obtained 212 days prior to the treatment start date (standard deviation of 256 days). On average, posttreatment counts were obtained 185 days following initiation of treatment (standard deviation of 108 days). Patients with significant lymphopenia were excluded from the study.

Cell culture (human monocytes, macrophages, and microglia)

PBMCs were isolated from whole blood using Ficoll-Pacque density gradient centrifugation (GE Healthcare, Baie d'Urfe QC, Canada). CD14+ cell isolation was done using CD14 immunomagnetic bead selection according to manufacturer's protocols to 95–99% purity (Miltenyi Biotech, San Diego CA, USA). Monocytes were either lysed immediately in Trizol reagent or cultured at 5×10^5 cells mL^{-1} in Roswell Park Memorial Institute (RPMI) media supplemented with 10% Fetal Bovine Serum (FBS), 0.1% penicillin/streptomycin (P/S) and 0.1% glutamine in 5 mL polypropylene tubes. MDMs were differentiated *in vitro* by culturing at 5×10^5 cells mL^{-1} in 10% RPMI with 25 ng mL^{-1} macrophage- colony stimulating factor (M-CSF) for 5 days in six well culture plates.[22] All functional assays were performed on freshly isolated cells.

Human microglia were isolated from fetal or adult brain tissue using previously described protocols.[22,23] Adult microglia were derived from brain tissue obtained following surgery for pharmacologically intractable epilepsy. Fetal microglia were isolated from 16 to 18 week old fetal brain tissue obtained from the fetal tissue repository of Albert Einstein School of Medicine. Briefly, brain tissue was mechanically dissociated, and underwent enzymatic digestion using trypsin and DNAse prior to mechanical separation through a nylon mesh filter. Adult tissues underwent an additional ultracentrifugation step to remove myelin. Dissociated cells were then centrifuged, counted, and plated at either 6×10^6 cells mL^{-1} in Dulbecco's Modified Eagle Medium (DMEM) with 5% FBS and 0.1% P/S, and 0.1% glutamine (fetal) or 2×10^6 cells mL^{-1} in minimum essential media with 5% FBS and 0.1% P/S, and 0.1% glutamine (adult). Microglia were grown for 10–14 days with one media replacement after 5–7 days. Purified microglia were then collected and plated at 1×10^5 cells mL^{-1} and maintained in culture for 5 days before treatments. More than 95% of these cells expressed CD11c. For experiments involving HCAR2

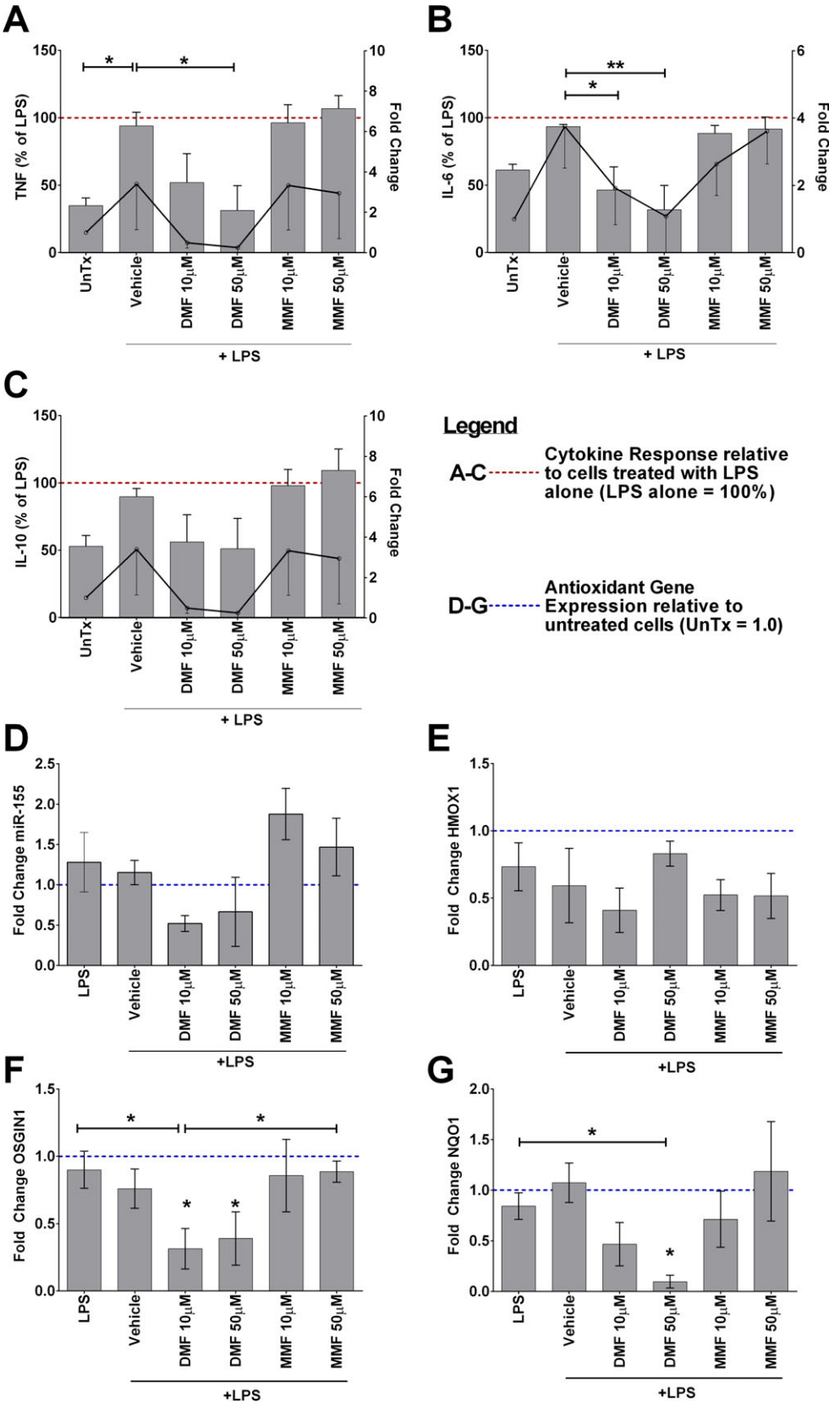

Figure 2. DMF but not MMF treatment *in vitro* reduces LPS-induced cytokine production and antioxidant gene expression in monocytes – Monocytes isolated from PBMCs of healthy controls were pretreated for 1 h with either DMF or MMF before addition of LPS (100 ng mL^{-1}) to the culture medium for 6 h. (A–C) DMF inhibits LPS-induced TNF, IL-6, and IL-10 protein production (grey bars) and mRNA expression (bold black line) in monocytes. Protein levels are expressed as percent of LPS-only condition (dotted red horizontal line); error bars indicate SEM. mRNA measurements correspond to right vertical axis, SEM shown ($n = 3$); data are expressed as fold change relative to untreated controls. Significance of changes in protein expression was assessed by repeated-measures one-way ANOVA; Holm–Sidak multiple comparisons test compares all columns versus vehicle + LPS column; $\alpha = 0.05$; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$; $n = 4$. (D) DMF treatment *in vitro* was also associated with a nonsignificant decrease in miR-155; miR-155 was not significantly regulated by LPS alone. (E–G) Antioxidant gene expression (NQO1, OSGIN1) was decreased in the presence of DMF. Grey bars indicate mean RNA level versus untreated condition (dotted blue horizontal line). Repeated-measures one-way ANOVA; Tukey's multiple comparisons test compares all columns; $\alpha = 0.05$; *$P < 0.05$, **$P < 0.01$; $n = 3$. DMF, dimethyl fumarate; MMF, monomethyl fumarate; LPS, lipopolysaccharide; TMF, tumor necrosis factor; ANOVA, analysis of variance.

expression, astrocytes isolated from the fetal human tissue specimens and cultured as described in[24] were included as controls.

All studies were approved by the institutional review board of McGill University; all subjects provided informed consent.

In vitro drug treatments

2 mmol L^{-1} DMF and MMF (Sigma Aldrich, Oakville ON, Canada) aliquots were prepared on the same date and frozen at −80°C with aliquots of vehicle dimethyl sulfoxide (DMSO). Initial dose-response studies conducted over a broad concentration range (DMF/MMF 0.5–100 μmol L^{-1}, BG00012 10–100 μmol L^{-1}) *in vitro* indicated that DMF from Sigma Aldrich that was used for the study had identical effects on lipopolysaccharide (LPS)-induced TNF production compared to DMF from Biogen Idec (BG00012, Biogen Idec, Cambridge MA, USA) – Figure S1. Cells were pretreated with either compound 1 h prior to the addition of 100 ng mL^{-1} LPS. LPS stimulation was carried out for either 6 or 24 h as indicated before supernatant collection, live/dead cell analysis, or RNA extraction procedures. In all studies, DMF and MMF were left in the cultures for the duration of the experiments.

Quantitation of gene and miRNA expression by quantitative, real-time polymerase chain reaction

Collected cells were lysed in Trizol reagent and stored at −80°C. Total RNA extraction was performed using standard protocols followed by DNAse treatment according to manufacturer's instructions (Qiagen, Valencia, CA). For gene expression analysis, standard reverse transcription (RT) using random hexaprimers and moloney murine leukemia virus reverse transcriptase was done. For miRNA expression analysis, multiplexed RT reactions were performed using a mix of miRNA-specific RT primers and a TaqMan® MicroRNA RT kit (Life Technologies, Burlington ON, Canada). Individual gene expression or miRNA expression assays were performed using specific TaqMan® or miRNA TaqMan® probes to assess expression relative to 18s or RNU48, abundant and stable housekeeping RNAs for gene and miRNA expression analysis, respectively. Fold Change calculations were performed using the $-\Delta\Delta CT$ method.

Quantitation of cytokine secretion by ELISA

Cell culture supernatants were collected following *in vitro* experiments and stored at −80°C. enzyme-linked immunosorbance assay (ELISAs) for TNF, IL-6, and IL-10 were performed in duplicate following manufacturer's protocols (BD Biosciences, Mississauga ON, Canada).

Live/dead cell assays

Initially, cell viability of monocytes was evaluated using trypan blue exclusion. Subsequent studies of the different myeloid cells were done using Calcein AM/propidium iodide (PI) based assays, either by flow cytometry or fluorescence microscopy.

Flow cytometry

Monocytes were incubated with 0.2 μmol/L Calcein AM for 20 min at 37°C and 5% CO_2 before a single wash in flow-activated cell sorting (FACS) buffer, and application of 0.5 μmol/L PI for 15 min prior to acquisition using an LSR Fortessa flow cytometer (BD Biosciences).

Fluorescent microscopy

A 4 μmol/L Calcein AM and 1 μmol/L PI were added to wells of MDMs, and microglia cultures (20 min at 37°C and 5% CO_2) that had been exposed to DMF or MMF. Following incubation, cells were washed twice in phosphate buffered saline (PBS) and directly imaged using a fluorescence microscope (Leica, Wetzlar, Germany).

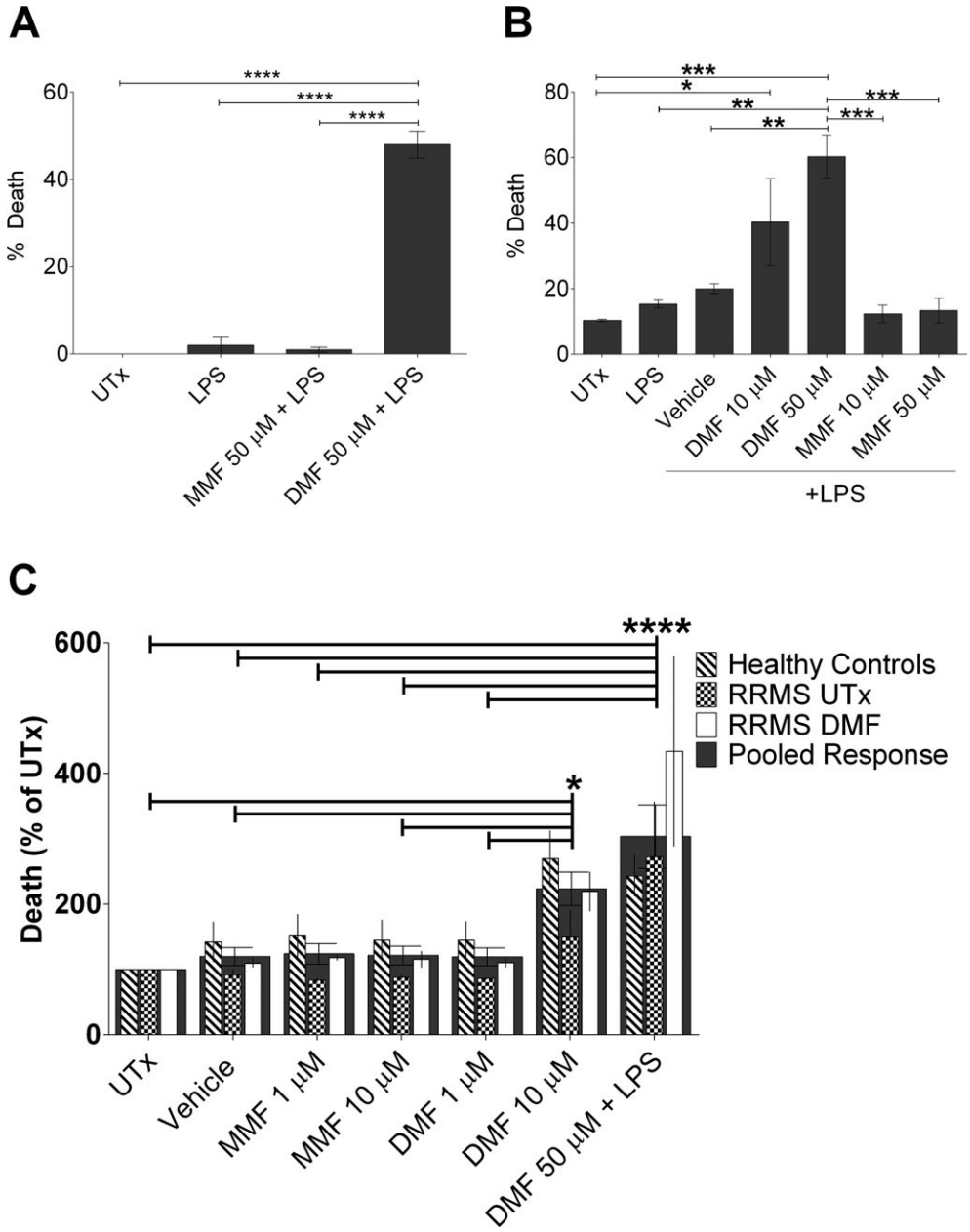

Figure 3. DMF *in vitro* is cytotoxic to monocytes from healthy controls and patients. Monocytes were cultured immediately following positive selection with anti-CD14 immunomagnetic beads in the presence of DMF and MMF ±LPS. (A) 6 h study- as assessed by trypan blue exclusion, 50 μmol/L DMF + LPS treatment was observed to significantly increase cell death (48%) over untreated, LPS-treated, and 50 μmol/L MMF + LPS-treated monocytes. Regular one-way ANOVA with Tukey's multiple comparisons test, $n = 3$, $\alpha = 0.05$; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$. (B) Twenty-four hours study- as assessed by trypan blue exclusion, DMF induced a dose-dependent increase in monocyte cell death under basal culture conditions. LPS, vehicle, and MMF-treated cells were not observed to undergo significant cell death relative to untreated cells (~10% cell death). Regular one-way ANOVA with Tukey's multiple comparisons test, $n = 3$, $\alpha = 0.05$; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$. (C) As assessed by flow cytometry using Calcein AM/PI staining, both 10 μmol/L DMF ($P < 0.05$) and 50 μmol/L DMF + LPS treatment ($P < 0.01$) induced significant cytotoxicity after 24 h. No toxicity was observed with MMF. One-way ANOVA with Tukey's multiple comparisons test compares means of pooled response group. There was no differential cytotoxicity of monocytes derived from untreated or DMF-treated patients, or healthy controls as assessed by regular two-way ANOVA with Tukey's multiple comparisons test. Healthy controls $n = 4$, RRMS Utx $n = 3$, RRMS DMF $n = 3$; $\alpha = 0.05$; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$. DMF, dimethyl fumarate; MMF, monomethyl fumarate; LPS, lipopolysaccharide; ANOVA, analysis of variance; PI, propidium iodide; RRMS, relapsing-remitting multiple sclerosis.

Live cell imaging

Microglia were plated directly onto the glass window of glass-bottom cell culture dishes (MatTek, Ashland MA, USA) and culture media was replenished after 24 h. Cells were pretreated with DMF or MMF at the indicated concentration for 20 min prior to imaging. Dishes were transferred to an incubated live-imaging microscope (VivaView, Olympus, Richmond Hill ON, Canada) and imaged every 12 min over 6 h using differential interference contrast at 20× magnification. Images were compiled to create a movie using standard ImageJ (Bethesda MD, USA) functions.

HCAR2 expression on microglia, astrocytes, and MDMs

For quantitation of HCAR2 expression, microglia, MDMs, or astrocytes were detached and collected from six well culture plates using 2 mmol/L EDTA in warm PBS for 5 min. Cells were blocked in 10% normal human serum and normal mouse immunoglobulin G (3 ng mL^{-1}) in FACS buffer (1% FBS in PBS) and then incubated with 0.1 ng anti-HCAR2 or concentration matched isotype APC-conjugated antibody (R&D Systems, Minneapolis MN, USA) for 40 min, followed by one wash in FACS buffer. To confirm the identity of astrocytes, these cells were then permeabilized and fixed with 10% saponin and 1% formaldehyde, and further incubated with anti-GFAP or isotype Alexa-488 conjugated antibody for 20 min. Events were acquired using an LSR Fortessa flow cytometer (BD Biosciences).

Results

Effects of DMF therapy on circulating monocytes

Overall numbers of circulating monocytes were within normal range for all the DMF-treated patients in the study. However, as expected there was a trend for an on-treatment relative reduction in lymphocyte counts (mean 1.71×10^6 per mL^{-1} blood for pretreatment counts vs. 1.47×10^6 per mL^{-1} blood for posttreatment counts) and a relative increase in monocytes (mean 4.60×10^5 per mL^{-1} blood for pre-treatment counts vs. 5.29×10^5 per mL^{-1} blood for posttreatment counts).

We observed significantly lower levels of miR-155 expression in the monocytes of patients treated with DMF relative to monocytes from untreated relapsing-remitting multiple sclerosis (RRMS) patients (Fig. 1A). Similar reductions were seen in patients receiving FTY720 and natalizumab when compared to untreated patients (Fig. 1A).

As regards antioxidant gene expression, we observed that HMOX1 expression was significantly higher in untreated MS patients relative to healthy controls (Fig. 1B: mean = 435.5 fold-induction, standard deviation = 133.6, $P < 0.0001$), followed by OSGIN1 (Fig. 1C: mean = 4.8 fold-induction, standard deviation = 3.5, $P = 0.0004$); NQO1 was not elevated in untreated RRMS patients (Fig. 1D). HMOX1 levels in DMF-treated patients were reduced compared to untreated MS patients but remained significantly elevated compared to healthy controls ($P < 0.0001$), DMF treatment was also associated with decreases in OSGIN1 relative to untreated patients ($P < 0.0001$); levels in treated patients were not significantly different from healthy controls ($P = 0.4941$). As with the miR-155 findings, antioxidant gene expression reductions similar to those seen with the DMF-treated cohort were also observed in patients receiving FTY720 and natalizumab when compared to untreated patients (Fig. 1B–D).

Effects of DMF and MMF on CD14+ monocytes *in vitro*

We initially observed that DMF added *in vitro* inhibited LPS-induced TNF, IL-6, and IL-10 expression in monocytes at the protein and mRNA level (Fig. 2A–C). DMF treatment was also associated with a nonsignificant decrease in basal miR-155 (Fig. 2D); miR-155 was not significantly regulated by LPS alone. Antioxidant gene expression (NQO1 and OSGIN1, but not HMOX1) was decreased in the presence of DMF (Fig. 2E–G).

We concurrently evaluated cell viability of monocytes treated *in vitro* with DMF or MMF + LPS. Using trypan blue exclusion, we found that at 6 h (time corresponding to functional assays) DMF but not MMF induced significant toxicity of LPS-treated control donor monocytes (Fig. 3A); LPS alone had no toxic effect. When examined at 24 h following exposure to DMF, we observed dose-dependent toxicity of control donor monocytes treated with DMF and not MMF (Fig. 3B). As assessed by counting PI-positive cells using flow cytometry, we confirmed the toxicity of low-dose DMF and that there were no differences in levels of toxicity between monocytes derived from healthy controls, DMF-treated, and untreated MS patients, under basal conditions or upon DMF exposure by regular two-way analysis of variance (ANOVA). MMF had no significant effects on any of the above cytotoxicity measures (Fig. 3C).

Effects of fumarates on MDMs

When we tested DMF and MMF on MDMs *in vitro* we observed a significant DMF-associated down-regulation of IL-6 (Fig. 4B) but not of TNF (Fig. 4A) or IL-10 (Fig. 4C). HMOX1 (Fig. 4E) and OSGIN1 (Fig. 4F) were significantly up-regulated by 50 μmol L^{-1} DMF. There

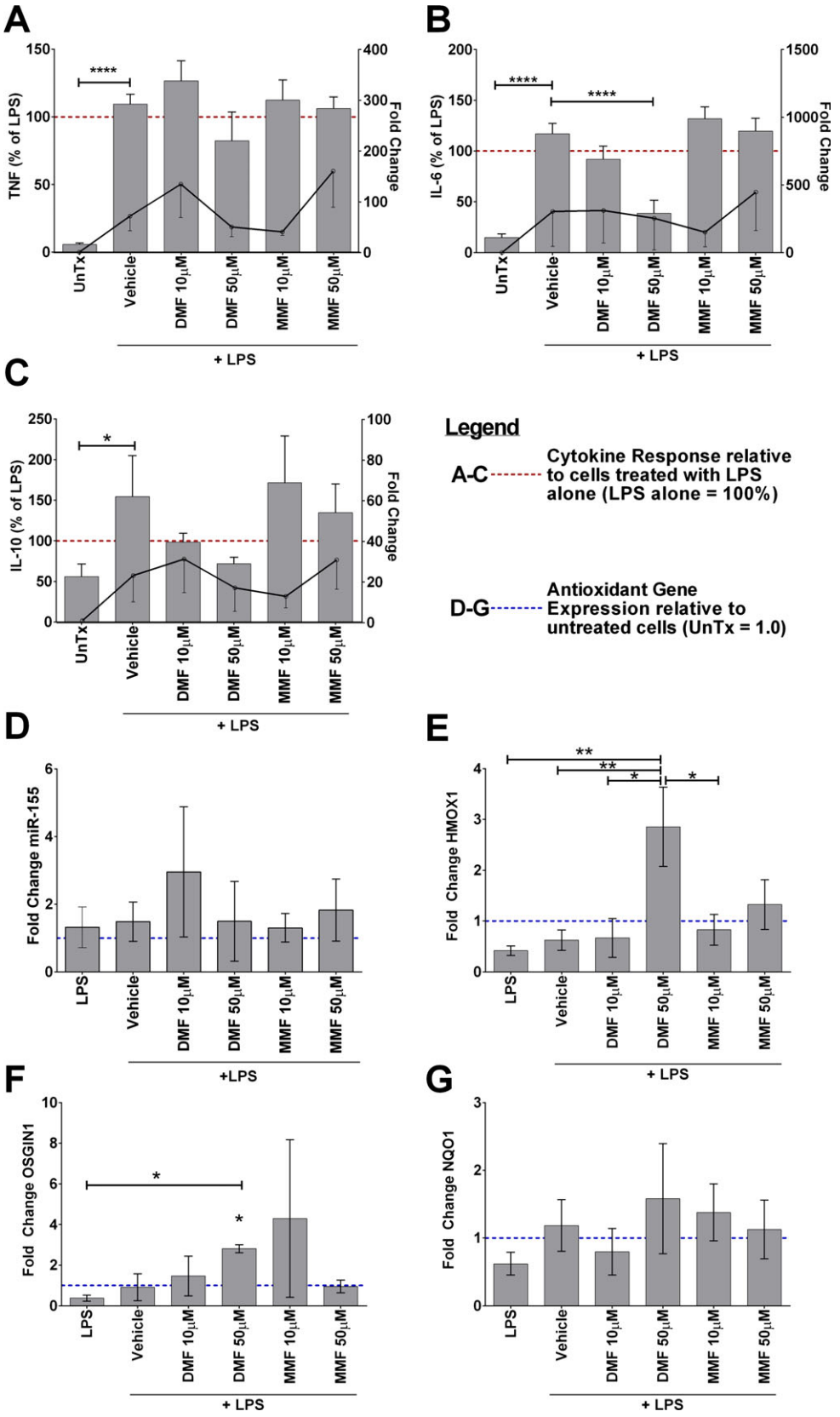

Figure 4. DMF treatment *inhibits* LPS-induced IL-6 and *induces* HMOX-1 expression in MDMs in the absence of cytotoxicity. MDMs from healthy controls were pretreated for 1 h with either DMF or MMF before addition of LPS (100 ng mL^{-1}) to the culture medium for 6 h. (A–C) DMF-treatment produced down-regulation of IL-6 (B) but not of TNF (A) or IL-10 (C). Protein levels are expressed as percent of LPS-only condition (dotted red horizontal line); error bars indicate SEM. mRNA measurements correspond to right vertical axis, SEM shown ($n = 3$); data are expressed as fold change relative to healthy controls. Significance of changes in protein expression was assessed by repeated-measures one-way ANOVA; Dunnett's multiple comparisons test compares all columns versus vehicle + LPS column; $\alpha = 0.05$; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$; $n = 8$. (D) miR-155 – no significant regulation of miR-155 in response to LPS, DMF or MMF ($n = 3$). (E–G) HMOX1 (E) and OSGIN1 (F) were significantly up-regulated by 50 μmol L^{-1} DMF, whereas NQO1 (G) was not. Grey bars indicate mean RNA level versus untreated condition (dotted blue horizontal line). Repeated-measures one-way ANOVA; Dunnett's multiple comparisons test compares all columns versus each other; $\alpha = 0.05$; $^{*}P < 0.05$, $^{**}P < 0.01$, $n = 7$ except OSGIN1 $n = 3$. DMF, dimethyl fumarate; LPS, lipopolysaccharide; MDMs, monocyte-derived macrophages; MMF, monomethyl fumarate; ANOVA, analysis of variance.

was no significant regulation of miR-155 (Fig. 4D), or NQO1 (Fig. 4G) in MDMs. We did not observe any effect of MMF on these functional response measures. We did not observe cytotoxicty to MDMs under any of the conditions tested.

Effects of fumarates on adult and fetal microglia

Overall, we observed that microglia were more sensitive to inhibition of cytokine and induction of HMOX1 by DMF treatment compared to peripheral myeloid cells. We observed significant inhibition of LPS-induced TNF, IL-6, and IL-10 (Fig. 5A–C) production in human fetal microglia pretreated with 50 μmol L^{-1} DMF, but not MMF. Pretreatment with 50 μmol L^{-1} DMF also inhibited LPS-induced miR-155 production in human fetal microglia (Fig. 5D). Similarly, we observed inhibition of TNF, IL-6, and IL-10 by 50 μmol L^{-1} DMF in human adult microglia (Fig. 6A–C). Levels of miR-155 expression in LPS- treated human adult microglia were decreased in the 50 μmol L^{-1} DMF pretreatment condition (Fig. 6D). 50 μmol L^{-1} DMF significantly induced HMOX1 expression in human fetal (Fig. 5E) and adult (Fig. 6E) microglia. DMF but not MMF application trended to increase NQO1 and OSGIN1 expression in fetal microglia (Fig. 5F and G) but not in adult microglia (Fig. 6F and G). Microglia appeared to exhibit normal motility while exposed to 50 μmol L^{-1} DMF and MMF over 6 hours (Videos S1–S3) and were not susceptible to cell death by DMF or MMF *in vitro* as observed by PI and Calcein AM staining at 6 or 24 hours (<1% cell death in any condition – data not shown). Phagocytosis of labeled human myelin by fetal microglia was not significantly altered by 50 μmol L^{-1} DMF or MMF treatment (Fig. S2).

HCAR2 expression by human microglia, astrocytes, and MDMs

Microglia (Fig. 7A) express the putative high-affinity MMF receptor HCAR2 (GP109A) as do astrocytes (Fig. 7B) and MDMs (Fig. 7C).

Discussion

Our combined analysis of circulating monocytes from DMF-treated MS patients and of the *in vitro* responses of blood and brain-derived myeloid cells to DMF and MMF provides insights and challenges regarding defining the mechanism of action of this therapy. Our study focused on both inflammation and oxidant-related responses. Compared to controls, untreated RRMS patients had elevated levels of the antioxidant genes HMOX1 and OSGIN1 in monocytes. Previous reports described a decrease in HMOX1 in the total PBMC population of such MS patients versus healthy controls[25,26] with a further decrease in both peripheral blood and CSF during relapse. We did not observe an increase in NQO1 expression, which indicates a specificity of antioxidant genes in the response of myeloid cells to the MS disease process. We did not specifically examine other cell constituents comprising the PBMC population in our study. Based on reports that HMOX1 can serve an antiinflammatory function,[14–16] we speculate that its increased expression in MS patients reflects a response to the chronic inflammatory state and an attempt to provide negative feedback and down-regulate this activity.

The relative increase in circulating monocytes observed in our DMF-treated patients, accompanied by a reduction in lymphocytes is consistent with previous studies.[27] We found no evidence that monocytes derived from DMF-treated patients had a shorter life span ex vivo or were more sensitive to exposure to MMF or DMF. We observed a significant down-regulation of miR-155 and of HMOX1 and OSGIN1 expression in CD14+ monocytes of DMF-treated patients compared to untreated individuals. Similar changes were seen in patients receiving FTY720 or natalizumab. Addition of FTY720 *in vitro* did not reproduce this effect (data not shown). We propose that the *in vivo* antioxidant and antiinflammatory effects of these agents reflect an indirect response to overall reduction in inflammatory activity rather than a direct effect on these cellular pathways.

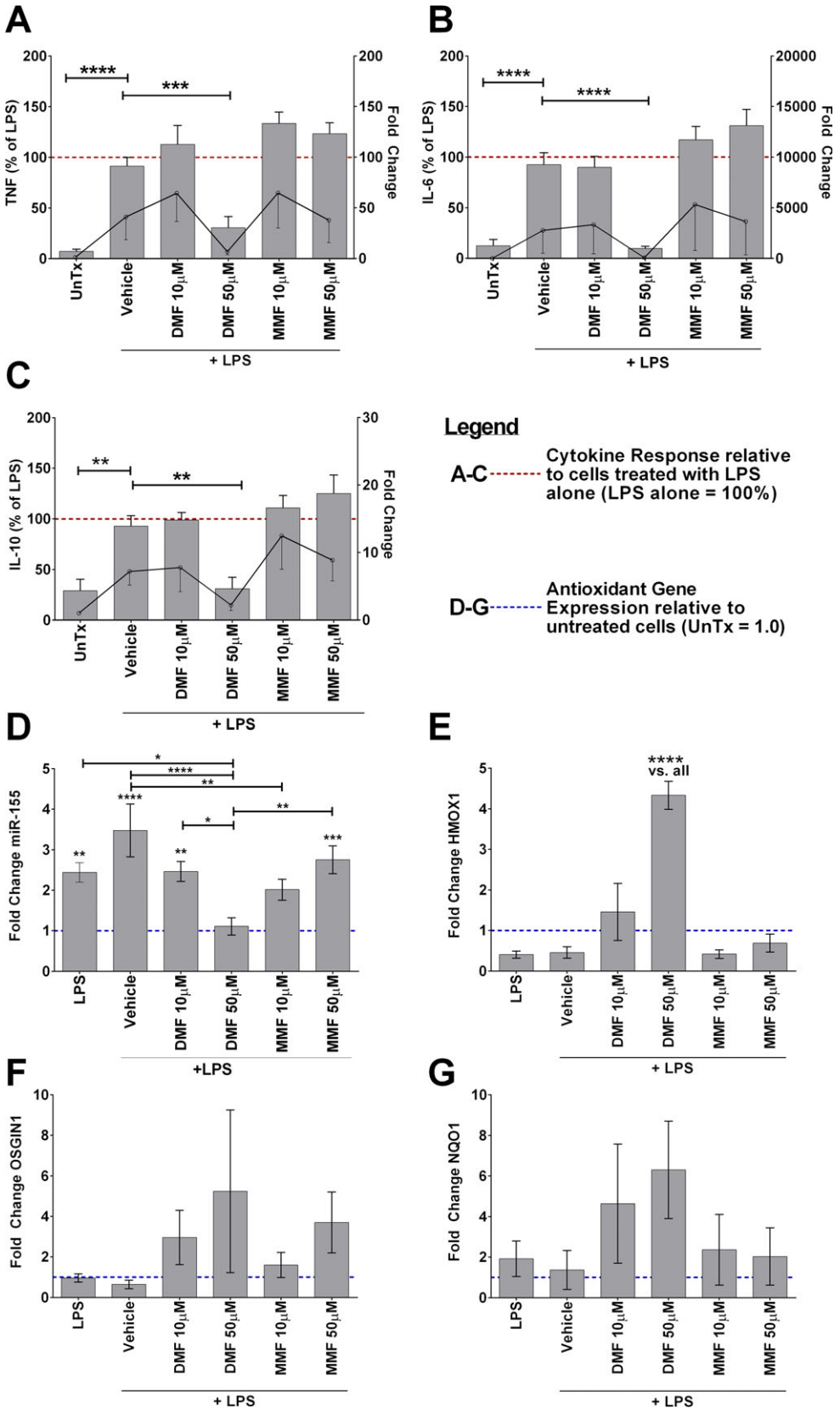

Figure 5. DMF treatment *inhibits* LPS-induced TNF, IL-6, IL-10 production and miR-155 expression and *induces* HMOX1 expression in human fetal microglia. Human fetal microglia were pretreated for 1 h with either DMF or MMF before addition of LPS (100 ng mL^{-1}) to the culture medium for 6 h. (A–C) – 50 μmol/L DMF but not MMF pretreatment inhibits LPS-induced TNF (A), IL-6 (B) and IL-10 (C) production in fetal microglia. Protein levels are expressed as percent of LPS-only condition (dotted red horizontal line); error bars indicate SEM. mRNA measurements correspond to right vertical axis, SEM shown ($n = 3$); data are expressed as fold change relative to untreated controls. Significance of changes in protein expression was assessed by repeated-measures one-way ANOVA; Dunnett's multiple comparisons test compares all columns versus vehicle + LPS column; $\alpha = 0.05$; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$; $n = 8$. (D) 50 μmol/L DMF pretreatment of human fetal microglia inhibits LPS-induced miR-155 expression. Significance of changes in expression were assessed by repeated-measures one-way ANOVA; Dunnett's multiple comparisons test compares all columns to each other; $\alpha = 0.05$; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$; $n = 4$. (E–G) DMF induced up-regulation of HMOX1 (E), but not OSGIN1 (F) or NQO1 (G) in human fetal microglia. Grey bars indicate mean RNA level versus untreated condition (dotted blue horizontal line). One-way ANOVA with Tukey's multiple comparisons test compares all columns versus each other; $\alpha = 0.05$; *$n = 5$ except OSGIN1 $n = 4$. DMF, dimethyl fumarate; LPS, lipopolysaccharide; TNF, tumor necrosis factor; MMF, monomethyl fumarate; ANOVA, analysis of variance.

To gain more direct insights into the mechanism of action of DMF therapy, we added DMF or MMF to human monocytes *in vitro*. Over a wide concentration range, we observed no effect of MMF on cell survival or LPS-induced cytokine production and antioxidant gene expression. This lack of effect of MMF contrasts with the report of Asadullah et al., which found that MMF (100 μmol L^{-1}) induced TNF and IL-10 in human monocytes.[11] *In vitro*, MMF is also reported to enhance IL-4 and IL-5 production in total PBMCs and isolated primed T cells.[28] As mentioned, Lehman et al. also found that DMF, but not MMF, decreased inflammatory cytokine production in PBMCs.[12] However, our observation that DMF beginning at 10 μmol L^{-1} induces cytotoxicity in monocytes makes it difficult to ascribe the observed reduction in LPS-induced cytokine production, and/or antioxidant gene expression to drug effect on a specific cellular signaling pathway.

In contrast to the results using human monocytes, we could observe effects of DMF on cytokine production and antioxidant gene expression in the absence of overt cell cytotoxicity on MDMs and microglia. We did not observe such effects with MMF although we could demonstrate expression of the HCAR2 receptor on microglia and MDMs.[8,9] The observed effect of DMF is unlikely to be mediated via this receptor as the HCAR2 receptor is specific for MMF in the concentration range used (0.5–100 μmol L^{-1}).[9] Both MMF and DMF, once added, were present throughout the time course of the studies making the exposure time comparable to that used by Parodi et al., suggesting there is no delayed MMF effect. Albrecht et al. and Gillard et al. also found that DMF rather than MMF is the active molecule in *in vitro* studies performed using rodent cortical neurons and an array of human and murine immune cells using BioMAP analysis, respectively.[4,17] Both DMF and its primary metabolite MMF have a reactive pi bond. This bond undergoes Michael-type addition reactions making it an effective glutathione depletion reagent, while oxidizing other cellular thiols such as those found in accessible cysteine residues of proteins.[5,19,29] Depletion of cellular glutathione can induce a multitude of antioxidant responses including induction of the Nrf2 response and HMOX1[12,29–32]; For these activities, DMF is shown to be significantly more potent than MMF.[3,19,29,32]

For both fetal and adult human microglia, we observed enhanced DMF responses compared to MDMs and monocytes. The precise mechanism underlying this differential susceptibility is not yet defined. We note that *in vivo* microglia are long lived cells compared to monocytes and have a distinct molecular signature.[33] The MDMs generated *in vitro* receive M-CSF whereas monocytes do not. Microglia, unlike their rodent counterparts, survive long term in culture even in the absence of growth factor supplementation.[34] The DMF-induced inhibition of LPS-induced TNF, IL-6, and IL-10 in human microglia was also associated with decreased mRNA expression for these cytokines, indicating transcriptional-level regulation.[13] DMF has previously been reported to decrease TNF and IL-6 mRNA in rat microglia *in vitro*.[13] The observation that miR-155 was inhibited by DMF pretreatment in microglia indicates that inhibition of cytokines may include transcriptional and posttranscriptional control. DMF-induced glutathione depletion has been shown to induce transcription of HMOX1 and may involve Nrf2; however, we did not observe consistent upregulation of NQO1 in response to DMF treatment either *in vivo* or *in vitro*.[3,4,12,14,30,31]

Conclusion

Our findings that DMF, FTY720, and natalizumab all reduce systemic myeloid cell inflammatory and antioxidant gene expression *in vivo* suggest that these results reflect an overall reduction in disease activity. Our *in vitro* results using human MDMs and microglia indicate that fumarate therapy can induce a noncytotoxic effect on cytokine and antioxidant gene expression on

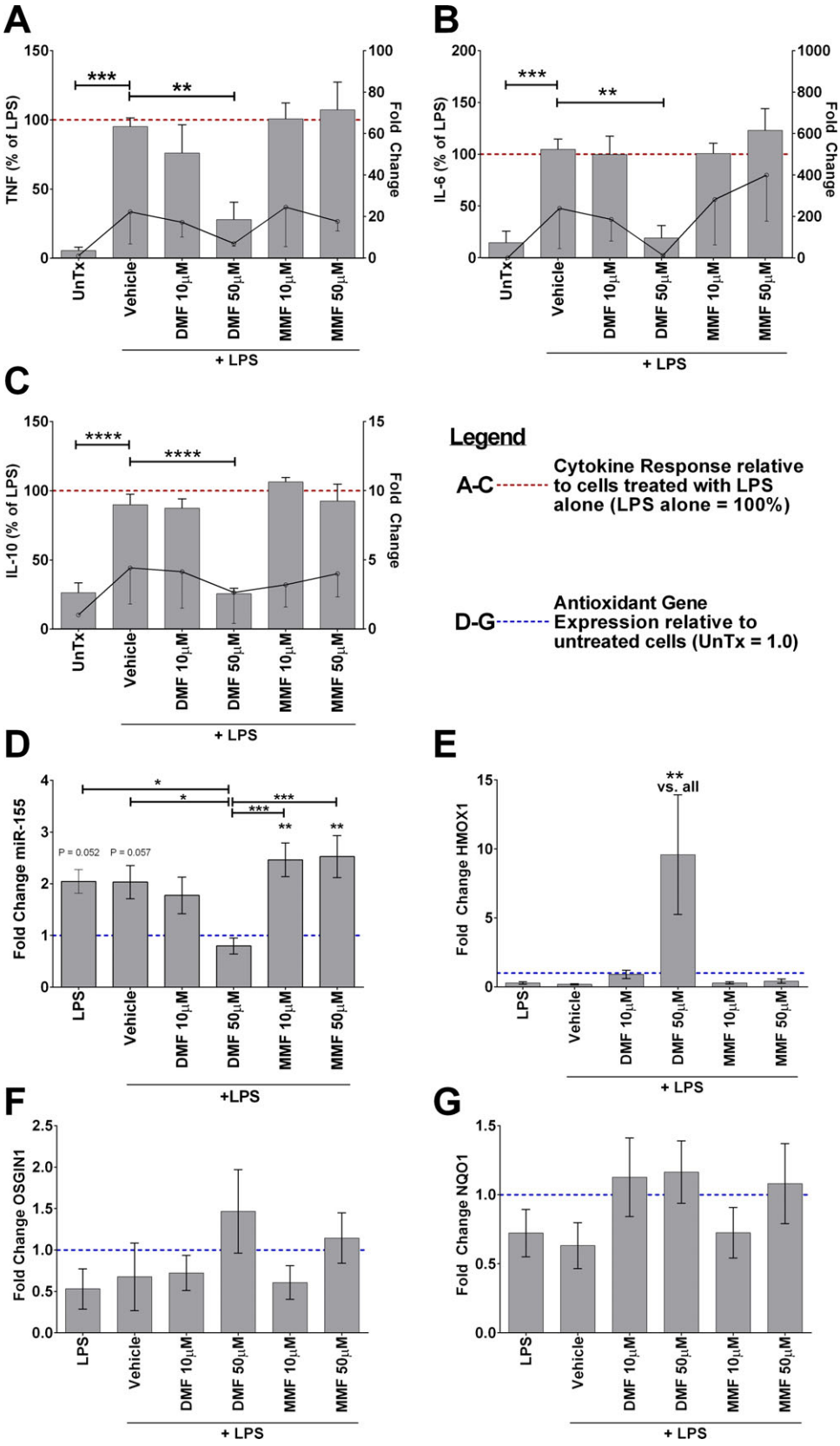

Figure 6. DMF treatment *inhibits* LPS-induced TNF, IL-6, IL-10 production and miR-155 expression, and *induces* HMOX1 expression in human adult microglia. Human adult microglia were pretreated for 1 h with either DMF or MMF before addition of LPS (100 ng mL^{-1}) to the culture medium for 6 h. (A–C) – 50 μmol/L DMF but not MMF pretreatment inhibits LPS-induced TNF (A), IL-6 (B) and IL-10 (C) production in adult microglia. Protein levels are expressed as percent of LPS-only condition (dotted red horizontal line); error bars indicate SEM. mRNA measurements correspond to right vertical axis, SEM shown (n = 3); data are expressed as fold change relative to untreated controls. Significance of changes in protein expression was assessed by repeated-measures one-way ANOVA with Geisser–Greenhouse correction; Dunnett's multiple comparisons test compares all columns versus vehicle + LPS column; α = 0.05; *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001; n = 8. (D) – 50 μmol/L DMF pretreatment of human adult microglia inhibits LPS-induced miR-155 expression. (E–G) DMF induced up-regulation of HMOX1 (E), but not OSGIN1 (F), or NQO1 (G) in human adult microglia. Grey bars indicate mean RNA level versus untreated condition (dotted blue horizontal line). Repeated-measures one-way ANOVA; Dunnett's multiple comparisons test compares all columns versus each other; α = 0.05; *n = 7 except OSGIN1 n = 3. DMF, dimethyl fumarate; LPS, lipopolysaccharide; TNF, tumor necrosis factor; MMF, monomethyl fumarate; ANOVA, analysis of variance.

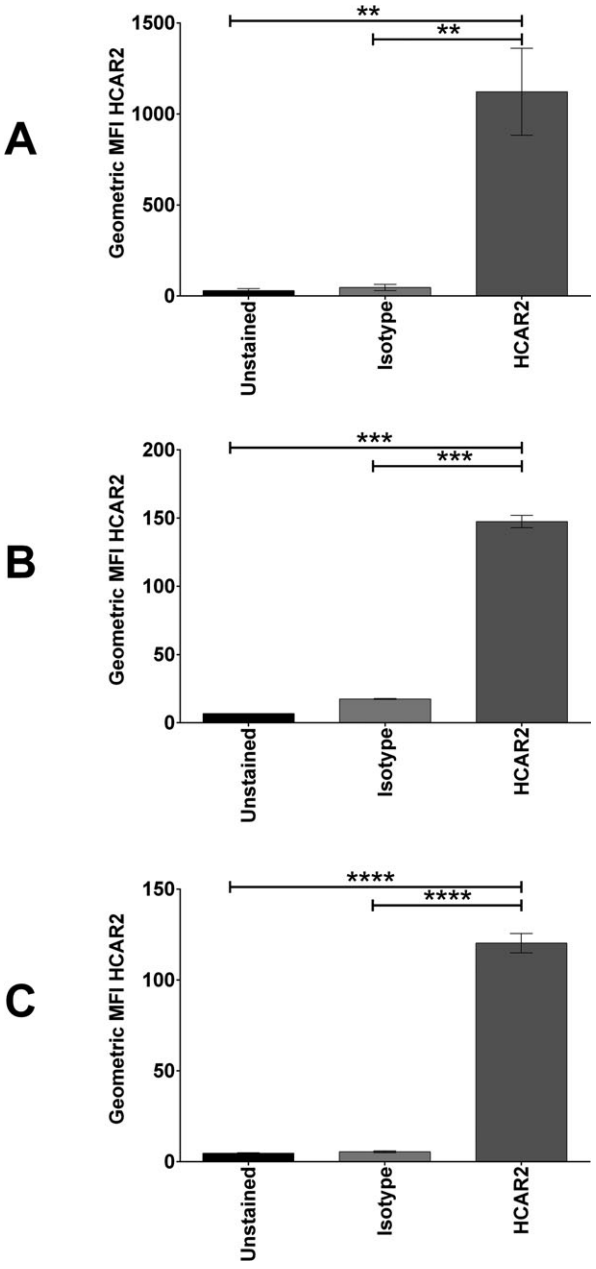

Figure 7. HCAR2 expression in human fetal microglia, astrocytes, and MDMS. Human fetal microglia (n = 4) (A), astrocytes (n = 2) (B), and MDMs (n = 4) (C) display specific HCAR2 staining by flow cytometry. Expression levels appear relatively higher in microglia than in astrocytes. Adult human microglia also express HCAR2 (data not shown). Repeated-measures one-way ANOVA with Tukey's multiple comparisons test all columns versus each other; α = 0.05; **P < 0.01, ***P < 0.001, ****P < 0.0001. HCAR2, hydroxycarboxylic acid receptor 2; MDMS, monocyte-derived macrophages; ANOVA, analysis of variance.

myeloid cells found within the CNS in MS. These effects were only mediated by DMF (not MMF), questioning the mechanisms whereby DMF therapy would modulate myeloid cell properties within the CNS.

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Author Contributions

M. A. M.-R.: Contributed or completed bench work for all figures, manuscript drafting, analysis and figure preparation, editing/revisions. L. M. H.: flow cytometry and project direction, microglia live imaging, editing/revisions. L. A. O.: micro-RNA qPCR assays, editing/revisions. N. Z.: micro-RNA qPCR assays, editing/revisions. V. G.: statistical analysis, editing/revisions. H. T. quality control and technical assistance. L. P.-L.: patient recruitment, editing/revisions. A.T.: patient recruitment; editing/revisions. C. S. M.: Project direction, patient recruitment, cell culture and isolation, qPCR, manuscript drafting and editing, data analysis and figure preparation, editing/revi-

sions. P. S. G.: patient recruitment; editing/revisions. A. B. O.: Project direction, patient recruitment, manuscript drafting and editing/revisions. J. P. A.: Project direction, patient recruitment, manuscript drafting and editing/revisions.

Conflict of Interest

Dr. Antel reports grants from Biogen Idec, during the conduct of the study. Dr. Bar-Or reports other from GlaxoSmithKline, during the conduct of the study; personal fees from Amplimmune,Bayhill Therapeutics, Berlex/Bayer, Biogen Idec, Diogenix, Genentech, GlaxoSmithKline, Guthy-Jackson/GGF, Merck/EMD Serono,Medimmune,Mitsubishi Pharma, Novartis, Ono Pharma, Receptos, Roche, Sanofi-Genzyme,Teva Neuroscience, outside the submitted work.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. DMF, MMF, and BG00012 human microglia dose-responses *in vitro*. Dose-response curves showing comparable effects of DMF obtained from Sigma Aldrich with BG00012 obtained from Biogen Idec on LPS induced TNF production by human fetal microglia.

Figure S2. DMF and MMF do not significantly alter microglial phagocytosis of human myelin *in vitro*. Microglia phagocytosis was live imaged as described (see Methods: Live Cell Imaging). Human myelin was labeled with pHRohdamine (Invitrogen) and added to microglial cultures to a final concentration of (20 μg mL^{-1}) immediately prior to imaging. pHRhodamine-labeled human myelin fluoresces upon acidification in phagolysosomes, thus mean fluorescence in images taken over 30 min intervals was used as a measure of phagocytosis. Mean fluorescence increased over a period of ~3 h before reaching a plateau. 50 μmol L^{-1} DMF or MMF did not significantly increase microglial phagocytosis of fluorescently labeled human myelin *in vitro*. Regular two-way ANOVA compares all treatment groups against each other at each time point ($\alpha = 0.05$, $n = 3$).

Videos S1–S3. DMF and MMF are not cytotoxic to human microglia. Human fetal microglia treated with 50 μmol L^{-1} DMF (Video S1), 50 μmol L^{-1} MMF (Video S2), display normal motility and morphology characteristics relative to untreated microglia (Video S3) over 6 hours in culture.

