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*J Immunol* 2011; 187:5015-5025; Prepublished online 5 October 2011;
doi: 10.4049/jimmunol.1101868

http://www.jimmunol.org/content/187/10/5015

Supplementary Material: http://www.jimmunol.org/content/suppl/2011/10/05/jimmunol.1101868.DC1

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Dimethyl Fumarate, an Immune Modulator and Inducer of the Antioxidant Response, Suppresses HIV Replication and Macrophage-Mediated Neurotoxicity: A Novel Candidate for HIV Neuroprotection

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Despite antiretroviral therapy (ART), HIV infection promotes cognitive dysfunction and neurodegeneration through persistent inflammation and neurotoxicity release from infected and/or activated macrophages/microglia. Furthermore, inflammation and immune activation within both the CNS and periphery correlate with disease progression and morbidity in ART-treated individuals. Accordingly, drugs targeting these pathological processes in the CNS and systemic compartments are needed for effective, adjunctive therapy. Using our in vitro model of HIV-mediated neurotoxicity, in which HIV-infected monocyte-derived macrophages release excitatory neurotoxins, we show that HIV infection dysregulates the macrophage antioxidant response and reduces levels of heme oxygenase-1 (HO-1). Furthermore, restoration of HO-1 expression in HIV-infected monocyte-derived macrophages reduces neurotoxin release without altering HIV replication. Given these novel observations, we have identified dimethyl fumarate (DMF), used to treat psoriasis and showing promising results in clinical trials for multiple sclerosis, as a potential neuroprotectant and HIV disease-modifying agent. DMF, an immune modulator and inducer of the antioxidant response, suppresses HIV replication and neurotoxin release. Two distinct mechanisms are proposed: inhibition of NF-κB nuclear translocation and signaling, which could contribute to the suppression of HIV replication, and induction of HO-1, which is associated with decreased neurotoxin release. Finally, we found that DMF attenuates CCL2-induced monocyte chemotaxis, suggesting that DMF could decrease recruitment of activated monocytes to the CNS in response to inflammatory mediators. We propose that dysregulation of the antioxidant response during HIV infection drives macrophage-mediated neurotoxicity and that DMF could serve as an adjunctive neuroprotectant and HIV disease modifier in ART-treated individuals. The Journal of Immunology, 2011, 187: 5015–5025.

Human immunodeficiency virus-1 infection of the CNS can result in cognitive, motor, and behavioral abnormalities, collectively known as HIV-associated neurocognitive disorders (HAND) (1, 2). Early in the course of infection, HIV traffics into the brain via infected monocytes and lymphocytes (3) and, despite antiretroviral therapy (ART), persists in parenchymal microglia and perivascular macrophages (4–6). HIV infection of the CNS results in the immune activation of resident glia and because HIV cannot infect neurons, neuronal damage is mediated by neurotoxins released by these infected and/or activated macrophages, microglia, and astrocytes. Although the severity of HAND has been significantly reduced through the widespread use of ART, the prevalence and associated morbidity remain high (~50%) (7, 8). The persistence of HAND in individuals effectively controlled for systemic viral load is incompletely explained, although recent evidence suggests that prolonged inflammation in both the CNS and periphery may be responsible (9–11).

Chronic systemic inflammation is tightly linked to morbidity and mortality in ART-treated patients, which suggests that adjunctive anti-inflammatory drugs or immune modulators may improve clinical outcomes. Despite undetectable plasma viral loads, measures of systemic inflammation correlate to cerebral spinal fluid (CSF) immune activation, CNS inflammation, and HAND (9–11). It has been proposed that elevated peripheral inflammation mediates neurocognitive decline by increasing the transendothelial migration of infected and/or activated monocytes into the brain (10, 12). An increased number of microglia and macrophages in the CNS correlates with the severity of premortem HAND, demonstrating the importance of these cell types in mediating neurologic impairment (4, 13, 14). Some of the most striking evidence linking peripheral inflammation to HAND derives from the strong association between early and persistent damage caused to GALT by HIV infection (or SIV infection in macaques), increased microbial translocation, systemic immune/monocyte activation, and HAND progression (9, 10, 15, 16). Therefore, reducing inflammation in the periphery as well as within the CNS is expected to improve neurocognitive impairment in HIV-infected patients.
Fumaric acid esters, including dimethyl fumarate (DMF) and its primary in vivo metabolite monomethyl fumarate (MMF), are a class of compounds that have anti-inflammatory and immunomodulating effects in vitro and in vivo. Fumaderm, a formulation of DMF and other fumaric acid esters, has been used in Europe since 1995 as an effective treatment for psoriasis; its mechanism of action is attributed to modulation of T cell activation and infiltration into plaques (17). DMF is currently under investigation for use in multiple sclerosis and a recently completed phase III study demonstrated a significant benefit in suppressing relapses, disease progression, and brain lesion inflammation (18). Using the rodent model of multiple sclerosis, experimental allergic encephalomyelitis (EAE), it was shown that DMF reduces the recruitment of monocytes into areas of active demyelination in the brain (19). In vitro model systems, DMF has been shown to inhibit proinflammatory cytokine production and NF-κB signaling via inhibition of nuclear translocation (19–22). Furthermore, DMF induces the expression of NF E2-related factor 2 (Nrf2)-driven antioxidant response genes, including heme oxygenase-1 (HO-1) and NAD(P)H quinone oxidoreductase 1 (NQO1) (23, 24). Notably, induction of HO-1 expression in human monocytes by heme has been associated with suppression of HIV-1 replication (25).

Because HIV replication can be strongly driven by NF-κB activation and nuclear translocation, we hypothesized that DMF treatment of HIV-infected monocyte-derived macrophages (HIV/MDM) would result in attenuation of HIV replication, immune activation, and neurotoxicity production. Our in vitro system models macrophage-mediated neurotoxicity during HIV infection by using human MDM and rat cerebrocortical neuronal cultures. In this system, HIV infection of MDM results in the release of low m.w. excitotoxins that injure neurons through excessive activation of N-methyl-D-aspartate receptors (NMDAR) (26–28). In this study, we demonstrate that DMF attenuates HIV replication, nuclear translocation of NF-κB subunits, and TNF-α production in human MDM. Furthermore, supernatants from DMF- and MMF-treated HIV/MDM cultures are markedly less neurotoxic to primary neurons than those from nontreated HIV/MDM cultures. Suppression of neurotoxicity production is mediated by induction of HO-1 in HIV/MDM and this suppression of neurotoxicity production can occur even without suppression of HIV replication. Finally, DMF and MMF also reduce CCL2-induced chemotaxis in human monocytes. This study demonstrates that DMF inhibits key steps in HAND pathogenesis through distinct effects on HIV replication and macrophage-mediated neurotoxin production and DMF should be considered as an adjunctive therapeutic for ameliorating the neurologic complications of HIV infection.

Materials and Methods

Reagents

Stock solutions of DMF and MMF (Sigma-Aldrich, St. Louis, MO) were prepared in DMSO and stored at −20°C until use. Tin (IV) mesoporphyrin IX dichloride (SnMP) and cobalt (III) protoporphyrin IX chloride (CoPP; Frontier Scientific, Logan, UT) were prepared in 1 N NaOH and stored at −20°C until use. Stock solutions of Ara-C (Sigma-Aldrich), PHA (Sigma-Aldrich), TNF-α (R&D Systems, Minneapolis, MN), and CCL2 (PeproTech, Rocky Hill, NJ) were prepared in filter-sterilized distilled water and stored at −20°C. Stock solutions of efavirenz (EFZ; National Institutes of Health [NIH] AIDS Research and Reference Reagent Program, Germantown, MD) were prepared in DMSO and frozen at −80°C until use.

Isolation and culture of human monocyte-derived macrophages

All human studies were reviewed and approved by the Institutional Review Board at the University of Pennsylvania. Human monocytes were prepared from PBMCs of healthy donors and isolated by Ficoll density gradient centrifugation as previously described (26, 29). Monocytes were plated at 10^6 cells/well to Cell-Bind six-well plates (Corning, Lowell, MA) and cultured in DMEM supplemented with 10% FBS, 10% horse serum, and 1% nonessential amino acids with 50 U/ml penicillin/streptomycin at 37°C, 6% CO2. Cells were cultured for 7 to 8 d and visually inspected for MDM differentiation before use in HIV-infection experiments. MDM were cultured for 7–10 d before use in noninfectious experiments.

HIV infection of MDM

Prior to infection, MDM were treated with E7F (5 or 20 nM), MMF, and/or DMF (1–100 μM), as indicated, for 1 h. All wells were normalized for the vehicles appropriate for drug treatments (DMSO and/or NaOH). Differentiated MDM were exposed to 50 ng (p24 ELISA, equivalent to 1.82 ± 0.22 kcpm/ml) by reverse transcriptase (RT) activity assay of HIV-1 Jago (R5 strain) or 89.6 (R5/X4 strain) for 24 h. HIV-Jago is a macrophage-tropic CSR isolate from a patient with confirmed HIV-associated dementia (29). Virus stocks were prepared by the University of Pennsylvania Center for AIDS Research Virology Core. Supernatants from HIV-infected or noninfected (mock) MDM were collected every 2–4 d and stored at −80°C. Supernatants were monitored for HIV replication by quantifying viral RT activity, as analyzed by the amount of radiolabeled deoxythymidine incorporation.

Subcellular fractions and Western blot analysis

For whole-cell lystate collection, cells were rinsed twice with ice-cold PBS, lysed in 75 mM Tris-HCl (pH 6.8), 15% glycerol, 3.75 mM EDTA, and 3% SDS, and supplemented with Complete Protease Inhibitor Cocktail (Roche Applied Science, Indianapolis, IN) and PhosSTOP phosphatase inhibitor mixture (Roche Applied Science).

To assess for nuclear translocation of NF-κB proteins, differentiated MDM were treated with DMF for 24 h, exposed to TNF-α (1 ng/ml) for 10 min, and fractionated. To prepare nuclear extracts, cells were rinsed twice in ice-cold PBS and lysed on ice for 10 min in 10 mM HEPES (pH 7.9), 10 mM KCl, 10 mM EDTA, 1 mM DTT, protease and phosphatase inhibitors, and incubated at 4°C on an orbital platform at 200 rpm for 2 h. After centrifugation at 16,000 × g for 5 min, supernatants (nuclear fractions) were collected and stored at −20°C. All protein concentrations were determined by the Detergent Compatible protein assay (Bio-Rad, Hercules, CA).

Cell lysates were subjected to SDS-PAGE as previously described (26) using the following Abs: rabbit anti–HO-1 (Stressgen/Enzo Life Sciences, Farmingdale, NY), mouse anti–MDM2 (Sigma-Aldrich), rabbit anti–Nrf2 (R&D Systems), rabbit anti–RelB (Cell Signaling Technologies, Danvers, MA), rabbit anti–NF-κB p65 (Cell Signaling Technology), rabbit anti–NF-κB p50 (Cell Signaling Technology), rabbit anti-poly(ADP-ribose) polymerase (Cell Signaling Technology), mouse anti–GAPDH (Advanced Immunoochemical, Long Beach, CA), and species-specific HPV-conjugated secondary Abs (Jackson ImmunoResearch Laboratories, West Grove, PA or Cell Signaling Technology). For densitometry analysis, films were scanned and a fixed cursor area centered over each band was assessed for pixel density using ImageJ (NIH, Bethesda, MD).

MDM-mediated neurotoxicity

Rat cerebrocortical neuronal cultures were prepared from embryos of Sprague–Dawley rats at day 17 of gestation, as previously described (26). All procedures were within the Animal Research: Reporting In Vivo Experiments guidelines for animal research and in accordance with protocols approved by the University of Pennsylvania Institutional Animal Care and Use Committee. Cells were plated in tissue-culture dishes precoated with poly-l-lysine (Peptides International, Louisville, KY) and maintained in neurobasal media plus B27 supplement (Invitrogen, Carlsbad, CA) at 37°C and 5% CO2. Forty-eight hours after plating, cells were treated with 10 μM Ara-C. After 7 d in vitro, approximately one-half volume of fresh media was added to the cells to counteract effects of evaporation. All cultures were exposed between 14 and 16 d in vitro.

Cell-based microtubule-associated protein 2 (MAP2) ELISAs were performed on primary rat cerebrocortical cells plated at a density of 6 × 10^5 cells/well in 96-well plates. Following a 24-h exposure to HIV/MDM supernatant, cultures were fixed and fluorescein labeled as described (30, 31) using the following reagents: mouse anti-MAP2 (Covance, Princeton, NJ), goat anti-mouse β-lactamase TEM-1 conjugate (Invitrogen), and Fluoroexcel (intensity). Fluorescence intensity was measured using a fluorometric plate reader with the 480/520-nm filter set. Macrophage supernatant was applied at a 1:10–1:50 dilution; the dilution that gave values within the linear range of the assay is presented.
Immunofluorescence
Primary rat cerebrocortical cells were plated at a density of $2 \times 10^5$ cells per 35-mm dish with glass coverslips. Following exposure to HIV/MDM supernatant for 24 h, cultures were fixed and fluorescently labeled as described (26) using the following reagents: mouse anti-MAP2 (Sigma-Aldrich), species-specific Cy3-conjugated secondary Ab (Jackson Immunoresearch Laboratories), and Hoechst 33342 (Invitrogen).

Lactate dehydrogenase assay
Soluble lactate dehydrogenase (LDH) in HIV/MDM culture supernatant was measured using the Cytotoxicity Detection KitPLUS (Roche Applied Science) according to the manufacturer’s instructions.

EMSA
Following 24 h of pretreatment with DMF, human MDM were exposed to 1 ng/ml TNF-α for 10 min and nuclear protein extracts were isolated as described. A total of 8 μg nuclear protein was assessed for NF-κB–DNA binding with an EMSA kit (Panomics, Santa Clara, CA), used according to the manufacturer’s directions. The labeled oligonucleotide for NF-κB p50 binding, 5'-AGTTGAGGGGACTTTTCCCCAGGC-3', was used.

Cytokine detection in culture supernatants
The concentration of TNF-α in culture supernatants was detected using an ELISA kit (Invitrogen) and used according to the manufacturer’s instructions. Uninfected MDM were treated with 0.067% DMSO (vehicle) or DMF for 24 h prior to exposure to 10 μg/ml PHA for 6 h. Supernatants were collected and frozen at −80°C until assayed.

Chemotaxis assay
Monocyte chemotaxis was assayed using the Chemicon QCM 96-well (5-μm pore size) Migration kit (Millipore, Temecula, CA) according to the manufacturer’s directions. Freshly isolated human monocytes were plated at a density of $2 \times 10^5$ cells/well in serum- and growth-factor-free culture media to the upper chamber in the presence of DMF, MMF, or vehicle (0.02% DMSO). CCL2 (300 ng/ml) was added to the lower chamber and cells were incubated at 37°C and 5% CO2 for 6 h (32–34). Exposure of monocytes to 300 ng/ml CCL2 for 6 h most consistently induced chemotaxis, with an average of 35.3 ± 20.2% above baseline. All cells that had migrated through the insert, including those adhered to the bottom of the membrane, were collected. For quantification, cells were lysed and labeled with CyQuant GR dye (Molecular Probes, Invitrogen). Fluorescence was read with the 480/520 nm filter set on a fluorometric plate reader.

Flow cytometry
Human PBMCs were cultured in RPMI 1640 supplemented with 10% FBS and 50 U/ml penicillin/streptomycin at 37°C and 5% CO2. Following 6 h of treatment with the indicated concentrations of DMF or DMSO vehicle, cells were washed with ice-cold FACS buffer (PBS, 1% BSA, and 0.1% NaN₃) and stained with CD11b-PE (clone ICRF44; ebBioscience, San Diego, CA), CD14-PE/Cy7 (M5E2; BioLegend, San Diego, CA), and CCR2-PerCP/Cy5.5 (TG5; BioLegend) Abs. Mouse IgG2a-PerCP/Cy5.5 (MOPC-173; BioLegend) was used as the isotype control for CCR2 staining. Ab-stained cell suspensions were pretreated with DAPI to identify dead cells. Flow cytometry was performed on an LSR II (BD Biosciences, Franklin Lakes, NJ). Doublets were excluded using forward side scatter-height versus forward side scatter-width and side scatter-height versus side scatter-width parameters. Data were analyzed using FlowJo (Tree Star, Ashland, OR). Monocytes were identified as CD11b+CD14+ cells.

Statistics
All quantifications are expressed as mean ± SE of mean. Statistical comparisons were made by Student t test, one-way ANOVA plus Newman–Keuls post hoc testing, or post hoc test for linear trend, as indicated. All graphs were generated and statistical analyses were performed using GraphPad Prism software (GraphPad, San Diego, CA) and values of $p < 0.05$ were considered significant.

Results
DMF and MMF inhibit HIV replication in human MDM
DMF and its in vivo primary metabolite MMF inhibit NF-κB signaling, suppress the production of inflammatory mediators, and induce an antioxidant response in a variety of cell types (19–22, 24, 35, 36). NF-κB signaling has been established as a major pathway of HIV transcriptional regulation and recent studies have implicated the antioxidant response enzyme HO-1 as a negative regulator of HIV replication in monocytes (25, 37). Therefore, we hypothesized that DMF could modulate HIV replication in human macrophages through one or both of these mechanisms. Human MDM infected with HIV-1 were treated with DMF or MMF and then examined for virus replication. As shown in Fig. 1, exposure of MDM to DMF (Fig. 1A) or MMF (Fig. 1B) attenuated HIV replication in a dose-dependent manner, as determined by culture supernatant RT levels. Suppression of replication in MDM was seen with the R5 CSF HIV strain Jago (Fig. 1) and the prototypic R5/X4 strain 89.6 (Supplemental Fig. 1). As shown in Supplemental Table I, HIV replication was inhibited by an average of 30% at MMF concentrations achieved in vivo after single-dose administration (4.4 μM in CSF and 6.5 μM in plasma) (23, 38).
No drug toxicity was detected at concentrations up to 100 μM in HIV-infected MDM (HIV/MDM) (Fig. 1C, 1D) and noninfected MDM (data not shown). DMF demonstrated additive effects in attenuating HIV replication when used in combination with EFZ, a nonnucleoside RT inhibitor (Supplemental Fig. 2A). There was no observed cellular toxicity when DMF was used in combination with EFZ (Supplemental Fig. 2B).

**DMF and MMF reduce HIV/MDM-mediated neurotoxicity**

We and others (26, 29, 39) have shown that HIV-infected MDM release potent neurotoxins that injure neurons through overactivation of NMDAR and that this excitotoxicity is mediated by glutamate and other low m.w. NMDAR agonists. Although the mechanisms underlying neurotoxin production in HIV/MDM are not fully understood, suppression of HIV replication in MDM generally suppresses such neurotoxicity, as demonstrated by treatment with EFZ (Fig. 2). Similarly, in addition to suppressing HIV replication (Fig. 1), DMF (Fig. 2A) and MMF (Fig. 2B) also reduce HIV/MDM neurotoxin production in a dose-dependent manner, as assessed by neuronal survival in our in vitro HIV neurotoxicity model. Representative images of HIV/MDM-mediated neurotoxicity and the protective effects of DMF and MMF are shown (Fig. 2C), in which surviving neurons are labeled for MAP2.

**FIGURE 2.** DMF and MMF reduce HIV/MDM-mediated neurotoxicity. Rat cerebrocortical cultures were exposed to supernatant from HIV-infected macrophages that were treated with DMF (A) or MMF (B) at the indicated concentrations (1–30 μM) during the course of infection. Neuronal survival was assessed by MAP2 ELISA and expressed as a percentage of untreated (UT) cultures (n = 6). C. Representative images of rat cerebrocortical cultures immunofluorescently stained for MAP2 (red) and Hoechst 33324 (blue) following 24 h treatment with the indicated HIV/MDM supernatant. Scale bar, 50 μm. All statistical comparisons were made by one-way ANOVA plus Newman–Keuls post hoc testing. ***p < 0.001 versus vehicle.

**FIGURE 3.** DMF inhibits NF-κB nuclear translocation, DNA binding, and TNF-α production in human MDM. DMF (A) and MMF (B) inhibit the nuclear translocation of the NF-κB proteins RelB, p65, and p50 in human MDM in a dose-dependent manner. Cells were treated with DMF or MMF for 24 h, exposed to TNF-α (10 min), separated into cytoplasmic and nuclear fractions, and analyzed by Western blotting. Results of densitometry analysis are presented numerically under each panel as the ratio of NF-κB protein to poly(ADP-ribose) polymerase, a nuclear marker, and loading control. Blots are representative of four to six independent experiments, with each replicate performed on cell preparations from different donors. C. DMF inhibits nuclear NF-κB p50 binding to DNA in TNF-α–stimulated MDM, as assessed by EMSA. Results of densitometry analysis were normalized to vehicle. D, DMF inhibits the production of TNF-α in MDM stimulated with PHA (10 μg/ml). Values are expressed as percent TNF-α production relative to vehicle-treated cells (227 ± 11.9 pg/ml TNF-α in vehicle). Data are expressed as mean ± SEM and averaged from four different donors. E, TNF-α production in HIV/MDM is inhibited by DMF treatment. HIV/MDM were treated with DMF (1–30 μM) or 20 nM EFZ over the course of infection and culture supernatants from days 14 to 15 postinfection were assayed for TNF-α by ELISA. Values represent the mean ± SEM of data averaged from four different donors. All statistical comparisons were made by one-way ANOVA plus Newman–Keuls post hoc testing. *p < 0.05, **p < 0.01, ***p < 0.01 versus vehicle.
the suppression of macrophage-mediated neurotoxicity (Supplemental Fig. 2C), demonstrating that DMF may successfully reduce HIV replication and macrophage-mediated neurotoxicity that is not fully suppressed by ART. This neuroprotection is due to drug effects on the macrophages, as DMF and MMF do not prevent HIV/MDM-mediated neurotoxicity when applied directly to the neurons prior to addition of HIV/MDM supernatants (data not shown).

**DMF inhibits NF-κB nuclear entry, DNA binding, and TNF-α production in human MDM**

NF-κB and TNF-α are part of a positive-feedback loop that regulates the transcriptional activity of the HIV long terminal repeat (LTR). In unstimulated cells, NF-κB is unable to bind DNA due to its association with IκB proteins, which sequester NF-κB in the cytoplasmic compartment (40–42). Following exposure to an activating stimulus such as TNF-α, NF-κB is rapidly freed from the inhibitory complex and translocates into the nucleus to induce transcriptional activation of viral and host genes. NF-κB proteins are major modulators of the HIV LTR and are among the most potent activators of proinflammatory and inflammatory genes.

Five members of the mammalian NF-κB/Rel family have been described, including c-Rel, NF-κB1 (p50/p105), NF-κB2 (p52/p100), RelA (p65), and RelB. Functional NF-κB complexes are composed of heterodimer complexes containing p65, c-Rel, or RelB bound to p50 or p52 (40, 43, 44). Exposure to activating stimuli, such as TNF-α, induces the nuclear accumulation of NF-κB proteins, DNA binding by NF-κB p50, and transcription from the HIV LTR (45).

To determine if DMF and MMF inhibit the nuclear translocation of NF-κB proteins in MDM, DMF- and MMF-treated MDM were stimulated with TNF-α and subjected to subcellular fractionations before detection of NF-κB subunits by Western blotting. DMF and MMF each inhibited TNF-α–induced nuclear accumulation of RelB, p65, and p50 in a dose-dependent manner (Fig. 3A, 3B). We also demonstrate that DMF inhibited the formation of the NF-κB p50–DNA complex, as assessed by EMSA (Fig. 3C). Because NF-κB signaling also induces expression of inflammatory mediators, we assessed the effects of DMF treatment on TNF-α release from MDM. In agreement with previous reports of DMF decreasing the release of inflammatory mediators from multiple cell types, including TNF-α, IL-1β, and IL-6 (24, 46), we found that DMF suppresses release of TNF-α from PHA-activated MDM (Fig. 3D).

Furthermore, DMF also markedly suppressed HIV-induced TNF-α release from MDM (Fig. 3E). Thus, DMF and its primary metabolite, MMF, inhibit NF-κB translocation and signaling events that contribute to the positive-feedback loop that modulates HIV transcription in infected and activated MDM.

**DMF restores the antioxidant response suppressed by HIV infection in MDM**

The antioxidant response is one of the cellular adaptive stress responses that can modulate virus replication and host cell survival, as shown in hepatitis B and dengue 2 infection models (47, 48). The antioxidant response maintains redox balance and counteracts oxidative damage through induction of proteins that are involved in detoxification of reactive oxygen species (ROS). These proteins are produced by genes with a common promoter element, the antioxidant response element (ARE), and ARE transcription is mediated by Nrf2. Under conditions of low oxidative stress, Nrf2 is kept transcriptionally inactive by Kelch-like ECH-associated protein 1 (Keap1), which sequesters Nrf2 in the cytoplasmic compartment (49). Following exposure to ROS or electrophiles, Keap1...
is degraded by the proteasome and Nrf2 translocates to the nucleus to drive expression of numerous genes, including HO-1, NQO1, glutathione peroxidase 1 (GPX1), and genes responsible for glutathione synthesis (glutamate cysteine ligase modifier, glutamate cysteine ligase catalytic subunit, and glutathione synthetase). HIV infection is associated with increased ROS production and depressed levels of glutathione, the major intracellular antioxidant (50). We observed a marked reduction in the level of HO-1 expression in HIV/MDM across multiple human donors, with a more modest but nonetheless consistent reduction in GPX1 levels (Fig. 4A, 4B). The effects of DMF on Nr2 and NQO1 levels were more variable among HIV/MDM cultures from different donors, but trended toward increased expression relative to uninfected mock/MDM (Fig. 4A, 4B).

Upon exposure of HIV/MDM to DMF, expression of Nr2, HO-1, GPX1, and NQO1 increased with increasing doses of DMF (Fig. 4C), suggesting a restoration of antioxidant responses in HIV-infected MDM. Both HIV infection and DMF increase total levels of Nr2, suggesting that although HIV infection stabilizes or induces total cellular Nr2 levels, this is not sufficient for the coordinated transcriptional activation of ARE-regulated genes, such as HO-1 and GPX1. DMF and MMF treatment activates transcription of these ARE-regulated genes in HIV-infected macrophages, possibly by disrupting inhibitory Nr2–Keap1 interactions (23). DMF restores levels of HO-1 and GPX1 to those observed in uninfected MDM, whereas NQO1, which is not suppressed during HIV infection, is induced to levels exceeding those in uninfected MDM (Fig. 4D). We have also confirmed that MFM can induce the antioxidant response in HIV/MDM (Fig. 4E, 4F) and that both DMF and MMF induce the antioxidant response in uninfected MDM (data not shown). DMF induction of antioxidant responses in MDM occurs independently of HIV infection, which is consistent with previous findings describing induction of the antioxidant response by DMF in multiple cell types, including glia and neurons (23, 24).

**DMF inhibition of HIV replication and NF-κB signaling is not mediated by HO-1**

HIV infection of human MDM results in alterations to the antioxidant response with a striking reduction in HO-1 levels (Fig. 4A, 4B). Induction of HO-1 by hemin has been reported to decrease HIV replication in human monocytes, suggesting that DMF’s induction of HO-1 may underlie its antiviral effects (25). We used a pharmacologic inhibitor of HO-1 enzymatic activity, SnMP, to assess the role of HO-1 in DMF inhibition of HIV replication in human monocytes, as assessed by Western blotting. Human MDM were treated with 10 μM SnMP from days 6 to 15 postinfection. Culture supernatants were collected every 3 d and assessed for RT activity. RT curves are representative of three independent experiments, with each replicate performed on cell preparations from different donors. Although previous studies found that increased HO-1 activity is associated with decreased HIV replication in MDM (25, 37), we found that neither inhibition of HO-1 activity by SnMP treatment of MDM (Fig. 6A) nor induction of HO-1 expression by CoPP (Fig. 6D, 6F) altered HIV replication. Remarkably, however, SnMP treatment significantly increased the neurotoxicity of MDM supernatant (Fig. 6B), even when HIV replication was low or absent (Fig. 6C). The increase in MDM-mediated neurotoxicity was a consequence of inhibiting HO-1 activity in the macrophage because SnMP was not toxic when added directly onto neurons (data not shown). Although CoPP does not attenuate HIV replication or inhibit NF-κB signaling, supernatant from CoPP-treated HIV/MDM is significantly less neurotoxic than untreated controls with a similar level of HIV replication at 1 ng/ml TNF-α (10 min) and subcellular fractionation. Western blot is representative of three independent experiments, with each replicate performed on cell preparations from different donors.

**DMF and MMF inhibit CCL2-induced chemotaxis in human monocytes**

The recruitment of activated and infected monocytes to the CNS in response to CCL2 is a key step in the pathogenesis of HAND (51, 52). In a previous DMF study using the mouse EAE model, DMF reduced macrophage infiltration into the spinal cord in areas of inflammation in the early stages of the disease.
We hypothesized that DMF could inhibit chemotaxis of human monocytes in response to chemotactic cytokines, such as CCL2. We found that DMF and MMF inhibited chemotaxis in freshly isolated human monocytes in response to CCL2 in a dose-dependent manner (Fig. 7A, 7B). Furthermore, we found that DMF reduced the expression of the CCL2 receptor CCR2 in freshly isolated human CD11b+CD14+ monocytes within 6 h of treatment (Fig. 7C, 7D), without causing death (Fig. 7E). These results indicate that DMF and MMF can decrease monocyte chemotaxis in response to CCL2 and that this effect is associated with downregulation of CCR2 expression.

Discussion
Monocytes and macrophages are major reservoirs for HIV in both the periphery and CNS and they facilitate the spread of virus to target cells, allow for viral persistence, and serve as major contributors to inflammation-mediated pathology. Despite current ART, latently infected monocytes and CD4+ T lymphocytes persist, resulting in inflammation in the periphery and in the CNS in up to 50% of patients on ART (7, 8). Although ART will remain the mainstay of HIV therapy, effective adjunctive therapies that suppress inflammation, improve morbidity, and improve long-term cognitive outcomes are greatly needed. The immunomodulator DMF, which is effective for the treatment of psoriasis and shows promising results for multiple sclerosis treatment in recent clinical trials, is an attractive candidate as a safe adjunctive neuroprotectant against HIV. We have demonstrated that physiologically relevant doses of DMF and its primary metabolite MMF (23, 38) affect key steps in the pathogenesis of HAND in our in vitro model system by inhibiting HIV replication, neurotoxin production, NF-κB signaling, and TNF-α production in human MDM and by reducing monocyte chemotaxis in response to CCL2. These results suggest that DMF could serve as an effective neuroprotectant in HAND and have beneficial effects on systemic HIV disease progression as well.

We have shown that DMF and MMF attenuate macrophage-mediated neurotoxicity following HIV infection by simultaneously attenuating viral replication and inducing HO-1 expression. Furthermore, induction of HO-1 can significantly decrease macrophage-mediated neurotoxicity even without decreasing...
HIV replication. Consequently, DMF may be an especially relevant therapeutic in patients who have relatively good virologic control but still suffer from neurologic complications of HIV. We have shown that HIV infection of MDM results in a dysregulation of the antioxidant response with an especially prominent reduction in HO-1 levels associated with supernatant neurotoxicity and that DMF treatment restores HO-1 levels and reduces neurotoxin production in macrophages. In activated microglia, an oxidative burst is required for the release of excitotoxic glutamate (53), demonstrating that alterations to cellular oxidative state can mediate the production and/or release of MDM neurotoxins. DMF’s ability to decrease HIV replication and neurotoxin production by distinct mechanisms makes it an especially attractive therapeutic candidate for HAND. Furthermore, macrophage- and microglia-mediated neurotoxicity contribute to many other neurologic disorders including multiple sclerosis, Alzheimer’s disease, amyotrophic lateral sclerosis, Huntington’s disease, and stroke/reperfusion injury, for which therapeutics for restoring oxidative balance resultant from the disease state have been investigated/prescribed (54).

Numerous proinflammatory factors contribute to HIV disease pathogenesis in both the peripheral and CNS compartments. TNF-α, IL-6, IL-1β, IFN-γ, and other proinflammatory cytokines are elevated in the blood and CSF of HIV-infected patients (55–58). Among these, TNF-α is the most potent mediator of inflammation and is induced early after HIV monocyte infection and its expression continues to increase over the course of infection (59–61). It is well established that TNF-α exposure upregulates HIV replication by initiating a signaling cascade that activates the nuclear translocation of NF-κB (62–65). We have shown that DMF and MMF attenuate TNF-α-mediated NF-κB signaling in human macrophages and reduce nuclear NF-κB levels, which are expected to decrease transcription from the HIV LTR. However, the NF-κB and TNF-α signaling loop may not entirely mediate DMF’s antiviral activity. Attenuation of HIV replication occurs at low concentrations, as does induction of the ARE, whereas inhibition of NF-κB signaling may be more relevant at concentrations of ≥15 μM. Future studies are necessary to assess the role of the antioxidant response, including NQO1 and the cellular redox state, in mediating HIV infection and replication. DMF may alter the expression of the HIV coreceptors CXCR4 and CCR5, similarly to the observed downregulation of cell-surface CCR2. It has been reported that antioxidants decrease the stability of mRNA transcripts for CXCR4 and CCR5 in human monocytes, suggesting that DMF treatment may directly reduce HIV entry into human monocytes (66).

However, DMF’s ability to inhibit NF-κB and TNF-α signaling following both PHA stimulation and HIV infection has clear implications for the physiologic reduction of neuroinflammation and cytokine-induced neuronal injury. Elevated TNF-α levels increase monocyte entry into the brain, promote HIV replication, and drive inflammatory cascades, thereby enhancing the production of neurotoxins in the CNS from MDM, microglia, and astrocytes (67). Therefore, dampening TNF-α–driven processes might also afford neuroprotection against HIV. Indeed, TNF-α is linked to glutamine synthetase and glutamate import in macrophages (68) and DMF’s inhibition of TNF-α–driven processes may further decrease the release of excitatory neurotoxins, such as glutamate, in HIV/MDM. In human macrophages, we have shown that DMF is a potent suppressor of NF-κB and TNF-α signaling. DMF’s antioxidant activity may be more relevant at concentrations of ≥15 μM. Future studies are necessary to assess the role of the antioxidant response, including NQO1 and the cellular redox state, in mediating HIV infection and replication. DMF may alter the expression of the HIV coreceptors CXCR4 and CCR5, similarly to the observed downregulation of cell-surface CCR2. It has been reported that antioxidants decrease the stability of mRNA transcripts for CXCR4 and CCR5 in human monocytes, suggesting that DMF treatment may directly reduce HIV entry into human monocytes (66).

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Although not directly dependent upon HO-1, DMF’s antioxidant properties are likely mediating the inhibition of NF-κB activity.
We hypothesize that such effects are due to DMF’s modulation of the macrophage intracellular redox state as activation of the antioxidant response has been shown to block NF-κB activity and HIV transcription (69–71). Furthermore, classical (α and β), novel (δ), and atypical (ζ) protein kinase C (PKC) isotypes can modulate the nuclear translocation and transcriptional activity of NF-κB and PKC is activated by oxidative stress and inhibited by antioxidants (72–76). In addition to potential effects on PKC, DMF may also affect the phosphorylation of IkB kinases and subsequent phosphorylation and degradation of IkB proteins (21, 36). Finally, DMF may affect NF-κB-dependent transcription by modulating the preferred composition of NF-κB homo- and heterodimers that form after nuclear translocation has occurred. The intracellular oxidative state can affect levels of NF-κB phosphorylated after nuclear translocation has occurred. The intracellular oxidative state can affect levels of NF-κB phosphorylated and degraded of IκB, which do not possess transactivation domains and are thought to act as transcriptional repressors of NF-κB heterodimer-responsive genes (77–79). We are currently examining the role of DMF and MMF in modulating the activation state of the macrophage, which would affect the cell’s relative sensitivity to proinflammatory signals and thereby contribute to decreased NF-κB signaling.

Although other antioxidants have been considered as potential therapeutics for HAND through direct effects on macrophages or neurons, DMF is unique in its ability to inhibit CCL2-induced monocyte chemotaxis. Monocyte transmigration across the blood–brain barrier is dependent upon production of chemokines, such as CCL2, in the CNS and the activation of monocytes in the periphery. Levels of CCL2 in the CSF correlate with CSF viral load and with the clinical severity of HAND (51, 52, 80–83) and CCL2 is produced by brain macrophages, astrocytes, and endothelial cells in response to inflammatory mediators and HIV proteins (84–86). Not only does DMF decrease TNF-α production and NF-κB signaling in MDM, both of which have been implicated in CCL2 production, but DMF and MMF also inhibit CCL2-driven monocyte chemotaxis, possibly by modulation of CCR2 expression. DMF and MMF may modulate the cell-surface expression of CCR2 by inducing the antioxidant response and consequently altering the redox state of the cell. It has been demonstrated that direct antioxidants are capable of reducing the transcript stability of CCR2, which has been linked to decreased cell-surface expression and CCL2-induced chemotaxis in human monocytes (66). These findings in our in vitro model system predict suppression of transendothelial migration of monocytes into the CNS during HIV infection. Furthermore, it has been reported that DMF modulates adhesion molecule expression in human endothelial cells by inhibiting TNF-α-induced expression of ICAM-1, VCAM-1, and E-selectin (35). Expression of each of these adhesion molecules has been linked to monocyte entry into the CNS after HIV infection and downregulation by DMF is expected to further inhibit monocyte entry into the CNS. Given these findings, DMF should be considered as a potential therapeutic for other neuroinflammatory diseases associated with CCL2-induced recruitment of leukocytes to the CNS.

With this study, we identify DMF as a candidate adjunctive therapy and potential neuroprotectant against HIV. We believe this is the first demonstration that HIV infection dysregulates components of the antioxidant response in human macrophages and that restoration of HO-1 levels, specifically, can reduce macrophage-mediated neurotoxicity. To our knowledge, DMF is the first proposed neuroprotectant that reduces CCL2-mediated monocyte chemotaxis as a component of its mechanism of action. Furthermore, we have shown that DMF attenuates HIV replication associated with decreased TNF-α and NF-κB signaling. Given these findings, we propose that DMF should be considered a relevant therapeutic candidate for neurologic disorders and other complications of HIV-infection mediated by monocyte and macrophage inflammation.

Acknowledgments

We thank Dr. Samantha S. Soldan for technical assistance, intellectual contributions, and critical review of the manuscript. We also thank Dr. Stefan Lanker of Biogen Idec and Dr. Francisco González-Scarano for helpful discussions, Margaret Maronski for expert preparation of primary rodent neuronal cultures, and Dr. Natalia Nedelsky for thoughtful critique and editing of the manuscript. The following reagent was obtained through the National Institutes of Health AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health (EFZ, catalog number 4624).

Disclosures

D.L.K. has served as a paid consultant to Teva Neurosciences and Biogen Idec, Inc. and serves on the advisory committee to the National Institutes of Health/National Institute of Mental Health National NeuroAIDS Tissue Consortium. The authors have no financial conflicts of interest.

References


Supplemental Table I: Reduction in RT activity, relative to vehicle control, with increasing concentrations of DMF and MMF among individual donors.

<table>
<thead>
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<th>Donor #</th>
<th>5μM DMF</th>
<th>15μM DMF</th>
<th>30μM DMF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>16.7 ± 11.0%</td>
<td>27.8 ± 9.8%</td>
<td>64.0 ± 13.4%</td>
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<td>68.0 ± 3.6%</td>
<td>52.7 ± 15.4%</td>
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<td>65.2 ± 9.2%</td>
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<table>
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<tbody>
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<td>40</td>
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<td>57.0 ± 8.3%</td>
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<tr>
<td>38</td>
<td>29.5 ± 9.9%</td>
<td>36.7 ± 2.9%</td>
<td>66.1 ± 3.7%</td>
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Values were calculated from RT data from the peak of infection and are presented as mean ± SD. For combined average (AVG) data, n = number of replicates averaged (number of unique donors). Only individual donors assayed more than once are included in the table.
Supplemental Figure 1. Dimethyl fumarate attenuates 89.6 HIV replication in human MDM. Human MDM infected with HIV-89.6 were treated with DMF (A) or MMF (B) over the course of infection at the indicated concentrations (1-30μM) or with the non-nucleoside reverse transcriptase inhibitor, efavirenz (EFZ). Culture supernatants were collected every 2-4 days, as indicated, and assessed for RT activity. Values indicate the mean ± SEM. RT curves are representative of 2-3 independent experiments, with each replicate performed on cell preparations from different donors.
Supplemental Figure 2. Dimethyl fumarate and efavirenz have additive effects on attenuation of HIV replication and neurotoxin production in human MDM. A, DMF and efavirenz (EFZ), a non-nucleoside reverse transcriptase inhibitor, have additive effects on the attenuation of HIV replication in human MDM. MDM were infected with HIV-Jago and treated with DMF and/or EFZ over the course of infection. Culture supernatants were collected every 3 days and assessed for RT activity. Values indicate the mean ± SEM. RT curves are representative of 2 independent experiments, with each replicate performed on cell preparations from a different donor. B, DMF and EFZ cause no cytotoxicity in HIV/MDM as assessed by LDH assay of supernatants harvested at
day 15 post infection. Maximum (Max) LDH release represents the soluble LDH release following cell lysis. LDH assays represent data averaged from 2 individual donors. C, DMF and EFZ used in combination reduce macrophage-mediated neurotoxicity to a greater extent than either drug alone. Neuronal survival was assessed by MAP2 ELISA following exposure to supernatant of HIV-infected macrophages, which were treated with DMF and/or EFZ over the course of infection. MAP2 data is expressed as a percentage of untreated (UT) cultures ($n = 6$). All statistical comparisons were made by one-way ANOVA plus Newman-Keuls post hoc testing, **$p<0.01$ and ***$p<0.001$ vs. Vehicle.