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Dimethyl fumarate prevents HIV-induced lysosomal dysfunction and cathepsin B release from macrophages

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Abstract

HIV-associated neurocognitive disorders (HAND) are prevalent despite combined antiretroviral therapy, affecting nearly half of HIV-infected patients worldwide. During HIV infection of macrophages secretion of the lysosomal protein, cathepsin B, is increased. Secreted cathepsin B has been shown to induce neurotoxicity. Oxidative stress is increased in HIV-infected patients, while antioxidants are decreased in monocytes from patients with HIV-associated dementia (HAD). Dimethyl fumarate (DMF), an antioxidant, has been reported to decrease HIV replication and neurotoxicity mediated by HIV-infected macrophages. Thus, we hypothesized that DMF will decrease cathepsin B release from HIV-infected macrophages by preventing oxidative stress and enhancing lysosomal function. Monocyte-derived macrophages (MDM) were isolated from healthy donors, inoculated with HIV-1_{ADA}, and treated with DMF following virus removal. After 12 days post-infection, HIV-1 p24 and total cathepsin B levels were measured from HIV-infected MDM supernatants using ELISA; intracellular reactive oxygen and nitrogen species (ROS/RNS) were measured from MDM lysates, and functional lysosomes were assessed using a pH-dependent lysosomal dye. Neurons were incubated with serum-free conditioned media from DMF-treated MDM neurotoxicity was determined using TUNEL assay. Results indicate that DMF reduced HIV-1 replication and cathepsin B secretion from HIV-infected macrophages in a dose-dependent manner. Also, DMF decreased intracellular ROS/RNS levels, prevented HIV-induced lysosomal dysfunction and neuronal apoptosis. In conclusion, the improvement in lysosomal function with DMF treatment may represent the possible mechanism to reduce HIV-1 replication, and cathepsin B secretion. DMF represents a potential therapeutic strategy against HAND.

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Ethical Approval: All procedure performed in studies involving human subjects were in accordance with the ethical standards the institutional review board and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all subjects included in this study. This article does not contain any studies with animals performed by any of the authors.

Keywords

Cathepsin B; HIV; DMF; Lysosomes; MDM; HIV-associated neurocognitive disorders

Introduction

HIV-associated neurocognitive disorders (HAND) can occur during advanced HIV-1 infection despite effective combined antiretroviral therapy (cART). After the implementation of cART, the incidence of HAD has decreased, but the prevalence of milder forms has increased due to longer life expectancy of patients and the presence of virus in CNS reservoirs (Neuenburg et al. 2002; Saylor et al 2016; Anderson et al 2016). Oxidative stress has been implicated in the onset of HAND (Sacktor et al. 2004; Saha and Pahan 2007; Kallianpur et al. 2016). The mechanisms and consequences of oxidative stress during HIV infection have been reviewed recently (Ivanov et al. 2016). Excess ROS production leads to dysfunction of the CNS homeostasis, affecting the permeability of the blood brain barrier. This results in increased access of blood soluble factors and entry of peripheral blood monocytes to brain tissues (Mollace et al. 2001; Price et al. 2005). In the brain, active replication of HIV occurs in perivascular macrophages and microglia. Although neurons are not infected, there is neuronal loss in distinct regions of the brain during HIV infection (Crews et al. 2009). Antioxidants including SOD-1, catalase, glutathione peroxidase, thioredoxin, and peroxiredoxin are decreased in monocytes from patients with HAND (Velázquez et al. 2009; Kraft-Terry et al. 2010). Glutathione (GSH) is also decreased in HIV-infected patients, mainly as result of chronic overproduction of pro-inflammatory cytokines, such as, IL-1, TNF- α and IL-17, that affect its biosynthesis (Morris et al. 2012). In HIV infection, LPS translocation from the gut increases inflammation, activates monocytes and promotes their trafficking into the brain (Ancuta et al 2008). The neuronal injury can be direct or indirect; the direct mechanism includes shedding of the viral neurotoxic proteins gp120, Tat, and Vpr. The indirect mechanism includes secretion of inflammatory products by activated macrophages and microglia, (Ghafouri et al. 2006; Louboutin et al. 2010). The inflammation process is promoted by increased secretion of IL-1 β and TNF- α from HIV-infected macrophages, that stimulate astrocytosis, ROS and other potentially neurotoxic substances, including cathepsin B (Werneburg et al. 2002). Cathepsin B is a lysosomal cysteine protease enzyme that plays an important role in antigen processing and presentation by macrophages and is normally controlled by its innate inhibitors, cystatins B and C. HIV infection of macrophages stimulates increased cathepsin B secretion that promotes neuronal apoptosis (Rodriguez-Franco et al. 2012; Zenón et al. 2014; Cantres-Rosario et al. 2015; Zenón et al. 2015). Cathepsin B is also increased in plasma and post-mortem brain tissues of patients with HAD. Interactions of cathepsin B with its inhibitors are interrupted during oxidative stress (Yamashima and Oikawa 2009) and HIV infection (Rodriguez-Franco et al., 2012).

Since oxidative stress has been implicated in the progression to AIDS, different antioxidant therapies could be used in conjunction with cART to offer protection against the development of HIV-associated pathologies (Aquaro et al. 2008; Schifitto et al. 2009; Cross et al. 2011). Studies using a system of antioxidant delivery, have demonstrated that

antioxidant enzymes have a protective effect against gp120-induced toxicity when injected in the brain (Louboutin et al. 2009; Louboutin et al. 2010). Others have used N-acetylcysteine amide, to prevent gp120- and tat-induced oxidative stress in a blood brain barrier system (Price et al. 2006). Interestingly, N-acetylcysteine can also replenish GSH levels in HIV-infected patients (De Rosa et al. 2000). Dimethyl fumarate (DMF), another antioxidant, acts by promoting nuclear translocation of nuclear factor-erythroid 2-related factor 2 (Nrf-2) (Wilms et al. 2010; Cross et al. 2011; Brennan et al. 2015). DMF can suppress HIV replication and MDM-induced neurotoxicity (Cross et al. 2011). During LPS-induced inflammation in the brain, DMF decreases the mRNA expression of iNOS, IL-1 β , IL-6 and TNF- α in astrocytes (Wilms et al. 2010). DMF is currently approved for psoriasis and multiple sclerosis treatment, and its effects include reduction of chronic immune activation (Hoefnagel et al. 2003; Kappos et al. 2008; Reich et al. 2009; Fox et al. 2012; Gold et al. 2012). Also, DMF has been proposed as adjunctive therapy with cART against systemic and CNS HIV-disease pathogenesis (Gill & Kolson 2013). This study was designed to explore if DMF reduces cathepsin B secretion from HIV-infected MDM by decreasing oxidative stress and enhancing lysosomal function. Our results indicate that DMF can decrease cathepsin B secretion from HIV-infected MDM. Also, HIV-induced oxidative stress, lysosomal dysfunction and neuronal apoptosis were prevented by DMF treatment in these macrophages.

Materials and Methods

Monocyte isolation, culture, and infection

Peripheral blood mononuclear cells were isolated from healthy blood donors in accordance with the University of Puerto Rico Medical Sciences Campus Institutional Review Board protocol #0720109. MDM were selected by adherence after 7 days in culture at a concentration of 5×10^6 cells/well. MDM were cultured in RPMI supplemented with 20% FBS, 10% human serum, and 100U/mL pen/strep (Sigma; St Louis, MO). At day 7, MDM were infected with HIV-1_{ADA} stock (University of Nebraska) at 0.1 M.O.I. and were incubated overnight. Thereafter, the media containing residual virus was removed and DMF (Sigma; St Louis, MO) was added at different concentrations (15, 30, and 60 μ M), using 100% DMSO (Sigma; St Louis, MO) as a vehicle control. Treatments were maintained until 12 days post-infection (dpi), changing media every three days. To generate the macrophage conditioned media (MCM) used in the neurotoxicity experiments, the same methodology described above was repeated, but this time, treatments were maintained until day 11pi, then, complete media was replaced with serum-free media (RPMI only) and supernatants were collected at day 12pi.

Cell Viability

Cell viability after DMF treatment was assessed by MTT assay (Sigma; St Louis, MO), following manufacturer instructions. Cells were seeded in a 96-well plate in triplicate/condition at a concentration of 2.5×10^5 /well. This assay consists on the reduction of tetrazolium MTT (3-(4, 5-dimethylthiazolyl)-2, 5-diphenyltetrazolium bromide) by metabolically active cells, in part by the action of dehydrogenase enzymes, to generate

reducing equivalents such as NADH and NADPH. The resulting intracellular purple formazan was measured by photometry using a Varioskan Flash (Thermo Fisher Scientific).

Quantification of HIV-1 p24 and secreted total cathepsin B

HIV-1 p24 levels were determined from supernatants of 3, 6, 9, and 12dpi with HIV-1 p24 ELISA (XpressBio) following manufacturer instructions. Secretion of Total cathepsin B was determined from MDM supernatants at 12dpi by ELISA (R&D Systems), following manufacturer instructions. The optical density of both tests was measured using a Varioskan Flash (Thermo Fisher Scientific).

Assessment of ROS/RNS levels in MDM

The total reactive oxygen/nitrogen species (ROS/RNS) present in MDM lysates at 12dpi were determined using OxiSelect *In Vitro* ROS/RNS Assay Kit (Cell Biolabs). This assay considers the cumulative amount of ROS/RNS, using dichlorodihydrofluorescein-DiOxyQ (DCFH-DiOxyQ) fluorogenic probe. ROS/RNS react with DCFH, which is oxidized to the highly fluorescent 2', 7'-dichlorodihydrofluorescein (DCF). Fluorescence intensity is proportional to the total ROS/RNS levels present in the sample. Fluorescent signal was recorded using a Varioskan Flash (Thermo Fisher Scientific).

Functional Lysosomes

MDM were cultured in Permanox chamber slides (Fisher, Suwannee, GA) and infected with HIV-1_{ADA}. At 12dpi, slides were washed twice with PBS, and Lysopainter dye was added according to manufacturer instructions (Abcam, Cambridge, UK). Lysopainter is a pH-dependent dye and is visualized as red fluorescence only when lysosomal pH is acidic. Since normal function of lysosomes depends on acidic pH (Kawai et al. 2007), lack of red fluorescence can be used as an indicator of lysosomal dysfunction. Cell nuclei were stained with DAPI (1:500, Sigma) and visualized using a Nikon Eclipse E400, camera SPOT Insight QE and Fluorescence X-Cite Series 120 with the software Spot Imaging 5.1. Intensity of signals was analyzed using the ImageJ 1.49V software

Assessment of neuronal apoptosis

SK-N-SH (ATCC) cells were cultured with EMEM, 10% FBS, 1% sodium pyruvate, 1% non-essential amino acids (Sigma). After cells reached an approximately 80% confluence they were exposed for 24hr to a 1:4 dilution of macrophage-conditioned media (MCM), collected at 12dpi. Cells were fixed with 4% paraformaldehyde, and permeabilized with 0.1% Triton in 0.1% sodium citrate. Auto-fluorescence was quenched using 3% hydrogen peroxide in methanol. To assess neuronal apoptosis, *in situ* TUNEL assay (ROCHE®) was performed, according to manufacturer instructions. For the positive control, DNase I was used at a concentration of 30U/mL for 10min at room temperature. Cells were visualized using a Nikon Eclipse E400, camera SPOT Insight QE and Fluorescence X-Cite Series 120 with the software Spot Imaging 5.1. Cells were quantified using ImageJ 1.49V Software with the cell counter analysis tool.

Statistical analysis

For all of the statistical analysis, we used the Graph Pad Prism 7.0 Software. All graphs are presented using the mean and the $\pm$ standard error of mean (SEM). Parametric and non-parametric analyses were used based on normality tests using Shapiro-Wilk. Two-way ANOVA with a Tukey's or Sidak's post-test was used for HIV-1 p24, total cathepsin B, and cell viability analyses. Student's t-tests were used for comparisons of intracellular ROS/RNS levels and functional lysosomes. Correlations of ROS/RNS vs. total cathepsin B levels were done using Pearson test. One-way ANOVA was used for neuronal apoptosis comparisons. Significant difference was considered at $p < 0.05$.

Results

Cell viability and HIV-1 replication after DMF treatment

Macrophages were treated with different concentrations of DMF (15, 30, 60 μ M) to determine if this antioxidant affects MDM viability and HIV replication. DMF treatment did not affect the cell viability of HIV-infected macrophages (Fig. 1a). HIV replication was assessed measuring p24 antigen levels at 3, 6, 9, and 12 dpi from MDM supernatants. DMF decreased HIV-1 p24 levels significantly in a dose dependent manner when compared to vehicle-treated HIV-infected macrophages (Fig. 1b). This was expected since HIV reverse transcriptase activity in macrophages can be inhibited with DMF treatment (Cross et al. 2011).

Total cathepsin B secretion after DMF treatment

To determine the effect of DMF on total cathepsin B secretion, we measured total cathepsin B on supernatants of DMF-treated HIV-infected MDM at 12dpi. DMF effects on cathepsin B secretion were variable. Since MDM cultures were not differentiated towards classical or alternative activation states by using macrophage colony stimulation factors (i.e. M-CSF or GM-CSF), we compared cultures with increased cathepsin B after HIV infection with those with decreased cathepsin B secretion. DMF at 15, 30 and 60 μ M concentrations reduced total cathepsin B levels from HIV-infected MDM supernatants in a dose-dependent manner in the cultures with increased cathepsin B secretion after HIV infection, (Fig. 1c). In the group with decreased cathepsin B secretion after HIV infection, DMF reduced total cathepsin B levels only at 60 μ M (**Online Resource 1**). Based on the viability, the p24 antigen reduction, and results in the MDM cultures with increased cathepsin B secretion in HIV, DMF concentration of 30 μ M was selected for the next experiments. This was the minimum concentration that showed a significant decrease ($p < 0.01$) in total cathepsin B levels in comparison with vehicle control samples and did not affect cell viability.

HIV-induced Oxidative Stress after DMF treatment

Since DMF is an antioxidant, we wanted to determine if in our system of HIV-infected MDM oxidative stress was decreased after DMF treatment and if this resulted in reduced cathepsin B secretion. To answer that question, whole cell lysates from HIV-infected MDM treated with DMF (30 μ M) were used for assessment of the intracellular ROS/RNS production. Since we had a small number of lysates from HIV-infected MDM with increased

cathepsin B secretion after HIV infection (n=3), and no significant differences were observed between untreated (NoTx) and vehicle-treated groups in ROS/RNS levels (**Online Resource 2**), we grouped untreated and vehicle-treated samples into two different pool groups ("Uninfected pool" and "HIV+ pool" group) to compare ROS/RNS levels between uninfected and HIV-infected MDM. Results demonstrated that MDM cultures with increased cathepsin B secretion after HIV infection also had increased intracellular ROS/RNS levels (Fig. 2); whereas MDM cultures with decreased cathepsin B levels after HIV infection had decreased ROS/RNS levels (**Online Resource 3**). DMF treatment decreased intracellular ROS/RNS levels from HIV-infected macrophages (Fig. 2), confirming its role as an antioxidant. These results suggest that intracellular ROS/RNS play a role in cathepsin B secretion from MDM with increased cathepsin B secretion after HIV infection.

HIV-induced lysosomal dysfunction after DMF treatment

Lysosomal dysfunction was measured in HIV-infected MDM with increased cathepsin B secretion after HIV infection using a pH-dependent lysosomal dye. Since increased lysosomal pH is an indicator of lysosomal dysfunction (Kawai et al. 2007), the lack of red fluorescence staining was used as an indicator of lysosomal dysfunction. As expected, HIV-infected untreated and vehicle-treated macrophages showed a significant decrease in lysosomal red fluorescence (Fig. 3, $p<0.05$), suggesting that HIV infection promotes lysosomal dysfunction. Interestingly, DMF treatment in HIV-infected MDM rescued HIV-induced lysosomal dysfunction significantly, in comparison with HIV+ untreated (NoTx) group ($p<0.01$). These results suggest that DMF protects lysosomes from HIV-induced lysosomal dysfunction.

HIV/MDM-induced neuronal apoptosis after DMF treatment

Cathepsin B is considered a neurotoxin and, since DMF treatment reduced secretion of total cathepsin B, we were interested in determining if cathepsin B reduction will prevent neurotoxicity. We tested the effect of DMF in HIV/MDM-induced neuronal apoptosis using TUNEL assay. Our results showed that exposing human neuroblastoma cells to macrophage conditioned media (MCM) derived from HIV-infected MDM serum free supernatants (untreated and vehicle-treated) increased neuronal apoptosis significantly in comparison to uninfected untreated MCM, whereas exposure of neurons to MCM from HIV-infected MDM treated with DMF prevented HIV/MDM-induced neuronal apoptosis (Fig. 4). These results are consistent with previous studies that show that MCM from HIV-infected MDM treated with DMF prevented HIV/MDM-induced loss of microtubule-associated protein 2 (MAP2), an important component of neuronal survival, in rat neuronal cells (Cross et al. 2011).

Discussion

Previous studies from our laboratory have demonstrated that HIV infection of macrophages and microglia induces secretion of cathepsin B and neurotoxicity (Rodriguez-Franco et al. 2012; Cantres-Rosario et al. 2015; Zenón et al. 2015). However, the mechanism of cathepsin B release in HIV-1 infected macrophages is not known. We hypothesized that increased secretion of cathepsin B resulted from HIV-induced oxidative stress and lysosomal dysfunction. In this study, we tested whether the antioxidant DMF could reduce cathepsin B

secretion by reducing oxidative stress. We found that treatment of macrophages with DMF was able to reduce HIV replication and cathepsin B secretion in a dose-dependent manner in MDM cultures. In addition, DMF treatment prevented HIV-induced oxidative stress, lysosomal dysfunction, and neuronal apoptosis.

HIV infection is known to induce endolysosomal dysfunction by different mechanisms. HIV induces NLRP3 inflammasome activation in macrophages, a process known to promote lysosomal membrane permeabilization, by promoting oxidative stress and cathepsin B activity at early time-points after infection (Guo et al. 2014). Also, lysosomal disruption induced by HIV-Tat has been reported in neurons and astrocytes (Chen et al, 2013; Fan and He, 2016). Endolysosomal de-acidification increases HIV replication in primary macrophages and astrocytes (Carter et al. 2011; Chauhan et al. 2014). Jouve et al. (2007) demonstrated that HIV virions are present inside non-acidic endosomes in HIV-infected macrophages, presumably to maintain their viability. Also, they observed that HIV-infected macrophages contained acidic lysosomes. However, it is not known whether this effect occurs in HIV-infected single cells or syncytia, or if all lysosomes in HIV-infected macrophages are acidic. Thus, we performed observations using whole MDM culture lysates and supernatants with productive HIV-1 replication to explain the overall effect observed in cathepsin B and ROS/RNS.. It would have been ideal to correlate lysosomal dysfunction with the intracellular HIV-p24 levels in macrophages but co-staining was not possible. The lysosomal dysfunction method requires cells to be alive and lysosomes to be acidic, and adding an antibody to stain for HIV-1 24 antigen would require cell permeabilization, fixation, long-time incubations, and addition of different solutions that could alter pH. However, the productivity of HIV infection in these cultures was determined by HIV-1 p24 ELISA, as well as by the presence of syncytia in these cultures, a common phenotype of HIV-infected macrophages.

Campbell and collaborators (2015) demonstrated that endosomal acidification is also important for HIV-induced autophagy at early time-points, and at seven days post-infection, autophagy is inhibited by HIV-1 Nef through cytosolic sequestration of transcription factor EB (TFEB) to promote viral replication and host survival. Impaired TFEB activity also results in lysosomal-autophagy pathway dysfunction in other neurodegenerative diseases (Martini-Stoica et al. 2016). TFEB modulates lysosomal exocytosis, and de-acidification of lysosomes located at the cell periphery is thought to contribute to this process (Medina et al. 2011; Sbano et al. 2017). A recent study found that HIV infection relocates lysosomes to the periphery of macrophages (Cinti et al. 2017). Lysosomes located at the cell periphery lose their luminal acidity and proteolytic activity, and their principal role is to fuse with the plasma membrane and promote their exocytosis in a calcium-dependent manner (Xu & Ren 2015; Johnson et al. 2016). In line with these studies, He et al. (2016) found that HIV infection induces cathepsin B secretion from astrocytes by promoting lysosomal exocytosis. Moreover, studies have shown that ROS can act as second messengers and induce lysosomal exocytosis (Ravi et al. 2016).

Given the fact that our observations were performed at a relatively late infection time-point (12dpi), it is possible that the observed HIV-induced lysosomal dysfunction in our experiments is a result of lysosomal de-acidification and autophagy inhibition. Thus, future

studies will focus in further dissect the mechanism of lysosomal dysfunction and cathepsin B release, including lysosomal disruption, deacidification, exocytosis and autophagy inhibition, performing observations at earlier time points in culture.

Our results supports previous findings by Cross and collaborators (2011), showing that DMF reduces HIV replication and HIV/MDM neurotoxicity. DMF was demonstrated to reduce HIV replication by decreasing the translocation of NF- κ B into the nucleus (Lin et al. 2011; Cross et al. 2011). Cross et al (2001) also showed that DMF prevented HIV-infected macrophage neurotoxin production by inducing the heme-oxygenase 1 (HO-1) expression through the activation of Nrf2. Once DMF activates Nrf2, it promotes the expression of genes involved in the antioxidant response such as NQO1 and HO-1, leading to an increase in reduced GSH and ultimately promoting neuroprotection (Albrecht et al. 2012; Scannevin et al. 2012; Wang et al. 2015). Inhibition of Nrf2 in astrocytes exposed to gp120 results in increased expression of NOX-2, NF- κ B, and TNF- α , suppressing HIV-gp120 induced ROS (Reddy et al. 2012).

The consequences of increased cathepsin B secretion by macrophages have been extensively studied in our laboratory (Rodriguez-Franco et al. 2012; Zenon et al. 2014; Cantres-Rosario et al. 2015; Zenón et al. 2015). The MDM cultures with decreased cathepsin B secretion levels after HIV infection observed in our experiments have captured our attention since they also have low levels of ROS/RNS after HIV infection. Since we do not differentiate MDM cultures towards a classical (M1) or alternative (M2) activation states using macrophage colony stimulation factors (i.e. M-CSF or GM-CSF), these differences in activation states may account for the phenotypic differences observed in our cultures. It is known that differentiation with GM-CSF, LPS, and IFN- γ induces an M1 phenotype (pro-inflammatory), while differentiation with M-CSF and IL-4 induces an M2 phenotype (anti-inflammatory) (Fleetwood et al. 2009; Herbein and Varin 2010; Cassol et al. 2010; Eligini et al. 2015). As reviewed recently by Tan HY et al. (2016), M2 activation induces arginase-1 activity and reduces ROS and NO production. The differences in macrophage phenotype are also reflected in its morphology, as round cells express proteins typical of M1 phenotype while spindle cells express those of M2 phenotype (Eligini et al. 2015). In our macrophage cultures, we observed mixed morphologies, round and spindle, indicating distinct phenotypes. These differences in phenotypes are important since an M1 phenotype is more neurotoxic than an M2 phenotype (Ni et al. 2015). The effect of macrophage activation states on cathepsin B secretion will be studied in future experiments.

Overall, in this study not only confirmed that DMF was able to reduce HIV replication in MDM; it demonstrated that DMF decreased cathepsin B secretion, intracellular ROS/RNS levels, improved lysosomal function, and prevented HIV/MDM-induced neuronal apoptosis. These results indicate that increased cathepsin B secretion from HIV-infected MDM is a result of lysosomal dysfunction and increased oxidative stress.

Therefore, DMF represents a potential therapy against HIV-induced cathepsin B neurotoxicity and HAND.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Cell viability, HIV replication and cathepsin B secretion after DMF treatment

MDM were isolated from healthy donors, infected with HIV-1_{ADA} and treated with DMF (15, 30, 60 μ M). (a) Cell viability of MDM was assessed at 12dpi. (b) HIV-1 p24 levels were measured in MDM supernatants from 3, 6, 9, and 12dpi. Figures (a and b) are representative of four different donors (n=4). (c) Total cathepsin B levels were measured from MDM supernatants at 12dpi. This figure is representative of six different donors that showed increased cathepsin B secretion after HIV infection in vehicle-treated controls (n=6). Graphs are presented using the mean and the $\pm$ standard error of mean (SEM). Two-way ANOVA was used for statistical analyses. *p<0.05, **p<0.01, ***p<0.001 vs. vehicle control (b), ****p<0.0001

Fig. 2. Intracellular ROS/RNS after DMF treatment

Total ROS/RNS levels were measured from MDM lysates at 12dpi. Since no significant differences in cathepsin B levels were observed between untreated and vehicle-treated groups, these data were pooled into single control groups (Uninfected Pool and HIV+ Pool) for statistical analyses. Paired t-test was used to compare uninfected and HIV+ Pool groups, and unpaired t-test was used to compare HIV+ Pool vs. HIV+ DMF. Graph is presented using the mean and the $\pm$ standard error of mean (SEM). This figure is representative of data derived from three different donors ($n=3$) that showed increased cathepsin B secretion after HIV infection in either untreated and/or vehicle-treated conditions. * $p<0.05$.

Fig. 3. Lysosomal function assessment after DMF treatment

Lysosomal function was assessed using the Cytopainter Lysosomal dye, which is pH-dependent. If lysosomes are intact, and their acidic pH is undisturbed they will be visualized as red (functional lysosomes). Lack of red fluorescence will indicate lysosomal dysfunction. (a) Representative immunofluorescence images of uninfected and HIV-infected MDM untreated and treated with vehicle and DMF (30 μM). (b) Functional lysosomes were determined by normalizing red fluorescence intensity density by the number of nuclei (blue) in at least 2 fields per treatment. Paired t-tests were used for comparisons. Graph is presented using the mean and the $\pm$ standard error of mean (SEM). These figures are representative of three different donors (n=3) that showed increased cathepsin B secretion after HIV infection. * $p < 0.05$ vs. Uninfected untreated control, ^{##} $p < 0.01$ vs. HIV+ NoTx

Fig. 4. HIV/MDM-induced neuronal apoptosis after DMF treatment

Serum-free supernatants from 12dpi were used to determine the effect of DMF treatment in HIV/MDM-induced neuronal apoptosis. (a) Representative immunofluorescence images of human neuroblastoma cells that were exposed to MCM from uninfected and HIV-infected MDM that were untreated or treated with vehicle and DMF (30 μ M). Green fluorescence indicates TUNEL positive cells (apoptotic neurons), whereas nuclei stained with DAPI are shown in blue. DNase and No TdT were included as positive and negative controls, respectively. (b) Green and blue nuclei were counted in at least 3 fields per condition and results are shown as %TUNEL positive cells. These figures are representative of five different donors (n=5) that showed increased cathepsin B secretion after HIV infection. Graph is presented using the mean and the $\pm$ standard error of mean (SEM). One-way ANOVA was used for statistical analyses. *p<0.05 vs. uninfected control.