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Δ^9 -tetrahydrocannabinol treatment during human monocyte differentiation reduces macrophage susceptibility to HIV-1 infection

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Abstract

The major psychoactive component of marijuana, Δ^9 -tetrahydrocannabinol (THC), also acts to suppress inflammatory responses. Receptors for THC, CB₁, CB₂, and GPR55, are differentially expressed on multiple cell types including monocytes and macrophages, which are important modulators of inflammation *in vivo* and target cells for HIV-1 infection. Use of recreational and medicinal marijuana is increasing, but the consequences of marijuana exposure on HIV-1 infection are unclear. *Ex vivo* studies were designed to investigate effects on HIV-1 infection in macrophages exposed to THC during or following differentiation. THC treatment of primary human monocytes during differentiation reduced HIV-1 infection of subsequent macrophages by replication competent or single cycle CCR5 using viruses. In contrast, treatment of macrophages with THC immediately prior to or continuously following HIV-1 exposure failed to alter infection. Specific receptor agonists indicated that the THC effect during monocyte differentiation was mediated primarily through CB₂. THC reduced the number of p24 positive cells with little to no effect on virus production per infected cell, while quantitation of intracellular viral *gag* pinpointed the THC effect to an early event in the viral life cycle. Cells treated during differentiation with THC displayed reduced expression of CD14, CD16, and CD163 and donor dependent increases in mRNA expression of selected viral restriction factors, suggesting a fundamental alteration in phenotype. Ultimately, the mechanism of THC suppression of HIV-1 infection was traced to a reduction in cell surface HIV receptor (CD4, CCR5 and CXCR4) expression that diminished entry efficiency.

Keywords

Macrophage; THC; cannabinoid receptor; HIV; differentiation

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Introduction

Self-administered marijuana use is common and can exceed 50% among human immunodeficiency virus -1 (HIV-1) positive adolescents (2012; Nichols *et al*, 2013; Rotheram-Borus *et al*, 1997). In addition, Dronabinol, a synthetic form of delta-9-tetrahydrocannabinol (THC), the major psychoactive constituent of marijuana, is an FDA approved drug prescribed to diminish AIDS-associated weight loss (Ben Amar, 2006).

THC interacts with cells primarily via cannabinoid receptors 1 and 2 (CB₁ and CB₂, respectively). CB₁ and CB₂ are G-protein coupled seven-transmembrane molecules that normally function as receptors for endocannabinoids (Devane *et al*, 1992). More recently a third G-protein coupled receptor, GPR55, which binds THC and the endogenous ligand lysophosphatidylinositol, was identified (Henstridge *et al*, 2011). Receptor expression is complex and can be modulated by anatomical location, cell lineage, and stage of differentiation. For example, CB₁ is distributed throughout the brain (Matsuda *et al*, 1990), but is also detected on peripheral immune cells (Galiègue *et al*, 1995; Han *et al* 2009). CB₂ is predominantly expressed on immune cells (Castaneda *et al*, 2013; Galiègue *et al*, 1995; Han *et al*, 2009), but appears on microglia cells during inflammation and HIV-1 infection (Cabral and Marciano-Cabral, 2005; Ramirez *et al*, 2013). GPR55 is expressed most abundantly in brain, intestine, and spleen but is also found on macrophages (Henstridge *et al*, 2011). Monocytes express CB₂ and GPR55, but little if any CB₁, although differentiation into macrophages increases levels of CB₁ and reduces levels of CB₂ compared with monocytes (Galiègue *et al*, 1995; Han *et al*, 2009; Whyte *et al*, 2009).

THC exhibits immune suppressive and anti-inflammatory effects on murine and human cells of monocyte/macrophage lineage (Chang *et al*, 2001; Coffey *et al*, 1996; Shivers *et al*, 1994). Macrophages provide long-lived environments for HIV-1 infection, which can prime infected cells leading to hyper-responsiveness to activation. As regulators of cellular immune responses, macrophages contribute to generalized immune dysfunction associated with HIV-1 infection. Macrophage activation resulting from HIV-1 infection persists for years even after initiation of antiretroviral therapy (ART) (Wallet *et al*, 2010) and is likely a significant contributor to chronic inflammation and inflammation-related pathogenesis (Hunt, 2012).

Cannabinoids modulate a number of virus infections, in some cases enhancing pathogenesis, but in other cases inhibiting reactivation from latency. For example, THC administration in murine models enhances disease severity and duration by vaccinia virus infection (Huemer *et al*, 2011), while attenuating cellular infiltration and increasing influenza viral load (Buchweitz *et al*, 2007). Kaposi's sarcoma virus (KSV) infection of human microvascular endothelial cells is enhanced by THC treatment (Zhang *et al*, 2007), but THC inhibits KSV or Epstein Barr virus reactivation in immortalized B cells (Medveczky *et al*, 2004). *In vitro*, HIV-1 infection of human CD4⁺ T cells or macrophages is attenuated by CB₂ specific agonists (Costantino *et al*, 2012; Ramirez *et al*, 2013), while chronic THC administration to rhesus macaques prior to simian immune deficiency virus (SIV) infection suppresses IFN- γ levels in lymph nodes, attenuates SIV levels in plasma and cerebral spinal fluid, and prolongs the life of the animals (Molina *et al*, 2011a; Molina *et al*, 2011b). Although THC

appears to have a suppressive effect on virus infection in a number of studies, data can be conflicting and mechanisms of THC effect, particularly on HIV-1 infection of macrophages, are unclear.

Since, HIV-1 infected individuals use marijuana, and are thus exposed to THC, and macrophages are targets for HIV-1 infection and express cannabinoid receptors, THC may impact HIV-1 infection of macrophages, although whether the outcome is harmful or beneficial, remains unclear. Therefore, we designed a study to investigate the effects of THC *ex vivo* on macrophage susceptibility to HIV-1 infection.

Materials and Methods

Cell culture

Monocyte derived macrophages (MDM) were generated from elutriated human monocytes provided by Dr. Howard Gendelman at the University of Nebraska (Gendelman *et al*, 1988) or Dr. Mark Wallet at the University of Florida. Use of human cells was approved by the institutional review boards of the University of Nebraska and University of Florida. MDM were cultured as previously described (Wallet *et al*, 2012).

THC treatment

THC (Sigma Aldrich, St Louis, MO) was obtained under the Federal Drug Enforcement Administration (DEA) license of our collaborator Dr. Bruce Goldberger at the University of Florida. Vehicle control for THC is ethanol (ETOH). Cannabinoid receptor specific agonists, JWH-133, ACEA, and O-1602, were obtained from Tocris (Bristol, UK). Vehicle control for JWH-133 is Tocrisolve (Tocris, Bristol, UK); for ACEA, ETOH; for O-1602, methyl acetate. Cells were exposed to THC using 3 different treatment protocols (Figure 1). In treatment 1, monocytes received THC or vehicle control on days -7 and -5 concurrent with MCSF treatment during differentiation into MDM. In treatment 2, MDM were differentiated and treated with THC or vehicle control 30 minutes prior to infection. Finally, in treatment 3, MDM were treated with THC or vehicle control on post infection days 1, 4, and 7. Toxicity of THC or cannabinoid receptor agonists was evaluated by MTS viability assays; briefly, MDM were incubated with CellTiter 96 Aqueous One Solution (Promega, Madison, WI) for 3 hours and absorbance measured at a wavelength of 490 nm using a universal microplate reader (BioTek Instrument Inc, Winooski, VT). Cell viability after THC treatments was 92-105% compared to untreated or vehicle treated cells.

HIV-1 infection of macrophages

Virus stocks of the CCR5 using, macrophage tropic, replication competent HIV-1_{AD} were prepared by transfecting plasmid DNA from a molecular clone into 293T cells and titered as described previously (Theodore *et al*, 1996). MDMs were infected for 24 hours with HIV-1_{AD} (MOI = 0.005). On post-infection day (PID) 1 media was removed, cells washed with PBS, and fresh media added. Supernatants were collected on PID 4, 7, and 10 and levels of p24 antigen were assessed using p24 ELISA kits (Sino Biological Inc., Beijing, China). Single cycle, luciferase-tagged HIV-1_{JRFL} envelope pseudotyped virus particles were produced as previously described, titered on TZM-bl cells (Tuttle *et al*, 2002), and

used to infect MDM for 24 hours (MOI = 0.0005). On PID 1, media was removed, cells washed with PBS, and fresh media added. On PID 4, media was removed, cells lysed, and luciferase activity was determined as relative light units (RLU) using the luciferase assay system (Promega, Madison, WI) and the microplate luminometer Monolight 3096 (BD Biosciences, San Jose, CA).

Flow cytometry

Macrophages were differentiated in UpCell tissue culture plates (Thermo Scientific Nunc, Rochester, NY). After indicated times, cells were released from plates according to manufacturer's protocol and stained in 0.5% BSA and 1 mM EDTA in PBS using the following antibodies, anti-CCR5 APC, anti-CXCR4 PE, anti-CD14 Pacific Blue, anti-CD16 PE-Cy7 (BD Biosciences, San Jose, CA), anti-CD4 FITC, anti-CD163 APC (ebioscience, San Diego CA), or anti-p24 PE antibody (KC57-RD1) (Beckman Coulter Indianapolis, IN). For intracellular p24 staining, cells were permeabilized and stained using the BD Cytofix/Cytoperm kit according to manufacturer's instructions (BD Biosciences San Jose, CA). Flow cytometry was performed using a LSRII flow cytometer (BD Biosciences, San Jose, CA). Data were analyzed using Flow Jo software (Treestar, Ashland, OR).

Quantification of supernatant

Flow cytometry was used to quantitate infected cells based on the presence of intracellular p24, while picogram (pg) of p24 released into the supernatant was quantified by ELISA (Biological Inc., Beijing, China). Amount of supernatant p24 was divided by total number of infected cells in the well, and this ratio was converted to pg of p24 released per 1000 infected cells.

Quantitative Real Time PCR

Total RNA from MDM was extracted using the Ambion RNAqueous-4PCR Kit (Invitrogen, Life Technologies, Grand Island, NY) according to the manufacturer's instructions. Samples were treated with DNase 1 and RNA concentrations were quantified. cDNA was synthesized using equal concentrations of RNA and SuperScript III First-Strand Synthesis SuperMix for qRT-PCR (Invitrogen, Grand Island, NY) per manufacturer's instructions. The qRT-PCR was performed using SYBER Select Master Mix (Applied Biosystems, Life Technologies, Grand Island, NY) and primers specific for APOBEC3G, SAMHD1, or Cyclophilin A, actin (Qiagen, Germantown, MD) or TRIM5 α or Tetherin (IDT, Coralville, Iowa). Data was first normalized to the endogenous control, actin, and then expressed as a relative fold change compared to results from vehicle treated cells.

For HIV-1 gag and ApoB copies, DNA was extracted from cells 24 hours post infection by HIV-1_{AD} using the DNeasy blood and tissue kit from Qiagen (Germantown, MD) and quantified by qRT-PCR using a TaqMan assay as previously described (Coberley *et al*, 2004). All qRT-PCR was performed using the ABI 7500 FAST instrument (Applied Biosystems, Life Technologies, Grand Island, NY).

Hypermutation Analysis

Genomic DNA was isolated from MDM infected with HIV-1_{AD} using DNeasy Blood and Tissue DNA isolation kit (Qiagen, Germantown, MD). A 460 base pair portion of viral DNA, corresponding to HXB2 coordinates 2147-2610, was amplified with the following program 95°C for 2 minutes, 30 cycles of 95°C for 15 sec, 55°C for 15 sec, 45°C for 45 seconds, followed by 1 cycle of 68°C for 5 minutes using Platinum Taq (Invitrogen, Life Technologies, Grand Island, NY) and the following forward and reverse primers, CAGAGCCAACAGCCCCACCAG and CTTTGGGGCCATCCATTCCTGGC, respectively. PCR products were ligated in TOPO-TA pCR 2.1 vector (Invitrogen, Life Technologies, Grand Island, NY). Samples were transformed and colonies were grown overnight. Sanger sequencing was performed by the UF ICBR Sequencing core facility. Quality sequences [at least 50 per treatment] were aligned and hypermutation was assessed using the Hypermutter tool (Los Alamos National laboratory).

Statistical analysis

Statistical analyses were performed using Graph Pad Prism software (La Jolla, CA). One or two way ANOVA and Bonferroni's post-test or t-tests were used where appropriate as indicated in figure legends. p-values less than 0.05 were considered statistically significant. Three to six replicate wells were examined in each experiment. Each experiment was performed in cells from multiple independent donors as indicated in figure legends (Bol *et al*, 2009).

Results

THC treatment during differentiation reduces HIV-1 replication in MDM

To determine if THC modulates HIV-1 replication, monocytes or MDM were treated with THC using three different treatment schemes (described in materials and methods, Figure 1) and infected by macrophage tropic, CCR5 using, replication competent, HIV-1_{AD}. Monocytes treated during differentiation with THC at 1, 10 or 30 μ M concentrations (Treatment 1) showed a concentration dependent decrease in supernatant p24 levels relative to vehicle controls (Figure 2A). THC at 30 μ M significantly reduced, but failed to completely suppress, p24 production on days 7 or 10 of infection. Although p24 production varied amongst donors, 30 μ M THC mediated suppression ranged from 2 to 25 fold, (mean of 6 fold) on PID 7 and 10. In contrast to THC treatment during differentiation, no differences in supernatant p24 levels were detected when MDM were exposed to a single THC treatment 30 minutes prior to infection (Treatment 2) (Figure 2B) or a series of THC treatments following HIV-1 infection (Treatment 3) (Figure 2C). Results indicate that THC treatment of monocytes during differentiation subsequently altered HIV-1 infection in resultant MDM.

To determine if THC reduces initial infection or diminishes virus production per cell, the percent of infected MDM was evaluated by intracellular p24 staining using flow cytometry. Significantly fewer p24 positive macrophages were observed on PID 7 and 10 in the cells treated with THC during differentiation (Treatment 1) compared to untreated or vehicle treated cells (Figure 2G). Average percent p24⁺ reduction across experiments from 3 donors

was 3.5 fold for PID 7 and 5.5 fold on PID 10. While the percent of p24⁺ cells in untreated or vehicle treated cells increased over 15-fold from PID 4 to 10, the percent p24⁺ macrophages treated with THC during differentiation increased about 3-fold (Figure 2G). However, when infection was examined as release of p24 into the supernatant per 1000 infected cells, similar values were observed across treatments (Figure 2H). Results suggest that THC reduces the potential of cells to be infected with little to no effect on virus replication or production within an infected cell.

THC inhibits early events of HIV-1 infection in MDM

To examine first round replication, monocytes were differentiated in the presence of THC (Treatment 1) and infected by single-cycle CCR5-envelope pseudotyped viruses (HIV-1_{JRFL}). A dose dependent reduction, to a maximum of 70%, in luciferase activity was observed (Figure 2D). Across 5 donors, the average RLU reduction induced by 10 μ M THC was 2.1 fold while 30 μ M was 16 fold; in contrast, no differences were found when MDM were treated with any concentration of THC either immediately prior to (Treatment 2) or following infection (Treatment 3) (Figure 2E and 2F respectively). To examine initial infection, viral *gag* copies were quantified after 24 hours of HIV-1_{AD} infection. MDM cultures treated with 30 μ M THC during differentiation had significantly reduced *gag* copies (4 to 5 fold fewer) compared to vehicle treated cells (Figure 2I). Taken together, these data support a model of reduced cell infection that could reflect modulation of entry and/or first round replication by THC treatment during differentiation.

CB₂, but not CB₁ or GPR55, specific agonists suppresses HIV-1 replication in MDM

To determine if the THC effect on viral infection was mediated through cannabinoid receptors CB₁, CB₂, or GPR55, monocytes were differentiated into MDM in the presence of cannabinoid receptor agonists, vehicle alone, or left untreated prior to infection (Treatment 1). MDM differentiated in the presence of 10 μ M CP55,940 (pan cannabinoid receptor agonist) showed significantly lower supernatant p24 levels compared to vehicle control by days 7 and 10 post-infection (Figure 3A). MDM treated during differentiation with the CB₂ specific agonist, JWH-133, also showed a decrease in viral production, with an average reduction on day 10 post infection of 7.1 fold at the 10 μ M dose across experiments from 3 donors (Figure 3B). In contrast, MDM treated during differentiation with ACEA, a CB₁ specific agonist, or O-1602, a GPR55 specific agonist, showed no decrease in p24 levels (Figure 3C and D). Overall, results indicate that activation of the CB₂ receptor plays a significant role in suppression of HIV-1 replication in MDM treated with THC during differentiation.

Differentiation of MDMs in the presence of THC inhibits HIV-1 receptor expression

Since the inhibitory mechanism of THC treatment appears to diminish entry and/or first round replication, cell surface levels of CD4, CCR5, and CXCR4, receptors used by HIV-1 for entry, were evaluated. Compared to treatment by vehicle control or untreated cells, monocytes treated with 30 μ M THC during differentiation into MDM (Treatment 1) displayed reduced cell surface expression of each HIV-1 receptor after 7 days of differentiation (Figure 4A-C). To determine the kinetics of the THC effect on HIV-1

receptors during differentiation, cell surface expression of CD4, CCR5 and CXCR4 was evaluated over the course of differentiation. Surface expression of CD4 and CCR5 was significantly reduced after only 1 day of exposure to THC (Figure 4D and E). CD4 levels continued to decline to a maximum of approximately 50% of untreated on days 5 and 7 (Figure 4D), while maximal reduction in CCR5 expression to approximately 50% of untreated persisted over time (Figure 4E). CXCR4 surface expression was significantly reduced on days 3 and 5 of differentiation with maximal reduction at approximately 65% of untreated (Figure 4F). Overall, monocyte exposure to THC during differentiation reduced HIV-1 receptor CD4 and CCR5 cell surface protein levels, which would limit viral entry and diminish infection of macrophages.

Differentiation of MDM in the presence of THC alters cell surface marker phenotype

Since HIV receptors are modulated during monocyte to macrophage differentiation, we next examined the phenotype of macrophages differentiated from monocytes in the presence of THC. *In vivo*, the circulating monocyte/macrophage phenotype, CD14⁺ CD16⁺ CD163⁺, correlates with viral load (Fischer-Smith *et al*, 2008). Therefore, CD14, CD16 and CD163 surface expression levels were assessed and all were reduced by differentiation in the presence of THC (Treatment 1) (Figure 5A-C). To investigate the kinetics of the THC effect, cell surface expression of CD14, CD16 and CD163 was evaluated after 1, 3, 5 and 7 days of differentiation. Expression of CD14 declined over time and was significantly reduced on days 3, 5 and 7 of differentiation with a maximal reduction to approximately 45% of untreated cells (Figure 5D). Expression of CD16 and CD163 rapidly declined to approximately 30% of untreated cells, and remained suppressed over time (Figure 5E and F). The THC effect on phenotype was selective, in that cell surface receptors CD11b, ICAM-1 and intracellular marker Mac 387 were not altered by THC treatment (Figure 5 G, H and I). Results indicate that THC treatment during monocyte differentiation selectively modifies MDM phenotype.

THC treatment during differentiation results in elevated levels of viral restriction factors

Host viral restriction factors are expressed at higher levels in monocytes and downregulated during differentiation (Bergamaschi and Pancino, 2010), therefore we examined the effect of THC on mRNA levels of host cell viral restriction factors, including cyclophilin A, tetherin, TRIM5 α , SAMHD1, and APOBEC3G (Figure 6). THC treatment of differentiating macrophages (Treatment 1) produced no differences in steady state mRNA levels for cyclophilin A or tetherin. In contrast, steady state levels of APOBEC3G, SAMHD1, and TRIM5 α were increased by about 1.5 to 2.5-fold over vehicle with evident donor variability ($p < 0.05$, $p < 0.01$, $p = 0.18$ respectively). Results support alteration of MDM phenotype by THC that might restrict virus replication.

To determine if elevated APOBEC3G mRNA corresponded with increased hypermutation of HIV-1 genomes, frequency of G to A substitutions in sequences of a 400 bp region of HIV-1 that includes protease were evaluated in infected THC treated, vehicle treated or untreated macrophages. Fewer than 5% of sequences showed one or more G to A transitions, with no differences in frequency of G to A substitutions between THC or control

treated cells. In addition, less than 3% of sequences displayed an APOBEC3G hypermutation signature independent of THC treatment.

Discussion

Our study uncovered a novel inhibitory function of THC in HIV-1 infection of macrophages. Administration of THC during macrophage differentiation rendered the cells less susceptible to HIV-1 infection concomitant with a dramatic reduction in surface expression of HIV receptors CD4 and CCR5. THC treatment of monocytes primarily modifies entry steps of HIV infection, with little to no effect post entry, and modulates macrophage phenotype.

Monocyte/macrophage differentiation state alters HIV receptor expression and infection (Bergamaschi and Pancino, 2010; Tuttle *et al*, 1998). Functional receptors are required for HIV infection of MDM as anti-receptor antibodies, pharmacologic inhibitors, such as Maraviroc, and soluble CD4 severely diminish infection (O'Connell *et al*, 2013; Surdo *et al*, 2013; Tuttle *et al*, 1998). Similarly, a number of studies have demonstrated that HIV-1 receptor expression levels modulate entry kinetics on a variety of target cells, including lymphocytes and macrophages (Anderson and Akkina, 2005; Anderson *et al*, 2003; Chikere *et al*, 2013; Johnston *et al*, 2009; Keele *et al*, 2008). Despite an increase in host restriction factor mRNA, a role for THC in the control of viral infection post entry was not apparent in our study. Moreover, infected cells released similar levels of p24 into the supernatant independent of THC treatment. Results taken together led us to conclude that THC mediated reduction in HIV-infected cells is due to a reduction in HIV receptor cell surface expression.

Our study focused on CCR5-using viruses, including replication competent HIV-1_{AD} and single cycle HIV-1_{JRFL} enveloped virions, because of the persistence of macrophage tropic CCR5-using viruses throughout the course of infection. Given the importance of CD4 expression for infection and dramatic reduction in CD4 surface expression by THC, we expect that infection of macrophages differentiated in the presence of THC by a CXCR4 using virus would be similarly reduced.

Doses of THC used in our study to achieve suppression of HIV-1 infection are consistent with other publications with human cells (Shivers *et al*, 1994; Watzl *et al*, 1991; Zheng *et al*, 1992), but are nonetheless greater than THC levels detected in the circulation following marijuana use (Grotenhermen, 2003). This disparity is partially explained by our use of 10% human serum in the monocyte cultures and the findings of a number of investigators that increasing the serum concentration in the tissue culture medium shifts the THC dose response to the right (Klein *et al*, 1985; Tang *et al*, 1993). Over the past 40 years concentrations of THC in marijuana have increased more than 10 fold (Sevigny, 2013), and levels in the blood of drivers suspected of DUI have also increased (Vindenes *et al*, 2013), so previous estimates of the concentration of THC and its metabolites in the circulation may not be reflective of levels in current chronic users. Cannabinoids and their metabolites accumulate at higher concentrations in fat, spleen and lymph nodes, compared to the brain and blood after administration in animal models (Ho *et al*, 1970; Kreuz and Axelrod, 1973; Lynn and Herkenham, 1994; Ryrfeldt *et al*, 1973). Consequently, current marijuana users

may well have greater accumulation of THC in immune tissues compared to cells in circulation, which has implications for immune modulation. Ultimately ex vivo findings provide a framework to investigate the effects of marijuana use on HIV-1 infection in vivo.

The suppressive effect of exposure to THC during differentiation of MDM on HIV-1 infection is primarily mediated through CB₂, consistent with monocytes expressing CB₂, but little if any CB₁ (Galiègue *et al*, 1995; Han *et al*, 2009). In addition, CP55,940 (a full CB₁/CB₂ agonist) and THC (a partial CB₁/CB₂ agonist) appear to have the same potency in suppressing p24 (Figures 2 and 3) suggesting that THC might have non-receptor effects on the MDM as has been suggested by a number of previous studies (Newton and Klein, 2012; Springs *et al*, 2008). Suppression of HIV-1 infection using the CB₂ agonist, JWH-133, has been observed in MDM (Ramirez *et al*, 2013), although in contrast to our study with THC, expression of HIV-1 receptors was unaltered. Differences between THC and JWH-133 as CB₂ ligands, multiplicity of infection, as well as infection and treatment schemes may explain our divergent results and point to multiple mechanisms, substantiating that the differentiation state of the target cells can impact THC responses.

Treatment with a CB₁ agonist during differentiation failed to reduce infection in MDMs reflecting a lack of CB₁ expression on monocytes (Han *et al*, 2009). A role in our study for signaling via GPR55 can also be ruled out, as no decrease of infection in MDMs differentiated in the presence of a GPR55 specific agonist occurred even though cells express GPR55 (Henstridge *et al*, 2011; Whyte *et al*, 2009). Differences in downstream signaling between CB₂ and GPR55 receptors can be used to identify signaling pathways critical for THC mediated suppression of HIV infection. CB₂ receptor signaling is associated with a G_{i/o} protein, while GPR55 receptor signaling is mediated through activation of a G_α₁₃ protein (Cabral and Griffin-Thomas, 2009; Henstridge *et al*, 2011). Previous studies show that THC signaling via CB₂ and the associated G_{i/o} protein leads to activation of signaling pathways involving cAMP, release of intracellular calcium and activation of the MAPK pathway (Bouaboula *et al*, 1996; Cabral and Griffin-Thomas, 2009). CB₂ mediated MAPK activation induces EGR1, a transcriptional regulator critical for macrophage differentiation and functioning (Bouaboula *et al*, 1996; McMahon and Monroe, 1996). Although, intracellular signaling pathways facilitating suppression of HIV-1 receptors and subsequent infection remains unclear; THC treatment prevents chemotaxis of mouse peritoneal macrophages to the CCR5 ligand, CCL5, in a CB₂ dependent manner; suggesting that THC can modulate signaling or expression of CCR5 (Cabral and Griffin-Thomas, 2009; Raborn *et al*, 2008). Similar signaling interactions by THC may modulate surface expression levels of CD4 or other chemokine receptors.

Our study is especially novel in that we discovered a treatment scheme in which exposure of monocytes to a drug alters the phenotype of the resultant macrophages. Others have found phenotypic alterations of MDM derived from monocytes infected by *Mycobacterium tuberculosis* (Castaño *et al*, 2011) or treated with corticosteroids (Rinehart *et al*, 1982) or butyrate, which diminishes macrophage expression of CD14 and CD16 (Millard *et al*, 2002). Ultimately, it is conceivable that exposure to THC during differentiation may elicit global or selective changes to the cell, resulting in phenotypic reprogramming.

THC mediated reduction in CD14, CD16, and CD163 could affect HIV-1 associated neurocognitive disorders (HAND). Cells expressing high levels of these markers can be infected *in vivo* (Ellery *et al*, 2007), are associated with alterations in brain metabolism (Lentz *et al*, 2011) and migrate through the blood brain barrier (Buckner *et al*, 2011; Williams *et al*, 2012). Ex vivo, THC reduces migration of macrophages to HIV-1 proteins (Raborn and Cabral, 2010). Taken together with our results, THC exposure may reduce HIV-1 infection in the brain by modulating CD14, CD16 and CD163 expressing cells and limiting migration.

Results from our study raise the possibility that extended marijuana use might decrease macrophage susceptibility to HIV-1 infection. Chronic exposure to marijuana may also fundamentally alter macrophage differentiation, which could result in functional differences in macrophages as regulators of immune responses and contributors to immune dysfunction. Increasing approval of marijuana and its derivatives for medical indications and elevating levels of THC in recreational marijuana necessitates further studies investigating the immunological effects of marijuana use.

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Figure 1. Study Design

Cells were treated with THC in 3 different ways. Treatment 1: monocytes were treated with THC during differentiation and then infected with HIV-1 as macrophages; Treatment 2: macrophages differentiated from monocytes (MDM) were treated with THC 30 minutes prior to HIV-1 infection; Treatment 3: MDM were treated with THC on days 1, 4 and 7 following infection.

Figure 2. THC treatment of monocytes during differentiation into MDMs suppresses HIV-1 infection

A, D, G, H, I) Treatment 1; B and E) Treatment 2; C and F) Treatment 3. A-C) Cells were infected with replication competent HIV-1_{AD} and supernatant p24 was measured by ELISA. D-F) Light units were observed via luciferase assay from cells were infected with single cycle HIV-1_{JRFL} Env-pseudotyped, luciferase-tagged virus particles. G) Percent of cells expressing intracellular p24 on days 4, 7, and 10 after infection was measured by flow cytometry. H) Amount of virus production from 1000 infected cells. Calculation described in materials and methods. I) 24 hours after infection, DNA was collected and subjected to real-time PCR analysis for viral gag and ApoB. Relative gag expression is graphed. Symbols, ■ Untreated, □ Vehicle, ▲ 30 μM THC, ▼ 10 μM THC, ◆ 1 μM THC. * p<0.05, ** p<0.01, *** p<0.001 vs. vehicle control using Bonferroni's post-test. Data are means with SD for 6 wells. Graphs are of a single experiment and are representative of at least 3 donors with similar results. A is representative of 9 donors, B, C, E, F, G, and H are representative of 3 donors, D and I are representative of 5 donors.

Figure 3. CB₂ mediates suppression of HIV-1 infection

Monocytes were differentiated using Treatment 1 scheme with specific cannabinoid receptor agonists. A) pan cannabinoid receptor agonist CP55,940; B) CB₂ agonist JWH-133; C) CB₁ agonist ACEA, or D) GPR55 agonist O-1602 at 0.1, 1.0 or 10 μ M. Cells were infected with HIV-1_{AD} and supernatant p24 levels were measured after 4, 7 and 10 days of infection. Symbols, ■ Untreated, □ Vehicle, ▼ 10 μ M agonist, ◆ 1 μ M agonist, ● 0.1 μ M agonist. *** $p < 0.001$ vs. vehicle control using Bonferroni's post-test. Data are means with SD for 6 wells. Graphs are of a single experiment and are representative of 3 donors with similar results.

Figure 4. THC treatment of monocytes during differentiation into macrophages results in decreased HIV-1 receptor levels

Monocytes were differentiated into macrophages the presence of 30 μ M THC or vehicle control or left untreated (Treatment 1). A-C) After 7 days of differentiation, cells were stained for CD4, CCR5, and CXCR4 and analyzed by flow cytometry. A) CD4, B) CCR5, C) CXCR4. Figures are representative of experiments from at least 4 donors with similar results. D-F) Cells were stained on day 1, 3, 5 and 7 of differentiation, D) CD4, E) CCR5, F) CXCR4. MFI of receptor expression of vehicle and THC treated MDM were normalized to untreated cells. Data graphed are mean and SD from at least 4 donors. Dashed black lines correspond to unstained cells, solid black lines correspond to untreated cells, green lines correspond to vehicle treatment, and red lines correspond to THC treatment. No statistically significant differences in MFI between vehicle and untreated at any time point. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs. vehicle control using Bonferroni's post-test.

Figure 5. THC treatment of monocytes during differentiation into macrophages alters cell surface receptor phenotype

Monocytes were differentiated into macrophages the presence of 30 μ M THC or vehicle control or untreated (Treatment 1). A-C) After 7 days of differentiation, cells were stained for A) CD14, B) CD16, C) CD163, G) CD11b, H) ICAM-1, I) Mac 387 and analyzed by flow cytometry. Figures are a representative of experiments from at least 3 donors with similar results D-F) Cells were stained on day 1, 3, 5 and 7 of differentiation, D) CD14, E) CD16 F) CD163. MFI of receptor expression was normalized to untreated cells. Data graphed are mean and SD from at least 4 donors. No statistical differences in MFI between vehicle and untreated in any receptor. **p<0.01, ***p<0.001 vs. vehicle control using Bonferroni's post-test.

Figure 6. THC treatment during monocyte differentiation increases expression of selected viral restriction factors

Monocytes from 5 to 6 donors were differentiated for 7 days in the presence of 30 μ M THC or vehicle control. mRNA was isolated and analyzed for a panel of HIV-1 restriction factors by quantitative real-time PCR. Each symbol represents results from an individual donor. Values are graphed relative to vehicle control which is represented as a dotted line at 1. Paired t-test was used to evaluate significant differences between THC-treated and untreated cells $p < 0.05$ for APOBEC3G and SAMHD1.