CANNABINOID INHIBITION OF MACROPHAGE MIGRATION TO THE TAT PROTEIN OF HIV-1 IS LINKED TO THE CB2 CANNABINOID RECEPTOR

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RUNNING TITLE: Cannabinoids Modulate Macrophage Migration to HIV-1 Tat

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NUMBER OF TEXT PAGES: 35

NUMBER OF FIGURES: 7

NUMBER OF TABLES: 0

NUMBER OF REFERENCES: 40

NUMBER OF WORDS IN ABSTRACT: 219

NUMBER OF WORDS IN INTRODUCTION: 687

NUMBER OF WORDS IN DISCUSSION: 1486

RECOMMENDED SECTION: Inflammation, Immunopharmacology, and Asthma
LIST OF NON-STANDARD ABBREVIATIONS: 2-AG, 2-arachidonylglycerol; ACEA, N-(2-Chloroethyl)-5Z,8Z,11Z,14Z-eicosatetraenamide; AIDS, Acquired Immunodeficiency Syndrome; CB, cannabinoid receptor; CCR5, CC chemokine receptor 5; CP55940, (-)-cis-3-[2-Hydroxy-4-(1,1-dimethylheptyl)phenyl]-trans-4-(3-hydroxypropyl)cyclohexanol; CXCR4, CXC chemokine receptor 4; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFAP, glial fibrillary acidic protein; Gp120, HIV glycoprotein 120; HAD, HIV-associated dementia; HIVE, HIV-associated encephalitis; ICAM-1, intercellular adhesion molecule-1; MIP-1α/CCL3, macrophage inflammatory protein-1α, CC chemokine ligand 3; MCP-1/CCL2, monocyte chemoattractant protein-1, CC chemokine ligand 2; O-2137-2, ((1R, 3R)-1-[4-(1,1-Dimethylheptyl)-2,6-dimethoxyphenyl]-3-methylcyclohexanol); RANTES/CCL5, regulated upon activation normal T cell expressed and secreted, CC chemokine ligand 5; SR141716A, 5-(4-Chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-N-(1-piperidyl)pyrazole-3-carboxamide hydrochloride; SR144528, (1S-endo)-5-(4-Chloro-3-methylphenyl)-1-((4-methylphenyl)methyl)-N-(1,3,3-trimethylbicyclo(2.2.1)hept-2-yl)-1H-pyrazole-3-carboxamide; Tat, trans activating protein; THC, delta-9-tetrahydrocannabinol; TNF-α, tumor necrosis factor-α; VCAM-1, vascular cell adhesion molecule-1; VEGFR-1, vascular endothelial growth factor receptor-1
Abstract

Macrophages and macrophage-like cells are important targets of human immunodeficiency virus type 1 (HIV-1) infection at peripheral sites and in the CNS. Following infection, these cells secrete a plethora of toxic factors, including the viral regulatory protein Tat. This protein is highly immunogenic and also serves as a potent chemoattractant for monocytes. In the present study, the exogenous cannabinoids delta-9-tetrahydrocannabinol (THC) and CP55940 were shown to significantly inhibit migration of human U937 macrophage-like cells to the Tat protein in a concentration-related manner. The CB₁ receptor selective agonist ACEA had no effect on Tat-mediated migration. In contrast, the CB₂ receptor selective agonist O-2137 exerted a concentration-related inhibition of U937 cell migration in response to Tat. Pharmacological blockage of CB₁ receptor signaling using the antagonist SR141716A had no effect on CP55940-mediated inhibition of macrophage migration to Tat, while treatment with the CB₂ receptor antagonist SR1444528 reversed the CP55940-mediated inhibition of migration. In addition, THC had no inhibitory effect on U937 migration to Tat following siRNA knockdown of the CB₂ receptor. Collectively, the pharmacological and biochemical knockdown data indicate that cannabinoid-mediated modulation of macrophage migration to the HIV-1 Tat protein is linked to the CB₂ cannabinoid receptor. Furthermore, these results suggest that the CB₂ cannabinoid receptor has potential to serve as a therapeutic target for ablation of HIV-1 associated untoward inflammatory response.
Introduction

Macrophage-like cells are primary targets for infection by the human immunodeficiency virus type-1 (HIV-1) (Cassol et al., 2006). Once infected, these cells produce and secrete chemokines, cytokines, and other toxic factors which include the viral proteins Tat and Gp120 that collectively play critical roles in the progression of HIV infection by directly altering immune homeostasis. HIV-1 Tat (Trans Activating Factor) is a regulatory protein that is indispensible for viral replication and gene expression (reviewed in Pugliese et al., 2005). This protein, once secreted, is highly immunogenic and induces the production of pro-inflammatory cytokines and chemokines in astrocytes (El-Hage et al., 2006), monocytes (Lafrenie et al., 1997), microglia (reviewed in Minghetti et al., 2004), and T lymphocytes (Kim et al., 2004). Tat-activated monocytes have been shown to upregulate cell surface adhesion molecules including vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) (Pu et al., 2003). Additionally, it has been reported that Tat is a potent chemoattractant for monocytes (Albini et al., 1998; Mitola et al., 1997).

HIV-1 invades the Central Nervous System (CNS) presumably through the transmigration of infected peripheral leukocytes across the Blood Brain Barrier (BBB) (Buckner et al., 2006). HIV-1 infection in this compartment is associated with progressive neurological impairments including changes in cognitive and motor function, and in behavior (Lu et al., 2008). The degree of neurological impairment in HIV-associated dementia (HAD) and encephalitis (HIVE) does not correlate with viral load in the CNS, but rather with the degree of monocyte infiltration and microglial activation (Glass et al., 1993). In this context, the secretion of viral proteins from HIV-infected cells has a multiplicity of effects on neighboring cells in the CNS, ultimately
resulting in the dysregulation of normal immune homeostasis and promotion of a pro-inflammatory environment and subsequent monocyte infiltration. Both Tat and the viral envelope protein Gp120 are neurotoxic (reviewed in Pugliese et al., 2005). In a transgenic mouse model, Kim et al. (2003) demonstrated that mice expressing Tat under the control of an inducible glial fibrillary acidic protein (GFAP) promoter developed neuropathologies similar to those seen in infected patients with HAD or HIV. The combination of neuronal loss, the induction of pro-inflammatory cytokines and chemokines by glial cells, the upregulation of adhesion molecules and chemokine receptors by microglia and monocytes, and the subsequent induction of monocyte migration by Tat and other viral proteins results in profound pathophysiological symptoms commonly associated with neuroAIDS.

Opiates, cocaine, amphetamines and cannabinoids have been reported to decrease host resistance to bacteria, viruses and fungi by exerting direct, adverse effects on cellular immunity (reviewed in Cabral and Staab, 2005; Friedman et al., 2006; Pelligrino and Bayer, 1998; Yu et al., 2002). Therefore, it has been suggested that various drugs of abuse increase susceptibility to infection with HIV-1 and promote subsequent progression to AIDS (reviewed in Ugen and Nyland, 2006). This drug-related increase in HIV-1 infection seems, likely due to a combination of the intrinsic immunosuppressive properties of drugs of abuse, as well as to co-mitigating risk factors which enhance exposure to the virus (Santibanez et al., 2006).

There is a paucity of information regarding the direct effects of cannabinoids on HIV-1 infection. Recent reports indicate that cannabinoids inhibit viral expression, and downregulate CCR5, a chemokine receptor which is a co-receptor for HIV-1 entry (Rock et al., 2007). Also, cannabinoid receptor agonists have been reported to decrease significantly the permeability of
human brain microvascular endothelial cells induced by Gp120 by preventing the down-regulation of integral tight junction proteins (Lu et al., 2008). Given the importance of macrophage infiltration into the CNS in the pathology of HIV infection and that cannabinoids have been shown to have anti-inflammatory properties (reviewed in Cabral and Staab, 2005) that include inhibition of macrophage migration (Raborn et al., 2008), the effect of cannabinoids on macrophage migration to the HIV-1 protein Tat was evaluated. In the present study we demonstrate that the exogenous cannabinoids delta-9-tetrahydrocannabinol (THC) and CP55940 inhibit the migratory response of human macrophage-like cells to Tat. This inhibitory response was linked to the CB2 receptor, suggesting that this cannabinoid receptor may provide an important therapeutic target for ablating immunopathological processes associated with HIV-1 infection.
Material and Methods

Cell Culture

The human leukemic monocyte lymphoma cell line U937 was obtained from the American Type Culture Collection (ATCC CRL-1593.2). Cells were cultured in RPMI 1640 medium (Cellgro, Herndon, VA) containing 10% Fetal bovine serum and supplemented with 1% L-glutamine, 1% nonessential amino acids, 1% MEM vitamins, 0.01M HEPES and penicillin [100 U/ml]/streptomycin [100 μg/ml]/fungizone [0.25 μg/ml](Cellgro).

Drugs

Delta-9-tetrahydrocannabinol (THC; Ki = 40.7nM), a partial agonist for the cannabinoid receptors CB1 and CB2, was obtained from the National Institute on Drug Abuse (NIDA, Rockville, MD). Additional cannabinoid analogs included the CB1 and CB2 receptor full agonist CP55940 ((-)-cis-3-[2-Hydroxy-4-(1,1-dimethylheptyl)phenyl]-trans-4-(3-hydroxypropyl)cyclohexanol)(Ki = 0.92 nM) and the highly selective CB2 receptor ligand O-2137-2 ((1R, 3R)-1-[4-(1,1-Dimethylheptyl)-2,6-dimethoxyphenyl]-3-methylcyclohexanol) (CB1 Ki = 2,700nM, CB2 Ki = 11nM). The highly selective CB1 receptor agonist ACEA (N-(2-Chloroethyl)-5Z,8Z,11Z,14Z-eicosatetraenamide) (K1 = 1.4 nM) that displays > 1,400-fold selectivity over the CB2 receptor was purchased from Tocris Cookson, Inc. (Ellisville, MO). The CB1 and CB2 receptor antagonists SR141716 (5-(4-Chlorophenyl)-1-(2,4-dichloro-phenyl)-4-methyl-N-(piperidin-1-yl)-1H-pyrazole-3-carboxamide)(CB1 Ki = 2nM, CB2 Ki > 1000nM) and SR144528 ((1S-endo)-5-(4-Chloro-3-methylphenyl)-1-((4-methylphenyl)methyl)-N-(1,3,3-trimethylbicyclo(2.2.1)hept-2-yl)-1H-pyrazole-3-carboxamide)(CB1 Ki = 400nM, CB2 Ki =
0.6nM, respectively, were obtained from Sanofi Recherche (Montpellier, France). Stock solutions of cannabinoids (10^{-2}M) were prepared in 100% ethanol and stored at –20°C. Experimental concentrations were obtained by dilution of cannabinoid stock solutions in assay medium (RPMI-1640) to yield a final ethanol concentration of 0.01%. Vehicle controls consisted of 0.01% ethanol in medium for cannabinoid treatment, equal volume of sterile PBS for Tat treatments, or 0.01% ethanol plus PBS for cannabinoid/Tat co-treatments.

Tat

Recombinant human HIV-1 Tat_{1-86} protein was obtained from Immunodiagnostics, Inc. (Woburn, MA). Tat was dissolved in sterile PBS (10 µM) and frozen in aliquots at -80°C until converted to a working solution, according to the manufacturer’s recommendation. For in vitro administration, special low retention microfuge tubes (Fisher, Pittsburgh, PA) and low binding pipette tips (VWR, Suwanee, GA) were used to minimize Tat loss.

Migration Assay

Cell migration was measured using transwell inserts pre-loaded in 35 mm standard tissue culture plates (Corning Inc., Corning, NY), in which the upper and lower compartments were separated by a polycarbonate filter with 8 µm pores (Corning Inc., Corning, NY). U937 macrophages (1x10^7 /ml) were pre-incubated in RPMI 1640 lacking serum and containing vehicle (0.01% ethanol) or cannabinoid (1µM – 1nM) for 3 h at 4°C. This time regimen for drug exposure was obtained through initial optimization experiments. Serum was omitted from the culture medium since it contains lipids and other factors that have the capacity to stimulate macrophage migration that could confound interpretation of migratory responses as attributable to Tat. For
experiments using antagonists, cells were exposed to SR141716A (SR1)(1μM) or SR144528 (SR2)(1μM) for 30 min prior to treatment with CP55940 for 3h. Following vehicle or cannabinoid treatment, 100 μl of drug- or vehicle-treated cell suspension (10^6 cells) was placed in the upper chamber of the transwell insert. Tat or serum-free complete RPMI plus vehicle was included in the lower chamber of the transwell apparatus. The assembled migration plate chamber system then was incubated (2 h) at 37°C in a 5% CO₂ atmosphere. To determine the number of cells that migrated to the bottom chamber, the upper chamber (i.e., polycarbonate filter) was removed and video still images (1mm²) in five random fields of each bottom chamber were captured using an Olympus CK2 inverted microscope (Opelco, Washington, DC) with an attached XV-GP230 digital video camera (Panasonic, Yokohama, Japan) interfaced to a Dell Dimension XPS1450 computer using Videum 100 hardware and Window NT software (Winnov, Sunnyvale, CA). The number of cells migrating into the bottom compartment/transwell plate was manually enumerated and calculated as the sum of the five 1 mm² fields and was represented as cells X 5mm²/well. Each sample group was run in duplicate and each experiment was performed in triplicate. Migration for each sample group was represented as the mean (±SD) of the total number of migrating cells counted in five fields of duplicate wells. A greater than 2-fold increase in cell migration to the chemoattractant in the lower compartment as compared to that in the absence of Tat in the lower compartment was indicative of a positive response. The effective inhibitory concentration (EC₅₀) was defined as the concentration of cannabinoid that resulted in 50% reduction of the maximum migratory response. To determine the EC₅₀ for THC and CP55940, the data were normalized to percentage of maximum Tat-induced migration after subtracting the number of vehicle treated cells that migrated into the bottom well in the absence of Tat (baseline migration).
Knockdown of CB2 receptor expression using siRNA

Cellular transfection to knockdown CB2 receptor protein expression was performed using TransIT-TKO Transfection Reagent according to the manufacturer’s protocol (Mirus, Madison, WI). Briefly, 24 hours prior to transfection U937 cells (2.5x10^5) were plated in 0.25 ml complete RPMI containing 10% FBS and allowed to grow overnight at 37°C and 5% CO2. The siRNA transfection complex, formed by combining the transfection reagent (3 µl) (Mirus) and 25 or 50 nM siRNA (Qiagen, Valencia, CA; Allstars Negative control 1 or Hs_CNR2_1- target sequence TTCCGGAATCATCTACACCTA) in serum free RPMI-1640 (50 µl), was added dropwise to the cells. To verify CB2 receptor protein knockdown, cells were collected after 48h incubation at 37°C and 5% CO2 and subjected to Western immunoblot analysis using an affinity-purified anti-human CB2 receptor antibody (Nowell et al., 1998). Since 25 nM siRNA yielded the optimal suppression of CB2 receptor protein expression this concentration of siRNA was used in subsequent experiments.

Real-time Reverse Transcriptase Polymerase Chain Reaction (Real-time RT-PCR)

Real-time Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR), using SYBR Green for detection and oligonucleotide primers for the CB1 and CB2 receptor and for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was used to assess for the presence of CB1 and CB2 receptor mRNA and for constitutively expressed GAPDH mRNA, respectively. Total RNA from cells was prepared using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. The RNA then was isolated by chloroform:isopropanol extraction and resuspended in 50 µl PCR grade water. The isolated RNA was treated with RNase-free DNase I Amplification grade (Invitrogen) to remove residual genomic DNA. The reverse transcription
(RT) step was performed in a Bio-Rad iCycler (BioRad, Richmond, CA) using the SuperScript III First-Strand Synthesis System (Invitrogen) that included random hexamers as primer to generate complementary DNA (cDNA). SYBR Green real-time PCR was performed using the RT² PCR Primer Set for human CB₁ receptor (PPH01504A) or CB₂ receptor (PPH02723A) and GAPDH (PPH00150E) as described by the manufacturer (SA Bioscience Corp., Frederick, MD). Briefly, each 25 μl PCR mix consisted of 12.5 μl 2X RT² Real-Time SYBR Green PCR Master Mix (SA Biosciences), 2.0 μl first strand cDNA template, and 1.0 μl RT² PCR Primer Set brought to a final volume of 25 μl with DEPC-treated water. Tubes containing the PCR mix were placed in a SmartCycler (Cepheid, Sunnyvale, CA) and PCR was performed using the following program: 95°C, 15 min; 40 cycles of (95°C, 30 sec; 55°C, 30 sec; and 72°C, 30 sec). The resulting PCR products were visualized by electrophoresis (100V) using 4% OmniPur Agarose PCR Plus (VWR, West Chester, PA) gel in 1X Tris-Borate-EDTA (TBE) buffer. Using this approach, amplification products of 175 bp and 185 bp were generated for GAPDH and CB₂ receptor, respectively.

**SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western Immunoblotting**

U937 cells were collected and centrifuged (3000xg, 10 min, 4°C) and the pellets were resuspended in cell lysis buffer containing sterile water and a Protease Inhibitor Cocktail (100:1; Sigma, St. Louis, MO; 4-(2-aminoethyl) benzenesulfonyl fluoride, pepstatin A, E-64, bestatin, leupeptin, aprotinin). The cell lysates were subjected to 3 freeze-thaw cycles (liquid nitrogen for 1 min, 37°C water bath for 2 min, followed by thorough mixing) and the protein concentration was determined by the Bradford assay (Bradford, 1976). U937 cell lysates (30 µg/sample) were prepared in sample buffer containing sodium dodecyl sulfate (SDS) and were separated by 10%
SDS-polyacrylamide gel electrophoresis (SDS-PAGE; Bio-Rad, Hercules, CA). The protein samples were transferred to a Transblot Transfer Nitrocellulose membrane (Bio-Rad), which then was probed with affinity purified rabbit polyclonal anti-human CB₂ receptor primary antibody (rabbit polyclonal anti-human CB₂.CV)(Nowell et al., 1998)(1:100 dilution) overnight at 4°C. Membranes then were probed with goat anti-rabbit secondary antibody conjugated to horseradish peroxidase (1:5000 dilution). To verify equal loading of the samples, gels were stained with Coomassie blue following transfer of proteins to nitrocellulose membrane. In addition, duplicate membranes were probed with anti-mouse actin primary antibody (1:400)(MP Biomedicals; Solon, OH) followed by goat anti-mouse secondary antibody conjugated to horseradish peroxidase (1:5000 dilution). Protein bands were detected using Enhanced Chemiluminescence (ECL Western Immunoblotting Detection Reagents, Amersham, GE Healthcare Biosciences, Piscataway, NJ) followed by autoradiography.

Statistical Analysis

Analysis of variance (ANOVA) was performed using a Dunnett’s test. Student’s t-tests were performed to allow for comparison of each drug treated sample to the vehicle control. To assess for differences between treatment groups, a Bonferroni t-test was performed.
Results

U937 human macrophage-like cells express the cannabinoid receptor CB2

To confirm the cannabinoid receptor expression profile of the U937 macrophage-like cells, SYBR Green RT-PCR was utilized to assess for CB1 and CB2 receptor mRNA expression. A 185 bp amplicon was detected from U937 whole cell total RNA, which is consistent with the predicted fragment size for the CB2 receptor (Fig. 1A). However, expression of CB1 receptor mRNA by U937 cells was not detected by Real Time RT-PCR (Fig. 1A). Western immunoblot analysis was performed to confirm the expression of the CB2 receptor at the protein level in U937 cells exposed in vitro to THC (1 μM), Tat (50nM) or a combination of THC and Tat (4h)(Fig. 1B). No major effect on CB2 receptor protein levels was observed following any of the treatment conditions.

HIV-1 protein Tat induces U937 migration

The HIV-1 protein Tat has been shown to be a potent chemoattractant for primary monocytes isolated from human blood (Albini et al. 1998). In order to determine whether Tat also acted as a chemoattractant for U937 cells, transwell migration assays were performed. Tat significantly stimulated macrophage migration, with maximal induction observed at a concentration 50 nM (2h migration)(Fig.2). All further migration assays were performed using a concentration of 50 nM Tat as the chemoattractant factor.

Treatment with THC and CP55940 in vitro inhibits U937 migration to Tat

To evaluate whether THC had a direct effect on macrophage migration to Tat, in vitro migration experiments were performed. THC treatment significantly inhibited U937 migration to Tat.
Vehicle-treated cells exhibited a baseline level of migration (i.e., approximately 500 cells in 5 X 1 mm² fields per well) through the pores of the membrane and into the bottom compartment of the transwell apparatus in the absence of Tat. Following addition of Tat to the bottom compartment of the transwell plate, an increase in the migration of vehicle-treated cells was observed (i.e., approximately 2000 cells in 5 X 1 mm² fields per well) after 2 hours. In vitro treatment with the partial agonist THC (1μM- 100 nM) resulted in significant inhibition of U937 migration to Tat by approximately 50%. Migration experiments were replicated using CP55940, a full agonist at the CB₁ and CB₂ receptors (Fig.4). Again, in the absence of Tat a minimal level of cell migration was observed (i.e., baseline migration of approximately 500 cells in 5 X 1 mm² fields per well). When vehicle-treated U937 cells were exposed to Tat, a maximal level of migration was recorded (i.e., approximately 1900 cells in 5 X 1 mm² fields per well). Treatment of cells with CP55940 (1μM – 50 nM) resulted in a significant, concentration-related decrease in macrophage migration to Tat. Greater than 50% inhibition of migration to Tat was observed when U937 cells were treated with CP55940 at 1μM and 100nM, compared to the positive migration control of vehicle-treated cells exposed to Tat. The EC₅₀ was determined for THC and CP55940 (Fig. 4B). Treatment with CP55940 more effectively inhibited U937 migration to Tat as compared to THC, with significant inhibition occurring over a wider concentration range and a shift in the EC₅₀ of approximately one-half of a log (e.g. THC EC₅₀ ≈ 100nM and CP55940 EC₅₀≈ 30nM).

The CB₂ receptor-selective ligand O-2137 inhibits U937 migration to Tat

The concentration-related inhibitory effect of cannabinoids on U937 human macrophage-like cells to Tat implied a role for a cannabinoid receptor in this process. To investigate which
cannabinoid receptor may be linked to the inhibitory effect on macrophage migration, U937 cells were treated in vitro with synthetic cannabinoid compounds with highly selective binding to either the CB₁ or CB₂ receptor prior to assessment of migration. Treatment of macrophages with O-2137, a CB₂ receptor-selective ligand, resulted in significant, concentration-related inhibition of migration to Tat (Fig. 5A). For drug concentration ranging from 1μM to 100nM, greater than 50% inhibition of migration to Tat was observed, as compared to vehicle-treated cells. In contrast, treatment with the CB₁ receptor-selective ligand ACEA had no effect on U937 cell migration to Tat (Fig 5B).

The CB₂ receptor-specific antagonist SR2 (SR144528) reverses the inhibitory effect of CP55940 on U937 human macrophage-like cell migration to Tat

To confirm involvement of the CB₂ receptor, pharmacological blockade of cannabinoid receptor signaling was achieved using CB₁ and CB₂ receptor-specific antagonists. Treatment of U937 cells with the CB₂ receptor-specific antagonist, SR2 (SR144528) alone had no effect on macrophage migration to Tat (1μM and 10nM)(Fig. 6B). At equimolar concentrations (i.e., 1μM) of antagonist and agonist, CP55940 treatment significantly inhibited macrophage migration to Tat (approximately 37% inhibition). However, when compared to inhibition of migration observed when macrophages were treated with CP55940 (1μM) alone (approximately 58% inhibition), the addition of SR2 significantly reversed the inhibitory effect of CP55940 (Fig. 6B). At lower concentrations of CP55940 (10nM -1nM), the inhibitory effect of the agonist was completely reversed by SR2 (1μM). Thus, addition of SR2 resulted in reversal of inhibition at each CP55940 concentration tested, when compared to macrophage migration following CP55940 treatment alone.
These results were in contrast to those observed when U937 cells were pretreated with the CB1 receptor-specific antagonist, SR1 (SR141716A). Treatment with antagonist SR1 (1μM and 10nM) alone had no effect on macrophage migration to Tat (Fig. 6A). Pretreatment with SR1 did not block or reverse the inhibitory effect of CP55940 (Fig. 6A). The addition of the antagonist SR1 did not significantly alter CP55940-mediated inhibition of macrophage migration to Tat at any concentration tested.

**Knockdown of CB2 receptor expression using siRNA**

To further address the possible functional role of the CB2 receptor in inhibiting macrophage migration, an siRNA approach was used to knockdown CB2 receptor expression by U937 cells. Transient transfection (48h) of U937 cells with CB2 receptor siRNA (25nM) resulted in the down-modulation of CB2 receptor protein expression as confirmed by Western immunoblot analysis (Fig 7A).

Transfected U937 cells were treated with THC (3h) and then assessed for migration to Tat (Fig. 7B). Macrophages treated with either vehicle (TransIT TKO Transfection reagent)(Data not shown) or Negative control siRNA (Fig. 7B) exhibited a baseline level of migration (i.e., approximately 475 (Fig. 7B, top panel) and 400 cells (Fig. 7B, bottom panel) in 5 X 1 mm² fields per well, respectively) through the pores of the membrane and into the bottom compartment of the transwell apparatus in the absence of Tat. Following addition of Tat to the bottom compartment of the transwell plate, a dramatic increase in the migration of vehicle (Data not shown) or Negative control siRNA-treated cells was observed (i.e., approximately 1100 cells (Fig. 7B, top panel) and 900 cells (Fig. 7B, bottom panel) in 5 X 1 mm² fields per well) after 2 hours. Treatment of the CB2 receptor siRNA knockdown U937 cells (∆CB2) with THC had no
significant effect on macrophage migration to Tat (Fig. 7B, top panel), consistent with the CB2 receptor not being available for THC binding.
Discussion

HIV-1 infected cells produce many viral proteins, including Tat, a regulatory protein that is essential for viral replication and gene expression (reviewed in Pugliese et al., 2005). Tat has been found in sera and brain of HIV-infected individuals (Westendorp et al., 1995) and is thought to be a key factor in the development of HIV-1 associated pathologies. In vivo studies have shown that intraventricular injection of Tat causes inflammation and gliosis, similar to histological pathologies observed in brains of patients with HAD (Jones et al., 1998). Pu et al. (2003) demonstrated that intracerebral administration of Tat upregulates expression of inflammatory cytokines and adhesion molecules (MCP-1, TNF-α, VCAM-1, ICAM-1) that results in dramatic infiltration of monocytes into brains of C57Bl/6 mice. Weiss et al. (1999) indicated that Tat-induced migration of monocytes across a co-culture model of the BBB is dependent on astrocyte-derived MCP-1 (monocyte chemoattractant protein-1; CCL2). Tat also has been shown to induce a migratory phenotype in human fetal microglia, with in vitro Tat treatment resulting in actin rearrangement and membrane ruffling, key indicators of migration (Eugenin et al., 2005). Furthermore, Tat facilitated microglial migration through the autocrine induction of CCL2, suggesting that Tat causes immune cell migration indirectly through induction of astrocyte and/or microglial-derived chemokines.

Chemokines are small molecular weight cytokines that play a key role in regulating the inflammatory process by directing migration of inflammatory cells to sites of injury or infection and activating resident immune cells. These proteins function by binding specific receptors that span the cell membrane seven times and are coupled to heterotrimeric G proteins. Chemokines and their cognate receptors play integral roles in HIV-1 infection and progression of disease. For
example, the chemokine receptors CCR5 and CXCR4 are major co-receptors for HIV-1 entry into host cells (Dragic et al., 1996). It has been reported that chemokine levels in brain and sera of HIV-1 infected individuals are increased (Conant et al., 1998).

Tat contains a “chemokine-like” region (Cys-Cys-Phe sequence), characteristic of many β-chemokines (Albini et al., 1998). Albini et al. reported (1998) that Tat-induced monocyte migration and Ca\(^{2+}\) fluxes appear to occur primarily through G\(_i\) protein-coupled receptors, as both were sensitive to treatment with pertussis toxin. Furthermore, cross-desensitization experiments demonstrated that Tat displaces binding of the β-chemokines MCP-1, MCP-3, and eotaxin, indicating that monocyte migration to Tat may be due to signaling through the chemokine receptors CCR2 or CCR3 (Albini et al., 1998). Therefore, it seems likely that Tat also may directly induce monocyte migration, perhaps through chemokine receptor signaling. Reports also indicate that Tat induces monocyte migration through the activation of Vascular Endothelial Growth Factor Receptor -1 (VEGFR-1) (Mitola et al., 1997). Additionally, it was demonstrated that Tat signals through VEGFR-2 to mediate endothelial cell migration and angiogenesis, suggesting that Tat interacts with VEGF receptors as a result of homology within the basic region of Tat (amino acids 42-64) to the basic sequence of the growth factor family of proteins (Fibroblast growth factor, vascular-endothelial growth factor, hepatocyte growth factor, and heparin binding growth factor) (Albini et al., 1996; Mitola et al., 1997). Thus, Tat may be capable of inducing the migration of multiple cell types through the interaction of different domains of the protein with various cellular receptors.

The U937 promonocytic cell line has been used extensively as an in vitro model of HIV-1 infection and upon differentiation into a macrophage-like phenotype is permissive for HIV-1
infection (Cassol et al., 2006). Comparable to human monocytes, these cells express cannabinoid receptor CB2 (Galiegue et al., 1995; Maccarrone et al., 2000) and upregulate cytokine expression upon productive infection with HIV (Mengozzi et al., 1999) or exposure to exogenous recombinant Tat protein (Leghmari et al., 2008). In this study we demonstrate that Tat induces a migratory response in U937 human macrophage-like cells but whether this migration is the result of autocrine chemokine release or direct chemokine receptor activation is not known. Zella et al. (1998) reported that U937 cells express multiple chemokine receptors, including CCR1, CCR3, CCR5, and CXCR4, of which CCR1, CCR3, and CCR5 can be upregulated upon macrophage activation. In this context, Tat has been shown to upregulate CCR5 expression on primary human monocytes, but not to alter CXCR4 or CCR2 expression (Weiss et al., 1999).

THC, the major psychoactive component in marijuana, has been shown to inhibit macrophage phagocytosis and antigen processing and to alter production of cytokines and chemokines (reviewed in Cabral and Staab, 2005). THC, and other exogenous cannabinoids, also inhibits the macrophage migratory response to a variety of chemical stimuli, primarily through the activation of the CB2 receptor (Jordá et al., 2002). We have reported that THC and select cannabinoids activate the CB2 receptor to inhibit the migration of murine peritoneal macrophages to the chemokine CCL5 (RANTES)(Raborn et al. 2008). Similarly, Montecucco et al. (2008) reported that the CB2 receptor agonist JWH-015 inhibited human monocyte migration to the chemokines CCL2 and CCL3 as the result of CCR1 and CCR2 downregulation. However, CB2 activation also can stimulate the migration of macrophage-like cells. For example, the endocannabinoid 2-arachidonylglycerol (2-AG) induced the migration of microglia (Walter et al., 2003) and monocytes (Montecucco et al., 2009) in a mode linked to the CB2 receptor. Thus,
endocannabinoid versus plant-derived or synthetic cannabinoid signaling through the CB2 receptor may result in differential modulation of the migratory response.

In the present study, in vitro exposure of U937 cells to the partial cannabinoid receptor agonist, THC, resulted in inhibition of macrophage migration to the Tat protein. A similar outcome was obtained for cells exposed to the full CB1/CB2 receptor agonist, CP55940. Upon comparison of the EC50 of THC and CP55940, it was found that a higher concentration of THC (100nM) was required to achieve 50% inhibition of the maximal migratory response, as compared to 30nM for CP55940. The more potent agonist CP55940 also was effective at inhibiting macrophage migration to Tat over a wider range of concentrations, with significant inhibition occurring from 1μM–50nM for CP55940-treated cells compared to 1μM-100nM for THC-treated cells. To determine if this observed inhibition of migration was linked to a cannabinoid receptor, experiments were performed utilizing cannabinoid receptor-selective ligands and cannabinoid receptor-specific antagonists to selectively target the CB1 or CB2 receptor. Treatment of U937 human macrophage-like cells in vitro with the CB2 receptor-selective agonist O-2137 resulted in a significant concentration-related inhibition of macrophage migration to Tat. In contrast, treatment of macrophages with ACEA, a CB1 receptor-selective compound, had no significant effect on migration. Experiments were replicated using the antagonists SR144528 (SR2) and SR141716A (SR1) to block CB2 or CB1 receptor signaling, respectively. Treatment of U937 cells with SR2 blocked CP55940-mediated inhibition of migration while treatment with SR1 did not. The pharmacological studies were complemented with those using U937 cells transiently transfected with siRNA to knockdown CB2 receptor expression. THC did not inhibit the migration of siRNA-transfected U937 cells in response to Tat, consistent with an absence of a sufficient number of CB2 receptors for THC binding. Collectively, the pharmacological and CB2
receptor knockdown data indicate that the cannabinoids THC and CP55940 inhibit the migration of U937 macrophage-like cells in response to Tat in a mode linked to the CB$_2$ cannabinoid receptor.

There is a paucity of information regarding the role of cannabinoids and their cognate receptors on HIV-1 infection or progression of HIV-1 disease. Esposito et al. (2002) reported that activation of the CB$_1$ receptor protects rat glioma cells from nitric oxide-mediated cell toxicity induced by the Tat protein. Rock et al. (2007) demonstrated that the CB$_2$ receptor is involved in cannabinoid-mediated inhibition of viral replication in microglial cell cultures and that cannabinoids downregulated the HIV-1 co-receptor CCR5. Cannabinoid agonists also have been reported to significantly decrease the permeability of human brain microvascular endothelial cells induced by the HIV-1 envelope glycoprotein Gp120 by preventing the down-regulation of the tight junction proteins, claudin-5 and zonula occludens-1 (ZO-1)(Lu et al., 2008). In the present study it is demonstrated that cannabinoids that activate the CB$_2$ receptor inhibit the migratory response of a human macrophage-like cell line to the HIV-1 protein Tat. Whether this inhibition is due to modulation of chemokines produced in response to Tat or to direct desensitization and/or downregulation of chemokine receptors that bind Tat has yet to be determined. Regardless of the mode of action, the inhibition of migration of uninfected macrophages towards focal sites of released Tat could be beneficial on multiple levels, as it could curtail dissemination of HIV-1 as well as dampen the over-production of macrophage/microglial-derived proinflammatory mediators released in response to Tat. Furthermore, since CB$_2$ receptor protein expression was shown to be unaffected by Tat or cannabinoid treatment, the CB$_2$ receptor has the potential to serve as a molecular target for ablating hyperinflammatory responses of macrophage-like cells while avoiding untoward
psychotropic effects due to activation of the CB₁ receptor. In conclusion, the immunosuppressive and anti-inflammatory properties of select cannabinoids may have profound therapeutic potential in moderating HIV-associated immunopathology including microglial activation, chemokine/cytokine dysregulation, and monocyte infiltration in the CNS.
References


Footnotes

This work was supported, in part, by award [DA005832] from the National Institute on Drug Abuse/National Institutes of Health.
Legends for Figures

Figure 1- U937 human macrophage-like cells express cannabinoid receptor CB2.  **A.** Products from Real time SYBR Green RT-PCR demonstrating the presence of message for the CB2 receptor (middle panel), and the absence of message for the CB1 receptor (top panel) in U937 cells. An amplification product of 185 bp was generated for the CB2 receptor. Constitutively expressed GAPDH product was used as a positive internal control (bottom panel). **B.** Western immunoblot analysis (top panel) illustrating that CB2 receptor protein levels are not altered by treatment with Tat (50 nM), THC (1μM), or THC + Tat (4h). The bottom panel designates the 43 kDa actin band on the Coomassie-stained gel used for electroblotting that served as a loading control.

Figure 2– HIV-1 protein Tat induces migration of U937 human macrophage-like cells. Migration of U937 cells to Tat (25-100 nM) was assessed in vitro (2h) using transwell tissue culture inserts. Results are representative of three experiments and are presented as the mean ±SD. ** p<0.01.

Figure 3 – Treatment in vitro with THC results in inhibition of macrophage migration to the HIV-1 Tat protein. Migration of U937 human macrophage-like cells to Tat (50 nM) was assessed using transwell migration assay following in vitro treatment (3h) with THC (1μM-10nM) or vehicle (0.01% ethanol). Statistical analysis was performed by comparing the number of migrating cells in THC treatment conditions to vehicle-treated cells exposed to Tat in the bottom chamber (positive migration control, designated by solid horizontal line). Solid box at abscissa =0 represents the baseline migratory control of vehicle treated cells exposed to serum-free
complete assay medium in the bottom chamber. Results are representative of three experiments and are presented as the mean ±SD.* p<0.05.

**Figure 4**– Treatment in vitro with CP55940 results in inhibition of macrophage migration to the HIV-1 Tat protein. **A.** Migration of U937 human macrophage-like cells to Tat (50 nM) was assessed using transwell migration assay following in vitro treatment (3h) with CP55940 (1μM-1nM) or vehicle (0.01% ethanol). Statistical analysis was performed by comparing the number of migrating cells treated with CP55940 to vehicle-treated cells exposed to Tat in the bottom chamber (positive migration control, designated by the solid horizontal line). Solid box at abcissa=0 represents the baseline migratory control of vehicle treated cells exposed to serum-free complete assay medium in the bottom chamber. Results are representative of three experiments and are presented as the mean ±SD.* p<0.05, ** p<0.01. **B.** The EC50 of THC and CP55940 was determined by subtracting the baseline migratory control from each group, followed by normalizing each drug treatment group to represent percent maximal migration designated by vehicle-treated cells exposed to Tat in the bottom chamber.

**Figure 5**– Effect of CB1 and CB2 receptor-selective agonists on macrophage migration to Tat. **A.** Treatment with the CB2 receptor-selective agonist O-2137 (3h) significantly inhibits U937 migration to Tat. Migration of U937 human macrophage-like cells to Tat (50 nM) was assessed using transwell migration assay following in vitro treatment (3h) with O-2137 (1μM-1nM) or vehicle (0.01% ethanol). Results are representative of three experiments and are presented as the mean ±SD. **B.** Treatment with the CB1 receptor-selective ligand ACEA has no effect on Tat-induced macrophage migration. Statistical analysis was performed by comparing the number of migrating cells treated with cannabinoid to vehicle-treated cells exposed to Tat in the bottom chamber.
chamber (positive migration control, designated by solid horizontal line). Solid box at abscissa=0 represents the baseline migratory control of vehicle treated cells exposed to serum-free complete assay medium in the bottom chamber. Results are representative of three experiments and are presented as the mean ±SD.* p<0.05, ** p<0.01.

Figure 6- Effect of cannabinoid receptor antagonists on migration to Tat. A. Treatment with the CB$_1$ receptor antagonist SR1 (SR141716A) has no effect on CP55940-mediated inhibition of macrophage migration to Tat. U937 macrophage-like cells were pretreated with antagonist (1μM, 30 min) before being treated with CP55940 (1μM-1nM, 3h). Migration to Tat (50 nm) was assessed using transwell migration assay (2h). Results are representative of three experiments and are presented as the mean ±SD. B. The CP55940-mediated inhibition is reversed by treatment with the CB$_2$ receptor antagonist SR2 (SR144528). Statistical analysis was performed by comparing the number of migrating cells treated with cannabinoid (●, closed circle; significant difference designated by asterisks), antagonist alone (▲, closed triangle), or cannabinoid plus antagonist (■, closed square; significant difference designated by delta) to vehicle-treated cells exposed to Tat in the bottom chamber (positive migration control, designated by solid horizontal line), as well as comparing the number of cannabinoid-treated migrating cells (●) to those pretreated with antagonist plus cannabinoid (□, SR1; ■, SR2; significant difference designated by alpha). Inverted solid triangle (▼) at abscissa=0 represents the baseline migratory control of vehicle treated cells exposed to serum-free complete assay medium in the bottom chamber. Results are representative of three experiments and are presented as the mean ±SD.*, α, δ, p<0.05; **, αα, δδ p<0.01.
Figure 7- RNA interference of the CB₂ receptor blocks THC-mediated inhibition of migration to Tat.  

A. Knockdown of CB₂ receptor protein expression. U937 cells were transfected with CB₂ receptor (ΔCB₂) or negative control (Neg) siRNA (25 nM and 50 nM) and assessed for CB₂ receptor protein expression by Western immunoblot analysis at 48 h post-transfection (top panel). Cells treated with serum-free medium (Veh) or transfection reagent (TKO) were used to establish baseline CB₂ receptor protein expression. The middle panel designates the 43 kDa actin band on the Comassie–stained gel used for electroblotting that served as a loading control. The bottom panel designates the actin band identified on the same Western immunoblot that was used to identify immunoreactive product for the CB₂ receptor protein that served as an additional loading control. 

B. Knockdown of CB₂ receptor protein expression reverses THC-mediated inhibition of macrophage migration to Tat. U937 cells were transfected (48h) with CB₂ receptor (ΔCB₂)(top panel) or negative control (Neg) siRNA (25 nM)(bottom panel) and assessed for migration to Tat (50 nM) using the transwell migration assay. Macrophages were pre-treated (3h) with THC (1μ -10nM) or vehicle (0.01% ethanol) then migration to Tat was analyzed. Statistical analysis was performed by comparing the number of migrating cells in THC treatment conditions to vehicle-treated cells exposed to Tat in the bottom chamber (positive migration control, designated by solid horizontal line). Solid box at abscissa=0 represents the baseline migratory control of vehicle treated cells exposed to serum-free complete assay medium in the bottom chamber. Results are representative of three experiments and are presented as the mean ±SD.
Fig. 4

A

Number of cells (cells x 5mm²/well) vs. CP55940 concentration (nM).

B

Percent maximal migration vs. cannabinoid concentration (nM) for CP55940 and THC.
Fig. 5

A

![Graph A](image)

B

![Graph B](image)