

Cannabinoid Inhibition of Macrophage Migration to the Trans-Activating (Tat) Protein of HIV-1 Is Linked to the CB₂ Cannabinoid Receptor

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ABSTRACT

Macrophages and macrophage-like cells are important targets of HIV-1 infection at peripheral sites and in the central nervous system. After infection, these cells secrete a plethora of toxic factors, including the viral regulatory trans-activating protein (Tat). This protein is highly immunogenic and also serves as a potent chemoattractant for monocytes. In the present study, the exogenous cannabinoids δ -9-tetrahydrocannabinol (THC) and (-)-*cis*-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-trans-4-(3-hydroxypropyl)cyclohexanol (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 *N*-(2-chloroethyl)-5Z,8Z,11Z,14Z-eicosatetraenamide (ACEA) had no effect on Tat-mediated migration. In contrast, the CB₂ receptor-selective agonist (1*R*,3*R*)-1-[4-(1,1-dimethylheptyl)-2,6-dimethoxyphenyl]-3-methylcyclohexanol (O-2137) exerted a concentration-related inhibition of U937 cell migration in response to Tat. Pharmacological blockage of CB₁ receptor signaling using

the antagonist 5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-*N*-(1-piperidyl)pyrazole-3-carboxamide hydrochloride (SR141716A) had no effect on CP55940-mediated inhibition of macrophage migration to Tat, whereas treatment with the CB₂ receptor antagonist (1*S*-endo)-5-(4-chloro-3-methylphenyl)-1-((4-methylphenyl)methyl)-*N*-(1,3,3-trimethylbicyclo(2.2.1)hept-2-yl)-1*H*-pyrazole-3-carboxamide (SR144528) reversed the CP55940-mediated inhibition of migration. In addition, THC had no inhibitory effect on U937 migration to Tat after small interfering RNA 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.

Macrophage-like cells are primary targets for infection by HIV-1 (Cassol et al., 2006). Once infected, these cells produce and secrete chemokines, cytokines, and other toxic factors, including the viral trans-activating protein (Tat) and HIV glycoprotein 120 (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 indispensable for viral replication

and gene expression (reviewed in Pugliese et al., 2005). This protein, once secreted, is highly immunogenic and induces the production of proinflammatory 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 up-regulate cell surface adhesion molecules, including vascular cell adhesion molecule-1 and intercellular adhesion molecule-1 (Pu et al., 2003). In addition, it has been reported that Tat is a potent chemoattractant for monocytes (Mitola et al., 1997; Albini et al., 1998).

HIV-1 invades the central nervous system (CNS) presumably through the transmigration of infected peripheral

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ABBREVIATIONS: Tat, trans-activating protein; Gp120, HIV glycoprotein 120; CNS, central nervous system; HAD, HIV-associated dementia; CCR5, CC chemokine receptor 5; THC, δ -9-tetrahydrocannabinol; CP55940, (-)-*cis*-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-trans-4-(3-hydroxypropyl)cyclohexanol; O-2137, (1*R*,3*R*)-1-[4-(1,1-dimethylheptyl)-2,6-dimethoxyphenyl]-3-methylcyclohexanol; ACEA, *N*-(2-chloroethyl)-5Z,8Z,11Z,14Z-eicosatetraenamide; SR141716A/SR1, 5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-*N*-(1-piperidyl)pyrazole-3-carboxamide hydrochloride; SR144528/SR2, (1*S*-endo)-5-(4-chloro-3-methylphenyl)-1-((4-methylphenyl)methyl)-*N*-(1,3,3-trimethylbicyclo(2.2.1)hept-2-yl)-1*H*-pyrazole-3-carboxamide; PBS, phosphate-buffered saline; siRNA, small interfering RNA; RT-PCR, reverse transcriptase-polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; bp, base pair; MCP, monocyte chemoattractant protein; CCL2, CC chemokine ligand 2; CXCR4, CXC chemokine receptor 4; VEGFR, vascular endothelial growth factor receptor; JWH-015, (2-methyl-1-propyl-1*H*-indol-3-yl)-1-naphthalenylmethanone.

leukocytes across the blood-brain barrier (Buckner et al., 2006). HIV-1 infection in this compartment is associated with progressive neurological impairments, including changes in cognitive and motor function and behavior (Lu et al., 2008). The degree of neurological impairment in HIV-associated dementia (HAD) and encephalitis 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 proinflammatory 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) showed that mice expressing Tat under the control of an inducible glial fibrillary acidic protein promoter developed neuropathologies similar to those seen in infected patients with HAD or HIV-associated encephalitis. The combination of neuronal loss, the induction of proinflammatory cytokines and chemokines by glial cells, the up-regulation 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 (Pellegrino and Bayer, 1998; Yu et al., 2002; reviewed in Cabral and Staab, 2005; Friedman et al., 2006). 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 as a result of a combination of the intrinsic immunosuppressive properties of drugs of abuse, as well as comitigating risk factors that 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 down-regulate CC chemokine receptor 5 (CCR5), a chemokine receptor that is a coreceptor for HIV-1 entry (Rock et al., 2007). In addition, 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 show that the exogenous cannabinoids δ -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.

Materials and Methods

Cell Culture. The human leukemic monocyte lymphoma cell line U937 was obtained from the American Type Culture Collection (CRL-1593.2; Manassas, VA). 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% minimal essential medium vitamins, 0.01 M HEPES, and penicillin (100 U/ml)/streptomycin (100 μ g/ml)/fungizone (0.25 μ g/ml) (Cellgro).

Drugs. THC ($K_i = 40.7$ nM), a partial agonist for the cannabinoid receptors CB₁ and CB₂, was obtained from the National Institute on Drug Abuse (Rockville, MD). Additional cannabinoid analogs included the CB₁ and CB₂ receptor full agonist CP55940 ($K_i = 0.92$ nM) and the highly selective CB₂ receptor ligand O-2137 (CB₁ $K_i = 2700$ nM; CB₂ $K_i = 11$ nM). The highly selective CB₁ receptor agonist ACEA ($K_i = 1.4$ nM) that displays >1400-fold selectivity over the CB₂ receptor was purchased from Tocris Bioscience (Ellisville, MO). The CB₁ and CB₂ receptor antagonists SR141716 (CB₁ $K_i = 2$ nM; CB₂ $K_i >1000$ nM) and SR144528 (CB₁ $K_i = 400$ nM, CB₂ $K_i = 0.6$ nM), respectively, were obtained from sanofi-aventis (Bridgewater, NJ). 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 medium) 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 phosphate-buffered saline (PBS) for Tat treatments, or 0.01% ethanol plus PBS for cannabinoid/Tat cotreatments.

Tat. Recombinant human HIV-1 Tat₁₋₈₆ protein was obtained from Immunodiagnosics, Inc. (Woburn, MA). Tat was dissolved in sterile PBS (10 μ M) 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 (Thermo Fisher Scientific, Waltham, MA) and low-binding pipette tips (VWR, West Chester, PA) were used to minimize Tat loss.

Migration Assay. Cell migration was measured using Transwell inserts preloaded in 35-mm standard tissue culture plates (Corning Life Sciences, Lowell, MA), in which the top and bottom compartments were separated by a polycarbonate filter with 8- μ m pores (Corning Life Sciences). U937 macrophages (1×10^7 /ml) were preincubated in RPMI 1640 medium lacking serum and containing vehicle (0.01% ethanol) or cannabinoid (1 μ M–1 nM) 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 because 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) (1 μ M) or SR144528 (SR2) (1 μ M) for 30 min before treatment with CP55940 for 3 h. After vehicle or cannabinoid treatment, 100 μ l of drug- or vehicle-treated cell suspension (10^6 cells) was placed in the top chamber of the Transwell insert. Tat or serum-free complete RPMI 1640 medium 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 top chamber (i.e., polycarbonate filter) was removed, and video still images (1 mm²) in five random fields of each bottom chamber were captured using an Olympus CK2 inverted microscope (Olympus America, Center Valley, PA) with an attached XV-GP230 digital video camera (Panasonic, Yokohama, Japan) interfaced to a Dell (Round Rock, TX) Dimension XPS1450 computer using Videum 100 hardware (Winnov, Sunnyvale, CA) and Windows NT software (Mi-

crossoft, Redmond, WA). 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 \times 5 mm²/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 (\pm S.D.) 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 bottom compartment compared with that in the absence of Tat in the bottom compartment indicated 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 Small Interfering RNA. Cellular transfection to knockdown CB₂ receptor protein expression was performed using TransIT-TKO Transfection Reagent according to the manufacturer's protocol (Mirus, Madison, WI). In brief, 24 h before transfection U937 cells (2.5×10^5) were plated in 0.25 ml of complete RPMI 1640 medium containing 10% fetal bovine serum and allowed to grow overnight at 37°C and 5% CO₂. The small interfering RNA (siRNA) transfection complex, formed by combining the transfection reagent (3 μ l) (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 medium (50 μ l), was added dropwise to the cells. To verify CB₂ receptor protein knockdown, cells were collected after 48-h 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). Because 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 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, Carlsbad, CA) according to the manufacturer's instructions. The RNA then was isolated by chloroform/isopropanol extraction and resuspended in 50 μ l of PCR-grade water. The isolated RNA was treated with RNase-free DNase I Amplification Grade (Invitrogen) to remove residual genomic DNA. The reverse transcription step was performed in a Bio-Rad Laboratories (Hercules, CA) iCycler 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). In brief, each 25 μ l of PCR mix consisted of 12.5 μ l of 2 \times RT² Real-Time SYBR Green PCR Master Mix (SA Biosciences), 2.0 μ l of first-strand cDNA template, and 1.0 μ l of RT² PCR Primer Set brought to a final volume of 25 μ l with diethyl pyrocarbonate-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 and 40 cycles of 95°C, 30 s; 55°C, 30 s; and 72°C, 30 s. The resulting PCR products were visualized by electrophoresis (100 V) using 4% OmniPur Agarose PCR Plus (VWR) gel in 1 \times Tris/borate/EDTA buffer. Using this approach, amplification products of 175 base pairs (bp) and 185 bp were generated for GAPDH and CB₂ receptor, respectively.

SDS-Polyacrylamide Gel Electrophoresis and Western Immunoblotting. U937 cells were collected and centrifuged (3000g, 10

min, 4°C), and the pellets were resuspended in cell lysis buffer containing sterile water and a protease inhibitor mixture [100:1; 4-(2-aminoethyl) benzenesulfonyl fluoride, pepstatin A, E-64, bestatin, leupeptin, and aprotinin; Sigma-Aldrich, St. Louis, MO]. The cell lysates were subjected to three 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 μ g/sample) were prepared in sample buffer containing SDS and were separated by 10% SDS-polyacrylamide gel electrophoresis (Bio-Rad Laboratories). The protein samples were transferred to a Transblot transfer nitrocellulose membrane (Bio-Rad Laboratories), which then was probed with affinity-purified rabbit polyclonal antibody to a human CB₂ receptor peptide (rabbit polyclonal CB₂CV; 1:100 dilution) (Nowell et al., 1998) 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 after 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; GE Healthcare, Little Chalfont, Buckinghamshire, UK) followed by autoradiography.

Statistical Analysis. Analysis of variance was performed using a Dunnett's test. Student's *t* tests were performed to allow for comparison of each drug-treated sample with 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 used 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 μ M), Tat (50 nM), or a combination of THC and Tat (4 h) (Fig. 1B). No major effect on CB₂ receptor protein levels was observed after 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). 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 of 50 nM (2-h 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 (Fig. 3). Vehicle-treated cells exhibited a baseline level of migration (i.e., approximately 500 cells in 5 \times 1-mm² fields/well) through the

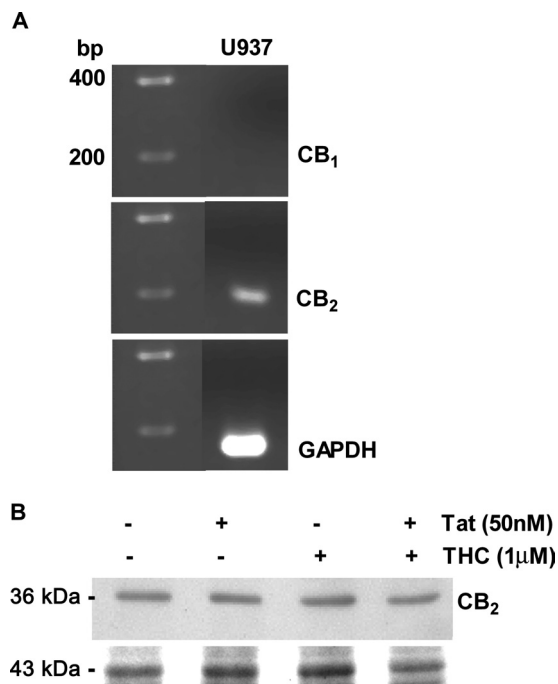


Fig. 1. U937 human macrophage-like cells express cannabinoid receptor CB₂. **A**, products from real-time SYBR Green RT-PCR showing the presence of message for the CB₂ receptor (middle) and the absence of message for the CB₁ receptor (top) in U937 cells. An amplification product of 185 bp was generated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

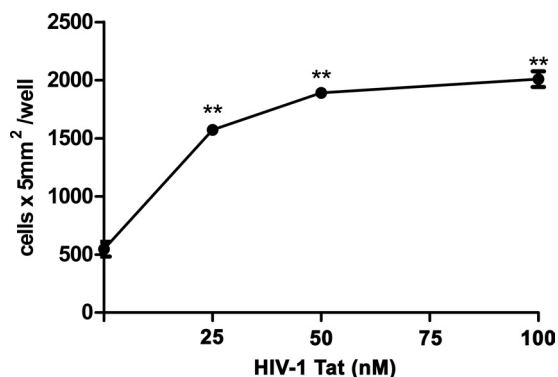


Fig. 2. HIV-1 protein Tat induces migration of U937 human macrophage-like cells. Migration of U937 cells to Tat (25–100 nM) was assessed in vitro (2 h) using Transwell tissue culture inserts. Results are representative of three experiments and are presented as the mean ± S.D. **, $p < 0.01$.

pores of the membrane and into the bottom compartment of the Transwell apparatus in the absence of Tat. After 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 $5 \times 1\text{-mm}^2$ fields/well) after 2 h. In vitro treatment with the partial agonist THC (1 μM–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 $5 \times 1\text{-mm}^2$

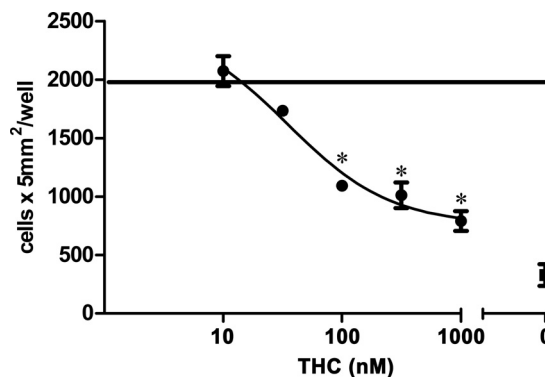


Fig. 3. Treatment in vitro with THC results in inhibition of macrophage migration to the HIV-1 Tat protein. Migration of U937 human macrophage-like cells to Tat (50 nM) was assessed using Transwell migration assay after in vitro treatment (3 h) with THC (1 μM–10 nM) or vehicle (0.01% ethanol). Statistical analysis was performed by comparing the number of migrating cells in THC treatment conditions with 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 ± S.D. *, $p < 0.05$.

fields/well). When vehicle-treated U937 cells were exposed to Tat, a maximal level of migration was recorded (i.e., approximately 1900 cells in $5 \times 1\text{-mm}^2$ fields/well). Treatment of cells with CP55940 (1 μM–50 nM) resulted in a significant, concentration-related decrease in macrophage migration to Tat. More than 50% inhibition of migration to Tat was observed when U937 cells were treated with CP55940 at 1 μM and 100 nM compared with 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 compared with 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 ≈ 100 nM and CP55940 EC₅₀ ≈ 30 nM).

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 before 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 1 μM to 100 nM, more than 50% inhibition of migration to Tat was observed compared with 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 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

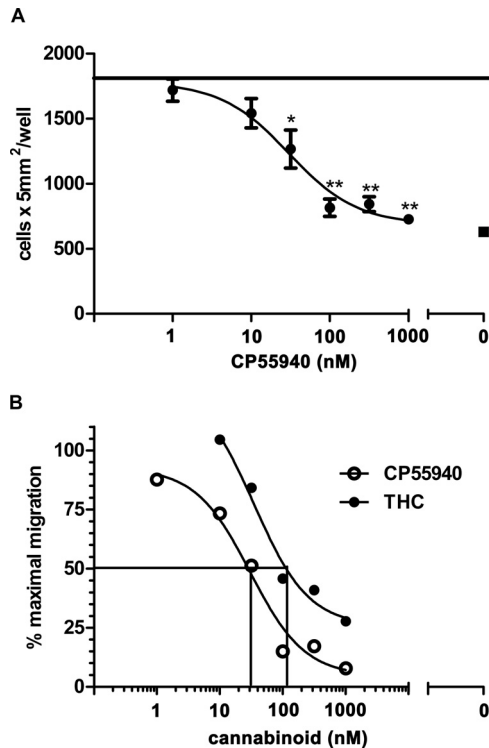


Fig. 4. Treatment in vitro with CP55940 results in inhibition of macrophage migration to the HIV-1 Tat protein. A, migration of U937 human macrophage-like cells to Tat (50 nM) was assessed using Transwell migration assay after in vitro treatment (3 h) with CP55940 (1 μ M–1 nM) or vehicle (0.01% ethanol). 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 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 \pm S.D. *, $p < 0.05$; **, $p < 0.01$. 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 percentage of maximal migration designated by vehicle-treated cells exposed to Tat in the bottom chamber.

had no effect on macrophage migration to Tat (1 μ M and 10 nM) (Fig. 6B). At equimolar concentrations (i.e., 1 μ M) of antagonist and agonist, CP55940 treatment significantly inhibited macrophage migration to Tat (approximately 37% inhibition). However, compared with inhibition of migration observed when macrophages were treated with CP55940 (1 μ M) alone (approximately 58% inhibition), the addition of SR2 significantly reversed the inhibitory effect of CP55940 (Fig. 6B). At lower concentrations of CP55940 (10 nM–1 nM), the inhibitory effect of the agonist was completely reversed by SR2 (1 μ M). Thus, addition of SR2 resulted in reversal of inhibition at each CP55940 concentration tested compared with macrophage migration after CP55940 treatment alone.

These results were in contrast to those observed when U937 cells were pretreated with the CB₁ receptor-specific antagonist, SR1. Treatment with antagonist SR1 (1 μ M and 10 nM) 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.

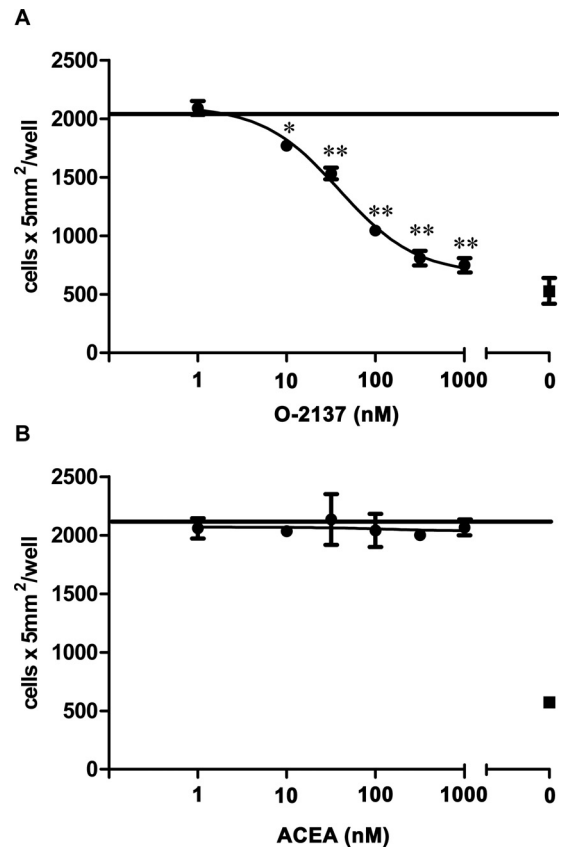


Fig. 5. Effect of CB₁ and CB₂ receptor-selective agonists on macrophage migration to Tat. A, treatment with the CB₂ receptor-selective agonist O-2137 (3 h) significantly inhibits U937 migration to Tat. Migration of U937 human macrophage-like cells to Tat (50 nM) was assessed using Transwell migration assay after in vitro treatment (3 h) with O-2137 (1 μ M–1 nM) or vehicle (0.01% ethanol). Results are representative of three experiments and are presented as the mean \pm S.D. 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 (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 \pm S.D. *, $p < 0.05$; **, $p < 0.01$.

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 knock down CB₂ receptor expression by U937 cells. Transient transfection (48 h) of U937 cells with CB₂ receptor siRNA (25 nM) 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 (3 h) and then assessed for migration to Tat (Fig. 7B). Macrophages treated with either vehicle (TransIT TKO Transfection Reagent; Mirus) (data not shown) or negative control siRNA (Fig. 7B) exhibited a baseline level of migration [i.e., approximately 475 (Fig. 7B, top) and 400 cells (Fig. 7B, bottom) in 5 \times 1-mm² fields/well, respectively] through the pores of the membrane and into the bottom compartment of the Transwell apparatus in the absence of Tat. After addition of Tat to the bottom compartment of the Transwell plate, a dramatic increase in the migration of vehicle (data not shown) or

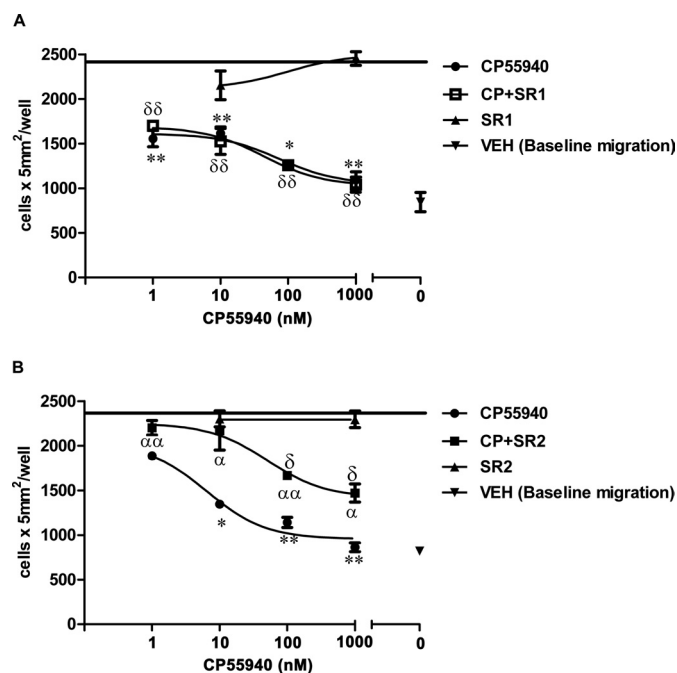


Fig. 6. Effect of cannabinoid receptor antagonists on migration to Tat. A, treatment with the CB₁ receptor antagonist SR1 has no effect on CP55940-mediated inhibition of macrophage migration to Tat. U937 macrophage-like cells were pretreated with antagonist (1 μM, 30 min) before being treated with CP55940 (1 μM–1 nM; 3 h). Migration to Tat (50 nM) was assessed using Transwell migration assay (2 h). Results are representative of three experiments and are presented as the mean ± S.D. B, the CP55940-mediated inhibition is reversed by treatment with the CB₂ receptor antagonist SR2. Statistical analysis was performed by comparing the number of migrating cells treated with cannabinoid (●; significant difference designated by asterisks), antagonist alone (▲), or cannabinoid plus antagonist (■; significant difference designated by δ) 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 (●) with those pretreated with antagonist plus cannabinoid (□, SR1; ■, SR2; significant difference designated by α). 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 ± S.D. *, α, δ, *p* < 0.05; **, αα, δδ, *p* < 0.01.

negative control siRNA-treated cells was observed [i.e., approximately 1100 cells (Fig. 7B, top) and 900 cells (Fig. 7B, bottom) in 5 × 1-mm² fields/well] after 2 h. Treatment of the CB₂ receptor siRNA knockdown U937 cells (ΔCB₂) with THC had no significant effect on macrophage migration to Tat (Fig. 7B, top), 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) showed that intracerebral administration of Tat up-regulates expression of inflammatory cytokines and adhesion molecules [monocyte chemoat-

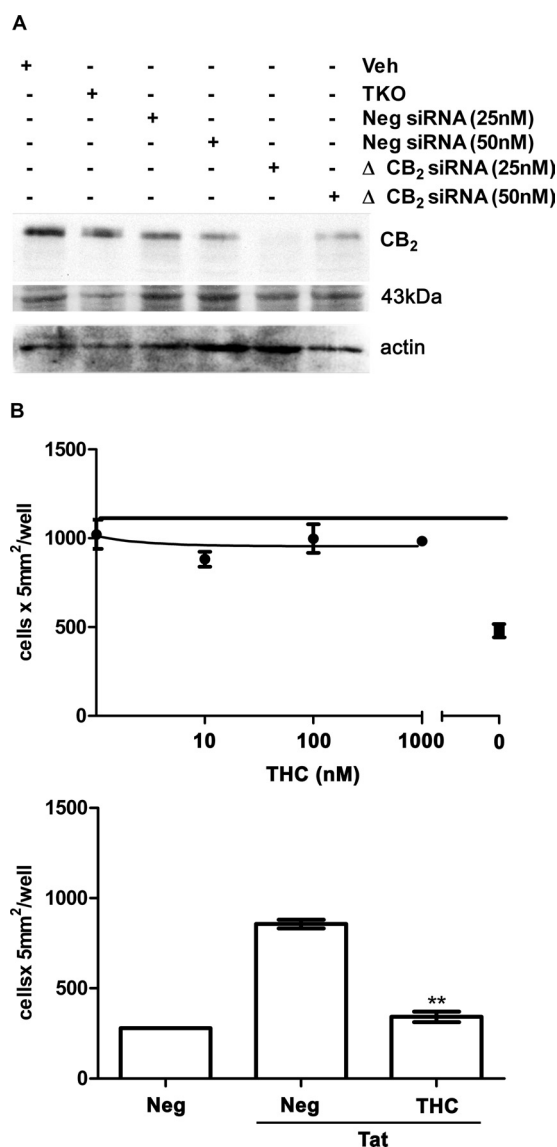


Fig. 7. RNA interference of the CB₂ receptor blocks THC-mediated inhibition of migration to Tat. A, knockdown of CB₂ receptor protein expression. U937 cells were transfected with CB₂ receptor (ΔCB₂) or negative control (Neg) siRNA (25 and 50 nM) and assessed for CB₂ receptor protein expression by Western immunoblot analysis at 48 h post-transfection (top blot). Cells treated with serum-free medium (Veh) or transfection reagent (TKO) were used to establish baseline CB₂ receptor protein expression. The middle blot designates the 43-kDa actin band on the Coomassie-stained gel used for electroblotting that served as a loading control. The bottom blot 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 (48 h) with CB₂ receptor (ΔCB₂) (top) or negative control (Neg) siRNA (25 nM) (bottom) and assessed for migration to Tat (50 nM) using the Transwell migration assay. Macrophages were pretreated (3 h) with THC (1 μM–10 nM) or vehicle (0.01% ethanol) and then migration to Tat was analyzed. Statistical analysis was performed by comparing the number of migrating cells in THC treatment conditions with 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 ± S.D.

tractant protein (MCP)-1, tumor necrosis factor-α, vascular cell adhesion molecule-1, intercellular adhesion molecule-1], which 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 coculture model of the blood-brain barrier depends on astrocyte-derived MCP-1 [CC chemokine ligand 2 (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 coreceptors 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. (1998) reported 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 showed that Tat displaces binding of the β -chemokines MCP-1, MCP-3, and eotaxin, indicating that monocyte migration to Tat may be caused by 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 (VEGFR)-1 (Mitola et al., 1997). In addition, it was shown that Tat signals through VEGFR-2 to mediate endothelial cell migration and angiogenesis, suggesting that Tat interacts with VEGFRs 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 on differentiation into a macrophage-like phenotype is permissive for HIV-1 infection (Cassol et al., 2006). Comparable with human monocytes, these cells express cannabinoid receptor CB_2 (Gallègue et al., 1995; Maccarrone et al., 2000) and up-regulate cytokine expression on productive infection with HIV (Mengozzi et al., 1999) or exposure to exogenous recombinant Tat protein (Leghmarri et al., 2008). In this study, we show 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 up-regulated on macrophage activation. In this context, Tat has been shown to up-regulate 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, as well as other exogenous cannabinoids, also inhibits the macrophage migratory response to a variety of chemical stimuli, primarily through the activation of the CB_2 receptor (Jordà et al., 2002). We have reported that THC and select cannabinoids activate the CB_2 receptor to inhibit the migration of murine peritoneal macrophages to the chemokine CCL5 (regulated on activation normal T cell expressed and secreted) (Raborn et al., 2008). Likewise, Montecucco et al. (2008) reported that the CB_2 receptor agonist (2-methyl-1-propyl-1*H*-indol-3-yl)-1-naphthalenylmethanone (JWH-015) inhibited human monocyte migration to the chemokines CCL2 and CCL3 as the result of CCR1 and CCR2 down-regulation. However, CB_2 activation also can stimulate the migration of macrophage-like cells. For example, the endocannabinoid 2-arachidonylglycerol induced the migration of microglia (Walter et al., 2003) and monocytes (Montecucco et al., 2009) in a mode linked to the CB_2 receptor. Thus, endocannabinoid versus plant-derived or synthetic cannabinoid signaling through the CB_2 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 CB_1/CB_2 receptor agonist, CP55940. On comparison of the EC_{50} of THC and CP55940, it was found that a higher concentration of THC (100 nM) was required to achieve 50% inhibition of the maximal migratory response compared with 30 nM 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 1 μM to 50 nM for CP55940-treated cells compared with 1 μM to 100 nM for THC-treated cells. To determine whether this observed inhibition of migration was linked to a cannabinoid receptor, experiments were performed using cannabinoid receptor-selective ligands and cannabinoid receptor-specific antagonists to selectively target the CB_1 or CB_2 receptor. Treatment of U937 human macrophage-like cells *in vitro* with the CB_2 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 CB_1 receptor-selective compound, had no significant effect on migration. Experiments were replicated using the antagonists SR2 and SR1 to block CB_2 or CB_1 receptor signaling, respectively. Treatment of U937 cells with SR2 blocked CP55940-mediated inhibition of migration, whereas treatment with SR1 did not. The pharmacological studies were complemented with those using U937 cells transiently transfected with siRNA to knock down CB_2 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 CB₂ 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 CB₁ receptor protects rat glioma cells from nitric oxide-mediated cell toxicity induced by the Tat protein. Rock et al. (2007) showed that the CB₂ receptor is involved in cannabinoid-mediated inhibition of viral replication in microglial cell cultures and that cannabinoids down-regulate the HIV-1 coreceptor 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 (Lu et al., 2008). In the present study it is shown that cannabinoids that activate the CB₂ receptor inhibit the migratory response of a human macrophage-like cell line to the HIV-1 protein Tat. Whether this inhibition is caused by modulation of chemokines produced in response to Tat or by direct desensitization and/or down-regulation of chemokine receptors that bind Tat has yet to be determined. Regardless of the mode of action, the inhibition of migration of uninfected macrophages toward focal sites of released Tat could be beneficial on multiple levels, as it could curtail dissemination of HIV-1 and dampen the overproduction of macrophage/microglial-derived proinflammatory mediators released in response to Tat. Furthermore, because CB₂ receptor protein expression was shown to be unaffected by Tat or cannabinoid treatment, the CB₂ receptor has the potential to serve as a molecular target for ablating hyperinflammatory responses of macrophage-like cells while avoiding untoward psychotropic effects caused by 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.

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