Attenuation of HIV-1 replication in macrophages by cannabinoid receptor 2 agonists

Servio H. Ramirez,*†,1 Nancy L. Reichenbach,* Shongsan Fan,* Slava Rom,* Steven F. Merkel,* Xu Wang,* Wen-zhe Ho,*† and Yuri Persidsky*,†,1

*Department of Pathology and Laboratory Medicine and †Center for Substance Abuse Research, Temple University School of Medicine, Philadelphia, Pennsylvania, USA

ABSTRACT

Infiltrating monocytes and macrophages play a crucial role in the progression of HIV-1 infection in the CNS. Previous studies showed that activation of the CB2 can attenuate inflammatory responses and affect HIV-1 infectivity in T cells and microglia. Here, we report that CB2 agonists can also act as immunomodulators on HIV-1-infected macrophages. First, our findings indicated the presence of elevated levels of CB2 expression on monocytes/macrophages in perivascular cuffs of postmortem HIV-1 encephalitic cases. In vitro analysis by FACS of primary human monocytes revealed a step-wise increase in CB2 surface expression in monocytes, MDMs, and HIV-1-infected MDMs. We next tested the notion that up-regulation of CB2 may allow for the use of synthetic CB2 agonist to limit HIV-1 infection. Two commercially available CB2 agonists, JWH133 and GP1a, and a resorcinol-based CB2 agonist, O-1966, were evaluated. Results from measurements of HIV-1 RT activity in the culture media of 7 day-infected cells showed a significant decrease in RT activity when the CB2 agonist was present. Furthermore, CB2 activation also partially inhibited the expression of HIV-1 pol. CB2 agonists did not modulate surface expression of CXCR4 or CCR5 detected by FACS. We speculate that these findings indicate that prevention of viral entry is not a central mechanism for CB2-mediated suppression in viral replication. However, CB2 may affect the HIV-1 replication machinery. Results from a single-round infection with the pseudotyped virus revealed a marked decrease in HIV-1 LTR activation by the CB2 ligands. Together, these results indicate that CB2 may offer a means to limit HIV-1 infection in macrophages.


Introduction

Despite immune recovery in individuals on combination antiretroviral therapy, the frequency of HAND remains high for reasons that are not well understood [1]. The current understanding is that HIV-1-associated neurodegeneration is driven by chronic inflammatory responses in the brain, secondary to a low level of HIV-1 replication in the CNS reservoir cells (macrophages, microglia) [2, 3]. Treatment approaches targeting inflammation and HIV-1 replication should be beneficial for amelioration of HAND.

Cannabis has been recognized for its psychoactive properties for centuries and more recently for medicinal activities. Cannabinoids, the primary psychoactive compounds of cannabis, act through two well-characterized receptors that generate distinct physiological effects when activated. The psychoactive effects of cannabinoids are associated with the CB1 receptor, whereas the CB2 receptor (further in the text referred as CB2) mainly mediates anti-inflammatory and immunomodulatory actions [4]. Cannabinoids have emerged recently as potential treatments for HAND. For example, cannabinoid receptor ligands are able to attenuate tissue inflammation and significantly reduce morbidity and mortality of SIV-infected macaques [5]. Medicinal cannabis reduces neurologic complications (neuropathic pain) in HIV-infected patients [6]. It has been proposed that the effects of medicinal cannabis may be associated with activation of the nonpsychoactive CB2 receptor. In fact, up-regulation of CB2 expression has been shown on perivascular macrophages/microglia, astrocytes, and brain endothelial cells in HIVE [7, 8]. Furthermore, CB2 ligands suppress HIV-1 replication in microglia, decrease microglial migration toward HIV-1 Tat, protect neurons and endothelial cells against gp120 toxicity, and diminish neuroinflammation in a rodent model of HIVE [9–13]. However, thus far, the role of CB2-selective activation in HIV-1 infection of macrophages has not been examined comprehensively.

1. Correspondence: Dept. of Pathology and Laboratory Medicine, Temple University School of Medicine, 3401 N. Broad St., Philadelphia, PA 19140, USA. E-mail: yuri.persidsky@tuhs.temple.edu or servio.ramirez@temple.edu
In this study, we explore the use of highly specific CB$_2$ receptor agonists to attenuate HIV-1 infection in macrophages. Our results indicate that there is a significant up-regulation of CB$_2$ expression upon monocyte differentiation to macrophages that is enhanced further by HIV-1 infection. Synthetic CB$_2$ receptor agonists markedly decreased HIV-1 replication (measured by RT activity). CB$_2$ activation may not appear to interfere with viral entry, as there was a lack of regulation by CB$_2$ receptor agonists on gene or surface protein expression.

MATERIALS AND METHODS

Reagents, cell culture, and HIV-1 infection

The following CB$_2$ receptor ligands were used: JWH133 ([6aR, 10aR]-3-(1,1-dimethylbutyl)-6a,7,10,1a-tetrahydro-6,6,9-trimethyl-6H-dibenzo[b,d][pyran], SR144528 [5-(4-chloro-5-methylphenyl)-1-(4-methylphenyl)methyl]-N-[(1S,2R,8R)-3,5,3-trimethyl[bicyclo(2.2.1)hept-2-yl]-1H-pyrazole-5-carboxamide],), and GP1a [N-(quinolin-1-yl)-1-(2,4-dichlorophenyl)-4,4-dihydro-6-methylindeno[1,2-c]pyrazole-3-carboxamide] were purchased from Toecis Bioscience (Ellville, MO, USA), and O-1966 {N-(1,3-dimethyl-1H-indolyl-5-yl)-4-methoxy-phenyl}-3-methyl-cyclohexanol} was acquired from Organix (Woburn, MA, USA) and synthesized as described previously [14]. Unless otherwise specified, all other reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Primary human monocytes derived from six donors were acquired from the Human Immunology Core at the University of Pennsylvania (Philadelphia, PA, USA). The facility isolates monocytes using counterflow centrifugal elutriation, as described previously [15]. The monocytes were placed in 24-well plates within 24 h of isolation. Monocytes were maintained in DMEM, supplemented with 10% heat-inactivated, pooled human serum, 1% glutamine, 50 µg/ml gentamicin, 10 µg/ml ciprofloxacin, and 1000 U/ml highly purified human rM-CSF, which was present in the medium for the first 5 days of culture to promote differentiation and was then removed. Culture medium was changed every 3 days. After 7 days in suspension culture, MDMs were infected with HIV-1$_{SAD}$ (a macrophage tropic strain; obtained from the AIDS Research and Reference Reagent Program, National Institutes of Health, Germantown, MD, USA) at a m.o.i. of 0.1 infectious virus particle/target cell. In pretreatment experiments, MDMs were exposed to the experimental treatment for 24 h before introduction of the virus. For post-treatments, the MDMs were infected with virus for 4 h, washed with DMEM, and then treated with the CB$_2$ ligand. Treatments were maintained for 7 days, with one-half medium replacement on Days 3, 5, and 7 after HIV was added to the cells. On Days 3, 5 and 7, culture medium was collected and stored at ~80°C until it was assayed for HIV-1 RT activity. The reporter cell line TZM-bl was obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases; TZM-bl cells were generated by Dr. John C. Kappes, Dr. Xiaoyun Wu, and Tranzyme (Durham, NC, USA). The facility isolates monocytes using counterflow centrifugal elutriation, as described previously [15]. The monocytes were placed in 24-well plates within 24 h of isolation. Monocytes were maintained in DMEM, supplemented with 10% heat-inactivated, pooled human serum, 1% glutamine, 50 µg/ml gentamicin, and 1000 U/ml highly purified human rM-CSF, which was present in the medium for the first 5 days of culture to promote differentiation and was then removed. Culture medium was changed every 3 days. After 7 days in suspension culture, MDMs were infected with HIV-1$_{SAD}$ (a macrophage tropic strain; obtained from the AIDS Research and Reference Reagent Program, National Institutes of Health, Germantown, MD, USA) at a m.o.i. of 0.1 infectious virus particle/target cell. In pretreatment experiments, MDMs were exposed to the experimental treatment for 24 h before introduction of the virus. For post-treatments, the MDMs were infected with virus for 4 h, washed with DMEM, and then treated with the CB$_2$ ligand. Treatments were maintained for 7 days, with one-half medium replacement on Days 3, 5, and 7 after HIV was added to the cells. On Days 3, 5 and 7, culture medium was collected and stored at ~80°C until it was assayed for HIV-1 RT activity. The reporter cell line TZM-bl was obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases; TZM-bl cells were generated by Dr. John C. Kappes, Dr. Xiaoyun Wu, and Tranzyme (Durham, NC, USA). The cells were grown in six-well plates in DMEM, 10% FBS, 100 U penicillin, and 0.1 mg/ml streptomycin. All cell-culture reagents (media, antibiotics, etc.) were purchased from Invitrogen/Life Technologies (Carlsbad, CA, USA).

Immunohistochemistry

Immunohistochemistry was performed on serial sections from postmortem paraffin-embedded brain tissue [16]. The analyzed tissue originated from frontal cortical regions from four cases of HIVE and from four seronegative, age-matched controls. The cases were provided by the National NeuroAIDS Tissue Consortium (Washington, DC, USA). With the use of standard immunohistochemistry methods, serial sections (5 µm in thickness) were generated and then baked at 65°C for 20 min, followed by deparaffinization and rehydration. The tissue was then subjected to antigen retrieval by incubating the sections at 100°C in 10 mM sodium citrate buffer (pH 6.0) for 20 min. Endogenous peroxidase activity was quenched by treating with Peroxidazed 1 (Biocare Medical, Concord, CA, USA) for 15 min. To block nonspecific antibody binding, the slides were incubated with 1% goat serum in 1X PBS and 0.1% Triton X-100 (Sigma-Aldrich). For immunolabeling, the following primary antibodies were used: polyclonal antibodies against human CB$_2$ (Thermo Scientific, Rockford, IL, USA; diluted at 1:100 with overnight incubation), polyclonal antibodies to Iba-1 (Wako Chemicals USA, Richmond, VA, USA; diluted at 1:200 with overnight incubation), and mAb to HIV-1 p24 (Clone Kal-1; Dako, Carpenteria, CA, USA; diluted at 1:10 with overnight incubation). Detection was performed with anti-mouse or anti-rabbit secondary antibodies (Biocare Medical) in combination with the Mach 3 HRP-polymer system and the 3,3’-di-aminobenzidine substrate (Biocare Medical). Normal rabbit or mouse IgG at the same concentrations as the primary antibodies served as negative controls. Counterstaining of the nuclei was performed using hematoxylin (Biocare Medical). Representative images shown in the first figure originate from previously published cases [16]. The control image is from Case Number 8, HIVE (middle) is from Case Number 2, and HIVE (bottom) is from Case Number 3. Clinical history of the cases used did not indicate treatment with antiretroviral therapy.

Real-time qPCR

Total cellular RNA was isolated from macrophages (1×10$^6$ cells) on Day 7 post-HIV infection using the RNAqueous 4PCR kit, according to the manufacturer’s directions (Ambion, Austin, TX, USA). RNA purity and concentration were determined with a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Fair Lawn, NJ, USA). Total RNA (2 µg) was subjected to RT using the high-capacity cDNA RT kit, according to the manufacturer’s directions (Applied Biosystems/Life Technologies, Carlsbad, CA, USA). The cDNA (diluted 1:20) template was then mixed with the Maxima Probe/ROX qPCR Master Mix (Fermentas, Hanover, MD, USA) and the corresponding human TaqMan gene expression assay (Applied Biosystems/Life Technologies): CNR2: Hs00361490_m1; CXCR4: Hs00607978_s1; and CCR5: Hs99999149_s1. Maxima Probe/ROX qPCR Master Mix (2%; Fermentas) was used to measure HIV-1$_{SAD}$ Pol gene expression with the following oligonucleotide primers: 5'GAATTTGGTACGAAATGGAAGAGGAAGGAGGA 3' (forward) and 5'TGAGTTCTTCTTATTAAGTCCAGAATGACCTAC 3' (reverse) and FAM-5'TGGGGCTGAAATCCATACCTGCATACATCCTCACATCGTAC 3'TAMRA (Taqman probe). All controls and samples were run in triplicate in the same culture plate. GAPDH mRNA was measured with the Taqman gene expression assay, Hs02758991_g1, and was used as a control to normalize the mRNA content in the samples tested. qPCR was performed on a StepOnePlus real-time PCR system (Applied Biosystems/Life Technologies). Raw data were analyzed with DataAssist software (Applied Biosystems/Life Technologies) using the ΔΔCt method (relative quantification). Results are expressed in relative gene expression levels (fold) compared with the untreated control or the HIV-1-only condition.

Flow cytometry

Analysis of surface expression of CB$_2$ and HIV-1 coreceptors CXCR4 and CCR5 was determined by flow cytometry. Briefly, 1×10$^6$ cells were placed in staining solution (2% BSA in PBS with 0.5% NaN$_3$) containing fluorophore-conjugated antibodies to CXCR4 (anti-CD184-allophycocyanin; BD Biosciences, Franklin Lakes, NJ, USA) and CCR5 (anti-CD195-PE; BD Biosciences) for 30 min on ice. For detection of CB$_2$ expression, the cells were incubated for 1 h with polyclonal antibodies to human CB$_2$ (Thermo Scientific), followed by detection with donkey anti-rabbit Alexa-488-conjugated antibodies. Cells were then washed and fixed in 2% methanol-free formaldehyde (Thermo Scientific) in 1X PBS. Acquisition and analysis of the labeled cells were then performed using a FACSCanto II flow cytometer (BD Biometrix) in count mode.
Acquisition parameters and gating were controlled by FACSDiva software (BD Biosciences). Data analysis was performed with FlowJo software (Tree Star, San Carlos, CA, USA). The data represent the fluorescence intensity of gated populations (as determined by isotype-matched controls) of at least 100,000 events recorded in a single experiment that was repeated at least three times.

RT assay
The HIV-1 RT assay is a radiometric assay designed for the quantitative measurement of RT activity in cell culture, as described previously [17]. This assay is used to determine the propagation of HIV-1 in infected MDMs. Please note that the RT assay (as used in this study) was not in the format used in screening for RT inhibitors, which involves challenging purified HIV-1 RT directly with the test compound. Rather, the results are representative of HIV-1 RT activity present in the medium of HIV-1-infected MDMs, untreated or treated with the indicated experimental compound at the given time-point. Briefly, 10 μl culture supernatant was mixed with 50 μl reaction mixture containing 5 μg/ml poly(A) (GE Healthcare, Piscataway, NJ, USA) and oligo dt (1.57 μg/ml; USB, Cleveland, OH, USA) in 50 mM Tris, pH 7.8, 7.5 mM KCl, 2 mM DTT, 5 mM MgCl₂, 0.05% Nonidet P-40, and 0.5 μCi [α-32P]dTTP (Perkin Elmer, Waltham, MA, USA) and incubated for 16 h at 37°C. Each reaction mixture was then spotted onto a Whatman DE81 (Thermo Fisher Scientific) paper, dried, and washed five times with 2X sodium citrate buffer (200 ml/wash) and once with 95% ethanol (150 ml) to remove unincorporated [α-32P]dTTP. Radioactivity on the air-dried filter paper was determined in a Tri-Carb 2810TR liquid scintillation spectrometer (Perkin Elmer).

Single-round HIV-1 infection and β-gal assay
Recombinant luciferase-encoding HIV-1 virions were pseudotyped with the envelope from HIV-1ADA as described previously [18]. The pseudotyped virus was used for single-round infectivity assays in T2Z-bl cells. Detection of β-gal was performed using the β-gal staining assay (Invitrogen/Life Technologies). After staining, images from each experimental condition were acquired using an Axio Observer Z1 microscope configured with an AxioCam HR camera (Carl Zeiss MicroImaging, Thornwood, NY, USA). Analysis was performed using AxioVision (v4.7) imaging software (Carl Zeiss MicroImaging). The number of stained cells was counted and averaged (three replicates) for each condition from at least three independent experiments.

Cell viability assay
After monocyte differentiation to macrophages (see above), the cells were infected with HIV-1ADA at a m.o.i. of 0.1. Two hours after the initial infection, we examined the CB2 agonist, AZT or vehicle control was added. Seven days postinfection, cell viability was assessed using calcein-AM (Invitrogen/Life Technologies), according to the manufacturer’s recommendations. The assay is based on the principle that intracellular esterase activity (in live cells) converts the nonfluorescent and cell-permeable calcein-AM into polyatomic calcein, which is retained and intensely fluorescent (excitation at ~495 nm and emission at ~515 nm). Calcein-AM was added at 1 μM, and the cells were incubated for 30 min, followed by three rinses with 1X PBS. Fluorescence emitted from live cells was measured at 495 nm (excitation)/500 nm (emission) on an M5 fluorescence plate reader (Molecular Devices, Sunnyvale, CA, USA). All measurements were performed in triplicate, and the data are represented as the percent viability from the uninfected control.

Statistical analysis
Results are presented as mean ± SD or SEM, and P values < 0.05 are considered significant. Data were analyzed using the Prism v5 software (GraphPad Software, La Jolla, CA, USA), and statistical significance was determined by performing unpaired two-tailed Student’s t-test or ANOVA with the Dunnett’s post-test. All in vitro studies were performed with cells from at least three different donors with multiple replicates (at least three).

RESULTS
Up-regulation of CB2 expression in infiltrating monocytes/macrophages in cases of HIVE
It is well established that immune cells express CB2; however, how this expression may change during HIV-1 infection remains unknown. Therefore, we first examined CB2 expression in HIV-1-infected monocytes/macrophages in perivascular cuffs present in HIVE. Autopsy tissues derived from six patients with HIVE, four HIV-positive controls, and five control seronegative patients were immunostained for CB2. Clinical, pathologic, and demographic details of these cases have been published before [16, 19]. Figure 1 shows representative images of immunostains in two HIVE cases and one HIV-1-seronegative control. HIVE cases demonstrated strong staining for CB2 in perivascular monocytes/macrophages and some microglia cells (Fig. 1B and C). Macrophages and microglial cells were detected by immunoreactivity with antibodies to Iba-1 (Fig. 1E and F). Interestingly, a number of the Iba-1-positive cells showed CB2 immunoreactivity. Immunolabeling for the HIV-1 core antigen, p24, showed variable staining in many of the CB2-positive cells (Fig. 1H and I). In addition to the monocytes/macrophages and microglia, microvascular endothelial cells featured enhanced CB2 staining in the HIVE cases (Fig. 1, compare A with B and C). In control cases, only marginal staining was observed for the endothelium and resident perivascular macrophages and rarely in microglial cells (Fig. 1A and D). None of the cells in control cases demonstrated HIV-1 p24 positivity. Also, HIV-1-positive cases (without signs of encephalitis) featured an increased number of activated microglia and macrophages with variable intensity for CB2 reactivity (data not shown). These results suggest that the increased expression of CB2 in macrophages and microglia is associated with HIV-1 infection and neuroinflammation.

Expression of CB2 in HIV-1-infected MDMs
To evaluate whether CB2 expression changes as a function of differentiation (monocyte to macrophage) and HIV-1 infection, we examined the gene and protein expression profile of CB2 in primary human monocytes. Isolated human peripheral blood monocytes from six healthy donors were used within 24 h or allowed to differentiate for 7 days by adhesion and exposure to M-CSF. Differentiated MDMs were uninfected or infected with the macrophage-tropic HIV-1ADA for 7 days. qPCR and analysis by the ΔΔCt method revealed a step-wise increase in CB2 expression with the following pattern: monocytes < MDMs < HIV-1-infected MDMs (Fig. 2A). Relative (fold regulation) expression showed MDMs with a 5.47 ± 1.07 increase over that observed for undifferentiated monocytes (set at 1). Infection of MDM with HIV-1ADA significantly increased the CB2 receptor gene expression even further, resulting in 7.41 ± 0.94-fold up-regulation compared with monocytes and a 1.35-fold increase over uninfected MDMs. With the use of a similar experimental approach, we evaluated surface expression of CB2.
CB2 by FACS analysis. With the use of antibodies for CB2, which recognize epitopes in the extracellular N-terminus segment of CB2, FACS analysis showed a similar detection pattern as observed in the gene-regulation profile. Figure 2B shows representative histograms for detection of CB2 along with MFI values. A fold induction of 1.76 in MDMs compared with monocytes was observed and a 2.28-fold increase in infected MDMs, in contrast to uninfected MDMs. Together, these results are complimentary in that the increase in gene regulation translates into an increase in CB2 protein expression. Furthermore, the analysis in primary cells appears consistent with the observations in Fig. 1, suggesting that HIV-1 infection induces further elevation in CB2 expression.

CB2 receptor agonists inhibit HIV-1
Given the anti-inflammatory role that CB2 plays in immune cells and the observations that the CB2 receptor is not only present in HIV-infected MDMs but also appears up-regulated when compared with uninfected cells (Figs. 1 and 2), we examined whether CB2 ligands may affect HIV-1 infection. Differentiated MDMs were infected with HIV-1ADA for 4 h (viral incubation period). The media were changed, and the infection was allowed to continue for 7 days (see Materials and Methods). Where indicated (Fig. 3), infected MDMs were exposed to the potent CB2 receptor agonist JWH133; $K_i$ = 1.8 nM with ~200-fold selectivity for the CB2 receptor over CB1 [20]. Performed in parallel and with the same donor, the activity of HIV-1 RT activity was measured from the culture medium of infected cells at the end of 7 days. JWH133 in the “pretreated” groups were introduced 24 h prior to infection, whereas in the “post-treated” groups, JWH133 was added following the viral incubation period (Fig. 3A, pre- and post-treated). The addition of the CB2 agonist resulted in a dose-dependent decrease of RT activity. JWH133 at 1 μM reduced
Ramirez et al. CB2 receptor agonists inhibit HIV-1 in macrophages

CB2 activation in MDMs does not affect the expression of CXCR4 or CCR5

In light of the inhibitory effect that CB2 receptor agonists have on HIV-1 RT activity, it is possible that CB2 signaling alters the expression of HIV-1 coreceptors, thereby preventing re-entry of HIV-1 virions into the cells. To test this possibility, expression profiles for CXCR4 and CCR5 were evaluated by FACS and gene expression (Fig. 4). Figure 4A and B shows representative histograms for the expression of CXCR4 and CCR5 (respectively) by FACS in MDMs that were treated with JWH133 (10 μM), GP1α (10 μM), or O-1966 (10 μM) for 24 h. As indicated, there was no change in the level of these HIV-1 coreceptors in the presence of the agonists. Interestingly, HIV-1 infection (7 days) did not change gene expression for CXCR4 (Fig. 4C) or CCR5 (Fig. 4D) in MDMs. Therefore, CB2-mediated inhibition of HIV-1 in primary human macrophages does not involve down-regulation of CXCR4 or CCR5.

CB2 agonists inhibit the activity of the HIV-1 LTR

To evaluate whether CB2 signaling may affect the activity of the HIV-1 LTR, single-round infection of the TZM-bl reporter cell line was used. Pseudotyped envelope-deficient HIV-1 virions were produced by cotransfection in 293T cells of a NL4-3 HIV-1 backbone DNA construct, along with a separate DNA construct encoding for the HIV-1 envelope. Purified, pseudotyped viruses were then introduced to TZM-bl cells, which stably express CXCR4 and CCR5 and also contain the HIV-1 viral backbone.

Figure 2. Monocyte differentiation and HIV-1 infection increase CB2 receptor expression. (A) Relative expression (RQ) of the CB2 gene was determined by real-time qPCR in monocytes, MDMs, and MDMs infected with HIV-1 (7 days postinfection at an m.o.i. of 0.1). The fold change in monocytes was assigned a value of 1.0. The results are represented as the mean value ± SD with P values shown for the indicated comparisons (brackets). (B) Primary human monocytes, differentiated MDMs, and HIV-1-infected MDMs, immunostained for CB2, were analyzed by FACS, as described (see Materials and Methods). The MFI values for surface expression are shown ± SD. Gene expression and FACS analysis were performed in replicates from at least three different donors.

The detectable RT activity in the medium by 34.6% and 68.9% when the agonist was introduced, pre- and post-treated, respectively. JWH133 at 10 μM resulted in an 82.8% decrease in RT activity in the pretreated group and similarly, when the cells were post-treated. Administration of the CB2-inverse agonist/antagonist SR144528 had no significant effect on RT activity; however, when coadministered with JWH133, it counteracted CB2 agonist effects completely (Fig. 3A). Of note, the RT inhibitor, AZT (10 μM), served as control for suppression of HIV-1 replication, thus resulting in decreased levels of RT activity in the medium of treated cells. The results also suggest that the effect of the CB2 agonist on viral replication (RT assay) may not be a result of interference with viral entry. This is surmised from the post-treated group, where the agonist was added following the 4-h virus incubation period. The results also show that the inhibition observed in the presence of the CB2 agonist requires a continued and prolonged exposure, as the effect is limited at earlier time-points (Fig. 3B). Although a downward trend was observed when JWH133 was present, RT activity at Day 3 was not reduced significantly. A similar trend was observed at Day 5, with the only significant difference (36% inhibition) apparent at the higher concentration of JWH133. Two additional synthetic CB2 agonists were also investigated, GP1α (K0 = 0.037 nM) and O-1966 (K0 = 23 nM) [14, 21]. As JWH133 appeared more effective when added as a post-treatment (after the introduction of HIV-1), the addition of GP1α or O-1966 was done in a similar fashion (Fig. 3C and D, respectively). The results indicated a marked decrease in the degree of RT activity when either of the two agonists was added to the cells. GP1α resulted in a 87.4% (2 μM) and 88.7% (10 μM) decrease in detectable RT activity. Likewise, the addition of the novel resorcinol-based compound O-1966 provided a 73.3% (1 μM), 85% (5 μM), and 87.6% (10 μM) attenuation in RT activity present in the culture medium. Interestingly, like JWH133, GP1α and O-1966 had no significant effect on RT activity at Days 3 and 5 (data not shown). Cell viability does not appear to be a factor contributing to the decreased level of RT activity. As reported by others [22, 23], the assessment of JWH133 on cell viability did not compromise cell survival (data not shown). GP1α and O-1966 did not change the viability of infected MDMs at the concentrations used above (Fig. 3E). A decrease in viability (20%) was evident only when GP1α was used at a much higher concentration (Fig. 3E). The results from these RT assays suggest that the up-regulation in CB2 expression in infected cells offers the possibility to use CB2 agonists to suppress HIV-1 replication.
LTR-driven β-gal reporter gene. The infection was allowed to occur for 6 h, at which point, the medium-containing virus was removed and changed to media containing JWH133 (1 μM) or O-1966 (1 μM). The agonist was added after the initial 4 h viral incubation (post-treated), and the RT assay was performed on medium from Day 7. All values are represented as the mean ± sd. (E) Analysis from viability assays using the fluorescence indicator calcein-AM. The cells were infected and treated as indicated in B and C. At the end of 7 days, the viability assay was performed as described in Materials and Methods. The results are shown as the percent mean ± sd from the untreated control. The P values are provided in the figure above the noted group comparisons (brackets). P values shown without brackets, compare each treatment with the HIV-1-only control or untreated control, as in the case of the viability results. The results were acquired from replicates of differentiated primary cells from at least three different donors.

DISCUSSION

The CB2 and the CB1 receptors comprise the cannabinoid receptor family. Unlike the CB1, the activation of the CB2 recep-
tor does not elicit psychoactive effects but rather promotes anti-inflammatory responses [24]. To date, the effect of CB2 on HIV-1-infected macrophages has remained largely unknown. Such information is of great interest in the context of HIV-1 neuropathogenesis, as infection of monocytes/macrophages is thought to be the primary cause of how HIV-1 infiltrates the CNS [25, 26]. Thus far, several studies have demonstrated CB2 expression in microglial cells, perivascular macrophages, and T cells in SIV encephalitis [27]. More recently, a study of HIVE from postmortem cases also showed a marked increase in CB2 receptor expression in microglia, astrocytes, and perivascular cells [8]. Another example of the CB2 receptor up-regulation in HIVE can be found in the relevant murine model of neuroAIDS [13]. The authors showed that the CB2 receptor, but not the CB1 or the orphan cannabinoid receptor GPR55, was up-regulated in HIVE brains in an animal model [13]. Our results are in agreement with these previous studies but also further add that in HIVE, microvessels and HIV-1-infected monocytes/macrophages in the perivascular space show up-regulated levels of the CB2. The up-regulation of the CB2 on microglial cells and on infiltrating immune cells has prompted the notion that up-regulation of the receptor and binding to endocannabinoids may act as a triggering mechanism to attenuate inflammation. Moreover, regional increases in cannabinoid receptor expression have been shown to increase potency and efficacy of exogenous agonists at sites of end-organ injury [28].

Up-regulation of CB2 is a key observation that could allow for targeting of the CB2 receptor to 1) minimize the inflammatory effects of HIV-1 harboring mononuclear cells and 2) reduce viral infection (the hypothesis presented in this study). Therefore, with the increasing availability of novel synthetic, highly specific CB2 agonists, we investigated whether HIV-1 could be inhibited upon CB2 activation on macrophages infected with the M-tropic HIV-1 ADA strain. We first examined the expression of CB2 in primary human monocytes, MDMs, and MDMs infected with HIV-1. The results for protein and gene expression showed the following increased order of CB2 receptor expression: monocytes/MDMs/HIV-1-infected MDMs. These results, obtained from multiple donors, suggest that CB2 expression is altered as a function of monocyte differentiation and activation. Up-regulation of the receptor by activation appears to be a common phenomenon in inflammation [28, 29]. Earlier, we reported on the increase of CB2 receptor levels in brain endothelial cells after exposure to IL-1β, TNF-α, and LPS [19]. Others have reported on increased CB2 receptor expression on activated microglia as result of Acanthamoeba culbertsoni-mediated neuroinflammation [30]. In terms of HIV-1-induced activation, our study is the first (that we are aware of) to show that HIV-1 infection up-regulates the expression of CB2 in macrophages. Interestingly, Raborn and Cabral [31] used U937 (a monocytic cell line) to show that the HIV-1 Tat has no effect on CB2 expression. Therefore, HIV-1 accessory proteins, such as the transactivator Tat, may not cause induc-
tion in CB2 receptor levels, but rather, cellular responses to direct viral infection do. We next examined whether the enhanced presence of the CB2 receptor could offer a therapeutic opportunity to curb HIV-1 infection. Indeed, the CB2 agonist JWH133 demonstrated a dose-dependent inhibition (up to 82%) in HIV-1 RT activity in MDMs infected for 7 days. Importantly, the addition of JWH133 prior to or after HIV-1 infection provided comparable levels of inhibition. These results are complementary with those by Costantino et al. [32], showing >40% inhibition in HIV-1 infection in primary CD4+ T cells using a similar concentration (1 μM) of JWH133. Similar observations were also found in HIV-1-infected microglial cells and CD4+ T cells using WIN55,212-2 [33]. A dual agonist for CB1 and CB2 receptors, WIN55,212-2 was shown to limit HIV-1 p24 expression by ~60% in microglia and CD4+ T cells [33]. A follow-up study from the same group using the CB2 receptor agonist JWH015 reported a 40% inhibition in HIV-1 p24 expression in infected microglial cells [9], ascertaining that the HIV-1 suppression by WIN55,212-2 in microglial cells was, in part, a result of CB2-related effects. To ensure CB2 receptor specificity, our analysis included the reverse agonist/antagonist SR144528. Indeed, SR144528, whether coadministered with the agonist before or after the onset of HIV-1 infection, completely eliminated the effects of the agonist. In addition to JWH133, two recently discovered and potent CB2 agonists, GP1a and O-1966, were also tested for HIV-1 inhibition. GP1a and O-1966, added after HIV-1 introduction and replenished during a 7-day infection, revealed an inhibition of up to 88% and 85% in RT activity, respectively. Thus, the efficacy of reducing HIV-1 RT activity can be achieved to similar levels with CB2 receptor agonists of varied chemical composition.

To understand the mechanism behind how the agonist affects HIV-1 infection in macrophages, we turned to evaluation of the HIV-1 coreceptors. Previously, it has been suggested that one possible consequence of CB2 signaling is that it may affect the expression of HIV-1 coreceptors, CXCR4 and CCR5 [9]. Our observations in macrophages support results by other groups showing no effect of CB2 on CXCR4 expression in CD4+ T cells [32]. Here, we provide evidence that the CB2 agonist does not alter surface protein expression of CXCR4 or its gene regulation in MDMs. Similarly, the CB2 agonist did not change surface protein or gene expression of CCR5 in MDMs. These results are in contrast to the inhibitory effect that WIN55,212-2 has on CCR5 expression in microglia and that reported for GP1a in CD4+ T cells (human PBLs) [9, 13]. However, in the same study with GP1a, CCR5 expression in CD8+ cells was unchanged. Perhaps, unlike CXCR4, CCR5 could be a regulatory target of CB2 receptor signaling in certain cell types but not others. Our results therefore suggest that in MDMs, there may not be interference in HIV-1 viral entry, at least not at the level of coreceptor expression, as a result of CB2 activation.

As the effect on the HIV-1 coreceptors did not seem to provide a plausible explanation for how the CB2 agonist inhibits HIV-1 infection, we next evaluated the activity of the HIV-1 LTR. With the use of single-round infections on the reporter cell line TZM-bl, which features the HIV-1 LTR-driven β-gal gene, the effects of CB2 agonists were evaluated. The results

![Figure 5. CB2 agonist inhibits HIV-1 LTR activity.](image-url)
indicated a clear diminution in the number of β-gal-positive cells upon treatment with JWH133 or O-1966. Moreover, results of HIV-1 pol copy number substantiate the observations of the LTR, as relative expression of pol was reduced significantly by all CB2 agonists tested. In light of these results, it is plausible that CB2 signaling could interfere with full activation of the HIV-1 LTR. Admittedly, how the HIV-1 LTR may be inhibited by the CB2 receptor is not known at this time, but it is the subject of ongoing studies in our laboratory. It is reasonable to speculate that a possible explanation may come from cellular transcription factors that share participation in proinflammatory responses and activation of the HIV-1 LTR. In fact, studies using ΔTHC, a partial agonist of CB1 and CB2, have shown inhibition of NF-κB in the macrophage cell line Raw 264.7 [34]. Furthermore, with the use of reporter assays, Borner and colleagues [35] demonstrated inhibition of NFAT and NF-κB by ΔTHC or JWH015 (a CB2 receptor agonist) in naïve and stimulated Jurkat cells. Accordingly, it is conceivable that transcription factors involved in the transactivation of the HIV-1 LTR could be targeted for inhibition upon stimulation of the CB2.

The effects of CB2 activation have been well characterized as a means to reduce inflammatory responses in various cell types, particularly those of the immune system [24]. To date, however, no study has exclusively focused on whether CB2 receptor activation may affect HIV-1 expression in macrophages. The study presented in this report invites the possibility of using CB2 receptor agonists as a means to limit HIV-1 replication in infected macrophages. We show that synthetic CB2 agonists provide a similar effect on HIV-1 infection, whether applied before or after the onset of infection. The findings also seem to implicate attenuation of the HIV-1 replication machinery rather than a hindrance to viral entry upon addition of the agonists. It should be noted that aside from affecting HIV-1 infection in primary macrophages, as reported here, CB2 receptor agonists have pleiotropic effects in macrophages that may further lessen the burden of HIV-1. These effects include a decrease in overall proinflammatory response and possible inhibition in monocyte/macrophage migration into the CNS [13, 31]. Although immunoregulatory, it is also important to point out that activation of the cannabinoid receptor system is not immunosuppressive. Systemic administration of GP1a (in a mouse model of HIV) and the now U.S. Food and Drug Administration-approved use of ΔTHC for AIDS-related wasting has not been shown to have adverse consequences on immune function [36, 37]. Therefore, the role of the CB2 receptor in HIV-1 infection merits further investigation and testing of novel, synthetic CB2 agonists.

ACKNOWLEDGMENTS

This study was supported (in part) by research funding from the U.S. National Institutes of Health (RO1MH065151 and R37AA015913 to Y.P.). We thank Dr. Ronald Tuma for kindly providing the O-1966 compound.

REFERENCES


tenuates leukocyte-endothelial cell interactions and blood-brain barrier dysfunction under inflammatory conditions. J. Neurosci. 32, 4004–4016.


KEY WORDS: neuroinflammation · JWH133 · CXCR4 · CCR5 · encephalitis · CB2