PLoS one

Cannabinoid Receptor 2-Mediated Attenuation of CXCR4-Tropic HIV Infection in Primary CD4+ T Cells

Cristina Maria Costantino[1,2], Achla Gupta[2], Alice W. Yewdall[1], Benjamin M. Dale[1], Lakshmi A. Devi[2]*, Benjamin K. Chen[1]*

1 Department of Infectious Diseases, Department of Medicine, Immunology Institute, Mount Sinai School of Medicine, New York, New York, United States of America, 2 Department of Pharmacology and Systems Therapeutics, Mount Sinai School of Medicine, New York, New York, United States of America

Abstract

Agents that activate cannabinoid receptor pathways have been tested as treatments for cachexia, nausea or neuropathic pain in HIV-1/AIDS patients. The cannabinoid receptors (CB_1R and CB_2R) and the HIV-1 co-receptors, CCR5 and CXCR4, all signal via $G\alpha i$-coupled pathways. We hypothesized that drugs targeting cannabinoid receptors modulate chemokine co-receptor function and regulate HIV-1 infectivity. We found that agonism of CB_2R, but not CB_1R, reduced infection in primary CD4+ T cells following cell-free and cell-to-cell transmission of CXCR4-tropic virus. As this change in viral permissiveness was most pronounced in unstimulated T cells, we investigated the effect of CB_2R agonism on to CXCR4-induced signaling following binding of chemokine or virus to the co-receptor. We found that CB_2R agonism decreased CXCR4-activation mediated G-protein activity and MAPK phosphorylation. Furthermore, CB_2R agonism altered the cytoskeletal architecture of resting CD4+ T cells by decreasing F-actin levels. Our findings suggest that CB_2R activation in CD4+ T cells can inhibit actin reorganization and impair productive infection following cell-free or cell-associated viral acquisition of CXCR4-tropic HIV-1 in resting cells. Therefore, the clinical use of CB_2R agonists in the treatment of AIDS symptoms may also exert beneficial adjunctive antiviral effects against CXCR4-tropic viruses in late stages of HIV-1 infection.

Introduction

Cannabinoid agonists are currently under investigation for the treatment of AIDS-associated cachexia, nausea, and neuropathic pain [1–3]. One such drug, dronabinol (Δ^9-THC; Marinol®), has won Food and Drug Administration (FDA) approval for treatment of HIV-associated anorexia [4]. Additionally, the prescription of smoked or ingested cannabis (marijuana) for treatment of AIDS-related symptoms has been approved in 14 states [5]. Despite the use of cannabinoids by HIV/AIDS patients, few studies have investigated the impact of such drugs in regard to viral pathogenesis or immune regulation. Early studies conducted in the pre-HAART era suggested a positive correlation between development of opportunistic infection, progression to AIDS, and marijuana use [6,7]. Yet recent analysis of HIV/AIDS patients enrolled a randomized, placebo-controlled clinical trial designed to study the outcome of cannabinoid administration have indicated that cannabinoid use does not result in significant immunosuppression [1]. Indeed, both smoked marijuana and dronabinol were reported to increase total CD4+ T cell number [1] and naïve T cell number [8] over a 21-day period. A decrease in viral load was also observed in these patients [1]. Similarly, in SIV infected rhesus macaques, Δ^9-THC exposure reduced viral load and CD4+ T cell depletion, significantly increasing animal survival over an 11 month period [9]. Despite these findings, the mechanisms by

which cannabinoid drugs can influence viral replication or pathogenicity remain unknown.

Cannabinoid agonists activate the CB_1R and CB_2R cannabinoid receptors. Like the HIV chemokine co-receptors CXCR4 and CCR5, CB_1R and CB_2R are members of the $G\alpha i$-coupled family A GPCRs [10]. CB_2R is highly expressed on all CD4+ T cells [11], whereas CB_1 expression is found in activated, memory subsets [12]. CB_1 and CB_2 have been classified as immunosuppressive receptors on CD4+ T cells [13], although antagonism of CB_1R and CB_2R does not enhance immune activation and knock-out mice do not exhibit differences in T cell frequency or increases in autoimmune pathogenesis [14]. The mechanism(s) by which cannabinoid agonists can modulate CD4+ T cell function remain unclear. Activation of CB_2R has been shown to inhibit inflammatory cytokine production in CD4+ T cells [11], which may account for the decrease in autoimmune pathogenesis observed in therapeutic trials of cannabinoid agonists in animal models of multiple sclerosis [14,15]. CB_2R may also function as a chemotactic modulator, as CB_2R activation inhibits CXCR4-induced chemotaxis in transformed lymphocytes [16]. CB_2R has further been shown to regulate lymphocyte egress from the bone marrow in a role previously attributed largely to CXCR4 [17,18]. These findings suggest that CB_2R may play a role in regulating chemokine receptor signaling, specifically the activity of CXCR4. Such cross-talk between CB_2R and CXCR4 may have implica-

tions for AIDS patients who take cannabinoid-derived agents for therapeutic purposes.

Although coreceptor signaling is not essential for HIV-1 infection, several recent studies have suggested that chemokine receptor signaling enhances infection of resting CD4+ T cells [19–21]. These cells express CXCR4, but not CCR5, whose expression is restricted to a small subset of memory CD4+ T cells [22]. In patients, the emergence of CXCR4-tropic virus usually occurs after years of infection and correlates with more rapid progression to AIDS [23,24,25]. Viral conversion to CXCR4-tropism increases the number of targets available to the virus [26]. Additionally, as HIV-1 can establish latency in resting T cells [27], a switch to CXCR4-tropism could enhance the establishment of a latent pools of virus within lymphoid tissues. The increased number of new targets may explain the rapid decline in CD4+ T cell numbers and increased viral load in late-stage AIDS patients with CXCR4-tropic virus [1,24]. The late-stage patients who frequently harbor CXCR4-tropic virus are also the most likely to benefit from cannabinoid drug use. It is therefore relevant to study the potential for cannabinoid signaling to modulate CXCR4 activity and alter the course of HIV infection, Interactions between GPCRs like CB_2R and CXCR4 can cause cross-desensitization, allosteric modulation, dimerization, changes in receptor localization, and alteration of physiological function among GPCR pairings [28]. Given that direct antagonism of chemokine receptor function can block viral infection [19,29,30], it is possible that allosteric modulation of CXCR4 through a GPCR partner may also reduce HIV-1 permissiveness. Indeed, oligomerization of the chemokine co-receptors including CXCR4 using conformationally specific monoclonal antibodies can inhibit HIV-1 entry into target cells [31,32]. These experiments demonstrate signaling-independent modulation of coreceptor function. Allosteric agents that disrupt HIV-1 infection by modulating chemokine receptor signaling have not yet been identified. Should CB_2R-induced signaling alter CXCR4 co-receptor function, this would represent first known example of a signaling-dependent GPCR interaction leading to viral inhibition.

To test this hypothesis, we examined the effect of cannabinoid receptor activation on HIV viral transmission and productive infection in CD4+ T cells using a GFP-expressing, CXCR4-tropic HIV-1 variant. We found that activation of CB_2R on CD4+ T cells significantly inhibited viral infection in a CB_2R-selective and dose-dependent manner. Viral inhibition was more pronounced in resting cells that were activated after infection. We investigated signaling in these cells and found that CB_2R agonism significantly decreased SDF-1α-induced CXCR4 activation. Furthermore, CB_2R agonism altered HIV-induced cytoskeletal rearrangement, associated with productive HIV infection in resting cells. We found that CB_2R was not sufficient to block viral transfer or fusion, but did significantly diminish productive viral infection. We conclude that CB_2R is a novel modulator of CXCR4-tropic HIV infection in CD4+ T cells.

Materials and Methods

Cell culture reagents

Anonymous blood donations were obtained from New York Blood Center. Cells were cultured as previously reported [33]. The purified monoclonal antibodies (mAbs) ⟨CD2 (OKT3) and ⟨CD28 (28.2) were purchased from eBioscience. Phytohemagglutinin (PHA) and carboxyfluorescein succinimidyl ester (CFSE) was purchased from Sigma. Recombinant human IL-2 and CXCR4 antagonist AMD3100 were obtained through the AIDS Research and Reference Reagent Program (NIH). Fluorophore-conjugated

CXCR4 (12GS), CCR5 (T21/8), CD25 (BC96), and CD45RO were purchased from Biolegend. The CB2 agonists JWH-133, JWH-150, Ser160, 2-arachidonoylglycerol, and anandamide and the CB2 antagonist AM630 were purchased from Tocris.

Cell purification and sorting

Total CD4+ T cells were isolated from healthy HIV/hepatitis B virus-seronegative donors as described previously [33]. For FACS-sorting, CD4+ T cells were labeled with Live/Dead for viability (Invitrogen), stained for CD45RO, and sorted with a FACS Aria (BD Biosciences).

Viral constructs

HIV NL-GI and Gag-iCherry are NL4-3 based CXCR4-tropic HIV-1 molecular clones that have been described previously [34]. NL-GI expresses green fluorescent protein (GFP) in place of the viral early gene nef, and nef expression is maintained by insertion of an internal ribosome entry site (IRES) [35]. Gag-iCherry carries the GFP variant Cherry inserted internally into Gag between the MA and CA domains. For CCR5-tropic virus, a variant of NL-GI expressing the *Env* gene from the molecular clone JRFL [36] was used. Virus was produced in HEK293T cells and p24 concentration was calculated by ELISA prior to use.

Cell-Free Infection Assay

CD4+ T cells were thawed, resuspended in RPMI medium containing 20 U/ml recombinant IL-2 and stimulated with 1 μg/ml PHA (Sigma) overnight. Cells were cultured for four days and reseeded into 96 well flat-bottom plates (Costar) at a density of 10^5 cells/well prior to treatment and infection. Treated cells were incubated with antagonist or vehicle (DMSO) for one hour at 37°C followed by incubation with agonist or vehicle (DMSO or 0.1% ethanol) for another three hours. Following this treatment, triplicate cultures were infected with 10 ng/well HIV NL-GI. To assess HIV infection, fluorescence was assessed at day 4 post-treatment. Harvested cells were stained for viability and fixed with 4% paraformaldehyde, prior to acquisition on a FACSCalibur (BD Biosciences) and analysis with FloJo software (TreeStar).

Cell-Associated Infection Assay

HIV-expressing Jurkat donor cells were generated by transfection using HIV Gag-iCherry, as described previously [34]. To generate infected CD4+ T cell donors, PHA activated CD4+ T cells were spinoculated with either HIV NL-GI or for 90 minutes at 1200×g. After 24–48 hours, approximately 10–30% of donor cells were infected. Donor cells were labeled with 10 μM CellTracker blue (CMF2HC) fluorescent dye (Invitrogen) and then co-cultured with unstimulated and antagonist and/or agonist-treated target cells in a 1:1 ratio for 3 hours at 37°C, as described previously [33]. Virus transfer was terminated by washing with PBS and treatment with 0.05% trypsin-EDTA (Invitrogen) for 5 minutes. Cells were stained for viability and for CD45RO prior to fixation and acquisition on a LSRII (BD Biosciences). Sorted GFP⁻Cherry⁻ CD45RO⁺ or CD45RO⁻ targets were seeded into 96-well plates coated with 2.5 μg/ml anti-CD3 in RPMI media containing 1 μg/ml anti-CD28 and 20 U/ml rIL-2 for activation. After 4 days, cells were harvested, fixed, and analyzed for fluorescence.

Quantitation of Viral Membrane Fusion

Cell-free viral fusion was measured using a method described previously [37]. BlaM-VPR was a gift from Michael Miller (Merck Research Laboratories). Viral infections were done with 20 ng of

virus at a concentration of 200 ng/ml for 2 hrs at 37°C. Cells were analyzed on an LSRII flow cytometer (Becton Dickinson).

Signaling Studies

For GTPγS binding, JWH-133 treated CD4+ T cells were permeabilized with CHAPS and incubated with increasing concentrations of SDF-1α. Incorporation of radiolabelled GTPγS was assayed as described [38]. For kinase phosphorylation and western blotting, primary CD4+ T cells were treated with antagonist and/or agonist in serum-free RPMI for a total of 4 hours prior to treatment with SDF-1α (PeproTech) or NL4.3 HIV at various times indicated. Levels of phosphorylated MAP kinase and total MAP kinase were determined as described [38].

Data Analysis

Data was analyzed with GraphPad Prism using a paired t-test. Mean and standard error indicate variance between multiple donors, with n as indicated.

Results

CB$_2$-specific agonists inhibit HIV-1 infection in primary CD4+ T cells

As activation of the cannabinoid receptor CB$_2$R has been shown to modify chemokine receptor activity [16] and cannabinoid use in HIV-1 infected individuals is associated with reductions in viral load [1], we hypothesized that CB$_2$R agonism may alter the course of HIV-1 infection. Using a GFP-expressing variant of the CXCR4-tropic lab isolate NL4.3 (NL-GI), in which GFP is expressed in place of the viral gene nef [33], we assayed HIV-1 infectivity in primary CD4+ T cells pre-treated with CB$_2$R agonist. Purified, blood-derived CD4+ T cells were stimulated with low-dose mitogen for four days prior to a three-hour cannabinoid treatment. To directly test the capacity for CB$_2$R activation to inhibit viral infection, we pretreated activated CD4+ T cells with a potent and selective CB$_2$R agonist that is approximately 200-fold selective for CB$_2$R over CB$_1$R (Ki = 3.4 nM) [39] (JWH-133) prior to HIV-1 exposure. Treated cells were then washed and exposed to virus in suspension (10 ng/ 2×10^5 cells) for four days. After this time, the frequency of GFP+ infected cells was measured and the frequency of inhibition, as compared to control (DMSO treated) infection, was calculated.

When cells were treated with 100 nM of JWH-133 prior to viral exposure, we observed an approximately 40% reduction in HIV-1 infected cells after four days (**Figure 1A–B**). This inhibition was significantly reduced when CB$_2$R activity was selectively blocked using the antagonist AM630, indicating that the antiviral activity of JWH-133 is indeed CB$_2$R-specific (**Figure 1A–B**). JWH-133-mediated blockade of HIV-1 infectivity was dose-dependent, with an EC50 of 7.59±0.1 nM and a plateau of efficacy at approximately 50% inhibition (**Figure 1B**). Further, the action of this drug was CXCR4-specific, as JWH-133 treatment was not sufficient to inhibit infection with an isogenic virus that carried a CCR5-tropic Env from molecular clone JRFL (**Figure 1C**). Therefore, CB$_2$ activation reduces CXCR4-tropic HIV-1 infection in primary CD4+ T cells in a dose-dependent and receptor-specific fashion.

To further confirm this novel role for CB$_2$R, we tested other CB$_2$R-selective agonists (JWH-015 and Ser016) to see if treatment was sufficient to reduce viral infectivity. Both of the highly specific CB$_2$R agonists we tested proved to be antiviral at concentrations of 1 μM; these were JWH-015 (35.9±11.96% inhibition) and Ser016 (30.7±13.72% inhibition) (mean±SEM, n = 8 donors) (**Figure 1D**). Treatment with 1 μM of the pan-cannabinoid agonist Hu210 also

significantly reduced HIV-1 infection, although to a lesser extent than with CB$_2$R-selective agonists (23.9±7.45%, mean±SEM, n = 8 donors) (**Figure 1D**). The reduction in infection efficiency observed with Hu210 was CB$_2$R-specific and was not observed when cells were pretreated with the CB$_2$R-selective antagonist AM630 (**Figure 1D**). Consistent with these findings, 1 μM pretreatment with the CB$_1$R selective agonist arachidonyl-2′-chloroethylamide (ACEA) did not significantly reduce viral infection (4.76±3.13%, mean±SEM, n = 5 donors) (**Figure 1D**). These results indicate that CB$_2$R-selective agonists, but not CB$_1$R-selective agonists, can inhibit HIV-1 permissiveness.

Given that the pan-cannabinoid agonist Hu210 possessed antiviral activity, we predicted that naturally occurring endogenous ligands of the cannabinoid receptors, the endocannabinoids 2-arachidonoylglycerol (2-AG) and anandamide (AEA) could reduce infectivity via CB$_2$R activation. We found that pretreatment with either 2-AG or AEA significantly inhibited HIV-1 infection in a dose-dependent and CB$_2$-specific manner (**Figure 1E–F**). AEA, which is reported to have higher affinity for CB$_2$R than 2-AG (Ki = 371 nM versus 1400 nM, respectively) [40], proved to be a more potent inhibitor of virus, with an EC50 of 8.59±0.09 nM, as compared to 1.82±0.25 μM for 2-AG. The antiviral activity of both of these endocannabinoid agents was abrogated by treatment with 1 μM of the CB$_2$R-selective antagonist AM630. These data demonstrate that, like synthetic cannabinoid agonists, the endocannabinoids can activate CB$_2$R to inhibit HIV-1 infectivity in CD4+ T cells.

CB$_2$ activation does not alter CXCR4 expression or T cell activation

We next sought to determine the mechanism of HIV-1 inhibition via CB$_2$R. Previous reports have indicated that cannabinoid treatment of immune cells can lead to changes in cell surface chemokine receptor expression [41], loss of proliferative capacity [11], and reduction in effector function [42,43]. We examined whether CB$_2$R activation in our infection model led to either a reduction in co-receptor expression or in host fitness, rendering the cells incapable of harboring productive viral infection. To test this possibility, we treated CD4+ T cells with the CB2 agonist JWH-133 with a concentration of drug sufficient to inhibit viral infection (100 nM). We found that this treatment did not lead to significant reduction of CXCR4 cell surface expression (**Figure 2A–B**) or total CXCR4 protein expression (data not shown).

Likewise, pretreatment with up to 1 μM of JWH-133 prior to TCR-mediated activation did not reduce T cell activation, as measured by an increase in CD25 expression, or proliferation, as indicated by CFSE dilution after stimulation with anti-CD3 and anti-CD28 antibodies (**Figure 2C–E**). Concentrations of JWH-133 ranging from 1 μM and below did not lead to a change in cell viability as compared to DMSO treated controls (data not shown). Higher concentrations of JWH-133 or Hu210 (10 μM and above) did lead to apoptosis and cell death, consistent with published observations [11]. Our findings indicate that low doses of the CB$_2$R agonist JWH-133 are sufficient to inhibit viral infection without significant disruption of CD4+ T cell co-receptor expression, CD4 expression, CD25 upregulation, or proliferation. Further, CB$_2$R agonism did not alter the ability of the CD4+ T cell to support viral infection, as JWH-133 treated cells are readily infected by the CCR5-tropic JRFL virus (**Figure 1C**). Taken together, these data suggest that CB$_2$R activation is altering a pathway specifically required for CXCR4-tropic infection.
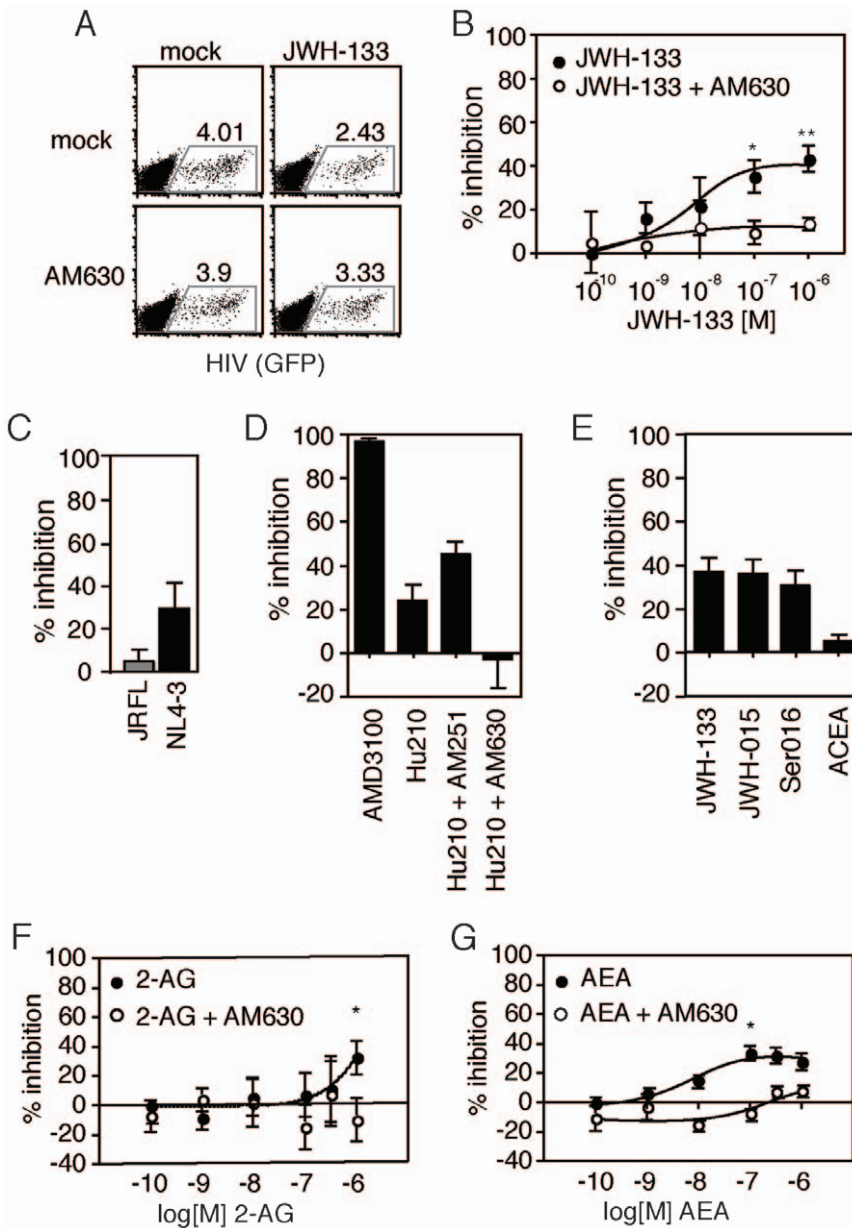
Figure 1. Cannabinoid inhibition of CXCR4-tropic HIV-1 infection is dose-dependent and CB$_2$-selective. (A) HIV-1 (GFP) expression 4 days post-infection in primary CD4+ T cells pretreated with 100 nM of the CB2-specific agonist JWH-133 and/or the CB$_2$-specific antagonist AM630 in a representative donor. HIV expression (GFP; FL1) is shown on the x-axis, and an empty channel (FL3) is shown on the y-axis. (B) Dose-dependent inhibition of viral infection by JWH-133 in primary CD4+ T cells is blocked by 100 nM of the CB$_2$ antagonist AM630 (mean±SEM; n = 7 donors; **p = 0.0032, *p<0.03). (C) Inhibition of CXCR4-tropic (NL4-3) but not CCR5-tropic (JRFL) HIV-1 after 4 days in primary CD4+ T cells pretreated with 100 nM of JWH-133 (mean±SEM; n = 5 donors). (D) Inhibition of viral infection in cells pretreated with the CXCR4 antagonist AMD3100 (which is predicted to block viral binding [57]), or 1 µM of cannabinoid agonists ACEA, Hu210, JWH-133, JWH-015 and Ser016 with or without pretreatment with the CB$_1$ antagonist AM251 or the CB$_2$ antagonist AM630. (E–F) Dose-dependent inhibition of NL-G1 infection in primary CD4+ T cells pretreated with (F) 2-arachidonoylglycerol (2-AG) or (G) anandamide (AEA) is blocked by 1ìM of the CB$_2$ antagonist AM630 (mean±SEM; n = 5 donors; *p<0.03).
doi:10.1371/journal.pone.0033961.g001

The antiviral activity of CB2 agonist JWH-133 is enhanced in unstimulated CD4+ T cells

Despite intense study, the importance of GPCR-mediated signaling during CXCR4-tropic HIV-1 infection remains unclear. Recently, several lines of study have demonstrated a role for chemokine receptor signaling in resting CD4+ T cells [44]. Activation of Gαi-coupled chemokine receptors enhances infectivity in resting CD4+ T cells that are stimulated with gp120 or chemokine agonist after exposure to HIV-1 [19,20]. Conversely,

inhibition of GPCR function by pertussis toxin inhibits viral infection [19]. This has been described as a model for latent infection in resting cells [19,45]. We tested to see if the CB$_2$ agonist JWH-133 differentially inhibited HIV-1 infection in resting versus activated cells.

Cells were activated with mitogen four days before (activated) (**Figure 3A**) or four days after (resting) (**Figure 3B**) treatment with 100 nM JWH-133 or 1 µM pertussis toxin and exposure to virus. We found that JWH-133 and pertussis toxin partially
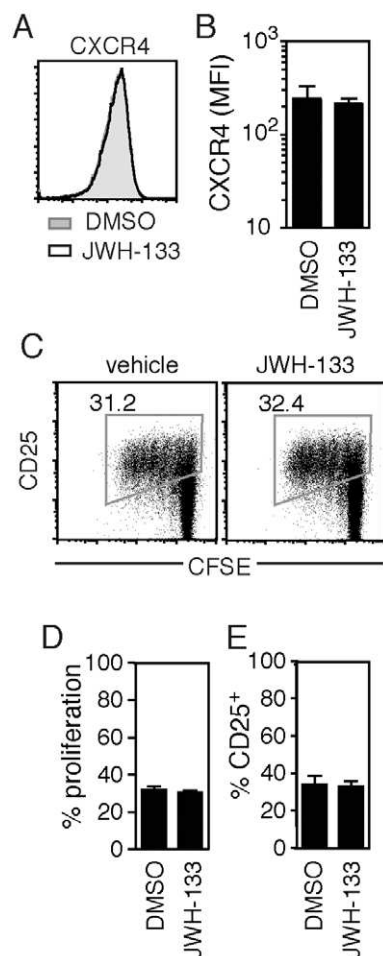
Figure 2. CB$_2$ agonism at concentrations sufficient to inhibit HIV-1 infection does not significantly alter cell-surface CXCR4 expression or inhibit cellular proliferation in primary CD4+ T cells. (A–B) Cell surface expression of CXCR4 in CD4+ T cells pretreated with either DMSO or 100 nM of JWH-133 (A) in a representative donor and (B) in multiple donors (mean±SEM; n = 4 donors). (C–E) Proliferation, as indicated by CFSE dilution; and activation, as measured by CD25 expression; in CD4+ T cells, stimulated with cross-linking anti-CD3 and anti-CD28 mAb, 4 days after pretreatment with 100 nM of JWH-133 (C) in a representative donor and (D–E) in multiple donors (mean±SEM; n = 5 donors).
doi:10.1371/journal.pone.0033961.g002

inhibited viral replication in both activated and resting cultures (**Figure 3**). Consistent with previous reports [19], inhibition by pertussis toxin was augmented in resting cultures as compared to activated cultures (59.38±29.5% versus 41.16±20.6%, respectively, *p<0.05) (mean±SEM, n = 4). Similarly, we found that the efficacy of JWH-133–mediated viral inhibition was significantly increased in resting, as compared to activated, cultures (63.34± 31.7% versus 36.31±18.2%, respectively, *p<0.05 (mean±SEM, n = 4) (**Figure 3C**). These data are consistent with the premise that alteration of GPCR signaling predominantly alters viral infection in resting cells.

CB$_2$R agonism inhibits SDF-1α mediated CXCR4 signaling

Our data suggested that CB$_2$R activation specifically inhibited CXCR4-tropic virus and that this effect was greatest in resting cells. Given a previous report, which indicated that CXCR4 signaling enhances HIV-1 infection in resting cells [19], we chose

to investigate the functional consequence of CB$_2$R stimulation on CXCR4-mediated signaling. To assay for CXCR4 activity, we first measured changes in a G protein activity in CD4+ T cells activated by the cytokine SDF-1α (CXCL12). SDF-1α treatment led to a robust, dose-dependent increase in [^{35}S]GTPγS binding in CD4+ T cells (**Figure 4A**). Pretreatment with 100 nM JWH-133 significantly decreased this effect at higher doses (16.7±8.37%, **p = 0.0034) (mean±SEM, n = 4 donors) (**Figure 4B**). In contrast, pretreatment with SDF-1α did not inhibit JWH-133 induced [^{35}S]GTPγS binding at any concentration tested (**Figure 4C**). CB2 agonism therefore decreases G-protein activation in response to the CXCR4 agonist SDF-1α.

The CB$_2$R-induced inhibition of CXCR4 signaling was also indicated by a decrease in phosphorylation of downstream kinases following SDF-1α treatment (**Figure 5**). We assayed SDF-1α-induced Akt and p32/44 MAP kinase (ERK 1/2) phosphorylation in CD4+ T cells following treatment with or without CB$_2$R agonist JWH-133. SDF-1α-mediated phosphorylation of both Akt (**Figure 5A–B**) and ERK 1/2 (**Figure 5C–D**) was significantly inhibited by pretreatment with JWH-133. These results suggest that the activation of CB$_2$R is sufficient to inhibit downstream CXCR4-mediated signaling pathways.

The CB$_2$R agonist JWH-133 decreases F-actin in CD4+ T cells

Down-regulation of CXCR4 signaling with pertussis toxin has been shown to decrease actin dynamics, disrupting the remodeling of the cortical actin barrier required for HIV-1 infection [19]. Given the capacity for CB$_2$R agonism to inhibit upstream CXCR4-mediated signaling events, we hypothesized that CB$_2$R could suppress CXCR4-induced actin polymerization. To test for this possibility, we assayed the ability of JWH-133 to inhibit SDF-1α mediated actin polymerization, as visualized by phalloidin binding. While a significant difference in the rate of SDF-1α induced F-actin accumulation was not detected (data not shown), we did find that JWH-133-treatment led to a significant decrease in phalloidin binding as compared to untreated control cells (**Figure 6A–B**) (at time 0, 46.2±17.46 versus 64.36±24.34 MFI, respectively; after 30 minutes of SDF-1α treatment, 23.36±11.68 versus 31.3±15.65 MFI, respectively) (mean±SEM, n = 7, **p = 0.0021, *p = 0.04). Although CB$_2$R agonism was found to disrupt SDF-1α induced G protein binding and signaling (**Figure 4 and 5**), this decrease did not translate into acute disruption of the rate of actin polymerization following SDF-1α treatment. Therefore it is possible that CB$_2$R exerts its effect at the level of F-actin formation as treatment with JWH-133 reduces the level of F-actin in the steady state.

During HIV-1 infection, the virus itself acts as an agonist to stimulate CXCR4 and induce actin remodeling in resting cells [46,47]. We tested to see whether changes in actin reorganization caused by viral binding were altered by CB$_2$R agonist pretreatment. To do so, we pretreated CD4+ T cells with JWH-133, incubated these cells with HIV-1 viral particles, and then measured changes in phalloidin binding to F-actin over time. We found that HIV-1 induced transient upregulation in phalloidin binding activity peaking at approximately 1 minute after addition in both control and JWH-133 treated cells (**Figure 6C**). Like with SDF-1α treatment, the rate of increase in phalloidin binding was not significantly altered in CB$_2$R agonist pretreated cells. Consistent with our previous observations, we found that that basal phalloidin binding was significantly reduced in JWH-133 treated cells. The reduction in F-actin formation in JWH-133 treated cells was consistent over time despite the transient upregulation of phalloidin stain induced by virus (**Figure 6C**).
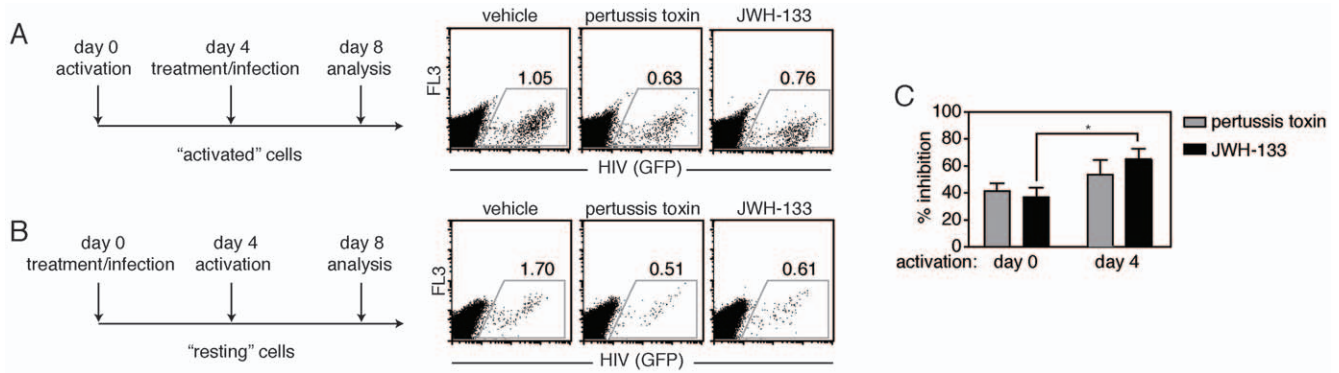
Figure 3. Inhibition of HIV-1 infection by CB$_2$ activation is enhanced in CD4+ T cells infected prior to activation. (A–C) Infection, as measured by GFP expression, in primary CD4+ T cells activated with mitogen (A) for 4 days prior or (B) 4 days after treatment with pertussis toxin or 100 nM of the CB2 agonist JWH-133 (A, B) in a representative donor and (C) in multiple donors (mean±SEM; n = 4 donors; *p<0.05).
doi:10.1371/journal.pone.0033961.g003

Taken together, these data suggest that reduction of F-actin induced by CB$_2$R agonism results in the reduction of total F-actin over time, despite addition of CXCR4 agonists such as SDF-1α or HIV-1 viral particles. The rate of actin polymerization remained constant after acute CXCR4 activation, but the total amount of F-actin induced was significantly lower in cells pretreated with CB$_2$R agonist. Indeed, the amount of F-actin induced by wild type virus in JWH-133 pretreated cells was similar to that induced by Env-null viral particles in control treated cells (at 1 minute, 107.5±53.7 versus 95.9±47.9) (mean±SEM, n = 4) (**Figure 6D**). Therefore, JWH-133 treatment reduces F-actin concentration to background levels, that is, the same level as non-specific induction of actin by Env-null virus. This basal reduction in actin polymerization via CB$_2$R may reduce actin rearrangement sufficiently to block viral infection.

CB$_2$R agonism decreases HIV-induced cofilin activation

HIV-triggered actin rearrangement is regulated in part by the severing protein cofilin, which dissociates and facilitates depolymerization of actin filaments thereby promoting actin dynamics [45]. In the inactive state, cofilin is phosphorylated; agonism of Gαi-coupled chemokine receptors initiates cofilin de-phosphorylation and activity [20]. Induction of cofilin de-phosphorylation increases HIV infection [19], we therefore hypothesized that JWH-133 treatment, which decreases HIV infection, would lead to increased cofilin phosphorylation. To identify changes in cofilin regulation, we measured phosphorylated cofilin (p-cofilin) expression in CD4+ T cells pretreated with JWH-133 and exposed to HIV. We detected a significant increase of p-cofilin over time in JWH-133 treated cells, as compared to control treated cells (216.42±124.95 versus 146.83±84.77, respectively, p-cofilin
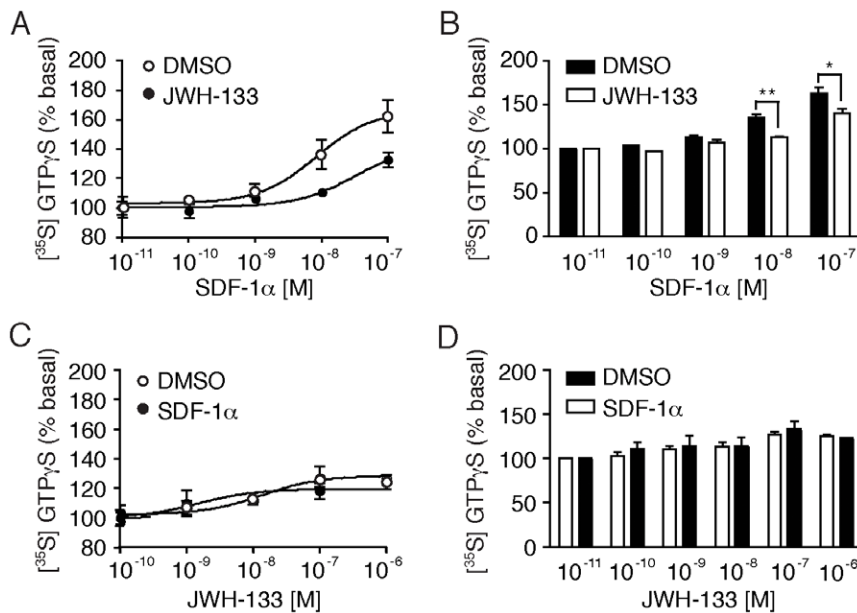

Figure 4. CB$_2$ agonism inhibits SDF-1á mediated G-protein coupling to CXCR4. (A–B) GTPáS binding following addition of the CXCR4 agonist SDF-1á to permeabilized primary CD4+ T cells pretreated with either DMSO or 100 nM of the CB$_2$ agonist JWH-133 (A) in a representative donor and (B) in multiple donors (mean±SEM; n = 4 donors; **p = 0.0034, *p<0.02). (C–D) GTPáS binding following addition of the CB$_2$ agonist JWH-133 to permeabilized CD4+ T cells pretreated with either DMSO or 100 nM of the CXCR4 agonist SDF-1á (C) in a representative donor and (D) in multiple donors (mean±SEM; n = 4 donors).
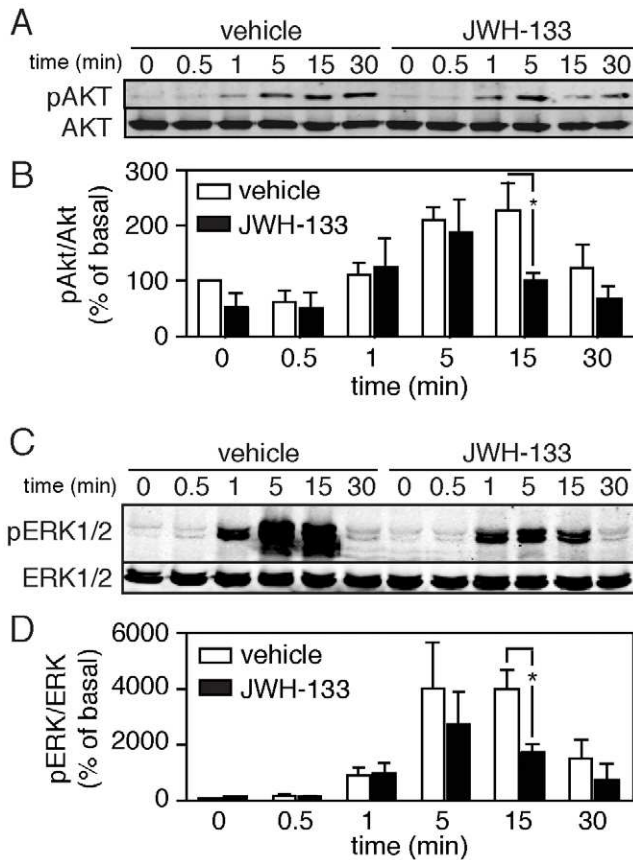doi:10.1371/journal.pone.0033961.g004

Figure 5. CB₂ agonism inhibits acute SDF-1á mediated signaling in primary CD4+ T cells. (A–B) SDF-1á induced phosphorylation of Akt and (C–D) p42/44 MAP kinase is downregulated in CD4+ T cells pretreated with 100 nM of the CB2 agonist JWH-133. (A, C) Representative western blots of phospho-kinase expression following SDF-1á treatment for 0, 0.5, 1, 5, 15 or 30 minutes. (B, D) Quantification of kinase phosphorylation in multiple donors (mean±‴ SEM; n = 3 donors; *p<0.05) taken over basal, defined as signal at time 0.
doi:10.1371/journal.pone.0033961.g005

density normalized to actin at 120 minutes) (mean±SEM, n = 3, *p<0.05) (**Figure 6E–F**). This data suggests that CB₂R agonism not only blocks HIV-induced cofilin de-phosphorylation, but also enhances p-cofilin. Indeed, CB₂R-induced p-cofilin was also observed in cells exposed to a control, VSV-envelope pseudotyped virus (183.11±91.56 JWH-133 versus 138.32±69.16 control, p-cofilin density normalized to actin at 120 minutes) (mean±SEM, n = 4, *p<0.05) (data not shown). We did not observe changes in total cofilin levels in cells treated with HIV and JWH-133 (Figure 6G). Taken together, our data indicate that CB₂R agonist pretreatment leads to accumulation of p-cofilin as well as the inhibition of cofilin dephosphorylation, i.e. activation in the presence of HIV; findings consistent with a known requirement for cofilin activation in HIV infection [19]. The increase of inactive cofilin in JHW-133 treated cells is a likely mechanism for the reduction of actin dynamism and subsequent inhibition of viral infection in these cells.

CB₂R agonism inhibits productive infection, but not viral fusion

Alteration of actin dynamics has been shown to inhibit productive viral infection but not viral binding or fusion [46].

To confirm that CB₂R-mediated changes in the actin cytoskeleton did not inhibit viral fusion, we assessed levels of fusion using a β-lactamase (BlaM)-Vpr fusion assay [37]. NL4.3 virions that incorporated BlaM-VPR were used to infect JWH-133 and control treated cells loaded with the β-lactamase substrate CCF2-AM. Upon viral membrane fusion, BlaM-Vpr is released into the cytoplasm where it is able to cleave CCF2-AM. Infection of primary, resting CD4+ T cells induced fusion in approximately 4% of cells (**Figure 7A**). We found that blockade of HIV binding with the CXCR4 antagonist AMD3100 significantly reduced viral fusion and CCF2-AM cleavage, but pretreatment with JWH-133 had no effect (**Figure 7 A–B**). This data indicates that CB₂R antagonism of CXCR4 function does not block the early stages of viral infection binding and fusion.

CB₂R agonism inhibits cell-associated viral infection, but not transfer

Our findings suggest that CB₂R agonism strongly inhibits post-fusion events during viral infection in resting cells exposed to cell-free virus. Given these observations, we wanted to determine the capacity for CB₂R agonism to block viral transmission and infectivity in a cell-associated model of infection. Cell-associated viral infection is hundreds to thousands-fold more efficient than cell-free infection [33,48]. During cell-associated transmission, a synaptic structure, called the viral synapse, is formed between the infected ("donor") and non-infected ("target") cell [49]. Significant actin rearrangements accompany formation of the virological synapse [50], and these actin structures have been hypothesized to regulate viral penetration into the target cell [51]. Unlike cell-free viral infection, transfer of virus between cells is co-receptor independent; blockade of viral binding to CXCR4 with a selective antagonist, AMD3100, does not inhibit passage of virus [34]. Once virus is captured within a target cell, however, co-receptor binding is still required for viral fusion. We hypothesized that CB₂R agonism, like the CXCR4 antagonist AMD3100, would not inhibit viral transfer, but would block productive infection.

To assay the impact of CB₂R agonism on cell-associated viral transfer, we used a CXCR4-tropic NL4-3-based reporter virus called HIV Gag-iGFP, which carries an interdomain insertion of green fluorescent protein (GFP) in the core structural protein Gag [52]. Each mature viral particle contains ~5000 GFP molecules[53], so viral transmission can be measured with high sensitivity [34]. We pretreated CD4+ T cell targets with AMD3100 or JWH-133, and then co-cultured these cells with dye-labeled Jurkat donors infected with the HIV Gag-iCherry reporter virus. After three hours of co-culture, we assessed viral transmission to the target population (**Figure 7C–D**). As previously reported, we found no difference in expression of the HIV Gag-iCherry reporter virus in CD4+ T cells pretreated with AMD3100 as compared to control treated cells. Likewise, pretreatment with the CB2 agonist JWH-133 did not impair viral transfer into target cells (**Figure 7D**). Within this T cell population, transmission to memory (CD45RO+) CD4+ T cells was approximately 50% more efficient than transfer into naive (CD45RO-) cells. Transfer to both T cell subsets was as efficient in AMD3100 and JWH-133 cells as control treated cells. These findings confirm that CB₂R-mediated inhibition of CXCR4 function does not impair cell-associated HIV-1 transfer to either naive or memory T cells.

We next sought to determine whether CB₂R agonist pretreatment blocked productive infection following cell-to-cell transfer of virus. We pretreated cells with either AMD3100 or JWH-133 and then conducted a 3-hour transfer experiment using dye-labeled donor CD4+ T cells infected with the NL-GI virus, the same as
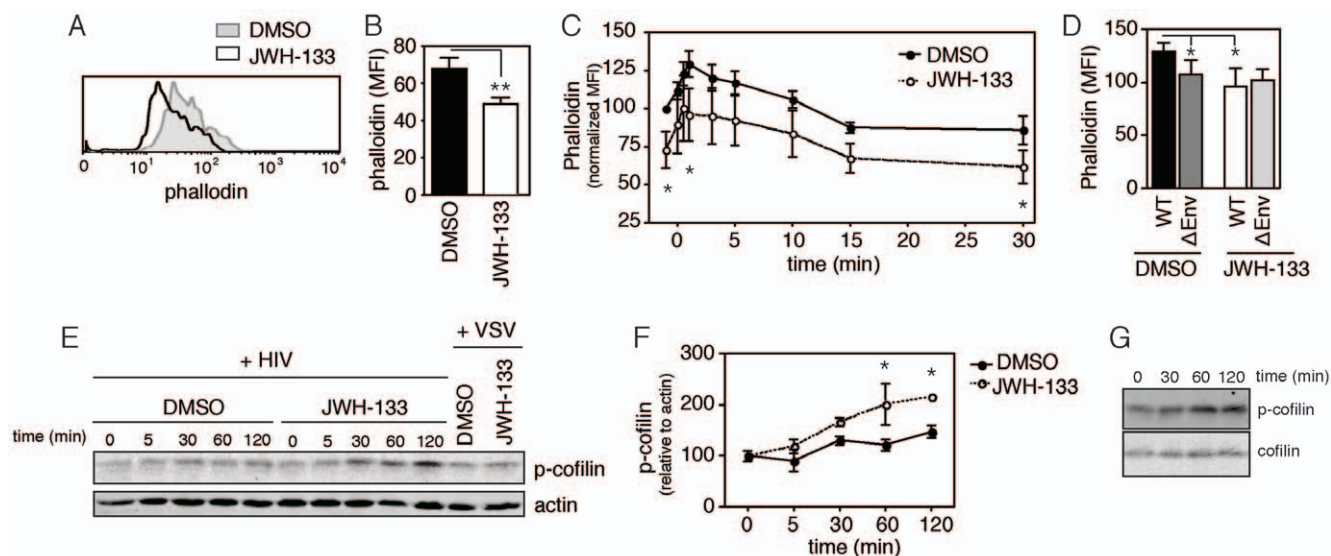
Figure 6. Alteration of actin organization in primary CD4+ T cells pretreated with CB$_2$ agonist. (A–B) Total F-actin, as measured by phalloidin binding, following 3 hours of pretreatment with 100 nM of the CB2 agonist JWH-133 in (A) a representative donor and (B) multiple donors (mean±SEM; n = 7 donors; **p = 0.0021). (C) Normalized change in phalloidin binding as a result of HIV-1-mediated CXCR4 activation in CD4+ T cells pretreated with DMSO or JWH-133 (mean±SEM; n = 4 donors; *p<0.05). (D) Mean phalloidin binding in DMSO or JWH-133 pretreated CD4+ T cells treated for 1 minute with wild type (WT) or Env null (ÄEnv) HIV-1 (mean±SEM; n = 4 donors; *p<0.05). (D–E) Cofilin phosphorylation following HIV-1 or VSV-pseudotyped viral exposure in CD4+ T cells pretreated with DMSO or JWH-133 in (D) a representative donor and (E) in multiple donors (mean±SEM; n = 3 donors; *p<0.05). (F) Cofilin phosphorylation and total cofilin following HIV-1 exposure in CD4+ T cells treated with JWH-133.
doi:10.1371/journal.pone.0033961.g006

was used for cell-free assessment of productive infection (**Figure 1**). We then sorted the naïve (CD45RO-) targets from the memory (CD45RO+) target T cell population and activated both subsets to initiate viral replication. We found that in both naive and memory T cells, both AMD3100 and JWH-133 pretreatment inhibited productive viral infection (**Figure 7E–G**). This inhibition was significant in the memory population, with CB$_2$R agonist pretreatment resulting in an approximately 50% decrease in infected cells after four days of culture (4.02±2.0% versus 7.47±3.7% control) (mean±SEM, n = 4, *p<0.05) (**Figure 7F**). Although naive cells overall exhibited productive infection at a much lower frequency than memory cells, JWH-133 pretreatment reduced the number of infected cells (0.02±0.01% versus 0.2±0.1%) (mean±SEM, n = 4) (**Figure 7E**). These results indicate that CB$_2$R agonism blocks productive viral infection after cell-associated viral exposure, just as it does with cell-free virus. The capacity for CB$_2$R agonism to block infection following viral transfer is consistent with our finding that CB$_2$R-mediated inhibition infection occurs after binding, and indeed fusion, in a cell-free system (**Figure 7A–B**). Taken together, these results are consistent with the idea that actin rearrangements inhibited by CB$_2$R, while not required for cell-associated viral transfer, are required for productive viral infection following cell-associated transfer.

Discussion

Human immunodeficiency virus type 1, (HIV-1) infection in T cells requires viral binding to two receptors, CD4+ and a chemokine co-receptor, either CXCR4 or CCR5 [54]. These co-receptors are members of the highly conserved family A of G-protein coupled receptors (GPCRs). Absence of co-receptors, or blockade of HIV-1 binding to one of these co-receptors, are both sufficient to abrogate de novo viral infection in a target cell [55]. Similarly, manipulation of these GPCRs with pharmacological

ligands that alter co-receptor recycling [56], binding pocket occupancy [57], or co-receptor activity [19] also inhibit viral replication.

Here, we report that cannabinoid activation of CB$_2$R inhibits CXCR4-tropic HIV infection by altering CD4+ T cell actin dynamics. We find that selective CB$_2$ activation blocks both cell-free and cell-associated viral infection, reducing the frequency of infected cells by 30-60% (**Figures 1,7**). This inhibition is pronounced in resting cells, which are a target of CXCR4-tropic HIV [44]. Additionally, this inhibition is mediated post-transfer during cell-to-cell infection and post-fusion in the target cell following infection with cell-free virus (**Figure 7**). We further investigated the mechanism by which CB$_2$R agonism altered HIV permissiveness. Our findings demonstrate that CB$_2$R activation at concentrations sufficient to inhibit virus does not alter CXCR4 levels of surface expression, but does significantly reduce CXCR4-mediated G-protein binding and downstream signaling (**Figure 4 and 5**). This inhibition of CXCR4 signaling is accompanied by a loss in F-actin accumulation (**Figure 6**), which may prevent the cortical actin rearrangements required for reverse transcription and migration of the viral preintegration complex to the nucleus [58]. Taken together, our results suggest that CB$_2$ cross-regulates CXCR4 and that this inhibitory cross-talk is sufficient to decrease viral infection.

Although we here identify cross-talk between the CB$_2$R and CXCR4 receptors and downstream impairment of actin dynamics, the possibility remains that CB$_2$R activation results in induction of unknown anti-viral host factors. Arguing against the possible induction of unknown anti-viral factors, CB$_2$R agonism did not block HIV infection by virus bearing the CCR5-tropic JRFL envelope (**Figure 1C**). The inability of CB$_2$ to inhibit CCR5-tropic virus suggests that CB$_2$-mediated alterations to the target cell are negligible in the predominantly memory CCR5+ CD4+ T cell subset. This effect is unlikely to be specific to memory cells as a whole, as CB$_2$R treatment efficiently blocked productive
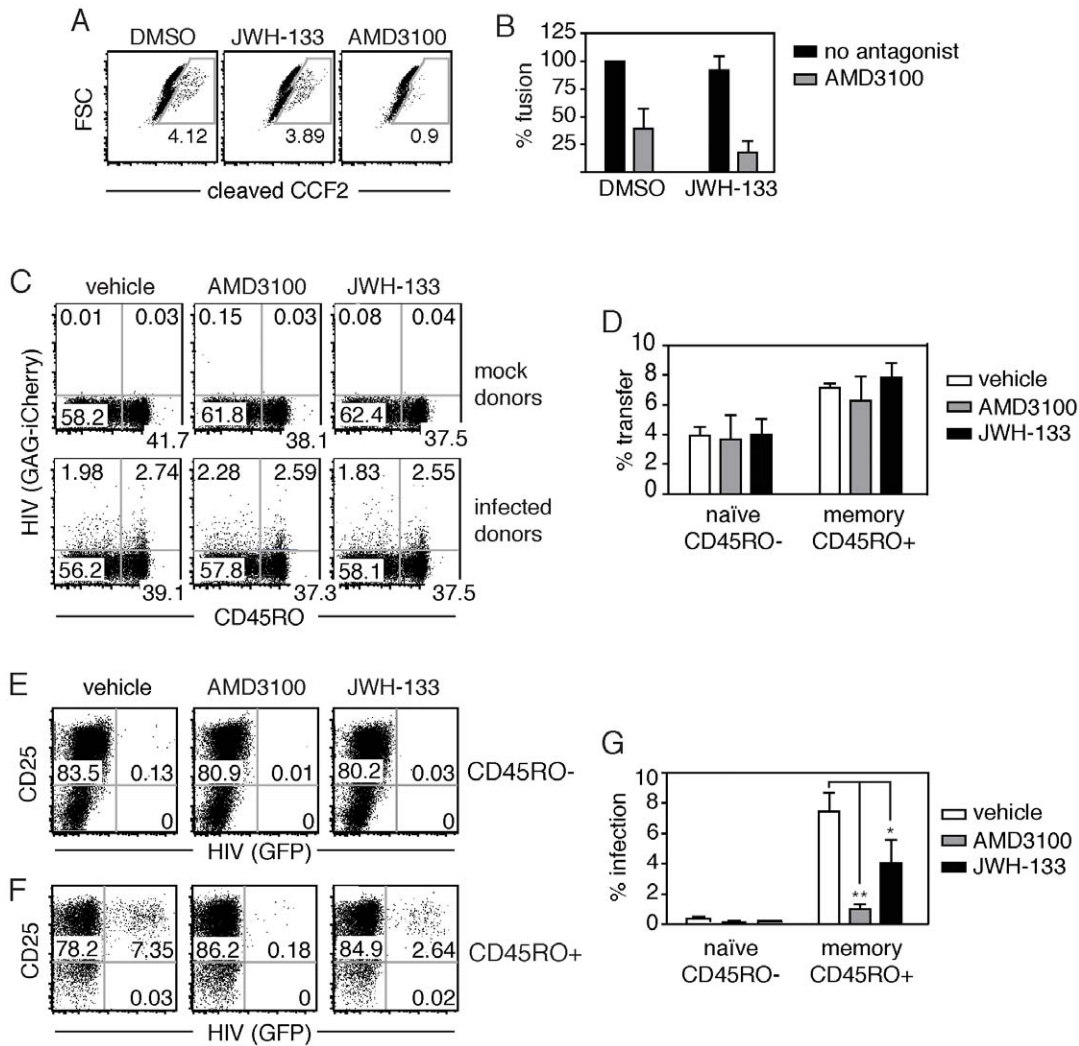
Figure 7. CB$_2$ agonism inhibits productive infection of CXCR4-tropic HIV-1, but not cell-associated transfer or viral fusion. (A–B) Viral fusion, as indicated by â-lactamase cleavage, in primary CD4+ T cells pretreated with either JWH-133 or AMD3100 in (A) a representative donor and (B) in multiple donors (mean±SEM; n = 4 donors). (C) Transfer of HIV (Gag-iCherry) from infected Jurkat donors to memory (CD45RO+) or naive (CD45RO-) primary CD4+ T cells pretreated with 100 nM of the CB2 agonist JWH-133 or the CXCR4 antagonist AMD3100, in a representative donor. (D) Quantification of viral HIV (Gag-iCherry) transfer from Jurkat donors after 3 hours in CD45RO+ and CD45RO- cells pretreated with JWH-133 or AMD3100 (mean±SEM; n = 4 donors). (E–G) Productive infection following HIV transfer from primary CD4+ donors infected with HIV (GFP). Pretreated CD4+ targets were cocultured with infected CD4+ donor for 3 hours, sorted by CD45RO expression, and then activated. HIV (GFP) expression is shown in (E) CD45RO- naive or (F) CD45RO+ memory CD4+ T cells after 4 days of activation with IL-2 and anti-CD3/CD28 or anti-CD3 alone, respectively. (G) Quantification of productive infection post-activation in sorted CD45RO- or CD45RO+ CD4+ T cells pretreated with JWH-133 or AMD3100 (mean±SEM; n = 4 donors; **p = 0.0099; *p<0.05).
doi:10.1371/journal.pone.0033961.g007

infection in both memory and naive cell subsets following infection with cell-associated X4-tropic HIV-1 (**Figure 7F–G**). Rather, these findings may indicate that infection with CCR5-tropic virus is less dependent on chemokine-receptor mediated signaling and *de novo* cytoskeletal rearrangement for productive infection.

The CB$_2$R may be considered as an adjunct therapeutic target for inhibition of CXCR4-tropic viral spread to resting T cell populations in patients with AIDS. A particularly compelling therapeutic rationale for the evaluation of antiviral affects of CB$_2$R agonists may be to address severe symptoms of cachexia or neuropathic pain which may also present in patients with AIDS, without the adverse neurological or behavioral side effects associated with CB$_1$R agonism [59]. Although the effect of CB$_2$R agonists on HIV infection is moderate, an accumulated effect in patients treated daily for pain could explain positive

effects on viral load over time. We find that CB$_2$R agonist pretreatment of resting cells inhibits viral spread in a receptor-selective manner in resting cells and does broadly inhibit T cell activation (**Figure 2**). Although previous studies have indicated that pan-cannabinoid agonists can possess an immunosuppressive function *in vitro* [11] and *in vivo* [42,43], ablation of CB$_2$R in mice was not found to increase T cell number, proliferation, or apoptosis in the periphery [14]. Immunosuppression by CB$_2$R may be attributed in part to drug toxicity at high concentrations of cannabinoid agonist. Indeed, the use of cannabinoid drugs in patients with HIV is associated with an increase, rather than a decrease, in CD4+ T cell number [1] and has been shown to reduce viral load in SIV infected rhesus macaques [9]. It is possible that novel CB$_2$R-specific agonists and allosteric modulators that exert potent anti-viral activity without inducing immunosuppres-

sion could be identified. Further study of cannabinoids and other neuroendocrine regulators that selectively modulate immune function may result in the discovery of new anti-viral drugs that can also mitigate AIDS-associated symptoms.

Acknowledgments

Author Contributions

References

1. Abrams DI, Hilton JF, Leiser RJ, Shade SB, Elbeik TA, et al. (2003) Short-term effects of cannabinoids in patients with HIV-1 infection: a randomized, placebo-controlled clinical trial. Ann Intern Med 139: 258–266.

2. Ben Amar M (2006) Cannabinoids in medicine: A review of their therapeutic potential. J Ethnopharmacol 105: 1–25.

3. Abrams DI, Jay CA, Shade SB, Vizoso H, Reda H, et al. (2007) Cannabis in painful HIV-associated sensory neuropathy: a randomized placebo-controlled trial. Neurology 68: 515–521.

4. ElSohly MA, deWit H, Wachtel SR, Feng S, Murphy TP (2001) Delta9-tetrahydrocannabivarin as a marker for the ingestion of marijuana versus Marinol: results of a clinical study. J Anal Toxicol 25: 565–571.

5. Hoffmann DE, Weber E (2010) Medical marijuana and the law. N Engl J Med 362: 1453–1457.

6. Newell GR, Mansell PW, Wilson MB, Lynch HK, Spitz MR, et al. (1985) Risk factor analysis among men referred for possible acquired immune deficiency syndrome. Prev Med 14: 81–91.

7. Tindall B, Cooper DA, Donovan B, Barnes T, Philpot CR, et al. (1988) The Sydney AIDS Project: development of acquired immunodeficiency syndrome in a group of HIV seropositive homosexual men. Aust N Z J Med 18: 8–15.

8. Bredt BM, Higuera-Alhino D, Shade SB, Hebert SJ, McCune JM, et al. (2002) Short-term effects of cannabinoids on immune phenotype and function in HIV-1-infected patients. J Clin Pharmacol 42: 82S–89S.

9. Molina PE, Winsauer P, Zhang P, Walker E, Birke L, et al. (2011) Cannabinoid Administration Attenuates the Progression of Simian Immunodeficiency Virus. AIDS Res Hum Retroviruses.

10. Demuth DG, Molleman A (2006) Cannabinoid signalling. Life Sci 78: 549–563.

11. Cencioni MT, Chiurchiu V, Catanzaro G, Borsellino G, Bernardi G, et al. (2010) Anandamide suppresses proliferation and cytokine release from primary human T-lymphocytes mainly via CB2 receptors. PLoS One 14; 5(1): e8688.

12. Borner C, Hollt V, Sebald W, Kraus J (2007) Transcriptional regulation of the cannabinoid receptor type 1 gene in T cells by cannabinoids. J Leukoc Biol 81: 336–343.

13. Klein TW, Newton CA (2007) Therapeutic potential of cannabinoid-based drugs. Adv Exp Med Biol 601: 395–413.

14. Maresz K, Pryce G, Ponomarev ED, Marsicano G, Croxford JL, et al. (2007) Direct suppression of CNS autoimmune inflammation via the cannabinoid receptor CB1 on neurons and CB2 on autoreactive T cells. Nat Med 13: 492–497.

15. Downer EJ, Clifford E, Gran B, Nel HJ, Fallon PG, et al. (2011) Identification of the Synthetic Cannabinoid R(+)WIN55,212-2 as a Novel Regulator of IFN Regulatory Factor 3 Activation and IFN-{beta} Expression: RELEVANCE TO THERAPEUTIC EFFECTS IN MODELS OF MULTIPLE SCLEROSIS. J Biol Chem 286: 10316–10328.

16. Ghosh S, Preet A, Groopman JE, Ganju RK (2006) Cannabinoid receptor CB2 modulates the CXCL12/CXCR4-mediated chemotaxis of T lymphocytes. Mol Immunol 43: 2169–2179.

17. Pereira JP, An J, Xu Y, Huang Y, Cyster JG (2009) Cannabinoid receptor 2 mediates the retention of immature B cells in bone marrow sinusoids. Nat Immunol 10: 403–411.

18. Jiang S, Zagozdzon R, Jorda MA, Parmar K, Fu Y, et al. (2010) Endocannabinoids are expressed in bone marrow stromal niches and play a role in interactions of hematopoietic stem and progenitor cells with the bone marrow microenvironment. J Biol Chem 285: 35471–35478.

19. Yoder A, Yu D, Dong L, Iyer SR, Xu X, et al. (2008) HIV envelope-CXCR4 signaling activates cofilin to overcome cortical actin restriction in resting CD4 T cells. Cell 134: 782–792.

20. Cameron PU, Saleh S, Sallmann G, Solomon A, Wightman F, et al. (2010) Establishment of HIV-1 latency in resting CD4+ T cells depends on chemokine-induced changes in the actin cytoskeleton. Proc Natl Acad Sci U S A 107: 16934–16939.

21. Saleh S, Solomon A, Wightman F, Xhilaga M, Cameron PU, et al. (2007) CCR7 ligands CCL19 and CCL21 increase permissiveness of resting memory CD4+ T cells to HIV-1 infection: a novel model of HIV-1 latency. Blood 110: 4161–4164.

22. Bleul CC, Wu L, Hoxie JA, Springer TA, Mackay CR (1997) The HIV coreceptors CXCR4 and CCR5 are differentially expressed and regulated on human T lymphocytes. Proc Natl Acad Sci U S A 94: 1925–1930.

23. Connor RI, Sheridan KE, Ceradini D, Choe S, Landau NR (1997) Change in coreceptor use correlates with disease progression in HIV-1–infected individuals. J Exp Med 185: 621–628.

24. Fenyo EM (1996) HIV-1 biological phenotype varies with severity of infection across HIV-1 genetic subtypes. Antibiot Chemother 48: 49–55.

25. Blaak H, van't Wout AB, Brouwer M, Hooibrink B, Hovenkamp E, et al. (2000) In vivo HIV-1 infection of CD45RA(+)CD4(+) T cells is established primarily by syncytium-inducing variants and correlates with the rate of CD4(+) T cell decline. Proc Natl Acad Sci U S A 97: 1269–1274.

26. Eckstein DA, Penn ML, Korin YD, Scripture-Adams DD, Zack JA, et al. (2001) HIV-1 actively replicates in naive CD4(+) T cells residing within human lymphoid tissues. Immunity 15: 671–682.

27. Zack JA, Arrigo SJ, Weitsman SR, Go AS, Haislip A, et al. (1990) HIV-1 entry into quiescent primary lymphocytes: molecular analysis reveals a labile, latent viral structure. Cell 61: 213–222.

28. Hur EM, Kim KT (2002) G protein-coupled receptor signalling and cross-talk: achieving rapidity and specificity. Cell Signal 14: 397–405.

29. Doranz BJ, Grovit-Ferbas K, Sharron MP, Mao SH, Goetz MB, et al. (1997) A small-molecule inhibitor directed against the chemokine receptor CXCR4 prevents its use as an HIV-1 coreceptor. J Exp Med 186: 1395–1400.

30. Berger EA, Murphy PM, Farber JM (1999) Chemokine receptors as HIV-1 coreceptors: roles in viral entry, tropism, and disease. Annu Rev Immunol 17: 657–700.

31. Vila-Coro AJ, Mellado M, Martin de Ana A, Lucas P, del Real G, et al. (2000) HIV-1 infection through the CCR5 receptor is blocked by receptor dimerization. Proc Natl Acad Sci U S A 97: 3388–3393.

32. Rodriguez-Frade JM, del Real G, Serrano A, Hernanz-Falcon P, Soriano SF, et al. (2004) Blocking HIV-1 infection via CCR5 and CXCR4 receptors by acting in trans on the CCR2 chemokine receptor. EMBO J 23: 66–76.

33. Chen P, Hubner W, Spinelli MA, Chen BK (2007) Predominant mode of human immunodeficiency virus transfer between T cells is mediated by sustained Env-dependent neutralization-resistant virological synapses. J Virol 81: 12582–12595.

34. Hubner W, McNerney GP, Chen P, Dale BM, Gordon RE, et al. (2009) Quantitative 3D video microscopy of HIV transfer across T cell virological synapses. Science 323: 1743–1747.

35. Cohen GB, Gandhi RT, Davis DM, Mandelboim O, Chen BK, et al. (1999) The selective downregulation of class I major histocompatibility complex proteins by HIV-1 protects HIV-infected cells from NK cells. Immunity 10: 661–671.

36. O'Brien WA, Koyanagi Y, Namazie A, Zhao JQ, Diagne A, et al. (1990) HIV-1 tropism for mononuclear phagocytes can be determined by regions of gp120 outside the CD4-binding domain. Nature 348: 69–73.

37. Cavrois M, De Noronha C, Greene WC (2002) A sensitive and specific enzyme-based assay detecting HIV-1 virion fusion in primary T lymphocytes. Nat Biotechnol 20: 1151–1154.

38. Gomes I, Filipovska J, Devi LA (2003) Opioid receptor oligomerization. Detection and functional characterization of interacting receptors. Methods Mol Med 84: 157–183.

39. Huffman JW (1999) Cannabimimetic indoles, pyrroles and indenes. Curr Med Chem 6: 705–720.

40. Lovinger DM (2008) Presynaptic Modulation by Endocannabinoids. Springer, Berlin.

41. Rock RB, Gekker G, Hu S, Sheng WS, Cabral GA, et al. (2007) WIN55,212-2-mediated inhibition of HIV-1 expression in microglial cells: involvement of cannabinoid receptors. J Neuroimmune Pharmacol 2: 178–183.

42. Zhu LX, Sharma S, Stolina M, Gardner B, Roth MD, et al. (2000) Delta-9-tetrahydrocannabinol inhibits antitumor immunity by a CB2 receptor-mediated, cytokine-dependent pathway. J Immunol 165: 373–380.

43. Klein TW, Newton CA, Nakachi N, Friedman H (2000) Delta 9-tetrahydro-cannabinol treatment suppresses immunity and early IFN-gamma, IL-12, and IL-12 receptor beta 2 responses to Legionella pneumophila infection. J Immunol 164: 6461–6466.

44. Wu Y, Yoder A (2009) Chemokine coreceptor signaling in HIV-1 infection and pathogenesis. PLoS Pathog 5: e1000520.

45. Bukrinsky M (2008) How to engage Cofilin. Retrovirology 5: 85.

46. Wu Y, Yoder A, Yu D, Wang W, Liu J, et al. (2008) Cofilin activation in peripheral CD4 T cells of HIV-1 infected patients: a pilot study. Retrovirology 5: 95.

47. Vorster PJ, Guo J, Yoder A, Wang W, Zheng Y, et al. (2011) LIM Kinase 1 Modulates Cortical Actin and CXCR4 Cycling and Is Activated by HIV-1 to Initiate Viral Infection. J Biol Chem 286: 12554–12564.

48. Dimitrov DS, Willey RL, Sato H, Chang LJ, Blumenthal R, et al. (1993) Quantitation of human immunodeficiency virus type 1 infection kinetics. J Virol 67: 2182–2190.

49. Piguet V, Sattentau Q (2004) Dangerous liaisons at the virological synapse. J Clin Invest 114: 605–610.

50. Jolly C, Kashefi K, Hollinshead M, Sattentau QJ (2004) HIV-1 cell to cell transfer across an Env-induced, actin-dependent synapse. J Exp Med 199: 283–293.

51. Vasiliver-Shamis G, Tuen M, Wu TW, Starr T, Cameron TO, et al. (2008) Human immunodeficiency virus type 1 envelope gp120 induces a stop signal and virological synapse formation in noninfected CD4+ T cells. J Virol 82: 9445–9457.

52. Hubner W, Chen P, Del Portillo A, Liu Y, Gordon RE, et al. (2007) Sequence of human immunodeficiency virus type 1 (HIV-1) Gag localization and oligomerization monitored with live confocal imaging of a replication-competent, fluorescently tagged HIV-1. J Virol 81: 12596–12607.

53. Briggs JA, Simon MN, Gross I, Krausslich HG, Fuller SD, et al. (2004) The stoichiometry of Gag protein in HIV-1. Nat Struct Mol Biol 11: 672–675.

54. Alkhatib G (2009) The biology of CCR5 and CXCR4. Curr Opin HIV AIDS 4: 96–103.

55. Simmons G, Reeves JD, Hibbitts S, Stine JT, Gray PW, et al. (2000) Co-receptor use by HIV and inhibition of HIV infection by chemokine receptor ligands. Immunol Rev 177: 112–126.

56. Amara A, Gall SL, Schwartz O, Salamero J, Montes M, et al. (1997) HIV coreceptor downregulation as antiviral principle: SDF-1alpha-dependent internalization of the chemokine receptor CXCR4 contributes to inhibition of HIV replication. J Exp Med 186: 139–146.

57. Wu B, Chien EY, Mol CD, Fenalti G, Liu W, et al. (2011) Structures of the CXCR4 chemokine GPCR with small-molecule and cyclic peptide antagonists. Science 330: 1066–1071.

58. Yu D, Wang W, Yoder A, Spear M, Wu Y (2009) The HIV envelope but not VSV glycoprotein is capable of mediating HIV latent infection of resting CD4 T cells. PLoS Pathog 5: e1000633.

59. Moreira FA, Grieb M, Lutz B (2009) Central side-effects of therapies based on CB1 cannabinoid receptor agonists and antagonists: focus on anxiety and depression. Best Pract Res Clin Endocrinol Metab 23: 133–144.