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Chronic Δ -9-tetrahydrocannabinol Administration Increases Lymphocyte CXCR4 Expression in Rhesus Macaques

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Abstract

Cannabinoids have been reported to produce various immunomodulatory effects, which could potentially impact the host response to bacterial or viral infection. We have recently demonstrated that chronic Δ -9-tetrahydrocannabinol (THC; 0.32 mg/kg i.m., BID) decreased early mortality in rhesus macaques infected with simian immunodeficiency virus (SIV). However, the possibility that prolonged THC administration affects lymphocyte counts, phenotype, and proliferation indices has not been addressed. We examined expression of proliferative and phenotypic markers in circulating lymphocytes of male young adult rhesus macaques chronically-treated with THC (i.m. twice daily 0.32 mg/kg) for 12 months. Chronic THC administration did not alter lymphocyte subtypes, naïve and memory subsets, proliferation, or apoptosis of T lymphocytes when compared to time-matched vehicle-treated controls. However, chronic THC increased T lymphocyte CXCR4 expression on both CD4+ and CD8+ T lymphocytes compared to control. These results show that chronic THC administration produces changes in T cell phenotype, which can potentially contribute to host immunomodulation to infectious challenges.

Keywords

Flow cytometry; Lymphocytes; Cannabinoids; CXCR4; Rhesus macaques

Introduction

Cannabinoids, including Δ -9-tetrahydrocannabinol (THC), have been shown to alter several aspects of immune function, including cytokine production and lymphocyte phenotype, function and survival (Yuan et al. 2002; Klein et al. 2003). In addition, cannabinoids have been reported to decrease cell-mediated immunity and the suppression of T cell proliferation (Nahas et al. 1977; Shay et al. 2003). Overall, results from in vivo and in vitro studies suggest that cannabinoids produce generalized immunosuppressive effects (Klein et al. 2003), which in some clinical studies have been reported to be associated with increased incidence of infection (Tindall et al. 1988). Recent studies from our laboratory have demonstrated decreased early mortality from simian immunodeficiency virus (SIV) infection in THC-treated rhesus macaques (Molina et al. 2010). This was not associated with significant alterations in the SIV-induced decrease in total lymphocyte counts, CD4+/CD8+, or markers of proliferation or apoptosis. Moreover, no significant differences in lymphocyte phenotypes were detected following 28 days of THC administration. The possibility that more prolonged THC administration can differentially modulate lymphocyte phenotype, and thereby host response to infections, has been proposed but not examined in a controlled in vivo setting. This study investigated the consequences of 12 months of chronic (twice daily 0.32 mg/kg) intramuscular administration of THC to rhesus macaques on white blood cell counts, leukocyte differentials, and lymphocyte phenotype.

Materials and methods

All experiments were approved by the Institutional Animal Care and Use Committee at Louisiana State University Health Sciences Center in New Orleans and adhered to the NIH guidelines for the use of experimental animals. Housed individually in aluminum cages (BREC, Inc., Bryan, TX, USA) in a Biosafety level-2 containment room maintained on a 12 h/12 h light–dark cycle at approximately 22°C with 30–70% relative humidity are 4–5-year-old male rhesus monkeys (*Macaca mulatta*) obtained from the New Iberia Primate Center, LA, USA. Animals were assigned to either: vehicle (VEH)-treated; $N=3$, or cannabinoid-treated (THC), $N=4$. Animals were fed standard primate chow (Formula 2050, Harlan Teklad, Madison, WI, USA), vitamins, and fruits.

After a 3-month quarantine period, THC or VEH treatments were initiated. THC was obtained from the National Institute on Drug Abuse (Research Technical Branch, Rockville, MD, USA). THC solution was lyophilized, stored frozen in aliquots, and prepared as an emulsion using alcohol, emulphor, and saline (1:1:18) as vehicle prior to use. Chronic administration of THC was initiated with 0.18 mg/kg BID and increased over 2 weeks to the final dose of 0.32 mg/kg BID. Intramuscular administration was chosen to reduce experimental variability. This protocol of THC administration produces similar neurobehavioral defects as seen following smoked marijuana in humans and produces tolerance over time (Winsauer et al. 2011).

Blood samples were obtained prior to initiation of chronic THC administration and at the 12 month time-point from both THC-treated and VEH-treated animals. Our previous studies have examined the impact of chronic THC administration, starting 28 days prior to SIV infection. THC-treated animals showed significant improvement in morbidity and mortality. However, prolonged chronic THC treatment may alter the immune system and, in turn, impact on the ability of the host to respond to infection. Thus, we chose to assess lymphocyte populations in the peripheral blood after 12 months of chronic THC treatment. Complete and differential blood counts were performed using a Beckman Coulter LH755 for total leukocyte counts and Wright–Giemsa staining of blood smears for leukocyte differentials. Blood lymphocyte subsets were determined by flow cytometric analysis using monoclonal antibodies and isotype controls. All antibodies were purchased from BD Biosciences (San Jose, CA, USA), with the exception of Pacific Blue[®] anti-human CD4 (eBioscience, San Diego, CA, USA). The Basic Profile and Ki67 (index of proliferation) assays were performed on 100 μ l of EDTA-treated whole blood. For the Basic Profile assay, PE-Cy-7 anti-CD3, PerCP anti-CD8, APC-Cy-7 anti-CD20, PerCP-Cy 5.5 anti-CD28, FITC anti-CD95, PE anti-CD184, APC anti-CD195, and Pacific Blue anti-CD4 were used. Erythrocytes were lysed using RBC Lysis Buffer (BD Biosciences) according to the manufacturer's instructions. Samples were fixed with 1% paraformaldehyde. For the Ki67 assay, PE anti-CD3e, APC anti-CD4, and PerCP anti-CD8 were used and erythrocytes lysed. Samples were permeabilized, incubated with FITC anti-Ki67, and fixed with 1% paraformaldehyde. For the caspase 3 assay (index of apoptosis), PBMCs were isolated by standard Ficoll-Paque Plus protocol (GE Healthcare, Piscataway, NJ, USA), and resuspended in RPMI-1640+10% FCS (RPMI). A caspase detection kit (APO LOGIX/FAM-DEVD-FMK) was used according to the manufacturer's instructions, and cell surface marker staining was performed as previously described for Ki67 assay. Samples were fixed with provided fixative. Cells were analyzed in the LSUHSC Comprehensive Alcohol Research Center Core Laboratory on a BD FACS Aria flow cytometer for the Basic Profile assay, and the BD FACSCalibur for the Ki67 and Caspase3 assays.

All data are presented as mean $\pm$ SEM for VEH-treated ($N=3$) and THC-treated ($N=4$) groups. The percent of gated lymphocytes expressing the indicated markers was assessed.

Treatment effects were established by *t*-test with significance set at $p \leq 0.05$, using SigmaStat 3.1 (Systat Software, Inc., San Jose, CA, USA).

Results

Chronic THC administration did not result in significant changes in total white blood cell or differential counts (Table 1). In addition, chronic THC administration did not alter the population expressing CD20 or CD3 (markers for B and T lymphocytes, respectively), nor did it change the percent of CD4+ or CD8+ lymphocytes (helper T and cytotoxic T lymphocytes, respectively) (Table 2). Similarly, no alterations in the percent of CD4+ or CD8+ naïve (CD95–CD28+), effector memory (CD95+CD28–), and central memory (CD95+CD28+) cell subsets were noted. Chronic THC did not alter the percent of lymphocytes expressing Ki67 or caspase 3 in either CD4+ or CD8+ subsets (Table 2).

In contrast, chronic THC administration resulted in a higher frequency of CXCR4 expression in CD4+ and CD8+ lymphocytes as compared to controls. The frequency of CCR5 expression in either lymphocyte subpopulation was not altered by chronic THC administration (Table 3).

Discussion

We examined the impact of chronic THC administration on peripheral blood lymphocyte counts and phenotype in rhesus macaques. Chronic THC administration resulted in increased frequency of CXCR4 expression in CD4+ and CD8+ lymphocytes. This modulation of CXCR4 expression was not associated with significant alterations in CCR5 expression, circulating lymphocyte counts, frequency of B and T lymphocyte marker expression, T lymphocyte memory subset markers, or markers of proliferation or apoptosis in T lymphocytes.

Our results did not show significant differences in the percent of either white blood cells or white blood cell subtypes. However, there was a trend for a decreased percentage of lymphocytes in the THC-treated animals vs. control (Table 1). There was no difference in B cells or CD4+ and CD8+ T cells between groups (Table 2). Kawakami et al. (1988) showed decreased proliferation of cloned NK cells with THC treatment. We did not measure NK cells in this study and do not know how chronic *in vivo* THC affects this population. However, if chronic THC decreases NK cell proliferation, it would explain the trend for decreased lymphocytes in this group. Previous studies have reported modest or transient changes in lymphocyte populations (Wallace et al. 1994), which we speculate to bear relatively minor, if any, significance on the overall immune response. This is based on the overall lack of significant modulation in lymphocyte counts and populations in chronic THC-treated SIV-infected macaques, as reported recently (Molina et al. 2010). It is quite possible that differential responses may be dependent on the dose of THC, the circulating and tissue concentrations achieved, as well as the route of administration. Our results also showed a trend for an increased percentage of neutrophils in the THC-treated monkeys vs. control (Table 1). Studies have shown that CB₂ receptor agonists inhibit neutrophil migration and recruitment *in vitro* and *in vivo* (Kurihara et al. 2006; Murikinati et al. 2010). Thus, a possible explanation for the trend for increased neutrophils in the blood may be that THC treatment decreased neutrophil migration from the peripheral blood to tissues. This remains to be explored.

Chronic THC treatment of rhesus macaques did not change circulating lymphocyte proliferative or apoptotic markers. This observation is also in contrast with previous reports in the literature describing decreased mitogen-induced proliferation in isolated peripheral

blood mononuclear cells from marijuana smokers (Petersen et al. 1976), as well as, reports indicating suppressed proliferative response in lymphocytes isolated from naïve animals or humans and incubated in the presence of micromolar concentrations of THC (Nahas et al. 1977; Pross et al. 1990). However, our results are in agreement with those of others that have failed to demonstrate an inhibitory effect of THC on lymphocyte proliferation (White et al. 1975; Zhu et al. 1998). In contrast to previous reports in the literature describing cannabinoid-induced increased apoptosis (Zhu et al. 1998; Jia et al. 2006), our results did not show any significant effects of chronic THC administration on lymphocyte apoptosis. Similar results have been obtained following in vitro THC exposure (0.32 µg/ml for 12 days) of peripheral blood mononuclear cells isolated from naïve rhesus monkeys (unpublished observations). Moreover, our previous studies have shown that the increased lymphocyte proliferative and apoptotic responses seen during the initial phase of SIV infection are not inhibited by chronic THC administration (Molina et al. 2010).

Our results did not show significant alteration in lymphocyte expression of CCR5. In contrast, studies have reported that microglia exposed to the cannabinoid receptor agonist WIN55, 212-2 for 48 h results in approximately 40% inhibition of CCR5 expression compared to cells exposed to an inactive enantiomer (Rock et al. 2007). We did, however, find that expression of CXCR4, particularly in CD8+ T lymphocytes, was markedly upregulated in THC-treated animals. The chemokine receptors CCR5 and CXCR4 have been demonstrated to be the most important coreceptors in HIV-1 pathogenesis. Most HIV-1 variants isolated from newly infected individuals use CCR5 as a coreceptor to enter activated CD4+ lymphocytes and macrophages. Over the course of the disease, some HIV-1 variants that utilize CXCR4 and target naïve and resting CD4+ cells may emerge in some (~50%) infected individuals (Schuitemaker et al. 1992). One could speculate that the increased CD4+ CXCR4+ lymphocyte population could impact infectivity with CXCR4 variants. However, previous studies from our laboratory have demonstrated that chronic THC administration to SIV-infected rhesus macaques decreases viral load in plasma and cerebrospinal fluid and decreases early mortality. In addition, we have also demonstrated that THC reduces viral replication in a MT4 cell line (Molina et al. 2010), a setting in which CXCR4 co-receptor expression is not able to be modulated by THC. Thus, these results would suggest that chronic THC-mediated modulation of CXCR4 expression is not required for THC-mediated suppression of viral replication in infected T cells.

Alternatively, the observed increase in CXCR4 on CD4+ and CD8+ cells may reflect greater efficacy in chemokine-induced lymphocyte recruitment to sites of inflammation. CXCL2, also known as stromal derived factor-1 (SDF-1), the ligand for CXCR4, has been reported to promote transendothelial migration of T cells and could thus contribute to naïve T cell recruitment to sites of infection (Phillips and Ager 2002). It is also important to note that naïve T lymphocytes have higher expression of CXCR4 than do memory T lymphocytes, and thus are more responsive to chemokine-mediated chemotaxis (Bleul et al. 1997).

We found that CXCR4 mean channel fluorescence on CD4+ and CD8+ T cells was increased in THC-treated animals (VEH 436 ± 228 and THC 591 ± 52 , $p=0.057$ and VEH 622 ± 50 and THC 905 ± 115 , $p=0.114$, respectively; data not shown). These data indicate that CXCR4 receptor density is greater on T cells from chronic THC-treated animals. This increased expression of CXCR4 may indicate that THC is either upregulating CXCR4 or that thymic output of naïve T cells is greater in these animals. It has been shown that upregulation of CXCR4 in human T cells is partially mediated by cAMP (Cristillo et al. 2002). Interestingly, signaling through both cannabinoid receptor subtypes CB₁ and CB₂ leads to decreased adenylate cyclase activity and intracellular cAMP accumulation (Condie et al. 1996). However, these measurements were made at 10 min post-THC treatment. Borner et al. (2009) showed that THC treatment caused an initial decrease (up to 1 hour) in

cAMP that was followed by a sustained (at 48 hours) and profound increased in cAMP (up to 10-fold). Together, these findings indicate that chronic THC treatment may increase cAMP in T cells, thus driving the expression of CXCR4. Alternatively, chronic THC may have increased the output of naïve T cells from the thymus. CXCR4 is expressed on naïve T cells, and when activated, the naïve T cells differentiate into memory T lymphocytes and downregulate CXCR4. HIV and SIV decrease naïve T cell output from the thymus (Dion et al. 2004; Sopper et al. 2003), and thus, it is possible that by increasing T cell output, THC may help to maintain naïve T cell populations during HIV/SIV infection. During SIV infection, we did not find changes in CXCR4 expression on CD4+ and CD8+ T cells in VEH-vs. THC-treated animals when viral load was lower in THC-treated macaques (2, 3, 4, and 5 months; unpublished data). However, in that study, animals were pre-treated with THC for only 28 days before SIV infection. While not significant, we currently show a trend for decreased proliferation of T cells in chronic THC-treated animals. These data indicate that chronic THC may lead to less activation of T cells, resulting in more cells expressing a naïve phenotypes. The decreased activation, resulting in decreased proliferation, may be due to THC's effects on antigen presenting cells. We have not measured this, but will do so in the future, as it may provide mechanistic insight into THC's impact on SIV disease progression.

In summary, we demonstrate that chronic THC treatment in an in vivo non-human primate model has minimal impact on changes in lymphocyte subpopulations, naïve and memory subsets, and lymphocyte functional activities including proliferation and apoptosis at doses tested. Chronic THC administration increased CD8+ and CD4+ lymphocyte expression of CXCR4, which may potentially contribute to T cell homing to sites of infection. Because chronic THC administration did not alter T cell phenotype toward resistance to viral entry, we speculate that THC-mediated suppression of viral replication in infected T cells may play a central role in attenuation of viral load and disease progression.

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Table 1

White blood cell complete and differential counts in VEH- and THC-treated rhesus macaques

Complete blood count	VEH-treated	THC-treated	<i>p</i>
White blood cells ($\times 10^3/\mu\text{l}$)	5.29 $\pm$ 1.63	5.60 $\pm$ 0.40	NS
% Neutrophils	39.33 $\pm$ 10.89	61.85 $\pm$ 8.06	NS
% Lymphocytes	50.73 $\pm$ 10.27	31.20 $\pm$ 8.93	NS
% Monocytes	5.43 $\pm$ 0.66	4.58 $\pm$ 0.57	NS
% Eosinophils	1.57 $\pm$ 0.85	2.25 $\pm$ 1.22	NS
% Basophils	2.90 $\pm$ 1.80	0.15 $\pm$ 0.03	NS
Hemoglobin (g/dL)	13.43 $\pm$ 0.24	12.90 $\pm$ 0.35	NS
Hematocrit (%)	41.00 $\pm$ 0.85	38.38 $\pm$ 0.92	NS
Platelets ($\times 10^3/\mu\text{l}$)	352.00 $\pm$ 21.13	256.25 $\pm$ 34.99	NS

Table 2

Blood lymphocyte subsets, memory subtypes, proliferation, and apoptotic indices in cells isolated from VEH- and THC-treated rhesus macaques

	VEH-treated	THC-treated	P
Lymphocyte type (%)			
CD20+	14.20±4.45	17.60±3.02	NS
CD3+	64.63±11.38	71.87±5.226	NS
CD3+ CD4+	58.13±1.48	67.2±4.384	NS
CD3+ CD8+	41.53±1.49	32.20±4.276	NS
CD4/CD8	1.41±0.08	2.50±0.73	NS
CD4+ memory subtypes			
CD95+ CD28+	22.30±12.43	18.70±2.95	NS
CD95+ CD28−	32.83±3.64	26.83±5.98	NS
CD95− CD28+	1.50±0.46	2.55±0.93	NS
CD8+ memory subtypes			
CD95+ CD28+	13.17±5.98	15.23±2.23	NS
CD95+ CD28−	43.93±4.42	37.90±4.12	NS
CD95− CD28+	2.70±1.03	5.18±2.07	NS
Proliferation			
CD4+ Ki67+	2.28±0.86	0.88±0.60	NS
CD8+ Ki67+	2.50±0.89	0.79±0.60	NS
Apoptosis			
CD4+ Casp3+	0.17±0.06	0.15±0.07	NS
CD8+ Casp3+	0.16±0.07	0.19±0.07	NS

Values are mean ± SEM for VEH-treated (N=3) and THC-treated (N=4) animals

Table 3

CXCR4 and/or CCR5 expression in CD4+ and CD8+ T lymphocytes of VEH- and THC-treated rhesus macaques

	VEH-treated	THC-treated	<i>P</i>
CD4+ chemokine receptors (%)			
Total CCR5+	13.20±3.70	10.08±5.60	NS
CCR5+ CXCR4−	10.53±2.76	5.30±1.80	NS
CCR5+ CXCR4+	2.67±1.04	2.73±0.93	NS
CCR5− CXCR4+	35.20±5.62	60.37±5.01	0.020
Total CXCR4+	37.87±6.44	61.40±3.35	NS
CD8+ chemokine receptors (%)			
Total CCR5+	30.77±6.27	26.80±5.47	NS
CCR5+ CXCR4−	28.20±5.69	15.47±3.31	NS
CCR5+ CXCR4+	2.57±0.95	6.47±0.62	0.024
CCR5− CXCR4+	23.57±5.19	53.30±4.00	0.010
Total CXCR4+	26.13±5.59	57.6±7.35	0.004

Values are mean ± SEM for VEH-treated (*N*=3) and THC-treated (*N*=4) animals