Cannabinoid Administration Attenuates the Progression of Simian Immunodeficiency Virus

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Running title: chronic THC decreases early mortality from SIV

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Abstract

Δ^9-tetrahydrocannabinol (Δ^9-THC), the primary psychoactive component in marijuana, is FDA-approved to ameliorate AIDS-associated wasting. Because cannabinoid receptors are expressed on cells of the immune system, chronic Δ^9-THC use may impact HIV disease progression. We examined the impact of chronic Δ^9-THC administration (0.32mg/kg i.m., 2 X daily), starting 28 days prior to inoculation with simian immunodeficiency virus (SIV_{mac251}; 100 $TCID_{50}$/ml, i.v.), on immune and metabolic indicators of disease during the initial 6 month asymptomatic phase of infection in rhesus macaques. $SIV_{mac251.}$ Inoculation resulted in measurable viral load, decreased lymphocyte CD4+/CD8+ ratio, and increased CD8+ proliferation. Δ^9-THC treatment of SIV-infected animals produced minor to no effects in these parameters. However, chronic Δ^9-THC administration decreased early mortality from SIV infection (p=0.039), and this was associated with attenuation of plasma and CSF viral load and retention of body mass (p=NS). In vitro, Δ^9-THC (3.2μg/ml) decreased SIV (10 $TCID_{50}$) viral replication in MT4-R5 cells. These results indicate that chronic Δ^9-THC does not increase viral load or aggravate morbidity and may actually ameliorate SIV disease progression. We speculate that reduced levels of SIV, retention of body mass, and attenuation of inflammation are likely mechanisms for Δ^9-THC-mediated modulation of disease progression that warrant further study.

The cannabinoids including cannabidiol, cannabinol, and Δ^9-tetrahydrocannabinol (Δ^9-THC) exert their effects by binding to two major subtypes of cannabinoid receptor, CB1 and CB2.[1] The CB1 receptor is preferentially expressed in the brain where it mediates neurobehavioral effects. The CB2 receptor is expressed primarily in peripheral tissues, particularly in immune cells where they have been shown to affect cytokine production, lymphocyte phenotype, function and survival, cell-mediated immunity, and balance of Th1/Th2 cells.[2] With the advent of highly active antiretroviral therapy (HAART), human immunodeficiency virus (HIV) infection has become a chronic disease frequently co-existing with chronic use of drugs of abuse, including marijuana.[3] In addition, Dronabinol (Δ^9-THC; Marinol), is approved by the Food and Drug Administration for treatment of HIV-associated anorexia.[4] Although this approval has gained strong support from the lay public, little scientific evidence exists to support the efficacy of such an intervention. To date, no clinical studies have rigorously addressed the impact of chronic Δ^9-THC use on the course and progression of HIV infection. Only one study has examined the impact of short-term (21 day) Δ^9-THC administration (3 daily 0.9 g marijuana cigarettes; 3.95% Δ^9-THC) on HIV viral load. The results from that study did not show substantial elevation in viral load in HIV-infected individuals receiving stable antiretroviral regimens containing nelfinavir or indinavir,[5] and thus, were considered to reflect the relative safety of short-term Δ^9-THC use in this patient population. The long-term effects of cannabinoid administration on progression of HIV infection had not been previously examined.

Using a well established nonhuman primate model of HIV disease, we examined the impact of chronic intramuscular Δ^9-THC (provided by National Institute on Drug Abuse; Research Technical Branch, Rockville, MD) administration on the early phase of simian immunodeficiency virus (SIV) infection in age (4-6 year old) - and body weight-matched healthy male Indian-derived rhesus macaques. Chronic administration of Δ^9-THC (or 0.05 ml/kg vehicle (VEH)) was

initiated prior to SIV with 0.18 mg/kg, a dose that eliminated responding in a complex operant behavioral task in almost all of the subjects. The dose was subsequently increased for each subject to 0.32 mg/kg, over a period of approximately 2 weeks, and maintained for the duration of the study. Almost all of the animals demonstrated tolerance to the behavioral effects of this dose prior to SIV inoculation, reported elsewhere.[6] Approximately one month after initiating chronic Δ^9-THC administration, eight animals were inoculated intravenously with 100 times the $TCID_{50}$ (50% tissue culture infective dose) of SIV_{MAC251}. Pathogenicity of this SIV isolate is similar to HIV.[7] The progression of SIV disease was monitored through clinical and biochemical parameters, and viral load in plasma and CSF.

Contrary to what we expected, chronic cannabinoid administration did not significantly increase viral load or exacerbate immune dysfunction. Mean viral load in VEH/SIV^+ animals was 6.56±0.16 log copies at 7 days, and 6.04±0.6 log copies/ml at 2 months post infection (Figure 1A). Viral load remained elevated (6.35±0.76 and 5.8±0.06 log copies/ml at 3 and 6 months, respectively) throughout the duration of the study. Viral load in THC/SIV^+ was lower (4.83±0.56 log copies/ml) as compared to VEH/SIV^+ beginning at 2 months post SIV infection (p=NS). Viral mRNA in CSF peaked at day 14 post-infection, and remained elevated up until 2 months post infection in the VEH/SIV^+ and THC/SIV^+ animals (Figure 1B). Thereafter, CSF viral load fluctuated between levels below the limit of detection and low counts in both SIV-infected groups. Across all time points, 60% of VEH/SIV^+ CSF samples were above the limit of detection for the assay. In contrast, only 40% of CSF samples from THC/SIV^+ animals had viral loads above the limit of detection.

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 cytometry as previously described.[8] The

CD4+ to CD8+ ratio decreased significantly from pre-infection values (p=0.0001) in both VEH- and THC-treated animals (Figure 2B). The marked decrease in CD4+ lymphocyte counts, reached statistical significance (p=0.023) at 0.5 and 1 months post SIV infection in VEH/SIV$^+$ animals, and beginning at 2 months in THC/SIV$^+$ (p=0.037). In contrast, CD8+ counts showed a short-lived increase at 2 weeks post infection, which reached statistical significance only in THC/SIV$^+$ animals (p=0.029) (Figure 2D). CD4+ and CD8+ lymphocyte expression of Ki67 and Caspase 3 (Cas3) were measured as indices of proliferation and apoptosis. No significant changes were noted in the percent of neither Ki67+ nor Cas3+ on CD4+ lymphocytes in either of the experimental groups (data not shown). In contrast, both markers were increased significantly in CD8+ lymphocytes at 2 weeks post infection, suggesting increased activation of these cells during the acute response to infection (Figure 3A). The CD8+ Ki67 expression was increased 10-fold at this time point, and was significantly higher in THC/SIV$^+$ animals when compared to the VEH/SIV$^+$ infected animals (p=0.049). This rise in CD8+ lymphocyte proliferation was transient in both groups with only values for THC/SIV$^+$ animals remaining higher (p=0.05) than controls at 1 month post SIV infection. Values for VEH/SIV$^+$ and THC/SIV$^+$ groups were not different from control beyond one month post-infection. The changes in the number of Ki67+ CD8+ lymphocytes were paralleled by those in number of Cas3+ CD8+ lymphocytes. Cas3+ CD8+ lymphocytes showed a transient and significant (p=0.001) 7-8-fold increase that peaked at 2 weeks post-SIV infection in both VEH/SIV$^+$ and THC/SIV$^+$ animals (Figure 3B). Although a similar pattern of higher levels of Cas3+ CD8+ lymphocytes was observed in the THC/SIV$^+$ animals, this difference failed to reach statistical significance in comparison to the VEH/SIV$^+$ animals (p=0.06). This rise in CD8+ lymphocyte apoptosis was short-lived, and values after 1 month post-SIV infection were not different from uninfected control values.

No deaths were noted during the first 5 months post SIV infection in VEH/SIV$^+$ or THC/SIV$^+$ animals (Figure 4). Two of the VEH/SIV$^+$ animals succumbed to SIV infection shortly after 5 months, and a third reached end-stage at 7 months. Among the THC/SIV$^+$ animals, the first animal did not reach end stage until 11 months post-SIV inoculation. A summary of the clinical indications for euthanasia and the most salient necropsy findings is presented in Table 1.

Notably different patterns in weight gain were observed between the initial 3 month post SIV-infection period and the subsequent 3-6 month post-SIV period (Figure 5). This was particularly true for the VEH/SIV$^+$ animals, which showed a tendency for greater (p=NS) weight loss than the THC/SIV$^+$ animals. Echocardiography (Toshiba Aplio at 8.5 MHz) performed prior to (baseline), and 3 and/or 6 months after SIV infection in the two SIV-infected groups (VEH and THC) failed to show significant alterations in cardiac systolic or diastolic function (data not shown).

Cannabinoids, including Δ^9-THC, have been shown to have immunomodulatory effects[9,10] on cytokine production and lymphocyte phenotype, function and survival[11,12,13] as well as cell-mediated immunity.[14] Similar immunosuppressant effects on lymphocyte[15] and alveolar macrophage[16] function have been reported in nonhuman primates. Furthermore, the potential of cannabinoids to regulate the activation and balance of human Th1/Th2 cells by a CB2 receptor-dependent pathway has been supported by findings from several studies.[17,18] More recent studies have provided evidence that the synthetic cannabinoid WIN 55,212-2 can potently inhibit HIV-1 expression in CD4+ lymphocytes and microglial cell cultures in a time- and concentration-dependent manner.[19] Moreover, Rock et al.[20] demonstrated the involvement of CB2 receptors in cannabinoid antiviral activity in microglial cells. To examine the possibility that the improved survival of SIV-infected rhesus could have been due to direct suppression of viral replication, we examined the in vitro effects of 1 and 10 μM Δ^9-THC on viral replication in MT4-

R5 cells (human T-cell line)[21] infected with SIV_{MAC251} (10 $TCID_{50}$). Pretreatment with 10 μM of Δ^9-THC (3.2 μg/ml) resulted in a significant decrease in cell-associated viral load in cells collected at 3 and 6 days post-SIV inoculation (Figure 6). Several possible mechanisms could be involved in this suppression of viral replication, including effects on cell cycle, interaction of cannabinoids with viral co-receptor, and viral integration into the host genome. Moreover, the contribution of the specific cannabinoid receptor subtypes to the overall effects also remains to be examined.

In summary, this study is the first to report *in vivo* experimental data demonstrating that chronic Δ^9-THC initiated prior to, and continued throughout the asymptomatic phase of SIV infection, does not impair the host's ability to control viral load, and does not increase morbidity and mortality from the infection. While the small groups and natural variation in SIV disease is a limitation of the study, the vehicle-treated group is representative of typical rhesus infections with SIV_{mac251}. Δ^9-THC treatment clearly did not increase disease progression, and indeed resulted in generalized attenuation of the classic markers of SIV disease (set point viral load/ viral level in general). The mechanisms underlying the decreased mortality in cannabinoid-treated SIV-infected animals remain to be elucidated. However, based on our results and reports in the literature, we speculate that retention of body mass, attenuation of viral replication, and an overall immunosuppressant effect of cannabinoids may contribute to the amelioration of SIV disease progression seen in our study. The cellular mechanisms, including the potential role of the recently identified interaction between the HIV coreceptor CXCR4 and the cannabinoid system[22] as potential sites of pharmaco-immuno-modulation remain to be determined.

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Table 1. Clinical indications for euthanasia and necropsy findings.

Animals were studied during the pre-treatment period (prior to initiating the Δ^9-THC administration) and following SIV infection during the "asymptomatic phase of infection" prior to any of them reaching criteria for euthanasia. This period was defined as the period of time following SIV infection during which none of the animals showed clinical signs of disease including weight loss greater than 5% of basal, secondary infections that required antibiotic treatment, neurological manifestations of disease, severe diarrhea and dehydration. Animals were sacrificed and necropsy performed when they met pre-defined clinical indications including presence of three or more of the following: weight loss greater than 15%, hypoalbuminemia (<3 mg/dl) in the presence of edema, anemia and thrombocytopenia, three days of complete anorexia, major organ failure or medical conditions unresponsive to treatment (including respiratory distress, intractable diarrhea, or persistent vomiting, secondary infections that required antibiotic treatment), neurological manifestations of disease, severe diarrhea and dehydration. Survival was tracked throughout the first year post infection, at which time all animals still on study were euthanized and necropsy performed.

FIGURE LEGEND

Figure 1. Viral load detected in (A) plasma and (B) CSF (log SIV RNA copies/ml) over time in relation to SIV infection. Shown are individual viral counts for each infected animal in the vehicle-treated (VEH/SIV$^+$; solid triangles) and THC-treated (THC/SIV$^+$; empty squares) groups as well as geometric means (lines). SIV copy number in plasma and CSF was measured by a real-time RT-PCR assay using gag primers and probe as previously described. Virus was isolated from 500 µl of plasma or 100 µl of CSF by high-speed centrifugation (20,000 g for 60 min.) at 4°C. The limit of detection for this assay (50 copies/ml plasma and 100 copies/ml CSF) is shown in the dotted lines. The contribution of the values below the limit of detection in the CSF assay was assessed as limit of detection divided by the square root of two.[23]

Figure 2. (A) Lymphocyte count (cells X 10^3/µl), CD4+/CD8+ lymphocyte ratio, CD4+ and CD8+ counts determined by flow cytometry in vehicle-treated uninfected (VEH/SIV$^-$), THC-treated uninfected (THC/SIV$^-$), vehicle-treated infected (VEH/SIV$^+$), THC-treated infected (THC/SIV$^+$) rhesus macaques expressed as means over time post SIV infection. Change from baseline was analyzed by a model that included baseline value, as a covariate, and factor effects for Study Period (SPno), THC (+/-), SIV (+/-), and all SPno, THC, and SIV interaction effects. The PROC MIXED procedure in SAS (version 9.1) was selected to perform the analyses. As these are repeated measures on the same subjects over the eight study periods, the within-subjects covariance structure was modeled as compound symmetry; the Kenward-Roger approximation was used to compute p-values for tests of significance. Values for experimental groups N=3 in VEH/SIV$^-$ and N=4 in all others. *$p<0.05$ vs. time- and treatment-matched uninfected controls.

Figure 3. Numbers of (A) Ki67+, and (B) Caspase 3+ CD8+ lymphocytes as determined by flow cytometry in vehicle-treated uninfected (VEH/SIV$^-$), THC-treated uninfected (THC/SIV$^-$), vehicle-treated infected (VEH/SIV$^+$), THC-treated infected (THC/SIV$^+$) rhesus macaques expressed as means over time post SIV infection. Values for experimental groups N=3 in VEH/SIV$^-$ and N=4 in all others. *$p<0.05$ vs. time- and treatment-matched uninfected control. + vs. time-matched VEH/SIV$^+$ values.

Figure 4. Percent survival of vehicle-treated infected (VEH/SIV$^+$) and THC-treated infected (THC/SIV$^+$) rhesus macaques over the initial 11 months post SIV infection, the timepoint when the first THC/SIV$^+$ animal reached end-stage criteria. Log rank statistic for the survival curve (Kaplan Meier Survival Analysis) is greater than would be expected by chance ($p=0.037$).

Figure 5. Body weight change (kg) during the initial 3 months post SIV infection (left panels) and during the subsequent 3-6 month period of infection. Top panels depict change in body weight in vehicle-treated uninfected (VEH/SIV$^-$), THC-treated uninfected (THC/SIV$^-$), vehicle-treated infected (VEH/SIV$^+$), THC-treated infected (THC/SIV$^+$) rhesus macaques expressed as means ± SEM over time post SIV infection. Because of the individual variability, data are shown for each animal during the initial 3 months post SIV infection and during the later 3-6 month post SIV infection in the lower panels, showing the trends in body-weight changes. The symbols in the line graphs represent individual animals in the specified treatment group and carry over across time.

Figure 6. MT4-R5 cells (human T-cell line) were seeded at a cell density of 5 X 10^5/ml in RPMI 1640 containing 10% heat – inactivated fetal calf serum, 2mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 1 µg/ml puromycin in T25 flasks and maintained at 37° C with 5 % CO_2 in the presence of Δ^9-THC (1 or 10µM) or vehicle for 11 days. Culture media was replaced at 3-4 day intervals. After 11 days, 1.5 X10^6 MT4-R5 cells from each treatment group were inoculated with 10 $TCID_{50}$ SIV_{mac} in fresh media containing Δ^9-THC or vehicle, plated in 96 well dishes at a density of 1 X 10^5 cells/well, and maintained at 37° C with 5% CO_2. Uninfected cultures of Δ^9-THC- and vehicle-treated cells were maintained identically for use as controls. One-half of the replicate cultures were harvested 3 days post-SIV-infection, with the remainder harvested 6 days after infection. Supernatant and cell pellets were fractionated by centrifugation at 700 x g for 5 minute at room temperature. Cell viability was monitored through cell counts using trypan blue. DNA was prepared and the quantity of proviral SIV DNA in the cell pellets was determined by real-time PCR. DNA viral load (log SIV DNA copies/well) was quantified in cells harvested at 3 and 6 days post inoculation. Expression of CB1 and CB2 receptors was confirmed in these cells prior to using them in the study. Values are mean ± SEM of 8 replicate wells. Results were analyzed with two-way ANOVA followed by pair-wise multiple comparison procedures (Holm-Sidak method) *p≤0.008.

References

Table 1. Clinical indications for euthanasia and necropsy findings

Subject ID	Time days post-SIV	Clinical findings	Necropsy findings
VEH/SIV$^+$			
A2L046	148	Head pressing, ataxia, inactivity, lack of a palpebral reflex and decreased papillary light reflex in the right eye, serum albumin < 3 mg/dl, and dehydration, unresponsive to treatment.	Cytomegalovirus meningitis with cerebral edema and uncal herniation Acute bilateral bronchopneumonia and pulmonary edema
A1R047	166	Unilateral right facial nerve paralysis, lethargic, ventral recumbence, dyspnea	Giant cell pneumonia with mycobacterium sp. Pneumocystis jiroveci, Meningoencephalitis and cerebral vasculitis
A1R058	220	Decreased platelet count, hematuria, hematochezia, pyuria. Collapsed and did not respond to CPR	Pulmonary thromboembolism
A2R065	503	none	Pulmonary congestion, hepatic steatosis
THC/SIV$^+$			
A1R044	343	Chronic progressive intermittent diarrhea, nonresponsive to treatment, chronic intermittent rectal prolapse, gradual body weight loss (> 25%), serum albumin < 3 mg/dl.	Mycobacterium sp. Involving lungs, liver, spleen, kidneys, gastrointestinal tract and mesenteric lymph nodes with granulomatous inflammation
A1R022	351	none	Pulmonary edema, congestion, mild hepatic microsteatosis
A1R005	461	none	Steatohepatitis, pulmonary granulomatous inflammation, chronic pancreatitis
A2L094	503	none	Mild cerebral edema, mild acute

			steatohepatitis

Figure 1

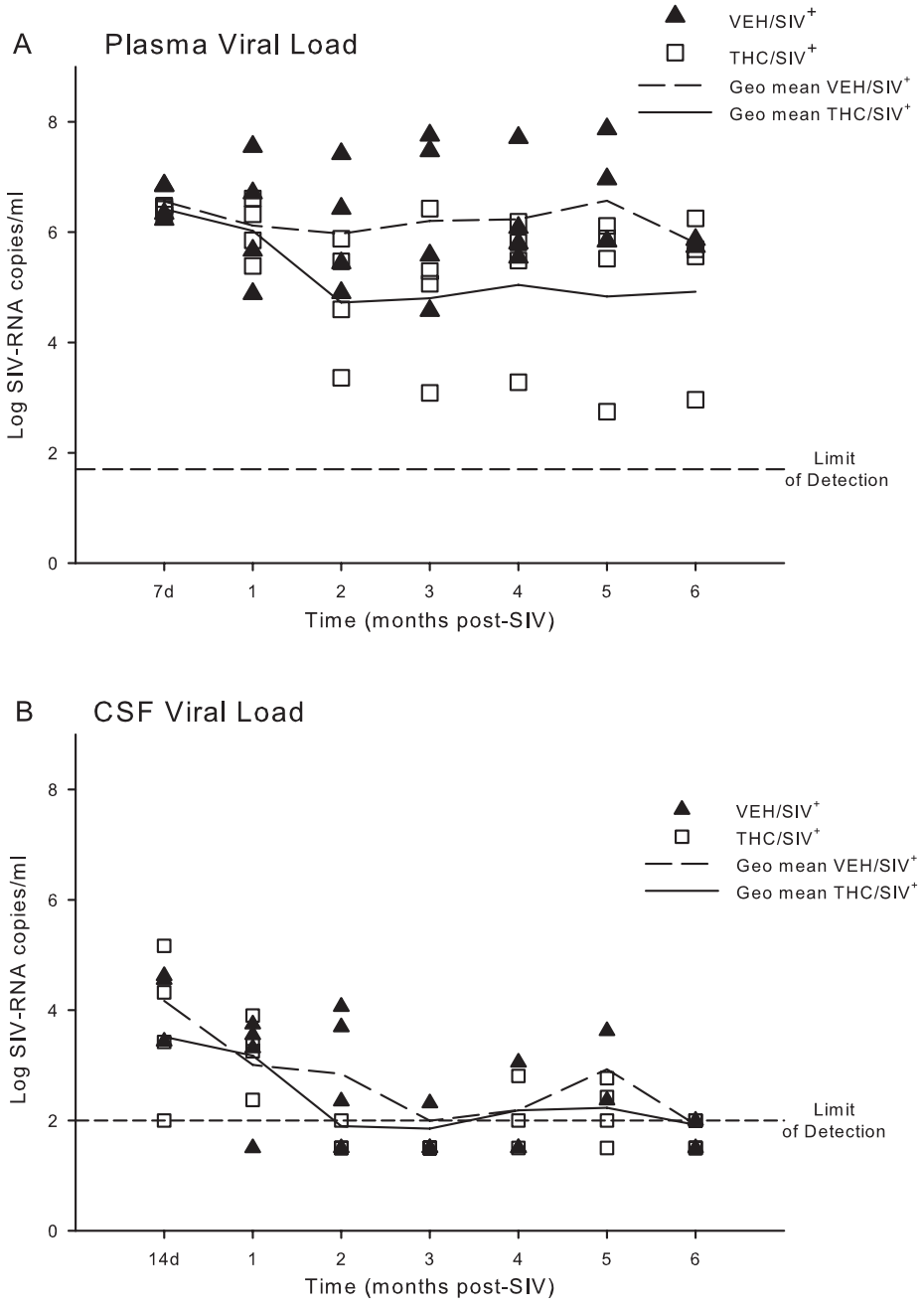


Figure 2

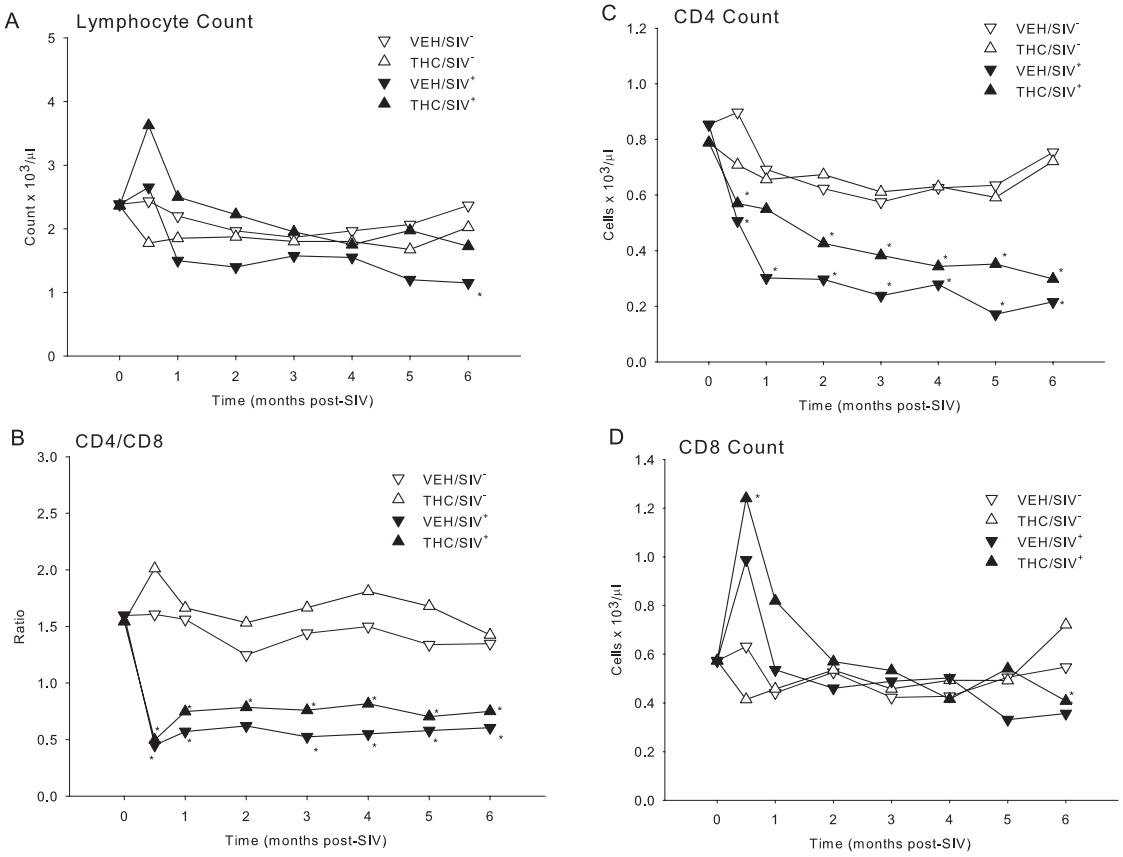


Figure 3

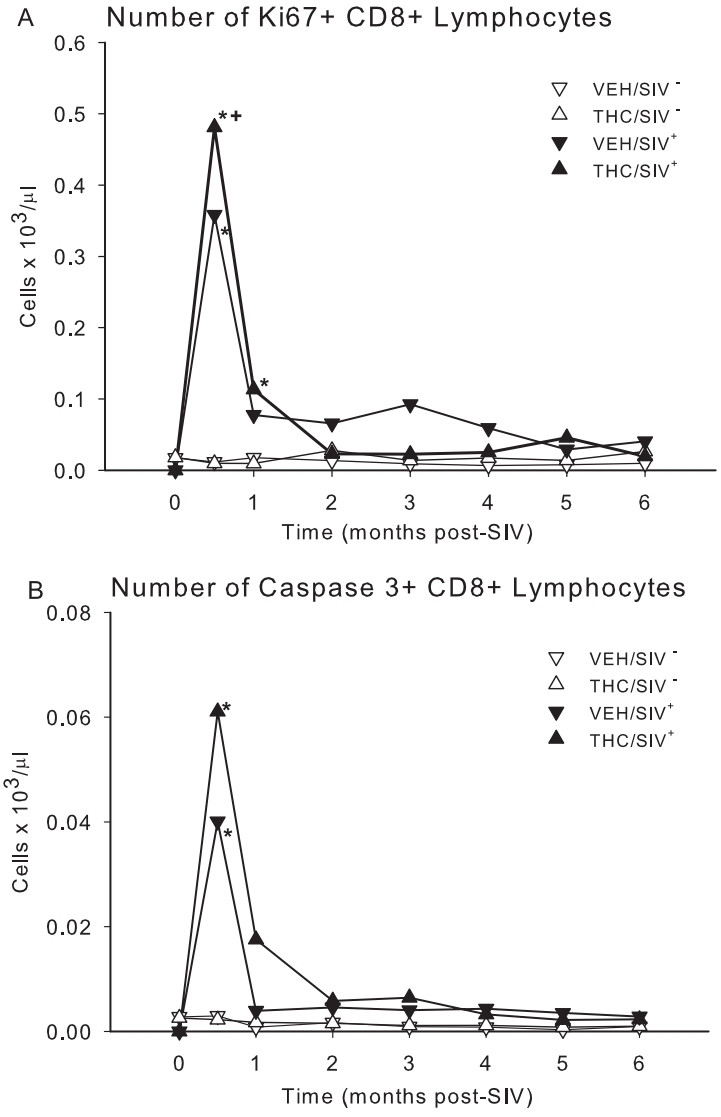


Figure 4

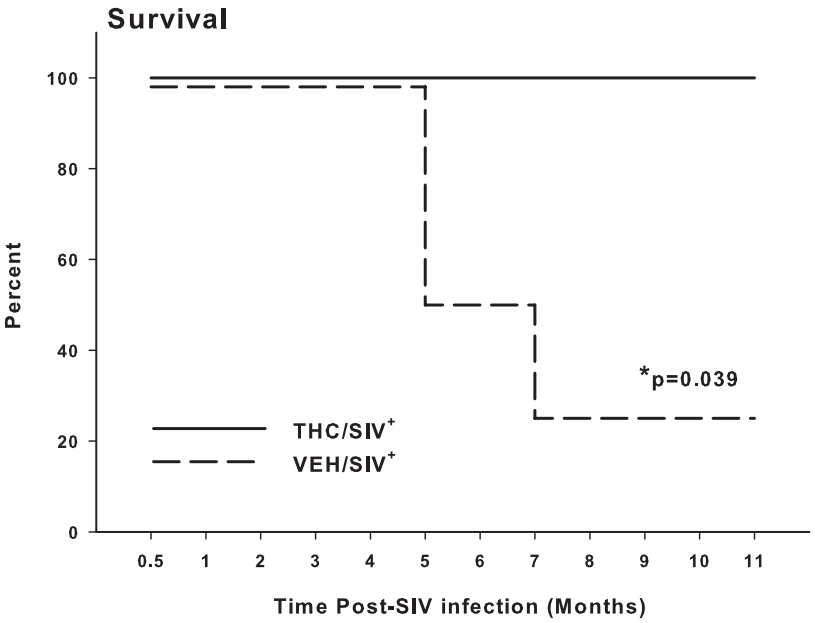


Figure 5.

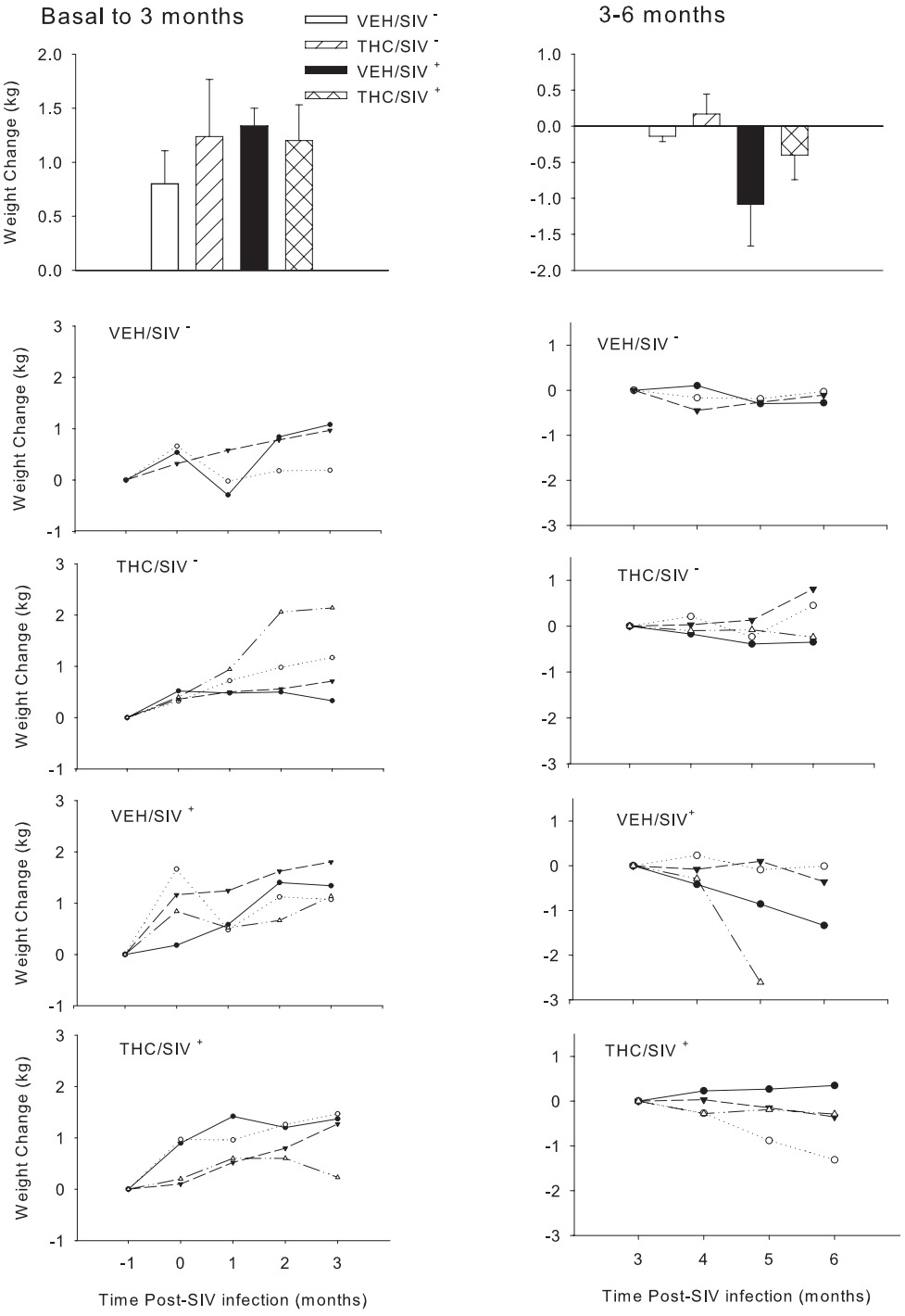
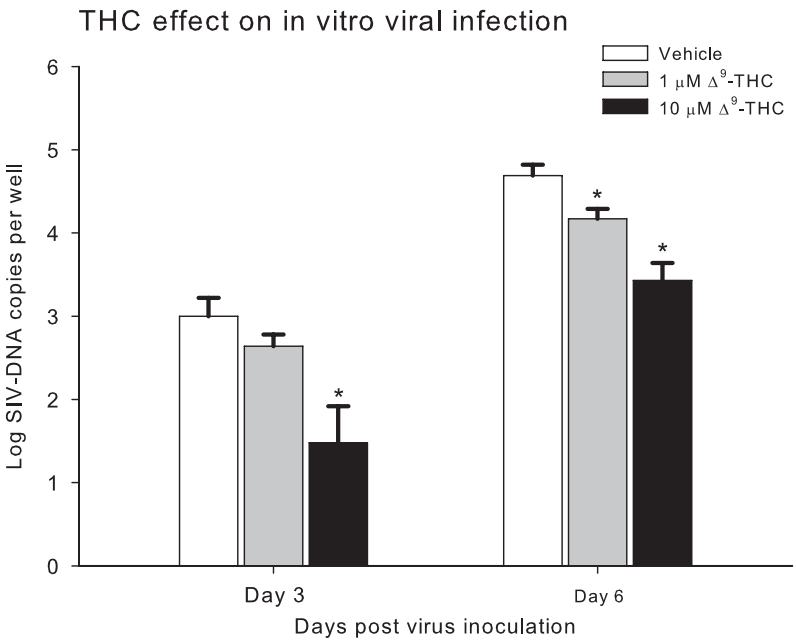


Figure 6



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