Perinatal exposure to \(\Delta^9\)-tetrahydrocannabinol (THC) triggers profound defects in T cell differentiation and function in fetal and post-natal stages of life including decreased responsiveness to HIV antigens

Catherine Lombard, Venkatesh L. Hegde, Mitzi Nagarkatti and Prakash S. Nagarkatti

Department of Pathology and Microbiology (C.L., V.L.H., M.N., P.S.N.), University of South Carolina, School of Medicine, Columbia, SC 29208
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b) Corresponding author:
   Dr. Prakash Nagarkatti,
   Associate Dean and Carolina Distinguished Professor
   Department of Pathology and Microbiology,
   University of South Carolina School of Medicine,
   6311 Garners Ferry Road, Building 3,
   Columbia, SC 29208.
   Telephone: 803- 216-3315
   Fax: 803- 216-3330
   Email: pnagark@uscmed.sc.edu

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d) List of nonstandard abbreviations used in the paper:
   CB, cannabinoid receptor; CFA, complete Freund’s adjuvant; ConA, concanavalin A;
   FITC, fluorescein isothiocyanate; FTOC, fetal thymic organ cultures; GD, gestation day;
   huPBL, human peripheral blood lymphocytes; mAb, monoclonal antibody; PD, post-
   gestation day; PE, phycoerythrin; THC, delta-9-tetrahydrocannabinol; TUNEL, terminal
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Abstract

Marijuana abuse is very prominent among pregnant women. Although, marijuana cannabinoids have been shown to exert immunosuppression in adults, virtually nothing is known about the effects of marijuana use during pregnancy on the developing immune system of the fetus and during post-natal life. We noted that murine fetal thymus expressed high levels of CB1 and CB2 receptors. Moreover, perinatal exposure to delta-9-tetrahydrocannabinol (THC), had a profound effect on the fetus as evidenced by a decrease in thymic cellularity on gestational day 16, 17, 18 and post-gestational day 1, and marked alterations in T cell subpopulations. These outcomes were reversed by CB1/ CB2 antagonists, suggesting that THC-mediated these effects through cannabinoid receptors. Thymic atrophy induced in the fetus correlated with caspase-dependent apoptosis in thymocytes. Thymic atrophy was the result of direct action of THC and not based on maternal factors inasmuch as THC was able to induce T cell apoptosis in vitro in fetal thymic organ cultures. Interestingly, perinatal exposure to THC also had a profound effect on the immune response during post-natal life. Peripheral T cells from such mice showed decreased proliferative response to T cell mitogen as well as both T cell and antibody response to HIV-1 p17/p24/gp-120 antigens. Together, our data demonstrate for the first time that perinatal exposure to THC triggers profound T cell dysfunction thereby suggesting that the offspring of marijuana abusers who have been exposed to THC in utero may be at a higher risk of exhibiting immune dysfunction and contracting infectious diseases including HIV.
Introduction

Marijuana, or *Cannabis sativa*, is one of the most commonly used drugs of abuse worldwide (Berdyshev, 2000). In particular, it is the illegal drug of choice among pregnant women in the United States (Hurd et al., 2005). In addition, marijuana has been used for its medicinal properties for centuries (Voth and Schwartz, 1997). More recently, cannabinoids, the major ingredients in marijuana, including Δ⁹-tetrahydrocannabinol (THC), the major psychoactive component, have been suggested as therapeutic agents in the treatment of ailments ranging from intra-ocular pressure due to glaucoma to multiple sclerosis and certain types of cancers (Voth and Schwartz, 1997; Berdyshev, 2000). The ability of marijuana and THC to alleviate nausea and loss of appetite in AIDS and during chemotherapy in cancer patients (Beal et al., 1995; Schwartz et al., 1997) has led some to suggest their medicinal use to relieve morning sickness in pregnant women (Westfall et al., 2006). However, studies from our laboratory and elsewhere have shown that THC and other cannabinoids can trigger immunosuppression by inducing apoptosis in lymphoid organs such as the thymus and spleen (McKallip et al., 2002b). Other mechanisms of immunosuppression elicited by cannabinoids include induction of Foxp3⁺ T regulatory cells and myeloid-derived suppressor cells (Hegde et al., 2008; Hegde et al., 2010), as well as alterations in the cytokine profiles (Berdyshev, 2000), which may render the host more susceptible to infections and cancer (Cabral and Dove Pettit, 1998; McKallip et al., 2005).

These findings suggest that the use of marijuana or cannabinoids during pregnancy, whether for recreational or medicinal purposes, might put the unborn child at a higher risk for developing immune dysfunction and susceptibility to infections. Maternal use of marijuana during pregnancy has been linked with impaired fetal growth (Hurd et al., 2005) and lower birth weight (Zuckerman et al., 1989; Hurd et al., 2005). In addition, studies in rodents have shown
that perinatal exposure to THC may affect fetal brain development and therefore alter behavioral responses of the offspring later in life (Bonnin et al., 1995; Vela et al., 1995). A few studies even suggested that children exposed to marijuana in utero may be at a higher risk of developing certain cancers, such as neuroblastoma (Bluhm et al., 2006) and leukemia (Robison et al., 1989), although those findings are still somewhat controversial. Nonetheless, virtually nothing is known about the consequences of perinatal exposure to THC on the offspring’s immune system.

In addition, several reports suggest that use of marijuana and other cannabinoids may lead to a higher risk of contracting Human Immunodeficiency Virus (HIV), both directly and indirectly. Indirectly, the “high” experienced by marijuana abusers makes them more likely to adopt risky behavior such as having unprotected sex with HIV-positive individuals (Brodbeck et al., 2006; Drumright et al., 2006). In addition, studies performed using severe combined immunodeficient mice implanted with human peripheral blood lymphocytes (huPBL-SCID mice) show that THC both induces immunosuppression and increases HIV replication (Roth et al., 2005), suggesting a more direct link between cannabinoid use and HIV infection. Clearly, more research is needed to determine the consequences of perinatal exposure to cannabinoids on the immune system of the unborn offspring, as well as the risk that the progeny faces of contracting diseases such as HIV.

In the current study, we demonstrate that perinatal exposure to THC can alter T cell development and functions in the neonatal and post-natal stages of life which in turn could potentially have a major impact on susceptibility to infections as well as other immune disorders.
Methods

Mice
Timed pregnant C57BL/6 mice were purchased from the National Cancer Institute, National Institute of Health (Frederick, MD) and maintained on a 12h-light: 12h-dark lighting schedule in our animal facility. All animal experiments were conducted following standard procedures and after obtaining approval from the Institutional Animal care and Use Committee (IACAUC) of University of South Carolina.

Reagents

Δ⁹-tetrahydrocannabinol (THC) was obtained from the National Institute of Drug Abuse (Rockville, MD) and was initially dissolved in dimethyl sulfoxide (DMSO, Sigma, St Louis, MO) to a concentration of 20 mM and stored at −20°C. For in vivo experiments, THC was further diluted in warm phosphate buffered saline (PBS). For in vitro experiments, THC was further diluted in RPMI 1640 (Invitrogen, Carlsbad, CA) medium supplemented with 5% fetal calf serum. The CB1 antagonist SR141716A (Rimonabant) was obtained from Sanofi Research (Montpellier, France), and the CB2 antagonist AM630 (6-Iodopridovadoline) was purchased from Tocris Cookson (Ellisville, MO). Both compounds were dissolved in DMSO.

Detection of CB1 and CB2 expression by RT-PCR
Fetal thymi were harvested on gestational day (GD) 16 and single cell suspensions were prepared and erythrocytes were lysed. RNA was then isolated using the Trizol method according to the manufacturer’s protocol (Invitrogen, Carlsbad, CA). Because CB1 and CB2 are encoded by single exons, we included a DNase digestion step to limit contamination from genomic DNA.
cDNA was prepared using the iScript cDNA synthesis kit (BioRad, Hercules, CA). CB1 and CB2 were amplified using the following primer sequences from 5’ to 3’: CB1 forward primer: GAGTGGTGCCCTTGTTGTAAT; and reverse primer: GTGGTATCTGCAAGGCCGTCTAAG; CB2 forward primer: AGGGGCTGACAGGAGGATAATA, for product sizes of 252bp and 253bp respectively. 18S RNA was used as a control and amplified with the following primers, from 5’ to 3’: forward primer: GCCCGAGCCGCCTGAATAC; and reverse: CCGGCGGGTCATGGGAATAAC, for a product size of 299bp. After holding the PCR reactions at 95 °C for 1 minute, PCR was carried out using the following parameters: 95 °C for 10 seconds, 58 °C for 20 seconds, and 72 °C for 45 seconds for 35 cycles; followed by a final minute at 72 °C, in a GeneAmp 9700 (Applied Biosystems, Foster City, CA). The resulting PCR products were separated on a 1% agarose gel.

**Acute in vivo exposure to THC**

Pregnant mice were treated intraperitoneally (i.p.) with 20 mg/kg or 50 mg/kg body weight THC or the vehicle on gestational day 16 (GD16) as this corresponds to the time of T cell receptor rearrangement, appearance of CD8 single positive T cells, and expression of characteristic markers such as MHC antigens on thymic stromal cells (Kingston et al., 1984; Haars et al., 1986; Pardoll et al., 1987). We used two pregnant mice each for vehicle or THC treatment groups. Each pregnant mouse had an average of 10 pups that were pooled separately and analyzed. In some experiments, the mothers were sacrificed on GD17 or GD18 and pups were harvested. In other experiments, the mothers were allowed to deliver the pups, and we used pups on post-natal day (PD) 1, or at 2 weeks of age.
Cell preparation

We harvested and pooled the thymi from the fetuses or pups from each mother in each treatment group on GD17, GD18 and PD1 due to low yield of thymocytes. On an average, 10 pups were obtained from each mother. Single cell suspensions were then prepared using a laboratory homogenizer (Stomacher, Tekmar, Cincinnati, OH). Contaminating erythrocytes were lysed by suspending the cells in 3 ml RBC lysing buffer (Sigma Aldrich). After 2 washes in complete RPMI medium, the cellularity was determined using the trypan blue dye exclusion method. The data were expressed as mean viable thymic cellularity per fetus/pup. This was calculated by dividing the total number of cells in each pooled sample by the total number of fetuses/pups used.

Subchronic in vivo exposure to THC

Pregnant mice (two per group) were treated i.p. with 25 mg/kg THC or vehicle on GD16, and with 10 mg/kg THC or vehicle every day thereafter until they delivered the pups (for a total of 4 injections or 55 mg/kg of THC). One week after the pups were born, the thymi and spleens were harvested, made into single cell suspension. Contaminating RBCs were lysed and cellularity was determined as described above.

Detection of THC-induced apoptosis in vivo

Thymocytes obtained from the fetuses of THC- and vehicle-treated pregnant mice were cultured in vitro in 96-well flat-bottomed plates (1x10^6 cells/well in 0.2 ml of medium) overnight at 37°C as this method prevents apoptotic cells from being engulfed by phagocytic cells and therefore allows a better detection of apoptosis. The cells were then harvested, washed twice with PBS,
fixed at room temperature with 100 μl 4% paraformaldehyde for 30 min, and subsequently stained for apoptosis using the TUNEL method.

**Blockade of THC-induced apoptosis in vivo using CB1 and CB2 inhibitors**

Pregnant mice (two per group) were treated i.p. with 20 mg/kg of the CB1 antagonist SR141716A or 40 mg/kg of the CB2 antagonist AM630 on GD16. After 1 h, the mice received 50 mg/kg of THC or the vehicle i.p. On GD17, the mothers were sacrificed and the fetuses were harvested and pooled from each mother. The thymocytes were prepared and cultured overnight at 37°C as described above. The cells were then harvested, fixed and stained for apoptosis using the TUNEL method.

**Fetal thymic organ cultures (FTOCs)**

FTOCs were prepared by the standard procedure. Briefly, thymic lobes were aseptically removed from 16-day-old fetuses from several pregnant mice. Up to six randomly chosen thymic lobes were placed on each nitrocellulose filter with 45 μm pore size (Millipore, Bedford, MA) and each filter was set in a 24-well plate culture well with 300 μl complete RPMI 1640 medium supplemented with 5% FBS, 2 mM L-glutamine, 100 μM non-essential amino-acids, 1 mM sodium pyruvate, 50 μM β-mercaptoethanol, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. Three wells were used for each treatment. THC was added to a final concentration of 5 μM, 10 μM or 20 μM. Control lobes were cultured in medium containing vehicle (DMSO). The thymic lobes were incubated at 37°C and 6% CO2 in a water-saturated atmosphere on day 0. Cultures were fed on day 3 by transferring the filters to new wells containing fresh medium with THC or DMSO. On day 6, thymic lobes from each well were harvested, pooled and single cell
suspensions were prepared. After lysis of the erythrocytes and two washes, the cell viability was determined using trypan blue exclusion. The cells were then analyzed for apoptosis using the TUNEL method.

Detection of apoptosis using the TUNEL method

Apoptotic events were quantified at the single cell level using the TUNEL method (Boehringer-Mannheim, Indianapolis, IN). Briefly, cells were washed with PBS twice and permeabilized on ice for 2 min using 100 μl of 0.1% Triton X-100 in 0.1% sodium citrate. After two additional washes, the cells were incubated with 25 μl of TUNEL reaction composed of FITC-dUTP and TdT at 37°C with 5% CO₂. After 1 h, the cells were washed two more times, resuspended in PBS, and fluorescence was determined by flow cytometry.

Assessment of THC-induced activation of caspase-3 and/or –7

Fetal thymocytes harvested from THC- and vehicle-treated mothers were cultured in vitro in 96-well flat-bottomed plates (1x10⁶ cells/well in 0.1 ml of medium) overnight at 37°C. The induction of apoptosis was then detected using the Apo-ONE homogeneous caspase-3/7 assay (Promega Corporation, Madison, WI) according to the manufacturer’s recommendation. Briefly, 100 μl of a 1:100 substrate / buffer solution was added to the plate. The plate was then shaken for 30 sec and incubated at room temperature for at least 30 min before reading the results on a Wallac Victor² instrument (Perkin Elmer, Boston, MA).
Detection of phenotypic markers on thymocytes

Thymocytes (1x10^6) derived from pups of THC- and vehicle-treated mothers were washed with PBS and incubated with anti-CD16/CD32 mAb on ice for 10 min to block the Fc receptors. The cells were subsequently washed and double-stained for CD4 and CD8, using fluorescein isothiocyanate (FITC)-conjugated anti-CD4 mAb and R-phycoerythrin (PE)-conjugated anti-CD8 mAb (Pharmingen, San Diego, CA), by incubating on ice for 30 min. The cells were then analyzed using a flow cytometer. Absolute numbers for each marker were calculated based on total cell numbers determined by trypan blue exclusion and frequency of each cell population determined by flow cytometry.

Effect of perinatal exposure to THC on the proliferative response of splenocytes and thymocytes to mitogens

Splenocytes and thymocytes collected from pups born to vehicle- or THC-treated mothers (5x10^5 in 100 μl) were cultured in 96-well plates with complete RPMI 1640 medium containing 10% FBS, 2 mM L-glutamine, 50 μM β-mercaptoethanol, 100 U/ml penicillin, and 0.1 mg/ml streptomycin and either left unstimulated or stimulated with 2 μg/ml concanavalin A (Con A) (Sigma Aldrich) or LPS (5 μg/ml) or 5 μg/ml anti-CD3 mAbs for 48 h. Eight hours prior to the end of the assay, the cells were pulsed with 2 μCi of [3H]-thymidine (GE Healthcare, Piscataway, NJ). DNA synthesis was determined by β-scintillation counting.

Effect of perinatal exposure to THC on post-natal (at adult stage) immune response to HIV-1 p17/p24/gp-120
Pregnant mice were treated *i.p.* with 50 mg/kg THC or vehicle on GD16 as described above. Five weeks after the pups were born, they were injected with 5 μg of HIV-1 p17/p24/gp-120 (Prospec Technogene) emulsified in complete Freunds adjuvant (CFA) in each rear footpad. One week later, the mice were sacrificed and draining popliteal lymph nodes (LNs) were harvested and made into a single cells suspension. The viable cellularity was determined using the trypan blue dye exclusion method. The LN cells were then plated and left unstimulated or re-stimulated *in vitro* with 25 μg/ml p17/p24/gp-120 for 72 h. Eight hours prior to the end of the assay, the cells were pulsed with 2 μCi of ³H-thymidine. DNA synthesis was determined by β-scintillation counting.

**Detection of HIV-1 p17/p24/gp-120-specific Abs by ELISA in mice exposed prenatally to THC**

Pregnant mice were treated *i.p.* with 50 mg/kg THC or the vehicle on GD16. Five weeks after the pups were born, they were injected with 5 μg of HIV-1 p17/p24/gp-120 emulsified in complete Freunds adjuvant (CFA) in each rear footpad. One week later, serum samples were screened for Abs using ELISA. High binding (2HB) Immulon microtiter plates (VWR) were coated with 50 μl of HIV-1 p17/p24/gp120 peptide (200 ng/ml) in 100mM NaHCO₃ coating buffer, pH 9.6, for 2 h at room temperature and incubated overnight at 4°C. After blocking for 1 h with 2% BSA, serum samples diluted in 1% BSA and 0.05% Tween 20 (100 μl/well) were then plated and incubated at room temperature for 2 h. p17/p24/gp-120-specific Abs were then detected by incubating with Horseradish Peroxidase (HRP)-conjugated anti-mouse IgG and ABTS (Sigma Aldrich). The colorimetric change was measured at 405 nm on a Wallac Victor² instrument.
Statistical analysis

All statistical analyses were performed using GraphPad prism 4. One-way and two-way ANOVA analyses were carried out to compare experimental groups with controls as detailed in figure legends. Two-tailed unpaired $t$-test was also used where appropriate. The statistical significance was indicated by asterisk as follows: $^*p \leq 0.05$; $^{**}p \leq 0.01$ and $^{***}p$ values $\leq 0.001$. 
Results

Thymocytes from gestational day (GD) 16 fetuses express CB1 and CB2 mRNA

To investigate if perinatal exposure to THC affects the fetal thymus, we first examined whether GD16 fetal thymocytes exhibit CB1 and CB2 cannabinoid receptors. To this end, we performed RT-PCR using RNA extracted from thymocytes of GD16 fetuses, and used RNA from adult thymocytes for comparison. As shown in Fig. 1, we found a similar pattern of expression of CB1 and CB2 in fetal thymocytes when compared to adult thymocytes, with CB2 being expressed at much higher levels than CB1.

Acute perinatal exposure to THC induces apoptosis and alterations in T cell subsets of the fetal thymus

In order to determine whether THC has an influence on thymic development, we injected pregnant C57BL/6 mice intraperitoneally with THC (20 mg/kg or 50 mg/kg) or vehicle on GD16. Analysis of the fetal thymi on GD17 revealed a dose-dependent decrease in thymic cellularity (Fig. 2A) indicative of thymic atrophy. In addition, THC treatment led to a decrease in the percentages of single positive (SP) CD8+ T cells at both doses and double-positive (DP) T cells only at the higher dose (Fig. 2B). Moreover, we noted an increase in the proportions of SP CD4+ T cells and double-negative (DN) T cells at higher doses of THC (Fig. 2B). When we enumerated the absolute numbers of various T cell subpopulations (Fig 2C), we found that all subsets were decreased following THC exposure in a dose-dependent manner except the SP CD4+ T cells.

Analysis of the samples by the TUNEL method revealed a dose-dependent increase in the level of apoptosis detected in the fetal thymocytes upon exposure to THC (Fig. 2D). At lower
doses of THC, the percentage of apoptosis was modest, which could be because the cells that undergo apoptosis in vivo are rapidly phagocytosed and cleared (Kamath et al., 1997). It has to be noted that thymocytes from vehicle-treated animals show significant levels of apoptosis. This is not surprising as thymocytes, which comprise a majority of immature double-negative T cells spontaneously undergo apoptosis when they are cultured in vitro (Kamath et al., 1997).

Together these data suggested that acute perinatal exposure to THC affects T cell development in the thymus of the fetus. Also, these data suggested that THC may mediate its effect, at least in part, through induction of apoptosis.

**THC-induced apoptosis in fetal thymocytes is cannabinoid receptor-mediated**

Next, we investigated whether THC mediated its effects on the fetus through CB1 and CB2 receptors or through non-specific mechanisms such as maternal-derived factors. To this end, pregnant mice were pre-treated with CB1 (Fig. 3A & B) or CB2 (Fig. 3C and D) antagonist, and subsequently treated with THC (50 mg/kg) or the vehicle on GD16. Analysis of the fetal thymocytes on GD17 revealed that both CB1 and CB2 antagonists were able to block, at least in part, the THC-induced fetal thymic atrophy (Fig. 3A and C) and apoptosis (Fig. 3B and D). Together these data suggested that THC-induced apoptosis was resulting from activation of both the CB1 and the CB2 receptors.

**Alterations of thymic cellularity and T-cell subpopulations persist several days after acute perinatal exposure to THC**

In order to determine whether the effects of THC on T cell development in the thymus were transient or if they persisted beyond the first 24 hours following exposure, pregnant mice...
received a single injection of THC (50 mg/kg) on GD16 and fetal thymi were harvested on GD18 and PD1. Fetuses from vehicle-treated mice showed an increase in thymic cellularity from GD17 to PD1 consistent with normal thymic development (Fig. 4A). Thymi harvested from the fetus of THC-treated mothers showed decreased cellularity at all days tested (Fig. 4A). In addition, THC-induced apoptosis was still detectable on GD18 (Fig. 4D) and PD1 (Fig. 4H), by TUNEL staining. We also noted that the THC-induced apoptosis was mediated by caspases as shown by use of caspase-3/7 assay (Fig. 4E, I). Although, we couldn’t detect any major changes in the percentages of cells found in each T cell subset on GD 18, there were significant changes seen on PD1 (Fig. 4B, F). However, when we investigated the absolute number of T cell subsets, we noted a consistent decrease in the absolute numbers of SP CD8⁺, DP CD4⁺CD8⁺, and DN T cells both on GD18 (Fig. 4C) and PD1 (Fig. 4G), similar to GD 16 (Fig 2C). Together, these data suggested that the a single perinatal exposure to THC around the time of T cell development can have a profound effect on T cell development which persists until the birth of the pups.

**Exposure of Fetal Thymic Organ Cultures (FTOCs) to THC results in decreased cellularity and induction of apoptosis.**

In order to exclude the possibility that THC-induced apoptosis was the result of the stress or due to maternal derived factors, we investigated the direct effects of THC on developing T cells using FTOCs. Fetal thymi were harvested on GD16 and cultured in the presence of various concentrations of THC or the vehicle for 6 days with a media change on day 3. Analysis at the end of the treatment showed a dose-dependent decrease in thymic cellularity upon exposure to THC (Fig. 5A), which correlated with an increase in apoptosis, particularly at higher doses of
THC (Fig. 5B). At lower doses, the apoptosis was not demonstrable which suggested that in FTOC, the low levels of apoptosis may not be detectable due to phagocytosis of apoptotic cells or it is also likely that THC may inhibit T cell differentiation. Together, these data suggested that the effects seen in vivo on the fetus may result from direct exposure to THC leading to apoptosis.

Acute perinatal exposure to THC results in decreased immune response to HIV-1 p17/p24/gp-120

Illegal drug abuse, including marijuana abuse, has been linked to a higher risk of contracting HIV. However, little is known about the risk faced by children exposed to marijuana in utero. In order to shed some light on this question, C57BL/6 pregnant mice were treated with an acute dose of THC or the vehicle on GD16 and allowed to deliver their pups. Five weeks after birth, the pups were injected in each rear footpad with HIV-1 p17/p24/gp-120 emulsified in CFA or left untreated (naïve) and allowed to rest for one week. Serum and draining lymph nodes were then collected and used to measure HIV-1 p17/p24/gp-120 specific antibodies and the proliferative response to re-stimulation with HIV-1 p17/p24/gp-120 in vitro respectively. THC-exposed animals showed lower serum titers of HIV-1 p17/p24/gp-120-specific antibodies when compared to animals exposed to vehicle (Fig. 6A). In addition, THC-exposed lymphocytes had a lower proliferative response to HIV-1 p17/p24/gp-120 re-stimulation in vitro, when compared to their vehicle-exposed counterpart (Fig. 6B). Together, these data suggested that perinatal exposure to THC decreases the immune response to HIV antigens.
Effect of subchronic perinatal exposure to THC on the thymus and spleen of 1-week old pups

To this point, we noted that acute perinatal exposure to THC has a negative effect on the immune system of the progeny that can still be detected up to five weeks into life. However, in humans, perinatal exposure to THC is more likely to be chronic than acute. Therefore, we investigated the effects of subchronic perinatal exposure to THC on the thymus and spleen of the progeny. To this end, C57BL/6 pregnant mice were injected with 25 mg/kg THC or the vehicle on GD16, and 10 mg/kg THC or the vehicle every day thereafter until the pups were born, for a total of four injections or 55 mg/kg THC. One week after the pups were born, thymi and spleens were harvested. The results showed that subchronic perinatal exposure to THC leads to thymic (Fig. 7A) and splenic (Fig. 8A) atrophy in 1-week old animals. Although there was no marked changes in the percentage of T cell subsets upon THC treatment (Fig. 7B), there was a significant decrease in the absolute number of most T cell subsets in the thymus of THC-exposed animals (Fig. 7C). Similarly, splenocytes from THC-exposed animals showed a significant decrease in proliferative response to ConA, LPS, and anti-CD3 mAb when compared to splenocytes from vehicle-exposed animals (Fig. 8B). Together, these data demonstrated that subchronic perinatal exposure to THC, like acute perinatal exposure to THC, negatively affects the immune system of the progeny.
Discussion

In this paper, we demonstrated that exposure to THC in utero can have long-term consequences on the immune system of the offspring. Indeed, we found that acute perinatal exposure to THC triggered fetal thymic atrophy, via CB1 and CB2 signaling and induction of apoptosis. There were significant changes in the fetal thymic subpopulations, leaving the offspring with lower absolute numbers of DP T cells. We found that this effect was dose-dependent, and could still be detected on PD1. In addition, we demonstrated that acute perinatal exposure to THC leads to decreased immune response to HIV-1 p17/p24/gp-120. Finally, subchronic exposure to THC also led to impairment of the development and functions of thymus and the spleen.

Marijuana has been used recreationally for centuries, and research shows that it is the more commonly used drug of abuse in women of childbearing age worldwide (Hurd et al., 2005). Out of the 4% of US women acknowledging the use of illegal drugs during pregnancy, 75% admit to using marijuana (Hurd et al., 2005). On the other hand, more and more studies are suggesting the use of marijuana and cannabinoids as therapeutic agents in the treatment of ailments ranging from multiple sclerosis and epilepsy (Voth and Schwartz, 1997; Berdyshev, 2000) to certain types of cancer (Schwartz et al., 1997; Berdyshev, 2000; McKallip et al., 2002a). In addition, the approval of oral THC by the FDA to treat nausea in AIDS and cancer therapy patients (Schwartz et al., 1997) has encouraged some to suggest the use of marijuana to relieve morning sickness during pregnancy (Westfall et al., 2006). However, surveys in humans and studies in rodents suggest detrimental effects stemming from prenatal exposure to cannabinoids. Some studies report that children exposed to marijuana during pregnancy have a slower gestational growth rate (Hurd et al., 2005) and lower birth weight (Zuckerman et al.,
1989; Hurd et al., 2005), as well are reduced gestational length (Fried et al., 1984; Hurd et al., 2005). In addition, perinatal exposure to THC has been shown to affect brain development, resulting in an alteration in behavioral responses, both in rodents and in humans (Bonnin et al., 1995; Vela et al., 1995; de Moraes Barros et al., 2006). Still, very little is known about the effects of perinatal exposure to cannabinoids on the developing immune systems. Perinatal exposure to HU-210, a cannabinoid agonist, caused altered distribution of lymphocyte subpopulations in the spleen and peripheral blood of Wistar rats. Also, there was a reduction in the T-helper subpopulation in the spleen and a decrease in the rate of T helper/T cytotoxic cells in peripheral blood (del Arco et al., 2000).

Studies from our laboratory and others have shown that THC and other cannabinoids induce apoptosis, and alter the proliferative response as well as effector functions of a variety of adult immune cells, such as thymic T cells (McKallip et al., 2002b), splenic B and T cells (McKallip et al., 2002b), NK cells (Patrini et al., 1997), macrophages (Sacerdote et al., 2000), and bone marrow-derived dendritic cells (DCs) (Do et al., 2004), resulting in overall immunosuppression of the host (McKallip et al., 2002b; Do et al., 2004). Such studies suggest that cannabinoids may serve as a double-edged sword, on one hand exhibiting the potential to treat inflammatory diseases, while on the other hand, potentially increasing the susceptibility to cancer and infections (Nagarkatti et al., 2009; Rieder et al.; Nagarkatti et al., 2010). In this paper, we show that perinatal exposure to THC negatively affects the immune system of the offspring, potentially compromising its response to infections. In particular, there is some evidence linking the use of marijuana to a higher risk of contracting HIV (Roth et al., 2005). However, not much work has been done to study how maternal use of marijuana during pregnancy affects the offspring’s risk of getting infected with HIV. Our studies demonstrate that
animals that have been exposed to THC in utero have a lower immune response to HIV at five weeks of age, as evidenced by a decreased proliferative response to HIV-1 p17/p24/gp-120 re-stimulation in vitro and lower HIV-1 p17/p24/gp-120 specific antibody titer in the serum. These data suggest that perinatal exposure to THC may increase the child’s risk of contracting infections such as HIV.

Maternal transmission of HIV still remains a global concern. According to the Joint United Nations Program on HIV/AIDS, at the end of 2003, an estimated 2.5 million children, under age 15, all over the world, were living with HIV/AIDS. Also, approximately 500,000 children under 15 had died from HIV/AIDS or associated causes in that year alone. It is striking that >90% of all HIV-infected children acquire the virus from their mothers before or during birth, or through breastfeeding. The precise mechanisms of HIV transmission and how the children’s immune response fights against the HIV infection are not clear. Moreover, it is likely that intrauterine environmental factors that the offspring is exposed to during pregnancy could have a major impact on HIV transmission, immunosuppression and consequent increased susceptibility to disease in the children. It is therefore expected that the risk of HIV transmission increases with drug abuse by the mother. Our studies indicate for the first time that prenatal exposure to marijuana THC may cause immunosuppression in the fetus and the neonate thereby decreasing the ability of the newborn or neonate to fight against HIV infection acquired from maternal transmission.

Our initial experiments were conducted with an acute dose of THC due to the short gestation period of mice when compared to humans. However, we realize that perinatal exposure is more likely to be chronic due to repeated consumption of marijuana over an extended period of time during the pregnancy. Therefore we conducted experiments in which mice were exposed
to lower doses of THC every day from GD16 to the day that they delivered the pups, in order to better mimic a situation that may be encountered in humans. We found that even in this setting, perinatal exposure to THC resulted in immunosuppression of the offspring, confirming the potential dangers of abusing marijuana during pregnancy.

In most experiments, we observed significant effects with a single dose of 20 mg/kg injected on GD16. This translates to human equivalent dose of 60 mg/m² (1.6 mg/kg) based on the body surface area normalization (Reagan-Shaw et al., 2008). The recommended dose of THC (dronabinol or Marinol) in cancer patients can be as high as 20 mg/m²/day (0.54 mg/kg), as an anti-emetic during chemotherapy. Rats injected with 50 mg/kg body weight of THC had a serum concentration of 10 mM THC within 10 h of administration (Chan et al., 1996). In humans, levels as high as 1 mM in the plasma after recreational use of marijuana has been reported (Azorlosa et al., 1992), which can preferentially get concentrated 15-20 fold in some tissues (Johansson et al., 1989). Such levels of THC may cause significant impairment of immune cell function, and increased susceptibility to infections. An experimental dose of 8 mg/kg in mice has been shown to significantly suppress the response to *Legionella pneumophila* infection (Klein et al., 2000). Overall, our results suggest that THC exposure during pregnancy at close to pharmacological or recreational dose can have significant effect and can impair the developing immune system of fetus.

We chose to treat the animals from GD16 onwards because this time-point corresponds to the initial stages of fetal T cell development, with TCR rearrangement, appearance of CD8 single positive T cells and expression of MHC on the stromal cells (Kingston et al., 1984; Haars et al., 1986; Pardoll et al., 1987), and exposure to toxicants at this stage may greatly influence the development of the immune system.
THC and other cannabinoids are believed to act through two distinct mechanisms. Because of its lipophilic properties, THC was initially thought to be acting via intercalation into the cell membrane. THC has now been shown to bind and signal through at least two G-protein coupled receptors, namely CB1 and CB2, although other receptors may exist (Berdyshev, 2000; Fride et al., 2003). In this paper, we found that both CB1 and CB2 antagonists could prevent, at least in part, THC-induced thymic atrophy and apoptosis, suggesting the involvement of both receptors. It is still unclear whether THC affects the pups directly or indirectly by affecting the mother or placental transport. Cannabinoids have been shown to cross the placenta. Samples from the newborns such as hair, meconium, and plasma are often used to determine whether the child was exposed to marijuana in utero (Hutchings et al., 1989; Vinner et al., 2003). Moreover, studies performed with pregnant dogs injected with radioactive THC intravenously showed that THC could be detected in the brains of both mother and offspring 30 min after the injection, suggesting that once it reaches the mother’s bloodstream, THC can quickly reach the fetus (Martin et al., 1977). In addition, our studies involving fetal thymic organ cultures confirmed the induction of apoptosis following exposure to THC in vitro, suggesting that perinatal exposure to THC may affect the fetal thymus directly.

The idea that perinatal insult may carry long lasting effects is not new. Relationship between low birth weight, which is the most common measure of fetal development, and the onset of high blood pressure, diabetes, stroke and cardiovascular diseases into adulthood, a phenomenon commonly known as the “fetal origins of adult disease” is known (Holladay, 1999). However, more and more studies are now focusing on the direct effects of perinatal exposure to toxicants on the immune system, showing that exposure during fetal development may have more severe consequences than exposure during adulthood (Holladay, 1999). Studies from our
laboratory and others have shown that perinatal exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) induces thymic atrophy and alterations in thymic subsets that can still be detected several days after exposure (Holladay, 1999; Camacho et al., 2004). In addition, others have shown that mice that had been exposed to chlordane during fetal development had decreased delayed-type hypersensitivity and mixed-lymphocyte reactivity as adults (Urso and Gengozian, 1984). The current study demonstrates for the first time that marijuana-abuse during pregnancy may impact the immune response of the fetus that could last into the adulthood.

Together, our studies suggest that pregnant women should be cautious about the decision to use cannabinoids whether for recreational or medicinal purposes as these could potentially have lifelong consequences for the health of their child. This is especially true if the mother is infected or at risk of being infected with HIV, inasmuch as our study suggests that perinatal exposure to THC could increase the risk of contracting the disease.
Authorship Contributions

Participated in research design: Nagarkatti M, Nagarkatti PS

Conducted experiments: Lombard C

Performed data analysis: Lombard C, Nagarkatti M, Nagarkatti PS

Wrote or contributed to the writing of the manuscript: Lombard C, Hegde VL, Nagarkatti M, Nagarkatti PS
References


Footnotes

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Address reprint requests to:

Dr. Prakash Nagarkatti
Department of Pathology and Microbiology
University of South Carolina School of Medicine
6311 Garners Ferry Road, Building 3
Columbia, SC 29208.
Telephone: 803- 216-3315
Fax: 803- 216-3330
Email: pnagark@uscmed.sc.edu
Legends for Figures

Figure 1: Fetal thymocytes express cannabinoid receptors. Fetal thymocytes were harvested on GD16. RNA was extracted and used for RT-PCR to check the expression of CB1 and CB2. The amplicons were run on a 1% agarose gel and visualized with ethidium bromide. Beta-actin was used as an internal control. RNA from adult thymocytes was used as a positive control for CB1 and CB2 expression.

Figure 2: Perinatal exposure to THC alters fetal thymic development. Groups of two C57BL/6 pregnant mice (n=2) were injected on GD16 with THC (20 or 50 mg/kg) or the vehicle. On GD17, the thymi from the fetuses were harvested. Thymi of fetuses from each pregnant mouse (average 10) were pooled separately for analysis. A) Thymic cellularity was determined by trypan blue dye exclusion. The data represent the mean thymic cellularity per fetus ± SEM, p=0.0062, one-way ANOVA. B and C) The thymocytes were double-stained with FITC-anti-CD4 and PE-anti-CD8 mAbs and analyzed by flow cytometry. Representative dot plots are shown in B where the percentage of cells in each subset has been depicted on each dot plot. Absolute numbers of cells for each subset per fetus are shown in C and expressed as mean ± SEM. Asterisk indicates statistically significant difference (p<0.05) in the mean cellularity of THC-exposed thymocytes when compared to the vehicle control. D) The thymocytes were analyzed for apoptosis using the TUNEL method followed by flow cytometric analysis as described in Methods. The percentage of apoptotic cells has been depicted in each histogram.

Figure 3: THC-induced apoptosis in the fetal thymus is mediated through both CB1 and CB2. On GD16, groups of two C57BL/6 pregnant mice (n=2) were pre-treated with CB1
(SR141716A) (A and B) or CB2 (AM630) (C and D) antagonist followed by injection with 50 mg/kg THC or the vehicle. On GD17, the thymi from the fetuses of each mother (average 10) were pooled and analyzed for viable cells (A and C) as described in Fig 2. The data represent the mean thymic cellularity per fetus ± SEM. In panels B and D, the thymocytes were analyzed for apoptosis using the TUNEL followed by flow cytometric analysis. The percentage of apoptotic cells has been depicted in each histogram. Asterisk indicates statistically significant difference (p<0.05) in the mean cellularity in THC + antagonist group when compared to THC-treated group, and in THC-treated group when compared to vehicle-treated group. A) p<0.0001, one-way ANOVA, C) p=0.0062 one-way ANOVA.

**Figure 4: THC-induced thymic atrophy and alteration of T cell subsets persist until birth.**

Groups of two C57BL/6 pregnant mice (n=2) were injected on GD16 with 50 mg/kg THC or the vehicle. On GD17 (A), GD18 (A–E), and PD1 (A, F–I), the thymi from the fetuses or pups (average 10) from each mother were harvested and pooled. A) Viable thymic cellularity was determined by trypan blue dye exclusion. The data represent the mean thymic cellularity per fetus/pup ± SEM. B, C, F and G) The thymocytes were double-stained with FITC-anti-CD4 and PE-anti-CD8 mAbs and analyzed by flow cytometry. Representative dot plots are shown in (B) and (F) where the percentage of cells in each subset has been depicted on each dot plot. Absolute numbers of cells found in each subset are shown in (C) and (G). D and H) thymocytes were analyzed for apoptosis using the TUNEL followed by flow cytometric analysis. The percentage of apoptotic cells has been depicted in each histogram. E and I) The thymocytes were analyzed for levels of caspase-3 and caspase-7 activity as described in Methods. The results are depicted
as mean ± SEM. In panels A, C, E, G and I, asterisk denotes statistically significant difference (p<0.05) between vehicle control and THC treatment group by two-tailed unpaired Student t-test.

**Figure 5: Effects of THC on Fetal Thymic Organ Cultures (FTOCs).** FTOCs were prepared and exposed to THC or the vehicle as described in Methods. Thymi from fetuses of each mother were plated with up to 6 thymic lobes/well and at the time of harvesting, lobes from each well were pooled to prepare cell suspensions. Three such wells were used for each in vitro treatment. On day 6, thymic lobes were harvested and pooled. A) Cell viability was determined using the trypan blue dye exclusion method. The data represent the mean thymic cellularity per organ ± SEM. Asterisk indicates statistically significant difference in the mean cellularity of THC-exposed FTOCs when compared to the vehicle control. P=0.0002 by one-way ANOVA. B) The cells were analyzed for apoptosis using the TUNEL method followed by flow cytometry. The percentage of apoptotic cells has been depicted on each histogram.

**Figure 6: Effect of acute perinatal exposure to THC on the immune response of post-natal five-weeks of age to gp-120.** Groups of two C57BL/6 pregnant mice (n=2) were injected with 50 mg/kg THC or the vehicle on GD16. Five weeks after the pups were born, they were injected in each rear footpad with 5 μg of HIV-1 p17/p24/gp-120 emulsified in CFA. After one week, sera (A) and draining LNs (B) were collected. A) Sera were analyzed for the presence of HIV-1 p27/p24/gp-120 specific IgG by ELISA. B) The draining LN cells were left unstimulated or restimulated with 25 μg/ml of HIV-1 p17/p24/gp-120 for 72 h. During the final 8 h, the cells were pulsed with 2 μCi of [3H]-thymidine. Thymidine incorporation was determined by β-scintillation counting. The data represent mean ± SEM of triplicate cultures. Asterisk denotes
statistically significant differences. **A)** One-way ANOVA, interaction: \( p=0.0005 \); DMSO vehicle vs THC: \( p=0.017 \); untreated vs gp-120: \( p<0.0001 \). **B)** One-way ANOVA, interaction not significant; DMSO vs THC: not significant; untreated vs gp-120: \( p=0.0016 \).

**Figure 7: Effect of subchronic perinatal exposure to THC on the thymus of one-week old pups.** Groups of two C57BL/6 pregnant mice (n=2) were injected with 25 mg/kg THC or the vehicle on GD16, and 10 mg/ml THC or the vehicle every day thereafter until they delivered the pups (for a total of 4 injections, or 55 mg/kg THC). One week after the pups were born, the thymi were harvested and pooled for each mother (average 10 pups per mother). **A)** Thymic cellularity was determined by trypan blue dye exclusion method. The data represent mean thymic cellularity per pup + SEM. Asterisk denotes statistically significant difference when compared to the vehicle control. \( p=0.0196 \) by two-tailed unpaired Student \( t \)-test. **B** and **C)** The thymocytes were double-stained with FITC-anti-CD4 and PE-anti-CD8 mAbs and analyzed by flow cytometry. Representative dot plots are shown in (B) where the percentage of cells in each subset has been depicted on each dot plot. Absolute numbers of cells found in each subset are shown in (C). Asterisk denotes statistically significant difference when compared with the vehicle control by two-tailed unpaired \( t \)-test. CD4: \( p=0.0116 \); CD8: \( p=0.0178 \); DP: \( p=0.0206 \); DN: \( p=0.0362 \).

**Figure 8: Effect of subchronic perinatal exposure to THC on the spleen of one-week old pups.** Groups of two C57BL/6 pregnant mice (n=2) were injected with 25 mg/kg THC or the vehicle on GD16, and 10 mg/ml THC or the vehicle every day thereafter until they delivered the pups (for a total of 4 injections, or 55 mg/kg THC). One week after the pups were born, the spleens were harvested and pooled for each mother (average 10 pups per mother). **A)** Splenic...
cellularity was determined by trypan blue dye exclusion method. The data represent mean thymic cellularity per pup ± SEM. Asterisk denotes statistically significant difference when compared to the vehicle control. p=0.0201 by two-tailed unpaired Student’s t-test. **B** The cells were left unstimulated or stimulated with Con A, LPS or anti-CD3 mAb for 48 h. During the final 8 h, the cells were pulsed with 2 μCi of [³H]-thymidine. Thymidine incorporation was determined by β-scintillation counting. The data represent mean ± SEM of triplicate cultures. Asterisk denotes statistically significant difference. Two-way ANOVA, overall interaction not significant; medium vs stimulus: p<0.0001, DMSO vs THC: p=0.021.
Figure 2

A

Cell number per fetus (million cells)

<table>
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<th>Treatment</th>
<th>Cell Number</th>
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</thead>
<tbody>
<tr>
<td>Vehicle</td>
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<tr>
<td>THC 20 mg/kg</td>
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</tr>
<tr>
<td>THC 50 mg/kg</td>
<td>1.0</td>
</tr>
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</table>

Mother’s treatment

Vehicle THC 20 mg/kg THC 50 mg/kg

B

CD8

<table>
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<th>CD8 24H</th>
<th>CD4 24H</th>
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<td>Vehicle</td>
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<td>42.26%</td>
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<tr>
<td>THC 20 mg/kg</td>
<td>26.24%</td>
<td>48.52%</td>
</tr>
<tr>
<td>THC 50 mg/kg</td>
<td>19.26%</td>
<td>22.46%</td>
</tr>
</tbody>
</table>

% of Total Cells
Figure 2

A

Cell number per fetus (million cells)

Vehicle
THC 20 mg/kg
THC 50 mg/kg
Mother’s treatment

B

Vehicle
THC 20 mg/kg
THC 50 mg/kg

CD8

32.92% 42.26%
24.06% 0.76%
19.26% 22.46%

CD4

26.24% 48.52%
24.04% 1.20%
51.64% 6.64%
Figure 3

A

Cell number per fetus (million cells)

Vehicle
THC
THC+CB1 antagonist

B

Vehicle
THC
THC+CB1 antagonist

Fluorescence intensity

22.92%
32.52%
18.84%
C

Cell number per fetus (million cells)

* * *  

Vehicle  THC  THC+CB2 antagonist

D

Vehicle  THC  THC+CB2 antagonist

Cell number

31.52%  42.66%  30.32%

Fluorescence intensity

This article has not been copyedited and formatted. The final version may differ from this version.
Figure 4

A

Cell number per fetus (million cells)

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<tr>
<td>PD1</td>
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</table>

B

Vehicle

CD8

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<tr>
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<tr>
<td>17.60%</td>
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<td>72.88%</td>
<td>0.81%</td>
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</table>

THC

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<tbody>
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<td>15.54%</td>
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<td>70.92%</td>
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C

Absolute cell number (x10^5)

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<th></th>
<th>CD4</th>
<th>CD8</th>
<th>DP</th>
<th>DN</th>
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<td>Vehicle</td>
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<td>**</td>
<td>*</td>
<td>**</td>
</tr>
<tr>
<td>THC</td>
<td>*</td>
<td>*</td>
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</table>
**Figure 5**

A

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<th>THC 10 μM</th>
<th>THC 20 μM</th>
</tr>
</thead>
<tbody>
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<td><img src="#" alt="Bar graph with asterisks" /></td>
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</tbody>
</table>

B

<table>
<thead>
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<th>THC 5 μM</th>
<th>THC 10 μM</th>
<th>THC 20 μM</th>
</tr>
</thead>
<tbody>
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<td><img src="#" alt="Histogram" /></td>
<td><img src="#" alt="Histogram" /></td>
</tr>
</tbody>
</table>

- **Vehicle**: 40.08%
- **THC 5 μM**: 41.90%
- **THC 10 μM**: 50.3%
- **THC 20 μM**: 96.8%
**Figure 6**

**A**

- **Vehicle**
- **THC**

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<tr>
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<tr>
<td>1</td>
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</tbody>
</table>

**B**

- **Unstimulated**
- **gp-120**

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<th>untreated</th>
<th>gp-120 in vivo</th>
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</table>

* indicates statistical significance.
Figure 7

A

Cell number (x10⁶)

Vehicle

THC

B

Vehicle

THC

CD8

3.92%  79.29%

5.47%  11.32%

CD4

3.71%  81.58%

7.05%  7.66%
C

Absolute cell number (x10^5)

- Vehicle
- THC

CD4  CD8  DP  DN

* * *
A

Figure 8

Cell number (x10^6)

Vehicle  THC

B

DMSO

THC

CPM

Medium  Con A  LPS  CD3

*  **