

Targeting CB2 Cannabinoid Receptors as a Novel Therapy to Treat Malignant Lymphoblastic Disease¹

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**Running Title: Targeting CB2 Cannabinoid Receptors to treat Malignancies of the
Immune System.**

**Key Words: Apoptosis, Cannabinoid, CB2 receptor, lymphoma, Primary Acute
lymphoblastic leukemia**

¹This work was supported in part by grants from National Institutes of Health (DA
0114885, and ES 09098)

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Abstract:

In the current study we examined whether ligation of CB2 receptors would lead to induction of apoptosis in tumors of immune origin and whether CB2 agonist could be used to treat such cancers. Exposure of murine tumors, EL-4, LSA, and P815 to delta-9-tetrahydrocannabinol (THC) *in vitro* led to a significant reduction in cell viability and increase in apoptosis. Exposure of EL-4 tumor cells to the synthetic cannabinoid, HU-210 and endogenous cannabinoid, anandamide led to significant induction of apoptosis, while exposure to WIN55212 was not effective. Treatment of EL-4 tumor-bearing mice with THC *in vivo* led to a significant reduction in tumor load, increase in tumor cell apoptosis and increased survival of tumor bearing mice. Examination of a number of human leukemia and lymphoma cell lines including, Jurkat, Molt-4, and Sup-T1 revealed that they expressed CB2 receptors but not CB1. These human tumor cells were also susceptible to apoptosis induced by THC, HU-210, anandamide and the CB2 select agonist JWH-015. This effect was mediated at least in part through the CB2 receptors as pretreatment with the CB2 antagonist SR144528 partially reversed the THC-induced apoptosis. Culture of primary acute lymphoblastic leukemia cells with THC *in vitro* reduced cell viability and induced apoptosis. Together, the current study demonstrates that CB2 cannabinoid receptors expressed on malignancies of the immune system may serve as potential targets for the induction of apoptosis. Also, because CB2 agonists lack psychotropic effects, they may serve as novel anticancer agents to selectively target and kill tumors of immune origin.

Introduction

Marijuana is one of the oldest drugs of abuse although its medicinal value has been known for several centuries. Delta-9-tetrahydrocannabinol (THC) is the major psychoactive component in marijuana ¹. THC and other synthetic cannabinoids have been used as potential therapeutic agents in alleviating such complications as intraocular pressure in glaucoma, cachexia, nausea and pain ². Interest in the potential medicinal use of cannabinoids grew recently with the discovery of two cannabinoid receptors, CB1 and CB2 ^{3,4}. CB1 receptors are predominantly expressed in the brain, whereas CB2 receptors are primarily found in the cells of the immune system ^{1,4}. Furthermore, endogenous ligands for these receptors capable of mimicking the pharmacological actions of THC have also been discovered. Such ligands were designated endocannabinoids and included anandamide and 2-arachidonoyl glycerol (2-AG) ⁵⁻⁷. The physiological function of endocannabinoids and cannabinoid receptors remains unclear.

Recently, anandamide was shown to inhibit the proliferation of human breast cancer cell lines, MCF-7 and EFM-19 *in vitro* ⁸. Also, THC was shown to induce apoptosis in human prostate PC-3 cells and in C6 glioma cells in culture ^{9,10}. THC-induced apoptosis involved cannabinoid receptor-dependent ^{8,11} or independent pathways ^{9,10}. Such studies have triggered interest in targeting cannabinoid receptors *in vivo* to induce apoptosis in transformed cells. To this end, cannabinoids were shown recently to inhibit the growth of C6 glioma cells *in vivo* ^{12,13}.

Cells of the immune system express high levels of CB2 receptors. It is not clear whether CB2 receptor ligation can induce apoptosis in normal or transformed immune cells. If CB2 receptor agonists can induce apoptosis in transformed immune cells, it could lead to development of a novel class of anticancer agents. In the current study, we investigated this possibility by using both murine and human leukemia and lymphoma lines as well as primary acute

lymphoblastic leukemia (ALL) cells. We demonstrate that ligation of CB2 receptors can induce apoptosis in a wide range of cancers of immune cell origin. Furthermore, we also demonstrate that THC can inhibit the growth of murine lymphoma cells *in vivo* by inducing apoptosis and cure ~ 25% of the mice bearing the tumor. Together, the current study suggests that CB2 agonists that are devoid of psychotropic effects may constitute a novel and effective modality to treat malignancies of the immune system.

Materials and Methods

Mice. Adult female (6-8 weeks of age) C57BL/6 mice were purchased from the National Institutes of Health (NIH). The mice were housed in polyethylene cages and given rodent chow and water ad libitum. Mice were housed in rooms maintaining a temperature of $74 \pm 2^{\circ}\text{F}$ and on a 12 h light/dark cycle.

Reagents. THC was obtained from the National Institute on Drug Abuse (Rockville, MD) and was initially dissolved in DMSO (Sigma, St. Louis, MO) to a concentration of 20 mM and stored at -20°C . THC was further diluted with tissue culture medium for *in vitro* studies and PBS for *in vivo* studies. SR141716A, SR144528, were obtained from Sanofi Recherche (Montpellier, France). HU-210, anandamide, WIN-55212 and JWH-015 were obtained from Tocris Cookson Inc. (Ellisville, MO).

Cell lines. The murine lymphomas (EL-4 and LSA) the murine mastocytoma (P815), the murine melanoma (B16F10), Sup-T1, a T lymphoblastic leukemia cell line developed from a 8 year old male with T-LL, Jurkat, an acute T lymphoblastic leukemia cell line generated from a 14 year old male, Molt-4, an acute T lymphoblastic leukemia cell line established from a 19 year old male and human glioma U251 cell line were all maintained in RPMI 1640 (Gibco Laboratories, Grand Island, NY) supplemented with 5% fetal calf serum (FCS), 10 mM HEPES, 1mM glutamine, 40 $\mu\text{g/ml}$ gentamicin sulfate, and 50 μM 2-mercaptoethanol. In assays examining the effect of cannabinoid agonists on tumor cell viability and apoptosis, the concentration of FCS ranged from 0-5%.

Primary Leukemic Cells. Peripheral blood samples were obtained from 2 patients diagnosed with acute lymphoblastic leukemia (ALL). The samples were referred to as ALL #1 and ALL #2. ALL #1 was obtained from a male patient newly diagnosed with CALLA (CD10) positive non-B, non-T acute lymphoblastic leukemia. All #2 was obtained from a female patient newly diagnosed with terminal deoxynucleotidyl transferase (TdT) positive T cell acute lymphoblastic leukemia. Informed consent was obtained following institutional guidelines. The content of the lymphoblasts was greater than 70% as determined by flow cytometric analysis. Mononuclear cells were isolated by Ficoll-Paque density gradient centrifugation. In this study the samples were cryopreserved and stored in liquid nitrogen before use. Viability after thawing was determined by trypan blue dye exclusion and was greater than 90%.

Measurement of the effect of cannabinoid receptor agonists on tumor cell viability *in vitro*.

Tumor cells were adjusted to 1×10^6 cells/ml in medium containing either 5% FCS or serum free medium. The cells (1×10^6) were cultured in 24 well plates in 2 ml medium in the presence or absence of various concentrations of cannabinoid receptor agonists for 2-24 hours. Finally, the cells were harvested, washed twice in PBS and the viable cell count was determined by trypan blue dye exclusion.

Detection of cannabinoid-induced apoptosis *in vitro*. Tumor cells (1×10^6 cells/well) were cultured in 24 well plates in the presence or absence of various concentrations of THC or other cannabinoid receptor agonists for 2-24 h as described above. Next, the cells were harvested, washed twice in PBS and analyzed for the induction of apoptosis using either the TUNEL or Annexin V/propidium iodide (PI) methods as described elsewhere^{14,15}. To detect apoptosis

using the TUNEL method the cells were washed twice with PBS and fixed with 4% p-formaldehyde for 30 minutes at room temperature. The cells were next washed with PBS, permeabilized on ice for 2 minutes and incubated with FITC-dUTP and TdT (Boehringer Mannheim, Indianapolis, IN) for 1 hour at 37°C and 5% CO₂. To detect apoptosis using the AnnexinV/PI method, the cells were washed twice with PBS and stained with AnnexinV and PI for 20 minutes at room temperature. The cells were washed twice with PBS. The levels of apoptosis in both the TUNEL and Annexin/PI assays were determined by measuring the fluorescence of the cells by flow cytometric analysis. Five thousand cells were analyzed per sample.

Measurement of tumor cell viability and induction of apoptosis in vivo. Groups of 5 C57BL/6 mice were injected with 1×10^6 EL-4 tumor cells i.p suspended in 0.2 ml PBS. The control mice received PBS alone. Ten days later, the mice were injected with various concentrations of THC (0, 1, 3, or 5 mg/kg) i.p. The mice were sacrificed 24 hours later and the EL-4 tumor cells were harvested from the peritoneal cavity by injecting 5.0 ml PBS, followed by aspiration of the peritoneal fluid from the cavity. The contaminating red blood cells were removed using red blood lysing solution (Sigma, St. Louis, MO) and the tumor cells were washed twice with PBS. The number of viable cells was determined by trypan blue dye exclusion and apoptosis was determined using the TUNEL assay. The presence of tumor cells in the peritoneal cavity was confirmed by the ability of the cells to grow *in vitro* and by the phenotype (Thy1⁺, CD4⁻, CD8⁻)

Effect of THC on survival of EL-4 challenged mice. Groups of 8 C57BL/6 mice were injected i.p. with 1×10^6 EL-4 tumor cells in a volume of 100 μ l PBS. One day following tumor injection, the mice received daily injections for 14 days i.p. with 5 mg/kg THC in a volume of 500 μ l PBS. Control mice received injections with the vehicle control. The mice were observed daily for signs of morbidity and were euthanized. Mice that survived for more than 60 days were rechallenged with live EL-4 (1×10^6) cells and tested for their ability to reject tumor and survive.

RNA isolation and RT-PCR. RNA was isolated from $\sim 1 \times 10^7$ cells using the RNeasy Mini Kit (Qiagen, Valencia, CA). As CB1 and CB2 are encoded by single exons, a DNase digestion was included in the isolation procedure to limit the possibility of PCR amplification of CB1 and CB2 from genomic DNA. cDNA was prepared with the Qiagen OmniScript RT kit using 1 μ g RNA as template for first strand synthesis. Mouse and human CB1 was amplified using primers H CB1 U (5'- CGTGGGCAGCCTGTTCCTCA – 3') and H CB1 L (5'- CATGCGGGCTTGGTCTGG – 3'), which yield a product of 403 bp. Human CB2 was amplified using primers H CB2 U (5'- CGCCGGAAGCCCTCATACC – 3') and H CB2 L (5' - CCTCATTCGGGCCATTCCTG – 3'), which yield a product of 522 bp. Mouse CB2 was amplified using M CB2 (5'- CCGGAAAAGAGGATGGCAATGAAT-3') and M CB2 (5'- CTGCTGAGCGCCCTGGAGAAC-3') which yields a product of 479 bp. β -actin was used as a positive control [primers M BA U (5'- AAGGCCAACCGTGAAAAGATGACC – 3') and M BA L (5'-ACCGCTCGTTGCCAATAGTGATGA – 3'), product size of 427 bp]. PCR reactions were carried out using the following parameters: 95°C for 15 s, 58°C for 15 s, and 72°C for 30 s for 35 cycles, followed by a final 5 min. at 72°C in a Applied Biosystems GeneAmp 9700 (Foster City, CA). The resulting PCR products were separated on a 1% agarose gel.

Results

Expression of CB1 and CB2 receptors in EL-4, LSA, and P815 murine tumor cells. The expression of CB1 and CB2 cannabinoid receptor mRNA was determined by RT-PCR (Figure 1). Such an analysis revealed that all three murine tumor cell lines expressed both CB1 and CB2 mRNA.

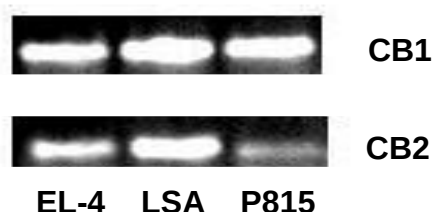


Figure 1. The expression of CB1 and CB2 mRNA in EL-4, LSA, and P815 tumor cells. The expression of CB1 and CB2 mRNA was determined by RT-PCR analysis. Total RNA was isolated from EL-4, LSA, and P815 tumor cells. mRNA was reverse transcribed and amplified by PCR with primers specific for CB1 and CB2. A photograph of ethidium bromide-stained amplicons is depicted.

Exposure of EL-4, LSA, and P815 tumor cells to THC leads to a reduction in viability and induction of apoptosis *in vitro*. We examined whether THC-exposure had an effect on the viability of EL-4, LSA, and P815 tumor cells *in vitro*. To this end, the tumor cells were cultured in medium containing 5% FCS and exposed to various concentrations of THC (0, 1, 10 and 20 μ M) for 24 hours and the viability was determined by trypan blue dye exclusion (Figure 2A). The results showed that exposure to THC at concentrations of 10 μ M or greater led to a significant reduction in the number of viable cells. Next, we analyzed the THC-treated tumor cells for induction of apoptosis by TUNEL staining (Figure 2B). The results demonstrated that THC induced significant apoptosis in all three cell lines *in vitro*. Together these results suggested that exposure of EL-4, LSA, and P815 tumor cells to THC *in vitro* led to significant cell killing by induction of apoptosis.

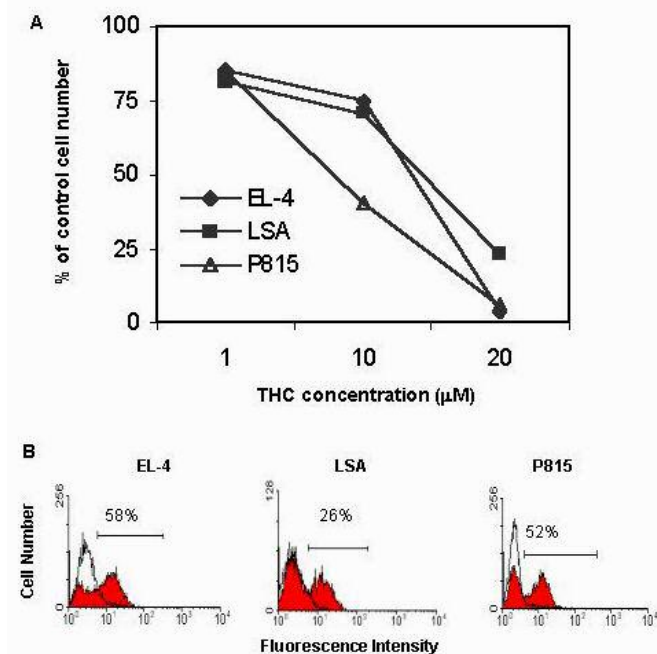


Figure 2. Exposure of murine tumor cells of immune origin to THC *in vitro* leads to a reduction in cell viability and induction of apoptosis. (A) The effect of THC on tumor cell viability was determined by culturing EL-4, LSA and P815 tumor cells for 24h in medium containing 5% FCS in the presence of various concentrations of THC (1, 10, and 20 μM) or the vehicle. The viable cell number was determined by trypan blue dye exclusion. The data were expressed as percent of control viable cell number. (B) The effect of THC on the induction of apoptosis in EL-4, LSA, and P815 tumor cells was determined by culturing the tumor cells for 24 hours in medium containing 5% FCS in the presence of 20 μM THC (filled histogram) or the vehicle (empty histogram). Apoptosis was quantified using the TUNEL method and the cells were analyzed using a flow cytometer.

THC-induced effect on cellularity is dependent on exposure time and serum concentration.

Previous studies suggested that the efficacy of THC may be directly related to the concentration of serum^{16,17}. Therefore, we examined whether culturing tumor cells in serum free medium would have an effect on THC-induced killing of tumor cells. This was accomplished by exposing EL-4 tumor cells to various concentrations of THC (1, 3, and 5 μM) or the vehicle for 4, 8, or 12 h in serum free medium and determining the cell viability. The results showed that by culturing the cells in serum-free medium the concentration of THC needed to decrease tumor cell viability was dramatically reduced (Figure 3A). For example, exposure of EL-4 tumor cells to as low as 5 μM THC for 4 h led to a significant decrease in tumor cell viability (Figure 3A) and increase in the induction of apoptosis (Figure 3B and C). Also, at 12 hours, THC at a concentration of 3 μM was able to cause a significant decrease in tumor cell viability (Figure 3A). The data shown in Fig 3B and 3C demonstrated that apoptosis induced by THC was

demonstratable using both TUNEL and Annexin/PI methods. Previous studies have shown that cells positive for Annexin only represent early apoptotic cells, whereas those positive for both Annexin and PI are late apoptotic/necrotic cells, and cells positive for PI alone are necrotic cells¹⁵. Thus, the majority of THC-treated cells appeared to be in early or late apoptotic stage of death (Figure 3C). It was also noted that in serum free medium, the time required to induce tumor cell killing was decreased significantly to 4 hrs at a concentration of 5 μ M of THC (Figure 3A). Because serum interfered with THC-induced apoptosis, all subsequent experiments were performed in serum-free medium.

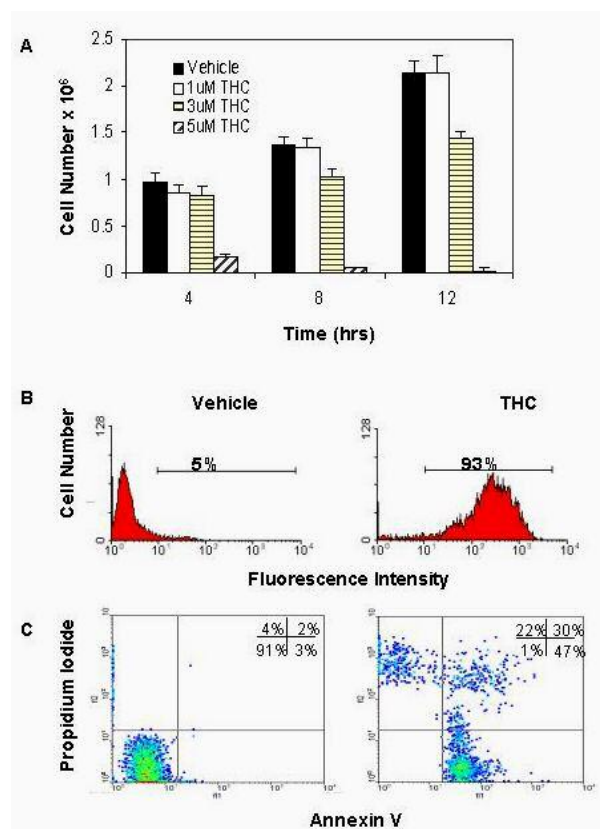


Figure 3. THC is more effective in serum free medium. (A) EL-4 tumor cells were cultured in serum free medium in the presence of various concentrations of THC (1, 3, and 5 μ M) or the vehicle for 4, 8, or 12 h. The number of viable cells was determined by trypan blue dye exclusion. The data represent the mean $\pm$ SEM of duplicate wells. (B) EL-4 tumor cells were cultured in the serum free medium in the presence of vehicle control (DMSO) or THC (5 μ M) for 4 h. The level of apoptosis induction was determined using the TUNEL method and (C) EL-4 cells cultured with THC as described above were stained with Annexin V/PI and analyzed using a flow cytometer.

HU-210 and anandamide but not WIN-55212 induce apoptosis in EL-4 tumor cells *in vitro*.

Three additional cannabinoid receptor agonists were tested for their ability to induce apoptosis in EL-4 tumor cells. In figure 4A, EL-4 tumor cells were exposed to 3 μ M THC, WIN-55212 and HU-210 for 4 hours. The cells were then analyzed for apoptosis using the Annexin/PI method

(Figure 4A). The results showed that exposure to THC or HU-210 led to a significant increase in apoptosis when compared to the controls. In contrast, exposure to 3 μ M of WIN-55212 had no significant effect on the induction of apoptosis. In addition, we examined the effects of anandamide exposure on the induction of apoptosis (Figure 4B). EL-4 tumor cells were exposed to 5 and 10 μ M anandamide for 4 h. The cells were analyzed for apoptosis using the TUNEL assay. The results showed that exposure to 5 μ M led to a slight increase in apoptosis, however exposure to 10 μ M anandamide led to significant levels of apoptosis.

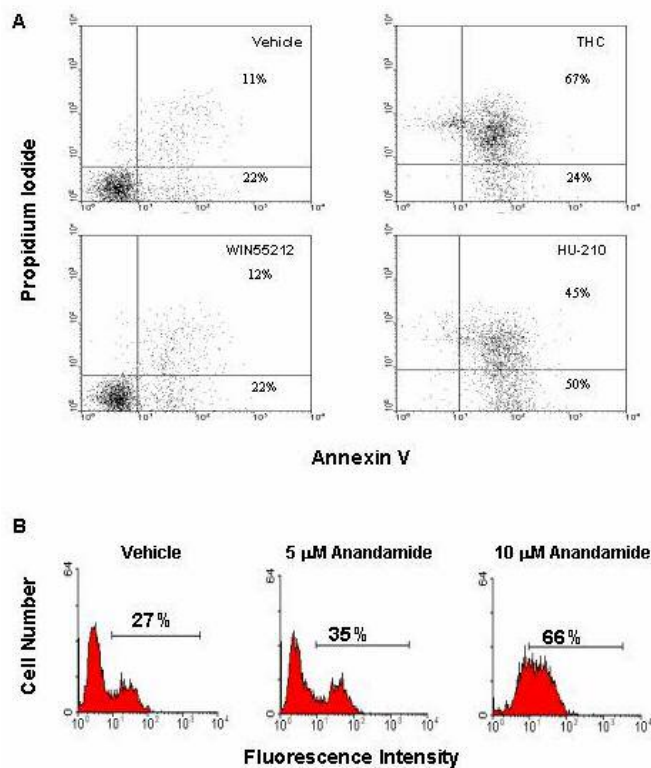


Figure 4. THC, HU-210 and anandamide but not WIN-55212 induce apoptosis in EL-4 tumor cells *in vitro*. (A) EL-4 tumor cells were cultured in serum free medium for 4 h in the presence of vehicle, THC (3 μ M), WIN55212 (3 μ M), HU-210 (3 μ M). The level of apoptosis was quantified by Annexin/PI staining as described in Fig 3. (B) EL-4 tumor cells were cultured in serum free medium for 4 h in the presence of vehicle or anandamide (5 and 10 μ M). The level of apoptosis was quantified by TUNEL assay.

THC treatment leads to reduced tumor burden and apoptosis *in vivo*. We examined whether treatment of tumor-bearing mice with THC was effective at killing tumor cells *in vivo*. To this end, C57BL/6 mice were injected with EL-4 tumor cells (1×10^6). On day 10 of tumor growth, the mice were injected i.p. with various doses of THC (1, 3, or 5 mg/kg) or the vehicle. One day later, the mice were sacrificed and injected with 5 ml PBS into the peritoneal cavity. The

peritoneal fluid was aspirated out and analyzed for viable tumor cells and for apoptosis. The data demonstrated that THC caused a dose-dependent decrease in the viable tumor cell number found in the peritoneal cavity (Figure 5A). THC failed to cause a decrease in cellularity at 1 mg/kg while it was effective at 3 and 5 mg/kg. Furthermore, when cells collected from mice treated with 5 mg/kg were analyzed for apoptosis, a significant proportion (77.3%) of the tumor cells showed apoptosis (Figure 5B). These data suggested that THC was effective *in vivo* to induce apoptosis and kill the EL-4 tumor cells.

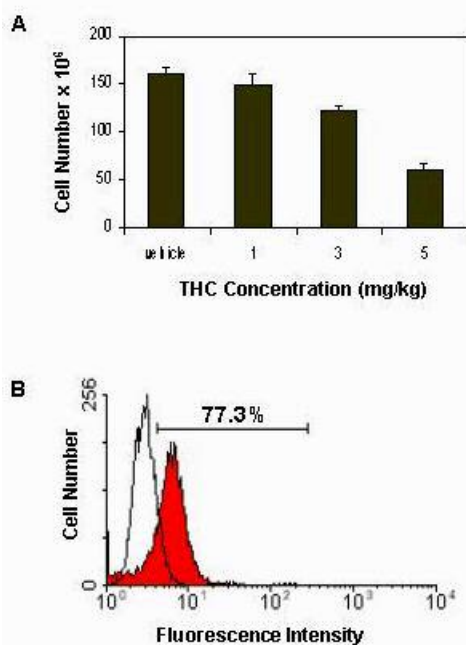


Figure 5. THC treatment leads to reduced tumor burden and tumor cell apoptosis *in vivo*. C57BL/6 mice were injected on day 0 with 1×10^6 EL-4 tumor i.p. On day 10, the mice were treated with various doses of THC (1, 3, or 5 mg/kg i.p.) or the vehicle. One day later, the peritoneal cavity was flushed with 5 ml PBS and the tumor cells were collected by aspiration. (A) The cell number was determined by trypan blue dye exclusion. The data represent the mean $\pm$ SEM from groups of 3 mice. (B) The tumor cells recovered from the peritoneal cavity were tested for apoptosis using the TUNEL method. Filled histogram shows tumor cells exposed to THC and open histogram shows cells exposed to the vehicle.

THC-treatment can cure tumor-bearing mice. Next we tested whether THC treatment can cure EL-4 tumor bearing mice. To this end, mice were injected with EL-4 (1×10^6) tumor cells and then received a daily injection of 5 mg/kg THC for 14 days. The mice were observed for survival and upon exhibiting signs of morbidity were immediately euthanized. The results showed that treatment with THC led to a significant increase in survival (Figure 6). Interestingly, 25 percent of the mice survived the tumor challenge (Figure 6). Also, they were completely cured inasmuch as they were resistant to rechallenge with the specific tumor (data not

shown). Taken together these results suggested that THC can exert anti-cancer properties *in vivo*.

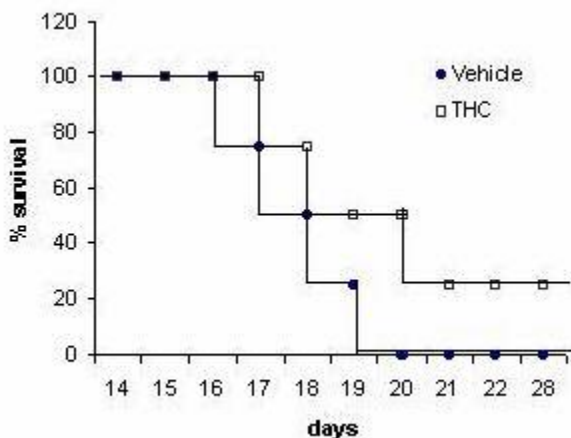


Figure 6. Treatment with THC increases survival of EL-4 tumor-bearing mice . C57BL/6 mice (8/group) were injected i.p. with 1×10^6 EL-4 tumor cells on day 0. From day 1 onwards, the mice were treated daily for 14 days with THC (5 mg/kg) or the vehicle control by the i.p. route. The mice were observed daily for survival and signs of morbidity. The data depicted is a representative of three separate experiments.

Expression of CB1 and CB2 cannabinoid receptors on human Molt-4, Jurkat, and Sup-T1 tumor cell lines. Next, we tested whether human leukemia/lymphoma cell lines express cannabinoid receptors. The expression of CB1 and CB2 cannabinoid receptor mRNA was determined using RT-PCR analysis (Figure 7). The results showed that all 3 cell lines screened expressed significant levels of CB2 mRNA. However, unlike the murine tumor cell lines, CB1 mRNA was not detected in these 3 cell lines. In these experiments, we used a human glioma cell line, U251 as a positive control for CB1 expression.



Figure 7. The expression of CB1 and CB2 mRNA in Molt-4, Jurkat, Sup-T1, and U251 human tumor cells. The expression of CB1 and CB2 was determined by RT-PCR analysis. Total RNA was isolated from Molt-4, Jurkat, Sup-T1 and U251 tumor cells. mRNA was reverse transcribed and amplified by PCR with primers specific for CB1 and CB2. A photograph of ethidium bromide-stained amplicons is depicted.

THC, HU-210, and anandamide induce apoptosis in human leukemia and lymphoma cell lines *in vitro*. Next, we examined whether exposure of human leukemia and lymphoma cell lines to THC or HU-210 would lead to induction of apoptosis. To this end, human tumor cell lines, Jurkat, Molt-4, and Sup-T1 were exposed to various concentrations of THC, HU-210 (2.5, 5, and 10 μ M) or the vehicle for 4 hours, and the induction of apoptosis was determined using the TUNEL method. The results showed that exposure of the Jurkat, Molt-4 and Sup-T1 cell lines to ≥ 5 μ M THC or HU-210 led to significant levels of apoptosis (Figure 8A). THC at 10 μ M and HU-210 at 5 μ M concentrations caused greater than 80 percent apoptosis (Figure 8A). Fig 8B shows a representative experiment using TUNEL assay. In addition, we examined the effects of anandamide exposure on the induction of apoptosis in Molt-4 tumor cells. Molt-4 tumor cells were cultured for 4 h in the absence or presence of various concentrations of anandamide (5, 10, 20 and 40 μ M). The level of apoptosis was quantified using the TUNEL method (Figure 9). The results showed that anandamide at concentrations of 20 μ M or greater induced significant levels of apoptosis in Molt-4 tumor cells. Together, these data suggested that THC, HU-210, and anandamide can induce apoptosis in various human leukemia and lymphoma cell lines.

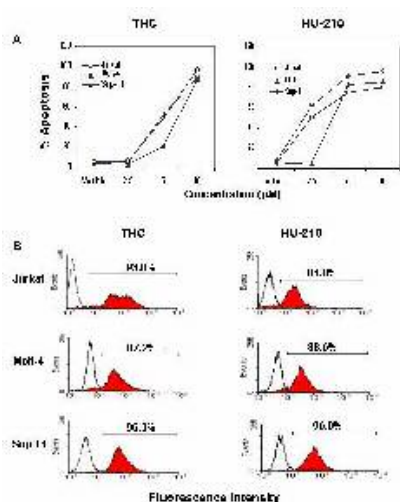


Figure 8. THC and HU-210 exposure leads to the induction of apoptosis in human lymphoid tumors *in vitro*. Human tumors, Molt-4, Jurkat, and Sup-T1 were cultured in serum free medium in the presence or absence of various concentrations of THC, HU-210 (2.5, 5, and 10 μ M), or the vehicle for 4 h. (A) The induction of apoptosis was determined by TUNEL method and the percentage of apoptotic cells was plotted. (B) A representative experiment in which human tumor cells cultured with 10 μ M of THC or HU-210 (filled histograms) or the vehicle (open histograms) were analyzed for apoptosis using TUNEL assay.

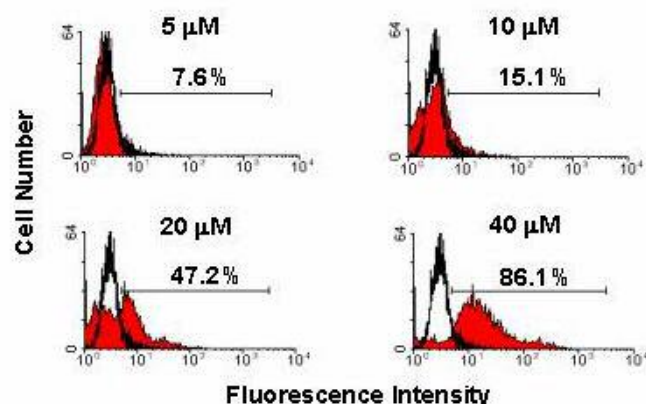


Figure 9. Anandamide exposure leads to the induction of apoptosis in Molt 4 tumor cells *in vitro*. Molt-4 tumor cells were cultured in serum free medium in the presence or absence of various concentrations of anandamide (5, 10, 20, and 40 μ M) or the vehicle for 4 h. The induction of apoptosis was determined by TUNEL method. A representative experiment in which Molt-4 tumor cells were cultured with anandamide (filled histogram) or the vehicle (open histogram) is depicted.

THC-induced reduction in viable cell number is mediated through the CB1 and CB2 cannabinoid receptors. Because the human tumor cell lines screened exhibited CB2 but not CB1 receptors, we tested whether THC was acting through CB2 receptors to induce apoptosis. To this end, Jurkat and Sup-T1 cells were incubated with 5 μ M THC in the presence of CB2 antagonists or the vehicle. After 4 h, the viable cell number was determined by trypan blue dye exclusion (Figure 10). The results showed that exposure to THC led to a dramatic reduction in the number of viable tumor cells. However, when the cells were co-cultured with the CB2 antagonist the viable cell numbers increased significantly thereby reversing the effect of THC. Together, these results suggested that THC-induced reduction in viable cell number and increase in the induction of apoptosis was mediated through the CB2 cannabinoid receptors.

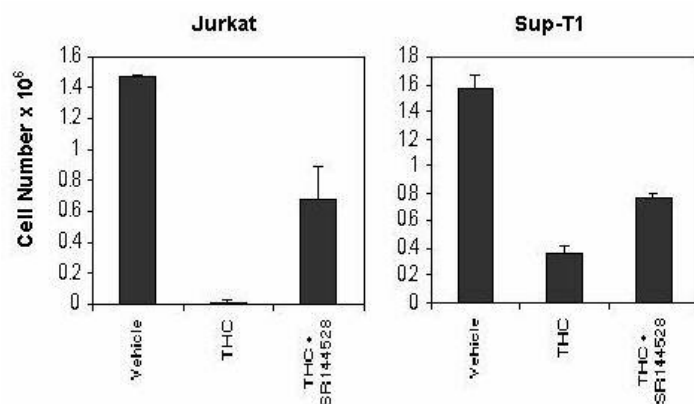


Figure 10. CB2 receptor antagonists can reverse the toxicity of THC. Jurkat and Sup-T1 human tumor cells were cultured for 4 hrs in the presence of THC (10 μ M) or the vehicle. In addition, the cultures received the CB2 antagonist (5 μ M). The viable cell number was determined by trypan blue dye exclusion. The data represent mean $\pm$ S.E.M of triplicate cultures.

Exposure of Jurkat and Molt-4 tumor cells to CB2 receptor agonist, JWH-015 leads to a reduction in viability and induction of apoptosis *in vitro*. Next we examined whether exposure to a CB2 select agonist would lead to tumor cell death and induction of apoptosis. Jurkat and Molt-4 tumor cells were exposed to various concentrations of the CB2 select agonist JWH-015 (1, 5, 10, and 20 μ M) or the vehicle in serum free medium for 24 hours. The results showed that exposure of Molt-4 and Jurkat tumor cells to 5 μ M or greater concentrations of JWH-015 led to a significant decrease in the number of viable tumor cells (Figure 11A). Next, we examined whether exposure to JWH-015 would lead to the induction of apoptosis. Jurkat and Molt-4 tumor cells were exposed to JWH-015 for 24 hours and apoptosis was determined by TUNEL assay (Figure 11B). The results showed that exposure of Jurkat and Molt-4 tumor cells to 5 μ M JWH-015 led to significant induction of apoptosis. Together these results suggested that treatment of Jurkat and Molt-4 tumor cells with CB2 select agonist can lead to a significant reduction in cell viability and induction of apoptosis.

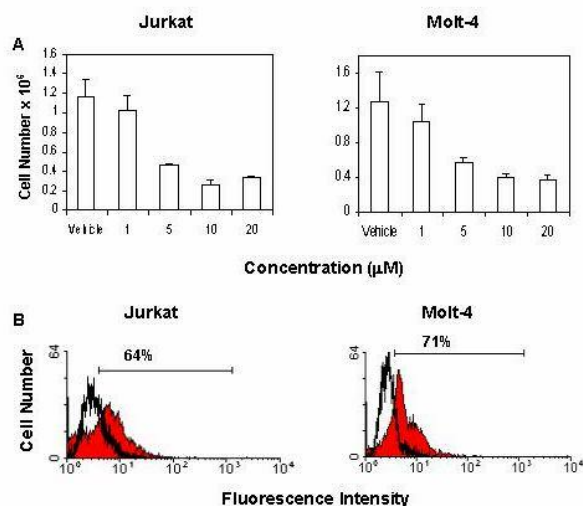


Figure 11. Exposure to the CB2 select agonist JWH-015 leads to reduced cell viability and induction of apoptosis in Jurkat and Molt-4 tumor cells *in vitro*.

(A) The effect of JWH-015 on tumor cell viability was determined by culturing Jurkat and Molt-4 tumor cells for 24h in serum-free medium in the presence of various concentrations of JWH-015 (1, 5, 10, and 20 μM) or the vehicle. The viable cell number was determined by trypan blue dye exclusion. (B) The effect of JWH-015 on the induction of apoptosis in Jurkat and Molt-4 tumor cells was determined by culturing the tumor cells for 24 hours in serum-free medium in the presence of 5 μM JWH-015 (filled histogram) or the vehicle (empty histogram). Apoptosis was quantified using the TUNEL method and the cells were analyzed using a flow cytometer.

THC induces apoptosis in primary acute lymphoblastic leukemia cells *in vitro*. Next we examined whether exposure of primary acute lymphoblastic leukemia cells to THC would have any effect on tumor cell viability or induction of apoptosis. To this end, lymphoblasts isolated from peripheral blood of two patients (ALL #1 and ALL #2) with acute lymphoblastic leukemia (ALL) were cultured in the presence of various concentrations of THC (1, 5, and 10 μM) or vehicle (DMSO) for 2 hours. The viable cellularity was determined by trypan blue dye exclusion and showed that exposure of the ALL samples to 5 μM or greater concentrations of THC resulted in significant reduction in viability (Figure 12A). In addition, we examined the effect of THC exposure on the induction of apoptosis using the TUNEL method and observed that exposure of cells from both ALL patients to 5 μM or greater concentrations of THC led to significant induction of apoptosis (Figure 12B). These results were further corroborated by staining the ALL cells with Annexin V and propidium iodide (data not shown). Together, these

results suggested that exposure of primary ALL cells to THC can lead to significant tumor killing mediated by the induction of apoptosis.

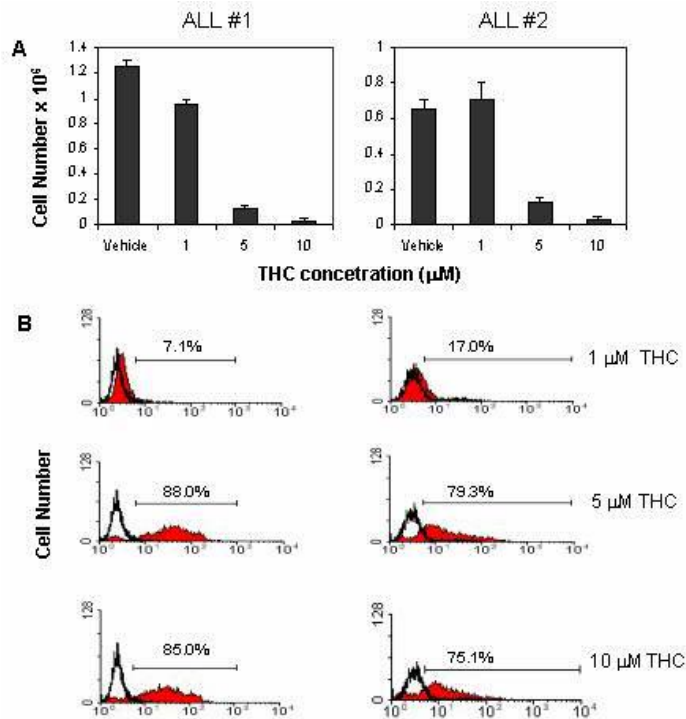


Figure 12. THC induces apoptosis in primary acute lymphoblastic leukemia cells *in vitro*. (A) The effect of THC on primary acute lymphoblastic leukemia cell viability was determined by culturing the cells for 2 hours in serum-free medium in the presence of various concentrations of THC (1, 5, and 10 μM) or the vehicle. The viable cell number was determined by trypan blue dye exclusion. (B) The effect of THC on the induction of apoptosis in primary acute lymphoblastic leukemia cells was determined by TUNEL assay as described in Fig 2. Tumor cells were cultured as described above with THC (filled histogram) or the vehicle (empty histogram). Apoptosis was quantified using the TUNEL method and the cells were analyzed using a flow cytometer. The percentage of apoptotic cells following THC exposure has been depicted in each histogram. The percentage of apoptotic cells following THC exposure has been depicted in each histogram.

Discussion

In the current study we demonstrated that THC and other cannabinoids can induce apoptosis in murine and human leukemia and lymphoma cell lines as well as primary acute lymphoblastic leukemia cells. The human tumor cell lines screened expressed CB2 but not CB1 receptors while the murine tumors expressed both CB1 and CB2 receptors. Ligation of CB2 receptors was sufficient to induce apoptosis inasmuch as CB2 selective agonists could induce apoptosis in tumor cells. Also, THC-induced apoptosis in human tumor cell lines was reversed by CB2 antagonists. THC was not only effective *in vitro* but also *in vivo* as demonstrated by its ability to induce apoptosis and decrease the tumor load. Moreover, THC treatment could cure ~25% of the mice bearing a syngeneic tumor. Together our data suggest for the first time that targeting CB2 receptors on tumor cells of immune origin may constitute a novel approach to treat such cancers.

The interactions between cannabinoids and their receptors in regulating neurobehavioral functions have been extensively studied. Cannabinoids have also been shown to alter immune functions although the precise mechanisms remain unclear. Also, the physiological functions of cannabinoid receptors on immune cells and the role played by endocannabinoids in immune cell regulation remains unresolved. Recent studies from our laboratory demonstrated that administration of THC into C57BL/6 mice led to marked decrease in the cellularity of the thymus and spleen which resulted from the induction of apoptosis in immune cells (manuscript submitted for publication). Recently cannabinoids were also shown to induce apoptosis in tumor cells *in vitro*^{9,10,12,18,19}. Together, such studies suggest the possible use of cannabinoids as anticancer agents.

The exact mechanism by which THC induces apoptosis in normal and transformed lymphocytes remains unclear. It is believed that THC and other cannabinoids can act by two distinct mechanisms. Because of its lipophilic properties it was thought that THC acted through direct intercalation into the cell membrane. However, it was soon realized that the activity of cannabinoids was highly stereospecific, suggesting that the lipophilic properties were not solely responsible for the cannabinoid's activity. Since then, receptors for cannabinoids were characterized. These receptors only share 44% homology but most cannabinoids tested show similar binding affinity to both receptors²⁰. Both receptors are coupled to G-protein suggesting that endogenous cannabinoids may play a role in cell signaling¹. Therefore, it is possible that the observed effects of THC on the immune response, including the induction of apoptosis, may be mediated by signals initiated through these receptors. For example Galve-Roperh et al¹² demonstrated that apoptosis induced by THC in C6 glioma cells *in vivo* involved cannabinoid receptor-dependent pathway. In contrast, others have shown in C6 glioma or a prostate cancer cell model that THC-induced apoptosis was independent of the involvement of the CB1 and CB2 receptors.

In the current study, several observations suggested that ligation of CB2 receptor can induce apoptosis in tumors of immune origin. For example, the human tumor cells such as Jurkat and Sup-T1 expressed only CB2 receptors and the THC-induced apoptosis in these tumor cells was inhibited at least in part by CB2 antagonists. These studies however did not rule out the possibility that ligation of CB1 receptors on murine tumors of immune origin would also induce apoptosis. In fact we have observed that addition of CB1 antagonist to the EL-4 tumor cells can also inhibit the apoptosis induced by THC (unpublished results). It should however be noted that the cannabinoid receptor antagonists can act as inverse agonists^{21,22} and thereby

prevent apoptosis through an alternate pathway. It is for this reason that we used in the current study human cell lines that expressed only the CB2 receptors and showed using CB2 antagonists that ligation of CB2 receptors alone is sufficient to induce apoptosis.

THC is well known for its impact on the cytokine network²³. For example, the presence of THC or activation of the CB1/CB2 receptors can block forskolin-induced accumulation of cAMP²⁴⁻²⁶ and reduced cAMP levels correlate with the repression of IL-2 transcription and secretion²⁷. IL-2 plays an important role in the regulation of apoptosis²⁸⁻³⁰. Therefore, reduction in the levels of IL-2 or other cytokines following exposure to THC may partly account for increased apoptosis. In fact, previous studies from our laboratory have demonstrated that IL-2 can act as an autocrine growth factor in the autonomous proliferation of transformed T cells^{31,32}. Thus, inhibition of IL-2 production by THC could lead to decreased proliferation and apoptotic cell death.

CB1 receptors are expressed in the central nervous system as well as the pituitary gland, immune cells, reproductive tissues, gastrointestinal tissues, heart, lungs, urinary bladder and adrenals (reviewed in¹). In contrast, CB2 receptors are primarily found in immune cells including T cells, B cells, NK cells, macrophages, neutrophils and mast cells^{33,34}. Thus, the selective expression of CB2 receptors on the immune cells provides a unique opportunity to target the malignancies of the immune system by using CB2 agonists to induce apoptosis and thereby provide new avenues to treat such cancers. The advantage in using CB2-selective agonists also stems from the fact that such a treatment is devoid of psychotropic effects that are characteristic of CB1 agonists. Thus, it is possible to synthesize new ligands for CB2 receptors and test them for their efficacy against tumors of immune origin. It should be noted that in the current study we randomly selected a few murine and human tumor cell lines of immune origin

which were all found to be sensitive to cannabinoid-induced apoptosis. Recently, however, we have identified a human cell line that was resistant and further studies are in progress to address whether this cell line lacks physical or functional cannabinoid receptors and/or signaling molecules that trigger apoptosis.

The dose of THC that induced apoptosis *in vitro*, in the current study, was found to be 10 μ M or greater using serum containing medium and 3 μ M or higher in serum-free medium. Similar observations were also made by others who noted that THC was less effective in inducing cell death in the presence of serum¹⁷. This is believed to be the result of direct interactions between serum proteins, such as albumin, with cannabinoids¹⁶. The doses of THC used *in vitro* in the current study were pharmacologically relevant because in an earlier study, rats injected with 50 mg/kg of THC were shown to exhibit 10 μ M of THC in the serum within 10 hours of administration³⁵. Also, in these studies, mice were given as high as 500 mg/kg five times a week for two years. Interestingly, despite such high doses, the survival of dosed rats was higher than controls. Also, the incidence of a wide range of cancers in mice and rats treated with THC was reduced in a dose-dependent manner³⁵. In the current study, we also observed that cannabinoid agonist WIN55212-2 failed to induce apoptosis similar to findings of Ruiz et. al. (1999) in the human prostate tumor model. The reason why WIN55212-2 fails to induce apoptosis is not clear. However, it can be speculated to be due to differences in the structure and binding abilities to the cannabinoid receptors when compared to the classical cannabinoids such as THC and HU-210²⁰. In most previous studies, the effect of THC or other cannabinoids to induce apoptosis in non-lymphoid tumor cell lines was seen only after exposure for 2 or more days^{9,10,12,13}. In contrast, in the current study, we were able to demonstrate marked induction of apoptosis in lymphoid tumors as early as 4 hours following culture with THC. These data

suggested that lymphoid tumors may be highly sensitive to THC-induced apoptosis. Anandamide was shown recently to induce apoptosis in human neuroblastoma (CHP100) and lymphoma (U937) cells¹⁸. These authors demonstrated that anandamide-induced apoptosis was independent of cannabinoid receptors and was induced through vanilloid receptors. In the current study we also observed that anandamide was effective at inducing apoptosis in lymphoid cell lines that were screened. Further studies are in progress to determine the involvement of vanilloid or cannabinoid receptors in anandamide-induced apoptosis.

In the current study we observed that THC was not only able to induce apoptosis in tumor cells *in vitro* but also *in vivo*. Furthermore, THC was effective in reducing the tumor load, prolonging the mean survival time of tumor bearing mice as well curing a significant proportion of such mice. Because THC is immunosuppressive and EL-4 is an immunogenic tumor³⁶ it is possible that the immunosuppressive effects of THC may have interfered with the host's anti-tumor immunity which may account for lower percentage of cures. Thus, further manipulations of the dose of THC that would induce significant apoptosis without causing significant suppression of antitumor immunity may lead to development of a treatment regimen that may cure a larger percentage of tumor-bearing mice. Such studies are currently in progress.

Together, the current study demonstrates that targeting CB2 receptors to induce apoptosis may constitute a novel approach to treat malignancies of the immune system. The advantage in using CB2 receptor agonists is that they do not exhibit psychoactive properties. Also, because CB2 receptors are expressed exclusively on immune cells, use of CB2 receptor agonists will not be toxic to non-immune cells. Thus, further research on CB2 receptor agonists to target transformed immune cells could lead to discovery of a new class of highly selective anti-cancer agents.

References:

1. Berdyshev EV. Cannabinoid receptors and the regulation of immune response. *Chem Phys Lipids*. 2000;108:169-190.
2. Watson SJ, Benson JA, Jr., Joy JE. Marijuana and medicine: assessing the science base: a summary of the 1999 Institute of Medicine report. *Arch Gen Psychiatry*. 2000;57:547-552.
3. Devane WA, Dysarz FA, 3rd, Johnson MR, Melvin LS, Howlett AC. Determination and characterization of a cannabinoid receptor in rat brain. *Mol Pharmacol*. 1988;34:605-613.
4. Munro S, Thomas KL, Abu-Shaar M. Molecular characterization of a peripheral receptor for cannabinoids. *Nature*. 1993;365:61-65.
5. Devane WA, Hanus L, Breuer A, Pertwee RG, Stevenson LA, Griffin G, Gibson D, Mandelbaum A, Etinger A, Mechoulam R. Isolation and structure of a brain constituent that binds to the cannabinoid receptor. *Science*. 1992;258:1946-1949.
6. Felder CC, Briley EM, Axelrod J, Simpson JT, Mackie K, Devane WA. Anandamide, an endogenous cannabimimetic eicosanoid, binds to the cloned human cannabinoid receptor and stimulates receptor-mediated signal transduction. *Proc Natl Acad Sci U S A*. 1993;90:7656-7660.
7. Mechoulam R, Ben-Shabat S, Hanus L, Ligumsky M, Kaminski NE, Schatz AR, Gopher A, Almog S, Martin BR, Compton DR, et al. Identification of an endogenous 2-monoglyceride, present in canine gut, that binds to cannabinoid receptors. *Biochem Pharmacol*. 1995;50:83-90.
8. De Petrocellis L, Melck D, Palmisano A, Bisogno T, Laezza C, Bifulco M, Di Marzo V. The endogenous cannabinoid anandamide inhibits human breast cancer cell proliferation. *Proc Natl Acad Sci U S A*. 1998;95:8375-8380.
9. Sanchez C, Galve-Roperh I, Canova C, Brachet P, Guzman M. Delta9-tetrahydrocannabinol induces apoptosis in C6 glioma cells. *FEBS Lett*. 1998;436:6-10.

10. Ruiz L, Miguel A, Diaz-Laviada I. Delta9-tetrahydrocannabinol induces apoptosis in human prostate PC-3 cells via a receptor-independent mechanism. *FEBS Lett.* 1999;458:400-404.
11. Campbell VA. Tetrahydrocannabinol-induced apoptosis of cultured cortical neurones is associated with cytochrome c release and caspase-3 activation. *Neuropharmacology.* 2001;40:702-709.
12. Galve-Roperh I, Sanchez C, Cortes ML, del Pulgar TG, Izquierdo M, Guzman M. Anti-tumoral action of cannabinoids: involvement of sustained ceramide accumulation and extracellular signal-regulated kinase activation. *Nat Med.* 2000;6:313-319.
13. Sanchez C, de Ceballos ML, del Pulgar TG, Rueda D, Corbacho C, Velasco G, Galve-Roperh I, Huffman JW, Ramon YCS, Guzman M. Inhibition of glioma growth in vivo by selective activation of the cb(2) cannabinoid receptor. *Cancer Res.* 2001;61:5784-5789.
14. Kamath AB, Xu H, Nagarkatti PS, Nagarkatti M. Evidence for the induction of apoptosis in thymocytes by 2,3,7,8- tetrachlorodibenzo-p-dioxin in vivo. *Toxicol Appl Pharmacol.* 1997;142:367-377.
15. Chen D, McKallip RJ, Zeytun A, Do Y, Lombard C, Robertson JL, Mak TW, Nagarkatti PS, Nagarkatti M. CD44-deficient mice exhibit enhanced hepatitis after concanavalin A injection: evidence for involvement of CD44 in activation-induced cell death. *J Immunol.* 2001;166:5889-5897.
16. Zheng ZM, Specter S, Friedman H. Serum proteins affect the inhibition by delta-tetrahydrocannabinol of tumor necrosis factor alpha production by mouse macrophages. *Adv Exp Med Biol.* 1993;335:89-93

17. Jacobsson SO, Rongard E, Stridh M, Tiger G, Fowler CJ. Serum-dependent effects of tamoxifen and cannabinoids upon C6 glioma cell viability. *Biochem Pharmacol.* 2000;60:1807-1813.
18. Maccarrone M, Lorenzon T, Bari M, Melino G, Finazzi-Agro A. Anandamide induces apoptosis in human cells via vanilloid receptors. Evidence for a protective role of cannabinoid receptors. *J Biol Chem.* 2000;275:31938-31945.
19. Sarker KP, Obara S, Nakata M, Kitajima I, Maruyama I. Anandamide induces apoptosis of PC-12 cells: involvement of superoxide and caspase-3. *FEBS Lett.* 2000;472:39-44.
20. Pertwee RG. Pharmacology of cannabinoid receptor ligands. *Curr Med Chem.* 1999;6:635-664.
21. Landsman RS, Burkey TH, Consroe P, Roeske WR, Yamamura HI. SR141716A is an inverse agonist at the human cannabinoid CB1 receptor. *Eur J Pharmacol.* 1997;334:R1-2.
22. Portier M, Rinaldi-Carmona M, Pecceu F, Combes T, Poinot-Chazel C, Calandra B, Barth F, le Fur G, Casellas P. SR 144528, an antagonist for the peripheral cannabinoid receptor that behaves as an inverse agonist. *J Pharmacol Exp Ther.* 1999;288:582-589.
23. Klein TW, Lane B, Newton CA, Friedman H. The cannabinoid system and cytokine network. *Proc Soc Exp Biol Med.* 2000;225:1-8.
24. Koh WS, Crawford RB, Kaminski NE. Inhibition of protein kinase A and cyclic AMP response element (CRE)- specific transcription factor binding by delta9-tetrahydrocannabinol (delta9-THC): a putative mechanism of cannabinoid-induced immune modulation. *Biochem Pharmacol.* 1997;53:1477-1484.

25. Schatz AR, Lee M, Condie RB, Pulaski JT, Kaminski NE. Cannabinoid receptors CB1 and CB2: a characterization of expression and adenylate cyclase modulation within the immune system. *Toxicol Appl Pharmacol.* 1997;142:278-287.
26. Herring AC, Koh WS, Kaminski NE. Inhibition of the cyclic AMP signaling cascade and nuclear factor binding to CRE and kappaB elements by cannabinal, a minimally CNS- active cannabinoid. *Biochem Pharmacol.* 1998;55:1013-1023.
27. Novak TJ, Chen D, Rothenberg EV. Interleukin-1 synergy with phosphoinositide pathway agonists for induction of interleukin-2 gene expression: molecular basis of costimulation. *Mol Cell Biol.* 1990;10:6325-6334.
28. Lenardo MJ. Interleukin-2 programs mouse alpha beta T lymphocytes for apoptosis. *Nature.* 1991;353:858-861.
29. Deng G, Podack ER. Suppression of apoptosis in a cytotoxic T-cell line by interleukin 2-mediated gene transcription and deregulated expression of the protooncogene bcl-2. *Proc Natl Acad Sci U S A.* 1993;90:2189-2193.
30. Zhang X, Giangreco L, Broome HE, Dargan CM, Swain SL. Control of CD4 effector fate: transforming growth factor beta 1 and interleukin 2 synergize to prevent apoptosis and promote effector expansion. *J Exp Med.* 1995;182:699-709.
31. Nagarkatti M, Hassuneh M, Seth A, Manickasundari K, Nagarkatti PS. Constitutive activation of the interleukin 2 gene in the induction of spontaneous in vitro transformation and tumorigenicity of T cells. *Proc Natl Acad Sci U S A.* 1994;91:7638-7642.
32. Hassuneh MR, Nagarkatti PS, Nagarkatti M. Evidence for the participation of interleukin-2 (IL-2) and IL-4 in the regulation of autonomous growth and tumorigenesis of transformed cells of lymphoid origin. *Blood.* 1997;89:610-620.

33. Pertwee RG. Pharmacology of cannabinoid CB1 and CB2 receptors. *Pharmacol Ther.* 1997;74:129-180
34. Klein TW, Newton C, Friedman H. Cannabinoid receptors and immunity. *Immunol Today.* 1998;19:373-381.
35. Chan PC, Sills RC, Braun AG, Haseman JK, Bucher JR. Toxicity and carcinogenicity of delta 9-tetrahydrocannabinol in Fischer rats and B6C3F1 mice. *Fundam Appl Toxicol.* 1996;30:109-117.
36. Iezzi G, Rivolta L, Ronchetti A, Martin-Fontecha A, Rosato A, Protti MP, Sabbadini MG, Bellone M. The immunogenicity of experimental tumors is strongly biased by the expression of dominant viral cytotoxic T-lymphocyte epitopes. *Cancer Res.* 1997;57:2564-2568.

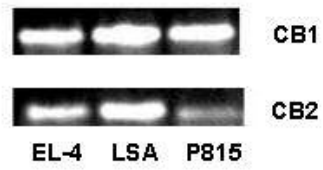


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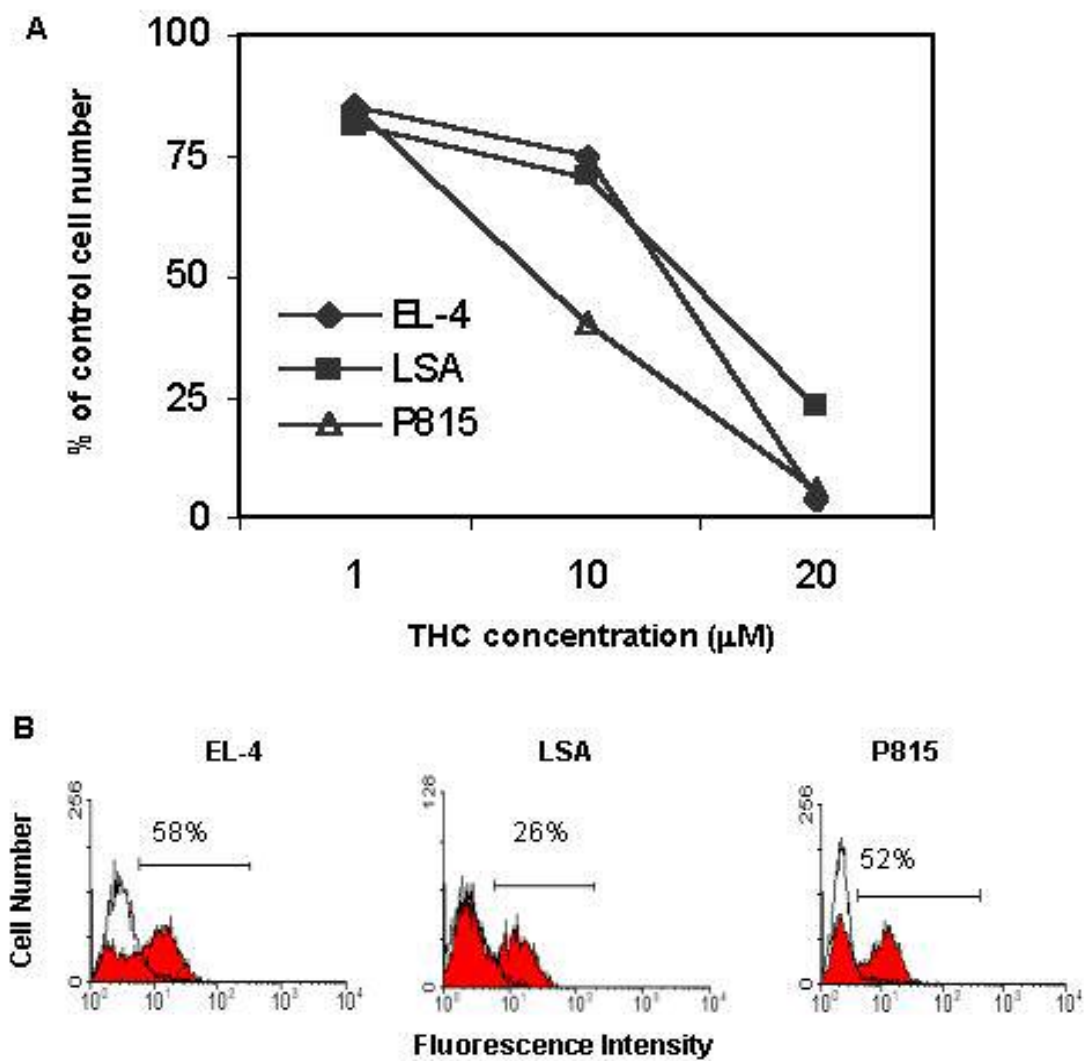


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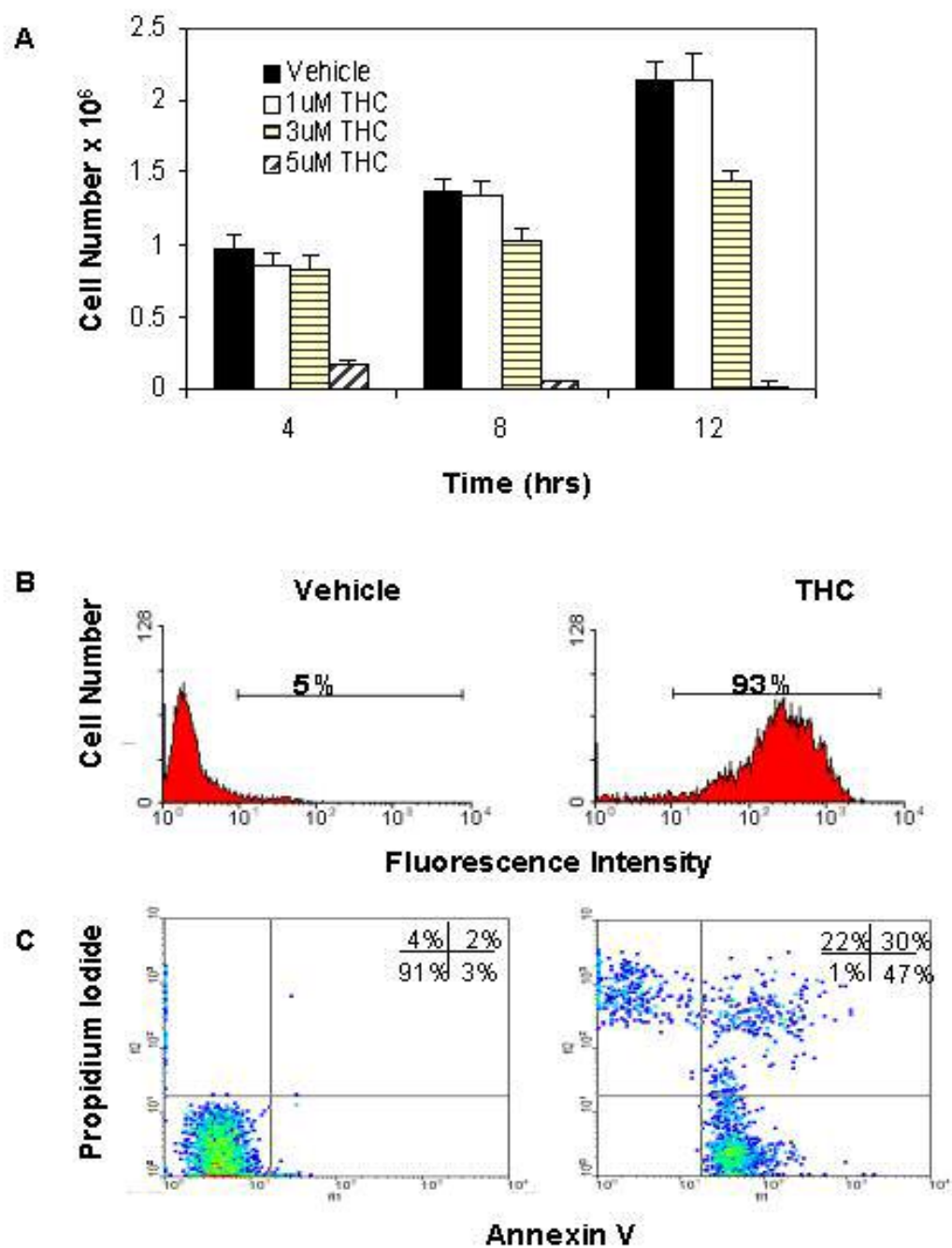


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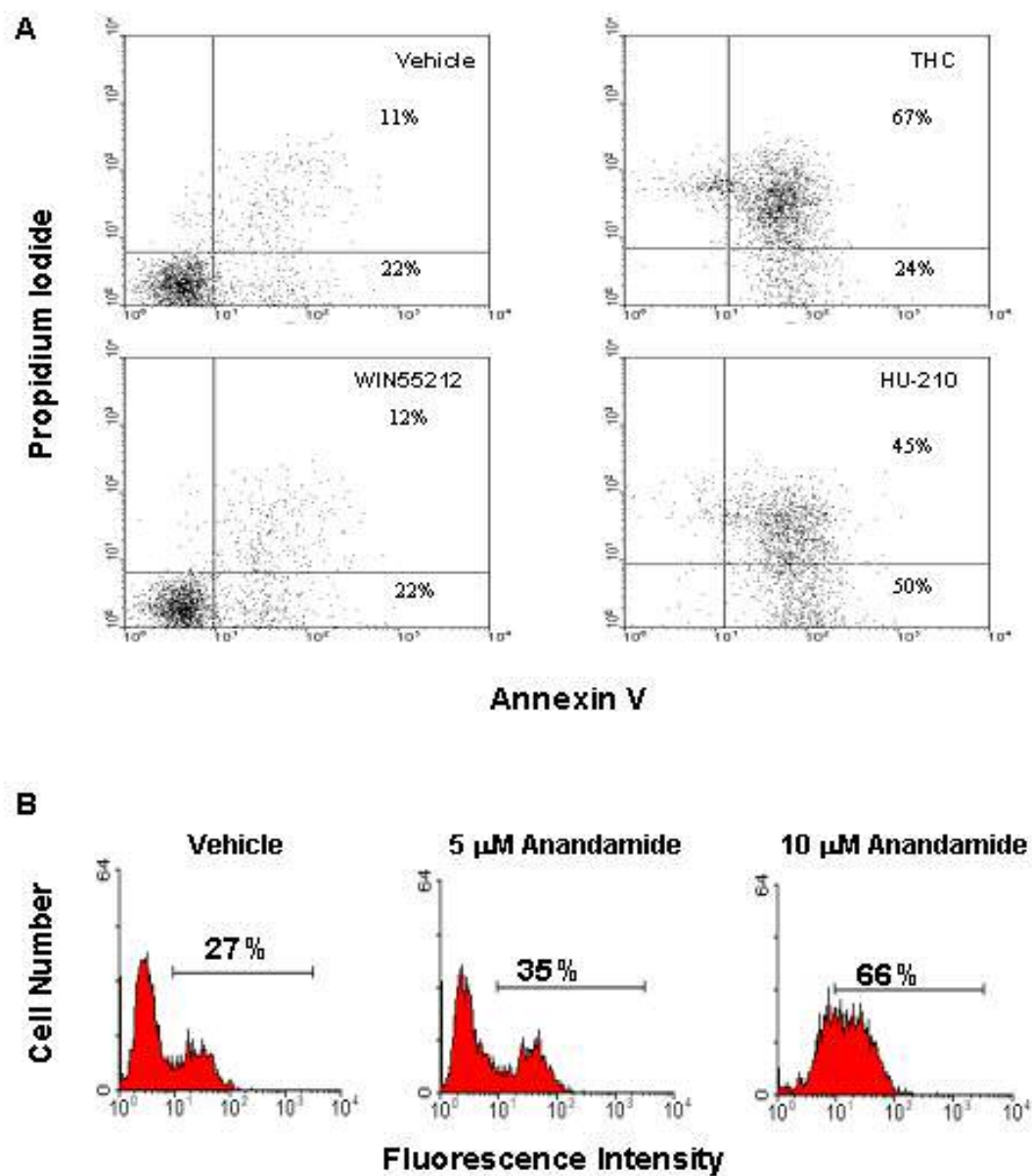


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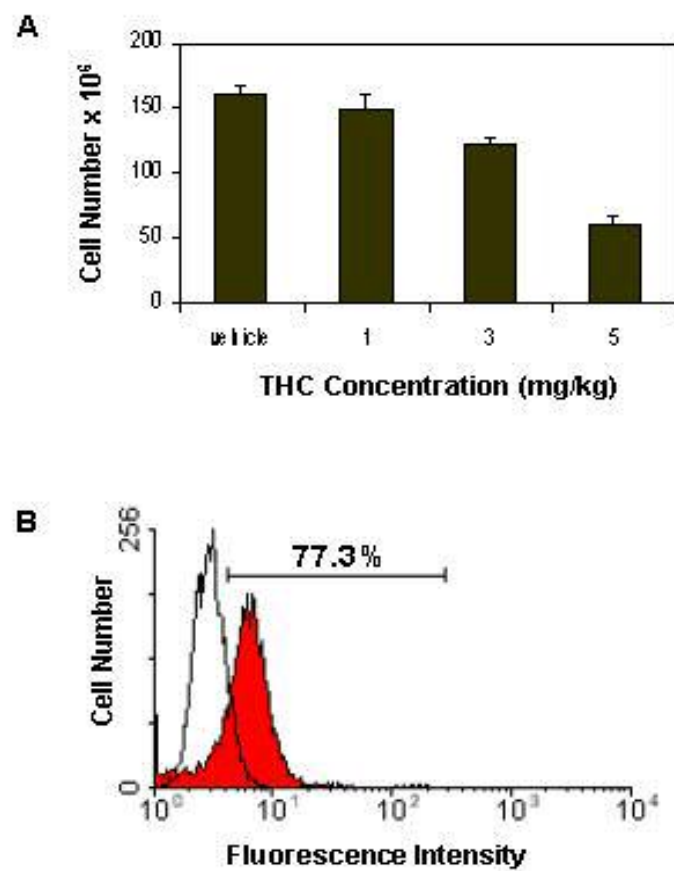


figure 5

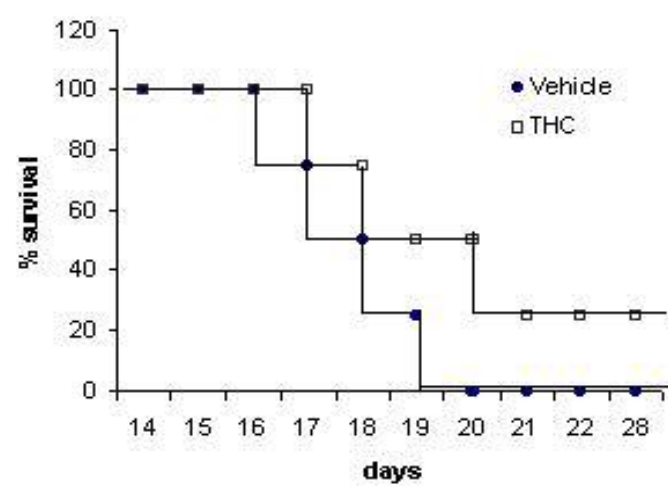


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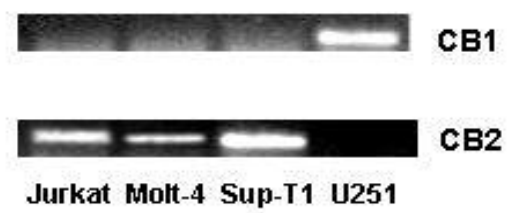


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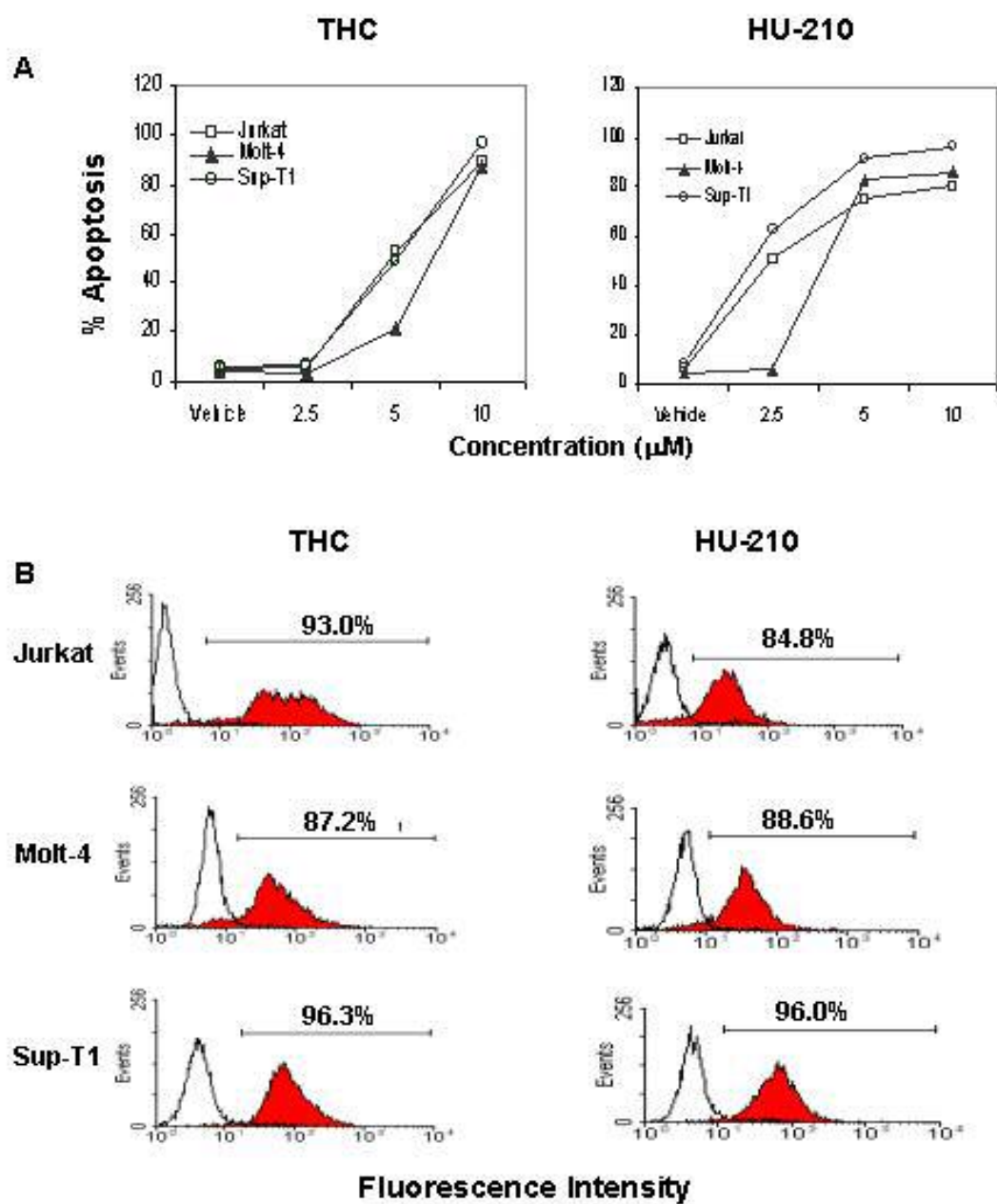


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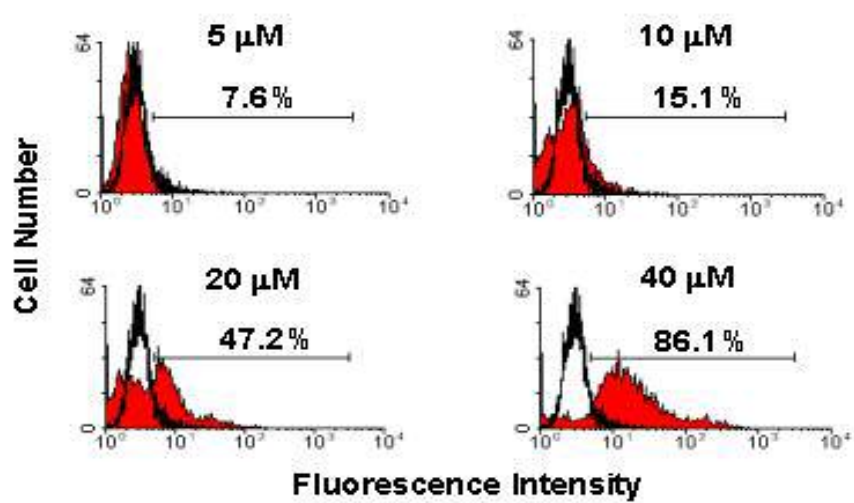


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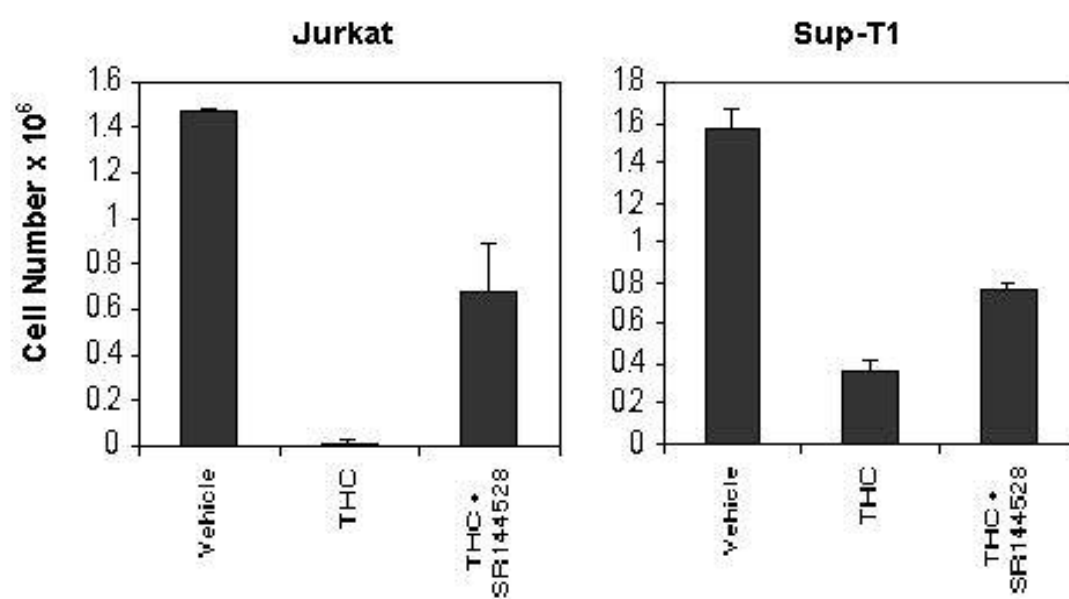


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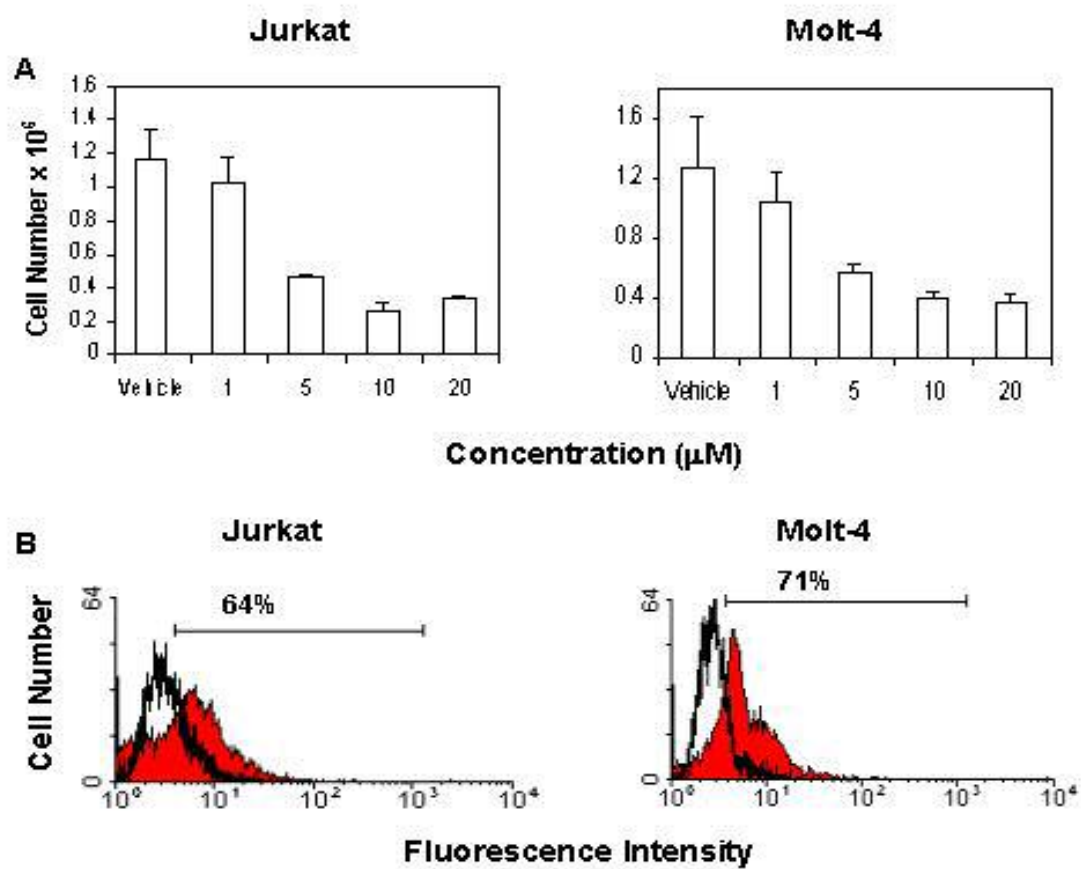


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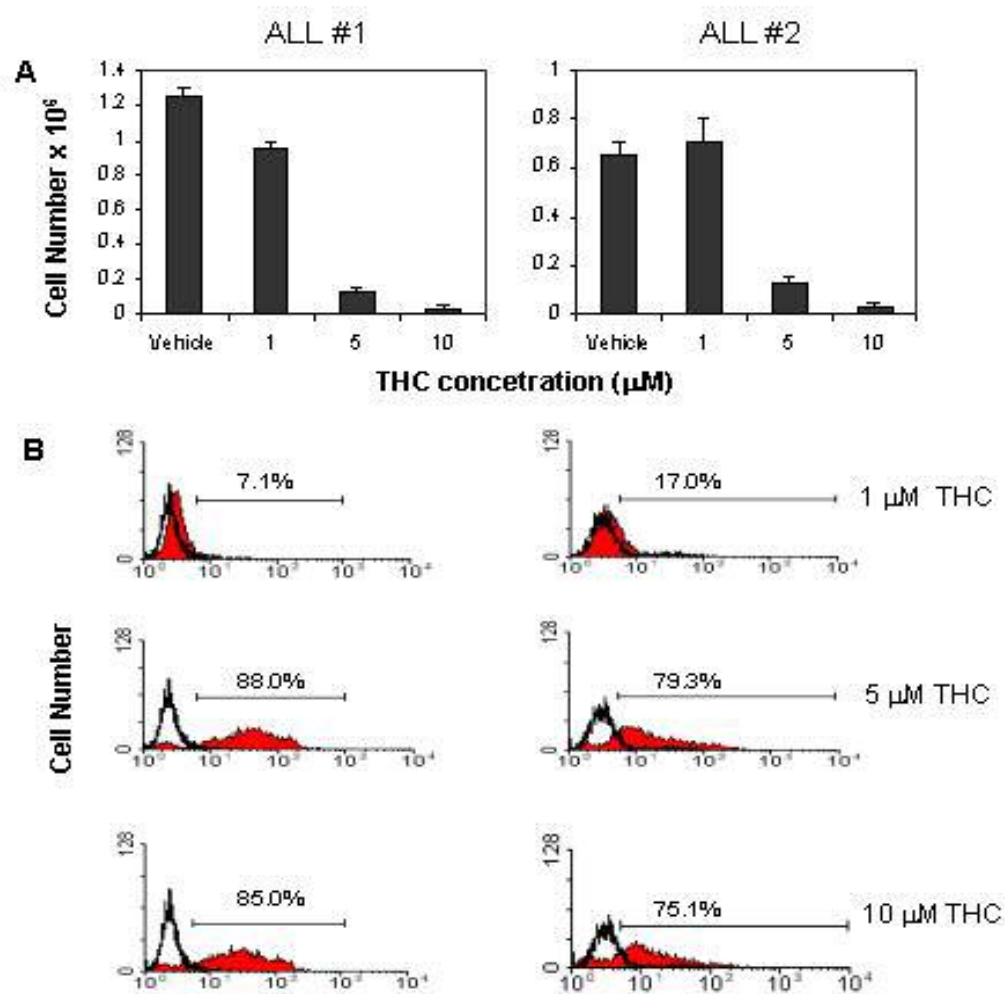


Figure 12