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# Δ-9-Tetrahydrocannabinol Enhances Breast Cancer Growth and Metastasis by Suppression of the Antitumor Immune Response[1]

**Robert J. McKallip,[2]* Mitzi Nagarkatti,* and Prakash S. Nagarkatti†**

**In the current study, we tested the central hypothesis that exposure to Δ-9-tetrahydrocannabinol ($\Delta^9$-THC), the major psychoactive component in marijuana, can lead to enhanced growth of tumors that express low to undetectable levels of cannabinoid receptors by specifically suppressing the antitumor immune response. We demonstrated that the human breast cancer cell lines MCF-7 and MDA-MB-231 and the mouse mammary carcinoma 4T1 express low to undetectable levels of cannabinoid receptors, CB1 and CB2, and that these cells are resistant to $\Delta^9$-THC-induced cytotoxicity. Furthermore, exposure of mice to $\Delta^9$-THC led to significantly elevated 4T1 tumor growth and metastasis due to inhibition of the specific antitumor immune response in vivo. The suppression of the antitumor immune response was mediated primarily through CB2 as opposed to CB1. Furthermore, exposure to $\Delta^9$-THC led to increased production of IL-4 and IL-10, suggesting that $\Delta^9$-THC exposure may specifically suppress the cell-mediated Th1 response by enhancing Th2-associated cytokines. This possibility was further supported by microarray data demonstrating the up-regulation of a number of Th2-related genes and the down-regulation of a number of Th1-related genes following exposure to $\Delta^9$-THC. Finally, injection of anti-IL-4 and anti-IL-10 mAbs led to a partial reversal of the $\Delta^9$-THC-induced suppression of the immune response to 4T1. Such findings suggest that marijuana exposure either recreationally or medicinally may increase the susceptibility to and/or incidence of breast cancer as well as other cancers that do not express cannabinoid receptors and are resistant to $\Delta^9$-THC-induced apoptosis.** *The Journal of Immunology,* **2005, 174: 3281–3289.**

Marijuana is one of the most common drugs of abuse and it medicinal use is the subject of current debate. Δ-9-tetrahydrocannabinol ($\Delta^9$-THC),[3] the major psychoactive component in marijuana (1), 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 with the discovery of two cannabinoid receptors, CB1 and CB2 (3, 4). CB1 is predominantly expressed in the brain, whereas CB2 is primarily found in the cells of the immune system (1, 4). Furthermore, endogenous ligands for these receptors capable of mimicking the pharmacological actions of $\Delta^9$-THC have also been discovered. Such ligands were designated endocannabinoids and include anandamide and 2-arachidonoyl glycerol (5–7). The physiological function of endocannabinoids and cannabinoid receptors remains unclear. Recent work from our laboratory and others suggest that cannabinoids, including $\Delta^9$-THC, may be effective in treating a variety of cancers including lymphomas, leukemias, and gliomas (8–10).

In contrast to these potentially beneficial properties, the use of marijuana has been associated with unwanted effects such as increased susceptibility to infections (11–13), and increased incidence of head and neck cancers in humans (14) and lung cancer in mice (15). $\Delta^9$-THC possesses significant immunomodulatory properties. For example, exposure of macrophages to $\Delta^9$-THC led to decreased production of TNF-$\alpha$ and NO in response to LPS (16). Additionally, exposure of macrophages to $\Delta^9$-THC caused an impairment of their Ag-presenting capabilities (17). Exposure to cannabinoids can also lead to significant reductions in the proliferative and cytolytic response of T lymphocytes and Ab production by B cells (18–21). In addition, other studies conducted in vivo have shown that exposure to $\Delta^9$-THC can lead to increased susceptibility to infections with various pathogens including *Herpes simplex* and Friend leukemia virus (12, 13). Furthermore, exposure to $\Delta^9$-THC has been shown to suppress the immune response to lung cancers in mice (15).

Both the innate and adaptive immune responses are believed to be involved in controlling the growth of many cancers. Coordination of the two arms of the immune system is largely controlled by cytokines produced by cells such as dendritic cells. In addition, T regulatory cells and NKT cells have been implicated in the control of the antitumor immune response (22–24). In general, it is believed that a Th1 response is necessary for an effective immune response to be mounted against most tumors (25). IL-2 and IFN-$\gamma$ are two cytokines that promote a Th1 response, while IL-4 and IL-5 promote a Th2 response. In addition, a number of cytokines possess suppressive activity. For example, IL-10 has been shown to suppress the Th1 response. In previous work, it was demonstrated that exposure to $\Delta^9$-THC could lead to alterations in cytokine production which resulted in suppression of the immune response to *Legionella pneumophila* (26) as well as a lung cancer cell line (15).

Departments of *Microbiology and Immunology, and †Pharmacology and Toxicology, Medical College of Virginia Campus, Virginia Commonwealth University, Richmond, VA 23298

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[2] Address correspondence and reprint requests to Dr. Robert J. McKallip, Department of Microbiology and Immunology, Massey Cancer Center, Virginia Commonwealth University Medical Center, Box 980678, Richmond, VA 23298-0678. E-mail address: rjmckall@hsc.vcu.edu

[3] Abbreviations used in this paper: $\Delta^9$-THC; Δ-9-tetrahydrocannabinol; SOCS, suppressor of cytokine signaling; MMTV, mouse mammary tumor virus.



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Current work examining the potential use of $\Delta^9$-THC and other cannabinoids for the treatment of cancers relies on the expression of CB1 and/or CB2 by the targeted tumor. However, little is known about the effect of $\Delta^9$-THC exposure on the generation, growth, or response to tumors with low to no expression of CB1 and/or CB2. Because CB1 and CB2 are primarily expressed by tumor of neural and immune origins, respectively, it is possible that the majority of tumors originating in other tissues would be significantly less sensitive to $\Delta^9$-THC-mediated killing and because $\Delta^9$-THC is highly immunosuppressive, such tumors may find a favorable environment for growth and progression.

The immune system is suggested to play a key role in controlling the development of cancers as suggested by the findings that immunosuppressed individuals are at a higher risk for developing cancer. For example, there is an increased incidence of Karposi sarcoma, non-Hodgkins lymphoma, Burkitt lymphoma, and cervical cancer in AIDS patients (27). In addition, there have been reports of increased susceptibility to various lymphomas and cutaneous neoplasms following organ transplantation (28–30). Interestingly, there is evidence that de novo breast cancer incidence may increase following liver transplantation (31), suggesting the possibility that the immune system can play an important role in the development of this type of cancer. Therefore, in the current study, using a breast cancer model, we examined the effect of $\Delta^9$-THC exposure on the immune response to and the growth of cancer cells that expresses low to undetectable levels of cannabinoid receptors.

## Materials and Methods

### *Mice*

Adult female BALB/c mice were purchased from the National Institutes of Health. SCID-NOD mice were purchased from The Jackson Laboratory. 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 ± 2°F and on a 12-h light/dark cycle.

### *Reagents*

$\Delta^9$-THC was obtained from the National Institute on Drug Abuse and was initially dissolved in DMSO (Sigma-Aldrich) to a concentration of 20 mM and stored at −20°C. $\Delta^9$-THC was further diluted with tissue culture medium for in vitro studies and PBS for in vivo studies. SR141716A and SR144528 were obtained from Sanofi Recherche. Anti-IL-4 mAbs (11B.11) were obtained from the Biological Resources Branch, National Cancer Institute-Frederick Cancer Research and Development Center. Anti-IL-10 mAbs were obtained from BD Pharmingen.

### *Cell lines*

The murine mammary cell carcinomas 4T1 and EMT6 syngeneic to BALB/c mice, the human breast cancer cell lines, MCF-7 and MDA-MB-231, the human T lymphoblastic leukemia cell line, Jurkat, and the human glioma U87 were maintained in RPMI 1640 (Life Technologies Laboratories) supplemented with 5% FCS, 10 mM HEPES, 1 mM glutamine, 40 $\mu$g/ml gentamicin sulfate, and 50 $\mu$M 2-ME.

### *RNA isolation and RT-PCR*

RNA was isolated from ~$1 \times 10^7$ cells using the RNeasy Mini kit (Qiagen). 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 $\mu$g of 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. $\beta$-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 were conducted 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 an Applied Biosystems GeneAmp 9700. The resulting PCR products were separated on a 1% agarose gel.

### *Detection of $\Delta^9$-THC-mediated cell death in vitro*

Tumor cells or splenocytes ($1 \times 10^6$ cells/well) were cultured in 24-well plates in the presence or absence of various concentrations of $\Delta^9$-THC for 24 h. Next, the cells were harvested, washed twice in PBS and analyzed for cell viability by trypan blue dye exclusion.

### *Quantification of the effect of $\Delta^9$-THC exposure on 4T1 tumor growth and metastasis in vivo*

Groups of BALB/c or SCID-NOD mice were injected s.c. with $3 \times 10^5$ 4T1 tumor cells. Three days later, the mice then were exposed every other day for ~3 wk to various doses of $\Delta^9$-THC (12.5, 25, or 50 mg/kg body weight) or vehicle (DMSO) control. The tumor volume was observed, recorded, and calculated using the following equation: tumor volume = length × width$^2$ × 0.52. In addition, the level of metastasis was determined by directly quantifying the number of metastatic nodules located in the lungs, by H&E staining of lung sections, and by assessing tumor burden by determining the increase in lung weight.

### *In vivo antitumor immune response*

BALB/c mice were first sensitized to 4T1 by injecting them i.p. with $1 \times 10^6$ irradiated 4T1 tumor cells twice at 2-wk intervals. Two weeks following the final sensitization, the mice were injected s.c. into their rear footpads with $1 \times 10^5$ irradiated 4T1 tumor cells or $1 \times 10^5$ irradiated EMT6 tumor cells (negative control). Groups of mice were then treated i.p. with various doses of $\Delta^9$-THC (0, 12.5, 25, or 50 mg/kg) daily for 4 days. Four days following the challenge with the irradiated tumor cells the immune response was determined by aseptically removing the draining lymph node and quantifying the increase in lymph node mass, cell number, and lymphocyte DNA synthesis. In experiments examining the role of anti-IL-4 mAbs and anti-IL-10 mAbs, mice received a single injection of 5 mg or 5 mg/kg of mAbs, respectively, which was previously shown to effectively reduce IL-4 and IL-10 concentrations (15, 32).

### *In vitro proliferation assay*

The spleens and lymph nodes from control or $\Delta^9$-THC-treated mice were placed into 10 ml of RPMI 1640 (Life Technologies Laboratories) supplemented with 10% FCS, 10 mM HEPES, 1 mM glutamine, 40 $\mu$g/ml gentamicin sulfate, and 50 $\mu$M 2-ME, referred to as complete medium. The spleens and lymph nodes were prepared into a single cell suspension using a laboratory homogenizer, washed twice, and adjusted to $5 \times 10^6$/ml in complete medium. The splenocytes and lymph node cells ($5 \times 10^5$ in 100 $\mu$l/well) were cultured in 96-well flat-bottom plates and stimulated with various concentrations of irradiated 4T1 tumor cells for 4 days. During the final 8 h of culture, the cells were pulsed with 2 $\mu$Ci of [$^3$H]thymidine. DNA synthesis was determined by beta scintillation counting (33, 34).

### *Cytokine detection*

BALB/c mice were first sensitized to 4T1 by injecting them i.p. with $1 \times 10^6$ irradiated 4T1 tumor cells twice at 2-wk intervals. Two weeks following the final sensitization injection the mice were injected s.c. into their rear footpads with $1 \times 10^5$ irradiated 4T1 tumor cells. Groups of mice were then treated daily for 4 days with vehicle control or $\Delta^9$-THC (50 mg/kg i.p.). Four days following the challenge with the irradiated tumor cells the draining lymph node were removed and adjusted to $2.5 \times 10^6$/ml in RPMI 1640 containing 10% FCS. The lymph node cells were cultured in a 96-well flat-bottom plate (200 $\mu$l/well) for 24 h, after which the levels of IFN-$\gamma$, TGF-$\beta$, IL-4, IL-10, and TNF were determined using the methods described in the Quantikine M ELISA kits (R&D Systems).

### *Microarray analysis of gene expression*

Total RNA was isolated from lymph node cells isolated from 4T1-immunized mice that were stimulated in their rear footpads with irradiated 4T1 tumor cells ($1 \times 10^5$ s.c.) and treated i.p. for 4 days with vehicle or 50 mg/kg $\Delta^9$-THC using the RNeasy Mini kit (Qiagen). Labeled cDNA probes were synthesized from the RNA samples using the Ampolabeling-LPR kit (SuperArray). The labeled cDNA probes were hybridized to individual GEArray Q series mouse Th1, Th2, Th3 array membranes overnight at 60°C with continuous agitation at 5–10 rpm. The membranes were washed twice for 10 min at 60°C with 2× SSC, 1% SDS solution, and twice for 10

min at 60°C with 0.1× SSC, 0.5% SDS. Nonspecific binding was blocked by incubating the membranes with GEAblocking solution for 40 min. The membranes were labeled with alkaline phosphatase-conjugated streptavidin alkaline phosphatase for 10 min. Excess alkaline phosphatase was removed by washing the membranes four times with Buffer F (SuperArray) for 5 min and rinsing the membranes with Buffer G. Gene expression was detected using CDP-Star chemiluminescent substrate and exposing the membranes to x-ray film. The data were analyzed by converting the x-ray image into a grayscale TIFF file and using the ScanAlzye software program to convert the data into numerical data. Finally, data analysis was performed using the GEArray Analyzer data analysis software (SuperArray). Data was normalized using housekeeping genes including $\beta$-actin, GAPDH, cyclophilin A, and ribosomal protein L13a.

### *Statistical analysis*

Student's *t* test or Tukey Kramer test was used to compare vehicle and $\Delta^9$-THC-treated groups. $p < 0.05$ was considered to be statistically significant.

## Results

### *Expression of CB1 and CB2 in human and murine breast cancer cells*

The expression of CB1 and CB2 mRNA was determined by RT-PCR. The results showed that splenocytes expressed both receptors, while in the 4T1 breast cancer cells, CB1 and CB2, mRNA was not detectable (Fig. 1*A*). Similar results were seen when we examined the expression of CB1 and CB2 in the human breast cancer cell lines MCF-7 and MDA-MB-231 (Fig. 1*C*). In this experiment, Jurkat cells were used as a positive control for CB2 expression and the human glioma U87 was used as a positive control for CB1 expression. The results demonstrated that in both human breast cancer cell lines there was very low detectable expression of CB1 while CB2 expression was not detected.

### *Sensitivity of 4T1 and MCF-7 to $\Delta^9$-THC-induced cell death*

Next we examined whether 4T1 or MCF-7 were sensitive to $\Delta^9$-THC-induced cytotoxicity compared with other cells reported to be sensitive to $\Delta^9$-THC. To this end, 4T1 breast cancer cells and splenocytes from BALB/c mice were cultured for 24 h in RPMI 1640 containing 5% FCS in the presence of various concentrations of $\Delta^9$-THC (0, 5, 10, or 20 $\mu$M). The viable cell number was determined by trypan blue dye exclusion (Fig. 1*B*). The results demonstrated that although the splenocytes were highly sensitive to $\Delta^9$-THC-induced killing, the 4T1 cells were relatively resistant. No decrease in viable cell number in the 4T1 breast cancer cells was observed even at the highest concentration of $\Delta^9$-THC tested. In contrast, splenic culture showed a significant reduction in viable cell number following exposure to concentration of $\Delta^9$-THC as low as 5 $\mu$M. In addition, we examined whether the human breast cancer cell line MCF-7 was sensitive to $\Delta^9$-THC-mediated cell death. To this end, MCF-7 and Jurkat cells were cultured for 24 h in RPMI 1640 containing 5% FCS in the presence of various concentrations of $\Delta^9$-THC (0, 5, 10, or 20 $\mu$M) and the viable cell number was determined by trypan blue dye exclusion (Fig. 1*D*). The results showed that while the Jurkat cells were sensitive to $\Delta^9$-THC-mediated killing at concentrations as low as 5 $\mu$M, the MCF-7 cells were resistant to $\Delta^9$-THC-induced toxicity. Together, these data suggested that both the murine 4T1 and the human MCF-7 human breast cancer cell lines are resistant to killing mediated by $\Delta^9$-THC exposure.

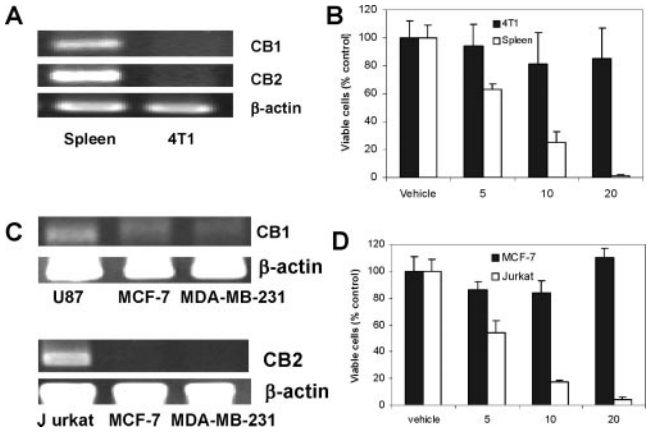


**FIGURE 1.** Human and murine breast cancer cells expression of CB1 and CB2 and sensitivity to $\Delta^9$-THC-induced cytotoxicity. The expression of CB1 and CB2 mRNA was determined by RT-PCR analysis. Total RNA was isolated from mouse spleen cells, 4T1, Jurkat, MCF-7, MDA-MB-231, and U87 tumor cells. mRNA was reverse transcribed and amplified by PCR with primers specific for CB1, CB2, and $\beta$-actin. A photograph of ethidium bromide-stained amplicons is depicted (*A* and *C*). The effect of $\Delta^9$-THC on cell viability was determined by culturing the cells with various concentrations of $\Delta^9$-THC for 24 h in medium containing 5% FCS. The cell viability was determined by trypan blue dye exclusion. The data were expressed as percent of control viable cell number (*B* and *D*).

### *$\Delta^9$-THC-exposure leads to increased growth of the 4T1 breast cancer in vivo*

Next, we examined whether exposure to $\Delta^9$-THC had any effect on the local growth of the 4T1 tumor (Fig. 2*A*). To this end, BALB/c mice were injected s.c. with $3 \times 10^5$ 4T1 tumor cells. Three days following the tumor injection, the mice were exposed every other day for 18–21 days to either vehicle or various doses of $\Delta^9$-THC (12.5, 25, or 50 mg/kg). Tumor growth was monitored and the data revealed that exposure to 25 mg/kg $\Delta^9$-THC led to a significant increase in tumor mass. This effect was even more pronounced in mice treated with 50 mg/kg $\Delta^9$-THC.

### *$\Delta^9$-THC-exposure leads to increased metastasis of 4T1 tumor to the lung*

In addition to examining the effects of $\Delta^9$-THC on the local growth of the 4T1 tumor, we examined whether exposure to $\Delta^9$-THC would have any effect on the level of metastasis in the lungs. To this end, BALB/c mice were injected s.c. with $3 \times 10^5$ 4T1 tumor cells. Three days following the tumor injection, the mice were exposed every other day to either vehicle control or various doses of $\Delta^9$-THC (12.5, 25, or 50 mg/kg). The lungs from the tumor-bearing mice were harvested 18–21 days following tumor injection and the level of metastasis was quantified (Fig. 2, *B* and *C*). The results showed that exposure to 25 or 50 mg/kg $\Delta^9$-THC led to a significant increase in the number of tumor nodules located in the lungs. H&E staining of lung sections revealed that $\Delta^9$-THC-treatment led to a dose-dependent increase in the size of the metastatic nodules (Fig. 2*D*). In addition, tumor burden in the lungs was quantified by determining the increase in lung mass in tumor bearing mice vs control mice and the results show that $\Delta^9$-THC-treatment led to a significant increase in lung mass (Fig. 2*E*). Together, these results suggested that $\Delta^9$-THC-exposure increased the metastasis of 4T1 tumor to the lungs.

### *The effect of $\Delta^9$-THC exposure on 4T1 tumor growth in SCID-NOD mice*

Next, the role of the immune system in the observed increase in 4T1 tumor growth and metastasis following $\Delta^9$-THC was evaluated using the SCID-NOD model. SCID-NOD mice are devoid of an antitumor immune response. Therefore, any effect of $\Delta^9$-THC

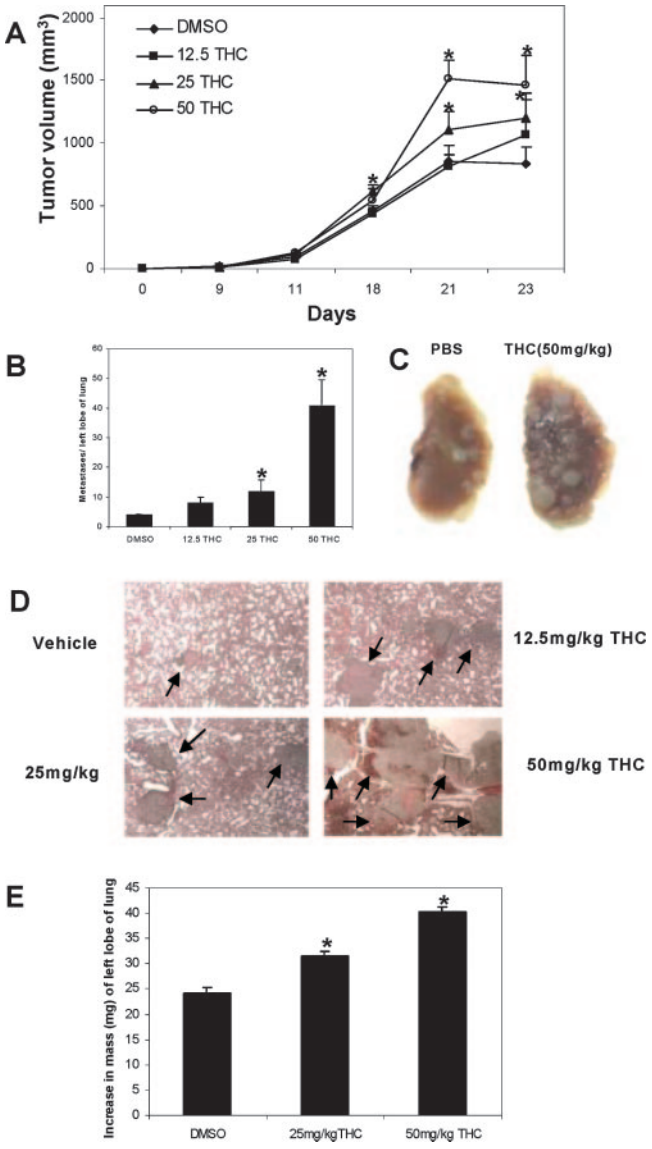


**FIGURE 2.** Δ9-THC exposure leads to an increase in number and size of 4T1 tumors metastasizing to the lungs in vivo. Mice injected s.c. with $3 \times 10^5$ 4T1 tumor cells were treated with various concentration of Δ9-THC every other day for 21 days. Local tumor volume was determined (*A*). The lungs were harvested and the metastases were quantified (*B* and *C*). Sections of the lungs were stained with H&E (*D*). The arrows indicate the sites of tumor growth. ∗, Statistically significant difference ($p < 0.05$) when compared with the controls. The results are representative data of experimental groups containing four mice. The experiment has been repeated three times with similar results. Tumor burden was quantified by determining the increase in the weight of the lungs from tumor bearing mice compared with control mice (*E*).

on tumor growth in these mice would be independent of an effect on the immune response. To this end, SCID-NOD mice were injected s.c. with 4T1 tumor cells. The mice were then treated with the vehicle or 25 mg/kg Δ9-THC every other day for 19 days. Local tumor growth and metastasis were recorded. The results revealed that Δ9-THC exposure did not result in a significant increase in tumor growth (Fig. 3*A*) or metastasis (Fig. 3*B*), suggesting that the effects of Δ9-THC on the growth of the 4T1 tumor in immunocompetent mice may be directly related to an effect on the immune system.

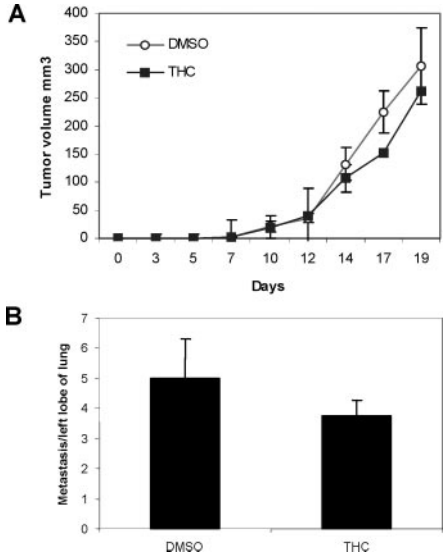


**FIGURE 3.** The effect of Δ9-THC exposure on 4T1 tumor growth in SCID-NOD mice. SCID-NOD mice were injected s.c. with $3 \times 10^5$ 4T1 tumor cells. The mice were then treated i.p. with the vehicle control or 25 mg/kg Δ9-THC every other day for 19 days. Local tumor growth (*A*) and metastasis were recorded (*B*). The results are representative data of experimental groups containing four mice. The experiment was repeated three times with similar results.

### *Δ9-THC exposure directly suppresses the immune response to 4T1 in vivo*

To directly examine the effect of Δ9-THC exposure on the antitumor immune response, we used a modified version of the popliteal lymph node assay. More specifically, mice were first sensitized to 4T1 by injecting them twice, separated by 2 wk, with irradiated 4T1 tumor cells. Two weeks following the final sensitization injection, the mice were rechallenged in their rear footpads with irradiated 4T1 tumor cells and either received daily i.p. injections of Δ9-THC (25 or 50 mg/kg) or vehicle. The immune response was determined 4 days following rechallenge by harvesting the lymph nodes, draining the site of tumor injection, and assessing the increase in lymph node mass (Fig. 4*A*) and cell number (Fig. 4*B*) compared with the same lymph nodes from mice not receiving the rechallenge. The results showed that rechallenge with 4T1 led to a significant and measurable immune response and that exposure to Δ9-THC at concentrations as low as 25 mg/kg significantly suppressed the antitumor immune response against 4T1. In addition, groups of mice sensitized against 4T1 received a challenge with an unrelated syngeneic mammary carcinoma (EMT6), and such mice showed no significant immune response (data not shown), demonstrating that the immune response in the sensitized mice was specific for 4T1. Next, the effect of Δ9-THC exposure on the growth and metastasis of 4T1 tumor cells in 4T1-sensitized mice was examined and the data showed that exposure to Δ9-THC led to quicker appearance of detectable tumors (Fig. 4*C*), an increase in tumor size (Fig. 4*D*), and an increase in the level of metastatic lesions in the lungs (Fig. 4*E*). Together, the results from these experiments demonstrated 4T1 tumor can be immunogenic and that exposure to Δ9-THC can suppress the immune response against 4T1 tumor, which may account for enhanced tumor growth and metastasis.

### *Δ9-THC exposure leads to suppression of the tumor-specific proliferative response*

To further examine the effect of Δ9-THC on the antitumor immune response, we determined the effect of Δ9-THC exposure on the

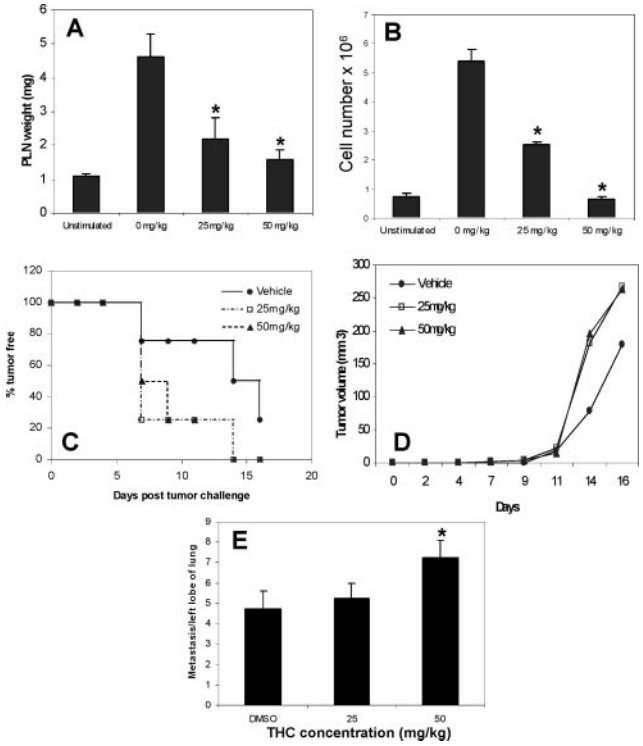


**FIGURE 4.** $\Delta^9$-THC exposure directly suppresses the immune response to 4T1 in vivo. 4T1-sensitized mice were challenged s.c. in their footpads with irradiated 4T1 ($1 \times 10^5$ cells). After which, the mice were treated daily for 4 days with CB1 (SR141716A), CB2 (SR144528) antagonists, or vehicle 1 h before exposure to $\Delta^9$-THC (25 mg/kg). The immune response was assayed by determining the increase in lymph node mass (*A*) and cell number (*B*) compared with unchallenged mice. ∗, Statistically significant difference ($p < 0.05$) when compared with the controls. The effect of $\Delta^9$-THC exposure on the growth and metastasis of 4T1 tumor cells in 4T1-sensitized mice was examined. To this end, 4T1-sensitized mice were injected s.c. with $1 \times 10^5$ live 4T1 cells. The mice were treated every other day for 16 days with vehicle or $\Delta^9$-THC (25 or 50 mg/kg). The tumor incidence (*C*), tumor mass (*D*), and number of metastatic lesions in the lungs were determined (*E*). The results are representative data of experimental groups containing four mice. The experiment was repeated three times with similar results.

proliferative response to 4T1. To this end, sensitized mice were treated for 4 days with various concentrations of $\Delta^9$-THC (0, 25, and 50 mg/kg). Next, the splenocytes and lymph node cells were harvested and cultured in vitro for 4 days with irradiated 4T1 tumor cells. The proliferative response was determined by [$^3$H]thymidine uptake and the results revealed that in vivo exposure to $\Delta^9$-THC led to a significant suppression of the proliferative response of splenocytes (Fig. 5*A*) and lymph nodes cells (Fig. 5*B*) to 4T1.

### *The effect of CB1 and CB2 antagonist on $\Delta^9$-THC-induced suppression of the immune response to 4T1 in vivo*

To investigate the role of CB1 and CB2 in $\Delta^9$-THC-induced suppression of the antitumor immune response to 4T1, sensitized mice were challenged s.c. in their footpads with irradiated 4T1. Next, the mice were treated daily for 4 days with CB1 (SR141716A), CB2 (SR144528) antagonists (20 mg/kg), or vehicle 1 h before exposure to $\Delta^9$-THC (25 mg/kg). The immune response was assayed by determining the increase in lymph node mass (Fig. 6*A*), cell number (Fig. 6*B*), and proliferation (Fig. 6*C*) compared with unchallenged mice. The results demonstrated that treatment with the CB2 antagonist, but not the CB1 antagonist, could significantly

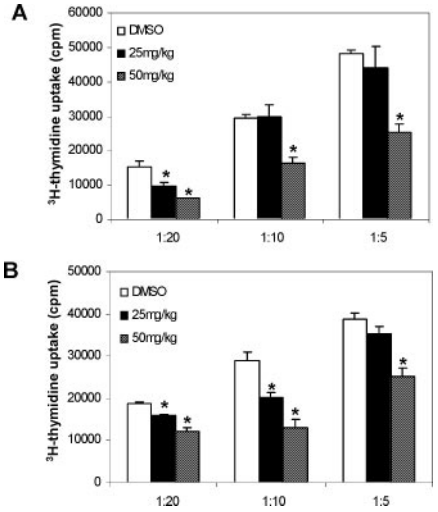


**FIGURE 5.** $\Delta^9$-THC exposure leads to suppression of the tumor-specific proliferative response. 4T1-sensitized mice were treated for 4 days with vehicle or $\Delta^9$-THC (25 or 50 mg/kg). Next, spleens (*A*) and lymph nodes (*B*) were harvested and cultured in vitro for 4 days with irradiated 4T1 tumor cells. Various responder (splenocytes or lymph node cells) to stimulator (irradiated 4T1 tumor cells) were tested. The proliferative response was determined by [$^3$H]thymidine uptake. The experiment was repeated three times with similar results. ∗, Statistically significant difference ($p < 0.05$) when compared with the controls.

reverse the $\Delta^9$-THC-induced suppression of the immune response against 4T1, suggesting a prominent role for CB2 in the observed $\Delta^9$-THC-mediated suppression of the antitumor immune response.

### *$\Delta^9$-THC exposure alters cytokine production*

Previous reports have indicated that exposure to $\Delta^9$-THC can alter the production of various cytokines (35, 36). Because the antitumor immune response is primarily mediated by Th1-directed immune response, we examined whether exposure to $\Delta^9$-THC had any effect on the production of Th1 vs Th2 cytokines. To this end, 4T1-sensitized mice were challenged s.c. with irradiated 4T1 tumors and then received daily injection with various doses of $\Delta^9$-THC (vehicle, 25, and 50 mg/kg). Four days following the challenge with 4T1, the lymph node cells draining the site of injection were harvested, counted, and cultured ($1 \times 10^6$ cells/well) for 24 h in 96-well plates. Next, the supernatants were tested for the presence of various Th1 and Th2 cytokines (Fig. 7*A*). The results showed that exposure to 25 mg/kg $\Delta^9$-THC led to a dramatic increase in the Th2 cytokines IL-4 and IL-10, suggesting that at this concentration, $\Delta^9$-THC enhances the Th2 response. In addition, levels of IFN-$\gamma$ were found to be elevated following exposure to $\Delta^9$-THC. Interestingly, exposure to 50 mg/kg $\Delta^9$-THC led to a significant reduction in IL-4, IFN-$\gamma$, and IL-10 compared with the vehicle or 25 mg/kg $\Delta^9$-THC groups, suggesting the possibility that at a higher concentration, $\Delta^9$-THC was leading to a more generalized suppression of the antitumor immune response, possibly due to the induction of apoptosis (21).

### *Anti-IL-4 and anti-IL-10 mAbs partially prevent $\Delta^9$-THC-induced suppression of the immune response to 4T1*

Next, we examined the effects of Abs against IL-4 or IL-10 on the $\Delta^9$-THC-induced suppression of the immune response to 4T1. To this end, 4T1-sensitized mice were first challenged in their rear footpads with irradiated 4T1 cells. Groups of mice were then treated with vehicle + isotype control mAbs, $\Delta^9$-THC (25 mg/kg/day) + isotype control mAbs, $\Delta^9$-THC (25 mg/kg/day) + anti-

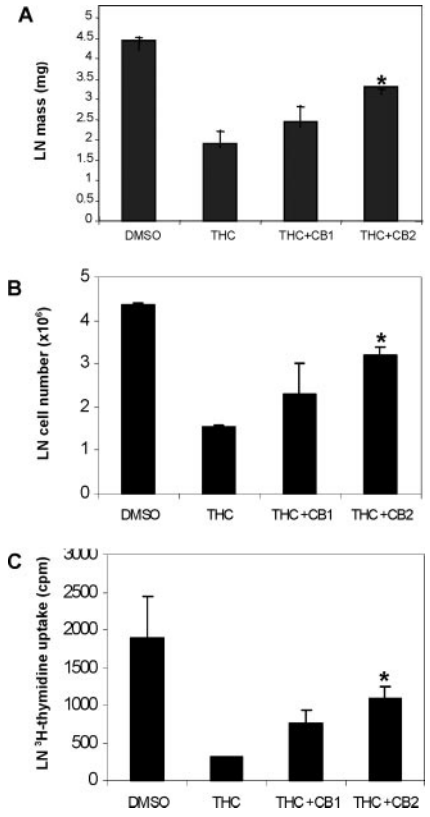


**FIGURE 6.** The effect of CB1 and CB2 antagonist on $\Delta^9$-THC-induced suppression of the immune response to 4T1 in vivo. 4T1-sensitized mice were challenged s.c. in their footpads with irradiated $1 \times 10^5$ irradiated 4T1 cells. Next, the mice were treated daily for 4 days with CB1 (SR141716A), CB2 (SR144528) antagonists (20 mg/kg), or vehicle 1 h before exposure to $\Delta^9$-THC (25 mg/kg). The immune response was assayed by determining the increase in lymph node mass (*A*), cell number (*B*), and DNA synthesis (*C*) compared with lymph nodes from unchallenged mice. The results are representative data of experimental groups containing four mice. The experiment was repeated three times with similar results. *, Statistically significant difference ($p < 0.05$) when compared with the controls.

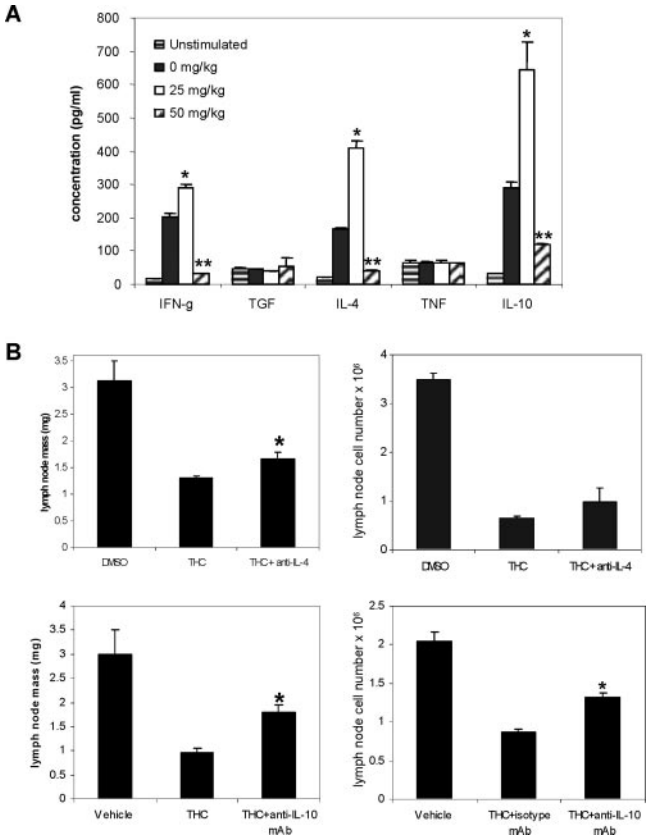


**FIGURE 7.** $\Delta^9$-THC exposure leads to alteration in cytokine production. 4T1-sensitized mice were challenged s.c. with $1 \times 10^5$ irradiated 4T1 cells and then received daily injection with various doses of $\Delta^9$-THC (vehicle, 25, and 50 mg/kg). Four days following the challenge with 4T1, the lymph node cells draining the site of injection were harvested, counted and cultured ($1 \times 10^6$ cells/well) for 24 h in 96-well plates. Next, the supernatants were tested for the presence of various Th1 and Th2 cytokines (*A*). *, Statistically significant increase ($p < 0.05$) when compared with the untreated control. **, Statistically significant decrease ($p < 0.05$) when compared with the untreated control. To examine the effects of Abs against IL-4 or IL-10 on $\Delta^9$-THC-induced suppression of the immune response, 4T1-sensitized mice were first challenged in their rear footpads with irradiated 4T1 cells ($1 \times 10^5$ cells). Groups of mice were then were treated with vehicle control + isotype control mAbs, $\Delta^9$-THC (25 mg/kg/day) + isotype control mAbs, $\Delta^9$-THC (25 mg/kg/day) + anti-IL-4 mAbs, or $\Delta^9$-THC (25 mg/kg/day) + anti-IL-10 mAbs. The immune response was assayed 4 days later by determining the mass and cell number of the lymph nodes draining the site of 4T1 injection (*B*). *, Statistically significant differences ($p < 0.05$) when compared with mice treated with $\Delta^9$-THC alone.

IL-4 mAbs, or $\Delta^9$-THC (25 mg/kg/day) + anti-IL-10 mAbs. The immune response was assayed 4 days later by determining the mass and cell number of the lymph nodes draining the site of 4T1 injection (Fig. 7*B*). The results showed that exposure to $\Delta^9$-THC led to a significant reduction in the lymph node mass. However, if the mice were treated with anti-IL-10 mAbs, or to a lesser extent, anti-IL-4 mAbs, the $\Delta^9$-THC-induced reduction in lymph node mass could be partially reversed. Together, these results further suggested a role for IL-4 and IL-10 in the $\Delta^9$-THC-induced suppression of the immune response to 4T1.

### *The effect of $\Delta^9$-THC exposure of gene expression in lymph node cells draining the site of 4T1 injection*

Next, using cDNA array analysis, we screened for alterations in the expression of genes involved in the Th1 and Th2 response in lymph node cells draining the site of 4T1 injection following exposure of 4T1-sensitized mice to 25 mg/kg $\Delta^9$-THC. Of the 96 genes screened, the expression of 18 genes was significantly (>2-fold) altered in the lymph nodes cells from the $\Delta^9$-THC-treated mice. The expression of 6 genes was reduced, while the expression of 12 was increased in the lymph node cells isolated from the $\Delta^9$-THC-treated mice (Table I). Included in the group of down-regulated genes were the Th1-associated genes, IL-1R, and the TNFR superfamily members DR6 and 4-1BB. In addition, the expression of a number of transcriptional regulators was reduced. Analysis of the genes that were up-regulated following exposure to $\Delta^9$-THC revealed several Th2-associated genes, including C2ta, eotaxin, IL-13R, IL-4, IL-4R, IL-5, GATA binding protein 3, and growth factor independent 1. In addition, the expression of a number of transcriptional regulators including, suppressor of cytokine signaling (SOCS)2, SOCS5, SOCS7, and Fos-like Ag 2 was increased. Taken together, the results from the cDNA analysis further suggested that exposure to $\Delta^9$-THC leads to suppression of genes regulating Th1 response and an increase in the Th2 response genes leading to an inefficient immune response against the 4T1 tumor in vivo.

## Discussion

In the current study, we demonstrated that exposure to $\Delta^9$-THC can enhance the growth and metastasis of the 4T1 mammary

Table I. *cDNA array analysis of Th1/Th2-associated gene expression in 4T1-stimulated lymph node cells from vehicle- or $\Delta^9$-THC-treated 4T1-sensitized mice*[a]

| Gene Name | Description | Function | Accession Number | Fold Change[b] vs Vehicle |
|---|---|---|---|---|
| *C2ta* | Class II transactivator | Th2 | NM_007575 | +2.1 |
| *Scya11/eotaxin* | Small chemokine ligand 11 | Th2 | U26426 | +19.4 |
| *IL13RA2* | Interleukin-13 receptor, $\alpha$2 | Th2 | U65747 | +4.4 |
| *IL-4* | Interleukin-4 | Th2 | M25892 | +4.8 |
| *IL-4ra* | Interleukin-4 receptor, $\alpha$ | Th2 | NM_010557 | +4.4 |
| *IL-5* | Interleukin-5 | Th2 | NM_010558 | +2.9 |
| *GATA3* | GATA binding protein 3 | Th2 | NM_008091 | +2.9 |
| *Gfi1* | Growth factor independent 1 | Th2 | NM_010278 | +3.5 |
| *SOCS2* | SOCS2 | Trans. reg. | NM_007706 | +2.0 |
| *SOCS5* | SOCS5 | Trans. reg. | NM_019654 | +6.8 |
| *SOCS7* | SOCS7 | Trans. reg. | NM_080843 | +2.6 |
| *Fosl2* | Fos-like antigen 2 | Trans. reg. | NM_008037 | +3.2 |
| *IL-1R* | Interleukin-1 receptor | Th1 | U43673 | −14.6 |
| *DR6* | TNF receptor superfamily member | Th1 | AF322069 | −5.3 |
| *4–1BB* | TNF receptor superfamily member | Th1 | J04492 | −5.7 |
| *Jund1* | Jun proto-oncogene related gene d1 | Trans. reg. | NM_010592 | −11.7 |
| *JNKK2* | MAP kinase kinase MKK7 | Trans. reg. | U74463 | −2.5 |
| *JNK1* | Mitogen-activated protein kinase 8 | Trans. reg. | AB005663 | −2.9 |

[a] Summary of gene expression that was found to be increased or decreased in splenic cells isolated from $\Delta^9$-THC-treated preimmunized mice following stimulation with irradiated 4T1 cells mice compared to the gene expression in splenic cells isolated from vehicle-treated preimmunized mice following stimulation with irradiated 4T1.
[b] Fold change represents the change in gene expression following normalization with $\beta$-actin gene expression.
[c] Trans. Reg., Transcriptional regulator.

carcinoma. This is in contrast to our previous finding in which we demonstrated that treatment with $\Delta^9$-THC led to the elimination of the EL-4 leukemia in vivo (8). This stark contrast suggests that some tumors may be more resistant to $\Delta^9$-THC-mediated killing and that the effects of $\Delta^9$-THC on the immune system may play an important role in tumor growth and host survival in such tumor models. More specifically, we hypothesize that the degree of sensitivity of a tumor to $\Delta^9$-THC may be directly related to the level of CB1 and CB2 expression. Importantly, these results would suggest that, although $\Delta^9$-THC may be effective at killing tumors that express cannabinoid receptors, $\Delta^9$-THC-exposure may actually lead to increased growth and metastasis of tumors with low to no expression of cannabinoid receptors due to suppression of the antitumor immune response.

The use of cannabinoids for the treatment of a number of cancers is currently under investigation (8, 10, 37, 38). However, little is known about the relationship between the level of cannabinoid expression and the sensitivity to $\Delta^9$-THC killing. In the current study, we proposed that tumors that express little to no cannabinoid receptors would be relatively resistant to the cytotoxic effects of $\Delta^9$-THC. This was shown in both a mouse and human breast cancer cell line. However, previous studies have shown that exposure to cannabinoids can lead to a decrease in the growth of some breast cancer cell lines in vitro. For example, exposure to anandamide inhibited the proliferation of the MCF-7 and EFM-19 human breast cancer cell line in vitro (38). It should be noted that although the use of CB1 antagonists led to the partial reversal of the anandamide-induced suppression of the proliferation of the EFM-19 cell line, the expression of CB1 or CB2 was not directly examined. To date, little has been reported about the expression and/or role of cannabinoid or vanilloid receptors in either human or mouse breast cancer cell lines. In this report, we demonstrated that the human breast cancer cell lines MCF-7 and MDA-MB-231 express only low levels of CB1 and undetectable levels of CB2 and that neither receptor was detectable in the mouse 4T1 mammary cell carcinoma. In addition, we demonstrated that 4T1 cells express high levels of vanilloid receptor 1 (data not shown). Therefore, because anandamide is also know to act as potent agonist for the vanilloid receptor 1 (39–41), it is possible that the breast cancer cells may be more sensitive to anandamide compared with $\Delta^9$-THC due to the expression of vanilloid receptors. In addition, most previous studies did not directly examine the effects of anandamide on the growth of tumors in an in vivo setting. Therefore, depending on the role of the immune system in the control of growth of the specific tumor tested, it is still possible that the antitumor effects of anandamide and other cannabinoids may be offset by their immunosuppressive properties, ultimately leading to increased tumor growth as seen in our study.

In the current study, we used doses of $\Delta^9$-THC up to 50 mg/kg. Importantly, there is evidence to suggest that the doses of $\Delta^9$-THC used in the current study are pharmacologically relevant. Azorlosa et al. showed that levels as high as 1 $\mu$M could be obtained in the plasma of humans (42), and in separate report it was shown that $\Delta^9$-THC can be concentrated 15- to 20-fold in some tissues (43). Therefore, it might be possible to reach levels as high as 20 $\mu$M in some tissues after recreational use. In an earlier study, Chan et al. showed that rats injected with 50 mg/kg body weight of $\Delta^9$-THC led to a serum concentration of 10 $\mu$M of $\Delta^9$-THC within 10 h of administration (44). Moreover, it has been proposed that the use of higher doses may be necessary in order for $\Delta^9$-THC to be effective medicinally. Therefore, use of up to 50 mg/kg of $\Delta^9$-THC should lead to physiologically relevant concentrations that correlate to the potential concentrations following recreational use and may also correlate with the concentrations necessary for some of the proposed clinical uses.

The immune response to tumors is believed to be mediated primarily by the Th1 response. Skewing of the immune response from the cell-mediated Th1 response to the humoral-mediated Th2 response may lead to a positive environment for tumor growth and development. In the current study, we showed that exposure to $\Delta^9$-THC led to increased production of IL-4 and IL-10, and importantly, administration of Abs against these cytokines reversed the $\Delta^9$-THC-mediated suppression of antitumor immunity. Increased levels of these cytokines have been associated with a number of cancers. For example, increased levels of IL-4 and IL-10 have been reported in patients with breast cancer and this was directly correlated to suppression of the immune response (45). In a separate study examining the immune response in patients with

breast and lung cancer, a shift toward the Th2 immune response was observed (46). Furthermore, increased levels of IL-10 secreting T-regulatory cells have been associated with the inability to mount an effective immune response to Hodgkins lymphoma (47). These studies highlight the potential involvement of the immune system in the development and progression of various tumors, including breast cancer, and suggest that skewing of the immune response to the Th2 phenotype may enhance the tumor's chances of survival. Therefore, the induction of a Th2 response following $\Delta^9$-THC exposure may significantly increase tumor cell survival and ultimately facilitate tumor growth. Interestingly, in this study we also observed an increase in IFN-$\gamma$ following $\Delta^9$-THC exposure. This may suggest that, in the current study, $\Delta^9$-THC led to an incomplete Th2 skewing of the response as seen in other tumor models (48) or to the activation of cells such as NKT or T regulatory cells (49, 50).

A number of other reports suggest that exposure to cannabinoids may affect the immune system by altering cytokine production in mice (35). For example, exposure to $\Delta^9$-THC leads to inhibition of the Th1 response following *L. pneumophila* infection (26). Exposure of mice to cannabinoids in the concanavalin A-induced hepatitis model led to increased production of Th2-associated cytokines IL-10 and IL-6 and a reduction in the Th1-associated cytokines IL-2 and IFN-$\gamma$ (51). Similar results were seen when examining the immune response to a murine lung cancer in which it was shown that the $\Delta^9$-THC-induced suppression of the antitumor immune response was due to a $\Delta^9$-THC-mediated shifting of cytokine production (15). Also, a recent study demonstrated that individuals who smoked marijuana on an occasional (eventual to monthly use) or regular basis (weekly to daily use) had abnormal T cell and NK cell functions and increased levels of TGF-$\beta$ and IL-10 (52), suggesting a possible Th2 bias in humans, similar to what we reported in the current study. In addition, previous studies from our laboratory have shown that $\Delta^9$-THC at doses of 50 mg/kg can lead to the induction of apoptosis in the thymus and spleen of naive mice. Previously, we demonstrated that concanavalin A-activated splenocytes and LPS-activated dendritic cells are relatively resistant to $\Delta^9$-THC-induced apoptosis when compared with their naive counterparts and that the sensitivity correlated with the level of cannabinoid receptor expression (21, 53). Little is know about the expression of cannabinoid receptors in cells involved in the immune response to tumors or the effect of $\Delta^9$-THC on their viability. Therefore, it is possible that $\Delta^9$-THC may suppress the tumor-specific immune response by inducing apoptosis in Th1-associated cells reacting to the tumor challenge, resulting in the observed shift to the Th2 response.

Work using the 4T1 has shown that the immune response to this tumor is primarily mediated by $CD8^+$ cells (54). Additional studies suggested that NKT cells may play a negative role in the response to this tumor (55). For example, $CD1d^{-/-}$ mice had a significantly elevated response to the 4T1 tumor in vivo (55). Following stimulation, NKT can rapidly produce large quantities of IL-4 and IL-10 and have been implicated as possible negative or positive regulators of the antitumor immune response. Another cell that may play an important role in controlling the immune response is the $CD4^+CD25^+$ regulatory T cell. Interestingly, $CD4^+CD25^+$ regulatory T cells have been reported to suppress the antitumor immune response and this suppression was associated with the increased production of IL-10 (47, 56). To date, little is known about the effect of cannabinoids on NKT or $CD4^+CD25^+$ regulatory T cell functions. However, it is possible that $\Delta^9$-THC exposure may directly lead to altered NKT and/or $CD4^+CD25^+$ regulatory T cell activity, resulting in the observed suppression of the antitumor immune response. In addition, it is possible that the observed suppression of the tumor-specific immune response may be mediated through alterations in dendritic cell function. This possibility is supported by work from our laboratory in which we demonstrated that dendritic cells are sensitive to $\Delta^9$-THC-mediated apoptosis (53). The exact role of these cells in the $\Delta^9$-THC-induced suppression of the antitumor immune response is currently being investigated in our laboratory.

Although, the importance of the immune system in protection against many of the common epithelial cancers remains controversial, it is becoming clear that the immune system plays a considerable role in the protection against virally induced or virus-associated tumors. For example, there is an increased rate of Karposi sarcoma, non-Hodgkins lymphoma, Burkitt lymphoma, and cervical cancer in AIDS patients (27). In addition, there have been reports of increased incidences of various lymphomas, cutaneous neoplasms, and de novo breast cancers following organ transplantation (28–31). Although, the immune response to 4T1 has not been fully elucidated, it has been postulated that the immune response may be directed against mouse mammary tumor virus (MMTV) Ags expressed by the tumor (57). Interestingly, a number of studies suggest a possible role of an MMTV-like virus in the etiology of a large proportion of human breast cancers (58, 59). Although direct epidemiological data linking marijuana exposure to increased incidence of breast cancers is not currently available, it is intriguing to speculate that immunocompromised individuals may become increasingly susceptible to MMTV-like infection and to the subsequent development of breast cancers. Therefore, the possibility exists that exposure to marijuana, either through recreational or medicinal use, may lead to increased incidence of immunogenic tumors.

## Disclosures

The authors have no financial conflict of interest.

## References


1. Berdyshev, E. V. 2000. Cannabinoid receptors and the regulation of immune response. *Chem. Phys. Lipids 108:169.*
2. Watson, S. J., J. A. Benson, Jr., and J. E. Joy. 2000. Marijuana and medicine: assessing the science base: a summary of the 1999 Institute of Medicine report. *Arch. Gen. Psychiatry 57:547.*
3. Devane, W. A., F. A. Dysarz, 3rd, M. R. Johnson, L. S. Melvin, and A. C. Howlett. 1988. Determination and characterization of a cannabinoid receptor in rat brain. *Mol. Pharmacol. 34:605.*
4. Munro, S., K. L. Thomas, and M. Abu-Shaar. 1993. Molecular characterization of a peripheral receptor for cannabinoids. *Nature 365:61.*
5. Devane, W. A., L. Hanus, A. Breuer, R. G. Pertwee, L. A. Stevenson, G. Griffin, D. Gibson, A. Mandelbaum, A. Etinger, and R. Mechoulam. 1992. Isolation and structure of a brain constituent that binds to the cannabinoid receptor. *Science 258:1946.*
6. Felder, C. C., E. M. Briley, J. Axelrod, J. T. Simpson, K. Mackie, and W. A. Devane. 1993. Anandamide, an endogenous cannabimimetic eicosanoid, binds to the cloned human cannabinoid receptor and stimulates receptor-mediated signal transduction. *Proc. Natl. Acad. Sci. USA 90:7656.*
7. Mechoulam, R., S. Ben-Shabat, L. Hanus, M. Ligumsky, N. E. Kaminski, A. R. Schatz, A. Gopher, S. Almog, B. R. Martin, D. R. Compton, et al. 1995. Identification of an endogenous 2-monoglyceride, present in canine gut, that binds to cannabinoid receptors. *Biochem. Pharmacol 50:83.*
8. McKallip, R. J., C. Lombard, M. Fisher, B. R. Martin, S. Ryu, S. Grant, P. S. Nagarkatti, and M. Nagarkatti. 2002. Targeting CB2 cannabinoid receptors as a novel therapy to treat malignant lymphoblastic disease. *Blood 100:627.*
9. Sanchez, C., I. Galve-Roperh, C. Canova, P. Brachet, and M. Guzman. 1998. Δ9-tetrahydrocannabinol induces apoptosis in C6 glioma cells. *FEBS Lett. 436:6.*
10. Sanchez, C., M. L. de Ceballos, T. G. del Pulgar, D. Rueda, C. Corbacho, G. Velasco, I. Galve-Roperh, J. W. Huffman, Y. C. S. Ramon, and M. Guzman. 2001. Inhibition of glioma growth in vivo by selective activation of the cb(2) cannabinoid receptor. *Cancer Res. 61:5784.*
11. Morahan, P. S., P. C. Klykken, S. H. Smith, L. S. Harris, and A. E. Munson. 1979. Effects of cannabinoids on host resistance to *Listeria monocytogenes* and herpes simplex virus. *Infect. Immun. 23:670.*
12. Cabral, G. A., E. M. Mishkin, F. Marciano-Cabral, P. Coleman, L. Harris, and A. E. Munson. 1986. Effect of δ9-tetrahydrocannabinol on herpes simplex virus type 2 vaginal infection in the guinea pig. *Proc. Soc. Exp. Biol. Med. 182:181.*
13. Specter, S., G. Lancz, G. Westrich, and H. Friedman. 1991. Δ-9-tetrahydrocannabinol augments murine retroviral induced immunosuppression and infection. *Int. J. Immunopharmacol. 13:411.*

14. Zhang, Z. F., H. Morgenstern, M. R. Spitz, D. P. Tashkin, G. P. Yu, J. R. Marshall, T. C. Hsu, and S. P. Schantz. 1999. Marijuana use and increased risk of squamous cell carcinoma of the head and neck. *Cancer Epidemiol. Biomarkers Prev. 8:1071*.
15. Zhu, L. X., S. Sharma, M. Stolina, B. Gardner, M. D. Roth, D. P. Tashkin, and S. M. Dubinett. 2000. Δ-9-tetrahydrocannabinol inhibits antitumor immunity by a CB2 receptor-mediated, cytokine-dependent pathway. *J. Immunol. 165:373*.
16. Jeon, Y. J., K. H. Yang, J. T. Pulaski, and N. E. Kaminski. 1996. Attenuation of inducible nitric oxide synthase gene expression by δ9-tetrahydrocannabinol is mediated through the inhibition of nuclear factor-κB/Rel activation. *Mol. Pharmacol. 50:334*.
17. McCoy, K. L., M. Matveyeva, S. J. Carlisle, and G. A. Cabral. 1999. Cannabinoid inhibition of the processing of intact lysozyme by macrophages: evidence for CB2 receptor participation. *J. Pharmacol. Exp. Ther. 289:1620*.
18. Pross, S. H., T. W. Klein, C. A. Newton, J. Smith, R. Widen, and H. Friedman. 1990. Differential suppression of T-cell subpopulations by thc (δ-9-tetrahydrocannabinol). *Int. J. Immunopharmacol. 12:539*.
19. Klein, T. W., Y. Kawakami, C. Newton, and H. Friedman. 1991. Marijuana components suppress induction and cytolytic function of murine cytotoxic T cells in vitro and in vivo. *J. Toxicol. Environ. Health 32:465*.
20. Kaminski, N. E., W. S. Koh, K. H. Yang, M. Lee, and F. K. Kessler. 1994. Suppression of the humoral immune response by cannabinoids is partially mediated through inhibition of adenylate cyclase by a pertussis toxin-sensitive G-protein coupled mechanism. *Biochem. Pharmacol. 48:1899*.
21. McKallip, R. J., C. Lombard, B. R. Martin, M. Nagarkatti, and P. S. Nagarkatti. 2002. Δ(9)-tetrahydrocannabinol-induced apoptosis in the thymus and spleen as a mechanism of immunosuppression in vitro and in vivo. *J. Pharmacol. Exp. Ther. 302:451*.
22. Wei, W. Z., G. P. Morris, and Y. C. Kong. 2004. Anti-tumor immunity and autoimmunity: a balancing act of regulatory T cells. *Cancer Immunol. Immunother. 53:73*.
23. Terabe, M., and J. A. Berzofsky. 2004. Immunoregulatory T cells in tumor immunity. *Curr. Opin. Immunol. 16:157*.
24. Smyth, M. J., K. Y. Thia, S. E. Street, E. Cretney, J. A. Trapani, M. Taniguchi, T. Kawano, S. B. Pelikan, N. Y. Crowe, and D. I. Godfrey. 2000. Differential tumor surveillance by natural killer (NK) and NKT cells. *J. Exp. Med. 191:661*.
25. Nishimura, T., M. Nakui, M. Sato, K. Iwakabe, H. Kitamura, M. Sekimoto, A. Ohta, T. Koda, and S. Nishimura. 2000. The critical role of Th1-dominant immunity in tumor immunology. *Cancer Chemother. Pharmacol. 46(Suppl.): S52*.
26. Klein, T. W., C. A. Newton, N. Nakachi, and H. Friedman. 2000. $\Delta^9$-tetrahydrocannabinol treatment suppresses immunity and early IFN-γ, IL-12, and IL-12 receptor β2 responses to *Legionella pneumophila* infection. *J. Immunol. 164:6461*.
27. Scadden, D. T. 2003. AIDS-related malignancies. *Annu. Rev. Med. 54:285*.
28. Penn, I. 1993. Incidence and treatment of neoplasia after transplantation. *J. Heart Lung Transplant. 12:S328*.
29. Abu-Elmagd, K. M., M. Zak, J. M. Stamos, G. J. Bond, A. Jain, A. O. Youk, M. Ezzelarab, G. Costa, T. Wu, M. A. Nalesnik, et al. 2004. De novo malignancies after intestinal and multivisceral transplantation. *Transplantation 77:1719*.
30. Bhatia, S., A. D. Louie, R. Bhatia, M. R. O'Donnell, H. Fung, A. Kashyap, A. Krishnan, A. Molina, A. Nademanee, J. C. Niland, et al. 2001. Solid cancers after bone marrow transplantation. *J. Clin. Oncol. 19:464*.
31. Oruc, M. T., A. Soran, A. K. Jain, J. W. Wilson, and J. Fung. 2004. De novo breast cancer in patients with liver transplantation: University of Pittsburgh's experience and review of the literature. *Liver Transpl. 10:1*.
32. Ohara, J., and W. E. Paul. 1985. Production of a monoclonal antibody to and molecular characterization of B-cell stimulatory factor-1. *Nature 315:333*.
33. Dean, T. N., V. N. Kakkanaiah, M. Nagarkatti, and P. S. Nagarkatti. 1990. Immunosuppression by aldicarb of T cell responses to antigen-specific and polyclonal stimuli results from defective IL-1 production by the macrophages. *Toxicol. Appl. Pharmacol. 106:408*.
34. McKallip, R. J., M. Nagarkatti, and P. S. Nagarkatti. 1995. Immunotoxicity of AZT: inhibitory effect on thymocyte differentiation and peripheral T cell responsiveness to gp120 of human immunodeficiency virus. *Toxicol. Appl. Pharmacol. 131:53*.
35. Klein, T. W., B. Lane, C. A. Newton, and H. Friedman. 2000. The cannabinoid system and cytokine network. *Proc. Soc. Exp. Biol. Med. 225:1*.
36. Klein, T. W., C. Newton, and H. Friedman. 1998. Cannabinoid receptors and the cytokine network. *Adv. Exp. Med. Biol. 437:215*.
37. Ruiz, L., A. Miguel, and I. Diaz-Laviada. 1999. Δ9-tetrahydrocannabinol induces apoptosis in human prostate PC-3 cells via a receptor-independent mechanism. *FEBS Lett. 458:400*.
38. De Petrocellis, L., D. Melck, A. Palmisano, T. Bisogno, C. Laezza, M. Bifulco, and V. Di Marzo. 1998. The endogenous cannabinoid anandamide inhibits human breast cancer cell proliferation. *Proc. Natl. Acad. Sci. USA 95:8375*.
39. Melck, D., D. Rueda, I. Galve-Roperh, L. De Petrocellis, M. Guzman, and V. Di Marzo. 1999. Involvement of the cAMP/protein kinase A pathway and of mitogen-activated protein kinase in the anti-proliferative effects of anandamide in human breast cancer cells. *FEBS Lett. 463:235*.
40. Smart, D., M. J. Gunthorpe, J. C. Jerman, S. Nasir, J. Gray, A. I. Muir, J. K. Chambers, A. D. Randall, and J. B. Davis. 2000. The endogenous lipid anandamide is a full agonist at the human vanilloid receptor (hVR1). *Br. J. Pharmacol. 129:227*.
41. Zygmunt, P. M., I. Julius, I. Di Marzo, and E. D. Hogestatt. 2000. Anandamide: the other side of the coin. *Trends Pharmacol. Sci. 21:43*.
42. Azorlosa, J. L., S. J. Heishman, M. L. Stitzer, and J. M. Mahaffey. 1992. Marijuana smoking: effect of varying δ9-tetrahydrocannabinol content and number of puffs. *J Pharmacol. Exp. Ther. 261:114*.
43. Johansson, E., K. Noren, J. Sjovall, and M. M. Halldin. 1989. Determination of δ1-tetrahydrocannabinol in human fat biopsies from marihuana users by gas chromatography-mass spectrometry. *Biomed. Chromatogr. 3:35*.
44. Chan, P. C., R. C. Sills, A. G. Braun, J. K. Haseman, and J. R. Bucher. 1996. Toxicity and carcinogenicity of δ9-tetrahydrocannabinol in Fischer rats and B6C3F1 mice. *Fundam. Appl. Toxicol. 30:109*.
45. Pockaj, B. A., G. D. Basu, L. B. Pathangey, R. J. Gray, J. L. Hernandez, S. J. Gendler, and P. Mukherjee. 2004. Reduced T-cell and dendritic cell function is related to cyclooxygenase-2 overexpression and prostaglandin E2 secretion in patients with breast cancer. *Ann. Surg. Oncol. 11:328*.
46. Caras, I., A. Grigorescu, C. Stavaru, D. L. Radu, I. Mogos, G. Szegli, and A. Salageanu. 2004. Evidence for immune defects in breast and lung cancer patients. *Cancer Immunol. Immunother.*
47. Marshall, N. A., L. E. Christie, L. R. Munro, D. J. Culligan, P. W. Johnston, R. N. Barker, and M. A. Vickers. 2004. Immunosuppressive regulatory T cells are abundant in the reactive lymphocytes of Hodgkin lymphoma. *Blood 103:1755*.
48. Lathers, D. M., N. J. Achille, and M. R. Young. 2003. Incomplete Th2 skewing of cytokines in plasma of patients with squamous cell carcinoma of the head and neck. *Hum. Immunol. 64:1160*.
49. Godfrey, D. I., and M. Kronenberg. 2004. Going both ways: immune regulation via CD1d-dependent NKT cells. *J. Clin. Invest. 114:1379*.
50. O'Garra, A., and P. Vieira. 2004. Regulatory T cells and mechanisms of immune system control. *Nat. Med. 10:801*.
51. Lavon, I., T. Sheinin, S. Meilin, E. Biton, A. Weksler, G. Efroni, A. Bar-Joseph, G. Fink, and A. Avraham. 2003. A novel synthetic cannabinoid derivative inhibits inflammatory liver damage via negative cytokine regulation. *Mol. Pharmacol. 64:1334*.
52. Pacifici, R., P. Zuccaro, S. Pichini, P. N. Roset, S. Poudevida, M. Farre, J. Segura, and R. De la Torre. 2003. Modulation of the immune system in cannabis users. *J. Am. Med. Assoc. 289:1929*.
53. Do, Y., R. J. McKallip, M. Nagarkatti, and P. S. Nagarkatti. 2004. Activation through cannabinoid receptors 1 and 2 on dendritic cells triggers NF-κB-dependent apoptosis: novel role for endogenous and exogenous cannabinoids in immunoregulation. *J. Immunol. 173:2373*.
54. Ostrand-Rosenberg, S., M. J. Grusby, and V. K. Clements. 2000. Cutting edge: STAT6-deficient mice have enhanced tumor immunity to primary and metastatic mammary carcinoma. *J. Immunol. 165:6015*.
55. Ostrand-Rosenberg, S., V. K. Clements, M. Terabe, J. M. Park, J. A. Berzofsky, and S. K. Dissanayake. 2002. Resistance to metastatic disease in STAT6-deficient mice requires hemopoietic and nonhemopoietic cells and is IFN-γ dependent. *J. Immunol. 169:5796*.
56. Seo, N., S. Hayakawa, M. Takigawa, and Y. Tokura. 2001. Interleukin-10 expressed at early tumour sites induces subsequent generation of $CD4^+$ T-regulatory cells and systemic collapse of antitumour immunity. *Immunology 103:449*.
57. Jensen, S. M., S. L. Meijer, R. A. Kurt, W. J. Urba, H. M. Hu, and B. A. Fox. 2003. Regression of a mammary adenocarcinoma in $STAT6^{-/-}$ mice is dependent on the presence of STAT6-reactive T cells. *J. Immunol. 170:2014*.
58. Wang, Y., J. F. Holland, I. J. Bleiweiss, S. Melana, X. Liu, I. Pelisson, A. Cantarella, K. Stellrecht, S. Mani, and B. G. Pogo. 1995. Detection of mammary tumor virus env gene-like sequences in human breast cancer. *Cancer Res. 55:5173*.
59. Levine, P. H., B. G. Pogo, A. Klouj, S. Coronel, K. Woodson, S. M. Melana, N. Mourali, and J. F. Holland. 2004. Increasing evidence for a human breast carcinoma virus with geographic differences. *Cancer 101:721*.