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*J Immunol* 2005; 174:3281-3289; doi: 10.4049/jimmunol.174.6.3281
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Δ-9-Tetrahydrocannabinol Enhances Breast Cancer Growth and Metastasis by Suppression of the Antitumor Immune Response

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 (Δ9-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 Δ9-THC-induced cytoxicity. Furthermore, exposure of mice to Δ9-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 Δ9-THC led to increased production of IL-4 and IL-10, suggesting that Δ9-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 Δ9-THC. Finally, injection of anti-IL-4 and anti-IL-10 mAbs led to a partial reversal of the Δ9-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 Δ9-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 (Δ9-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 Δ9-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 Δ9-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). Δ9-THC possesses significant immunomodulatory properties. For example, exposure of macrophages to Δ9-THC led to decreased production of TNF-α and NO in response to LPS (16). Additionally, exposure of macrophages to Δ9-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 Δ9-THC can lead to increased susceptibility to infections with various pathogens including Herpes simplex and Friend leukemia virus (12, 13). Furthermore, exposure to Δ9-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-γ 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 Δ9-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).
Current work examining the potential use of Δ9-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 Δ9-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 Δ9-THC-mediated killing and because Δ9-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 Kaposi sarcoma, non-Hodgkin 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 development of this type of cancer. Therefore, in the current study, using a breast cancer model, we examined the effect of Δ9-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 22 °C and on a 12-h light/dark cycle.

Reagents

Δ9-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. Δ9-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–anti-IL-10 mAbs were obtained from BD Pharmingen.

Cell lines

The murine mammary carcinoma 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 μg/ml gentamicin sulfate, and 50 μM 2-mercaptoethanol. Mice were housed in polyethylene cages and given rodent chow and water ad libitum. Mice were housed in rooms maintaining a temperature of 22 °C and on a 12-h light/dark cycle.

RNA isolation and RT-PCR

RNA was isolated from ~1 × 10⁶ 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 Quagen Omniscript RT kit using 1 μg of RNA as template for first strand synthesis. Mouse and human CB1 was amplified using primers H CB1 U (5'-CGTGGGACGCGCTTCCCTCA-3') and H CB1 L (5'-CAT GGGTCCTGCTGGTG-3'), which yield a product of 403 bp. Human CB2 was amplified using primers H CB2 U (5'-CGCCGAGACCT CATAACC-3') and H CB2 L (5'-CCTTCTTCTGGCCATTCCTG-3'), which yield a product of 522 bp. Mouse CB2 was amplified using M CB2 (5'-GGGAAAAGGGATGGCAATGAAT-3') and M CB2 (5'-CTGCT GAGCGCCCTGGAAGAC-3') which yields a product of 479 bp. β-Actin was used as a positive control (primers M BA U (5'-AAGGCCAACCG GAAAGATGACC-3') and M BA L (5'-ACCGCTCTGTGTCGAAT

AGTGTAGA-3')). PCR were performed under the following conditions: 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 Δ9-THC-mediated cell death in vitro

Tumor cells or splenocytes (1 × 10⁶ cells/well) were cultured in 24-well plates in the presence or absence of various concentrations of Δ9-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 Δ9-THC exposure on 4T1 tumor growth and metastasis in vivo

Groups of BALB/c or SCID-NOD mice were injected s.c. with 3 × 10⁶ 4T1 tumor cells. Three days later, the mice were then exposed every other day for ~3 wk to various doses of Δ9-THC (12.5, 25, or 50 mg/kg body weight) or vehicle (DMSO) control. The tumor volume was recorded, and calculated using the following equation: tumor volume = length × width² × 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 × 10⁶ 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 × 10⁶ irradiated 4T1 tumor cells or 1 × 10⁶ irradiated EMT6 tumor cells (negative control). Groups of mice were then treated i.p. with various doses of Δ9-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 Δ9-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 μg/ml gentamicin sulfate, and 50 μM 2-mercaptoethanol, to refer to as complete medium. The spleen cells and lymph node cells (5 × 10⁶ in 100 μl/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 μCi of [3H]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 × 10⁶ 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 × 10⁶ irradiated 4T1 tumor cells. Groups of mice were then treated daily for 4 days with vehicle control or Δ9-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 × 10⁶ cells/ml. The lymph node cells were cultured in a 96-well flat-bottom plate (200 μl/well) for 24 h, after which the levels of IFN-γ, TNF-α, 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 × 10⁶ s.c.) and treated i.p. for 4 days with vehicle or 50 mg/kg Δ9-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 minutes.
min at 60°C with 0.1× SSC, 0.5% SDS. Nonspecific binding was blocked by incubating the membranes with GEArray blocking 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 ScanArray 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 β-actin, GAPDH, cyclophilin A, and ribosomal protein L13a.

Statistical analysis

Student’s t test or Tukey Kramer test was used to compare vehicle and ∆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. 1A). 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. 1C). 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 ∆9-THC-induced cell death

Next we examined whether 4T1 or MCF-7 were sensitive to ∆9-THC-induced cytotoxicity compared with other cells reported to be sensitive to ∆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 ∆9-THC (0, 5, 10, or 20 μM). The viable cell number was determined by trypan blue dye exclusion (Fig. 1B). The results demonstrated that although the splenocytes were highly sensitive to ∆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 ∆9-THC tested. In contrast, splenic culture showed a significant reduction in viable cell number following exposure to concentration of ∆9-THC as low as 5 μM. In addition, we examined whether the human breast cancer cell line MCF-7 was sensitive to ∆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 ∆9-THC (0, 5, 10, or 20 μM) and the viable cell number was determined by trypan blue dye exclusion (Fig. 1D). The results showed that while the Jurkat cells were sensitive to ∆9-THC-mediated killing at concentrations as low as 5 μM, the MCF-7 cells were resistant to ∆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 ∆9-THC exposure.

∆9-THC-exposure leads to increased growth of the 4T1 breast cancer in vivo

Next, we examined whether exposure to ∆9-THC had any effect on the local growth of the 4T1 tumor (Fig. 2A). To this end, BALB/c mice were injected s.c. with 3 × 105 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 ∆9-THC (12.5, 25, or 50 mg/kg). Tumor growth was monitored and the data revealed that exposure to 25 mg/kg ∆9-THC led to a significant increase in tumor mass. This effect was even more pronounced in mice treated with 50 mg/kg ∆9-THC.

∆9-THC-exposure leads to increased metastasis of 4T1 tumor to the lung

In addition to examining the effects of ∆9-THC on the local growth of the 4T1 tumor, we examined whether exposure to ∆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 × 105 4T1 tumor cells. Three days following the tumor injection, the mice were exposed every other day to either vehicle control or various doses of ∆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. 2B and C). The results showed that exposure to 25 or 50 mg/kg ∆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 exposure to ∆9-THC-mediated cell death. To this end, BALB/c mice were injected s.c. with 3 × 105 4T1 tumor cells. Three days following the tumor injection, the mice were exposed every other day to either vehicle control or various doses of ∆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. 2B and C). The results showed that while the Jurkat cells were sensitive to ∆9-THC-mediated killing at concentrations as low as 5 μM, the MCF-7 cells were resistant to ∆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 ∆9-THC exposure.

The effect of ∆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 ∆9-THC was evaluated using the SCID-NOD model. SCID-NOD mice are devoid of an antitumor immune response. Therefore, any effect of ∆9-THC...
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. 3A) or metastasis (Fig. 3B), 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.

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 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. 4C), an increase in tumor size (Fig. 4D), and an increase in the level of metastatic lesions in the lungs (Fig. 4E). 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 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. 4A) and cell number (Fig. 4B) 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. 4C), an increase in tumor size (Fig. 4D), and an increase in the level of metastatic lesions in the lungs (Fig. 4E). 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-INDUCED SUPPRESSION OF ANTITUMOR IMMUNITY
was assayed by determining the increase in lymph node mass (Fig. 6A) compared with unchallenged mice. 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 Δ⁹-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 × 10⁶ live 4T1 cells. The mice were treated every other day for 16 days with vehicle or Δ⁹-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 Δ⁹-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 [³H]thymidine uptake and the results revealed that in vivo exposure to Δ⁹-THC led to a significant suppression of the proliferative response of splenocytes (Fig. 5A) and lymph node cells (Fig. 5B) to 4T1.

The effect of CB1 and CB2 antagonist on Δ⁹-THC-induced suppression of the immune response to 4T1 in vivo

To investigate the role of CB1 and CB2 in Δ⁹-THC-induced suppression of the antitumor immune response to 4T1, sensitized mice were challenged s.c. in their footpads with irradiated 4T1 (1 × 10⁶ cells). After which, the mice were treated daily for 4 days with CB1 (SR141716A), CB2 (SR144528) antagonists, or vehicle 1 h before exposure to Δ⁹-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 Δ⁹-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 × 10⁶ live 4T1 cells. The mice were treated every other day for 16 days with vehicle or Δ⁹-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.

Δ⁹-THC exposure leads to suppression of the tumor-specific proliferative response. 4T1-sensitized mice were treated for 4 days with vehicle or Δ⁹-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 [³H]thymidine uptake. The experiment was repeated three times with similar results. *, Statistically significant difference (p < 0.05) when compared with the controls.

Δ⁹-THC exposure alters cytokine production

Previous reports have indicated that exposure to Δ⁹-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 Δ⁹-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 Δ⁹-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 × 10⁶ cells/well) for 24 h in 96-well plates. Next, the supernatants were tested for the presence of various Th1 and Th2 cytokines (Fig. 7A). The results showed that exposure to 25 mg/kg Δ⁹-THC led to a dramatic increase in the Th2 cytokines IL-4 and IL-10, suggesting that at this concentration, Δ⁹-THC enhances the Th2 response. In addition, levels of IFN-γ were found to be elevated following exposure to Δ⁹-THC. Interestingly, exposure to 50 mg/kg Δ⁹-THC led to a significant reduction in IL-4, IFN-γ, and IL-10 compared with the vehicle or 25 mg/kg Δ⁹-THC groups, suggesting the possibility that at a higher concentration, Δ⁹-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 Δ⁹-THC-induced suppression of the immune response to 4T1

Next, we examined the effects of Abs against IL-4 or IL-10 on the Δ⁹-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, Δ⁹-THC (25 mg/kg/day) + isotype control mAbs, Δ⁹-THC (25 mg/kg/day) + anti-
The results showed that exposure 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.

The effect of $\Delta^9$-THC exposure on 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 downregulated genes were the Th1-associated genes, IL-1R, and the 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. 7B). 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 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. The results are representative data of experimental groups containing four mice. The experiment was repeated three times with similar results. * 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.

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...
carcinoma. This is in contrast to our previous finding in which we demonstrated that treatment with Δ⁹-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 Δ⁹-THC-mediated killing and that the effects of Δ⁹-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 Δ⁹-THC may be directly related to the level of CB1 and CB2 expression. Importantly, these results would suggest that, although Δ⁹-THC may be effective at killing tumors that express cannabinoid receptors, Δ⁹-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 Δ⁹-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 Δ⁹-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 known 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 Δ⁹-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 Δ⁹-THC up to 50 mg/kg. Importantly, there is evidence to suggest that the doses of Δ⁹-THC used in the current study are pharmacologically relevant. Azorlosa et al. showed that levels as high as 1 μM could be obtained in the plasma of humans (42), and in separate report it was shown that Δ⁹-THC can be concentrated 15- to 20-fold in some tissues (43). Therefore, it might be possible to reach levels as high as 20 μM in some tissues after recreational use. In an earlier study, Chan et al. showed that rats injected with 50 mg/kg body weight of Δ⁹-THC led to a serum concentration of 10 μM of Δ⁹-THC within 10 h of administration (44). Moreover, it has been proposed that the use of higher doses may be necessary in order for Δ⁹-THC to be effective medicinally. Therefore, use of up to 50 mg/kg of Δ⁹-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 Δ⁹-THC led to increased production of IL-4 and IL-10, and importantly, administration of Abs against these cytokines reversed the Δ⁹-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

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### Table I. cDNA array analysis of Th1/Th2-associated gene expression in 4T1-stimulated lymph node cells from vehicle- or Δ⁹-THC-treated 4T1-sensitized mice

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Description</th>
<th>Function</th>
<th>Accession Number</th>
<th>Fold Change&lt;sup&gt;ab&lt;/sup&gt; vs Vehicle</th>
</tr>
</thead>
<tbody>
<tr>
<td>C2ta</td>
<td>Class II transactivator</td>
<td>Th2</td>
<td>NM_007575</td>
<td>+2.1</td>
</tr>
<tr>
<td>ScyA1/eotaxin</td>
<td>Small chemokine ligand 11</td>
<td>Th2</td>
<td>U26426</td>
<td>+19.4</td>
</tr>
<tr>
<td>IL-13RA2</td>
<td>Interleukin-13 receptor, α2</td>
<td>Th2</td>
<td>U65747</td>
<td>+4.4</td>
</tr>
<tr>
<td>IL-4</td>
<td>Interleukin-4</td>
<td>Th2</td>
<td>M23892</td>
<td>+4.8</td>
</tr>
<tr>
<td>IL-4ra</td>
<td>Interleukin-4 receptor, α</td>
<td>Th2</td>
<td>NM_010557</td>
<td>+4.4</td>
</tr>
<tr>
<td>IL-5</td>
<td>Interleukin-5</td>
<td>Th2</td>
<td>NM_010558</td>
<td>+2.9</td>
</tr>
<tr>
<td>GATA3</td>
<td>GATA binding protein 3</td>
<td>Th2</td>
<td>NM_008091</td>
<td>+2.9</td>
</tr>
<tr>
<td>Gpl1</td>
<td>Growth factor independent 1</td>
<td>Th2</td>
<td>NM_010278</td>
<td>+3.5</td>
</tr>
<tr>
<td>SOCS2</td>
<td>SOCS2</td>
<td>Trans. reg.</td>
<td>NM_007706</td>
<td>+2.0</td>
</tr>
<tr>
<td>SOCS5</td>
<td>SOCS5</td>
<td>Trans. reg.</td>
<td>NM_019654</td>
<td>+6.8</td>
</tr>
<tr>
<td>SOCS7</td>
<td>SOCS7</td>
<td>Trans. reg.</td>
<td>NM_008043</td>
<td>+2.6</td>
</tr>
<tr>
<td>FoxA2</td>
<td>Fox-like antigen 2</td>
<td>Trans. reg.</td>
<td>NM_008037</td>
<td>+3.2</td>
</tr>
<tr>
<td>IL-1R</td>
<td>Interleukin-1 receptor</td>
<td>Th1</td>
<td>U43673</td>
<td>−14.6</td>
</tr>
<tr>
<td>DR6</td>
<td>TNF receptor superfamily member</td>
<td>Th1</td>
<td>AF322069</td>
<td>−5.3</td>
</tr>
<tr>
<td>4-1BB</td>
<td>TNF receptor superfamily member</td>
<td>Th1</td>
<td>J04492</td>
<td>−5.7</td>
</tr>
<tr>
<td>JunD1</td>
<td>Jun proto-oncogene related gene d1</td>
<td>Trans. reg.</td>
<td>NM_010592</td>
<td>−11.7</td>
</tr>
<tr>
<td>JNK2</td>
<td>MAP kinase kinase MKK7</td>
<td>Trans. reg.</td>
<td>U74463</td>
<td>−2.5</td>
</tr>
<tr>
<td>JNK3</td>
<td>Mitogen-activated protein kinase 8</td>
<td>Trans. reg.</td>
<td>AB005663</td>
<td>−2.9</td>
</tr>
</tbody>
</table>

<sup>a</sup> Summary of gene expression that was found to be increased or decreased in splenic cells isolated from Δ⁹-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.

<sup>b</sup> Fold change represents the change in gene expression following normalization with β-actin gene expression.

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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 Hodgkin’s 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 Δ9-THC exposure may significantly increase tumor cell survival and ultimately facilitate tumor growth. Interestingly, in this study we also observed an increase in IFN-γ following Δ9-THC exposure. This may suggest that, in the current study, Δ9-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 Δ9-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-γ (51). Similar results were seen when examining the immune response to a murine lung cancer in which it was shown that the Δ9-THC-induced suppression of the antitumor immune response was due to a Δ9-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-β 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 Δ9-THC at doses of 50 mg/kg can lead to the induction of apoptosis in the thymus and spleen of naïve mice. Previously, we demonstrated that concanavalin A-activated splenocytes and LPS-activated dendritic cells are relatively resistant to Δ9-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 Δ9-THC on their viability. Therefore, it is possible that Δ9-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 Δ9-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 Δ9-THC-mediated apoptosis (53). The exact role of these cells in the Δ9-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 Kaposi sarcoma, non-Hodgkin’s 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