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Δ^9-Tetrahydrocannabinol-Induced Apoptosis in the Thymus and Spleen as a Mechanism of Immunosuppression in Vitro and in Vivo

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ABSTRACT

Δ^9-Tetrahydrocannabinol (THC), the main psychoactive component of marijuana has been shown to suppress the immune response. However, the exact mechanism of THC-induced immunosuppression remains unclear. In the current study, we tested the hypothesis that exposure to THC leads to the induction of apoptosis in lymphocyte populations. Splenocytes of C57BL/6 mice cultured in the presence of 10 μM or greater concentrations of THC showed significantly reduced proliferative response to mitogens, including anti-CD3 monoclonal antibodies (mAbs), concanavalin A (Con A), and lipopolysaccharide (LPS) in vitro. Thymocytes and naive and activated splenocytes exposed to 10 μM or 20 μM THC showed significantly increased levels of apoptosis. Treatment with CB2 antagonist inhibited THC-induced apoptosis in thymocytes and activated splenocytes. Administration of 10 mg/kg body weight of THC into C57BL/6 mice led to thymic and splenic atrophy as early as 6 h after treatment. This effect could be partially inhibited by treatment with a caspase inhibitor in vivo. THC exposure led to reductions in the numbers of all subpopulations of splenocytes and thymocytes examined. Functional studies revealed that splenocytes from THC-treated mice had significantly reduced proliferative response to anti-CD3 mAbs, Con A, and LPS in vitro. Finally, thymocytes and splenocytes exposed to THC in vivo exhibited apoptosis upon in vitro culture. Together, these results suggest that in vivo exposure to THC can lead to significant suppression of the immune response by induction of apoptosis.

The use of marijuana for recreational and medicinal purposes has received increased attention in recent years. As a medicine, marijuana has been implicated as a potent therapeutic agent alleviating such complications as intraocular pressure in glaucoma, and cachexia, nausea, and pain in AIDS and cancer patients. In addition to these potentially beneficial effects, the use of marijuana, especially for recreational purposes, has been associated with some unwanted effects such as increased susceptibility to infections (Morahan et al., 1979; Cabral et al., 1986; Specter et al., 1991).

These observations have led to studies examining the effects of marijuana and its derivatives on the immune response. Δ^9-Tetrahydrocannabinol (THC) is the major active component of marijuana. Initial studies demonstrated that THC possesses significant immunomodulatory properties using both in vitro and in vivo models (for review, see Berdyshev, 2000). For example, exposure of macrophages to THC led to decreased production of tumor necrosis factor-α and nitric oxide in response to lipopolysaccharide (Jeon et al., 1996). In addition, exposure of macrophages to THC caused an impairment of their antigen presenting capabilities (McCoy et al., 1999). THC and other cannabinoids also directly affect the responses of T and B lymphocytes. Exposure to cannabinoids leads to significant reductions in the proliferative and cytolytic response of T lymphocytes and antibody production by B cells (Pross et al., 1990; Klein et al., 1991; Kaminski et al., 1994). Studies conducted in vivo have shown that exposure to THC can lead to increased susceptibility to infections with various pathogens including *Herpes simplex* and Friend leukemia virus (Cabral et al., 1986; Specter et al., 1991).

The immunomodulatory effects of THC were initially be-

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ABBREVIATIONS: FCS, fetal calf serum; LPS, lipopolysaccharide; Con A, concanavalin A; THC, Δ^9-tetrahydrocannabinol; mAb, monoclonal antibody; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline; PE, phosphatidylethanolamine; DMSO, dimethyl sulfoxide; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling; IL-2, interleukin 2; SR141716A, *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide hydrochloride; SR144528, 1-[2-(naphth-2-yl)ethy]-4-(3-trifluoromethyl phenyl)-1,2,5,6-tetrahydropyridine hydrochloride.

lieved to be mediated primarily through the lipophilic properties of THC leading to direct intercalation into the cell membrane. However, it was soon realized that the activity of cannabinoids was highly stereospecific, suggesting that the lipophilic properties were not solely responsible for the cannabinoid's activity. This finding led to an intensive search for specific cannabinoid receptors. In 1988, the first cannabinoid receptor, known as CB1, was isolated from rat brain (Devane et al., 1988). CB1 is primarily found in brain tissue, and low expression has been reported in cells of the immune system, small intestine, testis, urinary bladder, and uterus (for review, see Berdyshev, 2000). A second cannabinoid receptor (CB2) was cloned from a human promyelocytic cell line and was found to be primarily expressed on immune cells (Munro et al., 1993). Although immune cells can express both receptors, the expression of CB2 is believed to be around 100 times higher than CB1 (Munro et al., 1993).

It is now generally believed that at physiological concentrations, THC acts through binding of CB1 or CB2. However, the exact consequences of the interaction between THC and its ligand remain unclear. In the current study, we investigated the possibility that the interaction between THC and CB1 or CB2 on immune cells in vivo and in vitro leads to the induction of apoptosis. The data demonstrated that treatment of mice with THC led to marked decrease in the cellularity of thymus and spleen and decreased immune responsiveness to mitogens. The ability of THC to induce immunosuppression correlated with induction of apoptosis in immune cells.

Materials and Methods

Mice. Adult female (6–8 weeks of age) C57BL/6 mice were purchased from the National Institutes of Health. 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. THC was obtained from the National Institute on Drug Abuse (Rockville, MD) and was initially dissolved in DMSO (Sigma-Aldrich, St. Louis, MO) to a concentration of 20 mM and stored at −20°C. THC was further diluted with tissue culture medium for in vitro studies and PBS for in vivo studies. The CB1 (SR141716A) and CB2 (SR144528) antagonists were obtained from Sanofi Recherche (Montpellier, France).

In Vitro Proliferation Assay. The spleens were harvested from euthanized C57BL/6 mice and placed into 10 ml of RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 5% FCS, 10 mM HEPES, 1 mM glutamine, 40 μg/ml gentamicin sulfate, and 50 μM 2-mercaptoethanol, referred to as complete medium. The spleens were prepared into a single-cell suspension using a laboratory homogenizer, washed twice, and adjusted to 5×10^6/ml in complete medium. The splenocytes (5×10^5 in 100 μl/well) were cultured in 96-well flat-bottomed plates in the presence of various concentrations of THC (0, 1, 5, 10, and 20 μM) and either left unstimulated or stimulated with 5 μg/ml anti-CD3 mAbs (PharMingen, San Diego, CA), 5 μg/ml LPS (Sigma-Aldrich), or 2 μg/ml Con A (Sigma-Aldrich) for 48 h. We found that the mitogen-induced cell proliferation assay peaks at 48 h, and therefore, this time point was used. During the final 8 h of culture, the cells were pulsed with 2 μCi of ^{3}H-thymidine. DNA synthesis was determined by β-scintillation counting (Dean et al., 1990; McKallip et al., 1995).

Analysis of THC-Induced Apoptosis in Vitro. The spleens and thymi were aseptically harvested from C57BL/6 mice and prepared into a single-cell suspension, as described above. The splenocytes were adjusted to 5×10^6/ml in complete medium and added to 96-well plates (100 μl/well) containing various concentrations of THC (0, 10, or 20 μM). The splenocytes were either left unstimulated or stimulated with LPS or Con A for 16 h for Annexin V/PI and 24 h for TUNEL assay. We used 24-h incubation to detect apoptosis in splenocytes using the TUNEL assay because at this time point, we can detect significant levels of apoptosis. If we wait for 48 h, a large proportion of cells cultured with medium alone undergo spontaneous apoptosis, thereby making the THC-induced apoptosis difficult to detect. In some assays using the Annexin V/PI, we used a 16-h incubation to detect apoptosis because this assay is designed to detect early apoptosis. Thymocytes (5×10^5/well) were cultured in 96-well flat-bottomed tissue culture plates for 16, 24, or 48 h in the presence of various concentrations of THC (0, 5, 10, and 20 μM). After the designated time, the splenocytes and thymocytes were harvested and analyzed for apoptosis using both the Annexin/PI and TUNEL methods (Vermes et al., 1995; Kamath et al., 1997). To detect apoptosis using the TUNEL method, the cells were washed twice with PBS and fixed with 4% *p*-formaldehyde for 30 min at room temperature. The cells were next washed with PBS, permeabilized on ice for 2 min, and incubated with FITC-dUTP and terminal deoxynucleotidyl transferase (Roche Applied Science, Indianapolis, IN) for 1 h at 37°C and 5% CO_2 (Kamath et al., 1997). To detect apoptosis using the AnnexinV/PI method, the cells were washed twice with PBS and stained with AnnexinV and PI for 20 min at room temperature (Vermes et al., 1995). The cells were washed twice with PBS. The levels of apoptosis in both the TUNEL and Annexin/PI assays were determined by measuring the fluorescence of the cells by flow cytometric analysis. Five thousand cells were analyzed per sample.

Analysis of THC-Induced Thymic and Splenic Atrophy in Vivo. C57BL/6 mice were treated i.p. with a single dose of THC (0, 1, 5, 10, 20, or 50 mg/kg in 200 μl of PBS) or the vehicle. The thymus and spleen were harvested at various time points after THC injection (4, 6, 24, or 72 h). The cells were prepared into a single-cell suspension, as described above, and the total number of viable cells was determined by trypan blue dye exclusion.

Flow Cytometric Analysis of Splenic and Thymic Subsets. Splenocytes and thymocytes from vehicle or THC-treated mice were stained with various fluorescence-conjugated mAbs and analyzed using a flow cytometer. In brief, splenocytes were preincubated with FcR Block (PharMingen) to prevent nonspecific binding and were subsequently stained with PE-conjugated anti-CD3, anti-CD4, anti-CD8, anti-CD19, and Mac-3 mAbs (PharMingen). Thymocytes were stained with PE-conjugated anti-CD8 and FITC-conjugated anti-CD4 mAbs (PharMingen). The cells were incubated for 30 min on ice and then washed twice with PBS. Negative controls consisted of cells that were stained with appropriate fluorescence-conjugated normal isotype antibodies. Five thousand cells were analyzed per sample.

Determination of Effect of in Vivo THC Exposure on in Vitro Splenocyte Proliferation. C57BL/6 mice were treated with a single dose of THC (10 mg/kg in 200 μl of PBS), and 6 h later, the spleen cells were harvested. The spleen was prepared into a single-cell suspension, as described above, and adjusted to 5×10^6 cells/ml in complete medium. The cells (100 μl/well) were cultured in 96-well flat-bottomed plates and either left unstimulated or stimulated with 5 μg/ml anti-CD3, 5 μg/ml LPS, or 2 μg/ml Con A for 48 h. The cells were cultured with 2 μCi of ^{3}H-thymidine for the final 8 h, and DNA synthesis was determined by β-scintillation counting.

Analysis of THC-Induced Apoptosis in Vivo. C57BL/6 mice were treated with a single dose of THC (10 mg/kg, i.p. in 200 μl of PBS). At various time points after THC treatment, the mice were sacrificed and the thymus and spleen were harvested. The organs were prepared into a single-cell suspension, as described above. The cells were suspended at 5×10^6 cells/ml in complete medium and analyzed for apoptosis directly or after in vitro culture in 96-well flat-bottomed plates (1×10^6 cells/well in 0.2 ml of medium) for 24 h at 37°C. The cells were harvested and washed twice in PBS, and apoptosis was determined using the TUNEL method described above. In studies examining the effect of caspase inhibitors on

splenic and thymic cellularity, the mice were injected with 40 mg/kg Z-VAD-FMK (R & D Systems, Minneapolis, MN) i.p. in 400 μl of PBS 1 h before treatment with THC.

RNA Isolation and RT-PCR. RNA was isolated from 1×10^7 cells using the RNeasy Mini Kit (Qiagen, Valencia, CA). Because 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. The cDNA was prepared with the Qiagen OmniScript RT kit using 1 μg of RNA as template for first-strand synthesis. Mouse CB2 was amplified using M CB2 (5′-CCGGAAAAGAG GATGGCAATGAAT-3′) and M CB2 (5′-CTGCTGAGCGCCCTGGAGAAC-3′), which yields a product of 479 base pairs. β-Actin was used as a positive control [primers M BA U (5′-AAGG CCAACCGTGAAAAGATGACC-3′) and M BA L (5′-ACCGCTCGTTGCCAAT AGTGATGA-3′); product size of 427 base pairs]. PCR reactions were carried out using the following parameters: 95°C for 15 s, 58°C for 15 s, and 72°C for 30 s for 35 cycles, followed by a final 5 min at 72°C in a GeneAmp 9700 (Applied Biosystems, Foster City, CA). The resulting PCR products were separated on a 1% agarose gel.

Statistical Analysis. Student's t test or Tukey-Kramer test was used to compare vehicle and THC-treated groups. $p < 0.05$ was considered to be statistically significant.

Results

Exposure to THC Leads to Significant Reduction in Splenocyte Proliferation in Vitro. Splenocytes from C57BL/6 mice were exposed to various doses of THC (0, 1, 5, 10, and 20 μM) and either left unstimulated or stimulated with anti-CD3 mAbs, LPS, or Con A for 48 h in vitro. The data demonstrated that exposure of splenocytes to greater than 10 μM THC led to a significantly decreased cell proliferative response to all mitogens tested (Fig. 1). In contrast, lower doses of THC did not alter significantly the proliferative response. The observation that THC exposure reduced the response to both the T-cell (Con A and anti-CD3 mAbs) and B-cell mitogens (LPS) suggested that THC may act on both subsets of lymphocytes.

THC Induces Apoptosis in Naive and Mitogen-Activated Splenocytes in Vitro. To test whether the THC-induced reduction in splenocyte responsiveness to mitogens resulted from induction of apoptosis, we determined the level of apoptotic cells in naive and activated splenocytes. To this end, splenocytes from C57BL/6 mice were cultured with medium alone or with mitogens, in the presence or absence of 10 or 20 μM THC for 16 (Annexin/PI) or 24 (TUNEL) h. The cells were harvested, and apoptosis was measured by Annexin/PI staining and by using the TUNEL assay. Annexin/PI staining detects early apoptosis, and therefore, we analyzed the cells after 16 h of THC exposure. Using Annexin/PI staining procedure, it has been shown previously that cells stained with Annexin V alone are indicative of early apoptotic cells, those stained for both Annexin V and PI represent late apoptotic/necrotic cells, and cells positive for PI alone suggest necrotic phenotype (Vermes et al., 1995). The data shown in Fig. 2A demonstrated that culture of splenocytes with medium + vehicle induced significant levels of background apoptosis. This is expected because a certain percentage of cells when cultured in medium undergo spontaneous apoptosis, as shown previously in our studies (Kamath et al., 1997; Camacho et al., 2001). Interestingly, splenocytes cultured with medium + 10 μM THC showed significant increase in apoptosis. Furthermore, splenocytes cultured with medium + 20 μM THC exhibited even greater levels of apoptosis. It was interesting to note that at lower concentrations (10 μM), the cells were predominantly Annexin V^+, whereas at higher concentrations of THC (20 μM), the cells moved to mainly Annexin V^+PI^+ phenotype. These data suggested that the Annexin V^+PI^+ seen at the higher doses of THC may represent late apoptotic cells. Together, these results indicated that culture of splenocytes with medium + THC caused significant increase in

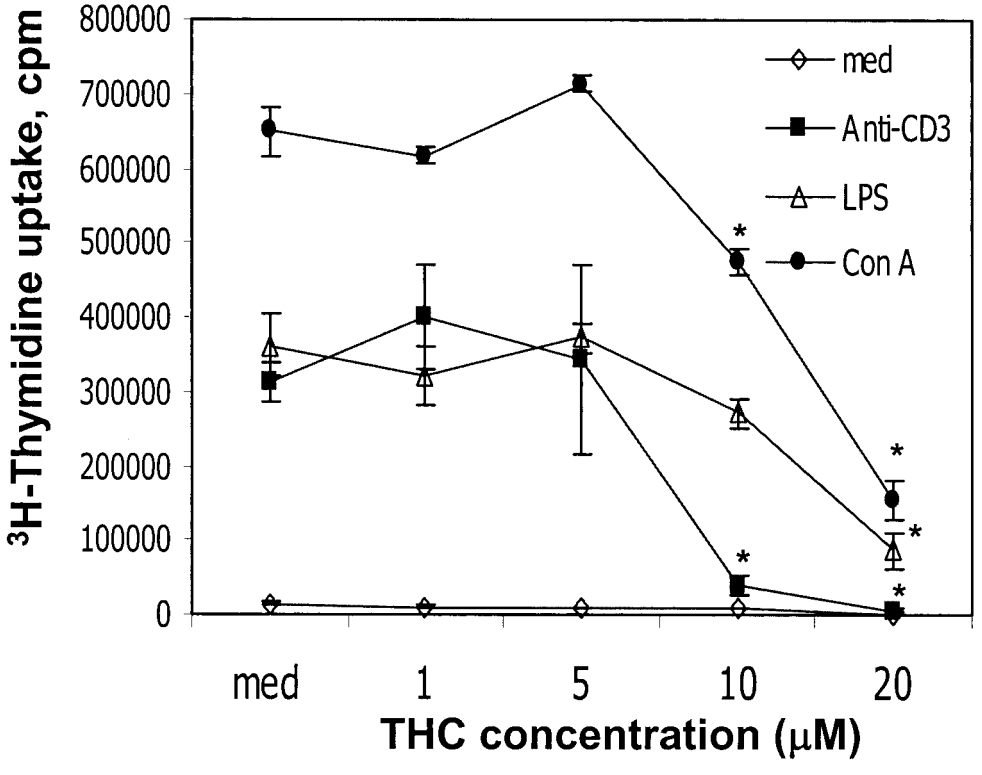

Fig. 1. The effect of THC on the proliferative response of splenocytes to stimulation with Con A, anti-CD3 mAbs, and LPS in vitro. Splenocytes (5×10^5) from C57BL/6 mice were incubated with various concentrations of THC (0, 1, 5, 10, and 20 μM) in 5% FCS RPMI in the absence or presence of 2 μg/ml Con A, 5 μg/ml anti-CD3 mAbs, or 5 μg/ml LPS for 48 h. During the final 8 h, the cells were pulsed with 2 μCi of [^{3}H]thymidine. Thymidine incorporation was determined by β-scintillation counting. The data points represent the mean ± S.D. of triplicate cultures. Asterisk denotes statistically significant difference ($p < 0.05$) when compared with the controls.

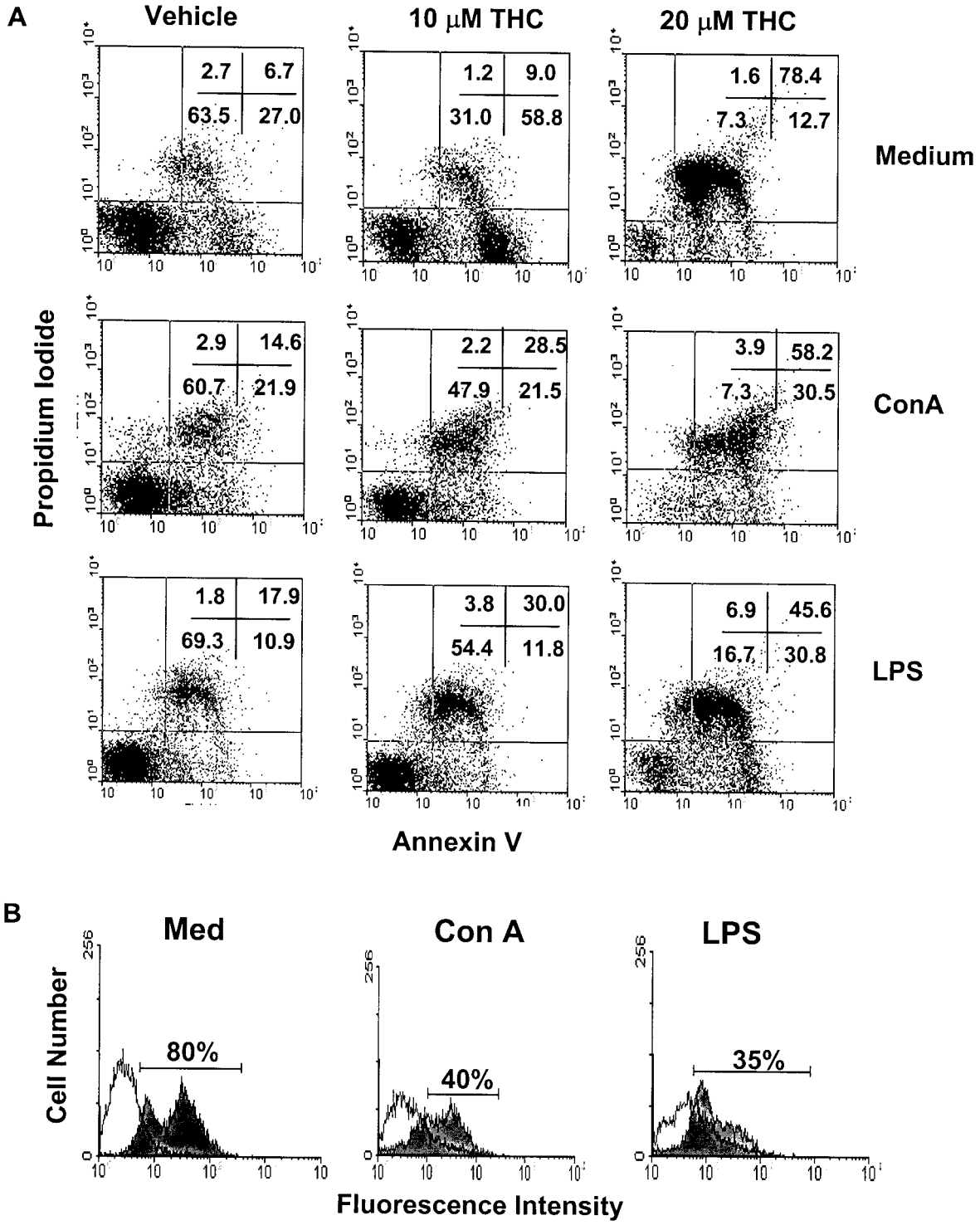

Fig. 2. The effect of THC on the induction of apoptosis in naive and activated splenocytes. Splenocytes (5×10^5) from C57BL/6 mice were cultured in medium alone or in the presence of 2 μg/ml Con A or 5 μg/ml LPS for 16 (A) or 24 (B) h. Also, these cultures received THC (10 or 20 μM in A and 10 μM in B) or the vehicle used for dissolving THC. The cells were harvested and analyzed for apoptosis by Annexin V/PI staining (A) or by TUNEL assay (B). In A, the percentage of cells found in each quadrant of the dot plot has been depicted. B shows increase in apoptosis in splenocytes exposed to THC (filled histogram) when compared with splenocytes incubated with vehicle alone (empty histogram). The percentage of apoptotic cells obtained by subtracting the empty histogram from the filled histogram showing percentage of apoptosis after exposure to THC has been depicted in each histogram.

apoptosis when compared with cells cultured with medium + vehicle. Similarly, when splenocytes were cultured with mitogens such as Con A or LPS along with THC, increased percentage of apoptotic cells were detected when compared with splenocytes incubated with mitogens + vehicle. The induction of apoptosis by THC was dose-related. To further corroborate the induction of apoptosis by THC, splenocytes cultured with medium or mitogens + THC (10 μM) for 24 h were analyzed for

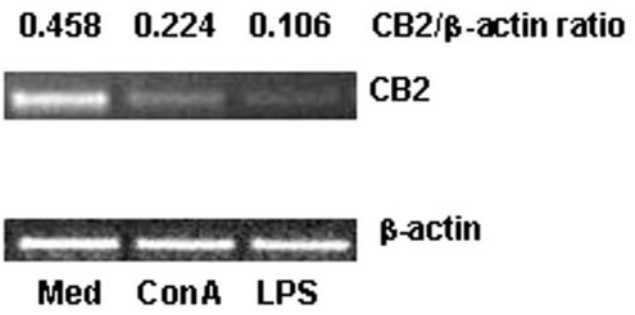

Fig. 3. CB2 mRNA expression in naive and mitogen-activated lymphocytes. The expression of CB2 mRNA in naive, LPS-, and Con A-activated splenocytes was determined by RT-PCR. Splenocytes from C57BL/6 mice were cultured in the absence or presence of 2 μg/ml Con A or 5 μg/ml LPS for 24 h. Total RNA was isolated from the cells. mRNA was reverse transcribed and amplified by PCR with primers specific for CB2 and β-actin. A photograph of ethidium bromide-stained amplicons is depicted.

apoptosis using TUNEL assay. The results showed that splenocytes cultured with medium, Con A, or LPS + THC (filled histogram) showed elevated levels of apoptosis when compared with cell cultured with medium, Con A, or LPS + vehicle (open histogram) (Fig. 2B). It should be noted that splenocytes cultured with medium + THC showed higher percentages of apoptosis when compared with cells cultured with mitogens + THC using both assays to detect apoptosis. These data suggested that naive lymphocytes may be more susceptible to induction of apoptosis by THC than mitogen-activated lymphocytes.

To further investigate whether the increased susceptibility of naive lymphocytes to THC-induced apoptosis was dependent on the levels of expression of cannabinoid receptors, we used semiquantitative RT-PCR to measure the levels of CB2 mRNA expression in naive and activated cells. The CB2 expression was compared with the levels of β-actin in various groups, and the data were expressed as CB2/β-actin ratio (Fig 3). These results demonstrated that CB2 expression in cells cultured with medium alone was much stronger than that seen with cells cultured with mitogens (Fig. 3). These data suggested that CB2 expression may be down-regulated after lymphocyte activation, which may explain why mitogen-activated lymphocytes showed lower levels of THC-induced apoptosis than naive cells (Fig. 2).

Culture with CB2 Antagonist Inhibits THC-Induced Effects on Splenocyte Proliferation and Apoptosis. Next, we tested whether the effects induced by THC on the

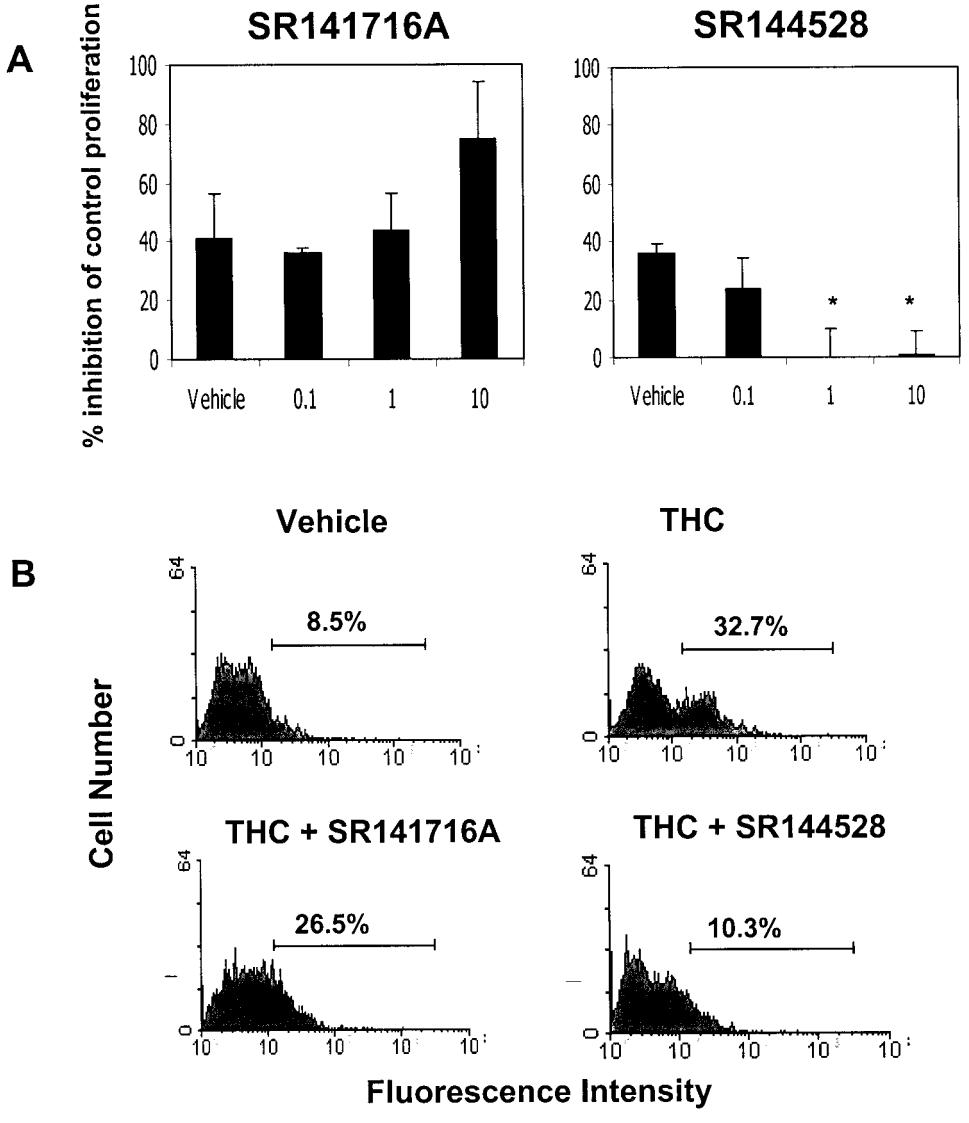

Fig. 4. The effect of cannabinoid receptor antagonists on THC-induced suppression of the proliferative response and induction of apoptosis in vitro. A splenocytes (5×10^5/well) stimulated with Con A were cultured with medium or 10 μM THC for 24 h. In addition, the cultures received various concentrations of CB1 (SR141716A) or CB2 antagonists (SR144528) or the vehicle. The proliferative response was determined by pulsing the cells during the final 8 h, with 2 μCi of [^{3}H]thymidine. The data points represent the percentage inhibition ± S.D. of the proliferative response of triplicate cultures in the presence of THC when compared with control cultures. Asterisk denotes statistically significant difference ($p < 0.05$) when compared with the controls. In B, splenocytes were cultured with 10 μM THC or vehicle and stimulated with Con A for 24 h. In addition, the cultures received 1 μM of SR141716A, SR144528, or vehicle. The cells were harvested, and apoptosis was determined using the TUNEL assay (B). The data depicted are representative histograms showing the level of apoptosis.

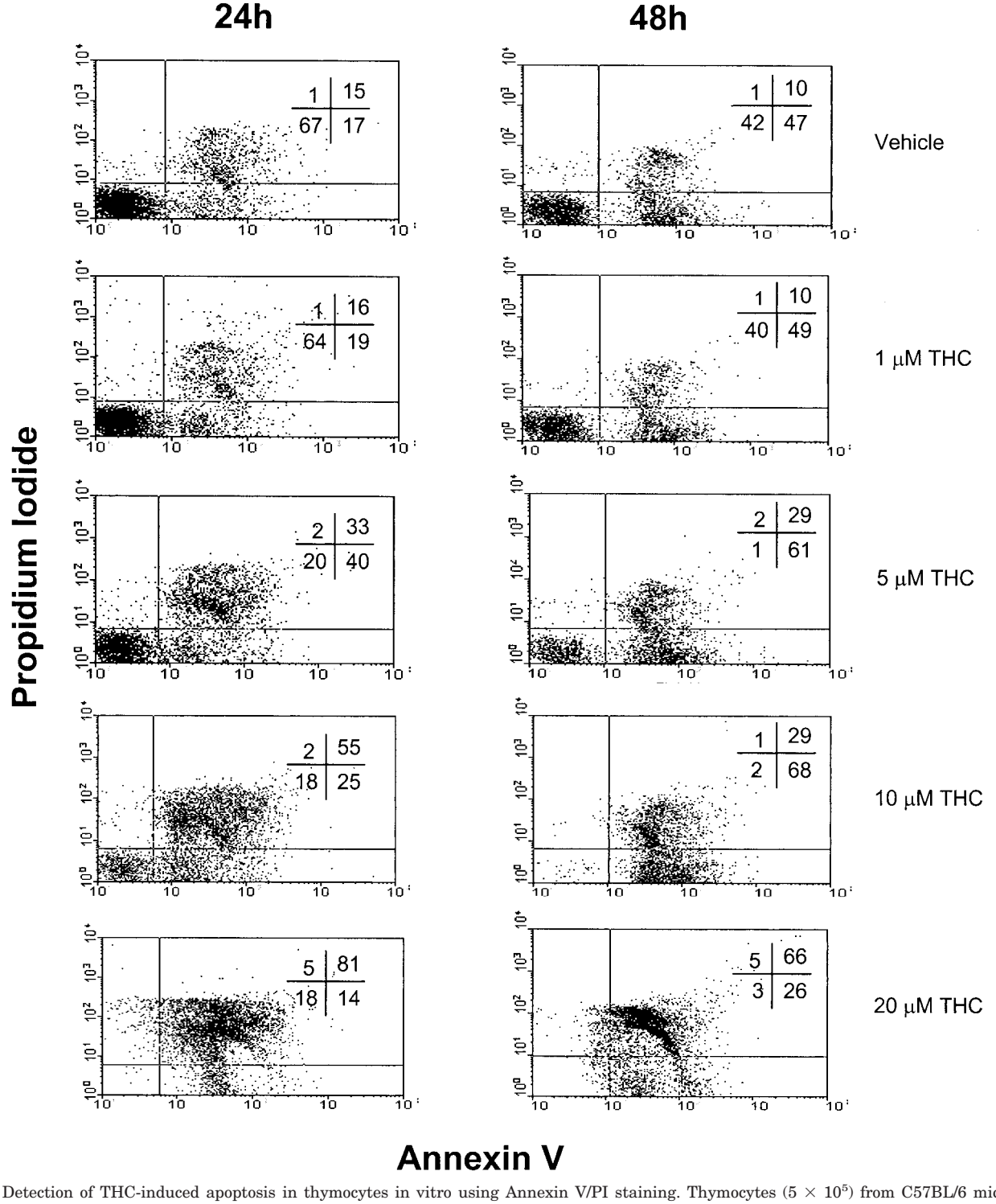

Fig. 5. Detection of THC-induced apoptosis in thymocytes in vitro using Annexin V/PI staining. Thymocytes (5×10^5) from C57BL/6 mice were incubated with various concentrations of THC (1, 5, 10, and 20 μM) or the vehicle for 24 or 48 h in RPMI containing 5% FCS, as described in Fig. 4. The cells were harvested and analyzed for apoptosis by Annexin V/PI assay. The percentage of cells found in each quadrant of the dot plot has been depicted.

proliferative response and induction of apoptosis were mediated through CB1 and CB2. To this end, splenocytes were cultured with various concentrations of CB1 or CB2 antagonists (SR141716A and SR144528, respectively) or the vehicle and exposed to 10 μM THC. The splenocytes were stimulated with Con A, and 24 h later, the proliferative response and induction of apoptosis were determined. The results showed that culturing splenocytes with 1 or 10 μM CB2 antagonist reversed the THC-induced suppression of the proliferative response (Fig. 4A) and induction of apoptosis (Fig. 4B). In contrast, culturing splenocytes with CB1 antagonist had no significant effect on the THC-induced suppression of the proliferative response (Fig. 4A) and only a slight effect on apoptosis (Fig. 4B). Together, these data suggested that the

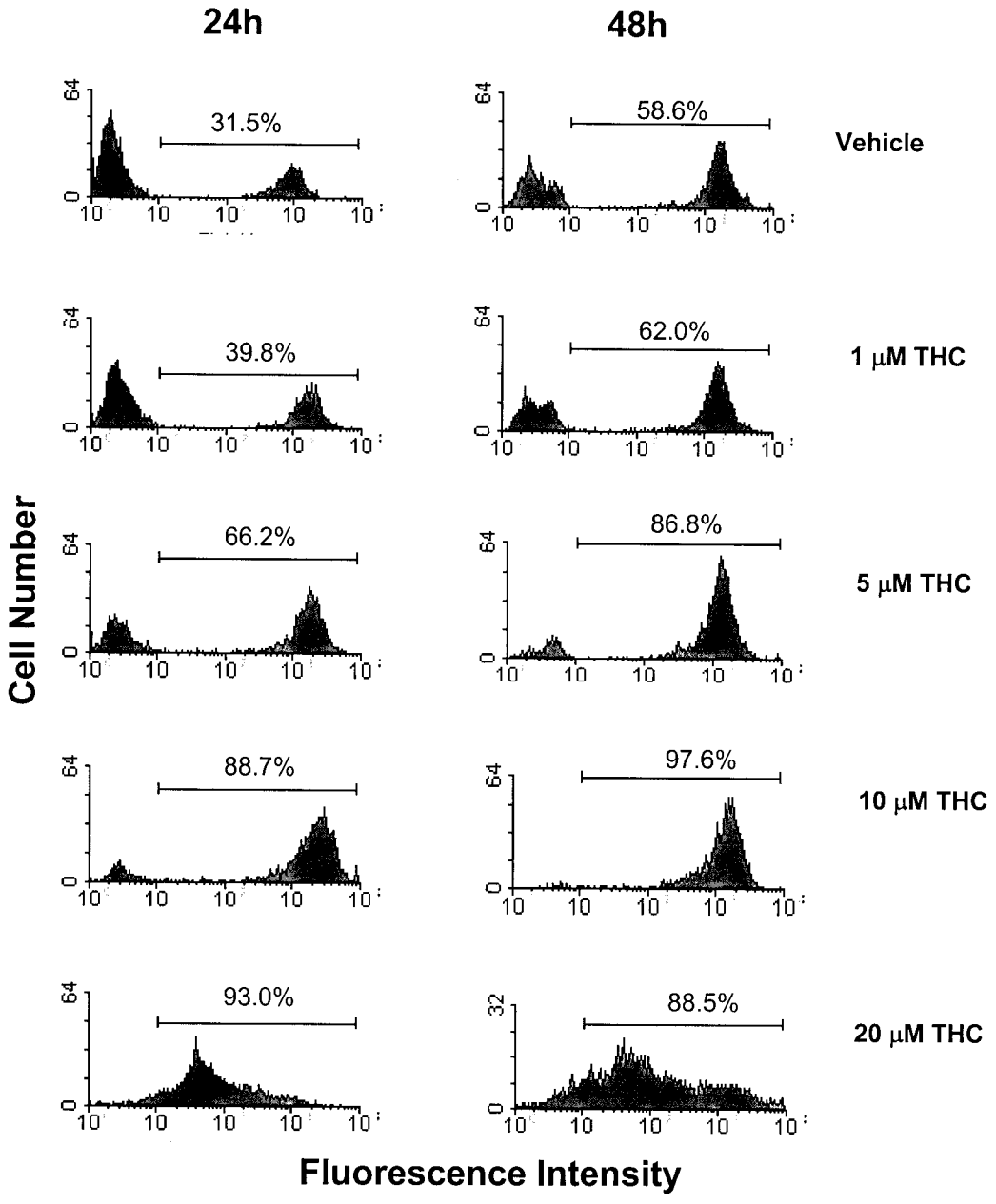

Fig. 6. Detection of THC-induced apoptosis in thymocytes in vitro using the TUNEL assay. Thymocytes (5×10^5) from C57BL/6 mice were cultured with various concentrations of THC (1, 5, 10, and 20 μM) or the vehicle for 24 or 48 h in RPMI containing 5% FCS. The cells were harvested and analyzed for apoptosis by TUNEL assay. The data depicted are representative histograms showing the level of apoptosis.

effects of THC on the proliferative response and induction of apoptosis are mediated through CB2.

Culture of Thymocytes with THC in Vitro Triggers Apoptosis. Next, we tested whether THC would also induce apoptosis in thymocytes. To this end, thymocytes from C57BL/6 mice were cultured in the presence of various concentrations of THC (1, 5, 10, and 20 μM) or the vehicle (DMSO) for 24 and 48 h and subsequently stained for apoptosis using the Annexin/PI (Fig. 5) and TUNEL methods (Fig. 6). The results demonstrated that culture of thymocytes with vehicle alone for 24 h induced a significant percentage of cells to undergo apoptosis as reported previously by us (Kamath et al., 1997). Interestingly, exposure of thymocytes to THC led to dose-related increase in apoptosis as indicated by Annexin V^+ cells (Fig. 5). Similar results were seen at 48 h of culture. It should be noted that at lower concentrations of THC, the cells were predominantly Annexin V^+ and PI^- whereas at higher doses, the cells were mainly Annexin V^+ and PI^+. These data were similar to that seen with splenocytes (Fig. 2) and suggested that at higher doses of THC, the cells were moving from apoptotic to late apoptotic/necrotic phenotype. To corroborate these results, we also performed the TUNEL assay, which gave similar results (Fig. 6). Together, these data suggested that THC induces apoptosis in thymocytes.

Effects of CB1 and CB2 Antagonists on THC-Induced Apoptosis in Thymocytes. To investigate the role of CB1 and CB2 in THC-induced apoptosis, thymocytes were cultured for 16 h with the vehicle or THC and in the presence of CB1 (SR141716A) and CB2 (SR144528) antagonists. The results showed that THC-induced apoptosis was reversed to a significant extent by CB2 antagonist as indicated by determination of viable cells (Fig. 7A) and Annexin/PI staining (Fig. 7B). In contrast, CB1 antagonist failed to reverse the decrease in viable cell number (Fig. 7A) and partially reversed the apoptosis induced by THC (Fig. 7B). It should be

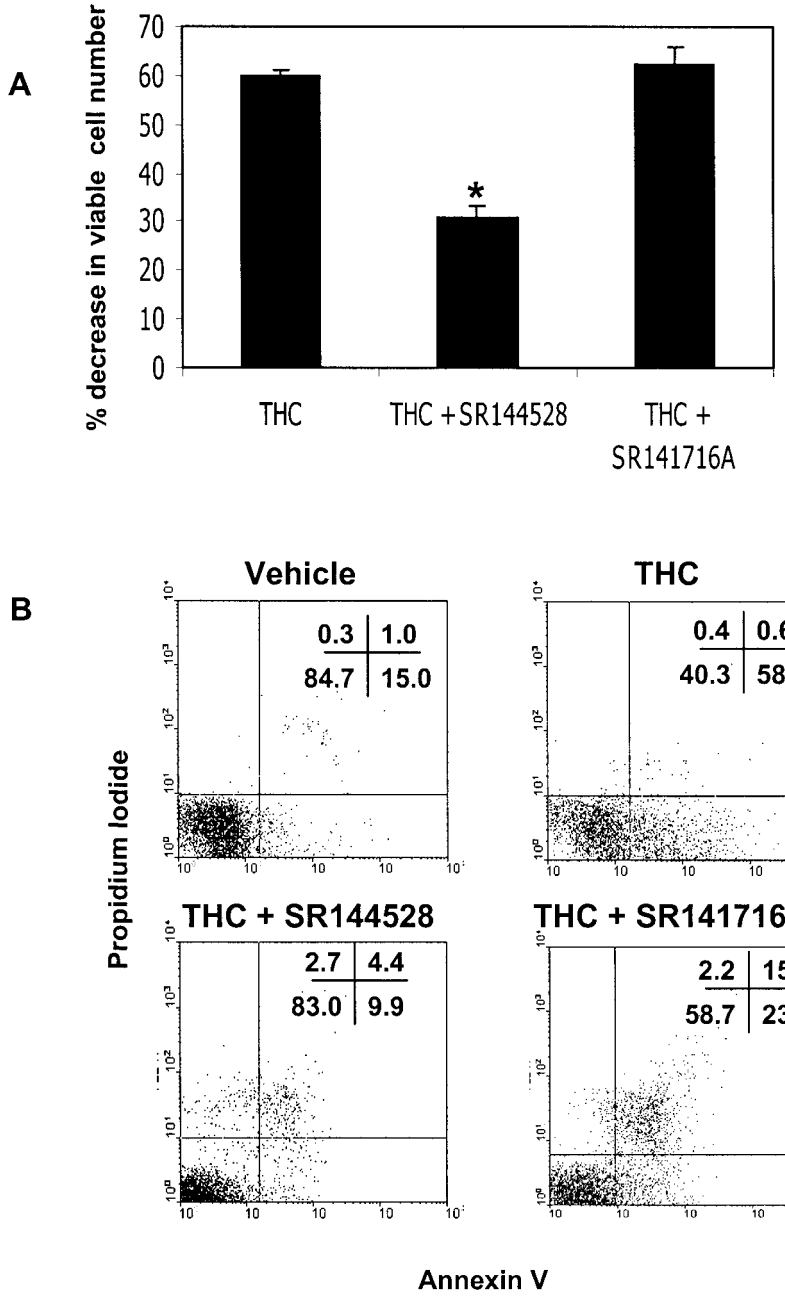

Fig. 7. Effect of CB1 and CB2 antagonists on THC-induced thymocyte viability and apoptosis in vitro. Thymocytes were cultured with the vehicle alone, 10 μM THC, 1 μM THC + SR144528, or 1 μM THC + SR141716A for 16 h. The cells were harvested, and the viable cell number was determined by trypan blue dye exclusion (A) and apoptosis by Annexin V/PI (B). In A, the data were expressed as percentage decrease in cell viability when compared with the vehicle control. The data depicted in A are the mean ± S.D. of triplicate cultures. Asterisk denotes statistically significant difference ($p < 0.05$) between THC and THC + SR144528 treatment groups.

noted that thymocytes cultured with CB1 or CB2 antagonists alone failed to undergo increased apoptosis when compared with vehicle controls (data not shown). Together, the data suggested that CB2 but not CB1 receptors play a key role in THC-induced apoptosis of thymocytes.

Exposure to THC Results in Thymic and Splenic Atrophy in Vivo. We carried out a series of studies to investigate whether exposure to THC in vivo would cause decreased cellularity of the lymphoid organs. Groups of four C57BL/6 mice were injected with various doses of THC (0, 1, 5, 10, 20 and 50 mg/kg body weight). The thymi and spleens were harvested 24 h later. Single-cell suspensions were prepared from these organs and enumerated by trypan blue dye exclusion. The results demonstrated that the injection of 10 mg/kg body weight or higher doses of THC resulted in a significant reduction in the total cellularity of the thymus and the spleen (Fig. 8). To determine the time kinetics of THC-induced toxicity, C57BL/6 mice were injected i.p. with 10 mg/kg THC, and at various time points after injection (4, 6, 24, and 72 h), the cellularity of the spleen and thymus was determined. The data demonstrated that THC-treatment caused a marked decrease in the cellularity of the thymus and spleen, particularly at 6 and 24 h after THC-exposure, and that the cellularity began recovering by 72 h (Fig. 8).

Effect of THC on T-Cell Subsets in the Thymus. We next examined whether exposure to THC (10 mg/kg for 24 h) led to alterations in T-cell maturation in the thymus by examining the distribution of the various T-cell subsets (Table 1). To this end, thymocytes from DMSO (vehicle) and THC-treated mice were stained with PE-conjugated anti-CD8 and FITC-conjugated anti-CD4 mAbs, and the percentage of the four subpopulations was examined. The results

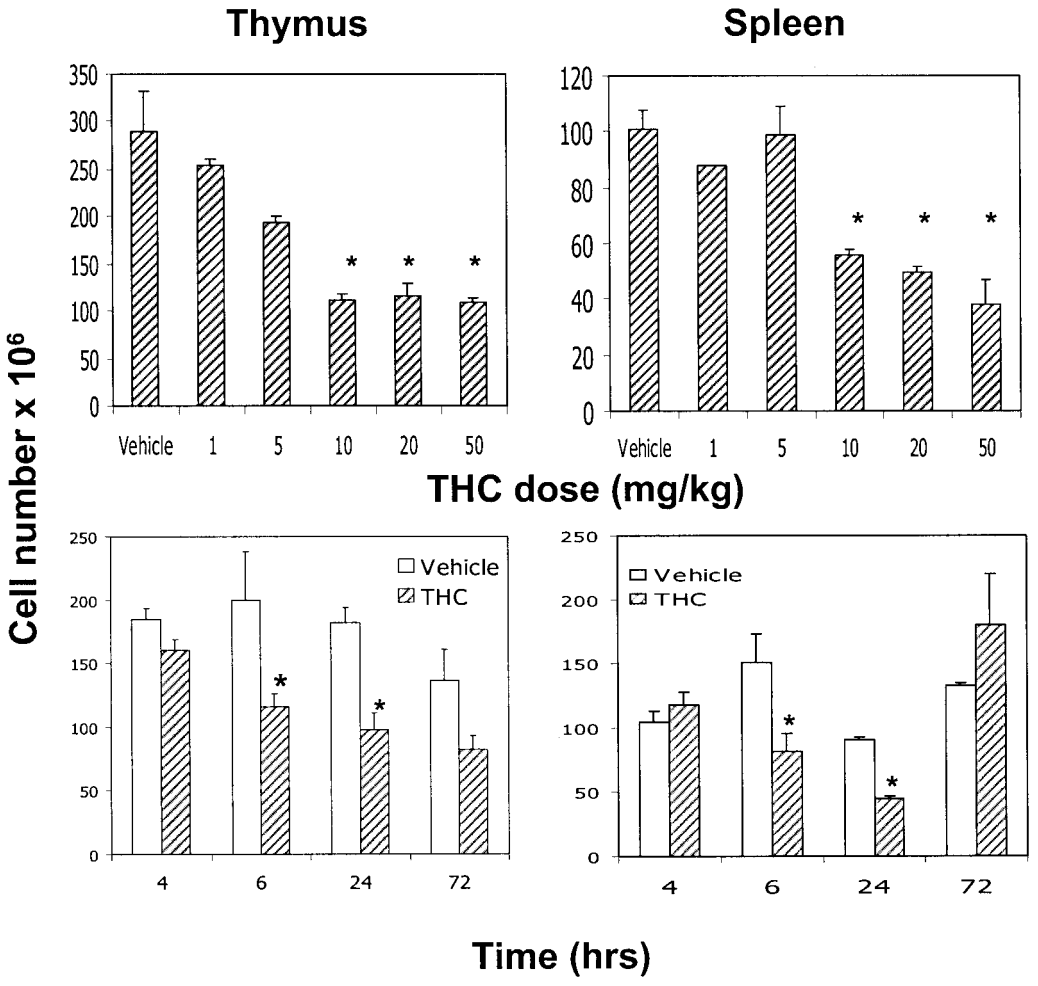

Fig. 8. The effect of THC exposure on thymic and splenic cellularity in vivo. A, C57BL/6 mice were injected with the vehicle or various concentrations of THC (1, 5, 10, 20, and 50 mg/kg body weight) i.p. The thymus and spleen were harvested 24 h later, and the cellularity was determined by trypan blue dye exclusion. The data represent the mean ± S.E.M. of groups of four mice. B, the kinetics of the response to THC exposure was examined by injecting C57BL/6 mice with 10 mg/kg body weight of THC, harvesting the thymus and spleen at various time points (4, 6, 24, and 72 h), and determining the cellularity by trypan blue dye exclusion. The data represent the mean ± S.E.M. of groups of four mice. Asterisk denotes statistically significant difference ($p < 0.05$) when compared with the controls.

TABLE 1
Effects of THC on the percentage of thymic subsets in vivo

	Cellularity[a]	T-Cell Subsets[c]			
		$CD4^-$ $CD8^-$	$CD4^+$ $CD8^+$	$CD4^+$ $CD8^-$	$CD4^-$ $CD8^+$
	cell no. $\times 10^6$*/thymus*				
Vehicle	182 ± 12[b]	4.13 ± 0.09 (7.52)	80.92 ± 0.87 (147.27)	10.77 ± 1.21 (19.60)	4.18 ± 0.29 (7.61)
THC	98 ± 13	4.80 ± 0.79 (4.70)	79.63 ± 4.21 (78.04)	10.50 ± 4.50 (10.29)	5.10 ± 1.21 (4.99)

[a] C57BL/6 mice were treated with THC (10 mg/kg body weight) or the vehicle. One day after treatment with THC, the thymocytes were harvested, and the total cellularity was determined.
[b] Data represent mean ± S.E.M. obtained from six mice.
[c] T-cell subsets were determined using flow cytometry. The data represent mean ± S.E.M. of the percentage of T-cell subsets in the thymus obtained from six mice. The numbers in parentheses represent the mean total cellularity $\times 10^6$ of each T-cell subset found in the thymi.

from this experiment, summarized in Table 1, showed that THC treatment did not lead to significant alteration in the percentage of the individual thymic subpopulations, when compared with vehicle-treated control mice. However, because the total thymic cellularity was decreased (Fig. 8), the total number of individual T-cell subsets was also significantly decreased in THC-treated mice (Table 1).

Effect of THC on Lymphocyte Populations in the Spleen. We also examined whether the reduction in splenic cellularity after THC treatment was due to an effect on specific lymphocyte and macrophage populations (Table 2). More specifically, we examined whether THC treatment led to specific reduction in the T-cell, B-cell, and macrophage populations. Splenocytes from mice treated for 24 h with the vehicle or THC (10 mg/kg, i.p.) were stained with fluorescence-conjugated mAbs specific for CD3 (T cell), CD4 (T helper), CD8 (T cytotoxic), CD19 (B cell), and Mac-3 (macrophage) cell surface markers, and the percentage of the corresponding lymphocyte populations was determined by flow cytometric analysis. The results summarized in Table 2 showed that exposure to THC in vivo did not specifically affect any one lymphocyte population but led to equal reduction in total cell number in each population examined.

In Vivo Exposure to THC Leads to Inhibition of the in Vitro Proliferative Response of Splenocytes to Mitogens. To examine whether in vivo exposure to THC

TABLE 2
Effects of THC on the percentage of splenic subsets in vivo

	Cellularity[a]	Splenocyte Populations[c]				
		$CD3^+$	$CD4^+$	$CD8^+$	$CD19^+$	Mac-3^+
	cell no. $\times 10^6$*/spleen*					
DMSO	91.20 ± 2.2[b]	45.20 ± 4.34 (41.13)	23.45 ± 0.56 (21.34)	18.05 ± 3.18 (16.43)	47.95 ± 6.11 (43.63)	16.50 ± 1.82 (15.02)
THC	45.12 ± 2.0	47.30 ± 5.96 (21.29)	26.45 ± 5.30 (11.90)	19.80 ± 2.73 (8.91)	47.45 ± 0.30 (21.35)	17.60 ± 0.10 (7.92)

[a] C57BL/6 mice were treated with THC (10 mg/kg body weight) or the vehicle. One day after treatment with THC, the spleens were harvested, and the total cellularity was determined.
[b] Data represent mean ± S.E.M. obtained from six mice.
[c] The percentage of each splenocyte subset was determined using flow cytometry. The data represent mean ± S.E.M. of percentage of lymphocyte and macrophage subsets in the spleens obtained from six mice. The numbers in parentheses represent the mean total cellularity $\times 10^6$ of each cell population found in the spleens.

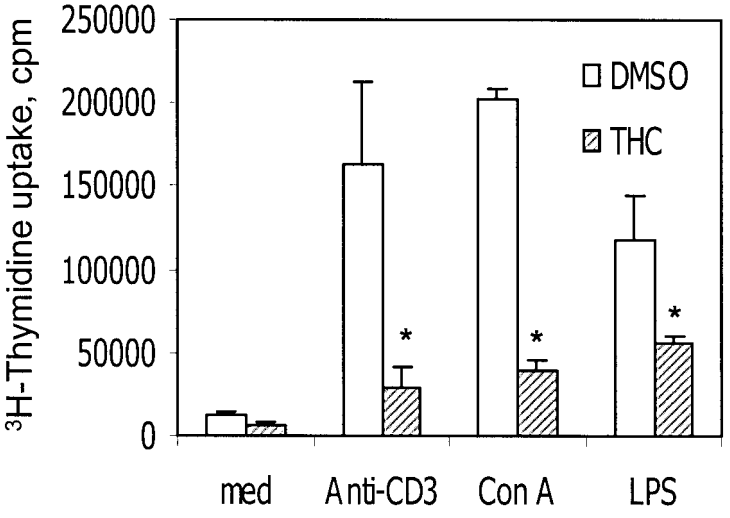

Fig. 9. The effect of in vivo exposure to THC on the activation of splenocytes in vitro. C57BL/6 mice were injected i.p. with 10 mg/kg body weight of THC or the vehicle. The spleens from vehicle or THC-treated mice were harvested 6 h later and prepared into a single-cell suspension. The splenocytes (5×10^5) were cultured in medium containing anti-CD3 mAb (5 μg/ml), Con A (2 μg/ml), or LPS (5 μg/ml) for 48 h. The proliferative response was determined by pulsing the cells during the final 8 h of culture with 2 μCi of [^{3}H]thymidine. Thymidine incorporation was determined by β-scintillation counting. The data points represent the mean ± S.D. of triplicate cultures. Asterisk denotes statistically significant difference ($p < 0.05$) when compared with the controls.

would alter the proliferative response of splenocytes, mice were exposed to 10 mg/kg THC for 6 h, after which the splenocytes were harvested and stimulated in vitro for 48 h with various polyclonal mitogens (anti-CD3 mAbs, LPS, and Con A). The results from this experiment showed that in vivo exposure to THC led to a significant suppression of splenocyte responsiveness to T-cell and B-cell mitogens in vitro (Fig. 9).

THC Exposure Induces Apoptosis in Splenocytes and Thymocytes in Vivo. Next, the possibility that the observed thymic and splenic atrophy after THC treatment was due to the induction of apoptosis was investigated. Mice were treated with 10 mg/kg THC for various time periods (4–72 h), the thymus and spleen were harvested and prepared into a single-cell suspension, and apoptosis was determined using the TUNEL method. It was observed that thymocytes and splenocytes from THC-treated mice when analyzed directly showed no significant levels of apoptosis when compared with the cells obtained from vehicle-treated mice (data not shown). Previous studies from our laboratory have demonstrated that detection of apoptosis induced by drugs or chemicals in vivo is difficult because such cells are rapidly cleared by the phagocytic cells (Kamath et al., 1997). However, we have also shown that when such cells are cultured in vitro, they exhibit increased levels of apoptosis (Kamath et al., 1997). In the current study, therefore, we incubated the thymocytes from THC-injected mice in vitro for an additional 24 h in medium and analyzed the cells for apoptosis. The data demonstrated that thymocytes from mice injected with 10 mg/kg THC when harvested 16 h after exposure and cultured for an additional 24 h in vitro exhibited a significantly higher proportion of apoptotic cells when compared with similar cells from vehicle-treated mice (Fig. 10A). Also, splenocytes collected from mice 6 h after THC-treatment and cultured in vitro showed increased levels of apoptosis when compared with the controls (Fig. 10B). Examination of splenocytes from mice treated for more than 6 h and thymocytes from mice treated for more than 16 h failed to show increased levels of apoptosis upon in vitro culture (data not shown). We have shown earlier that chemical/drug-induced apoptosis in vivo is best detected before onset of thymic or splenic atrophy (Kamath et al., 1997). This is because once the atrophy in the organs begins, the apoptotic cells are rapidly cleared by phagocytic cells and the remaining cells may represent those that escaped apoptosis. For this reason, we used early time points such as 6 and 16 h to study apoptosis. The decrease in spleen cellularity was most dramatic at 6 h, and the effect on thymus was most significant at 24 h. Therefore, the apoptosis in the thymus was studied at 16 h. We investigated whether apoptosis in splenocytes can be detected at time points earlier than 6 h, but failed to observe significant levels, because of which, we used the 6-h time point. These data together suggested that THC-induced apoptosis in thymocytes and splenocytes is detectable only at early time points and only after in vitro culture.

Treatment with Caspase Inhibitors Reduces THC-Induced Thymic and Splenic Atrophy in Vivo and in Vitro. To further confirm that apoptosis was involved in the observed THC-induced thymic and splenic atrophy, we examined the

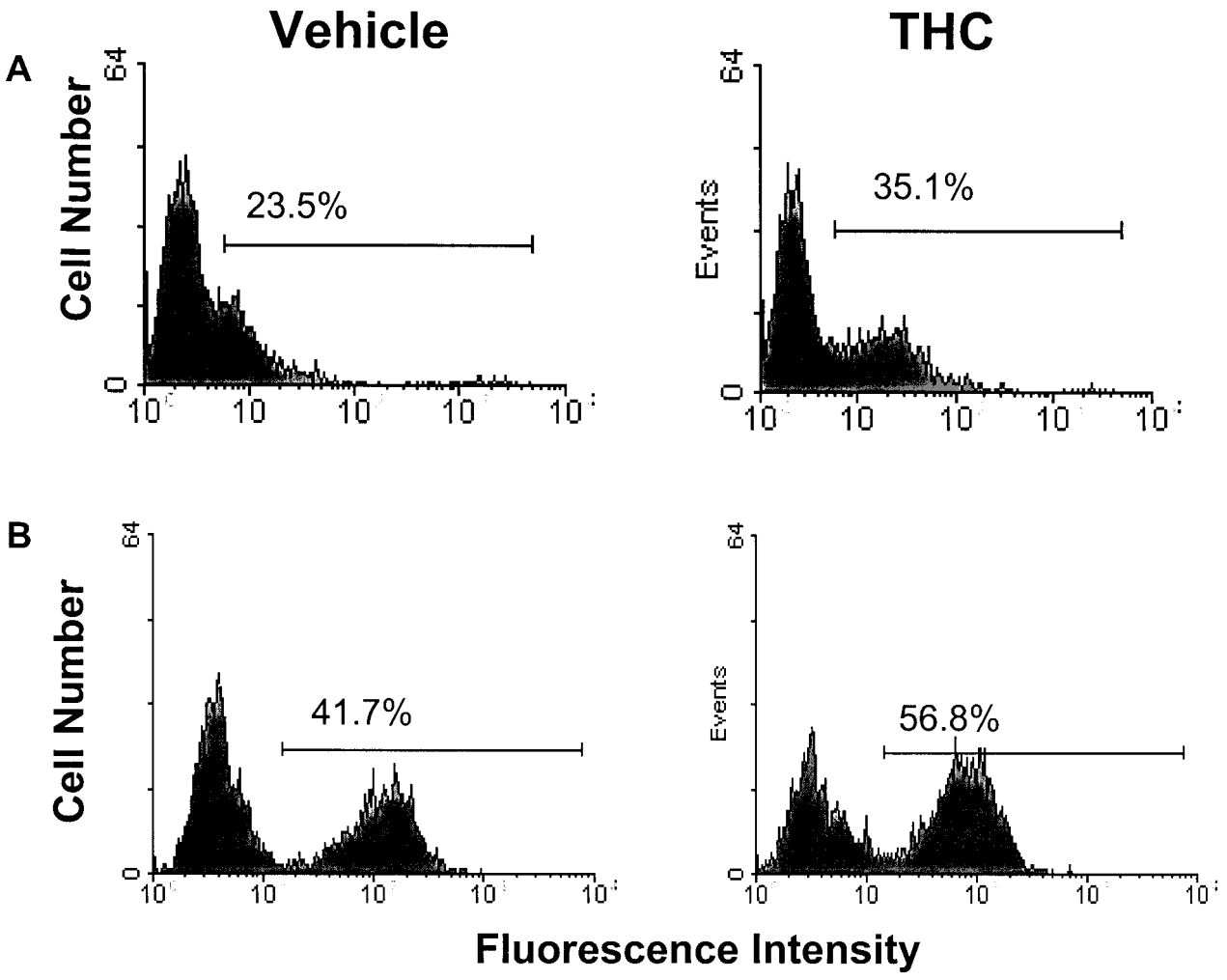

Fig. 10. Analysis of apoptosis in the thymus and spleen of mice injected with THC. C57BL/6 mice were injected with 10 mg/kg body weight of THC i.p. The spleen and thymus were removed 6 or 16 h later, respectively, and prepared into a single-cell suspension. The thymocytes and splenocytes (5×10^5) were cultured in RPMI containing 10% FCS for 24 h and stained for apoptosis using the TUNEL assay. The data depicted are representative histograms showing the level of apoptosis in splenocytes (A) and thymocytes (B) from mice exposed to vehicle or THC.

effect of pretreatment with caspase inhibitors on the cellularity of the thymus and spleen after THC injection. To this end, mice were injected i.p. with 40 mg/kg pan caspase inhibitor (Z-VAD-FMK) or vehicle. One hour later, the mice were injected with 10 mg/kg THC i.p. Six hours later, the thymic and splenic cellularity was determined (Fig. 11). The results showed that THC treatment led to significant reduction in cell number in both the thymus and spleen. However, when the mice were injected with the caspase inhibitor, the effect of THC on the thymic and splenic cellularity was partially reversed. These data suggested that the THC-induced decrease in thymic and splenic cellularity was caused at least in part by activation of caspases.

The effect of caspase inhibitor on THC-induced apoptosis in lymphocytes was also tested in vitro. To this end, spleen cells and thymocytes were cultured in vitro with 10 μM THC in the presence or absence of 50 μM Z-VAD-FMK. After 16 h, the viable cell number was determined, and data were expressed as percentage decrease in cell number and compared with the vehicle controls. Also, the cells were stained with Annexin/PI to detect apoptosis. The data demonstrated that Z-VAD-FMK reversed the decrease in viable cell number (Fig. 12A) and apoptosis (Fig. 12B) induced by THC.

Discussion

In the present study, we demonstrated for the first time that THC can induce apoptosis in vivo in thymocytes and splenocytes. Also, we found that THC-induced apoptosis was regulated by CB2 and was reversed, at least in part, by caspase inhibitors. Previous studies have suggested that THC may induce apoptosis in lymphocytes and macrophages (Schwarz et al., 1994; Zhu et al., 1998). However, in these studies, the ability of THC to induce apoptosis was studied only in vitro but not in vivo. Zhu et al. (1998) demonstrated that THC at 15 to 30 μM concentrations caused an increase in apoptosis in splenocytes cultured with Con A or peritoneal macrophages cultured with LPS. Also, THC was shown to induce apoptosis in cultured transformed neural cells (Galve-Roperh et al., 2000), cortical neurons (Campbell, 2001), human prostate PC3 cells (Ruiz et al., 1999), and C6 glioma cells (Sanchez et al., 1998). Although the mechanism by which THC triggers apoptosis is not clear, preliminary studies have suggested the involvement of mitochondria, cytochrome *c*, caspases, and Bcl-2 (Zhu et al., 1998; Campbell, 2001).

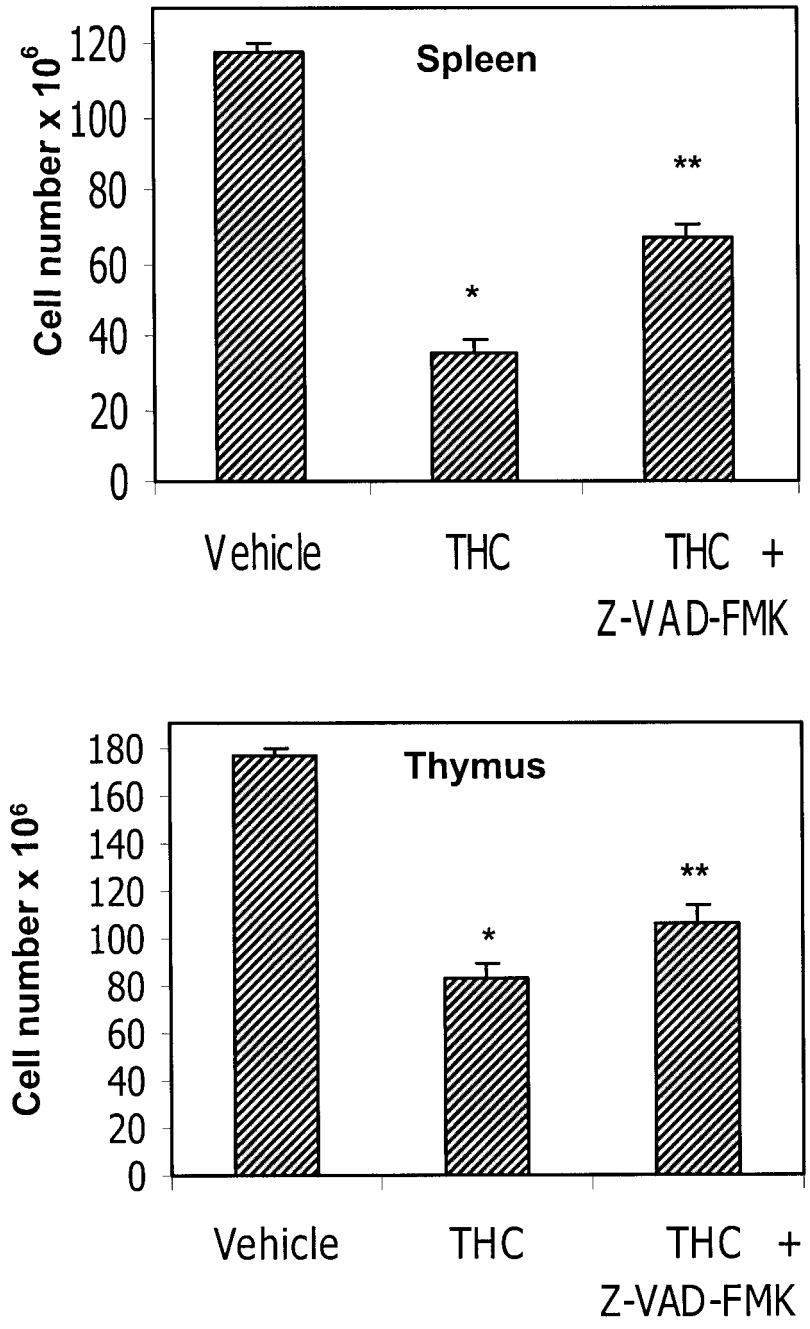

Fig. 11. The effect of caspase inhibitor on THC-induced thymic and splenic atrophy. C57BL/6 mice were injected with 40 mg/kg body weight of Z-VAD-FMK. One hour later the mice were injected with 10 mg/kg body weight of THC or the vehicle i.p. The spleen and thymus were removed 6 h later and the cellularity was determined by trypan blue dye exclusion. The data represent the mean ± S.E.M. of groups of three mice. Single asterisk denotes statistically significant difference ($p < 0.05$) between vehicle and THC treatment groups. Double asterisk denotes statistically significant difference ($p < 0.05$) between THC and THC + Z-VAD-FMK treatment groups. Statistical differences were determined using the Tukey-Kramer test.

The exact mechanism by which THC induces apoptosis in lymphocytes remains unclear. It is believed that THC and other cannabinoids can act by two distinct mechanisms. Because of its lipophilic properties, it was thought that THC acted through direct intercalation into the cell membrane. However, it was soon realized that the activity of cannabinoids was highly stereospecific, suggesting that the lipophilic properties were not solely responsible for the cannabinoid's activity. Since then, receptors for cannabinoids were characterized. These receptors only share 44% homology but most cannabinoids tested show similar binding affinity to both receptors (Pertwee, 1999). Both receptors are coupled to G-protein, suggesting that endogenous cannabinoids may play a role in cell signaling (Berdyshev, 2000). Therefore, it is possible that the observed effects of THC on the immune response, including the induction of apoptosis, may be mediated by signals initiated through these receptors. For example, Galve-Roperh et al. (2000) demonstrated that apoptosis induced by THC in C6 glioma cells in vivo involved cannabinoid receptor-dependent pathway. In contrast, others have shown in C6 glioma or a prostate cancer cell model that THC-induced apoptosis was independent of the involvement of CB1 and CB2. The present study suggests that THC-induced immunosuppression is primarily mediated through CB2 in that culture with the CB2 antagonist, but not the CB1 antagonist, inhibited the effect of THC on the proliferative response and induction of apoptosis.

THC is well known for its impact on the cytokine network (Klein et al., 2000a). For example, the presence of THC or activation of CB1/CB2 can block forskolin-induced accumu-

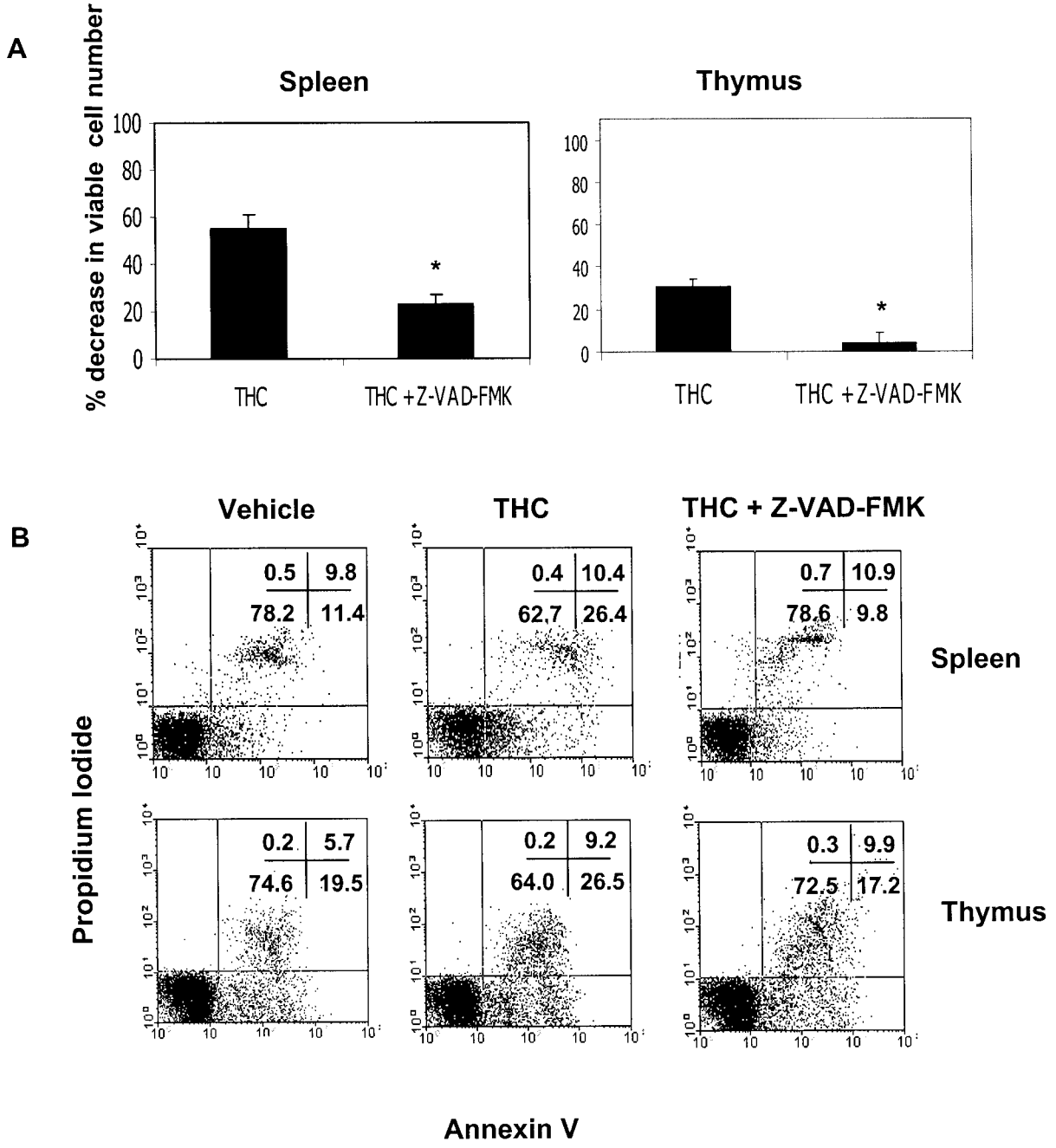

Fig. 12. Effect of caspase inhibitor on THC-induced apoptosis in splenocytes and thymocytes in vitro. Spleen cells and thymocytes were cultured with the vehicle, 10 μM THC, or 50 μM THC + Z-VAD-FMK for 16 h. The cells were harvested, viable cell number was determined by trypan blue dye exclusion (A), and apoptosis was studied by Annexin/PI staining (B), as described in Fig. 7. In A, the percentage decrease in viable cell number was determined by comparing with the vehicle controls, as described in Fig. 7. The data depicted in A are the mean ± S.D. of triplicate cultures. Single asterisk denotes statistically significant difference ($p < 0.05$) between THC and THC + Z-VAD-FMK treatment groups.

lation of cAMP (Koh et al., 1997; Schatz et al., 1997; Herring et al., 1998), and reduced cAMP levels correlate with the repression of IL-2 transcription and secretion (Novak et al., 1990). IL-2 plays an important role in the regulation of apoptosis (Lenardo, 1991; Deng and Podack, 1993; Zhang et al., 1995). Therefore, reduction in the levels of IL-2 or other cytokines after exposure to THC may partly account for increased apoptosis. Although this may account for the induction of apoptosis in activated lymphocytes, in the current study, we also noted that THC caused apoptosis in naive lymphocytes. Thus, exposure to THC may directly trigger activation of genes involved in apoptosis. Additional studies are clearly needed to fully elucidate the series of events leading to increased apoptosis both in vitro and in vivo.

Marijuana is currently used as a therapeutic agent alleviating such complications as intraocular pressure in glaucoma, and cachexia, nausea, and pain in AIDS and cancer patients. Many of these effects have been attributed to the psychoactive effects of THC (Watson et al., 2000). In addition, the potential use of marijuana and its derivatives in the treatment of autoimmune disorders, cancer, and neurodegenerative diseases has received a great deal of attention in

recent years (Watson et al., 2000). For example, as an agent in treating cancer, cannabinoids have shown antiproliferative effects on transformed neural (Sanchez et al., 1998), breast (De Petrocellis et al., 1998), and prostate cells (Ruiz et al., 1999), and in vivo treatment of glioma in a rat model more recently resulted in a significant increase in survival (Galve-Roperh et al., 2000). Studies also suggest that cannabinoids may constitute a powerful tool in treating autoimmune disorders. For example, cannabidiol has been shown to alleviate collagen-induced arthritis (Malfait et al., 2000). Thus, the ability of THC to induce apoptosis in activated T lymphocytes may offer new avenues to treat autoimmune diseases and allograft rejection. If agonists specific for CB2 can be shown to induce apoptosis, they may constitute an excellent tool to deplete activated T cells in disease states. The CB2 agonists have the advantage in that they lack psychosomatic effects.

There is evidence to suggest that the doses of THC used in the current study are pharmacologically relevant. In an earlier study, Chan et al. (1996) showed that rats injected with 50 mg/kg body weight of THC had a serum concentration of 10 μM THC within 10 h of administration. Also in these studies, mice were given as much as 500 mg/kg five times a week for 2 years. Interestingly, despite such high levels, the survival of those mice was higher than controls, suggesting that such doses are well tolerated. In addition, THC administered to mice at 150 mg/kg for 11 days suppressed streptozotocin-induced autoimmune diabetes in CD1 mice (Li et al., 2001). Moreover, based on our studies, cannabinoids offer new treatment modalities to treat autoimmune diseases and cancer. In fact, we have demonstrated recently that malignancies of the immune system are highly susceptible to apoptosis induced by THC and CB2 agonists (McKallip et al., 2002). Also in these studies, we were able to successfully cure a significant proportion of mice bearing lymphomas. Thus, the use of higher concentrations of THC may be pharmacologically relevant in the treatment of a wide number of diseases involving the immune system. The experiments in which we demonstrated that CB2 antagonist can block apoptosis induced by 10 μM THC in vitro suggest that induction of apoptosis is receptor mediated and may be independent of lipophilic properties of THC. In addition to the possible use of THC and other CB2 agonist for therapeutic purposes, there is evidence to suggest that the doses and concentrations used in this study may be obtainable during recreational use. Azorlosa et al. (1992) showed that levels as high as 1 μM could be obtained in the plasma of humans, and in a separate report, it was shown that THC can be concentrated 15- to 20-fold in some tissues (Johansson et al., 1989). Therefore, it may be possible to reach levels as high as 20 μM in some tissues after recreational use. Such levels of THC may lead to significant suppression of the immune response leading to increased susceptibility to opportunistic infections and cancer. In fact, it was shown that treatment of mice with doses of 8 mg/kg significantly suppressed the responses of mice to infection with *Legionella pneumophila* (Klein et al., 2000b). Therefore, additional studies are necessary to examine the effects of a chronic low dose of THC on the immune system of recreational users and to examine the potential use as a chronic treatment in which long-term immunosuppression is desirable such as after allogeneic organ transplantation. However, determining how quickly and to what degree a single dose of THC altered the immune response may have significant clinical relevance in treating such complications as shock induced by bacterial enterotoxins or in the treatment of immediate hypersensitivity reactions. These types of immune responses occur rapidly and treatment of such clinical disorders would require rapid down-regulation of the immune response.

In summary, the current study clearly demonstrates that exposure to THC leads to suppression of the immune response characterized by reduction in the response to polyclonal mitogens, reduced cellularity in the thymus and spleen, and increased induction of apoptosis. The demonstration that the THC-induced suppression of the immune response is directly related to the induction of apoptosis is an important step in understanding the mechanism of toxicity induced by THC and its potential medicinal use.

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