The Journal of Immunology

RESEARCH ARTICLE | AUGUST 15 2004

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Yoonkyung Do; ... et. al

J Immunol (2004) 173 (4): 2373–2382.
https://doi.org/10.4049/jimmunol.173.4.2373

Activation through Cannabinoid Receptors 1 and 2 on Dendritic Cells Triggers NF-κB-Dependent Apoptosis: Novel Role for Endogenous and Exogenous Cannabinoids in Immunoregulation[1]

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

The precise role of cannabinoid receptors (CB)1 and CB2, as well as endogenous ligands for these receptors, on immune cells remains unclear. In the current study, we examined the effect of endogenous and exogenous cannabinoids on murine bone marrow-derived dendritic cells (DCs). Addition of Δ^9-tetrahydrocannabinol (THC), a major psychoactive component found in marijuana or anandamide, an endogenous cannabinoid, to DC cultures induced apoptosis in DCs. DCs expressed CB1 and CB2 receptors and the engagement of both receptors was necessary to trigger apoptosis. Treatment with THC induced caspase-2, -8, and -9 activation, cleavage of Bid, decreased mitochondrial membrane potential, and cytochrome *c* release, suggesting involvement of death-receptor and mitochondrial pathways. DCs from Bid-knockout mice were sensitive to THC-induced apoptosis thereby suggesting that Bid was dispensable. There was no induction of p44/p42 MAPK, p38 MAPK, or stress-activated protein/JNK pathway in THC-treated DCs. However, THC treatment induced phosphorylation of IκB-α, and enhanced the transcription of several apoptotic genes regulated by NF-κB. Moreover, inhibition of NF-κB was able to block THC-induced apoptosis in DCs. Lastly, in vivo treatment of mice with THC caused depletion of splenic DCs. Together, our study demonstrates for the first time that endogenous and exogenous cannabinoids may suppress the immune response through their ability to induce apoptosis in DCs. ***The Journal of Immunology,*** **2004, 173: 2373–2382.**

Cannabinoids are compounds derived from the *Cannabis sativa* (marijuana) plant, as well as produced endogenously in the brain and by immune cells (1). Cannabinoids mediate their effect through cannabinoid receptors (CB),[3] designated CB1 and CB2, which belong to a superfamily of G-protein-coupled receptors (1). CB1 receptors are expressed at high levels in CNS, where they regulate psychoactivity. CB1 receptors are also expressed on immune cells. In contrast, the CB2 receptors are primarily expressed on immune cells and do not contribute to the psychoactivity (1). The presence of endogenous CB-ligand systems in immune cells suggests that they may play a critical physiological role, the precise nature of which remains to be characterized.

Cannabinoids can decrease the immune response and impair the host defense against viral, bacterial, or protozoan infections as discussed in several reviews (2–4). Cannabinoids have also been widely used in the treatment of pain and inflammation (5). Moreover, preliminary studies have shown the possible use of cannabinoids in the treatment of autoimmune diseases such as multiple sclerosis (6). Although the precise mechanism of cannabinoid-induced immune dysfunction is not clear, studies have indicated that cannabinoids may cause dysregulation in cytokine production by immune cells (7). Recent studies from our lab demonstrated that Δ^9-tetrahydrocannabinol (THC) can trigger apoptosis in vivo in thymocytes and splenocytes, which may account for immunosuppression (8).

DCs are the most potent APCs that play a crucial role in the presentation of self and non-self Ags to T cells (9). We demonstrate for the first time that THC and endocannabinoids such as anandamide can induce apoptosis in DCs through activation of CB1 and CB2 receptors. These studies provide the basis for understanding the mechanism by which THC triggers immunosuppression and mediates anti-inflammatory properties. Moreover, the expression of CB1 and CB2 receptors on DCs and their modulation during DC activation suggests that the endocannabinoid systems may play a critical role in the regulation DC growth and maturation.

Materials and Methods

Mice

Adult (6–8 wk of age) female C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Bid-knockout (KO) mice were generated as described elsewhere (10).

Reagents

THC and anandamide were obtained from the National Institute on Drug Abuse (Rockville, MD) and dissolved in DMSO (Sigma-Aldrich, St. Louis, MO). CB1 antagonist, SR141716A, and CB2 antagonist, SR144528, were obtained from Sanofi-Synthélabo (Montpellier, France) and were used as described (8, 11).

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

Received for publication December 16, 2003. Accepted for publication May 24, 2004.

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[1] This work was supported in part by National Institutes of Health Grants R01 ES09098, R01 DA016545, R01 AI053703, R01 HL058641, R01 AI058300, and R21 DA014885.

[2] Address correspondence and reprint requests to Dr. Prakash Nagarkatti, Department of Pharmacology and Toxicology, Medical College of Virginia Campus, Virginia Commonwealth University, Richmond, VA 23298. E-mail address: pnagark@hsc.vcu.edu

[3] Abbreviations used in this paper: CB, cannabinoid receptor; THC, Δ^9-tetrahydrocannabinol; KO, knockout; DC, dendritic cell; PTX, pertussis toxin; PI, propidium iodide; $\Delta\psi_M$, mitochondrial membrane potential; SAPK, stress-activated protein kinase; TPCK, *N-p*-tosyl-L-phenylalanine chloromethyl ketone; FAAH, fatty acid amide hydrolase; DR, death receptor; $DiOC_6$, 3,3[prime]-diehexiloxadicarbocyanine iodide.

0022-1767/04/$02.00

Dendritic cell (DC) culture

DCs were cultured from mouse bone marrow cells with murine recombinant GM-CSF (5 ng/ml; BD Pharmingen, San Diego, CA) for 6 days (12). In brief, bone marrow was obtained from the femurs and tibias, depleted of RBCs, and placed on petri dishes with RPMI 1640 supplemented with 10% FCS, 10 mM HEPES, 1 mM glutamine, and 50 μg/ml gentamicin. The $CD4^+CD8^+$ T cells, as well as $B220^+$ B cells, were depleted by using specific mAbs and complement. The cells (1×10^6 per well) were placed in 24-well culture plates supplemented with GM-CSF. The cultures were fed every 2 days to remove nonadherent monocytes by aspirating 75% of the medium and adding fresh medium with GM-CSF. The DCs activated with LPS (10 μg/ml; Sigma-Aldrich) were used as mature DCs (13). Phenotypic characterization of DCs revealed that they expressed CD40, CD80, CD86, and MHC class II molecules and exhibited the characteristic dendrites.

Detection of cell viability and apoptosis in DC cultures

DCs were placed in 24-well plates in serum-free RPMI 1640, devoid of any supplementation with serum or serum proteins, and in the presence or absence of various concentrations of THC for 2 h. The cells were harvested, washed twice with PBS, and analyzed for the induction of apoptosis in three ways: 1) Annexin V/propidium iodide (PI) method, 2) TUNEL assay using FITC-dUTP, and 3) 3,3′-diehexiloxadicarbocyanine iodide ($DiOC_6$) to detect altered mitochondrial membrane potential ($\Delta\psi_M$). The details of these assays have been previously described (11, 14). In addition, cell viability was also determined in DCs cultured with THC for ~3 h, using trypan blue dye exclusion.

To detect anandamide-induced apoptosis, DCs were cultured as described above. Various concentrations of anandamide were added to the cells with or without various concentrations of methyl arachidonyl fluorophosphonate (Sigma-Aldrich) as fatty acid amide hydrolase (FAAH) inhibitor and incubated for ~4 h. The cells were harvested, washed twice with PBS, and analyzed for the induction of apoptosis using a TUNEL assay. In some experiments, various concentrations of CB1 or CB2 antagonists were added to the DCs (see below).

Effect of CB1 or CB2 receptor antagonists on survival of DC in the presence of THC

Cells were preincubated with various concentrations of CB1 (SR141716A) or CB2 (SR144528) antagonists for 30 min at 37°C and then the cells were treated with either DMSO or 5 μM THC for 2 h (11). The cell viability was determined using trypan blue dye exclusion and the induction of apoptosis was measured using annexin V/PI or TUNEL as described above.

Role of G-protein-coupled receptor in THC-induced apoptosis

DCs were preincubated with various concentrations of pertussis toxin (PTX, 50–100 ng/ml; List Biological Laboratories, Campbell, CA) for overnight at 37°C. On the next day, the cells were thoroughly washed with 10% RPMI 1640 then treated with either DMSO or 5 μM THC for 2 h and analyzed for apoptosis.

Detection of CB1 and CB2 receptor expression by semiquantitative RT-PCR

To detect CB1 and CB2 receptor gene expression, RT-PCR was conducted as described earlier (11).

cDNA microarray

Total RNA was prepared as described above and cDNA microarray analysis was conducted using a panel of 96 key genes involved in apoptosis as described by the manufacturer (Super Array, Bethesda, MD). The level of expression or induction of a gene in control and THC- or *N-p*-tosyl-L-phenylalanine chloromethyl ketone (TPCK)-treated cells was normalized and the changes in gene expression following different treatments were presented as a fold increase or decrease when compared with expression profiles seen in the control group.

Western blot analysis

Cells were lysed by repeated freeze/thawing,and protein concentration in the supernatant was determined using the bicinchonic acid (BCA) protein assay kit (Pierce, Rockford, IL). Total protein (15–40 μg) was separated by SDS-PAGE and transferred to a membrane. Blots were blocked with 5% (w/v) nonfat dry milk in TBS-T (0.5 M NaCl, 20 mM Tris-HCl, 0.1% (v/v) Tween 20, pH 7.6) and probed with the primary Ab. Next, blots were washed and incubated with HRP-conjugated secondary Ab and visualized with an ECL reagent (Amersham Biosciences, Piscataway, NJ).

Caspase inhibition

Cells were preincubated with various concentrations of caspase-8 (Z-IETD-FMK), caspase-9 (Z-LEHD-FMK), or pan-caspase inhibitors (Z-VAD-FMK; R&D Systems, Minneapolis, MN) for 30 min at 37°C. The cells were then treated with either DMSO or 5 μM THC for 2 h and analyzed for apoptosis.

p44/p42 MAPK, p38 MAPK, or stress-activated protein kinase (SAPK)/JNK inhibition

Cells were preincubated with various concentrations of PD98059, SB203580 (Calbiochem, San Diego, CA), or wortmanin (Sigma-Aldrich) for 30 min at 37°C and then the cells were treated with either DMSO or 5 μM THC for 2 h and analyzed for apoptosis.

NF-κB inhibition

Cells were preincubated with various concentrations of TPCK (Sigma-Aldrich) for 30 min at 37°C and then the cells were treated with either DMSO or 5 μM THC for 2 h and analyzed for apoptosis.

In vivo injection of THC-C57BL/6 mice were injected with a single dose of THC (1, 10, 25, or 50 mg/kg body weight, i.p.) or the vehicle. The spleens were harvested 24 h after THC injection and a single-cell suspension was prepared, as described elsewhere (8, 14). Next, Fc receptors were blocked using the Fc-blocking reagent (BD Pharmingen), the cells were double-stained with FITC-conjugated anti-CD11c and PE-conjugated anti-MHC class II mAbs (BD Pharmingen) and analyzed using a flow cytometer.

Statistical analysis

The statistical comparisons between different treatment groups were conducted using a Student's t test and differences with $p < 0.05$ were considered to be significant. All experiments were repeated at least three times.

Results

THC induces apoptosis in DCs

To study the effect of THC on DCs, various concentrations of THC such as 1, 5, 10, and 20 μM or the vehicle (DMSO) were added to bone marrow-derived DCs in serum-free medium. THC treatment changed the morphology of DCs dramatically as shown in Fig 1*A*, where most of them lost their typical dendrites and some of them showed blebbed surface. As shown in Fig. 1*B*, 5 μM and greater concentrations of THC caused a dramatic decrease in the viability of DCs within 3 h of incubation. Next, we used a combination of annexin V and PI which detects apoptotic ($annexin^+$ only), late apoptotic/necrotic ($annexin^+PI^+$) and necrotic (PI^+ only) cells (8). DCs treated for 2 h with 5 μM or greater concentrations of THC showed a significant increase in early apoptotic and late apoptotic/necrotic cells (Fig. 1*C*). To further confirm THC-induced apoptosis, we used $DiOC_6$ to detect $\Delta\psi_M$ loss (15). The data shown in Fig. 1*D* demonstrated that THC induced a loss in $\Delta\psi_M$ at all concentrations tested when compared with the vehicle controls. Taken together, these data suggested that THC treatment reduced cell number by inducing apoptosis in murine bone marrow-derived DCs. It should be noted that THC was also able to induce apoptosis in DC in the presence of serum (data not shown). However, we used serum-free medium to prevent the interference from endocannabinoids present in the serum and the possible interactions between THC and serum proteins (16). When we tested DCs at 20 μM of THC in RPMI 1640 supplemented with 5% FBS, we observed >30% of apoptosis after 3 h and >60% apoptosis after 18 h, while the vehicle-treated cells showed <5% and <10% apoptosis respectively (data not shown).

The effect of CB1 and CB2 receptor antagonists on DC survival/apoptosis

THC binds to both CB1 and CB2 with similar affinities. To further address the involvement of CB1 and CB2 receptors in THC-induced apoptosis of DCs, we tested the ability of well characterized

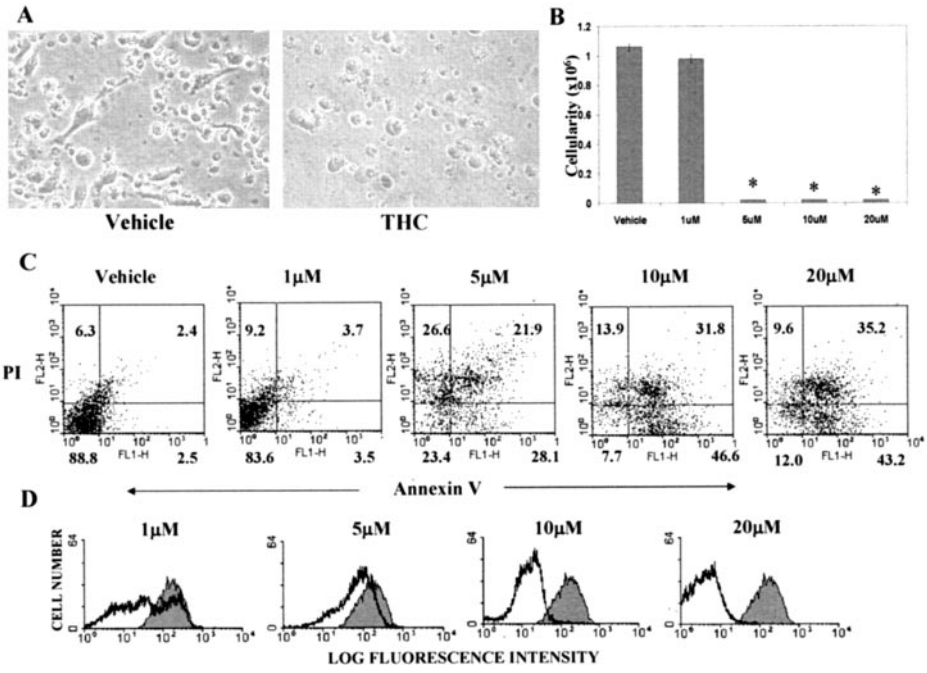

FIGURE 1. THC triggers apoptosis in bone marrow-derived DCs. *A*, Morphology of bone marrow-derived DC cultured with THC (5 μM) or vehicle (DMSO) for 2 h and observed under a phase contrast microscope. *B*, Viable cellularity of DCs following THC treatment. The vertical bars represent the total number of viable cells recovered from triplicate cultures (mean $\pm$ SEM). Asterisk denotes statistically significant difference ($p < 0.05$) between vehicle and treatment. *C*, The induction of apoptosis in DCs was analyzed using annexin/PI double-staining. *D*, The loss of $\Delta\psi_M$ of DCs was detected by $DiOC_6$ staining. The filled histograms represent cells incubated with vehicle and the empty histograms represent cells incubated with various concentrations of THC.

CB1 and CB2 receptor antagonists to block THC-induced apoptosis. Addition of various concentrations of CB1 (SR141716A) or CB2 (SR144528) or SR141716A + SR144528 receptor antagonists to DC cultures caused minimal or no significant apoptosis/loss of cell viability (Fig. 2). Addition of 5 μM THC alone to DC cultures caused 53.8% apoptosis (Fig. 2) and furthermore, addition of either SR141716A or SR144528 to DC cultures treated with THC caused a dose-dependent inhibition in THC-induced apoptosis. Moreover, the addition of a combination of SR141716A + SR144528 was more effective in blocking THC-induced apoptosis than either of these compounds independently. The fact that either SR141716A or SR144528 alone, when tested at higher concentrations, could completely reverse the apoptosis induced by THC, suggested that simultaneous ligation of both CB1 and CB2 receptors may be necessary to induce apoptosis.

Role of G proteins in THC-induced apoptosis in DCs

Because CBs are coupled to PTX-sensitive $G_{i/o}$ proteins, we tested the effect of PTX on induction of apoptosis by THC. To this end, DCs were preincubated with or without 50 ng/ml PTX overnight and then incubated with 5 μM THC for 2 h and the cells were analyzed for apoptosis using annexin/PI staining. The data shown in Fig. 3 demonstrated that PTX was able to partially inhibit the induction of apoptosis by THC.

Effect of an endogenous cannabinoid on DCs and the role of CB1 or CB2 receptors

To investigate whether endogenous cannabinoids such as anandamide could induce apoptosis in DCs, various concentrations of anandamide were added to DCs. It was observed that concentrations of 20 μM caused marked apoptosis while lower concentrations were not effective (Fig. 4). The lesser efficacy of anandamide when compared with THC might be due to rapid hydrolysis of anandamide by endogenous enzymes such as FAAH (17). To prevent the activity of FAAH, various concentrations of a FAAH inhibitor were added to the culture in the presence or in the absence of anandamide and then the induction of apoptosis was determined (Fig. 4*A*). The data showed that the addition of a FAAH inhibitor could increase the induction of apoptosis by anandamide in DCs significantly. Furthermore, anandamide-induced apoptosis was also inhibited by selective antagonists of CB1 (SR141716A) or CB2 (SR144528), suggesting that anandamide was acting through these receptors to induce apoptosis (Fig. 4*B*).

Mature DCs are more resistant to THC-induced apoptosis when compared with immature DCs

DCs show distinct phenotypic as well as functional differences depending on their activation/maturation status. In this study, we investigated whether the maturation status of DCs could have a differential effect on THC-induced apoptosis. To this end, the DCs obtained from the bone marrow were either untreated or treated with LPS and cultured with 1, 5, or 10 μM of THC. After 2 h, the cells were analyzed by TUNEL assay to detect apoptosis. The data shown in Fig. 5 suggested that DCs that were not treated with LPS representing immature DC, showed apoptosis at 5 and 10 μM but not at 1 μM of THC. These data corroborated the earlier findings (Fig. 1) that THC can induce apoptosis in immature DCs at 5 μM or greater concentrations, although the sensitivity of the four assays used varied somewhat. Interestingly, DCs activated with LPS were found to be more resistant to apoptosis as indicated by their inability to undergo apoptosis at 5 μM THC and showed sensitivity only at 10 μM concentrations (Fig. 5, *A* and *B*). RT-PCR analysis of CBs revealed that murine bone marrow-derived DCs expressed both CB1 and CB2 receptors mRNA (Fig. 5*C*). When DCs were activated with LPS, they were shown to modestly downregulate the expression of CB1 and to a lesser extent, CB2 receptors.

Mechanism by which THC triggers apoptosis in DCs

We next determined whether THC-induced apoptosis was mediated by caspases. Western blot analysis of DC lysates following THC treatment showed that THC treatment induced cleavage of caspase-8, -9, and -2 within 15 min of exposure (Fig. 6*A*). Furthermore, cleavage of Bid was seen at 30 min. Also, cytochrome *c* was detected at all time points tested. These data suggested that THC triggers apoptosis in DC using both death receptor (DR) and mitochondrial pathways and possibly involving cross-talk. When

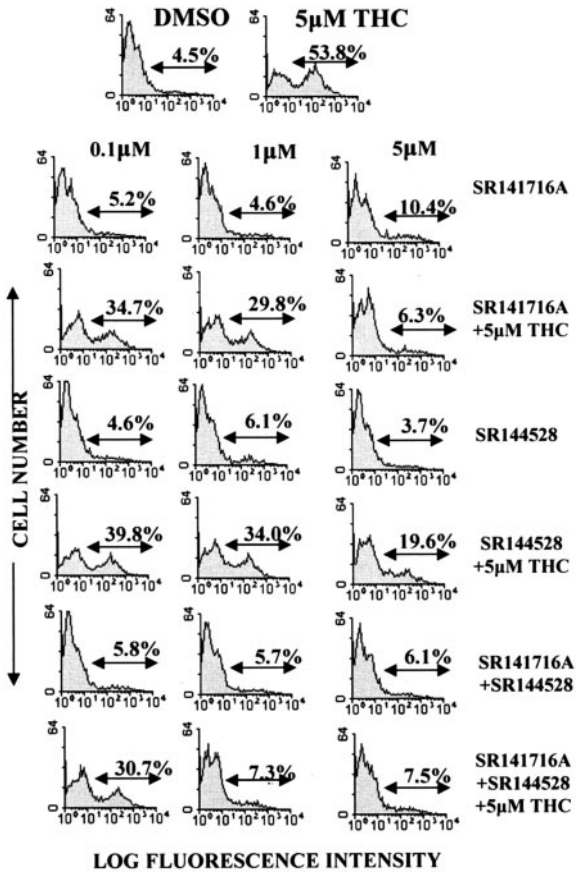

FIGURE 2. Use of CB1 (SR141716A) and CB2 (SR144528) antagonists to study the role of CBs in THC-induced apoptosis. Bone marrow-derived DCs were incubated with various concentrations of antagonists against CB1 or CB2 receptors alone or in the presence of 5 μM THC as described in Fig. 1. The figure depicts percentage of annexin$^+$ DCs.

caspase inhibitors were used to test their ability to inhibit THC-induced apoptosis, addition of pan-caspase inhibitor blocked the apoptosis completely in a dose-dependent fashion (Fig. 6*B*). Moreover, addition of caspase-8 or -9 inhibitors caused partial inhibition of apoptosis in DCs. These data confirmed Western blotting data, suggesting the involvement of both intrinsic and extrinsic pathways in DCs following THC treatment.

To further address the cross-talk between these two pathways of apoptosis, we tested whether the cleavage of Bid seen in Fig. 6*A* would be essential for THC-induced apoptosis in DCs. To this end, we generated bone marrow-derived DCs from Bid-KO or wild-type mice and treated them with 5 μM THC or the vehicle (DMSO) and induction of apoptosis was measured. The data showed that DCs with Bid deficiency underwent apoptosis to the same extent as the wild-type DCs, suggesting that Bid cleavage is not a critical step for induction of apoptosis in DCs (Fig 6*C*).

Involvement of p44/p42 MAPK, p38 MAPK, or SAPK/JNK pathways in DCs following THC treatment

To further study the down-stream pathways evoked in DCs following THC treatment, Western blot analysis for MAPK or JNK pathway was conducted. As shown in Fig. 7, there was no significant change in phosphorylated or nonphosphorylated form of these pathways, suggesting that these pathways were not activated in DCs following THC treatment. Such findings were further confirmed by the data that THC-induced apoptosis could not be

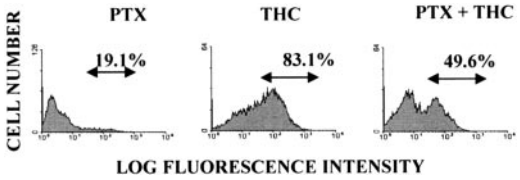

FIGURE 3. Effect of PTX on THC-induced apoptosis on DCs. Bone marrow-derived DCs were treated with PTX (50 ng/ml) overnight and next day, the cells were untreated or treated with THC (5 μM) for 2 h at 37°C. Cells treated with THC alone were also included. Annexin$^+$ cells were depicted as described in Fig. 1.

blocked by the addition of pathway-specific inhibitors including PD98059, SB203580, and wortmanin (data not shown).

Involvement of NF-κB pathway in DCs following THC treatment

Activation of NF-κB is considered to play an important role in cell death and apoptosis. It is also known that NF-κB is critical in DC maturation. To study the effect on NF-κB in THC-induced apoptosis in DCs, first, Western blot analysis was performed (Fig. 8*A*). With increasing time, there was a decrease of nonphosphorylated form as well as an increase of the phosphorylated form of IκB-α, which indicated the involvement of NF-κB activation following THC treatment in DCs. In addition, when the NF-κB inhibitor, TPCK, was used in the presence of THC or vehicle treatment, 5 μM and greater concentrations of TPCK almost completely inhibited THC-induced apoptosis in DCs (Fig. 8*B*). Next, we incubated DCs with THC in the presence or in the absence of TPCK, and then conducted Western blotting for caspase-3 (Fig. 9). The data showed that the cleaved form of caspase-3 was not detected in the protein extract obtained from DCs treated with THC plus TPCK at 15 min and furthermore, at 45 min, the cleaved caspase-3 was significantly less than that seen in DCs treated with THC alone. These data first, confirmed our result showing that NF-κB inhibition rescued DCs from THC-induced apoptosis, and second, they also suggested that NF-κB activation may be up-stream of caspase activation.

THC treatment up-regulates apoptotic genes regulated by NF-κB

To investigate the mechanism by which THC-induced NF-κB activation triggers apoptosis in DCs, cDNA microarray analysis was conducted. The data showed that several apoptotic genes were significantly up-regulated in THC-treated DCs when compared with control cells and furthermore, most of the up-regulated genes showed putative NF-κB binding site in their promoter, suggesting that NF-κB may act as proapoptotic molecule in THC-induced apoptosis in DCs (Table I). In addition, we observed down-regulation of some of the very same genes following TPCK treatment thereby indicating that such enhanced gene expression was indeed NF-κB-dependent (Table II).

In vivo effect of THC on DCs

To test whether THC would deplete DCs in vivo, mice were injected with various concentrations of THC or vehicle and 24 h later, splenic DC population was enumerated by using double-stain for CD11c and MHC-II. THC treatment led to a decrease in the total splenic cellularity at doses of 10, 25, and 50, but not at 1 mg/kg (data not shown), similar to our previous observation (8). Furthermore, THC at all concentrations tested caused a significant decrease in both the percentage and the total numbers of class II MHC-expressing DCs found in the spleen (Fig. 10).

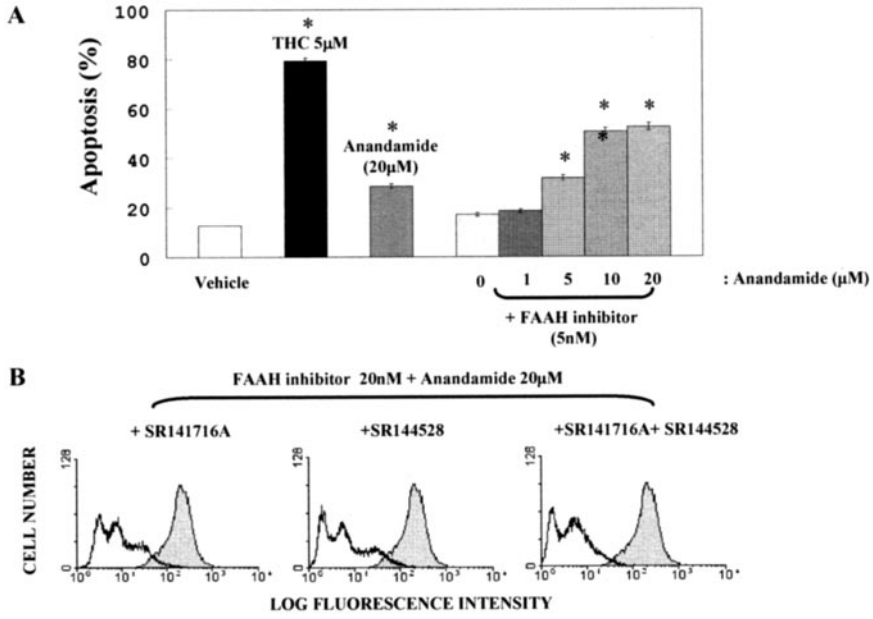

FIGURE 4. Effect of an endogenous cannabinoid on DCs and the role of CB1 and CB2. Bone marrow-derived DCs were incubated with anandamide in the presence or in the absence of a FAAH inhibitor for 4 h. The induction of apoptosis was measured by TUNEL. The vertical bars represent mean ± SEM from three experiments. Asterisk denotes statistically significant difference ($p < 0.05$) between vehicle and treatment groups. *B*, CB1 (SR141716A) and CB2 (SR144528) antagonists (1 μM) were added to the culture containing DCs incubated with anandamide (20 μM) and a FAAH inhibitor as described in *A*. The cells were analyzed by TUNEL. The filled histograms represent cells incubated with anandamide and a FAAH inhibitor. The empty histograms represent cells treated with anandamide plus a FAAH inhibitor and were additionally incubated with CB1 and/or CB2 antagonists.

Discussion

Recently, we demonstrated that both normal and malignant immune cells that express CBs undergo apoptosis when activated through these receptors (8, 11). Based on these studies, we suggested that the THC-mediated immunosuppression in vivo may result from its ability to induce apoptosis in immune cells, particularly in thymocytes and peripheral T and B cells (8). Moreover, inasmuch as transformed immune cells also expressed CBs and were susceptible to apoptosis induced by THC and other cannabinoids, we suggested that CB agonists may serve as novel anticancer agents (11). Furthermore, in the above studies, we had not addressed the mechanism(s) of apoptosis. Therefore, the overall objective of this investigation was to delineate the signaling pathways of apoptosis induced by THC on an important component of the immune system, the DCs. In the current study, we demonstrated that THC-mediated apoptosis in DCs occurs, at least in part, through G-protein signaling. Also, THC mediates apoptosis in DCs through activation of several caspases including caspase-2, -8, and -9 and the release of cytochrome *c*. These data suggested the involvement of both extrinsic and intrinsic apoptotic pathways. The sensitivity of DCs from Bid-KO mice to THC-induced apoptosis suggested that Bid did not play a critical role and that cross-talk between these pathways was not necessary to induce apoptosis. There was no involvement of MAPK or SAPK/JNK pathways in apoptosis induction; however, we noted that treatment of DCs with THC led to NF-κB activation. cDNA microarray analysis showed the induction of several apoptotic genes regulated by NF-κB in DCs following THC treatment. Moreover, preventing NF-κB activation effectively blocked the induction of apoptosis thereby suggesting that NF-κB played a critical role in this process. Treatment of mice with THC in vivo could also decrease DC population in the spleen. Although in our earlier study (8) we had

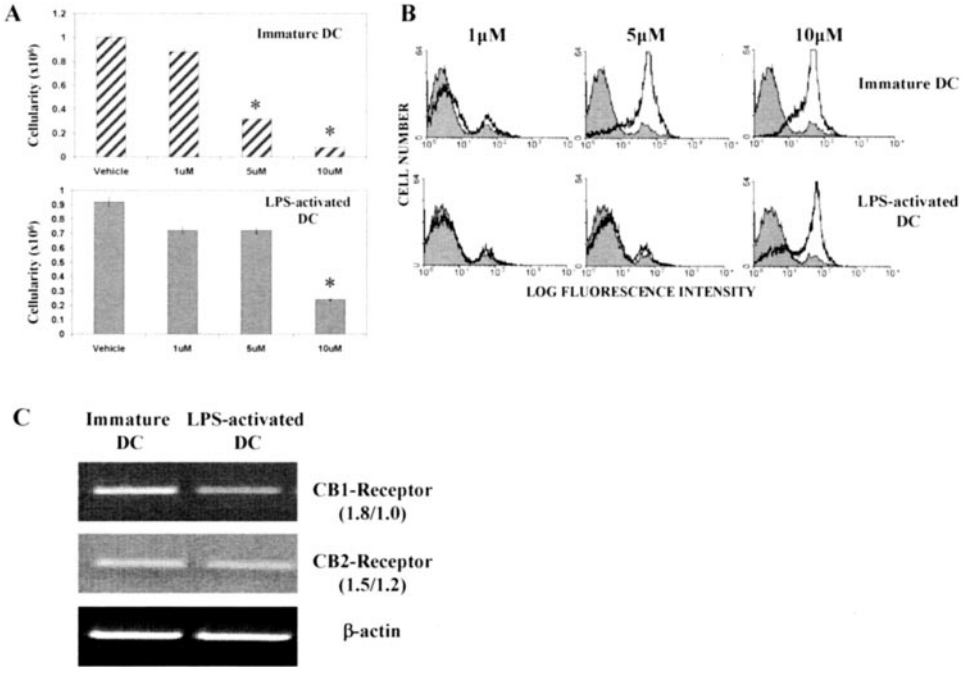

FIGURE 5. Effect of THC on immature and mature DCs. *A*, LPS-activated DCs or immature DCs (not activated) were incubated with THC or vehicle as described in Fig. 1, and the viable cell numbers were calculated by trypan blue dye exclusion. The vertical bars represent the total number of viable cells recovered from triplicate cultures (mean ± SEM). Asterisk denotes statistically significant difference ($p < 0.05$) between vehicle and treatment groups. *B*, Similar cells were analyzed for apoptosis using TUNEL. The filled histograms represent cells incubated with vehicle and the empty histograms represent cells incubated with THC. *C*, Total RNA was extracted from LPS-activated DC and immature DC and the cDNA was amplified with primers specific for CB1 and CB2 receptors, and β-actin as internal control. The numbers in parenthesis represent a relative intensity of the bands obtained from densitometer for immature vs LPS-activated cells and were normalized to β-actin.

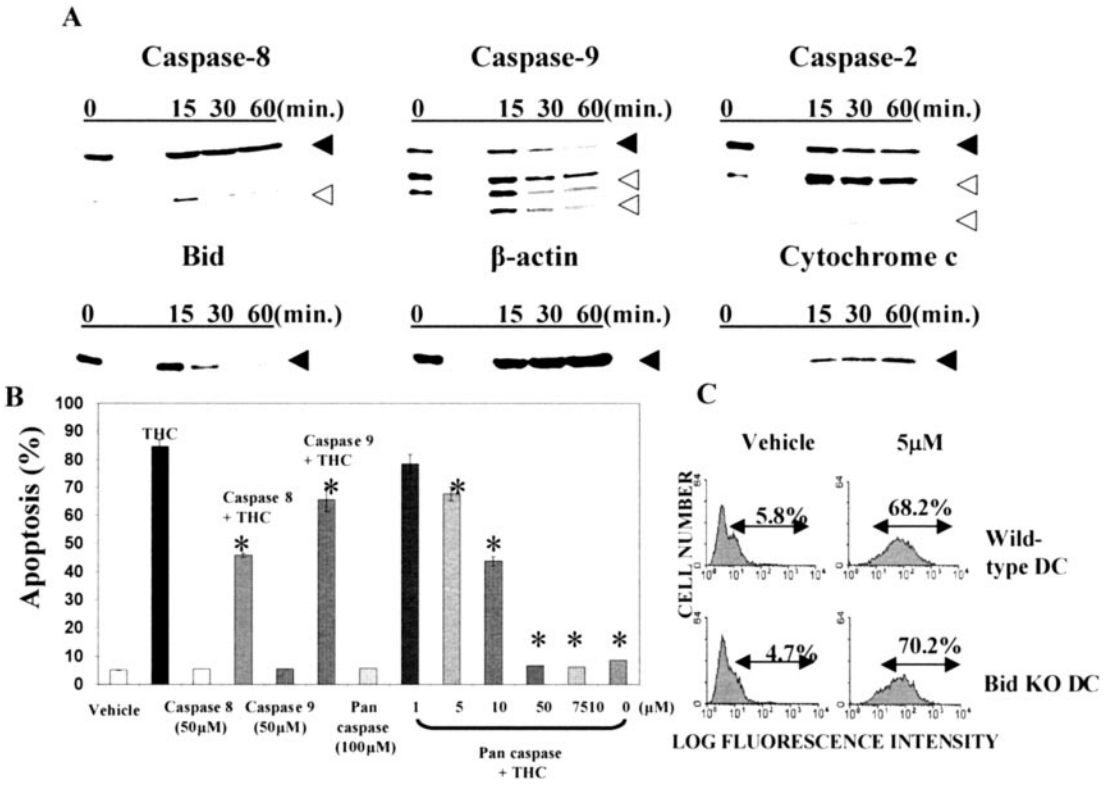

FIGURE 6. Apoptotic signaling pathways in DCs following THC treatment. *A*, Bone marrow-derived DCs were incubated with THC (5 μM) and total protein was extracted at each indicated time point. Fifteen (caspase-8, -9, -2, Bid, and β-actin) or 40 μg (cytochrome *c*) of lysate was used for Western blot analysis. Filled arrowheads indicate full length of the protein and open arrowheads indicate cleaved/activated form of the proteins. *B*, Various caspase inhibitors were added to DCs in the presence or in the absence of THC (5 μM) and the induction of apoptosis was measured by TUNEL. The vertical bars represent mean $\pm$ SEM from three experiments. Asterisk denotes statistically significant difference ($p < 0.05$) between THC alone and THC plus caspase inhibitor treatment. *C*, Bone marrow-derived DCs from wild-type mice and Bid-KO mice were incubated with 5 μM THC or vehicle as described in Fig. 1. The induction of apoptosis in DCs was analyzed using annexin/PI staining and only annexin$^+$ cell population was depicted.

not investigated the effect of anandamide on immune cells, in the current study, we found that this endocannabinoid could also induce apoptosis in DCs. Taken together, our data demonstrate for the first time that endogenous and exogenous cannabinoids can induce apoptosis in DCs.

The physiological functions of CBs expressed on immune cells and the role played by endocannabinoids produced by leukocytes in the regulation of immune response remain unclear. A recent study reported the presence of endocannabinoids, anandamide, and 2-arachidonoylglycerol, as well as CB1 and CB2 receptors, on human DCs (18). The general consensus is that endocannabinoids act as antiproliferative agents thereby down-regulating the immune response (19). In the current study, use of CB1 and CB2 antagonists showed that either antagonist when added alone was able to significantly inhibit apoptosis in DCs. These data not only suggested the involvement of both CB1 and CB2 receptors in apoptosis but also indicated that simultaneous activation through both receptors may be necessary to induce apoptosis. It should be noted that THC-induced apoptosis in tumor cells is mediated through CB-dependent (20) or -independent pathways (21).

Apoptosis is triggered primarily through two pathways: the extrinsic pathway (DR pathway) involving caspase-8 and -10, and the intrinsic pathway (mitochondrial pathway) involving caspase-2 and -9 (22). Moreover, in type II cells, there is a cross talk between these two pathways involving Bid (23). The mechanism by which THC triggers apoptosis may involve multiple pathways based on the nature of cells. THC and other cannabinoids were shown to induce apoptosis of glioma cells via ceramide generation (24). THC also promoted the mitochondrial pathway of apoptosis in cultured cortical neurons as evidenced by cytochrome *c* release

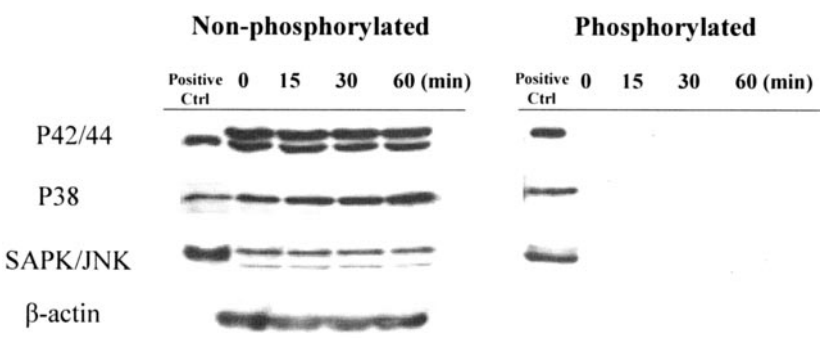

FIGURE 7. MAPK (p44/p42), p38 MAPK, or SAPK/JNK pathways in DCs following THC treatment. Bone marrow-derived DCs were incubated with THC (5 μM) and total protein was extracted at each indicated time point. Fifteen micrograms of lysate was used for Western blot analysis.

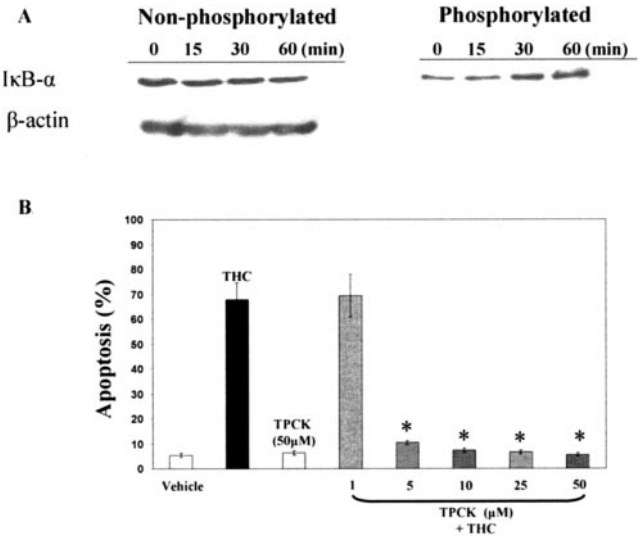

FIGURE 8. NF-κB pathway in DCs following THC treatment. *A*, Bone marrow-derived DCs were incubated with THC (5 μM) and total protein was extracted at each indicated time point. Fifteen micrograms of lysate was used for Western blot analysis. *B*, Various concentrations of the NF-κB inhibitor, TPCK, were added to the DCs in the presence or in the absence of THC, and the induction of apoptosis was measured by TUNEL. The vertical bars represent mean ± SEM from three experiments. Asterisk denotes statistically significant difference ($p < 0.05$) between THC alone and THC plus TPCK treatment.

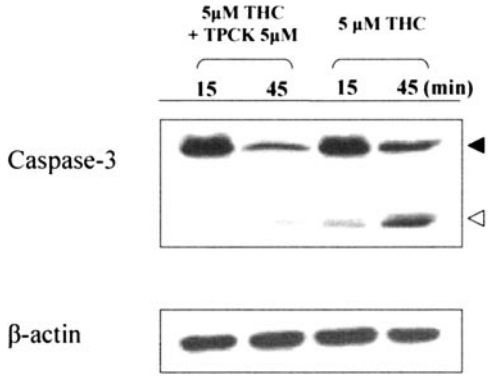

FIGURE 9. The relationship between NF-κB and caspase activation in DCs. Bone marrow-derived DCs were incubated with THC (5 μM) in the presence or in the absence of TPCK (5 μM) and total protein was extracted at each indicated time point for Western blotting. Open arrowheads indicate cleaved/activated form of the proteins.

and activation of caspase-3 (25). Others have suggested that THC caused sphingomyelin hydrolysis in C6.9 glioma cells resulting in apoptosis (26). In addition, THC treatment of splenocytes was shown to cause a decrease in Bcl-2 mRNA and protein expression (27). In the current study, we demonstrated that THC-induced apoptosis in DCs involved both the DR and mitochondrial pathways. The cleavage of Bid suggested the possible cross-talk between the two pathways. However, the susceptibility of DCs from Bid-KO mice to THC-induced apoptosis suggested that Bid was dispensable in THC-induced apoptosis.

The CB1 and CB2 are G-protein-coupled receptors that are linked to a variety of signaling pathways including the cAMP pathway (28) and MAPK pathway (29, 30) in a PTX-sensitive manner. In addition, it was shown that THC-induced apoptosis in malignant glioma induce sustained ceramide accumulation and Raf/extracellular signal regulated kinase activation (21). However, in the current study, our data showed that THC-induced apoptosis in DC did not induce MAPK nor JNK pathways by Western blot analysis. Moreover, specific inhibitors for each pathway could not block apoptosis in DCs after THC treatment. Also, addition of PTX only partially inhibited THC-induced apoptosis in DCs. Previous studies on apoptosis induced in neurons suggested that G proteins may exert both proapoptotic (31) and anti-apoptotic effects (32). Other studies have suggested that PTX-sensitive G proteins may act as facilitators of THC-induced apoptosis in neurons, with potential effectors being members of the Bcl family of mitochondria-associated proteins (25). Although the precise signaling mechanisms involved in G-protein-mediated apoptosis in the cannabinoid system are not clear, our data are consistent with the above studies and demonstrate that PTX-sensitive G proteins may at least play a partial role in THC-induced apoptosis in DC.

NF-κB is a transcription factor that regulates inflammation, immunity, cell death, or survival (33). In general, NF-κB exists as an inactive form in cytoplasm, where it binds to IκB inhibitory proteins. Extracellular signals induce a phosphorylation of IκB, leading to its degradation by proteasome. This results in the rapid translocation of NF-κB to the nucleus where NF-κB can stimulate transcription of various NF-κB-responsive target genes (33). In general, NF-κB functions as an anti-apoptotic transcription factor, but conversely, it also has been proposed as a proapoptotic protein depending on cell type and the nature of apoptotic inducers (34).

Table I. *List of genes that are up-regulated following THC treatment of DCs*

	Gene Name	GenBank	Description	Fold Increase	NF-κB Binding Site[a]
15 min	*Bcl-w*	AF030769	Bcl2-like 2	5.1 ± 0.8	+
	Bid	NM_00754 4	BH3 interacting domain death agonist	7.6 ± 1.2	+
	IAP1	U88908	*Mus musculus* inhibitor of apoptosis protein 1	4.9 ± 0.7	+
	Casper	U97076	*Mus musculus* FLICE-like inhibitory protein	3.9 ± 0.2	+
	Caspase-7	Y13088	*Mus musculus* caspase-7	4.4 ± 0.3	+
	Caspase-8	AF067834	Caspase 8-	5.7 ± 0.6	+
	CRADD	AJ224738	Raidd/Cradd	9.0 ± 2.8	+
	TNF-β	MI6819	Mouse TNF-β mRNA	6.5 ± 1.3	−
	OX40L	UI2763	*Mus musculus* OX40 ligand (ox401) mRNA	2.8 ± 0.2	+
45 min	*DFFA*	NM_01004 4	DNA fragmentation factor, α subunit	2.8 ± 0.4	
	Trail	U37522	TNF-related apoptosis-inducing ligand	8.1 ± 2.5	−
	1BB	LI5435	Receptor 4–1BB ligand mRNA	7.3 ± 2.3	+
	p53	K01700	Transformation* related protein 53, tumor antigene	5.7 ± 1.9	+

[a] Putative NF-κB binding site based on MatInspector (available at www.genomatix.de).

Table II. *List of genes that are down-regulated following TPCK (5 μM) treatment of DCs*

	Gene Name	GenBank	Description	Fold Decrease	NF-κB Binding Site[a]
	Bcl-w	AF030769	Bcl2-like 2	−6.3 ± 1.4	+
	Bid	NM_007544	BH3-interacting domain death agonist	−8.8 ± 2.3	+
	Caspase-8	AF067834	Caspase-8	−2.9 ± 0.2	+
	Caspase-6	NM_009811	Caspase-6	−6.6 ± 2.2	−
15 min	*Caspase-3*	U19522	Caspase-3	−3.6 ± 1.2	−
	Caspase-1	L03799	Caspase-1	−2.9 ± 0.6	−
	CRADD	AJ224738	Raidd/Cradd	−3.4 ± 0.8	+
	1BB	L15435	Receptor 4-1BB ligand mRNA	−2.3 ± 1.0	+
	IAP2	U88909	*Mus musculus* inhibitor of apoptosis protein-2	−2.5 ± 0.6	+
	p53	K01700	Transformation-related protein 53, tumor antigene	−3.9 ± 0.7	+
	Bax	L22472	Bcl2-associated X protein	−3.3 ± 1.2	−
	hrk	D83698	BH3-interacting domain, apoptosis agonist	−5.8 ± 1.2	+
	CIDE-B	AF041377	Cell death-inducing DNA fragmentation factor, α subunit-like effector B	−4.0 ± 1.1	+
45 min	*Nop30-like*	AK021023	Homology with human nucleolar protein 3, Nop 30	−2.3 ± 0.1	+
	CD40	M83312	TNFR superfamily, member 5	−7.3 ± 1.2	+
	TNFSF12/ A PO3L	AF030100	TNFR superfamily, member 12	−2.5 ± 0.3	−
	TRAF6	D84655	*Mus musculus* mRNA for TRAF6	−4.7 ± 0.7	−

[a] Putative NF-κB binding site based on MatInspector (available at www.genomatix.de).

The anti-apoptotic function of NF-κB has been strongly supported by large body of literature although most of the data were generated using knockout mice (35, 36). Interestingly, a few recent reports suggest that NF-κB may contribute to induction of proapoptotic molecules such as DR6, DR4, DR5, or Fas/FasL (37–40). Moreover, it was shown that NF-κB is activated and promotes cell death in focal cerebral ischemia (41). Regarding the induction of NF-κB following THC treatment, a previous study showed that cannabinol treatment inhibits IL-2 secretion by activated thymocytes, probably due to inhibition of NF-κB and CREB (42). Some studies have suggested that the immunosuppressive function of cannabinoid might be related to the inhibition of NF-κB (43). In another study, the activity of NF-κB was directly measured by the NF-κB-responsive reporter gene, which was integrated into PC12 cells. Treatment with 6-hydroxydopamine or the CB1-agonist, CP55,940, increased NF-κB activity, but it was not an integral part of the apoptotic cascade in PC12 cells because attenuated NF-κB activity was not related to the reduction in TUNEL-positive cells (44). On the contrary, in the current study, we found a potential link between the THC-induced apoptosis in DCs and the activation of NF-κB. Increased phosphorylation of IκB-α was detected following THC treatment in a time-dependent manner. Moreover, when the activity of NF-κB was inhibited by TPCK, DCs were almost completely prevented from undergoing apoptosis. It is also worthy to point out that ubiquitination-mediated proteolysis of IκB-α by the 26S proteasome is considered to be a critical step in NF-κB activation (45). The exact mechanism by which attenuated NF-κB activity was effective to prevent DCs from THC-induced apoptosis remains unclear and further study is necessary. Interestingly, it is well known that NF-κB also plays an important role in DC development as well as functional maturation (46, 47). Three general models by which NF-κB transcription factors may regulate apoptosis have been proposed, which include first, the direct regulation of genes involving anti- or proapoptosis, and second, the regulation of cell cycle, and lastly, the protein-protein interactions (34). Our cDNA microarray data showed the induction of several apoptotic genes, which may be regulated by NF-κB, in DCs following THC treatment. Moreover, the induction of some of these genes was down-regulated following TPCK, thereby suggesting that direct gene regulation by NF-κB may play a role in promoting apoptosis in DCs. Taken together, this study provides evidence for the unique role of NF-κB in DC death.

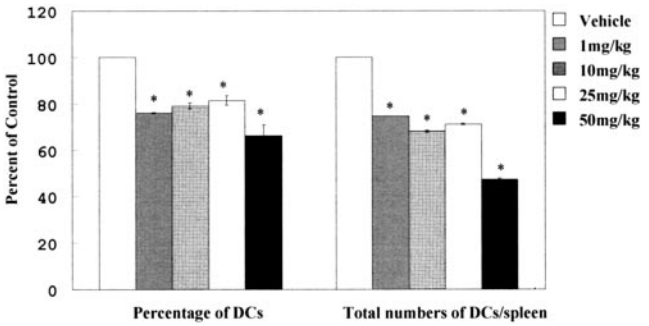

FIGURE 10. THC-induced apoptosis in splenic DCs in vivo. Mice were injected with the vehicle or various concentrations of THC and 24 h later, splenocytes were harvested and double-stained with anti-CD11c and anti-MHC class II mAbs. The cells were analyzed using a flow cytometer and the percentage of DCs was determined. Next, total number of DCs/spleen was calculated from the total cellularity of the spleen and percentage of DC. The data generated from THC-treated groups were depicted as percent of control, considering the vehicle control as 100%. The vehicle-treated mice had 4.43 ± 0.11% $CD11c^+MHC\text{-}II^+$ cells and 5.95 ± 0.01 × 10^6 total DC number per spleen. The vertical bars represent mean ± SEM from three experiments. Asterisk denotes statistically significant difference ($p < 0.05$) between vehicle and THC treatment.

In the current study, we used GM-CSF to derive DC from the murine bone marrow cells as originally described by Inaba et al. (48). Subsequently, several reports have been published on the differentiation of DCs from human peripheral blood monocytes or $CD34^+$ hemopoietic progenitors, using combinations of GM-CSF, IL-4, and TNF-α (49, 50). It is not known whether the DCs generated using different cytokines would exhibit differential sensitivity to THC-induced apoptosis. Cytokines such as IL-4 and TNF-α have been shown to activate NF-κB (51, 52) and inasmuch as we demonstrated that THC-induced activation of NF-κB regulates apoptosis in DCs, it would be interesting to further investigate the effect THC on DCs generated using GM-CSF and IL-4 or TNF-α. Such reports also suggest that our studies may have limitations in extrapolating the results to human DCs.

The dose of THC that induced apoptosis in vitro in the current study was found to be 5 μM or greater. The relevance of this dose to the recreational use of marijuana remains to be established. It is noteworthy that Azorlosa et al. (53) showed that marijuana smoking could lead to plasma levels as high as 1 μM in humans. Moreover, THC can be concentrated 15- to 20-fold in some tissues (54). Therefore, it may be possible to reach levels as high as 20 μM in some tissues after recreational use. It is not clear whether THC in lymphoid organs will increase by 20-fold, and additional studies are necessary in this regard. However, it should be noted that in our study, as low as 1 mg/kg of body weight of THC could deplete DCs in vivo. Moreover, 1 μM THC was able to cause significant $\Delta\psi_M$ loss in DCs in vitro, although we did not study whether this effect on mitochondria alters DC functions. In an earlier study, rats injected with 50 mg/kg of THC were shown to exhibit 10 μM THC in the serum within 10 h of administration (55). Also in these studies, rats were given THC at 50 mg/kg 5 times a week for 2 years while mice were given as much as 500 mg/kg for a similar duration. Interestingly, despite such high doses, the rats survived for a longer time than the controls and both the rats and mice had decreased tumor incidence. Thus, additional studies are necessary to address the consequences of THC-induced apoptosis on immune functions and its relevance to marijuana abuse and pharmacological use in cancer and AIDS patients.

In the current study, we injected 1, 10, 25, and 50 mg/kg of body weight of THC into mice and found that the total spleen cellularity was decreased at 10 mg/kg or higher doses but not at 1 mg/kg, similar to our earlier studies (8). In the previous study, we also noted that 10 mg/kg of THC did not cause a significant decrease in the percentage of T and B cells and macrophages, although this dose caused a significant decrease in the total numbers of each of these subpopulations in the spleen due to an overall decrease in spleen cellularity (8). Although, in the previous study, we had not investigated the effect of THC on DCs, our data suggested that T and B cells and macrophages may exhibit similar susceptibility to THC-induced apoptosis at 10 mg/kg (8). Interestingly, in the current study, we noted that THC at all concentrations tested, including 1 mg/kg, caused a significant decrease in the percentage of DCs as well as a decrease in the total numbers of DCs in the spleen. These data suggested that DCs may be more sensitive to THC-induced apoptosis when compared with other immune cells. Clearly, additional studies are necessary to evaluate this possibility, including the mechanisms of differential susceptibility to THC. It should be noted that DCs represent a small proportion of the splenocytes and thus it is not clear whether a decrease in their number would result in suppression of an Ag-specific immune response. Such studies are necessary to understand the consequences of THC-induced depletion of DCs on immune response. In our previous study, while we had shown that anandamide could induce apoptosis in transformed immune cells (11), we had not investigated whether a similar action is demonstrable in normal immune cells (11). In the current study, we noted that anandamide was able to induce apoptosis in DCs only at 20 μM but not at lower doses. This can be explained by the fact that anandamide is rapidly hydrolyzed by enzymes such as FAAH (17). Therefore, when we used a FAAH inhibitor, even lower concentrations of anandamide (5 μM) could induce significant levels of apoptosis in DCs.

Many studies have suggested the use of THC or related cannabinoids in the treatment of autoimmune diseases. THC administered to mice at 150 mg/kg for 11 days suppressed streptozotocin-induced autoimmune diabetes in CD1 mice (56). Dexanabinol (HU-211), a synthetic nonpsychotropic cannabinoid, significantly reduced clinical symptoms of experimental allergic encephalomyelitis and suppressed the inflammation (57). There is also clinical evidence to suggest that THC-treatment may benefit multiple sclerosis patients (58). Because DCs actively participate in presenting self-Ag to trigger autoimmunity, whether the effectiveness of THC and other cannabinoids against autoimmune diseases results from their ability to induce apoptosis in DCs remains a possibility that needs further investigation.

Acknowledgments

We thank Dr. Michael Fisher for his help in the analysis of NF-κB binding sites on the promoter of various genes using MatInspector.

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