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Cannabinoids suppress dendritic cell-induced T helper cell polarization

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Cannabinoids Suppress Dendritic Cell-Induced T Helper Cell Polarization

by

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ABSTRACT

Cannabinoids suppress Th1 immunity in a variety of models including infection with the intracellular pathogen *Legionella pneumophila* (Lp). To examine the cellular mechanism of this effect, mouse bone marrow-derived dendritic cells (DCs) were studied following infection and drug treatment. DCs produced high levels of IL-12p40 following Lp infection. THC suppressed this cytokine response in a concentration-dependent manner and the endocannabinoids 2-arachidonoyolglycerol and virodhamine less potently suppressed cytokine production. DCs expressed mRNA for cannabinoid receptor 1 (CB_1), CB_2, and transient receptor potential vanilloid type 1 (TRPV1); furthermore, inhibition of G_i signaling by adding pertussis toxin completely attenuated the suppression induced by low concentrations of THC but not at high concentrations. In addition, the THC suppression was partially attenuated in DC cultures from CB_1 and CB_2 knockout mice and in cultures from normal mice co-treated with THC and cannabinoid receptor antagonists. Cytokine suppression was not attenuated by pretreatment with the TRPV1 antagonist capsazepine,

suggesting that G_i signaling and cannabinoid receptors, but not TRPV1, are involved in THC-induced suppression of DC potential to polarize the development of naïve T cells to be Th1 cells. Besides IL-12, THC suppressed other DC polarizing characteristics such as the expression of MHC class II and co-stimulatory molecules CD86 and CD40, as well as the Notch ligand Delta 4. However, THC treatment did not affect other DC functions such as intracellular killing of Lp and Lp-induced apoptosis. Testing the capacity of THC to suppress DC polarizing function with T cells showed that DCs infected *in vitro* with Lp were able to immunize mice when injected prior to a lethal Lp infection; however, the immunization potential along with Th1 cytokine production was attenuated by THC treatment of the cells at the time of *in vitro* infection. In addition, THC-treated and Lp-infected DCs poorly stimulated primed splenic CD4 T cells in culture to produce IFN-gamma (IFN-γ); however, this stimulating deficiency was reversed by adding recombinant IL-12p40 protein to the cultures. In conclusion, the data suggest that THC inhibits Th1 polarization by targeting essential DC functions such as IL-12p40 secretion and the maturation and expression of co-stimulatory and polarizing molecules.

INTRODUCTION

Cannabis products and cannabinoids

Cannabis is one of the oldest psychotropic drugs known in human history and has been used from the earliest records. In 2737 BC, Shen Nung, an emperor of ancient China, had described the properties and therapeutic uses of cannabis in his compendium of Chinese medicinal compounds (98). Two main preparations derived from cannabis are marijuana and hashish. Marijuana is a green, brown, or gray mixture of dried leaves, stems, seeds, and flowers of the hemp plant (cannabis sativa) while hashish is the viscous resin of the Indian hemp plant (97). Despite the fact that cannabis and its products have been widely noted for their effects as an analgesic, appetite stimulant, antiemetic, muscle relaxant and anticonvulsant for centuries (178), it was not until the 1940s that scientists were able to purify and define the structures of the cannabis plant, including more than 60 dibenzpyrene components known as cannabinoids. The major psychoactive ingredient of cannabis is delta-9-tetrahydrocannabinol, usually termed as THC. Other cannabinoids in cannabis such as delta-8-tetrahydrocannabinol (Δ^8THC), cannabinol (CBN), cannabidiol (CBD), cannabicyclol (CBL), cannabichromene (CBC) and cannabigerol (CBG) are

present in small quantities and have little psychoactive effects compared to THC (14); however, it has been suggested they may have synergistic effects in combination with THC (8).

Cannabinoid receptors

Cannabinoids usually exert their actions by binding to specific receptors and two types of cannabinoid receptor have been decribed to date. These receptors are found in mammals, birds, fish, and reptiles (49). Cannabinoid receptor type 1 (CB_1), originally cloned by Matsuda et al. in 1990 from a rat brain cDNA library by a probe derived from the sequence of bovine substance-K receptor, exhibits 97 to 99% amino acid sequence identity across species (30, 57, 114, 115). CB_1 has been demonstrated in high levels in the central nervous system (CNS) and is predominantly found presynaptically expressed, and associated with the behavioral effects following cannabinoid usage, such as loss of short-term memory, dizziness, ataxia and sedation (34). Peripheral expression of CB_1 has also been found in many peripheral tissues including heart, vascular endothelium, small intestine, liver (144, 176) and in the cells of immune system such as splenocytes (78, 136), mast cells (163) and DCs (43, 113). Cannabinoid receptor type 2 (CB_2) cloned in 1993 by Munro and associates from a human HL60 promyelocytic cell line library, exhibits 48% homology with CB_1 (129). CB_2 expression differs from CB_1 in that it is relatively undetectable in the CNS (129, 164) except for microglia that express both CB_2

and CB_1 (54, 183) (26). CB_2 is expressed in a high level in the tissues of lymphoid system including the thymus, tonsils, bone marrow and spleen (56, 87, 105). Both receptors are coupled to G_i proteins to negatively regulate adenylyl cyclase and cAMP accumulation. This G_i protein-induced signal can be blocked by pertussis toxin (71).

Endocannabinoids and synthesized cannabinoids

Discovery of the cannabinoid receptors in humans and animals led first to the prediction and later to the actual findings of endogenous ligands termed endocannabinoids. Anandamide (Arachidonoylethanolamide; AEA) (40) and 2-arachidonylglycerol (2-AG) (119), both derivatives of arachidonic acid, are the most studied endocannabinoids. These endocannabinoids participate in the regulation of neurotransmission (10, 41, 69) and many biological effects associated with marijuana connabinoids. 2-AG is a full and potent agonist for both CB_1 and CB_2, while AEA is more selective to CB_1 than to CB_2 (70). AEA has also been shown to bind vanilloid receptors that are heat-gated, cation channels, sensitive to the vanilloid compound capsaicin and its analogues (196). Many studies have shown that upon stimulation with agents such bacteria-derived lipopolysaccharide (LPS), immune cells, including macrophages (42), DCs (113) and peripheral-blood mononuclear cells (PBMCs) (107) release the endocannabinoids 2-AG and AEA that may act as chemotactants for leukocytes and take part in immune regulation (83). A novel endocannabinoid, Virodhamine,

also a derivative of arachidonic acid, has been described recently as an agonist acting on both CB_1 and CB_2. Virodhamine was shown to be highly produced in spleen tissues suggesting its potential role in immunomodulation (148). Other endogenous cannabinoids including N-arachidonyldopamine (NADA) and Docosatetraenylethanolamide (DEA) have also been described. NADA is produced in mammalian nervous tissue and acts at vanilloid and cannabinoid receptors with more selectivity toward CB_1 than CB_2 (16, 36, 73). DEA is an endogenous ligand selectively activating CB_1, and produced by astrocytes. It acts as a cannabimimetic *in vivo*, causing hypothermia, analgesia, motor activity inhibition and catalepsy (12, 61).

In addition to these naturally occurring cannabinoids and endocannabinoids, also many structural analogues of cannabinoids have been synthesized in a number of laboratories. Some of these analogues include arachidonyl-2-chloroethylamide (ACAE), ajulemic acid (AJA), methyl arachidonyl fluorophosphonate (MAFP), JWH-133 and CP55, 940. Among these analogues, ACEA has high selectivity for CB_1 (64, 70). AJA is a nonpsychoactive, synthetic analog of a metabolite of THC with relatively low affinity for cannabinoid receptors but some demonstrated efficacy in animal models of chronic pain (46) and inflammatory diseases (24). MAFP is a potent, irreversible inhibitor of AEA amidase, the enzyme responsible for AEA hydrolysis, and a selective ligand for CB_1 (39, 110). JWH-133 is a highly selective agonist for CB_2 while CP55, 940 has high affinity for both CB_1 and CB_2 (71). Some of these analogues of cannabinoids possess low CB_1 binding resulting in low psychoactivity. Therefore,

these analogues may have potential therapeutic usage in immune inflammatory diseases associated with dysregulation of cannabinoid receptor signaling.

Receptors involved in cannabinoid effects

Since both CB_1 and CB_2 are G_i protein-coupled receptors, the signaling mechanisms involved in cannabinoid effects are associated with activation of G_i proteins. It is well known that these heterotrimeric proteins are activated by ligand binding to a seven-transmembrane G protein-coupled receptor (GPCR) causing a conformational change, promoting an exchange of GDP for GTP by the G_α subunit, and the dissociation of G_α from the $G_{\beta\gamma}$ dimmer. Activated G_α and $G_{\beta\gamma}$ subunits subsequently modulate specific downstream signaling pathways, and therefore relay information intracellularly in response to various extracellular receptor stimulants (117). Dysregulated G-protein signaling leads to pathologies in numerous organ systems and many important classes of medications can modify GPCR signaling pathways either directly or indirectly (155). A number of studies suggested that G_i signaling was involved in cannabinoid effects mainly through binding to either CB_1 or CB_2 (69). However, although currently there are only two known cannabinoid receptors, other known or yet to be identified receptors or non-receptor mediated mechanisms may be involved in cannabinoid effects. For example, the synthetic cannabinoid AJA, which has potent anti-inflammatory effects but low affinity to CB_2, is known to bind directly to and activate the peroxisome proliferator-activated receptor gamma (PPAR-gamma), a

pharmacologically important member of the nuclear receptor superfamily (24, 101). Similarly, the plant-derived cannabinoid CBD has immunosuppressive effects and binds weakly to both CB_1 and CB_2. One recent study demonstrated that the CBD effect could be reversed by an A2a adenosine receptor antagonist and abolished in A2a receptor knockout mice providing a non-cannabinoid receptor mechanism mediated by cannabinoids (27). Also, it has been shown that the endocannabinoid AEA can activate TRPV1 receptors (102, 108). However, some cannabinoid effects seemed to be mediated by non- CB_1, non-CB_2 and non-TRPV1 receptor mechanisms that remain to be elucidated (65, 124, 143). Overall, the signaling pathway involved in cannabinoids is more diverse than originally speculated and some effects are not mediated by the already known cannabinoid receptors.

Effects of cannabinoids on innate immunity

Innate immunity is critical in immune surveillance against pathological infection agents (188). Macrophages and neutrophils are mediators of innate immunity and can recognize, phagocytize and kill microbes through the activation of several enzymes including oxidases and inducible nitric oxide synthase (iNOS); these enzymes produce the toxic reactive oxygen intermediates (ROI) and nitric oxide (NO) that not only kill microbes but cause inflammation and tissue damage (162). It has been shown that THC, through inhibition of cAMP signaling, inhibits iNOS and NO production by macrophages stimulated with LPS

(77). In similar studies, lung alveolar macrophages collected from marijuana smokers exhibited limited antimicrobial activity. However, treatment with granulocyte/macrophage colony-stimulating factor (GM-CSF) or IFN-γ restored these cells to produce NO and antibacterial efficiency (159, 165). In addition, the endogenous cannabinoid AEA has been shown to suppress, though not as strong as THC, the expression of cytokines such as IL-1, IL-6 and TNFα by rat microglial cells, the macrophage cell type in brain (149). Gongora *et al.* recently showed that synthetic cannabinoid CP55, 940 blocked the expression of MHC class II molecules induced by IFN-γ on the surface of microglial cells (58). Also CP55, 940, but not AEA treatment, inhibited superoxide production in neutrophils (94), while macrophage proteolytic and lysosome processing could be suppressed by THC (116). It was recently reported that JWH-133 inhibited the production of IL-12p40 and enhanced IL-10 by LPS- or Theiler's virus -activated macrophages (32). In addition, it has been demonstrated that the main nonpsychoactive component of marijuana cannabidiol (CBD) significantly modulated murine macrophage cytokine production and chemotaxis (160).

Natural killer (NK) cells, which are a class of innate immune cells, can rapidly respond to intracellular infections with viruses or bacteria by direct killing of the infected cells (60). It has been shown that THC can suppress NK cell function in both animal models (85, 142) and humans (169). A recent study using marijuana users demonstrated that cannabis induced a significant decrease in the absolute number of NK cells as well as T and B cells in peripheral blood (48). In other studies, CB_1 and CB_2 antagonists were shown to

partially reverse the THC- inhibited NK cytolytic activity in mice (111). In addition, the endogenous cannabinoid 2-AG, but not AEA, has been shown to induce the migration of KHYG-1 cells, an NK leukemia cell line, and human peripheral blood NK cells (82). These studies demonstrated that various cannabinoids are capable to significantly modulate (mostly suppress) the innate immune cell functions which include migration, phagocytosis and processing foreign pathogens, cytokine production, and killing of target cells. Innate immunity can also highly impact the development of adaptive immunity. Therefore cannabinoid modulation of innate immune cells might also modulate the activation and development of T cells and B cells.

Effects of cannabinoids on adaptive immunity

T helper cells (Th) are CD4+ T cells that through a variety of mechanisms provide help for activating adaptive immunity. Th cells generate their effects by releasing cytokines and/or by direct cell-cell interactions. T helper cytokines and co-stimulatory molecules interact with macrophages, B cells and CD8+ killer cells to produce the effector mechanisms of adaptive immunity such as activated macrophages, antibodies and killer T cells to clear the invading pathogens (137). Based on the types of cytokines Th cells produce, they are classified into two subtypes, i.e., T helper cell type 1 (Th1) and type 2 (Th2). Th1 cells produce IL-2, IFN-γ and TNF-β, which promote the development of cell-mediated immunity, while Th2 cells produce IL-4, IL-5, IL-10 and IL-13, and can activate humoral

immunity, mainly directed against extracellular infections (4, 45). Recent studies show that many immune disorders are attributable to the collapse of the system controlling the proportion of Th1 and Th2 cells. For example, allergy, multiple sclerosis, and organ-specific autoimmune disease have pathology associated with aberrant Th1 and Th2 polarization (92, 100, 128). Moreover, restoration of the proper balance between Th1 and Th2 cells is generally considered essential in the treatment of tumors, which are generated when cellular immunity is affected by immunosuppressive factors (93).

Marijuana smoking increases susceptibility to infections (84) and is a risk factor in cancers of the respiratory system (174). Many studies indicate that cannabinoids have a Th biasing effect that shifts Th1 to Th2 response. Our group previously examined the effect of THC on host immune resistance to infection with *Legionella pneumophila* (Lp) (89, 134). Lp is a facultative, Gram-negative, intracellular bacterial pathogen that causes Legionnaires' disease in healthy as well as especially immunocompromised individuals (55). Host resistance to this pathogen depends on activation of Th1 cells, cell-mediated immunity, and acute phase cytokine mobilization (17, 133, 175), and THC was shown to suppress Th1 immunity and concomitantly to enhance Th2 development that could not mediate the protection against Lp infection (89, 134). These studies suggested that cannabinoids may have the unique character of biasing immune responses away from Th1 and toward Th2. The mechanism of the T helper biasing effect is unclear, but in mice involves activation of cannabinoid receptors, suppression of serum interleukin-12 (IL-12) and splenic

IL-12 receptor expression, suppression of serum interferon γ (IFN-γ) (89), and an increase in the Th2 biasing transcription factor GATA3 (86).

Also the effect of THC on Th basing has been observed in other animal models. Zhu *et al.* demonstrated THC decreased the production of Th1 cytokine IFN-γ and increased the immunosuppressive cytokines IL-10 and TGF-β, disrupted host anti-tumor immunity and promoted lung tumor growth (192). Similarly, a recent paper by Mckallip *et al.* demonstrated THC enhanced breast cancer growth and metastasis along with increased Th2 but decreased Th1 related gene expression (118). It was not clear in this study if the suppression of cytokines was due to the enhanced Th2 response or the activation of T regulatory cells, which are characterized as CD4+CD25+Foxp3+ and able to produce IL-10 and TGF-β (146). The alteration effect of THC on the balance of Th1 and Th2 cytokines was also observed in human T cell cultures stimulated with allogeneic DCs (191) and in peripheral blood mononuclear cells (PBMCs) isolated from marijuana smokers (140). Therefore, it has been implicated that cannabinoids have T helper biasing effect. However, the precise molecular and cellular mechanisms for these effects are far from defined; also the involvement of cannabinoid receptors (CBRs) remains unclear.

Effects of cannabinoids on DCs

DCs are professional antigen-presenting cells (APCs) that are generated in the bone marrow and migrate as precursor cells to sites of potential entry of

pathogens. During the past decade, intensive studies have demonstrated that DCs are central to the integration of innate and adaptive immunity (11). In contrast to B and T lymphocytes, DCs express many pattern recognition receptors including various Toll-like receptors (TLRs) and are therefore uniquely able to sense stimuli such as bacterial and viral infection as well as tissue damage and necrosis (47). Immature DCs residing in tissues respond to antigenic signals in the environment, leading to their maturation and migration to lymphoid organs. During this process, the phenotypic characteristics and functions of these cells change, including reduced phagocytic capacity and increased secretion of high levels of immunostimulatory cytokines such as IL-12 and expression of MHC and co-stimulatory molecules (79). Also, expression of the polarizing Notch ligands, Jagged and/or Delta, is increased (6). Matured DCs then acquire the ability to direct the development of adaptive immunity including shaping the type of Th cell response (79). In addition, it is becoming evident that DCs also play a critical role in amplifying the innate immune response, either directly by stimulating NK cells and other innate immune cells (37) or indirectly through orchestrating Th development. Studies have delineated the role of DCs in immune responses to a variety of pathogens, including bacteria, viruses, and protozoan parasites as well as to tumors (13, 126).

Little is known, however, concerning the role of cannabinoids on DCs. Only recently it has been shown that both CB_1 and CB_2 receptors are expressed on human and murine DCs (43, 113); and several endocannabinoids including AEA and 2-AG were found to be present in lipid extracts from immature DCs

(113). These findings suggest the possible involvement of DCs in cannabinoid modulatory effects on immunity including THC induced shift from Th1 to Th2 effect. The current project, therefore, studys the immunomodulatory effect of cannabinoids on mouse bone marrow-derived DCs during Lp infection.

Project significance

The current study examines a model of infection using Lp infected DCs which were treated with THC and related pharmacological agents. This study uncovers detailed mechanisms of the immunomodulatory effects of cannabinoids on DCs especially during the primary stages of infection and biasing toward T helper immunity. Moreover, even though a Th shift might be detrimental in the case of Lp and other intracellular pathogenic infections in mice and human because Th1 immunity is critical to recovery from these infections, in certain autoimmune diseases, for example systemic lupus erythematosus (SLE), the enhanced expression of Th1 immunity to self proteins can be a major cause of the development of disease (125). In this instance, cannabinoids might have therapeutic potential by suppressing Th1 immunity. The traditional focus for immunosuppressive drugs has been on lymphocytes as the primary cellular target. However, it is now understood that several classical and newly established immunosuppressive drugs interfere with immune responses in the early stages by suppressing DC differentiation, maturation and activation (59). Moreover, DCs have been suggested as immunotherapy for a number of cancers

(21, 132) and as adjuvants to induce Th1 and Th2 immunity (81). This study provides a close examination of cannabinoids on DC biology and clues to the use of these drugs in the pharmacological manipulation of immune responses. Overall, the results gathered will better define the public health risk of smoking marijuana and exposure to other cannabinoid agents as well as provide potential uses for these agents as immunomodulating and anti-inflammatory therapeutics.

OBJECTIVES

The studies to be conducted will investigate the impact of cannabinoids on mouse bone marrow-derived DCs. Previous studies suggested that cannabinoids bias Th polarization and we believe that cannabinoids influence the functions of DCs which are key to the polarizing event. Preliminary results from our laboratory showed that THC treatment of murine bone marrow-derived DCs, infected with Lp, suppressed the production of IL-12p40 (Figure 1), a key protein involved in Th1 polarization. These findings led to the **hypothesis** that cannabinoids, such as THC, suppress immunity against Lp infection by inhibiting the Th1 polarization function of DCs. In order to verify this hypothesis, the following aims are proposed.

Aim 1. To determine the effect of cannabinoids on the polarizing phenotype of DCs infected with Lp

Evidence is accumulating that endocannabinoids are physiologically essential molecules in various biological systems including the immune system (83, 87). However, the specific immunomodulatory effects of cannabinoids on DCs have not been fully investigated (84). DCs are the major source of IL-12

production in the early stages of an infection, and IL-12 is the key cytokine to direct Th naive cell development to Th1 cells (137). We will study the kinetics of IL-12p40 production in Lp-infected DC cultures and will determine the effect of THC treatment on the quantitative and qualitative aspects of IL-12p40 production. Similar experiments will be done using LPS as stimulant. In addition, we will examine the effect of other cannabinoid receptor agonists on IL-12p40 including the endocannabinoids 2-AG and Virodhamine, as well as CB_1-selective agonists ACEA, AJA, Methanandamide, MAFP, DEA and NADA, and the CB_2-selective agonist, JWH-133. A variety of DC characteristics in addition to IL-12p40 production are known to influence T cell subset development (79). Therefore, we will also examine the effect of THC on the expression of DC maturation markers including MHC class II and the co-stimulatory molecules CD86 and CD40, and the Notch ligand Delta 4 (6). In addition, DC functions such as Lp intracellular killing ability and Lp induced-apoptosis will also be studied.

Aim 2. To determine the role of cannabinoid receptors in drug effects on DC polarization

Both CB_1 and CB_2 have been reported to be involved in the THC-induced attenuation of IL-12 production in THC-treated mice infected with Lp (89). We detected the mRNA expression of both cannabinoid receptors in DCs by using

semi-quantitative RT-PCR. CB_1 and CB_2 are G_i protein-coupled receptors. G_i signaling is sensitive to pertussis toxin inhibition and has been suggested to be able to suppress IL-12 production (19, 89). We therefore will examine if the role of G_i-mediated signaling pathways in the suppression of IL-12p40 by THC in experiments using the G_i inhibitor, pertussis toxin. Also, experiments will be performed using $CB_1^{-/-}$ mice (194) and $CB_2^{-/-}$ (23) mice in combination with receptor antagonists to fully examine the involvement of cannabinoid receptors. Moreover, other receptors, such as TRPV1, have also been reported to mediate cannabinoid effects (180). We, therefore, will examine its role in THC effects on IL-12p40 production by pretreatment with the TRPV1 antagonist capsazepine. In addition, the intracellular activation of MAP kinases has been shown to regulate IL-12 production (18, 179, 186); by using specific antagonists MAP kinase involvement in Lp induced IL-12p40 and suppression byTHC will be studied.

Aim 3. To determine the effect of THC treatment on the T helper polarizing function of DCs

Effective protection from infections with intracellular microorganisms needs the induction of cell-mediated immune responses requiring the activation of Th1 cells stimulated by the interaction between naïve T cells and polarized DCs (123). To examine the mechanism of the effect of THC on the polarizing function of DCs with antigen (Ag)-specific T cells, Lp-infected DCs will be treated with THC and then co-cultured with Lp-primed splenic CD4+ T cells to detect the

protein levels of IFN-γ and IL-12p40 in supernatants. Furthermore, we will examine the immunization effect of Lp-infected DCs treated in culture with either DMSO or THC to induce protective immunity in mice when injected prior to Lp. In these experiments, mice mortality will be monitored and Th1 and Th2 cytokines measured in cultures of splenocytes from mice administrated DCs treated under different conditions.

MATERIALS AND METHODS

Mice

BALB/c and C57BL/6 mice, 7 week of age, were obtained from NCI (Fredericksburg, MD). Cannabinoid CB_1 receptor and CB_2 receptor gene deficient mice ($CB_1^{-/-}$ and $CB_2^{-/-}$) on C57BL/6 background were bred by USF animal facility staff from stocks provided by Dr. Andreas Zimmer ($CB1^{-/-}$, University of Bonn) and Dr. Nancy Buckley ($CB_2^{-/-}$, California State Polytechnic U.). The mice were housed and cared for in University of South Florida Health Sciences animal facility, which is fully accredited by the American Association for Accreditation of Laboratory Animal Care.

Reagents

THC, cannabinoid CB_1 receptor antagonist N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide hydrochloride (SR141716A), and cannabinoid CB_2 receptor antagonist N-[(1S)-endo-1,3,3-trimethyl bicycle [2.2.1] heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide (SR144528) were obtained from the

Research Technology Branch of the National Institute on Drug Abuse (Rockville, MD). SR141716A, SR144528 and THC were first diluted in dimethyl sulfoxide (DMSO) at 20 mg/ml and then in 5% fetal calf serum RPMI 1640 medium to a concentration of 0.01-10 μM. 2-Arachidonoylglycerol (2-AG), Virodhamine, Methanadamide, ACEA, MAFP, DEA, NADA and CP55,940 were purchased from Tocris (Bristol, UK). AJA was obtained from Dr. Sumner Burstein (University of Massachusetts, MA) and JWH-133 from Dr. John Huffman (Clemson University, Clemson, South Carolina). Pertussis toxin (G_i signaling inhibitor) was purchased from Sigma (St. Louis, MO). Capsazepine (TRPV1 antagonist) was purchased from ALEXIS (San Diego, CA). Pertussis toxin was dissolved in medium to a concentration of 0.01-1.0 ng/ml. Other cannabinoids and capsazepine were dissolved in medium to a concentration of 0.01-10 μM. MAP kinases antagonists UO126, SP600125 and SB203580 were purchased from Tocris (Bristol, UK).

Bacteria

A virulent strain of Lp (M124), serogroup 1, was obtained from a case of Legionellosis from Tampa General Hospital (Tampa, FL) and cultured on BCYE medium (Difco, Detroit, MI) as described previously (89). Bacteria from colonies of 48 hr cultures were suspended in pyrogen free saline and adjusted spectrophotometrically to a working concentration.

Preparation and treatment of bone marrow derived DCs

Bone marrow cells were collected from femurs and tibias of the BALB/c and C57BL/6 wildtype as well as $CB_1^{-/-}$ and $CB_2^{-/-}$ mice at 8 to 12 weeks age. Cells were suspended at 1.0 x 10^6/ml and cultured overnight in 6-well cell culture plates (GIBCO-Costar, Cambridge, MA) in RPMI1640 medium supplemented with 5 μM 2-mercaptoethamol, 2 mM L-glutamine, 1% antibiotic/antimycotic solution (Sigma), 5% heat-inactivated fetal calf serum (HyClone, Logan, UT) and 10ng/ml granulocyte/macrophage colony-stimulating factor (GM-CSF) (BD-Pharmingen, San Diego, CA). Non-adherent cells were removed and the adherent cells were incubated with fresh GM-CSF-containing medium for an additional 7-9 days, during which time the bone marrow-derived DCs became nonadherent and were harvested. The purity of the obtained DCs was determined by flow cytometry staining using fluorochrome-conjugated monoclonal antibodies to CD11b and CD11c (BD-Pharmingen). The purity was about 100% $CD11b^+$ and greater than 75% $CD11c^+$ cells. These DCs were either uninfected or infected with Lp at ratio 10:1 for 30 to 35 min. DCs were then washed two times to remove non-internalized Lp and re-suspended to10^6 cells/ml. The cells were treated with different cannabinoids at various concentrations or with the highest concentration of DMSO or ethanol (vehicle control). To study the mechanisms involved in THC effect, the DCs were pretreated with SR141716A or SR144528 at 0.01, 0.05, 0.1 and 0.5 μM or with capsazepine at 0.01, 0.1, and 1.0 μM. In the study with pertussis toxin, cells

were cultured for 18 hr with 0.01-1.0 ng/ml pertussis toxin before infected with Lp. When LPS (Sigma) was used as stimulator, DCs were incubated with LPS 0.01-1 μg/ml and supernatants were collected for cytokine detection at different time points as indicated. In studies with CD4 T cells, cells were obtained from mice intravenously (iv) infected (primed) with a sub-lethal dosed of Lp (7×10^6) and the spleens were removed 5 days post-infection. The T cells were isolated from the splenocytes by mouse T cell Enrichment Columns (R&D system, Minneapolis, MN) and CD4+ T cells were negatively selected from the purified T cells with CD4 enrichment magnetic bead kits (BD-Pharmingen). Isolated CD4 T cells were then dispensed in 24-well cell culture plates (GIBCO-Costar) and co-cultured with DCs (CD4: DC = 10:1) in either the absence or presence of recombinant IL-12p40 (BD-Pharmingen) for 24 hr followed by cytokine analysis.

Cell surface marker analysis by flow cytometry

To evaluate the effects of THC on MHC class II, CD86 and CD40 expression on DCs, cells, either uninfected or infected with Lp, were treated with DMSO or THC 10 μM for 48 hr. Following incubation, the cells were treated with fluorochrome-conjugated mAbs (BD-Pharmingen) at 4°C for 30 min, and then washed in PBS containing 2% BGS, and fixed in 1% paraformaldehyde. Cells were analyzed using FACScan (Becton Dickinson, Mountain View, CA). The instrument is equipped with lasers tuned to 488 nm and to 635 nm. The following fluorochrome-conjugated mouse mAbs were used for DC surface marker

staining: PE-conjugated anti-MHC class II; PE-conjugated anti-CD86; and PE-conjugated anti-CD40 (BD Pharmingen).

Bacteria growth determined by CFU assay

After 24 hr infection, spleens from infected mice were homogenized in Hanks balanced salt solution (HBSS). In studies of Lp growth in DC cultures, cells were lysed by with 0.1% saponin (Sigma) and diluted in HBSS. Homogenized spleens or lysed DCs were plated on BCYE agar plates and incubated at 37°C for 72 hr. CFU counts were determined on an AutoCount apparatus (Dynatech Labs, Chantilly, Va.).

Cell viability and apoptosis detection

DC viability and apoptosis were detected using the Annexin V-FITC kit (BD-Pharmingen). Briefly, uninfected or infected cells (10^5), treated with DMSO (LpDC/DMSO) or THC 10 μM for 24 hr (LpDC/THC), were washed twice with PBS, and incubated with Annexin V-FITC (5 μl) and propidium iodide (5 μl) in binding buffer for 15 min. Early apoptotic cells (Annexin V positive and propidium negative) and late apoptotic or dead cells (Annexin V positive and propidium positive) were quantitated by flow cytometry.

Cytokine detection by ELISA

IL-12p40, IL-4, IL-23 and IL-10 were determined using a sandwich ELISA with antibody pairs from BD Pharmingen. In 96-well enzyme immunoassay plates (GIBCO-Costar), each well was coated with 50 µl of anti-murine antibody in 0.1 M NaHCO3, pH 8.2 (anti-IL-12 p40 for IL-12p40 and IL-23; 5 µg/ml) or in PBS (anti-IL-4 and anti-IL-10; 2 µg/ml) overnight at 4^{o}C. The wells of the plate were blocked with 150 µl of 3% BSA/0.05% Tween 20 in PBS (IL-12p40 and IL-23) or 0.5% BSA/0.05% Tween 20 in PBS (IL-4, IL-10) and incubated for 1 hr. The culture supernatants or serial dilutions of cytokine standards were added and incubated for 1-2 hr, followed by biotinylated detection antibodies (2ug/ml, 50ul) for 1 hr, and streptavidin-horseradish peroxidase (HRP) (1:1000 in 50ul) for 30 min. The plates were washed between each addition. The tetramethyl benzidine (TMB; Sigma) substrates were developed for 5-30 min; the reaction was stopped with 1 N sulfuric acid and read at 450 nm on an E_{max} microplate reader (Molecular Devices; Menlo Park, CA). The concentrations of sample cytokines were calculated from standard curves that were done for each plate. The levels of IFN-γ and IL-12p70 in supernatants were measured using BD OptEIA^{TM} Sets (BD Pharmingen) according to the manufacturer instructions.

Cell-based ELISA

Phosphorylation of p38, JNK and ERK1/2 were measured by Fast Activated Cell-Based ELISA kits from Active Motif (Carlsbad, CA). Briefly, 96-well culture plates were treated with 100 µl 10 µg/ml poly-L-Lysine for 30 min at 37^{o}C and then washed twice with PBS. uninfected DCs or Lp-infected DCs were then seeded into 96-well plates with culture medium containing THC (6µM) for indicated time. Cells were fixed by replacing the culture medium with 100 µl of 8% formaldehyde in PBS. After 20 min incubation at room temperature, plates were washed three times with wash buffer (PBS containing 0.1% Triton X-100). Cells were then incubated with quenching buffer (wash buffer containing 1% H_2O_2 and 0.1% Azide) to inactivate the cells endogenous peroxidase activation. After 20 min, cells were washed twice and incubated with antibody blocking buffer 100 µl for 1 hr and incubated overnight with primary antibody for phospho-MAP kinase protein or total-MAP kinase at 4°C. Next day, cells were washed three times incubated with horseradish peroxidase-conjugated secondary antibody for 1 hr at room temperature and washed three times with wash buffer and twice with PBS. Subsequently the cells were incubated with 100 µl developing solution for 2-20 min at room temperature; the reactions were stopped by adding 100 µl stop solution and absorbance was read on an E_{max} microplate reader (Molecular Devices). Levels of MAP kinase activation were expressed as the ratios of phosphorylated MAP kinase to total MAP kinase.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from dendritic cell cultures by standard techniques using TriReagent (Sigma) and quantitated using RiboGreen RNA Quantitation Kit (Molecular Probes, Eugene, OR). The extracted RNA was treated with DNase using DNA-free kit from Ambion (Austin, TX). 1μg of total RNA were used for cDNA synthesis at 42^{o}C for 45 min by priming with 0.5 μg oligo $(dT)_{15}$ primer, 20 nmol each deoxynucleoside triphosphate, 0.5 U RNase inhibitor, and 15 U avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI) in a total volume of 25 μl. 2 μl of the reverse transcriptase product was used for PCR, which was carried out in PCR buffer (TaKaRa, Fisher; Atlanta, GA) containing 250 μM dNTP, 1.0 μM each primer and 2.5 U Tag DNA polymerase (TaKaRa). The primer pairs used were as follows: cannabinoid CB_1 receptor forward primer, 5'-TCACCACAGACCTCCTCCTCTAC-3'; reverse primer, 5'-CTCCTGCCGTCATCTTTTC-3' (149 bp product); cannabinoid CB_2 receptor forward primer, 5'-GTACATGATCCTGAGCAGTGG-3'; reverse primer 5'-TGAACAGGTACGAGGGCTTTCT-3' (147 bp product); TRPV1 forward primer, 5'-AATTTGGGATGTGGAGCAAG-3'; reverse primer, 5-GATCCCCCGAGTATCCATTT-3' (176 bp product); β-actin forward primer, 5'-GGGAATGGGTCAGAAGAACT-3'; reverse primer, 5'-AGGTGTGGTGCCAGATCTTC-3' (133 bp product); Jagged1, forward primer, 5'-AGAAGTCAGAGTTCAGAGGCGTCC-3', reverse primer, 5'-AGTAGAAGGCTGTCACCAGCAAC-3' (113 bp product); Delta4, forward primer,

5'-AGGTGCCACTTCGGTTACACAG-3', reverse primer 5'-CAATCACACACTCGTTCCTCTCTTC-3' (123 bp product); and β-actin, forward primer, 5'-ATGGATGACGATATCGCT-3', reverse primer, 5'-ATGAGGTAGTCTGTCAGGT-3' (530 bp product). All PCR were performed in a Mastercycler (Eppendorf, Westbury, N.Y.) at 60^{o}C for annealing. The number of cycles in each PCR was as follows: CB_1, CB_2, TRPV1 and β-actin (133bp), 40 cycles; Jagged1 and Delta4, 35 cycles; and β-actin (530bp), 28 cycles. PCR products were analyzed on ethidium bromide-stained, 2% agarose gels. RT-negative amplifications were also done to control for contaminating genomic DNA.

Animal injections and tissue sampling

Mice were immunized iv with 0.3-0.5 x 10^6 treated DCs suspended in PBS, and 7-9 days later spleens were isolated from mice. Single-cell suspensions of splenocytes (2 x 10^6 cells/ml) were cultured with formalin-killed Lp (10^7/ml) for 24 hr and supernatants collected for cytokine detection. Or, DC-treated mice were challenged iv with live Lp (sublethal dose, 7 x 10^6) diluted in pyrogen free saline. Spleens were obtained after 24 hr and CFUs of Lp were counted. In other experiments, mice were immunized iv with DCs (0.5 x 10^6) for two or three times at 7 day interval, challenged iv with a lethal dose of Lp (1.7-2.0 x 10^7) and survival of mice was monitored.

Statistical analysis

Data were analyzed by one-way analysis of variance with Dunnett's test for comparing individuals using SigmaStat (Jandel Scientific, San Rafael, CA), or using the two-tailed Student's t test. A value of $p < 0.05$ was accepted as indicating significance.

RESULTS

Aim 1. To determine the effect of cannabinoids on the polarizing phenotype of DCs infected with Lp

Lp infection of DCs induced IL-12p40 production

IL-12 is a heterodimer produced by DCs and formed by the association of a 35-kDa light chain (p35) and a 40-kDa heavy chain (p40). Interestingly, microbial components alone seem to induce primarily p40 with relatively low amounts of IL-12p70 (28). Based on this, the kinetics of the IL-12p40 subunit production was investigated to determine the bone marrow-derived dendritic cell response to Lp infection. Dendritic cell cultures were infected, supernatants harvested at indicated time points, and supernatant IL-12p40 was measured by ELISA. As shown in Figure 2, there was a rapid increase in the secretion of the p40 protein reaching a peak by 44 hr following infection. These data indicate that cultured bone marrow-derived DCs produce IL-12p40 following infection with *Legionella*.

Suppression by THC of Lp-induced IL-12p40 secretion

To determine if cannabinoids can modulate the ability of DCs to produce IL-12p40, infected DCs were treated with increasing concentrations of THC (0, 1, 3, 6 and 10 μM) and incubated for 24 hr followed by IL-12p40 measurements. Figure 3 A shows that THC treatment led to a significant decrease in IL-12p40 at a concentration of 3 μM and higher while drug vehicle (DMSO) had no effect. In addition, Figure 3 B shows that the THC (10μM) suppression effect on DC IL-12p40 was observed as early as 6 hr after treatment and Lp infection. These data demonstrated THC consistently and significantly suppressed DC secretion of IL-12p40.

No suppressive effect by THC on LPS-induced IL-12p40 production

To examine if the THC effect is related to TLR4 signaling, we used LPS, a ligand for TLR4, as a stimulant in DC cultures. Compared with Lp infection, LPS induced a rapid increase in IL-12p40 (Figure 4 A) that reached maximum at 12 hr after stimulation. However, unlike with Lp infection, THC treatment had no significant effect on LPS-induced IL-12p40 production (Figure 4 B). These data suggest that Lp infection stimulates IL-12p40 through receptors other than TLR4 and that the THC suppressive effect is not generalized for all stimuli but selective for mechanisms related to Lp infection rather than the TLR4/LPS pathway.

Other non-selective agonists suppressed IL-12p40 production

In addition to the effect of THC on DC function, we also examined the effect of the endocannabinoids 2-AG and Virodhamine. 2-AG has been shown to be produced in DC cultures (113) and Virodhamine has been reported in rat peripheral tissues including immune organs such as spleen in higher levels than AEA, another endocannabinoid (148). 2-AG and Virodhamine are potent agonists for both CB_1 and CB_2 (119, 148, 172) and there are no reports examining their functional effects on DCs. We therefore treated Lp-infected DCs with various concentrations of 2-AG and Virodhamine. We found that 2-AG treatment significantly suppressed IL-12p40 at concentrations of 1 μM and 10 μM (Figure 5 A). No effect was observed with ethanol as the drug vehicle control. Virodhamine also showed significant suppression at 10 μM and the suppression level was comparable to 2-AG, though not as strong as THC used at the same concentration (Figure 5 B). In addition, we tested other endogenous ligands, such as NADA and DEA, that are both CB_1 selectvie and produced in nervous tissues (12, 16, 36, 61, 73). We also tested the synthetic cannabinoids including the CB_1 selective agonists ACEA, AJA, M-AEA (1, 46, 64, 80), and the CB_2 agonist JWH-133 (74). The results showed these agents did not significantly suppress IL-12p40 in Lp infected DCs (data not shown). Thus, nonselective cannabinoid receptor agonists, including THC, 2-AG and Virodhamine, but not other agonists, led to a significant inhibition of IL-12p40 production in our culture system.

THC suppressed the expression of DC maturation and polarizing markers

Upon exposure to microbes, DCs are activated to go through a maturation process characterized by an increase in surface expression of MHC class II proteins and co-stimulatory molecules contributing to initiation of an effective adaptive immune response (76). To determine if THC modulates this polarizing DC phenotype, we treated infected and non-infected DCs with either DMSO or THC and assessed the expression of these surface markers. After 48 hr, we observed by flow cytometry that Lp infection increased the surface expression of CD86 and CD40; however, THC treatment significantly suppressed the expression of both markers (Figure 6 and Table 1). Regarding MHC class II, we observed that although expression was high in all three groups, the intensity per cell of the marker was enhanced in the Lp infected-DCs but was significantly decreased by THC treatment (Table 1). From these results, it is possible that drug suppression of T helper polarization is due in part to a down-modulation of these phenotypic markers. Other surface proteins such as Notch receptors are known to regulate T cell development (151). Recently it was shown that the Notch ligands, Delta4 or Jagged1 on DCs promote induction of either Th1 or Th2 activity, respectively (6). We, therefore, examined the relative mRNA expression of these ligands in DCs loaded with Lp and treated with THC or vehicle for 18 hr. We observed that mRNA for both ligands was increased in DCs after Lp infection (LpDC/DMSO group; Figure 7) but that the Delta 4 band intensity relative to β-

actin was decreased following THC treatment suggesting that the message level of this Th1 polarizing ligand was decreased by drug treatment.

THC treatment did not affect Lp survival in DCs or enhance apoptosis of infected DCs

THC has been observed to induce apoptosis in macrophages and lymphocytes (193) and also in DCs (43); therefore, it could be argued that drug suppression of IL-12 production and marker expression could be due to a toxic effect on DCs. Also, antigen presentation to CD4 T cells by DCs requires internalization and procession of infectious agents by the DCs and drug-induced suppression of phagocytosis, therefore, might contribute to suppression of T cell activation. In order to test the THC effect on these other relevant DC functions, we studied the drug effect on the survival of Lp in DCs and the induction of apoptosis in Lp-infected DCs. DCs were infected with Lp for 30 min followed by washing to remove non-internalized bacteria. Infected cultures were then treated with THC or DMSO for 0, 24 and 48 hr and the number of cell-associated CFUs, as a measure of phagocytized bacteria, was determined by cell lysis and viable bacteria colony counts. The results (Figure 8) showed that the bacteria internalizing function as measured by intracellular survival was unaffected by THC treatment. Both drug-treated and vehicle treated cells restricted the growth of Lp in an equivalent manner over time. To determine if THC induced apoptosis in Lp-infected DCs, staining with propidium iodide and annexin V in treated DCs

was analyzed by flow cytometry. Compared with uninfected DCs, the percentage of apoptotic cells, assessed as single positive for annexin V, was enhanced after Lp infection (Figure 9), and treatment with THC did not increase the annexin positivity. Furthermore, analysis of propidium iodide staining, as indicative of necrotic cells, was similar in infected and infected plus THC treated cells. The data suggest THC treatment did not affect the degree of apoptosis or processing of bacteria in DCs following Lp infection.

Aim 2. To determine the role of cannabinoid receptors in drug effects on DC polarization

Expression of cannabinoid and vanilloid receptor mRNA in DCs

The cannabinoid receptors identified so far are CB_1 and CB_2. Both have been reported to be involved in the THC-induced attenuation of IL-12 production in drug-treated mice infected with *Legionella* (89). Moreover, TRPV1, has recently been shown to bind AEA and other endocannabinoids and mediate their effects (96, 180, 196). To examine receptor expression in dendritic cell cultures, RNA was isolated from DCs and analyzed by RT-PCR for CB_1, CB_2, and TRPV1 messages. Figure 10 shows mRNA of both cannabinoid receptors and TRPV1 was readily detected in DCs and the level of cannabinoid CB_2 receptor and TRPV1 messages appeared to be more abundant than CB_1 receptor (Ratios of

target genes to β-actin are: CB_2 receptor 0.76, TRPV1 0.84, and CB_1 receptor 0.48).

Pertussis toxin attenuated THC-induced suppression of IL-12p40

Cannabinoid receptors are G_i protein-coupled receptors. G_i signaling is suppressed by pertussis toxin and the toxin has also been reported to suppress IL-12 production (19, 89). To examine if THC -induced suppression of IL-12p40 is through G_i protein-coupled mechanisms, DCs were pretreated with different concentrations of pertussis toxin (0.01, 0.1, or 1.0 ng/ml) for 18 hr followed by Lp infection and THC (3, 6 or10 μM) treatment for 24 hr. Pertussis toxin (at 1.0 ng/ml) completely reversed the suppression effect of THC at low concentrations (3 or 6 μM) (Figure 11). However, the effect of THC at a higher concentration (10 μM) was only partially attenuated by pertussis toxin (Figure 11). This finding suggests the involvement of G_i protein-coupled mechanisms in the suppression of IL-12p40 at low THC concentrations, but other suppressive mechanisms at higher concentrations.

Role of cannabinoid receptors in THC-induced suppression of IL-12p40

To fully examine if cannabinoid receptors were involved in suppression of IL-12p40, experiments were performed using $CB_1^{-/-}$ (194) and $CB_2^{-/-}$ (23) mice. Because the knockout mice are on the C57BL/6 background and our previous

studies had been done with BALB/c mice, we first tested the response of bone marrow-derived DCs from wild-type C57BL/6 mice in terms of IL-12p40 production and suppression by THC. Comparing Figure 12 A, to Figure 3 A shows that cells from C57BL/6 mice displayed a comparable suppressive response to those from BALB/c. We next studied the response of DCs from $CB_1^{-/-}$ and $CB_2^{-/-}$ mice and the results showed that both were suppressed by THC similar to wild-type cells (Figure 12, panels B and C). Since THC is a non-selective receptor agonist and can bind both CB_1 and CB_2 receptors (51), it is possible that the drug could have a relatively unimpeded effect in cells from single receptor knockout mice. To examine this possibility, the specific antagonists for CB_1 (SR141716A) and CB_2 (SR144528) were used in combination with cells from knockout mice. $CB_1^{-/-}$ DCs, after Lp addition, were pretreated with SR144528 at different concentrations for 30 min prior to THC treatment and the reciprocal experiment was performed using $CB_2^{-/-}$ and SR141716A pretreatment. After 24 h, supernatant IL-12p40 was assessed by ELISA. As a control, cultures were treated with either SR141716A or SR144528 only followed by infection and no effect on IL-12p40 was observed (not shown). Table 2, shows the SR compounds attenuated the THC effect in knockout mice, especially at the lower drug concentrations. For example, the attenuating effect of SR144528 (at 0.1 μM) in $CB_1^{-/-}$ cells is nearly 70% of control at 3 μM THC but only 28% of control at 10 μM THC. A higher concentration of SR144528 (0.5 μM) had the identical attenuating effect (data not shown). Similar decreases in receptor antagonist efficacy with increasing receptor agonist concentration were seen using $CB_2^{-/-}$ cells (Table 2). These results suggest that

cannabinoid receptors are involved in the THC-induced suppression of p40, especially at the lower drug concentrations, but that other mechanisms become involved as the THC concentration is increased.

TRPV1 was not involved in THC effect

From the above, it appears that mechanisms other than cannabinoid receptors are involved in THC suppression of IL-12p40. Therefore, we tested for a possible role of TRPV1 in the THC effect by pre-treating with capsazepine, the specific receptor antagonist for TRPV1. Dendritic cell cultures were infected with Lp and treated with either THC alone (3 and 10 μM) or in combination with capsazepine at 0.01, 0.1, and 1.0 μM. As shown in Figure 13, there was no attenuating effect of capsazepine on the THC effect suggesting that TRPV1 receptors were not involved in the response.

The activation of p38 MAP kinase was modulated by THC

It is reported that MAP kinases can be activated in response to ligands for G protein-coupled receptors, and many studies have indicated kinase activation affects either positively or negatively IL-12 production (18, 179, 186). There are three major groups of MAP kinases in mammalian cells: the extracellular signal-regulated protein kinases (ERK), the p38 MAP kinases, and the c-Jun-NH2-terminal kinases (JNK)(44). Many studies have suggested the modulation effect

of various cannabinoids on MAP kinase activity (69). However, a role for these kinases in the THC effect on DC cytokine production has not been reported (158); therefore we examined the role of kinase activity in our system. Initially we treated Lp-infected DCs with specific antagonists for different kinases for 18 hr and then measured IL-12p40. We found that only the p38 inhibitor, SB203580, but not Erk inhibitor, UO128, nor JNK inhibitor, SP600125, was able to suppress IL-12p40 production suggesting only p38 kinase activation is required for Lp-induced IL-12p40 secretion (Figure 14). From this finding, we next examined the effect of THC on p38 activation in DCs during Lp infection. As shown in Figure 15, Lp infection induced an increase in phosphorylated p38 protein within 10 min after infection indicating an up-regulation in p38 activity. THC treatment, interestingly, initially increased phosphorylated p38 but then caused a drop in the level by 3 hr after infection. From these studies it is clear that p38 kinase is an important signaling component of the Lp induction of IL-12p40 and that modulation of p38 phosphorylation by THC might be a critical mechanism of drug action.

Aim 3. To determine the effect of THC treatment on the T helper polarizing function of DCs

THC treatment impaired the immunization potential of Lp-loaded DCs

Due to the pivotal role in stimulating T cells, DCs loaded with specific antigens have been utilized as immunizing vehicles in numerous studies of tumor therapies (29, 195) and infectious diseases (126). To test whether DCs loaded with Lp would induce a specific immune response and if THC would impair this ability, we treated Lp-infected DCs in culture with 10 μM THC (LpDC/THC) or drug vehicle DMSO (LpDC/DMSO) for 24 hr. DCs without infection and drug treatment were incubated for the same time as controls. Following treatment, DCs were injected iv into mice two to three times at 7 day intervals, and seven days after the last injection, the mice were challenged with a lethal dose ($1.7\text{-}2.0\text{x}10^7$) of bacteria and survival monitored. The results in Figure 16 showed that uninfected DCs failed to induce protection as none of the mice survived; however, Lp-loaded DCs (LpDC/DMSO) induced significant protection with a survival ratio of 66 percent (6/9). However, mice receiving loaded DCs treated with THC (LpDC/THC) showed no survival after 25 hr indicating a lack of immunizing potential similar to mice injected with non-loaded DCs. In other experiments to examine immunizing potential, mice were injected with DCs, LpDC/DMSO or LpDC/THC ($0.3\text{-}0.5\text{x}10^6$) and seven to nine days later challenged with a sublethal dose ($7\text{x}10^6$) of Lp rather than a lethal dose as in the

above experiments. After 24 hr, spleens were isolated and homogenized, and bacterial burdens measured by CFU analysis. The data showed that spleens from mice receiving Lp-loaded DCs had much lower CFUs than spleens from mice receiving either unloaded DCs or loaded DCs treated with THC (Figure 17). These findings together demonstrated that mice immunized with Lp-loaded DCs were able to induce immunization against Lp infection and that THC treatment significantly attenuated this effect.

THC treatment of Lp-loaded DCs inhibited Th1 activity in splenocytes from recipient mice

Type 1 cytokines, including IL-12 and IFN-γ, can be measured in immune organs and are a measure of the development of protective immunity against intracellular microbial infections (150). To determine polarization toward Th1 immunity, the cytokine profiles in splenocytes of mice immunized with the various DC populations were analyzed to determine whether THC treatment of DCs suppressed an upregulation of Th1 activity in recipient mice. As in the transfer experiments above, mice were immunized with DCs only, Lp-loaded DCs, or loaded DCs treated with THC, and seven to nine days later splenocytes were harvested from the recipient mice and stimulated *in vitro* for 24 hr with specific Lp antigens. Supernatants from these cultures were collected and analyzed for type1-associated cytokines by ELISA. As shown in Figure 18, the splenocytes from mice receiving Lp-loaded DCs treated with DMSO produced 1.5-2 fold

increases in IL-12p40 and IFN-γ as compared to splenocytes from mice treated with unloaded DCs. This suggested an upregulation of Th1 activity in the spleens of mice immunized with Lp-loaded DCs accounting for their enhanced resistance to Lp infection (see Figure 16 and 17). However, THC treatment of the DCs inhibited this upregulation of Th1 activity, suggesting an attenuation of the immunizing potential of these cells (Figure 18 A and B). IL-4 production by splenocytes was also examined and we observed it was suppressed following injection of Lp-loaded DCs either treated or not with THC (Figure 18 C). This suggested that the increase in Th1 activity in the spleens coincided with a decrease in the Th2 cytokine, IL-4; furthermore, it suggested that THC suppressed Th1 cytokines by mechanisms other than the upregulation of IL-4. The data overall suggested that THC treatment of antigen-loaded DCs can suppress the immunizing and Th1 polarizing potential of these cells when subsequently injected into mice.

IL-12p40 addition restored the polarizing function of THC-treated DCs

We have shown above that THC suppresses the production of IL-12p40 in Lp-infected DC cultures. Therefore, to examine if this attenuation is responsible for the impaired Th1 polarizing function of these cells, co-cultures of DCs with T cells from both unprimed and Lp-primed animals were prepared to examine the reconstitution efficacy of exogenously added IL-12p40. Figure19 A shows results from co-cultures of Lp-loaded DCs and unprimed CD4 T cells. Lp loading of DCs

induces the production of IL-12p40 as detected in culture supernatants by ELISA and THC treatment of the cells suppressed this response. The addition of unprimed T cells had little effect on IL-12 production (Figure 19 A) and in studies not shown, no IFN-γ was detected in these cultures. We next examined the accessory cell potential of drug treated DCs in cultures containing Lp-primed T cells and supplied with various concentrations of IL-12p40. Figure 19 B shows that DCs plus primed T cells (LpCD4) produced a relatively small amount of IL-12p40; however, when DCs were loaded with Lp (LpDC/DMSO), a robust IL-12p40 response was evident and this was significantly attenuated by THC treatment (LpDC/THC + LpCD4). Of interest was the finding that the addition of recombinant IL-12p40 protein to the cultures increased the IL-12 supernatant concentrations above the amounts added (Figure 19 B). For example, addition of 0.5ng/ml recombinant IL-12p40 resulted in an increase of supernatant IL-12 from 2ng to 6ng/ml. Furthermore, in contrast to co-cultures containing unprimed CD4 T cells, cultures containing primed T cells, produced in addition to IL-12p40, robust amounts of IFN-γ but only in the presence of Lp-loaded DCs (Figure 19 C) and this effect was attenuated by THC treatment of the DCs. However, the addition of recombinant IL-12p40 completely restored IFN-γ production suggesting a restoration of Th1 polarization by IL-12. In addition to IL-12p40, we also tested for the presence of IL-12p70, IL-23, and IL-10 in the culture supernatants. These cytokines were not detected suggesting that the suppression of IL-12p40 by THC treatment was primarily responsible for the reduced Th1 polarization.

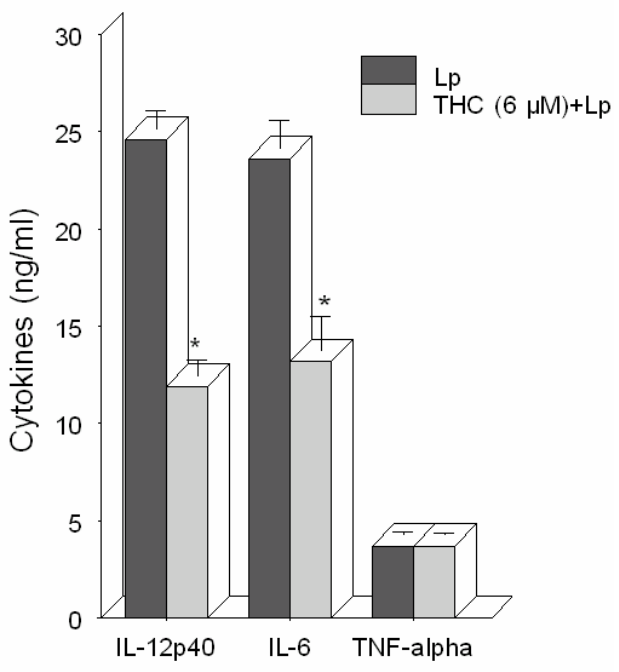


Figure 1. THC suppressed the production of IL-12p40 and IL-6 in bone marrow-derived DCs. *L. pneumophila* (Lp) infected DCs were treated with DMSO (Lp), or THC 6 μM for 24 hr. Supernatants were collected for cytokines detection. Data represent the mean of 3 experiments ± S.E.M.. *P< 0.05, compared to Lp group.

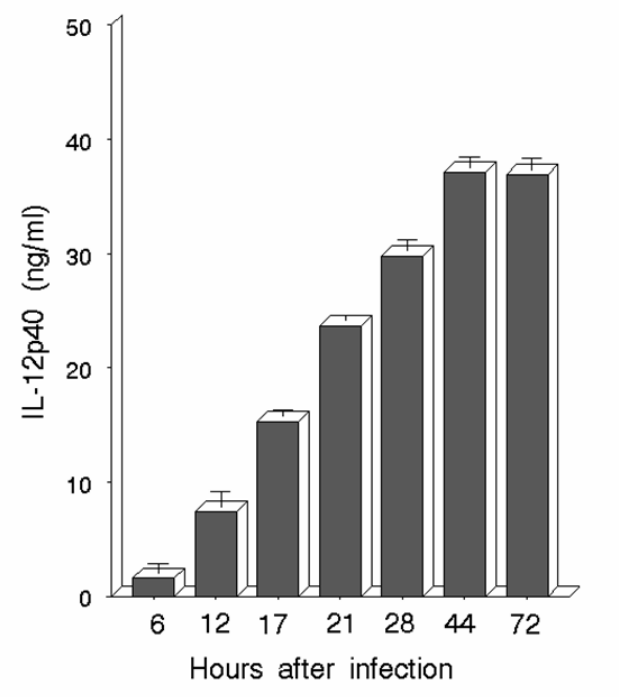


Figure 2. Lp infection induced IL-12p40 production in bone marrow-derived DCs from BALB/c mice. Immature bone marrow-derived DCs were infected as indicated in the methods. Supernatants were collected at indicated time points and IL-12p40 measured by ELISA. Data represent the mean of 4 experiments ± S.E.M..

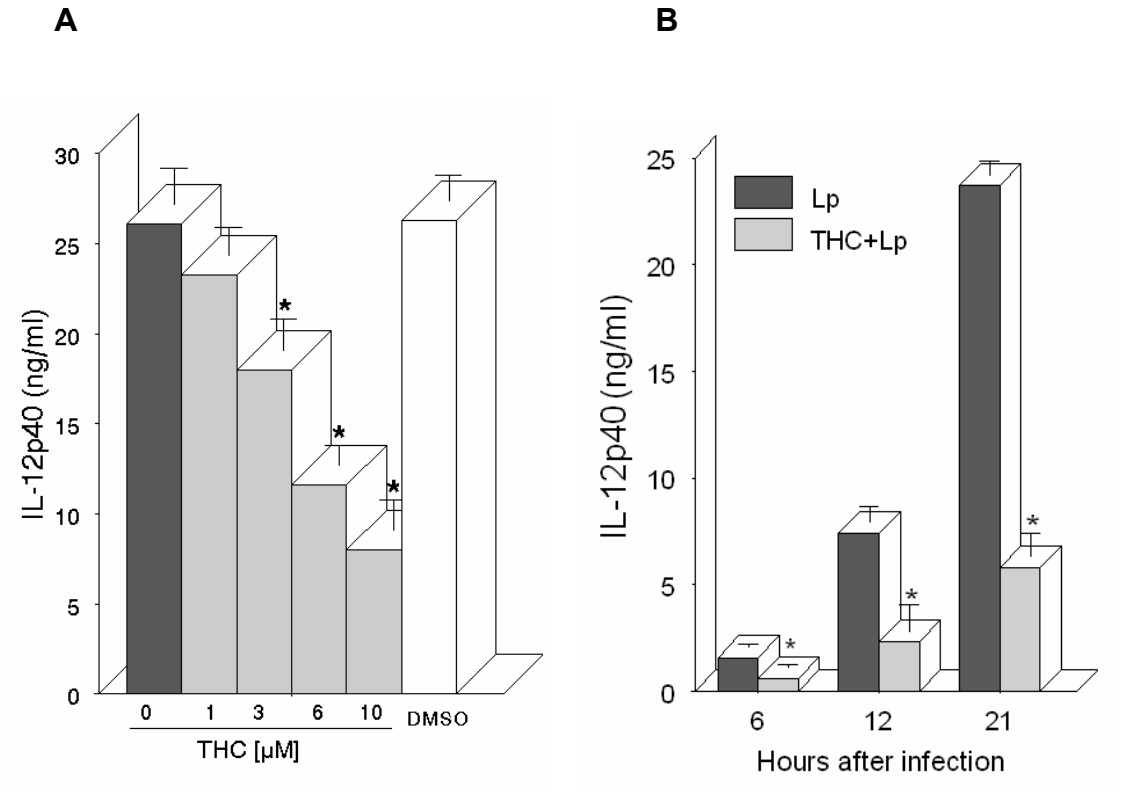


Figure 3. THC, in a concentration-dependent manner, suppressed IL-12p40 production in Lp-infected BM-DCs from BALB/c mice. (A) Lp-infected BM-DCs were treated with different concentrations of THC or the highest concentration of DMSO (vehicle control) for 24 hr. (B) Using THC at 10μM, THC suppresses IL-12p40 production at 6, 12, and 21 hr. Data represent the mean of 3-5 experiments ± S.E.M.. *P< 0.05, compared to Lp group (THC 0 μM).

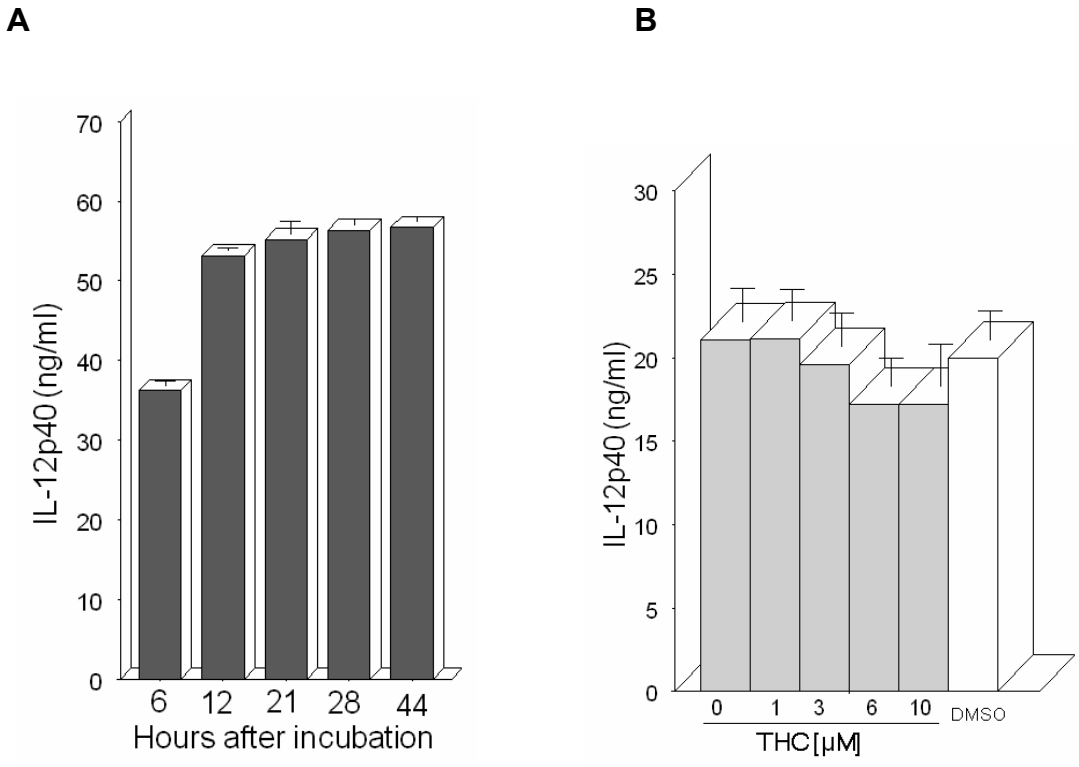


Figure 4. No significant effect of THC on LPS-induced IL-12p40 from DCs. (A) immature BM-DCs were incubated with LPS (1 µg/ml). Supernatants were collected at indicated time points and IL-12p40 measured by ELISA. (B) DCs were stimulated with LPS (10 ng/ml) and treated with different concentrations of THC or the highest concentration of DMSO (vehicle control) for 24 hr.

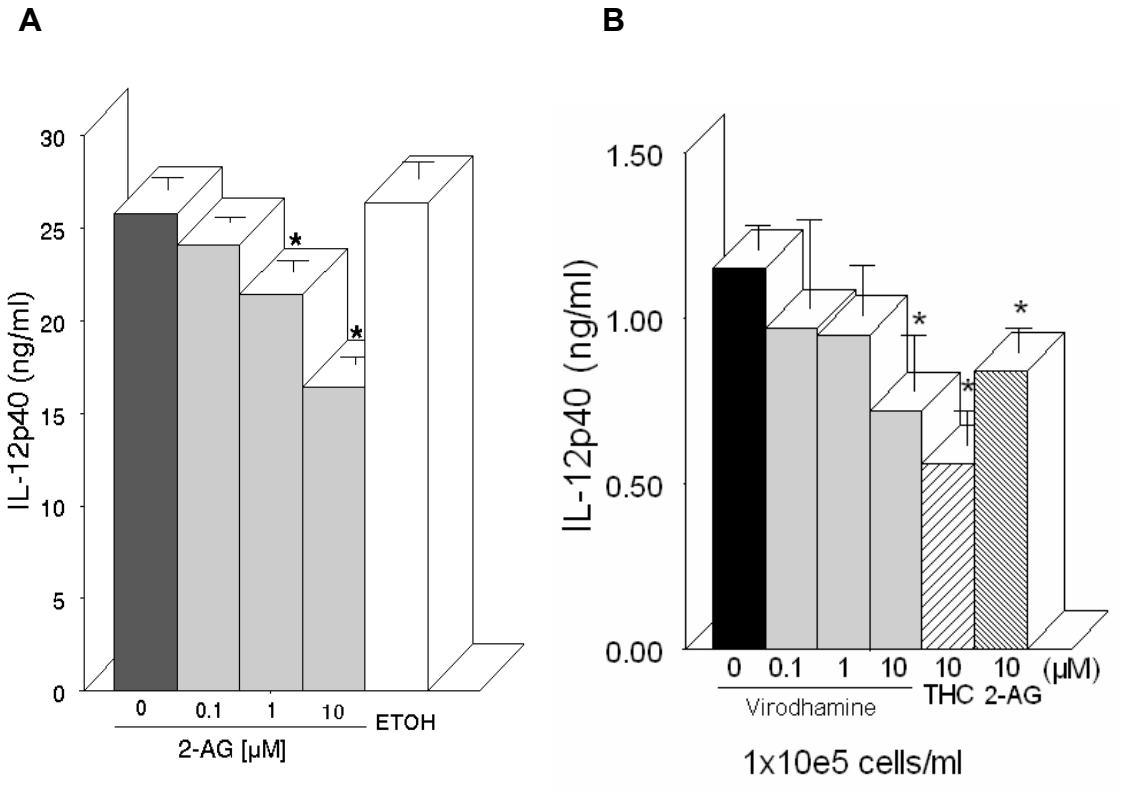


Figure 5. Cannabinoid receptor agonists 2-AG and Virodhamine in a concentration-dependent manner, suppressed IL-12p40 production in Lp-infected bone marrow-derived DCs from BALB/c mice. Lp-infected DCs were treated with different concentrations of 2-AG or the highest concentration of Ethanol (ETOH, vehicle control) (A) or Virodhamine with different concentrations or THC or 2-AG at 10 μM (B) for 24 hr. IL-12p40 was detected by ELISA and the data represent the mean of 3-5 experiments ± S.E.M.. *P< 0.05, compared to 2-AG 0 μM (A) or Virodhamine 0 μM (B).

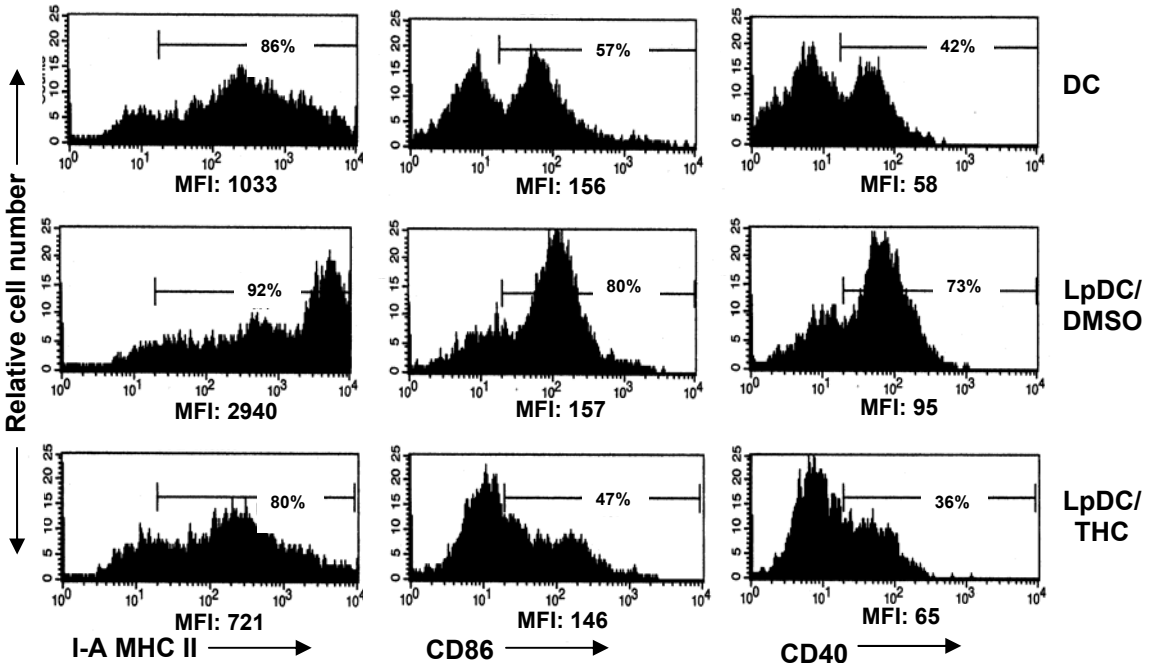


Figure 6. THC suppressed the expression of maturation markers on Lp infected-DCs. Cell surface markers were determined by flow cytometry on DCs treated for 48 hr in various ways: uninfected (DC); Lp-infected and DMSO treated (LpDC/DMSO); and Lp-infected and THC (10 μM) treated (LpDC/THC). Data are expressed as percent expression (%) of the surface marker and mean fluorescence intensity (MFI) of the population for the marker. Data are representative of 4 experiments.

Table 1. THC treatment suppressed DC maturation markers. Cell surface markers were determined in uninfected DCs (DC), Lp-infected and DMSO treated cells (LpDC/DMSO) or THC (10 μM) treated cells (LpDC/THC) by flow cytometry after 48 hr treatment.

Percent Expression

	DC	LpDC/DMSO	LpDC/ THC
MHC class II	**82.3 ± 11.2[a]**	**87.2 ± 6.0**	**79.4 ± 4.8**
CD86	**63.8 ± 3.1**	**81.7 ± 2.8[#]**	**53.7 ± 5.4***
CD40	**37.7 ± 7.9**	**70.1 ± 5.6[#]**	**36.5 ± 9.6***

Fluorescent intensity per cell

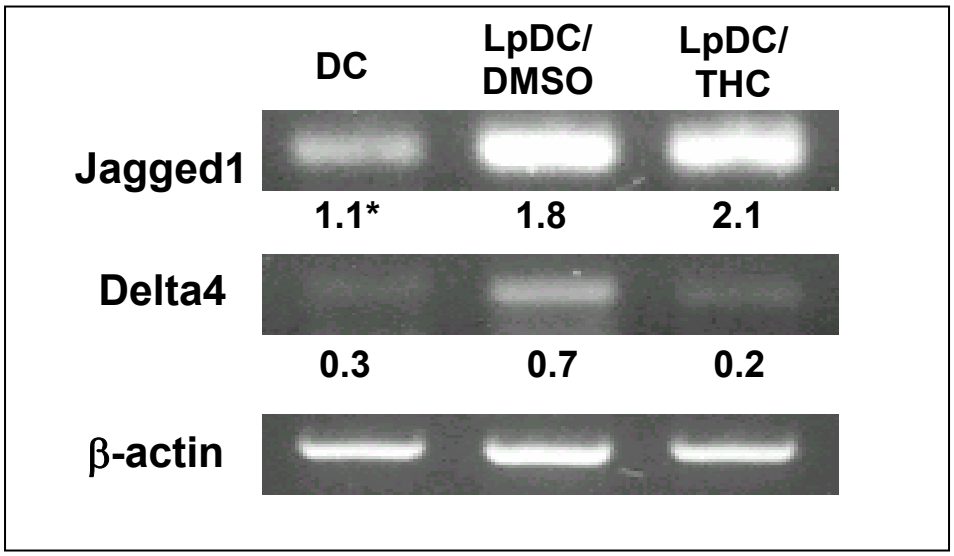
	DC	LpDC/DMSO	LpDC/ THC
MHC class II	**2212.1 ± 499.5 [b]**	**2693.7 ± 313.1**	**1433.5 ± 348.6***
CD86	**147.1 ± 8.6**	**183.7 ± 22.1**	**133.2 ± 12.7**
CD40	**83.4 ± 21.4**	**130.3 ± 29.6**	**81.0 ± 9.2**

a = Percent +/- SEM, n=4

b =Mean fluorescence intensity +/- SEM; n=4

= $p < 0.05$ versus the uninfected DC control

* = $p < 0.05$ versus LpDC/DMSO group



* Target to β –actin ratio

Figure 7. THC suppressed the expression of Delta 4 in Lp-infected DCs (LpDC/THC) as compared to infected DCs treated with DMSO (LpDC/DMSO). DCs were uninfected or infected with Lp and treated with DMSO or THC (10 μM) for 18 hr. Jagged1, Delta4, and β-actin mRNAs were amplified by RT-PCR. Data are representative of 3 experiments.

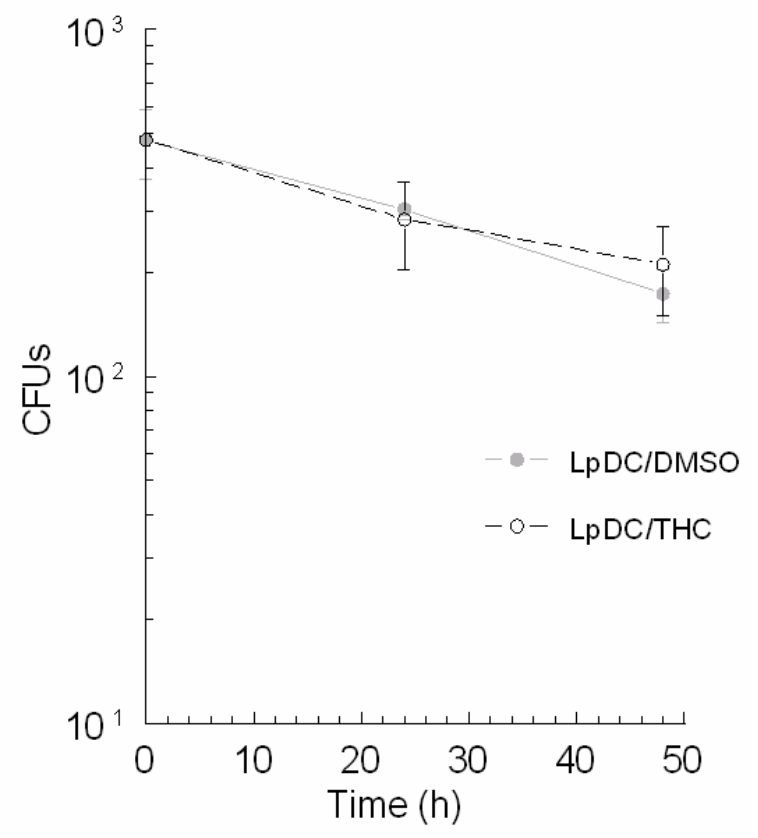
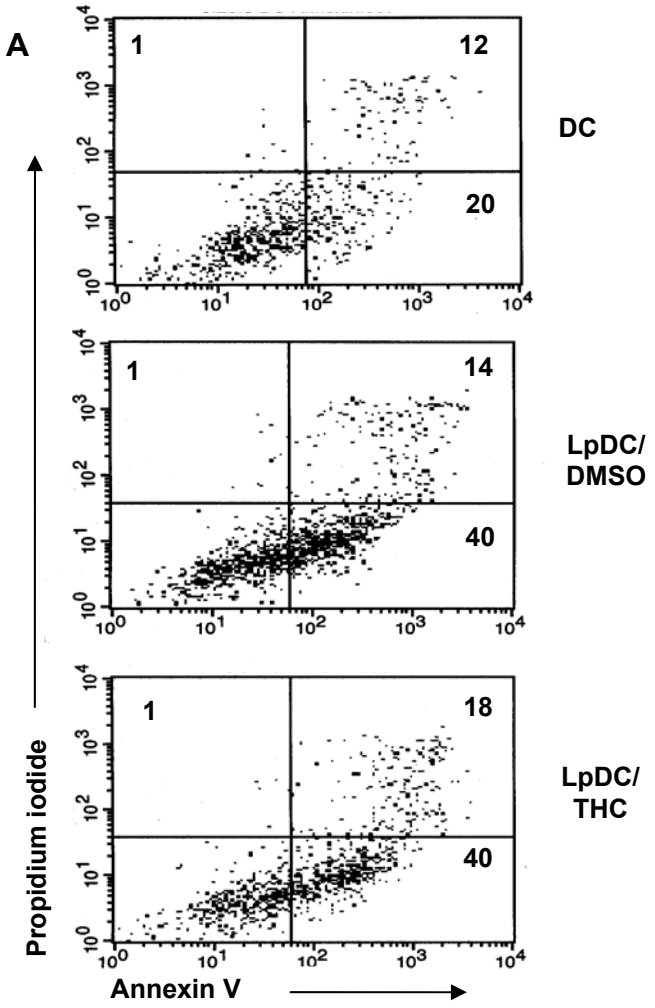
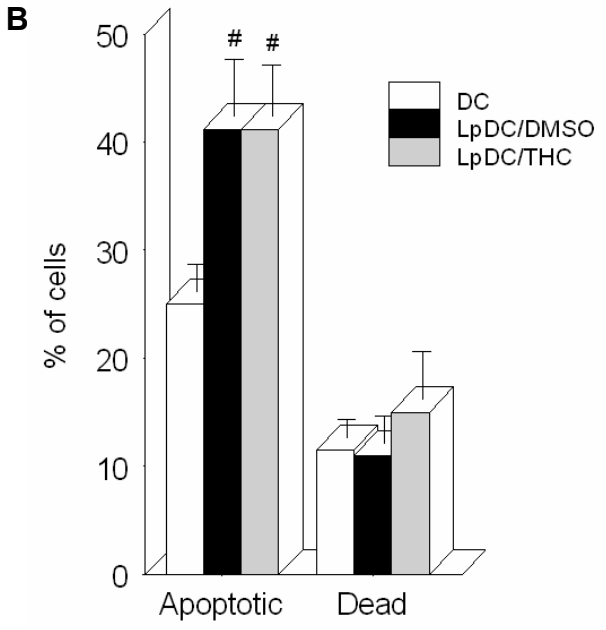


Figure 8. Lp uptake and survival were not affected by THC treatment of Lp infected-DCs. DCs infected with Lp for 30 min, washed twice to remove non-internalized bacteria and treated with DMSO or THC (10 μM) for 0, 24, 48 hr. At various time post-infection, cell lysates were harvested and plated on agar medium, and CFUs of Lp determined by plate counts at 72 hr. Data represent the mean of 3 experiments +/- SEM.

Figure 9. **Apoptosis and cell death were not affected by THC treatment.** Cultures of DCs were untreated (DC) or treated for 24 hr with either DMSO (LpDC/DMSO) or THC at 10 μM (LpDC/THC) and apoptosis and cell death were analyzed by staining with Annexin V and propidium iodide, respectively. (A) Dot plot of propidium iodide and Annexin V staining; representative of 3 similar experiments. (B) Percent of apoptotic cells (Annexin V+; propidium iodide-) and dead cells (propidium iodide+); mean +/- SEM , n=3. #, $p < 0.05$ versus DC control.


A
1
12
20
DC
1
14
40
LpDC/
DMSO
1
18
40
LpDC/
THC
Propidium iodide
Annexin V


B
50
40
30
20
10
0
% of cells
#
#
DC
LpDC/DMSO
LpDC/THC
Apoptotic
Dead

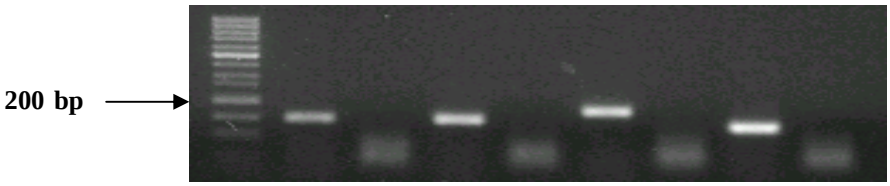


Figure 10. Demonstration by RT-PCR of cannabinoid receptor, TRPV1 and β-actin message in RNA from bone marrow-derived DCs. Negative RT represents PCR results when the reverse transcriptase is left out of reaction mix.

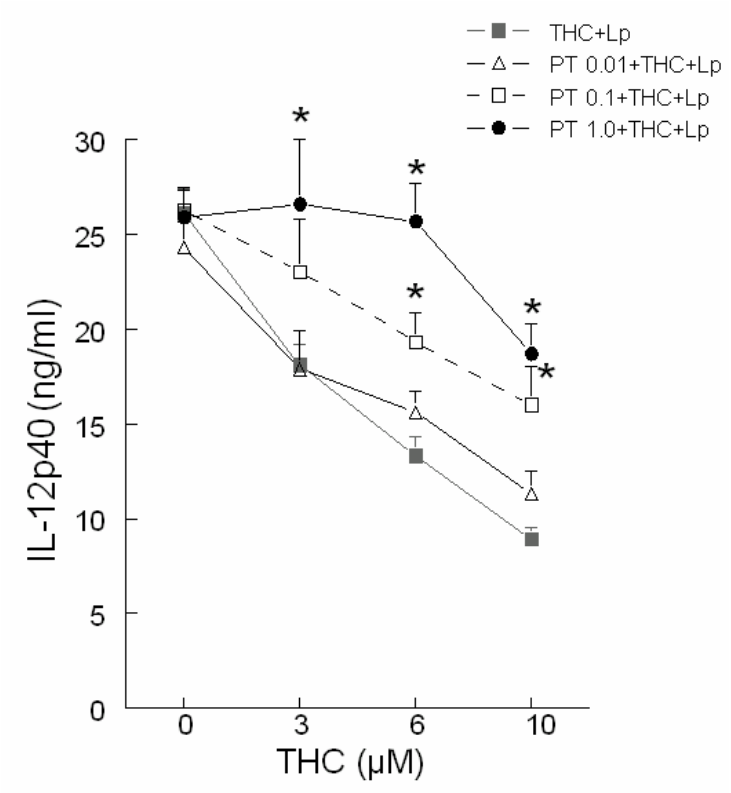
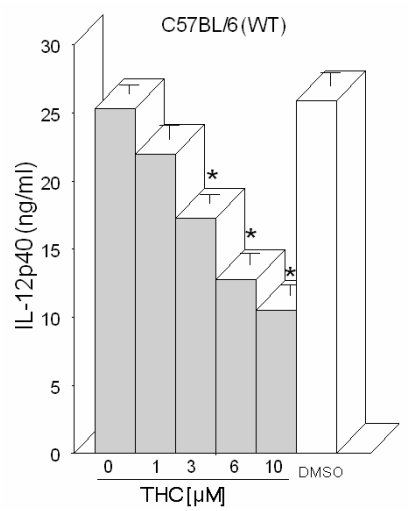


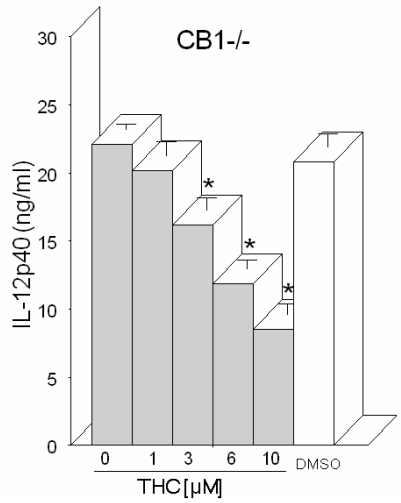
Figure 11. Pertussis toxin, the G_i signaling inhibitor, attenuated the suppression effect of THC on IL-12p40. Bone marrow-derived DCs were preincubated with pertussis toxin at 0.01, 0.1, 1.0 ng/ml 18 hr prior to Lp infection and treated with THC 0, 3, 6 and 10 μM. Cells were incubated for 24 hr and data represent the mean of 3 experiments ± S.E.M.. *P< 0.05, compared to THC + Lp group.

Figure 12. THC suppressed IL-12p40 production in Lp-infected bone marrow-derived DCs from C57BL/6 mice. (A) THC suppresses IL-12p40 in DCs from wild type (WT) mice. (B and C) THC suppresses IL-12p40 in cannabinoid CB_1 receptor knockout (CB_1-/-) and CB_2 receptor knockout (CB_2-/-) mice. Lp-infected DCs were treated with different concentrations of THC or the highest concentration of DMSO (vehicle control). Cells were cultured for 24 hr. Data represent the mean of 3 experiments ± S.E.M.. *P< 0.05, compared to THC 0 μM.

A


C57BL/6 (WT)
IL-12p40 (ng/ml)
30
25
20
15
10
5
0
0
1
3
6
10
DMSO
THC [µM]
*
*
*

B


CB1-/-
IL-12p40 (ng/ml)
30
25
20
15
10
5
0
0
1
3
6
10
DMSO
THC [µM]
*
*
*

C

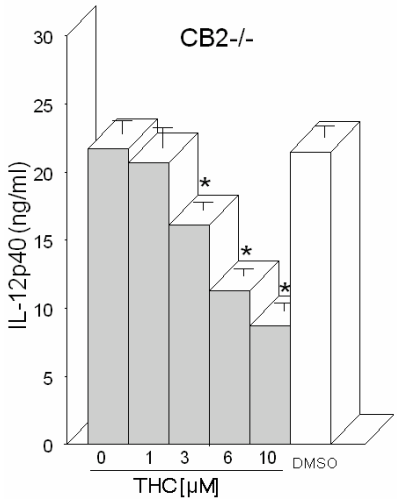

CB2-/-
IL-12p40 (ng/ml)
30
25
20
15
10
5
0
0
1
3
6
10
DMSO
THC [µM]
*
*
*

Table 2. Attenuation effect of SR compounds on THC-induced suppression of IL-12p40 in Lp-infected bone marrow-derived DCs from cannabinoid receptor knockout mice. Lp-infected DCs were pretreated with SR144528 ($CB_1^{-/-}$ DCs) or SR141716A ($CB_2^{-/-}$ DCs) at 0.01, 0.05, or 0.1 μM for 30 min prior to THC (3, 6 or 10 μM). Cells were cultured for 24 hr and the data represent the mean of 4 experiments ± S.E.M.. SR alone at these doses had no effect on IL-12p40 production (not shown). The percentage of attenuation is computed from 1-[IL-12p40(Lp-SR/THC/Lp)/Lp]/[IL-12p40(Lp-THC/Lp)/Lp].

CB_1-/-

THC (μM)	3	6	10
SR144528 (μM)		Attenuation (%)	
0.01	20.0 ± 6.0	18.2 ± 8.8	8.4 ± 1.5
0.05	59.5 ± 8.7	37.0 ± 5.7	15.8 ± 5.0
0.1	69.6 ± 7.0	44.7 ± 2.5	27.9 ± 2.0

CB_2-/-

THC (μM)	3	6	10
SR141716A (μM)		Attenuation (%)	
0.01	24.1 ± 7.8	18.9 ± 4.2	3.0 ± 1.6
0.05	38.0 ± 5.1	33.2 ± 4.1	14.7 ± 5.8
0.1	61.1 ± 8.5	39.9 ± 7.0	24.2 ± 5.7

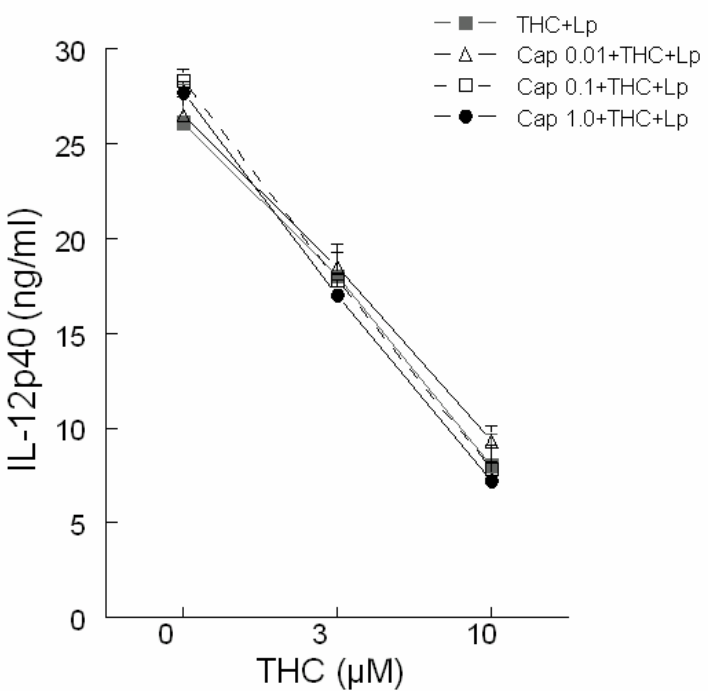


Figure 13. Vanilloid receptor inhibitor Capsazepine did not antagonize the suppression effect of THC on IL-12p40. Lp-infected DCs were pretreated with Capsazepine at 0.01, 0.1 and 1 μM for 30 min prior to THC (3 or 10 μM). Cells were cultured for 24 hr and the data represent the mean of 3 experiments ± S.E.M..

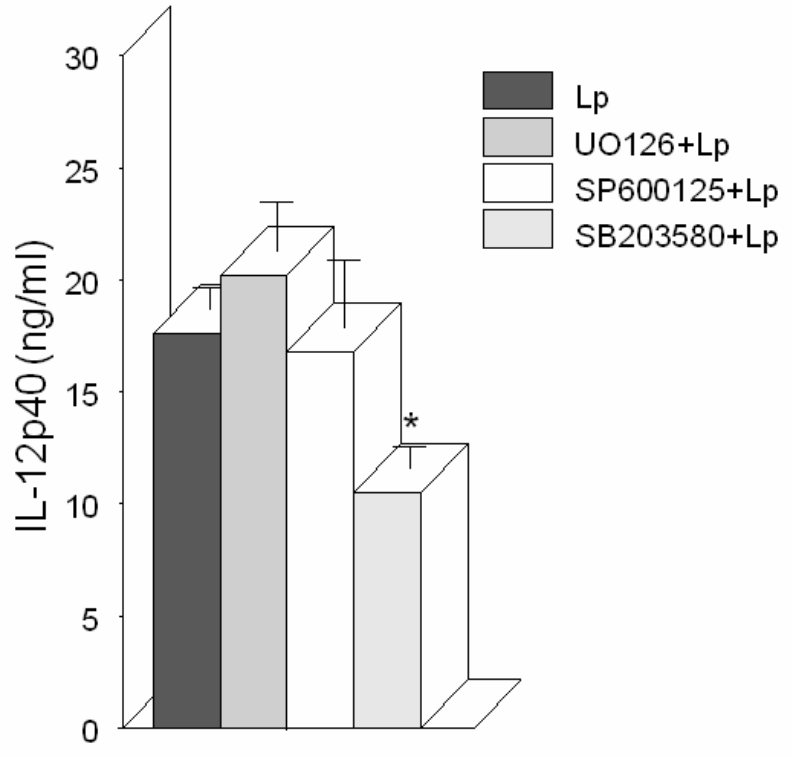


Figure 14. p38 MAP kinase, but not JNK or ERK was required for IL-12p40 production in Lp-infected DCs. Lp-infected DCs were treated with ERK inhibitor UO126 (5 μM), or JNK inhibitor SP600125 (5 μM) or p38 inhibitor SB203580 (5 μM) for 18 hr. IL-12p40 in supernatants was detected by ELISA and the data represent the mean of 3 experiments ± S.E.M.. *P< 0.05, compared to Lp group.

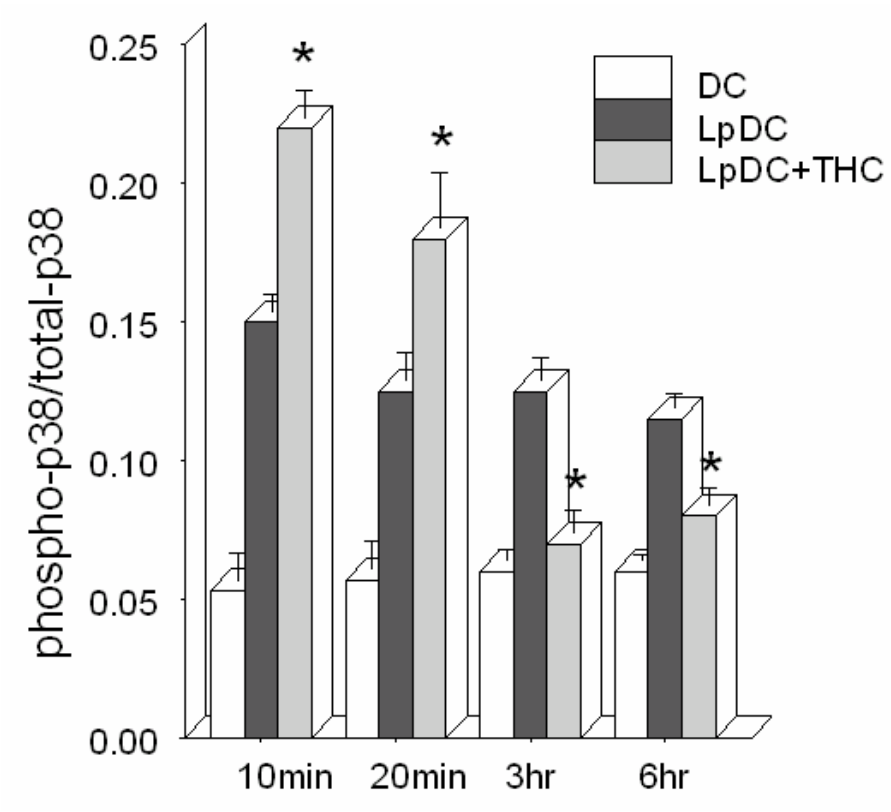


Figure 15. THC modulated p38 MAP kinase activation. DCs were either uninfected or infected with Lp or treated with THC (6 μM) following infection. Cells were fixed at indicated time points and the levels of p38 kinase expression were measured by cell-based ELISA. Data are expressed as the ratio of phosphorylated p38 to total p38 in DCs. Data represent the mean of 3 experiments ± S.E.M.. *P< 0.05, compared to Lp group.

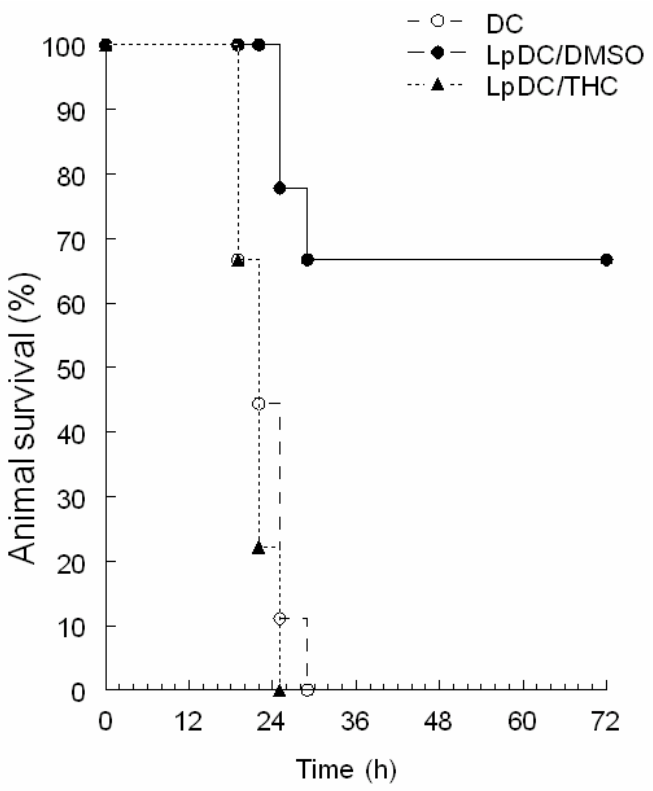


Figure 16. THC impaired immunization potential of Lp-loaded DCs. Naïve mice were iv immunized with DCs ($0.5x10^6$ cells/mouse) two to three times at 7 day intervals prior to being challenged with a lethal dose of Lp (1.7 - 2.0 $x10^7$/mouse). The DCs were either not loaded with Lp (DC group) or loaded with Lp and treated for 24 hr with either DMSO (LpDC/DMSO group) or THC, 10 μM (LpDC/THC group). Mice were monitored for survival and the data represent 9 mice per group from 3 experiments.

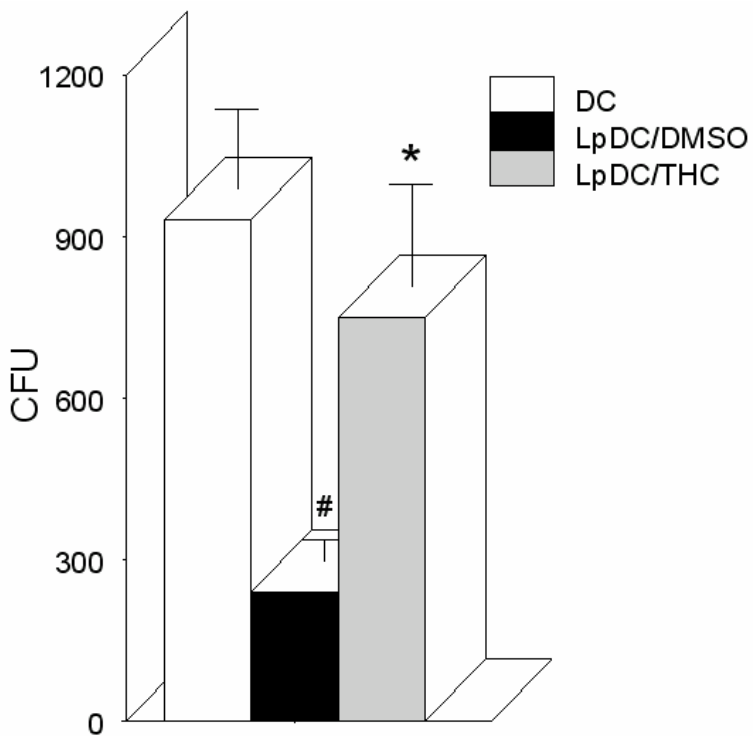


Figure 17. THC treatment of Lp-loaded DCs inhibited immunizing potential as evidenced by increased bacterial burden. Mice were iv injected with 0.3-0.5x10^6 DCs loaded or not in vitro with Lp and treated with DMSO (LpDC/DMSO), or THC at 10 μM (LpDC/THC) for 24 hr. Then, mice were challenged 7-9 days later with a sublethal dose of Lp (7x10^6 Lp/mouse), spleens isolated 24 hr post-infection, and colonies forming units (CFU) determined by plate counts. Data presented as the mean CFU +/- SEM for 4 mice per group. # and * ($p < 0.05$) versus the uninfected DC control and LpDC/DMSO group, respectively.

Figure 18. THC treatment of Lp-loaded DCs inhibited the expression of Th1 cytokines in splenocytes from immunized mice. Mice were iv injected with control DCs (0.3-0.5x10^6), Lp-loaded and DMSO treated DCs (LpDC/DMSO), and Lp-loaded and THC treated DCs (LpDC/THC) as in Figure17. Seven to 9 days post-injection, splenocytes were harvested and stimulated *in vitro* with killed Lp (10^7/ml) for 24 hr and cytokines (IL-12p40, IFN-gamma and IL-4) detected in supernatants by ELISAs (A, B and C). Data represent the mean of 5 experiments +/- SEM. # and * ($p < 0.05$) versus the control DC and Lp/DMSO group, respectively.

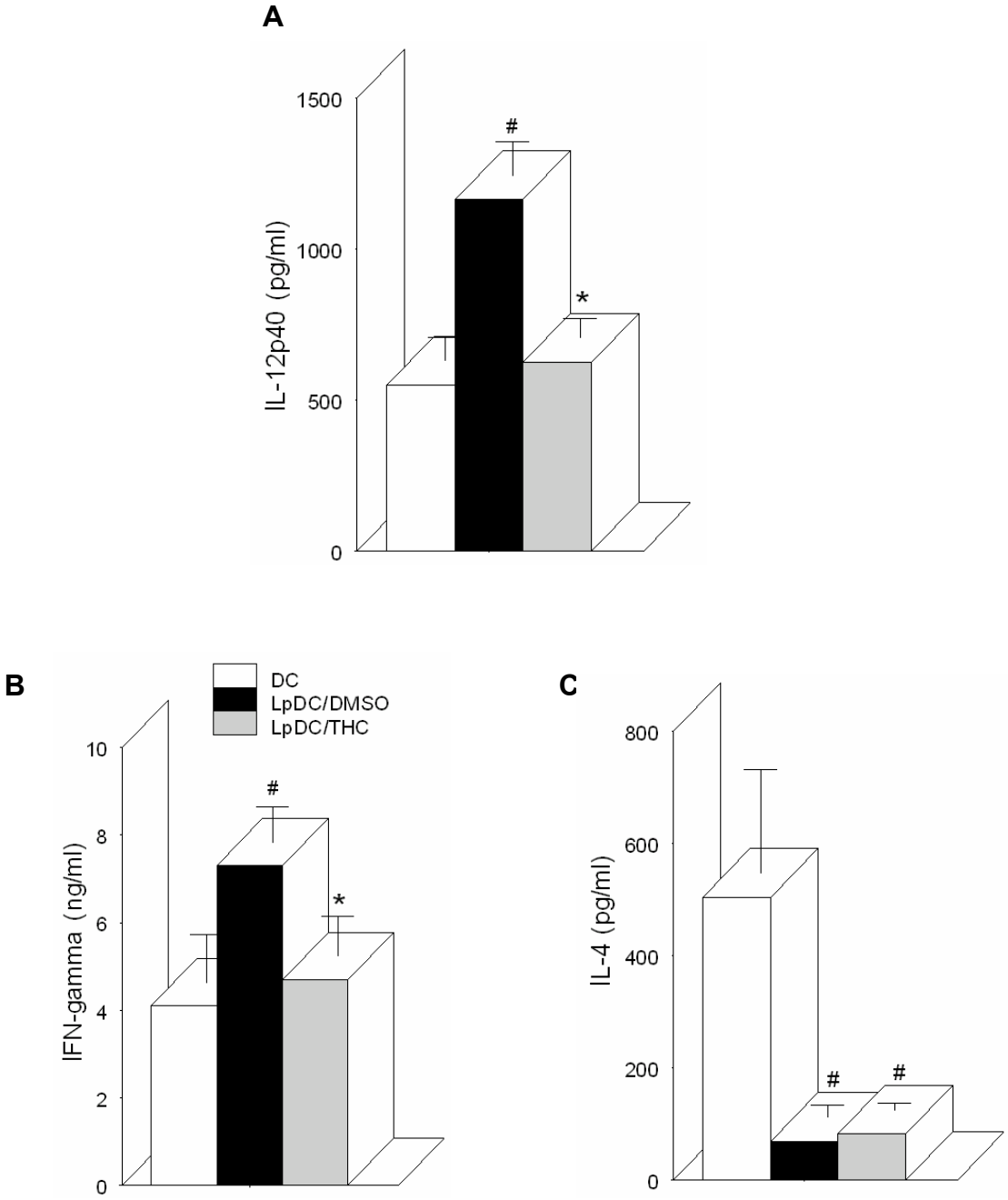
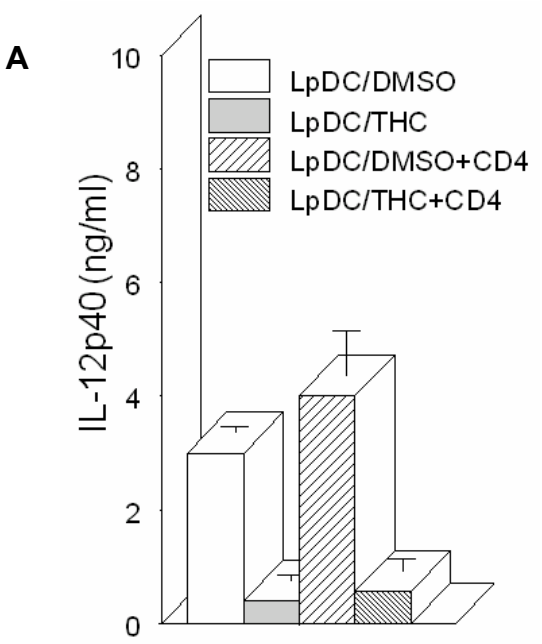
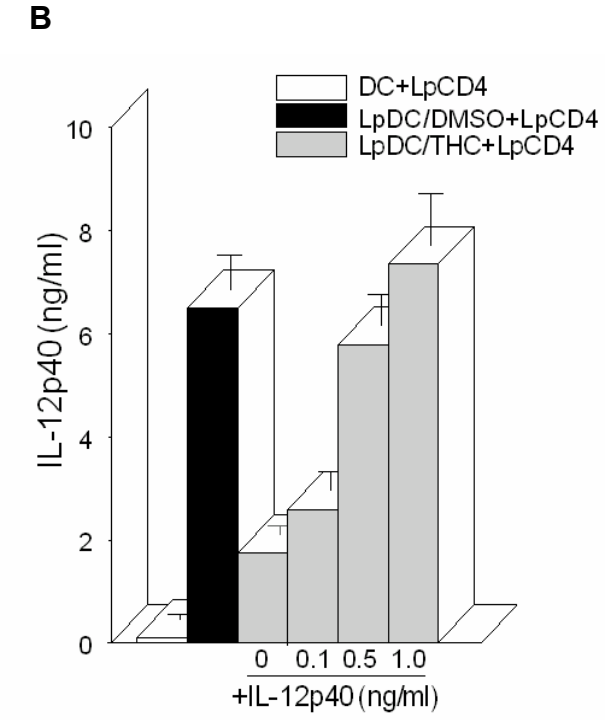
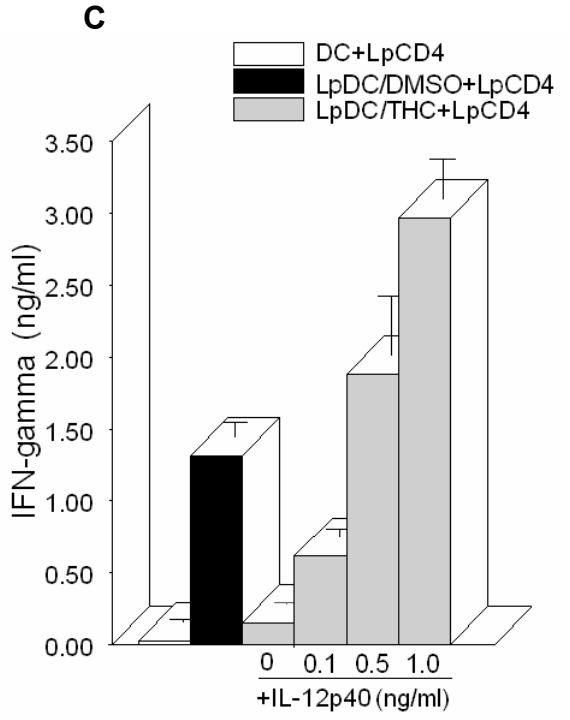

A
1500
1000
500
0
IL-12p40 (pg/ml)
#
*
B
DC
LpDC/DMSO
LpDC/THC
10
8
6
4
2
0
IFN-gamma (ng/ml)
#
*
C
800
600
400
200
0
IL-4 (pg/ml)
#
#

Figure 19. THC suppression of DC IL-12p40 production mediated loss of Th1 polarization of Lp-primed CD4+ T cells. Cytokines were measured in 24 hr supernatants of co-cultures containing DCs and either Lp-primed or unprimed CD4 T cells. Primed T cells were obtained 5 days post-infection from the spleens of mice infected with a sublethal dose of Lp. (A) IL-12p40 measured in co-cultures containing Lp-loaded DCs treated with DMSO or THC (10 μM) (LpDC/ DMSO or LpDC/THC) co-cultured with unprimed CD4 T cells. (B) IL-12p40 measured in co-cultures treated as in panel A and containing primed T cells; recombinant IL-12p40 was added in increasing amounts. (C) IFN-gamma measured in co-cultures as in panel B and treated with IL-12p40. Data are representative of 4 experiments.


A
LpDC/DMSO
LpDC/THC
LpDC/DMSO+CD4
LpDC/THC+CD4
IL-12p40 (ng/ml)
0
2
4
6
8
10


B
DC+LpCD4
LpDC/DMSO+LpCD4
LpDC/THC+LpCD4
IL-12p40 (ng/ml)
0
2
4
6
8
10
0 0.1 0.5 1.0
+IL-12p40 (ng/ml)


C
DC+LpCD4
LpDC/DMSO+LpCD4
LpDC/THC+LpCD4
IFN-gamma (ng/ml)
0.00
0.50
1.00
1.50
2.00
2.50
3.00
3.50
0 0.1 0.5 1.0
+IL-12p40 (ng/ml)

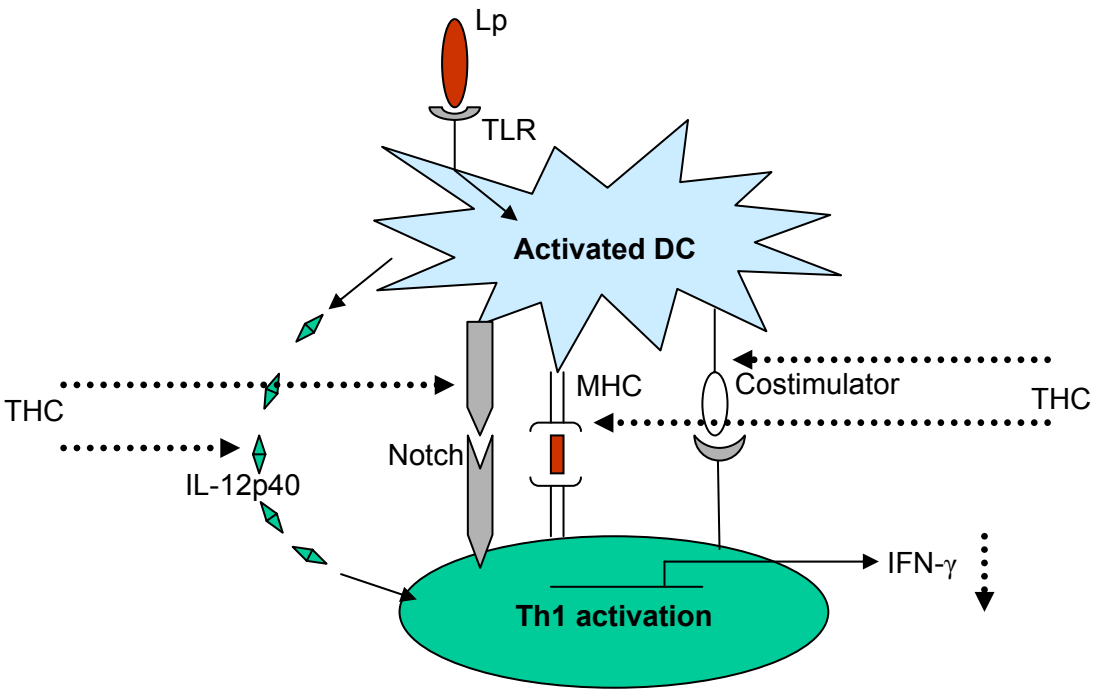


Figure 20. THC suppresses Th1 activation signals. Signals delivered by DCs are required for Th1 activation (2, 152). THC suppressed Th1 immune response is associated with THC impaired DC function leading to the insufficiency of (pointed by dot arrows):

1. Polarizing signals including suppressed IL-12p40 production and Notch ligand Delta 4 expression;

2. Antigen-specific signal through suppressed MHC class II expression;

3. Co-stimulatory signal as low expression of CD86 and CD40.

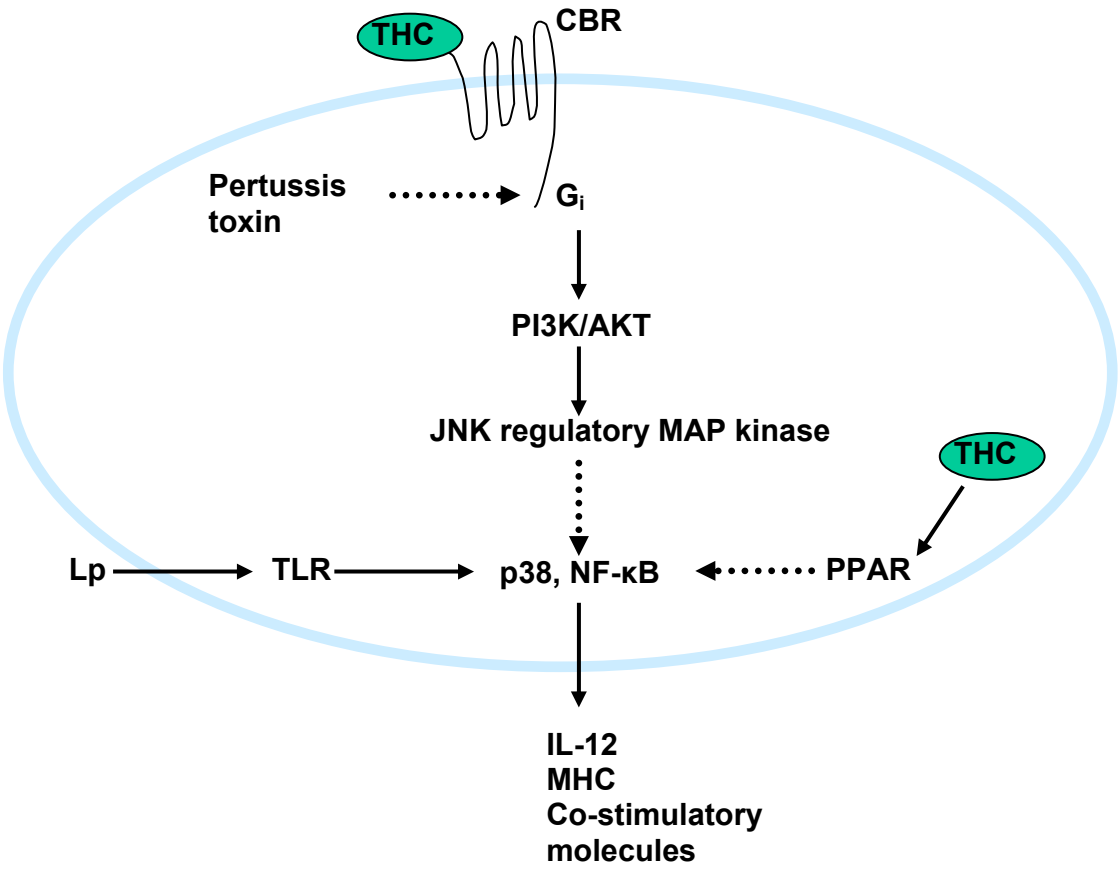


Figure 21. Postulated signaling pathways involved in THC suppression effect on DCs. Lp infection, through TLRs and activation of NF-kappaB and p38, leads to DC activation and maturation. THC may suppress these effects via G_i/PI3K/AKT pathway and subsequent regulatory JNK activation; and PPAR stimulation to suppress NF-kappaB transactivation. Normal arrow represents positive regulation; dot arrow represents negative regulation.

DISCUSSION

DCs are potential targets of cannabinoids

Cannabinoids, such as THC, have been shown to be immunosuppressive and anti-inflammatory (83) and can polarize adaptive immunity in mice away from Th1 immunity and toward Th2 immunity (89, 134). Polarization by cannabinoids has recently been reviewed (87, 158) and Th1 suppression occurs following treatment with endocannabinoids (112, 139), in drug-treated T cell cultures stimulated with allogenic DCs (191), following LPS and drug injection into *C. parvum*-primed mice (168), and in immune responses to tumor cells (192). In at least one of these studies (168), cannabinoids were shown to suppress production of the DC cytokine, IL-12. Because DCs are a primary link between innate and adaptive immunity, we postulated that DCs might be a target of cannabinoid action and that suppressing the function of these cells could be a key event in the drug-induced suppression of Th1 polarization.

Intracellular bacterial pathogens can modulate the host cell intracellular meilu thus providing an environment supportive of intracellular growth and survival. Lp, for example, a microbe ubiquitous in the aquatic environments and the causative agent for Legionnaires' disease in humans, can replicate within

eukaryotic host cells such as protozoa and human phagocytic and epithelial cells (68, 131). It has been demonstrated that T helper cell cytokines such as the type 1 cytokine IFN-γ can activate host cells to control Lp infection and other intracellular pathogens (25, 91, 130). *In vivo* experiments have shown that Lp causes severe infection in IFN-γ-deficient mice and IFN-γ treatment results in increased clearance of the bacteria from the lungs (166). Suppression of immunity to Lp in mice has also been reported by our group following injection of THC along with a decrease in Th1 acivity and associated cytokines such as IFN-γ and IL-12 (88). DCs are central in the orchestration of the formation of Th1 immunity from naïve CD4+ by providing multiple signals after pathogen priming. One set of these signals is provided by members of the IL-12 family shown to play a pivotal polarizing role in Th1 differentiation (106, 138, 145). IL-12p40 protein, which is a subunit shared by two IL-12 family members, IL-12p70 and IL-23, has been shown to be indispensable in Th1 development either by combining with other subunits or on its own (31, 66, 147, 184).

THC suppressed IL-12p40 production in Lp-infected DCs

DCs are a major cell source of IL-12. These cells can be purified from various mouse tissues such as spleen and bone marrow and studies with cultures of these cells have shown that a number of different bacteria induce IL-12p40 (79). We speculated that cultured DCs would also produce IL-12 in response to Lp infection since these bacteria have been shown to increase

serum levels of IL-12 in infected mice (89). We also wanted to study dendritic cell cultures in order to see if THC added to the cultures directly suppressed IL-12 production by these cells. Initially, we observed (Figure 2) Lp infection of DC cultures triggered a high level of IL-12p40 production that was significantly inhibited by THC treatment in a concentration-dependent manner (Figure 3). Thus, it appears that Lp like other microbes directly induces DCs to produce IL-12p40 (79). Also, because DCs are a major source for serum IL-12 levels in infected animals, the current *in vitro* results suggest THC is suppressing DC function resulting in decreased serum IL-12 production in treated animals (89).

THC did not suppress LPS-induced IL-12p40 secretion

The immediate recognition of microbes by the innate immune system plays a crucial role in host defense (121). This recognition process is based on the detection of unique pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors of the host such as TLRs. Activation of TLRs not only plays a critical role in activation of innate immunity but also in the activation of adaptive immune responses (120) through the production of IL-12p40 and other polarizing factors. One of the most commonly studied TLRs is TLR4 and we found that the TLR4 agonist LPS induced a significant amount of IL-12p40 in our DC cultures, which is consistent with previous reports (127). However, the LPS effect was not suppressed by THC as was the case with Lp infection (Figure 4). This finding is not surprising in light of recent studies showing that production

of IL-12p40 after infection with Lp was inhibited in macrophages from TLR2(-/-) but not inTLR4(-/-) mice suggesting that TLR2, but not TLR4, is involved in cytokine production by Lp (5). Archer *et al.* also reported that TLR2 but not TLR4 is involved in MyD88-dependent responses to Lp infection in mice (7). Besides TLR2, other TLRs might be stimulated by Lp, such as TLR9, which recognizes bacterial unmethylated CpG motifs and has been shown to be involved in the IL-12 response to Lp (135) and other bacteria such as *Brucella abortus* (72) and *Mycobacterium tuberculosis* (9). Thus, our data suggests that the mechanism of THC suppression of IL-12p40 does not involve the signaling cascades associated with all of the TLRs, for example TLR4, but might be associated with others such as TLR2 and 9.

THC suppressed DC maturation and polarizing molecules

Besides producing polarizing cytokines such as IL-12, DCs also promote the maturation of T helper cells by the production of helper cell-surface proteins such as MHC class II and co-stimulatory molecules (79). To further explore the basis of THC modulation of DC function, we examined THC effects on the expression of these surface markers. The results showed THC treatment markedly reduced the expression of MHC class II and the co-stimulatory molecules CD86 and CD40 (Figure 6 and Table 1). The mechanism for this is unclear at this time; however, signaling through cannabinoid receptors could be involved because ligation of similar receptors (i.e., G_i-linked) has been shown to

modulate DC maturation from human PBMCs (33). In addition to the above markers, Notch ligand expression on DCs has been reported to be critical for T cell differentiation and various stimuli have been shown to induce the expression of either Jagged1 (Th2 polarizing) or Delta4 (Th1 polarizing) (6). Our data showed mRNAs for Jagged1 and Delta4 were induced in DCs after Lp infection; however, THC treatment significantly suppressed the expression of the Th1 polarizing Delta4 ligand but had little effect on Jagged1 (Figure 7). These results, coupled with our other findings that the THC-treated DCs are deficient in promoting Th1 polarizitation, support the previous conclusion that the Delta ligands induce Th1 cells (6). The THC effect could involve cannabinoid receptors because G protein-coupled receptors activated through G_s have been shown to increase Jagged ligand expression and polarize to Th2 (6); therefore, cannabinoid receptors, coupled to G_i, might be expected to suppress Delta ligands and Th1 polarization as seen in our study along with inhibiting IL-12 as shown by others (95).

THC did not affect Lp survival and apoptosis in DCs

To initiate T helper activation, DCs must in addition to producing cytokines and co-stimulatory molecules, take up and process the bacteria for pathogen-related antigen presentation. The cells must also survive the bacterial infection and we wanted to see if antigen uptake by and survival of DC was also affected by cannabinoids. In contrast to macrophages, wherein intracellular growth of Lp

was observed (161), we observed that DCs restricted Lp growth with moderate killing over time (Figure 8). THC-treated DCs showed a similar restriction and time course of intracellular infection, suggesting Lp handling was the same in both groups. THC has been reported to induce apoptosis under certain conditions in mouse DCs (43) and Lp infection has also been shown to be apoptotic in macrophages and other cells (3, 53). To see if these treatments together were adversely affecting DC survival, we examined necrosis and apoptosis induction. Our results with Annexin V staining (Figure 9) showed that Lp infection induced limited apoptotic activity in DCs similar to that observed in other cell types (3, 53). THC treatment did not increase the level of apoptosis and both treatments had no effect on cell necrosis as measured by propidium iodide staining (Figure 9). The minimal effect of THC on apoptosis we observed is at variance with that observed previously. We used the drug at 10 μM and this concentration was shown previously to induce Annexin V positivity in 80% of the cells (43). However, the previous studies were done using serum-free medium while ours were done with medium containing fetal calf serum which is known to reduce the potency (and toxicity) of the added cannabinoids (90). From these results, it is concluded that THC treatment is not suppressing T helper polarization by either altering the intracellular life cycle of Lp or by causing enhanced apoptosis and death of the DCs.

The involvement of cannabinoid receptors and MAP kinases in THC effect

The previous studies showed that cannabinoid treatment of DCs altered the polarizing phenotype of the cells in terms of cytokine and co-stimulatory production. To uncover the mechanism of these effects, we wanted to see if cannabinoid receptors might be involved. One previouos report showed that CB_1 and CB_2 mRNA and protein were expressed in human blood-derived DCs but the function of these receptors was not studied (113). In the current study, we showed mRNA for both receptors was also expressed in mouse bone marrow-derived DCs and that CB_2 message appeared to be more abundant than CB_1 in these cells (Figure 10). Furthermore, using DCs from receptor knockout mice as well as using CB_1 and CB_2 antagonists, we observed that these receptors mediated suppression but only at the lowerTHC concentrations used in these studies (Figure 12 and Table 2). CB_1 and CB_2 are G_i protein-coupled receptors and activate genes by means of the heterotrimeric G protein complex containing the α-GTP subunit and the $\beta\gamma$ dimer (19). Stimulation of cannabinoid receptors with agonists has been shown to modulate various signaling and transcription factors; for example, cAMP (164) and AP1 and MAP kinase (50) were decreased in splenocytes following treatment with cannabinoids, and drug effects were attenuated by pertussis toxin which inactivates G_i (78). In the current study, we showed that pertussis toxin treatment completely attenuated the effect of low THC concentrations; however, complete attenuation could not be achieved at

higher drug concentrations (Figure 11). This finding along with the partial attenuation observed with cells from receptor knockout mice treated with receptor antagonists suggested that, at the low concentration of THC, the drug is suppressing cytokine production through activation of a G_i-coupled mechanism linked to both cannabinoid receptor and non-receptor pathways. The G_i-coupled mechanism might involve the $\beta\gamma$ dimer of the G_i that is reported to suppress IL-12 through MAP kinases (19, 52). Many studies have shown cannabinoids modulate the activation of MAP kinases (38). These protein kinases are widely used throughout evolution in many physiological processes and are involved in all aspects of immune responses, including the regulation of naïve Th cells differentiation into Th1 or Th2 cells (167) and the production of IL-12 either directly or indirectly through production of Th2 type cytokines (103, 179, 189). MAP kinase activation and IL-12 production have been shown to be induced by G protein-coupled receptors in response to ligands such as bacterial toxins, neuropeptides and chemoattractants (19). Our data showed that p38 MAP kinase, but not Erk or JNK, was involved in IL-12p40 production upon Lp infection (Figure 14). Furthemore, THC treatment enhanced p38 phosphorylation initially followed by suppression with time (Figure 15). Recently, using human monocytes, MAP kinase phosphorylation was observed to accompany the inhibition of LPS/IFN□-induced IL-12 production by the mediator C5a and this was shown to involve the PI3K/Akt signaling pathway (95). Together, these data suggest THC and other G_i ligands suppress IL-12 through MAP kinase phosphorylation.

TRPV1 was not involved in THC effect

Besides cannabinoid receptors, our data suggest that THC might also be working through other moieties (CBRX?) with affinity for THC but a relatively low sensitivity to cannabinoid receptor antagonist. For example, it has been reported that a non-CB_1 G protein-coupled receptor in mouse brain was activated by AEA and WIN55212-2 but not other cannabinoid receptor agonists and was relatively insensitive to antagonism with SR141716A (20). In addition, the attenuating activity of SR141716A on the antinociceptive effect of cannabinoids in mice was found to be receptor agonist-dependent (185), being most potent following CP55,940 injection and least potent following AEA injection. Vanilloid receptors, such as TRPV1, might also be involved because they respond to endocannabinoid treatment but are relatively insensitive to SR141716A (96, 180, 196). There are no previous reports of TRPV1 expression in DCs and we are the first to show in the current study that DCs readily express TRPV1 mRNA (Figure 10). Because we could find no reports that THC activates TRPV1 receptors on immune cells, we tested the possibility that TRPV1 was mediating the suppression of IL-12p40 in our system. DCs, pretreated with the selective TRPV1 antagonist, capsazepine, were still completely suppressed by THC treatment suggesting that TRPV1 was not involved in the drug effect on IL-12p40 production (Figure 13).

The above results show that Lp infection of bone marrow-derived DCs

leads to an increase in IL-12p40 and that co-treatment with THC significantly suppresses this effect. We also showed that inhibition of G_i signaling completely attenuates the THC effect at low cannabinoid concentrations; however, inhibiting CB_1 and CB_2 at low doses only partially attenuates the THC effect and inhibition of TRPV1 has no effect. These data suggest that THC is working through G_i signaling in DCs to suppress IL-12p40 and is also working partially through cannabinoid receptors in addition to possibly a third receptor with sensitivity to the effects of THC but low sensitivity to the action of SR compounds. In addition, we found that besides THC, two endocannabinoids, 2-AG and Virodhamine had a significant suppression effect on IL-12p40 production from Lp-DCs (Figure 5) suggesting the possible involvement of endocannabinoids in host anti-inflammatory responses. Other cannabinoids, either endogenous or synthetic, however, did not seem to have significant effect on DC IL-12p40 production. These observations support the speculation that other targets besides CB1 and CB2 may exist and that other signaling mechanisms may be involved in different cannabinoid-mediated actions.

THC impaired the immunization potential of Lp-loaded DCs

The results so far show that THC treatment can change the polarizing phenotype of DCs to one that is non-supportive of Th1 polarization. To further examine the drug effects and to functionally test the polarizing potential of the DCs, we utilized a cellular immune reconstitution model of infection with Lp. DCs

are potent antigen-presenting cells and loading them with microbial antigens has been shown to immunize mice against infection with various pathogens (104, 171, 181). In the current study, we showed that DCs loaded *in vitro* with Lp and injected into mice immunized and protected the mice to a subsequent lethal Lp infection (Figure 16). Evidence of immunity to Lp was documented because the number of CFUs in the spleens of the animals was reduced after injection with Lp-loaded DCs (Figure 17). Thus, it appears, that bone marrow-derived DCs have good immunizing potential when loaded or infected with Lp in culture and then injected into recipient mice. The immunity generated was probably Th1 because replication of Lp in the mice is known to depend on the activity of these helper cells (134). The next question involved what effect THC treatment had on the immunizing potential of the loaded DCs. These results showed that THC treatment attenuated the DC function as evidenced by the reduced ability to protect against infection and to reduce the number of CFUs in the spleens of infected mice (Figure 16 and 17).

THC inhibited Th1 activity induced by Lp-loaded DCs

The above results suggested that THC treatment suppressed the Th1 polarizing potential of Lp-loaded DCs. To test this more directly, splenocytes from immunized mice were analyzed for Th1 and Th2 cytokine production *in vitro* in response to Lp antigens. The results in Figure 18 showed that, as expected, immunization with Lp loaded DCs (LpDC/DMSO group) caused an increase in

the Th1 polarizing cytokines IL-12p40 and IFN-γ, probably produced by the antigen-presenting cells and lymphocytes in the splenocyte cultures; however, this effect was attenuated in splenocytes from animals immunized with THC-treated DCs (LpDC/THC group), suggesting drug treatment suppressed their polarizing potential. The mechanism surrounding the regulation of Th1 activity is controversial. IL-4, a key cytokine in promoting Th2 cells (173), was originally proposed to also inhibit Th1 development; however, more recently this has been challenged and IL-4 has been shown to actually promote Th1 development by inducing DCs to produce IL-12 (15). Because of these uncertainties and because we had previously observed a decrease in IL-12 and increase in IL-4 production in THC-treated and Lp-infected mice (89), we examined for IL-4 production by splenocytes from immunized mice. Figure 18 shows that immunization suppressed IL-4 production; furthermore, immunization with THC-treated cells had no effect on this suppression. These findings suggest several things. First, as expected, immunization by Lp led to a decrease in IL-4 producing splenocytes as confirmation of Th1 polarization in response to this agent (134). Second, it appears that immunization with drug-treated and Lp-loaded DCs, causes a decrease in Th1 activity with no concomitant increase in Th2 activity, at least as measured by IL-4 producing splenocytes. Third, these results would appear to be at odds with our previous finding that THC injection along with Lp infection led to an increase in IL-4. However, there are a several differences in the two models and the increase in IL-4 observed in our previous study (89) occurred within hours after infection and was transient in nature

whereas, in the current study, IL-4 was measured in splenocytes taken 7 - 9 days following immunization. Also, in the previous study, THC was injected into Lp-infected mice, whereas in the current model, mice were immunized with THC-treated and Lp-loaded DCs. It is perhaps not surprising that IL-4 production is regulated differently under these varying conditions of THC administration; furthermore, the studies suggest that Lp immunization under these conditions results in primarily a Th1 response and that the suppression of this response by THC is mediated by mechanisms independent of IL-4 production.

THC suppression of DC IL-12p40 production mediated loss of Th1 polarization

The previous studies suggested that THC treatment of DCs suppressed their Th1 polarizing function and we wanted to test this directly using an *in vitro* co-cultivation paradigm. Because IL-12 is potent in directing Th1 cell differentiation (109) and because we found that THC suppressed this cytokine in Lp-infected DC cultures, we evaluated the Th1 promoting potential (as measured by IFN-γ production) of both Lp-loaded DCs and drug-treated DCs in co-culture with Lp-primed CD4 T cells as well as the role of IL-12p40 in the response. The data showed that co-culturing Lp-loaded DCs with Lp-primed T cells led to enhanced IL-12p40 and IFN-γ production compared to co-culture with unprimed T cells, and that THC treatment of the DCs attenuated the production of polarizing cytokines (Figure 19). Furthermore, the addition of exogenous IL-

12p40 to the THC-treated cultures restored the robust production of both IL-12p40 and IFN-γ, suggesting that drug suppression of the p40 protein was responsible for inhibiting Th1 polarization in the cultures. IL-12p70 and IL-23 have also been shown to polarize toward Th1 (75), but drug effects on these cytokines were not involved because they could not be detected in the supernatants (data not shown). In addition, IL-10 has been shown to suppress Th1 polarization under various conditions (187), but again it was not detected in the supernatants so was probably not involved in the drug effect. These results show that IL-12p40 can be a major Th1 polarizing protein and that its suppression by THC is a key factor in the drug-induced inhibition of Th1 cell development. In this regard, several other reports have shown that the p40 protein has Th1 polarizing potential and affinity for IL-12 receptors (22, 31, 66).

In conclusion, our results show that a major cellular target of THC-induced immune suppression of Th1 immunity is the dendritic cell and that the drug attenuates polarizing function by suppressing IL-12p40 production and the expression of MHC class II, co-stimulatory molecules and Notch ligand Delta4 (Figure 20). Although, THC might compromise the host's ability to fight infection it also might be of use in the treatment of chronic inflammatory diseases such as coeliac disease and Crohn's disease (63, 153), rheumatoid arthritis (177, 182) and systemic lupus (62). The data reported here suggest that THC and other cannabinoids may belong to this group of anti-inflammatory drugs.

SUMMARY

The legalization of marijuana has been debated for years because many people consider it a relatively benign substance with possible beneficial medicinal properties. Indeed, it is not as addictive as are other drugs such as heroin, cocaine or nicotine (122). However, in the aspect of health impact, the potential risks and benefits of marijuana use remain to be further explored. The current study investigated the role of cannabinoids in the immune system and found multiple effects of THC on the cellular immune function of DCs.

Based on the data presented in this study, it appears that during Lp infection, immature DCs are capable of capturing Lp and its associated antigen. This leads to maturation and activation of DCs with up-regulation of MHC class II and other costimulatory molecules and the production of proinflammatory cytokines. Secretion of IL-12p40 by DCs plays a critical role in activation and differentiation of Th1 cells which are required for control and elimination of infection; activation of macrophages and B cells and the production of pathogen specific neutralizing antibodies such as IgG2a, which also contribute to host resistance and clearing of the infection. THC, however, suppresses IL-12p40 production in DCs after Lp infection and down-regulates DC MHC class II and several costimulatory molecules including CD86, CD40 and Notch ligand, leading

to the depressed Th1 development and immunosuppression against Lp challenge.

Current data suggest the involvement of G_i signaling and cannabinoid receptors in THC suppression of IL-12p40 and probably also MHC (67) and costimulatory molecules. The mechanism may involve the activation of phosphatidylinositol 3-kinase-protein 3 kinase B/Akt pathway and its downstream MAP kinases activation. It was recently reported that inhibition of IL-12 by ligands for G_i-protein-coupled receptors was mediated by the activation of PI3K/Akt signaling and the MAP kinase JNK (95). G_i receptors for THC may also stimulate Akt and JNK and consequently suppress the activation of NF-kappaB and p38 required for DC activation and maturation (190) (Figure 21). Our results also suggest the existence of mechanisms other than G_i signaling. One possible candidate is PPAR, which belongs to the nuclear receptor superfamily and can be activated by cannabinoid ligands such as 2-AG (154) and AJA (24). Recent studies showed that activation of PPARγ or PPARα may help to control inflammatory responses through inhibiting NF-kappaB transactivation, which is critical for DC immunostimulatory functions (141, 156, 190) (Figure 21).

Due to their immunomodulation effects, cannabinoids have been recently used in the treatment of several chronic inflammatory disorders. For example, diabetes is associated with the autoimmune destruction of pancreatic β cells and insulin deficiency. Studies show that THC attenuates the severity of disease in an animal model and is associated with the suppression of IFN-γ, TNF-α and IL-12 mRNA expression in pancreatic tissues from mice (99). More recently,

Steffens et al. reported that oral administration of THC significantly inhibited the progression of atherosclerosis in a murine model, suggesting a beneficial effect of cannabinoids in this chronic inflammatory disease of the vessels (157, 170). Moreover, in a model of multiple sclerosis, an autoimmune inflammatory disease of the CNS, the cannabinoid, Win55,212, showed suppression of disease associated with the suppression of T cell proliferation and IFN-γ secretion and other pro-inflammatory, Th1-type cytokines, such as IL-1β and TNF-α (35). All of these studies suggest the potential therapeutic uses of cannabinoids. Our studies reported here address possible cellular mechanisms of cannabinoid-induced suppression of Th1 immunity. By investigating the effect of cannabinoids on DCs controlling antigen-driven T-cell polarization, our results not only serve to expand our understanding of cannabinoid effects in the immune system, but also provide clues to the cellular mechanism involved and therefore offer more specific targets for future drug development in the treatment of chronic inflammatory diseases.

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