Cannabinoid Treatment Suppresses the T-Helper Cell-Polarizing Function of Mouse Dendritic Cells Stimulated with Legionella pneumophila Infection

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ABSTRACT

Marijuana cannabinoids, such as δ-9-tetrahydrocannabinol (THC), suppress type 1 T-helper 1 (Th1) immunity in a variety of models, including infection with the intracellular pathogen Legionella pneumophila (Lp). To examine the cellular mechanism of this effect, bone marrow-derived dendritic cells (DCs) were purified from BALB/c mice and studied following infection and drug treatment. DCs infected in vitro with Lp were able to protect mice when injected prior to a lethal Lp infection; however, the immunization potential of the Lp-loaded cells along with Th1 cytokine production was attenuated by THC treatment at the time of in vitro infection. In addition, THC-treated and Lp-loaded DCs were poorly stimulated in culture-primed splenic CD4⁺ T cells to produce interferon-γ; however, this stimulating deficiency was reversed by adding recombinant interleukin (IL)-12p40 protein to the cultures. Moreover, THC treatment inhibited the expression of DC maturation markers, such as major histocompatibility complex class II and costimulatory molecules CD86 and CD40 as determined by flow cytometry and suppressed the Notch ligand, Delta4, as determined by reverse transcription-polymerase chain reaction. However, THC treatment did not affect other DC functions, such as intracellular killing of Lp, determined by colony-forming unit counts of bacteria, and Lp-induced apoptosis, determined by annexin V staining. In conclusion, the data suggest that THC inhibits Th1 activation by targeting essential DC functions, such as IL-12p40 secretion, maturation, and expression of costimulatory and polarizing molecules.

Cannabinoid exposure increases susceptibility to infections (Klein and Cabral, 2006), and the mechanisms of this involve many factors, including suppression of Th1 immunity (Newton et al., 1994; Klein et al., 2000, 2005). The mechanism responsible for suppressing Th1 immunity and polarizing to the Th2 response, however, remains elusive. In the current study, we postulated that the cannabinoids suppress adaptive immunity and Th1 polarization by inhibiting the function of dendritic cells (DCs).

DCs are professional antigen-presenting cells and able to induce and regulate innate and adaptive immune responses. DCs reside in peripheral tissues, capturing and processing pathogens and microbial products and then migrating to lymphoid organs, wherein DCs present these antigens to quiescent T cells (Kapsenberg, 2003). 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 and expression of MHC and costimulatory molecules (Kapsenberg, 2003). Furthermore, expression of the Notch ligands, Jagged and/or Delta, is increased, which play a critical role in Th2 and Th1 polarization of naive CD4 T cells (Amsen et al., 2004). We recently reported that THC treatment significantly suppresses IL-12p40 production in mouse bone marrow-derived DC in culture (Lu et al., 2006). In the current study, we show that THC treatment of DCs impairs their immunizing and T-helper-polarizing function by inhibiting IL-12 production and the expression of essential costimulatory cell-surface proteins, such as MHC class II, CD86, CD40, and the Notch ligand, Delta4. This suggests that DCs might be a central target of THC-induced immune suppression.

Materials and Methods

Mice, Bacteria, and Drugs. BALB/c mice, 7 weeks of age, were obtained from NCI, National Institutes of Health (Fredericksburg, MD) and housed and cared for at the University of South Florida. This work was supported by National Institutes of Health Grants DA03646, DA10683, and AI45169. Article, publication date, and citation information can be found at http://jpet.aspetjournals.org. doi:10.1124/jpet.106.106381.

ABBREVIATIONS: Th1, type 1 T-helper cell; Th2, type 2 T-helper cell; THC, δ-9-tetrahydrocannabinol; DCs, dendritic cells; Lp, Legionella pneumophila; LpDC, Lp-loaded DC; IFN-γ, interferon-γ; IL, interleukin; MHC, major histocompatibility complex; DMSO, dimethyl sulfoxide; GM-CSF, granulocyte-macrophage colony-stimulating factor; PBS, phosphate-buffered saline; CFU, colony-forming unit; mAb, monoclonal antibody; ELISA, enzyme-linked immunosorbent assay; i.v., intravenous; bp, base pair; PE, phycoerythrin.
Health Sciences Center animal facility, which is fully accredited by the American Association for Accreditation of Laboratory Animal Care. Serogroup 1, *Legionella pneumophila* (Lp; M124), is a virulent strain and obtained from a case of legionellosis at Tampa General Hospital (Tampa, FL) and cultured on buffered charcoal-yeast extract medium (Difco, Detroit, MI) as described previously (Klein et al., 2000). After 48 h, bacteria were adjusted spectrophotometrically to a working concentration. THC, obtained from the Research Technology Branch of the National Institute on Drug Abuse (Rockville, MD), was first diluted in dimethyl sulfoxide (DMSO) at 20 mg/ml and then in 5% fetal calf serum RPMI 1640 medium to a working concentration of 10 μM.

**Preparation and Treatment of Bone Marrow-Derived Dendritic Cells.** Bone marrow cells were collected, as described previously (Lu et al., 2006), from femurs and tibias of the BALB/c mice at 8 to 12 weeks of age. Isolated cells were adjusted to 1.0 × 10⁷/ml in six-well cell culture plates (Corning Life Sciences, Acton, MA) in RPMI 1640 medium supplemented with 5 μM 2-mercaptoethanol, 2 mM L-glutamine, 1% antibiotic/antimycotic solution (Sigma, St. Louis, MO), 5% heat-inactivated fetal calf serum (HyClone; Logan, UT), and 10 ng/ml granulocyte/macrophage colony-stimulating factor (GM-CSF) (BD Biosciences PharMingen, San Diego, CA). After culture overnight, nonadherent cells were removed and the adherent cells were reincubated with fresh GM-CSF-containing medium for an additional 7 to 9 days, during which time the dendritic cells became nonadherent and were harvested. The purity of the cells was determined by flow-cytometry staining using fluorescence-conjugated monoclonal antibodies (mAbs) to CD11b and CD11c (BD Biosciences PharMingen). The purity was ~100% CD11b⁺ and greater than 75% CD11c⁺ cells. The dendritic cells were either uninfected or infected with Lp at a ratio 10:1 for 30 min. Dendritic cells were then washed twice to remove noninternalized Lp and resuspended to 10⁶ cells/ml and treated with either DMSO (vehicle control) or THC at 10 μM in the culture medium minus GM-CSF for 18 to 48 h. In studies with CD4 T cells, cells were obtained from mice intravenously (i.v.) infected (primed) with a sublethal dose of Lp (7 × 10⁷), and the spleens were removed 5 days postinfection. 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 Biosciences PharMingen). Isolated CD4 T cells were then dispensed in 24-well cell culture plates (Corning Life Sciences) and cocultured with DCs (CD4, DC = 10:1) in either the absence or presence of recombinant IL-12p40 (BD Biosciences PharMingen) for 24 h followed by cytokine analysis.

**Animal Injections and Tissue Sampling.** Mice were immunized i.v. with 0.3 to 0.5 × 10⁶-treated DCs suspended in PBS, and 7 to 9 days later, spleens were isolated from mice. Single-cell suspensions of splenocytes (2 × 10⁶ cells/ml) were cultured with formalin-killed Lp (10⁷/ml) for 24 h, and supernatants were collected for cytokine detection. In contrast, DC-treated mice were challenged i.v. with live Lp (sublethal dose, 7 × 10⁶) diluted in pyrogen-free saline. Spleens were obtained after 24 h, and CFUs of Lp were counted. In other experiments, mice were immunized i.v. with DCs (0.5 × 10⁶) for two or three times at 7-day interval and challenged i.v. with a lethal dose of Lp (1.7–2.0 × 10⁷), and survival of mice was monitored.

**Cytokine Detection by ELISA.** IL-12p40, IL-4, IL-23, and IL-10 were determined using a sandwich ELISA with antibody pairs from BD Biosciences PharMingen. In 96-well enzyme immunoassay plates (Corning Life Sciences), 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°C. The wells of the plate were blocked with 150 μl of 3% bovine serum albumin/0.05% Tween 20 in PBS (IL-12p40 and IL-23; 5 μg/ml), or in PBS (anti-IL-4 and anti-IL-10; 2 μg/ml) overnight at 4°C. The wells of the plate were blocked with 150 μl of 3% bovine serum albumin/0.05% Tween 20 in PBS (IL-12p40 and IL-23) or 0.5% bovine serum albumin/0.05% Tween 20 in PBS (IL-4, IL-10) and incubated for 1 h. The culture supernatants or serial dilutions of cytokine standards were added and incubated for 1 to 2 h followed by biotinylated detection antibodies (2 μg/ml, 50 μl) for 1 h and streptavidin-horseradish peroxidase (1:1000 in 50 μl) for 30 min. The plates were washed between each addition. The tetramethyl benzidine (Sigma) substrates were developed for 5 to 30 min; the reaction was stopped with 1 N sulfuric acid and read at 450 nm on an E₅₀₅ macroplate 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 OptiEIA sets (BD Biosciences PharMingen) according to the manufacturer’s instructions.

**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 at 10 μM for 48 h. After incubation, the cells were treated with fluorochrome-conjugated mAbs (BD Biosciences PharMingen) at 4°C for 30 min and then washed in PBS containing 2% bovine growth serum and fixed in 1% paraformaldehyde. Cells were analyzed using flow cytometry. 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 Biosciences PharMingen).

**mRNA Measurement by Reverse Transcription-Polymerase Chain Reaction.** Total RNA was extracted from DC cultures by standard techniques using TRIzol (Sigma) and quantitated using RiboGreen RNA QuantiKation Kit (Invitrogen). The extracted RNA was treated with DNase using a DNA-free kit from Ambion (Austin, TX). Total RNA (1 μg) was reverse-transcribe with avian myeloblastosis virus reverse transscriptase (Promega, Madison, WI). The cDNA product was amplified using TaKaRa TaqDNA polymerase (Fisher; Atlanta, GA). The primer pairs were used as follows: Jagged1, forward primer, 5′-AGAAGTCAGAGTTCAAGGGCGTTCC-3′, reverse primer, 5′-AGTAAGGACCTGGCTACACAGCAAC-3′ (113-bp product); Delta4, forward primer, 5′-AGTTGCACTTGGTTTACAGC-3′, reverse primer, 5′-CAATACACACTCTTCCTC-CTTC-3′ (123-bp product); and β-actin, forward primer, 5′-ATGGA-TGACGATATGCCTG-3′, reverse primer, 5′-ATGAGATTGTGTCGTA-GGT-3′ (530-bp product). Polymerase chain reaction was performed in a Mastercycler (Eppendorf; Westbury, NY) for 35 cycles (Jagged1 and Delta4) or 28 cycles (β-actin) and 60°C annealing. Polymerase chain reaction products were visualized with ethidium bromide in 2% agarose gels.

**Bacteria Growth Determined by CFU Assay.** After 24-h infection, spleens from infected mice were homogenized in Hanks’ balanced salt solution. In studies of Lp growth in DC cultures, cells were lysed by with 0.1% saponin (Sigma) and diluted in Hanks’ balanced salt solution. Homogenized spleens or lysed DCs were plated on buffered charcoal-yeast extract agar plates and incubated at 37°C for 72 h. CFU counts were determined on an AutoCount apparatus (Dyntech Labs, Chantilly, VA).

**Cell Viability and Apoptosis Detection.** DC viability and apoptosis were detected using the annexin V-fluorescein isothiocyanate (BD Biosciences PharMingen). In brief, uninfected or infected cells (10⁶), treated with DMSO (LpDC/DMSO) or THC (LpDC/THC) at 10 μM for 24 h, were washed twice with PBS and incubated with annexin V-fluorescein isothiocyanate (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.

**Statistical Analysis.** Comparisons between groups were performed using the two-tailed Student’s t test. A value of p < 0.05 was accepted as indicating significance.

**Results**

THC Impairs Immunization Potential of Lp-Loaded DCs. Because of their pivotal role in stimulating T cells, DCs loaded with specific antigens have been used as immunizing vehicles in numerous studies of tumor therapies (Zitvogel et
al., 1996; Celluzzi and Falo, 1998) and infectious diseases (Moll and Berberich, 2001). To test whether DCs loaded with Lp would induce an immune response to Lp or whether THC would impair this ability, we treated Lp-infected DCs with THC (LpDC/THC) or drug vehicle DMSO (LpDC/DMSO) for 24 h. The concentration of THC used in this study was 10 μM, which we previously showed to be the lowest concentration exerting a significant effect on IL-12p40 production in Lp-infected DC cultures (Lu et al., 2006). DCs without infection and drug treatment were incubated for the same time as infected DC cultures (Lu et al., 2006). To test whether DCs loaded with Lp would induce an immune response to Lp or whether THC suppression of DC IL-12p40 production mediated an attenuation of the immunizing potential of these cells (Fig. 1). As in Fig. 2, mice were immunized with DCs only, LpDC/DMSO, or LpDC/THC, and 7 to 9 days later, splenocytes from mice treated with unloaded DCs or loaded DCs treated with THC (Fig. 2). 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 the Expression of Th1 Type Cytokines in Splenocytes from Immunized Mice.** Type 1 cytokines, including IL-12 and IFN-γ, are critical for the initiation and development of protective immunity against intracellular microbial infections (Polendran, 2004). The cytokine profiles from splenocytes of immunized mice were analyzed to determine whether THC treatment of DCs suppressed an up-regulation of Th1 activity in recipient mice. As in Fig. 2, mice were immunized with DCs only, LpDC/DMSO, or LpDC/THC, and 7 to 9 days later, splenocytes from mice treated with unloaded DCs or loaded DCs treated with THC (Fig. 2). 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 Suppression of DC IL-12p40 Production Mediates Loss of Th1 Polarization of Lp-Primed CD4+ T Cells.** We have previously reported that THC suppresses the production of IL-12p40 in Lp-infected DCs in vitro (Lu et al., 2006). Therefore, to examine whether this attenuation is

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**Fig. 1.** THC impairs immunization potential of Lp-loaded DCs. Mice were i.v. immunized with DCs (0.5 × 10^6 cells/mouse) two to three times at 7-day intervals before being challenged with a lethal dose of Lp (1.7–2.0 × 10^7) of bacteria. The DCs were either not loaded with Lp (DC group) or loaded with Lp and treated for 24 h with either DMSO (LpDC/DMSO group) or THC (LpDC/THC group) at 10 μM. Mice were monitored for survival, and the data represent nine mice per group from three experiments.

**Fig. 2.** THC treatment of Lp-loaded DCs inhibited immunizing potential as evidenced by increased bacterial burden. Mice were i.v. injected with 0.3 to 0.5 × 10^6 DCs loaded or not in vitro with Lp and treated with DMSO (LpDC/DMSO) or THC (LpDC/THC) at 10 μM for 24 h. Then, mice were challenged 7 to 9 days later with a sublethal dose of Lp (7 × 10^6 Lp/mouse), spleens were isolated 24 h postinfection, and CFU were determined by plate counts. Data are presented as the mean CFU ± S.E.M. for four mice per group. # and * are p < 0.05 versus the uninfected DC control and LpDC/DMSO group, respectively.
responsible for the impaired Th1-polarizing potential of drug-treated and Lp-loaded DCs, cocultures of DCs with T cells from both unprimed and Lp-primed animals were prepared to examine the reconstitution efficacy of exogenously added IL-12p40. Figure 4A shows results from cocultures of Lp-loaded DCs and unprimed CD4 T cells. As reported previously, 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 (Fig. 4A), and no IFN-γ was detected in these cultures (data not shown). 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 4B shows that DCs plus primed T cells (LpCD4) produced little 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 (Fig. 4B). For example, the addition of 0.5 ng/ml recombinant IL-12p40 resulted in an increase of supernatant IL-12 from 2 ng to 6 ng/ml. Furthermore, in con-

Fig. 3. THC treatment of Lp-loaded DCs inhibited the expression of Th1 cytokines in splenocytes from immunized mice. Mice were i.v. injected with control DCs (0.3–0.5 × 10^6), Lp-loaded and DMSO-treated DCs (LpDC/DMSO), and Lp-loaded and THC-treated DCs (LpDC/THC) as in Fig. 2. Seven to 9 days postinjection, splenocytes were harvested and stimulated in vitro with killed Lp (10^7/ml) for 24 h, and IL-12p40 (A), IFN-γ (B), and IL-4 (C) were detected in supernatants by ELISA. Data represent the mean ± S.E.M. of five experiments. # and *, p < 0.05 versus the control DC and Lp/DMSO group, respectively.

Fig. 4. THC suppression of DC IL-12p40 production mediates loss of Th1 polarization of Lp-primed CD4^+ T cells. Cytokines were measured in 24-h supernatants of cocultures containing DCs and either Lp-primed or unprimed CD4^+ T cells. Primed T cells were obtained 5 days postinfection from the spleens of mice infected with a sublethal dose of Lp. A, IL-12p40 measured in cocultures containing Lp-loaded DCs treated with DMSO or THC (LpDC/DMSO or LpDC/THC) cocultured with unprimed CD4^+ T cells. B, IL-12p40 measured in cocultures treated as in A and containing primed CD4^+ T cells; recombinant IL-12p40 was added in increasing amounts. C, IFN-γ measured in cocultures as in B and treated with IL-12p40. Data are representative of four experiments.
contrast to cocultures containing unprimed CD4 T cells, cultures containing primed T cells produced robust amounts of IFN-γ but only in the presence of Lp-loaded DCs (Fig. 4C), and this effect was attenuated by THC treatment of the DCs. However, as seen in Fig. 4C, 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 from DCs by THC treatment was primarily responsible for the reduced Th1 polarization.

**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 and costimulatory molecules contributing to initiation of an effective adaptive immune response (Iwasaki and Medzhitov, 2004). To determine whether THC modulated these markers, we treated infected and noninfected DCs with either DMSO or THC. After 48 h, we observed by flow cytometry that Lp loading increased the surface expression of CD86 and CD40; however, THC treatment significantly suppressed the expression of both markers (Fig. 5). Regarding MHC class II, we observed that, although expression was high in all three groups, the mean fluorescence intensity of the marker was enhanced in the Lp-infected DCs but was significantly decreased by THC treatment (Fig. 5). From these results, it is possible that drug suppression of T-helper polarization is due in part to a down-modulation of these markers. Other surface proteins, such as Notch receptors, are known to regulate T-cell development (Radtke et al., 2004). Recently, it was shown that the Notch ligands, such as Delta4 and Jagged1, on DCs promote induction of either Th1 or Th2 activity, respectively (Amsen et al., 2004). Therefore, we examined relative mRNA expression of these ligands in DCs loaded with Lp and treated with THC or vehicle for 18 h. We observed that message for both ligands was increased in DCs after Lp infection (LpDC/DMSO group; Fig. 6) but that the Delta4 band intensity relative to β-actin intensity was decreased after THC treatment, suggesting that the message level of the Th1-polarizing ligand was decreased by drug treatment.

**THC Treatment Did Not Affect Lp Survival in DCs or Enhance Apoptosis in Lp-Infected-DCs.** THC has been observed to induce apoptosis in macrophages and lymphocytes (Zhu et al., 1998) and DCs (Do et al., 2004), and it could be argued that drug suppression of IL-12 production and marker expression could be due to a toxic effect on DCs. Furthermore, antigen presentation to CD4 T cells by DCs requires internalization and procession of infectious agents by the DCs. To test the THC effect on other relevant DC functions, we studied 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 noninternalized bacteria. Infected cultures were then treated with THC or DMSO for 0, 24, and 48 h, and the cell-associated CFUs were determined by cell lysis and viable bacteria plate counts. The results (Fig. 7) showed that the bacteria handling function, as measured by intracellular survival, was

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**Fig. 5.** THC suppressed the expression of maturation markers on Lp infected-DCs. Cell surface markers were determined by flow cytometry on DCs treated for 48 h in various ways: uninfected (DC); Lp-infected and DMSO-treated (LpDC/DMSO); and Lp-infected and THC-treated (LpDC/THC). Data are expressed as percentage expression (%) of the surface marker and mean fluorescence intensity of the population for the marker. Data are representative of four similar experiments.
unaffected by THC treatment. Both drug-treated and vehicle-treated cells restricted the growth of Lp in an equivalent manner over time. To determine whether 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 single positive for annexin V was enhanced after Lp infection (Fig. 8), and treatment with THC did not increase the annexin positivity. Furthermore, analysis of propidium iodide staining as indicative of dead cells was similar in infected and infected plus THC-treated cells. The data suggest that THC treatment did not affect the degree of apoptosis or processing of bacteria in DCs after Lp infection.

### Discussion

In the current study, we examined the THC effect on the Th1-polarizing potential of DCs in a model system involving adaptive immunity to *Legionella*. Loading of microbial antigens into DCs has been shown to immunize mice against infection with various pathogens (Ludewig et al., 1998; Su et al., 1998; von Stebut et al., 2000), and we showed that DCs loaded in vitro with Lp and injected into mice immunized and protected the mice from a subsequent lethal Lp infection (Fig. 1). Immunization was confirmed, because the number of splenic CFUs in vaccinated mice was reduced after challenge injection with Lp (Fig. 2). The next question involved what effect THC treatment had on the immunizing potential of the loaded DCs. We showed that exposure to THC decreased the capacity of these cells to protect against infection and to decrease the number of CFUs in the spleens of infected mice (Figs. 1 and 2).

Next, splenocytes from immunized mice were analyzed for Th1 and Th2 cytokine production in vitro in response to Lp antigens. The results in Fig. 3 showed that, as expected, immunization with Lp-loaded DCs caused an increase in the Th1-polarizing cytokines IL-12p40 and IFN-γ; however, this was attenuated in splenocytes from animals immunized with THC-treated DCs, suggesting that drug treatment suppressed DC-polarizing potential. The mechanism surrounding the regulation of Th1 activity by IL-4 is controversial (Swain et al., 1990; Biedermann et al., 2001); therefore, we examined for IL-4 production by splenocytes from immunized mice. Figure 3 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 (Newton et al., 1994). Second, it seems 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. Finally, 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.

The previous studies suggested that drug treatment suppressed the Th1-polarizing function of DCs, and we wanted to explore this further using an in vitro paradigm. Because IL-12 is potent in directing Th1 cell differentiation (Manetti et al., 1993) and because we had shown that THC suppressed IL-12p40 in Lp-infected DC cultures (Lu et al., 2006), we evaluated the Th1-promoting potential (as measured by IFN-γ production) of both Lp-loaded DCs and drug-treated DCs in coculture with Lp-primed CD4+ T cells and the role of IL-12p40 in the response. The data showed that coculturing Lp-loaded DCs with Lp-primed T cells led to enhanced IL-12p40 and IFN-γ production by the cultures compared with coculture with unprimed T cells and that THC treatment of the DCs attenuated cytokine production (Fig. 4). Furthermore, the addition of IL-12p40 to the THC-treated cultures restored the robust production of both IL-12p40 and IFN-γ, suggesting that suppression of the p40 protein plays a major role in inhibiting Th1 polarization. However, suppression of p40 protein is not the only factor involved. For example, the addition of IL-12p40 to cultures of purified Lp-primed CD4+ T cells in the absence of DCs resulted in very little IFN-γ production (<300 pg/ml) (data not shown), supporting previous findings that full Th1 polarization requires both stimulation by cytokines, such as IL-12, and costimulation by

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**Fig. 6.** THC suppressed the expression of Delta 4 in Lp-infected DCs (LpDC/THC) compared with infected DCs treated with DMSO (LpDC/DMSO). DCs were uninfected or infected with Lp and treated with DMSO or THC for 18 h. Jagged1, Delta4, and β-actin mRNAs were amplified by reverse transcription-polymerase chain reaction. Data are representative of three experiments.

**Fig. 7.** Lp uptake and survival in DCs was not affected by THC treatment. DCs were infected with Lp for 30 min, washed twice to remove noninternalized bacteria, and treated with DMSO or THC for 0, 24, and 48 h. At various times postinfection, cell lysates were harvested and plated on agar culture medium, and CFUs/culture of Lp were determined by plate counts at 72 h. Data represent the mean ± S.E.M. of three experiments.
contact with DCs (Kapsenberg, 2003). We looked at cytokines other than IL-12p40, such as IL-12p70 and IL-23, that have been shown to polarize toward Th1 (Hunter, 2005); however, these cytokines were probably 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 (Yao et al., 2005); however, again it was not detected in the supernatants and thus was probably not involved in the drug effect. Together, these results show that IL-12p40 is a major Th1-polarizing protein, as reported by others (Holscher et al., 2001; Cooper et al., 2002; Brombacher et al., 2003), and that its suppression by THC is a key factor in the inhibition of Th1 cell development.

Besides IL-12 production, DCs also promote the maturation of Th1 cells by the production of helper cell-surface proteins, such as MHC class II and costimulatory molecules (Kapsenberg, 2003). To further explore the basis of THC-suppressing DC function, we examined THC effects on the expression of these surface markers. The results showed that THC treatment markedly reduced the expression of MHC class II and the costimulatory molecules CD86 and CD40 (Fig. 5). The mechanism for this is unclear at this time; however, signaling through cannabinoid receptors could be involved because G protein-coupled receptors activated through Gs have been shown to increase Jagged ligand expression and polarize to Th2 (Amsen et al., 2004); cannabinoid receptors are coupled to Gi, and it is possible that these types of receptors might suppress Th1 by inhibiting Delta ligands as seen in our study along with inhibiting IL-12 as shown by others (la Sala et al., 2005).

To initiate an immune response against Lp infection, DCs must take up and process the intact bacteria and then transition through cellular maturation, cytokine production, and pathogen-related antigen presentation. In contrast to macrophages, wherein intracellular growth of Lp was observed (Salins et al., 2001), we showed in the current report that DCs restricted Lp growth with moderate killing over time (Fig. 7). THC-treated DCs showed a similar growth pattern and time course, suggesting that Lp handling was the same in both groups. THC has been reported to induce apoptosis in mouse DCs (Do et al., 2004), and Lp infection has been shown to be apoptotic in macrophages and other cells (Abu-Zant et al., 2005; Fischer et al., 2006). Our results with annexin V staining (Fig. 8) showed that Lp infection induces limited apoptotic activity in DCs as observed in other cell types (Abu-Zant et al., 2005;

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**Fig. 8.** Apoptosis and cell death were not affected by THC treatment. Cultures of DCs were untreated (DC) or infected with Lp and treated for 24 h with either DMSO (LpDC/DMSO) or THC (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 three similar experiments. B, percentage of apoptotic cells (annexin V+/propidium iodide−) and dead cells (propidium iodide+); mean ± S.E.M., n = 3. #, p < 0.05 versus DC control.
Fischer et al., 2006); however, we also showed that THC did not increase apoptosis over infection only and that, in addition, both treatments had no effect on actual cell death, as measured by propidium iodide staining (Fig. 8). Our results with THC and apoptosis seem to differ from previous results, wherein 10 μM THC was shown to induce annexin V positivity in 80% of the cells (Do et al., 2004). However, these studies were done using serum-free medium, whereas ours were done with medium containing fetal calf serum, which is known to reduce the potency of the added cannabinoids (Klein et al., 1985). Because THC at concentrations of 15 to 30 μM induced apoptosis in splenocytes and macrophages in medium containing fetal calf serum (Zhu et al., 1998), we expect that apoptosis would have been induced in the current studies at these high drug concentrations. It is concluded that THC treatment under the current conditions is not suppressing DC function by either altering the intracellular life cycle of Lp or by causing enhanced apoptosis and death of the cells.

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 polarization function by suppressing IL-12p40 production and the expression of MHC class II and costimulatory molecules. 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 celiac disease and Crohn's disease (Hart et al., 2005; Rimoldi et al., 2005), rheumatoid arthritis (Thomas and Lipsky, 1996; Walker et al., 2006), and systemic lupus (Hardin, 2005), and therefore might be in the class of anti-inflammatory drugs regulated by the crosstalk between epithelial cells and dendritic cells.

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