Δ9-Tetrahydrocannabinol Impairs the Inflammatory Response to Influenza Infection: Role of Antigen-Presenting Cells and the Cannabinoid Receptors 1 and 2

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Δ9-tetrahydrocannabinol (Δ9-THC) has potent immune modulatory properties and can impair pathogen-induced immune defenses, which in part have been attributed to ligation of the cannabinoid receptors 1 (CB1) and 2 (CB2). Most recently, dendritic cells (DC) were identified for their potential to enhance influenza-induced immunopathology in mice lacking CB1 and CB2 (CB1−/−CB2−/−). This study focused on the modulation of the inflammatory immune response to influenza by Δ9-THC and the role of CB1 and/or CB2, as receptor targets for Δ9-THC. C57Bl/6 (wild type) and CB1−/−CB2−/− mice were administered Δ9-THC (75 mg/kg) surrounding the intranasal instillation of A/PR/8/34 influenza virus. Three days post infection (dpi), Δ9-THC broadly decreased expression levels of mRNA induced by the innate immune response to influenza, suppressed the percentage of interferon-gamma (IFN-γ)–producing CD4+ and interleukin-17–producing CD8+ cells, and reduced the influx of antigen-presenting cells (APC), including inflammatory myeloid cells and monocytes/macrophages, into the lung in a CB1− and/or CB2-dependent manner. Δ9-THC had little effect on the expression of CD86, major histocompatibility complex I (MHC I), and MHC II by APC isolated from the lung. In vitro studies demonstrated that lipopolysaccharide (LPS)–induced maturation was suppressed by Δ9-THC in bone marrow–derived DC (bmDC). Furthermore, antigen-specific IFN-γ production by CD8+ T cells after coculture was reduced by Δ9-THC treatment of bmDC in a CB2− and/or CB2-dependent manner. Collectively, these studies suggest that Δ9-THC potently suppresses myeloid cell immune function, in a manner involving CB1, and/or CB2, thereby impairing immune responses to influenza infection.

Key Words: Δ9-tetrahydrocannabinol; cannabinoid receptors; immune modulation; antigen-presenting cells; influenza.

Δ9-tetrahydrocannabinol (Δ9-THC) is the primary psychoactive constituent in the marijuana plant. Its biological activity can be partially attributed to the G protein-coupled receptors (GPCR), CB1 and CB2 (Howlett et al., 2002). In addition to eliciting psychotropic effects, predominantly mediated by CB1 (Huestis et al., 2001), Δ9-THC exerts potent effects on the immune system through CB1− and CB2-dependent and -independent mechanisms, depending on the immune stimulus (Do et al., 2004; Lu et al., 2006a, b; Rao and Kaminski, 2006; Rao et al., 2004). Expression of CB1 and CB2 varies among leukocytes and can change in response to immune stimulation, albeit CB2 expression is greater than CB1 in cell of the immune system (Bouaboula et al., 1993; Galiègue et al., 1995; Nong et al., 2001). Although pharmacological effects by Δ9-THC have been extensively investigated on isolated immune cells in vitro, the effects of Δ9-THC on the immune system in vivo are more complex. Factors contributing to this complexity include the type of stimulus used, site of the immune response, the interaction between multiple cell types, and the kinetics of the cellular arms of immune response, none of which can be completely recapitulated in vitro. To date, a few studies have focused on the effects of Δ9-THC on multiple immune cell populations, such as in host resistance models to infection and tumor challenges (Buchweitz et al., 2007, 2008; Cabral et al., 1986; Klein et al., 2000; Mishkin and Cabral, 1985; Morahan et al., 1979; Newton et al., 1994; Zhu et al., 2000). Many of these studies show that Δ9-THC impairs host immune surveillance, thereby increasing adverse immune outcomes.

Our laboratory has characterized an influenza host challenge model in order to investigate the effects of Δ9-THC on the in vivo antiviral immune responses (Buchweitz et al., 2007, 2008). Infection with influenza virus induces a broad immune response within the airways of the lungs and associated lymph nodes, which is classically a T helper type 1 (Th1) response as characterized by production of interferon-gamma (IFN-γ) by antigen-specific T cells. More recently, a role for interleukin-17 (IL-17) in the immunopathology of influenza infection has been identified, which is, in part, responsible for an influenza-induced
neutrophilia (Crowe et al., 2009). The heterogeneous population of dendritic cells (DC) are important in the initiation of the aforementioned T-cell immune responses (Lee and Iwasaki, 2007). Conventional DC (cDC) present antigen to elicit effector immune cells, thereby dictating the magnitude and phenotype of the effector response (e.g., Th1) (Banchereau and Steinman, 1998). By contrast, plasmacytoid DC (pDC) primarily produce type I IFNs (Asselin-Paturel et al., 2001). Other APC, such as alveolar macrophages (AM), are involved in phagocytosis and cytokine production (Kobzik et al., 1993). The function of inflammatory myeloid cells, also termed terigrated or monogenic DC or inflammatory monocytes, is less well- understood but is thought to contribute to the inflammatory milieu (Drutman et al., 2012). Also macrophages/monocytes (Mac/ MΦ) are involved in cytokine production (Auffray et al., 2009).

Recently, we demonstrated that signaling via CB1 and CB2 suppressed the magnitude of the immune response, at least in part, by regulating DC function. In turn, the lack of CB1 and CB2 exacerbated inflammation and tissue damage after influenza infection, with the greatest inflammation observed at 3 days post infection (dpi) (Karmaus et al., 2011). Therefore, the objective of this study was to determine the effect of Δ9-THC on APC populations involved in the immune response to influenza at 3 dpi and to identify whether CB1 and CB2 are involved in modulating this anti-influenza response. Collectively, our results suggest that Δ9-THC suppresses DC, Mac/MΦ, and inflammatory myeloid cell responses to a greater extent via CB1 and/or CB2, thereby impairing cellular immune responses induced by influenza infection.

MATERIALS AND METHODS

Mice. Age-matched C57Bl/6 wild type (WT) and CB1−/−CB2−/− mice bred on a C57Bl/6 background were used for in vivo experiments. WT mice were purchased from the National Cancer Institute (Frederick, MD). CB1−/−CB2−/− mice were a kind gift of Dr. Andrew Zimmern from the University of Bonn (Karask et al., 2007). They were bred and housed at the animal care facilities at Michigan State University. All mice were given food (Purina Certified Laboratory Chow) and spring water ad libitum and were housed at 40–60% relative humidity and room temperature (21°C–24°C) with a 12-h light/dark cycle. Female mice were used for experiments between the ages of 8–12 weeks and randomly assigned to experimental groups. Female mice were used exclusively in this study primarily due to the dominant behavior exhibited by group housed male mice (i.e., fighting) and the potential confounding effects on immune competence associated with stress. Prior to experiments, mice were transferred to plastic cages containing sawdust bedding and quarantined for 1 week. Mice transgenic for T-cell receptor (αβ) and Ter β (OT-I), generating CD8+ T cells specific for chicken ovalbumin (OVA323–351, amino acid sequence: SLFEPKL), were purchased from Jackson Laboratories (Bar Harbor, ME). All CB1−/−CB2−/− breeders, experimental WT and CB1−/−CB2−/− mice, and sentinel mice were subjected to rigorous veterinary exams and were found negative for pathogens tested. All animal housing, handling, and procedures were approved by and performed following the guidelines of the Institutional Animal Care and Use Committee at Michigan State University.

Virus infection. Mice were randomly assigned to a treatment group 1 week prior to initiation of the experiment. A nonlethal dose of 50 plaque-forming units of A/PR/8/34 (PR8) influenza, a generous gift from Dr. Allen Harmsen (Montana State University, Bozeman, MT), or 0.9% saline (by weight of NaCl dissolved in water; SAL) was instilled at 25 µl per nostril per mouse under anesthesia with 4% isoflurane.

Δ9-THC treatment. The National Institute on Drug Abuse (NIDA, Bethesda, MD) provided Δ9-THC for experimental use. For in vivo experiments, mice received corn oil (CO) vehicle (VH) or Δ9-THC (75 mg/kg/day per mouse) by oral gavage at 0.1 ml/g body weight for five consecutive days (~2 to 2 dpi) surrounding the installation of PR8. This dose was chosen based on historical data, and although there is low absorption through the gastrointestinal tract following this clinically relevant route of administration, serum levels of Δ9-THC in the mice are comparable with serum Δ9-THC levels in humans after smoking marijuana. Specifically, after five consecutive days of treatment by oral gavage with 75 mg/kg Δ9-THC, mouse serum reached concentrations of 66.2 ng/ml of Δ9-THC 4 h after the last Δ9-THC dose (Buchweitz et al., 2007, 2008), whereas human serum Δ9-THC concentrations have ranged between 57 and 268 ng/ml (Aazorlosa et al., 1992).

Necropsy and tissue collection. Three dpi, mice were anesthetized with 250 mg/kg pentobarbital (Fatal-Plus, Vortech, Dearborn, MI) followed by euthanasia via exsanguination of the abdominal aorta. Lung lobes were excised and placed on ice block and either immersed in TRI Reagent (Sigma, St. Louis, MO) for RNA isolation (n = 5) or stored in ice-cold RPMI and disrupted using a cell dissociation kit (CD-1, Sigma) to obtain a single cell suspension (n = 5) for cytometric analysis. The experiments presented were repeated twice with similar results using n = 5/group.

RNA isolation and low density microarray mouse immune panel. RNA was isolated from whole lungs using manufacturer’s instructions for TRI Reagent (Sigma). RNA was converted into cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA) using 2 µg in a total reaction volume of 50 µl according to manufacturer’s instructions. TaqMan assay–based mouse immune panel low density microarrays were obtained from Applied Biosystems, and reactions were set up according to manufacturer’s instructions. In an 8 × 48 design, each well was loaded with 50 ng cDNA and centrifuged twice at 1100 rpm using a Sorvall Legend T (Thermo Fisher, Waltham, MA). Analysis was performed with an Applied Biosystems 7900HT Real-Time PCR System. Baseline fluorescence was set at 2–5 cycles for the internal control, 18S rRNA gene, and 2–13 cycles for target genes to determine thresholds for Ct values. Fold change was calculated by the ΔΔCt method (Livak and Schmittgen, 2001) compared with WT-CO-SAL, which was arbitrarily assigned a value of 1. All data were normalized with Blom transformation, and statistical analyses were performed with a factorial ANOVA 2 × 2 × 2 (genotype, immune stimulation, drug treatment) design using Statistical Analysis System (SAS) version 9.1.3 (Cary, NC). Gene expression values were log transformed, mean centered, and normalized within genes with Cluster v2.11 and visualized using Treeview v1.60 (Eisen et al., 1998). Restimulation of immune cell populations isolated from lungs. After mechanical disruption of lung tissue, cells were restimulated with phorbol myristate acetate (PMA; 40nM) and ionomycin (Io; 0.5µM) (both from Sigma) for 5h in 2% bovine calf serum (BCS) RPMI and 1× Brefeldin A (Biolegend, San Diego, CA).

Surface antibody labeling for flow cytometry. All staining protocols were performed in 96-well round bottom plates (BD Falcon, Franklin Lakes, NJ). Cells isolated from the lungs were washed with FACS buffer (1× HBSS, 1% bovine serum albumin, 0.1% sodium azide, pH 7.6), and surface Fc receptors were blocked with anti-mouse CD16/CD32 (BD Biosciences, Franklin Lakes, NJ) for 15 min at 4°C. Cells were labeled for 30 min at 4°C with the following antibody clones, all from Biolegend: CD4 (clone RM4-5), CD8 (53–6.7), NK1.1 (CD161, PK136) for lymphocyte analysis, and CD11b (M1/70), CD11c (N418), CD80 (16-10A1), CD86 (GL-1), Gr-1 (RB6-8C5), major histocompatibility complex I (MHC I; H-2Kb/H-2Db, 28-8-6), and MHC II (I-A/II-E, M5/114.15.2) for analysis of APC. Subsequently, cells were washed thrice with FACS buffer, fixed with Cytofix (BD Biosciences) for 15 min, and resuspended...
in FACS buffer. Compensation and voltage settings of fluorescent parameters were performed using single color staining controls. Fluorescent staining was analyzed using a BD Biosciences FACSCanto II flow cytometer.

**Intracellular antibody labeling for flow cytometry.** After surface staining (described above), cells were washed twice with 1× Perm/Wash (BD) and incubated with 1× Perm/Wash for 30 min at room temperature in 96-well round bottom plates. Fluorescently labeled antibodies were added at 0.25–0.5 μg/ml in 200 μl for 30 min. The following fluorescently labeled antibody clones were used (both from Biologend): IFN-γ (XMG1.2) and IL-17 (TC11-18H10.1). Cells were washed twice with 1× Perm/Wash and subsequently resuspended in FACS buffer. After intracellular staining, cells were analyzed the same day. Cells were identified as positive for cytokine secretion after gating on the negative population in unstimulated samples (without PMA/Io or Brefeldin A).

**Generation of bone marrow-derived DC in vitro.** Femurs and tibias of 8–12 week old WT and CB1−/−CB2−/− mice were flushed for bone marrow, and 1×10^6 cells were grown in 4ml of RPMI media supplemented with 100 units/ml penicillin and 100 μg/ml streptomycin (Gibco, Invitrogen) in a 6-well dish for 9 days in the presence of granulocyte-macrophage colony stimulating factor (20 ng/ml; Peprotech, Rocky Hill, NJ), with 50% of media replaced on days 3, 6, and 8. After 9 days, bone marrow–derived DC (bmdC) were left unstimulated (naive; NA) or stimulated with toll-like receptor (TLR) ligands: resiquimod (R848; 5 μg/ml) or LPS (1 μg/ml) in the presence of Δ9-THC (10μM) or VH (0.1% ethanol) in 2% BCS RPMI. After 24h, bmdC were stained using the same antibodies as for the previously mentioned in vivo experiment and analyzed by flow cytometry with the same gating scheme.

**Coculture of bmdC and OT-1 cells in vitro.** Splenocytes from OT-1 mice were isolated and labeled with Cell Trace (Invitrogen) proliferation dye to track loss of fluorescent staining as an indicator for proliferation. NA or TLR-stimulated and Δ9-THC- or VH-treated bmdC were incubated with SIINFEKL for 24h and washed thrice with RPMI before incubation with OT-1 splenocytes. Four days after coculture, cells were restimulated with SIINFEKL in the presence of Brefeldin A for 5h in 2% fetal bovine serum RPMI. Subsequently, cells were stained with LIVE/DEAD dye (Invitrogen), fluorescent antibodies to CD8 and IFN-γ, and analyzed by flow cytometry as described previously. The gating was performed in the following order: singlets, LIVE/DEAD, and CD8 for graphs showing proliferation or proliferation and IFN-γ production.

**Statistical analysis.** Graphing and statistical analyses were performed using GraphPad Prism v4.03. The mean ± SE is expressed in all bar graphs. For gene expression data, SAS version 9.1.3 and factorial ANOVA were used. To determine statistically significant changes between WT and CB−/−CB−/− mice, in uninfected and influenza-challenged mice, two-way ANOVA for parametric data or Kruskal-Wallis test for nonparametric data was used. Bonferroni’s post hoc test was used to compare select groups of samples: SAL versus PR8 (indicated by + in Figures), CO versus Δ9-THC (+), and WT versus CB−/−CB−/− (−). Statistical analysis of SAL–versus PR8-instilled mice treated with Δ9-THC was not performed in either WT or CB−/−CB−/− mice because an immune response was expected as a result of PR8 instalation.

**RESULTS**

**Δ9-THC Reduced Steady-State mRNA Expression of Genes Induced by Influenza in the Lungs of WT but Not CB−/−CB−/− Mice**

Previously, the effects of CB1 and CB2 deletion were assessed on the host immune response to PR8 challenge. These studies identified 3 dpi as the peak time of the pulmonary inflammatory response (Karmaus et al., 2011). To investigate the effects of Δ9-THC on the anti-influenza immune response in the lungs of WT and CB−/−CB−/− mice, a 96-plex immune panel was employed to assess gene expression changes. 18S rRNA served as the loading control, and all samples were normalized to 18S rRNA gene expression. In SAL-treated mice, mRNA levels of very few genes were changed by Δ9-THC treatment alone (Fig. 1A). In contrast, PR8 challenge induced broad changes in the steady-state mRNA levels for many genes in the 96-gene panel, which were attenuated by concomitant Δ9-THC treatment in WT, but not CB−/−CB−/− mice, suggesting CB−/− and/or CB2-dependent modulation (Fig. 1B). In addition, mRNA levels for a few genes (Fas and IL13 in SAL group and Col4a5, Cyp7a1, and Ifng in the PR8 group) were upregulated in CB−/−CB−/− mice as a result of Δ9-THC treatment, suggesting the potential for CB−/− and CB2-independent mechanisms of Δ9-THC–mediated activity (Figs. 1A and 1B).

**Δ9-THC Suppressed Influenza-Induced Cytokine Production by Effector Lymphocyte Populations**

Influenza infection significantly increased the total number of cells in the BALF (Fig. 2A). We observed statistically significant changes in total cell numbers, but due to experimental variation between mice, only trends are described in BALF subpopulations. Little effect was observed on the recruitment of eosinophils and lymphocytes (both shown at 10^5 cells/ml) into the airways, but increased numbers of neutrophils and Mac/Φ (both shown at 10^5 cells/ml) were identified, compared with SAL-treated mice (Fig. 2A). The presence of inflammatory cells in the BALF, total cells in lung tissue (Figs. 2A and 2B), and percentages of T lymphocytes and NK cells in whole-lung isolates (Fig. 3) were not affected by Δ9-THC. Greater BALF cellularity was observed in CB−/−CB−/− mice compared with WT mice and consisted mainly of neutrophils and Mac/Φ (Fig. 2A), but as opposed to BALF, overall cell numbers in the lung tissue did not differ between the genotypes (Fig. 2B). The percentages of lung CD4+, CD8+, and NK1.1+ did not change as a result of influenza infection by 3 dpi, suggesting that recruitment of these populations to the lung had not reached its peak. By contrast, lower percentages of CD8+ cells were found in the lungs of CB−/−CB−/− compared with WT mice, which likely occurred due to an overall increase in recruited inflammatory cells thereby decreasing the percentage of CD8+ cells (Fig. 3). Effector function was assessed in lymphocyte populations by measuring cytokine secretion in CD4+, CD8+, and NK1.1+ cells. Similar to our previous study (Karmaus et al., 2011), percentages of cytokine-producing cells were increased in uninfected lungs of CB−/−CB−/− mice compared with WT mice (Figs. 4 and 5). Influenza infection significantly reduced the percentage of IFN-γ-producing CD4+ cells in WT mice, but not CB−/−CB−/− mice, and Δ9-THC suppressed the percentage of IFN-γ-producing cells only in WT mice. The percentage of the IL-17–producing cells was also induced by influenza infection in WT mice, especially in NK1.1+ cells. The percentage of IL-17–producing NK1.1+ cells was significantly suppressed by Δ9-THC (Fig. 5). Taking into account the percent of cells positive for cytokine production, CD8+ T cells contributed little
to IFN-γ and IL-17 production at 3 dpi compared with CD4+ and NK1.1+ cells (Figs. 4 and 5). Consistent with the profile of inflammation, as observed by Hematoxylin and Eosin (H&E) staining in previous experiments (Buchweitz et al., 2008), Δ9-THC enhanced the percentages of IFN-γ–producing CD4+ and NK1.1+ cells and IL-17–producing CD8+ and NK1.1+ cells in CB1−/−CB2−/− mice, which was significantly different from Δ9-THC–treated WT samples (Figs. 4 and 5). Overall, these results demonstrate that Δ9-THC suppressed the percentages of functional effector CD4+ and NK1.1+ cells 3 dpi in a CB1- and/or CB2-dependent manner.

Reduced Presence of APC With Δ9-THC Treatment After Influenza Infection

To become functional effectors, lymphocytes depend on the presence of, and interaction with, APC. In the lung, several immune cell populations including APC are present and can be identified according to their surface expression of CD11b, CD11c, and Gr-1 (Fig. 6A) (Asselin-Paturel et al., 2001; Auffray et al., 2009; Drutman et al., 2012; Gonzalez-Juarrero et al., 2003). For this investigation, we identified granulocytes as CD11b+CD11c−, Mac/MΦ as CD11b−CD11c−, cDC as CD11b+CD11c−Gr-1−, and inflammatory myeloid cells as CD11b+CD11c−Gr-1−, whereas AM are CD11c−CD11b−Gr-1− and pDC are CD11b−CD11c−Gr-1−. It is important to note that there might be some limitations and exceptions to the identification strategy using surface antigens due to the heterogeneity of myeloid cell populations (one such example is in the pDC compartment as outlined in the Discussion section). The presence of inflammatory myeloid cells, Mac/MΦ, and neutrophils was significantly increased in WT mice as a result of influenza infection (Fig. 6B). Furthermore, greater percentages

FIG. 1. Reduction of gene expression associated with influenza infection as a result of Δ9-THC treatment. Mice (n = 4) were treated with CO VH or Δ9-THC (75 mg/kg) for 5 consecutive days surrounding the intranasal instillation of influenza (PR8) or saline (SAL). Lung RNA was isolated 3 dpi and converted into cDNA, and gene expression levels were analyzed using a TaqMan low density gene array. Fold change values were normalized with Blom transformation, log transformed, and mean and median were centered across genes with cluster analysis using Treeview. Shown are genes differentially regulated by at least 1.5-fold between CO and Δ9-THC samples, with a value of at least p ≤ 0.1 in SAL (A) and PR8-instilled (B) groups 3 dpi. Data were analyzed using factorial ANOVA in SAS version 9.1.3 as described in the Materials and Methods section, and p values are indicated in the left most column and represent statistical differences as a result of Δ9-THC treatment.
of inflammatory myeloid cells and pDC were found in lungs of CB₁⁻/⁻ CB₂⁻/⁻ compared with WT mice, in the absence of influenza infection. The increase of inflammatory myeloid cells and Mac/MΦ after influenza infection was attenuated in Δ⁹-THC–treated WT mice, but not CB₁⁻/⁻ CB₂⁻/⁻ mice, suggesting CB₁ and/or CB₂ dependence of the Δ⁹-THC effect (Fig. 6B). The pattern of reduced presence of inflammatory myeloid cells and Mac/MΦ is similar to the reduction of cytokine secretion in CD4⁺ and NK1.1⁺ cells after Δ⁹-THC treatment as it only occurs in WT mice infected with influenza. Thus, it is plausible that these APC populations contribute to the effect of Δ⁹-THC on effector cell populations in a CB₁⁻ and/or CB₂⁻ dependent manner.

Enhanced Maturation of APC From CB₁⁻/⁻ CB₂⁻/⁻ Mice Compared With WT Mice and the Effect of Δ⁹-THC on APC Maturation

The maturation markers MHC I, MHC II, and CD86 were assessed in several APC populations: cDC, pDC, inflammatory myeloid cells, AM, and Mac/MΦ (Fig. 7 and summary in Table 1). In the absence of influenza infection, CB₁⁻/⁻ CB₂⁻/⁻ mice exhibited higher MHC II expression on cDC, lower MHC I expression on pDC, and higher expression of MHC I on Mac/MΦ. Influenza infection increased expression of MHC I on cDC; MHC II on pDC and AM; and CD86 on cDC, pDC, inflammatory myeloid cells, and Mac/MΦ in WT mice. Influenza infection also increased expression of MHC I on cDC and inflammatory myeloid cells; MHC II on pDC, inflammatory myeloid cells, AM, and Mac/MΦ; and CD86 on all five identified APC subsets in CB₁⁻/⁻ CB₂⁻/⁻ mice. Δ⁹-THC did not alter the expression of MHC I, MHC II, or CD86 in any of the cell types of WT or CB₁⁻/⁻ CB₂⁻/⁻ mice (the only exception was an increase in CD86 expression in WT mice). Taken together, these results suggest that the suppression of lymphocyte effector function by Δ⁹-THC occurs by reducing the influx of APC into the lungs, rather than suppressing the maturation of APC present in the lungs.

**FIG. 2.** Δ⁹-THC does not alter BALF cell counts or composition or total lung cells after influenza infection. (A) BALF was isolated by flushing lungs at 3 dpi twice with 0.9 ml SAL (n = 5). Total cell numbers were counted using a hemacytometer, then BALF was centrifuged onto slides, dried, and differential cell counts were performed after Diff-Quick staining of slides. Shown are cell number per milliliters of differentially stained cells out of the total BALF. Statistical analyses indicate comparisons between total cells obtained from the BALF. As indicated on the right of the Figure, Mac/MΦ and neutrophils are in the order of 10⁵, whereas eosinophils are in the order of 10⁴ cells/ml. (B) Total cells were isolated from mechanically disrupted lung tissue and counted using a Coulter Counter. Data were analyzed using an ANOVA comparing total cells as indicated by the horizontal bar: +++ (p ≤ 0.001), difference between SAL and PR8; ### (p ≤ 0.001), difference between WT and CB₁⁻/⁻ CB₂⁻/⁻.Δ⁹-THC SUPPRESSES INFLUENZA IMMUNITY VIA APC

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Attenuation of bmDC Maturation by Δ^9-THC In Vitro

Maturation and migration of APC are inter-related processes such that changes in maturation might affect migration. Indeed, only mature DC are able to migrate; thus it is plausible that the site of infection is enriched for mature DC as immature DC do not migrate to the site of infection (De Vries et al., 2003; Lin et al., 1998). In light of maturation-dependent migration, Δ^9-THC might elicit its effects on the maturation of APC prior to migration, thereby reducing the number of mature APC present in the lung after influenza infection. Thus, we examined the effect of Δ^9-THC on maturation induced by TLR ligation of bmDC in vitro (Lutz et al., 1999). The in vitro-generated bmDC were identified as CD11b^+CD11c^- and are therefore similar to the DC/inflammatory myeloid phenotype (Fig. 6A). LPS (1 µg/ml), a TLR4 ligand, significantly induced maturation in WT and CB1^-/-CB2^-/- bmDC, as evidenced by MHC II, CD80, and CD86 expression (Figs. 8B and 8C). In contrast, R848 (5 µg/ml), a TLR 7/8 agonist, did not induce bmDC maturation to the same extent as LPS, especially regarding CD86 expression (Figs. 8B and 8C). As previously observed (Karmaus et al., 2011), greater maturation levels, as measured by MHC I and MHC II expression, were observed in unstimulated bmDC generated from CB1^-/-CB2^-/- mice compared with those obtained from WT mice. Δ^9-THC (10 µM) reduced LPS-induced maturation, but not R848-induced maturation, as evidenced by lower MHC II in CB1^-/-CB2^-/- and CD86 expression in WT and CB1^-/-CB2^-/- mice (Figs. 8B and 8C). Collectively, bmDC maturation was suppressed by Δ^9-THC independently of CB1 and/or CB2.

Impaired Elicitation of T Cells After Δ^9-THC Treatment of bmDC

To determine the functional consequences of Δ^9-THC–mediated suppression of TLR-stimulated maturation of bmDC, a T-cell coculture model was used. This model system recapitulates the interaction between DC and naïve T cells, which occurs after antigen capture in the lung parenchyma by DC and subsequent migration to the lung draining lymph nodes. However, this in vitro assay bypasses any need for migration and provides an indirect measurement of DC activity as a result of interaction with T cells. Again, bmDC were CD11b^+CD11c^- (DC/inflammatory myeloid phenotype). Splenocytes from OT-1 mice with a transgenic T-cell receptor specific to cognate epitope OVA257-264 (SIINFEKL) were incubated with bmDC pulsed with SIINFEKL, which is an MHC I–restricted peptide, thus eliciting only CD8^+ T cells. Four days after coculture, OT-1 splenocytes were restimulated with SIINFEKL to induce IFN-γ secretion (Fig. 9). No proliferation or IFN-γ was observed in T-cell cocultures with NA (unstimulated) WT bmDC. In contrast, bmDC from
CB1−/−CB2−/− mice readily elicited T cells without the need for prior TLR stimulation as previously reported (Karmaus et al., 2011). Δ9-THC (10 µM) potently suppressed proliferation and IFN-γ production following stimulation of WT bmDC with either LPS or R848. Due to the high magnitude of T-cell proliferation and IFN-γ production in response to naive bmDC from CB1−/−CB2−/− mice, neither TLR stimulation nor Δ9-THC had any effect on the ability of CB1−/−CB2−/− bmDC to elicit T cells. These in vitro results provide evidence for immunomodulation of DC by Δ9-THC, resulting in impaired lymphocyte responses in a CB1 and/or CB2-dependent manner as observed after influenza infection in vivo. The impairment of lymphocyte responses after Δ9-THC cotreatment during influenza infection is likely a consequence of attenuated maturation-dependent migration of DC. A comprehensive summary of results is presented in Table 1.

FIG. 4. Δ9-THC decreased IFN-γ production in CD4+ cells after influenza infection. At 3 dpi, lungs (n = 5) were mechanically disrupted, and single cell suspensions were restimulated in vitro with PMA/Io (40nM/0.5µM) to induce cytokine secretion and in the presence of Brefeldin A in 2% serum RPMI for 5 h to allow for intracellular accumulation of cytokines. After restimulation, cells were stained for CD4, CD8, and NK1.1 surface expression. On the day of flow cytometric analysis, cells were stained for intracellular IFN-γ and analyzed for fluorescence intensity. Data were analyzed using Kruskal-Wallis’ test for nonparametric data: * (p ≤ 0.05), difference between CO and Δ9-THC; ** (p ≤ 0.01), difference between SAL and PR8; ### (p ≤ 0.01), #### (p ≤ 0.001), difference between WT and CB1−/−CB2−/−. Shown are samples concatenated (n = 5) within treatment groups.

DISCUSSION

Despite the identification of CB1 and CB2, the effects of cannabinoids on the immune system in health and disease have remained elusive. It has been difficult to determine the contribution of Δ9-THC–induced CB1 and CB2 signaling in adverse immune outcomes. Furthermore, only an experimental model that induces several arms of the immune system allows for the identification of leukocyte populations sensitive to Δ9-THC treatment. In this study, an influenza model was used to stimulate the immune system, and the inflammatory response 3 dpi was investigated after cotreatment with the nonselective CB1 and CB2 partial agonist, Δ9-THC, in WT and CB1−/−CB2−/− mice. This time point was selected based on our previous study in which the kinetics of the immune response to influenza was compared in WT and CB1−/−CB2−/− mice, demonstrating that
inflammation peaked at 3 dpi and T-cell responses occurred earlier in $\text{CB}_1^{-/-}\text{CB}_2^{-/-}$ compared with WT mice (Karmaus et al., 2011). Even during this early response to infection, immune parameters were suppressed by $\Delta^9$-THC in WT mice as evidenced by reduced cytokine secretion by CD4$^+$ T cells and NK cells, lower percentages of APC populations in the lung, and decreased expression of genes associated with the immune response to influenza. Furthermore, $\Delta^9$-THC reduced T-cell responses through modulation of DC function in WT mice, but not $\text{CB}_1^{-/-}\text{CB}_2^{-/-}$ mice. Previous studies support that both $\text{CB}_1$ and $\text{CB}_2$ contribute to the modulation of DC function by $\Delta^9$-THC with the use of $\text{CB}_1$- and $\text{CB}_2$-specific antagonists (Do et al., 2004) and that $\text{CB}_1$ specifically diminishes $K^+$ outward currents through $K_v$ channels to suppress DC function (Wacnik et al., 2008). In contrast, pertussis toxin, which blocks the $G_\alpha_i$ subunit necessary for $\text{CB}_1$ and $\text{CB}_2$ signaling (Howlett et al., 1986; Kaminski et al., 1994), did not completely abrogate the $\Delta^9$-THC–mediated suppression of IL-12p40 production in bmDC (Lu et al., 2006b). Also, selective antagonists to either $\text{CB}_1$ or $\text{CB}_2$ did not block the suppression of IL-12p40 by $\Delta^9$-THC (Lu et al., 2006b). These results suggest that although $\text{CB}_1$ and $\text{CB}_2$ are involved in $\Delta^9$-THC modulation of DC, it is not the exclusive or sole mechanism. In this study, $\Delta^9$-THC suppressed LPS-induced CD86 expression of bmDC generated from $\text{CB}_1^{-/-}\text{CB}_2^{-/-}$ mice; however, this suppression of maturation did not alter elicitation of T-cell responses in cocultures.

**FIG. 5.** $\Delta^9$-THC decreased IL-17 production in NK1.1$^+$ cells after influenza infection. Lungs were mechanically disrupted and restimulated in vitro with PMA/Io as described in the Figure 4 ($n = 5$). CD4, CD8, and NK1.1 were stained on the surface of isolated cells, and intracellular staining for IL-17 and flow cytometry were performed. Nonparametric percentage data were analyzed using Kruskal-Wallis’ test: * ($p \leq 0.05$), difference between CO and $\Delta^9$-THC; ** ($p \leq 0.01$), difference between NA (SAL) and PR8; # ($p \leq 0.05$), ## ($p \leq 0.01$), ### ($p \leq 0.001$), difference between WT and $\text{CB}_1^{-/-}\text{CB}_2^{-/-}$. Displayed are concatenated samples ($n = 5$) of each treatment group.
Collectively, these results suggest that the maturation level of bmDC, although suppressed by Δ⁹-THC, was still sufficient to elicit T-cell responses in samples of CB¹⁻/⁻CB²⁻/⁻, but not WT, mice. Other factors aside from the maturation markers assessed on bmDC might be involved in the suppression of maturation, independent of CB¹ and CB². In support of this argument, R848 did not induce maturation to the same magnitude as LPS in bmDC, yet R848-treated DC were capable of eliciting effector T cells in cocultures. Moreover, cytokine production by lymphocytes was enhanced in vivo as a result of Δ⁹-THC treatment of CB¹⁻/⁻CB²⁻/⁻ mice, further supporting the view that targets in addition to CB¹ and CB² contribute to the Δ⁹-THC-mediated effects on DC.

Several APC populations were identified in the lung after influenza infection by CD11b, CD11c, and Gr-1 expression. Although these three surface markers provide a simple strategy to identify different APC subsets, some exceptions must be noted. For example, pDC (identified here as CD11c⁻CD11b⁻Gr-1⁺) can exhibit reduced CD11c expression (Asselin-Paturel et al., 2001). In fact in this report, CD11c expression is lower in pDC than cDC, but our gating strategy ignores CD11c⁻ pDC, which are a minor population. The correct identity of lung myeloid cell populations is a highly debated topic, and interpretations of our data are confined to our described gating strategy. The greatest sensitivity to Δ⁹-THC was observed by changes in the percent of lung-associated inflammatory myeloid cells and Mac/MΦ. Migration of these APC populations is regulated by several chemokines and chemokine receptors (Penna et al., 2001; Randolph et al., 2008). From the low density microarrays conducted, Δ⁹-THC reduced steady-state mRNA levels of proinflammatory cytokines, chemokine receptors, but not chemokines. Proinflammatory cytokines are involved in the maturation of DC and their precursors and positively influence their migration to the site of infection (Randolph et al., 2008). Thus, indirect effects of reduced tissue inflammation may be
Δ^9-THC does not alter the maturation status of lung-isolated myeloid cells after PR8 infection. Following the gating scheme in Figure 6, MHC I, MHC II, and CD86 expression was determined on cDC (CD11b^+CD11c^+Gr-1^−), pDC (CD11b^-CD11c^+Gr-1^+), inflammatory myeloid cells (CD11b^-CD11c^+Gr-1^+), AM (CD11b^-CD11c^-Gr-1^-), and Mac/MΦ (CD11b^-CD11c^+). Bar graphs show mean fluorescence intensities (MFI) for indicated maturation markers (n = 5). Statistical analysis was performed using ANOVA: * (p ≤ 0.05), ** (p ≤ 0.01), *** (p ≤ 0.001), difference between Δ^9-THC and VH (CO); + (p ≤ 0.05), ++ (p ≤ 0.01), +++ (p ≤ 0.001), difference between SAL and PR8; # (p ≤ 0.05), ## (p ≤ 0.01), ### (p ≤ 0.001), difference between WT and CB1^−/−CB2^−/−.
### TABLE I
Summary of Major Findings

<table>
<thead>
<tr>
<th>Cells</th>
<th>Basal WT</th>
<th>CB₁⁻⁻/CB₂⁻⁻</th>
<th>PR8 WT</th>
<th>CB₁⁻⁻/CB₂⁻⁻</th>
<th>PR8 + Δ⁹-THC WT</th>
<th>CB₁⁻⁻/CB₂⁻⁻</th>
</tr>
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<tbody>
<tr>
<td>BALF total cells</td>
<td>—</td>
<td>—</td>
<td>++</td>
<td>+++</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>CD4⁺IFN-γ⁺</td>
<td>—</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>NK1.1⁺IL-17⁺</td>
<td>—</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cDC</td>
<td>++</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
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<tr>
<td>pDC</td>
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<td>++</td>
<td>++</td>
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<tr>
<td>Inflammatory myeloid cells</td>
<td>—</td>
<td>++</td>
<td>++</td>
<td>++</td>
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</tr>
<tr>
<td>AM</td>
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<td>+++</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Mac/MΦ</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Maturation markers</td>
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<td></td>
</tr>
<tr>
<td>cDC</td>
<td>↑ MHC II</td>
<td>↑ MHC I</td>
<td>↑ MHC I</td>
<td>↑ CD86</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pDC</td>
<td>↓ MHC I</td>
<td>↑ MHC II</td>
<td>↑ MHC I</td>
<td>↑ CD86</td>
<td>↑ CD86</td>
<td>↑ MHC II</td>
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<tr>
<td>Inflammatory myeloid cells</td>
<td>↑</td>
<td>↑ CD86</td>
<td>↑ MHC I</td>
<td>↑ MHC I</td>
<td>↑ CD86</td>
<td>↑ CD86</td>
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<tr>
<td>AM</td>
<td>↑ MHC II</td>
<td>↑ MHC II</td>
<td>↑ CD86</td>
<td>↑ CD86</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mac/MΦ</td>
<td>↑ MHC I</td>
<td>↑ CD86</td>
<td>↓ MHC I</td>
<td>↓ CD86</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

| bmDC                  |          |              |        |              |                |              |
| bmDC maturation markers | ↑ MHC I | ↑ MHC II     | ↑ MHC II| ↓ CD86      | ↓ MHC II      | ↓ CD86       |
| bmDC-stimulated T-cell function | —     | +++          | ++     | +++          | —              | +++          |
involved in the suppression of maturation and maturation-induced migration of inflammatory myeloid cells and Mac/MΦ. Taken together with the reduced percentages of APC populations in the lung, the broad changes in mRNA levels might be reflective of a reduced influx of immune cells into the lung. It has been demonstrated that DC maturation is necessary for migration (De Vries et al., 2003; Lin et al., 1998). This would suggest that infected lungs are enriched for mature DC, but the level of expression of maturation markers on DC does not differ from uninfected lungs. Indeed, aside from minor changes of MHC II on cDC, no overt changes in the maturation status of the APC subsets were observed as a result of Δ9-THC treatment. In vitro, Δ9-THC treatment impaired the LPS-induced maturation of DC as evidenced by lower expression of CD86. Therefore, it is possible that Δ9-THC reduces maturation of DC as demonstrated in vitro, thereby decreasing their ability to migrate and accumulate in the lung as observed in vivo, which ultimately would result in lower numbers of mature DC after infection. Furthermore, leukocyte trafficking, to a large extent, is dependent on chemokine receptors, which are part of the GPCR family (Allen et al., 2007). Studies on cellular migration in vitro suggest a mechanism involving CB2-mediated diminution of signaling through chemokine receptors (Kishimoto et al., 2003; Montecucco et al., 2008).

Alternately, it has been proposed that ligation of CB1 and CB2 induces apoptosis in DC, which could account for the lower percentage of DC observed in the lung (Do et al., 2004). In this study, differences in bmDC viability in vitro using the LIVE/DEAD stain were not observed (data not shown). However, in contrast to our study, Do et al. (2004) used a

![Image](http://toxsci.oxfordjournals.org/)

**FIG. 8.** Δ9-THC suppresses TLR-stimulated bmDC maturation independent of CB1 and CB2. Bone marrow was used to generate bmDC in the presence of granulocyte-macrophage colony stimulating factor (20 ng/ml) for 9 days. bmDC were washed and incubated for 24h in the presence or absence (NA) of LPS (1 µg/ml) or R848 (5 µg/ml) and cotreated with VH (0.1% ethanol) or Δ9-THC (10 µM). After incubation, cells were stained for CD11b, CD11c, MHC I, MHC II and CD86 was performed as previously described. (A) Cells obtained after culture were CD11b+CD11c+, indicating cDC phenotype. (B) Graphs shown are concatenated samples (n = 3). (C) ANOVA tests were performed. ** (p ≤ 0.01), difference between Δ9-THC and VH (ethanol); ++ (p ≤ 0.01), +++ (p ≤ 0.001), difference between NA and PR8; ## (p ≤ 0.01), difference between WT and CB1−/−CB2−/−. The experiment is representative of three identical repeat experiments.
higher LPS concentration (10 \(\mu\)g/ml), longer preincubation periods with \(\Delta^9\)-THC (2 h), and serum-free media. Serum-free media alone is known to induce stress in cultured immune cells (Perandones et al., 1993). This suggests that apoptosis is not the primary mechanism by which \(\Delta^9\)-THC reduces DC function.

Other studies suggest that the mechanism by which cannabinoids suppress proinflammatory cytokine production might involve IRF3-dependent upregulation of IFN-\(\beta\) (Downer et al., 2011). Whole-lung steady-state mRNA levels of type I IFNs were not enhanced by \(\Delta^9\)-THC treatment (Supplementary fig. 1). Therefore, it is unlikely that in this influenza model the mechanism by which \(\Delta^9\)-THC impairs host immunity and reduces inflammation is by the upregulation of production of type I IFNs.

Collectively, the present studies demonstrate that \(\Delta^9\)-THC suppressed the host immune response against influenza virus challenge. Furthermore, after influenza infection, among the APC populations, inflammatory myeloid cells and Mac/\(\Phi\) were the most sensitive to \(\Delta^9\)-THC–mediated immune suppression, which required the presence of \(\text{CB}_1\) and/or \(\text{CB}_2\). In vitro, it was demonstrated that DC-like bmDC play a crucial role in suppressing T cell responses after treatment with \(\Delta^9\)-THC, which was also found to be dependent on \(\text{CB}_1\) and/or \(\text{CB}_2\). Importantly, not all the immune modulatory properties of \(\Delta^9\)-THC can be attributed to ligation of \(\text{CB}_1\) and/or \(\text{CB}_2\) as...
LPS-induced CD86 expression on bmDC was suppressed by Δ9-THC in WT and CB1−/−CB2−/− mice in vitro. In conclusion, signaling initiated through CB1 and CB2 expressed on APC is critically involved in the Δ9-THC–mediated reduction of inflammatory responses in the lung after influenza infection.

**SUPPLEMENTARY DATA**

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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