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# $\Delta^9$-Tetrahydrocannabinol Impairs the Inflammatory Response to Influenza Infection: Role of Antigen-Presenting Cells and the Cannabinoid Receptors 1 and 2

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**$\Delta^9$-tetrahydrocannabinol ($\Delta^9$-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 ($CB_1$) and 2 ($CB_2$). Most recently, dendritic cells (DC) were identified for their potential to enhance influenza-induced immunopathology in mice lacking $CB_1$ and $CB_2$ ($CB_1^{-/-}CB_2^{-/-}$). This study focused on the modulation of the inflammatory immune response to influenza by $\Delta^9$-THC and the role of $CB_1$ and/or $CB_2$ as receptor targets for $\Delta^9$-THC. C57Bl/6 (wild type) and $CB_1^{-/-}CB_2^{-/-}$ mice were administered $\Delta^9$-THC (75 mg/kg) surrounding the intranasal instillation of A/PR/8/34 influenza virus. Three days post infection (dpi), $\Delta^9$-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 $NK1.1^+$ cells, and reduced the influx of antigen-presenting cells (APC), including inflammatory myeloid cells and monocytes/macrophages, into the lung in a $CB_1$- and/or $CB_2$-dependent manner. $\Delta^9$-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 $\Delta^9$-THC in bone marrow–derived DC (bmDC). Furthermore, antigen-specific IFN-γ production by $CD8^+$ T cells after coculture was reduced by $\Delta^9$-THC treatment of bmDC in a $CB_1$- and/or $CB_2$-dependent manner. Collectively, these studies suggest that $\Delta^9$-THC potently suppresses myeloid cell immune function, in a manner involving $CB_1$ and/or $CB_2$, thereby impairing immune responses to influenza infection.**

***Key Words:*** **$\Delta^9$-tetrahydrocannabinol; cannabinoid receptors; immune modulation; antigen-presenting cells; influenza.**

$\Delta^9$-tetrahydrocannabinol ($\Delta^9$-THC) is the primary psychoactive constituent in the marijuana plant. Its biological activity can be partially attributed to the $G_i$ protein-coupled receptors (GPCR), $CB_1$ and $CB_2$ (Howlett *et al.*, 2002). In addition to eliciting psychotropic effects, predominantly mediated by $CB_1$ (Huestis *et al.*, 2001), $\Delta^9$-THC exerts potent effects on the immune system through $CB_1$- and $CB_2$-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 $CB_1$ and $CB_2$ varies among leukocytes and can change in response to immune stimulation, albeit $CB_2$ expression is greater than $CB_1$ in cell of the immune system (Bouaboula *et al.*, 1993; Galiègue *et al.*, 1995; Nong *et al.*, 2001).

Although pharmacological effects by $\Delta^9$-THC have been extensively investigated on isolated immune cells *in vitro*, the effects of $\Delta^9$-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 $\Delta^9$-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 $\Delta^9$-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 $\Delta^9$-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 ($Th_1$) 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., $Th_1$) (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 inflammatory or monocytic 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 $CB_1$ and $CB_2$ suppressed the magnitude of the immune response, at least in part, by regulating DC function. In turn, the lack of $CB_1$ and $CB_2$ 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 $\Delta^9$-THC on APC populations involved in the immune response to influenza at 3 dpi and to identify whether $CB_1$ and $CB_2$ are involved in modulating this anti-influenza response. Collectively, our results suggest that $\Delta^9$-THC suppresses DC, Mac/MΦ, and inflammatory myeloid cell responses to a great extent via $CB_1$ and/or $CB_2$, thereby impairing cellular immune responses induced by influenza infection.

## MATERIALS AND METHODS

***Mice.*** Age-matched C57Bl/6 wild type (WT) and $CB_1^{-/-}CB_2^{-/-}$ mice bred on a C57Bl/6 background were used for *in vivo* experiments. WT mice were purchased from the National Cancer Institute (Frederick, MD). $CB_1^{-/-}CB_2^{-/-}$ mice were a kind gift of Dr Andreas Zimmer from the University of Bonn (Karsak *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 (Tcr) α and Tcr β (OT-1), generating $CD8^+$ T cells specific for chicken ovalbumin ($OVA_{257-264}$; amino acid sequence: SIINFEKL), were purchased from Jackson Laboratories (Bar Harbor, ME). All $CB_1^{-/-}CB_2^{-/-}$ breeders, experimental WT and $CB_1^{-/-}CB_2^{-/-}$ mice, and sentinels 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 instillation.*** 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.

***$\Delta^9$-THC treatment.*** The National Institute on Drug Abuse (NIDA, Bethesda, MD) provided $\Delta^9$-THC for experimental use. For *in vivo* experiments, mice received corn oil (CO) vehicle (VH) or $\Delta^9$-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 instillation 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 $\Delta^9$-THC in the mice are comparable with serum $\Delta^9$-THC levels in humans after smoking marijuana. Specifically, after five consecutive days of treatment by oral gavage with 75 mg/kg $\Delta^9$-THC, mouse serum reached concentrations of 66.2 ng/ml of $\Delta^9$-THC 4 h after the last $\Delta^9$-THC dose (Buchweitz *et al*., 2007, 2008), whereas human serum $\Delta^9$-THC concentrations have ranged between 57 and 268 ng/ml (Azorlosa *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 *en bloc* 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 sieve 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 5 h 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; $H\text{-}2K^b/H\text{-}2D^b$, 28-8-6), and MHC II (I-A/I-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 Biolegend): 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 $CB_1^{-/-}CB_2^{-/-}$ mice were flushed for bone marrow, and $1 \times 10^6$ cells were grown in 4 ml of RPMI media supplemented with 10% serum containing 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 24 h, 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 2 h 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 5 h 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 $CB_1^{-/-}CB_2^{-/-}$ 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 $CB_1^{-/-}CB_2^{-/-}$ (#). Statistical analysis of SAL-versus PR8-instilled mice treated with Δ9-THC was not performed in either WT or $CB_1^{-/-}/CB_2^{-/-}$ mice because an immune response was expected as a result of PR8 instillation.

## RESULTS

### *Δ9-THC Reduced Steady-State mRNA Expression of Genes Induced by Influenza in the Lungs of WT but Not $CB_1^{-/-}CB_2^{-/-}$ Mice*

Previously, the effects of $CB_1$ and $CB_2$ 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 $CB_1^{-/-}CB_2^{-/-}$ mice, a 96-gene 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 $CB_1^{-/-}CB_2^{-/-}$ mice, suggesting $CB_1$- and/or $CB_2$-dependent modulation (Fig. 1B). In addition, mRNA levels for a few genes (*Fas* and *Il13* in SAL group and *Col4a5*, *Cyp7a1*, and *Ifnγ* in the PR8 group) were upregulated in $CB_1^{-/-}CB_2^{-/-}$ mice as a result of Δ9-THC treatment, suggesting the potential for $CB_1$- and $CB_2$-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 $10^4$ cells/ml) into the airways, but increased numbers of neutrophils and Mac/MΦ (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 $CB_1^{-/-}CB_2^{-/-}$ mice compared with WT mice and consisted mainly of neutrophils and Mac/MΦ (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 $CB_1^{-/-}CB_2^{-/-}$ 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 $CB_1^{-/-}CB_2^{-/-}$ mice compared with WT mice (Figs. 4 and 5). Influenza infection significantly induced the percentage of IFN-γ–producing $CD4^+$ cells in WT mice, but not $CB_1^{-/-}CB_2^{-/-}$ mice, and Δ9-THC suppressed the percentage of IFN-γ–producing cells only in WT mice. The percentage of the IL-17–producing population 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

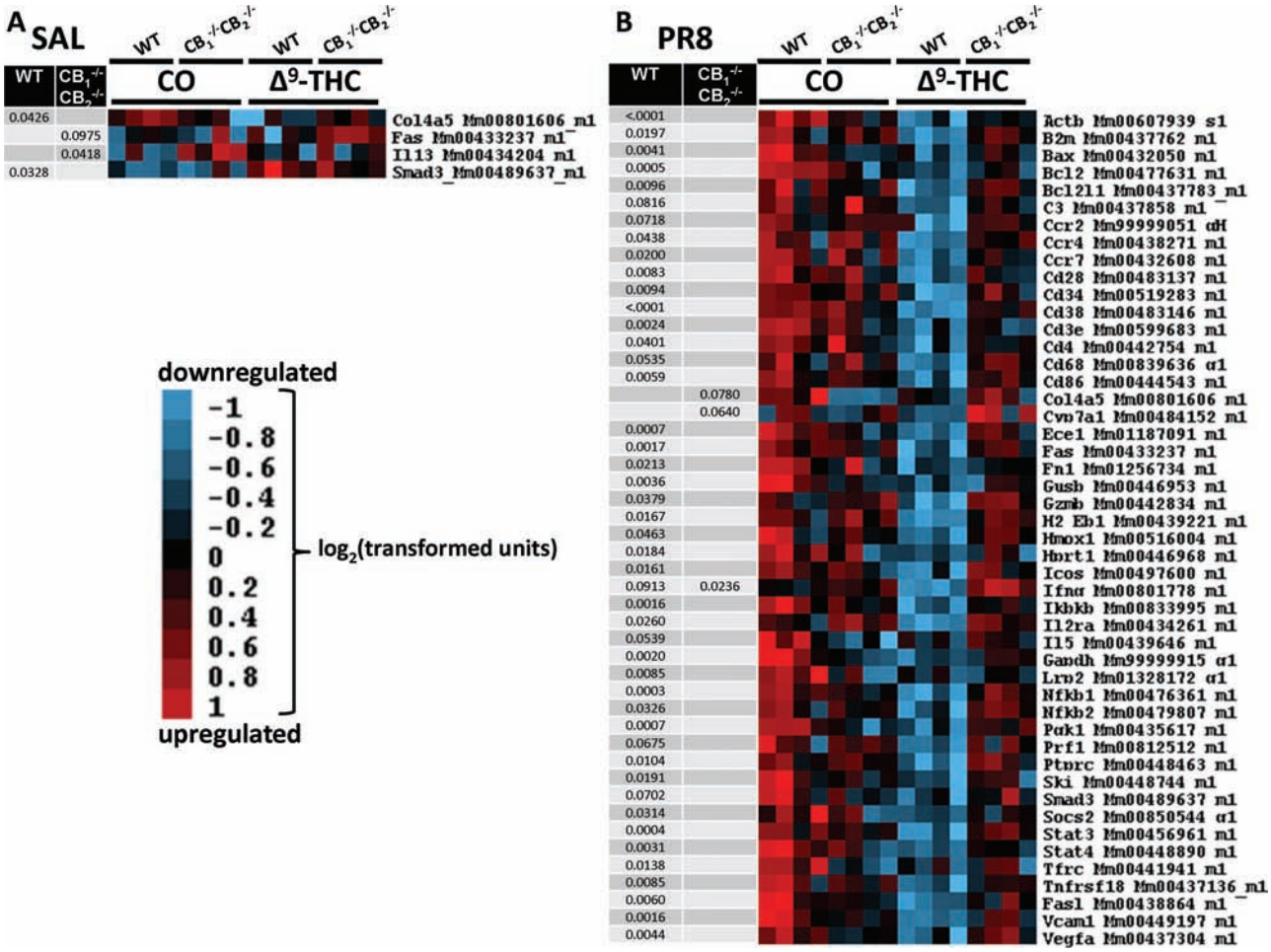


**FIG. 1.** Reduction of gene expression associated with influenza infection as a result of $\Delta^9$-THC treatment. Mice ($n = 4$) were treated with CO VH or $\Delta^9$-THC (75 mg/kg) for 5 consecutive days surrounding the intranasal instillation of influenza (PR8) or saline (SAL). Lung RNA was isolated 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 and visualized using Treeview. Shown are genes differentially regulated by at least 1.5-fold between CO and $\Delta^9$-THC samples, with a value of at least $p \leq 0.1$ 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 $\Delta^9$-THC treatment.

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), $\Delta^9$-THC enhanced the percentages of IFN-γ–producing $CD4^+$ and $NK1.1^+$ cells and IL-17–producing $CD8^+$ and $NK1.1^+$ cells in $CB_1^{-/-}CB_2^{-/-}$ mice, which was significantly different from $\Delta^9$-THC–treated WT samples (Figs. 4 and 5). Overall, these results demonstrate that $\Delta^9$-THC suppressed the percentages of functional effector $CD4^+$ and $NK1.1^+$ cells 3 dpi in a $CB_1$- and/or $CB_2$-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 $CD11b^{lo}CD11c^{lo}$, cDC as $CD11b^+CD11c^+Gr\text{-}1^-$, and inflammatory myeloid cells as $CD11b^+CD11c^+Gr\text{-}1^+$, whereas AM are $CD11c^+CD11b^-Gr\text{-}1^-$ and pDC are $CD11b^-CD11c^+Gr\text{-}1^+$. 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

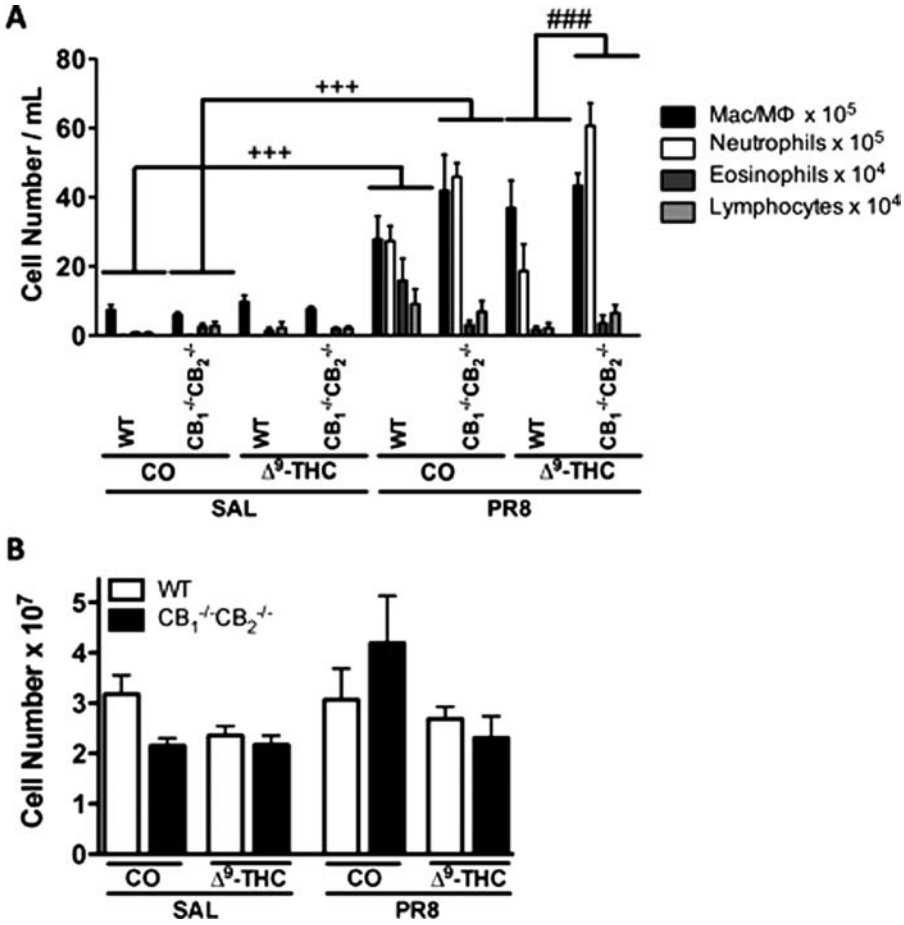


**FIG. 2.** Δ9-THC does not alter BALF cell counts or composition or total lung cells after influenza infection. (A) BALF was isolated by flushing lungs at 3 dpi twice with 0.9 ml SAL ($n = 5$). Total cell numbers were counted using a hemacytometer, then BALF was centrifuged onto slides, dried, and differential 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 $10^5$, whereas eosinophils are in the order of $10^4$ 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: +++ ($p \leq 0.001$), difference between SAL and PR8; ### ($p \leq 0.001$), difference between WT and $CB_1^{-/-}CB_2^{-/-}$.

of inflammatory myeloid cells and pDC were found in lungs of $CB_1^{-/-}CB_2^{-/-}$ 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 $CB_1^{-/-}CB_2^{-/-}$mice, suggesting $CB_1$ and/or $CB_2$ 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 $CB_1$- and/or $CB_2$-dependent manner.

### *Enhanced Maturation of APC From $CB_1^{-/-}CB_2^{-/-}$ 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, $CB_1^{-/-}CB_2^{-/-}$ 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 $CB_1^{-/-}CB_2^{-/-}$ mice. Δ9-THC did not alter the expression of MHC I, MHC II, or CD86 in any of the cell types of WT or $CB_1^{-/-}CB_2^{-/-}$ 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.

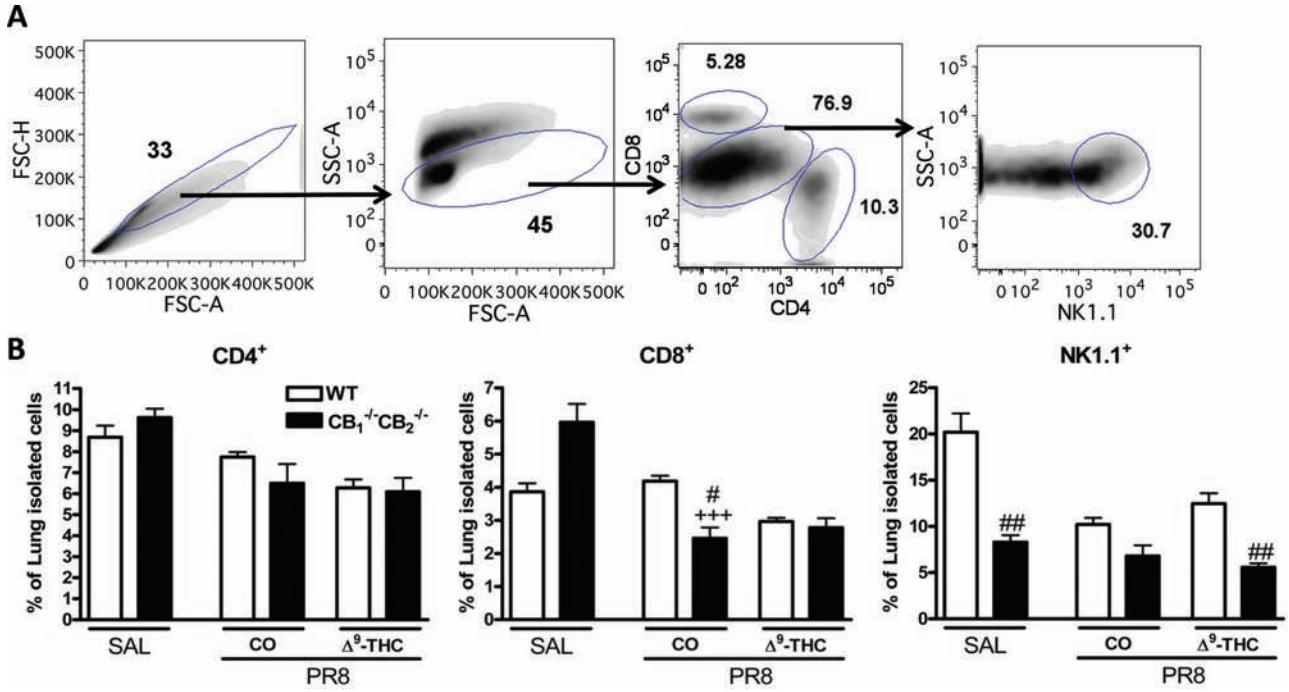


**FIG. 3.** No change in percent lymphocyte composition in lungs of mice treated with $\Delta^9$-THC. Lungs ($n = 5$) were mechanically disrupted at 3 dpi, and single cell suspensions were obtained. Cells were stained with fluorescently labeled antibodies for surface markers CD4, CD8, NK1.1 and analyzed by flow cytometry. (A) Cells were gated on single cells, size, $CD4^+$, $CD8^+$, or $CD4^-CD8^-NK1.1^+$. (B) The percent of cells in the lung with respect to prior gate is shown for each treatment group ($n = 5$). Data were analyzed using Kruskal-Wallis' test for nonparametric data: +++ ($p \leq 0.001$), difference between SAL and PR8; # ($p \leq 0.05$), ## ($p \leq 0.01$), difference between WT and $CB_1^{-/-}CB_2^{-/-}$.

*Attenuation of bmDC Maturation by $\Delta^9$-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, $\Delta^9$-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 $\Delta^9$-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 $CB_1^{-/-}CB_2^{-/-}$ 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 $CB_1^{-/-}CB_2^{-/-}$ mice compared with those obtained from WT mice. $\Delta^9$-THC (10 μM) reduced LPS-induced maturation, but not R848-induced maturation, as evidenced by lower MHC II in $CB_1^{-/-}CB_2^{-/-}$ and CD86 expression in WT and $CB_1^{-/-}CB_2^{-/-}$ mice (Figs. 8B and 8C). Collectively, bmDC maturation was suppressed by $\Delta^9$-THC independently of $CB_1$ and/or $CB_2$.

*Impaired Elicitation of T Cells After $\Delta^9$-THC Treatment of bmDC*

To determine the functional consequences of $\Delta^9$-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 $OVA_{257-264}$ (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

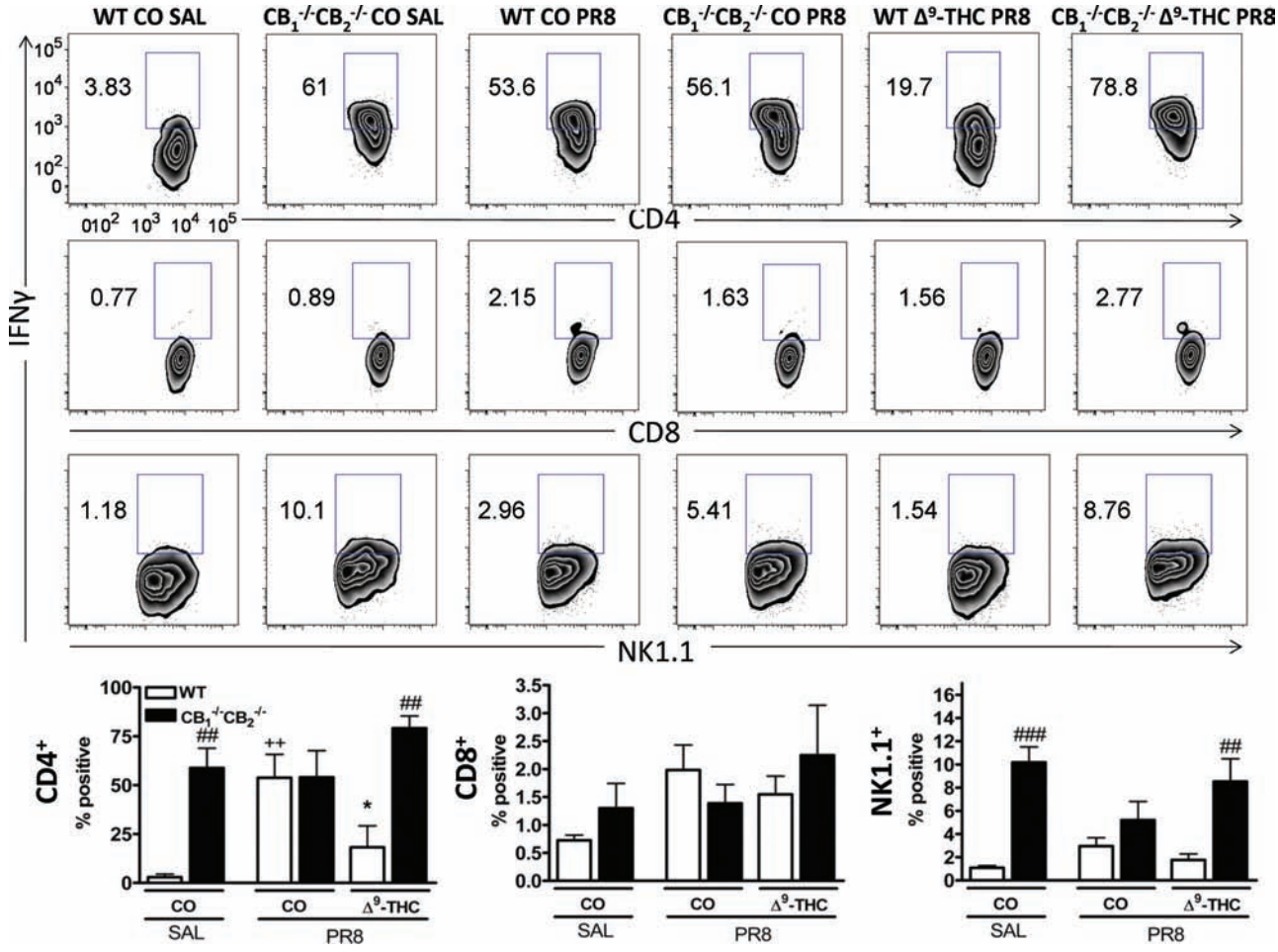


**FIG. 4.** Δ9-THC decreased IFN-γ production in CD4+ cells after influenza infection. At 3 dpi, lungs ($n = 5$) were mechanically disrupted, and single cell suspensions were restimulated *in vitro* with PMA/Io (40nM/0.5μM) to induce cytokine secretion and in the presence of Brefeldin A in 2% serum RPMI 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: *($p \leq 0.05$), difference between CO and Δ9-THC; ++ ($p \leq 0.01$), difference between SAL and PR8; ## ($p \leq 0.01$), ### ($p \leq 0.001$), difference between WT and $CB_1^{-/-}CB_2^{-/-}$. Shown are samples concatenated ($n = 5$) within treatment groups.

$CB_1^{-/-}CB_2^{-/-}$ 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 $CB_1^{-/-}CB_2^{-/-}$ mice, neither TLR stimulation nor Δ9-THC had any effect on the ability of $CB_1^{-/-}CB_2^{-/-}$ 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 $CB_1$- and/or $CB_2$-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.

## DISCUSSION

Despite the identification of $CB_1$ and $CB_2$, 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 $CB_1$ and $CB_2$ 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 $CB_1$ and $CB_2$ partial agonist, Δ9-THC, in WT and $CB_1^{-/-}CB_2^{-/-}$ 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 $CB_1^{-/-}CB_2^{-/-}$ mice, demonstrating that

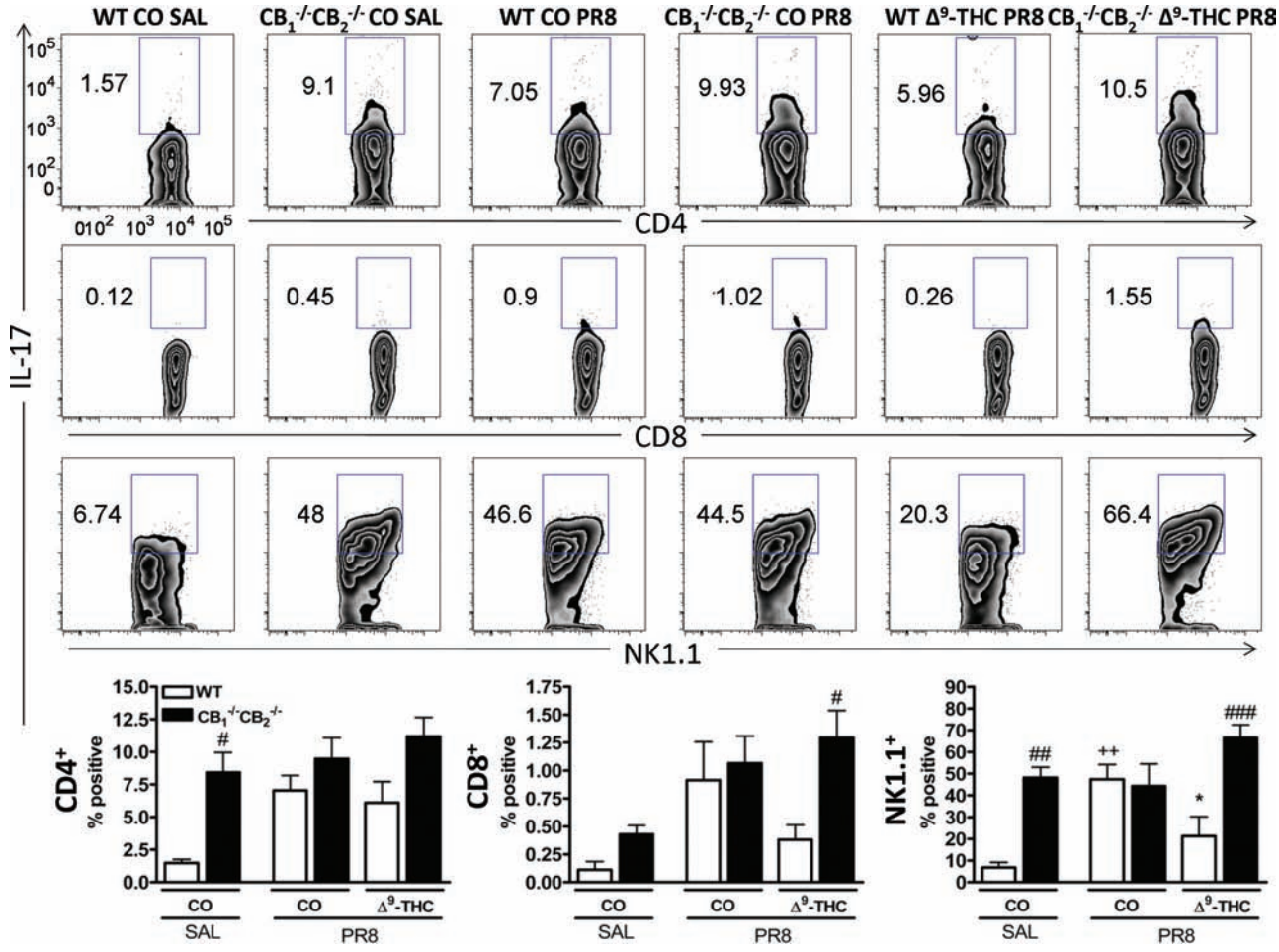


**FIG. 5.** Δ⁹-THC decreased IL-17 production in NK1.1⁺ cells after influenza infection. Lungs were mechanically disrupted and restimulated *in vitro* with PMA/Io as described in the Figure 4 ($n = 5$). CD4, CD8, and NK1.1 were stained on the surface of isolated cells, and intracellular staining for IL-17 and flow cytometry were performed. Nonparametric percentage data were analyzed using Kruskal-Wallis' test: *($p \le 0.05$), difference between CO and Δ⁹-THC; ++ ($p \le 0.01$), difference between NA (SAL) and PR8; # ($p \le 0.05$), ## ($p \le 0.01$), ### ($p \le 0.001$), difference between WT and $CB_1^{-/-}CB_2^{-/-}$. Displayed are concatenated samples ($n = 5$) of each treatment group.

inflammation peaked at 3 dpi and T-cell responses occurred earlier in $CB_1^{-/-}CB_2^{-/-}$ compared with WT mice (Karmaus *et al.*, 2011). Even during this early response to infection, immune parameters were suppressed by Δ⁹-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, Δ⁹-THC reduced T-cell responses through modulation of DC function in WT mice, but not $CB_1^{-/-}CB_2^{-/-}$ mice. Thus, although other targets for Δ⁹-THC exist, $CB_1$ and/or $CB_2$ expression on myeloid cells is important in mediating the immunomodulatory effects of Δ⁹-THC on effector cell populations such as NK cells and T cells involved in the immune response to influenza virus infection.

It was evident from the *in vitro* bmDC studies that direct modulation of DC by Δ⁹-THC is possible. Much of our *in vivo* evidence suggested that the immunomodulatory effect of Δ⁹-THC was mediated via $CB_1$ and/or $CB_2$; in contrast, Δ⁹-THC also suppressed maturation of bmDC generated from $CB_1^{-/-}CB_2^{-/-}$ mice. Previous studies support that both $CB_1$ and $CB_2$ contribute to the modulation of DC function by Δ⁹-THC with the use of $CB_1$- and $CB_2$-specific antagonists (Do *et al.*, 2004) and that $CB_1$ 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 $CB_1$ and $CB_2$ signaling (Howlett *et al.*, 1986; Kaminski *et al.*, 1994), did not completely abrogate the Δ⁹-THC–mediated suppression of IL-12p40 production in bmDC (Lu *et al.*, 2006b). Also, selective antagonists to either $CB_1$ or $CB_2$ did not block the suppression of IL-12p40 by Δ⁹-THC (Lu *et al.*, 2006b). These results suggest that although $CB_1$ and $CB_2$ are involved in Δ⁹-THC modulation of DC, it is not the exclusive or sole mechanism. In this study, Δ⁹-THC suppressed LPS-induced CD86 expression of bmDC generated from $CB_1^{-/-}CB_2^{-/-}$ mice; however, this suppression of maturation did not alter elicitation of T-cell responses in cocultures.

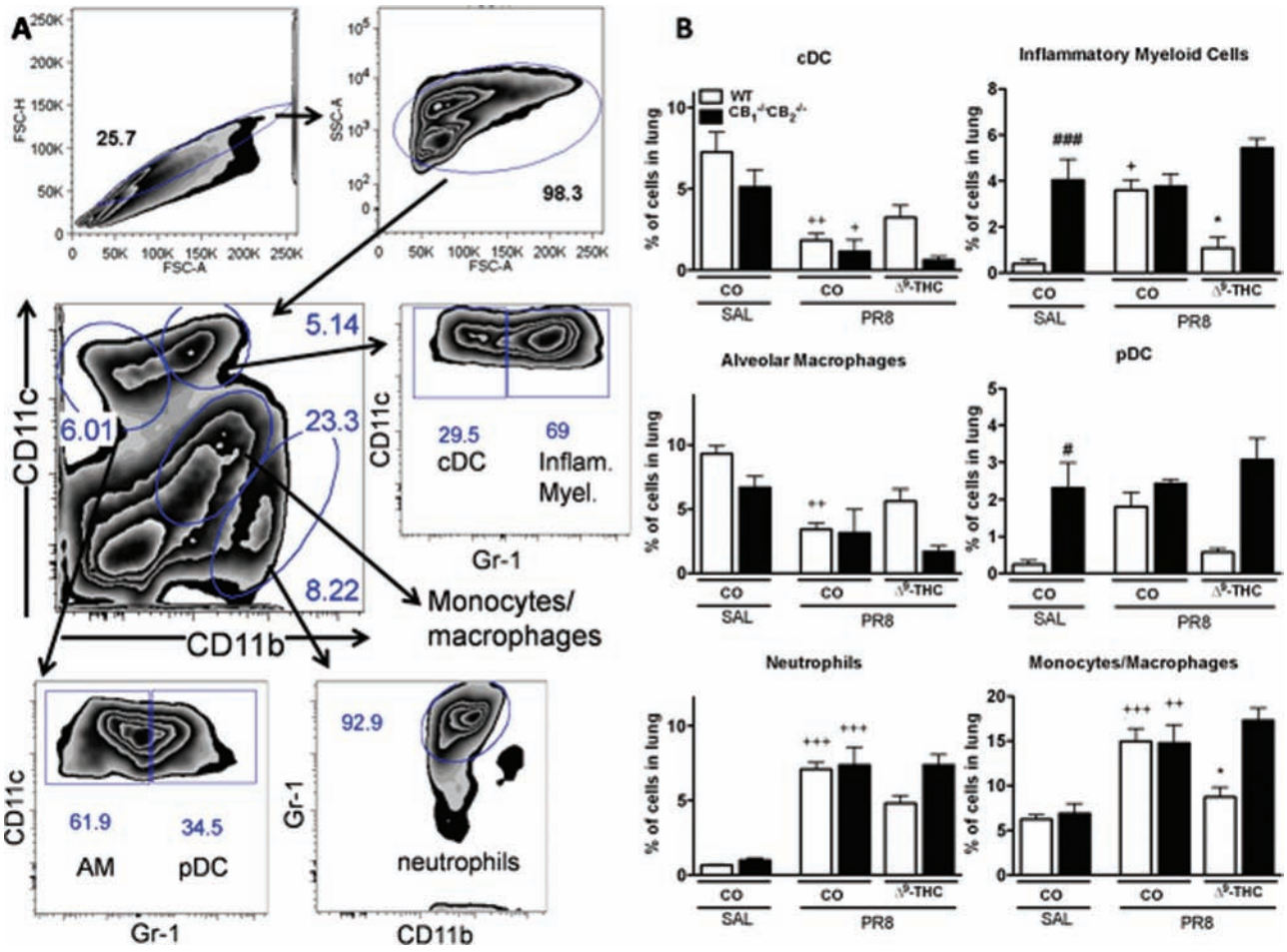


**FIG. 6.** Δ9-THC reduces recruitment of inflammatory myeloid cells and Mac/MΦ into the lungs of PR8-infected mice in a $CB_1$- and/or $CB_2$-dependent manner. At 3 dpi, lungs ($n = 5$) were mechanically disrupted. Single cell suspensions obtained from the lung were stained for CD11b, CD11c, Gr-1, MHC I, MHC II, and CD86. CD11b, CD11c, and Gr-1 were used as markers to identify separate cell populations as shown in the gating scheme (A), which were then enumerated by percentage (B). Kruskal-Wallis' test was used to perform statistics on nonparametric percentage data: ** ($p \le 0.01$), * ($p \le 0.05$), difference between CO and Δ9-THC, ++ ($p \le 0.01$), +++ ($p \le 0.001$), difference between SAL and PR8; ## ($p \le 0.01$), difference between WT and $CB_1^{-/-}CB_2^{-/-}$.

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 $CB_1^{-/-}CB_2^{-/-}$, but not WT, mice. Other factors aside from the maturation markers assessed on bmDC might be involved in the suppression of maturation, independent of $CB_1$ and $CB_2$. 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 $CB_1^{-/-}CB_2^{-/-}$ mice, further supporting the view that targets in addition to $CB_1$ and $CB_2$ 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 $CD11c^+CD11b^-Gr\text{-}1^+$) 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

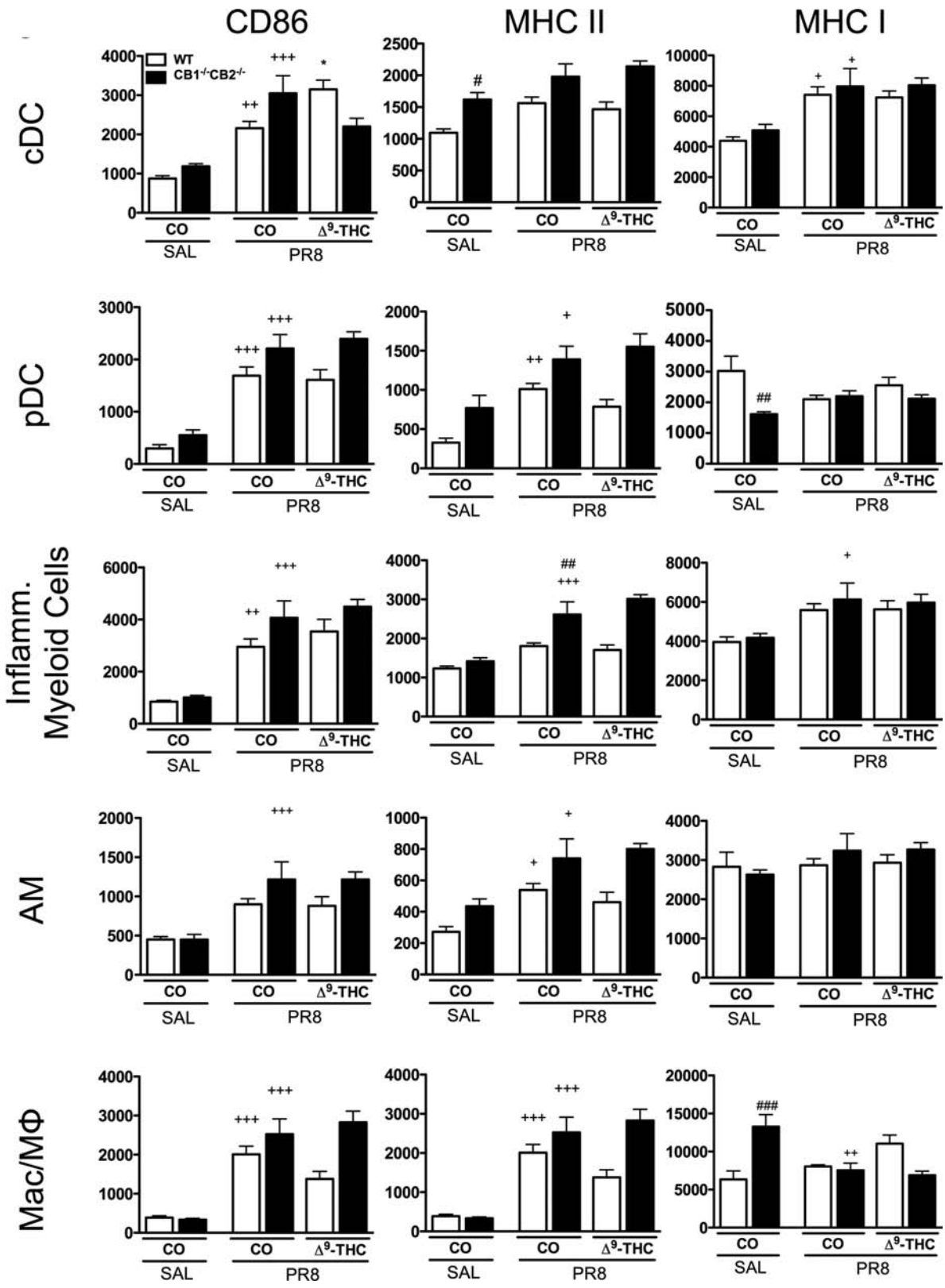


**FIG. 7.** $\Delta^9$-THC does not alter the maturation status of lung-isolated myeloid cells after PR8 infection. Following the gating scheme in Figure 6, MHC I, MHC II, and CD86 expression was determined on cDC ($CD11b^+CD11c^+Gr\text{-}1^-$), pDC ($CD11b^-Cd11c^+Gr\text{-}1^+$), inflammatory myeloid cells ($CD11b^+Cd11c^+Gr\text{-}1^+$), AM ($CD11b^-CD11c^+Gr\text{-}1^-$), and Mac/MΦ ($CD11b^{lo}CD11c^{lo}$). Bar graphs show mean fluorescence intensities (MFI) for indicated maturation markers ($n = 5$). Statistical analysis was performed using ANOVA: * ($p \le 0.01$), difference between $\Delta^9$-THC and VH (CO); + ($p \le 0.05$), ++ ($p \le 0.01$) +++ ($p \le 0.001$), difference between SAL and PR8; # ($p \le 0.05$), ## ($p \le 0.01$), ### ($p \le 0.001$), difference between WT and $CB_1^{-/-}CB_2^{-/-}$.

**TABLE 1**
**Summary of Major Findings**

| | Basal | | PR8 | | PR8 + Δ⁹-THC | |
|---|---|---|---|---|---|---|
| | WT | $CB_1^{-/-}/CB_2^{-/-}$ | WT | $CB_1^{-/-}/CB_2^{-/-}$ | WT | $CB_1^{-/-}/CB_2^{-/-}$ |
| **Cells** | | | | | | |
| BALF total cells | — | — | ++ | +++ | ++ | +++ |
| $CD4^+IFN\text{-}\gamma^+$ | — | ++ | ++ | ++ | + | +++ |
| $NK1.1^+IL\text{-}17^+$ | — | ++ | ++ | ++ | + | +++ |
| **Cell presence** | | | | | | |
| cDC | ++ | — | — | — | — | — |
| pDC | — | ++ | ++ | ++ | + | ++ |
| Inflammatory myeloid cells | — | ++ | ++ | ++ | — | + |
| AM | +++ | +++ | ++ | ++ | ++ | + |
| Mac/MΦ | + | + | ++ | ++ | + | ++ |
| **Maturation markers** | | | | | | |
| cDC | | ↑ MHC II | ↑ MHC I<br>↑ CD86 | ↑ MHC I<br>↑ CD86 | ↑ CD86 | |
| pDC | | ↓ MHC I | ↑ MHC II<br>↑ CD86 | ↑ MHC II<br>↑ CD86 | | |
| Inflammatory myeloid cells | | | ↑ CD86 | ↑ MHC I<br>↑ MHC II<br>↑ CD86 | | |
| AM | | | ↑ MHC II | ↑ MHC II<br>↑ CD86 | | |
| Mac/MΦ | | ↑ MHC I | ↑ CD86 | ↓ MHC I<br>↑ MHC II<br>↑ CD86 | | |

| | NA | | LPS | | LPS + Δ⁹-THC | |
|---|---|---|---|---|---|---|
| | WT | $CB_1^{-/-}/CB_2^{-/-}$ | WT | $CB_1^{-/-}/CB_2^{-/-}$ | WT | $CB_1^{-/-}/CB_2^{-/-}$ |
| **bmDC** | | | | | | |
| bmDC maturation markers | | ↑ MHC I<br>↑ MHC II | ↑ MHC II<br>↑ CD86 | ↑ MHC II<br>↑ CD86<br>↑ CD80 | ↓ CD86 | ↓ MHC II<br>↓ CD86 |
| bmDC-stimulated T-cell function | — | +++ | ++ | +++ | — | +++ |

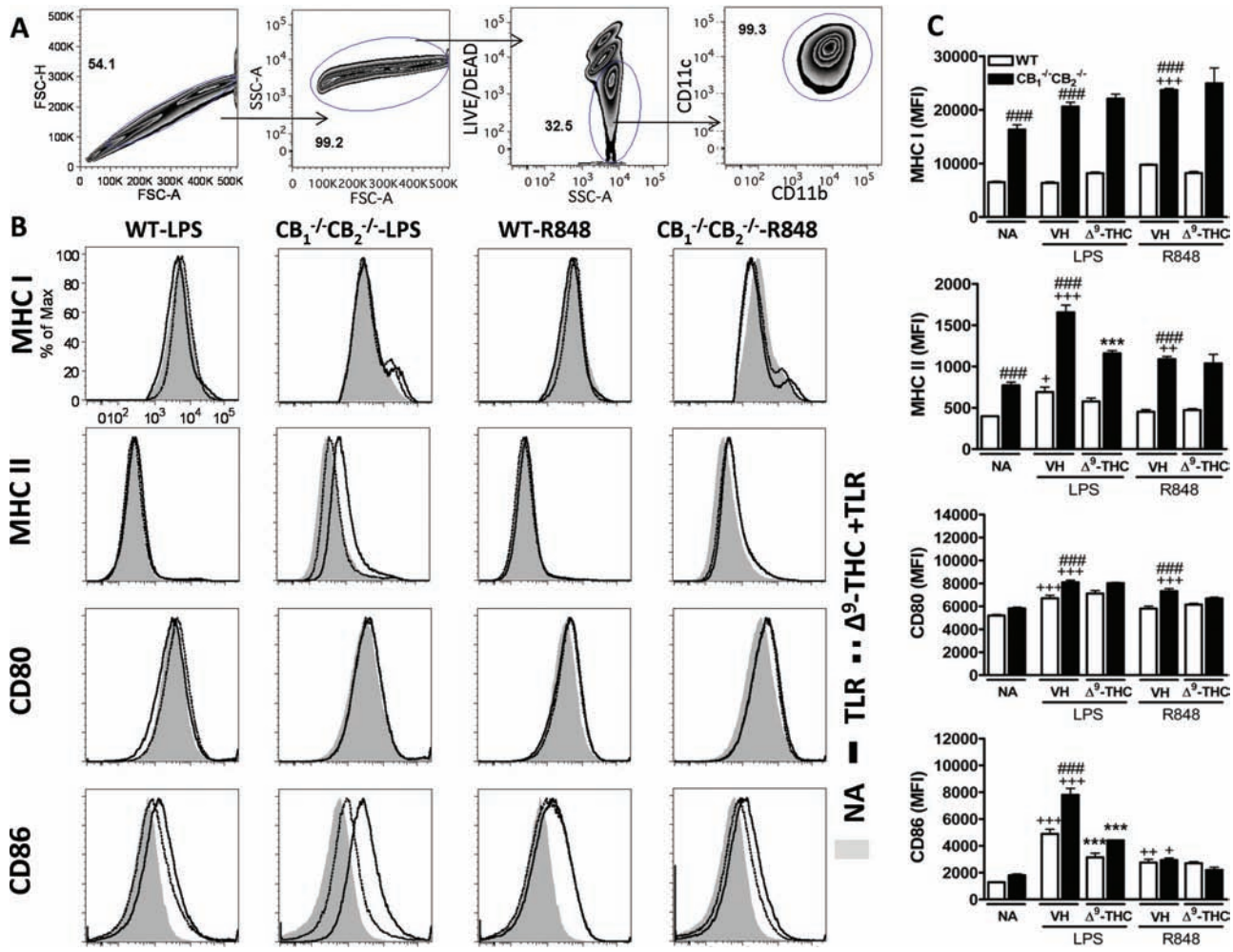


**FIG. 8.** $\Delta^9$-THC suppresses TLR-stimulated bmDC maturation independent of $CB_1$ and $CB_2$. Bone marrow was used to generate bmDC in the presence of granulocyte-macrophage colony stimulating factor (20 ng/ml) for 9 days. bmDC were washed and incubated for 24 h in the presence or absence (NA) of LPS (1 μg/ml) or R848 (5 μg/ml) and cotreated with VH (0.1% ethanol) or $\Delta^9$-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. ** ($p \leq 0.01$), difference between $\Delta^9$-THC and VH (ethanol); ++ ($p \leq 0.01$), +++ ($p \leq 0.001$), difference between NA and PR8; ## ($p \leq 0.01$), difference between WT and $CB_1^{-/-}CB_2^{-/-}$. The experiment is representative of three identical repeat experiments.

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 $\Delta^9$-THC treatment. *In vitro*, $\Delta^9$-THC treatment impaired the LPS-induced maturation of DC as evidenced by lower expression of CD86. Therefore, it is possible that $\Delta^9$-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 $CB_2$-mediated diminution of signaling through chemokine receptors (Kishimoto *et al*., 2003; Montecucco *et al*., 2008).

Alternately, it has been proposed that ligation of $CB_1$ and $CB_2$ 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

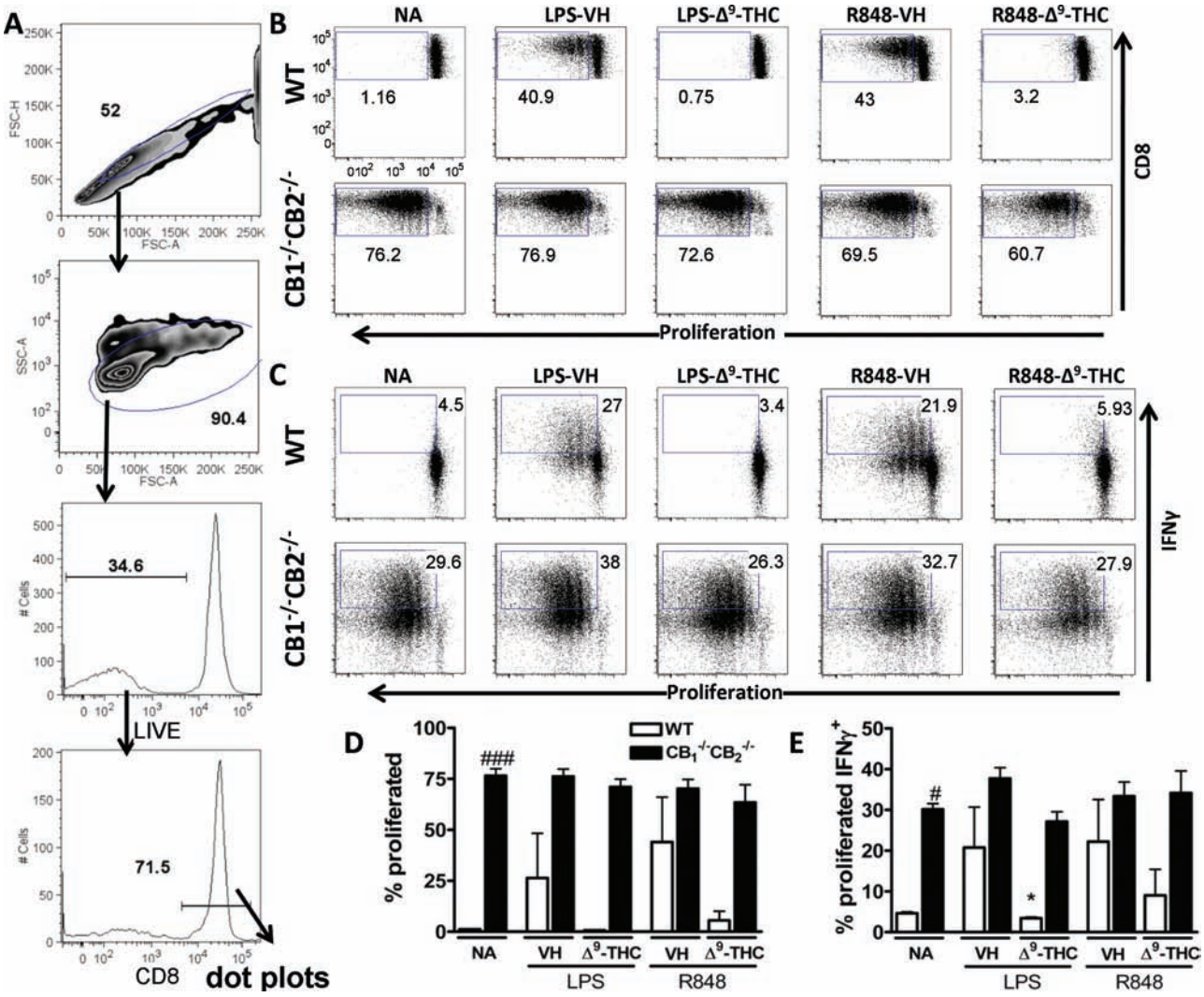


**FIG. 9.** Δ9-THC impairs antigen-specific bmDC-elicited T-cell responses. bmDC (NA or TLR-treated) were treated with VH (0.1% ethanol) or Δ9-THC (10 μM) and pulsed with the OT-1 TCR-specific peptide SIINFEKL for 1 h and washed three times prior to incubation with Cell Trace–labeled OT-1 splenocytes for 4 days. Cells were restimulated with SIINFEKL, stained for CD8 and IFN-γ, and gated as depicted in the scheme to obtain dot plots (A). bmDC and then gated as described in Figure 3A. Dot plots with Cell Trace loss indicating proliferation on the x-axis and CD8 staining on the y-axis are shown. Gate shown indicates proliferation (loss of Cell Trace fluorescence compared with control) and CD8 staining (B) or IFN-γ staining (C). Shown are concatenated samples of each group ($n = 3$). Kruskal-Wallis tests were performed on samples from Figures 9B and C, shown in D and E, respectively, * ($p \le 0.05$), difference between Δ9-THC to VH (ethanol); ++ ($p \le 0.01$), # ($p \le 0.05$), ### ($p \le 0.001$), difference between WT and $CB_1^{-/-}CB_2^{-/-}$. The experimental data are representative of two identical repeat experiments.

higher LPS concentration (10 μg/ml), longer preincubation periods with Δ9-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 Δ9-THC reduces DC function.

Other studies suggest that the mechanism by which cannabinoids suppress proinflammatory cytokine production might involve IRF3-dependent upregulation of IFN-β (Downer *et al.*, 2011). Whole-lung steady-state mRNA levels of type 1 IFNs were not enhanced by Δ9-THC treatment (Supplementary fig. 1). Therefore, it is unlikely that in this influenza model the mechanism by which Δ9-THC impairs host immunity and reduces inflammation is by the upregulation of production of type I IFNs.

Collectively, the present studies demonstrate that Δ9-THC suppressed the host immune response against influenza virus challenge. Furthermore, after influenza infection, among the APC populations, inflammatory myeloid cells and Mac/MΦ were the most sensitive to Δ9-THC–mediated immune suppression, which required the presence of $CB_1$ and/or $CB_2$. *In vitro*, it was demonstrated that DC-like bmDC play a crucial role in suppressing T cell responses after treatment with Δ9-THC, which was also found to be dependent on $CB_1$ and/or $CB_2$. Importantly, not all the immune modulatory properties of Δ9-THC can be attributed to ligation of $CB_1$ and/or $CB_2$ as

LPS-induced CD86 expression on bmDC was suppressed by $\Delta^9$-THC in WT and $CB_1^{-/-}CB_2^{-/-}$ mice *in vitro*. In conclusion, signaling initiated through $CB_1$ and $CB_2$ expressed on APC is critically involved in the $\Delta^9$-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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## REFERENCES

Allen, S. J., Crown, S. E., and Handel, T. M. (2007). Chemokine: Receptor structure, interactions, and antagonism. *Annu. Rev. Immunol.* **25,** 787–820.

Asselin-Paturel, C., Boonstra, A., Dalod, M., Durand, I., Yessaad, N., Dezutter-Dambuyant, C., Vicari, A., O'Garra, A., Biron, C., Brière, F., *et al.* (2001). Mouse type I IFN-producing cells are immature APCs with plasmacytoid morphology. *Nat. Immunol.* **2,** 1144–1150.

Auffray, C., Sieweke, M. H., and Geissmann, F. (2009). Blood monocytes: Development, heterogeneity, and relationship with dendritic cells. *Annu. Rev. Immunol.* **27,** 669–692.

Azorlosa, J. L., Heishman, S. J., Stitzer, M. L., and Mahaffey, J. M. (1992). Marijuana smoking: Effect of varying delta 9-tetrahydrocannabinol content and number of puffs. *J. Pharmacol. Exp. Ther.* **261,** 114–122.

Banchereau, J., and Steinman, R. M. (1998). Dendritic cells and the control of immunity. *Nature* **392,** 245–252.

Bouaboula, M., Rinaldi, M., Carayon, P., Carillon, C., Delpech, B., Shire, D., Le Fur, G., and Casellas, P. (1993). Cannabinoid-receptor expression in human leukocytes. *Eur. J. Biochem.* **214,** 173–180.

Buchweitz, J. P., Karmaus, P. W., Harkema, J. R., Williams, K. J., and Kaminski, N. E. (2007). Modulation of airway responses to influenza A/PR/8/34 by Delta9-tetrahydrocannabinol in C57BL/6 mice. *J. Pharmacol. Exp. Ther.* **323,** 675–683.

Buchweitz, J. P., Karmaus, P. W., Williams, K. J., Harkema, J. R., and Kaminski, N. E. (2008). Targeted deletion of cannabinoid receptors CB1 and CB2 produced enhanced inflammatory responses to influenza A/PR/8/34 in the absence and presence of Delta9-tetrahydrocannabinol. *J. Leukoc. Biol.* **83,** 785–796.

Cabral, G. A., Mishkin, E. M., Marciano-Cabral, F., Coleman, P., Harris, L., and Munson, A. E. (1986). Effect of delta 9-tetrahydrocannabinol on herpes simplex virus type 2 vaginal infection in the guinea pig. *Proc. Soc. Exp. Biol. Med.* **182,** 181–186.

Crowe, C. R., Chen, K., Pociask, D. A., Alcorn, J. F., Krivich, C., Enelow, R. I., Ross, T. M., Witztum, J. L., and Kolls, J. K. (2009). Critical role of IL-17RA in immunopathology of influenza infection. *J. Immunol.* **183,** 5301–5310.

De Vries, I. J., Krooshoop, D. J., Scharenborg, N. M., Lesterhuis, W. J., Diepstra, J. H., Van Muijen, G. N., Strijk, S. P., Ruers, T. J., Boerman, O. C., Oyen, W. J., *et al.* (2003). Effective migration of antigen-pulsed dendritic cells to lymph nodes in melanoma patients is determined by their maturation state. *Cancer Res.* **63,** 12–17.

Do, Y., McKallip, R. J., Nagarkatti, M., and Nagarkatti, P. S. (2004). Activation through cannabinoid receptors 1 and 2 on dendritic cells triggers NF-kappaB-dependent apoptosis: Novel role for endogenous and exogenous cannabinoids in immunoregulation. *J. Immunol.* **173,** 2373–2382.

Downer, E. J., Clifford, E., Gran, B., Nel, H. J., Fallon, P. G., and Moynagh, P. N. (2011). Identification of the synthetic cannabinoid R(+)WIN55,212-2 as a novel regulator of IFN regulatory factor 3 activation and IFN-beta expression: Relevance to therapeutic effects in models of multiple sclerosis. *J. Biol. Chem.* **286,** 10316–10328.

Drutman, S. B., Kendall, J. C., and Trombetta, E. S. (2012). Inflammatory spleen monocytes can upregulate CD11c expression without converting into dendritic cells. *J. Immunol.* **188,** 3603–3610.

Eisen, M. B., Spellman, P. T., Brown, P. O., and Botstein, D. (1998). Cluster analysis and display of genome-wide expression patterns. *Proc. Natl. Acad. Sci. U.S.A.* **95,** 14863–14868.

Galiègue, S., Mary, S., Marchand, J., Dussossoy, D., Carrière, D., Carayon, P., Bouaboula, M., Shire, D., Le Fur, G., and Casellas, P. (1995). Expression of central and peripheral cannabinoid receptors in human immune tissues and leukocyte subpopulations. *Eur. J. Biochem.* **232,** 54–61.

Gonzalez-Juarrero, M., Shim, T. S., Kipnis, A., Junqueira-Kipnis, A. P., and Orme, I. M. (2003). Dynamics of macrophage cell populations during murine pulmonary tuberculosis. *J. Immunol.* **171,** 3128–3135.

Howlett, A. C., Barth, F., Bonner, T. I., Cabral, G., Casellas, P., Devane, W. A., Felder, C. C., Herkenham, M., Mackie, K., Martin, B. R., *et al.* (2002). International Union of Pharmacology. XXVII. Classification of cannabinoid receptors. *Pharmacol. Rev.* **54,** 161–202.

Howlett, A. C., Qualy, J. M., and Khachatrian, L. L. (1986). Involvement of Gi in the inhibition of adenylate cyclase by cannabimimetic drugs. *Mol. Pharmacol.* **29,** 307–313.

Huestis, M. A., Gorelick, D. A., Heishman, S. J., Preston, K. L., Nelson, R. A., Moolchan, E. T., and Frank, R. A. (2001). Blockade of effects of smoked marijuana by the CB1-selective cannabinoid receptor antagonist SR141716. *Arch. Gen. Psychiatry* **58,** 322–328.

Kaminski, N. E., Koh, W. S., Yang, K. H., Lee, M., and Kessler, F. K. (1994). Suppression of the humoral immune response by cannabinoids is partially mediated through inhibition of adenylate cyclase by a pertussis toxin-sensitive G-protein coupled mechanism. *Biochem. Pharmacol.* **48,** 1899–1908.

Karmaus, P. W., Chen, W., Crawford, R. B., Harkema, J. R., Kaplan, B. L., and Kaminski, N. E. (2011). Deletion of cannabinoid receptors 1 and 2 exacerbates APC function to increase inflammation and cellular immunity during influenza infection. *J. Leukoc. Biol.* **90,** 983–995.

Karsak, M., Gaffal, E., Date, R., Wang-Eckhardt, L., Rehnelt, J., Petrosino, S., Starowicz, K., Steuder, R., Schlicker, E., Cravatt, B., *et al.* (2007).

Attenuation of allergic contact dermatitis through the endocannabinoid system. *Science* **316,** 1494–1497.

Kishimoto, S., Gokoh, M., Oka, S., Muramatsu, M., Kajiwara, T., Waku, K., and Sugiura, T. (2003). 2-arachidonoylglycerol induces the migration of HL-60 cells differentiated into macrophage-like cells and human peripheral blood monocytes through the cannabinoid CB2 receptor-dependent mechanism. *J. Biol. Chem.* **278,** 24469–24475.

Klein, T. W., Newton, C. A., Nakachi, N., and Friedman, H. (2000). Delta 9-tetrahydrocannabinol treatment suppresses immunity and early IFN-gamma, IL-12, and IL-12 receptor beta 2 responses to Legionella pneumophila infection. *J. Immunol.* **164,** 6461–6466.

Kobzik, L., Huang, S., Paulauskis, J. D., and Godleski, J. J. (1993). Particle opsonization and lung macrophage cytokine response. In vitro and in vivo analysis. *J. Immunol.* **151,** 2753–2759.

Lee, H. K., and Iwasaki, A. (2007). Innate control of adaptive immunity: Dendritic cells and beyond. *Semin. Immunol.* **19,** 48–55.

Lin, C. L., Suri, R. M., Rahdon, R. A., Austyn, J. M., and Roake, J. A. (1998). Dendritic cell chemotaxis and transendothelial migration are induced by distinct chemokines and are regulated on maturation. *Eur. J. Immunol.* **28,** 4114–4122.

Livak, K. J. and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* **25,** 402–408.

Lu, T., Newton, C., Perkins, I., Friedman, H., and Klein, T. W. (2006a). Cannabinoid treatment suppresses the T-helper cell-polarizing function of mouse dendritic cells stimulated with Legionella pneumophila infection. *J. Pharmacol. Exp. Ther.* **319,** 269–276.

Lu, T., Newton, C., Perkins, I., Friedman, H., and Klein, T. W. (2006b). Role of cannabinoid receptors in Delta-9-tetrahydrocannabinol suppression of IL-12p40 in mouse bone marrow-derived dendritic cells infected with Legionella pneumophila. *Eur. J. Pharmacol.* **532,** 170–177.

Lutz, M. B., Kukutsch, N., Ogilvie, A. L., Rössner, S., Koch, F., Romani, N., and Schuler, G. (1999). An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. *J. Immunol. Methods* **223,** 77–92.

Mishkin, E. M., and Cabral, G. A. (1985). delta-9-Tetrahydrocannabinol decreases host resistance to herpes simplex virus type 2 vaginal infection in the B6C3F1 mouse. *J. Gen. Virol.* **66** (Pt 12), 2539–2549.

Montecucco, F., Burger, F., Mach, F., and Steffens, S. (2008). CB2 cannabinoid receptor agonist JWH-015 modulates human monocyte migration through defined intracellular signaling pathways. *Am. J. Physiol. Heart Circ. Physiol.* **294,** H1145–H1155.

Morahan, P. S., Klykken, P. C., Smith, S. H., Harris, L. S., and Munson, A. E. (1979). Effects of cannabinoids on host resistance to Listeria monocytogenes and herpes simplex virus. *Infect. Immun.* **23,** 670–674.

Newton, C. A., Klein, T. W., and Friedman, H. (1994). Secondary immunity to Legionella pneumophila and Th1 activity are suppressed by delta-9-tetrahydrocannabinol injection. *Infect. Immun.* **62,** 4015–4020.

Nong, L., Newton, C., Friedman, H., and Klein, T. W. (2001). CB1 and CB2 receptor mRNA expression in human peripheral blood mononuclear cells (PBMC) from various donor types. *Adv. Exp. Med. Biol.* **493,** 229–233.

Penna, G., Sozzani, S., and Adorini, L. (2001). Cutting edge: Selective usage of chemokine receptors by plasmacytoid dendritic cells. *J. Immunol.* **167,** 1862–1866.

Perandones, C. E., Illera, V. A., Peckham, D., Stunz, L. L., and Ashman, R. F. (1993). Regulation of apoptosis in vitro in mature murine spleen T cells. *J. Immunol.* **151,** 3521–3529.

Randolph, G. J., Ochando, J., and Partida-Sánchez, S. (2008). Migration of dendritic cell subsets and their precursors. *Annu. Rev. Immunol.* **26,** 293–316.

Rao, G. K., and Kaminski, N. E. (2006). Cannabinoid-mediated elevation of intracellular calcium: A structure-activity relationship. *J. Pharmacol. Exp. Ther.* **317,** 820–829.

Rao, G. K., Zhang, W., and Kaminski, N. E. (2004). Cannabinoid receptor-mediated regulation of intracellular calcium by delta(9)-tetrahydrocannabinol in resting T cells. *J. Leukoc. Biol.* **75,** 884–892.

Wacnik, P. W., Luhr, K. M., Hill, R. H., Ljunggren, H. G., Kristensson, K., and Svensson, M. (2008). Cannabinoids affect dendritic cell (DC) potassium channel function and modulate DC T cell stimulatory capacity. *J. Immunol.* **181,** 3057–3066.

Zhu, L. X., Sharma, S., Stolina, M., Gardner, B., Roth, M. D., Tashkin, D. P., and Dubinett, S. M. (2000). Delta-9-tetrahydrocannabinol inhibits antitumor immunity by a CB2 receptor-mediated, cytokine-dependent pathway. *J. Immunol.* **165,** 373–380.