NIH Public Access
Author Manuscript
J Neuroimmune Pharmacol. Author manuscript; available in PMC 2012 December 11.

Published in final edited form as:
J Neuroimmune Pharmacol. 2012 December ; 7(4): 843–855. doi:10.1007/s11481-011-9293-4.

Δ^9-Tetrahydrocannabinol Suppresses Cytotoxic T Lymphocyte Function Independent of CB_1 and CB_2, Disrupting Early Activation Events

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Abstract

Previously, $CD8^+$ T cells were found to be a sensitive target for suppression by Δ^9-tetrahydrocannabinol (Δ^9-THC) in a murine model of influenza infection. To study the effect of Δ^9-THC on $CD8^+$ cytotoxic T lymphocytes (CTL), an allogeneic model of MHC I mismatch was used to elicit CTL. In addition, to determine the requirement for the cannabinoid receptors 1 (CB_1) and 2 (CB_2) in Δ^9-THC-mediated CTL response modulation, mice null for both receptors were used ($CB_1^{-/-}CB_2^{-/-}$). Δ^9-THC suppressed CTL function independent of CB_1 and CB_2 as evidenced by reduction of ^{51}Cr release by CTL generated from $CB_1^{-/-}CB_2^{-/-}$ mice. Furthermore, viability in $CD4^+$ and $CD8^+$ cells was reduced in a concentration-dependent manner with Δ^9-THC, independent of CB_1 and CB_2, but no effect of Δ^9-THC on proliferation was observed, suggesting that Δ^9-THC decreases the number of T cells initially activated. Δ^9-THC increased expression of the activation markers, CD69 in $CD8^+$ cells and CD25 in $CD4^+$ cells in a concentration-dependent manner in cells derived from WT and $CB_1^{-/-}CB_2^{-/-}$ mice. Furthermore, Δ^9-THC synergized with the calcium ionophore, ionomycin, to increase CD69 expression on both $CD4^+$ and $CD8^+$ cells. In addition, without stimulation, Δ^9-THC increased CD69 expression in $CD8^+$ cells from $CB_1^{-/-}CB_2^{-/-}$ and WT mice. Overall, these results suggest that CB_1 and CB_2 are dispensable for Δ^9-THC-mediated suppression and that perturbation of Ca^{2+} signals during Tcell activation plays an important role in the mechanism by which Δ^9-THC suppresses CTL function.

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Keywords

Δ^9-tetrahydrocannabinol; Cannabinoid receptors; Cytotoxic T lymphocytes; Immune modulation; Ca^{2+}; T cell activation

Introduction

Several cannabinoid compounds including Δ^9-THC have been implicated in the modulation of immune responses (Howlett et al. 2002; Klein et al. 2003). Cannabinoid compounds exert their activity, in part, by ligation of two identified targets, CB_1 and CB_2 (Maresz et al. 2007). However, studies using antagonists to these receptors, low affinity cannabinoid agonists, and mice lacking CB_1 and/or CB_2, have demonstrated that not all aspects of immune modulation by cannabinoid compounds can be attributed to CB_1 and/or CB_2 (Kaplan et al. 2003; Buchweitz et al. 2008; Springs et al. 2008; De Petrocellis and Di Marzo 2010). Thus, despite the ability to bind CB_1 and CB_2, the mechanism and cellular target(s) for Δ^9-THC within the immune system remain elusive. In particular, recent studies revealed that in T cells, the effect of Δ^9-THC on immune competence was found to occur independent of CB_1 and CB_2 (Kaplan et al. 2003; Rao and Kaminski 2006), an observation concordant with CB_1 and CB_2 expression, which is much lower on T cells compared to other leukocyte subpopulations (Galiegue et al. 1995). To further investigate the effects of Δ^9-THC on Tcell responses, an in vivo influenza challenge model was used and showed that the $CD8^+$ T cell population exhibited marked sensitivity to modulation by Δ^9-THC (Buchweitz et al. 2007). These results suggested that $CD8^+$ T cells might be a particularly sensitive T cell subset to cannabinoid-mediated immune modulation and might also represent a useful cellular target to gain novel insights into the molecular mechanisms of cannabinoid action.

The $CD8^+$ surface marker distinguishes a lineage of T cells that are capable of interaction with major histocompatibility complex I (MHC I). To become fully functional, naïve $CD8^+$ T cells must be activated, proliferate, and differentiate into CTL effectors. CTL are important in antiviral and antitumor immune responses. In addition to releasing cytokines pleiotropically involved in immune defense, CTL release cytotoxic granules or interact by cell contact with target cells to induce apoptosis in order to rid tissues of virus-infected or neoplastic cells (Boissonnas et al. 2007; Stinchcombe and Griffiths 2007). Lysis of target cells and the production of IFNγ are most often used as indicators of CTL function (Slifka et al. 1999; Sun et al. 2003). To experimentally elicit CTL from naïve $CD8^+$ T cells, several models have been developed, among them is an allogeneic model using P815 (DBA2-derived) tumor cells to activate lymphocytes from a haplotype mismatched donor, such as C57Bl/6 mice. Using this model, CTL are generated in response to a T cell receptor-MHC mismatch and upon subsequent encounter, their effector function can be assayed (Engers et al. 1975). The objective of this study was to determine the effect of Δ^9-THC and the involvement of CB_1 and CB_2 on CTL elicitation and function.

Methods

Animals

C57Bl/6 mice were ordered from the National Cancer Institute (NCI) and $CB_1^{-/-}CB_2^{-/-}$ mice on C57Bl/6 background were bred in-house. $CB_1^{-/-}CB_2^{-/-}$ mice were a kind gift of Dr. Andreas Zimmer at the Universität of Bonn, Germany. Mice were housed at Michigan State University in a pathogen free animal research housing facility at 21 to 24°C and 40 to 60% relative humidity with a 12-h light/dark cycle. Water and food (Purina Lab Chow) were available ad libitum. All protocols and procedures were performed in accordance with

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guidelines set forth by the Institutional Animal Care and Use Committee at Michigan State University. $CB_1^{-/-}CB_2^{-/-}$ mice were routinely genotyped to validate absence of CBs using commercial primers for *cnr1* and custom primers for *cnr2* from Applied Biosystems (Kaplan et al. 2010).

Chemicals and reagents

Δ^9-THC was obtained from the National Institute on Drug Abuse (Bethesda, MD). Ethanol was purchased from Decon Labs (King of Prussia, PA). Ionomycin (Io) and dimethylsulfoxide (DMSO) were purchased from Sigma Chemical Co. (St. Louis, MO). RPMI media was obtained from Gibco Invitrogen (Carlsbad, CA), and ^{51}Cr as sodium chromate was obtained from Perkin Elmer (Waltham, MA).

T cell elicitation for generation of functional CTL

C57Bl/6 (WT) and $CB_1^{-/-}CB_2^{-/-}$ mice were euthanized, the spleens isolated in a sterile environment, and splenocytes enumerated using a Coulter Counter (Beckman Coulter, Brea, CA). P815 cells were irradiated with 3000 rads to prevent proliferation, washed 3 times with RPMI and counted using a hemacytometer. Splenocytes and irradiated P815 cells were combined at 1×10^6 and 1×10^5 cells, respectively, in RPMI 1640 supplemented with 5% bovine calf serum (BCS) in a total volume of 200 μL in a round bottom 96 well plate. The plates were incubated in a humidified incubator with 5% CO_2 at 37°C for the indicated amounts of time.

Drug treatment

At the time of co-culture of splenocytes and irradiated P815, Δ^9-THC (1, 5, 10 μM), vehicle (VH, 0.1% ethanol) or RPMI (NA) was added. All Δ^9-THC treatments had the same ethanol content (0.1%) as vehicle control.

^{51}Cr release assay

After elicitation, cells were harvested and washed twice with RPMI 1640 media without serum. P815 cells were washed once and 1×10^6 cells were incubated in the presence of $Na_2\,^{51}CrO_4$ for 1 h in 10% fetal bovine serum (FBS) supplemented RPMI 1640 media in a volume of less than 50 μL. After incubation P815 cells were washed 3 times using RPMI 1640 media without serum. ^{51}Cr-labeled P815 cells were adjusted to 1×10^5 cells/mL in 2% FBS RPMI media. Elicited CTL were adjusted in 2% FCS complete RPMI media to ratios ranging from 50 (5×10^5) to 1 (1×10^4 cells) : 1 (1×10^4) P815 cells, depending on the experimental design, in a volume of 200 μL. After co-culture, elicited CTL and P815 were added to a 96 well round bottom plate and centrifuged at 200 g for 1 min to force cellular interactions. Control wells for spontaneous release (200 μL of P815 only) and total release (1% Triton-X 100 in 200 μL of P815 cells in RPMI) were used to determine the range of experimental release. After 5 h of co-culture in a humidified incubator with 5% CO_2 at 37°C, cell lysis was assessed by aliquoting 100 μL of supernatant from each well, which represents the experimental release. The cytolytic activity was calculated as follows: % Release = (experimental release − spontaneous release)/(experimental release − total release) × 100.

IFNγ T cell functional analysis

CTL were elicited as described above for generation of CTL. After 5 days, cells were harvested and co-cultured with P815 at a ratio of 10:1 (see above) for 12 h in the presence of brefeldin A to prevent IFNγ release and allow for detection by fluorescently labeled antibody. After co-culture, cells were prepared for fluorescent antibody staining (described below).

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Proliferation assay

Prior to elicitation, splenocytes were incubated with Cell Trace carboxyfluorescein succinimidyl ester (CFSE) dye (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. CTL were elicited as described above for generation of CTL. Dilution of dye staining is indicative of proliferation and staining profiles fromelicited samples were compared to a 24 h SPLC only control, incubated without P815.

T cell activation assays

CTL were elicited as described for generation of CTL; however, RPMI supplemented with 2% FCS was used instead of 5%. For direct stimulation, 0.5 μM Io dissolved in 0.01% DMSO in RPMI was used as the Ca^{2+} ionophore to induce intracellular Ca^{2+} levels, thereby increasing CD69 expression. After 6 h, CD69 surface staining was performed using antibodies specific for CD69 and after 24 h for CD25 (staining described in detail below).

Procedure for immunofluorescent staining for flow cytometry

CTL were elicited as described above. Cells were washed in 96 well round bottom plates with HBSS once and incubated with Near IR LIVE/DEAD (Invitrogen) dye to assess viability according to manufacturer's instructions. This dye stains cells that have lost membrane integrity. Cells were washed twice with FACS buffer (1X HBSS, 1% bovine serum albumin (BSA), 0.1% sodium azide). Subsequently, Fc receptors were blocked using Purified Rat Anti-Mouse CD16/CD32 (BD Pharmingen, San Diego, CA). To stain surface proteins, cells were incubated for 20 min with 0.25–0.5 μg of CD4 (clone RM4-5, Biolegend, San Diego, CA) and CD8 (53–6.7, Biolegend). For the T cell activation experiments, cells were additionally stained with CD69 (H1.2 F3, Biolegend) and CD25 (PC61, Biolegend). Cells were then washed twice and fixed with Cytofix (BD, Franklin Lakes, NJ). After re-stimulation (described above), on the day of flow cytometric analysis, cells were washed once, then permeabilized with Perm/Wash Buffer (BD, Franklin Lakes, NJ) by incubation for 20 min and stained with IFNγ (XMG1.2, Biolegend) for 30 min. All experiments included single stain controls to compensate for fluorescence overlap between detectors. Samples were individually analyzed for percent positive staining as defined by a gate to perform statistical analysis and concatenated by treatment group for graphing using FlowJo v.8.8.6. for Macintosh.

Statistical analysis

Samples were analyzed using ANOVA or non-parametric equivalent Kruskal-Wallis. A p-value of 0.05 or less was deemed statistically significant between experimental and relative control. Regression analysis was performed treating values of each independent variable as a single datum point. All Δ^9-THC treated samples were compared to vehicle (VH, 0.1% ethanol) control and comparisons between WT and $CB_1^{-/-}CB_2^{-/-}$ were performed in naïve (NA) groups. Stimulation effect was determined between resting (NA–0 h) and NA at the indicated time point of sample collection. Statistical analyses were performed using Graph Pad Prism v4.03.

Results

Kinetics of CTL response

In order to determine the kinetics of the P815-induced CTL response, CTL were elicited for 3, 5, 7, and 9 days after co-culture with irradiated P815 cells. Splenocytes from WTand $CB_1^{-/-}CB_2^{-/-}$ mice were used as the source of naïve $CD8^+$ T cells. Elicited CTL were assayed for CTL activity by ^{51}Cr release assay using ^{51}Cr-labelled P815 target cells. A ratio of 50:1 CTL:P815 cells was used to maximize release, thereby increasing sensitivity on non-

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peak days. ^{51}Cr release is a result of CTL-dependent lysis of P815 cells and the peak day of CTL activity occurred at day 5 post elicitation (Fig. 1). At the peak day of CTL elicitation, $CB_1^{-/-}CB_2^{-/-}$ splenocytes had a statistically significant reduction in CTL activity, when compared to WT ($p \leq 0.01$). The same trend was observed for non-peak days, although it was not deemed statistically significant. Subsequent experiments determining CTL effector activity under various conditions were performed at day 5 post elicitation.

Δ^9-THC suppressed CTL responses during elicitation but not during the effector response

To determine the sensitivity of CTL function to Δ^9-THC treatment in the P815 allogeneic model, Δ^9-THC and/or vehicle (0.1% ethanol) were added directly to culture during either the elicitation or effector phase of the CTL response. A concentration of 10 μM Δ^9-THC was used to determine sensitivity of CTL to immune modulation, a concentration that historically impaired T cell immune responses in the absence of direct cytotoxicity (Springs et al. 2008). Δ^9-THC suppressed CTL activity only when added prior to, but not after, the elicitation phase (Fig. 2). Likewise, repeated addition of Δ^9-THC during elicitation and effector phases did not further reduce CTL activity below single addition of the drug during the elicitation phase (Fig. 2).

Concentration-dependent suppression of CTL effector function

Next, we assessed whether CTL responses were suppressed by Δ^9-THC in a concentration-dependent manner. At E:T ratios ranging from 10:1 to 1:1, Δ^9-THC suppressed CTL responses in a concentration-dependent manner with statistically significant suppression at concentrations as low as 1 μM (Fig. 3a). Also, lower E:T ratios resulted in lower CTL activity, demonstrating that the elicited cell populations are responsible for the cytolytic activity and that Δ^9-THC suppressed CTL activity independent of the E:T ratio (Fig. 3a). Regression analysis was performed on CTL activity results and a statistically significant difference was observed from zero regression values for E:T ratios 10:1 (r^2= 0.8650, p<0.0001), 5:1 (r^2=0.7246, p=0.0004), 2.5:1 (r^2= 0.7461, p=0.0003), and 1:1 (r^2=0.7692, p=0.0002). Next, we assessed whether the effect by Δ^9-THC was dependent on the presence of CB_1 and/or CB_2. Δ^9-THC suppressed CTL activity in a concentration-dependent manner in CTL elicited from $CB_1^{-/-}CB_2^{-/-}$ splenocytes with significantly reduced CTL activity at concentrations of 5 μM or more as in WT (Fig. 3b). Regression analysis revealed a statistically significant difference from zero regression of E:T ratio of 10:1 in WT (r^2=0.6893, p=0.0008) and showed the same trendin$CB_1^{-/-}CB_2^{-/-}$ albeit not significant (r^2=0.2905, p=0.0706). Consistent with previous observations, CTL activity of $CB_1^{-/-}CB_2^{-/-}$ splenocytes was lower in magnitude compared to WT, although differences in genotypes were not deemed statistically significant.

Δ^9-THC-mediated suppression of CTL generation

Although MHC I mismatch is a strong stimulus for driving the expansion of differentiated $CD8^+$ T cell effectors, the overall number of viable Tcells within control cultures (NA or VH treatment groups) on day 5 of co-culture with P815 allogenic target cells was modest. This demonstrated that T cell proliferation and clonal expansion in response to MHC I-restricted alloantigens in this assay system induced a relatively small number of Tcells from the spleen. To assess the effects of Δ^9-THC on its ability to impair alloantigen-induced expansion of T cell effectors, the number of viable $CD4^+$ and $CD8^+$ T cells was enumerated on Day 5 of the elicitation phase of the CTL response. Viability was determined using LIVE/DEAD dye (Invitrogen), which permeates dead cells and thus low staining is observed in viable cell populations. The percentage of viable $CD4^+$ (Fig. 4a and b) and $CD8^+$ Tcells (Fig. 4c and d) was markedly reduced by Δ^9-THC treatment 5 days after elicitation with concentrations as low as 1 μM in both WTand $CB_1^{-/-}CB_2^{-/-}$ samples. No significant differences were found in the percent of viable $CD8^+$ cells between $CB_1^{-/-}CB_2^{-/-}$ and WT

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splenocytes (Fig. 4c and d); however, a greater number of viable $CD4^+$ T cells was observed in $CB_1^{-/-}CB_2^{-/-}$ compared to WT splenocytes (Fig. 4a and b).

IFNγ production by $CD8^+$ T cells is often used as a surrogate for CTL activity. After gating for single live cells (Fig. 5a), $CD4^+$ cells did not produce significant amounts of IFNγ as P815 cells are MHC I-mismatched to C57Bl/6 cells (Fig. 5b). However, significant IFNγ production was detected by $CD8^+$ cells, which was enhanced in CTL elicited from WTcompared to $CB_1^{-/-}CB_2^{-/-}$ (Fig. 5c, d and e). In the presence of Δ^9-THC, enhanced IFNγ production was observed in $CD8^+$ T cells derived from WT and the same trend was observed at higher Δ^9-THC concentrations in $CB_1^{-/-}CB_2^{-/-}$ mice, although not deemed statistically significant (Fig. 5c, d and e). Also, IFNγ production was reduced in samples from $CB_1^{-/-}CB_2^{-/-}$ compared to WT mice as previously observed in ^{51}Cr release assays (Fig. 5c, d and e). Analysis of $CD8^+$ IFNγ-secreting cells, as a percentage of viable cells, takes into account the reduced viability as a result of Δ^9-THC addition (see Fig. 4). However, while there was an enhancement of IFNγ-secreting cells with Δ^9-THC treatment, there were also fewer viable cells after Δ^9-THC treatment, thus overall the percentage of $CD8^+$ cells secreting IFNγ within the total population did not change (Fig. 5f).

Δ^9-THC does not affect $CD8^+$ T cell proliferation induced by P815 co-culture

Prior to becoming functional CTL, proliferation occurs in T cells to expand the effector pool. Thus, we focused on proliferation as a potential endpoint leading to decreased viability and decreased CTL response. Splenocytes were labeled with CFSE dye to determine proliferation by dye dilution. No statistically significant differences in proliferation were observed between VH and Δ^9-THC-treated groups neither in $CD8^+$ nor in $CD4^+$ cells from WTor $CB_1^{-/-}CB_2^{-/-}$ mice (Fig. 6a, b, c and d). In comparison to WT, $CD4^+$ cells obtained from $CB_1^{-/-}CB_2^{-/-}$ spleens underwent much greater proliferation. $CD4^+$ T cells were not directly stimulated in this model, suggesting that greater proliferation in $CB_1^{-/-}CB_2^{-/-}$ $CD4^+$ cells might either be due to higher basal proliferation in $CB_1^{-/-}CB_2^{-/-}$ cells or a result of the cytokines produced by $CD8^+$ cells. In contrast, notably more $CD8^+$ cells were elicited in samples from WT compared to $CB_1^{-/-}CB_2^{-/-}$ spleens (Fig. 6c and d).

Increase of CD69 expression on $CD8^+$ cells with Δ^9-THC treatment

In an effort to determine events leading to the Δ^9-THC-mediated reduction in cell viability and CTL activity seen at day 5, without any apparent effects on proliferation, we focused on T cell activation. Two markers of cellular activation found on both $CD4^+$ and $CD8^+$ T cells are the expression of CD69 and CD25. Whereas CD69 is thought to be one of the earliest markers of activation and is rapidly upregulated, CD25 expression increases gradually on activated T cells and is observed later than CD69 on activated T cells (Lopez-Cabrera et al. 1995; Brenchley et al. 2002). We focused on CD69 expression at 6 h and CD25 expression at 12 h after co-culture with irradiated P815 cells. Cells were gated on singlets, by size, and then on CD4 or CD8 (Fig. 7a). In $CD4^+$ cells, CD69 expression was unaffected by co-culture with P815 cells; however, cells from $CB_1^{-/-}CB_2^{-/-}$ mice showed higher basal expression compared to WT mice (Fig. 7b and c). In addition, no effect with Δ^9-THC treatment was observed on CD69 expression on $CD4^+$ cells (Fig. 7b and c). In contrast, the expression of CD69 on $CD8^+$ was increased by co-culture with P815 cells and $CD8^+$ T cells from $CB_1^{-/-}CB_2^{-/-}$ expressed higher levels of CD69 than WT $CD8^+$ T cells (Fig. 7d and e). CD69 expression was increased in a concentration-dependent manner by Δ^9-THC in $CD8^+$ T cells from both WT and $CB_1^{-/-}CB_2^{-/-}$ spleens, which was significant at concentrations as low as 5 μM in $CB_1^{-/-}CB_2^{-/-}$-derived cells (Fig. 7d and e).

CD25 expression on $CD4^+$ T cells was increased as a result of stimulation with P815 cells and increased levels of CD25 expression were observed in NA unstimulated samples from

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$CB_1^{-/-}CB_2^{-/-}$ compared to WT mice (Fig. 8a and b). There was a trend towards a Δ^9-THC-dependent increase in CD25 expression in both WT and $CB_1^{-/-}CB_2^{-/-}$ $CD4^+$ cell populations, but this trend was not statistically significant (Fig. 8b). In $CD8^+$ cells, the expression of CD25 was much lower than in $CD4^+$ cells (Fig. 8a, b, c and d). Expression of CD25 in $CD8^+$ cells of both genotypes was increased by co-culture with P815 cells (Fig. 8c and d). In $CD8^+$ cells from $CB_1^{-/-}CB_2^{-/-}$ mice, a concentration-dependent decrease with Δ^9-THC treatment was observed, while in WT Δ^9-THC had no effect (Fig. 8c and d).

Δ^9-THC synergizes with Io to induce CD69 expression

In light of the rapid Δ^9-THC-mediated increase in CD69 expression of $CD8^+$ T cells and its inverse correlation with Δ^9-THC-mediated suppression of CTL activity, we focused on intracellular signals leading to CD69 protein expression. Previously, it was determined that independent of CB_1 and CB_2, Δ^9-THC partially mediated its effects through the TRPC1 channel, which causes an increase in intracellular Ca^{2+} levels (Rao and Kaminski 2006). Using the calcium ionophore, Io, we tested whether a rise in intracellular Ca^{2+} might contribute to the observed increase in CD69 expression. In addition, the effect of Δ^9-THC on Io-induced CD69 expression was evaluated. Again, cells were gated as described above (Fig. 7a). In $CD4^+$ cells, Δ^9-THC increased expression of CD69 moderately in the absence of an activating stimulus when isolated from $CB_1^{-/-}CB_2^{-/-}$, but not WT mice (Fig. 9a and c). Δ^9-THC increased CD69 expression in $CD8^+$ splenocytes from both WT and $CB_1^{-/-}CB_2^{-/-}$ mice in the absence of an activating stimulus (Fig. 9a and e). After stimulation with Io, CD69 expression was dramatically increased in $CD4^+$ and $CD8^+$ cells from both genotypes (Fig. 9b, d and f). While CD69 expression was similar between WTand $CB_1^{-/-}CB_2^{-/-}$ $CD8^+$ cells (Fig. 9b and f), a marked reduction in CD69 expression was observed in $CB_1^{-/-}CB_2^{-/-}$ $CD4^+$ cells, when compared to WT $CD4^+$ cells (Fig. 9b and d). Most importantly, Δ^9-THC synergized with Io to further increase CD69 surface expression in $CD4^+$ and $CD8^+$ cells derived from both genotypes (Fig. 9b, d and f).

Discussion

Collectively, these studies provide evidence that Δ^9-THC-mediated suppression of $CD8^+$ T cell function occurs independent of CB_1 and CB_2. Moreover, Δ^9-THC alters early signaling events that involve putative changes in intracellular Ca^{2+} levels, eventually resulting in lower numbers of CTL effectors. Finally, this study introduces an inherent role for CB_1 and/or CB_2 in the elicitation of CTL, because elicitation and function of CTL was reduced in $CB_1^{-/-}CB_2^{-/-}$ mice in vitro.

In contrast to other reports describing enhanced immune responses in $CB_1^{-/-}CB_2^{-/-}$ mice (Karsak et al. 2007; Buchweitz et al. 2008), in this in vitro model of alloantigen-induced CTL elicitation and effector function, CTL activity in cells from $CB_1^{-/-}CB_2^{-/-}$ mice was reduced when compared to WT mice. Although this was consistent with our previous study in which we observed a reduction in the T cell-dependent humoral immune response to sheep erythrocytes in vitro in $CB_1^{-/-}/CB_2^{-/-}$ as compared to WT mice (Springs et al. 2008), there are several studies demonstrating enhanced immune function in $CB_1^{-/-}CB_2^{-/-}$ mice (Karsak et al. 2007; Buchweitz et al. 2008). In a previous study from our laboratory (Buchweitz et al. 2008), it was demonstrated that $CB_1^{-/-}CB_2^{-/-}$ mice, in addition to responding with a greater magnitude to immune stimuli, have altered immune homeostasis compared to WT mice as evidenced by greater numbers of $CD4^+$ T cells and higher levels of TNF-α in the bronchoalveolar lavage fluid in the absence of influenza challenge. In addition, there appeared to be mechanisms in $CB_1^{-/-}CB_2^{-/-}$ mice that might compensate for the loss of CB_1 and/or CB_2, as evidenced by greater levels of the endocannabinoids, 2-arachidonoyl glycerol and anandamide (Karsak et al. 2007), and increased transcripts of *cnr2* using primers specific for the undeleted portion of the gene (Liu et al. 2009). Although

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$CB_1^{-/-}CB_2^{-/-}$ mice seem to be particularly vulnerable to proinflammatory insult, there were no drastic differences observed in an adjuvant-free airway hypersensitivity model between $CB_1^{-/-}CB_2^{-/-}$ and WT mice (Kaplan et al. 2010). Reasons for reduced function of CTL from $CB_1^{-/-}$ $CB_2^{-/-}$ mice compared to WT mice, although not altogether clear, might be due to the present studies being conducted in vitro, and/or the nature in which the immune response was initiated, in this case by direct stimulation of $CD8^+$ T cells with an alloantigen.

Greater proliferation was detected in $CD4^+$, but lower proliferation in $CD8^+$ cells, from $CB_1^{-/-}$ $CB_2^{-/-}$ compared to WT mice. This lower proliferation in $CD8^+$ cells might contribute to the reduced CTL activity in $CB_1^{-/-}$ $CB_2^{-/-}$ compared to WT mice. Furthermore, higher percentages of $CD4^+CD25^+$ were observed in unstimulated splenocytes of $CB_1^{-/-}$ $CB_2^{-/-}$ compared to WT mice. These $CD4^+CD25^+$ cells are thought to be regulatory T cells (Treg) (Suri-Payer et al. 1998), which are involved in the suppression of the immune responses. It has been demonstrated that an increase in $CD4^+CD25^+$ cells leads to reduced CTL responses; thereby Tregs could potentially contribute to the reduced activity in vitro in CTL generated from $CB_1^{-/-}$ $CB_2^{-/-}$ mice (Piccirillo and Shevach 2001). This in vitro model, due to direct stimulation of CTL, lacks a proinflammatory stimulus and does not produce IL-6, which has been implicated in overriding Treg responses, thus Treg responses might be maintained during the in vitro culture (Bettelli et al. 2006). As our focus was on the Δ^9-THC-mediated suppression of CTL responses, we did not further characterize these $CD4^+CD25^+$ cells for expression of Foxp3 or other cellular markers of the Treg lineage (Fontenot and Rudensky 2005). Additional studies are required to determine the role of Tregs in $CB_1^{-/-}/CB_2^{-/-}$ mice.

Addition of Δ^9-THC during the elicitation and effector response showed that only NA $CD8^+$ cells, but not differentiated CTL, were sensitive to Δ^9-THC-mediated suppression of CTL function as assessed by ^{51}Cr release assay, suggesting Δ^9-THC-mediated immune modulation during elicitation. Arguably, the concentrations of Δ^9-THC at which altered CTL activity was observed in this investigation are higher than detected in the serum of recreational marijuana smokers. However, it is important to emphasize two points. The first is that in addition to Δ^9-THC, marijuana smoke contains over 60 other structurally-related cannabinoids, a number of which are also well established immune modulators and the majority of which remain to be evaluated for immunomodulatory activity (Ashton 2001; Friedman et al. 2003). Second, and equally important, the goal of this study was to relate the decrease in viral clearance observed after Δ^9-THC treatment of animal with influenza-infected airways (Buchweitz et al. 2007) and the possibility that this may be due to cannabinoid-mediated suppression in CTL activity. In the case of inhaled marijuana smoke, the cannabinoid concentrations attained in the lung are significantly higher than systemic serum Δ^9-THC concentrations and may be more closely related to those used in the current study (Azorlosa et al. 1992; Huestis and Cone 2004). The timeframe of sensitivity to Δ^9-THC might reflect a high level of T cell plasticity prior to differentiation and reduced plasticity after differentiation (Sundrud et al. 2003). Other immunomodulatory compounds such as 2,3,7,8-tetrachlorodibenzo- p-dioxin and glucocorticoids exert their greatest effect on early stages of immune cell differentiation and tend to have a lower efficacy if added after the onset of the differentiation program (Piccolella et al. 1985; Tucker et al. 1986). Specifically, cyclosporin A is very efficacious in suppressing in vitro culture of allogeneic cells only when added prior to elicitation (Hess and Tutschka 1980). Overall, this suggests a critical window of sensitivity for immunomodulatory compounds, including Δ^9-THC, for immune suppression, specifically during the transition of NA $CD8^+$ T cells to CTL during elicitation.

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Using IFNγ as a surrogate for CTL activity demonstrated that $CD8^+$ cells are the sole producers of IFNγ after allogeneic stimulation, suggesting that only CTL directly contribute to the ^{51}Cr release from labeled target cells. Although it appeared that Δ^9-THC increased IFNγ expression in CTL, the cells were gated on the live cell populations. Upon examination of the results from the total population, there was no effect of Δ^9-THC on IFNγ, demonstrating alternative interpretations of the data. Taken together, our results suggest that while there seemed to be a population of CTL whose activity cannot be suppressed by Δ^9-THC, which become the IFNγ-secreting cells, the remaining cells are sensitive to Δ^9-THC and were not capable of surviving the five-day culture period. Thus, the suppression by Δ^9-THC does not appear to reduce the amount of IFNγ each cell produces, but rather impaired the differentiation of a select pool of naïve cells from becoming effector CTL.

No effects of Δ^9-THC treatment on the proliferation of $CD4^+$ or $CD8^+$ cells in WT or $CB_1^{-/-}$ $CB_2^{-/-}$ mice were observed. To link the functional outcome of Δ^9-THC treatment to earlier T cell signaling, the expression of activation markers, CD25, forming the high affinity IL-2 receptor and CD69, the earliest known lymphocyte activation marker (Lopez-Cabrera et al. 1995) were assessed. While CD25 expression was increased as a result of P815 co-culture, only $CD8^+$ cells from $CB_1^{-/-}$ $CB_2^{-/-}$ mice had reduced CD25 expression as a result of Δ^9-THC treatment. CD69 expression was also increased as a result of co-culture with P815 cells in both WT and $CB_1^{-/-}$ $CB_2^{-/-}$ $CD8^+$ cells and Δ^9-THC treatment further enhanced the P815-induced rise in CD69 expression in both genotypes. The ability of Δ^9-THC to increase CD69 expression on the surface of $CD8^+$ T cells from WT and $CB_1^{-/-}$ $CB_2^{-/-}$ after P815 cell stimulation provides further evidence for CB_1 and/or CB_2-independent mechanism(s) of Δ^9-THC immune modulation. Notably, expression of CD69 was inversely correlated with CTL activity. For example, the highest CD69 expression and lowest CTL activity were observed in cells treated with the highest concentration of Δ^9-THC (10 μM) from $CB_1^{-/-}$ $CB_2^{-/-}$ mice.

CD69 expression is controlled by several transcription factors, including AP-1 in the proximal promoter (Castellanos et al. 1997) as well as EGR, ATF/CREB (Castellanos Mdel et al. 2002), and NFκB (Lopez-Cabrera et al. 1995). However, the pathways regulating the expression of CD69 are different depending on the cell type (Vazquez et al. 2009). It is not entirely clear what cellular signals contribute to CD69 induction in $CD8^+$ T cells, but it is known that Ca^{2+} influx into T cells increases CD69 expression (Testi et al. 1994) and, at least in thymocytes, extracellular Ca^{2+} was found to be critical for CD69 induction (Rodrigues Mascarenhas et al. 2003). In these studies Δ^9-THC synergized with Io to increase CD69 expression suggesting that Ca^{2+} plays an important role in the Δ^9-THC-mediated increase of CD69 expression. Previously, our laboratory reported that Δ^9-THC strongly induced intracellular Ca^{2+} in T cells, which was mediated by TRPC1 (Rao et al. 2004; Rao and Kaminski 2006) independent of the requirement for T cell activation. These studies, taken together with previous results, suggest that modulation of an activation stimulus by concurrent induction of Ca^{2+} through Δ^9-THC-mediated opening of TRPC1 channels contributes directly to the suppression of CTL activity. In addition, there are reports indicating that Δ^9-THC has a high binding affinity for GPR55 (Ryberg et al. 2007) and causes increases in intracellular Ca^{2+} levels via GPR55 (Lauckner et al. 2008), but there are also conflicting reports demonstrating that Δ^9-THC possesses a low affinity for GPR55 (Oka et al. 2007). The role of GPR55 in the present study is unknown; however, due to the expression of GPR55 in the spleen (Ryberg et al. 2007) and its ability to modulate Ca^{2+} currents it is tempting to speculate that GPR55 contributes to alterations in Ca^{2+} signaling induced by Δ^9-THC. Ca^{2+} is a ubiquitous intracellular signaling molecule and in addition to activation, Ca^{2+} is central to the induction of anergy and cell death, which are seemingly separate processes, yet involve shared mechanisms and endpoints (Macian et al. 2002;

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Parish et al. 2009). Thus, due to the role of Ca^{2+} in the generation of CTL, it seems plausible that Δ^9-THC-mediated alterations in Ca^{2+}-signaling are involved in the suppression of CTL responses.

Collectively, the reduced cytolytic activity, decreased number of CTL effectors, and the increase in CD69 expression, although temporally distinct, might be interrelated, and a consequence of early Δ^9-THC-induced changes in Ca^{2+} signaling. Concordantly, immune cells appear to initially undergo greater activation (CD69), but become unresponsive to secondary stimulation; in essence, anergic. The critical role of calcium in leukocyte activation and subsequent differentiation and effector function is well established. Δ^9-THC treatment prior to cellular activation significantly elevates intracellular Ca^{2+} levels, which in turn interferes with either entry or execution of the T cell differentiation program. The specific downstream signaling pathways affected through the Δ^9-THC-mediated elevation of intracellular Ca^{2+} levels affecting T cell function remain to be elucidated but likely involve p38 MAPK, JNK, and ERK. Indeed, prior studies by our laboratory demonstrated decreased ERK phosphorylation by cannabinol, another cannabinoid acting independently of CB_1 and CB_2 (Faubert Kaplan and Kaminski 2003). Thus, the immediate perturbation of intracellular Ca^{2+} levels by cannabinoids, including Δ^9-THC, seems to be the initial event triggering a cascade of events, eventually resulting in immune suppression. Signaling by cannabinoids seems to alter the regulation of Ca^{2+} that is central to the initiation of a T cell response to impair immune function.

Acknowledgments

The authors would like to thank Mr. Robert Crawford for excellent technical assistance with flow cytometer and Mrs. Kimberly Hambleton for assistance with submission of the manuscript.

Support: NIH Grants RO1DA12740 and RO1DA07908

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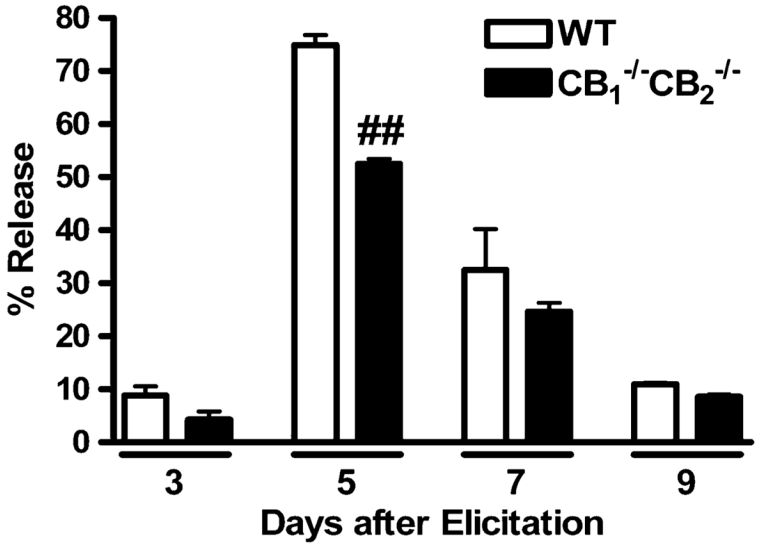

Fig. 1.

CTL activity peaks at day 5 after elicitation. To determine the peak day of CTL activity, splenocytes from WT and $CB_1^{-/-}CB_2^{-/-}$ mice were co-cultured with irradiated P815 cells to elicit an allogeneic CTL response. On various days after elicitation, CTL were harvested and assayed for target (P815) lysing activity by reincubation at a ratio of 50:1 (Effector CTL : P815 Targets) with ^{51}Cr labeled P815 cells. Percent release was calculated as described in Methods (n=4). The experiment was performed once, while more replicate experiments were performed for WTonly. ## $p \leq 0.01$ indicates difference between WT and $CB_1^{-/-}CB_2^{-/-}$

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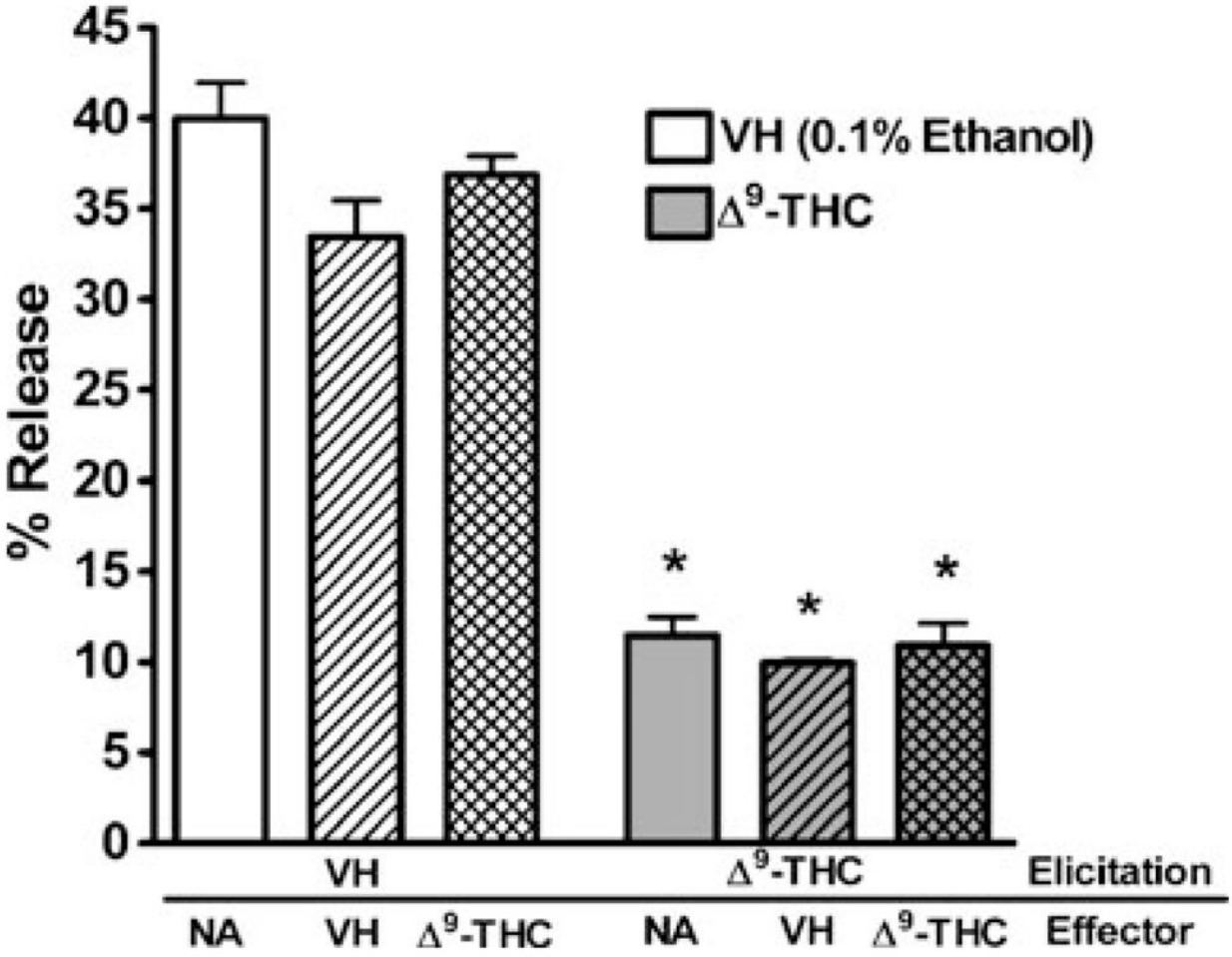

Fig. 2.

Δ^9-THC suppresses CTL activity during elicitation but not effector phase. Splenocytes were treated with Δ^9-THC and/or VH (0.1% ethanol) and stimulated with irradiated P815 cells for 5 days. After harvest, CTL were reincubated at a 10:1 ratio with ^{51}Cr labeled P815 cells in the presence of Δ^9-THC and appropriate controls (NA–naïve: no treatment; VH–0.1% ethanol) (n=4). Data shown are representative of two repeat experiments. * $p \leq 0.05$ indicates difference as compared to VH

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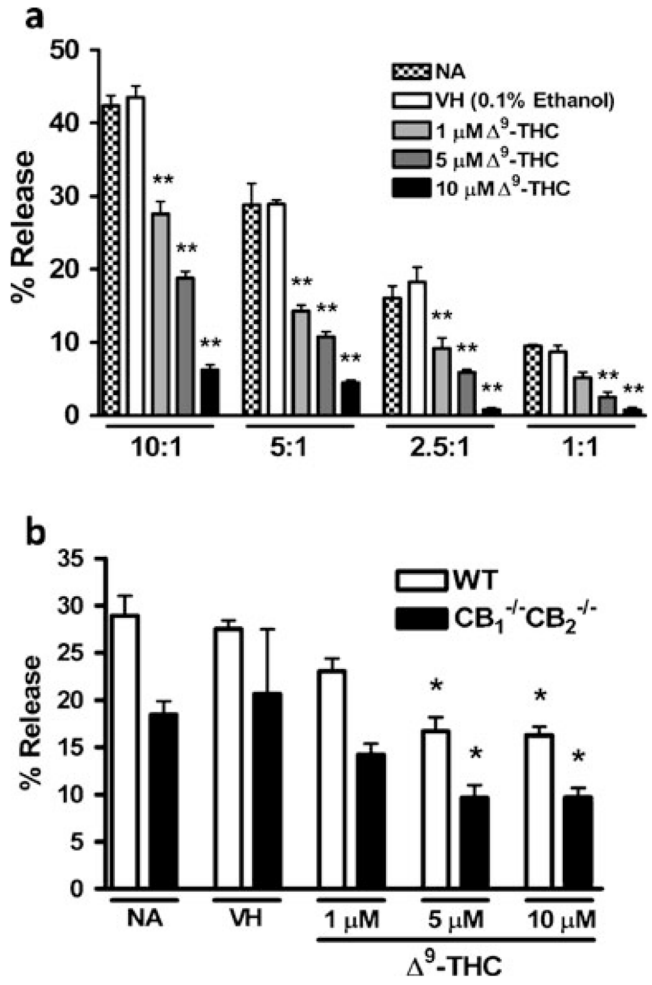

Fig. 3.

Δ^9-THC suppresses CTL activity in a concentration-dependent manner. Splenocytes from WT mice were treated with 1, 5 and 10 μM of Δ^9-THC and/or VH (control) and co-cultured for 5 days with irradiated P815 cells. After harvest, CTL were reincubated with ^{51}Cr labeled P815 cells at indicated ratios of 10:1 to 1:1 (**a**) (n=4). In a second experiment splenocytes from WTand $CB_1^{-/-}CB_2^{-/-}$ mice were elicited and restimulated with ^{51}Cr labeled P815 cells at a ratio of 10:1 (**b**) (n=4). Data shown are representative of two repeat experiments. * $p \leq 0.05$, ** $p \leq 0.01$ indicate differences as compared to VH

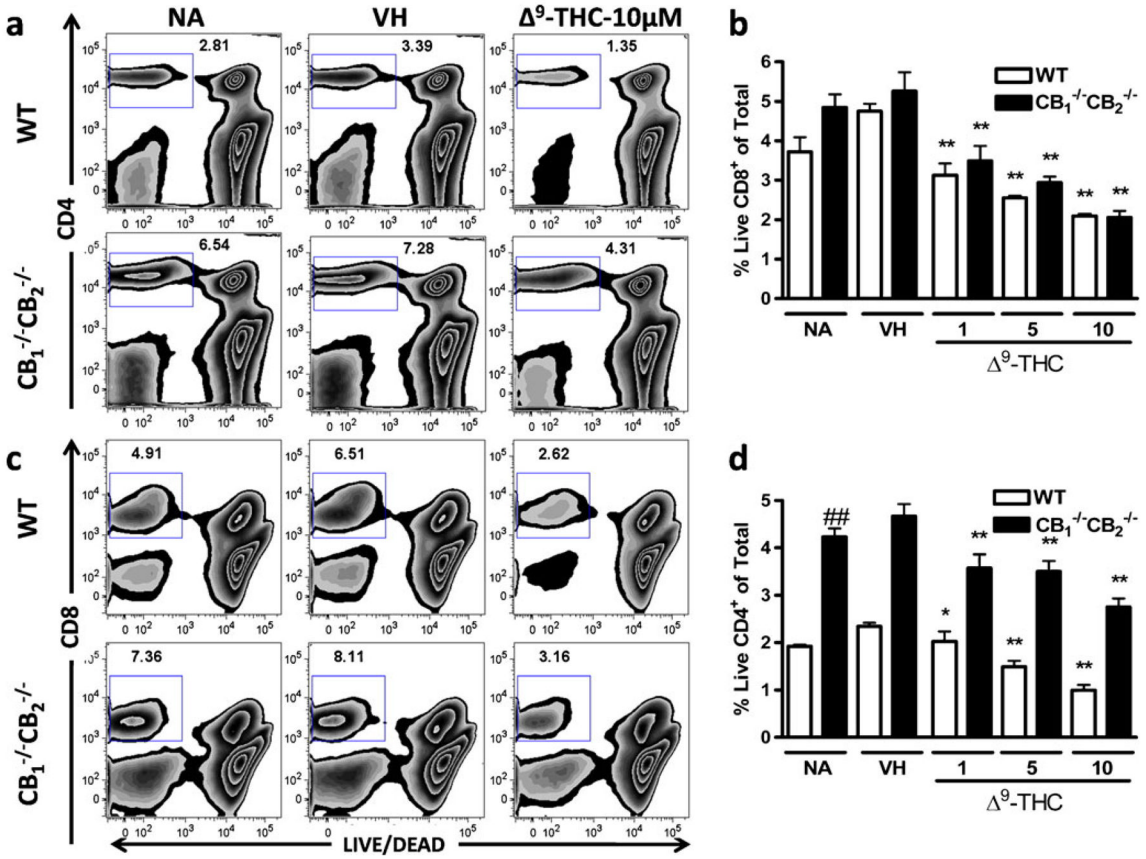

Fig. 4.

Lower viability of $CD4^+$ and $CD8^+$ cells following Δ^9-THC treatment. CTL were elicited from splenocytes of WTand $CB_1{}^{-/-}CB_2{}^{-/-}$ mice in the presence or absence of VH (0.1% ethanol) or Δ^9-THC (1, 5, and 10 μM). After 5 days in culture, CD4 and CD8 surface staining was performed and viability was assessed using LIVE/DEAD staining. Viability was determined within $CD4^+$ (**a**, **b**) and $CD8^+$ (**c**, **d**) cells after singlet and lymphocyte size gating (n=4). Data shown are representative of four repeat experiments. * $p \leq 0.05$, ** $p \leq 0.01$ indicate differences as compared to VH, ## $p \leq 0.01$ as compared to WT

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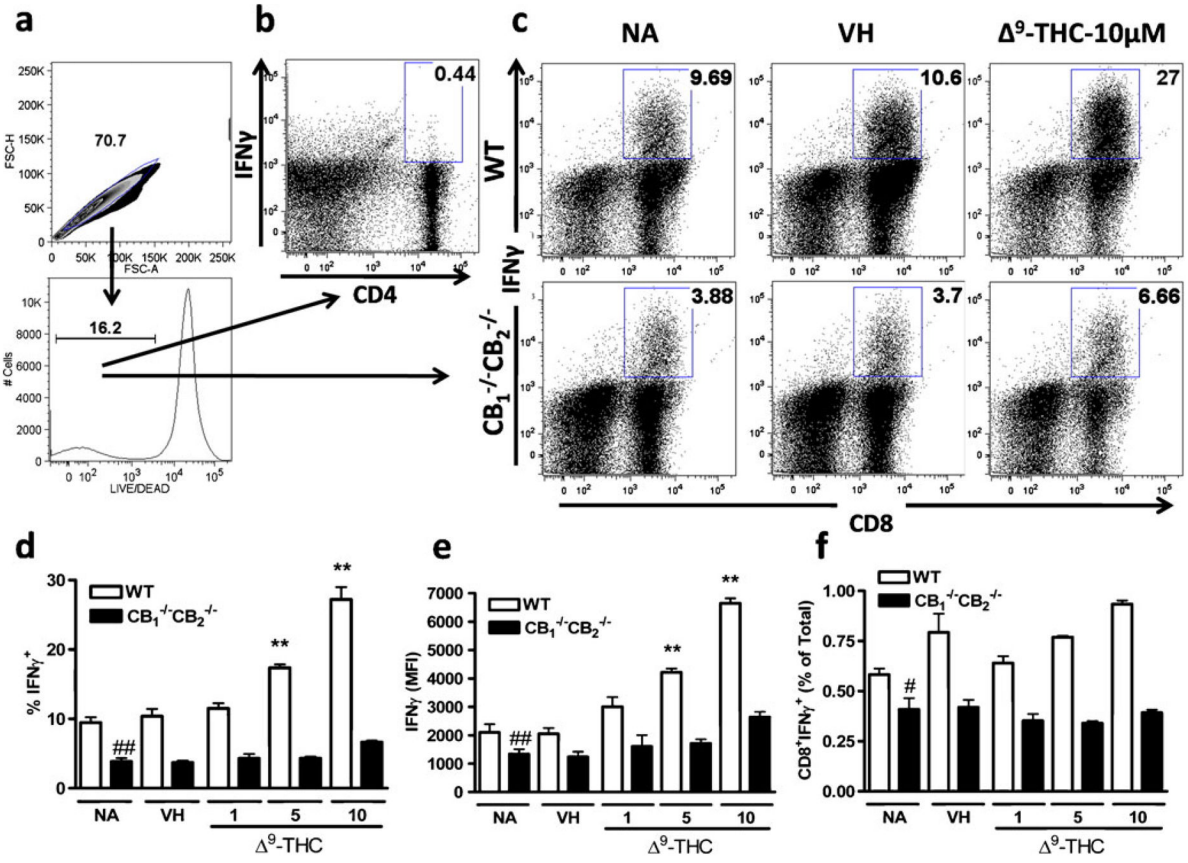

Fig. 5.

Increased IFNγ production in live but not total cells after Δ^9-THC treatment. CTL of WTand $CB_1^{-/-}CB_2^{-/-}$ were elicited as before with and without VH (0.1% ethanol) or Δ^9-THC (1, 5, 10 μM) treatment for 5 days. Cells were restimulated for 12 h in the presence of Brefeldin A and stained for $CD4^+$, $CD8^+$, LIVE/DEAD and IFNγ. Gating scheme is shown (**a**) for populations of $CD4^+$ (**b**) and $CD8^+$ (**c**) cells and dot plots indicate concatenated samples (n=4). Bar graph for $CD8^+$ cells are shown within live populations % (**d**), MFI (**e**), and in % of total populations (**f**) (n=4). Data shown are representative of three repeat experiments. ** $p \leq 0.01$ indicate differences as compared to VH, ## $p \leq 0.01$ as compared to WT

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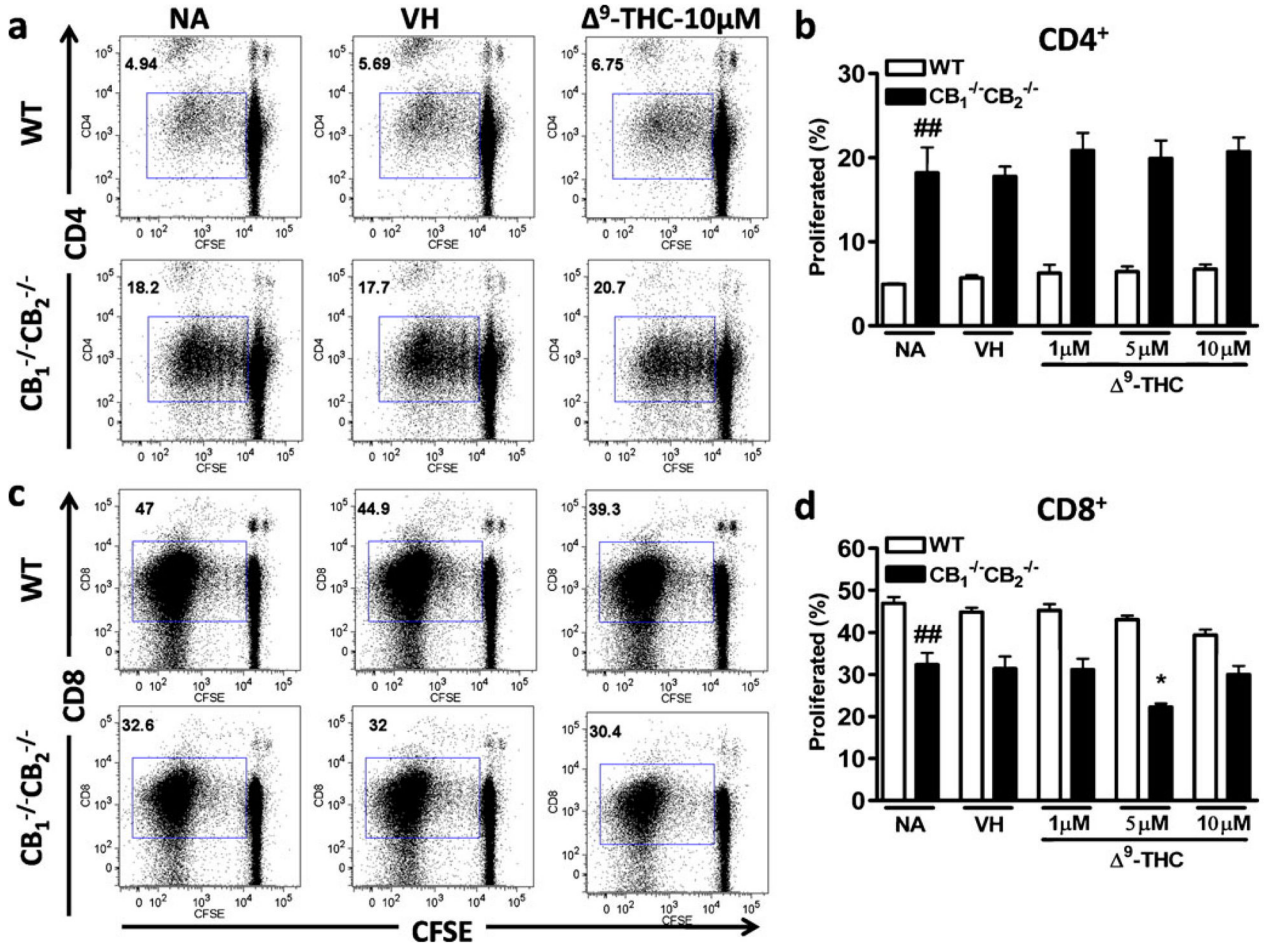

Fig. 6.

No effect of Δ^9-THC on proliferation. Splenocytes were isolated from WT and $CB_1{}^{-/-}CB_2{}^{-/-}$ mice and labeled with Cell Trace CFSE according to manufacturer's instructions. Four days after co-culture with irradiated P815 in the presence or absence of VH (0.1% ethanol) and Δ^9-THC (1, 5, 10 μM), surface staining with CD4 and CD8 were performed and CFSE fluorescence was assessed by FACS. Shown are concatenated samples (n=4) of CFSE staining as a result of proliferation in $CD4^+$ (**a**, **b**) and $CD8^+$ cells (**c**, **d**). Data shown are representative of two repeat experiments. * $p \leq 0.05$ indicate differences as compared to VH, ## $p \leq 0.01$ as compared to WT

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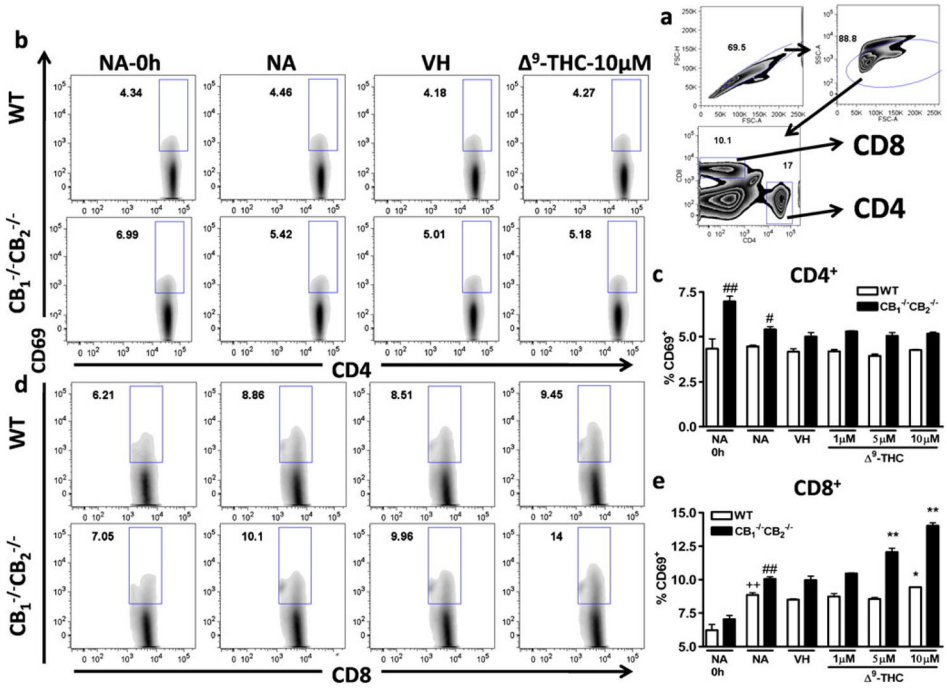

Fig. 7.

Δ^9-THC increases CD69 expression on $CD8^+$ cells in a concentration-dependent manner. Splenocytes from of WT and $CB_1^{-/-}$ $CB_2^{-/-}$ mice were incubated with irradiated P815 cells for 6 h to induce CD69 surface expression, in the presence or absence of VH (0.1% ethanol) or Δ^9-THC (1, 5, and 10 μM). Cells were gated on singlets and lymphocyte populations by size and then on CD4 and CD8 (**a**). $CD4^+$ (**b**, **c**) and $CD8^+$ (**d**, **e**) cells positive as defined by the *box* gate for CD69 in % are shown (n=4). Data shown are representative of three repeat experiments. Difference due to stimulation by P815 is indicated by ++ $p \leq 0.01$, due to genotypes by ## $p \leq 0.01$, due to Δ^9-THC by * $p \leq 0.05$,** $p \leq 0.01$

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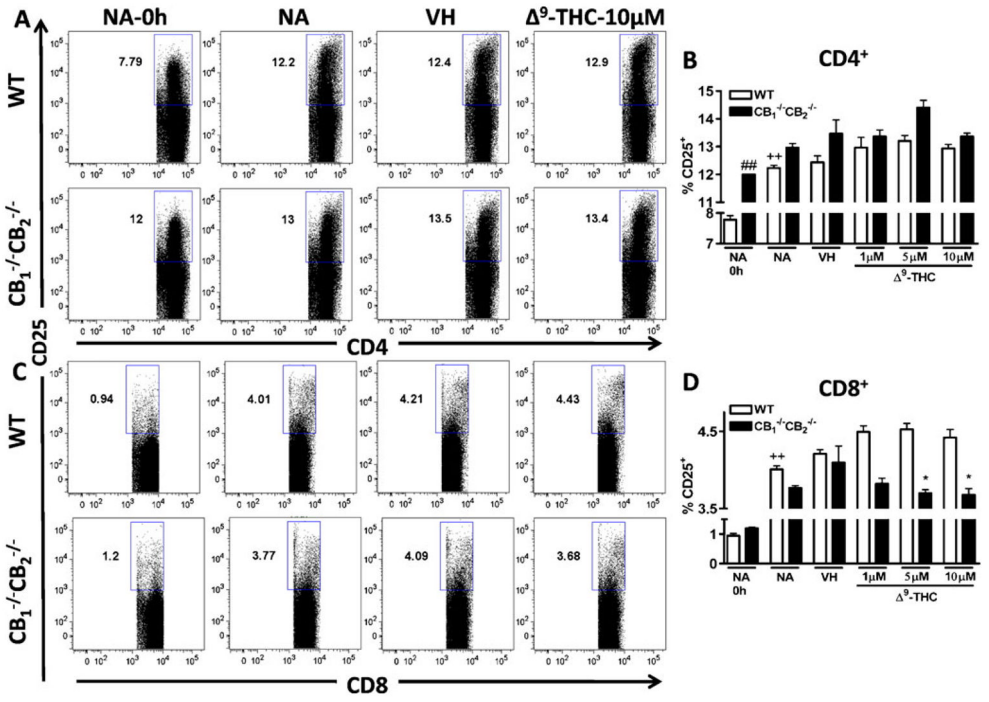

Fig. 8.

Δ^9-THC decreases CD25 expression on $CD8^+$ cells from $CB_1^{-/-}$ $CB_2^{-/-}$ mice in a concentration-dependent manner. Splenocytes from WT and $CB_1^{-/-}$ $CB_2^{-/-}$ mice were co-cultured with irradiated P815 cells for 12 h to induce CD25 levels, in the presence or absence of VH (0.1% ethanol) or Δ^9-THC (1,5, and 10 μM). Cells were gated on singlets and lymphocyte populations by size and cells positive for CD25 in % are shown (n=4). Data shown are representative of two repeat experiments. Difference due to stimulation by P815 is indicated by ++ $p \leq 0.01$, due to genotypes by ## $p \leq 0.01$, due to Δ^9-THC by * $p \leq 0.05$

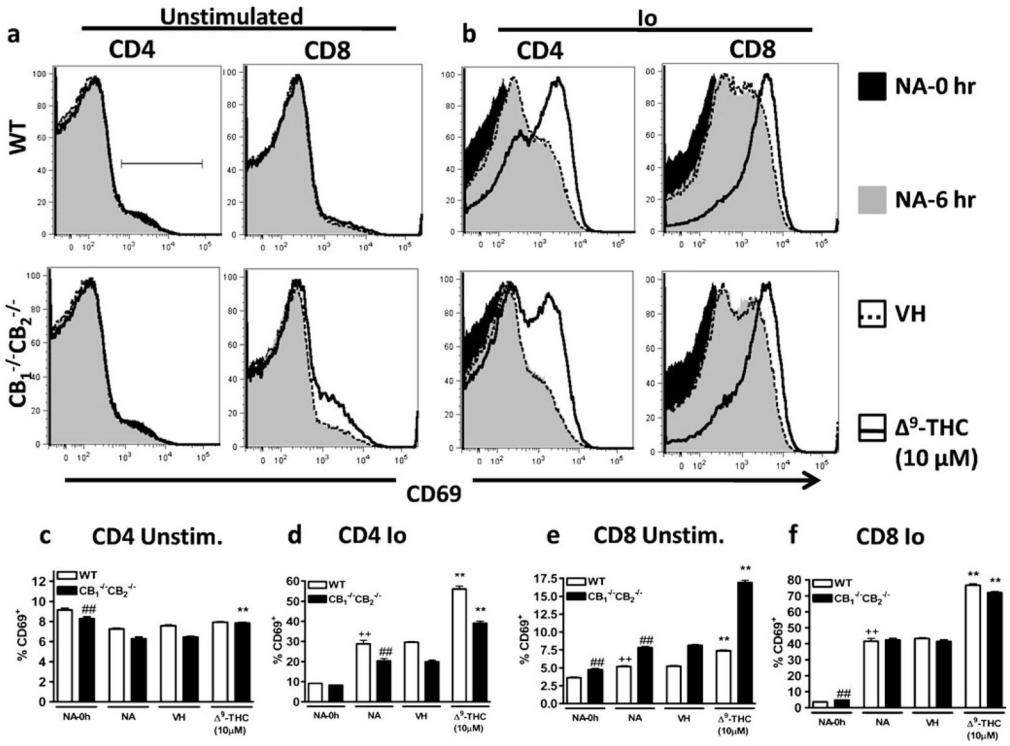

Fig. 9.

Δ^9-THC synergizes with Io to upregulate CD69 expression. Splenocytes were incubated in the presence of Io (0.5 μM) or VH (0.01% DMSO) in the presence or absence of VH (0.1% ethanol) or Δ^9-THC (1, 5, and 10 μM). For NA-0 h, CD69 staining was performed after isolation of a single cell suspension from the spleen otherwise 6 h after co-culture. Cells were gated on singlet, lymphocytes and within CD4 (**a**–**d**) or CD8 (**a**, **b**, **e**, **f**) positive populations. Data shown in histograms are concatenated (n=4) and are representative of two repeat experiments. Difference due to stimulation by Io is indicated by ++ $p \leq 0.01$, due to genotypes by ## $p \leq 0.01$, due to Δ^9-THC by ** $p \leq 0.01$

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