Published in final edited form as:

Cell Immunol. 2017 February ; 312: 25–34. doi:10.1016/j.cellimm.2016.11.006.

Cannabidiol (CBD) Induces Functional Tregs in Response to Low-Level T Cell Activation

Saphala Dhital¹, John V. Stokes², Nogi Park², Keun-Seok Seo², and Barbara L.F. Kaplan^{1,*}

¹Center for Environmental Health Sciences, Department of Basic Sciences, College of Veterinary Medicine, Mississippi State University, Mississippi State, MS 39762, USA

²Department of Basic Sciences, College of Veterinary Medicine, Mississippi State University, Mississippi State, MS 39762, USA

Abstract

Many effects of the non-psychoactive cannabinoid, cannabidiol (CBD), have been described in immune responses induced by strong immunological stimuli. It has also been shown that CBD enhances IL-2 production in response to low-level T cell stimulation. Since IL-2, in combination with TGF- β 1, are critical for Treg induction, we hypothesized that CBD would induce CD4⁺CD25⁺FOXP3⁺ Tregs in response to low-level stimulation. Low-level T cell stimulation conditions were established based on minimal CD25 expression in CD4⁺ cells using suboptimal PMA/Io (4 nM/0.05 μ M, S/o), ultrasuboptimal PMA/Io (1 nM/0.0125 μ M, Us/o) or soluble anti-CD3/28 (400-800 ng each, s3/28). CBD increased CD25⁺FOXP3⁺ cells from CD4⁺, CD4⁺CD25⁺, and CD4⁺CD25⁻ T cells, as well as in CD4⁺ T cells derived from FOXP3-GFP mice. Most importantly, the Us/o + CBD-induced CD4⁺CD25⁺ Tregs robustly suppressed responder T cell proliferation, demonstrating that the mechanism by which CBD is immunosuppressive under low-level T cell stimulation involves induction of functional Tregs.

Keywords

CD4⁺CD25⁺FOXP3⁺ Tregs; CBD; immunosuppression

*To whom correspondence should be addressed: Barbara L.F. Kaplan, Center for Environmental Health Sciences, Department of Basic Sciences, College of Veterinary Medicine, Mississippi State University, Mississippi State, MS 39762, USA, Tel 662-325-1113, barbara.kaplan@msstate.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

AUTHORSHIP

S. D. wrote the manuscript, designed and performed experiments. J. S. performed flow cytometry and reviewed the manuscript. N.P. and K.S.S. performed proliferation assays, participated in data interpretation and experimental design, and reviewed the manuscript. B. L. F. K. designed the study, wrote and reviewed manuscript, and performed experiments.

Compliance with Ethical Standards

The studies were carried out with approval from the Mississippi State University Institutional Animal Care and Use Committee (IACUC) in accordance with AAALAC guidelines (IACUC protocol number 13-110 to BLFK). Euthanasia via cervical dislocation was performed. This method is approved by the American Veterinary Medical Association for mice.

Conflict of Interest

The authors declare that they have no conflict of interest.

1. Introduction

Cannabidiol (CBD) is a non-psychoactive compound derived from *Cannabis sativa* [1, 2]. Studies with CBD are important since evidence suggests it can be used as a therapeutic agent for a variety of disease states [3]. For instance, CBD has exhibited anxiolytic, antiemetic, anti-tumorigenic and immune suppressive actions [4]. Specifically, CBD has been used for the management of seizures in severe epilepsy [3, 5]. CBD and its derivative dimethylheptyl-CBD have demonstrated efficacy as anti-inflammatory agents [6-14] and CBD also possesses anti-tumor activity [15, 16]. Moreover, in combination with the psychoactive cannabinoid, Δ^9 -tetrahydrocannabinol (THC) (a cannabinoid combination therapy known as Sativex®), CBD has been assessed for its efficacy to treat tumorigenic pain [4, 17] or spasticity induced by multiple sclerosis [18].

Although there are multiple studies and clinical trials investigating the use of CBD for immune-related diseases, its immunosuppressive mechanism is still unclear [19]. For instance, none of the studies have considered how the magnitude of cellular activation might alter CBD's effects. Studies such as these are important for many reasons. First, suboptimal T cell stimulation has been shown to contribute to persistent diseases, such as *M. tuberculosis* [20] or *T. cruzi* [21], so determination of the effects and mechanisms of CBD under low-level stimulation conditions will contribute to information on its putative therapeutic usefulness. Second, suboptimal T cell stimulation can be influenced by the presence of optimal stimulation of a distinct antigen, in what has been termed extended antigen priming [22], so studying low-level stimulation in the absence and presence of other antigens is key to understanding complex immune responses. Third, our previous study demonstrated that CBD either inhibited or enhanced IL-2 and IFN- γ production in response to optimal or suboptimal T cell activation, respectively [23], demonstrating that cellular activation dictates the CBD response. We were particularly interested in the consequences of enhanced IL-2 production by CBD in response to low-level T cell activation since IL-2, along with TGF- β 1, are key components for inducing and maintaining CD4⁺CD25⁺FOXP3⁺ Tregs [24]. Thus, we hypothesized that CBD would induce CD4⁺CD25⁺FOXP3⁺ cells under low-level stimulation of T cells. To address this hypothesis, we established low-level T cell stimulation conditions based on minimal expression of CD25 in order to evaluate CBD-induced CD25 and FOXP3 expression. Comparisons were made between naïve whole splenocytes and purified CD4⁺ T cells, including assessment of the effect of CBD on low-level stimulation of purified CD4⁺CD25⁺ (which likely contains a natural Treg population) and CD4⁺CD25⁻ T cells (potentially inducible Tregs). Finally, the functionality of CBD-induced Tregs was evaluated via examination of their ability to suppress naïve responder T cell proliferation. Together these data demonstrate that CBD induces functional CD4⁺CD25⁺FOXP3⁺ Tregs under low-level stimulation conditions, suggesting that CBD maintains its immunosuppressive actions regardless of magnitude of stimulation.

2. Materials and Methods

2.1 CBD

CBD was provided by the National Institute on Drug Abuse. CBD was prepared as a 10 mM solution in 99.5% pure ethanol and stored in aliquots at -80°C until use. All experiments include a 0.1% ethanol vehicle (VH) control.

2.2 Mice

Specific pathogen free 5 - 8 week old C57BL/6 mice were purchased from Envigo (Indianapolis, IN) and B6.129(Cg)-Foxp3^{tm3(DTR/GFP)Ayr/J} (FOXP3-GFP) mice were purchased from Jackson Labs (Bar Harbor, ME). Mice were housed 3-5 per cage, at 22-24°C, 40-55% humidity and 12-hr light/dark light cycle. The studies were carried out with approval from the Mississippi State University Institutional Animal Care and Use Committee (IACUC) in accordance with AAALAC guidelines (IACUC protocol numbers 13-110 and 15-077 to BLFK). Euthanasia via cervical dislocation was performed. This method is approved by the American Veterinary Medical Association for mice. All experiments were conducted *in vitro* using primary mouse splenocytes from female mice. Typically cells from 1-2 spleens were used for splenocyte experiments, and 3-4 spleens were pooled for RNA studies, enriched or purified T cells, and induction of Tregs for use in the functional assay.

2.3 Preparation of splenocyte cultures

Splenocytes were prepared as a single cell suspension by mechanical disruption in 1X RPMI media (Gibco/Life Technologies, Grand Island, NY). Splenocytes were enumerated with a Coulter Counter (Beckman Coulter, Indianapolis, IN). Splenocytes were cultured in complete medium containing bovine calf serum (BCS), 1% penicillin/streptomycin and 50 μM 2-mercaptoethanol. Cells were cultured in 2% BCS-containing medium for overnight cultures. For kinetic studies in which incubation periods included overnight cultures and longer periods (i.e., cultures for 1, 3 or 5 days), 5% BCS-containing medium was used. In some experiments, CD4⁺ T cells were enriched from splenocytes by negative selection using a mouse T cell CD4 subset column kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. Briefly, erythrocytes were lysed with ammonium-chloride-potassium (ACK) buffer, and up to 2×10^8 splenocytes were incubated with an antibody cocktail that allows for negative selection of CD4⁺ T cells. Cells were enriched on a prewashed column for 10 min at RT and eluted. CD4-enriched cells were collected, washed and adjusted in complete medium as required for assays. Cells were pretreated with CBD in 0.1% ethanol VH for 30 min then stimulated with suboptimal PMA/Io (4 nM/0.05 μM ; S/o), ultrasuboptimal PMA/Io (1 nM/0.012 μM ; Us/o) or soluble anti-CD3/CD28 (400-800 ng each; sCD3/28) for 1-5 days at 37°C at 5% CO₂. CBD was not washed out prior to stimulations in any experiment.

2.4 Purification of CD4⁺CD25⁺ Tregs and CD4⁺CD25⁻ T cells

Cells were purified either from fresh naive splenocytes or following culture of splenocytes with Us/o + CBD. For cultured cells, splenocytes (6×10^6 cells/ml, 6 ml/well) were seeded

in a 6-well flat bottom plate and treated with CBD (10 μ M) for 30 min followed by Us/o stimulation for 5 days. CD4⁺CD25⁺ Tregs were isolated using the Mouse CD25 Regulatory T cell Positive Selection Kit (Stemcell, Vancouver, BC, Canada) and CD4⁺CD25⁻ T cells were obtained using Mouse CD4⁺ T Cell Isolation Kit (Stemcell) according to the manufacturer's protocol. Briefly, CD4⁺CD25⁺ cells were initially purified by positive selection, followed by purification of the CD4⁺CD25⁻ T cells from the decanted cells. The purity of CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells was consistently higher than 80% as assessed by immunofluorescence staining for viability, CD4 and CD25.

2.5 Immunofluorescent staining

Splenocytes (1×10^6 cells/ml, 0.8 ml/well) were seeded in 48-well flat bottom plate and treated with CBD (0.5 - 10 μ M) or VH (0.1% ethanol) for 30 min. Cells were treated with S/o, Us/o or CD3/28 stimulation for 1-5 days. In some studies, CD4-enriched cells, CD4⁺CD25⁺ Tregs or CD4⁺CD25⁻ T cells were used instead of, or in addition to, splenocytes. For intracellular cytokine analysis cells were treated with Brefeldin A (BFA; Biolegend) for the last 4 hr of culture to block protein release from the cell. Cells were washed with PBS, centrifuged at $500 \times g$ for 5 min at RT. Cells were then treated with fixable viability dye (FVD; FITC or eFluor 780; BioLegend or eBioscience, San Diego, CA) as appropriate for 30 min at RT. Cells were washed with FACS buffer [Hank's Balanced Salt Solution (HBSS) with 1% bovine serum albumin (BSA), pH 7.3] and incubated with mouse Fc block (purified anti-mouse CD16/CD32, clone 2.4G2, BD Biosciences, San Jose, CA) for 15 min at RT to prevent non-specific binding. Cells were stained with extracellular antibodies (CD4 or CD25) for 30 min at RT, followed by fixation and permeabilization. For IL-2, TGF- β 1, IL-10 or FOXP3, cells were permeabilized using the FOXP3 staining kit (eBioscience, San Diego, CA) according to the manufacturer's instructions. Following extracellular staining, cells were washed, then incubated in fixation-permeabilization buffer (eBioscience) for 30 min at RT in the dark. Cells were washed and incubated with antibodies for intracellular markers for 30 min at RT. Immunofluorescent antibodies from BioLegend were: CD4 (FITC clone GK15-5; PECy7 clone GK1.5; or PE clone RM4-4), CD25 (FITC clone PC61), IL-2 (APC clone JES6-5H4), IL-10 (PeCy 7 clone JES5-16E3 or APC clone JES5-16E3) and TGF- β 1 (PE clone TW7-20B9). Antibodies purchased from eBioscience were: CD25 (PE clone PC61.5) and FOXP3 (APC clone FJK-16s). For the FOXP3-GFP cells, Sytox-Red (Invitrogen) was used to assess viability since these cells were analyzed without fixation or permeabilization. Stained cells were assayed using a FACSCalibur flow cytometer (BD Biosciences). Compensation was adjusted using single stain bead controls and gating was performed using fluorescence minus one (FMO) controls for each fluorochrome. Data were analyzed using FlowJo (FlowJo LLC, Ashland, OR). The gating strategy was initial dead cell exclusion using FSC versus FVD or Sytox, inclusion of lymphocytes using FSC versus SSC, then inclusion of CD4⁺ T cells using a histogram for CD4. FOXP3 versus CD25 in live CD4 lymphocytes was then analyzed from a dot plot. Representative gating strategies and viability percentages are provided in Supplemental Figs. 1-2.

2.6 Enzyme linked immunosorbent assay (ELISA)

Splenocytes (1×10^6 cells/ml, 0.8 ml/well) were seeded in 48-well flat bottom plates and treated with CBD (0.5 - 10 μ M) or VH (0.1% ethanol) for 30 min. Cells were stimulated with S/o, Us/o or CD3/28 stimulation for 1-5 days. Cytokine production in the culture supernatant was assessed by ELISA. Wells were coated overnight at 4°C with 1.0 μ g/ml anti-mouse IL-2 (clone JES6-1A12, BioLegend) in coating buffer (0.1 M carbonate-bicarbonate buffer, pH 9.6). Wells were washed three times each with 0.05% Tween-20 in PBS (PBST, pH 7.4) and deionized water (DW), then blocked with 3% BSA in PBS for 1 h at RT. After washing the wells, samples were added to each well and incubated for 1 h at RT. Again after washing, wells were incubated with 1.0 μ g/ml biotin-conjugated anti-mouse IL-2 (clone JES6-5H4, BioLegend) for 1 h at RT. Wells were washed and treated with 100 μ l of Avidin-conjugated with horseradish peroxidase (HRP-Avidin; 1:500, BioLegend) for 1 h at RT in the dark. For color development, wells were washed then 100 μ l of 3,3',5,5'-tetramethylbenzidine (TMB) substrate set (BioLegend) was added for 30 min followed by the addition of 100 μ l of 2 N sulfuric acid (H_2SO_4) to terminate the reaction. Optical density (OD) was measured at 450 nm within 30 min of reaction termination. Quantification of cytokine was performed by generation of a standard curve.

2.7 Reverse Transcriptase Quantitative Polymerase Chain Reaction (RTQPCR)

Splenocytes (5×10^6 cells/ml, 5 ml/well) were seeded in a 6-well flat bottom plate and treated with CBD (10 μ M) or VH (0.1% ethanol) for 30 min and treated with Us/o stimulation for 1-5 days. Cells were collected and resuspended in 1 ml TRI Reagent (Sigma, St. Louis, MO). RNA was isolated with RNeasy (Promega, Madison, WI) columns following TRI reagent (St. Louis, MO) phase separation and nucleic acid precipitation. Equal amounts of RNA were reverse transcribed using a high capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). RT-QPCR amplification was performed on an Mx3005P instrument (Stratagene) using a Taqman primer/probe for *Tgfb1* (Mm01178820_m1) or *Il10* (Mm00439614_m1) (Applied Biosystems) in a total reaction volume of 20 μ l. Relative gene expression in terms of fold-change values were determined using the $\Delta\Delta\text{Ct}$ method using 18s rRNA as the internal reference and Day 1 VH as the control [25].

2.8 Treg functional assay

$\text{CD4}^+\text{CD25}^+$ and $\text{CD4}^+\text{CD25}^-$ T cells were purified from Us/o + CBD (10 μ M)-treated splenocytes for 5 days. Purified cells were treated with mitomycin C (MMC, Sigma) at 10 μ g/ 10^6 cells for 2 hr and washed four times prior to use in the co-culture. Freshly isolated naïve splenocytes (responder cells; 1×10^5 cells/well) were mixed with an equal number of MMC-treated $\text{CD4}^+\text{CD25}^+$ and $\text{CD4}^+\text{CD25}^-$ T cells. Cultures were then stimulated with anti-CD3 plus anti-CD28 coated beads (Life Technologies) at 0.3 μ l/well. Co-cultures were incubated in a 96-well flat bottom plate in a final volume of 200 μ l for 4 days. Cells were treated with 1 μ Ci of titrated ^3H -thymidine for the last 16 hr of culture. Cells were harvested on glass fiber filters (Perkin Elmer) and ^3H -thymidine incorporation (degree of proliferation) was measured by scintillation counting.

2.9 Statistical analysis

Statistical analysis was performed using GraphPad Prism 6 (GraphPad software, San Diego, CA). Comparisons were performed by one-way or two-way ANOVA as appropriate. A post-hoc test for multiple comparisons was performed and the significance test was determined as $p < 0.05$. Logarithmic transformation was conducted prior to statistical analysis for PCR data using natural log (fold-change+1) for statistical analysis.

3. Results

3.1 Low-level PMA/Io stimulation as defined by CD25 expression

Previously we determined that cannabinoid effects differed depending on the magnitude to which cells were stimulated. Specifically, S/o stimulation was defined as 4 nM PMA/0.05 μ M Io based on the fact that no IL-2 was produced in response to S/o stimulation alone, and CBD enhanced IL-2 production under these conditions [23]. Initially, this seemed inconsistent with the notion that CBD is anti-inflammatory and immune suppressive [7, 11, 19, 26]. On the other hand, increased IL-2, in combination with TGF- β 1, can drive Treg development *in vitro* [27]. Thus, we hypothesized that CBD maintains its immune suppressive actions under low-level stimulation conditions through induction of Tregs. We first established low-level immunological stimulation conditions based on CD25 expression. Using the previously-defined S/o conditions of 4nM PMA/0.05 μ M Io in which little IL-2 was detected in the absence of CBD [23], CD25 was still readily induced following S/o stimulation overnight (Fig. 1), indicating that CD25 is very sensitive to upregulation by PMA/Io. Thus, a lower level of stimulation (1 nM PMA/0.0125 μ M Io, designated Us/o) was examined in order to identify a PMA/Io concentration that produced minimal expression of CD25. Indeed, no CD25 was induced at Day 1 in response to Us/o stimulation. The incubation time was also extended in order to allow time for Treg differentiation upon CBD treatment in subsequent studies. The results confirmed that CD25 expression was only modestly increased in response to Us/o stimulation at Day 5 (Fig. 1).

3.2 CBD increased CD25 and FOXP3 on CD4⁺ cells

3.2.1 CBD induced CD25 and FOXP3 in response to S/o and Us/o stimulation

—Initially, the effect of CBD on CD25 and FOXP3 after an overnight S/o stimulation was conducted. CBD upregulated the expression of CD25 and FOXP3 on CD4⁺ cells (Fig. 2A). In order to evaluate the possibility that CBD could induce Treg differentiation, as opposed to transient CD25 upregulation, the effect of CBD over an extended time period in response to the lower Us/o stimulation was examined. Indeed, increasing concentrations of CBD resulted in an increased percentage of CD4⁺CD25⁺FOXP3⁺ cells as compared to VH in response to Us/o stimulation for 5 days (Fig. 2B). A detailed analysis of gating strategies revealed that CBD produces modest suppression on viable cells after S/o stimulation for 1 day and Us/o stimulation for 5 days (Supplemental Figs. 1-2). Interestingly, CD4 expression was decreased by CBD after S/o stimulation for 1 day (Supplemental Fig. 1), but increased by CBD after Us/o stimulation for 5 days (Supplemental Fig. 2). It is important to note that we do not attribute the modest effect of CBD on viable cell percentages to direct cytotoxicity, but to apoptosis or differential cell survival (especially in splenocyte cultures) over the 5-day period.

3.2.2 CBD modestly induced CD25 and FOXP3 in response to s3/28

stimulation—Next, the effect of CBD specifically on T cells was examined in response to anti-CD3/CD28. It is important to note that we also wanted low-level stimulation with anti-CD3/28, similar to our previous work in which IL-2 production was induced by low-level anti-CD3/28 stimulation [23]. Thus we purposefully administered anti-CD3 in soluble form (as opposed to plate-bound) and only used 400-800 ng each of anti-CD3 and anti-CD28. The low-level stimulation conditions with anti-CD3/CD28 are designated as s3/28. As seen in Fig. 2C, CBD also increased CD25 and FOXP3 expression on CD4⁺ cells at Day 5 in s3/28-stimulated cells, albeit to a lower magnitude than that observed with Us/o stimulation.

3.2.3 CBD induced CD25 and FOXP3 in enriched CD4⁺ T cells—Given the observation that FOXP3 expression was lower and only modestly induced by CBD in response to s3/28 in whole splenocytes, the effect of CBD was determined using CD4-enriched cells. CD4 enrichment increased the CD4⁺ population to ~76% from 25% in total splenocytes. Treatment with CBD for 5 days resulted in a robust increase in the percentage of the CD25⁺FOXP3⁺ population on CD4⁺ cells stimulated with Us/o and a modest increase on CD4⁺ cells stimulated with sCD3/28 at 800 ng each (Fig. 3). The CBD-induced CD25⁺FOXP3⁺ population was not increased over VH in response to s3/28 at 400 ng each, but CD25 was robustly upregulated by CBD suggesting that CD25 is a very sensitive target of CBD. It should be noted that CBD alone produced only a small increase in the CD25⁺FOXP3⁺ population on CD4-enriched cells, indicating that CBD was more effective at enhancing T cell signaling initiated by Us/o or s3/28, rather than producing robust induction of CD25 and FOXP3 alone (Fig. 3).

3.2.4 CBD robustly induced CD25 and FOXP3 in FOXP3-GFP mice—To verify that CBD induced FOXP3, splenocytes derived from GFP-FOXP3 mice were treated with CBD or VH plus Us/o stimulation for 5 days. Us/o + CBD robustly increased the CD25⁺FOXP3-GFP⁺ population (Fig. 4). These results also verify that CBD increased FOXP3 expression, regardless of whether it was measured with a fluorescently-conjugated antibody during intracellular staining or directly assessed using the FOXP3-GFP mice.

3.3 Effect of CBD on early IL-2 and TGF-β1 production

In light of the previous observation that CBD enhanced S/o-stimulated IL-2 production [23] and, as shown here, CBD induced S/o- or Us/o-stimulated CD25 and FOXP3 expression in CD4⁺ cells, the CBD-induced cytokine milieu was examined to determine if CBD could induce the appropriate environment early to induce Treg differentiation. Thus, IL-2 and TGF-β1 were examined after an overnight stimulation since these two cytokines have been shown to promote Treg differentiation [27]. Cells were treated with CBD and stimulated with S/o and Us/o for 1, 3, or 5 days and IL-2 production was assessed in the supernatants by ELISA. CBD induced IL-2 production at all times in response to S/o stimulation, with a peak response at Day 1. However, in response to Us/o stimulation, IL-2 production was not detectable by ELISA at any time (Fig. 5A). However, CBD did induce IL-2 and TGF-β1, plus a CD4⁺IL-2⁺TGF-β1⁺ population, in response to both S/o and Us/o stimulation after 1 day as assessed by intracellular staining (Fig. 5B and 5C). s3/28 also modestly induced IL-2 and TGF-β1 after 1 day (Fig. 5C). Together these results suggest that IL-2 and TGF-β1

produced by CD4⁺ cells treated with CBD in response to S/o, Us/o and s3/28 stimulation create a favorable microenvironment for the induction of CD4⁺CD25⁺FOXP3⁺ cells [24, 27, 28].

3.4 CBD elevated IL-10 production

Another hallmark of Tregs is IL-10 [29-31], so the effect of CBD on IL-10 production was examined. In these and all subsequent studies, Us/o stimulation was used exclusively since it induced the most robust CD25 and FOXP3 expression with CBD treatment by Day 5. IL-10 was significantly elevated in Us/o + CBD-treated total lymphocytes at Day 5 (Fig. 6A). We also measured the FOXP3⁺IL-10⁺ percentage of CD4⁺ cells and found the percentage of CD4⁺IL-10⁺ cells was modestly increased by CBD in Us/o-stimulated cells (Fig. 6B), although most were FOXP3⁻. It should be noted that the IL-10⁺FOXP3⁺ results were more variable as compared to other endpoints, which is reflected in the discrepancy between percent gated numbers in the representative dot plot and the average provided above it. Next the effect of CBD on *Il10* and *Tgfb1* mRNA gene expression in response to Us/o stimulation was assessed for 1, 3 or 5 days. While CBD did not affect *Tgfb1* mRNA expression, CBD significantly upregulated the expression of *Il10* mRNA at Day 5 in Us/o-stimulated cells (Fig. 7A and 7B). It is important to note that the magnitude of CBD-induced *Il10* mRNA expression at Day 5 was also variable. A comparison of 3 separate experiments indicated the average increase was 67.1 ± 22.7 with at least a 2-fold increase in response to US/o + CBD over Us/o + VH in all experiments. Despite this variability in the magnitude of CBD-induced *Il10* expression, the consistent increase suggests that CBD-induced changes in IL-10 are mediated at the transcriptional level.

3.5 CBD increased FOXP3 in both CD4⁺CD25⁻ and CD4⁺CD25⁺ cells

To determine whether CBD increased FOXP3 on CD4⁺CD25⁻ or CD4⁺CD25⁺ populations, cells were purified directly from naïve splenocytes followed by treatment with CBD and Us/o stimulation. CBD robustly converted CD4⁺CD25⁻ T cells into CD4⁺CD25⁺FOXP3⁺ Tregs (Fig. 8). CBD also converted CD4⁺CD25⁺ into CD4⁺CD25⁺FOXP3⁺ Tregs. As a comparison, the average conversion to CD4⁺CD25⁺FOXP3⁺ Tregs was 82% from CD4⁺CD25⁻ cells, 45% from CD4⁺CD25⁺ cells, and 44% from naïve splenocytes. These data suggest CBD increased expression of CD25 and FOXP3 in both inducible and natural Treg populations (at least as defined as CD4⁺CD25⁻ or CD4⁺CD25⁺, respectively) in response to low-level stimulation.

3.6 CBD-induced CD4⁺CD25⁺ and CD4⁺CD25⁻ cells suppress responder cell proliferation

To assess the functionality of T cells induced by CBD, proliferation of fresh naïve responder splenocytes as targets in the presence of putative Tregs was assessed. To stimulate the responder splenocytes, anti-CD3 plus anti-CD28 bead (b3/28) stimulation was used (as opposed to low-level soluble antibodies) since we wanted significant stimulation in order to assess the ability of Us/o + CBD-induced cells to suppress proliferation. Us/o + CBD-induced CD4⁺CD25⁺ or CD4⁺CD25⁻ cells were purified after 5 days and mixed with b3/28-stimulated responder splenocytes. Classical CD4⁺CD25⁺ Tregs robustly suppressed the proliferation of naïve responder splenocytes. CD4⁺CD25⁻ cells only modestly suppressed b3/28-stimulated proliferation of naïve responder splenocytes (Fig. 9).

4. Discussion

Despite cannabinoid compounds exhibiting anti-inflammatory and immunosuppressive effects, there have been many reports that they possess some immune stimulatory effects, especially on cytokine production [23, 32-36]. Specifically, we previously demonstrated that IL-2 was sensitive to differential regulation by cannabinoids that depended on the magnitude to which the cells were activated [23, 32, 33]. In this study, we sought to determine the effects of CBD on T cell function following low-level stimulation conditions and found that CBD enhanced CD25 and FOXP3 on CD4⁺ cells. Most importantly, Us/o + CBD-induced CD4⁺CD25⁺ Tregs robustly suppressed proliferation of naive responder splenocytes, demonstrating for the first time that the mechanism by which CBD suppresses immune function involves induction of functional Tregs in response to low-level T cell activation.

Initially we utilized S/o conditions, which were originally identified as conditions under which little IL-2 was produced, and could be robustly enhanced with CBD [23]. While CBD induced a CD4⁺CD25⁺FOXP3⁺ population in response to S/o overnight, the results also showed that S/o conditions were not minimal for CD25 expression, suggesting that CD25 is much more sensitive to PMA/Io than IL-2. Thus, we characterized CBD-induced IL-2 production in response to S/o and Us/o over an extended time course. While IL-2 was not significantly detected by ELISA in response to Us/o stimulation, both IL-2 and TGF-β1 were detected intracellularly after 1 day suggesting CBD induced the appropriate cytokine milieu for Treg development. Indeed, CBD induced CD25 and FOXP3 expression in CD4⁺ cells in response to Us/o stimulation after a 5-day culture and the effect was enhanced when CD4 cells were enriched prior to culture initiation or with the increased sensitivity of the FOXP3-GFP cells.

In order to verify the CBD-induced CD25 and FOXP3 expression on CD4⁺ cells response to S/o or Us/o stimulation, cells were stimulated with s3/28 both in splenocytes and CD4-enriched cells. We purposefully used both anti-CD3 and anti-CD28 in soluble form at ng levels since we had previously demonstrated that use of anti-CD3 in soluble form (as opposed to plate bound) resulted in low-level T cell stimulation [23]. The effect of CBD in s3/28-stimulated cells was not as robust as in response to Us/o stimulation using splenocytes, but CD25 and FOXP3 were robustly upregulated by s3/28 in CD4-enriched cells. Previous studies demonstrated that CBD produced more robust IL-2 production in response to S/o stimulation as opposed to soluble anti-CD3/28 antibodies [23]. The reason that CBD produces more robust effects in response to PMA/Io as opposed to anti-CD3/28 is not clear, but could involve different intracellular signals since PMA/Io bypasses the TCR. Perhaps calcium signaling is involved since Io is a stronger inducer of intracellular calcium than anti-CD3/28. Alternatively, it is possible that CBD requires stimulation of an accessory cell (i.e., antigen presenting cell) for effective Treg induction that only gets stimulated with PMA/Io, but not anti-CD3/28. It is clear from the study in which CBD alone was used that CBD does require some kind of cellular stimulation for CD25 and FOXP3 induction.

CBD induced expression of CD25 and FOXP3, suggesting that CBD induced functional Tregs. Indeed, CBD increased *Il10* mRNA and intracellular IL-10 and TGF-β1 in response to Us/o stimulation. These results are consistent with another study in which CBD induced

Il10 mRNA in anergy-inducing T cells, defined as CD4⁺CD25⁻LAG⁺CD69⁺ cells [8]. It was interesting that while CBD induced *Il10* mRNA, it had no effect on *Tgfb1* mRNA, suggesting that the mechanism by which CBD induced IL-10 was at the level of transcription, while TGF- β 1 upregulation might be post-translational. For instance, perhaps CBD alters the ability of free TGF- β 1 to be released from its latent form [37]. We also determined that CBD induced FOXP3 in both CD4⁺CD25⁻ and CD4⁺CD25⁺ cells, although it was more effective at inducing FOXP3 in CD25⁻ cells. Most importantly, Us/o + CBD-induced CD4⁺CD25⁺ Tregs were functional as shown by their ability to robustly suppress b3/28-stimulated naïve responder cell proliferation. Us/o + CBD-induced CD4⁺CD25⁻ T cells also modestly suppressed naïve responder T cell proliferation, although the possibility that the CD4⁺CD25⁻ cells were contaminated with a small proportion of CD4⁺CD25⁺ Tregs exists. It should be noted that the intracellular IL-10 was detected predominantly in CD4⁺FOXP3⁻ cells, suggesting that the mechanism of suppression of responder splenocyte proliferation is likely IL-10-independent and perhaps cell contact-dependent. Regardless, the data do demonstrate robust suppressive function by Us/o + CBD-induced CD4⁺CD25⁺ Tregs, and suggests that CBD exhibits several mechanisms to suppress immunity, similar to other reports for Tregs [38-40].

While this is the first report that CBD induced functional Tregs in response to low-level stimulation, other reports have shown that cannabinoids induce Tregs. In response to Concanavalin-A to induce hepatitis, THC increased *Foxp3* mRNA expression in liver and increased the number of CD4⁺CD25⁺ cells and CD4⁺FOXP3⁺ cells in the liver [41]. THC also increased FOXP3 expression in CD4⁺ T cells in the lung of staphylococcal enterotoxin B-infused mice [42]. The synthetic cannabinoid agonist, WIN-55212-2, induced a CD4⁺CD25⁺FOXP3⁺ population in the CNS as late as 110 days post EAE initiation [43]. A CB₂-selective agonist, O-1966 increased the proportion of CD4⁺CD25⁺FOXP3⁺ cells and IL-10 production in a mixed lymphocyte response [44]. Finally, one study examined the effect of CBD on Treg induction using encephalitogenic T cells restimulated with MOG-loaded antigen presenting cells [8]. In contrast to our findings, CBD did not alter the CD4⁺CD25⁺ population, but CBD did induce a CD69⁺LAG⁺ population in CD4⁺CD25⁻ cells, and increased expression of genes associated with anergy in CD4⁺ T cells [8]. In the same study, CBD also induced *il10* mRNA expression in the T cells purified from the APC-T cell co-culture [8].

Interestingly, another recent study has demonstrated that there exists a T cell activation threshold for maximal induction of Tregs [45]. Specifically the authors identified a role for the E3 ubiquitin ligase Cbl-b to control TCR strength, and found that CD25 and FOXP3 expression were maximal with relatively low concentrations of anti-CD3 antibody [45].

5. Conclusions

Immune responses to pathogens are complex, likely involving T cell populations that are stimulated to various degrees by multiple antigens. Thus, an evaluation of the effects of a putative immune modulator such as CBD is critical under low-level stimulation conditions. These studies demonstrate for the first time that CBD maintains its immunosuppressive activity under conditions of low-level stimulation through induction of functional Tregs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

Studies were funded by Mississippi State University College of Veterinary Medicine and NIH P20GM103646.

Abbreviations

BCS	bovine calf serum
BFA	brefeldin A
CBD	cannabidiol
CB₁	cannabinoid receptor 1
CB₂	cannabinoid receptor 2
cpm	counts per minute
DW	deionized water
ELISA	enzyme linked immunosorbent assay
FOXP3	forkhead box P3
FVD	fixable viability dye
IFN	interferon
IL	interleukin
iTregs	inducible T regulatory T cells
MMC	mitomycin C
MOG	myelin oligodendrocyte glycoprotein
PMA	phorbol 12-myristate 13-acetate
RT-PCR	reverse transcription-polymerase chain reaction
s3/28	soluble anti-CD3 plus anti-CD28 treatment
S/o	suboptimal (4 nM phorbol ester/0.05 μ M ionomycin)
TCR	T cell receptor
TGF- β1	transforming growth factor- β 1
THC	⁹ -tetrahydrocannabinol
Us/o	ultrasuboptimal (1 nM phorbol ester/0.012 μ M ionomycin)

VH vehicle

References

1. Iuvone T, Esposito G, De Filippis D, Scuderi C, Steardo L. Cannabidiol: a promising drug for neurodegenerative disorders? *CNS Neurosci Ther.* 2009; 15:65–75. [PubMed: 19228180]
2. Scuderi C, Filippis DD, Iuvone T, Blasio A, Steardo A, Esposito G. Cannabidiol in medicine: a review of its therapeutic potential in CNS disorders. *Phytother Res.* 2009; 23:597–602. [PubMed: 18844286]
3. Iannotti FA, Hill CL, Leo A, Alhusaini A, Soubrane C, Mazzarella E, Russo E, Whalley BJ, Di Marzo V, Stephens GJ. Nonpsychotropic plant cannabinoids, cannabidivarin (CBDV) and cannabidiol (CBD), activate and desensitize transient receptor potential vanilloid 1 (TRPV1) channels in vitro: potential for the treatment of neuronal hyperexcitability. *ACS Chem Neurosci.* 2014; 5:1131–1141. [PubMed: 25029033]
4. Jensen B, Chen J, Furnish T, Wallace M. Medical Marijuana and Chronic Pain: a Review of Basic Science and Clinical Evidence. *Curr Pain Headache Rep.* 2015; 19:50. [PubMed: 26325482]
5. Porter BE, Jacobson C. Report of a parent survey of cannabidiol-enriched cannabis use in pediatric treatment-resistant epilepsy. *Epilepsy Behav.* 2013; 29:574–577. [PubMed: 24237632]
6. Juknat A, Kozela E, Kaushansky N, Mechoulam R, Vogel Z. Anti-inflammatory effects of the cannabidiol derivative dimethylheptyl-cannabidiol -studies in BV-2 microglia and encephalitogenic T cells. *J Basic Clin Physiol Pharmacol.* 2015
7. Malfait AM, Gallily R, Sumariwalla PF, Malik AS, Andreacos E, Mechoulam R, Feldmann M. The nonpsychoactive cannabis constituent cannabidiol is an oral anti-arthritis therapeutic in murine collagen-induced arthritis. *Proc Natl Acad Sci U S A.* 2000; 97:9561–9566. [PubMed: 10920191]
8. Kozela E, Juknat A, Kaushansky N, Ben-Nun A, Coppola G, Vogel Z. Cannabidiol, a non-psychoactive cannabinoid, leads to EGR2-dependent anergy in activated encephalitogenic T cells. *J Neuroinflammation.* 2015; 12:52. [PubMed: 25880134]
9. Costa B, Colleoni M, Conti S, Parolaro D, Franke C, Trovato AE, Giagnoni G. Oral anti-inflammatory activity of cannabidiol, a non-psychoactive constituent of cannabis, in acute carrageenan-induced inflammation in the rat paw. *Naunyn Schmiedeberg Arch Pharmacol.* 2004; 369:294–299. [PubMed: 14963641]
10. Costa B, Trovato AE, Comelli F, Giagnoni G, Colleoni M. The nonpsychoactive cannabis constituent cannabidiol is an orally effective therapeutic agent in rat chronic inflammatory and neuropathic pain. *Eur J Pharmacol.* 2007; 556:75–83. [PubMed: 17157290]
11. Kaplan BL, Springs AE, Kaminski NE. The profile of immune modulation by cannabidiol (CBD) involves deregulation of nuclear factor of activated T cells (NFAT). *Biochem Pharmacol.* 2008; 76:726–737. [PubMed: 18656454]
12. El-Remessy AB, Tang Y, Zhu G, Matragoon S, Khalifa Y, Liu EK, Liu JY, Hanson E, Mian S, Fatteh N, Liou GI. Neuroprotective effects of cannabidiol in endotoxin-induced uveitis: critical role of p38 MAPK activation. *Mol Vis.* 2008; 14:2190–2203. [PubMed: 19052649]
13. Mukhopadhyay P, Rajesh M, Horvath B, Batkai S, Park O, Tanchian G, Gao RY, Patel V, Wink DA, Liaudet L, Hasko G, Mechoulam R, Pacher P. Cannabidiol protects against hepatic ischemia/reperfusion injury by attenuating inflammatory signaling and response, oxidative/nitrative stress, and cell death. *Free Radic Biol Med.* 2011; 50:1368–1381. [PubMed: 21362471]
14. Ribeiro A, Ferraz-de-Paula V, Pinheiro ML, Vitoretti LB, Mariano-Souza DP, Quinteiro-Filho WM, Akamine AT, Almeida VI, Quevedo J, Dal-Pizzol F, Hallak JE, Zuardi AW, Crippa JA, Palermo-Neto J. Cannabidiol, a non-psychotropic plant-derived cannabinoid, decreases inflammation in a murine model of acute lung injury: role for the adenosine A(2A) receptor. *Eur J Pharmacol.* 2012; 678:78–85. [PubMed: 22265864]
15. Massi P, Valenti M, Vaccani A, Gasperi V, Perletti G, Marras E, Fezza F, Maccarrone M, Parolaro D. 5-Lipoxygenase and anandamide hydrolase (FAAH) mediate the antitumor activity of cannabidiol, a non-psychoactive cannabinoid. *J Neurochem.* 2008; 104:1091–1100. [PubMed: 18028339]

16. McAllister SD, Christian RT, Horowitz MP, Garcia A, Desprez PY. Cannabidiol as a novel inhibitor of Id-1 gene expression in aggressive breast cancer cells. *Mol Cancer Ther.* 2007; 6:2921–2927. [PubMed: 18025276]
17. Johnson JR, Burnell-Nugent M, Lossignol D, Ganae-Motan ED, Potts R, Fallon MT. Multicenter, double-blind, randomized, placebo-controlled, parallel-group study of the efficacy, safety, and tolerability of THC:CBD extract and THC extract in patients with intractable cancer-related pain. *J Pain Symptom Manage.* 2010; 39:167–179. [PubMed: 19896326]
18. Serpell MG, Notcutt W, Collin C. Sativex long-term use: an open-label trial in patients with spasticity due to multiple sclerosis. *J Neurol.* 2013; 260:285–295. [PubMed: 22878432]
19. Cabral GA, Rogers TJ, Lichtman AH. Turning Over a New Leaf: Cannabinoid and Endocannabinoid Modulation of Immune Function. *J Neuroimmune Pharmacol.* 2015; 10:193–203. [PubMed: 26054900]
20. Bold TD, Banaei N, Wolf AJ, Ernst JD. Suboptimal activation of antigen-specific CD4⁺ effector cells enables persistence of *M. tuberculosis* in vivo. *PLoS Pathog.* 2011; 7:e1002063. [PubMed: 21637811]
21. Vasconcelos JR, Bruna-Romero O, Araujo AF, Dominguez MR, Ersching J, de Alencar BC, Machado AV, Gazzinelli RT, Bortoluci KR, Amarante-Mendes GP, Lopes MF, Rodrigues MM. Pathogen-induced proapoptotic phenotype and high CD95 (Fas) expression accompany a suboptimal CD8⁺ T-cell response: reversal by adenoviral vaccine. *PLoS Pathog.* 2012; 8:e1002699. [PubMed: 22615561]
22. Shibuya M, Fujio K, Shoda H, Okamura T, Okamoto A, Sumitomo S, Yamamoto K. A new T-cell activation mode for suboptimal doses of antigen under the full activation of T cells with different specificity. *Eur J Immunol.* 2015; 45:1643–1653. [PubMed: 25789709]
23. Chen W, Kaplan BL, Pike ST, Topper LA, Lichorobiec NR, Simmons SO, Ramabhadran R, Kaminski NE. Magnitude of stimulation dictates the cannabinoid-mediated differential T cell response to HIVgp120. *Journal of leukocyte biology.* 2012; 92:1093–1102. [PubMed: 22899554]
24. Zorn E, Nelson EA, Mohseni M, Porcheray F, Kim H, Litsa D, Bellucci R, Raderschall E, Canning C, Soiffer RJ, Frank DA, Ritz J. IL-2 regulates FOXP3 expression in human CD4⁺CD25⁺ regulatory T cells through a STAT-dependent mechanism and induces the expansion of these cells in vivo. *Blood.* 2006; 108:1571–1579. [PubMed: 16645171]
25. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2⁻(Delta Delta C(T)) Method. *Methods.* 2001; 25:402–408. [PubMed: 11846609]
26. Kozela E, Lev N, Kaushansky N, Eilam R, Rimmerman N, Levy R, Ben-Nun A, Juknat A, Vogel Z. Cannabidiol inhibits pathogenic T cells, decreases spinal microglial activation and ameliorates multiple sclerosis-like disease in C57BL/6 mice. *Br J Pharmacol.* 2011; 163:1507–1519. [PubMed: 21449980]
27. Zheng SG, Wang J, Wang P, Gray JD, Horwitz DA. IL-2 Is Essential for TGF- β to Convert Naive CD4⁺CD25⁻ Cells to CD25⁺Foxp3⁺ Regulatory T Cells and for Expansion of These Cells. *The Journal of Immunology.* 2007; 178:2018–2027. [PubMed: 17277105]
28. Fantini MC, Becker C, Monteleone G, Pallone F, Galle PR, Neurath MF. Cutting edge: TGF-beta induces a regulatory phenotype in CD4⁺CD25⁺T cells through Foxp3 induction and down-regulation of Smad7. *J Immunol.* 2004; 172:5149–5153. [PubMed: 15100250]
29. Berg DJ, Zhang J, Lauricella DM, Moore SA. IL-10 Is a Central Regulator of Cyclooxygenase-2 Expression and Prostaglandin Production. *The Journal of Immunology.* 2001; 166:2674–2680. [PubMed: 11160331]
30. Cezmi AA, Kurt B. Mechanisms of interleukin-10-mediated immune suppression. *Immunology.* 2011; 103:131–136.
31. Couper KN, Blount DG, Riley EM. IL-10: The Master Regulator of Immunity to Infection. *The Journal of Immunology.* 2008; 180:5771–5777. [PubMed: 18424693]
32. Jan TR, Kaminski NE. Role of mitogen-activated protein kinases in the differential regulation of interleukin-2 by cannabinol. *J Leukoc Biol.* 2001; 69:841–849. [PubMed: 11358994]
33. Jan TR, Rao GK, Kaminski NE. Cannabinol enhancement of interleukin-2 (IL-2) expression by T cells is associated with an increase in IL-2 distal nuclear factor of activated T cell activity. *Mol Pharmacol.* 2002; 61:446–454. [PubMed: 11809870]

34. Jbilo O, Derocq JM, Segui M, Le Fur G, Casellas P. Stimulation of peripheral cannabinoid receptor CB2 induces MCP-1 and IL-8 gene expression in human promyelocytic cell line HL60. *FEBS Lett.* 1999; 448:273–277. [PubMed: 10218491]
35. Pross SH, Nakano Y, Widen R, McHugh S, Newton CA, Klein TW, Friedman H. Differing effects of delta-9-tetrahydrocannabinol (THC) on murine spleen cell populations dependent upon stimulators. *Int J Immunopharmacol.* 1992; 14:1019–1027. [PubMed: 1330945]
36. Karmaus PW, Wagner JG, Harkema JR, Kaminski NE, Kaplan BL. Cannabidiol (CBD) enhances lipopolysaccharide (LPS)-induced pulmonary inflammation in C57BL/6 mice. *J Immunotoxicol.* 2013; 10:321–328. [PubMed: 23173851]
37. Xu P, Liu J, Derynck R. Post-translational regulation of TGF-beta receptor and Smad signaling. *FEBS Lett.* 2012; 586:1871–1884. [PubMed: 22617150]
38. Sakaguchi S, Wing K, Yamaguchi T. Dynamics of peripheral tolerance and immune regulation mediated by Treg. *Eur J Immunol.* 2009; 39:2331–2336. [PubMed: 19662638]
39. Uhlig HH, Coombes J, Mottet C, Izcue A, Thompson C, Fanger A, Tannapfel A, Fontenot JD, Ramsdell F, Powrie F. Characterization of Foxp3+CD4+CD25+ and IL-10-secreting CD4+CD25+ T cells during cure of colitis. *J Immunol.* 2006; 177:5852–5860. [PubMed: 17056509]
40. Ulges A, Klein M, Reuter S, Gerlitzki B, Hoffmann M, Grebe N, Staudt V, Stergiou N, Bohn T, Bruhl TJ, Muth S, Yurugi H, Rajalingam K, Bellinghausen I, Tuettenberg A, Hahn S, Reissig S, Haben I, Zipp F, Waisman A, Probst HC, Beilhack A, Buchou T, Filhol-Cochet O, Boldyreff B, Breloer M, Jonuleit H, Schild H, Schmitt E, Bopp T. Protein kinase CK2 enables regulatory T cells to suppress excessive TH2 responses in vivo. *Nat Immunol.* 2015; 16:267–275. [PubMed: 25599562]
41. Hegde VL, Hegde S, Cravatt BF, Hofseth LJ, Nagarkatti M, Nagarkatti PS. Attenuation of experimental autoimmune hepatitis by exogenous and endogenous cannabinoids: involvement of regulatory T cells. *Mol Pharmacol.* 2008; 74:20–33. [PubMed: 18388242]
42. Rao R, Nagarkatti PS, Nagarkatti M. Delta(9) Tetrahydrocannabinol attenuates Staphylococcal enterotoxin B-induced inflammatory lung injury and prevents mortality in mice by modulation of miR-17-92 cluster and induction of T-regulatory cells. *Br J Pharmacol.* 2015; 172:1792–1806. [PubMed: 25425209]
43. Arevalo-Martin A, Molina-Holgado E, Guaza C. A CB(1)/CB(2) receptor agonist, WIN 55,212-2, exerts its therapeutic effect in a viral autoimmune model of multiple sclerosis by restoring self-tolerance to myelin. *Neuropharmacology.* 2012; 63:385–393. [PubMed: 22561283]
44. Robinson RH, Meissler JJ, Fan X, Yu D, Adler MW, Eisenstein TK. A CB2-Selective Cannabinoid Suppresses T-Cell Activities and Increases Tregs and IL-10. *J Neuroimmune Pharmacol.* 2015; 10:318–332. [PubMed: 25980325]
45. Qiao G, Zhao Y, Li Z, Tang PQ, Langdon WY, Yang T, Zhang J. T cell activation threshold regulated by E3 ubiquitin ligase Cbl-b determines fate of inducible regulatory T cells. *J Immunol.* 2013; 191:632–639. [PubMed: 23749633]

Highlights

- CBD induces CD25 and FOXP3 on CD4⁺ T cells in response to Us/o, S/o and s3/28 stimulation
- CBD is most effective in inducing CD4⁺CD25⁺FOXP3⁺ T cells in response to Us/o
- Us/o + CBD increased FOXP3 expression on CD4⁺ cells derived from FOXP3-GFP mice
- Us/o + CBD induced FOXP3 in pre-purified CD4⁺CD25⁻ and CD4⁺CD25⁺ T cells
- Us/o + CBD-induced CD4⁺CD25⁺ T cells suppressed responder cell proliferation

Fig. 1. Expression of CD25 in response to S/o and Us/o stimulation

Splenocytes were treated with S/o and Us/o stimulation and cultured for 1 or 5 days. Cells were stained for viability, CD4 and CD25. Only CD25 is depicted here to emphasize that Us/o stimulation produces low levels of CD25 as compared to S/o. Cells were gated on live CD4⁺ cells and gating was set based on FMO controls. Histograms are representative of triplicate results of at least three separate experiments for each stimulation. NA, naïve (untreated).

Fig. 2. Induction of CD4⁺CD25⁺FOXP3⁺ cells by CBD

Splenocytes were treated with CBD (0.5-10 μM) or VH (0.1% ethanol) for 30 min followed by low-level stimulation. Cells were stained for viability, CD4, CD25 and FOXP3. Cells are gated on live CD4⁺ cells. Numbers above plots are average percent gated numbers ± SE of triplicate results for the quadrant. Dot plots are representative of triplicate results of at least three separate experiments for each stimulation. **(A)** S/o stimulation for 1 day; **(B)** Us/o stimulation for 5 days; **(C)** s3/28 for 5 days. * $p < 0.05$ as compared to VH within each stimulation group. NA, naïve (untreated); Stim, stimulated.

Fig. 3. Effect of CBD on CD25 and FOXP3 expression on CD4-enriched cells

CD4-enriched T cells were treated with CBD (10 μM) or VH (0.1% ethanol) for 30 min followed by Us/o or s3/28 stimulation for 5 days. Cells were stained for viability, CD4, CD25 and FOXP3. Cells are gated on live CD4⁺ cells. Numbers above plots are average percent gated numbers ± SE of triplicate results for the quadrant. Dot plots are representative of triplicate results of at least three separate experiments. * $p < 0.05$ as compared to VH within each stimulation group. Stim, stimulated.

Fig 4. CBD increased FOXP3-GFP

Splenocytes from FOXP3-GFP mice were treated with CBD (10 μ M) or VH (0.1% ethanol) for 30 min followed by Us/o stimulation for 5 days. Cells were stained for viability, CD4 and CD25 without fixation. Cells are gated on live CD4⁺ cells. Numbers above plots are average percent gated numbers $\pm$ SE of triplicate results for the quadrant. Dot plots are representative of triplicate results of at least two separate experiments. * $p < 0.05$ as compared to VH.

Fig. 5. Effect of CBD on IL-2 and TGF- β 1 production

Splenocytes were treated with CBD (5 or 10 μ M) or VH (0.1% ethanol) for 30 min followed by low-level stimulation. **(A)** Cells were stimulated with S/o or Us/o stimulation for 1, 3 or 5 days. IL-2 was assessed by ELISA. Data are average of triplicate wells $\pm$ SD with * $p < 0.05$ as compared to VH within group; # $p < 0.05$ as compared to Day 1. **(B, C)** Cells were stimulated with S/o **(B)** or Us/o or s3/CD28 **(C)** for 1 day. Cells were treated with BFA for the last 4 hr of culture then stained for viability, CD4, IL-2 and TGF- β 1. Numbers above plots are average percent gated numbers $\pm$ SE of triplicate results for the quadrant. Dot plots represent triplicate results of at least three separate experiments. Cells are gated on live CD4⁺ cells. * $p < 0.05$ as compared to VH within each stimulation group. Stim, stimulated.

Fig. 6. CBD increased IL-10 and FOXP3 expression

Splenocytes were treated with CBD (1-10 μ M) or VH (0.1% ethanol) and Us/o stimulation for 5 days. Cells were treated with BFA for the last 4 hr of culture then stained for viability CD4, IL-10 and/or FOXP3. **(A)** Intracellular IL-10 in live lymphocytes. Data are average of triplicate wells $\pm$ SD with * $p < 0.05$ as compared to VH. **(B)** Intracellular IL-10 and FOXP3 in CD4⁺ T cells. Numbers above plots are average percent gated numbers $\pm$ SE of triplicate results for the quadrant. Dot plots represent triplicate results of at least three separate experiments.

Fig. 7. CBD upregulated the expression of *Il10* mRNA

Splenocytes were treated with VH or CBD (10 μM) and Us/o stimulation for 1, 3 or 5 days. Total RNA was isolated and quantitative RT-PCR was performed for (A) *Tgfb1* and (B) *Il10* mRNA. Data are relative gene expression as compared to Us/o + VH at Day 1 $\pm$ SD of triplicate wells. Data are representative of triplicate results from at least three separate experiments. * $p < 0.05$ as compared to expression at Day 1.

Fig. 8. Induction of CD4⁺CD25⁺FOXP3⁺ cells from CD4⁺CD25⁻ or CD4⁺CD25⁺ T cells by CBD CD4⁺CD25⁺ Tregs and CD4⁺CD25⁻ T cells from naive mouse splenocytes were purified and treated with CBD (10 μM) plus Us/o stimulation for five days. Cells were stained for viability, CD4, CD25 and FOXP3. Numbers above plots are average percent gated numbers ± SE of triplicate results for the quadrant. Cells are gated on live CD4⁺ cells. Dot plots are representative of triplicate results from two separate experiments. Average conversion to CD4⁺CD25⁺FOXP3⁺ cells from CD4⁺CD25⁻ or CD4⁺CD25⁺ T cells was 82% or 45%, respectively. Conversion from naive splenocytes was 44%. Conversion rates were calculated as (percentage of CD25⁺FOXP3⁺ cells produced by CBD – percentage of CD25⁺FOXP3⁺ cells produced by VH)/percentage of CD25⁺FOXP3⁺ cells produced by CBD) X 100%. * $p < 0.05$ as compared to VH within each cell population.

Fig. 9. Treg functional assay

Us/o + CBD-induced CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells were purified and treated with MMC to prevent proliferation. Fresh responder splenocytes were activated with anti-CD3/28 beads (b3/28) and incubated with MMC-treated, Us/o + CBD-induced cells. The effectiveness of MMC was assessed using fresh splenocytes treated with MMC followed by b3/28 stimulation. Co-cultures were incubated for 4 days. ³H-thymidine was added for last 16 hr of culture. Cells were lysed and ³H-thymidine was collected on glass fiber filters and counted using a scintillation counter. Data are average counts per minute (CPM) of quadruplet wells $\pm$ SD. Results are representative of quadruplicate cultures from two separate experiments. a, $p < 0.05$ as compared to b3/28-stimulated SPLC; b, $p < 0.05$ as compared to MMC-treated CD4⁺CD25⁻ T cells plus b3/28-stimulated SPLC.