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Research paper

Cannabidiol (CBD) induces functional Tregs in response to low-level T cell activation

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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-β1, 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 μM, S/o), ultrasuboptimal PMA/Io (1 nM/0.0125 μM, 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.

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 infections, 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

Abbreviations: BCS, bovine calf serum; BFA, Brefeldin A; CBD, cannabidiol; CB_1, cannabinoid receptor 1; CB_2, 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 M ionomycin); TCR, T cell receptor; TGF-β1, transforming growth factor-β1; THC, Δ^9-tetrahydrocannabinol; Us/o, ultrasuboptimal (1 nM phorbol ester/0.012 M ionomycin); VH, vehicle.

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TGF-β1, 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-h 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 to 2 spleens were used for splenocyte experiments, and 3 to 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% CO_2. 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 μM) 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 a 48-well flat bottom plate and treated with CBD (0.5–10 μM) 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 h of culture to block protein release from the cell. Cells were washed with PBS, centrifuged at 500×*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-β1, 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-β1 (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 and 2.