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The profile of immune modulation by cannabidiol (CBD) involves deregulation of nuclear factor of activated T cells (NFAT)☆

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ABSTRACT

Cannabidiol (CBD) is a cannabinoid compound derived from *Cannabis Sativa* that does not possess high affinity for either the CB1 or CB2 cannabinoid receptors. Similar to other cannabinoids, we demonstrated previously that CBD suppressed interleukin-2 (IL-2) production from phorbol ester plus calcium ionophore (PMA/Io)-activated murine splenocytes. Thus, the focus of the present studies was to further characterize the effect of CBD on immune function. CBD also suppressed IL-2 and interferon-γ (IFN-γ) mRNA expression, proliferation, and cell surface expression of the IL-2 receptor alpha chain, CD25. While all of these observations support the fact that CBD suppresses T cell function, we now demonstrate that CBD suppressed IL-2 and IFN-γ production in purified splenic T cells. CBD also suppressed activator protein-1 (AP-1) and nuclear factor of activated T cells (NFAT) transcriptional activity, which are critical regulators of IL-2 and IFN-γ. Furthermore, CBD suppressed the T cell-dependent anti-sheep red blood cell immunoglobulin M antibody forming cell (anti-sRBC IgM AFC) response. Finally, using splenocytes derived from $CB1^{-/-}$/$CB2^{-/-}$ mice, it was determined that suppression of IL-2 and IFN-γ and suppression of the *in vitro* anti-sRBC IgM AFC response occurred independently of both CB1 and CB2. However, the magnitude of the immune response to sRBC was significantly depressed in $CB1^{-/-}$/$CB2^{-/-}$ mice. Taken together, these data suggest that CBD suppresses T cell function and that CB1 and/or CB2 play a critical role in the magnitude of the *in vitro* anti-sRBC IgM AFC response.

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1. Introduction

Cannabinoids are a group of structurally related compounds derived from the *Cannabis Sativa* plant, which is commonly known as marijuana. The primary psychoactive congener in marijuana is tetrahydrocannabinol (THC) [1]. Although THC is currently approved for medical use as Marinol®, there exists an ongoing debate in the United States as to whether smoking crude marijuana could be a medical necessity. This debate has sparked interest in determining the physiological properties of some of the other plant-derived cannabinoid compounds. One such compound is cannabidiol (CBD), which is one of the most abundant cannabinoids in the plant.

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Abbreviations: AFC, antibody-forming cell; CBD, cannabidiol; THC, delta-9-tetrahydrocannabinol; IL-2, interleukin-2; IFN-γ, interferon-gamma.

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CBD possesses low affinity for both CB1 and CB2 cannabinoid receptors and therefore, does not produce the ''high'' associated with marijuana use [2,3]. Despite this, CBD does exhibit immunosuppressive properties. In particular, CBD decreased IL-8 and the chemokines MIP-1α and MIP-1β from a human B cell line [4]. CBD has also been shown to suppress collagen-induced arthritis [5], and carrageenan-induced inflammation [6]. Importantly, CBD has been efficacious in combination with THC in treating neuropathic pain in multiple sclerosis, an autoimmune disease [7,8].

Despite these reports that CBD possesses immunosuppressive actions, its effects on T lymphocytes have not been fully characterized. With our previous demonstration that CBD was one of the more potent plant-derived cannabinoids in suppressing IL-2 from PMA/Io-stimulated splenocytes [9], the focus of the present studies was to further investigate the effects of CBD on T lymphocyte function. The immunological endpoints include the determination of the effect of CBD on cytokine production (IL-2 and IFN-γ) from splenocytes activated through the T cell receptor, T and B cell proliferation, AFC responses, and direct effects on purified splenic T cells. As many reports in the literature suggest the involvement of a yet unidentified putative third cannabinoid receptor [10,11], cannabinoid actions via other receptors [12–14], and that some effects of CBD can be reversed by the CB1 and CB2 receptor antagonists [15], we utilized splenocytes derived from $CB1^{-/-}/CB2^{-/-}$ mice to address the role of CB1 and CB2 in the effects of CBD in T lymphocytes. Our results suggest that CBD suppresses T cell function via a mechanism that involves AP-1 and NFAT, and we have also discovered a putative critical role for CB1 and/or CB2 in the magnitude of the *in vitro* anti-sRBC IgM AFC response.

2. Materials and methods

2.1. Reagents

CBD and THC were provided by the National Institute on Drug Abuse (Bethesda, MD). All other reagents were obtained from Sigma (St. Louis, MO) unless otherwise noted.

2.2. Animals

Pathogen-free female B6C3F1 or C57BL/6 mice, 6 weeks of age, were purchased from Charles River Breeding Laboratories (Portage, MI). On arrival, mice were randomized, transferred to plastic cages containing sawdust bedding (5 animals/cage), and quarantined for 1 week. $CB1^{-/-}/CB2^{-/-}$ mice were kindly provided by Dr. Andreas Zimmer (University of Bonn) and were bred at Michigan State University. Mice were given food (Purina Certified Laboratory Chow) and water *ad libitum* and were not used for experimentation until their body weight was 17–20 g. Animal holding rooms were kept at 21–24 °C and 40–60% relative humidity with a 12-h light/dark cycle. All procedures involving mice were performed in accordance with guidelines set forth by the Institutional Animal Care and Use Committee at Michigan State University.

2.3. Preparation of lymphocyte cultures

Mice were sacrificed and spleens were aseptically removed. Single cell suspensions were prepared and cells were cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, 5×10^{-5} M 2-mercaptoethanol, and 2–10% bovine calf serum (BCS; Hyclone, Logan, UT). For immunofluorescence analysis, erythrocytes were lysed with ACK solution (150 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA). Jurkat cells (clone E6-1, ATCC, Manassas, VA) were maintained in RPMI 1640 medium supplemented with 2–10% BCS, 100 units/ml penicillin, 100 μg/ml streptomycin, 1× solutions of non-essential amino acids and sodium pyruvate (Invitrogen, Carlsbad, CA).

2.4. ELISA

Splenocytes (8×10^5 cells) were treated with CBD (0.1–15 μM) for 30 min at 37 °C, followed by cellular activation for 24 h in complete medium containing 2% BCS in 48-well culture plates at 0.8 ml/well. Cells were activated with either 40 nM/0.5 μM PMA/Io or 100 ng immobilized anti-CD3 plus 1 μg/ml soluble anti-CD28 (BD Biosciences, San Jose, CA). Alternatively, Jurkat cells (5×10^4 cells) were treated with CBD (0.1–10 μM) for 30 min at 37 °C, followed by cellular activation for 24 h in complete medium containing 2% BCS in 48-well culture plates at 0.25 ml/well. Jurkat cells were activated with 40 nM/0.5 μM PMA/Io. Cells were harvested and supernatants were collected and assayed for human IL-2, or murine IL-2 or IFN-γ production by ELISA. Recombinant purified human IL-2 or mouse IL-2 or IFN-γ (BD Biosciences, San Jose, CA) served as standards from which the amount of cytokine in the samples could be determined. Capture antibodies were purified anti-human IL-2 or anti-mouse IL-2 or IFN-γ and detection antibodies were biotinylated anti-human IL-2 or anti-mouse IL-2 or IFN-γ (BD Biosciences, San Jose, CA). Color development was performed using streptavidin peroxidase followed by tetramethylbenzidine (Fluka/Sigma, St. Louis, MO). Reactions were stopped with 6N H_2SO_4, after which samples were read at 450 nm.

2.5. Real time polymerase chain reaction (PCR)

Splenocytes (5×10^6 cells) were treated with CBD (0.5–10 μM) for 30 min at 37 °C, followed by cellular activation for 6 h in complete medium containing 2% BCS in 6-well culture plates at 5 ml/well. Cells were activated with 40 nM/0.5 μM PMA/Io. Cells were harvested and placed in TRI Reagent (Sigma, St. Louis, MO). Following phase separation with bromochlorophenol, RNA was precipitated from the aqueous phase with isopropanol. The remainder of the extraction, purification and DNase treatment was done using the Promega SV Total RNA Isolation System (Promega, Madison, WI). Total RNA was reversed transcribed using random primers with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). cDNA was amplified with Taqman primers and probe sets purchased from Applied Biosystems and analyzed using a 7900 HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA).

2.6. Immunofluorescence analysis

Splenocytes (8×10^5 cells) were treated with CBD (0.2–20 μM) for 30 min at 37 °C, followed by cellular activation with 40 nM/0.5 μM PMA/Io for 24 h in complete medium containing 2% BCS in 48-well culture plates at 0.8 ml/well. Cells were harvested, stained with antibodies directed against CD3 or CD25 (CD3-FITC or CD25-PE; BD Biosciences, San Jose, CA), and analyzed using a FACSCalibur (BD Biosciences, San Jose, CA). Cells were gated based on forward and side scatter (FSC/SSC) and data were analyzed using CellQuest software (BD Biosciences, San Jose, CA).

2.7. Lymphoproliferation assays

Splenocytes (2×10^5 cells) were treated with CBD (0.1–10 μM) for 30 min at 37 °C, followed by cellular activation in complete medium containing 2% (48 h cultures) or 5% (72 h cultures) BCS in 96-well culture plates at 0.2 ml/well. Cells were activated with either 40 nM/0.5 μM PMA/Io, 100 ng immobilized anti-CD3 plus 1 μg/ml soluble anti-CD28, or 10 μg/ml lipopolysaccharide (LPS). Splenocytes that were activated with LPS were cultured for 72 h; splenocytes that were activated with PMA/Io or anti-CD3/CD28 were cultured for 48 h. Cultures were pulsed with 1 μCi/well of [^{3}H]-thymidine 18 h prior to harvest, and the cells were harvested onto glass fiber filters using a PHD cell harvester (Cambridge Technology, Inc., Watertown, MA). Tritium incorporation was measured using a Packard Tri-Carb 2100TR Liquid Scintillation Analyzer (Packard Biosciences/PerkinElmer, Wellesley, MA).

2.8. Mixed lymphocyte response (MLR)

Splenocytes (1×10^5 cells) were treated with CBD (0.2–10 μM), followed by cellular activation with mitomycin C-treated non-self (DBA/2) splenocytes in complete medium containing 5% FBS in 96-well round bottom culture plates at 0.2 ml/well. The DBA/2 splenocytes were treated with 40 μg/ml mitomycin C for 60 min at 37 °C, washed 4 times with RPMI and adjusted to the appropriate cell density such that the stimulator:responder ratio was 4:1 (4×10^5 mitomycin C-treated DBA/2 splenocytes: 1×10^5 B6C3F1 splenocytes). Cells were cultured for 96 h. 18 h prior to harvest, cultures were pulsed with 1 μCi/well of [^{3}H]-thymidine and tritium incorporation was measured as described above.

2.9. Transient transfections

Jurkat cells (5×10^5 cells) were transfected using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) in complete medium containing 2% BCS. NFAT-luc, AP-1-luc and pTA-luc plasmids were purchased from Clontech (Mountain View, CA). Briefly, for every 5×10^5 cells to be transfected, pooled cells were incubated with 1.5 μg plasmid DNA and 3 μl Lipofectamine 2000 reagent, each delivered in 50 μl RPMI 1640. Plasmid DNA and Lipofectamine 2000 reagent were incubated in RPMI 1640 for 5 min, combined, and allowed to complex for 20 min prior to addition to cells. Transfected, pooled cells were then distributed to 48-well plates at 1 ml/well. Three hours post-transfection and plating, cells were treated with CBD (1–10 μM) for 30 min at 37 °C, followed by cellular activation with 40 nM/0.5 μM PMA/Io for 24 h. Supernatants were harvested and assessed for IL-2, and the cells were assessed for luciferase activity.

2.10. Luciferase assays

Luciferase activity was determined using the Luciferase Assay System and Reporter Lysis Buffer (RLB) from Promega (Madison, WI). Briefly, cells were washed once in PBS, then resuspended in 50 μl RLB per 5×10^5 cells. Cells were then frozen at −80 °C for 10 min, thawed and directly transferred to opaque 96-well plates for luciferase activity determination. Luciferase substrate (100 μl) was added to each well using the Bio-Tek Synergy HT instrument with KC4 version 3.4 software (Winooski, VT). After a 2 s delay, luciferase activity was detected over a 10 s period and data are presented as relative light units (RLU) in counts per second (CPS). Protein determinations were performed using a Bicinchoninic Acid Assay (BCA; Sigma, St. Louis, MO).

2.11. T cell purifications

T cells were purified from whole splenocyte preparations using the Pan T Cell Isolation Kit according to the manufacturer's instructions (Miltenyi Biotec, Auburn, CA). Briefly, spleens were collected and a single cell suspension was generated in MACS buffer (PBS, 0.5% BSA and 2 mM EDTA). Cells were then incubated with antibody cocktail and magnetic beads that allow for negative selection of T cells in the presence of a magnetic column. Purified T cells were subsequently used for ELISA analysis and proliferation. Purity of T cells was determined using immunofluorescence analysis with antibodies directed against CD3, and generally exceeded 98%.

2.12. In vitro antibody forming cell response (AFC)

Splenocytes ($2.5–5 \times 10^6$ cells) were treated with CBD (1–10 μM), followed by cellular activation in complete medium containing 10% heat-inactivated BCS in 48-well culture plates at 0.5 ml/well. Cells were activated with either 6.5×10^6 sheep erythrocytes (sRBC; Colorado Serum, Denver, CO) or 100 μg/ml LPS. Splenocytes were cultured (sRBC, 5 days; LPS, 3 days) in a Bellco stainless steel tissue culture chamber pressurized to 5.5 psi with a blood–gas mixture containing 10% O_2, 7% CO_2, and 83% N_2. The culture chamber was incubated at 37 °C and rocked continuously for the duration of the culture period. Enumeration of the antibody forming cells was based on the Jerne plaque assay [16,17]. Briefly, 50 or 100 μl aliquots of the cultured splenocytes were combined with 0.5% melted agar (Difco/BD, Franklin Lakes, NJ), guinea pig complement (Gibco/Invitrogen, Carlsbad, CA) and sheep erythrocytes, plated, covered with a 24 mm × 50 mm glass cover slip, and allowed to solidify. Plates were incubated for at least 3 h at 37 °C, after which AFCs were enumerated at 6.5× magnification using a Bellco plaque viewer (Bellco Glass Co., Vineland, NJ).

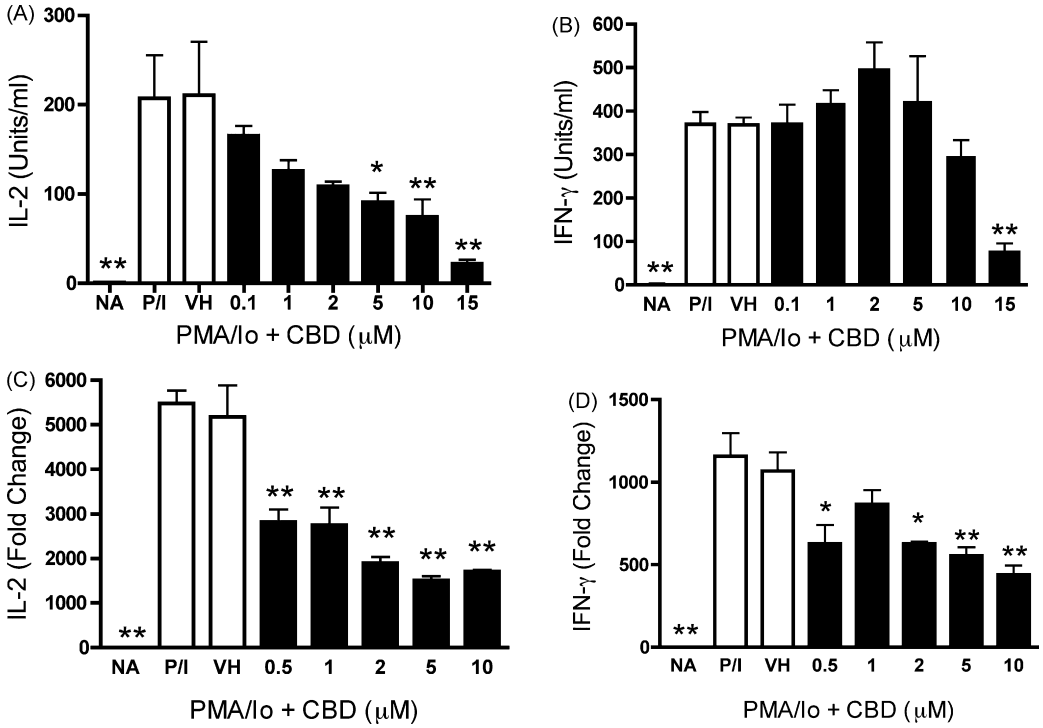

Fig. 1 – CBD suppressed cytokine production in PMA/Io-stimulated B6C3F1 splenocytes. (A and B) Splenocytes (8×10^5 cells) were treated with CBD (0.1–15 μM) for 30 min, followed by cellular activation with PMA/Io for 24 h. Supernatants were harvested and the amount of IL-2 (A) or IFN-γ (B) was determined by ELISA. The data are expressed as the mean units/ml ± S.E. of triplicate cultures. (C and D) Splenocytes (5×10^6 cells) were treated with CBD (0.5–10 μM) for 30 min, followed by cellular activation with PMA/Io for 6 h. Real time PCR was performed for IL-2 and IFN-γ. Fold change was calculated as compared to NA. * or ** denotes values that are significantly different from the vehicle control at $p < 0.05$ or 0.01. Results are representative of at least two separate experiments. NA, naïve (untreated); VH, vehicle (0.1% ethanol).

2.13. *In vivo antibody forming cell response (AFC)*

Mice (B6C3F1, 5 per treatment group) were administered CBD (25, 50 or 100 mg/kg) or THC (50 mg/kg) in corn oil by oral gavage for 3 days. On the second day, mice were sensitized with 5×10^8 sRBC per mouse by i.p. injection. Four days after sRBC sensitization, mice were sacrificed and total body, spleen, thymus and kidney weights were recorded. Single cell suspensions of splenocytes were then generated and used to determine the *in vivo* AFC response as described above.

2.14. *Statistical analysis*

The mean ± S.E. was determined for each treatment group. Differences between means were determined with a parametric analysis of variance. When significant differences were detected, treatment groups were compared to the appropriate control using Dunnett's two-tailed *t*-test. Following a two-way analysis of variance, all groups were compared using Bonferroni's test. A two-tailed *t*-test was used to determine statistical significance between stimulated groups in the anti-sRBC IgM AFC response between C57BL/6 and $CB1^{-/-}/CB2^{-/-}$ mice. Statistical analyses were performed using GraphPad Prism version 4.0a for Macintosh OS X, GraphPad Software (San Diego, CA).

3. Results

3.1. *CBD suppressed cytokine production in PMA/Io-stimulated splenocytes in a CB1 and CB2 receptor-independent manner*

Previous work from our laboratory demonstrated that CBD was one of the most potent cannabinoids for suppression of PMA/Io-induced IL-2 production in splenocytes [9]. As seen in Fig. 1B, CBD also suppressed PMA/Io-induced IFN-γ production, although the potency with which CBD suppressed IFN-γ was not as marked as for IL-2 (shown in Fig. 1A as a comparative control). The CBD-induced suppression of both cytokines occurred at the level of mRNA (Fig. 1C and D). With the demonstration that IL-2 is a sensitive target of suppression by CBD, we next determined the effect of CBD on expression of the IL-2 receptor α chain (CD25). CBD suppressed cell surface expression of CD25 in a concentration-dependent manner in PMA/Io-stimulated splenocytes (Fig. 2). Interestingly, there was not a large population of $CD25^+$ cells in the absence of stimulation, suggesting the primary effect of CBD on CD25 occurs during T cell activation as opposed to an effect on the T regulatory cell population. Finally, there was no difference in the ability of CBD to suppress PMA/Io-stimulated IL-2 and IFN-γ from splenocytes derived from either C57BL/6 wild type or $CB1^{-/-}/CB2^{-/-}$ mice (Fig. 3).

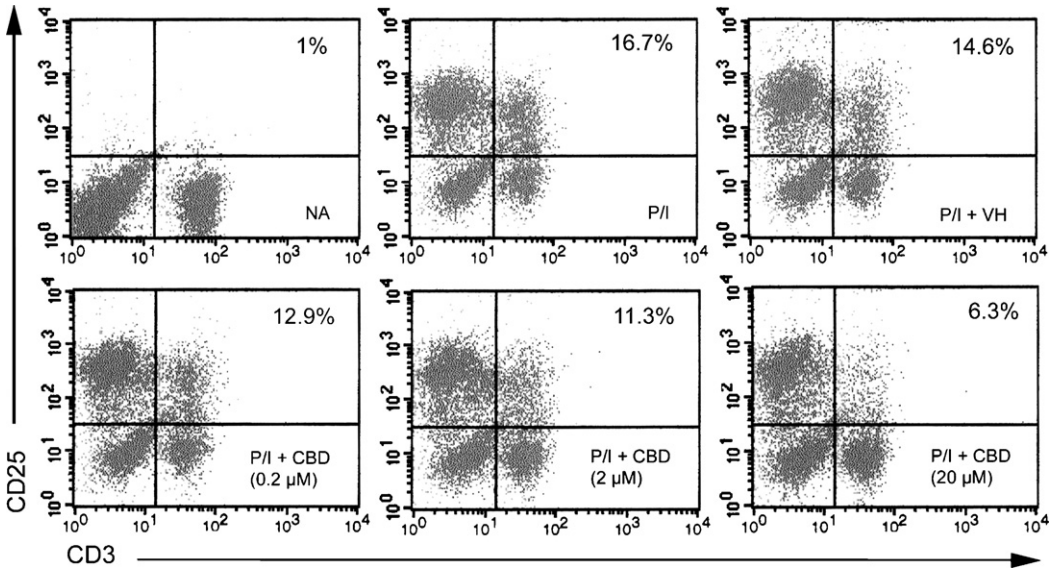

Fig. 2 – CBD suppressed CD25 cell surface expression in PMA/Io-stimulated B6C3F1 splenocytes. Splenocytes (8×10^5 cells) were treated with CBD (0.2–20 μM) for 30 min, followed by cellular activation with PMA/Io for 24 h. Cells were harvested and stained with fluorescent antibodies directed against CD3 (FITC) or CD25 (PE). Cells were gated on FSC/SSC. Numbers denote percent gated events. Results are representative of three separate experiments. NA, naïve (untreated); VH, vehicle (0.1% ethanol).

3.2. *CBD suppressed cytokine production in T cells*

In order to determine whether splenic T lymphocytes are direct targets of inhibition by CBD, splenocytes were activated with anti-CD3/anti-CD28, which exclusively stimulates T lymphocytes via the T cell receptor. Although not as marked as the inhibition of cytokines produced in response to PMA/Io, CBD also suppressed IL-2 and IFN-γ produced in response to

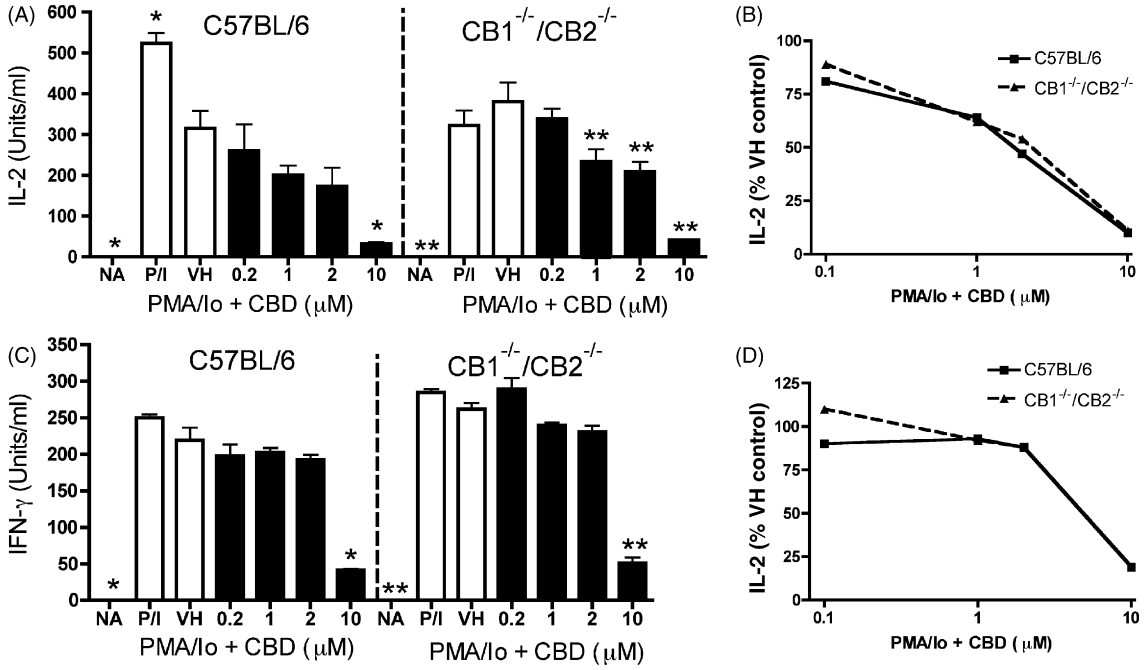

Fig. 3 – CBD suppressed cytokine production in wild type C57BL/6 and $CB1^{-/-}/CB2^{-/-}$ splenocytes. Splenocytes (8×10^5 cells) were treated with CBD (0.2–10 μM) for 30 min, followed by cellular activation with PMA/Io for 24 h. Supernatants were harvested and the amount of IL-2 (A and B) or IFN-γ (C and D) in wild type C57BL/6 and $CB1^{-/-}CB2^{-/-}$ was determined by ELISA. The data are expressed as the mean units/ml ± S.E. of triplicate cultures. * or ** denotes values that are significantly different from the respective vehicle control at $p < 0.05$ or 0.01. Data are presented as % VH control in (B) and (D). Results are representative of two separate experiments. NA, naïve (untreated); VH, vehicle (0.1% ethanol).

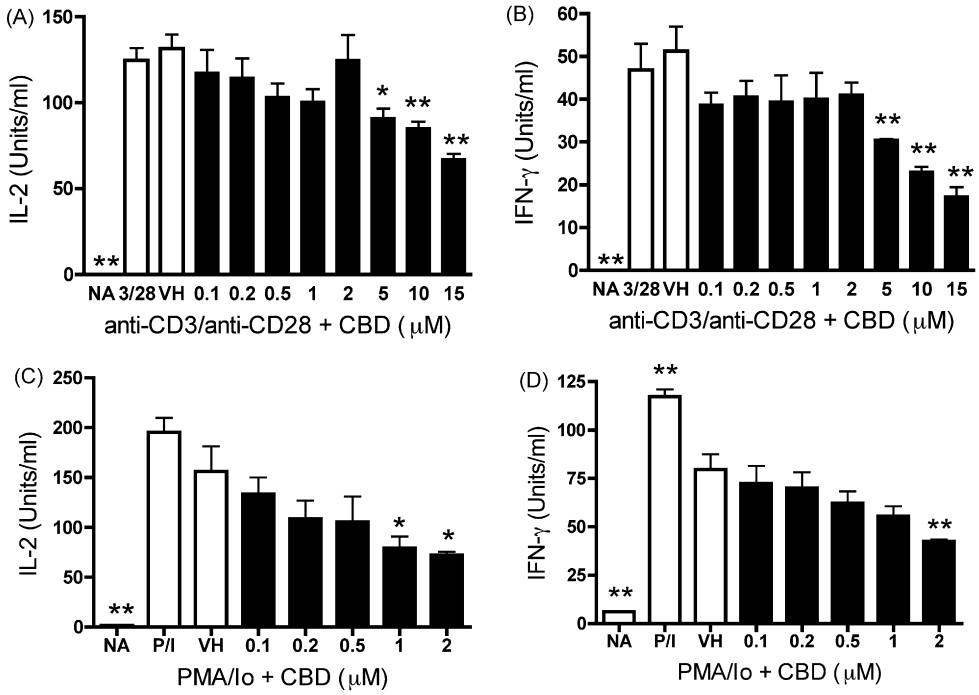

Fig. 4 – CBD suppressed cytokine production in B6C3F1 splenic T cells. (A and B) Splenocytes (8×10^5 cells) were treated with CBD (0.1–15 μM) for 30 min, followed by cellular activation with immobilized anti-CD3 plus soluble anti-CD28 for 24 h. Supernatants were harvested and the amount of IL-2 (A) or IFN-γ (B) was determined by ELISA. (C and D) T cells purified from splenocytes (8×10^5 cells) were treated with CBD (0.1–2 μM) for 30 min, followed by cellular activation with PMA/Io for 24 h. Supernatants were harvested and the amount of IL-2 (C) or IFN-γ (D) was determined by ELISA. The data are expressed as the mean units/ml ± S.E. of triplicate cultures. * or ** denotes values that are significantly different from the vehicle control at $p < 0.05$ or 0.01. Results are representative of at least two separate experiments. NA, naïve (untreated); VH, vehicle (0.1% ethanol).

anti-CD3/anti-CD28 from splenic T lymphocytes (Fig. 4A and B). Furthermore, PMA/Io-induced IL-2 and IFN-γ production from purified splenic T cells (i.e., >95% purity) was also suppressed by CBD (Fig. 4C and D). It is noteworthy that purified T cells were particularly sensitive to CBD in the presence of PMA/Io and therefore, lower concentrations of CBD were used for these studies.

3.3. *CBD suppressed cellular proliferation in several cell types*

IL-2, which acts via the IL-2 receptor, is a critical cytokine for T cell proliferation; therefore we determined the effect of CBD on cellular proliferation. CBD suppressed PMA/Io-stimulated proliferation in a concentration-dependent manner (Fig. 5A). However, as PMA/Io likely induces proliferation in most splenic cell types, various stimuli were utilized to target specific cell populations. Cellular proliferation in response to LPS, which predominantly activates B cells, was also suppressed in a concentration-dependent manner by CBD (Fig. 5B). In order to address the effect of CBD on T cell proliferation, two different stimuli were used: anti-CD3/anti-CD28, or mitomycin C-treated allogeneic lymphocytes (MLR). In response to either anti-CD3/anti-CD28 or mitomycin C-treated allogeneic lymphocytes, both of which activate T cells via the T cell receptor, CBD suppressed T cell proliferation (Fig. 5C and D).

3.4. *CBD suppressed the T cell-dependent AFC response*

One functional immune endpoint that is sensitive to suppression by other plant-derived and synthetic cannabinoids is the T cell-dependent AFC response [18,19]. As seen in Fig. 6A, and consistent with THC, CBD suppressed the *in vitro* T cell-dependent anti-sRBC IgM AFC response, but did not affect the *in vitro* IgM AFC response to the polyclonal B cell activator, lipopolysaccharide (LPS; Fig. 6B).

As CBD suppressed the *in vitro* anti-sRBC IgM AFC response, the effect of CBD *in vivo* was determined. Oral administration of CBD produced a modest modulation of the IgM AFC response to sRBC (Fig. 6C). Although none of the CBD treatments produced statistically significant modulation (at $p < 0.05$), the trend for CBD-induced suppression of the *in vivo* AFC response to sRBC at 100 mg/kg was consistent in two separate replicates of the experiment. The magnitude of suppression by 100 mg/kg CBD was similar to that produced by 50 mg/kg THC. There was no significant change in total body weight, spleen or thymus weights, or in the weight of the kidneys, presumably non-targets of immunosuppression by cannabinoids (data not shown).

Although CBD has been reported to possess low affinity for both CB1 and CB2 cannabinoid receptors [2,3], we next attempted to discern and/or confirm the lack of a role for CB1 and CB2 in CBD-induced suppression of the anti-sRBC IgM

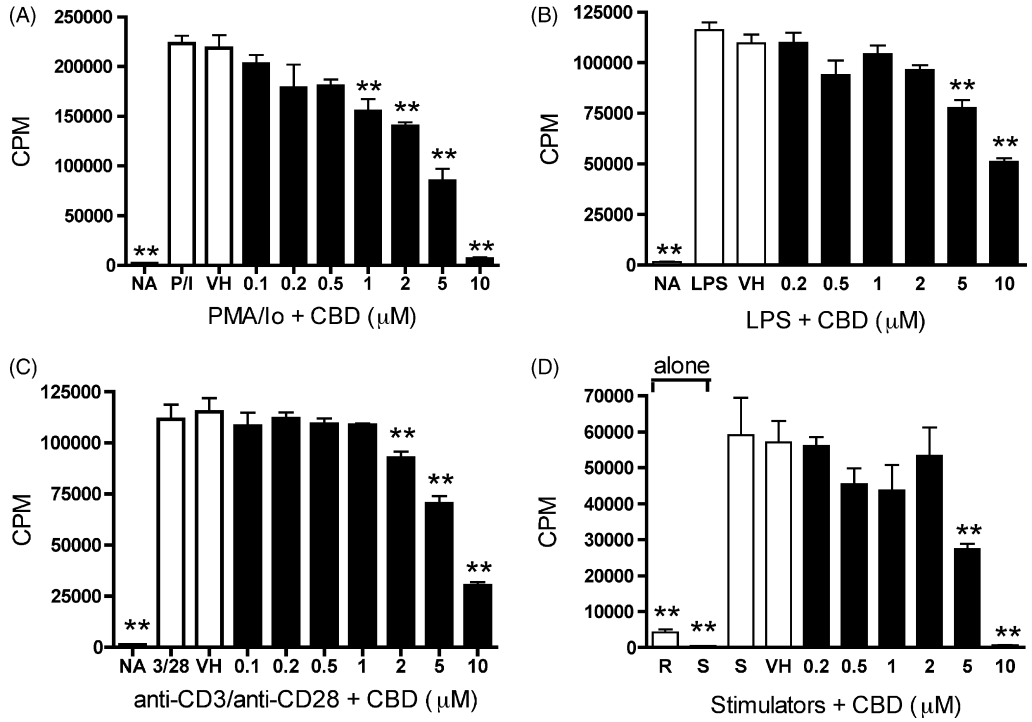

Fig. 5 – CBD suppressed cellular proliferation in B6C3F1 splenocytes in response to various stimuli. (A–D) Splenocytes (2×10^5 cells) were treated with CBD (0.2–10 μM) for 30 min, followed by cellular activation. 18 h prior to harvest, cells were pulsed with 1 μCi ^{3}H-thymidine. Cells were harvested onto glass fiber filters and tritium incorporation was measured with a liquid scintillation counter. Splenocytes were activated with (A) PMA/Io for 48 h; (B) LPS for 72 h; (C) immobilized anti-CD3 plus soluble anti-CD28 for 48 h; (D) mitomycin C-treated allogeneic lymphocytes for 96 h. The data are expressed as the mean CPM ± S.E. of quadruplicate cultures. * or ** denotes values that are significantly different from the vehicle control at $p < 0.05$ or 0.01. Results are representative of at least three separate experiments. R, responders; S, stimulators; NA, naïve (untreated); VH, vehicle (0.1% ethanol).

AFC response by using splenocytes derived from CB1$^{-/-}$/CB2$^{-/-}$ mice. As seen in Fig. 7, CBD robustly suppressed the anti-sRBC IgM AFC response in C57BL/6 mice. There was a significant decrease ($p < 0.001$) in the overall magnitude of AFC induced by sRBC in the CB1$^{-/-}$/CB2$^{-/-}$ mice versus C57BL/6 mice (379.7 ± 65.7 versus 3829 ± 443, respectively, over 4 separate experiments; one representative graph depicted in Fig. 7A). Since there was such a large discrepancy in the magnitude of the immune response to sRBC in the CB1$^{-/-}$/CB2$^{-/-}$ mice, the data are also expressed as percent of vehicle control for four separate experiments (Fig. 7B). There was no difference in CBD-induced suppression of the anti-sRBC IgM response in either genotype with the exception of the 5 μM concentration, which was highly variable in the CB1$^{-/-}$CB2$^{-/-}$ mice. It is likely that CB1 and/or CB2 are not involved in CBD-induced suppression of the *in vitro* anti-sRBC IgM AFC response. On the other hand, it is evident that either CB1 or CB2, or both, contribute to the overall magnitude of the *in vitro* anti-sRBC IgM AFC response.

3.5. *CBD suppressed NFAT and AP-1 reporter gene activity in PMA/Io-stimulated Jurkat cells*

CBD suppressed IL-2 and IFN-γ cytokine production in various T cell preparations, both of which are regulated by several transcription factors, including AP-1 and NFAT [20–23]. Interestingly, both AP-1 and NFAT have been shown to be sensitive targets of inhibition by many cannabinoids [24–26]. Thus, we determined whether NFAT and AP-1 were also targeted by CBD using AP-1- and NFAT-driven luciferase reporter genes in human Jurkat T cells. As shown in Fig. 8A, CBD did suppress human IL-2 production from PMA/Io-stimulated Jurkat T cells. Furthermore, the mechanism involves suppression of transcription as CBD suppressed PMA/Io-induced AP-1- and NFAT-luciferase expression in a concentration dependent manner (Fig. 8B and C, respectively). There was no effect on the overall protein content (data not shown) in the various treatment groups, indicating, in fact, that the suppression of luciferase activity was due to CBD. Consistent with other cannabinoids [26], suppression of NFAT-luciferase was more robust than AP-1-luciferase.

4. Discussion

CBD suppressed several immunological endpoints, with a profile of activity similar to other plant-derived, synthetic and endogenous cannabinoids [9,18,27–29]. Specifically, CBD suppressed cytokine production from activated primary mouse splenocytes in a concentration-dependent manner. Of note

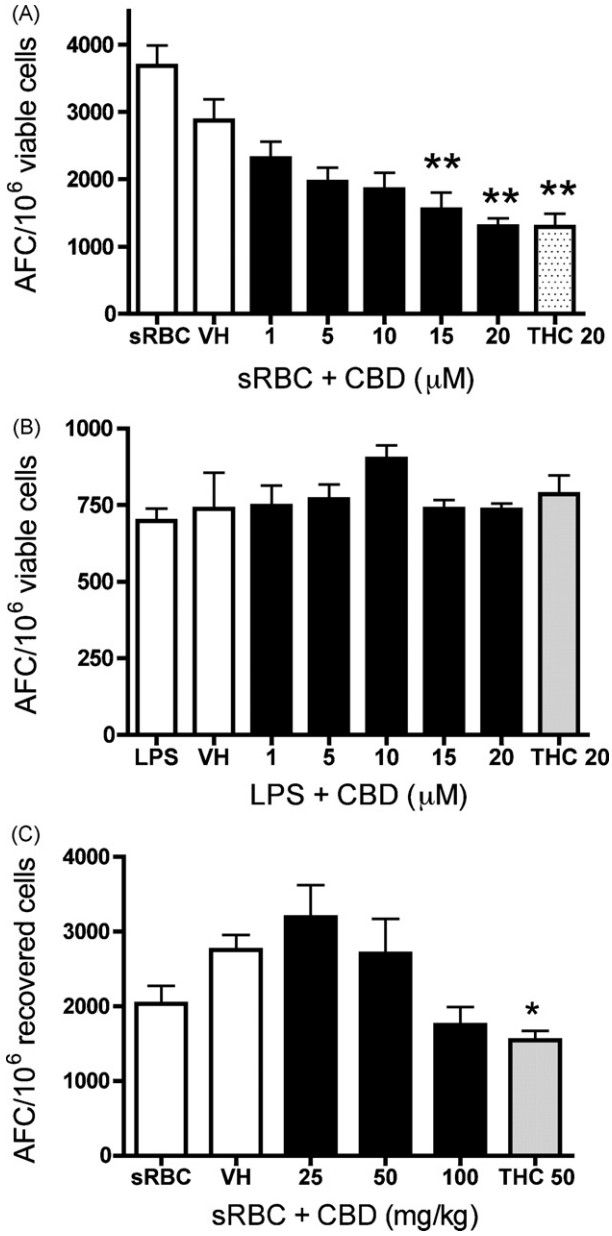

Fig. 6 – Effect of CBD on the IgM AFC response in B6C3F1 splenocytes. (A and B) Splenocytes (5×10^6 cells for sRBC; 2.5×10^6 cells for LPS) were treated with CBD (1–20 μM) or THC (20 μM) for 30 min, followed by stimulation with (A) sRBC for 5 days or (B) LPS for 72 h. Cells were then incubated in a Bellco stainless steel tissue culture chamber pressurized to 5.5 psi with a gas mixture consisting of 10% O_2, 7% CO_2 and 83% N_2. The culture chamber was placed at 37 °C with constant rocking for the duration of the culture period. Enumeration of the AFC response was performed as described in Section 2. The data are expressed as the mean AFC/10^6 viable cells ± S.E. of quadruplicate cultures. (C) B6C3F1 mice received CBD (25–100 mg/kg) or THC (50 mg/kg) by oral gavage for 3 days. On day 2, in addition to drug, mice received a single i.p. injection of sRBC (5×10^8 cells/mouse). Mice were sacrificed on day 6, after which the AFC response was enumerated as described in Section 2. The data are expressed as the mean AFC/10^6 viable or recovered cells ± S.E. Results are pooled from two separate experiments. * or ** denotes values that are significantly different from the vehicle control at $p < 0.05$ or 0.01.

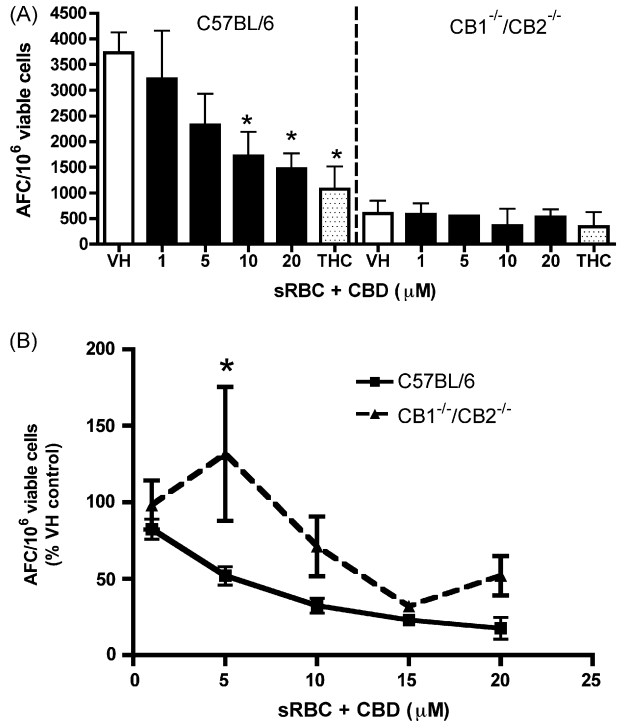

Fig. 7 – Effect of CBD on the *in vitro* AFC response in wild type C57BL/6 and CB1$^{-/-}$/CB2$^{-/-}$ splenocytes. Splenocytes (5×10^6 cells) from C57BL/6 or CB1$^{-/-}$/CB2$^{-/-}$ were treated with CBD (1–20 μM) or THC (20 μM) for 30 min, followed by stimulation with sRBC for 5 days. Cells were then cultured and the AFC were enumerated as stated in Fig. 1. The data are expressed as the mean AFC/10^6 viable cells ± S.E. of quadruplicate cultures for one representative experiment (A) or % VH results for CBD from four experiments are pooled, with the exception of the 15 μM group, which represents a single experiment (B). * or ** denotes values that are significantly different from the respective vehicle control at $p < 0.05$ or 0.01.

was the observation that this cytokine suppression occurred independently of either CB1 or CB2 as demonstrated in splenocytes derived from CB1$^{-/-}$/CB2$^{-/-}$ mice. The major advantages of evaluating immunological endpoints using CB1$^{-/-}$/CB2$^{-/-}$ mice, rather than the currently available CB1 and CB2 antagonists, are the absence of potential inverse agonism and/or direct effects of the antagonists, as has been reported [30,31]. Despite potential activity with antagonists under certain conditions, the antagonists have been used to suggest that CBD might exert some of its effects via CB1 and/or CB2 [15]. There is also recent evidence that CBD might exert its effects via a yet unidentified cannabinoid receptor or the newly identified putative cannabinoid receptor, GPR55 [32]. Furthermore, there are reports that CBD binds the vanilloid receptor, VR1 [33], is an agonist at the serotonergic 5HT-1a receptor [34,35], and is an agonist at A2a receptors in

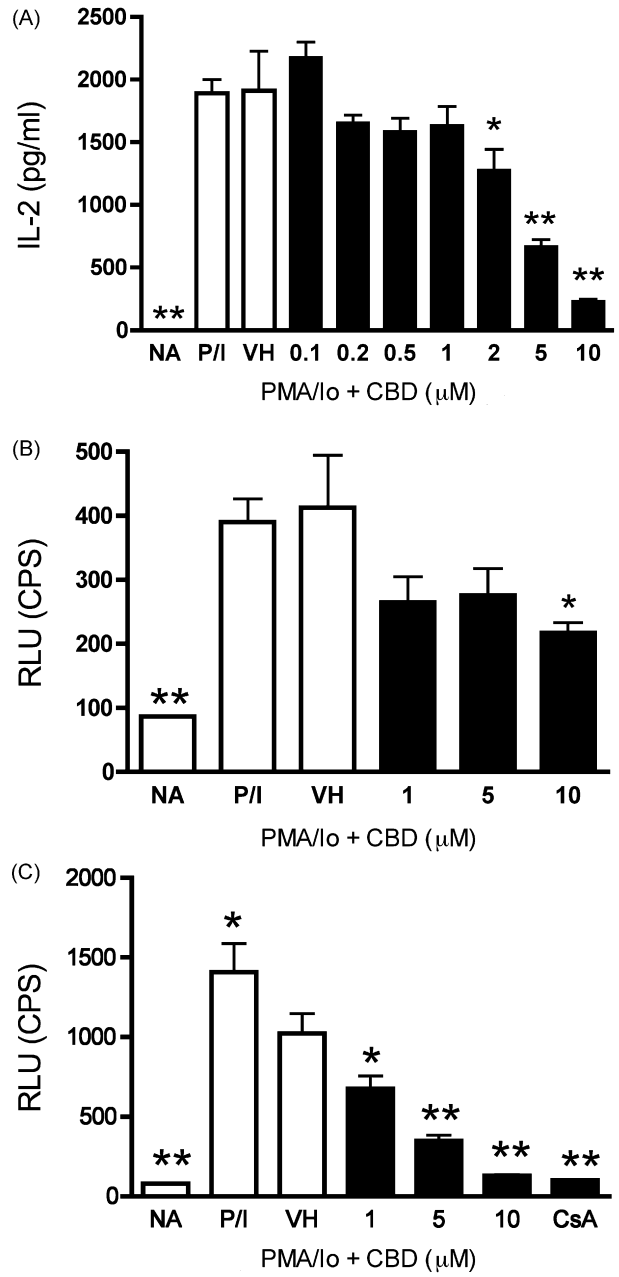

Fig. 8 – CBD suppressed IL-2 production and AP-1 and NFAT activity in PMA/Io-stimulated human Jurkat T cells. (A) Jurkat cells (5×10^4 cells) were treated with CBD (0.1–10 μM) for 30 min, followed by cellular activation with PMA/Io for 24 h. Supernatants were harvested and the amount of IL-2 was determined by ELISA. The data are expressed as the mean units/ml ± S.E. of triplicate cultures. (B and C) Jurkat cells (5×10^5 cells) were transiently transfected with either AP-1-luciferase (B) or NFAT-luciferase (C). Three hours later, cells were treated with CBD (1–10 μM) for 30 min, followed by cellular activation with PMA/Io for 21 h. Luciferase activity was determined as described in Section 2. The data are expressed as the mean CPS of triplicate cultures. * or ** denotes values that are significantly different from the vehicle control at $p < 0.05$ or 0.01. Results are representative of at least two separate experiments. NA, naïve (untreated); VH, vehicle (0.1% ethanol).

microglial cells [36]. In addition, Drysdale et al. demonstrated that the CBD-induced elevation in intracellular calcium in hippocampal cells was exacerbated in the presence of either a CB1 receptor antagonist or a VR1 receptor antagonist, suggesting signaling interactions between CBD and these receptors through an unknown mechanism [30]. Although we demonstrate that CBD-induced suppression of cytokine production from PMA/Io-stimulated splenocytes was independent of CB1 and CB2, we cannot exclude the possibility that CBD exerts its effects through one or more of the other aforementioned receptors at this time.

In addition to suppression of PMA/Io-stimulated IL-2 production, CBD suppressed IFN-γ production. The effect of CBD was more robust on IL-2 than IFN-γ from PMA/Io-stimulated splenocytes. However, in splenocytes that were stimulated through the T cell receptor using anti-CD3/anti-CD28, CBD exhibited similar potency on suppression of IL-2 and IFN-γ. The difference in sensitivity of CBD-induced suppression of IL-2 might be associated with the rapid elevation of intracellular calcium seen with PMA/Io, with PMA/Io-stimulated IL-2 being more sensitive to a rapid calcium rise than anti-CD3/anti-CD28-stimulated IL-2. This is supported by our previous observation that pretreatment of splenocytes with agents that elevate intracellular calcium resulted in suppression of PMA/Io-stimulated IL-2 [9], whereas the endogenous cannabinoid 2-arachidonoyl-glycerol (2-AG)-induced suppression of PMA/Io-stimulated IFN-γ could be partially reversed by increasing intracellular calcium [29]. The sensitivity of both IL-2 and IFN-γ, however, does suggest that there exists a common target of inhibition by CBD. One likely common target might be NFAT since NFAT is critical for both IL-2 and IFN-γ [37], and because NFAT DNA binding activity is markedly decreased in activated T cells when cultured in the presence of two other cannabinoids, CBN or 2-AG [24–26]. Indeed, CBD suppressed AP-1- and NFAT-luciferase gene expression. Although the CBD-induced suppression of NFAT-luciferase gene expression was more robust, it is notable that AP-1 proteins bind cooperatively with NFAT at many NFAT responsive elements in both IL-2 and IFN-γ promoters [21,23].

The demonstration that CBD suppresses NFAT activity is further supported by our observation that CBD suppressed cell surface expression of CD25, which is also regulated by NFAT [38]. Interestingly, this effect appeared to be T cell-specific. Although CBD suppressed cell surface expression of CD25 on the $CD3^+$ population, there was no effect on the $CD3^-$ (i.e., non-T cell, and presumably, mainly B cell) population. These results were also consistent with the observation that CBD suppressed the IgM AFC response to the T cell-dependent antigen, sRBC, but had no effect on the IgM AFC response to the polyclonal B cell activator, LPS. This is in contrast to our observation that CBD also suppressed LPS-induced proliferation, which has been classically identified as a B cell response. However, there is much evidence to suggest that T cells will proliferate in response to LPS and that T cells express the appropriate toll-like receptors important for this response [39,40]. Alternatively, these results suggest that the mechanism of immunosuppression by CBD, and likely other cannabinoids for which this dichotomy has been observed [18], might involve a generalized suppression of cellular prolifera-

tion to certain stimuli. Overall, the ability of CBD to suppress PMA/Io-, LPS-, anti-CD3/anti-CD28-induced proliferation, in combination with suppression of the MLR, provides more evidence that CBD targets T cells.

The profile with which CBD targeted T cell cytokine production and proliferation was very similar to that previously reported for two other plant-derived cannabinoid compounds, THC and CBN [18,19]. These results suggest that the mechanisms by which these three plant-derived cannabinoid compounds suppress cytokine production, at least *in vitro*, is similar and does not require CB1 or CB2. However, CBD only modestly suppressed the *in vivo* anti-sRBC IgM AFC response. These data support previous studies conducted in male CD-1 mice in which CBD (≤25 mg/kg) did not suppress the *in vivo* anti-sRBC IgM AFC response [41]. With the demonstration that CBD is efficacious *in vivo* in a variety of model systems [6,7,15,42], these results suggest that the AFC response *in vivo* is rather refractory to inhibition by CBD. One explanation for the absence of suppression of the *in vivo* AFC response is that CBD might be rapidly metabolized *in vivo* to a non-functional metabolite, particularly because CBD was administered orally. The discrepancy between CBD and THC *in vivo* versus *in vitro* does implicate the involvement of one or both cannabinoid receptors in the anti-sRBC IgM AFC response. Further evidence of the requirement for CB1 and/or CB2 in the *in vivo* anti-sRBC IgM AFC is provided by our observations that the THC-induced suppression of this response was abolished in $CB1^{-/-}/CB2^{-/-}$ mice (Springs et al., [43]), suggesting that cannabinoids must possess affinity for either CB1 or CB2 to suppress the AFC response *in vivo*.

We also attempted to discern the role of CB1 and CB2 in CBD-induced suppression of the *in vitro* anti-sRBC IgM AFC response using splenocytes derived from $CB1^{-/-}/CB2^{-/-}$ mice. Interestingly, the control anti-sRBC IgM AFC response by splenocytes isolated from $CB1^{-/-}/CB2^{-/-}$ mice was remarkably low as compared to that observed with splenocytes isolated from wild type C57BL/6 mice, which limited our ability to absolutely conclude whether CB1 and/or CB2 played a critical role in CBD-induced suppression of the *in vitro* anti-sRBC IgM AFC response. However, upon examination of the data as percent of vehicle control, it appears unlikely that CB1 and/or CB2 are involved. Although there was a difference between genotypes at lower concentrations of CBD, the response was highly variable, which is likely related to the degree to which the $CB1^{-/-}/CB2^{-/-}$ cells were stimulated *in vitro* with sRBC.

The above results also strongly suggest that CB1 and/or CB2 play a critical role in the *in vitro* anti-sRBC IgM AFC response. Currently, the reasons underlying the marked difference in the magnitude of the immune response to sRBC between splenocytes derived from $CB1^{-/-}/CB2^{-/-}$ and wild type C57BL/6 mice remain to be fully elucidated. Part of the mechanism might involve compromised accessory cell function (e.g. macrophages) in $CB1^{-/-}/CB2^{-/-}$ mice since there was no difference in the magnitude of the *in vitro* IgM AFC response by purified B cells activated with CD40 ligand-expressing L cells, a stimulation which does not require T cells or macrophages [43]. Notably, no difference in the magnitude of the *in vivo* anti-sRBC IgM AFC response between $CB1^{-/-}/CB2^{-/-}$ and wild type C57BL/6 mice was observed [43], suggesting the existence of additional compensatory mechanisms *in vivo*.

Overall, CBD did not significantly alter the anti-sRBC IgM AFC response *in vivo*, but CBD did suppress a number of immune responses *in vitro*, specifically involving T cells as primary effectors. Using splenocytes derived from $CB1^{-/-}/CB2^{-/-}$ mice, it was determined that CBD-induced suppression of cytokine production and suppression of the *in vitro* anti-sRBC IgM AFC response was CB1 and CB2 receptor-independent. More importantly, the observation of the significant difference in the control anti-sRBC IgM AFC response in $CB1^{-/-}/CB2^{-/-}$ mice as compared to wild type C57BL/6 mice suggests that CB1 and/or CB2 play a critical role in the magnitude of the response to sRBC *in vitro*. These data provide a detailed characterization of CBD's effects on various immunological endpoints and provide further evidence that the mechanisms by which cannabinoids modulate immune function is both cannabinoid receptor-dependent and -independent. Finally, these data demonstrate that transcription factors in the IL-2 promoter are sensitive targets of inhibition by CBD.

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