

Experimental Methods to Study the Role of the Peripheral Cannabinoid Receptor in Immune Function

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Summary

Marijuana components, such as Δ -9-tetrahydrocannabinol, and endogenous cannabinoids, such as anandamide and 2-arachidonoylglycerol, alter diverse immune functions. Two cannabinoid receptors have been discovered to date, the central cannabinoid receptor (CB₁R) and the peripheral cannabinoid receptor (CB₂R). The CB₁R is expressed predominantly in the central nervous system. The CB₂R is expressed mainly in cells of the immune system, suggesting that the CB₂R is involved in immunoregulatory events. Cannabinoids have been shown to modulate diverse immune functions including cytokine production, lymphocyte proliferation, and humoral and cell-mediated immune responses. In addition, cannabinoids have been shown to induce different signal transduction pathways. However, the role of cannabinoids and their receptors in the immune system remains unclear. The objective of the experimental methods described herein is to investigate the role of CB₂R activation in specific splenocyte and macrophage functions using a mouse lacking the CB₂R. Interestingly, our findings, thus far suggest that basal CB₂R activation modulates lymphocyte proliferation and cytokine secretion and macrophage phagocytic activity. Therefore, data obtained using the methodology described in this chapter will help us elucidate the role of cannabinoids and the CB₂R in the immune system.

Key Words: Cannabinoids; immune function; splenocytes; T cells; lymphocyte proliferation; macrophages; cytokines; mitogen-activated kinase; CB₂R knockout mouse.

1. Introduction

Cannabinoids are marijuana components known to modulate immune functions. Δ -9-tetrahydrocannabinol (Δ^9 -THC), the major psychoactive compound of marijuana, has been the most widely studied cannabinoid. However, synthetic cannabinoids and the recently discovered endogenous cannabinoids

arachidonoyl ethanolamine (anandamide) and 2-arachidonoyl glycerol are also known to have immunoregulatory effects. THC and other exogenous cannabinoids affect lymphocytes, macrophages, and natural killer cells in a variety of functions such as cellular proliferation, cytokine activity, and cell and humoral immunity (reviewed in refs. 1–13).

1.1. Cannabinoid Receptors

Although the observations described here have suggested a role for cannabinoids in immunomodulation, the mechanisms by which cannabinoids induce these immunomodulatory effects remain elusive. To date, two receptors with high affinity for cannabinoid ligands are known (14–16). The central cannabinoid receptor (CB₁R) was first cloned in 1990 by Matsuda et al. (14). This receptor is predominantly found in the central nervous system (CNS) (17–19) and to a much lesser degree in cells of the immune system (20–23). Because of its high expression in the CNS, the CB₁R is thought to mediate most, if not all, of the effects of the cannabinoids in the CNS. The peripheral cannabinoid receptor (CB₂R), cloned by Munro et al. in 1993 (15), is predominantly expressed in cells of the immune system (15,22,23). Therefore, the immunomodulatory effects of cannabinoids are thought to be mediated by the CB₂R. Human blood cell subpopulations have different degrees of CB₂R expression with the following rank order: B cells > natural killer cells > monocytes > neutrophils > T8 cells > T4 cells. CB₂R has been demonstrated in rat spleen, mouse spleen and thymus, and a number of immune-system-derived cell lines (20,22). Thus, several immunoregulatory effects of cannabinoids may be mediated by CB₂R present on immune cells.

1.2. Cannabinoid Receptors and Cell Signaling

Cannabinoid receptors are G protein-coupled receptors. Besides the modulation of adenylate cyclase (23–26), G proteins link cannabinoid receptors to the mitogen-activated protein kinase (MAPK) signaling cascade. Activation of the MAPK cascade is independent of regulation of adenylate cyclase (5,27–30). The CB₂R, specifically, is known to be coupled through a G_i. Thus, the CB₂R is a G protein-coupled receptor that is both positively and negatively coupled to MAPK and cAMP pathways, respectively, through a *Bordetella pertussis* toxin-sensitive G protein.

1.3. CB₂R Knockout Mouse

To investigate the role of the CB₂R in immunomodulation, we created a mouse deficient for the CB₂R using homologous recombination (CB₂R knockout or CB₂R^{-/-} mouse) (31). The CB₂R^{-/-} gene is encoded by a single exon. Upon homologous recombination, 341 bp from the coding region's 3' end was

replaced by the neomycin gene. This mouse model as well as cell lines derived from these mice have been successfully used to answer questions regarding the role of the CB₂R^{-/-} in the immune system (32). In addition, a CB₂R antagonist, SR144528 (33), has provided several investigators with a tool to study the role of the CB₂R in immune function (35). However, the CB₂R antagonist is also an inverse agonist (35–37). The CB₂R has a basal activity in the absence of ligand. This basal activity is reduced or blocked by SR144528 (37).

In our CB₂R^{-/-} mouse model, the CB₂R gene has been mutated to render the receptor incapable of binding cannabinoids and coupling to G proteins. Therefore, this animal model is unique in that it can provide us with different information from that obtained by using the CB₂R antagonist. The CB₂R^{-/-} mice have been backcrossed to c57BL/6 mice more than 12 times to ensure c57BL/6 genetic background strain. Therefore, the control (wild-type, CB₂R^{+/+}) mice are c57BL/6.

1.4. Conclusions

It is now known that the cannabinoid system includes at least two receptors and several endogenous ligands widely distributed throughout the body (16). Despite these findings, the role of the cannabinoid system in immune function remains unclear. It is very tempting to speculate that the cannabinoid system has a role in immunity due to the diverse effects of cannabinoids on immune functions and the reversion of some of these effects using cannabinoid receptor antagonists. However, we have yet to show direct evidence implicating cannabinoid receptor activation in immune events and specifically on immune cells.

The primary objective of the experimental methods described herein is to help us determine the role of CB₂R activation in cannabinoid-induced immune responses; specifically, the role of CB₂R activation on cell proliferation and cytokine production from lymphocytes, macrophage phagocytic activity, and putative signal transduction pathways mediating the immune responses to cannabinoids. To do this we use a mouse lacking a functional CB₂R receptor, the CB₂R knockout mouse (31).

2. Materials

2.1. Cell Cultures

2.1.1. Splenocyte Cell Cultures

1. Complete media: RPMI-1640 medium (stored at 4°C, wrapped in aluminum foil to protect it from light) supplemented with a 100 U/mL penicillin, 100 µg/mL streptomycin mixture, 4 mM L-glutamine and 7.5% heat-inactivated fetal calf serum (FCS) (all from BioSource International, Camarillo, CA). Allow the RPMI medium and the reagents to reach 37°C in a warm water bath before the reagents are added to the media. This complete media can be stored at 4°C for a maximum of 2 wk.

2. Concanavalin A (ConA): 5 mg powder stock of ConA lectin from *Canavalia ensiformis*, 5-mg powder vial (Sigma, St. Louis, MO). ConA was reconstituted in 1 mL RPMI medium (5 mg/mL stock), aliquotted into 50- μ L fractions, and stored at -20°C .
3. Sterile 70-m nylon mesh filter screen (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ).
4. Red blood cell lysis buffer (ACK lysing buffer from BioSource International, Camarillo, CA): 156 mM NH_4Cl , 10 mM KHCO_3 , and 1 mM Na_2EDTA .
5. Trypan blue: 0.4% stock concentration of trypan blue (Sigma, St. Louis, MO).
6. Sterile flat-bottom, tissue-culture grade 24-well plates (Corning Inc., Corning, NY).
7. Sterile flat-bottom, tissue-culture-grade 96-well microtiter plates (Corning Inc., Corning, NY).

2.1.2. T-Cell Cultures

1. RPMI-1640 medium: RPMI-1640 medium (BioSource International, Camarillo, CA) supplemented with 100 units/mL penicillin, 100 $\mu\text{g/mL}$ streptomycin, and 4 mM L-glutamine.
2. Complete media: RPMI-1640 medium (stored at 4°C , wrapped in aluminum foil to protect it from light) supplemented with a 100 U/mL penicillin, 100 $\mu\text{g/mL}$ streptomycin mixture, 4 mM L-glutamine and 7.5% heat-inactivated FCS (all from BioSource International, Camarillo, CA). Allow the RPMI medium and the reagents to reach 37°C in a warm water bath before the reagents are added to the media. This complete media can be stored at 4°C for a maximum of 2 wk.
3. ConA: 5 mg powder stock of ConA lectin from *Canavalia ensiformis*, 5 mg powder vial (Sigma, Saint Louis, MO). Con was reconstituted in 1 mL RPMI medium (5 mg/mL stock), aliquoted into 50- μ L fractions and stored at -20°C .
4. Plate-bound anti-CD3 antibody: Purified hamster anti-mouse CD3e (145-2C11, 0.5 mg/mL (BD Biosciences, San Diego, CA) is plated 5–0 $\mu\text{g/mL}$ in PBS. For proliferation assays, 50 μL are plated in 96-well plates. For cytokine assays, 250 μL are added to 24-well plates.
5. Sterile 70- μm nylon mesh filter screen (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ).
6. Red blood cell lysis buffer (ACK lysing buffer from BioSource International, Camarillo, CA): 156 mM NH_4Cl , 10 mM KHCO_3 , and 1 mM Na_2EDTA .
7. Phosphate-buffered saline (PBS): Prepare 10X stock with 1.37 M NaCl, 27 mM KCl, 100 mM Na_2HPO_4 , 18 mM KH_2PO_4 , adjust to pH 7.4 with HCl. Autoclave before storing at room temperature. Before using, dilute to 1X PBS.
8. Trypan blue: 0.4% stock concentration of trypan blue (Sigma, St. Louis, MO).
9. Sterile flat-bottom, tissue-culture-grade 24-well plates (Corning Inc., Corning, NY).
10. Sterile flat-bottom, tissue-culture-grade 96-well microtiter plates (Corning Inc., Corning, NY).
11. Pan T-Cell Isolation Kit (Miltenyibiotec, Auburn, CA).

- a. MidiMACS Separation Unit.
 - b. MACS MultiStand.
 - c. MACS High Gradient Magnetic Separation Columns of type LS.
 - d. Biotin-Antibody Cocktail.
 - e. Anti-Biotin MicroBeads.
12. Column buffer: PBS, pH 7.2 supplemented with 0.5% FBS and 2 mM EDTA. Degas by applying vacuum and maintain at 4–8°C.

2.1.3. Macrophage Cell Cultures

1. Thioglycolate broth: 3% thioglycolate broth (Becton Dickinson, Franklin Lakes, NJ) in sterile glass-distilled water. The bottle is placed in a boiling water bath to ensure complete dissolution of the powder. Autoclave the solution at 15 psi, 121°C for 15 min. Store at room temperature and use within 1 d.
2. Lavage medium: 0.95% NaCl in glass-distilled H₂O. Autoclave the solution at 15 psi, 121°C, 15 min, following dissolution.
3. Macrophage complete media: RPMI-1640 medium (stored at 4°C, wrapped in aluminum foil to protect it from light) supplemented with a 100 units/mL penicillin, 100 µg/mL streptomycin mixture, 4 mM L-glutamine and 5% heat-inactivated FCS (all from BioSource International, Camarillo, CA). Allow the RPMI medium and the reagents to reach 37°C in a warm water bath before the reagents are added to the media. This complete media can be stored at 4°C for a maximum of 2 wk.
4. Sterile 70-µm nylon mesh filter screen (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ).
5. Red blood cell lysis buffer (ACK lysing buffer from BioSource International, Camarillo, CA): 156 mM NH₄Cl, 10 mM KHCO₃, and 1 mM Na₂EDTA.
6. Trypan blue: 0.4% stock concentration of trypan blue (Sigma, St. Louis, MO).
7. Sterile flat-bottom, tissue-culture-grade 12-well plates (Corning Inc., Corning, NY).

2.2. Splenocyte and T-Cell Proliferation Assay

1. BrdU cell proliferation ELISA kit from Roche Molecular Biochemicals (Indianapolis, IN):
 - a. BrdU labeling reagent (1000X) (10 mM 5'-bromo-2'-deoxyuridine in PBS, pH 7.4) (provided in kit).
 - b. FixDenaturant (ready to use) (provided in kit).
 - c. Anti-BrdU-POD lyophilisate, stabilized. Monoclonal antibody from mouse-mouse hybrid cells (clone BMG 6H8, Fab fragments) conjugated with peroxidase (POD) (provided in kit).
 - d. Antibody dilution solution (ready to use) (provided in kit).
 - e. Washing buffer (10X) in PBS (provided in kit).
 - f. Substrate solution TMB (tetramethyl-benzidine) (provided in kit).
2. SpectraMax Plus Microplate Spectrophotometer plate reader (Molecular Devices, Sunnyvale, CA).

2.3. Cytokine Assays

2.3.1. Splenocyte Cytokine Assay

1. Bio-Plex cytokine assay kit (Bio-Rad Laboratories, Hercules, CA).
 - a. Anti-cytokine conjugated beads (25X), 2.5×10^6 beads/mL per cytokine (provided in kit).
 - b. Cytokine detection antibody 8-plex (50X) = 0.05 mg/mL per cytokine (provided in kit).
 - c. Cytokine standard (lyophilized), 25 ng per cytokine (provided in kit).
 - d. Bio-Plex assay buffer A (provided in kit).
 - e. Bio-Plex wash buffer A (provided in kit).
 - f. Bio-Plex detection antibody diluent A (provided in kit).
 - g. Streptavidin-PE (100X) (provided in kit).
 - h. Sterile filter plate (96-well) with cover, tray, and sealing tape (provided in kit).
2. Luminex 100 microplate platform (Luminex Corporation, Austin, TX).

2.3.2. T-Cell Cytokine Assays

1. Mouse interleukin (IL)-2 ELISA set from BD Biosciences (San Diego, CA).
 - a. Anti-mouse IL-2 monoclonal capture antibody (provided in kit).
 - b. Biotinylated anti-mouse IL-2 detection antibody (provided in kit).
 - c. Streptavidin-horseradish peroxidase conjugate enzyme reagent (provided in kit).
 - d. Lyophilized recombinant mouse IL-2 standards (provided in kit).
2. Mouse IL-4 ELISA set from BD Biosciences (San Diego, CA).
 - a. Anti-mouse IL-4 monoclonal capture antibody (provided in kit).
 - b. Biotinylated anti-mouse IL-4 detection antibody (provided in kit).
 - c. Streptavidin-horseradish peroxidase conjugate enzyme reagent (provided in kit).
 - d. Lyophilized recombinant mouse IL-4 standards (provided in kit).
3. Coating buffer: 0.1 M sodium carbonate, pH 9.5. 8.40 g NaHCO_3 , 3.56 g Na_2CO_3 , to 1.0 L; pH to 9.5. Freshly prepare or use within 7 d of preparation, stored at 2–8°C.
4. Assay diluent: PBS: 80.0 g NaCl, 11.6 g Na_2HPO_4 , 2.0 g KH_2PO_4 , 2.0 g KCl, to 10 L; pH to 7.0. with 10% FBS (Sigma, St. Louis, MO), pH 7.0. Freshly prepare or use within 3 d of preparation, with 2–8°C storage.
5. Wash buffer: PBS: 80.0 g NaCl, 11.6 g Na_2HPO_4 , 2.0 g KH_2PO_4 , 2.0 g KCl, to 10 L; pH to 7.0. with 0.05% Tween-20. Freshly prepare or use within 3 d of preparation, stored at 2–8°C.
6. Substrate solution: Tetramethylbenzidine (TMB) and hydrogen peroxide (BD Pharmingen™ TMB Substrate Reagent Set).
7. Stop solution: 2 N H_2SO_4 .

8. Immulon 2HB 96-well flat-bottom microtiter plates (Thermo Labsystems, Franklin, MA).
9. SpectraMax Plus Microplate Spectrophotometer plate reader (Molecular Devices, Sunnyvale, CA).

2.4. Phagocytosis Assay

2.4.1. Foreign Antigens

2.4.1.1. MICROSPHERES

1. Red-dyed polystyrene latex particles (0.8 μm) (Bangs Laboratories, Fishers, IN): 2.5% solids in 5% heat-inactivated FCS and incubated at 37°C for 30 min immediately prior to use. Incubation with FCS allows optimal opsonization.
2. Fluorescent microspheres: Yellow-green Fluoresbrite polystyrene latex particles (1 μm) (Polyscience, Inc., Warrington, PA): 1% solids in 5% heat-inactivated FCS and incubated at 37°C for 30 min immediately prior to use. Incubation with FCS allows optimal opsonization.

2.4.1.2. *ESCHERICHIA COLI*

1. Bacteria expressing a fluorescent marker: *E. coli* transformed and induced to express green fluorescent protein (GFP) using EDVO-kit no. 223 (Edvotek, Bethesda, MD) kit reagents.
2. Opsonization of bacteria: Incubate *E. coli* expressing GFP in 5% heat-inactivated FCS for 30 min at 37°C, immediately prior to use.

2.4.2. Cell Fixation and Permeabilization

1. Fixative: 4% formaldehyde in PBS containing 4% sucrose. Filter-sterilize and store at room temperature. For best results, the fixing medium should be prepared fresh or used within 1 wk of preparation.
2. Permeabilization solution: 0.25% Triton-X (BioRad Laboratories, Hercules, CA) in PBS. Filter-sterilize and store at room temperature. For best results, the fixing medium should be prepared fresh or used within 1 wk of preparation.

2.4.3. Microscopy

1. Mounting media: 13.3% polyvinyl alcohol (PVA, elvanol), 33% glycerol, and 0.5% 1,4-diazabicyclo-2,2,2-octane (DABCO) in 0.132 *M* Tris. Gradually dissolve 8 g PVA (J.T. Baker) in a beaker containing 30 mL 0.2 *M* Tris, pH 8.5 at 50°C, with stirring, over a 1- to 2-h period. Following complete dissolution of the PVA, turn off the heat and gradually add 20 mL glycerol in a thin stream over 5 min while stirring (see **Note 1**). In a separate container, dissolve 0.3 g DABCO (Sigma) in 10 mL 0.2 *M* Tris, pH 8.5. Once completely dissolved, add the DABCO solution to the PVA/glycerol stock. Cover the viscous solution with foil to protect it from ambient light and wrap with parafilm. Allow to stand overnight to remove bubbles. Finally, aliquot the solution and centrifuge in a microcentrifuge at 6000g for 20 min to

eliminate all bubbles. Immediately store at -20°C . Because of its viscosity, elvanol is kept in a 60°C heating block while in use.

2. Light microscope with 40X magnification capability to visualize the red-dyed microspheres.
3. Phase-contrast microscopy with a green fluorescent filter (Nikon Eclipse TE300 microscope): Fluorescent-dyed microspheres and GFP-expressing *E. coli* can be viewed under 20X or 40X magnification.
4. Metamorph Imaging Software, version 6.2r2 (Universal Imaging Corporation).
5. Microscope slides double frosted ($75\text{ mm} \times 25\text{ mm} \times 1.2\text{ mm}$) (Fisher, Pittsburgh, PA).
6. Round microscope cover glass (18 mm diameter) (Fisher, Pittsburgh, PA).

2.5. Mitogen-Activated Protein Kinase Assay

2.5.1. Cell Lysis and Treatment

1. RPMI-1640 medium: RPMI-1640 medium (BioSource International, Camarillo, CA).
2. Red blood cell lysis buffer (ACK lysing buffer from BioSource International, Camarillo, CA): $156\text{ mM NH}_4\text{Cl}$, 10 mM KHCO_3 , and $1\text{ mM Na}_2\text{EDTA}$.
3. Sterile $70\text{-}\mu\text{m}$ nylon mesh filter screen (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ).
4. Sterile flat-bottom, tissue-culture-grade 12-well plates (Corning Inc., Corning, NY).
5. Lipopolysaccharide (LPS): LPS derived from *E. coli* 055:B55 (Sigma, St. Louis, MO), 1 mg/ml in complete media. Special precaution should be taken when handling LPS as it is highly pyrogenic. Stored at -20°C .
6. Myelin basic protein (Sigma, St. Louis, MO) is dissolved in deionized water at a concentration of 1 mg/mL and stored in aliquots at -70°C .
7. Cell lysis buffer and Mg^{2+} /ATP cocktail: 20 mM HEPES-NaOH at pH 7.2, 1% (v/v) Triton X-100, 10% glycerol (v/v), 1 mM [ethylene-bis-(oxyethylenenitrilo)]-tetraacetic acid (EGTA), 50 mM sodium fluoride, 75 mM magnesium chloride, 1 mM sodium orthovanadate, $100\text{ }\mu\text{g/mL}$ leupeptin, 1 mM dithiothreitol (DTT), 0.05 mM phenyl methylsulfonyl fluoride (PMSF), 0.5 mM adenosine triphosphate (ATP). Store at room temperature (see **Note 2**).

2.5.2. Enzyme-Linked Immunosorbent Assay (ELISA)

1. Blocking buffer (10X): 10X PBS , 20% bovine serum albumin (BSA) (w/v), 0.1% Tween-20 (v/v), 0.02% sodium azide. Store at 4°C .
2. Dilution buffer (5X): 100 mM HEPES-NaOH at pH 7.2, $125\text{ mM } \beta\text{-glycerophosphate}$, 25 mM EGTA , 5 mM sodium orthovanadate, 5 mM DTT . Store at 4°C .
3. Wash buffer (10X): 10X PBS , 0.5% Tween-20 (v/v). Store at room temperature.
4. Capture and primary antibody: Mouse anti-human phosphorylated myelin basic protein (MBP) (US Biological, MA) (see **Note 3**).
5. Detection antibody: Affinity-purified sheep anti-mouse IgG conjugated to alkaline phosphatase (Sigma, St. Louis, MO).

6. Substrate for alkaline phosphatase: Alkaline phosphatase yellow *p*-nitro-phenylphosphate (pNPP) substrate system for ELISA (Sigma, St. Louis, MO). Stored at 4°C (see **Note 4**).
7. Custom-made peptide, standard: MBP phosphorylated at Thr98, FFKNIVTPRpTPPPSQGK, (City of Hope, Duarte, CA) is dissolved in PBS at a concentration of 1 mg/mL with 0.02% sodium azide (w/v) and stored at 4°C. Lyophilized peptide is stored at -70°C (see **Note 5**).
8. Immulon 2HB 96-well flat-bottom microtiter plates (Thermo Labsystems, Franklin, MA).

2.6. Cannabinoids

1. (-)-*trans*- Δ^9 -Tetrahydrocannabinol (THC) (NIDA-Research Triangle Institute) 5 mg/mL in absolute ethanol, MW 314.45. Prepare THC aliquots in silica-coated vials. Purge vials containing THC with N₂ gas and store in a locked freezer at -20°C. Do not use the cannabinoid solutions if there is suspicion of oxidation. After aliquotting, use within 6 mo. Working solutions are prepared by dilution in absolute ethanol.
2. *R*-(+)-[2,3-Dihydro-5-methyl-3-(4-morpholynylmethyl)pyrrolo[1,2,3-DE]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone mesylate (WIN55,212-2) (Tocris, Ellisville, MO) 10 mM in absolute ethanol, MW 531.62. Prepare WIN55,212-2 aliquots in silica-coated vials. Purge vials containing WIN55,212-2 with N₂ gas and store at -70°C. Do not use the cannabinoid solutions if there is suspicion of oxidation. After aliquotting, use within 6 mo. Working solutions are prepared by dilution in absolute ethanol.
3. 2-Arachidonyl ethanolamide (2-AG) (NIDA-Research Triangle Institute) 26.4 mM in absolute ethanol, MW 378.5. Prepare 2-AG aliquots in silica-coated vials. Purge vials containing 2-AG with N₂ gas and store at -70°C. Do not use the cannabinoid solutions if there is suspicion of oxidation. After aliquotting, use within 6 mo. Working solutions are prepared by dilution in absolute ethanol.

3. Methods

3.1. Cell Cultures: Splenocyte Cell Cultures

1. First, sacrifice the mice by CO₂ asphyxiation. Working in a sterile hood, cut into the animal's skin below the front legs, and pull away the skin to expose the peritoneum and the underlying internal organs. Make a sterile incision into the peritoneal membrane on the animal's left side. Then remove the spleen with forceps. Pool together three spleens from CB₂R^{+/+} and CB₂R^{-/-} mice groups and process them for primary splenocyte culture preparations.
2. Transfer the three spleens to a sterile 70- μ m nylon mesh filter screen covering a 50-mL conical centrifuge tube containing 5 mL of complete media. Release the splenocytes from the spleens by gently massaging the spleens with the end of a 3-cc tuberculin syringe.

3. Carefully rinse the cell suspension in the filter screen with 15 mL of complete media to harvest all the spleen cells.
4. Centrifuge the cell suspension at 150g for 10 min at 15–18°C (CRU-500 Centrifuge, International Equipment Co., Neeham Heights, MA).
5. Discard the supernatant and add 5 mL of ACK lysing buffer to the cell pellet to lyse all red blood cells. Swirl the solution gently and place the tube on ice for 5 min with occasional swirling.
6. At the end of the 5 min, add 10 mL of complete media to the solution to stop cell lysis and centrifuge the cell suspension again at 150g for 10 min.
7. Discard the supernatant and add 20 mL of complete media to the cell pellet to remove any remaining lysis buffer. Gently resuspend the cells thoroughly.
8. To perform a cell count, a 1:20 dilution of the cell solution is needed. This is done by making a 1:10 dilution with complete media and then a 1:2 dilution with 0.4% stock concentration of trypan blue.
9. Count the cells using a hemacytometer. Then centrifuge the cell suspension at 150g for 5 min. Resuspend the cell pellet in 5 mL of serum-free media and dilute to 2×10^6 cells/mL in complete media.
10. For proliferation studies, plate the cells in complete media at 2×10^5 cells/0.1 mL/well into sterile flat-bottom, tissue-culture-grade 96-well microtiter plates. For cytokine studies, plate the cells in complete media at 2×10^6 cells/mL/well into sterile flat-bottom, tissue-culture-grade 24-well plates.
11. Treat specified wells with various final concentrations of the cannabinoid agonists (0, 0.01 μ M, 0.1 μ M, 1 μ M, 5 μ M). Using a repeater pipetor, treat the 24-well plates with 10- μ L aliquots of the cannabinoid reagents (or 10 μ L absolute EtOH as the non-cannabinoid-treated control), and treat the 96-well plates with 1 μ L of the cannabinoid reagents (or 1 μ L absolute EtOH as the non-cannabinoid-treated control). Set up the wells in the 24-well plates in triplicates. Set up the wells in the 96-well plates in quadruplicates.
12. After cannabinoid treatment, incubate the plates for 1 h in a humidified 5% CO₂ incubator at 37°C. After 1 h of incubation, treat specified wells with the T-cell mitogen ConA at a final concentration of 2.5 μ g/mL. Treat each well of the 24-well plates with 10 μ L of ConA (or 10 μ L RPMI as the non-ConA-treated control). And treat every well of the 96-well plates with 1 μ L of ConA (or 1 μ L RPMI as the non-ConA-treated control).
13. Incubate the plates at 37°C in a humidified 5% CO₂ incubator for 72 h.

3.1.2. T-Cell Cultures

1. After obtaining the CB₂R^{+/+} and CB₂R^{-/-} transfer the spleens to a sterile 70- μ m nylon mesh filter screen covering a 50-mL conical centrifuge tube containing 5 mL of RPMI medium. Release the splenocytes from the spleens by gently pressing the spleens with the end of a 3 cc tuberculin syringe.
2. Carefully rinse the cell suspension in the filter screen with 15 mL of serum-free media to harvest all the spleen cells.

3. Perform a cell count using a hemacytometer by making a 1:10 dilution of the splenocyte suspension with serum-free media and then a 1:2 dilution with 0.4% stock concentration of trypan blue.
4. Transfer 5×10^8 total cells to a fresh 50-mL conical centrifuge tube.
5. Centrifuge the cell suspension at 300g for 10 min at 4–8°C.
6. Pipette off supernatant completely and resuspend cell pellet in 2 mL of column buffer.
7. Add 500 μ L of biotin-antibody cocktail and incubate for 10 min at 4–8°C.
8. Add 1.5 mL of buffer and 1000 μ L of anti-biotin microBeads and incubate for an additional 15 min at 4–8°C.
9. Wash cells with 10 mL of column buffer and centrifuge at 300g for 10 min.
10. Pipette off supernatant completely and resuspend cell pellet in 2500 μ L of column buffer.
11. Place an LS column in the magnetic field of a suitable MACS Separator.
12. Prepare column by rinsing with 3 mL of column buffer.
13. Apply cell suspension onto the column. Allow the cells to pass through and collect effluent as fraction with unlabeled cells, representing the enriched T-cell fraction.
14. Wash the column with 12 mL of column buffer. Collect entire effluent in the same tube as effluent of previous step.
15. Perform a cell count by making a 1:10 dilution of the T-cell suspension with serum-free media and then a 1:2 dilution with 0.4% stock concentration of trypan blue.
16. Count the cells using a hemacytometer and determine the total number of T cells.
17. Centrifuge the cell suspension at 300g for 10 min at 4–8°C.
18. Resuspend pellet in a suitable volume of warm complete media to obtain a 2×10^6 cell/mL T-cell suspension.
19. Prepare a 5–10 μ g/mL solution of anti-CD3 in sterile PBS.
20. Dispense 50 μ L of the antibody solution in each well of the sterile flat-bottom, tissue-culture grade 96-well microtiter plates or 250 μ L in each well of the sterile flat-bottom, tissue-culture-grade 24-well plates.
21. For the control unstimulated wells, add sterile PBS.
22. Tightly cover the plate with Parafilm to avoid sample evaporation and incubate at 4°C overnight.
23. Just before adding cells, remove the antibody solution with a multichannel pipettor.
24. Rinse each well two times with sterile PBS and discard PBS.
25. For proliferation studies, plate the cells in complete media at 2×10^5 cells/0.1 mL/well into sterile flat-bottom, tissue-culture-grade 96-well microtiter plates. For anti-CD3 stimulation, plate the cells in complete media at 2×10^5 cells/0.1 mL/well into the anti-CD3 prebound plates. For cytokine studies, plate the cells in complete media at 2×10^6 cells/mL/well into sterile flat-bottom, tissue-culture-grade 24-well plates for ConA stimulation. For anti-CD3 stimulation, plate the cells in complete media at 2×10^6 cells/mL/well into the anti-CD3 prebound plates.
26. Treat specified wells with various final concentrations of cannabinoids (0 μ M, 0.01 μ M, 0.1 μ M, 1 μ M). Using a repeater pipettor, treat the 24-well plates with 10- μ L

aliquots of the cannabinoid reagents (or 10 μ L absolute EtOH as the non-cannabinoid-treated control), and treat the 96-well plates with 1 μ L of the cannabinoid reagents (or 1 μ L absolute EtOH as the non-cannabinoid-treated control). Set up the wells in the 24-well plates in triplicates. Set up the wells in the 96-well plates in quadruplicates.

27. After cannabinoid treatment, incubate the plates for 1 h (in the case of ConA stimulation) in a humidified 5% CO₂ incubator at 37°C. After 1 h of incubation, treat specified wells with ConA to a final concentration of 2.5 μ g/mL. Treat each well of the 24-well plates with 10 μ L of ConA (or 10 μ L RPMI as the non-ConA treated control). And treat every well of the 96-well plates with 1 μ L of ConA (or 1 μ L RPMI as the non-ConA-treated control).
28. Incubate the plates at 37°C in a humidified 5% CO₂ incubator 24, 48, and 72 h.

3.1.3. Macrophage Cell Cultures

1. A 3% thioglycolate broth is prepared by dissolving 1.5 g Bacto-Thioglycollate dehydrated medium (Becton Dickinson, Franklin Lakes, NJ) in a 100-mL sterile glass bottle containing 50 mL of deionized water. The bottle is placed in a boiling water bath to ensure complete dissolution of the powder. Autoclave the solution at 15 psi, 121°C, for 15 min. Allow the thioglycolate broth to cool to 37°C. Upon cooling, inject 3 mL of the broth intraperitoneally using a 5 mL syringe and 27-gauge needle. Injections should be administered in the lower left section of abdominal area to avoid intestinal or internal organ puncture.
2. Four days following injection, sacrifice the animals via CO₂ asphyxiation. Peritoneal macrophages are obtained by peritoneal lavage with 9 mL of cold, sterile 0.95% NaCl. For experimental purposes, put the solution on ice in order to minimize macrophage activity prior to culturing. To do the lavage, make a slight cut in the abdominal area and pull back the fur to expose the peritoneum. Inject the cold lavage medium with a 20-gage needle at the fatty spot on the left side of the animal. Massage the inflated abdominal area and incubate for 5 min to obtain a maximum yield of macrophages. Place the animal on a 50-cc centrifuge tube and use a 20-gage needle to pierce the right ventral lateral section of the peritoneum, allowing the fluid to drain directly into the tube. Pool macrophages from CB₂R^{+/+} and CB₂R^{-/-} genotypes in order to obtain higher cell counts.
3. Pooled cells are then filtered through a 70- μ m nylon mesh filter screen to remove chunks of tissue and cell debris. Gently centrifuge for 5 min at 327g and 4°C. Pour off the supernatant and lyse the red blood cells for 1 min with 1 mL ACK lysing buffer. After 1 min add 19 mL of PBS to stop the lysis of red blood cells and to maintain macrophage integrity. Pellet cells for 5 min at 327g and 4°C. Pour off the supernatant and resuspend in complete medium.
4. Determine cell viability using trypan blue exclusion dye.
5. Prior to plating the thioglycolate-elicited macrophages, round cover slips are sterilized with ethanol and flamed. Subsequently, the cover slips are added to the bottom of Costar 12-well cluster microplates (Corning, Inc., Acton, MA).

Macrophages are then plated, 1 mL per well, at $0.5\text{--}1 \times 10^6$ cells per well and allowed to adhere to the topside of the cover slips for 1–2 h.

6. Adherent cells are then rinsed with 1 mL of complete medium for enrichment. Cannabinoids (0, 0.01 μM , 0.1 μM , 1 μM) are then added to plated macrophages for 1 h.

3.2. Splenocyte and T-Cell Proliferation Assays

1. The protocol described in the BrdU cell proliferation ELISA kit is used to assess cell proliferation.
2. Forty-eight hours after the cells were stimulated with ConA or plate-bound anti-CD3 antibody, treat each well in the 96-well microtiter plates with 10 μL of bromodeoxyuridine (BrdU)-labeled stock solution. Return the plates to the same 5% CO_2 incubator as described in the kit. After incubating for 24 h more, remove the 96-well microtiter plates containing the BrdU-labeled cells from the incubator. Centrifuge the plates in a plate centrifuge (Heraeus Model no. Megafuge 2.0 R table-top centrifuge) at 300g for 10 min to pellet the cells.
3. Remove the supernatant by suction using a sterile cannula and dry the plates containing the cell pellets in a 60°C oven for 30 min. Proceed as the kit protocol describes to fix the cells, detect the denatured BrdU containing DNA with anti-BrdU-peroxidase antibody, and visualize the BrdU incorporation by the colorimetric reaction. The colorimetric reaction is stopped by adding 25 μL of 1 M H_2SO_4 to each well. Gently tap the plates to allow the H_2SO_4 to mix thoroughly with the contents of the wells.
4. Within 5 min of adding the stop solution, quantify the colorimetric reaction product by measuring the absorbance of the samples at 450 nm (reference wavelength 690 nm) using the SpectraMax Plus Microplate Spectrophotometer plate reader (Molecular Devices, Sunnyvale, CA). The developing color and the corresponding absorbance values directly correlate to the amount of DNA synthesis, which is an indicator of the number of proliferating cells in the cell culture samples.
5. As seen in **Fig. 1**, cell proliferation is enhanced in the presence of THC (10–100 nM) in $\text{CB}_2\text{R}^{+/+}$ cells, but not in $\text{CB}_2\text{R}^{-/-}$ cells. Interestingly, cell proliferation in $\text{CB}_2\text{R}^{-/-}$ cells is greater than in $\text{CB}_2\text{R}^{+/+}$ cells (**Fig. 1**). Effects similar to those of THC were observed with WIN55,212-2 and 2-AG on splenocyte proliferation (data not shown).

3.3. Cytokine Assays

3.3.1. Splenocyte Cytokine Assay

3.3.1.1. BIORAD ASSAYS

1. Seventy-two hours postculture, remove the 24-well plates from the incubator and collect 120 μL of supernatant from each well and place into a sterile, flat-bottom, non-tissue-culture-treated 96-well microtiter plate. Store the plate containing the supernatant in the -70°C Revco freezer until it is assayed for specific cytokines using the Bio-Plex cytokine assay as indicated in the kit. The protocol included in

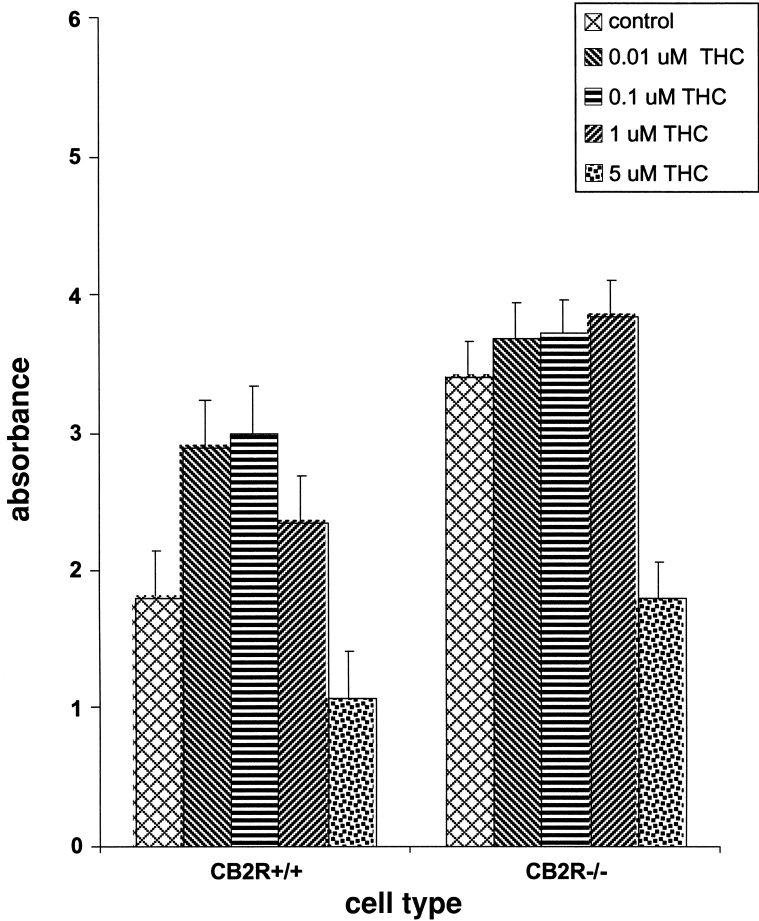


Fig. 1. Δ^9 -THC enhances CB₂R^{+/+} splenocyte proliferation, but not CB₂R^{-/-} splenocyte proliferation. Splenocyte proliferation was determined by BrdU incorporation assay as described in the text. Cells were plated at 2×10^5 cells/0.1 mL/well in complete media. Cells were treated with 2.5 μ g/mL ConA and the indicated Δ^9 -THC concentration for 72 h. Data are expressed as the mean of quadruplicate samples \pm standard deviation. Vehicle is absolute ethanol.

the kit is followed to assess the concentration of IL-1 β , IL-2, IL-4, IL-5, IL-10, granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon (IFN)- γ and tumor necrosis factor (TNF)- α in the supernatant.

2. The Luminex 100 microplate reader (Luminex Corporation, Austin, TX) is used to determine cytokine concentration using the Bio-Plex cytokine kit.

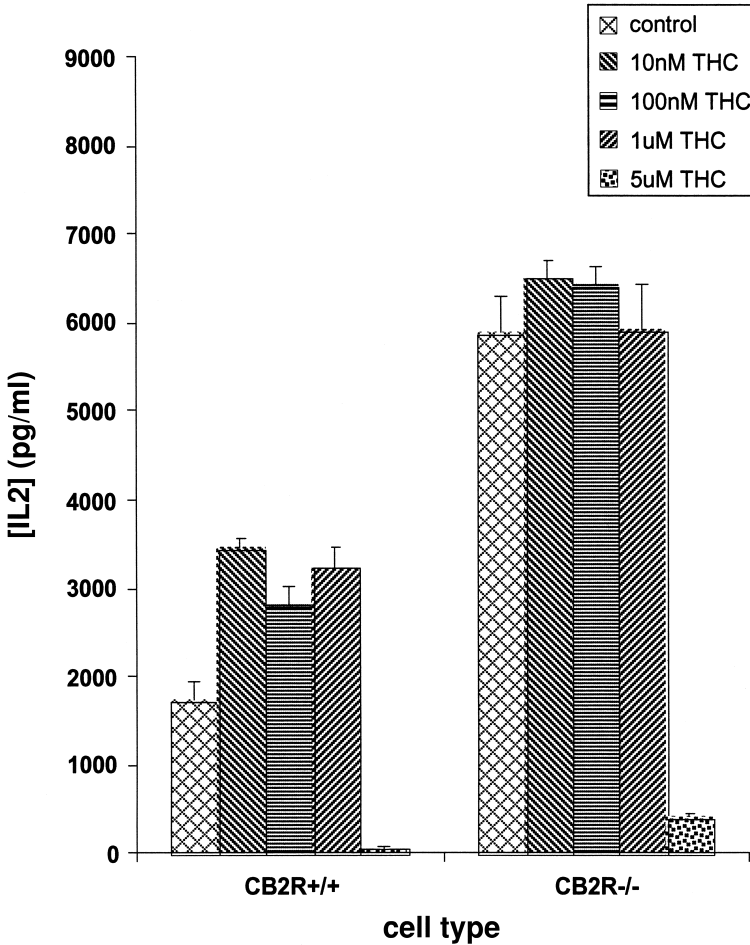


Fig. 2. Δ^9 -THC enhances CB₂R^{+/+} splenocyte IL-2 cytokine secretion, but not CB₂R^{-/-} splenocyte IL-2 secretion. Splenocyte proliferation was determined by BrdU incorporation assay as described in the text. Cells were plated at 2×10^5 cells/0.1 mL/well in complete media. Cells were treated with 2.5 μ g/mL ConA and the indicated Δ^9 -THC concentration for 72 h. Data are experiments and are expressed as the mean of quadruplicate samples \pm standard deviation. Vehicle is absolute ethanol.

3. As seen in **Fig. 2**, the CB₂R mediates THC-induced splenocyte IL-2 cytokine secretion, because this effect is not seen in the CB₂R^{-/-} cells. Perhaps more interesting is the finding that in CB₂R^{-/-} splenocytes IL-2 secretions are enhanced over those of CB₂R^{+/+} cells (**Fig. 2**). Effects similar to those of THC were observed with WIN55,212-2 and 2-AG on splenocyte cytokine secretion (data not shown).

3.3.2. T-CELL CYTOKINE ASSAYS

1. Twenty-four, 48, and 72 h after the cells are stimulated with ConA or plate-bound anti-CD3 antibody, remove 225 μ L of supernatant from each well and place into a sterile, flat-bottom, non-tissue-culture-treated 96-well microtiter plate. Store the plate containing the supernatant in the -70°C Revco freezer until it is assayed for specific cytokines using IL-2 and IL-4 BD Bioscience kits. The protocol included in the kit is followed to assess the concentration of IL-2 or IL-4 in the supernatant.
2. Quantify the colorimetric reaction product by measuring the absorbance of the samples at 450 nm (reference wavelength 570 nm) using the SpectraMax Plus Microplate Spectrophotometer plate reader (Molecular Devices, Sunnyvale, CA).
3. As seen in **Fig. 3**, the CB₂R mediates WIN55,212-2-induced IL-2 cytokine secretion, because this effect is not seen in the CB₂R^{-/-} cells. Furthermore, in CB₂R^{-/-} T-cell IL-2 secretions are enhanced over those of CB₂R^{+/+} cells (**Fig. 3**).

3.4. Phagocytosis Assay

Phagocytosis is one of the first lines of defense against invading microorganisms. Macrophages play a central role in this process as a host defends against intruding foreign bodies. This phagocytosis assay was developed in order to further elucidate the level at which macrophage function is compromised, if at all, in the presence of cannabinoids.

3.4.1. Macrophage Treatment With Foreign Antigen

1. Thioglycolate-elicited peritoneal macrophages from CB₂R^{+/+} or CB₂R^{-/-} are plated and treated with cannabinoids as indicated in **Subheading 3.1.3**. After incubating with cannabinoids for 1 h, in order to reduce cannabinoid interference, media is removed and 1 mL of fresh complete media is added prior to the addition of the foreign particles. Ten to 25 μ L of opsonized microspheres or 100:1 bacteria to macrophage are added and allowed to incubate for 1 h at 37°C .
2. Following the 1-h incubation, decant the complete medium to remove non-phagocytized particles. Phagocytosis is stopped by applying several rinses of 0.5-mL cold PBS to each well.
3. Next, fix macrophages onto the coverslips with 0.25 mL warm fixing medium and incubate for 15 min at 55°C . Rinse cells several times with 0.5 mL warm PBS.
4. Permeabilize macrophages with 0.25 mL of warm permeabilization medium for 10 min at 37°C . Wash cells two times with 0.5 mL of warm PBS, with a 5-min incubation step between washes, to ensure complete elimination of the detergent, Triton X-100. Do a final wash with 0.25 mL of sterile distilled water in order to remove all the salts prior to mounting.
5. To mount the cover slips, one drop of warm mounting medium is dropped onto a clean slide. Immediately pry the cover slip from the bottom of the well using an 18-gage needle and sterile tweezers. Flip the cover slip so that the adherent macrophages are preserved and mounted directly on top of the mounting medium.

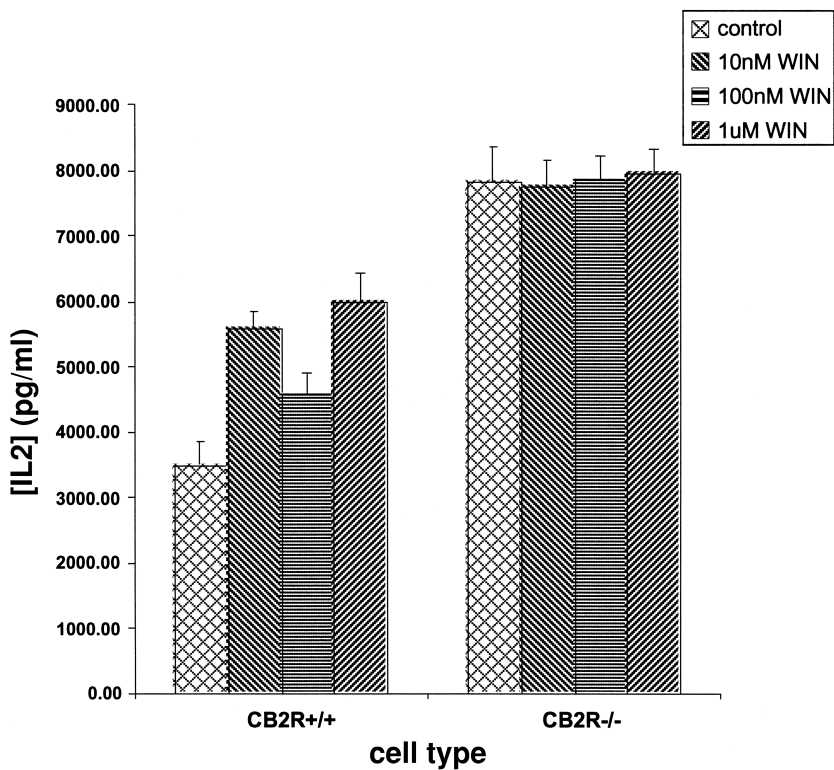


Fig. 3. WIN55-212,2 enhances CB₂R^{+/+} T cell IL-2 secretion, but not CB₂R^{-/-} T cell IL-2 secretion. IL-2 secretion was determined using the IL-2 kit from BD Biosciences as indicated in **Subheading 3**. Cells were plated at 2×10^5 cells/0.1 mL/well in complete media. Cells were treated with 2.5 μ g/mL ConA and the indicated Δ^9 -THC concentration for 72 h. Data are expressed as the mean of quadruplicate samples \pm standard deviation. Vehicle is absolute ethanol.

Finally, secure the cover slips with nail polish and allowed to dry overnight prior to viewing under the microscope.

3.4.2. Microscopic Analysis

1. The red-dyed latex beads are viewed with light microscopy under 40X magnification. The percent phagocytosis is determined by comparing the number of phagocytosing macrophages vs total macrophages present in the field. Macrophages with 5 or more red-dyed microspheres are considered positive for phagocytosis. A total of 200 macrophages are counted per mount.
2. Phagocytosis of microspheres and bacteria is determined using phase-contrast microscopy with a green fluorescent filter. Fluorescent-dyed microspheres and

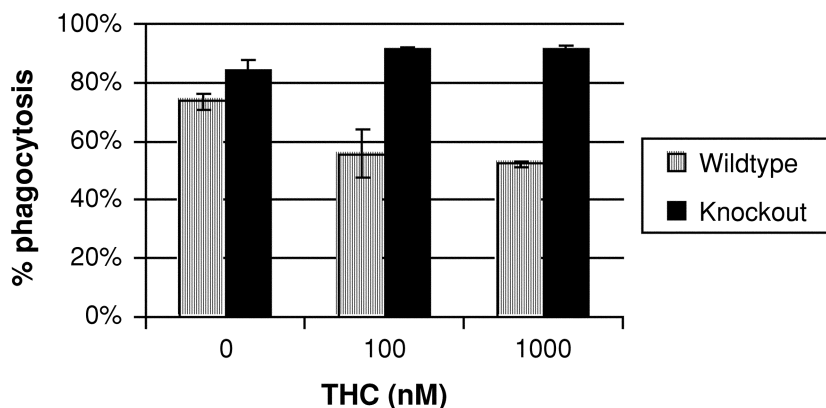


Fig. 4. Δ^9 -THC inhibits phagocytosis by $CB_2R^{+/+}$ macrophages, but not of $CB_2R^{-/-}$ macrophages. Thioglycolate-induced peritoneal macrophages were plated at a density of $0.5\text{--}1 \times 10^6$ cells/well as indicated in text and treated with the indicated concentrations of THC or 1% ethanol as vehicle control for 1 h. After 1 h, the cells were challenged with opsonized red-dyed microspheres. The cells were then processed for light microscopy as indicated in the text. Phagocytic activity, expressed at % phagocytosis, was determined by counting cells that had engulfed five or more red-dyed microspheres out of a field of 200 cells. Data are representative of three experiments and are expressed as the mean of triplicate samples \pm standard deviation.

GFP-expressing *E. coli* can be viewed under 20X or 40X magnification using a Nikon Eclipse TE300 microscope.

3. A field of interest is first visualized and photographed with the fluorescent filter. The same field is then photographed with the fluorescent filter. Resulting photographs are digitally overlaid using Metamorph Imaging Software, version 6.2r2 (Universal Imaging Corporation). An acceptable threshold of fluorescence inside and outside of the cell is then established using Metamorph. Based on the established parameters, quantification of phagocytosis is possible.
4. As seen in **Fig. 4**, THC inhibits phagocytic activity of thioglycolate-induced peritoneal $CB_2R^{+/+}$ macrophages, but not of $CB_2R^{-/-}$ macrophages, suggesting that THC is, at least partially, acting through the CB_2R . Similar results are obtained when using WIN55-212-2 (data not shown).

3.5. Mitogen-Activated Protein Kinase Assay

3.5.1. Cell Lysis and Treatment

1. After obtaining the $CB_2R^{+/+}$ and $CB_2R^{-/-}$ spleens, transfer the spleens to a sterile 70 μ m nylon mesh filter screen covering a 50-mL conical centrifuge tube containing 5 mL of RPMI medium. Release the splenocytes from the spleens by gently pressing the spleens with the end of a 3-cc tuberculin syringe.

2. Carefully rinse the cell suspension in the filter screen with 15 mL of RPMI medium to harvest all the spleen cells.
3. Perform a cell count using a hemacytometer by making a 1:10 dilution of the splenocyte suspension with serum-free media and then a 1:2 dilution with 0.4% stock concentration of trypan blue.
4. Resuspend cells at a starting working concentration of 0.9×10^7 cells/mL. Further serially dilute into 0.6×10^7 cells/mL and 0.3×10^7 cells/mL. Plate two sets, in a total volume of 500 μ L, into a 24-well plate in triplicates.
5. Add 20 μ g/mL LPS, in the presence or absence of cannabinoids (0, 0.01 μ M, 0.1 μ M, 1 μ M), for 4 min at 37°C. LPS stimulates the MAPK pathway in B cells.
6. Lyse splenocytes by adding 500 μ L of cell lysis buffer and Mg^{2+} /ATP cocktail plus 4 μ g MBP and incubating for 20 min at 37°C with agitation.
7. Add 200 μ L of cell lysate to mouse anti-human phosphorylated MBP antibody-coated 96-well microtiter plates (*see below*) at 4°C, overnight.

3.5.2. Enzyme-Linked Immunosorbent Assay

1. In triplicates, bind 100 ng/well, at a final volume of 100 μ L, mouse anti-human phosphorylated MBP to a 96-well microtiter plate at 4°C, overnight. Include wells for 5 standards in triplicates and an extra triplicate set for the detection of background signal.
2. Using an immuno-wash, wash twice with 1X wash buffer.
3. Add 100 μ L of blocking buffer for 60 min at room temperature.
4. Wash twice with 1X wash buffer.
5. Add 200 μ L of cell lysate to mouse anti-human phosphorylated MBP antibody-coated 96-well microtiter plates at 4°C overnight. For the standards, add 0, 1, 2, 4, and 8 μ g of the custom-made peptide (phosphorylated MBP). Add 1X PBS to wells for background signal detection.
6. Wash five times with 1X wash buffer.
7. Add 100 μ L of mouse anti-human phosphorylated MBP, diluted 1:1000 in dilution buffer, for 60 min at room temperature.
8. Wash five times with 1X wash buffer.
9. Add 100 μ L of affinity-purified sheep anti-mouse IgG conjugated to alkaline phosphatase, diluted 1:1000 in dilution buffer, for 60 min at room temperature.
10. Wash five times with 1X wash buffer.
11. Add 100 μ L of the pNPP substrate system for ELISA and wait until a yellow color develops. The reaction is normally completed within 10 min. Read absorbance at 405 nm with a microplate reader.

4. Notes

1. The viscosity of the mounting media will decrease if PVA is dissolved over longer periods of time (i.e., 8 h).
2. It is strongly recommended that leupeptin, DTT, PMSF, and ATP be freshly added before usage.
3. This antibody can be used for both immunoblots and ELISAs. It should be stored at 20°C for prolonged usage, although it also can be stored at 4°C for a short peri-

od of time. Numerous competitive reagents are available from other commercial sources.

4. *p*-Nitrophenyl phosphate is the preferred substrate for enzyme immunoassays using microtiter plates because the reaction product is water-soluble, whereas substrates that form precipitates are not recommended.
5. Custom-made peptides can be purchased from various commercial sources. Storage conditions are dependent on the characteristics of the peptide. Low concentrations of sodium azide are sufficient to prevent fungal contamination. At high concentrations the enzymatic activity of alkaline phosphatase will be inhibited.

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