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Immunomodulation by cannabinoids is absent in mice deficient for the cannabinoid CB_2 receptor

Nancy E. Buckley [a,*,1], Kathleen L. McCoy [b,1], Éva Mezey [a], Tom Bonner [c], Anne Zimmer [c,2], Christian C. Felder [d], Michelle Glass [e], Andreas Zimmer [c]

[a] *Basic Neuroscience Program, NINDS, National Institutes of Health, Bethesda, MD 20892, USA*
[b] *Department of Microbiology and Immunology, Medical College of Virginia, Virginia Commonwealth University, Richmond, VA 23298, USA*
[c] *Laboratory of Genetics, Room 3D06, Building 36, NIMH, Bethesda, MD 20892, USA*
[d] *Eli Lilly Research Laboratories, Lilly Corporate Center, Indianapolis, IN 46285, USA*
[e] *Laboratory of Cell Biology, National Institutes of DCD, Rockville, MD 20850, USA*

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Abstract

Cannabinoids have immunomodulatory as well as psychoactive effects. Because the central cannabinoid receptor (cannabinoid CB_1 receptor) is highly expressed in many neuronal tissues and the peripheral cannabinoid receptor (cannabinoid CB_2 receptor) is highly expressed in immune cells, it has been suggested that the central nervous system effects of cannabinoids are mediated by cannabinoid CB_1 receptors and that the immune effects are mediated by cannabinoid CB_2 receptors. To test this hypothesis, we have generated the first mouse strain with a targeted mutation in the cannabinoid CB_2 receptor gene. Binding studies using the highly specific synthetic cannabinoid receptor agonist (−)-*cis*-3-[2-Hydroxy-4-(1,1-dimethylheptyl)phenyl]-*trans*-4-(3-hydroxypropyl)cyclohexanol ([^{3}H]CP 55,940) revealed no residual cannabinoid binding sites in the spleen of the cannabinoid CB_2 receptor knockout mice, while binding in the central nervous system was unchanged. Cannabinoid CB_2 receptor knockout mice, which appear healthy, are fertile and care for their offspring. Fluorescence activated cell sorting (FACS) analysis showed no differences in immune cell populations between cannabinoid CB_2 receptor knockout and wildtype mice. We investigated the immunomodulatory effects of cannabinoids in cannabinoid CB_2 receptor deficient mice using a T cell co-stimulation assay. Δ^9Tetrahydrocannabinol inhibits helper T cell activation through macrophages derived from wild type, but not from knockout mice, thus indicating that this effect is mediated by the cannabinoid CB_2 receptor. In contrast, central nervous system effects of cannabinoids were not altered in these mice. Our results suggest that cannabinoid CB_2 receptor-specific ligands may be clinically useful in the modulation of macrophage immune function while exhibiting no central nervous system activity. Furthermore, we conclude that the cannabinoid CB_2 receptor knockout mouse is a useful animal model in which to study the role of the cannabinoid system in immunoregulation.

Keywords: Knockout mouse; Cannabinoid receptor; Δ^9Tetrahydrocannabinol; Macrophage

1. Introduction

Cannabinoids have been shown to modulate immune functions. The most widely studied cannabinoid is Δ^9tetrahydrocannabinol, the major psychoactive compound of marijuana. Within the immune system, Δ^9tetrahydrocannabinol and other cannabinoids affect lymphocytes, macrophages, and natural killer cells in a variety of functions such as cellular proliferation, cytokine activity, humoral responses, and cell mediated immunity (reviewed in Klein et al., 1998a,b,c).

Although these observations suggest a role for cannabinoids in immunomodulation, the mechanisms by which cannabinoids induce these immunomodulatory effects has remained elusive. However, the discovery of two cannabinoid receptors (Matsuda et al., 1990; Munro et al., 1993) has shed some understanding on the mechanisms of

* Corresponding author. Current address: Biological Sciences Department, California State Polytechnic University, 3801 W. Temple Avenue, Pomona, CA 91768, USA. Tel.: +1-909-869-4057; fax: +1-909-869-4078.

E-mail addresses: nebuckley@csupomona.edu (N.E. Buckley), zimmer@codon.nih.gov (A. Zimmer).

[1] These two authors contributed equally to the value of this work.

[2] Requests for materials and animals: Tel.: +1-301-496-8160; fax: +1-301-435-5465.

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cannabinoid action. The central cannabinoid receptor (cannabinoid CB_1 receptor), cloned in 1990 by Matsuda et al. (Matsuda et al., 1990), is predominantly found in the central nervous system (Herkenham et al., 1991). This receptor is found to a much lesser degree in cells of the immune system (Bouaboula et al., 1993; Galiegue et al., 1995; Daaka et al., 1995; Schatz et al., 1997). The peripheral cannabinoid receptor (cannabinoid CB_2 receptor) (Munro et al., 1993) is predominantly expressed in cells of the immune system, such as B cells, T cells, and macrophages (Munro et al., 1993; Galiegue et al., 1995; Schatz et al., 1997). This latter finding has led investigators to postulate that the immunoregulatory effects of cannabinoids may be cannabinoid CB_2 receptor mediated, although very few studies support this notion.

Recently, there has been direct evidence indicating that cannabinoids act through cannabinoid CB_2 receptor to alter immune function. Using the newly developed cannabinoid CB_2 receptor antagonist *N*-[(1*S*)-endo-1,3,3-trimethyl bicyclo[2.2.1] heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide (SR 144528) (Rinaldi-Carmona et al., 1998), cannabinoid-induced B cell proliferation (Derocq et al., 1995) was shown to be dependent on activation of the cannabinoid CB_2 receptor (Carayon et al., 1998). Furthermore, the cannabinoid CB_2 receptor is down-regulated during B cell differentiation as assessed by cannabinoid CB_2 receptor mRNA expression and anti-cannabinoid CB_2 receptor antibody binding (Carayon et al., 1998).

Here we report the development of another useful research tool to study the role of cannabinoid CB_2 receptor activation in the immune system. Using homologous recombination, we created the first mouse deficient for the cannabinoid CB_2 receptor (cannabinoid CB_2 receptor knockout mice or $CB_2^{-/-}$ mice). Here, we demonstrate that this mouse model is an effective system in which to investigate the role of the cannabinoid CB_2 receptor in cannabinoid-induced immunomodulation. In the present study, we have investigated the role of the cannabinoid CB_2 receptor on Δ^9tetrahydrocannabinol inhibition of macrophage co-stimulatory activity.

Previously, McCoy et al. observed that Δ^9tetrahydrocannabinol, at nanomolar concentrations, inhibits helper T cell activation by a macrophage cell line (McCoy et al., 1995, 1999; Clements et al., 1996, 1998). T cell stimulation by macrophages or dendritic cells is one of the first and most important steps in initiating an acquired immune response. Activation of T cells requires their physical interaction with an antigen-presenting cell or accessory cell. This requirement is partially due to the specificity of the T cell receptor (TCR), because its ligand is a molecular complex composed of a peptide fragment of the antigen bound to major histocompatibility complex molecules expressed on the surface of accessory cells (Germain, 1994; Watts, 1997). The cellular interaction between T cells and accessory cells also involves several cell adhesion molecules and serves to prolong the length of time of the primary signal through the TCR (Altman et al., 1990; Hynes, 1992). A second co-stimulatory signal delivered by accessory cells, which is independent of the TCR, is also critical for maximal proliferation and cytokine secretion by T cells (Linsley and Ledbetter, 1993; Janeway and Bottomly, 1994; Mondino and Jenkins, 1994). Once activated, the T cell proliferates and produces cytokines (most notably interleukin-2). These cytokines can then act on the same T cells or on other immune cells such as B cells and macrophages to induce different immune functions.

We have utilized macrophages derived from the cannabinoid CB_2 receptor knockout mice to investigate whether cannabinoids affect macrophage co-stimulatory functions via the cannabinoid CB_2 receptor. Our results show that cannabinoids inhibit macrophage co-stimulatory activity and hence T cell-activation via the cannabinoid CB_2 receptor.

2. Materials and methods

2.1. Homologous recombination

The targeting vector was derived from the plasmid pPNT (Tybulewicz et al., 1991) by introducing upstream of the neomycin gene, 731 base pairs (bp) of the cannabinoid CB_2 receptor genomic fragment containing the 5′ region of the exon which has the entire coding sequence. Downstream of the neomycin gene, we introduced 5.4 kb of the non-coding cannabinoid CB_2 receptor gene sequence. Upon homologous recombination, 341 bp of the cannabinoid CB_2 receptor gene is replaced by the neomycin gene (Fig. 1A). Chimeric mice were generated by morula aggregation or blastocyst injection with the targeted embryonic stem cell line 129. Chimeric animals were backcrossed with C57BL/6 mice. Genotyping of the F_2 generation to identify cannabinoid CB_2 receptor knockout mice was done by Southern blot hybridization and subsequently by polymerase chain reaction (PCR) analysis of tail DNA (Fig. 1B). The tail DNA was obtained by digesting a 1 cm long piece of mouse tail with proteinase K (0.5 mg/ml 50 mM, pH 7.5 Tris buffer), and by sequential protein extraction with phenol/chloroform (1:1) and chlorophorm/iosamyl alcohol (24:1) solutions. The DNA was then precipitated with ethanol from the aqueous phase. The primers used for the PCR analysis of the tail DNA were the following: Primer 1 (5′ AAATG CTTGA TTGGT GTCAG-CTCTC 3′), Primer 2 (5′ GGCTC CTAGG TGGTT TTCAC ATCAG CCTCT 3′), and Primer 3 (5′ TAAAG CGCAT GCTCC AGACT GCCTT 3′).

The PCR amplification was done at 95°C for 1 min, 60°C for 1 min, 72°C for 2 min, and was carried out for 30 cycles using Taq Polymerase (Boehringer Mannheim, Indi-

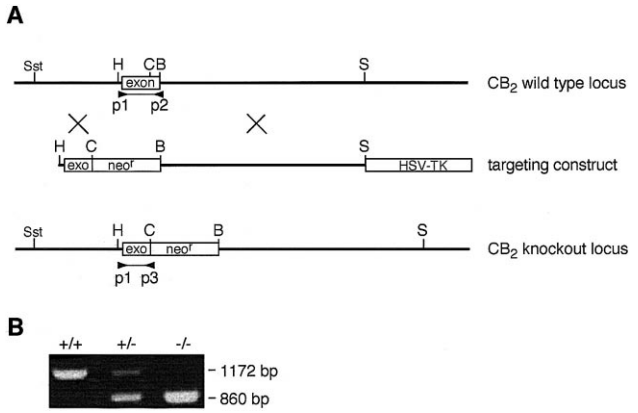

Fig. 1. Disruption of the cannabinoid CB_2 receptor gene by homologous recombination. (A) Structure of the wild type cannabinoid CB_2 receptor gene, the cannabinoid CB_2 receptor targeting construct and the mutated locus following homologous recombination. The coding exon is depicted as a closed box ''EXON''. Binding sites for tail DNA PCR primers 1, 2 and 3 are indicated as P1, P2 and P3, respectively. Relevant restriction enzyme sites are shown: H, HpaI; C, Crf10; B, BamHI; S, Sse. (B) PCR of tail DNA from $CB_2^{+/+}$, $CB_2^{+/-}$, and $CB_2^{-/-}$ mice. The extracted DNA was subjected to PCR as described under experimental procedures. $^{+/+}$ indicates wild type mice, $^{+/-}$ indicates heterozygous mice, and $^{-/-}$ indicates cannabinoid CB_2 receptor knockout mice.

anapolis, IN). The fragments were extended by incubating at 72°C for 10 min, and were later stored at 4°C.

2.2. Binding studies

Autoradiograms were generated by incubating 12 μm thick cryostat spleen sections with 10 nM (−)-*cis*-3-[2-Hydroxy-4-(1,1-dimethylheptyl)phenyl]-*trans*-4-(3-hydroxypropyl)cyclohexanol ([^{3}H]CP 55,940) (Dupont/NEN; specific activity 126 Ci/mmol) as previously described (Herkenham et al., 1991). Briefly, cryostat sections were incubated for 2 h with 10 nM [^{3}H]CP 55,940 in a Tris–HCl buffer (50 mM, pH 7.4) containing 5% bovine serum albumin (BSA) at 37°C. Non-specific binding was determined with the addition of 10 μM HU 210 ((6a*R*)-*trans*-3-(1,1-dimethylheptyl)-6a,7,10,10a-tetrahydro-1-hydroxy-6,6-dimethyl-6*H*-dibenzo[*b*,*d*]pyran-9-methanol). Incubation was terminated and unbound ligand removed by washing twice in Tris-buffer with 1% BSA at 4°C for 2 h. The slides were placed in X-ray cassettes and exposed to tritium sensitive Hyperfilm for 2 weeks. The films were developed manually in Kodak D19 for 5 min at 20°C, washed and fixed.

For saturation binding studies, brains and spleens from knockout and wild type animals were removed immediately following cervical dislocation and homogenized in a sucrose buffer (200 mM sucrose, 50 mM Tris–HCl, 5 mM $MgCl_2$, 2.5 mM EDTA, pH 7.4). A P2 membrane fraction was prepared by repeated centrifugation, and the P2 pellet resuspended in sucrose buffer, snap frozen, and stored at −70°C until use. Saturation binding assays were performed by incubating 100–150 μg protein/tube with 1×10^{-12}–5×10^{-7} M [^{3}H]CP 55,940 for 1 h at 30°C. Non-specific binding was defined by the addition of the cannabinoid receptor agonist (*R*)-(+)-2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone (WIN 55212-2) at 10 μM. All cannabinoid ligands were diluted in Eagles Modified Media containing 5% BSA, and added to the incubation reaction to produce a final concentration of 0.5% BSA. All reactions were performed in 50 mM Tris–HCl buffer with 5 mM $MgCl_2$, 2.5 mM EDTA (pH 7.4), at a total volume of 250 μl. Incubation was terminated by the addition of 2 ml ice cold wash buffer (50 mM Tris, 0.05% BSA, pH 7.4), and the membranes were rapidly filtered through Iontch type H filters pretreated with 0.1% polyethylenimine (pH 7.4). Filters were soaked overnight in 0.1% Triton X-100 prior to addition of scintillation fluid. Saturation isotherms were fitted, and binding parameters calculated using a non-linear regression program (Graph-Pad Prism).

2.3. Fluorescence activated cell sorting (FACS) analysis

Single cell suspensions from the spleen, lymph nodes or thymus from 6-week old wild type or cannabinoid CB_2 receptor knockout mice were prepared and stained as described previously (Giese and Davidson, 1995). Before staining, cells were pretreated with unlabeled monoclonal antibody to CD32 to prevent Fc receptor (FcR)-mediated binding of labeled antibodies. The analysis was done on a FACScan or FACStar^{Plus} (Becton Dickinson, Sunnyvale, CA). To analyze the T lymphocyte population in the spleen and lymph nodes, phycoerythrin (PE)-labeled anti-CD4, anti-CD8, and anti-CD69 antibodies, and fluorescein isothiocyanate (FITC)-labeled anti-CD8 and anti-TCR αβ, (PharMingen, San Diego, CA) were used. To determine the B lymphocyte population in spleen and lymph nodes, PE-labeled anti-CD19 antibodies, FITC-labeled anti-CD23 antibodies (PharMingen), and Tri Color (TC)-labeled anti-CD45/B220 antibodies (Caltag, San Francisco, CA) were used. To determine the macrophage population, FITC-labeled anti-Mac1 antibodies (Pharmingen) were used. To analyze thymic cells, the cells were stained with PE-labeled anti-CD8, anti-CD69, and anti-CD5 antibodies and FITC-labeled anti-CD4 and anti-TCR αβ antibodies.

2.4. THC exposure and T cell co-stimulation assay

Peritoneal macrophages were induced in mice by intraperitoneal administration of 4 mg latex beads of 1.0 μm diameter (Sigma Chemical, St. Louis, MO), followed by 2.0 ml of 3% Brewer's thioglycolate broth (Difco Laboratories, Detroit, MI), 24 h later. Administration of latex beads renders wild type peritoneal macrophages sensitive

to cannabinoid inhibition of co-stimulatory activity (data not shown). Cells were harvested by peritoneal lavage after an additional 4 days and used as accessory cells. Cells were pooled from two mice per group. Wells of 96-well flat bottom microtiter plates were coated with culture supernatant containing monoclonal anti-CD3 antibody (American Type Culture Collection, Rockville, MD) or complete medium overnight at 4°C. Before the addition of peritoneal cells, the supernatant and medium were removed from the wells. Δ^9Tetrahydrocannabinol (National Institute on Drug Abuse) was prepared in ethanol and utilized from 0.1 to 1000 nM in 0.1% ethanol. This concentration of ethanol was the vehicle control and did not affect cell viability. Peritoneal cells in replicates at 1.25×10^5/ml in complete medium were preincubated with Δ^9tetrahydrocannabinol or ethanol for 4 h at 37°C in antibody- or medium-coated wells (McCoy et al., 1995). Helper T cell hybridoma 2B4.11 (Samelson et al., 1983) at 1.5×10^5/ml was then added to the cultures and incubated with or without macrophages in complete medium in replicates at 37°C for an additional 24 h. Before Δ^9tetrahydrocannabinol or vehicle exposure, peritoneal cells were irradiated with 3000 rads from a Cs source to prevent the cells from depleting cytokines secreted by 2B4.11 cells into the culture supernatant. Cell-free culture supernatants were collected and assayed for the presence of interleukin-2 by enzyme linked immuno assay (R&D Systems, Minneapolis, MN). Absorbance at 450 nm was measured with a SpectraMax 250 microplate reader (Molecular Devices, Sunnyvale, CA). The quantity of interleukin-2 in the culture supernatants was calculated from standard curves.

2.5. Behavioral testing

The behavioural and physiological effects of cannabinoid drugs in rodents are commonly evaluated by measuring body and ring catalepsy. Body temperature was measured using a rectal probe before and 56 min after drug injection (Model BAT-12, Physitemp Instruments, Clifton, NJ). Immobility in the ring-catalepsy test was measured 50 min after injection of vehicle or drug essentially as described (Zimmer et al., 1999). Animals were placed on a vertical tube (5.5 cm diameter). The time that the animal spent motionless during the 4 min test session on the ring was recorded. If an animal fell down or jumped off the ring, it was immediately placed on the ring again. A maximum of four such escapes was permitted.

3. Results

3.1. Homologous recombination

All of the cannabinoid CB_2 receptor gene coding sequence is contained within a single exon. To inactivate the gene, we replaced the 3′ region of this coding exon with the phosphoglycerate kinase (PGK)-neomycin sequences through homologous recombination in embryonic stem cells (Fig. 1A). This mutation eliminates part of intracellular loop 3, transmembrane domains 6 and 7, and the carboxy terminus. Homozygous CB_2^{tm1}/CB_2^{tm1} mice (henceforth referrred to as $CB_2^{-/-}$ mice) were obtained with the expected Mendelian frequency from heterozygous $CB_2^{tm1/+}$ breedings. Homozygous knockout mice appeared healthy, were of similar size and weight as their wild type ($CB_2^{+/+}$) littermates and had no gross morphological defects. Mutant mice were fertile and cared for their offspring.

3.2. Cannabinoid receptor binding studies

To confirm the absence of cannabinoid CB_2 receptors in $CB_2^{-/-}$ animals, we performed receptor autoradiography in spleen tissues and homogenate binding assays with brain and spleen. A high level of [^{3}H]CP 55,940 binding was observed in the marginal zone of the spleen of wild type mice (Fig. 2, $+/+$), as had previously been reported (Lynn and Herkenham, 1994). In contrast, no specific binding was detected in corresponding sections of spleen from the knockout mice (Fig. 2, $-/-$). The spleen of wild type mice demonstrated high affinity saturable specific binding to [^{3}H]CP 55,940 ($B_{max} = 79$ fmol/mg protein and $K_d = 0.36$ nM) (Fig. 3). No specific binding was detected in spleen of knockout animals (data not shown), even at [^{3}H]CP 55,940 concentrations of up to 30 nM. In contrast, no significant difference was observed in the binding of [^{3}H]CP 55,940 in the brain ($B_{max} = 1.2$ fmol/mg protein and $K_d = 1.66$ nM) of knockout and

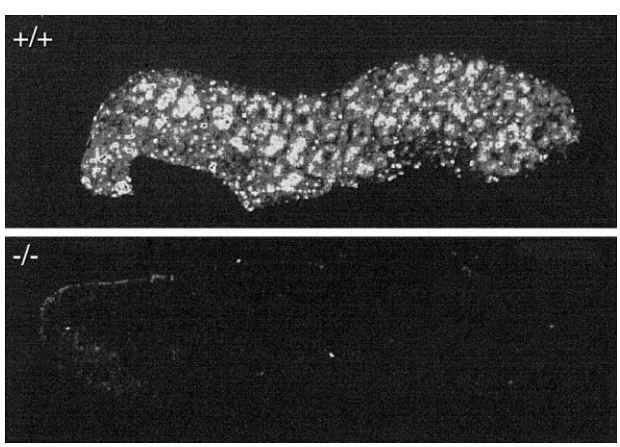

Fig. 2. Cannabinoid CB_2 receptor ligand binding is absent in $CB_2^{-/-}$ mice. Twelve micrometer spleen sections were incubated with 10 nM [^{3}H]CP 55940, and non-specific binding was determined with addition of 10 μM HU 210. The figures show representative autoradiographs for three different experiments. $^{+/+}$ indicates wild type mice and $^{-/-}$ indicates knockout mice.

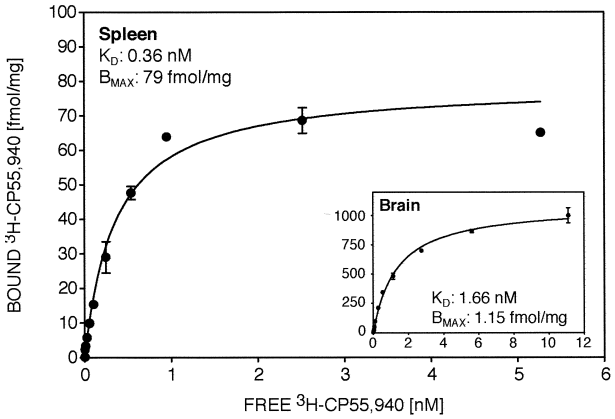

Fig. 3. Homogenate binding assay. Saturation binding curve of [^{3}H]CP 55,940 binding in $CB_2^{+/+}$ mice. Spleens or brain were homogenized in a sucrose buffer and a P2 membrane fraction was prepared as described under Section 2. Binding assays were done by incubating 100–150 μg protein with the indicated concentrations of [^{3}H]CP 55,940. Non-specific binding was determined using 10 μM WIN 55,212-2. The graph shown is representative of three experiments performed in duplicate.

wild type animals, indicating that the expression of the cannabinoid CB_1 receptor was not affected in cannabinoid CB_2 receptor knockout animals.

3.3. FACS analysis

Histological analysis of the spleen, in which the cannabinoid CB_2 receptor is most strongly expressed, did not reveal any morphological defects in $CB_2^{-/-}$ mice. In additon, cell counts from the spleen and thymus of the $CB_2^{-/-}$ mice did not differ from those of the wild type mice. To determine if the cannabinoid CB_2 receptor plays an essential role in the development and differentiation of immune cells, we therefore performed FACS analysis with cells isolated from spleens, lymph nodes, and thymi of adolescent mice (6 weeks of age). As seen in Table 1, the immune cell composition of the spleen and lymph nodes was not significantly different between naive, wild type and $CB_2^{-/-}$ mice. The number of T cells expressing the cell surface markers $CD4^+TCR\alpha\beta^+$ or $CD8^+TCR\alpha\beta^+$ was similar in wild type and cannabinoid CB_2 receptor knockout mice. Basal level of T cell activation in the spleen and lymph nodes was assessed by staining with anti-CD69 antibodies (Table 1). The percentage of CD69 expressing T lymphocytes was essentially the same in spleens from mice of both genotypes. Similarly, the percentage of activated T cells in lymph nodes is identical in cannabinoid CB_2 receptor wild type and knockout mice.

The B cell population was analyzed by anti-B220, anti-CD19, and anti-CD23 antibody staining (Table 1), and the macrophage population was determined by anti-Mac-1 antibody staining. When analyzing B cells derived from spleen and lymph nodes, we also observed that the percent of B cells positive for B220, CD19 and CD23, was essentially the same for wild type and $CB_2^{-/-}$ mice (Table 1). The number of macrophages in $CB_2^{-/-}$ mice did not differ from the number found in wild type animals. The tissue macrophages detected by the anti-Mac-1 antibody corresponded to approximately 5–6% of the cell population in the spleens and to about 1.3% in lymph nodes (Table 1).

T lymphocyte populations in the thymus were assessed by anti-CD4, anti-CD8, anti-TCRαβ, and anti-CD5 antibody staining (Table 2). Again, no differences were found between wild type and knockout animals (Table 2).

3.4. Δ^9Tetrahydrocannabinol exposure and T cell co-stimulation assay

Although immobilized anti-CD3 antibody crosslinks the TCR complex, T cell stimulation requires a second signal mediated by co-stimulatory molecules expressed on accessory cells (Linsley and Ledbetter, 1993; Janeway and Bottomly, 1994; Mondino and Jenkins,1994). Activation of a $CD4^+$ helper T cell hybridoma using peritoneal macrophages as accessory cells was assessed by interleukin-2 secretion. Monoclonal anti-CD3 antibody alone did not induce the T cells to secrete interleukin-2 (Fig. 4, figure legend). Production of interleukin-2 by these T cells

Table 1
FACS analysis of cells from the spleen and lymph nodes

Cell marker[a]	Spleens		Lymph nodes	
	Wild type[b]	$CB_2^{-/-}$[b]	Wild type[b]	$CB_2^{-/-}$[b]
$CD4^+$ TCR^+	20.73±3.09	16.47±0.90	45.41±1.05	38.47±2.67
$CD8^+$ TCR^+	12.25±0.84	10.82±0.26	26.59±0.57	30.35±2.65
$CD69^+$	1.87±0.35	1.89±0.4	7.82±0.43	6.55±1.51
$B220^+$	54.73±5.28	58.77±1.17	24.97±0.52	26.35±2.31
$CD19^+$ $CD23^+$	32.47±16.4	38.52±0.23	21.1±4.35	25.46±1.58
$CD19^+$	55.19±5.08	59.11±0.50	24.56±0.60	27.68±1.20
Mac-1	7.16±2.24	6.21±0.07	1.33±0.05	1.44±0.20

[a]The cells were prepared and stained with the indicated antibodies for FACS analysis.
[b]Percent positive cells from representative wt and CB_2 ko mice.

Table 2
FACS analysis of cells from the thymus of wild type and $CB_2^{-/-}$ mice

Cell markers[a]	Wild type[b]	$CB_2^{-/-}$[b]
$CD4^+$ $CD8^+$	84.59±1.97	87.67±2.07
$CD4^-$ $CD8^-$	2.38±1.06	2.09±0.34
$CD4^+$	9.7±1.15	7.6±1.29
$CD8^+$	3.32±0.65	2.64±1.45
$TCR^+\alpha\beta$	17.80±2.26	13.64±1.11
$CD5^+$	98.3±0.11	98.75±0.62

[a]The cells were prepared and stained with the indicated antibodies for FACS analysis.
[b]Percent positive cells from representative wt and CB_2 ko mice.

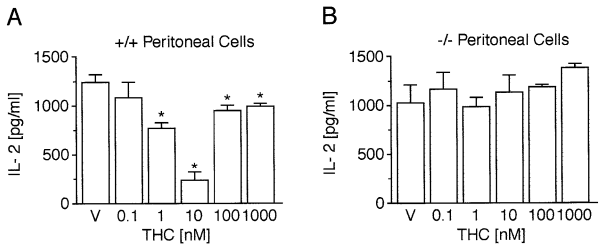

Fig. 4. Lack of cannabinoid CB_2 receptor renders peritoneal cells refractory to Δ^9tetrahydrocannabinol inhibition of co-stimulatory activity. Mice were administered latex beads intraperitoneally and 3% thioglycolate, 24 h later. Peritoneal cells were harvested after another 4 days. Wells were coated with monoclonal anti-CD3 antibody or medium. Peritoneal macrophages as accessory cells were pre-incubated with 0.1% ethanol (vehicle control) or various concentrations of Δ^9tetrahydrocannabinol (THC) for 4 h at 37°C. Helper T cell hybridoma 2B4.11 was added to the cultures, and interleukin-2 (IL-2) secretion by the T cells was measured by enzyme linked immunoassay after an additional 24 h. Values are the mean IL-2 in pg/ml ± SD from triplicate cultures. Both medium controls and cultures lacking macrophages were below the detection level. (A) Peritoneal cells from $^{+/+}$ mice. * Denotes significantly different from vehicle control by a two-tailed Student's *t*-test for unmatched pairs, $P < 0.01$. (B) Peritoneal cells from $^{-/-}$ mice from the same experiment as in panel A. The experiment is representative of four.

required the presence of macrophages. The possible influence of Δ^9tetrahydrocannabinol on the ability of peritoneal macrophages from wild-type mice to deliver co-stimulatory signals was investigated. Macrophages were pre-incubated with Δ^9tetrahydrocannabinol for 4 h before the addition of the T cells to allow time for the drug to exert its influence. Δ^9Tetrahydrocannabinol significantly decreased interleukin-2 production by the T cells compared to that in vehicle control cultures (Fig. 4A). Maximal suppression of approximately 80% was observed at 10 nM Δ^9tetrahydrocannabinol, whereas higher drug concentrations were less inhibitory. A loss of cell viability, as assessed by trypan blue exclusion, did not account for the diminished T cell response.

Analogous experiments were performed with peritoneal cells from $CB_2^{-/-}$ mice. The total number of peritoneal cells from $CB_2^{-/-}$ mice was not significantly different from the number of cells from wild type mice (6.3×10^6 vs. 4.8×10^6 cells per mouse for $CB_2^{+/+}$ and $CB_2^{-/-}$ mice, respectively). Macrophages in the peritoneal population were quantified by their Mac-1 expression as assessed by immunofluorescence staining and flow cytometry. The mean percent of Mac-1^+ cells was similar for both groups of mice (74.6 ± 2.3% for $CB_2^{-/-}$ mice vs. 75.0 ± 7.2% for $CB_2^{+/+}$ mice). The other cells consisted of neutrophils, eosinophils, and B cells. Their frequency did not differ between the mice (data not shown). In contrast to the preceding findings, Δ^9tetrahydrocannabinol had no significant impact on interleukin-2 secretion in cultures containing macrophages from $CB_2^{-/-}$ mice (Fig. 4B). These cells were completely resistant to the drug's inhibition, which cannot be explained by an altered frequency of macrophages, indicating that Δ^9tetrahydrocannabinol interference with co-stimulation is mediated via the cannabinoid CB_2 receptor.

3.5. Δ^9Tetrahydrocannabinol-induced hypothermia and catalepsy

In order to determine if the central nervous system effects of Δ^9tetrahydrocannabinol, believed to be cannabinoid CB_1 receptor mediated, were altered in $CB_2^{-/-}$ mice, we tested the hypothermic and cataleptic activities of Δ^9tetrahydrocannabinol. We measured the body temperature and immobility in the ring catalepsy test after intraperitoneal injection of 50 mg/kg Δ^9tetrahydrocannabinol or vehicle. The baseline body temperature was similar in knockout and wild type animals (Fig. 5A). Injection of Δ^9tetrahydrocannabinol resulted in a significant reduction in body temperature that was similar in animals of both genotypes. Furthermore, we did not find significant differences in the ring catalepsy test, which is commonly used to measure Δ^9tetrahydrocannabinol-induced catalepsy. As shown in Fig. 5B, mice of both genotypes displayed similar baseline activities, and Δ^9tetrahydrocannabinol in-

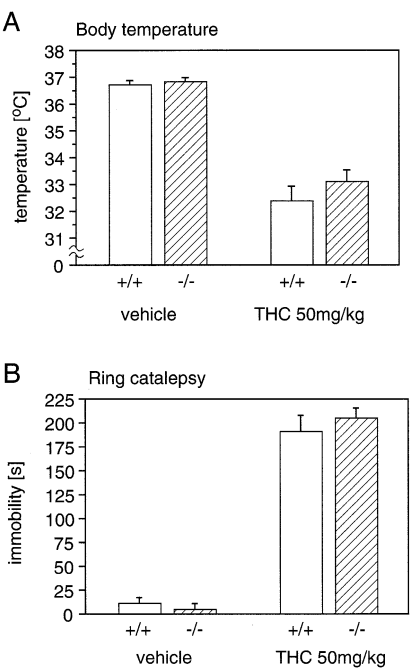

Fig. 5. Δ^9Tetrahydrocannabinol induces hypothermia and catalepsy in $CB_2^{-/-}$ mice. (A) The body temperature of $CB_2^{+/+}$ and $CB_2^{-/-}$ mice was measured using a rectal probe before (vehicle) and 56 min after an intraperitoneal injection of Δ^9tetrahydrocannabinol (THC) (50 mg/kg). (B) Catalepsy was measured in $CB_2^{+/+}$ and $CB_2^{-/-}$ mice before (vehicle) and after an intraperitoneal injection of THC (50 mg/kg). The mice were placed on a vertical tube 5.5 cm in diameter. The time in seconds (s) the mice spent motionless on the beam was recorded as described under Section 2.

jection produced a similarly profound catalepsy in mice of both genotypes.

4. Discussion

During the last decade, interest in the potential medicinal use of cannabinoid drugs has greatly increased. This renewed interest was stimulated in part by the molecular cloning of two cannabinoid receptors, the cannabinoid CB_1 and the cannabinoid CB_2 receptors. These two receptors, which belong to the large family of G-protein coupled receptors, are quite divergent in structure (44% overall homology) and in their pattern of expression. The cannabinoid CB_1 receptor is thought to mediate most of the central nervous system effects of cannabinoid drugs, while the cannabinoid CB_2 receptor is thought to be responsible for their immunomodulatory functions. Endogenous ligands for these receptors were also recently identified and include the eicosanoids anandamide and 2-arachidonylglycerol. In addition, several synthetic cannabinoid agonists with high affinity for these receptors, including some with a good selectivity for the cannabinoid CB_1 receptor, have been developed. Unfortunately, no specific cannabinoid CB_2 receptor ligands are currently available, and the most widely used antagonists, the cannabinoid CB_2 receptor selective antagonist SR 144528 (Rinaldi-Carmona et al., 1998) and the cannabinoid CB_1 receptor specific antagonist *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide hydrochloride (SR 141716A) are also inverse agonists (Portier et al., 1999; Bouaboula et al., 1999).

To complement the pharmacological analysis of the cannabinoid system, we and others have recently begun to use a genetic approach and generated mice with targeted deletions in the cannabinoid receptor genes (Zimmer et al., 1999; Ledent et al., 1999). Cannabinoid CB_1 receptor knockout mice display an increased mortality, alterations in their open field behavior, and are hypoalgesic (Zimmer et al., 1999). Importantly, most central nervous system-effects of cannabinoid drugs are lost in cannabinoid CB_1 receptor knockout mice (Zimmer et al., 1999; Ledent et al., 1999).

This paper examines, for the first time, the phenotype of mice with a mutation in the cannabinoid CB_2 receptor. This mouse has no cannabinoid ligand binding in the spleen as seen by autoradiography and radioligand binding studies. The cannabinoid CB_2 receptor has been suggested as mediating the immunoregulatory effects of cannabinoids. Since no cannabinoid CB_1 receptor specific binding sites could be detected with the cannabinoid CB_1 receptor specific antagonist [^{3}H]SR 141716A in the spleen of wild type animals (data not shown) and since no residual CB_2 binding sites were found in $CB_2^{-/-}$ mice, the cannabinoid CB_2 receptor must account for most, if not all, cannabinoid binding sites in the spleen. Other investigators, using the sensitive method of reverse transcriptase–PCR (RT–PCR), have reported the presence of cannabinoid CB_1 receptor mRNA in the spleen. RT–PCR is such a sensitive technique that it is capable of amplyfying cDNA from a very small mRNA copy number. However, using in situ hybridization histochemistry, we were unable to detect cannabinoid CB_1 receptor mRNA in the spleen (data not shown). In addition, the fact that we are unable to detect binding by [^{3}H]SR 141716A, implies that the cannabinoid CB_1 receptor mRNA may not be present in the spleen in relevant amounts or stable enough to be translated.

To investigate whether cannabinoid CB_2 receptor activation is involved in differential development of immune cells, we performed FACS analysis of spleen, lymph node and thymic cells derived from $CB_2^{-/-}$ mice. We investigated whether the immune cells from the tissues of $CB_2^{-/-}$ mice differed phenotypically from those of wild type mice. We found that the immune cell composition was similar in knockout and wild type mice at 6 weeks of age. These results suggest that, at least at this age, cannabinoid CB_2 receptor activation does not affect immune cell development and differentiation in the lymphoid tissues studied.

To directly assess the functional role of cannabinoid CB_2 receptor activation on immunomodulation, we studied the co-stimulatory activity of macrophages derived from $CB_2^{-/-}$ mice. Cannabinoids are known to depress the co-stimulatory activity of a macrophage cell line (Clements et al., 1996, 1998). In the present report, we investigated the consequence of Δ^9tetrahydrocannabinol on $CB_2^{+/+}$ and $CB_2^{-/-}$ peritoneal macrophages to deliver co-stimulatory signals to a helper T cell hybridoma. The primary signal was provided by immobilized anti-CD3 antibody that bypasses TCR specificity (Linsley and Ledbetter, 1993; Janeway and Bottomly, 1994; Mondino and Jenkins, 1994). Interleukin-2 secretion by the T cells required a second signal provided by the macrophages. Δ^9Tetrahydrocannabinol diminished the level of T cell activation when the accessory cells were peritoneal macrophages from $CB_2^{+/+}$ mice. However, Δ^9tetrahydrocannabinol had no effect on the T cell response in the presence of macrophages from $CB_2^{-/-}$ mice. These results cannot be explained by differences in the proportion of macrophages within the peritoneal population. If Δ^9tetrahydrocannabinol exerted its inhibition via the T cells, decreased interleukin-2 production should have occurred in cultures with cells from $CB_2^{-/-}$ mice, which was not observed. We previously reported that Δ^9tetrahydrocannabinol does not diminish surface expression of the CD3 complex on these T cells (McCoy et al., 1995) and does not decrease co-stimulation provided by immobilized fibronectin in the absence of macrophages (Clements et al., 1996). Our findings indicate that the drug's suppressive effect on co-stimulation is mediated through the cannabinoid CB_2 receptor expressed by macrophages.

Δ^9Tetrahydrocannabinol at 10 nM caused maximal inhibition with $CB_2^{+/+}$ macrophages, while higher drug con-

centrations were less suppressive. The drug's dose profile is virtually identical to that observed for a macrophage cell line (Clements et al., 1996). Similarly, other investigators reported a biphasic effect of Δ^9tetrahydrocannabinol on proinflammatory cytokine production by human monocytes (Berdyshev et al., 1997) and on lymphocyte proliferation (Luo et al., 1992; Pross et al., 1992); low Δ^9tetrahydrocannabinol doses are inhibitory while higher ones are not. The signal transduced through the cannabinoid CB_2 receptor inhibits adenylate cyclase activity, and decreases production and accumulation of intracellular cyclic AMP (Schatz et al., 1997). Δ^9Tetrahydrocannabinol has either an inhibitory or stimulatory consequence on leukocytes, in part, depending on the extent to which cyclic AMP is decreased (Klein et al., 1998a,b), and this incongruity may possibly account for the drug's dose profile on co-stimulatory activity of wild type cells.

Co-stimulation is an essential second signal for T cell activation and is delivered to T cells by co-stimulatory molecules expressed by macrophages or dendritic cells (Linsley and Ledbetter, 1993; Janeway and Bottomly, 1994; Mondino and Jenkins, 1994). Disruption or lack of co-stimulatory signals during TCR occupancy causes long-lasting T cell unresponsiveness or anergy (Enk and Katz, 1994; Chambers and Allison, 1997). Furthermore, some pathogens evade immune responses by down-regulating co-stimulatory molecules on macrophages (Kaye et al., 1994; Saha et al., 1994). Hence, Δ^9tetrahydrocannabinol suppression of co-stimulation mediated through the cannabinoid CB_2 receptor may potentially compromise immune responsiveness. Δ^9Tetrahydrocannabinol diminishes co-stimulatory activity of a macrophage cell line by down-regulating expression of heat-stable antigen, but not B7-1 or B7-2 molecules (Clements et al., 1998). Although Δ^9tetrahydrocannabinol may have a similar impact on peritoneal macrophages, additional experiments are needed to determine the co-stimulatory molecules that may be affected by Δ^9tetrahydrocannabinol.

While cannabinoid CB_2 receptor activation is involved in cannabinoid-induced immunomodulation as demonstrated in the present report, our studies also establish that this receptor is not involved in central nervous system events, as $CB_2^{-/-}$ mice displayed normal hypothermia and catalepsy after Δ^9tetrahydrocannabinol administration. These results further support the idea that cannabinoid effects on the central nervous system are mediated by the cannabinoid CB_1 receptor and they are consistent with the absence of cannabinoid effects in cannabinod CB_1 receptor knockout mice (Zimmer et al., 1999). Therefore, cannabinoid CB_2 receptor knockout mice provide a unique animal model in which to study the role of cannabinoid CB_2 receptor activation in immunomodulation.

In summary, mice with targeted mutations in cannabinoid receptor genes are useful tools to delineate the specific biological function of these receptors and their role in cannabinoid pharmacology. The data suggest that it may be possible in the future to separate central and peripheral effects of cannabinoid drugs with new receptor-specific agonists. Cannabinoid CB_2 receptor specific agonists should lack all of the unwanted cannabinoid CB_1 receptor-mediated side effects of cannabinoid drugs, and yet may be useful in the treatment of immunological disorders including autoimmune diseases.

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