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CANNABINOID MODULATION OF CHEMOTAXIS OF MACROPHAGES AND
MACROPHAGE-LIKE CELLS

A dissertation submitted in partial fulfillment of the requirements for the degree of
Doctor of Philosophy at Virginia Commonwealth University.

by

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Table of Contents

	Page
List of Tables	v
List of Figures	vi
Abstract	xi
Introduction.....	1
Materials and Methods.....	11
Amoeba.....	11
Cells.....	12
Mice.....	13
Drugs.....	14
Chemotaxis Assay.....	15
Immunoprecipitation of CCR5.....	19
Isolation of Cannabinoid Receptor DNA from Plasmid Constructs.....	19
Multiprobe Ribonuclease Protection Assay.....	20
Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR).....	21
Scanning Electron Microscopy.....	22
SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Immunoblotting.....	23
Statistical Analysis.....	23
Results.....	24

Discussion.....	73
Literature Cited.....	92

List of Tables

	Page
Table 1: CC Chemokine Nomenclature.....	7
Table 2: CC Chemokines and Chemokine Receptors.....	8
Table 3: Cannabinoid Receptor Ligands.....	16
Table 4: <i>Acanthamoeba</i> Secreted Factors.....	54

List of Figures

	Page
Figure 1: Transwell Migration Assay	18
Figure 2: CCR1 and CCR5 mRNA Are Expressed by Peritoneal Macrophages	25
Figure 3: Peritoneal Macrophages Express CCR1, CCR5, and CB ₂ at the Protein Level.	26
Figure 4: Generation of Real-time RT PCR CB ₁ and CB ₂ positive control DNA.....	28
Figure 5: Cannabinoid Receptor mRNA Expression by Peritoneal Macrophages.	29
Figure 6: Treatment <i>in vivo</i> with THC Results in Inhibition of the Chemotactic Response to RANTES.....	30
Figure 7: Treatment <i>in vitro</i> with THC Results in Inhibition of the Chemotactic Response to RANTES.....	32
Figure 8: Treatment <i>in vitro</i> with CP55940 Results in Inhibition of the Chemotactic Response to RANTES.....	34
Figure 9: Effect of the CB ₂ -selective Ligand on the Chemotactic Response to RANTES.	36
Figure 10: Effect of the CB ₁ -selective Ligand on the Chemotactic Response to RANTES..	37
Figure 11: Effect of CB ₁ Cannabinoid Receptor Antagonist on Chemotaxis to RANTES	39
Figure 12: Effect of CB ₂ Cannabinoid Receptor Antagonist on Chemotaxis to RANTES	40

Figure 13: Effect of THC on the Chemotactic Response of Peritoneal Macrophages from CB ₂ Knockout Mice to RANTES	41
Figure 14: Effect of THC on the Chemotactic Response of Peritoneal Macrophages from the Wild-type Counterpart	42
Figure 15: THC Alters the Morphology of Peritoneal Macrophages Migrating to RANTES	44
Figure 16: Effect of THC on Levels of CC Chemokine Receptor mRNA	46
Figure 17: Effect of Select Cannabinoids on Levels of CC Chemokine Receptor mRNA	47
Figure 18: Stimulation with RANTES and Cannabinoid Receptor Agonist CP55940 Induces CCR5 Phosphorylation	49
Figure 19: The Endogenous Cannabinoid 2-AG Induces Peritoneal Macrophage Chemotaxis	50
Figure 20: THC Inhibits Peritoneal Macrophage Chemotaxis to 2-AG	52
Figure 21: Hematoxylin and Eosin (H&E) Stained Section of Paraffin-embedded Brain Tissue Demonstrating Granuloma Formation in <i>Acanthamoeba</i> infection	53
Figure 22: Peritoneal Macrophages Migrate in Response to Amoebic Conditioned Medium	55
Figure 23: THC Inhibits Peritoneal Macrophage Chemotaxis to <i>Acanthamoeba</i> Conditioned Medium (JH1CM)	57
Figure 24: THC Inhibits Peritoneal Macrophage Chemotaxis to <i>Acanthamoeba</i> Conditioned Medium (AcCM)	58

Figure 25: <i>In vivo</i> Exposure to THC Inhibits Peritoneal Macrophage Chemotaxis to <i>Acanthamoeba</i> Conditioned Medium (AcCM).....	60
Figure 26: CP55940 Inhibits Peritoneal Macrophage Chemotaxis to <i>Acanthamoeba</i> Conditioned Medium (JH1CM).....	61
Figure 27: The CB ₂ Selective Agonist O-2137 Inhibits Peritoneal Macrophage Chemotaxis to <i>Acanthamoeba</i> Conditioned Medium (JH1CM).....	63
Figure 28: The CB ₁ Selective Agonist ACEA Has No Effect on Peritoneal Macrophage Migration to <i>Acanthamoeba</i> Conditioned Medium (JH1CM).....	64
Figure 29: Effect of Cannabinoid Receptor Antagonist on Chemotaxis to <i>Acanthamoeba</i> Conditioned Medium (JH1CM).....	65
Figure 30: THC Alters the Morphology of Peritoneal Macrophages Migrating to <i>Acanthamoeba</i> Conditioned Medium	67
Figure 31: THC and CP55940 Inhibit Chemotaxis of Microglia to <i>Acanthamoeba</i> Conditioned Medium (AcCM).....	69
Figure 32: The CB ₂ agonist O-2137 Inhibits Microglial Migration to <i>Acanthamoeba</i> Conditioned Medium (JH1CM), but not the CB ₁ Agonist ACEA.....	71
Figure 33: The CB ₂ agonist O-2137 Inhibits Microglial Migration to <i>Acanthamoeba</i> Conditioned Medium (AcCM), but not the CB ₁ Agonist ACEA	72
Figure 34: The CB ₂ Antagonist Reverses CP55940-mediated Inhibition of Primary Microglial Migration to <i>Acanthamoeba</i> Conditioned Medium (AcCM).....	73

Figure 35: Pretreatment with the CB₁ antagonist SR1 Does Not Alter CP55940-Mediated
Inhibition of Primary Microglial Migration to *Acanthamoeba* conditioned
medium75

Abstract

CANNABINOID MODULATION OF CHEMOTAXIS OF MACROPHAGES AND MACROPHAGE-LIKE CELLS

By Erinn S. Raborn, Ph.D.

A dissertation submitted in partial fulfillment of the requirements for the degree of
Doctor of Philosophy at Virginia Commonwealth University.

Virginia Commonwealth University, 2007

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Exogenous and endogenous cannabinoids have been reported to modulate functional activities of macrophages. It is recognized that macrophages express primarily the CB₂ cannabinoid receptor, but recent studies indicate that its expression is differential in relation to activation state with maximal levels occurring when cells are in “responsive” and “primed” states. The functional activities of macrophages when in these states of activation are the most susceptible to the action of cannabinoids, at least in terms of a functional linkage to the CB₂. To assess the effect of cannabinoid treatment on macrophage chemotaxis and test the hypothesis that cannabinoids inhibit the chemotactic response of macrophages and microglia to endogenous and exogenous, pathogen-derived

stimuli, primary murine peritoneal macrophages and neonatal rat microglia were used. Chemotaxis assays and scanning electron microscopy studies demonstrated that cannabinoids inhibit chemotaxis, a signature activity attributed to “responsive” macrophage-like cells, to the endogenous chemokine RANTES (Regulated upon Activation Normal T-cell Expressed and Secreted) and to *Acanthamoeba* conditioned medium containing secreted proteases. The partial agonist delta-9-tetrahydrocannabinol (THC), administered *in vitro*, inhibited the chemotactic response of peritoneal macrophages to the chemokine RANTES and to *Acanthamoeba* conditioned medium. *In vivo* treatment with THC also resulted in inhibition of the *in vitro* chemotactic response of murine peritoneal macrophages to RANTES and amoebic conditioned medium. Pharmacological studies employing cannabinoid receptor agonists and antagonists demonstrated the involvement of CB₂ in cannabinoid-mediated inhibition of peritoneal macrophage chemotaxis to RANTES and *Acanthamoeba* conditioned medium, implying that signaling through cannabinoid receptors may desensitize chemokine receptors. Treatment with cannabinoids had no apparent effect on chemokine receptor mRNA levels, but did enhance CCR5 protein phosphorylation. Macrophage migration to *Acanthamoeba* conditioned medium may involve activation and signaling through protease activated receptors (PARs), as pathogen-derived proteases have been shown to activate PARs and initiate cellular migration; however, further studies are required to demonstrate PAR activation by amoebic conditioned medium and to assess the effects of cannabinoids on PAR signaling.

Acanthamoeba are opportunistic pathogens that cause Granulomatis amoebic encephalitis, an infection of the CNS that is often fatal. THC treatment has been shown to increase mortality to *Acanthamoeba* infections and is characterized by an absence of granuloma formation. We hypothesize that inhibitory effect of THC on macrophage migration may be a key factor in cannabinoid-mediated immunosuppression. To assess the effect of cannabinoids on microglial migration to *Acanthamoeba* conditioned medium, chemotaxis assays were performed using primary rat microglia treated with cannabinoids. These studies demonstrated that cannabinoids inhibit microglial chemotaxis to amoebic conditioned medium.

Furthermore, the studies demonstrate that cannabinoids, acting through cannabinoid receptors, may cross-talk with a diverse array G-protein coupled receptors so as to modulate responsiveness of macrophage and macrophage-like cells.

Introduction

Cannabis is one of the oldest and most widely used drugs in human history, with references to its use in ancient Chinese civilization dating to 2737 BC (Li, 1974). Medicinal use of cannabis continued freely in Western countries throughout the 1800s and into the mid-1900s. However, in 1942, cannabis was withdrawn from the United States Pharmacopoeia (USP), a compendium published annually by the United States Pharmacopoeia Convention providing standardization of therapeutic drugs. Great Britain and many other European countries prohibited cannabis use in 1971, by adopting policies proposed by the United Nations-led Convention on Psychotropic Substances (Ben Amar, 2006).

The marijuana plant (*Cannabis sativa*) contains over 450 known chemicals including more than 60 pharmacologically active compounds called cannabinoids. Cannabinoids have been shown to elicit a variety of physiological effects including impairments of short-term memory, attention span, and motor function, in addition to numerous psychological effects including paranoia, altered perception, and hallucinations (Martin, 1986). Delta-9-tetrahydrocannabinol (THC) is the primary psychoactive component in marijuana. This exogenous cannabinoid exerts a variety of modulatory effects on the immune system, the majority of which have been reported to be immunosuppressive (Klein *et al.*, 1998; Cabral and Dove Pettit, 1998; Cabral and Staab, 2005). In this capacity, THC affects a diverse array of immune cell types, including B

lymphocytes (Klein *et al.*, 1985), Natural Killer cells (Specter *et al.*, 1986), T lymphocytes (Zimmerman *et al.*, 1977), macrophages (Raz and Goldman, 1976; Friedman *et al.*, 1986) and macrophage-like cells (Puffenbarger *et al.*, 2000). Cannabinoid effects on cellular systems can occur by both receptor-mediated and non-receptor-mediated modes (Makriyannis *et al.*, 1990; Felder *et al.*, 1992; Berdyshev *et al.*, 2001; Price *et al.*, 2004). In terms of receptor-mediated action, two cannabinoid receptors have been identified and linked to THC effects on immune function. The first of these, CB₁, is found at highest levels in the central nervous system (CNS) with expression in the hippocampus, basal ganglia, cerebral cortex, amygdala and cerebellum correlating with observed neurological effects of cannabinoids (Matsuda *et al.*, 1990; Galiégue *et al.*, 1995; Herkenham *et al.*, 1991). CB₁ is present in the testis (Galiégue *et al.*, 1995) and also at low levels in various immune cells (Galiégue *et al.*, 1995; Daaka *et al.*, 1996; Waksman *et al.*, 1999). The second receptor, CB₂, is found primarily in immune cells (Munro *et al.*, 1993; Galiégue *et al.*, 1995) and appears to play a major role in immune modulation (Klein *et al.*, 1998; Cabral and Dove Pettit, 1998; Cabral and Staab, 2005). Both cannabinoid receptors are members of a large receptor superfamily known as G-protein coupled receptors (GPCRs), and characteristically have seven transmembrane domains and initiate cellular signal transduction through coupling with G proteins. Both CB₁ and CB₂ receptors are coupled to G_{i/o} proteins. Cannabinoid receptor signaling through these G proteins inhibits cyclic adenosine 3',5'-monophosphate (cAMP) and subsequent activation of protein kinase A (PKA) and activates mitogen activated protein kinase (MAP kinase)(Berdyshev, 2000). Cannabinoid receptor

signaling involves numerous second messengers and converges with multiple signal transduction pathways that are critical in the immune response.

Of the various immune cell populations affected by THC and other cannabinoids, macrophages and macrophage-like cells appear to be a major target (Munro *et al.*, 1993; Cabral *et al.*, 1995; Waksman *et al.*, 1999; Puffenbarger *et al.*, 2000). Ultrastructural abnormalities have been observed in alveolar macrophages of humans who have been heavy users of marijuana (Mann *et al.*, 1971) and in peritoneal macrophages of mice exposed *in vitro* to various concentrations of THC (Raz and Goldman, 1976). Various functional defects of alveolar and peritoneal macrophages from humans, rats or mice following *in vivo* or *in vitro* exposure to marijuana or THC also have been reported. These alterations have included decreases in cell motility, ability to spread *in vitro*, release of β -glucuronidase, phagocytosis of yeast particles, and inactivation of *Staphylococcus aureus* and *S. albus* (Huber *et al.*, 1975; Chari-Britton, 1976; McCarthy *et al.*, 1976; Drath *et al.*, 1979; Huber *et al.*, 1978; Lopez-Cepero *et al.*, 1986; Specter *et al.*, 1991; Tang *et al.*, 1992). In addition, THC has been reported to affect macrophage processing of soluble protein antigens (McCoy *et al.*, 1995; 1999). THC and other cannabinoids also have been shown to modulate the production of cytokine and chemokines by peripheral macrophages as well as microglia, the resident macrophages within the CNS (Klein *et al.*, 2000; McCoy *et al.*, 1995; Puffenbarger *et al.*, 2000; Srivastava *et al.*, 1998; Waksman *et al.*, 1999; Zheng *et al.*, 1992).

Macrophages play a critical role in both innate immunity as well as cell-mediated immunity. The primary functions of these cells are to navigate to sites of tissue damage

or infection, phagocytose cellular debris or pathogens, and stimulate lymphocytes and other immune cells to respond to the pathogen. Integral to this process is the recruitment of macrophages, which occurs early in the inflammatory process and is mediated by specific chemical stimuli. This migratory activity is distinctive from that of stimulus-independent random cellular motion (Lauffenburger and Horwitz, 1996; Mitchison and Cramer, 1996). The two major modes of stimulus-dependent cellular motility are chemokinesis and chemotaxis. Chemokinesis is a process whereby cells exhibit random, non-directional motion that is dependent on a chemical stimulant (Becker, 1977; Keller *et al.*, 1978). On the other hand, chemotaxis is a process in which cell motility is directed toward a concentration gradient of a chemical stimulant (Harris, 1953, 1954; Jin and Hereld, 2006; Kehrl, 2006). In this chemotactic process, macrophage interaction with chemoattractants not only initiates rapid and directed movement, but also is associated with a complex array of cellular events that includes changes in ion fluxes, alterations in integrin expression and avidity, production of superoxide anions, and secretion of lysosomal enzymes (Murdoch and Finn, 2000). “Classical” chemoattractants include bacterial-derived N-formyl peptides, the complement fragment peptides C5a and C3a, and lipids such as leukotriene B₄ and platelet-activating factor (Schiffman *et al.*, 1975; Goldman and Goetzl, 1982; Hanahan, 1986; Gerard and Gerard, 1994). Chemokines represent a second group of chemoattractants. These secreted cytokines range from 8- to 17-kD molecular mass and are selective for leukocytes *in vitro*, in addition to eliciting the accumulation of inflammatory cells *in vivo* (Baggiolini *et al.*, 1994, 1997; Kim, 2004; Le *et al.*, 2004). Chemokines have been categorized into four groups on the basis of their N-

terminal cysteine motifs - CXC (α -chemokines), CC (β -chemokines), CX₃X (δ -chemokines), and C (γ -chemokines) (Murphy, 2000) (**Table 1**). As in the case for cannabinoid receptors, the specific effects of chemokines on target cells are mediated by G protein-coupled receptors (Murdoch and Finn, 2000; Charo and Ransohoff, 2006). Ligation of chemokines with their cognate receptors initiates a series of signal transductional events that results in regulation of leukocyte trafficking in inflammation, tissue injury, tumor development and host response to infection (Charo and Ransohoff, 2006). Correlative to chemokine nomenclature, four families of chemokine receptors have been defined based on the chemokines they bind (CC, CXC, CX₃C, or C), followed by R for receptor and a number indicating the order in which they were discovered (Murphy, 2002) (**Table 2**).

G protein coupled receptors have been reported to cross-talk through a process known as heterologous desensitization. Chemokine receptor activity has been shown to be inhibited by the activation of numerous classes of GPCRs including opioid receptors and adenosine receptors (Zhang and Oppenheim, 2005). Activation of one type of GPCR can result in the phosphorylation of cytosolic C-terminal residues of other GPCRs by G protein coupled receptor kinases (GRKs) or other second messenger kinases. Phosphorylated receptors are unable to couple to G proteins; therefore, subsequent ligand binding does not initiate signal transduction. GPCR crosstalk may play an important role in the integration of diverse systems and the overall maintenance of immune homeostasis. Alternately, dysregulation of this crosstalk through the addition of exogenous compounds may constitute a significant element of drug-related immunosuppression. For example,

opioid desensitization of chemokine receptors has been directly implicated in immunosuppression consequent of opioid use (Rogers *et al.*, 2000). Macrophages also play an important role in pathogen recognition and clearance, and appear to be the primary effector cell in the immune response against *Acanthamoeba* infection (Marciano-Cabral *et al.*, 2003). The free-living amoebae of the genus *Acanthamoeba* are opportunistic pathogens with ubiquitous distribution. These microorganisms have been isolated world-wide from varied environments including soil, sewage, hospitals, seawater, drinking water treatment plants, and contact lenses (Marciano-Cabral *et al.*, 2003). *Acanthamoeba* spp. are also the causative agents of granulomatous amebic encephalitis (GAE), amebic keratitis and cutaneous amebiasis in humans.

Granulomatous amebic encephalitis is a progressive infection of the CNS that is often fatal, especially in the immunocompromised. *Acanthamoeba* access to the CNS may occur through the olfactory neuroepithelium following inhalation through the nasal passages or via hematogenous spread from a cutaneous lesion (Martinez *et al.*, 1985). Pathological findings from fatal cases of GAE reveal inflammation and severe hemorrhagic necrosis. The latter is presumably caused by a combination of direct destruction of brain tissue by feeding amoeba, secretion of lytic factors by amoeba, and prolonged induction of inflammatory cytokines such as tumor necrosis factor-alpha (TNF- α) and interleukin-1 (IL-1) (Marciano-Cabral *et al.*, 2000).

Table 1 – CC Chemokine Nomenclature

Symbol	Alternate Name(s)
CCL1	TCA3, I-309
CCL2	MCP-1 (monocyte chemotactant protein-1)
CCL3	MIP-1 α (macrophage inflammatory protein1-alpha)
CCL4	MIP-1 β (macrophage inflammatory protein1-beta)
CCL5	RANTES (Regulated upon activation normal T cell expressed and secreted)
CCL6	C10, MRP-1 (MIP-related protein-1)
CCL7	MCP-3 (monocyte chemotactant protein-3)
CCL8	MCP-2 (monocyte chemotactant protein-2)
CCL9	MIP-1 γ (macrophage inflammatory protein1-gamma), MRP-2 (MIP-related protein-2)
CCL10	now CCL9
CCL11	eotaxin
CCL12	MCP-5 (monocyte chemotactant protein-5)
CCL13	MCP-4 (monocyte chemotactant protein-4)
CCL14	HCC1 (human CC chemokine 1)
CCL15	MIP-5 (macrophage inflammatory protein-5), HCC-2
CCL16	LEC (liver expressing chemokine), Mnt-1 (monotactin-1)
CCL17	TARC (thymus and activation related chemokine)
CCL18	MIP-4 (macrophage inflammatory protein-4), PARC (pulmonary and activation regulated chemokine)
CCL19	MIP-3 β (macrophage inflammatory protein3-beta)
CCL20	MIP-3 α (macrophage inflammatory protein3-alpha), LARC (liver activation regulated chemokine)
CCL21	6Ckine, SLC (secondary lymphoid-tissue chemokine)
CCL22	MDC (macrophage derived chemokine)
CCL23	MIP-3 (macrophage inflammatory protein-3), MPIF-1 (myeloid progenitor inhibitory factor-1)
CCL24	eotaxin-2, MPIF-2 (myeloid progenitor inhibitory factor-2)
CCL25	TECK
CCL26	MIP-4 α (macrophage inflammatory protein4-alpha), eotaxin-3
CCL27	CTAK (cutaneous T-cell attracting chemokine)
CCL28	MEK (mucosal-associated epithelial chemokine)

Table 2- CC Chemokines and Chemokine Receptors

Receptor	Ligand(s)	Primary Functions
CCR1	CCL3, CCL5, CCL7, CCL8, CCL13-16, CCL23	T cell and monocyte migration, inflammation
CCR2	CCL2, CCL8, CCL7, CCL13	T cell and monocyte migration, inflammation
CCR3	CCL5, CCL7, CCL8, CCL11, CCL13, CCL15, CCL24, CCL26	Eosinophil, basophil, T cell migration
CCR4	CCL17, CCL22	T cell and monocyte Migration
CCR5	CCL3, CCL4, CCL5, CCL8, CCL14	T cell and monocyte migration
CCR6	CCL20	Dendritic cell migration
CCR7	CCL19, CCL21	T cell and dendritic cell migration
CCR8	CCL1, CCL4, CCL14	T cell trafficking
CCR9	CCL25	T cell trafficking
CCR10	CCL26-28	T cell trafficking

Adapted from Murphy *et al.*, 2002.

THC has been shown to increase host susceptibility to a wide variety of pathogenic bacteria, protozoa, and viruses (reviewed in Cabral and Dove Pettit, 1998). Experiments using an *in vivo* animal model of *Acanthamoeba* infections performed by Marciano-Cabral *et al.* (2001) demonstrated that (B₆C₃)F₁ mice injected with THC and exposed to *Acanthamoeba* intranasally had a higher incidence of mortality compared with vehicle treated animals. While macrophages are thought to be the primary immune cell involved in host response to *Acanthamoeba* infection, the specific effect of THC on macrophage function has not been fully defined. Histopathological analysis of brain slices from mice and humans with GAE show the formation of immune cell granulomas, containing macrophages and neutrophils, surrounding the amoebae (Marciano-Cabral *et al.*, 2003; Cabral *et al.*, in press 2007). These granulomas are conspicuously absent in THC-induced immunosuppression, indicating that THC may impair the macrophage migratory response.

The goal of the present study was divided into three main objectives. Initially, we sought to assess the effect of THC on the chemotactic response of murine peritoneal macrophages to RANTES/CCL5 (Regulated upon Activation Normal T cell Expressed and Secreted/ chemokine (C-C motif) ligand 5). Next, we proposed to examine the migratory response of peritoneal macrophages to *Acanthamoeba*, and determine whether THC affected this response. Finally, to expand the relevance of the previous studies we assessed the effect of THC on the migration of primary rat microglial cells to *Acanthamoeba*, thereby more closely modeling the process of a natural infection and

identifying a potential mechanism of THC-induced immunosuppression in CNS infections

We demonstrate that THC inhibits the chemotactic response of murine peritoneal macrophages to RANTES, a cognate chemokine receptor ligand. The inhibitory effect was shown to be linked functionally to the CB₂, suggesting that cannabinoids can signal through this receptor to trans-deactivate the chemokine receptor-mediated migratory response. Additionally, we show that peritoneal macrophages and microglia, the macrophages of the CNS, exhibit migration to *Acanthamoeba* and that this response is inhibited by THC. While the immunosuppressive effects of THC are extensive and likely involve numerous cell types and complex mechanisms and/or pathways, these studies serve to define, at least in part, the mechanism by which THC suppresses macrophage function.

Materials and Methods

Amoeba

Acanthamoeba culbertsoni (American Type Culture Collection, ATCC 30171, Manassas, VA) were cultured axenically in PYG medium containing 2% proteose peptone, 0.2% yeast extract, and 0.1M glucose at 37 °C.

Acanthamoeba sp. strain JH1 was isolated from an immunosuppressed patient at Johns Hopkins Hospital (Marciano-Cabral *et al.*, 2003b). This clinical isolate was obtained postmortem from a cutaneous lesion of an immune suppressed patient who had undergone a prior renal transplant. Transmission electron microscopy studies identified the presence of gram negative rods within the cytoplasmic vacuoles of the amoeba.

Acanthamoeba JH1 were cultured in Oxoid medium with serum and hemin and without antibiotics.

Amoeba conditioned medium (ACM) was obtained by culturing amoeba in PYG medium in 1L flasks at 37°C with constant agitation for 4 days. The amoebae were harvested by centrifugation. The pelleted amoebae (10^9) were suspended in 5 ml Hank's Balanced Salt Solution (HBSS) and incubated at 37°C for 24 h. The following day the cultures were centrifuged at 489 x g (2,500 RPM, Eppendorf 5810 R) for 20 min. The supernatant was decanted and centrifuged again at 16100 x g (13,100 RPM, Eppendorf 5415 D) for 10 min to remove any remaining debris and was designated amoeba

conditioned medium (ACM). Protein concentration of ACM was determined by Bradford assay (Bradford, 1976).

Cells

Thioglycollate-elicited peritoneal macrophages were obtained by injecting B₆C₃(F₁) or C57BL/6 mice intraperitoneally with 1 ml 10% Brewer's yeast thioglycollate. Five days later, cells were harvested by aseptic peritoneal lavage with HBSS supplemented with penicillin [200U/ml] and streptomycin [200 µg/ml]. The peritoneal exudate cells were screened for purity for macrophages by FACScan analysis using monoclonal antibody for the murine macrophage marker F4/80 (Serotec, Kidlington, Oxford, UK). Cells that were greater than 95% positive for F4/80 were used in studies. Macrophages (10⁷ /ml) in RPMI 1640 medium (Cellgro, Herndon, VA) lacking serum and supplemented with 1% L-glutamine, 1% nonessential amino acids, 1% MEM vitamins, 0.01M HEPES and penicillin [100 U/ml]/streptomycin [100 µg/ml]/fungizone [0.25 µg/ml]) were used in chemotaxis assays.

Primary microglia cultures were prepared from neonatal Sprague-Dawley (Zivik-Miller Laboratory, Zeleinope, PA) rat pups (1-2 days postpartum). Following sacrifice, the cerebral cortices were isolated and placed in dissection saline containing 2.8% (v/v) stock dissection HEPES (352mM HEPES in dH₂O), 5% stock dissection saline (137 mM NaCl, 5.3mM KCl, 0.17mM Na₂PO₄-7H₂O, 0.22 mM KH₂PO₄, and 0.0012g/L Phenol Red in dH₂O), 5% stock Glucose/Sucrose solution (6g/L glucose, 15 g/L sucrose), and penicillin [100 U/ml]/streptomycin [100 µg/ml]. Subsequently, the surrounding

meninges were removed, the remaining tissue manually disrupted and incubated with porcine pancreas derived trypsin (Sigma) for 10 min. The tissue homogenate was suspended in DMEM containing 10% FBS and supplemented with 1% L-glutamine, 1% nonessential amino acids, 1% MEM vitamins, 0.01M HEPES, and penicillin (100U/mL)/streptomycin (100µg/mL) and fungizone (0.25 µg/mL) (complete DMEM) and filtered through a 70µm nylon cell strainer. The strained suspension then was centrifuged at 1,000 RPM for 30 min at 4°C. The mixed glial cell suspension containing astrocytes and microglia were seeded in 172cm² tissue culture flasks (Greiner, Monroe, NC) and cultured at 37°C and 5% CO₂ in complete DMEM medium. The medium was replaced the following day with warmed complete DMEM and the cells allowed to grow for 14-21 days. To recover primary microglia, the mixed glial cultures were agitated at 180 RPM on an orbital shaker for 2 h at 37°C. Alternately, primary rat mixed glial cultures containing astrocytes and microglia were obtained from Dr. Jameel Dennis, Department of Anatomy and Neurobiology following immunopanning using anti-oligodendrocyte antibodies to isolate oligodendrocytes from cortical cultures (Fox *et al.*, 2003).

Mice

Six to eight-week old female (B₆C₃)F₁ and C57BL/6 mice were obtained from Taconic Laboratories (Hudson, NY). CB₂ (-/-) mice on a C57BL/6 background were obtained from Dr. Nancy E. Buckley (California Polytechnic University, Pomona, CA). CB₂ deficiency was confirmed by polymerase chain reaction (PCR) as described (Buckley *et*

al., 2000; Chuchawankul *et al.*, 2004). Phenotypic characterization was performed by Buckley *et al.* (2000) and reportedly CB₂ knockout mice are healthy and fertile with no significant alterations in immune cell populations as measured by FACS analysis, indicating that the knockout did not affect immune cell development or differentiation. Therefore, peritoneal macrophages from these knockout mice should be phenotypically comparable to wild type peritoneal macrophages and any effect, or absence thereof, of cannabinoids on macrophage immune function should be the result of a measurable scientific phenomenon and not an artifact.

Animals were quarantined for one week prior to initiation of experiments and were used as a source of peritoneal macrophages. All animal procedures were conducted in accordance with guidelines established by the Virginia Commonwealth University Institutional Animal Care and Use Committee (IACUC).

Drugs

Delta-9-tetrahydrocannabinol (THC; CB₁ K_i = 40.7nM; CB₂ K_i= 36.4 nM), a low efficacy agonist for CB₁ and CB₂, was obtained from the National Institute on Drug Abuse (Rockville, MD). Additional cannabinoid analogs included the CB₁ and CB₂ high efficacy agonist CP55940 (CB₁ and CB₂ K_i = 1.37 nM) and the highly selective CB₂ ligand O-2137-2 (CB₁ K_i = 2,700nM, CB₂ K_i = 11nM). The highly selective CB₁ agonist ACEA (CB₁ K_i = 1.4 nM) that displays > 1,400-fold selectivity over CB₂ was purchased from Tocris Cookson, Inc. (Ellisville, MO). The CB₁ and CB₂ antagonists SR141716 (CB₁ K_i =11.8 nM, CB₂ K_i = 13,200 nM) and SR144528 (CB₁ K_i = 437nM, CB₂ K_i = 0.6nM), respectively, were obtained from Sanofi Recherche (Montpellier, France).

The review by Howlett *et al.* (2002) provides a comprehensive summary of cannabinoid receptor ligand binding affinities and methodologies and was utilized in preparation of **Table 3**. Ligand binding data was obtained from *in vitro* radiolabeled ligand displacement assays performed on membrane preparations from either from cell lines transfected with cloned receptors or tissues (brain tissue preparations for CB₁ or spleen tissue preparations for CB₂).

Stock solutions of cannabinoids (10^{-2} M) were prepared in 100% ethanol and stored at -20°C . Experimental concentrations were obtained by dilution of cannabinoid stock solutions in assay medium (RPMI-1640 for peritoneal macrophages or DMEM for primary microglia) to yield a final ethanol concentration of 0.01%. Vehicle controls consisted of 0.01% ethanol in medium.

Chemotaxis Assay

Chemotaxis was measured using transwell inserts pre-loaded in 35 mm standard tissue culture plates (Corning Inc., Corning, NY), in which the upper and lower compartments were separated by a polycarbonate filter with 8 μm pores (Corning Inc., Corning, NY). Peritoneal macrophages (1×10^7 /ml) were pre-incubated in RPMI 1640 lacking serum and containing vehicle (0.01% ethanol) or cannabinoid ($10^{-6}\text{M} - 10^{-12}\text{M}$) for 3 h at 4°C . This time regimen for drug exposure was obtained through initial optimization experiments. Serum was omitted from the culture medium since it contains lipids and other factors that have the capacity to stimulate macrophage migration that could confound interpretation of migratory responses as attributable to RANTES. For

Table 3- Cannabinoid Receptor Ligands

Cannabinoid	Classification	Dissociation Constant (K_i)	
		$CB_1 k_i$	$CB_2 k_i$
THC	Low efficacy CB_1/CB_2 agonist	40.7nM	36.4 nM
CP55940	High efficacy CB_1 and CB_2 agonist	1.37nM	1.37nM
ACEA	CB_1 selective agonist	1.4nM	>2000nM
O-2137	CB_2 selective agonist	2,700nM	11nM
SR141716A (SR1)	CB_1 selective antagonist	11.8nM	13200nM
SR144528 (SR2)	CB_2 selective antagonist	437nM	0.6nM

Abbreviations- THC, (δ^9 -tetrahydrocannabinol); CP55940, ((-)-cis-3-[2-Hydroxy-4-(1,1-dimethylheptyl)phenyl]-trans-4-(3-hydroxypropyl)cyclohexanol); ACEA, (*N*-(2-Chloroethyl)-5*Z*,8*Z*,11*Z*,14*Z*-eicosatetraenamamide); O-2137, ((1*R*,3*R*)-1-[4-(1,1-Dimethylheptyl)-2,6-dimethoxyphenyl]-3-methylcyclohexanol); SR141716A, (5-(4-Chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-*N*-(1-piperidyl)pyrazole-3-carboxamide); SR144528, ((1*S*-endo)-5-(4-Chloro-3-methyl-phenyl)-1-((4methylphenyl)methyl)-*N*-(1,3,3-trimethylbicyclo(2.2.1)hept-2-yl)-1*H*-pyrazole-3-carboxamide)

Adapted from Howlett *et al.*, 2002

experiments using antagonists, cells were exposed to SR141716A (SR1)(10^{-6} M) or SR144528 (SR2)(10^{-6} M) for 30 min prior to treatment with THC or CP55940 for 3h. Following vehicle or cannabinoid treatment, 100 μ l of drug- or vehicle-treated cell suspension (10^6 cells) were placed in the upper chamber of the transwell insert. For assessment of chemotaxis (directed migration against a chemokine concentration gradient) the lower compartment was loaded (600 μ l) with medium containing murine RANTES (1 ng/ml; R&D Systems, Minneapolis, MN). This concentration of RANTES was selected based on preliminary titration for a chemoattractant response that approximated a mid-point in the linear phase of the dose-response curve. For assessment of chemokinesis (enhanced random migration to chemokine), RANTES (1 ng/ml) was included in both the top and bottom chambers to eliminate the chemoattractant concentration gradient. In addition, for a select number of experiments, RANTES was eliminated from both chambers (**Fig. 1**). The assembled migration plate chamber system was incubated (1-2 h) at 37°C in a 5%CO₂ atmosphere. To determine the number of cells that migrated to the bottom chamber, the upper chamber (i.e., polycarbonate filter) was removed and video still images (1mm²) in five random fields of each bottom chamber were captured using an Olympus CK2 inverted microscope (Opelco, Washington, DC) with an attached XV-GP230 digital video camera (Panasonic, Yokohama, Japan) interfaced to a Dell Dimension XPS1450 computer using Videum 100 hardware and Window NT software (Winnov, Sunnyvale, CA). The number of cells migrating into the bottom compartment/transwell plate was manually enumerated and calculated as the sum of the five 1 mm² fields and was represented as cells/mm²/well. Each sample group was

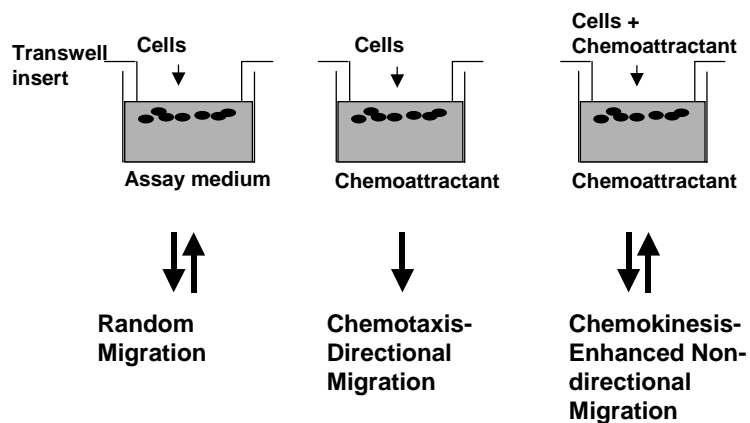


Figure 1- Transwell Migration Assay. Cells are loaded into transwell inserts and assessed for migration to the bottom well. When the top and bottom wells contain assay medium only, any migration is random. When the bottom well contains a chemoattractant, cell migration occurs in response to a concentration gradient. This migration, chemotaxis, is compared to migration in which the chemoattractant substance is placed in both the top and bottom wells. Under these conditions, no concentration gradient exists and any migration that occurs as a response to exposure to a chemostimulus is called chemokinesis.

run in duplicate and each experiment was performed in triplicate. Migration for each sample group was represented as the mean (\pm SD) of the total number of migrating cells counted in five fields of duplicate wells. A greater than 2-fold increase in cell migration to the chemoattractant RANTES in the lower compartment as compared to that in the absence of RANTES in the lower compartment was indicative of a positive response.

Immunoprecipitation of CCR5

Whole cell protein lysate (400 μ g) in NP-40 lysis buffer containing protease inhibitors was precleared for 30 min using 0.25 μ g normal mouse control IgG (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and 20 μ l Protein A/G Plus Agarose (Santa Cruz Biotechnology). Precleared protein lysates were incubated with 2 μ g CCR5 antibody (CCR5 D6, Santa Cruz Biotechnology) for 2h at 4°C, followed by 12h incubation (4°C) with Protein A/G Plus Agarose. The beads were collected by centrifugation (3000 RPM, 4°C, 1 min) and washed four times in room temperature phosphate-buffered saline. The precipitates were resuspended in 40 μ l electrophoresis sample buffer, heated for 3 min at 95°C, and resolved on a 10% SDS-polyacrylamide gel.

Isolation of Cannabinoid Receptor DNA from Plasmid Constructs

In order to obtain CB₁ DNA to serve as a positive control in Real Time RT-PCR assays, DH5 α *E.coli* transfected with a pCD mammalian expression vector containing SKR6 (rat CB₁ DNA sequence) from Dr. L. Matsuda (Medical University of South Carolina, Matsuda *et al.*, 1990) were cultured in LB broth containing ampicillin (100

$\mu\text{g/ml}$). The pCD-SKR6 plasmid was isolated using the Midi Prep Kit (Qiagen, Valencia, CA). Following a restriction digest with EcoRI (Invitrogen) and BamHI (Invitrogen), the cut plasmid was subjected to agarose gel electrophoresis (0.8% agarose gel; OmniPure Agarose). The 2.4 kb fragment containing the rat CB₁ DNA sequence was cut from the agarose gel, isolated and purified using the QIAquick Gel Extraction Kit (Qiagen).

Purified CB₂ DNA from the pUC18 vector containing murine CB₂ sequence from Dr. T. Bonner (NIMH, Bethesda, MD) was similarly obtained. Both purified CB₁ and CB₂ DNA products were stored at -20°C in dH₂O and the concentration determined using a BioPhotometer (Eppendorf, Westbury, NY). Each product was sequenced at the MCV-VCU Nucleic Acids Research Facility core lab using an Automated DNA sequencer. DNA samples were further analyzed by BLAST (nucleotide-nucleotide BLAST, NCBI, Bethesda, MD) to confirm sequence identity.

Multiprobe Ribonuclease Protection Assay

Total RNA prepared from peritoneal macrophages using TRIzol reagent (Invitrogen, Carlsbad, CA) was redissolved after isopropanol precipitation directly in 1X hybridization buffer (BD Biosciences/PharMingen, San Diego, CA). A Riboquant Multiprobe Ribonuclease Protection Assay (RPA) was used to assess for levels of murine chemokine receptor mRNA (mcr-5 probe template set; BD Biosciences/PharMingen). The ribo-probes were labeled with ^{32}P [UTP] (MP Biomedicals, Aurora, OH) to a specific activity of greater than 3,000Ci/mmol. The isolated RNA samples then were hybridized with the probe overnight at 56°C and the protected fragments were resolved on a 6%

polyacrylamide gel containing 6M urea. Imaging of the protected fragments was performed using a 445 SI Phosphorimager (Molecular Dynamics, Sunnyvale, CA). The pixel intensity of each band was quantified using ImageQuant 4.1 software (Molecular Dynamics) and the amount of chemokine receptor mRNA was normalized for loading by dividing the pixel value for the chemokine receptor band by the sum of the pixel values for the mRNAs of the housekeeping genes, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and a ribosomal protein, L32.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Real-time Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR), using SYBR Green for detection and primers for CB₁ and CB₂ and GAPDH, was used to assess for the presence of CB₁ and CB₂ mRNA, and for constitutively expressed GAPDH mRNA, respectively. Total RNA from peritoneal macrophages was prepared using TRIzol reagent (Invitrogen) according the manufacturer's instructions. The RNA then was isolated by chloroform:isopropanol extraction and resuspended in 50 µl PCR grade water. The isolated RNA was treated with RNase-free DNase I Amplification grade (Invitrogen) to remove residual genomic DNA. The reverse transcription (RT) step was performed in a Bio-Rad iCycler (BioRad, Richmond, CA) using the SuperScript III First-Strand Synthesis System (Invitrogen) that included random hexamers as primer to generate complementary DNA (cDNA). SYBR Green real-time PCR was performed using the RT² PCR Primer Set for mouse CB₁ (Cnr 1: PPM04603A) or CB₂ (Cnr 2: PPM04826A) and GAPDH (GAPDH:PPM02946A) as described by the manufacturer

(SuperArray Bioscience Corp., Frederick, MD). Briefly, each 25 μ l PCR mix consisted of 12.5 μ l 2X RT² Real-Time SYBR Green PCR Master Mix (SuperArray), 1.0 μ l first strand cDNA template, and 1.0 μ l RT² PCR Primer Set brought to a final volume of 25 μ l with DEPC-treated water. Tubes containing the PCR mix were placed in a SmartCycler (Cepheid, Sunnyvale, CA) and PCR was performed using the following program: 95°C, 15 min; 40 cycles of (95°C, 30 sec; 55°C, 30 sec; and 72°C, 30 sec). The resulting PCR products were visualized by electrophoresis (100V) using 4% OmniPur Agarose PCR Plus (VWR, West Chester, PA) gel in 1X Tris-Borate-EDTA (TBE) buffer. A pCD- and a pUC18-mCB₂ plasmid template served as positive PCR controls for CB₁ and CB₂, respectively. Using this approach, amplification products of 167 bp and 207 bp were generated for CB₁ and CB₂, respectively.

Scanning Electron Microscopy

Transwell inserts containing polycarbonate filters with 8 μ m pores were removed from the chemotaxis plates. The top and bottom sides of the filter were washed gently to remove non-adherent cells and fixed with 2.5% glutaraldehyde. Post-fixation with 2%OsO₄ was followed by dehydration in a graded series of alcohol washes (Pettit *et al.*, 1996). The filters containing adherent macrophages were subjected to critical point drying, coated with gold and viewed with a JEOL scanning electron microscope (20kV).

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Immunoblotting

Peritoneal macrophages were washed with room temperature PBS (2x) then incubated in NP-40 lysis buffer containing Protease Inhibitor Cocktail (100:1) (Sigma, St. Louis, MO; 4-(2-aminoethyl) benzenesulfonyl fluoride, pepstatin A, E-64, bestatin, leupeptin, aprotinin) for 30 min on ice. The cell lysates were centrifuged at 10,000 x g for 15 min, at 4⁰C. The pellets then were discarded, the supernatants containing cellular proteins saved, and concentrations were determined by the Bradford assay. Protein samples (40 µg/sample) were separated on a 12% polyacrylamide gel and transferred to a Transblot Transfer nitrocellulose membrane (BioRad, Hercules, CA). The membranes were incubated individually with anti-CCR1, anti-CCR5, and anti-CB₂ antibody. The antibody to CCR1 (CKR1 H-52 rabbit polyclonal IgG, Santa Cruz Biotechnology) was directed against the extracellular amino terminus whereas the antibody to CCR5 (CKR5 D-19 mouse monoclonal IgG, Santa Cruz Biotechnology) was directed against the carboxy terminal domain. The antibody to CB₂ (CB₂ M15 goat polyclonal IgG, Santa Cruz Biotechnology) was raised against peptide mapping of the C terminus of mouse CB₂.

Statistical Analysis

Analysis of variance (ANOVA) was performed using Dunnett's test and was followed by a Student's t-test to allow for comparison of each sample to the vehicle. Comparisons between treatment groups were performed using Bonferroni's t test.

Results

Murine Thioglycollate-Elicited Peritoneal Macrophages Express the Chemokine Receptors CCR1 and CCR5 and the Cannabinoid Receptor CB₂

RANTES binds to the chemokine receptors CCR1, CCR3, and CCR5 (Murphy, 2002; Bajetto *et al.*, 2002; Charo *et al.*, 2006). Thus, in order to determine the CC chemokine receptor gene expression profile of (B₆C₃)F₁ and C57BL/6 murine thioglycollate-elicited peritoneal macrophages, a multiprobe RNase Protection assay was employed. Using a template set for CC chemokine receptors, it was demonstrated that the predominant chemokine receptor mRNAs detected for (B₆C₃)F₁ mice were those for CCR1, CCR2 and CCR5 (**Fig. 2**). C57BL/6 mice contained approximately equal levels of mRNA for CCR1 and CCR5 but, in contrast to (B₆C₃)F₁ mice, contained low levels of mRNA for CCR2. Because RANTES is a major agonist for CCR1 and CCR5, but not CCR2, the presence of protein for the former two receptors also was determined (**Fig. 3**). Consistent with the mRNA data, approximately equivalent levels of protein for CCR1 and CCR5 were detected in thioglycollate-elicited macrophages of (B₆C₃)F₁ and C57BL/6 mice.

SYBR Green RT-PCR was employed to assess for the presence of CB₂ mRNA in peritoneal macrophages from (B₆C₃)F₁ and C57BL/6 mice. Prior to performing PCR, CB₁ and CB₂ cDNA was isolated from plasmid preparations of DH5 α *E. coli* that had been transfected with mammalian expression vectors containing CB₁ and CB₂ DNA sequences (pCD-rSKR6 and pUC18-mCB₂, respectively) for use as positive controls in Real-time RT-PCR. The isolated plasmids were digested with restriction enzymes EcoRI

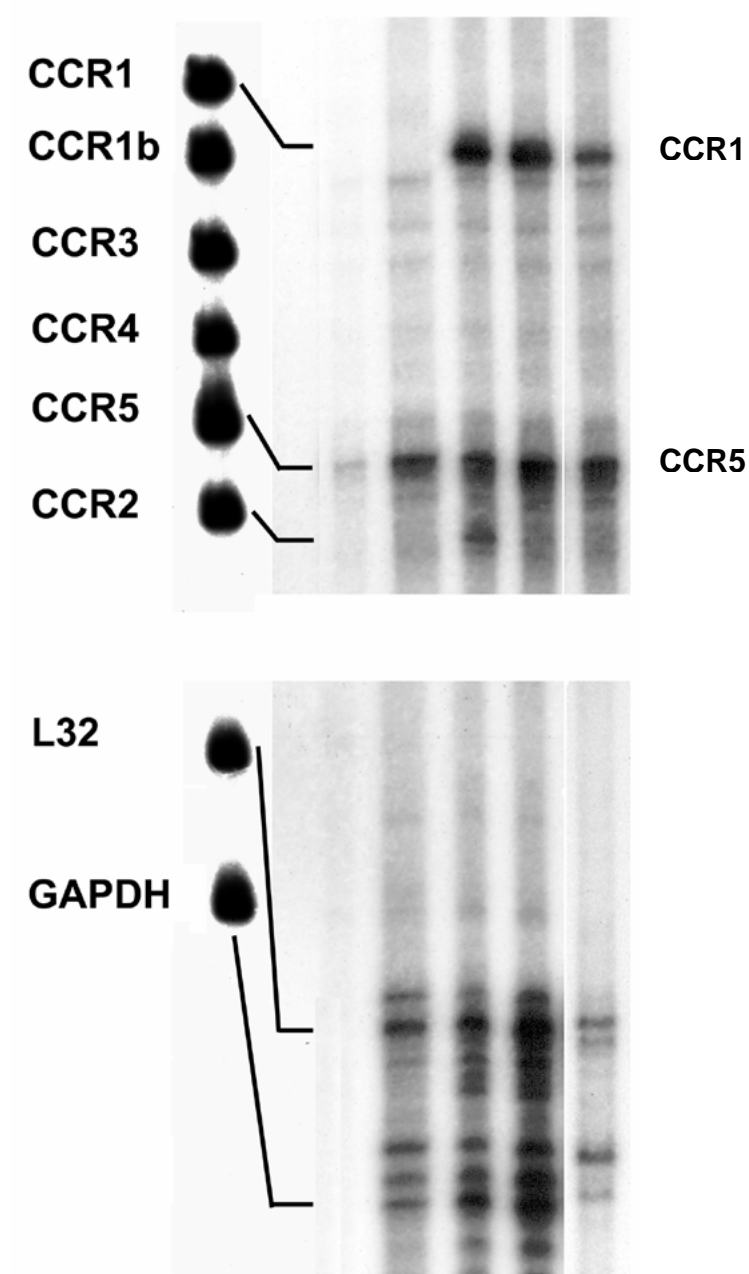


Figure 2 – CCR1 and CCR5 mRNA Are Expressed by Peritoneal Macrophages. RNase Protection Assay demonstrated that peritoneal macrophages express predominantly CCR1 and CCR5, with low levels of CCR2 expression. P- Undigested probe, Y- yeast control (-), M- mouse control (+), 1-(B₆C₃)F₁ peritoneal macrophages, 2- C57BL/6 peritoneal macrophages, 3- CB2^{-/-} BM cell line (Raborn *et al.*, 2007).

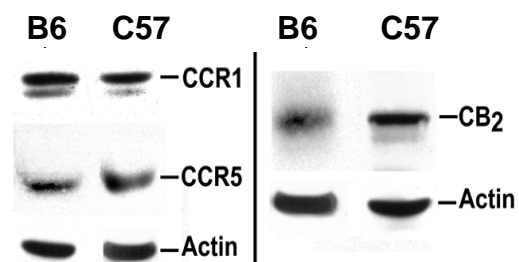


Figure 3– Peritoneal Macrophages Express CCR1, CCR5, and CB₂ at the Protein Level. Whole cell lysates from (B₆C₃)F₁ and C57BL/6 murine peritoneal macrophages were separated by SDS-PAGE followed by Western immunoblot analysis with antibodies to CCR1, CCR5, and Actin. B6- (B₆C₃)F₁, C57- C57BL/6

and BamHI to release cannabinoid receptor DNA sequences. The digests were subjected to agarose gel electrophoresis and CB₁ and CB₂ DNA was extracted from the gel and purified (**Fig. 4**)(CB₁ fragment 2.4kb, CB₂ fragment 1.3kb). A 207 bp amplicon, consistent with the fragment size predicted for the CB₂, was detected from total RNA of peritoneal macrophages (**Fig. 5**). Western immunoblot analysis using a murine CB₂ domain-specific antibody confirmed the presence of CB₂ in murine peritoneal macrophages at the protein level (**Fig. 3**). Furthermore, using SYBR Green RT-PCR (**Fig. 5**) and Western immunoblot analysis (Data not shown) the absence of CB₁ mRNA or protein expression in thioglycollate-elicited peritoneal macrophages from (B₆C₃)F₁ and C57BL/6 mice was demonstrated.

Treatment with THC in vivo Results in Inhibition of the Chemotactic Response of Murine Peritoneal Macrophages to RANTES in vitro.

(B₆C₃)F₁ mice were inoculated with thioglycollate and 5 days later were administered a single intraperitoneal injection of vehicle (ethanol:emulphor:saline, 1:1:18) or THC (25 mg/kg or 50 mg/kg). Peritoneal macrophages were harvested 24h later and were subjected to migration assay. *In vivo* administration of 25 mg/kg or 50 mg/kg THC resulted in a significant and greater than 50% inhibition of cell migration in response to RANTES as compared to that observed for cells of mice receiving vehicle (**Fig. 6**). No significant differences in migration were obtained between vehicle and drug treated cells when RANTES was placed in both the top and bottom compartments to

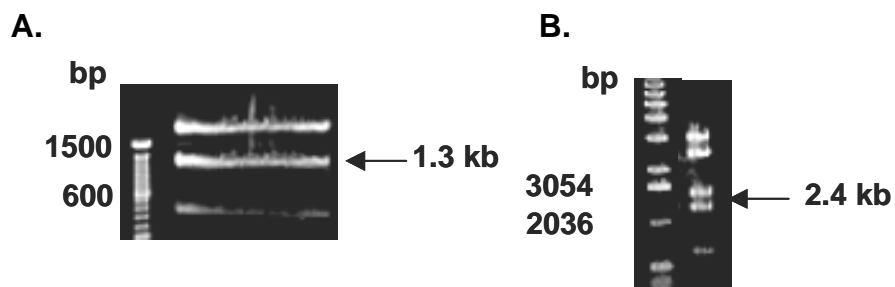


Figure 4- Generation of Real-time RT-PCR CB₁ and CB₂ positive control DNA. pUC18-mCB₂ was digested with EcoR1 and BamH1 to release mCB₂ DNA (1.3 kb). B. pCD-sKR6 (rCB₁) was digested with EcoR1 and BamHI to release 2.4 kb fragment of rCB₁ DNA.

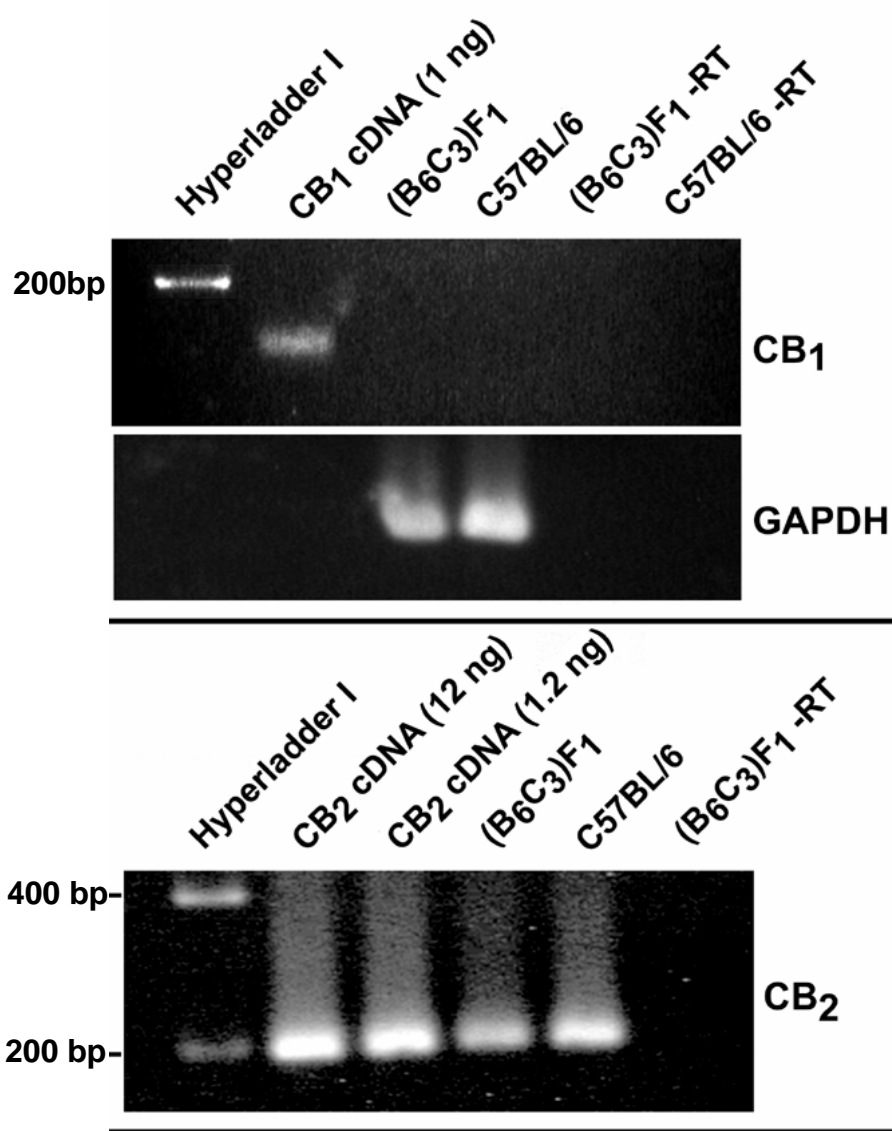


Figure 5- Cannabinoid Receptor mRNA Expression by Peritoneal Macrophages. Real-time RT-PCR demonstrated that peritoneal macrophages express primarily CB₂ mRNA. CB₁ and CB₂ DNA as well as GAPDH were used as positive controls.

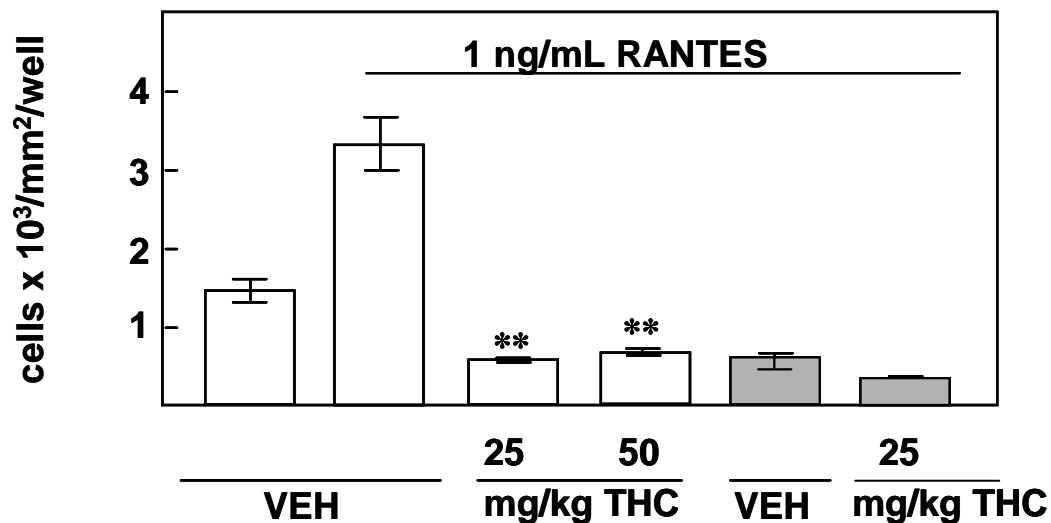


Figure 6- Treatment *in vivo* with THC Results in Inhibition of the Chemotactic Response to RANTES. (B₆C₃)F₁ mice were injected intraperitoneally with 10% thioglycollate to elicit macrophages. Five days later, the mice were injected intraperitoneally with vehicle (VEH) (1:1:18, ethanol:emulphor:saline) or THC (25 mg/kg or 50 mg/kg). Migration of macrophages to 1ng/ml RANTES was assessed *in vitro* using transwell tissue culture inserts. Results are presented as the mean ± SD. For RANTES placed only in the bottom chamber, SD was compared with that of vehicle-treated macrophages exposed to RANTES in the bottom chamber. For RANTES placed in both chambers (shaded bars), SD was compared with that of vehicle-treated macrophages exposed to RANTES in both chambers. When RANTES was added only to the bottom compartment, THC as compared to the vehicle control exerted a major inhibitory effect on cell migration to the bottom compartment. When RANTES was added to both the upper and lower compartments to eliminate the chemoattractant concentration gradient, THC as compared to the vehicle control did not result in significant inhibition of cell migration to the bottom well. These results indicate that THC inhibits directed migration (i.e., chemotaxis) to a RANTES concentration gradient rather enhancement of random movement (i.e., chemokinesis) to RANTES. **p<0.01.

eliminate the RANTES concentration gradient. These results are consistent with THC as exerting an inhibitory effect on the macrophage chemotactic response to RANTES.

Treatment with THC and CP55940 in vitro Results in Inhibition of the Chemotactic Response of Murine Peritoneal Macrophages to RANTES

In order to determine whether THC exerted a direct effect on macrophages, *in vitro* exposure experiments were performed. THC treatment of (B₆C₃)F₁ murine peritoneal macrophages *in vitro* resulted in a significant inhibition of the chemotactic response to RANTES (**Fig. 7**). Cells treated with vehicle exhibited a minimal level of migration (i.e., approximately 1,600 cells/mm²/well) to the bottom compartment in the absence of RANTES. In contrast, when RANTES was added to the bottom compartment to establish a chemokine concentration gradient, a nearly five-fold increase (i.e., in excess of 5,000 cells/mm²/well) was obtained for macrophages treated with vehicle. Treatment of macrophages with THC (10⁻⁶M – 10⁻¹²M) resulted in a significant inhibition of migration in response to RANTES. THC, at a concentration as low as 10⁻¹²M, exerted a major inhibitory effect on cell migration, with numbers of cells in the bottom compartment approximating those for cells treated with vehicle and not exposed to RANTES. Again, the inhibitory effect of THC on macrophage migration was at the level of chemotaxis rather than chemokinesis. When RANTES was added to both the upper and lower compartments to eliminate the chemoattractant concentration gradient to allow for assessment of random migration to chemokine, approximately 1,000 cells/mm²/well were obtained for peritoneal macrophages treated with vehicle. Treatment of these cells

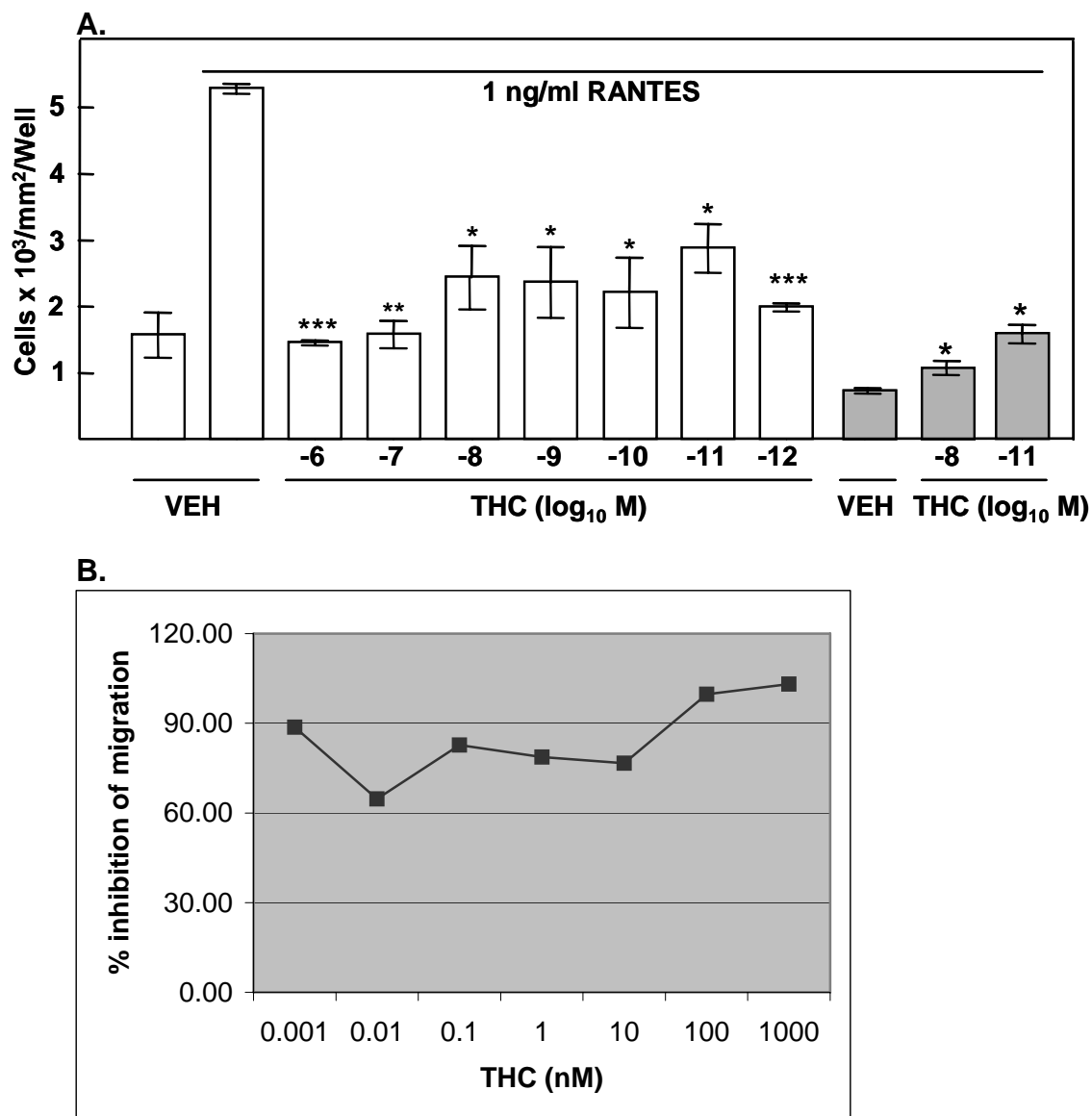


Figure 7 - Treatment *in vitro* with THC Results in Inhibition of the Chemotactic Response to RANTES. A. Migration of peritoneal macrophages to 1ng/ml RANTES was assessed following *in vitro* treatment (3h) with THC (10^{-6} to 10^{-12} M) or vehicle (VEH) (0.01% ethanol). Treatment with the partial agonist THC resulted in inhibition of chemotaxis. Results are presented as the mean \pm SD. For RANTES placed only in the bottom chamber, SD was compared with that of vehicle-treated macrophages exposed to RANTES in the bottom chamber. For RANTES placed in both chambers (shaded bars), SD was compared with that of vehicle-treated macrophages exposed to RANTES in both chambers. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. B. Results presented as Percent Inhibition of migration.

with 10^{-8}M or 10^{-11}M THC did not result in significant inhibition of this random movement. Rather, a slight augmentation in random migration to the bottom compartment was recorded.

Experiments performed with THC were replicated using CP55940, a high efficacy agonist at CB_1 and CB_2 (**Fig. 8**). Again, a minimal level in cell migration was observed for control wells. Approximately 1,500 cells/ mm^2 /well were recorded when vehicle-treated cells were placed in the top compartment in the absence of RANTES in the bottom compartment. An approximate four-fold increase in the number of peritoneal macrophages treated with vehicle was obtained when RANTES was placed in the bottom compartment to establish a chemoattractant gradient. Treatment of cells with CP55940 ($10^{-6}\text{M} - 10^{-12}\text{M}$) resulted in a significant concentration-related decrease in migration in response to RANTES. A greater than 50% inhibition in migration was obtained for cells treated with CP55940 at $10^{-6}\text{M} - 10^{-9}\text{M}$ as compared to vehicle control. CP55940 as compared to vehicle did not affect macrophage migration when RANTES was placed both in the top and bottom compartments to eliminate the chemoattractant gradient, indicating that the effect of CP55940 on migration was at the level of chemotaxis rather than chemokinesis.

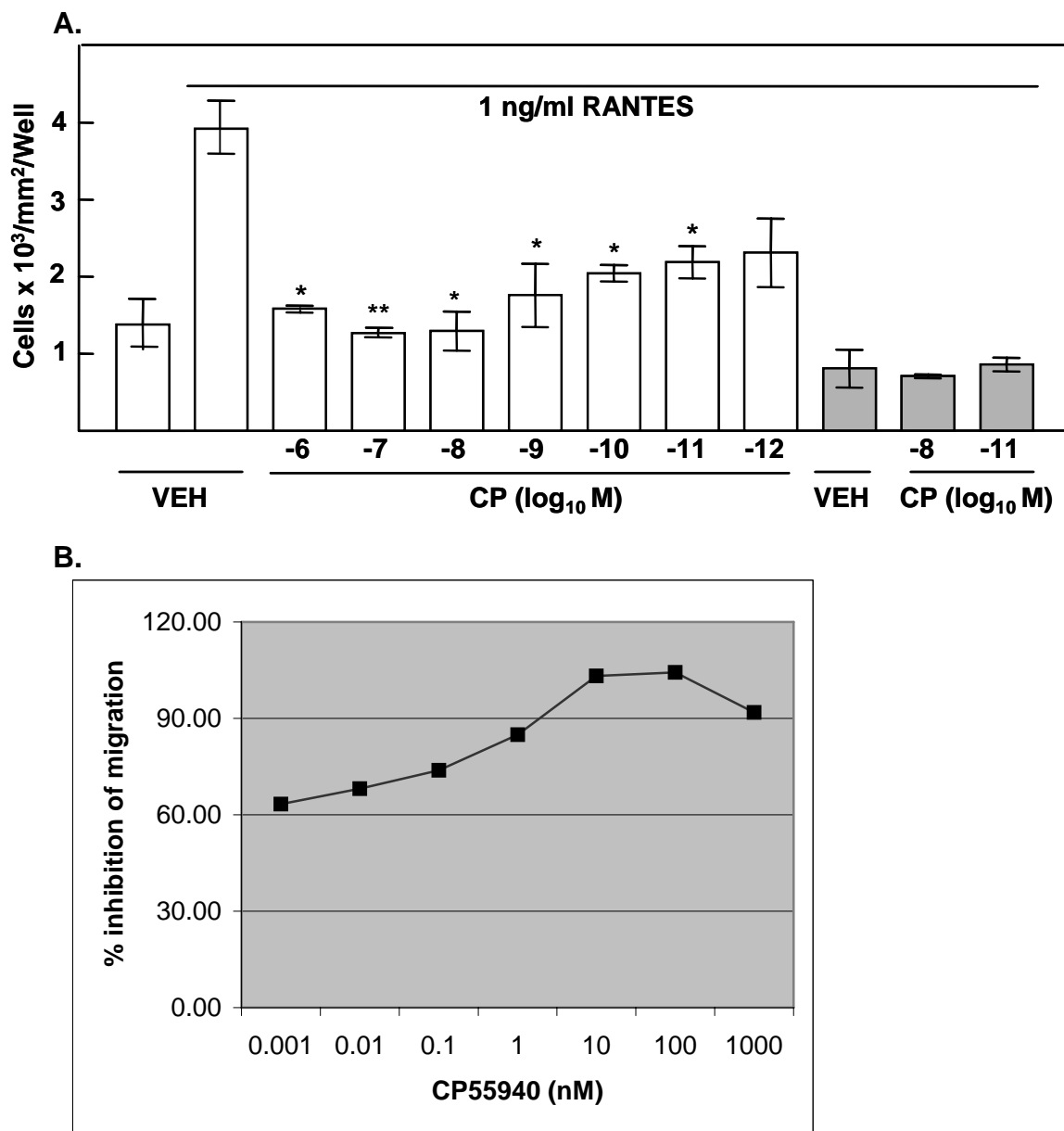


Figure 8- Treatment *in vitro* with CP55940 Results in Inhibition of the Chemotactic Response to RANTES. A. Migration of peritoneal macrophages to 1ng/ml RANTES was assessed following *in vitro* treatment (3h) with CP55940 (CP) (10^{-6} to 10^{-12} M) or vehicle (VEH) (0.01% ethanol). Treatment with the full agonist CP55940 resulted in a robust dose-related inhibition of chemotaxis to RANTES. Results are presented as the mean \pm SD. For RANTES placed only in the bottom chamber, SD was compared with that of vehicle-treated macrophages exposed to RANTES in the bottom chamber. For RANTES placed in both chambers (shaded bars), SD was compared with that of vehicle-treated macrophages exposed to RANTES in both chambers. * $p < 0.05$, ** $p < 0.01$. B. Results presented as Percent Inhibition of Migration.

The CB₂-selective Ligand O-2137 Exerts a Robust Inhibitory Effect on the Murine Peritoneal Macrophage Chemotactic Response to RANTES

The concentration-related inhibitory effect most evident using CP55940 on the chemotactic response of murine peritoneal macrophages to RANTES implicated a role for a cannabinoid receptor in this process. In order to obtain insight as to the cannabinoid receptor linked to the inhibitory effect, macrophages from (B₆C₃)F₁ mice were treated with compounds exhibiting selective high affinity binding to the CB₁ or the CB₂ prior to assessment of the chemotactic response to RANTES. Treatment of macrophages with the highly selective CB₂ ligand O-2137 resulted in a profound and significant concentration-related inhibition in the chemotactic response to RANTES (**Fig. 9**). For drug concentrations of 10⁻⁶M – 10⁻⁸M, a greater than 50% inhibition, as compared to vehicle control, was observed. In contrast, the CB₁ specific ligand ACEA (10⁻⁶M – 10⁻¹²M) exerted a minimal inhibitory effect on the peritoneal macrophage chemotactic response to RANTES (**Fig. 10**).

The CB₂-specific Antagonist SR144528 Reverses the Inhibitory Effect of CP55940 on the Murine Peritoneal Macrophage Chemotactic Response to RANTES

In order to confirm the data indicating that activation of the CB₂ with a cannabinoid receptor selective ligand exerted a major inhibitory effect on the chemotactic response to RANTES, cannabinoid receptor agonist-antagonist experiments were performed. For these experiments, the CB₁ or CB₂ antagonist was used at a concentration of 10⁻⁶M. Treatment of (B₆C₃)F₁ murine peritoneal macrophages with the CB₂-specific

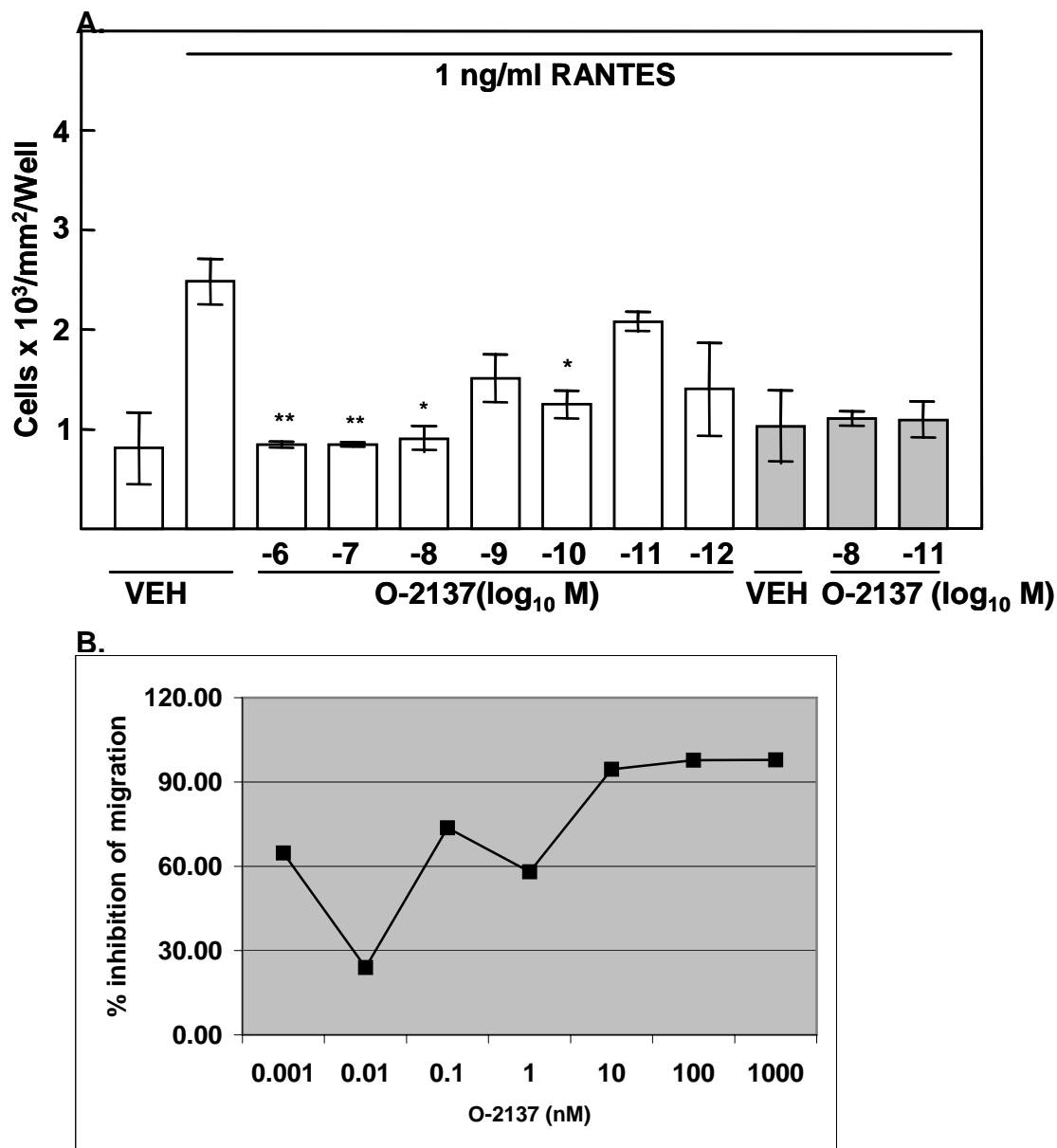


Figure 9- Effect of the CB₂-selective Ligand on the Chemotactic Response to RANTES. A. Treatment (3h) with the CB₂-selective ligand O-2137 resulted in a robust and significant inhibition of chemotaxis. Results are presented as the mean \pm SD. For RANTES placed only in the bottom chamber, SD was compared with that of vehicle-treated macrophages exposed to RANTES in the bottom chamber. For RANTES placed in both chambers (shaded bars), SD was compared with that of vehicle (VEH)-treated macrophages exposed to RANTES in both chambers. * $p < 0.05$, ** $p < 0.01$. B. Results presented as Percent Inhibition of migration.

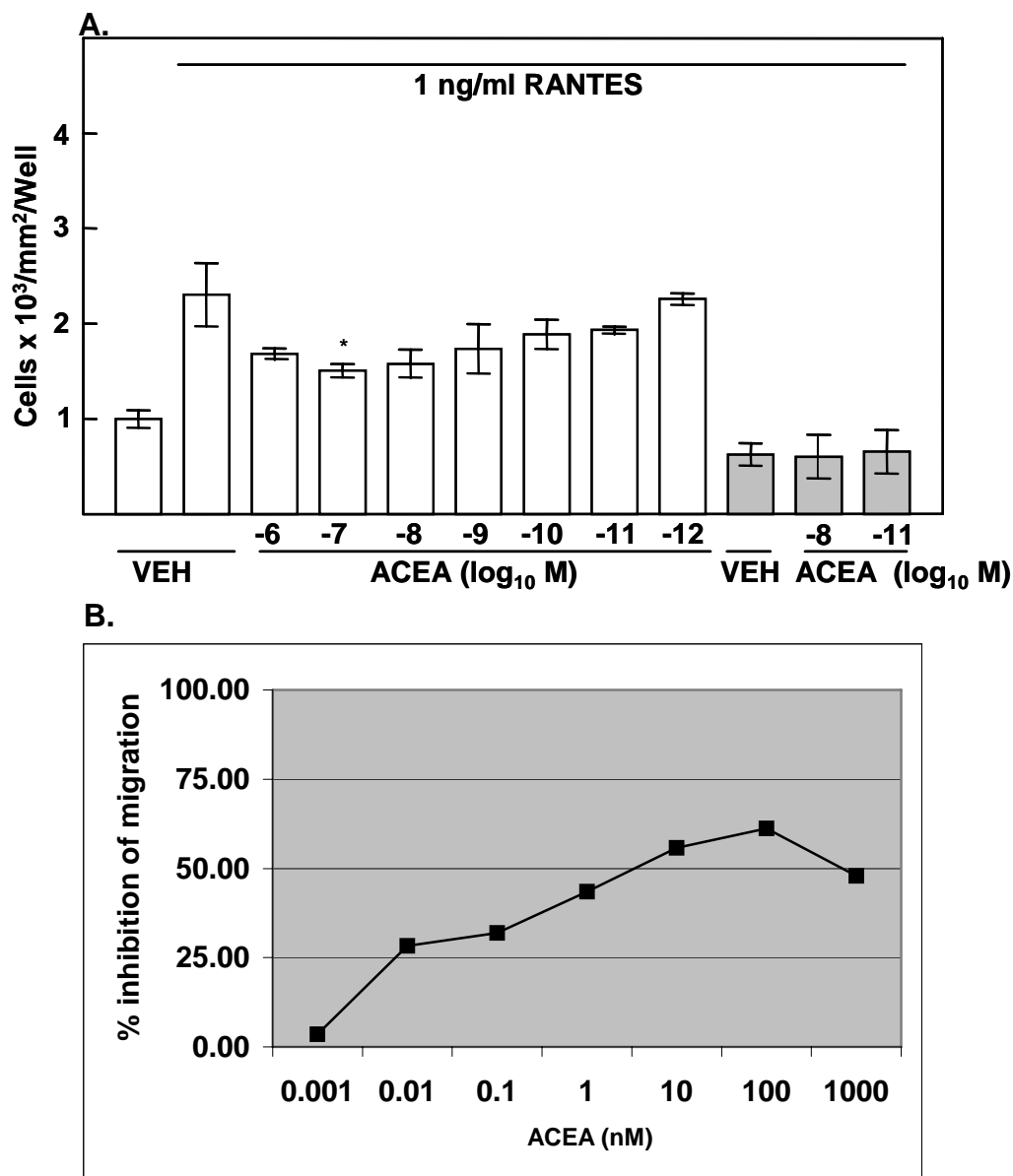


Figure 10- Effect of the CB₁-selective Ligand on the Chemotactic Response to RANTES. A. Treatment with the CB₁-selective ligand ACEA had a minimal effect on RANTES-induced migration of peritoneal macrophages. Results are presented as the mean \pm SD. For RANTES placed only in the bottom chamber, SD was compared with that of vehicle (VEH)-treated macrophages exposed to RANTES in the bottom chamber. For RANTES placed in both chambers (shaded bars), SD was compared with that of vehicle-treated macrophages exposed to RANTES in both chambers. * $p < 0.05$. B. Results presented as Percent Inhibition of migration.

antagonist SR144528 (SR2) alone had no major effect on the chemotactic response to RANTES. At equimolar concentrations (i.e., 10^{-6}M) of antagonist and agonist, CP55940 inhibited macrophage chemotaxis to RANTES. However, at lower concentrations of CP55940 ($10^{-7}\text{M} - 10^{-11}\text{M}$), the inhibitory effect of the agonist was reversed by the CB_2 antagonist SR144528 (**Fig. 12**). These results were in direct contrast to those obtained when the CB_1 antagonist SR141716A (SR1) was used (**Fig. 11**). Treatment with SR1 ($10^{-6}\text{M} - 10^{-12}\text{M}$) did not block the inhibitory effect of CP55940.

THC Does Not Inhibit the Chemotactic Response to RANTES of Peritoneal Macrophages from CB_2 Knockout Mice

To confirm the pharmacological data implicative of a functional linkage of the CB_2 to cannabinoid-mediated inhibition of macrophage chemotaxis to RANTES, experiments were performed using thioglycollate-elicited peritoneal macrophages from C57BL/6 CB_2 knockout mice. THC ($10^{-5}\text{M} - 10^{-9}\text{M}$) had no significant effect on either the chemotactic or chemokinetic response of macrophages from the knockout mice (**Fig. 13**). Since these CB_2 null animals were generated on a C57BL/6 genetic background, replicate migration experiments were performed using thioglycollate-elicited peritoneal macrophages from their C57BL/6 CB_2 (+/+) wild-type counterparts. Consistent with the data obtained using $(\text{B}_6\text{C}_3)\text{F}_1$ mice, THC exerted a concentration-related inhibition of the chemotactic response of peritoneal macrophages to RANTES (**Fig. 14**).

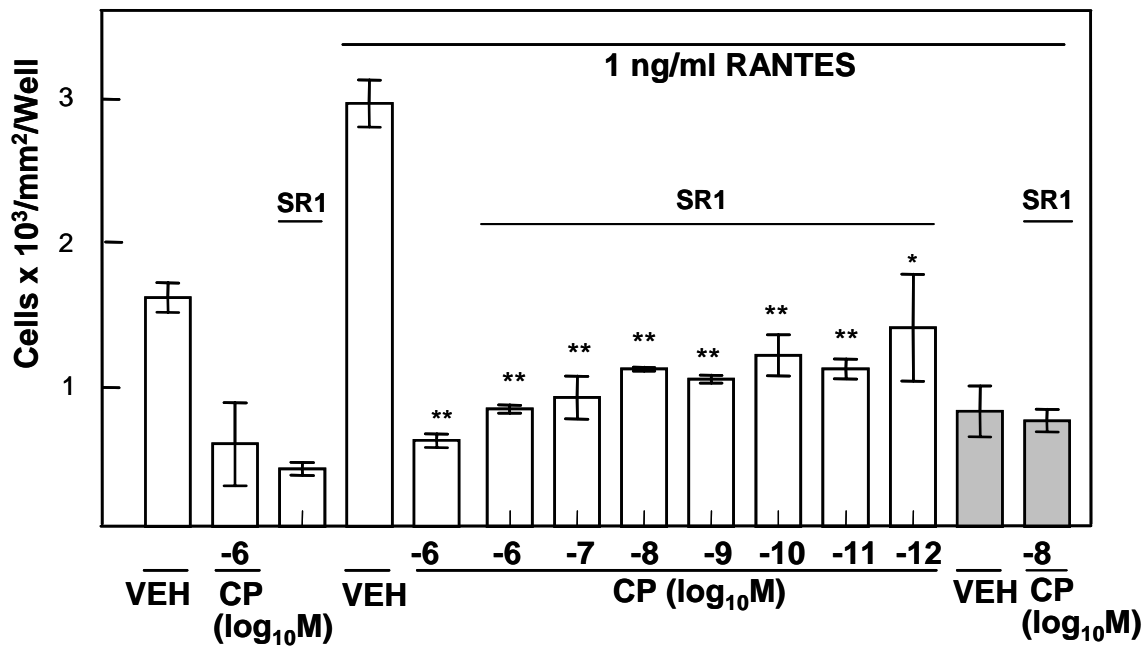


Figure 11- Effect of CB₁ Cannabinoid Receptor Antagonist on Chemotaxis to RANTES. Treatment with the CB₁ antagonist SR141716A (SR1)(10⁻⁶ M) did not block CP55940 (CP)-mediated inhibition of chemotaxis. Macrophages were treated (30 min) with antagonist prior to treatment (3h) with cannabinoid. Results are presented as the mean ± SD. For RANTES placed only in the bottom chamber, SD was compared with that of vehicle (VEH)-treated macrophages exposed to RANTES in the bottom chamber. For RANTES placed in both chambers (shaded bars), SD was compared with that of vehicle-treated macrophages exposed to RANTES in both chambers. *p<0.05, **p<0.01.

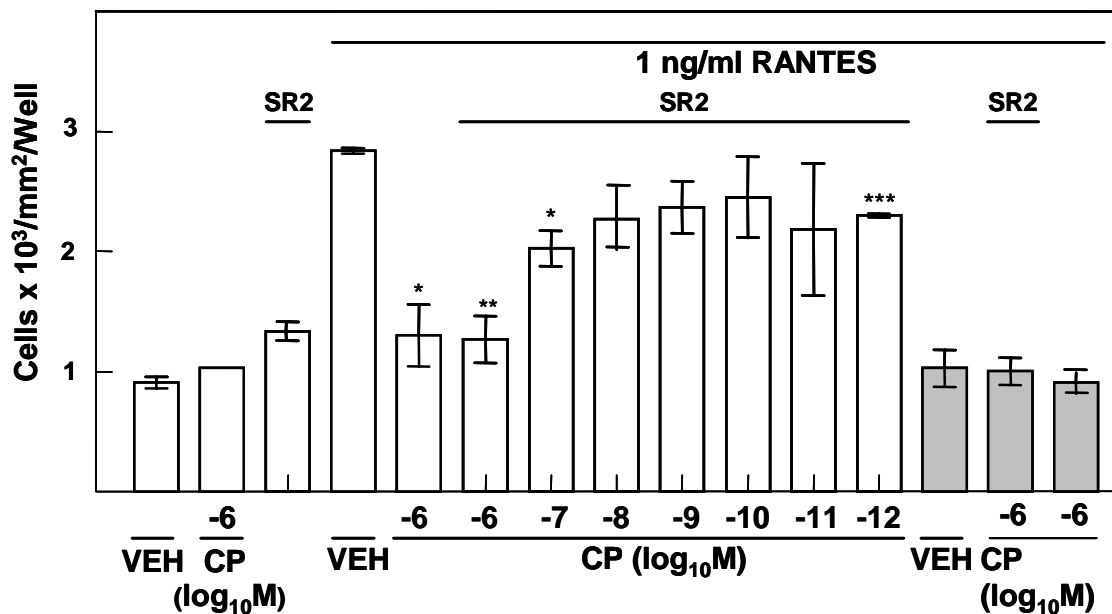


Figure 12- Effect of CB₂ Cannabinoid Receptor Antagonist on Chemotaxis to RANTES. The CP55940 (CP)-mediated inhibition of chemotaxis was reversed by SR144528 (SR2)(10⁻⁶ M). Macrophages were treated (30 min) with antagonist prior to treatment (3h) with cannabinoid. Results are presented as the mean ± SD. For RANTES placed only in the bottom chamber, SD was compared with that of vehicle (VEH)-treated macrophages exposed to RANTES in the bottom chamber. For RANTES placed in both chambers (shaded bars), SD was compared with that of vehicle-treated macrophages exposed to RANTES in both chambers. *p<0.05, **p<0.01, ***p<0.001.

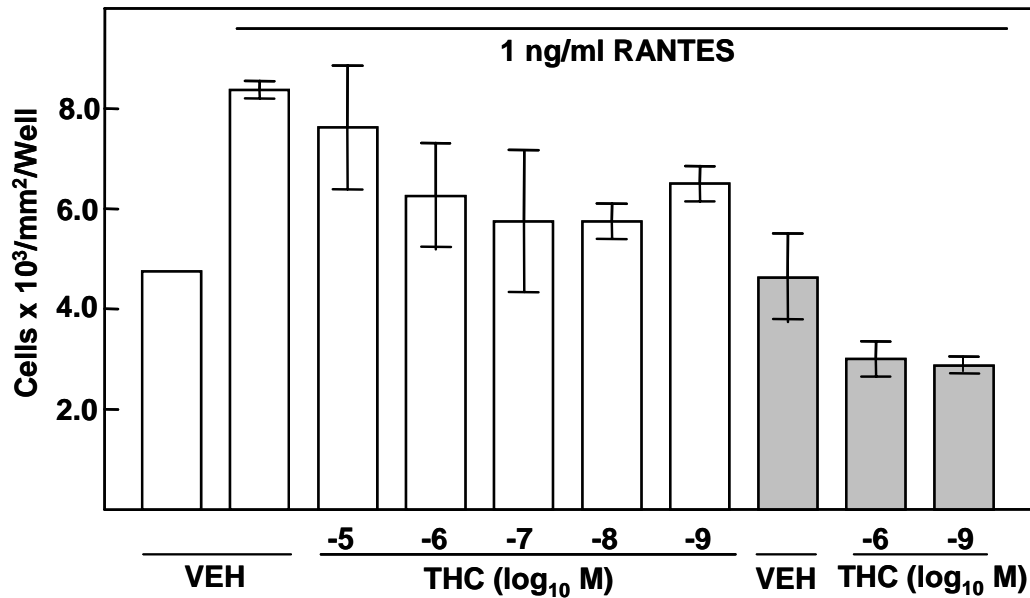


Figure 13- Effect of THC on the Chemotactic Response of Peritoneal Macrophages from CB₂ Knockout Mice to RANTES. *In vitro* THC treatment (3h) did not have a significant effect on RANTES-induced migration by peritoneal macrophages from CB₂ receptor knockout mice. Results are presented as the mean ± SD. For RANTES placed only in the bottom chamber, SD was compared with that of vehicle (VEH)-treated macrophages exposed to RANTES in the bottom

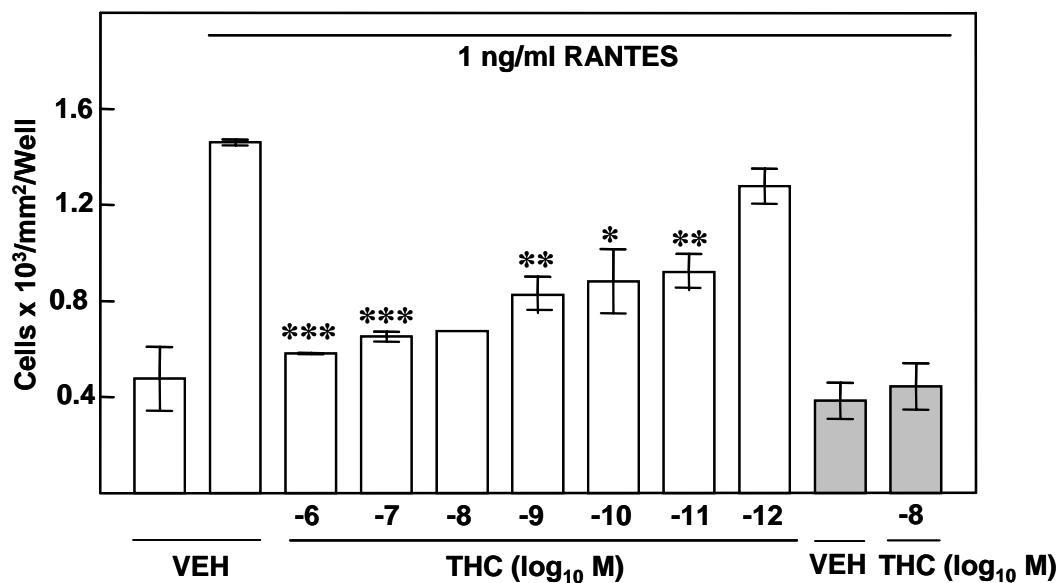


Figure 14- Effect of THC on the Chemotactic Response of Peritoneal Macrophages from the Wild-type Counterpart. THC treatment (3h) resulted in a concentration-related inhibition of the chemotactic response of peritoneal macrophages to RANTES from the C57BL/6 CB₂ (+/+) wild-type counterpart. Results are presented as the mean ± SD. For RANTES placed only in the bottom chamber, SD was compared with that of vehicle (VEH)-treated macrophages exposed to RANTES in the bottom. *p<0.05, **p<0.01, ***p<0.001

THC Alters Functional Morphology of Murine Peritoneal Macrophages Undergoing Chemotaxis to RANTES

Transwell chemotaxis assays revealed that THC inhibits peritoneal macrophage migration to RANTES. Scanning electron microscopy analysis of the transwell membranes demonstrated that cells treated with THC exhibited altered morphology and were apparently impaired in their ability to migrate through the pores into the bottom chamber containing the chemoattractant. Vehicle-treated (0.01% Ethanol) peritoneal macrophages exposed to RANTES exhibited characteristics indicative of cell migration including cell membrane ruffling and lobose cellular extensions (pseudopodia formation) (**Fig. 15**). Additionally, numerous vehicle-treated macrophages were found in or in close proximity to the membrane pores with pseudopodia extending toward the pore (**Fig. 15**). In contrast, peritoneal macrophages treated with THC were rounded in appearance, with the absence of cellular extensions. Few THC-treated macrophages were observed close to or in filter pores.

THC Does Not Alter mRNA Levels of CC Chemokine Receptors in Thioglycollate-Elicited Murine Peritoneal Macrophages

Chemotaxis to RANTES results from a complex series of signal transductional activities following ligation of the chemokine to its cognate G protein-coupled receptor. THC treatment of macrophages could affect activation of chemokine receptors and alter their expression and/or compartmentalization. Thus, in order to obtain initial insight as to

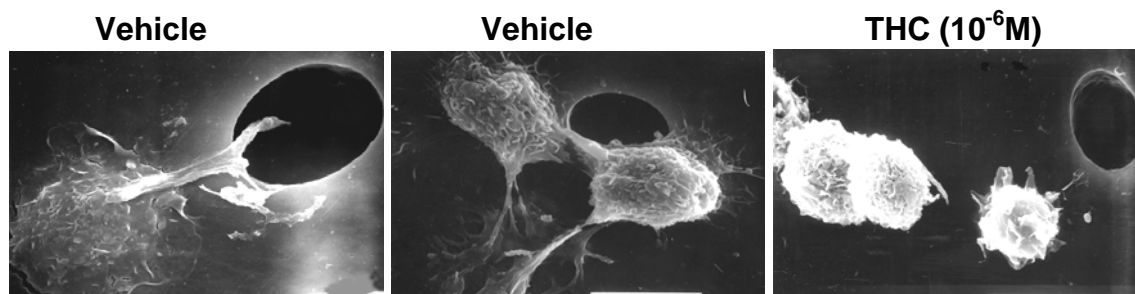


Figure 15– THC Alters the Morphology of Peritoneal Macrophages Migrating to RANTES. Scanning electron microscopy of transwell inserts of peritoneal macrophages treated with Vehicle (0.01% Ethanol) migrating towards 1ng/ml RANTES (note multiple cellular projections). Cells treated with THC are rounded in appearance and do not seem to be migrating toward RANTES. Scale bars 1, 10, and 10 mm left to right.

the mode of action through which THC treatment results in inhibition of chemotaxis, experiments were performed to assess for levels CC chemokine mRNA in peritoneal macrophages. THC (10^{-6} M - 10^{-12} M) treatment of (B₆C₃)F₁ peritoneal macrophages (3h) had no major effect on total mRNA levels of CCR1, CCR2 or CCR5 (**Fig. 16**).

Additionally, treatment with a battery of cannabinoid receptor agonists and antagonists (THC, CP55940, SR1, and SR2 at 10^{-6} M) also had no major effect on chemokine receptor mRNA levels (**Fig. 17**). Chemokine receptor mRNA levels also were assessed for peritoneal macrophages exposed to THC *in vivo* (50mg/kg THC for 24h prior to harvest of peritoneal macrophages). Consistent with results obtained *in vitro*, THC treatment had no major effect on the expression of CCR1, CCR2, or CCR5 at the level of total mRNA (Data not shown). Similarly, at this concentration range THC had no major effect on total mRNA levels of CB₂ (Data not shown).

Heterologous Desensitization of Chemokine Receptors by Cannabinoids

G-protein coupled receptors display reduced responsiveness with prolonged or repeated agonist stimulation, a process known as receptor desensitization. Upon phosphorylation by G-protein receptor kinases (GRKs) receptor internalization and recycling is initiated. In cell types expressing numerous classes of G-protein coupled receptors, signaling through one class of GPCR has been shown to trans-deactivate other receptor types through heterologous desensitization. It is therefore possible that cannabinoid treatment could affect chemokine receptor function, resulting in reduced responsiveness to chemokines. In order to obtain insight regarding whether cannabinoid

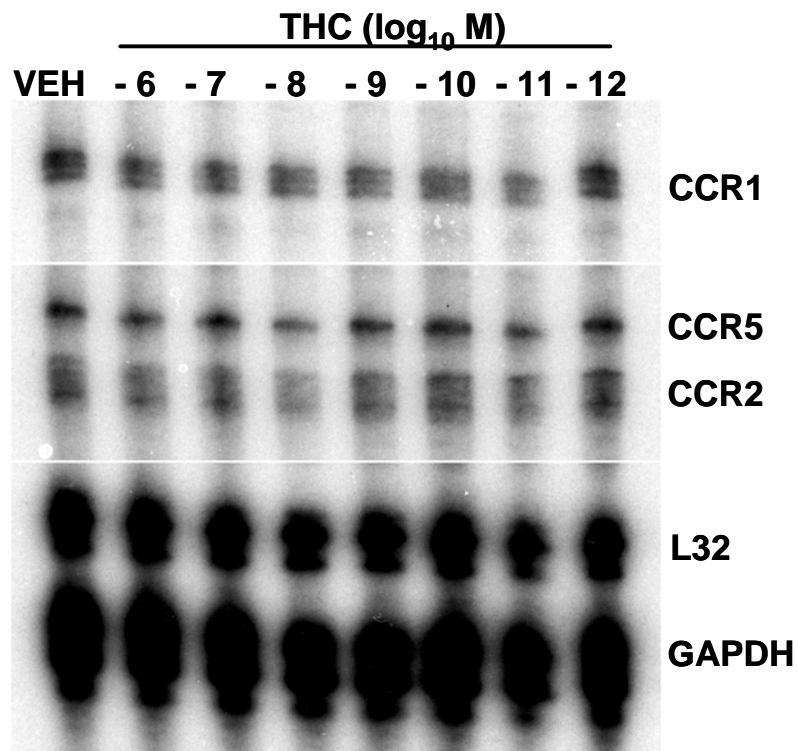


Figure 16- Effect of THC on Levels of CC Chemokine Receptor mRNA. Multiprobe ribonuclease protection assay demonstrated that THC (10^{-6} M – 10^{-12} M) treatment (3h) had no major effect on levels of CCR1, CCR2, and CCR5 mRNA in thioglycollate-elicited (B₆C₃)F₁ murine peritoneal macrophages.

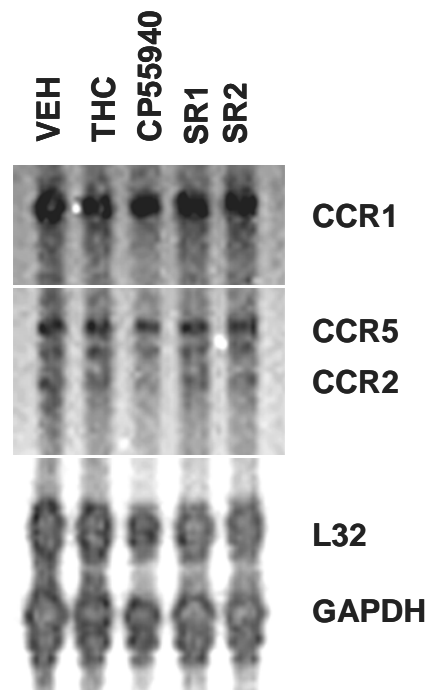


Figure 17- Effect of Select Cannabinoids on Levels of CC Chemokine Receptor mRNA. Multiprobe ribonuclease protection assay demonstrated that THC, CP55940, SR1, or SR2 (10^{-6} M) treatment (3h) had no major effect on levels of CCR1, CCR2, and CCR5 mRNA in thioglycollate-elicited (B₆C₃)F₁ murine peritoneal macrophages.

treatment caused heterologous desensitization of chemokine receptors, experiments were performed to assess levels of CCR5 phosphorylation following stimulation with RANTES or CP55940. Treatment of peritoneal macrophages with cognate ligand RANTES (1ng/ml) (1h) resulted in increased CCR5 phosphorylation compared to vehicle (**Fig. 18**). CP55940 treatment also enhanced CCR5 phosphorylation, though not to the same extent as the cognate agonist, indicating that heterologous desensitization of chemokine receptors by cannabinoids may be occurring.

Endogenous Cannabinoid 2-Arachidonylglycerol (2-AG) Induces Murine Peritoneal Macrophage Migration

Numerous groups have reported that select cannabinoids are capable of inducing migration in a variety of immune cell types including microglia and cell lines of myeloid origin (Walter *et al.*, 2003; Jordá *et al.*, 2002; Kishimoto *et al.*, 2005); however, to date there have been no published reports specifically utilizing primary macrophages from the periphery. To address this, experiments were performed to assess the ability of the endogenous cannabinoid, 2-AG (1 nM to 5 μ M) to induce peritoneal macrophage migration. Maximal macrophage migration was observed using 1 μ M 2-AG (**Fig. 19**), consistent with data obtained using other cell types.

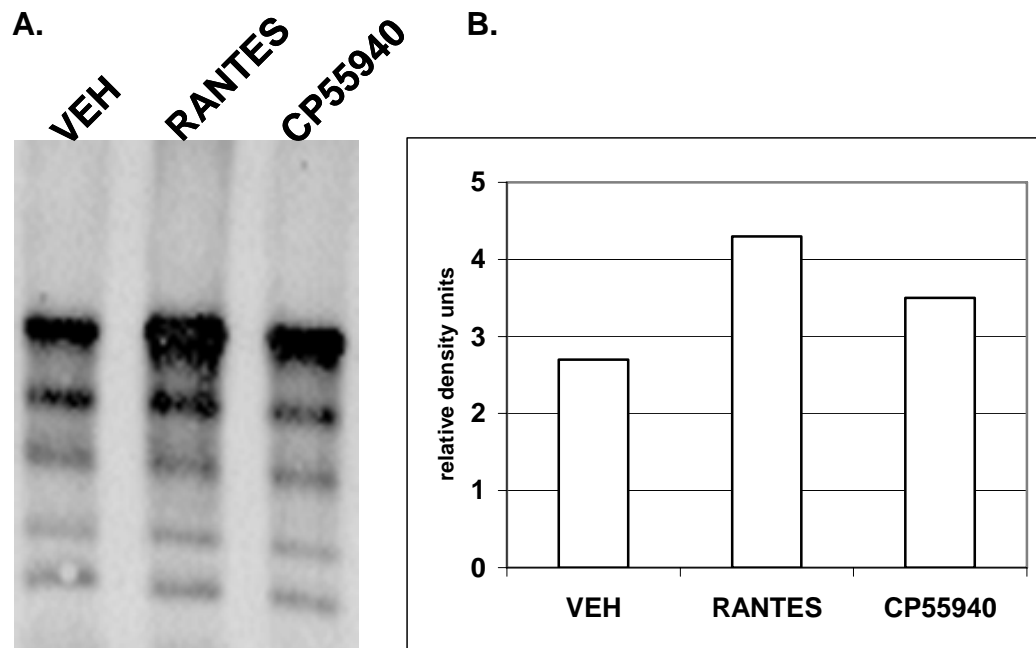


Figure 18– Stimulation with RANTES and Cannabinoid Receptor Agonist CP55940 Induces CCR5 Phosphorylation. Peritoneal macrophages were treated for 1h with vehicle, RANTES (1ng/ml), or CP55940 (10^{-6} M) and harvested for protein. Whole cell protein lysates of peritoneal macrophages were precleared with normal mouse IgG. A CCR5 antibody was used to immunoprecipitate CCR5 from the precleared protein lysate. Following SDS-PAGE, the immunoblot (A.) was probed with an antiphosphoserine antibody to detect CCR5 phosphorylation. B. Densitometric analysis of CCR5 phosphorylation. V-vehicle, R- RANTES, CP-CP55940.

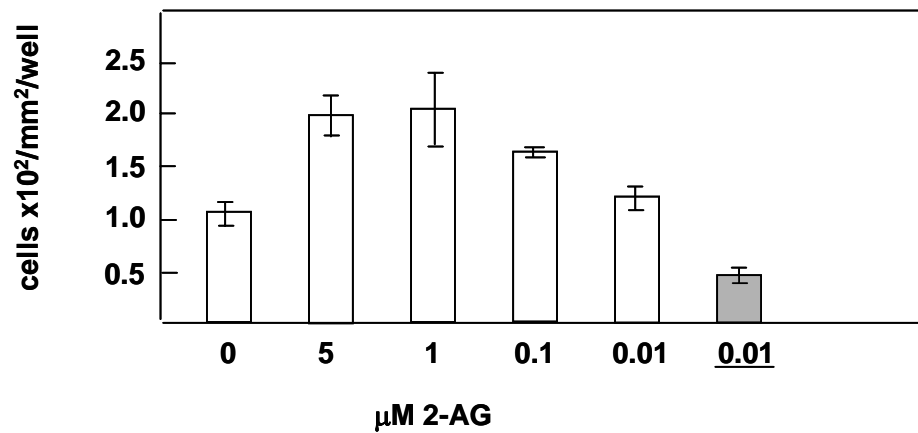


Figure 19 – The Endogenous Cannabinoid 2-AG Induces Peritoneal Macrophage Chemotaxis. Transwell chemotaxis assay was utilized to assess chemoattractant properties of 2-AG (4h) (0.01-5 µM). Results are presented as mean ± SD.

Treatment with THC Inhibits Murine Peritoneal Macrophage Migration to 2-AG

Further experiments were performed to address whether treatment with THC could inhibit peritoneal macrophage chemotaxis to 2-AG. Treatment of macrophages with THC ($10^{-6}\text{M} - 10^{-8}\text{M}$) resulted in a significant inhibition of migration in response to 2-AG (**Fig 20**).

Migration of Murine Peritoneal Macrophages to Amoebic Conditioned Medium

In studies using an *in vivo* murine model of *Acanthamoeba* infection, Marciano-Cabral and Cabral (2003) demonstrated that macrophage-like cells are the primary immune cell component in granulomas, which are formed in response to amoebic infection. It has been proposed that these granulomas may serve to sequester the amoeba, thereby preventing further dissemination (**Fig. 21**). Additionally, exposure to THC exacerbated *Acanthamoeba* infection, characterized by increased mortality and numbers of amoeba present in brain sections, as well as the absence of granuloma formation (**Fig. 21**). *Acanthamoeba* infection notably results in dramatic and extensive tissue damage, which occurs through a combination of direct contact and the secretion of numerous lytic enzymes (**Table 4**). Experiments, therefore, were performed to assess peritoneal macrophage migration to amoebic conditioned medium (0-72 μg) obtained from cultures incubated with *Acanthamoeba culbertsoni* (AcCM) and *Acanthamoeba* (JH1) (JH1CM), a clinical isolate containing intracellular bacteria. Amoebic conditioned medium from *A. culbertsoni* (**Fig. 22**) and JH1 isolate (similar results, data not shown) induced

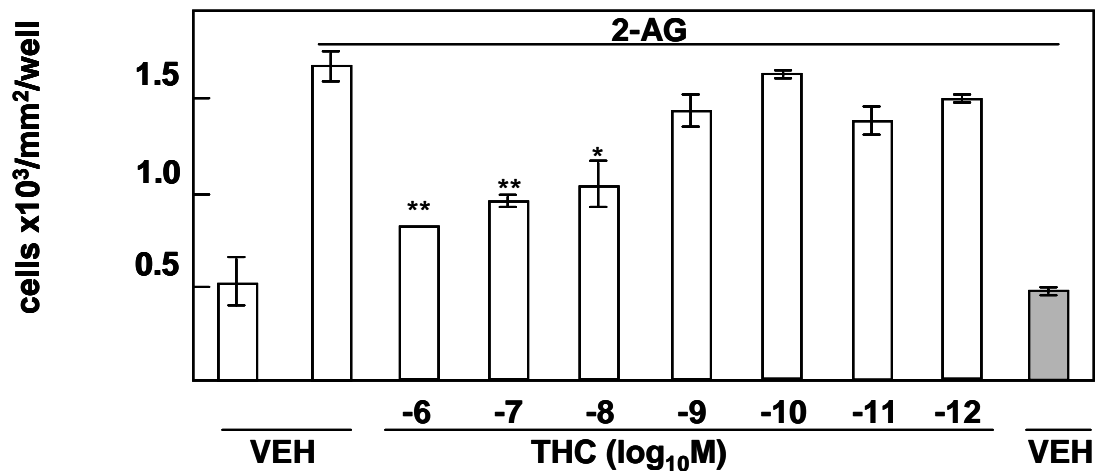


Figure 20– THC Inhibits Peritoneal Macrophage Chemotaxis to 2-AG. Peritoneal macrophages were treated with THC (10^{-6} - 10^{-12} M) for 3 h and then assessed for chemotaxis to 1 μ M 2-AG (2h). VEH- vehicle (0.01% ethanol). * $p < 0.05$, ** $p < 0.01$.

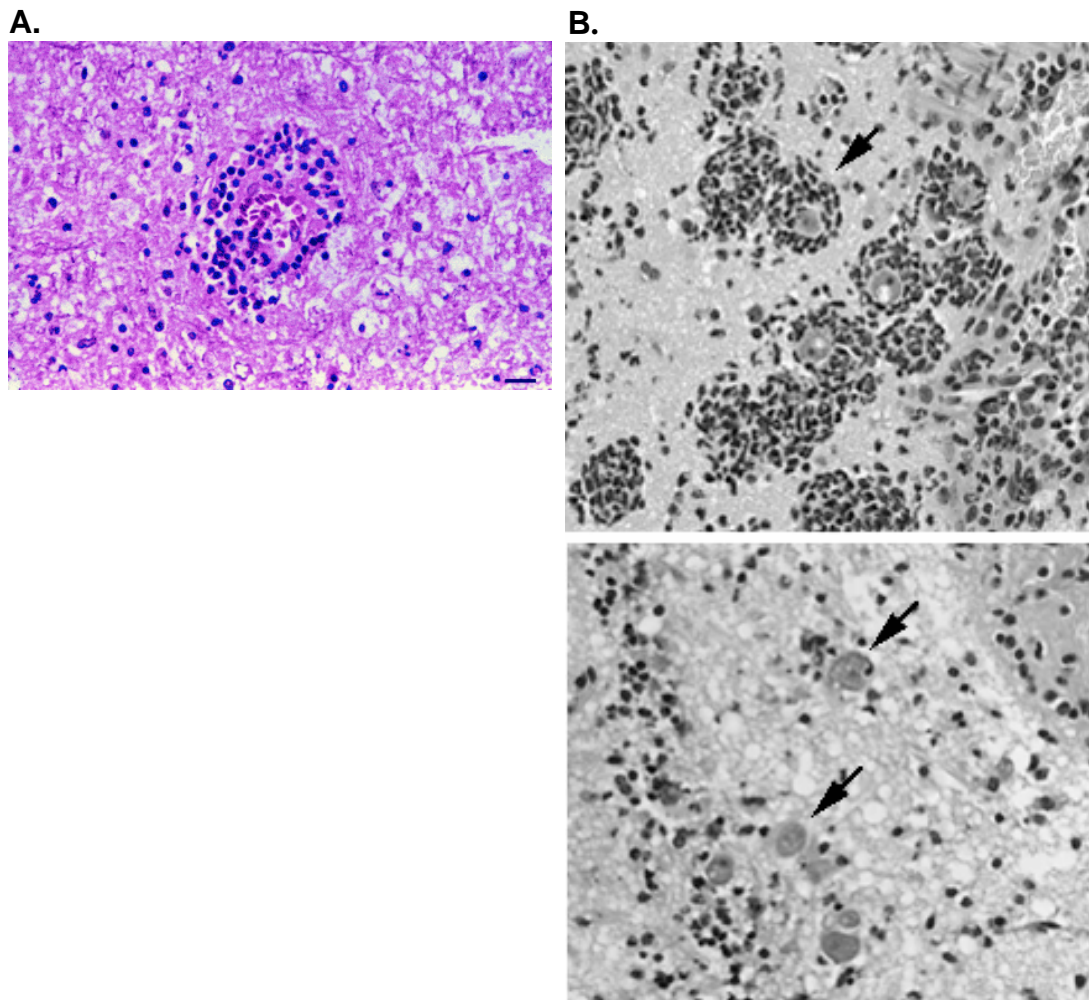


Figure 21– A. Hematoxylin and Eosin (H&E) Stained Section of Paraffin-embedded Brain Tissue Demonstrating Granuloma Formation in *Acanthamoeba* infection. B. Sections of paraffin-embedded brain tissue demonstrating granuloma formation in *Acanthamoeba* infection. Top panel- Vehicle treated mouse. Bottom panel- THC-treated mouse. Images courtesy of Marciano-Cabral and Cabral 2003 and Cabral *et al.*, 2007.

Table 4- *Acanthamoeba* Secreted Factors

Product	Action	Reference
Serine Protease (33 kD)	degrades IgG and IgA	Kong <i>et al.</i> , 2000
Lytic enzymes	involved in invasion	Moore <i>et al.</i> , 1991 Alfieri <i>et al.</i> , 2000
Serine and cysteine proteinases	markers for pathogenicity	Hadas and Mazur 1993
Serine, cysteine and metalloproteinases	degrade type I collagen	Mitro <i>et al.</i> , 1994
Serine protease (42kD)	degrades collagen	Cho <i>et al.</i> , 2000
Serine protease (33kD)	implicated in virulence	Kim <i>et al.</i> , 2006
Serine protease (85,130kD)	degrades types I and III collagen elastin, plasminogen, casein, and hemoglobin	Sissons <i>et al.</i> , 2006
Metalloprotease (150kD)	degrades types I and III collagen elastin, plasminogen, casein, and hemoglobin	

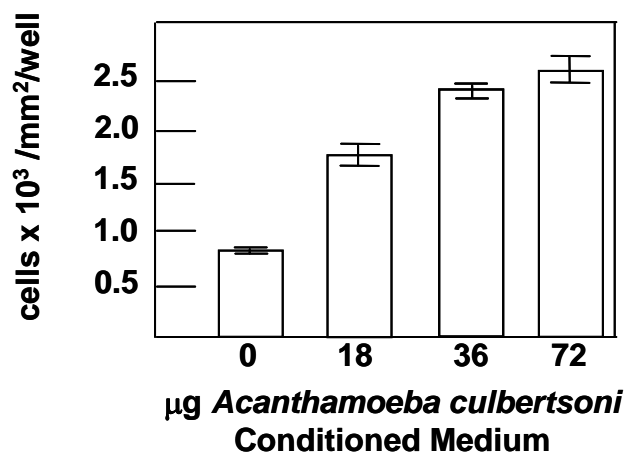


Figure 22 – Peritoneal Macrophages Migrate in Response to Amoebic Conditioned Medium. Transwell migration assays (2h) were performed to assess peritoneal macrophage migration to *Acanthamoeba* conditioned medium (*Acanthamoeba culbertsoni*- 0-72 μg).

macrophage migration with a greater than 2-fold increase observed at concentrations of conditioned medium as low as 36µg.

Treatment with THC Inhibits the Chemotactic Response of Murine Peritoneal Macrophages to Amoebic Conditioned Medium

In order to determine whether THC exerted a direct effect on macrophage migration to amoebic conditioned medium, *in vitro* exposure experiments were performed. A minimal level of cell migration was observed for control wells. An approximate three-fold increase in the number of peritoneal macrophages treated with vehicle was obtained when amoebic conditioned medium was placed in the bottom compartment to establish a chemoattractant gradient. Treatment of cells with THC (10^{-6} - 10^{-10} M) resulted in a significant concentration-related decrease in migration in response to *Acanthamoeba* (JH1) conditioned medium (JH1CM; 36µg) (**Fig. 23**). Similarly, treatment of cells with THC (10^{-6} - 10^{-11} M) resulted in a significant concentration-related decrease in migration in response to *Acanthamoeba culbertsoni* conditioned medium (AcCM; 36µg) (**Fig. 24**).

Treatment with THC in vivo Results in Inhibition of Murine Peritoneal Macrophage Migration to Amoebic Conditioned Medium in vitro

(B₆C₃)F₁ mice were inoculated with vehicle (ethanol:emulphor:saline, 1:1:18) or THC (5, 10, 25 or 50 mg/kg) (one injection per day for 4 days). On the last day of injections, thioglycollate was administered and 5 days later peritoneal macrophages were harvested. Migration assays were performed to assess the effect of *in vivo* THC exposure

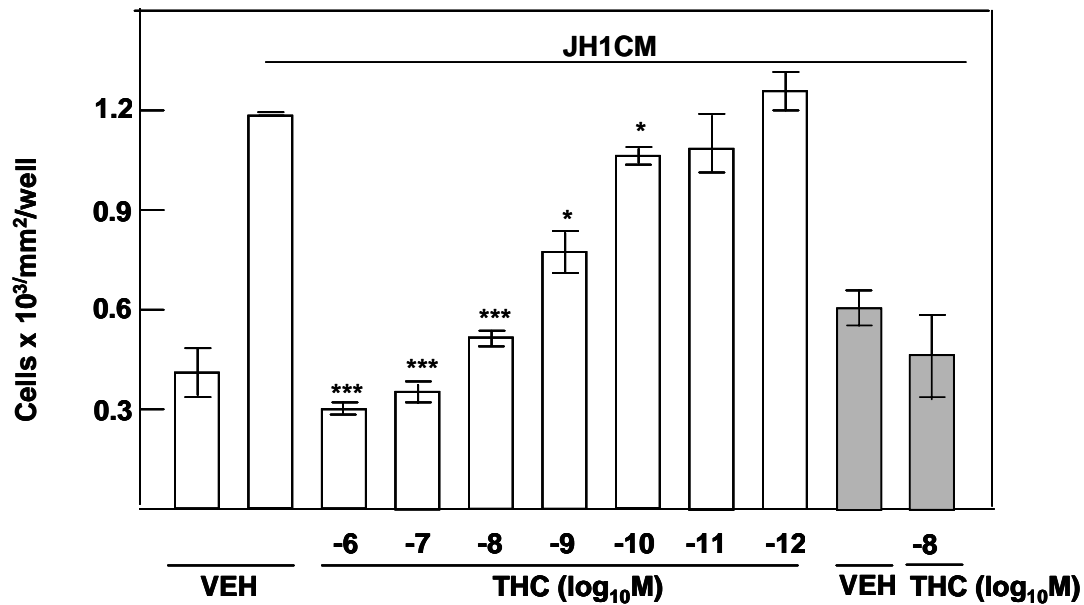


Figure 23– THC Inhibits Peritoneal Macrophage Chemotaxis to *Acanthamoeba* Conditioned Medium. Peritoneal macrophages were treated with THC (10^{-6} - 10^{-12} M) for 3h and then assessed for migration to 36 μg *Acanthamoeba* (JH1) conditioned medium (1h). * $p < 0.05$, *** $p < 0.001$. VEH- vehicle (0.01% ethanol).

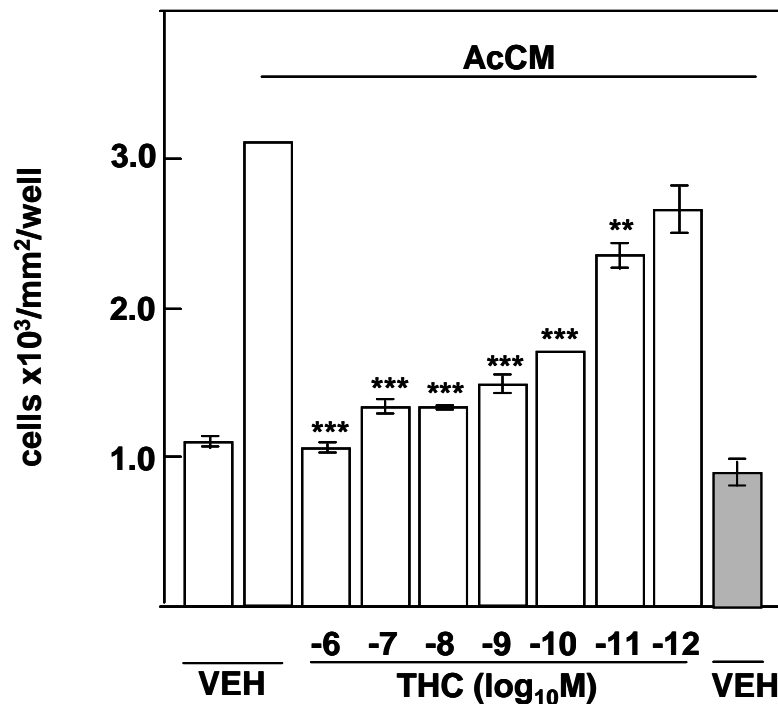


Figure 24– THC Inhibits Peritoneal Macrophage Chemotaxis to *Acanthamoeba* Conditioned Medium. Peritoneal macrophages were treated with THC (10^{-6} - 10^{-12} M) for 3h then assessed for migration to 36 μ g *Acanthamoeba culbertsoni* conditioned medium (AcCM) for 1h. Results are presented as mean \pm SD. ** $p < 0.01$, *** $p < 0.001$. VEH-vehicle (0.01% ethanol).

on *in vitro* migration to amoebic conditioned medium. *In vivo* administration of 10 mg/kg or 25 mg/kg THC resulted in a significant and greater than 50% inhibition of cell migration to *Acanthamoeba culbertsoni* conditioned medium (AcCM; 36µg) (**Fig. 25**).

Treatment with CP55940 Inhibits the Chemotactic Response of Murine Peritoneal Macrophages to Amoebic Conditioned Medium

Experiments performed with THC were replicated using CP55940, a high efficacy agonist at CB₁ and CB₂. Treatment of cells with CP55940 (10⁻⁶-10⁻¹⁰ and 10⁻¹² M) resulted in a significant concentration-related decrease in chemotaxis in response to *Acanthamoeba* (JH1) conditioned medium (JH1CM; 36µg) (**Fig. 26**). Greater than 50% inhibition of cell migration to amoebic conditioned medium was observed with CP55940 treatment at concentrations of 10⁻⁶-10⁻¹⁰M compared to vehicle-treated macrophages.

The CB₂-selective Ligand O-2137 Exerts a Robust Inhibitory Effect on the Murine Peritoneal Macrophage Chemotactic Response to Amoebic Conditioned Medium

In order to determine whether a cannabinoid receptor was linked to the observed inhibitory effect, macrophages from (B₆C₃)F₁ mice were treated with compounds exhibiting selective high affinity binding to the CB₁ or the CB₂ prior to assessment of the chemotactic response to amoebic conditioned medium. Treatment of macrophages with

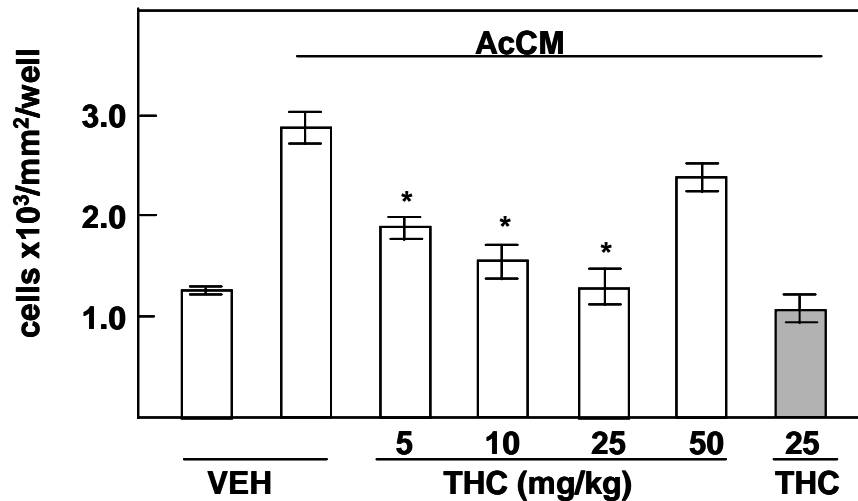


Figure 25- *In vivo* Exposure to THC Inhibits Peritoneal Macrophage Chemotaxis to *Acanthamoeba* Conditioned Medium. (B₆C₃)F₁ mice were inoculated with vehicle (VEH) (ethanol:emulphor:saline, 1:1:18) or THC (5, 10, 25 or 50 mg/kg) (one injection per day for 4 days) and peritoneal macrophages harvested 5 days later. Migration to *Acanthamoeba culbertsoni* conditioned medium (AcCM; 36µg) was assessed. Results are presented as mean ± SD. *p<0.05.

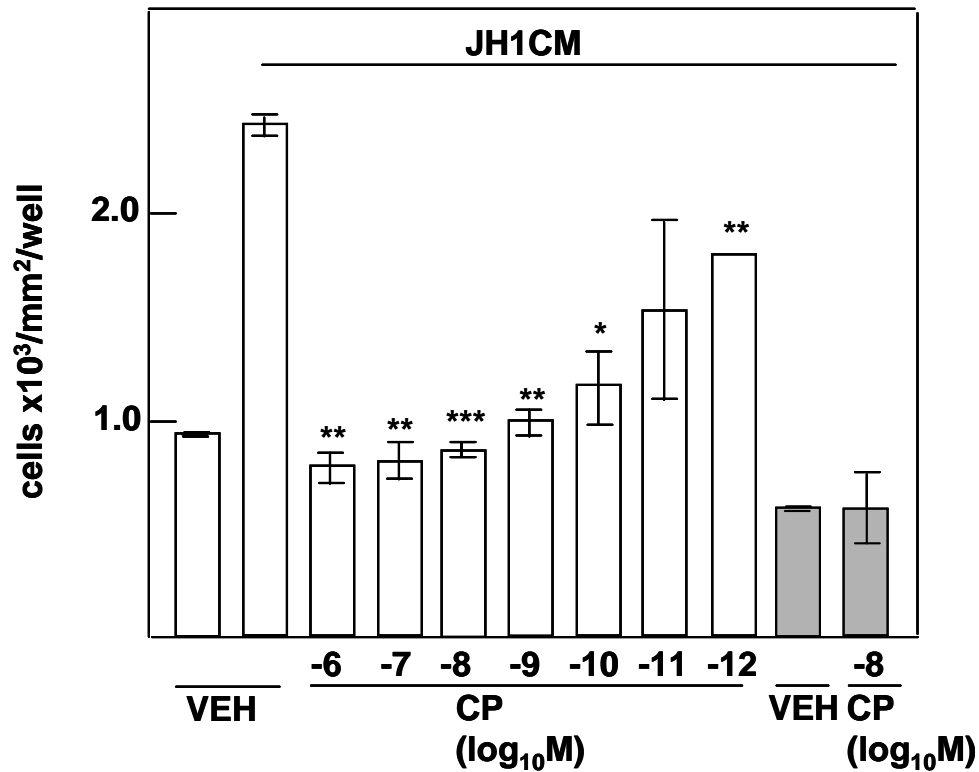


Figure 26– CP55940 Inhibits Peritoneal Macrophage Chemotaxis to *Acanthamoeba* Conditioned Medium. Peritoneal macrophages were treated with CP55940 (CP) (10^{-6} - 10^{-12} M) for 3h then assessed for migration to 36 μ g *Acanthamoeba* (JH1) conditioned medium (JH1CM) for 1h. Results are presented as mean \pm SD. * p <0.05, ** p <0.01, *** p <0.001. VEH- vehicle (0.01% ethanol).

the highly selective CB₂ ligand O-2137 resulted in a profound and significant concentration-related inhibition in the chemotactic response to *Acanthamoeba* (JH1) conditioned medium (JH1CM; 36 µg) (**Fig. 27**). For drug concentrations of 10⁻⁶M – 10⁻⁸M, a greater than 50% inhibition, as compared to vehicle control, was observed. In contrast, the CB₁ specific ligand ACEA (10⁻⁶M – 10⁻¹²M) had no inhibitory effect on the peritoneal macrophage chemotactic response to *Acanthamoeba* (JH1) conditioned medium (JH1CM; 36 µg) (**Fig. 28**).

CB₂-specific Antagonist SR144528 (SR2) Reverses the Inhibitory Effect of CP55940 on the Murine Peritoneal Macrophage Chemotactic Response to Amoebic Conditioned Medium

Cannabinoid receptor agonist-antagonist experiments were performed to further confirm the previous data indicating that activation of the CB₂ with a cannabinoid receptor selective ligand exerted a major inhibitory effect on the chemotactic response to amoebic conditioned medium. For these experiments, the CB₂ antagonist SR144528 (SR2) was used at a concentration of 10⁻⁶M. Treatment of (B₆C₃)F₁ murine peritoneal macrophages with the CB₂-specific antagonist SR2 alone had no significant effect on the chemotactic response to *Acanthamoeba* (JH1) conditioned medium (JH1CM; 54 µg), as there was no significant difference in migration to JH1CM by SR2- or vehicle-treated cells (**Fig. 29**). At equimolar concentrations (i.e., 10⁻⁶M) of antagonist and agonist,

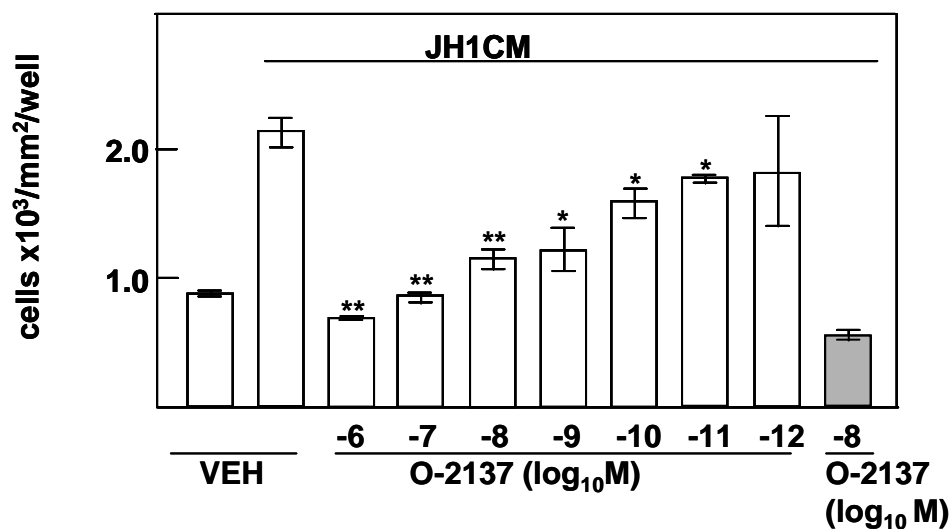


Figure 27 – The CB₂ Selective Agonist O-2137 Inhibits Peritoneal Macrophage Chemotaxis to *Acanthamoeba* Conditioned Medium. Peritoneal macrophages were treated with O-2137 (10⁻⁶- 10⁻¹² M) for 3h then assessed for migration to 36 μg *Acanthamoeba* (JH1) conditioned medium (JH1CM) for 1h. Results are presented as mean ± SD. *p<0.05, **p<0.01. VEH- vehicle (0.01% ethanol).

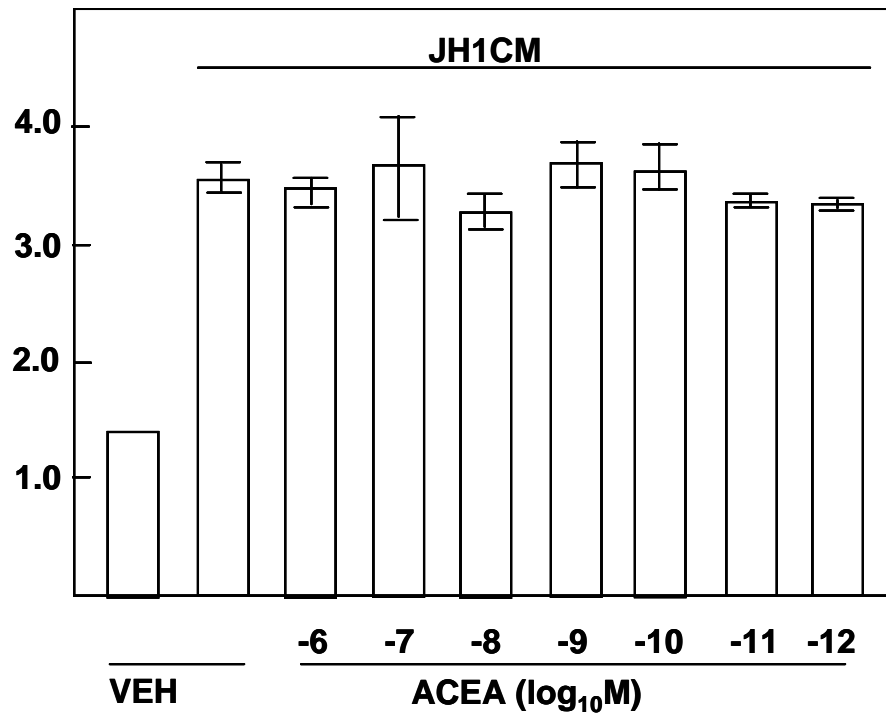


Figure 28 – The CB₁ Selective Agonist ACEA Has No Effect on Peritoneal Macrophage Migration to *Acanthamoeba* Conditioned Medium. Peritoneal macrophages were treated with ACEA (10⁻⁶ - 10⁻¹² M) for 3h then assessed for migration to 36 µg *Acanthamoeba* (JH1) conditioned medium (JH1CM) for 1h. Results are presented as mean ± SD. VEH- vehicle (0.01% ethanol).

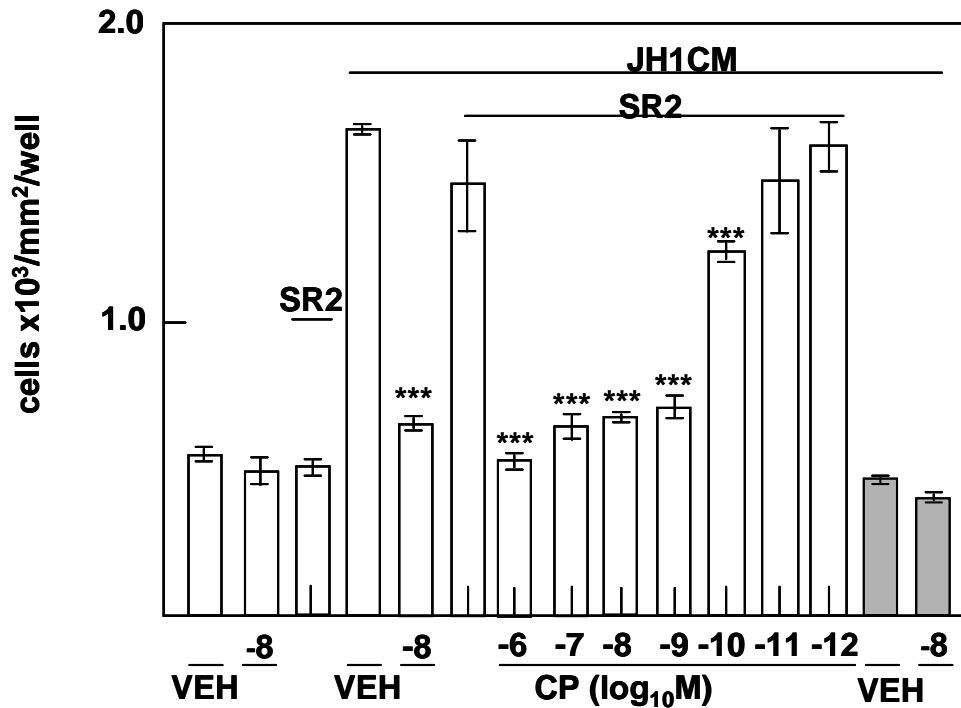


Figure 29- Effect of Cannabinoid Receptor Antagonist on Chemotaxis to *Acanthamoeba* Conditioned Medium. The CP55940 (CP)-mediated inhibition of chemotaxis was reversed by the CB₂ antagonist (SR2) SR144528 (10⁻⁶ M). Macrophages were treated (30 min) with antagonist prior to treatment (3h) with cannabinoid and assessed for migration to 54 μg *Acanthamoeba* (JH1) conditioned medium (JH1CM)(1h). Results are presented as the mean ± SD. For cannabinoid-treated cells exposed to amoeba conditioned medium placed only in the bottom chamber, SD was compared with that of vehicle-treated macrophages exposed to conditioned medium in the bottom chamber. For cannabinoid-treated cells exposed to conditioned medium placed in both chambers (shaded bars), SD was compared with that of vehicle (VEH)-treated macrophages exposed to conditioned medium in both chambers. ***p<0.001.

CP55940 inhibited macrophage migration to JH1CM. Additionally, referencing Fig. 26, CP55940-mediated inhibition of migration extended through concentrations as low as 10^{-12} M, with greater than 50% inhibition observed through 10^{-10} M. However, the inhibitory effect of the agonist began to be reversed by SR2 in cells treated with 10^{-10} M CP55940 (approximately 25% inhibition compared to 50%) and was totally reversed in cells treated with 10^{-11} and 10^{-12} M CP55940.

THC Alters Functional Morphology of Murine Peritoneal Macrophages Undergoing Chemotaxis to Amoebic Conditioned Medium

Transwell chemotaxis assays revealed that THC inhibits peritoneal macrophage migration to *Acanthamoeba* conditioned medium. Similar to results seen of macrophages migrating to RANTES, scanning electron microscopy analysis of the transwell membranes demonstrated that cells treated with THC were impaired in their ability to migrate through the pores into the bottom chamber containing the chemoattractant. Vehicle-treated (0.01% Ethanol) peritoneal macrophages exposed to amoeba conditioned medium exhibited characteristics indicative of cell migration including lobose cellular extensions (**Fig. 30**). Additionally, numerous vehicle-treated macrophages were found in or near the membrane pores with pseudopodia extending toward the pore (**Fig. 30**). In contrast, peritoneal macrophages treated with THC were rounded in appearance, with the absence of cellular extensions. Few THC-treated macrophages were observed in filter pores and those cells that were proximal did not appear to be moving toward the pore.

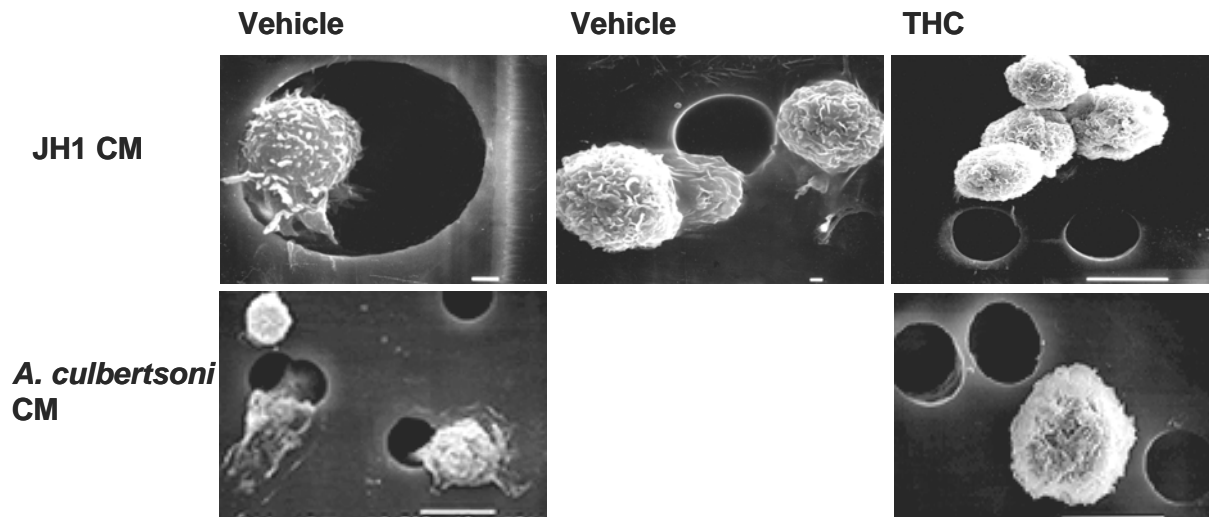


Figure 30– THC Alters the Morphology of Peritoneal Macrophages Migrating to *Acanthamoeba* Conditioned Medium. Scanning electron microscopy of transwell inserts of peritoneal macrophages treated with Vehicle (0.01% Ethanol) or THC (10^{-6} M) (3h) migrating towards *Acanthamoeba* conditioned medium (top row- *Acanthamoeba* JH1 conditioned medium (JH1CM), bottom row- *Acanthamoeba culbertsoni* conditioned medium). Note the cellular projections of the vehicle-treated macrophages migrating to amoebic conditioned medium, whereas the cells treated with THC are rounded in appearance and do not appear to be migrating toward the pores. Scale bars top row left to right 1, 1, and 10 μ m; bottom row both 10 μ m.

Treatment with THC and CP55940 Inhibits Rat Primary Microglial Migration to Amoeba Conditioned Medium

As one of the primary sites of *Acanthamoeba* infection occurs in the CNS, it was important to assess the effect of cannabinoids on migration of primary microglial cells to amoebic conditioned medium. Treatment of primary neonatal rat microglia with THC (10^{-6} - 10^{-8} M) resulted in significant inhibition of migration to *Acanthamoeba culbertsoni* conditioned medium (AcCM; 54 μ g) (**Fig. 31**). Replicate experiments using CP55940 also resulted in significant concentration-related inhibition of microglial migration to *Acanthamoeba culbertsoni* conditioned medium (AcCM; 54 μ g) (**Fig. 31**). Similar experiments assessing the effect of THC or CP55940 (10^{-6} M) on microglial migration to *Acanthamoeba* (JH1) conditioned medium also revealed cannabinoid-mediated inhibition (data not shown).

The CB₂-selective Ligand O-2137 Inhibits Rat Primary Microglial Migration to Amoebic Conditioned Medium

The inhibitory effect of THC and CP55940 on the migratory response of primary microglia to amoebic conditioned medium implicated a role for a cannabinoid receptor. Cannabinoid receptor selective agonists therefore were employed to determine the cannabinoid receptor associated with this inhibitory effect. Treatment with O-2137, a highly selective CB₂ ligand resulted in significant inhibition of microglial migration to *Acanthamoeba* (JH1) conditioned medium (JH1CM; 72 μ g) conditioned medium

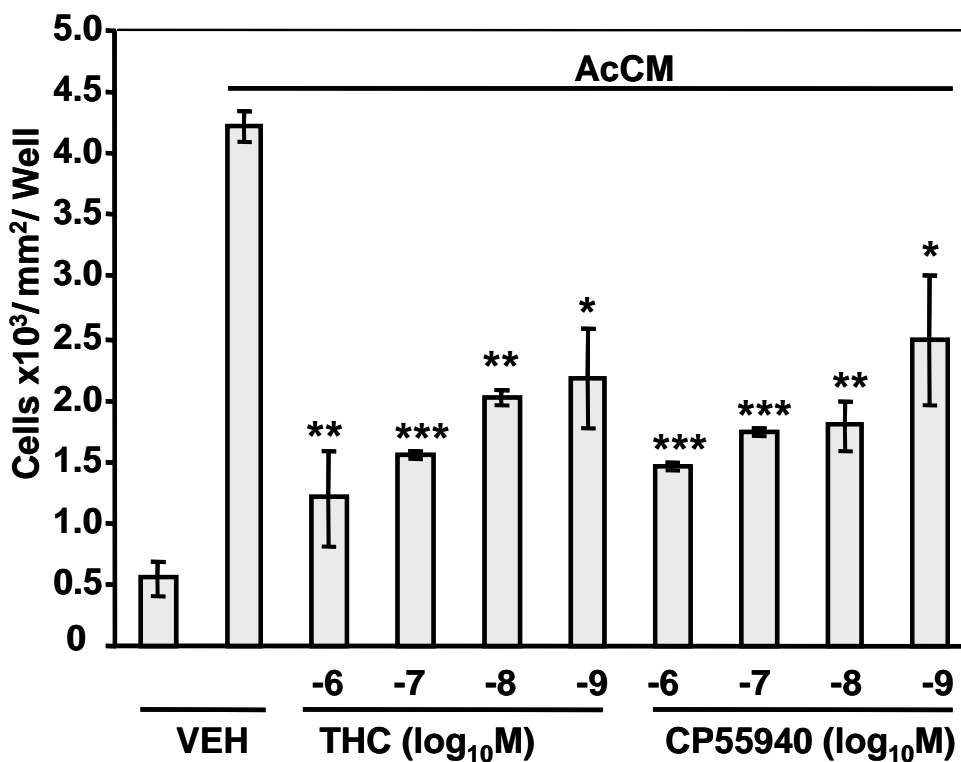


Figure 31- THC and CP55940 Inhibit Chemotaxis of Microglia to *Acanthamoeba* Conditioned Medium. Purified rat microglia were treated for 3 h with cannabinoid or vehicle (VEH) (0.01% ethanol) and assessed (2h) for migration to *Acanthamoeba culbertsoni* conditioned medium (AcCM; 54 μ g). Results are presented as the mean \pm SD. * p <0.05, ** p <0.01, *** p <0.001.

and *Acanthamoeba culbertsoni* conditioned medium (AcCM; 54 µg) (**Figures 32** and **33**, respectively). In contrast, treatment with the CB₁ selective ligand had no effect on microglial migration to *Acanthamoeba* (JH1) conditioned medium (JH1CM; 54 µg) conditioned medium and *Acanthamoeba culbertsoni* conditioned medium (AcCM; 54 µg) (**Figures 32** and **33**, respectively).

The CB₂-specific Antagonist SR144528 Reverses the Inhibitory Effect of CP55940 on Rat Primary Microglial Migration to Amoebic Conditioned Medium

To confirm the cannabinoid receptor associated with the inhibitory effect on microglial migration, cannabinoid receptor agonist-antagonist experiments were performed. For these experiments, the CB₂ antagonist SR144528 (SR2) was used at a concentration of 10⁻⁶M. Treatment of primary rat microglia with the CB₂-specific antagonist SR2 alone had no effect on the chemotactic response to *Acanthamoeba culbertsoni* conditioned medium (AcCM; 54 µg), as the number of SR2-treated cells migrating to AcCM was equivalent to the number vehicle-treated cells migrating to AcCM (**Fig. 34**). CP55940 treatment alone (10⁻⁶ and 10⁻⁸M) significantly inhibited microglial migration to AcCM. At equimolar concentrations (i.e., 10⁻⁶M) of antagonist and agonist, CP55940 inhibited macrophage migration to AcCM. However at lower concentrations of agonist (i.e. 10⁻⁸M), SR2 reversed CP55940-mediated inhibition.

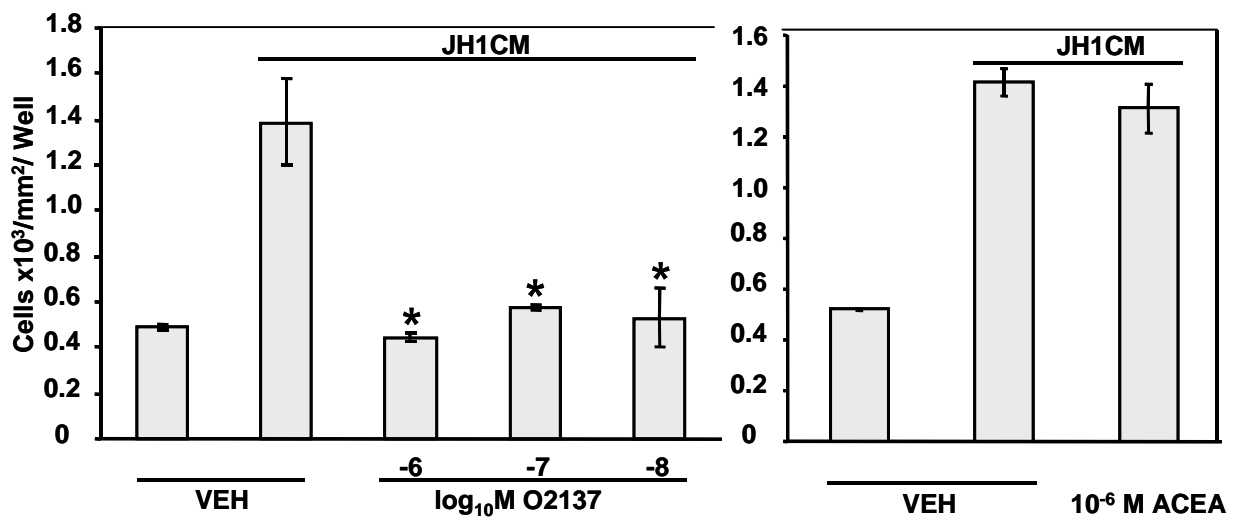


Figure 32- The CB₂ Agonist O-2137 Inhibits Microglial Migration to *Acanthamoeba* Conditioned Medium, but not the CB₁ Agonist ACEA. Microglia were treated (3h) with cannabinoid or vehicle (VEH) (0.01% ethanol) and assessed for migration to *Acanthamoeba* (JH1) conditioned medium (2h) (JH1CM; 54 µg). Results are presented as the mean ± SD. *p<0.05.

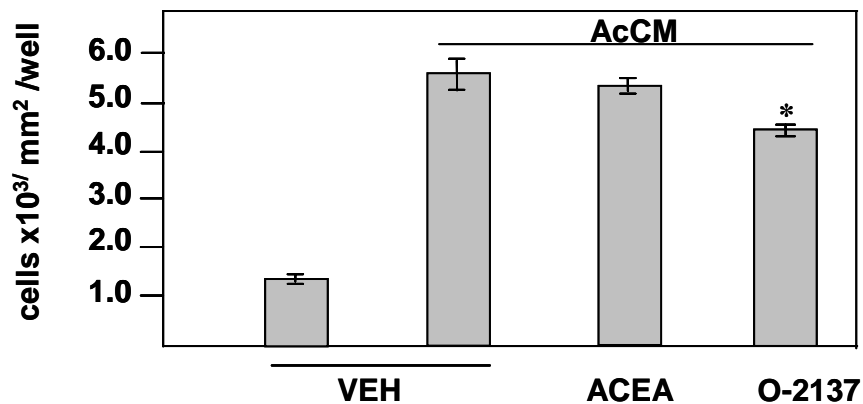


Figure 33-The CB₂ Agonist O-2137 Inhibits Microglial Migration to *Acanthamoeba* Conditioned Medium, but not the CB₁ Agonist ACEA. Microglia were treated (3h) with cannabinoid (10⁻⁶M) or vehicle (VEH) (0.01% ethanol) and assessed for migration to *Acanthamoeba culbertsoni* conditioned medium (2h) (AcCM; 54 µg). Results are presented as the mean ± SD. *p<0.05.

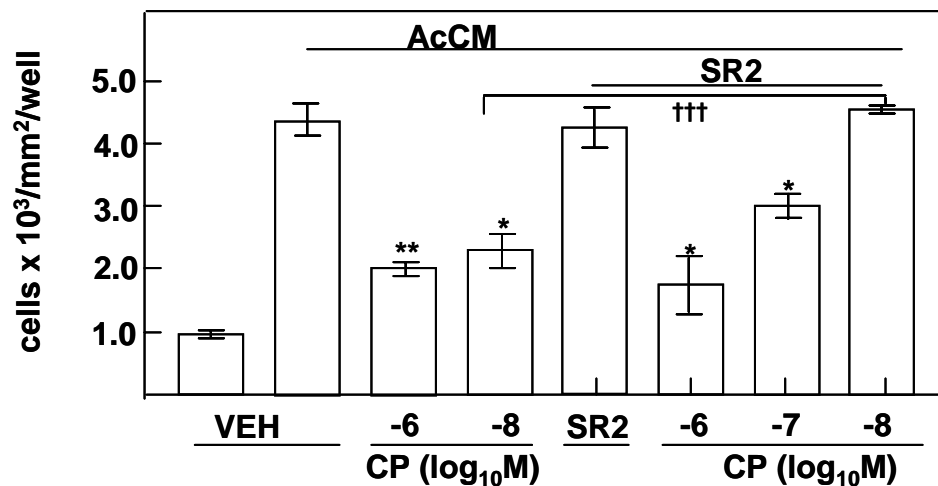


Figure 34 – The CB₂ Antagonist Reverses CP55940-mediated Inhibition of Primary Microglial Migration to *Acanthamoeba* Conditioned Medium. Primary rat microglial cells were pretreated (30 min) with CB₂ antagonist SR2 (10⁻⁶ M) followed by treatment with 10⁻⁶- 10⁻⁸M CP55940 (CP) (3h). Migration to 54 µg *A. culbertsoni* conditioned medium (AcCM) was assessed (2h). Results are presented as the mean ± SD. Cannabinoid (CP55940, SR2, and CP55940+SR2) treated groups were compared with vehicle (VEH)-treated microglia exposed to conditioned medium in the bottom chamber. *p<0.05, **p<0.01. Further statistical analysis using Bonferroni's t-test compared groups treated with CP55940 (10⁻⁶ M) or CP55940+SR2 (10⁻⁶ M) to SR2 (10⁻⁶ M). ††† p <0.001.

Bonferroni's test was performed for comparisons between treatment groups and further confirmed a significant difference in migration to amoebic conditioned medium between microglia treated with CP55940 (10^{-8} M) and those treated with CP55940 (10^{-8} M) and SR2 (10^{-6} M).

These experiments were complemented with those using the CB₁ antagonist SR141716A (SR1). For these experiments, CB₁ antagonist SR141716A (SR1) was used at a concentration of 10^{-6} M. Treatment of primary rat microglia with the CB₁-specific antagonist SR1 alone had no effect on the chemotactic response to *Acanthamoeba culbertsoni* conditioned medium (AcCM; 54 μ g), as the number of SR1-treated cells migrating to AcCM was equivalent to the number of vehicle-treated cells migrating to AcCM (**Fig. 35**). As observed in the previous experiment using SR2 (Fig. 34), CP55940 treatment alone (10^{-6} and 10^{-8} M) significantly inhibited microglial migration to AcCM. At equimolar concentrations (i.e., 10^{-6} M) of antagonist and agonist, CP55940 inhibited macrophage migration to AcCM. However, treatment with the CB₁ antagonist SR1 was unable to block the inhibitory effect of CP55940.

Discussion

THC, the major psychoactive component in marijuana, has been shown to alter the activities of macrophages and macrophage-like cells, including phagocytosis (Friedman *et al.*, 1986; Lopez-Cepero *et al.*, 1986; Tang *et al.*, 1992; Ehrhart *et al.*, 2005), antigen processing (McCoy *et al.*, 1995; McCoy *et al.*, 1999), and production of

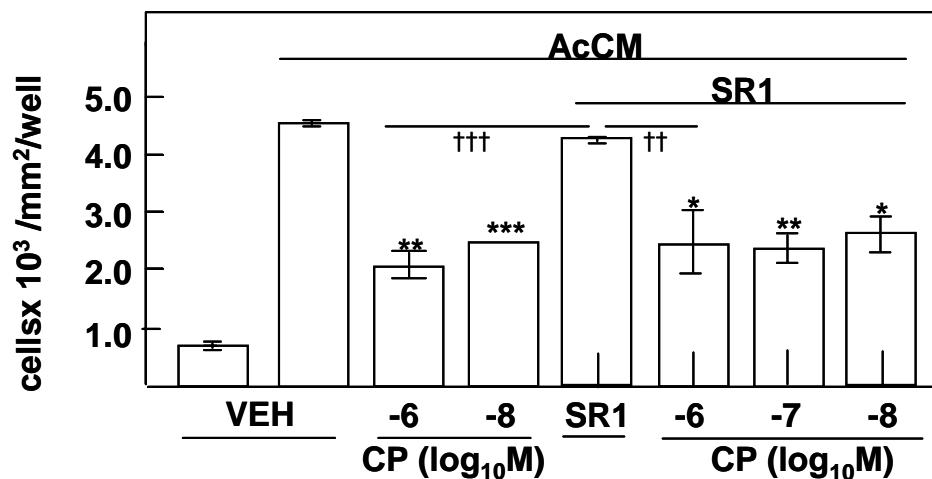


Figure 35– Pretreatment with the CB₁ antagonist SR1 Does Not Alter CP55940-Mediated Inhibition of Primary Microglial Migration to *Acanthamoeba* conditioned medium. Primary microglia were pretreated with SR1 (30 min) followed by treatment with CP55940 (CP) (3h). Migration to 54 μ g *A. culbertsoni* conditioned medium (AcCM) was assessed (2h). Results are presented as the mean \pm SD. Cannabinoid (CP55940, SR1, and CP55940+SR1) treated groups were compared with vehicle (VEH)-treated microglia exposed to conditioned medium in the bottom chamber. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Further statistical analysis using Bonferroni's t-test compared groups treated with CP55940 (10^{-6} M) or CP55940+SR1 (10^{-6} M) to SR1 (10^{-6} M). ††† $p < 0.001$ and †† $p < 0.002$.

chemokines and cytokines (Watzl *et al.*, 1991; Zheng *et al.*, 1992; Puffenbarger *et al.*, 2000). Recent studies indicate that this exogenous cannabinoid, as well as other cannabinoids, also affects the migratory activities of macrophages. Stefano *et al.* (1998) reported that acute exposure to the endogenous cannabinoid (endocannabinoid) anandamide resulted in transformation of macrophages from an amoeboid and motile state to that of a rounded and non-motile conformation. These investigators proposed that the transforming events were linked to the CB₁ receptor since the CB₁-specific antagonist SR141716A blocked the transformation. Sacerdote *et al.* (2000) demonstrated that *in vivo* and *in vitro* treatment of rat peritoneal macrophages with CP55940, a high efficacy agonist at both CB₁ and CB₂ receptors, resulted in decreased migration *in vitro* to the peptide formal-methionyl-leucine-phenylalanine (fMLP). It was indicated that, while both the CB₁ and CB₂ receptors appeared to be involved in this process, the cannabinoid-mediated effect was linked primarily to the CB₂. The chemotactic response of murine macrophages to fMLP also has been shown to be decreased by cannabidiol (Sacerdote *et al.*, 2005), a cannabinoid that binds weakly to CB₂. The CB₂ antagonist SR144528 prevented this decrease, suggesting a functional linkage to this receptor.

On the other hand, Walter *et al.* (2003) found that the endocannabinoid 2-arachidonylglycerol (2-AG) triggered migration of microglia, macrophages that are resident in the brain, and that the CB₂ was involved in this effect. Additionally, these investigators and others (Jorda *et al.*, 2002; Kishimoto *et al.*, 2005) have demonstrated that THC did not induce a migratory cellular response in natural killer cells or cells of myeloid origin and, further, inhibited migration of these cell types to 2-AG. Collectively,

these studies suggest that exogenous cannabinoids exert inhibitory effects on macrophage migration while endocannabinoids elicit an opposite effect.

Consistent with these observations, in the present study we demonstrated that THC inhibits the chemotactic or directed migratory response of murine peritoneal macrophages to RANTES, a chemokine that can signal through the chemokine receptors CCR1 and CCR5. This effect was exerted on peritoneal macrophages from mice administered THC *in vivo* or on peritoneal macrophages that were exposed directly to THC *in vitro*. In the latter context, the inhibition occurred over a wide concentration range (i.e., 10^{-6} M – 10^{-12} M). These results are consistent with THC as having a direct effect on macrophages which results in inhibition of chemotaxis. Indeed, scanning electron microscopic analysis revealed dramatic alterations in cellular morphology following treatment with THC, indicating that these cells had reduced migratory responsiveness. Whereas vehicle-treated cells migrating to RANTES displayed morphological characteristics of migration including membrane ruffling and numerous cellular projections extending toward or into membrane pores, the cells treated with THC were rounded and appeared to be non-motile.

The results obtained with THC were replicated using the high efficacy CB₁/CB₂ agonist CP55940. Treatment of murine macrophages *in vitro* with CP55940 resulted in inhibition of chemotaxis to RANTES over the same concentration range (i.e., 10^{-6} M – 10^{-12} M) of THC. In order to establish whether the cannabinoid-mediated inhibition was linked to a cannabinoid receptor, a series of experiments was performed in which cannabinoid receptor-selective agonists as well as cannabinoid receptor-specific

antagonists were used. Treatment of macrophages *in vitro* with O-2137, a compound that exhibits high selectivity for the CB₂, resulted in a robust inhibition of macrophage chemotaxis. In contrast, the CB₁ selective compound ACEA had a minimal effect. In addition, the CB₂ antagonist SR144528 blocked CP55940-mediated inhibition of macrophage chemotaxis while the CB₁ antagonist SR141716A had a minimal effect. Finally, THC was not able to inhibit the chemotactic response to RANTES of peritoneal macrophages obtained from CB₂ knockout mice. Collectively, the results of experiments in which a pharmacological approach was complemented with that using macrophages from CB₂ null (i.e., CB₂ ^{-/-}) mice support the proposition that the CB₂ is linked functionally to the THC-mediated inhibition of chemotaxis to RANTES.

RANTES, for which the current International Union of Pharmacology nomenclature is CCL5 (Murphy, 2002), is one of many chemotactic cytokines that direct the migration of leukocytes to sites of infection and inflammation. In this capacity, these small molecular weight proteins constitute a critical component of innate immune defenses. Four subfamilies of chemokines have been identified based on the relative position of their N terminal cysteine residues. All chemokines bind specific receptors that have seven transmembrane domains and are coupled to heterotrimeric G_i proteins, a feature that is shared with cannabinoid agonists. However, binding within a chemokine subfamily is somewhat promiscuous. In addition, multiple chemokine receptor types have been identified on individual immune cells and their expression may vary in relation to cell differentiation and activation. These characteristics confer multiple levels of regulation and exquisitely sensitive responses to the chemokine/chemokine receptor

system. RANTES, for example, can bind CCR1, CCR3, and CCR5, receptors that have specialized roles in leukocyte trafficking (Murdoch and Finn, 2000; Murphy, 2002; Charo *et al.*, 2006). Monocytes have been reported to express a variety of chemokine receptors, particularly CCR1, CCR2, and CCR5 (Mantovani *et al.*, 2004). It has been demonstrated also that differentiation of monocytes into tissue macrophages is associated with the upregulation of CCR1 and CCR5 and loss of CCR2 expression (Mantovani *et al.*, 2004). In the present study, we examined thioglycollate-elicited peritoneal macrophages from (B₆C₃)F₁ and C57BL/6 mice for their CC chemokine receptor expression profile. These cells were shown to express CCR1 and CCR5, receptors that can bind RANTES. Thus, in the context of our experimental paradigm it is possible that RANTES acted through one or both receptors to induce chemotactic activity. In turn, THC may have affected the functionality of one or both chemokine receptors. Regardless of which of the chemokine receptors found on macrophages is functionally relevant in RANTES-mediated signaling, the results of this study suggest that cannabinoid activation of the CB₂ can result in deactivation of other members of the G protein-coupled family such as chemokine receptors. Further studies utilizing chemokine receptor-specific antagonists should serve to identify the CC receptor type that is linked to RANTES-mediated chemotactic activity that is targeted by cannabinoids.

The mode by which THC and other analogs that signal through cannabinoid receptors to deactivate CCR1 and/or CCR5 chemokine receptor migratory responsiveness to RANTES remains to be defined. THC and other cannabinoids, as highly lipophilic molecules, can perturb cellular membranes (Martin, 1986; Makriyannis *et al.*, 1990;

Cabral and Staab, 2005). Such perturbation could alter conformational strictures requisite for ligand-receptor interaction, disrupt receptor-G protein complexes, and disturb intracellular membranous compartments that are linked to biochemical events in the cascade of signal transduction. However, as suggested by the present study, cannabinoids also may trans-deactivate chemokine receptors and affect their ability to elicit a signal transductional cascade that culminates in the chemotactic migratory response. Indeed, it has been reported that members of the G protein coupled receptor superfamily can associate with each other, forming homodimers and heterodimers that results in alteration in the functionality of one of the involved receptors (Rios *et al.*, 2001). Opioid receptors, for example, have been reported to interact with chemokine receptors to alter their function. Grimm *et al.* (1998) indicated that this interaction resulted in trans-deactivation of chemokine receptors and that it occurred through a process of receptor-mediated heterologous desensitization. Desensitization is the functional result of receptor phosphorylation by G protein coupled receptor kinases (GRKs) or other second messenger kinases (i.e., protein kinase C), which prevents further coupling to G proteins. Following arrestin binding, the phosphorylated receptor undergoes internalization and recycling.

In their studies, Grimm *et al.* (1998) demonstrated that met-enkephalin and morphine inhibited interleukin (IL)-8-induced chemotaxis of human neutrophils and macrophage inflammatory protein (MIP)-1 α , RANTES, and monocyte chemoattractant protein (MCP)-1-mediated chemotaxis of human monocytes. This inhibition was indicated as mediated by δ - and μ -opioid receptors, the activation of which led to

phosphorylation of the chemokine receptors CXCR1 and CXCR2 resulting in heterologous desensitization. Rogers *et al.* (2000) reported that activation of opioid and chemokine receptors could lead to reciprocal down-regulation of leukocyte migratory activities. These observations have been extended using a number of experimental paradigms (Szabo and Rogers, 2001; Szabo *et al.*, 2001; Szabo *et al.*, 2002; Suzuki *et al.*, 2003; Zhang *et al.*, 2003). Indeed, it has been proposed that cross-desensitization of chemokine receptors by opioids represents a significant element in opioid-mediated immunosuppression (Zhang *et al.*, 2003). The process of heterologous desensitization may also apply to cannabinoid receptors, and these studies may serve to elucidate, in part, the mechanism of cannabinoid-mediated immunosuppression. Ghosh *et al.* (2006) reported that the CB₁/CB₂ agonist CP55940, as well as the CB₂-selective agonist JW-015, caused significant inhibition of chemokine CXCL12-induced chemotaxis of CD4⁺ and CD8⁺ T lymphocytes. These investigators also found that these cannabinoids inhibited CXCL12 induced chemotaxis and transendothelial migration of Jurkat T cells. Rios *et al.* (2006) reported recently that the μ opioid receptor also interacts with the CB₁ to affect a reciprocal inhibition of receptor signaling and receptor-induced neuritogenesis.

In the present study, we propose that heterologous desensitization may articulate a mode of action by which cannabinoids mediate inhibition of the murine peritoneal macrophage chemotactic response to RANTES. Thus, in order to obtain initial insight as to the process by which THC and other cannabinoids cross-deactivate this macrophage activity, a multiprobe RNase protection assay was performed to assess for levels of CC chemokine receptor mRNAs. THC over a concentration range of 10^{-6} M to 10^{-12} M had no

effect on macrophage mRNA levels of CCR1 and CCR5. Likewise, treatment with other cannabinoid receptor agonists and antagonists including CP55940, SR1 and SR2 (10^{-6} M) had no effect on CCR1 or CCR5 mRNA as assessed by multiprobe RNase protection assay. These results are consistent with THC-mediated inhibition of the chemotactic process as occurring at a level of regulation other than gene expression of the cognate receptors at the mRNA level. Immunoprecipitation experiments followed by Western blot analysis using a primary antibody specific for phosphorylated serine residues were performed to assess for effects of cannabinoids on protein expression and phosphorylation of CCR5, which notably undergoes serine phosphorylation. Stimulation with the cognate ligand RANTES induced CCR5 phosphorylation as compared to cells treated with vehicle. Further, treatment with CP55940 (10^{-6} M) resulted in the induction of CCR5 phosphorylation. The level of CCR5 phosphorylation induced by CP55940 was less than the level of phosphorylation following stimulation with RANTES, results which are consistent with reports by Chen *et al.* (2004) who demonstrated that treatment with μ -opioid receptor agonist DAMGO induced CCR5 phosphorylation but to a lesser extent than RANTES.

Cannabinoids can also affect migration to chemotactic molecules other than chemokines including bioactive lipids such as 2-AG. 2-Arachidonoylglycerol (2-AG) is an endogenous cannabinoid (endocannabinoid) that is a native ligand to cannabinoid receptors in the CNS and in the periphery. Formation of 2-AG occurs rapidly through the cleavage of membrane phospholipids by phospholipases and diacylglycerol lipase. It is produced by numerous cell types under a wide variety of stimulatory conditions including

LPS stimulation of rat macrophages (DiMarzo *et al.*, 1999), ATP stimulation of mouse microglia (Witting *et al.*, 2004), cholera toxin-treated mouse small intestine (Izzo *et al.*, 2003), macrophage colony stimulating factor treated rat microglia (Carrier *et al.*, 2004), and mouse brain following traumatic brain injury (Panikashvili *et al.*, 2001). Indeed, it has been proposed that 2-AG plays a physiological role in the regulation of neurotransmitter release (Sugiura and Waku, 2000), the cardiovascular system (reviewed in Sugiura *et al.*, 2006), and the proliferation and invasion of certain types of cancer cells (reviewed in Sugiura *et al.*, 2006). It seems very likely, therefore, that 2-AG plays an essential role in the regulation of a variety of biological systems.

Accumulating evidence also suggests that 2-AG is involved in immunomodulation. Kishimoto *et al.* (2004) reported that 2-AG induced production of interleukin-8 (IL-8) and monocyte chemoattractant protein-1 (MCP-1), and that this effect was linked to CB₂. Further, addition of LPS to 2-AG synergistically augmented IL-8 and MCP-1 production. 2-AG has also been shown to modulate other activities associated with the inflammatory response inducing changes in cellular morphology through rapid actin rearrangement (Gokoh *et al.*, 2005), enhancing cellular adhesion (Gokoh *et al.*, 2005), and stimulating cell migration. Walter *et al.* (2003) demonstrated that 2-AG induced migration in microglia, and that this migration was linked to activation of CB₂. Further, localization of CB₂ to the leading edge of lamellipodia implicated a role for CB₂ in migration (Walter *et al.*, 2003). Additional studies (Jorda *et al.*, 2002; Kishimoto *et al.*, 2005) demonstrated that 2-AG induces migration in multiple immune cell types and that treatment with THC can inhibit this migration. It has recently been

proposed by Cabral *et al.* (2007) that cannabinoids alter macrophage migration through activation of CB₂, with endocannabinoids such as 2-AG exerting a stimulatory effect and exogenous cannabinoids eliciting an opposite, inhibitory effect. Endogenous cannabinoids such as 2-AG, signaling through CB₂, may stimulate or support an inflammatory response through the increase in cell adhesion and migration, the activation of mitogen-activated protein kinase (MAP kinase), induction of [Ca²⁺] release, and upregulation of chemokine production whereas other CB₂ ligands may block these effects. This block could be the result of competition for receptor binding involving limiting factors such as ligand accessibility (generation and short half-life of endogenous ligands) or ligand affinity.

As a critical component in innate immunity, macrophages play a key role in recognition and clearance of bacterial, protozoan, and viral pathogens. Integral to this recognition process are a host of cellular receptors. For example, pattern recognition receptors recognize pathogen associated molecular pattern (PAMPs), which include bacterial carbohydrate moieties like mannose or LPS; bacterial, viral, protozoan RNA or DNA; and viral glycoproteins. Host cells also express a class of G-protein coupled receptors designated Protease-activated receptors (PARs1-4) that can be activated by immune cell-derived or microorganism-derived serine proteases (Steinhoff *et al.*, 2005; Traynelis and Trejo, 2007). PAR₁ and PAR₂ can be coupled to G α_q , G α_i , G $\alpha_{12/13}$, and G $\beta\gamma$ and induce multiple signal transduction cascades. PAR_{1/2} activation induces protein kinase C (PKC) and MAP kinase activation, Rho/Rac signaling, mobilization of intracellular calcium [Ca²⁺] through activation of phospholipase C (PLC), and activation

of receptor tyrosine kinases (RTKs) (Traynelis and Trejo, 2007). Activation of these signaling networks results in alterations in cell shape, adhesion, secretion of inflammatory mediators, and motility. PAR₁ is expressed in a variety of cells including platelets, endothelial cells, CD8+ T cells, monocytes, microglia, astrocytes, neurons, mast cells, and certain tumor cells (reviewed in Steinhoff *et al.*, 2005). PAR₂ is expressed by immune cells including dendritic cells, eosinophils, macrophages, and neutrophils (Miike *et al.*, 2001; Colognato *et al.*, 2003; Howells *et al.*, 1997; Moormann *et al.*, 2006). Serine proteases and PARs have been implicated in immune and inflammatory regulation; however, the majority of the data to date is limited to PAR modulation of cell adhesion molecules, chemokine/cytokine production, and cell migration. Colotta *et al.* (1994) reported that thrombin, acting through PAR₁, induced expression of MCP-1 by human peripheral blood mononuclear cells (PBMC). Additionally, treatment of endothelial cells with thrombin, which signals through PAR₁, induced IL-8, E-selectin (Kaplanski *et al.*, 1997), intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1) expression (Kaplanski *et al.*, 1998). PAR₁ activation with thrombin has also been shown to induce human neutrophil chemotaxis (Mariano-Oliveira *et al.*, 2007). Morris *et al.* (2006) demonstrated that PAR₂ plays a critical role in breast cancer cell migration and invasion.

Many bacteria and amebae that are pathogenic in humans produce serine proteases. It is reasonable, therefore, to postulate that these pathogen-derived proteases may trigger specific immune or inflammatory responses through direct interaction with host cell PARs. Amebae of the genus *Acanthamoeba* have been shown to secrete

multiple serine, cysteine, and matrix metalloproteases that have been implicated in the virulence of the pathogen (Marciano-Cabral and Cabral, 2003). These proteases are thought to play a role in tissue destruction and pathogen invasion, and are capable of degrading host IgA and IgG, multiple extracellular matrix components including types I and III collagen, and serum proteins hemoglobin and fibrinogen (Kong *et al.*, 2000; Mitro *et al.*, 1994; Cho *et al.*, 2000; and Sissons *et al.*, 2006). In the present study we demonstrated that peritoneal macrophages migrate towards media conditioned with two strains of *Acanthamoeba* (*Acanthamoeba culbertsoni* and *Acanthamoeba* JH1, which contains intracellular gram negative bacteria). *Acanthamoeba* conditioned medium has been shown to contain a variety of proteases including serine and metalloproteases (Sissons *et al.*, 2006). The chemoattractants in the amoebic conditioned medium seem to be directly produced by the amoeba, as there was no major difference in cell migration to the conditioned medium derived from culture with *Acanthamoeba culbertsoni* or from medium cultured with *Acanthamoeba* JH1 harboring intracellular bacteria. We also found that THC inhibits the chemotactic response of murine peritoneal macrophages to *Acanthamoeba* conditioned medium. This inhibitory effect was exerted on peritoneal macrophages from mice administered THC *in vivo* or on peritoneal macrophages that were exposed directly to THC *in vitro*. Scanning electron microscopic analysis revealed dramatic alterations in macrophage morphology following treatment with THC, indicating that these cells had reduced migratory responsiveness similar to previous results obtained using RANTES as the chemoattractant. Vehicle-treated cells migrating to *Acanthamoeba* conditioned medium displayed morphological characteristics of

migration including membrane ruffling and numerous cellular projections extending toward or into membrane pores; however, as demonstrated in previous studies the macrophages treated with THC appeared to be rounded and non-motile.

We then employed a strategy similar to the one previously utilized in the RANTES studies to determine whether a cannabinoid receptor was involved in inhibition of macrophage migration to amoebic conditioned medium. Experiments were repeated using the high efficacy CB₁/CB₂ agonist CP55940. Treatment of peritoneal macrophages *in vitro* with CP55940 resulted in significant inhibition of migration to *Acanthamoeba* conditioned medium. In addition, we performed experiments using compounds exhibiting selective high affinity binding to the CB₁ or the CB₂ prior to assessment of the chemotactic response to amoebic conditioned medium (ACEA and O-2137, respectively). Treatment of macrophages with the selective CB₂ ligand O-2137 resulted in significant concentration-related inhibition in the migration to *Acanthamoeba* conditioned medium. The CB₁ specific ligand ACEA had no effect on peritoneal macrophage migration to *Acanthamoeba* conditioned medium. Further experiments employing cannabinoid receptor antagonists used in concert with CP55940 were performed. The CB₂ antagonist SR144528 blocked CP55940-mediated inhibition of macrophage migration to amoebic conditioned medium while the CB₁ antagonist SR141716A had a minimal effect. Together, these results imply that CB₂ is linked to cannabinoid-mediated inhibition of macrophage migration to *Acanthamoeba* conditioned medium.

For many years the brain was believed to be an immune privileged site, however accumulating evidence suggests that, like the periphery, the CNS is under constant immune surveillance. Microglia are a resident population of cells in the CNS that are morphologically, phenotypically, and functionally related to macrophages (Aloisi, 2001; Gehrmann *et al.*, 1995). Upon activation by inflammatory or infectious stimuli, these cells undergo proliferation and functional maturation with alterations in receptor expression and the production of inflammatory mediators like cytokines, chemokines, and reactive oxygen species (Aloisi, 2001). Dysregulation of this response, or chronic activation, has been implicated in neuropathological diseases like Multiple Sclerosis (MS), Alzheimer's disease (AD), Parkinson's disease, and Acquired Immune Deficiency (AIDS) dementia (reviewed in Bajetto *et al.*, 2002).

A signature activity of activated microglia is migration to sites of inflammation or infection. Using a mouse model of *Acanthamoeba* infection in the CNS, Cabral *et al.* (2007) demonstrated the involvement of macrophage-like (microglia) cells in the formation of immune cell granulomas surrounding *Acanthamoeba culbertsoni* cysts. Granulomas are believed to sequester pathogens preventing further dissemination. Mice administered THC *in vivo* experienced higher rates of mortality following infection with the amoebae which may be due, in part, to the observed inability to form granulomas. We postulated that the absence of granulomas following THC might be the consequence of a cannabinoid-mediated inhibition of microglial migration. In the final part of the study, we utilized an *in vitro* migration system with primary rat microglial cells and *Acanthamoeba* conditioned medium to model an *in vivo* infection and assess the effect of

THC on microglial migration. Treatment of primary neonatal rat microglia with THC or CP55940 (10^{-6} - 10^{-8} M) resulted in significant concentration-related inhibition of microglial migration to *Acanthamoeba* conditioned medium.

Carlisle and Cabral (2002) reported that microglia constitutively express very low levels of CB₁, whereas CB₂ is expressed differentially in relation to cell activation state. CB₂ is expressed at high levels in microglia when they are in responsive or primed states. These activation states are characterized by differential gene expression and certain functional activities. In responsive and primed states macrophages and microglia are capable of chemotaxis, phagocytosis, and antigen presentation, activities that correlate with early inflammatory responses. We performed migration assays using compounds exhibiting selective binding to the CB₁ or the CB₂ prior to assessment of the chemotactic response to amoebic conditioned medium. Treatment of macrophages with the CB₂ ligand O-2137 resulted in significant concentration-related inhibition in the migration to *Acanthamoeba* conditioned medium; however, the CB₁ specific ligand ACEA had no effect on microglial migration to *Acanthamoeba* conditioned medium. Further experiments using CB₁ and CB₂ specific antagonists in combination with CP55940 were also performed. Treatment with the CB₂ antagonist SR144528 blocked CP55940-mediated inhibition of microglial migration to amoebic conditioned medium while the CB₁ antagonist SR141716A had no effect. These data suggest cannabinoid-mediated inhibition of microglial migration to *Acanthamoeba* conditioned medium is linked to the CB₂ cannabinoid receptor, which is consistent with the known cannabinoid receptor expression profile of microglia, as well as the results from previous models used in our

studies. Furthermore, since migration can be elicited by protease activation of PARs on microglia, it is possible that THC-mediated inhibition of the chemotactic response to amoebic conditioned medium may be due to CB₂ “cross-talk” with PARs. Studies to establish such a functional linkage should serve to clarify whether the CB₂ receptor can “cross-communicate” with a diverse array of G-protein coupled receptors so as to modulate responsiveness by macrophages and macrophage-like cells.

In summary, we have demonstrated that THC and other exogenous cannabinoids that activate the CB₂ inhibit murine peritoneal macrophage chemotaxis to RANTES/CCL5. This inhibitory effect was linked functionally to the CB₂ receptor. Furthermore, since this chemokine serves as a ligand for CCR1 and CCR5, these results suggest that activation of the CB₂ leads to trans-deactivation of these G protein-coupled receptors of the CC chemokine subfamily that have specialized roles in leukocyte trafficking. Thus, as has been suggested for opioid receptors, CB₂ “cross-talk” with chemokine receptors may constitute an integrative component of a network of intercommunicating G protein-coupled receptors that regulate immune responses. In addition, we have demonstrated that THC and other exogenous cannabinoids that activate the CB₂ inhibit murine peritoneal macrophage and rat primary microglial chemotaxis to *Acanthamoeba* conditioned medium. Cannabinoids, as acting through cannabinoid receptors, may “cross-communicate” with a diverse array of G-protein coupled receptors, thereby affecting activation of receptors such as CCR5 and PARs. Although much more work needs to be done to link the chemotactic response of macrophages and microglia to amoebic conditioned medium to PAR activation and to establish whether heterologous

desensitization is occurring between CB₂ and PARs, this study provides initial insight to the mechanism of THC-mediated immunosuppression in CNS infections with amoeba. We also have shown that the endogenous cannabinoid, 2-AG induces migration of peritoneal macrophages and that this effect can be inhibited by the exogenous cannabinoid, THC. We have proposed a model in which endogenous cannabinoids signaling through CB₂ exert a positive or stimulatory effect on the inflammatory response, whereas exogenous cannabinoids elicit an inhibitory effect. These studies demonstrate a critical role for CB₂ in immunoregulation and inflammation in the CNS and the periphery.

Literature Cited

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1. Alfieri SC, Correia CE, Motegi SA, Pral EM (2000) Proteinase activities in total extracts and in medium conditioned by *Acanthamoeba polyphaga* trophozoites. *J Parasitol* 86:220-227.
2. Aloisi F (2001) Immune function of microglia. *Glia* 36:165-179.
3. Bajetto A, Bonavia R, Barbero S and Schettini G (2002) Characterization of chemokines and their receptors in the central nervous system: physiopathological implications. *J Neurochem* 82:1311-1329.
5. Baggiolini M, Dewald B and Moser B (1994) Interleukin-8 and related chemotactic cytokines CXC and CC chemokines. *Adv Immunol* 55:97-179.
6. Baggiolini M, Dewald B and Moser B (1997) Human chemokines: an update. *Annu Rev Immunol* 15:675-705.
7. Ben Amar M (2006) Cannabinoids in medicine: A review of their therapeutic potential. *J Ethnopharmacol* 105:1-25.
8. Berdyshev EV (2000) Cannabinoid receptors and the regulation of immune response. *Chemistry and Physics of Lipids* 108:169-190.
9. Berdyshev EV, Schmid PC, Krebsbach RJ, Hillard CJ, Huang C, Chen N, Dong Z and Schmid HO (2001) Cannabinoid-receptor-independent cell signaling by N-acylethanolamines. *Biochem J* 360:67-75.

10. Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248-254.
11. Buckley NE, McCoy KL, Mezey E, Bonner T, Zimmer A, Felder CC, Glass M and Zimmer A (2000) Immunomodulation by cannabinoids is absent in mice deficient for the cannabinoid CB(2) receptor. *Eur J Pharmacol* 396:141-149.
12. Cabral GA, Toney DM, Fischer-Stenger K, Harrison MP and Marciano-Cabral FM (1995) Anandamide inhibits macrophage-mediated killing of tumor necrosis-sensitive cells. *Life Sci* 56:23-24.
13. Cabral GA and Dove Pettit DA (1998) Drugs and immunity: cannabinoids and their role in decreased resistance to infectious disease. *J Neuroimmunol* 83:116-123.
14. Cabral GA and Staab A (2005) Effects on the immune system, in *Handbook on Experimental Pharmacology: Cannabinoids* (Pertwee R ed) pp 85-423, Springer-Verlag, Berlin.
15. Cabral GA, Raborn ES, Griffin L, Dennis J, Marciano-Cabral FM (2007) CB₂ receptors in the brain: role in central immune function. *Br J Pharmacol*. In press.
16. Carlisle SJ and Cabral GA (2002) Differential expression of the CB₂ cannabinoid receptor by rodent macrophages and macrophage-like cells in relation to cell activation. *International Immunopharmacol* 2:69-82.
17. Carrier EJ, Kearn CS, Barkmeier AJ, Breese NM, Yang W, Nithipatikom K, Pfister SL, Campbell WB, Hillard CJ (2004) Cultured rat microglial cell lines synthesize the

- endocannabinoid 2-arachidonoylglycerol, which increases proliferation via CB₂ receptor-dependent mechanism. *Mol Pharmacol* 65:999-1007.
18. Chari-Bitron A (1976) Effect of δ^1 -tetrahydrocannabinol on red blood cell membranes and alveolar macrophages, in *Marihuana: Chemistry, Biochemistry, and Cellular Effects* (Nahas GG ed) pp 273-281, Springer-Verlag, New York.
 19. Charo IF and Ransohoff RM (2006) Mechanisms of disease: The many roles of chemokines and chemokine receptors in inflammation. *N Engl J Med* 354:610-621.
 20. Cho JH, Na BK, Kim TS, and Song CY (2000) Purification and characterization of an extracellular serine proteinase from *Acanthamoeba castellanii*. *IUBMB Life* 50:209-214.
 21. Chuchawankul S, Shima M, Buckley NE, Hartmann CB and McCoy KL (2004) Role of cannabinoid receptors in inhibiting macrophage costimulatory activity. *Int Immunopharmacol* 4:265-278.
 22. Colotta F, Sciacca FL, Sironi M, Luini W, Rabiet MJ, Mantovani A (1994) Expression of monocyte chemotactic protein-1 by monocytes and endothelial cells exposed to thrombin. *Am J Pathol* 144:975-985.
 23. Colognato R, SlupskyJR, Jendrach M, Burysek L, Syrovets T, Simmet T (2003) Differential expression and regulation of protease-activated receptors in human peripheral blood monocytes and monocyte-derived antigen-presenting cells. *Blood* 102:2645-2652.

24. Daaka Y, Friedman H and Klein TW (1996) Cannabinoid receptor proteins are increased in Jurkat, human T-cell line after mitogen activation. *J Pharmacol Exp Ther* 276:776-783.
25. DiMarzo V, Bisogno T, De Petrocellis L, Melck D, Orlando P, Wagner JA, Kuno G (1999) Biosynthesis and inactivation of the endocannabinoid 2-arachidonoylglycerol in circulating and tumoral macrophages. *Eur J Biochem* 264:258-267.
26. Dove Pettit D, Harrison MP, Spencer RF and Cabral GA (1998) Immunohistochemical localization of the neural cannabinoid receptor in rat brain. *J Neurosci Res* 51:391-402.
27. Drath DB, Shorey JM, Price L and Huber GL (1979) Metabolic and functional characteristics of alveolar macrophages recovered from rats exposed to marijuana smoke. *Infect Immun* 25:268-272.
28. Ehrhart J, Obregon D, Mori T, Hou H, Sun N, Bai Y, Klein T, Fernandez F, Tan J and Shytle RD (2005) Stimulation of cannabinoid receptor 2 (CB2) suppresses microglial activation. *J Neuroinflammation* 2:29-41.
29. Felder CC, Veluz JS, Williams HL, Briley EM and Matsuda LA (1992) Cannabinoid agonists stimulate both receptor- and non-receptor-mediated signal transduction pathways in cells transfected with and expressing cannabinoid receptor clones. *Mol Pharmacol* 42:838-845.
30. Friedman M, Cepero ML, Klein T and Friedman H (1986) Suppressive effect of delta 9-tetrahydrocannabinol in vitro on phagocytosis by murine macrophages. *Proc Soc Exp Biol Med* 182:225-228.

31. Galiégue S, Mary S, Marchand J, Dussossoy D, Carrier D, Carayon P, Bouaboula M, Shire D, Le Fur G and Casellas P (1995) Expression of central and peripheral cannabinoid receptors in human immune tissues and leukocyte subpopulations. *Eur J Biochem* 232:54-61.
32. Gehrman J, Matsumoto Y, Kreutzberg GW (1995) Microglia: intrinsic immune effector cell of the brain. *Brain Res Rev* 20:269-287.
33. Gerard C and Gerard NP (1994) C5A anaphylatoxin and its seven transmembrane-segment receptor. *Rev Immunol* 12:775-808.
34. Ghosh S, Preet A, Groopman JE and Ganju KG (2006) Cannabinoid receptor CB₂ modulates the CXCL12/CXCR4-mediated chemotaxis of T lymphocytes. *Mol Immunol* 43:2169-2179.
35. Gokoh M, Kishimoto S, Oka S, Mori M, Waku K, Ishima Y, Sugiura T (2005) 2-Arachidonoylglycerol, an endogenous cannabinoid receptor ligand, induces rapid actin polymerization in HL-60 cells differentiated into macrophage-like cells. *Biochem J* 386:583-589.
36. Gokoh M, Kishimoto S, Oka S, Metani Y, Sugiura T (2005) 2-Arachidonoylglycerol, an endogenous cannabinoid receptor ligand, enhances the adhesion of HL-60 cells differentiated into macrophage-like cells and human peripheral blood monocytes. *FEBS Lett* 579:6473-6478.
37. Goldman DW and Goetzl EJ (1982) Specific binding of leukotriene B₄ to receptors on human polymorphonuclear leukocytes. *J Immunol* 129:1600-1604.

38. Grimm MC, Ben-Baruch A, Taub DD, Howard OM, Resau JH, Wang JM, Ali H, Richardson R, Snyderman R and Oppenheim JJ (1998) Opiates transactivate chemokine receptors: delta and mu opioid receptor-mediated heterologous desensitization. *J Exp Med* 188:317-325.
39. Hadas E and Mazur T (1993) Proteolytic enzymes of pathogenic and non-pathogenic strains of *Acanthamoeba* spp. *Trop Med Parasitol* 44:197-200.
40. Hanahan DJ (1986) Platelet activating factor: A biologically active phosphoglyceride. *Annu Rev Biochem* 55:483-509.
41. Harris H (1953) Chemotaxis of monocytes. *Br J Exp Pathol* 34:276-279.
42. Harris H (1954) Role of chemotaxis in inflammation. *Physiol Rev* 34:529-562.
43. Herkenham M, Lynn AB, Johnson MR, Melvin LS, de Costa BR and Rice KC (1991) Characterization and localization of cannabinoid receptors in rat brain: A quantitative in vitro autoradiographic study. *J Neurosci* 11:563-583.
44. Howells GL, Macey MG, Chinni C, Hou L, Fox MT, Harriott P, Stone SR (1997) Proteinase-activated receptor-2: expression by human neutrophils. *J Cell Sci* 110:881-887.
45. Howlett AC, Barth F, Bonner TI, Cabral G, Casellas P, Devane WA, Felder CC, Herkenham M, Mackie K, Martin BR, Mechoulam R, and Pertwee RG (2002) International Union of Pharmacology. XXVII. Classification of Cannabinoid Receptors. *Pharmacol Rev* 54:161-202.

46. Huber GL, Simmons GA, McCarthy CR, Cutting MB, Laguarda R and Pereira W (1975) Depressant effect of marihuana smoke on antibactericidal activity of pulmonary alveolar macrophages. *Chest* 68:769-773.
47. Huber GL, Shea JW, Hinds WC, Pochay VE, and Weker RT, First MW and Sornberger GC (1978) An experimental animal model for quantifying the biological effects of marijuana on the defense system of the lung. *Adv Biosci* 22-23:301-328.
48. Izzo AA, Capasso F, Costagliola A, Bisogno T, Marsicano G, Legresti A, Matias I, Capasso R, Pinto L, Borrelli F, Cecio A, Lutz B, Mascolo N, Di Marzo V (2003) An endogenous cannabinoid tone attenuates cholera toxin-induced fluid accumulation in mice. *Gastroenterology* 125:765-774.
49. Jin T and Hereld D (2006) Moving toward understanding eukaryotic chemotaxis. *Eur J Cell Biol* In press.
50. Jordá MA, Verbakel SE, Valk PJ, Vankan-Berjoudt YV, Maccarrone M, Finazzi-Agró A, Löwenberg B, Delwel (2002) Hematopoietic cells expression the peripheral cannabinoid receptor migrate in response to the endocannabinoid 2-arachidonoylglycerol. *Blood* 99:2786-2793.
51. Kaplanski G, Fabrigoule M, Boulay V, Dinarello CA, Bongrand P, Kaplanski S, Farnarier C (1997) Thrombin induces endothelial type II activation in vitro: IL-1 and TNF-alpha-independent IL-8 secretion and E-selectin expression. *J Immunol* 158:5435-5441.
52. Kaplanski G, Marin V, Fabrigoule M, Boulay V, Benoliel AM, Bongrand P, Kaplanski S, Farnarier C (1998) Thrombin-activated human endothelial cells support monocyte

- adhesion in vitro following expression of intercellular adhesion molecule-1 (ICAM-1; CD54) and vascular cell adhesion molecule-1 (VCAM-1; CD106). *Blood* 92:1259-1267.
53. Kehrl JH (2006) Chemoattractant receptor signaling and the control of lymphocyte migration. *Immunol Res* 34:211-228.
54. Kim CH (2004) Chemokine chemokine-receptor network in immune cell trafficking. *Curr Drug Target Immune Endoc Metabol Disord* 4:343-361.
55. Kim WT, Kong HH, Ha YR, Hong YC, Jeong HJ, Yu HS, and Chung DI (2006) Comparison of specific activity and cytopathic effects of purified 33 kDa serine proteinase from *Acanthamoeba* strains with different degree of virulence. *Korean J of Parasitol* 44:321-330.
56. Kishimoto S, Kobayashi Y, Oka S, Gokoh M, Waku K, Sugiura T (2004) 2-Arachidonoylglycerol , an endogenous cannabinoid receptor ligand, induces accelerated production of chemokines in HL-60 cells. *J Biochem* 135:517-524.
57. Kishimoto S, Muramatsu M, Gokoh M, Oka S, Waku K, Sugiura T (2005) Endogenous cannabinoid receptor ligand induces the migration of human natural killer cells. *J Biochem* 137:217-223.
58. Klein TW, Friedman H and Specter S (1998) Marijuana, immunity and infection. *J Neuroimmunol* 83:102-115.
59. Klein TW, Newton CA, Widen R and Friedman H (1985) The effect of delta-9-tetrahydrocannabinol and 11-hydroxy-delta-9-tetrahydrocannabinol on T-lymphocyte and B-lymphocyte mitogen responses. *J Immunopharmacol* 7:451-466.

60. Klein TW, Lane B, Newton CA, FreidmanH (2000) The cannabinoid system and cytokine network. *ProSoc Exp Biol Med* 225:1-8.
61. Kong HH, Park JH, Chung DI (2000) Purification and characterization of a secretory serine proteinase of *Acanthamoeba healyi* isolated from GAE. *J Parasitol* 86:12-17.
62. Lauffenburger DA and Horwitz AF (1996) Cell migration: A physically integrated molecular process. *Cell* 84:359-369.
63. Le Y, Zhou Y, Iribarren P and Wang J (2004) Chemokines and chemokine receptors: their manifold roles in homeostasis and disease. *Cell Mol Immunol* 1:95-104.
64. Lehner T (2002) The role of CCR5 chemokine ligands and antibodies to CCR5 coreceptors in preventing HIV infection. *TRENDS in Immunol* 23:347-351.
65. Li HL (1974) An archeological and historical account of cannabis in China. *Economic Botany* 28:437-448.
66. Lopez-Cepero M, Friedman M, Klein T and Friedman H (1986) Tetrahydrocannabinol-induced suppression of macrophage spreading and phagocytic activity in vitro. *J Leukoc Biol* 39:679-686.
67. Makriyannis A, Yang DP, Griffin RG and Das Gupta SK (1990) The perturbation of model membranes by (-)-delta 9-tetrahydrocannabinol. Studies using solid-state 2H- and 13C-NMR. *Biochim Biophys Acta* 1028:31-42.
68. Mann PEG, Cohen AB, Finley TN and Ladman AJ (1971) Alveolar macrophages. Structural and functional differences between non-smokers and smokers of marijuana in vitro. *Lab Invest* 25:111-120.

69. Mantovani A, Sica A, Sozzani S, Allavena P, Vecchi A and Locati M (2004) The chemokine system in diverse forms of macrophage activation and polarization. *TRENDS in Immunol* 25:677-686.
70. Marciano-Cabral F, Puffenbarger R, Cabral G (2000) The increasing importance of *Acanthamoeba* infections. *J Eukaryot Microbiol* 47:29-6.
71. Marciano-Cabral, Ferguson T, Bradley S, Cabral G (2001) Delta-9-tetrahydrocannabinol (THC), the major psychoactive component of marijuana, exacerbates brain infection by *Acanthamoeba*. *J Eukaryot Microbiol* 48:4S-5S.
72. Marciano-Cabral F and Cabral G (2003) *Acanthamoeba* spp. as agents of disease in humans. *Clin Micro Rev* 16:273-307.
73. Marciano-Cabral F, Han K, Powell E, Ferguson T, Cabral G (2003) Interaction of an *Acanthamoeba* human isolate harboring bacteria with murine peritoneal macrophages. *J Eukaryot Microbiol* 50:516-519.
74. Mariano-Oliveira A, De Freitas MS, Monteiro RQ, Barja-Fidalgo C (2007) Prothrombin fragments containing kringle domains induce migration and activation of human neutrophils. *Int J Biochem Cell Biol* September, epublish ahead of print.
75. Martin BR (1986) Cellular effects of cannabinoids. *Pharmacol Rev* 1:45-74.
76. Martinez AJ and Janitschke K (1985) *Acanthamoeba*, an opportunistic microorganism: a review. *Infection* 13:251-256.
77. Matsuda LA, Lolait SJ, Brownstein MJ, Young AC and Bonner TI (1990) Structure of a cannabinoid receptor and functional expression of the cloned CDNA. *Nature* 346:561-564.

78. McCarthy CR, Cutting MB, Simmons GA, Pereira W, Laguarda R, and Huber G L (1976) The effect of marihuana on the in vitro function of pulmonary alveolar macrophages, in *Pharmacology of Marihuana, Vol. 1* (Braude MC and Szara S eds) pp 211-216, Raven Press, New York.
79. McCoy K, Gainey D and Cabral G (1995) Delta-9-tetrahydrocannabinol modulates antigen processing by macrophages. *J Pharmacol Exp Ther* 273:1216-1223.
80. McCoy KL, Matveyeva M, Carlisle SJ and Cabral GA (1999) Cannabinoid inhibition of the processing of intact lysozyme by macrophages: evidence for CB2 receptor participation. *J Pharmacol Exp Ther* 289:1620-1625.
81. Miike S, McWilliam AS, Kita H (2001) Trypsin induces activation and inflammatory mediator release from human eosinophils through protease-activated receptor-2. *J Immunol* 167:6615-6622.
82. Mitchison TJ and Cramer LP (1996) Actin-based cell motility and cell locomotion. *Cell* 84:371-379.
83. Mitro K, Bhagavathiammai A, Zhou OM, Bobbett G, McKerrow JH, Chokshi R, Chokshi B, and James ER (1994) Partial characterization of the proteolytic secretions of *Acanthamoeba polyphaga*. *Exp Parasitol* 78:377-385.
84. Moore MB, Ubelaker JE, Marin JH, Silvany R, Dougherty JM, Meyer DR, and McCulley JP (1991) In vitro penetration of human corneal epithelium by *Acanthamoeba castellanii*: a scanning and electron transmission microscopy study. *Cornea* 10:291-298.

85. Moormann C, Artuc M, Pohl E, Varga G, Buddenkotte J, Vergnolle N, Brehler R, Henz BM, Schneider SW, Luger TA, Steinhoff M (2006) Functional characterization and expression analysis of the protease-activated receptor-2 in human cutaneous mast cells. *J Invest Dermatol* 126:746-755.
86. Morris D, Ding Y, Ricks T, Gullapalli A, Wolfe B, Trejo J (2006) Protease-activated receptor-2 is essential for factor viia and xa-induced signaling, migration, and invasion of breast cancer cells. *Cancer Res* 66:307-314.
87. Munro S, Thomas KL and Abu-Shaar M (1993) Molecular characterization of a peripheral receptor for cannabinoids. *Nature* 365:61-65.
88. Murdoch C and Finn A (2000) Chemokine receptors and their role in inflammation and infectious disease. *Blood* 95:3032-3043.
88. Murphy PM (2002) International Union of Pharmacology. XXX. Update on Chemokine Receptor Nomenclature. *Pharmacol Rev* 54:227-229.
89. Panikashvili D, Simeonidou C, Ben Shabat S, Hanus L, Breuer A, Mechoulam R, Shohami E (2001) An endogenous cannabinoid (2-AG) is neuroprotective after brain injury. *Nature* 413:527-531.
90. Pettit DA, Williamson J, Cabral G, and Marciano-Cabral F (1996) *In vitro* destruction of nerve cell cultures by *Acanthamoeba* spp.: a transmission and scanning electron microscopy study. *J Parasitol* 82:769-777.
91. Price TJ, Patwardhan A, Akopian AN, Hargreaves KM and Flores CM (2004) Cannabinoid receptor-independent actions of the aminoalkylindole WIN 55,212-2 on trigeminal sensory neurons. *Br J Pharmacol* 142:257-266.

92. Puffenbarger R, Boothe C and Cabral G (2000) Cannabinoids inhibit LPS-inducible cytokine mRNA expression in rat microglial cells. *Glia* 29:58-69.
93. Raz A and Goldman R (1976) Effect of hashish compounds on mouse peritoneal macrophages. *Lab Invest* 34:69-76.
94. Rios CD, Jordan BA, Gomes I and Devi LA (2001) G-protein-coupled receptor dimerization: modulation of receptor function. *Pharmacol Ther* 92:71-87.
95. Rios CD, Gomes I and Devi LA (2006) Mu and CB1 cannabinoid receptor interactions: reciprocal inhibition of receptor signaling and neuritogenesis. *Br J Pharmacol* 148:387-395.
96. Rogers TJ, Steele AD, Howard OMZ and Oppenheim JJ (2000) Bidirectional heterologous desensitization of opioid and chemokine receptors. *Ann NY Acad Sci* 917:19-28.
97. Sacerdote P, Martucci C, Vaccani A, Bariselli F, Panerai AE, Colombo A, Parolaro D and Massi P (2005) The nonpsychoactive component of marijuana cannabidiol modulates chemotaxis and IL-10 and IL-12 production of murine macrophages both in vivo and in vitro. *J Neuroimmunol* 159:97-105.
98. Sacerdote P, Massi P, Panerai AE and Parolaro D (2000) In vivo and in vitro treatment with the synthetic cannabinoid CP55, 940 decreases the in vitro migration of macrophages in the rat: involvement of both CB1 and CB2 receptors. *J Neuroimmunol* 109:155-163.
99. Schiffmann E, Corcoran BA and Wahl SM (1975) N-formyl-methionyl peptides as chemoattractants for leucocytes. *Proc Natl Acad Sci USA* 72:1059-1062.

100. Sissons J, Alsam S, Goldsworthy G, Lightfoot M, Jarroll E, Khan N (2006) Identification and properties of proteases from an *Acanthamoeba* isolate capable of producing granulomatous encephalitis. *BMC Microbiology* 6:42.
101. Specter SC, Klein TW, Newton C, Mondragon M, Widen R and Friedman H (1986) Marijuana effects on immunity: suppression of human natural killer cell activity of delta-9-tetrahydrocannabinol. *Int J Immunopharmacol* 8:741-745.
102. Specter S, Lancz G and Goodfellow D (1991) Suppression of human macrophage function in by delta-9-tetrahydrocannabinol. *J Leukocyte Biol* 50:423-426.
103. Srivastava MD, Srivastava BI, Brouhard B (1998) Delta-9-tetrahydrocannabinol and cannabidiol alter cytokine production by human immune cells. *Immunopharmacology* 40:179-185.
104. Stefano GB, Salzet M, Rialas CM, Mattocks D, Fimiani C and Bilfinger TV (1998) Macrophage behavior associated with acute and chronic exposure to HIV GP120, morphine and anandamide: endothelial implications. *Int J Cardiol* 64 Suppl 1:S3-13.
105. Steinhoff M, Buddenkotte J, Shpacovitch V, Rattenholl A, Moormann C, Vergnolle N, Luger T, and Hollenberg M (2005) Proteinase-Activated receptors: transducers of proteinase-mediated signaling in inflammation and immune response. *Endocrine Reviews* 26:1-43.
106. Suzuki S, Chuang I, Yau P, Doi R and Chuang R (2002) Interactions of opioid and chemokine receptors: oligomerization of mu, kappa, and delta with CCR5 on immune cells. *Exp Cell Res* 280:192-200.

107. Szabo J, Chen XH, Xin L, Adler MW, Howard OMZ, Oppenheim JJ and Rogers TJ (2002) Heterologous desensitization of opioid receptors by chemokines inhibits chemotaxis and enhances the perception of pain. *Proc Natl Acad Sci USA* 99:10276-10281.
108. Szabo J and Rogers TJ (2001) Crosstalk between chemokine and opioid receptors results in downmodulation of cell migration. *Adv Exp Med Biol* 493:75-79.
109. Szabo J, Wetzel M, McCarthy L, Steele A, Henderson E, Howard OMZ, Oppenheim Z and Rogers TJ (2001) Interactions of opioid receptors, chemokines and chemokine receptors. *Adv Exp Med Biol* 493:69-74.
110. Tang JL, Lancz G, Specter S and Bullock H (1992) Marijuana and immunity: tetrahydrocannabinol-mediated inhibition of growth and phagocyte activity of the murine macrophage cell line, P388D1. *Int J Immunopharmacol* 14:253-262.
111. Traynelis S and Trejo J (2007) Protease-activated receptor signaling: new roles and regulatory mechanisms. *Curr Opin Hematol* 14:230-235.
112. Waksman Y, Olson JM, Carlisle SJ and Cabral GA (1999) The central cannabinoid receptor (CB1) mediates inhibition of nitric oxide production by rat microglial cells. *J Pharmacol Exp Ther* 288:1357-1366.
113. Walter L, Franklin A, Witting A, Wade C, Xie Y, Kunos G, Mackie K and Stella N (2003) Nonpsychotropic cannabinoid receptors regulate microglial cell migration. *J Neurosci* 23:1398-1405.

114. Watzl B, Scuderi P and Watson RR (1991) Marijuana components stimulate human peripheral blood mononuclear cell secretion of interferon-gamma and suppresses interleukin-1 alpha in vitro. *Int J Immunopharmacol* 13:1091-1097.
115. Witting A, Walter L, Wacker J, Moller T, Stella N (2004) P2X7 receptors control 1-arachidonoylglycerol production by microglial cells. *Proc Natl Acad Sci USA* 101:321-324.
116. Zhang N, Hodge D, Rogers TJ and Oppenheim JJ (2003) Ca²⁺-independent protein kinase Cs mediate heterologous desensitization of leukocyte chemokine receptors by opioid receptors. *J Biol Chem* 278:12729-12736.
117. Zhang N and Oppenheim JJ (2005) Crosstalk between chemokines and neuronal receptors bridges immune and nervous system. *J Leuk Biol* 78:1210-1214.
118. Zheng ZM, Specter S and Friedman H (1992) Inhibition by delta-9-tetrahydrocannabinol of tumor necrosis factor alpha production by mouse and human macrophages. *Int J Immunopharmacol* 14:1445-1452.
119. Zimmerman S, Zimmerman AM, Cameron IL and Laurence HL (1977) Delta1-tetrahydrocannabinol, cannabidiol and cannabiniol effects on the immune response of mice. *Pharmacology* 15:10-23.