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dissertation entitled

MODULATION BY CANNABINOL OF INTERLEUKIN (IL)-2 AND IL-4 EXPRESSION IS CLOSELY CORRELATED WITH THE REGULATION OF NUCLEAR FACTOR OF ACTIVATED T CELLS (NF-AT)

presented by

Tong-Rong Jan

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By

Tong-Rong Jan

A DISSERTATION

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ABSTRACT

MODULATION BY CANNABINOL (CBN) OF INTERLEUKIN (IL)-2 AND IL-4 EXPRESSION IS CLOSELY CORRELATED WITH THE REGULATION OF NUCLEAR FACTOR OF ACTIVATED T CELLS (NF-AT)

By

Tong-Rong Jan

Plant-derived cannabinoids have been well established as immune modulators. Previous studies have demonstrated that T cell accessory function is involved in the inhibition of humoral immune responses by cannabinoids. To further investigate the underlying mechanism for cannabinoid-mediated immune modulation, the effect of CBN on the expression of IL-2 and IL-4, two critical cytokines involved in antibody response against T cell-dependent antigens was characterized. CBN elicited contrasting effects on IL-2 protein secretion by T cells, which was dependent on the magnitude of the T cell activation stimulus. IL-2 secretion induced by optimal activation stimuli was suppressed, whereas IL-2 secretion induced by sub-optimal stimuli was enhanced. The CBN-mediated enhancement of IL-2 secretion was closely associated with an enhancement in IL-2 steady state mRNA expression, and with an increase in ERK MAP kinase activation. More in depth studies identified the distal NF-AT site in the IL-2 promoter as one downstream response element involved in the increased transcription of IL-2 by CBN.

In contrast, CBN and Δ^9-THC treatment inhibited IL-4 secretion by T cells under all T cell activation conditions tested. The IL-4 steady state mRNA expression by PMA/Io-activated EL4 T cells was also inhibited by cannabinoids. Concordantly, DNA binding activity to the IL-4 P0 NF-AT site was diminished in the presence of cannabinoids. Additionally, RNase protection assays demonstrated that Δ^9-THC suppressed the expression of three separate cytokine genes that are regulated by NF-AT,

IL-2, IL-4 and IL-5, under the conditions of optimal T cell activation. The *in vitro* studies were extended to an *in vivo* model of allergic airway disease critically dependent on T cell cytokines, including IL-2 and IL-4. In this model, both IL-2 and IL-4 mRNA expression in the lungs of A/J mice sensitized and challenged with ovalbumin was attenuated by administration of CBN or Δ^9-THC. The level of ovalbumin-specific serum IgE, an important mediator of the allergic airway response, was concordantly attenuated by both cannabinoids. Collectively, these results confirm that CBN and Δ^9-THC-mediated inhibition of IL-2 and IL-4 can be produced both *in vivo* and *in vitro.*

The putative role of cannabinoid receptors and the cAMP signaling pathway to which CB1 and CB2 negatively couple, in CBN-mediated modulation of IL-2 and IL-4 expression was investigated. The CBN-mediated effects were not attenuated by (1) dibutyryl-cAMP treatment, (2) pre-incubation of T cells with pertussis toxin, and (3) pretreatment of T cells with cannabinoid receptor antagonists. Furthermore, comparative studies employing the cannabinoid congeners cannabidiol, CP55,940 and WIN55212 demonstrated a lack of correlation between their activity of cytokine modulation and affinity of CB1 and CB2 binding. In spite of this, the WIN55212 enantiomers produced stereo-selective inhibition of IL-4.

Collectively, the present studies demonstrate that cannabinoid-mediated modulation of IL-2 and IL-4 expression by T cells is closely associated with the regulation of NF-AT and ERK activation. In addition, these studies suggest that cannabinoid modulation of IL-2 and IL-4 is apparently not mediated through the negative modulation of the cAMP pathway via cannabinoid receptors.

TO

MY WIFE, STACY, AND
MY CHILDREN, JENNIFER, PIN-PIN AND MICHAEL

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There are many people I wish to acknowledge. Foremost, I would like to thank my mentor, Dr. Norb Kaminski, whose sagacious guidance has led me to the vivid world of science. His encouragement, support and patience to me are remarkable and unforgettable.

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αCD3	anti-CD3 monoclonal antibody
αCD28	anti-CD28 monoclonal antibody
Δ^9-THC	delta-9-tetrahydrocannabinol
AFC	antibody forming cell response
AP-1	activator protein-1
APC	antigen presenting cell
B cell	B lymphocytes
BCS	bovine calf serum
BSA	bovine serum albumin
CaM	calmodulin
CaM kinase	calcium/calmodulin-dependent protein kinase
cAMP	cyclic adenosine 3',5'-monophosphate
CB1	cannabinoid receptor 1
CB2	cannabinoid receptor 2
CBD	cannabidiol
CBN	cannabinol
CD	cluster of differentiation
CD28RE	CD28 response element
cDNA	complimentary DNA
CHO cell	Chinese hamster ovary cell
CNS	central nervous system
CRE	cAMP response element
CREB	cAMP-response element binding protein
D,B-cAMP	dibutyryl-cAMP
DMSO	dimethyl sulfoxide

DNA	deoxyribonucleic acid
DNP-Ficoll	dinitrophenyl haptenated ficoll
DTT	dithiothreitol
ELISA	enzyme-linked immunosorbent assay
EMSA	electrophoretic mobility shift assay
ERK	extracellular signal-regulated kinase
EtOH	ethanol
g	gravity
G_i/G_o	inhibitory G-protein
G-protein	guanine-nucleotide-binding protein
G_s	stimulatory G-protein
GTP	guanosine triphosphate
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid
iCD3	immobilized αCD3
iCD3/CD28	immobilized αCD3 plus soluble αCD28
Ig	immunoglobulin
IL	interleukin
IFN-γ	interferon-γ
Io	ionomycin
IP_3	1,4,5-triphosphate
IS	internal standard
kD	kilodalton
LPS	lipopolysaccharide
mAb	monoclonal antibody
MAP kinase	mitogen-activated protein kinase
MEK	MAP kinase kinase
mRNA	messenger RNA

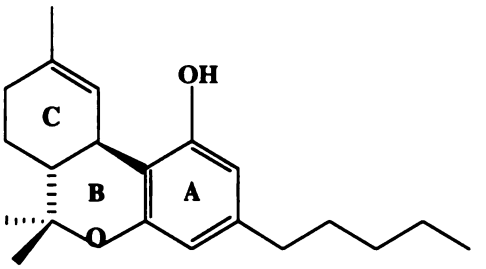
NA	naïve
NF-AT	nuclear factor of activated T-cells
NF-ATc	cytosolic component of NF-AT
NF-ATn	nuclear component of NF-AT
NF-κB	nuclear factor-κ light chain of B-cells
Oct	octamer protein
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PHA	phytohemagglutinin
PI 3-K	phosphoinositide 3-kinase
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
PMA	phorbol 12-myristate 13-acetate
PMSF	phenylmethylsulfonyl fluoride
PTK	protein tyrosine kinase
RNA	ribonucleic acid
RNase	ribonuclease
RPMI	Roswell Park Memorial Institute
RT-PCR	reverse transcription-polymerase chain reaction
sCD3	soluble αCD3
sCD3/CD28	soluble αCD3 plus soluble αCD28
SDS	sodium dodecyl sulfate
SE	standard error
SEAP	secreted alkaline phosphatase
sRBC	sheep red blood cell
TBE	tris-boric acid-EDTA

TBS	tris-buffered saline
T cell	T lymphocyte
TCR	T cell receptor
Th	helper T cell
TRE	TPA response element
tRNA	transfer RNA
VH	vehicle

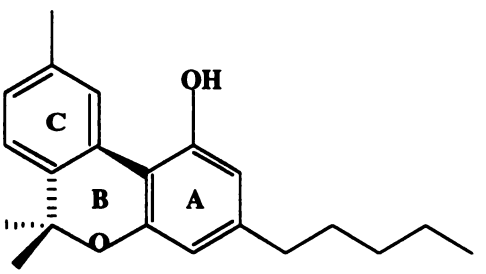
INTRODUCTION

I. Cannabinoid background

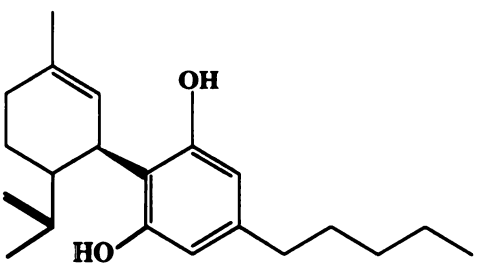
Plant-derived cannabinoid compounds are present in *cannabis sativa* plant which is also known as marijuana. More than 60 structurally-related compounds termed cannabinoids have been identified in cannabis smoke, including Δ^9-tetrahydrocannabinol (Δ^9-THC), cannabidiol (CBD) and cannabinol (CBN) (structure illustrated in Fig. 1). Some, but not all of the members, of this class of compounds are well established as being psychoactive and/or immunosuppressive. With the discovery of two types of cannabinoid receptors (CB1 and CB2), the putative mechanism for the diverse effects induced by cannabinoids has been proposed to be mediated through cannabinoid receptors. Both receptors are coupled to inhibitory GTP-binding protein (G_i/G_o) which negatively regulates adenylate cyclase-cAMP signaling cascade. Coupling of CB1 to stimulatory GTP-binding protein (G_s) has also been reported in cultured striatal neurons (Glass and Felder, 1997). To date, the distribution of cannabinoid receptors has not yet been comprehensively characterized. CB1 has been found to be expressed primarily in the central nervous system and CB2 predominantly expressed within the immune system (reviewed by Matsuda, 1997; Klein *et al.*, 1998). The primary psychoactive component of marijuana is Δ^9-THC which is also immunosuppressive, presumably because it binds with similar affinity to both CB1 and CB2 receptors. By comparison, CBN, which is also immunosuppressive, exhibits only minimal CNS activity due to a 10-fold higher affinity for CB2 than CB1 (Munro *et al.*, 1993). In contrast to Δ^9-THC and CBN, the affinity of CBD for cannabinoid receptors is much lower than that of Δ^9-THC and CBN. Therefore,



Δ^9-tetrahydrocannabinol (Δ -THC)



Cannabinol (CBN)



Cannabidiol (CBD)

Figure 1. Structure of plant-derived cannabinoids. Δ^9-THC, CBN and cannabidiol are natural cannabinoid compounds derived from *Cannabis sativa*. Δ^9-THC is the primary psychoactive component of marijuana. By comparison, CBN is minimally CNS-active and cannabidiol is a CNS-inactive cannabinoid congener.

CBD is generally considered as a CNS-inactive cannabinoid congener. CNS-inactive or minimally active cannabinoids possessing immunomodulatory activity, such as CBN and CBD, represent a potentially novel class of therapeutic agents. This unique property renders CBN an appropriate candidate to study the mechanism of cannabinoid-mediated immunomodulation and the role of CB2 receptors in the immune system. In light of this, a major focus of this dissertation project has been to elucidate the molecular mechanisms responsible for IL-2 and IL-4 modulation by CBN.

A. Biological effects of cannabinoids

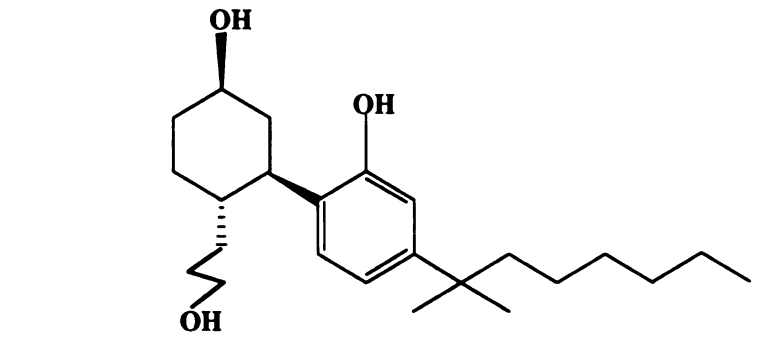
Cannabinoid compounds produce a wide variety of biological effects in animals and humans, among which the effects on the CNS and immune system have been most extensively characterized. Many CNS associated functions are altered by cannabinoids, including changes in mood, psychomotor skill, and cognition and memory (Dewey, 1986; Pertwee, 1988). Acute administration of Δ^9-THC leads to a decrease in stimulus-controlled behavior in several animal models (Black *et al.,* 1970; Carlini, 1968; Ferraro and Grilly, 1973), whereas an amotivational syndrome characterized by general apathy is associated with chronic marijuana use (Hollister, 1986). In addition to the CNS effects, immune modulation is another well established action of cannabinoids which is the focus of this dissertation research and will be discussed in more detail in the third section of the introduction. Due to the diverse biological effects, several therapeutic applications of marijuana have been proposed, including analgesia, decreasing intraocular pressure, appetite stimulation, and anti-emesis for chemotherapy. Recently, the use of marijuana for medical purposes has been legalized in Alaska, Arizona, Nevada, Oregon and

Washington. Hence, a more comprehensive understanding of cannabinoid pharmacology is of great interest as it relates to the therapeutic application of marijuana.

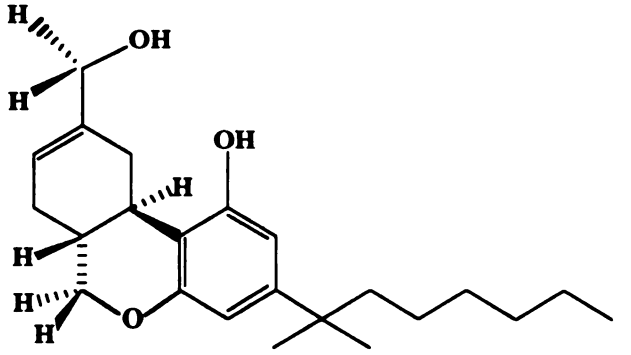
In addition to plant-derived cannabinoids, several non-classical synthetic cannabinoid analogs have been developed, which possess potent cannabimimetic activity and high binding affinity to cannabinoid receptors. CP55,940 is a synthetic cannabinoid that is structurally-related to Δ^9-THC with the exception that it has an extended aliphatic side chain (structure illustrated in Fig. 2). The dimethyl heptyl HU-210/HU-211 analogs are also structurally similar to Δ^9-THC but differ in that they possess a branched aliphatic side chain. In addition, a novel series of synthetic aminoalkylindole compounds have been developed which are high affinity CB1 and CB2 ligands. Although they are structurally different from plant-derived cannabinoids, these molecules exhibit potent cannabimimetic activity in all models thus far tested including the CNS and immune system. Of the aminoalkylindole-based cannabinoids, WIN55212 has been most extensively investigated.

B. Cannabinoid receptors and modulation of intracellular signaling pathways by cannabinoids

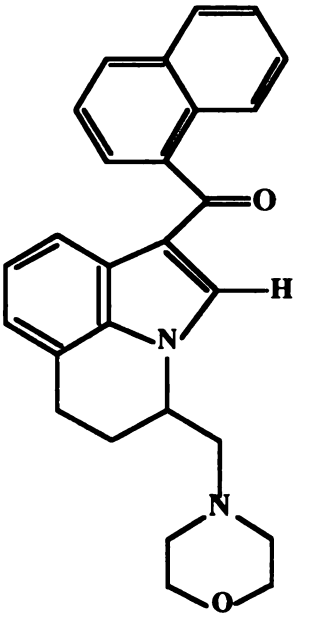
Due to their high lipophilicity, the mechanism of action for cannabinoids has been historically attributed to cell membrane intercalation and disruption (Makriyannis and Rapaka, 1990). However, stereo-selective difference in biological activity of cannabinoid enantiomers was observed, indicating that lipophilicity did not correlate with the biological activity of cannabinoids (Thomas *et. al.*, 1990). Several lines of evidence suggest the involvement of receptors in the mechanism of action by cannabinoids: (1)



CP-55,940



HU-210/HU-211



WIN55212

Figure 2. Structure of the synthetic cannabinoids. CP55,940 is a bicyclic cannabinoid analog which structure is similar to Δ^9-THC with extended aliphatic side chain. The dimethyl heptyl HU-210/HU-211 analogs are also structurally similar to Δ^9-THC with a branched aliphatic side chain. The aminoalkylindole WIN55212-2, although the structure is completely different from natural cannabinoids, exhibits potent cannabimimetic activity and high binding affinity to cannabinoid receptors.

cannabinoid compounds displayed specific and saturable binding to neuronal tissues and leukocytes as demonstrated by radioligand binding analysis (Harris *et al.*, 1978; Kaminski *et al.*, 1992); (2) cannabinoids negatively regulated the activity of adenylate cyclase in neuronal and immune cells (Howlett, 1985; Howlett *et al.*, 1986; Kaminski *et al.*, 1992); (3) the genes for two cannabinoid receptors, CB1 and CB2, have been identified and cloned (Matsuda *et al.*, 1990; Munro *et al.*, 1993); and (4) both CB1 and CB2 were coupled to G_i/G_o which negatively regulated adenylate cyclase-cAMP signaling cascade (Matsuda *et al.*, 1990; Slipetz *et al.*, 1995). According to these findings, the putative mechanism for the action of cannabinoids has been proposed to be mediated through cannabinoid receptors and the subsequent inhibition of adenylate cyclase-cAMP signaling (Fig. 3). The cAMP signaling cascade comprises several components. Upon ligand binding to G protein coupled receptors, a stimulatory (form G_s) or inhibitory (form G_i/G_o) signal is transduced to adenylate cyclase resulting in up or down-regulation of the synthesis of cAMP, respectively. cAMP is a widely distributed intracellular secondary messenger which regulates many cellular functions through the activation of cAMP-dependent protein kinase (PKA). PKA is a serine/threonine kinase that can modulate the activity of its target proteins by phosphorylation. For example, phosphorylation of cAMP-responsive element binding protein (CREB) by PKA is the key step for the activation of CREB and the subsequent regulation of cAMP responsive genes.

The adenylate cyclase-cAMP pathway is one of the most extensively characterized signal transduction pathways modulated by cannabinoids. Ligand binding to either CB1 or CB2 markedly inhibits the activity of adenylate cyclase as evidenced by

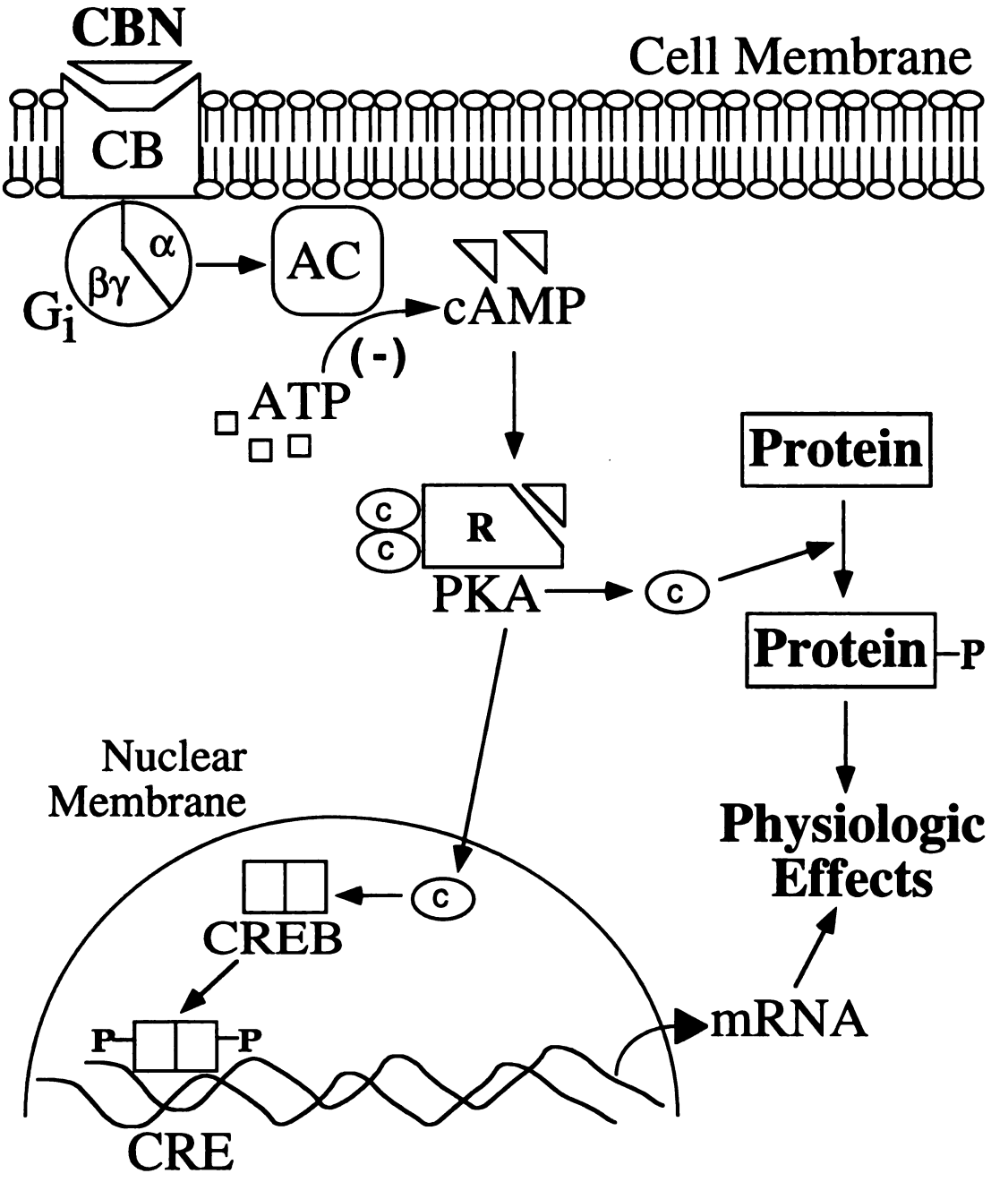


Figure 3. Schematic representation of the cannabinoid receptor-coupled cAMP signaling pathway. Upon the binding of CBN to cannabinoid receptors (CB), the α-subunit of G_i protein will be released and act to inhibit the activity of adenylate cyclase (AC), resulting in decreased production of cAMP. The subsequent activation of PKA by cAMP and CREB by PKA will be suppressed, which consequently leads to the down-regulation of cAMP-mediated physiological effects .

the decrease in intracellular cAMP accumulation (Howlett and Fleming, 1984; Howlett, 1985; Condie *et al.*, 1996; Jeon *et al.*, 1996). The inhibition of cAMP accumulation can be abrogated by pre-incubation of cells with pertussis toxin, indicating the involvement of G_i/G_o protein (Howlett *et al.*, 1986; Kaminski *et al.*, 1994). Consistent with the inhibition of cAMP accumulation, the suppression by cannabinoids of protein kinase A activity and DNA binding by CREB has been demonstrated in a number of cell types, including primary lymphoid cells and cell lines (Condie *et al.*, 1996; Herring *et al.*, 1998). In addition to the cAMP pathway, other cellular signaling events have been shown to be modulated by cannabinoid compounds. Positive coupling of cannabinoid receptors to the ERK MAP kinase pathway has been described in CHO cells transfected to express high levels of either CB1 or CB2 (Bouaboula *et al.*, 1995, 1996). Conversely, CBN produces an inhibition of ERK activation in murine primary splenocytes stimulated with PMA/Io (Faubert and Kaminski, 2000). Additionally, cannabinoids have been demonstrated to decrease calcium influx through calcium channels in neuronal cells and to mobilize arachidonic acid in CHO cells (Mackie and Hill, 1992; Felder *et al.*, 1995; Hunter *et al.*, 1997).

The role of CB1 and CB2 in cannabinoid-mediated biological effects has been recently assessed using transgenic approaches. Consistent with the predominant expression of CB1 in the CNS, studies with CB1 knockout mice have shown that most of cannabinoid-mediated effects on the CNS are mediated through the CB1 receptor, such as analgesia, hypotension, catalepsy, hypomobility and hypothermia (Ledent *et al.*, 1999; Steiner *et al.*, 1999; Zimmer *et al.*, 1999). In contrast, although CB2 is predominantly expressed in the immune system, the involvement of CB2 has only been recently

demonstrated in cannabinoid-mediated effects primarily on some of the functions of macrophages, including antigen processing and presentation to stimulate T cells. (McCoy *et al.*, 1999; Buckley *et al.*, 2000). Examination of the role of CB1 and/or CB2 in cannabinoid-mediated effects on T and B cell function is still lacking. Therefore, whether CB1 and/or CB2 play a role in cannabinoid-mediated modulation of T cell functions remains to be determined.

II. T cell background

A. T cells and the immune system

T cells are important effector cells of acquired immunity. They participate in a widely variety of immune responses through a complicated cytokine network and by interaction with other immune competent cells. According to the diverse functions and distinct cell surface markers, T cells can be classified as cytotoxic T cells (Tc, $CD8^+$) and helper T cells (Th, $CD4^+$). Tc cells are effector cells involved in cell-mediated immunity, and Th cells are critical for both cell-mediated and humoral immunity. Th cells can be further categorized into two subsets, Th1 and Th2. Th cells produce cytokines that are required for the activation, proliferation and differentiation of other immune competent cells involved in acquired immune responses. For instance, Th1 cells produce IFN-γ, TNF-β and IL-2, which mediate activation of Tc cells and macrophages and thereby promote cell-mediated immunity. On the other hand, Th2 cells express IL-4, IL-5, IL-10 and IL-13, and these cytokines, in turn, stimulate the proliferation and differentiation of B cells, resulting in antibody production by plasma cells. In light of the key role of Th2 cytokines in B cell function, the activation of T cells and the subsequent differentiation

into Th2 subset is an important component for the development of humoral immunity. T cell activation and differentiation is a complex process which, similar to B cells, is controlled by cytokines derived from T cells themselves and other leukocytes. IL-2, a cytokine expressed primarily by T cells, is a hallmark of T cell activation. IL-2 functions as an autocrine/paracrine T cell growth factor mediating the clonal expansion of T cells. Following clonal expansion, T cells may undergo differentiation which is also dictated by the cytokine environment. In the presence of IL-4, T cells differentiate toward the Th2 phenotype. Th2 cells then stimulate humoral immunity by secreting the Th2 battery of cytokines.

The effector function of T cells requires the activation of T cells by engagement of specific antigen to the T cell receptors (TCR). The TCR is expressed on the surface of mature T cells. It is a heterodimer comprised of an α and β chain, and is intimately associated with the muti-unit CD3 complex. Each unit of the CD3 molecule consists of a dimer of delta, epsilon, gamma or zeta polypeptide chains. The antigen specificity of TCR is dictated by α/β heterodimer while the transmission of activation signals is primarily mediated through the CD3 molecules.

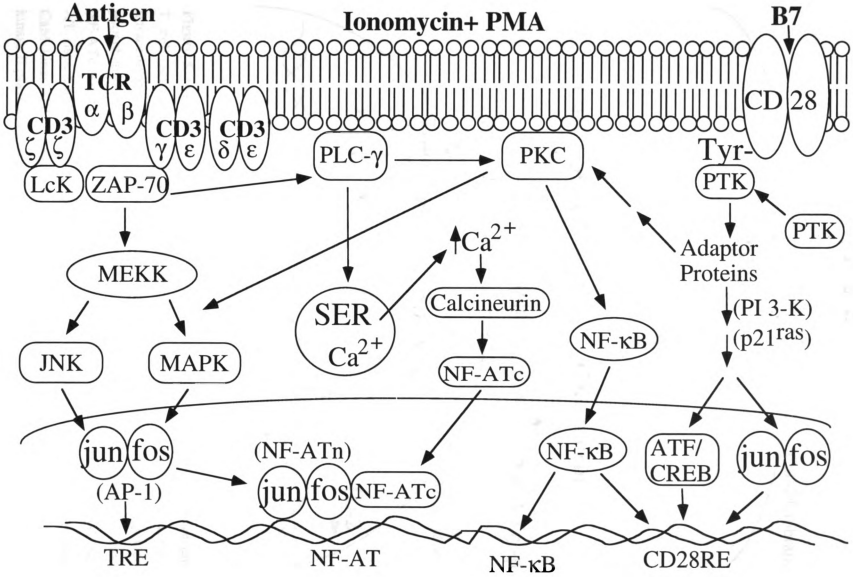
B. T cell activation and associated signaling pathways

Full activation of mature T cells requires two distinct signals, a primary stimulatory signal and a co-stimulatory signal. The primary signal is initiated by the engagement of the TCR/CD3 complex with a processed antigenic peptide presented by an antigen presenting cell (APC). The co-stimulatory signal is primarily mediated by the interaction between CD28 molecules on T cell membrane and B7 molecules on APCs.

The early consequence of T cell activation by antigen stimulation is the up-regulation of high affinity IL-2 receptors on the T cell surface and secretion of IL-2. IL-2 then stimulates the clonal expansion of the activated T cells. Although the precise molecular mechanisms linking antigen recognition by the TCR/CD3 complex on the cell membrane to initiation of gene transcription in the nucleus are not fully understood, a number of signaling cascades involved in this process have been identified (Fig. 4). The initial signaling event mediated through TCR/CD3 complex is the activation of src-family tyrosine kinases, including $p56^{lck}$ and $p59^{fyn}$ (Chan *et al.*, 1994). Upon activation, these kinases phosphorylate tyrosine residues located in the intracellular domain of CD3 molecules, which will increase recruitment of additional signaling components to the TCR/CD3 complex, such as ZAP-70 (also a tyrosine kinase) and phosphoinositide 3-kinase (PI 3-kinase). Further signal-transduction events are thereby triggered by these kinases. For example, phospholipase Cγ (PLCγ) and the small GTP-binding protein $p21^{ras}$ (Ras) are activated by the aforementioned tyrosine kinases, and mediate a complex cascade of biochemical events. PLCγ plays a vital role for T-cell activation because it catalyzes the synthesis of inositol 1,4,5-triphosphate (IP_3) and diacylglycerol, two important intracellular messengers for T cell activation.

In one pathway, IP_3 induces an increase in intracellular calcium and the subsequent activation of calcium/calmodulin-dependent enzymes, including calcineurin and CaM kinases. Calcineurin is a calcium-dependent phosphatase that mediates the activation and translocation of NF-AT transcription factors into the nucleus, thereby stimulating the expression of NF-AT sensitive genes (Fig. 5). The importance of CaM

Figure 4. Schematic representation of the signaling pathways associated with T cell activation. Dual activation signals are required for full activation of T cells. The primary signal is initiated by the engagement of the TCR/CD3 complex with a processed antigen, and the co-stimulatory signal is mediated by the interaction between CD28 and B7 molecules. Distinct and multiple signaling cascades are induced by the dual signals, which activate a number of downstream transcription factors and ultimately induce the expression of cytokine genes. Using mAbs against CD3 and CD28 is a commonly employed approach to specifically activate T cells. Alternatively, a combination of phorbol ester (i.e., PMA) and calcium ionophore (i.e., ionomycin) is also used to activate T cells. PMA activates PKC and ionomycin increases intracellular calcium levels, which mimic signaling through the TCR.


Antigen
Ionomycin+ PMA
B7
TCR
α
β
CD3
ζ
ζ
CD3
γ
ε
CD3
δ
ε
CD 28
PLC-γ
PKC
Tyr-
PTK
PTK
LcK
ZAP-70
Ca2+
Adaptor
Proteins
MEKK
SER
Ca2+
Calcineurin
(PI 3-K)
(p21ras)
NF-κB
JNK
MAPK
NF-ATc
jun
fos
(AP-1)
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CREB
jun
fos
TRE
NF-AT
NF-κB
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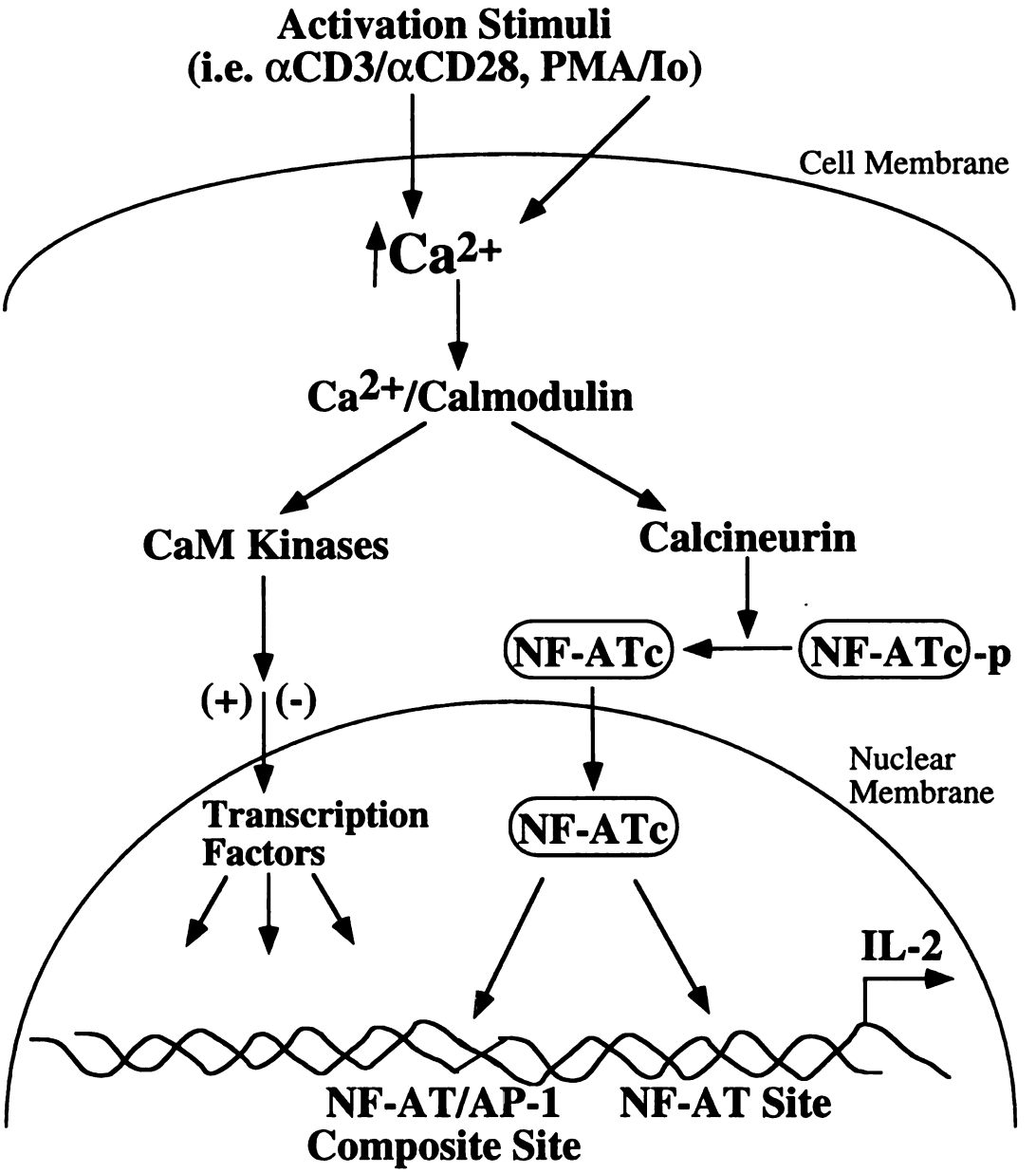


Figure 5. Schematic representation of the calcium signaling in T cell activation. T cell activation by TCR/CD3 engagement and CD28 co-stimulation increases intracellular calcium, which leads to the activation of the calcium-dependent phosphatase calcineurin, and activation of CaM kinases. Calcineurin dephosphorylates NF-ATc resulting in nuclear translocation of NF-ATc and subsequently binding to NF-AT sites. CaM kinases are also invovled in signal transduction of T cell activation. CaM kinase IV up-regulates IL-2 expression via activation of AP-1, whereas CaM kinase II plays a negative role in the IL-2 regulation.

kinases, specifically CaM kinase IV, in T cell activation has been recently demonstrated by several laboratories (Gringhuis *et al.*, 1998; Means *et al.*, 1997; Anderson *et al.*, 1997). An increase in the activity of CaM kinase IV was observed following T cell activation (Gringhuis *et al.*, 1998). Expression of an inactive form of CaM kinase IV in thymocytes results in attenuation of IL-2 production induced by PMA/Io (Anderson *et al.*, 1997). Mechanistic studies have identified AP-1 as the possible downstream transcription factor targeted by CaM kinase IV (Ho *et al.*, 1996a). Activation of ERKs by CaM kinases has also been demonstrated in neuronal and vascular smooth muscle cells (Enslen *et al.*, 1996; Abraham *et al.*, 1997), suggesting a potential mechanism for the activation of AP-1 by CaM kinases. In addition to CaM kinase IV, CaM kinase II has also been identified in T cells. Interestingly, CaM kinase II plays a negative role in the regulation of T cell activation. Transfection of a constitutive active mutant of CaM kinase II into Jurkat T cells robustly suppressed IL-2 reporter gene activity (Nghlem *et al.*, 1994). The inhibitory effect of CaM kinase II on IL-2 promoter was subsequently shown to be associated with down-regulation of calcineurin and PKC-mediated pathways (Hama *et al.*, 1995). Together these findings demonstrate a critical but complicated role for CaM kinases in T cell activation. In the other pathways, diacylglycerol activates protein kinase C (PKC) which then activate downstream AP-1 and NF-κB transcription factors. Multiple isoforms of PKC have been identified in a number of cell types, including T cells (Mellor and Parker, 1998). Stable overexpression of either PKCα or PKCθ promotes an increase in IL-2 production following stimulation with αCD3 plus PMA (Baier-Bitterlich *et al.*, 1996). Several isoforms of PKC that activate AP-1, NF-κB and NF-AT in T cells have been identified (Baier-Bitterlich *et al.*, 1997; Genot *et al.*,

1995; Altman *et al.*, 2000). The NF-κB activation induced by CD28 co-stimulation has been demonstrated to be mediated by PKCθ (Coudronniere *et al.*, 2000). Moreover, PKCα activates Raf-1 by direct phosphorylation (Kolch *et al.*, 1993). Taken together, although precise T cell activation signaling pathways associated with PKC are not completely elucidated yet, PKC is essential in the regulation of cytokine gene expression by T cells and is involved in activation of multiple transcription factors. As discussed earlier, cytokines secreted by Th cells are critical for the activation, proliferation and differentiation of both B and T cells. The activation of NF-AT, AP-1 and NF-κB transcription factors through the aforementioned signaling cascades is therefore a crucial event in T cell activation, because the expression of many cytokine genes by T cells is tightly regulated at the transcriptional level. In light of this, phorbol ester plus calcium ionophore (PMA/Io), a stimulus that mimics signaling events mediated by TCR/CD3 complex, is often used to activate T cells pharmacologically *in vitro* (figure 4).

Activation of Ras by protein tyrosine kinases transmits T cell activation signals into the nucleus through a series of kinase phosphorylation cascades composed of Raf-1, MEK and the ERK MAP kinases (Fig. 6). At the cell membrane, active GTP-bound Ras directly binds and, by phosphorylation, activates Raf-1. The activated Raf-1 then activates MEK1 and MEK2 by phosphorylation on their serine residues. The MEKs are unique kinases that can phosphorylate both tyrosine and threonine residues of ERK1 and ERK2. The enzymatic activity of ERKs is greatly increased by the dual phosphorylation as compared to the mono-phosphorylation forms. The final event of the Ras cascade is the translocation of activated ERKs into the nucleus where ERKs activate AP-1 and Elk-1 proteins (Reviewed by Seger and Krebs, 1995). The Ras pathway has been described

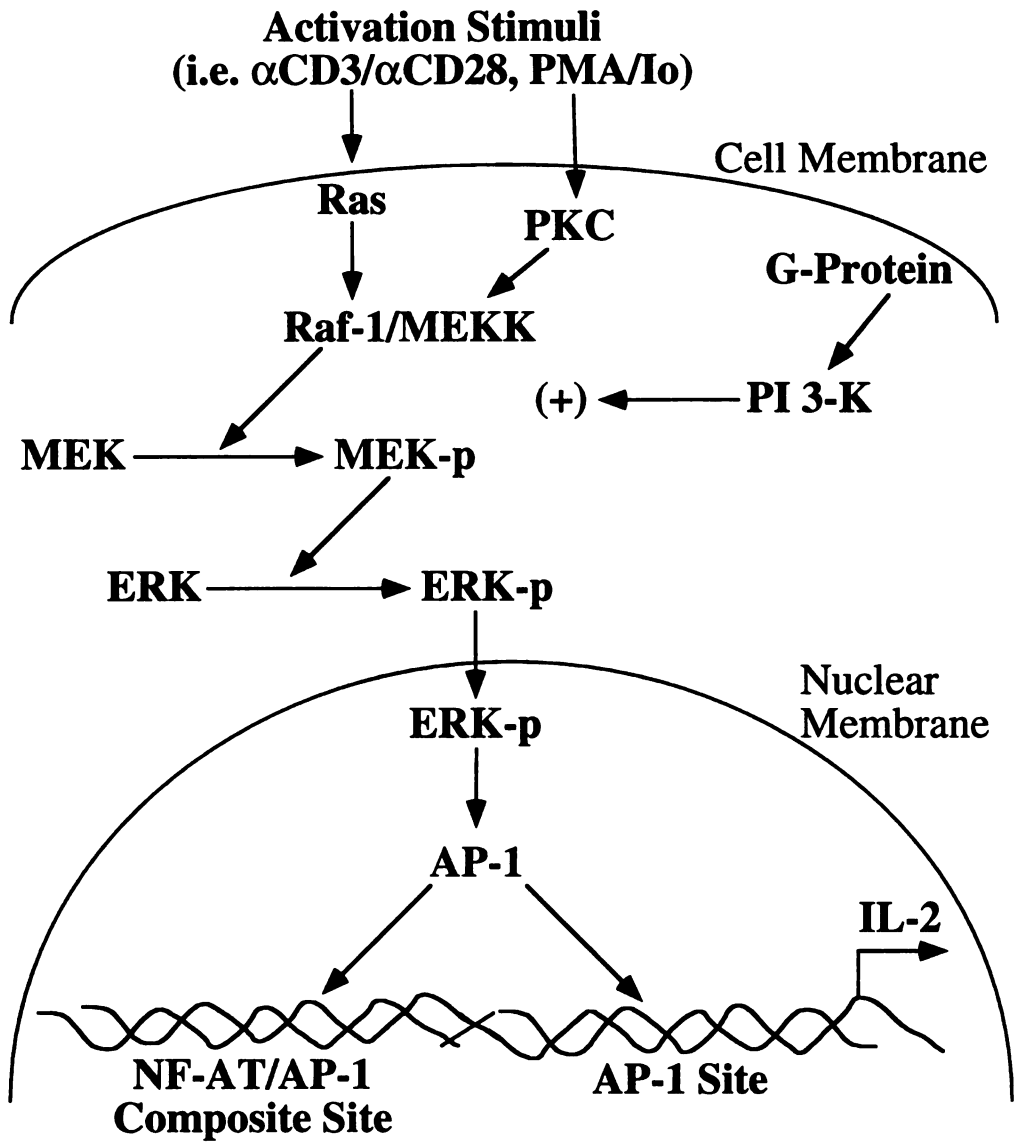


Figure 6. Schematic representation of the ERK MAP kinase signaling pathway. T cell activation by TCR/CD3 engagement and CD28 co-stimulation leads to activation of Ras. Ras can direct bind Raf-1, and, through phosphorylation, activate Raf-1. A sequential kinase phosphorylation event is triggered by Raf-1, and ultimately results in the activation of ERKs. The phospho-ERKs translocate into the nucleus and up-regulate IL-2 transcription through the activation of AP-1 transcription factor. In addition to the Ras pathway, PKC and PI 3-kinase are also capable of up-regulating the ERK MAP kinase pathway.

as a critical signaling component in T cell activation. Ras activity has been shown to be induced in T cells activated by mitogens or αCD3 (Downward, 1990). The activities of Ras and Raf-1 are required for both IL-2 production and ERK activation in T cells (Baldari *et al.*, 1993; Owaki *et al.*, 1993; Izquierdo *et al.*, 1994). Transfection of activated Ras in the presence of increased intracellular calcium stimulates IL-2 promoter activity (Rayter *et al.*, 1992), whereas expression of a dominant negative mutant of ERK1 results in suppression of mitogen-stimulated IL-2 production by T cells (Li *et al.*, 1999). Expression of constitutively active Raf-1 or MEK1 also enhances IL-2 promoter activity in Jurkat T cells stimulated with PMA/Io (Whitehurst and Geppert, 1996). Consistent with the premise that AP-1 is the down-stream target for the Ras pathway, it has been demonstrated that Ras can synergize with calcium signals to activate NF-AT resulting in IL-2 transcription (Woodrow et al., 1993). Taken together, experimental evidence has established an important role for the Ras-ERK MAP kinase pathway in T cell activation and IL-2 expression.

In addition to the signals mediated by TCR/CD3 complex, complete activation of T cells requires a second co-stimulatory signal delivered by CD28. Activation of T cell via TCR in the absence of the co-stimulatory signal induces T cell anergy, a state of non-responsiveness to antigenic stimulation (Harding *et al.*, 1992; June *et al.*, 1994). Ligation of CD28 with B7 or monoclonal antibodies provides distinct signals from TCR/CD3 complex. For example, CD28, in conjunction with PMA, can induce IL-2 production and T cell proliferation which is not sensitive to cyclosporin A, a calcineurin inhibitor (June *et al.*, 1987). Moreover, CD28 provides signals which can dramatically enhance the production of IL-2 induced by TCR/CD3 engagement due to the stabilization of IL-2

mRNA and increasing transcription of IL-2 gene. A CD28 responsive element has been identified in the IL-2 promoter, in which AP-1, CREB and NF-κB are involved in the binding (McGuire and Iacobelli, 1997). To date, the signaling pathways triggered by CD28 have not yet been clearly understood. However, a number of signaling cascades have been demonstrated to be targeted by CD28, including the PLCγ pathway, the MAP kinase pathway, c-Jun N-terminal kinase, src-family of protein tyrosine kinases, and PI 3-kinase (reviewed by Ward, 1996). Among them, some signaling pathways are also induced by TCR/CD3 engagement and some are not. Therefore, it appears that CD28 might produce its effects by inducing distinct signals from TCR/CD3 or by increasing the strength and duration of TCR/CD3-mediated signals, which ultimately achieve full activation of T cells. The cognate T cell-APC interactions leading to full T cell activation via TCR/CD3 and CD28 can be mimicked using antibodies directed against these molecules (i.e., αCD3/αCD28). In fact, αCD3/αCD28 treatment more closely mimics physiological activation of T cells than PMA/Io treatment. Nevertheless both activation stimuli induce robust IL-2 expression by T cells.

C. Regulation of IL-2 and IL-4 gene expression in T cells

The hallmark of T cell activation is the expression and secretion of IL-2. IL-2 is a 15 kD glycoprotein produced primarily by activated Th cells and is critical for the activation and differentiation of several types of immune cells, including T cells, B cells, NK cells and macrophages (Stern and Smith, 1986; Forman and Pure, 1991; Gomez *et al.*, 1998). Expression of IL-2 gene is tightly regulated at the transcriptional level. Naïve resting T cells exhibit almost no basal level expression of IL-2 (< 100 transcripts/cell),

whereas T cell activation by mitogens, PMA/Io or through TCR/CD3 engagement and CD28 co-stimulation readily induces IL-2 production by initiating the transcription of IL-2 gene. The proximal promoter/enhancer region, which is -300 bp of the transcriptional start site, of the IL-2 gene has been well characterized (Serfling *et al.*, 1989). Multiple cis-acting elements involved in IL-2 regulation have been identified in the IL-2 promoter, including NF-AT, AP-1, NF-κB, Oct and CD28 response elements (Fig. 7). Coordinate binding of multiple trans-activating factors to these cis elements is required for full promoter activity (Rothenberg and Ward, 1996). The diversity of multiple DNA binding proteins reflects the integration and cooperation of multiple signaling pathways for IL-2 regulation.

One essential trans-activating factor for IL-2 transcription is NF-AT. This point is evidenced by the fact that inhibition of NF-AT by cyclosporin A ablates IL-2 production. It is for this reason that cyclosporin A is one of the most effective immunosuppresive agents in preventing rejection of organ transplantation. Two binding sites for NF-AT have been identified in the IL-2 promoter, the proximal (-130 to –140) and the distal (-260 to –290) site. Site-directed mutagenesis has confirmed that at least one of the two NF-AT sites is required for IL-2 promoter activity (Thompson *et al.*, 1992), and mutation of both NF-AT sites completely abrogates the IL-2 promoter activity (Boise *et al.*, 1993). Moreover, stability of NF-AT DNA binding to the distal NF-AT site is markedly increased by cooperation with AP-1 proteins (Jain *et al.*, 1993). It was subsequently found that a weak AP-1 like site adjacent 3' to the NF-AT motif is responsible for the recruitment of AP-1 to the NF-AT/AP-1 binding complex. The weak AP-1 sequence does not bind AP-1 proteins in the absence of NF-AT; however, it is

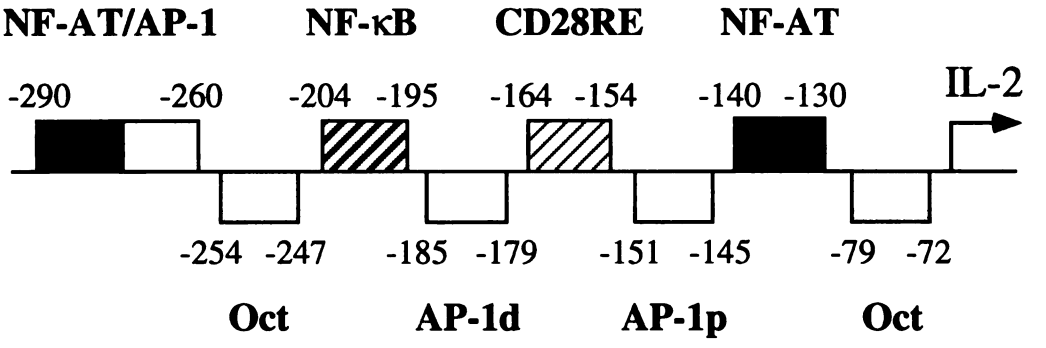


Figure 7. Minimal essential region of the IL-2 promoter. The region of the IL-2 promoter required for gene expression is approximately 300 bp upstream from the transcriptional start site. Multiple cis-elements responsible for the DNA binding of a number of transcription factors, including NF-AT, NF-κB, AP-1 and Oct are located within this region of the IL-2 promoter.

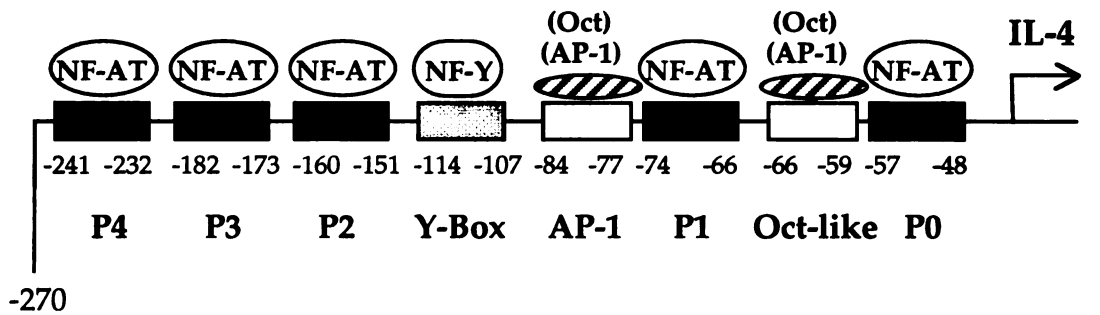
required for assembly of the NF-AT/AP-1 complex, and, in conjunction with the NF-AT motif, mediates the cooperative binding (Boise *et al.*, 1993; Jain *et al.*, 1993).

The AP-1 family of transcription factors is also critical for IL-2 promoter activity. Two AP-1 like sites exist in the IL-2 promoter region termed the proximal (-145 to –151; AP-1p) and distal (-179 to –185; AP-1d) elements. DNA footprinting assays have revealed the binding of fos and jun to the AP-1p site (Serfling *et al.*, 1989). Mutation of the AP-1p motif results in marked suppression of IL-2 production by PMA/Io-activated T cells (Jain *et al.*, 1992). Conversely, investigation of the AP-1d site in these studies failed to detect changes in IL-2 expression. These results indicate that the AP-1p element is critical for IL-2 regulation. In addition, AP-1 also participates in DNA binding to the Oct and CD28 response elements (CD28RE) in the IL-2 promoter (Ullman *et al.*, 1991; Ullman *et al.*, 1993; Fraser *et al.*, 1991). The binding of Oct-1 to the Oct response element of IL-2 promoter has been found to be closely associated with an octomer-associated protein (OAP^{40}) consisting of a heterodimer of fos and jun. Protein components of AP-1, NF-AT and NF-κB families participate in the DNA binding to the adjacent CD28RE/AP-1p element in the IL-2 promoter (McGuire and Iacobelli, 1997; Maggirwar *et al.*, 1997). Collectively, it has been widely demonstrated that AP-1 plays a substantial role in the regulation of the IL-2 gene by binding to the AP-1p element, and through interaction with other transcription factors resulting in binding to multiple cis elements, including distal NF-AT, Oct and CD28RE. In addition to NF-AT and AP-1, the family of NF-κB transcription factors also plays a significant role in IL-2 regulation. The recognized NF-κB response element is located between -195 to –204 of the IL-2 promoter, which is a functional regulatory element for IL-2 gene expression (Hughes and

Pober, 1996). As discussed earlier, another mechanism by which NF-κB regulates IL-2 transcription is mediated through interactions with CD28RE.

In summary, expression of the IL-2 gene in T cells is tightly controlled at the transcriptional level by several cis elements located in the IL-2 proximal enhancer/promoter region. Coordinate binding of multiple transcription factors, including NF-AT, NF-κB, AP-1 and Oct, to these cis elements is required for full activation of IL-2 gene transcription. As the activation of these transcription factors is mediated through a number of different signaling pathways, the involvement of multiple transcription factors reflects the diversity and complexity in IL-2 regulation.

IL-4 is a 13.5 kD protein secreted by activated Th2 cells (the principal cellular source), as well as by basophils and mast cells. The proximal IL-4 promoter has been found to possess multiple regulatory elements that control the transcription of the IL-4 gene in response to activating signals transduced from TCR/CD3 complex and CD28 molecules on the cell membrane. However, as compared to the IL-2 gene, the regulation of IL-4 gene expression is less extensively characterized and therefore the detailed mechanism for IL-4 regulation remains to be fully elucidated. To date, binding sites for NF-AT, AP-1, Oct, c-Maf and nuclear factor Y have all been identified in the proximal IL-4 promoter (Fig. 8; Rao, 1994; Szabo *et al.*, 1993; Li-Weber *et al.*, 1994; Ho *et al.*, 1996; Li-Weber *et al.*, 1998). Among these cis-acting elements, the role of NF-AT response elements is the best characterized. The IL-4 promoter contains at least five NF-AT binding sites (P elements), designated as P0, P1, P2, P3 and P4. These P elements are essential for the transcription of IL-4 gene as they confer the sensitivity of the IL-4 gene to calcium signals. Treatment with calcium ionophore or transfection with a

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P4          TATGGTGTAATTTCCTATGCTTGA
P3          GGTGTTTCATTTTCCAATTTGTCT
P2          ACAGGTAAATTTTCCTGTGAAATC
P1          GTAATAAAATTTTCCAATGTAAAC
P0          GTAAACTCATTTTCCCTTGGTTTC
P CONSENSUS      ATTTTCCNNT
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Figure 8. Schematic representation of the promoter region of mouse IL-4 gene. A consensus sequence for NF-AT binding (P element) is repeated five times within the IL-4 promoter, designated as P0, P1, P2, P3 and P4. The sequences of P elements are aligned in the lower panel in which P consensus motifs are underlined.

constitutively active form of calcineurin is sufficient to induce IL-4 expression, and the induction of IL-4 can be abrogated by cyclosporin A which is a specific inhibitor of calcineurin (Todd *et al.*, 1993). These results clearly demonstrate the unique property of the IL-4 gene and the crucial role NF-AT plays in its regulation. The NF-AT family of transcription factors is involved in the expression of many cytokines, such as IL-2, IL-3, IL-4, IL-5, IL-13, IFN-γ and TNF-α. As discussed earlier, the regulation of IL-2 by NF-AT has been extensively studied in which NF-AT binds to the distal NF-AT/AP-1 composite site cooperatively with AP-1 (Rao *et al.*, 1997). Therefore, NF-AT transcription factors are often referred to as the cytosolic component of NF-AT (NF-ATc), whereas AP-1 proteins are sometimes termed the nuclear component of NF-AT (NF-ATn). The activation of NF-ATc and its entry into the nucleus are mediated by calcineurin which is activated by calcium signals; within the nucleus, NF-ATc binds to NF-AT/AP-1 composite sites of target genes in conjunction with NF-ATn. Although IL-4 can be induced by increasing intracellular calcium, the contribution of AP-1 should not be minimized. Indeed, the P1 and adjacent AP-1 sites in the IL-4 promoter have been demonstrated to be a composite site, and the cooperative involvement of both NF-AT and AP-1 is required for full transcriptional activity of this composite site (Rooney *et al.*, 1995). Moreover, similar results have been reported for P0 and adjacent octamer-like sites as well (Li-Weber *et al.*, 1998). Hence, both NF-AT and AP-1 are likely involved in the regulation of IL-4 gene transcription.

D. The role of T cells in allergic airway disease

Allergic airway diseases, such as asthma, are characterized by hyperresponsiveness of the airways to provocative stimuli, recruitment of eosinophils, elevation of serum IgE, and over-production of mucus. Recently, it has been demonstrated that T cells, more specifically the Th2 subset, play a pivotal role in the pathophysiology of allergic airway disease (reviewed by Marone, 1998; Anderson and Coyle, 1994). In the murine model of allergic airway diseases induced by allergen (i.e., ovalbumin), Th2-derived cytokines are essential for the development of allergic responses. Blockade of IL-4 effectively prevents the induction of respiratory allergic responses. Interestingly, it was observed that anti-IL-4 antibodies prevented the allergic reactions, including hyperresponsiveness of the airways, and IL-5 and IgE production, only when it was administered to the animals before allergen sensitization. After the sensitization phase, administration of anti-IL-4 antibodies, either before or during allergen challenge, did not alter any features of the allergic response (Coyle *et al.*, 1995; Corry *et al.*, 1996). These results indicate that IL-4 is involved in the priming of immunocompetent cells in the early phase of immune reactions, which later differentiate toward the Th2 phenotype to promote the subsequent development of allergic responses by expressing Th2 cytokines. However, IL-4 itself appears not to act directly on the airways to trigger the allergic reactions. The importance of IL-4 for the priming and induction of allergic airway diseases is further supported by the experimental results demonstrating that IL-4 deficient mice have an attenuated allergic response elicited by antigen challenge as compared to wild type mice (Brusselle *et al.*, 1995; Coyle *et al.*, 1995; Kips *et al.*, 1995). In light of these observations, it has been proposed that IL-4 is a

key mediator for the pathogenesis of airway allergic disease, such as asthma. Specifically, IL-4 is thought to mediate two important biological effects. IL-4 is the determining factor for directing the differentiation of Th cells to Th2 phenotype which promotes B cell differentiation and immunoglobulin production. The Th2 cytokine IL-5 is an effector mediator well known as being involved in the recruitment of eosinophils into the airways in response to antigen challenge (Anderson and Coyle, 1994), and IL-13 has been demonstrated to be a central effector cytokine directly mediating airway hyperresponsiveness and mucous over-production (Wills-Karp *et al.*, 1998). In addition, IL-4, in conjunction with IL-13 (also a Th2 cytokine), mediates immunoglobulin isotype switching from IgM to IgE, which acts as a trigger of allergic reactions. The elevation of serum IgE is in fact a hallmark of allergic asthma. Collectively, experimental evidence strongly suggests that T cells are critical for the pathophysiology of allergic airway disease. Th2-derived cytokines are important mediators of the allergic response. IL-4 is essential for the priming of early immune reactions associated with allergic airway diseases, whereas the other Th2 cytokines are mediators involved in the antigen-induced allergic reactions.

III. Immune modulation by cannabinoids

The immunomodulatory activity of cannabinoids has been demonstrated in a variety of model systems. Both acquired and innate immunity are sensitive to cannabinoid compounds, including cytotoxic T cell activity, natural killer cell activity, phagocytosis and antigen processing and presentation by macrophages, and T cell dependent antibody forming cell responses (Kaminski, 1994; Klein *et al.*, 1998). The

effects of Δ^9-THC on antibody production have been extensively studied using IgM antibody forming cell (AFC) responses. Both the *in vivo* and *in vitro* sheep red blood cell (sRBC)-induced IgM AFC responses are inhibited by Δ^9-THC and CBN (Schatz *et al.*, 1993; Herring *et al.*, 1998). Since the sRBC is a T cell-dependent antigen, the elicited AFC response requires the participation of three major types of leukocyte, the T cell, B cell and macrophage. Notably, AFC responses induced by T cell-independent antigens (i.e., DNP-Ficoll) or polyclonal B cell activators (i.e., lipopolysaccharide), which do not require T cells and macrophages as accessory cells, are not inhibited by cannabinoids (Schatz *et al.*, 1993). These findings suggest that T cell accessory function (i.e., cytokine production) is involved in the inhibition of AFC response by cannabinoids. Cannabinoids also possess anti-proliferative activity which is likely linked to the inhibition of cytokine expression and results in a suppression of T cell clonal expansion. (Pross *et al.*, 1987; Schatz *et al.*, 1993; Herring *et al.*, 1998). In contrast to the inhibitory effects on T cell proliferation, both positive and negative effects on B cell proliferation by cannabinoids have been reported in the literature. For example, nanomolar concentrations of Δ^9-THC and the synthetic cannabinoids, CP55,940 and WIN55212, elicit a modest stimulatory effect on B cell proliferative responses induced by cross-linking of surface immunoglobulins (Derocq *et al.*, 1995). Conversely, LPS-stimulated proliferation of splenocytes has been shown to be suppressed by micromolar concentrations of Δ^9-THC (Klein *et al.*, 1985). These findings suggest that cannabinoids elicited contrasting effects on B cell proliferation, in which the concentration of cannabinoids is at least one contributing factor. Collectively, although B cells are the primary effector cells of humoral immunity, experimental evidence suggests that the

inhibition of IgM AFC responses by Δ^9-THC appears to be mediated through the inhibition of T cell accessory function and not through direct effects on the B cell. Cannabinoids may interfere with the activation and proliferation of T cells resulting in suppression of the T cell-dependent AFC responses.

In addition to T cells, macrophages appear to be another sensitive cellular target for cannabinoids. Macrophages participate in both innate and acquired immunity. The cytolytic activity of these cells, which is mediated by the release of hydrolytic enzymes, reactive oxygen species and nitric oxide (NO), is critical for the innate immunity. Macrophages are also involved in acquired immunity by presenting antigens to T cells. Notably, a variety of functional endpoints related with macrophage activation have been demonstrated to be inhibited by Δ^9-THC, including phagocytosis, cytolysis, antigen presentation, protein expression and NO production (Lopez-Cepero *et al.*, 1986; Cabral *et al.*, 1991; Zheng *et al.*, 1992; Jeon *et al.*, 1996).

In summary, considerable evidence exists for the immunomodulatory activity of cannabinoids. Cannabinoid compounds, such as Δ^9-THC, modulate the function of various immune competent cells. Among them, T cells and macrophages are sensitive to the alterations by cannabinoids, whereas B cells appear to be less sensitive.

A. Cannabinoid effects on IL-2 gene expression

T cells are involved in various immune reactions through a complicated cytokine network. As already discussed, a hallmark of T cell activation is the production of IL-2. IL-2 also contributes to the proliferation and differentiation of other cell types, including B cells and NK cells. For example, *in vitro* humoral immune responses are increased in

the presence of IL-2 (Watson *et al.*, 1979). In light of the fact that both cell-mediated immunity and the T cell accessory function in T-dependent AFC responses are sensitive to the inhibition by cannabinoids, the effect of cannabinoids on IL-2 expression has been extensively investigated. In a variety of experimental cell culture systems employing primary lymphoid cells and cell lines, cannabinoids inhibited IL-2 expression by T-cells activated with T cell mitogens or PMA/Io (Nakano *et al.*, 1993a; Condie *et al.*, 1996; Herring *et al.*, 1998). More in depth studies have revealed that Δ^9-THC and CBN produced a significant inhibition in the DNA binding activity of two nuclear factors critical to the transcriptional regulation of IL-2, NF-AT and AP-1 (Condie et al., 1996; Faubert and Kaminski, 2000; Yea *et al.*, 2000). Reporter gene activity of plasmids driven by the IL-2 promoter or multiple consensus sequences for NF-AT was suppressed by cannabinoids in transiently transfected EL4 T cells. A relatively transient attenuation by CBN of the promoter activity driven by multiple consensus AP-1 motifs was also observed in the same system (Yea *et al.*, 2000). In addition, PMA/Io-mediated activation of the ERK MAP kinases (ERK1 and ERK2), the upstream signaling molecules for AP-1 activation, was found to be down-regulated by CBN in murine primary splenocytes (Faubert and Kaminski, 2000). As ERKs are critical for the activation of AP-1 DNA binding, these findings suggest a potential mechanism for the inhibition of IL-2 expression by cannabinoids through the disruption of MAP kinase associated signaling resulting in suppression of AP-1 and NF-AT activation and subsequently IL-2 expression.

It is notable, as well as paradoxical, that both positive and negative regulation of IL-2 by cannabinoids has been reported (Nakano *et al.*, 1993a; Pross *et al.*, 1992; Snella *et al.*, 1995). For example, Nakano *et al.* have shown that Δ^9-THC inhibited mitogen-

induced IL-2 production but also enhanced αCD3-induced IL-2 production and proliferation of murine splenocytes. Several factors have been implicated in mediating these differential effects of cannabinoids on IL-2, including the method of T cell activation and age (Nakano *et al.*, 1993a; Pross *et al.*, 1993). A subsequent study by Nakano and coworkers found an increase in intracellular calcium in splenocytes activated with soluble αCD3 in the presence of Δ^9-THC, which was postulated as a possible mechanism for Δ^9-THC mediated enhancement of IL-2 production by splenocytes (Nakano *et al.*, 1993b). One striking feature concerning the various investigations of IL-2 modulation by cannabinoids which makes comparisons between these studies difficult are the differences in cell preparations, cell activation stimuli and culture conditions employed. To date, a comprehensive study aimed at deciphering the underlying mechanism for the diverging effects of cannabinoids on IL-2 regulation is still lacking. Hence, the elucidation of the mechanism responsible for cannabinoid-induced differential modulation of IL-2 expression was one of the goals of this dissertation.

B. Cannabinoid effects on IL-4 gene expression

In addition to IL-2, other cytokines derived from T cell are also involved in humoral immunity. Specifically, cytokines produced by Th2 cells, including IL-4, IL-5, IL-10 and IL-13, are critical for the proliferation and differentiation of B cells (Parker, 1993). Among these Th2 cytokines, IL-4 appears to be a key regulator for humoral immune responses. IL-4 is primarily produced by activated Th2 cells. It has a broad range of effects on T, B and mast cells, and other immune cells. In particular, IL-4 plays a major role in directing Th2 cell development. Subsequently, Th2 cells produce an array

of Th2 cytokines that promote B cell development into antibody secreting plasma cells. Interestingly, the effect of Δ^9-THC on IL-4 production has been examined in an *in vivo* model where mice were challenged with *Legionella pneumophila* (Klein *et al.*, 1995). It was found that Δ^9-THC disrupted the balance between Th1 and Th2 activity, as evidenced by the observations that the level of Th1 cytokine IFN-γ was suppressed, while the level of Th2 cytokine IL-4 was enhanced by Δ^9-THC in the *L. pneumophila* model. Immunity against *L. pneumophila,* an intracellular parasite, is primarily cell-mediated and therefore relies on the participation of Th1 cells. The mechanism responsible for the enhancing effect by Δ^9-THC on IL-4 levels observed in the *L. pneumophila* model is presently unknown but could be a consequence of secondary effects resulting from the disruption of Th1/Th2 balance. In fact, Berdyshev and coworkers have reported an opposite effect by Δ^9-THC on the IFN-γ and IL-4 production in PHA-stimulated human mononuclear cells *in vitro* (Berdyshev *et al.*, 1997). Under the *in vitro* culture system, the production of IFN-γ by PHA-stimulated leukocytes was enhanced, whereas IL-4 was diminished by Δ^9-THC. These findings and others already discussed serve to underscore numerous conflicting reports concerning cannabinoid-mediated immune modulation. It is likely that a great deal of the seemingly contradictory results can be attributed to differences in assay systems and models used in the respective studies. Results presented in this dissertation in fact help reconcile some of the conflicting results currently present in the literature.

In summary, only limited reports in the literature describe the effect of Δ^9-THC on the expression of IL-4. Unfortunately, the investigation by Klein *et al.* employed a model system that is not a measurement of humoral immune responses, and the report by

Berdyshev *et al.* does not provide mechanistic information on the inhibition of IL-4 secretion by Δ^9-THC. Hence, studies with model systems more relevant to the assessment of humoral immunity are required to further understand the effect of cannabinoids on IL-4 expression. In addition, more comprehensive studies would also be needed to elucidate the underlying mechanism for cannabinoid-mediated modulation of IL-4 expression by T cells.

IV. Objective and specific aims of thesis

The objective of this dissertation project was to examine the effects of CBN on the expression of IL-2 and IL-4 by T cells, and to elucidate underlying mechanisms responsible for CBN-mediated effects on the expression of these two cytokines. Specifically, the present studies were designed to address a series of related specific aims, including (1) to more critically investigate influencing factors that dictate the differential modulation of IL-2 gene expression by CBN, (2) to elucidate the molecular mechanism by which CBN enhances IL-2 transcription, (3) to characterize the effect of CBN on IL-4 expression by T cells, (4) to examine whether cannabinoid receptors are involved in CBN-mediated modulation of IL-2 and IL-4 expression, and (5) to investigate whether CBN modulates the expression of IL-2 and IL-4 *in vivo*, by employing a murine model of allergic airway disease induced by ovalbumin. The rationale for focusing on IL-2 and IL-4 is based on previous findings that T cell accessory function involved in humoral antibody responses is likely the component targeted by cannabinoids (Schatz *et. al.*, 1993). T cells participate in acquired immunity through a complicated cytokine network. IL-2 is a cytokine essential for T cell activation, and IL-4 is critical in directing Th0 to Th2 differentiation, thereby facilitating humoral immunity. In light of the key roles played by IL-2 and IL-4 in T cell-mediated immune responses, we hypothesize that IL-2 and IL-4 are important target genes modulated by cannabinoid compounds. Understanding the underlying mechanism of cannabinoid-mediated modulation of IL-2 and IL-4 will provide insights toward elucidating the mechanism for the apparent sensitivity of T-dependent AFC responses to cannabinoids, and pinpoint specific intracellular signaling pathways targeted by cannabinoids in T cells.

The experimental results of this thesis research are presented in three sections. The first section describes the CBN-mediated differential modulation of IL-2 expression. The second section describes the effects produced by Δ^9-THC and CBN on IL-4 expression. Lastly, the effect of Δ^9-THC and CBN on the IL-2 and IL-4 expression associated with allergen-induced airway inflammatory responses was presented in the third section.

MATERIALS AND METHODS

I. Reagents

All reagents were purchased from Sigma Chemical (St. Louis, MO) unless otherwise stated. Cannabinol (CBN), cannabidiol (CBD), Δ^9-tetrahydrocannabinol (Δ^9-THC), SR141716A and SR144528 were provided by National Institute on Drug Abuse; purity of these compounds were determined to be greater than 99% by GC-Mass spectrometric analysis.. CP55,940 was a gift from Pfizer Inc. (Groton, CT). CBN, CBD and Δ^9-THC were reconstituted in absolute ethanol, aliquoted, and stored at -80°C. CP55,940, WIN55212-1, WIN55212-3, SR141716A and SR144528 were reconstituted in dimethylsulfoxide, aliquoted, and stored at -80°C. To ensure the activity of all chemicals used in the present studies, stock solutions were always kept at -80°C, and working solutions were prepared freshly just before addition to cell cultures. H89, Ro-31-8220, and KN93 were purchased from Calbiochem (La Jolla, CA).

II. Animals and cell cultures

Female B6C3F1 mice, 6 weeks of age were purchased from the Charles River (Portage, MI). On arrival, mice were randomized, transferred to plastic cages containing a saw-dust bedding (5 mice per cage) and quarantined for 1 week. Mice were given food (Purina Certified Laboratory Chow) and water *ad libitum* and were not used for experimentation until their body weight was 17 - 20g; approximately 8 – 14 weeks old. Animal holding rooms were kept at 21 - 24°C and 40-60% relative humidity with a 12-hour light/dark cycle. Spleens were isolated aseptically and made into single cell

suspensions as described previously (Kaminski *et al.*, 1994). The splenocytes were cultured in RPMI 1640 medium (GIBCO BRL, Gaithersburg, MD) supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, 50 μM 2-mercaptoethanol and 2% bovine calf serum (BCS, Hyclone, Logan, UT). The C57BL/6 mouse T cell lymphoma line, EL4, was obtained from American Type Culture Collection (Rockville, MD). The EL4 cells were cultured in RPMI 1640 medium supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, 50 μM 2-mercaptoethanol, 2 mM L-glutamine, and 10% BCS. In all cases leukocytes were cultured at 37°C in 5% CO_2.

III. Antibodies and reporter plasmids

Rabbit polyclonal anti-phospho-ERK1/ERK2 was purchased from Promega (Madison, WI). Goat polyclonal anti-ERK1/ERK2, rabbit anti-c-jun/AP-1 and anti-c-fos were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-c-jun/AP-1 antibody recognizes c-jun, jun-B and jun-D, and the anti-c-fos antibody recognizes c-fos, fos-B, fra-1 and fra-2. Anti-NF-ATc1 mouse monoclonal antibody (clone 7A6) was purchased from Affinity BioReagents, Inc. (Golden, CO). Purified hamster anti-mouse CD3ε (145-2C11) and anti-mouse CD28 (37.51) monoclonal antibodies were purchased from PharMingan (San Diego, CA). The IL-2 distal NF-AT reporter gene pNFAT-SEAP plasmid was purchased from Clontech (Palo Alto, CA). This reporter plasmid is under the control of 3 consecutive copies of IL-2 distal NF-AT motifs and contains the reporter vector secreted alkaline phosphatase (SEAP) gene.

IV. Plasmid transfection and assessment of reporter gene activity

EL4 cells were transfected with pNFAT-SEAP plasmids using Cytofectene Transfection Reagent (Bio-Rad, Melville, NY). Briefly, cells (2 x 10^5 cells/mL) were harvested and resuspended in RPMI 1640 medium with 2% BCS in 60-mm cell culture dishes (5 mL/dish) and incubated with the transfection buffer (0.3 mL RPMI, 7.5 μg plasmids and 15 μL Cytofectene) for 16-20 hr. The transfected cells were washed, resuspended with 10 mL of RPMI with 2% BCS and received various treatments in triplicate in 48-well cell culture plates (0.2 mL/well; Corning Inc., Corning, NY). After 48 hr of culture, supernatants were collected and the SEAP activity in the supernatants was assayed using SEAP chemiluminescence detection kit (Clontech) following the instruction of manufacturer. In some cases the supernatant was also quantified for IL-2 by ELISA as previously described (Ouyang *et al.*, 1995).

V. Quantitative competitive RT-PCR

A. Preparation of internal standard for RT-PCR

A recombinant IL-2 internal standard (IS) was prepared as previously described (Condie *et al.*, 1996). A recombinant IL-4 IS was prepared to quantify IL-4 mRNA expression by quantitative/competitive RT-PCR. Briefly, an artificial/recombinant RNA (rcRNA) was used as an IS containing specific PCR primer sequences for IL-4 that were added to RNA samples in a series of dilutions. A rat ß-globin sequence was used as the spacer gene for the IL-4 IS. This method, developed by Vanden Heuvel (Vanden Heuvel *et al.*, 1993), avoids sample-to-sample variation of reference gene expression (e.g. ß-actin) as well as gene-to-gene differences in amplification efficiency. The primer

sequences from 5' to 3' for IL-4 are: forward primer = AACGAGGTCACAGGAGAAG, and reverse primer = GTCTATCGATGAATCCAGGC. The IS primer design from 5' to 3' is as follows: IS forward primer = T7 promoter (TAATACGACTCACTATAGG), IL-4 forward primer (as stated above), and rat ß-globin forward primer (AAGCCTGATGCTGTAGAGCC); and IS reverse primer = $(dT)_{18}$, IL-4 reverse primer (as stated above), and rat ß-globin reverse primer (AACCTGGATACCAACCTGCC). PCR reaction conditions for making the internal standard were performed as stated previously using 100 ng of rat tailed-genomic DNA (Vanden Heuvel *et al.*, 1993). PCR-amplified products were purified using the Wizard PCR Prep DNA Purification System (Promega Co, Madison, WI) and transcribed into RNA using Promega's Gemini II *In Vitro* Transcription System. The rcRNA was subsequently treated with RNase-free DNase to remove the DNA template. After quantifying, the following calculations were performed in order to determine the molecules/µl of the IL-4 IS:

[(µg/µl RNA) / (330 µg/µmol/bp x bp IS)] x 6.02 x 10^{17} molecules/µmole

Concentration of IS (µg/µl RNA) is derived from 260 nm reading; 330 x bp is an approximation for the molecular weight of the IS; the length of IL-4 IS (bp IS) is 346.

B. Quantification of steady state IL-2 mRNA expression by RT-PCR

Total RNA was isolated using TRI Reagent (Sigma). IL-2 steady state mRNA expression was quantified by quantitative competitive RT-PCR as described previously (Condie *et al.*, 1996) with minor modifications. All isolated RNA samples were confirmed to be free of DNA contamination as determined by the absence of product after PCR amplification in the absence of RT (data not shown). Briefly, known amounts

of total RNA and internal standard mRNA for IL-2 were reverse-transcribed simultaneously, in the same reaction tube, into cDNA using oligo$(dT)_{15}$ as primers. A PCR master mixture consisting of PCR buffer, 4 mM $MgCl_2$, 6 pmol each of the forward and reverse primers, and 1.25 units of *Taq* DNA polymerase was added to the cDNA samples. Samples were heated to 94 °C for 4 min and cycled 28 – 32 times at 94 °C for 15 sec, 60 °C for 30 sec, and 72 °C for 30 sec, after which an additional extension step at 72 °C for 5 min was included. PCR products were electrophoresed in 3% NuSieve 3:1 gels (FMC Bioproducts, Rackland, ME) and visualized by ethidium bromide staining. Quantification was performed by assessing the optical density for both of the DNA bands (internal standard and IL-2 mRNA) using a Gel Doc 1000 video imaging system (BioRad, Melville, NY). The number of transcripts was calculated from a standard curve generated from the density ratio between the gene of interest (IL-2) and the different amounts of internal standard used.

C. Quantification of steady state IL-4 mRNA expression by RT-PCR

All reagents used for RT-PCR were of molecular biological grade and were purchased from Promega (Madison, WI) unless otherwise noted. Competitive RT-PCR was performed as described by Gilliland et al. (Gilliland *et al.*, 1990), except that rcRNA was used as an IS instead of genomic DNA with 8 aliquots of rcRNA from 10^3 to 10^{10} molecules made for each RNA treatment group. Briefly, total RNA and IS rcRNA of known amounts were reverse transcribed into cDNA using oligo$(dT)_{15}$ as primers. A PCR master mixture consisting of PCR buffer, 4 mM $MgCl_2$, 6 pmole each of IL-4 forward and reverse primers, and 2.5 U of *Taq* DNA polymerase was added to the cDNA

samples. Samples were then heated to 94°C for 4 min and cycled 27 – 32 times at 94°C for 15 sec, 58°C for 30 sec, and 72°C for 45 sec after which an additional extension step at 72°C for 5 min was included. PCR products were electrophoresed in 3% NuSieve 3:1 gels (FMC Bioproducts, Rockland, ME) and visualized by ethidium bromide staining. The IL-4 primers produce a 228-bp amplified product from the cellular RNA and a 346-bp product from the IS rcRNA. Quantification was performed using the Gel Doc 1000 (Bio-Rad) where the amount of IL-4 mRNA present is determined as described by Gilliland *et al.* (Gilliland *et al.*, 1990). Briefly, the ratio of the volume of the IS rcRNA to IL-4 mRNA is plotted against the amount of IS rcRNA (in molecules) added to each reaction. The point at which the ratio of IS (rcRNA) to IL-4 mRNA is equal to 1 signifies the "cross-over" point which represents the amount of IL-4 molecules present in the initial RNA sample. After performing the 10^3 to 10^{10} range-finding experiment, a second set of much narrower internal standard dilutions were examined in order to accurately quantify IL-4 steady state mRNA expression in the various treatment groups.

VI. Western blotting

Nuclear proteins were isolated as previously described (Francis *et al.*, 1995). Briefly, cells were lysed with a hypotonic buffer (10 mM HEPES, 1.5 mM $MgCl_2$, pH 7.5) and the nuclei were pelleted by centrifugation at 3000 x g for 5 min. Nuclear lysis was performed using a hypertonic buffer (30 mM HEPES, 1.5 mM $MgCl_2$, 450 mM NaCl, 0.3 mM EDTA, and 10% glycerol) which contained 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, and 1 μg/mL each of

aprotinin and leupeptin, for 15 min on ice. Following lysis, samples were centrifuged at 17,500 x g for 15 min, and the supernatant was retained for use in the Western blotting. 25 μg of nuclear protein was loaded in each lane of a mini-gel apparatus and resolved on a 8% SDS-PAGE gel and transferred to nitrocellulose by electroblotting. The blot was incubated with the primary antibody for phospho-ERK1/ERK2, rabbit polyclonal anti-phospho-ERK1/ERK2 (Promega, Madison, WI), or the primary antibody for total ERK1/ERK2, goat polyclonal anti-ERK1/ERK2 (Santa Cruz Biotechnology, Santa Cruz, CA). After washing, the blot was incubated with an anti-rabbit horseradish peroxidase-linked immunoglobulin for detection of phospho-ERK1/ERK2 or incubated with anti-goat horseradish peroxidase-linked immunoglobulin for detection of total ERK1/ERK2, followed by exposure to enhanced chemiluminescence (ECL) Western blotting detection reagents (Amersham, Arlington Heights, IL). Bands were quantified using a densitometer visual imaging system (Bio-Rad, Hercules, CA).

VII. RNase protection assay (RPA)

The expression of multiple cytokine mRNAs, including IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-13, IL-15 and IFNγ was simultaneously detected using a RiboQuant MuitiProbe RPA system (PharMingen, San Diego, CA) following the instructions of the supplier. Briefly, RNA samples obtained from each group of EL4 cells (5 μg) or from right lung lobes of A/J mice (50 - 75 μg) receiving various treatments was hybridized overnight to ^{32}P-labeled riboprobes which have been synthesized from the supplied template set (mCK-1 from PharMingen). Single strand RNA and free probes were digested by RNase, and protected probes (double strand RNA) purified and

electrophoresed in a 6% polyacrylamide-Tris-borate-EDTA-urea gel. Following electrophoresis, the gel was dried and subjected to autoradiography. A standard curve of migration distance versus log nucleotide length was obtained using the undigested probes as markers. The nucleotide length of protected bands was determined by plotting their migration distance in the standard curve. The identity of each protected band was then established according to its nucleotide length.

VIII. Enzyme-linked immunosorbent assay (ELISA)

Mouse recombinant IL-2 and IL-4 standard, purified rat anti-mouse IL-2 and IL-4 antibody and biotinylated anti-mouse IL-2 and IL-4 antibody were purchased from PharMingen (San Diego, CA). Splenocytes (2 x 10^6 cells/mL) and EL4 cells (2 x 10^5 cells/mL) were cultured in triplicate in 48-well cell culture plates (0.2 mL/well; Corning Inc., Corning, NY). The supernatant fluids were collected 24 – 48 hr after T cell activation and quantified for IL-2 or IL-4 by ELISA as previously described (Ouyang *et al.*, 1995).

For measurement of ovalbumin-specific IgE, ELISA plates (Dynex Technologies, Inc., Chantilly, VA) were coated overnight at 4°C with 100 µL/well of 0.05% ovalbumin in 0.1 M $NaHCO_3$ buffer (pH 8.2). Wells were blocked with 200 µL/well of 3% bovine serum albumin in phosphate-buffered saline containing 0.02% Tween 20 (BSA-PBST) for 1 hr at 37 °C. After washing with PBST, serum samples at appropriate dilutions were added into wells (100 µL/well) and incubated for 1 hr at 37 °C. Wells were again washed and a rat biotinylated anti-mouse IgE (PharMingen) was added at 50 µL/well (2 µg/mL in 3% BSA-PBST) followed by incubation for 1 hr at room temperature. After another

washing with PBST, 50 μL/well of streptavidin peroxidase (1.5 μg/mL in 3% BSA-PBST) was added and incubated for 1 hr at room temperature. Lastly, the plates were washed and the bound peroxidase conjugate was detected by addition of substrate solution (100 μL/well) containing 0.1 M citric-phosphate buffer (pH 5.5), 0.1 mg/mL tetramethylbenzidine (Fluka Chemical Corp., Ronkonkoma, NY), and 1% H_2O_2. The reaction was terminated by adding 100 μL/well of 6N H_2SO_4 solution, and optical density was measured at 450 nm using a microplate reader (Bio-Tek Instrument, Inc., Winooski, VT).

IX. Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared as described (Francis *et al.*, 1995). Briefly, cells were lysed with a hypotonic buffer (10 mM HEPES, 1.5 mM $MgCl_2$, pH 7.5) and the nuclei were pelleted by centrifugation at 3000 x g for 5 min. Nuclear lysis was performed using a hypertonic buffer (30 mM HEPES, 1.5 mM $MgCl_2$, 450 mM NaCl, 0.3 mM EDTA, and 10% glycerol) which contained 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml each of aprotinin and leupeptin, for 15 min on ice. Following lysis, samples were centrifuged at 17,500 x g for 15 min, and the supernatant was retained for use in the DNA binding assay. Double-stranded deoxyoligonucleotides containing the IL-4 P0 NF-AT sequence (-70 ~ -45 of mouse IL-4 promoter; 5'-CCAATGTAAACTCA-TTTTCCCTTGGT-3'), the mouse IL-2 distal NF-AT sequence (5'-AGAGGAAAATTTGTTTCATACAGAAGGCG-3'), consensus AP-1 sequence (5'-GATCCGCTGACTCATCAGTA-3'; Novak *et al.*, 1990) and consensus NF-κB sequences (5'-GGGGACTTTCC-3'; Herring *et al.*, 1998) were synthesized and

end-labeled with [γ-^{32}P]-dATP. Nuclear extracts (5 μg) were incubated with 0.4 - 1 μg poly (dI-dC) and the ^{32}P-labeled DNA probe in the binding buffer (100 mM NaCl, 30 mM HEPES, 1.5 mM $MgCl_2$, 0.3 mM EDTA, 10% glycerol, 1 mM DTT, 1 mM PMSF, and 1 μg/ml of each aprotinin and leupeptin) for 20 min at room temperature. DNA binding activity was separated from free probe using a 4% polyacrylamide gel in 0.25 X TBE (1 X TBE: 89 mM Tris, 89 mM boric acid, and 2 mM EDTA). Following electrophoresis, the gel was dried and subjected to autoradiography.

X. *In vivo* animal model of allergic airway diseases induced by ovalbumin

Male A/J mice (6-week old) were purchased from Jackson Laboratory (Bar Harbor, Maine). On arrival, mice were randomized, transferred to plastic cages containing a saw-dust bedding (5 mice per cage) and quarantined for 1 week. Mice were either left untreated (NA) or sensitized on day 0 by intraperitoneal injection (i.p.) with 250 μL per mouse of sensitization solution containing 100 μg ovalbumin (Ova) and 1 mg aluminum potassium sulfate (as adjuvant) in saline. Mice of the control group for sensitization were i.p. injected with 250 μL of saline per mouse. On day 14, mice were challenged with either an aerosolized Ova solution (1% in saline) or aerosolized saline (control group for Ova challenge) for 30 min (single challenge protocol; Fig. 9A). Ova or saline aerosol was generated using Kegel-Inhalator model KU2000 (Werner Kegel GmbH, Lengerich, Germany). Δ^9-THC or CBN (50 mg/kg) was administered daily by i.p. for 3 consecutive days immediately prior to Ova sensitization and then before Ova challenge as depicted in figure 10A. Mice were sacrificed 24 hr or 48 hr after ovalbumin challenge by i.p. injection of 0.1 mL per mouse of 12% pentobarbital solution. Total

RNA from the lungs was isolated by TRI reagent. The steady state cytokine mRNA expression in the RNA samples was determined by competitive RT-PCR and RNase protection assay.

For measurement of serum Ova-specific IgE, Ova-sensitized mice were subjected to the second challenge with either aerosolized Ova or saline (control group) 10 days after the first challenge (Fig. 9B). Δ^9-THC or CBN (50 mg/kg) was administered daily by i.p. for 3 consecutive days immediately prior to Ova sensitization and then before each Ova challenge as depicted in figure 10B. Mice were sacrificed 96 hr after the second Ova challenge. Blood samples were collected from the brachial artery, and serum was obtained by centrifugation at 3,000 x g for 15 min. The level of Ova-specific IgE in serum was measured by ELISA.

XI. Statistical analysis

The mean ± standard error was determined for each treatment group in the individual experiments. Homogeneous data were evaluated by a parametric analysis of variance, and Dunnett's two-tailed *t-test* was used to compare treatment groups to the vehicle control when significant differences were observed (Dunnett, 1955). $p < 0.05$ was defined as statistical significance.

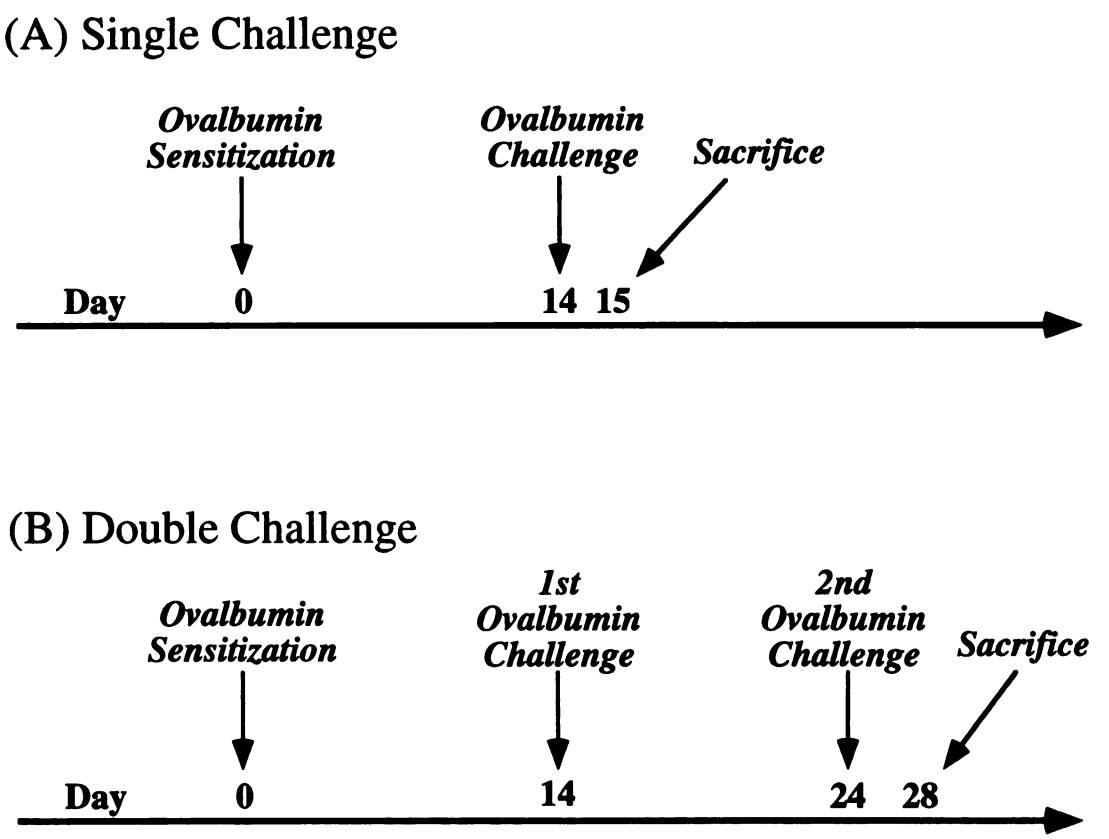


Figure 9. Protocol of ovalbumin-induced allergic airway disease. A/J mice (6-week old) were sensitized by intraperitoneal injection (i.p.) with ovalbumin (Ova; 100 μg/mouse) plus aluminum potassium sulfate (as adjuvant; 1 mg/mouse) on day 0. (A) Mice were challenged one time with an aerosolized Ova solution (1% in saline) for 30 min on day 14. Mice received single Ova challenge were sacrificed 24 or 48 hr after the Ova challenge. Total RNA from the lungs was isolated by TRI reagent (Sigma), and the steady state cytokine mRNA expression was measured by RT-PCR and RNase protection assay. (B) After the first Ova challenge, mice received a second Ova challenge10 days after the first challenge (day 24). Mice received double Ova challenge were sacrifice 96 hr after the second Ova challenge. Serum samples from each mouse were collected, and the level of Ova-specific IgE was quantified by ELISA.

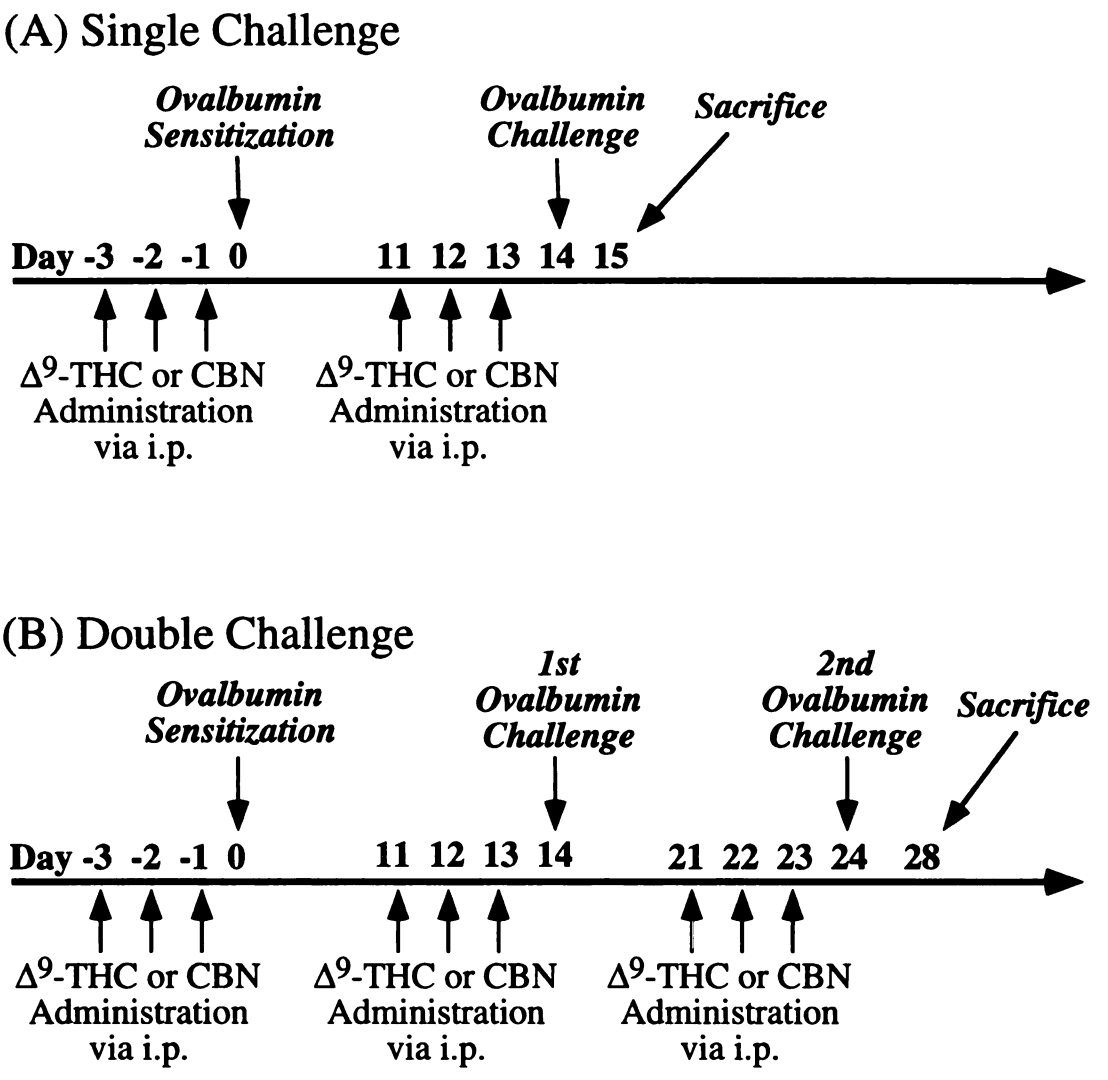


Figure 10. Protocol for administration of Δ^9-THC and CBN in A/J mice sensitized and challenged with ovalbumin. A/J mice were sensitized on day 0 by i.p. with ovalbumin plus aluminum potassium sulfate (100 μg/1 mg). Mice were either challenged one time with Ova aerosol on day 14 (single challenge) or challenged twice with Ova aerosol on day 14 and 24 (double challenge). Δ^9-THC (50 mg/kg), CBN (50 mg/kg) and/or VH (5% ethanol and 0.5% Tween 20 in saline) was administered daily by i.p. for 3 consecutive days immediately prior to sensitization and then before Ova challenge as indicated in the above cartoon.

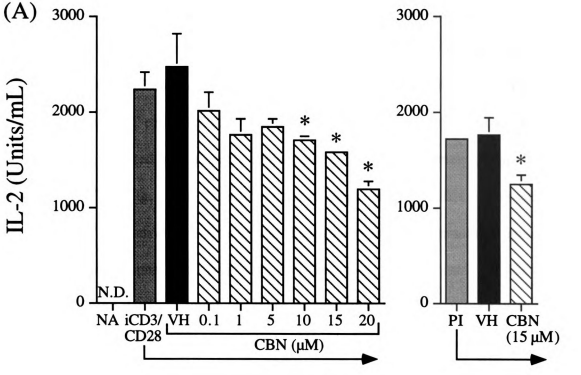
EXPERIMENTAL RESULTS

I. Differential modulation of IL-2 expression by CBN

A. Role of T cell activation in the differential modulation of IL-2 by CBN

Paradoxically, IL-2 expression has been reported to be positively and negatively regulated by cannabinoid treatment. The objective of this series of experiments was to examine the role of T cell activation (magnitude and type of stimulus) on this differential effect. Toward this end murine splenocytes were activated with either soluble or immobilized αCD3 alone or in combination with soluble αCD28. Stimulation of splenic T cells with iCD3 (2 μg/mL coated for overnight) alone for 48 hr induced only modest IL-2 secretion (131 ± 2 units/mL of IL-2 activity in the culture supernatant), which could be dramatically potentiated by addition of soluble αCD28 (2 μg/mL; iCD3/CD28; 3062 ± 161 units/mL of IL-2). Likewise, activation of splenic T cells with sCD3 (2 μg/mL) alone or in combination with αCD28 (sCD3/CD28; 2 μg/mL of each mAb) was also capable of inducing IL-2 secretion (101 ± 10 and 109 ± 8 units/mL of IL-2, respectively); however, the magnitude of stimulation was modest as compared to iCD3/CD28. These control studies demonstrate the magnitude of IL-2 induction by iCD3/CD28, a strong (optimal) activation stimulus, and by relatively weak (sub-optimal) stimuli (i.e., iCD3, sCD3 and sCD3/CD28) under the experimental conditions employed in the present investigation. The effect of CBN on IL-2 secretion induced by the various stimuli, either mAbs or PMA/Io (80 nM/1 μM), was examined in splenocytes. Cells were pretreated with CBN and/or vehicle (0.1% ethanol) for 30 min followed by activation with mAbs or PMA/Io. As illustrated in figure 11A, the magnitude of IL-2 induced by stimuli that had

Figure 11. Comparison of the effects of CBN on IL-2 secretion by splenocytes treated with various activation stimuli. Splenocytes (2 x 10^6 cells/mL) were either untreated (NA), or pretreated with CBN and/or vehicle (VH; 0.1% ethanol) for 30 min followed by stimulation with (A) the optimal activation stimulus immobilized anti-CD3 plus anti-CD28 (iCD3/CD28; 2 μg/mL) or PMA plus ionomycin (PI; 80 nM/1 μM), or (B) the sub-optimal stimulus soluble anti-CD3 (sCD3; 2 μg/mL), soluble anti-CD3 plus anti-CD28 (sCD3/CD28; 2 μg/mL of each antibody), or immobilized anti-CD3 alone (iCD3). After 48 hr of culture, supernatants were harvested and IL-2 was assayed by ELISA. Data are expressed as the mean ± standard error of triplicate cultures. *, $p <$ 0.05 as compared to the VH control group. *N.D.*, IL-2 protein was below the level of quantification. Results are representative of three independent experiments.


(A)
IL-2 (Units/mL)
3000
2000
1000
0
N.D.
NA
iCD3/ CD28
VH
0.1
1
5
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15
20
CBN (μM)
PI
VH
CBN (15 μM)
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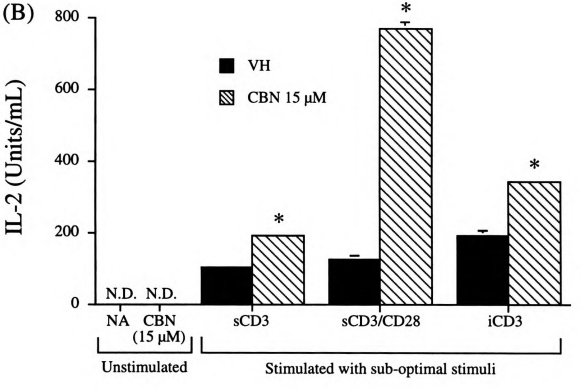

(B)
IL-2 (Units/mL)
800
600
400
200
0
VH
CBN 15 μM
N.D.
N.D.
NA
CBN (15 μM)
sCD3
sCD3/CD28
iCD3
Unstimulated
Stimulated with sub-optimal stimuli
*

been previously optimized for maximum IL-2 expression was significantly inhibited by CBN. For example iCD3/CD28-induced IL-2 secretion was suppressed by CBN in a concentration-dependent manner (10 - 20 μM), and is similar with previous reports demonstrating the inhibition of IL-2 by CBN in PMA/Io-activated T cells (Condie *et al.*, 1996). In contrast, T cells activated with a sub-optimal stimulus, sCD3, sCD3/CD28 or iCD3 alone, exhibited significantly enhanced IL-2 secretion when cultured in the presence of CBN (Fig. 11B). It is important to emphasize that CBN treatment in the absence of a T cell activation stimulus was incapable of inducing detectable amounts of IL-2 in the culture supernatants (Fig. 11B, 12). Moreover, enhancement of IL-2 by CBN was concentration-dependent with the enhancing effect by CBN being more pronounced in the presence of αCD28 (Fig. 12). IL-2 enhancement by CBN treatment was also demonstrated in activated EL4 cells, a murine thymoma widely used in studies of IL-2 regulation and expression (Condie *et al.*, 1996; Yea *et al.*, 2000). Although a widely employed model, EL4 cells differ from primary T cells in several way which is notable for the present studies. First, expression of IL-2 cannot be readily induced by αCD3/ αCD28 treatment. Second, IL-2 expression can be strongly induced by phorbol ester (i.e., PMA) alone or in combination with calcium ionophore (i.e., Io). In order to sub-optimally activate EL4 cells, sub-optimal concentrations of PMA (2 – 10 nM) were employed that induced modest production of IL-2 as compared to a high concentration of PMA (100 nM) in the presence and/or absence of ionomycin (Fig. 13). Preliminary concentration response experiments demonstrated that treatment of EL4 cells with PMA concentrations below 2 nM resulted in no measurable IL-2 activity in the culture supernatant (data not shown). Based on these preliminary experiments, 2 – 10 nM of

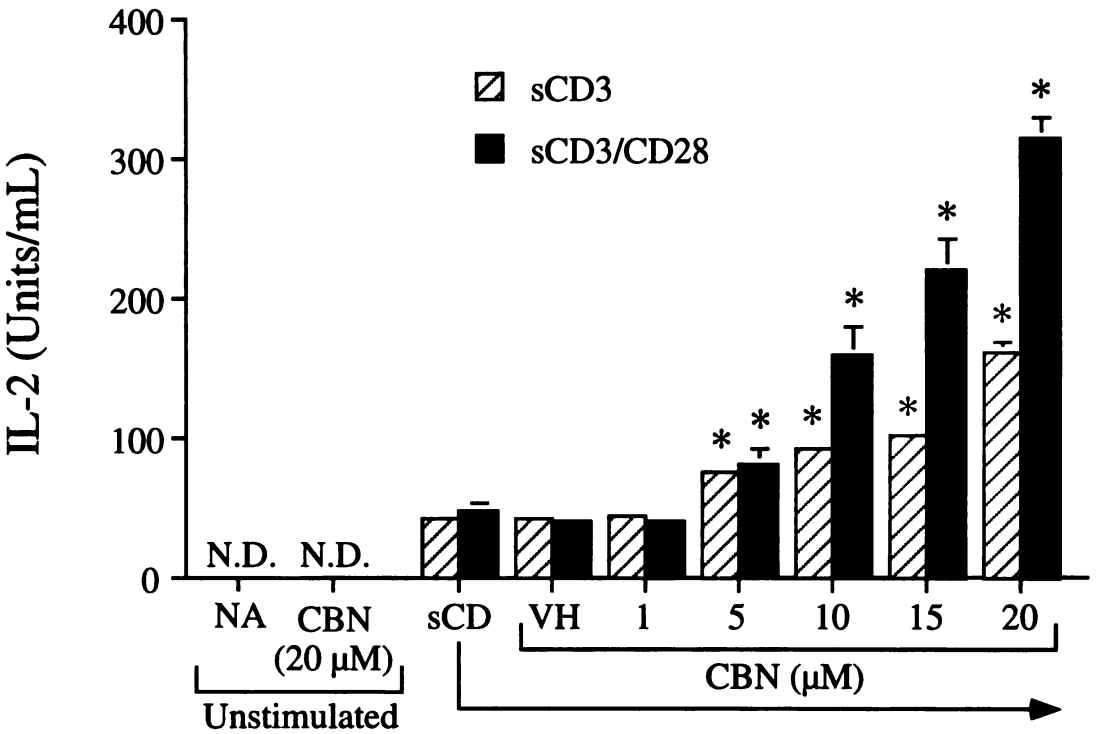
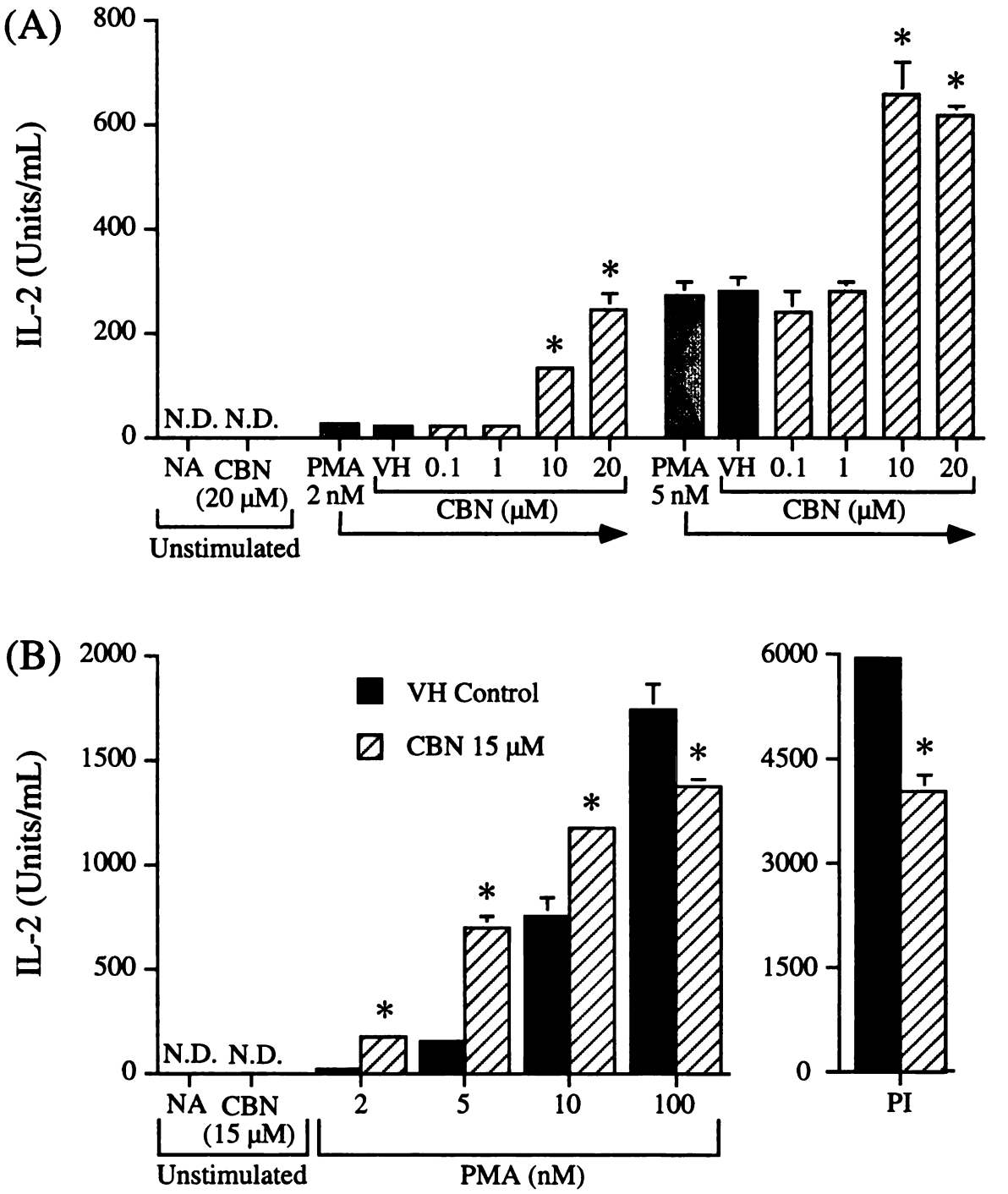


Figure 12. Concentration-dependent enhancement by CBN of IL-2 secretion by sCD3 or sCD3/CD28 activated splenic T cells. Splenocytes (2 x 10^6 cells/mL) were either untreated (NA), or pretreated with CBN (1 - 20 μM) and/or VH (0.1% ethanol) for 30 min followed by treatment with sCD3 (2 μg/mL) or sCD3/CD28 (2 μg/mL of each antibody). After 48 hr of culture, supernatants were harvested and IL-2 was assayed by ELISA. Data are expressed as the mean ± standard error of triplicate cultures. *, $p < 0.05$ as compared to the VH control group. *N.D.*, IL-2 protein was below the level of quantification. Results are representative of three independent experiments.

Figure 13. The effects of CBN on the IL-2 secretion induced by PMA or PMA plus ionomycin in EL4 cells. (A) EL4 cells (2 x 10^5 cells/mL) were either untreated (NA), or pretreated with CBN (0.1 - 20 μM) and/or VH (0.1% ethanol) for 30 min followed by stimulation with PMA (2 and 5 nM) for 24 hr at 37°C. (B) EL4 cells (2 x 10^5 cells/mL) were pretreated with CBN (15 μM) or VH for 30 min followed by stimulation with PMA (2, 5, 10 and 100 nM) or PMA plus ionomycin (PI; 80 nM/1 μM) for 24 hr at 37°C. IL-2 in the supernatants was quantified by ELISA. Data are expressed as the mean ± standard error of triplicate cultures. *, $p < 0.05$ as compared to the VH control group. *N.D.*, IL-2 protein was below the level of quantification. Results are representative of three independent experiments.


(A)
IL-2 (Units/mL)
800
600
400
200
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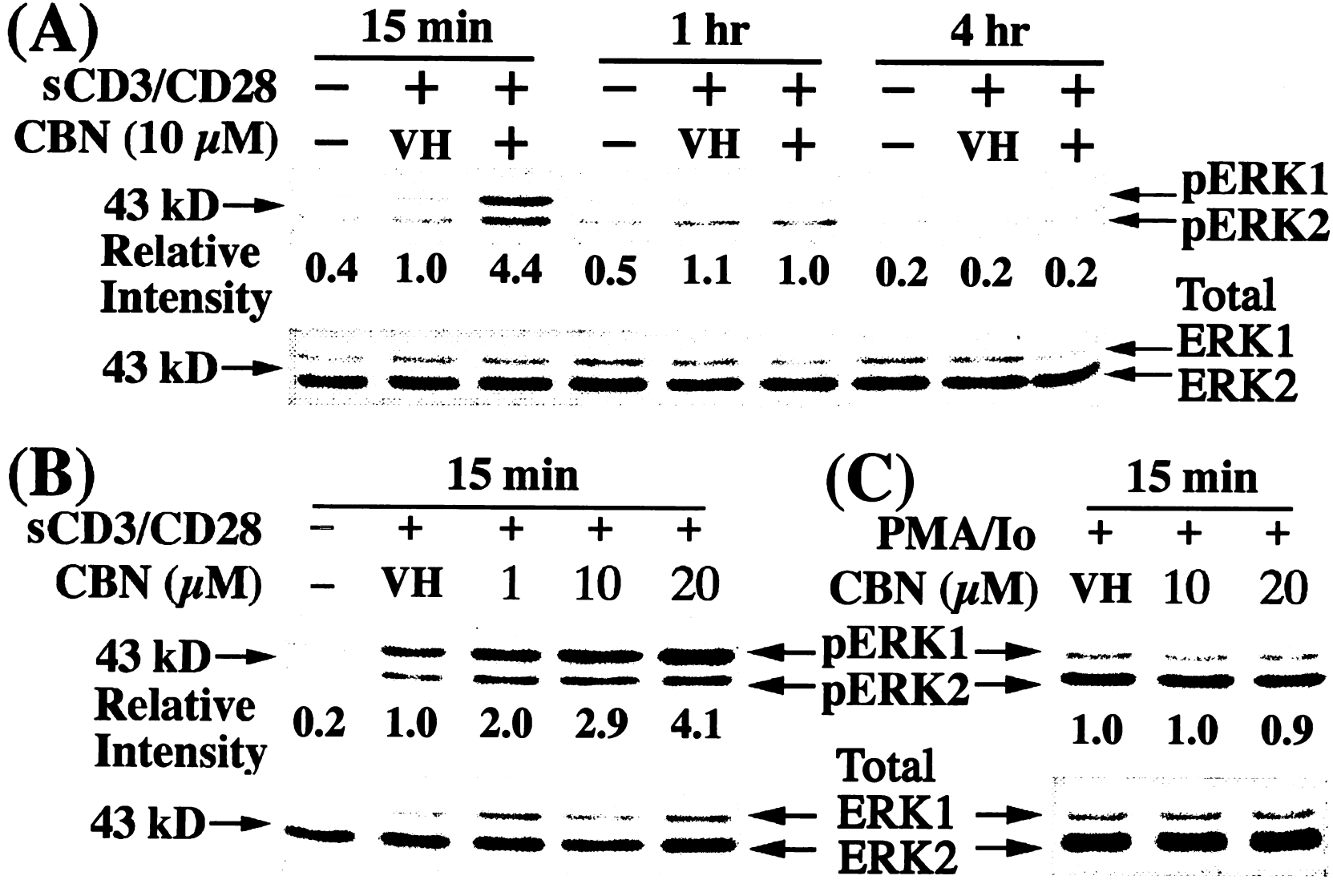
N.D. N.D.
NA CBN
(20 μM)
Unstimulated
PMA
2 nM
VH 0.1 1 10 20
CBN (μM)
PMA
5 nM
VH 0.1 1 10 20
CBN (μM)
(B)
IL-2 (Units/mL)
2000
1500
1000
500
0
VH Control
CBN 15 μM
N.D. N.D.
NA CBN
(15 μM)
Unstimulated
2 5 10 100
PMA (nM)
6000
4500
3000
1500
0
PI

PMA was functionally defined as a sub-optimal stimulus for IL-2 secretion by EL4 cells. Interestingly, pretreatment of EL4 cells with CBN (10 or 20 μM) 30 min prior to activation by sub-optimal concentrations of PMA (2 and 5 nM) resulted in a significant increase in IL-2 secretion (Fig. 13A). Conversely, EL4 cells activated with a high concentration of PMA (100 nM) or PMA/Io and cultured in the presence of CBN exhibited an inhibition of IL-2 secretion (Fig. 13B). Similar to the results with splenocytes, CBN alone did not induce measurable amounts of IL-2 secretion by EL4 cells (Fig. 13). Collectively, these results confirm that CBN-mediated enhancement or inhibition of IL-2 expression is governed by the magnitude of the T cell activation stimulus rather than the mode of activation.

B. Involvement of the ERK MAP kinases in the differential modulation of IL-2 by CBN

The ERK MAP kinases, which have been implicated as an intracellular target responsible for contributing to certain biological actions produced by cannabinoids, also play a critical role in the regulation of IL-2 gene expression through the activation of c-fos. In light of these previous findings the effect of CBN on ERK1 and ERK2 activation was examined in the context of IL-2 modulation. Western blot analysis using antibodies specific for phospho-ERK1/ERK2 and total ERKs revealed an up-regulation by CBN of both phosphorylated ERKs in the nucleus of splenocytes activated by sCD3/CD28 (sub-optimal stimulus), but not total ERKs which served as an internal loading control for the Western blotting (Fig. 14). The increase in phosphorylated nuclear ERKs in CBN treated cells was concentration dependent and transient. Peak enhancement of ERK

Figure 14. The effect of CBN on the ERK MAP kinase activation induced by sCD3/CD28 or PMA/Io in splenocytes. Splenocytes (2 x 10^6 cells/mL) were pretreated with VH (0.1% ethanol) or CBN for 30 min and then activated with either sCD3/CD28 (2 μg/mL of each) or PMA/Io (80 nM/1 μM). At the end of the culture period the cells were harvested, nuclear proteins for each treatment group isolated and assayed for phospho-ERK1/ERK2 (pERK) and total ERKs by immunoblotting. (A) Time course analysis (15 min - 4 hr) of the effect of CBN (10 μM) on sCD3/CD28-induced activation of ERK1 and ERK2. (B) Concentration-response by CBN (1, 10, and 20 μM) measuring the activation of ERK1 and ERK2 after a 15 min sCD3/CD28 treatment of splenocytes. (C) The effect of CBN (10 and 20 μM) on the activation of ERK1 and ERK2 after a 15 min PMA/Io (80 nM/1 μM) treatment of splenocytes. Molecular mass markers are indicated on the left; the molecular mass for ERK1 and ERK2 are 44 and 42 kD, respectively. The intensity of pERK1 and pERK2 bands in combination was quantified using a densitometer visual imaging system (Bio-Rad). Densitometry values were calculated relative to the matched VH control group at 15 min time point. Results are representative of three independent experiments.


(A)
15 min
1 hr
4 hr
sCD3/CD28 – + + – + + – + +
CBN (10 μM) – VH + – VH + – VH +
43 kD→
Relative Intensity 0.4 1.0 4.4 0.5 1.1 1.0 0.2 0.2 0.2
43 kD→
←pERK1
←pERK2
Total
←ERK1
←ERK2
(B)
15 min
sCD3/CD28 – + + + +
CBN (μM) – VH 1 10 20
43 kD→
Relative Intensity 0.2 1.0 2.0 2.9 4.1
43 kD→
←pERK1
←pERK2
Total
←ERK1
←ERK2
(C)
15 min
PMA/Io + + +
CBN (μM) VH 10 20
pERK1→
pERK2→
1.0 1.0 0.9
ERK1→
ERK2→

phosphorylation was observed 15 min after sCD3/CD28 stimulation (Fig. 14A and 14B). Importantly, CBN up-regulation of ERK1/ERK2 activation was only observed in splenocytes treated with a sub-optimal activation stimulus, and not in resting cells (data not shown) or those cells activated by a robust activation stimuli. For example, CBN (10 and 20 μM) did not exhibit marked effects on PMA/Io (80 nM/1 μM)-induced phospho-ERK1/ERK2 15 min post activation (Fig. 14C). Similar experiments were conducted in EL4 cells. As shown in figure 15, the CBN-mediated enhancement of ERK1/ERK2 activation was also demonstrable in PMA (2 nM) activated EL4 cells. Interestingly, enhancement of ERK1/ERK2 activation was more pronounced at 4 hr post activation than at 15 min in the PMA activated EL4 cells. These results suggest the involvement of ERK MAP kinase activation in CBN-mediated enhancement of IL-2 expression in sub-optimally activated T cells.

C. Involvement of PKC and CaM kinases, but not PI-3 kinase, in the CBN-mediated enhancement of IL-2

Since both PI 3-kinase and PKC have been widely established as upstream activators of MAP kinases in T cells (reviewed by Lopez-Ilasaca, 1998; Seger and Krebs, 1995), experiments were designed to investigate whether either of these two kinases is involved in CBN-mediated enhancement of IL-2 secretion by sCD3/CD28-activated splenocytes. The specific PI 3-kinase inhibitor, wortmannin, and PKC inhibitor, staurosporine, were used for these studies. Because the IC_{50} for PI 3-kinase inhibition by wortmannin in neutrophils was reported to be less than 10 nM, concentrations between 1 and 100 nM were utilized for these studies (Ward *et al.*, 1996). As illustrated in figure

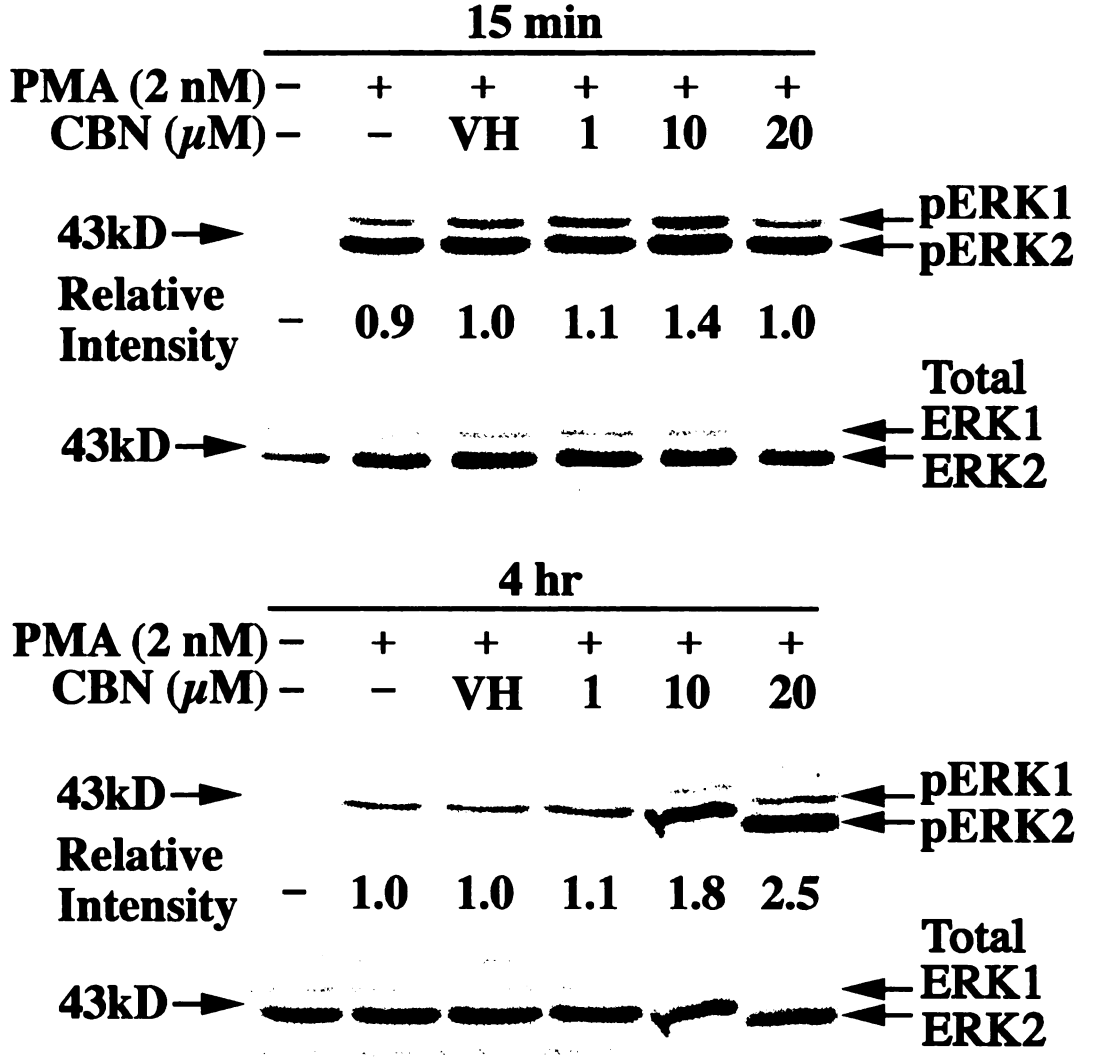
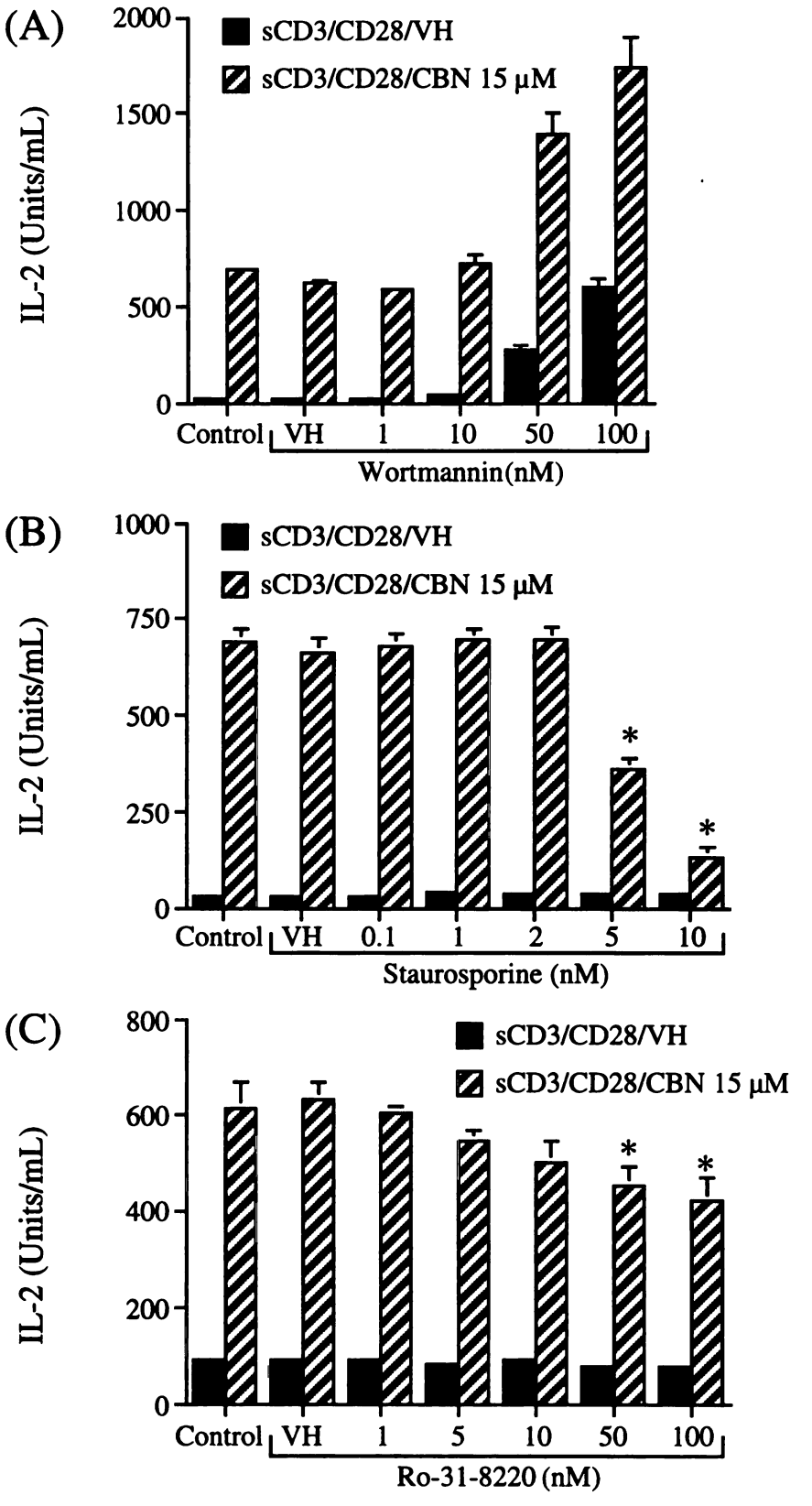


Figure 15. The effect of CBN on ERK MAP kinase activation in PMA (2 nM) treated EL4 cells. EL4 cells (2 x 10^5 cells/mL) were pretreated with VH (0.1% ethanol) or CBN (1, 10, and 20 μM) for 30 min and then activated with PMA (2 nM) for either 15 min or 4 hr. Cells were then harvested, nuclear proteins from each group isolated and assayed for phospho-ERK1/ERK2 and total ERKs by immunoblotting. Molecular mass markers are indicated on the left; the molecular mass for ERK1 and ERK2 are 44 and 42 kD, respectively. The intensity of pERK1 and pERK2 bands in combination was quantified using a densitometer visual imaging system (Bio-Rad). Densitometry values were calculated relative to the matched VH control group. Results are representative of three independent experiments.

16A, wortmannin (10 - 100 nM) alone increased the magnitude of IL-2 secretion by sCD3/CD28-activated splenic T cells (Fig. 16A, black columns). These results suggest a negative role by PI 3-kinase in the induction of IL-2 by sCD3/CD28. Nevertheless, CBN was still capable of enhancing IL-2 secretion in the presence of wortmannin indicating that PI 3-kinase was not involved in the CBN-mediated enhancement of IL-2. In contrast, when splenocytes were pretreated with staurosporine (0.1 - 10 nM) prior to CBN treatment, the CBN-mediated enhancement of IL-2 was remarkably attenuated by 5 and 10 nM of staurosporine (Fig. 16B). Because the IC_{50} for PKC inhibition by staurosporine was determined to be approximately 9 nM (Bradshaw *et al.*, 1993), a concentration range between 0.1 and 10 nM was used to ensure its selectivity for PKC. Notably, staurosporine at these low concentrations did not interfere with the sCD3/CD28-induced IL-2 secretion (Fig. 16B, black columns), indicating that the ability of splenocytes to produce IL-2 in response to sCD3/CD28 was not affected by the presence of staurosporine. In addition to staurosporine, the role of PKC in CBN-mediated enhancement of IL-2 secretion was also examined by employing Ro-31-8220 (Ro), a more specific PKC inhibitor that is a structural analog of staurosporine. Ro possesses an IC_{50} for PKC between 5 - 27 nM (Wilkinson *et al.*, 1993). Similar to staurosporine, Ro partially attenuated CBN-mediated enhancement of IL-2 secretion (Fig. 16C), further supporting the involvement of PKC. However, the extent of the attenuation by Ro (50 –100 nM) was rather modest compared to staurosporine. It is notable that staurosporine has also been reported to inhibit other calcium-dependent protein kinases, such as CaM kinases (Yanagihara *et al.*, 1991). Thus, the putative role of CaM kinases in CBN-mediated enhancement of IL-2 secretion was also investigated using KN93, a competitive CaM kinase inhibitor possessing an IC_{50} of 13 μM (Abraham *et al.*, 1997). As illustrated

Figure 16. Reversal by staurosporine and Ro-31-8220, but not by wortmannin, of CBN-mediated enhancement of the IL-2 secretion induced by sCD3/CD28 in splenocytes. Splenocytes (2 x 10^6 cells/mL) were pretreated with (A) wortmannin (1 - 100 nM) or VH (0.001% of DMSO), (B) staurosporine (0.1 - 10 nM) or VH (0.002% DMSO), (C) Ro-31-8220 (1 – 100 nM) or VH (0.001%DMSO), or untreated (Control) for 15 min. Cells in each group were then incubated with CBN (15 μM) or VH for CBN (0.1% ethanol) for 30 min and then activated with sCD3/CD28 (2 μg/mL of each) for 48 hr at 37°C. IL-2 in the supernatants was quantified by ELISA. Data are expressed as the mean ± standard error of triplicate cultures. *, $p < 0.05$ as compared to the matched VH group treated with sCD3/CD28 and CBN. Results are representative of three independent experiments.


(A)
IL-2 (Units/mL)
2000
1500
1000
500
0
sCD3/CD28/VH
sCD3/CD28/CBN 15 µM
Control
VH
1
10
50
100
Wortmannin(nM)
(B)
IL-2 (Units/mL)
1000
750
500
250
0
sCD3/CD28/VH
sCD3/CD28/CBN 15 µM
*
*
Control
VH
0.1
1
2
5
10
Staurosporine (nM)
(C)
IL-2 (Units/mL)
800
600
400
200
0
sCD3/CD28/VH
sCD3/CD28/CBN 15 µM
*
*
Control
VH
1
5
10
50
100
Ro-31-8220 (nM)

in figure 17A, KN93 (5 – 10 μM) robustly attenuated CBN-mediated enhancement of IL-2. Interestingly, 1 μM KN93 in combination with 10 nM Ro significantly attenuated CBN-mediated enhancement of IL-2, even though neither 1 μM KN93 or 10 nM Ro alone was effective (Fig. 17A and 17B). Moreover, the magnitude of reversal induced by KN93 was further increased in the presence of 50 nM Ro (Fig. 17A and 17C). These results suggest that both PKC and CaM kinases are likely involved in the mechanism by which CBN enhances IL-2 secretion by sCD3/CD28-activated splenocytes.

D. Concentration-dependent enhancement of steady state IL-2 mRNA expression by CBN in sub-optimally activated T cells

In light of the above results showing an increase in IL-2 protein secretion by CBN in sub-optimally activated T cells, the effect of CBN on steady state IL-2 mRNA expression was examined in EL4 T cells activated by low concentrations of PMA (2 – 10 nM). Based on kinetics studies demonstrating peak steady state IL-2 mRNA expression occuring 4 – 8 hr after T cell activation (Jain *et al.*, 1995), EL4 cells were harvested and total RNA was isolated at 6 hr post PMA stimulation. The magnitude of IL-2 mRNA expression was quantified by competitive RT-PCR. Pretreatment of EL4 cells with CBN (15 μM) for 30 min significantly enhanced the steady state IL-2 mRNA expression induced by PMA (2 – 10 nM; Fig. 18A). The CBN-mediated enhancement of IL-2 steady state mRNA expression was further demonstrated to be concentration-dependent (Fig. 18B), and is concordant with CBN-mediated enhancement of IL-2 protein secretion. It is notable that CBN treatment alone, in the absence of PMA, was incapable of inducing detectable steady state IL-2 mRNA expression in EL4 cells (Fig. 18).

Figure 17. Reversal by KN93 alone or in combination with Ro-31-8220 of CBN-mediated enhancement of the IL-2 secretion induced by sCD3/CD28 in splenocytes. Splenocytes (2 x 10^6 cells/mL) were pretreated with (A) KN93 (0.1 - 10 μM), (B) KN93 plus Ro-31-8220 (10 nM), (C) KN93 plus Ro-31-8220 (50 nM) or vehicle (0.0005% DMSO; Control group) for 15 min. Cells in each group were then incubated with CBN (15 μM) or VH for CBN (0.1% ethanol) for 30 min and then activated with sCD3/CD28 (2 μg/mL of each) for 48 hr at 37°C. IL-2 in the supernatants was quantified by ELISA. Data are expressed as the mean ± standard error of triplicate cultures. *, $p < 0.05$ as compared to the matched VH group treated with sCD3/CD28 and CBN. Results are representative of three independent experiments.

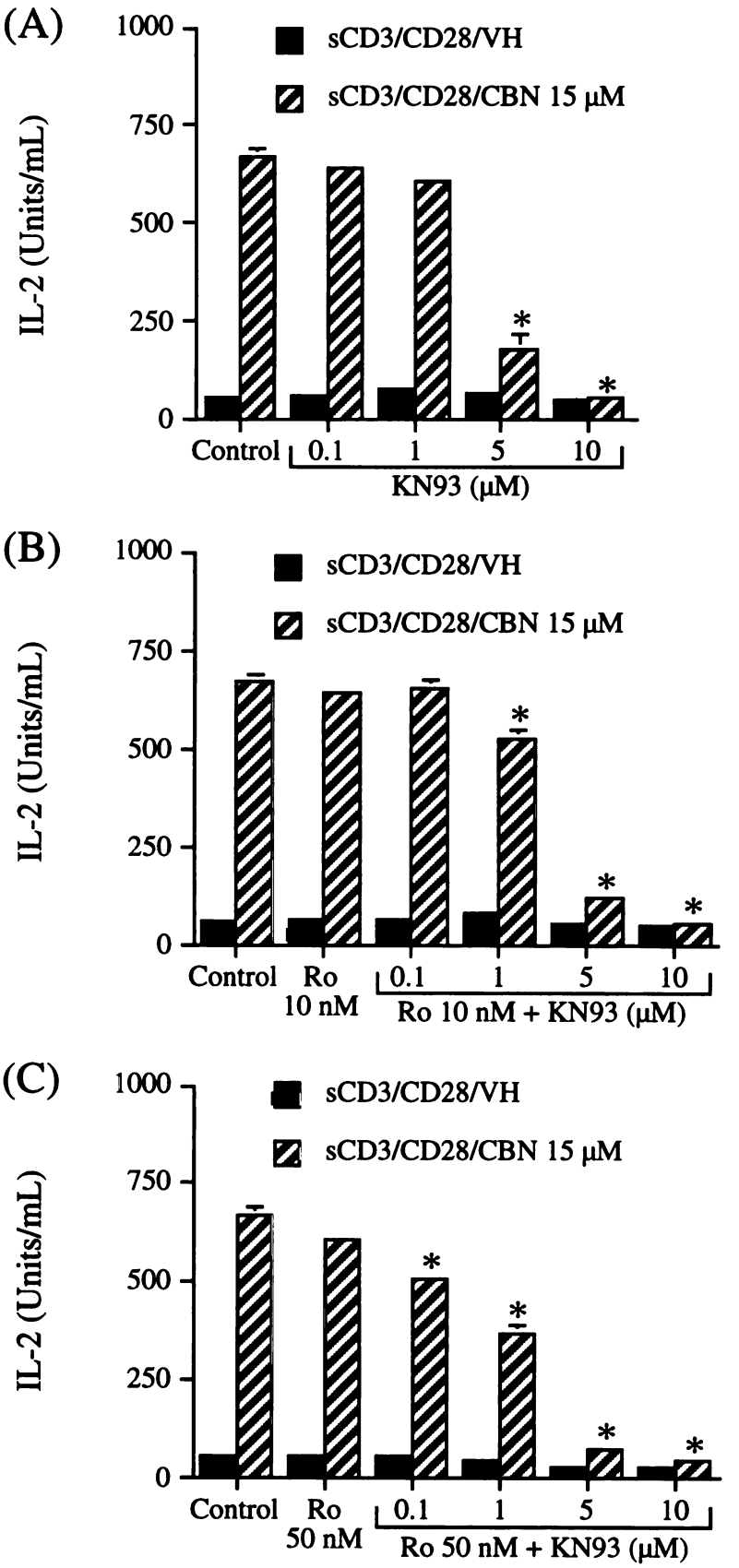
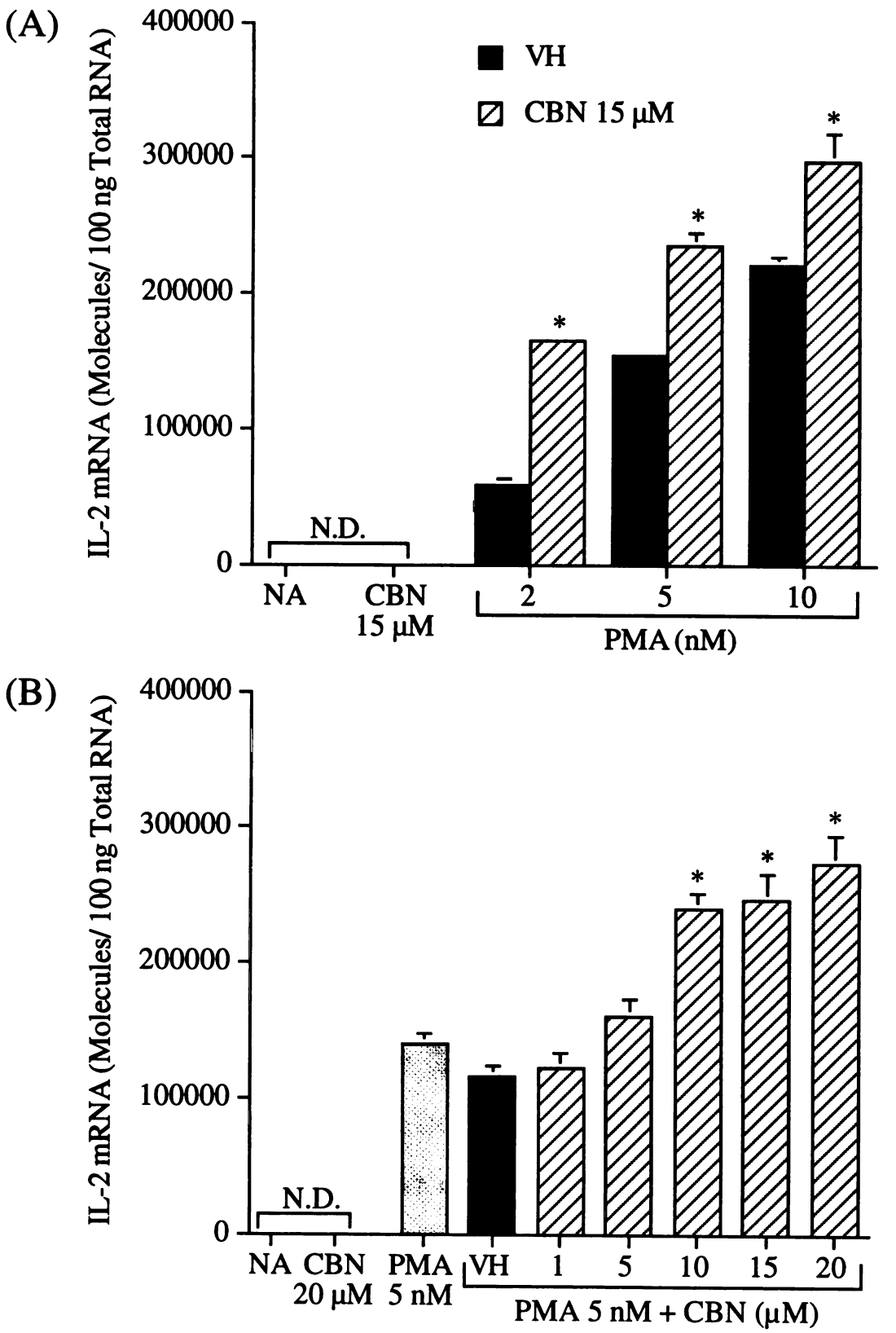

(A)
1000
750
500
250
0
IL-2 (Units/mL)
sCD3/CD28/VH
sCD3/CD28/CBN 15 μM
*
*
Control
0.1
1
5
10
KN93 (μM)
(B)
1000
750
500
250
0
IL-2 (Units/mL)
sCD3/CD28/VH
sCD3/CD28/CBN 15 μM
*
*
*
Control
Ro
10 nM
0.1
1
5
10
Ro 10 nM + KN93 (μM)
(C)
1000
750
500
250
0
IL-2 (Units/mL)
sCD3/CD28/VH
sCD3/CD28/CBN 15 μM
*
*
*
*
Control
Ro
50 nM
0.1
1
5
10
Ro 50 nM + KN93 (μM)

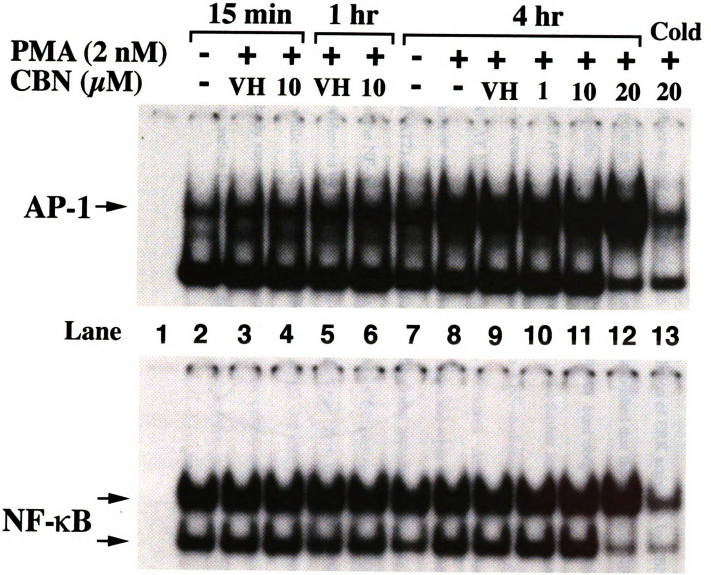
Figure 18. CBN-mediated enhancement of the steady state IL-2 mRNA expression in sub-optimally activated EL4 cells. (A) EL4 cells (2 x 10^5 cells/mL) were either untreated (NA) or pretreated with CBN (15 μM) and/or VH (0.1% ethanol) for 30 min followed by stimulation with the sub-optimal activation stimulus PMA (2 - 10 nM) for 6 hr at 37°C. (B) EL4 cells (2 x 10^5 cells/mL) were either untreated (NA) or pretreated with CBN (1 - 20 μM) and/or VH (0.1% ethanol) for 30 min followed by stimulation with PMA (5 nM) for 6 hr at 37°C. The total RNA was isolated and IL-2 mRNA determined by competitive RT-PCR. The data are expressed as the mean ± standard error of triplicate cultures. *, $p < 0.05$ as compared to the VH control group. *N.D.*, IL-2 mRNA was below the level of quantification. Results are representative of three independent experiments.


(A)
IL-2 mRNA (Molecules/ 100 ng Total RNA)
400000
300000
200000
100000
0
VH
CBN 15 μM
*
N.D.
NA
CBN 15 μM
2
5
10
PMA (nM)
(B)
IL-2 mRNA (Molecules/ 100 ng Total RNA)
400000
300000
200000
100000
0
N.D.
NA
CBN 20 μM
PMA 5 nM
VH
1
5
10
15
20
PMA 5 nM + CBN (μM)

E. CBN-mediated enhancement of DNA binding to the IL-2 distal NF-AT site, but not NF-κB or AP-1 consensus motifs in sub-optimally activated T cells

As previously discussed, transcription of IL-2 is regulated by several trans-acting factors, such as AP-1, NF-κB, NF-AT and Oct (Jain *et al.*, 1995). In light of the above findings which implicate a role by ERK MAP kinases and CaM kinases in CBN-mediated enhancement of IL-2 secretion, the present series of experiments focused on characterizing the effect of CBN on three potential transcription factors critical for IL-2 regulation, AP-1, NF-κB and NF-AT. These three families of transcription factors are known to be regulated by either ERKs or CaM kinases (Jain *et al.*, 1995). Moreover, it has been previously shown that, under optimal T cell activation conditions, cannabinoids modulate both the activation of these transcription factors and IL-2 gene expression (Condie *et al.*, 1996; Herring *et. al.*, 1998; Yea *et al.*, 2000). The same experimental conditions utilized in mRNA studies were employed, and the DNA binding activity of AP-1, NF-κB and NF-AT was examined by EMSA. Nuclear proteins were isolated 15, 60 or 240 min post PMA stimulation. As illustrated in figure 19, although AP-1 binding was induced by PMA (2 nM) in a time-dependent manner, CBN pretreatment did not significantly alter AP-1 binding (Fig. 19, upper panel). Likewise, NF-κB binding was not markedly influenced by CBN either (Fig. 19, lower panel). In contrast, the DNA binding activity to the IL-2 distal NF-AT motif was markedly enhanced (1.7-fold) by

Figure 19. Effect of CBN on AP-1 and NF-κB DNA binding in sub-optimally activated EL4 cells. EL4 cells (2 x 10^5 cells/mL) were either untreated, or pretreated with CBN (1 - 20 μM), and/or VH (0.1% ethanol) for 30 min followed by stimulation with PMA (2 nM). EL4 cells were cultured for 15 min, 1 hr or 4 hr at 37°C and nuclear proteins isolated as described in the "Materials and Methods". Nuclear proteins (5 μg) were incubated with 1μg of poly(dI-dC) and ^{32}P-labeled AP-1 or NF-κB consensus DNA probe in binding buffer at room temperature for 20 min followed by separation on a 4% polyacrylamide gel. *Lane 1*, free probe; *lane 2* and *7*, naïve cells. The competitor lane (*lane 13*) included 50-fold molar excess of the unlabeled DNA probe as cold competitors. Results are representative of three independent experiments.


15 min
1 hr
4 hr
Cold
PMA (2 nM) - + + + + - + + + + +
CBN (μM) - VH 10 VH 10 - - VH 1 10 20 20
AP-1
Lane 1 2 3 4 5 6 7 8 9 10 11 12 13
NF-κB

CBN (10 and 20 μM) at the 4 hr time point post PMA activation (Fig. 20). This enhancement in NF-AT DNA binding activity is paralleled with CBN-mediated enhancement of IL-2 protein secretion and mRNA expression and is consistent with the aforementioned observations demonstrating an up-regulation of ERK activation by CBN at the same time point in EL4 cells. It has been well established that the IL-2 distal NF-AT site is a composite site where both AP-1 and NF-AT bind cooperatively to the adjacent NF-AT and AP-1 like motifs (Rao *et al.*, 1997). To further investigate whether AP-1 and/or NF-AT transcription factors are involved in CBN-mediated enhancement of the IL-2 distal NF-AT binding, supershift assays were performed. Antibodies directed against components of AP-1 transcription factors (i.e., c-fos and c-jun family members) or NF-ATc1 (NF-AT2) were employed. Both AP-1 associated proteins and NF-ATc1 were identified in the NF-AT binding complex induced by CBN pretreatment and PMA stimulation, as evidenced by the fact that anti-c-fos polyclonal rabbit IgG inhibited the NF-AT binding while anti-c-jun/AP-1 polyclonal rabbit IgG and anti-NF-ATc1 mouse monoclonal antibody supershifted the binding (Fig. 21). In contrast, control rabbit IgG and control mouse ascites fluid did not alter the DNA binding to the IL-2 distal NF-AT site.

F. CBN-mediated enhancement of IL-2 distal NF-AT transcriptional activity in sub-optimally activated T cells

To further evaluate the significance of the increase in DNA binding activity to the IL-2 distal NF-AT site by CBN treatment, the effect of CBN on the transcriptional activity of a reporter gene driven by multiple IL-2 distal NF-AT motifs was assessed by

Figure 20. Effect of CBN on murine IL-2 distal NF-AT binding in sub-optimally activated EL4 cells. EL4 cells (2 x 10^5 cells/mL) were either untreated, or pretreated with CBN (1 - 20 μM), and/or VH (0.1% ethanol) for 30 min followed by stimulation with PMA (2 nM). EL4 cells were cultured for 15 min, 1 hr or 4 hr at 37°C and nuclear proteins isolated. Nuclear proteins (5 μg) were incubated with 1μg of poly(dI-dC) and ^{32}P-labeled murine IL-2 distal NF-AT DNA probe in binding buffer at room temperature for 20 min followed by separation on a 4% polyacrylamide gel. *Lane 1*, free probe; *lane 2* and *7*, naïve cells. The competitor lane (*lane 13*) included 50-fold molar excess of the unlabeled DNA probe as competitors. Results are representative of three independent experiments.

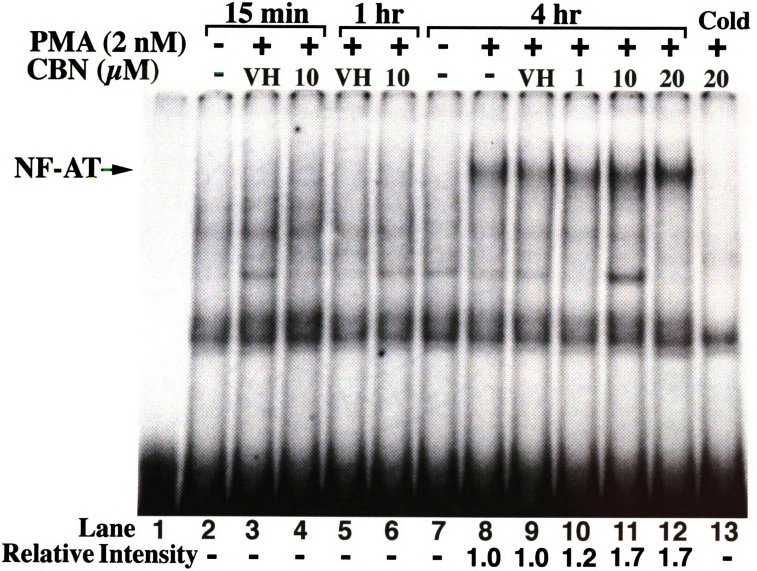
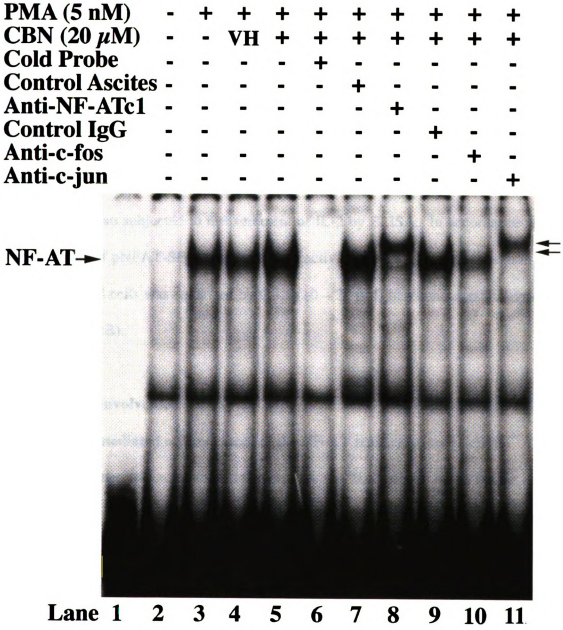

15 min
1 hr
4 hr
Cold
PMA (2 nM) - + + + + - + + + + + +
CBN (μM) - VH 10 VH 10 - - VH 1 10 20 20
NF-AT
Lane 1 2 3 4 5 6 7 8 9 10 11 12 13
Relative Intensity - - - - - - 1.0 1.0 1.2 1.7 1.7 -

Figure 21. Supershift analysis of CBN-mediated enhancement of NF-AT binding. EL4 cells (2 x 10^5 cells/mL) were either untreated, or pretreated with CBN (20 μM), and/or VH (0.1% ethanol) for 30 min followed by stimulation with PMA (5 nM) for 4 hr at 37°C. The nuclear proteins were isolated and 5 μg of nuclear proteins were incubated with 1 μg of poly(dI-dC) and ^{32}P-labeled murine IL-2 distal NF-AT DNA probe in binding buffer at room temperature for 20 min, and then incubated with supershift antibodies (2 μL at 2 μg/μL) for another 20 min followed by separation on a 4% polyacrylamide gel. *Lane 1*, free probe; *lane 2*, naïve cells; *lane 3*, PMA-stimulated cells; *lanes 4*, VH-pretreated and PMA-stimulated cells; *Lane 5 – 11*, CBN-pretreated and PMA-stimulated cells; *lane 6*, incubated with 50-fold molar excess of cold probe; *lane 7*, incubated with control ascites fluid; *lane 8*, incubated with anti-NF-ATc1 monoclonal antibody; *lane 9*, incubated with control rabbit IgG; *lane 10*, incubated with anti-c-fos rabbit IgG; *lane 11*, incubated with anti-c-jun/AP-1 rabbit IgG. The arrows on the right indicate the supershifted complexes induced by anti-NF-ATc1 and anti-c-fos. Results are representative of two independent experiments.


PMA (5 nM) - + + + + + + + + +
CBN (20 μM) - - VH + + + + + + +
Cold Probe - - - - + - - - - -
Control Ascites - - - - - + - - - -
Anti-NF-ATc1 - - - - - - + - - -
Control IgG - - - - - - - + - -
Anti-c-fos - - - - - - - - + -
Anti-c-jun - - - - - - - - - +
NF-AT
Lane 1 2 3 4 5 6 7 8 9 10 11

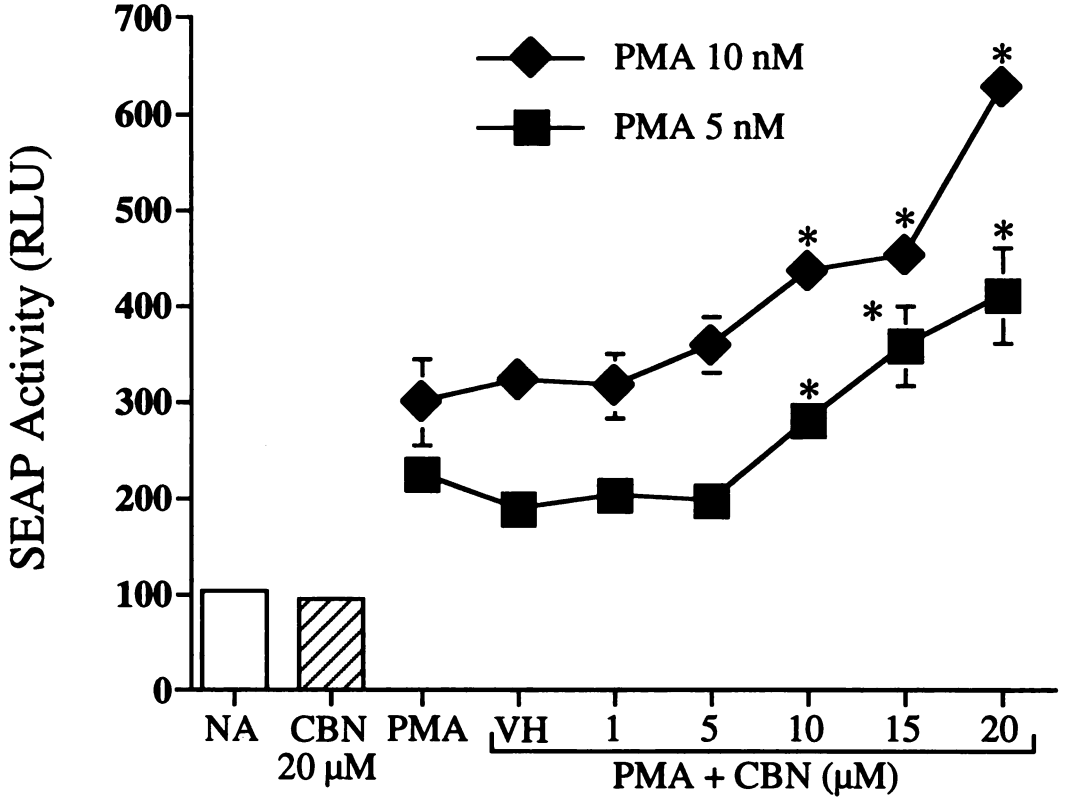
reporter gene assays. In this series of studies, EL4 cells were transiently transfected with the pNFAT-SEAP reporter gene whose expression is under the control of the IL-2 distal NF-AT site. The transfected cells were pretreated with CBN (1 – 20 μM) for 30 min followed by the sub-optimal activation with low concentrations of PMA (5 or 10 nM). The SEAP activity in the supernatants was measured after 48 hr of PMA stimulation. Consistent with the DNA binding results from EMSA studies, the transcriptional activity of pNFAT-SEAP reporter gene induced by PMA (5 and 10 nM) was significantly enhanced by pretreatment with CBN (10 – 20 μM) in a concentration-dependent manner (Fig. 22A). In addition, the same supernatants utilized for determination of SEAP activity were also subjected to measurement of IL-2 by ELISA. In accordance with the enhancement of pNFAT-SEAP reporter gene activity, the IL-2 protein secretion by the transfected EL4 cells was enhanced by CBN (10 – 20 μM) in a concentration-dependent manner (Fig. 22B).

G. Involvement of the ERK MAP kinases and CaM kinases on CBN-mediated enhancement of the NF-AT transcriptional activity

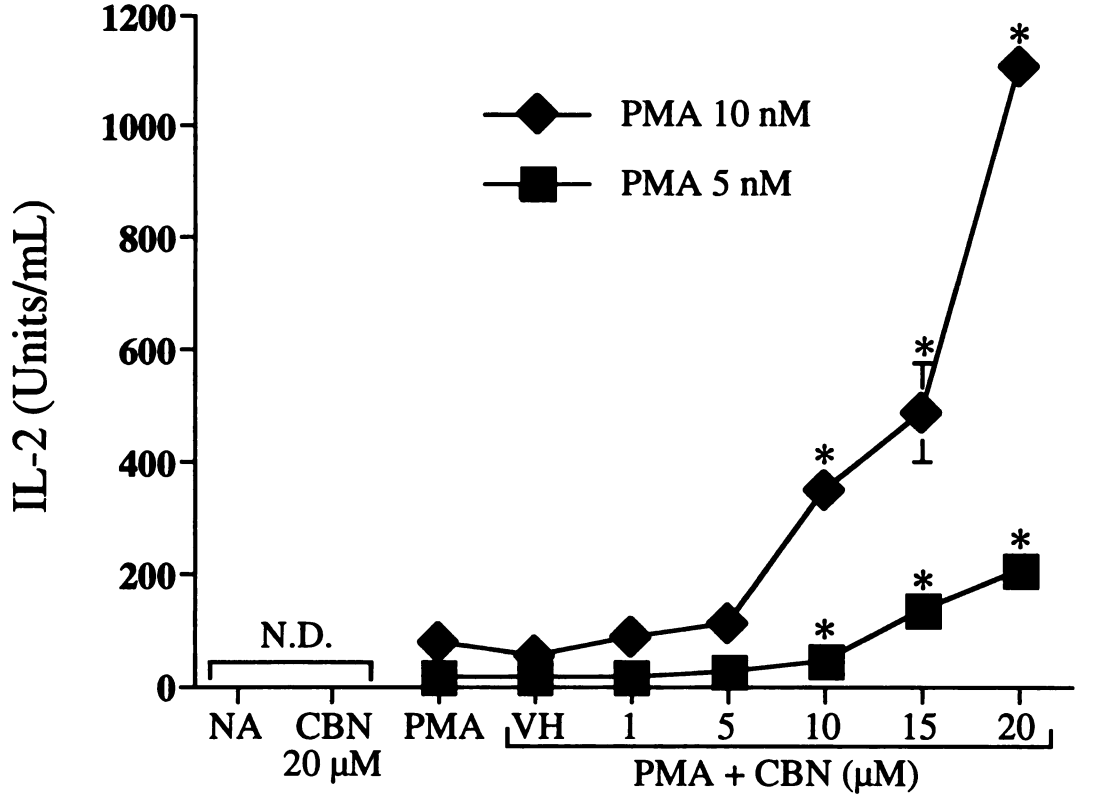
The transcriptional activation through the IL-2 distal NF-AT site requires cooperative interaction between AP-1 and NF-AT transcription factors. Because ERK MAP kinases are well-known upstream activators for the AP-1 associated fos family of proteins, coupled with the identification of AP-1 components in the IL-2 distal NF-AT binding (Fig. 21), experiments were designed to examine whether ERKs are involved in the transcriptional activation of the IL-2 distal NF-AT reporter gene. As illustrated in figure 23, the specific ERK kinase (MEK) inhibitor UO126 (Favata *et al.*, 1998) robustly

Figure 22. Effect of CBN on (A) IL-2 distal NF-AT reporter gene activity and (B) IL-2 protein secretion in sub-optimally activated EL4 cells. EL4 cells (2 x 10^5 cells/mL) were transiently transfected with pNFAT-SEAP reporter plasmids as described in the "Materials and Methods". The transfected cells were either untreated (NA) or pretreated with CBN (1 - 20 μM) and/or VH (0.1% ethanol) for 30 min followed by stimulation with PMA (5 or 10 nM) for 48 hr at 37°C. The activity of IL-2 and SEAP in the supernatants was quantified by ELISA and chemiluminescent assay, respectively. Data are expressed as the means ± standard error of triplicate cultures. *N.D.*, the IL-2 activity was below the level of quantification. *, $p < 0.05$ as compared to the matched VH group. Results are representative of three separate experiments.

(A) IL-2 Distal NF-AT Transcriptional Activity



(B) IL-2 Protein Secretion



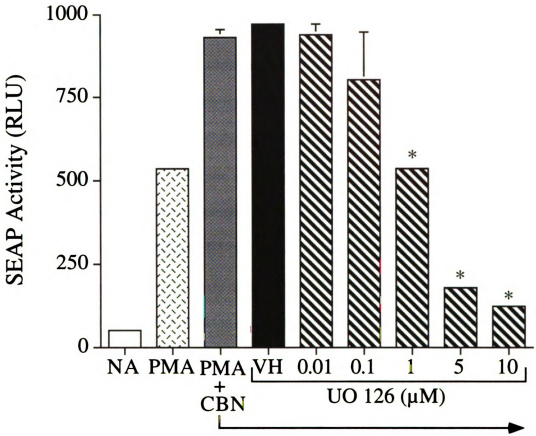


Figure 23. Attenuation by UO126 of CBN-mediated enhancement of IL-2 distal NF-AT reporter gene activity in sub-optimally activated EL4 cells. EL4 cells (2 x 105 cells/mL) were transiently transfected with pNFAT-SEAP plasmids. The transfected cells were either untreated (NA) or pretreated with CBN (15 μM) for 30 min in the absence or presence of UO126 (0.01 – 10 μM). Following the pretreatment, NA cells were left unstimulated and the others were stimulated with PMA (10 nM) for 48 hr at 37°C. The SEAP activity in the supernatants was quantified by chemiluminescent assay. Data are expressed as the means ± standard error of triplicate cultures. *, $p < 0.05$ as compared to the VH group. Results are representative of three separate experiments.

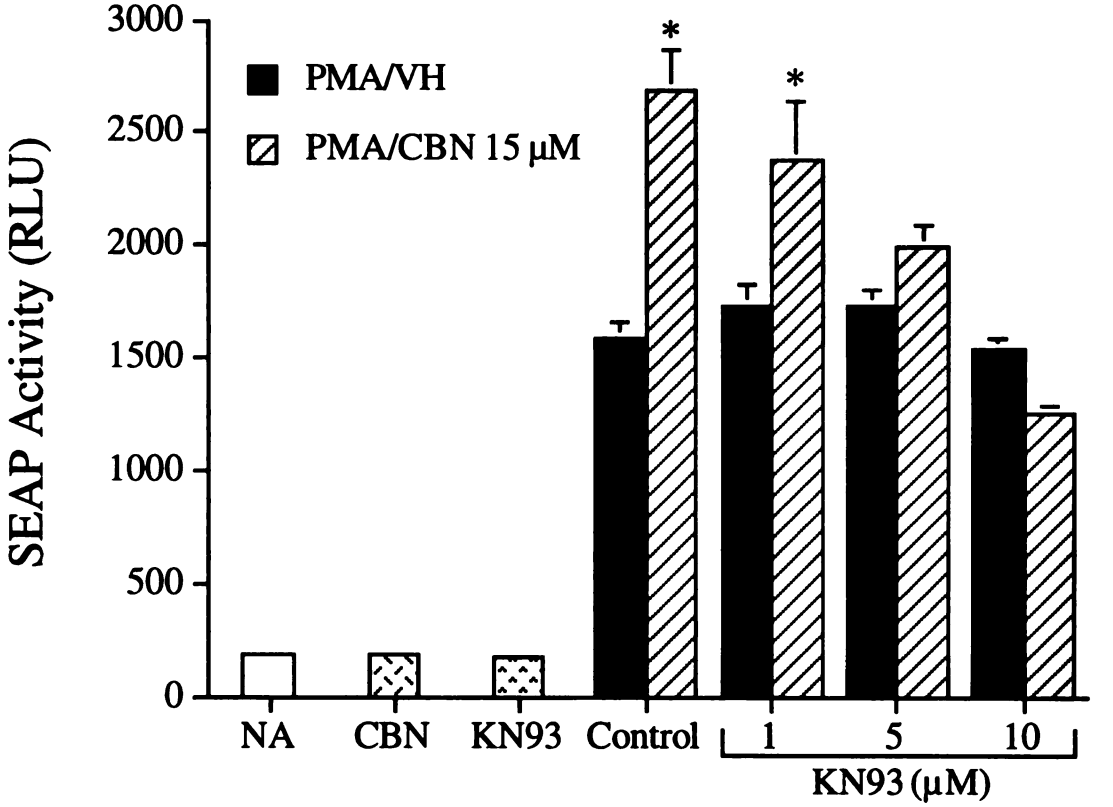
suppressed the pNFAT-SEAP activity in transfected EL4 cells pretreated with CBN (15 μM) and activated with PMA (10 nM). These observations are consistent with the previous results demonstrating a role of ERK MAP kinases in the CBN-mediated enhancement of IL-2 secretion. In addition to ERK MAP kinases, CaM kinases were also found to be involved in the CBN-mediated enhancement of IL-2 secretion (Fig. 17). In light of this, the effect of KN93, a CaM kinase inhibitor on CBN-mediated enhancement of the IL-2 distal NF-AT reporter gene activity was studied. Consistent with the previous finding, both CBN-mediated enhancement of the IL-2 distal NF-AT reporter gene activity and IL-2 secretion by transfected EL4 cells was completely attenuated by the presence of KN93 (1 – 10 μM) in a concentration-dependent manner (Fig 24). These results further implicate a role by ERK MAP kinases and CaM kinases in the enhancement by CBN of NF-AT and subsequently IL-2 expression.

H. Role of CB2 in CBN-mediated enhancement of IL-2 distal NF-AT transcriptional activity

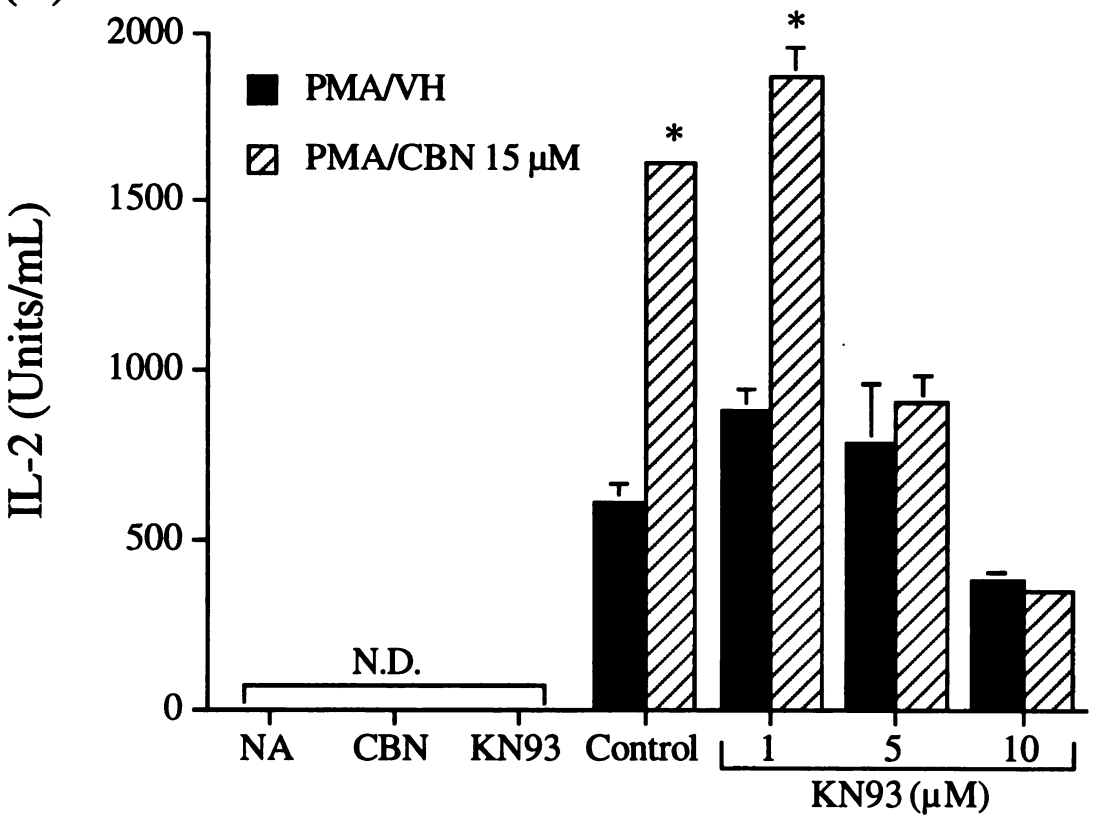
Cannabinoid compounds are ligands for cannabinoid receptors, CB1 and CB2, that are believed to mediate, at least some of cannabinoid-induced biological effects (Matsuda *et al.*, 1990; Munro *et al.*, 1993). In light of the fact that CB2 is predominantly expressed in the immune system (Munro *et al.*, 1993; Bouaboula *et al.*, 1996; Schatz *et al.*, 1997) and CB2 mRNA transcripts have been detected in EL4 cells (Condie *et al.*, 1996), the CB2 antagonists SR144528 was employed to investigate whether CB2 is involved in CBN-mediated enhancement of the IL-2 distal NF-AT transcriptional activity. Because the binding affinity of SR144528 to CB2 has been determined to be in

Figure 24. Reversal by KN93 of CBN-mediated enhancement of IL-2 distal NF-AT transcriptional activity and IL-2 protein secretion in sub-optimally activated EL4 cells. EL4 cells (2 x 10^5 cells/mL) were transiently transfected with pNFAT-SEAP plasmids. The transfected cells were either untreated (NA) or pretreated with CBN (15 μM) for 30 min in the absence (control) or presence of KN93 (0.1 – 10 μM). Following the pretreatment, NA cells were left unstimulated and the others were stimulated with PMA (10 nM) for 48 hr at 37°C. The activity of IL-2 and SEAP in the supernatants was quantified by ELISA and chemiluminescent assay, respectively. Data are expressed as the means ± standard error of triplicate cultures. *N.D.*, the IL-2 activity was below the level of quantification. *, $p < 0.05$ as compared to the matched PMA/VH group. Results are representative of three separate experiments.

(A) IL-2 Distal NF-AT Transcriptional Activity



(B) IL-2 Protein Secretion



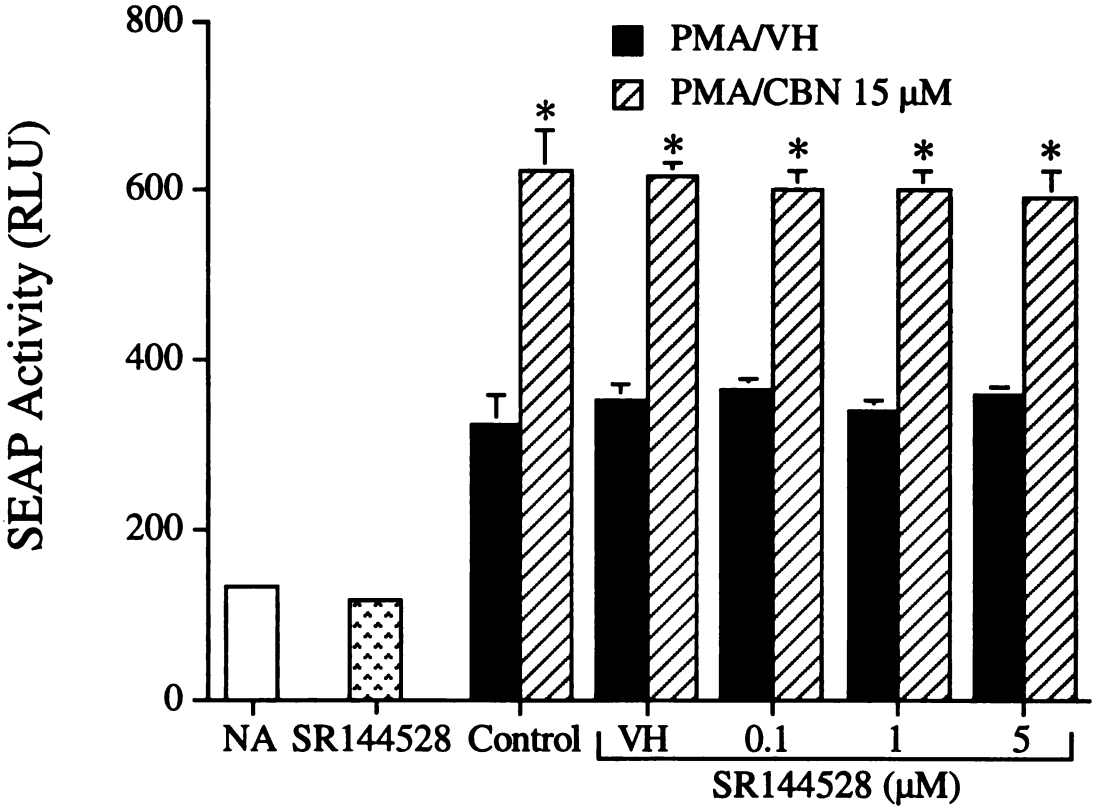
nanomolar range (Rinnaldi-Carmona *et al.*, 1998), a concentration range between 0.1 – 5 μM was employed to ensure the blockage of CB2 receptors. Notably, CBN-mediated enhancement of the IL-2 distal NF-AT transcriptional activity, as well as the enhancement of IL-2 secretion in transfected EL4 cells were not attenuated by the presence of SR144528 (Fig. 25). The role of cannabinoid receptors and the associated cAMP signaling pathway in CBN-mediated enhancement of IL-2 expression was further investigated using primary splenocytes in the following section.

I. CBN-mediated enhancement of IL-2 protein secretion is not sensitive to dibutyryl-cAMP and pertussis toxin

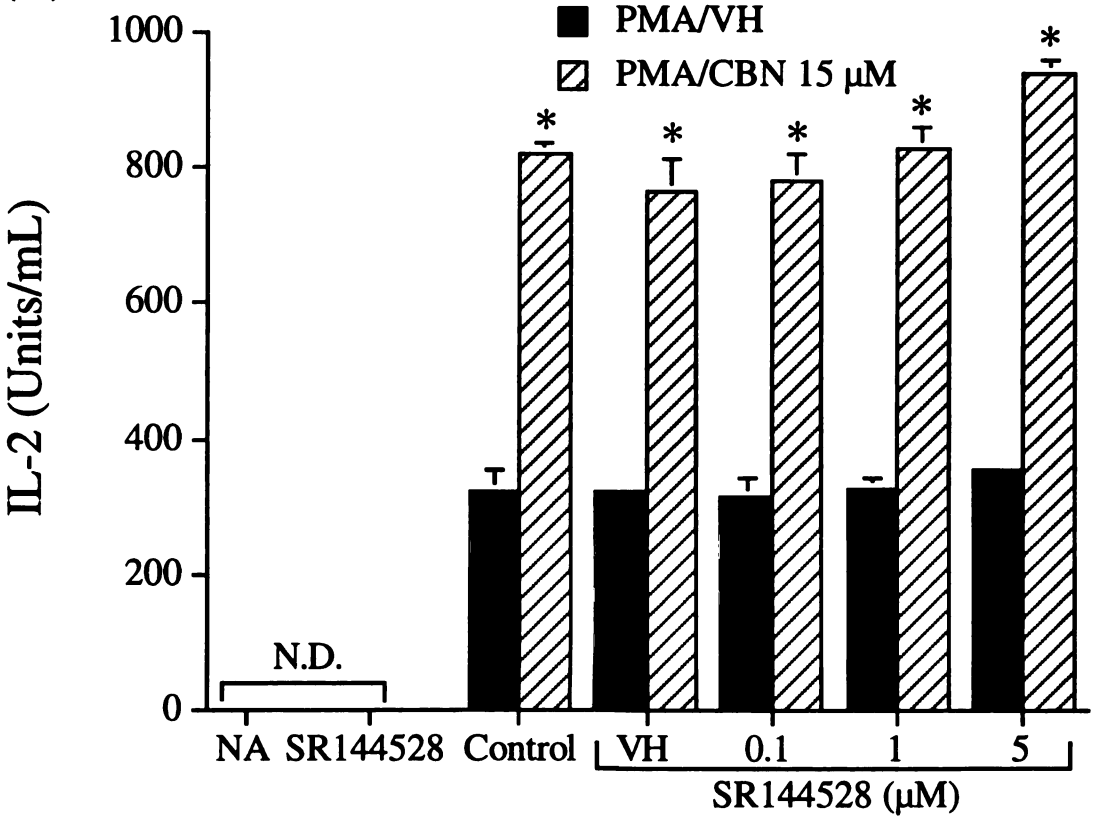
Cannabinoid receptors negatively couple to the pertussis toxin-sensitive GTP binding protein G_i/G_o to inhibit the adenylate cyclase-cAMP signaling cascade. To investigate the involvement of the GTP binding protein G_i/G_o and the cAMP signaling pathway in CBN-mediated enhancement of IL-2 secretion, dibutyryl-cAMP (a membrane-permeable cAMP analog), pertussis toxin and H89 (a protein kinase A inhibitor) were employed. Consistent with previous reports, control studies demonstrated that treatment of splenocytes with CBN (5 – 20 μM) for 30 min prior to T cell activation with sCD3/CD28 (2 μg/mL of each antibody) markedly enhanced the magnitude of IL-2 secretion in a concentration-dependent manner (Fig. 26). The same experiments were also conducted in the presence of dibutyryl-cAMP. Notably, the enhancing effect of CBN on IL-2 secretion was not reversed by co-treatment with dibutyryl-cAMP (1 and 10 μM; Fig. 26). At the highest concentration of dibutyryl-cAMP, 100 μM, IL-2 secretion was inhibited in all of the treatment groups (Fig. 26). Preincubation of splenocytes with

Figure 25. No reversal by SR144528 of CBN-mediated enhancement of IL-2 distal NF-AT transcriptional activity and IL-2 protein secretion in sub-optimally activated EL4 cells. EL4 cells (2 x 10^5 cells/mL) were transiently transfected with pNFAT-SEAP plasmids. The transfected cells were either untreated (NA) or pretreated with CBN (15 μM) for 30 min in the absence (VH; 0.05% DMSO) or presence of SR144528 (0.1 – 5 μM). Following the pretreatment, NA cells were left unstimulated and the others were stimulated with PMA (10 nM) for 48 hr at 37°C. The activity of IL-2 and SEAP in the supernatants was quantified by ELISA and chemiluminescent assay, respectively. Data are expressed as the means ± standard error of triplicate cultures. *N.D.*, the IL-2 activity was below the level of quantification. *, $p < 0.05$ as compared to the matched PMA/VH group. Results are representative of three separate experiments.

(A) IL-2 Distal NF-AT Transcriptional Activity



(B) IL-2 Protein Secretion



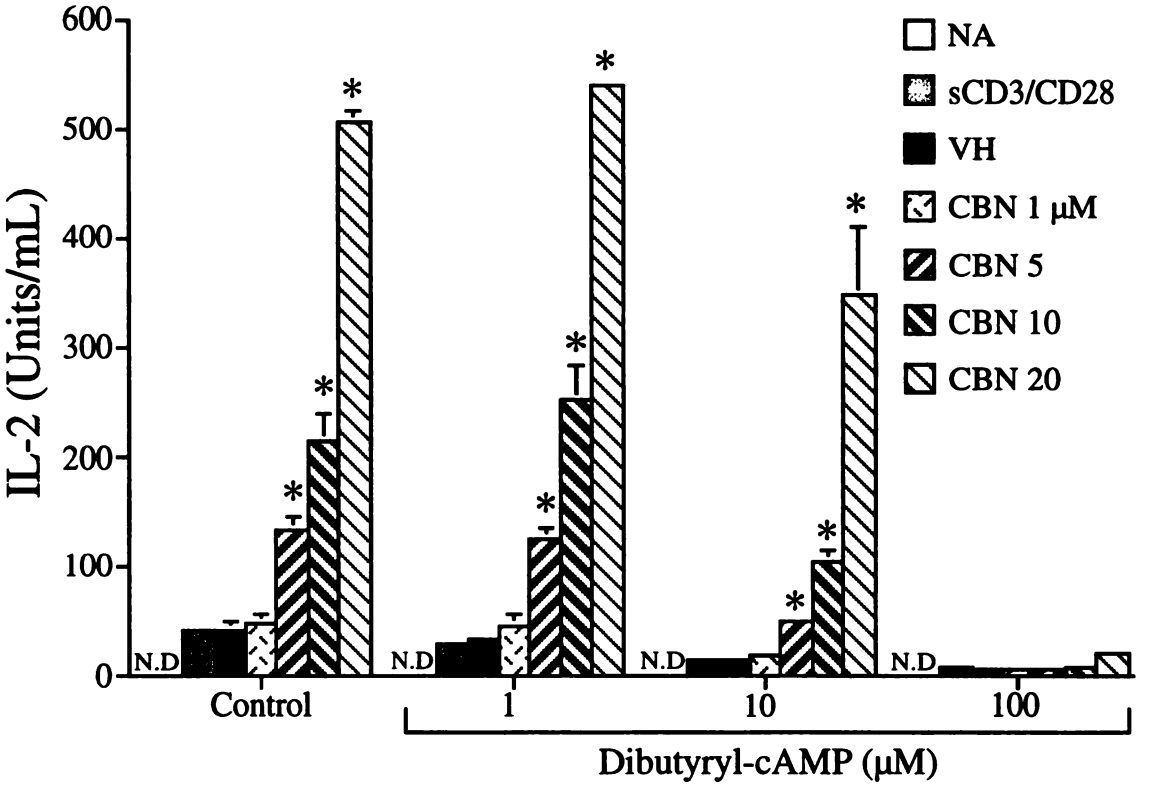


Figure 26. No reversal of CBN-mediated enhancement of sCD3/CD28-induced IL-2 secretion by dibutyryl-cAMP. Splenocytes (2 x 10^6 cells/mL) were either untreated (NA) or pretreated with CBN (1 - 20 μM) and/or VH (0.1% ethanol) for 30 min in the absence (control group) or presence of dibutyryl-cAMP (1 - 100 μM). Following the pretreatment, NA cells were left unstimulated and the others were stimulated with sCD3/CD28 (2 μg/mL of each mAb) for 48 hr at 37°C. IL-2 in the supernatants was quantified by ELISA. Data are expressed as the means ± standard error of triplicate cultures. *, $p < 0.05$ as compared to the VH control group. *N.D.*, IL-2 protein was below the level of quantification. Results are representative of two separate experiments.

increasing concentrations of pertussis toxin (1 – 100 ng/mL) for 24 hr prior to CBN treatment and sCD3/CD28 activation also failed to reverse the CBN-mediated enhancement of IL-2 secretion (Fig. 27). These results suggest that G_i/G_o and the cAMP pathway are either not involved or not solely responsible for CBN-mediated enhancement of the IL-2 secretion by sCD3/CD28-activated splenocytes. To further substantiate these observations, the role of the cAMP signaling cascade in sCD3/CD28-induced IL-2 secretion was further investigated using the specific protein kinase A (PKA) inhibitor H89. As the reported IC_{50} for inhibition of PKA by H89 is 48 nM (Chijiwa *et al.*, 1990), a concentration range between 10 nM – 1 μM was employed. In accordance with above results, H89 failed to exhibit any marked effect on the magnitude of IL-2 secretion by sCD3/CD28-activated splenic T cells (Fig. 28).

J. CBN-mediated enhancement of IL-2 protein secretion is not reversed by cannabinoid receptor antagonists

As both CB1 and CB2 mRNA transcripts have been detected in primary splenocytes (Schatz et al., 1997), receptor antagonists for CB1 and CB2, SR141716A and SR144528 respectively, were employed in combination to investigate their ability to attenuate CBN-mediated enhancement of IL-2 secretion by splenocytes. Interestingly, pretreatment of splenocytes with SR141716A in combination with SR144528 (0.1 - 1 μM of each antagonist) 15 min prior to CBN treatment did not attenuate CBN-mediated enhancement of IL-2 secretion (Fig. 29). The effect of SR141716A and SR144528 alone on sCD3/CD28-induced IL-2 secretion by splenocytes was also examined. Both antagonists at concentrations ≤ 1 μM exhibited no effects on sCD3/CD28-induced IL-2

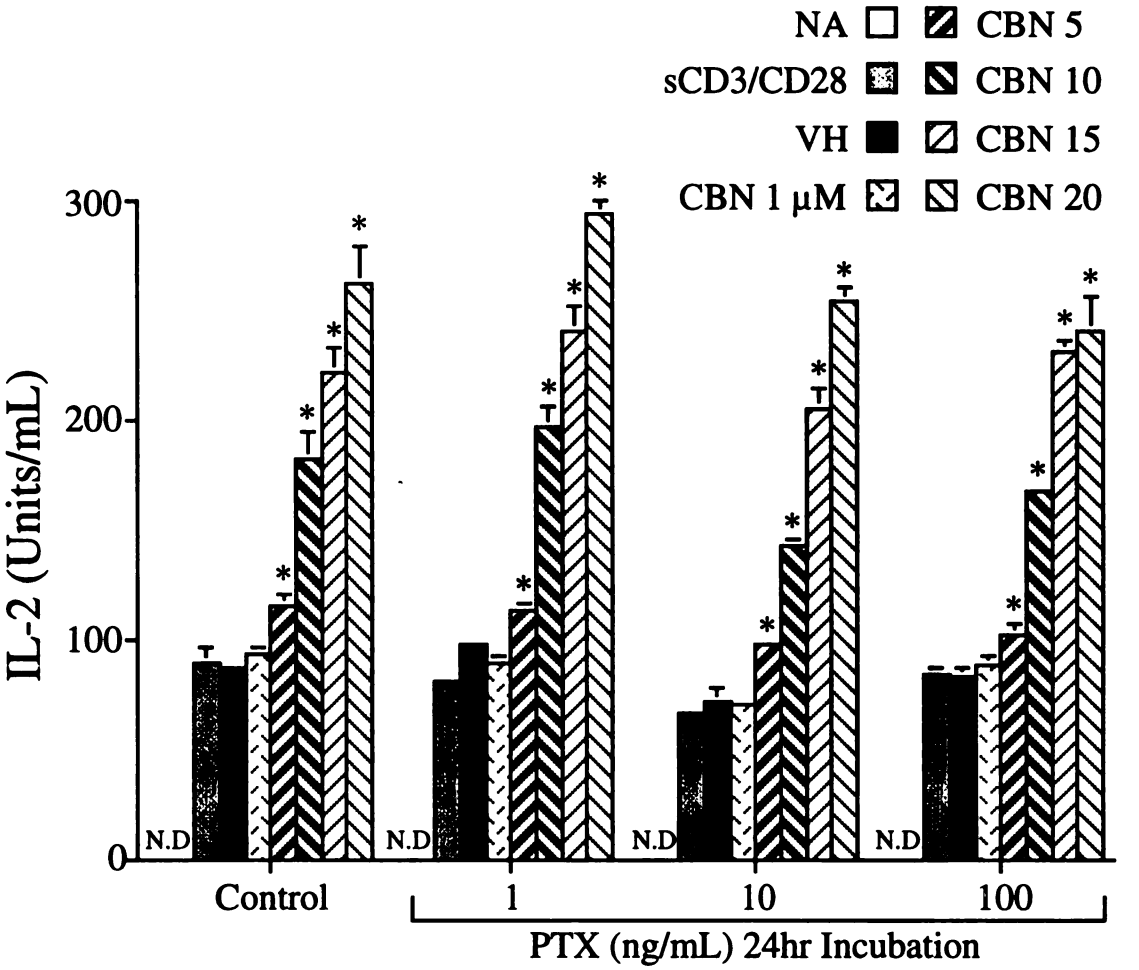


Figure 27. **No reversal of CBN-mediated enhancement of sCD3/CD28-induced IL-2 secretion by pertussis toxin (PTX).** Splenocytes (2 x 10^6 cells/mL) were incubated with the culture medium (control group) or PTX (1 - 100 ng/mL) for 24 hr. After PTX incubation, cells were washed twice with fresh medium and adjusted to 2 x 10^6 cells/mL. Cells in each group were then left untreated (NA) or pretreated with CBN (1 - 20 μM) and/or VH (0.1% ethanol) for 30 min followed by stimulation with sCD3/CD28 (2 μg/mL of each mAb) for 48 hr at 37°C. IL-2 in the supernatants was quantified by ELISA. Data are expressed as the means ± standard error of triplicate cultures. *, $p < 0.05$ as compared to the matched VH control group. *N.D.*, IL-2 protein was below the level of quantification. Results are representative of two separate experiments.

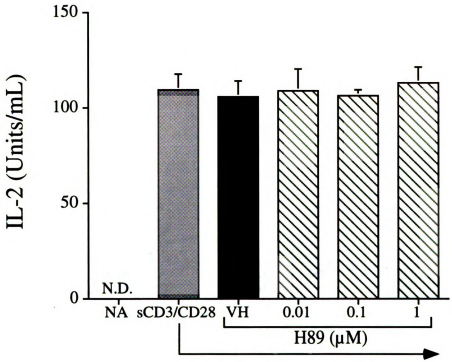


Figure 28. The effect of H89 on IL-2 secretion by sCD3/CD28-activated splenocytes. Splenocytes (2 x 10^6 cells/mL) were either untreated (NA) or pretreated with H89 (0.01 - 1 μM) and/or VH (0.01% DMSO) for 30 min. Following the pretreatment, NA cells were left unstimulated and the others were stimulated with sCD3/CD28 (2 μg/mL of each antibody) for 48 hr at 37°C. IL-2 in the supernatants was quantified by ELISA. Data are expressed as the means ± standard error of triplicate cultures. *N.D.*, IL-2 protein was below the level of quantification. Results are representative of two separate experiments.

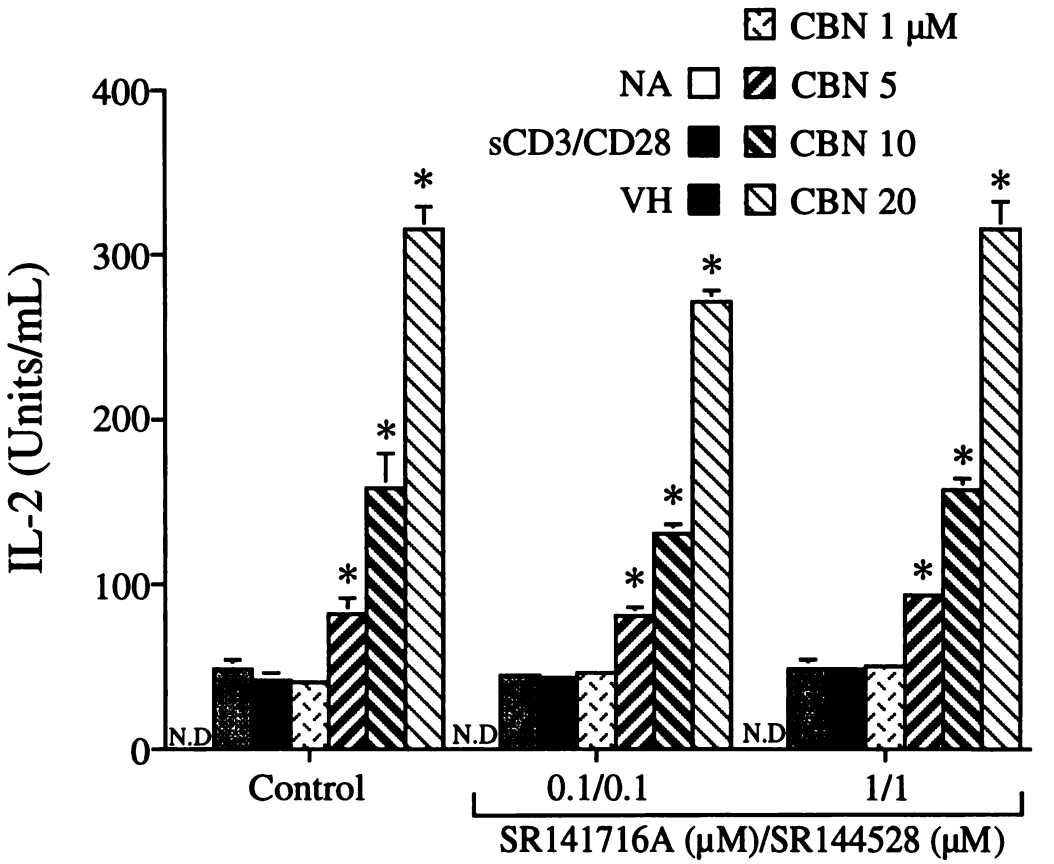


Figure 29. No reversal of CBN-mediated enhancement of sCD3/CD28-induced IL-2 secretion by CB1 and CB2 antagonists (SR141716A and SR144528). Splenocytes (2 x 10^6 cells/mL) were either untreated (NA) or pretreated with CBN (1 - 20 μM) and/or VH (0.1% ethanol) for 30 min in the absence (control group) or presence of SR141716A plus SR144528 (0.1 or 1 μM of each antagonist). Following the pretreatment, NA cells were left unstimulated and the others were stimulated with sCD3/CD28 (2 μg/mL of each) for 48 hr at 37°C. IL-2 in the supernatants was quantified by ELISA. Data are expressed as the means ± standard error of triplicate cultures. *, $p < 0.05$ as compared to the matched VH control group. *N.D.*, IL-2 protein was below the level of quantification. Results are representative of two separate experiments.

secretion. However, at 5 and 10 μM both SR141716A and SR144528 augmented the magnitude of IL-2 secretion by sCD3/CD28-activated splenic T cells (Fig. 30). These data indicate that SR141716A and SR144528 alone modestly potentiated the IL-2 responses under the present experimental condition and suggest that they may be acting as partial agonists at higher concentrations.

K. The effect of cannabidiol, CP55,940, WIN55212-2 and WIN55212-3 on IL-2 protein secretion by sCD3/CD28-activated splenocytes

To further explore the potential involvement of CB1 and/or CB2 cannabinoid receptors in the CBN-mediated enhancement of IL-2 secretion by splenocytes, comparative experiments using several model cannabinoid compounds, specifically, two structurally related cannabinoid compounds, cannabidiol and CP55,940, which exhibit low and high affinities to cannabinoid receptors, respectively, were preformed. Despite marked differences in the binding affinity to cannabinoid receptors between cannabidiol and CP55,940, both were similarly effective in enhancing IL-2 secretion by sCD3/CD28-activated splenocytes under identical assay conditions utilized with CBN (Fig 31). The observed effective concentration range of these two cannabinoid compounds was between 5 – 20 μM which was comparable to that of CBN (Fig. 31). In addition to cannabidiol and CP55,940, the effect of stereo isomers of WIN55212-2 and WIN55212-3 on IL-2 secretion by sCD3/CD28-activated splenic T cells was also examined. As illustrated in figure 32, both compounds were capable of enhancing the secretion of IL-2 by splenocytes, though the efficacy of these two enantiomers was lower than that of CBN. The maximum enhancement of IL-2 secretion induced by either WIN55212

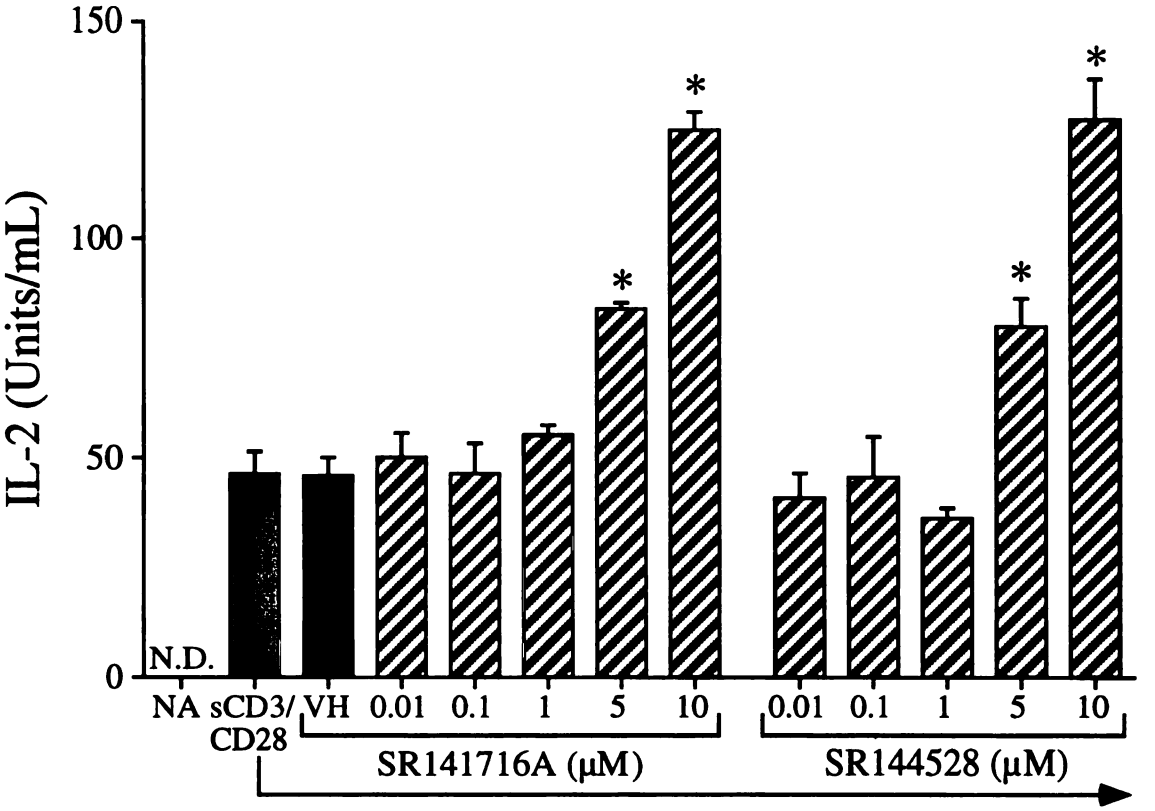
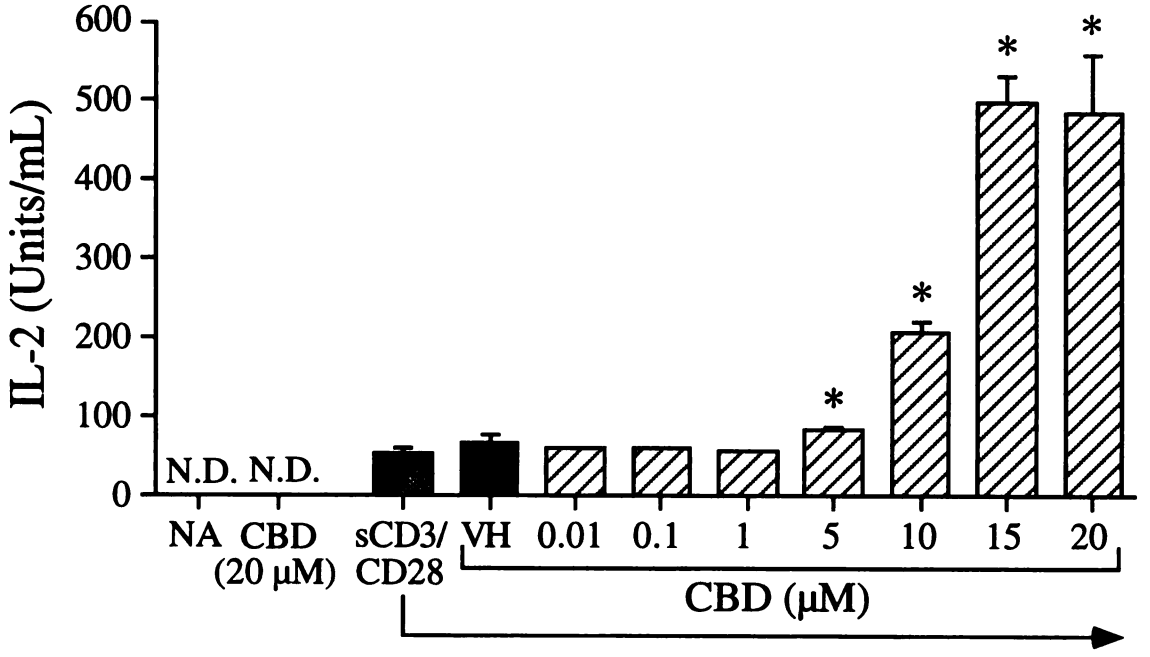


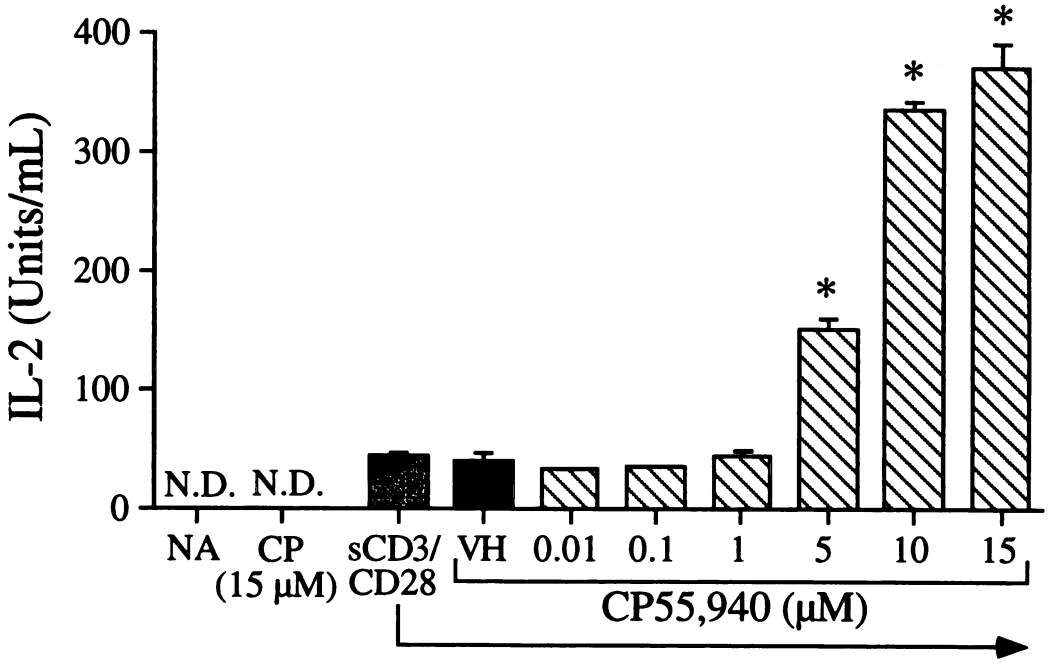
Figure 30. The effect of SR141716A and SR144528 on IL-2 secretion by sCD3/CD28-activated splenocytes. Splenocytes (2 x 10^6 cells/mL) were either untreated (NA) or pretreated with SR141716A (0.01 - 10 μM), SR144528 (0.01 – 10 μM) and/or VH (0.1% DMSO) for 30 min. Following the pretreatment, NA cells were left unstimulated and the others were stimulated with sCD3/CD28 (2 μg/mL of each antibody) for 48 hr at 37°C. IL-2 in the supernatants was quantified by ELISA. Data are expressed as the means ± standard error of triplicate cultures. *, $p < 0.05$ as compared to the VH control group. *N.D.*, IL-2 protein was below the level of quantification. Results are representative of two separate experiments.

Figure 31. Concentration-dependent enhancement by cannabidiol (CBD) and CP55,940 of IL-2 secretion by sCD3/CD28-activated splenocytes. (A) Splenocytes (2 x 10^6 cells/mL) were either untreated (NA), or pretreated with CBD (0.01 - 20 μM) and/or VH (0.1% ethanol) for 30 min followed by activation with sCD3/CD28 (2 μg/mL of each antibody). (B) Splenocytes (2 x 10^6 cells/mL) were either untreated (NA), or pretreated with CP55,940 (0.01 - 15 μM) and/or VH (0.15% DMSO) for 30 min followed by activation with sCD3/CD28 (2 μg/mL of each antibody). After 48 hr of culture, supernatants were harvested and IL-2 was assayed by ELISA. Data are expressed as the mean ± standard error of triplicate cultures. *, $p < 0.05$ as compared to the VH control group. *N.D.*, IL-2 protein was below the level of quantification. Results are representative of three independent experiments.

(A)


600
500
400
300
200
100
0
IL-2 (Units/mL)
N.D. N.D.
*
*
*
*
NA
CBD (20 μM)
sCD3/ CD28
VH
0.01
0.1
1
5
10
15
20
CBD (μM)

(B)


400
300
200
100
0
IL-2 (Units/mL)
N.D. N.D.
*
*
*
NA
CP (15 μM)
sCD3/ CD28
VH
0.01
0.1
1
5
10
15
CP55,940 (μM)

enantiomer is approximately 40% of that induced by 10 μM of CBN (Fig. 32). The observed peak of the enhancing effect on IL-2 secretion by the high affinity cannabinoid receptor ligand WIN55212-2 was between 5 and 10 μM. In contrast, WIN55212-3 was less active than WIN55212-2 at 5 μM; however, WIN55212-3 modestly enhanced sCD3/CD28-induced IL-2 at higher concentrations in a concentration dependent manner (10 – 20 μM; Fig. 32).

II. Cannabinoid-mediated inhibition of IL-4 expression by T cells

A. Inhibition of IL-4 protein secretion and steady state mRNA expression by cannabinoids

Based on the sensitivity of T cell-dependent humoral immune responses to inhibition by cannabinoids and the important role IL-4 plays in B cell differentiation, the effect of two immunomodulatory cannabinoids, Δ^9-THC and CBN, were investigated on IL-4 protein secretion in primary splenocytes and in EL4 T cell line. Splenocytes were activated with either soluble or immobilized αCD3 in the absence or presence of αCD28 to induce IL-4 protein secretion. As illustrated in figure 33, all the employed stimuli were able to induce IL-4 secretion by splenocytes, and more importantly, the magnitude of IL-4 secretion induced by the various stimuli was inhibited by pretreatment of splenocytes with 15 μM of CBN. The CBN-mediated inhibition of IL-4 secretion by sCD3/CD28-activated splenocytes was concentration-dependent within 5 – 20 μM (Fig. 33B). Likewise, CBN also produced a concentration-dependent inhibition of IL-4 secretion by EL4 cells stimulated with Io (1 μM) alone or in combination with PMA (80 nM; Fig. 34). In addition to CBN, the effect of Δ^9-THC on IL-4 secretion was also

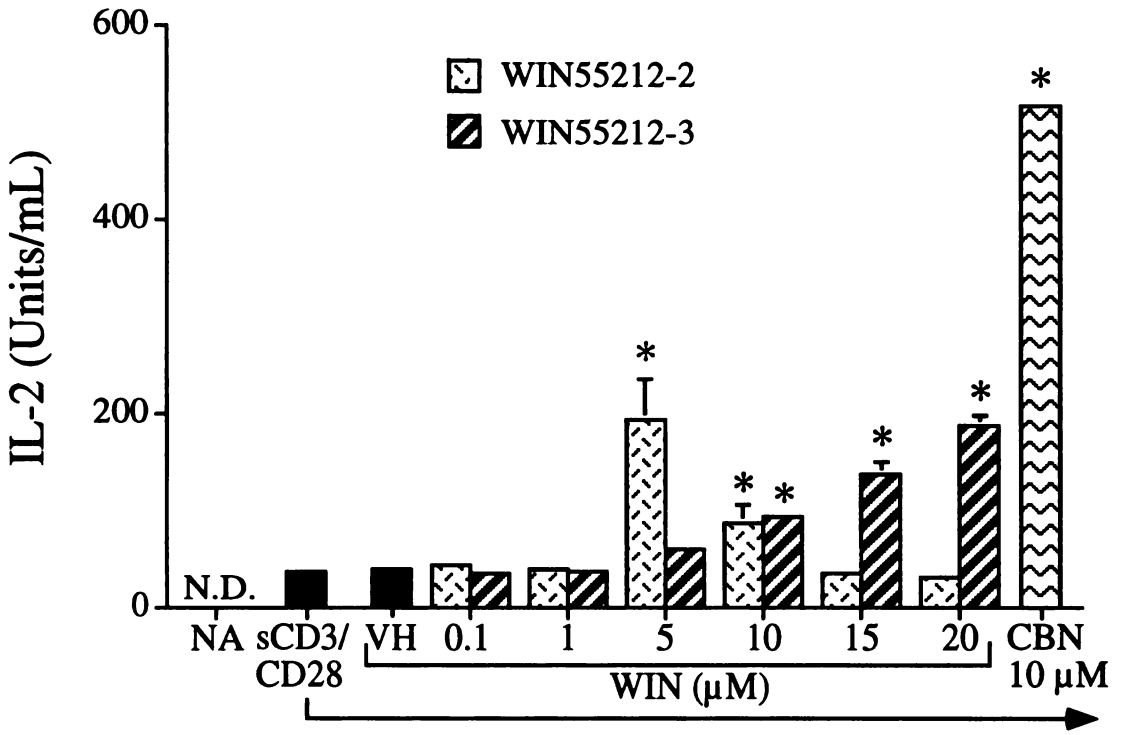
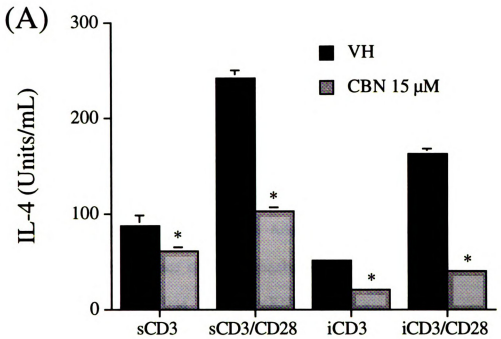
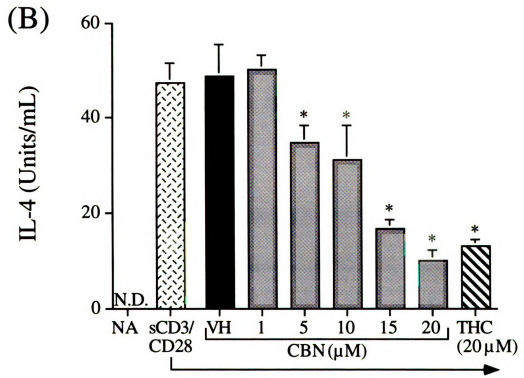


Figure 32. The effects of WIN55212-2 and WIN55212-3 on IL-2 secretion by sCD3/CD28-activated splenocytes. Splenocytes (2 x 10^6 cells/mL) were pretreated with WIN55212-2 (0.1 - 20 μM), WIN55212-3 (0.1 – 20 μM), CBN (10 μM), and/or VH (0.1% ethanol) for 30 min followed by activation with sCD3/CD28 (2 μg/mL of each antibody). After 48 hr of culture, supernatants were harvested and IL-2 was assayed by ELISA. Data are expressed as the mean ± standard error of triplicate cultures. *, $p < 0.05$ as compared to the VH control group. Results are representative of three independent experiments.

Figure 33. Inhibition by CBN and Δ^9-THC of IL-4 protein secretion by splenocytes. (A). Splenocytes (2 x 10^6 cells/ml) were either pretreated with CBN (15 μM) and/or VH (0.1% ethanol) for 30 min followed by stimulation with soluble anti-CD3 (sCD3; 2 μg/mL), soluble anti-CD3 plus anti-CD28 (sCD3/CD28; 2 μg/mL of each antibody), immobilized anti-CD3 alone (iCD3), or immobilized anti-CD3 plus anti-CD28 (iCD3/CD28; 2 μg/mL). (B) Splenocytes (2 x 10^6 cells/ml) were either untreated (NA), or pretreated with CBN (1 - 20 μM), Δ^9-THC (20 μM) and/or VH (0.1% ethanol) for 30 min followed by stimulation with sCD3/CD28. Splenocytes were cultured for 48 hr at 37°C and the culture supernatants harvested. The supernatants were assayed for IL-4 by ELISA. Data are expressed as the mean ± standard error of triplicate cultures. *, $p <$ 0.05 as compared to the VH control group. *N.D.*, IL-4 protein was below the level of quantification. Results are representative of three independent experiments.


(A)
IL-4 (Units/mL)
300
200
100
0
VH
CBN 15 μM
*
sCD3
sCD3/CD28
iCD3
iCD3/CD28

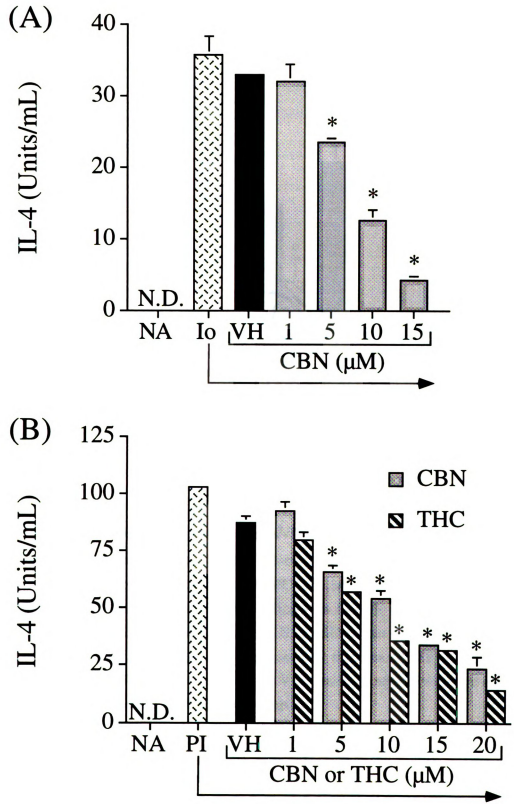

(B)
IL-4 (Units/mL)
60
40
20
0
N.D.
*
NA
sCD3/
CD28
VH
1
5
10
15
20
THC
(20μM)
CBN(μM)

examined. Δ^9-THC inhibited IL-4 secretion by sCD3/CD28-activated splenocytes or PMA/Io-activated EL4 cells (Fig. 33B, 34B). To further characterize cannabinoid-mediated inhibition of IL-4 expression by T cells, the effect of CBN and Δ^9-THC on the steady state IL-4 mRNA expression by EL4 cells was examined. Kinetic studies demonstrated that pretreatment of EL4 cells with Δ^9-THC (20 μM), prior to activation with PMA/Io, produced a marked inhibition of IL-4 steady state mRNA at all the time points tested (1, 3, 6 and 12 hr; Fig. 35). Additional concentration response studies were performed in the presence of both cannabinoids (1 – 20 μM) after a 6 hr PMA/Io stimulation, the peak time of IL-4 mRNA expression. Both Δ^9-THC and CBN diminished IL-4 steady state mRNA expression by EL4 cells in a concentration-dependent manner (Fig 36). Both Δ^9-THC and CBN exhibited comparable potency and efficacy. Moreover, a good correlation existed between the effective concentration range required for inhibition of mRNA expression and protein secretion for both cannabinoids (5 – 20 μM; Fig. 34 and 36). No effect on cell viability was observed at any of the cannabinoid concentrations utilized in the present studies as assessed by trypan blue exclusion.

B. Inhibition of P0 DNA binding by cannabinoids

To date, five NF-AT binding sites named P elements have been identified in the proximal promoter region of the murine IL-4 gene (Szabo *et al.*, 1993). These *cis*-acting P elements have been demonstrated to be critical for IL-4 transcription and confer the calcium sensitivity of the IL-4 gene (Szabo *et al.*, 1993). Studies were designed to investigate whether Δ^9-THC and/or CBN influenced the DNA binding of nuclear proteins

Figure 34. Concentration-dependent inhibition by CBN and Δ^9-THC of IL-4 protein secretion by EL4 cells. (A) EL4 cells (2 x 10^5 cells/ml) were either untreated (NA), or pretreated with CBN (1 – 15 μM) and/or VH (0.1% ethanol) for 30 min followed by stimulation with Io (1 μM). (B) EL4 cells (2 x 10^5 cells/ml) were either untreated (NA), or pretreated with CBN (1 – 20 μM), Δ^9-THC (1 - 20 μM) and/or VH (0.1% ethanol) for 30 min followed by stimulation with PMA/Io (80 nM/1 μM). EL4 cells were cultured for 24 hr at 37°C and the culture supernatants harvested. The supernatants were assayed for IL-4 by ELISA. Data are expressed as the mean ± standard error of triplicate cultures. *, $p < 0.05$ as compared to the VH control group. *N.D.*, IL-4 protein was below the level of quantification. Results are representative of three independent experiments.


(A)
IL-4 (Units/mL)
40
30
20
10
0
N.D.
NA
Io
VH
1
5
10
15
CBN (μM)
(B)
IL-4 (Units/mL)
125
100
75
50
25
0
N.D.
CBN
THC
NA
PI
VH
1
5
10
15
20
CBN or THC (μM)

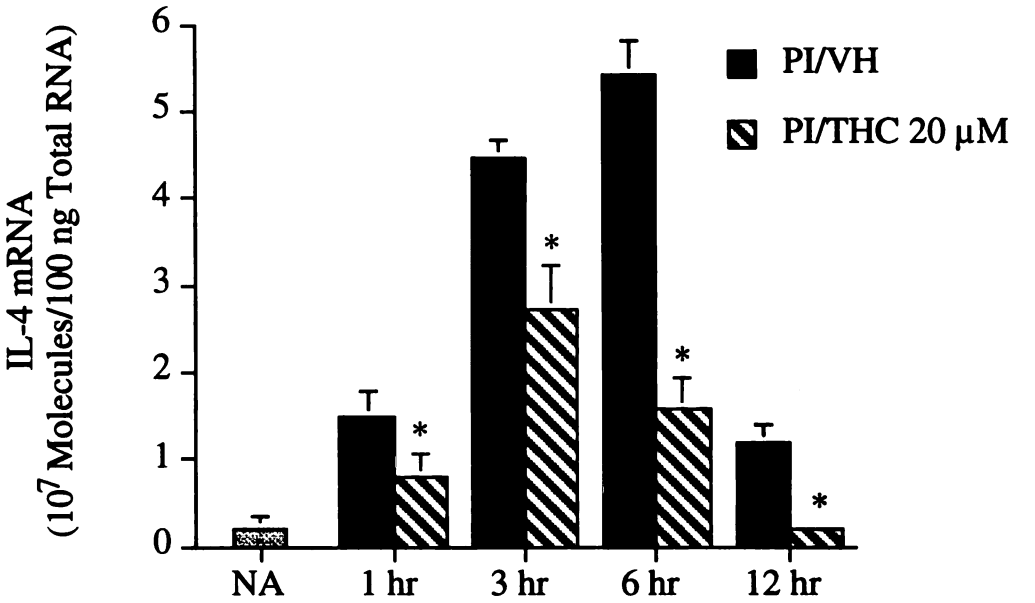


Figure 35. Time course analysis of Δ^9-THC-mediated inhibition of IL-4 mRNA expression in EL4 cells. EL4 cells (2 x 10^5 cells/ml) were either untreated (NA), or pretreated with Δ^9-THC (20 μM) and/or vehicle (VH; 0.1% ethanol) for 30 min followed by stimulation with PMA/Io (80 nM/1 μM) for 1 - 12 hr. The total RNA was isolated and IL-4 mRNA was determined by competitive RT-PCR. The data are expressed as the mean ± SE of triplicate cultures. *, $p < 0.05$ as compared to the VH control group. Results are representative of three independent experiments.

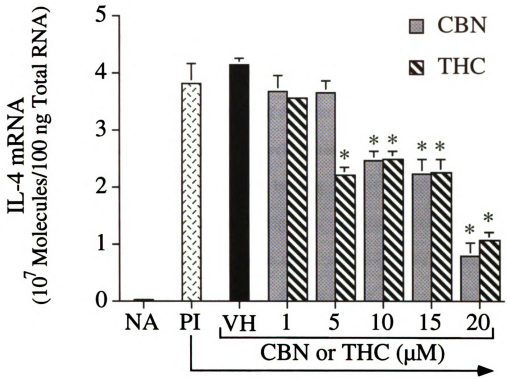


Figure 36. Concentration-dependent inhibition by CBN and Δ^9-THC of IL-4 mRNA expression in EL4 cells. EL4 cells (2 x 10^5 cells/ml) were either untreated (NA), or pretreated with cannabinoids (CBN or Δ^9-THC; 1 – 20 μM) and/or VH (0.1% ethanol) for 30 min followed by stimulation with PMA/Io (80 nM/1 μM) for 6 hr. The total RNA was isolated and IL-4 mRNA was determined by competitive RT-PCR. The data are expressed as the mean ± SE of triplicate cultures. *, $p < 0.05$ as compared to the VH control group. Results are representative of three independent experiments.

to the P0 element in the IL-4 promoter. Initially EMSA time course studies revealed that PMA/Io activation of EL4 cells for 1 – 4 hr markedly induced DNA binding activity to the P0 motif. Maximal P0 binding occurring at approximately 2 hr (data not shown). The 2 hr time point was then selected to examine the effect of Δ^9-THC and CBN on P0 DNA binding activity. Although multiple binding complexes were resolved using the P0 probe as previously reported (Hodge *et al.*, 1995), only one major PMA/Io-inducible binding complex was observed (Fig. 37). In light of this, our investigation primarily focused on this major PMA/Io-inducible complex. Cyclosporin A, which was used as a positive control for the inhibition of NF-AT DNA binding, readily inhibited PMA/Io-induced DNA binding activity to the P0 site (Fig 37). At the lowest Δ^9-THC (1 μM) concentration tested, which also produced no effect on IL-4 expression, PMA/Io-induced P0 DNA binding was modestly enhanced. Conversely, Δ^9-THC (10 and 20 μM) and CBN (20 μM) at concentrations where IL-4 expression was inhibited at the mRNA and protein level, P0 DNA binding activity was concordantly diminished. To further investigate possible mechanisms underlying cannabinoid-mediated suppression of the P0 binding, the protein components in the major PMA/Io-inducible P0 DNA binding complex were characterized employing antibodies directed against components of AP-1 transcription factors (i.e., c-fos and c-jun family members) or NF-ATc1 (NF-AT2). As shown in figure 38, both AP-1 associated proteins and NF-ATc1 were identified in the P0 binding complex as evidenced by the fact that anti-c-fos, anti-c-jun/AP-1 polyclonal rabbit IgG, and anti-NF-ATc1 mouse monoclonal antibody inhibited the PMA/Io-induced P0 binding complex. In contrast, control rabbit IgG (control for anti-c-fos and anti-c-jun

Figure 37. Inhibition of P0 NF-AT binding by Δ^9-THC, CBN and cyclosporin A. EL4 cells (2 x 10^5 cells/ml) were either untreated, or pretreated with Δ^9-THC (1, 5, 10, and 20 μM), CBN (20 μM), cyclosporin A (CsA; 1 μM), and/or VH (0.1% ethanol) for 30 min followed by stimulation with PMA/Io (80 nM/1 μM). EL4 cells were cultured for 2 hr at 37°C and nuclear proteins were isolated as described in Materials and Methods. Nuclear proteins (5 μg) were incubated with 0.4 μg of poly(dI-dC) and ^{32}P-labeled P0 NF-AT DNA probe in binding buffer at room temperature for 20 min followed by separation on a 4% polyacrylamide gel. The arrow indicates the major PMA/Io-inducible binding complex with the relative intensity values for this complex provided at the bottom of each lane. *Lane 1*, free probe; *lane 2*, naïve cells; *lanes 3 – 12*, PMA/Io-stimulated cells. The competitor lanes included 15-fold (*lane 11*) or 75-fold (*lane 12*) molar excess of the unlabeled P0 DNA probe as competitors. Results are representative of three independent experiments.

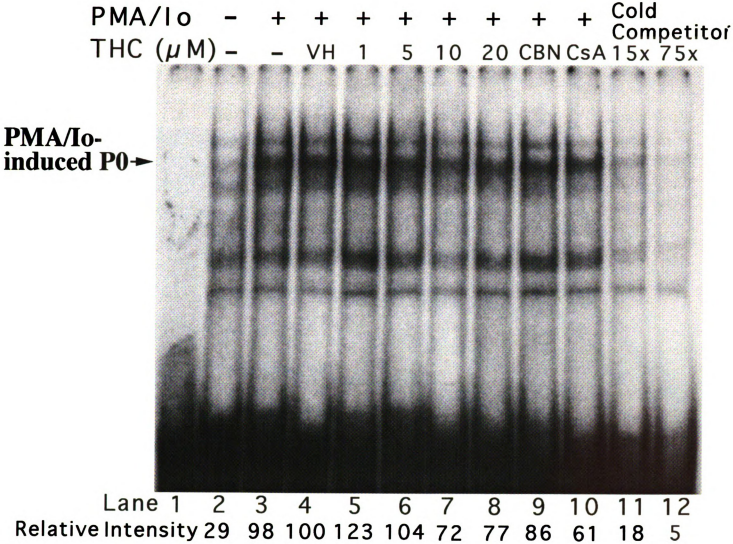
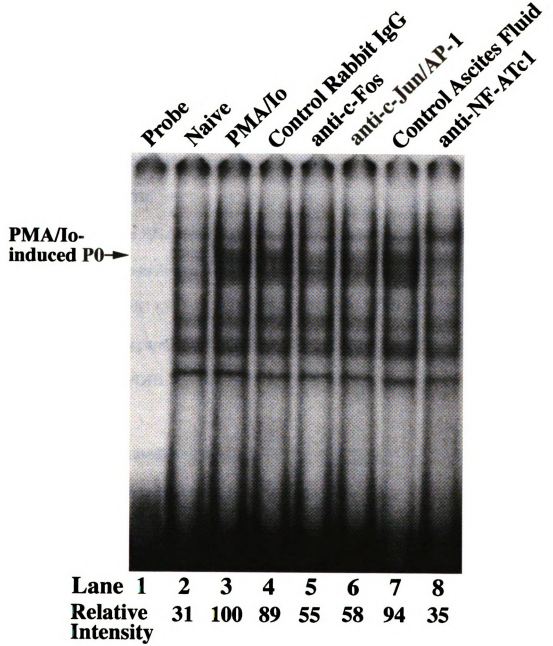

PMA/Io - + + + + + + + + Cold Competitor
THC (μM) - - VH 1 5 10 20 CBN CsA 15x 75x
PMA/Io-induced P0
Lane 1 2 3 4 5 6 7 8 9 10 11 12
Relative Intensity 29 98 100 123 104 72 77 86 61 18 5

Figure 38. Identification of protein components binding to the P0 NF-AT motif. EL4 cells (2 x 10^5 cells/ml) were untreated or stimulated with PMA/Io (80 nM/1 μM) for 2 hr at 37°C. The nuclear proteins were isolated as described in Materials and Methods. Nuclear proteins (5 μg) were incubated with 0.4 μg of poly(dI-dC) and ^{32}P-labeled P0 NF-AT DNA probe in binding buffer at room temperature for 20 min and then incubated with antibodies (2 μl at 2 μg/μl) for another 20 min followed by separation on a 4% polyacrylamide gel. The arrow indicates the major PMA/Io-inducible binding complex with the relative intensity values for this complex provided at the bottom of each lane. *Lane 1*, free probe; *lane 2*, naïve cells; *lanes 3 - 8*, PMA/Io-stimulated cells; *lane 4*, incubated with control rabbit IgG; *lane 5*, incubated with anti-c-fos rabbit IgG; *lane 6*, incubated with anti-c-jun/AP-1 rabbit IgG; *lane 7*, incubated with control ascites fluid; *lane 8*, incubated with anti-NF-ATc1 monoclonal antibody. Results are representative of two independent experiments.


Probe
Naive
PMA/Io
Control Rabbit IgG
anti-c-Fos
anti-c-Jun/AP-1
Control Ascites Fluid
anti-NF-ATc1
PMA/Io-induced P0
Lane 1 2 3 4 5 6 7 8
Relative Intensity 31 100 89 55 58 94 35

antibodies) and control mouse ascites fluid (control for NF-ATc anti-sera) did not alter PMA/Io-induced binding to the P0 site.

C. Inhibition of IL-2, IL-4 and IL-5 mRNA expression by Δ^9-THC

The EL4 T cell line expresses three major cytokines, IL-2, IL-4 and IL-5 in response to PMA/Io stimulation. NF-AT is a major transcriptional regulator of all three of these cytokines (Rao *et al.*, 1997). In light of the aforementioned results suggesting that both Δ^9-THC and CBN attenuate IL-4 expression by inhibiting NF-AT regulation, the effect of Δ^9-THC on the expression of all three cytokines was examined simultaneously by an RNase protection assay (RPA). As demonstrated in figure 39, the PMA/Io-induced expression of IL-2, IL-4 and IL-5 steady state mRNA was all markedly suppressed by Δ^9-THC (20 μM) treatment. In contrast, Δ^9-THC did not influence the expression of two separate housekeeping genes, L32 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

D. CBN-mediated inhibition of IL-4 protein secretion is not sensitive to dibutyryl-cAMP and pertussis toxin

The putative role of the cAMP signaling pathway and cannabinoid receptors in CBN-mediated inhibition of IL-4 secretion by T cells was investigated with the same experimental approaches employed in aforementioned IL-2 studies. The inhibitory effect of CBN on IL-4 secretion was not abrogated by the presence of dibutyryl-cAMP (1 - 10 μM) in splenocytes stimulated with sCD3/CD28 (Fig. 40A). In addition to primary splenocytes, dibutyryl-cAMP (10 - 500 μM) also failed to reverse CBN-induced

Figure 39. Effect of Δ^9-THC on cytokine mRNA expression by EL4 cells. EL4 cells (2×10^5 cells/ml) were either untreated (NA), or pretreated with Δ^9-THC (20 μM) and/or VH (0.1% ethanol) for 30 min followed by stimulation with PMA/Io (80 nM/1 μM) for 3 or 6 hr. The total RNA was isolated and cytokine mRNA expression was determined by RPA as described in Materials and Methods. *Lane 1*, undigested ^{32}P-labeled riboprobes; *Lane 2*, yeast tRNA as a background control; *lane 3*, control mouse RNA as an integrity control for the RPA procedure; *lanes 4 - 5*, NA; *lanes 6 – 7*, pretreated with VH and activated with PMA/Io for 3 hr; *lanes 8 – 9*, pretreated with Δ^9-THC (20 μM) and activated with PMA/Io for 3 hr; *lanes 10 – 11*, pretreated with VH and activated with PMA/Io for 6 hr; *lanes 12 – 13*, pretreated with Δ^9-THC (20 μM) and activated with PMA/Io for 6 hr. The position of undigested free probes was indicated on the left, and the protected probes on the right. Because of its strong signals as compared to those of IL-4 and IL-5, the IL-2 mRNA expression is also shown on the bottom graph with a shorter time exposure of autoradiography.

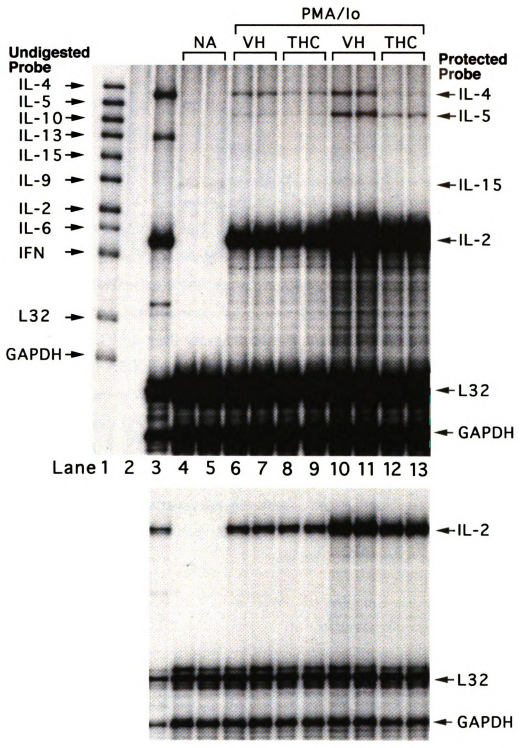
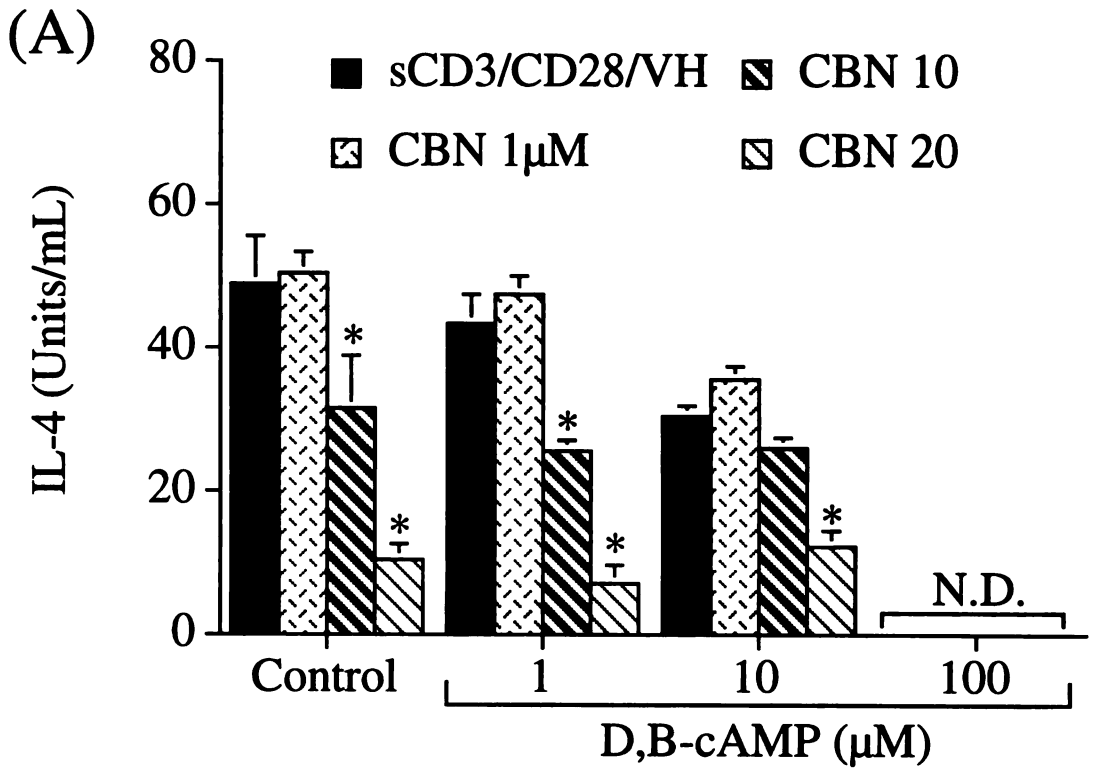
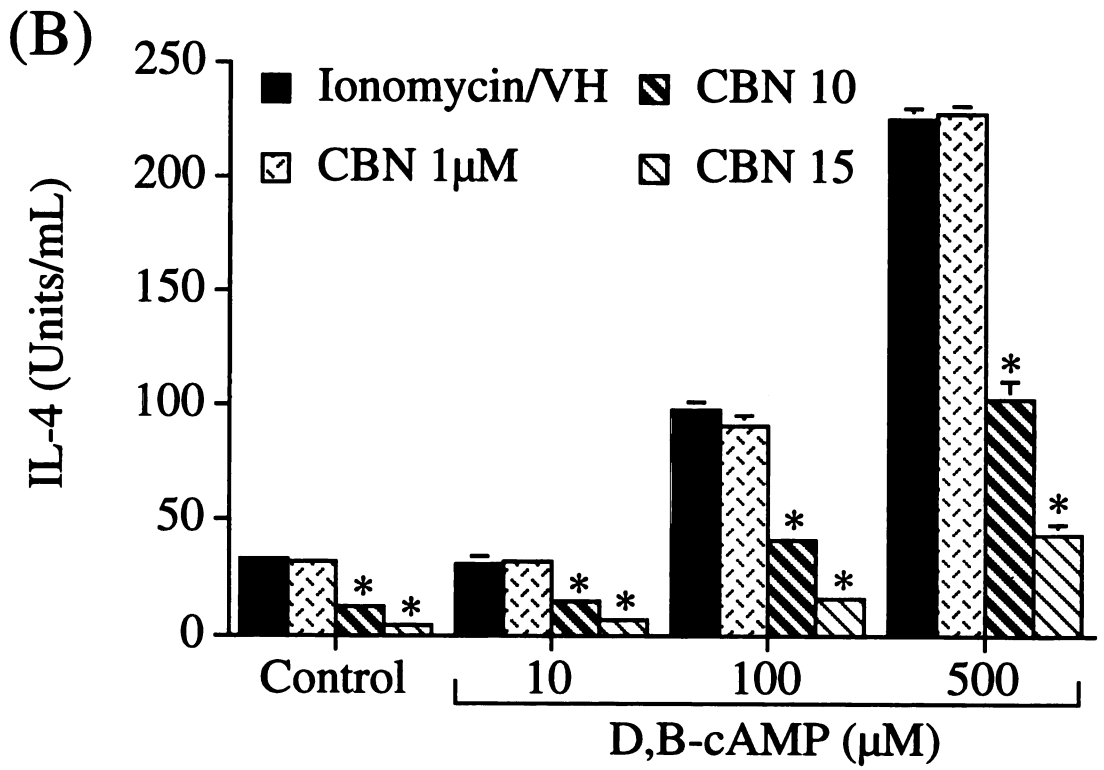

PMA/Io
NA
VH
THC
VH
THC
Undigested Probe
IL-4
IL-5
IL-10
IL-13
IL-15
IL-9
IL-2
IL-6
IFN
L32
GAPDH
Protected Probe
IL-4
IL-5
IL-15
IL-2
L32
GAPDH
Lane 1 2 3 4 5 6 7 8 9 10 11 12 13
IL-2
L32
GAPDH

Figure 40. No reversal of CBN-mediated inhibition of sCD3/CD28-induced IL-4 secretion by dibutyryl-cAMP (D,B-cAMP). (A) Splenocytes (2 x 10^6 cells/mL) were either untreated (NA) or pretreated with CBN (1 - 20 μM) and/or VH (0.1% ethanol) for 30 min in the absence (control group) or presence of D,B-cAMP (1 – 100 μM). Following the pretreatment, NA cells were left unstimulated and the others were stimulated with sCD3/CD28 (2 μg/mL of each mAb) for 48 hr at 37°C. (B) EL4 cells (2 x 10^5 cells/mL) were either untreated (NA) or pretreated with CBN (1 - 15 μM) and/or VH (0.1% ethanol) for 30 min in the absence (control group) or presence of D,B-cAMP (10 – 500 μM). Following the pretreatment, NA cells were left unstimulated and the others were stimulated with ionomycin (1 μM) for 24 hr at 37°C. IL-4 in the supernatants was quantified by ELISA. Data are expressed as the means ± standard error of triplicate cultures. The IL-4 activity in NA supernatants was below the level of quantification. *, $p < 0.05$ as compared to the matched VH group. Results are representative of two separate experiments.


(A)
80
60
40
20
0
IL-4 (Units/mL)
sCD3/CD28/VH
CBN 10
CBN 1μM
CBN 20
*
N.D.
Control
1
10
100
D,B-cAMP (μM)

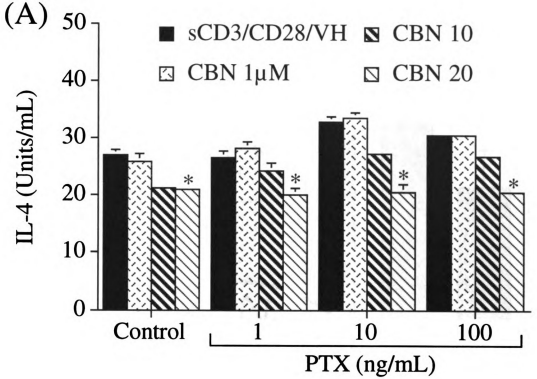

(B)
250
200
150
100
50
0
IL-4 (Units/mL)
Ionomycin/VH
CBN 10
CBN 1μM
CBN 15
*
Control
10
100
500
D,B-cAMP (μM)

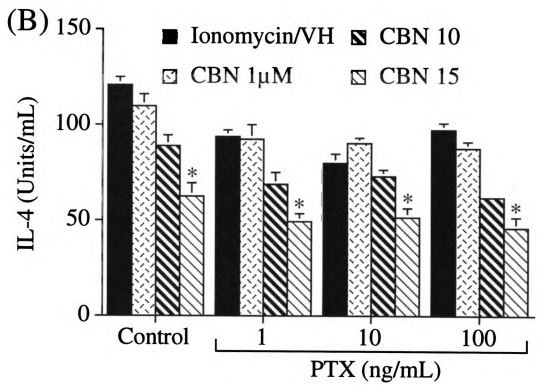
inhibition of IL-4 secretion by ionomycin-stimulated EL4 T cells (Fig. 40B). Interestingly, the highest concentration of dibutyryl-cAMP, 100 μM, tested in splenocytes suppressed IL-4 secretion in all treatment groups (Fig. 40A), whereas 100 and 500 μM of dibutyryl-cAMP markedly enhanced the secretion of IL-4 by EL4 cells (Fig. 40B). Moreover, preincubation of splenocytes and EL4 cells with increasing concentrations of pertussis toxin (PTX; 1 – 100 ng/mL) for 24 hr prior to CBN treatment failed to reverse the CBN-mediated inhibition of IL-4 secretion (Fig 41). Notably, in control studies where cells were incubated in RPMI culture medium in the absence of T cell stimulation for 24 hr, both splenocytes and EL4 cells were less sensitive to the inhibitory effect by CBN (Fig 41). Nevertheless, CBN was still capable of inhibiting IL-4 secretion in both control and PTX-pretreated groups. These results suggest that G_i/G_o and the cAMP pathway may not be solely responsible for CBN-mediated inhibition of the IL-4 secretion by T cells. To substantiate these observations, the role of the cAMP signaling cascade in sCD3/CD28-induced IL-4 secretion was further investigated using the specific protein kinase A (PKA) inhibitor H89. Consistent with the aforementioned results, H89 failed to exhibit any marked effect on the magnitude of IL-4 secretion by sCD3/CD28-activated splenic T cells (Fig 42).

E. CBN-mediated inhibition of IL-4 protein secretion is not reversed by cannabinoid receptor antagonists.

Pretreatment of splenocytes with SR141716A in combination with SR144528 (0.1 - 1 μM of each antagonist) 15 min prior to CBN treatment did not reverse CBN-mediated inhibition of IL-4 secretion (Fig. 43A). In the presence of higher concentrations of both

Figure 41. No reversal of CBN-mediated inhibition of sCD3/CD28-induced IL-4 secretion by pertussis toxin (PTX). (A) Splenocytes (2 x 10^6 cells/mL) were incubated with the culture medium (control group) or PTX (1 - 100 ng/mL) for 24 hr. After PTX incubation, cells were washed twice with fresh medium and adjusted to 2 x 10^6 cells/mL. Cells in each group were then left untreated (NA) or pretreated with CBN (1 - 20 μM) and/or VH (0.1% ethanol) for 30 min followed by stimulation with sCD3/CD28 (2 μg/mL of each mAb) for 48 hr at 37°C. (B) EL*4* cells (2 x 10^5 cells/mL) were incubated with the culture medium (control group) or PTX (1 - 100 ng/mL) for 24 hr. After PTX incubation, cells were washed twice with fresh medium and adjusted to 2 x 10^5 cells/mL. Cells in each group were then left untreated (NA) or pretreated with CBN (1 - 15 μM) and/or VH (0.1% ethanol) for 30 min followed by stimulation with ionomycin (1 μM) for 24 hr at 37°C. IL-4 in the supernatants was quantified by ELISA. Data are expressed as the means ± standard error of triplicate cultures. The IL-4 activity in NA supernatants was below the level of quantification. *, $p < 0.05$ as compared to the matched VH group. Results are representative of two separate experiments.


(A)
sCD3/CD28/VH
CBN 10
CBN 1μM
CBN 20
IL-4 (Units/mL)
50
40
30
20
10
0
Control
1
10
100
PTX (ng/mL)


(B)
Ionomycin/VH
CBN 10
CBN 1μM
CBN 15
IL-4 (Units/mL)
150
100
50
0
Control
1
10
100
PTX (ng/mL)

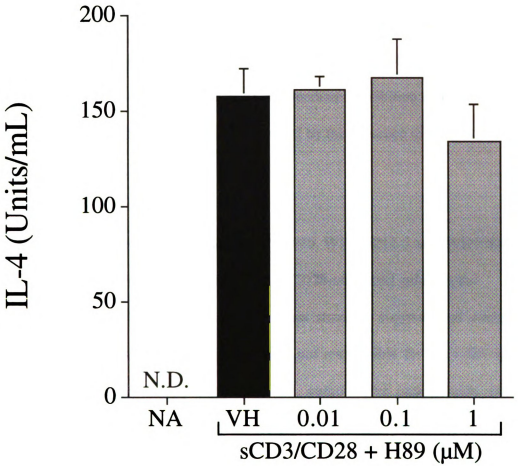


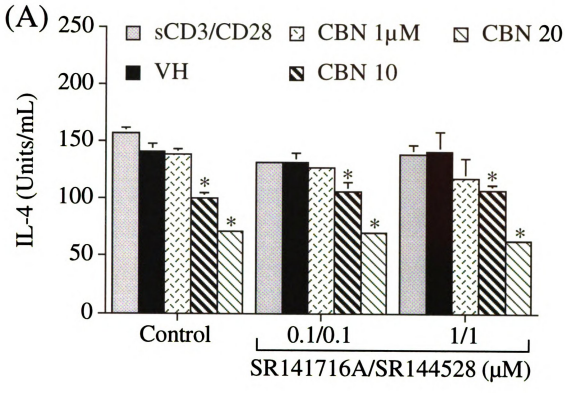
Figure 42. The effect of H89 on IL-4 secretion by sCD3/CD28-activated splenocytes. Splenocytes (2 x 10^6 cells/mL) were either untreated (NA) or pretreated with H89 (0.01 - 1 μM) and/or VH (0.01% DMSO) for 30 min. Following the pretreatment, NA cells were left unstimulated and the others were stimulated with sCD3/CD28 (2 μg/mL of each mAb) for 48 hr at 37°C. IL-4 in the supernatants was quantified by ELISA. Data are expressed as the means ± standard error of triplicate cultures. *N.D.*, IL-4 protein was below the level of quantification. Results are representative of three separate experiments.

antagonists (5 – 10 μM), CBN still significantly inhibited IL-4 secretion (data not shown). The effect of SR141716A and SR144528 alone on sCD3/CD28-induced IL-4 secretion by splenocytes was also examined. Both antagonists at concentrations ≤ 1 μM in the absence of CBN did not produce marked effects on sCD3/CD28-induced IL-4 secretion (data not shown). Likewise, the CBN-mediated inhibition of IL-4 secretion by ionomycin-stimulated EL4 cells were not reversed by the presence of the CB2 antagonist SR144528 (0.1 – 1 μM; Fig. 43B).

F. The effect of cannabidiol, CP55,940, WIN55212-2 and WIN55212-3 on IL-4 protein secretion by sCD3/CD28-activated splenocytes

Results from the preceding experiments strongly suggest that cannabinoid receptors and the cAMP signaling pathway are not responsible for the CBN-mediated inhibition of IL-4 secretion by T cells. Thus, comparative experiments using two structurally related cannabinoid compounds, cannabidiol and CP55,940, which exhibit low and high affinities to cannabinoid receptors, respectively, were performed. As the sensitivity to CBN-mediated inhibition of IL-4 secretion between splenocytes and EL4 cells is similar, the remainder of the experiments were conducted only in splenocytes. Despite marked differences in the binding affinity to cannabinoid receptors between cannabidiol and CP55,940, both produced a similar magnitude of inhibition on IL-4 secretion in sCD3/CD28-activated splenic T cells under identical assay conditions as those utilized for CBN (Fig 44). The observed effective concentration range of these two cannabinoid compounds was between 5 – 20 μM which was comparable to that of CBN. In addition to cannabidiol and CP55,940, the effect of WIN55212 enantiomers

Figure 43. No reversal of CBN-mediated inhibition of sCD3/CD28-induced IL-4 protein secretion by SR141716A and SR144528. (A) Splenocytes (2 x 10^6 cells/mL) were either untreated (NA) or pretreated with CBN (1 - 20 μM) and/or VH (0.1% ethanol) for 30 min in the absence (control group) or presence of SR141716A plus SR144528 (0.1 or 1 μM of each antagonist). Following the pretreatment, NA cells were left unstimulated and the others were stimulated with sCD3/CD28 (2 μg/mL of each mAb) for 48 hr at 37°C. (B) EL4 cells (2 x 10^5 cells/mL) were either untreated (NA) or pretreated with CBN (1 - 15 μM) and/or VH (0.1% ethanol) for 30 min in the absence (control group) or presence of SR144528 (0.1 or 1 μM). Following the pretreatment, NA cells were left unstimulated and the others were stimulated with ionomycin (1 μM) for 24 hr at 37°C. IL-4 in the supernatants was quantified by ELISA. Data are expressed as the means ± standard error of triplicate cultures. The IL-4 activity in NA supernatants was below the level of quantification. *, $p < 0.05$ as compared to the matched VH group. Results are representative of three separate experiments.


(A)
sCD3/CD28
CBN 1μM
CBN 20
VH
CBN 10
IL-4 (Units/mL)
250
200
150
100
50
0
*
Control
0.1/0.1
1/1
SR141716A/SR144528 (μM)

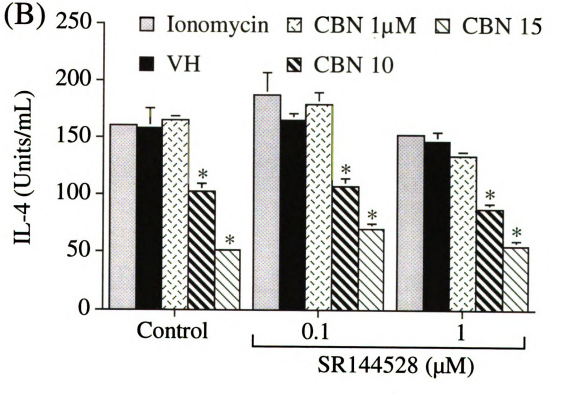
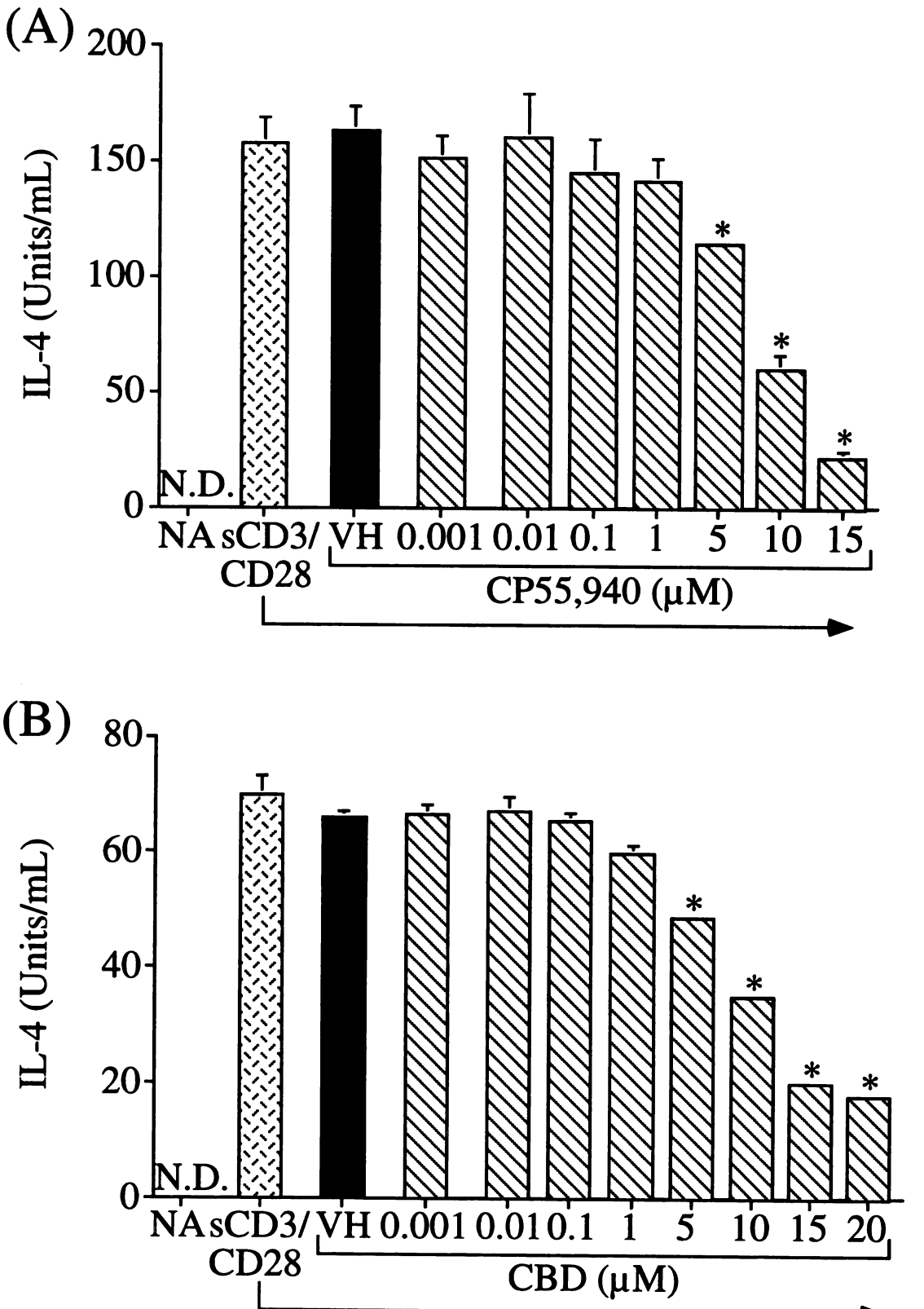

(B)
Ionomycin
CBN 1μM
CBN 15
VH
CBN 10
IL-4 (Units/mL)
250
200
150
100
50
0
*
Control
0.1
1
SR144528 (μM)

Figure 44. The effect of CP55,940 and cannabidiol on IL-4 secretion by splenocytes activated with sCD3/CD28. Splenocytes (2 x 10^6 cells/mL) were either untreated (NA) or pretreated with (A) CP55,940 (0.001 - 15 μM) and/or VH (0.15% DMSO), or (B) cannabidiol (CBD; 0.001 – 20 μM) and/or VH (0.1% ethanol) for 30 min. Following the pretreatment, NA cells were left unstimulated and the others were stimulated with sCD3/CD28 (2 μg/mL of each mAb) for 48 hr at 37°C. IL-4 in the supernatants was quantified by ELISA. Data are expressed as the means ± standard error of triplicate cultures. *N.D.*, IL-4 protein was below the level of quantification. *, $p < 0.05$ as compared to the matched VH group. Results are representative of three separate experiments.


(A)
IL-4 (Units/mL)
200
150
100
50
0
N.D.
NA
sCD3/ CD28
VH
0.001
0.01
0.1
1
5
10
15
CP55,940 (μM)
(B)
IL-4 (Units/mL)
80
60
40
20
0
N.D.
NA
sCD3/ CD28
VH
0.001
0.01
0.1
1
5
10
15
20
CBD (μM)

(WIN55212-2 and WIN55212-3) on IL-4 secretion by splenic T cells was also examined. As illustrated in figure 45, WIN55212-2 (5 – 20 μM) produced a robust concentration-dependent inhibition of IL-4 secretion by sCD3/CD28-activated splenocytes. In contrast, WIN55212-3 was inactive at the corresponding concentration range (Fig. 45). The stereo-selective inhibition of IL-4 by WIN55212 is especially intriguing because it suggests that the cellular target for WIN55212 is highly specific and it's modulation is critically dependent on the tertiary conformation of the cannabinoid.

G. WIN55212-2 mediated inhibition of IL-4 protein secretion is not reversed by cannabinoid receptor antagonists

As stated above, the stereo-selective inhibition of IL-4 secretion by WIN55212 suggests that the effect by WIN55212-2 is critically dependent on the tertiary structure of the cannabinoid, thus implying a potential involvement of cannabinoid receptors in the inhibitory effect by WIN55212-2. However, pretreatment of splenocytes with SR141716A and SR144528 (0.1 and 1 μM of each), in combination, failed to abrogate WIN55212-2 mediated inhibition of IL-4 secretion (Fig. 46). These results appear to rule out the involvement of either CB1 and CB2 or could be interpreted as suggesting that CB1 and CB2 are not the mechanism solely responsible for the isomer-selective activity of WIN55212-2.

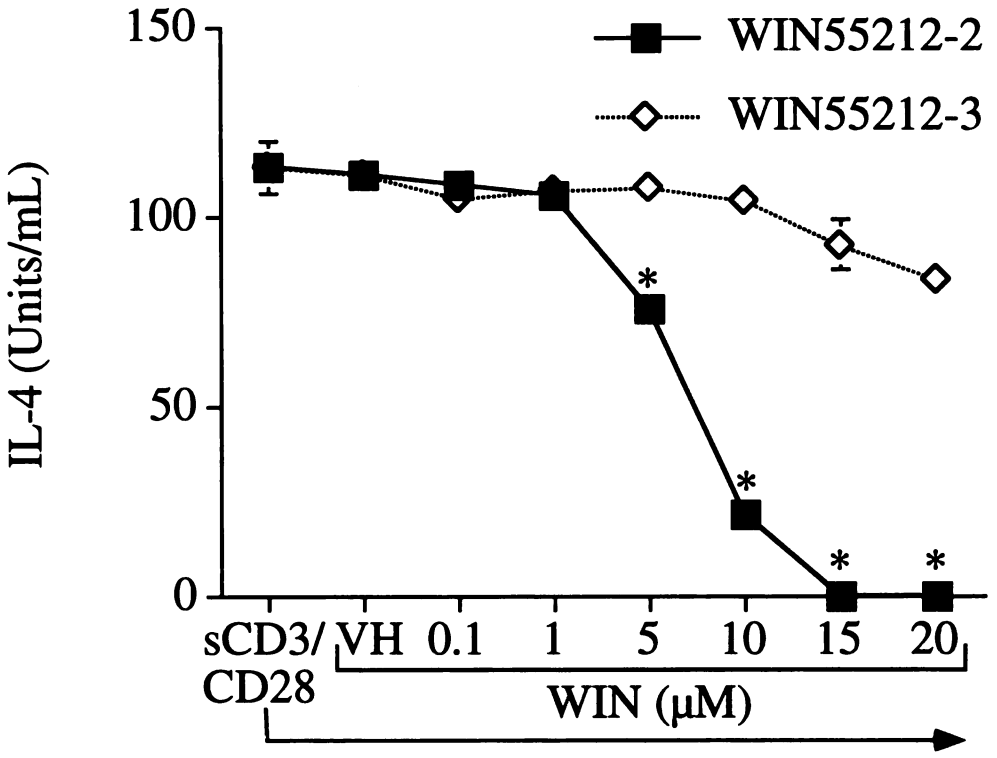


Figure 45. The effect of WIN55212 enantiomers on IL-4 secretion by splenocytes activated with sCD3/CD28. Splenocytes (2 x 10^6 cells/mL) were either untreated (NA) or pretreated with WIN55212-2, WIN55212-3 (0.1 - 20 μM) and/or VH (0.1% DMSO) for 30 min. Following the pretreatment, NA cells were left unstimulated and the others were stimulated with sCD3/CD28 (2 μg/mL of each mAb) for 48 hr at 37°C. IL-4 in the supernatants was quantified by ELISA. Data are expressed as the means ± standard error of triplicate cultures. The IL-4 activity in NA supernatants was below the level of quantification. *, $p < 0.05$ as compared to the VH group. Results are representative of three separate experiments.

Figure 46. No reversal of WIN55212-2 mediated inhibition of sCD3/CD28-induced IL-4 secretion by SR141716A and SR144528. Splenocytes (2 x 10^6 cells/mL) were either untreated (NA) or pretreated with WIN55212-2 (1 - 20 μM) and/or VH (0.1% DMSO) for 30 min in the absence (control group) or presence of SR141716A plus SR144528 (0.1 or 1 μM of each antagonist). Following the pretreatment, NA cells were left unstimulated and the others were stimulated with sCD3/CD28 (2 μg/mL of each mAb) for 48 hr at 37°C. IL-4 in the supernatants was quantified by ELISA. Data are expressed as the means ± standard error of triplicate cultures. *N.D.*, IL-4 protein was below the level of quantification. *, $p < 0.05$ as compared to the matched VH group. Results are representative of three separate experiments.

IL-4 (Units/mL)
80
60
40
20
0
NA
sCD3/CD28/VH
WIN 1 μM
WIN 5
WIN 10
WIN 15
WIN 20
N.D.
N.D.
N.D.
Control
0.1/0.1
1/1
SR141716A/SR144528 (μM)

III. Cannabinoid-mediated attenuation of IL-2 and IL-4 expression associated with ovalbumin (Ova)-induced allergic airway disease

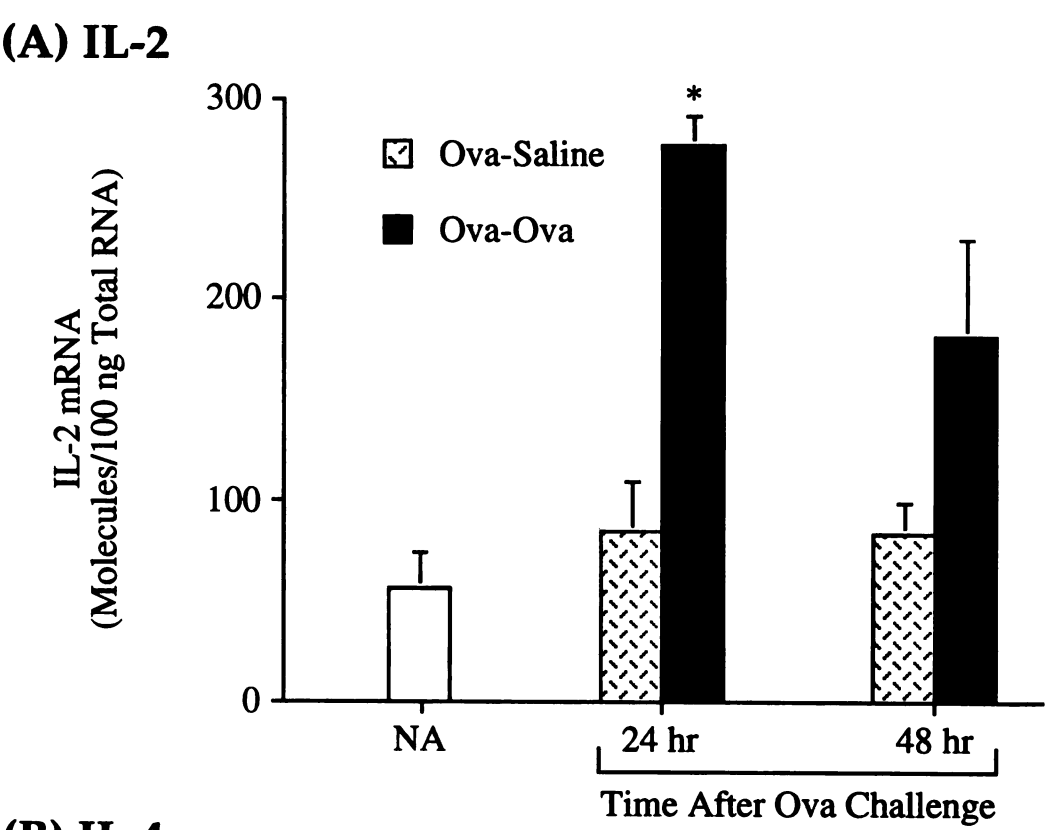
A. Elevation of the steady state IL-2 and IL-4 mRNA expression in the lungs of Ova-sensitized and challenged A/J mice

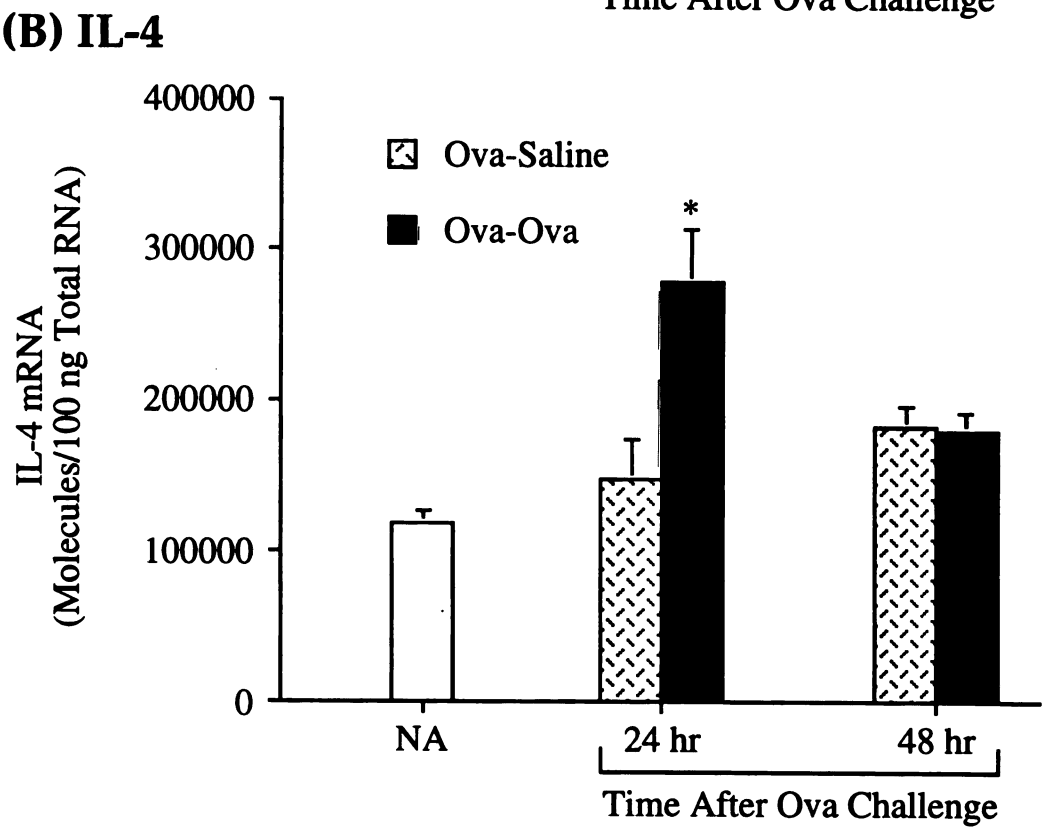
Helper T cells that produce a Th2 pattern of cytokines have been shown to play a pivotal role in the pathophysiology of allergic airway disease (reviewed by Anderson and Coyle, 1994; Wills-Karp, 1999). To further examine the effect of CBN and Δ^9-THC on IL-2 and IL-4 expression *in vivo*, a murine model of allergic airway disease induced by Ova was employed. A/J mice were sensitized with Ova plus aluminum potassium sulfate and then challenged with aerosolized Ova 14 days after sensitization. The steady state IL-2 and IL-4 mRNA expression in the lungs was measured by competitive RT-PCR. As illustrated in figure 47, Ova challenge elicited a marked increase in both IL-2 and IL-4 steady state mRNA expression in the lungs of A/J mice sensitized with Ova as compared to the control group in which Ova-sensitized mice were challenged with saline, instead of Ova aerosol. The peak elevation of IL-2 and IL-4 mRNA expression was detected at 24 hr post Ova challenge, which decreased in the magnitude of expression levels at 48 hr post Ova challenge (Fig. 47).

B. Attenuation by CBN and Δ^9-THC of IL-2 and IL-4 mRNA expression in the lungs of Ova-sensitized and challenged A/J mice

To examine the effect of cannabinoids on T cell cytokine expression in the employed animal model, CBN or Δ^9-THC (50 mg/kg) was administered via i.p. route daily for 3 consecutive days immediately prior to Ova sensitization and then before Ova

Figure 47. Elevation of the steady state IL-2 and IL-4 mRNA expression by ovalbumin (Ova) challenge in the lungs of Ova sensitized A/J mice. A/J mice were either left untreated (NA) or sensitized with Ova and then challenged with aerosolized saline or Ova 14 days after sensitization as described in the Materials and Methods section (Fig. 9). Total RNA from the lungs was isolated by TRI reagent and the level of IL-2 and IL-4 steady state mRNA expression was measured by quantitative RT-PCR. Data are expressed as the mean $\pm$ SE for three samples. One of three representative experiments is shown. * $p < 0.05$ with comparison to the matched Ova-Saline group.

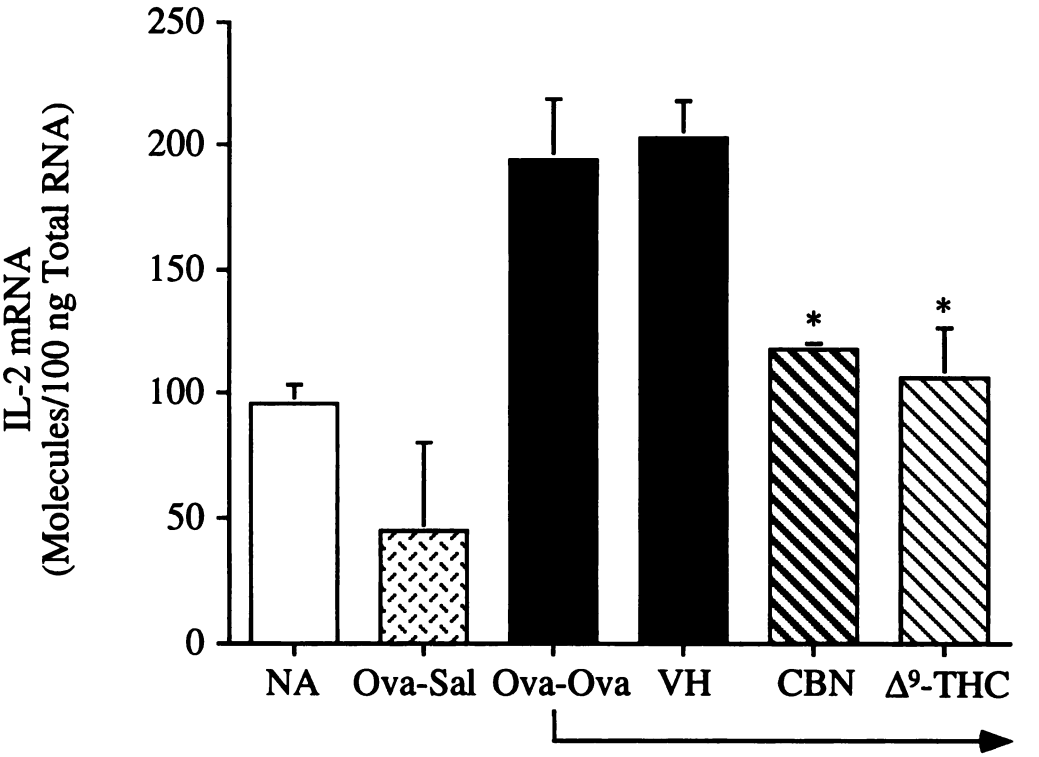

(A) IL-2
300
200
100
0
IL-2 mRNA
(Molecules/100 ng Total RNA)
Ova-Saline
Ova-Ova
*
NA
24 hr
48 hr
Time After Ova Challenge


(B) IL-4
400000
300000
200000
100000
0
IL-4 mRNA
(Molecules/100 ng Total RNA)
Ova-Saline
Ova-Ova
*
NA
24 hr
48 hr
Time After Ova Challenge

challenge. A significant attenuation of both IL-2 and IL-4 steady state mRNA expression was observed by the regimen with either CBN or Δ^9-THC as compared to the VH control group (Fig. 48). The efficacy was comparable between these two plant-derived cannabinoids in the attenuation of cytokine responses. To further elucidate which phase, sensitization and/or challenge, of the immune responses was involved in cannabinoid-mediated attenuation of IL-2 and IL-4 expression in the animal model, phase analysis studies were conducted. Ova-sensitized and challenged mice were administered with CBN (50 mg/kg) daily for 3 consecutive days before sensitization, before challenge, or before both sensitization and challenge. Interestingly, CBN treatment significantly attenuated the IL-2 and IL-4 mRNA expression only in the group that received CBN before both sensitization and challenge. CBN treatment did not produce a significant effect on IL-2 and IL-4 mRNA expression when it was administered either before sensitization or before challenge (Fig. 49). The modest attenuation of IL-2 and IL-4 mRNA expression by the administration of CBN before Ova sensitization was not statistically significant. In addition to measurements of IL-2 and IL-4 expression by RT-PCR, the expression of other T cell-derived cytokines in the lungs of A/J mice was further examined by an RNase protection assay that can simultaneously detect IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-13 and IFN-γ. In accordance with previous reports that IL-5 and IL-13 are critical mediators derived from Th2 cells and involved in the inflammatory reactions of allergic airway disease, the Ova sensitized and challenged mice exhibited an increased expression of IL-5 and IL-13 in their lungs as compared to control groups (Fig. 50). This increased expression of IL-5 and IL-13 was markedly attenuated by the regimen of CBN administered either prior to Ova sensitization or before both

Figure 48. Attenuation by CBN and Δ^9-THC of the IL-2 and IL-4 mRNA expression induced by Ova challenge in the lungs of Ova sensitized A/J mice. A/J mice were either left untreated (NA) or sensitized with Ova and then challenged with aerosolized saline or Ova 14 days after sensitization. CBN, Δ^9-THC (50 mg/kg) and/or vehicle (VH; 5% ethanol and 0.5% Tween 20 in saline) was administered daily via i.p. injection for 3 consecutive days immediately prior to Ova sensitization and then before challenge as described in the Materials and Methods section (Fig. 10). Total RNA from the lungs was isolated by TRI reagent and the level of IL-2 and IL-4 steady state mRNA expression was measured by quantitative RT-PCR. Data are expressed as the mean $\pm$ SE for three samples. One of three representative experiments is shown. * $p < 0.05$ with comparison to the VH group.

(A) IL-2


IL-2 mRNA
(Molecules/100 ng Total RNA)
250
200
150
100
50
0
*
*
NA
Ova-Sal
Ova-Ova
VH
CBN
Δ9-THC

(B) IL-4

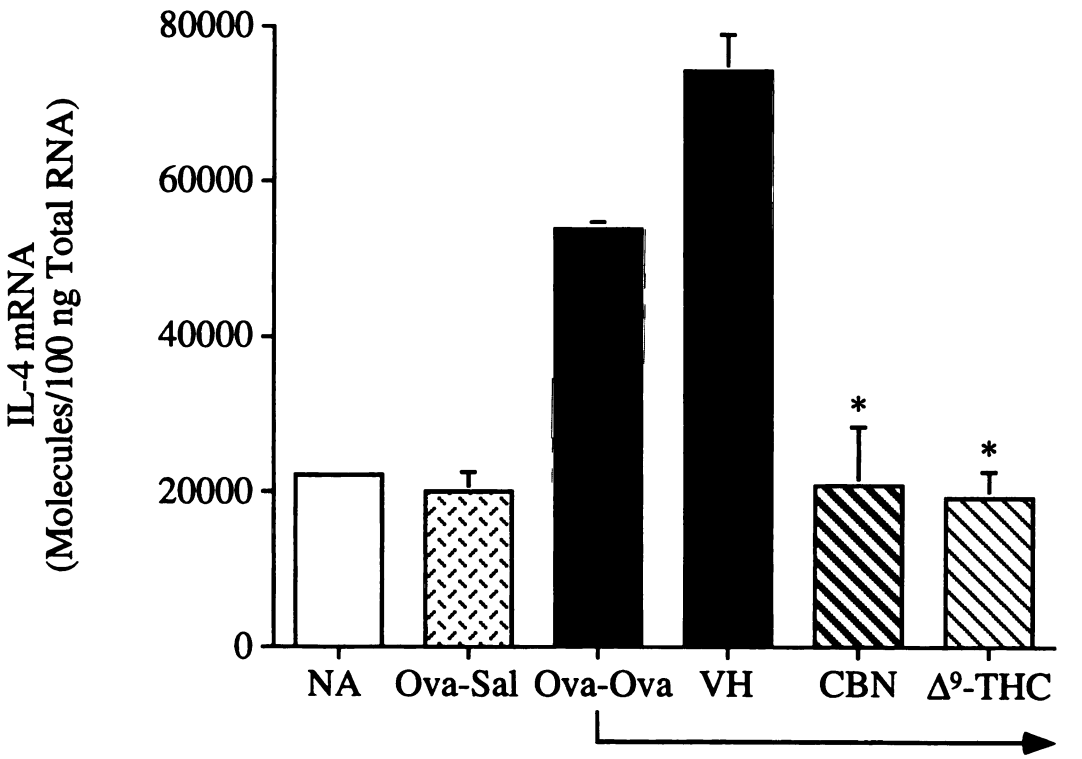
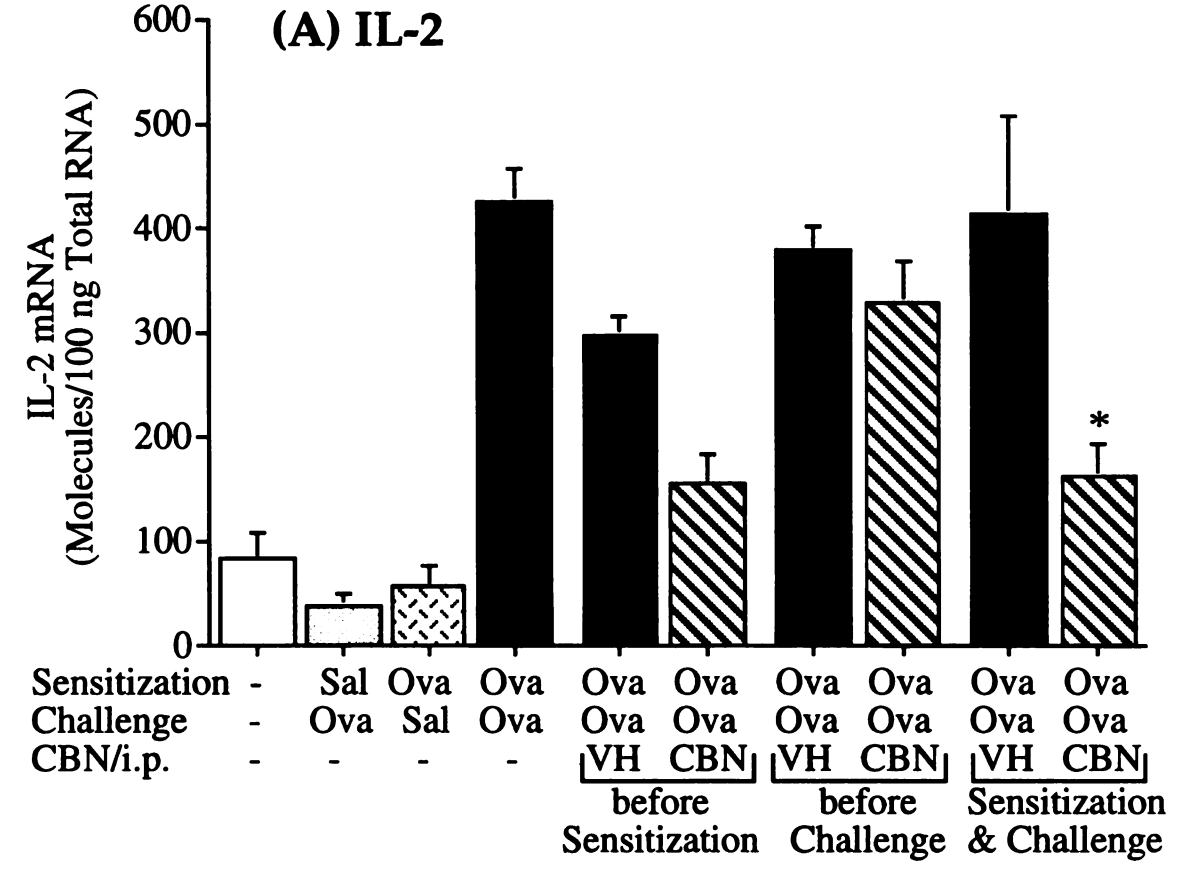

IL-4 mRNA
(Molecules/100 ng Total RNA)
80000
60000
40000
20000
0
*
*
NA
Ova-Sal
Ova-Ova
VH
CBN
Δ9-THC

Figure 49. Phase analysis of CBN-mediated attenuation of the IL-2 and IL-4 mRNA expression induced by Ova challenge in the lungs of Ova sensitized A/J mice. A/J mice were either left untreated or sensitized with Ova and then challenged with aerosolized saline or Ova 14 days after sensitization. An additional control group was included in which mice were injected with saline and then challenged with Ova aerosol 14 days after saline injection. CBN (50 mg/kg) and/or vehicle (VH; 5% ethanol and 0.5% Tween 20 in saline) was administered daily via i.p. injection for 3 consecutive days immediately before Ova sensitization, before Ova challenge, or before both sensitization and challenge. Total RNA from the lungs was isolated by TRI reagent and the level of IL-2 and IL-4 steady state mRNA expression was measured by quantitative RT-PCR. Data are expressed as the mean $\pm$ SE for five samples. One of two representative experiments is shown. * $p < 0.05$ with comparison to the matched VH group.


(A) IL-2
IL-2 mRNA
(Molecules/100 ng Total RNA)
600
500
400
300
200
100
0
*
Sensitization - Sal Ova Ova Ova Ova Ova Ova Ova Ova Ova
Challenge - Ova Sal Ova Ova Ova Ova Ova Ova Ova
CBN/i.p. - - - - VH CBN VH CBN VH CBN
before Sensitization
before Challenge
Sensitization & Challenge

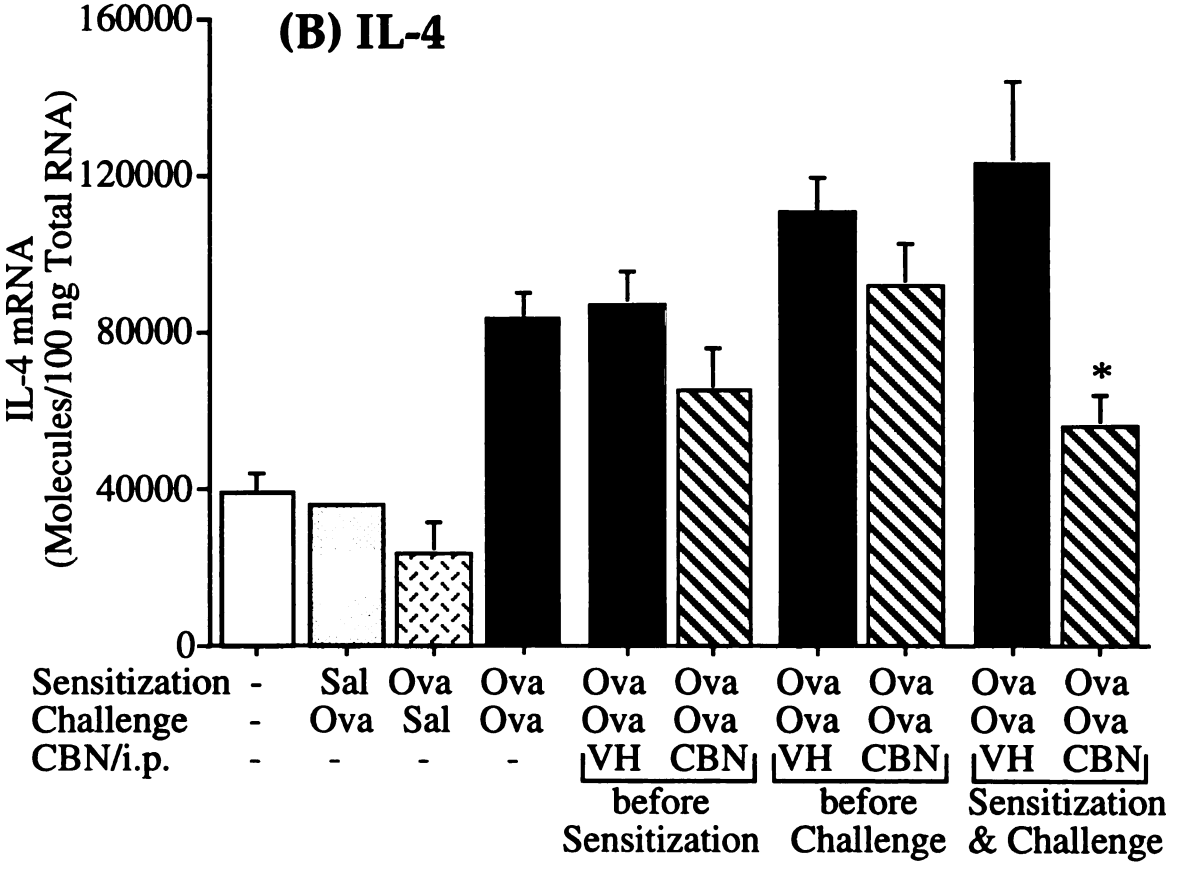
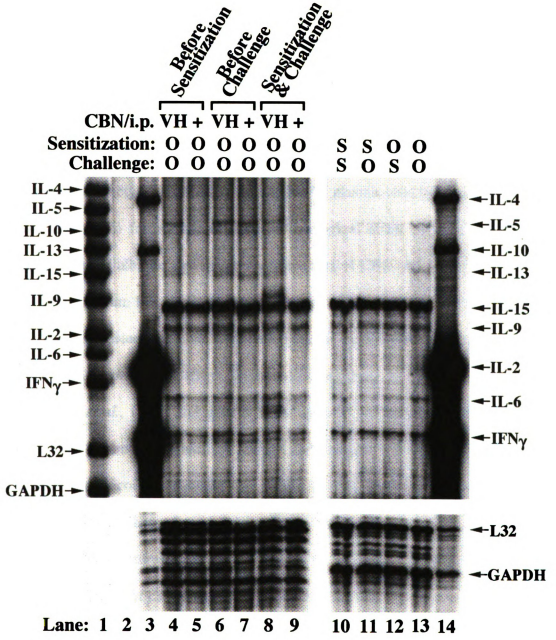

(B) IL-4
IL-4 mRNA
(Molecules/100 ng Total RNA)
160000
120000
80000
40000
0
*
Sensitization - Sal Ova Ova Ova Ova Ova Ova Ova Ova Ova
Challenge - Ova Sal Ova Ova Ova Ova Ova Ova Ova
CBN/i.p. - - - - VH CBN VH CBN VH CBN
before Sensitization
before Challenge
Sensitization & Challenge

Figure 50. Attenuation by CBN of the IL-5 and IL-13 mRNA expression induced by Ova challenge in the lungs of Ova sensitized A/J mice. A/J mice were sensitized with Ova and then challenged with aerosolized saline or Ova 14 days after sensitization. CBN (50 mg/kg) and/or vehicle (VH; 5% ethanol and 0.5% Tween 20 in saline) was administered daily via i.p. injection for 3 consecutive days immediately before Ova sensitization, before Ova challenge, or before both sensitization and challenge. Total RNA from the lungs was isolated by TRI reagent and the level of multiple cytokine mRNA expression was detected by an RNase protection assay (RPA) as described in the Materials and Method section. *Lane 1*, undigested ^{32}P-labeled roboprobes; *Lane 2*, yeast tRNA as a background control; *Lane 3 and 14*, control mouse RNA as integrity controls for the RPA procedure; *Lane 4 – 9 and 13,* mice sensitized and challenged with Ova (O); *Lane 10,* sensitized and challenged with saline (S); *Lane 11,* sensitized with saline and challenged with Ova; *Lane 12,* sensitized with Ova and challenged with saline; *Lane 4 – 9,* mice were administered with CBN and/or VH before sensitization and/or challenge as indicated in the figure. The position of undigested free probes was indicated on the left, and the protected probes on the right. Because of their strong signals as compared to those of cytokines, the expression of house keeping genes, L32 and GAPDH, was shown on the bottom graph with a shorter time exposure of autoradiography.


Before Sensitization
Before Challenge
Sensitization & Challenge
CBN/i.p. VH + VH + VH +
Sensitization: O O O O O O S S O O
Challenge: O O O O O O S O S O
IL-4
IL-5
IL-10
IL-13
IL-15
IL-9
IL-2
IL-6
IFNγ
L32
GAPDH
Lane: 1 2 3 4 5 6 7 8 9 10 11 12 13 14

sensitization and challenge, as detected by the RNase protection assay. Consistent with IL-2 and IL-4 expression, CBN administered only prior to Ova challenge failed to inhibit IL-5 and IL-13 steady state mRNA expression (Fig. 50).

C. Attenuation by CBN and Δ^9-THC of Ova-specific serum IgE in A/J mice sensitized and challenged with Ova

One of the hallmarks of allergic airway disease is the elevation of serum IgE (Anderson and Coyle, 1994). Th2 cytokines, including IL-4 and IL-13, are key mediators that switch the isotype of antibody production by plasma cells from IgM to IgE (Wills-Karp. 1999). To further characterize the biological effect of CBN and Δ^9-THC on allergen-induced allergic airway disease, the effect of CBN and Δ^9-THC on the level of Ova-specific serum IgE was investigated. In this series of studies, mice were sensitized and challenged twice with Ova, and serum samples were collected 96 hr after the second challenge. This protocol is adapted from a previous report by Wills-Karp and coworkers (Wills-Karp *et al.,* 1998). As illustrated in figure 51, a marked increase in the level of Ova-specific serum IgE in mice sensitized and challenged twice with Ova (O-O-O group) was observed as compared to the Ova sensitized and saline challenged (O-S-S) or NA treatment group. These observations confirm that elevation of allergen-specific serum IgE is one of the features associated with the allergic airway disease induced by Ova. To examine the effect of cannabinoids on the production of Ova-specific serum IgE in this animal model, CBN or Δ^9-THC (50 mg/kg) was administered via i.p. injection daily for 3 consecutive days immediately prior to Ova sensitization and then before each of the two challenges with Ova. Consistent with the inhibitory effect of cannabinoids on the

expression of T cell cytokines, both CBN and Δ^9-THC significantly attenuated the amount of Ova-specific serum IgE in mice sensitized and challenged twice with Ova as compared to the VH control group (Fig. 51).

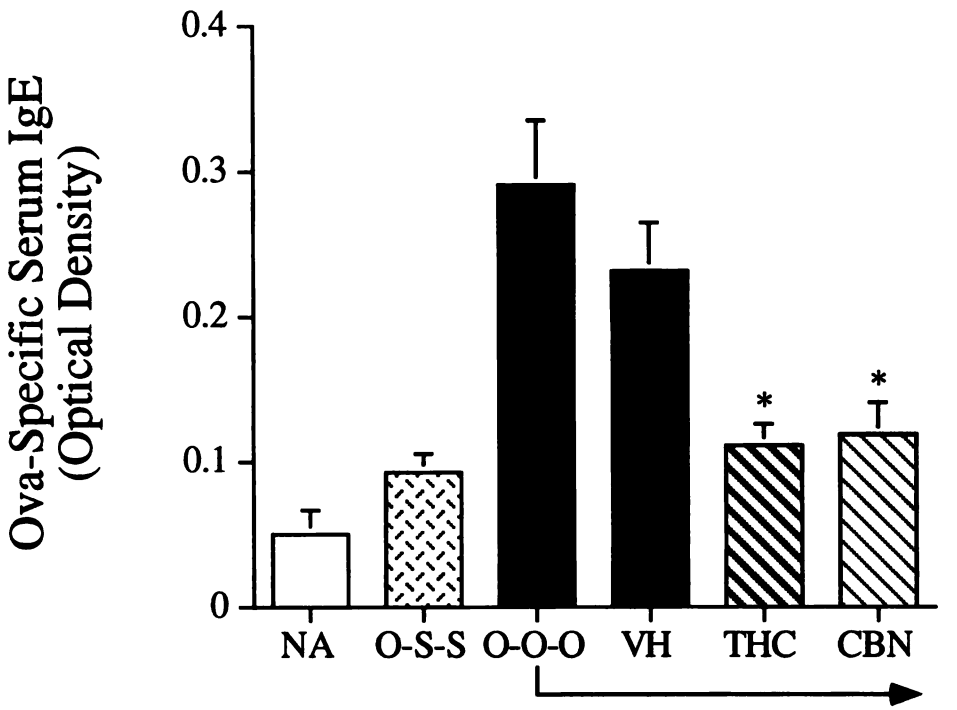


Figure 51. Attenuation by CBN and Δ^9-THC of Ova-specific serum IgE in A/J mice sensitized and challenged with Ova. A/J mice were either left untreated (NA) or sensitized with Ova on day 0. The Ova-sensitized mice were challenged twice with aerosolized saline (O-S-S) or Ova (O-O-O) on day 14 and 24. CBN, Δ^9-THC (50 mg/kg) and/or vehicle (VH; 5% ethanol and 0.5% Tween 20 in saline) was administered daily via i.p. injection for 3 consecutive days immediately prior to Ova sensitization and then before each Ova challenge as described in the Materials and Methods section (Fig. 10). Mice were sacrificed 96 hr after the second Ova challenge. Blood samples were collected and serum was obtained by centrifugation at 3,000 x g for 15 min. The level of Ova-specific IgE in serum was measured by ELISA. Data are expressed as the mean $\pm$ SE for 9 samples pooled from two experiments. * $p < 0.05$ with comparison to the VH group.

DISCUSSION

I. CBN-mediated differential modulation of IL-2 expression

Plant-derived cannabinoids have been reported to both positively and negatively modulate IL-2 expression by T cells (Condie *et al.*, 1996; Herring *et al.*, 1998; Nakano *et al.*, 1993a; Snella *et al.*, 1995). Although the mechanism responsible for cannabinoid-mediated differential modulation of IL-2 is unknown, several factors have been implicated including the method of T cell activation and the age of animals used in the studies (Nakano *et al.*, 1993a; Snella *et al.*, 1995). Previously, our laboratory has reported that PMA/Io-induced IL-2 expression by T cells was markedly inhibited by CBN and Δ^9-THC (Condie *et al.*, 1996; Herring *et al.*, 1998). In contrast, Nakano *et al.* demonstrated that the IL-2 activity induced in splenic T cells by concanavalin A was inhibited by Δ^9-THC whereas T cells activated by sCD3 exhibited enhanced IL-2 production in the presence of Δ^9-THC. In light of these diverging results, one of the focuses of the present studies was to critically evaluate whether the mode and/or magnitude of T cell activation are influencing factors contributing to the differential effects of cannabinoids on the IL-2 expression. αCD3 and αCD28 mAbs or PMA/Io were employed under various conditions to differentially activate primary splenic T cells. As expected, iCD3/CD28 was a strong activation stimulus for IL-2 induction. Conversely, in the absence of αCD28, the efficacy of iCD3, alone, to activate IL-2 was weak. Most striking was the observation that sCD3 and sCD3/CD28 were also capable of inducing IL-2 secretion, albeit the magnitude of induction was significantly lower as compared to iCD3/CD28. Using this model system, we demonstrated that CBN elicits

contrasting effects on IL-2 secretion by splenocytes isolated from adult mice (8 – 14 weeks) and provide further insights into the results reported by Nakano and coworkers (Nakano *et al.*, 1993a). Specifically, the present studies show that the contrasting effect of CBN on IL-2 secretion is dependent on the magnitude rather than the mode of T cell activation. Several lines of evidence support this premise. First, CBN significantly enhanced IL-2 secretion by splenocytes activated with stimuli that alone only produced a sub-optimal induction of IL-2 (i.e., iCD3, sCD3 or sCD3/CD28). Second, CBN significantly attenuated IL-2 secretion by splenocytes activated with strong inducers of IL-2 (i.e., iCD3/CD28 or PMA/Io). Third, CBN significantly enhanced IL-2 secretion by EL4 cells that were activated with low concentrations of PMA and markedly inhibited IL-2 secretion by EL4 cells that were activated by a high concentration of PMA with or without Io. It is important to emphasize that CBN alone was incapable of inducing a detectable amount of IL-2 production by splenocytes or EL4 cells.

Previous studies from this laboratory sought to characterize the biochemical mechanism responsible for the decrease in IL-2 gene expression by cannabinoids under the conditions of robust T cell activation (Condie *et al.*, 1996; Yea *et al.*, 2000). Those studies demonstrated that EL4 cells and splenic T cells activated by high concentration of PMA (80 nM) plus Io (1 μM) in the presence of CBN exhibited a significant inhibition in the DNA binding activity of two nuclear factors critical to the transcriptional regulation of IL-2, NF-AT and AP-1 (Condie *et al.*, 1996; Faubert and Kaminski, 2000; Yea *et al.*, 2000). Follow-up studies in which the inhibition of AP-1 by CBN was further investigated revealed a marked and concomitant inhibition of ERK MAP kinase activation in PMA/Io-activated splenocytes (Faubert and Kaminski, 2000). These

findings were in contrast to several reports from other laboratories employing primarily CHO cells artificially transfected with high levels of cannabinoid receptors or cell lines with non-immune origins. In those models cannabinoid treatment induced a positive activation of ERKs which occurred in the absence of any additional activation stimuli (Bouaboula *et al.*, 1995, 1996; Sanchez *et al.*, 1998a). Based on the important role of MAP kinases in IL-2 regulation and the strong correlation between decreased IL-2 expression and the inhibition of ERK activation in our previous studies, ERK regulation was evaluated under conditions where CBN produced enhanced IL-2 expression. Remarkably, a parallel up-regulation of nuclear phospho-ERKs by CBN was observed in conjunction with enhanced IL-2 secretion by both splenocytes and EL4 cells. However, CBN treatment of resting splenocytes or EL4 cells, in the absence of activation stimuli did not produce detectable modulation of ERK (data not shown). These findings are in contrast to those demonstrating the activation of MAP kinases by cannabinoids in transfected cell systems where cannabinoid receptors have been greatly over expressed (Bouaboula *et al.*, 1995, 1996). The present results suggest that in leukocytes CBN can positively and negatively modulate ERK activation but not in the absence of activators of the MAP kinase cascade. Similar to the enhancing effects of cannabinoids on IL-2 secretion, an increase in human tonsillar B cell proliferation after cross-linking of surface immunoglobulins in the presence of low concentrations of cannabinoids has been reported and may also be influenced by an up-regulation of ERK activity (Derocq *et al.*, 1995). The present studies suggest that in leukocytes, CBN-mediated modulation of ERKs occurs through effects on upstream regulators outside of the MAP kinase cascade. The other possibility is that the effect of CBN on ERKs in resting cells is direct but so

modest that it is below the level of detection. Although possible, the latter scenario seems unlikely since the magnitude of CBN-mediated enhancement on nuclear ERKs under certain conditions appears to be quite profound. Collectively, these independent lines of evidence imply that the activation of ERKs is modulated indirectly by CBN and that this may represent a common signaling mechanism by which cannabinoids influence biological activity.

It has been widely established that the induction of the MAP kinase signaling cascade, as assessed through the phosphorylation and activation of ERKs, can be up-regulated through direct activators of PKC such as phorbol esters, or by mitogens that activate the small GTP-binding protein $p21^{ras}$ (reviewed by Seger and Krebs; 1995). Recently, agonists for G-protein coupled receptors have also been implicated in the indirect activation of ERKs via the activation of PI 3-kinase (reviewed by Lopez-Ilasaca, 1998). In fact PI-3 kinase has been identified as a critical mediator bridging signaling between G-proteins and the MAP kinases. In light of this, the role of PI 3-kinase and PKC in the CBN-mediated enhancement of IL-2 was examined. These studies showed that pretreatment with the PI-3 kinase inhibitor, wortmannin, alone enhanced IL-2 production by sCD3/CD28 activated splenic T cells. However, wortmannin pretreatment did not attenuate the CBN-mediated enhancement of sCD3/CD28-induced IL-2 secretion. We interpreted these results as suggesting that PI-3 kinase is not involved in the CBN-mediated enhancement of IL-2. A second series of studies focused on the role of PKC in the CBN-mediated enhancement of IL-2. Interestingly, staurosporine a broad calcium-dependent protein kinase inhibitor with some selectivity for PKC, at low concentrations (5 – 10 nM) produced no effect on sCD3/CD28-induced IL-2 but significantly attenuated

the CBN-mediated enhancement of IL-2 secretion. Employment of the PKC inhibitor, Ro-31-8220, a staurosporine-derived analog with greater selectivity for PKC only partially attenuated the CBN-mediated IL-2 enhancement. These data suggested that PKC and/or possibly other calcium-dependent protein kinases are likely involved in CBN-mediated enhancement of IL-2 secretion.

In light of the above findings implicating the involvement of other calcium-dependent protein kinases, experiments were performed to investigate the role of CaM kinases. The CaM kinase inhibitor, KN93, was found to effectively attenuate CBN-mediated enhancement of IL-2 secretion by splenocytes or in EL4 cells transiently transfected with pNFAT-SEAP. Concordantly, CBN-mediated enhancement of transcriptional activity of the IL-2 distal NF-AT reporter plasmid was abrogated by KN93. These findings support a role for CaM kinases in the signaling pathway associated with CBN-mediated enhancement of IL-2 expression. As activation of ERKs by CaM kinases has been shown in cultured vascular smooth muscle cells and transfected PC12 cells (Enslen *et al.*, 1996; Abraham *et al.*, 1997), whether CaM kinases can activate ERKs in T cells remains to be elucidated. Nevertheless, results from the present studies implicate a potential link between CaM kinases and ERKs, at least, in CBN-mediated enhancement of IL-2 expression by sub-optimally activated T cells. In addition, the present data also suggest a synergistic role between CaM kinases and PKC in CBN-mediated enhancement of IL-2 as evidenced by the observations that the magnitude of reversal by KN93 was potentiated by Ro-31-8220. It is notable that, in addition to PKC and PI-3 kinase, elevated intracellular calcium can also activate the ERK MAP kinase cascade in certain cell types (Chao *et al.*, 1992; Zohn *et al.*, 1995; Frolin *et al.*, 1995).

Moreover, CaM kinases have been demonstrated as being the signaling molecules responsible for the activation of ERKs by elevated intracellular calcium (Enslen *et al.*, 1996; Abraham *et al.*, 1997). In light of the well-established role of PKC and calcium-associated signaling as positive regulators of ERKs, our results suggest that the CBN-induced enhancement of IL-2 described in this investigation is mediated, at least in part, through an up-regulation of ERK MAP kinase associated signaling. Consistent with this premise, cannabinoids have been identified as being capable of mobilizing intracellular calcium (Felder *et al.*, 1992). Most pertinent to the present studies, Δ^9-THC was previously reported to enhance the rise in intracellular calcium in splenocytes activated with sCD3 (Nakano *et al.*, 1993b). In addition, PKC was also implicated by others as being positively modulated by cannabinoids and involved in cannabinoid-mediated induction of the growth-related gene Krox-24 (Bouaboula *et al.*, 1996), and in isolated preparations from rat forebrain. These results were subsequently confirmed ***in vitro*** where it was demonstrated that the plant-derived cannabinoids, CBN, Δ^9-THC and cannabidiol all enhanced PKC activity (Hillard and Auchampach, 1994). Collectively, these studies suggest that PKC and calcium-associated signaling pathways can be positively regulated by cannabinoids and that they are cellular targets involved in CBN-induced enhancement of the IL-2 secretion.

The present studies further characterize the underlying molecular mechanism by which CBN up-regulates IL-2 expression. The results suggest that the IL-2 distal NF-AT site is likely one of the cis elements in the IL-2 promoter responsible for CBN-mediated enhancement of IL-2 expression by sub-optimally activated EL4 cells. Several lines of evidence support this premise. First, under the same experimental condition where CBN

enhanced IL-2 protein secretion and steady state mRNA expression, a paralleled increase in the DNA binding activity to the IL-2 distal NF-AT site was observed in EMSA studies. Second, transient transfection of EL4 cells with the reporter gene pNFAT-SEAP driven by multiple IL-2 distal NF-AT motifs revealed a concomitant enhancement of both pNFAT-SEAP transcriptional activity and IL-2 secretion in the presence of CBN. Lastly, the enhancement by CBN of both IL-2 secretion and the NF-AT reporter gene activity was concordantly abrogated by KN93.

Multiple transcription factors, including NF-AT, NF-κB, AP-1 and Oct, are critical for the regulation of IL-2 gene transcription (Jain *et al.*, 1995; Serfling *et al.*, 1995). The activation of these transcription factors is mediated by various signaling pathways, such as the activation of NF-AT by a calcium-triggered cascade, NF-κB by protein kinases (i.e., PKC, PKA and Raf-1) and AP-1 by the MAP kinase associated cascades. As CBN-mediated enhancement of IL-2 secretion is closely correlated with the ERK MAP kinase signaling pathway, the present studies sought to elucidate which downstream transcription factors are involved in CBN-mediated enhancement of IL-2 expression. EMSA analyses were employed to examine three potential candidates, including NF-AT, NF-κB and AP-1, that are critical for IL-2 transcription and known to be regulated by the ERK MAP kinases (Jain *et al.*, 1995; Serfling *et al.*, 1995; May and Ghosh, 1998). Interestingly, only the DNA binding activity to the IL-2 distal NF-AT, but not NF-κB or AP-1, motif was markedly enhanced by CBN in sub-optimally activated EL4 cells, suggesting that the IL-2 distal NF-AT site may be the cis element through which CBN enhances IL-2 transcription. To further test this hypothesis, reporter gene assays were performed to examine the effect of CBN on the transcriptional activation of

the IL-2 distal NF-AT reporter gene pNFAT-SEAP. Consistent with the EMSA results, CBN markedly enhanced the transcriptional activity of the IL-2 distal NF-AT reporter gene, and, equally importantly, IL-2 secretion by the pNFAT-SEAP transfected EL4 cells. These observations demonstrate that, in the same model system, CBN enhanced not only the expression of the endogenous IL-2 gene, but also the expression of an exogenous reporter plasmid driven by the IL-2 distal NF-AT site. Taken together, these results strongly suggest that CBN-induced enhancement of the IL-2 gene expression is mediated, at least in part, through the IL-2 distal NF-AT site.

It is notable that none of the AP-1 response elements (proximal or distal AP-1 site) in the IL-2 proximal promoter/enhancer region are consensus AP-1 binding sequences (Serfling *et al.*, 1995). Nevertheless, the AP-1 transcription factor is crucial for the regulation of the IL-2 gene as it stabilizes the binding of NF-AT transcription factor to the distal NF-AT site (Rao *et al.*, 1997). The cooperativity between NF-AT and AP-1 reflects the requirement of dual stimulatory signals from calcium and PKC associated signaling pathways for the induction of IL-2 expression by primary T cells (Altman *et al.*, 1990). To further examine the proteins which bind to the distal NF-AT motif, supershift assays were employed. The results confirm that both NF-ATc1 and fos and jun members of AP-1 are present in the EMSA binding complex. These findings in conjunction with the aforementioned enhancement of NF-AT reporter gene activity by CBN suggest that the IL-2 distal NF-AT site is likely the downstream physiological target for the ERK MAP kinase pathway that mediates CBN-induced enhancement of IL-2 expression in T cells. This notion is further supported by the observation that the MEK specific inhibitor UO126 robustly suppressed the transcriptional activity of the IL-2 distal

NF-AT reporter gene in EL4 cells pretreated with CBN and sub-optimally activated by PMA. Interestingly, ligands for cannabinoid receptors have been found to modulate the activation of ERKs. In CB1- or CB2-transfected CHO cells, cannabinoids activate ERKs in the absence of activation stimuli (Bouaboula *et al.*, 1995, Bouaboula *et al.*, 1996). Conversely, inhibition of ERK activation, as well as inhibition of transcriptional activity driven by multiple AP-1 or NF-AT motifs, by cannabinoids has been demonstrated as being the underlying mechanism for cannabinoid-induced inhibition of IL-2 expression in T cells robustly activated by PMA/Io (Condie *et al.*, 1996; Faubert and Kaminski, 2000; Yea *et al.*, 2000). In addition, the present studies also show that the signaling cascade associated with ERKs and the downstream IL-2 distal NF-AT site is involved in the enhancement of IL-2 by CBN under the condition of sub-optimal T cell activation. Taken together, it appears that the differential effect on the regulation of ERK MAP kinases and NF-AT is closely correlated with the differential modulation on IL-2 by CBN. As the ERK MAP kinase pathway regulates a variety of cellular events and NF-AT is involved in multiple cytokine gene expression, the ERKs and NF-AT may be critical intracellular targets for cannabinoids to mediate the diverse biological effects induced by cannabinoids in the immune system, including IL-2 modulation.

The putative role of cannabinoid receptors, which negatively regulate the cAMP signaling cascade, was investigated in CBN-mediated enhancement of IL-2. The cAMP pathway is thus far the most extensively characterized intracellular signaling pathway coupled to cannabinoid receptors and known to be modulated by cannabinoid compounds (Howlett *et al.*, 1985; Rowley and Rowley, 1989). Cannabinoids inhibit adenylate cyclase activity resulting in inhibition of cAMP accumulation in a number of cell types,

including lymphoid cells (Schatz *et al.*, 1992; Condie *et al.*, 1996). In light of this, the exogenous membrane permeable cAMP analog, dibutyryl-cAMP, was used to explore whether the cAMP signaling pathway plays a role in CBN-mediated enhancement of IL-2 secretion. Interestingly, dibutyryl-cAMP failed to reverse CBN-mediated enhancement of IL-2 secretion by sCD3/CD28-activated splenocytes. It was notable that the highest concentration of dibutyryl-cAMP (100 μM) tested in the current study suppressed IL-2 secretion in all of the treatment groups thus providing evidence that dibutyryl-cAMP was active under the experimental condition.

As cannabinoid receptors are coupled to G_i/G_o GTP-binding protein to down-regulate the activity of adenylate cyclase, pertussis toxin was employed to investigate whether G_i/G_o is involved in CBN-mediated enhancement of IL-2. Preincubation of splenocytes with pertussis toxin (1 – 100 ng/mL) for 24 hr failed to block the CBN-mediated enhancement of IL-2 secretion. Pertussis toxin is a commonly employed agent to inhibit the function of G_i/G_o α-subunit, which can block the signal transduction initiated from the G_i/G_o protein-coupled receptors (Gilman, 1987). Pertussis toxin has been successfully employed by our laboratory in the past to abrogate Δ^9-THC-mediated inhibition of cAMP accumulation in splenocytes (Kaminski *et al.*, 1994). The results hence suggest that G_i/G_o may not be involved in CBN-mediated enhancement of IL-2 secretion in our experimental system. To further substantiate these findings, the role of PKA in sCD3/CD28-induced IL-2 secretion by splenocytes was examined. In accordance with aforementioned results, sCD3/CD28-induced IL-2 secretion was not influenced by the specific PKA inhibitor H89. Collectively, although it has been well

established that ligand binding to cannabinoid receptors causes a decrease in adenylate cyclase activity and cAMP accumulation in lymphoid cells, the G_i/G_o protein-adenylate cyclase-cAMP signaling pathway is apparently not involved in CBN-mediated enhancement of IL-2 secretion by sCD3/CD28-activated splenic T cells.

To further investigate the potential involvement of cannabinoid receptors, specific receptor antagonists for CB1 and CB2 were employed. Based on the fact that mRNA expression of both CB1 and CB2 has been detected in splenocytes by RT-PCR, suggesting coexpression of both receptors on leukocytes (Schatz *et al.*, 1997), receptor antagonists for CB1 and CB2 (SR141716A and SR144528, respectively) were employed in combination. The results showed that CBN-mediated enhancement of IL-2 secretion was not influenced by the presence of the cannabinoid receptor antagonists (0.1 – 1 μM of each antagonist). As the binding affinity of both antagonists to cannabinoid receptors has been determined to be in nanomolar range (Rinaldi-Carmona *et al.*, 1994; Rinaldi-Carmona *et al.*, 1998), the finding that the antagonists, even micromolar concentrations, failed to block the effect of CBN suggests that CB1 and/or CB2 cannabinoid receptors are not solely responsible for the CBN-mediated enhancement of IL-2 secretion under the present experimental condition.

Lastly, the role of cannabinoid receptors in CBN-mediated enhancement of IL-2 secretion was investigated by comparing the activities of several model cannabinoids possessing different cannabinoid receptor binding affinities. The rationale for this approach was based on the hypothesis that the potency among the various cannabinoids would correlate with cannabinoid receptor affinity if the effect was mediated exclusively through cannabinoid receptors. Most striking was the observations that both cannabidiol

and CP55,940 produced a similar magnitude of enhancement of IL-2 at comparable concentrations as CBN despite marked differences in their binding affinities to cannabinoid receptors (Munro *et al.*, 1993; Felder *et al.*, 1995; Thomas *et al.*, 1998). Moreover, the synthetic stereo isomers of WIN55212, WIN55212-2 and WIN55212-3, which exhibit high isomer-selectivity between their cannabimimetic activities (Compton *et al.*, 1992), were both capable of enhancing the sCD3/CD28-induced IL-2 secretion. Interestingly, the efficacy of these two stereoisomers was much lower than that of CBN. In addition, the low affinity cannabinoid receptor ligand WIN55212-3 produced greater enhancing effect than the high affinity ligand WIN55212-2 between 10 – 20 μM. Based on these studies, it appears that the affinity of cannabinoid ligands to cannabinoid receptors does not correlate with the efficacy of their enhancing effect on IL-2 secretion. These results further substantiate that cannabinoid enhancement of IL-2 secretion is either not mediated or not solely mediated through CB1 and/or CB2.

It has been known that cannabinoids modulate cellular signaling through both cannabinoid receptor-dependent and -independent mechanisms. In CHO cells, inhibition of cAMP accumulation by cannabinoids was demonstrated to be receptor-mediated, as this effect was only observed in cannabinoid receptor-transfected cells and, as expected, it required nanomolar concentrations of cannabinoids. Conversely, the release of arachidonic acid and increase in intracellular calcium were cannabinoid receptor-independent, as similar effects were observed in transfected and non-transfected cells, and it required micromolar concentrations of agonists (Felder *et al.*, 1992; Felder *et al.*, 1993). In addition to CHO cells, cannabinoid receptor-independent effects by cannabinoids were also observed in other preparations, such as the induction of apoptosis

by Δ^9-THC in prostate PC-3 cells and C6 glioma cells (Sanchez *et al.*, 1998b; Ruiz *et al.*, 1999), and the potentiation of cell growth by anandamide in hematopoietic cell lines (Derocq et al., 1998). Similar with these findings, data from the present studies also suggest a CB1/CB2-independent mechanism for the IL-2 enhancement by cannabinoids. Moreover, Hillard and Auchampach have reported that PKC could be activated by high affinity (i.e., Δ^9-THC) and low affinity (i.e., cannabidiol) cannabinoid receptor ligands (Hillard and Auchampach, 1994). The present studies show that CBN, CP55,940 and cannabidiol all enhance IL-2 with similar potency which correlate with the findings demonstrating a role of PKC in CBN-mediated enhancement of IL-2 secretion. Additionally, an increase in intracellular calcium was also reported to be a potential mechanism mediating soluble anti-CD3 induced enhancement of IL-2 production by splenocytes (Nakano *et al.*, 1993b). Our results also showed that CBN-mediated enhancement of IL-2 secretion by splenocytes was dependent on CaM kinases. As discussed earlier, transfection studies have demonstrated that cannabinoid-mediated increase in intracellular calcium was likely a cannabinoid receptor-independent phenomenon. Finally, cannabinoid-mediated enhancement of IL-2 requires cannabinoids at micromolar concentrations (5 – 20 μM) that are known to induce cannabinoid receptor-independent effects. Collectively, these several lines of independent evidence support a CB1/CB2-independent mechanism for the enhancement of IL-2 secretion by cannabinoids.

II. CBN-mediated inhibition of IL-4 expression

In addition to the modulation of IL-2, the present studies also demonstrate that cannabinoid treatment significantly diminished IL-4 expression by primary splenic T cells and in the thymoma derived EL4 T cell line. This effect by cannabinoids appears to be closely correlated with a down-regulation of transcription factor DNA binding activity to the P0 element in the IL-4 promoter. The P0 element is a composite DNA binding site for NF-AT and AP-1 proteins which are believed to bind coordinately to induce IL-4 transcription. Although the regulation of IL-4 gene expression has not yet been fully elucidated, several trans-acting factors are involved including NF-AT, AP-1, GATA-3 and c-Maf. Likewise their corresponding cis elements have been located in the proximal promoter region of IL-4 gene (Szabo *et al.*, 1993; Todd *et al.*, 1993; Hodge *et al.*, 1995; Ho *et al.*, 1996b; Zheng *et al.*, 1997). To date at least five NF-AT binding sites, named P elements, have been characterized in the IL-4 promoter and are believed to play a substantial role in the transcription of the IL-4 gene. It is these NF-AT sites that confer the sensitivity of the IL-4 gene to calcium signals in Th2 clones (Szabo *et al.*, 1993; Bruhn *et al.*, 1993; Li-Weber *et al.*, 1998). As a result cyclosporin A, a potent inhibitor of the calcium regulated phosphatase, calcineurin, is capable of blocking the transcriptional activity of the IL-4 promoter (Todd *et al.*, 1993; Kubo *et al.*, 1994; Hodge *et al.*, 1995). NF-AT is also a critical regulator of IL-2 transcription as evidenced by the profound inhibition cyclosporin A produces on IL-2 expression. Recent studies by our laboratory have demonstrated that IL-2 expression is markedly inhibited by plant-derived cannabinoids. The mechanism responsible for this inhibition is closely correlated with the disruption of NF-AT DNA binding within the IL-2 promoter through affects both on

NF-AT and its binding partner AP-1. In light of these previous finding, we investigated the effects of cannabinoids on IL-4 expression and the involvement of NF-AT regulation in order to provide additional insight into the sensitivity of T cell dependent antibody responses to inhibition by cannabinoids.

The present studies demonstrate that both Δ^9-THC and CBN markedly inhibited IL-4 secretion by T cells under various T cell activation conditions. The cannabinoid-mediated inhibition of IL-4 was concentration dependent and coincided with a concomitant inhibition of steady state mRNA expression, as determined by quantitative RT-PCR. It is also notable that the concentrations of Δ^9-THC and CBN required to produce inhibition of IL-4 correlated closely with those previously demonstrated to inhibit the anti-sRBC IgM antibody forming cell response. To gain additional insight into the potential mechanism of IL-4 inhibition by cannabinoids, the DNA binding activity of nuclear extracts from activated EL4 cells to the P0 site was examined by EMSA using an oligomer corresponding to the sequence from –70 to –45 of the mouse IL-4 promoter. The P0 site was chosen for the present studies because it is the P element closest to the transcriptional start site of IL-4 gene and it has been established as being critical for optimal transcriptional activity of the IL-4 promoter (Hodge *et al.*, 1995). In addition, the P0 site has also been implicated in Th2-specific expression of IL-4 as P0 site DNA binding activity was observed in Th2, but not in Th1 clones (Li-Weber *et al.*, 1998). The EL4 cell nuclear extracts, when analyzed by EMSA, exhibited multiple binding complexes which is similar to the results reported by Hodge and coworkers using the murine Th2 clone D10 cells (Hodge *et al.*, 1995). However, in spite of the formation of multiple complexes only one major binding complex was induced by PMA/Io in EL4

cells, and the binding activity of this complex was inhibited by cannabinoid treatment as well as by cyclosporin A (Fig 37).

The protein components which bind to the P0 site are NF-AT and AP-1 family members as evidenced by EMSA and supershift assays (Li-Weber *et al.*, 1998). This cooperative binding between NF-AT and AP-1 is likely due to the presence of P0 NF-AT binding site (-57 to –51) and the adjacent upstream octomer-like sequence (-67 to -60) in the IL-4 promoter (Li-Weber *et al.*, 1998). Consistent with these previous findings, results from the supershift assay in the present studies showed that in EL4 cells both NF-ATc1 and AP-1 are components of the major PMA/Io-inducible P0 DNA binding complex which was sensitive to cannabinoid-mediated inhibition. In addition, employment of RPA in the present studies also showed that IL-2 and IL-5, both of which are NF-AT regulated Th2-derived cytokine were also inhibited by Δ^9-THC and CBN in PMA/Io-activated EL4 cells. Collectively and in concordance with our past investigation of IL-2 regulation, these results suggest that signaling events proximal to the activation of NF-AT and AP-1 are likely intracellular targets for cannabinoids.

The inhibition by Δ^9-THC and CBN of IL-4 expression demonstrated in the present studies is in agreement with previous results by Berdyshev and coworkers where Δ^9-THC was reported to suppress IL-4 production in response to phytohemagglutinin-stimulation by human peripheral mononuclear cells (Berdyshev *et al.*, 1997). Inhibition of IL-4 by cannabinoids is also mechanistically consistent with suppression of humoral immunity. It is noteworthy that Δ^9-THC has also been reported to increase IL-4 production under certain conditions. Specifically, Δ^9-THC was found to concomitantly increase the Th2 cytokines, IL-4 and IL-10, while decreasing the Th1 cytokine,

interferon-γ, in mice infected with the intracellular parasite, *L. pneumophila*, (Newton *et al.*, 1994). The reason for the contrasting activity by Δ^9-THC on IL-4 expression in this latter model is unclear but may be related to the fact that host defense against *L. pneumophila* is primarily a cell-mediated response requiring Th1 cytokines (i.e. interferon-γ). Because cannabinoids inhibit the expression of interferon-γ (Blanchard *et al.*, 1986; Berdyshev *et al.*, 1997; Massi *et al.*, 2000), it is possible that the increased IL-4 production by Δ^9-THC observed in the *L. pneumophila* model is a secondary effect due to a shift in Th1/Th2 balance. In contrast, experiments conducted by Berdyshev *et al.* and the present studies using *in vitro* cell culture systems where T cells are activated directly under less diverse conditions, both Δ^9-THC and CBN inhibited IL-4 expression.

The putative role of the cAMP signaling pathway and cannabinoid receptors in cannabinoid-mediated inhibition of IL-4 expression was investigated with similar approaches employed for the IL-2 studies. The results suggest that cannabinoid compounds, plant-derived and synthetic, inhibit IL-4 secretion by activated T cells through a CB1- and CB2-independent mechanism. Several lines of evidence support this argument. First, pretreatment of splenocytes with SR141716A and SR144528 in combination failed to reverse either CBN or WIN55212-2 mediated inhibition of IL-4. Similar results with SR144528 were also observed in EL4 cells. Second, comparative studies employing cannabidiol and CP55,940, which possess low and high binding affinity for cannabinoid receptors, respectively, showed that both are approximately equally effective in their inhibition of IL-4. Hence, the binding affinity of cannabinoid compounds to cannabinoid receptors clearly has no correlation with the activity of these cannabinoids to inhibit IL-4. Third, preincubation of splenocytes and EL4 cells with

pertussis toxin for 24 hr failed to prevent CBN-mediated inhibition of IL-4 secretion by T cells. Finally, CBN-mediated inhibition of IL-4 secretion was not influenced by direct addition of dibutyryl-cAMP to cell cultures. To further examine the role of cAMP signaling pathway, the protein kinase A inhibitor, H89, was utilized to determine whether the downstream effector molecule PKA is involved in the regulation of IL-4 secretion. Consistent with the above findings, H89 alone did not produce any significant effects on sCD3/CD28-induced IL-4 secretion by splenocytes. Collectively, these data strongly suggest that under the present experimental condition the cAMP signaling pathway, and CB1 and CB2 receptors do not play a significant role in cannabinoid-mediated inhibition of IL-4 secretion by T cells.

Notably, dibutyryl-cAMP alone in the absence of CBN suppressed IL-4 secretion by sCD3/CD28-stimulated splenocytes, whereas ionomycin-induced IL-4 secretion by EL4 cells was robustly enhanced by dibutyryl-cAMP. These results are consistent with reports in the literature showing that cAMP exhibits marked but differing effects on IL-4 expression depending on the preparation of T cells, or the mode of T cell activation (Borger *et al.*, 1996; Wirth *et al.*, 1996). Nevertheless, dibutyryl-cAMP did not attenuate the inhibition of IL-4 secretion by CBN in either of the leukocyte preparations employed in the present studies.

To further assess the functional role of cannabinoid receptors in cannabinoid-mediated inhibition of IL-4, the effect of WIN55212 enantiomers on IL-4 secretion by splenocytes was assayed. As expected, the high affinity CB1/CB2 ligand WIN55212-2 produced robust inhibitory effect on IL-4 secretion by splenocytes. Conversely, the stereoisomer WIN55212-3 that is not a ligand for cannabinoid receptors was inactive in

the corresponding concentration range. The stereo-selective inhibition of IL-4 by the WIN55212 enantiomers strongly suggested that the mechanism by which WIN55212-2 inhibits IL-4 is highly specific and is dependent on the tertiary structure of the cannabinoid. Interestingly as with CBN, SR141716A and SR144528 did not antagonize WIN55212-2 mediated inhibition of IL-4 secretion. In addition, similar to the inhibition of IL-4, WIN55212-2 mediated suppression of cAMP accumulation was not reversed by CB1 and CB2 antagonists (data not shown). These data suggest that CB1 and CB2 are not solely responsible for the inhibition of IL-4 secretion and cAMP accumulation and, more importantly, imply a specific mechanism other than CB1 and CB2 for the stereo-selective effects by WIN55212 enantiomers on the inhibition of IL-4 secretion. In accordance with these findings, WIN55212-2 has been reported to inhibit cAMP formation through G protein-coupled receptors distinct from CB1 in cultured astrocytes (Sagan *et al.*, 1999). As the effect of WIN55212-3 was not studied by Sagan and coworkers, it is not known whether stereo-selectivity exists between the WIN55212 enantiomers on the inhibition of cAMP in astrocytes. Due to the high lipophilicity, one possible mechanism for cannabinoid-mediated biological effects has been historically proposed to be through direct interaction with cell membranes and membrane-associated proteins (Makriyannis and Rapaka, 1990). However, this clearly can not explain the cannabinoid-mediated inhibition of IL-4 in light of the stereo-selective effects observed by WIN55212.

The chemical structure of WIN55212 is strikingly different from that of plant-derived cannabinoids (Compton *et al.*, 1992). Presumably due to this structural property, interaction through distinct points between WIN55212 and cannabinoid receptors has

been described (Song and Bonner, 1996). In addition, WIN55212-2 has also been reported to inhibit calcium channels through both CB1 dependent and independent mechanisms in cultured hippocampal neurons (Shen and Thayer, 1998). Submicromolar concentrations WIN55212-2 inhibited calcium channels through CB1 receptor dependent mechanism as evidenced by attenuation with SR141716A, whereas concentrations greater than 1 μM inhibited calcium channels in a manner independent of CB1. As discussed earlier, the cannabinoid receptor-independent mechanism at micromolar concentrations of cannabinoid ligands has also been demonstrated in several other experimental systems. It is therefore possible that specific intracellular targets other than CB1 and CB2 are responsible for the observed stereo-selective effect in the present studies and yet to be elucidated.

III. CBN-mediated inhibition of IL-2 and IL-4 expression *in vivo*

The investigation of the effects by cannabinoids on IL-2 and IL-4 expression in cell culture studies was extended to an *in vivo* model of allergic airway disease induced by the aeroallergen, ovalbumin (Ova). It has been well characterized that $CD4^+$ Th2 cells play a pivotal role in the morphologic and immunologic changes associated with the allergic airway response induced by Ova (Anderson and Coyle, 1994; Wills-Karp, 1999). Th2 cytokines, including IL-4, IL-5 and IL-13, which are primarily produced by Th2 cells, are critical mediators involved in both the early priming of immune competent cells (i.e., IL-4), and the inflammatory reactions associated with the allergic airway disease (i.e., IL-5 and IL-13). Using this *in vivo* model, the present investigation demonstrated that both IL-2 and IL-4 steady state mRNA expression in the lungs of Ova-sensitized

mice was elevated 24 hr after challenge with aerosolized Ova. In addition, analysis of cytokine mRNA expression in the lungs by the RNase protection assay (RPA) revealed a concomitant increase in two critical Th2 cytokines, IL-5 and IL-13. These observations confirm that T cells, most likely Th2 cells, are closely associated with the allergic airway response induced by Ova. More importantly, these results indicate that T cell activation and cytokine gene expression in the lungs are measurable events in the murine airway allergen model employing Ova. We believe that the present animal model provides an experimental system to study some of the hallmark pathophysiologic properties of allergic airway disease as well as the ability to modulate these properties with potential therapeutic agents.

Consistent with the latter application of the model, the effect of CBN and Δ^9-THC on cytokine gene expression was investigated in mice administered CBN or Δ^9-THC (50 mg/kg) daily for 3 consecutive days immediately prior to Ova sensitization and then before Ova challenge. The expression of both IL-2 and IL-4 steady state mRNA in the lungs was significantly attenuated by the CBN or Δ^9-THC administration, as measured by competitive RT-PCR. Likewise, the expression of IL-5 and IL-13 was also markedly attenuated by CBN or Δ^9-THC treatment, as detected by RPA. The marked attenuation of IL-2 expression by cannabinoids in the Ova model correlates with the inhibition of IL-2 expression observed *in vitro* when T cells were activated with strong inducers of IL-2.

Examination of the sensitization and elicitation of the Ova response demonstrated that CBN produced greater inhibitory effect on the IL-2 and IL-4 expression when the drug was administered before both sensitization and challenge phase. In addition, results from the RNase protection assay suggest that the sensitization phase was apparently more

sensitive than the challenge phase as evidenced by the marked attenuation of IL-5 and IL-13 expression when CBN was administered to mice prior to Ova sensitization, but not prior to Ova challenge. With respect to the critical role of IL-4 in the early priming of immune competent cells in the animal model, these observations are consistent with the results from *in vitro* studies showing marked inhibition of IL-4 expression by cannabinoids. Since the detection of mRNA expression by RPA is rather qualitative, more accurate quantification of the expression level of individual cytokine mRNA by other methods, such as competitive RT-PCR, would be necessary to further address this issue. Nevertheless, results from this series of *in vivo* studies demonstrating the attenuation of IL-2 and Th2 cytokine gene expression by cannabinoids correlate with the aforementioned result that Δ^9-THC down-regulate the expression of IL-2, IL-4 and IL-5 in PMA/Io-activated EL4 cells. Collectively, data from these studies confirm that cannabinoids possess inhibitory effects on cytokine gene expression not only in cell culture systems, but also *in vivo* in our respiratory allergen model. Moreover, these results are concordant with the notion that NF-AT is likely a critical target for cannabinoids, since IL-2, IL-4, IL-5 and IL-13 are all regulated by NF-AT (Rao *et. al.*, 1997).

As Th2 cytokines are important mediators in the pathophysiology of allergic airway disease, the marked inhibition by cannabinoids on IL-2, IL-4, IL-5 and IL-13 expression implicates that cannabinoids may also suppress the downstream immune responses mediated by Th2 cytokines, such as the production of IgE. It is well established that both IL-4 and IL-13 are the determining factors that mediate the isotype switching of antibody production by plasma cells from IgG to IgE. Clinical studies have

shown a close correlation between asthma and serum IgE levels (Burrows *et al.*, 1989). IgE-mediated immune reactions have been recognized as an important mechanism in triggering the release of mediators from inflammatory cells (i.e., mast cells), which contributes to the pathophysiology associated with allergic disease (Fred Wong and Koh, 2000). More recently, immune complexes of IgE and the antigen Ova have also been shown to be more potent than antigen alone in the induction of allergic inflammation in Ova-sensitized mice, further supporting an important role for IgE in allergic airway disease (Zuberi *et al.*, 2000). In light of the key role of IgE in allergic diseases, the effect of CBN and Δ^9-THC on the production of Ova-specific IgE was examined in Ova sensitized and challenged mice. Consistent with the inhibition of IL-4 and IL-13 mRNA expression, both CBN and Δ^9-THC significantly attenuated the level of Ova-specific serum IgE in mice that were sensitized with Ova and subsequently challenged twice with Ova. These observations clearly show that, in addition to cytokine expression, IgE antibody production which is a downstream consequence of the T cell-derived cytokines, was also suppressed by cannabinoids. Interestingly, with recent advances in understanding the pivotal role of Th2 cytokine in the pathophysiology of asthma, specific antibodies against IL-4, IL-5 and IgE have been suggested as potential therapeutic agents for treatment of asthma (Fred Wong and Koh, 2000). In light of the inhibitory effect of CBN and Δ^9-THC on both T cell cytokine expression and Ova-specific IgE production, plant-derived cannabinoid compounds may represent a novel class of therapeutic agents for the treatment of allergic airway diseases, such as asthma and allergic rhinitis.

SUMMARY AND CONCLUSIONS

I. Summary

The present dissertation research demonstrates that CBN, a plant-derived cannabinoid with modest effect on the CNS, possesses immunomodulatory activity on cytokine gene expression by T cells in culture and in a murine model of allergic airway disease. CBN elicits both enhancing and inhibitory effects on IL-2 expression by T cells, which is dependent on the magnitude of T cell activation. The contrasting effects of CBN on IL-2 appear to be closely correlated with the regulation of the activation of ERKs and NF-AT. More in depth studies have elucidated that the underlying mechanism for CBN-induced enhancement of IL-2 expression involves CaM kinases, PKC, ERKs, and the IL-2 distal NF-AT site as the downstream cis element responsible for increased transcription. Additionally, investigation of the effect by cannabinoids on IL-4 expression demonstrated that CBN and Δ^9-THC produced marked inhibition of IL-4 protein secretion by T cells under various activation conditions. The IL-4 steady state mRNA expression by PMA/Io-activated EL4 cells was also suppressed by cannabinoids. A parallel attenuation of nuclear factor binding to the P0 NF-AT motif of the IL-4 promoter was also observed in T cells pretreated with cannabinoids, suggesting that the inhibition of IL-4 expression is, at least in part, mediated by the down-regulation of NF-AT activation. Interestingly, steady state mRNA expression for IL-5, a gene also critically regulated by NF-AT was similarly inhibited by Δ^9-THC treatment, further supporting a role of NF-AT in cannabinoid-mediated inhibition of T cell cytokine expression.

The results in cell culture studies were extended to an *in vivo* murine model of allergic airway disease induced by Ova. The steady state IL-2, IL-4, IL-5 and IL-13 mRNA expression in the lungs of Ova-sensitized mice was markedly elevated by Ova challenge. This elevation of cytokine expression was attenuated by administration of CBN or Δ^9-THC prior to Ova sensitization and then before Ova challenge. Consistent with the suppression of cytokine expression, the level of Ova-specific serum IgE in mice sensitized and challenged with Ova was also significantly attenuated by CBN or Δ^9-THC. Taken together, these results confirm a similar profile of immune modulation on cytokine gene expression *in vivo* and *in vitro*. The ability of CBN to suppress the expression of multiple cytokines regulated by NF-AT, including IL-2, IL-4, IL-5 and IL-13, further supports the notion that NF-AT may be a critical intracellular target for cannabinoids. In addition, the suppression of cytokine gene expression and IgE production by CBN and Δ^9-THC demonstrated in the Ova model suggests that molecules that are structurally-related to plant-derived cannabinoids may represent a novel class of therapeutic agents for the treatment of allergic diseases.

Although the cAMP cascade is the best characterized signaling pathway coupled to cannabinoid receptors, results from the present investigation suggests that cannabinoid-induced modulation of cytokine expression by T cells is likely not mediated or exclusively mediated by cannabinoid receptors via negative coupling to cAMP pathway. Several lines of evidence support this argument. First, CBN-mediated modulation of cytokine expression was not reversed by dibutyryl-cAMP. Second, preincubation of T cells with pertussis toxin did not alter the CBN-mediated effects on cytokine expression. Finally, CB1 and CB2 antagonists (SR141716A and SR144528,

respectively) failed to block the CBN-mediated effects. In addition, the PKA specific inhibitor, H89, exhibited modest if any influence on either IL-2 or IL-4 expression under the present experimental conditions thus further ruling out the involvement of cAMP signaling pathway. However, it is notable that WIN55212 enantiomers exhibited stereo-selective inhibition of IL-4, indicating a highly specific molecular target for cannabinoid-mediated inhibition of IL-4 expression. Collectively, although CB2 has been implicated in mediating cannabinoid effects on antigen presentation and on the accessory cell functions of macrophages to stimulate T cells (Buckley *et al.*, 2000; McCoy *et al.*, 1999), the direct effect by cannabinoids on cytokine expression by T cells appears to be independent of CB1 and CB2. These data indicate that both CB1- and/or CB2-dependent and independent mechanisms are involved in cannabinoid-mediated immune modulation.

In conclusion, the present research project has demonstrated that plant-derived cannabinoid compounds produce marked influences on cytokine gene expression by T cells. In addition to IL-2, several other Th2-derived cytokines are also sensitive to the inhibition by cannabinoids both *in vitro* and *in vivo*. Based on the integral role of T cell cytokines, including IL-2, IL-4, IL-5 and IL-13, in B cell proliferation and differentiation, these findings provide new mechanistic insights concerning the apparent preferentially sensitivity of T cell-dependent humoral immune responses to inhibition by cannabinoids. In addition, the current studies suggest that the modulation of cytokine gene expression by cannabinoids is closely correlated with the regulation of signaling pathways associated with NF-AT activation. This work is also the first to demonstrate the molecular mechanism for CBN-mediated enhancement of IL-2 expression.

II. Concluding discussion

It is notable that CBN differentially modulated IL-2 expression depending on the magnitude of T cell activation stimuli, whereas IL-4 expression induced by all tested activation stimuli was strongly inhibited by CBN. One factor that may account for this discrepancy is the difference between the transcriptional regulation of these two cytokines. Preliminary results of the present dissertation research revealed that the magnitude of IL-2 secretion induced by different T cell activation stimuli in splenocytes was sCD3/CD28/PMA ≥ iCD3/CD28 ≥ sCD3/PMA ≥ PMA/Io >> sCD3/CD28/Io ≥ sCD3/Io ≥ sCD3/CD28 ≥ sCD3. These results implicate that the ERK MAP kinase signaling cascade plays a substantial role in the regulation of IL-2 transcription. In fact, PMA-triggered signaling events are required for IL-2 expression by primary T cells, and, in the presence of Io or αCD3 mAb, PMA robustly enhances the magnitude of IL-2 gene expression (Altman *et al.*, 1990). In contrast, the ERK MAP kinase pathway appears not to play a significant role in the activation of IL-4 gene transcription, as evidenced by the observations that sCD3/CD28 was a stronger stimulus for the induction of IL-4 expression than the other aforementioned stimuli. Because CBN markedly up-regulated the ERK MAP kinase activation under conditions where ERKs were only modestly activated by sub-optimal T cell activation stimuli (i.e., sCD3/CD28), it is therefore not surprising that CBN elicited an enhancing effect on IL-2, but not IL-4, expression via an up-regulation of ERK activation.

The enhancement of IL-2 expression induced by CBN demonstrated in the present dissertation studies is intriguing in that an up-regulation of ERK and NF-AT activation was identified to be the underlying molecular mechanism. Interestingly, previous reports

from our laboratory showing that cannabinoids suppressed PMA/Io-induced IL-2 expression by down-regulating ERK, AP-1 and NF-AT activation (Condie *et al.*, 1996; Yea *et al.*, 2000; Faubert and Kaminski, 2000). Together these results suggest that signaling pathways associated with ERK and NF-AT activation are important intracellular targets for cannabinoids. Because CBN-mediated enhancement of both IL-2 expression and the distal NF-AT transcriptional activity was attenuated by KN93, CaM kinases and intracellular calcium are therefore potential signaling molecules involved in the enhancing effects by CBN on IL-2 expression. To further explore the role of CaM kinases and calcium in cannabinoid-mediated modulation of IL-2 and IL-4 expression, the effect of KN93, as well as other CaM kinase inhibitors, on CBN-mediated inhibition of IL-2 and IL-4 expression would need to be investigated. In light of the fact that expression of the IL-4 gene is critically regulated by calcium associated signaling pathways, experimental approaches employing CaM kinase inhibitors would provide more insight into the molecular mechanism of cannabinoid-induced immune modulation. Additionally, a more comprehensive characterization of the effect of cannabinoids on CaM kinase activation and intracellular calcium mobilization is also needed to further understand the role of calcium associated signaling pathways in cannabinoid-induced modulation of T cell function.

In addition to elucidating the downstream molecular mechanism responsible for CBN-induced IL-2 enhancement, the present dissertation research investigated possible upstream targets that may mediate the effect of CBN. As two major types of cannabinoid receptors have been identified and cloned, the primary focus of the present studies was to characterize the role of CB1 and/or CB2 receptors in CBN-mediated modulation of IL-2

and IL-4 expression. It is somewhat surprising that both IL-2 enhancement and IL-4 inhibition by CBN were not attenuated by receptor antagonists for CB1 and CB2. In spite of this, the WIN55212 enantiomers produced stereo-selective inhibition of IL-4. However, the inhibition of IL-4 secretion by WIN55212-2 was not attenuated by CB1 and CB2 antagonists. These results suggest a highly specific molecular target other than CB1 and CB2 for WIN55212-2-induced inhibition of IL-4 expression. Although it is currently unknown whether WIN55212-2 and CBN inhibited IL-4 expression via the same mechanism, two lines of evidence suggest that WIN55212-2 and plant-derived cannabinoids may act similarly. First, ligand binding assays demonstrated that specific binding of WIN55212-2 to CB2 is attenuated by the presence of CBN, Δ^9-THC and cannabidiol (Munro *et al.*, 1993). Second, similar to other synthetic cannabinoids (i.e., CP55,940), WIN55212-2 possesses potent cannabimimetic activity (Compton *et al.*, 1992). Therefore, the inhibition of IL-4 expression by plant-derived cannabinoids and by WIN55212-2 observed in the present studies may be mediated by a common molecular target that is not CB1 or CB2, and yet to be determined. One potential target that is currently being explored by this laboratory is the glucocorticoid receptor (GR). In fact, it has been previously reported that CBN and Δ^9-THC exhibit specific binding to the GR (Eldridge and Landfield, 1990). Concordantly, preliminary results of our current studies also demonstrated that CBN increased nuclear translocation of GR in sCD3/CD28-activated splenocytes. Our current hypothesis is that cannabinoids may inhibit IL-4 expression by acting as dissociated glucocorticoids. Two critical approaches will be employed to further test this hypothesis. One is to characterize the binding of

cannabinoids to GR by ligand binding assays. The other is to investigate protein-protein interactions between cannabinoid-activated GR and NF-AT.

Investigation of the effect of CBN and Δ^9-THC on cytokine mRNA expression *in vivo* confirms that the expression of IL-2 and Th2 cytokines associated with Ova-induced allergic airway response was markedly attenuated by cannabinoid administration before Ova sensitization and then before Ova challenge. Interestingly, phase analysis studies showed that the challenge phase is apparently less sensitive to the inhibition of cytokine expression by cannabinoids. It is not clear at present why cannabinoid administration before Ova challenge failed to attenuate the cytokine response. One potential factor relevant to this point is the dose of cannabinoids. As mice administered with cannabinoids before either Ova sensitization or Ova challenge received only 50% of the total dose of cannabinoids as compared to those mice administered with cannabinoids before Ova sensitization and then before Ova challenge. As a result, CBN and Δ^9-THC failed to attenuate IL-2 and IL-4 expression when they were administered before either Ova sensitization or Ova challenge due to the insufficient dose. Nevertheless, a modest but statistically insignificant attenuation of IL-2 and IL-4 expression by cannabinoid administration was observed when CBN or Δ^9-THC was administered prior to Ova sensitization. Moreover, this modest effect on IL-2 and IL-4 expression by cannabinoids may contribute to the cannabinoid-mediated marked suppression of IL-5 and IL-13 expression as evidenced by RPA. Conversely, the expression of IL-5 and IL-13 was not influenced by cannabinoid treatment in mice administered with CBN or Δ^9-THC before Ova challenge only. These results clearly demonstrated that cannabinoids elicited differential effects on IL-5 and IL-13 expression between the sensitization and the

challenge phase. One important aspect related with the expression of IL-5 and IL-13 is that these two Th2 cytokines are expressed by differentiated Th2 cells, whereas Th cells at earlier stages of differentiation (i.e., Th0 cells) express IL-2, IL-4 and IFN-γ. Accordingly, one possibility concerning the different sensitivity between sensitization and challenge phase to the inhibition of IL-5 and IL-13 expression by cannabinoids would be the differential sensitivity of Th0 and Th2 cells to cannabinoids. It is possible that T cells at earlier stages of differentiation are more sensitive to the modulation by cannabinoids than T cells at latter stages of differentiation. Notably, in contrast to the modulation of IL-2 expression by cannabinoids, which has been extensively studied by several laboratories including our own, the effect of cannabinoids on IL-5 and IL-13 expression has not yet been systemically characterized. More comprehensive investigations of the effect of cannabinoids on IL-5 and IL-13 expression by T cells are needed to answer the question whether cannabinoids differentially modulate the expression of cytokines by T cells at different stages of differentiation.

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