



COMMENTARY

# Immune Regulation by Cannabinoid Compounds Through the Inhibition of the Cyclic AMP Signaling Cascade and Altered Gene Expression

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**ABSTRACT.** Immune modulation by cannabinoid compounds, although established for several decades, has remained up until recently mechanistically obscure. The identification of a novel class of G-protein coupled receptors that negatively regulate the cyclic adenosine 3':5'-monophosphate (cAMP) cascade, bind cannabinoids, and are expressed on cells within the immune system has provided new insights into the mechanism for their biologic activity. Although the role of the cAMP cascade in the regulation of immune responses is itself highly controversial, a number of laboratories recently demonstrated that aberrant regulation of this signaling pathway leads to alterations in the expression of critical immunoregulatory genes, cell cycle arrest, and decreased immune function. This profile of effects is strikingly similar to that which is induced in leukocytes in the presence of cannabinoid compounds. In the present commentary, a putative mechanism of immune regulation by cannabinoids is proposed. This mechanism is discussed in the context of decreased cAMP signaling, the transcription factors that are consequently adversely regulated, and immunologically relevant genes that ultimately exhibit altered expression. *BIOCHEM PHARMACOL* 52;8:1133–1140, 1996.

**KEY WORDS.** cannabinoids; immune suppression; cAMP; protein kinase A; cAMP responsive element binding protein; AP-1; NK- $\kappa$ B; interleukin-2; inducible nitric oxide synthase

The immunoinhibitory activity of cannabinoid compounds has been well established over the past three decades using a variety of experimental model systems (reviewed in Refs. 1–3). These studies have demonstrated clearly that cannabinoids produce a broad array of effects on the immune system including inhibition of innate, humoral, and cell-mediated immune responses. As will be discussed, the mechanism for immune modulation by cannabinoids, although not characterized extensively, has been convincingly demonstrated to involve cannabinoid receptors. This class of receptors has been identified on most of the major cell-types within the immune system. Although the teleologic role for cannabinoid receptors is presently unknown, several endogenous molecules have been identified recently which exhibit low affinity binding to this family of receptors. Interestingly, the putative endogenous cannabinoid receptor ligands identified thus far are structurally related to arachidonic acid [4–7]. The physiologic significance of cannabinoid receptor binding by arachidonic acid derivatives is unclear and presently under investigation by a number of laboratories. In spite of the recent identification of these endogenous molecules, the best characterized canna-

binoid receptor ligands to date are the plant-derived cannabinoids. The prototypic cannabinoid in this group,  $\Delta^9$ -THC $\dagger$  is the primary psychoactive constituent in the cannabis sativa plant, more commonly known as marijuana.  $\Delta^9$ -THC is one of over 60 structurally related congeners possessing varied degrees of CNS and immunological activity in marijuana. The objective of this commentary will be to discuss one putative mechanism by which cannabinoids modulate the immune system, that being through their interaction with cannabinoid receptors. Included in this commentary will be a critical discussion of the signal transduction pathways involved in this receptor-associated mechanism that lead to the aberrant regulation of two immunologically relevant genes, IL-2, a cytokine responsible for the clonal expansion of T-cells, and iNOS, the

$\dagger$  Abbreviations:  $\Delta^9$ -THC,  $\Delta^9$ -tetrahydrocannabinol; G-protein, guanine nucleotide-binding protein; PMA, phorbol-12-myristate-13-acetate; I $\alpha$ , ionomycin; RT-PCR, reverse transcription-polymerase chain reaction; CB1, cannabinoid receptor type 1; CB2, cannabinoid receptor type 2; iNOS, inducible nitric oxide synthase; NO, nitric oxide; cAMP, cyclic adenosine 3':5'-monophosphate; CRE, cAMP response element; NF- $\kappa$ B, nuclear factor for immunoglobulin  $\kappa$  chain in B cells; I $\kappa$ B, inhibitor protein of NF- $\kappa$ B; LPS, lipopolysaccharide; sRBC, sheep erythrocytes; PKA, protein kinase A; PKC, protein kinase C; IL-1, interleukin-1; IL-2, interleukin-2; IL-5, interleukin-5; INF $\gamma$ , interferon- $\gamma$ ; CREB, cAMP response element binding protein; CREM, cAMP response element modulator; ATF, activating transcription factor; NF-AT, nuclear factor of activated T-cells; and MAP, mitogen-activated protein.

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enzyme responsible for the production of NO, a secretory factor which mediates cytolytic activity by mononuclear cells.

### CANNABINOID RECEPTORS

Structurally, cannabinoid receptors possess seven transmembrane regions and belong to the G-protein receptor superfamily. Upon ligand binding, cannabinoid receptors negatively regulate adenylate cyclase to inhibit the conversion of ATP to cAMP through a pertussis toxin-sensitive GTP $\alpha$ -inhibitory protein. To date two major forms of cannabinoid receptors have been isolated and cloned, CB1 (brain-type) [8], which is expressed most abundantly within the CNS with low level expression in peripheral tissues including the immune system, and CB2 (peripheral-type) [9], which thus far has been shown to be expressed primarily on leukocytes with no expression in the brain. Interestingly, the two forms of the cannabinoid receptor are strikingly dissimilar, sharing only 44% identity with this increasing to 68% when exclusively comparing the transmembrane regions [9], that portion of the receptor involved in ligand binding. Equally surprising is that in spite of the structural differences, most cannabinoid receptor ligands studied to date exhibit remarkable similarity in binding characteristics to both forms of the receptor. One exception to this rule is the plant-derived cannabinoid, cannabitol, which possesses significantly greater binding affinity for CB2 than for CB1 [9]. Moreover, cannabitol exhibits good binding affinity to mouse spleen cells<sup>3</sup> and has immunomodulatory activity in a number of leukocyte preparations [10] (unpublished observations) as would be expected with cells that primarily express CB2 receptors.

Since first being identified in rat brain [8], examination of CB1 within the immune system has revealed its expression in human B-cells, T-cells, and monocytes [11], and in mouse spleen [12], while being below the level of quantitation in mouse thymus even as assayed by RT-PCR.‡ The more recently identified cannabinoid receptor, CB2, has also been partially characterized with respect to gene expression within the immune system. These studies have demonstrated CB2 transcripts in rat spleen [9], mouse spleen and thymus, and a number of immune system-derived cell lines including the T-cell lines, EL4.IL-2 [10], HPB-ALL<sup>3</sup>, Jurkat E6-1<sup>3</sup>, the monocytic lines, HL60 [9] and RAW264.7 [13], and the mast cell line, RBL-2H3 [14]. Extensive mapping of CB1 and CB2 protein expression in primary tissues is awaiting the development of receptor-specific antibodies.

### DOES THE cAMP SIGNALING CASCADE POSITIVELY REGULATE IMMUNE RESPONSES?

The role of cannabinoid receptors in mediating immune inhibition through negative regulation of the cAMP sig-

naling pathway has been somewhat puzzling in light of the fact that this signaling pathway has long been perceived as negatively regulating lymphocytic and monocytic responses. This premise is based on numerous studies that have clearly demonstrated the negative influence of high concentrations of cAMP analogs (>100  $\mu$ M) on leukocyte function in culture. Under these conditions, immune inhibition has been demonstrated in B- and T-cells [15–17] and most often is also associated with an arrest in cell cycle progression [18]. The latter observation pertaining to altered cell cycle is especially interesting in light of the recent finding that the cAMP-dependent kinase, PKA, participates in the regulation of cell cycle progression as demonstrated in a *Xenopus* model system. Results from the aforementioned studies showed that: (a) inhibition of PKA induces cell cycle arrest while simultaneously blocking the degradation of the M phase promoting factor, cyclin B-p34<sup>cdc2</sup> complex; (b) intracellular cAMP concentrations and PKA activity coordinately increase and decrease at specific phases of the cell cycle; (c) PKA activators, including low cAMP concentrations (<50  $\mu$ M) of 8-bromo-cAMP, reverse cell cycle arrest induced by a recombinant PKA regulatory subunit that blocks endogenous PKA activation; and, as previously demonstrated in lymphoid cells, (d) high cAMP concentrations (>50  $\mu$ M) markedly inhibit cell cycle progression [19]. The authors attributed the inhibitory effects associated with high cAMP concentrations to be coupled to the induction of excessive nonphysiological amounts of PKA activity, which in turn mediate a significant amount of nonspecific phosphorylation. Almost without exception, high concentrations of intracellular cAMP (e.g. membrane-permeable cAMP analogs at concentrations >100  $\mu$ M) produce inhibition of immune function. This result is not surprising in light of the fact that most lymphocyte-associated effector functions require cell proliferation.

In contrast to the aforementioned reports, the inhibition of adenylate cyclase and, consequently, the decreased cAMP signaling that is produced by cannabinoids in leukocytes are closely correlated with decreased immune function and not immune enhancement. Furthermore, this observation is consistent with an increasing number of studies that have convincingly shown not only a positive but in many cases an obligatory role for cAMP as a mediator of cellular responses in immunocompetent cells. From these findings it is now clear that to generalize that the cAMP signaling pathway is strictly involved in negative regulation of immunological responses is an oversimplification and likely a consequence of nonphysiologic experimental conditions. It is notable that the same cAMP analogs that have been shown to be inhibitory at high concentrations are immunostimulatory at lower, physiologically relevant, concentrations (<100  $\mu$ M) in a variety of assay systems [20–22]. The critical role of cAMP in lymphoid cell function is further supported by the fact that there is a rapid transient burst in adenylate cyclase activity within the first 5 min

‡ Schatz AR, Lee M, Condie R, Pulaski JT and Kaminski NE, Manuscript submitted for publication.

following lymphocyte activation by treatment with mitogens or phorbol ester plus calcium ionophore [21, 23–27], strongly suggesting positive lymphocyte regulation through this mechanism. Moreover, it is clear that the inhibitory effects produced by cannabinoids on immunocompetent cells can be abrogated by blocking or reversing the cannabinoid-induced decrease in intracellular cAMP. This has been demonstrated in several ways. For example, antibody responses requiring helper T-cells and macrophages as accessory cells, as in the case with the T-cell dependent antigen, sRBC, are markedly sensitive to inhibition by cannabinoid compounds [28]. However, inhibition of this response can be abrogated *in vitro* by direct addition of low concentrations (50–100  $\mu$ M) of membrane-permeable cAMP analogs (i.e. dibutyryl- or 8-bromo-cAMP) to the cell cultures. It is notable that this reversal of the antibody forming cell responses can only be achieved if the cAMP analogs are added to culture within the first 30–60 min following antigen stimulation [21]. This observation is consistent with the hypothesis that immune dysfunction by cannabinoids is due to the inhibition of an early T-cell activation event and/or mediated through the disruption of critical signaling events induced by macrophage–T-cell interactions. It is important to emphasize that, as described by many laboratories, these studies also demonstrated that high concentrations (>100  $\mu$ M) of either dibutyryl-cAMP or 8-bromo-cAMP, alone in the absence of cannabinoids, are markedly inhibitory to spleen cell function [21]. Similarly, cannabinoid-mediated inhibition of the anti-sRBC antibody response as well as inhibition of lymphocyte proliferation induced by phorbol ester and calcium ionophore can also be attenuated by pretreating immunocompetent cells overnight with pertussis toxin, an agent that ADP-ribosylates GTP-inhibitory proteins [21]. By doing so, the transduction of signaling from the receptor to adenylate cyclase via GTP-binding proteins is blocked. Consistent with the above observations which suggest a direct correlation between a decrease in immune function and an inhibition of cAMP signaling, the inhibitory effects of  $\Delta^9$ -THC on the anti-sRBC antibody response are attenuated by concomitant treatment of immunocompetent cells with the hormone, glucagon [29]. Glucagon acts by producing an increase in intracellular cAMP through its own respective G-protein-coupled receptors that positively regulate adenylate cyclase. Under these conditions, the inhibition of cAMP formation by  $\Delta^9$ -THC is presumably offset by an increase in intracellular cAMP formation initiated through glucagon receptors. Likewise, treatment of spleen cells with dideoxyadenosine, an adenylate cyclase inhibitor, induces a marked inhibition of the anti-sRBC response and, as demonstrated with cannabinoid treatment, this inhibition can be attenuated similarly by glucagon [29]. As will be discussed in more detail below, cannabinoids also inhibit LPS-induced NO formation by mouse-derived macrophages and RAW264.7 cells, a macrophage-derived line [13]. The inhibition of this macrophage-mediated response is abrogated

by concomitant treatment of the cells with 8-bromo-cAMP (50–100  $\mu$ M). All of the examples described above are consistent with a positive regulatory role for the cAMP signaling cascade in immune cell function. Further, these findings support the conclusion that the cAMP signaling pathway is one of the primary intracellular targets responsible for alterations in immunocompetence by cannabinoids.

#### **CANNABINOID-MEDIATED INHIBITION OF HELPER T-CELL FUNCTION THROUGH A DECREASE IN cAMP-ASSOCIATED SIGNALING**

More recently, efforts by a number of laboratories have been directed toward investigating the effects of cannabinoids on specific cell-types as well as subpopulations within the immune system in an attempt to further elucidate the mechanism by which this class of compounds is capable of altering immunocompetence. One cell-type being investigated extensively with respect to cannabinoid modulation is the helper T-cell. This effort is based on previous findings suggesting that helper T-cells exhibit a marked sensitivity to inhibition by cannabinoids [28] coupled with the role of this T-cell subpopulation in the maintenance of immunocompetence. The primary function of helper T-cells is the secretion of lymphokines that regulate T- and B-cell proliferation and differentiation during cell-mediated and humoral immune responses. One of the best characterized lymphokines with respect to regulation at the transcriptional level is IL-2. Furthermore, it has been suggested by findings from a number of laboratories that the cAMP signaling pathway may play an important role in the regulation of IL-2. Not surprisingly, based on the inhibitory effects exerted on adenylate cyclase, cannabinoid compounds markedly inhibit IL-2 production by T-cells [10, 30]. Recently, the extensively characterized IL-2-secreting murine-derived thymoma, EL4.IL-2, was used as a model to investigate the role of cannabinoid-mediated inhibition of cAMP signaling on the regulation of IL-2. EL4.IL-2 cells, which express RNA transcripts only for CB2 receptors and not CB1, exhibit inhibition of forskolin-stimulated cAMP accumulation following cannabinoid treatment. This strongly suggests that only the CB2 form of the receptor is expressed by this cell line and that these receptors are, in fact, functional [10]. The cAMP cascade is regulated by the formation of cAMP which binds to the regulatory subunits of PKA resulting in the release and activation of PKA-catalytic subunits. These catalytic subunits go on to phosphorylate a variety of intracellular proteins including the CREB/ATF family of transcriptional regulators which consist of CREB, ATF, and CREM. CREB, which is the best characterized member of this family, is activated by PKA-mediated phosphorylation at Ser residue 133 [31] and forms either homo- or heterodimers with a number of other transcription factors capable of binding CRE DNA sequences present in the promoter region of a variety of genes. Upon stimulation of EL4.IL-2 cells with forskolin in the presence

of  $\Delta^9$ -THC, PKA is inhibited concentration dependently. Moreover, examination of CREB/ATF family member activation by gel shift assays, using a CRE consensus motif, demonstrated that forskolin treatment significantly up-regulates DNA binding by 30 min, exhibiting peak binding activity at 60 min followed by a rapid decrease at 90 min. This finding is consistent with a rapid activation of the cAMP cascade following forskolin treatment. In the presence of cannabinol, CREB/ATF binding was found to be inhibited at all of the time points described above [10]. It is notable that although the mechanism for the transient activation of CRE DNA binding, even in the presence of forskolin which provides sustained activation of cAMP, has not been fully characterized, the Ser/Thr protein phosphatase, PP-1, which is activated by PKA, has been reported recently to be a major down-regulator of CREB activity following cAMP stimulation [32]. We (unpublished observation) and others [32] have observed that this decrease in CRE binding at later time points ( $\geq 90$  min) following forskolin stimulation is blocked by okadaic acid, a protein phosphatase inhibitor, in the absence of cannabinoid treatment.

Measurements of IL-2 expression following PMA/Io stimulation in EL4.IL-2 cells as well as primary mouse splenocytes, in the presence of either  $\Delta^9$ -THC or cannabinol, showed a marked inhibition of IL-2 secretion that was closely correlated with a significant decrease in IL-2 gene transcription [10]. It is known that IL-2 gene transcription is highly regulated through a number of well-characterized recognition sites in the promoter region for inducible and noninducible regulatory factors. The regulatory elements present in the minimal essential portion of the IL-2 promoter include AP-1, NF-AT, NF- $\kappa$ B, and CD28RE but do not contain any known CRE sites [33]. However, forskolin has been demonstrated by gel shift assays, in EL4 cells, to enhance phorbol ester/calcium ionophore-induced AP-1 binding in the IL-2 promoter [34]. Moreover, several laboratories have also shown that both Fos and Jun family members can dimerize with CREB and that these "chimeric" heterodimers, in turn, are capable of binding to AP-1 sites [35, 36]. Recently, this phenomenon has been demonstrated employing anti-CREB and anti-Fos/Jun gel shifts that identified all three protein types bound to an AP-1-like site (AP-1 proximal site: AP-1p) in the IL-2 promoter, suggesting that CREB family proteins help to regulate IL-2 transcription through the formation of heterodimers with Fos and Jun [37]. Concordant with a role for CREB protein binding at the AP-1p site in the IL-2 promoter, cannabinol markedly inhibited PMA/Io-induced AP-1p binding in EL4.IL-2 cells [10]. Interestingly, this convergence of the PKA and PKC regulatory pathways is further supported by the fact that forskolin augmented PMA/Io-induced AP-1p binding in this same cell line [10]. In addition to the down-regulation of IL-2 expression by cannabinoids through an inhibition of nuclear regulatory factor binding at AP-1 sites, we have also observed that cannabinoids markedly

inhibit the activation of NF- $\kappa$ B in a variety of T-cell models (unpublished observation) as well as in the macrophage line RAW264.7 [13]. This almost certainly is an additional contributing factor to the inhibition of IL-2 gene expression.

The fact that cannabinoids markedly inhibit the activation of CREB/ATF proteins, as demonstrated by a decrease in CRE binding, is especially interesting in light of the recent studies by Barton and coworkers [38] in which they developed a transgenic mouse expressing a dominant negative form of CREB under the specific control of the T-cell specific CD2 promoter/enhancer. CREB in transgenic mouse T-cells lacked a serine residue which when phosphorylated activates this nuclear binding factor. Moreover, this modified form of CREB was incapable of activating CRE-regulated genes. Interestingly, T-cell development appeared to be normal in these transgenic mice. In contrast, thymocytes and T-cells from the CREB transgenic mice exhibited a profound inhibition of proliferative responses to a variety of T-cell stimuli (PMA/Io, anti-CD3, and concanavalin A), greater than 99% inhibition of IL-2 production and G<sub>1</sub> cell-cycle arrest. Furthermore, activated T-cells from these transgenic mice exhibited decreased induction of *c-jun*, *c-fos*, *Fra-2* and *FosB* which code for AP-1 associated proteins. This decrease is likely due, at least in part, to the fact that some of these genes (i.e. *c-jun*, *c-fos*) are known to possess CRE sites within their regulatory regions. One notable point made by the authors, although the data were not shown, was that in their experiments H-8, a PKA inhibitor, did not inhibit the phosphorylation of CREB in normal thymocytes. This finding which suggests that CREB is regulated through a PKA-independent pathway is intriguing in light of the fact that PKA is well established as being the primary kinase that mediates CREB phosphorylation/activation. Unfortunately, the conditions under which the studies with H-8 were performed in the aforementioned studies were not provided. However, the anomalies described in the transgenic-derived T-cells possessing the mutant CREB are strikingly similar to those observed with EL4.IL-2 cells and mouse splenic T-cells in which PKA and CRE binding activity has been inhibited by either  $\Delta^9$ -THC or cannabinol [10].

A number of recently published reports have shed additional light on the positive role played by modest transient increases in cAMP during T-cell activation. Interesting, unlike for a variety of cell types including fibroblasts, adipocytes, and muscle cells in which cAMP antagonizes the Raf-MAP kinase pathway [39-45], in T-cells this pathway is resistant to the negative influences normally associated with short-term increases in cAMP [17]. This is evidenced by the fact that cAMP did not inhibit ERK2, the dominant form of MAP kinase in T cells [17]. Similarly, the newly identified c-Jun N-terminal kinase which defines a T-cell antigen receptor-independent activation pathway was also resistant to short-term increases (<30 min) in cAMP [17]. Conversely, sustained (2 hr) treatment of T-cells with 0.5

mM dibutyryl-cAMP induced an antagonism of c-Jun N-terminal kinase that was not due to a decrease in the synthesis of the kinase but was dependent on protein synthesis [17].

As already mentioned, the accessory function played by helper T-cells in antibody responses is especially sensitive to inhibition by cannabinoid compounds [28]. This observation is very consistent with recent findings which suggest that the cAMP signaling cascade may serve as a switching mechanism between the helper T-cell subpopulations, Th1, which facilitate cell-mediated immune responses, and Th2, the helper T-cell subtype that regulates humoral immune responses. Several recent studies indicate that high intracellular concentrations favor Th2 responses. For example, high concentrations of cAMP (1 mM) activate the binding of nuclear factors to GATA-3 and the conserved lymphokine element (CLE0) to enhance the expression of the B-cell differentiation factor, IL-5 [46]. Moreover, high sustained concentrations of cAMP inhibit IL-2 expression by Th1 cells [47]. This finding, coupled with the fact that T-cells show a marked inhibition in a number of responses following cannabinoid treatment, suggest that cAMP is essential for both Th1 and Th2 lymphokine gene expression; however, low and perhaps transient intracellular cAMP concentrations favor the activation of Th1 lymphokines, whereas high and sustained cAMP concentrations appear to favor the induction of Th2-associated cytokines. Further, the apparent requirement for high cAMP concentrations for IL-5 expression provides an explanation for the marked sensitivity of T-cell-dependent humoral responses to inhibition by cannabinoids since these compounds are potent inhibitors of adenylate cyclase.

In summary, the adverse regulation of helper T-cells by cannabinoids appears to be directly linked to the inhibition of CREB/ATF, AP-1, and NF- $\kappa$ B/Rel activation. These nuclear factors are known to be essential regulators of the lymphokines which help to control cell-mediated and humoral immune responses.

#### **INHIBITION OF THE cAMP SIGNALING CASCADE BY CANNABINOIDS LEADS TO THE DOWN-REGULATION OF iNOS EXPRESSION IN MACROPHAGES**

As part of the innate immune response, cells of the myeloid lineage have been shown to release NO to facilitate killing or to inhibit the growth of tumor cells, bacteria, fungi, and parasites [48-50]. The production of NO by myeloid cells is mediated by iNOS which catalyzes the production of large amounts of NO from L-arginine and molecular oxygen [51]. Transcription and synthesis of iNOS are induced rapidly in macrophages by a number of stimuli including LPS or tumor necrosis factor with the magnitude of stimulation being further increased by IFN $\gamma$ . In addition to IFN $\gamma$ , iNOS production is also potentiated by agents that increase adenylate cyclase such as ligands for  $\beta_2$ -adrenoreceptors [52]. In spite of much current interest in the role of iNOS in host

resistance to pathogens and its involvement in the very closely related process, inflammation, its regulation is only partially understood. Recently, much insight pertaining to the transcriptional regulation of iNOS has emerged from the sequencing of the regulatory region of this gene. Interestingly, the promoter region for iNOS contains two  $\kappa$ B binding sites, one at position -79 and the second at position -962 [53]. Protein binding at the  $\kappa$ B site is necessary to confer iNOS inducibility by LPS [54].

The NF- $\kappa$ B/Rel family of transcription factors are pleiotropic regulators of many genes involved in immune and inflammatory responses, including iNOS [54, 55]. In unstimulated cells, NF- $\kappa$ B/Rel proteins remain quiescent in the cytoplasm and bound to their cognate inhibitor, I $\kappa$ B. Activation of macrophages by a variety of external stimuli induces the phosphorylation of I $\kappa$ B. Although the specific kinase(s) that mediates I $\kappa$ B phosphorylation has not been characterized extensively, PKA is one of several kinases implicated in the activation of NF- $\kappa$ B. Phosphorylation and the less well understood process of ubiquitination [56] mark I $\kappa$ B for degradation, which then leads to the release of the active DNA-binding form of NF- $\kappa$ B/Rel family members. Once activated, NF- $\kappa$ B translocates to the nucleus and binds  $\kappa$ B motifs in the regulatory region of a variety of genes [57]. LPS treatment of macrophages activates both PKC and PKA, the latter being induced by an elevation in intracellular cAMP [58, 59]. Additionally, IL-1, which is induced by LPS, also contributes to the elevation of cAMP. The coordinate activation mediated through LPS and IL-1 is followed by a rapid increase in iNOS expression and nitrite formation [60, 61]. Based on the role played by cAMP signaling in the regulation of NF- $\kappa$ B/Rel proteins and the ability of cannabinoids to inhibit NF- $\kappa$ B DNA binding in lymphoid cell preparations (unpublished observation), the ability of cannabinoids to inhibit LPS-induced iNOS expression by macrophages was investigated recently. The results summarized below come from studies using primary mouse peritoneal macrophages and the macrophage-derived line RAW264.7 [13].

RAW264.7 cells express CB2 but not CB1 RNA transcripts [13]. The magnitude of basal CB2 RNA expression, as determined by quantitative RT-PCR, is significantly greater in RAW264.7 cells than observed in the previously discussed T-cell line, EL4.IL-2 [10, 13]. Based on a number of studies that are beyond the scope of this article and as suggested by quantitation of CB2 RNA transcripts, it is likely that macrophages express a markedly greater number of CB2 receptors than are present on T-cells. However, this conclusion will need to be confirmed in isolated and purified primary leukocytes either by western blotting with CB2-specific antibodies or by radioligand binding analysis with high affinity, CB2 selective, agonists. The treatment of RAW264.7 cells with  $\Delta^9$ -THC was found to produce a concentration-related inhibition in forskolin-stimulated cAMP accumulation, confirming the functional expression of cannabinoid receptors by the RAW264.7 cell line. Can-

nabinoid treatment of RAW264.7 cells also produced a concentration-dependent inhibition of LPS-induced NO production which was correlated closely with a decrease in iNOS RNA. A similar inhibition in LPS-induced NO production was also observed in resident (unelicited) mouse peritoneal macrophages. Interestingly, cannabinoid-mediated inhibition of LPS-induced NO production was reversed by concomitant treatment of RAW264.7 cells with 8-bromo-cAMP. This observation is consistent with previous studies that have demonstrated an enhancement of NO production in the presence of agents that increase intracellular cAMP (e.g.  $\beta_2$ -adrenoreceptor ligands and IL-1). Gel shift assays demonstrated that both LPS and forskolin treatment of RAW264.7 cells alone significantly increased protein binding to  $\kappa$ B and CRE DNA motifs. However, nuclear protein binding activity to either CRE or  $\kappa$ B was decreased significantly in the presence of  $\Delta^9$ -THC following either stimulus (i.e. forskolin or LPS). These results further support previous reports which have shown that: (a) the cAMP signaling cascade is a major regulator of the NF- $\kappa$ B/Rel family of DNA binding proteins; and (b) NF- $\kappa$ B/Rel activation is required for iNOS gene expression in response to LPS in macrophages.

## CONCLUSIONS

From this discussion of biological events that are believed to be initiated following ligand binding to cannabinoid receptors, it is not surprising that cannabinoids mediate a very broad array of effects on immune function. It has now been established that the inhibitory influence that cannabinoids produce on the cAMP signaling pathway in leukocytes, primarily on helper T-cells and macrophages, leads to a decrease in the activation of three major families of transcriptional regulators, CRE/ATF, AP-1 (Fos/Jun), and NF- $\kappa$ B/Rel. All are intimately involved in the regulation of immunologically relevant genes, most notably those for cytokines and related secretory factors. The significance of these findings pertains to two general areas. First, based on the influence that cannabinoids exhibit on gene regulation in leukocytes, the CB2 receptor appears to be a potential target for a novel class of immunomodulatory therapeutic agents. Plant-derived cannabinoids have been useful biological probes to demonstrate this potential; however, because they are relatively weak agonists for cannabinoid receptors, high concentrations of these plant-derived products are generally required to produce many of the effects described above. In light of this, exploitation of the CB2 receptor for therapeutic use will require the development of high affinity synthetic CB2 selective ligands. Second, and in addition to the therapeutic potential of these agents, studies with cannabinoid compounds have contributed significantly to elucidating the role of the cAMP signaling pathway in the regulation of leukocyte function. Recently, the critical evaluation of the cAMP signaling pathway by a number of laboratories has helped to dispel a long-standing

myth that this is a signaling cascade responsible for the down-regulation of immune responses. As is now evident, only extremely high and physiologically irrelevant intracellular concentrations of cAMP under *in vitro* conditions evoke immunoinhibitory effects on leukocyte function. Moreover, because cannabinoids are potent inhibitors of adenylate cyclase, they will continue to serve as excellent biological probes for further characterizing the role of the cAMP signaling cascade in immune regulation.

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