

Cannabinoids, Immune System and Cytokine Network

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Abstract: How cannabinoids influence immune function has been examined extensively in the last 30 years. Studies on drug-abusing humans and animals, as well as *in vitro* models employing immune cell cultures, have shown that marijuana, natural and endogenous cannabinoid compounds are immunomodulators. These substances modulate host resistance to bacterial, protozoan and viral infections as well as they can profoundly affect the Th1/Th2 response. Recently, two types of cannabinoid receptor, CB1 and CB2, have been discovered. While CB1 is expressed primarily in the brain, CB2 is peculiar of the immune cells. Cannabinoid receptors have been shown to be involved in some but not all of immune effects. Nevertheless, their identification provides a specific mechanism of action in the attempting to find out how exogenous cannabinoids and endogenous cannabinoid system affect the immune apparatus, strengthen the hypothesis of cannabinoids as immunomodulators. As support to this theory, enough evidence exists to suggest that the cannabinoid system significantly affects almost every component of the immune response machinery and impacts the functioning also of the cytokine network. The evaluation of the biological consequences of these drug-induced cytokine changes has also dramatically become important considering not only the impact of cytokines on immune system *per se* but also envisaging their influence in cancer, inflammation, autoimmune disease, brain injury, hematopoietic colony formation in which cannabinoids have demonstrated a clear role as important modulators.

Key Words: Cannabinoids; Cannabinoid receptors; Signaling; Tetrahydrocannabinol; Endogenous cannabinoids; Immune response; Cytokines.

INTRODUCTION

Marijuana is one of the most common drugs of abuse and its medicinal value has also been known by many cultures throughout human history. The plant, *Cannabis sativa*, contains over 400 chemical entities including alkaloid derivatives of spermidine, sterols, terpenes, flavonoid glucosides, and 60 or more cannabinoids which are predominantly found in the flowering tops of the plant (marijuana) possessing either psychoactive or non psychoactive properties. Recent works from many laboratories suggest that natural cannabinoids, as well as synthetic cannabinoids compounds, may be effective in alleviating various diseases such as glaucoma, cachexia, nausea, pain, inflammation, multiple sclerosis and in treating a variety of cancers including lymphomas, leukemias and gliomas. In contrast to these potentially beneficial properties, studies on the effects of marijuana smoking and cannabinoid unwanted effects, have evolved into the discovery that they can decrease host resistance to bacterial, protozoan and viral infection in experimental animal models and *in vitro* systems. Recent immune epidemiological studies suggest that marijuana may also influence the outcome of viral infections in humans as well as it can increase the incidence of cancer in humans and mice, influencing the immune system as whole.

The discovery of membrane receptors that bind cannabinimimetic compounds in 1990s helped the understanding

of the pharmacology of marijuana derivatives. Nearly concurrently with this discovery, the isolation of endogenous cannabinoid receptor ligands (endocannabinoids), the most important of these being anandamide (arachidonoyl ethanolamide, AEA) and 2-arachidonoyl glycerol (2-AG), and the identification in the greater part of cells of specific degrading enzymes and membrane transport proteins corroborate the existence of an endocannabinoid system (Fig. 1). This evidence suggests a novel modulatory and ubiquitous system whose the physiological role forewarns to be complex and widespread. The arachidonic acid derivatives (AEA and 2-AG) are exciting not just for depicting a new "endocannabinoid system", but because they also represent a novel class of "modulators" derived from membrane fatty acids that may be very important in neuromodulation and in brain-immune axis regulation, mimicking in a similar way the pan-action already described for opioid endogenous system.

CANNABINOID RECEPTORS IN THE IMMUNE SYSTEM

Cannabinoids have been shown to induce their biological effects mainly by binding to specific cannabinoid receptors (CB). Presently, two main subtypes of cannabinoid receptors have been identified, designated cannabinoid receptor 1 (CB1) and cannabinoid receptor 2 (CB2). Although CB1 is predominantly expressed in the brain, it has also been detected in the periphery. Conversely, CB2 receptor subtype appears to be the principal form of cannabinoid receptor within the immune system, with no quantifiable expression in the brain, and its level is higher than that of CB1 in immune cells (Fig. 2).

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CB1 RECEPTOR

Cannabinoid receptor type 1 (CB1) is the most abundant G-protein-coupled receptors in brain and was cloned from rat and human brain specimens in 1990 [1, 2]; it is highly expressed in brain tissue and to a lesser extent in the adrenal gland, reproductive organs, bone marrow, and immune cells. CB1 is very conserved throughout evolution, 97% of mouse CB1, 84% of amphibian CB1, and 72% of fish CB1 being identical with human CB1 protein.

The CB1 receptor was first identified within the immune system in mouse spleen cells [3]. This preparation showed saturable specific binding of the high-affinity synthetic cannabinoid receptor radioligand [H^3]-CP55,940, with a K_d in the picomolar range. This data was confirmed by reverse transcription-polymerase chain reaction (RT-PCR) that detected RNA transcripts for CB1.

The expression of CB1 receptors, abundant in the central nervous system (CNS), was reported to be very limited in the immune system [3, 4, 5]. The expression and distribution of CB1 receptor mRNA were analyzed in human immune tissues and leukocyte subsets, and in monocytic and leukemia cell lines. CB1 transcripts were found in human spleen, tonsils and peripheral blood leukocytes [4, 6] but were below the level of quantization in mouse thymus even by RT-PCR [3].

The pattern of distribution showed wide variations in the mRNA levels in the main human blood cells, the order of the levels in B cells being >natural killer>polymorphonuclear neutrophils>T8 cells>monocytes>T4 cells. Several leukocyte lines also express CB1 receptor mRNA, including Daudi and THP1 monocytes and cultured rat microglial cells [7]. Jurkat cells did not have detectable levels of CB1 mRNA transcript in the unstimulated state but expressed this receptor after mitogen activation [8]. It has also been reported [9] that both lipopolysaccharide (LPS)-stimulated Raji and PMA-stimulated THP-1 human monocytic leukemia cell lines showed elevated levels of CB1 receptor mRNA and protein. Changes in CB1 mRNA expression were contrasting in mouse splenocytes stimulated with the T-cell mitogens PMA/Io and with anti-CD3a, where CB1 mRNA content decreased. In contrast, cultures stimulated with the B-cell mitogen showed an increase in its message. In addition, exposure of the cells to IL-2 led to an increase in CB1. These important observations have suggested that the CB1 gene is differently expressed and it has biological relevance in immune cells during certain stages of cell activation [7-9].

The level of cannabinoid receptors, as already reported for the central CB1 receptors, were strongly regulated by exposure to cannabinoids themselves. Massi *et al.* [10] reported a significant loss of cannabinoid binding in spleen coronal sections in chronic cannabinoid-treated, tolerant rats *in vivo* after administration of CP-55,940.

Finally, since CB1 receptors are expressed primarily in the CNS, it was also suggested that stimulation of CB1 receptors by tetrahydrocannabinol (THC) acts on the hypothalamic-pituitary-adrenal axis, resulting in secondary immunoregulation by corticosteroids [11].

CB2 RECEPTOR

In 1993, the second cannabinoid receptor, CB2, was cloned by PCR from a human promyelocytic cell line (HL60) cDNA library [12]. The CB2 receptor showed 44% amino acid identity with the rat CB1 protein within the transmembrane regions. The cDNA encoded a protein of only 360 amino acids (substantially shorter than CB1) although this included the typical seven-transmembrane structure of the G-protein-coupled receptor.

The most intriguing aspect of CB2 is its predominant expression in the periphery, rather than in the brain, and particularly in cells of the immune system, with no quantifiable expression in the brain, as determined by Northern blot analysis and quantitative RT-PCR. The level of expression of the CB2 gene in immune tissue appears to be 10-100 times higher than CB1.

Nong *et al.* [13] used semi-quantitative RT-PCR to evaluate the expression of mRNA encoding for both CB1 and CB2 in human volunteers. Expression of mRNA for CB2 was three times the expression of transcripts encoding for CB1 and this distribution was preserved across individuals of different sex, age, and race. Galiègue *et al.* [6], examining the expression of the CB2 receptor in human immune tissue and leukocyte subpopulation, showed that the receptor mRNA content in spleen and tonsils was the same as CB1 mRNA in the CNS. This finding strongly suggests that, by analogy with CB1, CB2 can be considered a tissue-selective antigen and its receptor should have important physiological functions in immunity. The rank order of CB2 mRNA in human blood was B cells>natural killer cells>monocytes>polymorphonuclear neutrophils>CD8 T-cells>CD4 T-cells. The same order was established in human cell lines from myeloid, monocytic and lymphoid lineages [6].

These results have been reproduced by Carayon *et al.* [14] using flow-cytometry techniques to detect cell-surface receptors and by Marchand *et al.* [15] using quantitative RT-PCR to detect CB2 transcripts. Immunohistological analysis of CB2 receptors in the tonsil showed that only B cells, not T cells, stained in the mantle of follicles. This restricted expression of the protein was confirmed by Lynn and Herkenham [16] who, using [3H]CP55,940 as ligand, showed that the B lymphocyte was the predominant cell type bearing the CB2 receptor.

The high CB2 expression in B and NK cells may be related to the ability of cannabinoids in altering their functions. However, CB2 expression has been demonstrated in rat and mouse spleen and thymus and a number of immune system-derived cell lines including the T-cell lines ELA.IL-2, HPB-ALL, Jurkat E6-1, the monocytic lines HL60 and RAW264.7, and the mast cell line RBL-2H3 [17, 22]. Lee *et al.* [18] reported a similar pattern of CB2 mRNA distribution in murine immune cell subpopulations. CB2 was most abundant in splenic B cells, followed by macrophages and T cells. Messenger RNA for CB2 has also been identified in neonatal rat brain cortical microglia maintained *in vitro*, at levels more than ten times those for CB1 [19]. The CB2 receptor has been also found in dendritic cells, which are the most potent antigen-presenting cells of the immune system, able to po-

larize the immune response towards a Th1 and Th2 profile [20].

Changes in the level of CB2 receptors or their mRNA have been reported after treatment with various immune modulators or activators. Levels of CB2 mRNA in peritoneal macrophages differ depending on the cell activation state. Lee *et al.* [18] and Carlisle *et al.* [19] found CB2 mRNA in thioglycolate-elicited murine peritoneal macrophages but not in resident peritoneal macrophages, suggesting again that its level can vary in relation to the cell's state of activation.

One interesting point about the CB2 receptor is that CB2 mRNA transcripts in mouse lymphoid tissues (about 4 kb) are significantly larger than in rat spleen and man (2.5 kb), probably reflecting a mutation at the polyadenylation site, or perhaps even alternative splicing in the mouse CB2 gene [12].

Finally, stereoselective binding sites for anandamide were also found in invertebrate immunocytes and microglia, indicating that the endogenous cannabinoid system, represented by the cannabinoid receptors, endogenous cannabinoid receptor ligands and enzymes for the biosynthesis and degradation of these ligands, is conserved throughout evolution from coelenterates to man [21].

ENDOGENOUS LIGANDS

The presence of cannabinoid receptors in the immune system predicted the existence of an endogenous ligand system. Anandamide (AEA), 2-arachidonoylglycerol (2-AG) and palmitoylethanolamide (PEA) were synthesized by different immune cells, that all have the machinery to produce, transport and catabolize endocannabinoids although their precise function in the immune system is still being determined [22]. Because it is often present at higher levels than AEA in immune tissue, it has been suggested that 2-AG is the "true" endocannabinoid [23,24]. However, it seems that the endocannabinoid signaling system plays a generally negative part in the onset of the immune response, but its exact role in the maintenance of immune system homeostasis and the development of immune system disorders still needs to be defined.

SIGNAL TRANSDUCTION ASSOCIATED WITH CANNABINOID RECEPTORS IN THE IMMUNE SYSTEM

Cannabinoid receptors belong to a superfamily of G-protein-coupled receptors. They are single polypeptides with seven transmembrane helices and an extracellular N-terminus and intracellular C-terminus. Activation of either receptor blocks forskolin-induced accumulation of intracellular cyclic adenosine 3',5'-monophosphate (cAMP) and involves linkage to Gi- and/or Go-proteins. This has been amply demonstrated in virtually every tissue using both cannabinoids and endocannabinoids as ligands and cells with naturally expressed CB1/CB2 receptors [25] as well as in cell lines lacking either CB1 and CB2 but successfully transfected with one of the two receptor genes (CHO or AtT20 cells) (Fig. 2) [5, 26, 27].

The importance of the action of cannabinoids on adenylyl cyclase was demonstrated in the immune system in mouse

splenocytes [28], purified mouse splenic T and B cells [5] and a number of immune system-derived cell lines, including T-cell lines, EL4.IL-2 [29] and HPB-ALL [5] and the monocytic line RAW264.7 [30]. The intracellular cAMP level is critical for protein kinase A (PKA)-mediated signaling as cAMP regulates PKA activation and the release of a catalytic subunit which phosphorylates multiple intracellular targets including the cAMP-response element-binding protein/activation transcription factor (CREB/ATF) family of transcriptional regulators.

The PKA-dependent signaling cascade has major importance for gene regulation in immune cells, and the cannabinoid receptors' effects on the cAMP-dependent immune response was shown with the example of interleukin-2 (IL-2) gene transcription. In EL4.IL-2 cells expressing CB2 cannabinoid receptors, cannabinoil and ⁹-THC inhibited forskolin-induced cAMP formation and PKA activation and this inhibition was closely related with the repression of IL-2 transcription and secretion [29]. The inhibition was mediated by a steep, sustained down-regulation of nuclear factor of activated T cells (NF-AT) and by the transient inhibition of AP-1 DNA binding [31].

Cannabinoid receptor activation by ⁹-THC inhibited forskolin-induced binding of PKA-dependent transcription factor to the cAMP-response element (CRE) in the cAMP-responsive gene of mouse splenocytes [26]. The down-regulation of adenylyl cyclase and PKA activity reduced transcription factor binding to CRE and B motifs were also seen in mouse splenocytes and thymocytes [27, 32]. In the same way the inhibition of CREB/ATF and NF- κ B/rel DNA binding by ⁹-THC was responsible for the down-regulation of inducible NO synthase in the macrophage cell line RAW264.7 (Fig. 2) [30].

One important question arising from these various findings is how important is inhibition of the cAMP-dependent signaling pathways in the immune response. A transient burst in adenylyl cyclase activity has been reported within five minutes after lymphocyte activation by mitogens, implying positive lymphocyte regulation through this mechanism [33, 34]. cAMP-dependent signaling pathways may positively or negatively regulate cytokine mRNA transcription in macrophages, depending on the cytokine tested [35]. There is ample evidence that the immune response is inhibited by high levels of cAMP analogs (>100 μ M) and stimulated at more physiological concentrations (<100 μ M) [33,36]. On the other hand, the inhibition of LPS-induced nitrite production in RAW264.7 by ⁹-THC could be reversed by the addition of 8-bromo-cAMP [30] or dibutyryl cAMP or cholera toxin [37]. Similarly, the permeable cAMP analogs reverse the inhibition of T-cell-dependent antibody responses by cannabinoids [33].

All these results suggest that the cAMP signaling cascade has a positive regulatory role in immune cell function and that cannabinoid receptor stimulation might antagonize the early events in immune cell activation. Mechoulam's group, using CHO and COS cell lines transfected with CB2 receptors, reported that, unlike synthetic cannabinoids, natural cannabinoids may behave as inverse agonists [38] or even antagonists [39] of adenylyl cyclase, confirming that much

remains to be clarified about the exact nature of the signaling process of the cannabinoid receptors in immune cells.

There are also reports of a dual linkage of the CB1 but not the CB2 cannabinoid receptor to Gi- and Gs- binding proteins, leading to more complicated intracellular signals triggered by cannabinoid receptors [40]. Although immune cells express significantly less CB1 than CB2 receptors, this potential dual link of CB1 receptors should be taken into account when evaluating the immunomodulatory properties of cannabinoid receptor ligands [40].

Besides down-regulation of cAMP formation, CB1/CB2 cannabinoid receptors either raise or lower MAP kinase (MAPK) activity depending on the cell type and receptor ligand studied [41, 42]. Kobayashi *et al.* [43] found that 2-AG induced rapid phosphorylation of p42/44 MAPK in HL60 cells. Adding the selective CB2 receptor cannabinoid antagonist SR144528 to the cells before 2-AG abolished the response induced by 2-AG, indicating that the CB2 receptor is involved in the response. Presumably a Gi-Go protein is also involved, because pertussis toxin treatment of the cells nullified the response induced by 2-AG. CP-55,940 and AEA also activated p42/44 MAPK, although AEA had less effect than 2-AG or CP-55,940 [43]. In contrast to these findings, Faubert and Kaminski [44] reported the inhibitory effect of cannabinol on the extracellular signal-regulated kinase (ERK) family of MAPKs in PMA/Io-stimulated mouse splenocytes grown in the presence of serum. It is evident that the real picture of cannabinoid receptor ligand regulation of MAPK in the immune system is complex and the exact nature of this signaling process is far from clear.

In conclusion, cannabinoid receptor stimulation in the immune system triggers a very complex regulation of DNA binding of different nuclear factors only partially linked to an effect on cAMP pathway. The cannabinoid receptor stimulation of a Gi-mediated and cAMP-independent stimulation of MAPK is also a player in immune homeostasis and control.

THE EFFECT OF NATURAL, SYNTHETIC AND ENDOGENOUS CANNABINOIDS ON IMMUNE RESPONSE

The immune system comprises several components, including lymphoid tissue, such as the spleen and lymph nodes, the bone marrow and thymus where lymphocytes and other immune cells are made, and circulating lymphocytes. Immunity is either innate or acquired. Innate immunity involves immune responses that do not require previous sensitization and exposure to foreign substances whereas acquired immunity does. Actions of macrophages and NK cells are part of the host's innate immunity while the responses mediated by B and T cells are part of the acquired immunity.

Generally, cannabinoids have a deleterious effect on a variety of immune parameters. Ample literature about marijuana and immune modulation dates back to the 1970s. At that time, some reports suggested that cannabis use was associated with an increase in the incidence of viral infections and allergic symptoms. More specific studies were subsequently designed to clarify the cannabinoids' action after *in vivo* and *in vitro* exposure in various human and animal cell lines.

LYMPHOCYTES AND HEMATOPOIETIC CELLS

T lymphocytes are particularly important in immune responses to viruses and microbes. A fundamental consequence of immune activation is the proliferation of various lymphocyte antigen reactive clones. The antigen-induced proliferation can be mimicked using lectin proteins and certain microbial products that are lymphocyte mitogens. It was already suggested in 1970 that marijuana or cannabinoids suppressed immune function, changing the number and function of T cells. Findings regarding the proliferation response of peripheral blood T cells from marijuana smokers were generally inhibitory although the evidence is sometimes conflicting, with T-cell proliferation responses either suppressed or unaffected [45].

Taskin *et al.* [46] discussed the consequences of regular marijuana use for lung function and reported altered cannabinoid receptor expression in leukocytes from chronic smokers. There was a measurable inhibitory effect after acute or chronic relatively high doses of cannabinoids to experimental animals, or high concentrations *in vitro*. Mouse splenocyte proliferation in response to T-cell mitogens *in vitro*, and the B-cell mitogen LPS, was suppressed by THC concentrations in the 10 μ M range and B cells appeared to be more sensitive than T cells. Interestingly, non-psychoactive cannabinoids were slightly more potent than THC, suggesting that at least in some situations, suppression of T-cell proliferation involves mechanisms other than cannabinoid receptors [47]. However, like in human culture, proliferation of mouse cells was not always suppressed and was even increased by low cannabinoid concentrations or in response to anti-CD3 as mitogenic agent [45].

Patrini *et al.* [48] reported that in rats an acute *i.p.* injection of the synthetic cannabinoid CP55,940, at a dose inducing analgesia, significantly reduced the splenocytes' proliferative response to PHA, and this was not antagonized by *in vivo* pretreatment with the CB1 cannabinoid receptor antagonist SR141716A, suggesting the involvement of CB2 receptors. The inhibitory effect on splenocyte proliferation was no longer seen after chronic CP55,940 treatment, suggesting that tolerance developed, probably due to down-regulation of the cannabinoid receptor in the spleen [10]. In another study, an acute *in vivo* injection of THC in mice at a dose inducing analgesia did not alter the splenocytes' proliferative response to Con A, but after seven days' treatment, proliferation was markedly reduced [49].

Some groups have studied the T-cell rosetting capacity of lymphocytes in CD4 and CD8 subsets, besides lymphoproliferation [13]. Rosette formation was impaired in peripheral blood cells from marijuana users. Others examined the effects on the number of lymphocytes in CD4 and CD8 subsets. The percentage of CD4 T cells was increased in peripheral blood cells from marijuana smokers, with a mean CD4/CD8 ratio of 1.95 as opposed to 1.27 in controls. The number of CD8 cells was also low in drug-treated, mitogen-driven mouse spleen cell cultures, suggesting these might be more sensitive to the cannabinoid's suppressive effects.

Finally, a few studies have examined the cannabinoids' effects on cytotoxic T lymphocytes (CTL), which can lyse and destroy potentially harmful cellular elements in the

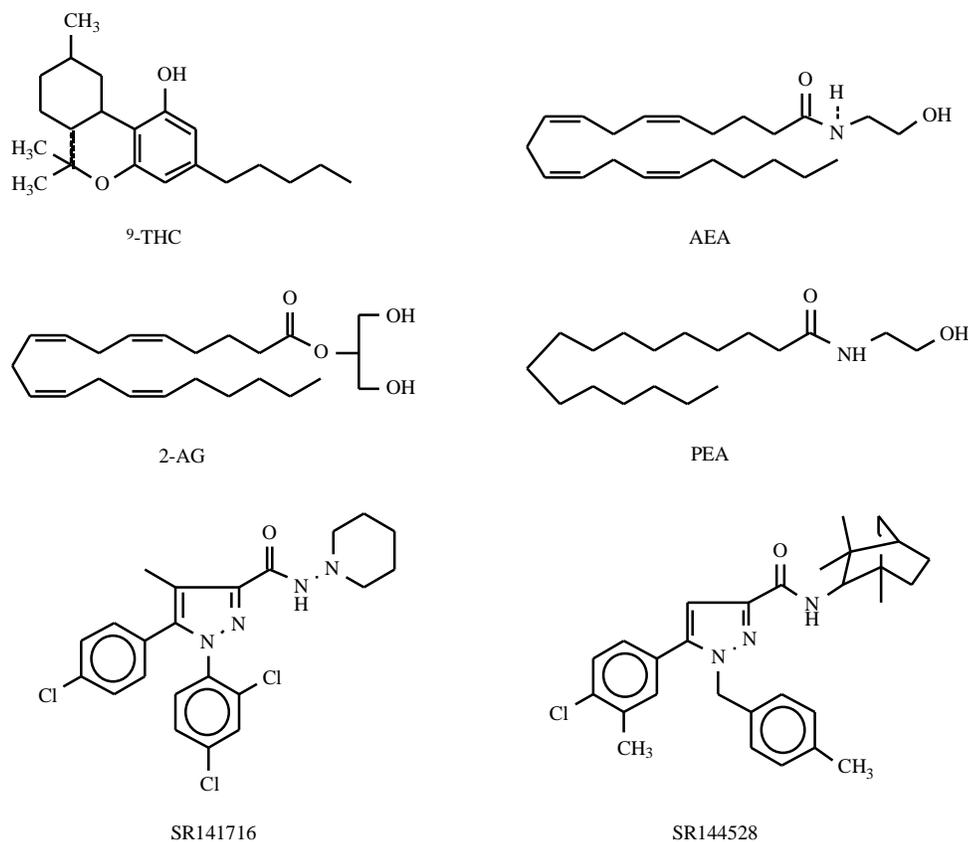


Fig. (1). Chemical structure of the psychotropic plant derived ⁹-tetrahydrocannabinol (THC) and the principal endocannabinoids anandamide (AEA), 2-arachidonoylglycerol (2-AG), and palmitoylethanolamide (PEA). At the bottom, the selective CB1 antagonist (SR141716A) and CB2 antagonist (SR144528) are represented.

body. After incubation with THC or 11-OH-THC, the cytolytic activity of murine splenocyte CTL was depressed by about 60% [50]. THC did not inhibit CTL binding to the target cell but lowered the cytolytic activity subsequent to binding. In another study, THC injected into mice at the time they were primed with a sublethal *Herpes simplex* virus type 1 infection suppressed the development of HSV1-specific CTL when the splenocytes were removed and tested *in vitro* against an HSV1-infected target. This deficiency appears to be due to suppression of the CTL lytic process at a stage beyond target cell binding [51].

Another major function of immune cells examined in the context of cannabinoid treatment is antibody formation. Studies in humans and animals have measured the levels of serum immunoglobulins after cannabinoid treatment. In chronic marijuana smokers given cigarettes for two months, the levels of IgG, A, and M were the same as in controls, but IgE appeared to be greatly elevated [52]. In a subsequent study by Nahas and Osserman [53], cannabis use was associated with a drop in serum IgG and an increase in IgD but no changes in IgA and IgM.

Cannabinoid ligands either injected into mice or added to splenocyte cultures suppressed the development of the antibody response to SRBC (sheep erythrocyte) [3, 45]. When the synthetic cannabinoids HU-210 and HU-211 were tested

on the anti-SRBC antibody response, HU-210 significantly suppressed serum hemagglutination titers and reduced the number of splenocytes and plaque-forming cells. Little is known about the cellular mechanism underlying this action. However, findings by Kaminski *et al.* [33] supported a role for a G protein-coupled receptor mechanism in the suppression of antibody formation of IgM induced by THC in splenocyte cultures.

In the last few years much work has been done to clarify the endocannabinoid system's role in relation to immune cells. The first studies by Schwarz *et al.* [54] examined the immunoregulatory effects of AEA on mitogen-induced T and B human lymphocyte proliferation. AEA caused dose-dependent inhibition in a concentration range where it regulates neuronal responses. AEA was respectively three and ten times less potent than ⁸-THC and CP-55940. AEA's effects on DNA synthesis in T and B lymphocytes were rapid as exposure during the last 4h of culture was enough to achieve > 40% inhibition. Low doses of AEA, which significantly inhibited lymphocyte proliferation, caused DNA fragmentation in parallel, demonstrated by immunohistochemistry, FACS analysis and Southern blotting.

Lee *et al.* [55], however, found that 2-AG but not AEA had biological activity in mouse splenocytes. 2-AG showed strong immunomodulatory activity on mitogen-induced lym-

phocyte proliferation, the mixed lymphocyte response and on antibody-forming cell (AFC) responses to T-cell-dependent and T-cell-independent antigen. 2-AG's inhibitory effects on proliferation were at least partly dependent on cell density. At high cell density, 2-AG enhanced lymphoproliferation whereas at low density it had marked inhibitory activity. The authors suggested that the immunoenhancing effect might be due to an increase in 2-AG catabolism with cell density, leading to the formation of an immunostimulatory bioproduct. One possible setting for this type of mechanism might be the rapid degradation of 2-AG to arachidonic acid (AA) at high cell density. AA might then serve as a precursor for prostaglandin E₂, a well-characterized activator of adenylyl cyclase, which is an important mediator of lymphocyte proliferation [55].

Derocq *et al.* [56] reported that AEA potentiated the growth of two cytokine-dependent murine hematopoietic cell lines, the IL-3-dependent myeloblastic cells FDC-P1 and the IL-6-dependent lymphoid cells B9, with a low level of serum. Neither the CB1 antagonist SR141716 nor the CB2 antagonist 144528 were able alone or together to prevent AEA's effect. This result provided evidence of a non-CB1- or CB2-mediated process. Whatever the precise mechanism of the non-receptor-mediated effect of endogenous AEA, it was clear that this messenger directly activated a fundamental biological process such as cell growth by bypassing its receptors. However, the equipotent (CB1 and CB2) cannabinoid ligand CP-55940, which binds cannabinoid receptors with 100-fold potency compared to AEA, was inactive in these models [56].

In line with these reports, Valk *et al.* [57] demonstrated that AEA but not other natural or synthetic cannabinoid compounds (all added at concentrations ranging from 0.1 to 10 $\mu\text{mol/L}$) stimulated proliferation of hematopoietic cell lines in synergy with other growth stimuli such as IL-3, Epo, GM-CSF and G-CSF. The addition of AEA alone to the cell cultures had no effect on the proliferation. The synergistic effect of AEA was seen only when cells were cultured without fetal calf serum (FCS), leading the authors to suggest that FCS may contain AEA or other cannabinoid ligands. This was further supported by more recent findings of AEA and other N-acylethanolamides at significant levels in commercial FBS preparations, suggesting that their presence in culture media must be taken into account in studies on cell growth *in vitro* [58, 17].

NATURAL KILLER CELLS

NK cells are important in host defences against tumors and microbes. They kill infected target cells and are a source of cytokines for upregulating immune function. Cannabinoids did not affect NK activity in humans [45]. Although there was a tendency for the blood cell NK activity to be lower the day after the last drug dose, the difference from controls was not significant. However, *in vitro* investigations on purified human peripheral blood lymphocytes [45] indicated that THC suppressed NK activity at concentrations (30 μM) that were not toxic for the cells, and suggested that THC might directly suppress the cells' cytolytic potential.

THC or synthetic cannabinoids injected in either mice or rats reduced splenic NK activity [45] in a concentration-

dependent manner. The effect was independent of cellular calcium mobilization and the binding of the NK cell to the target cell was not suppressed. As reported by Patrini *et al.* [48], the effect of CP55,940 on NK activity in rats was not antagonized by *in vivo* pretreatment with the CB1 antagonist SR141716A but the CB2 antagonist SR144528 partially reversed the inhibitory effect of THC in mice [59]. Both CB1 and CB2 receptors were reported to be involved in cannabinoid-induced inhibition of NK activity, since when THC is administered *in vivo* to mice, both cannabinoid antagonists prevent its inhibitory effect on NK cytolytic activity [59].

From these findings, it appears that cannabinoids can disrupt proliferation and cytolytic activity in these cells, like the activation of other lymphocyte functions.

MACROPHAGES

Macrophages are involved in innate and acquired immunity to infection. They participate in many events of innate immunity through the production of acute-phase cytokines and phagocytosis and by killing microbes and secreting inflammatory mediators such as NO and AA metabolites. These cells also interact in a vital way with lymphocytes to start the acquired immune response, acting as antigen-presenting cells and releasing cytokines. During activation, for instance by treatment with the bacterial endotoxin LPS, macrophages are a major source of the various inflammatory mediators that contribute to the local inflammatory response. As with other immune cells, cannabis and cannabinoids may significantly affect the host response to microbes by altering macrophage functions through either a receptor- or non-receptor-mediated mechanism, although macrophages express CB2 receptors and low levels of the CB1 receptor [6, 4].

Studies with pulmonary alveolar macrophages obtained by lavage from patient groups including non-smokers, marijuana smokers and tobacco smokers showed that tobacco rather than marijuana smoking resulted in lung changes and suppression of macrophage superoxide production [60]. However, when administered intranasally to mice, followed by an aerosol challenge with LPS, THC and WIN-55,212-2 significantly lowered the level of tumor necrosis factor- (TNF) in the bronchoalveolar lavage. There are reports of effects of cannabinoids *in vitro* on various macrophage functions including cell spreading and phagocytosis, protein expression, cytolysis and antigen presentation [45, 17]. McCoy *et al.* [61, 62] demonstrated that THC influenced the ability of macrophages to process antigens necessary for the activation of CD4⁺ T lymphocytes. Sacerdote *et al.* [63] reported that *in vivo* and *in vitro* treatment with the synthetic cannabinoid CP-55,940 reduced the spontaneous and formyl-methionyl-leucine-phenylalanine-induced chemotaxis of macrophages in the rat and that this effect involved both CB1 and CB2 receptors, although the CB2 antagonist was more potent in reversing it.

It also appears that cannabinoids affect the production of arachidonic acid metabolites in macrophage cultures, this effect being related to cannabinoid receptor function [17]. Mouse peritoneal macrophages present specific, saturable, high-affinity binding to THC, and several phospholipases are involved in eicosanoid mobilization. Whatever the cannabi-

noid receptor or cellular mechanism involved, the enhanced mobilization of these substances from macrophages may have important immunomodulatory potential considering the eicosanoids' important role in macrophage-mediated resistance to infection.

NO generation by macrophages is an obligatory element of cellular attack on bacterial pathogens. THC suppresses NO production in mouse peritoneal macrophages, but the cannabinoid ligand may increase NO production in human monocytic culture [45]. This effect was antagonized by the CB1 antagonist SR141716A, suggesting the CB1 receptor was involved. Although these findings indicate that cannabinoids inhibit a variety of important macrophage functions,

the relevance of these effects to resistance to infection is still unclear.

Endocannabinoids such as AEA and 2-AG are also synthesized by macrophages [64, 65, 66] but the endocannabinoids' actions on macrophage signal transduction still largely need clarifying. AEA inhibits macrophage-mediated killing of TNF-sensitive murine L929 fibroblasts [67]. Ross *et al.* [68] assessed the inhibition of LPS-stimulated NO production in RAW264.7 macrophages by cannabinoids and the putative cannabinoid CB2-like receptor ligand PEA, but this effect did not appear to be mediated by cannabinoid receptors. Chang *et al.* [69] also found that THC, AEA and 2-AG reduced LPS-induced NO, PGE₂ and IL-6 production in a

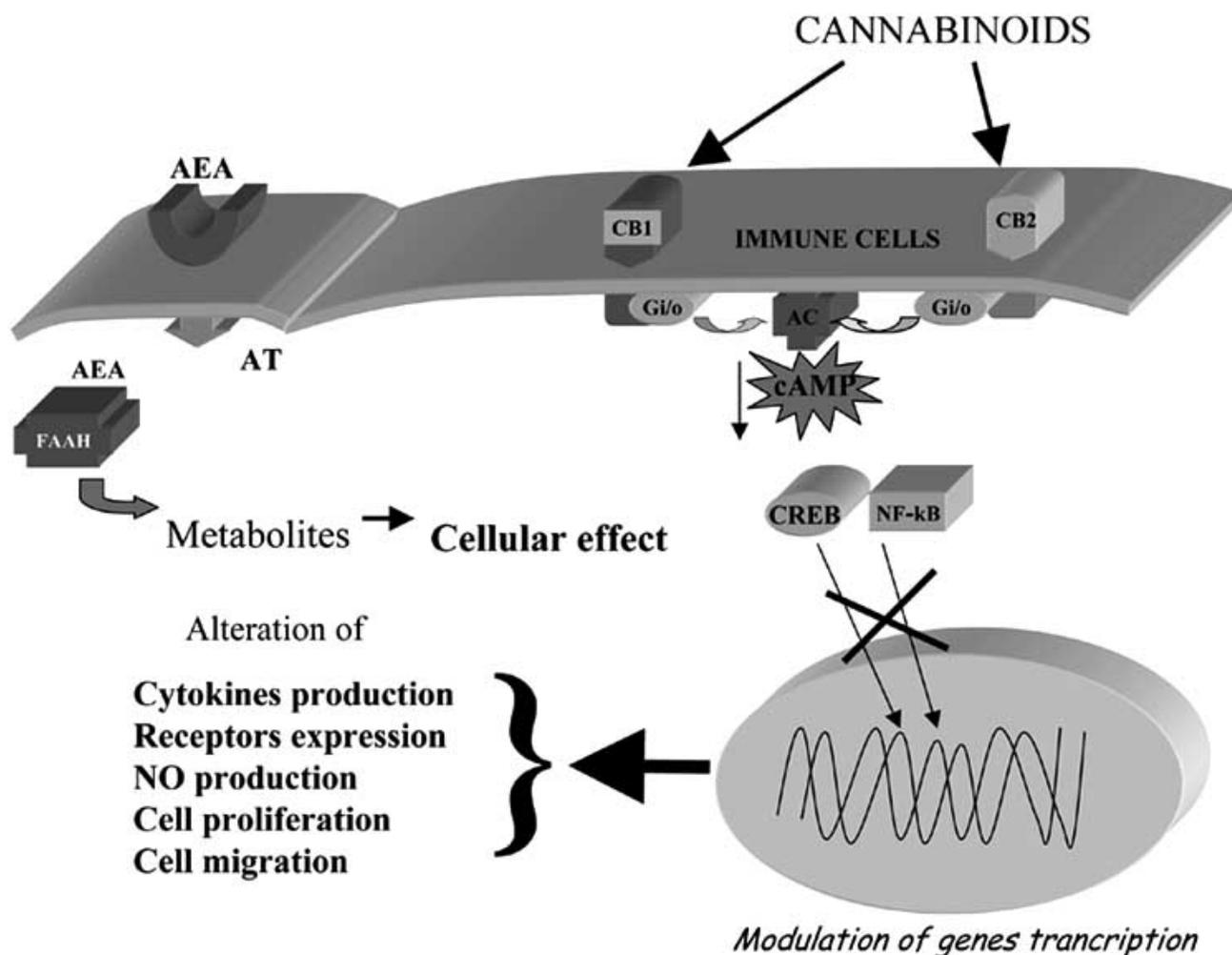


Fig. (2). The putative signalling pathway associated with cannabinoid receptor in immune cells.

Binding of cannabinoids to CB receptors induces the interaction of G proteins to adenylyl cyclase resulting in a decrease of intracellular cAMP. Decreased cAMP formation leads to the inhibition of the translocation in the nucleus of two important gene transcription factors CREB/ATF and NF-kB. The consequences of this event is an altered gene transcription of important genes involved in immune cell functions.

Endogenous cannabinoids (i.e AEA) can bind to CB1 and CB2 receptors or can be transported inside the cell by specific transporters (i.e. AT: anandamide transporter) or it can diffuse passively through the cell membrane. The endocannabinoids are then degraded by the fatty acid amide hydrolase (FAAH) and their metabolites can exert some biological action.

concentration-dependent manner through CB2 activation. In contrast, 2-AG but not AEA, induced migration of HL-60 cells differentiated into macrophage-like cells, suggesting a role for this endocannabinoid in inflammatory and immune competent cells [24].

MAST CELLS AND NEUTROPHILS

Tissue mast cells are multifunctional immune cells present in connective tissues of various organs and in the nervous system. In 1995 Facci *et al.* [70] showed that rat peritoneal mast cells (RPMC) and the cognate cell line RBL-2H3 expressed both the gene and functional CB2 receptor protein, with negative regulatory effects on mast cell activation. Although both PEA and AEA bound to the CB2 receptors, only PEA down-modulated mast cell activation *in vitro* and this action was efficiently antagonized by AEA. These results led to speculation that PEA might be the real ligand for peripheral cannabinoid receptors [70]. However, several authors failed to find any PEA binding to CB2 receptors, including the binding to these receptors expressed in RBL-2H3 cells [71, 23].

The effects of the cannabinoid receptor agonist WIN-55,212-2, 9 -THC, AEA, and PEA on LPS-induced bronchopulmonary inflammation in mice were also investigated. WIN-55,212-2 and 9 -THC induced a concentration-dependent reduction in TNF in bronchoalveolar lavage fluid. This was accompanied by moderately reduced neutrophil recruitment. PEA lowered the level of TNF by 31.5% but had no effect on neutrophil recruitment. AEA did not influence the inflammatory process but reduced the TNF level and neutrophil recruitment [68].

CANNABINOIDS' ACTION ON CYTOKINES

The regulation of the complex immune response is regulated by cytokines-signaling proteins synthesized and secreted by immune cells upon stimulation. Cytokines, together with their membrane-associated and soluble receptors, constitute a complex network with positive and negative regulatory roles, which plays a major role in the development of Th1 and Th2-dependent immune responses. Acute phase cytokines such as IL-1, TNF, and IL-6 produced by macrophages and other cells are an important part of natural immunity and help to control resistance to microbes during the early phases of an infection. Immune cytokines, on the other hand, such as IL-2, IL-4 and IFN, are produced by activated Th cells and help to eliminate microbes from the body by regulating powerful cell-mediated and antibody-mediated immune mechanism.

An increasing number of publications confirm that natural, endogenous and synthetic cannabinoids can alter the cytokine production in immune cells.

Early work on the modulation of cytokine production was performed by Blanchard *et al.* [72] who found that cannabinoids caused an inhibition of IFN production in splenocytes isolated from chronically THC-treated animals upon stimulation by the mitogens phytohemagglutinin (PHA), concanavalin A (ConA) or *Escherichia coli* LPS. On the other hand THC and other non-psychotropic drugs as cannabidiol were shown either decrease or increase IFN pro-

duction depending on the concentrations used. At low concentrations (e.g. <0.1 μ M) cannabinoids increase IFN production while they decrease it at highest concentrations (e.g. 30 μ M) [73].

Besides IFN, several other cytokines were modulated by cannabinoid treatment. THC is also able to induce an inhibition of TNF production in cultured mouse peritoneal macrophages, as demonstrated by Zheng *et al.* [74]. The decrease in TNF release was due to impaired processing of the presecreted to secreted form of TNF but not due to the decrease in expression of its mRNA. The alteration in cytokine protein processing rather than transcription and translation was confirmed later by two other groups [51, 75].

Moreover Klein and Friedman [76] reported that THC in the range of 10-30 μ M increased IL-1 bioactivity in the supernatant of cultured mouse peritoneal macrophages. IL-1 is an inflammatory and immunomodulatory protein produced by macrophages and other cells in response to tissue injury and infection. As already reported for IFN, subsequent studies showed this occurred because THC alters the processing and release of IL-1 rather than cellular production of the protein [77].

However, the immunomodulatory properties of cannabinoids were shown to be complex. THC *in vivo* given to mice 24 h before and 24 h after injection of sublethal dose of *L. pneumophila* presented acute collapse and death resembling cytokine-mediated shock [78]. Measurement of acute phase cytokines TNF and IL-6 in blood showed a significant increase in their content in the treated animals [78]. The mechanism involved in this mobilization is still not clear but it is reasonable to assume that in whole animals the drug affects several levels of control over cytokine mobilization.

A good source of IFN is the NK population and these cells are impaired in their function by the exposure to THC [79]. Also lymphocytes are limited in their proliferation by cannabinoids. Proliferation of these cells is regulated, at least in part, by the IL-2/IL-2 receptor system. Nakamo *et al.* [80] showed that IL-2 secretion by lymphocytes was modulated by cannabinoids, thus accounting for some of the inhibitory drug effects on the cell growth. In accordance with this finding, Zhu *et al.* [81] provided also evidence that THC can alter the expression of IL-2R proteins in a manner consistent with a decrease in receptor function/presence on the surface of immune cells [82].

A clear evidence that cannabinoids can affect the development of a Th1/Th2 immune response, was first given by Newton *et al.* [83]. Mice treated with a single dose of THC were more susceptible to a challenge infection with *L. pneumophila* and presented several manifestations of Th1 deficiency, such as reduced lymphoproliferation, IgG2a antibody production and IFN production. In subsequent experiments, THC was examined for its impact on cytokine production during the initial immunization phase [84]. The pretreatment with THC resulted in lower serum concentrations of IL-12 and IFN within hours after sub-lethal infection with *L. pneumophila* and induced a higher levels of secretion of IL-4. Both of these results are consistent with the conclusion that cannabinoids can act as Th2 inducer.

Massi *et al.* [49], using an *in vivo* chronic treatment paradigm, provided further support for the assumption that THC in mice lowering IFN and IL-2 level in splenocytes, can direct the cytokine network away from cell-mediated immunity, provoking a shift towards Th2 humoral responses. This would be interesting, considering that it might reduce the host's resistance to certain pathogens such as viruses, intracellular bacteria and parasites.

However, some discrepancy was observed when studies were conducted on murine macrophages exposed to the non-psychoactive natural cannabinoid compound cannabidiol (CBD) [85]. In this context, CBD appeared to increase IL-12 and decrease IL-10 cytokines thus resulting in a pro-inflammatory rather than anti-inflammatory phenotype.

The more general observed shift in the Th1/Th2 balance, however, might be important considering that cannabis has been proposed for the treatment of autoimmune diseases like multiple sclerosis (MS), where it is known to be present a prevalence of a Th1 response. Myelin destruction in MS is mediated largely by the production of the pro-inflammatory cytokines, IFN and TNF, secreted from autoreactive T cells and macrophages. Croxford and Miller [86] described in a mouse model of chronic-progressive multiple sclerosis, a therapeutic effect of the synthetic cannabinoid WIN55,212 associated with a reduced capacity of CD4⁺ T cells to differentiate to Th1 effector cells. This effect was accompanied with inhibited levels of spinal cord mRNA coding for a number of inflammatory mediators (IL-1, IL-6, TNF and IFN) strictly associated to the induction/progression of MS. These "anti-inflammatory" effects of cannabinoids were proved also in other papers [87] concerning experimental animal model of MS, although a recent paper of Killestein *et al.* [88] which evaluated the immune function in MS patients treated orally with a combination of THC and cannabidiol, showed a significant increase rather than decrease in plasma pro-inflammatory cytokines TNF and IL-12.

However, the unbalance of a Th1/Th2 pattern provoked by the exposure to cannabinoids is largely confirmed also in human leukocytes, where THC caused a decreased production of IL-2 and IFN cytokines accompanied with a decreased steady-state levels of mRNA encoding for Th1 cytokines and increased mRNA levels for Th2 cytokines [89].

Since the disruption of Th1/Th2 cytokine balance could play a role in promoting tumor growth, studies concerning the antitumoral/protumoral action of cannabinoid were done. Zhu *et al.* [90] examining the effects of THC on the host response to a lung tumor challenge, found that the animals receiving THC experienced a more rapid rate of tumor growth. Since in parallel there was no effect of THC when tumors were implanted into immunodeficient mice, these studies suggested that the cannabinoid enhanced tumor growth by disrupting immune function *in vivo*. Since they found that THC augmented the immunosuppressive cytokines IL-10 and TGF, while IFN was down regulated at both the tumor site and in the spleens, they suggested that THC promotes tumor growth by inhibiting anti-tumor activity by a cytokine-dependent pathway. These studies suggested for the first time that THC regulated anti-tumor immunity primarily by increasing the production of suppressive cytokines and

not simply causing a passive state of immunosuppression due to a lack of cells producing IFN.

Srivastava *et al.* [91] found that THC and non-psycho-tropic cannabidiol had widespread, lineage- and derivative-specific effects on cytokine expression, in line with previous animal studies. They concluded that these effects, while offering potential benefit in some autoimmune/inflammatory diseases, might worsen HIV infection, tumorigenesis, and metastases and exacerbate allergic responses.

Since animal and cellular models do not always predict human responses, epidemiological studies were performed to clarify the real impact of marijuana smoking on the development of opportunistic infections and cancer. Tindall *et al.* [92] observed a more rapid progression from HIV infection to AIDS in marijuana users and more recently its use was found to be an independent risk factor for the development of cancer [93]. In order to evaluate whether human immune responses are similar as in animal and cellular studies, T cells were collected from healthy volunteers. It was found [94] that *in vitro* THC down-regulated the expression and release of Th1 cytokines, increased the expression of Th2 cytokines, and altered normal Th1/Th2 balance in a dose-dependent manner.

The normal host response to an immunologic challenge involves dendritic cells, specialized antigen-presenting cells that activate and expand antigen-specific T cell clones. The relative production of IL-12 (Th1) versus IL-10 (Th2) by dendritic cells and the relative balance of other Th1 and Th2 cytokines (i.e., IFN and IL-4) in the local environment results in differentiation of the activated T cells towards either a Th1 and Th2 phenotype. Roth *et al.* [94] investigating the effects of an exposure of dendritic cells to *in vitro* THC, found IFN concentration reduced of about 50% while IL-4 levels were increased on average by 110%, resulting in a dramatic shift in Th1/Th2 cytokine balance.

Cytokine inhibition appears to be dependent on specific cannabinoid stimulation. Massi *et al.* [59], using an *in vivo* protocol, reported that either the CB1 or the CB2 receptor was involved in the inhibition of IFN release by THC; Derocq *et al.* [95] clearly demonstrated the participation of CB2 receptors in the stimulatory effect of nanomolar concentrations of CP-55,940 on the production of several cytokines in human promyelocytic HL-60 cells. In accordance with these data, Ihenetu *et al.* [96] also showed that the inhibition of IL-2 release from human peripheral blood mononuclear cells by WIN55,212-2 and JWH-015 is mediated by CB2 receptors. However, evidence has also been provided that some of the immunomodulatory effects of cannabinoids are not mediated by receptors. Puffenbarger *et al.* [97] found that SR144528 and SR141716A did not reverse the inhibitory effect of cannabinoids on mRNA expression of IL-1, IL-6 and TNF. Thus, it might be possible that "non-specific binding sites" are responsible for the described properties of cannabinoids.

Finally, an interesting field of research is represented by the understanding of the role of the endogenous cannabinimimetics on immunity and regulation of cytokines. AEA has been reported to stimulate proliferation of mouse bone marrow cells in the presence of IL-3 [57], leading the authors to

the conclusion that AEA is a synergistic growth stimulator for hematopoietic cells. Also in other studies AEA and PEA acted as stimulators of growth cells in the presence of cytokines [56], although without a clear linkage of this effect to cannabinoid receptor stimulation. This is not surprising for fatty acid compounds that can readily penetrate the cell membrane, as suggested in other studies [98]. AEA, in addition to modulating cellular responsiveness to various cytokines, has also been reported to increase *per se* the production of cytokines under different conditions. It has been reported that cortical astrocytes infected with Theiler's murine encephalomyelitis virus in the presence of AEA produced more IL-6 [99], a pleiotropic anti-inflammatory cytokine that can have a role in the neuroprotective effects already described for endocannabinoids.

CONCLUSION

The cannabinoids are by now well established as immune modulators. CB2 receptors appear to be readily expressed by immune cells, while CB1 receptors are probably up-regulated in the immune system depending on the level of cell differentiation or activation. The cannabinoids' role in immune system regulation, health and disease needs to be clarified not only in marijuana smokers but in non-users as well. In addition, a better understanding of how the endogenous cannabinoid system works may help clarify its part in homeostasis of the normal immune system and in pathologies like autoimmune disorders, cancer, inflammation and viral and bacterial infections. Considering the high expression of the CB2 receptor, there is probably considerable potential for further research and for the development and application of novel, highly selective CB2 ligands based on knowledge of the intrinsic role of endocannabinoids in the immune system.

ABBREVIATIONS

THC	=	Tetrahydrocannabinol
AEA	=	Arachidonylethanolamide
2-AG	=	2-arachidonoylglycerol
CB	=	Cannabinoid receptors
PEA	=	Palmitoylethanolamide

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