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MARIJUANA, RECEPTORS AND IMMUNOMODULATION

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INTRODUCTION

It is now widely accepted that drugs of abuse, such as opiates, cocaine and marijuana, as well as other abuse drugs, both recreational and psychoactive, are potent immunomodulators, suppressing and sometimes enhancing the immune response. A wide variety of models have been used both *in vivo* and *in vitro* to demonstrate the immunomodulatory properties of such drugs and the mechanisms involved (Bacynsky et al., 1983a, b, Cushman, and Khurana, 1976, Friedman et al., 1991, 1994, Hollister, 1988, Petersen, 1976, Zimmerman, et al., 1977). In recent years it has been shown in various studies that morphine, cocaine, marijuana and also alcohol have marked effects on various parameters of immunity, including those mediated by T cells, i.e., cell mediated immunity, B cells, i.e., humoral immunity such as antibody formation, as well as natural killer cells, macrophages and PMN cell mediated killing of microbes like bacteria, viruses and fungi. For example, recent studies in this laboratory have shown that marijuana and its components are markedly immunomodulatory (Blanchard et al., 1986, Djeu et al., 1991, Kawakami, 1988 a, b, Klein et al., 1985, 1987, 1993, 1994, Lopez-Cepero, et al., 1986, Pross et al., 1990, 1992, Shivers et al., 1994, Zhu et al., 1993, 1994).

Investigations with many drugs of abuse were initially performed in humans, but more recently in experimental animal models, both *in vivo* and *in vitro*. In regards to marijuana, it is now recognized that this drug, especially psychoactive components, have effects on immune responses. This abuse substance is also often used legally for potential therapeutic purposes. For example, marijuana may be used as an anti-emetic agent in cancer patients and in patients being treated with antimetabolites, which often induce nausea and loss of appetite. However, it is now widely recognized that marijuana and especially its major component, tetrahydrocannabinol (THC), is an immunomodulator. This has been shown both *in vivo* as well as *in vitro*. Although controversial, a number of studies have been performed with peripheral blood monocytes from the blood of heavy marijuana users and provide evidence of altered immune responses (Nahas et al., 1974, Gupta et al., 1974, Sherman et al., 1991, Wallace et al., 1988, White et al., 1975). Drugs of abuse such as THC, when added to normal human peripheral blood lymphocytes, may suppress a number of normal immune responses of the cells, but again this is controversial (Lau et al., 1992, Nahas et al., 1977).

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Although some reports with human blood monocytes have been compelling, such results have had little impact on public health policy because results of such studies were often based upon investigations in which the drug concentrations used were considered outside the range of human experience. However, reports in the 1970's showed that injection of THC into rodents such as mice or rats could alter a variety of immune responses (Baczynski and Zimmerman, 1983a,b,) and also increase susceptibility to various infectious agents such as Listeria, *Escherichia coli* or Salmonella (Bradley et al., 1977, Morahan, et al., 1979). Later reports showed similar effects in animals treated with THC and challenged with Herpes simplex virus or a murine retrovirus (Cabral et al., 1986, 1987, 1992; Mishkin and Cabral, 1985b, Lancz et al., 1991, Specter et al., 1991). Furthermore, THC has been shown to suppress interferon production *in vivo* and *in vitro* ((Blanchard et al., 1986; Cabral et al., 1986). In addition, THC also was reported to suppress *in vitro* production of tumor necrosis factor (Fischer Stenger 1993, Zeng 1992), but enhanced as well as suppressed production of IL1, and IL2 receptor alpha and beta proteins (Shivers et al., 1994, Zhu et al., 1993, 1994). Thus THC, as well as other drugs of abuse, are now considered immunomodulators rather than only immunosuppressants.

THC Effect on Cytokines

Studies in this laboratory have shown that production of IL1 by macrophages is enhanced rather than suppressed by THC exposure (Shivers et al., Zhu et al., 1994). Within a few hours of culturing peritoneal exudate macrophages with a stimulant such as bacterial lipopolysaccharide and co-treatment with graded doses of THC, enhanced levels of this cytokine are present in culture supernatants as determined by bioassay (Fig 1). Furthermore, by a standard ELISA procedure it was found that the IL1α and IL1β protein component of this interleukin was enhanced in cultures treated with both LPS and THC. However, examination of mRNA for cytokine production failed to show an alteration in production of the genetic message for this cytokine. Thus, such experiments suggested that treatment of macrophages with THC augmented release of IL1 and not its production at the cellular level.

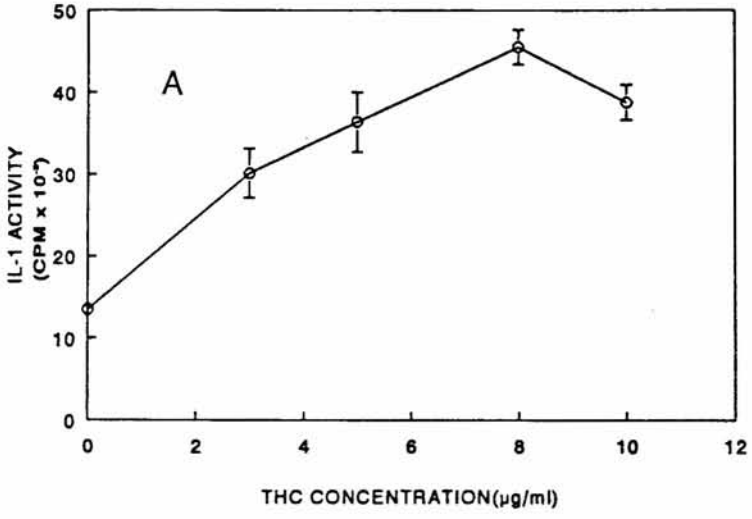

Figure 1. The effect of THC on IL1 bioactivity in cultures of mouse macrophages stimulated with LPS *in vitro*. Each point represents average for 3-5 cultures treated with indicated concentration of THC for 24 hr.

THC and Legionella Infection

The cytokine IL1 is considered to be a pro-inflammatory protein which can mediate fever as well as other physiological parameters following infection of an individual, including infection by opportunistic gram negative bacteria. Thus we examined in detail whether THC could affect susceptibility of mice to infection with an opportunistic intracellular pathogen such as *Legionella pneumophila*, which causes pneumonia and acute systemic infection, mainly in immunocompromised individuals, including those with immunodeficiency induced by the AIDS virus. Previously we had reported that THC injection into mice co-infected with both a murine AIDS virus, i.e., FLV, as well as a common opportunistic virus, i.e., Herpes virus, resulted in greater fatality in the animals than treatment with THC only, THC plus FLV alone, or THC plus Herpes virus alone (Specter et al., 1991).

An advantage of an animal infection model, such as that studied with the AIDS virus and THC, or Legionella infection and THC, is that one can readily study both primary and secondary infection. Thus in the studies with THC and Legionella we initially studied the effect of THC injection on the primary intravenous infection of mice with this bacterium. BALB/c mice readily tolerate a sublethal infection with Legionella, i.e., 7×10^6 bacteria per mouse. We tested whether relatively low doses of THC (1-4 mg/Kg) given either a day before or the day after infection increased susceptibility to infection. We found mortality of the mice was not altered by drug treatment under these conditions (Klein et al., 1993). Mice given a sublethal primary infection with these bacteria normally develop increased immunity to a second lethal dose given several weeks later. To test the drug effect on such secondary immunity to Legionella, THC treated and Legionella primed mice were given a higher secondary dose of the bacteria, i.e., 5×10^7 CFU. Control mice not treated with THC survived this challenge infection. However, unlike the primary infection, mortality after the challenge infection was increased in THC treated mice, indicating that administration of this cannabinoid at the time of primary infection suppressed development of secondary immunity (Klein, et al., 1993).

Since marijuana is usually taken more than once, we also studied resistance to infection in mice treated with two doses of THC. When a dose range of 1-5 mg/Kg was given one day before and one day after primary infection, no alterations in survival rate occurred, although morbidity (i.e., malaise, etc.) appeared to be greater in animals given two injections of the drug. However, by increasing the drug dose to 8 mg/Kg the day before and the day after infection, the mortality of the animals increased dramatically and was evident as early as 30 minutes following the second THC injection (Table 1). Non-primed mice, which were not immune to Legionella, challenged with a lethal dose of the bacteria but not exposed to THC begin to die only after 1-2 days. Thus, the very rapid mortality of the animals given

Table 1. The effect of THC on primary infection of mice with Legionella

	Legionella injected per animal[b]	
THC reatment[a]	8×10^7[c]	1×10^7[c]
None (Control)	0/11	0/10
THC (-24 and +24 hrs)	11/12	23/23
THC (-24 and -48 hrs)	0/12	0/15

[a]Mice injected with THC (8 mg/Kg) -24 and -48 hrs before infection or -24 before and +24 hr after infection.
[b]Total number of mice surviving per number challenged.
[c]Concentration of bacteria injected i.v. per mouse.

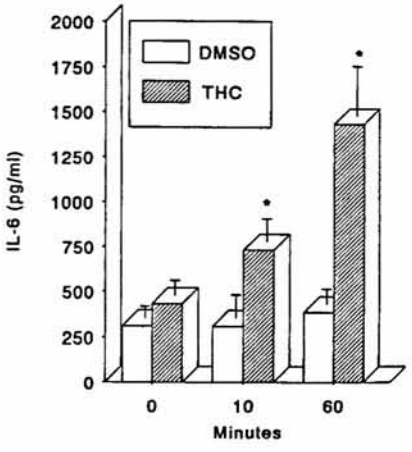

Figure 2. TNF activity in serum of mice treated with THC 24 hr before and 24 hr after i.v. infection with 8 x 10^7 Legionella. Each bar represents average serum TNF titers for for 5-10 mice per group given either THC or vehicle, i.e., DMSO, at 10 or 60 minutes after infection.

Legionella and THC one day before and one day after infection appeared to be a toxic shock like death.

Pro-Inflammatory Cytokines in Legionella Infected Mice Given THC

Since THC injection significantly modified the course of primary as well as secondary infection, studies were performed to determine the role played by the cytokine network in these observations, because high levels of pro-inflammatory cytokines such as TNFα and IL-6 are associated with a toxic shock like death in individuals infected with microorganisms like Legionella as well as other opportunistic bacteria. We found that the primary infection of mice with Legionella induced mobilization of acute phase cytokines. Therefore, it seemed likely that these cytokines were being mobilized at toxic levels by THC treatment and related to the rapidly developing toxic shock like death observed. Shock and collapse are known to be due to acute phase cytokine mobilization. Therefore we examined for this in THC treated mice infected with sublethal amounts of Legionella.

The acute phase cytokines TNFα and IL6 were found increased in serum of the mice following THC injection (Figs. 2 and 3). To show that these cytokines were important in the THC induced death of the Legionella infected mice, we found that injection of antibodies to either TNFα, IL6 or IL1 alpha/beta into the mice protected them from the drug induced mortality. Although it was not clear how mobilization of these cytokines in THC treated animals resulted in increased death to Legionella, it is possible that arachidonic acid metabolism, which has been shown to be increased by THC and also modulates cytokine mobilization, may be involved. In this regard, the endogenous-like ligand for THC has been shown to be an arachidonic acid-like substance termed anandamide.

THC Modulation of Th1 vs. Th2 Cell Function

It appeared likely that the THC was suppressing development of T cells involved in cellular immunity to Legionella, important in resistance to bacteria. Two sets of CD4 helper cells, i.e., Th1 and Th2 cells, have been defined based on the cytokine profiles of CD4 clones. These subsets have contrasting roles in the immune response. For example Th1 cells produce IL2 and interferon γ and are primarily involved in cellular immunity while Th2 cells produce IL4, IL5, IL6 and IL10 and are prominently involved in humoral immunity. Additonally, these two T cell subsets appear to regulate the production of different IgG isotypes by B

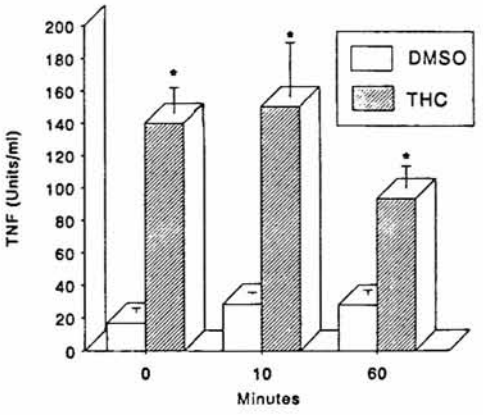

Figure 3. Average IL6 serum activity on 5-8 mice 10 or 60 minutes after primary infection with Legionella and treated 24 hr before and +24 hr after with 8 mg/Kg mouse and THC or, as a control, with DMSO vehicle. Each bar represents average titer at indicated times of the sera of THC treated and Legionella infected mice.

cells. For example, Th1 cells stimulate more IgG2a antibodies, while Th2 cells are involved in stimulating more IgG1 antibodies. Several studies have recently demonstrated the importance of Th1 cells and the cytokines they produce. This subset of helper T cells is thought to be important in mediating cellular immune resistance to bacteria, including Legionella. Thus we hypothesized that THC injection suppressed cellular immunity to Legionella by causing a change in the relative balance of Th1 vs. Th2 cells. To examine for this possibility, mice were infected with Legionella and treated with a single i.v. injection of THC (4 mg/Kg). Spleen cells from these mice and their serum were obtained. The spleen cells were cultured with killed Legionella to determine their specific lymphoproliferative response.

As expected, the cultured lymphocytes from Legionella infected mice showed a vigorous proliferation following stimulation with bacteria. The response was most vigorous when the mice were also immunized previously to the Legionella. However, mice infected with Legionella and treated with THC showed a significantly reduced antigen-specific proliferation. Thus THC given one day prior to a primary infection suppressed development of cell mediated immunity as measured by the antigen specific proliferation test with their cultured lymphocytes. Development of specific serum antibodies to Legionella following infection and drug treatment was also affected. Mice injected with THC and infected with Legionella as above were bled for analysis of anti-Legionella serum antibody, using a standard ELISA assay. When total anti-Legionella IgG antibody was measured, both non-drug and drug treated mice showed a good response. However, when antibodies of either the IgG2a or IgG1 isotype were measured, increased production of the IgG1 antibody class was found in the drug treated mice. These results supported the possibility of a shift from Th1 to Th2 activity in the THC treated mice infected with Legionella.

Several recent studies have shown a shift of Th1 to Th2 activity by lymphocytes from animals stimulated in culture with mitogens and determination of the relative production of either Th1 cytokines (i.e., IFN gamma) or Th2 cytokines (i.e., IL4). Thus, we performed such assays in THC treated and Legionella infected mice. For these studies, mice were treated with either THC or drug vehicle (i.e., DMSO) and then infected one day later with Legionella. The splenocytes from the mice were stimulated in culture with mitogen for 24 hr and the resulting supernatants assayed by ELISA for IFN protein. The spleen cells from the drug treated mice were found deficient in production of IFNγ as compared to spleen cells from animals infected only but not treated with THC. This suggested a shift from Th1 to Th2 cellular activity in the spleen of drug treated animals. The exact mechanism for such a drug induced shift from Th1 to Th2 cell activity is not known at this time. However, the balance of these two cells is known to be controlled by cytokines such as IL4 and IL12 and also by the number of ThO cells in lymphoid tissue. Thus it seems likely that THC affects these

events following infection with an opportunistic microorganism which is controlled mainly by cellular immunity.

THC Receptors and Lymphoid Cells

Cannabinoids such as THC are now known to influence cell function through the action of specific cannabinoid receptors as well as receptor independent mechanisms (Bouaboula et al., 1993, Devane, et al., 1988, Felder, et al., 1992, Gerard et al., 1991, Howlett, 1988, Kaminski, et al., 1992, Matsuda, 1990, Munro et al., 1993). A cannabinoid receptor was first demonstrated in rat brain membrane preparations by showing stereospecific binding of various cannabinoid agonists. Agonist binding studies also showed an inhibition of cAMP accumulation by means of a G; protein cDNA encoding the binding activity was cloned from a rat cerebral cortex library using a probe derived from the sequence of bovine substance K receptor. Although the cannabinoid receptor protein has not yet been isolated and characterized, a translated sequence of the cDNA suggest a protein of 473 amino acids in length, with several transmembrane brain regions and to be a member of the G protein coupled family of receptors. The human counterpart of this gene activity has also been cloned from a human brain cDNA library using a probe based on conserved sequences in the G protein receptor family. The deduced amino acid sequence suggests a protein of 472 residues that is 97% identical to the rat brain protein and 100% identical in the transmembrane regions.

Recently a second gene has been cloned from a human leukemic cell line (HL60) cDNA library using a probe based upon the G protein receptor family. The deduced amino acid sequence was only 44% identical to the brain receptor and unlike the brain gene is not expressed in the brain but rather in macrophages in the spleen. This and the fact that the binding potency of various receptor agonist differed from the brain receptors suggest the possibility that at least two separate receptors exist and their distribution is tissue specific. Multiple receptors are also suggested by the demonstration of a cannabinoid-regulatedly N type calcium channel which is coupled to a pertussis toxin sensitive G protein but is independent of intracellular cAMP. However, cannabinoid effects on cells, including lymphoid cells, are believed to occur also by receptor independent mechanisms. The high lipid solubility of cannabinoids promoting the partition of the drug into cell membranes may represent a way for these agents to disrupt the function of membrane proteins, and therefore, cell function. These effects would be expected to occur at drug concentrations higher than those causing receptor mediated changes and, indeed, this has recently been observed in CHO cells transfected with cannabinoid receptor cDNA's. Stereo selectivity has been demonstrated for both cannabinoid binding and inhibition of cAMP accumulation, but not for release of arachidonic acid and intracellular calcium. The IC_{50} doses for inhibition of cAMP accumulation were found to be in the low nM range while the release of arachidonic acid and calcium occurred at higher doses and also occurred in untransfected CHO cells.

The recent demonstration of cannabinoid binding sites and receptor gene expression in lymphoid tissue suggests a role for the receptor in immune cell function and immunomodulation by THC. Thus, studies in this laboratory showed that different splenocyte subsets had the brain gene mRNA. This gene message was found to be highest in B cells followed by T cells and macrophages. For these studies, purified mouse splenic B and T cells, as well as macrophage populations, were analyzed by RT-PCR for this brain receptor message. Although the level of amplified cDNA for β-actin mRNA was found to be equivalent among the three cell types examined, the level of the cannabinoid receptor message was found to be varied. We found also that stimulated lymphocytes expressed higher levels of this messenger RNA than unstimulated cells. Furthermore, a mouse macrophage cell line RAW 264.7, following stimulation with endotoxin, showed that the receptor message increased, with a maximum at 6 hr after stimulation, and declined by 24 hrs, suggesting that the brain

receptor gene for THC is regulated during leukocyte activation. Such data raise important biological questions of the role of this receptor in immune cells and suggest the level of gene expression varies from one leukocyte subset to another and that gene expression also varies with extensive cell activation.

DISCUSSION AND CONCLUSIONS

It is clear from the results of studies in this laboratory, as well as others, that THC, the major psychoactive cannabinoid in marijuana, can modulate the function of immune cells, including the production and/or release of cytokines. Furthermore, it is clear that the effects of the drug on immune cells and cytokine production or release are important in individuals infected with an opportunistic microorganism such as Legionella. Thus, our recent results with an infection model in THC treated animals, as well as studies of the receptor for cannabinoids, extend the results from various laboratories over the last two decades which have shown that THC, like other drugs of abuse, may affect the function of lymphocytes and macrophages, both *in vivo* and *in vitro*. It is already known that *in vivo* the size and architecture of lymphoid organs can be affected by treatment with such drugs and these changes can be related to drug induced alterations in a variety of cell functions, including cytokine production. The addition of drugs of abuse, such as THC, to cultures of mouse or rat lymphocytes modulates cell proliferation in a dose dependent manner. Besides, treatment of lymphoid cells *in vitro* with a drug of abuse such as THC depresses the ability of the cells to produce antibodies, respond to IL2 and produce cytokines, especially interferon. Thus it is accepted that drugs of abuse, including THC, may interfere with early events of lymphocyte signal transduction.

Injection of THC or other cannabinoids into mice or rats can also result in suppression of their antibody producing capacity as well as the capacity of the animal to develop cellular immunity. Such treated animals are unable to resist challenge by sublethal infection with a bacterium or virus. Most studies concerning the effect of drugs of abuse such as THC on immunity have been performed with adults and in subjects who displayed normal immune function. Recently, however, it has been found in this laboratory that THC is more markedly suppressive for the immune responsiveness of immature individuals (Pross, et al., 1990, 1992). For example, THC has been found to be more immunosuppressive for one or two week old mice, equivalent to children who are newborn or infants. In addition, THC is also markedly immunomodulatory for lymphoid cells from older mice, equivalent to humans who are 50-70 years old or more. This drug has also been shown to be more suppressive for lymphocytes in the thymus as compared to lymphocytes in the spleen or lymph nodes. The thymus is thought to be more important for immunoregulation than peripheral lymphoid organs. The greater sensitivity of thymus cells to the immunosuppressive effects of THC suggest that this component of marijuana has a greater immunomodulatory potential for immature lymphocytes.

As found in this laboratory, treatment of animals or lymphoid cells from these animals with marijuana and its psychoactive components does not always result in immunosuppression. In some cases, an immunoenhancement occurs and this is often related to relatively low concentrations of the drug. Production of the cytokine IL1 is augmented in animals or macrophage cultures following exposure to THC. TNFα production is also enhanced under certain circumstances, both *in vivo* and *in vitro,* following THC exposure. However, it is important to note that the clinical information obtained to date indicates that marijuana or other drugs of abuse does not inevitably result in a marked increase of acute disease incidence or marked depression of immunity in normal individuals. However, use of marijuana, as well as other drugs of abuse, may increase susceptibility to chronic diseases. For example, the

observed alterations of immune function by concentrations of THC close to those readily found in the blood of drug abusers probably has little impact on development of systemic acute disease in otherwise normal healthy individuals. However, the observation made by a number of investigators over the last two decades, including ourselves, indicate it is likely that localized disease in areas in the body where drugs of abuse concentrate may be significantly impacted.

Observations such as that described above suggest that the biological impact of a drug of abuse such as marijuana on immune mechanisms may have especially negative effects on a host by increasing susceptibility to chronic infections and/or tumors (Donald, 1991, Endicott, et al., 1991). More importantly, individuals with an already compromised immune system due to the presence of existing tumors or with immunosuppressed functions caused by infection may be at higher risk for these drug effects vs. healthy individuals. For example, individuals with HIV infection could have an increased risk for developing active AIDS when smoking marijuana or taking other drugs of abuse, including heroin, cocaine or alcohol. Furthermore, individuals using drugs of abuse may be at higher risk for infection by certain opportunistic bacteria, fungi or viruses. It should also be noted that cancer patients who use marijuana to negate the nauseating effects of chemotherapy could be at increased risk for immunosuppression, creating a greater risk of tumor reemergence during therapy. Likewise, bone marrow transplant patients given a THC-containing product to relieve nausea may be at an increased risk for opportunistic infections.

It should be noted that in some cases the immunosuppressive effects of THC might also have desirable effects by increasing the potential of host resistance to disease. For example, by downgregulating the production of an antitumor blocking factor, which may protect a tumor from immune recognition, THC treatment may actually allow the destruction of a tumor. Furthermore, it has recently been reported that THC given to experimental rats blocked the induction of allergic encephalomyelitis resulting from sensitization with neurologic tissue (Lyman et al., 1989). This experimental disease is considered an animal model for multiple sclerosis. It has been postulated that THC suppression of the experimental disease occurs because this drug is immunosuppressive and may inhibit the autoimmune response to neurological tissue which normally would induce the autoimmune disease. However, no studies have yet been performed to determine whether marijuana or other drugs of abuse given in a clinical setting has a beneficial effect on the general well being of a patient.

Most studies to date have provided only minimal information about the mechanisms of immunomodulation induced by drugs of abuse. Nevertheless, newer studies, including those reviewed here with marijuana concerning the effects of this drug of abuse on molecular aspects of the immune responses, as well as involvement of receptors to the drugs, are now emerging. Future investigation will undoubtedly focus more on the molecular mechanisms by which drugs of abuse affect immune cell function not only in experimental animal model systems but also in humans.

SUMMARY

THC, the major psychoactive component of marijuana, has been shown both in humans and experimental animals to have immunomodulatory properties. For example, marijuana smokers may show impaired immunological functions, including deficiency of blood leukocyte blastogenesis to mitogens. Detailed studies with mice have shown that animals given THC can show marked immunomodulation, including suppression of antibody formation, deficient cytokine production, etc. However, recent studies have also shown that lymphoid cells evince enhanced production or release or IL1, but suppression of IL2

and interferon production. Such lymphoid cells treated *in vitro* with THC also show suppressed blastogenesis to antigens and mitogens, suppressed NK activity, etc. In contrast, it has recently been shown that THC can enhance production or release of pro-inflammatory cytokines. This includes release of these cytokines from macrophages, including augmented release of IL1, TNFα, and IL6 activity. Susceptibility of mice to infection with opportunistic organisms such as *L. pneumophila* has been found and this increased susceptibility can be modulated by THC. A toxic shock-like death to Legionella has been induced by THC treatment given one day before and one day after infection. Receptors to THC have been detected in the brain as well as in peripheral tissues, including lymphoid cells. Thus, immunomodulation induced by THC may be related to receptor effects as well as unrelated to such receptors. It is clear that THC and other cannabinoids are excellent tools for studying the mechanisms of immune modulation, especially altered susceptibility to microbial infection.

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