Università degli Studi di Milano

Dipartimento di Scienze Farmacologiche e Biomolecolari

Scuola di Dottorato in Scienze Farmacologiche

Ciclo XXVI BIO 14

Exposure of adolescent mice to Δ^9-Tetrahydrocannabinol shapes immune response in adulthood

Tutor: Prof.ssa Paola Sacerdote

Direttore della Scuola: Prof. Alberto Panerai

Tesi di Dottorato di Ricerca

Dott.ssa Sarah Moretti

R09323

Anno Accademico 2012-2013

ABSTRACT

ABSTRACT

Marijuana, also called Cannabis, is the illicit drug most frequently used by human adolescents; this is facilitated by the fact that users generally perceive these drugs as relatively harmless.

Recent data estimate that cannabis use starts in the period from 12 to 18 years of age, though even earlier use (9–10 years) is now anecdotally reported. Among the many consequences related to the cannabis use, it is increasingly emerging as the Δ^9-THC, as well as endogenous cannabinoids, are able to affect certain functions of the immune system.

In fact, studies conducted in experimental animals indicate that cannabis impairs the immune response by altering the homeostasis of the immune system.

The adolescence is a particularly vulnerable period of individual life, both for physical and psychological maturity; also the immune system during this development period might be particularly vulnerable to the effects induced by this drug. However, there aren't currently available studies to investigate whether the cannabis intake during adolescence period can have a negative impact on some immune functions such as the individual's response to infection, allergic and autoimmune pathologies, both during the period of drug intake and in adulthood, thus long time from the last direct cannabis exposure.

The purpose of this study was to investigate whether the use of cannabis during adolescence may induce immediate and delayed effects on the immune system that may then persist in adulthood.

As immune parameters we considered both innate and acquired immunity, measuring T-cells and macrophage cytokines production.

We used Balb C/J male mice, 33 post natal days (PND) as model of adolescence age and 80 PND as model of adult age. Δ^9-THC was administerd with increasing subcutaneous (s.c.) doses, starting from 5 mg/kg and gradually reaching 15mg/kg in 10 days.

Control animals were treated with the vehicle for the same duration of time.

Some groups of animals were sacrificed, for the immune evaluation, immediately at the end of the treatment, while other groups of mice were housed until 90 PND before the immune evaluation.

We recently added a fourth study group consisting of adult animals treated with the drug, for 10 days, and housed for 47 days, up to 137 PND, when the evaluation of immune parameters were performed. The data regarding this last study group are not completed, due to the long times required for the implementation of the experimental protocol and refer only to the innate and acquired immunity.

In order to study acquired immunity, young and adult mice were immunized with KLH protein to induce an antigen-specific reaction and Th1/Th2 balance was assessed by measuring the production of IFN-γ, IL-4 and IL-10 by splenic lymphocytes.

In order to assess macrophage function we used peritoneal macrophages stimulated in vitro with LPS endotoxin; IL-1β and TNF-α were assessed as pro-inflammatory cytokines, while IL-10 was evaluated as anti-inflammatory cytokine.

The cytokine production was measured by specific ELISA and Real-Time PCR.

At the end of Δ^9-THC treatment in both young and adult mice, after immunization, a significant reduction in the production of IFN-γ was observed, while the Th2 cytokines IL-4 and IL-10 were significantly increased.

When we measured immune response of adult mice treated with the drug in adolescence, we observed that the production of IFN-γ was still lower and that, in contrast to what observed immediately at the end of treatment, also the Th2 cytokine levels were decreased compared with vehicle mice.

In adult animals, assessed 47 days after the end of treatment, there were no significant differences between Δ^9-THC or vehicle groups.

We also evaluated the ability of the animals to develop an antibody response by measuring IgM anti-KLH titers in the serum; in all study groups, the antibody titers were significantly reduced compared to the vehicle.

Regarding the macrophage function, the Δ^9-THC treatment induced in both young and adult mice a significant increase of IL-10 while TNF-α and IL-1β are decreased. Particularly interesting were the results obtained in mice treated in adolescent age with Δ^9-THC respect to vehicle; in fact, we observed an opposite effect since IL-1β and TNF-α levels were significantly increased while IL-10 production was lower, indicating a switch towards a pro-inflammatory phenotype of the macrophage.

These effects on cytokines are at transcriptional level since we observed similar result by measuring IL-10, IL-1β and TNF-α mRNA with RT-PCR. In adult animals evaluated 47 days after the end of treatment, by analyzing the pro- and anti-inflammatory cytokine levels and their relative mRNA expression, no significant differences were found between treatments considered. In order to understand whether the Δ^9-THC effects might be due to an alteration of circulating hormones, we also assessed the modulation of hypothalamic-pituitary-adrenal axis (HPA) axis activity, after sub-acute and chronic Δ^9-THC treatment, by measuring the corticosterone plasma levels in the three study groups.

The corticosterone concentrations were lower in all groups, indicating a long lasting dysregulation of HPA.

Using a method HPLC/ Mass Spectrometry, we also evaluated blood levels of Δ^9-THC and its main metabolites in the experimental groups considered, showing that at the end of 10 days treatment they were comparable to those that can be found in human heavy smokers. We also demonstrated that in animals treated as adolescents and studied as adults, plasma Δ^9-THC was not more present, further assuring that the effects observed were due to a long lasting modulation of immunity

In addition, to understand the mechanisms underlying the observed effects, we began to investigate whether the Δ^9-THC treatment may affect hematopoietic cells development.

Preliminary data obtained by evaluating the number of colonies formed by hematopoietic precursors have not highlighted differences between the different treatment groups.

Finally, we started to study also the Δ^9-THC effects on neuroinflammation, by evaluating the basal production of pro- and anti-inflammatory cytokines in brain areas that are particularly rich in cannabinoid receptors, such as hippocampus and hypothalamus.

Our data indicate that chronic treatment with the drug is able to reduce the basal levels of IL-1β and TNF-α in the brain areas considered, in adolescent and adult mice assessed immediately at the end of treatment.

The basal production of IL-10 instead appears to be increased compared to relative vehicles in mice evaluated immediately at the end of the treatment.

In adult animals, treated with the drug as adolescents, the basal production of pro-inflammatory cytokines, in hypothalamus and in hippocampus, appears to be increased with respect to vehicles and IL-10 decreased, indicating a predisposition to a pro-inflammatory status.

Although these data are preliminary they indicate a parallel modulation of brain and peripheral cytokines by Δ^9-THC.

In conclusion, these results indicate that the immune system is profoundly altered by treatments with Δ^9-THC, with the presence of a dysregulated response.

In particular we can affirm that the administration of Δ^9-THC in adolescent animals has significant effects on the immune response that last long after its intake.

INTRODUCTION

INTRODUCTION

HISTORY OF CANNABIS

The cannabis plant has been known since antiquity and grows in almost all parts of the world, but has been known principally as a source of useful fibre for the manufacture of textiles and rope. The use of the plant has origins in Asia, in particular in China, where archaeological and historical findings indicate that Cannabis was cultivated for fibres since 4000 B.C.

Kalant (Kalant, 2001) and Zuardi (Zuardi, 2006) have reported also an important use of cannabis in Asia for the treatment of disorders like rheumatic pain, intestinal constipation, disorders of the female reproductive system, malaria and others.

In India the use of cannabis was disseminated, both as a medicine and recreational drug, probably around 1000 B.C. (Zuardi, 2006). From Asia, cannabis spread to the Western World where several references reported the use of this plant in Ancient Egypt (Figure 1).
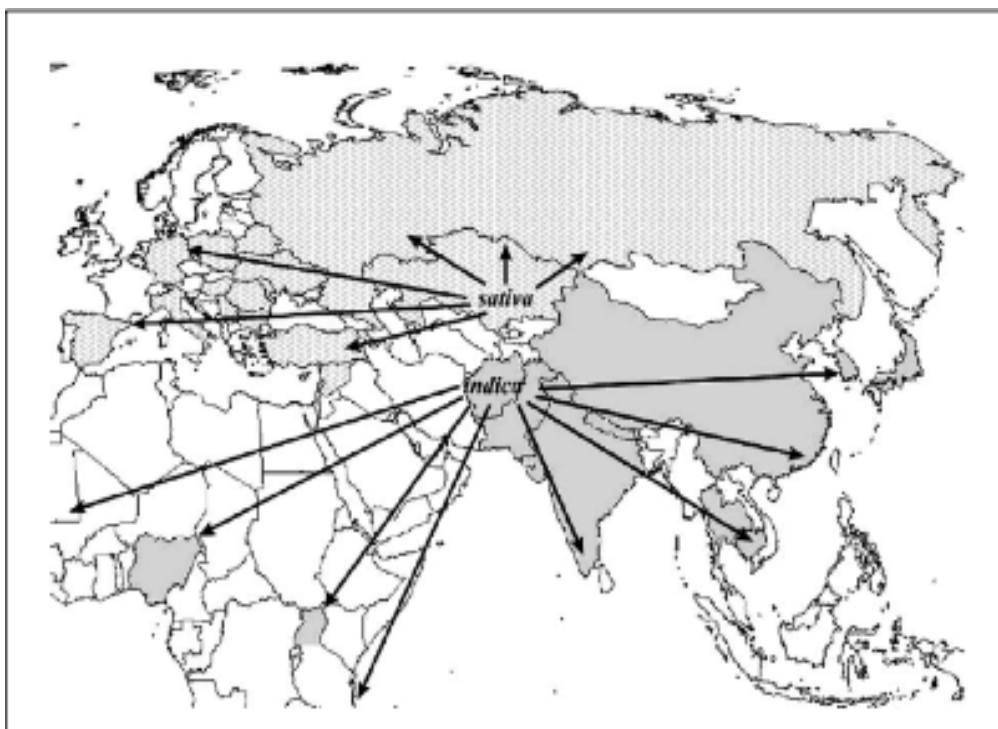
Figure 1 Map showing the countries of origin Cannabis indica and sativa. Figure taken from Hilling 2005

The scarce literary references regarding the use of cannabis by the Greeks and Romans suggest that it was little used by these populations.

In Africa the use of cannabis began around the 15th century where it was reported useful for snake bite, to facilitate childbirth, malaria, fever, asthma and dysentery. In the 16th century, Cannabis reached the Americas, where probably African slaves brought the plant's seeds to Brazil. Here, cannabis was above all used in popular religious rituals. In the middle of 19th century, cannabis was introduced in Western Medicine by O'Shaughnessy, an Irish physician, and Moreau, a French psychiatrist.

Their contributions had a deep impact on Western medicine, especially due to the scarcity of therapeutic options for infectious diseases (Zuardi, 2006).

In the 20th century, several laboratories started to market cannabis extracts or tinctures, such as Merk, Burroughs-Wellcome, Parke-Davis and Eli Lilly (Fankhauser, 2002).

During this period, the medical indications of cannabis, as reported in Sajous's Analytic Cyclopaedia of Practical Medicine (1924)(Aldrich, 1997), were summarized in three areas: sedative or hypnotic, analgesic and in appetite and digestion dysfunctions.

After this period, the use of cannabis significantly decreased, because of the difficulty to obtain replicable effects, due to the varying efficacy of different samples of the plant.

Moreover, the introduction of many legal restrictions limited the medicinal use and experimentation with cannabis. In the second half of 20th century, cannabis was mainly used as a drug for recreational use by intellectuals groups, and later spread among the younger generation throughout the Western World.

In 1964, Gaoni (Gaoni, 1964) isolated and characterized the main psychoactive component of cannabis, Δ^9-tetrahydrocannabinol, thus contributing to a proliferation of studies about the active components of the plant.

In the last 30 years three drugs have been developed from cannabis or on the basis of active components derived from cannabis:

1. Nabilone, synthetic analogue of Δ^9-THC; it is used for suppression of nausea and vomiting produced by chemotherapy.

2. Dronabinol; it is the synthetic (-)-trans-Δ^9-tetrahydrocannabinol; this drug finds applications as an anti-emetic and appetite stimulant.

3. Sativex®; it contains equal amounts of Δ^9-THC and the non-psychoactive plant cannabinoid, cannabidiol (CBD), and is prescribed for the symptomatic relief of spasticity and neuropathic pain in adults with multiple sclerosis, and as an adjunctive analgesic treatment in advanced cancer (Pertwee, 2009).

Cannabis is currently the most recreationally used illicit substance across the globe; the prevalence of use has increased markedly over the past decade in young people in the Europe although patterns of consumption vary between different social groups.

TAXONOMY OF CANNABIS

Cannabis sativa is an annual, dioecious, wind-pollinated herb, with male and female flowers developing on separate plants. It propagates from seed, grows in open sunny environments with well drained soils and has an abundant need for nutrients and water. It can reach up to 5 meters in height in a 4-6 month growing season.

According to current botanical classification, Cannabis belongs, with Humulus (hops), to the family of *Cannabinaceae* (also *Cannabaceae* or *Cannabidaceae*).

As regards the exact taxonomy of the genus Cannabis (family Cannabaceae) there are different opinions depending on considering the polytypic or monotypic species.

The binomial nomenclature (Linnaeus, 1735; Small, 1976) considers this genre as monotypic, the only species known as Cannabis sativa L.

In 1785 Lamarck described a second species of Cannabis, which he called Cannabis indica Lam.: this species, of lesser importance than the Cannabis sativa as a fiber plant, shows a more substantial stimolant use. With the name of Cannabis indica it joined Pharmacopoeia of many countries, thus indicating the species designated for medicinal preparations.

In 1924 the Russian botanist Janichevsky described a third species, which he identified as Cannabis ruderalis; this species extended from the north of European Russia to Central Asia (Schultes, 1976).

In 1976, Small in his taxonomic revision defined a single species of Cannabis with two subspecies: C. sativa L. subsp. sativa, and C. sativa L. subsp. Indica.
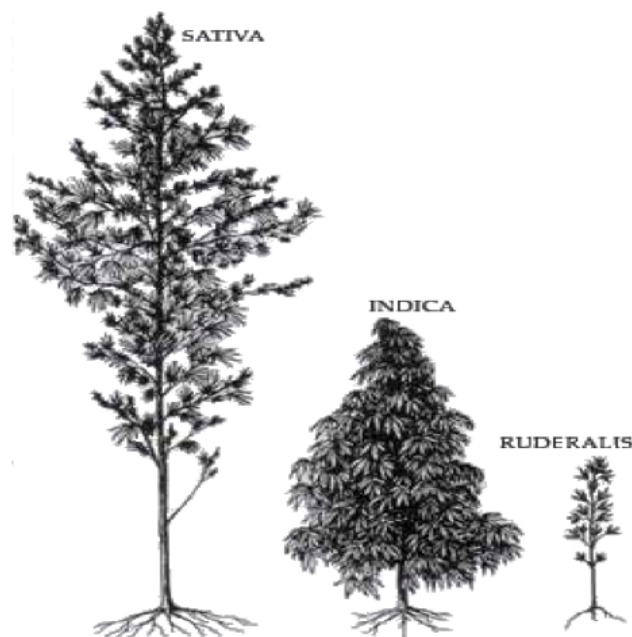
Figure 2 Morphology of Cannabis species

The hypothesis was that the two subspecies differentiated substantially as a result of selection by man: the Cannabis sativa subsp. Sativa was selected for the production of fiber or seeds, while Cannabis ativa subsp indica had been selected for medical and stimolant use.

Taxonomic studies conducted in the same year led the botanist Schultes to identify, on the basis of morphological differences, at least three species, Cannabis sativa, Cannabis indica, and Cannabis ruderalis so rejecting monotypic theories (Schultes, 1976).

The traditional forms in which the substance is consumed are: Marijuana (inflorescences, dried leaves), in which the percentage of Δ^9-THC can vary between 0.5 and 5% in seedless varieties and between 7 and 14% in seeded varieties, called "sinsemilla", while even higher concentrations are reported for the "Nederweed marijuana"; Hashish (cannabis resin and pressed flowers) with Δ^9-THC concentrations ranging from 2 to 20%, Hashish oil at concentrations of 15-50%.

Cannabis is smoked in special devices (chilum, hookah) or hand-rolled cigarettes (joints, canes, joint) with or without the addition of tobacco.

CANNABIS COSTITUENS

Cannabis is composed by a vast number of compounds (approximately 538), including mono- and sesquiterpenes, sugars, hydrocarbons, steroids, flavonoids, nitrogenous compounds and amino acids, among others. The more known and the most specific class of cannabis constituents is represented by cannabinoids, which are characterized by a C21 terpenophenolic constituent's structure (ElSohly and Slade, 2005). There are ten main types of cannabinoids and fourteen different cannabinoids subtypes, based on their chemical structure (Figure 3).

The first natural cannabinoid to be discovered was cannabinol (CBN), which was extracted in 1899 and chemically characterized in 1940 (Adams et al., 1940).

In 1963, Mechoulam's group isolated cannabidiol (CBD), the main non-psychoactive constituent of cannabis (Mechoulam and Shvo, 1963), and one year later they isolated and characterized Δ^9-THC, the main psychoactive component of cannabis (Gaoni, 1964). In more recent years, other phytocannabinoids have been isolated and characterized, but it is possible

that some of them may not have been discovered yet.

Phytocannabinoids are synthesised in cannabis by glandular trichomes as carboxylic acids from geranyl pyrophosphate and olivetolic acid to yield the parent phytocannabinoid compound, cannabigerolic acid (CBGA). Subsequent reactions involving different enzymes catalyze the transformation of CBGA into other phytocannabinoids. The presence of these enzymes differs between various strains and species of cannabis, resulting in a different content of phytocannabinoids (Russo, 2007).
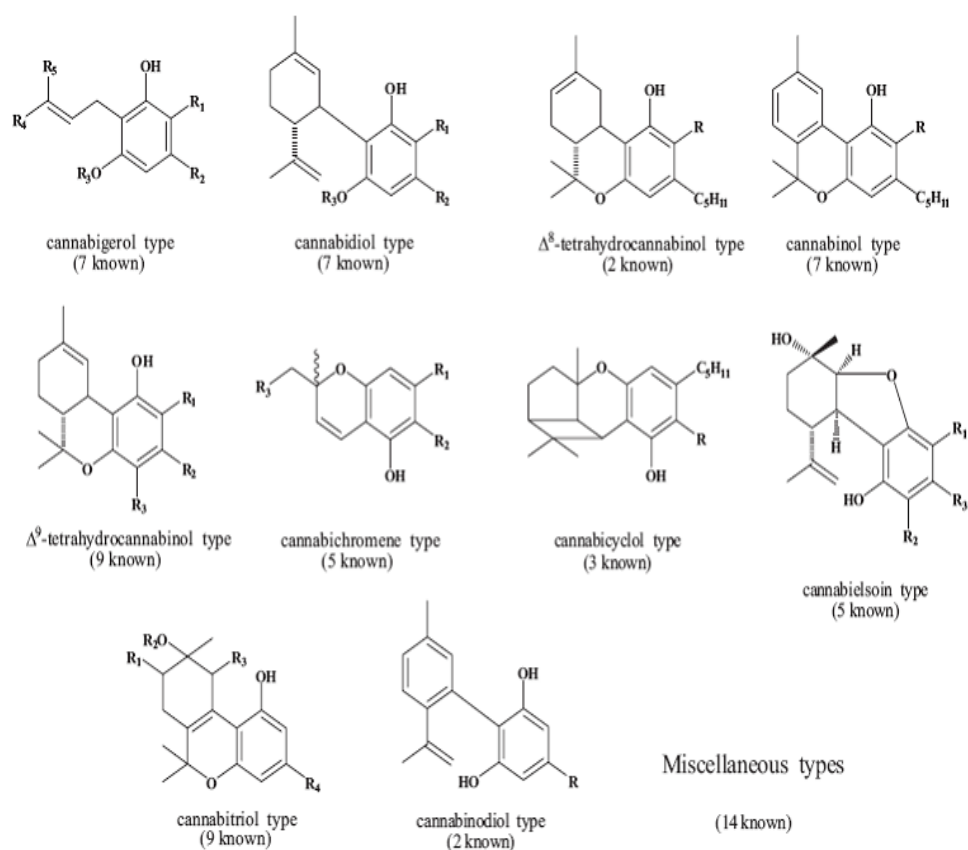

Figure 3 The phytocannabinoids can be subclassified into 10 main groups depending on their chemical structure. Figure taken from Hanus, 2009

Δ^9-THC is the main psychoactive constituent of cannabis; chemically, Δ^9-THC is a poorly water soluble, amorphous substance which is sticky, resin-like and highly viscous which makes it difficult to handle and process (Thumma et al., 2008).

As a cannabis constituent, Δ^9-THC is assumed to be involved in self-defence, perhaps against herbivores. Besides, Δ^9-THC has been found to posses high UV-B (280-315 nm) absorption properties, which have been speculated to protect the plant from harmful UV radiation exposure (Pate, 1994).

Δ-9-tetrahydrocannabinol (THC)
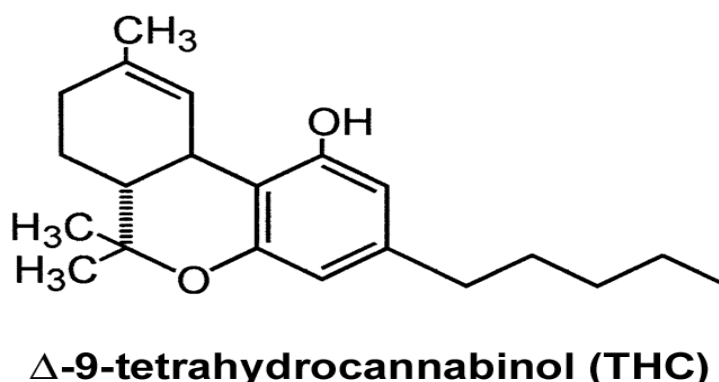
Figure 4 The molecur structure of Δ^9-THC

Dronabinol is the synthetic analogue of tetrahydrocannabinol, in particular the (-)-trans-Δ^9-tetrahydrocannabinol isomer that is the main isomer of as a cannabis constituent, (Pate, 1994). Dronabinol is sold as Marinol (a registered trademark of Saolvay Pharmaceuticals); in this formulation, the drug has limited stability and has to be stored at low temperatures (4°C). Furthermore, Δ^9-THC has been reported to be unstable in acid solutions or when exposed to heat, air, and light. Hence, in the last few years researchers have been challenging the development of Δ^9-THC as a prodrug, to avoid the pharmacokinetics and physico-chemical limitation properties of the pure drug (Thumma et al., 2008).

PHARMACOKINETICS OF Δ^9-THC

ABSORPTION

Smoking is the principal route of cannabis administration and provides a rapid and higly efficient method of drug delivery. Approximately 30% of Δ^9-THC in marijuana or hashish cigarettes is destroyed by pyrolysis during smoking (Abrams et al., 1985).

Smoked drugs are highly abused in part because of the efficiency and speed of delivery of the drug from the lungs to the brain.

Bioavailability of smoked Δ^9-THC is reported to be 18%-50% partly as a result of the intra- and inter-subject variability in smoking dynamics that contribute to uncertainty in dose delivery (Agurell et al., 1986).

The number, duration, and spacing of puffs, hold time, and inhalation volume greatly influence the degree of drug exposure (Azolorosa et al., 1992). Δ^9-THC can be measured in the plasma within seconds after inhalation of the first puff of marijuana smoke.

In some studies Δ^9-THC was measured in blood, and expected values were found to be about half those of plasma. Albumin and other proteins that bind Δ^9-THC and the poor penetration of Δ^9-THC into red blood cells contribute to these higher plasma concentrations.

If cannabis is ingested orally, absorption is slower and peak plasma Δ^9-THC concentrations are lower (Law et al., 1984).

Wall et al. (1983) found peak Δ^9-THC concentrations approximate 4-6 hours after ingestion of 15-20 mg of Δ^9-THC in a chocolate cookie. Oral bioavaialability has been reported to be 4-20%, in part as a result of degradation of drug in the stomach.

Also, there is significant first-pass metabolism to active 11-hydroxy-Δ^9-tetrahydrocannabinol (11-0H-THC) and inactive metabolites. Plasma 11-OH-THC concentrations range from 50 to 100% of Δ^9-THC concentrations following the oral route of cannabis administration compared to only about 10% after smoking (Wall et al., 1983).

11-OH-THC is equipotent to Δ^9-THC, explaining the fact that pharmacodynamic effects after oral cannabis administration appear to be greater that those after smoking Δ^9-THC at the same concentrations.

DISTRIBUTION

Cannabinoids rapidly penetrate into the tissues (liver, heart, lung, gut, kidney, spleen, mammary gland, placenta, adrenal cortex, thyroid, pituitary gland, fat and lower concentrations in brain, testis and foetus) because of their high lipophilicity. Therefore, the distribution volume is about 10 L/kg (Kelly and Jones, 1992). Δ^9-THC is strongly bound to plasma proteins (~97%). About 60% is bound to lipoproteins (the low-density fraction), about 9% to blood cells and the rest to albumin. The Δ^9-THC metabolite 11-hydroxy-Δ^9-THC (11-OH-THC) is even more strongly bound (~99%).

METABOLISM

About 100 metabolites have been identified for Δ^9-THC so far. All cannabinoids are good substrates of cytochrome P450. The hydroxylation sides of Δ^9-THC are at C11 and C8 as well as at all positions of the alkyl side-chain. The preferred hydroxylation site in man is C11. It has been shown that cytochrome P450 isoenzyme 2C9 catalysis the formation of the psychoactive 11-OH-THC metabolite of Δ^9-THC and that cytochrome P450 3A is responsible for the hydroxylation at the 8β-position (Watanabe et al., 1995).

Many of the hydroxyl groups undergo further oxidation after the hydroxylation.

Carboxylic groups at C11 and C5' (alkyl side-chain) are formed. 11-OH-THC is further oxidised, probably by alcohol dehydrogenase and of microsomal alcohol oxygenase, to the intermediate aldehyde 11-oxo-THC followed by oxidation to 11-nor-9-carboxy-THC catalysed by a microsomal aldehyde oxygenase, a member of the CYP2C subfamily (Watanabe et al., 1995; Nadulski et al., 2005)

After glucuronidation of the carboxy group, the THC-COOH is the main metabolite excreted and found in urine (Maurer et al., 2006).

Carbon atoms from the alkyl side-chain get lost after β-oxidation and related reactions of the C5'-acid. The predominant phase II metabolite of Δ^9-THC is the O-ester-glucuronide of THC-COOH. The formation of conjugates with long-chain fatty acids is another possible pathway. Although it is a phase II reaction, the lipophilicity is rather increased than decreased. It is possible that these conjugates with long-chain fatty acids may be retained within tissues for considerable periods of time because they are membrane constituents resembling compounds.
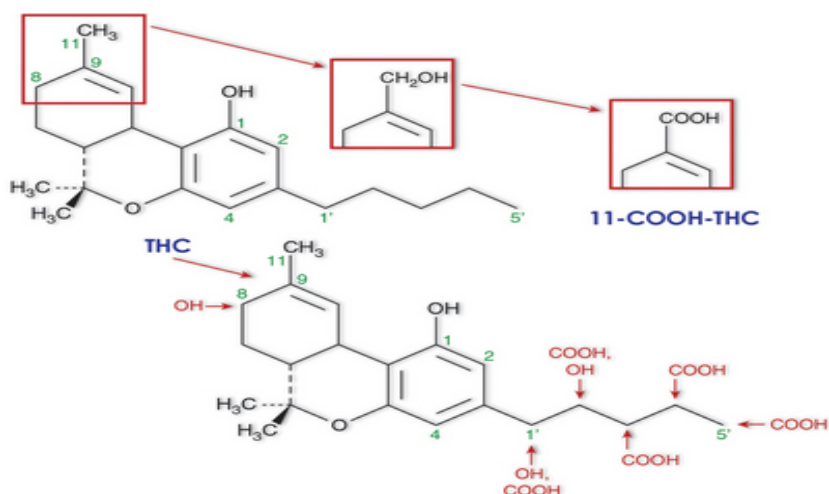
Figure 5 The metabolism of Δ^9-THC

ELIMINATION

Elimination of Δ^9-THC from the plasma is rather slow because of the limited re-diffusion of Δ^9-THC from body fat and other tissues into the blood. The results of this phenomenon are low plasma levels. The literature describes very variable half-life periods from 1 to 4 days. The absolute elimination time could require up to five weeks. Δ^9-THC metabolites are excreted in urine (~20%) and faeces (~80%). The distinctive enterohepatic cycle is another reason for the slow elimination (Kelly and Jones, 1992).

Most urinary metabolites are acids. The major metabolites found in urine are THC-COOH and THC-COOH-glucuronide.

Normalised to the drug creatinine ratio, THC-COOH and its glucuronide are used for detection and monitoring of drug abuse (Musshoff and Madea, 2006).

THE ENDOCANNABINOID SYSTEM

The endocannabinoid system refers to a group of endogenous lipid and their receptors that are involved in a variety of physiological process including neurotransmitter release, synaptic plasticity, inflammation and appetite stimulation. The endocannabinoid system is constitued by:

- The cannabinoid receptor type 1 and 2, (CB1 and CB2), primarily located in the Central Nervous System (CNS) and periphery respectively

- The endogenous lipids (endocannabinoids) that are synthesized " on demand" as ligands for the cannabinoid receptors

- Enzymes involved in the synthesis and degradation of endocannabinoids

CANNABINOID RECEPTORS

The two main subtypes of cannabinoid receptors (CBR) classically described (CB1 and CB2) are G protein coupled receptors, therefore constituted by a single polypeptides with an extracellular N-terminus, and intracellular C-terminus and seven transmembrane helices. Both mainly activate G proteins (Gi proteins) inhibitory to adenylate cyclase thus inhibiting the conversion of ATP to cyclic AMP (cAMP)(Howlett and Mukhopadhyay, 2000).

However they can also activate adenylate cyclase through stimulating G proteins (Gs proteins), and both are positively coupled to mitogen-activated protein kinase (MAPK)(Woelkart et al., 2008).

CB1 is coupled with ion channels, inhibiting D-type K+, N and P/Q-type Ca 2+ currents, and activating inward and A-type rectifying K+ currents (Croxford and Yamamura, 2005).

For CB2, the ion channel modulation is more variable; CB2 signaling mechanisms also involve the activation of phosphatidylinositol 3-kinase and Akt (PI3-Akt) pathway and increases the synthesis of the sphingolipid messenger ceramide, thus having a pro-survival and pro-apoptotic effect, respectively (Carracedo et al., 2006).

Besides the CB pathway, endocannabinoids also interact with other receptors, like the vanilloid receptor-type 1 (TRPV-1) activated by anandamide (AEA), and also K+ channels, 5-HT3 receptors and alpha 7nicotinic receptors (Mackie, 2008; Oz, 2006).

It is not clear which of these interactions are relevant for the physiologic effects of cannabinoids or just a consequences of their lipophilic character. Some effects of the classical cannabinoids such as anti-emesis may not be mediated only by CB1-CB2 receptor system; some evidence suggests the existence of additional cannabinoid receptor subtypes possibly responsible for these alternative mechanisms (Howlett et al., 2002).

Recent studies focus on GPR55, suggesting that this receptor can be activated by anandamide and 2-arachidonoylglycerol (Ryberg et al., 2007).

CB1 RECEPTOR

CB1 is the most abundant G-protein–coupled receptors in brain and was cloned from rat and human brain specimens in 1990 and encoded a protein with 472 amino acids.

It is highly expressed in brain tissue and to a lesser extent in the adrenal gland, reproductive organs, bone marrow and immune cells.

The mouse CB1 sequence has also been cloned, and showed 99% and 97% identity to rat and human CB1, respectively, at amino acid level (Bosier et al., 2010).

CB1 genes have been demonstrated in other species including the tetraodontoid fish, Fugu rubripes (Yamaguchi et al., 1996), suggesting the conservation and importance of the CB system in evolution (Klein et al., 1998).

Regarding the receptor signalling, CB1 receptor is coupled to Gi and Go proteins, and its activation involves the inhibition of adenylyl cyclase with a consequent decrease in cyclic AMP production. Activation of the CB1 receptor is also associated with the activation of MAPK, which regulates many cellular functions such as cell growth, transformation and apoptosis. The

CB1 receptor has also been associated with the modulation of voltage-gated ion channels, in particular N-and P/Q-type calcium channels and inwardly rectifying A-type potassium channels, which are thought underlie the cannabinoid-induced inhibition of neurotransmitter release at presynaptic level (Howlett et al., 2002).
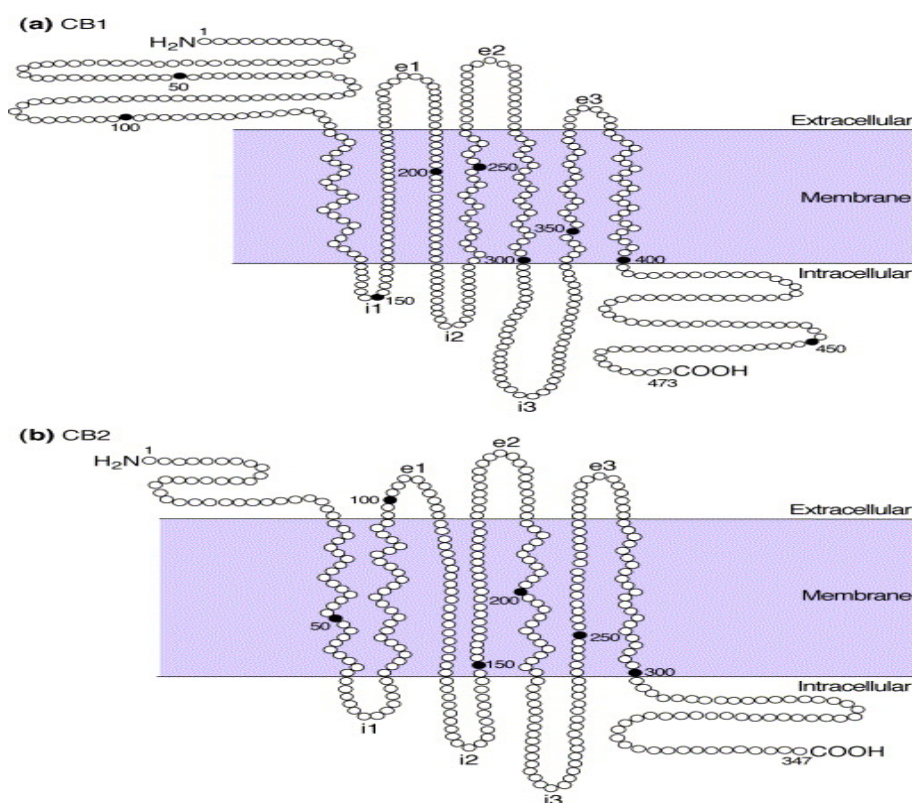
CB2 RECEPTOR

The gene encoding the human cannabinoid CB2 receptor was cloned in 1993 (Munro et al., 1993) and has now been isolated from several diverse species including mouse (Shire et al., 1996), rat (Brown et al., 2002), Japanese pufferfish (Elphick, 2002) and zebrafish (Rodriguez-Martin et al., 2007). In addition this receptor was revealed to exhibit 68% identity to the CB1 receptor within the transmembrane regions and 44% identity throughout the whole CB1 protein (Munro et al., 1993). Unlike the CB1 receptor nucleotide sequence, which appears to be highly conserved between the human, mouse and rat, the nucleotide sequence of the CB2 receptor exhibits less homology between these mammalian species. For example, the human and mouse CB2 receptor share 82% amino acid identity (Shire et al., 1996) whereas the mouse and rat 93% homologous (Brown et al., 2002).

CB2 receptors are prevalently found in immune tissues, most abundantly in the spleen and leukocytes (Ofek et al., 2006). At the cellular level, B cells express high levels of CB2 receptors, while moderate levels are found in natural killer cells, and low levels are found in T-cells (Galiegue et al. 1995). The presence of CB2 in dendritic cells, potent antigen-presenting cells, suggests a role for cannabinoids in modulating antigen presentation (Matias et al., 2002). Other tissues and cells also express CB2 receptors including enterocytes, osteoclasts and osteoblasts, and the liver (Mackie, 2008). Initially, northern analysis, quantitative Real Time-PCR analysis and autoradiography could not elicit the CB2 presence in the brain, but Western-blot analysis and immunohistochemistry demonstrated its presence in astrocytes and microglia.

Because CB2 expression on microglia is related to the cell activation status, it was also suggested that it may be induced by local inflammation, infection or stress (Croxford and Yamamura, 2005), but further studies demonstrated that CB2 is present in astrocytes, microglia, neural subpopulations and oligodendroglial progenitors in healthy brains (Van Sickle et al., 2005). Interestingly, CB2 receptor appears to be highly inducible, which makes this receptor an interesting tool for the treatment of diseases such as inflammation, pain, atherosclerosis, hepatic fibrosis, and bone remodelling, where CB2 receptors have been shown to be involved (Mackie, 2008).

Figure 6 Mouse cannabinoid receptor CB1(a) and CB2 (b). Figure taken Klein et al., 1998

PHARMACOLOGY OF Δ^9-THC AT NON-CB1, NON-CB2 RECEPTORS

It has been found that $\Delta 9$-THC interacts with several no cannabinoid receptors, targeting the orthosteric or allosteric sites of other GPCRs, transmitter-gated channels, ion channels or nuclear receptors. For example, $\Delta 9$-THC binds the allosteric sites of opioid receptors. GPR55 is an additional GPCR that has been found to be activated by Δ^9-THC (Pertwee, 2010).

Moreover Δ^9-THC acts as a potent antagonist on the ionotropic receptor 5-HT3, which belongs to the serotoninergic receptor family. 5-HT3 receptors are located prevalently in the central and peripheral neurons, where they trigger rapid depolarization due to the opening of non-selective cation channels (Na+, Ca++ influx, K+ efflux) (Hannon and Hoyer, 2008).

In particular, Δ^9-THC has been found to modulate the 3A subunit of the human 5-HT3 receptor. Δ^9-THC has also been shown to interact with some ion channels, in particular T-type Calcium (Cav3) and Potassium Voltage (Kv1.2) channels. Additional Δ^9-THC-target receptors are Transient Receptor Potential (TRP) channels, which are a family of ion channels with relative non-selective permeability for cations, including sodium, calcium and magnesium. In particular, Δ^9-THC activates both TRP Vanilloid-2 (TRPV2) and TRP Ankirin-1 (TRPA1) channels (Pertwee, 2010).

Finally, Δ^9-THC has been shown to interact with PPARγ (O'Sullivan et al., 2006).

PPARγ, also known as the glitazone receptor, is a nuclear receptor which regulates fatty acid storage and glucose metabolism by controlling the expression of specific target genes involved in adipogenesis, inflammatory responses, and lipid metabolism.

The discovery of the cannabinoid receptors suggested the existence of endogenous ligands, which could bind to these receptors and exert physiological effects.

The first endocannabinoid to be isolated was anandamide (arachidonoyl ethanolamide, AEA), which is the amide between arachidonic acid and ethanolamine (Devane et al., 1992). This finding was soon to be followed by the identification of 2-arachidonoylglycerol (2-AG), the arachidonate ester of glycerol (Mechoulam et al., 1995). More recently came the discovery of other endocannabinoids, 2-arachidonyl-glycerol ether (noladin ether), N-arachidonoyl-dopamine (NADA). These lipid compounds differ totally in structure from Δ^9-THC, the main exogenous cannabinoid.

Endocannabinoids are considered either neurotransmitters or neuromodulators: they have distinct synthetic pathway, are released from cells upon depolarization and calcium entry, and their synaptic action is rapidly terminated by reuptake and intracellular enzymatic degradation. AEA and 2-AG are produced from cleavage of two different phospholipid precursors present in the cell membranes of neurons and immune cells in particular. AEA is synthesized from the membrane phospholipid N-arachidonoyl phosphatidylethanolamine by a phosphodiesterase called phospholipase D, an enzyme stimulated by depolarization-induced increase in intracellular Ca^{2+}. The synthetic pathway is also indirectly stimulated by cAMP/protein kinase A, indicating possible receptor-mediated mechanism. The synthesis of 2-AG is also calcium-dependent. An interesting feature of AEA and 2-AG is the "on-demand" synthesis and release of these lipids, differentiating the endocannabinoids from classical neurotransmitters-hence the term "modulator".

AEA is then known to be transported into cells by carrier-mediated uptake, which does not depend on sodium or adenosine-5'-triphosphate (ATP), another difference from classical neurotransmitters, but similar to the structurally related prostaglandin E_2.

Both AEA and 2-AG are known to be rapidly hydrolyzed by the intracellular enzyme fatty acid amide hydrolase (FAAH).

Endocannabinoids may function physiologically as retrograde synaptic messengers.

When a postsynaptic neuron is strongly depolarized, it synthesizes and releases endocannabinoids through a non-vesicular mechanism. These molecules, in turn, bind the presynaptic neuron at CB1 receptors and inhibit its neurotransmitter release. It is a form of negative feedback. The chemical nature of the presynaptic neuron is important. If the release of an inhibitory transmitter like γ-aminobutyric acid (GABA) is decreased, it is called in electrophysiology depolarization-induced suppression of inhibition (DSI) and would result in exacerbation of postsynaptic transmission.

If the release of an excitatory neurotransmitter like glutamate is decreased, it is referred to as depolarization-induced suppression of excitation (DSE), and diminishes postsynaptic transmission (Di Marzo, 2008). Both DSI and DSE depend on rises in calcium and G_i proteins, which are also necessary for the synthesis and release of endogenous cannabinoids and a feature of their receptors. Furthermore, CB1 stimulation inhibits GABA release from hippocampal interneurons and glutamate from cerebellar basket cells (which synapse with Purkinje neurons) (Di Marzo, 2008).

The resistance to diseases, in particular infectious diseases is defined as immunity.

The set of cells, tissues and molecules that mediate such resistance takes the name of the immune system and the sequence of reactions that occur as a result of the meeting of these cells and molecules with infectious antigens is called the immune response.

The physiological function of the immune system is to prevent infections and eradicate once they are established in the body.

The defense mechanisms of the host on which it relies include innate and acquired immunity. Innate immunity is involved in an initial protection against infection and is in constant operation in healthy subjects, while acquired immunity develops more slowly and is involved the most effective defense against infectious agents. The main characteristics of acquired immunity, that better distinguish it from that innate immunity, are represented by the fine specificity for antigens and structurally different from the fact of developing memory in respect of a previous exposure to the antigen (Parkin and Cohen, 2001).

Innate immunity puts in place a first line of defense that consists of epithelial barriers, specialized cells and substances naturally equipped with power antibiotic, used as blocking the penetration of microbes into the tissue. If the pathogens manage to overcome the epithelia, are attacked by phagocytes and lymphocytes specialized cells called "natural killer" (NK), as well as numerous proteins present or plasma, including those of the complement system. The mechanisms of innate immunity are able to identify and respond to the microorganisms, but without reacting against foreign substances of non-infectious nature, moreover, these mechanisms are programmed to react against different classes of molecules produced by microbes and, in addition to providing a first embankment defense against infection, enhance immune responses acquired (MacPherson and Austyn, 2012).

Acquired immunity includes lymphocytes and some of their products, the antibodies.

The cells of this class of immunity express receptors that recognize specifically the products of the different microbes, as well as non-infectious molecules (antigens). The answers acquired will not start unless the microbes or antigens, derived from them, exceed epithelial barriers and make contact with the lymphocytes. There are two types of acquired immunity: humoral immunity and cellular immunity (or cell-mediated), they are based on the intervention of different cells and molecules, and are members of the defense, respectively, toward microbes extracellular and intracellular microbes. More precisely, the humoral immunity is mediated by antibodies produced by B-lymphocytes. These molecules are released into the circulation and mucosal secretions do and work to neutralize and eliminate microbes and microbial toxins in the blood and into the lumen of equipment, such as the gastro-intestinal tract and the respiratory system (MacPherson and Austyn, 2012).

Therefore, the antibodies are essential in preventing the penetration of foreign pathogens in the body and preventing the colonization of cells and connective tissues of the host.

Cellular immunity, however, rests on the intervention of cells called T-lymphocytes.

Some T-lymphocytes activate the phagocytes to destroy microorganisms, while others directly kill host cells in which there is the infectious agent.

T AND B LYMPHOCYTES

A key role is played, in the immune system, by the CD4 + helper T-cells, which mediate the immune response, both humoral and cell-mediated immunity.

The T-lymphocyte response to microbial antigens comprises a series of successive stages, that induce their numerical expansion and processing of virgin T-cells in effector cells.

The virgin T-lymphocytes express the TCR (T-cell receptor for the peptide-MHC complexes) and other molecules, forming the device for the recognition of the antigen, that promotes the differentiation of T-cells into effector cells (Abbas et al., 2003).

Simultaneously with the antigenic recognition, they receive further signals by microbes or products of the innate response, which induce the secretion of proteins called cytokines. Then, part of the activated lymphocytes undergoes differentiation, transforming into cells capable of eradicating pathogens strangers. The CD4 + T-helper lymphocytes can differentiate into subpopulations of effector cells, which produce a spectrum of different cytokines, equipped with various functions. The subpopulations of T-helper cells best characterized are called Th-1 and Th-2. Th-1 cells potentiate the killing of microorganisms mediated by phagocytic cells, producing IFN-γ. This protein is a potent activator of macrophages and stimulates the production of antibodies of an isotype that promotes phagocytosis, in addition to stimulating the expression of MHC (major histocompatibility complex) class II and macrophages. They also produce IL-2, a cytokine essential for their activation and subsequent clonal expansion.

Th-2 cells promote, instead, a type of immune response mediated by eosinophils and independently by phagocytes, producing IL-4, which stimulates the production of IgE antibodies (Abbas et al., 2003).

Among the cytokines produced by lymphocytes Th-2 there is also the IL-10, which has an important role in inhibiting of activating macrophage and therefore in the suppression of the responses mediated by Th-1 cells. The differentiation in cells Th-1 and Th-2 is a process finely regulated by stimuli that T-lymphocytes virgin receive when they encounter microbial antigens. B-lymphocytes instead develop and mature in the bone marrow, where through appropriate rearrangements develop numerous variants of B-cell receptors (BCR). These structures constitute the membrane precursors of future antibodies (which are also released in soluble form by plasma cells) and as these recognize antigens in the native form.

Selection mechanisms of B-lymphocytes are little known, although it is clear that it is a process less rigid than that of T-lymphocytes (MacPherson and Austyn, 2012).

This phenomenon can be explained by two reasons:

- B-lymphocytes depend on the interaction with the CD4+ T-lymphocytes for their activation in plasma cells. So if a B-lymphocyte is self-reactive, but is not triggered by T-lymphocyte, can not be activated and give autoimmunity.

- B-lymphocytes can recognize a much larger number of non-peptide antigens. So a rigid elimination of all self-reactive clones could restrict the antibody response in the event of aggression (Abbas et al., 2003).

CYTOKINES

The body's defense against foreign organisms is mediated by an innate immune response and an acquired immune response: the effector phase of both types of immune response is largely mediated by proteins called cytokines. These are a heterogeneous group of soluble proteins involved in intracellular transduction signal (Borish and Steinke, 2003). Although the cytokines are a family of proteins very different between them, these molecules have common characteristics:

- they are produced during the effector phase of both the innate and the acquired immunity and serve to mediate and regulate immune and inflammatory responses. In the Innate immunity, microbial products such as LPS directly stimulate mononuclear phagocytes to secrete cytokines, while cytokines derived from T-cells are above all produced in response to specific recognition of foreign antigen

- the secretion of cytokines is a phenomenon of short duration and auto-limited

- several cytokines are produced by many different cell type

- cytokine acts on many different cell types: this characteristic is defined pleiotropism

- often perform many different effects on the same target cells

- their activity is often redundant

- cytokines often influence the synthesis of other cytokines, and their interaction can have an antagonistic, additive or synergistic effect

- the action of cytokines may be autocrine, paracrine and endocrine

- the expression of many cytokine receptors is regulated by specific signals, constituted by other cytokines or from the same, which binding its receptor, generates amplification or inhibition circuits

On the basis of the function, the cytokines can be divided into 4 groups:

1. mediators of innate immunity products from mononuclear phagocytes

2. activators of growth and differentiation of lymphocyte, produced in consequence of the antigenic recognition by T-lymphocytes

3. regulators of immune-mediated inflammation, which activate inflammatory cells evoked as a result of non-specific antigenic recognition of T-lymphocytes

4. substances having a stimulant growth and differentiation action of immature leukocyte, derived from lymphocytes and other non-lymphoid cells

CYTOKINES THAT MEDIATE AND REGULATE INNATE IMMUNITY

TNF-α

This proinflammatory cytokine was first isolated in 1975, and its name is misleading in that it does not cause the necrosis of all tumors.

As a matter of fact, it may stimulate the growth of some tumors.

TNF-α is a 185-amino-acid glycoprotein, which is cleaved from a 212-amino-acid peptide, and the cleavage occurs on the cell surface of mononuclear phagocytes. The major cell source of TNF-α is the macrophage, specifically the endotoxin-activated mononuclear phagocyte. Other sources include endothelium after tissue damage, antigen-stimulated T-cells, activated NK cells and activated mast cells. IFN-γ augments TNF-α synthesis.

TNF-α is a mediator of both natural and acquired immunity (Borish and Steinke, 2003).

TNF-α induces the production of IL-1, IL-6, TNF-α itself and chemokines via stimulation of macrophages. It exerts an IFN-like protective effect against viruses and augments expression of MHC class I molecules. TNF-α is an endogenous pyrogen that acts on cells in hypothalamic regulatory regions of the brain to induce fever. It suppresses appetite.

The hypothalamic–pituitary–adrenal axis (HPA) is stimulated via the release of corticotrophin-releasing hormone by TNF-α.

TNF-α suppresses bone marrow stem cell division and reduces tissue perfusion by depressing myocardial contractility (Borish and Steinke, 2003).

IL-1

Interleukin-1 (IL-1) was originally discovered as a factor that induced fever, caused damage to joints and regulated bone marrow cells and lymphocytes, it was given several different names by various investigators.

Later, the presence of two distinct proteins, IL-1α and IL-1β, was confirmed, which belong to a family of cytokines, the IL-1 superfamily (Borish and Steinke, 2003).

IL-1 plays an important role in both innate and adaptive immunity and is a crucial mediator of the host inflammatory response in natural immunity. The major cell source of IL-1 is the activated mononuclear phagocyte. Other sources include dendritic cells, epithelial cells, endothelial cells, B-cells, astrocytes, fibroblasts and Large Granular Lymphocytes (LGL). Endotoxins, macrophage-derived cytokines such as TNF-α or IL-1 itself, and contact with CD4+ cells trigger IL-1 production.

IL-1 can be found in circulation following Gram-negative bacterial sepsis. It produces the acute-phase response in response to infection. IL-1 induces fever as a result of bacterial and viral infections (Dinarello and Wolff, 1993).

Activation of T-helper cells, resulting in IL-2 secretion, and B-cell activation are mediated by

IL-1. It is a stimulator of fibroblast proliferation, which causes wound healing. Autoimmune diseases exhibit increased IL-1 concentrations. It suppresses further IL-1 production via an increase in the synthesis of PGE2.

IL-10

First identified as an inhibitor of IFN-γ synthesis in Th1 cells, IL-10 is an important immunoregulatory cytokine. It is an anti-inflammatory cytokine that was first called human cytokine synthesis inhibitory factor (Del Prete et al., 1993).

IL-10 is secreted by macrophages, Th2 cells and mast cells.

Cytotoxic T cells also release IL-10 to inhibit viral infection- stimulated NK cell activity.

IL-10 is a 36-kDa dimer composed of two 160-aminoacid residue long chains. Its gene is located on chromosome 1 in humans and consists of five exons.

IL-10 inhibits the synthesis of a number of cytokines involved in the inflammatory process including IL-2, IL-3, GM-CSF, TNF-α and IFN-γ. Based on its cytokine-suppressing profile, it also functions as an inhibitor of Th1 cells and by virtue of inhibiting macrophages, it functions as an inhibitor of antigen presentation. Interestingly, IL-10 can promote the activity of mast cells, B-cells and certain T-cells. The major immunobiological effect of IL-10 is the regulation of the Th1/Th2 balance. Th1 cells are involved in cytotoxic T-cell responses whereas Th2 cells regulate B-cell activity and function. IL-10 is a promoter of Th2 response by inhibiting IFN-γ production from Th1 cells. This effect is mediated via the suppression of IL-12 synthesis in accessory cells. IL-10 is involved in assisting against intestinal parasitic infection, local mucosal infection by costimulating the proliferation and differentiation of B-cells. Its indirect effects also include the neutralization of bacterial toxins (Borish and Steinke, 2003). IL-10 is a potent inhibitor of IL-1, IL-6, IL-10 itself, IL-12, IL-18, CSF and TNF-α. It not only inhibits the production of proinflammatory mediators but also augments the production of anti-inflammatory factors including soluble TNF-α receptors and IL-1ra.

IL-4

In addition to T-helper lymphocytes, IL- 4 is derived from eosinophils, basophils, and possibly mast cells. In both eosinophils and mast cells, IL-4 exists as a preformed, granule-associated peptide and can be rapidly released in allergic inflammatory responses.

IL-4 stimulates MHC class II molecules, B7, CD40, surface IgM, and low-affinity IgE receptor (CD23) expression by B-cells, thereby enhancing the antigen-presenting capacity of B-cells.

IL-4 induces the immunoglobulin isotype switch from IgM to IgE (Romagnani, 1990).

Other B-cell–activating cytokines, such as IL-2, IL-5, IL-6, and IL-9, synergize with IL-4 to increase the secretion of IgE. IL-4 has been identified in the serum, bronchoalveolar lavage fluid, and lung tissue of asthmatic subjects, in nasal polyp tissue, and in the nasal mucosa of subjects with allergic rhinitis.

In addition to these effects on B-cells, IL-4 has important influences on T-lymphocyte growth, differentiation, and survival, producing important influences on allergic inflammation. As will be discussed later, IL-4 drives the initial differentiation of naïve T-helper type 0 (TH0) lymphocytes toward a Th2 phenotype.

IL-4 is also important in maintaining allergic immune responses by preventing apoptosis of T-lymphocytes (Borish and Steinke, 2003). The production of IL-4 by Th2 lymphocytes renders these cells refractory to the anti-inflammatory influences of corticosteroids.

Other activities of IL-4 include enhancing the expression of MHC molecules and low-affinity IgE receptors (CD23) on macrophages. In contrast to these proinflammatory effects on monocytes, IL-4 downregulates antibody-dependent cellular cytotoxicity (ADCC), inhibits expression of Fc receptors, inhibits their differentiation into macrophages, and downregulates production of nitric oxide, IL-1, IL-6, and TNF-α while stimulating production of IL-1ra.

The most important cytokine responsible for cell-mediated immunity is IFN-γ.

It is primarily produced by T-helper lymphocytes but is also derived from cytotoxic T cells and NK cells. IFN-γ mediates increased MHC class I and II molecule expression.

IFN-γ stimulates antigen presentation and cytokine production by monocytes and also stimulates monocyte effector functions, including adherence, phagocytosis, secretion, the respiratory burst, and nitric oxide production. The net result is the accumulation of macrophages at the site of cellular immune responses, with their activation into macrophages capable of killing intracellular pathogens (Borish and Steinke, 2003). In addition to its effects on mononuclear phagocytes, IFN-γ stimulates killing by NK cells and neutrophils. It stimulates adherence of granulocytes to endothelial cells through the induction of ICAM-1, an activity shared with IL-1 and TNF-α. As with other interferons, IFN-γ inhibits viral replication. As discussed later, IFN-γ is an inhibitor of allergic responses through its capacity to inhibit IL-4–mediated effects (Farrar and Schreiber, 1993).

TH1/TH2 BALANCE

Th1 and Th2 cells differ from the virgin T-helper CD4, their profile is influenced by the stimuli present in the microenvironment, during the early stages of the immune response. Each of the two subpopulations are self-amplifying, blocking the differentiation of the other (Romagnani et al., 1994). The differentiation in Th1 is stimulated by intracellular parasites and is characterized by the production of IFN-γ; the principal effector function is the defense mediated by phagocytes.

The differentiation in Th2 occurs in response to allergens and to helminths and is characterized by the production of IL-4 and IL-5, the main effector function is the response mediated by IgE, mast cells and eosinophilis.

Th2 cells can behave as suppressor cells, through the production of IL-4 and IL-13, which act as antagonists of IFN-γ and block the macrophage activation induced by T-lymphocytes.

The cytokines produced also limit the possible tissue damage caused by cell-mediated responses.

Th2 cells also mediate certain inflammatory reactions in which predominate eosinophils and mast cells, contributing to the elimination of helminths (Borish and Steinke, 2003).

The immune system comprises several components, including lymphoid tissue, such as the spleen and lymph nodes, the bone marrow and the 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 are part of the host's innate immunity while the responses mediated by B- and T-cells are part of the acquired immunity (Berdyshev, 2000).

Generally, Δ^9-THC has a deleterious effect on a variety of immune parameters.

Many studies in the literature suggested that cannabis use is associated with an increase in the incidence of viral infections and allergic symptoms.

More specific studies were subsequently designed to clarify the Δ^9-THC's action after in vivo and in vitro exposure in various human and animal cells.

MACROPHAGES

Cannabis use and effects on innate macrophage functions were initially studied in pulmonary models by Berdyshev et al. (1998) Alveoli macrophages are the principal immune-effector cells in the lung and are primarily responsible for protecting the lung against infectious microorganisms, inhaled foreign substances, and tumour cells. Cannabis smoking reduced the ability of alveoli macrophages to kills fungi, pathogenic bacteria, and tumour target cells. Furthermore, cannabis smoking depressed production of pro-inflammatory cytokines, which are important regulators of macrophage function. This cannabis-related decrease in inflammatory cytokine production might be a mechanism whereby cannabis smokers are less able to destroy fungal and bacteria organisms, as well as tumour cells.

The inability of alveolar macrophages from habitual cannabis smokers without apparent disease to destroy fungi, bacteria, and tumour cells, and to release pro-inflammatory cytokines, suggests that cannabis might be an immunosuppressant with clinically significant effects on host defense.

Studies by Tashkin et al. (1997) suggested that regular cannabis consumption reduces the respiratory immune response to invading organisms. Further, serious invasive fungal infections as a result of cannabis contamination have been reported among individuals who are immunocompromised, including patients who were infected by AIDS.

There are reports of effects of cannabinoids *in vitro* on various macrophage functions including cell spreading and phagocytosis, protein expression, cytolysis and antigen presentation (Berdyshev, 2000).

McCoy et al., (1995; 1999) demonstrated that Δ^9-THC influenced the ability of macrophages to process antigens necessary for the activation of CD4+ T-lymphocytes.

Sacerdote et al., (2000a) reported that *in vivo* and *in vitro* treatment with the synthetic cannabinoid CP-55, 940 reduced the spontaneous and stimulated chemotaxis of macrophages in the rat and this effect involved both CB1 and CB2 receptors.

NO generation by macrophages is an obligatory element of cellular attack on bacterial pathogens. Δ^9-THC suppresses NO production in mouse peritoneal macrophages, but the cannabinoid ligand may increase NO production in human monocytic culture (Klein, 1999).

Chang et al., (2001) also found that Δ^9-THC, AEA and 2-AG reduced LPS- induced NO, PGE_2 and IL-6 production in a concentration-dependent manner through CB2 activation.

T-cells and B-cells are the major cellular componentes of the adaptative immune response. Marijuana is able to suppress immune function, changing the number and function of T-cells (Massi et al., 2006). Finding 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 (Klein, 1999).

It is seen in fact a measurable inhibitory effect on leukocytes after acute and chronic treatment of relatively high doses of cannabinoids in experimental animals, and high concentrations *in vitro*.

It is also seen that the cell proliferation is not always suppressed, but rather appears to be enhanced by low concentrations of cannabinoids or in response to anti CD3 antigen used as a mitogen.

Massi et al. (1998) reported that, following acute treatment of Δ^9-THC, in mice *in vivo*, and with doses that induce analgesic effect, there was no alteration in the proliferative response of spleen cells to ConA, but after 7 days of treatment, proliferation was found to be markedly reduced.

Furthermore, a few studies have evaluated the Δ^9-THC effect on cytotoxic T-lymphocytes (CTL), which can lyse and destroy potentially harmful cellular elements in the body.

Klein and colleagues (1991) demonstrated that after incubation with Δ^9-THC the cytolilytic activity of murine splenocyte CTL was depressed by about 60%.

Δ^9-THC did not inhibit CTL binding to the target cell but lowered the cytolytic activity subsequent to binding. McKallip et al., (2002), based on studies of Klein et al., (1991), demonstrated that Δ^9-THC administration *in vivo* in animals, lowered the cytolytic activity subsequent to binding on lymphocytes, mediated by a rapid clearance of dead cells by phagocytic cells. After exposing C57BL/ 6 mice to 10 mg/kg body-weight Δ^9-THC, he isolated

lymphocytes from the thymus and spleen, and he detected that levels of Δ^9-THC-induced apoptosis in T-cells, B-cells and macrophages.

He has also demonstrated that Δ^9-THC induced higher levels of apoptosis in naive lymphocytes, when compared with mitogen-activated lymphocytes, because activated cells down-regulated the levels of CB2 on their cell surface. Another major function of immune cells examined in the context of cannabinoid treatment is antibody formation. Studies in humans and animals have examined the levels of serum immunoglobulins after treatment with cannabinoids. In chronic marijuana smokers given cigarettes for two months, the levels of IgG and A were not different from those of controls, but the IgE appeared significantly increased (Massi et al., 2006). Still, very little is known about the Δ^9-THC effect of prenatal exposure on the developing immune system; Lombard et al. (2011) has recently demonstrated that perinatal exposition to Δ^9-THC, in animals, negatively affects the immune system of offspring, potentially compromising its response to infections. In particular, her study has demonstrated that animals that have been exposed to Δ^9-THC in utero have a lower immune response to HIV at 5 weeks of age, with a decreased proliferative response to HIV restimulation *in vitro* and lower HIV-specific antibody titer in the serum.

Δ^9-TETRAHYDROCANNABINOL'S ACTION ON CYTOKINES

The regulation of the concerted work of different types of immune cells is controlled by cytokines-signalling 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 role in the development of Th1 and Th2 dependent immune responses (Borish and Steinke, 2003).

As previously reported, cytokines of acute phase such as IL-1, TNF-α, 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-γ, are produced by activated Th cells and help to eliminate microbes from the body by regulating powerful cell-mediated and antibody-mediated immune mechanism (Borish and Steinke, 2003).

An increasing number of publications confirm that Δ^9-THC can alter the cytokine production in immune cells.

The first work on the modulation of cytokine production belongs to Blanchard et al., (1986), who demonstrated that cannabinoids caused an inhibition of the production of IFN-γ in splenocytes isolated from animals chronically treated with Δ^9-THC, after *in vitro* stimulation with the mitogen PHA, ConA and LPS.

Moreover Δ^9-THC and other non-psychotropic drugs as cannabidiol, have been shown to induce both a decrease and an increase of the IFN-γ production, according to the concentrations used. Low concentrations (e.g, <0,1 μM) of cannabinoids increase the production of IFN-γ, whereas high concentrations diminish the production (e.g, 30 μM) (Watzl et al., 1991).

The Δ^9-THC is also able to induce an inhibition on the production of TNF-α in cultures of murine peritoneal macrophages as shown by Zheng et al., in 1992.

Moreover Klein and Friedman (1990) reported that Δ^9-THC in the range of 10-30 μM increased IL-1 bioactivity in the supernatant of cultured mouse peritoneal macrophages.

Zhu et al., (1994) showed that this occurred because Δ^9-THC alters the processing and release of IL-1 rather than cellular production of the protein.

Also lymphocytes are limited in their proliferation by cannabinoids. Proliferation of these cells is regulated, at least in part, by the system IL-2/IL-2 receptor.

Nakamo et al., (1992) showed that the secretion of IL-2 secretion by lymphocytes was modulated by cannabinoids, thus accounting for some of the inhibitory drug effects on the cell growth.

Using a chronic treatment *in vivo*, it is confirmed the thesis that Δ^9-THC lower in mice levels of IFN-γ and IL-2 in splenocytes, and can steer away from the cytokine network in cell-mediated immunity, causing a shift towards Th2 humoral response (Massi et al., 1998).

This reduces host resistance to pathogens such as viruses, bacteria and parasites.

The unbalance of Th1/Th2 pattern caused by the exposure to cannabinoids is also confirmed in human leukocytes, where the Δ^9-THC causes a decrease in production of IL-2 and IFN-γ accompanied by a decrease in basal levels of mRNA coding for Th1 cytokines while those for Th2 cytokines increase (Kertscher and Cohen B, 2002).

Zhu et al., (2000) examining the effects of Δ^9-THC on the host response to a lung tumor challenge, found that animals receiving Δ^9-THC experienced a more rapid rate of tumor growth. Since they found that Δ^9-THC augmented the immunosuppressive cytokines IL-10 and TGF-β, while IFN-γ was down-regulated at both the tumor site and in the spleens, they suggested that Δ^9-THC promotes tumor growth by inhibiting anti-tumor activity by a cytokine-dependent pathway.

The effects of Δ^9-THC and other cannabinoid compounds such as cannabidiol could be considered of benefit for some autoimmune and inflammatory diseases, but they seem to be at the basis of a worse tumorigenesis, metastasis, and some infections such as HIV besides exacerbating allergic responses.

Whereas the animal and cellular models do not always predict human responses, epidemiological studies have been aimed at clarifying the real impact of marijuana smoking on the development of opportunistic infections and cancer. In many studies it has been observed a more rapid progression from HIV infection to AIDS in marijuana users and, more recently, its use was found to be a risk factor for the development of cancer.

Roth et al., (2002) in order to evaluate whether human immune responses are similar to animal and cellular studies, collected T-cells from healthy volunteers.

It was found that in *in vitro* Δ^9-THC down-regulated the expression and release of Th1 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 relative balance of other Th1 Th2 cytokines in the local environment results in differentiation of the activated T-cells towards either a Th1 and Th2 phenotype (Massi et al., 2006).

Roth et al., (2002) studied the effects of an *in vitro* exposure of dendritic cells to Δ^9-THC, and found concentrations of IFN-γ reduced of about 50% while IL-4 levels were increased on average by 110%, resulting in a shift in Th1/Th2 cytokine balance.

The shift Th1/Th2 balance seems to be the cause of susceptibility of laboratory animals against many pathogens such as viruses, bacteria and parasites, against which normally the response of body is supported by Th1 cytokines (Roth et al., 2002).

The hypothalamic-pituitary-adrenal (HPA) axis is a complex neuroendocrine system involved in an important number of central and peripheral physiological functions, most of them related to appropriate adaptation to stressful situation. The corticotrophin-releasing factor (CRF), which is produced in the paraventricular nucleus (PVN) of the hypothalamus, represents the main driving force controlling HPA axis activation.

CRF induces the release of adrenocorticotrophic hormone (ACTH) from the anterior pituitary, which in turn regulates glucocorticoid production from the adrenal gland. Then, the glucocorticoids negatively regulate the release of both CRF and ACTH trough a feedback mechanism (Figure 7).
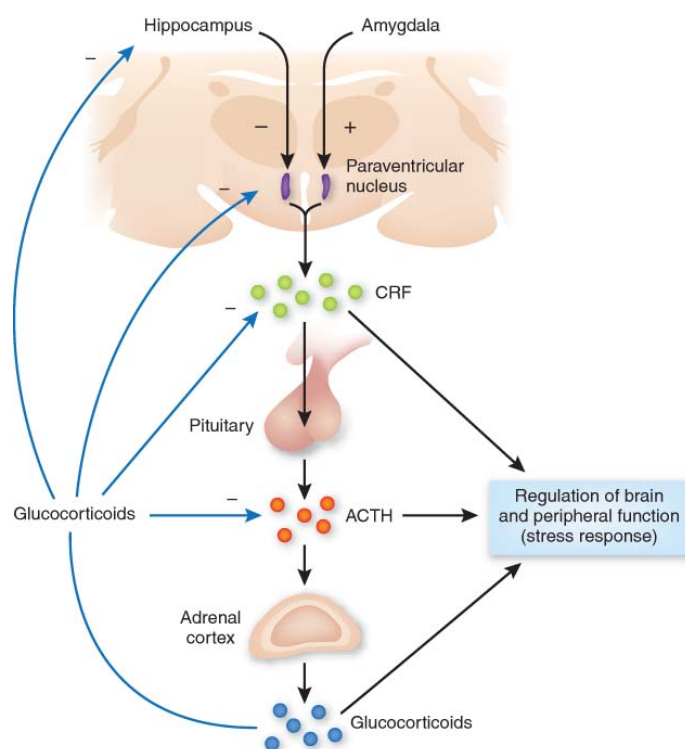
Figure 7 General organisation of the hypotalamic-pituitary-adrenal (HPA) axis and feedback inhibition of HPA axis fucntion by glucocorticoids (CORT), ACTH, adrenalcorticotrophic hormone.
Figure taken from Lightman and Heyman, 2009

Activation of the HPA axis is generally beneficial for the organism; however, when the stress response is inadequate or excessive and prolonged, the cost of maintaining homeostasis becomes too high and might thus put an individual at the risk of developing illness, including, depression, abdonimal obesity, affective disorders and immune diseases.

The smoking marijuana or administration of Δ^9-THC are both known to stimulate ACTH and glucocorticoid secretion in humans and experimental animals model. Several studies have actually demostrated that the action of exogenous cannabinoids on the HPA axis is dose-dependent and involves modulation of CB1 receptors.

In multiple animal studies, acute administration of cannabinoids increased both ACTH and glucocorticoids (GC) in a dose-related fashion, an effect that is likely mediated by an increase in CRH (Rodriguez de Fonseca et al., 1992).

Rodents administered potent CB1 agonist HU-210 had marked activation of the HPA axis, but at the highest doses, ACTH decreased while GC increased, suggestive of rapid negative feed-back by GC (Martin-Calderon et al., 1998). However, tolerance to these effects develops quickly with chronic administration (Rodriguez de Fonseca et al., 1992).

Human studies have shown variable effects of marijuana and component cannabinoids on the HPA axis. Similar to the effects in animals, increased cortisol levels have been reported after acute administration of marijuana (Brown and Dobs, 2002); conflict results were observed in human users of Δ^9-THC, after chronic oral administration for example abusing foods like cookies rich in marjiuana.

In their review, Brown and Dobs (2002) related that prolonged activation of the HPA axis, for marijuana consumption, may induce to a reduction in adrenocortical reserve.

CARDIOVASCULAR EFFECTS

The use of marijuana is associated with serious cardiovascular consequences; Δ^9-THC can induce tachycardia (Perez-Reyes, 1999) and increase cardiac output with increased cardiac work and oxygen demand.

It can also produce peripheral vasodilation, orthostatic hypotension and reduced platelet aggregation. There was no change of mean global cerebral blood flow after smoking cannabis, but increases and decreases in several regions (O'Leary et al., 2002).

Due to the development of tolerance, long-term use can lead to bradycardia.

Moreover, postural hypotension and fainting may occur. These and other cardiovascular effects may carry a risk for individuals with preexisting cardiac disease, and several cases of acute and sometimes fatal cardiac incidents have been reported in young cannabis smokers.

RESPIRATORY EFFECTS

The smoke from herbal cannabis preparations contains all the same constituents, excluding nicotine, as tobacco smoke, including carbon monoxide, bronchial irritants, tumour initiators (mutagens), tumour promoters and carcinogens. A single cannabis cigarette contains higher concentrations of benzanthracenes and benzpyrenes, both of which are carcinogens, than tobacco smoke. It has been estimated that smoking a cannabis cigarette results in approximately a five-fold greater increase in carboxyhaemoglobin concentration, a three-fold greater amount of tar inhaled and retention in the respiratory tract of one-third more tar than smoking a tobacco cigarette (Benson and Bentley 1995; Khalsa, 2007). This is mainly due to the way a cannabis joint is smoked, with deep and prolonged inhalation and no filter.

In addition, cannabis has a higher combustion temperature than tobacco.

Chronic heavy smoking of marijuana is associated with increased symptoms of chronic

bronchitis, coughing, and with impairment of pulmonary function, pulmonary responsiveness and bronchial cell characteristics in marijuana-only smokers (Tashkin et al., 1990).

It has been calculated that smoking 3-4 cannabis cigarettes a day is associated with the same evidence of acute and chronic bronchitis and the same degree of damage to the bronchial mucosa as 20 or more tobacco cigarettes a day (Khalsa, 2007).

Tashking and colleagues (1997) reported that marijuana smoking may predispose individuals to pulmonary infection, especially patients whose immune system is already compromised by HIV infection and/or cancer and related chemoterapy.

ENDOCRINE EFFECTS

Marijuana use affects endocrine and reproductive functions as well, since it inhibits the secretion of gonadotropins from the pituitary gland and may act directly on the ovary or testis. Cannabinoids affect multiple reproductive functions, from hormone secretion to birth of offspring (Schuel et al., 2002). Schuel and colleagues (2002) reported that endocannabinoid anandamide signaling regulates sperm functions required for fertilization in the human reproductive tract and the abuse of marijuana could affect these processes. Chronic administration of high doses of Δ^9-THC lowers testosterone secretions; impairs semen production, motility, viability and disrupts the ovulatory cycle in animals (Bloch, 1983). Furthermore, according to Harclerode (1984) Δ^9-THC lowers testosterone levels by lowering luteinizing hormone and follicle-stimulating hormone.

Marijuana depresses the levels of prolactin, thyroid function and growth hormone while elevating adrenal cortical steroids.

Chronic exposure of laboratory animals (rats, mice and monkeys) to marijuana altered the function of several accessory reproductive organs: reduced testosterone levels leads to reduced testicular function and reduced prostate and seminal vesicle weights.

Chronic administration of marijuana also produces testicular degeneration and necrosis in dogs (Khalsa, 2007).

EFFECTS ON CENTRAL NERVOUS SYSTEM

Numerous studies show that the phytocannabinoids can induce different degrees of damage to the cells of CNS. Recent discoveries have shown that just the Δ^9-THC is capable of inducing the death of the cells with DNA fragmentation in the hippocampus.

The areas of the brain primarily involved in cognitive functioning include the frontal cortex, hippocampus and cerebellum; it was demonstrated by Herkenham and colleagues (1990) that the use of cannabis alters the functioning of these brain areas rich in cannabinoid receptors.

Finally, changes in the activity of the endocannabinoid system during the phases of high neuronal plasticity, as the perinatal and adolescence period, can have behavioral consequences for long time.

IMPAIRMENT OF COGNITIVE BEHAVIOUR

Through studies of performance evaluation and neuropsychological testing in man, the cognitive functions found to be more affected by cannabis use are attention, learning and memory. Moreover, the executive functions, processing speed and psychomotor speed are also impaired. It has also been described that individuals who start using cannabis in early age may be more vulnerable to lasting neuropsychological deficits than those who have started to use it later (Chadwick et al., 2013).

Some studies show the appearance of deficit in sustained attention, in the memory, mental flexibility and speed of information processing (Wegener and Koch, 2009).

A recent study showed that the use of cannabis represents an important risk factor for the development of psychotic disorders especially in adolescence (Chadwick et al., 2013; Rubino and Parolaro, 2013). Although a small fraction of teens that use cannabis develop schizoaffective disorders, a number of epidemiological studies repeatedly demonstrate elevated risk to develop these psychiatric disorders in association with early-life cannabis use. Longitudinal studies assessing the relationship between early-life cannabis exposure and schizotypal personality disorder demonstrated that early adolescent use increases adulthood symptomatology (Page et al., 2007).

Although it is challenging to model schizophrenia in animals, phenotypes related to this disorder may be studied; rats exposed to cannabinoids during adolescence demonstrate increased schizoaffective-like phenotypes, such as impaired sensorimotor gating, which, similar to humans, results in decreased prepulse inhibition (Wegener and Koch, 2009).

Since not all cannabis users develop schizophrenia, early cannabis use likely interacts with other factors to facilitate the emergence of this disease (Casadio et al., 2011).

Recent data have shown that the association between early cannabis exposure and vulnerability to schizophrenia is related to individual genetics.

Caspi et al. (2005) demonstrated that the relationship between adolescent cannabis use and schizophreniform disorder, as well as the presence of various psychotic symptoms, was attributable to the presence of a functional polymorphism in the catechol-O-methyltransferase (COMT) gene.

This enzyme degrades catecholamines, such as dopamine, and this functional variant (COMTvaline158) catabolizes this neurotransmitter more rapidly than the methionine allele (Lachman et al., 1996).

In cannabis users, schizophreniform disorder is predominantly observed in persons with at least one copy of the polymorphic COMT gene (Costas et al., 2011). Moreover, clinical laboratory experiments show that Δ^9-THC's acute psychotomimetic effects are moderated by this COMT SNP with Δ^9-THC-induced psychotic-like experiences and cognitive impairments being more pronounced in individuals with the valine158 allele. Animal models also confirm a link between the genetic disturbance of COMT and developmental cannabis such that adolescent Δ^9-THC exposure in transgenic mice lacking endogenous COMT synergistically impacts behaviors relevant to schizophrenia (Henquet et al., 2006).

The CNS is a complex organ that consists of a diverse group of cell type, including neurons, oligodendrocytes, microglia and astrocytes. While astrocytes are the predominant cell type of the CNS, microglia are the resident macrophages of the brain and provide the first line of defence against injury, assault and /or infection. These myeloid lineage cells play an import role also in CNS remodelling and regeneration. The combined cellular functions of astrocytes and microglia form the innate immune system of the CNS (Carlisle and Cabral, 2002).

In the healthy adult brain, resting microglial cells are characterized by a small soma with fine, highly ramified branches and low expression of surface antigens. Following brain stress or injury, local changes in the extracellular milieu promote a change in the microglial phenotype, referred to as microglial activation.

Once activated, they produce various cytokines including IL-1, IL-6, TNF-α, and express major histocompatibility complex classes I and II antigens and the complement receptor, CR3 (Roche et al., 2006).

Several studies have shown that microglia and microglia-derived cells are the major cell types responsible for chronic inflammation that participates in Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis. Microglial cells also express both the CB1 and CB2 cannabinoid receptor; using *in vitro* model, Carlisle and Cabral (2002) have shown that levels of CB2 mRNA and protein are modulated differentially in relation to cell activation state.

The CB2 is not detected in "resting" cells, is present at high levels in "responsive" and "primed" cells, and is identified at greatly diminished levels in "fully" activated cells.

In contrast, CB1 is present in microglia at relatively low levels and is expressed constitutively in relation to the cell activation state.

Recently, it has been proposed that the role of CB2 in immunity in the CNS is primarily anti-inflammatory (Carrier et al., 2005); this receptor has the potential to serve as a therapeutic

target for appropriately designed CB2-specific ligands that could act as anti-inflammatory agents in neuropathological processes such as multiple sclerosis (MS).

Numerous studies are investigating the effects of cannabinoids in neuroinflammation: for example, in Theiler's virus infection of mouse CNS, an animal model for human MS, it was showed that the synthetic cannabinoids WIN55, 212-2 and JWH-015 improved neurological deficits and reduced microglial activation, MHC class II expression and T-lymphocyte infiltration (Arevalo-Martin et al., 2003). Moreover, Roche and colleagues (2006) have recently showed, with *in vivo* treatment in mice, that the cannabinoid receptor agonist, HU210 attenuated lipopolysaccharide (LPS)-induced increases in IL-1β and TNF-α in rat brain and IL-1β, TNF-α, IL-6 and IFN-γ in plasma and the involvement of CB1 was suggested as the CB1 receptor antagonist, SR141716A, attenuates the immunosuppressive effects of HU210 on IL-1β (Roche et al., 2006).

In the present study, we have started to investigate the effects of Δ^9-THC chronic treatment on the neuroinflammatory component of CNS in animal models of adolescence and adulthood, analyzing the basal production of pro and anti-inflammatory cytokines in the hypothalamus and in the hippocampus.The hypothalamus is the ventral portion of the diencephalon and includes numerous nuclei that activate, control and integrate peripheral autonomic mechanisms, endocrine activity, somatic functions such as thermoregulation, sleep, water-salt balance and food intake. Moreover, hypothalamus constitutes together with pituitary and adrenal glands, the neuroendocrine axis hypothalamic-pituitary-adrenal (HPA), actively involved in the stress response. The hippocampus plays an important role in the regulation of the processes of learning and memory and also has a lot of importance in the regulation of emotional behavior. Furthermore hippocampus participates in relation to HPA axis feedback, inhibiting the release of CRH and the subsequent release of ACTH when the corticosterone level in the circulation becomes too high.

Adolescence is the transition period from childhood to adulthood, characterized by increases in peer social behavior, risk-taking and novelty-seeking, and develop of many other functions, for example cognitive function and immune maturation. Adolescence in humans spans the age of 10-12 years old to approximately age 24. It includes, but is not defined by puberty. There is no specific endpoint of adolescence and adolescent behaviors may persist into the mid twenties (Spears, 2000).

During this time in humans, adolescents obtain the skills and knowledge they need to operate as functional adults in society. Animal models such as the rodent also have a defined adolescent period. The adolescence period in mouse is generally considered to be at post natal days 28 (PND 28). Like in humans, the transition to adulthood is not strictly defined. Some adolescent traits may remain until PND 55-60.

However, mice are generally characterized as early adults by PND 65 (Spears, 2000).

For the purpose of the present studies, adolescents were defined as PND33, and adults were defined as PND 80.

The educational, social and developmental demands upon adolescents are considerable; illicit drug use can acutely interfere with the progression of development, and therefore impose limits on how well adolescents transit into adulthood.

In adolesence age increased involvement in social situations, exposure to peer pressure and greater risk-taking and novelty-seeking behaviors all contribute to an increased likelihood of adolescent drug-taking. Adolescents are more sensitive to the impairing effects of many drugs, but less sensitive to the aversive effects. As such, adolescents are more likely to experiment with and escalate drug use at a more rapid rate than adults.

The purpose of the present thesis is to characterize the differences in adolescent and adult immune parameters after exposure to Δ^9-THC, the main psychoactive component of the commonly used illicit drug marijuana.

MARIJUANA USE DURING ADOLESCENCE

Marijuana is the most commonly used illicit drug in the world, third most used overall after the legal drugs alchool and tobacco. This use is especially prevalent among adolescents.

Adolescent use of marijuana may have long-lasting biological consequences.

Early-onset cannabis use increases the risk of developing schizophrenia (Arseneault et al., 2002; Fergusson et al., 2003).

It has been shown that subclinical positive and negative symptoms were more strongly associated with first time use before age 16 than after, and was independent of lifetime frequency of use (Stefanis et al., 2004). Adolescent cannabis exposure has been shown to also affect cognition, in terms of impaired attentional functions (Ehrenreich et al., 1999) and lower verbal IQ (Pope et al., 2003).

In addition, animal studies demonstrated that chronic exposure to cannabinoid in adolescent but not in adult rats produce long-lasting memory impairment and increased anxiety (O'Shea et al., 2004), impaired spatial and non-spatial learning (Cha et al., 2006) as well as impaired prepulse inhibition (Schneider and Koch, 2003; Hall, 2006).

These studies indicate an influence of cannabis exposure in adolescence age on neuropsychiatric functioning. Furthermore, adolescence cannabis use has been hypothesized to influence the subsequent abuse of other drugs; indeed, several epidemiological studies report that early regular use of cannabis increases the risk of initiation of use of other illicit drugs (Agrawal et al., 2004), supporting the cannabis gateway hypothesis (see below) of cannabis as a steppingstone towards abuse of other drugs.

A very delicate and controversial issue is the discussion whether cannabis may act as a gateway drug, and subsequently lead to increase intake of other illicit drug, and subsequently lead to increased intake of other illicit drugs.

The gateway hypothesis, introduced by Kandel in the 1975, proposes the thesis that developmental stages can be observed in drug use.

The sequence normally begins with the use of legal drugs, such as beer and wine, subsequently followed by hard liquor and cigarettes and proceeds in some adolescents progressively through the use of cannabis products (marijuana or hashish) to other illicit drugs such as psychostimulants and heroin (Hall, 2006).

There is consistent evidence that the use of cannabis almost invariably precedes the use of other illicit drugs. Additionally, there seems to be a strong association between early cannabis use and other illicit drug use, because cannabis users are more likely to use psychostimulants, hallucinogens or opiods than those who have never used cannabis.

Many population surveys in western societies show that those who started using cannabis at age 14 or younger had a higher percentage of present illicit drug abuse or dependence than those starting at an older age (SAMHSA 2004). It has been argued that the observed associations may simply reflect a combination of a genetic predisposition to substance use in general and levels of avaibility and access to different drug classes by contact to peer-groups or a social environment encouraging drug use and abuse. Therefore, whether or not there is a casual relationship between cannabis use and a progression to other illicit drug use is still heavily debated and clarification of this contentious issue definitely requires further research. Evidence from animal studies indicates that cannabinoids might induce lasting neuronal modulations that could alter the perception and/or reinforcing values of other drugs of abuse, independent of genetic, social or cultural factors. A recent study demonstrated that chronic Δ^9-THC

administration during early puberty of male rats enhanced heroin self-administration in adulthood and increased pre-proenkephalin mRNA exclusively in the nucleus accumbes shell (Ellgren et al., 2007). This study indicates that there is a link between pubertal cannabis experience and further drug intake.

Cannabis exposure during young ages might have a priming effect on the brain and render cannabis users more susceptible to the effects of other illicit drugs; although strong evidence suggests that cannabinoids induce neurobiological alterations in common reward pathways during this critical period, these findings do not exclude the possibility that other factors such as genetic predisposition, social structure and environment might influence these neurodevelopmental cannabinoid effects and either enhance or attenuate further progression into illicit drugs use (Rubino et al., 2012).

AIM OF THE PROJECT

AIMS OF THE PROJECT

The main aim of this study is to investigate whether the use of cannabis in adolescent age may have effects on the immune system that can persist over time, even in adulthood, altering the susceptibility of the individual to infections, allergic and autoimmune diseases.

The project is conducted using Balb C/J male mice as an experimental model.

The use of experimental models allows a better control of the study variables and to investigate the effects of the interaction between the consumption of cannabis in adolescent age and the immune system, independently of external factors (cultural, social and moral) that would be instead be present in a study conducted in human subjects.

Adolescent and adult mice are treated with increasing doses of Δ^9-THC for 10 days.

Some study groups are sacrificed immediately at the end of treatment, while other study groups treated with Δ^9-THC in adolescence or in adulthood are left undisturbed for 47 days and sacrificed respectively at 90 PND and 137 PND.

In order to study the long-term effects of Δ^9-THC on the immune system the following parameters are investigated:

- The macrophage activation analyzing the profile of pro and anti-inflammatory cytokines
- The ability of lymphocytes to produce Th1/Th2cytokines
- The ability to develop an antibody response to a protein antigen

In order to understand whether the Δ^9-THC effects might be due to an alteration of circulating hormones, we also assessed the alteration of HPA axis activity, after subacute and chronic Δ^9-THC treatment, by measuring the corticosterone plasma levels in the three study groups.

In order to identify the mechanisms underlying the long lasting immune effects observed, we started to investigate whether they were mediated through modulation of hematopoietic cells. We are aware that it is often difficult to translate animal studies to human.

This is particularly true for drug of abuse studies, where the route of administration of substances in the animals is different from the ones used by humans.

Therefore we wanted to be certain that Δ^9-THC plasma concentrations achieved in mice with our experimental protocol would be comparable to those that can be detected in human heavy smokers.

Therefore the blood concentrations of Δ^9-THC are verified analyzing the samples with Liquid Chromatography-Mass Spectrometry method (LC-MS/MS).

Since it is now recognized that pro and antinflammatory cytokines play a pivotal role also in neuroinflammation, and a few recent evidences seem to indicate that cannabinoids can modulate it, we started to study also the effects of Δ^9-THC administration in our experimental protocol on neuroinflammation, by evaluating the basal production of pro- and anti-inflammatory cytokines in brain areas that are particularly rich in cannabinoid receptors, such as hippocampus and hypotalamus.

METHODS

METHODS

DRUGS

Δ^9-Tetrahydrocannabinol (Δ^9-THC) was obtained from Sigma Aldrich (Milan, Italy) and was dissolved in ethanol, cremophor and saline (1:1:18).

Lipopolysaccaride (LPS) from *Escherichia coli* and Keyhole Limpet Hemocyanin (KLH) were purchased respectively from Sigma-Aldrich (Milan, Italy) and Calbiochem (La Jolla, CA, USA). For the Liquid Chromatography/Mass Spectrometry (LC-MS/MS) analysis, methanolic stock solutions of Δ^9-THC (0.1mg/mL), THC-OH (0.1mg/mL), THC-COOH (1mg/mL) and their deuterated analogues THC-D3 (0.1mg/mL), THC-OH-D3 (0.1mg/mL), THC-COOH-D3 (1mg/mL), used as the internal standards, were purchased from Cerilliant Corporation (Round Rock, TX, USA).

ANIMALS

Male mice Balb C/J, Post natal days 33 (PND 33) were used as model of adolescence age and 80 PND were used as model of adult age. The animals were obtained from Charles River (Calco, Italy). All animal experiments were conducted following the guidelines released by the Italian Ministry of Health (DL 116/92 and DL111/94-B), and the European Community directives regulating animal research (86/609/EEC). All efforts were made to minimize the number of animals used and their suffering.

TREATMENTS

Animals were housed in a room with controlled temperature (22±1°C), humidity (60±10%) and light (12h/day) for at least 1 week before being used. Food and water were available *ad libitum*. The treatment began at 33 PND, for the adolescent model and lasted until 42 PND.

During this period, mice received increasing subcutaneous (s.c) doses of Δ^9-THC once a day, for the following 10 days (5mg/kg 33-35 PND; 10mg/kg 36-38 PND; 15 mg/kg 39-42 PND) or its vehicle. For the adult model the treatment began at PND 80, and lasted until PND 89.

During this period, mice received increasing s.c. doses of Δ^9-THC once a day, for the following 10 days (5mg/kg 80-82 PND; 10mg/kg 83-85 PND; 15 mg/kg 86-89PND) or its vehicle.

The dose protocol was chosen on the basis of previous experiments from our and other group, and the dosage was increased over the ten days in order to counteract a possible development of tolerance (Massi et al., 1998; Lombard et al., 2011).

The treatment schedule is depicted in Figure 8.

For this study, the mice were divided into 3 main experimental groups:

1. **Adolescent animals**

   - **Group A** mice were treated with subcutaneous injection of vehicle once a day starting from 33 PND until 42 PND; at the end of treatment (43 PND) mice were sacrificed to assess immune parameters.

   - **Group B** mice were treated with subcutaneous injection with increasing doses of Δ^9-THC once a day starting from 33 PND until 42 PND; at the end of treatment (43 PND) mice were sacrificed to assess immune parameters.

2. **Adult animals**

   - **Group C** mice were treated with subcutaneous injection of vehicle once a day starting from 80 PND until 89 PND; at the end of treatment (90 PND) mice were sacrificed to assess immune parameters.

   - **Group D** mice were treated with increasing subcutaneous injections of Δ^9-THC once a day starting from 80 PND until 89 PND; at the end of treatment (90 PND) mice were sacrificed to assess immune parameters.

3. Adult animals treated when adolescents

- **Group E** mice were treated with subcutaneous injection of vehicle once a day starting from 33 PND until 42 PND; at the end of treatment, the mice were housed for 47 days, until they reached adulthood (90 PND). At this time, animals were sacrificed to assess immune parameters.

- **Group F** mice were treated with subcutaneous injection with increasing doses of Δ^9-THC once a day starting from 33 PND until 42 PND; at the end of treatment, the mice were housed for 47 days, until they reached adulthood (90 PND). At this time, animals were sacrificed to assess immune parameters.
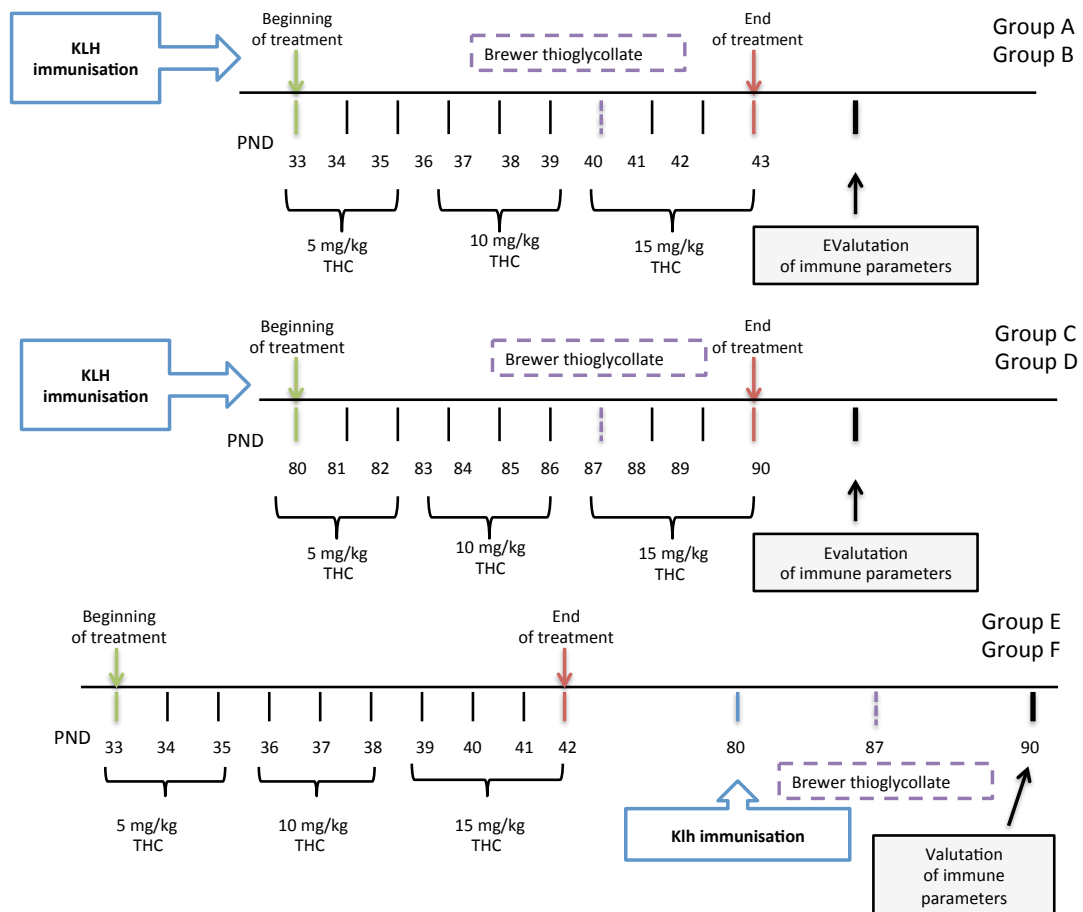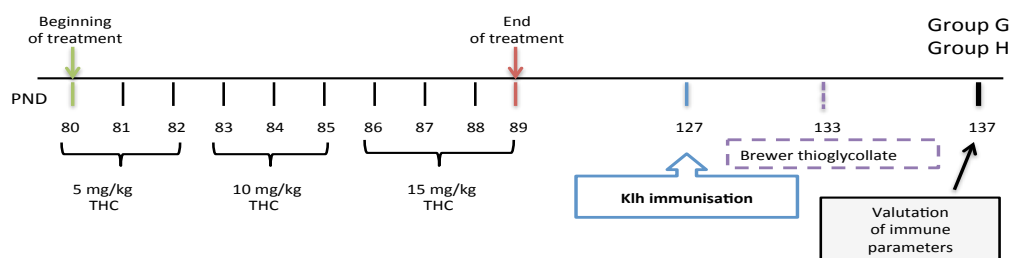

Figure 8 - Treatment schema of three study groups

Moreover in order to assess the sub-acute effect of Δ^9-THC treatment on corticosterone serum levels, a group of adult (83-84 PND) and adolescent (36-37 PND) mice were treated with 10 mg/Kg Δ^9-THC once daily for two days, and serum obtained 2 hours later the last dose of Δ^9-THC.

Recently, it has been added a fourth study group, where we started to assess the immune parameters, consisting of:

- **Group G** mice were treated with subcutaneous injection of vehicle once a day starting from 80 PND until 89 PND; at the end of treatment, the mice were housed for 47 days, until they reached 137 PND. At this time, animals were sacrificed to assess immune parameters.

- **Group H** mice were treated with subcutaneous injection with increasing doses of Δ^9-THC once a day starting from 80 PND until 89 PND; at the end of treatment, the mice were housed for 47 days, until they reached 137 PND. At this time, animals were sacrificed to assess immune parameters.

Figure 9 - Treatment schema of the recent study group

MACROPHAGES PURIFICATION AND STIMULATION

Mice were inoculated intraperitoneally (i.p.) with 2 ml of 3% Brewer thioglycollate medium (Fluka, Sigma-Aldrich, Italy) for macrophage elicitation.

Four days after thioglycollate treatment, at 43 PND in the adolescent model for the Groups A and B, at 90 PND in the adult models for the Groups C, D, E and F, and at 137 PND in the adult model for the Groups G and H, mice were sacrificed and macrophages isolated (Martucci et al., 2007). Briefly, peritoneal elicited cells were harvested in RPMI 1640 medium (Sigma-Aldrich, Milan, Italy) plus 10% FCS.

The viability of the cells was checked by the trypan blue exclusion test.

The cells were also counted in Turk's liquid to allow evaluation of nuclei for differential counting. Macrophages were resuspended at a concentration of 1×10^6/ml in the same harvesting medium and 1 ml/well aliquots were dispensed into 24-well culture plates. Isolation and purification of macrophages were carried out by 2 hours of adherence to plastic. As we previously demonstrated this procedure produces a population of macrophages with a 90%purity (Martucci et al., 2007). Non-adherent cells were removed and adherent cells were washed twice with PBS solution and incubated with 1μg/ml of LPS for IL-1β, IL-10 and TNF-α stimulation. The stimulus was added to the macrophage cultures in a final volume of 1 ml/well with RPMI 1640 plus 10% FCS, 1% glutamine, 2% streptomycin solution and 0,1% 2 Mercaptoethanol (2ME). After 24 hours of culturing the supernatant was removed and collected for cytokines evaluation. For mRNA quantification Trizol reagent was added to the adherent cells into the plate (see below). The samples were stored frozen at -80°C for subsequent analysis.

KLH IMMUNISATION AND COLLECTION OF SPLENOCYTES

Mice were inoculated intraperitoneally (i.p.) with 100 μg of the protein antigen KLH in a volume of 0.2 ml of saline; 10 days after immunisation, the animals were sacrificed and their spleens were aseptically removed; 20-Gauge sterile needles were used to tease the cells through an incision made in the spleen cuticle (Martucci et al., 2007; Sacerdote et al., 2000b).

The cells were plated at a concentration of 7×10^6 in 24-well plates containing a final concentration of 80 μg/ml KLH in a total volume of 1 ml.

The presence of *in vitro* KLH restimulates the KLH-specificT-lymphocyte clones previously activated by *in vivo* KLH. This is a frequently used method for inducing cytokine production by specific T-lymphocyte clones.

The plates were incubated at 37°C in 5% CO_2 and 95% air, and the supernatants were collected after 48 hours (in the case of IFN-γ) or 72 hours (in the case of IL-10 and IL-4), which are the times of maximum cytokine release (Sacerdote et al., 2000b); the supernatants were stored at -80°C for cytokine analysis.

The KLH concentration used *in vitro* was chosen on the basis of previous experiments showing that it induces sub-maximal but easily measurable cytokine production.

Times of *in vivo* immunisation with KLH correspond respectively to 33 PND and to 80 PND in adolescence and adult age models (Groups A, B, C and D). In the groups of adult animals treated when adolescent and in adult mice evaluated 47days after the end of treatment, KLH was administered 10 days before the animal sacrifice, corresponding respectively at 80 and 127 PND (Figure 8; Figure 9).

SERUM COLLECTION FOR ANTI-KLH AB AND CORTICOSTERONE EVALUATION

Ten days after KLH immunisation mice were sacrificed by decapitation at 10.30 AM. Blood was collected in tubes and left to clot at room temperature for 8 hours and centrifuged at 6000×g for 10 minutes. Serum was stored at −20 °C until assayed for Anti-KLH Ab.

In order to measure corticosterone levels, blood was collected at the end of 10 days treatment from adolescent and adult mice, and from adult mice treated as adolescent. Moreover in order to assess the sub-acute effect of Δ^9-THC treatment on corticosterone serum levels, a group of adult (83-84 PND) and adolescent (36-37 PND) mice were treated with 10 mg/Kg Δ^9-THC for two days, and serum obtained 2 hours after the last administration of Δ^9-THC.

anti-KLH Ab ELISA

The anti-keyhole-limpet hemocyanin antibody titer (Anti-KLH Ab) was measured in each study group with Enzyme-Linked Immunosorbent Assay (ELISA). The plates were coated overnight with 10 KLH in a carbonate coating buffer, pH 9.6. Mice sera were diluted 1:25, 1:75 and 1:225 in PBS/Tween containing 1M NaCl and incubated for 3 hours at 37°C. Alkaline phosphate-conjugated goat antimouse IgM [μ-chain-specific (Sigma-Aldrich), diluted 1:6000 in PBS/Tween] was then added and plates were incubated overnight at 4 °C. After washing, *p*-nitrophenyl-phosphate substrate at 1 mg/ml in carbonate buffer was added, and the colored product formed was measured at OD 405 nm.

CORTICOSTERONE EVALUATION

Corticosterone was measured with a commercially available ELISA kit (IBL International GmbH, Hamburg, and Germany) (see below).

Sensitivity of the method for Corticosterone evaluation was 5 nmol/ L.

DETERMINATION OF BLOOD Δ^9-THC LEVELS

In order to estimate Δ^9-THC concentrations, together with THC-COOH and THC-OH levels, blood was collected from adult and adolescent animals 24 hours after the last Δ^9-THC administration and from adult mice treated in adolescent age 30, 47 and 60 days after the end of treatment (Groups A, B, C, D, E and F).

The blood collected form each animal was transferred onto Whatman 903 Protein Saver Cards (Maidstone, UK) in order to obtain Dried Blood Spots (DBS) samples. DBS samples were stored at room temperature in a dark and dry place until the analysis.

The Dried Blood Spots were then sent to the laboratory of toxico-pharmacological analysis of University Alma Mater Studiorum of Bologna, directed by Professor Raggi for Δ9-THC analysis and its major metabolites. The Δ^9-THC being highly lipophilic has a rather long stay in the body. The THC-OH and THC-COOH are the major metabolites of Δ^9-THC. The THC-OH has psychoactive effects and a short half-life, is quickly transformed into THC-COOH that instead has a long half-life and for which no known psychoactive effects have been described.

The method of Liquid Chromatography and Mass Spectrometry (LC-MS/MS), newly developed by the group of Professor Raggi, has a high sensitivity and selectivity and allows to evaluate the presence of Δ^9-THC and metabolites in the same DBS samples.

Briefly, 10 mm of the spot are put into a vial in the presence of methanol, centrifuged and the supernatant dried. At this dry residue the solvents were then added and the injection of samples and standard solutions in HPLC-MS/MS system is performed.

The LC-MS/MS apparatus was a Waters (Milford, MA, USA) Alliance e2695 system coupled to a Waters Micromass Quattro Micro triple quadrupole mass spectrometer. Tandem mass spectrometry acquisition was carried out in multiple-reaction-monitoring (MRM) scan mode, using an electrospray ionisation source (Mercolini et al., 2013; Mercolini et al., submitted). The concentrations of Δ^9-THC and its metabolites were expressed as ng/ml.

The levels of IL-1β (R&D Systems, Minneapolis, Minnesota), IL-10, TNF-α, IL-4, and IFN-γ (eBioscience, San Diego, California) were determined by Enzyme-Linked-Immunosorbent-Assay (ELISA) using ultra-sensitive ELISA kit according to the manufacturer's instruction.

Sensitivity of the method for IL-1β evaluation was 15,625 pg/ml, for IL-10 was 30 pg/ml, 8 pg/ml for TNF-α, for IL-4 was 4 pg/ml and 15 pg/ml for IFN-γ.

Brieflly, a first anti-cytokine monoclonal antibody (Ab-primary) was adhered onto a polystyrene plate with U bottom 96 wells. The adhesion of the protein to the plastic polymer was favoured by a basic pH and occurs during an over-night incubation at 4 °C.

Several washings were then carried out so as to eliminate the excess antibody that was not bound. The nonspecific sites of the plate were blocked with blocking buffer solution and the samples were added.

The plates were incubated to ensure that any cytokine present in the sample binds to the antibody already immobilized to the wells.

After several washes, the secondary anti-cytokine biotinylated monoclonal antibody is added: this binds the immobilized cytokine already linked to primary Ab. Therefore a sandwich was formed with the substance in the center and on the sides the antibodies. After another series of washes, avidin peroxidase was added which has the task of binding the biotinylated antibody.

Finally, the enzyme substrate was added: a colored complex is formed of intensity proportional to the amount of cytokine present in the sample; the color development was stopped with the addition of H_2SO_4 (2N).

The result was obtained by reading in a spectrophotometer the absorbance of each well at a specific wavelength to each cytokine studied.

The amount of cytokine present in each sample is assessed with a calibration curve based on the values obtained from the analysis of scalar known concentrations of recombinant cytokine together with the samples subjected to ELISA.
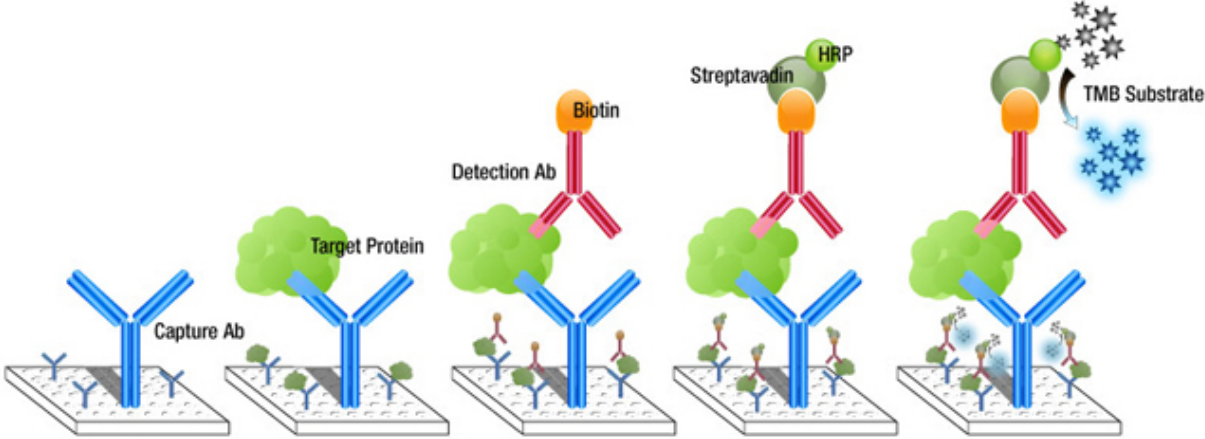

Figure 10 - ELISA sandwich

RNA EXTRACTION AND REAL-TIME PCR

Total RNA was extracted by adherent peritoneal macrophages, hypothalamus and hippocampus using Trizol reagent (Invitrogen Life Technologies, San Giuliano Milanese, Italy) according to the manufacturer's instructions and re-suspended in 10μl of DEPC-treated water (Fluka).

After removing DNA contaminations (DNA-free kit, Ambion, Applied Biosystems, Monza, Italy) mRNA was quantified and an equal amount of mRNA (1000 ng) underwent to Reverse Transcription (iScript Reverse Transcription Supermix, Bio-Rad, Segrate, Italy) to obtain cDNA.

For the process of reverse transcription, in a new series of tubes, for each sample, were added to 1000 ng of RNA, iScript RT Supermix and finally DEP H_20 up to the final volume of 20 μl. The tubes were then placed in a thermocycler for 40 minutes as follows: 5 minutes at 25°C (Priming), 30 minutes at 42°C (Reverse Transcription) and the last 5 minutes at a temperature of 85°C (RT Inactivation).

The cDNA samples obtained are used for the amplification reaction with Reverse transcriptase-polymerase chain reaction (Real-Time PCR) using ABI PRISM 7000 (Applied Biosystems, Foster City, CA). The reaction was performed in a 25μl volume using Real Master Mix Probe Rox (Eppendorf, Milano, Italy). Specific Taqman probes for mouse interleukins IL-1β (Mm 00434228_m1), IL-10 (Mm00439616_m1), TNF-α (Mm00443258_m1) and for glyceralde-hydes-3-phosphate dehydrogenase GAPDH (Mm99999915_g1) were purchased from Applied Biosystems.

All PCR assays were done in triplicate, and before starting, we performed a validation experiment to demonstrate that the efficiencies of target and reference are similar.

The reaction conditions were as follows: 95 °C for 2 minutes (initial denaturation), followed by 40 cycles at 95 °C for 15 seconds (cycled template denaturation) and at 60 °C for 60 seconds (annealing and extension); the reaction mixture without the cDNA was used as control.

The results were quantified using the comparative threshold method. The Ct value of the specific gene of interest was normalized to the Ct value of the endogenous control, GAPDH, and the comparative Ct method ($2^{-\Delta\Delta Ct}$) was then applied using adult mice treated with vehicle (Group C) as calibrator.

At the time of sacrifice, the femur of each animal was collected and the bone marrow cells were isolated by flushing with RPMI 1640 plus 10% FCS.

The cells thus obtained were brought to a concentration of $50*10^6$/ml and then were plated and incubated for 14 days in medium MethoCult ® (STEMCELL Techonologies, Voden Medical Instruments spa, Peschiera Borromeo, Italy) at 37° C and 5% CO_2.

The cells of each mouse were plated in quadruplicated. After 14 days of culture, the total number of colonies present for each mouse, in each of 4 wells, was counted by an operator unaware of the treatments. It then was made the average of colonies for each group of animals.
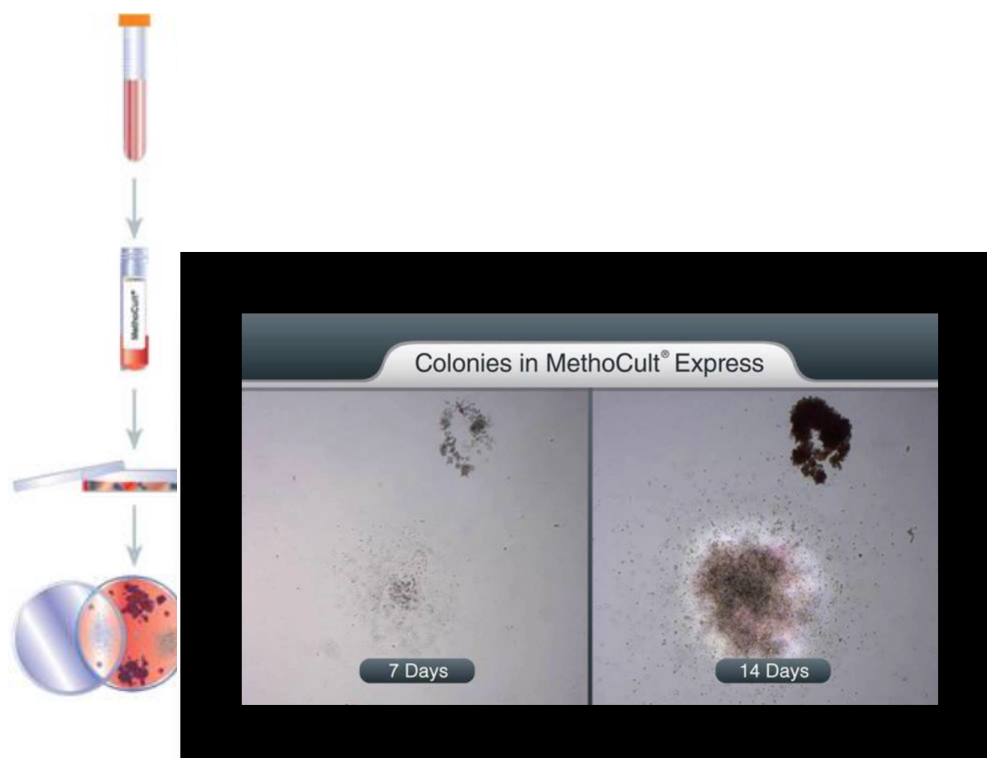
Figure 11 - Extraction and counts of hematopoietic cells

ASSESSMENT OF BASELINE LEVELS OF CYTOKINES IN THE HYPOTHALAMUS AND HIPPOCAMPUS

At the time of sacrifice the brain of animals were rapidly removed and hypothalamus and hippocampus dissected out on an ice- cold plate. The hypothalamus and hippocampus were then rapidly frozen on dry ice and stored at -80°C until processing for RNA extraction or ELISA cytokine analysis.

PREPARATION OF THE SAMPLES FOR THE ELISA ASSAY

Hypothalamus and hippocampus of each animal were sonicated with a sonicator (UP50H Compact Lab Homogenizer, Hielscher ultrasonic GmbH; Teltow, Germany) in 225 μl of lysis buffer composed as follows: PBS, Complete Mini EDTA-free protease inhibitor cocktail (Roche) and EDTA (5 mg/ml). After sonication all samples were centrifuged at 13000 g for 15 minutes at a temperature of 4 °C.

A part of the supernatant obtained was used for the protein assay, the remaining part was used for the ELISA assay.

PROTEIN ASSAY OF TOTAL PROTEIN CONTENT IN THE HYPOTHALAMUS AND HIPPOCAMPUS

The protein assay is required to relate the level of cytokine measured by ELISA assay, with the amount of total protein present in the samples.

The protein assay according to Lowry 's method involves the use of bovine serum albumin as standard. For the calibration line, bovine serum albumin was prepared at the stock concentration of 1 mg/ml in $H_2$0d. In each tube we added, in the following order 5, 10, 20, 40, 60, 80, 100 μl of the stock bovine serum albumin reaching a final volume of 100 μl with addition of $H_2$0 d.

200 μl of NaOH 1N and 2ml of Cooper reagent consisting of Na_2CO_3 2%, $CuSO_4$ (5 H_2O) 1% and Na-K- tartrate 2% (in $H_2$0 d), respectively in the proportion 100:1:1were also added.

The samples were incubated avoiding the light and at room temperature for 10 minutes and then were added 100 μl of Folin Ciocalteau reagent.

The tubes were then placed again in the dark for 45 minutes, to finally proceed with the reading of the absorbance at a wavelength of 750 nm using a spectrophotometer (Uvicon 941 PLUS, Kontron Instrument).

The controls were obtained by replacing the albumin with 100 μl of H_2O d.

The samples were prepared following an analogous procedure to that used for the standard curve, to each tube was added a fixed quantity of sample (15 μl) to which 85 μl of H_2O d were added to reach the final volume of 100 μl.

EVALUATION OF BASAL CYTOKINE PRODUCTION WITH ELISA METHOD

The levels of basal IL-1β, IL-10 and TNF-α cytokine production in hypothalamus and hippocampus from Δ^9-THC or vehicle treated mice were determined using ultra-sensitive ELISA kit according to the manufacturer's instruction (see paragraph " CYTOKINE ELISA").

Cytokines content were determined by interpolation with standard curves assayed on individual plates and normalized to protein content in each tissue.

The results are presented as means ± SEM of values. Statistical analyses were performed using one-way ANOVA for parametric results. Follow-up analysis was performed using the Tukey's test for multiple comparisons. In the antibody titers experiments TWO Way ANOVA was utilized, with treatment and serum dilutions as factors. Differences were considered significant at $p < 0.05$.

RESULTS

RESULTS

MACROPHAGES

To investigate the effects induced by Δ^9-THC treatment on innate immunity, we examined the cytokine production by peritoneal macrophages elicitated *in vivo* with thioglycollate and stimulated in vitro with 1 mg/ml LPS. Culture media and macrophages are collected after 24 hours. Spontaneous production of cytokines was always very low, at the limit of detection, and no effect of treatment was ever observed (data not shown).

Figure 12 shows the effect of chronic Δ^9-THC administration on IL-1β, TNF-α and IL-10 production by peritoneal macrophages obtained from adult and adolescent animals evaluated immediately at the end of treatment, from adult mice treated with Δ^9-THC when adolescent or from adult mice evaluated 47 days after the end of treatment.

IL-1β, TNF-α and IL-10 are measured as protein concentrations in culture media with ELISA method (Figure 12, panels a, b and c).

Panels a and b shows the results relating to the production of IL-1β and TNF-α in 4 study groups; in adolescents and adults mice treated with Δ^9-THC, we observe a decrease of pro-inflammatory cytokines respect to controls: in agreement with the literature the drug administration induces a decrease of inflammatory responses.

In adult animals treated with Δ^9-THC during adolescent period, on the contrary we can observe an increase of IL-1β and TNF-α levels in comparison to vehicles, indicating a tendency towards a pro-inflammatory state. No significant difference was detected in adult animals evaluated 47 days after the last administration of Δ^9-THC.

The protein levels of IL-10 after Δ^9-THC chronic administration, in the 4 study groups, are shown in Figure 12, panel c.

This cytokine was increased in young and adult mice treated with Δ^9-THC. However in adult mice treated in adolescent age and tested at the age of 90 PND, Δ^9-THC induced a reversed effect: IL-10 concentration decreased in Δ^9-THC treated mice.

Also in this case, no significant difference was detected in adult animals evaluated 47 days after the last injection of Δ^9-THC.

Figure 12

MACROPHAGES
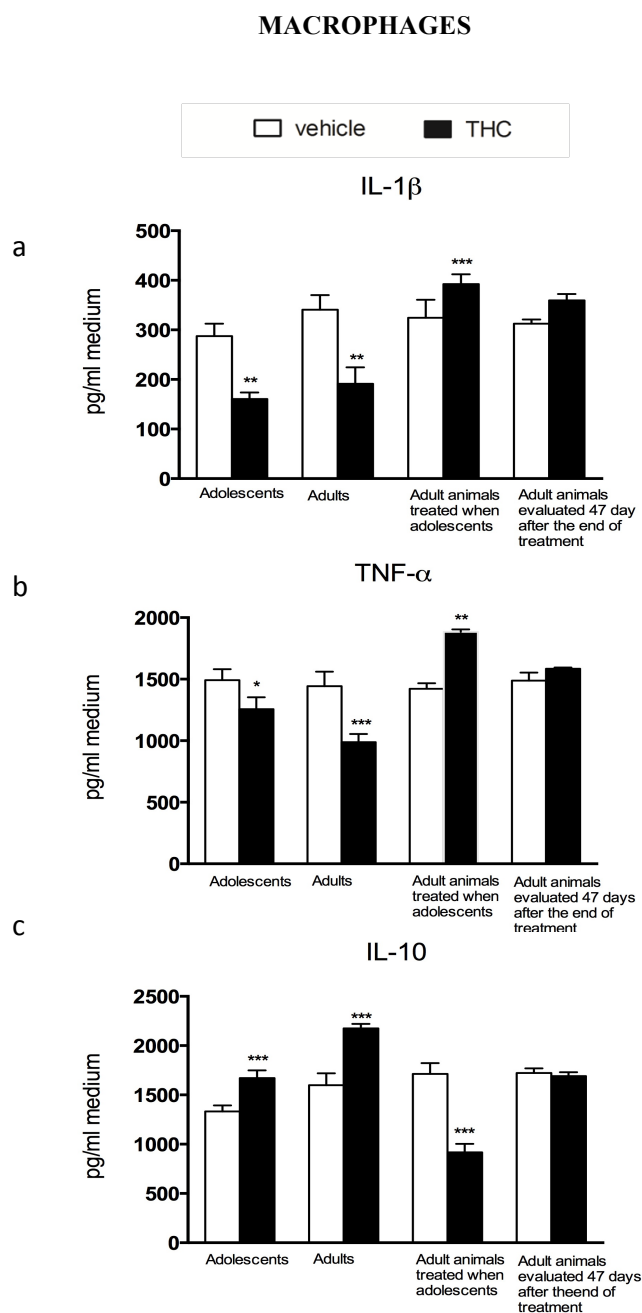

Figure 12 *Effect of chronic Δ⁹-THC administration on IL-1β, TNF-α and IL-10 production by peritoneal macrophages obtained from adult and adolescent animals immediatly at the end of treatment or from adult animals treated with Δ⁹-THC when adolescent and adult mice evaluetd 47 days after the end of treatment. IL-1β, TNF-α and IL-10 are measured as protein concentrations in culture media (panels a, b and c). Peritoneal macrophages, elicited in vivo by thioglycolate, were stimulated in vitro with 1 mg/ml LPS. Culture media and macrophages are collected after 24 hours. (see " Macrophages purification and stimulation" in Method section for details).*
Values are means ± SEM of 8-10 animals /group
* p <0.05 vs respective vehicle
** p <0.01 vs respective vehicle
*** p <0.001 vs respective vehicle

Figure 13 reports the mRNA levels of the pro-inflammatory cytokines IL-1β and TNF-α and of the anti-inflammatory cytokine IL-10 (panels a, b and c).

As shown in panel a, the treatment with Δ^9-THC induced a modulation at transcriptional level, in fact, the levels of IL-1β mRNA expression were decreased in young and adult mice, evalueted immediately at the end of treatment, while they were significantly increased in adult animals treated when adolescents.

In adult animals studied 47 days after the end of treatment, no significant differences were found between the two treatments considered.

Panel b shows the TNF-α mRNA expression in the four study groups.

The levels of mRNA were significantly decreased by Δ^9-THC treatment in young and adult mice, evaluated after 10 days of chronic treatment, whereas they were significantly increased in adult animals treated in adolescent age with the drug and evaluated 47 days after the end of treatment.

Also in this case, no significant differences were found between the two treatments considered, in the fourth study group. We then considered the anti-inflammatory cytokine IL-10 mRNA expression (Figure 13, panel c). Also the levels of mRNA expression measured by Real Time – PCR parallel those of IL-10 protein; treatment with Δ^9-THC induced an increase of mRNA expression in young and adult mice, while in adult mice treated in adolescent age the drug induced a significant decrease of mRNA expression.

In the fourth study group, no significant differences were found between the two treatments considered.

Figure 13

MACROPHAGES
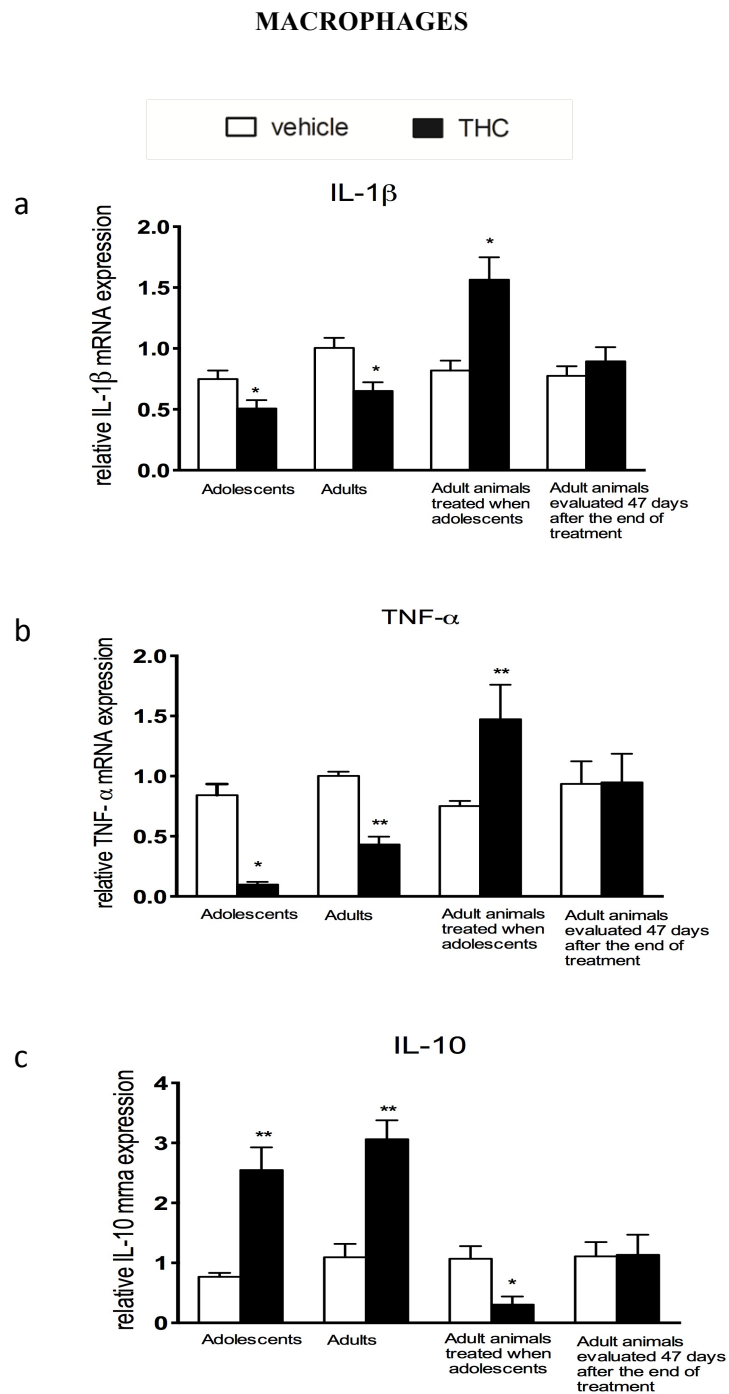

Figure 13 Effect of chronic Δ⁹-THC administration on IL-1β, TNF-α and IL-10 mRNA levels, determined by Real-Time PCR, expressed in relation to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and presented as fold of increase/decrease relative to vehicle treated animals.
Values are means ± SEM of 8-10 animals /group
* p <0.05 vs respective vehicle
** p <0.01 vs respective vehicle

Figure 14 shows the effect of Δ^9-THC chronic treatment on the production of T-helper cytokines IFN-γ, IL-4 and IL-10 by spleen cells obtained from mice immunized *in vivo* with 100 μg of KLH and restimulated *in vitro* with KLH at concentration of 80 μg/ml .

The presence of *in vitro* KLH stimulates the KLH-specific T-lymphocyte clones previously activated by *in vivo* KLH. This is a frequently used method to induce cytokine production by specific T-lymphocyte clones (Sacerdote et al., 2000b).

We report only the data relating to the cytokines production stimulated *in vitro* with KLH; we did not find significant differences in the spontaneous production of cytokines in the four study groups (data not shown). The production of the Th1 cytokine IFN-γ appears significantly lower in mice treated with Δ^9-THC respect to vehicle in all study groups (Figure 14, panel a); in fact the levels of this cytokine was significant reduced in adolescent and adult mice at the end of treatment with Δ^9-THC.

In animals treated in adolescence age and immunized as adults, the production of IFN-γ resulted still significantly lower 47 days after the last administration of the drug.

We found no difference in the IFN-γ levels, in adult animals evaluated 47 days after the last injection of the drug.

The effects of Δ^9-THC on Th2 cytokines IL-4 and IL-10 are reported respectively in panels b and c. The response of these cytokines to Δ^9-THC chronic treatment appears different from that observed with the cytokine Th1. The treatment with the drug induced a significant increase of IL-4 and IL-10 production in adolescent and adult mice respect to relative vehicles. However in the third study group, we observed a reversed effect, i.e. the production of IL-4 and IL-10 cytokines is significantly decreased respect to relative vehicle.

In the fourth study group, as regards the Th2 cytokine levels, no significant differences were found between the two treatments considered.

Figure 14

SPLEEN LYMPHOCYTES
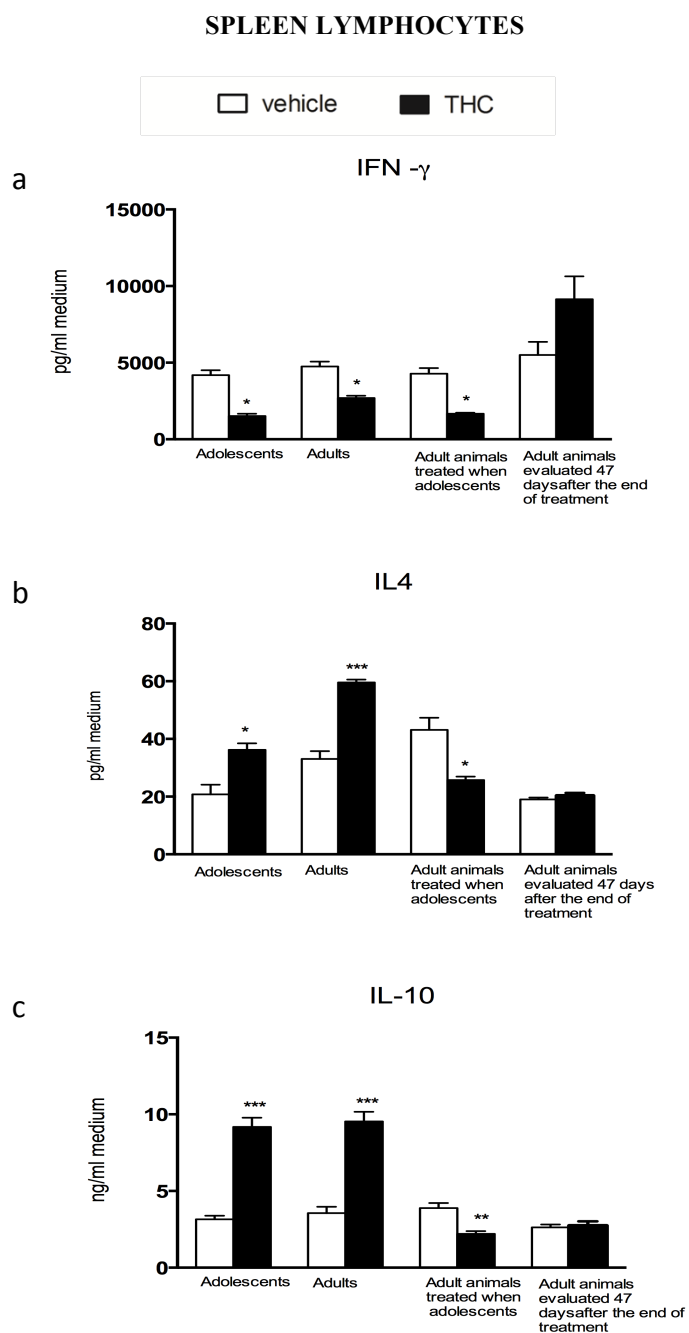
Figure 14 Effect of chronic Δ⁹-THC administration on IFN-γ, IL-4 and IL-10 production by splenocytes obtained from adult and adolescent animals evalueted immediately at the end of treatment or from adult animals treated with Δ⁹-THC when adolescents and adult mice evaluated 47 days after the end of treatment. Mice were immunized in vivo with KLH 10 days before of the immune parameters evaluation and cells were restimulated in vitro with 80 μg/ml KLH for 48 or 72 h (see " KLH immunisation and collection of splenocytes " in Method section for details).
Values are means ± SEM of 8-10 animals /group
* p <0.05 vs respective vehicle
** p <0.01 vs respective vehicle
*** p <0.001 vs respective vehicle

The titers are reported in Figure 15. The mice were immunized with the antigen protein KLH respectively at 33 PND (adolescents) and 80 PND at the beginning of Δ^9-THC treatment.

A third group of animals had received Δ^9-THC when adolescent and was immunized at 80 PND, i.e. 47 days after the last Δ^9-THC injection.

In adolescent and adult animals (Figure 15, panels a and b) the IgM anti-KLH titers measured in the sera after 10 days of chronic treatment with Δ^9-THC, are significantly reduced compared to respective controls. The decrease in the production of the IgM anti–KLH antibodies persists in the third study group, 47 days after the last administration of the drug (Figure 15, panel c).

In the three Δ^9-THC groups a low antibody titer than in animals treated with vehicle was therefore present, thus demonstrating that these animals were unable to develop an effective antibody response.

Figure 15
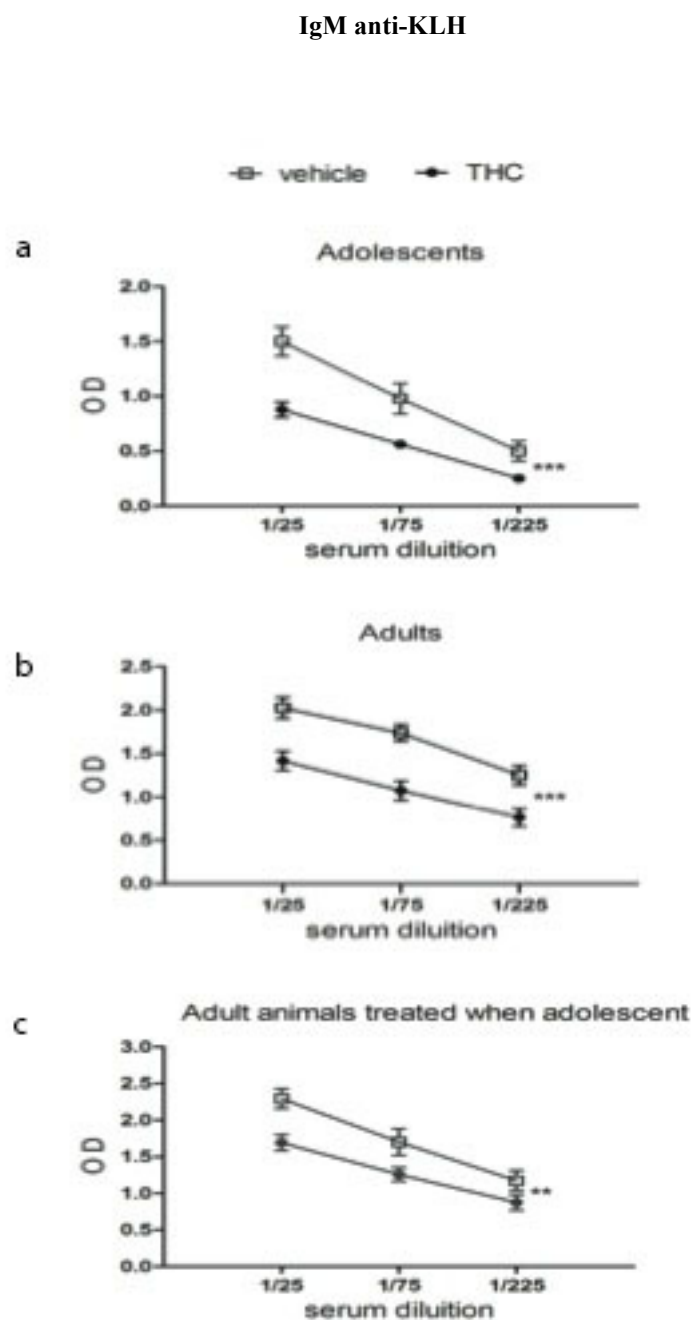
IgM anti-KLH

Figure 15 Effect of chronic Δ⁹-THC administration on IgM anti-KLH titers in sera from adult and adolescent animals immediately at the end of treatment or from adult animals treated with Δ⁹-THC as adolescents.
Adolescents (panel a) and adults (panel b) animals were immunized in vivo with KLH together with Δ⁹-THC treatment, while the third group was treated with Δ⁹-THC as adolescent and immunized with KLH in adult age, 10 days before the evaluation of immune parameters (panel c).
Values are means ± SEM of 8-10 animals /group (Two Way ANOVA)
** p <0.01 vs respective vehicle
*** p <0.001 vs respective vehicle

In order to evaluate a possible involvement of the HPA axis in the effects that we observed, we measured serum corticosterone in mice treated with both acute (10mg/Kg for 2 days) and chronic Δ^9-THC (10 days treatment).

As reported in Figure 16, panel a, 2 hours after the last administration of Δ^9-THC a significant increase of corticosterone levels was present both in adult and adolescent animals. In contrast the levels of corticosterone in adolescent and adult animals measured at the end of chronic treatment were significantly reduced compared to relative controls.

The significant decrease in the serum corticosterone levels persists even in the third group of study, 47 days from the last administration of the drug (Figure 16, panel b).

Figure 16
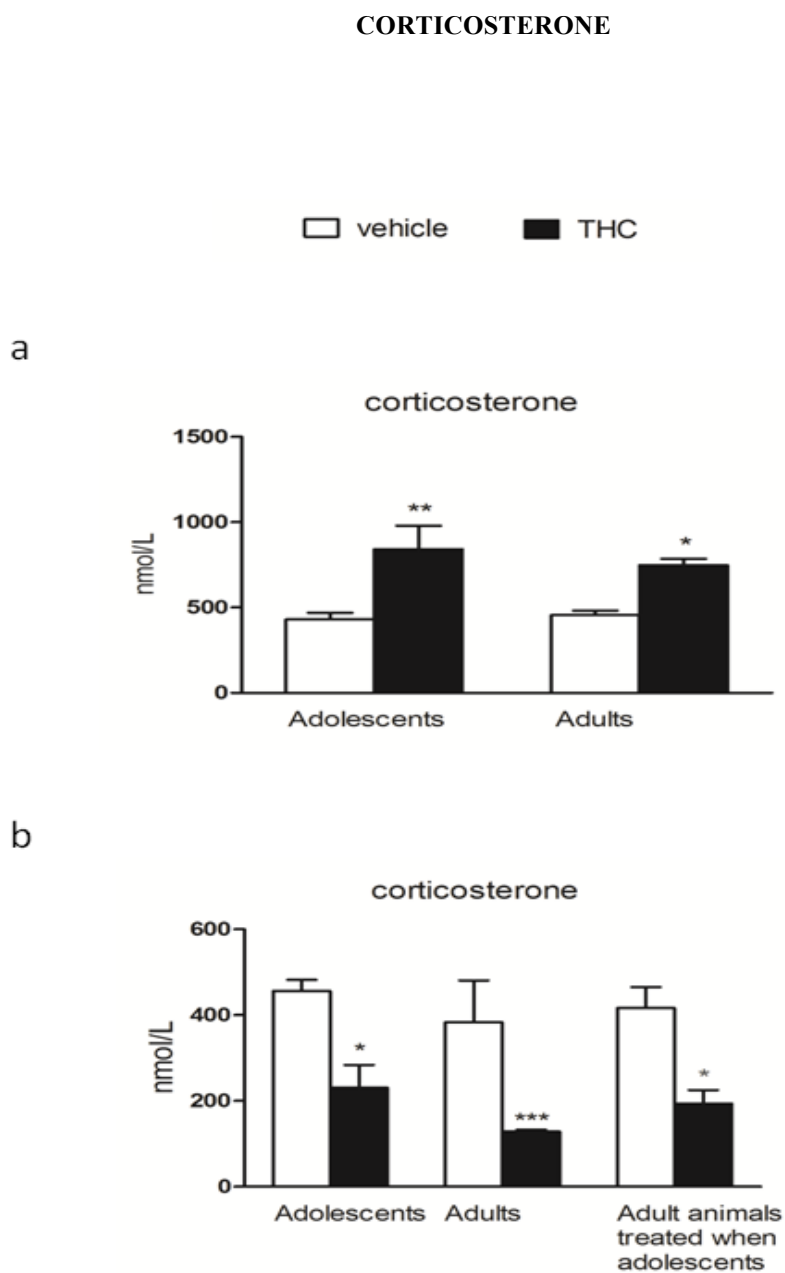
CORTICOSTERONE

Figure 16 Effect of Δ⁹-THC treatment on serum corticosterone levels.
Panel a: Δ⁹-THC was administered to adult and adolescent mice at the dose of 10 mg/Kg for 2 days, and serum obtained after 2 hours.
Panel b: Corticosterone levels were measured in adult and adolescent animals at the end of   chronic Δ⁹-THC administration (5mg/Kg for 3 days, 10 mg/Kg for 3 days and 15 mg/Kg for 4 days), and from adult animals treated with Δ⁹-THC when adolescent.
Values are means± SEM of 6 animals /group
* p <0.05 vs respective vehicle
** p <0.01 vs respective vehicle
*** p <0.001 vs respective vehicle

Table 1 shows the results relative to the count of the number of colonies formed by hematopoietic cells. The cells were obtained from bone marrow taken from the femur from adolescent and adult mice of the 3 study groups. The results show no significant differences in the number of colonies generated from animals treated with Δ^9-THC compared to animals treated with vehicle, in none of the three experimental groups considered.

Table 1

NUMBER OF COLONIES FORMED BY HEMATOPOIETIC CELLS

THC	Vehicle
Adolescents	
108,3 ± 12,68	**93,33 ± 7,88**
Adults	
99,33 ± 8,33	**103,7 ± 1,45**
Adult animals treated whan adolescents	
109,5 ± 6,89	**103,7 ± 1,45**

Table 1 Effect of chronic Δ^9-THC administration (5mg/Kg for 3 days, 10 mg/Kg for 3 days and 15 mg/Kg for 4 days) on the number of colonies formed by hematopoietic cells in adult and adolescent mice evaluated immediately at the end of the treatment and adult animals treated with Δ^9-TH in adolescent period.
The bone marrow was obtained from femur of adult and adolescent mice and the isolated cells were cultured in vitro for 14 days, for the count of colonies formed.
Values are means± SEM of 4 animals /group

In order to be sure that Δ^9-THC was not present in adult animals treated with the drug during adolescence, we measured the levels of Δ^9-THC and its major metabolites in blood.

Table 2 shows the blood levels of Δ^9-THC, THC-OH and THC-COOH in adolescent and adult animals 24 hours and 30,47, and 60 days after the end of treatment. This work was performed in collaboration with the team of Professor Raggi, Università Alma Mater Studiorum, Bologna. Δ^9-THC is metabolised to 11-hydroxy-Δ9-75-tetrahydrocannabinol (6aR,10aR)-9-hydroxymethyl-6,6-dimethyl-3-penthyl-6a,7,8,10a76tetrahydrobenzo[c]chromen-1-ol,THC-OH, by cytochrome P450, subtype 2C977 (CYP2C9). THC-OH is biologically active, since it binds CB receptors, and is in turn metabolised by CYP2C9 to 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol(6aR,10aR)-1-hydroxy-6,6-dimethyl-3-79pentyl-6a,7,8,10atetrahydrobenzo [c]chromen-9-carboxylic acid, THC-COOH. This latter metabolite is not psychoactive and has a very long half-life.

No traces of Δ^9-THC or metabolites were found in the blood of mice treated with the vehicle (data not shown). The results indicate that Δ^9-THC and THC-COOH were detectable in the blood of adolescent and adult mice evaluated after 10 days of chronic treatment, 24 hours after the last injection; at 30 days after the end of treatment Δ^9-THC was no longer present, but we still found the presence of the non-psychoactive metabolite THC-COOH; 47 and 60 days after the end of treatment neither Δ^9-THC, nor THC-COOH were detected .

The presence of the psychoactive metabolite THC-OH was never detected in mice evaluated at different time after the end of chronic treatment with the drug.

Table 2

DETERMINATION OF BLOOD CONCENTRATIONS OF Δ^9-THC AND ITS METABOLITES LEVELS

Blood concentrations ng/ml	Adult mice	Adolescent mice	Mice treated as adolescent		
	24 hours after last Δ^9-THC injection	24 hours after last Δ^9-THC injection	30 days after last Δ^9-THC injection	47 days after last Δ^9-THC injection	60 days after last Δ^9-THC injection
Δ^9-THC	18.47± 2.33	11.63 ± 5.44	<LOD	<LOD	<LOD
THC-OH	<LOD	<LOD	<LOD	<LOD	<LOD
THC-COOH	15.9 ± 1.38	8.55 ± 4.52	3.7 ± 0.32	<LOD	<LOD

LOD= limit of detection

Table 2 Presence of Δ^9-THC and its metabolites, THC-OH and THC-COOH, in the blood of adult and adolescent animals evaluated immediately at the end of the treatment, and of adult animals treated with Δ^9-THC when adolescent.
The blood was collected at the end of the treatment (24 hours after the last Δ^9-THC injection) and at 30, 47 and 60 days from the last direct exposure to the drug.
Values are means± SD of 4 animals /group

ASSESSMENT OF BASELINE LEVELS OF CYTOKINES IN THE HYPOTHALAMUS AND HIPPOCAMPUS

We started to study the effects induced by Δ^9-THC treatment on basal cytokine production, in hyppocampus and hypotalamus.

Figure 17 reports the concentrations, measured in the two neuronal tissues, of the pro-inflammatory cytokines IL-1β and TNF-α.

As shown in panel a, in both the tissues considered, the treatment with Δ^9-THC induced a significant decrease of IL-1β level in adolescent and adult mice, evaluated immediatly at the end of treatment. In contrast, a significant increase of IL-1β concentrations was present in adult mice treated as adolescents.

A similar pattern of modifications is present also when considering the production of TNF-α in hyppocampus and hypotalamus (Figure 17, panel b).

In adolescent and adult mice, evaluated immediatly at the end of treatment, a significant decrease of TNF-α concentrationsì was evident in both the neuronal area considered; as previously reported for IL-1β, in the third group, i.e. mice treated with Δ^9-THC during adolescent and studied when they had reached adulthood, the drug induced a reversed effect, since TNF-α concentrations appear to be increased 47 days after the end of treatment.

We then considered the basal production of the anti-inflammatory cytokine IL-10, in the two neuronal tissues, hippocampus and hypotalamus; this cytokine was increased in young and adult mice treated with Δ^9-THC (Figure 18, panel a).

However in adult mice treated in adolescent age and tested at the age of 90 PND, Δ^9-THC induced a reversed effect: IL-10 concentration decreased in Δ^9-THC treated mice.

Figure 17

BASELINE LEVELS OF CYTOKINES PRODUCTION IN THE HYPOTHALAMUS AND HIPPOCAMPUS
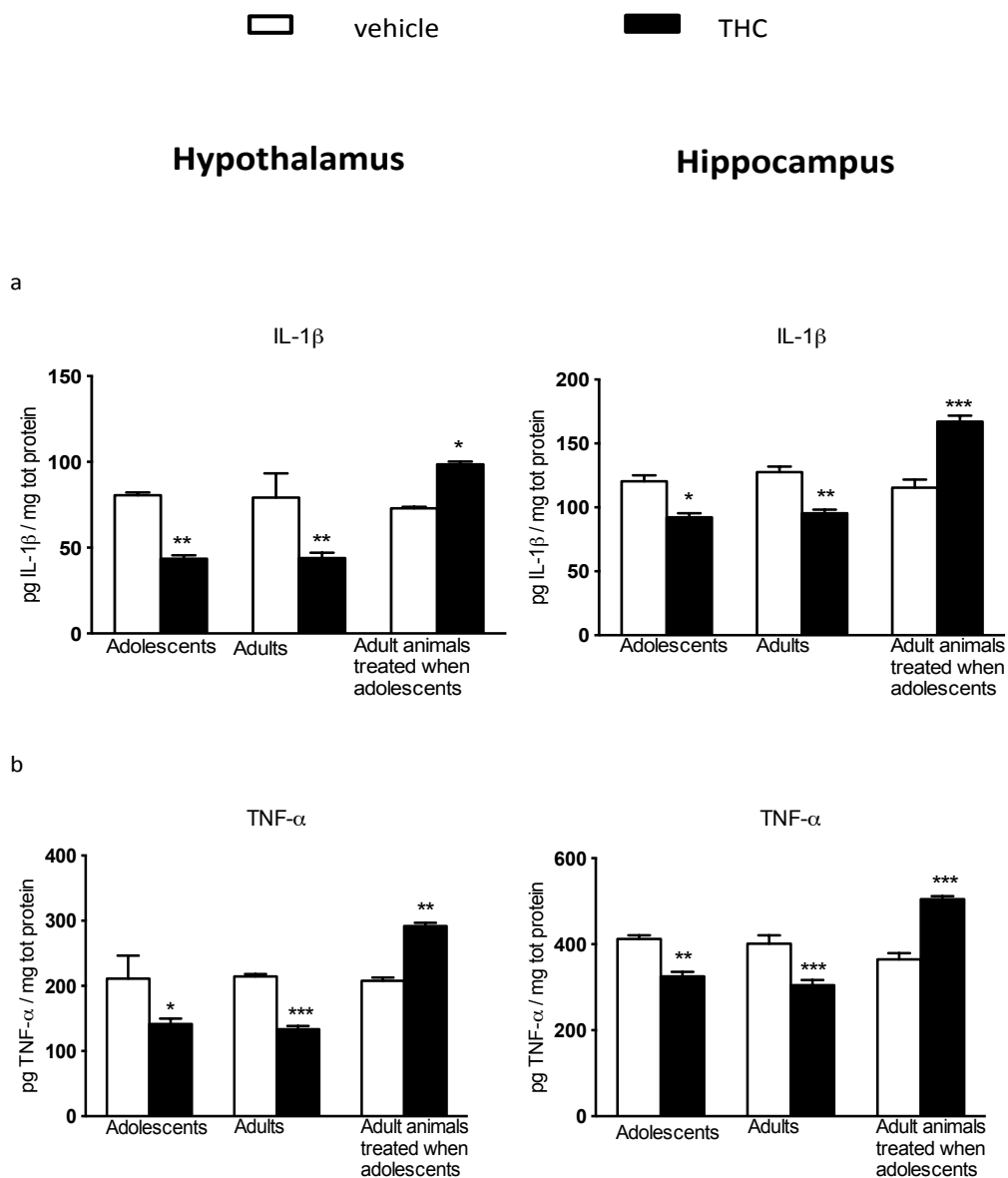
Figure 17 Effect of chronic Δ⁹-THC administration on IL-1β and TNF-α levels of cytokines in the hypothalamus and hippocampus obtained from adult and adolescent animals immediately at the end of treatment or from adult animals treated with Δ⁹-THC when adolescent.

IL-1β and TNF-α are measured as protein, and referred to total protein content.

Values are means ± SEM of 6 animals /group

* p<0.05 vs respective vehicle

** p<0.01 vs respective vehicle

*** p<0.001 vs respective vehicle

Figure 18

BASELINE LEVELS OF CYTOKINES PRODUCTION IN THE HYPOTHALAMUS AND HIPPOCAMPUS

Figure 18 Effect of chronic Δ⁹-THC administration on IL-10 levels of cytokine in the hypothalamus and hippocampus obtained from adult and adolescent animals immediately at the end of treatment or from adult animals treated with Δ⁹-THC when adolescent.

IL-10 is measured as protein, and referred to total protein content.

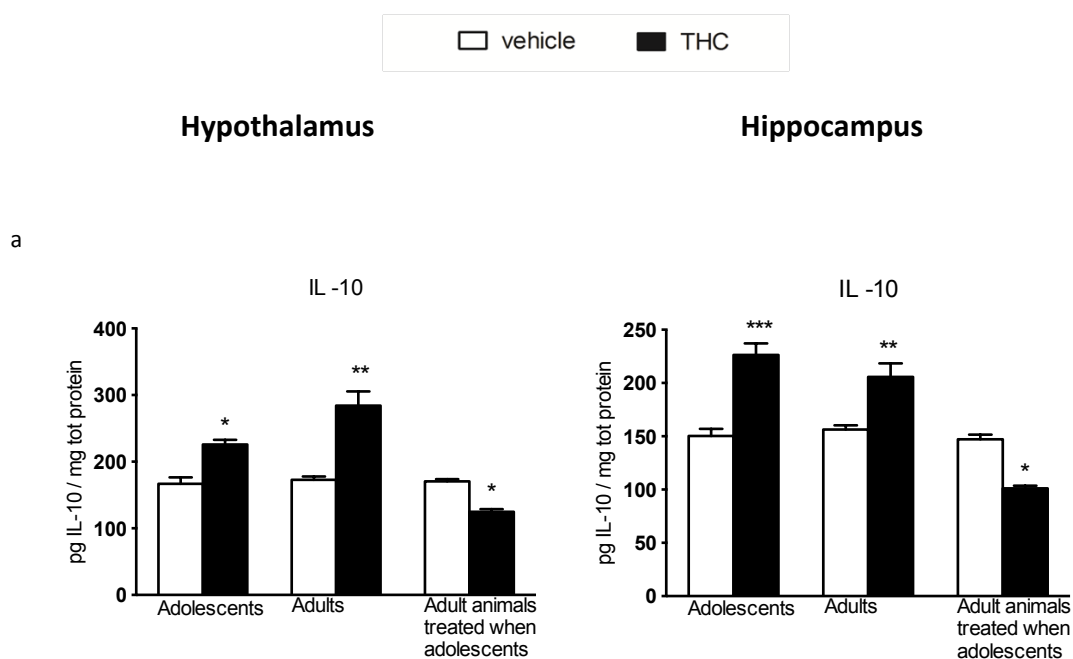*Values are means ± SEM of 6 animals /group*

* p<0.05 vs respective vehicle

** p<0.01 vs respective vehicle

*** p<0.001 vs respective vehicle

DISCUSSION

DISCUSSION

Cannabis or Marijuana is the most commonly used drug of abuse worldwide; marijuana use in Europe and the U.S. is increasing and the age of drug intake has very early onset and continues to fall. In the U.S. it is estimated that 46% of the population has tried marijuana at least once, while more than 5% use it continuously.

The cannabis intake begins very early, around 12 years and the percentage of patients who take it gradually increases up to 19 years of age. In Europe it is estimated that more than 23 million people have used cannabis, with an average of 7%; in Italy, more than 11% of the population appears to be involved. In Italy the use is particularly high among young people between 14 and 25 years of age (SAMSHA, 2004).

The immunosuppressive and anti-inflammatory properties of cannabis, including, Δ^9-THC have been well studied (Berdyshev, 2000; Klein, 2005; Massi et al., 2006); Δ^9-THC is known to cause immune suppression by various mechanisms including induction of apoptosis in effector T-cells, suppressing the inflammatory cytokine production, as well as arresting cell proliferation (McKallip et al., 2002; Klein and Cabral, 2006).

Furthermore, Δ^9-THC has been indicated to promote a shift in T-cell differentiation from Th1 to Th2 (Klein, 2005).

The immune modulating ability of cannabinoids suggests that these drugs might be useful as therapeutics in immune diseases and indeed their efficacy has been tested in several disease models (Mackie, 2006).

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 (Kaushik et al., 2011).

Marijuana remains the most commonly used drug of abuse worldwide (Berdyshev, 2000), especially by adolescents who are the main consumers of this drug.

In addition, the habit to attending large groups of peers and an attitude toward sex are often not aware/protected, predisposes young cannabis users to be more susceptible to bacterial and viral infection. Currently, in the literature there are studies indicating as Δ^9-THC intake during adolescence is able to influence the brain maturation and may modulate the behavior, emotion, affectivity and the incidence of psychiatric disorders in adulthood (Rubino et al., 2008; Realini et al., 2009; Tomasiewick et al., 2012) however there are no studies that assess this aspect in the immune system.

The aim of my thesis work was to study the effect on immune responses of Δ^9-THC intake in adolescent mice, demonstrating both short and long term immune dysfunctions.

The adolescence is a gradual period of transition from childhood to adulthood (Spear and Brake, 1983): this period in humans can be considered as the age range between 12 to 18 years. It is very difficult to identify in laboratory animals the period corresponding to the phase of human adolescence, such as some researchers have argued that adolescence is uniquely human and hence cannot be modeled in animals. However Spear and colleagues, (Spear and Brake, 1983; Odell, 1990; Spear, 2000) on the basis of behavioural and biochemical specific changes, have spotted in the period 28-42 post natal days (PND) a phase comparable to adolescence in human.

Starting from these premises, most of the works that analyzed the Δ^9-THC induced neurobehavioral modifications in adolescent, were performed using 30-32 PND mice (Rubino et al., 2008; Rubino et al., 2012).

In our experiments we followed this indication and started the Δ^9-THC treatment at 33 PND for 10 days, in order to have a Δ^9-THC exposure time covering the whole adolescence.

Our experimental protocol included the use of s.c. Δ^9-THC treatment or vehicle, for 10 days, with increasing doses of drug, as low as 5 mg/kg until to 15 mg/kg.

We previously conducted studies using a higher dose of Δ^9-THC (starting from 5 mg/kg until to 25 mg/kg) in the treatment of 10 days, getting a modulation of immune parameters similar to that illustrated in this work (data not shown). However, we have decided to show only the data related to Δ^9-THC treatment with doses in the range 5-15 mg/kg, because plasma concentrations of Δ^9-THC detectable at the end of our treatment in mice were comparable to those that can be found smoking in humans (Mercolini et al., 2013).

The first result that emerges from our study is the presence of a similar immunomodulation induced by Δ^9-THC chronic treatment in adult and adolescent mice. Both macrophage and spleen cytokines, evaluated 24 hours after the last Δ^9-THC administration, are in fact modulated in the same direction in the two age groups. Consistently with what reported in the literature for adult animals, we observed a clear decrease of macrophage pro-inflammatory cytokines IL-1β and TNF-α and increase in the anti-inflammatory cytokine IL-10 (Massi et al., 2006).

It is well known that several cytokines, and IL-1β in particular, are regulated at several post-transcriptional and post-translational levels (Ferrari et al., 2007), and previous studies suggested that Δ^9-THC could affect only secretion of pro-inflammatory cytokines but not transcription (Zheng and Specter, 1996). For these reasons, we measured both the released protein and the mRNA levels. The results obtained well correlate the cytokine mRNA and protein, suggesting that our Δ^9-THC treatment paradigm affects both gene expression and protein production.

In agreement with the data present in literature, we also confirm the ability of Δ^9-THC to alter the balance between the cytokines produced by T-helper subsets, Th1 and Th2, skewing it towards Th2 (Massi et al., 1998; Klein et al., 2000; Newton et al., 2004; Mackie, 2006).

Both in adult and adolescent animals in fact, at the end of 10 days treatment, we measured low levels of IFN-γ and high levels of IL-4. The ratio between these two cytokines is generally considered an affordable index of the Th1/Th2 status.

Moreover also a relevant overexpression of IL-10 production by splenocytes is observed, demonstrating that this potent anti-inflammatory cytokine is similarly modulated by Δ^9-THC in macrophages stimulated with LPS and splenocytes restimulated in vitro with the antigen KLH, independently on the stimulus utilized to induce its production.

We supported the hypothesis that also in the adolescent period Δ^9-THC directs the cytokine network away from cell-mediated immunity, provoking a shift towards Th2. This would be interesting, considering that it might reduce the host's resistance to certain pathogens such as viruses, intracellular bacteria and parasites (Massi et al., 2006).

In the whole the Δ^9-THC exposure during adolescent age, the drug can affect both innate and acquired immunity, with higher risk of morbidity for infectious disease.

This aspect is particularly important considering that adolescents often are exposed to dangerous habits that can increase the risk of infections such as unprotected sexual intercourses or attending events in very often crowded environments (Wang et al., 2012; Matkins, 2013).

However, the most interesting and surprising results of our studies are the demonstration that marijuana abuse during adolescence may have long-term impact and affect the immune responses into adulthood, with lifelong consequences.

In the same direction, a recent study conducted by Lombard and colleagues (2011) demonstrated that perinatal exposure to Δ^9-THC in mice, altered the immune function not only in the fetus, but also during postnatal life.

In this study, we showed that 47 days after the last Δ^9-THC injection in adolescent mice, a profound dysregulation of macrophage and splenocytes cytokine production is still present.

Indeed, the alterations that are present in adult animals treated as adolescent go in a different direction in comparison to what we and others observed immediately at the end of treatment.

While in adult and adolescent animals at the end of treatment an anti-inflammatory cytokine profile is present, in adult mice treated as adolescent a clear pro-inflammatory macrophage phenotype is induced. Also these modifications take place at transcriptional levels, as indicated by the observed parallelism between protein and mRNA cytokine concentrations.

Moreover in the third group of study, we found that both Th1 and Th2 cytokines are now significantly decreased, indicating a general dysfunction, rather than a specific Th1/Th2 bias.

Although the data are not fully completed, the absence of alterations in macrophage and splenocytes cytokine production measured in animals that received Δ^9-THC as adults and evaluated 47 days after the end of treatment, would seem to suggest that the alteration observed in the third study group are related to fact that treatment takes place in the adolescent period. Several differences between adult and adolescent animals are of course present, that can account for the high sensibility of adolescent immune system to cannabis. For example it is demonstrated that the HPA endocrine axis is particularly active during this stage of development and particularly vulnerable to drugs or environmental stressors (van Leewen et al., 2011). Since it is well known that an altered pro-inflammatory/anti-inflammatory balance is involved in many immune related diseases, in consideration of the high levels of IL-1β and TNF-α and low IL-10 that we measured in adult mice treated as adolescents, it can be speculated that individuals that abused marijuana in young age might be at higher risk of developing autoimmune or chronic inflammatory diseases in adulthood.

As already reported, the main indication for therapeutic marijuana utilization is just for treating autoimmune and inflammatory diseases. Therefore the history of drug abuse of each individual should be evaluated before proposing medicinal cannabis use for pathology such as multiple

sclerosis or inflammatory bowel diseases (Klein, 2005; Nagarkatti et al., 2009; Pertwee, 2009).

Moreover the constantly low IFN-γ levels can modulate the ability to fight infectious diseases.

In our experiments we also measured the IgM anti-KLH titers after primary immunization only, i.e 10 days after KLH vaccination. As expected the levels of IgG anti KLH were always extremely low and never affected by Δ^9-THC treatment (data not shown).

A significant decrease of serum IgM levels were indeed observed in all treatment groups.

In adult and adolescent animals, that were injected with KLH in the first day of Δ^9-THC treatment, the low Th1 response can account for the decreased ability to mount an adequate IgM responses, thus confirming previous *in vitro* and *in vivo* studies (Aguledo et al., 2008; Springs et al., 2008).

The profound dysfunction in cytokine balance that persists also long time after the end of Δ^9-THC treatment may be at the basis of the altered antibody response that is present when animals are immunized without concomitant Δ^9-THC treatment.

However, we know that a detailed analysis of other Ig isotypes would have been of interest, considering for example the strict dependency between IgE and IL-4 (Takeda et al., 1996).

The objective of the Ig studies was to evaluate the impact of Δ^9-THC treatment on the ability to mount an antibody response rather than analyze in detail the isotype switching. From these results we can hypothesize that the ability of the host to mount an efficacious Ig response to vaccination is altered both during the Δ^9-THC intake as well as long time after its abuse.

The mechanisms at the basis of the peculiar long-term effects induced by Δ^9-THC treatment in the adolescent mice still need to be identified. Similar shaping of the immune responses after exposure to pollutants or other drugs have been described (Gascon et al., 2013).

Interestingly cannabinoid receptors are expressed also by hematopoietic precursors in murine embryonic stem cells and CB activation can affect also hematopoietic stem cells (Lu et al., 2006; Jiang et al., 2007).

Recently Hedge et al. (2010) has shown that cannabinoid receptor activation leads to massive mobilization of myeloid-derived suppressor cells with potent immunosuppressive properties.

It can be hypothesized that an alteration of hematopoietic cells by Δ^9-THC in adolescence would affect also in the long term the differentiation and maturation of immune cells.

In this study, we began to test the hypothesis that Δ^9-THC can affects hematopoietic stem cells; in this direction, we simply counted the number of colonies formed after plating the cells obtained from bone marrow of mice treated with Δ^9-THC or vehicle. The preliminary results obtained showed no significant differences in the number of colonies formed.

However, we are aware that further experiments, which differentiate the type of colonies, such as myeloid towards lymphoid, and further flow cytometric analysis on the various precursors will be needed before reaching a proper conclusion.

However, a possibility is that Δ^9-THC can induce epigenetic modifications on hematopoietic cells able to induce long-term changes on the differentiation and maturation of immune cells; further analysis, to identify possible epigenetic modifications induced by treatment with the drug, should be performed. Morris and Tomasiewicz (Morris et al., 2011; Tomasiewicz et al., 2012) have recently suggested, that Δ^9-THC abuse in the adolescent mice induces alteration of neuronal circuits and to alters brain plasticity, probably through epigenetic regulation of neurotransmitter genes.

The possibility of an epigenetic regulation induced by Δ^9-THC for cytokine genes is an exciting one. Interestingly an epigenetic regulation of IL-10 gene by another drug of abuse, i.e. morphine, has been recently reported (Schwarz et al., 2011).

A further possibility would be that the observed effects might not be mediated by a direct activation of CB receptors on cells of the immune system, but by a general dysregulation of neuroendocrine functions.

In this study, we reported as acute treatment induces an increase of corticosterone levels, while a more prolonged treatment, i.e. 10 days, leads to a significant reduction of corticosterone levels in all groups of treatments.

Once again this effect of Δ^9-THC permains for very long time after the last drug administration. The relationship between Δ^9-THC abuse and HPA axis have been extensively studied, often reaching contrasting conclusion (Roche et al., 2006).

Both stimulation and tonic inhibition of HPA have been associated to CB activation.

In particular, while acute Δ^9-THC administration has been linked to HPA stimulation (Schramm-Sapyta et al., 2007; Steiner and Wotjak, 2008), prolonged Δ^9-THC exposure seems to lead to a lowered HPA responses (Roche et al., 2006; Armario, 2010).

In agreement with our low corticosterone results, two recent studies in the human stated for hypo-activity of the HPA axis in adolescents with early onset of cannabis use leading to deficient cortisol reactivity to stressors (Huizink et al., 2006; van Leewen et al., 2011).

The modified endocrine milieu consequent to Δ^9-THC treatment could in part be responsible of the long-term immunomodulatory effects.

Indeed since corticosteroid levels are consistently reduced in all chronic experimental groups of studies while the cytokine profile is differently affected throughout the groups, it seems difficult to directly correlate Δ^9-THC effects on HPA and cytokines.

The fluctuations that we observe during the 10 days treatment (early increase followed by decrease) can account for the effects observed immediately at the end of Δ^9-THC treatment. However, considering the well known immunomodulating properties of steroids (Visser et al., 1998; Miyaura and Iwata, 2002), the persistence of low corticosterone levels until adulthood are compatible with the pro-inflammatory cytokine pattern that we observe in adult animals treated as adolescents. The steroid hormones might therefore be involved in the long-term effects induced by Δ^9-THC.

It is known that Δ^9-THC, due to its high lipid solubility, can remain in the body for a rather long time. In collaboration with the group of Prof. Raggi, University of Bologna, we measured the levels of Δ^9-THC and its main metabolites in the blood of animals; Δ^9-THC and its metabolites measurements have been performed in order to rule out the possibility that in adult animals treated with Δ^9-THC as adolescents some residual Δ^9-THC could still be present, in order to be so certain that the immune effects observed were due to a long lasting modulation of immune responses.

In human Δ^9-THC plasma levels decrease drastically being metabolised to THC-OH, by cytochrome P450, subtype 2C9 (CYP2C9) (Watanabe et al., 2007).

THC-OH is biologically active and is in turn metabolised by CYP2C9 (Hunault et al., 2008) to THC-COOH.

This latter metabolite is not psychoactive and has a very long half-life (Gustafson et al., 2004). The relative concentrations of these three compounds can be considered together as markers of the time elapsed after the last cannabis intake.

Consistently, also in mouse we measured high levels of Δ^9-THC and THC-COOH 24 hours after the last Δ^9-THC injection. Δ^9-THC had completely disappeared 30 days after the last administration, while lower traces of the long half life metabolite were still present.

THC-COOH does not bind the classical CB receptors and it is therefore devoid of any psychoactive properties (Gustafson et al., 2004). Although it is not known its potential ability to modulate immunity we can state that 17 days later, at the time when we evaluated all the immune response, neither Δ^9-THC nor its metabolite were present.

From our measurements we can also define that the intermediate metabolite THC-OH has an extremely short half-life in the mouse, as we never detected it.

An other important point that is always addressed when Δ^9-THC administration is performed in animal models, is how the dosage translates to human equivalent doses.

However several recent studies from the group of Nagarkatti et al., (2009), (Lombard et al., 2001; Pandey et al., 2011), have demonstrated that dosages as high as 50 mg/Kg in mouse reach Δ^9-THC blood concentrations in the range of pharmacological or recreational dose in humans. Moreover we now without doubt can affirm that the dose regimen that we utilized in the mouse leads to blood Δ^9-THC and THC-COOH concentrations similar to the ones that are achieved in blood from human chronic smokers.

Mercolini et al. (2013), in fact, assessed the presence of these cannabinoids in a cohort of marijuana smokers and found plasma levels ranging from 3 to 11 ng/ml for Δ^9-THC and from 7 to 20 ng/ml for THC-COOH, in line with the mean Δ^9-THC and THC-COOH concentrations that we now detected in the mouse.

It is now known that, in CNS, cells such as astrocytes and microglia produce pro- and anti-inflammatory cytokines that play a role in many neurodegenerative diseases (Carrier et al., 2005). The factors that control and modulate these cytokines are many, for example μ-opioid and noradrenergic receptors, and it has recently emerged as the endocannabinoid system also appears to be involved (Carlisle and Cabral, 2002).

In the present study, we have started to investigate the effects of Δ^9-THC chronic treatment also on the neuroinflammatory component of CNS in our adolescent and adult animals, analyzing the production of pro and anti-inflammatory cytokines in the hypothalamus and in the hippocampus. These areas have been chosen since they are particularly rich in CB receptors and primarily involved in many Δ^9-THC central effects (Irving et al., 2002).

Our data, although preliminary, indicate that chronic administration of Δ^9-THC is able to induce an anti-inflammatory status in brain of adolescent and adult mice assessed immediately at the end of treatment, both in the hypothalamus and in the hippocampus.

In adult animals, treated with the drug as adolescents, the production of pro-inflammatory cytokines, in hypothalamus and in hippocampus, appears to be increased with respect to

vehicles, indicating a predisposition to a pro-inflammatory status. It is very interesting to note how the modulation exerted by Δ^9-THC on the peripheral cytokines is in parallel to that exerted on the brain. It could be then hypothesized that the factors that regulate the modulation exerted by the drug on the peripheral cytokines, are also able to modulate the cytokine production in the brain; we are aware that further studies are needed to identify the modulatory factors involved.

Interestingly we measured basal levels of cytokines, since neither Lypopolisaccaride or other activating stimuli were given to the animals. Therefore the results that we have obtained suggest the presence of a tonic regulation of the cannabinoid system on brain cytokines.

In the present study we did not evaluate the relative involvement of CB1/CB2 receptors in the observed effects. Obviously Δ^9-THC targets both these receptors as an agonist and our main aim was to demonstrate that marijuana abuse during adolescence is endangering the immune system/cytokine pattern. Several studies from our and other groups have anyhow demonstrated that both receptors are likely to be involved in Δ^9-THC induced immunomodulation (Sacerdote et al., 2000a; Klein et al., 2000; Lombard et al., 2011; Springs et al., 2008).

Moreover a third cannabinoid receptor GPR55 has been reported and may account for some of the immune effects observed in the relative absence of CB1 or CB2 (Newton and Klein, 2012; Ryberg et al., 2007). Further studies in this direction will be conducted by us in the adolescent mouse model. Moreover, the 10 days Δ^9-THC treatment that we performed could also modulate the receptor sensibility, leading to either down regulation or desensitization, as previously observed (Massi et al. 2006; Buchweitz et al., 2007).

Also this aspect will be taken into consideration in a future project.

In conclusion the present study demonstrates for the first time that Δ^9-THC exposure in adolescent can trigger immune alterations that persist into adulthood, time after the last Δ^9-THC administration.

These long term modifications are different from the one observed immediately after the end of Δ^9-THC intake, suggesting the presence of a proinflammatory cytokine pattern and a general blunted Th responses.

The altered immune system may condition the susceptibility of the host to immune related pathologies in adulthood and our observations draw the attention on the fact the history of drug abuse of each individual should be evaluated before proposing any immunomodulating therapy.

REFERENCES

REFERENCES

Abbas AK, Lichtman AH. (2013) Fondamenti di immunologia, funzioni e alterazioni del sistema immunitario. Piccin; chapters: 1; 2; 3; 5; 8

Abrams RM, Davis KH, Jaeger ML, Szeto HH. (1985) Marijuana smoke production and delivery system, in Marihuna '84 Proceedings of the Oxford Symposium on Cannabis (Harvey DJ, Paton SW, Nahas GG, eds.), IRL Press Limited, Oxford: 205-209

Adams R. (1940) Marihuana. Science 92: 115-119

Agrawal A, Neale MC, Prescott CA, Kendler KS. (2004) A twin study of early cannabis use and subsequent and abuse/dependence of other illicit drugs. Psychol Med 34: 1227-1237

Agudelo M, Newton C, Widen R, Sherwood T, Nong L, Friedman H, Klein TW. (2008) Cannabinoid receptor 2 (CB2) mediates immunoglobulin class switching from IgM to IgE in cultures of murine-purified B lymphocytes. J Neuroimm Pharmacol 3: 35-42

Agurell S, Halldin M, Lindgren JE, Ohlsson A, Widman M, Gillespie H, Hollister L. (1986) Pharmacokinetics and metabolism of delta1-tetrahydrocannabinol and other cannabinoids with emphasis on man. Pharmacol Rev 38: 21-43

Aldrich M. (1997) History of therapeutic cannabis, in Cannabis in medical practice, ed. Mathre ML: 35-55

Arevalo-Martin A, Vela JM, Molina-Holgado E, Borrell J, Guazza C. (2003) Therapeutic action of cannabinoids in a murine model of multiple sclerosis. J Neurosci 23: 2511-2516

Armario A. (2010) Activation of the hypothalamic-pituitary-adrenal axis by addictive drugs: different pathways, common outcome. Trend in Pharmacol Sci 31: 318-325

Arseneault L, Cannon M, Poulton R, Murray R, Caspi A, Moffitt TE. (2002) Cannabis use in adolescence and risk for adult psychosis: longitudinal prospective study. BMJ 325: 1212-1213

Azorlosa JL, Heishman SJ, Stitzer ML, Mahaffey JM. (1992) Marijuana smoking: effect of varying delta-9-tetrahydrocannabinol content and number of puff. J Pharmacol Exp Ther 261: 114-122

Benson MK, Bentley AM. (1995) Lung disease induced by drug addiction. Thorax 50: 1125-1127

Berdyshev E, Boichot E, Corbel M, Germain N, and Lagente V (1998) Effects of cannabinoid receptor ligands on LPS-induced pulmonary inflammation in mice. Life Sci 63: N Engl J Med 336: 1186-1187

Berdyshev EV. (2000) Cannabinoid receptors and the regulation of immune response. Chem Phys Lipids 108: 169–190

Blanchard DK, Newton C, Klein TW, Stewart WE, Friedman H. (1986) In vitro and in vivo suppressive effects of delta-9-tetrahydrocannabinol on interferon production by murine spleen cells. Int J Immunopharmacol 8: 819-824

Bloch E. (1983) Effects of marijuana and cannabinoids on reproduction, endocrine function, development and chromosomes, in Cannabis andHealth Hazards (Fehr KO and Kalant H, eds.), Addiction Research Foundantion, Toronto

Borish LC, Steinke JW. (2003) Cytokine and chemokine. J Allergy Clin Immunol 111: 464-474

Bosier B, Muccioli GG, Hermans E, Lambert DM. (2010) Functionally selective cannabinoid receptor signalling: Therapeutic implications and opportunities. BC Pharmacol 80: 1-12

Brown SM, Wager-Miller J, Mackie K. (2002) Cloning and molecular characterization of the rat CB2 cannabinoid recptor. Biochim Biophys Acta 1576: 255-264

Brown T, Dobs AS. (2002) Endocrine effects of marijuana. J Clin Pharmacol 42: 90-96

Buchweitz JP, Karmaus PW, Harkema JR, Williams KJ, Kaminski NE. (2007) Modulation of airway responses to influenza A/PR/8/34 by Delta9-tetrahydrocannabinol in C57BL/6 mice. J Pharmacol Exp Ther 323: 657-683

Carlisle SJ, Cabral GA. (2002) Differential expression of the CB2 cannabinoid receptor by rodent macrophages and macrophage-like cells in relation to cell activation. International Immunopharmacol 2: 69-82

Carracedo A, Lorente M, Egia A, Blazquez C, Garcia S, Giroux V, Malicet C, Villuendas R, Gironella M, Gonzalez-Feria L, Piris MA, Iovanna JL, Guzman M, Velasco G. (2006) The stress-regulated protein p8 mediates cannabinoids-induces apoptosis of tumor cells. Cancer Cell 9: 301-312

Carrier EJ, Patel S, Hillard CJ. (2005) Endocannabinoids in neuroimmunology and stress. Curr Drug Targets CNS Neurol Disord 4: 657-665

Casadio P, Fernandes C, Murray RM, Di Forti M. (2011) Cannabis use in young people: the risk for schizophrenia. Neurosci Biobehav Rev 35:1779–1787

Caspi A, Moffitt TE, Cannon M, McClay J, Murray R, Harrington H, Taylor A, Arseneault L, Williams B, Braithwaite A, Poulton R, Craig IW. (2005) Moderation of the effect of adolescent-onset cannabis use on adult psychosis by a functional polymorphism in the catechol-O-methyltransferase gene: longitudinal evidence of a gene X environment interaction. Biol Psychiatry 57: 1117–1127

Cha YM, White AM, Kuhn CM, Wilson WA, Swartzwelder HS. (2006) Differential effects of THC on learning in adolescent and adult rats. Pharmacol Biochem Behav 83: 448-455

Chadwick B, Miller ML, Hurd YL. (2013) Cannabis use during adolescent development: susceptibility of psychiatric illness. Front Psychiatry 14: 129

Chang YH, Lee ST, Linn WW. (2001) Effects of cannabinoids on LPS-stimulated inflammatory mediator release from macrofages: involvement of eicosanoids. J Cell Biochem 81: 715-723

Costas J, Sanjuán J, Ramos-Ríos R, Paz E, Agra S, Tolosa A, Paramo M, Brenila J, Arrojo M. (2011) Interaction between COMT haplotypes and cannabis in schizophrenia: a case-only study in two samples from Spain. Schizophr Res 127: 22–27

Croxford JL and Yamamura T. (2005) Cannabinoids and the immune system: potentialfor the treatment of inflammation diseases? J Neuroimmunol 166: 3-18

Del Prete G, De Carli M, Almerigogna F, Giudizi MG, Biagiotti R, Romagnani S. (1993) Human IL-10 is produced by both type 1 helper (Th1) and type 2 helper (Th2) T cell clones and inhibits their antigen-specific pro- liferation and cytokine production. J Immuno l50: 353-360

Devane W, Hanus L, Breuer A, Pertwee R, Stevenson L, Griffin G. (1992) Isolation and structure of a brain constituent that binds to the cannabinoid receptor. Science 258: 1946-1949

Di Marzo V. (2008) Endocannabinoids: synthesis and degradation. Rev Physiol Biochem Pharmacol 160: 1-24

Dinarello CA, Wolff SM. (1993) The role of interleukin-1 in disease. N Engl J Med 328: 106-113

Ehrenreich H, Rinn T, Kunert HJ, Moeller MR, Poser W, Schilling L, Gigerenzer G, Hoehe MR. (1999) Specific attentional dysfunction in adults following early start of cannabis use. Psychopharmacol 142: 295-301

Ellgren M, Spano SM, Hurd YL. (2007) Adolescent cannabis exposure alters opiate intake and opioid limbic neuronal populations in adult rats. Neuropsychopharmacol 32: 607-615

Elphick MR. (2002) Evolution of cannabinoid receptors in vertebrates: identification of a CB(2) gene in the puffer fish Fugu rubripes. Biol Bull 202: 104-107

ElSohly M, Slade D. (2005) Chemical constituents of marijuana: The complex mixture of natural cannabinoids. Life Sciences 78: 539

Fankhauser M. (2002) Chapter 4: history of cannabis in Western medicine. In: Grotenhermen, F., Russo, R. (Eds.), Cannabis and Cannabinoids: Phar- macology, Toxicology and Therapeutic Potential. The Haworth Integrative Healing Press, New York pp. 37–51

Farrar MA, Schreiber RD. (1993) The molecular cell biology of interferon-γ and its receptor. Ann Rev Immunol 11: 571-611

Fergusson DM, Horwood LJ, Beautrais AL. (2003) Cannabis and educational achievement. Addiction 98: 1681-1692

Ferrari D, Pizzirani C, Adinolfi E, Lemoli RM, Curti A, Idzko M, Panther E, Di Virgilio F. (2007) The P2X7 receptor: a key player in IL-1 processing and release. J Immunol 179: 8569

Galiègue S, Mary S, Marchand J, Dussossoy D, Carrière D, Carayon P, Bouaboula M, Shire D, Le Fur G, Casellas P. (1995) Expression of central and peripheral cannabinoid receptors in human immune tissues and leukocytes subpopulations. Eur J Biochem 232: 54-61

Gaoni Y. (1964) Isolation, structure, and partial synthesis of an active constituent of hashish. J American Chem Soc pp. 1646

Gascon M, Morales E, Sunyer J, Vrijheid M. (2013) Effects of persistent organic pollutants on the developing respiratory and immune systems: a systematic review. Environ Int 52: 51-65

Gustafson RA, Kim I, Stout PR, Klette KL, George MP, Moolchan ET, Levine B, Huestis MA. (2004) Urinary pharmacokinetics of 11-nor-9-carboxy-delta9-tetrahydrocannabinol after controlled oral delta9-tetrahydrocannabinol administration. J Anal Toxicol 28: 160-167

Hall WD. (2006) Cannabis use and the mental health of young people. Aust NZ J Psychiatry 40: 105–113

Hannon J, Hoyer D. (2008) Molecular biology of 5-HT receptors. Behav Brain Res 195: 198-213

Hanus LO. (2009) Pharmacological and therapeutic secrets of plant and brain (endo)cannabinoids. Med Res Rev 29: 213-271

Harclerode J. (1984) Endocrine effects of marijuana in the male: preclinical studies. NIDA Res Monogr 44: 46-64

Hegde VL, Nagarkatti M, Nagarkatti PS. (2010) Cannabinoid receptor activation leads to massive mobilization of myeloid-derived suppressor cells with potent immunosuppressive properties. Eur J Immunol 40: 3358-3371

Henquet C, Rosa A, Krabbendam L, Papiol S, Fananas L, Drukker M, Ramaekers JG, van OS J. (2006) An experimental study of catechol-o-methyltransferase Val158Met moderation of D9-THC-induced effects on psychosis and cognition. Neuropsychopharmacology 31: 2748–2757

Herkenham M, Lynn A, Little M, Johnson M, Melvin L, Decosta B. (1990) Cannabinoid receptor localization in brain. Proceedings of the NAS of USA 87: 1932- 1936

Heyman SE. (2009) The HPA axis is under the excitatory control of the amygdala and inhibitory control of the hyppocampus. Nature Neuroscience 12: 241-243

Hillig K. (2005) Genetic evidence for speciation in Cannabis (Cannabaceae). Genetic Resources and Crop Evolution 52: 161

Howlett AC, Barth F, Bonner TI, Cabral G, Casellas P, Devane WA, Felder CC, Herkenham M, Mackie K, Martin BR, Mechoulam R, Pertwee RG. (2002) International Union of Pharmacology XXVII. Classification of cannabinoid receptors. PharmacolRev 54: 161-202

Howlett AC, Mukhopadhyay S. (2000) Cellular signal transduction by anandamide and 2-arachidonoylglycerol. Chem Phys Lipids 108: 53-70

Huizink AC, Ferdnand RF, Ormel J, Verhulst FC. (2006) Hypothalamic–pituitary–adrenal axis activity and early onset of cannabis use. Addiction 101: 1581–1588

Hunault CC, Mensinga TT, de Vries I, Kelholt-Dijkman HH, Hoek J, Kruidenier M, leenders ME, Meulenbelt J. (2008) Delta-9-tetrahydrocannabinol (THC) serum concentrations and pharmacological effects in males after smoking a combination of tobacco and cannabis containing up to 69 mg THC. Psychopharmacology 201: 171-181

Irving AJ, Rae MG, Coutts AA. (2002) Cannabinoids on the brain. Scientific World J 2: 632-648

Jiang S, Fu Y, Williams J, Wood J, Pandarinathan L, Avraham S, Makriyannis A, Avraham S, Avraham HK. (2007) Expression and function of cannabinoid receptors CB1 and CB2 and their cognate cannabinoid ligands in murine embryonic stem cells. PLoS One 2: e641

Kalant H. (2001) Medicinal use of cannabis: history and current status. Pain Res Manag 6: 80-91

Kandel D. (1975) Stages in adolescent involvement in drug use. Science 190: 912-914

Kaushik KS, Kapila K, Praharaj AK. (2011) Shooting up: the interface of microbial infections and drug abuse. J Med Microbiol 60: 408-422

Kelly P, Jones RT. (1992) Metabolism of tetrahydrocannabinol in frequent and infrequent marijuana users. J Anal Toxicol 16: 228–235

Kertscher P, Cohen B. (2001) An overview of the immune system. Lancet 357: 1777-1789

Khalsa JH. (2007) Medical and health consequences of marijuana. Marijuana and Cannabinoids 10: 237-252

Klein TW, Cabral GA. (2006) Cannabinoid-induced immune suppression and modulation of antigen-presenting cells. J Neuroimmune Pharmacol 1: 50-64

Klein TW, Friedman H. (1990) In: Watson R. Ed. Drugs of Abuse and Immune Function. Modulation of murine immune cells functions by marijuana components. Boca Raton. CRC Press. 87-111

Klein TW, Kawakami Y, Newton C, Friedman H. (1991) Maarijuana component suppress induction and cytolitytic function of murine cytotoxic T cells *in vitro* and *in vivo*. J Toxicol Environ Health 32: 465-477

Klein TW, Newton C, Friedman H. (1998) Cannabinoid receptors and immunity. Immunol Today 19: 373-381

Klein TW, Newton CA, Nakachi N, Friedman H. (2000) Δ^9-Tetrahydrocannabinol treatment suppresses immunity and early IFN-γ, IL-12, and IL-12 receptor 2 responses to *Legionella pneumophila* infection. J Immunol 164: 6461–6466

Klein TW. (1999) Cannabis and Immunity. In: The health effect of cannabis. Center for addiction and mental health, Kalant H. Corrigal W. Eds. Canada pp. 349-373

Klein TW. (2005) Cannabinoid-based drugs as anti-inflammatory therapeutics. Nat Rev Immunol 5: 400–411

Lachman HM, Morrow B, Shprintzen R, Veit S, Parsia SS, Faedda G, Goldberg R, Kucherlapati R, Papolos DF. (1996) Association of codon 108/158 catechol-O-methyltransferase gene polymorphism with the psychiatric manifestations of velo-cardio-facial syndrome. Am J Med Genet 67: 468–472

Law B, Mason PA, Moffat AC, Gleadle RI, King LJ. (1984) Forensic aspects of the metabolism and excretion of cannabinoids following oral ingestion of cannabis resin. J Pharm Pharmacol 36: 289–294

Lombard C, Hegde VL, Nagarkatti M, Nagarkatti PS. (2011) Pernatal exposure to Δ(9)-tetrahydrocannabinol triggers profound defects in T-cell differentiation and function in fetal and postnatal stages of life, including decreased responsiveness to HIV Antigens. J Pharmacol Exp Therap 339: 607-617

Lu T, Newton C, Perkins I, Friedman H, Klein TW. (2006) Cannabinoid treatment suppresses the T-helper cell-polarizing function of mouse dendritic cells stimulated with *Legionella pneumophila* infection. J Pharmacol Exp Ther 319: 269-276

Mackie K. (2006) Cannabinoid receptors as therapeutic targets. Annu Rev Pharmacol Toxicol 46: 101–122

Mackie K. (2008) Cannabinoid receptors : where they are and what they do. J Neuroendocrinol 20: 10-14

MacPherson G, Austyn J. (2012) Exploring Immunology: Concepts and Evidence, First Edition.Wiley-VCH Verlag GmbH & Co. KGaA pp 5-47

Martin-Calderon JL, Munoz RM, Villanua MA, Arco I, Moreno JL, Rodriguez de Fonseca F, Navarro M.(1998) Characterization of the acute en- docrine actions of (–)-11-hydroxy-Delta-8-tetrahydrocannabinol- dimethylheptyl (HU-210), a potent synthetic cannabinoid in rats. Euro J Pharmacol 344: 77-86

Martucci C, Franchi S, Lattuada D, Panerai AE, Sacerdote P. (2007) Differential involvement of RelB in morphine-induced modulation of chemotaxis, NO, and cytokine production in murine macrophages and lymphocytes. J Leukoc Biol 81: 344-354

Massi P, Sacerdote P, Ponti W, Fuzio D, Manfredi B, Viganò D, Rubino T, Bardotti M, Parolaro D. (1998) Immune function alterations in mice tolerant to Delta9-tetrahydrocannabinol: functional and biochemical parameters. J Neuroimmunol 92: 60-66

Massi P, Vaccani A, Parolaro D. (2006) Cannabinoids, immune system and cytokine network. Curr Pharm Des 12: 3135-3146

Matias I, Pochard P, Orlando P, Salzet M, Pestel J, Di Marzo V. (2002) Presence and regulation of the endocannabinoid system in human dendritic cells. Eur J Biochem 269: 3771-3778

Matkins PP. (2013) Sexually transmitted infections in adolescents. N C Med J 74: 48-52

Maurer HH, Sauer C, Theobald DS. (2006)Toxicokinetics of drugs of abuse: current knowledge of the isoenzymes involved in the human metabolism of tetrahydrocannabinol, cocaine, heroin, morphine, and codeine. Ther Drug Monit 28: 447-453

McCoy KL, Gainey D, Cabral GA. (1995) delta9-tetrahydrocannabinol modulates antigen processing by macrophages. J Pharmacol Exp Ther 237: 1216-1223

McCoy KL, Matveyeva M, Carlisle SJ, Cabral GA. (1999) Cannabinoid inhibition of the processing of intact lysozyme by macrophages: evidence for CB2 receptor participation. J Pharmacol Exp Ther 289: 1620-1625

McKallip RJ, Lombard C, Martin BR, Nagarkatti M, Nagarkatti PS. (2002) Delta(9)THC-induced apoptosis in the thymus and spleen as a mechanism of immunosuppression in vitro and in vivo. J Pharmacol Exp Ther 302: 451-465

Mechoulam R, Benshabat S, Hanus L, Ligumsky M, Kaminski N, Schatz A. (1995) Identification of an endogenous 2-monoglyceride, present in canine gut, that binds to cannabinoid receptors. Biochemical pharmacol 50: 83

Mechoulam R, Shvo Y. (1963) Hashish: The structure of Cannabidiol, Tetrahedron 19: 2073-2078

Mercolini L, Mandrioli R, Sorella V, Somaini L, Giocondi D, Serpelloni G, Raggi MA. (2013) Dried blood spots: liquid chromatography-mass spectrometry analysis of Δ9-tetrahydrocannabinol and its main metabolites. J Chromatogr A 1271: 33-40

Miyaura H, Iwata M. (2002) Direct and indirect inhibition of Th1development by progesterone and glucocorticoids. J Immunol 168: 1087-1094

Morris CV, Di Nieri JA, Szutorisz H, Hurd YL. (2011) Molecular mechanisms of maternal cannabis and cigarette use on human neurodevelopment. Eur J Neurosci 34: 1574-1583

Munro S, Kerrie LT, Abu-Shaar M. (1993) Molecular characterization of a peripheral receptor for cannabinoids. Nature 365: 61-65

Musshoff F and Madea B. (2006) Review of biologic matrices (urine, blood, hair) as indicators of recent or ongoing cannabis use. Ther Drug Monit 28: 155-163

Nadulski T, Pragst F, Weinberg G, Roser P, Schnelle M, Fronk EM, Stadelmann AM. (2005) Randomized, double-blind, placebo- controlled study about the effects of cannabidiol (CBD) on the pharmacokinetics of Delta9-tetrahydrocannabinol (THC) after oral application of THC verses standardized cannabis extract. Ther Drug Monit 27: 799-810

Nagarkatti P, Pandey R, Rieder SA, Hegde VL, Nagarkatti M. (2009) Cannabinoids as novel anti-inflammatory drugs. Future Med Chem 1: 1333-1349

Nakamo Y, Pross SH, Friedman H. (1992) Modulation of IL-2 activity by Δ^9-tetrahydrocannabinol after stimulation with concanavalin A, phytoemagglutinin, or anti-CD3 antibody. Proc Soc E xp Biol Med 201: 165-168

Newton CA, Klein TW. (2012) Cannabinoid 2 (CB2) receptor involvement in the down-regulation but not up-regulation of serum IgE levels in immunized mice. J Neuroimmune Pharmacol 7: 591-598

Newton CA, Lu T, Nazian SJ, Perkins I, Friedman H, Klein TW. (2004) The THC-induced suppression of Th1 polarization in response to Legionella pneumophila infection is not mediated by increases in corticosterone and PGE2. J Leukoc Biol 76: 854-861

O'Shea M, Singh ME, McGregor IS, Mallet PE. (2004) Chronic cannabinoid exposure produces lasting memory impairment and increased anxiety in adolescent but not adult rats. J Psychopharmacol 18: 502-508

O'Sullivan SE, Kendall DA, Randall MD. (2006) Further characterization of the time-dependent vascular effects of delta9-tetrahydrocannabinol. J Pharmacol Exp Ther 317: 428-438

O'Leary DS, Block RI, Koeppel JA, Flaum M, Schultz SK, Andreasen NC, Ponto LB, Watkins GL, Hurting RR, Hichwa RD. (2002) Effects of smoking marijuana on brain perfusion and cognition. Neuropsychopharmacol 26: 802-816

Odell WD. (1990) Sexual maturation in the rat. In: Grumbach MM , Sizonenko PC, Aubert ML, eds. Control of the Onset of Puberty.Baltimore: Williams &Wilkins 183-210

Ofek O, Karsak M, Leclerc N, Fogel M, Frenkel B, Wright K, Tam J, Attar-Namdar M, Kram V, Shohami E, Mechoulam R, Zimmer A, Bab I. (2006) Peripheral cannabinoid receptor, CB2, regulates bone mass. Proc Natl Acad Sci 103: 696-701

Oz M. (2006) Receptor-indipendent effects of endocannabinoids on ion channels. Curr Pharm Des 12: 227-239

Page ME, Oropeza VC, Sparks SE, Qian Y, Menko AS, Van Bockstaele EJ. (2007) Repeated cannabinoid administration increases indices of noradrenergic activity in rats. Pharmacol Biochem Behav 86: 162–168

Pandey R, Hegde VL, Nagarkatti M, Nagarkatti PS. (2011) Targeting cannabinoid receptors as a novel approach in the treatment of graft-versus-host disease: evidence from an experimental murine model. J Pharmacol Exp Ther 338: 819-828

Parkin J, Cohen B. (2001) An overview of the immune system. Lancet 357: 1777-1789

Pate DW. (1994) Chemical ecology of Cannabis. JIHA 2: 32-37

Perez-Reyes M. (1999) The psychologic and physiologic effects of active cannabinoids. Marihuana and Medicine 245-252

Pertwee RG. (2009) Emerging strategies for exploiting cannabinoid receptor agonists as medicines. Br J Pharmacol 156: 397-411

Pertwee RG. (2010) Receptors and channels targeted by synthetic cannabinoid receptor agonists and antagonists. Curr Med Chem. 17: 1360-81

Pope HG Jr, Gruber AJ, Hudson JI, Cohane G, Huestis MA, Yurgelun-Todd D.Pope HG Jr, Gruber AJ, Hudson JI, Cohane G, Huestis MA, Yurgelun-Todd D. (2003) Early-onset cannabis use and cognitive deficits: what is the nature of the association? Drug Alcohol Depend 69: 303-310

Realini N, Rubino T, Parolaro D. (2009) Neurobiological alterations at adult age triggered by adolescent exposure to cannabinoids. Pharmacol Res 60: 132-138

Roche M, Diamond M, Kelly JP, Finn DP. (2006) In vivo modulation of LPS-induced alterations in brain and peripheral cytokines and HPA axis activity by cannabinoids. J Neuroimmunol 181: 57-67

Rodriguez deFonseca F, Murphy LL, Bonnin A, Eldridge JC, Bartke A, Fernandez-Ruiz JJ. (1992) Delta-9-THC administration affects anterior pituitary, corticoadrenal and adrenomedullary function in male rats. Neuroendocrinology 22: 147-156

Rodriguez-Martin I, de Velasco EM, Rodriguez RE.(2007) Characterization of cannabinoid-binding sites in zebrafish. Neurosci Lett 413: 249-254

Romagnani S, Del Prete G, Manetti R, Ravina A, Annunziato F, DeCarli M, Mazzetti M, Piccinni MP, D'Elios MM, Parronchi P, Sampognaro S, Maggi E. (1994) Role of Th1/Th2 cytokines in HIV infection. Immunological Rev 140:73-92

Romagnani S. (1990) Regulation and deregulation of human IgE synthesis. Immunol Today 11: 316-321

Roth MD, Baldwin GC, Tashkin DP. (2002) Effects of Δ9-tetrahydrocannabinol on human immune function and host defense. Chem Phys Lipids 121: 229-239

Rubino T, Parolaro D. (2013) Cannabis abuse in adolescence and the risk of psychosis: A brief review of the preclinical evidence. PNP & BP J pii: S0278-5846(13)00162-0 [Epub ahead of print]

Rubino T, Vigano' D, Realini N, Guidali C, Braida D, Capurro V, Castiglioni C, Cherubino F, Romualdi P, Candeletti S, Sala M, Parolaro D. (2008) Chronic Δ9-tetrahydrocannabinol during adolescence provokes sex-dependent changes in the emotional profile in adult rats: behavioral and biochemical correlates. Neuropsychopharmacology 33: 2760-2771

Rubino T, Zamberletti E, Parolaro D. (2012) Adolescent exposure to cannabis as a risk factor for psychiatric disorders. J Psychopharmacol 26: 177-188

Russo E. (2007) History of cannabis and its preparations in saga, science, and sobriquet. Chemistry biod 4: 1614

Ryberg E, Larsson N, Sjogreen S, Hjorth S, Hermansson NO, Leonova J, Elebring T, Nilsson K, Drmota T, Greasley PJ. (2007) The orphan receptor GPR55 is a novel cannabinoid receptor. B J Pharmacol 152: 1092-1101

Sacerdote P, Manfredi B, Gaspani L, Panerai AE. (2000b) The opioid antagonist naloxone induces a shift from type 2 to type 1 cytokine pattern in BALB/cJ mice. Blood 95: 2031-2036

Sacerdote P, Massi P, Panerai AE, Parolaro D. (2000a) In vivo and in vitro treatment with the synthetic cannabinoid CP55, 940 decreases the in vitro migration of macrophages in the rat: involvement of both CB1 and CB2 receptors. J Neuroimmunol 109:155–163

SAMHSA (2004) Center for Substance Abuse Treatment. Substance Abuse Treatment and Family Therapy. Rockville (MD): Substance Abuse and Mental Health Services Administration (US); Treatment Improvement Protocol (TIP) Series, n. 39

Schneider M, Koch M. (2003) Chronic pubertal, but not adult chronic cannabinoid treatment impairs sensorimotor gating, recognition memory, and the performance in a progressive ratio task in adult rats. Neuropsychopharmacol 28: 1760-1769

Schramm-Sapyta NL, Cha YM, Chaudhry S, Wilson WA, Swartzwelder HS, Kuhn CM. (2007) Differential anxiogenic, aversive, and locomotor effects of THC in adolescent and adult rats. Psychopharmacol 191: 867-877

Schuel H, Burkman LJ, Lippes J, Crickard K, Mahony MC, Giuffrida A, Picone RP, Makriyannis A. (2002) Evidence that anandamide-signaling relulates human sperm functions required for fertilization. Mol Reprod Dev 63: 367-387

Schultes RE. (1976) Indole alkaloids in plant hallucinogens. Planta Med 29: 330-342

Schwarz JM, Hutchinson MR, Bilbo SD. (2011) Early-life experience decreases drug-induced reinstatement of morphine CPP in adulthood via microglial-specific epigenetic programming of anti-inflammatory IL-10 expression. J Neurosci 31: 17835-17847

Shire D, Calandra B, Rinaldi-Carmona M, Oustric D, Pessègue B, Bonnin-Cabanne O, Le Fur G, Caput D, Ferrara P. (1996) Molecular cloning, expression and function of the murine CB2 peripheral cannabinoid receptor. Biochim Biophys Acta 1307: 132-136

SM, Cheng Q, Zoumalan R, Tashkin DP, Roth MD. (2002) Delta 9-tetrahydrocannabinol regulates Th1/Th2 cytokine balance in activated human Tcells. J Neuroimmunol 133: 124-131

Small E. (1976) The forensic taxonomic debate on Cannabis: semantic hokum. J Forensic Sci 21:239-251

Spear LP, Brake SC. (1983) Periadolescence: age-dependent behaviour and psychopharmacological responsivity in rats. Developmental Psychobiology 16: 83-109

Spear LP. (2000) The adolescent brain and age-related behavioral manifestations. Neurosci Biobehav Rev 24: 417-463

Springs AE, Karmaus PW, Crawford RB, Kaplan BL, Kaminski NE. (2008) Effects of targeted deletion of cannabinoid receptors CB1 and CB2 on immune competence and sensitivity to immune modulation by Delta9-tetrahydrocannabinol. J Leukoc Biol 84: 1574-15784

Stefanis NC, Delespaul P, Henquet C, Bakoula C, Stefanis CN, Van Os J. (2004) Early adolescent cannabis exposure and positive and negative dimensions of psychosis. Addiction 99: 1333-1341

Steiner MA, Wotjak CT. (2008) Role of the endocannabinoid system in regulation of the hypothalamic-pituitary-adrenocortical axis J Neuroimmunol 181: 57-67

Takeda K, Tanaka T, Shi W, Matsumoto M, Minami M, Kashiwamura S, Nakanishi K, Yoshida N, Kishimoto T, Akira S. (1996) Essential role of Stat6 in IL-4 signalling. Nature 380: 627-630

Tashkin DP, Fligiel S, Wu TC, Gong H Jr, Barbers RG, Coulson AH, Simmons MS, Beals TF. (1990) Effects of habitual use of marijuana and/or cocaine on the lung. NIDA Res Monogr 99: 63-87

Tashkin DP, Roth MD, Dubinett SM. (1997) Medical marijuana? N Engl J Med 336: 1186

Thumma S, Majumdar S, ElSohly M, Gul W, Repka M. (2008) Preformulation Studies of a Prodrug of Delta(9)-Tetrahydrocannabinol. AAPS PharmSciTech 9: 982-990

Tomasiewicz HC, Jacobs MM, Wilkinson MB, Wilson SP, Nestler EJ, Hurd YL. (2012) Proenkephalin mediates the enduring effects of adolescent cannabis exposure associated with adult opiate vulnerability. Biol Psychiatry 72: 803-810

van Leewen AP, Creemers HE, Greaves-Lord K, Verhulst FC, Ormel J, Huizink AC. (2011) Hypothalamic-pituitary-adrenal axis reactivity to social stress and adolescent cannabis use: the TRAILS study. Addiction 106: 1484-1492

Van Sickle MD, Duncan M, Kingsley PJ, Mouihate A, Urbani P, Mackie K, Stella N, Makriyannis A, Piomelli D, Davison JS, Marnett LJ, Di Marzo V, Pittman QJ, Patel KD, Sharkey KA. (2005) Identification and functional characterization of brainstem cannabinoid CB2 receptors. Science 310: 329-332

Visser J, van Boxel-Dezaire A, Methorst D, Brunt T, de Kloet ER, Nagelkerken L. (1998) Differential regulation of interleukin-10 (IL-10) and IL-12 by glucocorticoids in vitro. Blood 91: 4255-4264

Wall ME, Sadler BM, Brine D, Taylor H, Perez-Reyes M. (1983) Metabolism, disposition, and kinetics of Δ9-tetrahydrocannabinol in men and women. Clin Pharmacol Ther 34: 352–363

Wang K, Gill P, Perera R, Thomson A, Mant D, Harnden A. (2012) Clinical symptoms and signs for the diagnosis of Mycoplasma pneumoniae in children and adolescents with community-acquired pneumonia. Cochrane Database Syst Rev 10: CD009175

Watanabe K, Matsunaga T, Yamamoto I, Funae Y, Yoshimura H. (1995) Involvement of CYP2C in the metabolism of cannabinoids by human hepatic microsomes from an old woman. Biol Pharm Bul 18: 1138-1141

Watanabe K, Yamaori S, Funahashi T, Kimura T, Yamamoto I. (2007) Cytochrome P450 enzymes involved in the metabolism of tetrahydrocannabinols and cannabinol by human hepatic microsomes. Life Sciences 80: 1415–1419

Watzl B, Scuder P, Watson RR. (1991) Marijuana components stimulate human peripheral blood mononuclear cell secretion of interferon-gamma and suppress interleukin-1 alpha *in vitro*. Int J Immunopharm 13: 1091-1097

Wegener N, Koch M. (2009) Behavioural disturbances and altered Fos protein expression in adult rats after chronic pubertal cannabinoid treatment. Brain Res 1253: 81–91

Woelkart K, Salo-Ahen OM, Bauer R. (2008) CB receptor ligands from plants. Curr Top Med Chem 8: 173-186

Yamaguchi F, Tokuda M, Hatase O, Brenner S. (1996) Molecular cloning of the novel human G protein-coupled receptor (GPCR) gene mapped on chromosome 9. Biochem Biophys Res Commun 227: 608-614

Zheng ZM, Specter SC. (1996) Delta-9-tetrahydrocannabinol suppresses tumor necrosis factor alpha maturation and secretion but not its transcription in mouse macrophages. Int J Immunopharmacol 18: 53-68

Zhu LX, Sharma S, Stolina M, Gardner B, Roth MD, Tashkin DP, Dubinett SM. (2000) Delta 9-tetrahydrocannabinol inhibits antitumor immunity by a CB2 receptor-mediated, cytokine-dependent pathway. J Immunol 165: 373-380

Zhu W, Newton C, Daaka Y, Friedman H, Klein TW. (1994) Delta9- tetrahydrocannabinol enhances the secretion of interleukin 1 from endotoxin –stimulated macrophages. J Pharmacol Exp Ther 270: 1334-1339

Zuardi AW. (2006) History of cannabis as a medicine: a review.Rev Bras Psiquiatr 28:153-15

LIST OF PUBLICATIONS

Δ^9-Tetrahydrocannabinol treatment of adolescent mice induces a long lasting modulation of macrophage and lymphocyte cytokine production.
<u>Moretti S</u>, Castelli M, Franchi S, Raggi MA, Mercolini L, Protti M, Somaini L, Panerai AE, Sacerdote P.
Journal of Leucocyte Biology (under revision).

Changes of Substance P in the Crevicular Fluid in relation to Orthodontic Movement Preliminary Investigation.
Levrini L, Sacerdote P, <u>Moretti S</u>, Panzi S, Caprioglio A.
ScientificWorldJournal 2013; Articole ID 896874, 6 pages

Effects of NSAIDs and paracetamol (acetaminophen) on protein kinase C epsilon translocation and on substance P synthesis and release in cultured sensory neurons.
Vellani V, Franchi S, Prandini M, <u>Moretti S</u>, Castelli M, Giacomoni C, Sacerdote P.
J Pain Res. 2013; 6:111-20

Cytokine modulation is necessary for efficacious treatment of experimental neuropathic pain.
Sacerdote P, Franchi S, <u>Moretti S</u>, Castelli M, Procacci P, Magnaghi V, Panerai AE.
J Neuroimmune Pharmacol. 2013; 8(1): 202-11

Acute and late changes in intraarticular cytokine levels following anterior cruciate ligament injury.
Bigoni M, Sacerdote P, Turati M, Franchi S, Gandolla M, Gaddi D, <u>Moretti S</u>, Munegato D, Augusti CA, Bresciani E, Omeljaniuk RJ, Locatelli V, Torsello A.
J Orthop Res. 2013; 31(2): 315-21

Mu opioid receptor activation modulates Toll like receptor 4 in murine macrophages.
Franchi S, <u>Moretti S</u>, Castelli M, Lattuada D, Scavullo C, Panerai AE, Sacerdote P.
Brain Behav Immun. 2012; 26(3): 480-8

Nimesulide inhibits protein kinase C epsilon and substance P in sensory neurons - comparison with paracetamol.
Vellani V, Franchi S, Prandini M, <u>Moretti S</u>, Pavesi G, Giacomoni C, Sacerdote P.
J Pain Res. 2011; 4:177-87

The effects of alcoholism pharmacotherapy on immune responses in alcohol-dependent patients.
Franchi S, Sacerdote P, <u>Moretti S</u>, Gerra G, Leccese V, Tallone MV, Panerai AE, Somaini L.
Int J Immunopathol Pharmacol. 2010; 23(3): 847-55

Sodium N-(methylsulfonyl)-N-(4-nitro-2-phenoxyphenyl) sulfamate: a water-soluble nimesulide prodrug for parenteral use.
Rapposelli S, Digiacomo M, Franchi S, <u>Moretti S</u>, Pinza M, Sacerdote P, Balsamo A.
Mol Pharm. 2010; 7(5): 1871-6

1. **Δ^9-tetrahydrocannabinol -induced anti-inflammatory responses in adolescent mice switch to pro-inflammatory in adulthood**
Moretti S, Castelli M, Franchi S, Raggi MA, Mercolini L , Protti M, Somaini L, Panerai AE, SacerdoteP
Convegno monotematico SIF, Neuroimmune Pharmacology: Challenging Paradigms Beyond Boundaries Integrating pharmacology, immunology, and neurosciences in a therapeutic perspective Center of Research in Medical Pharmacology, University of Insubria November 15th-16th, 2013

2. **Δ^9-tetrahydrocannabinol treatment of adolescent mice induces a long lasting modulation of macrophage and lymphocyte cytokine production**
Moretti S, Castelli M, Franchi S, Raggi MA, Mercolini L , Protti M, Somaini L, Panerai AE, SacerdoteP
36° Congresso Nazionale Della Società Italiana Di Farmacologia: Il Ruolo Della Ricerca Farmacologica Per La Crescita E La Salute In Italia, 23-26 ottobre 2013, Torino (comunicazione orale)

3. **Exposure of adolescent mice to THC shapes immune response in adulthood**
Moretti S,Franchi S,Castelli M,Panerai A.E,Sacerdote P.
First Joint Spanish-Italian Meeting On Cannabinoid Research; 13a Reunion Anual De La Sociedad EspañOla De Investigacion Sobre Cannabinoides, 29th November-1st December 2012, Madrid

4. **Exposure of adolescent mice to THC shapes immune response in adulthood**
Moretti S,Franchi S,Castelli M,Panerai A.E,Sacerdote P.
Seminario SIF per Dottorandi e Assegnisti di Ricerca 16-19 settembre 2012, Rimini

5. **Exposure of adolescent mice to THC shapes immune response in adulthood**
Moretti S,Franchi S,Castelli M,Panerai A.E,Sacerdote P.
Convegno monotematico: "I cannabinoidi: presente e futuro" 14-15 Settembre 2012, Ferrara (comunicazione orale)

6. **Exposure of adolescent mice to THC shapes immune response in adulthood**
Moretti S,Franchi S,Castelli M,Panerai A.E,Sacerdote P.
Next step 3- la giovane ricerca avanza, 26 giugno 2012, Milano

7. **Short and long-term effect of delta-9-tetrahydrocannabinol on immune function in adolescent mice**
Moretti S,Franchi S,Rossi A,Panerai A.E,Sacerdote P.
Convegno monotematico: " I cannabinoidi: dalla biologia alla clinica "29-30 settembre 2011, Cagliari (comunicazione orale)

8. **Short and long-term effect of delta-9-tetrahydrocannabinol on immune function in mice**
   Moretti S,Franchi S,Rossi A,Panerai A.E,Sacerdote P.
   35° Congresso Nazionale della Società Italiana di Farmacologia, 14-17 Settembre 2011, Bologna

9. **Opioid and alcohol modulation of immune system: the effects of treatment**
   Sacerdote P, Franchi S, **Moretti S,** Gerra G, Panerai Ae, Somaini L.
   12 th International Society of Addiction Medicine (ISAM) Meeting, October 3-7 2010, Milano

10. **Toll lke receptor 4: a role in morphine immunomodulatory effect**
    Franchi S, **Moretti S**, Scavullo C, Valsecchi Ae, Panerai Ae,Sacerdote P.
    17 th Annual Meeting of The Psychoneuroimmunology Research Society, June 2-5, 2010, Dublin