Chapter 7

Unheated *Cannabis sativa* extracts and its major compound THC-acid have potential immuno-modulating properties not mediated by CB$_1$ and CB$_2$ receptor coupled pathways.

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Unheated Cannabis sativa extracts and its major compound THC-acid have potential immuno-modulating properties not mediated by CB₁ and CB₂ receptor coupled pathways.

There is a great interest in the pharmacological properties of cannabinoid like compounds that are not linked to the adverse effects of Δ⁹-tetrahydrocannabinol (THC), e.g. psychoactive properties. The present paper describes the potential immuno-modulating activity of unheated Cannabis sativa extracts and its main non-psychoactive constituent Δ⁹-tetrahydrocanabinoid acid (THCa). By heating Cannabis extracts, THCa was shown to be converted into THC. Unheated Cannabis extract and THCa were able to inhibit the tumor necrosis factor alpha (TNF-α) release from U937 macrophages and peripheral blood macrophages after stimulation with LPS in a dose-dependent manner. The inhibition of TNF-α release was prolonged over a longer period of time, whereas THC and heated extracts induced TNF-α release at longer incubation times. Furthermore we demonstrated that THCa and THC show distinct effects on phosphatidylcholine specific phospholipase C (PC-PLC) activity. Unheated Cannabis extract and THCa inhibit the PC-PLC activity in a dose-dependent manner, while THC induced PC-PLC activity at high concentrations. These results suggest that THCa and THC exert their immuno-modulating effects via different metabolic pathways. In order to study the possible in vivo implications of these findings, a pilot study in an Experimental Autoimmune Encephalomyelitis (EAE) mouse model was performed. Unheated Cannabis extract and THCa had a favourable effect on the clinical and histological signs of EAE. These preliminary results are auspicious, but further extensive investigation is necessary.

Introduction

Cannabis sativa and its primary psychoactive constituent, Δ⁹-tetrahydrocannabinol (THC) have shown therapeutic benefits in the relief of nausea and vomiting associated with cancer and its treatments, stimulation of appetite in AIDS patients and patients with anorexia and wasting syndromes, analgesia, muscle relaxation⁴⁻⁶. These positive effects are partially linked to the presence of the CB₁ receptor, but are over shadowed by the psychotropic effects which have thus far also been attributed to the CB₁ receptor⁷⁻⁹. Besides the psychotropic effect, THC and Cannabis exert several additional adverse effects on health. For example chronic inflammatory and precancerous changes in the airways have been demonstrated in Cannabis
smokers. Overdosing Cannabis manifests itself in anxiety and panic attacks, increased heart rate, and changes in blood pressure $^{1,10}$.

Cannabinoids are known to bind to cannabinoid receptors CB$_1$ and CB$_2$ with different affinity $^{4,5}$. The CB$_1$ receptor is predominantly expressed in the brain as well as several tissues of the periphery, whereas the CB$_2$ receptor is primarily expressed on cells of the immune system $^{4,6}$. Both receptors have shown to be involved in immuno-modulating actions $^{7,11-13}$, but not all actions could be linked to these receptors, suggesting that non-cannabinoid receptors and metabolic pathways are involved $^{1,8,14}$. The psychotropic effect of CB$_1$ receptor agonists and the stigma of cannabinoids as a recreational and addicting drug are still major obstacles to legalize the drug in certain countries for therapeutic use $^1$. So there is great interest in cannabinoids and other compounds that have a reduced ability to activate CB$_1$ receptors but maintain the therapeutic effects of THC and lack the unwanted effect of these drugs.

In the Cannabis plant, cannabinoids are synthesized and accumulated as cannabinoid acids $^1,15,16$. The cannabinoid acids are devoid of psychotropic effects and have to be decarboxylated to phenols to produce psychotropic effects e.g. by smoking the dried plant matter. MS patients using Cannabis preparations other than by smoking or that contain low THC content claim positive health effects and fewer side effects. For this reason we investigated the relation between cannabinoid content of heated and unheated Cannabis extracts and their inhibitory effect on tumor necrosis factor alpha (TNF-$\alpha$) production after stimulation of U937 macrophages with endotoxin lipopolysaccharide (LPS). TNF-$\alpha$ is a well known pro-inflammatory cytokine which plays an important role in inflammatory responses. It has been shown that TNF-$\alpha$ production can be inhibited by various pathways. In the present study we focussed on the role of the CB receptors in this process and investigated the involvement of adenosine-3’5’-cyclic monophosphate (cAMP) and phosphatidylcholine specific phospholipase C (PC-PLC).

Finally we studied the effect of THCa and unheated Cannabis extract in an Experimental Autoimmune Encephalomyelitis (EAE) mouse model. Cannabinoids have shown to have a positive effect on inhibiting the development of clinical signs of MS in some animal models $^9,17,18$. EAE is a demyelinating disease of the central nervous system (CNS) that can be induced by immunization with various myelin-derived antigens, including myelin basic protein (MBP) and proteolipid protein (PLP) $^{19,20}$. Evidence has been obtained both in rats and mice showing that infiltration of macrophages and CD4$^+$ T cells in the CNS play an important role in the pathogenesis of the disease through the secretion of a variety of cytokines, in particular TNF-$\alpha$, TNF-$\beta$, and IFN-$\gamma$ $^{21,22}$. 

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**Materials and Methods**

**Chemicals**

Lipopolysaccharide (Escherichia coli O111:B4) and D609 were obtained from Sigma-Aldrich (St. Louis, MO, USA). The pure cannabinoid compounds: Δ⁹-tetrahydrocannabinol (THC) and Δ⁹-tetrahydrocanabinoid acid (THCa) were purified as described previously and provided by the University of Leiden (Leiden, The Netherlands). The CB₁ antagonist, AM281 and CB₂ antagonist AM630 were obtained from Tocris Cookson Ltd. (Bristol, UK).

**Cannabis extracts**

The *Cannabis sativa* plant materials were obtained from a local grower (The Netherlands). About 3.1 g of dried flower tops were ground in liquid nitrogen and extracted with 40 mL chloroform/methanol/water (50:25:25) for 30 minutes. The mixture was centrifuged for 20 minutes (2000 g) allowing the separation of the polar from the non-polar liquid phase. The non-polar chloroform-phase that contained the cannabinoids was isolated. For cell based studies, the chloroform phase was evaporated using a vacuum centrifuge and re-dissolved in an equal volume of DMSO. This extract will be referred to as the ‘unheated’ extract. For the heated extracts, the chloroform extracts were evaporated, heated for 7 minutes at 200 °C and re-dissolved in an equal volume of DMSO. The concentration of THC, THCa, CBD and CBDa in Cannabis extracts were analysed using LC-IT-MS²⁴. Metabolite profiles were obtained using a gas chromatographic instrument from Agilent (6890N) with a mass spectrometric detector (5973). The extracts were dried using sodium sulphate and subsequently derivatized with N-Methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA). The derivatized extracts (1 µl) were separated on a HP-5MS column (30 m * 0.25 mm * 0.25 µm, Agilent technologies, Amstelveen, The Netherlands).

**Cell cultures**

Human monocyte-like histiocytic lymphoma cells U937²⁵ obtained from the ATCC (CRL-1593.2) were grown in RPMI-1640 medium, supplemented with 10% (v/v) fetal calf serum and 2 mM L-glutamine (Life technologies, Breda, The Netherlands) at 37 °C, 5% CO₂ in a humidified atmosphere. U937 monocyctic cells (1x10⁶ cells per well) were differentiated into macrophages using phorbol 12-myristate 13-acetate (PMA, 10 ng/ml, overnight, Omnilabo, Breda, The Netherlands) as described previously²⁶. The PMA-differentiated macrophages were allowed to recover from PMA treatment for 48 h, during which the culture medium was replaced daily.
Peripheral blood monocytes (PB-MO) were isolated from human EDTA-blood with Rosette Sep™ human monocyte enrichment cocktail (Stemcell Technologies Inc, Meylan, France) as described previously. The monocytes (5x10⁵ cells per well) were cultured in 24 well cell culture plates containing RPMI-1640 medium supplemented with 10% (v/v) human serum and 2 mM L-glutamine and were allowed to differentiate into peripheral blood macrophages (PB-MØ) for eight days. Following this procedure, the macrophage maturation has been described to give rise to the characteristically morphology and phenotype of primary macrophages.

**Macrophage activation and TNF-α assay**

PB-MØ and U937 cells were exposed to LPS (1 µg/ml) in the presence or absence of THC and THCa (12.5, 25, 50, 100 and 200 nM) or Cannabis sativa plant extracts (250, 500, 1000, 2000 and 4000 fold diluted) for 4 h. For the time course experiments U937 cells (1x10⁶ cells per well) were incubated with LPS (1 µg/ml) in combination with 80 nM THC, THCa or 500 fold diluted heated Cannabis extract or unheated Cannabis extract. Culture medium was collected at 1, 2, 4, 6, 8, 16 and 24 h respectively. All incubations were performed in triplicate.

The concentration of TNF-α in the culture supernatants was determined by ELISA using the cytoset antibody pair kit for TNF-α from Biosource (Etten-Leur, The Netherlands) according to the manufacturer’s protocol. The cells were lysed in 0.1 M NaOH and used for protein determination by the modified method of Bradford (Bio-Rad, Veenendaal, The Netherlands).

**CB₁ and CB₂ antagonism**

U937 macrophages were incubated with LPS (1 µg/ml) together with 80 nM THC, THCa or 500 fold diluted unheated Cannabis extract in combination with a concentration series (1x10⁻⁸ - 1x10⁻⁴ M) of AM281, a CB₁ antagonist or AM630, a CB₂ antagonist. After 4 h the culture medium was collected and the cells lysed in 1 ml 0.1 M NaOH.

**Measurement of intracellular cAMP formation**

U937 macrophages were cultured in 24 well cell culture plates at a concentration of 1x10⁶ cells per well. Cells were incubated for 10 minutes at 37 °C with a concentration series of THC, THCa (12.5, 25, 50, 100, and 200 nM) or unheated Cannabis extract (250, 500, 1000, 2000 and 4000 fold diluted) in combination with LPS (1 µg/ml). Zilpaterol (Intervet Inc., Millsboro, US), a β₂-adrenergic receptor agonist at a concentration of 1x10⁻⁶ M was used as a
positive control for cAMP induction. The culture medium was aspirated and the cells were placed directly on ice. The cells were lysed in 0.25 ml 0.1 M HCl. After centrifugation, the samples were assayed directly using the cyclic AMP EIA kit from Cayman Chemical (Ann Arbor, USA). The incubations were performed in duplicate.

Determination of phospholipase C activity
The medium of U937 macrophages (1x10^6 cells per well) was changed into RPMI-1640 medium, without fetal calf serum, 24 h before incubation with THC, THCa (25, 50, 100, and 200 nM), or unheated Cannabis extract (250, 500, 1000, and 2000 fold diluted). D609 at a concentration of 200 µM was used as a positive control for inhibition of PC-PLC activity. After 15 minutes LPS (1 µg/ml) was added and the cells were incubated for a further 5 minutes at 37 °C. Supernatant was aspirated and the cells were washed with ice cold PBS. The cells were lysed in 0.2 ml reaction buffer and 100 µg of cell lysate was further analyzed using the Amplex® Red Phosphatidylcholine-Specific Phospholipase C assay Kit (Molecular Probes, Eugene, USA) according to the manufacturer’s protocol.

EAE in SJL mice
Female SJL mice (Harlan, Horst, The Netherlands) 9 weeks of age, were randomized and divided over three groups (n = 10) and immunized subcutaneously with 75 µg of synthetic peptide comprising amino acid 139 through 151 from proteolipid protein (PLP_139-151) dissolved in PBS and emulsified with an equal volume of complete Freund’s adjuvant containing 1 mg/ml *Mycobacterium tuberculosis* H37Ra (Difco, Detroit, MI). After one and three days, mice were injected i.v. with 10^{10} heat-inactivated *Bordetella pertussis* organisms (Institute of Public Health, Bilthoven, The Netherlands). Development of EAE was monitored daily by assessment of bodyweight and a disability score. The score ranges from 0: no signs, 0.5: partial loss of tail tonus, 1: complete loss of tail tonus, 2: limb weakness, 2.5: partial paresis, 3: complete paralysis from diaphragm to hind limbs, 3.5: complete paralysis from diaphragm to hind limbs, incontinence, 4: moribund, to 5: death due to EAE. The animals received daily an oral dose of the following compounds for 21 consecutive days from immunization until day 20: group 1: placebo (olive oil), group 2: THCa (50 mg/kg) in olive oil and group 3: unheated Cannabis extract (50 mg THCa/kg) in olive oil. After 42 days the animals were sacrificed. Brains and spinal cords were fixed in 10 % formalin and paraffin embedded. Six sections per animal, each comprising three different areas of the nervous system (cerebellum, brain stem) and three sections of spinal cord were analyzed for infiltrates
after staining with haematoxylin. The severity of the inflammatory reaction was indicated using the following histological score; 0: no infiltrates, 1: sporadic, mild perivascular infiltration (less than two inflammatory lesions per section), 2: multifocal, mild perivascular infiltration, 3: multifocal, severe perivascular infiltration, 4: multifocal, severe perivascular infiltration accompanied by spreading into the parenchyma. The study protocol was approved by the Ethical Committee for experiments on Animals of our Institute.

Results

Cannabis extracts

Two different plant cultivars; a cultivar with a high THCa content (Table 1 A; no CBDa) and one with a low THCa content (Table 1, B; CBDa : THCa 1:1) were extracted with chloroform/methanol/water. The chloroform phase containing the cannabinoid acids is referred to as the unheated Cannabis extract. The same extract was heated at 200 °C for 7 minutes and this extract is referred to as the heated extract.

Table 1 shows the concentrations of the main cannabinoid acids (THCa and CBDa) and cannabinoid compounds (THC and CBD) in the two plant extracts (unheated) and when heated as determined using LC-IT-MS.

Table 1 Concentration of the main cannabinoids; THC, THCa, CBD and CBDa in unheated and heated plant extracts (3.1 g per 40 ml), determined by LC-MS. The concentrations are given in µg/ml.

<table>
<thead>
<tr>
<th>Plant extract</th>
<th>Treatment</th>
<th>THC</th>
<th>THCa</th>
<th>CBD</th>
<th>CBDa</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>unheated</td>
<td>90</td>
<td>14500</td>
<td>3.9</td>
<td>28</td>
</tr>
<tr>
<td>A</td>
<td>heated</td>
<td>10060</td>
<td>150</td>
<td>29</td>
<td>17</td>
</tr>
<tr>
<td>B</td>
<td>untreated</td>
<td>48</td>
<td>3300</td>
<td>26</td>
<td>3500</td>
</tr>
<tr>
<td>B</td>
<td>heated</td>
<td>1100</td>
<td>9.3</td>
<td>1900</td>
<td>0.9</td>
</tr>
</tbody>
</table>

In the unheated extracts the cannabinoid acids of THC and CBD (THCa and CBDa) were most dominant, whereas after heating these compounds were decarboxylated into THC and CBD. The metabolite profiles of the different Cannabis extracts (Fig.1) obtained by GC-MS show that THCa and THC are the main constituents of unheated and heated Cannabis extracts respectively.
Immuno-modulating properties of unheated Cannabis extracts and THCa

Macrophage activation and TNF-α assay

U937 macrophages were exposed for 4 h to LPS in the presence or absence of different concentrations of Cannabis extracts. The immuno-modulating effect of three plant extracts, unheated A (high THCa content), heated A (low THCa content) and unheated B (low THCa content) on LPS activated U937 cells was tested by measuring the TNF-α release in the cell culture media (Fig. 2). The release of TNF-α was clearly inhibited in a concentration dependent manner by all three plant extracts.

Figure 1 Total ion currents from unheated (black) and heated (grey) Cannabis extract A (A) and Cannabis extract B (B) obtained with GC-MS.

Figure 2 Release of TNF-α from U937 macrophages incubated for 4 h with LPS (black bars) with a dilution series (from left to right successively; 4000, 2000, 1000, 500 and 250 fold diluted) of unheated Cannabis extract A (high THCa content), heated Cannabis extract A (low THCa content), and unheated Cannabis extract B (low THCa content), respectively. The release of TNF-α is corrected for protein content. The results are presented as means ± SD of triplicate measurements.
The inhibition was not due to a toxic effect induced by the Cannabis extracts according to LDH activity determinations (data not shown). The inhibitory effect of the 250, 500 and 1000 fold dilutions of the unheated Cannabis extract A was larger than the effect of the heated Cannabis extract A and the unheated Cannabis extract B according to the Student’s *t*-test (*p* < 0.05).

Because the main difference between the three extracts was the THCa/THC content (Fig. 1), we investigated whether THCa could be responsible for the TNF-α-inhibitory effect. THCa (pure compound) was added to the heated Cannabis extract A as well as the unheated Cannabis extract B in such a way that the concentration THCa was equal to the level of THCa in the unheated Cannabis extract A. Figure 3 shows the effect of THCa addition on the TNF-α release.

The addition of THCa to the heated Cannabis extract A and the unheated Cannabis extract B augmented the inhibitory effect on the TNF-α release. The effect of the unheated Cannabis extract B + THCa addition was similar to the effect induced by the unheated Cannabis extract A, this may suggest that THCa is one of the main compounds involved in the inhibitory effect of the unheated Cannabis extracts. For this reason it was interesting to see if pure THCa also exhibited this TNF-α inhibitory property. From this point we only used the Cannabis extract containing a high amount of THCa (Cannabis extract A).

U937 macrophages and PB-MØ were exposed to LPS in the absence or presence of pure THC, THCa or unheated Cannabis extract. Figure 4 shows the results of the comparison of the effects of the pure compounds on the TNF-α release in U937 macrophages and PB-MØ. The concentration of the pure compounds was chosen in such a way that the concentration of the pure compound equals the concentration of THCa in the unheated Cannabis extract.
THC, THCa as well as unheated Cannabis extract were capable of inhibiting the TNF-α release from U937 macrophages and blood macrophages exposed to LPS in a dose-dependent manner. The inhibitory effect of pure THCa was comparable to the effect induced by the unheated Cannabis extract on both U937 macrophages and PB-MØ.

![Figure 4](image)

**Figure 4** Release of TNF-α from U937 macrophages (left panel) and PB-MØ (right panel) incubated for 4 h with LPS or LPS in combination with a concentration series of THC, THCa and unheated Cannabis extract A (from left to right 4000, 2000, 1000, 500, and 250 fold diluted respectively for U937 macrophages and 1000, 500, and 250 fold diluted respectively for PB-MØ). The concentrations of THC and THCa are equal to the concentration of THCa in the unheated Cannabis extract. The release of TNF-α is corrected for protein content. The results are presented as means ± SD of triplicate measurements.

The TNF-α release from U937 macrophages after treatment with THC showed an analogous effect to THCa, whereas the effect of THC on the TNF-α release from PB-MØ was markedly stronger. TNF-α release was more inhibited with respect to THCa. At higher THC concentration (200 nM), the inhibition of the TNF-α release tended to become weaker. Considerable information can be found on the anti-inflammatory effect of THC. Unfortunately these results are often contradictory, because different cells, incubation times and concentrations were used. We postulated that the incubation time could be an important factor for these discrepancies in the literature and therefore we investigated the TNF-α inhibitory effect of pure THC, THCa, unheated and heated Cannabis extract in time. U937 macrophages were incubated with LPS in the presence or absence of THC, THCa, unheated or heated Cannabis extract. Culture media was collected at different time points for the determination of TNF-α (Fig. 5). THC, THCa, unheated and heated Cannabis extract all inhibited the TNF-α release. As opposed to TNF-α production by cells stimulated in the presence of heated extract or THC, the TNF-α production in the presence of unheated extract or THCa was delayed and showed a lower maximum.
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**Figure 5** Left panel: release of TNF-α from U937 macrophages after exposure to LPS (1 µg/ml) alone and in combination with unheated extract A (500 fold diluted) or heated Cannabis extract A (500 fold diluted). Right panel: release of TNF-α from U937 macrophages after exposure to LPS (1 µg/ml) alone and in combination with THC (80 nM) or THCa (80 nM). The levels of TNF-α released by the U937 macrophages in the culture medium were determined at the time points indicated and are corrected for protein content. The results are presented as means ± SD of triplicate measurements.

**CB₁ and CB₂ antagonism**

To further investigate the possible differences in the TNF-α inhibitory properties of THC and THCa we investigated if the inhibition of TNF-α production was mediated via the cannabinoid receptors CB₁ and CB₂ (Fig. 6).

Western blot experiments with specific antibodies for CB₁ and CB₂ confirmed the presence of these receptors on the membranes of U937 cells (data not shown). This is in line with previous reports that mention the presence of CB receptors on human leukocytes. U937 macrophages were exposed to LPS plus a fixed concentration of THC, THCa or unheated Cannabis extract in combination with a concentration series of the CB₁ receptor antagonist AM281 or the CB₂ receptor antagonist AM630. The results presented in Figure 6 revealed that THC, THCa and the unheated Cannabis extract inhibited the TNF-α release induced by LPS. Both the CB₁ antagonist and the CB₂ antagonist were not able to reverse this inhibitory effect. The antagonists themselves had no effect on the TNF-α release induced by LPS (data not shown).

The results suggested that the inhibitory effect on the TNF-α release by THC, THCa and unheated Cannabis extract involved different mechanisms. We therefore investigated whether the TNF-α inhibitory properties were mediated via cAMP induction, which is also known to be linked to TNF-α inhibition.
Measurement of intracellular cAMP formation

The concentration of cAMP was measured after treatment of U937 macrophages with LPS in the presence or absence of a concentration series of THC, THCa and unheated Cannabis extract. From Figure 7 it can be concluded that cAMP is not induced by THC, THCa or unheated Cannabis extract according to the Student’s t-test. As a positive control we used zilpaterol, a β2-adrenergic receptor agonist, which induced the cAMP release more obviously, as determined by using the Student’s t-test (p < 0.05). Beta2-agonists inhibit the production of LPS induced TNF-α via the induction of cAMP.
As THC, THCa and unheated Cannabis extract did not mediate their inhibitory effect on TNF-α via cAMP, we examined if the inhibitory effect was established via the inhibition of PC-PLC activity.

**Determination of phospholipase C activity**

Tricyclodecan-9-yl-xanthogenate (D609), a selective inhibitor of phosphatidylcholine specific phospholipase C (PC-PLC), inhibits the activity of PC-PLC induced by LPS stimulation and subsequently inhibits the production of TNF-α via a complex signalling pathway. We therefore investigated if THC, THCa and unheated Cannabis extract inhibit TNF-α release after LPS exposure via the induction of PC-PLC. Macrophages were pre-incubated with THC, THCa and unheated Cannabis extract in serum free culture media. Subsequently the cells were exposed to LPS for 5 minutes after which the cells were lysed and the PC-PLC activity was determined directly. The results presented in Figure 8 revealed that THCa and unheated Cannabis extract both inhibited the PC-PLC activity in a dose-dependent manner. The induction of PC-PLC activity at high THC concentration (80 nM) correlates with the induction of TNF-α in Figure 4 (left panel).

**Effects of THCa and unheated Cannabis extract on EAE**

Like THC, THCa and unheated Cannabis extracts were able to inhibit the TNF-α release *in vitro*, although this effect was obviously regulated via different pathways it was interestingly to know if THCa and unheated Cannabis extracts were also immuno-modulating in *vivo*.

Previous studies have shown that THC had a positive effect on the clinical and histological signs of EAE in rat models. We therefore investigated the effect of THCa and unheated Cannabis extract on the severity of EAE immunized mice. Table 2 summarizes the clinical data on EAE incidence, day of disease onset, disease severity and loss of bodyweight. Both treatments resulted in a slightly more favourable disease course than treatment with placebo,
Immuno-modulating properties of unheated Cannabis extracts and THCa

evident from a lower incidence of disease, a lower maximal or cumulative EAE score or lower loss of bodyweight. However, these results failed to reach statistical significance when evaluated with the non-parametric Kruskall Wallis test for multiple groups.

Table 2 Clinical evaluation of in vivo treatment with THCa and unheated Cannabis extract on the development of EAE. The concentration of unheated Cannabis extract was chosen in such a way that the THCa concentration in the unheated Cannabis extract was equal to the THCa concentration of the pure compound (1mg). The results are presented as means ± SEM.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Incidence</th>
<th>Day of onset*</th>
<th>Max. clinical score day 0-20</th>
<th>Max. cumulative score day 0-20**</th>
<th>Max. clinical score day 21-42</th>
<th>Max. cumulative score day 21-42**</th>
<th>Max. % loss of bodyweight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placebo</td>
<td>9/10</td>
<td>11.4 ± 0.2</td>
<td>2.1 ± 0.2</td>
<td>13.4 ± 2.3</td>
<td>1.8 ± 0.4</td>
<td>16.6 ± 3.8</td>
<td>19.6 ± 1.9</td>
</tr>
<tr>
<td>THCa</td>
<td>8/9</td>
<td>12.8 ± 0.6</td>
<td>1.5 ± 0.3</td>
<td>9.0 ± 2.5</td>
<td>0.9 ± 0.2</td>
<td>11.8 ± 4.6</td>
<td>16.3 ± 2.6</td>
</tr>
<tr>
<td>Unheated Cannabis</td>
<td>6/10</td>
<td>12.0 ± 0.3</td>
<td>1.4 ± 0.4</td>
<td>9.8 ± 3.2</td>
<td>1.5 ± 0.5</td>
<td>17.0 ± 5.9</td>
<td>13.5 ± 3.0</td>
</tr>
</tbody>
</table>

* The mean day of onset is calculated for the animals that developed clinical signs of EAE (N=8 for THCa, N=6 for unheated Cannabis extract and N=9 for placebo)

** For each individual mouse the cumulative disability score (sum of daily disability scores) was calculated from day 0 up to day 20 and from day 21 up to day 42 and included in the mean.

To establish if THCa and unheated Cannabis extract had a sub-clinical effect on the inflammatory response within the CNS, different regions of the CNS were (cerebellum, brain stem and spinal cord) analysed using haematoxylin staining (Fig. 9). These areas of the brain were infiltrated by immune cells in the majority of the animals. In mice treated with THCa and unheated Cannabis extract the severity of the inflammation in the brainstem was decreased (Kruskall-Wallis, p < 0.05), a significant inhibition was observed for THCa (post-hoc Mann-Witney U-test, p = 0.004) and unheated Cannabis extract (post-hoc Mann-Whitney U-test, p = 0.006) as compared to the placebo group.

Figure 9 Effect of THCa and unheated Cannabis extract on the inflammatory response in the cerebellum (A), brain stem (B) and spinal cord (C). The severity of the inflammatory response was expressed on a scale of 0 (no infiltrates) to 4 (severe
inflammatory response) as indicated in Material and Methods. Each symbol (*) represents one individual mouse. ■ represents the mean value of histological score.

These data thus suggest that both THCa and unheated Cannabis extract have the potential to inhibit inflammatory responses within the CNS and may possibly inhibit the development of clinical disease when administered at a different dosage.

Discussion

In the Cannabis plant, cannabinoids are synthesized and accumulated as cannabinoid acids. The main constituent of Cannabis Sativa is Δ⁹-THCa. Δ⁹-THCa and other cannabinoid acids are devoid of psychotropic effects. When the plant is heated (e.g. by smoking), Δ⁹-THCa decarboxylizes into Δ⁹-THC, the main psychoactive compound of heated Cannabis extract. The bioavailability of Δ⁹-THC depends on the way of administration, but eventually Δ⁹-THC is metabolized into its psychoactive metabolite 11-hydroxy-Δ⁹-THC (11-OH-THC), which in turn is further oxidized into the non-psychoactive compound THC-COOH (11-nor-9-carboxy-Δ⁹-THC). THC-COOH is secreted after glucuronidation and must not be mistaken for THCa in the Cannabis plant. The psychoactive properties of Δ⁹-THC and 11-OH-THC are mediated by the CB₁ receptor. Cannabinoid receptors (CB₁ and CB₂) are the main receptors of the endogenous cannabinoid system. THCa is compared to THC, a weak agonist for the CB₁ receptor (Ki = 6.3 x 10⁻⁷ M vs. 3.5x10⁻⁹ M) and CB₂ receptor (Ki = 8.9x10⁻⁷ M vs. 3.2x10⁻⁹ M) as determined with receptor expressing membranes from cells transfected with cloned CB receptors (data not shown).

In this study we showed for the first time that THCa and unheated Cannabis sativa extracts were capable of inhibiting the production of TNF-α by LPS induced macrophages in a dose-dependent manner. Our results indicate that THCa is probably the major component responsible for this effect. However, other constituents might contribute as well but this remains to be investigated. The TNF-α inhibitory effect of the heated Cannabis extract was less pronounced, whereas the effects of the pure compounds (THC and THCa) were comparable. This discrepancy can be explained by the fact that the amount of THC in the heated extracts was lower with respect to the THCa content in the unheated extracts, but an effect of other metabolites in the unheated extract should not be ruled out beforehand. THCa as well as unheated Cannabis extract were also capable of inhibiting the TNF-α release from blood macrophages in a dose-dependent manner. However, the effect of THC on the TNF-α release from PB-MØ was more pronounced than observed in U937 macrophages. This discrepancy can be caused by the fact that U937 cells and PB-MO are originating from
different differentiation stages of the mononuclear myeloid cell line.\textsuperscript{28,35,36} The effect of THC concentration (Fig. 4) and its time-course (Fig. 5) on TNF-\(\alpha\) release differed from that of THCa. At the higher THC concentration tested, the inhibition of the TNF-\(\alpha\) release tended to become weaker. This phenomenon has been reported before, Berdyshec et al.\textsuperscript{37} reported that THC exerted a biphasic action on pro-inflammatory cytokines in human PB-MO. Low amounts of THC (nM) inhibited the TNF-\(\alpha\) production, whilst high concentrations (\(\mu\)M) stimulated TNF-\(\alpha\). After prolonged exposure time THC and heated Cannabis extract tend to induce the TNF-\(\alpha\) production. Cannabinoids have demonstrated effects \textit{in vivo} and \textit{in vitro} on the production and function of a variety of cytokines. Depending upon the model system used, these effects are often conflicting.\textsuperscript{11,14} The dose and duration of incubation time, as mentioned above are possible explanations for these discrepancies.

Using a selective antagonist AM281 for the CB\(_1\) receptor\textsuperscript{29,30} and a selective antagonist for the CB\(_2\) receptor, AM630\textsuperscript{31} we showed that the inhibitory effect on TNF-\(\alpha\) production by THC, THCa and unheated Cannabis extract is not mediated via these receptors (Fig. 6). From these results it can not be concluded whether THCa acts via another receptor or via non-receptor mediated mechanisms. There have been many speculations about a third type of CB receptor\textsuperscript{1,8,37} but many other options could be possible as well. As one pathway leading to TNF-\(\alpha\) inhibition is linked to cAMP induction\textsuperscript{38,39} the possibility that THCa acts via this route was investigated. Accumulation of cAMP may result from the stimulation of receptors which activate Gs proteins\textsuperscript{38,39} Figure 7 shows that cAMP was not significantly induced by THC, THCa or unheated Cannabis extract. Another possibility is that receptors that couple to Gq proteins are involved. Binding to this receptor will activate phospholipase C. LPS can induce TNF-\(\alpha\) production via the following pathway: LPS activates PC-PLC, which hydrolyses phosphatidylcholine resulting in the formation of cholinephosphate and diacylglycerol (DAG). DAG stimulates ceramide, which in turn activates successively raf1, MEK, ERK, and TNF-\(\alpha\).\textsuperscript{40} We therefore investigated if THC, THCa and unheated Cannabis extract had any effect on the PC-PLC activity. From Figure 8 we can conclude that there is indeed a difference in the effect on PC-PLC activity of THC and THCa. THCa and unheated Cannabis extract inhibited both the PC-PLC activity in a dose-dependent manner. This could be an explanation for the difference in TNF-\(\alpha\) inhibitory effect. D609 was used as a positive control for inhibition of PC-PLC activity. D609 is a selective PC-PLC inhibitor and inhibits the production of TNF-\(\alpha\) induced by LPS\textsuperscript{40-42} via a PC-PLC dependent pathway. The question is if THCa exerts its effect via a Gq receptor or directly on PC-PLC itself. These
results merit further investigation. Interesting is the induction of PC-PLC activity at high THC concentration (80 nM). This induction reflects the increase in TNF-α production shown in Figure 4. Ho et al.\(^\text{43}\) found that the CB\(_1\) receptor coupled positively to phospholipase C activity. We can only speculate that high levels of THC bind to the CB\(_1\) receptor, which couples to PC-PLC and via a complex pathway, induces TNF-α production. Further investigation would be necessary to support this.

The cannabinoids, \(\Delta^9\)-THC and \(\Delta^8\)-THC showed in previous \textit{in vivo} studies to have a positive effect on the onset, incidence and severity of EAE in Lewis rats immunized with myelin basic protein or autologous spinal cord.\(^\text{17, 18}\) \(\Delta^9\)-THC, not \(\Delta^8\)-THC also inhibited the inflammatory infiltrates in brain tissue. In this study we investigated if THCa and unheated Cannabis extract are able to reflect the positive effect of THC on the clinical and histological signs of EAE. In this pilot study we used, the EAE-SJL mice model system. EAE induced by PLP\(_{139-151}\) in SJL mice follows a reliable relapsing-remitting course with acute clinical signs first appearing about 11 days after immunization and relapses first appear after 28 days\(^\text{21, 44}\). During the relapsing period (20-42 days) the animals were not treated with medication, so the effect over a longer period can be investigated. Table 2 revealed that unheated Cannabis extract and THCa had a slight effect on the severity of the disease, which failed to reach significance. Since no information on the pharmacokinetics of THCa and unheated Cannabis extract was available for mice, the dosing schedule could be further optimized. Nevertheless, both treatments resulted in significantly less inflammatory infiltrates in the brain stem, supporting that THCa and unheated Cannabis extract may have the potential to inhibit inflammatory responses \textit{in vivo} and to suppress clinical symptoms when administered at a different dosage. More detailed analysis of inflammatory infiltrates is required at earlier time points for a better appreciation of the efficacy of treatment on sub-clinical aspects of the disease.

**Concluding remarks**

This is the first report describing the immuno-modulating properties of THCa and unheated Cannabis extracts, which lack the psychoactive properties of THC. Our results suggest that THCa and unheated Cannabis extracts are acting via different metabolic pathways than THC, but we can only speculate if they are devoid of the adverse effects of THC and heated Cannabis extracts on health. The immuno-modulating effect of THCa and unheated Cannabis extracts was not only observed \textit{in vitro}, but also \textit{in vivo}. In a pilot EAE animal study the effects of THCa and unheated Cannabis extracts on the clinical and histological signs of EAE
Immuno-modulating properties of unheated Cannabis extracts and THCa collectively suggest that these may have therapeutic potential, but more attention has to be paid to the way and amount of dosing, because little is known about the pharmacokinetics of THCa and unheated Cannabis extracts.

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References
Chapter 7


