**The anti-inflammatory effect of cannabinoid administration in graft versus host disease may be hampered by a suppressive effect on lymphocyte reconstitution – comparison of D9 tetrahydrocannabinol (THC), cannabidiol (CBD) and cannabis extract treatment in murine models for bone marrow transplantation.**

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**Introduction**

Bone marrow transplantation (BMT) is a well-established treatment for malignant and non-malignant hematological diseases (1). Allogeneic transplantation comes with the risk of graft versus host disease (GVHD), a major cause of morbidity and mortality in BMT patients (2). In addition, the toxicity of the conditioning protocol that precedes BMT impairs innate and adaptive immunity, making transplanted patients very susceptible to both common and rare infections. The early post-engraftment period is categorized by a progressive recovery of cell mediated immunity, however, full reconstitution of the hematological components may take years (3).

In recent years, numerous publications have suggested the potential of cannabis-based medicines for the treatment of various conditions (4). Among the patients who can benefit from such treatment are BMT patients, who often suffer from nausea and chronic pain. Cannabiscontains numerous molecules, including more than 60 chemical compounds classified as cannabinoids, and different sub-strains vary in their cannabinoid content. Two cannabinoids have been the focus of most of the studies examining medical uses: D9 tetrahydrocannabinol (THC) and cannabidiol (CBD). THC and some of the other cannabinoids mediate their actions primarily through the Gi protein-coupled seven transmembrane cannabinoid receptors: cannabinoid receptor 1 (CB1), which is mainly expressed in the brain and to some extent in peripheral tissues such as the immune tissues, and cannabinoid receptor type 2 (CB2), which is expressed at high density in immune cells. CBD has a very weak affinity to CB1 and CB2 (5). Several reports demonstrated CBD signaling through non-cannabinoid receptor mechanisms, such as TRP channels and the nuclear receptor, peroxisome proliferator-activated receptor gamma (PPAR-γ) (6).

In addition to their effects on the nervous system, both phyto- and endocannabinoids have important immunological effects. They possess a wide range of anti-inflammatory properties as they induce the production of anti-inflammatory cytokines, such as IL-4, IL-5 and IL-10, and they affect the differentiation and function of several immune cells (7). The involvement of cannabinoid receptor signaling in the biology of hematopoietic stem and progenitor cells has also been reported (8,9). Importantly, different cannabinoids were shown to differentially affect immune cell function (10).

Although there is much information regarding the influence of cannabis and cannabinoids on the immune system, the effect of these drugs on rehabilitation of the hematological system after BMT and their efficacy in GVHD patients is largely unknown. THC treatment was shown to reduce GVHD in a mouse model that did not include BMT (11) and a recent publication demonstrated the beneficial effect of the cannabinoid CBD in GVHD prophylaxis in patients (12), but the differential effects of cannabinoids was not examined.

We hypothesize that each cannabinoid has selective effects on hematopoietic and immune cells and hence a different impact on hematopoiesis and on GVHD.

In our study we compared the treatment of pure THC, pure CBD and high THC/high CBD cannabis extracts in murine BMT models. We show here that all the treatments reduced activated lymphocyte proliferation *in vitro*, but pure cannabinoids, particularly CBD, had a stronger inhibitory effect. We also found that CBD and THC utilize different signal transduction pathways to cause these effects. Using a syngeneic transplantation model, we demonstrated that all treatments, but pure THC in particular, inhibit lymphocyte reconstitution after transplantation. Although pure cannabinoids had a superior effect *in vitro,* cannabis extracts were better at reducing the severity of disease and improving survival in the GVHD model than pure cannabinoids.

Our results emphasize the similarities and differences when using various cannabis-based drugs in BMT. As different strains of cannabis contain a wide range of cannabinoids and other molecules that may influence the clinical outcome of the treatment, a better understanding of the effects of each molecule on hematological recovery and GVHD pathology will allow the use of a specific cannabinoid drug for each patient, as a type of personalized medicine.

**Materials and Methods**

**Cannabis extracts and cannabinoids**

**Inhibitors**

SR144528, a CB2 receptor antagonist, was purchased from Sigma-Aldrich, Israel; A967079, a TRPA1 receptor antagonist and BCTC, a TRPV1 receptor antagonist, were purchased from Alomone Laboratories, Israel; GSK2193874, a TRPV4 antagonist, was purchased from Sigma-Aldrich; and GW9662, a PPARɣ antagonist, was purchased from Enzo Life Sciences, New York, USA.

**Mice**

Female 8- to 11-week-old C57BL/6 and BALB/c mice were purchased from Envigo, Jerusalem, Israel and CB2 knockout mice ( ) were bred in the specific pathogen-free (SPF) facility of the Authority for Biological and Biomedical Models at the Hebrew University of Jerusalem. The study was approved by the Institutional Animal Care and Use Committee of the Hebrew University of Jerusalem in accordance with national laws and regulations for the protection of animals and the mice were housed under SPF conditions.

**Syngeneic BMT model**

C57BL/6 or CB2 knockout mice underwent lethal whole-body irradiation by single exposure to 10 Gy and were reconstituted with 8 x 106 donor C57BL/6 or CB2 knockout BM cells the following day. Cannabis extracts/cannabinoids (5 mg/kg) were administered intraperitoneally (IP) every other day, from the day of transplantation for two weeks. Once a week, blood was collected from the mice tail into ethylenediaminetetraacetic acid (EDTA) coated capillary tubes. A complete blood count (CBC) with differentials was performed using a validated BC-2800Vet Auto Hematology Analyzer (Mindray).

**Allogeneic BMT model**

BALB/c mice underwent lethal whole-body irradiation by single exposure to 8 Gy and were reconstituted with 8 x 106 donor C57BL/6 BM cells and 2 x 106 spleen cells the following day. Cannabis extracts/cannabinoids (5 mg/kg) were administered intraperitoneally (IP) every other day, from the day of transplantation for two weeks. For GVHD evaluation, mice were monitored daily for weight loss, diarrhea, ruffled skin, and survival (rated on a scale of 0-4) to give a GVHD score, calculated as previously described (13).

**Lymphocyte activation assays**

A total of 1 x 106 carboxyfluorescin diacetate succinimidyl ester (CFSE)-labeled C57BL/6 splenocytes cells/well were plated in 96-well flat bottom plates with RPMI 1640 medium supplemented with 10% FCS, 1% penicillin/streptomycin, and 1% L-glutamine (Biological Industries, Beit Haemek, Israel). Splenocytes were activated with anti-CD3 antibodies (Biolegend, USA) in the presence of the indicated concentrations of cannabis extracts/cannabinoids for 4 days. For the proliferation assay, CFSE levels in the cells were determined using FACS analysis. Cytokine concentration in the culture media was quantified using ELISA Ready SET Go kits (eBioscience, San Diego, CA, USA), according to the manufacturer’s instructions. All determinations were made in triplicates.

**RNA extraction and PCR analysis**

Total cellular RNA was extracted using RNeasy® Mini Kit columns (QIAGEN, add location) according to the manufacturer's protocols. 1 µg of total RNA was used to synthesize cDNA using the High-Capacity cDNA kit (Applied Biosystems, add location) following the manufacturer’s instructions. Detection of transcript levels of PPAR-γ were performed using the TaqMan Gene Expression Assay Kit (Applied Biosystems), using HPRT-1 as a reference. All primers were purchased from Applied Biosystems. Real-Time PCR reactions were conducted using StepOne Plus (Applied Biosystems). Data was analyzed by StepOne Software version 2.2 (Applied Biosystems).

**Statistical analysis**

Data from the BMT studies are described as mean values on dot plot showing individual values (lymphocyte and platelet count) at the indicated time points. Data from *in vitro* studies are represented as mean ± SE. Single comparisons to control were made using two-tailed Student’s t-test, with P value <.05 considered statistically significant.

**Results**

**CBD is a stronger inhibitor of *in vitro* lymphocyte activation than THC.**

We decided first to utilize *in vitro* methods in order to learn about the effects of pure CBD/THC and cannabis extracts on lymphocyte function. Cannabis extracts with high CBD or THC content were named CBD botanical drug substance (BDS) or THC BDS respectively. We used these extracts in addition to the pure cannabinoids for two reasons: first, most patients are currently treated with cannabis-based medications and not with pure cannabinoids. Second, it has been suggested that the combination of cannabinoids with other active molecules in the plant may achieve better clinical results (known as the entourage effect) (Russo 2011).

The effect of cannabis/cannabinoids on the proliferation of activated lymphocytes was analyzed. Succinimidyl ester (CFSE)-labeled C57Bl/6 mouse splenocytes were activated with anti-CD3 antibodies in the presence of pure cannabinoids, CBD BDS or THC BDS at various concentrations. Cell proliferation was assessed using CFSE FACS analysis. Interestingly, *in vitro* the inhibitory effect of pure cannabinoids on lymphocyte activation was stronger than that of cannabis extract. Whether in the form of pure cannabinoids or cannabis extract, CBD inhibited proliferation significantly better than THC **(Figure 1A).** Similar results were obtained using Balb/C splenocytes **(Supplementary Figure 1A)** or human peripheral blood mononuclear cells (PBMC) **(Supplementary Figure 1B).**

Next,we used the supernatant from the same experiments to test the effect of cannabinoid treatment on cytokine secretion upon lymphocyte activation. We tested four different cytokines: IL-17, secreted in the Th17 reaction; IL-10, secreted from Tregs; TNFα, secreted in the Th1 reaction; and IL-5, secreted in the Th2 reaction. The levels of secreted cytokines were examined using an ELISA assay. We show here the results of 3 µg/ml treatment with pure cannabinoids and 10 µg/ml treatment with the cannabis extracts, which contain approximately 30% of the designated cannabinoid. The results for IL-17 and IL-10 after treatment with various other concentrations can be found in the supplementary data.

All treatments significantly reduced IL-17 secretion **(Figure 1B, Supplementary Figure 2).** CBD BDS had the strongest effect with 16 times less IL-17 in the supernatant compared to untreated activated lymphocytes (control). IL-10 secretion was significantly increased by all treatments **(Figure 1C, Supplementary Figure 2).** Pure CBD had the strongest effect, with a 360-fold increase (compared to control). Notably, pure CBD had a stronger effect then pure THC, but CBD BDS had less effect than THC BDS. All treatments led to a small increase in TNFα secretion **(Figure 1D)**, which was significant in all treatments except THC BDS. The levels of IL-5 secretion were affected by THC BDS and pure CBD treatments **(Figure 1E).**

Overall these results show that the cannabinoids CBD and THC have an inhibitory effect on lymphocyte activation, associated with a reduction in the secretion of the inflammatory IL-17 cytokine and an elevation in the secretion of the regulatory cytokine IL-10.

**THC and CBD affect lymphocyte activation by different mechanisms.**

The cannabinoid receptor CB2 is highly expressed in immune cells ( ). To elucidate whether CB2 is involved in the effects of THC and CBD on lymphocytes, we used CB2 knock-out mice (CB2 KO) and the CB2 inverse agonist SR144528. First, we used splenocytes extracted from CB2 KO mice **(Supplementary Figure 3A-B)** in a CFSE lymphocyte proliferation assay, similar to the assay whose results are shown in Figure 1A. The inhibitory effect of pure THC, but not pure CBD, was abolished in CB2 KO-derived splenocytes **(Figure 2A)**. Interestingly, the inhibitory effect of THC BDS was maintained. THC’s effect was partially reversed also by addition of 1 µM SR144528 to the culture in a proliferation assay using C57Bl splenocytes **(Figure 2B)**.

Our results indicate that the CB2 receptor is the main mediator for THC’s effect on lymphocytes; whereas CBD’s effect clearly does not involve CB2 signaling. Several molecules have been proposed as mediators for CBD’s effects on mammalian cells (refs?). We used inhibitors of some of these together with CBD in a CFSE lymphocyte proliferation assay. A967079, BCTC and GSK2193874 are antagonists of TRP channels TRPA1, TRPV1 and TRPV4 respectively, which have been demonstrated to mediate CBD signaling ( ). However, we found that none of these antagonists interfered with CBD’s inhibitory effect on lymphocyte activation **(Supplementary Figure 4A-C)**. Another potential mediator of CBD signaling is the nuclear receptor PPAR-γ ( ). We found that GW9662, the antagonist for PPAR-γ, could partially reverse the effect of CBD on lymphocyte proliferation **(Figure 2C)**.

**Cannabinoid treatment alters hematologic rehabilitation after bone marrow transplantation.**

To investigate the effect of THC and CBD and cannabis extracts on hematopoiesis after BMT, we utilized a syngeneic transplantation model. C57BL/6 mice underwent lethal whole-body irradiation and were reconstituted with 8 x 106 donor C57BL/6 BM cells the following day (**Figure 3A)**. 5 mg/kg of cannabis extracts/pure cannabinoids/vehicle were administered intraperitoneally (IP) from the day of transplantation, every other day, for two weeks. Once a week, starting one week after transplantation, blood was collected from mice tails and CBC with differentials was performed. Both pure cannabinoids and cannabis extracts had a significant inhibitory effect on lymphocyte recovery (**Figure 3B and C**). Among the tested compounds, pure THC had the strongest effect with a mean of 39% inhibition compared to vehicle-treated mice (control), three weeks after transplantation **(Figure 3B, right)**. The inhibitory effect of CBD treatment was significantly lower. Interestingly there was no significant difference between CBD BDS and THC BDS treatment **(Figure 3C, right)**. The number of monocytes and granulocytes was not affected by the treatment (data not shown). Platelet recovery was significantly improved only in the group that received THC BDS treatment, with a mean of 10% improvement compared to control, two weeks after transplantation **(Figure 3D and E)**.

These results demonstrate that cannabis/cannabinoids treatment affect hematological reconstitution after BMT and that different cannabinoids have different effects.

**CB2 receptor has an inhibitory effect on lymphocyte recovery.**

Since THC had the strongest inhibitory effect on lymphocyte recovery, we wanted to examine the involvement of CB2 in this process. First, we administered syngeneic BMT mice with the CB2 antagonist SR144528 once a day for one week from the day of transplantation. Once a week, starting one week after transplantation, blood was collected from mice tails and CBC with differentials was performed. Our results demonstrate significantly improved lymphocyte recovery in the treated group **(Figure 4A)**.

To clarify whether this improvement is due to an effect on the grafted cells or on the accepting environment, we used CB2 KO mice as donors/acceptors in BMT experiments. The normal blood counts of CB2 KO female mice were similar to the WT C57BL/6 counts **(Supplementary Figure 3B).** C57BL/6 mice underwent lethal whole-body irradiation and were reconstituted with 8 x 106 donor CB2 KO or C57BL/6 BM cells the following day. There were a significantly higher number of lymphocytes in the group that received CB2 KO transplant compared to control, starting from the second week after transplantation **(Figure 4B)**. When C57BL/6 BM cells were transplanted to CB2 KO or C57BL/6 recipient mice, lymphocyte counts were not significantly different **(Figure 4C)**.

Altogether, these experiments demonstrate the inhibitory role of CB2 in rehabilitation of blood lymphocytes after bone marrow transplantation.

**Cannabis/cannabinoids administration for GVHD prophylaxis**

Several studies, as well as our *in vitro* assays **(Figure 1)**, indicate that cannabinoids are anti-inflammatory ( ). Yeshurun *et al*. demonstrated the beneficial effect of the cannabinoid CBD in GVHD prophylaxis in patients (12). We therefore decided to compare the immunosuppressive effect of CBD/THC and cannabis extracts on GVHD prophylaxis in a murine model.

Balb/C mice underwent whole-body irradiation followed by allogeneic BMT from C57BL/6 donor mice. 5 mg/kg of cannabis extracts/pure cannabinoids/vehicle were administered IP from the day of transplantation, every other day, for two weeks (**Figure 5A**). Mice chimerism was not affected by the treatment **(Supplementary Figure 5).**  In our model, both CBD BDS and THC BDS significantly improved survival (**Figure 5B, right**), while pure cannabinoids had a smaller effect (**Figure 5B, left**). Moreover, GVHD scores were significantly lower in mice administered cannabis extracts **(Figure 5C)**.

These results demonstrate *in vivo* that modulation of allogeneic activation due to cannabis extracts administration is better than that due to administration of pure THC or CBD.

**Discussion**

Cannabis contains hundreds of chemical compounds. Different sub-strains of cannabis comprise unique sets of cannabinoids and other molecules, which influence the clinical outcome of the treatment. The scientific data regarding the use of a specific strain or isolated cannabinoid for the treatment of each disease is currently very limited.

The increased demand for medical cannabis around the world results in an urgent need for scientific evaluation of cannabis-based medicines for medical treatments. n this research, we decided to compare the effect of the most abundant cannabinoids, THC and CBD, as well as cannabis extracts from THC- and CBD-rich plants. We used the extracts because these drugs are those most commonly used by patients and also because of the suggested entourage effect (Russo 2011). We have used *in vitro* assays as well as syngeneic and allogeneic murine models to test the effect of these cannabis-based treatments on BMT. Our results demonstrate that all of these cannabis-based treatments suppress lymphocyte activation and influence cytokine secretion. In accordance with its known anti-inflammatory activity ( ), CBD had the most profound effect on cell proliferation. The induction of IL-10 together with inhibition of IL-17 secretion by all treatments may indicate an influence on the Th17/Treg balance. Th17 cells are known to participate in the pathophysiology of GVD (van der wart 2014) and several autoimmune diseases, and therefore this effect is most clinically relevant. Notably, our results correspond to previous data for CBD treatment in the experimental autoimmune encephalomyelitis (EAE) mice model for multiple sclerosis and in an animal model of asthma (vuolo 2015, kozela). Interestingly, we did not find any correlation between the effect of the treatment on cytokine secretion and its effect on proliferation. For example, 10 µg/ml THC BDS reduced cell proliferation by only 25%, but induced a relatively high secretion of the regulatory cytokine IL-10.

We utilized CB2 knockout mice and antagonists of different receptors to screen for signal transduction pathways used by CBD and THC to inhibit lymphocyte activation. We found that CB2 is the main mediator of THC’s effect but is not involved in the effect of CBD. The TRP channels we examined were also not found to participate in CBD’s inhibitory function. The only receptor that was found to mediate part of CBD’s inhibitory effect on lymphocyte activation is PPARγ. PPARγ is a nuclear hormone receptor widely expressed in adipose tissue and in immune/inflammatory cells, colonic mucosa, and the placenta ( ). PPARγ activation attenuates inflammatory processes associated with several diseases ( ), and it was found to be involved in inhibition of Th17 differentiation ( ). The involvement of PPARγ in CBD signaling has bene shown in various tissues ( ). For example in biopsies from patients with ulcerative colitis, CBD treatment *ex vivo* reduces signs of inflammation that can be blocked with a PPARγ antagonist (De Filippis et al., 2011). The involvement of other receptors in CBD-related signaling in lymphocytes is as yet unknown.

There are several obstacles to a good clinical outcome for BMT. The toxicity of the conditioning protocol leads to a period of low hematological counts which makes the patients susceptible to common and unusual infections ( ). Our results demonstrate that all the cannabis-based treatments we have used significantly delay lymphocyte reconstitution after transplantation. This finding is of great importance since delayed lymphocyte re-constitution may have a deleterious effect on the clinical outcome. On the other hand, THC-BDS treatment improved platelet rehabilitation. The involvement of endocannabinoids in thrombogenesis was previously demonstrated (Patinkin 2008, grambow 2016, gasperi 2014). However, it is not known yet which component is responsible for this effect in our model and if this result can be repeated with THC-BDS from different sources.

The inhibitory effect of the cannabis-based treatments on lymphocyte recovery contradicted our expectations. Patinkin *et al*. demonstrated that endocannabinoids increase the number of several hematopoietic cell's colony forming units (CFU) *in vitro* (Patinkin 2008) and Jiang *et al*. showed elevation of CFU in bone marrow of sub-lethally irradiated mice treated with the CB2 agonist AM1241 (Jiang 2011). Importantly, our results clearly identify CB2 as an inhibitory receptor of lymphocyte recovery. We demonstrate that THC, a CB2 agonist, has the strongest inhibitory effect on lymphocyte recovery. CB2 antagonist treatment in syngeneic transplanted mice improves lymphocyte recovery and similarly, CB2 KO bone marrow transplanted into WT mice resulted in improved rehabilitation of lymphocytes. Wild type bone marrow transplanted into CB2 KO mice did not affect the recovery rate indicating a role for CB2 expression on the transplanted cells rather than on the cells of the accepting environment. Our results can possibly be explained by the role of cannabinoids in hematopoietic stem and progenitor cell homing to the bone marrow niche. Pereira *et al*. proved that CB2 has a role in the retention of immature B cells in the bone marrow (Pereira 2009) and Hoggatt *et al*. demonstrated a significant decrease in CXCR4 in bone marrow cells treated with the CB1/CB2 agonist CP55940 (Hoggatt 2010).

In contrast to the greater effect of the pure cannabinoids *in vitro* and in the syngeneic transplantation model, the cannabis extracts, particularly the THC-BDS, had more effect on GVHD prophylaxis. This result together with the cytokine results from our *in vitro* experiments and the syngeneic model experiments demonstrate that the effects of the extracts are different from the effects of pure cannabinoids. There are two explanations for this phenomenon. The unique effects of the extract could result either from other molecules in the plant (not THC/CBD) or from a synergistic function of THC/CBD with other molecules.

Overall, our results demonstrate the complexity of using cannabis-based drugs and the need for accumulating comparative scientific results. The results of this study may influence the treatment of BMT patients with cannabis-based medicines. They should facilitate the choice of which particular cannabis-based drug should be used in the therapy of BMT patients according to their specific clinical condition.

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