**The anti-inflammatory effect of cannabinoids administration in Graft versus Host Disease may be hampered by suppressive effect on lymphocyte reconstitution – comparison of D9 tetrahydrocannabinol (THC), cannabidiol (CBD) and cannabis extracts 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 which precedes BMT impairs innate and adaptive immunity, making transplanted patients very susceptible to both common and unusual infections. The early post-engraftment period is categorized by progressive recovery of cell mediated immunity. However, full reconstitution of the hematological components may take years (3).

In the 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 the different sub-strains differ in their cannabinoid contents. Two cannabinoids have been subjects 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 toward the CB1 and CB2 cannabinoid receptors (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 effect on the nervous system, both fyto and endo-cannabinoids impart important immunological effects. They possess a wide range of anti-inflammatory properties by induction of anti-inflammatory cytokine production e.g.IL-4, IL-5 IL-10 and affect differentiation and function of several immune cells (7). The involvement of cannabinoid receptors signaling in the biology of hematopoietic stem and progenitor cells was also reported (8,9). Importantly, different cannabinoids were shown to affect differently immune cell function (10).

Although a lot of information has been collected regarding the influence of cannabis and cannabinoids on the immune system, the effect of these drugs on rehabilitation of the hematologic 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 as GVHD prophylaxis in patients (12) but the differential effect of different cannabinoids was not examined.

We hypothesize that each cannabinoid have selective effects on hematopoietic and immune cells and therefore different cannabinoids would have different influence on hematopoiesis and on GVHD.

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

Our results stress out the similarities and the differences in using different cannabis based drugs in BMT. As different strains of cannabis contain wide range of cannabinoids and other molecules which may influence the clinical outcome of the treatment, a better understanding of the effects of each molecule on hematologic recovery and GVHD pathology will allow the use of specific cannabinoid drug for each patient: as individualized medicines.

**Materials and Methods**

**Cannabis extracts and cannabinoids**

**Inhibitors**

SR144528- antagonist for CB2 receptor was purchased from SIGMA- ALDRICH, A967079- antagonist for TRPA1 Receptor and BCTC- antagonist for TRPV1 Receptor were purchased from alomone labs, Israel, GSK2193874- antagonist for TRPV4 was purchased from SIGMA- ALDRICH and GW9662- antagonist for PPARɣ was purchased from Enzo

**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 SPF facility of the authority of biological and biomedical models in 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 specific pathogen-free (SPF) conditions.

**Syngeneic BMT model**

C57BL/6 or CB2 knockout mice underwent lethal whole-body irradiation by single exposure to 10Gy and were reconstituted with 8\*106 donor C57BL/6 or CB2 knockout BM cells the following day. Cannabis extracts/cannabinoids (5 mg/kg) were administered intraperitoneally (IP), from the day of transplantation, every other day, for two weeks. Once a week, blood was collected from the mice tail into Ethylenediaminetetraacetic acid (EDTA) coated capillary tubes. 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 8Gy and were reconstituted with and were reconstituted with 8\*106 donor C57BL/6 BM cells and 2\*106 spleen cells the following day. Cannabis extracts/cannabinoids (5 mg/kg) were administered intraperitoneally (IP), from the day of transplantation, every other day, for two weeks. For GVHD evaluation, mice were monitored daily for weight loss, diarrhea, ruffled skin, and survival. GVHD score, based on all of the aforementioned factors (rated on a scale of 0-4), was calculated as previously described (13).

**Lymphocyte activation assays**

A total of 1\*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) at the presence of indicated concentrations of cannabis extracts/cannabinoids for 4 days. For proliferation test, CFSE levels on the cells were determined using FACS analysis. Cytokine concentration in the culture media was quantified using the 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) according to the manufacturer's protocols. 1µg of total RNA was used to synthesize cDNA using High-Capacity cDNA kit (Applied Biosystems) following the supplier’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) in the indicated time point. 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, as compared to THC.**

First, we decided to utilize in vitro methods in order to learn about the influence of pure CBD/THC and cannabis extracts on lymphocyte function. Cannabis extracts with high content of CBD or THC were named CBD BDS/ THC BDS (Botanical Drug Substance). We used these extracts, in addition to the pure cannabinoids, for two reasons: first, most of the patients are currently treated with cannabis based treatment and not with pure cannabinoids. Second, it was suggested that the combination of cannabinoids with other active molecules in the plant may have better results in medical use (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 different concentrations. Cell proliferation was assessed using CFSE FACS analysis. Interestingly, *in vitro* the inhibitory effect of pure cannabinoids on lymphocyte activation was stronger as compared to cannabis extract. In both pure cannabinoids assay and cannabis extract assay, CBD inhibited proliferation significantly better than THC **(Figure 1A).** Similar results were obtained using Balb/C splenocytes **(Supplementary figure 1A)** or human PBMC **(Supplementary figure 1 B).**

Next,we used the supernatant from the same experiments to test the effect of cannabinoids treatment on cytokine secretion upon lymphocyte activation. We tested 4 different cytokines: IL17, secreted in Th17 reaction, IL10, secreted from Treg, TNFα, secreted in Th1 reaction and IL5, secreted in Th2 reaction. The levels of secreted cytokines were examined using ELISA assay. We show here the results of 3µg/ml treatment with pure cannabinoids and 10µg/ml treatment with the cannabis extracts, containing approximately 30% of the designated cannabinoid. IL17 and IL 10 results with treatment in different concentrations can be found in the supplementary.

All treatments significantly reduced IL17 secretion **(Figure 1B, Supplementary figure 2).** CBD BDS had the strongest effect with 16 times less IL17 in the supernatant as compare to untreated activated lymphocytes (control). IL10 secretion was significantly elevated by all treatments **(Figure 1C, Supplementary figure 2).** Pure CBD had the strongest effect, 360 times higher than the control. Notably, pure CBD had a stronger effect then pure THC, but CBD BDS had less effect then THC BDS. All treatments led to a small elevation in the secretion of TNFα **(Figure 1D).** In all treatments but THC BDS, this elevation was significant. The levels of IL5 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 reduction in the secretion of the inflammatory IL17 cytokine and an elevation in the secretion of the regulatory cytokine IL10.

**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 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 effect was partially reversed also by addition of 1µM of SR144528 to the culture in a proliferation assay using C57Bl splenocytes **(Figure 2B)**.

Our results point on CB2 receptor as the main mediator for THC influence on lymphocytes. However, the effect CBD clearly does not involve CB2 signaling. Several mediators were suggested for CBD effects on mammalian cells. In search for the molecule that mediates CBD’s effect on lymphocyte activation, we used inhibitors for some of these molecules together with CBD in a CFSE lymphocyte proliferation assay. A967079, BCTC and GSK2193874 are antagonist to TRP channels TRPA1, TRPV1 and TRPV4 respectively, which were demonstrated to mediate CBD signaling ( ). However, our results demonstrate that none of these antagonists could interfere with CBD’s inhibitory effect on lymphocyte activation **(Supplementary figure 4A-C)**. Another molecule which was suggested as mediator for CBD signaling is the nuclear receptor PPAR-γ ( ). Indeed 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 tests the influence 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\*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 the mice tail 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 as compare to vehicle treated mice (control), 3 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 counts of monocytes and granulocytes were not affected by the treatment (data not shown). Platelets recovery was significantly improved only in the group that received THC BDS treatment, with a mean of 10% improvement as compare to control, 2 weeks after transplantation **(Figure 3D and E)**.

These results demonstrate that cannabis/cannabinoids treatments have an influence on hematological reconstitution after bone marrow transplantation and that different cannabinoid drugs have different effect.

**CB2 receptor is has an inhibitory effect on lymphocytes 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 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 the mice tail and CBC with differentials was performed. Our results demonstrate significantly improved lymphocytes recovery in the treated group **(Figure 4A)**.

To elucidate whether this effect is on the grafted cells or the accepting environment, we used CB2 KO mice as donors/acceptors in BMT experiments. The normal blood counts of CB2 KO female mice are similar to the WT C57BL/6 counts **(Supplementary figure 3B).** C57BL/6 mice underwent lethal whole-body irradiation and were reconstituted with 8\*106 donor CB2 KO or C57BL/6 BM cells the following day. We found significantly higher lymphocyte count in the group that received CB2 KO transplant as compare 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 evidences, as well as our in vitro assays **(Figure 1)**, indicate the anti-inflammatory function of cannabinoids ( ). Yeshurun, et.al demonstrated the beneficial effect of the cannabinoid CBD as 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 chemerism 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 smaller effect (**Figure 5B, left**). Moreover, GVHD scores were significantly lower in mice receiving cannabis extracts **(Figure 5C)**.

These results demonstrate *in-vivo* better modulation of allogeneic activation by cannabis extracts in comparison to 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. In this research, we have decided to compare the effect of the most abundant cannabinoids, THC and CBD, as well as cannabis extracts from THC rich plants and CBD rich plants. We used the extracts because these drugs are most commonly used by patients and also for their 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 bone marrow transplantation. 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 IL10 together with inhibition of IL17 secretion by all treatments, may indicate an influence on the Th17/Treg balance. Th17 cells are known to participate in the pathophysiology of graft versus host disease (van der wart 2014), as well as several autoimmune diseases and therefore this effect is most clinically relevant. Notably, our results are in correlation with previous data with CBD treatment in EAE mice model for multiple sclerosis and in animal model of asthma ( vuolo 2015, kozela). Interestingly, we did not find correlation between the effect of the treatment on cytokine secretion and its effect on proliferation. For example, 10µg/ml THC BDS reduced proliferation only by 25%, but induced relatively high secretion of the regulatory cytokine IL10.

We have utilized CB2 knockout mice and antagonists for 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 effect but it is not involved in the effect of CBD. The tested TRP channels were also not found to participate in CBD inhibitory function. The only receptor that was found to mediate part of CBD 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 was shown in different 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 lymphocytes CBD related signaling is yet to be found.

Several obstacles may interfere with the clinical outcome of bone marrow transplantation. 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 high importance since delayed lymphocyte re-constitution may affect the clinical outcome. On the other hand, THC BDS treatment improved platelets rehabilitation. The involvement of endocannabinoids in thrombogenesis was previously demonstrated (Patinkin 2008, grambow 2016, gasperi 2014). However, it is yet to be discovered 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 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 for 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 transplant, rather on the cells of the environment. Our results can possibly be explained by the role of cannabinoids in hematopoietic stem and progenitor cells 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 opposite to the higher effect of the pure cannabinoids in vitro and in the syngeneic transplantation model, the cannabis extracts, particularly the THC BDS, showed better effect in GVHD prophylaxis. This result joins the cytokine results from our *in vitro* experiments and the syngeneic model experiments to demonstrate that the effects of the extracts are different than the effect of pure cannabinoids. Two explanations can be made for this phenomenon. The unique effects of the extract can result from other molecules in the plant (not THC/CBD) or they can result from synergistic function of THC/CBD with other molecules.

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

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