Title: Cannabidiol (CBD) inhibits glioblastoma progression through regulation of tumor microenvironment in a murine model

Authors: Hesam Khodadadi[1,2], Évila Lopes Salles[1,2], Ahmet Alptekin[3], Daniel Mehrabian[4], Martin Rutkowski[5], Ali S Arbab[3], W.Andrew Yeudall[1,2], Jack C Yu MD[6], John C Morgan[7], David C Hess[8], Kumar Vaibhav[5], Krishnan M Dhandapani[5], and Babak Baban[1,2,6,8].

Affiliations:

1- Department of Oral Biology and Diagnostic Sciences, Dental College of Georgia, Augusta University, Augusta, GA, USA.
2- Center for Excellence in Research, Scholarship and Innovation, Dental College of Georgia, Augusta, Augusta University, Augusta, GA, USA.
3- Georgia Cancer Center, Augusta University, Augusta, 30912, GA.
4- Medical College of Georgia, Augusta University, Augusta, 30912, GA.
5- Department of Neurosurgery, Medical College of Georgia, Augusta University, Augusta, GA, USA.
6- Department of Surgery, Medical College of Georgia, Augusta University, Augusta, GA, USA.
7- Parkinson's Foundation Center of Excellence, Movement Disorders, Program, Department of Neurology, Medical College of Georgia, Augusta University, Augusta, GA, USA.
8- Department of Neurology, Medical College of Georgia, Augusta University, Augusta, GA, USA.

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Corresponding author: Babak Baban, Ph.D., MPH, MBA, FAHA.
-Department of Oral Biology and Diagnostic Sciences, DCG.
-DCG Center for Excellence in Research, Scholarship and Innovation (CERSI) Augusta University,
Augusta, GA, USA.
bbaban@augusta.edu

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Abstract

Introduction: Glioblastoma multiforme (GBM) is the most common invasive brain tumor composed of diverse cell types with poor prognosis. The highly complex tumor microenvironment (TME) and its interaction with tumor cells play important roles in the development, progression, and durability of GBM. Angiogenic and immune factors are two major components of TME of GBM, their interplay is a major determinant of tumor vascularization, immune profile, as well as immune unresponsiveness of GBM. Given the ineffectiveness of current standard therapies (surgery, radiotherapy, and concomitant chemotherapy) in managing patients with GBM, it is necessary to develop new ways of treating these lethal brain tumors. Targeting TME, altering tumor ecosystem may be a viable therapeutic strategy with beneficial effects for patients in their fight against GBM.

Methods: Given the potential therapeutic effects of cannabidiol (CBD) in a wide spectrum of diseases including malignancies, we tested for the first time, whether inhalant CBD can inhibit GBM tumor growth using a well-established orthotopic murine model. Optical imaging, histology, immunohistochemistry, and flow cytometry were employed to document the outcomes.

Results: Our findings showed that inhalant CBD was able to limit not only the tumor growth, but also to alter the dynamics of TME by repressing P-selectin, apelin, and IL-8, as well as blocking a key immune checkpoint -- indoleamine 2,3 dioxygenase (IDO). In addition, CBD enhanced the CD103 expression, indicating improved antigen presentation, promoted CD8 immune responses, and reduced innate Lymphoid Cells (ILCs) within the tumor.

Conclusion: Overall, our novel findings support the possible therapeutic role of inhalant CBD as an effective, relatively safe and easy to administer treatment adjunct for GBM with significant impacts on the cellular and molecular signaling of TME, warranting further research.

Introduction

Glioblastoma multiform (GBM), the most common malignant brain tumor, is highly invasive locally, recurs often, and has poor prognosis (1-3). Despite advances in cancer therapies, GBM remains incurable, with a median survival of only 15 months (1-5). Current standards of care for GBM, including surgery, radiotherapy, and chemotherapy, produce only limited responses (4). Therefore, an urgent need exists for the development of novel, more effective alternative therapeutic modalities in the treatment of GBM.

Tumor microenvironment (TME), a complex network of many cell types, blood vessels, lymphatics, and immune signaling, and extracellular matrices, plays significant and integral role in the progression of cancer (5-8). The interplay between angiogenic and immunogenic compartments within TME is of fundamental importance to tumor survival and, thus poor patient outcomes (7,9). Therefore, targeting angiogenesis and immunologic components may alter the ecosystem of TME with beneficial outcomes for patients with GBM. Recent studies have suggested a central role for apelin, an inotropic peptide with proangiogenic features during the progression of GBM (10-13). While there was a minimal level of apelin expression in normal brain tissues, however, apelin expression was significantly elevated in GBM. Inhibition of apelin have resulted in the decrease in the growth rate of GBM tumor volume(12-13). Several studies have reported a very distinctive immune profile within TME of glioblastoma, characterized by heightened immune checkpoint signaling, accumulated suppressive myeloid cells, and a decrease in effector lymphoid cells. There is a particularly low frequency of cytotoxic T cells (7,9,14). The reciprocal communication between immune compartment and non-immune components of TME (e.g., tumor cells, endothelial cells, vascular system) in GBM not only determines the status of the immune profile of TME, but also affects the vascularization, angiogenesis, and ultimately the longevity and viability of the tumor itself (7,9,15-16). Importantly, cellular Immunity, mediated by effector T cells is the major arm of immune system against tumor antigens and cancer progression (17-18). Inhibiting T cell activation, particularly auto-reactive CD8+ cytotoxic T cells, is a central immunomodulatory strategy by which immune checkpoints exert their suppressive role against anti-tumor immunity within GBM (5, 19-21). As an immune checkpoint, indoleamine 2,3,dioxygenase (IDO), a rate-limiting enzyme with

inhibitory effects on T cells, has emerged as a very attractive potential target in the immunotherapy of several types of cancer including GBM (4, 22-25). Due to its unique potential immunomodulatory function, IDO is considered a non-conventional immune checkpoint with overarching effects on chronic inflammation, antigen presentation, and immune-tolerance for tumor ecosystem. Along with IDO, recent studies have demonstrated that P-selectin may serve as an immune checkpoint within TME, promoting tumor growth in GBM (26-27). P-selectin is a transmembrane protein acting as a cell adhesion molecule on the surfaces of activated endothelial cells and platelets, providing the foundation for interplay between tumor cells and cellular components of the blood (28).

Further, the phylogenetically ancient, but newly discovered members of TME are Innate Lymphoid Cells (ILCs) (29-31). These special lymphoid cells mirror T-helper cells but possess neither T cell receptors nor lymphoid surface markers except CD45 (32-34). The role of ILCs in tumor development and cancer progression is controversial and yet to be elucidated (30-31,35). However, increasing evidence suggests a central role for ILCs (including natural killer, NK, cells) in GBM (36). Given the complexity and heterogeneity of glioblastoma, an alternative treatment to alter the TME by inhibiting Immune checkpoints may be a potential therapeutic modality with significant beneficial effects for patients with GBM.

Cannabidiol (CBD) is a relatively safe, non-psychoactive phytocannabinoid produced by cannabis plants. Recent work by our laboratory and others suggest a beneficial effect of CBD alone or in combination with other cannabinoids in the treatment of malignancies (37-41); however, few studies have investigated the efficacy of CBD as mono-therapy and/or as an adjunct with other, conventional, anti-cancer medications in the treatment of GBM (42-45).

In this study, we tested for the first time, the potential effect of inhalant CBD in the progression of glioblastoma in a murine model, and whether such treatment could alter the TME of glioblastoma. Our findings demonstrated the potential of inhalant CBD in the inhibition of tumor growth with alterations of TME in glioblastoma.

Materials and methods

Animals

Wild type (WT), 9–11 week old C57BL/6 mice (obtained from Jackson Laboratories, Bar Harbor, ME USA) were used to generate the orthotopic GBM model. The animals were housed in the laboratory animal facilities of the Augusta University with free access to food and water. All the experiments were performed according to the National Institutes of Health (NIH) guidelines and regulations. The Institutional Animal Care and Use Committee (IACUC) of Augusta University (protocol #2011–0062) approved all the experimental procedures.

Tumor cells and orthotopic animal model of GBM

To generate the orthotopic GBM model in mice, syngeneic GL261 cells were used as described previously (46). In brief, luciferase positive GL261 cells were grown in standard growth media (RPMI-1640 plus 10% FBS) and collected in serum-free media on the day of implantation. Mice were anesthetized using 3% isoflurane and maintained with 1.5%–2% isoflurane throughout all surgical procedures. After preparation and drilling a hole at 2.25 mm to the right and 1 mm posterior to the bregma, taking care not to penetrate the dura, a 10 µL Hamilton syringe with a 26G-needle containing tumor cells (30,000) in a volume of 3 µl was lowered to a depth of 4 mm and then raised to a depth of 3 mm. During and after the injection, a careful note was made for any reflux from the injection site. The needle was withdrawn 1 mm at a time in a stepwise manner 2–3 minutes after completing the injection. The surgical hole was sealed with bone wax. Finally, the skull was swabbed with betadine before suturing the skin. Postoperative analgesia was provided with a single injection of buprenorphine (1 mg/kg sc). Tumor growth was determined by optical imaging (bioluminescence imaging after injecting luciferin) on day 8 post-implantation.

Treatment protocol

The animals were further subdivided to receive either inhalant CBD or placebo (10 mg/day), delivered through an inhaler (ApelinDx, TM Global Bioscience, USA). Inhalant CBD or placebo was applied to the animals every day for a period of 8 days. At day 17 post-implantation, another set of imaging was performed before all animals were sacrificed, tumor tissues were harvested for histology and Immunohistochemical analysis as well as all flow cytometry-based assays.

Histology and Immunohistochemistry

Freshly harvested GBM tumor tissues were fixed with 10% neutral buffered formalin (Sigma HT50-1-128), processed and then embedded with conventional dehydrated paraffin. All subsequent procedures were performed at room temperature. Fixed paraffin-embedded tumoral tissues were cut in 4 µm sections and stained with hematoxylin and eosin (H&E) based on standard protocol of H&E staining, observed and analyzed by a bright field light Zeiss microscope. ImageJ Java-based image processing program was used for visualization and quantification of tumor size. Further Immunohistochemical assessment was carried out as described previously (47). In short, all slides were rehydrated and endogenous peroxidase activity was blocked using hydrogen peroxide diluted 1:10 with distilled water for 10 min. Sections were treated with Proteinase K for 10 min and washed twice in PBS. Next, slides were incubated with antibodies against apelin (Bioss cat# BS-2425R-A750), IL-8 (Biorbyt Cat# Orb360891), P-selectin (Biolegend Cat# 148309), IDO (SantaCruz Biotechnology Cat# SC-53978 AF594), CD103 (Biolegend Cat# 121415) and CD8 (BD Biosciences Cat# 553032) for two hours at room temperature. Biotinylated immunoglobulins (Biogenex cat# HK340-9K) were added to all slides for 20 min. After two washes with PBS, all slides were incubated with peroxidase-conjugated streptavidin

(Biogenex cat# HK330-9K) for 20 min followed by two washes in PBS. All slides then treated with chromogen (Dako cat# K3461) until the desired color appeared. Excess chromagen was decanted and all slides were washed by distilled water. All preparations were counterstained with Hematoxylin (ANATECH Ltd cat# 812) for 3 minutes and mounted in aqueous mountant (LERNER laboratories cat# 13800) prior to the analysis using bright field Zeiss (AXIO Imager M2) light microscope.

Analytical flow cytometry

For flow cytometry analysis, tumor tissues were placed in a tissue culture dish with 1 mL PBS + 2% FCS, 2 mg/mL of collagenase type II, and 1 mg/mL of DNase type I for 30 minutes at 37°C. Samples were then sieved through a cell strainer (BD Biosciences), followed by centrifugation (252g, 5 minutes, 4°C) to prepare single-cell suspensions. Cells then were subjected to flow cytometry analysis using a NovoCyte Quanteun and analyzed by FlowJo analytical software. Briefly, cells were gated as Lin−CD45+(mouse, catalog 103114, clone 30-F11) lymphocytes and a lineage cocktail of antibodies (all antibodies from BioLegend, unless otherwise noted) included FITC-conjugated anti-CD3 (mouse, catalog 100204, clone 17A2), anti-CD4 (mouse, catalog 100406, clone GK1.5), anti-CD14 (mouse, catalog 123308, clone Sa14-2), anti-CD16 (mouse, catalog 101305, clone 93), anti-CD19 (mouse, catalog 152404, clone 1D3/CD19), anti-CD8 (mouse, catalog 140404, clone 53-5.8), anti-CD15 (human/mouse, catalog 125611, clone MC-480), anti-CD20 (mouse, catalog 152108, clone SA271G2) were used for negative selection. Subsequently, ILC1s were identified as mouse (Lin−CD127+IL-12Rβ2+ [mouse/human, R&D Systems, catalog FAB1959P-100, clone 305719]) cells, ILC2s were identified as mouse (Lin−CD127+GATA3+) cells, and ILC3s were identified as mouse (Lin−CD127+RORγt+; mouse/human, Thermo Fisher Scientific catalog 17-6988-82, clone AFKJS-9) cells (all antibodies from BioLegend). Isotype-matched controls were analyzed to set the appropriate gates for each sample. For each marker, samples were analyzed in duplicate. To minimize false-positive events, the number of double-positive events detected with the isotype controls was subtracted from the number of double-positive cells stained with corresponding antibodies (not isotype control). Cells expressing a specific marker were reported as a percentage of the number of gated events. A population was considered positive for a specific marker if the population exceeded a 2% isotypic control threshold.

Statistics

For statistical analysis, Brown-Forsythe and Welch ANOVA was used to establish significance ($P<0.05$) among groups and for statistical analysis.

Results

- **Inhalant CBD inhibited tumor growth in GBM**

Tumor establishment was shown by optical imaging at pre-treatment stage (Fig 1a) prior to dividing mice into two groups of placebo or CBD treated groups. As demonstrated in figure 1b, photon intensities of optical imaging demonstrated that inhalant CBD was able to inhibit tumor growth in GBM compared to the placebo group. Additionally, histological assessment using hematoxylin and eosin staining (H&E) revealed a significant inhibition of tumor growth ($p<0.05$) in CBD treated mice versus placebo control group (Fig 1c).

- **Inhalant CBD suppressed angiogenic factors within GBM tumor**

Immunohistochemistry staining showed that inhalant CBD was able to repress the expression of apelin (Fig 2a) and P-selectin (Fig 2b) as well as Interleukin (IL)-8 (Fig 2c), in CBD treated group compared to the placebo group. Down-regulation of these proteins can potentially alter the TME, affecting the tumor angiogenesis negatively.

- **Inhalant CBD blocked Immune checkpoint signaling, altering the immune profile in GBM**

Further Immunohistochemistry analysis showed that inhalant CBD blocked the IDO expression (Fig 3a) while enhanced CD8 and CD103 expression compared to placebo treated group (Fig 3b-c). IDO reduction might have promoted anti-tumor immunity by increasing frequency of CD8+ cells and improving antigen presentation through heightened CD103 expression. Besides IDO, P-selectin is a non-classic Immune checkpoint which CBD was able to repress its expression, enhancing anti-tumor Immunity.

- **Inhalant CBD regulated ILCs, affecting local proliferation and activation of ILCs within TME**

Flow cytometry analysis showed that ILCs were down regulated significantly ($p<0.05$) in group treated with inhalant CBD compared to the placebo group (Fig 4). Since each cytokine produced by each class of ILCs can affect TME in a specific way, therefore reduction in ILCs would affect the interaction between ILCs and TME, altering the intra-tumor vascularization and immune profile.

Discussion

Our findings are significant and novel at several levels. This is the first study in which CBD is applied in an experimental model for the treatment of GBM. CBD is a relatively safe and naturally occurred compound (48-51). Therefore, such inhibitory effect of CBD on tumor growth offers a novel therapeutic modality with high relevance and significant clinical values in the treatment of GBM, the most fatal primary brain tumor. More importantly, the use of inhaler to deliver the CBD in non-invasive, precisely metered doses is highly translational to human trials.

Glioblastoma is a hypervascular type of tumor, highly depending on angiogenesis and vascularization (46,52). Therefore, targeting angiogenic factors has emerged as an attractive and potentially effective therapeutic modality in the treatment of GBM. In addition to cellular heterogeneity and matrix complexity

of TME in glioblastoma, the strategic location of GBM at central nervous system (CNS) within the brain blood barrier (BBB) zone has added to the intricacy and existing challenges of therapy for GBM. Results of our current studies showed that inhalant CBD could penetrate into the BBB, blocking angiogenic factors and altering the balance between stimulating versus inhibitory forces during the tumor angiogenesis within TME, resulting in the tumor growth inhibition. Our findings revealed for the first time, that inhalant CBD could block apelin, P-selectin and IL-8, all are able to influence angiogenesis and vascularization in GBM (). Apelin, a neuro-angiogenic peptide, promotes cancer metastasis through contribution to the tumor angiogenesis, promoting cancer stem cells and drug resistance (53). Further, the role of IL-8 and its receptors CXCR1/2 in the progression of several cancers including GBM have been demonstrated (54). Accordingly, as a potent angiogenic factor, IL-8 plays a crucial role in the progression as well as invasion of GBM, altering TME in glioblastoma, affecting angiogenesis process in an autocrine/endocrine fashion. Regulation of NF-κB, NO (nitric oxide) signaling, as well as the inhibition of crosstalk between IL-8 and the intra-tumor IL-6/VEGF are examples of potential mechanisms by which targeting IL-8 may limit the tumor growth in GBM (55-58). While several previous studies had shown the suppressive effect of CBD on IL-8 (59), however, our findings for the first time showed the down-regulation of IL-8 in GBM by inhalant CBD, supporting the notion that CBD may be used as an immunotherapeutic agent in the treatment of GBM. The other novel finding of our studies here was the blockade of P-selectin expression in GBM after CBD treatment. Several reports have already indicated the role of P-selectin in the progression of GBM (26-27). P-selectin is a vascular adhesion molecule contributing to the cancer development by facilitating the cancer-endothelial cells interactions, enhancement of myeloid cell recruitment and promoting crosstalk between cancer cells and platelets (28,60). By blocking P-Selectin, inhalant CBD not only interrupted the basic P-selectin functions, but it also re-structured the glioblastoma TME. Additionally, besides the traditional role of P-selectin as a cell adhesion molecule and conciliator of cellular recruitment, several recent studies have reported that P-selectin may function as an Immune checkpoint through its receptor, PSGL-1, by regulating T cells and curtailing the Immuno-inflammatory responses (61). Interestingly, our findings revealed that in addition to P-selectin, CBD was able to reduce the IDO expression within TME. IDO functions as an Immune checkpoint by depletion of tryptophan, an essential amino acid, regulating T effector cells and promoting Tregs induction. Given the previous reports indicating the significance of IDO inhibition in limiting GBM development (4, 62-63), therefore, the potential of CBD in down-regulation of IDO may be an effective immunotherapeutic strategy in the treatment of GBM, requires further investigations. Importantly, suppression of Immune checkpoints in CBD treated animals was associated with higher CD103 and CD8 expression. As a member of integrins family, Integrin E (ITGAE) also known as CD103 (cluster of differentiation 103) plays crucial roles in a variety of biological processes including limiting tumor growth (64-65). Several studies have demonstrated the association between heightened intratumoral level of CD103 and improved outcomes for cancer patients (65-66). Consistent with our findings, CD103-expressing dendritic cells have been shown to possess higher quality of antigen presentation, resulting in more effective recruitment of CD8+ T cells and greater anti-tumor immunity with better prognosis as well as more favorable clinical consequences (65-66).

Additionally, our studies here showed that CBD reduced the frequencies of ILCs within TME of glioblastoma. Due to their fast-reacting feature to the microenvironmental stimulators, ILCs are considered as central modulatory cells during inflammatory responses (67-68). While increasing evidence indicates crucial roles for ILCs in cancer, however, the exact roles of ILCs in cancer is controversial and not fully understood (35, 68-70). Several studies have reported that TME of certain cancers are enriched with ILCs compared to the scant numbers of ILCs in normal tissues and circulation (68). Our findings here in this study are the first report demonstrating the high frequencies of ILCs within TME in glioblastoma. Importantly, CBD treatment was able to reduce ILCs significantly in GBM compared to the placebo group. Further, ILCs are heterogenic and plastic innate cells with high capabilities in crosstalking with all components of TME (68-70). Therefore, CBD-induced regulation of ILCs in GBM tmours has the potential to be considered as an effective immunotherapeutic strategy in the treatment of GBM.

In conclusion, our findings suggest that inhalant CBD can inhibit the tumor growth of GBM by re-shaping and establishing an anti-tumor dynamic within TME of GBM. Given the urgency to explore new therapeutic strategies for more effective treatment of GBM, leveraging the modulatory and protective capacities of CBD seems a potent option to help patients with GBM. Our data definitively warrants further research in this area.

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Figure Legends:

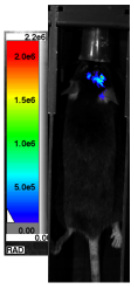
Figure 1. Inhalant CBD inhibits tumor growth in GBM a) Optical imaging (bioluminescence imaging after injecting luciferin) showed the establishment and growth of GBM tumor at day 8 post implantation. b) Tumor growth was inhibited after 8 consecutive daily treatments of inhalant CBD compared to the placebo treated group. c) H&E staining (left panel) demonstrated significant decrease in tumor size after CBD treatment compared to placebo. The difference in tumor size between CBD and placebo treated groups ($p<0.05$) was visualized and quantified using ImageJ Java-based image processing program (middle and right panels).

Figure 2. Inhalant CBD alters tumor microenvironment (TME), repressing angiogenic factors in GBM. Immunohistochemical staining of paraffin embedded GBM tumor tissues showed inhalant CBD decreased expression of angiogenic factors: a) P-Selectin, b) apelin and c) IL-8 significantly compared to the placebo treated group. All images have been obtained using bright field Zeiss (AXIO Imager M2) light microscope, magnifications of 200X and 630X.

Figure 3. Inhalant CBD modulates immune checkpoints within TME in glioblastoma, altering the intra-tumoral immune profile. Immunohistochemical staining of paraffin embedded GBM tumor tissues showed inhalant CBD blocked the immune checkpoint, IDO (a) while enhanced CD8 (b) and CD103 (c) expression compared to the placebo treated group. All images have been obtained using bright field Zeiss (AXIO Imager M2) light microscope, magnifications of 200X and 630X.

Figure 4. Inhalant CBD decreased innate lymphoid cells frequencies in glioblastoma, regulating local proliferation and activation of ILCs within TME. Flow cytometry analysis showed that inhalant CBD was able to reduce frequencies of ILCs within TME of glioblastoma significantly ($p<0.05$), potentially improving anti-tumor immunity. ILCs were characterized as $CD45^{+}$Lineage negative (CD3ε, CD11b, CD24, CD5, CD11c, CD19, NK1.1, Gr-1, TER119 and gd TCR), were gated from the live forward scatter/side scatter (FSC/SSC) leukocytes (CD45+) as $Thy1^{+}CD90.2^{+}CD127^{+}$.

No Treatment



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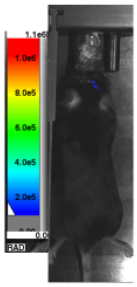
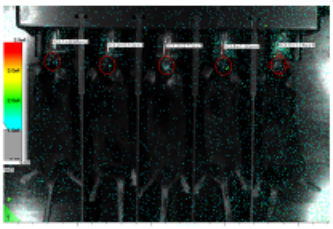
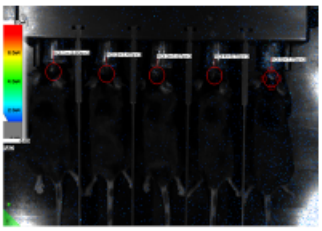


No Treatment

CBD

After 1 week





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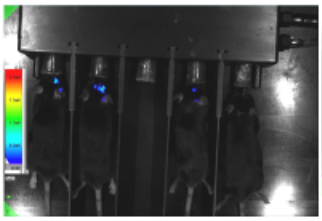

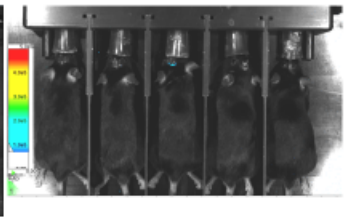

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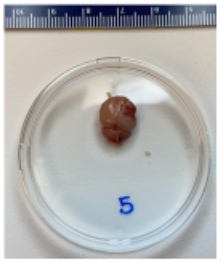

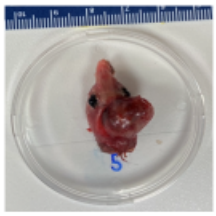

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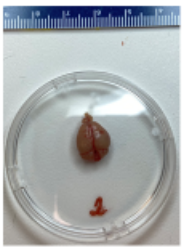
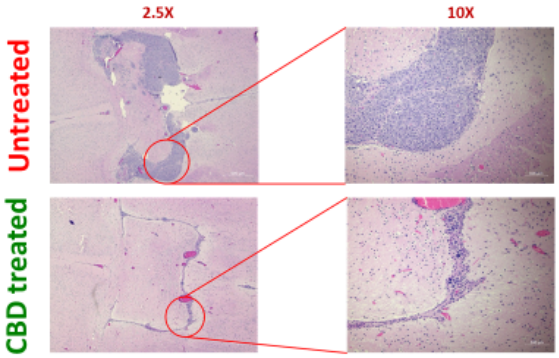
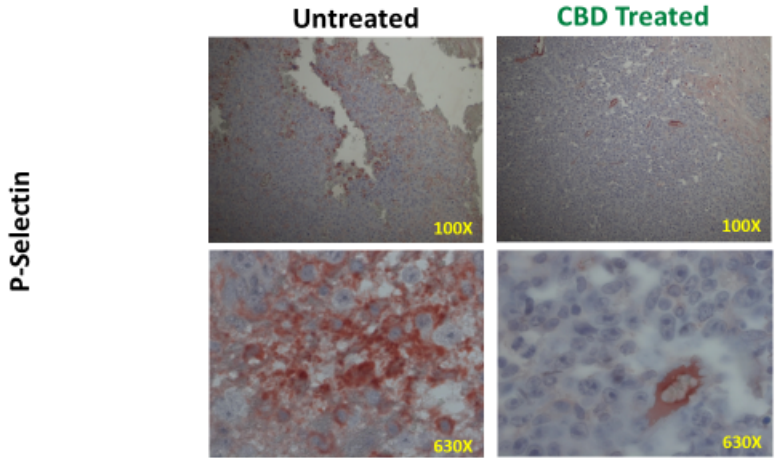
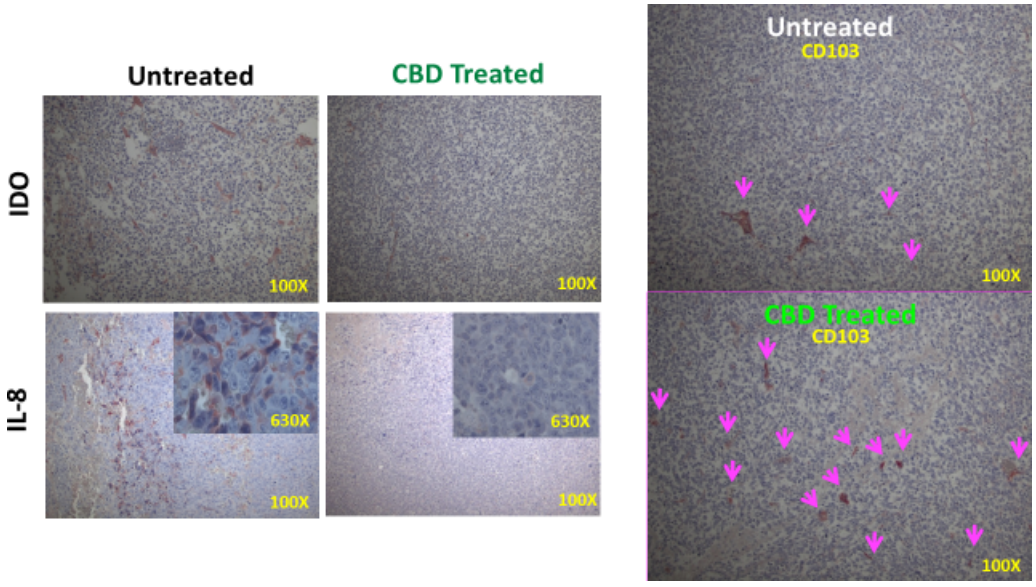



2.5X
10X
Untreated
CBD treated


Untreated
CBD Treated
P-Selectin
100X
100X
630X
630X


Untreated
CBD Treated
IDO
IL-8
100X
100X
630X
630X
100X
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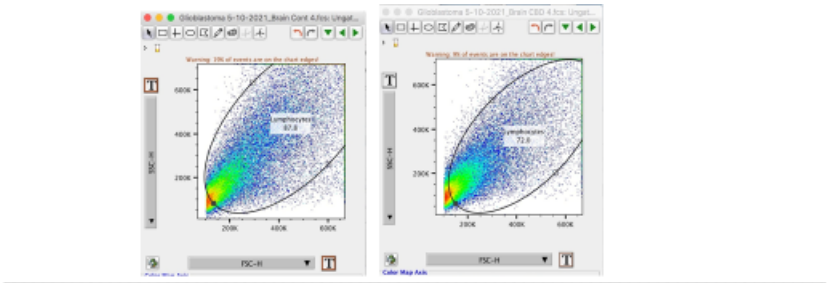
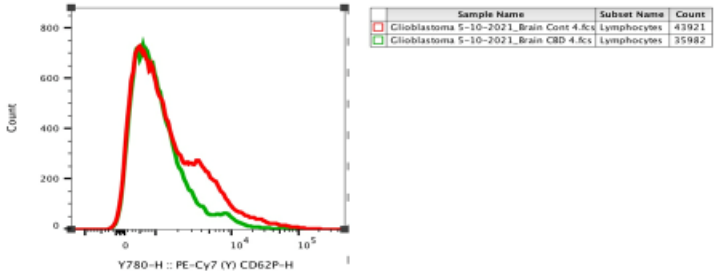
Untreated
CD103
100X
CBD Treated
CD103
100X





Supplemental