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Cannabidiol Enhances Intestinal Cannabinoid Receptor Type 2 Receptor Expression and Activation Increasing Regulatory T Cells and Reduces Murine Acute Graft-versus-Host Disease without Interfering with the Graft-versus-Leukemia Response[S]

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ABSTRACT

Cannabidiol (CBD) is a highly lipidic phytocannabinoid with remarkable anti-inflammatory effects. The aim of this study was to evaluate CBD's effects and mechanisms of action in the treatment of mice subjected to acute graft-versus-host disease (aGVHD). aGVHD was induced by the transplantation of bone marrow cells and splenocytes from C57BL-6j to Balb-c mice. The recipient mice were treated daily with CBD, and the treatment reduced mouse mortality by decreasing inflammation and injury and promoting immune regulation in the jejunum, ileum, and liver. Analysis of the jejunum and ileum showed that CBD treatment reduced the levels of C-C motif chemokine ligand (CCL) 2, CCL3, CCL5, tumor necrosis factor α, and interferon γ (IFNγ). CCL3 and IFNγ levels were also decreased in the liver. Mechanistically, CBD also increased the number of cannabinoid receptor type 2 (CB_2) receptors on $CD4^+$ and forkhead box $P3^+$ cells in the intestine, which may explain the reduction in proinflammatory cytokines and chemokines. Antagonists of the CB_2 receptor reduced the survival rates of CBD-treated mice, suggesting the participation of this receptor in the effects of CBD. Furthermore, treatment with CBD did not interfere with the graft-versus-leukemia response. CBD treatment appears to protect aGVHD mice by anti-inflammatory and immunomodulatory effects partially mediated by CB_2 receptor interaction. Altogether, our study suggests that CBD represents an interesting approach in the treatment of aGVHD, with potential therapeutic applications in patients undergoing bone marrow transplantation.

SIGNIFICANCE STATEMENT

This study provides for the first time a mechanism by which cannabidiol, a phytocannabinoid with no psychoactive effect, induces immunomodulation in the graft-versus-host disease. Enhancing intestinal cannabinoid receptor type 2 (CB_2) receptor expression on $CD4^+$ and forkhead box $P3^+$ cells and increasing the number of these regulatory cells, cannabidiol decreases proinflammatory cytokines and increases graft-versus-host disease mice survival. This effect is dependent of CB_2 receptor activation. Besides, cannabidiol did not interfere with graft-versus-leukemia response, a central response to avoid primary disease relapse.

Introduction

The main complication after allogeneic hematopoietic cell transplantation (alloHSCT) is graft-versus-host disease (GVHD), a systemic inflammatory condition secondary to alloHSCT that leads to significant morbidity and mortality in humans (Major-Monfried et al., 2018). AlloHSCT is the transplantation of cells between two genetically nonidentical individuals (Beatty et al., 1995; Ljungman et al., 2006) and is a curative therapy for several malignant and nonmalignant diseases, including leukemia (Gale, 1981), lymphoma (Curtis et al., 1997; Hartmann et al., 1997), thalassemia (Lucarelli et al., 1990), aplastic anemia (Locasciulli et al., 2007), and autoimmune diseases, such as lupus (Marmont, 1994; Gladstone et al., 2017).

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The authors declare no conflict of interest in this study.

This work was previously presented in the following scientific meetings: 4th International Symposium on Inflammatory Diseases (INFLAMMA IV), International Congress In Cannabinoid Medicine (CannX), XXVI Encontro de Pesquisa em Fisiologia and Farmacologia; Brazilian Congress of Pharmacology and Experimental Therapeutics (SBFTE), and The 5th Congress on Controversies in Stem Cell Transplantation and Cellular Therapies (COSTEM), with the work being rewarded in the last two.

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GVHD occurs because of histoincompatibilities identified by T cells that react against host tissues causing extensive damage (Ho and Soiffer, 2001). Classically, acute GVHD (aGVHD) occurs within 100 days after transplantation, and chronic GVHD occurs after this period. However, nowadays, aGVHD is defined mainly by erythematous rash, gastrointestinal symptoms, cholestatic hepatitis, immune suppression, T cell infiltration, and tissue apoptosis (Zeng, 2018). The pathophysiology of aGVHD is complex and involves several inflammatory cytokines whose release starts during the conditioning regimen (irradiation and/or chemotherapy), leading to a proinflammatory environment that promotes T cell activation, proliferation, and differentiation against target organs (Schroeder and DiPersio, 2011). About 50% of patients with GVHD will die because of disease complications, and this is matched only by aGVHD's high incidence rate among patients who undergo alloHSCT (Barton-Burke et al., 2008; Yang et al., 2017). Although alloHSCT leads to a higher chance of developing aGVHD, it also provides a stronger graft-versus-tumor (GVT) reactivity by which the new immune system recognizes and mediates the immunologic eradication of reminiscent cancer cells (Noriega et al., 2015; van Bergen et al., 2017). Therefore, GVT reactivity represents a strong plea in the recommendation of alloHSCT as a curative therapy for malignant conditions (Kolb, 2008).

The current treatment of aGVHD includes high doses of steroids, thus promoting host immune suppression but also leading to several adverse effects, such as reduced GVT reactivity, so new therapeutic approaches are urgently needed (Devetten and Vose, 2004; Shlomchik, 2007; Devergie and Janin, 2008; (Poetker and Reh, 2010); Peppa et al., 2011; Jamil and Mineishi, 2015). Meanwhile, cannabinoids emerge as strong pharmacological candidates for several pathologies, such as multiple sclerosis (Kozela et al., 2011), colitis (Borrelli et al., 2009), and inflammatory bowel disease (Schicho and Storr, 2012), but their psychoactive effects restrict their widespread therapeutic use. Cannabidiol (CBD), an abundant phytocannabinoid of *Cannabis sativa* with well known anti-inflammatory effects, is not psychoactive (Häußermann and Wagner, 2017; Adams et al., 2018) and is currently in a phase II study for the treatment of GVHD in humans with reports of benefits from CBD treatment, such as increased survival (Yeshurun et al., 2015). Furthermore, although there have been attempts to identify CBD's mechanisms of action in murine models (Khuja et al., 2019), several gaps remain regarding the alterations triggered by CBD in the host organism and immune system. Therefore, the aim of this study was to investigate how CBD treatment can lead to an increase in mouse survival and by what mechanisms it can modulate the immune response regarding murine aGVHD.

Materials and Methods

Animals. All the experiments were performed on 8-week-old male Balb-c mice, and the donor group consisted of 8-week-old male C57BL/6j mice from the Center of Bioterism, Federal University of Minas Gerais, Belo Horizonte, Brazil. The animals were housed under a 12-hour light/dark cycle with food and water available *ad libitum*. The animal procedures were approved by the Ethics Committee on Animal Experimentation of the Federal University of Minas Gerais (337/2014) and were carried out according to the Guide for the Care and Use of Laboratory Animals. The mice were euthanized through cervical displacement.

The sample size was defined based on the sample variance of three different GVHD-induction experiments previously performed by our group (Castor et al., 2010)(Castor et al., 2011). For the sample size, we used the following equation:

$$n = \left(\frac{\left[\frac{z\alpha}{2} * \sigma\right)}{e}\right)^2$$

in which n represents the sample size, $z\alpha/2$ is the relative value from the z table, e is the absolute error, and σ is the standard deviation. To obtain a reliability of 95%, we needed a sample size of five mice per group for our experiments.

Disease Induction. For bone marrow ablation, the mice were irradiated with 7 Gy of γ radiation before transplantation and received 3×10^7 splenocytes and 1×10^7 bone marrow cells intravenously either from the C57BL/6j donor mice (and thus developed GVHD) or from the Balb-c donor (and thus did not develop GVHD); the mice receiving cells from the Balb-c donor were considered the control group. The cells were acquired according to Castor et al. (2010). The mice were randomly divided into the groups. After induction, the mice were monitored daily using a clinical standard scoring system consisting of seven points: hunching/posture, activity, fur texture, weight loss, skin integrity, diarrhea, and occult blood in the feces. The scoring was given according to the grade of clinical manifestation, with 0 = normal, 1 = minor alteration, 1.5 = moderate alteration, and 2 = severe alteration, as described previously (Castor et al., 2010). To avoid animal suffering, we standardized 10 points as the cutoff point.

Drugs. The mice receiving C57BL/6j cells (allogeneic transplant) were treated either with CBD (THC-Pharm, Frankfurt, Germany and STI-Pharm, Brentwood, UK) in doses of 10, 30, and 60 mg/kg or its vehicle (5% Tween80 in sterile PBS-1×). The participation of cannabinoid receptors was assessed by a treatment with AM251 (Tocris) 1 mg/kg or AM630 (Tocris) 1 mg/kg (35), being CB_1 and cannabinoid receptor 2 (CB_2) antagonists, respectively. The vehicle of both these antagonists contained 2.5% DMSO in PBS-1× and CBD's vehicle. All drugs were administered by a daily intraperitoneal injection starting on the day of disease induction and continuing for the duration of the protocols.

Histopathology. On day 7, the intestine and liver were fixed and stained with H&E. The entire extension of the jejunum and ileum walls and the liver were analyzed blindly by a fellow pathologist under an optical microscope (BX51; Olympus, Tokyo, Japan). Numerical values were assigned to changes observed in each of the intestinal layers (the mucosa, lamina propria, muscular, and serosa layers). The epithelium was scored as follows: 0 = without alterations, 1 = reactive and aspecific alterations, 2 = erosion or loss of the architecture of the region of crypts or the surface of the epithelium, and 3 = nuclear changes and hyperplasia in the crypt or surface epithelium, with or without evidence of ulceration and loss of globet cells. The other layers were scored as follows: 0 = normal aspect; 1 = discrete inflammatory cell infiltration; 2 = moderate inflammatory cell infiltration, swelling, and congestion; 3 = severe inflammatory infiltration and villous enlargement with swelling and congestion in the lamina propria; and 4 = ischemic necrosis and intense inflammatory alterations in the muscular and serosa layers. Each animal received a total score

ABBREVIATIONS: aGVHD, acute GVHD; alloHSCT, allogeneic hematopoietic cell transplantation; CB_1, cannabinoid receptor type 1; CB_2, cannabinoid receptor type 2; CBD, cannabidiol; CCL, C-C motif chemokine ligand; CFU, colony-forming unit; DAPI, 4,6-diamidino-2-phenylindole; FACS, fluorescence-activated cell sorting; FITC, fluorescein isothiocyanate; Fox, forkhead box; GVHD, graft-versus-host disease; GVT, graft-versus-tumor; IFNγ, interferon γ; IL, interleukin; LSD, least significant difference; PE, Phycoerythrin; PerCP, Peridinin-chlorophyll-protein; TNFα, tumor necrosis factor α; Treg, regulatory T cell.

generated by the summation of the three individual scores (maximum index 9). In the liver, there were degenerative alterations in the parenchyma, and a numerical value was assigned to the changes observed: 0 = normal; 1 = discrete cytoplasmatic vacuolization and focal eosinophilia; 2 = diffuse vacuolization, alterations in hepatocyte form, and moderate nucleus alterations; and 3 = hepatocyte necrosis and diffuse vacuolization, alterations in hepatocyte form, and accentuated nucleus alterations. Inflammatory infiltration of the liver was scored as follows: 0 = none or rare, 1 = discrete presence of infiltrates in the periportal area, 2 = discrete or moderate presence of infiltrates in the periportal and intralobular areas, and 3 = accentuated presence of infiltrates in the periportal and intralobular areas. Each animal received a total score generated by the summation of the two individual scores (maximum index 6).

Bacterial Translocation. Intraperitoneal wash was performed on the 7th day. The mice were anesthetized with 80 μl of ketamine/xylazine (2:1), and the washing was performed with 1 ml of a sterile solution of PBS 1×. The wash was incubated overnight in a Müller-Hilton agar medium at 37°C, and the number of colony-forming units (CFUs) was counted. The mean values of the CFUs are shown.

ELISA. On the 7th day, the mice were euthanized, and 100 mg of the intestine and liver were collected for the ELISA assay. The samples were then processed following the manufacturer's instructions (R&D System) and were read in a wavelength of 490 nm in a spectrophotometer, and the absorbance value was determined for each sample. We evaluated CCL2, CCL3, CCL5, TNFα, and IFNγ.

Flow Cytometry. Seven days after disease induction, the intestine, spleen, liver, and bone marrow were collected and processed as previously described (Resende et al., 2017). Cells were counted, and a total of 1×10^6 cells were stained with the following antibodies: CD3 [Pacific Blue (RRID-17-A2), BioLegend], CD4 [PE-Cy7 (RRID-RM45), BioLegend], CD8 [FITC (RRID-53-6.7), BioLegend], CD25 [allophycocyanin (RRID-PC61), BioLegend], and CD28 [PE-A (RRID-37.51), BioLegend]. For cell surface markers, we used FoxP3 [PE-A (RRID-MF14), BioLegend], $H2D^b$ [AlexaFluor 647 (RRID-KH95), BioLegend], and $H2D^d$ [PE (RRID-34,212), BioLegend) for the bone marrow to evaluate chimerization. A total of 100,000 events were considered in the FACS analysis for each sample, and the cells were acquired with a BD FACSCanto II cytometer and analyzed using FlowJo V10 software (BD Immunocytometry Systems, San Jose, CA).

Immunofluorescence. At day 7 after transplant, intestinal slices were processed to immunofluorescence assay. The antibodies to CD4, CD8, FoxP3, and CB_2 described on supplemental material (Supplemental Fig. 1) were used to investigate the presence of CB_2 on these cells and whether cannabidiol treatment could induce CB_2 expression on them. The nuclei were marked with 4,6-diamidino-2-phenylindole (DAPI) (D1306-Carlsbad; Life Technology). Slides were mounted using 60% glycerol. Zen microscope was used to acquire images with suitable filters for selectively detecting the fluorescence of FITC (green), AlexaFluor 488 (green), AlexaFluor 594 (red), PE (red), or DAPI (blue) (Gonzaga et al., 2017). For each tissue section, 10 regions were randomly captured by an independent experiment-blinded user at 100× magnification, totaling 50 pictures per group (n = 5). Images were analyzed using ImageJ software v 1.53c 1.41 (National Institutes of Health, public domain).

Graft-versus-Tumor Response. On the day of GVHD induction, after radiation, the mice received murine mastocytoma $P815^+$ cells intravenously (H-2d; American Type Culture Collection, Rockville, MD–RRID TIB-64) transduced with an EF1aGFP vector (National Institute of Cancer, Rio de Janeiro, Brazil) in a concentration of 5×10^4 cells. The mice were treated with a vehicle or CBD for 7 days. The spleen and lymph nodes (mesenteric and inguinal) were separately processed in the FACS analysis by flow cytometry (FacsScaibur). The number of tumor cells was evaluated by the percentage of GFP^+ fluorescence.

Statistical Analysis. Statistical analysis was performed with GraphPad Prism 8.0.2 software. Normality was tested by the Shapiro-Wilk test, and outliers were identified by the Grubbs test (α = 0.05). The samples were tested by one-way ANOVA followed by Fisher's LSD post hoc test to address differences between groups. In the survival curves, the differences were assessed by the log-rank (Mantel-Cox) test. Samples were considered different when $P < 0.05$.

Results

Engraftment and Mouse Survival. Major histocompatibility complex (MHC) disparities between animals led to the development of GVHD (Fig. 1A). The most effective dose of CBD was chosen by a daily treatment with 10, 30, or 60 mg/kg. Survival was assessed daily (Fig. 1B). We found no difference between CBD 10 mg/kg and the vehicle. Regarding the other doses, the CBD–30 mg/kg group displayed the highest survival (80%), with the CBD–60 mg/kg group showing a survival rate of 40%. CBD 30 mg/kg was clearly the most effective dose when compared with CBD 10 mg/kg (P = 0.0127), but there was no statistically significant difference between CBD 30 mg/kg and CBD 60 mg/kg (P = 0.2207). The clinical parameters of the mice were evaluated for 20 days and translated into a score (Fig. 1C). In this way, a dose of 30 mg/kg was chosen to be used for the next experimental procedures. During our experiments, the CBD–30 mg/kg group displayed a variation from 60% to 80% in survival, and the mortality in the vehicle group varied from 12 to 18 days.

Furthermore, the bone marrow samples collected 7 days after disease induction revealed that the CBD–30 mg/kg treatment did not interfere with engraftment, as assessed by the percentages of the histocompatibility antigens $H2D^b$ and $H2D^d$ in the CBD and vehicle groups. The vehicle group presented 75% of $H2D^b$, and the mice treated with CBD presented 69% of $H2D^b$ (Fig. 1D).

Cellular Recruitment, Activation, and Damage. The spleen, intestine, and liver were collected for analysis. Flow cytometry of the spleen (Supplemental Fig. 2) revealed no difference in the total number of lymphocytes (Fig. 2A) or in the number of $CD8^+$ cells among the groups (Fig. 2C). Additionally, we found an increased number of $CD4^+$ cells in the vehicle group when compared with the control group, but there was no difference between the CBD and the vehicle group in this respect (Fig. 2B). Moreover, we found no difference in the activation of $CD4^+$ cells (Fig. 2D), but $CD8^+$ cells were found to be increased in the vehicle group when compared with the control group. This profile was not altered by CBD treatment (Fig. 2E), and the number of $CD4^+$, $CD25^+$, and $FoxP3^+$ cells, collectively known as Tregs, was not different between the groups (Fig. 2F).

The H&E slices of the small intestine revealed that CBD treatment reduced damage to the tissue, preserving intestinal crypts, reducing inflammatory infiltration, preventing edema, and improving the general tissue score when compared with the vehicle group (Fig. 3, A–D). Supportive of this improvement in the small intestine, intraperitoneal washing also revealed that although mice from the vehicle group, as compared with the control group, had an increased number of CFUs translocating to the peritoneal cavity, CBD treatment prevented translocation, protecting the intestinal barriers (Fig. 3E).

Flow cytometry of the small intestine (Supplemental Fig. 3) revealed an increased number of total lymphocytes in the CBD group in comparison with the vehicle group; however,

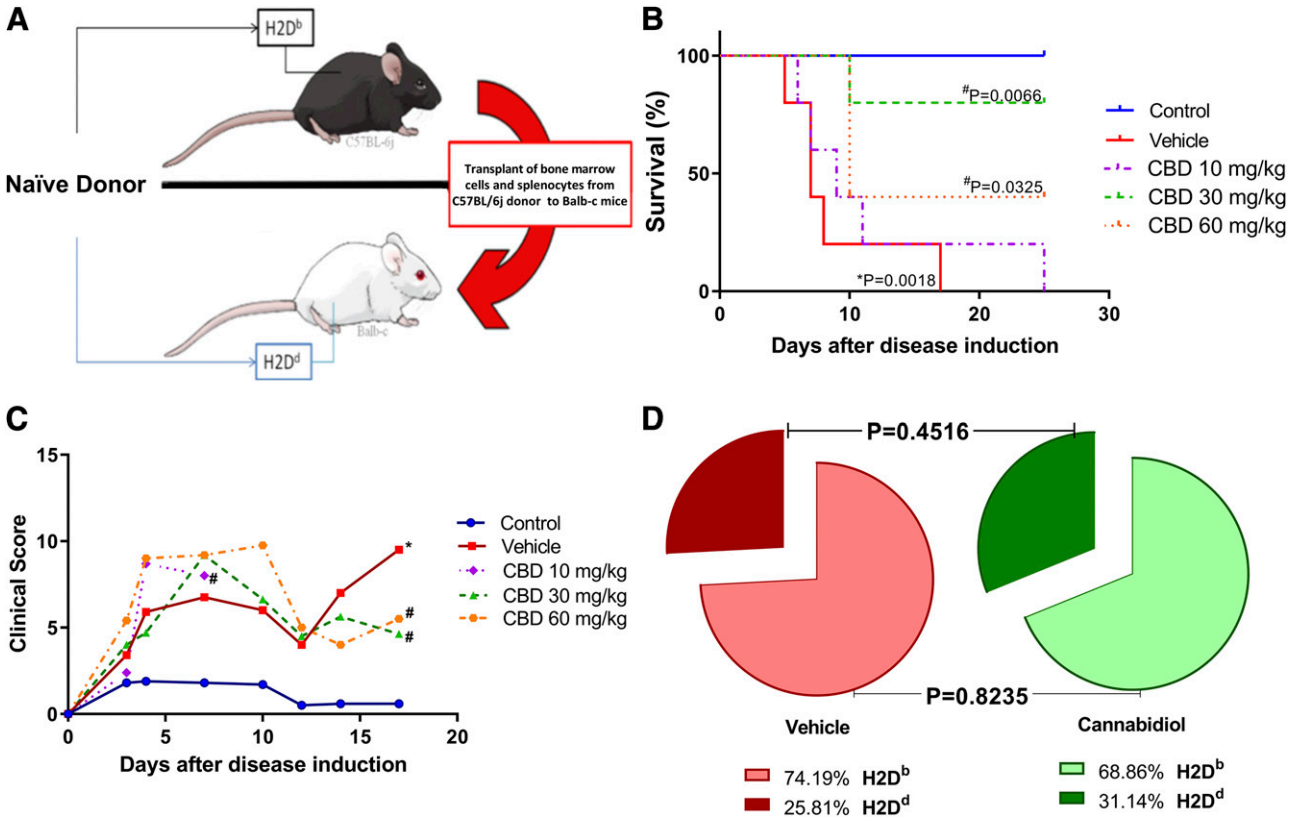

Fig. 1. Clinical aspects of experimental aGVHD. (A) Allogeneic transplant was performed from C57BL6j mice with $H2D^b$ phenotype to Balb-c mice with $H2D^d$ phenotype. (B) Mice were treated with CBD 10 mg/kg, CBD 30 mg/kg, CBD 60 mg/kg, or vehicle, and their survival was assessed daily. (C) Daily assessment of mice through clinical score. (D) Bone marrow engraftment was verified in vehicle and CBD 30 mg/kg 7 days after the transplant through flow cytometry. * indicates $P < 0.05$ when compared with control group and # indicates $P < 0.05$ when compared with the vehicle. Data are expressed in dispersion with mean, and statistical difference was determined by Student's t test for engraftment and log-rank (Mantel-Cox) test for survival. $n = 5$ for all groups

the vehicle group had not an increased number of total lymphocytes in comparison with the control group (Fig. 4A).

Both $CD4^+$ cells (Fig. 4B) and $CD8^+$ cells (Fig. 4C) were increased in the CBD group when compared with the vehicle group, but there was no difference between the vehicle and the control group. Interestingly, there was no difference in the activation of $CD4^+$ cells between the CBD and the vehicle group, but in the latter group, the activation of $CD4^+$ cells was increased in comparison with the control group (Fig. 4D). As for $CD8^+$ cells, the vehicle group revealed an increase in activation when compared with the control group, and CBD did not prevent this activation (Fig. 4E). Interestingly, the CBD group displayed a higher number of Tregs when compared with the vehicle group, and the latter group showed no difference from the control group in this respect (Fig. 4F). Next, at the same time point, we verified the cytokine and chemokine profiles of the mice. The vehicle group displayed an increase in CCL2, CCL3, TNFα, IFNγ, and IL-10, whereas CBD, surprisingly, led to a reduction in CCL2, CCL3, CCL5, TNFα, IFNγ, and IL-10 in the intestine (Fig. 4, G–L).

The same parameters were evaluated in the mice liver 7 days after disease induction. Liver slices stained with H&E showed that CBD, as compared with the vehicle, was able to reduce inflammatory infiltration in the periportal and intralobular regions near the centrilobular veins of the liver (Fig. 5, A–D). In addition, ELISA in the liver showed that CBD decreased the levels of CCL2, CCL3, and IFNγ (Fig. 5 E, F, and I). Flow cytometry of the liver (Supplemental Fig. 4) revealed an increased number of lymphocytes in the CBD group when compared with the vehicle group (Fig. 5K), and this was also the case for $CD4^+$ cells (Fig. 5L). However, we found no difference in $CD8^+$ cells among the groups (Fig. 5M). Nevertheless, when activation was assessed by $CD28^+$ staining, the $CD4^+$ cells were found to be increased in the vehicle group when compared with the control group, whereas in the CBD group the activation was higher than in the vehicle group (Fig. 5N). The $CD8^+$ cells presented the same low level of activation in all groups (Fig. 5O). Moreover, in the liver, the CBD group did not display a difference in the number of Treg cells when compared with the vehicle group, and neither did the vehicle group when compared with the control group (Fig. 5P).

Graft-versus-Leukemia Response. The mice received $P815GFP^+$ mastocytoma cells on the day of disease induction. After 7 days, the response was assessed by flow cytometry. The control group received only tumor cells and a syngeneic transplant. In this group, we observed an increase in $P815GFP^+$ cells in the spleen and lymph nodes, indicating tumor growth (Fig. 6, A and B). The vehicle group received tumor cells and allogeneic bone marrow and splenocytes, leading to a reduction in the number of GFP^+ tumor cells compared with the control group (Fig. 6, A and B). In the same way, CBD treatment did not interfere with the allograft response against tumor cells ($P815GFP^+$) since the presence

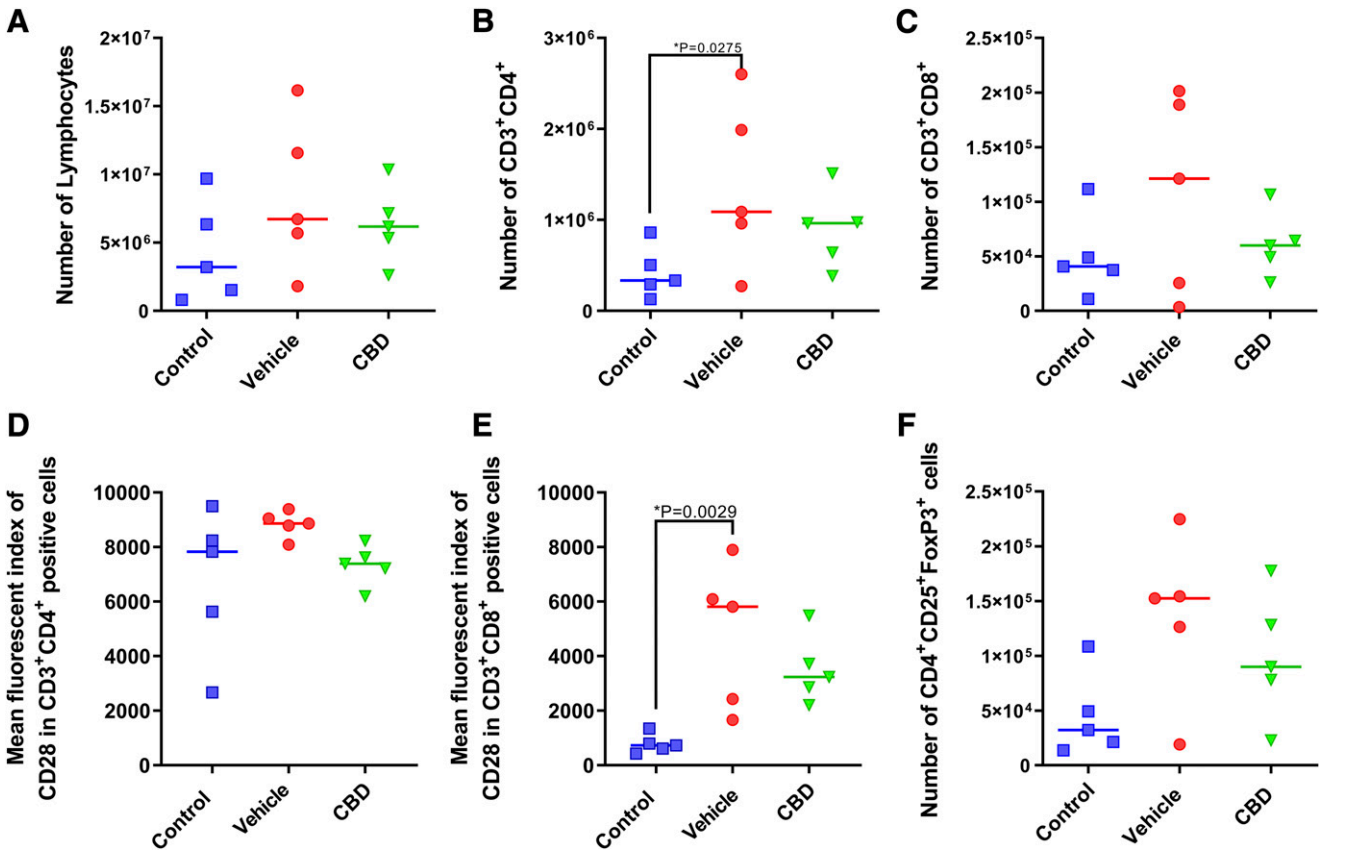

Fig. 2. Flow cytometry of mice spleen 7 days after disease induction. (A) Total lymphocytes; (B) $CD3^+CD4^+$ cells; (C) $CD3^+CD8^+$ cells; (D) $CD3^+CD4^+CD28^+$; (E) $CD3^+CD8^+CD28^+$; and (F) $CD4^+CD25^+FoxP3^+$. * indicates $P < 0.05$ when compared with control group and # when $P < 0.05$ when compared with vehicle. Data are expressed in dispersion with mean, and statistical difference was determined by ANOVA with Fisher's LSD post hoc. $n = 5$ for all groups

of tumor cells was severely reduced in the spleen (Fig. 6A) and lymph nodes (Fig. 6B) of the mice that received CBD when compared with the mice in the control group, preserving the graft-versus-tumor response.

CBD's Effect Is Dependent on the CB_2 Receptor. The mice were treated with CBD, CBD + AM630, CBD + AM251 or AM630, and AM251 alone, with AM630 being a selective antagonist of the CB_2 receptor and AM251 being a selective antagonist of the CB_1 receptor, and the survival of the mice was assessed. Interestingly, the mice that received AM251 with CBD survived, as did the mice from the CBD group (Fig. 7 A). Furthermore, both the vehicle group and the vehicle CBD + vehicle antagonist group were different from the control group ($P < 0.001$). The CBD group was different from its vehicle ($P = 0.0043$) but was not different from the CBD + AM251 group, and this last group was different from the vehicle CBD + vehicle antagonist group ($P = 0.0054$).

Surprisingly, we found that the mice treated with AM630 together with CBD exhibited a lower survival rate than the mice treated with CBD alone (Fig. 7B). Moreover, the vehicle group's survival was significantly different from the control group's ($P < 0.001$); the same was found when the control group was compared with the vehicle CBD + vehicle AM630 group ($P < 0.001$). There was no difference between the vehicle CBD + vehicle AM630 group and the CBD + AM630 group. Finally, the CBD + AM630 group was different from the CBD group ($P = 0.034$). Additionally, the mice treated with either AM251 (Fig. 7C) or AM630 (Fig. 7D) were not different from their respective vehicle groups and did not display the same increase in survival as the mice that received CBD treatment.

To verify CB_2 receptor expression in the target organs, we performed an immunofluorescence assay in the intestine, a major target organ of GVHD. CB_2 expression is not altered by GVHD induction. However, CBD treatment increases CB_2 receptor expression in the intestinal cells (Fig. 7E). As FACS analysis, immunofluorescence showed an increased number of $CD4^+$ cells in the CBD group compared with GVHD and control group (Fig. 7E). Positive colocalization of CD4 and CB_2 staining was increased in CBD group in comparison with both control and GVHD groups (Fig. 7E; Supplemental Fig. 5). This result was also found regarding FoxP3 with CB_2 staining (Fig. 7F; Supplemental Fig. 6). However, although CD8 was also increased in CBD when compared with the other groups, CB_2 receptor expression was found to be increased only in comparison with control and not GVHD group ($P = 0.1232$), and there was no difference between CBD and GVHD groups remarking colocalization (Fig. 7 G; Supplemental Fig. 7). Pearson's coefficient was found to be mainly positive in CD4/CB_2 analysis (control: 0.197 ± 0.127; GVHD: 0.161 ± 0.103; CBD: 0.333 ± 0.143; with no difference between groups), CD8/CB_2 analysis (control: -0.0393 ± 0.169; GVHD: 0.275 ± 0.162; CBD: 0.253 ± 0.311; with no difference between groups), and regarding FoxP3 (control: 0.119 ± 0.075; GVHD: 0.179 ± 0.081; CBD: 0.233 ± 0.081; with statistical difference between control and CBD: $P = 0.0418$).

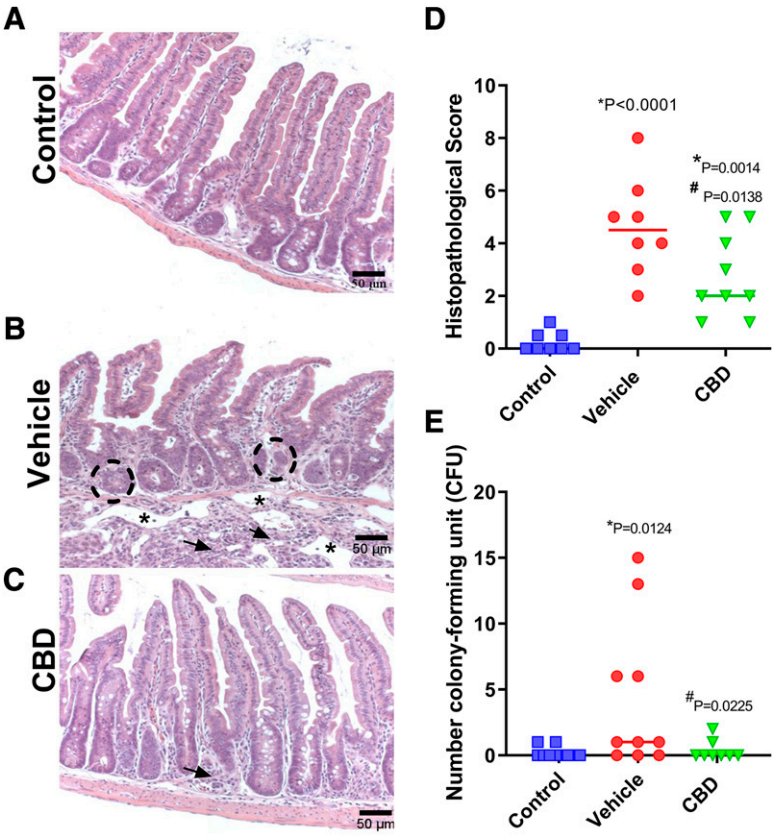

Fig. 3. Intestines stained with H&E, (A) control, (B) vehicle, and (C) CBD; stars represent edema, the dashed circle represents intestinal crypt, and arrows point to mononuclear infiltrate. (D) Quantification of the histopathological score; the preservation of the intestine morphology is translated in (E) reduced bacteria translocation. Data are expressed in dispersion with mean, and statistical difference was determined by ANOVA with Fisher's LSD post hoc; $n = 5$ for all groups.

Discussion and Conclusions

First, we evaluated three different doses of CBD (10, 30, and 60 mg/kg) based on the published literature (De Filippis et al., 2011; Campos et al., 2015; Vilela et al., 2015; Ahmed and Katz, 2016) to determine the most appropriate dose for the treatment of aGVHD. The mice treated with CBD 30 mg/kg, however, were not free from the disease, as assessed by the clinical score, but displayed a reduced disease progression and mortality when compared with the vehicle group. Not only that, but when chimerism was assessed, CBD did not affect bone marrow engraftment, as the animals recovered to display the same percentage of bone marrow cells as the animals in the vehicle group, which was verified both in the bone marrow itself and in the spleen, a secondary lymphoid organ.

The absence of a functional immune system would have left the mice susceptible to opportunistic diseases and is proven to be of the highest importance when considering possible treatments for GVHD in humans (Jamil and Mineishi, 2015; Admiraal et al., 2017). Therefore, in the present study, the presence of active and proliferating T cells in the spleen reinforces that CBD does not lead to an immunosuppressive status, as it does not lead to a reduction in T $CD4^+$ and $CD8^+$ cells when compared with the vehicle group. Jamil and Mineishi (2015) highlighted the importance of not entirely suppressing GVHD because it is related to a higher GVT response, which will protect the recipient from reminiscent tumor cells. The presence of transplanted cells in secondary lymphoid organs is important for the protective effect of the GVT response; however, it also relates to aGVHD development (Tkachev et al., 2018). In our study, CBD treatment was able to protect target organs against damage without interfering with the GVT response. These first two results are of the utmost importance when considering CBD usage in both prevention and treatment of patients who undergo alloHSCT.

$CD4^+$ cells are responsible for generating both thymic-derived and inducible Tregs (Ferrara et al., 2017). Tregs are characterized by the presence of $CD4^+$, $CD25^+$, and $FoxP3^+$ markers and promote tolerability, suppressing alloreactive lymphocytes. This regulation is key to aGVHD suppression/reduction (Blazar et al., 2012). Like our findings, the results reported by Edinger et al. (2003) of an experimental model of Treg transfer showed that even though Tregs are able to reduce aGVHD, they did not affect the GVT response, probably because of cytolytic effector T cells, especially $CD8^+$ cells in the lymphoid organs (Schneidawind et al., 2013). $CD8^+$ cells or T cytotoxic cells are vital to antitumoral immunologic responses (Uri et al., 2018). In this regard, we attested that CBD treatment does not reduce the migration of $CD4^+$ or $CD8^+$ cells to lymphoid and target organs. In our study, the main organ evaluated was the intestine, which is a major target organ of aGVHD and is regarded as one of the main environments for antigen presentation, acting as a barrier against commensal microbiota (Peled et al., 2016). CBD protected the intestinal barriers by preventing the migration of bacteria into the peritoneal cavity, whereas in the vehicle group, the intestinal barrier was inefficient in preventing this translocation; owing to intestinal damage, bacteria were found in the peritoneal cavity (Fig. 3E).

In the intestine, CBD led to an increase in $CD4^+$ and $CD8^+$ populations, although there was no increase in the activation status of these cells. Another important difference between

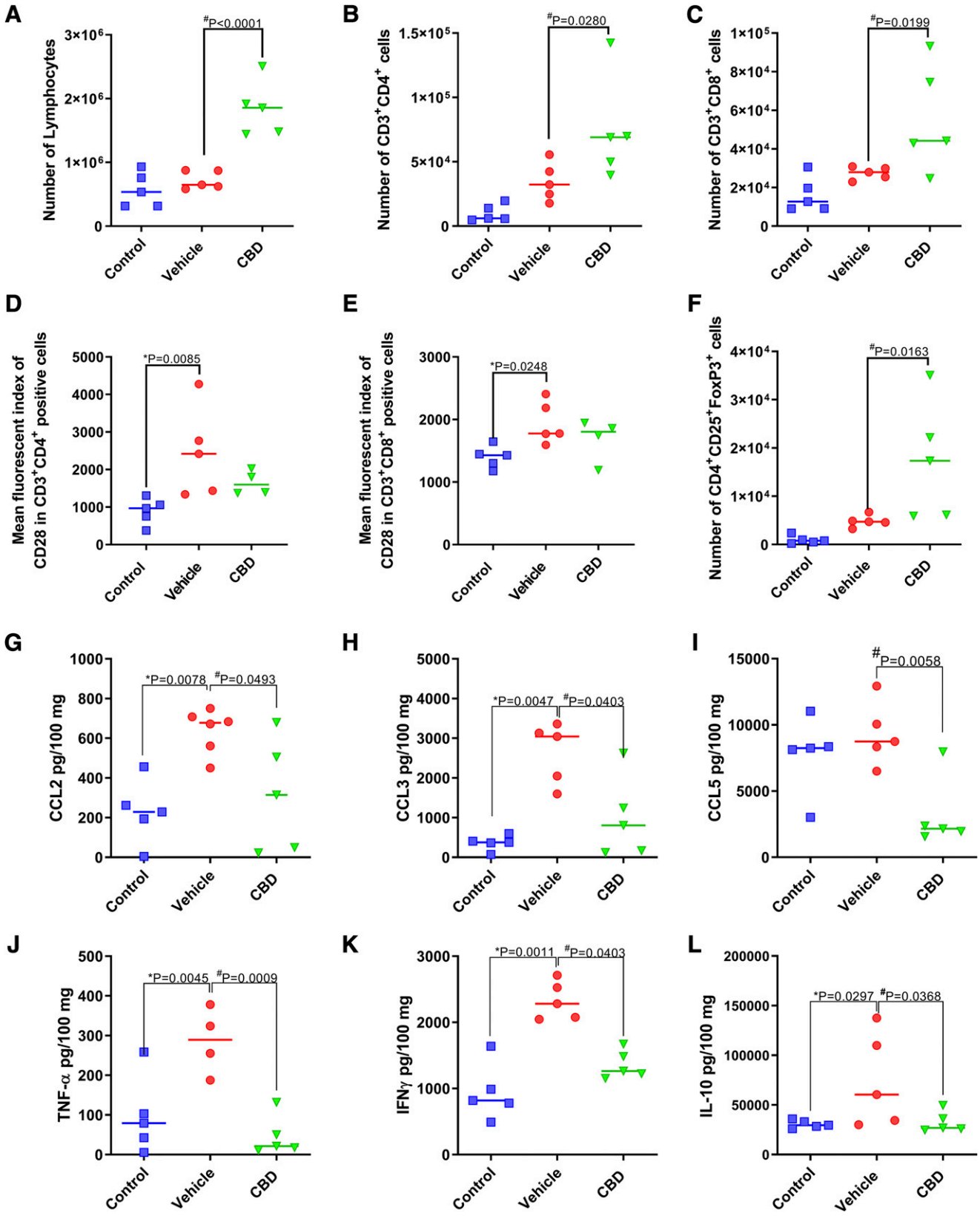

Fig. 4. Flow cytometry and ELISA of mice small intestines 7 days after disease induction. (A) Total lymphocytes; (B) $CD3^+CD4^+$ cells $n = 5/5/5$; (C) $CD3^+CD8^+$ cells $n = 5/5/5$; (D) $CD3^+CD4^+CD28^+$ $n = 5/5/4$; (E) $CD3^+CD8^+CD28^+$ $n = 5/5/4$; (F) $CD4^+CD25^+FoxP3^+$ $n = 5/5/5$ cells; (G) CCL2, (H) CCL3, (I) CCL5, (J) TNFα, (K) IFNγ, and (L) IL-10. Data are represented in dispersion graphs with mean; $n = 5$ for all groups, except when specified. * indicates $P < 0.05$ when compared with control group and # when $P < 0.05$ when compared with vehicle. Data are expressed in dispersion with mean, and statistical difference was determined by ANOVA with Fisher's LSD post hoc.

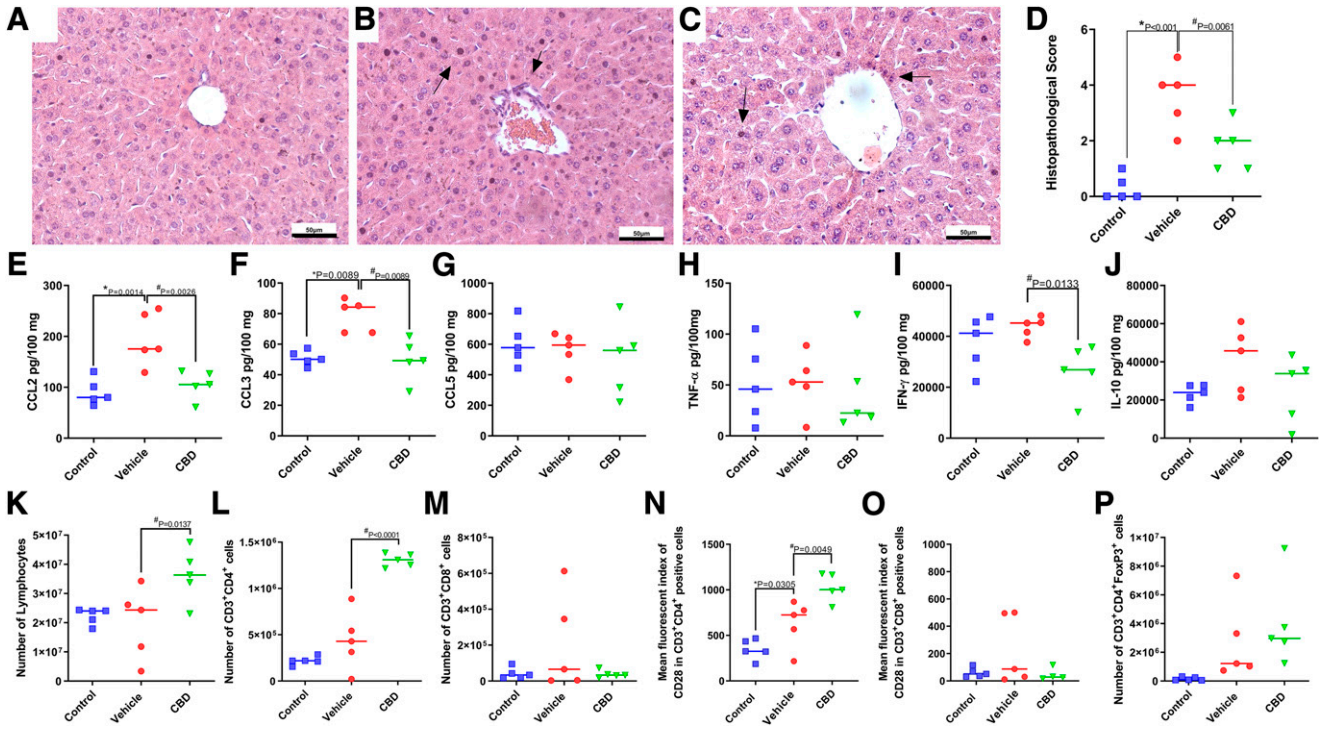

Fig. 5. Liver analysis. H&E from groups (A) control, (B) vehicle, and (C) CBD; dark arrows represent mononuclear infiltrate. (D) Quantification of histopathological score; ELISA of (E) CCL2, (F) CCL3, (G) CCL5, (H) TNFα, (I) IFNγ, and (J) IL-10; flow cytometry displaying (K) total lymphocytes; (L) $CD3^+CD4^+$ cells; (M) $CD3^+CD8^+$ cells; (N) $CD3^+CD4^+CD28^+$ cells; (O) $CD3^+CD8^+CD28^+$ cells; and (P) $CD4^+CD25^+FoxP3^+$ cells. * indicates $P < 0.05$ when compared with control group and # when $P < 0.05$ when compared with vehicle. Data are expressed in dispersion with mean, and statistical difference was determined by ANOVA with Fisher's LSD post hoc; $n = 5$ for all groups.

CBD and the vehicle is that CBD promotes Treg cells in the intestine, a factor that may elucidate the reduction in intestinal damage and the level of proinflammatory cytokines (TNFα and IFNγ) and chemokines (CCL2, CCL3, and CCL5). The chemokines CCL2, CCL3, and CCL5 are mandatory for recruitment of inflammatory cells to the target organs (Castor et al., 2010; Blazar et al., 2012; Ferrara et al., 2017). In this way, we have investigated whether the recruitment process was impaired in CBD-treated mice. Using an intravital assay, the rolling and adhesion of inflammatory cells on intestinal microvasculature were examined. There was no difference between vehicle- and CBD-treated mice (Supplemental Fig. 8).

Based on the findings by Panoskaltsis-Mortari et al. (2004), effector cells migrate first to the intestine and later to the liver. We hypothesize that $CD4^+$ activation and the further differentiation of $CD4^+$ cells into $FoxP3^+$ cells would also occur later in the liver and that, therefore, the number of $CD4^+$ cells were not increased on the 7th day of the disease in vehicle. The timing of the disease in the liver explains why we found that CCL2, CCL3, and IFNγ were increased only in the vehicle group; interestingly, this increase was prevented by CBD. Tregs have been described to suppress cytokine production of both $CD4^+$ and $CD8^+$ effector cells (Helling et al., 2015), a process that elucidates our findings regarding the ELISA assay in the intestine. Additionally, the presence of Tregs is necessary for the promotion of immune tolerance, preventing an overwhelming graft immune response after alloHSCT (Becker et al., 2007; Beres and Drobyski, 2013). To our

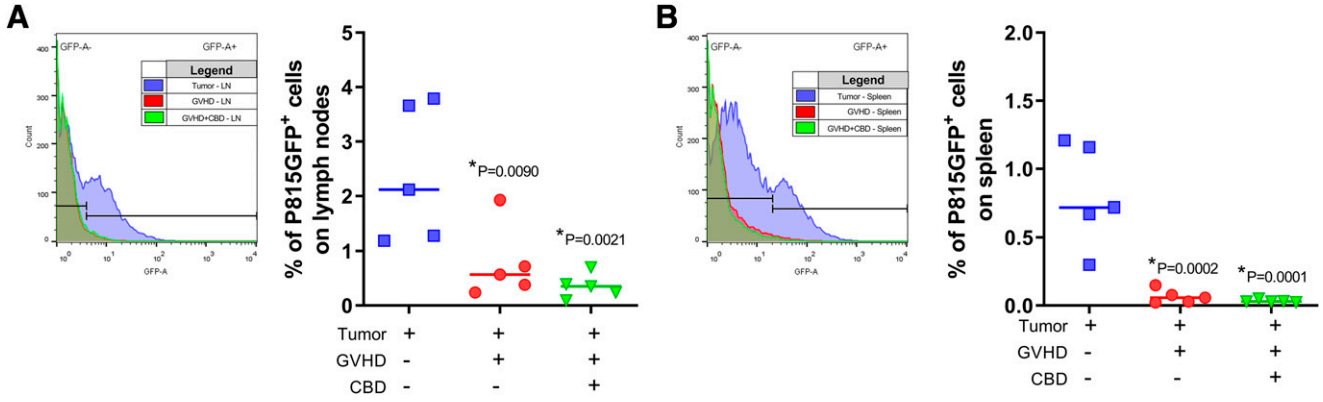

Fig. 6. Flow cytometry of mice spleen and lymph nodes (LNs) 7 days after disease induction and $P815GFP^+$ cells transplant. (A) Percentage of $P815^+$ cells in lymph nodes. (B) Percentage of $P815^+$ cells in spleen. Absolute number of $P815^+$ cells are represented in dispersion graphs next to flow-cytometry shards; $n = 5$ for all groups.

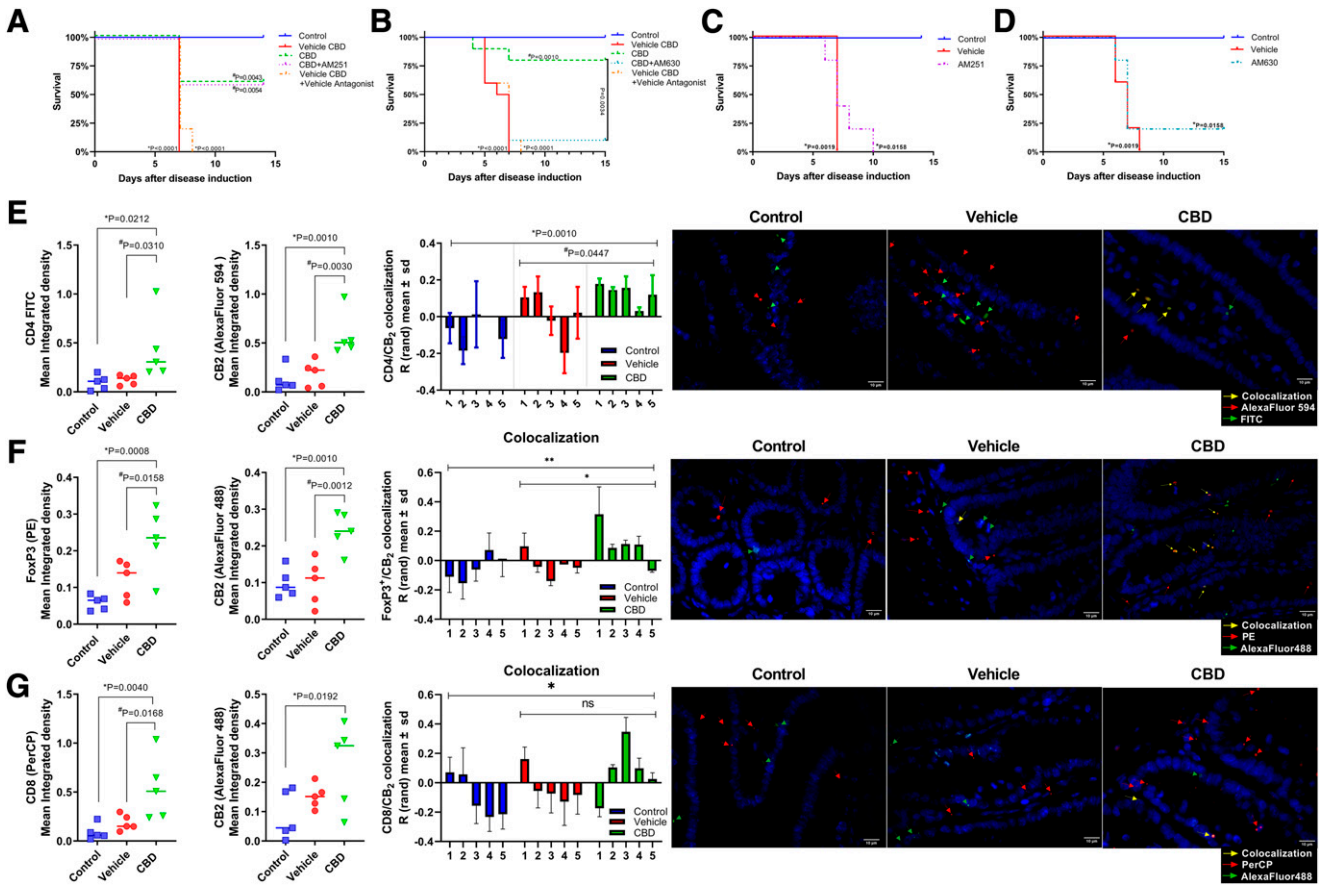

Fig. 7. Role of CB_2 in the protective effect of CBD. (A) CBD in the presence of CB_1 antagonist; (B) CBD in the presence of CB_2 antagonist; (C) treatment with CB_1 antagonist without CBD; (D) treatment with CB_2 antagonist without CBD. (E) CD4 and CB_2 mean integrated density, colocalization of CD4/CB_2, and representative figures of groups (CD4 FITC, CB_2 AlexaFluor 594, and DAPI); (F) CD8 and CB_2 mean integrated density, colocalization of CD8/CB_2, and representative figures of groups (CD8 PerCP, CB_2 AlexaFluor 488, and DAPI); (G) FoxP3 and CB_2 mean integrated density, colocalization of FoxP3/CB_2, and representative figures of groups (FoxP3, CB_2 AlexaFluor 488, and DAPI). Differences between groups were assessed with log-rank (Mantel-Cox) test for $P < 0.05$; n = 10/10/5/5 for survival and ANOVA with Fisher's LSD post hoc; n = 5 for all groups in immunofluorescence. ns, not significant.

knowledge, this is the first time that CBD has been shown to increase $CD4^+$, $CD25^+$, and $FoxP3^+$ cells in an experimental model of aGVHD, an effect that elucidates part of the modulatory mechanism of the inflammatory response and the increase in mouse survival and organ protection.

Another interesting mechanism of CBD is that in our model the CB_2 but not the CB_1 receptor is partially responsible for mouse survival, and we believe that it may also be responsible for the immunomodulatory effects of CBD. Both receptors are classified as cannabinoid receptors, as they are sensitive to

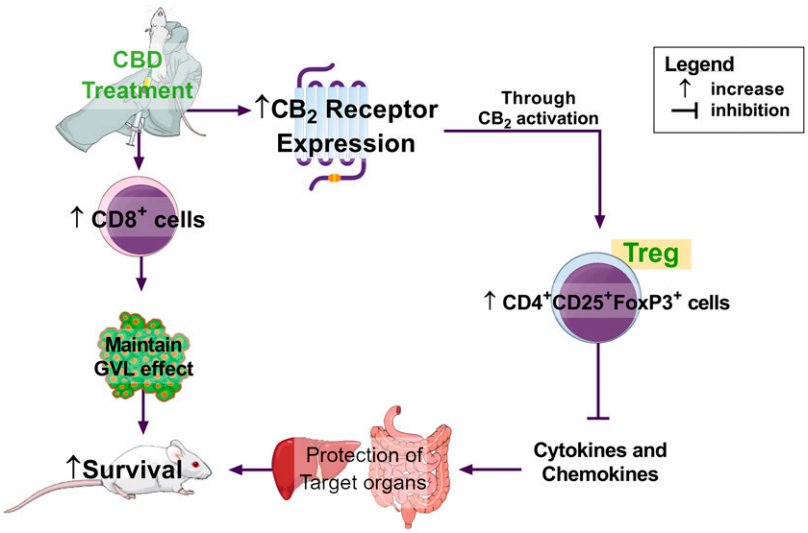

Fig. 8. Schematic representation of our findings. CBD treatment, partially by CB_2 receptor activation, increased the number of Tregs in the intestines and reduced cytokine/chemokine release. Together these factors led to the protection of target organs and the increase in mice survival.

both endogenous cannabinoids and some phytocannabinoids; however, they unleash different effects in the host organism. The CB_1 receptor is present mainly in the central nervous system, whereas the CB_2 receptor is more abundant in the peripheral nervous system and immune cells (Adams et al., 2018). The presence of CB_2 in immune cells is correlated with its modulatory effects, including cytokine inhibition and reduction in antigenic presentation (Ehrhart et al., 2005)(Horváth et al., 2012). CB_2 receptor is expressed on nearly all immune cells, and its absence on donor $CD4^+$ or $CD8^+$ T cells or administration of a selective CB_2 pharmacological antagonist exacerbated acute GVHD lethality (Yuan et al., 2021). CBD has about 74% higher affinity for CB_2 than for CB_1 (Rosenthaler et al., 2014). In this way, we investigated the effect of CBD on CB_2 receptor expression on intestine. CBD treatment was able to induce an increase of CB_2 receptor expression on $CD4^+$ and $FoxP3^+$ cells in the intestine of mice subject to GVHD. The correlation between CD4 and CB_2 but also that of FoxP3 and CB_2 further reinforce our hypothesis that cannabidiol might stimulate FoxP3 differentiation by interaction with CB_2 receptor. These findings reinforce CB_2's role in regulation of GVHD and suggest an interesting pharmacological therapeutic targeting.

In the present study, the effects of CBD may be associated with CB_2 activation. However, it is important to highlight that CBD can exert different effects upon a vast number of different receptors (Pertwee, 2006) and that, therefore, additional mechanisms may underlie the full modulatory effects of CBD. Lastly, in our model of aGVHD, CBD treatment was able to increase survival and reduce the damage of target organs. The increase of CB_2 receptor in the intestine leads to an immunoregulatory environment with an enhanced number of Treg cells and a reduction in the major GVHD proinflammatory mediators. Moreover, CBD increases the number of $CD8^+$ cells maintaining the graft-versus-leukemia effect. Therefore, it may be concluded that CBD represents an interesting approach in the treatment of aGVHD (Fig. 8).

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Authorship Contributions

Participated in research design: Berg, Romero, Pinho, Teixeira, Castor.

Conducted experiments: Berg, Soares, Paiva, Castor.

Contributed new reagents or analytic tools: Rezende, Cau, Romero, Pinho, Teixeira.

Performed data analysis: Berg, Soares, Rezende, Rachid, Castor.

Wrote or contributed to the writing of the manuscript: Berg, Pinho, Teixeira, Castor.

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Supplemental Data

This are the supplemental figures from the manuscript number **MS ID#: JPET/2019/263699**

The Journal of Pharmacology and Experimental Therapeutics (JPET)

Cannabidiol Enhances Intestinal CB2 Receptor Expression and Activation Increasing Regulatory T Cells and Reduces Murine Acute Graft-Versus-Host Disease without Interfering with The Graft-Versus-Leukemia Response

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Supplemental Figures

Supplemental Figure 1. Antibodies used in immunofluorescence. Information regarding antibodies used in immunofluorescence assay, their dilution and source.

Supplemental Figure 2. Flow cytometry analysis of spleen. (A) Mix 1 regards the analysis of cell surface markers, such as CD3, CD4, CD8 and the activation marker CD28. (B) Mix 2 includes CD4 and CD25 as cell surface markers, and the intracellular FoxP3 for the characterization of Tregs.

Supplemental Figure 3. Flow cytometry analysis of small intestine. (A) Mix 1 comprehends CD3, CD4, CD8 and CD28, all cellular surface markers. (B) Mix 2 includes CD4, CD25 and FoxP3 in order to characterize T regulatory lymphocytes.

Supplemental Figure 4. Flow cytometry analysis of liver. (A) Mix 1 included CD3, CD4 and CD8 for the characterization of local lymphocytes, and CD28 as an activation marker. (B) Mix 2 regards T regs and was composed of CD4, CD25 and FoxP3 markers.

Supplemental Figure 5. Immunofluorescence of CD4/CB2 in small intestine. CD4 marker is stained with FITC (green arrow) and CB2 receptor with AlexaFluor594 (red arrow). In yellow it is possible to perceive colocalization of these markers (yellow arrow). For the staining of cell nuclei DAPI was used (blue marker). The last column displays a zoomed detailed part of the Merge image.

Supplemental Figure 6. Immunofluorescence of FoxP3/CB2 in small intestine. FoxP3 marker is stained with PE (red arrow) and CB2 receptor with AlexaFluor488 (green arrow). In yellow it is possible to perceive colocalization of these markers (yellow arrow). For the staining of cell nuclei DAPI was used (blue marker). The last column displays a zoomed detailed part of the Merge image.

Supplemental Figure 7. Immunofluorescence of CD8/CB2 in small intestine. CD8 marker is stained with PerCP (red arrow) and CB2 receptor with AlexaFluor488 (green arrow). In yellow it is possible to perceive colocalization of these markers (yellow arrow). For the staining of cell nuclei DAPI was used (blue marker). The last column displays a zoomed detailed part of the Merge image.

Supplemental Figure 8. Intravital analysis after GVHD was induced in Balb/c mice using C57BL/6J GFP+ mice as donors. At day 7 after transplant, mice were anesthetized intraperitoneally with 15 mg/Kg of xylazine and 80mg/Kg of Ketamine diluted in PBS autoclaved, and the mesentery exposed in a perfusion system containing PBS (pH 7.4) at 37^{o} C. Only, 30 minutes before intravital assay, mice that received C57BL/6J GFP+ splenocytes were treated with 200µL of CBD vehicle (5% of Tween 80) or CBD 30 mg/kg. An intravital confocal microscope (Nikon, ECLIPSE 50i, 20x *objective lens*) was used to examine the mesenteric microcirculation. The images were recorded for playback analysis using Fiji-ImageJ with *plugin* NIS Elements-Nikon Imaging software (NIS ELEMENTS-NIKON). Intestinal venules (±40 µm) were selected and the number of rolling and adherent leukocytes determined offline during video playback analysis. Rolling leukocytes were defined as those cells moving at velocity less than that of erythrocytes within a given vessel. The flux of rolling cells was measured as the number of rolling cells passing by a given point in the venule per minute. A leukocyte was adherent if it remained stationary for at least 30s, and total leukocyte adhesion was quantified as the number of cells in the intravascular space within area of 100 µm. Number of rolling cells per minute and number of adherent cells per 100um is presented as the mean ± SEM (n= 6-8). * for $P<0.05$ when compared to the vehicle and CBD treated group.

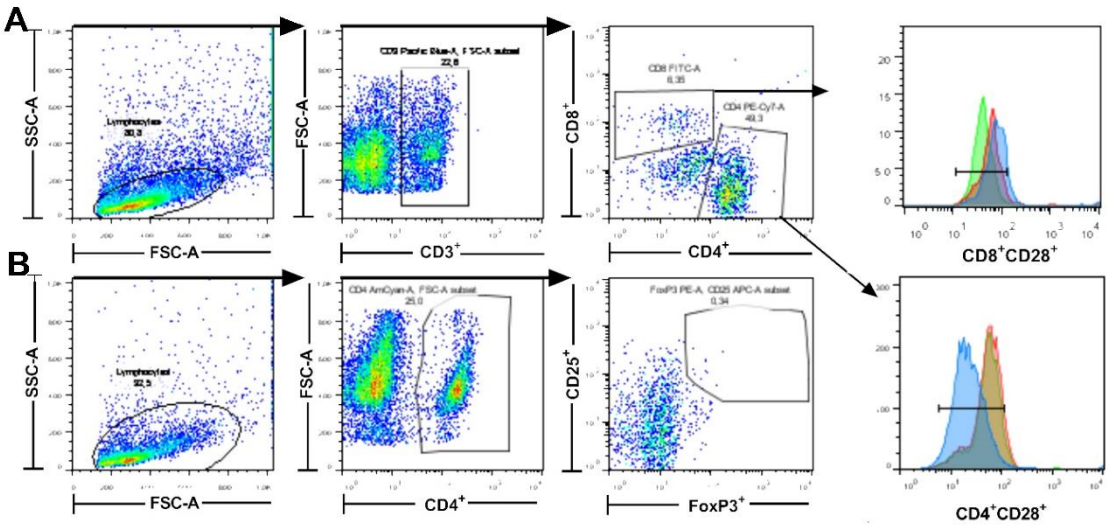
Supplemental Figure 1. Antibodies used in immunofluorescence.

Figure S1

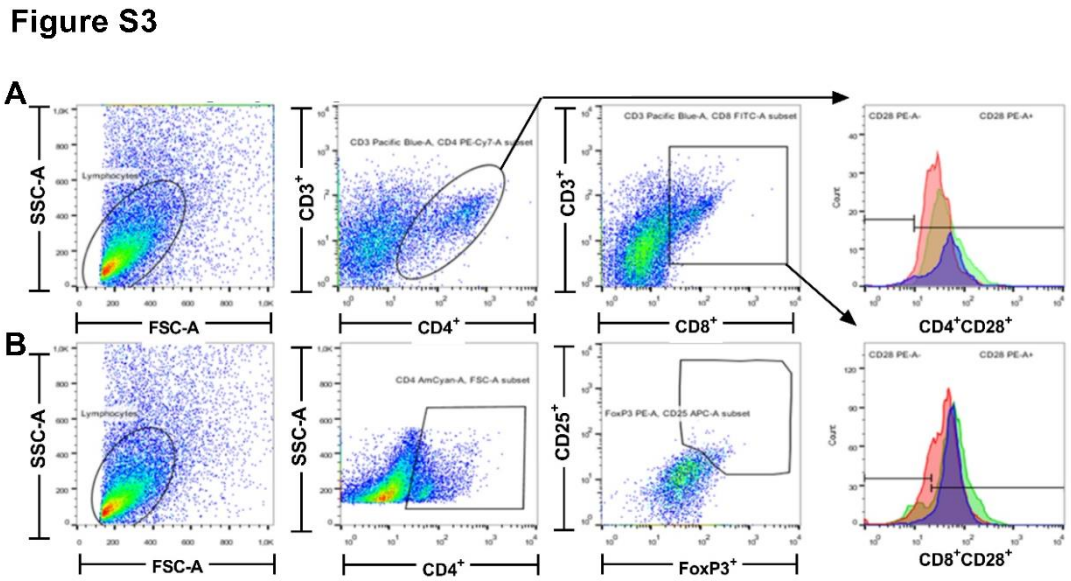
		Primary		
Antibody	**Species**	**Source**	**Catalog Number**	**Dilution**
CB2	goat	Santa Cruz	(M-15): sc-10076	1:300
CD4 FITC	rat	BD Pharmingen™	553730	1:200
CD8 PerCP	rat	BD Pharmingen™	553036	1:200
FoxP3 PE	rat	eBioscience	12-5773-82	1:200
		Secondary		
AlexaFluor 488	donkey anti-goat	Invitrogen - ThermoFischer	A11055	1:500
AlexaFluor 594	donkey anti-goat	Invitrogen - ThermoFischer	A11058	1:500

Supplemental Figure 2. Flow cytometry analysis of spleen.

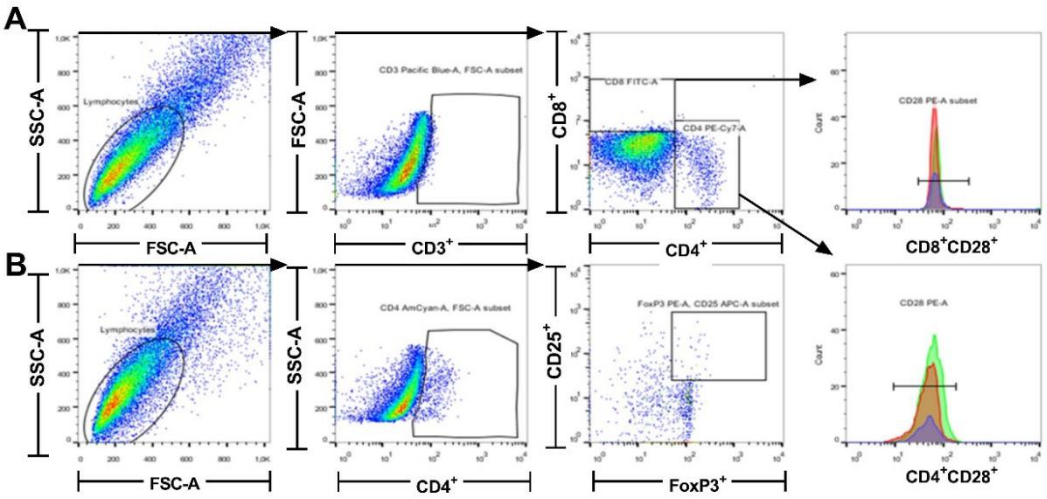
Figure S2



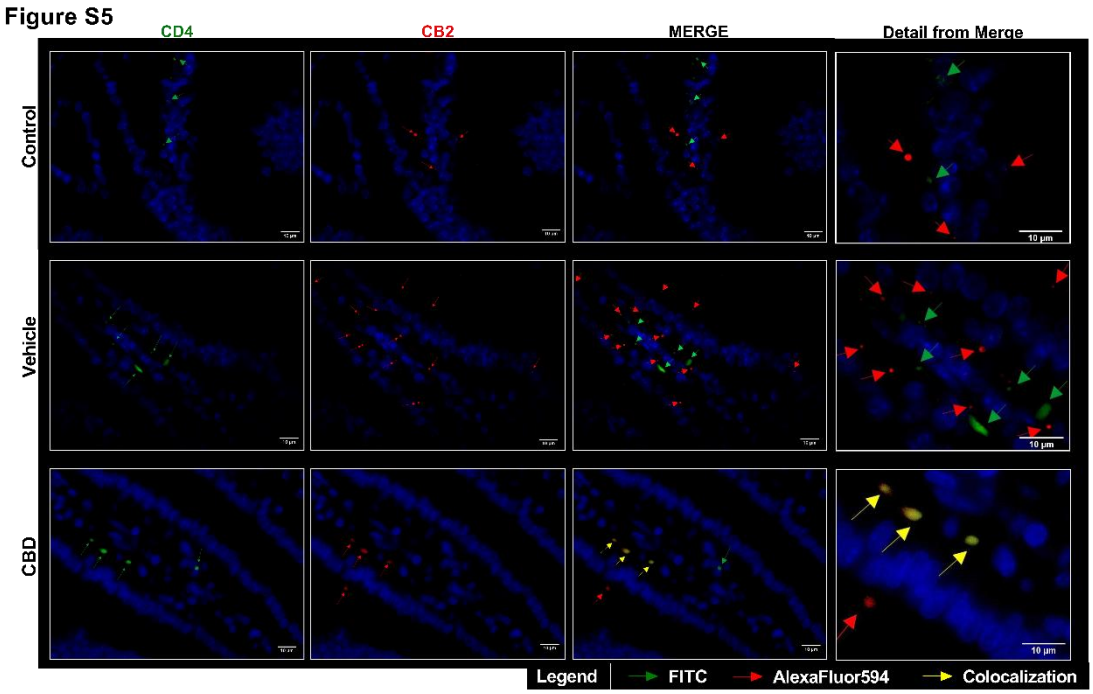
Supplemental Figure 3. Flow cytometry analysis of small intestine.



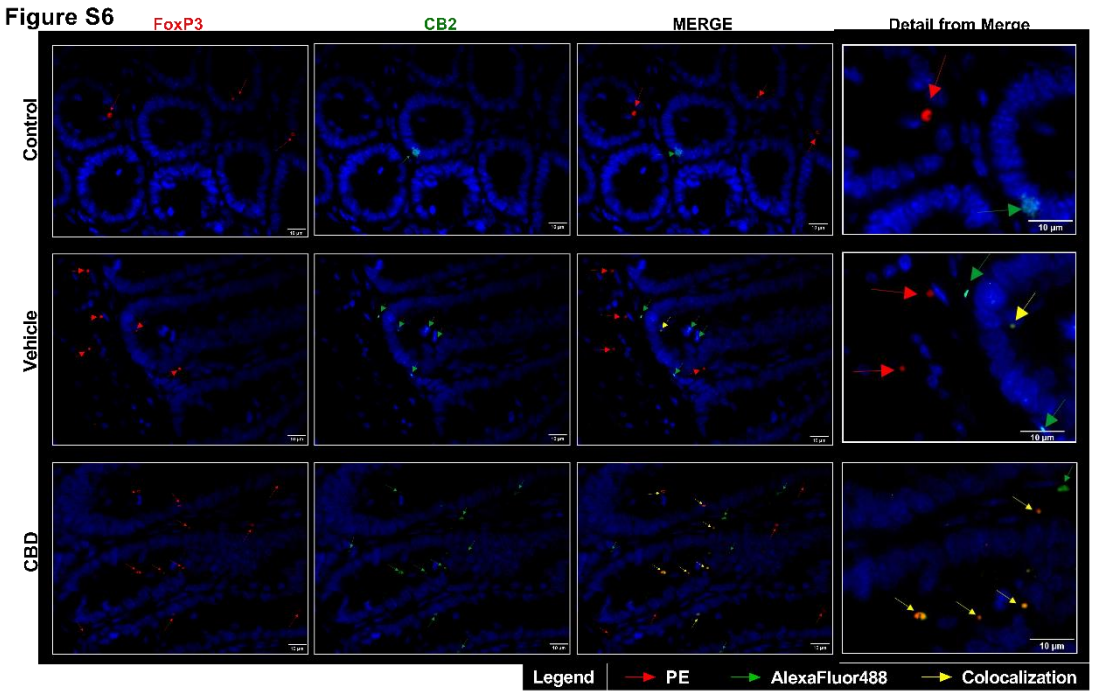
Supplemental Figure 4. Flow cytometry analysis of liver



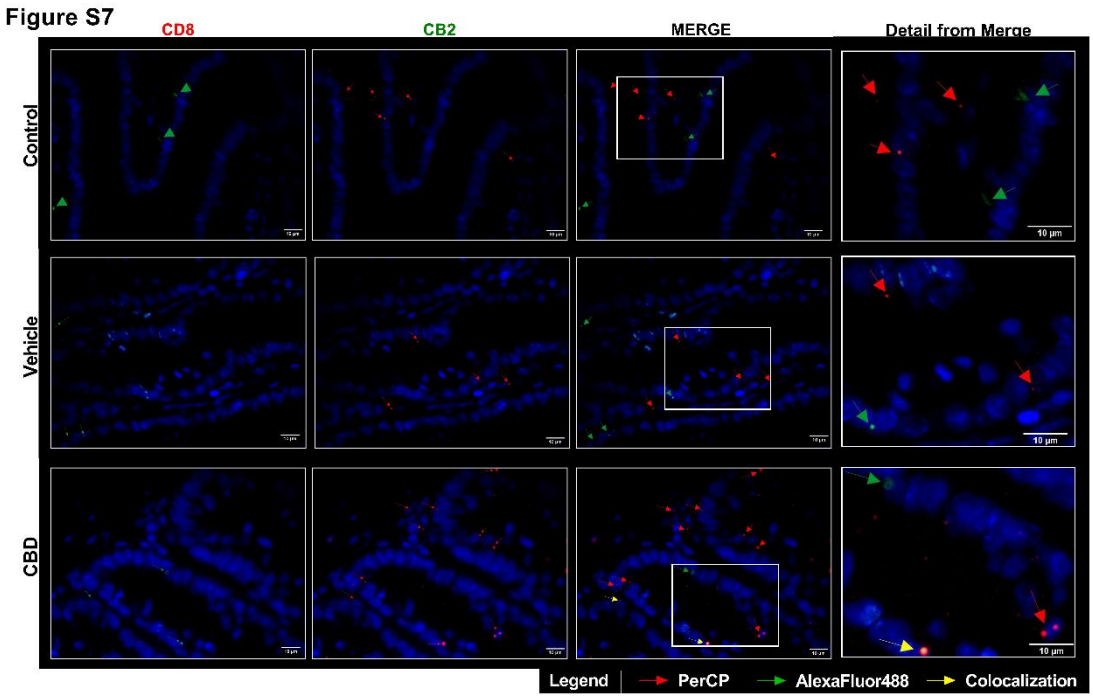
Supplemental Figure 5. Immunofluorescence of CD4/CB2 in small intestine.



Supplemental Figure 6. Immunofluorescence of FoxP3/CB2 in small intestine.



Supplemental Figure 7. Immunofluorescence of CD8/CB2 in small intestine.



Supplemental Figure 8. Intravital analysis after GVHD was induced in Balb/c mice using C57BL/6J GFP+ mice as donors.