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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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CBD ENHANCES TREG CELLS AND REDUCES GVHD BY CB2 ACTIVATION

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ABBREVIATIONS: ANOVA (analysis of variance); CB1 (cannabinoid receptor type 1); CB2 (cannabinoid receptor type 2); CBD (cannabidiol); CFU (colony forming units); ELISA (enzyme-linked immunosorbent assay); FACS (fluorescence activated cell sorting); GFP (green fluorescent protein); GVHD (graft-versus-host disease); GVT (Graft-versus-tumor); i.p. (intraperitoneal); i.v. (intravenous); IFNγ (interferon gamma); n (number of observations); PBS (Phosphate buffered saline); SD (standard deviation); TNFα (tumor necrosis factor alpha).

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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 CCL2, CCL3, CCL5, TNF-α, and IFN-γ. CCL3 and IFN-γ levels were also decreased in the liver. Mechanistically, CBD also increased the number of CB_2 receptor on $CD4^+$ and $FoxP3^+$ cells in the intestine, which may explain the reduction in pro-inflammatory cytokines and chemokines. Antagonists of the CB2 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 CB2 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.

KEYWORDS: graft-versus-host disease, cannabidiol, cannabinoid receptor, Treg cells.

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 CB2 receptor expression on $CD4^+$ and $Foxp3^+$ cells and increasing the number of these regulatory cells, cannabidiol decreases pro-inflammatory cytokines and increases

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GVHD mice survival. This effect is dependent of CB2 receptor activation. Besides, cannabidiol did not interfere with Graft-versus-leukemia, a central response to avoid primary disease relapse.

Introduction

The main complication following 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 non-identical 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).

GVHD occurs because of histo-incompatibilities identified by T cells that react against host tissues causing extensive damage (Ho *et al.*, 2001). Classically, acute GVHD (aGVHD) occurs within 100 days after transplantation, and chronic GVHD 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 pro-inflammatory environment that promotes T cell activation, proliferation, and differentiation against target organs (Schroeder *et al.*, 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

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immunological 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, promoting host immune suppression but also leading to several adverse effects, such as reduced GVT reactivity, so new therapeutic approaches are urgently needed (Devetten *et al.*, 2004; Shlomchik, 2007; Devergie *et al.*, 2008; Poetker *et al.*, 2010; Peppa *et al.*, 2011; Jamil *et al.*, 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 *et al.*, 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 *et al.*, 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.

1. Methods

1.1. Animals

All the experiments were performed on eight-week-old male Balb-c mice, and the donor group consisted of eight-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-h light–dark cycle with food and

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water available *ad libitum*. The animal procedures were approved by the Ethics Committee on Animal Experimentation of the Federal University of Minas Gerais (no. 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; Castor et al., 2012). For the sample size, we used the following equation:

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

where *n* represents the sample size, *z*α/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.

1.2. Disease induction

For bone marrow ablation, the mice were irradiated with 7 Gy of gamma radiation before transplantation and received 3 x 10^7 splenocytes and 1 x 10^7 bone marrow cells i.v., 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.

1.3. *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-1x). The participation of cannabinoid receptors was assessed by a treatment with AM251 (Tocris®) 1 mg/kg or AM630 (Tocris®) 1 mg/kg (35), being a CB1 and a CB2 antagonist, respectively. The vehicle of both these antagonists contained 2.5% DMSO in PBS-1x and CBD's vehicle. All drugs were administered by a daily intraperitoneal (i.p.) injection, starting on the day of disease induction and continuing for the duration of the protocols.

1.4. Histopathology

On day seven, 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 (Olympus BX51, 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 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).

1.5. Bacterial translocation

Intraperitoneal wash was performed on the seventh 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 1x. The wash was incubated overnight in a Müller–Hilton agar medium at 37 ºC, and the number of colony-forming units (CFUs) was counted. The mean values of the CFUs are shown.

1.6. ELISA

On the seventh 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.

1.7. 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 x 10^6 cells were stained with the following antibodies: CD3 (Pacific Blue [RRID-17-A2], BioLegend), CD4 (PE-

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Cy7 [RRID-RM45], BioLegend), CD8 (FITC [RRID-53-6.7], BioLegend), CD25 (APC [RRID-PC61], BioLegend), and CD28 (PE-A [RRID-37.51], BioLegend). For cell surface markers, we used FoxP3 (PE-A [RRID-MF14], BioLegend); $H2D^b$ (Alexa Fluor-647 [RRID-KH95], BioLegend); and $H2D^d$ (PE [RRID-34212], BioLegend) were used 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, USA).

2.8 Immunofluorescence

At day 7 after transplant intestinal slices were processed to immunofluorescence assay. The antibody to CD4, CD8, FoxP3 and cannabinoid receptor 2 (CB2), described on supplemental material (Supp. Fig. 1), were used to investigate the presence of CB2 on these cells and if cannabidiol treatment can induce CB2 expression on them. The Nuclei were marked with 4,6-diamidino-2-phenylindole (Life Technology, DAPI/D1306-Carlsbad). 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, Campolina-Silva et al. 2017). For each tissue section, ten regions were randomly captured, by an independent experiment-blinded user, at 100× magnification, totaling fifty pictures per group (n=5). Images were analyzed using ImageJ software v 1.53c 1.41 (National Institutes of Health, USA, public domain).

1.8.Graft-versus-tumor response

On the day of GVHD induction, after radiation, the mice received murine mastocytoma $P815^+$ cells i.v. (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 seven 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.

1.9. 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 (alpha = 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 $p < 0.05$.

2. Results

2.1. Engraftment and mouse survival

MHC disparities between animals led to the development of GVHD (Figure 1 A). The most effective dose of CBD was chosen by a daily treatment with 10, 30, or 60 mg/kg. Survival was assessed daily (Figure 1 B). 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 (Figure 1 C). 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 seven days after disease induction revealed that the

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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 $H2D^b$ (Figure 1D).

2.2. *Cellular recruitment, activation, and damage*

The spleen, intestine, and liver were collected for analysis. Flow cytometry of the spleen (Supp. Fig. 2) revealed no difference in the total number of lymphocytes (Figure 2 A) or in the number of $CD8^+$ cells among the groups (Figure 2 C). 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 (Figure 2 B). Moreover, we found no difference in the activation of $CD4^+$ cells (Figure 2 D), 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 (Figure 2 E), and the number of $CD4^+$, $CD25^+$, and $FoxP3^+$ cells, collectively known as Tregs, was not different between the groups (Figure 2 F).

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 (Figure 3 A-D). Supportive of this improvement in the small intestine, intraperitoneal washing also revealed that while 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 (Figure 3 E).

Flow cytometry of the small intestine (Supp. Fig. 3) revealed an increased number of total lymphocytes in the CBD group in comparison with the vehicle group; however, the vehicle group had not an increased number of total lymphocytes in comparison with the control group (Figure 4 A).

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Both $CD4^+$ cells (Figure 4 B) and $CD8^+$ cells (Figure 4 C) 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 (Figure 4 D). 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 (Figure 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 (Figure 4 F). 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, TNF-α, IFN-γ, and IL-10, while CBD, surprisingly, led to a reduction in CCL2, CCL3, CCL5, TNF-α, IFN-γ, and IL-10 in the intestine (Figure 4 G-L).

The same parameters were evaluated in the mice's liver seven 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 (Figure 5 A-D). In addition, ELISA, in the liver, showed that CBD decreased the levels of CCL2, CCL3, and IFN-γ (Figure 5 E, F, and I). Flow cytometry of the liver (Supp. Fig. 4) revealed an increased number of lymphocytes in the CBD group when compared with the vehicle group (Figure 5 K), and this was also the case for $CD4^+$ cells (Figure 5 L). However, we found no difference in $CD8^+$ cells among the groups (Figure 5 M). 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, while in the CBD group the activation was higher than in the vehicle group (Figure 5 N). The $CD8^+$ cells presented the same low level of activation in all groups (Figure 5 O). 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

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group when compared with the control group (Figure 5 P).

2.3. Graft-versus-leukemia response

The mice received P815GFP$^+$ mastocytoma cells on the day of disease induction. After seven 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 (Figure 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 (Figure 6 A and B). In the same way, CBD treatment did not interfere with the allograft response against tumor cells (P815GFP$^+$), as the presence of tumor cells was severely reduced in the spleen (Figure 6 A) and lymph nodes (Figure 6 B) of the mice that received CBD, as compared with the mice in the control group, preserving the graft-versus-tumor response.

2.4. CBD's effect is dependent on the CB2 receptor

The mice were treated with CBD, CBD + AM630, CBD + AM251 or AM630, and AM251 alone, with AM630 being a selective antagonist of the CB2 receptor and AM251 a selective antagonist of the CB1 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 (Figure 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 (Figure 7 B). 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

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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 (Figure 7 C) or AM630 (Figure 7 D) 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 CB2 receptor expression in the target organs we performed an immunofluorescence assay in the intestine, major target organ of GVHD. CB2 expression is not altered by GVHD induction. However, CBD treatment increase CB2 receptor expression in the intestinal cells (Figure 7 E). As FACS analysis, immunofluorescence showed an increased number of $CD4^+$ cells in the CBD group compared to GVHD and Control group (Figure 7 E). Positive colocalization of CD4 and CB2 staining was increased in CBD group, in comparison both to control and GVHD groups (Figure 7 E and Supp. Fig. 5). This result was also found regarding FoxP3 with CB2 staining (Figure 7 F and Supp. Fig. 6). However, although CD8 was also increased in CBD when compared to the other groups, CB2 receptor expression was found to be increased only in comparison to control and not GVHD group ($p= 0.1232$) and there was no difference between CBD and GVHD groups remarking colocalization (Fig 7 G and Supp. Fig. 7). Pearson's coefficient was found to be mainly positive in CD4/CB2 analysis (Control 0.197±0.127; GVHD 0.161±0.103; CBD 0.333±0.143; with no difference between groups); CD8/CB2 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 $p=0.0418$).

3. 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 *et al.*, 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, and 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 proved to be of the highest importance when considering possible treatments for GVHD in humans (Jamil *et al.*, 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 *et al.* (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 for 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

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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 anti-tumoral immunological 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, while in the vehicle group, the intestinal barrier was inefficient in preventing this translocation; owing to intestinal damage, bacteria were found in the peritoneal cavity (Figure 3 E).

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 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 pro-inflammatory cytokines (TNF-a and IFN-g) 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 and Ferrara *et al*., 2017). In this way, we have investigated if the recruitment process was impaired in CBD treated mice. Using an intravital assay, the rolling and adhesion of inflammatory cells on intestinal microvasculature was examined. There was no difference between vehicle and CBD treated mice (Supp. 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 seventh day of the disease in vehicle. The timing of the disease in the liver explains why we found that CCL2, CCL3, and IFN_γ were increased only in the vehicle group; interestingly, this

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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 *et al.*, 2013). To our 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 CB2 but not the CB1 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 both endogenous cannabinoids and some phytocannabinoids; however, they unleash different effects in the host organism. The CB1 receptor is present mainly in the central nervous system, while the CB2 receptor is more abundant in the peripheral nervous system and immune cells (Adams et al., 2018). The presence of CB2 in immune cells is correlated with its modulatory effects, including cytokine inhibition and reduction in antigenic presentation (Ehrhart et al., 2005; Horvath et al., 2012). CB2 receptor is expressed on nearly all immune cells and its absence on donor $CD4^+$ or $CD8^+$ T cells, or administration of a selective CB2 pharmacological antagonist, exacerbated acute GVHD lethality (Yuan, Belle et al. 2020). CBD has about 74% higher affinity for CB2 than for CB1 (Rosenthaler et al., 2014). In this way, we investigate the effect of CBD on CB2 receptor expression on intestine. CBD treatment was able to induce an increase of CB2 receptor expression on $CD4^+$ and $FoxP3^+$ cells in the intestine of mice subject to GVHD. The correlation between CD4 and CB2, but also of FoxP3 and CB2 further reinforces our hypothesis that cannabidiol might stimulates FoxP3 differentiation by interaction with CB2 receptor.

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These findings reinforce CB2 role in regulation of GVHD and suggests an interesting pharmacological therapeutic targeting.

In the present study, the effects of CBD may be associated with CB2 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 CB2 receptor in the intestine leads to an immunoregulatory environment with an enhanced number of Treg cells and a reduction in the major GVHD pro-inflammatory mediators. Moreover, CBD increase the number of $CD8^+$ cells maintaining the GVL effect. Therefore, it may be concluded that CBD represents an interesting approach in the treatment of aGVHD (Figure 8).

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Conflict of interest statement

The authors declare no conflict of interest in this study.

Author Contribution

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

Conducted experiments: Berg, Soares, Paiva and Castor.

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

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

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

All authors revised the manuscript and approved the final version.

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Footnotes

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Figure legends

Figure 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 10mg/kg, CBD 30 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 30mg/kg, seven days after the transplant, through flow cytometry. * indicates $P<0.05$ when compared to control group and # indicates $P<0.05$ when compared to the vehicle. Data were expressed in dispersion with mean and statistical difference was determined by Test t Student for engraftment and Log-rank (Mantel-Cox) test for survival. n=5 for all groups

Figure 2 - Flow Cytometry of mice spleen seven 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 to control group and # when $P<0.05$ when compared to vehicle. Data were expressed in dispersion with mean and statistical difference was determined by ANOVA with Fisher's LSD LSD *post hoc*. n=5 for all groups

Figure 3 - Intestines stained with H&E, (A) control, (B) vehicle and (C) CBD, in with 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 were expressed in dispersion with mean and statistical difference was determined by ANOVA with Fisher's LSD LSD *post hoc*; n=5 for all groups.

Figure 4 – Flow Cytometry and ELISA of mice small intestines seven 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 and (F) $CD4^+CD25^+FoxP3^+$ n=5/5/5 cells; (G) CCL2, (H) CCL3, (I) CCL5, (J) TNFα, (K) IFNγ and (L) IL-10. Data was represented in dispersion graphs with

mean; n=5 for all groups, except when specified. * indicates P<0.05 when compared to control group and # when P<0.05 when compared to vehicle. Data were expressed in dispersion with mean and statistical difference was determined by ANOVA with Fisher's LSD LSD *post hoc*.

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

Figure 6 - Flow Cytometry of mice spleen and lymph nodes, seven days after disease induction and $P815GFP^+$ cells transplant. (A) Percentage of $P815^+$ cells in spleen; (B) Percentage of $P815^+$ cells in lymph nodes. Absolute number of $P815^+$ cells were represented in dispersion graphs next to Flow Cytometry shards; n=5 for all groups.

Figure 7 – Role of CB2 in the protective effect of CBD. (A) CBD in the presence of CB1 antagonist; (B) CBD in the presence of CB2 antagonist; (C) Treatment with CB1 antagonist without CBD; (D) Treatment with CB2 antagonist without CBD. (E) CD4 and CB2 mean integrated density, colocalization of CD4/CB2 and representative figures of groups (CD4 FITC, CB2 AlexaFluor 594 and DAPI); (F) CD8 and CB2 mean integrated density, colocalization of CD8/CB2 and representative figures of groups (CD8 PerCP, CB2 AlexaFluor 488 and DAPI); (G) FoxP3 and CB2 mean integrated density, colocalization of FoxP3/CB2 and representative figures of groups (FoxP3, CB2 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.

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Figure 8 - Schematic representation of our findings. CBD treatment, partially by CB2 receptor activation, increased the number of Tregs in the in the intestines and reduced cytokine/chemokine release. Together these factors led to the protection of target organs and the increase in mice survival.

Figure 1

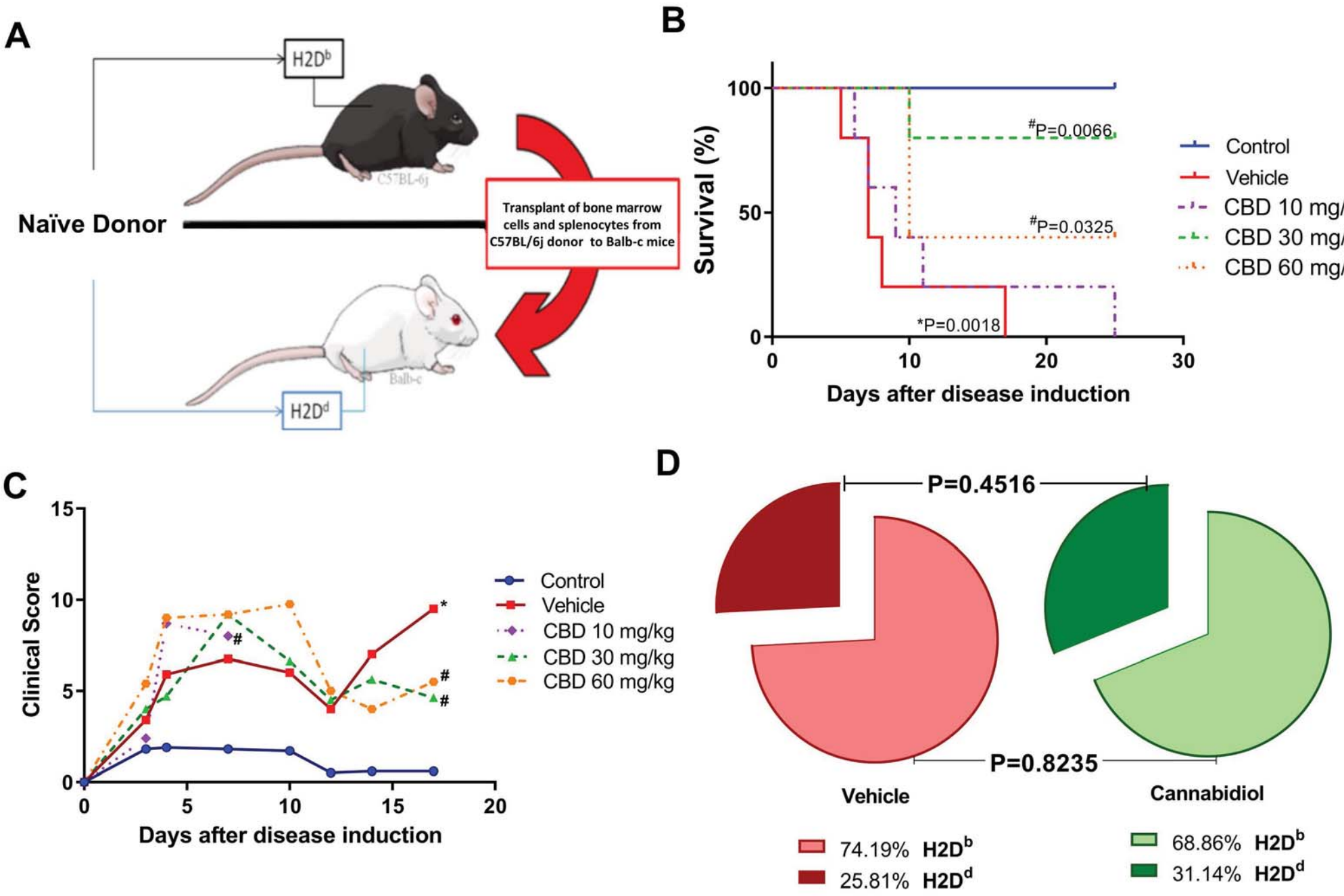

Figure 2

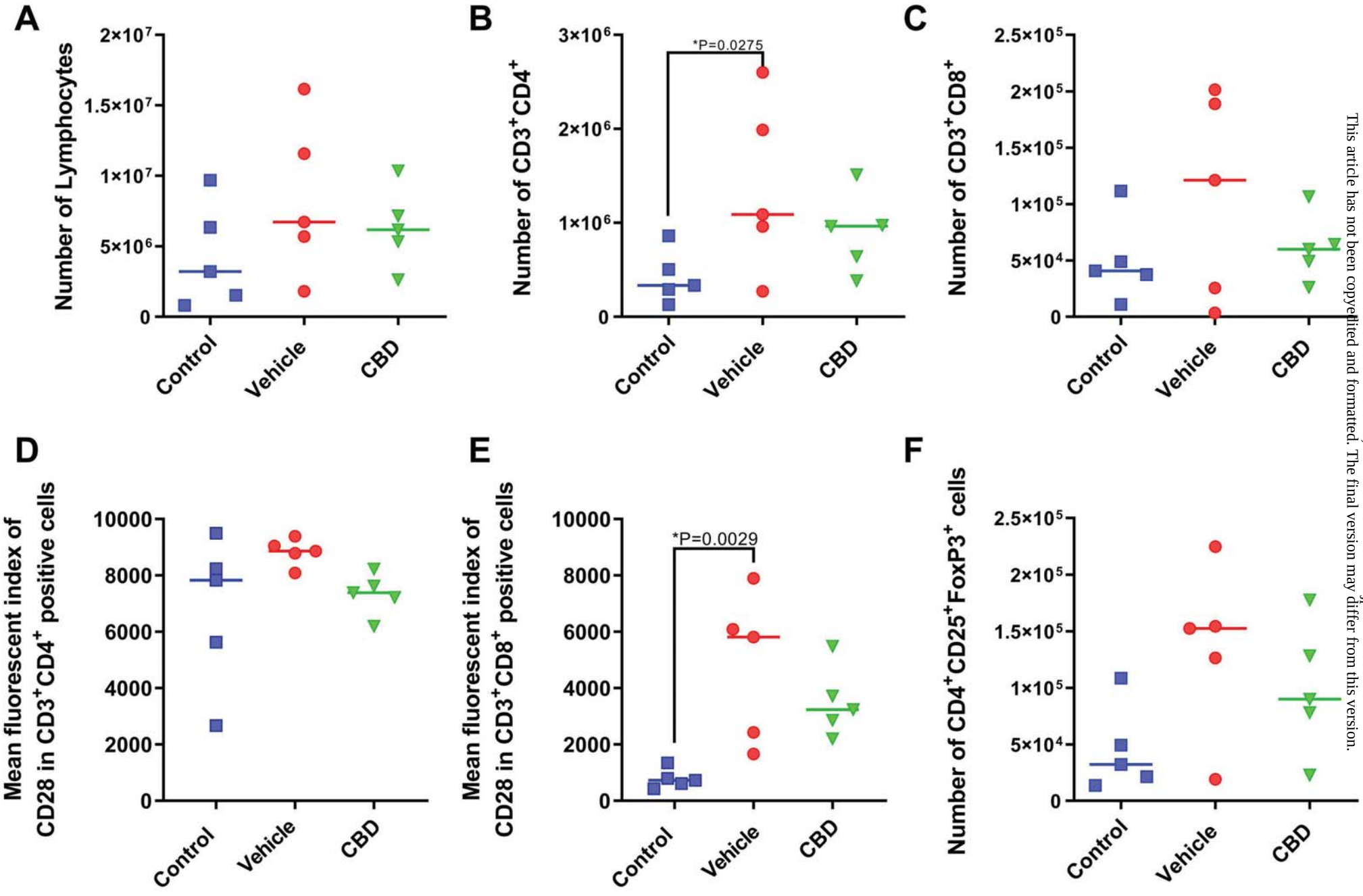

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Figure 3

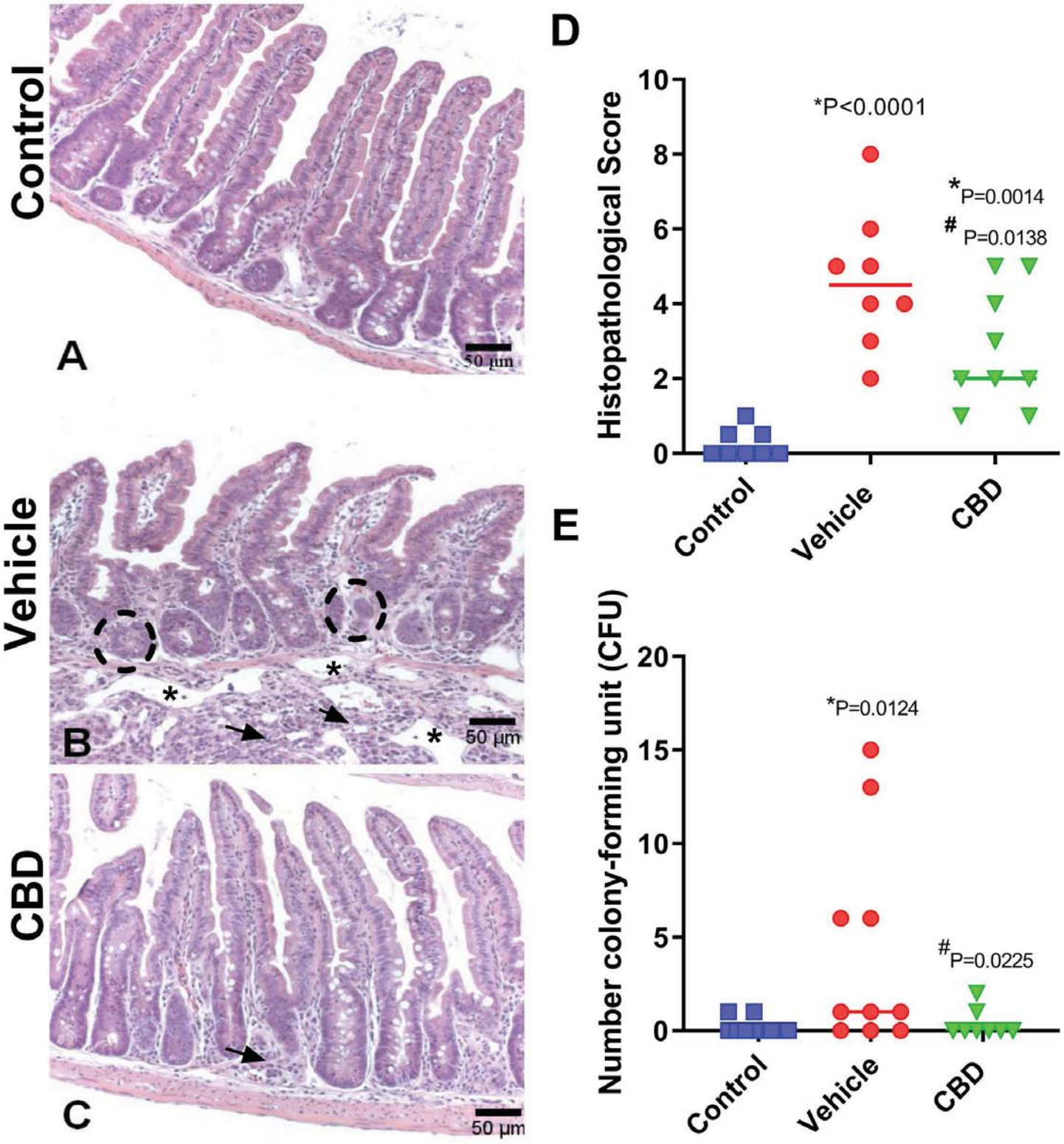

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Figure 4

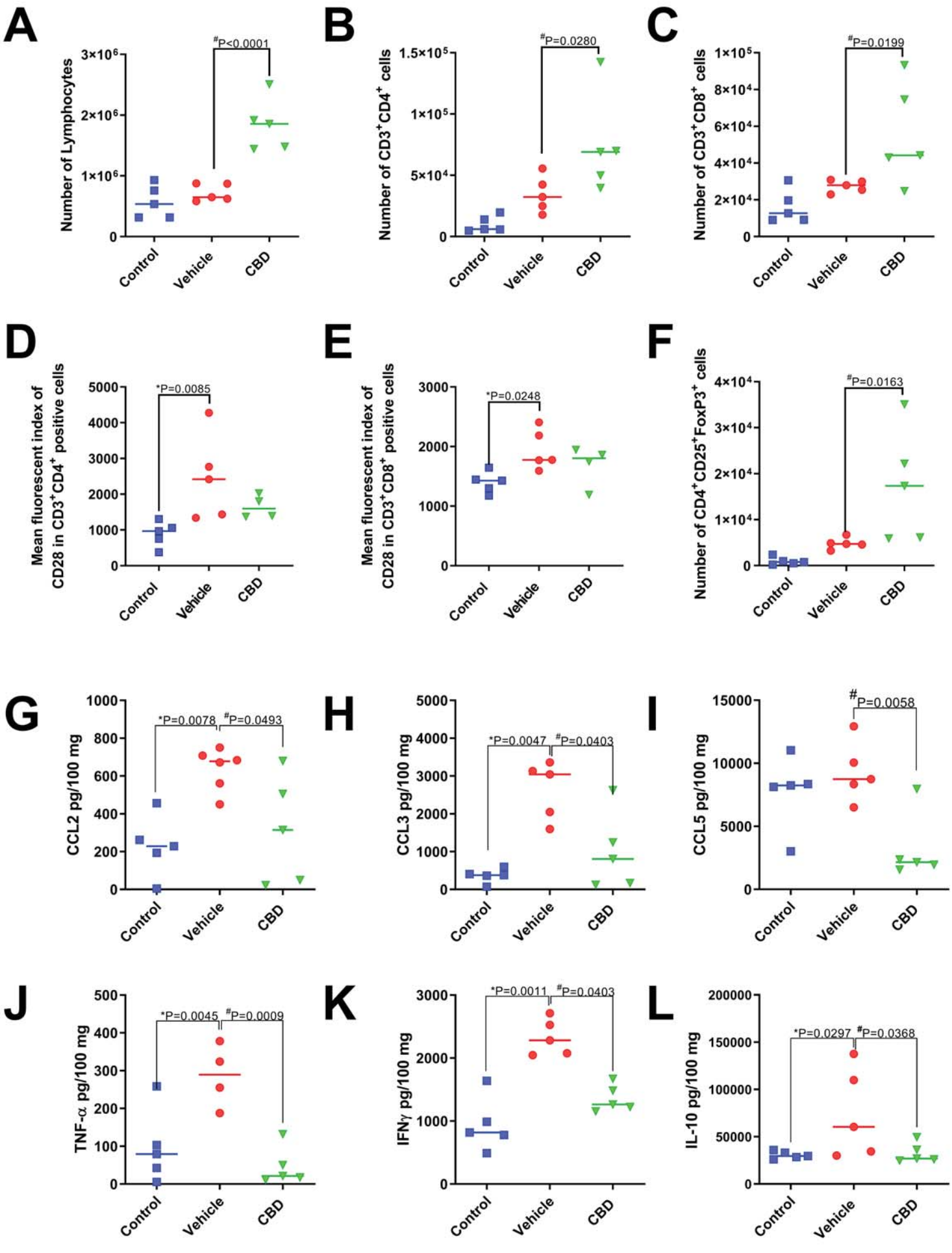

Figure 5

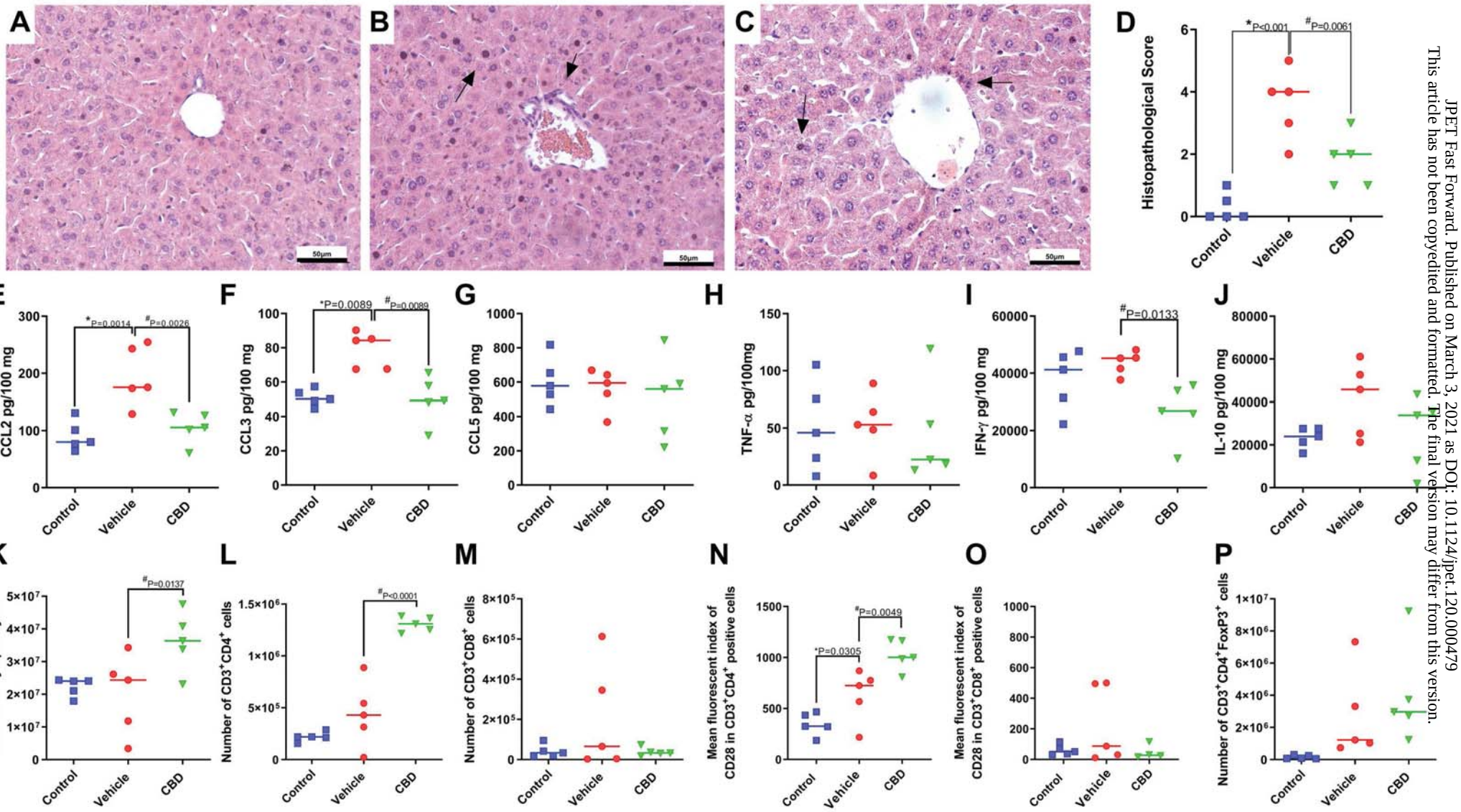

Figure 6

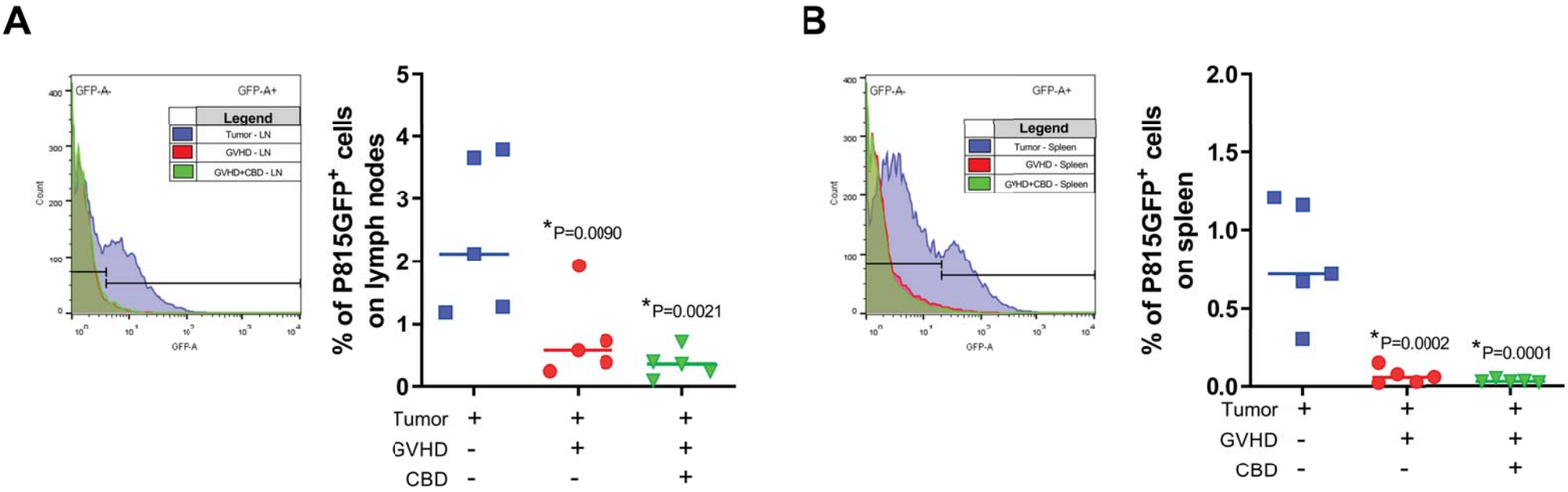

Figure 7

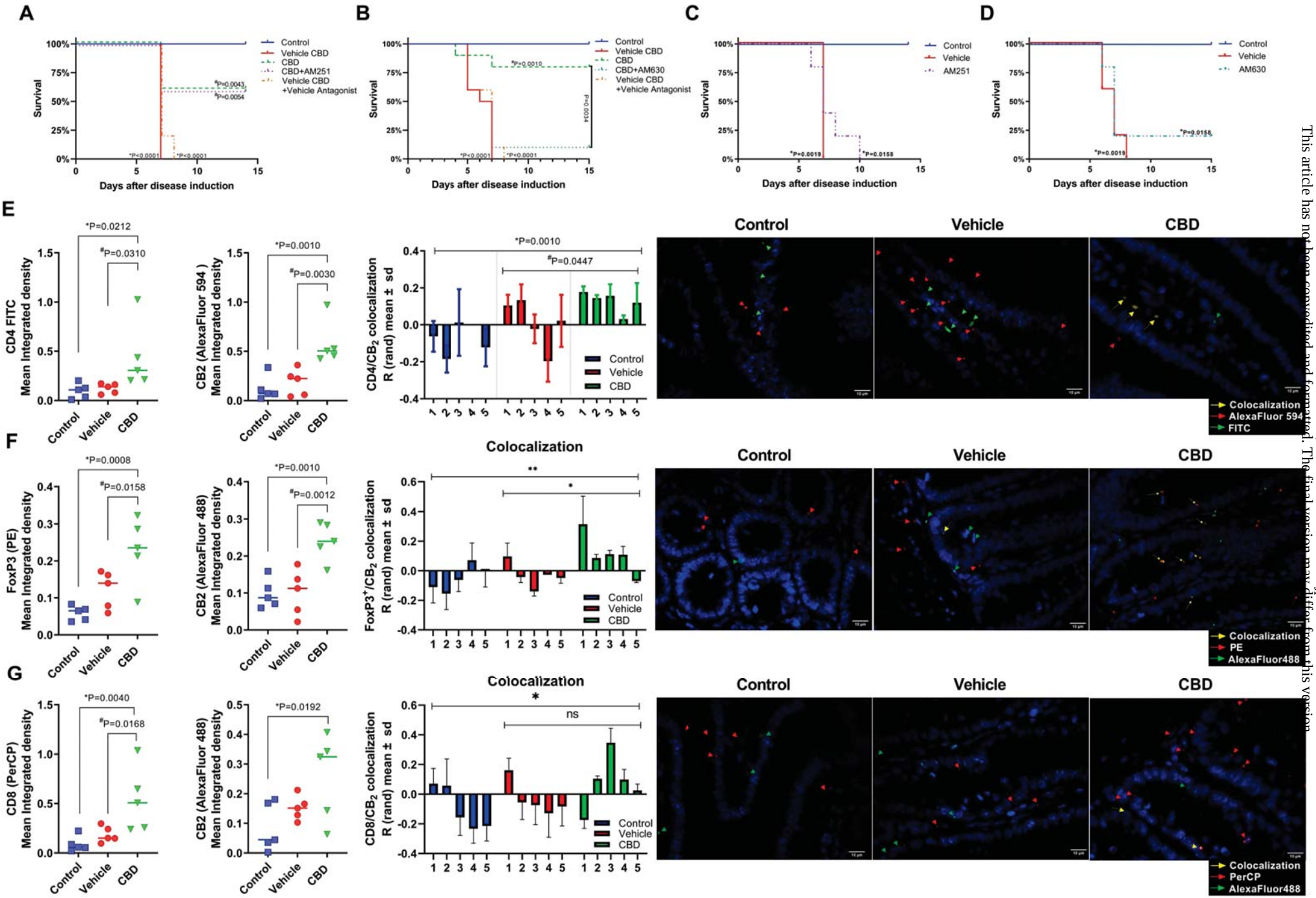

Figure 8