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RESEARCH ARTICLE

Cannabidiol improves lung function and inflammation in mice submitted to LPS-induced acute lung injury

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Abstract

We have previously shown that the prophylactic treatment with cannabidiol (CBD) reduces inflammation in a model of acute lung injury (ALI). In this work we analyzed the effects of the therapeutic treatment with CBD in mice subjected to the model of lipopolysaccharide (LPS)-induced ALI on pulmonary mechanics and inflammation. CBD (20 and 80 mg/kg) was administered (i.p.) to mice 6 h after LPS-induced lung inflammation. One day (24 h) after the induction of inflammation the assessment of pulmonary mechanics and inflammation were analyzed. The results show that CBD decreased total lung resistance and elastance, leukocyte migration into the lungs, myeloperoxidase activity in the lung tissue, protein concentration and production of pro-inflammatory cytokines (TNF and IL-6) and chemokines (MCP-1 and MIP-2) in the bronchoalveolar lavage supernatant. Thus, we conclude that CBD administered therapeutically, i.e. during an ongoing inflammatory process, has a potent anti-inflammatory effect and also improves the lung function in mice submitted to LPS-induced ALI. Therefore the present and previous data suggest that in the future cannabidiol might become a useful therapeutic tool for the attenuation and treatment of inflammatory lung diseases.

Keywords

Cannabidiol, cannabinoids, inflammation, lipopolysaccharide, pulmonary mechanics

History

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Introduction

Cannabidiol (CBD) is a major non-psychotropic cannabinoid found in marijuana (Cannabis sativa) and has immunosuppressive and anti-inflammatory properties in several rodent models of inflammation[1]. Acute lung injury (ALI) and its most severe form, acute respiratory distress syndrome (ARDS), are still a matter of public health concern. Nearly 200 000 hospitalizations and 75 000 deaths are associated with ALI and ARDS in the United States each year[2]. In Brazil, there is relatively little information regarding the incidence of ALI and ARDS in intensive care units. Nevertheless incidences of 2.3% for ARDS and 3.8% for ALI have been reported by the Hospital das Clínicas of Porto Alegre[3]; data from the Hospital das Clínicas of Ribeirão Preto showed incidences of 1% for ALI and 2.2% for ARDS[4]. ALI and ARDS result in persistent respiratory failure, prolonged dependence on mechanical ventilation and increased susceptibility to multi-organ dysfunction[2]. Unfortunately, despite numerous innovations in intensive care medicine, the mortality related to these conditions is reported to be approximately 40%[2,5,6].

Recently, we showed that prophylactic treatment with CBD was able to abrogate the inflammatory lung response observed in a murine model of lipopolysaccharide (LPS)-induced ALI[7]. These anti-inflammatory effects were found to be mediated by adenosine A2A receptor activation[7]. Since impaired lung function is one of the main issues in ALI and ARDS, we aimed to evaluate the therapeutic effects of CBD on airway hyperresponsiveness and pulmonary resistance induced by intra-nasal instillation of LPS. Additionally, we analyzed the therapeutic anti-inflammatory effects of CBD, i.e. given during an ongoing lung inflammatory response.

Material and methods

Animals

Male C57BL/6 mice from our own colony, weighing 22–26 g and approximately 8–10 weeks old, were used. The animals were housed in temperature-controlled (22–24 °C) and artificially lit rooms on a 12 h light/12 h dark cycle (lights were turned on at 7:00 a.m.). The mice were given free access to rodent chow and water. Sterilized, residue-free wood shavings were used as animal bedding. The experiments were performed in a different room that was maintained at the

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same temperature as the animal colony. The animals were maintained in their home cages during the experiments and housed and used in accordance with the guidelines of the Committee on Care and Use of Laboratory Animal Resources of the School of Veterinary Medicine, University of São Paulo (approval protocol # 2087/2010).

Drug treatment

CBD (THC-Pharm, Frankfurt, Germany and STI-Pharm, Brentwood, UK) was prepared in ethanol:Tween 20:saline (1:1:18) and administered intraperitoneally (i.p.) to mice at 20 and 80 mg/kg (0.1 mL/10 g weight). Animals in the control groups received a similar volume of CBD vehicle i.p. The CBD doses were chosen based on recently published data[7].

Acute lung injury

Mice were anesthetized with ketamine and xylazine i.p. (100 and 10 mg/kg, respectively) before intranasal instillation of LPS. *Escherichia coli* LPS (O55:B5 L2880, Sigma-Aldrich, St. Louis, MO) at a concentration of 100 μg/mL, or as a control, sterile saline (0.9%) was instilled at 1 μL/g of mouse body weight. Twenty four hours after instillation, pulmonary mechanics were evaluated or mice were anesthetized and killed by exsanguination through the inferior vena cava (Figure 1).

Assessment of pulmonary mechanics

Evaluation of pulmonary mechanics was performed according to Ferreira et al.[8] and Lino-dos-Santos-Franco et al.[9], 24 h after LPS instilation. Briefly, after anesthesia (ketamine and xylazine, 100 mg/kg and 10 mg/kg, respectively, i.p.), the animals were tracheostomized (18 gauge metal cannula) and linked with controlled small ventilator. The ventilator was connected to a computer system (flexiVent, Scireq Inc., Montreal, Canada). Mice were mechanically ventilated with a tidal volume of 10 mL/kg, at a frequency of 150 breaths/min, and the positive end expiratory pressure (PEEP) was set to 3 cm H_2O. A warming pad prevented cooling of the mice. Animals were paralyzed with 1.0 mg/kg pancuronium dibromide i.p. (Tocris Bioscience, Bristol, UK). In order to standardize lung volume history, the lungs were first inflated four times to a pressure of 30 cm H_2O (recruitment maneuvre). Mice were allowed to stabilize for 5 min before measurements commenced; we collected three measurements of regular ventilation data to establish the baseline for each animal. Mice received Methacholine (MCh, Acetyl-β-methylcholine chloride, Sigma-Aldrich, St. Louis, MO) intravenously (an external jugular vein was isolated for intravenous infusion of MCh) with increasing concentrations (8, 16 and 32 μg/kg) or its vehicle sterile saline 0.9%. Respiratory mechanics were assessed using the single-compartment linear model, which provides resistance (R_{rs}) and elastance (E_{rs}) of the total respiratory system[10,11]. Next, the lung mechanics were assessed using the constant phase model, which utilizes forced-oscillation technique allowing partitioning of the lung mechanics into central and peripheral components[12]. The obtained parameters are the Newtonian resistance (R_n), a close approximation of resistance in the proximal airways, tissue damping (G_{tis}), which reflects energy dissipation in the lung tissue and tissue elastance (H_{tis}), characterizing tissue stiffness and reflecting energy storage in the tissue[13–17]. After receiving each concentration of MCh, the measurements were delivered every 15 s, over 5 min duration. Between impedance measurements, regular ventilation was returned. The peak response after each concentration of MCh was determined. To the experiments, a coefficient of determination (COD) >0.80 was used (maximum COD is 1.0).

BAL analysis

The lungs were lavaged with 1.5 mL of phosphate-buffered saline (PBS) solution through a cannula inserted into the trachea. BAL was collected and centrifuged at 250 *g* for 5 min, and the remaining cell pellet was resuspended in 1 mL of PBS. Total leukocyte counts were performed after adding 10 μL of crystal violet to 90 μL of the cell suspension. Neubauer chambers were used for cell counting under a light microscope. Differential counts were performed on cytocentrifuge preparations (FANEM, São Paulo, Brazil) stained with Rosenfeld's dye using standard morphological criteria.

Myeloperoxidase activity

The lungs were perfused via the pulmonary artery with 10 mL of PBS. Samples were prepared according to Goldblum[18]. Briefly, a lung fraction was homogenized with 1 mL of PBS containing 0.5% of hexadecyl-trimethylammonium bromide and 5 mM EDTA, pH 6.0. Samples were homogenized and centrifuged at 30 000 *g* for 10 min. Aliquots of lung homogenates (10 μL) were then incubated for 5 min with a solution containing H_2O_2 (0.1%) and ortho-dianisidine. The reaction was stopped by the addition of 1% $NaNO_3$. The absorbance was determined at 450 nm using a microplate

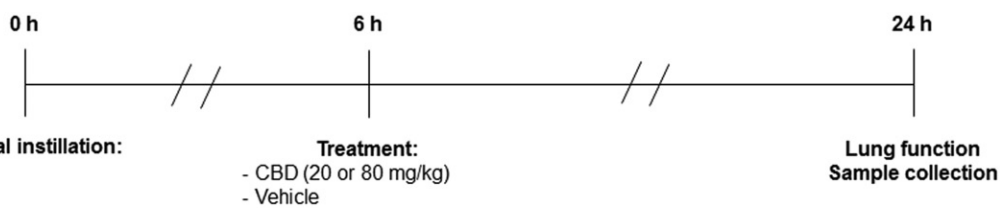

Figure 1. Schematic representation of the experimental design: C57BL/6 male mice (22–26 g) were anesthetized with ketamine and xilazine (100 and 10 mg/kg, respectively) and received LPS (100 μg/mL) or saline (NaCl 0.9%) through intranasal instillation (1 μL/g body weight). Following 6 h of intranasal instillation mice were treated with CBD (20 or 80 mg/kg) or Vehicle through i.p. injection (0.1 mL/10 g body weight). Twenty four (24) hours after instillation, pulmonary mechanics was evaluated or mice were anesthetized and killed by exsanguination through the inferior vena cava. Groups formation: Sal + Veh, LPS + Veh, LPS + CBD 20 and LPS + CBD 80.

reader (Bio-Tek Instruments®, Winooski, VT). The absorbance was standardized to lung weight to normalize pulmonary myeloperoxidase activity between groups.

Cytokine and chemokine analysis

A BD™ cytometric bead array (CBA) Mouse Inflammation kit (BD Biosciences, San Jose, CA) was used to measure IL-6, TNF and MCP-1 and an ELISA kit (R&D Systems, Minneapolis, MN) was used to measure MIP-2 levels in the BAL supernatant. The assays were performed according to the manufacturers' instructions.

Protein concentration

BAL aliquots were placed in a 96-well ELISA plate (10 µL/well), and 250 µL of Bradford reagent (Sigma-Aldrich, St. Louis, MO) was added to each well; the absorbance was measured at 595 nm. A standard curve using bovine serum albumin (BSA, Sigma-Aldrich, St. Louis, MO) was obtained by plotting the net absorbance versus the protein concentration (1500–100 µg/mL) to determine the protein concentration in the samples.

Histological analysis

Left lungs were prepared, cut into 5 µm and hematoxylin and eosin (H.E.) stained. From each sample representative photos were taken (magnification ×200).

Statistical analysis

GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA) was used for the statistical analysis. The parametric data were analyzed by a one-way ANOVA followed by a Tukey–Kramer test for multiple comparisons. In all experiments, $p \leq 0.05$ was considered significant. The data are presented as the mean ± S.E.M.

Results

Therapeutic treatment with CBD improves lung function in mice subjected to LPS-induced lung inflammation

Our primary goal was to investigate the therapeutic effects of CBD, i.e. given 6 h after the induction of lung inflammation, on lung function. LPS intranasal instillation was effective in impairing lung function, as observed by its effects after a dose–response methacholine challenge (8, 16 and 32 µg/kg). After various dose of methacholine, we observed a dose-responsive increase on lung resistance (R_{rs}), lung elastance (E_{rs}), Newtonian resistance (R_n) and tissue damping (G_{tis}) (Figure 2a–d) in LPS-treated mice (LPS + Veh) ($p \leq 0.05$); a trend but not a significant effect was observed on tissue stiffness and energy storage parameters (H_{tis}) (Figure 2e) after methacholine challenge in LPS-treated mice. Therapeutic treatment with CBD at 20 and 80 mg/kg (LPS + CBD 20 and LPS + CBD 80, respectively) decreased the effects of methacholine challenge on lung resistance (R_{rs}), lung elastance (E_{rs}), Newtonian resistance (R_n), tissue damping (G_{tis}) and tissue stiffness and energy storage parameters (H_{tis}) in LPS-treated mice ($p \leq 0.05$) (Figures 2a–e). These data clearly show that CBD was able to attenuate the effects of LPS on lung function after methacholine challenge.

Therapeutic treatment with CBD decreases LPS-induced lung inflammation

We have previously shown that the prophylactic treatment with CBD, i.e. prior to the induction of inflammation, decreases the inflammatory response induced by intranasal instillation of LPS[7]. We asked whether CBD would be able to decrease lung inflammation when given therapeutically, i.e. during an ongoing inflammatory process. We have chosen 6 h

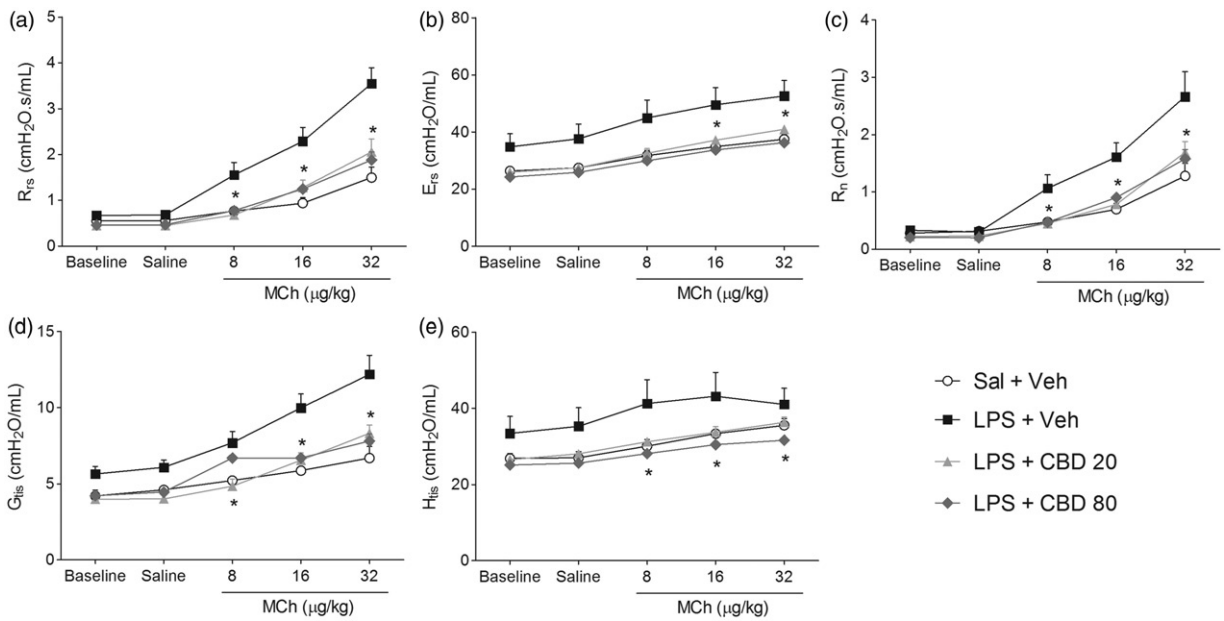

Figure 2. Effects of therapeutic treatment with CBD on assessment of pulmonary mechanics. (a) Total lung resistance (R_{rs}), (b) total lung elastance (E_{rs}), (c) Newtonian resistance (R_n), (d) tissue damping (G_{tis}) and (e) tissue stiffness ans energy storage (H_{tis}). Data are presented as the mean ± S.E.M., $n = 4$–5 mice/group. *$p < 0.05$ compared to LPS + Veh group. One-way ANOVA followed by a Tukey–Kramer multiple comparison test.

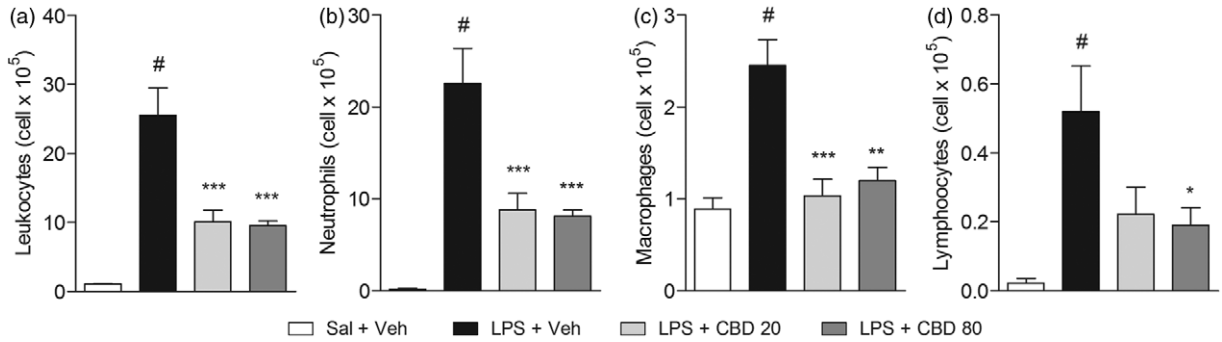

Figure 3. Effects of therapeutic treatment with CBD on leukocyte migration into the lungs. (a) Total leukocytes, (b) Neutrophils, (c) Macrophages and (d) Lymphocytes in the BAL. Data are presented as the mean ± S.E.M., $n = 4–5$ mice/group. #$p < 0.05$ compared to Sal + Veh group, *$p < 0.05$, **$p < 0.01$ and ***$p < 0.0001$ compared to LPS + Veh group. One-way ANOVA followed by a Tukey–Kramer multiple comparison test.

after LPS intranasal instillation because at this time point it was already possible to observe significant increased total cell number in the BAL, myeloperoxidase (MPO) activity in the lung tissue and protein in the BAL supernatant (data not shown).

LPS intranasal instillation was effective in inducing lung inflammation (LPS + Veh group), since 24 h after its intranasal instillation we observed an increase in the number of leukocytes in the BAL (Figure 3a–d), MPO activity in the lung tissue (Figure 4a), protein concentration (Figure 4b) and cytokines (TNF and IL-6)/chemokines (MCP-1 and MIP-2) in the BAL supernatant (Figure 4c–f) ($p < 0.05$). The therapeutic treatment with CBD (20 and 80 mg/kg), given 6 h after the induction of lung inflammatory response, decreased total leukocytes counting in the BAL ($p < 0.0001$) in LPS-treated mice (Figure 3a). We also performed a differential analysis of leukocytes in the BAL and we observed that CBD (20 and 80 mg/kg) treatment decreased neutrophil ($p < 0.0001$) (Figure 3b), macrophage ($p < 0.01$) (Figure 3c) and lymphocyte ($p < 0.05$) (Figure 3d) migration into the lungs.

We also analyzed the neutrophil presence in the lung tissue by MPO activity and vascular permeability by protein concentration in the BAL supernatant. We found that the therapeutic treatment with CBD (80 mg/kg) decreased myeloperoxidase activity ($p < 0.01$) (Figure 4a). Additionally, CBD (80 mg/kg) decreased the protein concentration in BAL ($p < 0.05$) (Figure 4b).

Finally, we investigated the effects of the therapeutic treatment with CBD (20 and 80 mg/kg) on pro-inflammatory cytokines/chemokines in the BAL supernatant. The therapeutic treatment with CBD 20 mg/kg decreased the TNF ($p < 0.05$) (Figure 3c) and MIP-2 ($p < 0.0001$) (Figure 3f), while CBD 80 mg/kg decreased TNF ($p < 0.0001$), IL-6 ($p < 0.05$), MCP-1 ($p < 0.05$) and MIP-2 ($p < 0.0001$) (Figure 4c–f) levels in the BAL supernatant.

Qualitative analysis of lung sections showed that LPS intranasal instillation (LPS + Veh, Figure 5b) stimulated alveolar congestion and, mainly, increase in thickness of the alveolar walls and infiltration or aggregation of inflammatory cells, when compared to control mice (Figure 5a). In contrast, therapeutic treatment with CBD (20 and 80 mg/kg) reduced LPS-induced lung damage as reflected by the reduction of these parameters (Figure 5c and d).

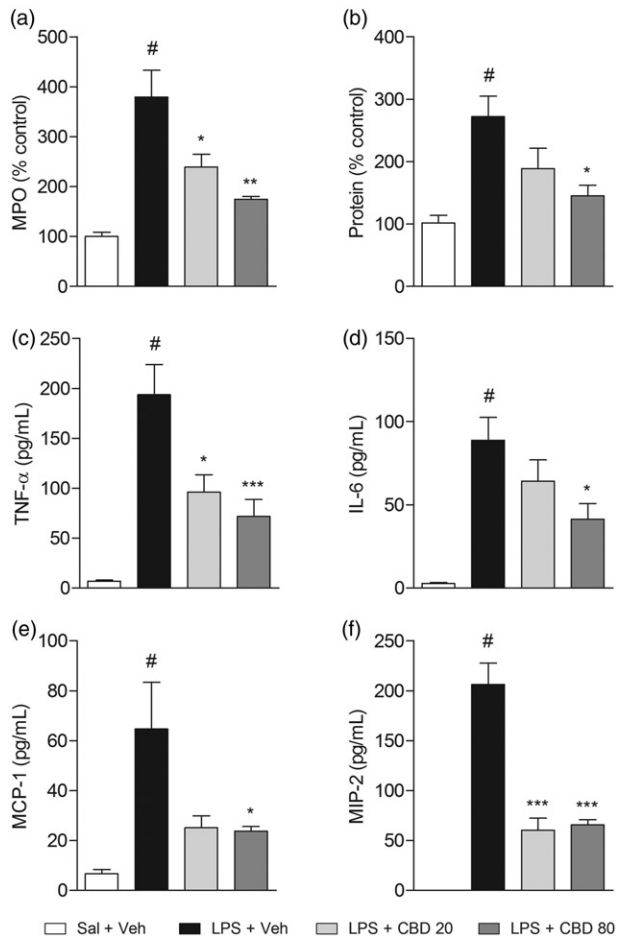

Figure 4. Effects of therapeutic treatment with CBD on inflammatory parameters. (a) Myeloperoxidase activity (MPO) in the lung tissue, (b) protein concentration, (c) TNF-α, (d) IL-6, (e) MCP-1 (CCL2) and (f) MIP-2 (CXCL2) levels in the BAL. Data are presented as the mean ± S.E.M., $n = 4–5$ mice/group. #$p < 0.05$ compared to Sal + Veh group, *$p < 0.05$, **$p < 0.01$ and ***$p < 0.0001$ compared to LPS + Veh group. One-way ANOVA followed by a Tukey–Kramer multiple comparison test.

Discussion

The therapeutic use of CBD was studied in a murine model of LPS-induced ALI. Results show that the treatment with CBD 6 h after the induction of inflammation improved the lung function, as observed by the decrease of the lung resistance and elastance induced by LPS after methacholine challenge.

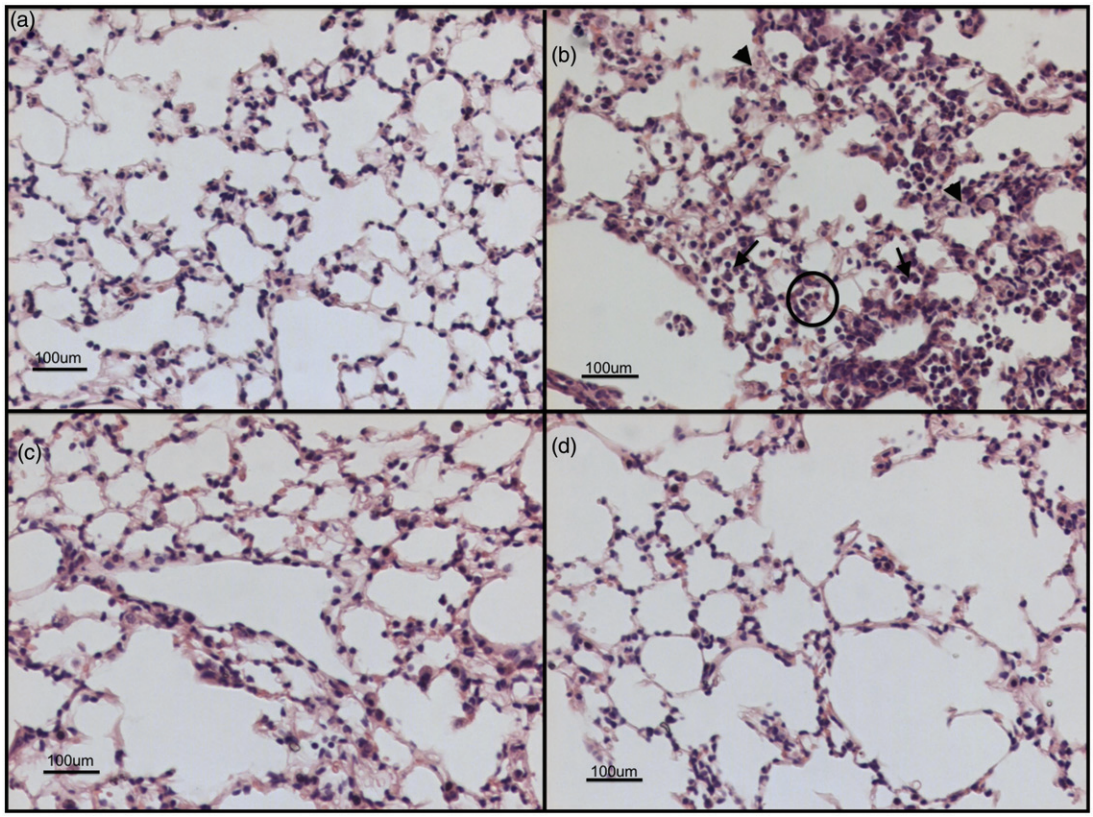

Figure 5. Representative images from lungs of each experimental group. (a) Sal + Veh, (b) LPS + Veh, (c) LPS + CBD 20 and (d) LPS + CBD 80. Circle: alveolar congestion; arrow head: thickness of alveolar walls; arrow: infiltration and aggregation of inflammatory cells. Sections from left lungs lobes were stained with hematoxylin and eosin (magnification = 200×).

We also observed that the therapeutic treatment with CBD decreased leukocyte migration (neutrophil, macrophages and lymphocytes) into the lungs, myeloperoxidase activity in the lung tissue, vascular permeability and production of pro-inflammatory cytokines/chemokines. Recently, we reported that prophylactic treatment with CBD, i.e. given before the induction of inflammation, decreased several inflammation parameters over time in LPS-induced ALI[7]. The present data strengthen our previous study and provide additional information about the beneficial effects of CBD on lung inflammation.

Our primary goal was to investigate the effects of CBD on pulmonary mechanics of mice submitted to LPS-induced lung inflammation. It is known that an important aspect of ALI is pulmonary dysfunction with alterations in the airway smooth muscle. Lung function is reduced in ALI and structural changes in the parenchyma, due the inflammatory process, might interfere in the respiratory mechanics and, consequently, in the function of the lung[19,20]. It was used as a pharmacological approach in order to investigate the effects of CBD on the LPS induced alterations in the lung function. The alterations in respiratory mechanics can be evaluated by the resistance and elastance measurements of lung after methacholine challenge. The results show that LPS intranasal instillation increased total lung resistance (reflects both narrowing of the conducting airways and parenchymal viscosity) and total lung elastance (considered to primarily reflect the elasticity of the lung parenchyma, but is also influenced by surface tension, smooth muscle contraction and peripheral airway inhomogeneity); also LPS-induced lung inflammation increased Newtonian resistance (a close approximation of resistance in the proximal airways) and tissue damping (reflects energy dissipation in the lung tissues)[13–17]. We observed that the therapeutic treatment with CBD decreased the previous effects induced by LPS and, therefore, improved the lung function.

Once we observed that CBD was able to improve lung function, our second goal was to investigate whether CBD given therapeutically, i.e. during an ongoing inflammatory process, would be able to decrease inflammation. One of the more important features of lung inflammation is an increase in leukocyte migration[21,22]; in addition to that is the production of pro-inflammatory cytokines and chemokines that attract the leukocytes to the site of inflammation. MCP-1, also known as CCL2, is commonly known to attract monocytes and lymphocytes[23] and neutrophils[24] to inflamed tissue. Similarly, MIP-2, also known as CXCL2, was reported as being an important factor to allow neutrophil attraction to the site of inflammation[25]. Regarding the effects of CBD on production of pro-inflammatory cytokines some efforts are being made to improve our understanding at the molecular level, and it is noteworthy to describe them here. It has been shown *in vitro* that CBD decreases the production and release of pro-inflammatory cytokines, such as IL1-β and IL-6, from LPS-activated microglia and that CBD reduces the activity of NF-κB, which regulates the expression of pro-inflammatory cytokines[26]. Moreover, it was shown that inhibition of pro-inflammatory cytokines induced by CBD is not mediated by the CB1 and CB2 cannabinoid receptors[26]. Together, these data strongly suggest that cytokines and chemokines are

relevant for the anti-inflammatory effects of CBD on ALI, either by decreasing the production of inflammatory mediators by resident cells and by the reduction in the number of inflammatory cells migrating to the site of inflammation.

Recently, it was reported that CBD (75 mg/kg *per os*) enhanced the pulmonary inflammation induced by LPS[27]; it should be noted that in their work more than increasing inflammation CBD was not able to decrease several inflammatory parameters. Discrepancies among these data and others reported in the literature probably rely on treatment protocol, since in Karmaus et al.[27] work animals received 75 mg/kg of CBD *per os* for 3 days prior to the induction of inflammation. Cannabinoids, including CBD, should be considered immune modulators rather than immune suppressive compounds[28,29], which is in accordance with a large body of evidence showing an increase in the immune response after the treatment with some cannabinoids. Nonetheless, it should not be forgotten there is overwhelming evidence supporting the anti-inflammatory effects of CBD[30–39].

It is noteworthy that our present and previous[7] results clearly show a dose-dependent effect for CBD on LPS-induced lung inflammation, which might be of clinical relevance. CBD has a large margin of safety when used for anxiety, psychosis and movement disorder management[40]. Currently, CBD and THC, delivered as an oromucosal spray, are being therapeutically used for the treatment of multiple sclerosis spasticity and currently being tested for cancer and neuropathic pain (Phase III and II, respectively). Safety studies of CBD were required before human tests. CBD was extensively investigated in laboratory animals to detect possible side or toxic effects[41]. CBD is well tolerated in humans, acute administration by the oral, inhalatory or intravenous route did not induce any significant toxic effect in humans; in addition, chronic administration of CBD for 30 days to healthy volunteers, at daily doses ranging from 10 to 400 mg, failed to induce any significant alteration in neurological, psychiatric or clinical exams[42,43]. In experimental reports showing anti-inflammatory effects of CBD in rodents, especially in mouse models of inflammation, the dose-range used is from 5 to 80 mg/kg. Considering the body surface area as suggested by Reagan-Shaw et al.[44], it is possible to calculate and translate the doses used in our study (20 and 80 mg/kg) from mice to humans; translating these doses it was obtained fixed-doses of 97.2 mg (1.62 mg/kg) and 388.8 mg (6.48 mg/kg), considering a 60 kg person, for 20 and 80 mg/kg dose in mice. This data provides us additional information and reinforces that CBD could be safely administered, over a wide dose range, and tested for the treatment of inflammatory diseases.

Conclusion

The data from the present study confirm and extend our understanding of the potential prophylactic and therapeutic use of CBD in inflammatory conditions, especially in lung inflammation. A variety of direct and indirect stimuli can induce ALI and ARDS, such as sepsis, pneumonia, acid aspiration, hyperoxia, high pressure ventilation, pulmonary contusion and ischemia/reperfusion events[45], with a high mortality rate and no specific treatment is still available. Therefore, our present and previous data suggest that CBD may become a relevant therapeutic tool for the management of inflammatory lung diseases. However, care should be taken when extrapolating data from laboratory animals to humans.

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Declaration of interest

The authors report no declarations of interest.

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