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Cannabidiol reduces airway inflammation and fibrosis in experimental allergic asthma

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ABSTRACT

Asthma is characterized by chronic lung inflammation and airway hyperresponsiveness. Asthma remains a major public health problem and, at present, there are no effective interventions capable of reversing airway remodelling. Cannabidiol (CBD) is known to exert immunomodulatory effects through the activation of cannabinoid-1 and − 2 (CB_1 and CB_2) receptors located in the central nervous system and immune cells, respectively. However, as the role of CBD on airway remodelling and the mechanisms of CB_1 and CB_2 aren't fully elucidated, this study was designed to evaluate the effects of cannabidiol in this scenario. Allergic asthma was induced in Balb/c mice exposed to ovalbumin, and respiratory mechanics, collagen fibre content in airway and alveolar septa, cytokine levels, and CB_1 and CB_2 expression were determined. Moreover, expressions of CB_1 and CB_2 in induced sputum of asthmatic individuals and their correlation with airway inflammation and lung function were also evaluated. CBD treatment, regardless of dosage, decreased airway hyperresponsiveness, whereas static lung elastance only reduced with high dose. These outcomes were accompanied by decreases in collagen fibre content in both airway and alveolar septa and the expression of markers associated with inflammation in the bronchoalveolar lavage fluid and lung homogenate. There was a significant and inverse correlation between CB^1 levels and lung function in asthmatic patients. CBD treatment decreased the inflammatory and remodelling processes in the model of allergic asthma. The mechanisms of action appear to be mediated by CB_1/CB_2 signalling, but these receptors may act differently on lung inflammation and remodelling.

1. Introduction

Asthma affects an estimated 300 million individuals worldwide and its prevalence has increased in the last years (Global Burden of Disease Study, 2015). Therefore, this disease is a serious global health problem that affects all age groups, with an increasing prevalence in many developing countries, rising treatment costs, and a rising burden for patients and the community (GINA – Global Initiative for Asthma, 2016). Currently, asthma is defined as a heterogeneous disease that is usually characterized by chronic airway inflammation (Reddel et al., 2015) and remodelling (Xisto et al., 2005). Airway remodelling is characterized by subepithelial fibrosis, mucous metaplasia, wall thickening, smooth muscle cell hypertrophy and hyperplasia, myofibroblast hyperplasia, vascular proliferation, and extracellular matrix modifications, such as collagen fibre deposition and elastic fibre fragmentation (Xisto et al., 2005; Holgate et al., 2004). Additionally, airway hyperresponsiveness (AHR) is one of the hallmarks of inflammation and remodelling in asthma (Aun et al., 2017).

Cannabinoids are components of the *Cannabis sativa* plant. The two most studied cannabinoids are Δ9-tetrahydrocannabinol (Δ9-THC) and cannabidiol (CBD). The latter is the primary non-psychotropic cannabinoid and is reported to have beneficial effects in many pathological conditions, including neuropsychiatric disorders, brain inflammatory diseases (Zuardi et al., 2006), colitis, sepsis-related encephalomyelitis,

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and inflammatory lung diseases (Burstein, 2015). Achieving and maintaining asthma control are widely recognized as the primary objectives of asthma management (GINA – Global Initiative for Asthma, 2016). Despite great advances in the understanding of asthma epidemiology, physiopathology and management, there is strong evidence suggesting that asthma control is still suboptimal in many patients, particularly in those with more severe asthma (Demoly et al., 2009). Thus, there is a clear need to explore new ways of managing and treating this respiratory disease (Chung, 2013).

CBD is known to exert anti-inflammatory, immunomodulatory, and analgesic effects through the activation of cannabinoid-1 and − 2 (CB_1 and CB_2) receptors located in the central nervous system (CNS) and immune cells, respectively (Klein, 2005; Pacher et al., 2006). Both CB_1 and CB_2 receptors have the ability to modulate the release of chemical messengers. By acting on CB_1 receptors, cannabinoids interact with a multitude of neurotransmitters in the CNS and can modulate their release (Marsicano et al., 2002). CB_2 controls the release of inflammatory cytokines, thereby regulating the immune system (Marinelli et al., 2016). Receptor-independent effects of CBD, such as antioxidant effects, have also been demonstrated (Rosenkrantz et al., 1981; Pertwee, 2008).

One possible mechanism of immune control by cannabinoids during inflammation is the dysregulation of cytokine production by immune cells and subsequent disruption of the well-regulated immune response (Klein et al., 2000). A previous study from our laboratory indicated that CBD treatment reduced the production of relevant cytokines in an animal model of chronic asthma (Vuolo et al., 2015). Recently, the involvement of CBD in the control of the inflammatory response, including in inflammatory lung diseases (Ribeiro et al., 2012), has been reviewed, demonstrating acute and chronic effects on inflammation (Nagarkatti et al., 2009). In addition, CBD is well tolerated without significant side effects, even when chronically administered in humans (Rosenkrantz et al., 1981; Pertwee, 2008). Therefore, CBD is a plausible option for treating chronic inflammatory diseases such as asthma. So far, to our knowledge, no study has evaluated the role of CBD on airway remodelling as well as the possible mechanisms of action of CB_1 and CB_2 receptors.

We hypothesized that the administration of CBD could mitigate lung inflammation and remodelling in experimental allergic asthma in mice and may be associated with CB_1 and/or CB_2 receptors. For this purpose, we investigated the effects of two different doses of CBD in ovalbumin induced asthma and whether CB blockade could revert the beneficial effects on lung inflammation and remodelling. Additionally, the relationship between CB_1 and CB_2 expressions in inflammatory cells obtained by induced sputum from asthmatic patients as well as airway inflammation and function were also evaluated.

2. Material and methods

The Ethics Committee of the University of Southern Santa Catarina and University Federal of Rio de Janeiro (CEUA-019) approved this study. All animals received care in compliance with the “Principles of Laboratory Animal Care” by the National Society for Medical Research and the “Guide for the Care and Use of Laboratory Animals” by the National Academy of Sciences, USA (Carbone, 2012).

2.1. Study design and protocol

One hundred and forty-seven Balb/c mice (20–25 g) were randomly assigned to two groups: CTRL and OVA. In the OVA group, mice were immunized using an adjuvant-free protocol by intraperitoneal injection of sterile ovalbumin (OVA, 10 μg of OVA in 100 μl saline) on 7 alternate days. Forty days after the beginning of sensitization, 20 μg of OVA in 20 μl saline was instilled intratracheally. This procedure was performed three times with 3-day intervals between applications (Xisto et al., 2005). In the CTRL, sterile saline solution was administered using the same protocol (n = 21). The OVA group was further randomized into three subgroups, which received vehicle saline (OVA – asthma control, n = 21) or cannabidiol (99.9% pure; STI-Pharm, Brentwood, UK) in two different doses, 5 or 10 mg/kg i.p. (OVA5 and OVA10, respectively, n = 21/each), at same time as the OVA challenge. Finally, the OVA10 group was further divided into three subgroups that received an antagonist of CB^1, CB^2 or of both receptors (OVA10-CB_1 – AntiCB_1 - AM251 – 3 mg/kg i.p., OVA10-CB^2 - AntiCB_2 - AM630 – 3 mg/kg, i.p., OVA10- CB_1/CB_2, n = 21/each) 30 min prior to CBD treatments (Hassanzadeh and Rostami, 2014; Mansouri et al., 2014). All treatments and vehicle were administered in a volume of 1 ml/kg. Forty-nine mice were used for assessment of airway responsiveness after methacholine challenge (n = 7/group). As methacholine and bronchoalveolar lavage technique may affect lung histological analysis, another 49 mice were used to evaluate lung mechanics and histology as well as levels of cytokines (n = 7/group), whereas 49 animals were used for analysis of pro-inflammatory mediators in bronchoalveolar lavage fluid (BAL fluid) (n = 7/group). Fig. 1 presents a schematic of the study design.

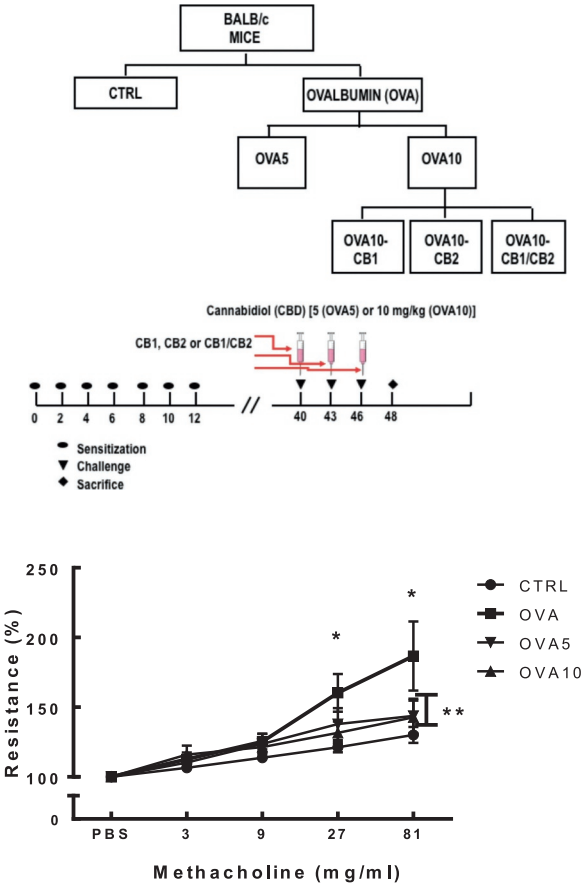

Fig. 1. Schematic flow chart and timeline of the study design. CTRL: Mice sensitized and challenged with saline; **OVA:** mice sensitized and challenged with ovalbumin; **OVA5**: mice sensitized, challenged with ovalbumin and treated with 5 mg/kg cannabidiol (CBD); **OVA10:** mice sensitized, challenged with ovalbumin and treated with 10 mg/kg CBD; **OVA10-CB_1**: mice sensitized, challenged with ovalbumin, treated with 10 mg/kg CBD and a CB_1 antagonist (3 mg/kg i.p.) 30 min before CBD therapy; **OVA10-CB_2**: mice sensitized, challenged with ovalbumin, treated with 10 mg/kg CBD and a CB_2 antagonist (3 mg/kg i.p.) 30 min before CBD therapy; **OVA10-CB_1/CB_2**: mice sensitized, challenged with ovalbumin, treated with 10 mg/kg CBD and CB_1 and CB_2 antagonists (3 mg/kg i.p.) 30 min before CBD therapy.

2.2. Airway responsiveness

Mice were sedated (diazepam, 1 mg/kg intraperitoneally),

anesthetized (thiopental sodium, 20 mg/kg intraperitoneally), tracheostomised, and paralysed (vecuronium bromide, 0.005 mg/kg intravenously). Briefly, airway responsiveness was assessed as a change in airway resistance 24 h after the last treatment following aerosolized methacholine in a FinePoint R/C Buxco Platform (Buxco Electronics, Sharon, CT, USA). Airflow and transpulmonary pressure were recorded using a Buxco Pulmonary Mechanics Processing System (Buxco Electronics, Wilmington, NC, USA). Analogue signals from the computer were digitized using a Buxco Analogue/Digital Converter (Buxco Electronics). Mice were stabilized for 5 min and increasing concentrations of methacholine (3, 9, 27, and 81 mg/ml in PBS; Sigma Chemical Co., St Louis, Mi, USA) were delivered by aerosol for 5 min each. Baseline resistance was assessed with aerosolized PBS. Response in airway resistance to inhaled methacholine was expressed as percentage of the response to the PBS aerosol (Abreu et al., 2014).

2.3. Lung mechanics

After the last methacholine challenge, animals were then ventilated with a constant flow ventilator (Samay VR15; Universidad de la Republica, Montevideo, Uruguay) set to the following parameters: respiratory rate: 100 breaths/min; tidal volume: 0.2 ml; and fraction of inspired oxygen: 0.21. The anterior chest wall was surgically removed, and a positive end-expiratory pressure of 2 cmH_2O was applied. Airflow and tracheal pressure were measured (Burburan et al., 2007). Static lung elastance (Est,L) was analysed by the end-inflation occlusion method (Bates et al., 1988). Briefly, after end-inspiratory occlusion, there is an initial fast drop in transpulmonary pressure from the pre-occlusion value down to an inflection point, followed by a slow pressure decay until a plateau is reached. This plateau corresponds to the elastic recoil pressure (Pel) of the lung and Est,L was calculated by dividing Pel by the tidal volume. All data were analysed using ANADAT data analysis software (RHT-InfoData, Inc., Montreal, QC, Canada).

2.4. Lung morphometry

A laparotomy was performed immediately after the determination of lung mechanics, and heparin (1000 IU) was intravenously injected in the vena cava. The trachea was clamped at end expiratory lung volume, and the abdominal aorta and vena cava were sectioned, yielding a massive haemorrhage that quickly killed the animals. The left lung was then removed, fixed in 3% buffered formaldehyde and embedded in paraffin. Slices (4 μm thick) were cut, deparaffinized, and stained with haematoxylin and eosin.

Lung morphometric analysis was performed with an integrating eyepiece with a coherent system consisting of a grid with 100 points and 50 lines (known length) coupled to a conventional light microscope (Olympus BX51, Olympus Latin America-Inc., Brazil). The volume fraction of collapsed and normal pulmonary areas, and the number of mononuclear cells (MN), eosinophils and total cells in pulmonary tissue were determined by the point-counting technique (Weibel, 1990; Hsia et al., 2010) across 10 random, non-coincident microscopic fields (Xisto et al., 2005; Burburan et al., 2007). Collagen (Picrosirius-polarization method) fibres were quantified in airways and alveolar septa (Xisto et al. (2005), Antunes et al. (2010) using Image-Pro Plus 6.0 software. All histological analyses were performed by a researcher (SCA) blinded to the experimental protocol.

2.5. Cytokine levels

IL-4, IL-5, IL-13 and eotaxin were measured in lung homogenate and bronchoalveolar lavage fluid with Luminex xMap™ Technology, and were expressed as pg/ml.

2.6. CB_1, CB_2 and metalloproteinase (MMP-2 and MMP-9) protein contents in Balb/c mice

Samples of lung tissue were fractionated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransferred to nitrocellulose membranes. Membranes were incubated with rabbit polyclonal anti-CB^1 (1:1000) (ABCAM - Brazil), anti-CB^2 (1:1000) (ABCAM - Brazil), anti-MMP-2 (1:1000) (ABCAM - Brazil) and anti-MMP-9 (1:1000) (ABCAM - Brazil) antibodies. Secondary anti-rabbit IgG was incubated with the membranes for 2 h (1:10000). The membranes were washed with TTBS, and the immunoreactivity was detected by chemiluminescence using enhanced chemiluminescent (ECL) (Bio-Rad – Brazil) substrates for the detection of horseradish peroxidase (HRP) enzyme activity. Densitometry analysis was performed with ImageJ® v.1.34 software. All results are expressed as the relative ratio between CB_1, CB_2, MMP-2 or MMP-9 and β-actin.

2.7. CB_1 and CB_2 protein contents in induced sputum

As a non-invasive biomarker (Pizzichini et al., 1996) in of CB pathway, CB_1 and CB_2 protein content was measured in induced sputum from asthma patients. To determine its putative relevance in asthma development, CB_1 and CB_2 sputum levels were correlated with sputum markers of inflammation and pulmonary function (post-bronchodilator forced expiratory volume in 1 s). Sputum was induced using a previously published protocol (Pizzichini et al., 1996) in six patients with well-controlled asthma according to the GINA guidelines (GINA – Global Initiative for Asthma, 2016) and in six healthy subjects. Total and differential cell counts were performed, and CB_1 and CB_2 protein contents were measured by western blot.

2.8. Statistical analysis

The statistical program GraphPad Prism 6.0 (Graph Pad software, Inc.) was used for statistical analysis. Parametric data were analysed by one-way ANOVA followed by Tukey's test for multiple comparisons; the data are reported as the mean ± S.E.M. Nonparametric data were analysed using the Kruskal-Wallis test followed by Dunn's multiple comparison test; the data are reported as the median with interquartile range. Two-way repeated measures ANOVA followed by Dunn's post-hoc test was used to compare methacholine dose ranges. Correlation between sputum CB_1 and CB_2 protein content and sputum cell counts and post-bronchodilator forced expiratory volume in 1 s was performed using Pearson correlation test. Differences were considered significant when $P < 0.05$.

3. Results

The increase in airway resistance elicited by methacholine was significantly augmented in the OVA group compared to CTRL [Fig. 2 (left panel)]. However, these changes were mitigated to CTRL levels in the groups treated with the two different doses of CBD. The effects of CBD appear to be mediated by CB_1 receptor since the administration of anti-CB_1, but not anti-CB_2, blocked the CBD effect, leading to increased airway resistance (Fig. 2 (right panel)). Interestingly, double blockade of CB_1 and CB_2 receptors did not have the same effect, suggesting a complex interaction between CB^1 -and CB^2 -mediated effects. This complex interplay between CB_1 and CB_2 receptors was further observed when studying Est,L (Fig. 2 (lower panel)). The increased Est,L observed in the OVA group was reduced by the highest dose of CBD. However, this effect of CBD was only reversed by the double blockade of CB_1 and CB_2.

To better understand the remodelling process in this experimental model, collagen fibre content was measured in airways and alveolar septa. Accordingly, the OVA group presented an increase in collagen fibre content in airways and alveolar septa compared to the CTRL

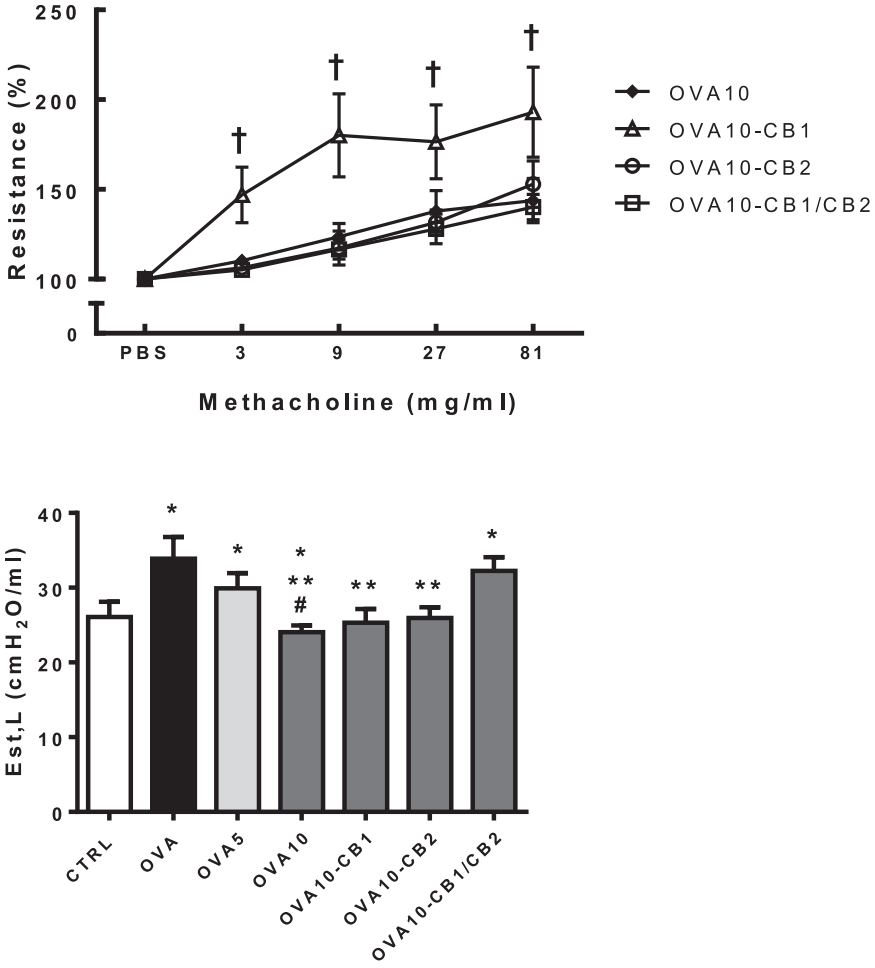

Fig. 2. Airway responsiveness and lung mechanics in experimental allergic asthma. Airway resistance (% PBS) after increasing doses of methacholine (Upper panels) and static lung elastance (Est, L – Lower panel). **CTRL:** mice sensitized and challenged with saline; **OVA:** mice sensitized and challenged with ovalbumin and treated with saline; **OVA5**: mice sensitized, challenged with ovalbumin and treated with 5 mg/kg cannabidiol (CBD); **OVA10:** mice sensitized, challenged with ovalbumin and treated with 10 mg/kg CBD; **OVA10-CB_1**: mice sensitized, challenged with ovalbumin, treated with 10 mg/kg CBD and a CB_1 antagonist; **OVA10-CB_2**: mice sensitized, challenged with ovalbumin, treated with 10 mg/kg CBD and a CB2 antagonist; **OVA10-CB_1/CB_2**: mice sensitized, challenged with ovalbumin, treated with 10 mg/kg CBD and CB_1 and CB_2 antagonists. *Significantly different from CTRL ($P < 0.05$). **Significantly different from OVA ($P < 0.05$). † Significantly different from OVA10.

group, and this was subsequently reduced by CBD treatment (Fig. 3A and B). Both single and double blockade of CB_1 and CB_2 receptors reversed collagen deposition in both airways and alveolar septa (Fig. 3A and B). In addition, single blockade of CB_1 receptor increased the collagen deposition in the alveolar septa compared to CTRL and OVA (Fig. 3A). No significant changes were observed in the content of metalloproteinase (MMP-2 and MMP-9) in the lung tissue between groups (*online* supplementary material).

Increased areas of alveolar collapse resulted in changes in static lung elastance in the OVA group (Table 1) that were reversed by the highest dose of CBD. The combination of CB^1 and CB^2 antagonists reversed this effect (Table 1). During asthma development, alterations in lung mechanics was associated with inflammation. In this context, we observed a significant increase in the number of eosinophils in the OVA compared to CTRL group (Table 1). This inflammatory cell infiltration in the lung tissue was reduced with 10 mg/kg CBD (Table 1) but was not influenced with CB_1 or CB_2 antagonism (Table 1).

We further assessed the levels of different cytokines involved in the OVA-induced inflammatory process in BAL fluid (Fig. 4) and lung homogenate (Fig. 5). OVA increased the levels of IL-4, IL-5 IL-13 and eotaxin compared to CTRL, and CBD generally decreased all measured cytokines when compared to OVA (Fig. 4 and 5). This effect was not reversed by blockade of either CB^1 or CB^2 receptor. However, double blockade of both CB_1 and CB_2 receptors not only reversed the CBD effects but also increased the levels of IL-4, IL-5 and IL-13 in BAL fluid compared to the OVA group (Fig. 4 A-C). Eotaxin in BAL fluid was also decreased by CBD treatment (5 mg/kg), and this reduction was reversed by the single blockade of either CB_1 or CB_2 receptor. The anti-inflammatory effect of CBD was also consistent in the lung homogenate and generally was not affected by blockade of CB_1 and CB_2 receptors (Fig. 5 A-D).

Since CBD was able to reduce inflammation and improve lung mechanics and antagonists could exacerbate some aspects of the lung response to OVA, we further evaluated whether OVA could up-regulate CB_1 and CB_2 protein contents. There was no significant effect of OVA on either CB_1 or CB_2 protein expression in the lung (*online* supplementary material).

In addition, we did not detect CB_1 or CB_2 protein in the induced sputum of healthy volunteers, but both were present in asthmatic patients (CB_1 levels: median, 1.74; minimum, 1.01; maximum, 3.37; CB_2 levels: median, 1.84; minimum, 1.6; maximum, 2.7; n = 6 patients). There was no significant correlation between CB receptor protein levels and induced sputum inflammatory cell levels (data not shown), but there was a significant and strong inverse correlation between CB_1 levels and the percentage of post-bronchodilator forced expiratory volume in 1 s ($r = -0.92$, $P = 0.026$).

4. Discussion

The results of the present study demonstrated that treatment with CBD was effective in minimizing the inflammatory response and structural changes that characterize the remodelling process of asthma in a murine model of ovalbumin induced allergic asthma. These effects appear to be mediated by an interaction involving CB_1 and CB_2 receptor

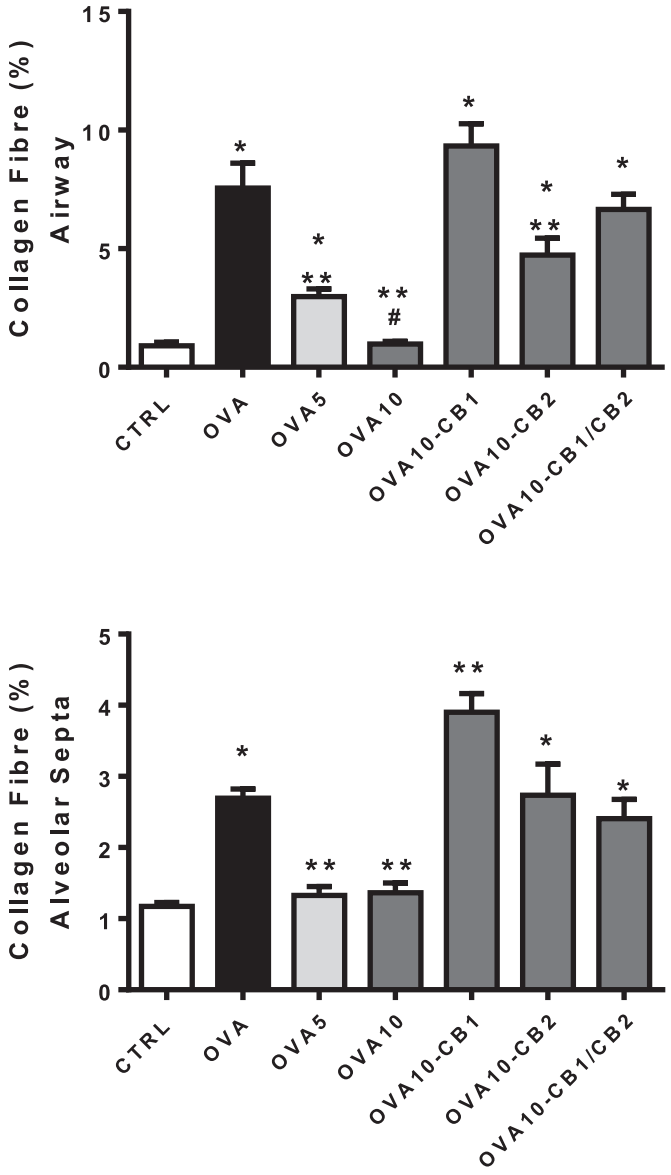

Fig. 3. Collagen fibre content in airways and alveolar septa in experimental allergic asthma. All values were computed in 10 random, non-coincident fields of view per mouse. **CTRL:** mice sensitized and challenged with saline; **OVA:** mice sensitized and challenged with ovalbumin and treated with saline; **OVA5**: mice sensitized, challenged with ovalbumin and treated with 5 mg/kg cannabidiol (CBD); **OVA10:** mice sensitized, challenged with ovalbumin and treated with 10 mg/kg CBD; **OVA10-CB_1**: mice sensitized, challenged with ovalbumin, treated with 10 mg/kg CBD and a CB_1 antagonist; **OVA10-CB_2**: mice sensitized, challenged with ovalbumin, treated with 10 mg/kg CBD and a CB_2 antagonist; **OVA10-CB_1/CB_2**: mice sensitized, challenged with ovalbumin, treated with 10 mg/kg CBD and CB_1 and CB_2 antagonists. *Significantly different from CTRL ($P < 0.05$). **Significantly different from OVA-SAL ($P < 0.05$). #Significantly different from OVA5.

activation.

This model, described by Xisto et al. (2005), reproduced many features of human asthma, including denudation of the bronchial epithelium, infiltration and activation of eosinophils, presence of T lymphocytes and macrophages in the lumen and airway mucosa, and hypertrophy and hyperplasia of smooth muscle cells and mucus glands (Xisto et al., 2005). However, such changes may vary depending on the duration of the protocol and the species used (Antunes et al., 2009). CD4 + T cells, Th2 cytokines, and allergen-specific IgE antibodies are the main inflammatory mediators involved in allergic asthma. The interaction of these mediators induces airway inflammation and hyperresponsiveness. Airways from asthmatic individuals are infiltrated with eosinophils, this inflammatory process has been considered relevant to the pathogenesis of the disease. Although the connection between the inflammatory process and fibrotic remodelling is well recognized, the role of CB receptors during this process is not well known. A recent study demonstrated that CBD was able to decrease inflammatory parameters in a murine model of acute lung injury induced by LPS (Ribeiro et al., 2015). Although the inflammation involved in LPS-induced acute lung injury differs from the inflammatory response observed in asthma models, this result suggests a positive effect of CBD in lung inflammation.

One of the effects observed after CBD treatment was a reduction in airway hyperresponsiveness, which may be associated with the anti-inflammatory potential of CBD. Additionally, these effects appear to be mediated by CB_1 receptor since the administration of anti-CB_1 blocked CBD effect. An intriguing fact is the observation that the administration of anti-CB_1 increases airway resistance with increased doses of methacholine doses. This suggests a possible relevant effect of endogenous CB_1 ligands, since CB_1 receptor activation inhibited airway contraction in both normal and inflamed tracheas, directly inhibiting cholinergic-induced contractions (Wang et al., 2016). It was also demonstrated that CB-mediated effect was associated with the improvement of static lung elastance and reduced collagen fibre content. Therefore, improvement in airflow resistance may be explained by the reduction in alveolar collapse and the content of collagen fibres in the lung tissue (Xisto et al., 2005; Abreu et al., 2011). Asthmatic airways have subepithelial collagen deposition and changes in the structure of airway following epithelial cell damage and airway inflammation lead to the development of chronic asthma and airway hyperresponsiveness (Boulet et al., 1997). The persistence of airway hyperresponsiveness despite CBD anti-inflammatory effects suggests that not only the inflammatory process results in airway hyperresponsiveness, but also the structural modifications of the airways (Boulet et al., 2000; Ward et al., 2002) that increases airway stiffness. Moreover, collagen fibre in alveolar septa may increase airway constriction through parenchymal tethering (Khan et al., 2010). In short, airway hyperresponsiveness, inflammation and remodelling are interrelated events (Bellini et al., 2012).

The lung homogenate of OVA-exposed animals showed a significant increase in IL-4 and IL-13, which are important inflammatory mediators involved in the pathophysiology of asthma. Some studies have demonstrated that both IL-4 and IL-13 stimulate fibrocytes to produce collagen fibres and other extracellular matrix components (Bellini et al., 2012), as well as recruit eosinophils into lung tissue (Rothenberg et al., 2011). Therefore, the reduction in IL-4 and IL-13 promoted by the administration of CBD may be associated with a decrease in both the number of eosinophils and the deposition of collagen fibres in the lung tissue, which may be related to the decreased in airway hyperresponsiveness after CBD treatment. CB_2 is found in cells of the immune system, including T and B cells, monocytes and eosinophils, and its effects are mediated by G-proteins. Interestingly, this mediation by G-proteins is a common feature of chemokine receptors. Some of the endogenous ligands of CB receptors are arachidonic acid derivatives. Additionally, endocannabinoids exert a small chemotactic effect upon eosinophils that is partially dependent on CB_2 (Larose et al., 2014). However, this effect seems to be complex, since it also depends on IL-5 and the 15-lipoxygenase pathways (Chiurchiù et al., 2015). This complexity is also found in the effects of CB_2 on other immune system cells (Giannini et al., 2008). Specifically, in the context of asthma, we previously demonstrated that CBD treatment decreases plasma Th2 cytokines in rats sensitized to OVA (Vuolo et al., 2015). Giannini *et al.* evaluated the effects of a dual CB_1/CB_2 receptor agonist on OVA-

Table 1
Lung Morphometry Fraction area of normal and collapsed alveoli in the lung parenchyma as well as the percentage of mononuclear cells (MN), eosinophils, and total cells in lung tissue. **CTRL:** mice sensitized and challenged with saline; **OVA:** mice sensitized and challenged with ovalbumin; **OVA5**: mice sensitized, challenged with ovalbumin and treated with 5 mg/kg CBD; **OVA10:** mice sensitized, challenged with ovalbumin and treated with 10 mg/kg CBD; **OVA10-CB1**: mice sensitized, challenged with ovalbumin, treated with 10 mg/kg CBD and a CB1 antagonist; **OVA10-CB2**: mice sensitized, challenged with ovalbumin, treated with 10 mg/kg and a CB2 antagonist; **OVA10-CB1/CB2**: mice sensitized, challenged with ovalbumin, treated with 10 mg/kg and CB1 and CB2 antagonists.

Groups	CTRL	OVA	OVA5	OVA10	OVA10-CB1	OVA10-CB2	OVA10-CB1/CB2
Normal (%)	94.8 ± 1.1	72.2 ± 1.0[a]	77.7 ± 1.8[a]	86.0 ± 1.6[a,b,c]	82.3 ± 1.1[a,b]	84.4 ± 1.1[a,b]	70.6 ± 6.1[a,b,c]
Collapse (%)	5.2 ± 1.1	27.8 ± 1.0[a]	22.3 ± 1.8[a]	14.0 ± 1.6[a,b,c]	17.7 ± 1.1[a,b]	15.6 ± 1.1[a,b]	29.4 ± 6.1[a,b,c]
MN (%)	16.1 ± 4.7	16.5 ± 4.3	18.8 ± 2.2	19.5 ± 1.6	19.2 ± 2.4	21.5 ± 3.4	17.6 ± 4.9
Eosinophils (%)	1.9 ± 0.9	9.1 ± 1.65[a]	5.3 ± 2.6[a]	3.2 ± 1.8[a,b]	4.7 ± 0.95[a,b]	3.1 ± 1.05[a,b]	4.9 ± 1.7[a,b]
Total Cells (%)	18.0 ± 4.6	25.6 ± 4.0	24.1 ± 2.7	22.7 ± 2.4	23.9 ± 2.3	24.6 ± 3.3	22.5 ± 5.0

[a] Significantly different from the CRTL group.
[b] Significantly different from the OVA group ($P < 0.05$).
[c] Significantly different from the OVA5 group ($P < 0.05$).

induced asthma in guinea pigs and concluded that both CB_1 and CB_2 receptors are involved in lung protection (Giannini et al., 2008). However, a more recent study demonstrated that activation of CB_2 aggravates allergic inflammation both *in vitro* and *in vivo* (Frei et al., 2016). It seems that CB_2 activation is of pivotal relevance to priming eosinophils, thereby increasing their responsiveness towards different chemoattractants (Frei et al., 2016). Therefore, activation of CB_2 receptor before OVA challenge worsens airway hyperresponsiveness to methacholine (Giannini et al., 2008). Nevertheless, the present study reported that CBD, an agonist of both CB_1 and CB_2 receptors, when administered at the same time as the OVA challenge, promoted different effects. In addition, some of our observed effects were dependent on single activation of CB_1 or CB_2 or on double activation of CB receptors. These results illustrate a complex role of CB receptors in allergic inflammation.

The effects of CB agonists could target not only immune cells. CB_2 receptors are also found in fibroblasts, and it was recently demonstrated that activation of CB_2 could improve wound healing by attenuating fibroblast accumulation and myofibroblast transformation (Del Rio et al., 2016). Adding further complexity to CBD, the antifibrotic effects of the CB_2 pathway also depend on the activation of peroxisome proliferator-activated receptor-gamma (Bozkurt et al., 2016b). These results could be relevant to understanding the effects of CBD on the lung mechanics demonstrated here. Serotonin-induced airway hyperresponsiveness was also decreased by CB_1 activation (Bozkurt et al., 2016a). It has also been demonstrated that the CBD effects could be mediated by TRPV receptors (Hedge et al., 2011) and the adenosine A2A receptor (Vuolo et al., 2015). However, suppression of

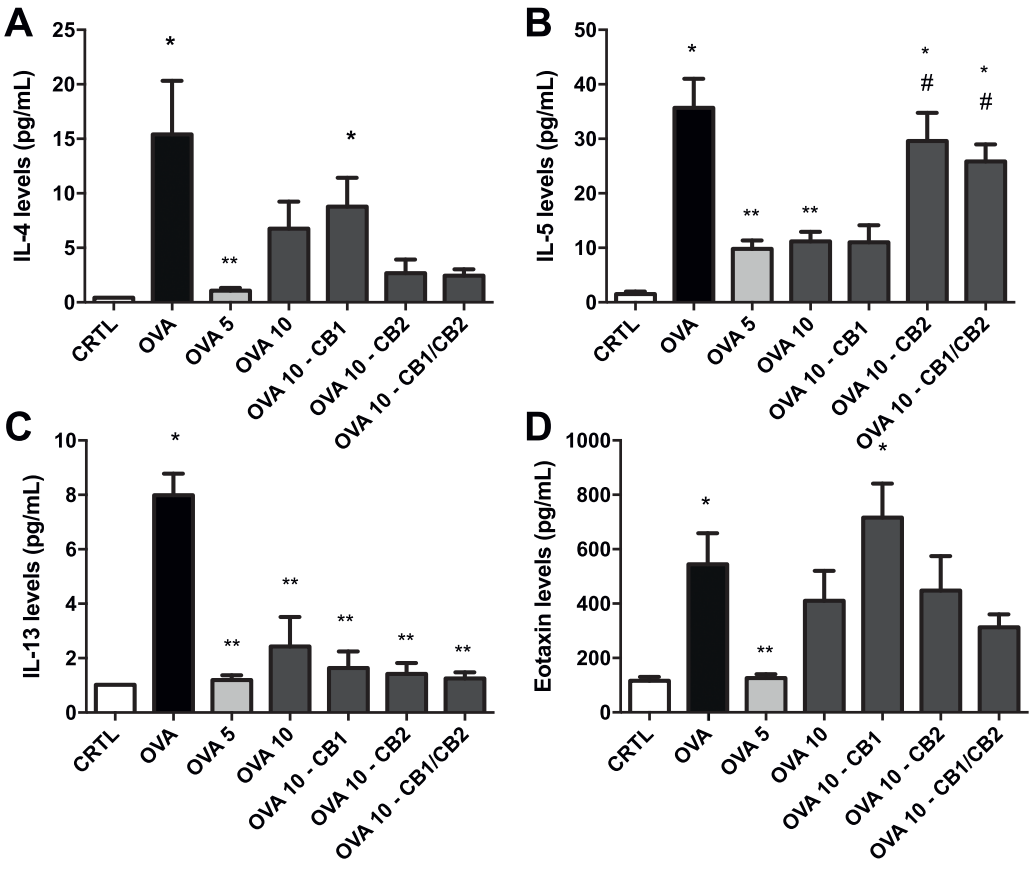

Fig. 4. Cytokine levels in bronchoalveolar lavage fluid in experimental allergic asthma. Levels of IL-4 (A), IL-5 (B), IL-13 (C) and eotaxin (D) in the bronchoalveolar lavage fluid. **CTRL:** mice sensitized and challenged with saline; **OVA:** mice sensitized and challenged with ovalbumin; **OVA5**: mice sensitized, challenged with ovalbumin and treated with 5 mg/kg CBD; **OVA10:** mice sensitized, challenged with ovalbumin and treated with 10 mg/kg CBD; **OVA10-CB_1**: mice sensitized, challenged with ovalbumin, treated with 10 mg/kg CBD and a CB^1 antagonist; **OVA10-CB_2**: mice sensitized, challenged with ovalbumin, treated with 10 mg/kg CBD and a CB^2 antagonist; **OVA10-CB_1/CB_2**: mice sensitized, challenged with ovalbumin, treated with 10 mg/kg and CB_1 and CB_2 antagonists. Bars represent the median with interquartile range, n = 7 for each group. *Significantly different from CTRL ($P < 0.05$). * *Significantly different from OVA ($P < 0.05$). #Significantly different from OVA5 ($P < 0.05$).

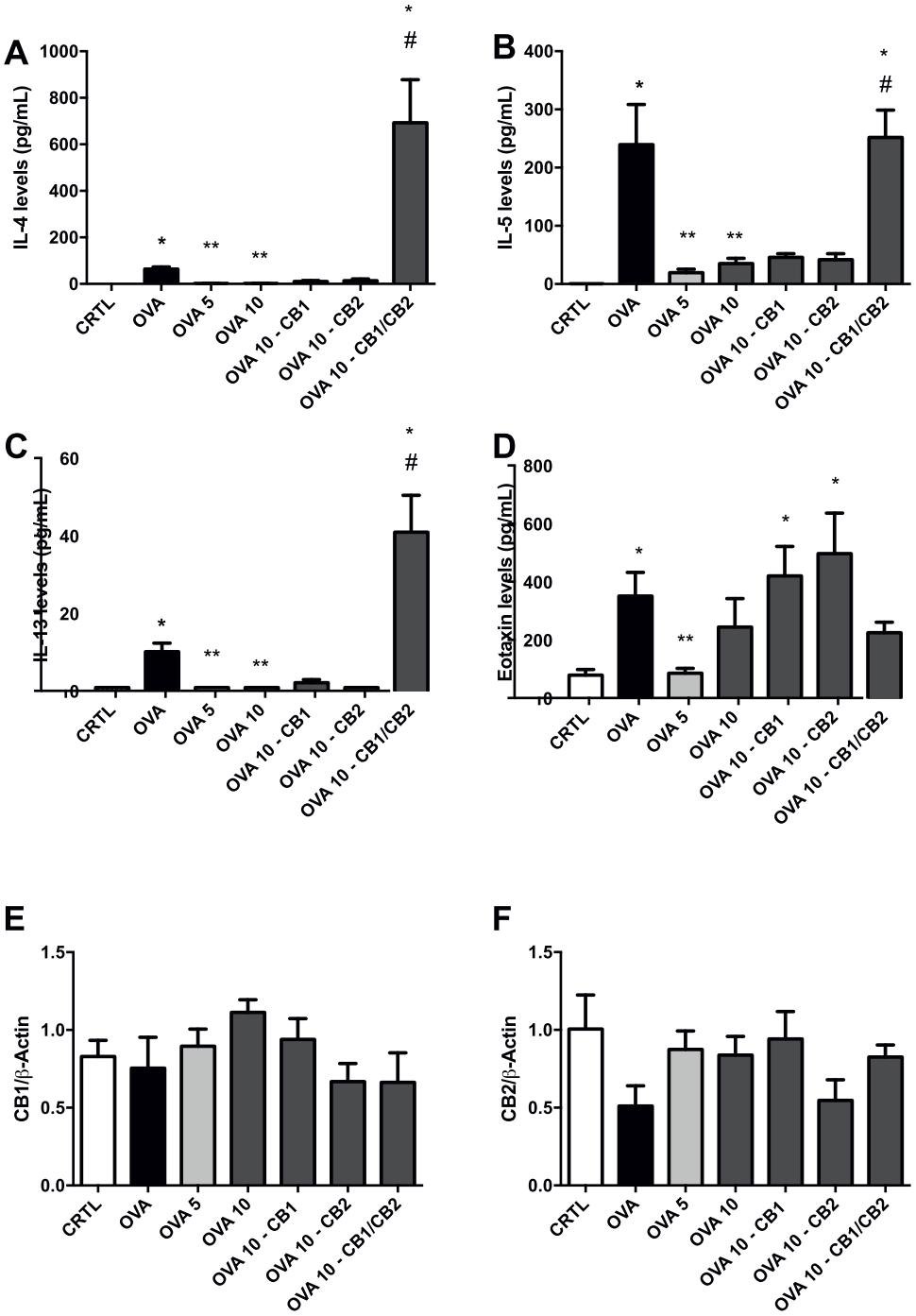

Fig. 5. Cytokine levels in lung tissue in experimental allergic asthma. Levels of IL-4 (A), IL-5 (B), IL-13 (C) and eotaxin (D) levels in the lung tissue. **CTRL:** mice sensitized and challenged with saline; **OVA:** mice sensitized and challenged with ovalbumin; **OVA5**: mice sensitized, challenged with ovalbumin and treated with 5 mg/kg CBD; **OVA10:** mice sensitized, challenged with ovalbumin and treated with 10 mg/kg CBD; **OVA10-CB_1**: mice sensitized, challenged with ovalbumin, treated with 10 mg/kg CBD and a CB_1 antagonist; **OVA10-CB_2**: mice sensitized, challenged with ovalbumin, treated with 10 mg/kg CBD and a CB2 antagonist; **OVA10-CB_1/CB_2**: mice sensitized, challenged with ovalbumin, treated with 10 mg/kg CBD and CB_1 and CB_2 antagonists. Bars represent the median with interquartile range, n = 7 for each group. *Significantly different from CTRL ($P < 0.05$). **Significantly different from OVA ($P < 0.05$). #Significantly different from OVA5 ($P < 0.05$).

hypersensitivity in rat vagal lung C-fibre afferents is dependent on the activation of CB_1 but not TRPV and adenosine receptors (Yeh et al., 2016). Combined, these findings could partially explain our results. Finally, testing a wider range of CBD doses would have helped determine whether the familiar bell-shaped response curve also occurs here.

Some limitations of our study must be acknowledged. First, our results did not reveal precisely the isolated role of CB_1 or CB_2 receptors in different aspects of asthma pathophysiology. Thus, further studies are needed to better understand the mechanism of the cannabinoid system in asthma development. However, our results are clinically relevant, as CBD is available for human use and was effective in our model. Second, it is not possible to determine if inhaled CBD would have the same protective effects as systemic administration did; to improve clinical relevance, this hypothesis must be tested. Third, it is possible that some endogenous ligands of CB receptors could have some role in the development of asthma, but our experimental design cannot answer precisely this issue.

In conclusion, CBD treatment decreases the inflammatory and remodelling processes in a murine model of ovalbumin induced allergic asthma. The beneficial effect upon airway hyperresponsiveness seems to be dependent on a receptor mediated effect of CBD. However, the protective CBD effects on the inflammatory response appears to be more complex, and generally independent of receptor agonism.

CRediT authorship contribution statement

Francieli Vuolo: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing - original draft. **Soraia C. Abreu:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology. **Monique Michels:** Investigation, Methodology. **Débora G. Xisto:** Investigation, Methodology. **Natália G. Blanco:** Investigation, Methodology. **Jaime EC Hallak:** Resources, Validation. **Antonio W. Zuardi:** Resources, Validation. **José A. Crippa:** Resources, Validation. **Cardine Reis:** Investigation, Methodology. **Marina Bahl:** Investigation, Methodology. **Emílio Pizzichinni:** Resources, Validation, Supervision. **Rosemeri Maurici:** Methodology, Validation, Supervision. **Marcia M.M. Pizzichinni:** Resources, Validation, Supervision. **Patricia R.M. Rocco:** Resources, Validation, Resources, Supervision, Writing - review & editing. **Felipe Dal-Pizzol:** Resources, Validation, Resources, Supervision, Writing - review & editing.

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Conflict of interest

JEH, AWZ and JAC are co-inventors on the patent “Fluorinated CBD compounds, compositions and uses thereof. Pub. No.: WO/2014/108899. International Application No.: PCT/IL2014/050023″. International Application No.: PCT/IL2014/050023″; Def. US no. Reg. 62193296; 29/07/2015; INPI em 19/08/2015 (BR1120150164927). The University of São Paulo licensed this patent to Phytecs Pharm (Resolução USP No. 15.1.130002.1.1). The University of São Paulo has an agreement with Prati-Donaduzzi (Toledo, Brazil): “Desenvolvimento de um produto farmacêutico contendo canabidiol sintético e comprovaç ão de sua segurança e eficácia terapêutica na epilepsia, esquizofrenia, doença de Parkinson e transtornos de ansiedade”. JAC received travel grant support from BSPG. The other authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ejphar.2018.11.029.

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