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Cannabidiol, a non-psychotropic plant-derived cannabinoid, decreases inflammation in a murine model of acute lung injury: Role for the adenosine A_{2A} receptor

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ABSTRACT

Acute lung injury is an inflammatory condition for which treatment is mainly supportive because effective therapies have not been developed. Cannabidiol, a non-psychotropic cannabinoid component of marijuana (*Cannabis sativa*), has potent immunosuppressive and anti-inflammatory properties. Therefore, we investigated the possible anti-inflammatory effect of cannabidiol in a murine model of acute lung injury. Analysis of total inflammatory cells and differential in bronchoalveolar lavage fluid was used to characterize leukocyte migration into the lungs; myeloperoxidase activity of lung tissue and albumin concentration in the bronchoalveolar lavage fluid were analyzed by colorimetric assays; cytokine/chemokine production in the bronchoalveolar lavage fluid was also analyzed by Cytometric Bead Arrays and Enzyme-Linked Immunosorbent Assay (ELISA). A single dose of cannabidiol (20 mg/kg) administered prior to the induction of LPS (lipopolysaccharide)-induced acute lung injury decreases leukocyte (specifically neutrophil) migration into the lungs, albumin concentration in the bronchoalveolar lavage fluid, myeloperoxidase activity in the lung tissue, and production of pro-inflammatory cytokines (TNF and IL-6) and chemokines (MCP-1 and MIP-2) 1, 2, and 4 days after the induction of LPS-induced acute lung injury. Additionally, adenosine A_{2A} receptor is involved in the anti-inflammatory effects of cannabidiol on LPS-induced acute lung injury because ZM241385 (4-(2-[7-Amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl)phenol) (a highly selective antagonist of adenosine A_{2A} receptor) abrogated all of the anti-inflammatory effects of cannabidiol previously described. Thus, we show that cannabidiol has anti-inflammatory effects in a murine model of acute lung injury and that this effect is most likely associated with an increase in the extracellular adenosine offer and signaling through adenosine A_{2A} receptor.

1. Introduction

The discovery of cannabinoid receptors (CB_1 and CB_2-cannabinoid receptors) and their major ligands N-arachidonoylethanolamide (anandamide) and 2-arachidonoylglicerol led to the characterization of the endocannabinoid system more than two decades ago (Devane et al., 1992; Matsuda et al., 1990; Mechoulam et al., 1995; Munro et al., 1993). The endocannabinoid system has become a topic of great interest in pharmacology due to its remarkable distribution in mammals and its capacity to play a modulating role in diverse physiological functions including immunomodulation and inflammation (Di Marzo and Petrocellis, 2006).

Cannabidiol is a major non-psychotropic cannabinoid component of marijuana (*Cannabis sativa*) (Zuardi, 2008). Cannabidiol has been shown to have potent immunosuppressive and anti-inflammatory properties in several rodent models of inflammation (Mechoulam et al., 2002). A single dose of cannabidiol has been shown to suppress serum TNF production induced by lipopolysaccharide (LPS) in mice (Carrier et al., 2006) and has been found to be beneficial in murine collagen-induced arthritis by inhibiting IFN-γ production and T cell

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proliferation (Malfait et al., 2000). It was also shown that cannabidiol orally administered was effective in reducing inflammation in a model of acute carrageenan-induced paw inflammation (Costa et al., 2004a), and that the anti-nociceptive effect was mediated by the transient receptor potential vanilloid ($TRPV_1$) receptor (Costa et al., 2004b). Moreover, it was shown that cannabidiol reduced not only acute but also chronic inflammatory responses (Costa et al., 2007).

Acute lung injury and its most severe form, acute respiratory distress syndrome, is a lung disease with acute onset that is characterized by bilateral pulmonary infiltrates (Grommes and Soehnlein, 2011). Acute lung injury is characterized by alveolar-capillary injury and inflammation with neutrophil accumulation as well as by the release of pro-inflammatory cytokines (Tsushima et al., 2009). Acute lung injury can result in persistent respiratory failure and prolonged dependence on mechanical ventilation, increased susceptibility to multi-organ dysfunction and mortality (Rubenfeld et al., 2005). Despite numerous innovations in intensive care medicine, the mortality related to these conditions is reported to be around 40% (Rubenfeld et al., 2005; Ware and Matthay, 2000).

Cannabidiol has a broad spectrum of pharmacological actions (Mechoulam et al., 2002; Zuardi, 2008), including inhibition of an equilibrative nucleoside transporter, which in turn leads to the increase of extracellular adenosine (Carrier et al., 2006). Increase in the adenosine signaling is thought to be the major mechanism by which cannabidiol decreases inflammation (Izzo et al., 2009). Adenosine signaling through adenosine A_2 receptors (A_{2A} and A_{2B}) has been reported to play an important role in the resolution of LPS-induced acute lung injury (Eckle et al., 2009). Thus, we hypothesized that, by increasing extracellular levels of adenosine, cannabidiol would decrease inflammation in a murine model of acute lung injury. Therefore, our goal was to investigate the potential anti-inflammatory effect of cannabidiol in a murine model of acute lung injury induced by intra-nasal instillation of LPS.

2. Materials and methods

2.1. Animals

Male C57BL/6 mice from our own colony, weighing 20–25 g and approximately 60 days old, were used. Animals were housed in temperature controlled (22–24 °C) and artificially lit rooms on a 12 h light/12 h dark cycle (lights on at 7:00 a.m.) with 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 same temperature as the animal colony from which the animals were transferred. Animals were maintained in their home cages for 7 days before the beginning of the experiments. Animals were 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 (Protocol # 2002/2010).

2.2. Drugs

Cannabidiol (THC-Pharm, Frankfurt, Germany and STI-Pharm, Brentwood, UK) was prepared in ethanol:tween 20:saline (1:1:18) and administered at 0.3, 1.0, 10, 20, 30, 40, and 80 mg/kg intraperitoneally (i.p.) (0.1 mL/10 g weight). Control animals received a similar volume of vehicle alone. Sixty minutes after treatment, acute lung injury was induced as described below. ZM241385 (4-(2-[7-Amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-ylamino] ethyl)phenol) (Tocris Bioscience, Missouri, USA), an antagonist of the adenosine A_{2A} receptor, was prepared in ethanol:tween 20:saline (1:1:18) and was administered i.p. at 5 mg/kg 30 min prior to cannabidiol treatment; this diluent was used in control and cannabidiol-treated mice.

2.3. Acute lung injury

Mice were anesthetized with ketamine and xylazine injected i.p. (100 and 10 mg/kg, respectively) before intranasal instillation of LPS. *Escherichia coli* LPS (O55:B5 L2880, Sigma-Aldrich, St. Louis, USA) at a concentration of 100 μg/mL, or as a control, sterile 0.9% saline was instilled at 1 μL/g of mouse body weight. At 1, 2, 4, and 7 days after instillation, mice were anesthetized and killed by exsanguination through the inferior vena cava.

2.4. Leukocyte trafficking

2.4.1. Bronchoalveolar lavage fluid

Briefly, mice were killed at 1, 2, 4, and 7 days after LPS instillation by exsanguination through the inferior vena cava after ketamine (100 mg/kg) and xylazine (10 mg/kg) i.p. anesthesia, and bronchoalveolar lavage fluid was collected. Lungs were lavaged with 1.5 mL of phosphate-buffered saline (PBS) solution through a cannula inserted into the trachea. Bronchoalveolar lavage fluid recovery was always around 1 mL. It 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.

2.4.2. Blood

White blood cells were counted with an automatic cell counter (ABC Vet®, São Paulo, Brazil).

2.4.3. Bone marrow

The total number of bone marrow cells was quantified from femoral marrow lavage. Both epiphyses of collected femurs were cut off, and the bone marrow was flushed with 5 mL ice-cold PBS. The cell suspension was centrifuged at 250×*g* for 5 min, and the remaining cell pellet was resuspended for total leukocyte counts in Neubauer chambers.

2.5. Myeloperoxidase activity

The lungs were perfused via the pulmonary artery with 10 mL of PBS. Samples were prepared according to Goldblum et al. (1985). 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 orthodianisidine. The reaction was stopped by the addition of 1% $NaNO_3$. The absorbance was determined at 450 nm using a microplate reader (Bio-Tek Instruments®, Winooski, VT, USA). To normalize pulmonary myeloperoxidase activity between groups, the absorbance was standardized to lung weight.

2.6. Cytokine and chemokine analysis

A BD™ cytometric bead array (CBA) mouse inflammation kit (BD Biosciences, San Jose, USA) was used to measure IL-6, IL-10, MCP-1, IFN-γ, TNF, and IL-12p70, and an ELISA kit (R&D Systems, Minneapolis, USA) was used to measure MIP-2 in the bronchoalveolar lavage fluid. The assays were performed according to the manufacturer's instructions.

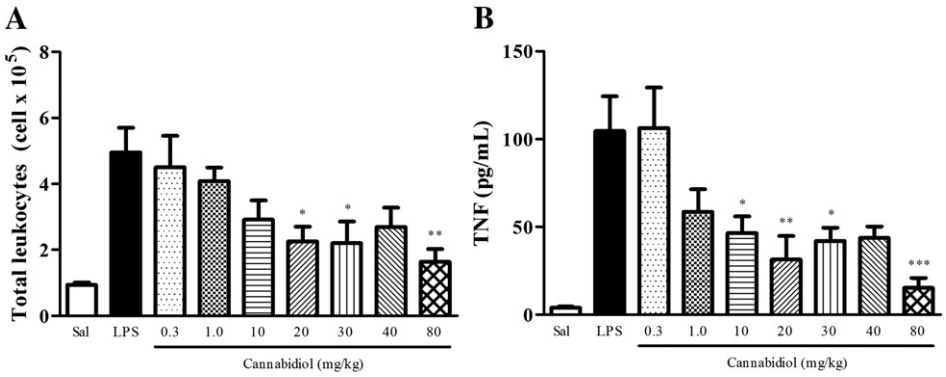

Fig. 1. Dose–response curve for the effects of cannabidiol in a murine model of acute lung injury. (A) Total leukocyte counts and (B) TNF concentration in the bronchoalveolar lavage fluid. Data are presented as the mean ± S.E.M., n = 4–5 mice/group. *P<0.05 when compared with the vehicle + LPS group; **P<0.001 when compared with the vehicle + LPS group; and ***P<0.0001 when compared with the vehicle + LPS group. Statistics were calculated by one-way ANOVA followed by a Tukey–Kramer test (parametric data).

2.7. Protein analysis in the bronchoalveolar lavage fluid

Bronchoalveolar lavage fluid aliquots were placed in a 96-well ELISA plate (10 μL/well), and 250 μL of Bradford reagent (Sigma-Aldrich, St. Louis, USA) was added to each well; after a 30 min incubation, the absorbance was measured at 595 nm. A standard curve using bovine serum albumin (BSA, Sigma-Aldrich, St. Louis, USA) was obtained plotting the net absorbance vs. the protein concentration (1.5–0.1 mg/mL) to determine protein concentration in the samples.

2.8. Statistical analysis

GraphPad Prism 5.0 (GraphPad Software, Inc.) was used for statistical analysis. Parametric data were analyzed by one-way ANOVA followed by a Tukey–Kramer test for multiple comparisons. A Kruskal–Wallis test followed by a Dunn's test for multiple comparisons was used to analyze non-parametric data. Myeloperoxidase activity and bronchoalveolar lavage fluid protein concentration data were analyzed by normalizing to percent of control (vehicle + Sal) and then comparing vehicle + LPS and cannabidiol + LPS groups using a Mann–Whitney *U* test. In all experiments, $P \leq 0.05$ was considered significant. Data are presented as the mean ± S.E.M.

3. Results

3.1. Cannabidiol has dose-dependent anti-inflammatory effects on acute lung injury

We first performed a dose–response curve for cannabidiol using leukocyte migration into the lungs and TNF production in the bronchoalveolar lavage fluid as readouts 1 day after the induction of LPS-induced acute lung injury. Importantly, LPS instillation was effective in inducing lung inflammation as can be observed by the number of leukocytes ($F(8,33) = 5.9$; $P<0.0001$) (Fig. 1A) and the concentration of TNF ($F(8,33) = 7.8$; $P<0.0001$) (Fig. 1B) observed in the bronchoalveolar lavage fluid 1 day after the induction of acute lung injury. We observed that cannabidiol at 20 ($P<0.05$), 30 ($P<0.05$), and 80 ($P<0.01$) mg/kg decreased leukocyte migration into the lungs ($F(8,33) = 5.9$) (Fig. 1A). We also observed that cannabidiol at 10 ($P<0.05$), 20 ($P<0.01$), 30 ($P<0.05$), and 80 ($P<0.0001$) mg/kg decreased TNF production in the bronchoalveolar lavage fluid ($F(8,33) = 7.8$) (Fig. 1B).

3.2. Cannabidiol treatment persistently decreases leukocyte migration into the lungs

Based on the previous results we selected the dose of 20 mg/kg of cannabidiol for the next experiments. We selected this dose because it was the minimal dose that effectively decreased both leukocyte migration into the lungs and TNF production in the bronchoalveolar lavage fluid. We then investigated the effects of cannabidiol (20 mg/kg) on leukocyte migration into the lungs 1, 2, 4, and 7 days after the induction of acute lung injury. We observed that cannabidiol treatment decreased leukocyte counts 1 ($F(2,15) = 27.4$; $P<0.0001$), 2 ($F(2,16) = 38.6$; $P<0.0001$), and 4 ($F(2,17) = 17.3$; $P<0.001$) days after the induction of lung inflammation (Fig. 2). Lung inflammation was resolved by 7 days after the induction of inflammation, and further changes due to cannabidiol were therefore not observed (Fig. 2).

We analyzed leukocyte distribution in the blood and bone marrow; however we did not observe any significant differences between groups at any time point analyzed with the exception of an increase in the bone marrow counts in the vehicle + LPS and the cannabidiol + LPS groups when compared with the vehicle + Sal group 7 days after the induction of inflammation ($F(2,17) = 4.9$; $P<0.05$) (Table 1).

We also performed differential analysis of leukocytes in the bronchoalveolar lavage fluid, and we found that cannabidiol decreased neutrophil, lymphocyte, and macrophage migration into the lungs 1, 2, and 4 days after the induction of ALI (Table 2). Seven days after the induction of acute lung injury, we found minimal neutrophilic inflammation in the lungs of control-treated mice (vehicle + LPS), and we observed that this was ablated by cannabidiol treatment ($F(2,17) = 5.519$; $P<0.05$) (Table 2).

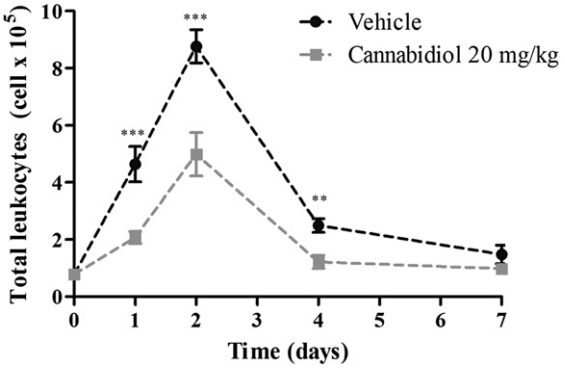

Fig. 2. Effects of cannabidiol on total leukocyte count in the bronchoalveolar lavage fluid 1, 2, 4, and 7 days after the induction of LPS-induced acute lung injury. Data are presented as the mean ± S.E.M., n = 5–9 mice/group. **P<0.001 when compared with the vehicle + LPS group; ***P<0.0001 when compared with the vehicle + LPS group. Statistics were calculated by one-way ANOVA followed by a Tukey–Kramer test (parametric data).

Table 1
Effects of cannabidiol on total leukocyte distribution in the bone marrow and blood.

Groups	Bone marrow (cells × 10^6)				Blood (cells × 10^3/mm^3)			
	1 day	2 days	4 days	7 days	1 day	2 days	4 days	7 days
Vehicle + Sal	3.3 ± 0.3	2.8 ± 0.5	4.7 ± 0.3	3.3 ± 0.3	6.5 ± 0.8	7.7 ± 1.0	5.2 ± 0.4	4.9 ± 0.3
Vehicle + LPS	2.9 ± 0.2	3.3 ± 0.3	4.7 ± 0.4	4.5 ± 0.3[a]	7.9 ± 0.8	10.8 ± 1.3	6.2 ± 0.4	6.1 ± 0.4
Cannabidiol + LPS	3.0 ± 0.2	2.8 ± 0.4	4.0 ± 0.3	4.4 ± 0.2[a]	7.3 ± 1.2	9.3 ± 1.5	5.4 ± 0.3	6.1 ± 0.5

Data are expressed as the mean ± S.E.M. Statistics were calculated by one-way ANOVA followed by Tukey–Kramer or Kruskal–Wallis followed by Dunn's multiple comparison tests (for parametric and non-parametric data, respectively).
[a] $P<0.05$ when compared with the vehicle + Sal group.

3.3. Cannabidiol decreases myeloperoxidase activity in the lung tissue

Because of the important role neutrophils play in the acute lung injury, we analyzed myeloperoxidase activity in the lungs of mice 1, 2, 4, and 7 days after the induction of inflammation. We observed that cannabidiol treatment significantly decreased myeloperoxidase activity 2 ($U=3.0$; $P<0.01$) and 4 days ($U=7.5$; $P<0.05$) after the induction of inflammation. By 7 days after the induction of acute lung injury, we did not observe any changes, most likely because the lung inflammation had already resolved (Fig. 3).

3.4. Cannabidiol decreases production of pro-inflammatory cytokines and chemokines in the bronchoalveolar lavage fluid

We next investigated inflammatory cytokines/chemokines in the bronchoalveolar lavage fluid 1, 2, 4, and 7 days after the induction of acute lung injury. One day after the induction of inflammation, we observed that cannabidiol decreased TNF concentration ($F(2,11)=15.7$; $P<0.01$) (Fig. 4A); no statistically significant differences were observed for IL-12p70, IL-10 (data not shown), IL-6, and MCP-1 (Fig. 4) between the groups, with the exception that there was a trend towards a decrease in MIP-2 production ($P=0.06$) (Fig. 4). Two days after the induction of acute lung injury, we observed that cannabidiol decreased TNF ($F(2,12)=5.5$; $P<0.05$), IL-6 ($F(2,12)=8.9$; $P<0.01$) (Fig. 4B), MCP-1 ($F(2,12)=14.3$; $P<0.0001$) (Fig. 4C), and MIP-2 ($KW=10.6$; $P<0.05$) (Fig. 4D) concentrations; the other cytokines analyzed (IL-12p70 and IL-10) remained unchanged at this time point. Four and 7 days following the induction of acute lung injury, no differences were observed in any of the cytokines/chemokines analyzed (data not shown). The levels of IFN-γ were below the limit of quantification in the bronchoalveolar lavage fluid samples.

3.5. Cannabidiol decreases vascular permeability

Finally, we investigated vascular permeability indirectly by measuring protein in the bronchoalveolar lavage fluid. We observed that cannabidiol decreased the protein concentration in the bronchoalveolar lavage fluid 1 ($U=5.5$; $P=0.05$) and 2 ($U=7.0$; $P=0.05$) days after the induction of acute lung injury (Fig. 5). Four and 7 days after the induction of acute lung injury, no differences were observed in protein concentration in the bronchoalveolar lavage fluid.

3.6. Adenosine A_{2A} receptor antagonism attenuates the anti-inflammatory effects of cannabidiol on acute lung injury

Cannabidiol is known to exert its actions through a number of different mechanisms (Zuardi, 2008). For example, it has been reported that blocking (with ZM241385) or knocking out the adenosine A_{2A} receptor, abrogated the ability of cannabidiol to decrease the TNF production induced by systemic administration of LPS (Carrier et al., 2006). Therefore, we investigated the role of the adenosine A_{2A} receptor in the anti-inflammatory effects of cannabidiol reported here by using ZM241385, a highly selective adenosine A_{2A} receptor antagonist. We observed that ZM241385 treatment abrogated cannabidiol inhibition of leukocyte migration into the lungs (Fig. 6A) and myeloperoxidase activity in the lung tissue (Fig. 6B) 1, 2, and 4 days after the induction of acute lung injury. Moreover, ZM241385 attenuated cannabidiol inhibition of inflammatory cytokines/chemokines in the bronchoalveolar lavage fluid (Fig. 6C–F) and vascular permeability (Fig. 6G) 1 and 2 days after the induction of acute lung injury. As expected, 7 days after the induction of inflammation, no differences were observed.

4. Discussion

Cannabidiol is a major non-psychoactive cannabinoid component of marijuana (*C. sativa*) (Zuardi, 2008) and has been shown to have potent immunosuppressive and anti-inflammatory properties (Mechoulam et al., 2002). Therefore, we wondered whether cannabidiol would be as effective in a murine model of acute lung injury, due to its potent anti-inflammatory property, as it has been reported in other models of inflammation (Mechoulam et al., 2002). We report here that a single dose of cannabidiol is able to induce a decrease in several lung inflammation parameters, such as leukocyte migration (neutrophil, macrophages, and lymphocytes), myeloperoxidase activity, pro-inflammatory cytokine/chemokine production, and vascular

Table 2
Effects of cannabidiol on differential cell analysis in the bronchoalveolar lavage fluid.

Groups	Neutrophil (cells × 10^5)				Macrophage/monocyte (cells × 10^5)				Lymphocyte (cells × 10^5)			
	1 day	2 days	4 days	7 days	1 day	2 days	4 days	7 days	1 day	2 days	4 days	7 days
Vehicle + Sal	.03 ± .01	0.2 ± 0.1	.04 ± .01	.03 ± .01	0.4 ± .04	0.4 ± 0.1	0.5 ± 0.1	0.9 ± 0.2	0.1 ± .03	0.2 ± .04	0.1 ± .01	0.1 ± .01
Vehicle + LPS	1.1 ± 0.2	3.2 ± 0.5	0.1 ± .01	0.2 ± 0.1	2.8 ± 0.4	3.8 ± 0.3	1.9 ± 0.2	1.0 ± 0.2	0.7 ± 0.1	1.5 ± 0.2	0.2 ± .03	0.3 ± 0.1
Cannabidiol + LPS	0.4 ± .03[b]	1.4 ± 0.3[b]	.05 ± .01[a]	0.1 ± .01[a]	1.5 ± 0.2[b]	2.2 ± 0.5[a]	1.0 ± 0.2[b]	0.7 ± 0.1	0.2 ± .03[c]	0.4 ± 0.1[c]	0.1 ± .02[a]	0.1 ± .01

Data are expressed as the mean ± S.E.M. Statistics were calculated by one-way ANOVA followed by Tukey–Kramer test (parametric data).
[a] $P<0.05$ when compared with the vehicle + LPS group.
[b] $P<0.001$ when compared with the vehicle + LPS group.
[c] $P<0.0001$ when compared with the vehicle + LPS group.

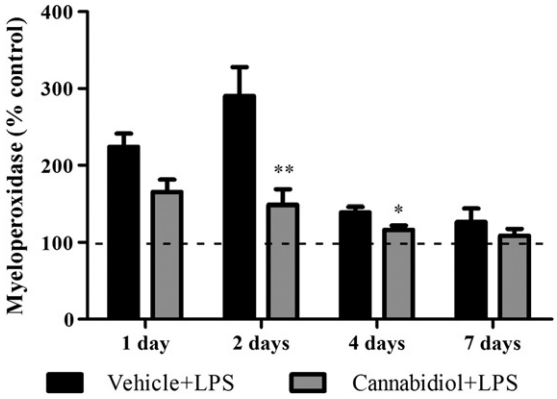

Fig. 3. Effects of cannabidiol on myeloperoxidase activity in the lung tissue 1, 2, 4, and 7 days after the induction of LPS-induced acute lung injury. Data are presented as the mean ± S.E.M., n = 6–8 mice/group. *P<0.05 when compared with the vehicle + LPS group; **P<0.001 when compared with the vehicle + LPS group. Statistics were calculated by normalizing the data to percent of the control group (vehicle + Sal); the vehicle + LPS and cannabidiol + LPS groups were then compared using the Mann–Whitney *U* test (non-parametric data).

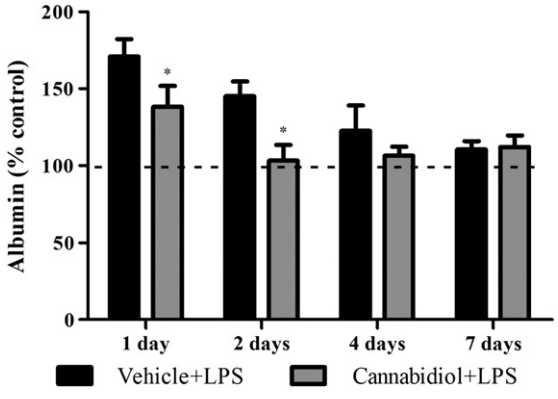

Fig. 5. Effects of cannabidiol on protein (albumin) concentration in the bronchoalveolar lavage fluid 1, 2, 4, and 7 days after the induction of LPS-induced acute lung injury. Data are presented as the mean ± S.E.M., n = 6–7 mice/group. *P<0.05 when compared with the vehicle + LPS group. Statistics were calculated by normalizing the data to percent of the control group (vehicle + Sal); the vehicle + LPS and cannabidiol + LPS groups were then compared by Mann–Whitney *U* test (non-parametric data).

permeability during the course of a murine model of LPS-induced acute lung injury. We also show that signaling through adenosine A_{2A} receptor is most likely the mechanism mediating the anti-inflammatory effects of cannabidiol in our work.

Neutrophil influx into the interstitium and the bronchoalveolar space is considered to play a key role in the progression of acute lung injury (Grommes and Soehnlein, 2011). We found that cannabidiol (20 mg/kg) decreases leukocyte migration into the lungs of mice 1, 2, and 4 days after the induction of acute lung injury, as shown by the

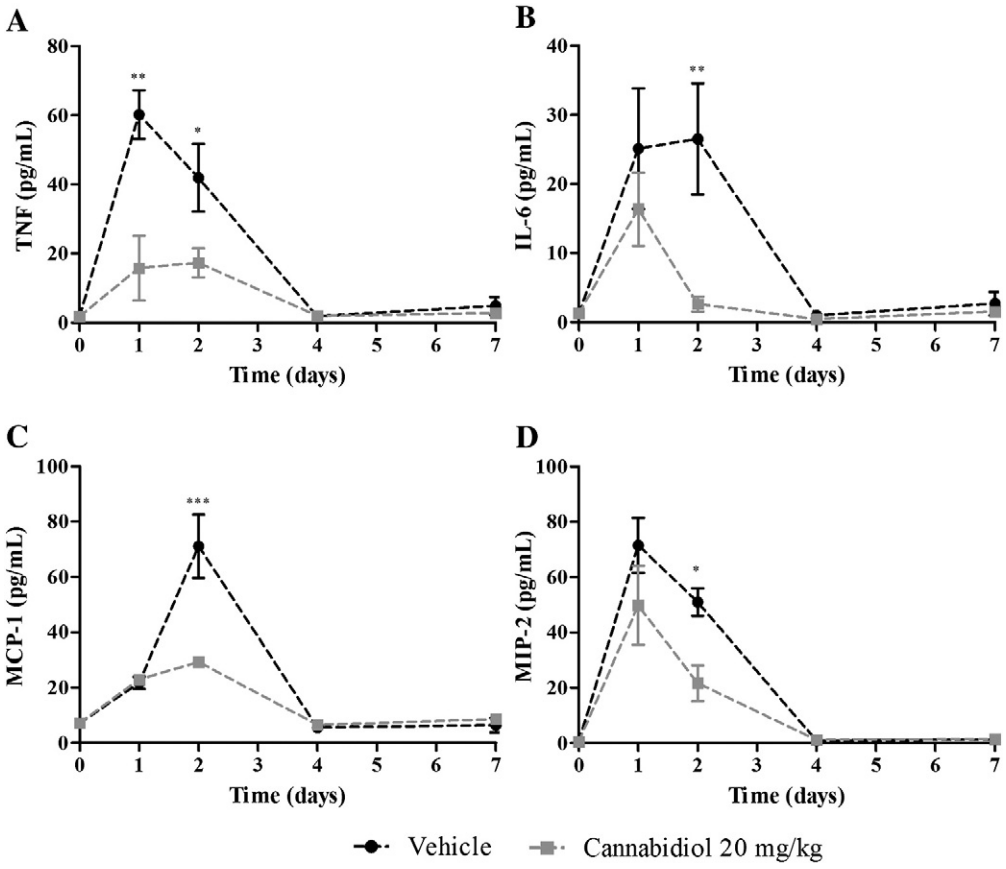

Fig. 4. Effects of cannabidiol on cytokine/chemokine production in the bronchoalveolar lavage fluid 1, 2, 4, and 7 days after the induction of LPS-induced acute lung injury. (A) TNF, (B) IL-6, (C) MCP-1 (CCL2), and (D) MIP-2 (CXCL2). Data are presented as the mean ± S.E.M., n = 4–7 mice/group. *P<0.05 when compared with the vehicle + LPS group; **P<0.001 when compared with the vehicle + LPS group; and ***P<0.0001 when compared with the vehicle + LPS group. Statistics were calculated by one-way ANOVA followed by a Tukey–Kramer test (parametric data).

bronchoalveolar lavage fluid leukocyte counts; specifically, we show that neutrophil migration is greatly decreased and that macrophage and lymphocyte numbers are also decreased in cannabidiol-treated mice. Leukocyte migration into the site of inflammation is extremely important in both tissue damage and resolution of tissue injury. Moreover, neutrophils are highly specialized for their primary function, which are phagocytosis and destruction of microorganisms (Klebanoff, 2005). Myeloperoxidase analysis is considered a good surrogate for the tissue neutrophil activity that occurs predominantly under pathological conditions. We show that myeloperoxidase activity is decreased in cannabidiol-treated mice with ongoing lung inflammation 1, 2 and 4 days after the induction of acute lung injury model. This effect may be important because myeloperoxidase-associated tissue damage will likely be inhibited, which could also contribute to decrease lung inflammation.

It is noteworthy that neutrophil migration into the lungs is not sufficient to cause acute lung injury, because neutrophils can migrate into the lungs without causing injury; therefore, neutrophil activation is required (Grommes and Soehnlein, 2011). Thus, we also analyzed the production of pro-inflammatory cytokines and chemokines in the bronchoalveolar lavage fluid of mice with ongoing LPS-induced acute lung injury. We show that TNF and IL-6 are decreased in cannabidiol-treated mice 1 and 2 days after the induction of acute lung injury. These results are extremely important because both cytokines are essential to the establishment of inflammation and to phagocyte activation. Consistent with our results, it has been reported that the inhibition of the TNF-converting enzyme reduces LPS-induced acute lung injury in rats (Shimizu et al., 2009). Additionally, we show that cannabidiol decreases MIP-2 (CXCL2) 1 and 2 days after the induction of acute lung injury and decreases MCP-1 (CCL2) 2 days after the induction of acute lung injury. These results may explain the dynamics of leukocyte migration into the lungs. MIP-2 promotes neutrophil migration and degranulation (Kobayashi, 2008); therefore, it is reasonable to suggest that cannabidiol, by reducing MIP-2 production in the bronchoalveolar lavage fluid, reduces the neutrophil count and also decreases myeloperoxidase activity during ongoing lung inflammation. Moreover, supporting our observations, it has been shown that blocking (Zarbock et al., 2008) or knocking out (Reutershan et al., 2006) the MIP-2 receptor (CXCR2) leads to the attenuation of neutrophil migration and inflammation in a model of acute lung injury. MCP-1 concentration in the bronchoalveolar lavage fluid may also explain the reduced numbers of macrophages and lymphocytes that we observed in the differential analysis of the bronchoalveolar lavage fluid. Therefore, these data are extremely important because cannabidiol modifies the cytokine/chemokine *milieu* in the lungs of mice submitted to experimental acute lung injury, which seems to be important to the pronounced anti-inflammatory effects in cannabidiol-treated mice that we observed in our work.

Cannabidiol is known to exert its effects in a number of different ways, as reviewed by Izzo et al. (2009) and Zuardi (2008). Among them, the inhibition of adenosine uptake and signaling through the adenosine A_{2A} receptor was shown to be involved in the anti-inflammatory actions of cannabidiol (Carrier et al., 2006). Indeed, adenosine signaling has been shown to play an important role in the attenuation of experimentally induced acute lung injury (Ngamsri et al., 2010; Reutershan et al., 2007; Schingnitz et al., 2010; Wagner et al., 2010). Therefore, we decided to investigate the involvement of activation of the adenosine A_{2A} receptors in the anti-inflammatory effects of cannabidiol using ZM241385, a highly selective antagonist of the adenosine A_{2A} receptors. Remarkably, ZM241385 treatment abrogated all anti-inflammatory effects of cannabidiol at all time points reported here; leukocyte migration into the lungs, myeloperoxidase activity, production of pro-inflammatory cytokines/chemokines, and vascular permeability were all reduced 1, 2, 4, and 7 days after the induction of inflammation. Corroborating with our data, it has been reported that the activation of adenosine A_{2A} receptor decreases neutrophil migration into the lung, cytokine/chemokine production, vascular permeability, etc., in murine models of acute lung injury (Impellizzeri et al., 2011; Reutershan et al., 2007). Although ZM241385 is a highly selective adenosine A_{2A} receptor antagonist, we cannot conclude that the abrogation of the anti-inflammatory effects of cannabidiol in the LPS-induced acute lung injury was specifically mediated by A_{2A} receptor since ZM241385 also binds to adenosine A_{2B} receptor (Poucher et al., 1995), especially at the dose we used in our work (5 mg/kg).

It should not be forgotten that the pharmacological profile of cannabidiol is complex and other mechanisms besides signaling through adenosine receptors might be involved. Cannabidiol has been shown to inhibit fatty acid amide hydrolase (FAAH) and anandamide reuptake (Bisogno et al., 2001). This effect could contribute to the anti-inflammatory effect of cannabidiol, since it has been shown in other model of inflammation, such as intestinal inflammation (Capasso et al., 2008). Cannabidiol has also been shown to have CB_2 inverse agonistic property, which could contribute to its documented anti-inflammatory properties (Thomas et al., 2007). Reduced activity of 5-lipoxygenase (5-LOX) was also attributed as a molecular mechanism of cannabidiol (Massi et al., 2008); although this effect was analyzed in the context of cannabidiol antitumoral activity, it might also contribute to cannabidiol anti-inflammatory effect. Recently, in an in vitro model of hypoxic-ischemic brain damage, it has been shown that cannabidiol decreased pro-inflammatory mediators, such as cyclooxygenase (COX-2) and inducible nitric oxide synthase (iNOS), as well as TNF and IL-6 (Castillo et al., 2010). They also showed that both CB_2 and especially adenosine A_{2A} receptor are involved in these anti-inflammatory effects (Castillo et al., 2010). Finally, some efforts are being made to improve our understanding of cannabidiol actions at the intracellular level, and it is noteworthy to describe them here. It was shown that cannabidiol decreases the production and release of pro-inflammatory cytokines such as IL1-β and IL-6 from LPS-activated microglial cells and that cannabidiol reduces the activity of NF-κB, which regulates the expression of pro-inflammatory cytokines. Cannabidiol also decreased the activation of STAT1 transcription factor, an important regulator of the IFNβ-dependent pro-inflammatory process. Moreover, it was shown that cannabidiol up-regulates the activation of the STAT3 transcription factor, a counteracting mechanism inducing anti-inflammatory events.

Finally, it was shown that inhibition of pro-inflammatory cytokines induced by cannabidiol is not mediated by the CB_1 and CB_2 cannabinoid receptors (Kozela et al., 2010).

Nevertheless, in our model of LPS-induced acute lung injury, inhibition of adenosine uptake and signaling through the adenosine A_{2A} receptor seems to be the most likely mechanism involved in the anti-inflammatory effect observed.

5. Conclusions

We show for the first time that a single dose of cannabidiol has anti-inflammatory effects in a murine model of LPS-induced acute lung injury. Additionally, we show that augmentation of adenosine signaling through the adenosine A_{2A} receptor is the most likely mechanism controlling the actions of cannabidiol in our work. Currently, we are investigating whether cannabidiol is able to decrease LPS-induced acute lung injury when the inflammatory process is already installed. Additionally, care should be taken when extrapolating these data to patients; nevertheless, in the future, cannabidiol may prove useful as a therapeutic tool for the treatment/attenuation of inflammatory lung diseases, such as acute lung injury and acute respiratory distress syndrome.

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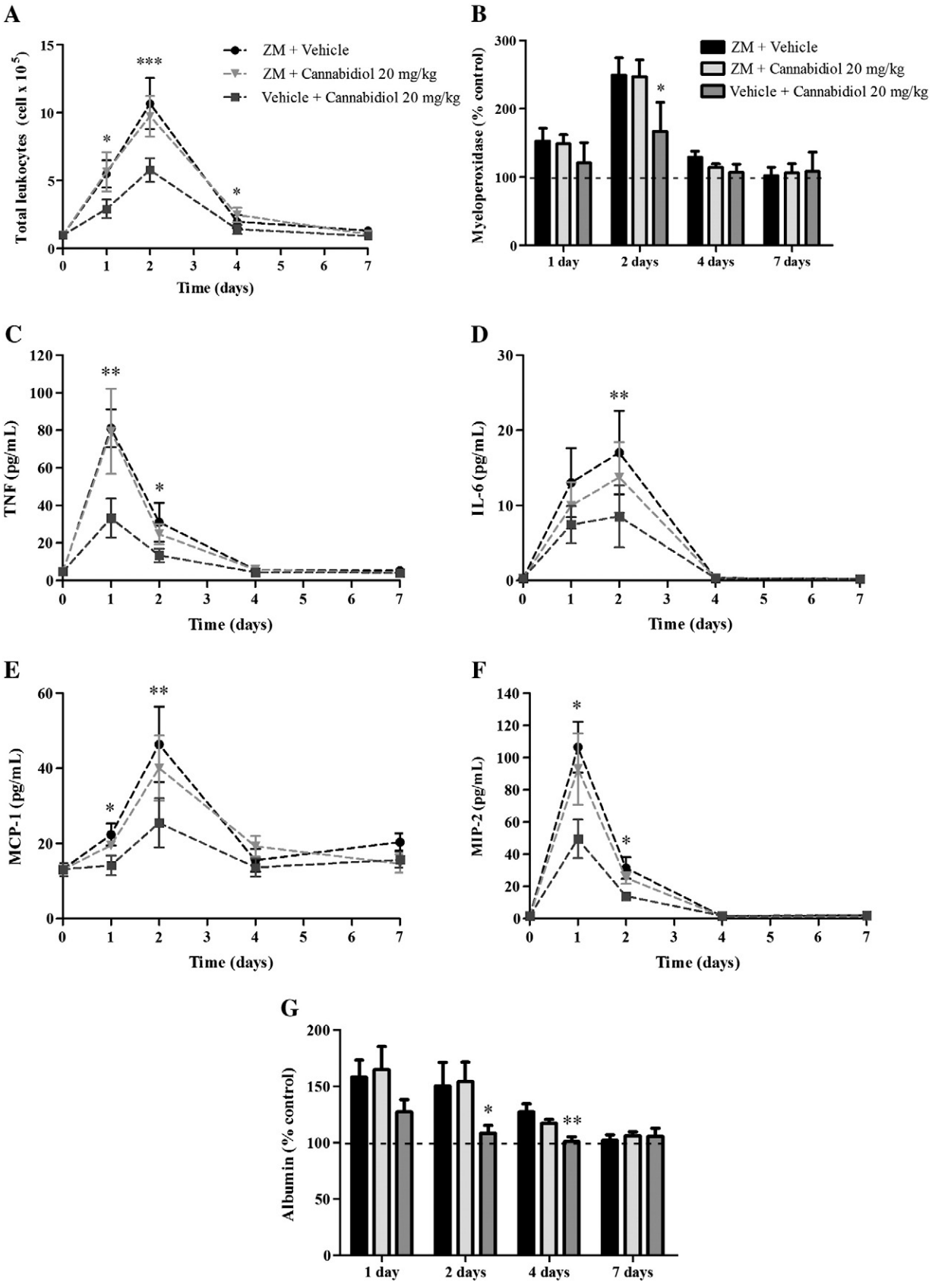

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Fig. 6. Effects of ZM241385 (adenosine A_{2A} receptors antagonist) on the anti-inflammatory effects of cannabidiol 1, 2, 4, and 7 days after the induction of LPS-induced acute lung injury. (A) Total leukocyte count in the bronchoalveolar lavage fluid, (B) myeloperoxidase activity in the lung tissue, (C) TNF, (D) IL-6, (E) MCP-1 (CCL2), (F) MIP-2 (CXCL2), and (G) albumin concentration in the bronchoalveolar lavage fluid. Data are presented as the mean ± S.E.M., n = 4–9 mice/group. *P<0.05 when compared with the ZM + cannabidiol group; **P<0.001 when compared with the ZM + cannabidiol group; and ***P<0.0001 when compared with the ZM + cannabidiol group. Statistics were calculated by one-way ANOVA followed by a Tukey–Kramer or a Kruskal–Wallis test followed by Dunn's multiple comparison tests (for parametric and non-parametric data, respectively) (A, C, D, E, and F) or by normalizing the data to percent of the control group (ZM + vehicle + Sal); ZM + vehicle + LPS, ZM + cannabidiol + LPS and vehicle + cannabidiol + LPS groups were then compared using Kruskal–Wallis test followed by Dunn's multiple comparison tests (non-parametric data) (B and G).