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THEMED SECTION: MEDIATORS AND RECEPTORS IN THE RESOLUTION OF INFLAMMATION

RESEARCH PAPER

Preventive and therapeutic anti-inflammatory properties of the sesquiterpene α-humulene in experimental airways allergic inflammation

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Background and purpose: α-Humulene and *trans*-caryophyllene are plant sesquiterpenes with pronounced anti-inflammatory properties. Here, we evaluated the effects of these compounds in an experimental model of airways allergic inflammation.
Experimental approach: Female BALB/c mice, sensitized to and challenged with ovalbumin received daily α-humulene or *trans*-caryophyllene (50 mg·kg^{-1}, orally) or α-humulene (1 mg·mL^{-1}, by aerosol) as either a preventive (for 22 days) or therapeutic (from the 18th to the 22nd day) treatment. Dexamethasone or budesonide was used as a positive control drug. Inflammation was determined on day 22 post-immunization by leukocyte recruitment, interleukin-5 (IL-5), CCL11, interferon-γ (IFN-γ) and leukotriene (LT)B_4 levels in bronchoalveolar lavage fluid (BALF). In addition, transcription factors [nuclear factor κB (NF-κB), activator protein 1 (AP-1)] and P-selectin in lung tissue were measured by immunohistochemistry and mucus secretion by histochemistry.
Key results: Preventive or therapeutic treatments with α-humulene, but not with *trans*-caryophyllene, significantly reduced the eosinophil recruitment to the BALF. In addition, α-humulene recovery INF-γ and reduced the IL-5, CCL11 and LTB_4 levels in BALF, as well as the IL-5 production in mediastinal lymph nodes (*in vitro* assay). Furthermore, α-humulene decreased the NF-kB and the AP-1 activation, the expression of P-selectin and the increased mucus secretion in the lung.
Conclusions and implications: α-Humulene, given either orally or by aerosol, exhibited marked anti-inflammatory properties in a murine model of airways allergic inflammation, an effect that seemed to be mediated via reduction of inflammatory mediators, adhesion molecule expression and transcription factors activation.
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Keywords: α-humulene; airways allergic inflammation; eosinophils; mouse

Abbreviations: AP-1, activator protein 1; BALF, bronchoalveolar lavage fluid; EPO, eosinophil peroxidase; IL, Interleukin; LTB_4, leukotriene B_4; MLN, mediastinal lymph node; NF-κB, nuclear factor κB; Th1, T-helper 1; Th2, T-helper 2

Introduction

In recent decades, the incidence of asthma has risen dramatically worldwide, especially, although not exclusively, in developed countries (Holgate, 1999; Carlsen, 2003). Allergic asthma is a complex inflammatory disorder characterized by airway hyperresponsiveness, eosinophilic inflammation and hypersecretion of mucus by goblet cells. This disease is

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frequently accompanied by high serum levels of immunoglobulin E and by intrapulmonary production of certain interleukins (IL), especially IL-4, IL-5 and IL-13 by allergen-specific T-helper 2 (Th2) cells (Neurath *et al.*, 2002; Elias *et al.*, 2003). Integrated signalling events between IL-4 and IL-13 regulate pulmonary eosinophilia by stimulating eosinophil-specific adhesion pathways and by modulating the local production of IL-5 and CCL11 (eotaxin), which in turn selectively drive eosinophil recruitment. Moreover, in particular, IL-5 plays a critical role in the regulation of bone marrow and blood eosinophilia (Foster *et al.*, 2001).

Recent evidence now indicates that some compounds are effective in inhibiting eosinophil function and/or eosinophil infiltration. Such inhibition is desirable for the treatment of patients with atopy/allergic disorders, hypereosinophilic syndromes and other eosinophil-associated diseases. A number of novel classes of drugs that inhibit eosinophil activation are currently under development and might be useful against a variety of therapeutic targets to control the eosinophilic inflammation (Barnes, 2006).

In folk medicine, plants have long been used as alternative treatments for a wide range of diseases, including inflammatory processes of diverse origins, and have provided symptomatic relief comparable to that obtained from allopathic medicines (Calixto *et al.*, 2003; 2004). *Cordia verbenacea* known popularly in Brazil as 'Erva Baleeira' or 'Salicina' is used in folk medicine for the treatment of several inflammatory processes. Early studies by our group have shown that both the essential oil of *C. verbenacea* and the main secondary active constituents, the sesquiterpenes α-humulene and *trans*-caryophyllene, also found in other plant species (Kanokmedhakul *et al.*, 2007; Rali *et al.*, 2007), exhibit oral and topical anti-inflammatory properties in different inflammatory models by reducing inflammatory cytokine levels and pro-inflammatory protein expression via inhibition of the nuclear factor κB (NF-κB) pathway (Fernandes *et al.*, 2007; Medeiros *et al.*, 2007; Passos *et al.*, 2007).

Herein, we have used the classical experimental model of ovalbumin-induced airway allergic inflammation together with some molecular and immunohistochemistry procedures in order to determine whether or not α-humulene or *trans*-caryophyllene, given in a preventive or therapeutic scheme of treatment, could interfere with the development and/or establishment of airway allergic inflammation.

Methods

Animals

All animal care and procedures used in the present study complied with the guidelines on animal care of the UFSC Ethics Committee on the Use of Animals, which follows the 'Principles of Laboratory Animal Care' from NIH publication N° 85-23. Experiments were conducted using female BALB/c mice (8 weeks old and weighing 20–25 g) kept in controlled room temperature (22 ± 2°C) and humidity (60–80%) under a 12:12 h light–dark cycle (lights on 06:00 h). At appropriate time intervals, mice were killed by isoflurane overdose.

Antigen immunization, booster and airway challenge

Mice were immunized on days 0 and 7 by subcutaneous injection of 4 μg of ovalbumin plus 1.6 mg of aluminium hydroxide in 0.4 mL of saline followed by two intranasal challenges (on post-immunization days 14 and 21) with 10 μg of ovalbumin in 50 μL of saline, delivered into the nostrils under light ether anaesthesia with the aid of a micropipette. The control group consisted of non-immunized mice that received two intranasal instillations of ovalbumin. All determinations were made 24 h after the second ovalbumin challenge (on post-immunization day 22) (Russo *et al.*, 2001; Rogerio *et al.*, 2008).

Treatment with α-humulene or trans-caryophyllene

To study the preventive anti-inflammatory effects, α-humulene or *trans*-caryophyllene were given orally by gavage with a volume varying between 200 and 250 μL according to the animals' weight, at a dose of 50 $mg \cdot kg^{-1}$ for 22 days, starting from the day of immunization. The choice of this dose was based on published values (Fernandes *et al.*, 2007). To study the therapeutic effects of α-humulene or *trans*-caryophyllene, mice were first immunized as previously described and orally treated once per day from day 18 to day 22 after the first immunization with α-humulene or *trans*-caryophyllene (50 $mg \cdot kg^{-1}$, p.o.) or vehicle (saline). As a positive control, mice in one group were treated preventively or therapeutically with dexamethasone (1 $mg \cdot kg^{-1}$, s.c. injection). In another set of experiments, unanaesthetized, unrestrained, animals were placed in a transparent perspex chamber (dimensions 20 cm × 20 cm × 20 cm) and exposed to an aerosol of α-humulene solution (1 $mg \cdot mL^{-1}$) or vehicle (5% Tween 80 in saline) daily from day 18 to day 22 after the first immunization (therapeutic treatment). Aerosols of the solution were generated with an ultrasonic nebulizer (NS, inalamax®; São Paulo, São Paulo, Brazil) at 0.36 $mL \cdot min^{-1}$. Mice aerolized with budesonide (1 $mg \cdot mL^{-1}$, 5% Tween 80 in saline) were used as positive control. The choice of the doses of budesonide and dexamethasone were based on previous studies (Gong *et al.*, 2005; Rogerio *et al.*, 2008). In all experiments, the animals were treated 1 h before starting the immunization and challenge with ovalbumin.

Evaluation of leukocyte influx into the bronchoalveolar space

Mice were killed by isoflurane overdose. Subsequently, a polyethylene cannula was introduced into the trachea, and phosphate-buffered saline (PBS) containing heparin (10 $UI \cdot mL^{-1}$) was instilled in three aliquots (0.3, 0.3 and 0.4 mL) to a total of 1 mL. The bronchoalveolar lavage fluid (BALF) was recovered and placed on ice. Total cell and differential leukocyte counts were made according to Rogerio *et al.* (2008). Following centrifugation (400 × *g*, 5 min, 4°C), supernatants of the BALF were collected and stored at −70°C for subsequent cytokine and chemokine determination.

Measurement of eosinophil peroxidase (EPO) in the lung
Eosinophil recruitment in the lung was indirectly measured by means of EPO activity. The lungs were removed and homogenized, and the assays were performed as described by Lee *et al.* (2007).

Histological analysis of mucus secretion in the lung
The lungs were removed, immersed in 4% phosphate-buffered formalin, and embedded in paraffin. Tissues were cut into 5 μm sections, which were then stained with periodic acid-Schiff (PAS) stain to evaluate mucus production. Mucus hypersecretion by goblet cells in the airway epithelium was analysed as previously described (Vargaftig and Singer, 2003) with minor modifications. Using NIH Image 1.59b5, six random, digital images were captured from the area surrounding the epithelium of the bronchioles, measuring the total area (labelled plus non-labelled) minus the non-labelled area with a 200 × magnification. Imaging for aerosol therapeutic treatment conditions was conducted sequentially during a single imaging session. The sum of the values of five fields per slide is provided for each animal ($n = 5$). The results are shown as optical density.

Measurement of IL-5, CCL11, interferon (IFN)-γ and leukotriene (LT)B_4 levels
LTB_4, IFN-γ, IL-5 and CCL11 levels were assayed according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA) by specific ELISA (RayBiotech Norcross, Georgia, USA). Sensitivities were >10 pg·mL^{-1}.

Draining lymph node derived cultures
This assay was carried out according to Schnyder-Candrian *et al.* (2006) with some modifications. Draining lymph nodes (mediastinal) of ovalbumin-immunized and challenged animals were passed through 70 μm mesh. The cells were washed with PBS and centrifuged for 10 min at 400 × *g* three times; afterwards the cells were counted and resuspended at 10^6 cells mL^{-1} in culture medium consisting of RPMI supplemented with 10% fetal bovine serum and 100 U·mL^{-1} penicillin/streptomycin. The cells were cultivated on 96-well plates, 200 μL cell suspension per well, with RPMI, OVA (50 μg·mL^{-1}) plus vehicle [0.05% dimethyl sulphoxide (DMSO)], ovalbumin plus α-humulene (10 μmol·L^{-1}, 0.05% DMSO), ovalbumin plus α-humulene (1 μmol·L^{-1}), ovalbumin plus α-humulene (0.1 μmol·L^{-1}) and ovalbumin plus dexamethasone (10 μmol·L^{-1}) for 24 h at 37°C in a humidified incubator with 5% CO_2.

Immunohistochemical studies
For the animals therapeutically treated by aerosol, immunohistochemical detection of p65 NF-κB, c-Jun activator protein 1 (AP-1) and P-selectin was carried out in the lung (5 μm slices) using polyclonal rabbit anti-phospho-p65 NF-κB (#3037, 1:100) and polyclonal rabbit anti-phospho-c-Jun AP-1 (#9164, 1:100) both from Cell Signaling Technology (Beverly, MA, USA) and polyclonal goat anti-P-selectin (#6943, 1:2000, from Santa Cruz Biotechnology, Santa Cruz, CA, USA). High temperature antigen retrieval was performed by immersion of the slides in a water bath at 95–98°C in 10 mmol·L^{-1} trisodium citrate buffer pH 6.0, for 45 min. After overnight incubation at 4°C with primary antibodies, the slides were washed with PBS and incubated with the secondary antibody Envision plus (DakoCytomation, Carpinteria, CA, USA), ready-to-use, for 1 h at room temperature. The sections were washed in PBS, and the visualization was completed by use of 3,3′-diaminobenzidine (DAB) (Dako) in chromogen solution and light counterstaining with Harris's haematoxylin solution. Images were obtained with a microscope (Nikon Eclipse 50i) and Digital Sight Camera (DS-5M-L1, Nikon, Melville, New York, USA). Control and experimental tissues were placed on the same slide and processed under the same conditions. Settings for image acquisition were identical for control and experimental tissues. For each mouse lung, three images were obtained. The images were transferred to a computer, and the average pixel colour intensity of phospho-p65 NF-κB, phospho-c-Jun AP-1 or P-selectin staining was calculated for each image using NIH ImageJ 1.36b imaging software (National Institute of Health, Bethesda, MD, USA) and the results were represented as arbitrary units.

Statistical analysis
The data are reported as mean ± SEM. The means from different treatments in each individual experiment were compared by ANOVA. When significant differences were identified, individual comparisons were subsequently made with the Tukey's test. Values of $P < 0.05$ were considered statistically significant.

Materials
α-Humulene (purity 98%), *trans*-caryophyllene (purity 98%), dexamethasone, budesonide, ovalbumin and PAS stain were purchased from Sigma–Aldrich (St Louis, MO, USA), tween-80 from Merck (Darmstadt, Germany), DMSO from Vetec Química Fina (Duque de Caxias, RJ, Brazil) and RPMI Medium 1640 from Invitrogen (Carlsbad, CA, USA). The drug/molecular target nomenclature was according to the BJP's Guide to Receptors and Channels (Alexander *et al.*, 2008).

Results

Effect of preventive treatment with α-humulene and trans-caryophyllene on leukocyte recruitment to BALF
We first examined the effect that a 22-day course with α-humulene or *trans*-caryophyllene (both 50 mg·kg^{-1}, given orally) had on eosinophilic airway inflammation at 24 h after the second ovalbumin challenge. Leukocyte, neutrophil, eosinophil and mononuclear cell totals were quantified in the BALF. As shown in Figure 1, the total number of cells recovered from the BALF of animals immunized and then challenged with ovalbumin was fivefold greater than that observed in control animals (non-immunized). Differential cell counts revealed that eosinophils accounted for 52%, neutrophils for 14% and mononuclear cells for 34% of the

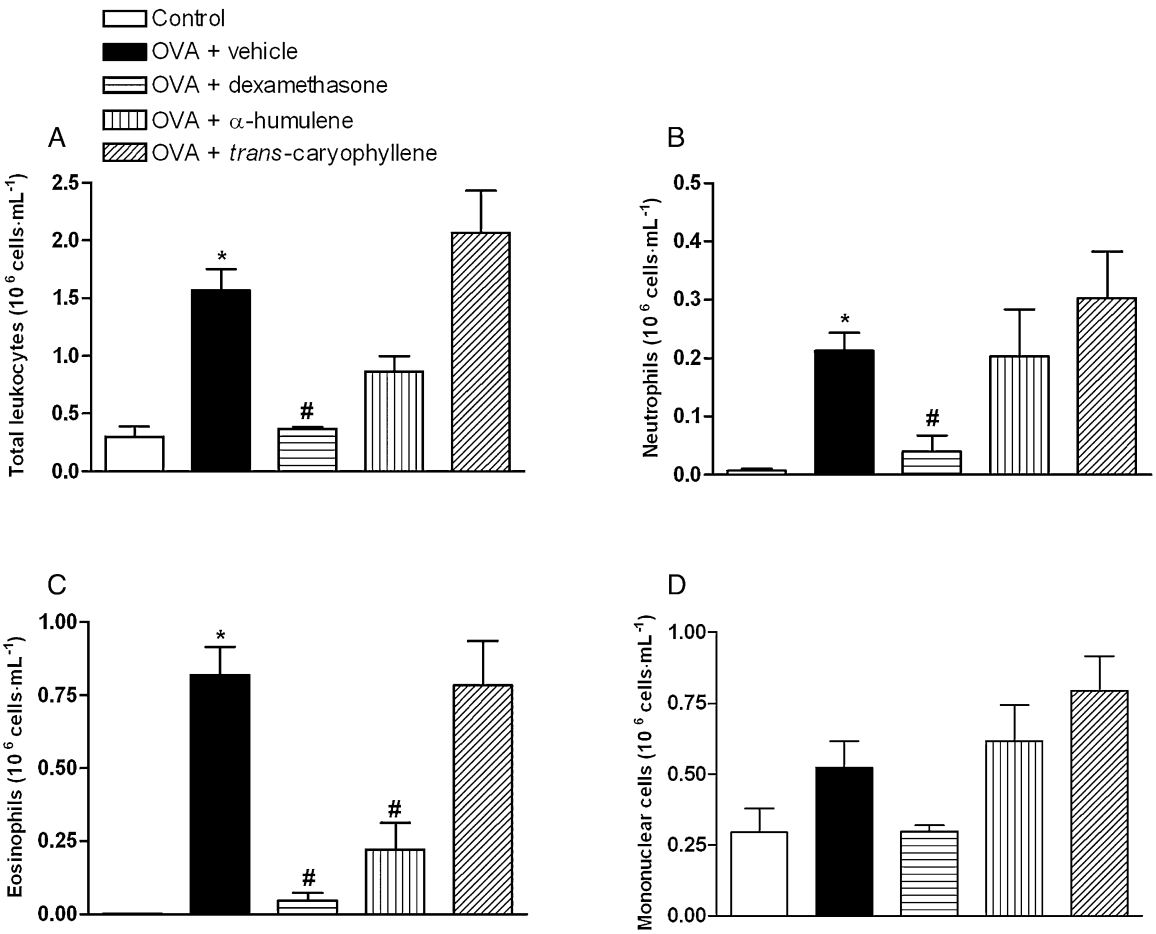

Figure 1 Effect of the preventive treatment (oral route) with α-humulene or *trans*-caryophyllene on the number of total leukocytes (A), neutrophils (B), eosinophils (C) and mononuclear cells (D) in BALF of mice immunized and then challenged with ovalbumin (OVA). Mice were treated with vehicle (saline), α-humulene (50 mg·kg^{-1}), *trans*-caryophyllene (50 mg·kg^{-1}) or dexamethasone (1 mg·kg^{-1}, s.c.) for 22 days starting from the day of immunisation. Samples were collected 24 h after the second ovalbumin challenge. Values are presented as mean ± SEM (n = 8 per treatment). *P < 0.05 compared with control group; $^{\#}P$ < 0.05 compared with OVA + vehicle group.

infiltrating cells. Oral treatment with α-humulene or dexamethasone for 22 consecutive days significantly reduced the numbers of eosinophils in comparison to the treatment with vehicle (Figure 1C). No statistical differences were observed in the group treated with *trans*-caryophyllene. Eosinophil numbers in the BALF of mice treated with α-humulene or dexamethasone were, respectively, 73% and 94% lower than in immunized mice treated with vehicle. Moreover, dexamethasone also significantly reduced total leukocyte (by 96%) and neutrophil (71%) counts. Nonetheless, there were no differences in mononuclear cell counts among the groups (Figure 1A–D).

Effect of therapeutic treatment with α-humulene and trans-caryophyllene on leukocyte recruitment to BALF

In order to investigate the possible therapeutic anti-inflammatory effects of the compounds, immunized mice were treated with α-humulene or *trans*-caryophyllene from day 18 to day 22. For this, we used two different routes of treatment (oral and aerosol). Figure 2C shows that the treatment with α-humulene or dexamethasone significantly reduced the numbers of eosinophils in the BALF by 52% and 72% respectively. No significant differences were observed in the group treated with *trans*-caryophyllene. Dexamethasone also significantly reduced total leukocyte (83%) and neutrophil (55%) counts and decreased the number of mononuclear cells to a level which was below the basal level (Figure 2A–D).

In a second set of experiments, animals received α-humulene (1 mg·mL^{-1}) by aerosol in the same period (days 18–22). Aerosolized budesonide (1 mg·mL^{-1}) was used for comparison. As *trans*-caryophyllene inhibition did not reach statistical significance when assessed orally, no additional experiments were conducted with this compound. Figure 3C shows that the treatment with α-humulene by aerosol exhibited some similarities to the oral route, decreasing the numbers of eosinophils by 82% in the BALF when compared with immunized and antigen challenged mice treated with vehicle. However, one difference from the results of oral treatment was that aerosol treatment with α-humulene also significantly reduced the total leukocyte counts (67%) (Figure 3A). The positive control, aerosolized budesonide

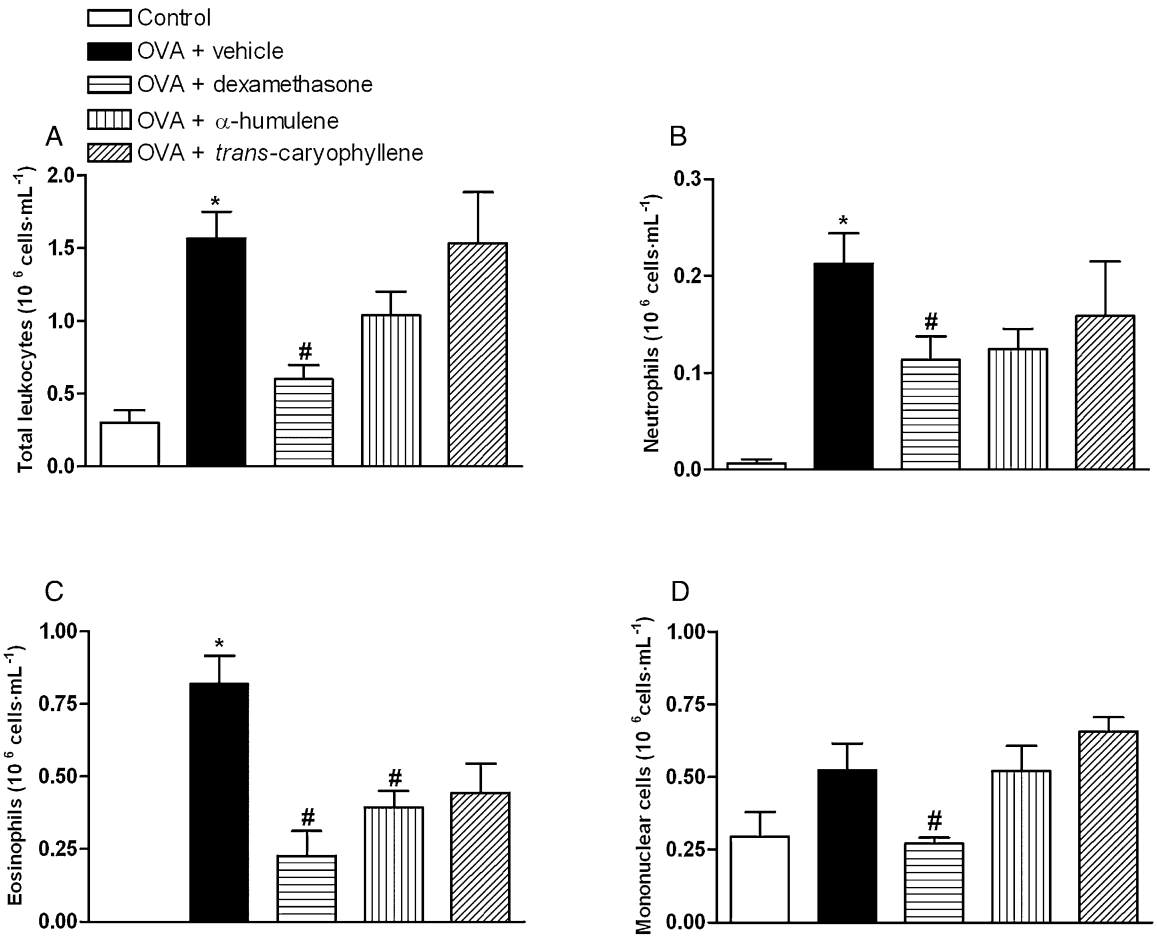

Figure 2 Effect of the therapeutic treatment (oral) with α-humulene or *trans*-caryophyllene on the number of total leukocytes (A), neutrophils (B), eosinophils (C) and mononuclear cells (D) in BALF of mice immunized and then challenged with ovalbumin (OVA). Mice were treated with vehicle (saline), α-humulene (50 mg·kg^{-1}), *trans*-caryophyllene (50 mg·kg^{-1}) or dexamethasone (1 mg·kg^{-1}, s.c.) daily from day 18 to day 22 after the first immunisation. Samples were collected 24 h after the second ovalbumin challenge. Values are presented as mean ± SEM ($n = 12$ per treatment). *$P < 0.05$ compared with control group; $^{\#}P < 0.05$ compared with OVA + vehicle group.

reduced the total leukocyte (95%), eosinophil (88%) and mononuclear cell (94%) counts (Figure 3A–D).

The effects of α-humulene on the eosinophil peroxidase activity in the lung

The EPO activity in the lung homogenate of immunized and ovalbumin-challenged mice treated with vehicle was greater than that observed in the control animals (Figure 4A–C). Preventive (oral, Figure 4A) or therapeutic (aerosol, Figure 4C) treatments with α-humulene consistently and significantly reduced the EPO activity (89% and 90% respectively). In contrast, animals treated therapeutically (oral) with α-humulene did not show a significant reduction of EPO activity (Figure 4B). No alteration of EPO activity was observed in animals treated with *trans*-caryophyllene (p.o.), either preventively or therapeutically (Figure 4A and B respectively). The positive control drugs dexamethasone (oral preventive or therapeutic treatment) and budesonide (aerosol therapeutic treatment) almost abolished the EPO activity (Figure 4A–C).

Effects of α-humulene on the IL-5, CCL11, IFN-γ and LTB$_4$ levels in the BALF

Next we assessed the effects of α-humulene on cytokine and chemokine levels in the BALF. In immunized mice, CCL11 and IL-5 levels were significantly elevated 24 h after ovalbumin challenge, when compared with control animals. The preventive treatment with α-humulene markedly decreased the CCL11 and IL-5 levels by 90% and 79%, respectively, when compared with vehicle-treated animals (Figure 5A and B). Animals treated orally with α-humulene (therapeutic treatment) also significantly decreased the CCL11 and IL-5 levels by 79% and 62% respectively. Likewise, the CCL11 and IL-5 levels were significantly reduced in the animals treated with α-humulene by aerosol (68% and 55% respectively) in comparison with mice treated with vehicle (Figure 5A and B). Dexamethasone (therapeutic and preventive treatment) and budesonide (aerosol therapeutic treatment) used as positive control drugs also significantly reduced the CCL11 (97%, 91% and 97% respectively) and IL-5 (65%, 63% and 90% respectively) levels (Figure 5A and B).

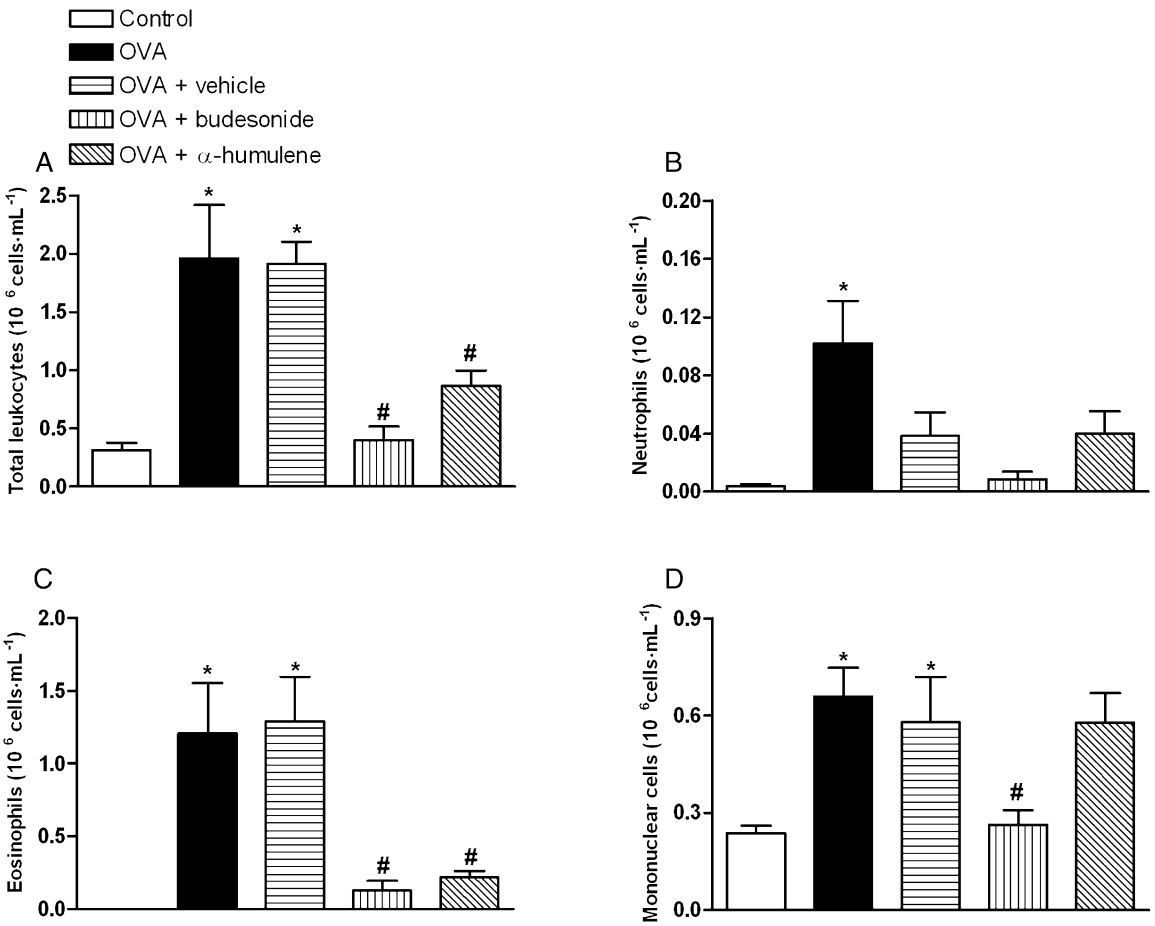

Figure 3 Effect of the therapeutic treatment (by aerosol) with α-humulene on the recruitment of total leukocytes (A), neutrophils (B), eosinophils (C) and mononuclear cells (D) in BALF of mice immunized and then challenged with ovalbumin (OVA). Mice were treated with budesonide (1 mg·mL^{-1}), α-humulene (1 mg·mL^{-1}) or vehicle (5% Tween 80 in saline), from day 18 to day 22 after the first immunisation. Samples were collected 24 h after the second ovalbumin challenge. Values are presented as mean ± SEM (n = 12 per treatment). *P < 0.05 compared with control group; $^{\#}P$ < 0.05 compared with OVA + vehicle group.

Next we assessed the LTB_4 and IFN-γ levels in the BALF following aerosol therapeutic treatment with α-humulene. Immunized and ovalbumin-challenged mice treated with vehicle demonstrated a significant increase of LTB_4 levels in comparison the control mice. LTB_4 levels in the BALF of mice treated with α-humulene or budesonide were, respectively, 52% and 58% lower than in immunized and ovalbumin-challenged mice treated with vehicle (Figure 5C). On the other hand, immunized and ovalbumin-challenged mice demonstrated a decrease of IFN-γ levels (30%) when compared with control animals and the treatment with α-humulene, but not budesonide, increased levels of IFN-γ above control values (Figure 5D).

Effects of α-humulene on IL-5 in mediastinal lymph node (MLN) cells

To analyse the effect of α-humulene on T-lymphocytes, the MLN cells were isolated and re-stimulated with ovalbumin in culture. Production of IL-5 was abolished by α-humulene at 10 μmol·L^{-1} and 1 μmol·L^{-1} (Figure 5E) as well as by dexamethasone (10 μmol·L^{-1}, positive control). However, α-humulene at 0.1 μmol·L^{-1} did not affect IL-5 levels.

Effects of α-humulene on mucus secretion in the lung

Sections of lung tissue were stained with PAS to analyse the effects of α-humulene on mucus secretion. The lung tissue obtained from immunized and ovalbumin-challenged mice treated with vehicle was characterized by mucus hyper-production by goblet cells within the bronchi as compared with control animals (Figure 6A, B and E). Aerosol therapeutic treatment with α-humulene or budesonide decreased the amount of mucus secretion by 51% and 93% respectively (Figure 6D and E).

Effects of α-humulene on NF-κB and AP-1 activation and expression of P-selectin in the lung

In order to assess the effects of treatment with aerosolized α-humulene on P-selectin expression and on the phosphorylation state of p65 NF-κB and c-Jun AP-1 subunits, immunohistochemical techniques were used. Under homeostatic conditions, no significant activation of p65 NF-κB (Figure 7A) or c-Jun AP-1 (Figure 8A) was found in the lung cells. However, a constitutive staining for P-selectin was observed (Figure 9A). In the lung tissue of immunized and ovalbumin-challenged mice treated with vehicle, we detected the

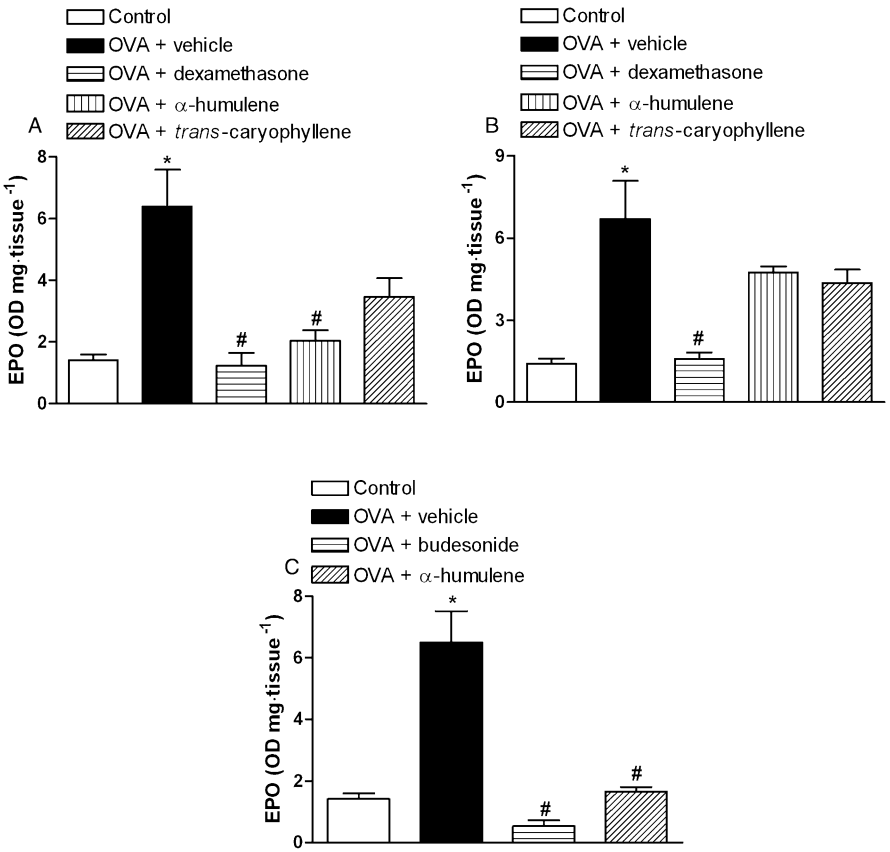

Figure 4 Effect of the preventive (A) and therapeutic (B) oral treatment and therapeutic treatment by aerosol (C) with α-humulene on eosinophil peroxidise (EPO) activity in the lung. Values are mean ± SEM (n = 5 per treatment). *P < 0.05 compared with control group; #P < 0.05 compared with OVA + vehicle group.

phosphorylated p65 subunit of NF-κB (Figure 7B) and phosphorylated c-Jun AP-1 (Figure 8B) in the nucleus of endothelial and inflammatory cells around the bronchiolar epithelium. P-selectin staining was also increased in these cells (Figure 9B). Of note, α-humulene significantly reduced the activation of p65 NF-κB (by 57%, Figure 7D and E) and c-Jun AP-1 (by 80%, Figure 8D and E), as well as the expression of P-selectin (by 87%, Figure 9D and E) in the mouse lung, in comparison to the vehicle-treated animals. Budesonide used as a positive control virtually abolished activation of p65 NF-κB and c-Jun AP-1, as well as the expression of P-selectin (Figures 7C–9C and 7E–9E).

Discussion

In recent decades, considerable progress has been made by using molecular and cellular assays together with knockout and transgenic animals in our understanding of the genetic, tissue-specific and immunological factors that contribute to the development of allergic disorders and allergic inflammation. However, despite that, the current therapy for the treatment of airway inflammation has not changed to the same degree, and inhaled corticosteroids and β_2-adrenoceptor agonists remain as the mainstay of asthma treatment. Thus, the identification of new molecules that are able to prevent or treat inflammatory airway diseases is highly desirable and constitutes a field of continuous research on the part of the major pharmaceutical companies.

Phytochemical and pharmacological studies have identified many potential anti-inflammatory substances, especially those derived from plants used in folk medicine. It has been shown that the essential oil derived from *C. verbenacea* and isolated compounds exhibits marked anti-inflammatory effects in many models of inflammation, even in some models of allergic disease (Fernandes *et al.*, 2007; Medeiros *et al.*, 2007; Passos *et al.*, 2007). It has been demonstrated that the essential oil of *C. verbenacea* reduces ovalbumin-induced pleurisy by inhibiting exudation and cell migration (eosinophil and mononuclear cells, but not neutrophils) (Passos *et al.*, 2007). The possible anti-allergic effects of the two major constituents of the essential oil of *C. verbenacea*, namely α-humulene and *trans*-caryophyllene, have been evaluated in ovalbumin-induced paw oedema in mice and been shown to inhibit this response (Fernandes *et al.*, 2007). This experimental evidence suggests that these compounds might be of potential interest for the treatment of allergy or related conditions. Therefore, in the present study we analysed the effects of these compounds in another model of allergy, that is ovalbumin-induced airways inflammation.

The strategy that has received much attention in the treatment of allergic diseases and asthma is the use of substances

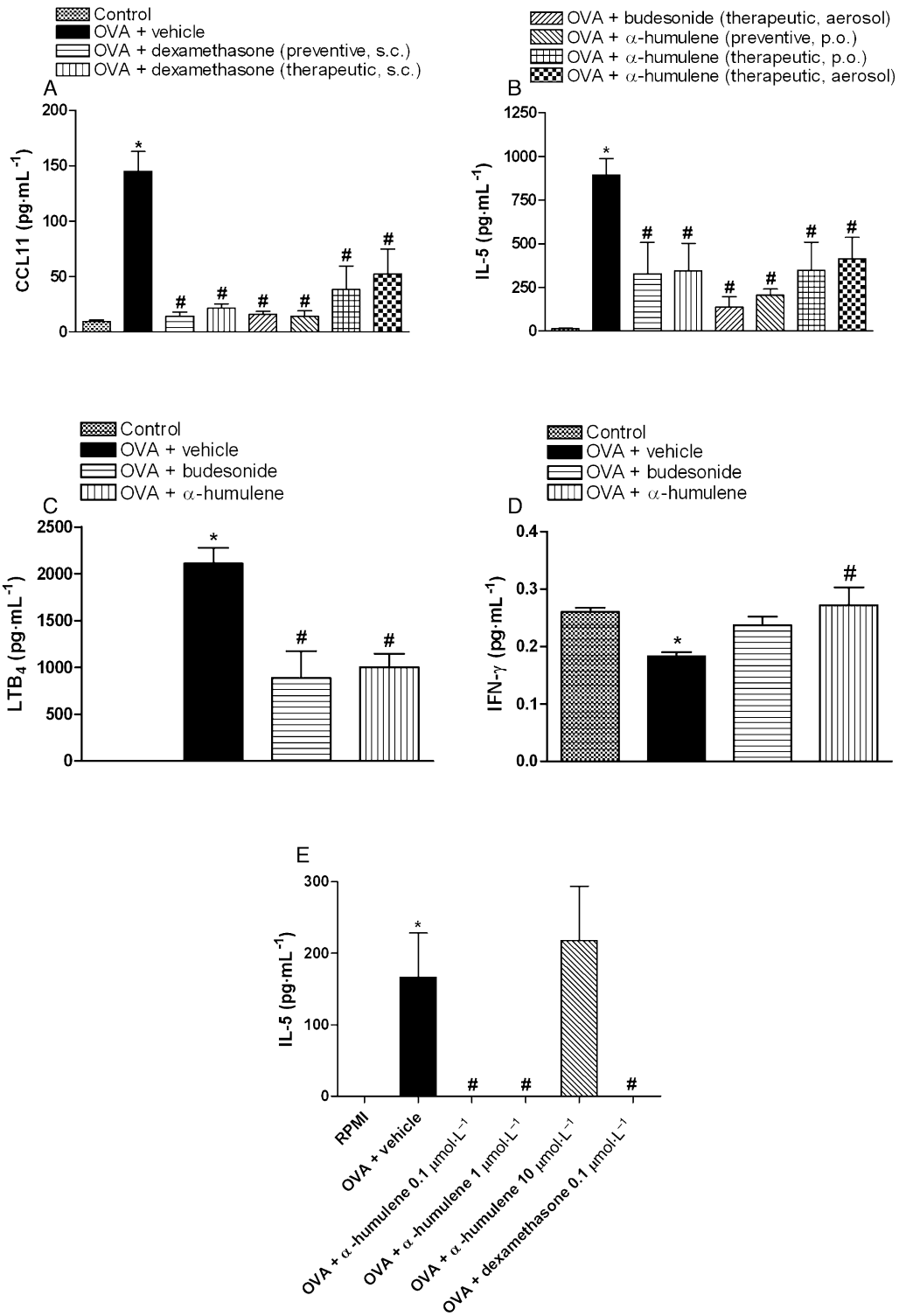

Figure 5 Effect of the preventive treatment and therapeutic treatment by oral or aerosol route with α-humulene on CCL11 (A), IL-5 (B) LTB_4 (C) and IFN-γ (D) levels in the bronchoalveolar lavage fluid (BALF). α-humulene inhibits IL-5 production in mediastinal lymph node (MLN) cells (E). MLN cell cultures were prepared from mice immunized and then challenged with ovalbumin (OVA). Cells were treated with RPMI, OVA (50 $\mu g \cdot mL^{-1}$) + vehicle, OVA + α-humulene (0.1 $\mu mol \cdot L^{-1}$, 1 $\mu mol \cdot L^{-1}$ and 10 $\mu mol \cdot L^{-1}$) and OVA + dexamethasone (0.1 $\mu mol \cdot L^{-1}$) for 24 h. Values are mean ± SEM ($n = 5$ per group). $^{*}P < 0.05$ compared with control group; $^{\#}P < 0.05$ compared with OVA + vehicle group.

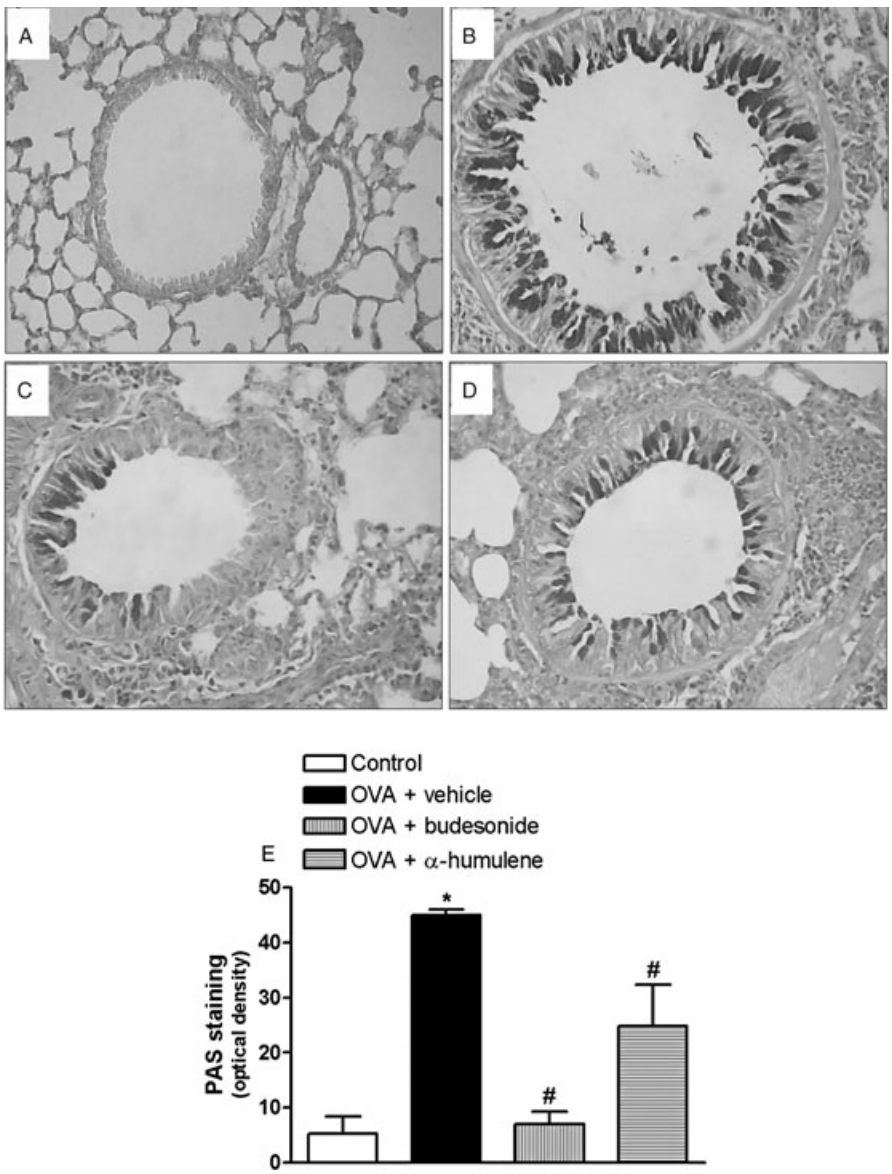

Figure 6 Effect of the therapeutic (aerosol route) treatment with α-humulene on the airway mucus. Representative lung tissue sections from control mice (A), immunized and ovalbumin (OVA)-challenged mice given vehicle (B), budesonide (C) or α-humulene (D) were stained with periodic acid-Schiff (original magnifications, ×200). Mucus staining was determined from image analysis and represented as arbitrary units (E). Values are mean ± SEM (n = 5 per group). *P < 0.05 compared with control group; $^{\#}P$ < 0.05 compared with OVA + vehicle group.

that can control the eosinophil influx, as these cells are important sources of various cytokines, chemokines and cationic proteins, which exhibit pronounced pro-inflammatory properties and induce damage in lung tissue (Holgate and Polosa, 2008).

The main result that emerges from our study is that the α-humulene was effective in preventing eosinophil recruitment to the BALF and lung, similar to that reported herein by corticosteroids. Interestingly, animals treated with dexamethasone for 22 days (preventive protocol) clearly demonstrated weight loss, while animals treated with α-humulene or *trans*-caryophylene gained weight similarly to control animals, suggesting a minor collateral effect. The inhibition of eosinophil recruitment was related to reduction of some of the most relevant asthma-related mediators, namely IL-5, CCL11 and LTB_4. These effects were associated with diminished P-selectin expression and inhibition of NF-kB and AP-1 pathways. On the other hand, *trans*-caryophyllene failed to exhibit either preventive or therapeutic anti-inflammatory properties in this murine airways allergic inflammatory model. Taken together these results suggest the potential of α-humulene as a candidate for the treatment of asthma and other allergic diseases.

Of particular interest, α-humulene not only had a preventive anti-inflammatory effect, but also an important therapeutic property, as it was able to attenuate eosinophil migration after the first challenge with OVA. In the therapeutic regimen, α-humulene was administrated orally or by aerosol. Although both treatments were able to reduce eosinophill influx in the BALF, aerosol treatment was more effective, as it diminished

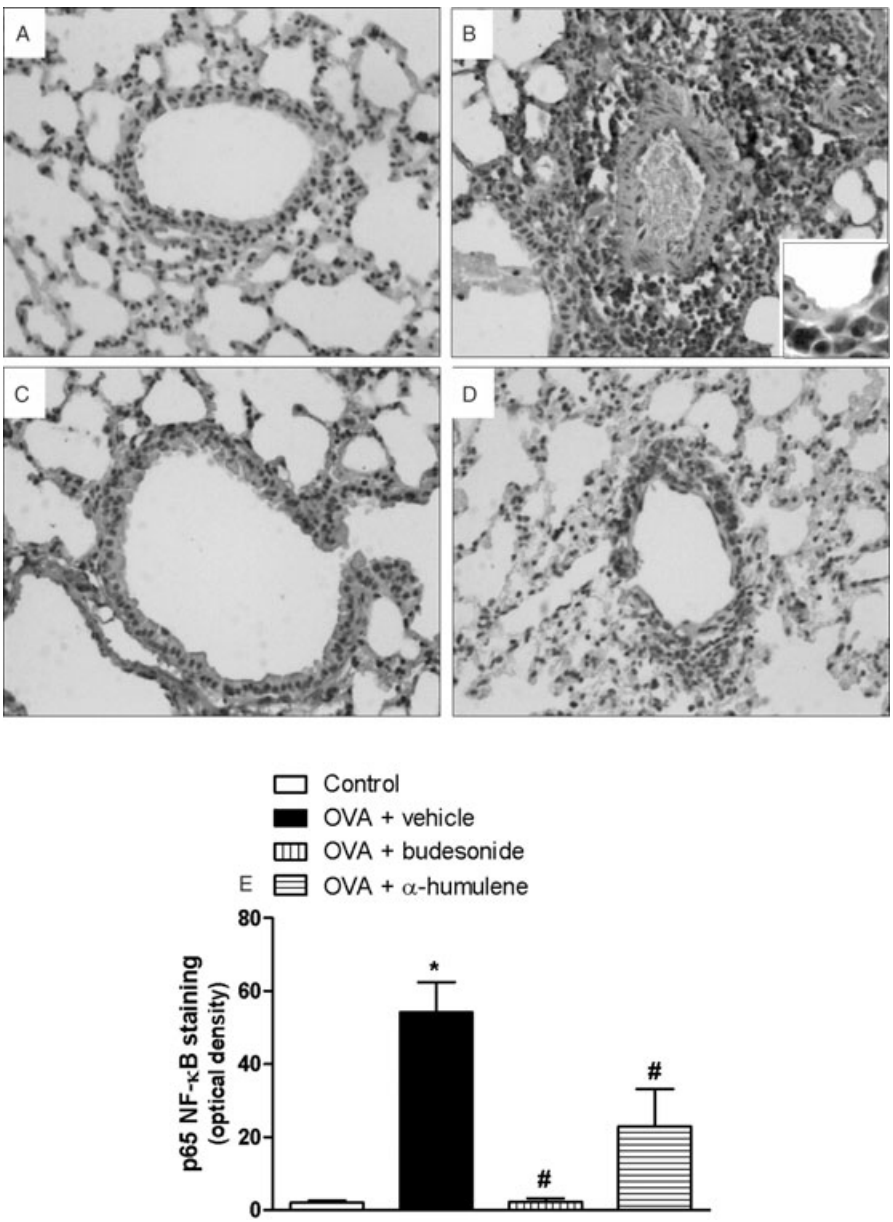

Figure 7 Effect of therapeutic treatment with aerosolized α-humulene on the activation of p65 NF-κB. Representative images of phospo-p65 NF-κB imunohistochemistry staining of control (A), OVA + vehicle (B), OVA + budesonide (C) and OVA + α-humulene (D) groups (magnification: ×200; inset magnification: ×1000). The mean intensity of phospho-p65 NF-κB staining was determined from image analysis and represented as arbitrary units (E). Values are mean ± SEM ($n = 3$ per group) for immunohistochemical analysis. $^{*}P < 0.05$ compared with control group; $^{\#}P < 0.05$ compared with OVA + vehicle group.

total leukocyte recruitment and EPO activity in the lung.

The selective drive of eosinophil recruitment observed in asthma is regulated by the production of different cytokines, especially IL-5. This cytokine is produced by Th2 lymphocytes and eosinophils and exerts a crucial role in orchestrating the eosinophilic influx to inflammation. Antibodies against IL-5 have been used extensively to inhibit eosinophil accumulation in different experimental models (Murali *et al.*, 1993; Tomaki *et al.*, 2000). Moreover, this cytokine specifically supports terminal differentiation and the proliferation of eosinophil precursors, and it is also able to activate mature eosinophils (Sanderson *et al.*, 1985; Yamaguchi *et al.*, 1988; Coeffier *et al.*, 1991). Of note, α-humulene greatly reduced IL-5 levels in the BALF. This effect could be a consequence of the reduction in eosinophil numbers. Nonetheless, we found that the production of IL-5 in re-stimulated regional lymph node cell cultures was also inhibited by α-humulene; therefore, this result suggests that α-humulene also acts on the Th2 lymphocyte response.

CCL11 is a potent inducer of eosinophil chemotaxis which binds to a specific receptor (CCR3) that is highly expressed in eosinophils (Das *et al.*, 1997). A growing amount of evidence indicates that, in animal models of asthma, the administration of an anti-CCL11 antibody, the genetic depletion of CCL11 or of its receptor, is able to inhibit eosinophil influx to

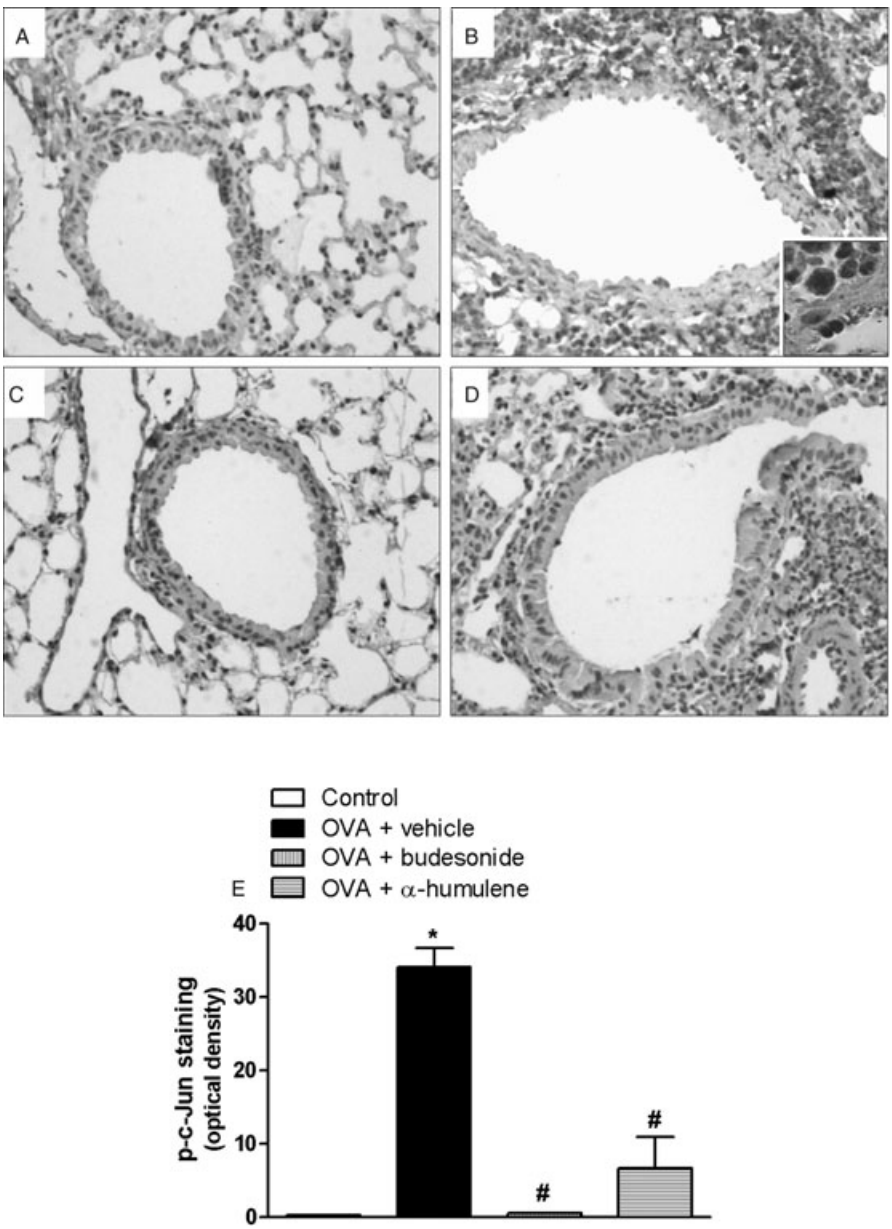

Figure 8 Effect of the therapeutic treatment with aerosolized α-humulene in the activation of phospho-c-Jun AP-1. Representative images of phospho-c-Jun AP-1 imunohistochemistry staining of control (A), OVA + vehicle (B), OVA + budesonide (C) and OVA + α-humulene (D) groups (magnification: ×200; inset magnification: ×1000). The mean intensity of phospho-c-Jun AP-1 staining was determined from image analysis and represented as arbitrary units (E). Values are mean ± SEM ($n = 3$ per group) for immunohistochemical analysis. *$P < 0.05$ compared with control group; $^{\#}P < 0.05$ compared with OVA + vehicle group.

the BALF and lung tissue (Campbell *et al.*, 1998; Humbles *et al.*, 2002; Schuh *et al.*, 2002). Our results demonstrated that α-humulene also reduced CCL11, suggesting another mechanism related to eosinophil influx reduction.

Another chemoattractant for eosinophils is LTB_4, which acts through two G-protein-coupled receptors, BLT_1 and BLT_2 (Yokomizo *et al.*, 1997; Yokomizo *et al.*, 2000). BLT_1-deficient mice do not develop airway hyperresponsiveness or eosinophilic inflammation in an experimental asthma model (Terawaki *et al.*, 2005). Therefore, the reduction observed in eosinophil influx could be related to the reduction of LTB_4 levels, but as eosinophils also produce LTB_4, the decrease of this lipid mediator could be associated with the diminished eosinophil numbers in the BALF. In a process that depends on a cascade effect, it is difficult to pinpoint the fundamental event being affected and what represents the consequences of the primary event.

It is well known that adhesion molecules are involved in leukocyte influx control. Notably, P-selectin is involved in the control of eosinophil influx to inflamed tissues, and therefore it is also considered to be an important target for the modulation of the eosinophilic influx (Wardlaw, 2001). Eosinophil recruitment is abolished in L- or P-selectin-deficient mice, and following administration of anti-P-selectin antibodies or its

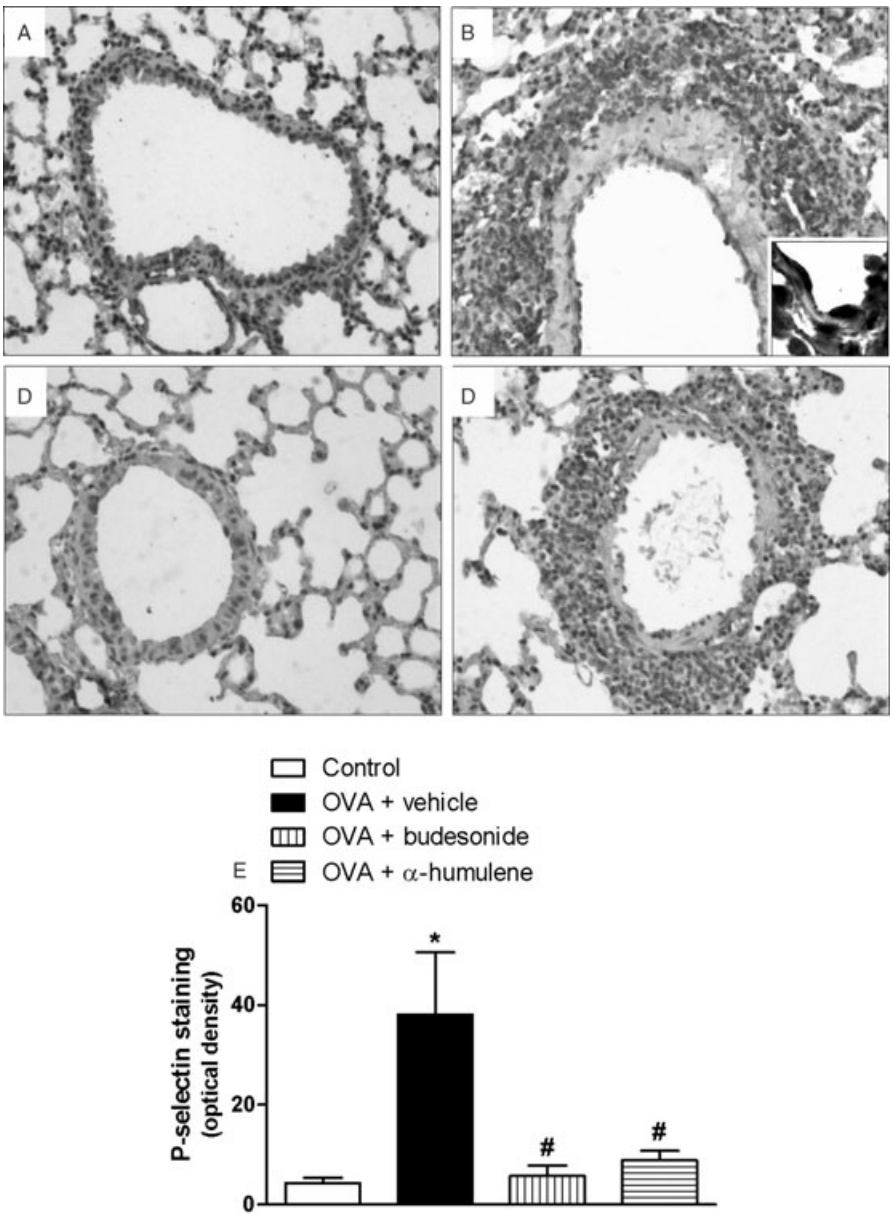

Figure 9 Effect of the therapeutic treatment with aerosolized α-humulene on the P-selectin expression. Representative images of P-selectin imunohistochemistry staining of control (A), OVA + vehicle (B), OVA + budesonide (C) and OVA + α-humulene (D) groups (magnification: ×200; inset magnification: ×1000). The mean intensity of P-selectin staining was determined from image analysis and represented as arbitrary units (E). Values are mean ± SEM ($n = 3$ per group) for immunohistochemical analysis. *$P < 0.05$ compared with control group; $^{\#}P < 0.05$ compared with OVA + vehicle group.

counter-ligand, P-selectin glycoprotein ligand-1 (PSGL-1) (Katayama *et al.*, 2000; Théorêt *et al.*, 2001; Ulfman *et al.*, 2003). In a murine model of allergy, P-selectin expression on the surfaces of platelets is required for pulmonary eosinophil and lymphocyte recruitment (Pitchford *et al.*, 2005). Our findings revealed that α-humulene, like budesonide, when given by aerosol, consistently prevented the over-expression of P-selectin in the endothelial and inflammatory cells around the bronchiolar epithelium of immunized and ovalbumin-challenged mice. Therefore, the reported inhibition of P-selectin expression is expected to contribute to the anti-allergic and anti-inflammatory actions of α-humulene.

Inflammatory diseases are controlled by pro-inflammatory transcription factors such as NF-κB and AP-1 (Handel, 1997; Atreya AtreyaN *et al.*, 2008). Mice deficient in the p50 subunit of NF-κB, as well as animals intratracheally treated with NF-κB decoy oligodeoxynucleotides, in an asthma model are incapable of mounting an eosinophilic airway response (Yang *et al.*, 1998; Desmet *et al.*, 2004). Evidence now suggests that in certain allergic diseases, the expression of some relevant genes encoding chemokines (CCL11), cytokines (IL-5), adhesion molecules (P-selectin) and other proteins such as specific 5-lipooxygenase-activating protein (FLAP) is controlled by NF-κB (Rothenberg *et al.*, 1995; Anrather *et al.*, 1997; Yang *et al.*, 1998; Serio *et al.*, 2005). Therefore, the inhibition of the NF-κB activation observed by us might be related to the reduction of CCL11, IL-5, P-selectin and LTB_4 in the lungs of antigen challenged animals, treated with α-humulene.

Here, we have corroborated and extended the previous observations of our group (Medeiros *et al*., 2007) which demonstrated that oral treatment with α-humulene was able to reduce activation of NF-κB in paw oedema induced by bacterial lipopolysaccharide.

The AP-1 pathway is also an important target for the control of eosinophil inflammation. In an experimental asthma model, the intratracheal administration of AP-1 decoy oligodeoxynucleotides resulted in a significant attenuation of all the pathophysiological features of experimental asthma (Desmet *et al*., 2005). In addition, AP-1 has been implicated in the promoter regions of many pro-inflammatory genes including Th2 cytokines (Nakamura *et al*., 1997; Dalton *et al*., 1999). Studies using knockout mice have shown that AP-1 (JunB and JunD) family members are required for full differentiation of Th2 cells, and are thus essential for the induction of allergic airway inflammation (Hartenstein *et al*., 2002; Meixner *et al*., 2004). Our results obtained with lymphocytes isolated from MLNs reinforced this data, demonstrating that α-humulene inhibited the production of the Th2 cytokine, IL-5. Aerosol treatment with α-humulene reduced AP-1 activation in the lung and our results obtained with lymphocytes isolated from MLNs and with BALF analysis reinforced these data, demonstrating that α-humulene inhibited production of the Th2 cytokine, IL-5.

Although CD4 T cells and IL-5 are important in the development of airway inflammation and eosinophilia, neither IL-5 nor eosinophils are essential for the induction of mucus hypersecretion, but signalling through the receptor, IL-4Rα, is critically important in Th2 cell stimulation of mucus production (Cohn *et al*., 1999). Therefore, the reduction of the Th2 cytokines, IL-4 and IL-13, whose receptors (IL-4R and IL-13R) share a common receptor chain (IL-4Rα), may decrease mucus hypersecretion. Reinforcing this hypothesis, our results have shown that α-humulene increased production of IFN-γ, a Th1 cytokine that inhibits Th2 immune responses, providing some rationale for the decreased mucus production.

In conclusion, the results presented here revealed that the sesquiterpene α-humulene, when given either preventively or therapeutically, reduced the eosinophilic migration into the BALF and lung tissue, when assessed in a murine model of airway allergic inflammation. Our data also revealed that α-humulene exerts its actions through mechanisms associated with the modulation of Th1/Th2 balance, decreased mucus production, inhibition of IL-5, CCL11 and LTB_4 levels and P-selectin expression, probably by inhibiting the activation of the transcription factors, NF-κB and AP-1. Such results confirm and also largely extend our previous findings and suggest that α-humulene might constitute an attractive molecule with potential interest for the treatment of asthma and related inflammatory and allergic diseases.

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Conflict of interest

The authors state no conflict of interest.

References

Alexander SPH, Mathie A, Peters JA (2008). Guide to Receptors and Channels (GRAC), 3rd edn. *Br J Pharmacol* **153** (Suppl. 2): S1–S209.

Anrather J, Csizmadia V, Brostjan C, Soares MP, Bach FH, Winkler H (1997). Inhibition of bovine endothelial cell activation *in vitro* by regulated expression of a transdominant inhibitor of NF-kappa B. *J Clin Invest* **99**: 763–772.

Atreya I, Atreya R, Neurath MF (2008). NF-kappaB in inflammatory bowel disease. *J Intern Med* **263**: 591–596.

Barnes PJ (2006). Corticosteroids: the drugs to beat. *Eur J Pharmacol* **533**: 2–14.

Calixto JB, Otuki MF, Santos AR (2003). Anti-inflammatory compounds of plant origin. Part I. Action on arachidonic acid pathway, nitric oxide and nuclear factor kappa B (NF-kappaB). *Planta Med* **69**: 973–983.

Calixto JB, Campos MM, Otuki MF, Santos AR (2004). Anti-inflammatory compounds of plant origin. Part II. Modulation of pro-inflammatory cytokines, chemokines and adhesion molecules. *Planta Med* **70**: 93–103.

Campbell EM, Kunkel SL, Strieter RM, Lukacs NW (1998). Temporal role of chemokines in a murine model of cockroach allergen-induced airway hyperreactivity and eosinophilia. *J Immunol* **161**: 7047–7053.

Carlsen KH (2003). Can asthma and allergy be prevented in real life? *Allergy* **58**: 730–732.

Coeffier E, Joseph D, Vargaftig BB (1991). Activation of guinea pig eosinophils by human recombinant IL-5. Selective priming to platelet-activating factor-acether and interference of its antagonists. *J Immunol* **147**: 2595–2602.

Cohn L, Homer RJ, MacLeod H, Mohrs M, Brombacher F, Bottomly K (1999). Th2-induced airway mucus production is dependent on IL-4Ralpha, but not on eosinophils. *J Immunol* **15**: 6178–6183.

Dalton TP, Shertzer HG, Puga A (1999). Regulation of gene expression by reactive oxygen. *Annu Rev Pharmacol Toxicol* **39**: 67–101.

Das AM, Flower RJ, Hellewell PG, Teixeira MM, Perretti M (1997). Eotaxin-induced eosinophil migration in the peritoneal cavity of ovalbumin-sensitized mice: mechanism of action. *J Immunol* **159**: 1466–1473.

Desmet C, Gosset P, Pajak B, Cataldo D, Bentires-Alj M, Lekeux P *et al*. (2004). Selective blockade of NF-kappa B activity in airway immune cells inhibits the effector phase of experimental asthma. *J Immunol* **173**: 5766–5775.

Desmet C, Gosset P, Henry E, Garzé V, Faisca P, Vos N *et al*. (2005). Treatment of experimental asthma by decoy-mediated local inhibition of activator protein-1. *Am J Respir Crit Care Med* **172**: 671–678.

Elias JA, Zheng T, Lee CG, Homer RJ, Chen Q, Blackburn M *et al*. (2003). Transgenic modeling of interleukin-13 in the lung. *Chest* **123**: 339–345.

Fernandes ES, Passos GF, Medeiros R, da Cunha FM, Ferreira J, Campos MM *et al*. (2007). Anti-inflammatory effects of compounds alpha-humulene and (-)-trans-caryophyllene isolated from the essential oil of *Cordia verbenacea*. *Eur J Pharmacol* **569**: 228–236.

Foster PS, Mould AW, Yang M, Mackenzie J, Mattes J, Hogan SP *et al.* (2001). Elemental signals regulating eosinophil accumulation in the lung. *Immunol Rev* **179**: 173–181.

Gong PH, Gao ZC, Hu P, Xu Y (2005). Investigation of the measurement of murine airways hyperresponsiveness and the therapeutic effects of budesonide on ovalbumin sensitized and challenge mice. *Chin Med J (Engl)* **118**: 1959–1964.

Handel ML (1997). Transcription factors AP-1 and NF-kappa B: where steroids meet the gold standard of anti-rheumatic drugs. *Inflamm Res* **46**: 282–286.

Hartenstein B, Teurich S, Hess J, Schenkel J, Schorpp-Kistner M, Angel P (2002). Th2 cell-specific cytokine expression and allergen-induced airway inflammation depend on JunB. *EMBO J* **21**: 6321–6329.

Holgate ST (1999). Genetic and environmental interaction in allergy and asthma. *J Allergy Clin Immunol* **104**: 1139–1146.

Holgate ST, Polosa R (2008). Treatment strategies for allergy and asthma. *Nat Rev Immunol* **8**: 218–230.

Humbles AA, Lu B, Friend DS, Okinaga S, Lora J, Al-Garawi A *et al.* (2002). The murine CCR3 receptor regulates both the role of eosinophils and mast cells in allergen-induced airway inflammation and hyperresponsiveness. *Proc Natl Acad Sci USA* **99**: 1479–1484.

Kanokmedhakul S, Kanokmedhakul K, Lekphrom R (2007). Bioactive constituents of the roots of *Polyalthia cerasoides*. *J Nat Prod* **70**: 1536–1538.

Katayama T, Ikeda Y, Handa M, Tamatani T, Sakamoto S, Ito M *et al.* (2000). Immunoneutralization of glycoprotein Ibalpha attenuates endotoxin-induced interactions of platelets and leukocytes with rat venular endothelium *in vivo*. *Circ Res* **86**: 1031–1037.

Lee JS, Lee CM, Jeong YI, Jung ID, Kim BH, Seong EY *et al.* (2007). D-pinitol regulates Th1/Th2 balance via suppressing Th2 immune response in ovalbumin-induced asthma. *FEBS Lett* **581**: 57–64.

Medeiros R, Passos GF, Vitor CE, Koepp J, Mazzuco TL, Pianowski LF *et al.* (2007). Effect of two active compounds obtained from the essential oil of *Cordia verbenacea* on the acute inflammatory responses elicited by LPS in the rat paw. *Br J Pharmacol* **151**: 618–627.

Meixner A, Karreth F, Kenner L, Wagner EF (2004). JunD regulates lymphocyte proliferation and T helper cell cytokine expression. *EMBO J* **23**: 1325–1335.

Murali PS, Kumar A, Choi H, Banasal NK, Fink JN, Kurup VP (1993). *Aspergillus fumigatus* antigen induced eosinophilia in mice is abrogated by anti-IL-5 antibody. *J Leukoc Biol* **53**: 264–267.

Nakamura H, Nakamura K, Yodoi J (1997). Redox regulation of cellular activation. *Annu Rev Immunol* **15**: 351–369.

Neurath MF, Finotto S, Glimcher LH (2002). The role of Th1/Th2 polarization in mucosal immunity. *Nat Med* **8**: 567–573.

Passos GF, Fernandes ES, da Cunha FM, Ferreira J, Pianowski LF, Campos MM *et al.* (2007). Anti-inflammatory and anti-allergic properties of the essential oil and active compounds from *Cordia verbenacea*. *J Ethnopharmacol* **110**: 323–333.

Pitchford SC, Momi S, Giannini S, Casali L, Spina D, Page CP *et al.* (2005). Platelet P-selectin is required for pulmonary eosinophil and lymphocyte recruitment in a murine model of allergic inflammation. *Blood* **105**: 2074–2081.

Rali T, Wossa SW, Leach DN, Waterman PG (2007). Volatile chemical constituents of *Piper aduncum* L and *Piper gibbilimbum* C. DC (Piperaceae) from Papua New Guinea. *Molecules* **12**: 389–394.

Rogerio AP, Fontanari C, Borducchi E, Keller AC, Russo M, Soares EG *et al.* (2008). Anti-inflammatory effects of *Lafoensia pacari* and ellagic acid in a murine model of asthma. *Eur J Pharmacol* **580**: 262–270.

Rothenberg ME, Luster AD, Leder P (1995). Murine eotaxin: an eosinophil chemoattractant inducible in endothelial cells and in interleukin 4-induced tumor suppression. *Proc Natl Acad Sci USA* **92**: 8960–8964.

Russo M, Nahori MA, Lefort J, Gomes E, de Castro Keller A, Rodriguez D *et al.* (2001). Suppression of asthma-like responses in different mouse strains by oral tolerance. *Am J Respir Cell Mol Biol* **24**: 518–526.

Sanderson CJ, Warren DG, Strath M (1985). Identification of a lymphokine that stimulates eosinophil differentiation *in vitro*. Its relationship to interleukin 3, and functional properties of eosinophils produced in cultures. *J Exp Med* **162**: 60–74.

Schnyder-Candrian S, Togbe D, Couillin I, Mercier I, Brombacher F, Quesniaux V *et al.* (2006). Interleukin-17 is a negative regulator of established allergic asthma. *J Exp Med* **203**: 2715–2725.

Schuh JM, Blease K, Kunkel SL, Hogaboam CM (2002). Eotaxin/CCL11 is involved in acute, but not chronic, allergic airway responses to *Aspergillus fumigatus*. *Am J Physiol Lung Cell Mol Physiol* **283**: L198–L204.

Serio KJ, Reddy KV, Bigby TD (2005). Lipopolysaccharide induces 5-lipoxygenase-activating protein gene expression in THP-1 cells via a NF-kappaB and C/EBP-mediated mechanism. *Am J Physiol Cell Physiol* **288**: 1125–1133.

Terawaki K, Yokomizo T, Nagase T, Toda A, Taniguchi M, Hashizume K (2005). Absence of leukotriene B_4 receptor 1 confers resistance to airway hyperresponsiveness and Th2-type immune responses. *J Immunol* **175**: 4217–4225.

Théorêt JF, Bienvenu JG, Kumar A, Merhi Y (2001). P-selectin antagonism with recombinant p-selectin glycoprotein ligand-1 (rPSGL-Ig) inhibits circulating activated platelet binding to neutrophils induced by damaged arterial surfaces. *J Pharmacol Exp Ther* **298**: 658–664.

Tomaki M, Zhao LL, Lundahl J, Sjostrand M, Jordana M, Linden A *et al.* (2000). Eosinophilopoiesis in a murine model of allergic airway eosinophilia: involvement of bone marrow IL-5 and IL-5 receptor alpha. *J Immunol* **165**: 4040–4050.

Ulfman LH, Joosten DP, van Aalst CW, Lammers JW, van de Graaf EA, Koenderman L *et al.* (2003). Platelets promote eosinophil adhesion of patients with asthma to endothelium under flow conditions. *Am J Respir Cell Mol Biol* **28**: 512–519.

Vargaftig BB, Singer M (2003). Leukotrienes mediate part of Ova-induced lung effects in mice via EGFR. *Am J Physiol Lung Cell Mol Physiol* **285**: L808–L818.

Wardlaw AJ (2001). Eosinophil trafficking in asthma. *Clin Med* **1**: 214–218.

Yang L, Cohn L, Zhang DH, Homer R, Ray A, Ray P (1998). Essential role of nuclear factor kappaB in the induction of eosinophilia in allergic airway inflammation. *J Exp Med* **188**: 1739–1750.

Yamaguchi Y, Suda T, Suda J, Eguchi M, Muira Y, Harada N *et al.* (1988). Purified interleukin-5 (IL-5) supports the terminal differentiation and proliferation of murine eosinophilic precursors. *J Exp Med* **167**: 43–56.

Yokomizo T, Izumi T, Chang K, Takuwa Y, Shimizu T (1997). A G-protein-coupled receptor for leukotriene B_4 that mediates chemotaxis. *Nature* **387**: 620–624.

Yokomizo T, Kato K, Terawaki K, Izumi T, Shimizu T (2000). A second leukotriene B (4) receptor, BLT2. A new therapeutic target in inflammation and immunological disorders. *J Exp Med* **192**: 421–432.