



Ferulic acid suppresses Th2 immune response and prevents remodeling in ovalbumin-induced pulmonary allergy associated with inhibition of epithelial-derived cytokines



Alessa Sin Singer Brugiolo*, Ana Cláudia Carvalho Gouveia, Caio César de Souza Alves, Flávia Márcia de Castro e Silva, Erick Esteves de Oliveira, Ana Paula Ferreira

IMUNOCET, Departamento de Parasitologia, Microbiologia e Imunologia, Instituto de Ciências Biológicas, Universidade Federal de Juiz de Fora, Juiz de Fora, Minas Gerais, Brazil

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ABSTRACT

Asthma is characterized by intermittent airway obstruction and chronic inflammation, orchestrated primarily by Th2 cytokines. There is a strong rationale for developing new asthma therapies, since current treatment protocols present side effects and may not be effective in cases of difficult-to-control asthma. The purpose of this study was to evaluate the effect of ferulic acid, a phenolic acid commonly present in plants, in the ovalbumin-induced pulmonary allergy murine model.

Methods: BALB/c mice were sensitized and challenged with ovalbumin, and treatments were provided by gavage. Six groups of mice ($n = 6$) were studied, labeled as: control, pulmonary allergy, dexamethasone, and 3 receiving ferulic acid (at 25, 50, and 100 mg/kg). Lung tissue, bronchoalveolar lavage fluid and serum were collected for analysis.

Results: Ferulic acid treatment inhibited an established allergic Th2-response by decreasing the key features of pulmonary allergy, including lung and airway inflammation, eosinophil infiltration, mucus production and serum levels of OVA-specific IgE. These results were associated with lower levels of CCL20, CCL11 and CCL5 chemokines and IL-4, IL-5, IL-13, TSLP, IL-25 and IL-33 cytokines in lung tissue homogenate.

Conclusions: In this study it was demonstrated for the first time that ferulic acid treatment is able to suppress one of the main features of the airway remodeling, indicated by reduction of mucus production, besides the Th2 pathogenic response on ovalbumin-induced pulmonary allergy. Taken together, results shows that the immunopathological mechanism underlying these effects is linked to a reduction of the epithelial-derived chemokines and cytokines, suggesting that ferulic acid may be useful as a potential therapeutic agent for asthma.

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1. Introduction

Asthma is a serious global health problem affecting more than 300 million people worldwide, imposing severe economic and social burdens [1]. This chronic airway inflammation disease is characterized by respiratory symptoms associated with

* Corresponding author.

E-mail addresses: alessa.brugiolo@ufjf.edu.br (A. Sin Singer Brugiolo), ana.bioquimica@gmail.com (A.C. Carvalho Gouveia), caiocsa@gmail.com (C.C. de Souza Alves), flaviamsilva@gmail.com (F.M. de Castro e Silva), erickestevesdeoliveira@gmail.com (E. Esteves de Oliveira), ana.paula@ufjf.edu.br (A.P. Ferreira).

expiratory flow limitation [2]. The immune response in asthma is initiated by exposure to inhaled allergens leading to the activation of epithelial cells that release CCL20 and cytokines, such as thymic stromal lymphopoietin (TSLP), IL-25, and IL-33, inducing the migration and activation of dendritic cells, which coordinate the Th2 pathogenic response [3]. In parallel, IL-25 and IL-33 activate type 2 innate lymphoid cells (ILC2) [4] which together with Th2 cells secrete IL-4, IL-5 and IL-13, contributing to allergen-specific IgE production, bronchial eosinophilia, airway hyperresponsiveness and mucus hypersecretion [5]. Prolonged or repetitive exposure to allergen results in chronic inflammation characterized by substantial changes in the extracellular matrix,

Abbreviations

BALF	bronchoalveolar lavage fluid
DEXA	dexamethasone
FA	ferulic acid
HE	hematoxylin and eosin
ILC2	type 2 innate lymphoid cells
LD50	lethal dose 50%
OVA	ovalbumin
PA	pulmonary allergy
PAS	periodic acid-Schiff
PBS	phosphate-buffered saline
PBST	Tween 20- phosphate-buffered saline
SEM	standard error of the mean
SR	Sirius red
TSLP	thymic stromal lymphopoietin

leading to airway remodeling, with goblet cell hyperplasia, sub-epithelial fibrosis and airway smooth muscle cell hypertrophy, causing changes in the structure and function of the lungs, which contribute to the airway obstruction [6]. Currently there is no cure for asthma and treatment aimed at controlling the disease and preventing the exacerbations, the association of anti-inflammatory and bronchodilator drugs being the most effective therapy currently available. However, these treatments present side effects and may not be effective in cases of difficult-to-control asthma [2], highlighting the need for the development of new effective compounds for asthma therapy. In this context, research on plant-derived drugs has been given high importance in recent years, and some have shown promising results in mouse models of pulmonary allergy [7,8].

Ferulic acid (FA; 4-hydroxy-3-methoxycinnamic acid) is a phenolic acid commonly present in plants, being a bioactive compound of many foods; including cereals, vegetables, fruits and drinks; besides its use as a food additive in many countries [9]. It is estimated that the daily intake of FA can reach 150–250 mg, however it is unclear whether this amount of intake is sufficient to generate biological effects [10]. FA presents efficient absorption and low toxicity, confirmed by several experimental studies, their acute oral LD50 (lethal dose 50%) in female and male F344 rats being 2.1 and 2.4 g/kg, respectively, whereas in mice it is 3.2 g/kg [11]. Due to its chemical structure, FA shows a great capacity to eliminate free radicals, being a potent antioxidant [9]. Other effects demonstrated in *in vitro* and *in vivo* studies include analgesic [12], antimicrobial [13], anticancer [14,15], photoprotective [16,17] and anti-inflammatory activity shown by decreasing levels of inflammatory mediators and cytokines [18]. Also, FA presented therapeutic effects in *in vivo* chronic disease models such as diabetes [19,20], metabolic syndrome [21,22], cardiovascular diseases [23] and Alzheimer's disease [24]. Concerning allergies, inhibition of cutaneous anaphylaxis, degranulation of mast cells, Arthus reactions and delayed hypersensitivity reaction in rats have been observed [25], besides improvement of airway allergic inflammation through the induction of Th1 immune response in an ovalbumin (OVA)-induced allergy model [26]. Despite the promising results obtained on allergic reactions, the mechanisms involved in this modulation have been little explored. The purposes of the present study were to further investigate the effect of FA in allergic inflammation of airways to better understand the underlying immunopathological mechanisms associated with the FA protective anti-allergic effect in an OVA-induced pulmonary allergy murine model.

2. Material and methods

2.1. Animals

Female BALB/c mice, aged 6–8 weeks, were obtained from the animal facilities of the Universidade Federal de Juiz de Fora and were housed under standard conditions in common cages in ventilated shelves (Alesco, Monte Mor, Brazil). All the protocols involving mice handling were approved by the Ethical Committee for Animal Research of the Universidade Federal de Juiz de Fora (073/2012-CEEA), and are in accordance with internationally accepted principles for laboratory animal use and care.

2.2. OVA-induced pulmonary allergy and treatments

Mice were sensitized intraperitoneally on days 0 and 14 with 3 µg of OVA (Grade V, Sigma-Aldrich Corp, St Louis, USA) in 1 mg of alum (Sigma-Aldrich Corp., St Louis, USA). The animals were challenged for 20 min with 1% aerosolized OVA (Inaltec Plus, Nevoni, São Paulo, Brazil) on days 21, 23, 25, 27, and 29. All treatments (0.1 ml/day/mouse) were performed daily by gavage from day 21 until day 29 (Fig. 1). A total of 6 groups of mice (n = 6) were studied: i) control (treatment with vehicle – filtered water); ii) PA (pulmonary allergy, sensitizations and challenges with OVA, treatment with vehicle); iii) DEXA (positive control, sensitizations and challenges with OVA, treatment with dexamethasone 3 mg/kg; Aché, Guarulhos, Brazil); iv) FA 25 (sensitizations and challenges with OVA, treatment with ferulic acid 25 mg/kg, Sigma-Aldrich Corp., St Louis, USA); v) FA 50 (sensitizations and challenges with OVA, treatment with ferulic acid 50 mg/kg); vi) FA 100 (sensitizations and challenges with OVA, treatment with ferulic acid 100 mg/kg).

2.3. Collection of blood, bronchoalveolar lavage fluid and lung samples

Twenty 4 h after the last OVA challenge mice were anesthetized intraperitoneally with ketamine (90 mg/kg, Syntec, São Paulo, Brazil) and xylazine (10 mg/kg, Calmion, Agener União, São Paulo, Brazil) and exsanguinated via the brachial plexus. Blood samples were collected and centrifuged at 7500g for 2 min (Centrifuge 5410, Eppendorf, Hamburg, Germany), and the serum was separated and stored at –80 °C. Subsequently, bronchoalveolar lavage fluid (BALF) was collected after intratracheal instillation of 1 ml of phosphate-buffered saline (PBS) and the cell pellets were resuspended in 1 ml of PBS containing 2% fetal bovine serum. Then, the chest cavity was opened, the lung tissue was washed by perfusion with 10 ml of PBS and the lung lobes were removed.

2.4. Total and specific cell counts

The total number of viable cells was determined by trypan blue exclusion using a Neubauer cell chamber (Kasvi, Curitiba, Brazil). Cytospins were prepared (Cytological Centrifuge 248, Fanem, São

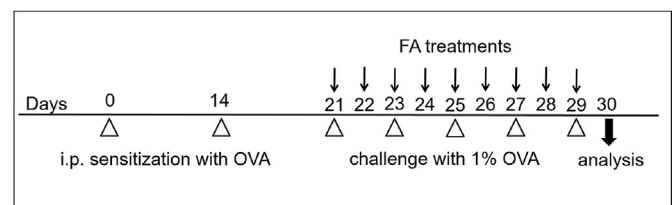


Fig. 1. Mouse model of OVA-induced pulmonary allergy and ferulic acid treatments. i.p., intraperitoneally; OVA, ovalbumin; FA, ferulic acid.

Paulo, Brazil) and the slides were stained with Panoptic (Laborclin Ltda, Pinhais, Brazil). Differential cell counts were determined from a total of 300 cells per slide using the standard morphologic criteria.

2.5. Histopathological analysis

The right upper lung lobe was fixed in 10% buffered formalin, and the samples were subjected to routine histological processing. The paraffin-embedded material was subjected to 4 μ m serial sections and stained with hematoxylin and eosin (HE, EasyPath, São Paulo, Brasil), periodic acid-Schiff (PAS, EasyPath, São Paulo, Brasil) or Sirius Red (SR, Direct Red 80 staining, Sigma-Aldrich Corp., St Louis, USA) and examined in a blinded fashion on an optical microscope (Zeiss, Hallbergmoos, Germany) at 50 $\times$, 100 $\times$, 400 $\times$, and 1000 $\times$ magnification. Peribronchiolar and perivascular areas were assessed according to the degree of inflammatory cell infiltrate of the HE-stained lungs, and at least ten fields were analyzed for each section. Scores of 0–5 were assigned as follows: 0, no inflammation; 1, a few inflammatory cells; 2, a layer of inflammatory cells around the structure evaluated; 3, a ring of inflammatory cells containing two to four layers; 4, focal inflammatory cells clustered around the structure; and 5, intense inflammatory infiltrate [27]. Mucus production was evaluated by the number of PAS-positive goblet cells quantified per 100 μ m² of the PAS-stained lungs, at 400 $\times$ magnification. Number of eosinophils was quantified per 100 μ m² of the SR-staining lungs, at 1000 $\times$ magnification.

2.6. Measurement of chemokines and cytokines in lungs

The levels of chemokines and cytokines in lung were assessed as follows: 100 mg of tissue was homogenized in 1 ml of PBS containing protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM benzethonium chloride, 10 mM ethylenediaminetetraacetic acid, and 2 μ l of aprotinin A) and 0.05% Tween 20. The samples were then centrifuged for 15 min at 500g (Thermo Electron CR312, Waltham, USA) and the levels of chemokines and cytokines in the supernatants were measured using commercially available ELISA kits. BD OptEIA kits (BD Bioscience, San Jose, USA) were used for IL-4, IL-5 and IFN- γ , and R&D Diagnostics kits (Minneapolis, USA) were used for IL-13, TSLP, IL-25, IL-33, CCL5, CCL11, and CCL20, in accordance with the manufacturer's instructions.

2.7. Measurement of anti-OVA-specific antibodies

Quantification of anti-OVA-specific IgE and IgG2a antibodies were performed using ELISA. In brief, 96-well plates (MaxiSorp high protein-binding, NUNC, Sigma-Aldrich Corp, St Louis, USA) were coated with OVA (10 μ g/ml, Grade V, Sigma-Aldrich Corp., St Louis, USA) and incubated for 18 h at 4 °C. Then, the plates were washed with 0.05% Tween 20-PBS (PBST) and blocked with 10% fetal bovine serum-PBST for 2 h at room temperature. Again, the plates were washed with PBST and 50 μ l of each serum sample (1:20 dilution for IgE and 1:100 dilution for IgG2a) was added to each well and incubated for 18 h at 4 °C. The plates were then washed with PBST and biotin-conjugated rat anti-mouse IgE or IgG2a (1:1000; Pharmingen, BD Bioscience, San Jose, USA) was added and incubated for 1 h at room temperature. This was followed by the addition of a substrate solution containing 0.1 M citric acid, 0.2 M sodium phosphate, distilled water, o-phenylenediamine, and 0.03% hydrogen peroxide. Finally, the reaction was stopped with 1 M sulfuric acid and the optical density was measured at 492 nm using a microplate reader (Spectramax 190, Molecular Devices, Sunnyvale, USA).

2.8. Statistical analysis

All statistical analyses were performed using GraphPad prism 5.0 software (GraphPad Software, San Diego, USA). Numerical data were analyzed by the normality test of Kolmogorov–Smirnov. Subsequently, one-way analysis of variance test was used followed by Bonferroni post-test for multiple comparisons for parametric data. The significance level accepted for the tests was $p \leq 0.05$. Data are expressed as mean and standard error of the mean (mean $\pm$ SEM).

3. Results

3.1. Ferulic acid inhibits the key features of OVA-induced pulmonary allergy

In order to address whether ferulic acid treatment was able to reduce the OVA-induced pulmonary allergy, the HE-, PAS- and SR-stained lung sections were evaluated for an overall semi-quantitative score of inflammation, mucus production and eosinophil infiltration, respectively. An increased number of infiltrated cells was observed in animals of the PA group (score 3.8), characterized by intense inflammatory infiltrate (Fig. 2A), when compared with the control group (score 0.1). The ferulic acid (25 mg/kg, 50 mg/kg and 100 mg/kg) and dexamethasone (3 mg/kg) treatments reduced the inflammatory cell infiltrate compared with the PA group and the inflammatory scores were 1.3, 2.0, 1.2, and 0.3, respectively (Fig. 2A and B). Other key features of Th2 pathogenic response in pulmonary allergy are the mucus hypersecretion because of the goblet cell hyperplasia, which is associated with airway remodeling, and the eosinophil infiltration in lungs. An increased amount of mucus production and eosinophil infiltration was observed in the PA group compared to the control group, while ferulic acid, at all doses evaluated, and dexamethasone treatments significantly decreased the mucus production and number of eosinophils in the lung tissue compared with the PA group (Fig. 2A, C and 2D). The OVA-specific IgE production in sera were assessed by ELISA, because this is an indirect measurement of the systemic allergic immune response. Pulmonary allergy increased OVA-specific IgE levels when compared with the control group, indicating allergic immunization, while ferulic acid treatments, at all doses tested, significantly downregulated the OVA-specific IgE levels, when compared with the PA group (Fig. 2E).

3.2. Ferulic acid reduces airway inflammation in association with lower chemokine levels in lung

Aiming to investigate the airway inflammation, the number of total and specific cells in the BALF were analyzed. In the PA group the number of total and specific cells was significantly increased compared to the control group, while ferulic acid and dexamethasone treatments significantly reduced the number of total cells, eosinophils, lymphocytes, neutrophils and macrophages in the BALF (Fig. 3). Additionally, to evaluate whether ferulic acid alters the chemokines in the lungs, the levels of CCL20, CCL11 and CCL5 were measured in the lung homogenate tissue. Results indicated that these chemokine levels increased in the PA group, when compared with the control group. On the other hand, when compared with the PA group, data demonstrated that ferulic acid, at all concentrations evaluated, and dexamethasone treatments significantly decreased the measured chemokines (Fig. 4).

3.3. Ferulic acid suppressed the epithelial-derived and Th2 inflammatory cytokines

To investigate the effect of ferulic acid treatment on the cytokine

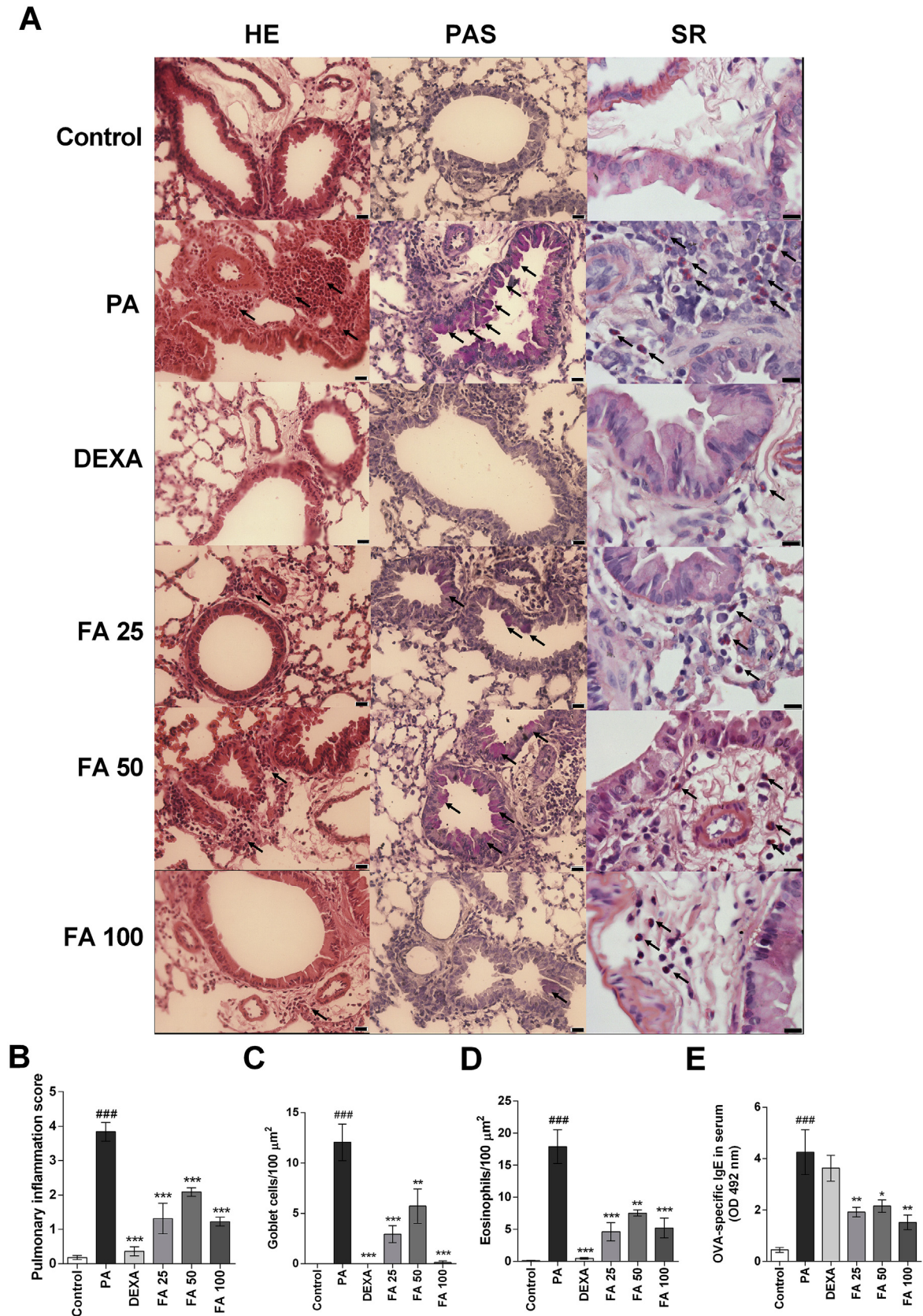


Fig. 2. Effect of ferulic acid treatments on the key features of OVA-induced pulmonary allergy. (A) Samples of lung parenchyma of BALB/c mice representative of the control; PA; DEXA; FA 25; FA 50; and FA 100 groups stained with HE (400 $\times$ magnification, bars represent 20 μm , arrows indicate inflammatory cells); PAS (400 $\times$ magnification, bars represent 20 μm , arrows indicate mucus production); or SR (1000 $\times$ magnification, bars represent 10 μm , arrows indicate eosinophils). (B) Pulmonary inflammation score, (C) number of goblet cells/100 μm^2 , and (D) number of eosinophils/100 μm^2 in HE-, PAS-, and SR-stained lung sections, respectively. (E) Levels of OVA-specific IgE in serum. PA, pulmonary allergy; DEXA, dexamethasone; FA, ferulic acid; HE, hematoxylin-eosin; PAS, periodic acid-Schiff; SR, Sirius Red; OD, optical density. ###P < 0.001 vs control group, *P < 0.05 vs PA group, **P < 0.01 vs PA group, ***P < 0.001 vs PA group. Data represent the mean $\pm$ SEM of at least three independent experiments (n = 6). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

secretion in the inflammatory site, the lung homogenate samples were used for cytokine analysis by ELISA. The induction of pulmonary allergy increased the levels of TSLP, IL-25, IL-33, IL-4, IL-5, and IL-13, when compared with the control group. Ferulic acid, at all doses tested, and dexamethasone treatments significantly suppressed the evaluated cytokine levels, when compared with the PA group (Fig. 5).

4. Discussion

During the past few years, extensive efforts were made to develop new active agents that improve current therapeutic regimens for asthma, aimed at effective low-cost therapy with reduced adverse effects. Plant-derived drugs arose from this effort as very promising for new drug discoveries [8,28,29]. In the current study, ferulic acid markedly suppressed the key features of allergic asthma by reducing the pulmonary inflammation, eosinophil infiltration, Th2 pathogenic immune response and OVA-specific IgE production as well by inhibiting important airway remodeling mediators including goblet cell hyperplasia and IL-13 production. All tested doses showed similar effects to those observed with dexamethasone treatment, a synthetic glucocorticoid widely used in the treatment of asthma [30]. The results obtained were encouraging, since both the inflammatory cell infiltration, particularly eosinophils, and the production of mucus are involved in the pathophysiological impairment in the airway structures contributing significantly to the limitation of expiratory airway flow and to chronic inflammatory pathology [6]. These pathogenic features have been well documented in several OVA-induced pulmonary allergy models and the improvement of these parameters is correlated with therapeutic effectiveness, which is an expected result of a promising therapy [7,31]. Especially in relation to the mucus overproduction, ferulic acid holds great promise for relieving allergic asthma symptoms, it being shown for the first time that this phenolic acid was able to prevent this important pathological feature, suggesting that ferulic acid therapy can be effective in both acute and chronic asthmatic symptoms and may have significant impact on asthma control. This assumption is supported on the fact that excessive mucus production results in the obstruction of airways with mucus plugs, making movement of gas difficult and contributing significantly to asthma morbidity and mortality [32]. Additionally, the airway mucus hypersecretion is related to the airway remodeling, characterized by structural changes in the airway wall responsible for a gradual decline in lung function [33]. Supporting the therapeutic efficacy of ferulic acid, pulmonary inflammation improvement was associated with lower serum levels of OVA-specific IgE, as has been recently demonstrated [26]. Since IgE has an important role in Th2 immune response development and is associated with the early airway obstruction, via the activation of mast cells and basophils [34,35] the present results suggest that the ferulic acid anti-allergic effect started early, during allergen sensitization, when the systemic response can be modulated. Despite there have been a previous report showing similar findings with ferulic acid treatment concerning the key features of allergic asthma, it is relevant highlight that the induction of the pulmonary allergy model was different. Lee et al. [26] did a protocol with duration of 48 days and five consecutive challenges. In the present study, the protocol has 30 days of duration and five challenges on alternate days. In addition, the dose of OVA and sensitization/challenge time course were different between the studies. These variations on the conventional models of antigen sensitization and challenge could reflect in the induction of different magnitudes of immune response. Moreover, in spite of the same doses and way of treatment, Lee et al. [26] did 27 days of treatment, whereas in the present study it was only 9

days of treatment, which represents the same suppressive effect in the key features of asthma with a 3-fold shorter treatment protocol.

Additionally, it is important to note that the reduction in inflammatory cells in BALF was associated with decreased levels of chemokines CCL11, CCL5 and CCL20 in the lungs. In asthma, chemokines are secreted primarily by the airway epithelial cells and alveolar macrophages, directly participating in the development and maintenance of allergic responses by recruiting some crucial cells to Th2 immune response establishment [36,37]. Among these chemokines, CCL11 is a highly potent chemoattractant for eosinophils [38] whose granule-derived bioactive substances have been associated with bronchospasm, edema and tissue injury observed in the allergic responses [39]. On the other hand, it has been shown that CCL5 also recruits eosinophil, neutrophil and monocyte to the airways, in addition to T lymphocytes [40], it being demonstrated in experimental models of asthma that this chemokine induces eosinophilic airway inflammation, antigen-specific IgE production and airway hyperresponsiveness [41] and is correlated with disease severity [42]. Furthermore, the decrease in CCL20 levels may have also contributed to dampening down the airway inflammation, probably by interfering with the onset of the asthmatic response, since this chemokine recruits dendritic cells from bone marrow to the lungs where they uptake and process the inhaled allergens and trigger the Th2 allergic response [3]. Hence, the reduction of chemokine levels could be one probable mechanism involved in the ferulic acid anti-asthmatic effect. However, further studies are needed to investigate these pathways, including the migration and activation of pulmonary dendritic cells, which may result in the induction of a tolerance response to the allergen, which is a desirable effect of a promising therapeutic agent.

An important finding of the present study, demonstrated for the first time, is that ferulic acid treatment inhibited the epithelial-derived cytokines. This effect looks very promising, since it has been well established that TSLP, IL-25 and IL-33 have key roles in driving and sustaining the allergic response and several model studies have shown that these innate cytokines are associated with mainly pathogenic features of the asthma, such as the airway eosinophilia, IgE synthesis, Th2-cytokine and mucus overproduction [43]. Also, they are involved in the activation of ILC2 and Th2 cells, enhancing the lung inflammation and the airway hyperresponsiveness [43–45]. Furthermore, TSLP and IL-33 are able to activate the mast cells directly, regardless of the presence of IgE and specific allergens [46]. On the other hand, it has been demonstrated that monoclonal antibody therapy using either anti-IL-25 or anti-IL-33 inhibits the pathogenic allergic response by suppressing Th2 cytokine production, lung cell infiltration, airway mucus production and hyperreactivity [47,48]. Since epithelial-derived cytokines link innate and adaptive immunity to promote Th2 pathogenic response [46], the ferulic acid therapy-induced reduction of innate cytokines may have contributed to the decreasing IL-4, IL-5 and IL-13 levels observed in this study. Recently it was demonstrated that ferulic acid treatment reduced the Th2 cytokines, however, the authors assign this effect to a switch to Th1-type immune response [26], which did not occur in this present study since the levels of IFN- γ in lung tissue homogenate and OVA-specific IgG2a in serum did not increase after treatment with ferulic acid (data not shown).

The role of these cytokines in driving and sustaining allergic responses is well known, including the fact that the epithelial responses to allergens are maintained through the action of these Th2 effector cytokines, which provide an important feedback loop that can perpetuate disease. Additionally, IL-4 and IL-13 are important for the production of allergen-specific IgE by B lymphocytes [5], levels of which decreased in this present work.

Altogether, these results emphasize the anti-inflammatory

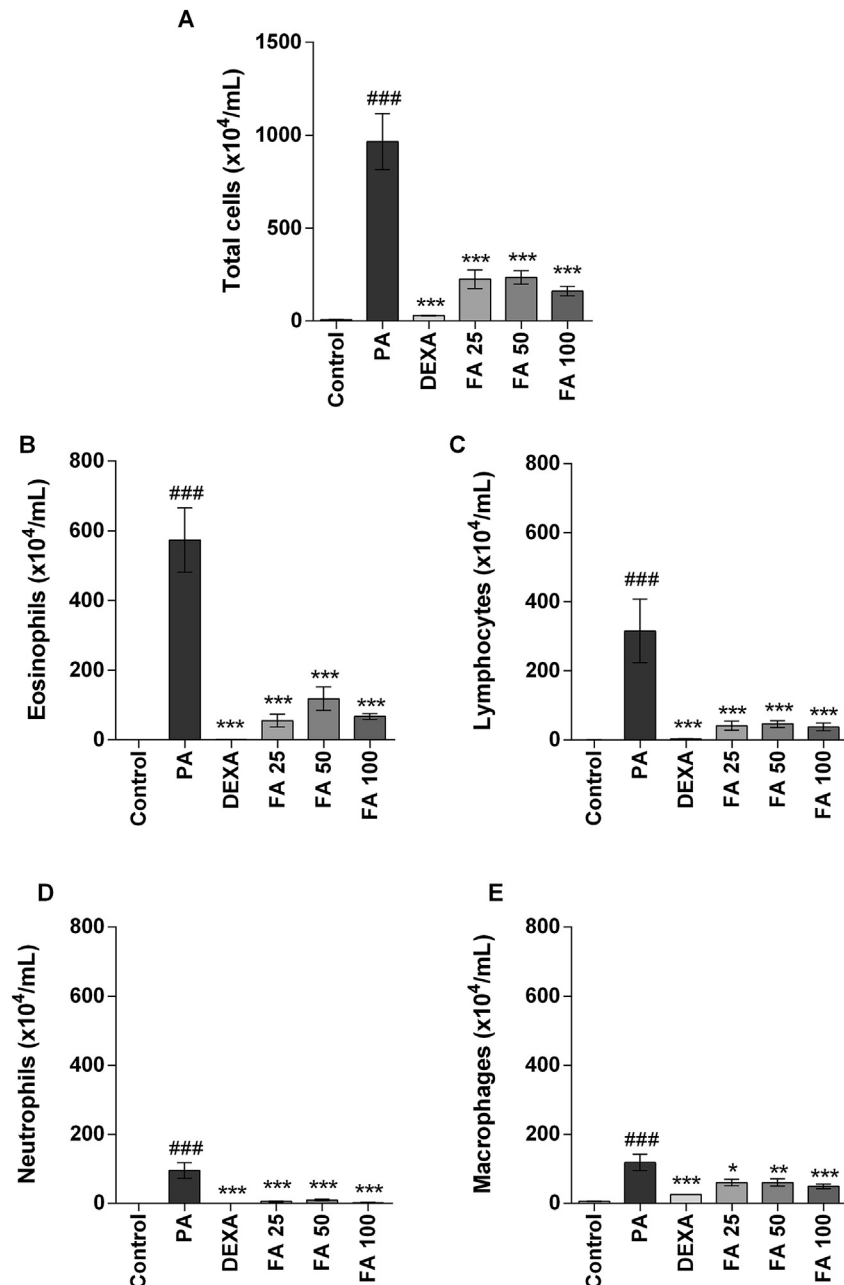


Fig. 3. Counting of total and specific cells in bronchoalveolar lavage fluid (BALF). (A) Total number of cells; (B) eosinophils; (C) lymphocytes; (D) neutrophils; and (E) macrophages ($\times 10^4/\text{mL}$). PA, pulmonary allergy; DEXA, dexamethasone; FA, ferulic acid. ### $P < 0.001$ vs control group, * $P < 0.05$ vs PA group, ** $P < 0.01$ vs PA group, *** $P < 0.001$ vs PA group. Data represent the mean $\pm$ SEM of at least three independent experiments ($n = 6$).

effects of ferulic acid treatment, which gave the optimal result using a lower dose, 25 mg/kg, since those higher doses do not significantly improved the protective effects on allergic response. Also in previous studies, the optimal results were obtained with similar doses of 20 mg/kg and 25 mg/kg in nicotine-induced toxicity [49] and pulmonary allergy models [26], respectively. It is important to note that ferulic acid presents a low toxicity after oral administration [11], far above the doses tested in this work. Currently, one possible limitation for clinical use of ferulic acid is associated with their low bioavailability and short half-life after oral administration, due to its rapid metabolism [10]. To overcome this, new drug delivery-systems containing ferulic acid, such as solid lipid nanoparticles and cyclodextrins, have been proposed to

allow its better absorption and concentration in peripheral organs [50,51]. Further, ferulic acid is more bioavailable than flavonoids, remaining in the blood for longer than other antioxidants, such as vitamin C [18]. Therefore, data above mentioned combined with its great features, such as being easy to get and low cost, make ferulic acid an extremely attractive immunomodulatory agent for asthma therapy. Further, results obtained give clues about the immunopathological pathways associated with the protective effect of ferulic acid in the OVA-induced pulmonary allergy model, providing an excellent starting point for future research aimed at elucidating the cellular mechanisms involved in the suppression of the Th2 pathogenic immune response.

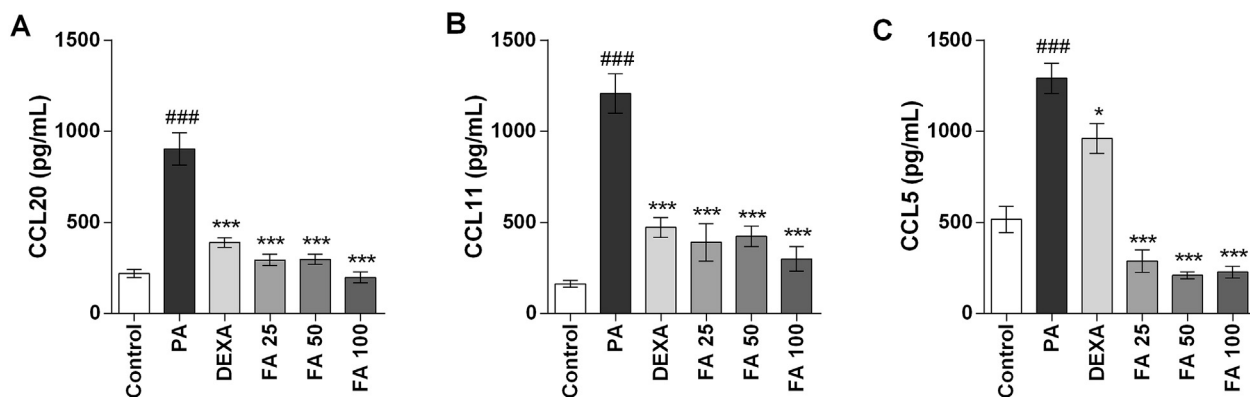


Fig. 4. Lung chemokine levels. (A) CCL20, (B) CCL11 and (C) CCL5 in the supernatant of the homogenate lung tissue. ###P < 0.001 vs control group, *P < 0.05 vs PA group, ***P < 0.001 vs PA group, PA, pulmonary allergy; DEXA, dexamethasone; FA, ferulic acid. Data represent the mean $\pm$ SEM of at least three independent experiments (n = 6).

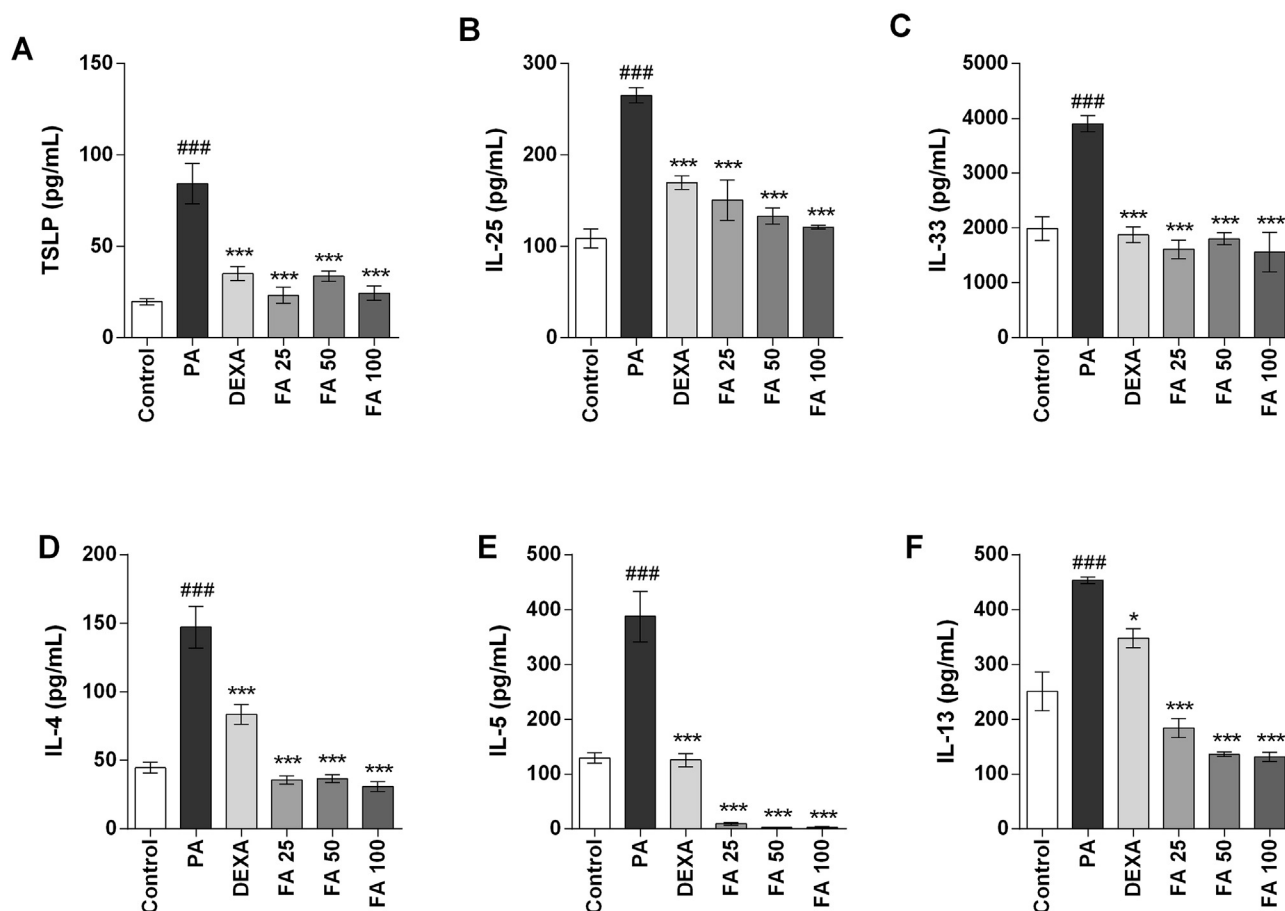


Fig. 5. Lung cytokine levels. (A) TSLP, (B) IL-25, (C) IL-33, (D) IL-4, (E) IL-5, and (F) IL-13 in the supernatant of the homogenate lung tissue. ###P < 0.001 vs control group, *P < 0.05 vs PA group, ***P < 0.001 vs PA group. PA, pulmonary allergy; DEXA, dexamethasone; FA, ferulic acid; TSLP, thymic stromal lymphopoietin. Data represent the mean $\pm$ SEM of at least three independent experiments (n = 6).

5. Conclusions

In conclusion, this study demonstrates that ferulic acid improved an allergen-driven inflammatory response by suppressing the development of the Th2 pathogenic immune response. This effect was associated with reduced levels of epithelial-derived chemokines and cytokines, suggesting that the immunopathological mechanism underlying these ferulic acid effects relies on the innate immune effector pathways, which in turn dampened down

the Th2 adaptive immune response development. However, further research is needed to clarify the specific mechanisms involved in ferulic acid anti-allergic effects. In spite of this, results obtained indicate that this phenolic acid has the potential to be an effective therapeutic agent for asthma, mainly regarding its suppressive effect on mucus overproduction, since current therapies have little effect on this parameter, which has a pivotal role in the development of the airway remodeling.

Conflict of interest

The authors declare that they have no conflict of interest.

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References

- [1] M. Masoli, D. Fabian, S. Holt, R. Beasley, Global Initiative for Asthma P. The global burden of asthma: executive summary of the GINA Dissemination Committee report, *Allergy* 59 (2004) 469–478.
- [2] Global Initiative for Asthma, Global strategy for asthma management and prevention. www.ginasthma.org, 2016. GINA.
- [3] S.T. Holgate, Innate and adaptive immune responses in asthma, *Nat. Med.* 18 (2012) 673–683.
- [4] M.J. Holtzman, D.E. Byers, J. Alexander-Brett, X. Wang, The role of airway epithelial cells and innate immune cells in chronic respiratory disease, *Nat. Rev. Immunol.* 14 (2014) 686–698.
- [5] P.J. Barnes, Immunology of asthma and chronic obstructive pulmonary disease, *Nat. Rev. Immunol.* 8 (2008) 183–192.
- [6] S.J. Galli, M. Tsai, A.M. Piliponsky, The development of allergic inflammation, *Nature* 454 (2008) 445–454.
- [7] J. Wang, R.G. Jin, L. Xiao, Q.J. Wang, T.H. Yan, Anti-asthma effects of synthetic salidroside through regulation of Th1/Th2 balance, *Chin. J. Nat. Med.* 12 (2014) 500–504.
- [8] C. Ma, Z. Ma, Q. Fu, S. Ma, Curcumin attenuates allergic airway inflammation by regulation of CD4+CD25+ regulatory T cells (Tregs)/Th17 balance in ovalbumin-sensitized mice, *Fitoterapia* 87 (2013) 57–64.
- [9] E. Graf, Antioxidant potential of ferulic acid, *Free Radic. Biol. Med.* 13 (1992) 435–448.
- [10] Z. Zhao, M.H. Moghadasian, Chemistry, natural sources, dietary intake and pharmacokinetic properties of ferulic acid: a review, *Food Chem.* 109 (2008) 691–702.
- [11] C. Mancuso, R. Santangelo, Ferulic acid: pharmacological and toxicological aspects, *Food Chem. Toxicol. Int. J. Publ. Br. Industr. Biol. Res. Assoc.* 65 (2014) 185–195.
- [12] W.H. Lv, L. Zhang, S.J. Wu, S.Z. Chen, X.B. Zhu, J.C. Pan, Analgesic effect of ferulic acid on CCI mice: behavior and neurobiological analysis, *Zhongguo Zhong yao za zhi = Zhongguo zhongyao zazhi = China J. Chin. materia medica* 38 (2013) 3736–3741.
- [13] S. Ou, K.-C. Kwok, Ferulic acid: pharmaceutical functions, preparation and applications in foods, *J. Sci. food Agric.* 84 (2004) 1261–1269.
- [14] V.R. Bandugula, R.P. Nagarajan, 2-Deoxy-D-glucose and ferulic acid modulates radiation response signaling in non-small cell lung cancer cells, *Tumour Biol. J. Int. Soc. Oncodevelopmental Biol. Med.* 34 (2013) 251–259.
- [15] C.C. Peng, C.C. Chyau, H.E. Wang, C.H. Chang, K.C. Chen, K.Y. Chou, et al., Cytotoxicity of ferulic acid on T24 cell line differentiated by different microenvironments, *BioMed Res. Int.* 2013 (2013) 579859.
- [16] F.H. Lin, J.Y. Lin, R.D. Gupta, J.A. Tournas, J.A. Burch, M.A. Selim, et al., Ferulic acid stabilizes a solution of vitamins C and E and doubles its photoprotection of skin, *J. Investig. dermatol.* 125 (2005) 826–832.
- [17] A. Saija, A. Tomaino, D. Trombetta, A. De Pasquale, N. Uccella, T. Barbuzzo, et al., In vitro and in vivo evaluation of caffeic and ferulic acids as topical photoprotective agents, *Int. J. Pharm.* 199 (2000) 39–47.
- [18] M. Srinivasan, A.R. Sudheer, V.P. Menon, Ferulic Acid: therapeutic potential through its antioxidant property, *J. Clin. Biochem. Nutr.* 40 (2007) 92–100.
- [19] R. Choi, B.H. Kim, J. Naowaboot, M.Y. Lee, M.R. Hyun, E.J. Cho, et al., Effects of ferulic acid on diabetic nephropathy in a rat model of type 2 diabetes, *Exp. Mol. Med.* 43 (2011) 676–683.
- [20] J. Azay-Milhaud, K. Ferrare, J. Leroy, J. Aubaterre, M. Tournier, A.D. Lajoix, et al., Antihyperglycemic effect of a natural chioric acid extract of chicory (*Cichorium intybus* L.): a comparative in vitro study with the effects of caffeic and ferulic acids, *J. Ethnopharmacol.* 150 (2013) 755–760.
- [21] O. Wang, J. Liu, Q. Cheng, X. Guo, Y. Wang, L. Zhao, et al., Effects of ferulic acid and gamma-oryzanol on high-fat and high-fructose diet-induced metabolic syndrome in rats, *PLoS One* 10 (2015) e0118135.
- [22] K. Senaphan, U. Kukongviriyapan, W. Sangartit, P. Pakdeecheote, P. Pannangpetch, P. Prachaney, et al., Ferulic acid alleviates changes in a rat model of metabolic syndrome induced by high-carbohydrate, high-fat diet, *Nutrients* 7 (2015) 6446–6464.
- [23] H.K. Kim, T.S. Jeong, M.K. Lee, Y.B. Park, M.S. Choi, Lipid-lowering efficacy of hesperetin metabolites in high-cholesterol fed rats, *Clin. chimica acta; Int. J. Clin. Chem.* 327 (2003) 129–137.
- [24] A. Sgarbossa, D. Giacomazza, M. di Carlo, Ferulic acid: a hope for Alzheimer's disease therapy from plants, *Nutrients* 7 (2015) 5764–5782.
- [25] H.J. Hu, B.Q. Hang, Effects of ferulic acid on allergic reaction, *Zhongguo yao li xue bao = Acta Pharmacol. Sin.* 12 (1991) 426–430.
- [26] C.C. Lee, C.C. Wang, H.M. Huang, C.L. Lin, S.J. Leu, Y.L. Lee, Ferulic acid induces Th1 responses by modulating the function of dendritic cells and ameliorates Th2-Mediated allergic airway inflammation in mice, *Evid.-Based Complement. Altern. Med. eCAM* 2015 (2015) 678487.
- [27] M. Lee, S. Kim, O.K. Kwon, S.R. Oh, H.K. Lee, K. Ahn, Anti-inflammatory and anti-asthmatic effects of resveratrol, a polyphenolic stilbene, in a mouse model of allergic asthma, *Int. Immunopharmacol.* 9 (2009) 418–424.
- [28] F. Alves, G.N. Angeli, D.C. Favarin, E.L. de Andrade, J.E. Chica, L.H. Faccioli, et al., The effects of proresolubilization of ellagic acid in an experimental model of allergic airway inflammation, *Mediat. Inflamm.* 2013 (2013) 863198.
- [29] A.S. Brugiolo, C.C. Alves, A.C. Gouveia, A.T. Dias, M.F. Rodrigues, L.G. Pacifico, et al., Effects of aqueous extract of *Echinodorus grandiflorus* on the immune response in ovalbumin-induced pulmonary allergy, *Ann. allergy, asthma & Immunol. off. Publ. Am. Coll. Allergy, Asthma, & Immunol.* 106 (2011) 481–488.
- [30] P. Jungsuwadee, G. Dekan, G. Stingl, M.M. Epstein, Inhaled dexamethasone differentially attenuates disease relapse and established allergic asthma in mice, *Clin. Immunol.* 110 (2004) 13–21.
- [31] H. Bae, Y. Kim, E. Lee, S. Park, K.H. Jung, M.J. Gu, et al., Vitex rotundifolia L. prevented airway eosinophilic inflammation and airway remodeling in an ovalbumin-induced asthma mouse model, *Int. Immunol.* 25 (2013) 197–205.
- [32] L.M. Kuyper, P.D. Pare, J.C. Hogg, R.K. Lambert, D. Ionescu, R. Woods, et al., Characterization of airway plugging in fatal asthma, *Am. J. Med.* 115 (2003) 6–11.
- [33] R.M. Pascual, S.P. Peters, Airway remodeling contributes to the progressive loss of lung function in asthma: an overview, *J. allergy Clin. Immunol.* 116 (2005) 477–486 quiz 87.
- [34] M. Averbach, C. Gebhardt, F. Emmrich, R. Treudler, J.C. Simon, Immunologic principles of allergic disease, *J. Dtsch. Dermatol. Ges.* 5 (2007) 1015–1028.
- [35] H.S. Bandukwala, B.S. Clay, J. Tong, P.D. Mody, J.L. Cannon, R.A. Shilling, et al., Signaling through Fc gamma RIII is required for optimal T helper type (Th)2 responses and Th2-mediated airway inflammation, *J. Exp. Med.* 204 (2007) 1875–1889.
- [36] B.J. Rollins, Chemokines, *Blood* 90 (1997) 909–928.
- [37] B.N. Lambrecht, H. Hammad, The airway epithelium in asthma, *Nat. Med.* 18 (2012) 684–692.
- [38] C.M. Lloyd, S.M. Rankin, Chemokines in allergic airway disease, *Curr. Opin. Pharmacol.* 3 (2003) 443–448.
- [39] J.S. Neves, P.F. Weller, Functional extracellular eosinophil granules: novel implications in eosinophil immunobiology, *Curr. Opin. Immunol.* 21 (2009) 694–699.
- [40] R.E. Marques, R. Guabiraba, R.C. Russo, M.M. Teixeira, Targeting CCL5 in inflammation, *Expert Opin. Ther. Targets* 17 (2013) 1439–1460.
- [41] N.W. Lukacs, Role of chemokines in the pathogenesis of asthma, *Nat. Rev. Immunol.* 1 (2001) 108–116.
- [42] Y. Kaneko, H. Masuko, T. Sakamoto, H. Iijima, T. Naito, Y. Yatagai, et al., Asthma phenotypes in Japanese adults - their associations with the CCL5 and ADRB2 genotypes, *Allergology Int. off. J. Jpn. Soc. Allergology* 62 (2013) 113–121.
- [43] K.R. Bartemes, H. Kita, Dynamic role of epithelium-derived cytokines in asthma, *Clin. Immunol.* 143 (2012) 222–235.
- [44] J.L. Barlow, R.J. Flynn, S.J. Ballantyne, A.N. McKenzie, Reciprocal expression of IL-25 and IL-17A is important for allergic airways hyperactivity, *Clin. Exp. allergy J. Br. Soc. Allergy Clin. Immunol.* 41 (2011) 1447–1455.
- [45] D. Kaur, E. Gomez, C. Doe, R. Berair, L. Woodman, R. Saunders, et al., IL-33 drives airway hyper-responsiveness through IL-13-mediated mast cell: airway smooth muscle cross-talk, *Allergy* 70 (2015) 556–567.
- [46] J. Deckers, F. Branco Madeira, H. Hammad, Innate immune cells in asthma, *Trends Immunol.* 34 (2013) 540–547.
- [47] J.L. Barlow, A.N. McKenzie, IL-25: a key requirement for the regulation of type-2 immunity, *BioFactors* 35 (2009) 178–182.
- [48] K.A. Verheijden, P. Akbari, L.E. Willemsen, A.D. Kraneveld, G. Folkerts, J. Garssen, et al., Inflammation-induced expression of the alarmin interleukin 33 can be suppressed by galacto-oligosaccharides, *Int. archives allergy Immunol.* 167 (2015) 127–136.
- [49] R.S. Adluri, D. Nagarajan, V. Periyaswamy, P.M. Venugopal, Dose-response effect of ferulic acid against nicotine-induced tissue damage and altered lipid levels in experimental rats: a pathohistological evaluation, *Fundam. Clin. Pharmacol.* 22 (2008) 557–567.
- [50] J. Qin, D. Chen, H. Hu, M. Qiao, X. Zhao, B. Chen, Body distribution of RGD-mediated liposome in brain-targeting drug delivery, *Yakugaku zasshi J. Pharm. Soc. Jpn.* 127 (2007) 1497–1501.
- [51] F.Q. Li, H. Su, J. Wang, J.Y. Liu, Q.G. Zhu, Y.B. Fei, et al., Preparation and characterization of sodium ferulate entrapped bovine serum albumin nanoparticles for liver targeting, *Int. J. Pharm.* 349 (2008) 274–282.