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From Cell Biology to Tissue Engineering

A plant proteinase inhibitor from *Enterolobium contortisiliquum* attenuates airway hyperresponsiveness, inflammation and remodeling in a mouse model of asthma

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Summary, Introduction. Proteinase inhibitors have been associated with anti-inflammatory and antioxidant activities and may represent a potential therapeutic treatment for asthma. Purpose. The aim of the present study was to evaluate the effects of Enterolobium contortisiliquum trypsin inhibitor (EcTI) on pulmonary mechanical function, eosinophilic recruitment, inflammatory cytokines, remodeling and oxidative stress in an experimental model of chronic allergic pulmonary inflammation. Methods. BALB/c mice were divided into 4 groups: C (saline i.p and inhalations with saline), OVA (ovalbumin i.p and inhalations with ovalbumin); C+EC (saline i.p, inhalations with s aline and treatment with EcTI); OVA+EC (ovalbumin i.p, inhalations with ovalbumin and treatment with EcTI). On day 29, we performed the following tests: resistance (Rrs) and elastance (Ers) of the respiratory system; (b) quantify eosinophils, 8-ISO-PGF2α, collagen and elastic fiber volume fractions; (c) IFN-γ, IL-4, IL-5, IL-13, MMP-9, TIMP-1, TGF-β, iNOS and p65-NFκB-positive cells in the airway and alveolar walls. Results. In OVA+EC group, there was an attenuation of the Rrs and Ers, reduction of eosinophils, IL-4, IL-5, IL-13, IFN-γ, iNOS and p65-NF κB-positive cells compared to OVA group. The 8-ISO-PGF2α, elastic and collagen fibers volume fractions as well as the positive cells for MMP-9, TIMP-1 and TGF-β positive cells were decreased in OVA+EC compared to the OVA group. Conclusion. EcTI attenuates bronchial hyperresponsiveness, inflammation, remodeling and oxidative stress activation in this experimental mouse model of asthma.

Key words: Asthma, Pulmonary inflammation, Plant proteinase inhibitor, *Enterolobium contortisiliquum Trypsin Inhibitor*

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Introduction

Asthma is a chronic inflammatory disease in which several inflammatory cells and mediators play a role. The care of asthma patients causes economic and psychosocial effects, and asthma is a serious worldwide public health problem (Pawankar, 2014). It is characterized by airway and alveolar wall inflammation, airflow limitation, hyper-reactivity and remodeling of the airways and alveolar walls (Righetti et al., 2014; GINA, 2017).

Asthma depends on the interaction of genetic and environmental factors featured by the activation of the

Th2 profile cells, eosinophils, mast cells, and neutrophils. Several mediators/modulators are involved in asthma pathogenesis and in hyperresponsiveness such asTh2 cytokines (IL-5, IL-4, and IL-13), leukotrienes and nitric oxide and others that were clearly evaluated in humans and animal models (Kudo et al., 2012; Pigati et al., 2015).

Airway inflammation, tissue injury, and subsequent abnormal repair lead to structural changes in the airway walls of asthmatic subjects collectively referred to as airway remodeling and that contribute to irreversibility of lung functions observed in some severe asthmatics (Phipps et al., 2004; Fattouh et al., 2008; Bergeron et al., 2010). Among structural changes can be noted subepithelial fibrosis, smooth muscle hypertrophy/hyperplasia, epithelial cell mucus metaplasia, and increased angiogenesis (Righetti et al., 2014; Pigati et al., 2015; GINA, 2017).

Corticosteroids are the gold standard for the treatment and control of asthma symptoms because of their powerful anti-inflammatory actions. However, the use of these drugs presents some problems, including heterogeneity in the responses of individual patients and steroid-resistant processes related to airway inflammation (GINA, 2017). Corticosteroids do not act directly on structural changes in the airways, so there is a growing search for new alternative therapeutic agents that better regulate the different processes involved in the pathophysiology of asthma (Fernandes et al., 2007; Royce et al., 2012).

Proteinase inhibitors can prevent inflammation and tissue injury and can subsequently promote a decrease in tissue remodeling (Shigetomi et al., 2010; Martins-Olivera et al., 2016; Theodoro-Júnior et al., 2017). Some proteinase inhibitors have been tested in asthma models, and their potential anti-inflammatory effects in airways have been demonstrated (Chen et al., 2006; Ishizaki et al., 2008; Lin et al., 2014). EcTI is a Kunitz-type proteinase inhibitor capable of inhibiting trypsin, chymotrypsin, plasma kallikrein, plasmin, human neutrophil elastase, and factor XIIa at stoichiometric ratios of 1:1 (Zhou et al., 2013). It is derived from the seeds of *Enterolobium contortisiliquum*, a native plant species of the *Leguminosae* family, subfamily Mimosoideae, that is found in South America (Batista et al., 2001). *EcTI* has been studied in human tumor cell lines, where it has been shown to block pro-metalloproteinase and FAK (focal adhesion kinase) (Nakahata et al., 2011; de Paula et al., 2013). In elastase models, EcTI showed control of several inflammatory and remodeling alterations (Theodoro-Júnior et al., 2017).

The present study aimed to evaluate the effects of *EcTI* on pulmonary mechanical function, eosinophilic recruitment, inflammatory cytokines, remodeling of the extracellular matrix and oxidative stress in an experimental model of chronic allergic pulmonary inflammation.

Materials and methods

Purification of EcTI

The purification of *EcTI* was performed as described previously (Batista et al., 1996; de Paula et al., 2012). The tegument of the seeds of *E. contortisiliquum* was removed manually, and the cotyledon was triturated in 0.05 M Tris/HCl buffer, pH 8.0, at a ratio of 1/40 (w/v). The material was centrifuged at 2254xg for 15 min at 4°C, and the supernatant was precipitated with 80% acetone (v/v). The protein precipitate was dried at room temperature and solubilized in 0.05 M Tris/HCl buffer, pH 8.0, and centrifuged under the same conditions as above. The supernatant was subjected to ion exchange chromatography on a DEAE Sepharose column previously equilibrated in 0.1 M Tris/HCl buffer, pH 8.0. The material not adsorbed in the column was washed out with the equilibration buffer. The adsorbed material was then removed with the equilibration buffer plus 0.15 M NaCl. Chromatography was performed with a flow rate of 30 ml/h to elute the proteins, followed by detection at 280 nm.

The fractions containing the *EcTI* were pooled and applied to a trypsin-Sepharose column and subsequently processed by molecular exclusion chromatography in a Superdex-75 10/300 (GE Healthcare) column on an Avant AKTA system (GE Healthcare). The column was equilibrated with 0.050 M Tris/HCl buffer, pH 8.0, at a flow rate of 0.5 ml/min, and 1 ml fractions were collected and the protein monitored as described below.

The inhibitor purity was confirmed by reverse phase chromatography in an Ultrasphere C18 protein/peptide column (0.46x15 cm) (Vydac - California, USA) on a Shimadzu HPLC system equilibrated with a 0.1% solution of trifluoroacetic acid (TFA) in water (v/v), and the samples were eluted in a linear gradient (0-100%) of 90% acetonitrile in 0.1% TFA at a flow rate of 0.7 ml/min.

The concentration of the inhibitor was determined using the Lowry assay, and the dose used (2 mg/kg) was the same as that previously used for the elastase inhibitor *BbCI* (*Bauhinia bauhinioides cruzipain inhibitor*), another proteinase inhibitor (Neuhof et al., 2003).

Homogeneity of the EcTI inhibitor

The chromatographic profile of the EcTI inhibitor is shown in Fig. 1. The main peak indicates that the preparation of the protein is homogenous and similar to preparations obtained in previous studies (de Paula et al., 2012).

Animals

Twenty-four young adult male pathogen-free BALB/c mice (6-7 weeks old) obtained from the University of São Paulo were used. The animals were

held in the laboratory vivarium for 2 weeks prior to the study. The animals were handled in accordance with the Guidelines for the Care of Laboratory Animals (NIH, 2002). The study was approved by the Ethics Committee for Analysis of Research Projects (CAPPesq) of the Clinical Hospital and of the Faculty of Medicine – University of Sao Paulo (protocol number: 187/12).

Experimental design

The animals were randomly divided into 4 groups based on the experimental protocol (Fig. 2), which lasted 29 days.

1. Control group (C): animals received saline (i.p.) and were inhaled with saline (n=6);
2. Ovalbumin group (OVA): animals received ovalbumin (i.p.) and were inhaled with ovalbumin (n=6);
3. Control group treated with *EcTI* (C+EC): animals received saline (i.p.), inhaled with saline and treated with *EcTI* (n=6);
4. Ovalbumin group treated with *EcTI* (OVA+EC): animals received ovalbumin (i.p.), inhaled with ovalbumin and treated with *EcTI* (n=6).

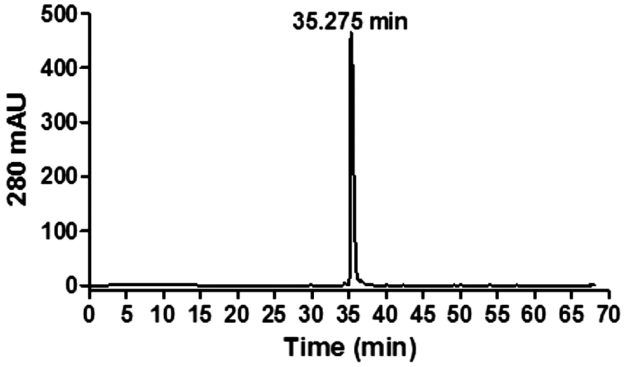

Fig. 1. Reverse phase chromatography. The EcTI obtained from molecular exclusion chromatography was further chromatographed on C18 protein/peptide. The column was equilibrated in 0.1% solution of TFA in water (v/v) (A) and samples eluted with a gradient of 90% acetonitrile in 0.1% TFA (B) under constant flow of 42 ml/h (t=0.1min, 5% B; t=5 min, 5% B; t=60 min 100% B; 68 min, 0% B).

Induction of chronic allergic pulmonary inflammation

Animals were immunized using 50 µg of OVA (OVA and OVA+EC groups), injected intraperitoneally (grade IV, Sigma Aldrich, St. Louis, MO), along with 6 mg of $Al(OH)_3$ adjuvant (Pepsamar, Sandei-Synthelabo SA, Rio de Janeiro, Brazil) diluted in 0.2 mL of saline, on days 1 and 14, as previously described (Arantes-Costa et al., 2008). Challenges of aerosolized OVA (1%) started one week after the second immunization, on days 22, 24, 26 and 28. For the challenge, mice were placed in a Plexiglas box (30x15x20 cm) coupled to an ultrasonic nebulizer (US-1000; ICEL, São Paulo, Brazil) and exposed to OVA (1%) for 30 min. Control mice (C and C+EC groups) received the saline intraperitoneally and were exposed to a nebulized aerosol of saline (0.9% NaCl) at the same time points (Fig. 2).

EcTI treatment

The mice received intraperitoneal (i.p.) injections (50 µL) of *EcTI* for seven consecutive days, starting two hours after the first inhalation exposure (day 22 to 28) to saline or ovalbumin. The dose was 2 mg/kg body weight and was diluted in saline (Theodoro-Júnior et al., 2017). The dose to administer to mice was the same as that used in an experimental model of emphysema (Theodoro-Júnior et al., 2017) and similar to the Bauhinia bauhinioides Kallikrein Inhibitor (BbKI) which is also a proteinase inhibitor used in the enphysema model (Martins-Oliveira et al., 2016). These authors showed this dose demonstrated therapeutic effects in the modulation of inflammation, extracellular matrix remodeling and oxidative stress.

Pulmonary mechanical function evaluation

Twenty-four hours after the last challenge, on day 29, the animals were anesthetized with sodium thiopental (70 mg/kg, intraperitoneally injected) and tracheotomized. A metal cannula was inserted through the hole of the tracheostomy and fixed with cotton thread around the trachea. The animals were mechanically ventilated (Harvard 687, Harvard Apparatus, Holliston, MA) in an acrylic plethysmograph

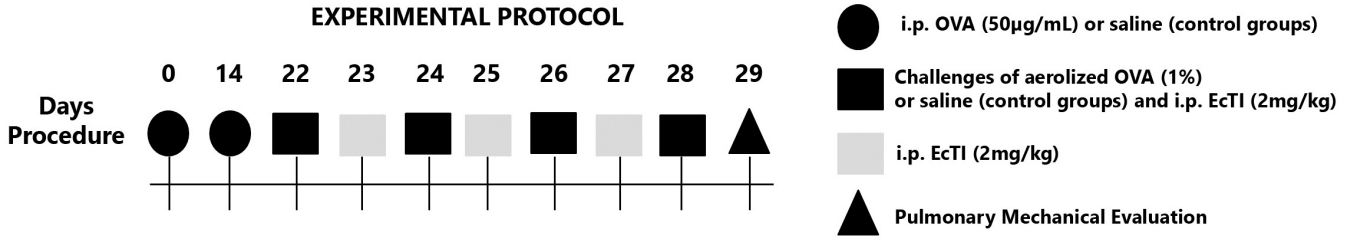

Fig. 2. Experimental protocol of induction of chronic allergic pulmonary inflammation and EcTI treatment.

(120 cycles per minute, 10 ml/kg). Data on tracheal pressure and lung volume were acquired through differential pressure transducers (Honeywell 163PC01D36, Freepot, IL) and converted with a digital analog board (DT01EZ, Data Translation, Marlboro, MA). The values of resistance and elastance of the respiratory system were calculated using the following equation for the motion of the respiratory system: Ptr(t)=Rrs.V'(t)+Ers.V(t). In this model, (t) is time, Ptr is tracheal pressure, Rrs is respiratory system resistance, Ers is respiratory system elastance, V' is airflow and V is lung volume. The values of Rrs and Ers were obtained at baseline and after administration of aerosolized methacholine (3, 30, and 300 mg/ml, for 1 min). We used the maximal response (300 mg/ml) of resistance (Rrs) and elastance (Ers) of respiratory system obtained after the methacholine challenge for the analysis (Arantes-Costa et al., 2008; Possa et al., 2012).

Hematoxylin-eosin, picro-sirius and resorcin-fuchsin staining

After pulmonary responsiveness measurements, mice were euthanized by exanguination the vena cava. The chest was opened, and the heart and lungs were removed *en bloc*. The lungs were fixed in a sufficient volume of formalin (4%) to completely immerse the tissue for 24 hours. After this period, the lung was dehydrated in a solution of 70% ethanol, xylol, and afterwards it was immersed in paraffin, and different regions of the lung were cut into slices to obtain 4-µm thick sections that were then mounted on slides. The slides were stained with hematoxylin-Eosin for analysis of eosinophils, Resorcin-Fuchsin for analysis of elastic fibers and with Picrosirius Red for analysis of collagen fibers.

To study the elastic fibers, sections were deparaffinized and hydrated in 95% alcohol and subsequently stained with Weigert's Resorcin-Fuchsin after oxidation. This was followed by two changes of 70% alcohol, each for 10 min. At this stage, the fibers were differentiated. They were then dehydrated, made transparent and mounted on slides. Picrosirius Red (Direct Red 80, C.I. 35780, Sigma Aldrich) staining was used to count the collagen fibers in the airway and in the alveolar walls. The sections were deparaffinized, rinsed with water and stained for 1 hour in picrosirius red at room temperature. Then, the slices were washed in running water for 5 min, and the sections were stained with Harris hematoxylin for 6 min and washed again in running water for 10 min (Possa et al., 2012).

Quantification of elastic fibers, collagen fibers and 8-ISO-PGF2α volume fractions

Optical density measurements were used for the analysis of elastic fibers, collagen fibers and 8-ISO-PGF2α. The images were acquired at a magnification of 400x using a Leica DM4000B microscope (Leica Microsystems, Wetzlar, Germany) and a digital camera (Leica DFC420 Leica Microsystems) connected to a computer. Ten airways and twenty fields of alveolar septa were photographed from each animal. The threshold color tones were selected and represented the positive areas. In each slide, the analyzed field was selected, and the software made the quantitative analysis of the volume fraction of positive cells for each field (ImageProPlus - Media Cybernetics, Bethesda, MD). The calculation was performed for each analyzed field. The area considered positive, according to the predetermined threshold color tones, was divided by the total area (Pinheiro et al., 2015). Volume fraction is the unit used to determine the positive area of lung tissue expressing for collagen fibers, elastic fibers and 8-ISO-PGF2α divided by the total tissue area. This method was used for airways and alveolar walls (Pinheiro et al., 2015; Camargo et al., 2018).

Immunohistochemistry

Additional slides were also prepared for immunohistochemical staining to determine the presence of antibodies. The slices were treated with Proteinase K for 20 min at 37°C followed by 20 min at room temperature, and washed with phosphate-buffered saline (PBS). Blocking of endogenous peroxidases was performed by incubation with 3% hydrogen peroxide (H_2O_2) 10 V (3x10 min). The sections of experimental

Table 1. Types of antibodies and dilutions used in the study.

Marker	Dilution	Primary antibody	Specification
IL-4	1:600	anti-goat goat	polyclonal; Santa Cruz Biotechnology, cod. SC-1260, Santa Cruz, CA, USA
IL-5	1:500	anti-rabbit rabbit	polyclonal; Santa Cruz Biotechnology, cod. SC-7887, Santa Cruz, CA, USA
IL-13	1:700	anti-goat goat	polyclonal; Santa Cruz Biotechnology, cod. SC-1776, Santa Cruz, CA, USA
IFN-γ	1:200	anti-rabbit rabbit	polyclonal; Santa Cruz Biotechnology, cod. SC-8308, Santa Cruz, CA, USA
MMP-9	1:500	anti-goat goat	polyclonal; Santa Cruz Biotechnology, cod. SC-6840, Santa Cruz, CA, USA
TIMP-1	1:200	anti-mouse mouse	monoclonal; lifeSpan BioScien, cod. MA5-13688, Inc, WA, USA
TGF-β	1:100	anti-rabbit goat	polyclonal; Santa Cruz Biotechnology, cod. SC-1836, Santa Cruz, CA, USA
iNOS	1:100	anti-mouse mouse	monoclonal; BD Transduction Lab, cod. N-32020, CA, USA
8-ISO-PGF2α	1:10000	anti-goat goat	polyclonal; Oxford Biomed. Resear, MI, USA
NF-κB	1:100	anti-rabbit rabbit	polyclonal; Santa Cruz Biotechnology, cod. SC-109, Santa Cruz, CA, USA

and control (positive and negative) tissue slides were incubated overnight with the indicated antibodies (Table 1). The following day, the slides were washed in PBS and incubated with a secondary antibody using ABCKit by Vectastain (Vector Elite-PK-6105 anti-goat), PK-6101 (anti-rabbit) and PK-6102 (anti-mouse). For visualization of positive cells, the slides were washed in PBS and proteins were visualized using 3,3'-diaminobenzidine chromosome (DAB) (Sigma Chemical Co., St. Louis, MO, USA). Slide sections were contrasted with Harris hematoxylin (Merck, Darmstadt, Germany) and assembled using Entellan microscopy resin (Merck) (Possa et al., 2012). The analyses were performed using the morphometric technique described below.

Morphometric analysis

Conventional morphometric analysis was performed with an optical microscope using a point-counting technique (Weibel, 1963; Osmanagic et al., 2010; Gonçalves et al., 2012; Camargo et al., 2018) with a reticle of 50 lines and 100 points for a total area of 10^4 μm^2. For the evaluation of the airways and alveolar walls, the reticle was oriented adjacent to the wall of the airway, in the bronchovascular axis, starting from the base of the epithelium or in the alveolar septa and the positive cells were determined by counting the number of positive cells divided by the number of points corresponding to the total area of tissue, under a magnification of 1000x.

Five airways were randomly selected from each animal, approximately three fields per airway. For the evaluation of the alveolar walls, ten fields were randomly selected. This technique was used to quantify eosinophils density and the number of IFN (interferon)-γ, IL (interleukin)-4, IL-5, IL-13, matrix metalloproteinases (MMP)-9, tissue inhibitor of metalloproteinase (TIMP)-1, Transforming growth factor (TGF)-β, inducible nitric oxide synthase (iNOS) and NF (nuclear factor)-ϰB-positive cells. The results are expressed as the number of cells per area (cells/10^4 μm^2). The positive cells are considered the inflammatory cells (polymorphonuclear and mononuclear cells) that showed reactions with the specific antibodies.

Passive cutaneous anaphylaxis test

Passive cutaneous anaphylaxis (PCA) tests were performed in Wistar rats to test for anti-IgE, and in Balb/c mice to test for anti-IgG1, as previously described (Mota and Perini, 1970). Seven days after the last inhalation of ovalbumin (as described above), the animals' backs were shaved and injected intradermally with different serum dilutions. Two and twenty-four hours after injection, for mice and rats, respectively, animals were challenged with 0.5 mg of OVA in 0.25% Evans Blue solution, i.v. The PCA titer was expressed as the reciprocal of the highest dilution that provoked an intradermic allergic reaction >5 mm in diameter in duplicate tests. The detection threshold of the technique was established at a 1:5 dilution (Possa et al., 2012; Righetti et al., 2014).

Data analysis

Statistical analysis was performed using the *SigmaStat* software (SPSS Inc, Chicago, IL). Multiple comparisons were made by *One-Way ANOVA*. For comparisons between groups, we used the *Holm-Sidak* test. Data are presented as the mean ± standard error. Bar graphs were made showing the standard errors. We also determined Pearson's correlation coefficients (R) to assess the associations of the Rrs and Ers scores with the markers for inflammation, remodeling and oxidative stress markers. Differences were considered significant at $P<0.05$.

Results

EcTI modulates airway hyperresponsiveness (AHR) to methacholine

Fig. 3A,B show the baseline response of resistance (Rrs) and elastance (Ers) of the respiratory system, respectively. There were no differences in Rrs and Ers among the experimental groups.

Fig. 3C,D show the dose response of Rrs and Ers, respectively. There was an increase in Rrs at doses of 3, 30 and 300 mg/ml of methacholine in the OVA group compared to the OVA-EC group and controls ($P<0.05$). In all experimental groups, the maximum response of Rrs and Ers was at the dose of 300 mg/ml methacholine.

Fig. 3E,F show the maximum response of Rrs and Ers after methacholine challenge (300 mg/ml), respectively. There was an increase in Rrs and Ers in the OVA group compared to the controls ($P<0.05$). There was a decrease in the maximal response Rrs and Ers in the OVA+EC group compared to the OVA group ($P<0.05$). There were no differences in the maximal responses of Rrs and Ers between the OVA+EC group and controls.

EcTI treatment of ovalbumin-sensitized animals attenuated the eosinophil density and the number of IL-4, IL-5, IL-13 and IFN-γ-positive cells

In OVA+EC group there was a reduction of the number of positive inflammatory cells. The absolute number of eosinophil number and of IL-4, IL-5, IL-13 and IFN-γ-positive cells in the airways and alveolar walls are shown in Table 2 and in Table 3, respectively, for all experimental groups. There was a significant increase in the expression of all cell types in the airways and alveolar walls in the OVA group compared to that in the controls ($P<0.05$) and a decrease of all cell types in the OVA+EC group compared to that of the OVA group ($P<0.05$).

After treatment with *EcTI*, the OVA+EC group

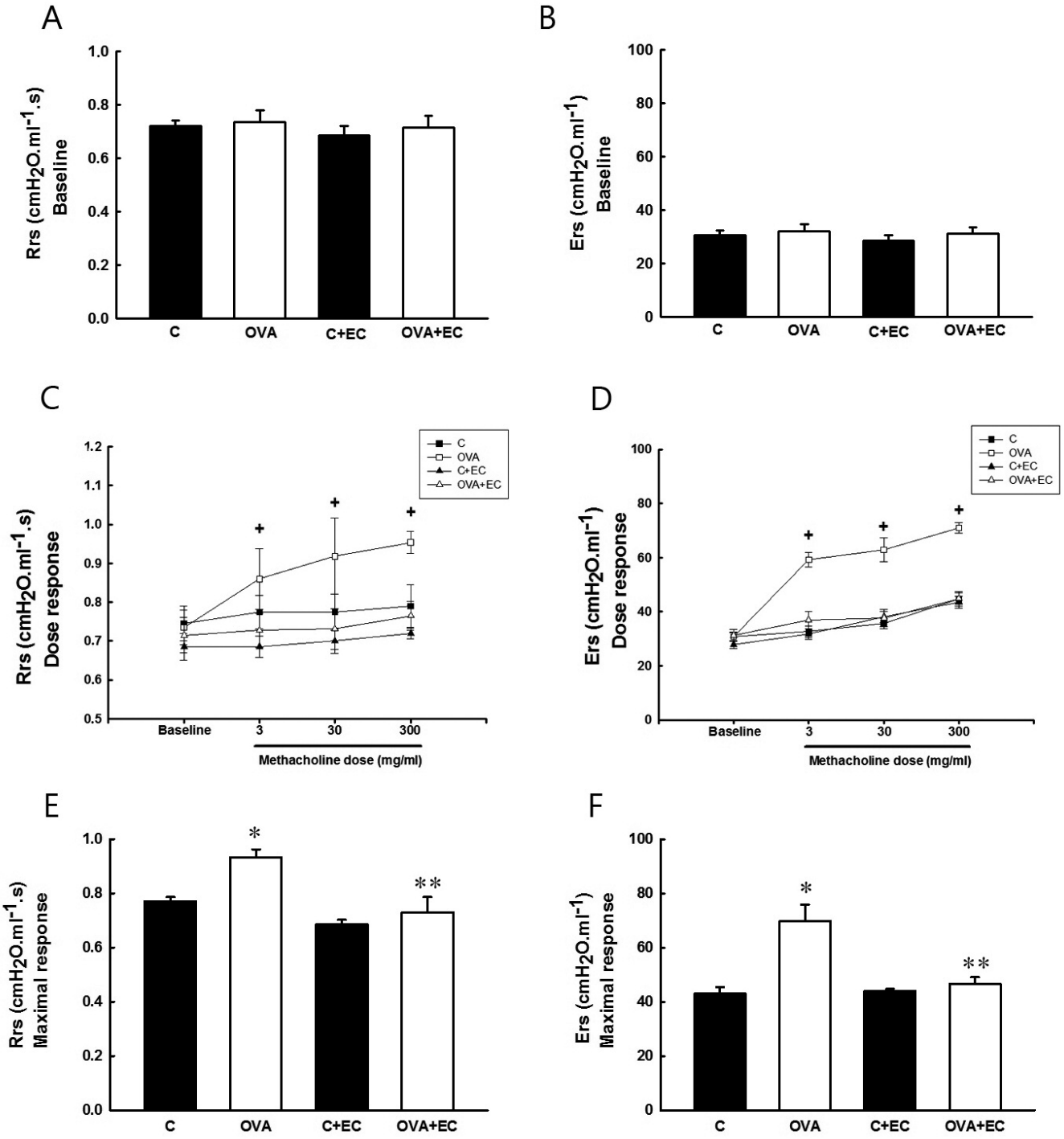

Fig. 3. A. Baseline response of Rrs: mean ± standard error values of the four experimental groups. **B.** Baseline response of Ers: mean ± standard error values of the four experimental groups. **C.** Metacholine dose response of Rrs. +P<0.05 compared to C, C+EC and OVA+EC groups. **D.** Metacholine dose response of Rrs. +P<0.05 compared to C, C+EC and OVA+EC groups. **E.** Maximum response of Rrs: mean ± standard error values of the four experimental groups. *P<0.05 compared to C and C+EC groups; **P<0.05 compared to OVA group. **F.** Maximum response of Ers: mean ± standard error values of the four experimental groups. *P<0.05 compared to C and C+EC groups; **P<0.05 compared to OVA group.

Table 2. Absolute values of the morphometric and measurement of optical density analysis for inflammatory, remodeling and oxidative stress markers in the airway.

	C	OVA	C+EC	OVA+EC
Inflammatory Markers				
Eosinophils (cells/10^4 μm^2)	0.64±0.28	18.69±1.50*	1.82±0.31	3.98±0.47**/*
IL-4 (cells/10^4 μm^2)	2.26±0.35	22.24±1.53*	1.90±0.22	3.17±0.54**
IL-5 (cells/10^4 μm^2)	3.12±0.40	22.61±0.70*	1.71±0.36	9.60±1.17**/*
IL-13 (cells/10^4 μm^2)	2.57±0.36	15.80±1.35*	1.42±0.25	6.09±0.35**/*
IFN-γ (cells/10^4 μm^2)	2.99±0.42	18.14±1.32*	1.68±0.25	5.95±0.46**/*
Remodeling Markers				
Colagen Fibers (%)	7.73±1.27	18.10±1.61*	5.26±0.69	13.70±0.93**
Elastic Fibers (%)	2.81±0.25	6.05±0.54*	3.27±0.29	2.91±0.20**
MMP-9 (cells/10^4 μm^2)	0.92±0.16	5.42±0.58*	0.55±0.12	1.28±0.24**
TIMP-1 (cells/10^4 μm^2)	1.63±0.31	10.32±0.86*	1.32±0.20	5.03±0.40**/*
TGF-β (cells/10^4 μm^2)	0.24±0.15	35.93±1.77*	0.83±0.20	9.09±0.61**/*
Oxidative Stress Markers				
iNOS (cells/10^4 μm^2)	0.90±0.19	9.70±0.68*	1.57±0.67	3.21±0.30**/*
8-ISO-PGF2α (%)	7.48±0.49	25.55±1.48*	7.37±0.73	8.99±0.48**/*

*P<0.05, compared with C and C+EC groups; **P<0.05, compared with the OVA group.

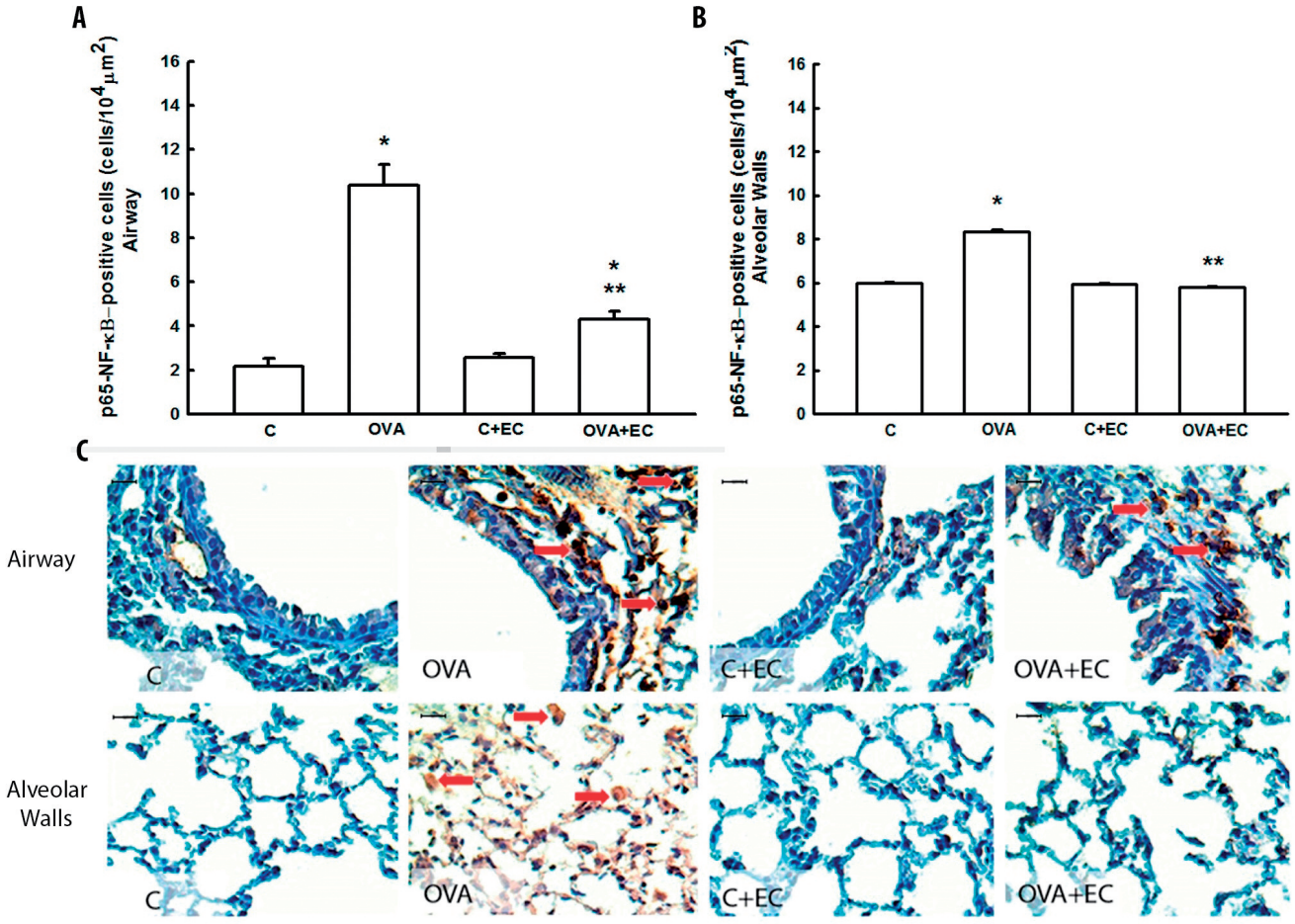

Fig. 4. p65-NF-κB in the lung: p65-NF-κB -possitive cells in the airway (**A**), alveolar walls (**B**) and photomicrographs of the results of the analyses of these processes in the airways and alveolar walls to detect p65-NF-κB (**C**). All the experimental groups are represented: C, OVA, C+EC and OVA+EC. *P<0.05 compared to C and C+EC groups; **P<0.05 compared to OVA group.

showed no difference from the C and C+EC groups in the number of IL-4-positive cells in the airways or of IL-13-positive cells in the alveolar walls (P<0.05). However, there was a difference between the OVA+EC group and the C and C+EC groups in the number of eosinophils and IL-5 -positive cells in the airway and alveolar walls as well as in the number of IL-13-positive cells in airway walls and the IL-4-positive cells in alveolar walls (P<0.05).

EcTI treatment of ovalbumin-sensitized animals reduced the elastic and collagen volume fractions and the expression of MMP-9, TIMP-1 and TGF-β-positive cells

In OVA+EC group we observed a reduction of all remodeling markers evaluated. The absolute values of the volume fraction of elastic and collagen fibers, and the number of MMP-9, TIMP-1, TGF-β-positive cells in the airway and alveolar walls are shown in Table 2 and in Table 3, respectively, for all experimental groups. There was an increase in the expression of all positive cells in the airway and alveolar walls in the OVA group compared with C and C+EC groups (P<0.05) and a decrease of those in the OVA+EC group compared with the OVA group (P<0.05).

After treatment with *EcTI*, the OVA+EC group showed no difference from the C and C+EC groups in the volume fraction of elastic fiber in the airways, or the volume fraction of collagen fibers in the airway and alveolar walls or the number of MMP-9-positive cells in the airways (P<0.05). However, there was a difference between the OVA+EC group and the C and C+EC

Table 3. Absolute values of the morphometric and measurement of optical density analysis for inflammatory, remodeling and oxidative stress markers in the alveolar walls.

	C	OVA	C+EC	OVA+EC
Inflammatory Markers				
Eosinophils (cells/10^4 μm^2)	0.34±0.21	5.67±0.60*	0.89±0.25	2.21±0.41**/*
IL-4 (cells/10^4 μm^2)	1.89±1.06	15.63±1.31*	1.10±0.52	6.93±0.76**/*
IL-5 (cells/10^4 μm^2)	0.71±0.43	12.37±0.40*	1.05±0.44	6.34±0.70**/*
IL-13 (cells/10^4 μm^2)	1.61±0.28	6.60±0.48*	1.63±0.34	2.76±0.36**
IFN-γ (cells/10^4 μm^2)	2.24±0.40	9.36±0.74*	1.49±0.36	5.71±0.36**/*
Remodeling Markers				
Colagen Fibers (%)	5.93±0.15	8.48±0.26*	5.97±0.14	5.72±0.18**
Elastic Fibers (%)	9.08±0.43	12.54±0.86*	9.01±0.46	10.70±0.41**/*
MMP-9 (cells/10^4 μm^2)	1.66±0.55	4.84±0.39*	1.65±0.31	3.16±0.31**/*
TIMP-1 (cells/10^4 μm^2)	1.22±0.35	5.71±0.96*	1.54±0.22	2.57±0.35**/*
TGF-β (cells/10^4 μm^2)	0.62±0.19	18.82±0.88*	2.17±0.28	5.89±0.46**/*
Oxidative Stress Markers				
iNOS (cells/10^4 μm^2)	2.11±0.45	10.39±0.76*	2.36±0.51	4.49±0.65**/*
8-ISO-PGF2α (%)	2.45±0.52	17.82±0.97*	2.73±0.59	8.24±0.88**/*

*P<0.05, compared with the C and C+EC groups; **P<0.05, compared with the OVA group.

Table 4. Pearson Correlation Rrs and Ers in relation to inflammatory, remodeling and oxidative stress markers in the airway walls.

	Rrs R value/ P value	Ers R value/ P value
Inflammation Markers		
Eosinophils	0.823/0.00102	0.964/0.000000428
IL-4	0.873/0.000208	0.968/0.000000266
IL-5	0.821/0.00106	0.898/0.0000728
IL-13	0.774/0.00312	0.823/0.000999
IFN-γ	0.900/0.0000663	0.962/0.000000603
NF-κB	0.681/0.0148	0.829/0.000858
Remodeling Markers		
Colagen Fibers	0.806/0.00156	0.749/0.00502
Elastic Fibers	0.709/0.00985	0.848/0.000495
TGF-β	0.764/0.00378	0.876/0.000186
MMP-9	0.718/0.00859	0.768/0.00354
TIMP-1	0.793/0.00209	0.871/0.000227
Oxidative Stress Markers		
iNOS	0.779/0.00285	0.843/0.000579
8-ISO-PGF2α	0.835/0.000724	0.958/0.000000992

Table 5. Pearson Correlation Rrs and Ers in relation to inflammatory, remodeling and oxidative stress markers in the alveolar walls.

	Rrs R value/ P value	Ers R value/ P value
Inflammatory Markers		
Eosinophils	0.826/ 0.000930	0.962/ 0.000000624
IL-4	0.804/ 0.00162	0.908/ 0.0000445
IL-5	0.798/ 0.00185	0.878/ 0.000175
IL-13	0.787/ 0.00238	0.882/ 0.000145
IFN-γ	0.738/ 0.00614	0.842/ 0.000598
NF-κB	0.712/ 0.00943	0.808/ 0.00148
Remodeling Markers		
Colagen Fibers	0.815/ 0.00125	0.934/ 0.00000898
Elastic Fibers	0.813/ 0.00129	0.857/ 0.000373
TGF-β	0.867/ 0.000260	0.960/ 0.000000742
MMP-9	0.776/0.00298	0.937/ 0.00000694
TIMP-1	0.852/ 0.000433	0.888/ 0.000115
Oxidative Stress Markers		
iNOS	0.878/ 0.000170	0.932/ 0.00000987
8-ISO-PGF2α	0.740/ 0.00590	0.864/ 0.000288

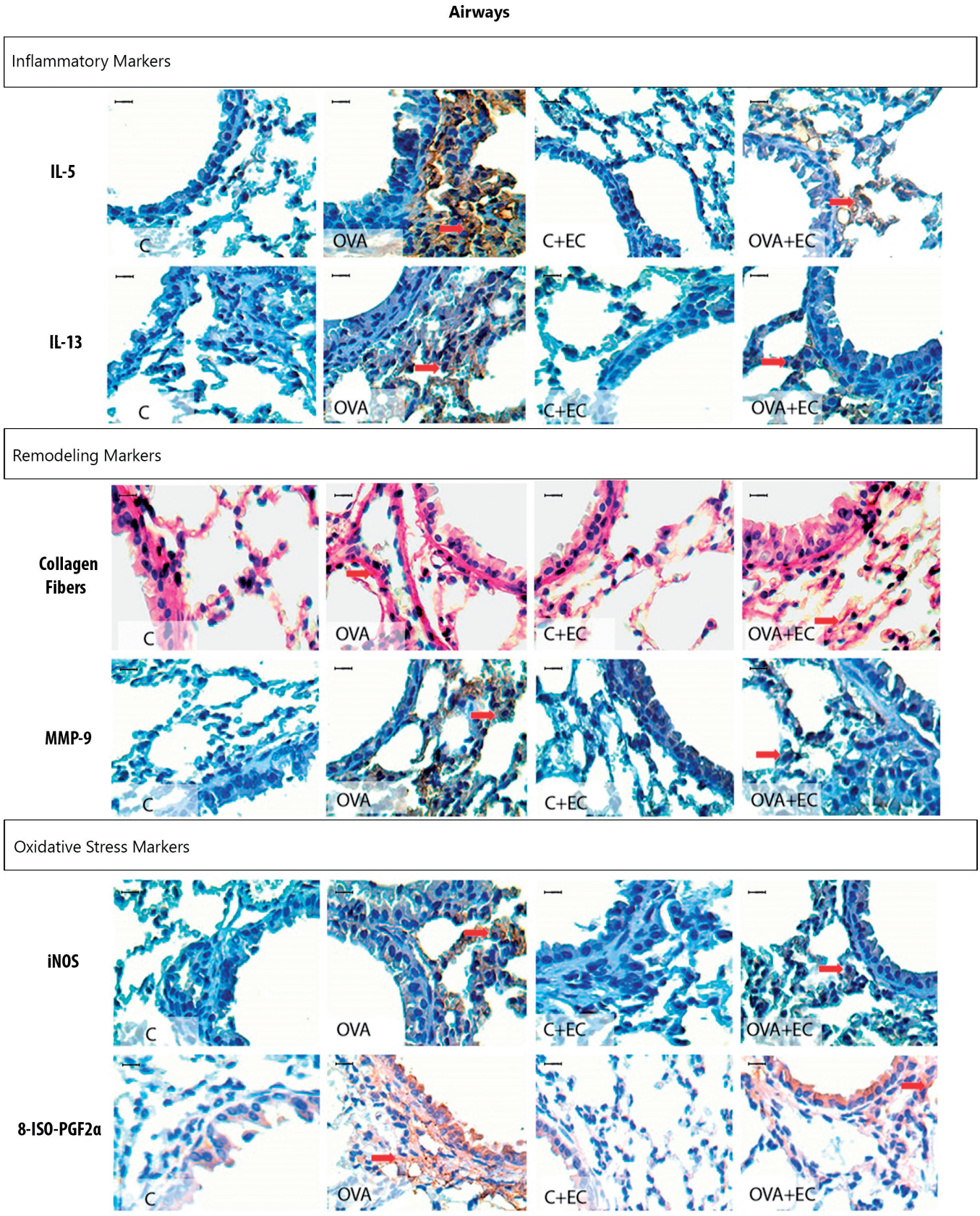

Fig. 5. Airway inflammatory, remodeling and oxidative stress markers: Photomicrographs of the results of the analyses of these processes in the airways to detect IL-5, IL-13, collagen fibers, MMP-9, iNOS-positive cells and 8-ISO-PGF2α. All the experimental groups are represented: C, OVA, C+EC and OVA+EC. Scale bars: 10 μm.

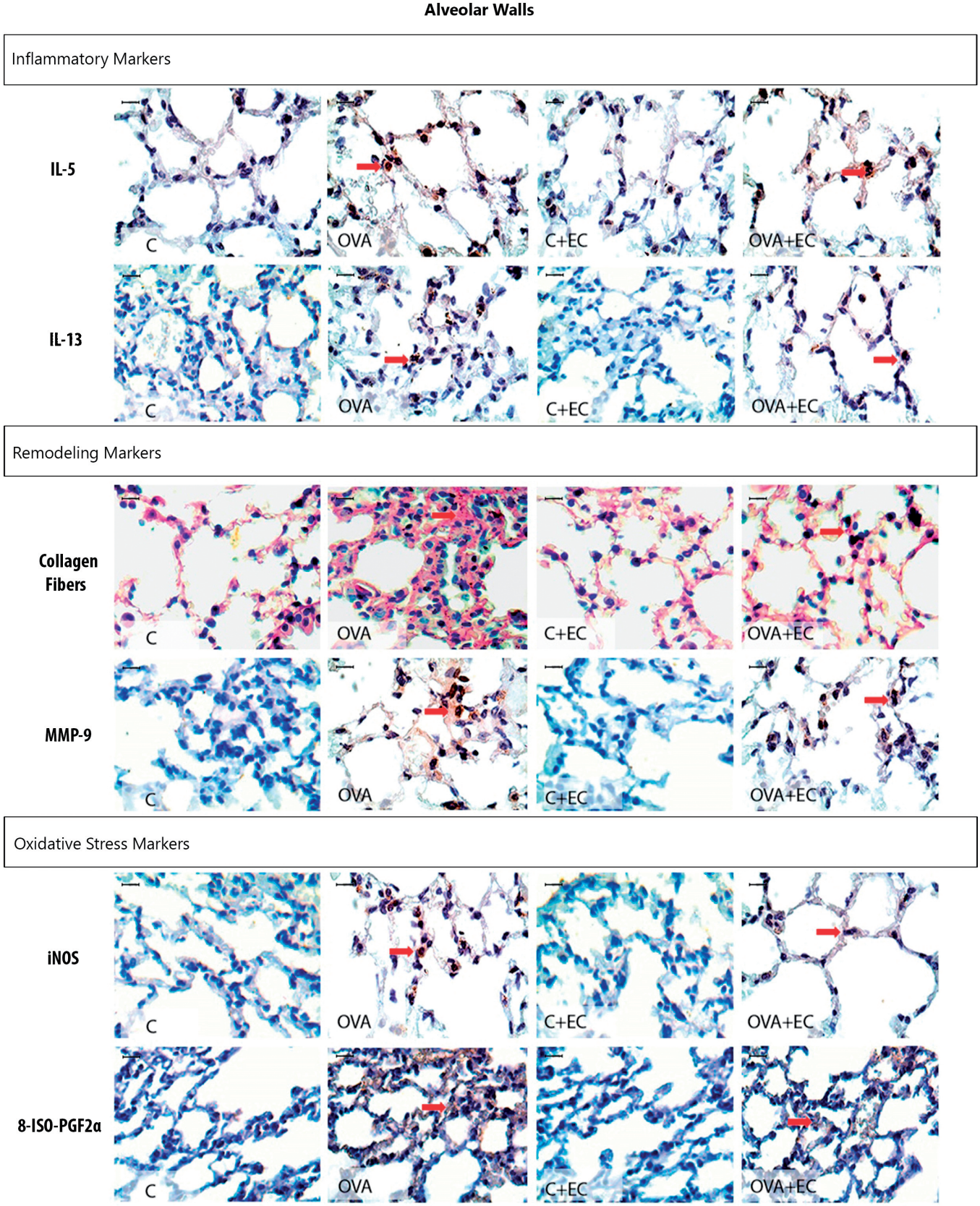

Fig. 6. Alveolar walls inflammatory, remodeling and oxidative stress markers. Photomicrographs of the results of the analyses of these processes in the alveolar walls to detect IL-5, IL-13, collagen fibers, MMP-9, iNOS-positive cells and 8-ISO-PGF2α. All the experimental groups are represented: C, OVA, C+EC and OVA+EC. Scale bars: 10 μm.

groups in the number of TIMP-1- and TGF-β-positive cells in the airway and alveolar walls and in the number of MMP-9-positive cells in the alveolar walls (P<0.05).

EcTI treatment of ovalbumin-sensitized animals reduced the number of iNOS-positive cells and the 8-iso-PGF-2α volume fraction

In OVA+EC group the oxidative stress markers were reduced. The absolute number of iNOS-positive cells and the 8-ISO-PGF2α volume fraction in airway and alveolar walls are shown in Table 2 and in Table 3, respectively, for all experimental groups. There was an increase in iNOS-positive cells and of the 8-ISO-PGF2α volume fraction in the OVA group compared with the controls (C and C+EC groups) (P<0.05). There was a decrease in these same parameters in the OVA+EC group compared to those in the OVA group (P<0.05).

After treatment with *EcTI*, the OVA+EC group showed no difference from the C and C+EC groups in the number of iNOS-positive cells or the volume fraction of 8-ISO-PGF2α in the airway (P<0.05). However, there was a difference between the OVA+EC group and C and C+EC groups in the number of iNOS-positive cells and the volume fraction of 8-ISO-PGF2α in the alveolar walls (P<0.05).

EcTI treatment of ovalbumin-sensitized animals on p65-NF-κB-positive cells

The absolute values of the NF-κB-positive cells in the airway and alveolar walls are shown in Fig. 4A,B, respectively, for all experimental groups. There was a significant increase in the NF-κB-positive cells in the airway and alveolar walls in the OVA group compared with C and C+EC groups (P<0.05) and a decrease of those in the OVA+EC group compared with OVA group (P<0.05). In Fig. 4C, we noted an increase in the number of NF-κB-positive cells expression in OVA group compared with C and C+EC in the airway and alveolar walls. A reduction in the positive cells was clearly observed in OVA+EC group compared with OVA group in the airway and alveolar walls.

Passive cutaneous anaphylaxis test

The results of the passive cutaneous anaphylaxis test showed an increase in the specific anaphylactic IgG1 and IgE antibody response in the ovalbumin sensitized animals (OVA group and OVA + EC group, maximum titer of 1:1280). No difference was observed after treatment with *EcTI*. As expected, there was no detection of IgG1 and IgE antibodies in C and C+EC groups.

Qualitative analysis of the EcTI response in the airway and alveolar walls

Microscopic analyses were performed at a magnification of 1000x to better highlight the differences between the groups. Representative photomicrographs of the inflammatory positive cells (stained for IL-5 and IL-13-positive cells), extracellular matrix remodeling (stained for collagen fibers volume fraction and MMP-9-positive cells) and oxidative stress (stained for iNOS-positive cells and the 8-ISO-PGF2α volume fraction) are shown in Fig. 5 for the airways and Fig. 6 for the alveolar walls. The OVA group showed important increases in these makers compared with those of the C, C+EC and OVA+EC groups. A reduction in the positive cells was clearly observed in OVA+EC group in the airway and alveolar walls compared with OVA group. All groups showed a similar anatomical composition of lung (alveolar septa) with approximately 90% of the tissue area composed of alveolar wall.

Correlation analysis

Correlation analysis of Rrs and Ers related to inflammation (eosinophils, IL-4, IL-5, IL-13, IFN-γ and NF-κB), remodeling (collagen and elastic fibers, TGF-β, MMP-9 and TIMP-1) and oxidative stress (iNOS and 8-ISO-PGF2α) in the airway walls is shown in Table 4, and for the alveolar walls, in Table 5. Significant correlations between all parameters were observed.

Discussion

In the present study, we evaluated the effect of a Kunitz-type proteinase inhibitor purified from the seeds of *Enterolobium contortisiliquum*, *EcTI*, on chronic allergic pulmonary inflammation in a mouse model of asthma. Ovalbumin-sensitized animals treated with *EcTI* showed an attenuation of pulmonary hyperresponsiveness, inflammation, oxidative stress and remodeling in their airway and alveolar walls.

Proteinase inhibitors have previously been studied in asthma mouse models, with promising results for their effects on airway inflammation (Chen et al., 2006; Ishizaki et al., 2008). *EcTI* and other proteinase inhibitors have been used in elastase animal models showing reduction of inflammation, remodeling and oxidative stress markers (Martins-Olivera et al., 2016; Theodoro-Júnior et al., 2017), but the effects of the trypsin inhibitor from *E. contortisiliquum* have not been clearly demonstrated in asthma. In the present study, we assessed the effects of EcTI treatment on chronic allergic pulmonary inflammation in a mouse model of asthma.

Airway hyperresponsiveness (AHR) is a characteristic feature of asthma, and chronic airway inflammation is thought to be responsible for this symptom (GINA, 2017). Methacholine challenge is used to document and quantify AHR (Pauwels et al., 1997; Leong and Huston, 2001). Ovalbumin-sensitized animals develop airway obstruction, and consequently have high levels of respiratory system resistance and elastance (Arantes-Costa et al., 2008). In the present study, treatment with *EcTI* decreased the Rrs by 21.5% and the Ers by 33% in OVA+EC group. This bronchodilatory

effect on AHR of *EcTI* treatment in ovalbumin-sensitized animals may be associated with the more than 50% reduction in the expression of all evaluated inflammatory markers and with the reduction in all remodeling markers. Lin et al. (2014) showed similar effects of serine protease inhibitors in an asthma model. In this case, the reduction in AHR was associated with a decrease in IL-5, IL-6 and IL-13 in the BALF (bronchoalveolar lavage fluid), which agrees with our findings.

IFN-γ in asthma is controversial; treatment with IFN-γ suppresses airway inflammation induced by subsequent allergen challenge in mice (Lack et al., 1996). However, Raundhal et al. (2015) suggest that severe asthma is characterized by high levels of the T helper 1 (Th1) and low levels of secretory leukocyte protease inhibitor (SLPI). This study shows that high IFN-γ levels in the airways promote AHR through the suppression of SLPI expression in bronchial epithelial cells and that this IFN-γ-mediated immune response differentiates severe asthma from mild–moderate asthma in both humans and mice. Corroborating with these results, our study showed that the reduction of IFN-γ-positive cells also showed a decrease of AHR. These findings are also found in animal models of asthma (Possa et al., 2012; Pigati et al., 2015; Camargo et al., 2018).

The endogenous basic pancreatic trypsin inhibitor (BPTI) mediates anti-inflammatory mechanisms via the protection of the high-affinity thrombin receptor, protease-activated receptor 1 (PAR-1). PAR-1 can be activated by trypsin, thrombin and matrix metalloproteinase-1, which leads to the production of various pro-inflammatory cytokines and chemokines (Shigetomi et al., 2010). In our study, the inhibition of trypsin by *EcTI* probably explains the observed reductions in the expression of IL-4, IL-5, IL-13 and IFN-γ by more than 50% in the airway and alveolar walls of ovalbumin-sensitized animals.

Eosinophils are a source of several molecules implicated in AHR and tissue remodeling processes, such as TGF-β, MMP-9, TIMP-1 and IL-13 (Wong et al., 1991; Okada et al., 1997; Levi-Schaffer et al., 1999; Schmid-Grendelmeier et al., 2002; Hogan, 2007; Cockcroft, 2010). TGF-β is a potent differentiation factor for the formation of myofibroblasts and has been shown to upregulate the expression by fibroblast of an array of ECM proteins (Fattouh et al., 2008). These same studies have shown that the reduction of eosinophils by anti-IL-5 treatment is associated with a significant decrease in the expression of ECM proteins, suggesting that eosinophil-derived TGF-β regulates airways remodeling (Flood-Page et al., 2003; Hogan, 2007; Fattouh and Jordana, 2008). In our study, *EcTI* treatment in ovalbumin-sensitized animals reduced TGF-β-positive cells in airway and alveolar walls by more than 68%, probably due to the reduction of inflammatory markers, which is in agreement with previous studies.

IL-5 regulates the growth, differentiation, activation and survival of eosinophils from the bone marrow into the lung following allergen exposure (Collins et al., 1995). Studies of anti-IL-5 treatments have demonstrated that IL-5 neutralization could block AHR and eosinophilia in response to antigen inhalation (Flood-Page et al., 2003). *EcTI* treatment in ovalbumin-sensitized animals reduced the number of IL-5-positive cells in airway and alveolar walls by more than 50%. This finding probably explains the reduction in eosinophilia and contributed to the decrease in AHR.

ECM remodeling refers to modifications in the normal composition and structural organization of the tissues, which usually occur in response to various mechanical and physiological forms of stress and can be observed in almost all tissues (Phipps et al., 2004; Vignola et al., 2003). In asthma, the ECM remodeling process involves collagen and elastic fiber deposition (Possa et al., 2012) and increases in MMPs, particularly MMP-9 (Flood-Page et al., 2003), TIMP-1 (Royce et al., 2012), TGF-β (Righetti et al., 2014) and other markers.

There was a significant reduction in the content of collagen and elastic fibers in ovalbumin-sensitized animals treated with *EcTI*, suggesting that the treatment affected ECM remodeling. There is evidence that an experimental model of chronic pulmonary inflammation is associated with an increased collagen and elastic fiber content in the airway and alveolar walls (Starling et al., 2009; Prado et al., 2011a,b; Camargo et al., 2018). These results are consistent with the idea that a process of degradation/turnover of collagen and elastic fibers in the lung is continuously occurring, and that this process is further induced by chronic inflammatory stimuli, as previously suggested by other authors (Possa et al., 2012; Flood-Page et al., 2003). Therefore, the *EcTI* appears to reduce remodeling by controlling inflammation.

In the present study, we observed an increase in MMP-9-positive cells in the airway and alveolar walls of ovalbumin-sensitized animals. The importance of the contribution of MMP-9 in the remodeling of asthmatic lung tissue has been demonstrated by studies with MMP-9-knockout mice. Sensitized knockout mice show significant reductions in total lung collagen fibers and eosinophil infiltration compared with mice that were only sensitized (Lim et al., 2006).

Theodoro-Júnior et al., (2017) showed in elastase treated mice that *EcTI* reduced MMP-9, TIMP-1 and other markers of remodeling. Nakahata et al., (2011) described the first evidence that the proteinase inhibitor *EcTI* was capable of blocking the activation of prometalloproteinases-9 (proMMP-9) based on the knowledge that MMP-9 is mainly expressed and secreted as zymogens requiring activation by serine or cysteine proteinases or by active MMPs (Shamamian et al., 2001). De Paula et al., (2012) demonstrated the effects of *EcTI* in inhibiting the formation of invadopodia through the integrin-mediated activation of Scr-FAK and decreasing the proteolytic activity and invasive potential of gastric cancer cells. FAK is a

nonreceptor-protein tyrosine kinase that is phosphorylated in response to Src transformation and has been shown to localize focal adhesion sites where the cells are attached to the ECM. FAK plays a critical role in the production of MMPs such as MMP-9 (Mon et al., 2006). In our model of asthma, *EcTI* likely acts as shown in previous studies and probably leads to a decrease in FAK activation, and consequently, a reduction in the production of MMP-9.

In the current study, we also observed that there was an augmentation of TIMP-1 in the airway and alveolar walls after ovalbumin exposure. TIMP-1 binds to MMP-9 and is the major inhibitor of MMP-9. It is believed that the balance between the levels of MMP-9 and TIMP-1 is another factor determining the degradation of the ECM (Royce et al., 2012).

The relationship between MMP-9 and TIMP-1levels in asthma is still being evaluated (Royce et al., 2012). Yao et al. (1999) showed that the MMP-9/TIMP-1 ratio and specific MMP-9 activity is increased in chronic allergic inflammation, while others have shown that a reduction in the MMP-9/TIMP ratio was associated with worsening airflow obstruction in chronic allergic inflammation and increased airway wall thickness, suggesting that lower MMP-9/TIMP-1 ratios may be associated with a profibrotic environment with reduced extracellular matrix turnover leading to extracellular matrix remodeling. We also emphasize that TIMP-1 also has been recognized as having biological effects independent of MMPs, such as inducing cellular apoptosis and plays an important role in the pathophysiology of asthma (Kelly and Jarjour, 2003; Lambert 2004; Camargo et al., 2018), explaining the increase of TIMP-1-positive cells in the OVA group in the present study. These findings are also found in animal models of asthma (Possa et al., 2012; Pigati et al., 2015; Camargo et al., 2018).

Treatment with *EcTI* in ovalbumin-sensitized animals reduced the number of MMP-9-positive cells by 76% in the airway walls and by 35% in the alveolar walls. It also reduced the number of TIMP-1-positive cells in the airway and alveolar walls by more than 47%. The reduction of TIMP-1 is probably due the reduction of MMP-9. Therefore, treatment with *EcTI* likely reduced ECM degradation because of decreased MMP-9 activation.

Another important pathogenic feature of asthma is the imbalance between oxidative stress and antioxidant capacity and the generation of reactive nitrogen and oxygen species, which are biomarkers of disease activity and effectors of disease progression. Nitric oxide (NO) and isoprostane are considered biomarkers of oxidative stress. The high levels of these markers increase the harmful effects on the lungs (Ricciardolo et al., 2004; Prado et al., 2011a,b; Voynow and Kummarapurugu, 2011; Righetti et al., 2014).

Inducible nitric oxide synthase (iNOS) can act in a variety of tissues and organs, and its expression is typically increased by cytokines and pro-inflammatory factors such as interferon gamma, TNF (tumor necrosis factor)-α, and IL1-β (Comhair and Erzurum, 2010; Voynow and Kummarapurugu, 2011). The NO derived from iNOS has a proinflammatory role in helping the recruitment of inflammatory cells to a site of injury. Another important role is that NO produced by inflammatory cells can contribute to the amplification of the inflammatory response (Prado et al., 2011a,b). The treatment with *EcTI* in ovalbumin-sensitized animals reduced the number of iNOS-positive cells in airway and alveolar walls by more than 56%, probably due to the reduction in the inflammatory markers. Our findings agree with the research of Molor-Erdene et al. (2005) that demonstrated the effects of another Kunitz inhibitor (bikunin) in a model of LPS-induced hypotension and showed that bikunin significantly inhibited both the increases in lung tissue levels of iNOS mRNA and iNOS activity.

These pro-inflammatory mediators can result in activation of intracellular nuclear factor-kappa B (NF-κB). The activation of NF-κB and their related signal pathways stimulates airway cell proliferation and production of cytokines, remodeling and mucin secretion (Schuliga, 2015). Subsequently, the NF-κB activation kinin receptor in the epithelium dysfunction results in AHR that are often seen in asthmatic and inflammatory airways (Ather et al., 2011). In the current study, we observed a reduction in NF-κB-positive cells. However the phosphorilation was unaffected. It is important to consider that there are several subunits of NF-κB, including p65-NF-κB (Ather et al., 2011). In the classical pathway, NF-κB/Rel proteins are bound and inhibited by IκB proteins. Proinflammatory cytokines, LPS, growth factors and atigen receptors activate IKK complex (IKKα, IKKβ, and NEMO), wich phosphorylates IκB proteins (Ather et al., 2011; Tang et al., 2018a,b). Phosphorylation of IκB leads to its ubiquitination and proteasomal degradation, freeing NF-κB/Rel complexes. It was previously described that Runt-related Transcription Factor 1 (RUNX1) is highly expressed in pulmonary epithelial cells and modulates NF-κB due to inhibition of IκB Kinase β pathway (Tang et al., 2018a,b). One hypothesis to explain our results was that IκB kinase β pathway was not modulated by *EcTI*. In our study, we assessed the expression of p65-NF-κB in inflammatory cells in airway and alveolar walls. The present study in fact showed that the effect of this dose of *EcTI* treatment controlling pulmonary inflammation, remodeling and oxidative stress pathway activation depended on NF-κB activation.

NO could also be involved in the increase in the resistance and elastance in lung mechanic function through the promotion of peroxynitrite production (Prado et al., 2011a,b). This agent is formed by the interaction of NO and superoxide, which leads to lipid peroxidation and isoprostane generation. Isoprostane is a prostaglandin-like compound derived from the free radical peroxidation of polyunsaturated fatty acids in tissues. 8-ISO-PGF2α is considered the predominant

form of isoprostane generated during free radical attacks on cell membranes in asthma (Montuschi et al., 1999; Voynow and Kummarapurugu, 2011). Isoprostanes regulate human airway smooth muscle (ASM) tone and hyperresponsiveness to stimuli and also regulate the chemokine production in ASM cells that contributes to the inflammatory cascade (Possa et al., 2012). Starling et al. (2009), showed that there was a significant decrease in 8-ISO-PGF2α density in lung tissue from guinea pigs that were repeatedly exposed to ovalbumin and treated with 1400W, a specific and highly selective iNOS inhibitor. In our study, the decrease of more than 53% in the volume fraction of isoprostane in the airway and alveolar walls was probably due to the reduction in the number of iNOS-positive cells and also may have contributed to the modulation of AHR in ovalbumin-sensitized animals treated with *EcTI*.

8-ISO-PGF2α is also increased following eosinophilic inflammation, allergen challenge, bronchitis and exercise-induced bronchospasms. Elevated levels of 8-ISO-PGF2α may directly reflect the exacerbation of inflammation associated with asthma due to infections or allergens (Voynow and Kummarapurugu, 2011). In the present study, the decrease in the volume fraction of isoprostane in ovalbumin-sensitized animals treated with *EcTI* may have also been due to the reduction in all evaluated inflammatory markers as well as the reduction in the iNOS-positive cells.

The reduction in the oxidative stress pathway caused by treatment with *EcTI* could also have contributed to the attenuation of ECM remodeling. Prado et al., (2011a,b), using another model of asthma, showed that iNOS inhibition reduced the number of MMP-9, TIMP-1 and TGF-β-positive cells. These mediators act in the production of collagen and elastic fibers, thus contributing to ECM remodeling. Theses findings are in agreement with ours.

In summary, we demonstrated that inflammatory markers were reduced by the action of *EcTI* and, as a consequence of this reduction, we found a decrease in remodeling and in the levels of oxidative stress markers.

Several studies have reported the importance of IgE and IgG1 in the pathophysiology of asthma (Holgate, 2008). The release of IgE by mast cell activation seems to contribute to the maintenance of Th2 responses (Galli et al., 2008). The results reported in the present study demonstrate the presence of these antibodies in animals sensitized with ovalbumin through the use of the PCA technique. There was no effect of EcTI in the titers of IgG1 and IgE. This result was expected since treatment with EcTI began after the sensitization process.

The present study had some limitations. *EcTI* was tested in a model of chronic allergic pulmonary inflammation, but we did not test its toxicity. This proteinase inhibitor comes from plant origin and is similar to compounds that are a part of normal human biochemistry. After our therapeutic intervention with *EcTI* was performed, we did not observe any differences between the OVA and OVA+EC groups in parameters such as hair loss, weight loss or death. In addition to, and because of this limitation, we cannot extrapolate our findings directly to human beings. In addition, we only tested one dose of EcTI. Perhaps different doses could contribute to the control of NF-κB activation, but we could not exclude the possibility that other controlling pathways may be involved in the EcTI responses observed in the present study. Future research is needed for the elucidation of all mechanisms involved. In conclusion, *EcTI* treatment contributes to improvements in airway hyperresponsiveness (AHR) to methacholine, inflammation, extracellular matrix remodeling processes and oxidative stress activation, suggesting that *EcTI* might constitute a potential pharmacological tool for controlling lung alterations due to asthma.

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