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ORIGINAL ARTICLE



Cysteinyl leukotriene induces eosinophil extracellular trap formation via cysteinyl leukotriene 1 receptor in a murine model of asthma

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ABSTRACT

Purpose: Eosinophils are one of the main cells responsible to the inflammatory response in asthma by the release of inflammatory molecules such as cytokines, reactive oxygen species (ROS), cytotoxic granule, eosinophil extracellular trap (EET), and lipid mediators as cysteinyl leukotriene (cysLT). The interconnections between these molecules are not fully understood. Here, we attempted to investigate the cysLT participation in the mechanisms of EET formation in an asthma model of OVA challenge.

Materials and methods: Before intranasal challenge with OVA, BALB/cJ mice were treated with a 5-lipoxygenase-activating protein (FLAP) inhibitor (MK-886), or with a cysLT1 receptor antagonist (MK-571) and the lung and bronchoalveolar lavage fluid (BALF) were analyzed.

Results: We showed that OVA-challenged mice treated with MK-886 or MK-571 had a decrease in inflammatory cells, goblet cells hyperplasia, and eosinophil peroxidase (EPO) activity in the airway. However, only OVA-challenged mice treated with MK-571 had an improvement in lung function. Also, treatments with MK-886 or MK-571 decreased Th2 cytokines levels in the airway. Moreover, we observed that OVA-challenged mice treated with MK-886 or MK-571 had a decrease in EET formation in BALF. We also verified that EET release was not due to cell death because the cell viability remained the same among the groups.

Conclusion: We revealed that the decrease in cysLT production or cysLT1 receptor inhibition by MK-886 or/and MK-571 treatments, respectively reduced EET formation in BALF, showing that cysLT regulates the activation process of EET release in asthma.

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KEYWORDS

Asthma; cysteinyl leukotriene; eosinophil extracellular trap; MK-886; MK-571

Introduction

Asthma is a chronic inflammatory pulmonary disease with high morbidity and mortality worldwide. Pathophysiologic features of asthma include chronic inflammation in the airway, airway hyperresponsiveness (AHR), mucus overproduction, and variable obstruction associated with narrowing of the airway.¹ In asthma, there is plasma extravasation, edema, and a high influx of inflammatory cells such as eosinophils, neutrophils, lymphocytes, macrophages, dendritic cells, and mast cells.² However, eosinophils are key cells involved in the pathophysiology of asthma which increases lung dysfunction. In addition,

eosinophils come equipped with preformed enzymatic and nonenzymatic cationic proteins, stored in and selectively secreted from their large secondary (specific) granules, such as eosinophil peroxidase (EPO). These proteins contribute to the functions of the eosinophil in airway inflammation and tissue damage.³

Therapeutic strategies to reduce eosinophilic inflammation in the lung have been related to a decrease in asthma symptoms.⁴ Eosinophils are able to release inflammatory cytokines, reactive oxygen species (ROS), cytotoxic granule, eosinophil extracellular trap (EET), and lipid mediators such as leukotrienes.^{5–8} Leukotrienes are synthesized from arachidonic acid through the 5-

lipoxygenase pathway and play multiple roles in inflammatory disorders, such as asthma.^{9,10} There are two classes of leukotrienes, leukotriene B₄ (LTB₄) and cysteinyl leukotriene (cysLT). The LTB₄ is involved in neutrophilic inflammation^{11,12}; and cysLT induce eosinophilic inflammation.¹³ The stimulation of leukocytes by allergens induces the liberation of arachidonic acid from the phospholipids membrane by phospholipase A₂. The 5-lipoxygenase, with the aid of the 5-lipoxygenase-activating protein (FLAP), catalyzes the conversion of arachidonic acid to 5-hydroperoxyeicosatetraenoic acid (5-HPETE) followed by the generation of leukotriene A₄ (LTA₄). The LTA₄ can be metabolized by LTA₄ hydrolase into LTB₄ or it can be conjugated to glutathione by leukotriene C₄ synthase (LTC₄S) to LTC₄, which may then be sequentially metabolized into leukotriene D₄ (LTD₄) and, finally, in leukotriene E₄ (LTE₄).⁹ The LTC₄, LTD₄, and LTE₄ are cysLTs due to the presence of cysteine in their structure.¹⁴ The major cellular sources of cysLT production are eosinophils.¹³ In eosinophils, synthesis of cysLT occurs in perinuclear membranes and lipid bodies in the cytoplasm. There are three types of cysLT receptors: cysteinyl leukotriene receptor 1 (cysLT₁), cysteinyl leukotriene receptor 2 (cysLT₂), and cysteinyl leukotriene receptor 3 (cysLT₃).¹⁵ The CysLT₁ receptor is expressed on airway smooth muscle cells, eosinophils, B cells, mast cells, monocytes, and macrophages.¹⁶ Activation of cysLT₁ receptor induces bronchoconstriction, mucus secretion, and airway edema in asthma.¹⁷ Moreover, there are two main therapeutic strategies to antagonize cysLT in patients with asthma. The first strategy is decreasing cysLT production by inhibiting 5-lipoxygenase or FLAP.¹⁸ The second strategy is the inhibition of cysLT₁ receptor with antagonists leading to the reduction of cysLT binding on target cells.¹⁸ Thus, cysLT plays an important role in asthma pathophysiology. However, cysLT participation in the mechanism of eosinophil extracellular trap (EET) formation in asthma is not completely understood.

Eosinophil extracellular traps (EETs) are formed by extracellular DNA and intact eosinophil granules.^{19,20} Stimulated eosinophils rapidly

release extracellular DNA fibers decorated with eosinophil granules forming EET that extracellularly bind and kill pathogens before they reach host cells.^{19,21} Furthermore, EET formation plays an important role in inflammatory respiratory diseases.^{6,7,21} Studies have shown EET formation in bronchial biopsy from asthmatic patients and airway from murine asthma models.^{6,7} EET formation also may contribute to the increase of the immunopathology of asthma. Studies have observed that EET release increases mucus viscosity and airway damage in asthma.^{22,23} However, mechanisms of EET formation in asthma are poorly understood. Previous studies of our group have demonstrated that ROS and autophagy regulate the process of extracellular traps released by eosinophils in asthma.^{24,25} Furthermore, studies have demonstrated that lipid mediators regulate the release of neutrophil extracellular trap (NET).^{26–29} Although EET and lipid mediators, such as cysLT play an key role in the increase of lung inflammation in asthma; there is no evidence of cysLT participation in mechanisms of EET formation in asthma.

Here, we analyzed the outcome of a FLAP inhibitor, MK-886, and a cysLT₁ receptor antagonist, MK-571, in EET formation in an asthma model of OVA challenge. We have chosen MK-886 and MK-571 because they are the two main therapeutic strategies to antagonize cysLT in asthma.¹⁸ Our study hypothesized that a decrease in cysLT production or cysLT₁ receptor inhibition may decrease EET formation. Therefore, the aim of this study was to investigate cysLT participation in mechanisms of EET formation in asthma.

Material and methods

Animals

We used 6–8 weeks old specific-pathogen-free (SPF) female BALB/cJ mice weighing approximately 20 g from the Center for Experimental Biological Models (CeMBE, PUCRS, Porto Alegre, Brazil). This asthma model used was standardized with female animals. The animals were fed standard ration for rodents and water *ad libitum*. The animals were housed in cages,

maintained on a 12-hour light/dark cycle, and controlled temperature ($21 \pm 1^\circ\text{C}$). All experiments followed the Guiding Principles in the Care and Use of Animals approved by the Council of the American Physiological Society. The experimental protocol was approved by Pontifícia Universidade Católica do Rio Grande do Sul Animal Ethics Committee (#14/00387).

Experimental asthma model and treatments with MK-886 or MK-571

Mice were sensitized subcutaneously with $20\ \mu\text{g}$ of OVA (Grade V, Sigma, Missouri, USA) without adjuvant diluted in $200\ \mu\text{L}$ of dulbecco's phosphate-buffered saline (DPBS; Gibco, Massachusetts, USA), on days 0 and 7. Afterward, mice were challenged intranasally with $100\ \mu\text{g}$ of OVA without adjuvant diluted in $50\ \mu\text{L}$ of DPBS, on days 14, 15, and 16.^{22,30} The negative control group received subcutaneous and intranasal DPBS. The treatments were performed 1 hour before each one of three intranasal challenges, with intranasal administration of $1\ \text{mg/kg}$ of MK-886 (Cayman Chemical, Michigan, USA) or $5\ \text{mg/kg}$ of MK-571 (Cayman Chemical, Michigan, USA) diluted in $50\ \mu\text{L}$ of DPBS.^{31,32}

Total and differential cell counts in BALF

Twenty-four hours after the last challenge with OVA, the animals were anesthetized with ketamine ($0.4\ \text{mg/g}$) and xylazine ($0.2\ \text{mg/g}$), and bronchoalveolar lavage fluid (BALF) was collected with $1\ \text{mL}$ of phosphate-buffered saline (PBS) and 2% fetal bovine serum (FBS). The BALF was centrifuged, and the pellet was resuspended in PBS with 2% FBS for performing total and differential cell counts. The total cell counts were determined in the Neubauer chamber (BOECO, Hamburg, Germany). Differential cell counts were done by cytopspin preparations (Eppendorf, Wesseling, Germany) and the section was stained with hematoxylin and eosin (H&E; Newprov, Paraná, Brazil). Four hundred cells were counted under an Olympus BX43 Microscope (Olympus, Tokyo, Japan).

Analysis of the EPO activity in BALF

EPO activity was determined by the colorimetric assay according to Strath and colleagues (1985). Briefly, the BALF supernatants were incubated with working reagent ($0.1\ \text{mM}$ O-phenylenediamine (OPD; Sigma, Missouri, USA), $0.05\ \text{M}$ Tris, Triton X-100, and $1\ \text{mM}$ hydrogen peroxide, pH 8.0). After that, the reaction was stopped with sulfuric acid $1.0\ \text{M}$. The absorbance was measured at a wavelength of $492\ \text{nm}$ in a microplate reader (Biochrom).

Lung histology

Lungs were fixed in 10% buffered formalin. Tissue was embedded in paraffin blocks and cut into $5\ \mu\text{m}$ sections. To evaluate the inflammatory infiltrate, sections were stained with H&E staining. For evaluating the extent of peribronchial and perivascular inflammatory infiltrate in μm , 10 measurements were performed starting from the beginning of the bronchial or vessel epithelium to the end of the inflammatory infiltrate using Olympus CellSens Standard software (Olympus, Tokyo, Japan).³⁰ Goblet cells hyperplasia and mucus production were evaluated by alcian blue dye. Images were taken in an Olympus BX43 Microscope (Olympus, Tokyo, Japan).

Assessment of respiratory system mechanics

Respiratory system mechanics testing was performed after cannulation of the trachea and the animals were connected to a ventilator (flexiVent, SCIREQ, Montreal, Canada). Three measurements of forced oscillation technique (FOT) were performed during a pause of the respirator (3 seconds). During the ventilation pauses, a frequency oscillatory signal ($4\text{--}38\ \text{Hz}$) was generated by a loudspeaker and passed through the tracheal cannula of the animal. A four-parameter model of tissue impedance with constant phase was fitted to Zrs data to obtain Newtonian resistance (R_n) measurements, which is equivalent to airway resistance in mice due to compliance of the chest wall. Tissue damping (G) represents the resistance of small airways where air movement occurs mainly by diffusion while that tissue elastance

(H) reflects the energy conservation in the alveolus. The data were analyzed in a specific software (flexiWare, SCIREQ, Montreal, Canada), where airway resistance and elastic properties (viscosity and elasticity) of the lung were measured through a pulmonary impedance.^{33,34}

Cytokine levels in BALF

Interleukin (IL)-5, interferon (IFN)- γ , and IL-10 levels were measured in BALF supernatants by a multiplex assay using a ProcartaPlex Multiplex immunoassay (Thermo Fisher Scientific, Massachusetts, USA) according to the manufacturer's recommendations. Results were analyzed using xPONENT® Software Solutions (Luminex Corporation, Texas, USA). The IL-4 levels were analyzed using a sandwich enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (Thermo Fisher Scientific, Massachusetts, USA).

Quantification of extracellular DNA traps and immunofluorescence in BALF eosinophils

Extracellular DNA concentration was measured in BALF supernatant using the Quant-iT dsDNA HS kit (Invitrogen, Carlsbad, USA) and measured in the Qubit 2.0 fluorometer (Invitrogen, Carlsbad, USA), according to the manufacturer's recommendations. To visualize the EET formation by immunofluorescence microscopy, BALF eosinophils ($2 \times 10^5/\text{mL}$) were plated in 8-chamber culture slides (BD Falcon, New Jersey, USA) and incubated at 37°C with 5% carbon dioxide for 1 hour. Afterward, cells were fixed with 4% paraformaldehyde (PFA) for 45 minutes and incubated with the primary antibody, anti-EPO (1:250, Santa Cruz Biotechnology, Dallas, USA) for 45 minutes. After this time, cells were incubated for 30 minutes with the secondary antibody, FITC anti-goat (1:100, Santa Cruz Biotechnology, Dallas, USA). Finally, the cells were stained with Hoechst 33342 DNA dye (1: 2000, Invitrogen, Carlsbad, USA) for 4 minutes. Images were performed in Zeiss LSM 5 Exciter confocal microscope (Zeiss LSM5, Oberkochen, Germany).

Analysis of cell death in BALF eosinophils

BALF eosinophils were analyzed for apoptosis by annexin V and necrosis by propidium Iodide (PI), according to the manufacturer's instructions (BD Pharmingen). Cells were incubated with annexin V and PI and analyzed by flow cytometry (FACS Canto II, BD Bioscience New Jersey, USA). Data were analyzed using FlowJo software version X.0.7 (TreeStar, Oregon, USA).

CysLT levels in lung

Lungs were homogenized in PBS (1:5 w/v) and cysLTs (LTC4/LTD4/LTE4) levels were measured using an ELISA Kit according to the manufacturer's instructions (Enzo Life Sciences, New York, USA). The lower limits of detection were 26.6 pg/mL. The cysLTs levels were corrected for protein levels in tissue.

Protein levels determination in lung

The protein contents in lungs were determined using the Qubit™ Protein Assay Kit (Invitrogen, Carlsbad, USA).

Statistical analysis

Data were expressed as mean $\pm$ standard deviation. The Shapiro-Wilk normality test was used to evaluate the normal distribution of the data. We used ANOVA followed by Tukey post-hoc and the adopted significance level was $p \leq 0.05$. Statistical analysis and graphs were performed using the Graphpad Prism Software, version 5 (GraphPad Software, San Diego, USA).

Results

Treatments with MK-886 or MK-571 decrease inflammatory cells, EPO activity, and goblet cells hyperplasia in airway

We observed that OVA-challenged mice had a significant increase in the total cell counts, as well as an increase in eosinophils, neutrophils, macrophages, and lymphocytes counts when compared to the control group (Figure 1A–E). Firstly, we verified the ability of MK-886 and MK-571 treatments in decreasing pulmonary

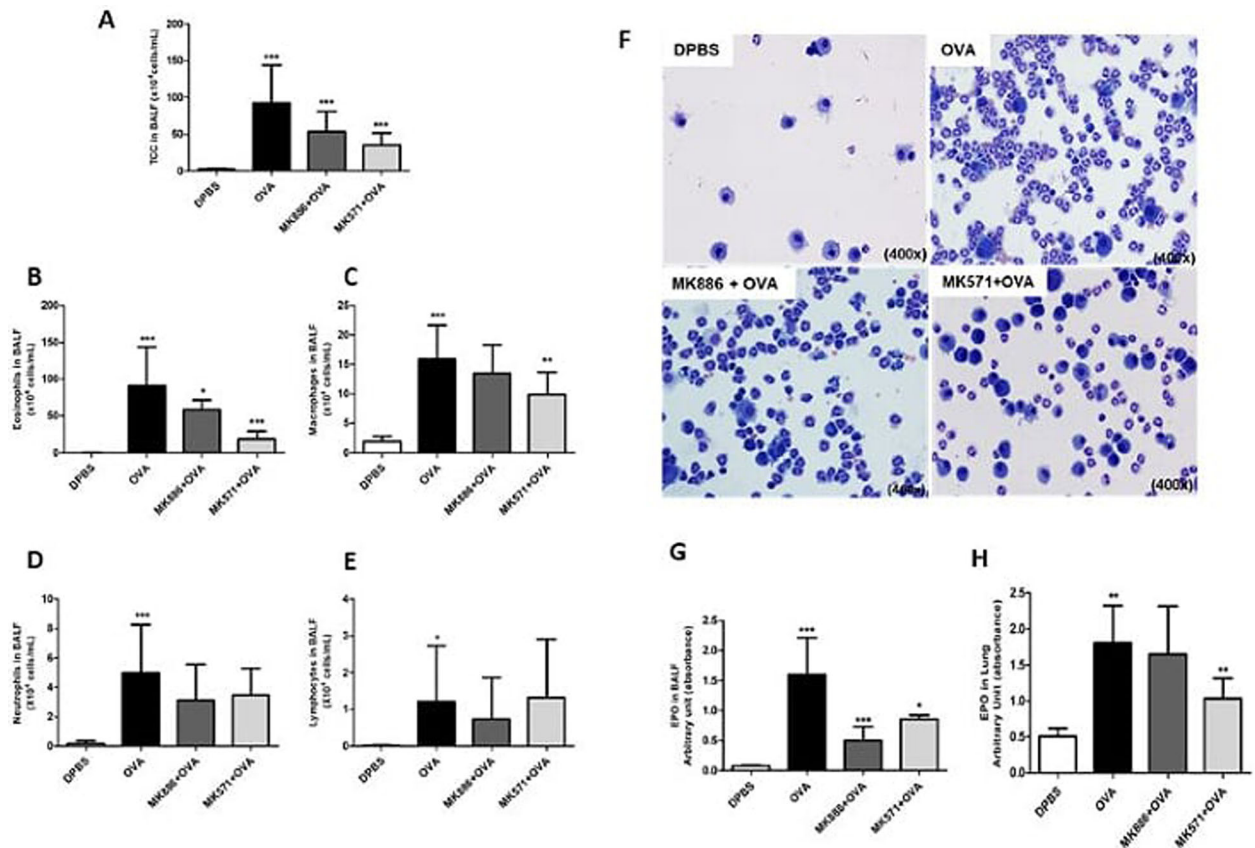


Figure 1. Total and differential cells count in BALF (A-E). Representative illustration of BALF cells (400 $\times$ magnification, arrows indicate eosinophils) (F). Analysis of the activity of the EPO enzyme (G and H). Data represent the mean $\pm$ SD, $n = 6-8$ animals per group. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ (one-way ANOVA followed by Tukey test). BALF: bronchoalveolar lavage fluid; DPBS: dulbecco phosphate buffered saline; OVA: ovalbumin; TCC: total cells count; EPO: eosinophil peroxidase; MK-886: FLAP inhibitor; MK-571: cysLT1 receptor antagonist.

inflammation in OVA-challenged mice. We showed that OVA-challenged mice treated with MK-886 or MK-571 had a decrease in total cell counts (Figure 1A) as well as a decrease in eosinophil counts in the BALF compared to the OVA group (Figure 1B). However, only treatment with MK-571 decreased macrophage counts in BALF (Figure 1C). We next evaluated whether the treatments with MK-886 and MK-571 were capable of decreasing EPO activity in the airway. OVA-challenged mice had an increase of EPO activity in the BALF. However, OVA-challenged mice and treated with MK-886 or MK-571 decreased EPO activity (Figure 1G). In addition, in lung tissue only treatment with MK-571 was able to decrease the EPO activity when compared to the OVA group (Figure 1H). Then, we decided to investigate the capacity of MK-886 and MK-571 to prevent the induction of histopathological changes in the lung, that are typical pathologic

features of asthma. We demonstrated an increase in inflammatory cell infiltration located in peribronchial and perivascular areas in OVA-challenged mice when compared to the DPBS control group. On the other hand, OVA-challenged mice treated with MK-886 or MK-571 had a reduction in the pulmonary influx of cells located in the peribronchial and perivascular areas when compared to the OVA group (Figure 2A). Another typical pathologic characteristic of asthma is an increase in goblet cells and mucus production. By the staining of lung sections with Alcian Blue, our data suggest that OVA-challenged mice had an increase in goblet cells and mucus production compared to the control group. On the other hand, staining with Alcian blue suggests that OVA-challenged mice and treated with MK-886 or MK-571 had a reduction in goblet cells and mucus production (Figure 2B).

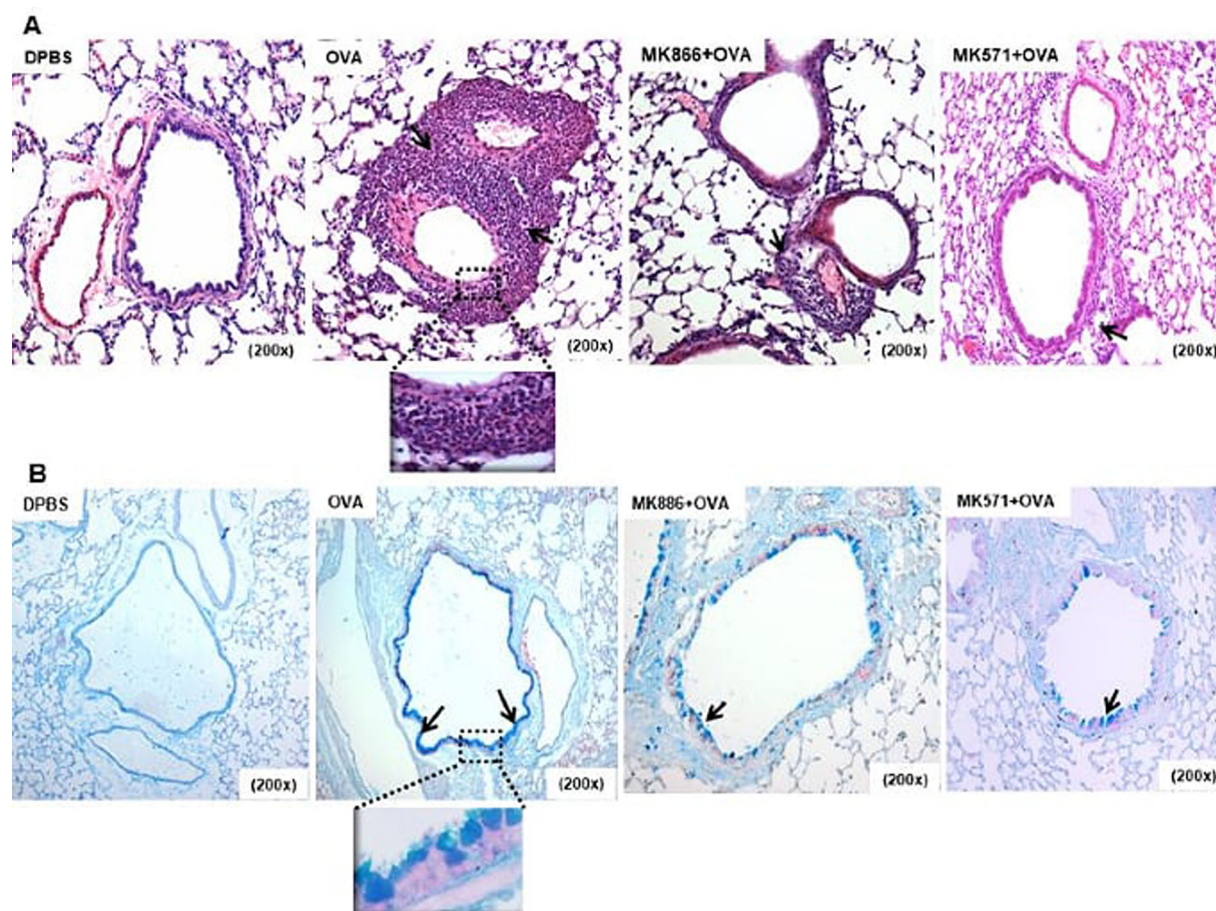


Figure 2. Histopathological analysis in lung. Representative photomicrographs of stained sections with H&E (200x and 1000x magnification, arrow indicate inflammatory infiltrate) (A). Representative photomicrographs of stained sections with alcian blue (200x and 1000x magnification, arrows indicate goblet cells) (B). Data represent the mean $\pm$ SD, $n=6-8$ animals per group. DPBS: dulbecco phosphate buffered saline; OVA: ovalbumin; MK-886: FLAP inhibitor; MK-571: cysLT1 receptor antagonist.

Treatment with MK-571 improves lung function

We evaluated whether treatments with MK-886 and MK-571 were capable of improving respiratory mechanics in an asthma model. Tissue damping and tissue elastance were significantly increased in the OVA-challenged mice when compared to the DPBS group (Figure 3B,C). We also showed that OVA-challenged mice treated with MK-571 had a reduction in tissue damping and tissue elastance when compared to the OVA group (Figure 3B,C). However, treatment with MK-886 did not improve lung function. We did not observe any difference among the groups in airway resistance.

Treatments with MK-886 or MK-571 decrease Th2 cytokines levels in BALF

Later, we evaluated the effects of treatments with MK-571 and MK-886 in inflammatory cytokines

in an asthma model. We showed that OVA-challenged mice had an increase in IL-4, IL-5, and IFN- γ in BALF when compared to the control group (Figure 4A–C). Moreover, we observed that OVA-challenged mice treated with MK-886 or MK-571 had attenuation in levels of IL-4 (Figure 4A) and IL-5 (Figure 4B) when compared to the OVA group. However, treatments with MK-886 or MK-571 did not alter IFN- γ in BALF (Figure 4C). We did not observe any difference among the groups in IL-10 levels (Figure 4D).

EET release in BALF depends on cysLT

As expected, OVA-challenged mice had an increase in extracellular DNA concentrations in BALF when compared to the DPBS group (Figure 5B). In immunofluorescence microscopy, we showed that eosinophils from OVA-challenged mice released EETs colocalized with EPO

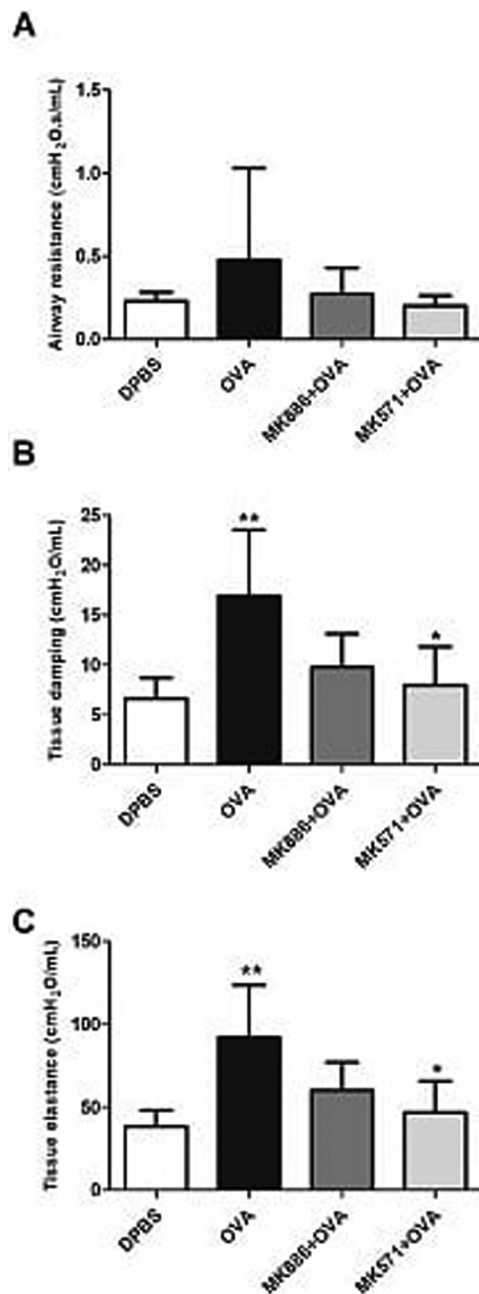


Figure 3. Assessment of respiratory system mechanics: resistance (Rn), tissue damping (G) and tissue elastance (H) (A–C). Data represent the mean $\pm$ SD, $n = 6$ –8 animals per group. * $p < 0.05$ and ** $p < 0.01$ (One-way ANOVA followed by Tukey test). DPBS: dulbecco phosphate buffered saline; OVA: ovalbumin; MK-886: FLAP inhibitor; MK-571: cysLT1 receptor antagonist.

(Figure 5A). Later, we investigated if treatments with MK-886 or MK-571 were able to reduce EETs formation in an asthma model. We showed that OVA-challenged mice treated with MK-886 or MK-571 had a decrease in extracellular DNA concentrations in BALF (Figure 5B). Similarly, in immunofluorescence microscopy, we did not

observe EET formation in BALF eosinophils in OVA-challenged mice treated with MK-886 or MK-571, confirming our findings (Figure 5A).

EET release do not alter BALF cell viability

To exclude the possibility of extracellular DNA derived from dead cells in BALF, we performed flow cytometric analysis of annexin-V and PI. EETs release was not due to cell death because we observed that cell viability remained high among the groups, showing that most cells had negative staining for annexin-V and PI (Figure 5C).

Treatment with MK-886 decreases cysLT levels in lung

Finally, we evaluated whether treatments with MK-886 or MK-571 were capable of modifying cysLT levels in the lung. We showed that OVA-challenged mice had an increase in cysLT levels in the lung when compared to the control group. Moreover, OVA-challenged mice treated with MK-886 had a reduction in cysLT levels in the lung when compared to the OVA group. In contrast, treatment with MK-571 did not alter cysLT levels in the lung (Figure 6).

Discussion

Recent studies have revealed that Th2-type cytokines that include IL-4, IL-5, and IL-13, as well as overproduction of cysteinyl leukotriene to be associated with persistent eosinophilia in asthmatic patients. It is known that eosinophilia contributes to the pathogenesis of disease through producing molecules such as EET, that are cytotoxic enough to induce tissue damage in asthmatic airways. It has already been demonstrated that the percentage of eosinophils secreting EET is negatively correlated with the baseline forced expiratory volume in 1 second, in other hand, have a positive correlation with the levels of eosinophil-derived neurotoxin in serum, suggesting that EET may play a crucial role in eosinophilic inflammation and airway obstruction.^{35,36} Interestingly, Wu, A et al showed that the Montelukast, a CysLT1 receptor antagonist,

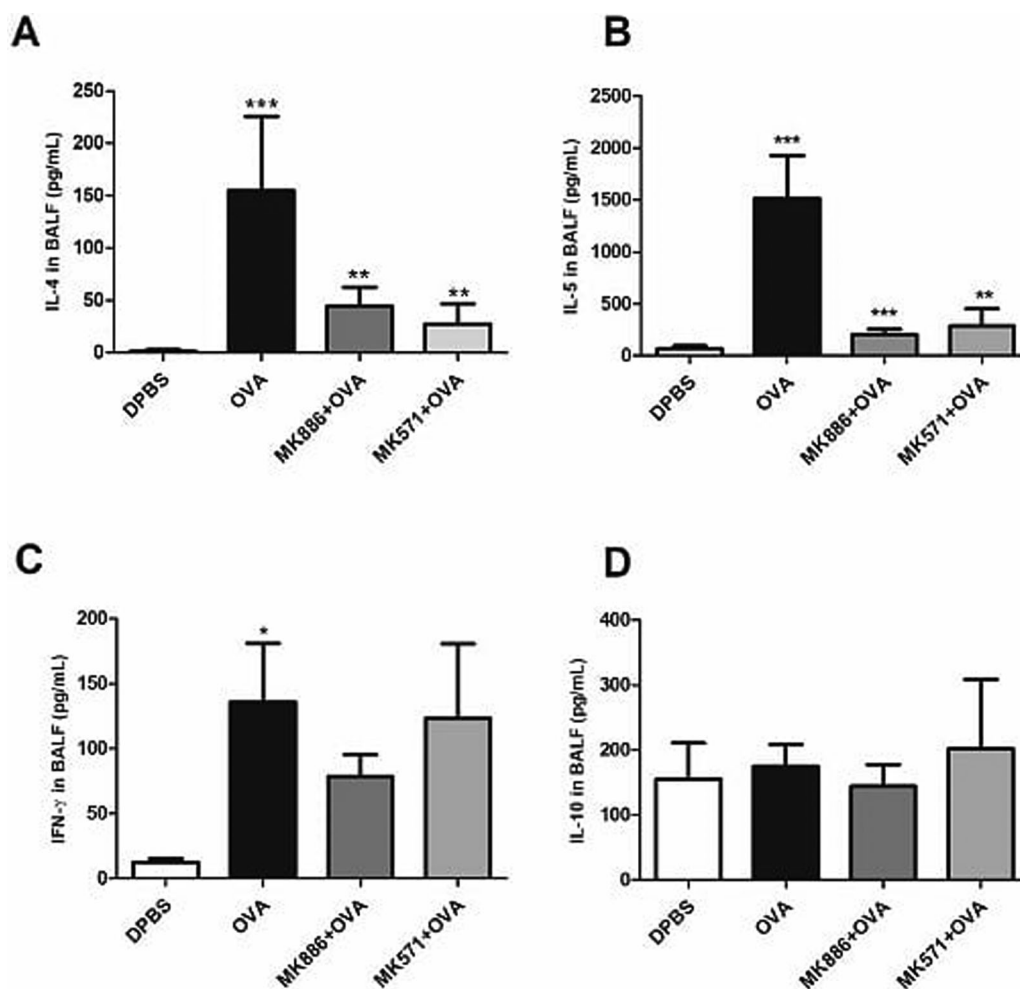


Figure 4. Cytokine levels in BALF (IL-4, IL-5, IFN- γ and IL-10) (A-D). Data represent the mean $\pm$ SD, $n = 6-8$ animals per group. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ (one-way ANOVA followed by Tukey test). BALF: bronchoalveolar lavage fluid; DPBS: dulbecco phosphate buffered saline; OVA: ovalbumin; MK-886: FLAP inhibitor; MK-571: cysLT1 receptor antagonist; IFN: interferon; IL: interleukin.

reduces eosinophil recruitment into the airway through the suppression of Th2 cytokines and chemokines.³⁷ Although it is an important finding, there is no knowledge about cysLT participation in mechanisms of EET formation. In this present study, our data showed the first evidence that cysLT is necessary for EET formation in an asthma murine model.

Studies have shown that CysLT regulates eosinophil recruitment and induces an eosinophilotactic activity.^{13,38} In our data, the cell counts as well as histopathological lung examination revealed that OVA-challenged mice treated with MK-886, a FLAP inhibitor, or MK-571, a cysLT1 receptor antagonist, had a significantly reduction in the recruitment of cells, mainly in eosinophil number into the lung. In asthmatic patients, an increase in cytotoxic proteins released from

eosinophil granules is indicative of its activation.³⁹ However, the literature showed that the eosinophil degranulation in the mouse model is controversial. Malm-Erfjelt and colleagues demonstrate that eosinophils recruited to the lungs and the nose of heavily allergen-exposed mouse airways do not exhibit a sign of degranulation.⁴⁰ On the other hand, more recent studies have reported that eosinophil from mice degranulates when stimulated and releases secondary granule proteins such as EPO.^{24,30,41} Our data showed that treatment with MK-886 or MK-571 significantly decreased EPO activity induced by OVA challenge. In agreement with our results, studies have shown that cysLT stimulate the release of eosinophil granule-derived.^{42,43}

Mucus hypersecretion and a decrease in lung function are features of respiratory diseases such

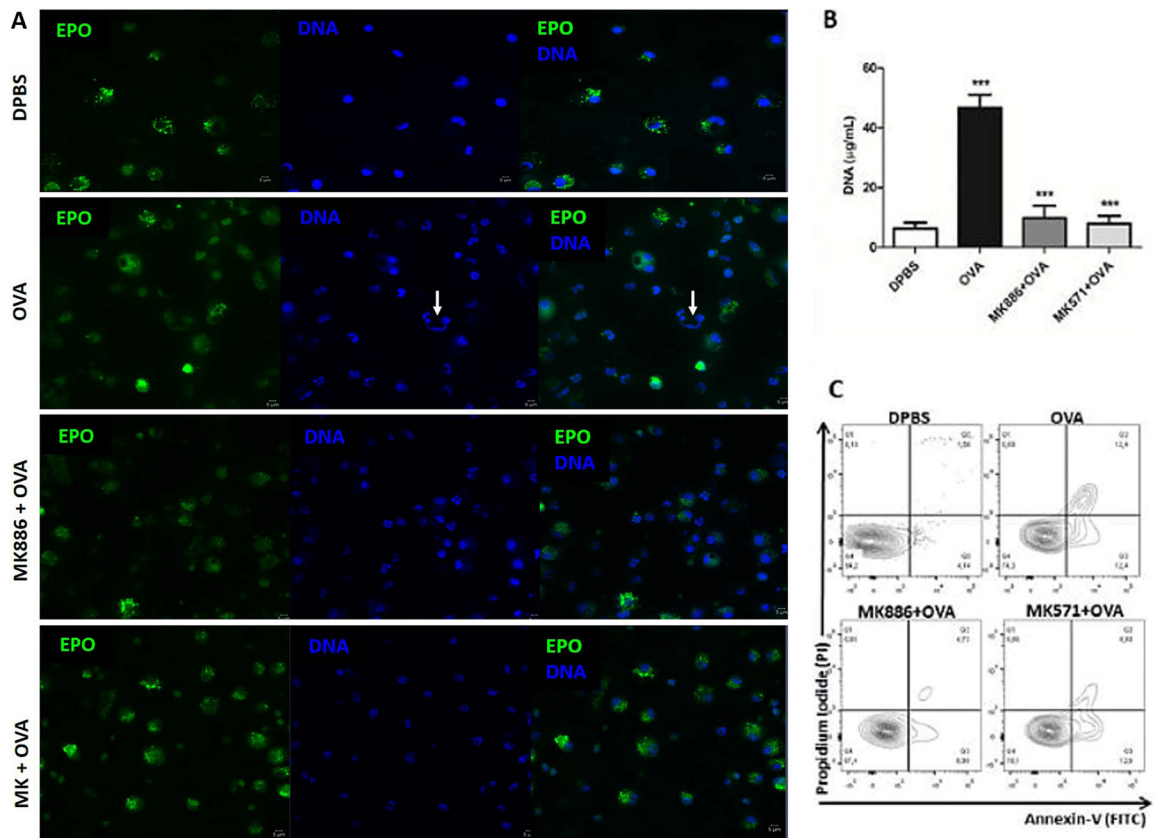


Figure 5. Immunofluorescence microscopy of EETs in BALF eosinophils (630x magnification, arrows indicate EETs formation) (A). Quantification of extracellular DNA traps in BALF (B). Analysis of cell death in BALF cells by Annexin V/PI (C). Data represent the mean $\pm$ SD, $n = 6-8$ animals per group. *** $p < 0.001$ (one-way ANOVA followed by Tukey test). BALF: bronchoalveolar lavage fluid; DPBS: dulbecco phosphate buffered saline; EETs: eosinophil extracellular traps; EPO: eosinophil peroxidase; OVA: ovalbumin; PI: propidium iodide; MK-886: FLAP inhibitor; MK-571: cysLT1 receptor antagonist.

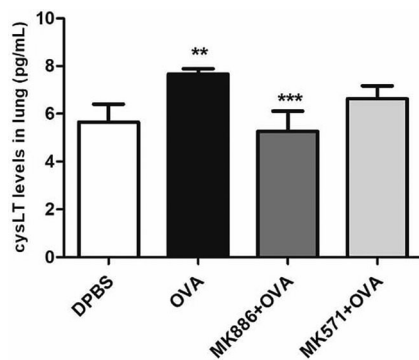


Figure 6. Analysis of CysLTs levels in lung tissue. Data represent the mean $\pm$ SD, $n = 6-8$ animals per group. ** $p < 0.01$ and *** $p < 0.001$ (one-way ANOVA followed by Tukey test). DPBS: dulbecco phosphate buffered saline; OVA: ovalbumin; MK-886: FLAP inhibitor; MK-571: cysLT1 receptor antagonist; CysLTs: cysteinyl leukotrienes.

as asthma.¹ CysLT is an important mediator for hyperplasia in goblet cells and epithelial mucus synthesis.^{44,45} Our data suggest that MK-886 or MK-571 had a capacity to reduce goblet cell numbers and mucus production. In agreement

with our result, studies have shown that cysLT inhibition or cysLT1 receptor antagonists may modulate mucus hypersecretion in asthma.^{44,46} The cysLT act on their specific receptors on the airway smooth muscle and cause airway narrowing and constriction.⁴⁷ We showed that MK-571 significantly improved lung function in OVA-challenged mice. However, treatment with MK-886 did not improve lung function. We did not observe any difference among the groups regarding airway resistance. Other studies have also observed that there are no significant differences between an acute asthma model and the control group.^{24,25,48} In contrast with our result, the blockade of 5-lipoxygenase inhibited bronchoconstriction in OVA-challenged mice.⁴⁹ On the other hand, cysLT1 receptor antagonists such as Montelukast and Zafirlukast are efficient in inhibiting the action of cysLT on the airway smooth muscle causing an improvement in lung function.⁵⁰

Inflammatory cytokines such as IL-4, IL-5, and IFN- γ stimulate eosinophils to synthesize and release cysLT.⁸ In addition, endogenous cysLT production is an important stimulator of inflammatory cytokines secretion.¹⁰ The IL-4, IL-5, and IFN- γ release in lung tissue contribute to IgE synthesis, airway inflammation, mucus hypersecretion, and AHR in asthma.¹ Our data showed that OVA-challenged mice treated with MK-886 or MK-571 had a decrease in levels of IL-4 and IL-5. FLAP increase may be one of the responsible for cysLT production mediated by IL-5.⁵¹ Moreover, IL-4, IL-5, and IFN- γ increase the expression of the cysLT1 receptor in eosinophils.^{52–54} The IFN- γ modulation of cysLT in airway smooth muscle cells is still not understood.⁵² In agreement with our data, Chen and colleagues observed that OVA-challenged mice treated with a 5-lipoxygenase inhibitor had lower levels of Th2 cytokines in the lung.⁵⁵ Thus, a decrease in Th2 cytokines production by MK-886 and MK-571 may contribute to a reduction of eosinophil migration into the airway, mucus hypersecretion, and alteration in lung function.

EET is formed by DNA and intact eosinophil granules, such as EPO.^{19,20} Moreover, EET formation plays an important role in several inflammatory diseases.^{6,7,19,20,56} Studies have shown an increase in EET levels in bronchial biopsy, BALF, and serum from asthmatic patients compared to healthy patients.^{6,57} Furthermore, our group has already shown that mice challenged with OVA induces EET formation in the BALF and the lung,⁷ once again we confirmed this data. Choi and colleagues have shown that excessive EET formation leads to epithelial damage and stimulates airway epithelium to induce type 2 immune responses in asthma.³⁵ Moreover, DNA traps represent major extracellular structural components scaffolding eosinophilic secretions and contribute to high viscosity of mucus,²⁰ increasing airway obstruction and decreasing lung function.²² In this context, a previous study of our group demonstrated in an asthma model that recombinant human deoxyribonuclease therapy improved lung function and reduced EET generation, showing that reducing the presence of extracellular DNA traps in the airway may be beneficial in asthma.²² Studies have shown that several lipid mediators

regulate the NET release.^{26–29} However, the role of lipid mediators in EET release is not understood. Therefore, we investigated cysLT participation in EET formation in a murine asthma model. We chose to analyze the cysLT effect in EET release in asthma because it is the lipid mediator most involved in asthma immunopathology. Initially, we analyzed if EET formation is dependent on cysLT. We observed that OVA-challenged mice treated with MK-886 had a decrease in extracellular DNA concentrations in BALF when compared to the OVA group. Similarly, by immunofluorescence microscopy we did not observe EET formation in BALF in asthmatic mice treated with MK-886, confirming our findings. Thus, we showed that EET formation in asthma is dependent on cysLT. Later, we analyzed if EET formation dependency on cysLT is via cysLT1 receptor activation. CysLT1 receptor is present in eosinophils and it is one of the most involved receptors in asthma immunopathology.¹⁶ We showed that cysLT1 receptor inhibition with MK-571 decreased extracellular DNA concentrations in BALF from OVA-challenged mice. Likewise, by immunofluorescence microscopy we did not observe EET formation on BALF in the OVA-challenged mice that received treatment with MK-571. Thus, we showed that EET formation dependent on cysLT is via cysLT1 receptor activation in asthma. A reduction in EET formation by treatments with MK-886 or MK-571 may contribute to decreasing in airway damage, inflammation, obstruction by mucus and consequently, impairment in lung function. We evaluated also if the DNA extracellular released by eosinophils was derived from dead cells. EET release was not due to cell death because cell viability remained high between the groups. Other studies also have demonstrated that in asthma the DNA extracellular release by eosinophils was not derived from dead cells.^{7,22,24,25}

Finally, we showed that OVA-challenged mice had an increase in cysLT levels in the lung. Moreover, OVA-challenged mice treated with MK-886 had a decrease in cysLT levels. The MK-886 decreases the cysLT levels via inhibition of the FLAP. FLAP inhibited there is no conversion of arachidonic acid to cysLT.⁹ The FLAP also may induce the production of LTB₄.⁹ However,

in our study, eosinophils are the predominant cells in BALF from OVA-challenged mice and eosinophils may produce only cysLT. Thus, we believe that FLAP inhibition with MK-886 mainly inhibited cysLT production in our eosinophilic asthma model. Therefore, FLAP inhibition is a manner effective to reduce cysLT effects on asthma. In contrast, OVA-challenged mice treated with MK-571 had no altering cysLT levels in the lung. The beneficial action of MK-571 in an asthma model is via antagonism of cysLT in cysLTs1 receptor and consequently, decreasing the cysLT pathological effect. Studies have suggested that exhaled nitric oxide and urinary LTE4/exhaled nitric oxide ratio may predict responsiveness to cysLT inhibitors or cysLTs1 receptor antagonists in children with mild-to-moderate asthma.^{58,59} We suggest that an increase in EET formation in the airway may predict therapeutic responses to decrease in cysLT formation or cysLTs1 receptor antagonists; however, other studies should investigate further this hypothesis.

In summary, this is the first study to report that viable eosinophils release EET dependent on cysLT via cysLT1 receptor activation in an asthma model. Moreover, our data showed that OVA-challenged mice treated with MK-886 or MK-571 had a decrease in airway inflammation, EPO activity, mucus production, inflammatory cytokines, and EET release. Furthermore, we also showed that it can improve lung function. Our data revealed that the decrease in cysLT production or cysLT1 receptor inhibition by MK-886 or MK-571 treatments respectively reduced EET formation in BALF, showing that cysLT regulates the activation process of EET release in asthma.

Disclosure statement

The authors declare that they have no commercial or financial conflict of interests.

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